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EPIDEMIOLOGY OF HOSPITAL-ACQUIRED AND COMMUNITY-ONSET METICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS

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Doctor of Philosophy

Aston University

July 2007

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THE UNIVERSITY OF ASTON IN BIRMINGHAM
Epidemiology of Hospital-Acquired and Community-Onset Meticillin-Resistant
Staphylococcus aureus
A thesis submitted by Jessica Rollason for the degree of Doctor of Philosophy
2007

Summary

Meticillin-resistant Staphylococcus aureus (MRSA) is a recognised cause of morbidity and mortality in healthcare settings, exacerbated over the last decade following the emergence of virulent MRSA strains within the community. This study has sought to investigate the epidemiology of hospital- and community-associated MRSA within the West Midlands region. Nasal swabs obtained from patients in a renal dialysis unit revealed 6% to be positive for MRSA and 23% positive for meticillin-sensitive S. aureus. From 14 central venous catheter (CVC) swabs, one patient was positive. All MRSA isolates from individual nasal samples displayed distinct antimicrobial resistance phenotypes and pulsed-field gel electrophoresis (PFGE) profiles. PFGE analysis of colonies from primary isolation plates suggested homogenous strain colonisation. In one patient, simultaneous isolates were indistinguishable from the nasal and CVC site suggesting intra-patient cross-contamination. In a parallel investigation 25% of patient’s anterior nares and tongue were simultaneously colonised with genetically indistinguishable MRSA. All MRSA isolates were negative for pvl. One MRSA isolate was obtained from the environment which was not represented in any of the clinical isolates suggesting this was not a significant reservoir compared to intra-patient contamination.

One hundred and ninety-nine MRSA isolates were obtained from community-onset infection; 87% harboured SCCmec IV and 8% SCCmec II, displaying PFGE profiles genetically related to EMRSA-15 and EMRSA-16 respectively. Eight isolates harboured a novel SCCmec IIIa-mec. All isolates were ascertained to be of nosocomial genetic lineage and un-typical of de-novo community origin. Hospital-onset bacteraemia isolates were characterised by SCCmec and antimicrobial sensitivity testing. Using Yates-corrected chi-square, isolates obtained from community onset were significantly associated with SCCmec IV and reduced resistance to non-β-lactams; in contrast, hospital isolates were associated with SCCmec II and a multi-drug resistant phenotype. In addition, as part of a methodological development, five virulence determinants encoded by fnbA, sdrE, hlg, cna and icaA were combined into a multiplex PCR assay to facilitate determination of these genes within various MRSA populations.

Application of SaTScan and the space-time scan statistic to 832 incidences of MRSA in the community identified two significant clusters correlating with the location of nursing care institutions. An increased relative risk of MRSA incidence was also observed in neighbouring Census Output Areas suggesting potential overspill from these community hotspots into adjacent areas.

Keywords: MRSA, PFGE, SCCmec, geographical information systems, SatScan.
For my grandparents;

Joe, Alice, Joseph and Jessie.
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ABBREVIATIONS

6-APA  6 aminopenicillanic acid
A      Absorbance
AAC    N-acetyltransferases
agr    Accessory gene regulator
ANT    O-nucleotidyltransferases
APH'   O-phosphotransferases
AP-PCR Arbitrarily primed-PCR
AST    Antimicrobial susceptibility testing
ATP    Adenosine Triphosphate
BHI    Brain heart infusion
bp     Base pairs
BSAC   British Society for Antimicrobial Chemotherapy
CA     Community-acquired
Ccr    cassette chromosome recombinase
CCs    Clonal complexes
CHEF   Contour clamped homogenous electrophoresis
Clf    Fibrinogen binding clumping factors
Cna    Collagen binding protein
CVC    Central venous catheter
D-ala-D-ala D-alanyl-D-alanine
D-ala-D-lac D-alanine-D-lactate
DHFR   Dihydrofolate reductase
<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>DHPS</td>
<td>Dihydropterate synthetase</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine-tetraacetic acid</td>
</tr>
<tr>
<td>EMRSA</td>
<td>Epidemic-MRSA</td>
</tr>
<tr>
<td>ET</td>
<td>Exfoliative toxin</td>
</tr>
<tr>
<td>FnBP</td>
<td>Fibronectin binding protein</td>
</tr>
<tr>
<td>GIS</td>
<td>Geographical Information System</td>
</tr>
<tr>
<td>GP</td>
<td>General practitioner</td>
</tr>
<tr>
<td>HA</td>
<td>Hospital-associated</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HI</td>
<td>Haemolysin</td>
</tr>
<tr>
<td>hVISA</td>
<td>Hetero-resistant vancomycin-intermediate <em>S. aureus</em></td>
</tr>
<tr>
<td>Ica</td>
<td>Intercellular adhesion protein</td>
</tr>
<tr>
<td>IS</td>
<td>Insertion sequence</td>
</tr>
<tr>
<td>Kbp</td>
<td>Kilo-base pairs</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Daltons</td>
</tr>
<tr>
<td>Luk</td>
<td>Leukocidin</td>
</tr>
<tr>
<td>Mbp</td>
<td>Mega-base pairs</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
</tr>
<tr>
<td>MLS</td>
<td>Macrolide, lincosamide and streptogramin B</td>
</tr>
<tr>
<td>MLST</td>
<td>Multi-locus sequence typing</td>
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</table>
MR  Muti-drug resistant
mRNA  Messenger ribonucleic acid
MRSA  Meticillin-resistant *Staphylococcus aureus*
MSCRAMMS  Microbial surface components recognising adhesive matrix molecules
MSSA  Meticillin-sensitive *Staphylococcus aureus*
NaCl  Sodium chloride
NAG  N-acetylglucosamine
NAM  N-acetylglucosamine
NCCLS  National Committee for Clinical Laboratory Standards
NCTC  National Collection of Type Cultures
NHS  National Health Service
NMR  Non-mutidrug resistant
OD  Optical density
ONS  Office of National Statistics
ORF  Open reading frame
PABA  P-aminobenzoic acid
PBP  Penicillin binding protein
PCR  Polymerase chain reaction
PFGE  Pulsed-field gel electrophoresis
PIA  Polysaccharide intercellular adhesin
PVL  Panton valentine leukocidin
RAPD  Random amplification of polymorphic DNA
RDU  Renal Dialysis Unit
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>sarA</td>
<td>Staphylococcal accessory regulator gene</td>
</tr>
<tr>
<td>SCCmec</td>
<td>Staphylococcal cassette chromosome mec</td>
</tr>
<tr>
<td>Sdr</td>
<td>Serine-aspartate repeat-containing surface protein</td>
</tr>
<tr>
<td>SDW</td>
<td>Sterile distilled water</td>
</tr>
<tr>
<td>SE</td>
<td>Staphylococcal enterotoxin</td>
</tr>
<tr>
<td>SSSSS</td>
<td>Staphylococcal scalded-skin syndrome</td>
</tr>
<tr>
<td>ST</td>
<td>Sequence type</td>
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<td>Tris, boric acid, EDTA</td>
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<td>Tris, EDTA</td>
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<td>TESS</td>
<td>Tris, EDTA, NaCl, sucrose</td>
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<td>Tm</td>
<td>Melting temperature</td>
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<tr>
<td>Tn</td>
<td>Transposon</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris [hydroxymethyl] aminomethane</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer ribonucleic acid</td>
</tr>
<tr>
<td>TSS</td>
<td>Toxic shock syndrome</td>
</tr>
<tr>
<td>UHB</td>
<td>University Hospital Birmingham</td>
</tr>
<tr>
<td>UPGMA</td>
<td>Un-weighted pair groups using mathematical average</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra-violet</td>
</tr>
<tr>
<td>VISA</td>
<td>Vancomycin-intermediate <em>S. aureus</em></td>
</tr>
<tr>
<td>VRSA</td>
<td>Vancomycin-resistant <em>S. aureus</em></td>
</tr>
</tbody>
</table>
CHAPTER 1 INTRODUCTION

1.1 Staphylococcus aureus

*Staphylococcus aureus* was first described by Ogsten in 1881. Following microscopic observation of a pus sample from the leg of a young male patient he observed grape like (staphule) bacterial clusters (kokkos). Three years later Rosenbach successfully cultured this bacterium and noted a yellow/orange pigmentation to the bacterial colonies and so derived the name *Staphylococcus aureus* from the Latin translation of golden (aureus). *Staphylococcus aureus* (*S. aureus*) is a member of the Micrococaceae bacterial family and is characterised as a Gram-positive, oxidase-negative, glucose fermentative, non-spore-forming, facultative anaerobe with a cell 1μm diameter. Routine clinical laboratory identification utilises the organism’s ability to acrobically ferment mannitol and to produce catalase, coagulase and deoxyribonuclease enzymes (Baird-Parker, 1963; Morton and Cohn, 1972; Tu and Palutke, 1976; Fung et al., 1984). *Staphylococcus aureus* will optimally grow at a temperature range between 35-37°C and is halo-tolerant typically surviving salt concentrations of up to 15%.

The human skin is a natural reservoir for *S. aureus* endogenous carriage, in particular the nasal passages, axillae, groin, vagina, pharynx and any point of skin damage (Williams, 1963; Kluytmans et al., 1997; Kampf and Kramer, 2004; Warner and Onderdonk, 2004). *Staphylococcus aureus* is estimated to inhabit 30-50% of the adult population, with 10-20% being persistently colonised and 60% being intermittent
carriers (Kluytmans et al., 1997). Natural carriers reportedly harbour an increased risk of staphylococcal infection which is further elevated in susceptible patient groups such as those with damaged mucosal skin barriers, those undergoing haemodialysis or those undergoing surgery (Kreft et al., 1998; Koontz, 2000; Saxena and Panhotra, 2005). *Staphylococcus aureus* causes a wide range of human infections ranging from skin infections such as boils, styes and furunculosis to the acquisition of deep-seated and blood-borne infections such as pneumonia, mastitis, phlebitis, meningitis, osteomyelitis, sepsis and endocarditis. This pathogenic diversity is largely governed by a wide range of virulence factors that work synergistically to facilitate host cell attachment, colonisation, cell-cell interactions, immune evasion and invasive tissue damage (Barbour, 1981; Kuroda *et al.*, 2001; Fueyo *et al.*, 2005).

The discovery and introduction of β-lactam antibiotics heralded a new era in medical intervention against staphylococcal disease. However, such success has continually been hampered by the countless ability of this micro-organism to adapt under selective pressure in the clinical setting with the acquisition of antimicrobial resistance mechanisms.
1.2 *Staphylococcus aureus* resistance mechanisms and the emergence of meticillin resistance

1.2.1 β-lactam mediated resistance

The antibacterial properties of the β-lactam compounds were first discovered in 1929 by Alexander Fleming who observed that fungal contamination of a bacterial plate substantially inhibited the growth of *S. aureus* colonies. This fungal organism was *Penicillium notatum* and the anti-bacterial agent produced was subsequently named penicillin. Florey and Chain later established the production of penicillin on a large commercial scale and this compound was first used therapeutically in 1941 against a number of Gram-positive infections (Dyke and Gregory, 1997).

The β-lactams are a broad class of antibiotics that include the penicillins, cephalosporins, carbapenems and monobactams. These compounds exert an antibacterial effect through the inhibition of peptidoglycan synthesis, a process essential in bacterial cell-wall construction. The bacterial cell-wall is composed of a rigid outer layer providing protection and shape to the bacterial cell, maintaining osmotic pressure and cell integrity. An approximate 90% of the cell-wall weight of Gram-positive bacteria is composed of peptidoglycan constituting a polymer chain of interlocking murein monomers (Lowy, 1998). Each murein monomer consists of two β-1-4 linked amino sugars, N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM). A pentapeptide attaches each murein monomer by a carboxyl residue and functions as a
cross linking bridge to form strength between peptidoglycan layers. Staphylococcal pentapeptides are composed of L-alanyl-D-isoglutaminyl-L-lysyl-D-alanyl-D-alanine. Individual glycan chains are cross-linked by a pentaglycine bridge between the L-lysine of one pentapeptide to the D alanine residue of an adjacent pentapeptide (Lowy, 1998).

Murein monomers are synthesised in the cell cytosol and are enzymatically inserted into peptidoglycan to construct the polymer chain during bacterial cell wall growth. Penicillin binding proteins (PBP’s), the targets of β-lactam antibiotics, are transpeptidase enzymes that are vital for cell-wall construction. In a process called transpeptidation, the PBPs bind to D-alanyl-D-alanine (D-ala-D-ala) on the pentapeptide and function to reform and facilitate peptide cross linkage between the peptidoglycan rows. β-lactam compounds are structural analogues of D-ala-D-ala and therefore covalently bind to the active site serine residue of PBP to form a penicilloyl–enzyme complex. This complex renders the PBPs inactive and halts peptidoglycan synthesis, subsequently initiating bacterial cell lysis. Staphylococcus aureus produces four types of PBP between 44-85kDa, each exhibiting a high affinity for β-lactam antibiotics (Canepari et al., 1985; Labischinski, 1992).

Penicillin resistance was first observed in the 1940s (Kirby, 1944) and by the 1950s 50% of S. aureus strains in UK hospitals were no longer sensitive to β-lactam compounds (Grundmann et al., 2006). Penicillin resistance is driven by the production of penicillinase, an extracellular enzyme which catalyses the inactivation of β-lactams through hydrolysis of the β-lactam ring. Penicillinase enzymes (from hereon referred to
as β-lactamases) have been classified into four classes: class A, C and D are active site serine β-lactamases and class B are zinc dependant mettaloenzymes (Bush et al., 1995; Peimbert and Segovia, 2003).

Over 90% of staphylococci produce β-lactamase through blaZ transcription, regulated by anti-repressor blaR1 and repressor blaI (Hackbarth and Chambers, 1993; Rosato et al., 2003). BlaR1 encodes BlaR1, a transmembrane protein that functions to detect β-lactams via an extracellular penicillin binding protein domain. Following penicillin binding, BlaR1 cleaves a protein protease that, in turn, cleaves repressor BlaI from blaZ to allow subsequent transcription of β-lactamase (Zhang et al., 2001; Lowy, 2003). This process is illustrated in figure 1.1.
(a) Regulation of the β-lactamase synthesis pathway. In the absence of penicillin, BlaI binds to the operator region controlling blaZ and blaR1-blaI, to repress transcription (I). Penicillin binds to the intracellular BlaR1 transmembrane sensor-transducer (II) initiating the cleavage of an active intracellular protein protease (III). Active BlaR1 protein protease, by site specific proteolytic cleavage, inactivates repressor BlaI (IV) allowing blaZ transcription and β-lactamase production (V). β-lactamase is released
from the cells and hydrolyses the β-lactam ring of penicillin (VI), rendering it inactive (VII).

The continued predominance of *S. aureus* β-lactam resistance in the clinical setting initiated the design and commercial production of semi-synthetic penicillins. Chemical removal of the β-lactam acyl group exposed 6-aminopenicillanic acid (6-APA) which could be utilised for the synthetic addition of acyl halide groups (Cole, 1966). These acyl groups had varied properties and bulky side chains and subsequently protected the β-lactam ring from β-lactamase hydrolysis. Meticillin was the first of the semi-synthetic penicillins to be designed.

**1.2.2 The rise of meticillin-resistant *Staphylococcus aureus***

Meticillin-resistant *Staphylococcus aureus* (MRSA) was first observed shortly after the introduction of meticillin in the 1960s (Eriksen, 1961; Jevons *et al.*, 1963) and by the 1970s had spread worldwide in a wave of clonal dissemination (Oliveira *et al.*, 2002; Wielders *et al.*, 2002; Grundmann *et al.*, 2006). The gene encoding meticillin resistance, *meca*, is localised on a 21-67 kb transposable chromosomal region known as the staphylococcal cassette chromosome (SCCmec) (Archer *et al.*, 1996; Ito *et al.*, 1999; Katayama *et al.*, 2000; Hiramatsu *et al.*, 2001). The discovery of an evolutionary homolog of *mecA* in *Staphylococcus sciuri*, (Wu *et al.*, 1996) and evidence for in vivo horizontal transfer of *mecA* DNA from *Staphylococcus epidermidis* to *S. aureus*, provides evidence for the role of coagulase negative staphylococci in the evolution of
MRSA (Wielders et al., 2001).

MecA encodes an altered 78kDa penicillin binding protein designated 2' (PBP2') or 2a (PBP2a) (Hartman and Tomasz, 1984; Reynolds and Brown, 1985), that has a low affinity for β-lactam antibiotics whilst continuing to function in cell-wall transpeptidation (Brown and Reynolds, 1980; Reynolds and Brown, 1985; Lim and Strynadka, 2002). Transcription of mecA is regulated by mecI and mecR1 which are genetic homologues of blaI and blaR1 (Lowy, 2003) (figure 1.1). MecR1 and mecI encode for anti-repressor and repressor proteins MecR1 and MecI. Meticillin binds to the membrane spanning MecR1 protein initiating the cleavage of an active intracellular protein protease that in turn cleaves repressor MecI and its repression upon mecA transcription (Hiramatsu et al., 1992). Characteristically, MRSA have mutations or a specific deletions in mecI to prevent repression of PBP2' synthesis and subsequently only pre-MRSA (meca-positive S. aureus) have fully functioning mecI repression and susceptibility to meticillin (Suzuki et al., 1993; Kobayashi et al., 1998; Weller, 1999). Additionally, BlaI has also been demonstrated to be a co-regulator of mecA transcription (Rosato et al., 2003).

The staphylococcal cassette chromosome is proposed to have been acquired through horizontal gene transfer from coagulase negative staphylococci, inserted into the S. aureus genome at a site - and orientation - specific chromosomal location near the origin of replication (orfX) (Ito et al., 1999; Kuroda et al., 2001). SCCmec can be classified into five distinct groups (figure 1.2) designated SCCmec I, II, III, IV and
more recently V (Ito et al., 2001; Ma et al., 2002; Ito et al., 2004). SCCmec, aside from mecA, also contains numerous insertion sequences, plasmids, transposons and genetic determinants that facilitate the acquisition of resistance to non-β-lactams (Ito et al., 1999) for example insertion element IS431 and transposon Tn554, (Oliveira and de Lencastre, 2002).

MecA forms part of a mec gene complex that can be classified into groups based upon its genetic structure: class A, mecI-mecR1-mecA-IS431; class B, ψIS1272-ΔmecR1-mecA-IS431; class C, IS431L-ΔmecR1-mecA-IS431 and class D, IS431- mecA- ΔmecR1 (Katayama et al., 2001). The ccr gene complex encodes cassette chromosome recombinase enzymes responsible for excision and integration of SCCmec into the staphylococcal chromosome (Katayama et al., 2000; Ito et al., 2004; Jansen et al., 2006). SCCmec can further be classified by allotypes for ccrA and ccrB as follows: ccrA1, ccrA2, ccrA3, ccrA4, ccrB1, ccrB2, ccrB3 and ccrB4. The combination of mec and ccr gene complex classifies the allotype of SCCmec. SCCmec type I comprises of the class B mec gene complex and ccr gene complex type 1, SCCmec type II comprises of the class A mec gene complex and ccr gene complex type 2, SCCmec type III comprises of the class A mec gene complex and ccr gene complex type 2, SCCmec type IV comprises of the class B mec gene complex and ccr gene complex type 2 (Ito et al., 2001; Ma et al., 2002). The fifth allotype of SCCmec (SCCmec V) harbours a novel mec gene complex (IS431-mecA-ΔmecR1-IS431) designated C2 mec and a single copy of a gene homolog encoding a cassette recombinase designated ccrC (Ito et al., 2004). In addition the recent report of an SCCmec type with class B mec complex, ccrAB
allotype 4, and a specific junkyard region (J1), has been characterised as SCC\textit{mec} type VI (Oliveira \textit{et al}., 2006).

\textbf{Figure 1.2} Illustrative representation of SCC\textit{mec} types I-V adapted from (Ito \textit{et al}., 2003).

Novel variants of SCC\textit{mec} have been reported based upon variations in junkyard regions outside of the \textit{mec} and \textit{ccr} gene complex (Oliveira and de Lencastre, 2002; Shore \textit{et al}., 2005). Isolates harbouring IA have an integrated \textit{pUB110} plasmid downstream of the \textit{mec} complex, IIIA have an absence of \textit{pT181} that flanks \textit{IS431}, IIIB lack the \textit{mer} operon, Tn554 and \textit{pT181} and IVA has been reported to incorporate an integrated \textit{pUB110} (Oliveira and de Lencastre, 2002). SCC\textit{mec} IV can be further
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categorised IVa–IVg through polymorphic variations in L-C regions between the left extremity and the ccr gene complex (Kwon et al., 2005; Shore et al., 2005; Zhang et al., 2005; Kondo et al., 2007) and more recently subtype IVh has been associated with EMRSA-15 strains (Milheirico et al., 2007). In addition a wide degree of variations in SCCmec types II, III and IV junkyard regions has recently been reported, further highlighting the potential diversity exhibited by these mobile genetic elements (Hisata et al., 2005; Shore et al., 2005; Kondo et al., 2007).

1.3 The worldwide dissemination of MRSA

With the 1980s came the emergence and dissemination of epidemic MRSA strains throughout hospitals in England and Wales (Cookson and Phillips, 1988; Duckworth et al., 1988). The Public Health Laboratory Service Staphylococcal Unit devised a numerical prefix for the recognition of epidemic MRSA strains and by the 1990s 16 EMRSA types had been identified. Epidemic MRSA-15 (EMRSA-15) and epidemic MRSA-16 (EMRSA-16) currently predominate in the UK and account for > 95% of blood stream infections in England and Wales (Johnson et al., 2001; Moore and Lindsay, 2002). Additionally, the recent emergence of multi-drug resistant EMRSA-17 in England and Wales has further increased the concern for UK public health (Aucken et al., 2002).

Inter-country and inter-continental dissemination of MRSA is a worldwide concern (Ayliffe, 1997; Aires de Sousa et al., 1998). Molecular analysis combining SCCmec
typing with multilocus sequence typing (MLST) has revealed a number of pandemic clones to be responsible for the majority of hospital MRSA infections. These predominant MRSA clones are predicted to have arisen from the introduction of SCCmec into five MSSA lineages and within each lineage SCCmec is thought to have been acquired on multiple occasions. Genetic lineages are observed as clonal complexes (CCs) (Robinson and Enright, 2003) and each CC is grouped by MLST sequence type (ST) based upon a common ancestral genotype. The five major lineages responsible for the majority of international nosocomial infections are CC5, CC8, CC22, CC30, and CC45 (Enright et al., 2002). Using CC, ST and SCCmec type analysis, the major MRSA clones have been designated: CC5, ST5-MRSA-II (New York/Japan) and ST5-MRSA-IV (Pediatric); CC8, ST239-MRSA-III A (Brazilian), ST239-MRSA-III (Hungarian), ST247-MRSA-IA (Iberian), ST250-MRSA-I (Archaic), ST8-MRSA-II (Irish-I), and ST8-MRSA-IV (EMRSA-2, EMRSA-6); CC22, ST22-MRSA-IV (EMRSA-15); CC30, ST36-MRSA-II (EMRSA-16); CC45, ST45-MRSA-IV (Berlin) (Oliveira et al., 2001a; Enright et al., 2002; Oliveira et al., 2002).

1.4 Mechanisms of antibiotic resistance and the emergence of multi-resistant MRSA

The evolution of multi-drug-resistant MRSA (MR-MRSA) has compromised antimicrobial therapy and international infection control, largely driven by the overuse of antibiotics in the clinical environment, insufficient infection control measures, increased invasive procedures, the widespread use of antibiotics in agriculture and an
increase in foreign travel, (Henwood et al., 2000; Schmitz et al., 2000a). Antimicrobial resistance mechanisms may be acquired through chromosomal gene mutation or by horizontal acquisition of resistance genes via mobile genetic elements (Ito et al., 2003). Horizontal gene transfer may be mediated via cell-cell contact (conjugation), bacteriophage-uptake (transduction) or incorporation of free DNA (transformation) (Tenover, 2006). In general antimicrobial resistance may be expressed by one of three mechanisms; (i) antibiotic is prevented from reaching the target site; (ii) antibiotic is rendered inactive; (iii) alteration of the target site inhibiting antibiotic-target interaction.

1.4.1 Tetracyclines

Tetracycline, first isolated in the 1940s from Streptomyces, are a broad-spectrum antibiotic with activity against both Gram-positive and Gram-negative bacteria (Chopra and Roberts, 2001). Classical tetracycline's inhibit protein synthesis by blocking aminoacyl-tRNA through reversible target site binding to the bacterial ribosomal acceptor A site (Schnappinger and Hillen, 1996; Chopra and Roberts, 2001) These compounds include chlortetracycline, minocycline and doxycycline.

Tetracycline resistance in S. aureus is mediated by the transcription of the tet gene locus. Efflux proteins are encoded by plasmid-based tetK and tetL and function to export tetracycline from the bacterial cell (Khan and Novick, 1983; Trzcinski et al., 2000; Chopra and Roberts, 2001; Ardic et al., 2005). A second resistance mechanism utilises ribosomal protection proteins encoded by tetM and tetO that prevent
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tetracycline binding to the ribosomal target site (Schwarz et al., 1998; Trzcinski et al., 2000; Ardic et al., 2005).

Tigecycline, a structural analogue of minocycline, is the first of a new class of antibiotics called the glyyclcyclines (Pankey, 2005). Bacterial resistance is avoided through the addition of an N-alkyl-glycylamido group to the tetracycline structure giving a broader spectrum of activity and the ability to overcome active drug efflux through steric hindrance (Pankey, 2005).

1.4.2 Fluoroquinolones

Fluoroquinolones, introduced in the 1980s, target two components essential for DNA replication; topoisomerase II (DNA gyrase) and topoisomerase IV (Drlica and Zhao, 1997). DNA gyrase is composed of two subunits encoded by gyrA and gyrB and mediates negative supercoiling of chromosomal DNA, a process responsible for initiating replication and releasing chromosomal topological pressure. Topoisomerase IV is also composed of two subunits encoded by grlA and grlB and is vital for decatenation and the separation of the daughter chromosome in the final stages of DNA replication (Hooper, 2000). Fluoroquinolones inhibit DNA synthesis by trapping DNA gyrase and DNA topoisomerase IV, inhibiting replication and initiating the introduction of DNA strand breaks and cell death (Drlica and Zhao, 1997).
Fluoroquinolone resistance is mediated by two mechanisms (Ferrero et al., 1995). The first is through chromosomal gene mutation in \textit{gyrA}, \textit{gyrB}, \textit{grlA} and \textit{grlB} leading to the subsequent alteration of target binding sites on DNA gyrase and topoisomerase IV. The second mechanism is initiated through the transcription of a membrane multi-drug efflux pump encoded by \textit{norA}. This facilitates the transportation of hydrophilic fluoroquinolones such as norfloxacin, enoxacin and ciprofl oxacin out of the bacterial cell but has little effect on more hydrophobic compounds such as nalidixic acid, oxolinic acid and sparfl oxacin (Yamagishi et al., 1996).

1.4.3 Rifampicin

Rifampicin is a semi-synthetic derivative of rifampicin B produced by \textit{Amycolatopsis mediterranae} and is used to treat serious \textit{S. aureus} infections as part of multi-drug therapy (Drancourt et al., 1993; Zimmerli et al., 1998). These compounds inhibit bacterial DNA transcription by targeting the $\beta$-subunit of RNA polymerase enzymes encoded by the $\text{rpoB}$ gene (Murphy et al., 2006). Combination therapy is recommended (Mandell and Moorman, 1980) due to a high mutation frequency of $\text{rpoB}$ (Aubry-Damon et al., 1998).
1.4.4 Fusidic Acid

Fusidic acid is a steroid-based compound first isolated from *Fusidium coccineum* (Godtfredsen *et al.*, 1962). Fusidic acid inhibits elongation factor G which is essential in the translocation step of bacterial protein synthesis (Chopra, 1976). Resistance is driven by mutations in the *fusA* gene resulting in the alteration of the elongation factor G antimicrobial target binding site (Besier *et al.*, 2003). Mutation frequencies are significantly reduced when fusidic acid is used in therapy with rifampicin (O'Neil *et al.*, 2001a; Brown and Thomas, 2002).

1.4.5 Mupirocin

Mupirocin (pseudomonic acid A) is derived from *Pseudomonas fluorescens* (Fuller *et al.*, 1971; Chain and Mellows, 1977) and is used topically for the treatment of skin infections and the eradication of nasal staphylococcal colonisation (Upton *et al.*, 2003). Mupirocin is a structural analogue of isoleucine, the natural substrate of isoleucyl tRNA synthetase, an essential enzyme in protein synthesis (Sutherland *et al.*, 1985; Farmer *et al.*, 1992). Mupirocin actively competes against isoleucine for the active site of isoleucyl tRNA synthetase and subsequently blocks bacterial protein synthesis (Farmer *et al.*, 1992).

Two years after the clinical introduction of mupirocin, resistance in *S. aureus* was reported (Anon, 1987). Resistance may be expressed at a low level (MIC 8-256 µg/ml)
or high level (MIC ≥ 512 μg/ml). Low level resistance results from a chromosomal mutation in the mupA gene that encodes an altered isoleucil tRNA synthetase (ileS) with reduced affinity for mupirocin (Farmer et al., 1992). High level resistance results in the acquisition of the ileS gene via plasmid incorporation (Gilbart et al., 1993; Perez-Roth et al., 2006).

1.4.6 Aminoglycosides

Aminoglycosides are potent antibacterial agents often combined with β-lactams or glycopeptides for synergistic bactericidal action against serious staphylococcal infections. Aminoglycosides bind to the 16S rRNA component of the 30S subunit of the S. aureus ribosome, inhibiting protein synthesis.

Aminoglycoside-modifying enzymes N-acetyltransferases (AAC), O-nucleotidyldtransferases (ANT) and O-phosphotransferases (APH) alter specific amino/hydroxyl groups on the aminoglycoside compounds reducing their binding affinity to the 16S rRNA target site (Ardic et al., 2006). The most frequent aminoglycoside-modifying enzymes found in staphylococci are AAC(6') and APH(2") encoded by aac(6') and aph(2"). These genes are located on plasmids and transposon Tn4001 (Ubukata et al., 1984; Ardic et al., 2006) conferring resistance to gentamicin, tobramycin and kanamycin. ANT 4' encoded by ant(4')-Ia, confers resistance to neomycin, tobramycin and amikacin and is located on smaller plasmids within SCCmec (Schmitz et al., 1999).
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1.4.7 Trimethoprim and sulphonamides

Trimethoprim and sulphonamides are inhibitors of bacterial folic acid synthesis, a process essential in metabolism and are often used in combination against *S. aureus* derived infections (Ellison *et al.*, 1984; Burman, 1986; Elwell *et al.*, 1986). As part of the folic acid synthesis pathway dihydropterate synthetase (DHPS) forms dihydropteroic acid by the catalysation of p-aminobenzoic acid (PABA). Sulphonamides are structural analogues of PABA thus blocking the folic acid synthesis pathway through competitive enzyme inhibition (Skold, 2000). Dihydrofolate, at a later step of folic acid synthesis, is required to form tetrahydrofolate catalysed by dihydrofolate reductase (DHFR). Trimethoprim is a structural analogue of dihydrofolate and consequently inhibits the action of DHFR.

Sulphonamide resistance is due to *dhrs* which encodes an amino acid alteration in DHPS that effectively decreases the affinity of sulphonamide target-binding (Skold, 2000). In addition, transposon Tn4003 harbors *dfra* which encodes a structurally altered DHFR which reduces the affinity of trimethoprim target-binding (Huovinen *et al.*, 1995; Dale *et al.*, 1997).

1.4.8 Macrolides, lincosamides and streptogramin B

Macrolides (erythromycin), lincosamides (lincomycin and clindamycin), and streptogramins (pristinamycin, dalfopristin and quinupristin) are used frequently in the
treatment of staphylococcal infections (Lina et al., 1999b). These structurally diverse compounds inhibit bacterial protein synthesis by binding to the 50S ribosomal subunit initiating the dissociation of peptidyl-tRNA (Tenson et al., 2003). *Staphylococcus aureus* may exhibit one of three resistance phenotypes; M-type, characterised by resistance to only the 14 and 15 membered ring of macrolides, MS-type, characterised by resistance to the 14 and 15 membered ring macrolides and streptogramin B and MLS-type, characterised by resistance to 14-15- and 16-membered ring macrolides, lincosamides and streptogramin B.

MLS resistance can be induced by one of three mechanisms. The first is by enzymatic de-methylation and target site alteration of an adenine residue on the ribosomal subunit (Weisblum, 1995). Five methylase genes have been identified in *S. aureus*: *ermA* (Murphy, 1985), *ermB* (Wu et al., 1999), *ermC*, (Projan et al., 1987), *ermF* (Chung et al., 1999; Matsuoka et al., 2002) and *ermY* (Matsuoka et al., 2002). *ErmA* is the predominant methylase gene in MRSA (Lina et al., 1999b; Schmitz et al., 2000b) encoded on transposon Tn554 and associated with SCCmec elements II and III (Oliveira and de Lencastre, 2002). Other methylase genes such as *ermC* have been identified on plasmid DNA (Westh et al., 1995). The second resistance mechanism, encoded by *msrA*, utilises an ATP-dependant efflux pump that actively eliminates 14- and 15-membered macrolides and type B streptogramins and therefore only confers MS-type resistance (Eady et al., 1993). The third resistance mechanism is driven by *ereA* and *ereB* encoding phosphotransferases that inactivate macrolides through
hydrolysis of the lactone ring of the macrocyclic nucleus (Wondrack et al., 1996). This mechanism is rarely reported in *S. aureus* isolates.

1.4.9 Quinupristin-dalfopristin

Quinupristin (streptogramin B) and dalfopristin (streptogramin A) are modified streptogramins structurally related to pristinamycin (Batts et al., 2001). Both agents are inhibitors of bacterial protein synthesis and high susceptibility rates have been reported for Gram-positive bacteria (Eliopoulos, 2004). In isolates with constitutive resistance to the macrolide-lincosamide-streptogramin B, quinupristin-streptogramin B resistance may occur (Fuchs et al., 2000). The use of these agents is limited.

1.4.10 Linezolid

Linezolid is a broad-spectrum oxazolidinone antimicrobial agent that can be administered orally or intravenously (Eliopoulos, 2004). Linezolid exerts a bacteriostatic effect through the inhibition of the initiation step in protein synthesis and prevents the formation between tRNA\(^{0\text{Met}}\), mRNA and the ribosome (Swaney et al., 1998). Resistance in *S. aureus* has already been observed driven by 23S ribosomal RNA mutation (Tsiodras et al., 2001; Wilson et al., 2003; Meka and Gold, 2004).
1.4.11 Daptomycin

Daptomycin is a lipopeptide antibiotic that is structurally unrelated to any other current group of antibiotics. Resistance is less likely to develop rapidly due to the absence of any previously encoded genetic mechanisms in the S. aureus chromosome (Eliopoulos, 2004). Daptomycin inhibits cell-wall peptidoglycan synthesis and disrupts the bacterial cytoplasmic membrane potential through a calcium ion effect (Allen et al., 1987; Petersen et al., 2002). Resistance has now begun to emerge in MRSA (Hayden et al., 2005; Marty et al., 2006).

1.4.12 Vancomycin

Vancomycin and teicoplanin are glycopeptide antibiotics that were first introduced in the 1950s. Vancomycin originates from Streptomyces orientalis (now known as Nocardia orientalis) and is active against most Gram-positive bacteria (Barna and Williams, 1984). Glycopeptides readily diffuse through the bacterial cell wall and bind to the D-ala-D-ala terminal dipeptide of the N-acetyl-muramyl-pentapeptide. This action inhibits cell-wall precursors (murein monomers) from being transported from the site of generation in the cytoplasm to the peptidoglycan cell-wall (Reynolds, 1989; Groves et al., 1994).

The rise of multi-drug resistant MRSA (MR-MRSA) has escalated the use of glycopeptides in the clinical environment and the increasing pressures of vancomycin
therapy have inevitably led to the evolution of resistance mechanisms. The guidelines used for identifying vancomycin resistance by antimicrobial minimum inhibitory concentrations (MIC) are defined by the US National Committee for Clinical Laboratory Standards (NCCLS) and are as follows: vancomycin-susceptible *S. aureus* (VSSA) produce an MIC of $\leq 4 \ \mu g/ml$, vancomycin-intermediate *S. aureus* (VISA) produce an MIC of 8 to 16 $\mu g/ml$ and vancomycin-resistant *S. aureus* (VRSA) produce an MIC of $\geq 32 \ \mu g/ml$ (Srinivasan et al., 2002). The British Society for Antimicrobial Chemotherapy however defines vancomycin-susceptible *S. aureus* (VSSA) to produce a MIC of 4 $\mu g/ml$ and vancomycin-resistant *S. aureus* (VRSA) to produce a MIC of 8 $\mu g/ml$ (Olsson-Liljequist et al., 1997; Anon, 1998). Intermediate-vancomycin resistance is now considered obsolete in the UK due to evidence that *S. aureus* strains with a MIC of 8 $\mu g/ml$ are in fact resistant to vancomycin therapy.

### 1.4.12.1 Vancomycin-intermediate *S. aureus*

Low-level vancomycin resistance is associated with the reorganisation of bacterial cell-wall metabolism expressed as an increase in bacterial cell-wall thickness with reduced peptidoglycan cross-linking and biofilm formation (Walsh and Howe, 2002; Howden et al., 2006). There are two reported mechanisms for low-level vancomycin-resistance: vancomycin-intermediate *S. aureus* (VISA) and hetero-resistant vancomycin-intermediate *S. aureus* (hVISA). Both phenotypes have become a worldwide clinical problem in vancomycin therapy failure (Fridkin et al., 2003; Charles et al., 2004; Howden et al., 2004).
Hetero-resistance is a phenomenon where variable vancomycin susceptibilities are observed among MRSA sub-populations that may potentially develop into VRSA following repeated exposure to vancomycin therapy (Tenover et al., 2001). Intermediate vancomycin resistance involves sequential mutations in a number of genes regulating peptidoglycan synthesis leading to the subsequent increase of D-ala-D-ala murcin monomers in the cell wall (Boyle-Vavra et al., 2001; Avison et al., 2002). As a result glycopeptides readily bind to additional D-ala-D-ala sites restricting glycopeptide movement through steric hindrance and inhibiting passage to the target destination in the cytoplasmic membrane (Hanaki et al., 1998; Sieradzki et al., 1999; Boyle-Vavra et al., 2001; Lambert, 2002; Lambert, 2005).

Workers in Japan were the first to report the emergence of vancomycin-intermediate S. aureus (VISA) (Hiramatsu et al., 1997b; Hiramatsu, 1998) in the clinical isolate Mu50. Since then, VISA have become a worldwide problem and have been reported in the USA (Smith et al., 1999; Boyle-Vavra et al., 2001), the UK (Howe et al., 1998; Woodford et al., 2000; Paton et al., 2001), France (Guerin et al., 2000), Belgium (Denis et al., 2002), the Netherlands (Van Griethuysen et al., 2003), South Korea (Kim et al., 2000), South Africa (Ferraz et al., 2000), Brazil (Oliveira et al., 2001b), Greece (Tsakris et al., 2002), Germany (Reipert et al., 2003), China (Lu et al., 2005a) and Australia (Howden et al., 2005).

Hetero-vancomycin-intermediate resistant S. aureus (h-VISA) was first identified in Japan (Hiramatsu et al., 1997a) in the clinical isolate Mu3. Exposure to vancomycin
selected sub-clones with resistance equal to that of Mu50 (MIC $\geq 8 \mu g/ml$) at a frequency of 1/1,000,000 (Hiramatsu et al., 1997a).

1.4.12.2 Vancomycin-resistant *S. aureus*

High-level vancomycin-resistant *S. aureus* (VRSA) was first reported in 2002 in Michigan, USA (Anon, 2002a; Weigel et al., 2003) shortly followed by reports in Pennsylvania (Anon, 2002c) and New York (Anon, 2004). All three cases were thought to originate from independent genetic events but all carried the *vanA* gene and displayed MIC $\geq 128 \mu g/ml$ (Tenover and McDonald, 2005; Fox et al., 2006). *VanA* is thought to have been acquired from vancomycin-resistant *Enterococcus faecalis* transmitted via a multi-resistance conjugative plasmid (pLw1043) within which Tn1546 (*vanA*) was incorporated (Noble et al., 1992; Woodford, 2001; Weigel et al., 2003). Reportably a sex pheromone in *S. aureus* promotes plasmid encoded transfer of *vanA* from *Enterococcus spp* further supporting the theory behind *vanA* staphylococcal acquisition of enterococcal origin (Showsh et al., 2001).

*VanA* confers resistance through the modification of the peptidoglycan synthesis pathway (figure 1.3). The D-ala-D-ala target site of vancomycin is replaced with D-alanine-D-lactate depsipeptide (D-ala-D-lac) and the removal of one essential hydrogen bond that is critical for glycopeptide binding (Gonzalez-Zorn and Courvalin, 2003). Instigation of *vanA* resistance may occur in the presence of low concentrations of
glycopeptides and can be turned on and off when needed through regulation of a two-component vanRS system (Arthur et al., 1996).

Figure 1.3 Mechanism of vancomycin resistance in *S. aureus*. Adapted from (Murray, 2000).
1.5 Community-associated MRSA

*Staphylococcus aureus* is a serious worldwide hospital pathogen, however, over the last decade MRSA clones have disseminated within the community setting following the horizontal transmission of *mecA* into MSSA strains circulating outside of the nosocomial environment. Community-acquired MRSA, was first reported in 1993 in the indigenous Australian aborigine population (Udo *et al.*, 1993). Following the deaths of four pediatric patients in Minnesota and North Dakota communities (Anon, 1999b; Anon, 1999a) community-acquired MRSA has become a worldwide public health issue (Adhikari *et al.*, 2002; Dufour *et al.*, 2002; Okuma *et al.*, 2002; Vandenesch *et al.*, 2003; Bratu *et al.*, 2006; Wijaya *et al.*, 2006).

Community-acquired MRSA (CA-MRSA) is primarily associated with virulent and invasive skin infections in previously healthy individuals with no recent hospital association. These infections may range from abscesses and furunculosis to more serious deep seated tissue infections such as necrotising fasciitis and necrotising pneumonia (Lina *et al.*, 1999a; Gillet *et al.*, 2002; Miller *et al.*, 2005). *De-novo* CA-MRSA often proves difficult to define as all hospital associated risk factors must be absent for true characterisation including recent hospitalisation, surgery, nursing home admission, antibiotic exposure and the use of indwelling medical devices (Salgado *et al.*, 2003).

Community-acquired MRSA isolates are associated with an increase in virulence and an increased antibiotic susceptibility to non β-lactam antibiotics. Following genomic
sequencing, CA-MRSA strain, MW2 was identified to harbour SCCmec IVa with no additional antibiotic resistance genes (Baba et al., 2002). Furthermore, an additional 19 virulence factors were also identified, including the locus for Panton-Valentine leukocidin (pvl), that were reported to be absent in the nosocomial associated strains N315 and Mu50. Multi-locus sequence type analysis has revealed CA-MRSA strains are likely to originate from diverse genetic backgrounds and different clonal lineages may possibly have co-evolved at different locations around the world (Okuma et al., 2002). Molecular typing studies using PFGE have demonstrated that USA 300 (MLST ST-8) harbouring pvl, SCCmec IV and resistance only to β-lactams and erythromycin, are the major cause of community-acquired MRSA infections in the USA (McDougal et al., 2003; Tenover et al., 2006). Table 1.1 summarises the current worldwide predominant CA-MRSA clones (Wijaya et al., 2006).
Table 1.1 A summary of the worldwide predominant CA-MRSA clones characterised by multi-locus sequence type (MLST), SCCmec type and pvl detection.

<table>
<thead>
<tr>
<th>Country</th>
<th>MLST type</th>
<th>SCCmec type</th>
<th>pvl</th>
</tr>
</thead>
<tbody>
<tr>
<td>UK</td>
<td>80</td>
<td>IV</td>
<td>Positive</td>
</tr>
<tr>
<td>USA</td>
<td>8</td>
<td>IV</td>
<td>Positive</td>
</tr>
<tr>
<td>Canada</td>
<td>1</td>
<td>IV</td>
<td>Positive</td>
</tr>
<tr>
<td>Brazil</td>
<td>30</td>
<td>IV</td>
<td>Positive</td>
</tr>
<tr>
<td>France</td>
<td>80</td>
<td>IV</td>
<td>Positive</td>
</tr>
<tr>
<td>Germany</td>
<td>80</td>
<td>IV</td>
<td>Positive</td>
</tr>
<tr>
<td>Belgium</td>
<td>80</td>
<td>IV</td>
<td>Positive</td>
</tr>
<tr>
<td>Greece</td>
<td>80</td>
<td>IV</td>
<td>Positive</td>
</tr>
<tr>
<td>Holland</td>
<td>80</td>
<td>IV</td>
<td>Positive</td>
</tr>
<tr>
<td>Australia</td>
<td>93/1</td>
<td>IV</td>
<td>Negative</td>
</tr>
<tr>
<td>New Zealand</td>
<td>30</td>
<td>IV</td>
<td>Positive</td>
</tr>
<tr>
<td>Taiwan</td>
<td>59</td>
<td>V</td>
<td>Positive</td>
</tr>
<tr>
<td>China</td>
<td>30</td>
<td>IV</td>
<td>Positive</td>
</tr>
</tbody>
</table>

Meticillin-resistant *Staphylococcus aureus* strains circulating in the community setting may also arise from the displacement of hospital clones, in particular EMRSA-15 (Salgado *et al.*, 2003; Coombs *et al.*, 2004; O'Brien *et al.*, 2004). In such cases disease onset may occur within the community setting however the MRSA infection will be termed community-associated or community-onset rather than *de-novo* community-acquired as previously discussed. Such strains also typically harbour SCCmec IV which obscures the opinion that SCCmec IV isolates are an indicator of true community origin (Adedeji *et al.*, 2007). The pvl locus has also been extensively used as a marker for CA-MRSA. However, following gene knock out therapy, pvl-negative USA 300 and 400 were found to be as lethal in sepsis and skin disease as the wild-type strains and subsequently the identification of pvl as a marker for CA-MRSA is now considered
unreliable (Voyich et al., 2006).

The lines between hospital-and-community MRSA are becoming increasingly blurred and determination of true community origin is further complicated due to the low prevalence of carriers with no hospital associated risk factors (Salgado et al., 2003). The identification of true de-novo CA-MRSA in the UK remains low, with the Health Protection Agency (Collindale, UK) only reporting 100 cases within three years of sampling (Anon, 2005a; Adedeji et al., 2007). Additionally, the frequency of pvl carriage in S. aureus strains has also remained low, occurring in only 1.6% of processed UK isolates (Holmes et al., 2005). Nevertheless, a reported pvl positive CA-MRSA clone in the West Midlands region (Anon, 2006a) and the recent re-emergence of a pvl-positive MSSA strain (phage type 80/81) in the UK community (Robinson et al., 2005) warrants continued surveillance for emerging virulent clones.

1.6 The genomic structure of S. aureus

The genetic plasticity of the S. aureus genome has played a major role in the evolution and worldwide dissemination of MRSA. With scientific advances and the advent of whole genomic sequencing, an evolutionary insight into the genetic diversity of the staphylococcal genome has now been provided (Kuroda et al., 2001). Complete genome sequences are currently publicly available for the following ten strains: MRSA252 (Johnson et al., 2001; Holden et al., 2004), MSSA476 (Holden et al., 2004),
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N315 (Kuroda et al., 2001), Mu50 (Kuroda et al., 2001), MW2, (Baba et al., 2002), COL, (Gill et al., 2005), NCTC8325, USA 300, RF122 and JH9 (table 1.2).

Table 1.2 Ten S. aureus strains with publicly available genome sequences - June 2007.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Origin</th>
<th>Comments</th>
<th>Genbank Accession №</th>
</tr>
</thead>
<tbody>
<tr>
<td>N315</td>
<td>Japan</td>
<td>HA-MRSA</td>
<td>BA000018</td>
</tr>
<tr>
<td>Mu50</td>
<td>Japan</td>
<td>HA-VISA</td>
<td>BA000017</td>
</tr>
<tr>
<td>MW2</td>
<td>USA</td>
<td>CA-MRSA</td>
<td>BA000033</td>
</tr>
<tr>
<td>MRSA252</td>
<td>Oxford, UK</td>
<td>HA-MRSA</td>
<td>BX571856</td>
</tr>
<tr>
<td>MSSA476</td>
<td>Oxford, UK</td>
<td>CA-MSSA</td>
<td>BX571857</td>
</tr>
<tr>
<td>COL</td>
<td>Colindale, UK</td>
<td>Progenitor MRSA</td>
<td>CP000046</td>
</tr>
<tr>
<td>NCTC8325</td>
<td>Colindale, UK</td>
<td>Lab reference strain</td>
<td>CP000253</td>
</tr>
<tr>
<td>RF122</td>
<td>USA</td>
<td>MSSA</td>
<td>AJ938182</td>
</tr>
<tr>
<td>USA 300</td>
<td>USA</td>
<td>CA-MRSA</td>
<td>CP000255</td>
</tr>
<tr>
<td>JH9</td>
<td>USA</td>
<td>HA-VISA</td>
<td>CP000703</td>
</tr>
</tbody>
</table>

Approximately 75% of the 2.8Mbp S. aureus genome is composed of conserved core genomic regions. Core genes evolve from common ancestors and are principally associated with housekeeping and metabolic functions essential for bacterial cell survival. Through comparative genomic analysis the conserved genomic regions of various strain lineages are evidently co-linear expressing 98-100% similarity at the amino acid level (Fitzgerald et al., 2001; Lindsay and Holden, 2004).

In addition to conserved genomic regions, an estimated 22% of the S. aureus genome is made up of variable genetic elements. These un-conserved domains are primarily associated with the horizontal transfer and integration of bacteriophages, chromosomal
cassettes, pathogenicity islands, transposons and plasmids that facilitate the incorporation of virulence and resistance genes not immediately essential for growth and survival (Lindsay and Holden, 2004). Of the six published MRSA genomes, all strains exhibit a diverse distribution of variable genomic islands, demonstrating the extensive exchange of mobile DNA within this species (Holden et al., 2004). MRSA 252, the predominating epidemic strain in UK hospitals (EMRSA-16), is the most divergent of the sequenced *S. aureus* strains containing up to 6% novel genetic material (Holden et al., 2004; Lindsay and Holden, 2004).

### 1.6.1 Accessory genetic elements of the *S. aureus* genome

Chromosomal cassettes are large genetic islands which transport antibiotic resistance genes into the *S. aureus* genome, of particular importance is SCCmec. Five distinct SCCmec islands (I-V) are thought to have been acquired on up to 20 separate occasions (Robinson and Enright, 2003) through horizontal transfer from coagulase-negative staphylococci. Col (SCCmec I), N315 (SCCmec II), Mu50 (SCCmec II), MRSA252 (SCCmec II), MW2 (SCCmec IV) and USA300 (SCCmec IV) all harbour SCCmec. MSSA476 contains a novel 22.8kb cassette element designated SCC476. Integration of SCC476 into the *S. aureus* genome occurs at the same integration site as SCCmec but instead of encoding meticillin resistance, a putative fusidic acid resistance protein, Far1, is transcribed (Holden et al., 2004).

Bacteriophage and phage conversion play an important role in the uptake of virulence genes into the *S. aureus* chromosome (Lindsay and Holden, 2004). Prophages are
latent bacteriophages incorporating viral genes into the host genome without a detrimental effect upon bacterial cell function. Three prophage families exist and are designated ΦSa1-ΦSa3 (Baba et al., 2004). Prophage ΦSa2mw is found in strain MW2 and harbors the pvl locus. Genomic islands that are unique to S. aureus and are not related to prophages or SCC are termed vSa islands. Two classes of v islands have been identified in all sequenced genomes of S. aureus strains and are designated vSaα and vSaβ (Kuroda et al., 2001; Baba et al., 2002). Plasmid DNA, either free or integrated may additionally provide a vector for the transfer of antibiotic resistance, heavy metal resistance, antiseptic resistance, virulence genes and super antigens into the S. aureus genome via transposons and insertion sequences (Lindsay and Holden, 2004).

1.7 Pathogenesis of S. aureus

Pathogenesis of S. aureus and host invasion is mediated by a number of virulence associated factors that govern the ability of S. aureus to bind to the extra cellular matrix in the host tissue and cause infection through invasion and circulatory dissemination (table 1.3).
Virulence determinants associated with *S. aureus* pathogenesis are separated into two main classes; surface associated factors and secreted factors. Cell surface associated factors facilitate binding to the host tissue through adherence, attachment and immune evasion. Secreted factors, on the other hand, initiate tissue degradation and subsequent invasive spread (Novick *et al.*, 1993; Frees *et al.*, 2005). The co-ordination of virulence factor expression is provided by the accessory gene regulator (*agr*) locus (Novick *et al.*, 1993; Chien and Cheung, 1998; Frees *et al.*, 2005) and a second regulatory locus called the staphylococcal accessory regulator (*sara*) (Cheung *et al.*, 1997).
1.7.1 Cell surface-associated factors and adhesins

Bacterial cell surface adhesion proteins are collectively known as microbial surface components recognising adhesive matrix molecules (MSCRAMMS). These proteins act as receptors to bind and adhere to the extra cellular matrix of host tissue cells and additionally aid in the avoidance of mediated host defenses (Kuroda et al., 2001; Clarke and Foster, 2006). Protein A (Spa) is a ubiquitous staphylococcal surface protein that binds to the Fc region of immunoglobulin G and acts to disguise \textit{S. aureus} from immune system mediated phagocytosis in the early stages of infection (Patel et al., 1987). In addition, protein A has been also shown to interact with the H-chain variable region (\(V_H\)) of human antibodies causing the elimination of B-cell subpopulations damaging the host innate immune response (Viau et al., 2005). The formation of capsular polysaccharides by the staphylococcal bacterial cell also may act to evade host immune response systems (O'Riordan and Lee, 2004). Capsular polysaccharide serotype 5 and 8 mask complement C3b inhibiting the initiation of opsonised phagocytosis (Cunnion et al., 2001; Watts et al., 2005).

Staphylococcal host invasion utilises the abundance of fibronectin and fibrinogen extracellular matrix proteins that are particularly apparent on indwelling medical devices (Francois et al., 1996). Fibronectin binding proteins (FnBP) are expressed in two forms on the bacterial cell surface, FnBPA and FnBPB (Foster and Hook, 1998). These cell surface proteins mediate the attachment of \textit{S. aureus} to fibronectin, fibrinogen, plasma clots and biomaterials such as catheters through binding to the integrin \(\alpha_5\beta_1\) cellular

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adhesion receptors of the host cell (Vaudaux et al., 1993; Foster and Hook, 1998; Sinha et al., 1999; Wann et al., 2000). Integrin–initiated internalisation of bacterial cells through the host cell endothelium occurs by host cell actin cytoskeleton rearrangement (Agerer et al., 2003) and leads to localised tissue infections of bones, organs and joints (Peacock et al., 1999b). Transmission of *S. aureus* into the host endothelium mediates alpha-toxin production and bacterial cell death but alpha-toxin deficient small colony variants may persist to allow the bacterial cell to survive and predominate in the intracellular domains (von Eiff et al., 1997).

Fibrinogen is an abundant protein in endothelial lesions. There are two fibrinogen binding clumping factors (Clf) in *S. aureus*, ClfA and ClfB. Clumping factors cause bacterial clumping in the host plasma and the adherence to fibrinogen coated surfaces such a catheters and indwelling medical devices (Cheung and Fischetti, 1990). Clumping factor A is also thought to mediate binding of bacterial cells to platelets, inhibiting platelet activated release of antimicrobial peptides (Siboo et al., 2001).

Collagen binding proteins (Cna) facilitate the adherence of the bacterial cell to collagen and cartilage in the host tissue. Cna is only expressed in up to 56% of *S. aureus* strains (Foster and Hook, 1998) but is associated with many staphylococcal infections including osteomyelitis (Elasri et al., 2002), endocarditis (Hienz et al., 1996), keratitis (Rhem et al., 2000) and arthritis (Patti et al., 1994).
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The intercellular adhesion locus (ica) encodes an adhesion molecule that plays a major role in biofilm formation (Cramton et al., 1999). The ica gene locus (icaADBC) mediates cell-cell adhesion and the production of enzymes required for the synthesis of polysaccharide intercellular adhesin (PIA) which plays a pivotal role in biofilm formation (Cramton et al., 1999). Infections from implants are difficult to treat as once a biofilm has formed, the cells on the bottom layer are protected from the host immune response and antimicrobial agents by an exopolysaccharide glycocalyx encasement (Gander, 1996; Beenken et al., 2004). However, it should be noted that there are conflicting views on the role of ica in biofilm formation due to the report of icaADBC-independent biofilm formation in MRSA clinical isolates (Fitzpatrick et al., 2005).

The serine-aspartate repeat-containing (Sdr) surface proteins are encoded by sdrC, sdrD, and sdrE genes of the sdr locus (Josefsson et al., 1998). At least two Sdr proteins are encoded in all S. aureus isolates with the presence of sdrC being constant (Josefsson et al., 1998; Peacock et al., 2002; Sabat et al., 2006). All three Sdr proteins have variable functions in S. aureus pathogenicity in particular SdrD has been associated with bone infections (Trad et al., 2004) and SdrE has been associated with invasiveness (Peacock et al., 2002).

1.7.2 Exoenzymes

Exoenzymes are secreted by S. aureus to enable tissue degradation and subsequent host invasion (Kuroda et al., 2001). Coagulase is an extra-cellular enzyme responsible for
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the coagulation of plasma and binds to prothrombin to form a complex which converts fibrinogen to fibrin (Panizzi et al., 2006). Coagulation of plasma may protect staphylococcal cells from host immune cells although this role in pathogenesis has not been fully explored.

*Staphylococcus aureus* produce a number of extra cellular proteases including metalloprotease and serine proteases. These enzymes have a number of functions, in particular degradation of damaged proteins produced under environmental stress, degradation of host proteins, degradation of staphylococcal adhesions for dis-attachment and degradation of antibacterial proteins to evade destruction (Shaw et al., 2004). Proteases also function to degrade staphylococcal toxins such as haemolysins for the down-regulation of virulence in bacterial nasal and skin colonisation (Lindsay and Foster, 1999). The serine protease V8 is the most explored of these enzymes (Karlsson et al., 2001; Rice et al., 2001) which functions to cleave FnBPs and protein A from their attachment sites on the host cell enabling bacterial dissemination (McGavin et al., 1997; Lowy, 1998; Karlsson et al., 2001). The metalloproteinase, aureolysin, functions in a similar way by cleaving clumping factor ClfB (McAleese et al., 2001).

Lipases produced by *S. aureus* enable bacteria to persist in the fatty secretions of the host skin (Rosenstein and Gotz, 2000) and interfere with human granulocyte function protecting *S. aureus* from phagocytosis (Rollof et al., 1988). Lipases also degrade bactericidal fatty acids produced during infection and dissemination.
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Staphylokinase interacts with plasminogen to form a complex that binds to fibrin, a constituent that abundantly surrounds infected or damaged tissue. Staphylokinase cleaves fibrin to facilitate deep tissue invasion and binds to bactericidal α-defensins inhibiting bacterial cell phagocytosis (Bokarewa et al., 2006).

1.7.3 Exotoxins

Pyrogenic exotoxins secreted by *S. aureus* function to damage and destroy host cells and tissues. Three main categories exist; enterotoxins, cytolytic toxins and exfoliative toxins.

1.7.3.1 Enterotoxins

Enterotoxins (SEA-SER) are the main agents of staphylococcal derived food poisoning and belong to a family of proteins called super antigens (Sergeev et al., 2004). Super antigens bind to the major histocompatibility complex class II proteins on antigen presenting cells and T-cell receptor molecules. This trimolecular interaction leads to a massive proliferation of T-cells and an over expressive release of inflammatory cytokines and interleukins that result in toxic shock syndrome (TSS) and death (Miethke et al., 1993; Kum et al., 2001). Toxic-shock syndrome toxin-1 (TSST-1) is the main cause of TSS (Schlievert et al., 2000; Kuroda et al., 2001). This super antigen is encoded by the *tst* gene found on the SaPIII pathogenic island (Lindsay et al., 1998; Kuroda et al., 2001).
1.7.3.2 Cytolytic toxins

Staphylococcal $\gamma$-haemolysin (Hlg), leukocidin (Luk), and Panton–Valentine leukocidin (PVL) are two-component membrane pore-forming toxins with cytolytic activity. Staphylococcal Hlg lyses erythrocytes, Luk (LukF/LukS) is cytolytic to polymorphonuclear leukocytes and PVL (LukF_PV and LukS-PV) is cytolytic to leukocytes through pore formation (Kaneko and Kamio, 2004). The Hlg locus transcribes LukF, LukS, and Hlg proteins that are present 99% of *Staphylococcus aureus* isolates. The PVL locus transcribes LukF-PV and LukS-PV proteins that are present in approximately 1.6% of the isolates processed here in the UK (Holmes *et al.*, 2005). The *pvl* locus is integrated into *S. aureus* by a PVL-phage (Zou *et al.*, 2000; Narita *et al.*, 2001) and is associated with virulent CA-MRSA causing severe staphylococcal infections and necrotising pneumonia (Lina *et al.*, 1999a; Labandeira-Rey *et al.*, 2007).

1.7.3.3 Exfoliative toxins

Exfoliative toxins are the predominating cause of *S. aureus* derived scalded-skin syndrome and impetigo. These toxins function to cleave the desmosomal cadherin protein that mediates cell-cell adhesion of keratinocytes in the epidermal granular layer (Amagai *et al.*, 2000). There are three types of exfoliative toxin; ETA and ETB are associated with scalded-skin syndrome (Yamasaki *et al.*, 2005) and ETD is associated with cutaneous abscesses and furuncles (Yamasaki *et al.*, 2006).
1.8 Colonisation and Infection

The abundance of glucoproteins, proteoglycans and mucin coated cells make the anterior nares a primary site for *S. aureus* colonisation. Persistent or intermittently colonised patients have an increased risk of developing *S. aureus* infection (von Eiff *et al.*, 2001) particularly susceptible patient groups such those with HIV (Tumbarello *et al.*, 2002), those undergoing surgery (Graffunder and Venezia, 2002), intravenous drug users (Bassetti and Battegay, 2004) and patients with indwelling devises (Fowler *et al.*, 2005). *Staphylococcus aureus* can cause a wide range of manifestations and toxinoses ranging from superficial skin infections to more deep seated tissue infections such as endocarditis and osteomyelitis following bacteraemic infection and sepsis.

1.8.1 Impetigo

Impetigo manifests on the upper layers on the epidermis. There are two forms of *S. aureus* derived impetigo, non-bullous and bullous (Nichols and Florman, 2001). Non-bullous is associated with a yellow- brown exudate that forms a golden coloured crust on the hands, feet and legs. Bullous impetigo is associated with a thin walled vesicle (papule) and is found on the warm areas of the body. Topical mupirocin treatment will be effective against mild cases but systemic complications such as cellulitis, septicemia, staphylococcal scalded-skin syndrome may occur (Nichols and Florman, 2001).
1.8.2 Folliculitis

*Staphylococcus aureus* can cause infections of the hair follicle and sub-epidermal regions of the skin. The most common of these infections is folliculitis, resulting in a furuncle, carbuncle or boil at the hair root (Nichols and Florman, 2001). Recurrent infection is often associated with defects in cell-mediated immunity (Forte et al., 2000; Gilad et al., 2006). Antibiotic treatment is often ineffective due to the closed nature of the boil and surgical drainage and oral administration of antibiotics is therefore the most effective measure (Nichols and Florman, 2001).

1.8.3 Cellulitis

Cellulitis is an acute infection of the skin developing into lymphangitis and in severe cases bacteraemia or necrotising fasciitis. Cellulitis commonly occurs in patients with skin trauma and immunosuppression (Nichols and Florman, 2001).

1.8.4 Staphylococcal scalded-skin syndrome

Staphylococcal scalded-skin syndrome (SSSS) is particularly associated with children and manifests as painful blistering on the skin. The condition is caused by epidermolytic toxins (exfoliating toxin A and B) with serine protease activity (Ladhani et al., 1999). Scalded-skin syndrome can range from localised blisters (bullous impetigo) to severe blistering covering 90% of the body.
1.8.5 Bacteraemia and sepsis

Bacteraemia occurs when an infected site inoculates and disseminates into the host bloodstream leading to infections in vulnerable anatomical sites such as bones, kidneys, joints, lungs and the heart (Yamashita et al., 2001; Gillet et al., 2002; Kao et al., 2003; Yuan et al., 2006). In the UK rates of bacteraemia have continued to rise with an approximate mortality of 30% following 30 days after diagnosis (Fatkenheuer et al., 2004; Wyllie et al., 2006). The proportion of MRSA bacteraemias have risen from 2% to greater than 40% between 1990 and 2000 with EMRSA-15 and EMRSA-16 strains being the most predominant cause of infection (Johnson et al., 2001; Melzer et al., 2003; Anon, 2005b; Johnson et al., 2005). Bacteraemia as a result of MRSA is significantly associated with an increase in mortality compared with MSSA-derived bacteraemia (Cosgrove et al., 2003), but this increase in mortality is most likely to be due to failed antibiotic therapy rather than increased virulence (Melzer et al., 2003).

Common sources of *S. aureus* bacteraemia are intra-vascular device infections, soft tissue infections and surgical infections (Carnicer-Pont et al., 2006; Jeyaratnam et al., 2006; Das et al., 2007). Patients in intensive care units are at a particular high risk, as are the elderly, the immuno-compromised and those with serious cardiac, neurological or respiratory disease (Lowy, 1998; Thompson, 2004; Jeyaratnam et al., 2006).
1.8.6 Endocarditis

*Staphylococcus aureus* is a leading cause of endocarditis, a condition characterised by a bacterial infection of the heart endothelial surface (Cabell *et al*., 2002; Nadji *et al*., 2005). *Staphylococcus aureus* infective endocarditis has a high morbidity rate (Nadji *et al*., 2005) and is usually co-associated with intravenous catheters, haemodialysis, malignancy, immuno-suppression, intravenous drug users and the elderly (Marr *et al*., 1998; Cabell *et al*., 2002; Nadji *et al*., 2005; Ruotsalainen *et al*., 2006). *Staphylococcus aureus* is the predominant cause of prosthetic valve endocarditis and a serious threat in valve replacement surgery (Wolff *et al*., 1995). Right-sided infective endocarditis is associated with drug use and has a more favourable prognosis however left-sided infective endocarditis has a less favourable outcome and is associated with high morbidity (Miro *et al*., 2005).

1.9 Characterisation of *S. aureus*

Phenotypic and genotypic analytical techniques are employed for the typing of *S. aureus* strains to aid in infection control and the analysis of bacterial populations in epidemiological investigations.

1.9.1 Phenotypic characterisation

Phenotypic techniques analyse the products of gene expression. These methods have a limited discriminatory capacity but nevertheless provide useful, fast and inexpensive
information for epidemiological investigation and infection control.

1.9.1.1 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing (AST) determines the sensitivity of *S. aureus* to a panel of antibiotics and is used to determine therapeutic treatment for *S. aureus* derived infection and colonisation. Clonally unrelated strains of *S. aureus* can be identified by AST, highlighting the varying antibiotic resistance phenotypes within *S. aureus* populations. The rapid and inexpensive qualities of AST make it a common procedure in clinical laboratories; however antibiotic resistance expression can be influenced by the surrounding environment and loss and gain of transposable elements may vary in clonally related strains (Weller, 2000). This method provides poor discriminatory capacity when compared with molecular techniques but remains an invaluable tool for clinical infection control.

1.9.1.2 Biotyping

Biotyping analyses the biological, morphological and environmental tolerance responses of a micro-organism to a panel of biochemical tests. Micro-organisms variably alter the expression of cellular products and processes and biotyping is therefore seen to yield too many subgroups within outbreak clusters to be useful as an epidemiological tool (Tenover et al., 1994).
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1.9.1.3 Phage typing

Phage typing has been used since the 1970s to characterise *S. aureus* strains by differential susceptibilities to bacteriophage infection. Twenty three standard phages are recommended by the International Sub-committee on Phage Typing of Staphylococci. Each standard phage is applied to an agar plate containing the test organism and the subsequent phage lysis pattern is observed. Phage typing is time-consuming, technical and not highly reproducible. With up to 30% of MRSA being non-typeable by this method, phage typing has now been replaced by molecular typing techniques (Tenover *et al.*, 1994; Bannerman *et al.*, 1995; Weller, 2000).

1.9.1.4 Serotyping

Serotyping identifies *S. aureus* strain variation through coagulase antigenic property testing and capsular polysaccharide identification. There are 11 capsular polysaccharide types displayed in divergent *S. aureus* strains but up to 90% of MRSA belong to just two of these (Weller, 2000); types 5 and 8 (Schlichting *et al.*, 1993). Serotyping is deemed to have a low discriminatory capacity and is therefore not employed in MRSA epidemiological studies.
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1.9.1.5 Protein Electrophoresis

Protein electrophoresis analyses cellular proteins and includes the following methods: whole cell protein electrophoresis; immunoblotting; multi locus enzyme electrophoresis and zymotyping (Weller, 2000). These methods are labour intensive and are not widely used in *S. aureus* characterisation.

1.9.2 Genotypic methods

Advances in molecular biology have allowed the development and application of genotypic techniques to utilise the structure of the *S. aureus* genome for epidemiological studies and evolutionary genetics. A variety of methods are currently employed.

1.9.2.1 Plasmid analysis

Analysis of plasmid DNA was the first genotypic method to be applied to *S. aureus* epidemiological studies (McGowan *et al.*, 1979). Plasmid analysis differentiates strains by the number and size of harboured plasmids which are visualised through gel-electrophoresis separation (Weller, 2000). This method is simple to perform and interpret but due to their mobility, the stability of staphylococcal plasmids are debated (Tenover *et al.*, 1994).
1.9.2.2 Southern hybridisation and ribotyping

Chromosomal DNA analysis requires enzymatic cleavage of the whole chromosome by restriction digestion. A common enzyme used in restriction digests is EcoRI which recognises and cleaves specific sequences leading to the fragmentation of the staphylococcal chromosome. Ribotyping uses ribosomal RNA (rRNA) probes that are designed and radioactively labeled to target and bind to multiple rRNA transcriptional sites on the staphylococcal chromosome. Following restriction digestion and electrophoresis, DNA fragments are transferred to a nylon membrane and labeled rRNA is used as a probe to bind to conserved rRNA transcriptional sequences. A typical S. aureus isolate may harbour between five and seven ribosomal operons that vary in sequence and restriction enzyme site position and differing hybridisation sites will consequently be visualised in unrelated strains. This method is reproducible and more discriminatory than phenotypic methods but in comparison to other DNA analysis techniques it has not been widely used in S. aureus genotyping due to its time consuming, complex and expensive protocol (Tenover et al., 1994; Weller, 2000).

1.9.2.3 Pulsed-Field Gel Electrophoresis

Pulse-Field Gel Electrophoresis (PFGE) is considered the 'gold standard' technique for genotyping MRSA strains. Bacterial cells are embedded in an agarose plug and lysed using lysozyme, lysostaphin and proteinase-K enzymes. The chromosome is then further digested using an infrequent cutting restriction endonuclease such as Smal,
generating large chromosomal fragments between 50-700kb. Agarose slices are then inserted into an agarose gel and the fragments are resolved by electrophoresis. Contour clamped homogenous electrophoresis (CHEF) uses six electrodes in a hexagonal pattern with a three directional current 120° apart. The current is applied in each of the three directions for short pulses of time causing a continual shift in the direction and orientation of the migration of the DNA fragments. Banding patterns from the chromosomal digest can then be visualised and compared by computer aided analysis (Tenover et al., 1995). A harmonised protocol was developed to enable reproducibility across the EU to allow successful tracking of European MRSA strains (Murchan et al., 2003). Pulse-Field Gel Electrophoresis is therefore highly reproducible with a high discriminatory capacity but it is also expensive and time consuming taking up to six days to generate a single profile.

1.9.2.4 SCCmec element assignment and multiplex PCR

The polymerase chain reaction (PCR) is used for the exponential enzymatic amplification and detection of target DNA. Application requires the following essential elements:

- Template DNA encoding the target gene sequence
- Complementary primer pairs to the 5' and 3' ends of the target sequence
- A thermostable polymerase enzyme for synthesis of target DNA
- dNTPs to provide the building blocks for DNA synthesis
- A buffer containing magnesium and potassium ions
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The polymerase chain reaction incorporates up to 30 cycles, including a DNA denaturation step at 94°C, a primer annealing step at 53°C and DNA extension/synthesis step at 72°C. Amplified fragments are visualised by gel electrophoresis and ethidium bromide staining (Dieffenbach et al., 1993; Baumforth et al., 1999).

The polymerase chain reaction (PCR) can been used to genetically distinguish MRSA strains through characterisation of SCCmec. Two techniques are predominantly employed; amplification of heterogeneous cer and mec complex genes (Ito et al., 2001; Okuma et al., 2002) and amplification of structural features of SCCmec (Oliveira and de Lencastre, 2002). The latter incorporates a multiplex PCR which encompasses 17 primers in a single tube reaction to distinguish between SCCmec I, II, III, and IV (Oliveira and de Lencastre, 2002). This typing method is limited in its discriminatory capacity and ability to detect recently discovered SCCmec types and subtypes such as SCCmec type V. However, when combined with PFGE or MLST it can add valuable epidemiological and evolutionary information regarding SCCmec lineage. Increasing reports of SCCmec diversity (Shore et al., 2005; Oliveira et al., 2006) has led to various proposals for new typing schemes and classification for SCCmec element identification (Zhang et al., 2005; Chongtrakool et al., 2006; Milheirico et al., 2007).

1.9.2.5 Random amplification of polymorphic DNA

Random amplification of polymorphic DNA (RAPD), also known as arbitrarily primed-PCR (AP-PCR), is a molecular genotyping technique that can obtain analytical results in a matter of hours. This method incorporates the polymerase chain reaction (PCR)
Chapter 1 Introduction

and has been successful in genotyping Gram-negative (Hilton et al., 1997) and Gram-positive bacteria (Grundmann et al., 2002a). Random amplification of polymorphic DNA uses a short primer (approximately 10bp) with no particular specificity to any designated sequence. The first round of PCR is carried out under low annealing temperatures to allow the primers to bind to not entirely complementary sequences. Subsequent rounds of high-stringency annealment then only allows binding to complementary sequences and the amplification of template products from the first round of cycling. This combination of low- and high-stringency annealment of the primer selects polymorphic differences between varying strains. This method is fast and cost effective and the discriminatory capacity can be increased by using two or more primers. The criticised lack of reproducibility of this method can be addressed if stringent optimisation of the protocol is carried out (Hilton et al., 1997).

1.9.2.6 Spa Typing

The spa gene encodes the S. aureus cell-wall constituent Protein-A. Spa typing uses PCR to amplify the X region of spa, a hypervariable region consisting of direct repeats with polymorphic domains generated from point mutations, deletions and insertions (Frenay et al., 1996; Strommenger et al., 2006). DNA sequencing determines the number and sequences of these repeat regions and an alpha-numerical code is allocated to specify spa type. Spa typing is a rapid a reproducible technique that is comparable with other molecular typing methods (Shopsin et al., 1999; Strommenger et al., 2006).
1.9.2.7 Multi-Locus Sequence Typing

Multi-locus sequence typing (MLST) is a technique used to study evolutionary genetics of *S. aureus* strain lineages incorporating ~450-bp internal fragments of seven chosen housekeeping genes (Maiden *et al.*, 1998). Each gene fragment is allocated an allelic profile or sequence type (ST) and isolates with the same ST are deemed of the same clone (Enright *et al.*, 2000). Multi-locus sequence typing can be used across laboratories and results can be compared over the internet (Spratt, 1999). This technique is highly discriminatory but technically demanding, expensive and not suited to typing nosocomial outbreaks or routine infection control (Robinson and Enright, 2003).

1.9.2.8 Variations in Staphylococcal Interspersed Repeat Units

Seven different tandem repeat sequences identified in the *S. aureus* genome are utilised in staphylococcal interspersed repeat unit typing (Hardy *et al.*, 2004). All variable tandem repeat sequences are unique in length, DNA sequence, copy number and organisation within a given *S. aureus* genome (Hardy *et al.*, 2004; Hardy *et al.*, 2006b) Determination of numbers of repeats at various locus points subsequently produces a digital profile that may be used and compared between laboratories. Staphylococcal interspersed repeat unit typing has recently been utilised to discriminate between epidemic meticillin-resistant *Staphylococcus aureus* strains within the UK (Hardy *et al.*, 2004; Hardy *et al.*, 2006b) This typing method is a rapid transportable technique that
may be used for the identification of transmission events within *S. aureus* populations (Hardy *et al.*, 2006b).

### 1.10 Geographical Information Systems

The application of disease mapping in public health analysis was evidently brought to the world’s attention by John Snow following the identification of the Broad Street water pump as the source of the London cholera outbreak in 1854. Today geographical information systems (GIS) offer an array of analytical and statistical programs to compute and determine the significance of disease clustering against underlying environmental and demographic variables of chosen geographical settings (Vine *et al.*, 1997; Camara and Monteiro, 2001; Cassa *et al.*, 2005; Aamodt *et al.*, 2006; Kulldorff, 2006). Since the early 1990s, the World Health Organisation has continued to promote and utilise GIS for the monitoring of public health issues and the implementation of targeted disease control strategies. The space- scan statistic, designed and implemented by Kulldorff in the early 1990s (Kulldorff, 1997), may be used to map and identify significant spatial and temporal disease clustering against the underlying population density of a target study region (Kulldorff, 1997). Kulldorff’s space-scan scan statistic has been applied to a range of disease surveillance investigations, some of which include: tuberculosis (Onozuka and Hagihara, 2007), malaria (Gaudart *et al.*, 2006), cancer (Kulldorff *et al.*, 1998), Creutzfeldt-Jakob disease (Cousens *et al.*, 2001) and meningitis (Elias *et al.*, 2006). Geographical Information Systems may play an important role in future national and international epidemiological investigations.
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1.11 Aims and objectives

Meticillin-resistant *Staphylococcus aureus* is a major cause of nosocomial and community morbidity and mortality exasperated by the emergence of multi-drug resistance and virulent CA-MRSA clones. Regardless of a devised search and destroy infection control strategy in the UK the rates of MRSA-associated disease have remained high. The recent report of *pvl*-positive CA-MRSA in the West Midlands and additional transmission into the hospital setting warrants continued surveillance for emerging virulent clones within the clinical and community population (Anon, 2006a). Staphylococcal infections are commonly of endogenous origin (von Eiff *et al.*, 2001) however, increased incidence is largely associated with immuno-compromised patient groups such as those on haemodialysis (Kirmani *et al.*, 1978; Yu *et al.*, 1986; Kluytmans *et al.*, 1997; Koziol-Montewka *et al.*, 2001). Establishment of MRSA carrier status and eradication of all potential anatomical MRSA reservoirs is therefore critical for effective infection control. The hospital environment also provides a niche reservoir for MRSA (Asoh *et al.*, 2005; Sexton *et al.*, 2006) and reducing nosocomial MRSA transmission may also rely upon hospital hygiene standards (Rampling *et al.*, 2001). With increasing evidence for the dissemination of hospital epidemic strains into the community populations (Salgado *et al.*, 2003; Adediji *et al.*, 2007), the lines defining hospital- and community- MRSA acquisition are increasingly losing clarity (Salgado *et al.*, 2003). Application of geographical information systems may provide a valuable tool for future monitoring of MRSA incidence, offering an insight into the
distribution of MRSA within the West Midlands population where the extent of the community as a reservoir for transmission remains largely unknown.

This thesis has sought to investigate the epidemiology of hospital and community MRSA within the West Midlands region.

The aims of this study were to:

- Determine and characterise the anatomical carriage of MRSA and MSSA in renal dialysis outpatients and the magnitude of environmental MRSA contamination within the study setting.

- Characterise by PFGE, MRSA in the anterior nares and tongues of haemodialysis patients and determine the clinical implications of co-colonisation.

- Phenotypically and genotypically characterise MRSA strains circulating within the Lichfield, Tamworth, Burntwood, North and East Birmingham community.

- Determine the phenotypic and genotypic characteristics of hospital-associated MRSA obtained from nosocomial-onset and community-onset infection in an attempt to define the driving factors of community dissemination.

- Assess the use of SaTScan for the identification of MRSA clustering within the Lichfield, Tamworth, Burntwood, North Birmingham and East Birmingham community setting.

- Incorporate primer gene targets for fnbA, cna, sdrE, sej, eta, hlg and icaA into a multiplex PCR protocol to provide an efficient, cost effective method for virulence gene detection in clinical MRSA isolates.
CHAPTER 2 BACTERIAL STRAINS

Within this thesis an isolate is defined as a single colony derived from a population of bacterial cells grown in pure culture identified to the species level. Alternatively a strain is identified as a group of isolates exhibiting phenotypic or genotypic traits that belong to the same genetic lineage, distinct from other isolates of the same species.

2.1 Common strains used within this thesis

*Staphylococcus aureus* strains from the National Collection of Type Cultures (NCTC) were used where stated.

*Staphylococcus aureus* NCTC 8325 strain was included as a molecular weight control for PFGE analysis.

Meticillin-resistant *Staphylococcus aureus*: COL, PER34, N315, ANS46, HU25 and MW2 were included as controls for SCCmec type element assignment (Oliveira *et al.*, 2001a; Oliveira and de Lencastre, 2002). The aforementioned strains were kindly supplied by Hermínia de Lencastre of the Rockefeller University, New York, 10021. Additionally MW2 was also included as a control strain for PVL gene detection (Lina *et al.*, 1999a).
2.2 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 agar (BHI) under aerobic conditions for 16 hours at 37°C unless otherwise stated.
CHAPTER 3 THE EPIDEMIOLOGY OF MRSA IN A
RENAL DIALYSIS SETTING

3.1 Introduction

Renal dialysis units encompass a dynamic outpatient environment with a varying number of patients dialysing up to three times weekly. *Staphylococcus aureus* plays a leading role in infection and morbidity within the renal dialysis setting (Koziol-Montewka et al., 2001) where patients receiving haemodialysis have an increased risk of acquiring entry site infections and access-related bacteraemia (Peacock et al., 1999a; Lentino et al., 2000; Koziol-Montewka et al., 2001; Dopirak et al., 2002; Troidle et al., 2007). In the healthy adult population the nasal carriage rate of *S. aureus* is approximately 27% (Wertheim et al., 2005a) and MRSA <2% (Shopsin et al., 2000; Kenner et al., 2003), however, the incidence is reported to increase in immunocompromised patient groups (Kirmani et al., 1978; Yu et al., 1986; Kluytmans et al., 1997; Koziol-Montewka et al., 2001). Staphylococcal infections are commonly of endogenous origin (von Eiff et al., 2001) and elimination of nasal carriage may contribute towards reducing cross-contamination to catheter entry sites and subsequent infection (Yu et al., 1986; Ena et al., 1994; Zimakoff et al., 1996). Establishment of MRSA carrier state of patients in the renal dialysis setting and the implementation of appropriate antimicrobial therapy is therefore critical for infection control.
The hospital environment also provides a niche reservoir for MRSA transmission through the colonisation of medical equipment and porous surfaces that are difficult to disinfect (Asoh et al., 2005; Sexton et al., 2006). A key strategy in reducing nosocomial MRSA transmission therefore relies heavily upon maintenance of hospital hygiene standards along with effective hand washing protocols, isolation of colonised patients and patient education (Rampling et al., 2001).

The aim of this study was to determine anatomical carriage of MRSA and MSSA in renal dialysis outpatients and the magnitude of environmental MRSA contamination within the study setting.
3.2 Materials and methods

3.2.1 Bacterial isolation

This study was undertaken over a 4 week period in the Renal Dialysis Unit (RDU), University Hospital Birmingham (UHB) NHS Foundation Trust UK, which provides 29 dialysis stations for outpatient care. Patients were recruited into the study following informed consent and ethical approval granted from the South Birmingham Ethics Committee.

A single nasal swab from the left and right anterior nares was taken from 81 patients undergoing long term haemodialysis. In addition, from 14 patients, a central venous catheter (CVC) entry site sample was also obtained. All samples were isolated on mannitol salt agar plates (Oxoid Ltd, UK) and mannitol salt agar plates containing oxacillin (4mg/l) (Oxoid Ltd, UK) and incubated aerobically for 48 hours at 37°C. Presumptive MSSA and MRSA colonies were further identified by standard laboratory techniques including coagulase, DNAase and Gram-stain analysis. Antibiotic resistance phenotypes for all MRSA isolates were determined by Vitek in the microbiology laboratory at the UHB (bioMerieux, Inc, France).
3.2.2 Pulsed-field gel electrophoresis analysis of MRSA

Preparation of cell blocks and subsequent lysis of staphylococcal cells was performed using previously published protocols (Lang et al., 1999; Caddick et al., 2005). Meticillin-resistant Staphylococcus aureus colonies were grown overnight on Brain Heart Infusion Agar (Oxoid Ltd, Basingstoke) for 16-24 hours at 37°C. Harvested cells taken from several colonies were re-suspended in 0.5ml sterile distilled water (SDW), centrifuged at 5,500g for 4 mins and the wet weight of the cell pellet was determined. The pellet was re-suspended in NET-100 (10mM Tris-HCl, pH 8, 100Mm EDTA, pH 8, and 100mM NaCl) to provide a final cell suspension of 20mg/ml. An aliquot of 0.5ml of cell suspension was added to 0.5ml chromosomal grade agarose (0.9%) (Biorad Laboratories Ltd, UK) at 50°C, vortexed, loaded into block wells (Biorad Laboratories Ltd, UK) and allowed to solidify at 4°C. The resulting agarose blocks were incubated at 37°C for 24 hours in 3ml lysis solution (6mM Tris pH 7.6, 100mM EDTA pH 8, 100mM NaCl, 1mg/ml lysozyme and 0.5% lauroyl sarcosine) and 20 units of lysostaphin. Following incubation the agarose blocks were re-suspended in 3ml ESP (0.5M EDTA pH 9, 1.5mg/ml proteinase K and 1% lauroyl sarcosine) then incubated at 50°C for 48 hours. After incubation the blocks were washed twice for two hours and twice for one hour at room temperature in TE buffer solution (10Mm Tris, pH 8 and 1M EDTA, pH8). The blocks were then ready to be stored at 4°C until required. Approximately 2mm portions were cut from the blocks and digested at 25°C for 18 hours in 20 units of SmaI, 0.1ml of supplied enzyme buffer (Roche Diagnostics Ltd, Lewes, UK) and 90μl of SDW.
Pulsed-Field Gel Electrophoresis grade agarose (Biorad Laboratories Ltd, UK) was prepared at 1% using 0.5x TBE Buffer (44.5 mM Tris, 44.5 mM Boric Acid and 1 mM EDTA, pH 8). The digested DNA samples were loaded into the prepared agarose gel with two wells of all gels containing the digest of *S. aureus* NCTC 8325 as the molecular weight marker strain (Tenover *et al.*, 1995; Murchan *et al.*, 2003). Each well was sealed with 0.5% agarose (PFGE certified Biorad Laboratories Ltd, UK). Electrophoresis was carried out in 0.5x TBE buffer (44.5 mM Tris, 44.5 mM Boric Acid and 1 mM EDTA, pH 8) by contour clamped homogenous electric field method with CHEF Mapper System (Biorad Laboratories Ltd, UK). The following parameters were applied; final pulse 40 seconds; voltage 200V or 6V/cm; time 20 hours; and temperature 12°C (Bannerman *et al.*, 1995). Following electrophoresis gels were stained for 30-45 minutes in 1μg/ml ethidium bromide and de-stained for 45 minutes in SDW. Gels were visualised under UV-illumination, photographed using Genesnap (Syngene, Synoptics, Cambridge, UK) and PFGE profiles visually compared using Gel Compar (Applied Maths, Belgium). Banding pattern similarities between isolates were calculated by the Dice co-efficient (tolerance of 1%) and represented by un-weighted pair groups using mathematical average (UPGMA) dendrogram. Letters were assigned to PFGE profiles according to dendrogram grouping (A-F).

### 3.2.3 Rapid DNA extraction by boiling

For all PCR protocols employed, chromosomal DNA was extracted by a rapid boiling extraction method (Caddick *et al.*, 2005). Isolates were retrieved from BHI agar plates
and re-suspended in 1 ml TESS buffer (50mM Tris-HCl, 5mM EDTA, 50mM NaCl and 50mM sucrose, pH 8) and centrifuged for 4 mins at 5500 x g. The supernatant was then discarded and the pellet was re-suspended in 1ml SDW and washed twice. Following re-suspension in 1ml SDW, the cells were transferred to a 1ml sterile cuvette where the A₆₀₀ of the cell suspension was adjusted to 1.7 OD with SDW. A 0.1ml sample was aliquotted into 0.2 ml microamp tubes (Applied Biosystems, Warrington UK) and boiled at 94°C for 12mins using a heating block. The samples were cooled to 4°C and centrifuged for 4mins at 1200 x g. The resulting supernatant containing extracted template DNA was cooled to 4°C to be used in further PCR reactions.

3.2.4 Multiplex PCR for SCCmec element assignment

Staphylococcal Cassette Chromosome mec (SCCmec) element assignment was determined for all MRSA isolates using primers and cycle conditions obtained from previously described methods (Oliveira and de Lencastre, 2002). Multiplex PCR was performed using Gene Amp PCR system 9700 (Applied Biosystems, UK) in a 25μl reaction volume comprising of 17.7μl SDW, 2.5μl of 10x primer mix (table 3.1), 0.2μl of 25mM dNTPs (Promega, UK), 0.1μl of 1.25 units/μl Taq DNA polymerase (Promega, UK) and 2μl template DNA (section 3.2.3). SCCmec band pattern validation was carried out using the positive control strains COL, PER34, N315, ANS46, HU25 and MW2 representing SCCmec element types I, Ia, II, III, IIIa and IV respectively. A 2% agarose gel containing 1μg/ml of ethidium bromide was used to separate amplified
fragments. Electrophoresis was performed in 1x TAE (40mM Tris, 1mM EDTA and 0.1% (v/v) glacial acetic acid) buffer at 100 volts for 1 hour.

Table 3.1 Primers and stock concentrations used in SCCmec multiplex PCR (Oliveira and de Lencastre, 2002).
3.2.5 Random amplification of polymorphic DNA

3.2.5.1 Primers

The primers used in this study for RAPD amplification are from previously published protocols; primer LAM 797 and primer SL 1254 (table 3.2).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAM 797</td>
<td>AGC GTC ACT G</td>
<td>(Pereira et al., 2002)</td>
</tr>
<tr>
<td>SL 1254</td>
<td>CCG CAG CCA A</td>
<td>(Akopyanz et al., 1992)</td>
</tr>
</tbody>
</table>

3.2.5.2 RAPD reaction

Sample DNA was obtained using the previously described DNA rapid boil extraction method (section 3.2.3). Each RAPD PCR reaction was carried out in a 25μl volume containing 19.15μl of SDW, 2.5μl of 10x PCR buffer 3 (10mM Tris HCl pH 8.3, 3.5mM MgCl₂, 25mM KCl), 0.6μl of 100μM primer (table 3.2), 0.5μl of 10mM dNTPs (Promega, UK), 0.25μl of 1.25units/μl Taq DNA polymerase (Promega UK) and 2μl of template DNA. Amplification was carried out on Gene Amp PCR system 9700 (Applied Biosystems, UK) using the following parameters: Four and a half minutes 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. The amplified reaction products were then stored with 5μl of 6x DNA loading buffer at 4°C until
required. A 2% agarose gel containing 1μg/ml of ethidium bromide was used to separate amplified fragments. Electrophoresis was performed in 1x TAE (40mM Tris, 1mM EDTA and 0.1% (v/v) glacial acetic acid) buffer at 100 volts for 1 hour.

3.2.6 *Pvl* gene locus detection

Panton Valentine Leukocidin gene locus detection was determined with primers (table 3.3) and cycle conditions obtained from a previously described method (Lina *et al.*, 1999a). Subsequent PCR was performed using Gene Amp PCR system 9700 (Applied Biosystems, UK) in a 25μl reaction volume comprised of 19.4μl of SDW, 2.5μl of 10x buffer 3 (10mM Tris HCl pH 8.3, 3.5mM MgCl₂, 25mM KCl) 0.6μl of primer mix (table 3.3), 0.5μl of 25mM dNTPs (Promega, UK), 0.25μl of 1.25 units/μl *Taq* DNA polymerase (Promega, UK) and 2μl of template DNA (section 3.2.3). Meticillin-resistant *Staphylococcus aureus* strain MW2 was used as a positive-control to amplify the 433bp product of *pvl* and N315 was used as a negative-control. A 2% agarose gel containing 1μg/ml of ethidium bromide was used to separate amplified fragments. Electrophoresis was performed in 1x TAE (40mM Tris, 1mM EDTA and 0.1% (v/v) glacial acetic acid) buffer at 100 volts for 1 hour.

Table 3.3 Primers used for PVL detection as described by (Lina *et al.*, 1999a).
3.2.7 Identification of a mecA negative isolate

3.2.7.1 16S ribosomal RNA gene sequencing

Universal oligonucleotide primers (RW01 5'-AACTGGAGGAGGTTGGGGAT-3' and DG74 5'-AGGAGGTTGATCAAACCGCA-3') were used to amplify two conserved regions which flanked a 370bp variable locus of the 16S rRNA gene (Teng et al., 2004). All PCR reactions were carried out in a 25 µl reaction containing 21.25µl of SDW, 2.5µl of 10x PCR Buffer (15 mM MgCl2), 0.5µl of 10nM dNTPs, 0.25µl of primer RW01 (100pm/µl), 0.5µl of primer DG74 (100pm/µl), 0.25µl of 5U/µl HotStar Taq Polymerase (Qiagen) and 1 colony of template DNA per reaction. Subsequent PCR was performed using Gene Amp PCR system 9700 (Applied Biosystems, UK) using the following cycling conditions; 15 min at 95°C, followed by 30 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute. The amplified reaction products were then stored with 5µl of 6x DNA loading buffer at 4°C until required. A 1% agarose gel containing 1µg/ml of ethidium bromide was used to separate amplified fragments. Electrophoresis was performed in 1x TAE (40Mm Tris, 1mM EDTA and 0.1% (v/v) glacial acetic acid) buffer at 100 volts for 1 hour. The gel was then photographed under UV (Genesnap, Syngene, Synoptics, Cambridge, UK).

Following the manufacturer’s instructions gel extraction was carried out using the QIAEX II Agarose Gel Extraction Protocol (Qiagen). The extracted DNA was stored in two 10µl mixes with one of each primer. Each reaction mix contained 3µl of Primer (100pm/µl), 2µl of amplified DNA and 5µl of SDW, stored at 4°C and submitted for
genomic sequencing at Birmingham University Genomics Laboratory. The genomic sequence obtained was then searched using Biology Workbench against sequences held in the Genbank Database for genus determination.

3.2.7.2 PBP2' detection

Detection of PBP2a was carried out using a slide agglutination kit (MRSA-Screen test Denka Seiken Co Ltd. Japan) and performed according to the manufacturer’s instructions. EMRSA-15 was used as a mec-positive control strain and Oxford Staphylococcus aureus NCTC 8325 as a mec-negative control strain. The sample colonies were taken from a fresh BHI agar plate and re-suspended in 4 drops (approximately 200µl) of extraction reagent no.1 (0.1M NaOH) and boiled for 3 min. Following boiling 1 drop (approximately 50µl) of extraction reagent no.2 (0.5M KH₂PO₄) was aliquoted into the suspension mix and vortexed for 30 seconds. The sample was then centrifuged at 1,500 × g for 5 min at room temperature. A 50 µl sample of the test isolate supernatant, the positive-control and the negative-control was placed on the provided slides. One drop (approximately 25 µl) of anti-PBP 2a monoclonal antibody-sensitized latex was added to each sample for the positive control and one drop (approximately 25 µl) of negative-control latex was added to each sample for a negative control. The slides were rotated by hand for 3 minutes and characteristic agglutination indicated a positive result for PBP2' production.
3.2.7.3 Production of β-lactamase

Three wells of a microtitre plate were allocated to a control (SDW), a negative-control (Oxford Staphylococcus aureus NCTC 8325) and the test sample. A nitrocefin solution was prepared by re-hydrating nitrocefin lyophilized powder (Oxoid, U.K) in 1.9ml of 0.1M phosphate buffer pH 7 and further diluted to 1:10 with phosphate buffered saline (50μg/ml). For each test a bacterial suspension was made containing 5μl of bacterial cell suspension with SDW and 100μl of 0.1M phosphate buffer pH7. A 50μl sample was then taken from the bacterial suspension along with 50μl of nitrocefin solution and combined in a single well. A negative result was indicated by no colour change (yellow) and a positive result for β-lactamase production was indicated by a pink colour change.

3.2.8 Multiple MRSA colony analysis from primary isolation plates

All colonies from MRSA primary isolation plates were re-inoculated onto separate blood agar plates. Each colony was identified as MRSA by standard laboratory techniques including coagulase, DNAase and Gram-stain analysis. Meticillin resistance was determined by SCCmec element assignment and the identification of the internal locus for mecA (section 3.2.4). All MRSA colonies retrieved from the primary isolation plate were genotyped by PFGE (section 3.2.2).
3.2.9 Environmental sampling for MRSA

A total of 233 swabs were used to sample 29 dialysis stations including the following areas in each station: dialysis chair, chair remote switch, table, dialysis cuff, dialysis machine switch, pump door, nurses call button, TV screen, TV remote control and the two computer key pads on the unit. A total of 28 curtains surrounding the dialysis stations on the RDU unit were analysed for the presence of MRSA. Mannitol salt agar plates containing oxacillin (4mg/l) (Oxoid Ltd, UK) were prepared and for each dialysis curtain. Contact with the agar plate was made six times along the central inside of the curtain where hand contact was predicted to occur most frequently.

3.2.10 Air sampling for MRSA contamination

Air sampling was carried out using 15 cm diameter mannitol salt agar plates containing oxacillin (4mg/l) (Oxoid Ltd, UK). A total of four MRSA positive patients provided written consent to have air sampled around their beds four times during the course of the day (before dialysis, during dialysis, after dialysis and when the unit was empty). The air sampler was set at to draw 700 L/min for 5 minutes gathering a total volume of 3500 litres of air. In addition, a total of 72 mannitol salt agar settle plates containing oxacillin (4mg/l) (Oxoid Ltd, UK) were distributed throughout the RDU including dialysis stations, waiting room, the staff room, store room, sinks and nurses stations.
3.3 Results

Of 81 nasal swabs, five (6%) were positive for MRSA and 19 (23%) were positive for MSSA. Of 14 central venous catheter entry site swabs, one patient was positive for MRSA.

3.3.1 Antibiotic resistance profiles of MRSA isolates

All MRSA isolates retrieved from individual patient nasal samples had distinct antimicrobial resistance phenotypes (table 3.4).

Table 3.4 Antibiotic resistance phenotypes, pulsed-field gel electrophoresis type and SCCmec element assignment for all MRSA isolates obtained with corresponding patient number and isolate source.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Isolate Source</th>
<th>Antibiotic resistance phenotype</th>
<th>PFGE</th>
<th>SCCmec</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Em</td>
<td>Tp</td>
<td>Rf</td>
</tr>
<tr>
<td>1</td>
<td>CVC</td>
<td>R</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>1</td>
<td>Nasal</td>
<td>R</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>2</td>
<td>Nasal</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>3</td>
<td>Nasal</td>
<td>S</td>
<td>S</td>
<td>S</td>
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<td>4</td>
<td>Nasal</td>
<td>R</td>
<td>R</td>
<td>S</td>
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<td>5</td>
<td>Nasal</td>
<td>R</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>Env†</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Antibiotic abbreviations: Em (Erythromycin); Tp (Trimethoprim); Rf (Rifampicin); Gm (Gentamicin); Te (Tetracycline); Mu (Mupirocin); Vm (Vancmycin); Fu (Fusidic acid) and Fl (Flucloxacillin).
† Env abbreviates the environmental MRSA isolate retrieved.
All MRSA isolates were resistant to flucloxacillin but had varied antimicrobial resistance patterns; five were resistant to erythromycin, three were resistant to trimethoprim, one was resistant to rifampicin, gentamicin and mupirocin, and two were resistant to fusidic acid. No MRSA isolates obtained in this study were resistant to tetracycline or vancomycin. The MRSA isolate obtained from patient four displayed a multi-resistant phenotype and was only sensitive to rifampicin, tetracycline and vancomycin. From patient three, simultaneous isolates were obtained from the nasal and CVC site which expressed identical antibiotic resistance profiles.

### 3.3.2 Molecular analysis of MRSA isolates

All PFGE profiles obtained for each MRSA isolate were designated A-F following dendrogram positioning. Figure 3.1 shows a dendrogram of the PFGE profiles, with corresponding patient number (1-5), isolate source, SCCmec element assignment and RAPD profile. Following RAPD analysis two profiles were obtained for each primer; LAM 795 generated profiles L1 and L2 and SL 1254 generated profiles S1 and S2. Isolates displaying PFGE profiles A-D harboured SCCmec IV elements and RAPD profile L1 and S1. This group of isolates possessed 68% similarity through PFGE profiling and were all related to EMRSA-15 by Tenover criteria (Tenover et al., 1995). Isolates displaying PFGE profiles E-F harboured SCCmec type II elements and RAPD profile L2 and S2. This group of isolates possessed 70% similarity through PFGE profiling and were all related to EMRSA-16 by Tenover criteria (Tenover et al., 1995) (figure 3.2 and figure 3.3). From patient one, indistinguishable MRSA isolates were
retrieved from both the nasal and CVC site (PFGE profile A, SCCmec IV, RAPD L1 and S1). All MRSA isolates retrieved from this study setting were negative for \textit{pvl} (figure 3.4).

A single isolate, identified as MRSA from standard laboratory procedures (3.2.1), was untypeable by SCCmec element assignment and was absent for the \textit{mecA} internal control (table 3.1).

Figure 3.1 PFGE profiles with corresponding patient number, SCCmec types, RAPD types and isolate source. Percentage relatedness was calculated by Dice coefficient and represented by un-weighted pair-group method with mathematical averages (UPGMA) clustering.
Figure 3.2 SCC mec type analysis of MRSA isolates. Isolates from patients 1-4 and the environmental isolate harbour SCC mec IV and isolates from patients 5-6 harbour SCC mec II. P represents the positive control N315/SCC mec II.

* designates a nasal isolate, **ve designates a central venous catheter entry site isolate and **nv designates the environmental isolate.
Figure 3.3 RAPD profiles obtained for all MRSA strains using primers LAM 795 with representative profiles L1 and L2 and SL 1254 with representative profiles S1 and S2.
Figure 3.4 PVL analysis of MRSA isolates from patients 1-5. P represents the positive control MW2 and N represents the negative control N315

\(^a\) designates a nasal isolate and \(^c\)\(^v\) designates a central venous catheter entry site isolate

3.3.3 Identification of a \textit{mecA}-negative isolate

An additional isolate from the renal dialysis unit was identified as MRSA through the phenotypic methods described in section 3.2.1. Further analysis by multiplex SCC\textit{mec} typing revealed an absence of the \textit{mecA} gene locus (section 3.3.2). The following methods were applied to ascertain the nature of this isolate and the basis for its meticillin resistance.
3.3.3.1 Ribosomal 16s RNA gene sequencing of the *mecA*-negative isolate

Ribosomal 16s RNA gene sequencing of the *mecA*-negative isolate indicated 99% identity to *Staphylococcus aureus* with 0% gaps. The isolate was therefore indicated to be *S. aureus* rather than a related *Staphylococcus* species.

3.3.3.2 PBP2 detection

The *mecA*-negative isolate was negative for PBP2a production through latex agglutination detection. This indicated that PBP2a was not responsible for meticillin resistance.

3.3.3.3 Microplate nitrocefin assay for the detection of β-lactamase production

The *mecA*-negative MSSA isolate was positive for β-lactamase production after 10 minutes incubation at 37°C with nitrocefin solution (figure 3.5).

![Figure 3.5 β-lactamase production of the *mecA*-negative isolate.](image)

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3.3.4 Multiple MRSA colony analysis from primary isolation plates

All MRSA colonies taken from individual primary isolation plates were indistinguishable by PFGE analysis (table 3.5).

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Isolation site</th>
<th>Number of colonies isolated for PFGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nose</td>
<td>8</td>
</tr>
<tr>
<td>1</td>
<td>CVC</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>Nose</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>Nose</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>Nose</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>Nose</td>
<td>8</td>
</tr>
</tbody>
</table>

3.3.5 Environmental sampling for MRSA

Of 233 environmental swabs, 28 dialysis curtain samples, 72 settle plates and 16 air samples, only one positive MRSA isolate was obtained from a dialysis curtain. This isolate was not represented in any of the clinical MRSA isolates by PFGE analysis.
3.4 Discussion

3.4.1 Phenotypic and genotypic analysis of MRSA isolates

Of 81 nasal swabs, five (6%) were positive for MRSA and 19 (23%) were positive for MSSA. The nasal carriage rate of MSSA was notably higher than MRSA nasal carriage in this study, which correlates well with previous UK studies that report a predominance of MSSA in endogenous colonisation (Dall'Antonia et al., 2005). Staphylococcal nasal carriage is of clinical importance in haemodialysis patients where anatomical colonisation may often prelude hospital acquired infection. A reported 80% of all bacteraemias are as a result of endogenous colonisation reflecting the importance of eliminating nasal MRSA carriage in susceptible patient groups (von Eiff et al., 2001; Davis et al., 2004; Wertheim et al., 2004).

All MRSA isolates were unique to each patient on the basis of PFGE and antibiogram analysis and thus provided no evidence for inter-patient MRSA transmission within the renal dialysis unit (table 3.1 and figure 3.1). Additionally, individual patient strains highlighted the possible acquisition of MRSA nasal isolates from diverse sources, indicative of a dynamic outpatient environment. All MRSA isolates obtained from this study were related to nosocomial epidemic strains EMRSA-15 or EMRSA-16 suggesting acquisition may have been of hospital origin. However, the community setting as a source of MRSA colonisation cannot be ruled out following the reports of
EMRSA subtypes circulating within the Birmingham community population (Adedeji et al., 2007).

The nasal MRSA isolate obtained from patient four exhibited a multi-drug resistant phenotype that was only sensitive to rifampicin, tetracycline and vancomycin (table 3.4). Meticillin-resistant *Staphylococcus aureus* colonisation of the anterior nares is typically treated with 2% mupirocin ointment which is particularly effective for *S. aureus* de-colonisation in haemodialysis patients (Tacconelli et al., 2003). The expression of mupirocin resistance in the isolate from patient four, may render eradication therapy ineffective with the colonising strain persisting following antimicrobial treatment (Walker et al., 2003). This underlines the importance of detecting multi-resistant MRSA colonisation in the anterior nares so that alternative antimicrobial therapy can be applied for successful de-colonisation.

Patient one simultaneously harboured MRSA isolates at both the nasal and CVC site. Both isolates were indistinguishable by molecular analysis and antimicrobial resistance profiling implying intra-patient cross-contamination of the catheter site from the nasal reservoir. This is further supported by the exclusivity of the MRSA genotype in this patient as it was not represented in any other patient or environmental sample. Indwelling catheters are a major risk factor for acquisition of hospital associated bacteraemia (Yoshida et al., 2005) along with metastatic infections such as osteomyelitis, septic arthritis and endocarditis (Robinson et al., 1997; Fowler et al., 2005; Saxena and Panhotra, 2005). Meticillin-resistant *Staphylococcus aureus*
associated bacteraemias are less likely to occur when colonisation is absent from the nares (Nielsen et al., 1998; Saxena et al., 2002) and the importance of eliminating nasal MRSA carriage should therefore not be under estimated in reducing the risk of cross-contamination to such portals of entry where the skin is breached.

All MRSA isolates from this study were absent of the pvl gene locus, in line with the current low prevalence of pvl-positive S. aureus strains in the UK (Holmes et al., 2005). It has been suggested that low numbers of S. aureus isolates harbouring pvl is due to only a few strains being susceptible to pvl converting phages (Narita et al., 2001). However, surveillance of pvl within circulating S. aureus populations should continue following the recent re-emergence of a pvl-positive MSSA strain (phage type 80/81) in the UK community (Robinson et al., 2005) and the presence of pvl in strains related to hospital epidemic EMRSA-15 and EMRSA-16 (Holmes et al., 2005).

3.4.2 RAPD and SCCmec analysis

Random amplification of polymorphic DNA is a simple and rapid molecular method which can be used for typing MRSA in hospital epidemiological investigations (Tambic et al., 1997). In this study, RAPD and SCCmec molecular analysis of MRSA isolates demonstrated an equal discriminatory capacity; all SCCmec IV isolates produced RAPD profiles L1/S1 and all SCCmec II isolates produced RAPD profiles L2/S2. From PFGE analysis all SCCmec IV isolates were designated subtypes of EMRSA-15 and all SCCmec II isolates were designated subtypes of EMRSA-16 deducing that the clonal relationship of isolates exhibiting the same RAPD profile may explain the low
Chapter 3 The Epidemiology of MRSA in a Renal Dialysis Setting

discriminatory capacity of this molecular technique in this study setting. Analysis by RAPD therefore may be more applicable to larger investigations which may include a more clonally diverse subset of isolates.

3.4.3 Identification of a mecA-negative isolate

Accurate detection of MRSA can be problematic and automated systems, such as Vitek, may report false-positives due to expression of borderline meticillin susceptibilities (mecA-negative, oxacillin MICs of 2 to 8 µg/ml). Borderline meticillin susceptible S. aureus isolates lack intrinsic mecA to confer PBP2a production but may still express low meticillin resistance. This is mediated by the constitutive over production of β-lactamase (Barg et al., 1991; Varaldo, 1993; Knapp et al., 1996) that causes the sufficient hydrolysis of penicillinase-resistant compounds such as meticillin, to make cells resistant in vitro (McDougal and Thornsberry, 1986). Although such isolates may express a reduced susceptibility to β-lactams, they are still often susceptible to clinical antibiotic therapy and are therefore termed MSSA (Varaldo, 1993). A single isolate retrieved from this study was confirmed MRSA by clinical laboratory analysis and automated Vitek validation. However, further analysis demonstrated this isolate to be mecA-negative by multiplex SCCmec element assignment, negative for the production of PBP2a from latex agglutination and a possible constitutive hyper β-lactamase producer through nitrocefin analysis. From this it was concluded that the isolate was expressing borderline meticillin susceptibility and determined MSSA for the remainder of the study.
3.4.4 Multiple MRSA colony analysis from primary isolation plates

An important question which arises from the dynamic nature of MRSA carriage is whether, at any one time, the anterior nares may be simultaneously colonised with multiple strains of MRSA. Previous studies have provided contrasting opinions on the clonality of *S. aureus* nasal carriage (Kluytmans *et al.*, 1997), reporting both homogenous (Hu *et al.*, 1995) and heterogeneous colonisation (Cespedes *et al.*, 2005). Molecular investigations often use single colony analysis to determine the characterisation of an MRSA population assuming a clonal nasal reservoir and excluding the detection of co-colonising strains (Kluytmans *et al.*, 1997). This study investigated the frequency and molecular characterisation of simultaneous MRSA carriage in the anterior nares. Molecular analysis revealed that multiple colonies from the primary isolation plate of each MRSA patient sample were indistinguishable by PFGE. This suggests that nasal MRSA colonisation within this patient subset was with a single strain and that antimicrobial therapy targeted towards the phenotypic analysis of these isolates should be effective in nasal eradication. The homogenous nature of MRSA colonisation demonstrated in this study is reassuring for the continuation of single colony analysis in the clinical microbiology laboratory and epidemiological research.
3.4.5 Environmental sampling for MRSA

The role of the hospital environment as a reservoir of MRSA contamination has been well documented (Bures et al., 2000; Shiomori et al., 2002; Hardy et al., 2006a). Typically surviving for prolonged periods of time on irregular hospital surfaces, medical utensils and cleaning mops, MRSA may often be difficult to eradicate. In the absence of an effective cleaning regime, cross contamination to patients and health care workers is imminent (Oie and Kamiya, 1996; Huang et al., 2006). From extensive environmental sampling of the RDU only one MRSA isolate was obtained from a curtain surrounding a dialysis station. The PFGE profile of this environmental isolate was not represented by any profile obtained from nasal clinical samples, which is in contrast to other studies where the contribution of the environment is much more apparent as a source of infection (Bures et al., 2000; Shiomori et al., 2002; Hardy et al., 2006a; Sexton et al., 2006).

The RDU is cleaned between 11am and 12 noon every day and every night at 12am when the unit has closed for the day. Cleaning is carried out using soap and water however, between each patient session the dialysis nurses clean the patient’s tables, chairs and dialysis machines with soap, water and a hypochlorite (bleach) solution. Following any blood spillage curtains are immediately changed and washed. It was therefore interesting to note in this study the relatively minor contribution of the environment as a source of MRSA transmission which suggests that the cleaning
regime currently employed in this RDU is effective in reducing its environmental persistence.

3.5 Conclusion

The results of this investigation have revealed that inter-patient MRSA transmission and contamination from the environment was not a major factor in this study setting, demonstrating that appropriate cleaning of the hospital environment will minimise the risk of MRSA exposure to patients undergoing haemodialysis treatment. Analysis of intra-patient nasal samples indicated that patients when colonised, were with one type of MRSA. Additionally, evidence supported the potential for contamination of CVC sites from nasal carriage sites. This study has therefore highlighted the importance of the elimination of nasal MRSA carriage and the prevention of cross-contamination in reducing the incidence of catheter related infections.

Eradication of nasal colonisation is essential for infection control. Future investigations should aim to assess the genetic diversity at nasal and extra-nasal endogenous sites and the effectiveness of de-colonisation in reducing the risk of entry site infections.
CHAPTER 4 THE TONGUE AS A RESERVOIR FOR MRSA IN A RENAL DIALYSIS SETTING

4.1 Introduction

Endogenous carriage of \textit{Staphylococcus aureus} is a predisposing risk for hospital acquired infection (von Eiff \textit{et al.}, 2001). The anterior nares are regarded as the primary reservoir for \textit{S. aureus} colonisation but recent reviews have highlighted the increasing prevalence of oral colonisation and its association with periodontal and systemic disease (Jackson \textit{et al.}, 1999; Smith \textit{et al.}, 2001; Smith \textit{et al.}, 2003b). Previous studies have identified the increasing importance of the throat and oral cavity as a site for MSSA and MRSA colonization (Smith \textit{et al.}, 2003b; Nilsson and Ripa, 2006) and in light of these investigations the UK Guidelines for the Control and Prevention of MRSA in Healthcare facilities now recommend that healthcare workers sample the throat as well as the nose when screening for MRSA carriage (Coia \textit{et al.}, 2006). Meticillin-resistant \textit{Staphylococcus aureus} is an opportunistic pathogen and colonisation of the oral cavity may provide a reservoir for subsequent horizontal transmission to distant anatomical sites, neighbouring patients and health care workers (Anon, 1998; Smith \textit{et al.}, 2001). Establishment of carrier state and eradication of MRSA from all anatomical sites is therefore critical for infection control in the clinical setting.
This study aims to evaluate by PFGE, the molecular profiles of MRSA in the anterior nares and tongues of haemodialysis patients and the clinical implications of co-colonisation.
4.2 Materials and methods

4.2.1 Patients

Twenty patients from the University Hospital Birmingham NHS Foundation Trust were enrolled onto this study following informed written consent and ethical approval granted from the South Birmingham Ethics Committee. All patients attended the Renal Dialysis Unit or had been registered as an in-patient on the renal wards. Of the twenty patients, thirteen were male and seven were female with a mean age of 65 years old (34-85). All patients had previously been reported as MRSA nasal carriers at least 12 months prior to sampling. Additionally, all patients had received treatment of 2% mupirocin nasal ointment, (Bactroban Nasal® Beecham) which was applied three times a day into the anterior nares for five days. During this time all patients also underwent daily whole body washing with 4% (w/v) aqueous chlorhexidine gluconate soap (Hydrex® Adams Healthcare). All patients were re-screened 2 days after treatment then twice further at weekly intervals. Three consecutive negative screens were required to confirm eradication.

4.2.2 Screening for nasal and tongue MRSA carriage

A nasal swab was obtained from the left and right anterior nares and the tongue of each patient. Each tongue sample was swabbed from the posterior to the tip along the entire accessible dorsum surface. All sample swabs were plated onto MRSA identification
chromogenic agar plates (bioMerieux®, Marcy-L’Etoile, France), and incubated aerobically at 37°C for 48 hours. Isolates were confirmed as MRSA following standard microbiological techniques and Vitek analysis (bioMerieux Basingstoke UK Ltd).

4.2.3 Characterisation of MRSA by PFGE

All isolates were genotypically characterised by PFGE as previously described (section 3.2.2).
4.3 Results

Five out of twenty patients were MRSA-positive in both the anterior nares and the tongue. All five MRSA patients had previously been positive for MRSA at least 12 months prior to sampling and had received eradication treatment but remained intermittently positive. The remaining 15 patients had previously been reported as MRSA nasal carriers but at the time of the study MRSA was not isolated from either the nose or the tongue.

4.3.1 PFGE analysis of meticillin-resistant Staphylococcus aureus

All MRSA nasal isolates obtained from each patient were genetically indistinguishable by PFGE to the MRSA isolates obtained from the tongue (figure 4.1). In total, four PFGE profiles were observed designated as A-D following dendrogram positioning. Patient two and three harboured MRSA isolates with identical PFGE profiles (profile B). Isolates displaying this profile were genetically indistinguishable from EMRSA-15 (O'Neill et al., 2001b). The remaining isolates, displaying PFGE profiles A, C and D, only differed to profile B by 1-3 band differences and were determined closely related to EMRSA-15 by Tenover criteria (Tenover et al., 1995).
Figure 4.1 PFGE profiles obtained from nasal and tongue MRSA isolates with corresponding patient number (1-5) and PFGE profile (A-D). Percentage relatedness was calculated by Dice coefficient and represented by unweighted pair-group method with mathematical averages (UPGMA) clustering.

* designates a nasal isolate and † designates a tongue isolate
4.4 Discussion

This preliminary investigation demonstrated that both the nose and the tongue provided sites for MRSA colonisation and that when a patient was nasally colonised with MRSA, a genetically indistinguishable isolate could also be retrieved from the tongue. Homogenous co-colonisation would therefore imply that MRSA horizontal transfer may be occurring between the anterior nares and oral cavity.

The potential horizontal transfer of MRSA between nasal and oral sites may have clinical implications in regards to antimicrobial eradication therapy. Meticillin-resistant Staphylococcus aureus colonisation of the anterior nares is typically treated with topical 2% mupirocin, but successful MRSA de-colonisation often fails due to re-colonisation with the pre-treated strain (Kluytmans et al., 1996; VandenBergh et al., 1999; Pena et al., 2004; Kluytmans and Wertheim, 2005; Wertheim et al., 2005b). Relapse following antimicrobial therapy may occur as a result of MRSA carriage at extra-nasal sites, such as the tongue, providing a subsequent reservoir for re-colonisation of the nose. Successful eradication of endogenous MRSA may therefore only be achieved following treatment of all colonisation sites (Watanabe et al., 2001; Pena et al., 2004; Kluytmans and Wertheim, 2005).

Meticillin-resistant Staphylococcus aureus de-colonisation programs often only target nasal and skin carriage sites (Watanakunakorn et al., 1992; Anon, 1998) and studies suggest that nasal mupirocin treatment in combination with a chlorhexidine body wash
Chapter 4 The Tongue as a Reservoir for MRSA in a Renal Dialysis Setting

may only be marginally effective in eradicating multi-site MRSA carriage (Harbarth et al., 1999; Wertheim et al., 2005b). Additionally, nasal mupirocin treatment may have a questionable effect upon throat colonisation where reported low levels of residual mupirocin in the oropharynx may select for mupirocin resistant *S. aureus* strains (Watanabe et al., 2001; Wertheim et al., 2005b). Recent investigations have demonstrated the efficiency of an over the counter 0.2% chlorhexidine gluconate w/v mouthwash in reducing *S. aureus* oral colonisation (Smith et al., 2003a). A combination of 2% mupirocin nasal ointment combined with a chlorhexidine mouth and body-wash therefore warrants further investigation in its efficacy in reducing MRSA endogenous carriage.

Meticillin-resistant *Staphylococcus aureus* is an opportunistic pathogen and colonisation of the tongue may provide an additional source for subsequent infection at distant anatomical sites (Anon, 1998; Smith et al., 2001). Patients receiving haemodialysis have an increased risk of acquiring access-related bacteraemia from MRSA colonisation and eradication of endogenous carriage is critical for infection control (Peacock et al., 1999a; Lentino et al., 2000; Koziol-Montewka et al., 2001; Döprr et al., 2002; Trojde et al., 2007). This study highlights the tongue as an additional site for MRSA colonisation which should be taken into consideration when establishing patient carrier state and de-colonisation programs.

All pulsed field types (A-D) were closely related to the UK epidemic strain EMRSA-15 as determined by the Tenover criteria (Tenover et al., 1995). PFGE profile B was
identical to the progenitor profile of EMRSA-15 and PFGE profiles A, C and D only differed by 1-3 band differences (O'Neill et al., 2001b). EMRSA-15 is a nosocomial epidemic strain and the related subtypes observed in this study may indicate patient MRSA colonisations were of hospital origin (Johnson et al., 2005). However, EMRSA-15 subtypes are also prevalent in the community setting and the source of MRSA acquisition cannot therefore be accurately identified (Adedeji et al., 2007).

4.5 Conclusion

This investigation provides evidence for the co-colonisation of genetically indistinguishable MRSA strains in the anterior nares and the tongue. Homogenous co-colonisation of the two anatomical sites highlights the importance of multi-site MRSA eradication in the prevention of relapse and re-colonisation following antimicrobial therapy.

Further studies are now required to determine the significance of the oral cavity as a reservoir for MRSA infection and the efficacy of oral screening and de-colonisation in the eradication of persistent and intermittent MRSA carriage.
CHAPTER 5 EPIDEMIOLOGY OF COMMUNITY MRSA OBTAINED FROM THE WEST MIDLANDS REGION

5.1 Introduction

Meticillin-resistant *Staphylococcus aureus* and its association with UK hospital-acquired infections has been well documented (Johnson *et al.*, 2001; Johnson *et al.*, 2005; Wyllie *et al.*, 2006; Das *et al.*, 2007). However, following four pediatric deaths in Dakota and Minnesota (Anon, 1999a), the emergence of MRSA in the community setting has become a major focal point of epidemiological research (Lina *et al.*, 1999a; Okuma *et al.*, 2002; Vandenesch *et al.*, 2003; Rossney *et al.*, 2005; King *et al.*, 2006).

Community-derived MRSA infections are a worldwide concern (Vandenesch *et al.*, 2003), predominantly associated with invasive skin and soft-tissue infections of non-hospitalised individuals with no established risk factor for MRSA acquisition (Lina *et al.*, 1999a; Gillet *et al.*, 2002; Miller *et al.*, 2005). Following MLST analysis, distinct genetic lineages have been associated with CA-MRSA infections (Wijaya *et al.*, 2006), further identified by the predominance of SCCmec IVa, the locus for the Panton-Valentine leukocidin and an increased susceptibility to non-β-lactams (Baba *et al.*, 2002; Tenover *et al.*, 2006).
Meticillin-resistant *Staphylococcus aureus* of true community origin are thought to arise from diverse genetic backgrounds through the horizontal transmission of *mecA* into MSSA strains circulating outside of the nosocomial environment. With increasing evidence for the dissemination of hospital epidemic strains into the community populations (Salgado *et al.*, 2003; Adedeji *et al.*, 2007), the lines defining hospital- and community- MRSA acquisition are becoming increasingly difficult to define (Salgado *et al.*, 2003).

This chapter aimed to characterise the epidemiology of MRSA strains circulating within the Lichfield, Tamworth, Burntwood, North and East Birmingham community.
5.2 Materials and methods

All mapping was undertaken in collaboration with Dr Lucy Bastin, lecturer in Geographical Information Systems at Aston University.

5.2.1 Bacterial isolates

Between January 2005 and December 2005, 199 MRSA isolates were obtained from non-hospitalised patients presenting skin and soft tissue infections to local general practitioners (GP). The study area incorporated 57 surgeries from three Primary Care Trusts in the Lichfield, Tamworth, Burntwood, North and East Birmingham region.

5.2.2 Patient details

From each MRSA-positive patient the following details were recorded, date of sample retrieval, GP, age and home postcode. Permission to undertake this study was granted by the Medical Director of Good Hope Hospital NHS Trust and the Director of Public Health of North Birmingham Primary Care Trust. Formal ethical approval was not required as patient identifiers, such as name and NHS registration number, were deleted from the datasets.
5.2.3 Antibiotic sensitivity testing

Isolate identification was undertaken at the Good Hope Hospital NHS Trust, Sutton Coldfield, following standard laboratory procedures. For all presumptive MRSA, sensitivity to a panel of antibiotics was determined using the British Society for Antimicrobial Chemotherapy (BSAC) disc diffusion method in the microbiology laboratory of Good Hope Hospital (Andrews, 2001b; Andrews, 2001a). Zones of inhibition for the determination of antibiotic sensitivity are illustrated in table 5.1.

Table 5.1 Antibiotic panel and size of zones of inhibition used for the determination of antibiotic sensitivity using the British Society for Antimicrobial Chemotherapy (BSAC) disc diffusion method (Andrews, 2001b; Andrews, 2001a).

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Concentration in disc (µg)</th>
<th>Resistant (&lt;mm)</th>
<th>Sensitive (&gt;mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythromycin</td>
<td>5</td>
<td>19</td>
<td>20</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>5</td>
<td>19</td>
<td>20</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>2</td>
<td>29</td>
<td>30</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>10</td>
<td>19</td>
<td>20</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>10</td>
<td>19</td>
<td>20</td>
</tr>
<tr>
<td>Mupirocin</td>
<td>5</td>
<td>21</td>
<td>22</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>5</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>Fusidic Acid</td>
<td>10</td>
<td>29</td>
<td>30</td>
</tr>
<tr>
<td>Meticillin</td>
<td>4</td>
<td>13</td>
<td>14</td>
</tr>
</tbody>
</table>
All antibiotic sensitivity patterns were characterised by a unique three digit antiobiogram code (table 5.2) and isolates with resistance to six and above antibiotics were designated multi-drug resistant MRSA (MR-MRSA). Resistance to an antimicrobial would score the value allocated in table 5.1 whereas sensitivity to an antimicrobial would score zero. Antimicrobial scores were grouped into three groups of three to attain a unique three digit code for the phenotype observed. All MRSA isolates were received at Aston University on nutrient agar slopes for further molecular analysis.

Table 5.2 An illustrated example of the assignment of antiobiogram code dependant upon antimicrobial sensitivity pattern.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Value assigned for resistance</th>
<th>Example</th>
<th>Antiobiogram code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythromycin</td>
<td>4</td>
<td>R</td>
<td>6</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>2</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>Rifampicin</td>
<td>1</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>Gentamicin</td>
<td>4</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>Tetracycline</td>
<td>2</td>
<td>R</td>
<td>7</td>
</tr>
<tr>
<td>Mupirocin</td>
<td>1</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>Vancomycin</td>
<td>4</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>Fusidic acid</td>
<td>2</td>
<td>R</td>
<td>3</td>
</tr>
<tr>
<td>Meticillin</td>
<td>1</td>
<td>R</td>
<td></td>
</tr>
</tbody>
</table>
5.2.4 Molecular characterisation of MRSA

5.2.4.1 Rapid DNA extraction by boiling

For all PCR protocols employed, chromosomal DNA was extracted by a rapid boil extraction method (Caddick et al., 2005) as previously described in section 3.2.3.

5.2.4.2 Multiplex PCR for SCCmec element assignment

Staphylococcal Cassette Chromosome mec (SCCmec) element assignment was performed as previously described in section 3.2.4. SCCmec validation was carried out using the positive-control strains COL, PER34, N315, ANS46, HU25 and MW2 (figure 5.1).

5.2.4.3 Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis was performed as previously described in section 3.2.2.

5.2.5 Mapping of SCCmec type to patient home postcode

All SCCmec types were mapped to patient home postcode determined by the UK All Fields Postcode Directory (Office of National Statistics, 2005).

5.2.6 Population mapping within the study bounds

Based upon the UK 2001 Census data, the population within the study bounds was determined to be approximately 374,883. Population density within the study region was geographically mapped to Census Output Area.
5.2.7 Pvl gene locus detection

Panton Valentine Leukocidin gene locus detection was performed as previously described in section 3.2.6.
5.3 Results

5.3.1 Antibiotic sensitivity testing

Antibiotic sensitivity testing (table 5.3) revealed that six MRSA isolates were resistant to all antibiotics investigated, with the exception of rifampicin, tetracycline and vancomycin. Five of these isolates harboured SCCmec II and one isolate harboured SCCmec IV. A further SCCmec IV isolate was resistant to seven antibiotics exhibiting sensitivity to only tetracycline and vancomycin. All isolates with resistance to six and above antibiotics were designated multidrug-resistant (MR-MRSA).

From the non-multidrug-resistant (NMR-MRSA) isolates, 37 were resistant to meticillin alone; 36 harbouring SCCmec IV and one harbouring SCCmec II. The remaining NMR-MRSA isolates were resistant to at least one non-β-lactam. In total, 126 NMR-MRSA expressed erythromycin resistance, 106 expressed trimethoprim resistance, six expressed gentamicin resistance, seven expressed mupirocin resistance and five expressed tetracycline resistance. All 199 MRSA isolates were resistant to meticillin and sensitive to vancomycin.
Table 5.3 Antibiotic resistance phenotypes and SCCmec types of 199 MRSA isolates obtained from the Lichfield, Tamworth, Burntwood, North and East Birmingham community.

<table>
<thead>
<tr>
<th>*MR</th>
<th>Em</th>
<th>Tp</th>
<th>Rf</th>
<th>Gm</th>
<th>Tc</th>
<th>Mu</th>
<th>Vm</th>
<th>Fu</th>
<th>Mt</th>
<th>Code</th>
<th>SCCmec II</th>
<th>SCCmec IIIa mecI</th>
<th>SCCmec IV</th>
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</thead>
<tbody>
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<td>S</td>
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<td>S</td>
<td>R</td>
<td>R</td>
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<td>0</td>
<td>0</td>
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<td>S</td>
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<td>S</td>
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<td>S</td>
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<td>R</td>
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<td>S</td>
<td>S</td>
<td>R</td>
<td>601</td>
<td>3</td>
<td>5</td>
<td>64</td>
</tr>
<tr>
<td>-</td>
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<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
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<td>S</td>
<td>S</td>
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<td>S</td>
<td>R</td>
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<td>S</td>
<td>S</td>
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<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>651</td>
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<td>R</td>
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<td>R</td>
<td>653</td>
<td>5</td>
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<tr>
<td>MR</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
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<td>R</td>
<td>753</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

MR designates multidrug-resistance i.e. isolates resistant to six or more antibiotics.

Antibiotic abbreviations are as follows; Em (erythromycin), Tp (trimethoprim), Rf (rifampicin), Gm (gentamicin), Tc (tetracycline), Mu (mupirocin), Vm (vancomycin), Fu (fusidic acid) and Mt (meticillin).

See section 5.3.2.1 below for definition of this mec type.
5.3.2 Molecular characterisation of MRSA

5.3.2.1 Multiplex PCR for SCCmec element assignment

All positive controls were assigned the correct SCCmec types following SCCmec multiplex PCR validation (figure 5.1).

![Typical profiles of SCCmec types following SCCmec multiplex validation using positive controls COL (SCCmec I), PER34 (SCCmec Ia), N315 (SCCmec II), ANS46 (SCCmec III), HU25 (SCCmec IIIa) and MW2 (SCCmec IV).](image)

Figure 5.1 Typical profiles of SCCmec types following SCCmec multiplex validation using positive controls COL (SCCmec I), PER34 (SCCmec Ia), N315 (SCCmec II), ANS46 (SCCmec III), HU25 (SCCmec IIIa) and MW2 (SCCmec IV).
Following SCCmec element assignment 173 (87%) of the 199 MRSA isolates were designated SCCmec IV, 18 (9%) were designated SCCmec II and 8 (4%) were identified as a novel SCCmec IIIa variant. All isolates harbouring SCCmec IIIa variant were characterised by the amplification of the locus determined by the E and F primer sets, specific for SCCmec IIIa (table 3.1), but minus the 209bp amplification product (locus C) internal to mecl (figure 5.2). Stains harbouring this mec element were therefore designated SCCmec IIIa\textsuperscript{\textit{mecI}}. All MRSA isolates were positive for \textit{meca} through the amplification of the 162bp internal fragment.

\textbf{Figure 5.2 SCCmec element assignment for the 8 isolates containing novel SCCmec IIIa\textsuperscript{\textit{mecI}}. ANS 46 was used as a positive control depicting a typical SCCmec III profile with corresponding amplicon sizes.}

\textsuperscript{a}designates locus F, \textsuperscript{b}designates locus H, \textsuperscript{c}designates locus E, \textsuperscript{d}designates locus C and \textsuperscript{e}designates the internal control to meca (table 3.1).
5.3.2.2 Pulsed-field gel electrophoresis analysis.

Pulsed-field gel electrophoresis analysis of the 199 MRSA isolates identified 29 variant band pattern profiles (figure 5.3). Thirteen distinct PFGE profiles groups were designated A-M. A further sixteen PFGE profiles were unique to individual isolates and were designated U1-U16. All SCCmec IV isolates produced PFGE profiles A-I and unique profiles U1-U12. All SCCmec II isolates produced PFGE profiles L-M and unique profiles U13-U16. All SCCmec IIIa

Profile B was identified in 21% of the isolate population and represented a typical EMRSA-15 progenitor profile (O’Neill et al., 2001b). PFGE profile M was identified in 2% of the isolates and represented a typical EMRSA-16 progenitor profile (Murchan et al., 2004). By Tenover criteria, PFGE patterns that displayed less than four fragment differences were considered the same strain and isolates that displayed four to six fragment differences were considered to be of the same genetic lineage (Tenover et al., 1995). All 173 SCCmec IV isolates differed by no more than 5 bands to profile B (representative of EMRSA-15) and accounted for 87% of the isolate population (figure 5.4). Sixteen SCCmec II isolates, with PFGE profiles L, M, U15 and U16, differed by no more than five bands to profile M (representative of EMRSA-16) and accounted for 8% of the isolate population (figure 5.4). The remaining SCCmec II isolates with PFGE profiles U13 and U14 were not related to EMRSA-16 by Tenover criteria. Eight isolates harboured novel SCCmec IIIa

124
six isolates produced PFGE profile K. Both profiles had 95% relatedness calculated by Dice coefficient and were closely related by Tenover criteria (Tenover et al., 1995).

Figure 5.3 Dendrogramatic representations of the range of different PFGE profiles obtained for 199 MRSA isolates with their corresponding SCCmec types. Relatedness was calculated by Dice co-efficient and represented by UPGMA clustering.
5.3.3 Mapping of SCCmec type to patient home postcode

All MRSA isolates were mapped by SCCmec type to patient home postcode. From the map produced in figure 5.5, a diverse distribution of SCCmec II and SCCmec IV was observed. Seven of the eight isolates harbouring SCCmec IIIa\textsuperscript{mecl} appeared to be localised in the north-west of the study region. The general isolate distribution of the 199 MRSA isolates (figure 5.5) was visually comparable to the population density of the study region as depicted in figure 5.6.
Figure 5.5 SCC\textit{mec} types mapped to patient home postcode for all 199 MRSA isolates (see figure 5.6 for geographical location in the UK).
Figure 5.6 Population densities of the Lichfield, Tamworth, Burntwood, North and East Birmingham study region.
5.3.4 pvl gene locus detection

The pvl gene locus was not detected in any of the 199 MRSA isolates analysed in this investigation.

5.4 Discussion

The worldwide emergence of CA-MRSA has generated considerable scientific interest. Meticillin-resistant Staphylococcus aureus MLST types ST1, ST8, ST30 and ST80 (Wijaya et al., 2006) dominate the US and mainland European communities, but in Birmingham and the UK the prevalence of true CA-MRSA remains low (Abudu et al., 2001; Anon, 2005a; Adedeji et al., 2007). However, the recent report of pvl positive CA-MRSA in the West Midlands and its detection in the nosocomial setting (Anon, 2006a) warrants continued surveillance for emerging virulent clones within the clinical and community population. Community-acquired MRSA can be derived from two sources; de-novo CA-MRSA from horizontal transfer of mecA into community MSSA lineages or the displacement of successful hospital epidemic clones into the community population (Salgado et al., 2003; Charlebois et al., 2004; Coombs et al., 2004; O'Brien et al., 2004; Tacconelli et al., 2004). With a reported low prevalence of patients with no associated hospital risk factors, the lines defining origin of MRSA acquisition are becoming increasingly blurred (Salgado et al., 2003; Kourbatova et al., 2005; David et al., 2006; Seybold et al., 2006; Adedeji et al., 2007). The aim of this chapter was to characterise phenotypically and genotypically 199 MRSA isolates obtained from the
Chapter 5 Epidemiology of Community MRSA obtained from the West Midlands Region

Lichfield, Tamworth, Burntwood, North and East Birmingham community to determine the epidemiology of these strains.

5.4.1 Interpretation of pulsed-field gel electrophoresis chromosomal restriction patterns

Guidelines have been established for the interpretation of chromosomal restriction patterns obtained from PFGE analysis (Tenover et al., 1995). These guidelines are typically used to define outbreak strains within a relatively short period of time, but previous reports suggest that PFGE profiles may be regarded as a continuum from progenitor profiles in temporally and spatially dispersed epidemic strains (O'Neill et al., 2001b). Genetic events such as point mutations, insertions and deletions may alter restriction enzyme sites on the bacterial chromosome, which in turn may alter the PFGE profile displayed. The guidelines for the interpretation of restriction banding patterns are therefore as follows:

- Strains with identical banding patterns are considered genetically indistinguishable
- Closely related strains will differ by two to three bands representing one genetic event
- Related strains of the same genetic lineage may differ by four to six bands representing two genetic events
- Un-related strains will differ by seven or more bands representing multiple genetic events.

Application of the aforementioned criteria aided analysis of complex PFGE pattern profiles obtained within this investigation.
5.4.2 Molecular analysis of MRSA

Following PFGE analysis, profile B was identified in 21% of the isolate population and represented a typical EMRSA-15 progenitor profile (O'Neill et al., 2001b) whereas profile M was identified in 2% of the isolates and represented a typical EMRSA-16 progenitor profile (Murchan et al., 2004). All 173 SCCmec IV isolates differed by no more than five bands to profile B (representative of EMRSA-15) and accounted for 87% of the isolate population (figure 5.4). Sixteen SCCmec II isolates with PFGE profiles L, M, U15 and U16 differed by no more than five bands to profile M (representative of EMRSA-16) and accounted for 8% of the isolate population (figure 5.4). Therefore, according to Tenover criteria, 95% of the isolates retrieved from the Lichfield, Tamworth, Burntwood, North and East Birmingham community, were representative of the genetic lineages from either EMRSA-15 or EMRSA-16 nosocomial strains (Tenover et al., 1995; Enright et al., 2000).

EMRSA-15 and EMRSA-16 represent the majority of MRSA strains residing in UK hospitals (Johnson et al., 2001) associated with invasive infections and accounting for >95% of MRSA associated bacteraemia (Enright et al., 2000; Johnson et al., 2001; Johnson et al., 2005). The overspill of epidemic strains into circulating community populations should therefore be perceived as a serious public health threat (Cox et al., 1995b; Caddick et al., 2005; Adedeji et al., 2007). Patients who acquire nosocomial MRSA whilst in hospital may remain undetected if symptoms fail to present. The subsequent discharge of MRSA-positive patients from the health care setting provides
an ideal mechanism for MRSA dissemination into community populations, where the spread of epidemic MRSA may then be further exuberated by inter-familial transmission and cross-contamination in homes and communal centres (Calfee et al., 2003; Huijsdens et al., 2006). Additionally, MRSA circulating within the community population may increase the number of patients transferring MRSA back into the health care setting (Karas et al., 2006) and control of MRSA in the UK might only be achieved by targeted action, effective screening and de-colonisation in both the clinical and community environment.

The international spread of EMRSA-15 and EMRSA-16 underlines the transmissible success of these strains to adapt to varied environments to become worldwide dominant clones (Witte et al., 2001; Moore and Lindsay, 2002; Murchan et al., 2004; Aires de Sousa et al., 2005; Hsu et al., 2005; Gosbell et al., 2006; Melter et al., 2006). The epidemic nature of EMRSA strains has been attributed to the expression of virulence factors that may favour persistent colonisation and host survival (Roberts and Gaston, 1987; Papakyriacou et al., 2000) although, such theories have been difficult to corroborate due to the complexity of regulatory factors within the S. aureus genome (Sabersheikh and Saunders, 2004). Through comparative genomics, both EMRSA-15 and EMRSA-16 display distinct core regions and unique accessory elements when compared to previous epidemic clones (Moore and Lindsay, 2002), which is speculated to play a role in the success, survival and spread of these widely disseminated strains.
Chapter 5 Epidemiology of Community MRSA obtained from the West Midlands Region

The lateral genetic transfer of transposable elements is fundamental to the evolution of *S. aureus*, particularly regarding the acquisition of antibiotic resistance and virulence determinants (Fitzgerald *et al.*, 2001; Moore and Lindsay, 2002). Using the Tenover criteria, 20 possible subtypes of EMRSA-15 were identified in the study population, demonstrating the genetic diversity that can be displayed by epidemic strains that have evolved temporally from a common genetic lineage (O'Neil *et al.*, 2001b; Moore and Lindsay, 2002). EMRSA-15 and EMRSA-16 were first observed in the early 1990s and it is therefore not surprising that these widely disseminated epidemic strains demonstrate divergent PFGE patterns in response to varying exposure to environmental pressures (Moore and Lindsay, 2002; Murchan *et al.*, 2004).

It is interesting to note that 87% of the isolate population harboured SCCmec IV and only 9% harboured SCCmec II, further underlining the view of Hiramatsu that the factor driving MRSA transmission in the community is the dissemination of a successful SCCmec IV (Hiramatsu *et al.*, 2001). Isolates harbouring SCCmec IV have been associated with increased fitness and transmissibility when compared to the larger, genetically burdened SCCmec II and III elements (Ito *et al.*, 2001) and evolutionary models generated by MLST have inferred that SCCmec type IV is the most frequently acquired mec element within the five major lineages of hospital-acquired MRSA (Robinson and Enright, 2003). This may account for the high predominance of EMRSA-15/SCCmec IV strains observed in this study setting.
Unlike the larger SCCmec II and III elements, SCCmec IV lacks any antibiotic resistance determinants other than mecA and MRSA strains harbouring SCCmec IV are therefore predominantly associated with sensitivity to non-β-lactams (Ma et al., 2002; Okuma et al., 2002). This study demonstrated that 79% of MRSA isolates harbouring SCCmec IV displayed resistance to at least one non-β-lactam, with two expressing multi-drug resistance. Following environmental pressures and antibiotic exposure, such strains may acquire multi-resistance through chromosomal mutation or the horizontal incorporation of genetic determinants (Livermore, 2000; Caddick et al., 2005; Rice, 2006). This would be a typical attribute for an isolate originating from a clinical setting (Okuma et al., 2002; Jung et al., 2006).

5.4.3 Molecular analysis of novel SCCmec IIIa mecI

From SCCmec type element assignment eight (4%) isolates harboured a novel SCCmec variant designated SCCmec IIIa meci. Novel SCCmec elements are reportedly widespread (Oliveira and de Lencastre, 2002; Shore et al., 2005) and through multiplex-PCR mec element assignment, multiple variants of SCCmec I, II, III and IV have been identified (Aires de Sousa and de Lencastre, 2003; Shukla et al., 2004b; Shore et al., 2005). The identification of SCCmec IIIa meci was not unique to this study and has previously been identified in hospital- and community-colonised patients in Taiwan (Lu et al., 2005b). Variants of SCCmec III, characterised by either the absence of locus E (between pl258 and Tn554) or locus F (between Tn554 and orfx) have also been reported (Shore et al., 2005; Szczepanik et al., 2007).
SCCmec IIIα-mecI is characterised by the absence of the amplification target (locus C), internal to mecI. The mecI locus transcribes a protein that acts to repress mecA transcription and substitutions, deletions and mutations in mecI are therefore not uncommon through negative pressure to select for meticillin resistance (Suzuki et al., 1993; Kobayashi et al., 1998; Katayama et al., 2001; Watson et al., 2003; Shukla et al., 2004a). A typical SCCmec IIIa element will contain a class A mec gene complex where the mec regulatory genes are present (mecI-mecRI-meca-IS431) and it therefore may be unlikely that mecI deletion has occurred in the case of SCCmec IIIα-mecI. Base mutations and substitutions internal to mecI may be a more plausible consideration (Kobayashi et al., 1998) reducing the affinity of target primers to bind to the mecI locus.

The identification of novel SCCmec types demonstrates the mobility and plasticity of this transferable genetic element (Daum et al., 2002). Variant SCCmec types in MRSA are reportably increasing (Chung et al., 2004; Perez-Roth et al., 2004; Shukla et al., 2004b; Shore et al., 2005; Szczepanik et al., 2007) and the continued evolution of novel structural types may further complicate the accurate typing of this evolutionary important genetic determinant (Zhang et al., 2005).

5.4.3.1 Mapping of SCCmec type to patient home postcode

All MRSA isolates and corresponding SCCmec types were mapped to patient home postcode (figure 5.5). The general distribution of MRSA isolates was visually comparable to the underlying population density gradient within the study region.
(figure 5.6). This indicated that there was no apparent sampling bias and the isolates obtained for this study were a fair representation of the underlying population structure.

From the map produced in figure 5.5, a diverse distribution of SCCmec II and SCCmec IV was observed. However, seven of the eight isolates harbouring SCCmec IIIa\(^{\text{mecl}}\) appeared to be localised in the north-west of the study region. Following PFGE analysis, isolates harbouring SCCmec IIIa\(^{\text{mecl}}\) produced banding profiles that were 95% related and genetically distinct from the remaining isolate population, which may denote a population of minor sporadic clones (Aires de Sousa and de Lencastre, 2003). Lack of additional molecular data on local hospital epidemiology prevented further investigation into the origins of these novel variants.

**5.4.4 Pvl gene locus detection**

The \(pvl\) gene locus was not detected in any of the 199 MRSA isolates retrieved, which is in line with previous UK study outcomes (Anon, 2002b; Klein \textit{et al.}, 2003; Holmes \textit{et al.}, 2005). However, the recent report of \(pvl\) positive CA-MRSA in the West Midlands and its detection in the nosocomial setting (Anon, 2006a) warrants continued surveillance for emerging clones.
5.5 Conclusion

In this investigation, all MRSA isolates retrieved from the Lichfield, Tamworth, Burntwood, North and East Birmingham community population were either genetically related to hospital associated EMRSA-15 and EMRSA-16 epidemic strains or harboured SCCmec elements indicative of nosocomial origin. The majority of the isolate population expressed resistance to non-β-lactams and all were negative for the pvl gene locus. It can therefore be concluded that none of the 199 MRSA isolates obtained from study setting was characteristic of de-novo CA-MRSA, highlighting the transmission of MRSA from the hospital setting into the surrounding community population. The overspill of hospital acquired MRSA strains into the community environment warrants the need for targeted infection control, effective screening and de-colonisation in both the clinical and community setting for the future control of MRSA within the UK population.

The predominance of SCCmec IV and the genetic diversity exhibited by strains of a common genetic lineage, demonstrates the transmissible success of SCCmec IV to prevail outside of the hospital setting and the importance of genetic transfer and mutation in the adaptable evolutionary success of the S. aureus genome. In addition, the identification of novel SCCmec types reveals the plasticity of the SCCmec genomic structure and the potential emergence of sporadic MRSA clones.
CHAPTER 6 COMPARATIVE ANALYSIS OF MRSA ISOLATED FROM COMMUNITY AND HOSPITAL INFECTION

6.1 Introduction

Between January and December 2005, 199 MRSA isolates were obtained from non-hospitalised patients presenting with skin and soft tissue infections to local practitioner surgeries in the Lichfield, Tamworth, Burntwood, North and East Birmingham community. Although obtained from the community setting, further molecular analysis demonstrated that all isolates were related to a hospital derived genetic lineage (chapter 5).

Hospital-acquired MRSA are generally associated with increased resistance to non-β-lactams (Fey et al., 2003; Jung et al., 2006). However, antibiotic resistance often carries a biological cost and the increased genetic burden of additional resistance determinants may reduce bacterial fitness and replication efficiency (Wichelhaus et al., 2002; Thouverez et al., 2003; Hurdle et al., 2004; Besier et al., 2005; McCallum et al., 2006). The dissemination of HA-MRSA into the community setting, in the absence of antimicrobial pressure, may therefore impose the redundancy of the multi-drug-resistant phenotype in favour of increased metabolic fitness.

Through comparative statistical analysis this investigation aimed to determine the phenotypic and genotypic characteristics of hospital-associated MRSA obtained from
Chapter 6 Comparative Analysis of MRSA Isolated from Community and Hospital Infection

nosocomial-onset and community-onset infection in an attempt to define the driving factors of community dissemination.
6.2 Methods

6.2.1 Bacterial isolates
A total of 199 MRSA isolates were obtained from non-hospitalised patients presenting with skin and soft tissue infections to local practitioner surgeries in the Lichfield, Tamworth, Burntwood, North and East Birmingham community. Previous analysis had demonstrated that all MRSA isolates were related to a hospital derived genetic lineage (chapter 5).

A total of 94 MRSA isolates were obtained from blood cultures collected within the University Hospital Birmingham, NHS Trust, UK. Isolates were obtained from patients with no evidence of infection within 48 hours of hospital admission.

6.2.2 Antibiotic sensitivity testing
Antibiotic sensitivity testing of the 199 MRSA isolates retrieved from the community was undertaken at the Good Hope Hospital NHS Trust, Sutton Coldfield, following standard laboratory procedures outlined in section 5.2.3.

Antibiotic sensitivity testing of the 94 MRSA isolates retrieved from the hospital setting was undertaken at the University Hospital Birmingham, NHS Foundation Trust UK using the British Society for Antimicrobial Chemotherapy (BSAC) disc diffusion method (Andrews, 2001b; Andrews, 2001a).
Zones of inhibition for the determination of antibiotic sensitivity are illustrated in table 5.2. All antibiotic sensitivity patterns were further characterised by a unique three digit antibiogram code (table 5.1). Isolates with resistance to six and above antibiotics were designated multi-drug resistant MRSA (MR-MRSA).

6.2.3 Multiplex PCR for SCCmec element assignment
Staphylococcal Cassette Chromosome mec (SCCmec) element assignment for all MRSA isolates was performed as described in section 3.2.4.

6.2.4 Chi-square statistical analysis
Yates'corrected chi-square ($\chi^2$) was used to determine the significance of any associations between antibiogram sensitivity patterns and SCCmec types of MRSA isolates obtained from hospital-onset and community-onset infection.

In chi-square analysis, the observed numerical differences between two data set populations are measured against a null-hypothesis which states that the expected frequencies will match the observed frequencies in a normal distribution. If the observed results obtained are sufficiently different to the expected results then the null-hypothesis will be rejected and a statistically significant relationship will be concluded to exist within the data. The observed frequencies are tabulated against the chosen variable parameters of a 2 x 2 contingency table. In the case of the provided working example (table 6.1), $\chi^2$ will determine whether there is a significant association between resistance to $\geq 5$ antibiotics and MRSA isolate source.
Table 6.1 A working example of a 2 x 2 contingency table for $\chi^2$ analysis. The analysis package used was designed by Dr Anthony Hilton and Dr Richard Armstrong, Aston University, Birmingham, UK.

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<tr>
<th></th>
<th>Resistance to 5+ antibiotics</th>
<th>Resistance to &lt; 5 antibiotics</th>
<th>Total</th>
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<tr>
<td>Hospital-onset MRSA</td>
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<td>6</td>
<td>83</td>
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<tr>
<td>Community-onset MRSA</td>
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<td>116</td>
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<tr>
<td>Total</td>
<td>88</td>
<td>111</td>
<td>Grand Total = 199</td>
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$\chi^2 = 132.711$

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<td>5.412</td>
<td>6.635</td>
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The expected frequencies on which to base the null-hypothesis are calculated by the following equation:

$$\text{(Row Total x Column Total)}/ \text{Grand Total}.$$  

The $\chi^2$ calculation measures the difference between the observed and expected frequencies. This is achieved by the following equation:

$$\chi^2 = \sum \frac{(\text{Observed frequency} - \text{Expected frequency})^2}{\text{Expected frequency}}$$

To achieve significance at the $<0.05$ (5% level of probability) with a 1 degree of freedom, the critical chi-value ($\chi^2$ crit) is set at 3.84. The $\chi^2$ value obtained must therefore be greater than or equal to 3.84 to reject the null hypothesis.
In this study Yates’ correction was applied to the chi-square statistic. Application of the Yates’ correction will take into account low observed values (<10) and improve the estimate of chi-square by reducing the difference between the observed and expected values by 0.5 before squaring.

In the given example the calculation of $\chi^2$ is 132.711, which is higher than the critical $\chi^2$ value tabulated at the 5% level of probability (3.84). This indicates that the $\chi^2$ value generated would occur rarely by chance in a normal distribution and the null-hypothesis would therefore be rejected.
6.3 Results

6.3.1 Multiplex PCR for SCCmec element assignment

Of the 199 MRSA isolates obtained from community-onset infection, 18 (9%) were designated SCCmec II, 8 (4%) were identified as a novel SCCmec IIIa variant and 173 (87%) were designated SCCmec IV, (section 5.2.1).

Of the 94 MRSA isolates derived from the hospital setting, one (1%) was designated SCCmec I, one (1%) was designated SCCmec IA, forty-four (47%) were designated SCCmec II, one (1%) was designated SCCmec III, one (1%) was designated SCCmec IIIA, forty-three (45%) were designated SCCmec IV and three (3%) were untypable by SCCmec element assignment (NEW1-NEW2).
Table 6.2 Antibiotic sensitivity patterns for all MRSA isolates obtained from community-onset infection.

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* Antibiotic abbreviations are as follows; Em (erythromycin), Tp (trimethoprim), Rf (rifampicin), Gm (gentamicin), Tc (tetracycline), Mu (mupirocin), Vm (vancomycin), Fu (fusidic acid) and Mt (meticillin).
Table 6.3 Antibiotic sensitivity patterns for all MRSA isolates obtained from hospital-onset infection.

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<thead>
<tr>
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<th>Em</th>
<th>Tp</th>
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<td>-</td>
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<td>-</td>
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<td>-</td>
<td>+</td>
<td>-</td>
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<td>IV</td>
<td>+</td>
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<td>-</td>
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<td>+</td>
<td>-</td>
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<td>IV</td>
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<td>+</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>703</td>
<td>2</td>
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<tr>
<td>NEW1</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>601</td>
<td>1</td>
</tr>
<tr>
<td>NEW2</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>401</td>
<td>2</td>
</tr>
</tbody>
</table>

* Antibiotic abbreviations are as follows: Em (erythromycin), Tp (trimethoprim), Rf (rifampicin), Gm (gentamicin), Tc (tetracycline), Mu (mupirocin), Vm (vancomycin), Fu (fusidic acid) and Mt (meticillin).
6.3.2 Chi-square analysis

Yates’-corrected $\chi^2$ was used to determine the significance of potential associations between antibiogram sensitivity patterns and SCCmec type of MRSA isolates obtained from the hospital and community setting. In general, MRSA isolates obtained from hospital-onset infection were significantly associated with the SCCmec II element (table 6.4) and MRSA isolates obtained from community-onset infection were significantly associated with the SCCmec IV element (table 6.5). Multi-drug resistance was significantly associated with isolates harbouring SCCmec II within both study settings when compared to isolates harbouring SCCmec IV (table 6.6-6.7).

On the whole, MRSA isolates derived from hospital-onset infection were more likely to have multi-drug resistance and resistance to 4> antibiotics when compared to MRSA isolates retrieved from community-onset infection (table 6.8-6.9).
Table 6.4 Chi-square analysis to determine if SCCmec II is associated with community-onset or hospital-onset MRSA infection.

<table>
<thead>
<tr>
<th></th>
<th>Community</th>
<th>Hospital</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCCmec II</td>
<td>18</td>
<td>47</td>
<td>65</td>
</tr>
<tr>
<td>Not SCCmec II</td>
<td>181</td>
<td>47</td>
<td>228</td>
</tr>
<tr>
<td>Total</td>
<td>199</td>
<td>94</td>
<td>293</td>
</tr>
<tr>
<td>$\chi^2$</td>
<td></td>
<td></td>
<td>59.681</td>
</tr>
<tr>
<td>P value</td>
<td></td>
<td></td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>5%</td>
</tr>
<tr>
<td>$\chi^2_{crit}$</td>
</tr>
<tr>
<td>Significant</td>
</tr>
</tbody>
</table>

Table 6.5 Chi-square analysis to determine if SCCmec IV is associated with community-onset or hospital-onset MRSA infection.

<table>
<thead>
<tr>
<th></th>
<th>Community</th>
<th>Hospital</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCCmec IV</td>
<td>173</td>
<td>43</td>
<td>216</td>
</tr>
<tr>
<td>Not SCCmec IV</td>
<td>26</td>
<td>51</td>
<td>77</td>
</tr>
<tr>
<td>Total</td>
<td>199</td>
<td>94</td>
<td>293</td>
</tr>
<tr>
<td>$\chi^2$</td>
<td></td>
<td></td>
<td>53.804</td>
</tr>
<tr>
<td>P value</td>
<td></td>
<td></td>
<td>&lt;0.0001</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Significance level</th>
</tr>
</thead>
<tbody>
<tr>
<td>5%</td>
</tr>
<tr>
<td>$\chi^2_{crit}$</td>
</tr>
<tr>
<td>Significant</td>
</tr>
</tbody>
</table>
Table 6.6 Chi-square analysis to determine if multi-drug resistance in community-onset MRSA is associated with SCCmec type.

<table>
<thead>
<tr>
<th></th>
<th>Community SCCmec II</th>
<th>Community SCCmec IV</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>MR-MRSA</td>
<td>5</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Not MR-MRSA</td>
<td>13</td>
<td>171</td>
<td>184</td>
</tr>
<tr>
<td>MR-MRSA</td>
<td>18</td>
<td>173</td>
<td>191</td>
</tr>
<tr>
<td>$\chi^2$</td>
<td>25.621</td>
<td></td>
<td></td>
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<td>P value</td>
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<td></td>
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</tr>
</tbody>
</table>

<table>
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<tbody>
<tr>
<td>5%</td>
</tr>
<tr>
<td>$\chi^2$ crit</td>
</tr>
<tr>
<td>Significant</td>
</tr>
</tbody>
</table>

Table 6.7 Chi-square analysis to determine if multi-drug resistance in hospital-onset MRSA is associated with SCCmec type.

<table>
<thead>
<tr>
<th></th>
<th>Hospital SCCmec II</th>
<th>Hospital SCCmec IV</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>MR-MRSA</td>
<td>42</td>
<td>0</td>
<td>42</td>
</tr>
<tr>
<td>Not MR-MRSA</td>
<td>2</td>
<td>43</td>
<td>45</td>
</tr>
<tr>
<td>Total</td>
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<td>87</td>
</tr>
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<td>$\chi^2$</td>
<td>75.578</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Significance level</th>
</tr>
</thead>
<tbody>
<tr>
<td>5%</td>
</tr>
<tr>
<td>$\chi^2$ crit</td>
</tr>
<tr>
<td>Significant</td>
</tr>
</tbody>
</table>
Table 6.8 Chi-square analysis to determine if multi-drug resistance is associated with community-onset or hospital-onset MRSA infection.

<table>
<thead>
<tr>
<th></th>
<th>Community</th>
<th>Hospital</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>MR-MRSA</td>
<td>7</td>
<td>42</td>
<td>49</td>
</tr>
<tr>
<td>Not MR-MRSA</td>
<td>192</td>
<td>52</td>
<td>244</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>199</strong></td>
<td><strong>94</strong></td>
<td><strong>293</strong></td>
</tr>
<tr>
<td>$\chi^2$</td>
<td></td>
<td></td>
<td>74.748</td>
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<tr>
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<td></td>
<td>&lt;0.0001</td>
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</table>

<table>
<thead>
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<th>Significance level</th>
</tr>
</thead>
<tbody>
<tr>
<td>5%</td>
</tr>
<tr>
<td>$\chi^2$ crit</td>
</tr>
<tr>
<td>Significant</td>
</tr>
</tbody>
</table>

Table 6.9 Chi-square analysis to determine if harbouring resistance to >4 antibiotics is associated with community-onset or hospital-onset MRSA infection.

<table>
<thead>
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<th></th>
<th>Community</th>
<th>Hospital</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;4</td>
<td>17</td>
<td>53</td>
<td>70</td>
</tr>
<tr>
<td>&lt;4</td>
<td>182</td>
<td>41</td>
<td>223</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>199</strong></td>
<td><strong>94</strong></td>
<td><strong>293</strong></td>
</tr>
<tr>
<td>$\chi^2$</td>
<td></td>
<td></td>
<td>77.749</td>
</tr>
<tr>
<td>P value</td>
<td></td>
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<tr>
<td>$\chi^2$ crit</td>
</tr>
<tr>
<td>Significant</td>
</tr>
</tbody>
</table>
Additional analysis revealed that resistance to erythromycin, gentamicin, mupirocin and fusidic acid was significantly associated with MRSA retrieved from hospital-onset infection. Resistance to trimethoprim, rifampicin and tetracycline was not associated with either hospital or community derived MRSA (table 6.10).

Table 6.10 Chi-square analysis to determine if antibiotic resistance is associated with community-onset or hospital-onset MRSA infection

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Community-onset</th>
<th>Hospital-onset</th>
<th>P value</th>
<th>Significant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resistant</td>
<td>Susceptible</td>
<td>Resistant</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Em</td>
<td>133</td>
<td>66</td>
<td>80</td>
<td>14</td>
</tr>
<tr>
<td>Gm</td>
<td>13</td>
<td>186</td>
<td>44</td>
<td>50</td>
</tr>
<tr>
<td>Mu</td>
<td>14</td>
<td>185</td>
<td>44</td>
<td>50</td>
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<tr>
<td>Fu</td>
<td>16</td>
<td>183</td>
<td>53</td>
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</tr>
<tr>
<td>Tp</td>
<td>113</td>
<td>86</td>
<td>61</td>
<td>33</td>
</tr>
<tr>
<td>Rf</td>
<td>1</td>
<td>198</td>
<td>2</td>
<td>92</td>
</tr>
<tr>
<td>Tc</td>
<td>5</td>
<td>194</td>
<td>3</td>
<td>91</td>
</tr>
</tbody>
</table>

* Antibiotic abbreviations are as follows; Em (erythromycin), Gm (gentamicin), Mu (mupirocin), Fu (fusidic acid), Tp (trimethoprim), Rf (rifampicin) and Tc (tetracycline).
6.4 Discussion

Meticillin-resistant *Staphylococcus aureus* isolates of nosocomial origin ordinarily carry resistance to non-β-lactam antibiotics following the selective pressures of increased antimicrobial use in the clinical environment (Fey *et al.*, 2003; Jung *et al.*, 2006). The transmission of HA-MRSA into the community, in the absence of antimicrobial pressure, may plausibly enforce the redundancy of non-β-lactam resistance in MRSA strains circulating outside of the clinical environment. Through the statistical comparison of SCCmec distributions and antimicrobial sensitivity patterns of MRSA isolates retrieved from community-onset and hospital-onset infections, this investigation aimed to determine the effect of community dissemination upon the prevailing phenotypic and genotypic characteristics of HA-MRSA.

6.4.1 Comparative analysis of SCCmec distributions between HA-MRSA isolated from community-onset and hospital-onset infections

Through application of the $\chi^2$ statistic, SCCmec II was found to be significantly associated with MRSA isolates retrieved from hospital-onset infections (table 6.4). As discussed further in section 6.4.2, multi-drug resistance is a common feature of MRSA isolates harbouring SCCmec II (Ito *et al.*, 2003). Increased antimicrobial use in the hospital setting may therefore account for the natural selection and predominance of SCCmec II, where expression of multiple resistance may be a selective advantage to survival and prevalence in the clinical setting (Johnson, 1998).
The SCCmec IV element was significantly associated with MRSA isolates retrieved from community-onset infection (table 6.5). The predominance of HA-MRSA harbouring SCCmec IV in the community setting could possibly be attributed towards the success of this genetic element to drive MRSA transmission out of the hospital and into surrounding community populations (Hiramatsu et al., 2001). In comparison to the larger genetically burdened SCCmec II element, isolates harbouring SCCmec IV are proposed to have increased metabolic fitness which may offer a competitive advantage for dissemination outside of the clinical environment (Ito et al., 2001).

6.4.2 Comparative analysis of antibiotic resistance patterns between HA-MRSA isolated from community-onset and hospital-onset infections

Further application of the Yates-corrected $\chi^2$ statistic revealed that in both the hospital and community setting, the multi-drug resistant phenotype was significantly associated with MRSA isolates harbouring SCCmec II (table 6.6 and 6.7). In addition to meca, the SCCmec II element may carry additional antibiotic resistance determinants facilitated by insertion sequences, plasmids and transposons e.g. pUB110 encoding kanamycin, tobramycin, bleomycin and gentamicin resistance and Tn554 encoding MLS and erythromycin resistance (Ito et al., 1999; Hiramatsu et al., 2001; Ito et al., 2001; Okuma et al., 2002; Oliveira and de Lencastre, 2002; Holden et al., 2004). Subsequently, the capacity of SCCmec II to incorporate additional genetic material may account for its significant association with multi-drug resistance in this study setting (Holden et al., 2004). In contrast, the smaller SCCmec IV element only harbours
determinants for $\beta$-lactam resistance (Okuma et al., 2002) and as a result, such isolates are generally resistant to fewer non-$\beta$-lactams (table 6.6-6.7). However, it should be noted that multi-drug resistance was not exclusive to SCCmec II and resistance to $6\geq$ antibiotics was also displayed in two MRSA isolates harbouring SCCmec IV. Multi-drug resistance in isolates harbouring SCCmec IV may occur through previous antibiotic exposure and the selective the uptake of resistance determinants in the nosocomial environment (Chambers, 2001; Charlebois et al., 2004; Tacconelli et al., 2004; Styers et al., 2006).

Meticillin-resistant Staphylococcus aureus isolates obtained from the hospital setting, had an increased association with the multi-drug resistant phenotype (table 6.8) and resistance to $4\geq$ antibiotics (table 6.9) when compared to isolates obtained from the community. The predominance of MR-MRSA in the clinical setting may be due to the abundance of SCCmec II found within the same bacterial pool. Additionally, MRSA isolates obtained from hospital-onset infection had a significant association with erythromycin, gentamicin, mupirocin and fusidic acid resistance (table 6.10) which may reflect the selective pressures imposed by the antibiotic prescribing regime applied within the hospital setting.

Meticillin-resistant Staphylococcus aureus isolates obtained from community-onset infections were not associated with multiple-resistance when compared to MRSA isolates obtained from hospital-onset infections. Multi-drug resistance, favourable for survival in the clinical environment, may not be an essential attribute for MRSA
dissemination within the community population, where the genetic burden of antimicrobial resistance may compromises bacterial fitness and replication (Laurent et al., 2001; Wichelhaus et al., 2002; Thouverez et al., 2003; Hurdle et al., 2004; Besier et al., 2005; McCallum et al., 2006). Consequently, the natural selection of MRSA strains with reduced antibiotic burden may be fundamental for the successful transmission and competitive survival of hospital derived MRSA strains in the community population.
6.5 Conclusion

Through comparative statistical analysis this study has demonstrated significant differences between hospital and community MRSA populations, suggesting that the environment may play a central role in the natural selection of residing HA-MRSA strains.

Meticillin-resistant *Staphylococcus aureus* isolates harbouring SCCmec II and multidrug resistance were statistically associated with hospital-onset infection illustrating the importance of antimicrobial resistance in the predominance and survival of MRSA within the challenging clinical environment. Meticillin-resistant *Staphylococcus aureus* isolates harbouring SCCmec IV and decreased antimicrobial resistance were statistically associated with community onset infection. This demonstrated that the natural selection of strains with reduced genetic burden may provide the basis for successful transmission of hospital derived MRSA into the community population.
CHAPTER 7 APPLICATION OF SATSCAN TO IDENTIFY MRSA CLUSTERING IN THE COMMUNITY SETTING

7.1 Introduction

Geographical information systems provide a valuable tool for monitoring disease incidence, revealing trends and inter-relationships that would be difficult to interpret on the basis of numerical data analysis alone (Vine et al., 1997; Camara and Monteiro, 2001; Cassa et al., 2005; Aamodt et al., 2006; Kulldorff, 2006). Kulldorff’s space-time scan statistic (Kulldorff, 1997) may be used to identify spatial and temporal disease clustering within a defined geographical setting and has been applied to a number of disease surveillance investigations which include: tuberculosis (Onozuka and Hagihara, 2007), malaria (Gaudart et al., 2006), cancer (Kulldorff et al., 1998), Creutzfeldt-Jakob disease (Cousens et al., 2001) and meningitis (Elias et al., 2006).

SaTScan, a publicly available statistical software package, incorporates the space-time scan statistic in the detection of disease clustering against a known geographical population distribution from which expected disease rates are generated (Kulldorff, 2006). Visualisations of MRSA incidence applied to a space-time context, may offer an insight into the distribution of MRSA within a community population, where the extent of the community as a reservoir for transmission remains largely unknown.
This chapter aims to assess the use of SaTScan for the identification of MRSA clustering within the Lichfield, Tamworth, Burntwood, North Birmingham and East Birmingham community setting.
Chapter 7 Application of SaTScan to Identify MRSA Clustering in the Community Setting

7.2 Methods

Geographical mapping and spatial-temporal analysis demonstrated in this investigation was undertaken in collaboration with Dr Lucy Bastin, Lecturer in Geographical Information Systems at the University of Aston.

7.2.1 Patient isolates

Between 01/09/2004 and 31/08/2005, 1867 meticillin-sensitive *Staphylococcus aureus* (MSSA) and 832 meticillin-resistant *Staphylococcus aureus* (MRSA) isolates were obtained from non-hospitalised patients presenting skin and soft tissue infections to local general practitioners. Home postcode and age was provided for each patient sample.

Permission to undertake this study was granted by the Medical Director of Good Hope Hospital NHS Trust and the Director of Public Health of North Birmingham Primary Care Trust. Formal ethical approval was not required as patient identifiers, such as name and NHS registration number, were deleted from the datasets.

7.2.2 Study area

The study area measured approximately 328.6 km², incorporating 57 general practitioners of three Primary Care Trusts in the Lichfield, Tamworth, Burntwood,
Chapter 7 Application of SaTScan to Identify MRSA Clustering in the Community Setting

North and East Birmingham region. The total population within the defined study bounds was 374,833 based upon the 2001 UK Census.

7.2.3 Isolate processing

Isolate identification was undertaken at the Good Hope Hospital NHS Trust, Sutton Coldfield, UK following standard laboratory procedures. Antibiotic sensitivity was determined using the British Society for Antimicrobial Chemotherapy (BSAC) disc diffusion method (Andrews, 2001b; Andrews, 2001a).

7.2.4 Underlying population density of the study region

The population density of the study region was determined and mapped according to 2001 Census data Output Area level.

7.2.5 Geographical mapping of MRSA incidence using patient home postcode

All 832 MRSA cases were located to postcode centroid co-ordinates using the All Fields Postcode Directory (ONS, 2005).

7.2.6 Preliminary analysis – identification of age as a co-variable for analysis

Patient age groups were categorised as follows; 0-4, 5-15, 16-29, 30-44, 45-59, 60-64, 65-74, 75-84 and 85+ years. The MRSA and MSSA prevalence rate for each age group was calculated by the following equation, where total population within each age category was derived from the 2001 Census:
MRSA prevalence rate = \( \frac{\text{Number of MRSA cases in a given age group}}{\text{Total population in a given age group}} \)

Preliminary analysis identified that MRSA and MSSA prevalence rate within the study population increased with age (figure 7.1). All further analysis within this investigation was therefore stratified to three age groups: 65, 65-85 and over 85 (table 7.1).

![MRSA and MSSA Prevalence Rate by Age](image)

**Figure 7.1** Prevalence rates of MRSA and MSSA in relation to age within the population of the study bounds.
Table 7.1 Categories for age related data stratification. Number of MRSA and MSSA cases are illustrated for each age category with calculated prevalence rate.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Under 65</th>
<th>65 to 85</th>
<th>85 and over</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSSA</td>
<td>N°. cases</td>
<td>1090</td>
<td>563</td>
</tr>
<tr>
<td></td>
<td>Prevalence rate</td>
<td>0.0034457</td>
<td>0.0109267</td>
</tr>
<tr>
<td>MRSA</td>
<td>N°. cases</td>
<td>196</td>
<td>429</td>
</tr>
<tr>
<td></td>
<td>Prevalence rate</td>
<td>0.0006196</td>
<td>0.008326</td>
</tr>
</tbody>
</table>

7.2.7 SatScan and the space-time scan statistic

The SatScan space-time scan statistic was applied to identify local MRSA clustering against the age structured population of the study setting (Kulldorff, 2006). Although data plotted to patient postcode data gave a fine spatial grain, the identified co-variables required for analysis (population structure and age structure), were only available at the spatially coarser Census Output Area level. Aggregation of the postcoded data to the Census Output Area level allowed the incorporation of an age structured population, ensuring that the expected number of cases at any given location was an accurate representation of geographical demographics (Kulldorff et al., 2005). All 832 cases of MRSA were therefore analysed as being located at the population centroids of the appropriate 2001 Census Output Areas.

The basis of Kulldorff's space-time scan statistic uses a cylindrical observation window which moves in steps across the study area. The circular base of the cylinder
corresponds to the geographical space and the height of the cylinder corresponds to time of the study period. The cylinder centres upon each data point in turn and from here on, the radius and height continue to increase until an upper limit is reached. This ensures that data from each possible geographical location, area size and time interval have been extrapolated (Kulldorff et al., 1998). Within each given cylindrical window the observed number of MRSA cases are statistically compared to the expected number of MRSA cases (Kulldorff, 1997). Under the null-hypothesis, expected MRSA incidence follows a Poisson distribution; where the probability of MRSA acquisition is proportional to the age and structure of the underlying population (Kulldorff, 1997). This distribution is used to generate Monte Carlo simulations of expected MRSA incidence, to which the observed value is compared to assess the significance of the observed pattern. Identified MRSA clusters that are significantly different from the expected distribution are allocated a P-value (Kulldorff, 1997; Bastin et al., 2007). If the P-value is less than or equal to 0.05 the null-hypothesis is rejected. Restrictions on cylinder overlaps ensure that for each identified disease cluster, the cylinder which most significantly differs from the expected norm is selected to represent that cluster.

7.2.8 Calculating the relative risk of MRSA acquisition located to Census Output Area

The relative risk represents how much more common disease incidence is in a given location and time compared to the overall incidence baseline across the whole study region (Kulldorff, 2006). The following equation standardises the rate of MRSA
observed at a given location (Census Output Area) against the base rate for the whole study area:

Relative risk = \[
\frac{\text{rate of MRSA at a given location}}{\text{rate of MRSA over the whole study region}}
\]

A value of 1 would represent the norm, a value of >1.5-5 would represent a low elevated risk, a value of >5-10 would represent a medium risk and a value of >10-26 would represent a high relative risk.

7.2.9 Identification of nursing homes within the study region

Nursing homes, residential homes and hospitals within the study region were located to postcodes and mapped using co-ordinates from the All Fields Postcode Directory 2005 (ONS, 2005).

7.2.10 Analysis of variable sampling effort

Analysis of variable sampling effort was performed to determine whether recorded MRSA incidence rates reflected sample bias at any geographical location. Using MSSA incidence data as a comparator population, both MSSA and MRSA cases were plotted to patient home postcode.
7.3 Results

The boundaries and the underlying population density of the study region are illustrated in figure 7.2, highlighting the number of people per hectare in each Census Output Area. The darker the shading the greater the population density as detailed in the key.

Figure 7.2 Population densities of the Lichfield, Tamworth, Burntwood, North and East Birmingham study region.
All 832 MRSA isolates retrieved within the one year study period were located to home postcode and mapped onto 2001 Census Output Areas using the All Fields Postcode Directory (figure 7.3). The size of each point represents the number of MRSA cases isolated at that location.

Figure 7.3 All 832 MRSA cases recorded within the one year study period located to postcode centroid level (1 m precision).
7.3.1 SaTScan and the space-time scan statistic

The application of the space-time scan statistic using SaTScan identified two significant MRSA clusters \((p<0.001)\) against the age-stratified population of the study region. The first cluster was located in the eastern region of the study setting (figure 7.4, inset 1) and contained thirteen cases of MRSA. The second cluster was located in the southern region of the study setting (figure 7.4, inset 2) and contained eleven cases of MRSA.

Further analysis identified one nursing home in the location of the eastern cluster where twelve of the thirteen patients were registered and two nursing homes in the location of the southern cluster where nine of the eleven patients were registered (figure 7.5).
Maps generated by Lucy Bastin,
University of Aston

Figure 7.4 Identification of two significant MRSA clusters (p<=0.001) using SaTScan-generated space-time cluster analysis.
Figure 7.5 Identified MRSA clusters, corresponding nursing homes (nursing homes marked by a star) and underlying relative risk of each Census Output area (grey shading).

7.3.2 Relative risk

A high MRSA relative risk was observed at the two Census Output Areas where MRSA clusters were identified (figure 7.5). It should also be noted that high relative risk levels were also observed in neighbouring Census Output Areas to the identified MRSA clusters.
7.3.3 Analysis of variable sampling effort

When mapped to home postcode, the observed incidence of MRSA was geographically comparable to both the observed incidence of MSSA and the underlying population density (figure 7.2 and figure 7.6); this illustrates that there was no apparent bias in MRSA sampling effort within the study setting. This visual conclusion was further supported by a formal random labelling analysis (Bastin et al., 2007) which treated MRSA as cases and MSSA as controls.

Figure 7.6 Geographical comparisons between MRSA and MSSA case distributions.
Chapter 7 Application of SaTScan to Identify MRSA Clustering in the Community Setting

7.4 Discussion

7.4.1 SaTScan and the space-time scan statistic

The application of the space-time scan statistic using SaTScan identified two significant MRSA clusters (p<0.001) in the eastern and southern region of the study area (figure 7.4). At these identified Census Output Areas the observed cases of MRSA were significantly higher than the numbers of cases which would be expected based upon the underlying age structure and population. It is interesting to note that neighbouring Output Areas to the eastern and southern MRSA clusters also displayed elevated relative risk of disease acquisition (figure 6.4 insets 1 and 2); illustrating MRSA clustering at medical care institutions may also be impacting upon the surrounding geographical area. Superimposing additional molecular characterisation onto the physical location of MRSA infection would be invaluable in determining the genetic relatedness of isolates at high risk locations and whether MRSA cluster ‘hotspots’ were truly representative of an outbreak situation.

The eastern MRSA cluster (figure 7.4, inset1), contained thirteen cases of MRSA and the southern MRSA cluster (figure 7.4, inset 2), contained eleven cases of MRSA. From the eastern cluster, twelve MRSA cases were obtained from patients registered at one nursing home. Equally from the southern cluster, seven MRSA cases were obtained from patients registered at one nursing home and two MRSA cases were obtained from patients registered at another (figure 7.5). The dissemination of
nosocomial MRSA to nursing and residential homes has been well documented (Fraise et al., 1997; Cookson, 2000; von Baum et al., 2002) where endemic strains may spread readily through the enclosed resident population (Drinka et al., 2005). Residents of long-term care institutions have an increased risk of MRSA acquisition (Fraise et al., 1997; O'Sullivan and Keane, 2000) following predisposition to underlying risk factors such as immunosuppression, antibiotic treatment, catheterisation, debilitating illness and previous hospitalisation (von Baum et al., 2002). With a reported increase in patient independence and mobility, nursing homes are seen to provide an ideal reservoir for MRSA transmission between medical facilities and even into the community setting (Cookson, 2000; Hoefnagels-Schuermans et al., 2002). Geographical information systems, such as SaTScan, may easily be used to identify MRSA clustering at medical care institutions, where subsequent application of eradication therapy in carrier patients may reduce the spread of MRSA strains between health care and community environments (Cookson, 2000). The use of SaTScan in the identification of MRSA clustering in nursing homes may therefore benefit MRSA surveillance mechanisms and the subsequent implementation of infection control.

Taking into account the underlying population (374, 883); MRSA was recovered from 0.22% of the study population and MSSA was recovered from 0.49% of the study population. This investigation provides an indication that the prevalence of MRSA within the Lichfield, Tamworth, Burntwood, North Birmingham and East Birmingham community population remains low, which is comparable to previous MRSA investigations undertaken within the Birmingham community region (Abudu et al.,
Chapter 7 Application of SaTScan to Identify MRSA Clustering in the Community Setting

2001). However, the results obtained for this investigation were restricted to patients presenting infection and does not account for asymptomatic MRSA carriage rates. The deduced rates within this investigation therefore may only be regarded as an estimate of the true extent of MRSA incidence and colonisation within the study setting.

7.4.2 Age as a co-variable for analysis

Following preliminary data analysis, an increase in age was identified as a predisposing risk factor for MRSA acquisition (figure 7.1), in line with the currently reported UK trend (Anon, 2006b). Elderly patients with underlying illness are often predisposed to MRSA colonisation and infection largely due to an increased association with hospitalisation, indwelling catheter use, recent antibiotic treatment, or residence in a long term health care facility (Rezende et al., 2002; Jernigan et al., 2003; Maudsley et al., 2004; Saxena et al., 2004; Hidron et al., 2005; Karas et al., 2006; Tacconelli et al., 2006; Gopal Rao et al., 2007). In this investigation, MRSA incidence was observed to dramatically increase in patients over the age of 75, with patients above the age of 85 being most at risk. However, the results from this study only included patients presenting infection and did not necessarily include the healthy elderly community population where MRSA prevalence is reported to be low (Grundmann et al., 2002b; Maudsley et al., 2004).
7.4.3 Analysis of variable sampling effort

The incidence of MRSA at any given location may be influenced by varying sample bias between participating surgeries. Using MSSA incidence as a comparator population, no variation in sampling effort was observed between the two bacterial populations (figure 7.6). Nevertheless, it should also be noted that sampling efforts of both bacterial populations may not be truly independent, as MSSA sampling will often be coupled with MRSA sampling as a follow up to eradication therapy.

7.4.4 Future recommendations

When geographically plotting MRSA incidence, using postcode alone suggests that an individual is adequately represented by their home address (Gatrell et al., 1996). This assumption excludes the identification of MRSA clustering at other locations where transmission may occur, such as the workplace (Allen et al., 1997), nurseries (Adcock et al., 1998), sports teams (Stacey et al., 1998), or communal centres (Cookson, 2000; Nguyen et al., 2005; Beam and Buckley, 2006). Locating MRSA incidence to patient home postcode may have therefore restricted this study to the identification of MRSA clustering amongst immobilised patients or those who are likely to contract MRSA at their home address i.e. nursing home care. In this instance, further ancillary data regarding employment, lifestyle and health care status would greatly enhance the power and resolution of MRSA cluster analysis within the community setting.
7.5 Conclusion

Application of geographical information systems to identify disease clustering provides a useful tool for targeted prioritisation within large epidemiological data sets. This study has demonstrated the successful application of SaTScan software to identify MRSA clusters amongst an age-stratified population within a defined community setting. By use of the space-time-scan statistic, two MRSA clusters were identified at nursing care institutions and these cases may now be prioritised for further investigation. This could involve detailed characterisation of MRSA isolates using discriminatory molecular techniques and a more thorough investigation of patient demographics. Additionally, an increased relative risk of MRSA incidence was identified in neighbouring Census Output Areas, implying that concentrations of MRSA infection at a particular location may have the potential to impact upon surrounding geographical areas.

As stated above, locating MRSA incidence to home postcode alone excludes the identification of other environmental locations as a basis for MRSA acquisition. It is therefore recommended that supplementary occupational and lifestyle data should be obtained in order to increase the power and resolution of cluster analysis, to give a more accurate representation of MRSA prevalence within the community setting.
With the aforementioned recommendations, SaTScan may provide a useful epidemiological tool for MRSA cluster identification and future targeted infection control within the community setting.
CHAPTER 8 MULTIPLEX PCR DESIGN FOR THE DETECTION OF VIRULENCE GENES IN MRSA

8.1 Introduction

Multiplex PCR, first described in the 1980s (Chamberlain et al., 1988), utilises multiple primer sets for the simultaneous detection and amplification of more than one target gene sequence (Stranden et al., 2003). The incorporation of a multiplex assay into a study design significantly reduces the time, the consumable costs and the risk of process contamination associated with single tube PCR (Edwards and Gibbs, 1994). However, design and implementation requires methodical optimisation to ensure effective amplification and resolution of all target gene locations (Baumforth et al., 1999). Common applications for multiplex PCR in bacterial genomics include: strain detection and identification (McClure et al., 2006; Bonnstetter et al., 2007); antibiogram typing (Perez-Roth et al., 2001; Strommenger et al., 2003); multiple-locus variable-number tandem repeat analysis (Sabat et al., 2003); SCCmec element assignment (Oliveira and de Lencastre, 2002; Zhang et al., 2005); epidemiological typing (Stranden et al., 2003) and virulence gene identification (Monday and Bohach, 1999; McClure et al., 2006).

Virulence determinants within the S. aureus genome govern the transition from a natural and harmless human skin commensal to an invasive host pathogen (Hienz et al.,
1996; Peacock et al., 2002). Peacock and colleagues identified seven virulence genes that are significantly associated with invasive S. aureus infections: fnbA encoding fibronectin binding protein A; cna encoding collagen binding protein; sdrE encoding serine-aspartate repeat containing protein E; sej encoding staphylocoecal enterotoxin J; eta encoding exfoliative toxin A; hlg encoding gamma-haemolysin and icaA encoding intracellular adhesin protein A (Peacock et al., 2002). This study aimed to incorporate primer gene targets for fnbA, cna, sdrE, sej, eta, hlg and icaA into a multiplex PCR protocol to provide an efficient, cost effective method for virulence gene detection in clinical MRSA isolates.
Chapter 8 Multiplex PCR Design for the Detection of Virulence Genes in MRSA

8.2 Methods

8.2.1 Primers

Primer sequences to amplify \( fnbA, hlg, sdrE, icaA, cna, eta \) and \( sei \) gene loci (table 8.1), were obtained from previously published studies (Lina et al., 1999a; Peacock et al., 2002; Becker et al., 2003). The primer sequences for amplification of \( cna \) were reported to produce variable results (Peacock et al., 2002) and therefore deemed unsuitable for inclusion in a multiplex PCR design. Primers to amplify a different target within the \( cna \) gene were therefore designed using SDSC Biology Workbench (www.workbench.sdsc.edu).

8.2.1.1 Primer design for amplification of the \( cna \) gene locus

The 2430bp \( cna \) gene sequence of \( Staphylococcus aureus \) (accession number AB266877) was uploaded from an NCBI nucleotide search (www.ncbi.nlm.nih.gov) and inserted into the ‘Primer 3’ program of Biology Workbench 3.2 (www.workbench.sdsc.edu). Two primers were designed to amplify a non-variable 531bp region of the \( cna \) gene (table 8.1), specified to include oligonucleotides between 18 and 24 bases with no intra-primer homology beyond 3 base pairs and an approximate GC content of 50%.
Table 8.1 Primer sequences and amplicon sizes for seven virulence gene targets.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer</th>
<th>Primer sequence (5'-3')</th>
<th>Amplicon bp</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>fnbA</td>
<td>Fnb1</td>
<td>CACAAAACGCAAATAATAG</td>
<td>1362</td>
<td>(Peacock et al., 2002)</td>
</tr>
<tr>
<td></td>
<td>Fnb2</td>
<td>CTGTGTGGATTCAATGTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hlg</td>
<td>Hlg1</td>
<td>GCCAATCCGTTTTAGAATAG</td>
<td>937</td>
<td>(Lina et al., 1999a)</td>
</tr>
<tr>
<td></td>
<td>Hlg2</td>
<td>CCATAGACGTAGCAACGGAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sdrE</td>
<td>Sdr1</td>
<td>CAGTAATGTTGCAAAAGA</td>
<td>767</td>
<td>(Peacock et al., 2002)</td>
</tr>
<tr>
<td></td>
<td>Sdr2</td>
<td>TTGACTAACCAGCTATATC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>icaA</td>
<td>Ica1</td>
<td>GATTATGTAATGTGCTTGG</td>
<td>770</td>
<td>(Peacock et al., 2002)</td>
</tr>
<tr>
<td></td>
<td>Ica2</td>
<td>ACTACTGTGCTGTTATAAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cna</td>
<td>Cna1</td>
<td>TCGTCAACAATCAAGTTGCC</td>
<td>531</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>Cna2</td>
<td>CCGTGAAAGATGGAAGGACG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sej</td>
<td>Sej1</td>
<td>CTCCCTGACCTGTTACTAATAAA</td>
<td>641</td>
<td>(Becker et al., 2003)</td>
</tr>
<tr>
<td></td>
<td>Sej2</td>
<td>TTGTCTGGATATGGACCTATAACCTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eta</td>
<td>Eta1</td>
<td>CTAATGCAATTTGATTACAGAC</td>
<td>119</td>
<td>(Becker et al., 2003)</td>
</tr>
<tr>
<td></td>
<td>Eta2</td>
<td>TGGATGGACACCTAGTATATAC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

8.2.2 Positive controls

Positive controls were determined for each primer pair using the Blast search on the NCBI nucleotide database. The precursor MRSA strain COL, was identified as a positive control for fnbA, sdrE, and hlg and the MRSA strain MW2, was identified as a positive control for cna and icaA. No positive control for eta and sej could be identified from published S. aureus genomes and these gene targets were therefore excluded from in the multiplex assay design.

8.2.3 Validation of cna primers

Validation of cna primers was achieved using cycle conditions obtained from previously published protocols (Oliveira and de Lencastre, 2002). Amplification was
performed using Gene Amp PCR system 9700 (Applied Biosystems, UK) in a 25µl reaction volume comprised of 19.7µl of SDW, 2.5µl of 10x buffer (10mM Tris HCl pH 8.3, 3.5mM MgCl₂, 25mM KCl) 0.4 µl of each primer at 25pmoles/µl (table 8.1), 0.2µl of 25mM dNTPs (Promega, UK), 0.2µl of 1.25 units/µl Taq DNA polymerase (Promega, UK) and 2µl of template DNA (10ng/25 µl). Meticillin-resistant Staphylococcus aureus strain MW2 was used as a positive-control to amplify the 531bp target of the cna gene locus. Precursor MRSA strain COL was used as a negative-control.

A 2% agarose gel containing 1µg/ml of ethidium bromide was used to separate amplified fragments. Electrophoresis was performed in 1x TAE (40mM Tris, 1mM EDTA and 0.1% (νν) glacial acetic acid) buffer at 100 volts for 1 hour.

8.2.4 Primer combinations and multiplex design

Two multiplex assays were designed to include suitable amplicon size ranges for effective electrophoretic resolution; the first assay incorporated primer targets for fnbA (1362bp), sdrE, (937bp) and hlg (767bp) with COL as a positive control and the second assay incorporated primer targets for cna (531bp) and icaA (770bp) with MW2 as a positive control.
8.2.5. Purification of genomic DNA by phenol extraction and ethanol precipitation

To ensure uniformity in template DNA concentrations during assay optimisation, genomic DNA from MW2 and COL was retrieved by phenol extraction and ethanol precipitation.

Bacterial cells were grown aerobically overnight at 37°C in 6ml brain heart infusion broth and harvested following centrifugation at 5,500g for 5 minutes. The resulting bacterial pellet was washed with SDW before being re-suspended in 270µl TE buffer (10Mm Tris, pH 8 and 1m EDTA, pH8). The re-suspended cells were heated for 10 minutes at 75°C prior to the addition of 30µl sodium dodecyl sulphate (10%). A 3µl volume of proteinase K (100mg/ml) was added to the suspension and incubated for 3 hours at 65°C. Following incubation the suspension was divided between two Eppendorf tubes (1.5ml) and diluted with 450µl SDW. A 600µl volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to each tube and sealed with silicon. Both tubes were centrifuged at 5,500g for one minute. A 240µl volume of the supernatant from each tube was transferred to a fresh Eppendorf tube with 48µl of sodium acetate (pH 5.2) and 960µl of ice cold ethanol. Both tubes were inverted five times and incubated (aerobically) overnight at -20°C. Following incubation, DNA was pelleted at 5,500g for 15 minutes at 4°C. The resulting pellet was washed in 70% ice-cold ethanol five times and left to air dry for 90 minutes. The pellet was re-hydrated in 100µl SDW and incubated at 37°C for one hour to assist dissolution. The quantity and purity of extracted DNA was determined using a UV spectrophotometer. A 5µl aliquot
of DNA sample was mixed with 995μl of SDW in a quartz crystal cuvette. The $A_{260}$ was measured (1 $A_{260}$ unit is equal to 50μg/ml DNA) to determine DNA quantity. The extracted DNA was adjusted to 5ng/μl with SDW. A measurement at $A_{280}$ was taken and the ratio of $A_{260}$ and $A_{280}$ was used to determine purity. Readings between 1.7-1.9 indicated DNA extracts of high purity to be taken forward for analysis. DNA was stored at -20°C until required.

8.2.5.1 Validation of extracted DNA from control strains MW2 and COL

DNA extracts from control strains MW2 and COL were validated by SCCmec element assignment to ensure DNA integrity (Oliveira and de Lencastre, 2002).

8.2.6 Multiplex PCR optimisation

8.2.6.1 Standard PCR protocol

Table 8.2 outlines a recommended standardised PCR protocol that was applied during multiplex optimisation (Henegariu et al., 1997).
Table 8.2 A standard reaction mix for multiplex optimisation.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (total volume 25μl)</th>
<th>Working concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile distilled water</td>
<td>17.7/16.9</td>
<td>-</td>
</tr>
<tr>
<td>10x PCR buffer *</td>
<td>2.5</td>
<td>1x</td>
</tr>
<tr>
<td>dNTPs (25mM each nucleotide)</td>
<td>0.2</td>
<td>200μM each nucleotide</td>
</tr>
<tr>
<td>Primer mix (25pmoles/μl each primer)</td>
<td>0.4 (each primer)</td>
<td>0.4μM each primer</td>
</tr>
<tr>
<td>DNA Taq polymerase</td>
<td>0.2</td>
<td>1 unit/25μl</td>
</tr>
<tr>
<td>Template DNA (5ng/μl)</td>
<td>2</td>
<td>10ng/25μl</td>
</tr>
</tbody>
</table>

*10x PCR buffer contains 10mM Tris HCl pH 8.3, 3.5mM MgCl2, 25mM KCl.

All PCR amplifications were performed in a DNA Gene Amp PCR system 9700 (Applied Biosystems, UK) using a previously published PCR protocol (Oliveira and de Lencastre, 2002): pre-denature for 4 min at 94°C; 30 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 1 min; post-extension for 4 min at 72°C and incubation at 4°C until needed. A 2% agarose gel containing 1μg/ml of ethidium bromide was used to separate amplified fragments. Electrophoresis was performed in 1x TAE (40Mm Tris, 1mM EDTA and 0.1% (v/v) glacial acetic acid) buffer at 100 volts for 1 hour.

8.2.6.2 Buffer selection

The optimum buffer for each multiplex assay was determined using the Opti-prime™ buffer matrix (Schoettlin et al., 1994). Each of the twelve buffers (table 8.3) were
analysed using template DNA from MW2 and COL and the standardised protocols described in section 8.2.6.1.

Table 8.3 Opti-prime™ buffer matrix (Schoettlin et al., 1994).

8.2.6.3 Annealing temperature

The hybridisation of a primer to a DNA target is temperature dependant (Brown, 1997). The melting temperature \( T_m \) of an oligonucleotide primer will be governed by base length and GC composition and optimisation of annealment temperature is therefore critical in a multiplex PCR design to ensure the efficient binding of all primers within the primer pool. The Wallace rule (Wallace et al., 1979) provides the simplest calculation for determining primer melting temperature:

\[
T_m = 2^\circ C(A+T) + 4^\circ C(G+C)
\]

The annealing temperature determined in a PCR design should be set at 2-5\(^\circ\)C below the calculated melting temperature of a primer pair. An annealment gradient between 42 \(^\circ\)C-64 \(^\circ\)C was chosen on the basis of the melting temperatures of individual primers
Chapter 8 Multiplex PCR Design for the Detection of Virulence Genes in MRSA

(table 8.4) and further increased to 64°C in an attempt to reduce unspecific binding that was noted at previous optimisation steps.

Table 8.4 The GC content and melting temperatures for all primers used in this study.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer</th>
<th>GC content (%)</th>
<th>Melting Temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>fnbA</td>
<td>Fnb1</td>
<td>38.9</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>Fnb2</td>
<td>42.1</td>
<td>47</td>
</tr>
<tr>
<td>hlg</td>
<td>Hlg1</td>
<td>39.1</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>Hlg2</td>
<td>50</td>
<td>52</td>
</tr>
<tr>
<td>sdrE</td>
<td>Sdr1</td>
<td>31.6</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>Sdr2</td>
<td>38.9</td>
<td>43</td>
</tr>
<tr>
<td>icaA</td>
<td>Ica1</td>
<td>35</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>Ica2</td>
<td>35</td>
<td>46</td>
</tr>
<tr>
<td>cna</td>
<td>Cna1</td>
<td>45</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Cna2</td>
<td>50</td>
<td>52</td>
</tr>
</tbody>
</table>

8.2.6.4 Concentration of dNTPs

The optimum concentration of dNTPs in each multiplex assay was determined using template DNA from MW2 and COL and the standardised protocols outlined in section 8.2.6.1. Each reaction was performed in duplicate using dNTP working concentrations of 200µM, 400µM, 600µM and 800µM per nucleotide.

8.2.6.5 Primer concentration

A low amplicon yield for the icaA gene locus was observed in previous optimisation steps. A series of primer concentration titrations was therefore applied to the icaA/cna
multiplex design using the standardised protocol outlined in section 8.2.6.1, and primer stock concentrations illustrated in table 8.5.

Table 8.5 Primer stock concentrations used in primer optimisation.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Stock 1</th>
<th>Stock 2</th>
<th>Stock 3</th>
<th>Stock 4</th>
<th>Stock 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ica1/Ica2</td>
<td>0.4</td>
<td>0.4</td>
<td>0.6</td>
<td>0.8</td>
<td>1.0</td>
</tr>
<tr>
<td>Cna1/Cna2</td>
<td>0.4</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.1</td>
</tr>
</tbody>
</table>

8.2.7 Multiplex assay inter-reproducibility

Inter-reproducibility for each multiplex assay was ascertained using six MRSA isolates (designated A-F), provided by the UHB from various nosocomial infections. For each test isolate, uniplex amplification of \( fnbA \), \( hlg \), \( sdrE \), \( cna \) and \( icaA \) single gene targets were previously determined (table 8.8). DNA extraction for each test isolate was carried out using the rapid boiling extraction method as previously described in section 3.2.3. Optimised multiplex assays, described in section 8.3.4, were applied to isolates A-F and observed virulotypes were compared to the results from single PCR amplification. Multiplex PCR was repeated one week later on the same isolates grown from separate cultures to ascertain reproducibility of the assay design.
8.3 Results

8.3.1 Validation of DNA from control strains MW2 and COL

Both DNA extracted from MW2 (SCCmec IV) and COL (SCCmec I) was successfully amplified following multiplex SCCmec element assignment (Oliveira and de Lencastre, 2002). This ensured the integrity of DNA positive controls for subsequent multiplex optimisation.

8.3.2 Validation of primers for the amplification of the cna gene locus

Using MW2 as a positive control, primers cna1 and cna2 successfully amplified the 531bp cna gene locus target (figure 8.1).
Figure 8.1 Validation of primers for the amplification of the 531bp cna gene locus using MW2 as a positive control and COL as a negative control.
8.3.3 Multiplex PCR optimization

8.3.3.1 Multiplex PCR validation using the standardised protocol

Both multiplex assays were validated using the standardised protocols described in section 8.2.7.1. Gene targets for fnbA, hlg and sdrE were amplified in both single PCR reactions and a multiplex reaction using COL as a positive control (figure 8.2). Single PCR amplifications for all gene targets matched multiplex PCR.

![Image of gel showing PCR results for fnbA, hlg, sdrE, and COL]  

Figure 8.2 Validation of fnbA, hlg and sdrE primers in both single and multiplex PCR reactions with COL as a positive control and SDW as a negative control.
Gene targets for *icaA* and *cna* were amplified in both single PCR reactions and a multiplex reaction using MW2 as a positive control (figure 8.3). Single PCR amplifications for all gene targets matched multiplex PCR.

Figure 8.3 Validation of *icaA* and *cna* primers in both single and multiplex PCR reactions with MW2 as a positive control and SDW as a negative control.
8.3.3.2 Effects of buffer titration on multiplex PCR

Each multiplex assay was optimised to the twelve PCR buffers illustrated in table 8.2. Successful amplification of the three locus products for \( fnbA \), \( hlg \) and \( sdrE \) was generally supported by buffers 3, 5, 7 and 11. Buffers 3, 7 and 11 exhibited low concentrations of KCl (25mM) and high concentrations of MgCl\(_2\) (3.5mM). Amplification of \( fnbA \), \( hlg \) and \( sdrE \) was achieved at pH levels 8.3-9.2 (figure 8.4).

![Image](image.png)

Figure 8.4 Buffer titration of \( fnbA \), \( hlg \) and \( icaA \) multiplex assay using COL as a positive control.
Successful amplification of the two locus products for \textit{icaA} and \textit{cna} were also supported by buffers 3, 5, 7 and 11. Buffers 3, 7 and 11 exhibited low concentrations of KCL (25mM) and high concentrations of MgCl\textsubscript{2} (3.5mM). Amplification of both \textit{icaA} and \textit{cna} was achieved at pH levels 8.3-9.2 (figure 8.5).

![Buffer titration of \textit{icaA} and \textit{cna} multiplex assay using MW2 as a positive control.](image)

Figure 8.5 Buffer titration of \textit{icaA} and \textit{cna} multiplex assay using MW2 as a positive control.
8.3.3.3 Effects of annealing temperature on multiplex PCR

The optimum amplification of the three locus products for fnbA, hlg and sdrE was achieved between annealing temperatures 44°C-58°C (figure 8.6). From 60°C upwards amplification of sdrE failed, followed by loss of fnbA and hlg amplification at 64°C.

![Annealing Temp °C](image)

Figure 8.6 Determination of optimum annealing temperature for fnbA, hlg and sdrE multiplex assay using COL as a positive control.
Optimum amplification of the two locus products for icaA and cna was achieved at all annealing temperatures between 42°C and 64°C (figure 8.7).

Figure 8.7 Determination of optimum annealing temperature for icaA and cna multiplex assay using MW2 as a positive control.
8.3.3.4 Effects of dNTP concentration on multiplex PCR

The optimum concentration of dNTPs for both multiplex assays was determined using dNTP working concentrations of 200μM, 400μM, 600μM and 800μM per nucleotide. Optimum amplification of the three locus products for fnbA, hlg and sdrE was achieved at dNTP concentrations of 200μM and 400μM (figure 8.8). At 600μM, a reduced amplification of fnbA and sdrE was observed, followed by no amplification of these two targets at 800μM.

Figure 8.8 Determination of optimum dNTP concentration for fnbA, hlg and sdrE multiplex assay using COL as a positive control.
Optimum amplification of the two locus products for icaA and cna was achieved at a dNTP concentration of 200μM (figure 8.8). At 400μM, a reduced amplification of cna was observed followed by no amplification at 600 μM and 800μM.

Figure 8.9 Determination of optimum dNTP concentration for icaA and cna multiplex assay using MW2 as a positive control.
8.3.3.5 Primer titration for the amplification of icaA and cna

During previous optimisation, it was observed that amplification of the icaA gene locus produced a low product yield. Further optimisation using a primer titration was therefore undertaken (figure 8.10). An increase in ica1 and ica2 primer concentration and a decrease in cna1 and cna2 primer concentration ultimately increased the yield of icaA amplification effectively balancing the product yields of both primer targets. The optimum product yield for amplification of both icaA and cna was therefore achieved at 1μM ica1/ica2 in combination with 0.1μM cna1/cna2.

![Figure 8.10 Determination of optimum primer concentrations for icaA and cna amplification using MW2 as a positive control.](image-url)

8.3.4 Optimised protocol for multiplex PCR

Following analysis of the results in section 8.3.3, optimised protocols were designed for the two multiplex assays as illustrated in table 8.6 and table 8.7.

Table 8.6 Optimised protocol for the amplification of fnbA, hlg and sdrE.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (total 25μl)</th>
<th>Working concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile distilled water</td>
<td>17.7</td>
<td>-</td>
</tr>
<tr>
<td>10x PCR buffer 3</td>
<td>2.5</td>
<td>1x</td>
</tr>
<tr>
<td>dNTPs (25mM each nucleotide)</td>
<td>0.2</td>
<td>200μM each nucleotide</td>
</tr>
<tr>
<td>Primer fnb1 (25pmoles/μl)</td>
<td>0.4</td>
<td>0.4μM</td>
</tr>
<tr>
<td>Primer fnb2 (25pmoles/μl)</td>
<td>0.4</td>
<td>0.4μM</td>
</tr>
<tr>
<td>Primer hlg1 (25pmoles/μl)</td>
<td>0.4</td>
<td>0.4μM</td>
</tr>
<tr>
<td>Primer hlg2 (25pmoles/μl)</td>
<td>0.4</td>
<td>0.4μM</td>
</tr>
<tr>
<td>Primer sdr1 (25pmoles/μl)</td>
<td>0.4</td>
<td>0.4μM</td>
</tr>
<tr>
<td>Primer sdr2 (25pmoles/μl)</td>
<td>0.4</td>
<td>0.4μM</td>
</tr>
<tr>
<td>DNA Tag polymerase</td>
<td>0.2</td>
<td>1 unit/25μl</td>
</tr>
<tr>
<td>Template DNA (5ng/μl)</td>
<td>2</td>
<td>10ng/25μl</td>
</tr>
</tbody>
</table>

Table 8.7 Optimised protocol for the amplification of icaA and cna.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (total 25μl)</th>
<th>Working concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile distilled water</td>
<td>17.9</td>
<td>-</td>
</tr>
<tr>
<td>10x PCR buffer 3</td>
<td>2.5</td>
<td>1x</td>
</tr>
<tr>
<td>dNTPs (25mM each nucleotide)</td>
<td>0.2</td>
<td>200μM each nucleotide</td>
</tr>
<tr>
<td>Primer ica1 (25pmoles/μl)</td>
<td>1.0</td>
<td>1.0μM</td>
</tr>
<tr>
<td>Primer ica2 (25pmoles/μl)</td>
<td>1.0</td>
<td>1.0μM</td>
</tr>
<tr>
<td>Primer cna1 (25pmoles/μl)</td>
<td>0.1</td>
<td>0.1μM</td>
</tr>
<tr>
<td>Primer cna2 (25pmoles/μl)</td>
<td>0.1</td>
<td>0.1μM</td>
</tr>
<tr>
<td>DNA Tag polymerase</td>
<td>0.2</td>
<td>1 unit/25μl</td>
</tr>
<tr>
<td>Template DNA (5ng/μl)</td>
<td>2</td>
<td>10ng/25μl</td>
</tr>
</tbody>
</table>
Chapter 8 Multiplex PCR Design for the Detection of Virulence Genes in MRSA

The standard cycle conditions described in section 8.2.6.1 continued to be used for both multiplex assays including an annealment temperature of 53°C.

8.3.5 Multiplex PCR inter-reproducibility

Inter-reproducibility for each multiplex assay was ascertained using six MRSA isolates (designated A-F). For each strain, single amplification of \( fnbA \), \( hlg \), \( sdrE \), \( cna \) and \( ica \) was previously determined (table 8.8).

<table>
<thead>
<tr>
<th>Test isolate</th>
<th>( Fnba )</th>
<th>( Hlg )</th>
<th>( SdrE )</th>
<th>( IcaA )</th>
<th>( Cna )</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>F</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Multiplex assays were carried out on all test isolates using the optimised protocols described in section 8.3.4. Multiplex assays for each test isolate was duplicated the following week from separately grown cultures. The results of these assays are illustrated in figures 8.11 and 8.12.
Figure 8.11 Duplicated multiplex assays from separate DNA preparations for amplification of *fnbA*, *hlg* and *sdrE* using MRSA isolates A-F. COL was used as a positive control and SDW as a negative control.
Figure 8.12 Duplicated multiplex assays from separate DNA preparations for amplification of *icaA* and *cna*, using MRSA isolates A-F. MW2 was used as a positive control and SDW as a negative control.
Both multiplex assays using isolates A-F, were consistent with the results produced from single gene locus amplification. Additionally, both multiplex assays demonstrated reproducibility using separately grown cultures one week after original analysis.
8.4 Discussion

Multiplex PCR designs are commonly applied to reduce the time constraints associated with single tube PCR amplification (Edwards and Gibbs, 1994). This study aimed to incorporate the amplification of *fnbA*, *cna*, *sdrE*, *hlg* and *icaA* in a two tube multiplex PCR to provide an efficient, cost effective protocol for determining the presence of virulence genes in clinical MRSA isolates.

8.4.1 Multiplex PCR optimisation

8.4.1.1 Effects of buffer titration on multiplex PCR

Buffers play an essential role in supporting the activity of *Taq* polymerase during the extension phase of PCR. *Taq* polymerase requires free magnesium to work effectively and insufficient magnesium chloride (MgCl₂) may reduce enzyme efficiency and product yield (Saiki, 1989; Schoettlin *et al.*, 1994). Determining the optimum balance of MgCl₂ may therefore be crucial for the success of a multiplex study design. Additionally, the concentration of potassium chloride (KCl) is necessary for effective primer annealing to target template DNA. Primers with long amplification products work efficiently at lower concentrations of KCl and primers with short amplification products work more efficiently at higher concentrations of KCl (Henegariu *et al.*, 1997). This further highlights the importance of effective buffer optimisation during multiplex PCR design.
Following a twelve buffer titration, successful amplification of all target products was achieved at low concentrations of KCl (25mM) and high concentrations of MgCl₂ (3.5mM) (figure 8.4-8.5). An increase in pH was shown to have no affect upon multiplex amplification, but in general a buffer exhibiting pH 8.3 at 25°C is largely recommended (Henegariu et al., 1997). Buffer 3 (pH 8.3, KCl 25mM and MgCl₂ 3.5mM) exhibited the optimum conditions for amplification of all target loci and was therefore selected for both multiplex assay designs.

8.4.1.2 Effects of annealing temperature on multiplex PCR

Hybridisation of an oligonucleotide primer to a DNA template is temperature dependant. If annealing temperature is set too high primers may remain dissociated. If on the other hand, the annealing temperature is set too low, unspecific primer binding will occur (Brown, 1997). Optimisation of annealing temperature is therefore critical in multiplex PCR design to ensure the efficient binding of all primers within the primer pool (Rychlik et al., 1990; Baumforth et al., 1999). In this investigation the optimum annealing temperature for each multiplex assay was determined using an annealing temperature gradient between 42°C-62°C. Optimum annealing temperatures for fnbA, hlg and sdrE gene target amplification was achieved between 44°C and 58°C (figure 8.6), whereas optimum annealing temperatures for icaA and cna gene target amplification was achieved between 42°C and 64 °C (figure 8.7). Annealing temperatures beyond 60°C may dramatically reduce amplicon product yield, although choosing a low annealing temperature may alternatively promote the tolerance of
single-base mismatches within the target genome. The recommended annealment temperature of 53°C from a previously published multiplex protocol (Oliveira and de Lencastre, 2002) was therefore applied to both multiplex assay designs.

8.4.1.3 Effects of dNTP concentration on multiplex PCR

The concentration of dNTPs in a 25μl reaction volume is essential for the synthesis of target DNA (Henegariu et al., 1997; Baumforth et al., 1999), however, un-utilised dNTPs will trap and chelate free magnesium subsequently inhibiting DNA Taq polymerase and target amplification (Henegariu et al., 1997; Baumforth et al., 1999). The optimum concentration of dNTPs for both multiplex assays was determined using working concentrations of 200μM, 400μM, 600μM and 800μM per nucleotide. Optimum amplification of fnbA, hlg and sdrE target products was achieved at dNTP concentrations of 200μM and 400μM (figure8.8) and optimum amplification of icaA and cna gene products was achieved at a dNTP concentration of 200μM (figure8.9). A concentration of 200μM for each nucleotide is generally recommended for multiplex assays incorporating up to eight primer pairs (Henegariu et al., 1997). Optimum dNTP concentration was therefore established at 200μM for both multiplex assays in this study design.
8.4.1.4 Primer concentration

During simultaneous amplification of two or more loci, shorter efficient target sites may out-compete longer target sites for enzyme and nucleotide resources (Henegariu et al., 1997). In this investigation, following the standardised protocol, amplification of cna (531bp target) produced an increased amplicon yield over amplification of icaA (770bp target). The optimum primer concentrations for amplification of icaA and cna were therefore ascertained following a 0.1-1.0μM primer titration. By gradually increasing the primer concentration for the weak loci, whilst simultaneously decreasing the primer concentration for the strong loci, amplification balance of the two target products was effectively resolved. The determined optimum primer concentration therefore incorporated 1.0μM icaA1/icaA2 and 0.1μM cna1/cna2 per 25μl reaction (figure 8.10).

8.4.2 Multiplex reproducibility

Inter-reproducibility for each multiplex assay was established using six MRSA isolates (designated A-F) where single amplification of fnbA, hlg, sdrE, icaA and cna had previously been determined (table 8.8). Application of the optimised multiplex PCR protocols to isolates A-F provided 100% concurrence with the single gene amplification (figure 8.11 and figure 8.12). Multiplex assays were then duplicated the following week from separately grown cultures where 100% concurrence was also observed (figure 8.11 and figure 8.12). These results demonstrate the reliability and
reproducibility of the two tube multiplex assay designed in this study to identify \textit{fabA}, \textit{hlg}, \textit{sdrE}, \textit{icaA} and \textit{cna} virulence gene targets in MRSA clinical isolates.

8.4.3 Future recommendations

Each generated amplicon of a multiplex assay will provide an internal control for PCR amplification (Edwards and Gibbs, 1994). However, the incorporation of \textit{S. aureus} and MRSA conserved genetic markers (\textit{femB} and \textit{meca}) in both multiplex assays would provide further validation of DNA integrity for each PCR cycle (Baumforth \textit{et al.}, 1999) and reduce the risk of false negative reports.

The application of PCR is limited to static gene detection and the presence of a gene target may not necessarily mean that the gene is being actively expressed within the bacterial cell. Future investigations may therefore consider multiplex reverse transcription PCR for the detection of mRNA gene transcripts from the five target gene locations. In addition, application of real-time PCR may enable quantification of target gene expression which may add further analytical dimension to the original multiplex study design.
8.5 Conclusion

This study has demonstrated the successful design and implementation of a two tube multiplex assay for the detection of fnbA, hlg, sdrE, icaA and cna virulence gene targets in MRSA clinical isolates. Stringent optimisation of critical PCR parameters has enabled the effective development and application of a robust and reproducible assay reducing the time, costs and contamination risks associated with uniplex gene amplification.

Due to time constraints, this multiplex assay had not been put into practice at the time of writing this thesis. However, it is envisaged that the designed protocol will be applied in future investigations comparing the virulence gene patterns of MRSA isolates retrieved from hospital- and community-onset infection.
CHAPTER 9 FINAL DISCUSSION

Meticillin-resistant *Staphylococcus aureus* is a major cause of morbidity and mortality in healthcare-associated disease (Wyllie *et al.*, 2006). Regardless of a devised and implemented search and destroy infection control strategy (Anon, 1998) the rates of MRSA-associated disease have remained high which is an increasing concern for UK public health. The role of MRSA as a serious worldwide pathogen has exacerbated over the last decade following the emergence of virulent MRSA strains within the community setting (Vandenesch *et al.*, 2003). The recent report of *pvl*-positive CA-MRSA in the West Midlands and its detection in the nosocomial setting (Anon, 2006a) warrants continued surveillance for emerging virulent clones within the clinical and community population. This thesis has sought to investigate the epidemiology of hospital and community MRSA within the West Midlands region.

Patients undergoing haemodialysis have an increased risk of acquiring MRSA entry site infections and access related bacteraemia (Peacock *et al.*, 1999a; Lentino *et al.*, 2000; Koziol-Montewka *et al.*, 2001; Dopirak *et al.*, 2002; Troidle *et al.*, 2007) and as a result *Staphylococcus aureus* is a leading cause of infection and morbidity within the renal dialysis setting. Following analysis of nasal swabs taken from 81 patients in a renal dialysis outpatient unit, 6 % were positive for MRSA and 23% were positive for MSSA. On the basis of PFGE and antibiogram analysis there was no evidence for inter-patient transmission highlighting possible MRSA acquisition from diverse sources, indicative of a dynamic outpatient environment. Of 349 environmental
samples, MRSA was only isolated from one dialysis curtain which was not represented in any of the clinical MRSA isolates following PFGE analysis. Additionally, evidence for intra-patient cross-contamination of a CVC entry site from the endogenous nasal reservoir was demonstrated in this investigation. The low incidence of environmental MRSA and evidence for a heterogeneous MRSA population suggested environmental and inter-patient contamination did not appear to be a contributing reservoir for MRSA transmission within this study setting. However, observed intra-patient MRSA transmission from the nasal reservoir to a CVC site further demonstrates the importance of MRSA eradication from the nasal cavity in the prevention of access site infections (Nielsen et al., 1998; Saxena et al., 2002).

The establishment of MRSA carrier status and the implementation of appropriate antimicrobial therapies are critical for intra-patient and inter-patient infection control. However, the dynamics of MRSA colonisation remains largely unresolved (Hu et al., 1995; Kluymans et al., 1997; Cespedes et al., 2005). Single colony identification obtained from the anterior nares may not account for the recognition of additional co-colonising strains, subsequently targeted eradication therapy may be compromised when based upon the antimicrobial sensitivity profile of an individual colonising isolate. This thesis reported homogenous MRSA colonisation within the anterior nares of renal dialysis patients, thus presenting supporting evidence for the practicality and continued use of single colony MRSA identification from the endogenous nasal reservoir.
The anterior nares are considered to be the primary site for MRSA colonisation (Kluuytmans et al., 1997) although increasing reports of MRSA prevalence in the oral cavity may question screening programs that are targeted towards nasal MRSA eradication (Smith et al., 2003b; Coia et al., 2006; Nilsson and Ripa, 2006). Meticillin-resistant Staphylococcus aureus colonisation of the oral cavity may provide a portal for horizontal transfer to distant anatomical sites, neighbouring patients and health care workers, thus compromising intra-patient and inter-patient infection control strategies based upon nasal MRSA eradication (Anon, 1998; Smith et al., 2001). This investigation identified that both the nose and tongue provided simultaneous sites for MRSA colonisation and, in addition, when a patient was nasally colonised with MRSA a genetically indistinguishable isolate could also be retrieved from the tongue. This observed homogenous co-colonisation provides supporting evidence towards the oral cavity as a site for re-colonisation of the anterior nares following eradication therapy which may possibly account for the high rates of MRSA relapse in patients with persistent colonisation (Kluuytmans et al., 1997; Pena et al., 2004). Consequently, co-colonisation of MRSA in the nose and on the tongue demonstrated in this thesis highlights the importance of multi-site MRSA eradication in the prevention of relapse and re-colonisation following antimicrobial therapy.
The emergence of EMRSA-15 and EMRSA-16 in the 1990s had a major impact upon the epidemiology of MRSA infections within the clinical environment. Currently accounting for 95% of MRSA isolates in the UK, EMRSA-15 and EMRSA-16 have widely spread throughout hospitals and across continents to become a worldwide epidemic problem (Richardson and Reith, 1993; Cox et al., 1995a; Johnson et al., 2001; Murchan et al., 2004; Perez-Roth et al., 2004; Johnson et al., 2005; Gosbell et al., 2006; Melter et al., 2006). Evidence for the dissemination of hospital epidemic strains into the circulating community population (Salgado et al., 2003; Adedeji et al., 2007) has led to increased difficulty in defining the origins of MRSA acquisition outside of the clinical environment (Salgado et al., 2003). Phenotypic and genotypic analysis determined 199 MRSA isolates obtained from the community-onset infection to be uncharacteristic of de-novo CA-MRSA. Following PFGE analysis the majority of isolates were of the same genetic lineage to EMRSA-15 and EMRSA-16 strains highlighting the transmission of HA-MRSA from the clinical setting into the surrounding community population. The predominance of SCCmec IV within this isolate population demonstrates the transmissible success of SCCmec IV to prevail outside of the hospital setting. Furthermore the identification of novel SCCmec types reveals the plasticity of the SCCmec genomic structure and the potential emergence of sporadic MRSA clones within localised geographical regions.

The discharge of MRSA-positive patients from the nosocomial setting provides the ideal mechanism for MRSA dissemination into community population where spread and cross-colonisation may be further exacerbated by inter-familial transmission.
(Calfee et al., 2003; Huijsdens et al., 2006). In addition, the subsequent increase of MRSA circulating within the community population may, indirectly, increase the number of patients transferring MRSA back into the health care setting (Karas et al., 2006). The evidential dissemination of HA-MRSA into the community therefore warrants the need for targeted screening and de-colonisation programs within both the hospital and community setting for future control of MRSA disease incidence within the UK population.

When MRSA isolates from community-onset infection were compared to MRSA isolates obtained from hospital-onset infection, significant phenotypic and genotypic differences were observed between the two bacterial populations. Meticillin-resistant Staphylococcus aureus isolates harbouring SCCmec II and multi-drug resistance were significantly associated with hospital-onset infection, exemplifying the importance of increased antimicrobial resistance in the prevalence of MRSA clones within the challenging hospital setting. However, the multi-drug resistant phenotype favourable within the clinical environment may not be an essential attribute for dissemination within the community setting where antibiotic exposure may be low. Subsequently, an increased sensitivity to non-β-lactams and the predominance of SCCmec IV was associated with HA-MRSA retrieved from community-onset infection. Surplus antimicrobial resistance may carry with it an unnecessary genetic burden compromising the bacterial fitness of an MRSA clone and strains with reduced antimicrobial resistance may possibly possess increased fitness and a competitive advantage for community dissemination (Laurent et al., 2001; Wielchelau et al., 2002; Besier et al.,
2005; McCallum et al., 2006). The phenotypic and genotypic differences observed between HA-MRSA isolated from community-onset and hospital-onset infections demonstrates the potential need for structured antimicrobial prescribing regimes dependant upon patient demographic status.

Geographical information systems, such as SaTScan, may be utilised for the identification of significant disease hotspots assisting in targeted research and infection control prioritisation. The application of the space-time scan statistic to MRSA incidence data from a given geographical location enabled the identification of MRSA cluster hotspots located to nursing care institutions. Stratification of the data to underling co-variables, such as population density and age, ensured plausible identification of significant disease clustering that would be impossible to attain from pure numerical analysis alone. In addition, increased relative risk of MRSA incidence was also detected in surrounding Census Output Areas illustrating MRSA clustering at medical care institutions may also be impacting upon the surrounding geographical area. With the addition of sufficient ancillary data and molecular characterisation, the space-time scan statistic may be applied to disease monitoring in a variety of epidemiological investigations. These may include disease transmission within medical care institutions, schools, places of work, sports clubs and even zoonotic disease transmission within farms and veterinary surgeries.

The transition of *S. aureus* from a harmless skin commensal to an invasive host pathogen is largely governed by the individual and synergistic action of an array of
virulence determinants (Hienz et al., 1996; Peacock et al., 2002). The final element of this thesis implemented the successful design of a two tube multiplex assay to identify
\( fnbA, \: hlg, \: sdrE, \: icaA \) and \( cna \) virulence gene targets in MRSA clinical strains, to reduce the time, and the risk of process contamination associated with single tube PCR (Edwards and Gibbs, 1994). The designed multiplex assay demonstrated in this study may be further utilised in future comparative investigations and the identification of pathogenic factors governing the predominance of MRSA in environmentally distinct settings. With the increasing predominance of multi-drug resistance, antimicrobial development is now being targeted towards novel functional genetic determinants. The identification of key virulence genes involved in various disease manifestations may assist in future research into novel therapeutic pathogenesis inhibitors or possible vaccine development targeted to essential cell surface adhesins (Maira-Litran et al., 2004; Dubin et al., 2005; Stranger-Jones et al., 2006; Zhou et al., 2006; Wright and Sutherland, 2007).

This thesis has demonstrated the epidemiology of MRSA within the West Midlands with particular focus upon the diverse distributions of HA-MRSA within community and nosocomial bacterial populations. Implementation of targeted infection control in both the community and clinical setting and identification and eradication of patient endogenous reservoirs will further enhance MRSA infection control within the West Midlands region.
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