Characterisation of Staphylococcus aureus from South West Wales: Comparison of SCCmec-orfX amplification methods and genotyping of clinical isolates including Panton-Valentine leukocidin-positive strains.

Bome-Mannathoko, Naledi Betsi

Characterisation of *Staphylococcus aureus* from South West Wales: Comparison of SCC*mec-orfX* amplification methods and genotyping of clinical isolates including Panton-Valentine Leukocidin-positive strains

by

Naledi Betsi Bome-Mannathoko

Swansea University
Prifysgol Abertawe

Medical Microbiology and Infectious Diseases
Institute of Life Science, School of Medicine
Swansea University
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Summary

Methicillin-resistant *Staphylococcus aureus* is a leading cause of hospital infections world-wide. Consecutive *S. aureus* wound isolates (n=561) were collected from PHW Microbiology ABM Laboratory, Swansea (PHW-ABM); 137 (24.4%) were *mecA*-positive; 424 (75.6%) were *mecA*-negative using real-time PCR. Audit revealed that 15 (10.9%) *mecA*-positive strains were not reported as MRSA. Genotyping was performed using pulsed-field gel electrophoresis (PFGE), *spa* typing and SCC*mec* typing. MRSA predominantly belonged to EMRSA-15 (89.1%) and EMRSA-16 (5.8%) clones. All *S. aureus* strains were included in an evaluation of three SCC*mec-orfX* PCR assays. The assays had high diagnostic sensitivity (>95%) and specificity (≥94%) but false negative and false positive results were obtained. A deletion at the SCC*mec-orfX* right junction was proposed as the probable cause of false negative results. SCC*mec*-associated loci *ccrAB1*, *ccrAB4*, *ccrC*, and *dcs* were detected in four false positive MSSA, respectively. MALDI Biotyper mass spectrometry was evaluated for identification of *S. aureus*.

Nineteen (3.4%) of the PHW-ABM wound isolates were Panton Valentine-Leukocidin (PVL)-positive *S. aureus*. The molecular epidemiology of these and PVL-positive *S. aureus* (n=61) from Specialist Antimicrobial Chemotherapy Unit, Cardiff (SACU) was investigated using *mecA* and arginine catabolic mobile element PCRs, PFGE, *spa* and SCC*mec* typing. The PHW-ABM strains were predominantly MSSA belonging to the CC159 (n=5; 26.3%), CC275 (n=4; 21.1%) and CC005 (n=2; 10.5%) *spa*-BURP clusters, affiliated to the ST121, ST30 and ST22 lineages. Within the SACU cohort the USA300 clone (n=16; 26.2%) was predominant, other genotypes included: t044-MRSA-IVc (n=5; 8.2%); t002-MRSA-IVc (n=3; 4.9%) and t127-MRSA-IVa (n=2; 3.3%), affiliated to the European (ST80), USA800 (ST5) and USA400 (ST1) clones. Susceptibility testing demonstrated statistically significant differences between the SACU and PHW-ABM cohorts for oxacillin 57%/5%, gentamicin 2%/16%, and tetracycline 10%/42% resistance ($p<0.05$). These observed differences highlight the importance of including unselected strains in addition to referred reference laboratory isolates in epidemiological investigations.

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Contents

Abbreviations

ATP	Adenosine triphophate
bp	Base pair(s)
BSAC	British Society for Antimicrobial Chemotherapy
BURP	Based Upon Repeat Pattern
BURST	Based Upon Related Sequence Types
CA-MRSA	Community acquired/associated associated infections
Ct	Cycle threshold
dH_2O	Distilled water
DNA	Deoxyribose nucleic acid
dNTP	Deoxyribosenucleoside triphosphate
DMSO	Dimethylsulphoxide
DTT	Dithiothreitol
EDTA	Ethylene Diamine Tetraacetic acid
g	Gram(s)
xg	Gravitational force
HAI	Hospital acquired/associated associated infections
HA-MRSA	Hospital acquired- Methicillin resistant *Staphylococcus aureus*
hr	Hour
HPLC	High performance liquid chromatography
kDa	Kilo Dalton
kb	Kilobase pair(s)
MALDI-TOF	Matrix-assisted laser desorption/ionisation-Time-of-Flight
min	Minute
MS	Mass spectroscopy
μ	Micro
MIC	Minimum inhibitory concentration
MRSA	Methicillin resistant *Staphylococcus aureus*
ng	Nanogram
NPV	Negative Predictive Value
PBP	Penicillin binding protein

PCR	Polymerase chain reaction
PFGE	Pulsed Field Gel Electrophoresis
pg	Picogram
PPV	Positive Predictive Value
OD	Optical density
ORF	Open reading frame
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
rpm	Revolutions per minute
SRU	Staphylococcus Reference Unit
SSTI	Skin and soft tissue infections
TAE	Tris acetic acid EDTA
TE	Tris EDTA
TBE	Tris Borate EDTA
UV	Ultra violet
SCC*mec*	Staphylococcal cassette chromosome *mec*
V	Volts

Chapter One: Introduction

1.1 Staphylococci

The *Staphylococcus* genus comprises bacteria that are Gram-positive cocci (0.5 to 1.5 μm in diameter) which occur singly, in pairs, tetrads, short chains of three to four cells and grape-like clusters (Bannerman and Peacock, 2007, Gotz et al., 2006). They are non-motile, non-spore forming, usually catalase positive and are typically unencapsulated or have limited capsule formation under laboratory conditions (Bannerman and Peacock, 2007, Gotz et al., 2006). Until the early 1970's the *Staphylococcus* genus comprised three species: the coagulase-positive species *S. aureus* and coagulase-negative species *S. epidermidis* and *S. saprophyticus* but currently 44 species and 24 subspecies have been described (http://www.bacterio.cict.fr/) (see Table 1.1). The members of the *Staphylococcus* genus are facultative anaerobes except for *S. saccharolyticus* and *S. aureus* subsp. *anaerobius*, which initially grow anaerobically but may become increasingly aerotolerant on subculture (Bannerman and Peacock, 2007, Gotz et al., 2006).

The genomes of several *Staphylococcus* species have been published, particularly *S. aureus* strains and some coagulase-negative staphylococci including *S. epidermidis*, *S. saprophyticus*, *S. haemolyticus*, *S. carnosus* and others (see Table 1.2). Currently there are 21 published complete genomes of *S. aureus* strains, twenty *S. aureus* genome sequencing projects are in progress and 48 are in draft assembly (http://www.ncbi.nlm.nih.gov/genomeprj/). The genome of *S. aureus* has a guanine-plus-cytosine (G+C) content of approximately 30 to 39% (Bannerman and Peacock, 2007, Gotz et al., 2006). These comprise a single chromosome which is approximately 2.5 Mb and carry 2,400 to 2,500 coding sequences (Bannerman and Peacock, 2007, Gotz et al., 2006). The *Micrococcus* and *Macrococcus* genera are also Gram-positive cocci and were initially regarded as staphylococci however these genera are distinguished from staphylococci by their G+C content and certain phenotypic characteristics. The *Micrococcus* genus comprises bacteria which are Gram-positive cocci (0.7 to 1.8 μm in diameter) occurring in clusters, tetrads or pairs and they are obligate aerobes (Bannerman and Peacock, 2007). Both the staphylococci and micrococci may reside on mucosal surfaces and may be present in clinical and veterinary specimens however, micrococci are less prevalent and are largely regarded as saprophytic contaminants (Bannerman and Peacock, 2007).

Table 1.1. *Staphylococcus* species and subspecies (http://www.bacterio.cict.fr/)

No.	***Staphylococcus species* and subspecies**
1.	*S. arlettae*
2.	*S. aureus* *S. aureus* subsp. *anaerobiu s; S. aureus* subsp. *aureus*
3.	*S. auricularis*
4.	*S. capitis* *S. capitis* subsp. *capitis; S. capitis* subsp. *urealyticus*
5.	*S. caprae*
6.	*S. carnosus* *S. carnosus* subsp. *carnosus*; *S. carnosus* subsp. *utilis*
7.	*S. caseolyticus*
8.	*S. chromogenes*
9.	*S. cohnii* *S. cohnii* subsp. *cohnii*; *S. cohnii* subsp. *urealyticus*
10.	*S. condimenti*
11.	*S. delphini*
12.	*S. epidermidis*
13.	*S. equorum* *S. equorum* subsp. *equorum*; *S. equorum* subsp. *linens*
14.	*S. felis*
15.	*S. fleurettii*
16.	*S. gallinarum*
17.	*S. haemolyticus*
18.	*S. hominis* *S. hominis* subsp. *hominis*; *S. hominis* subsp. *novobiosepticus*
19.	*S. hyicus* *S. hyicus* subsp. *chromogenes; S. hyicus* subsp. *hyicus*
20.	*S. intermedius*
21.	*S. kloosii*
22.	*S. lentus*
23.	*S. lugdunensis*
24.	*S. lutrae*
25.	*S. massiliensis*
26.	*S. microti*
27.	*S. muscae*
28.	*S. nepalensis*
29.	*S. pasteuri*
30.	*S. pettenkoferi*
31.	*S. piscifermentans*
32.	*S. pseudintermedius*
33.	*S. pulvereri*
34.	*S. rostri*
35.	*S. saccharolyticus*
36.	*S. saprophyticus* *S. saprophyticus* subsp. *bovis; S. saprophyticus* subsp. *saprophyticus*
37.	*S. schleiferi* *S. schleiferi* subsp. *coagulans; S. schleiferi* subsp. *schleiferi*
38.	*S. sciuri* *S. sciuri* subsp. *carnaticus; S. sciuri* subsp. *lentus; S. sciuri* subsp. *rodentium; S. sciuri* subsp. *sciuri*
39.	*S. simiae*
40.	*S. simulans*
41.	*S. succinus* *S. succinus* subsp. *casei; S. succinus* subsp. *succinus*
42.	*S. vitulinus*
43.	*S. warneri*
44.	*S. xylosus*

Table 1.2. Completely sequenced *S. aureus* genomes (http://www.ncbi.nlm.nih.gov/genomeprj/)

No.	Strains	Accession no.	Reference
1.	*S. aureus* 04-02981	CP001844	(Nubel et al., 2010)
2.	*S. aureus* ED98	NC 013450	(Lowder et al., 2009)
3.	*S. aureus* RF122	AJ938182	(Herron-Olson et al., 2007)
4.	*S. aureus* COL	CP000046	(Gill et al., 2005)
5.	*S. aureus* ED133	CP001996	(Guinane et al., 2010)
6.	*S. aureus* JH1	CP000736	(Mwangi et al., 2007)
7.	*S. aureus* JH9	CP000703	(Mwangi et al., 2007)
8.	*S. aureus* JKD6159	CP002114	(Chua et al., 2010)
9.	*S. aureus* MRSA252	BX571856	(Holden et al., 2004)
10	*S. aureus* MSSA476	BX571857	(Holden et al., 2004)
11.	*S. aureus* MW2	BA000033	(Baba et al., 2002)
12.	*S. aureus* Mu3	AP009324	(Neoh et al., 2008)
13.	*S. aureus* Mu50	BA000017	(Kuroda et al., 2001)
14.	*S. aureus* N315	BA000018	(Kuroda et al., 2001)
15.	*S. aureus* NCTC 8325	CP000253	(Gillaspy et al., 2006)
16.	*S. aureus* ST398 S0385	AM990992	(Schijffelen et al., 2010)
17.	*S. aureus* TW20	FN433596	(Holden et al., 2010)
18.	*S. aureus* USA300 FPR3757	CP000255	(Diep et al., 2006)
19.	*S. aureus* USA300 TCH1516	NC 010079	(Highlander et al., 2007)
20.	*S. aureus* JKD6008	CP002120	Stinear, T. P., Seemann, T. & Howden, B. P*
21.	*S. aureus* Newman	AP009351	(Baba et al., 2008)

* ST239 *S. aureus* strain associated with intermediate level vancomycin resistance (unpublished) (http://www.ncbi.nlm.nih.gov/)

Micrococcus luteus is the most common micrococcal species found in nature (Bannerman and Peacock, 2007). Furthermore they have a higher G+C genome content, 66 to 75% versus 30 to 39% in staphylococci and they are oxidase positive whereas staphylococci are oxidase negative, with the exception of *S. lentis, S. sciuri, S. vitulus, S. fleurettii* (Bannerman and Peacock, 2007, Gotz et al., 2006). Macrococci are marginal facultative anaerobes and they grow optimally under aerobic conditions. Members of this genus include *Macrococcus bovicus*, *Macrococcus caseolyticus*, *Macrococcus carouselicus* and *Macrococcus equipercicus*. They have a smaller genome size (1.5 to 1.8 Mb) than staphylococci but have a higher G+C genome content (38 to 45%) and a larger cell size diameter (1.3 to 2.5 μm) (Bannerman and Peacock, 2007, Kwok and Chow, 2003, Kloos et al., 1998). They can also be distinguished from staphylococci by virtue of being oxidase positive (Bannerman and Peacock, 2007, Kwok and Chow, 2003). The clinical significance of macrococci is yet to be established and these bacteria are largely associated with aquatic animals and some hoofed animals (Bannerman and Peacock, 2007).

1.2 Natural habitats of staphylococci

Staphylococcus species are widespread in nature and represent a major group of the bacteria inhabiting the skin and mucous membranes of mammalian and avian hosts (Bannerman and Peacock, 2007). Generally they have a symbiotic relationship with their hosts but can become pathogenic after gaining entry into host tissue (Bannerman and Peacock, 2007). *Staphylococcus* species which are affiliated with humans and other primates include *S. epidermidis*, *S. aureus*, *S. capitis*, *S. caprae*, *S. saccharolyticus*, *S. warneri*, *S. pasteuri*, *S. haemolyticus*, *S. hominis*, *S. lugdunensis*, *S. schleiferi*, *S. auricularis*, *S. saprophyticus*, *S. cohnii*, *S. xylosus* and *S. simulans* (Bannerman and Peacock, 2007).

Some of the species have particular niches on their hosts, for instance *S. aureus* primarily resides in the anterior nares of humans especially adults and other sites include the axilla, pharynx and perineum. On the other hand *S. epidermidis* is the most prevalent and persistent *Staphylococcus* species on human skin (Bannerman and Peacock, 2007, Gotz et al., 2006). It is especially present in greater populations in areas with high moisture content e.g. axillae, the inguinal perineal areas, anterior nares and between the toes. Infrequently this species may be found on other hosts such as domestic animals but this may be the result of human to animal transfer (Bannerman and Peacock, 2007, Gotz et al., 2006). *S. hominis* is also prevalent on the human skin and together with *S. haemolyticus* these species are found in greater populations in regions comprising numerous apocrine glands e.g. axillae and pubic areas (Bannerman and Peacock, 2007). *S. capitis* subsp. *capitis* is commonly present on the human head where sebaceous glands are numerous e.g. the scalp and forehead (Bannerman and Peacock, 2007). *S. auricularis* and *S. cohnii* are primarily isolated from the external auditory meatus and human feet, respectively, whereas *S. saprophyticus* is commonly present in the female genitourinary tract (Bannerman and Peacock, 2007). Other staphylococci rarely inhabit humans and primarily reside on animal hosts, such as *S. intermedius* and *S. felis* which are major species of domestic dogs and cats, respectively. Whereas *S. xylosus*, *S. kloosii* and *S. sciuri* are common residents on rodents (Gotz et al., 2006). *S. hyicus*, *S. chromogenes, S. sciuri*, *S. lentus* and *S. vitulus* are frequent residents of ungulates and have also been isolated from food products (Bannerman and Peacock, 2007).

1.3 Clinical relevance of *Staphylococcus aureus*

Staphylococcus aureus is the most common cause of staphylococcal infections in humans and these range in severity from self-limiting to life-threatening. Normally it colonises the anterior nares and carriage varies within the population: 10 to 35% of individuals are persistently colonised whereas 70% of the population are transiently colonised, however, colonisation is a risk factor for infection (Kooistra-Smid et al., 2009, Miller et al., 2009). *S. aureus* primarily causes skin and soft tissue infections (SSTIs) but it can also cause invasive infections and toxin-mediated diseases (Bannerman and Peacock, 2007). The latter include food poisoning, scalded skin syndrome and toxic shock syndrome whereas SSTIs include boils, ulcers, abscesses, cellulitis, furunculosis, wound infections and necrotizing fasciitis (Bannerman and Peacock, 2007). Invasive infections include bacteraemia, osteomyelitis, pneumonia and infective endocarditis. *S. aureus* also causes hospital-acquired infections including surgical wounds, ventilator-associated pneumonia and infections associated with intravenous devices and prosthetic materials e.g. prosthetic joints and vascular grafts (Bannerman and Peacock, 2007).

The success of *S. aureus* in clinical pathogenesis is attributed to its expression of virulence factors and the acquisition of antibiotic resistance determinants. Currently the most notorious resistance determinant is *mecA* which confers resistance to all beta (β)-lactam antibiotics i.e. methicillin resistance. Methicillin-resistant *S. aureus* (MRSA) have become a leading cause of hospital-acquired infections in the UK and in many countries world-wide. *S. aureus* can also express resistance to non β-lactam antibiotics via the acquisition of plasmids and transposons harbouring diverse resistance determinants. These include plasmid based *tet(K)* and *tet(L)* genes which confer resistance to tetracycline and *ermC* which confers resistance to macrolides (erythromycin), lincosamides (clindamycin) and streptogramin B. The *ileS* and *aac(6')/aph(2")* genes are also acquired via the acquisition of plasmids and these genes confer resistance to mupirocin, an antibiotic which is essential for MRSA de-colonisation and aminoglycosides, gentamicin, respectively (Diep et al., 2006). In cases of treatment failure glycopeptides (vancomycin, teicoplanin), linezolid and daptomycin are used as the last lines of antibiotic therapy however since 2002, reports describing clinical MRSA with decreased susceptibility to vancomycin have been published (Lindsay, 2009, Sung and Lindsay, 2007).

Vancomycin resistance is attributed to the acquisition of *vanA* via conjugative transposition from vancomycin resistant *enterococci* and it has also been suggested that this resistance may arise from chromosomal mutations resulting in the disruption of the accessory gene regulator. (Grundmann et al., 2006). Threats to linezolid and daptomycin treatment have also been reported through resistance to linezolid due to expression of the plasmid-mediated *cfr* gene, although daptomycin resistance has also been observed, where the mechanism of resistance is not yet clearly understood (Woodford and Livermore, 2009, Hayden et al., 2005, Qi et al., 2005).

1.4 Synthesis of the staphylococcal cell wall

1.4.1 Peptidoglycan

Staphylococci are gram-positive bacteria and therefore their cell walls are composed of peptidoglycan (murein), teichoic acids and cell-wall associated proteins (Dmitriev et al., 2004). Peptidoglycan is a heteropolymer comprising alternating β-1,4 linked amino sugars namely *N*-acetylglucosamine (Glc*N*ac) and *N*-acetylmuramic acid (Mur*N*ac) which are cross-linked by short peptides (Bannerman and Peacock, 2007, Gotz et al., 2006). The latter comprise: L-alanine, D-isoglutamine, L-lysine, D-alanyl-D-alanine and this pentapeptide is linked via an amide bond to the D-lactyl moiety of Mur*N*ac. The pentaptides are also cross-linked in a 2^{nd} dimension by bridges consisting of five glycines extending from the carboxy-terminal D-alanine to the ε-amino group of the L-lysine in position 3 of the adjacent pentapeptide (Bannerman and Peacock, 2007, Gotz et al., 2006, Stapleton and Taylor, 2002). Therefore the Glc*N*ac-MurNac glycan strands and the cross-linked peptides create a three-dimensional macromolecule which gives rigidity to the cell wall (Bannerman and Peacock, 2007, Gotz et al., 2006).

The first stage of peptidoglycan synthesis occurs within the cytoplasm and concerns the assembly of the glycan strand precursor: UDP-Mur*N*ac-pentapetide from UDP-Glc*N*ac, this involves six cytoplasmic steps mediated by MurA to MurF (see Figure 1.1.) (Bannerman and Peacock, 2007, Gotz et al., 2006). The second stage involves the transfer of UDP-Mur*N*ac-pentapeptide by the transferase MraY, to a membrane attached lipid carrier (undecaprenyl phosphate, C_{55}-P) and this yields lipid I (Bannerman and Peacock, 2007, Gotz et al., 2006) (see Figure 1.1).

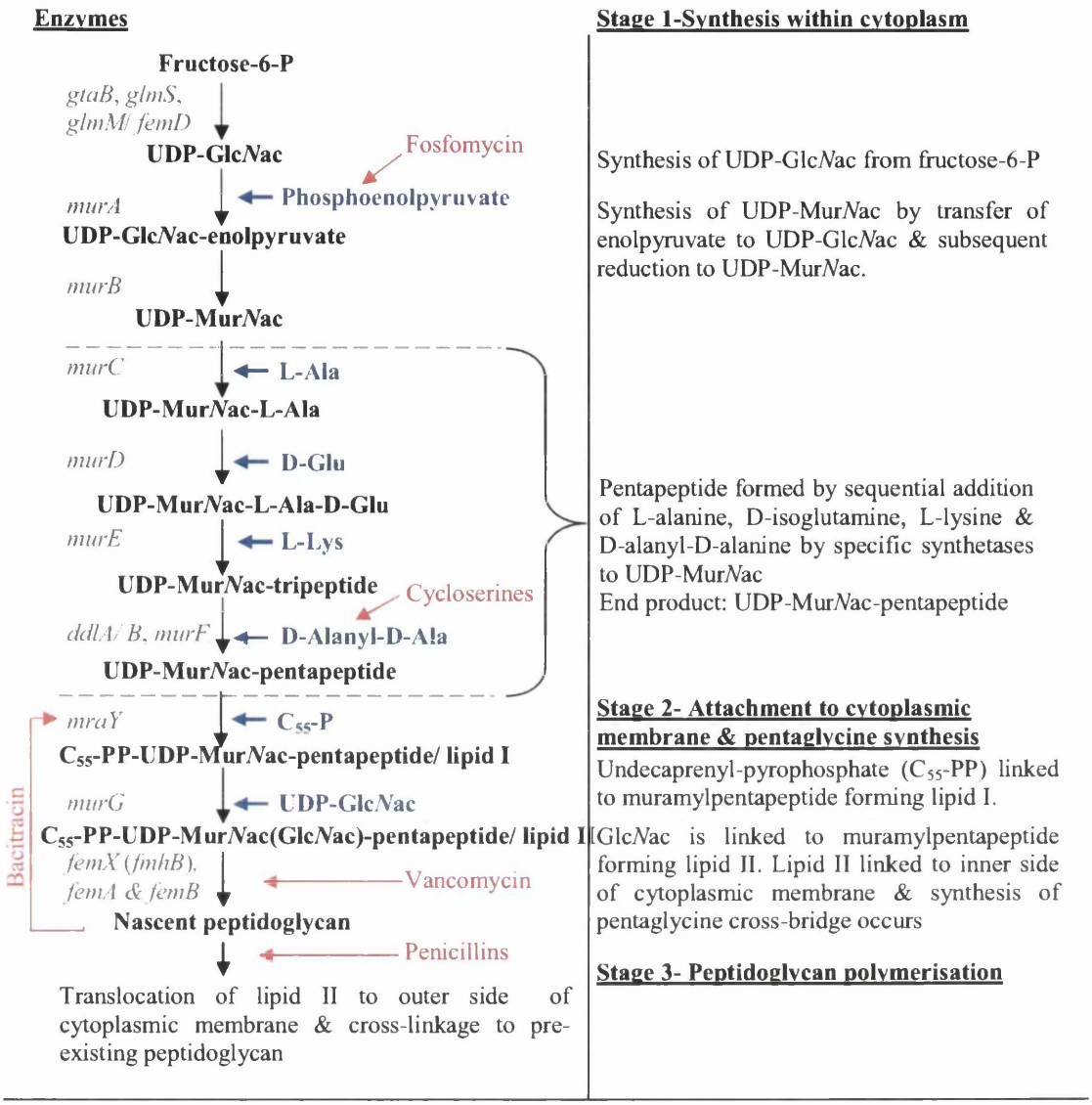


Figure 1.1. Pathway of peptidoglycan synthesis in staphylococci (Gotz et al., 2006). Red, antibiotics inhibiting specific stages of synthesis; Grey, enzymes mediating specific stages of synthesis; Blue, peptides added at different stages.

Subsequently Glc*N*ac is added to the C_{55}-UDP-Mur*N*ac-pentapeptide/ lipid I, forming the C_{55}-PP-disaccharide-pentapeptide/ lipid II. Subsequently the synthesis of the pentaglycine interbridge occurs by the sequential addition of glycine to ε-amino group of the L-lysine (position 3) on the stem pentapeptide. This is mediated by enzymes termed the Fem factors (Bannerman and Peacock, 2007, Gotz et al., 2006).

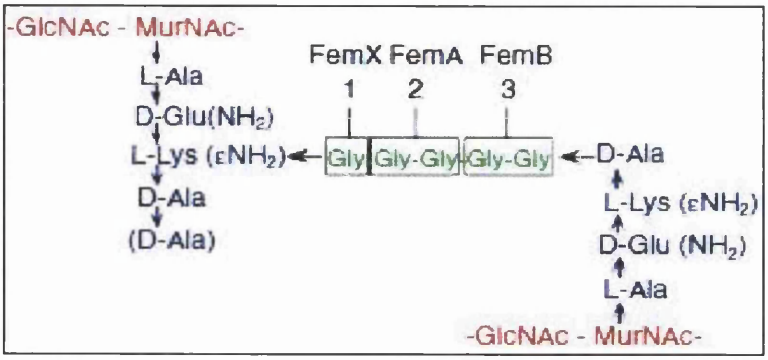


Figure 1.2. Synthesis of pentaglycin bridge between pentapeptide chains of peptidoglycan in *S. aureus* (Gotz et al., 2006).

The FemX (FmhB), FemA and FemB factors mediate the addition of the first glycine, then the second and third, and finally the fourth and fifth glycines, respectively (see Figure 1.2). The last stage of peptidoglycan synthesis involves the transportation of the subunits to the outside of the cytoplasmic membrane, incorporation of the subunits to the nascent peptidoglycan and the cross linking of peptidoglycan (Bannerman and Peacock, 2007, Gotz et al., 2006). The latter involves transpeptidation reactions which are catalysed by membrane bound penicillin-binding proteins (PBPs). *S. aureus* has four intrinsic PBPs: PBP1, PBP2, PBP3, PBP4, the first three are high molecular mass PBPs (HMM-PBPs) whereas PBP4 is a low molecular mass PBP (LMM-PBP). HMM-PBPs have multifunctional roles, the C-terminal has transpeptidase activity and the N-terminal has transglycosylase activity (Navratna et al., 2010). Interestingly most LMM-PBPs are monofunctional but PBP4 is unique as it is multifunctional, exhibiting transpeptidase and D,D-carboxypeptidase activities (Navratna et al., 2010). Transglycosylation involves the attachment of the last glycine residue of the pentaglycine bridge to the penultimate D-alanine residue on another stem pentapeptide. This is followed by the removal of the terminal D-alanine by the activity of D,D-carboxypeptidases (Kong et al., 2009).

1.4.2 Antimicrobials inhibiting peptidoglycan synthesis

Several antimicrobials have been developed for the inhibition of peptidoglycan synthesis at different stages. These include phosphonomycin (fosfomycin), cycloserines, bacitracin, glycopeptides and β–lactam antibiotics (see Figure 1.1) (Kong et al., 2009, Gotz et al., 2006).

Fosfomycin binds to the cysteine residue of the transferase which mediates the transfer of enolpyruvate from phosphoenolpyruvate to UDP-Glc*N*ac, thus it inhibits the earliest stage of peptidoglycan synthesis (Kong et al., 2009, Gotz et al., 2006). The cycloserines inhibit D-alanine metabolism by irreversibly binding to the alanine racemase and this hinders the incorporation of D-alanine into peptidoglycan (Kong et al., 2009, Gotz et al., 2006). Bacitracin inhibits the dephosphorylation of undecaprenyl pyrophosphate (C_{55}-PP) thus preventing further transfer of the muramylpentapeptides across the cytoplasmic membrane to the nascent peptidoglycan (Kong et al., 2009, Gotz et al., 2006). Vancomycin binds to the terminal D-alanyl-D-alanine moiety of pentapeptide thus preventing transglycosylation and cross linkage of the stem-pentapeptides (Kong et al., 2009, Gotz et al., 2006).

Penicillins are a group of natural and semisynthetic antibiotics which contain a β-lactam ring fused to a thiazolidine ring. These antibiotics are the most widely used antibiotic in the treatment of most Gram-positive and some Gram-negative bacteria. They are structural analogues of D-alanyl-D-alanine which irreversibly bind to the Ser_{403} residue within the active site of PBPs. This inhibits the transpeptidase activity of the PBPs and it is also thought that the activity of β-lactam antibiotics triggers the activation of membrane associated autolytic enzymes which destroy the cell wall (Bannerman and Peacock, 2007). However, resistance to penicillins is widely exhibited by *S. aureus* strains by the expression of penicillinases which hydrolyse the β-lactam ring. These enzymes are encoded and regulated by the *blaZ* operon which is frequently located on transposon Tn*552* (McKinney et al., 2001, Rowland and Dyke, 1990). Methicillin is a semi-synthetic β-lactam antibiotic which was introduced in clinical practice in 1960 but later replaced by less toxic derivatives e.g. flucloxacillin and dicloxacillin (Cunha, 2005). These antibiotics are poorly hydrolysed by penicillinases as they contain an ortho-dimethoxyphenyl group attached to a side chain carbonyl group and this sterically hinders the activity of penicillinases. However, MRSA express a fifth type of PBP termed PBP2a which is encoded by *mecA* and works cooperatively with PBP2. Beta-lactam antibiotics irreversibly bind to the transpeptidase domain of PBP2 but its transglycosylation domain continues its function of cross linking the stem pentapeptides of peptidoglycan. PBP2a which has very low affinity for β-lactam antibiotics then continues the transpeptidation of peptidoglycan (Berger-Bachi and Rohrer, 2002).

1.5 Regulation of PBP2a expression

Transcription of *mecA* is regulated by the regulatory genes *mecR1* and *mecI* which are located upstream and transcribed divergently from *mecA*. It can also be regulated by the homologous *blaR1* and *blaI* regulatory genes of *blaZ*. The *mecR1* gene encodes MecR1, a membrane bound β-lactam-sensing signal-transducer and *mecI* encodes MecI, a transcriptional repressor (Deurenberg and Stobberringh, 2008, Berger-Bachi and Rohrer, 2002, Stapleton and Taylor, 2002). In the absence of a β-lactam antibiotic, BlaI and MecI bind to the operator region and repress transcription of *blaZ*; *blaR1-blaI* and *mecA*; *mecRI-mecI*, respectively. When an inducing β-lactam antibiotic binds to the extracellular sensor domain of MecR1, cleavage of the sensor transducer is triggered, followed by the subsequent activation of an intracellular metalloprotease domain (see Figure 1.3). This protease cleaves the repressor (BlaI or MecI) enabling the transcription of the *blaZ*; *blaR1-blaI* and *mecA; mecR1-mecI* transcripts and subsequent expression of PBP2a (Deurenberg and Stobberringh, 2008, Berger-Bachi and Rohrer, 2002, Stapleton and Taylor, 2002, Katayama et al., 2001).

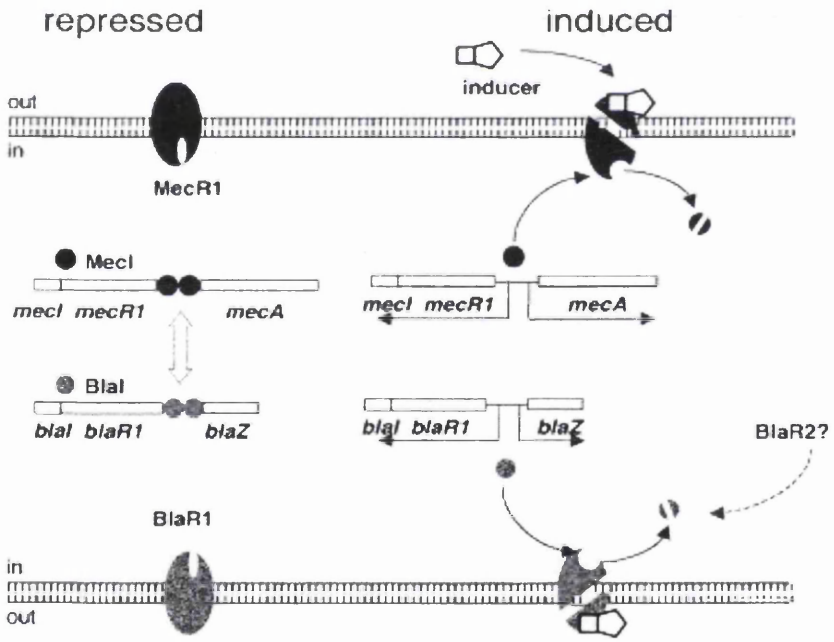


Figure 1.3 Schematic diagram of regulation of PBP2a and penicillinase expression (Berger-Bachi & Rohrer, 2002)

1.6 Phenotypic expression of methicillin resistance

Even though MRSA strains may exhibit high minimum inhibitory concentrations (MICs) for β-lactam antibiotics it is relatively rare that all the cells in the culture of a MRSA strain have identical high levels of resistance (Tomasz et al., 1991). Different MRSA strains may exhibit homogeneous or heterogeneous forms of methicillin resistance. With homogeneous strains all the cells of a single culture exhibit uniform high MICs in vitro whereas heterogeneous strains comprise two or more subpopulations of cells with varying MICs in vitro. There are different degrees of heterogeneous expression, at one end of the spectrum there are strains with up to 99.9% cells with MICs just above susceptibility and a very low frequency of highly resistant cells, whereas other strains may comprise multiple populations with intermediate degrees of resistance ranging from low MICs to very high MICs (Finan et al., 2002, Tomasz et al., 1991). Tomasz *et al.* (1991) defined four arbitrary classes of methicillin resistance expression in staphylococci (see Figure 1.4). In class 1 strains, the majority of cells had low methicillin MICs (1.3 to 3 μg/ml) and a very small population (10^{-7} to 10^{-8}) depicted MICs >25 μg/ml. In class 2 strains most cells had more substantial MICs (6 to 12μg/ml) and more cells (10^{-6} to 10^{-4}) could grow at higher concentrations of methicillin.

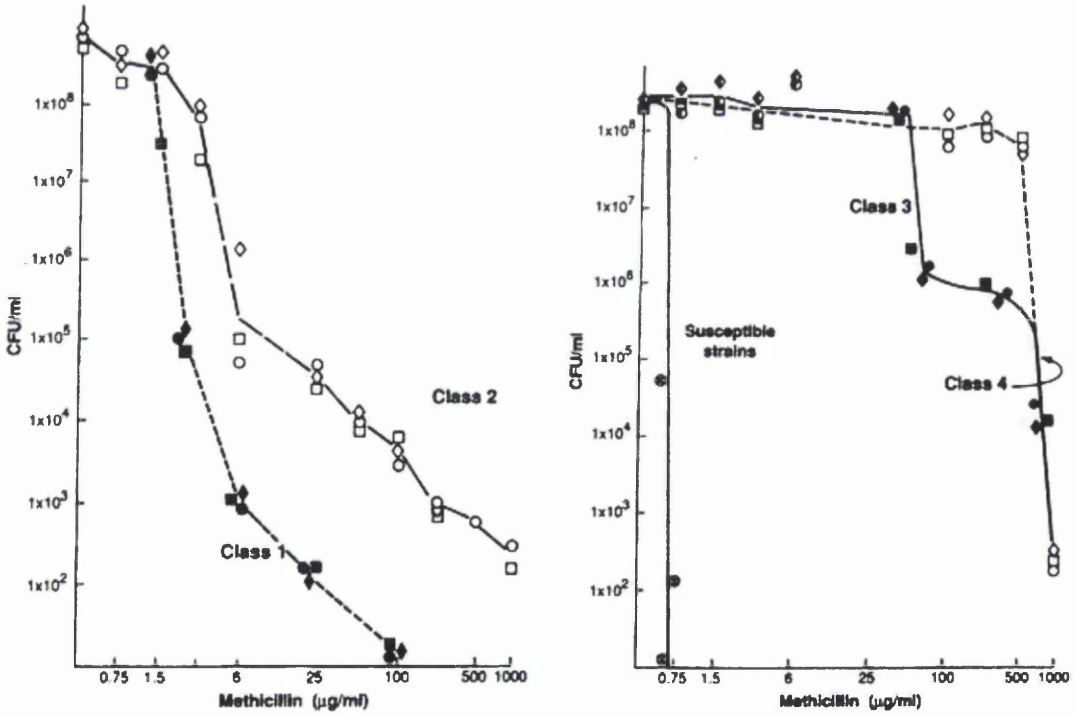


Figure 1.4. Growth curves of four MRSA strains with different degrees of expression of methicillin resistance (Tomasz et al., 1991)

In class 3 strains the majority of cells (99%) exhibited considerably high MICs (50 to 200 μg/ml) and a small population was highly resistant (see figure 1.4). Class 4 strains contained cells with uniform high resistance to methicillin (MICs of 400 to 1000 μg/ml) thus displaying homogenous methicillin resistance. In spite of the low frequency of cells with high MICs in heterogeneous strains, under conditions of antibiotic pressure these highly resistant subpopulations can grow extensively in culture (Balslev et al., 2005, Venezia et al., 2001, Tomasz et al., 1991).

Some MRSA strains may depict oxacillin susceptible phenotypes due to the strong suppression of *mecA* by the repressor MecI as has been observed in the MRSA N315 strain which was consequently referred to as a pre-MRSA strain. The de-activation of MecI by deletion or promoter mutations subsequently de-represses PBP2a production (Berger-Bachi and Rohrer, 2002, Kondo et al., 2001). Furthermore despite the carriage of *mecA* by all MRSA strains, the constitutive expression of PBP2a does not necessarily correlate with high MICs because other genetic determinants affect the expression and regulation of methicillin resistance e.g. the *fem* or *aux* factors (de Lencastre and Tomasz, 1994). The roles of the Fem factors in the synthesis of pentaglycine cross bridge during peptidoglycan synthesis are unique and there are no alternate pathways to compensate for them. Therefore mutations in the *fem* genes have significant impacts on resistance and ultimately compromise cell wall synthesis. The activity of PBP2a is dependent on the complete pentaglycine bridge, so modifications hinders its function and leads to hypersusceptibility to β-lactam antibiotics (Berger-Bachi and Rohrer, 2002). Many other chromosomal genetic determinants or auxiliary genes have been identified and these have varying effects on the expression of methicillin resistance (see Table 1.3) (de Lencastre and Tomasz, 1994, Hartman and Tomasz, 1986).

Table 1.3. Some of the genetic determinants with effects on the expression of methicillin resistance (Berger-Bachi and Rohrer, 2002)

Factor	Function and effect on methicillin resistance
fmhB (*femX*)	Addition of the first glycine to pentapeptide stem; inactivation lethal
femA	Addition of the 2nd and 3rd glycine to pentapeptide stem; inactivation abolishes methicillin resistance
femB	Addition of the 4th and 5th glycine to pentapeptide stem; inactivation reduces methicillin resistance
femC (*glnR*)	Glutamine synthetase repressor; inactivation reduces amidation of the D-glutamate of the pentapeptide; inactivation reduces methicillin resistance
femD (*glmM*) (*femR315*)	Phosphoglucosamine mutase; catalyzes the interconversion of glucosamine-6-phosphate to glucosamine-1-phosphate; a cytoplasmic peptidoglycan precursor; inactivation reduces methicillin resistance
femE	Function unknown; inactivation slightly reduces methicillin resistance
femF (*murE*)	Catalyzes incorporation of lysine into peptidoglycan pentapeptide stem; inactivation reduces methicillin resistance
fmtA	Membrane protein; inactivation decreases cross-linking and amidation of peptidoglycan, and reduces methicillin resistance
fmtB (*mrp*)	Cell surface protein; function unknown; inactivation reduces pentaglycyl-substituted monomer of the cell wall fraction while increasing the amount of unsubstituted pentapeptide and reduces methicillin resistance
fmtC (*mprF*)	Membrane-associated protein; inactivation reduces modification of phosphatidyl-glycerol with L-lysine, and reduces methicillin resistance
llm	Function unknown; inactivation increases Triton-X-100-induced autolysis and reduces resistance
lytH	Homologous to lytic enzymes; inactivation increases methicillin resistance
pbp2	Penicillin-binding protein 2; functional transglycosylase domain of PBP2 is needed for methicillin resistance
sigB	Alternate transcription factor; inactivation reduces methicillin resistance
hmrA	Putative aminohydrolase; over expression increases methicillin resistance
hmrB	Homologue of acyl carrier protein; over expression increases methicillin resistance
dlt operon	Transfer of D-alanine into teichoic acids; inactivation increases methicillin resistance

1.7 Identification of staphylococci at species level

The differentiation of *Staphylococcus* species is performed with a range of tests investigating the activity of diverse enzymes and the capacity of the strains to produce acid from carbohydrate substrates. The evaluation of colony morphology and pigment production is also used as a measure of identification. The ability to clot rabbit plasma due to the expression of coagulase is a major criterion for the identification of pathogenic staphylococci i.e. *S. aureus* in humans and *S. intermedius* and *S. hyicus* in animals (Bannerman and Peacock, 2007). Most of the other staphylococcal species do not produce coagulase and the term coagulase-negative staphylococci (CoNS) is used as a general reference for all of these species. Other key biochemical tests for the identification of *Staphylococcus* species include detecting the activity of the following enzymes: phosphatase, pyrrolidonyl arylamidase, ornithine decarboxylase, urease, β-galactosidase and acetoin production (Bannerman and Peacock, 2007). In addition the identification of novobiocin resistance differentiates a group containing *S. saphrophyticus* from the other CoNS species (Bannerman and Peacock, 2007). Polymixin B resistance is also used to differentiate staphylococci, as only a few are resistant namely *S. aureus, S. epidermidis*, *S. hyicus* and *S. chromogenes* (Bannerman and Peacock, 2007). The tests for the detection of acid production involve the use of the following carbohydrate substrates: D-Trehalose, D-Mannitol, D-Mannose, D-Turanose, D-Xylose, D-Cellobiose, maltose and sucrose (Bannerman and Peacock, 2007).

1.8 Identification of *S. aureus*

1.8.1 Phenotypic tests

S. aureus is the most common cause of staphylococcal infections in humans however certain CoNS species such as *S. epidermidis* can also cause severe infections, particularly bacteraemia and biomedical device-associated infections. In routine clinical testing the identification of *S. aureus* is based on the identification of certain distinct phenotypic characteristics. These include the examination of colony morphology. in contrast to the CoNS species, *S. aureus* typically produce staphyloxanthin, an orange carotenoid which confers a yellow colour to the colonies (Forbes, 2009). The production of coagulase is a major phenotypic determinant of *S. aureus* and the detection of this enzyme is an integral part of *S. aureus* identification. Other tests include the detection of specific cell wall-associated proteins i.e. clumping factor and

protein A and the secreted DNase enzyme (Kateete et al., 2010, Forbes, 2009, Brown et al., 2005, Goldstein and Roberts, 1982, Menzies, 1977).

Conventional detection of coagulase is performed with the tube test and the principle is based on the property of this enzyme to cause clumping of rabbit plasma. Coagulase binds to prothrombin in rabbit plasma and activates it via conformational change, the resultant coagulase:prothrombin complex binds to fibrinogen and triggers the cleavage of fibrinogen forming fibrin which causes the clotting of the plasma (Cheng et al., 2010). However false negative results are possible with some *S. aureus* strains due to the production of staphylokinase which may cause lysis of the clots, furthermore some CoNS species can generate false positive results i.e. *S. delphini*, *S. intermedius*, *S. lutrae*, *S. pseudointermedius* and *S. schleiferi* but these staphylococci are rarely implicated in human infections (Forbes, 2009, Jin et al., 2008, Lijnen et al., 1991). The detection of clumping factor and protein A is done with slide agglutination tests comprising latex particles coated with fibrinogen and immunoglobulin G which bind to clumping factor and protein A, respectively, leading to the clumping of the latex beads. Generally agglutination tests are rapid, generating results within several seconds and are frequently performed as *S. aureus* identification tests rather than the conventional coagulase tube tests which may take up to 24 hours (Davies et al., 2008, Weist et al., 2006, van Griethuysen et al., 2001). However, some CoNS species can cause false positive results and some MRSA strains express capsular type 5 and 8 antigens which mask clumping factor and protein A, causing false negative results with the agglutination tests (Forbes, 2009, Smole et al., 1998, Brakstad et al., 1993, Croize et al., 1993). Accordingly several agglutination tests have been developed for the detection of the capsular type 5 and 8 antigens and these are often used for the verification of presumptive *S. aureus* strains e.g. the Prolex Staph Xtra Latex Kit (Pro-Lab Diagnostics), MastaStaph (Mast Diagnostics), *S. aureus* Latex test (Denka-Seiken) (Forbes, 2009). The DNase enzyme depolymerizes deoxyribonucleic acid and its expression is detected with agar that contains DNA. Following growth of a presumptive *S. aureus* isolate on the DNase test agar the plate is flooded with hydrochloric acid, which precipitates the DNA making the medium opaque. If DNase has been expressed by the isolate a clear zone manifests around the growth of the isolate, signifying the area where the DNA has been degraded.

In routine clinical analysis testing for coagulase or clumping factor/ protein A and DNase will recognize most *S. aureus* strains but some strains may remain undetected. There are further commercially available diagnostic tests for the identification of staphylococcal species e.g. API / ID32 Staph range and RAPIDEC Staph strips by BioMerieux and the BBL crystal identification system Gram-positive ID kit (Becton Dickinson). These tests contain micro-tubes with dehydrated biochemical substrates for the detection of enzymes which are typically produced by staphylococci allowing identification. There are also automated microbial identification systems which are also designed for susceptibility testing of microorganisms e.g. Vitek 2 (BioMerieux), BD Phoenix (Becton Dickinson) and MicroScan Walk-Away (Siemens) (Forbes, 2009, Carroll et al., 2006). Alternative identification systems include the resolution of fatty acids with gas chromatography e.g. the MIDI Sherlock identification system (MIDI, Inc.) and the matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) system which combines the ionization of ribosomal proteins and the resolution of resultant mass ions with mass spectrometry (Rajakaruna et al., 2009, Forbes, 2009, Leonard et al., 1995).

1.8.2 Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a molecular technique based on the amplification of nucleic acids and due to its low limit of detection it is a powerful method for the identification of bacterial isolates from clinical specimens. PCR comprises three main temperature dependant stages: denaturing, annealing and extension. Denaturing is the separation of a double stranded DNA sequence into single strands and this occurs at high temperatures. At a lower temperature the primers (or oligonucleotides) anneal to complementary regions within the separated DNA sequences. An optimal temperature is vital at this stage because it determines the specificity (or stringency) of the reaction. During the subsequent stage the annealed primers are extended by the thermostable DNA polymerase as it synthesizes a DNA sequence which is complementary to the region of interest using the four desoxyribonucleotide triphosphates. In an efficient PCR reaction at the end of each cycle the quantity of generated DNA is double the amount of the preceding cycle. However, the successful amplification of nucleic acid sequences requires the optimisation of factors which affect the efficiency of the PCR i.e. the concentration of magnesium ions which is essential for robust activity of the DNA polymerase, the

annealing temperature, the DNA template concentration, the concentration of the primers and the number of cycles performed.

1.8.2.1 Real-time PCR

Conventionally at the end of the PCR the products are resolved in an agarose gel using electrophoresis and then visualised with Ultra-Violet transillumination. The development of real-time PCR has enabled the quantitative detection of the products as the reaction progresses and this also decreases the duration of the assay. Real-time PCR involves the use of fluorescent dyes which bind to the minor grooves of the DNA hybrid structure and fluoresce upon light excitation e.g. SYBR Green, Syto 9 or EvaGreen (Kubista et al., 2006, Monis et al., 2005). Since these bind to double-stranded DNA, as the reaction progresses the dye binds to each new copy of double-stranded DNA and the fluorescence increases in proportion to the quantity of generated DNA per cycle. The cycle number at which the fluorescence signal indicates progression of the reaction above the background noise is used as an indicator of successful target amplification and this is commonly referred to as the threshold cycle (Ct) or crossing point (C_P) (see Figure 1.5). In quantitative real-time PCR the Ct values are vital for the quantification of the PCR products, as determined by generated standard curves. Baustin *et al.* (2009) recently published guidelines for the 'Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) in which they have recommended the universal use of the term 'Cp value' (Bustin et al., 2009).

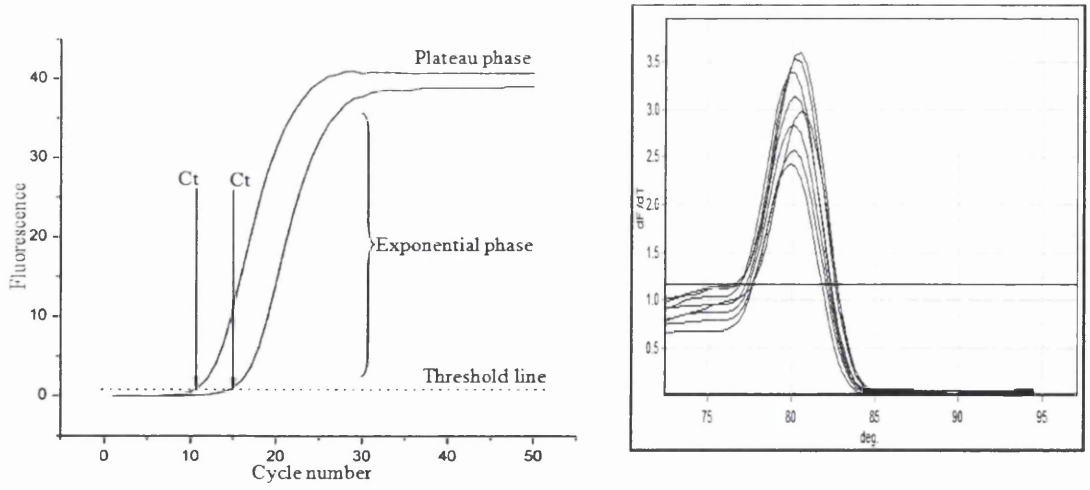


Figure 1.5. An amplification curve from a PCR reaction (adapted from Kubista *et al.*, 2006) and a melt curve showing similar T_m peaks of several specific PCR products

The chemistry of DNA-intercalating dyes is effective and simple but the disadvantage is that they also bind non-specific PCR products. Therefore the analysis of the dissociation or melting curves of the PCR products is important for the verification of specific fragments (see Figure 1.5). For instance melting curves with peaks at melting temperatures (T_m) lower than that of the specific PCR product indicate the formation of primer-dimers, while diverse peaks with different T_m values indicate production of non-specific products (Espy et al., 2006, Mackay, 2004, Cockerill, 2003).

DNA probes which are labelled with fluorophores are used in real-time PCR and the application of these enhances the specificity of the reaction. Several fluorescent probe technologies have been developed based on two modes of action: the hydrolysis probes e.g. Taqman probes and the hybridization probes e.g. the dual hybridization fluorescence resonance energy transfer (FRET) probes, which are also referred to as LightCycler probes (Bustin et al., 2009, Espy et al., 2006, Mackay, 2004, Cockerill, 2003). Fluorophores are excited and emit fluorescence at different wavelengths and this property is incorporated into the chemistry of the probes used in real-time PCR. For instance the Taqman probe is labelled at the 5' end with a reporter dye and at the 3' end with a quencher dye and these are in close vicinity, so the fluorescence of the reporter dye is suppressed by the quencher dye (see Figure 1.6). However during the extension stage the 5' to 3' exonuclease activity of the DNA polymerase causes cleavage of the annealed probe (Espy et al., 2006, Mackay, 2004, Cockerill, 2003, Holland et al., 1991).

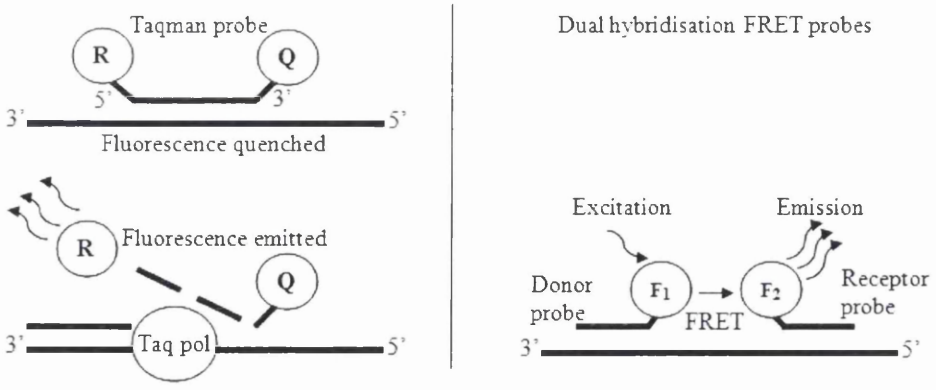


Figure 1.6. Real-time PCR technologies of the Taqman and dual hybridization FRET probes [adapted from Espy et al. (2006).]

This releases the reporter dye from its close proximity to the quencher dye and the fluorescence is freely emitted. The intensity increases in proportion to the generated product at each cycle. In the dual hybridization FRET probe system a donor and receptor probe anneal in close proximity to adjacent regions on the target sequence. The donor probe is labelled with fluorophore at the 3' end and the acceptor probe is labelled with fluorophore at 5' end (see Figure 1.6). When the fluorophore of the donor probe is excited at a specific wavelength the energy is transmitted to the fluorophore of the acceptor probe and this mechanism of transfer is referred to as FRET. The excited acceptor fluorophore then emits fluorescence at a different wavelength which is detected and measured. In contrast to the hydrolysis probes which are destroyed by the exonuclease activity of the polymerase, the FRET hybridization probe technology enables the annealing of the probes at the end of the reaction and therefore melt curve analysis of the amplified product is possible. There are various automated systems which have been developed for real-time PCR reactions, including the Rotor-gene RG-6000 (Corbett), the Light Cycler (Roche) and the iQ5 real-time PCR detection system (Bio-Rad). These instruments comprise thermo-cycling platforms, channels for fluorescence detection and in-built software for the analysis and graphical representation of results.

Several conventional PCR methods have been developed for the amplification of target sequences in *S. aureus*-specific genes and these have greatly contributed to the accurate identification of *S. aureus* isolates e.g. 16S rRNA, the thermonuclease gene (*nuc*), coagulase (*coa*), protein A (*spa*), *femA*, *femB*, *sa442*, *clfA* and *clfB* (Kilic et al., 2010, Ghebremedhin et al., 2008, Riyaz-Ul-Hassan et al., 2008, Maes et al., 2002, Martineau et al., 2001).

1.9 Susceptibility test methods: broth dilution and disc diffusion methods

The gold standard for antibiotic susceptibility testing is the determination of minimum inhibitory concentration (MIC) with broth or agar dilution methods (Forbes, 2009, Brown et al., 2005, Brown, 2001b). The MIC is the fundamental measurement that forms the basis of most susceptibility testing methods. In dilution methods antimicrobial agents are usually tested at $\log_2$ (two-fold) dilutions and the lowest concentration that inhibits visible growth of the microorganism is regarded as the MIC (Jorgensen and Turnidge, 2007). The general approaches for the dilution method include broth macrodilution in which the broth is in volumes of $\geq$1.0 ml in test tubes and broth microdilution whereby the antimicrobial dilutions are in volumes of $\leq$0.1 ml in wells of microdilution trays. The recommended inoculum in dilution testing is 5 x 10^5 CFU/ml and growth in the antimicrobial-containing tubes/ wells is indicated by turbidity or sedimented buttons of bacterial growth (Jorgensen and Turnidge, 2007). The lowest concentration that completely inhibits visible growth of the organism as detected by the unaided eye is defined as the MIC (Jorgensen and Turnidge, 2007).

Under standardized conditions MIC values provide a fixed reference point for the formulation of pharmacodynamic breakpoints with the power to predict efficacy in vivo. These can be applied directly to routine dilution testing methods and they are also used as a reference value for deriving breakpoints for disc diffusion methods (Turnidge et al., 2007). Breakpoints or interpretative criteria are the values that determine the categories for susceptible, intermediate and resistant. The approach to establishing these is established by national testing committees or institutes and these bodies also review them regularly e.g. British Society for Antimicrobial Chemotherapy (BSAC, UK), Clinical Laboratory Standards Institute (CLSI, USA), Comite de l'Antibiogramme de la Societe Francaise de Microbiologie (France) and Swedish Reference Group for Antibiotics (SRGA, Sweden). Since 2002 there have been on-going efforts by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) to harmonize guidelines and antimicrobial breakpoints across European countries (Brown and MacGowan, 2010). Regardless of the different testing committees the establishment of breakpoints requires the inclusion of certain essential data like the distribution of MIC values exhibited by a population of strains; the pharmacokinetic/ pharmacodynamic properties of the antibiotic and the clinical and bacteriological response rates of the organism for which the MICs of the novel antimicrobial agents have been determined

(Turnidge et al., 2007). Broth dilution methods represent a standardized reference method for susceptibility testing, however, they are laborious and commercially prepared microdilution testing panels and automated systems are alternatively used in most routine clinical laboratories (Turnidge et al., 2007).

Susceptibility testing is also performed with agar diffusion tests and these involve filter paper discs impregnated with a single concentration of an antimicrobial agent, placed on the surface of an agar medium that has been inoculated with the test organism (Jorgensen and Turnidge, 2007). The drug diffuses through the agar and as the distance from the disk increases, the concentration of the antimicrobial agent decreases logarithmically (Jorgensen and Turnidge, 2007). This creates a gradient of drug concentrations in the agar medium surrounding each disk. However, in areas where the concentration of the drug is inhibitory to the test organism, no bacterial growth occurs, resulting in a clear zone around each disk, which are of varying size depending on the susceptibility of the test organism (Jorgensen and Turnidge, 2007). The breakpoints for disc diffusion tests are determined from the MICs predetermined by broth dilution methods. These are calculated by plotting the inhibition zone diameters against the MICs derived from testing of a large number of strains of various species. Statistical evaluations are then used to determine the appropriate zone diameters for categorization in susceptible, resistant and intermediate/indeterminate (Turnidge et al., 2007).

1.10 Detection of methicillin resistance in *S. aureus*

1.10.1 Detection of methicillin resistance in *S. aureus* with phenotypic tests

According to the BSAC March 2010, version 9.1 susceptibility testing guidelines, the detection of methicillin resistance in *S. aureus* can be performed with either oxacillin or cefoxitin. Both these antibiotics are penicillinase resistant β-lactam antibiotics, however cefoxitin is a cephamycin, a cephalosporin that has a broader spectrum of activity against Gram-positive and Gram-negative bacteria. The accuracy of antibiotic susceptibility testing is challenged by the variability of *mecA* expression and certain *mecA*-positive strains may depict low-level resistance phenotypes, whilst some *mecA*-negative *S. aureus* strains depict borderline oxacillin resistance phenotypes due to mutations in their existent PBP genes or the hyper-production of penicillinase (Skinner et al., 2009, Keseru et al., 2005, Varaldo, 1993, Chambers et al., 1989). Furthermore

susceptibility tests are affected by test conditions and it is difficult to implement standard conditions for the unambiguous detection of heterogeneous MRSA strains and at the same time clearly distinguish *mecA*-negative penicillinase hyper-producers (Brown et al., 2005, Berger-Bachi and Rohrer, 2002, Berger-Bachi, 1995). Nevertheless test conditions have been modified to augment the expression of methicillin resistance and these include the addition of NaCl (2-5%) and the lowering of incubation temperatures. The benefits of increased NaCl concentrations are dependent on the type of growth media, inoculum size, the incubation temperature and duration of incubation (Skov et al., 2009, Brown et al., 2005, Berger-Bachi and Rohrer, 2002, Berger-Bachi, 1995, Madiraju et al., 1987). For instance the incorporation of 2% NaCl into Mueller-Hinton (MH) or Columbia agar has been shown to improve the detection of methicillin resistance, however, in Iso-Sensitest agar this is less beneficial (Forbes, 2009). With heterogeneous strains the smaller population of cells with higher MIC may grow more slowly and may be missed at higher incubation temperatures. Thus the reduction of the incubation temperature to 30°C rather than 35°C has been recommended in order to enhance the growth of the highly resistant subpopulation. However, a higher NaCl concentration in the growth medium i.e. 5% NaCl, may inhibit the growth of some MRSA. Slow growing MRSA strains may require up to 48 hours for accurate detection of methicillin resistance and if tests are read earlier the strains may be falsely regarded as MSSA. These represent some of the challenges of developing standardised methods which detect all MRSA strains with excellent accuracy.

In the UK the BSAC March 2010 version 9.1 susceptibility testing guidelines are used and these recommend 2% NaCl in either MH or Columbia agar for the oxacillin disc diffusion test and an incubation at 30°C for 24 hours, whereas Iso-Sensitest agar with no added NaCl and incubation at 35°C for 18 to 20 hours is recommended for the cefoxitin disc diffusion test. The loading amounts for the antimicrobial discs are 1 μg for oxacillin and 10 μg for the cefoxitin. However the option of oxacillin or cefoxitin and the concentrations vary depending on the guidelines of the respective national committees. For instance the CLSI M100-S17 performance standards for antimicrobial susceptibility testing recommend cefoxitin (30 μg) instead of oxacillin for disc diffusion or a plate containing 6 μg/ml of oxacillin in MH agar supplemented with NaCl (4% w/v) as alternative methods of testing for MRSA (http://www.cdc.gov/mrsa/).

Automated susceptibility systems are frequently employed especially in the high volume clinical laboratory environments such as the VITEK 2 system and the BD Phoenix. Several studies have investigated the diagnostic performance of these systems for the detection of methicillin resistance in *S. aureus* and CoNS strains which may also exhibit heterogeneous expression of methicillin resistance (Junkins et al., 2009, Horstkotte et al., 2004, Horstkotte et al., 2002). With *S. aureus* strains these systems have been shown to have improved diagnostic sensitivity for the detection of methicillin resistance when both the oxacillin and cefoxitin MICs were included in the interpretation of the results (Junkins et al., 2009). With the CoNS strains the BD Phoenix was shown to have excellent diagnostic sensitivity but lower diagnostic specificity (Horstkotte et al., 2004, Horstkotte et al., 2002).

Automated susceptibility systems generally comprise micro-dilution broth panels and serve the dual purpose of antibiotic susceptibility testing and species identification (Junkins et al., 2009, Kaase et al., 2009). For instance the BD Phoenix automated microbiology system is intended for the rapid identification and determination of MICs for isolates from pure cultures. It uses cassettes comprising an identification (ID) test panel and an antimicrobial susceptibility test (AST) panel. The ID panel comprises micro-wells containing lyophilized biochemical substrates based on microbial utilization and the degradation of the substrates which are detected with various chromogenic or fluorogenic indicator systems e.g. acid production from the hydrolysis of carbohydrates is detected with a phenol red indicator which changes to a yellow colour. The AST panel comprises micro-wells with various antimicrobial agents in two-fold doubling concentrations and a redox indicator is used for the detection of bacterial growth in the presence of the antimicrobial agent. In the Public Health Wales Abertawe Bro Morgannwg (PHW ABM) Microbiology Laboratory the BD Phoenix PMIC/ID-67 panel is used for identification and susceptibility testing of bacteria including presumptive MRSA strains. The AST panel includes a range of antibiotics including oxacillin and cefoxitin, the dilution range is 0.25 to 2 μg/ mL for oxacillin and 2 to 8 μg/ mL for cefoxitin. The determined species identification of the tested isolate is then used in the interpretation of the MIC values for evaluation of susceptibility or resistance to the respective antimicrobial agents. In the BSAC March 2010 version 9.1 susceptibility testing guidelines the MIC breakpoints for methicillin resistance in *S. aureus* are >2 μg/ mL for oxacillin and >4 μg/ mL for cefoxitin. There are several

commercially available latex agglutination tests which detect PBP2a, and these are often used for verification of presumptive MRSA strains in routine clinical laboratories e.g. PBP2' latex test (Oxoid), MRSA latex test (Denka-Seikin), Mastalex-MRSA test (Mast Diagnostics) and Slidex MRSA test (BioMerieux) (Horstkotte et al., 2001, Louie et al., 2000, Marriott et al., 1999).

1.10.2 Detection of methicillin resistance in *S. aureus* with molecular methods

The specificity of MRSA identification is based on the detection of the *mecA* gene and a *S. aureus*-specific gene. The application of real-time PCR methods has been particularly valuable in MRSA detection, whereby DNA-probes labeled with fluorophores have been used for the simultaneous detection of *mecA* and a *S. aureus*-specific gene e.g. *nuc, femA, femB* or Sa442 and *mecA* (Pasanen et al., 2010, Thomas et al., 2007, Costa et al., 2005, Hope et al., 2004, Fang and Hedin, 2003, Francois et al., 2003, Jonas et al., 2002, Reischl et al., 2000a, Pérez-Roth et al., 2001, Vannuffel et al., 1995). For instance Pasanen *et al.* (2010), Thomas *et al.* (2007), Hope *et al.* (2004) and Fang *et al.* (2003) used Taqman probe-based duplex real-time PCR methods for the detection of *mecA* and *nuc* to expedite the screening of MRSA from clinical specimens. Alternatively Francois *et al.* (2003) and Perez-Roth *et al.* (2001) targeted the *femA* and *femB* genes respectively in their methods. Real-time PCR has also been used for the detection of additional antibiotic resistance markers in MRSA e.g. *ermA* (erythromycin resistance), *aacA-aphD* (aminoglycoside resistance) and *ileS*-2 (mupirocin resistance) (Sabet et al., 2007a, Sabet et al., 2007b, Pérez-Roth et al., 2001). However, the application of PCR assays which target *mecA* and a *S. aureus*-specific gene is limited to clinical isolates or typically sterile clinical specimens e.g. blood cultures because in non-sterile or mixed flora clinical specimens e.g. wound swabs, contaminating CoNS species may also be present and since *mecA* may also be acquired by these staphycloccci, false positive results are possible with these duplex PCR assays.

A novel concept for the identification of MRSA from clinical specimens which potentially harbour mixed flora such as MRSA and methicillin resistant CoNS was pioneered by Huletsky *et al.* (2004). This method is based on the location of the *mecA* gene on a mobile genetic element termed the SCC*mec* element and the *orfX* locus which is unique to *S. aureus*. They developed a PCR method comprising oligonucleotides for the amplification of a sequence spanning from the right extremity of the SCC*mec*

element to *orfX*. Ideally the amplification of the SCC*mec-orfX* junction provides a surrogate marker for MRSA and allows unambiguous detection from non-sterile clinical specimens. Further SCC*mec-orfX* PCR methods were developed by Cuny and Witte (2005) and Hagen *et al.* (2005) and the original Huletsky *et al.* (2004) method was subsequently used in the development of a commercial test for detection of MRSA from clinical nasal swabs i.e. the BD GeneOhm MRSA test. Amplification of the SCC*mec-orfX* junction has been shown to be robust for MRSA detection and this concept serves as the principle of other commercial tests for MRSA detection in screening nasal swabs i.e. the GeneXpert MRSA (Cepheid), the GenoQuick MRSA test (Hain LifeScience) and the LightCycler MRSA Advanced test (Roche) (Kolman et al., 2010, Snyder et al., 2010, Bishop et al., 2006, Prere et al., 2006, Cuny and Witte, 2005, Hagen et al., 2005, Huletsky et al., 2004).

1.11 The Staphylococcal Cassette Chromosome *mec* (SCC*mec*)

The *mecA* gene is located on a mobile genetic element termed the Staphylococcal Cassette Chromosome *mec* (SCC*mec*) (Zhang et al., 2009, Berglund et al., 2008, Ito et al., 2004). These elements have characteristic features:

i) The *mec* gene complex and the *ccr* gene complex

ii) Direct and inverted repeats at the chromosome-SCC*mec* boundaries

The *mec* gene complex comprises *mecA*, its regulator genes and insertion sequences while the *ccr* gene complex contains *ccr* genes and surrounding open reading frames (Arakere et al., 2009).

1.11.1 The different *mec* gene complex classes

Presently there are five classes of *mec* gene complexes (A, B, C1, C2 and D):

i) Class A is the prototype and is structured as follows: *mecI-mecR1-mecA*-IS*431*. It comprises *mecA*, complete *mecR1* and *mecI* regulatory genes upstream of *mecA*, the hypervariable region (HVR) and an insertion sequence IS*431* downstream of *mecA*. Variants have also been described including those containing IS*431* or IS*1182* upstream of *mecI* (IWG-SCC, 2009, Ito et al., 2004, Katayama et al., 2001).

ii) Class B is structured as follows: ψIS*1272*-Δ*mecR1-mecA*-IS431, whereby there is a deletion of *mecI* and the deletion of the penicillin binding domain of *mecR1* due to the insertion of pseudo-IS*1272*. A variant has been described with an insertion of Tn*4001* upstream of *mecA* (IWG-SCC, 2009)

iii) Class C comprises two types (C1 and C2) which are structured as follows: IS*431*-Δ*mecR1-mecA*-IS*431*, whereby *mecR1* is truncated by the insertion of IS*431*. In class C1 the IS*431* upstream of *mecR1* is in the same orientation as IS*431* downstream of *mecA* whereas in class C2 the orientation of IS*431* upstream of *mecR1* is reversed. Also class C2 has a larger deletion of the penicillin binding domain in *mecR1* (IWG-SCC, 2009, Hanssen and Sollid, 2006, Katayama et al., 2001).

iv) Class D: Δ*mecR1-mecA*-IS*431* has a truncated *mecR1* (lacking an insertion sequence) due to a deletion of the penicillin binding domain. Shore *et al.* (2005) have also described class E which is similar to class D except, it has a 976 bp deletion in the membrane-spanning domain of *mecR1* (IWG-SCC, 2009).

1.11.2 The *ccr* gene complex

The *ccr* genes encode site-specific recombinases which mediate the chromosomal integration and excision of the SCC*mec* element in the *S. aureus* chromosome at a specific site termed *attB* which is located in *orfX* (Wang and Archer, 2010, Deurenberg and Stobberringh, 2008, Hanssen and Sollid, 2006). There are five allotypes of the *ccr* gene complex, the first four types are pairs of homologous *ccrAB* genes: type 1 (containing *ccrA1B1*), type 2 (containing *ccrA2B2*), type 3 (containing *ccrA3B3*), type 4 (containing *ccrA4B4*) and type 5 contains one recombinase gene, *ccrC* (IWG-SCC, 2009, Hanssen and Sollid, 2006, Ito et al., 2004). These have homology to integrases and recombinases of other bacteria e.g. an integrase of *Lactococcus lactis* acquired from bacteriophage TP901-1 and a putative integrase of *Bacillus cereus* acquired from bacteriophage TP21ply21. They also have homology to recombinase *SpoIVCA* of *Bacillus subtilis* and transposase *TnpX* of *Clostridium perfringens* but the SCC*mec* elements do not carry genes encoding bacteriophage head and tail proteins or the *tra* genes which are essential for conjugative transfer (Hanssen and Sollid, 2006).

1.11.3 The junkyard regions

Outside of the *mec* and *ccr* gene complexes, the SCC*mec* elements comprise three regions termed the junkyard (J) regions J1, J2 and J3. These may harbour additional antibiotic resistance genes, virulence genes, pseudogenes, non-coding regions, insertion sequences, plasmids or transposons (see Figure 1.7). J1 is the region between the left chromosomal junction and the *ccr* gene complex, J2 is between the *ccr* and *mec* gene complexes and J3 is between the *mec* gene complex and the right chromosomal junction (IWG-SCC, 2009).

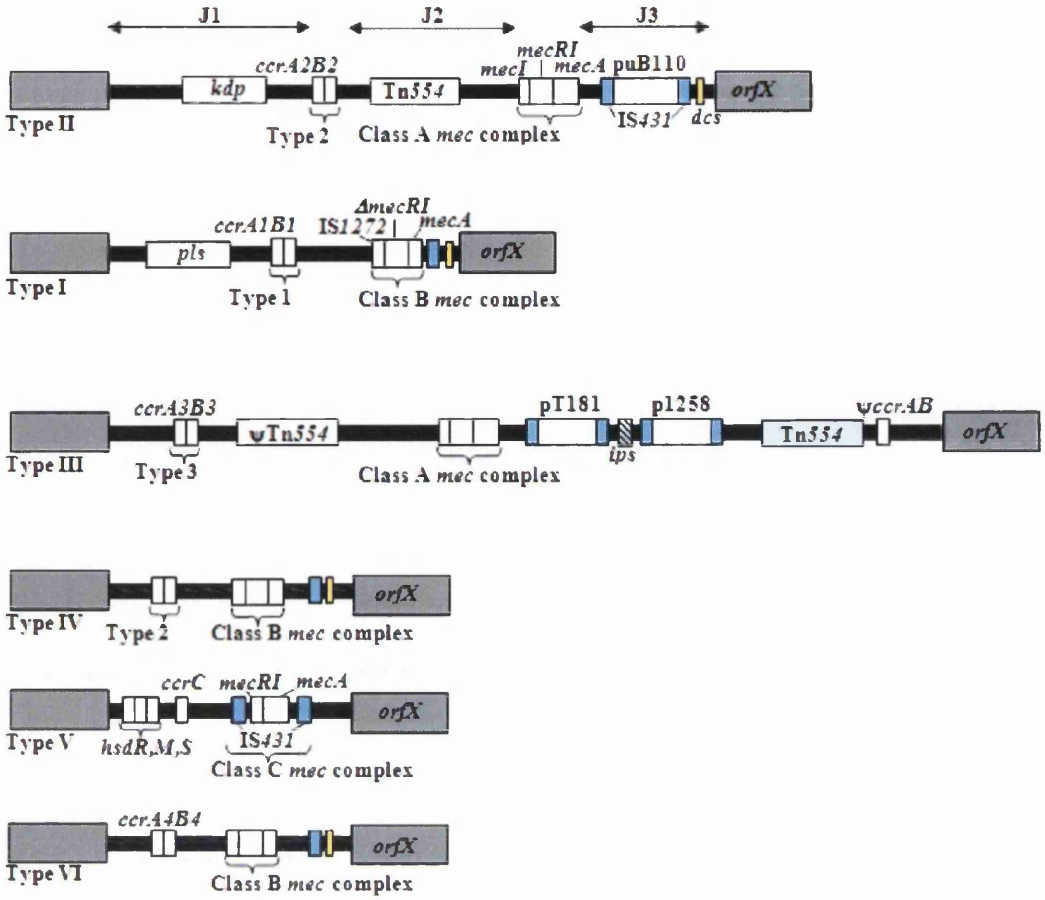


Figure 1.7. Schematic diagram of SCC*mec* type I to VI elements (not drawn to scale) [adapted from (de Lencastre et al., 2007, Hanssen and Sollid, 2006)]

1.11.4 Classification of SCC*mec* elements in MRSA

SCC*mec* types I to VIII have been described in MRSA and they range in size from approximately 21 to 67kb (see Figure 1.7). Recent recommendations by the International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC) state that the nomenclature of the elements should reflect the combination of the *ccr* and *mec* gene complexes within the elements. Therefore SCC*mec* type I would be termed '1B' to signify the type 1 *ccr* gene complex and the class B *mec* gene complex harboured by this element (see Figure 1.7). Similarly the other SCC*mec* types would be: type II (2A), type III (3A), type IV (2B), type V (5C2) and type VI (4B) (see Figure 1.7). SCC*mec* subtypes are defined by the presence of characteristic loci in the junkyard regions (see Table 1.4).

S. aureus especially MRSA strains can carry several copies of insertion sequences e.g. IS*431* and these are implicated in the trapping and clustering of resistance markers by homologous recombination. For instance the SCC*mec* type II and III elements contain plasmids pUB*110,* pT*181* and pI*256* respectively which are flanked by *IS431* (Hanssen and Sollid, 2006) (see Figure 1.7). The SCC*mec* type I, IV and V elements are typically smaller than SCC*mec* type II and III elements and usually only harbour the methicillin resistance genetic determinant, *mecA*. However, some variants of these elements carry additional resistance determinants such as the SCC*mec* I.2 and IVA subtypes which elements also harbour the pUB*110* plasmid (see Table 1.4). This plasmid carries *aadD/ble* which encodes kanamycin, tobramycin and bleomycin resistance (see Table 1.3). SCC*mec* type V is the only known SCC*mec* element which has a complete set of genes encoding the type I restriction-modification system i.e. *hsdR*, *hsdS*, and *hsdM* genes. These are also thought to be involved in the stabilisation of this element in the *S. aureus* chromosome (see Figure 1.7).

Pairs of SCC*mec* elements arranged in tandem, possibly arising from recombination events, have been described in *S. aureus* such as the ZH47, TSGH17 and PM1 strains. Strain ZH47 contains a composite element comprising a SCC element with *ccrC* and an additional SCC*mec* with a type 2 *ccr* gene complex and a class B2 *mec* complex (a class B *mec* complex with Tn*4001* integrated within it) (IWG-SCC, 2009). In these cases the composite elements are classified by the SCC*mec* element present within the combination i.e. the element of the ZH47 strain is designated SCC*mec* 2B&5 and regarded a type IV variant (IWG-SCC, 2009).

There is more structural diversity of SCC*mec* elements in methicillin resistant CoNS than in MRSA and CoNS species are postulated to harbour the reservoir of SCC elements. Unique *ccr* and *mec* gene classes and complex combinations i.e. one *mec* gene complex and up to three *ccr* gene complexes have been described in CoNS species. Therefore it has also been proposed that the classification of SCC*mec* elements should reflect the copy number of *ccr* genes (Ruppe et al., 2009, Hanssen and Sollid, 2006).

Table 1.4 SCC*mec* elements identified in MRSA (IWG-SCC, 2009).

SCC*mec* types	Reported names	Major characteristics of J-regions	Representative strain
I (1B)	I	J1, subtype 1 specific ORFs (*pls*); J3, *dcs*	NCTC10442, COL
	I.2	J1, subtype 2 specific ORFs; J3, *dcs* and pUB110	PL72
II (2A)	II	J1, subtype 1 specific ORFs (*kdp*); J2, subtype 1 specific ORFs; J3, *dcs* and pUB110	N315
	IIb	J1, subtype 2 specific ORFs; J2, subtype 1 specific ORFs and Tn*554*; J3, *dcs*	JCSC3063
	IIB	J1, subtype 3 specific ORFs; J2, subtype 1 specific ORFs; J3, *dcs* and pUB110	AR05/ 0.1345
	IIE	J1, subtype 3 specific ORFs; J2, short J2 region the same as subtype 1 and Tn*554*; J3, *dcs* and pUB110	AR13.1/ 3330.2
	II.4.1.1	J1, subtype 4 specific ORFs; J2, subtype 1 specific ORFs and Tn*554*; J3, *dcs* and pUB110	RN7170
III (3A)	III	J1, subtype 1 specific ORFs; J2, subtype 1 specific ORFs and ΨTn*554*; J3, subtype 1 specific ORFs and pT181	85/2082/ANS46
	IIIA	J1, subtype 1 specific ORFs; J2, subtype 1 specific ORFs and ΨTn*554*; J3, subtype 1 specific ORFs, pT181 and SCC_{Hg} carrying *ccrC*	HU25
IV (2B)	IVa	J1, subtype 1 specific ORFs; J3, *dcs*	CA05, MW2
	IVb	J1, subtype 2 specific ORFs; J3, *dcs*	8/6-3P
	IVc	J1, subtype 3 specific ORFs; J3, *dcs* and Tn*4001*	81/108
	IVc	J1, subtype 3 specific ORFs; J3, *dcs*	2314
	IVA	J1, subtype 3 specific ORFs; J3, *dcs* and pUB110	cml1
	IVE	J1, subtype 3 specific ORFs; J3, subtype 2 specific ORFs	AR43/3330.1
	IVd	J1, subtype 4 specific ORFs; J3, *dcs*	JCSC4469
	IVg	J1, subtype 5 specific ORFs; J3, *dcs*	M03-68
	IVh	J1, subtype 6 specific ORFs; J3, *dcs*	EMRSA-15
	IVi	J1, subtype 7 specific ORFs; J3, *dcs*	JCSC6668
	IVj	J1, subtype 8 specific ORFs; J3, *dcs*	JCSC6670
IV (2B&5)	IV variant	J1, subtype 3 specific ORFs; SCC carrying *ccrC*	ZH47
V (5C2)	V	J1, subtype 1 specific ORFs; J2, subtype 1 specific ORFs; J3, subtype 1 specific ORFs	WIS(WEG8318)
V (5C2&5)	V_T, VII	J1, subtype 2 specific ORFs; J2, subtype 2 specific ORFs; J3, SCC carrying *ccrC*	TSGH17, PM1
VI (4B)	IV	J1, subtype 1 specific ORFs; J3, *dcs*	HDE288
VII (5C1)	5C1	J1, subtype 1 specific ORFs; J2, subtype 1 specific ORFs; J3, subtype 1 specific ORFs	JCSC6082
VIII (4A)	VIII	J1, subtype 1 specific ORFs; J2, subtype 1 specific ORFs; J3, subtype 1 specific ORFs	C10682, BK20781

1.11.5 SCC elements that do not contain *mecA*

Several SCC elements lacking *mecA* have been described in *S. aureus* e.g. SCC*cap1*, SCC*Hg*, SCC*fur* and these carry genes for capsular polysaccharide 1 involved in the evasion of phagocytosis, a mercury resistance operon and fusidic acid resistance genes, respectively (IWG-SCC, 2009, Hanssen and Sollid, 2006). The SCC elements have characteristic features of SCC*mec* elements i.e. carriage of a *ccr* gene complex, integration at *orfX* and flanking direct repeats. The SCC_{476} element which is carried by the MSSA476 strain carries a gene encoding a protein similar to the plasmid-borne fusidic acid-resistance determinant (Holden et al., 2004). Is also has two site-specific recombinases which are homologous to the *ccrA* and *ccrB* genes of the non-*mecA* SCC element, SCC_{12263} which is harboured by a *S. hominis* strain (Holden et al., 2004). Other non-*mecA* SCC elements in CoNS strains include a large composite non-*mecA* element (57kb) termed SCCcomposite island, which is carried by the *S. epidermidis* ATCC 12228 strain. It comprises a SCC element carrying a type 2 and type 4 *ccr* complex and an additional element in tandem termed SCC*pbp4*. The latter element carries a homolog of the gene encoding PBP4, which is involved in peptidoglycan synthesis (Hanssen and Sollid, 2006).

1.12 The typing of *S. aureus* isolates

A comprehensive understanding of the epidemiology and evolution of *S. aureus* is enhanced by the application of typing techniques which enable the discrimination of unrelated isolates and discernment of clonal lineages. The discrimination of strains may be based on phenotypic or genetic factors but it is important that the techniques have robust discriminatory power and reproducibility in order to be effective methods.

1.12.1 Phenotypic typing methods

The earliest phenotypic typing methods included the analysis of antibiograms and phage typing which were based on the susceptibility of the isolates to antibiotics and bacteriophages (Weller, 2000, Gillepsie et al., 1990). However, antibiotic resistance patterns are influenced by environmental selective pressures and unrelated isolates may eventually have similar profiles. Phage typing involves the application of a selection of phages to a bacterial isolate on an agar plate and the examination of developed plaques. This method was commonly used and was the earliest method to be standardised by an international committee, however, inherent problems include the lack of susceptibility of certain strains to phages and poor reproducibility (Strommenger et al., 2008, Devriese, 1984, Blair and Williams, 1961). Other methods include cellular protein analysis after lysis of the bacterial isolate with lysostaphin. The proteins are then separated with polyacrylamide gel electrophoresis and the resultant patterns exhibit multiple bands. This method was further refined with immunoblotting, a process involving the further addition of anti-staphylococcal antibodies and the visualisation of resultant patterns labeled secondary antibodies (Weller, 2000, Pagani et al., 1991, Mulligan et al., 1988). However, in some outbreaks the use of these protein-based patterns did not adequately distinguish strains. Multi-locus enzyme electrophoresis (MLEE) has also been applied to *S. aureus* isolates. It involves the extraction of cell associated enzymes and their separation by electrophoresis. Since mutations in the genes encoding the enzymes can affect the amino acid composition of the enzymes, this can affect the electrophoretic mobility of the products and thus result in diverse MLEE patterns for respective test isolates (Weller, 2000, Tenover et al., 1994). However, the discriminatory power of MLEE is partly dependant on which enzymes are included and some have been found to be monomorphic across respective *S. aureus* isolates thus limiting the discriminatory capacity of the method.

1.12.2 Molecular typing methods

A plethora of molecular typing techniques have been developed and the earliest technique was plasmid analysis which differentiated strains by virtue of the number and size of harboured plasmids, as determined by plasmid extraction and subsequent electrophoresis. However, the success of this method is limited to strains which carry plasmids and since these are mobile elements they may be lost by certain strains and this affects the discriminatory power and reproducibility of this method (Weller, 2000). The digestion of chromosomal DNA with restriction enzymes e.g. *Bgl*II and *Eco*R1 was found to be an alternative means of discriminating *S. aureus* isolates. This method is referred to as restriction enzyme analysis (REA) and produces multiple fragments but conventional electrophoresis is not able to separate large fragments (>20kb), thus there is a likelihood of these overlapping and this masks existent differences between strains. The combination of REA and Southern blotting enabled the use of probes for the detection of different loci which are found in multiple copies in the chromosome e.g. 16S and 23S ribosomal proteins (ribotyping) and insertion sequences such as IS*431*, IS*256* and IS*1181* (Deplano et al., 1997, Derbise et al., 1994, Jensen et al., 1993). However, there will always be strains lacking insertion sequences making them untypeable with this method (Weller, 2000, Richardson et al., 1994, Jordens, 1991). Some unrelated MRSA strains have been shown to exhibit identical rRNA gene restriction fragment patterns, indicating that ribotyping is not a highly discriminatory typing tool for *S. aureus* (Prevost et al., 1992).

The analysis of restriction enzyme patterns has been enhanced by the application of pulsed field gel electrophoresis. This technique involves continual changes in the direction of the electric field during electrophoresis and this frequent re-orientation enables efficient separation of large chromosomal DNA fragments, resulting in patterns which have proved highly effective for the discrimination of strains. For *S. aureus* the use of the *Sma*I restriction enzyme has been found to produce the most discriminatory and reproducible PFGE patterns. Efforts have been undertaken to standardize this method and it is currently regarded as the gold standard for *S. aureus* typing (Murchan et al., 2003). However it is technically demanding, the results are inclined to subjective interpretation and inter-laboratory comparison of the results is difficult (Conceicao et al., 2009, Schouls et al., 2009, Faria et al., 2008, Strommenger et al., 2006).

As such molecular techniques are still being developed and advocated as alternative methods to expedite *S. aureus* typing. This has included the development of methods involving various PCR applications. For instance random amplified polymorphic DNA (RAPD) analysis involves a low stringency PCR with a pair or several pairs of short primers (Weller, 2000). The low stringency conditions enable the primers to anneal to random regions in the chromosome and the resulting fragments are resolved in an agarose gel. However although it is more rapid than PFGE it has been found to lack reproducibility in some studies (Reinoso et al., 2004, Telecco et al., 1999, Tambic et al., 1997).

The sequencing of several *S. aureus* genomes revealed the presence of polymorphic regions comprising variable numbers of highly similar repeated DNA sequences arranged consecutively, which are alternatively termed variable-number tandem repeats (VNTRs) (Hardy et al., 2006, Hardy et al., 2004). The amplification of VNTRs of several *S. aureus*-specific loci including the *sdr, clfA, clfB, ssp, coa* and *spa* genes, has been used as the basis for the differentiation of strains (Hardy et al., 2006, Harmsen et al., 2005, Hardy et al., 2004, Shopsin et al., 1999, Schwarzkopf and Karch, 1994, Goh et al., 1992). For instance *sdr, clfA, clfB* encode cell-surface proteins and contain a region with repeated Ser-Asp dipeptides encoded by an 18 bp sequence at the 3'- region of the genes. Whereas the *coa* and *spa* genes contain regions comprising a series of approximately 81 and 24 bp repeats which vary in number across *S. aureus* strains (Weller, 2000, Shopsin et al., 1999). In *coa* typing the PCR products are subsequently digested with a restriction enzyme e.g. *Alu*I and the patterns are used to discriminate strains (Goh et al., 1992). Although *spa* typing also initially involved restriction enzyme digestion, this method has since been modified and currently involves the sequencing of the amplified polymorphic region (Harmsen et al., 2003). This has increased the discriminatory capacity of this approach and it has been found to be comparable to the PFGE typing in several studies (Faria et al., 2008, Strommenger et al., 2006, Koreen et al., 2004, Harmsen et al., 2003). The DNA sequence between IS*431mec* and *mecA* is referred to as the hypervariable region (HVR) and is composed of direct repeat units (DRUs) each of which are approximately 40 bp in size (Nishi et al., 1995). Initially the HVR-PCR product sizes were used as the basis for discriminating *S. aureus* strains, however, sequencing of the products and the comparison of the variable *dru* sequences has been found to be a more discriminatory approach (Goering et al., 2008). Since the

dru locus is located on SCC*mec* elements this method is limited to MRSA and may be more appropriate as a subtyping technique. In several studies the DNA sequence analysis of multiple VNTR loci (MVLA) has been shown to be comparable to PFGE and is advocated as a potential alternative to this method (Pourcel et al., 2009, Schouls et al., 2009, Tenover et al., 2007, Sabat et al., 2003).

DNA sequencing-based methods are increasingly the preferred means of typing *S. aureus* because they offer the advantage of rapid, unambiguous, exportable data (Deurenberg and Stobberringh, 2008, Faria et al., 2008, Hallin et al., 2007, Aires-de-Sousa et al., 2006). Multi locus sequence typing (MLST) involves the generation of allelic profiles based on sequencing of 500 bp internal fragments of seven housekeeping genes, for *S. aureus* these are: *arc* (carbamate kinase), *aro* (shikimate dehydrogenase), *glp* (glycerol kinase), *gmk* (guanylate kinase), *pta* (phosphate acetyltransferase), *tpi* (triosephosphate isomerase) *yqi* (acetyl-coenzyme A-acetyltransferase (http://saureus.mlst.net/). MLST has become the gold standard for long-term epidemiological studies, however, it is too expensive and labour-intensive for routine surveillance or outbreak investigations (Faria et al., 2008, Mellmann et al., 2008, Enright et al., 2002, Enright et al., 2000). Alternatively, *spa* typing has been recommended as a potentially effective technique for long-term epidemiological studies. A further advantage of DNA sequence based genotyping, is the availability of accessible internet databases e.g. http://saureus.mlst.net/ and http://www.spaserver.ridom.de/. This facilitates data comparison and the monitoring of clone dissemination world-wide (Faria et al., 2008, Strommenger et al., 2008, Strommenger et al., 2006, Koreen et al., 2004).

The discrimination of SCC*mec* elements is integral to the nomenclature of MRSA strains and there are various SCC*mec* typing methods which have been developed for the discrimination of these elements into diverse types (Kondo et al., 2007, Milheirico et al., 2007b, Milheirico et al., 2007a, Oliveira and de Lencastre, 2002). The combination of the MLST sequence type and the SCC*mec* type is fundamental to the nomenclature of MRSA clones e.g. ST8-MRSA-IVa (USA300 clone), ST22-MRSA-IV (EMRSA-15 clone).

DNA hybridisation microarray technology is basically miniaturised DNA detection assays based on the hybridisation of a DNA target sequence to its complementary probe on a solid support. The number of oligonucleotide sequences which are used as the DNA probes on the microarray plate varies according to the application of the assay. This is a powerful molecular technique which has been used for the analysis of bacterial genomes (Lindsay et al., 2006, Witney et al., 2005) and also for the characterization of *S. aureus* strains, MRSA and PVL-positive *S. aureus* (Tissari et al., 2010, Garch et al., 2009, Goering et al., 2009, Monecke et al., 2009, Monecke et al., 2008, Monecke et al., 2007, Lindsay et al., 2006). Since this method enables the detection of multiple genetic determinants in a single test, e.g. virulence genes, pathogenicity related determinants, and antibiotic resistance markers, large volumes of data are obtained for the comparison and typing of strains. Thus with a larger population of test strains genes that are affiliated with particular clones can be identified.

In previous studies the cluster analysis of the data generated with whole genome microarrays has been shown to be concordant with the clonal lineages predetermined by MLST (Lindsay et al., 2006, Witney et al., 2005, Lindsay and Holden, 2004, Fitzgerald et al., 2001). Furthermore whole genome microarray analysis has granted insights into the structural composition of the *S. aureus* genome, revealing that a large proportion (>50%) is composed of the core genome which comprises genes that are highly conserved within clonal lineages (Lindsay and Holden, 2006). It is also composed of the accessory genome which is represented by mobile genetic elements (MGEs) such as bacteriophages, pathogenicity islands, genomic islands, plasmids and transposons. These vary extensively across the different strains and clonal lineages (Lindsay and Holden, 2006, Lindsay and Holden, 2004). Therefore strains from the same lineage have remarkably conserved genomes (with the exception of their MGEs) despite having diverse geographic, temporal and selective diversity. This has been shown by the comparison of whole genome sequences of the USA CA-MRSA MW2 strain and the UK MSSA476 strain which belong to the same lineage, CC1. Even though the former is an MRSA, only 285 point mutations were identified among approximately 2,500 genes that do not encode MGEs (Lindsay, 2010, Holden et al., 2004). Therefore, since microarrays provide a comprehensive analysis of the core and accessory genes, they can enable the elucidation of which genes are important in invasive infection and also identify genes that can serve as ideal targets for candidate antimicrobial therapies.

1.13 Emergence of methicillin resistant *Staphylococcus aureus*

In 1960 MRSA were first reported in the UK and were implicated in three cases in a Southern England hospital (Deurenberg and Stobberringh, 2008, Deurenberg et al., 2007, Grundmann et al., 2006). The causative strain (NCTC 10442), also referred to as the archaic clone, was later found to contain SCC*mec* type I (Deurenberg and Stobberringh, 2008, Grundmann et al., 2006, Ito et al., 2001). By 1967 MRSA which were resistant to multiple classes of antibiotics e.g. streptomycin, tetracycline and erythromycin, were reported from Switzerland, France, Denmark, England, Australia and India. From 1967 to 1971 approximately 15% of all *S. aureus* isolates in Denmark were methicillin resistant and belonged to the phage type 83A clone (Grundmann et al., 2006). Even though the rates of MRSA declined in the 1970s in Europe the 1980's brought a surge of MRSA in many countries including the UK where there was an increase in the prevalence of gentamicin-resistant strains (Grundmann et al., 2006). The New York/ Japan clone containing SCC*mec* type II also spread to various countries worldwide, followed by the discovery of the MRSA 85/ 2082 strain in New Zealand which harbours SCC*mec* type III (Deurenberg et al., 2007, Grundmann et al., 2006). In the UK a numerical prefix classification system based on phage typing was initiated in the 1980s and based on this definition sixteen epidemic types (EMRSA-1 to EMRSA-16) were identified in England and Wales until 1995 (Grundmann et al., 2006). In the early 1990s, EMRSA-3 was prevalent but then EMRSA-15 emerged in hospitals in the Midlands of England and in 1992 the EMRSA-16 strain was identified as the causative pathogen of an outbreak affecting 400 patients and 27 staff in a hospital in Northamptonshire, England (Murchan et al., 2004). EMRSA-17 emerged in hospitals along the south coast of England during the late 1990s but it remained largely restricted to this area (Aucken et al., 2006).

Currently the EMRSA-15 and EMRSA-16 clones are the predominant causes of hospital-acquired infections (HAIs) in the UK (Ellington et al., 2009, Lindsay, 2009, Aucken et al., 2006, Grundmann et al., 2006, Moore and Lindsay, 2002). Characteristically HA-MRSA exhibit multiple-antibiotic resistance and carry SCC*mec* type II and III elements, however the EMRSA-15 and EMRSA-16 clones exhibit resistance solely to ciprofloxacin and variable resistance to erythromycin and clindamycin, and EMRSA-15 harbours the SCC*mec* type IV element (Amorim et al., 2007, Aucken et al., 2006, Aucken et al., 2002, Shukla et al., 2004, Okuma et al., 2002).

The earliest reports of community-acquired MRSA (CA-MRSA) was in the 1980s, when MRSA strains were implicated in infections amongst intra-venous drug users in Michigan, USA (Millar et al., 2007a). In 1993 novel strains were isolated from skin and soft tissue infections of healthy individuals of the aboriginal community in Western Australia. The infected individuals had no known HAI risk factors and the MRSA had exclusively originated in the community (Boucher and Corey, 2008, Grundmann et al., 2006). In 1999, unanticipated deaths of four previously healthy children were reported in Minnesota and North Dakota in the USA. These cases also lacked any HAI risk factors and the causative virulent strain was designated the Midwest/MW2 clone (Shukla et al., 2004, Ma et al., 2002). Since these early reports CA-MRSA have become highly prevalent in the USA but variably present in other countries e.g. Australia and Asia (Boyle-Vavra and Daum, 2007, Millar et al., 2007b).

The Centers for Disease Control (CDC) drafted the initial demographic, clinical and epidemiologic factors for the differentiation of HA-MRSA from CA-MRSA (see Table 1.5). CA-MRSA are primarily associated with young healthy individuals or community populations with no HAI risk factors i.e. school children, sport athletes especially those in team or contact sports, military personnel and incarcerated individuals (see Table 1.5). Generally CA-MRSA cause self-limiting skin and soft tissue infections but certain virulent strains like USA300 and the European clone have been affiliated with severe infections e.g. necrotizing pneumonia and infective endocarditis. These cases exhibit rapid deterioration and are frequently fatal (Boyle-Vavra and Daum, 2007, Millar et al., 2007b, Maltezou and Giamarellou, 2006). CA-MRSA were originally susceptible to non-β-lactam antibiotics, however, the resistance profile of CA-MRSA is evolving and some strains express now resistance to several classes of antibiotics e.g. tetracycline, erythromycin and clindamycin, chloramphenicol, aminoglycosides and trimethoprim.

CA-MRSA primarily carry SCC*mec* type IV and some isolates carry SCC*mec* type V. It has been suggested that the carriage of these smaller SCC*mec* elements rather than the larger type II and III elements, imparts some dissemination advantage to CA-MRSA, enabling them to successfully outcompete other MRSA clones and colonise hosts (Ellington et al., 2009, Kobayashi and DeLeo, 2009, Boyle-Vavra and Daum, 2007, Maltezou and Giamarellou, 2006, Okuma et al., 2002).

Table 1.5. Clinical, epidemiological and genotypic characteristics of CA-MRSA and HA-MRSA (Millar et al., 2007a)

Factor	HA-MRSA	CA-MRSA
Population	• Hospital/healthcare/nursing home residents • Elderly • Pre-term neonate • Immunocompromised	• Young healthy individuals with no risk factors for HAIs • School children • Individuals in prisons • Military personnel • Athletes in contact and ball sports
Site of infection	• Skin and soft tissue infections • Bacteraemia and wound infections • Symptomatic infections of respiratory and urinary tract infections	• Mainly skin and soft tissue infections(abscesses, cellulitis, furunculosis) • Severe cases: necrotizing pneumonia, septic shock, bacteraemia
Risk factors	• Indwelling devices, catheter lines, haemodialysis • Prolonged hospitalization and long-term antibiotic use	• Close physical contact, abrasion, injuries. • Activities associated with poor communal hygiene
Transmission	• Person- to- person spread: healthcare staff (e.g. nurses, doctors, surgeons, physiotherapists), patients hospital visitors	• Person- to- person spread • Shared facilities • Environment-to-person spread, e.g. shared sports equipment
Susceptibility to methicillin	• No	• No
Susceptibility to other antibiotics	• No	• Yes (in most cases)
Presence of PVL[a]	• Low (<5%)	• High (>95%)
SCC*mec* type	• Predominantly I, II, III, IV	• Predominantly IV, V
agr [b] **genotype**	• Predominantly II	• Predominantly I and III

[a] PVL: Panton-Valentine Leukocidin; [b] *agr*: accessory gene regulator

1.14 Prevalent HA-MRSA and CA-MRSA clones

The molecular typing of *S. aureus* strains with MLST revealed that this species is highly clonal consisting of about ten dominant human lineages (CC1, CC5, CC8, CC12, CC15, CC22, CC25, CC30, CC45 and CC51) and many minor lineages (Feil et al., 2003, Enright et al., 2002, Enright et al., 2000). The lineages are often referred to by their clonal complex (CC) numbers which are identified by MLST but can also be identified with microarray analysis and *spa* typing (Lindsay, 2010). A selected number of HA-MRSA and CA-MRSA clones associated with specific clonal lineages have become established as the leading cause of infections worldwide (Lindsay, 2010, Deurenberg and Stobberringh, 2008, Diep and Otto, 2008, Tristan et al., 2007, Enright et al., 2002).It is suggested that the success of these clones is due to the horizontal transfer of MGEs encoding virulence and resistance genes into a lineage that can establish in a given environment. The acquired MGEs remain stable within the genome and they provide some selective advantage to enable the transmissibility and dissemination of the clone (Lindsay, 2010)

The common names of HA-MRSA and CA-MRSA clones often reflect the region or the communities in which they caused adverse or extensive infections (see Table 1.6 and 1.7). For instance the Midwest/MW2/USA400 clone (ST1-MRSA-IV) was originally prevalent in Western Australia. However, it was later implicated in fatal infections in the USA and became well known with reference to this region. McDougal *et al.* (2003) initially defined MRSA in the USA according to their PFGE patterns. They identified eight distinct clusters and designated them USA100 to USA800. PFGE types USA300 and USA400 were from CA-MRSA outbreaks, while the others were from HA-MRSA infections (Seybold et al., 2006, McDougal et al., 2003). The Southwest Pacific clone (ST30-MRSA-IV) has been frequently implicated in community outbreaks in Australia and the USA whereas the European clone (ST80-MRSA-IV) is largely restricted to sporadic community associated infections in some European countries i.e. France, Denmark, Norway. The Pacific clone or Taiwanese clone (ST59-MRSA-V_T) is highly prevalent in paediatric infections in Taiwan and is becoming established as a HA-MRSA clone in this country (Chen et al., 2009, Takano et al., 2008).

Table 1.6. Major HA-MRSA clones identified worldwide (Deurenberg and Stobberingh, 2008)

Clone	MLST profile	ST[a]	CC[b]	SCC*mec*	*spa* type[c]
Archaic	3-3-1-1-4-4-16	250	8	I	**t008**,t009,t914
Berlin (USA600)	10-14-8-6-10-3-2	45	45	IV	**t004**,t015,t026,t031,t038,t050, t065,t204,t230,t390
Brazilian/ Hungarian	2-3-1-1-4-4-3	239	8	III	t030,**t037**,t234,t387,t388
Iberian	3-3-1-12-4-4-16	247	8	I	**t008**,**t051**,t052,t054,t200
Irish-1	3-3-1-1-4-4-3	8	8	II	**t008**,t024,t064,t190,t206,t211
New York/Japan (USA100)	1-4-1-4-12-1-10	5	5	II	**t001**,**t002**,t003,t010,t045,t053,t062, t105,t178,t179,t187,t214,t311,t319, t389, t443
Paediatric (USA800)	1-4-1-4-12-1-10	5	5	IV	**t001**,**t002**,t003,t010,t045,t053,t062, t105,t178,t179,t187,t214,t311,t319, t389,t443
Southern Germany	1-4-1-4-12-24-29	228	5	I	**t001**,t023,t041,t188,t201
EMRSA-2/-6 (USA500)	3-3-1-1-4-4-3	8	8	IV	**t008**,t024,t064,t190,t206,t211
EMRSA-3	1-4-1-4-12-1-10	5	5	I	**t001**,**t002**,t003,t010,t045,t053,t062, t105,t178,t179,t187,t214,t311,t319, t389,t443
EMRSA-15/ Barnim	7-6-1-5-8-8-6	22	22	IV	t005,t022,**t032**,t223
EMRSA-16 (USA200)	2-2-2-2-3-3-2	36	36	II	**t018**,t253,t418,t419

[a]Sequence type; [b]Clonal complex, [c]*spa* types according to the Ridom SpaServer (most prevalent *spa* types in bold)

Table 1.7. Major CA-MRSA clones identified worldwide (Diep and Otto, 2008, Deurenberg and Stobberringh, 2008)

Clone	MLST profile	ST[a]	CC[b]	SCC*mec*	*spa* type[c]
Midwest/MW2/ USA400	1-1-1-1-1-1-1	1	1	IV	**t127**,t128,t174,t176 t386,t558
Southwest pacific/Oceania	2-2-2-2-6-3-2	30	30	IV	**t012**,t018,t019,t021 t038,t268
European	1-3-11-14-11-51-10	80	80	IV	**t044**,t131,t376,t416 t436 t455,t1109
Pacific	19-23-15-2-19-20-15	59	59	IV or V_T	**t216**,t199,t444
USA300	3-3-1-1-4-4-3	8	8	IV	**t008**,t024,t064,t190 t206,t211

[a]Sequence type; [b]Clonal complex, [c]*spa* types according to the Ridom SpaServer (most prevalent *spa* types in bold)

The most notorious CA-MRSA clone is USA300 (ST8-MRSA-IVa) because of its high prevalence in the USA. It is characterised by the PFGE profile designated USA300-0114 and first came to the attention of CDC in 2000 during an outbreak among football players in Pennsylvania, USA. In subsequent years it showed high transmissibility causing a series of outbreaks throughout the country e.g. among prisoners in California, Texas and Georgia, children in Texas, Illinois and Arkansas and athletes in Colorado and Indiana (Tenover and Goering, 2009, King et al., 2006).

In the UK, MRSA remain primarily associated with HAIs and the leading HA-MRSA clones belong to the ST22-MRSA-IV (EMRSA-15) and ST36-MRSA-II (EMRSA-16) clonal lineages. CA-MRSA represent <1% of MRSA but due to the high transmissibility of certain CA-MRSA clones, there is a possibility that some could be imported and established into the UK (Ellington et al., 2009, Elston and Barlow, 2009, Tenover and Goering, 2009).

1.15 Virulence factors of *S. aureus*

A plethora of secreted and cell-surface associated virulence factors have been described in *S. aureus* and these function at different stages of infection such as the adhesion to host cells and the evasion of innate and acquired immune responses (Foster, 2005, Rooijakkers et al., 2005). Adhesion to extracellular matrix proteins is mediated by Microbial Surface Components Recognising Adhesive Matrix Molecules which are convalently anchored to peptidoglycan by sortase (Otto, 2010, Foster, 2005). These include clumping factors A, B and Efb which bind fibrinogen and SdrC, SdrD and SasG which promote adhesion to squamous cells (Foster, 2005). On establishment during invasive infection within the host tissues formylated peptides are secreted by the growing bacterium. In addition the C3a and C5a peptide fragments are released by complement activation and these are recognised with high affinity by receptors on the surface of neutrophils (Foster, 2005). Consequently *S. aureus* strains can express chemotaxis inhibitory proteins which bind C5a- and formyl peptide-receptors on the surface of neutrophils, thus inhibiting the attraction of these phagocytes to the growing bacteria (Foster, 2005). Staphylococci complement inhibitor is also involved in the inhibition of complement activation. *S. aureus* strains also express protein A, which is cell-surface associated protein. It binds to the Fc region of IgG resulting in a coat of non-specific antibodies around the bacterium, thus inhibiting the recognition of the bacteria by phagocytes (Otto, 2010, Foster, 2005). Some strains also express a capsular polysaccharide to prevent phagocytosis and many strains also carry the *icaADBC* operon which encodes the polysaccharide intercellular adhesion (PIA) in *S. epidermidis*. PIA is a major mechanism of intercellular adhesion which is essential for the accumulation of multilayered biofilms. It has also been shown to confer virulence to *S. epidermidis* strains and also grant reduced susceptibility to certain combinations of antibiotics (Mack et al., 2006). Similarly, in *S. aureus* the *icaADBC* operon may confer similar properties.

S. aureus also harbours pathogenicity islands that encode several virulence genes, including genetic determinants for superantigens, toxic shock syndrome toxin-1 (*tst*) and enterotoxins B and C (*seb, sec*). These can elicit a non-specific T-cell response, leading to massive cell expansion and shock. Other *S. aureus* toxins include exfoliative toxins A and B, encoded by *eta, etb*, which cause scalded skin syndrome (Lindsay and Holden, 2006).

Toxins that lyse immune cells represent some of the most crucial weapons that *S. aureus* strains use in the evasion of the immune response. *S. aureus* exotoxins are involved in the lysis of erythrocytes or leukocytes, many are β-channel pore forming leukotoxins and are monomeric or have a bi-component structure. The archetype is α-toxin/α-haemolysin (Hla) which forms pores in the cytoplasmic membrane of several cell types including monocytes, platelets and erythrocytes. Panton-Valentine Leukocidin (PVL) also belongs to the pore-forming leukotoxin family. It is a bi-component toxin comprising LukF-PV and LukS-PV subunits encoded on an operon. *S. aureus* strains acquire PVL genes via various PVL-encoding bacteriophages ΦSa2958, ΦSa2mw, ΦPVL, Φ108PVL, ΦSLT, and ΦSa2USA (Boakes et al., 2010, Otto, 2010, Kobayashi and DeLeo, 2009). There is variability in the prevalence of virulence factors in *S. aureus* strains; while α-toxin is expressed by most strains, PVL is expressed by a much lower proportion of strains i.e. 2-3% (Loffler et al., 2010, Otto, 2010).

1.15.1 Role of PVL in *Staphylococcus aureus* pathogenesis

PVL genes are frequently present in established CA-MRSA clones and absent in the leading HA-MRSA clones. As such it has previously been used as an epidemiological marker for CA-MRSA. Furthermore since certain CA-MRSA clones have been implicated in severe necrotising infections e.g. necrotising pneumonia, necrotising fasciitis and sepsis, it has been considered that PVL could contribute directly to the virulence of these clones (Li et al., 2009, Boyle-Vavra and Daum, 2007). Several studies have employed *in vitro* cell culture experiments and different animal infection models to investigate the specific role of PVL in CA-MRSA pathogenesis, however, these have generated conflicting results and the role of PVL remains a point of contention (Loffler et al., 2010, Otto, 2010, Li et al., 2009, Kobayashi and DeLeo, 2009, Diep et al., 2008a).

In a recent study by Brown *et al.* (2009) mice were infected with $1x10^8$ CFUs of PVL-positive USA300 or with isogenic PVL-negative strains and examined for 8 days. Seventy two percent of the mice infected with the PVL-positive strains died 2 days post infection, compared to only 19% mortality of the mice infected with the isogenic PVL-negative mutant strains. Furthermore the PVL-positive strains caused more aggressive

lung damage and severe myositis beneath the site of infection on the skin. Similarly, Labandeira-Rey *et al.* (2007) used purified PVL and isogenic PVL-positive and negative *S. aureus* strains in murine pneumonia models. They determined that PVL had a key role in pulmonary infections and the expression of *lukF/lukS*-PV genes interfered with global regulatory factors (Labandeira-Rey et al., 2007). Diep *et al.* (2008) used isogenic USA300 PVL-positive and PVL-negative strains in a bacteraemia rabbit model and investigated bacterial densities in vital organs i.e. lungs, spleen and kidney. They found higher densities of the PVL-positive strain in the kidney at 24 and 48 hours but at 72 hours the densities of the isogenic PVL-positive and negative strains were comparable. So the they deduced that PVL has a minor and transient role in pathogenesis (Diep et al., 2008a).

Even though the former studies illustrate the virulence potential of PVL they are ardently contested by other studies with contrasting results. For instance Voyich *et al.* (2006) compared PVL-positive USA300 and USA400 strains to their isogenic PVL-negative mutants in sepsis and abscess mouse models and found that the wild type and the mutants caused comparable skin conditions (Voyich et al., 2006). Similarly, Wardenburg *et al.* (2007, 2008) used a USA300 strain and its isogenic PVL-negative mutant in murine models of staphylococcal skin infection and pneumonia. They also found that PVL did not increase the virulence of staphylococcal infection in mice (Wardenburg et al., 2008, Wardenburg et al., 2007). Furthermore Otto (2010) discusses these former studies in a comprehensive review and determines that PVL does not have a key role in *S. aureus* pathogenesis (see Table 1.8).

Table 1.8. Investigation of the role of PVL in animal infection models using isogenic deletion mutants (Otto, 2010)

Genes	Model/animal	Bacterial strain	Results	Reference
lukS, lukF	Necrotising pneumonia-mice Skin infection-mice	LAC (USA300)	PVL has prolonged effect on necrotizing pneumonia & skin infection in mice. Also anti-PVL antibodies protect from infection. Thus PVL is a virulence determinant in mouse pneumonia & skin infection	(Brown et al., 2009)
lukS, lukF	Bactaeraemia-rabbits	SF8300 (USA300) LAC (USA300) MW2 (USA400)	Significantly more PVL wild type bacteria in rabbit kidney, than the *lukSF* mutant. Therefore PVL has a modest virulence effect in rabbit bacteraemia model	(Diep et al., 2008a)
lukS, lukF	Abscess-mice Bacteraemia-mice	LAC (USA300) MW2 (USA400)	No significant differences between wild type & isogenic deletion mutant	(Voyich et al., 2006)
lukS, lukF	Pneumonia-mice	LAC (USA300)	No reduction of mortality in *lukSF* mutant in comparison to wild type, thus PVL not a virulence determinant in mouse pneumonia	(Wardenburg et al., 2007)
lukS, lukF	Pneumonia-mice	LAC (USA300) MW2 (USA400)	Anti-PVL antibodies provide no protection against pneumonia in mice	(Wardenburg et al., 2007)
lukS, lukF	Pneumonia-mice Skin infection-mice	LAC (USA300)	No significant effect of PVL in mouse pneumonia and skin infection	(Wardenburg et al., 2008)
lukS, lukF	Necrotising pneumonia-mice	LAC (USA300)	No significant effect of PVL in rat pneumonia & no significant effect of PVL on the expression of host inflammatory genes	(Montgomery and Daum, 2009)

Key: *lukS, lukF* genes encoding PVL

1.16 The Arginine Catabolic Mobile Element

The arginine catabolic mobile element (ACME) was initially identified in *S. aureus* by Diep *et al.* (2006) when they sequenced the complete genome of the USA300 FPR3757 strain (Montgomery et al., 2009, Diep and Otto, 2008, Ellington et al., 2008). The 30.9 kb genetic island was integrated into *orfX* at the same attachment site as the SCC*mec* element. As it was flanked by repeat sequences characteristic of SCC*mec* elements they postulated that it uses the SCC*mec* recombinase genes for integration and excision from the core genome. The ACME element comprises diverse genes and Diep *et al.* (2006) characterised two interesting gene clusters: *arc* and *Opp-3*. The *arc* gene cluster comprises six genes which encode a complete arginine deiminase pathway which mediates the conversion of L-arginine to carbon dioxide, ATP and ammonia. The generated ammonia could enable the strain to maintain pH homoeostasis on the acidic human skin (pH 4.2-5.9). As the ATP was induced under anaerobic conditions it could be important for energy production in wound environments which are low in oxygen (Diep et al., 2006). Therefore the ACME element could facilitate colonization and persistence within a host (Montgomery et al., 2009). All *S. aureus* strains carry a native *arc* cluster on the core genome which is induced under biofilm and mild acid conditions but it is different from the ACME-*arc* cluster by virtue of gene sequence and orientation. The second gene cluster identified in ACME, was the *Opp-3* operon which comprises five genes encoding a putative oligopeptide permease pathway (Diep et al., 2006). It was termed number three to differentiate it from the native *Opp-1* and *Opp-2* operons present in the core genome. Homologues of these operons are present in group A *Streptococcus* species and other gram positive and gram negative bacteria. They encode various functions including peptide nutrient uptake, quorum sensing, pheromone transport, chemotaxis, eukaryotic cell adhesion, binding of serum components and expression of virulence determinants (Podbielski et al., 1996).

Outside of the USA300 genome, ACME has been infrequently detected in other *S. aureus* genetic backgrounds. Diep *et al.* (2008) discovered variant allotypes in MRSA strains belonging to ST5 and ST59 lineages whereas Ellington *et al.* (2008) detected ACME in MRSA belonging to ST1 and ST97 lineages (Diep et al., 2008b, Ellington et al., 2008).

Many of the antibiotic resistance and virulence determinants harboured by *S. aureus* strains originate in *S. epidermidis* and other coagulase-negative staphylococci. Similarly, *S. haemolyticus* strains and many *S. epidermidis* strains also carry diverse ACME allotypes, including the allotype specifically found in the USA300 clone. It is thought that ACME was horizontally acquired by USA300 from *S. epidermidis* and that this transfer was central to the evolution of the USA300 clone (Miragaia et al., 2009, Pi et al., 2009).

The role of ACME in *S. aureus* pathogenesis has been investigated in several studies but with conflicting results (Montgomery et al., 2009, Diep et al., 2008b). In co-infection studies involving a rabbit bacteraemia model, Diep *et al.* (2008b) inoculated rabbits with both the wild type USA300 and isogenic ACME-deletion mutants and they found greater numbers of the wild type strain in the lungs, spleen and blood of the rabbits and concluded that the deletion of ACME attenuates the fitness of the USA300 strain. Montgomery *et al.* (2009) used murine models of necrotizing pneumonia and skin infection to investigate the role of ACME. They used isogenic ACME positive and deletion mutants of USA300 strains and also clinical isolates containing ACME and lacking the element. They found no significant difference between the virulence of the wild type USA300 and its isogenic deletion mutant in the murine models. There were also no differences between the clinical isolates containing or lacking ACME. So the role of ACME in the USA300 clone pathogenicity and the significance of its presence in the genetic background of some other *S. aureus* backgrounds remain unclear.

1.17 MRSA infections: societal effects and infection control measures

MRSA infections are a health-care problem in the UK and many countries worldwide so the control of these infections is essential (Grundmann et al., 2006). Surveillance systems such as the European Antimicrobial Resistance Surveillance System and the National Nosocomial Infection Surveillance System sponsored by the CDC in the USA, serve to monitor HAIs and provide national prevalence rates of MRSA. In the UK, a compulsory surveillance system was undertaken by acute National Health Service (NHS) Trusts from April 2001 requiring the reporting of MRSA causing bacteraemia (Pearson et al., 2009).

The MRSA prevalence rates in *S. aureus* bacteraemia peaked at 40-45% during 2001-2005 in the UK but decreased to 37% in 2007 and these rates continue to decline (see Figure 1.8) (Pearson et al., 2009, Woodford and Livermore, 2009, Gould, 2005). For instance recent data from the voluntary reporting scheme shows that there were 11,115 *S. aureus* bacteraemia reports in 2009, comprising 10,039 from England, 585 from Wales and 491 from Northern Ireland indicating a 10.8% decrease from rates in 2008 (www.hpa.org.uk/hpr/infections/bacteraemia.htm#saur).

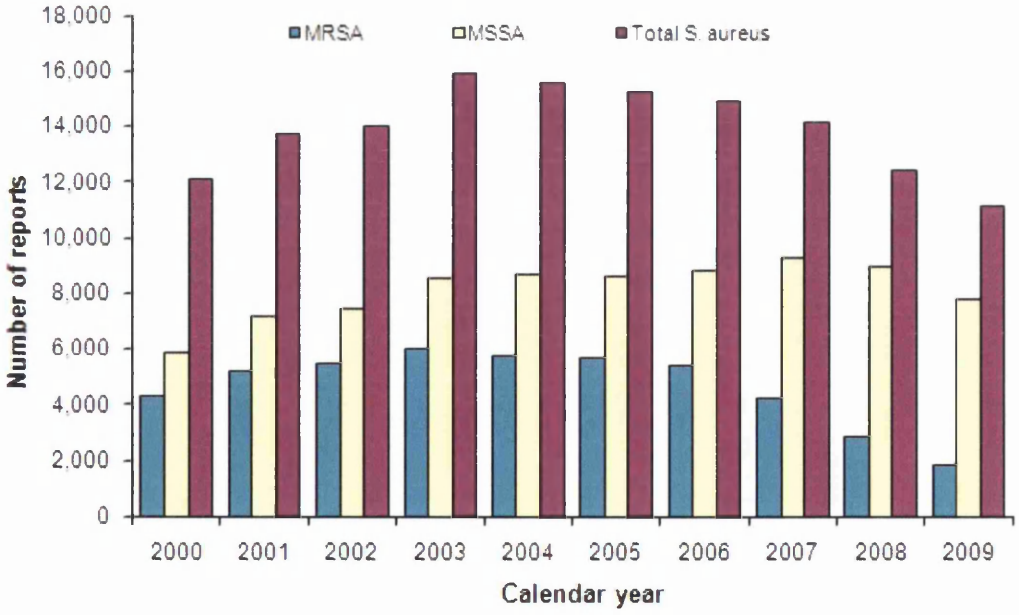


Figure 1.8. Trends in *S. aureus* bacteraemia laboratory reports (voluntary reporting scheme): England, Wales and Northern Ireland 2000-2009
http://www.hpa.org.uk/hpr/infections/bacteraemia.htm#saur

In certain other countries the prevalence rates of MRSA are considerably higher, e.g. in the USA MRSA accounts for >60% of *S. aureus* isolates in hospital intensive care units and >50% of *S. aureus* bacteraemia isolates (Boucher and Corey, 2008, Shukla et al., 2004). In European countries the prevalence varies considerably, for instance the Netherlands and Scandinavian countries have exceptionally low prevalence rates ≤1% but this is in contrast to other countries e.g. Italy, Spain, Greece and Portugal which have reported high rates (>30%) (van Rijen and Kluytmans, 2009, Boucher and Corey, 2008, Zetola et al., 2005).

Societal costs of MRSA infections accrue directly from extended hospital stays and additional antibiotic use or indirectly from reduced productivity, long-term disability and cases of mortality. Other financial repercussions include costs for the containment of outbreaks and changes of empirical antibiotic prescriptions (Grundmann et al., 2006). The economic burden of MRSA infections is not readily available in many countries but studies in the USA suggest that patients with MRSA infections have up to three times increased hospital stay; three times total charges and five times the risk of in-hospital mortality. National hospital costs are between $1.5 billion and $4.2 billion (Gould, 2006). In the UK, the results of a study conducted in an England NHS trust district hospital revealed an estimated 320 994 patients per annum have one or more infections presenting during the in-patient period, costing the hospital sector approximately £1 billion per annum and the hospital costs resulting from a MRSA outbreak have been estimated at £400 000 (Plowman et al., 2001).

In an effort to reduce the burden of MRSA infections, the UK Department of Health introduced the target to reduce MRSA bacteraemia in all acute and foundation trusts by 50% by 2008 (NAO Report: Reducing Healthcare Associated Infections in Hospitals in England, 2009). This was pursued by the implementation of several strategies including the robust training on infection control procedures (NAO Report: Reducing Healthcare Associated Infections in Hospitals in England, 2009). In May 2008 reports indicated that the NHS had attained a 57% reduction of MRSA bacteraemia against the initial target of 50%, indicating that the reduction of infections is attainable (NAO Report: Reducing Healthcare Associated Infections in Hospitals in England, 2009).

Rapid laboratory diagnosis is an important part of infection control but routine phenotypic assays involving the growth of cultures in media, biochemical and antibiotic susceptibility tests have long turnaround times of up to 24 to 48 hours, whereas molecular real-time PCR-based methods are more rapid (2 to 4 hours) and generally more sensitive. From a laboratory perspective the development and implementation of rapid and accurate screening methods is essential as this can benefit a more rapid implementation of infection control procedures and consequently facilitate the reduction of MRSA transmission.

In light of this the objectives of this study were the following:

i. To investigate the concordance of the *mecA* and *femA* real-time PCR results of *S. aureus* isolates to the reported results by the Public Health Wales (PHW) Microbiology ABM Swansea Laboratory.
ii. To determine the genotypic characteristics of the MRSA isolates with the following molecular typing techniques: PFGE, *spa* typing and SCC*mec* typing.
iii. To compare the diagnostic performance of three SCC*mec-orfX* PCR methods by determining the sensitivity, specificity and predictive values with a collection of MRSA and MSSA isolates.
iv. To further investigate the genotypic characteristics of false negative and false positive isolates of the SCC*mec-orfX* PCR methods by PFGE, *spa* typing and SCC*mec* typing.
v. To investigate the prevalence of PVL in unselected consecutive *S. aureus* isolates and to characterize the PVL-positive strain by PFGE, *spa* typing, SCC*mec* typing and detection of ACME.
vi. To compare the molecular epidemiology of two collections of PVL-positive *S. aureus* strains from PHW Microbiology ABM Swansea Laboratory and the Specialist Antimicrobial Chemotherapy Unit, Cardiff.
vii. To explore the discriminatory capacity of the Matrix Associated Laser Desorption Ionisation-Time Of Flight mass spectrometry technique with a collection 137 *S. aureus* isolates.

Chapter Two

Materials and Methods

2.1 Media

Media was prepared with distilled water (dH_2O) and sterilised at 121°C for 15 min in bench-top pressure vessels (Prestige Medical) or the front loading autoclave (Priorclave).

2.1.1 Tryptose Soy media (TSB)

TSB powder	30 g/ L

15g/ L Oxoid Bacteriological Agar was used for TSB agar

Make up to 1L with dH_2O

2.1.2 Oxoid ready-to-use agar plates (90 mm)

The following were obtained from PHW Microbiology ABM Laboratory.

2.1.2.1 Columbia Blood Agar with horse blood (CBA)

2.1.2.2 2% NaCl Columbia Salt Agar (CSA)

2.1.2.3 Iso-Sensitest Agar

2.2 Buffers and stock solutions

2.2.1 50X TAE Buffer

Tris Base	242 g
EDTA	22.6 g
Glacial acetic acid	57 ml

Make up to 1L with dH_2O, adjust to pH 8.0

2.2.2. DNA Enzymatic lysis buffer

Tris-HCl (pH8)	20 mM
EDTA	2 mM
Triton X-100	1.2 % (w/v)
100 µg/ml RNAse	10 µl
100 µg/ml Lysostaphin	10 µl

2.2.3 Buffers and stock solutions for Pulsed field gel electrophoresis (PFGE)

2.2.3.1 0.5M EDTA (stock)

Na-EDTA	93.06 g
dH_2O	400 ml

Adjust pH to 7.5 with NaOH. Make up to 500ml.

2.2.3.2 5M NaCl (stock)

NaCl	146.1 g

dH_2O to 500ml

2.2.3.3 Tris-HCl, pH 7.5 (stock)

Tris Base	2.36 g
Tris-HCl	12.70 g
dH_2O	89 ml

Make up to 100ml. Autoclave and check pH before use.

2.2.3.4 SE Buffer (75mM NaCl, 25mM EDTA, pH 7.5)

5M NaCl	15 ml
0.5M EDTA	50 ml

dH_2O to 1000ml

2.2.3.5 1.6% Low-melting point (LMP) agarose

LMP agarose (BIO RAD)	80 mg
SE buffer	5 ml

Melt in microwave oven and equilibrate to 50°C in a water bath before use. 2ml aliquots stored at -20°C.

2.2.3.6 TE Buffer (10mM Tris-HCl, 10mM Na-EDTA)

1M Tris-HCl (pH 7.5)	10 ml
0.5M Na-EDTA (pH 7.5)	20 ml
dH_2O	970 ml

2.2.3.7 Lysis Buffer (6mM Tris-HCl, 1M NaCl, 100mM EDTA, 0.2% Na-deoxycholate, 1% Na-lauryl sarcosine)

1M Tris-HCl (pH 7.5)	1.2 ml
5M NaCl	40 ml
0.5M EDTA (pH 7.5)	40 ml
Na-deoxycholate	400 mg
Na-lauryl sarcosine	2.0 g
dH_2O to 200ml	

2.2.3.8 Lysis solution

Lysis Buffer 1	1 ml
Lysostaphin (5mg/ml)	10 µl

2.2.3.9 Proteinase K buffer (pH 9)

Na-lauryl sarcosine	10 g
0.5M EDTA (pH 7.5)	1000 ml

Add 40-50 drops of NaOH to pH 9, make up to 1L

2.2.3.10 Proteinase K lysis solution

Proteinase K buffer	1ml
Proteinase K	2µl

2.2.3.11 Buffer J (10X)

Tris HCl (pH 7.5)	100 mM
$MgCl_2$	70 mM
KCl	500 mM
$_{DL}$-Dithiothreitol (DTT)	10 mM

2.2.3.12 0.5X TBE

10X TBE (bought stock)	100 ml
dH_2O	1900 ml

2.3 Agarose gels and gel staining solutions

2.3.1 2%Agarose gel

Agarose	1.5 g
1x TAE	75 ml

2.3.2 PFGE small agarose certified megabase gel

Agarose (BIO RAD)	1.2 g
0.5X TBE	120 ml

Melt at 60°C in microwave

2.3.3 PFGE large agarose certified megabase gel

Agarose (BIO RAD)	1.6 g
0.5X TBE	160 ml

2.3.4 Ethidium Bromide DNA staining solution

Ethidium bromide	20 µl
dH_2O	400 ml

2.3.5 SYBR safe DNA gel stain (Invitrogen)

SYBR safe DNA gel stain	1 µl
Melted agarose	10 µl

2.4 QIAGEN Buffers

2.4.1 Buffer AL

Supplied in QIAamp DNA Mini Kit (Qiagen, UK), details not provided

2.4.2 Buffer AW1

Supplied in QIAamp DNA Mini Kit as a concentrate and the appropriate volume of ethanol was added as stated on the label of the bottle.

2.4.3 Buffer AW2

Supplied in QIAamp DNA Mini Kit as a concentrate and the appropriate volume of ethanol was added as stated on the label of the bottle.

2.4.4 Buffer PBI

Supplied in QIAquick PCR Purification kit, details not provided.

2.4.5 Buffer PE

Supplied in QIAquick PCR Purification kit, details not provided.

2.5 Enzymes

Analytical grade enzymes and chemicals were obtained from Promega, Sigma, Fisher BioReagents and Qiagen:

- Protease (supplied in QIAamp DNA Mini kit)
- Lysostaphin (Sigma)
- 100mg/ ml Proteinase K (Fisher BioReagents)
- 100mg/ ml RNAse A (Qiagen)
- 10 U/ mg *SmaI* (Promega)

Lyophilised lysostaphin (Sigma, UK) was dissolved in dH_2O to 5 mg/ ml and stored at -20°C. DTT ($_{DL}$-Dithiothreitol) (Sigma) was dissolved in dH_2O to 100 mM and stored in the dark at -20°C.

2.6 Bacterial growth conditions

S. aureus cultures were grown under aerobic conditions at 37°C (unless otherwise stated) in laboratory incubators: the stationary incubator (Genlab, UK), the Lab Therm LT-X incubator with an inbuilt Kuhner shaker, the HT Inforse Minitron incubator and the Sartorius Certomat BS-1 incubator.

2.7 Storage and preparation of bacterial stocks

S. aureus isolates were preserved on Protect™ cryopreservation beads (Technical Service Consultants Ltd, UK) and stored at -80°C in an ultra low temperature freezer (New Brunswick Scientific, UK). Isolates were also preserved in glycerol stocks (0.7ml TSB and 0.3ml 50% glycerol) in 1.5 ml microcentrifuge tubes (Fisherbrand, UK) and stored at -20°C in freezers (Liebherr-Profiline, Germany). Fresh media plates and cultures were stored at 2 to 8°C in separate refrigerators (LEC, UK and Liebherr-Profiline, Germany) or in the laboratory cold room.

2.8 Vortex and centrifugation

Genie-2 vortexes (Scientific Industries Inc.) were used for the vigorous mixing of suspensions and solutions. Cells were harvested from liquid cultures or from suspensions by spinning at high speeds in the laboratory centrifuges: the Sanyo MSE MicroCentaur,the Eppendorf centrifuge 5418, the Eppendorf centrifuge 5415R and Eppendorf Mini Spin centrifuge.

2.9 Bacterial strains

The bacterial reference strains included MRSA strains: NCTC10442 (SCC*mec* type I/ accession number AB033763), N315 (SCC*mec* type II/ accession number D86934) and 85/2082 (SCC*mec* type III/ accession number AB037671), as described by (Ito et al., 2001). CA05 (SCC*mec* type IVa/ accession number AB063172) and JCSC1978 or 8/6-3P (SCC*mec* type IVb/ accession number AB063173) as described by (Ma et al., 2002). MR108 or 81/108 (SCC*mec* type IVc/ accession number AB096217) as described by (Ito et al., 2003) and WIS (SCC*mec* type V/ accession number AB121219) as described by (Ito et al., 2004) were kindly provided by T. Miethke, Munich, Germany. The UK epidemic MRSA strains, EMRSA-1 to EMRSA-17 were kindly provided by J. A. Lindsay, St. Georges Hospital Medical School, London, UK (Moore and Lindsay, 2002).

The MRSA reference strains which carried genes for Panton-Valentine Leukocidin (PVL) were: MW2 (provided by T. Miethke), the USA300 SF8300 and USA300 FPR3757 strains (provided by B. A. Diep, UCLA, Los Angeles, USA). The PVL-positive MSSA reference strains were: the Oxford strain (provided by the PHW Microbiology ABM Laboratory) and the MSSA476 strain (provided by J. A. Lindsay).

2.10 Clinical isolates

S. aureus isolates were initially identified from wound swabs in the PHW Microbiology ABM Laboratory and were collected as pure cultures on Iso-Sensitest agar plates. Colonies were collected directly from these cultures or were sub-cultured onto CBA plates and incubated at 37°C for 24 hours. Fresh cultures were prepared by inoculating isolates from frozen stocks onto CBA plates (or other media), these were then incubated at 37°C for 24 hours.

2.11 Extraction of genomic DNA

2.11.1 Crude genomic DNA

Colonies were picked directly from cultures and a bacterial suspension of two to three colonies in 400µl nuclease free water was prepared in 1.5 ml micro-centrifuge tubes and heated at 95°C for 10 min in a dry heat block (Techne, UK). The suspension was centrifuged at 8000 xg for 3 min and then 2 µl of the supernatant was transferred to 23µl PCR reaction mix.

2.11.2 Purified genomic DNA

Purified genomic DNA was extracted from *S. aureus* isolates with the QIAamp DNA Mini kit (QIAGEN, UK). Isolates were inoculated onto CBA plates from frozen stocks and incubated overnight at 37°C, a single colony was then suspended in 5 ml TSB broth and grown overnight at 37°C. Then 1.0 ml of bacterial culture was pipetted into a 1.5ml micro-centrifuge tube and centrifuged for 5 min at 13000 xg. The supernatant was discarded and the bacterial pellet was suspended in 180 µl of the enzymatic lysis buffer, with 10µl lysostaphin and 10µl RNase. The mixture was incubated for at least 30 min at 37°C (solution should go clear) and then 20 µl Proteinase K and 200 µl Buffer AL was added and mixed by vortexing. The mixture was incubated at 56°C for 30 min. The mixture was then briefly centrifuged and 200 µl ethanol was added, pulse-vortexed for 15 sec and briefly centrifuged to remove droplets from the inside of the lid. The mixture was carefully transferred to the QIAamp Spin Column (in a 2.0 ml collection tube) without wetting the rim and centrifuged at 6,000 xg (8000 rpm) for 1 min. The collection tube containing the flow through was discarded and the QIAamp spin column was placed in a clean 2 ml collection tube provided. 500 µl Buffer AW1 was carefully added into the QIAamp spin column and centrifuged at 6000 xg for 1 min. The collection tube containing the flow through was discarded and the QIAamp spin column was placed in a clean 2 ml collection tube. 500µl Buffer AW2 was carefully added into the QIAamp spin column and centrifuged at 20,000 xg (14000 rpm) for 3 min. The collection tube containing the flow through was discarded and the QIAamp spin column was placed in a clean 1.5 ml micro-centrifuge tube. 200 µl sterile dH_2O was carefully added into the QIAamp spin column and incubated at room temperature for 5 min and then centrifuged at 6,000 xg for 1min. The eluted DNA was stored at -20°C for long-

term storage and in subsequent PCR assays 1 µl was transferred to 24 µl PCR reaction mix.

2.12 Calculation of DNA concentration

The concentration (ng/ µl) of DNA in extracts or amplification products was ascertained with the Nanodrop ND-1000 spectrophotometer (Thermo Scientific, USA) which was performed according to the manufacturer's instructions. Only 2 µl of the samples was required and the concentrations were determined by the in-built software and were shown on the viewer display screen. On completion the system was shut down according to the manufacturer's instructions.

2.13 Polymerase Chain Reaction methods

The Sensimix No Ref (Quantace, UK) reaction mix was used for all the polymerase chain reaction (PCR) assays i.e. conventional and real-time PCR methods. The ready-to-use heat activated 2 x reaction mix containing: heat-activated DNA polymerase; dNTPs and $MgCl_2$ (6 mM). Separate vials of SYBR Green and 50 mM $MgCl_2$ were also provided. All the reactions were performed in a total volume of 25 µl in detached 0.2 mL thin-walled, flat capped tubes (StarLab, UK).

With the conventional PCR methods the reactions were performed in the DNA Engine Tetrad 2 Thermal cycler (BIO-RAD, UK) and products were resolved by electrophoresis in an agarose gel. They were prepared by dissolving agarose powder in 1 X TAE buffer by boiling in a 900W or 1200W microwave oven (Sanyo, UK). Then SYBR safe DNA gel stain (Invitrogen) was added (1 µl/ ml) and the mixture was gently swirled to mix it in the stain. The mixture was poured into a casting tray, the required sized comb was set in and the gel was cooled at room temperature. The agarose concentration was dependant on the size of the PCR products: ≤1000 bp products were resolved in a 2% gel and >1000 bp products were resolved in a 1% gel. Electrophoresis was performed in mini tanks (Mini Subcell GT, BIO-RAD) with 1 X TAE buffer used as the running buffer for 40 to 50 min at 110V. Visualisation and imaging of the PCR products was done with the BIO-RAD Molecular Imager Gel Doc XR system and the Quantity One 4.6.3 Basic software (BIO-RAD, UK).

With the real-time PCR methods the PCR products were detected with SYBR Green which was provided in the Sensimix No Ref kit. The reactions were performed in the Rotor-Gene RG-6000 (Corbett Life Science, Australia).

2.13.1 Amplification of target sequences in the *mecA* and *femA* genes

The amplification of target sequences in *mecA*, *femA* of *S. aureus* ($femA_{SA}$) and *femA* of *S. epidermidis* ($femA_{SE}$) was done with singleplex real-time PCR methods which were adapted from Francois *et al.* (2003). Since *S. epidermidis* is a common contaminant of staphylococci wound infections the $femA_{SE}$ PCR was included to detect the presence of *S. epidermidis* (see Table 2.1).

2.13.2 Amplification of sequences of the SCC*mec-orfX* right junction

Three methods: PCR-A, PCR-B and PCR-C were employed for the amplification of the SCC*mec-orfX* junction. The PCR-A method was adapted from the Huletsky *et al.* (2004) method, the PCR-B method was adapted from the Hagen *et al.* (2005) method and the PCR-C method adapted was from the Cuny and Witte (2005) assay (see Table 2.1).

Table 2.1. Oligonucleotides for *mecA*, $femA_{S.\ aureus}$, $femA_{S.epidermidis}$ and the SCC*mec-orfX* PCRs

Label	Sequence (5'-3')	Locus	Position	Reference
DM1	TATGATATGCTTCTCC	SCC*mec*	25210-25225[a]	(Cuny and Witte, 2005)
DM2	AACGTTTAGGCCCATACACCA	*orfX*	25611-25591[a]	(Cuny and Witte, 2005)
DM3	AAATGAAAGACTGCGG	SCC*mec*	25056-25071[a]	(Hagen et al., 2005)
DM4	AATTCCTCCACATCTCA	SCC*mec*	67723-67739[b]	(Hagen et al., 2005)
DM5	CTATCTTCCGAAGGATTG	*orfX*	25438-25421[a]	(Hagen et al., 2005)
DM6	GTCAAAAATCATGAACCTCATTACTTATG	SCC*mec*	25083-25111[a]	(Huletsky et al., 2004)
DM7	ATTTCATATATGTAATTCCTCCACATCTC	SCC*mec*	67710-67738[b]	(Huletsky et al., 2004)
DM8	CAAATATTATCTCGTAATTTACCTTGTTC	SCC*mec*	486-511[c]	(Huletsky et al., 2004)
DM9	CTCTGCTTTATATTATAAAATTACGGCTG	SCC*mec*	459-487[d]	(Huletsky et al., 2004)
DM10	CACTTTTTATTCTTCAAAGATTTGAGC	SCC*mec*	503-529[e]	(Huletsky et al., 2004)
DM11	GGATCAAACGGCCTGCACA	*orfX*	25360-25342[a]	(Huletsky et al., 2004)
DM12	CATTGATCGCAACGTTCAATTT	*mecA*	17966-17 987[a]	(Francois et al., 2003)
DM13	TGGTCTTTCTGCATTCCTGGA	*mecA*	18064-18044[a]	(Francois et al., 2003)
DM14	TGCCTTTACAGATAGCATGCCA	$femA_{S.\ aureus}$	1379239-260[f]	(Francois et al., 2003)
DM15	AGTAAGTAAGCAAGCTGCAATGACC	$femA_{S.\ aureus}$	1379380-356[f]	(Francois et al., 2003)
DM16	CAACTCGATGCAAATCAGCAA	$femA_{S.\ epidermidis}$	1068209-229[g]	(Francois et al., 2003)
DM17	GAACCGCATAGCTCCCTGC	$femA_{S.\ epidermidis}$	1068380-362[g]	(Francois et al., 2003)

Key: [a]AB063172 (MRSAIVa/CA05/JCSC1968); [b]AB037671 (MRSAIII/85/2082); [c]AY267374 (ATCC BAA-40 Portuguese clone of MRSA); [d]AY267381 (MRSA strain CCR1-2025); [e]AY267375 (MRSA strain ID-61880); [f]BA000018 (N315/MRSAII); [g]AE015929 (ATCC 12228/*S. epidermidis*)

The real-time PCR cycles were performed as following:

***mecA, femA$_{SA}$* and *femA$_{SE}$* PCR methods**

Singleplex PCR reaction mix		**PCR cycle**	
Sensimix *No Ref*	12.5 µl	S1	95°C / 10min
Forward primer	200nM	S2	95°C / 15s (denature)
Reverse primer	200nM		60°C / 20s (anneal)
SYBR Green	0.75µl		72°C / 20s (extension)
Crude DNA supernatant	2.0 µl	Repeat cycle S2 50 times	
Make up to 25µl with Nuclease free water		Melt	

PCR-A SCC*mec-orfX* method

Multiplex PCR (Master Mix)		**PCR cycle**	
Sensimix *No Ref*	12.5 µl	S1	95°C / 10min
Forward primer (DM6)	200nM	S2	95°C / 15s
Forward primer (DM7)	200nM		60°C / 20s
Forward primer (DM8)	200nM		72°C / 20s
Forward primer (DM9)	200nM	Repeat cycle S2 50 times	
Forward primer (DM10)	200nM	Melt	
Reverse primer (DM11)	200nM		
SYBR Green	0.75µl		
Crude DNA supernatant	2.0 µl		
Make up to 25µl with Nuclease free water			

Key: S1-step1, S2-step 2.

PCR-B SCC*mec-orfX* method

Multiplex PCR (Master Mix)		**PCR cycle**	
Sensimix *No Ref*	12.5 µl	S1	95°C / 10min
Forward primer (DM3)	200nM	S2	95°C / 15s
Forward primer (DM4)	200nM		56°C / 20s
Reverse primer (DM5)	200nM		72°C / 20s
SYBR Green	0.75µl	Repeat cycle S2 50 times	
Crude DNA supernatant	2.0 µl	Melt	
Make up to 25µl with Nuclease free water			

S1-step1, S2-step 2

PCR-C SCC*mec-orfX* method

Singleplex PCR (Master Mix)		PCR cycle	
Sensimix *No Ref*	12.5 µl	S1	95°C / 10min
Forward primer (DM1)	200nM	S2	95°C / 15s
Reverse primer (DM2)	200nM		55°C / 20s
SYBR Green	0.75µl		72°C / 20s
Crude DNA supernatant	2.0 µl	Repeat cycle S2 50 times	
Make up to 25µl with Nuclease free water		Melt	

S1-step1, S2-step 2.

2.14 Amplification of Panton-Valentine Leukocidin

The amplification of a sequence in the Panton-Valentine Leukocidin (PVL) gene was done with a real-time PCR method which was adapted from Deurenberg *et al.* (2004) (see Table 2.2).

2.15 Amplification of *arcA*-ACME sequence

The amplification of a sequence in the *arcA* gene of the *arcRADBC* locus located in the Arginine Catabolic Mobile Element (ACME) was performed with a conventional PCR, performed in a DNA Engine Tetrad 2 Thermal cycler (BIO-RAD, UK). The method which was adapted from Zhang *et al.* (2008) (see Table 2.3).

Table 2.2. Oligonucleotides for the PVL RT-PCR

Label	Sequence (5'-3')	Position	Reference
PVL-F	GCTGGACAAAACTTCTTGGAATAT	2666-2690 [a]	(Deurenberg et al., 2004)
PVL-R	GATAGGACACCAATAAATTCTGGATTG	2749-2723 [a]	(Deurenberg et al., 2004)

[a] *S.aureus V8 / ATCC* 49775

Table 2.3. Oligonucleotides for the *arcA*-ACME PCR

Label	Oligonucleotide sequence (5'-3')	Position	Reference
arcA-F	GCAGCAGAATCTATTACTGAGCC	74075-74053 [a]	(Zhang et al., 2008)
arcA-R	TGCTAACTTTTCTATTGCTTGAGC	73563-73586 [a]	(Zhang et al., 2008)

[a] *Staphylococcus aureus subsp. aureus* USA300, TCH1516, CP000730.1

The PVL PCR method

Singleplex PCR (Master Mix)		**PCR cycle**	
Sensimix *No Ref*	12.5 µl	S1	95°C / 10min
Forward primer	200nM	S2	95°C / 15s
Reverse primer	200nM		60°C / 20s
SYBR Green	0.75µl		72°C / 20s
Crude DNA supernatant	2.0 µl	Repeat cycle S2 40 times	
Make up to 25µl with Nuclease free water		Melt	

The *arcA*-ACME PCR method

Singleplex PCR (Master Mix)		**PCR cycle**	
Sensimix *No Ref*	12.5 µl	S1	95°C / 10min
arcA- Forward	400nM	S2	95°C / 30s
arcA- Reverse	400nM		60°C / 30s
Genomic DNA	1.0 µl		72°C / 45s
Make up to 25µl with Nuclease free water		S3	72°C / 4min
		Hold at 4°C	

2.16 Purification of PCR products

2.16.1 QIAquick PCR purification

The purification of the PCR products for subsequent DNA sequencing was done with the QIAquick PCR purification kit (QIAGEN, UK) according to the manufacturer's instructions. Five times the volume of Buffer PBI was added to one volume of the PCR reaction before mixing. The mixture was applied to the QIAquick column and centrifuged at 13000 rpm for 1 min, the flow through was discarded and the QIAquick column was placed back into the same collection tube. Then 0.75 ml Buffer PE was added to the QIAquick column and centrifuged at 13000 rpm for 1 min. The flow through was discarded and the QIAquick column was placed back in the same collection tube. The column was centrifuged again for 1 min at 13000 rpm in order to remove any remaining traces of Buffer PE. The QIAquick column was placed in a clean 1.5 ml micro-centrifuge tube and 30 µl sterile dH_2O was added. The column was left to stand for 1 min and then centrifuged for at 13000rpm for 1 min and the eluate was stored at -20°C.

2.16.2 Sequencing of PCR products

DNA sequencing was done by an external company, Eurofins MWG Operon (London, UK). The concentrations of the purified PCR products were prepared according to the size of the products as per the company's criteria:

- <300bp: 2ng/ µl in a minimum volume of 15 µl
- 300 to 1000bp: 5ng/ µl in a minimum volume of 15 µl
- >1000bp: 10ng/ µl in a minimum volume of 15 µl
- Oligonucleotide concentration: 2 pmol/µl in a minimum volume of 15 µl

Generally the PCR product sizes ranged within 300-1000 bp therefore they were prepared at a concentration of 5 ng/µl in a volume of 15 µl, duplicate samples were prepared for sequencing of forward and reverse sequences. Aliquots of the forward and reverse oligonucleotides (2 pmol/µl in a minimum volume of 15 µl) were also prepared. The samples and the olignucleotides were then dried in the Eppendorf Vacufuge Concentrator 5301 centrifuge (Cambridge, UK) or in the Vacuum Centrifuge Maxi Dry Plus (Heto, Allerod, Denmark) for 30 min to 1 hour, these were packaged and sent by normal mail. The DNA sequences were aligned and assessed with the Clustal X 1.81 and BioEdit sequence alignment programmes.

2.17 Identification of *S. aureus* Matrix Assisted Desorption/ ionization-time of flight mass spectrometry

The Matrix Assisted Desorption/ ionization-time of flight (MALDI-TOF) mass spectrometry assay was performed with the Bruker Daltonics Microflex LT system. (Bruker Daltonics, Germany). For *S. aureus* identification either colonies from pure cultures were tested directly or extracts were prepared with the ethanol formic acid method

2.17.1 Preparation of Matrix solution

The saturated solution of matrix solution was prepared in a 1.5 mL micro-centrifuge tube in a fume cupboard due to the potent vapours and corrosive nature of acetronitrile (ACN) and tri-fluor-acetic acid TFA. 500 µl of ACN was carefully added to 15 to 20 mg of alpha-4-cyano-hydrocycinnamic acid (HCCA), then 475 µl of sterile Millipore dH_2O and 25 µl TFA were carefully added. The solution was then ultra-sonicated for 10-15 min, until the HCCA had dissolved. The matrix solution was protected from light in order to keep it stable for several days.

2.17.2 Direct smear preparation

Pure cultures of *S. aureus* isolates were prepared on CBA plates (37°C for 24 hours) and a sterile loop was used to carefully touch and spread part of a single colony within a well of a clean steel target plate. The smears were dried at room temperature, overlaid with 0.5 to 1 µl matrix solution, then again dried at room temperature prior to analysis.

2.17.3 Protein extraction with the ethanol formic acid procedure

One or two colonies from a pure *S. aureus* culture were suspended in 100 µl of sterile Millipore dH_2O in a sterile 1.5 ml micro-centrifuge tube then 450µl of absolute ethanol was added and the suspension was centrifuged at 12000 xg for 2 min. The supernatant was decanted taking care to completely remove the supernatant. The pellet was vigorously re-suspended in 30 µl of formic acid (at least 70%) followed by the addition of 30 µl pure ACN and the suspension was vortexed. The suspension was centrifuged at 12,000 xg for 2 min and then 0.5 to 1 µl of the supernatant was placed onto a spot of a clean steel target plate; each extract was placed onto six successive spots on the target plate. These were dried at room temperature and then overlaid with 0.5 to 1 µl matrix

solution and then again dried at room temperature. The target plate was then loaded into the Bruker Daltonics Microflex LT system.

2.17.4 MALDI-TOF data analysis

The spectra were recorded within a positive linear mode of range 2000 to 20000Da and analysed with the MALDI Biotyper 2.0 software (Bruker Daltonics, Germany). Generated mass-ion peaks of the mass spectra profiles were automatically analysed against reference strains in the in-built MALDI Biotyper reference library. The software provides a log-score classification system for the identification of isolates whereby:

≥ 2.3	highly probable species identification
≤ 2.299 to ≥ 2.0	secure genus identification, probable species identification
≤ 1.999 to ≥ 1.7	probable genus identification
≤ 1.699	no reliable identification

2.18 Typing methods

2.18.1 Pulsed Field Gel Electrophoresis

Pure cultures of *S. aureus* isolates were prepared on CBA plates from frozen stocks and incubated 37°C for 24 hours. A single colony was suspended in 5 mL TSB broth and grown overnight at 37°C. The overnight cultures were vortexed and 800 µl was aliquoted into respective sterile micro-centrifuge tubes. The bacterial cultures were centrifuged at 12000 rpm for 2 min, the supernatant was discarded and the pellet was re-suspended in 1.0 ml SE Buffer (this step was repeated twice). Then 125 µl of the suspension was transferred to a clean micro-centrifuge tube, 125 µl LMP agarose was added to the suspension and mixed by vortexing. Approximately 100 µl of the mixture was then transferred into a plug mould (BIORAD) and these were placed at 4°C for at least 30 min.

The plugs were removed from the moulds, placed into 15.0 ml tubes and 1.0 ml of lysis solution was added to the plug. The samples were incubated at 37°C for 4 hours then the lysis solution was removed. Proteinase K solution (1.0 ml) was added and then the plugs were left in this solution at 56°C for at least 4 hours or overnight. The Proteinase K solution was removed and the plugs were washed four times with 10 ml TE buffer with gentle shaking. At the end of each hour the TE buffer was removed and 10 ml of fresh TE buffer was added prior to gentle shaking. After the last wash, 1.0 ml TE buffer was added to the plugs, at this stage the plugs could be left in the 1.0 ml TE Buffer for up to 1 year at 4°C.

The 10X Buffer J solution (specific for the *Sma*I restriction enzyme) was diluted to a 1:10 dilution with dH_20. After the TE buffer was removed from the plugs, 800 µl of the 1X Buffer J was added to the plugs and these were equilibrated in the buffer for 1 hour. The buffer was removed and 100 µl of 1X Buffer J was again added to each plug. Then 2 to 4 µl of *Sma*I was added to the plugs in 100 µl Buffer J and the plugs were incubated at 25°C overnight. Two litres of 0.5X TBE (100 ml 10X TBE and 1900 ml dH_20) was prepared and chilled at 4°C. The casting stand for the gel was set up; the desired comb was attached to the comb holder and placed into position in the slots of the casting stand. The agarose gel was prepared by adding 1.6 g agarose to 160 ml 0.5X TBE and melted in a microwave.

oven. Approximately 100 ml of agarose was poured into the casting stand for a thickness of approximately 5 to 6 mm and the gel was allowed to cool and solidify at room tcmperature for 30 min, then the comb and comb holder were carefully removed. The plugs were removed from the 25°C incubator and with the use of the spatula each plug was modified to the size of the well in the gel. Using the spatula the sample plugs were gently pressed into the bottom of the wells. Two wells were left free for the standards the lambda DNA ladders. Each well was filled with low melting point agarose, at a concentration equal to that of the gel and then allowed to solidify for 10 to 15 min.

The casting platform was placed at the centre of the contour clamped homogenous electrophoresis (CHEF-DR III) electrophoresis cell. The CHEF-DR III cell was levelled at each of the feet at each corner and then a levelling bubble placed on the casting platform. The platform was removed after levelling and the frame was positioned in the cell by placing the two pins into the bottom set of holes in the floor of the cell. 2.0 to 2.2 L of the chilled 0.5X TBE was poured into the cell and then the CHEF-DR III power module was switched on. The variable speed pump was adjusted to circulate the buffer at approximately 0.75 L/ min (a setting of approximately 70 on the pump regulator) in order to remove bubbles from the system. A constant flow rate was then maintained at a setting that did not disturb the cell and the attached cooling module was set at 14°C.
The following PFGE settings were set up for the run:

- Block 1: Initial switch time 5s, final switch time 15s, run time 10h.
- Block 2: switch time 15s, final switch time 60s, run time 13h.

The Volts/ cm (6.0V/ cm) and the angle (120°) were set and then the programme was started. The current was maintained at range of 115 to 135mA by draining (in order to reduce a high current) or adding more buffer (in order to elevate a low current). After the run was completed the gel was removed from the cell and the gel was slid off the platform and placed in 400 ml dH_2O into which 20 µl or 4 drops of 0.5 µg/ ml ethidium bromide were pipetted and the gel was left to stain for 20 to 30 min with gentle shaking. The solution was then poured into a bottle containing an ethidium bromide decontamination bag. The gel was de-stained by placing it into 400 ml of dH2O with gentle shaking for 1 to 3 hours. The gel was removed from the water and visualisation

and imaging of the PFGE patterns was done with the BIO-RAD Molecular Imager Gel Doc XR system and the Quantity One 4.6.3 Basic software.

2.18.2 *spa* typing

The *spa* typing technique involved an initial amplification of a sequence comprising polymorphic X region within the *spa* gene with the Ridom Bioinformatics (Ridom GmbH, Würzburg, Germany) on-line protocol, termed the DNA Sequencing of the *spa* gene (www3.ridom.de/doc/Ridom_spa_sequencing.pdf). The PCR was performed in the BIO-RAD DNA Engine Tetrad 2 Thermal cycler as (see Table 2.4).

The PCR products were resolved in a 2% agarose gel by electrophoresis with 1X TAE buffer at 110V for 40 min and were visualised with SYBR safe under UV illumination. The *spa* PCR products were then purified and prepared in duplicate for DNA sequencing of the forward and reverse sequences. Alignment of the sequences was done with the Clustal X 1.81 and BioEdit alignment tools and identification of the repeats within the sequences was done manually. The definition of the *spa* types was done via the on-line Ridom *spa* server (http://spaserver2.ridom.de) and novel *spa* types were verified by on-line submissions of the forward and reverse sequence chromatograms.

Table 2.4. Oligonucleotides of the *spa* PCR

Label	Oligonucleotide sequence (5'-3')	Position	Reference
*spa*1113F	TAAAGACGATCCTTCGGTGAGC	1113-1092[a]	(http://www.ridom.de/)
*spa*1514R	CAGCAGTAGTGCCGTTTGCTT	1534-1514[a]	(http://www.ridom.de/)

[a] *S.aureus* J01796 (http://www.ridom.de/)

The *spa* PCR method

Singleplex PCR (Master Mix)		**PCR cycle**	
Sensimix *No Ref*	12.5 µl	S1	95°C / 10min
spa 1113F	200nM	S2	95°C / 45s
spa 1514R	200nM		60°C / 45s
Genomic DNA	1.0 µl		72°C / 1min
Make up to 25µl with Nuclease free water		Repeat cycle S2 35 times	
		S3	72°C / 10min
		Hold at 4°C	

2.18.3 Typing of the Staphylococcal Chromosome Cassette *mec* elements

The characterisation of the Staphylococcal Chromosome Cassette *mec* (SCC*mec*) elements was performed with two multiplex PCRs. The first was the Milheirico *et al.* (2007b) PCR which involves the amplification of nine SCC*mec*-specific loci and the *mecA* gene for the identification of SCC*mec* types I to V (see Table 2.5).

The second typing method comprised two separate multiplex PCR methods by Kondo *et al.* (2007) i.e. MPCR-1 and MPCR-2 which were developed for detection of the different *ccr* and *mecA* gene classes respectively. The MPCR-1 method includes oligonucleotides for the amplification of loci in *ccrAB1, ccrAB2, ccrAB3, ccrAB4* and *ccrC*, whereas the MPCR-2 method includes oligonucleotides for the amplification of loci in the class A, class B and class C2 *mec* gene complexes (see Table 2.6).

For all methods 1 µl of purified genomic DNA was transferred into 24 µl of the PCR reaction mix and the reaction was performed in the DNA Engine Tetrad 2 Thermal cycler. The Sensimix was retained for the Milheirico *et al.* (2007) and MPCR-1 methods. However with the MPCR-2 method large PCR products ranging from 804bp to 2827 bp were generated, so the Expand High Fidelity PCR kit which amplifies products up to 10 kb was used (Roche, Hertfordshire, UK). For the Expand High Fidelity PCR system the PCR cycling conditions were set up according to the manufacturer's instructions. The recommended elongation times were: 45 sec for up to 0.75 kb, 1 min for 1.5 kb, 2 min for 3 kb, 4 min for 6 kb and 8 min for 10 kb and a final extension step of 7 min.

The multiple PCR products were resolved in a 2% agarose gel by electrophoresis in 1X TAE buffer at 100V for 50 min. Visualisation and imaging of the products was done with the BIO-RAD Molecular Imager Gel Doc XR system and the Quantity One 4.6.3 Basic software.

Table 2.5. Oligonucleotides of the SCC*mec* typing PCR

Label	Oligonucleotide sequence (5'-3')	Region	Product size(bp)
CIF2 F2	TTCGAGTTGCTGATGAAGAAGG	J1 region, locus A: downstream of *pls* (SCC*mec*I).	495
CIF2 R2	ATTTACCACAAGGACTACCAGC		
ccrC F2	GTACTCGTTACAATGTTTGG	*ccrC* complex(SCC*mec*V)	449
ccrC R2	ATAATGGCTTCATGCTTACC		
RIF5 F10	TTCTTAAGTACACGCTGAATCG	J3 region, locus F: between *Tn*554 & *orfX* (SCC*mec*III).	414
RIF5 R13	ATGGAGATGAATTACAAGGG		
SCC*mec*V J1F	TTCTCCATTCTTGTTCATCC	J1 region (SCC*mec*V)	377
SCC*mec*V J1R	AGAGACTACTGACTTAAGTGG		
dcs F2	CATCCTATGATAGCTTGGTC	J3 region, locus D: internal to downstream constant segment (SCC*mec*I, II, IV)	342
dcs R1	CTAAATCATAGCCATGACCG		
ccrB2	AGTTTCTCAGAATTCGAACG	II and IV *ccr* complex	311
ccrB2	CCGATATAGAAWGGGTTAGC		
kdp F1	AATCATCTGCCATTGGTGATGC	J1 region locus B: internal to the *kdp* operon specific to SCC*mec*II	284
kdp R1	CGAATGAAGTGAAAGAAAGTGG		
SCC*mec*III J1F	CATTTGTGAAACACAGTACG	J1 region(SCC*mec*III)	243
SCC*mec*III J1R	GTTATTGAGACTCCTAAAGC		
mecI P2	ATCAAGACTTGCATTCAGGC	Locus C, internal to the *mecI* of the *mec* complex (SCC*mec*II, III)	209
mecI P3	GCGGTTTCAATTCACTTGTC		
mecA P4	TCCAGATTACAACTTCACCAGG	*mecA* - internal positive control	162
mecA P7	CCACTTCATATCTTGTAACG		

The adapted Milheirico *et al.* (2007b) method

Multiplex PCR (Master Mix)		**PCR cycle**
Sensimix *No Ref*	12.5 µl	S1 95°C / 10min
*kdp*F1	200 nM	S2 95°C / 30s
*kdp*R1	200 nM	53°C / 30s
CIF2 F2	400 nM	72°C / 1min
CIF2 R2	400 nM	Repeat cycle S2 30 times
RIF5 F10	400 nM	S3 72°C / 4min
RIF5 R13	400 nM	Hold at 4°C
SCC*mec*III J1F	400 nM	
SCC*mec*III J1R	400 nM	
SCCmecV J1F	400 nM	
SCCmecV J1R	400 nM	
mecI P2	800 nM	
mecI P3	800 nM	
dcs F2	800 nM	
dcs R1	800 nM	
mecA P4	800 nM	
mecA P7	800 nM	
ccrB2 F2	800 nM	
ccrB2 R2	800 nM	
ccrC F2	800 nM	
ccrC R2	800 nM	
Genomic DNA	1.0 µL	
Make up to 25µl with Nuclease free water		

Table 2.6. Oligonucleotides for the MPCR-1 and MPCR-2 methods

Label MPCR-1	**Oligonucleotide sequence (5'-3')**	**Region**	**Expected product size (bp)**
mA1	TGCTATCCACCCTCAAACAGG	*mecA* - internal positive control	286 (mA1-mA2)
mA2	AACGTTGTAACCACCCCAAGA	*mecA*	
α1	AACCTATATCATCAATCAGTACGT	*ccrA1*	695 (α1- βC)
α2	TAAAGGCATCAATGCACAAACACT	*ccrA2*	937 (α2- βC)
α3	AGCTCAAAAGCAAGCAATAGAAT	*ccrA3*	1791 (α3- βC)
βC	ATTGCCTTGATAATAGCCITCT	*ccrB1*, *ccrB2*, *ccrB31*	
α4.2	GTATCAATGCACCAGAACTT	*ccrAB4*	1287 (α4.2 - β4.2)
β4.2	TTGCGACTCTCTTGGCGTTT	*ccrAB4*	
γR	CCTTTATAGACTGGATTATTCAAAATAT	*ccrC*	518 (γR - γF)
γF	CGTCTATTACAAGATGTTAAGGATAAT	*ccrC*	

Label MPCR-2	**Oligonucleotide sequence (5'-3')**	**Region**	**Expected product size (bp)**
mA16	CATAACTTCCCATTCTGCAGATG	*mecI*	1963 (mA7-m16)
IS7	ATGCTTAATGATAGCATCCGAATG	*IS1272*	2827 (mA7-IS7)
IS2 (iS-2)	TGAGGTTATTCAGATATTTCGATGT	*IS431*	804 (mA7-iS-2)
mA7	ATATACCAAACCCGACAACTACA	*mecA*	

The MPCR-1 and MPCR-2 methods

MPCR-1 (Master Mix)		**PCR cycle**	
Sensimix	12.5 µl	S1	95°C / 10min
mA1	100 nM	S2	94°C / 2min
mA2	100 nM		57°C / 1min
α 1	100 nM		72°C / 2min
α 2	100 nM	Repeat cycle S2 30 times	
α 3	100 nM	S3	72°C / 2min
β C	100 nM	Hold at 4°C	
α 4.2	100 nM		
β 4.2	100 nM		
γ R	100 nM		
γ F	100 nM		
Genomic DNA	1.0 µl		
Make up to 25µl with Nuclease free water			

MPCR-2 (Master Mix)		**PCR cycle**	
Make up to 25µl with Nuclease free water		S1	94°C / 2min
Expand High Fidelity Buffer (10X) without $MgCl_2$	2.5 µl	S2	94°C / 2min
$MgCl_2$ (25mM)	3.0 µl		58°C / 1min
PCR Grade Nucleotide Mix	0.5 µl		72°C / 3min
m16	100 nM	Repeat cycle S2 30 times	
IS7	100 nM	S3	72°C / 7min
IS2(iS-2)	100 nM	Hold at 4°C	
mA7	100 nM		
Expand High Fidelity Enzyme Mix	0.375 µl		
Genomic DNA	1.0 µl		

2.19 Amplification of remnant of SCC*mec* sequence, *att*L to *att*R

Amplification of a sequence spanning from the left chromosome-SCC*mec* junction (*att*L) to right chromosome-SCC*mec* junction (*att*R) was attempted with forward primers that were located upstream of *att*L and a reverse primer located in *orfX*. The PCR methods and the oligonucleotides that were used were developed by Katayama *et al.* (2000) and Jansen *et al.* (2006) (see Table 2.7). An oligonucleotide termed cR2 served as the reverse primer and the oligonucleotides termed cL1, E3 and E4 served as the forward primers. These aligned to regions upstream of *att*L in the following published sequences:

i) cL1-AB033763: SCC*mec* type I/ NCTC10442, SCC*mec* type II/ D86934 and SCC*mec* type IVb/ AB063173.
ii) E3- SCC*mec* type IVc/ AB096217; SCC*mec* type II/ BX571856
iii) E4- SCC*mec* type IVa/ JCSC1968

Purified genomic DNA (1µl) and the Expand High Fidelity PCR kit were used for the reactions which were performed as singleplex PCRs i.e. cL1 and cR2, E3 and cR2, and E4 and cR2. They were performed in the BIO-RAD DNA Engine Tetrad 2 Thermal cycler at different annealing temperatures i.e. the E4 and cR2; E3 and cR2 PCRs were performed at 56°C whereas the cL1 and cR2 PCR was performed at 53°C. The PCR products were resolved in a 1% agarose gel with electrophoresis in 1X TAE buffer at 100V for 50 min. Visualisation and imaging of the products was done with the BIO-RAD Molecular Imager Gel Doc XR system and the Quantity One 4.6.3 Basic software. The products were purified, sent for DNA sequencing and the DNA sequences were aligned and assessed with the ClustalX 1.81 and the BioEdit sequence alignment tools.

Table 2.7. Oligonucleotides for the amplification of the sequence spanning from *att*L to *orfX*

Label	Oligonucleotide sequence (5'-3')	Position	Reference
cL1	ATTTAATGTCCACCATTTAACA	4607-4628[a]	Katayama *et al.*, 2000
E3	TTTTGCTGTTTTTATCACCATATTGAA	6434-6460[c]	Jansen *et al.*, 2006
E4	AATTTACCAGACAGCCTGGTGC	836-857[b]	Jansen *et al.*, 2006
cR2	AAACGACATGAAAATCACCAT	58122-58102[a]	Jansen *et al.*, 2006

[a]SCC*mec* type II/ D86934; [b]SCC*mec* type IVa/ AB063172; [d]SCC*mec* type IVc/ AB096217

The *attL–orfX* PCR method

Master Mix		**PCR cycle**	
Make up to 25µl with Nuclease free water		S1	94°C / 2min
Expand High Fidelity Buffer (10X) without $MgCl_2$	2.5 µl	S2	94°C / 30s
$MgCl_2$ (25mM)	3.0 µl		53°C / 30s*
PCR Grade Nucleotide Mix	0.5 µl		72°C / 8min
cL1 or E4 or E3	200 nM	Repeat cycle S2 30 times	
cR2	200 nM	S3	72°C / 7min
Expand High Fidelity Enzyme Mix	0.375 µl	Hold at 4°C	
Genomic DNA	1.0 µl		

*PCRs with oligonucleotides E4 and cR2; E3 and cR2, were performed at annealing temperature 56°C, whereas cL1 and cR2 was performed at 53°C

2.20 Antibiotic susceptibility testing

Susceptibility testing of *S. aureus* was performed with the oxacillin and cefoxitin disc diffusion assays and the BD Phoenix automated microbiology system. These were performed and interpreted according to the British Society for Antimicrobial Chemotherapy (BSAC) version 9.1 methods for antimicrobial susceptibility testing. Pure cultures which were prepared on CBA plates and incubated at 37°C for 24 hour were used for testing and bacterial suspensions were prepared in 5 ml of sterile dH_2O to a 0.5 McFarland turbidity. Ready-to-use Columbia Salt Agar (CSA) plates (90 mm) with 2% NaCl (Oxoid, UK) and Iso-Sensitest media were used for the oxacillin and cefoxitin disc diffusion assays respectively.

2.20.1 Oxacillin disc diffusion assay

A sterile straight wire was used to touch a single colony and it was suspended in 2.0 ml of sterile dH_20. A sterile loop was used to transfer a 5 µl aliquot of the bacterial suspension onto the surface of the CSA plate and sterile cotton tipped swabs were used to spread the isolate in three directions over the quarter section of the plate. Then, using sterile tweezers, a 1 µg oxacillin disc (Oxoid) was placed firmly on the dried surface of the inoculated plate. Each plate accommodated four 1µg oxacillin discs without the overlapping of inhibition zones. Within 15 min of disc application the plates were incubated aerobically at 30°C for 24 hours,. Zone diameters were measured in good light and also examined for colonies within zones, if colonies within zones were considered contaminants the isolate was re-tested. For the 1µg oxacillin disc tests the sizes of the inhibition zones were interpreted as following: ≥15 mm susceptible and ≤14 mm resistant (see Table 2.8).

Table 2.8. Zone diameter breakpoints for *S. aureus* oxacillin and cefoxitin disc diffusion tests

	Zone diameter (mm)		
Antibiotic	**Disc**	**Resistant**	**Susceptible**
Oxacillin	1µg	≤14	≥15
Cefoxitin	10µg	≤21	≥22

(http://www.bsac.org.uk/_db/_documents/Version_9.1_March_2010_final.pdf)

2.20.2 Cefoxitin disc diffusion assay

A sterile straight wire was used to touch a single colony and to suspend it in 2.0 ml sterile dH_20. In order to obtain semi-confluent growth, a sterile 5 µl loop was used to transfer an aliquot of the bacterial suspension of the test isolate, onto the surface of the Iso-Sensitest agar plate and sterile cotton tipped swabs were used to spread the isolate in three directions over the plate. With sterile tweezers the 10 µg cefoxitin disc was placed firmly on the dried surface of the inoculated plate, two discs were accommodated per plate. Within 15 min of disc application the plates were incubated aerobically strictly at 35°C for 18 to 20 hours. Zone diameters were measured in good light and examined for colonies within zones e.g. for contaminants. A light haze of colonies may reflect the growth of hyper-producers of β-lactamase. For the 10 µg cefoxitin disc tests the sizes of the inhibition zones were interpreted as following: ≥22 mm susceptible, ≤21 mm resistant (see Table 2.8).

2.20.3 BD Phoenix automated microbiology system

The following BD Phoenix reagents and components were provided for susceptibility testing:

1) BD Phoenix PMIC/ID-67 gram positive panels, closures and an inoculation support
2) Phoenix ID broth for identification
3) Phoenix AST broth for antimicrobial susceptibility test
4) Phoenix AST indicator solution
5) BD PhoenixSpec™ Nephelometer

Prior to taking turbidity readings the BD PhoenixSpec™ Nephelometer was calibrated with prepared Phoenix 0.25 or 0.5 or 1.0 McFarland solutions. A panel was placed on an inoculation support with the ports of the panels at the top and the pad at the bottom. A Phoenix ID Broth tube was labelled with the specimen number and a single colony was aseptically selected with the tip of a sterile cotton swab (polyester swabs prohibited) and suspended in the broth. The tube was capped, vortexed for approximately 5 sec and allowed to rest for several seconds to allow the air bubbles to surface. The tube was inserted into the nephelometer and the turbidity/density readings were calculated by the instrument; a range of 0.5 to 0.6 is acceptable. If the density of the suspension was low, more of the selected colony was added, the suspension was re-

vortexed and re-read to confirm that the correct density had been achieved. The bacterial suspension in the ID broth had to be used within 60 min of preparation. The Phoenix AST indicator solution for gram positive panels was allowed to attain room temperature. A Phoenix AST broth was labelled with the specimen number and one drop of the indicator solution was added to the AST broth. The AST broth was inverted (not vortexed) and the indicator solution was returned to 2 to 8°C as soon as possible. Using a pipette and sterile tips, 25µl of the standardised inoculated ID broth was transferred to the AST broth and the tube was capped and inverted a few times. Several seconds were allocated for air bubbles to surface and it was gently tapped to aid elimination of bubbles. The ID tube inoculum was poured into the open port of the ID side of the panel and the inoculated AST broth was poured into the open port of the AST side. Panels were inoculated within 30 min after inoculation of the AST broth. The ports were capped and the panel was left on the inoculation support, to allow the flowing suspension to fill the wells sufficiently, this was indicated by the pad at the bottom of the panel turning blue. Inoculated ID tubes could be retained for optional purity checks. Using a sterile loop a small drop from the inoculum was recovered and spread on a fresh CBA plate. The plate was incubated at 37°C for 24 hours and then verified as a pure culture. The used ID and AST broth tubes were capped and discarded in a biohazard sharps disposal container. The panels were logged in and loaded into the BD Phoenix instrument as per instructions in the BD Phoenix System user's manual.In the instrument panels were continuously incubated at 35°C and tested every 20 min: on the hour; at 20 minutes past the hour and again at 40 min, for up to 16 hours (Phoenix panels are read only by the instrument and cannot be read manually). Results were viewed on a computer and printed from an external printer connected to the instrument. On the report, the identification of the organism with a probability percentage and MICs of antimicrobial agents (interpreted according to BSAC criteria) were displayed (see Table 2.9 for the oxacillin and cefoxitin MIC breakpoints).

Table 2.9. MIC breakpoints for oxacillin and cefoxitin susceptibility testing of *S. aureus*

	MIC breakpoint (mg/L)	
Antibiotic	**Resistant**	**Susceptible ≤**
Oxacillin	>2	≤2
Cefoxitin	>4	≤4

(http://www.bsac.org.uk/_db/_documents/Version_9.1_March_2010_final.pdf)

2.20.4 Testing for inducible macrolide-lincosamide-streptogramin B resistance in *S. aureus*

Inducible macrolide-lincosamide-streptogramin B (MLS_B) resistance was investigated with the dissociated test (D-test) in *S. aureus* isolates which were susceptible to clindamycin but resistant to erythromycin (see Table 2.10). An IsoSensitest plate was inoculated with 5 µl of the suspension of the test isolate, to give a semi-confluent growth. After the plate had dried a 5µg erythromycin disc and a 2µg clindamycin disc were placed 1.5 cm apart at the centre of the plate and these were then incubated at 37°C for 18 to 20 hours. Isolates that had no zones of inhibition with erythromycin but exhibited a blunted edge at the inhibition zone of the clindamycin disc were considered to have inducible MLS_B resistance (see Figure 2.1).

Table 2.10. MLS_B resistance: interpretation of susceptibility testing results

R≤ 16mm; S≥ 20mm Int. 17-20mm	**R≤ 22mm; S≥ 26mm Int. 23-25mm**	**Interpretation**
Erthromycin (5µg)	**Clindamycin (2µg)**	
S	S	Isolate susceptible to both erythromycin and clindamycin
R	R	Isolate resistant to both erythromycin and clindamycin (constitutive MLS_B resistance)
R	S	Isolate may have inducible MLS_B resistance, confirm with D-test

R-resistance; S-susceptible; Int-intermediate

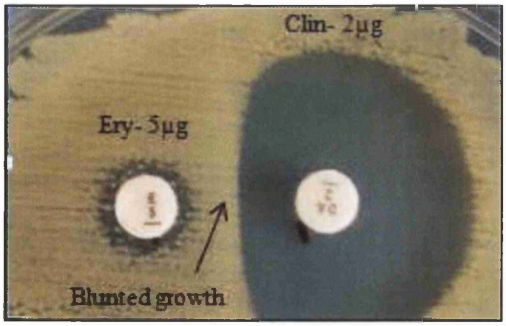


Figure 2.1. D-test result showing blunted zone of inhibition around the clindamycin disc signifying inducible MLS_B resistance

Chapter Three

A comparison of the real-time PCR results and the reported clinical microbiology laboratory results of *Staphylococcus aureus* clinical isolates

3.0 Introduction

Accurate detection of methicillin resistance in *Staphylococcus aureus* isolates is essential for the initiation of infection control measures and the implementation of effective therapy. The gold standard is the detection of the *mecA* gene by PCR however due to technical and financial constraints, molecular assays are infrequently implemented in clinical microbiology laboratories. Routinely, phenotypic assays which incorporate culture based identification and antibiotic susceptibility assays are performed but the variable expression of methicillin resistance poses challenges for these methods. MRSA exhibit heterogeneous or homogeneous expression of methicillin resistance whereby the heterogeneous strains comprise subpopulations of cells with different degrees of methicillin resistance. The majority of cells have low-level resistance and only a low frequency of cells have high level resistance. Conversely, in homogenous strains the majority of cells express high level resistance constitutively (Tomasz et al., 1991). The heterogeneous MRSA can express low methicillin resistance or methicillin susceptible phenotypes, therefore the application of phenotypic tests such as disc diffusion tests can result in the misidentification of *mecA* positive strains as MSSA. Additional challenges include certain *mecA* negative *S. aureus* strains which exhibit minimum inhibitory concentrations (MICs) close to the breakpoint for oxacillin resistance. Often they are hyper producers of penicillinase and are referred to as borderline oxacillin resistant *S. aureus* (BORSA). Although these strains are *mecA* negative they can be misidentified as MRSA with phenotypic assays. *S. aureus* isolates with these types of atypical resistance phenotypes often generate discrepant results with diagnostic assays and present ongoing challenges for accurate identification of MRSA.

For the purposes of this study the *mecA* and $femA_{SA}$ real-time PCR methods were performed as a reference method for the identification of MRSA. The objective was to perform the reference method on 561 *S. aureus* wound isolates and to compare these real-time PCR results to the reported results of the Public Health Wales (PHW) Microbiology Abertawe Bro Morgannwg (ABM) Laboratory.

3.1 Results

3.1.1 Comparison of PCR and reported PHW microbiology results

Five hundred and sixty one *S. aureus* clinical isolates were collected from the PHW Microbiology ABM Laboratory from April 2007 to September 2007. These were originally isolated from wound swab specimens and were collected as cultures on Iso-Sensitest agar, a medium used for antimicrobial susceptibility testing. By real time PCR the *S. aureus femA* gene (*femA*$_{SA}$) was detected in all 561 isolates. A real-time PCR for the detection of the *S. epidermidis femA* gene (*femA*$_{SE}$) was also performed in order to verify that the clinical isolates were not *S. epidermidis* and this gene was not detected in any of the isolates. One hundred and thirty seven (24.4%) isolates were *mecA* positive i.e. MRSA and 424 (75.6%) were *mecA* negative i.e. MSSA. The reported PHW Microbiology ABM Laboratory results were retrieved from the computerised data management system and out of the 137 *mecA* positive *S. aureus* only 122 were reported as MRSA. In addition one of the 424 *mecA* negative *S. aureus* was also reported as a MRSA. Furthermore in three cases *S. aureus* isolates were not reported at all but coagulase negative staphylococci (CoNS) were reported in two cases and a different bacterial species was reported in one case (see Table 3.1).

In total sixteen discrepancies were observed between the PCR results and the reported results in the PHW Microbiology ABM Laboratory system (see Table 3.2). In twelve cases MSSA was reported and in the case of isolate 2 the MSSA was co-identified with an *S. epidermidis*. In two cases MSSA were not reported at all rather, *S. epidermidis, Acinetobacter* species and *B. cereus* were reported in the case of isolate 1 whereas only *S. epidermidis* was reported for isolate 13. For isolate 12 a *Staphylococcus* sp. was not reported at all and only a *Streptococcus* species was reported. In the remaining case of isolate 16, MRSA, anaerobes and *Corynebacterium* were reported in the laboratory system (see Table 3.2).

Table 3.1. Comparison of real-time PCR results and reported PHW Microbiology ABM Laboratory results of 561 clinical isolates

Real-time PCR	***femA*$_{SA}$**		***femA*$_{SE}$**		***mecA***		**Other**
	POS	**NEG**	**POS**	**NEG**	**POS**	**NEG**	
	561	0	0	561	137	424	0
PHW Microbiology ABM	***Staphylococcus* species**						**Other**
Reported results	***S. aureus***		**CoNS**		**MRSA**	**MSSA**	
	558		2		123*	435	1

*n=123 includes the 122 MRSA and the *mecA* negative isolate reported as a MRSA

Table 3.2. Discrepancies between the PCR results and the reported PHW Microbiology ABM Laboratory results of sixteen *S. aureus* isolates

No.	Isolate	PCR			Reported PHW Microbiology ABM Laboratory results	
		mecA	*femA*$_{SA}$	Interp.	*Staphylococcus* species	Other bacteria isolated from same specimen
1.	113 015	Pos	Pos	MRSA	*S.epidermidis*	*Acinetobacter, B. cereus*
2.	122 126	Pos	Pos	MRSA	MSSA, *S. epidermidis*	
3.	124 051	Pos	Pos	MRSA	MSSA	--
4.	129 103	Pos	Pos	MRSA	MSSA	--
5.	176 065	Pos	Pos	MRSA	MSSA	--
6.	186 079	Pos	Pos	MRSA	MSSA	--
7.	189 009	Pos	Pos	MRSA	MSSA	--
8.	192 020	Pos	Pos	MRSA	MSSA	--
9.	205 092	Pos	Pos	MRSA	MSSA	--
10.	206 006	Pos	Pos	MRSA	MSSA	--
11.	208 506	Pos	Pos	MRSA	MSSA	--
12.	219 066	Pos	Pos	MRSA	None	*Streptococcus* species
13.	228 027	Pos	Pos	MRSA	*S.epidermidis*	--
14.	229 015	Pos	Pos	MRSA	MSSA	--
15.	241 132	Pos	Pos	MRSA	MSSA	--
16.	225 124	Neg	Pos	MSSA	MRSA	Anaerobes, Corynebacterium

Key: id.-identification; Interp.- Interpretation; -- no bacteria isolated

3.1.2 Comparison of the PCR results and results of susceptibility testing with disc diffusion tests and the BD Phoenix automated microbiology system

The 1μg oxacillin and 10μg cefoxitin disc diffusion tests were performed on the sixteen isolates according to BSAC version 9.1 methods for antimicrobial susceptibility testing criteria. Eleven of the isolates were resistant to oxacillin with no zone of inhibition whereas isolates 7, 9 and 10 showed clear susceptibility to oxacillin, exhibiting inhibition zone diameters of 23 mm, 20 mm and 20 mm respectively. They also depicted susceptibility in the repeat oxacillin disc test (see Table 3.3). One unusual isolate (8) depicted a zone of 15mm (the breakpoint for susceptibility) and then 10mm (exhibiting resistance) in the repeat test. Isolate 16 which was reported as an MRSA in the PHW Microbiology ABM Laboratory system also showed susceptibility to oxacillin and had zone diameters of 22 mm and 21mm in the duplicate tests (see Table 3.3).

Table 3.3. Results of sixteen *S. aureus* isolates with the *mecA* PCR, oxacillin and cefoxitin disc diffusion assays and the BD Phoenix

No	Isolate	*mecA* PCR	PHW reported result	Disc diffusion assay (mm)						BD Phoenix MIC (mg/L)		
				Oxacillin (1µg) S ≥15mm; R ≤14mm			Cefoxitin (10µg) S ≥22mm; R ≤21mm			Oxacillin R > 2mg/L	Cefoxitin R > 4mg/L	
				1st	2nd	Interp	1st	2nd	Interp	MIC	MIC	Interp
1.	113 015	pos	*S. epidermidis*	0	0	R	0	0	R	>4	>8	MRSA
2.	122 126	pos	MSSA	0	0	R	0	0	R	>4	>8	MRSA
3.	124 051	pos	MSSA	0	0	R	0	0	R	>4	>8	MRSA
4.	129 103	pos	MSSA	0	0	R	0	0	R	>4	>8	MRSA
5.	176 065	pos	MSSA	0	0	R	0	0	R	>4	>8	MRSA
6.	186 079	pos	MSSA	0	0	R	0	0	R	>4	>8	MRSA
7.	**189 009**	**pos**	**MSSA**	**23**	**18**	**S**	**12**	**8**	R	**≤1**	**≤2**	**MSSA**
8.	192 020	pos	MSSA	15	10	S/R	14	17	R	4	8	MRSA
9.	205 092	pos	MSSA	20	18	S	0	0	R	>4	>8	MRSA
10.	206 006	pos	MSSA	20	17	S	0	0	R	4	>8	MRSA
11.	208 506	pos	MSSA	0	0	R	0	0	R	>4	>8	MRSA
12.	219 066	pos	*Streptococcus sp.*	0	0	R	0	0	R	>4	>8	MRSA
13.	228 027	pos	*S. epidermidis*	0	0	R	0	0	R	>4	>8	MRSA
14.	229 015	pos	MSSA	0	0	R	0	0	R	>4	>8	MRSA
15.	241 132	pos	MSSA	0	0	R	0	0	R	>4	>8	MRSA
16.	**225 125**	**neg**	**MRSA**	**22**	**21**	**S**	**20**	**21**	**R**	**0.5**	**4**	**MSSA**

Key: Interp- Interpretation; S- susceptible, R – resistant.

In contrast to the oxacillin disc test, all sixteen isolates exhibited resistance to cefoxitin, with thirteen isolates displaying no zone of inhibition. Isolates 7 and 8 exhibited zones of 12 mm and 14 mm respectively, indicating resistance to cefoxitin and also there was a light haze of colonies within the zones of inhibition. Isolate 16 was more unusual because with the cefoxitin test it depicted inhibition zones of 20 mm and 21 mm (breakpoint for resistance), indicating tentative methicillin resistance however it had exhibited clear susceptibility with the oxacillin disc test.

With the BD Phoenix, fourteen isolates had MICs of ≥4 mg/L and ≥8 mg/L for oxacillin and cefoxitin respectively, thus were reported methicillin resistant. These included isolate 8 which had depicted ambiguous 'susceptible/ resistant' results with the 1μg oxacillin assay and then clear resistance with the 10μg cefoxitin assay (see Table 3.3). However isolate 7 had MICs of ≤1mg/L and ≤2mg/L for oxacillin and cefoxitin respectively and isolate 16 had MICs of 0.5 mg/L and 4 mg/L for oxacillin and cefoxitin respectively. These MICs clearly signified susceptibility to oxacillin and cefoxitin and thus these isolates were identified as a MSSA with the BD Phoenix. These two isolates presented two fascinating cases because although isolate 7 was *mecA* positive and isolate 16 was *mecA* negative by PCR, they both depicted similarly discordant results with the phenotypic susceptibility testing methods i.e. methicillin susceptibility with the 1 μg oxacillin disc diffusion test and the BD Phoenix versus methicillin resistance with the 10 μg cefoxitin disc diffusion assay (see Table 3.3).

3.1.3 Clinical history associated with the isolates with discrepant results

The clinical history of the patients from whom the sixteen isolates were identified was retrieved from the PHW Microbiology ABM Laboratory data management system, for the investigation of previous or later identification of MRSA (see Table 3.4). In the PHW Microbiology ABM Laboratory if a MRSA isolate is identified from a patient >3 months after a former MRSA isolate has been identified, the latter is considered a new MRSA isolate. Therefore the duration of time that elapsed before a MRSA isolate was reported from a similar clinical specimen, was assessed for all sixteen isolates. For isolates 1, 7, 9, 10 and 13, there were no prior (or subsequent) MRSA reports for the respective patients, although in these cases few or no further organisms of any type were isolated from the patients previously or afterwards (see Table 3.4 and 3.5).

Table 3.4. Bacteria identified prior or post the isolates with discrepant assay results

No.	Isolate	Date of result	Associated infection	Bacteria reported
	110 024	20/04/ 2007	Wound swab	*A. baumannii, B. cereus*
1.	**113 015***	22/04/ 2007	Fracture of femur. Groin oozing, infected	*S. epidermidis, B. cereus, A. baumannii*
	114 030	24/04/ 2007	Pus	*S. epidermidis, A. baumannii*
			(Patient deceased)	
	313 006	09/11/2006	Wound swab	*S. aureus*
2.	**122 126***	02/05/ 2007	Post ascitic drain	*S. aureus, S. epidermidis,*
	123 019	02/05/ 2007	Ascitic drain	MRSA
	203 003	22/07/ 2007	Ascitic drain	MRSA
	709 226	23/07/ 2007	Ascitic drain	MRSA
	703 317	26/07/ 2007	Screening swab	MRSA
	721 773	25/10/ 2007	Screening swab	MRSA
3.	**124 051***	04/05/2007	Post operation sternal wound infection	*S. aureus*
	128 022	05/05/2007	Wound swab	MRSA
	128 051	08/05/2007	Sternal swab	MRSA
	133 003	14/05/2007	Wound swab	MRSA
	156 028	05/06/2007	Wound swab	MRSA
	163 004	12/06/2007	Sternal swab	MRSA
	166 001	15/06/2007	Wound swab	MRSA
	109 235	18/04/2007	Screen: all swabs MRSA pos	MRSA
	128 297	07/05/2007	Screen:Hairline, nasal, axillae-MRSA pos	MRSA
4.	**129 103***	09/05/2007	Wound: graft failure to finger	*S. aureus*
	739 658	11/02/2008	Screen: Throat- MRSA pos	MRSA
	150 372	30/05/2007	Screening swab-throat positive for MRSA	MRSA
	717 036	18/06/2007	Screening swab-throat positive for MRSA	MRSA
5.	**176 065***	26/06/2007	Infection of hip post operation incision	*S. aureus*
	736 286	18/01/2008	Screening swab	MRSA
	703 589	12/03/2008	Screening swab-throat and umbilicus	MRSA
	166 054	15/06/2007	Wound swab amputation site	MRSA
	177 023	26/06/2007	Exit site:chronic renal failure on haemodialysis	MRSA
6.	**186 079***	05/07/2007	Stump suture line	*S. aureus*, Coliform
	191 146	10/07/2007	Wound swab	MRSA
	225 013	11/08/2007	Wound swab	MRSA
	232 024	20/08/2007	Wound swab	MRSA
	711 846	06/09/2007	Screening swab	MRSA
	166 001	15/06/2006	Skin swab	*S. aureus*
7.	**189 009***	08/07/2007	Nasal swab	*S. aureus*
	189 010	08/07/2007	Thigh swab	*S. aureus, Streptococcus* groupA

*Isolates with PHW Microbiology ABM Laboratory and PCR discrepant results.

Table 3.4 continued. Bacteria identified prior or post the isolates with discrepant assay results

No.	Isolate	Date of result	Associated infection	Bacteria isolated
	183 151	02/07/2007	Patient Type 1 Diabetic infected finger	*S. aureus, Streptococcus* groupB
8.	**192 020***	11/07/2007	Infected amputation site of finger	*S. aureus*
	310 027	05/11/2007	Infected amputated-serous fluid	*S. aureus*
	835 930	18/07/2008	Eye swab	MRSA
	819 543	21/08/2008	Eye swab	MRSA
	200 042	19/07/2007	Skin swab, infected eczema	*S. aureus*
9.	**205 092***	24/07/2007	Pre septal cellulitis	*S. aureus*
10.	**206 006***	25/07/2007	Face swab-weeping lesion to face	*S. aureus*
	700 388	04/07/2007	Screening swab	MRSA
	186 056	05/07/2007	Rheumatoid patient- foot ulcer	MRSA
	702 538	20/07/2007	Foot swab	MRSA
11.	**208 506***	27/07/2007	Left thigh wound	*S. aureus*
	236 0 16	23/08/2007	Odorous wound on foot	MRSA
	236 502	24/08/2007	Ankle swab	MRSA
			(Patient deceased)	
	071 261	11/03/2007	Screening swab	MRSA
	116 035	24/04/2007	Pressure sore on heel	MRSA
	184 057	02/07/2007	Ulcer swab	MRSA
12.	**219 066***	07/08/2007	Pressure sore, left heel not healing	*Streptocccus* species
13.	**228 027***	16/08/2007	Infected belly button	*S. epidermidis*
14.	**229 015***	17/08/2007	Wound to left leg	*S. aureus*
	298 020	25/10/2007	Hip swab: hardened black lump on leg	*S.epidermidis*
	338 050	04/10/2007	Hip swab- long standing wound	*S.aureus, Anaerobes*
	808 061	13/04/2008	Hip swab	MRSA
			(Patient deceased)	
	151 102	31/05/2007	Eye swab	MRSA
	172 001	21/06/2007	Eye swab	MRSA
	701 918	16/07/2007	Screening swabs	MRSA
15.	**241 132***	29/08/2007	Chronic conjunctivitis	*S. aureus*
	722 593	31/10/2007	Screening swabs	MRSA
	347 054	12/12/2007	Conjunctival swab	MRSA
	016 102	16/01/2007	Wound discharge	MRSA
	082 525	23/03/2007	Right leg ulcer	MRSA
	103 501	13/04/2007	Discharging ulcer	MRSA
	117 509	27/04/2007	Right leg ulcer	MRSA
16.	**225 214***	13/08/2007	Discharging ulcer	MRSA
	304 073	30/10/2007	Right leg ulcer	MRSA

*Isolates with PHW Microbiology ABM Laboratory and PCR discrepant results.

Table 3.5. Duration of time before MRSA reported in the sixteen cases

MRSA reported from same clinical specimen	**No. of cases**
≤ 1day	2
≤ 1 week	1
1 month	1
2 months	1
8 months	1
MRSA reported from different clinical specimen	
6 - 8 months	3
No further clinical isolates reported	5
Total cases	15*

*Isolate 16 not included as it was reported as an MRSA in the laboratory system

In five cases (isolates 2, 3, 6, 11 and 15) MRSA strains were reported within three months from clinical specimens similar to those of the isolates with the discrepant results and in three cases the MRSA had been reported within a week. In the case of isolate 2, even though MSSA had been reported, an MRSA was reported from a replicate clinical specimen (123019) collected on the same date (see Table 3.5 and 3.4). Therefore in these cases it seems the MRSA strains which were possibly initially missed were subsequently identified fairly quickly from the same wound infections. However in the case of isolate 14, an MRSA was reported approximately eight months after the isolate with the discrepant results, thus it would have been considered a new MRSA isolate even though it may have been a persistent strain which was not previously identified in the same wound infection. In three cases (isolates 4, 5 and 8) it seems MRSA may have been completely missed in earlier infections, as they were reported six to eight months after the isolate with the discrepant results but from different types of clinical specimens: no MRSA from finger wound versus MRSA in throat swab; no MRSA from hip infection versus MRSA in screening swab and no MRSA in finger amputation versus MRSA in eye swab (see Table 3.4 and 3.5).

Isolate 16 was the only case in which an MRSA was reported in the laboratory system but conversely the isolate was identified as a MSSA by PCR (see Table 3.4). It was reported from a patient with an apparent history of MRSA reports, including approximately three months prior and two months after isolate 16 was reported.

3.4 Discussion

In this study fifteen (10.9%) of 137 *mecA* positive *S. aureus* isolates were reported as MSSA, *S. epidermidis* or *Streptococcus* species and one (0.24%) of 424 *mecA* negative *S. aureus* isolates was reported as MRSA in the PHW Microbiology ABM Microbiology Laboratory data management system. These clinical isolates were initially identified from wound swabs and the factors that could have contributed to the discrepancies between the reported microbiology laboratory results and the PCR results are discussed below.

Infected wounds are often polymicrobial sites in which both pathogenic and commensal bacteria are present, the latter are often skin microflora i.e. *S. epidermidis*, *Corynebacterium*, *Propionibacterium* and micrococci. *Streptococcus* species may also be present even though these are usually commensal bacteria of the mouth and upper respiratory tract and less often found on the skin (Bowler *et al.*, 2001). The presence of these bacteria could lead to the misidentification of the actual pathogenic microorganism(s). Similarly in the fifteen cases whereby MRSA were identified by PCR but not reported in the laboratory system, this may have been because the MRSA were present in the original wound swabs but were then missed in the polymicrobial cultures in subsequent media. However in twelve cases, only one bacterial isolate was reported in the laboratory system, indicating that either there was misidentification during initial testing or typographical errors occurred during the entering of results.

In the PHW Microbiology ABM Microbiology Laboratory wound swabs are cultured with selective media: Columbia Colimycin Nalidixic acid (CNA) agar, Cystine Lactose Electrolyte Deficient (CLED) agar and Neomycin media (with a metronidazole 5µg disc) for the identification of fastidious anaerobic bacteria. The 'Staph latex' test for the detection of clumping factor or protein A and the DNAse test are performed on colonies depicting *S. aureus* morphology. Antibiotic susceptibility testing is subsequently performed on *S. aureus* isolates with the 1µg oxacillin disc diffusion assay, two panels of antibiotics or with the BD Phoenix automated microbiology system.

CNA agar is a blood-based media for the isolation of Gram-positive microorganisms from polymicrobial clinical samples. It supports the growth of staphylococci, haemolytic streptococci and enterococci and contains colistin and nalidixic acid to inhibit the growth of *Proteus, Klebsiella, Pseudomonas* species, Enterobacteriaceae and other gram negative bacteria (http://www.bd.com/resource.aspx?IDX=8969). On this media *S. aureus* typically grow as large, cream or yellow colonies, exhibiting beta-haemolysis. However, a limitation of using CNA plates is that streptococci e.g. *S. pyogenes* also grow as white colonies (though they are smaller) and typically exhibit β-haemolysis (Bessen, 2009). Although coagulase-negative staphylococci (CoNS) generally do not depict haemolysis, they also have similar colony morphology (usually they grow as smaller colonies) to *S. aureus*. Therefore there is a possibility that based on colony morphology and subjective interpretation, MRSA isolates on CNA agar could be misidentified as *S. epidermidis* or *Streptococcus* species in the case of isolates 1, 12 and 13.

However, on CLED agar the typical growth of *S. aureus* is more distinct because they are lactose fermenters (growing as deep yellow colonies) and produce acid via the fermentation process. Bromthymol blue which is incorporated in the media serves as a pH indicator and the presence of acid lowers the pH and changes the blue-green colour of the media to yellow. *S. epidermidis* do not ferment lactose and grow as pale yellow to white colonies thus the media retains its blue-green colour due to the absence of acid (http://www.bd.com/resource.aspx?IDX=8967). Although streptococci are lactose fermenters they do not grow sufficiently on CLED media. It is unlikely that the distinct growth of *S. aureus* on CLED plates could have been mistaken for a *S. epidermidis* or *Streptococcus* species.

Following the identification of colonies with *S. aureus* characteristic morphology, tests for the detection of DNase and coagulase production are performed to distinguish *S. aureus* from CoNS species. Coagulase is an *S. aureus* enzyme which is excreted extracellularly and causes the clotting of rabbit plasma, and traditionally tube tests were commonly employed for detection of this enzyme. However there are some rare *S. aureus* strains which do not express coagulase including a bacteraemia isolate described by Olver *et al.* (2005) which was a EMRSA-15 strain, a common strain in the UK

(Olver et al., 2005, Fukuda et al., 2002, Al Obaida et al., 1999, Mackay et al., 1993). In addition, due to the long duration of the coagulase tube tests, these are seldom used in clinical laboratories and alternatively rapid slide agglutination assays for the detection of other *S. aureus* specific factors i.e. clumping factor and protein A are performed as identification tests. In the PHW Microbiology ABM Microbiology Laboratory the Prolex™ Staph Latex kit is used for the rapid identification of *S. aureus* and it comprises buffers containing latex particles which have been sensitized with human fibrinogen which is bound by clumping factor or IgG which is bound by protein A (van Griethuysen et al., 2001). However there are rare *S. aureus* strains that do not express protein A, furthermore false-negative results are possible with slide tests due to the expression of capsular polysaccharides by certain MRSA strains; usually these are capsular serotypes 5 and 8, which mask clumping factor and protein A. To circumvent this further agglutination tests have been developed which also include antibodies against the capsular polysaccharides (van Griethuysen et al., 2001). Therefore it is possible that the initial slide agglutination results may have been negative in the cases of isolate 1 and 13, thus these isolates were subsequently reported as *S. epidermidis.*

Methicillin resistance in heterogeneous MRSA strains is notoriously difficult to identify unambiguously with phenotypic susceptibility tests because these often depict methicillin susceptible or low resistance phenotypes (Hososaka et al., 2007, Katayama et al., 2001, Weller, 1999). In this study isolate 7 was an ideal example of this phenomenon, as it was *mecA* positive by PCR but exhibited methicillin susceptibility with the phenotypic tests i.e. the oxacillin (1 μg) disc diffusion test and the BD Phoenix. Therefore it is highly likely that this isolate was reported as a MSSA in the PHW Microbiology ABM Microbiology Laboratory system because it exhibited the same phenotype in the original oxacillin 1μg disc diffusion test. Two further *mecA* positive isolates (9 and 10) depicted methicillin susceptibility with the oxacillin disc diffusion test and one isolate (8) had tentative resistance. Based solely on this test, these four isolates would be regarded MSSA strains and likewise it is possible that in the original oxacillin disc tests, the inhibition zones of these isolates portrayed susceptibility to methicillin and thus they were reported as MSSA isolates in the PHW Microbiology ABM Microbiology Laboratory system. In contrast isolate 16 was *mecA* negative by PCR but was reported as a MRSA isolate. Non-*mecA* mediated resistance i.e. mutations

in the binding site of native PBPs or hyperproduction of β-lactamase can cause borderline oxacillin resistance phenotypes in *S. aureus* strains (BORSA) (Croes et al., 2009, Skinner et al., 2009, Khorvash et al., 2008, Balslev et al., 2005, Keseru et al., 2005). Interestingly even though isolate 16 displayed methicillin susceptibility with the oxacillin disc diffusion test and the BD Phoenix it exhibited borderline methicillin resistance with the cefoxitin disc diffusion test. So it is possible that this *mecA* negative isolate is a BORSA strain which is a hyper producer of penicillinase and in the original oxacillin test it may have depicted resistance, resulting in this isolate being reported as a MRSA isolate.

The expression of methicillin resistance is also affected by the test conditions i.e. NaCl concentration in the test medium, temperature of incubation and the size of inoculum (Prere et al., 2006, Brown et al., 2005, Brown, 2001a). In the BSAC version 9.1, March 2010 antimicrobial susceptibility testing methods, it is stated that a heavy inoculum density can result in reduced inhibition zones whereas a light inoculum density can result in increased inhibition zones. Even time delays (more than 15 minutes) with plates at room temperature after application of the discs and the high stacking of plates in incubators can result in unequal distribution of heat and increased zones of inhibition. However the possibility of these factors having occurred on fifteen occasions in the PHW microbiology laboratory is unlikely. The detection of methicillin resistance with oxacillin disc tests has been shown to be improved when 2% NaCl is added to the medium and when the incubation temperature is lowered to 30°C (Berger-Bachi and Rohrer, 2002, Madiraju et al., 1987). The BSAC version 9.1, March 2010 antimicrobial susceptibility testing methods state the use of Columbia salt agar (CSA) or Mueller Hinton agar containing 2% NaCl and incubation at 30°C for 24 hours for oxacillin disc diffusion testing of staphylococci. However some *S. aureus* strains i.e. the EMRSA-16 strain are sensitive to salt and may be inhibited by increased NaCl concentrations (Bruins et al., 2007). In this study when the 1μg oxacillin disc diffusion test was performed on the isolates, methicillin resistance was detected in eleven of the fifteen *mecA* positive *S. aureus* isolates, demonstrating that the NaCl concentration in the CSA media did not inhibit the expression of methicillin resistance in these isolates and so this is an unlikely underlying cause of these isolates being reported as MSSA in the laboratory system.

With the 10 μg cefoxitin disc diffusion test, all fifteen isolates depicted methicillin resistance including the one *mecA* negative isolate, illustrating one case of non specificity with this assay. Two of the isolates i.e. 7 and 8, had a light haze of colonies within the zones of inhibition with the cefoxitin test, a typical feature of heterogeneous MRSA, whereby the colonies growing up to the cefoxitin disc are the population with high methicillin resistance and those growing further from the disc are the population with lower levels of methicillin resistance (Cauwelier et al., 2004). This implied that the cefoxitin test was more accurate in detecting methicillin resistance in these heterogeneous strains than the oxacillin test, whereby isolate 7 depicted susceptibility and isolate 8 had ambiguous results. The results of this study seemingly support data from other studies that suggests the cefoxitin test is more sensitive than the oxacillin test for detection of methicillin resistance in *S. aureus*. However the number of isolates (n=16) evaluated in this study was low and a larger collection of MSSA and MRSA isolates is required for a more comprehensive evaluation of the diagnostic performance of these tests.

Nevertheless several studies investigating methicillin resistance in *S. aureus* isolates generally indicate that cefoxitin assays have higher sensitivities and specificities than oxacillin disc tests (see Table 3.6) (Broekema et al., 2009, Junkins et al., 2009, Roisin et al., 2008, Andrews et al., 2005, Skov et al., 2005, Skov et al., 2003, Felten et al., 2002). Whereas moxalactam has been shown to be more sensitive than both cefoxitin and oxacillin for the detection of methicillin resistance in CoNS species (Pupin et al., 2007). Currently cefoxitin rather than oxacillin is recommended for disc diffusion tests by the Clinical and Laboratory Standards Institute (CLSI) and the Swedish Reference Group for Antibiotics (Swenson et al., 2009). However in the UK, as stated in the BSAC version 9.1, March 2010 methods for antimicrobial susceptibility testing, both oxacillin and cefoxitin discs are permitted for detection of methicillin resistance in *S. aureus*.

Table 3.6. Comparison of cefoxitin disc test, oxacillin disc test and automated systems for the detection of methicillin resistance

Reference	No. of isolates	Tests evaluated	Sens.	Spec.	Comment
(Felten et al., 2002)	MRSA (83) / MSSA (69)	Cef. DD (30μg) MH	100%	100%	Reference method- *mecA* PCR
		Mox. DD (30μg) MH	100%	100%	Cefoxitin and moxalactam tests have higher
		Vitek 2 system	94.0%	100%	sensitivities and specificities than oxacillin tests
		MRSA screen test (detection of PBP2a)	97.6%	100%	
		Ox. DD (1μg) MH	96.4%	97.1%	
		Ox. DD (5μg) MH	95.2%	100%	
		Ox. screen agar (Ox.6μg/ml) MH+2% NaCl	94.0%	100%	
		Ox. E-test on MH	91.6%	100%	
(Skov et al., 2003)	MRSA (190) / MSSA (267)	Cef. DD (30μg) ISA	100%	99.0%	Reference method- *mecA* probe based assay
		Ox. DD (1μg) ISA	78.0%	99.0%	Cefoxitin test more sensitive than oxacillin test
(Skov et al., 2005)	MRSA (380) / MSSA (261)	Cef. DD (5μg) ISA	99.5%	98.1%	Reference method- *mecA* probe based assay
		Cef. DD (5μg) MH	99.5%	98.1%	Cefoxitin tests on different media have higher
		Cef. DD (10μg) ISA	99.7%	98.1%	sensitivities than oxacillin test
		Cef. DD (10μg) MH	99.5%	98.9%	
		Ox. DD (1μg) ISA	82.3%	100%	
(Andrews et al., 2005)	MRSA (328) / MSSA (672)	Cef. DD (10μg) ISA	100%	99.7%	Reference method- *mecA* PCR Cefoxitin test comparable to *mecA* PCR
(Baddour et al., 2007)	MRSA (39) / MSSA (24)	Ox. screen agar (Ox.6μg/ml) MH+4% NaCl	92.3%	45.8%	Reference method- *mecA* PCR
		Ox. DD (1μg) MH	84.6%	79.2%	Cefoxitin tests comparable to PBP2a latex test
		Cef. DD (30μg) MH	89.7%	87.5%	and oxacillin tests
		Cef. E-test on MH	89.7%	91.7%	
		PBP2a latex test	92.3%	79.2%	

Key: Sens.-sensitivity; Spec.-specificity; dil.-dilution; Co.-company Cef- Cefoxitin; Ox- Oxacillin; Mox-Moxalactam; DD- disc diffusion; ISA-Iso-Sensitest; MH-Mueller Hinton

Table 3.6. contd. Comparison of cefoxitin disc test, oxacillin disc test and automated systems for the detection of methicillin resistance

Reference	No. of isolates	Tests evaluated	Sens.	Spec.	Comment
(Jain et al., 2008)	MRSA (73) / MSSA (24)	Cef. DD (30μg) MH	94.4%	95.8%	Reference method- *mecA* PCR
		Ox. DD (1μg) MH	100%	58.3%	Oxacillin tests have higher sensitivities but
		Ox. screen agar (Ox.4μg/ml) MH+4% NaCl	100%	58.3%	cefoxitin test has specificity comparable to
		Ox. screen agar (Ox.6μg/ml) MH+4% NaCl	98.6%	62.5%	oxacillin agar dilution test
		Ox. agar dil. (0.125-256 μg/ml) MH+2% NaCl	98.6%	98.5%	
(Perez et al., 2008)	MRSA (69) / MSSA (98)	Cef. agar dilution (0.5-256 μg/ml) MH	100%	99.0%	Reference method- *mecA* PCR
		Ox. agar dilution (0.5-256 μg/ml) MH	100%	99.0%	Cefoxitin test comparable to oxacillin tests
		Ox. screen agar (Ox.6μg/ml) MH+4% NaCl	98.5%	100%	
(Roisin et al., 2008)	MRSA (157) / MSSA (56)	Vitek 2 system	97.5%	100%	Reference method- *mecA* PCR and oxacillin
		Cef. DD (10μg) MH (by Rosco Co.)	99.6%	100%	screen agar. Cefoxitin tests have higher
		Cef. DD (30μg) MH (by Rosco Co.)	99.6%	100%	sensitivities than Vitek 2 system but have
		Cef. DD (60μg) MH (by Rosco Co.)	98.7%	100%	comparable specificities
		Cef. DD (60μg) MH (by Oxoid Co.)	98.7%	100%	
		Mox. DD (30μg) MH (by Rosco Co.)	98.7%	100%	
(Broekema et al., 2009)	MRSA (788) / MSSA (823)	Cef. DD (30μg) MH	97.3%	100%	Reference method- oxacillin DD (1μg) and PBP2a latex test, cefoxitin DD is comparable
(Junkins et al., 2009)	MRSA (448) / MSSA (172)	Cef. Microdil. BD Phoenix PMIC/ID-102 panel	99.8%	100%	Reference method- *mecA* PCR
		Ox. Microdil. BD Phoenix PMIC/ID-102 panel	97.8%	100%	Cefoxitin panel within BD Phoenix and Vitek
		Cef. Microdil. VITEK 2 AST-GP66 panel	99.1%	100%	has higher sensitivities than oxacillin panel
		Ox. Microdil. VITEK 2 AST-GP66 panel	98.2%	100%	
(Swenson et al., 2009)	MRSA (312) / MSSA (167)	Cation adjusted MH broth by 3 companies:			Reference method- *mecA* PCR
		Cef. broth dil (0.5-32 μg/ml) by BBL (18hour)	99.7%	100%	Cefoxitin panels of different companies
		Cef. broth dil (0.5-32 μg/ml) by Difco (18hour)	100%	99.4%	comparable to *mecA* PCR at 18hours. At 24hr
		Cef. broth dil (0.5-32 μg/ml) by Oxoid (18hour)	100%	100%	specificity of Oxoid panel dropped to 99.4%

Key: Sens.-sensitivity; Spec.-specificity; dil.-dilution; Co.-company; Cef- Cefoxitin; Ox- Oxacillin; Mox-Moxalactam; DD- disc diffusion; ISA-Iso-Sensitest; MH-Mueller Hinton

With broth dilution assays CLSI recommends the use of either oxacillin or cefoxitin for detection of *mecA*-mediated resistance in *S. aureus*. Manufacturers of the BD Phoenix and Vitek 2 automated susceptibility testing systems, which use microdilution assays, have adapted these systems to optimize detection of *mecA* mediated resistance by including both oxacillin and cefoxitin (Junkins et al., 2009). The in-built expert systems within the instruments interpret any *S. aureus* isolate that expresses MICs depicting resistance to either cefoxitin or oxacillin as methicillin resistant (Junkins et al., 2009, Mencacci et al., 2009).

In this study, with the BD Phoenix fourteen of the fifteen *mecA* positive isolates had MICs of ≥4mg/L and ≥8mg/L to oxacillin and cefoxitin respectively, showing clear resistance to both of these antibiotics and were identified as MRSA. However isolate 7 had very low MICs, depicting susceptibility to both oxacillin (≤1mg/L) and cefoxitin (≤2mg/L) and was identified as a MSSA. This isolate is clearly a low level resistance MRSA and so it is highly likely that a methicillin susceptible result was attained during the original testing of this isolate. Another interesting *mecA* positive *S. aureus* was isolate 8 because whereas most isolates depicted clear resistance to oxacillin (>4mg/L) and cefoxitin (>8mg/L), this isolate had MICs of 4 mg/L and 8 mg/L respectively. These MICs are merely one 2-fold dilution higher than the BSAC resistance breakpoints and it is generally held that the precision of a dilution susceptibility test method is plus minus one 2-fold dilution (Prakash et al., 2008). So it is possible that isolate 8 could have exhibited MICs depicting susceptibility on previous testing and was thus reported as a MSSA isolate.

Even though MRSA were not reported in fifteen of the sixteen cases evaluated in this study, in five cases (isolates 2, 3, 6, 11 and 15) MRSA strains were subsequently reported within three months and in three cases within a week. This relatively rapid detection of MRSA (from similar clinical specimens) after former isolates were reported as MSSA isolates supports the likelihood that MRSA were probably initially misidentified as MSSA isolates.

The retrospective evaluation of the reported PHW Microbiology ABM Laboratory results is an apparent limitation of this study and it is difficult to conclusively determine the causes of the discrepancies between these reported results and the *mecA* PCR results. However by repeating the oxacillin, cefoxitin and BD Phoenix susceptibility tests, it was evident that there were a few heterogeneous MRSA strains and a MSSA isolate with borderline methicillin resistance. These isolates constitute atypical MRSA and MSSA strains which could have exhibited susceptible phenotypes and a resistance phenotype in the original microbiology laboratory tests, thus contributing to the observed discordant reported and PCR results. Therefore this study also helps to highlight the limitations of exclusively using phenotypic susceptibility tests for the detection of methicillin resistance in *S. aureus*. Even though the conditions of these tests have been modified and optimised to facilitate the accurate detection of *mecA*-mediated resistance the misidentification of heterogeneous MRSA and BORSA strains is still possible. Therefore the molecular detection of the *mecA* gene i.e. by PCR which is the gold standard method, is the optimal method for the conclusive detection of methicillin resistance in *S. aureus*.

Chapter Four

Molecular characterisation of methicillin resistant *Staphylococcus aureus* isolates

4.0 Introduction

4.01 Pulsed field gel electrophoresis and *spa* typing

The typing of *S. aureus* isolates enables the discrimination of strains based on their phenotypic or genetic characteristics. In addition the application of typing methods enables the discernment of the clonal relatedness of strains which is valuable in outbreak investigations and in long-term evolutionary studies. Molecular techniques are increasingly employed and pulsed field gel electrophoresis (PFGE) is the gold standard. However, *spa* typing has also become a widely used technique. PFGE involves an initial digestion of the chromosomal DNA with a restriction endonuclease and the fragments are subsequently resolved with electrophoresis whereby the direction of the current is changed periodically. The resultant PFGE patterns are representative DNA fingerprints of each strain. However, since mutations modify nucleotide sequences consequently they can affect PFGE patterns. For instance a single nucleotide change may produce a novel restriction site in a sequence and this may cause the loss of a larger fragment and the gain of two smaller ones in the PFGE pattern (three changes in the pattern). Since PFGE rapidly indexes genetic variation closely related strains can exhibit dissimilar patterns within a short period. Therefore criteria have been developed for the visual analysis of PFGE patterns and for discerning the relatedness of strains (see Table 4.1) (Tenover et al., 1995).

The *spa* gene encodes protein A, a cell wall associated protein in *S. aureus*. Within the 3' coding region of *spa* there is a variable-number tandem repeat region referred to as the X-region. This region comprises 21-27 bp repeat sequences which have extensive polymorphism due to point mutations, duplications and insertions. The *spa* typing method involves the amplification and DNA sequencing of the polymorphic X-region (Koreen et al., 2004, Harmsen et al., 2003, Shopsin et al., 1999). The identified repeat sequences are assigned an alpha or numerical code and the order of these defines the *spa* type of the strain (see Figure 4.1) (Harmsen et al., 2003). An online *spa* server developed and maintained by Ridom GmbH includes a database containing current *spa* types and the associated epidemiological information (Harmsen et al., 2003).

Table 4.1. Criteria for interpreting PFGE patterns (Tenover et al., 1995)

Category	No. of genetic differences	No. of fragment differences	Interpretation
Indistinguishable	0	0	Belong to single clone
Closely related	1	2-3	Belong to single clone
Possibly related	2	4-6	Possibly belong to single clone
Different	≥ 3	≥ 7	Unrelated strains

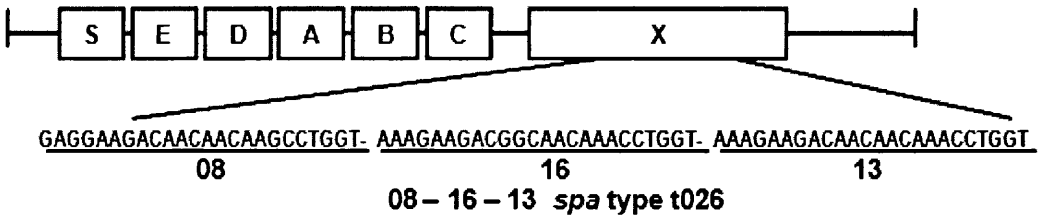


Figure 4.1. A schematic diagram of the *spa* gene. Boxes indicate sequences encoding: S, signal sequence; A-D, immunoglobulin G binding regions; E, sequence homologous to A-D; X, region comprising 21-26 bp repeats (Shopsin et al., 1999).

4.02 SCC*mec* typing

The typing of SCC*mec* elements is integral to the characterisation and nomenclature of MRSA strains. Several SCC*mec* elements (type I to VIII) have been described in MRSA and the discrimination of these is based on the detection of loci which are specific to the respective elements. The Milheirico *et al.* (2007b) method is a multiplex PCR assay which incorporates the detection of *mecA* as the internal control and the detection of nine additional loci for the characterisation of SCC*mec* types I to V. For instance, both SCC*mec* type I and II harbour *mecA* and *dcs* but *pls* is specific to SCC*mec* type I, whereas *ccrB2*, *kdp*, *mecI* are specific to SCC*mec* type II (see Figure 4.2). The loci are amplified with specific primers resulting in PCR fragments of different sizes. These are resolved in an agarose gel and the banding patterns are specific to the respective elements (see Table 4.2).

The characterisation of the *ccr* and *mec* complexes harboured by SCC*mec* elements is an alternative means of typing these elements. Kondo *et al.* (2007) developed a SCC*mec* typing method which includes a multiplex PCR method, MPCR-1 which comprises primers specific for five *ccr* types: types 1 to 5 (*ccrC*) and *mecA* (included as an internal control) (see Figure 4.3 and Table 4.3).

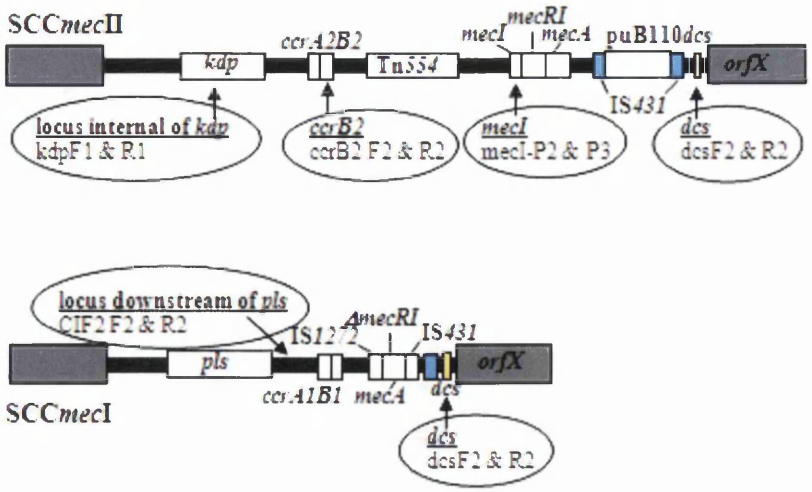


Figure 4.2. Diagrams of the SCC*mec* type I and II elements and the loci detected by the Milheirico *et al.* (2007b) SCC*mec* typing method in these elements

Table 4.2. PCR fragment sizes of the Milheirico *et al.* (2007) method for SCC*mec* types I to V (Milheirico et al., 2007b)

Loci	Size(bp)	SCC*mec*I	SCC*mec*II	SCC*mec*III	SCC*mec*IV	SCC*mec*V
pls	495bp	+				
ccrC	449bp					+
III- J3	414bp			+		
V- J1	377bp					+
dcs	342bp	+	+		+	
ccrB2	311bp		+		+	
kdp	284bp		+			
III- J1	243bp			+		
mecI	209bp		+	+		
mecA	162bp	+	+	+	+	+
No. of fragments		3	5	4	3	3

The Kondo *et al.* (2007) SCC*mec* typing method also includes another multiplex PCR termed MPCR-2 which includes primers for the detection of three different classes of *mec* complexes: class A, B and C2. The PCR fragments are resolved in an agarose gel and the *ccr* types and *mec* classes are identified by virtue of the expected fragment sizes (see Figure 4.3 and Table 4.3).

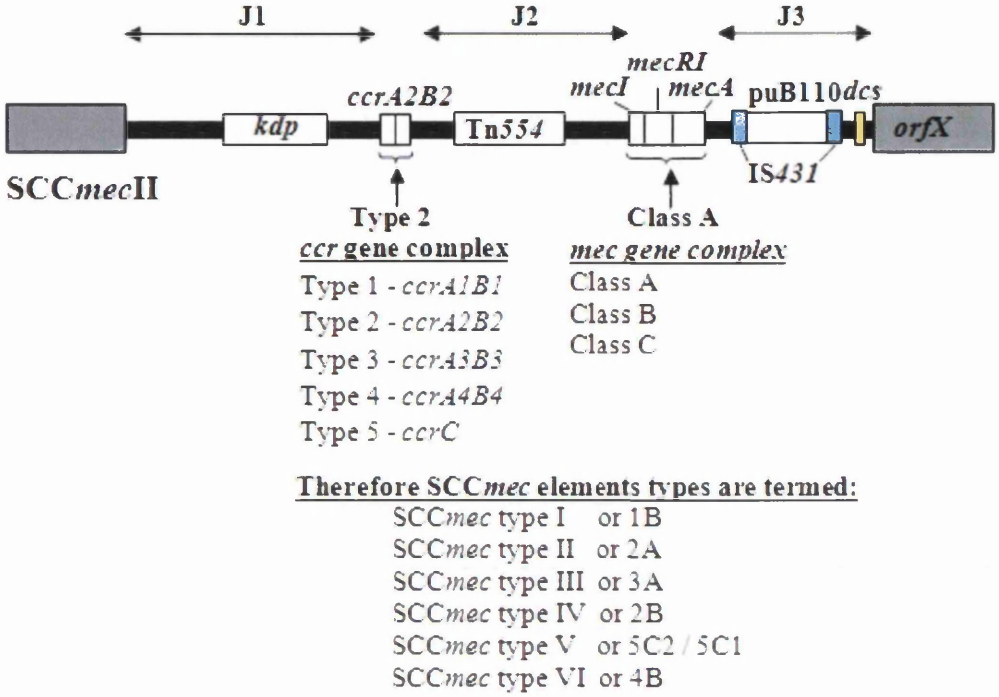


Figure 4.3. Diagram of SCC*mec* type II and the different *ccr* types and *mec* classes harboured in the MRSA I to VI strains

Table 4.3. PCR fragment sizes of *ccr* and *mec* loci of SCC*mec* type I to VI elements with MPCR-1 and MPCR-2 assays (Kondo et al., 2007)

Loci	Size(bp)	SCC*mec*I	SCC*mec*II	SCC*mec*III	SCC*mec*IV	SCC*mec*V
MPCR-1		**(1B)**	**(2A)**	**(3A)**	**(2B)**	**(5C)**
mecA	286bp	+	+	+	+	+
ccrA1B1	695bp	+				
ccrA2B2	937bp		+		+	
ccrA3B3	1791bp			+		
ccrA4B4	1287bp				(+)	
ccrC	518bp					+
MPCR-2						
mecA-mecI	1963bp		+	+		
mecA*-IS*1272	2827bp	+			+	
mecA*-IS*431	804bp					+

(+) SCC*mec*VI which contains *ccrAB4* has been described which is structurally similar to SCC*mec*IV

4.03 Subtyping of the SCC*mec* type IV elements

The definition of SCC*mec* subtypes is based on variations in the J-regions (J1, J2 and J3) of the elements. The type IV element is the most variable and subtypes IVa, IVb, IVc, IVd, IVE, IVF and IVg have been described in MRSA; recently Milheirico *et al.* (2007a) have also described an additional subtype IVh in the EMRSA-15 strain HAR22. Several subtyping methods have been developed including the MPCR-3 method by Kondo *et al.* (2007) and a method by Milheirico *et al.* (2007a). These are multiplex PCR assays which discriminate SCC*mec* type IV subtypes based on variations in the J1-region.

Table 4.4. PCR fragment sizes of SCC*mec* type IV subtypes with the Milherico *et al.* (2007a) and MPCR-3 methods

SCC*mec* type IV Subtypes	PCR fragment sizes	
	Milheirico *et al.* (2007a)	MPCR-3
IVa	278 bp	458 bp
IVb	336 bp[a]	726 bp
IVc	483 bp[b]	259 bp
IVd	575 bp	1, 242 bp
IVE	483 bp[b]	
IVF	336 bp[a]	
IVg	792 bp	
IVh	663 bp	
ccrB (internal control)	203 bp	

[a] J1 region of IVb and IVF is highly similar; [b] J1 region of IVc and IVE is highly similar

The MPCR-3 method involves four primer pairs which are specific for subtypes IVa, IVb, IVc and IVd, whereas the Milherico *et al.* (2007) method is more comprehensive and involves seven pairs of primers which are specific for the IVa, IVb, IVc, IVd, IVE, IVF, IVg and IVh subtypes (see Table 4.4). One of the primer pairs is for the amplification of an internal region in *ccrB2* which is characteristic of the SCC*mec* type IV and serves as an internal control. Both methods similarly identify IVa, IVb, IVc and IVd subtypes but the primer sets are specific for different open reading frames in the J1-regions.

The objective of this study was to determine the genetic characteristics of 137 MRSA clinical isolates with the following molecular typing techniques: PFGE, *spa* typing and the Milheirico *et al.* (2007b) SCC*mec* typing method. The MPCR-1 and MPCR-2 assays were performed on indefinite results and subtyping of the SCC*mec* type IV elements was performed with the Milheirico *et al.* (2007a) and the MPCR-3 methods.

4.1 Results

4.1.1 Pulsed Field Gel Electrophoresis

The pulsed field gel electrophoresis (PFGE) method was successfully performed on all of the MRSA clinical isolates (n=137) and a total of 22 patterns including six unique PFGE patterns were resolved with this assay. The PFGE patterns were compared to those of the EMRSA-15 and EMRSA-16 reference strains and 130 of the 137 isolates exhibited patterns that were comparable to these strains. Sixty-one of the isolates had PFGE patterns which were identical or had a 1-band difference to the EMRSA-15 reference strain, their patterns were designated EMRSA-15 and EMRSA-15i respectively (see lane 1 to 4 in Figure 4.4 and Table 4.5). Forty-three isolates had PFGE patterns with 2 to 3 band differences to the EMRSA-15 reference strain and these were termed EMRSA-15.1 to EMRSA-15.1.1i (see lane 5 to 13 in Figure 4.4 and Table 4.5). Since these isolates had a $\leq$3 band difference to the EMRSA-15 reference strain this signified that they were closely related to this strain according to the Tenover *et al.* (1995) criteria. Eighteen isolates had patterns which were more variable to the EMRSA-15 reference strain but collectively they had 4 to 6 band differences and they were termed EMRSA-15.1.1ii to EMRSA-15.4 (see lane 13 to 23 in Figure 4.4 and Table 4.5). According to the Tenover *et al.* (1995) criteria isolates with up to a 6-band difference belong to a single clone, therefore in total 122 (89.5%) of the 137 MRSA isolates belonged to the EMRSA-15 clone (see Table 4.4).

Eight of the MRSA clinical isolates had PFGE patters that were comparable to the pattern of the EMRSA-16 reference strain. Two isolates had patterns which were identical and the remaining six isolates exhibited patterns that were termed EMRSA-16i (n=5) and EMRSA-16ii (n=1) (see lane 24, 25, 26 in Figure 4.4 and Table 4.5). These patterns had a $\leq$3 band difference to the EMRSA-16 reference strain and according to the Tenover *et al.* (1995) criteria, this indicated that they were closely related to this strain. Therefore in total eight (5.8%) of the 137 MRSA isolates belonged to the EMRSA-16 clone (see Table 4.4). Seven (5.1%) isolates depicted a total of six unique PFGE patterns, two isolates of these isolates exhibited an identical pattern. These were not comparable to the patterns of the other MRSA isolates and had >6 band difference to the EMRSA-15 and EMRSA-16 reference strains.

Table 4.5. PFGE characteristics of the MRSA clinical strains (n=137)

PFGE pattern	No. of band differences	No. of isolates	PFGE group
EMRSA-15		**122 (89.1%)**	
EMRSA-15	0	30	A
EMRSA-15i	1	31	A
EMRSA-15.1	2	8	A
EMRSA-15.1i	3	26	A
EMRSA-15.1.1	2	3	A
EMRSA-15.1.1i	3	6	A
EMRSA-15.1.1ii	4	3	A
EMRSA-15.1.2	4	1	A
EMRSA-15.1.3	4	1	A
EMRSA-15.2	5	4	A
EMRSA-15.2i	6	2	A
EMRSA-15.3	6	6	A
EMRSA-15.4	4	1	A
EMRSA-16		**8 (5.8%)**	
EMRSA-16	0	2	B
EMRSA-16i	2	5	B
EMRSA-16ii	2	1	B
Unique 01-06	>6	**7 (5.1%)**	C to H
Total		**137**	

This signified that the seven isolates with the unique patterns did not belong to the EMRSA-15 or EMRSA-16 clones and their patterns were termed: Unique-01 (n=1) Unique-02 (n=1), Unique-03 (n=1), Unique-04 (n=1), Unique-05 (n=1) and Unique-06 (n=2) (see lane 27 to 30 in Figure 4.4 and Table 4.5). Based on the closely related or unique patterns the MRSA clinical isolates (n=137) were demarcated into eight PFGE groups (PFG), these were allocated by clustering isolates with PFGE patterns that had up to 6-band differences. Therefore the 122 clinical isolates that exhibited the EMRSA-15 related PFGE patterns were placed into PFG-A and the eight isolates that exhibited the EMRSA-16 related PFGE patterns were placed into PFG-B. The seven isolates that had unique patterns were placed into six groups i.e. PFG-C (n=1), PFG-D (n=1), PFG-E (n=1), PFG-F (n=1) and PFG-G (n=1) and PFG-H (n=2) (see Table 4.5).

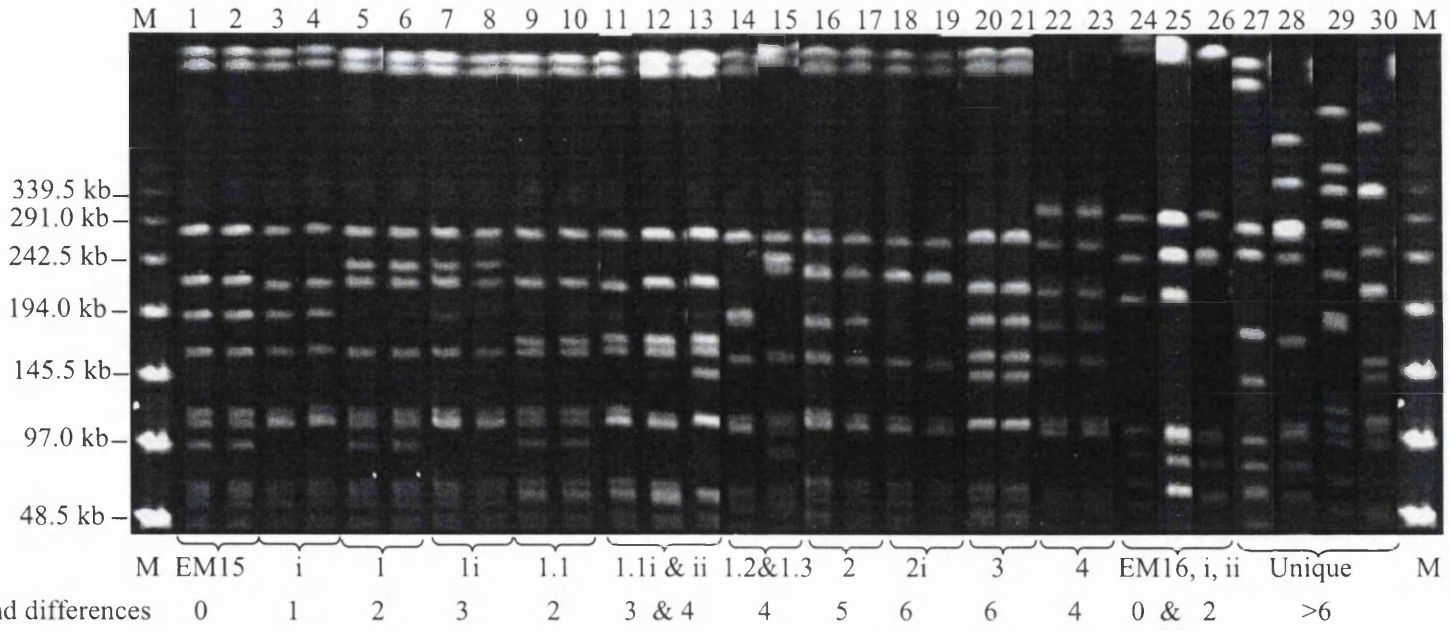


Figure 4.4. PFGE patterns exhibited by 137 MRSA clinical isolates (compiled from different gels).
M, lambda DNA marker. Lane 1, EMRSA-15 reference strain. Lanes 2-23, diverse EMRSA-15 patterns: lane 2-EMRSA-15; lane 3 & 4-EMRSA-15i; lane 5 & 6-EMRSA-15.1; lane 7 & 8-EMRSA-15.1i; lane 9 & 10-EMRSA-15.1.1; lane 11 & 12-EMRSA-15.1.1i; lane 13-EMRSA-15.1.1ii; lane 14-EMRSA-15.1.2; lane 15-EMRSA-15.1.3; lane 16 & 17-EMRSA-15.2; lane 18 & 19-EMRSA-15.2i; lane 20 & 21-EMRSA-15.3; lane 22 & 23-EMRSA-15.4.

Lane 24 - 26, EMRSA-16 patterns: lane 24-EMRSA-16 reference strain; lane 25-EMRSA-16i pattern; lane 26-EMRSA-16ii pattern.

Lane 27 – 30, four of the six unique patterns: lane 27, Unique-01; lane 28, Unique-02; lane 29, Unique-06; lane 30, Unique-05.

4.1.2 *spa* typing

The genetic background of the MRSA clinical isolates (n=137) was further investigated by performing *spa* typing on 49 selected isolates, including 35 isolates that represented diverse EMRSA-15 PFGE patters, all eight isolates with the EMRSA-16 PFGE patterns and six isolates representing the unique PFGE patterns. The *spa* typing method had excellent typeability except in one case where the amplification of the *spa* target sequence was unsuccessful with the isolate exhibiting the unique-05 PFGE pattern. In the remaining 48 isolates a total of fourteen *spa* types were attained, seven (t022, t032, t379, t879, t910, t4185, and t4424) were depicted by the selected EMRSA-15 strains (n=35). The majority exhibited *spa* type t032 (n=20). The remaining EMRSA-15 strains depicted *spa* types: t022 (n=5), t379 (n=1), t879 (n=1), t910 (n=4), t4185 (n=1) and t4424 (a new *spa* type, n=3) (see Table 4.6a). In comparison to *spa* type t032, these *spa* types were related variants exhibiting deletions of repeat(s) or nucleotide substitution(s) i.e. t022 has a deletion of repeat r23 in comparison to t032, whereas t379 and t910 have apparent deletions of more than one repeat i.e. r31-r29-r17 and r31-r29-r17-r25-r17 respectively (see Table 4.6a). The *spa* type t879 does not have deletions of repeats but has r20 in a position where r23 is present in t032. Similarly t4424 has r31 present in a location where r23 is present in t032. The *spa* type t4185 has both deletions of repeats and the presence of r16 where r23 is present in t032. The sequences of the r20, r31 and r16 repeats in t879, t4424 and t4185 have nucleotide substitution(s) distinguishing them from the sequence of r23.

The t012 (n=2) and t018 (n=6) *spa* types were depicted by the eight strains which belonged to the EMRSA-16 clone, these two *spa* types differed by the absence of the r24 repeat unit in t012. The five isolates with unique PFGE patterns had diverse *spa* types: t138, t359, t437, t878 and t1778, which were different to those depicted by the EMRSA-15 and EMRSA-16 clinical strains. In addition *spa* typing was performed on MRSA strains which have been previously epidemic in the UK i.e. EMRSA-1 to EMRSA-17 strains (see Table 4.6b). These EMRSA strains exhibited the t001, t009, t018, t025, t037, t051, t190 and t194 *spa* types. None of these *spa* types were exhibited by the unique clinical MRSA isolates, signifying that these were not related to the EMRSA-1 to EMRSA-17 strains (see Table 4.6b).

Correlation between the PFGE patterns and *spa* types was highly variable, especially with the EMRSA-15 strains. Four of the thirteen EMRSA-15 patterns were exclusively represented by t032 but in contrast the EMRSA-15 and EMRSA-15.1i patterns were represented by three *spa* types i.e. t022, t032 and t910 and t022, t032 and t4185 respectively. The remaining EMRSA-15 patterns were represented by variable pairs of *spa* types. Similarly, with the EMRSA-16 strains the EMRSA-16i pattern was represented by *spa* types t012 and t018 (see Table 4.6a).

Table 4.6a. PFGE and *spa* typing characteristics of a selection of clinical MRSA isolates (n=49)

PFGE pattern	No.	PFG	*spa* type	*spa* repeat	BURP cluster	Ridom info.
EMRSA-15	4	A	t022 (1)	26- -23-13-23-31-29-17-31-29-17-25-17-25-16-28	spa-CC379	ST-22
			t032 (2)	26-23-23-13-23-31-29-17-31-29-17-25-17-25-16-28	spa-CC379	ST-22
			t910 (1)	26-23-23-13-23-31-29-17- -25-16-28	spa-CC379	--
EMRSA-15i	5	A	t032 (3)	26- -23-13-23-31-29-17-31-29-17-25-17-25-16-28	spa-CC379	ST-22
			t022 (2)	26- -23-13-23-31-29-17-31-29-17-25-17-25-16-28	spa-CC379	ST-22
EMRSA-15.1	3	A	t032 (2)	26-23-23-13-23-31-29-17-31-29-17-25-17-25-16-28	spa-CC379	ST-22
			t910 (1)	26-23-23-13-23-31-29-17- -25-16-28	spa-CC379	--
EMRSA-15.1i	5	A	t022 (2)	26-23-23-13-23-31-29-17-31-29-17-25-17-25-16-28	spa-CC379	
			t032 (2)	26-23-23-13-23-31-29-17-31-29-17-25-17-25-16-28	spa-CC379	ST-22
			t4185 (1)	26-23-23-13-**16**- -17-25-16-28	Singleton	--
EMRSA-15.1.1	2	A	t032 (1)	26-23-23-13-23-31-29-17-31-29-17-25-17-25-16-28	spa-CC379	ST-22
			t910 (1)	26-23-23-13-23-31-29-17- -25-16-28	spa-CC379	--
EMRSA-15.1.1i	3	A	t032 (3)	26-23-23-13-23-31-29-17-31-29-17-25-17-25-16-28	spa-CC379	ST-22
EMRSA-15.1.1ii	2	A	t879 (1)	26-23-**20**-13-23-31-29-17-31-29-17-25-17-25-16-28	spa-CC379	--
			t910 (1)	26-23-23-13-23-31-29-17- -25-16-28	spa-CC379	--
EMRSA-15.1.2	1	A	t032 (1)	26-23-23-13-23-31-29-17-31-29-17-25-17-25-16-28	spa-CC379	ST-22
EMRSA-15.1.3	1	A	t032 (1)	26-23-23-13-23-31-29-17-31-29-17-25-17-25-16-28	spa-CC379	ST-22
EMRSA-15.2	2	A	t4424 (2) *-new*	26-23-**31**-13-23-31-29-17-31-29-17-25-17-25-16-28	spa-CC379	new
EMRSA-15.2i	2	A	t032 (1)	26-23-23-13-23-31-29-17-31-29-17-25-17-25-16-28	spa-CC379	ST-22
			t4424 (1) *-new*	26-23-**31**-13-23-31-29-17-31-29-17-25-17-25-16-28	spa-CC379	new
EMRSA-15.3	4	A	t032 (3)	26-23-23-13-23-31-29-17-31-29-17-25-17-25-16-28	spa-CC379	ST-22
			t379 (1)	26-23-23-13-23-31-29-17- -25-17-25-16-28	spa-CC379	--
EMRSA-15.4	1	A	t032 (1)	26-23-23-13-23-31-29-17-31-29-17-25-17-25-16-28	spa-CC379	ST-22
Subtotal	35					
EMRSA-16	2	B	t018 (2)	15-12-16-02-16-02-25-17-24-24-24	Cluster 2	ST-36(30,38)
EMRSA-16i	5	B	t012 (2)	15-12-16-02-16-02-25-17-24- -24	Cluster 2	ST-30
			t018 (3)	15-12-16-02-16-02-25-17-24-24-24	Cluster 2	ST-36(30,38)
EMRSA-16ii	1	B	t018 (1)	15-12-16-02-16-02-25-17-24-24-24	Cluster 2	ST-36(30,38)
Subtotal	8					

Key: *new*-new *spa* type identified from this study; Ridom info, data from www.spaserver.ridom.de; -- no information available; ST-sequence type; Untyp, untypeable

Table 4.6a. cont. PFGE and *spa* typing characteristics of a selection of clinical MRSA isolates (n=49)

PFGE pattern	No.	PFG	*spa* type	*spa* repeat	BURP cluster	Ridom info.
Unique-01	1	C	t138 (1)	08-16-02-25-17-24	Singleton	ST-30
Unique-02	1	D	t437 (1)	04-20-17-20-17-25-34	Singleton	--
Unique-03	1	E	t1778 (1)	26-23-21-17-13-34-16-34-33-13	Singleton	--
Unique-04	1	F	t878 (1)	26-23-17-34-21-25-33-16	Singleton	--
Unique-06	1	H	t359 (1)	07-23-12-21-17-34-34-33-34	Singleton	
Unique-05	1	G	Untyp. (1)	--	--	--
Subtotal	6					
Total	49					

Key: * Untyp, untypeable; Ridom info, data from www.spaserver.ridom.de; -- no information available; ST-sequence type; new-new *spa* type identified from this study

Table 4.6b. The *spa* types of the epidemic MRSA strains from the UK

Epidemic strain	*spa* type	*spa* repeat	Ridom info.
EMRSA-1, 4, 7 and 9	t037	15-12-16-02-25-17-24	ST239, ST240, ST241
EMRSA-2, 6, 11*, 12, 13 and 14	t190	11-17-34-24-34-22-25	ST 8
EMRSA-3	t001	26-30-17-34-17-20-17-12-17-16	ST5, ST222, ST228
EMRSA-5	t051	11-19-21-12-21-17-34-24-34-22-25	ST247, ST250
EMRSA-8	t194	11-19-12-12-12-34-34-24-34-22-25	ST250
EMRSA-10	t009	11-12-21-17-34-24-34-22-24-34-22-33-25	ST254
EMRSA-15	t025	26-23-23-13-23-29-17-31-29-17-25-17-25-16-28	
EMRSA-16	t018	15-12-16-02-16-02-25-17-24-24-24	ST30, ST36, ST38
EMRSA-17	t051	11-19-21-12-21-17-34-24-34-22-25	ST247, ST250

*EMRSA-11 *spa* type t037 in Ridom SpaServer

4.1.2.1 Cluster analysis of the *spa* types

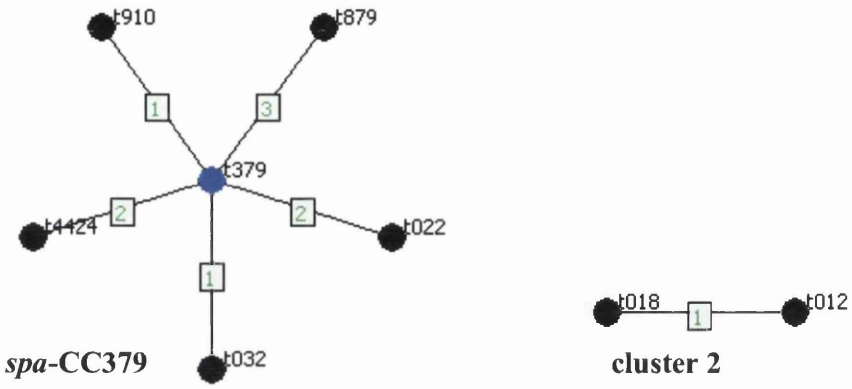


Figure 4.5. Minimum spanning trees generated with Ridom StaphType software. Circles represent respective *spa* types and the central circle depicts the *spa* type defined as the founder of the cluster i.e. t379 for *spa*-CC379. Note: no founder defined for cluster 2. Numerical values on branches represent the similarity (distance) between the respective *spa* types and the founder or between the *spa* types where there is no founder.

Cluster analysis of the *spa* types was performed with the Ridom StaphType version 1.5.2.1 software, using the Based Upon Repeat Pattern (BURP) algorithm. It excluded *spa* types that were shorter than five repeats and placed *spa* types in clusters if costs were ≤ 4. Two clusters: *spa*-CC379, cluster 2 and six singletons were generated. Cluster *spa*-CC379 comprised *spa* types: t022, t032, t379, t879, t910 and t4424, implying that all the isolates that exhibited these *spa* types were genetically related. Whereas cluster 2 comprised *spa* types: t012 and t018 again implying that the isolates with these *spa* types were genetically related. There were six singletons: t138, t359, t437, t878, t1778, t4185 and these implied that the isolates with these *spa* types were considered to be genetically unrelated to the other isolates (see Figure 4.5).

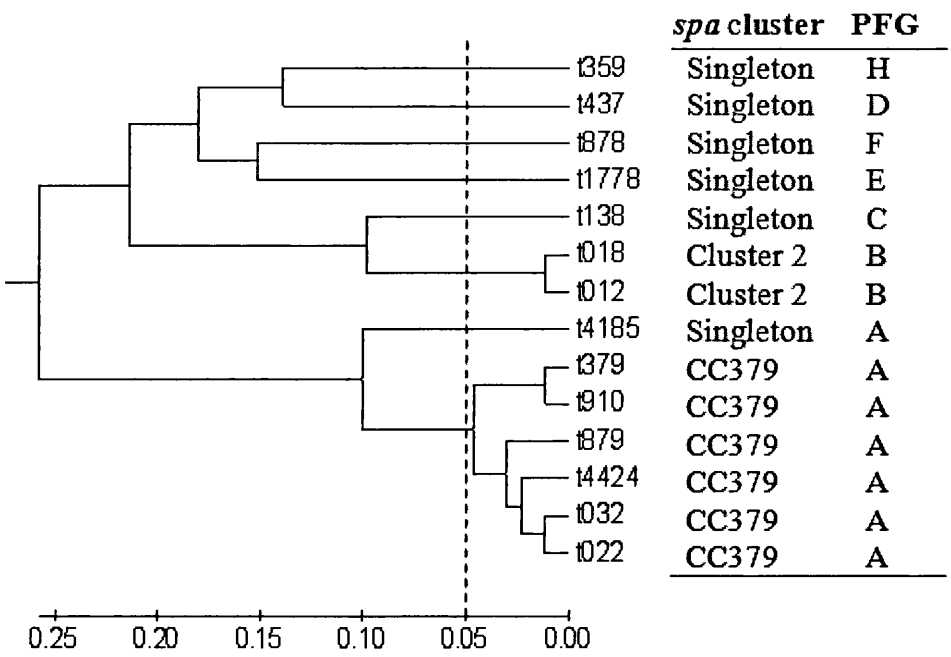
4.1.3 Discriminatory power of PFGE and *spa* typing and concordance of results

The discriminatory power of PFGE and *spa* typing was investigated by determining the number of types and groups (or BURP clusters with *spa* typing) generated with the selected isolates. In total the isolates (n=48, one untypeable with *spa* typing) were represented by 21 PFGE patterns versus fourteen *spa* types (see Table 4.7). The thirty-five EMRSA-15 strains were characterised by thirteen PFGE patterns in comparison to seven *spa* types (t022, t032, t379, t879, t910, t4185 and t4424) by typing (see Table 4.7). The eight EMRSA-16 strains were characterised by three PFGE patterns and two *spa* types (t012 and t018). The five isolates with the unique PFGE patterns similarly had five diverse *spa* types (t138, t359, t437, t878 and t1778). This indicated that on a types level the PFGE method was more discriminatory than *spa* typing for the EMRSA-15 and EMRSA-16 strains.

Table 4.7. Comparison of PFGE and *spa* typing results of 41 clinical MRSA strains

Typing Assay	EMRSA-15 (n=35)	EMRSA-16 (n=8)	Unique (n=5)	Total (n=48)
PFGE patterns	13	3	5	21
PFGE groups[b]	1	1	5	7
spa type	7	2	5	14
spa-BURP	2	1	5	8

Key: PFGE types[a]-strains differing by ≤3bands.
PFGE groups[b]- strains differing by ≤6 bands.



spa cluster	PFG
Singleton	H
Singleton	D
Singleton	F
Singleton	E
Singleton	C
Cluster 2	B
Cluster 2	B
Singleton	A
CC379	A
CC379	A
CC379	A
CC379	A
CC379	A
CC379	A

Figure 4.6. Dendrogram showing the genetic relatedness of *spa* types and correlation with PFGE groups. Generated with the Mega IV software, clustering by unweighted-pair group method using average linkages. At a cutoff of 0.05 (arbitrarily selected) two clusters and six singletons were evident.

Congruence between PFGE groups and *spa* clusters was investigated by comparing the strains assigned to the PFGE groups and those within the *spa* BURP clusters. The eight EMRSA-16 strains were clustered into one group by both assays i.e. PFG-B by PFGE and cluster 2 by *spa* BURP analysis (see Table 4.6). However the EMRSA-15 (n=35) strains were demarcated into one group (PFG-A) by PFGE but with *spa*-BURP analysis they were demarcated into two clusters. Most of the *spa* types (t022, t032, t379, t879, t910, t4185 and t4424) were clustered into *spa*-CC379 but the t4185 *spa* type was defined as a singleton (see Figure 4.6). This *spa* type was represented by one isolate which exhibited the EMRSA-15.1i PFGE pattern. Therefore by PFGE it was characterised as closely related to the EMRSA-15 clone but by *spa* typing it was defined as unrelated to the EMRSA-15 strains (see Figure 4.6). With regards to the isolates depicting unique PFGE patterns, all of these were demarcated into individual groups with PFGE and singletons by *spa*-BURP analysis. In summary, PFGE grouping and *spa* BURP analysis were comparable for the EMRSA-16 and unique strains but the latter was slightly more discriminatory than PFGE grouping for the EMRSA-15 strains.

4.1.4 SCC*mec* typing

SCC*mec* typing was performed on the MRSA clinical isolates (n=137) to determine the types of SCC*mec* elements contained in these isolates and the following MRSA reference strains were also included: NCTC10442 (SCC*mec* type I); N315 (SCC*mec* type II), 85/2082 (SCC*mec* type III), JCSC1978 (SCC*mec* type IVb) and WIS (SCC*mec* type V). With the Milheirico *et al.* (2007b) method all of the isolates (n=122) with the EMRSA-15 related PFGE patterns and four of the seven isolates with unique PFGE patterns exhibited the three expected SCC*mec* type IV fragments (*mecA*, 162 bp; *ccrB2*, 311 bp and *dcs*, 342 bp), therefore these were defined as MRSAIV strains (see Table 4.8 and lane 4 in Figure 4.7). The eight isolates with the EMRSA-16 related PFGE patterns exhibited all of the SCC*mec* type II fragments (*mecA*, 162 bp; *ccrB2*, 311 bp; *dcs*, 342 bp; *kdp*, 284 bp and *mecI*, 209 bp) thus these isolates were defined as MRSAII strains (see Table 4.8 and lane 2 in Figure 4.7). The remaining three isolates had unique PFGE patterns and they exhibited the *mecA* fragment (162 bp) but only had one additional fragment which was comparable in size to the expected *ccrC* fragment (449 bp). However unlike the MRSAV WIS reference strain the three isolates (termed isolate 7, 8 and 151) did not depict the fragment for the J1-region of SCC*mec* type V (377 bp) (see Table 4.8 and lane 5 in Figure 4.7). Furthermore in the banding pattern produced by the MRSAIII 85/ 2082 strain, the SCC*mec* type III-J3 fragment (414 bp) had similar electrophoretic mobility as the *ccrC* fragment of the MRSAV strain (see lane 3 and 5 in Figure 4.7). These results had several possible interpretations for isolates 7, 8 and 151:

i) The three isolates contained SCC*mec* type V elements that lacked the target sequence in the J1 region (377 bp).

ii) The three isolates contained SCC*mec* type III elements that lacked the target sequences in *mecI* (209 bp) and the J1 region (243 bp).

iii) The three isolates had atypical SCC*mec* elements that contained *ccrC* but did not contain the other loci of currently published SCC*mec* elements.

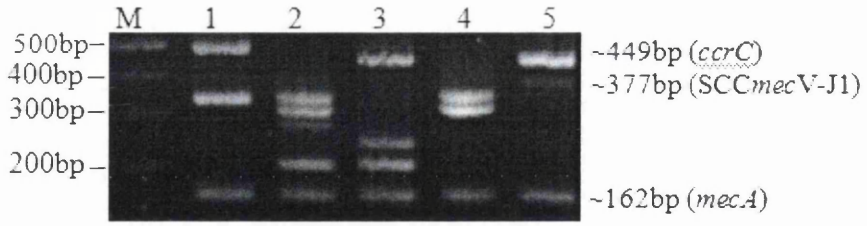


Figure 4.7. Milheirico *et al.* (2007b) SCC*mec* typing PCR product patterns for MRSA reference strains in a 2% agarose gel. M, 100bp molecular marker; lane 2, MRSAI; lane 3, MRSAII; lane 4, MRSAIII; lane 5, MRSAIVb and lane 6, MRSAV.

Table 4.8. SCC*mec* typing results of MRSA isolates (n=137) with Milheirico *et al.* (2007b) method

Locus	Size	SCC*mec* types II (2A)	IV (2B)	V (5C2)
pls	495bp			
ccrC	449bp			+
SCC*mec*III-J3	414bp			
SCC*mec*V- J1	377bp			*none?*
dcs	342bp	+	+	
ccrB2	311bp	+	+	
kdp	284bp	+		
SCC*mec*III-J1	243bp			
mecI	209bp	+		
mecA	162bp	+	+	+
Total number of clinical isolates		8	126	3

none?-expected product not amplified

The SCC*mec* typing method was repeated using the respective Milheirico *et al.* (2007b) primer sets of the SCC*mec* type III and V elements in separate PCRs and the MRSAIII 85/ 2082 and MRSAV WIS strains were included in the repeat assays. Like the MRSAV reference strain isolates 7, 8 and 151 generated the *mecA* and the *ccrC* fragments with the respective PCR assays but unlike the MRSAV reference strain they still did not exhibit the SCC*mec* type V-J1 fragment (see Table 4.9). The three isolates did not produce fragments with the PCR assays that amplified specific sequences in SCC*mec* type III element (see Table 4.9). Whereas the MRSAIII 85/ 2082 reference strain produced the *mecA*, *mecI* and SCC*mec* type III-J1 fragments but it did not exhibit the 414 bp fragment for the targeted SCC*mec* type III-J3 sequence located between Tn*554* and *orfX* (see Figure 4.8).

Table 4.9. Results of singleplex PCR assays for loci in SCC*mec* type III and V for MRSAIII, MRSAV, isolate 7, 8 and 151 with MPCR-1 and MPCR-2 assays

Locus	Size	MRSAIII	MRSAV	Isolate 7	Isolate 8	Isolate 151
ccrC	449bp	+	+	+	+	+
SCC*mec*III-J3	414bp	*none?*				
SCC*mec*V- J1	377bp		+			
SCC*mec*III-J1	243bp	+				
mecI	209bp	+				
mecA	162bp	+	+	+	+	+

none?-expected product not amplified

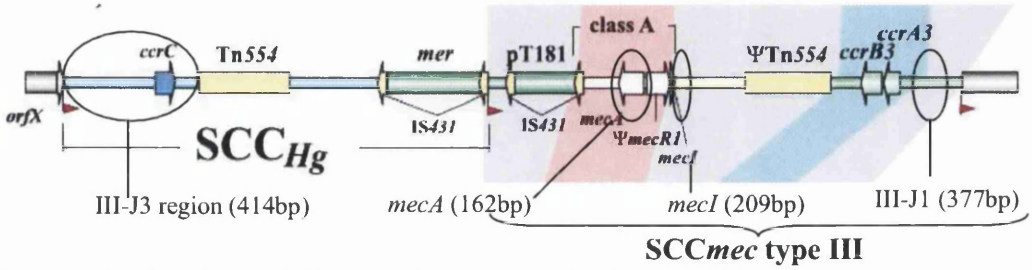


Figure 4.8. Structure of SCC*mec* type III (strain 85/ 2082) showing target sites of the Milheirico *et al.* (2007) typing method (IWG, 2009).

This MRSAIII 85/ 2082 strain also generated the *ccrC* fragment (449 bp) because it harbours a composite SCC*mec* element which comprises a $SCC_{Mercury}$ element which contains the *ccrC* gene and a SCC*mec* type III element (see Table 4.9 and Figure 4.8). The results of the singleplex PCR assays suggested that isolates 7, 8 and 151 were probably not MRSAIII strains because they did not produce any of the specific fragments however, since they also lacked the SCC*mec* type V-J fragment they could not be conclusively defined as MRSAV strains. Therefore the MPCR-1 and MPCR-2 assays were performed to determine the *ccr* types (types 1 to 5) and *mec* classes (classes A, B and C2) harboured by these isolates. The MRSAIII 85/ 2082 strain exhibited the *ccrA3B3* (1791 bp) and *mecA-mecI* (1963 bp) fragments with MPCR-1 and MPCR-2 respectively (see lane 3 in Figure 4.9a and b; see Table 4.10). This showed that it contained the class A *mec* complex and the type 3 *ccr* complex which are typical of the SCC*mec* type III (3A) element. Once again it also depicted the *ccrC* fragment (518 bp) (see lane 3 in Figure 4.9a). With the MPCR-1 assay isolates 7, 8 and 151 also produced the *ccrC* fragment but with MPCR-2 only isolates 7 and 8 exhibited the *mecA*-IS*431* (804bp) fragment whereas isolate 151 did not generate any fragments (see lane 5 in Figure 4.9a and b; see Table 4.10).

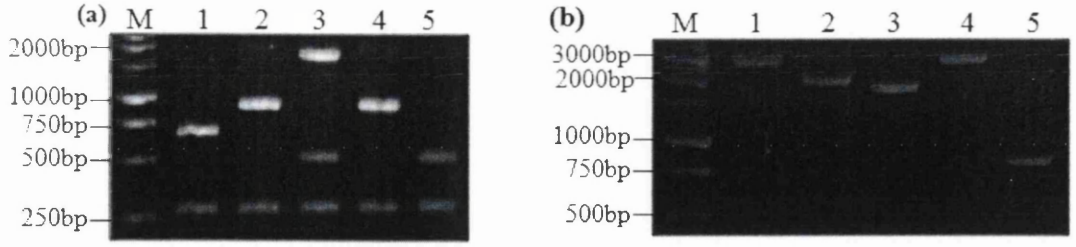


Figure 4.9. PCR product patterns for *ccr* types with MPCR-1 (a) and *mec* classes MPCR-2 (b). Lane M, 1kb molecular marker; lane 1, MRSAI; lane 2, MRSAII; lane 3, MRSAIII; lane 4, MRSAIVb; lane 5, MRSAV.

Table 4.10. MPCR-1 and MPCR-2 results of MRSAIII, MRSAV, isolate 7, 8 and 151

	MPCR-1						*ccr* type
	mecA 286bp	***ccrA1B1*** 695bp	***ccrA2B2*** 937bp	***ccrA3B3*** 1791bp	***ccrA4B4*** 1287bp	***ccrC*** 518bp	
MRSAIII	+			+		+	3 & 5
MRSAV	+					+	5
Isolate 7	+					+	5
Isolate 8	+					+	5
Isolate 151	+					+	5
	MPCR-2						***mec* class**
	mecA-mecI 1963bp		***mecA*-IS*1272*** 2827bp		***mecA*-IS*431*** 804bp		
MRSAIII	+						A
MRSAV					+		C2
Isolate 7					+		C2
Isolate 8					+		C2
Isolate 151					*none?*		?

none?-expected product not amplified

The carriage of *mecA*-IS*431* by isolates 7 and 8 indicated that these isolates harboured the class C2 *mec* complex and the MPCR-1 results indicated that they contained the type 5 *ccr* complex (*ccrC*). Since the MRSAV/ WIS strain also contains the type 5 *ccr* complex and the class C2 *mec* complex, this suggested that isolates 7 and 8 were MRSAV (5C2) strains. However, the results of isolate 151 were more ambivalent because it harboured *ccrC* but it did not generate any fragments with the MPCR-2 assay so its *mec* class could not be confirmed and consequently the SCC*mec* type of this isolate remained undefined.

The lack of amplification of the SCC*mec* type III-J3 fragment with the 85/ 2082 strain was further investigated by assessing the locations of the primers on the sequence of this strain. The Milheirico *et al.* (2007) method is an update of an assay originally developed by Oliveira and de Lencastre (2002) and it retains most of the original primer pairs, including RIF5 F10 (forward primer) and RIF5 R13 (reverse primer) which were developed for the amplification of a sequence in the J3 region of SCC*mec* type III. However, although the RIF5 F10 sequence is identical in both methods the RIF5 R13 sequence is different, but the expected product size is stated as 414 bp in both methods (see Table 4.11). In both studies the MRSA ANS46 strain was used in the optimisation of the methods. This strain contains a SCC*mec* element which is identical to the SCC*mec* type III (AB037671) element in the MRSAIII 85/ 2082 strain. In the SCC*mec* type III/ AB037671 sequence the location of the RIF5 R13 sequence as stated in the Milheirico *et al.* (2007b) method, is located much further downstream of the original reverse primer in the Oliveria and de Lencastre (2002) method and when analysed in silico resulted in a 2,983 bp fragment (see Table 4.11). However, when the RIF5 R13 sequence as stated in the Oliveira and de Lencastre (2002) method was analysed in silico on SCC*mec* type III/ AB037671 sequence it resulted in an expected 414 bp product (see Table 4.10). The same results were attained with two further published SCC*mec* type III sequences i.e. AF422696 (HU25 strain) and AF047089 (85/ 3097 strain) (see Table 4.11).

Table 4.11. Primers for the SCC*mec* type III-J3 region of the Milheirico *et al.* (2007b) and Olivera and Lencastre. (2002) methods

SCC*mec* typing method	Primer	Primer sequence (5'-3')	Location	Size
Oliveira *et al.* (2002)	RIF5 F10	TTCTTAAGTACACGCTGAATCG	59573-59594[a]	
	RIF5 R13	GTCACAGTAATTCCATCAATGC	59986-59965[a]	414bp
Milheirico *et al.* (2007b)	RIF5 F10	TTCTTAAGTACACGCTGAATCG	59573-59594[a]	
	*RIF5 R13	**ATGGAGATGAATTACAAGGG**	62556-62537[a]	*~2.9kb?*
Milheirico *et al.* (2007b)	*ccrC* F2	GTACTCGTTACAATGTTTGG	60662-60643[a]	
	ccrC R2	ATAATGGCTTCATGCTTACC	60214-60223[a]	448bp

[a] AB037671/ SCC*mec* type III (85/2082)

*AF422696/ SCC*mec* type III (HU25): RIF5 F10, 894-915 & *RIF5 R13, 3877-3858

*AF047089/ SCC*mec* type III (85/ 3097): RIF5 F10, 21370-21391 & *RIF5 R13, 24354-24335

It is highly likely that in the multiplex format, the fragment in the banding pattern of MRSA III 85/2082 which was assumed to be the SCC*mec* type III-J3 fragment (414 bp) was actually the *ccrC* fragment (449 bp) since this strain harbours this gene. This is supported by the location of the *ccrC* primers on the AB037671/ SCC*mec* type III (85/2082) sequence which result in a 448 nucleotide sequence (see table 4.10). Therefore with the Milheirico *et al.* (2007b) method the lack of amplification of the SCC*mec* type III-J3 fragment with the MRSAIII 85/2082 strain was probably due to the unusual location of the RIF5 R13 reverse primer which results in an unanticipated larger PCR product (approximately 2.9 kb). Such a large PCR product would not have been amplified under the PCR conditions of the multiplex and singleplex PCRs that were performed in this study.

In summary the SCC*mec* typing results indicate that out of the 137 MRSA isolates, 126 (92.0%) contained SCC*mec* type IV (2B), 8 (5.8%) contained SCC*mec* type II (2A) and 2 (1.5%) contained SCC*mec* elements which were related to the SCC*mec* type V (5C2) element of the MRSA WIS strain. However the SCC*mec* element harboured in one isolate (0.7%) remained undefined as it was not typeable with the typing methods employed in this study.

4.1.5 Subtyping of SCC*mec* type IV elements

One hundred and twenty-six MRSA isolates were found to contain the SCC*mec* type IV element and these comprised 122 isolates with EMRSA-15 related PFGE patterns (n=122) and four isolates with unique PFGE patterns. The Milheirico *et al.* (2007a) and the MPCR-3 methods were performed to determine the specific subtypes harboured by these isolates. The following MRSA reference strains were also included in these investigations: MRSAIVb/ JCSC1978 (SCC*mec* type IVb), MRSAIVc/ MR108 (SCC*mec* type IVc) and the EMRSA-15 reference strain. A PVL-positive USA300 clinical isolate was used as the MRSAIVa control (attained from the Specialist Chemotherapy Reference Unit, Cardiff, UK and identification and genotyping results presented in chapter 7).

With the MPCR-3 method the USA300 isolate, the MRSAIVb and MRSAIVc reference strains exhibited the expected PCR fragments i.e. IVa (458 bp), IVb (726 bp) and IVc (259 bp) respectively. Similarly with the Milheirico *et al.* (2007a) methods these strains depicted the IVa (278 bp), IVb (336 bp) and IVc (483 bp) fragments (see lanes 1, 2 and 3 in Figure 4.10a and b). However the EMRSA-15 reference strain did not produce any products with either of the two methods (see lane 8 in Figure 4.10a and b). Subtyping of the clinical isolates (n=126) was largely unsuccessful and only four produced PCR fragments (isolates 9, 10, 150 and 152). Interestingly these four had all depicted unique PFGE patterns. With the MPCR-3 method isolates 9, 10 and 152 exhibited the IVa PCR fragment (458 bp) and isolate 150 depicted the IVd PCR fragment (1, 242bp) (see lanes 4 to 7 in Figure 4.10a). With the Milheirico *et al.* (2007a) assay the *ccrB2* PCR product (203bp) was depicted by all of the clinical isolate (n=126), this served as an internal control for isolates containing a SCC*mec* type IV or type II elements. However, only isolate 150 which had exhibited the IVd PCR fragment with the MPCR-3 method once again produced a IVd fragment (575 bp) with the Milheirico *et al.* (2007a) assay, whereas isolates 9, 10 and 152 did not produce the expected IVa fragments (see lane 4, 5, 6 and 7 in Figure 4.10b).

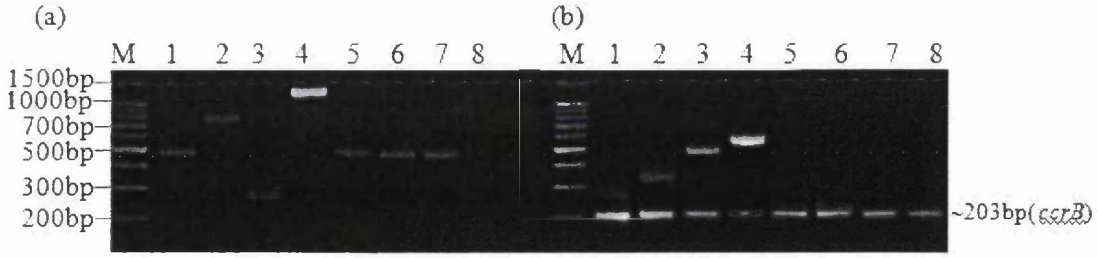


Figure 4.10. PCR products of MRSA reference strains and four clinical isolates.
(a) MPCR-3 method. Lane M, 100bp molecular marker. Lane 1, USA300 clinical isolate; lane 2, MRSAIVb; lane 3, MRSAIVc; lane 4, isolate 150; lane 5, isolate 9; lane 6, isolate 10; lane 7, isolate 152; lane 8, EMRSA-15 reference strain.

(b) Milheirico *et al.* (2007a) method. Lane M, 100bp molecular marker. Lane 1, USA300 clinical isolate; lane 2, MRSAIVb; lane 3, MRSAIVc; lane 4, isolate 150; lane 5, isolate 9; lane 6, isolate 10; lane 7, isolate 152; lane 8, EMRSA-15 reference strain.

In summary the MRSAIVb, IVc reference strains and a MRSAIVa clinical isolate were successfully subtyped with the two methods employed in this study however, the EMRSA-15 reference strain was not typeable with either of the methods. Similarly out of the 126 MRSA isolates that contained the SCC*mec* type IV elements, the 122 isolates with the EMRSA-15 related PFGE patterns did not generate any PCR products. Only the four isolates with unique PFGE patterns were subtyped with variable success i.e. isolate 150 was a IVd strain whereas isolate 9, 10 and 152 were possible IVa strains.

4.1.6 Detection of the arginine catabolic mobile element

Carriage of the arginine catabolic mobile element (ACME) was detected by amplification of the *arcA* locus located in ACME. It was detected in only two (isolate 14 and 68) of the MRSA clinical isolates (n=137). Both of them were EMRSA-15 strains and carried SCC*mec* type IV elements. Isolate 14 exhibited the EMRSA-15.1.2 PFGE pattern and the t032 *spa* type whereas isolate 68 exhibited the EMRSA-15i PFGE pattern (*spa* typing not done on this isolate). The *arcA*-ACME PCR products of the two clinical isolates were sequenced and aligned to the *arcA*-ACME sequence of the FPR3757/ USA300 strain (accession number CP000255.1). The sequence of isolate 68 was identical to the USA300 sequence whereas the sequence of isolate 14 had two point mutations (see Figure 4.11).

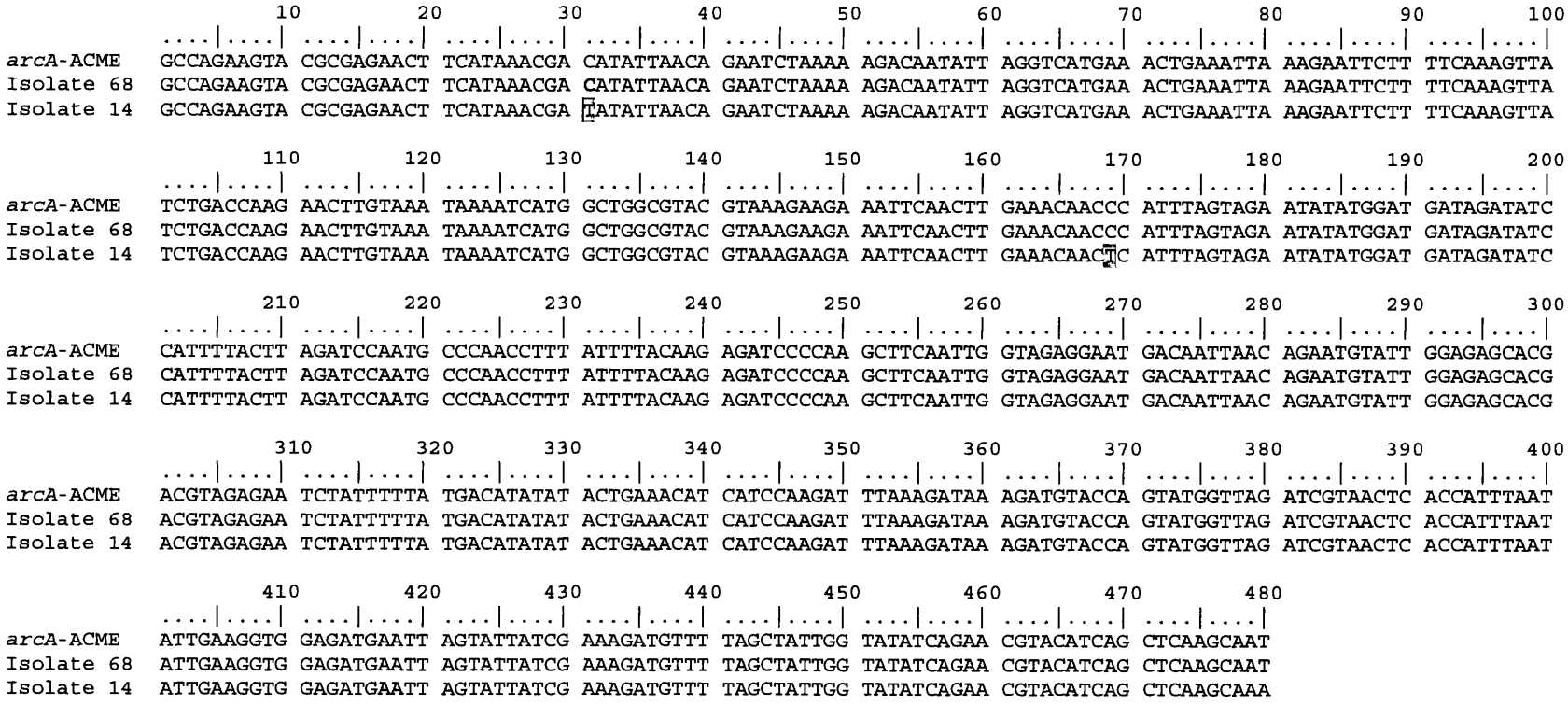


Figure 4.11. Alignment of *arcA*-ACME sequence of the USA300 FPR3757 strain, accession no. CP000255.1 and the clinical isolates 68 and 14. Blue- nucleotide differences.

4.1.7 Antibiotic susceptibility testing of selected MRSA clinical isolates

Antibiotic susceptibility testing was performed with the BD Phoenix on the two ACME-positive isolates (14 and 68) and since these isolates had depicted EMRSA-15 related PFGE patterns eleven additional clinical isolates which had also exhibited an EMRSA-15 PFGE pattern but were ACME negative were also tested. Susceptibility testing was also performed on seven isolates with EMRSA-16 related patterns and the seven isolates which had unique PFGE patterns. The EMRSA-15 and EMRSA-16 laboratory reference strains were also included in these investigations. The EMRSA-15 and EMRSA-16 reference strains were largely susceptible to non β-lactam antibiotics, showing resistance to ciprofloxacin only (see Table 4.12). Similarly the two ACME-positive isolates and all of the other EMRSA-15 clinical isolates were also resistant to this antibiotic and largely susceptible to other non β-lactam antibiotics. For instance very few were resistant to tobramycin (n=2) and clindamycin (n=1) and a variable number were resistant to erythromycin (n=6) and trimethoprim (n=4). Only one EMRSA-15 clinical isolate (75) was also resistant to gentamicin and mupirocin. However even though the EMRSA-16 reference strain was largely susceptible to non β-lactam antibiotics, there was an apparent trend of multiple antibiotic resistance amongst the EMRSA-16 clinical isolates (see Table 4.12). These seven isolates were extensively resistant to clindamycin (n=6), erythromycin (n=6), trimethoprim (n=7) and surprisingly mupirocin (n=6), with two isolates (144 and 145) exhibiting resistance to high level mupirocin. There was more variation within the seven unique isolates, three were completely susceptible to non β-lactam antibiotics, whereas the remaining four isolates were largely resistant to trimethoprim (n=4) and erythromycin (n=3). (As previously discussed in chapter 3, isolate 7 had multiple antibiotic resistance but it was susceptible to oxacillin).

Table 4.12. Antibiotic resistance profiles of ACME-positive isolates and selected ACME-negative EMRSA-15 and EMRSA-16 clinical isolates

Study ID	Antibiotic resistance	PFGE	*spa*	*spa* repeat sequence	SCC*mec*	ACME
EMRSA-15						
EMRSA-15 ref.	Ox, Cip	EMRSA-15	t025	26-23-23-13-23- -29-17-31-29-17-25-17-25-16-28	IV	neg
14	Ox, Cip	EMRSA-15.1.3	t032	26-23-23-13-23-31-29-17-31-29-17-25-17-25-16-28	IV	POS
68	Ox, Cip, FA, Tob	EMRSA-15i	nt.done		IV	POS
22	Ox, Cip, Ery	EMRSA-15.1i	nt.done		IV	neg
39	Ox, Cip	EMRSA-15	t022	26- -23-13-23-31-29-17-31-29-17-25-17-25-16-28	IV	neg
40	Ox, Cip, Clin, Ery	EMRSA-15i	nt.done		IV	neg
44	Ox, Cip, Ery, Trim	EMRSA-15i	nt.done		IV	neg
75	Ox, Cip, Ery, Mup, Mup-H, GM, Tob	EMRSA-15	t032	26-23-23-13-23-31-29-17-31-29-17-25-17-25-16-28	IV	neg
84	Ox, Cip	EMRSA-15	nt.done		IV	neg
92	Ox, Cip, Ery, Trim	EMRSA-15.1	t032	26-23-23-13-23-31-29-17-31-29-17-25-17-25-16-28	IV	neg
99	Ox, Cip, Ery, Trim	EMRSA-15.1i	nt.done		IV	neg
104	Ox, Cip	EMRSA-15.1	t910	26-23-23-13-23-31-29-17- -25-16-28	IV	neg
137	Ox, Cip, Trim	EMRSA-15.3	t032	26-23-23-13-23-31-29-17-31-29-17-25-17-25-16-28	IV	neg
138	Ox, Cip, Ery, Trim	EMRSA-15.3	nt.done		IV	neg
EMRSA-16						
EMRSA-16 ref.	Ox, Cip	EMRSA-16	t018	15-12-16-02-16-02-25-17-24-24-24	II	neg
21	Ox, Cip, Clin, Ery, Tob, Trim	EMRSA-16ii	t018	15-12-16-02-16-02-25-17-24-24-24	II	neg
144	Ox, Cip, Clin, Ery, Mup, Mup-H, Tob, Trim	EMRSA-16	t018	15-12-16-02-16-02-25-17-24-24-24	II	neg
145	Ox, Cip, Clin, Ery, Mup, Mup-H, Tob, Trim	EMRSA-16	t018	15-12-16-02-16-02-25-17-24-24-24	II	neg
146	Ox, Cip, Ery, Mup, Tob, Trim	EMRSA-16i	t012	15-12-16-02-16-02-25-17-24- -24	II	neg
147	Ox, Cip, Clin, Ery, Mup, Trim	EMRSA-16i	t018	15-12-16-02-16-02-25-17-24-24-24	II	neg
148	Ox, Cip, Clin, Ery, Mup, Trim	EMRSA-16i	t018	15-12-16-02-16-02-25-17-24-24-24	II	neg
149	Ox, Cip, Clin, Ery, Mup, Trim	EMRSA-16i	t018	15-12-16-02-16-02-25-17-24-24-24	II	neg

Ox, oxacillin; Cip, ciprofloxacin; Ery, erythromycin, FA, fusidic acid; GM, gentamicin; Mup, Mupirocin; Mup-H, mupirocin high level;Tob, tobramycin, Trim, trimethoprim; nt. done, not done; untype, untypeable; ref. reference strain; neg, negative; POS, positive

Table 4.12 contd. Antibiotic resistance profiles of ACME-positive isolates and selected ACME-negative EMRSA-15 and EMRSA-16 clinical isolates

Study ID number	Antibiotic resistance	PFGE	*spa*	*spa* repeat sequence	SCC*mec*	ACME
Unique strains						
7	Ox-S, Clin, Ery, Tet, Trim,	Unique-02	t437	04-20-17-20-17-25-34	V	neg
8	Ox, Cip, Ery, FA, Trim	Unique-03	t1778	26-23-21-17-13-34-16-34-33-13	V	neg
9	Ox	Unique-06	nt.done		IVa	neg
10	Ox	Unique-06	t359	07-23-12-21-17-34-34-33-34	IVa	neg
150	Ox, Trim	Unique-01	t138	08-16-02-25-17-24	IVd	neg
151	Ox,Ery, FA, Tet, Tob, Trim	Unique-04	t878	26-23-17-34-21-25-33-16	Untype.	neg
152	Ox	Unique-05	untyp.		IVa	neg

Ox, oxacillin; Cip, ciprofloxacin; Ery, erythromycin, FA, fusidic acid; Mup, Mupirocin; GM, gentamicin; Mup-H, mupirocin high level;Tob, tobramycin, Trim, trimethoprim; nt. done, not done; ref. reference strain; neg, negative; POS, positive; Untype, untypeable.

In the PHW Microbiology ABM Laboratory isolates that are identified from a patient within three months are considered to be the same strain. So consecutive isolates from the same patients within this period were identified from MRSA isolates of this study (n=137) and fifteen pairs and two triplicate isolates were identified. This enabled an assessment of any possible genotypic changes within this period, in most cases the consecutive isolates had the same PFGE pattern, *spa* type and SCC*mec* type (see Table 4.13).

Table 4.13. Genotypic data of MRSA isolates identified from consecutive (<3 months)

Isolate ID.	PHW result date	PFGE	*spa* type	SCC*mec*	ACME
46	30-Apr-07	EMRSA-15i	not done	IV	neg
61	22-May-07	EMRSA-15i	not done	IV	neg
2	02-May-07	EMRSA-15.2	not done	IV	neg
135	02-May-07	EMRSA-15.2	not done	IV	neg
3	04-May-07	EMRSA-15i	not done	IV	neg
52	08-May-07	EMRSA-15i	not done	IV	neg
57	14-May-07	EMRSA-15i	not done	IV	neg
97	04-May-07	EMRSA-15.1i	not done	IV	neg
102	25-Jun-07	EMRSA-15.1i	not done	IV	neg
53	09-May-07	EMRSA-15	not done	IV	neg
62	26-Jun-07	EMRSA-15	not done	IV	neg
144	13-May-07	EMRSA-16	t018	II	neg
145	16-May-07	EMRSA-16	t018	II	neg
143	13-May-07	EMRSA-16i	t012	II	neg
146	16-May-07	EMRSA-16i	t012	II	neg
129*	15-May-07	**EMRSA-15.1.1i**	t032	IV	neg
108*	27-Jun-07	**EMRSA-15.1i**	t032	IV	neg
63*	22-Jun-07	**EMRSA-15**	not done	IV	neg
83*	08-Aug-07	**EMRSA-15i**	not done	IV	neg
103	25-Jun-07	EMRSA-15.1	not done	IV	neg
107	27-Jun-07	EMRSA-15.1	not done	IV	neg
114	24-Jul-07	EMRSA-15.1	not done	IV	neg
64	02-Jul-07	EMRSA-15i	t022	IV	neg
12	07-Aug-07	EMRSA-15i	not done	IV	neg
111	10-Jul-07	EMRSA-15.1i	not done	IV	neg
115	27-Jul-07	EMRSA-15.1i	not done	IV	neg
67	17-Jul-07	EMRSA-15i	not done	IV	neg
85	08-Aug-07	EMRSA-15i	not done	IV	neg
71	20-Jul-07	EMRSA-15i	not done	IV	neg
90	24-Aug-07	EMRSA-15i	t032	IV	neg
9	24-Jul-07	Unique-06	t359	IVa?	neg
10	25-Jul-07	Unique-06	not done	IVa?	neg
77	01-Aug-07	EMRSA-15	not done	IV	neg
81	06-Aug-07	EMRSA-15	not done	IV	neg
149	08-Aug-07	EMRSA-16i	t018	II	neg
147	28-Aug-07	EMRSA-16i	t018	II	neg

*PFGE different but by <3 bands, so same clone (same *spa* type); PHW- PHW Microbiology ABM Laboratory

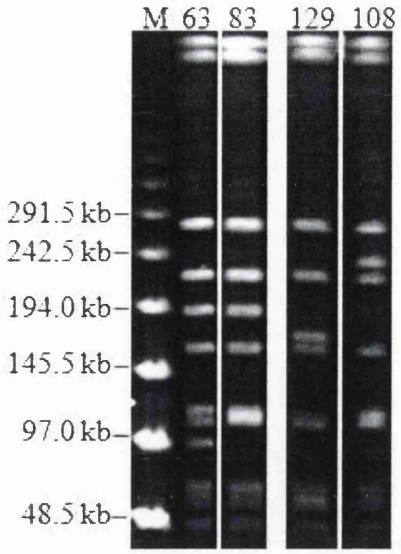


Figure 4.12. Different PFGE patterns exhibited by consecutive isolates identified from a single patient within one and a half months. Isolates 63 and 83 identified from one patient and isolates 129 and 103 identified from another patient.

However in two cases, there was some variability in the PFGE pattern i.e. isolates 129 and 108 were identified approximately one and half months apart but they exhibited the EMRSA-15.1.1i and EMRSA-15.1i PFGE patterns respectively (see Table 4.13 and Figure 4.12). These only had a 2-band difference, indicating that they were closely related and were probably the same strain furthermore their *spa* types were identical. Similarly isolates 63 and 83 were also identified approximately one and a half months apart and they depicted the EMRSA-15 and EMRSA-15i patterns. These only had a 1-band difference, indicating that they were closely related and were probably the same strain (see Table 4.13 and Figure 4.12).

4.2 Discussion

In this study 137 MRSA clinical isolates were collected from the PHW Microbiology ABM Laboratory from April 2007 to September 2007. These isolates were successfully typed with the PFGE typing method and twenty two patterns were resolved. One hundred and twenty-two (89.1%) isolates were determined to be EMRSA-15 strains, eight (5.8%) isolates were determined to be EMRSA-16 strains and seven (5.1%) were diverse MRSA strains. PFGE is renowned for its high discriminatory power however, EMRSA-15 and EMRSA-16 clones have highly conserved macro-restriction PFGE patterns. For instance Goering *et al.* (2008) observed that approximately 50% of EMRSA-15 and 30% of EMRSA-16 strains from Scotland are indistinguishable by PFGE (Holmes et al., 2010, Shore et al., 2010, Goering et al., 2008). Similarly in this study only thirteen PFGE patterns were resolved from the 122 EMRSA-15 strains and 30/122 (24.6%) strains had patterns that were identical to the EMRSA-15 reference strain. So even though this technique effectively identified two clones in this study, these results suggest that it had a limited capacity to discriminate EMRSA-15 and EMRSA-16 subtypes. The possible implication of this is that in geographical areas where these clones are endemic it may be difficult to identify and monitor specific subtypes which may be more predominant and have higher transmissibility within hospitals or the community (Holmes et al., 2010, Shore et al., 2010, Goering et al., 2008, Cookson et al., 2007). So it would be valuable to employ alternative or additional techniques that have the further capacity to discriminate these widely disseminated clones (Ghebremedhin et al., 2007).

The EMRSA-15 clone belongs to the ST22-MRSA-IV lineage and various techniques have been developed to address the limited discriminatory power of PFGE with regards to the discrimination of ST22-MRSA-IV strains. These include methods based on the amplification and sequencing of variable number tandem repeat (VNTR) such as *spa* typing, *dru* typing and multi locus VNTR analysis (Holmes et al., 2010, Shore et al., 2010, Goering et al., 2008, Nahvi et al., 2001). The *dru* typing method involves the sequencing of a hyper variable non-coding region located between *mecA* and IS*431* in SCC*mec* elements. This region comprises repeat sequences which vary in composition and quantity in different strains. The analysis and assignment of *dru* types is performed with a software and there is an on-line database that provides defined *dru* types.

This method has been investigated by Shore *et al.* (2010) and Goering *et al.* (2008) as an alternative to PFGE for the discrimination of EMRSA-15 strains. Goering *et al.* (2008) investigated EMRSA-15 (n=47) and EMRSA-16 (n=57) strains from hospitals in Scotland from 1998 to 2005. The EMRSA-15 and EMRSA-16 strains exhibited 13 and 12 *dru* types respectively and although there were highly prevalent types which were commonly present in all of the hospitals, they were also able to identify some minor EMRSA-15 and EMRSA-16 *dru* types that were specific to certain geographical regions. They suggested that this method may have the potential to trace specific subtypes of otherwise indistinguishable EMRSA-15 and EMRSA-16 strains. Shore *et al.* (2010) compared the efficacy of *dru* typing with PFGE and *spa* typing for the discrimination of ST22-MRSA-IV strains (n=173) from Ireland. In total, they attained 26 pulsed field types (patterns with ≤3 band differences) and found that these could be placed into only four pulsed field groups (≤6 band differences). Almost all of their 173 strains (n=168, 97%) belonged to one pulsed field group (PFG-01) which was comparable to this study whereby all the EMRSA-15 strains belonged to one pulsed field group (PFG-A). When they performed *dru* typing, PFGE and *spa* typing individually on the PFG-01 strains, PFGE was the most discriminatory method followed by *dru* typing. They also evaluated pair-wise combinations of the three typing methods and determined that *dru* typing and PFGE provided the most discriminatory combination method. However they recommended the combination of all three methods for an enhanced discrimination of the ST22-MRSA-IV strains. Although *dru* typing is not as widely used as PFGE or *spa* typing, it involves the same techniques as *spa* typing, such as the amplification and sequencing of a single VNTR locus. Therefore in clinical laboratories or reference laboratories that already perform DNA sequencing-based typing methods, the implementation of this technique would not require additional expertise or gravely increase testing costs (Shore et al., 2010).

Holmes *et al.*, 2010 performed two related techniques which are based on the amplification and analysis of seven VNTR loci, namely the MLVF and the MVLA methods. The former involves the resolution of the PCR fragments in a gel whereas the latter requires DNA-sequencing of the amplified VNTR regions and the assignment of a numerical profiles with a specific software. They compared MVLF and MVLA to PFGE for the discrimination of EMRSA-15 (n=41), EMRSA-16 (n=20) and MRSA (n=24) strains with variable PFGE patterns. They also evaluated 29 EMRSA-15 strains

with indistinguishable PFGE patterns which were from two different outbreaks (Holmes et al., 2010). Amongst the strains with the variable patterns, they found MVLF to be comparable to PFGE for discriminating the EMRSA-15 strains but less discriminatory with the EMRSA-16 strains. With the indistinguishable EMRSA-15 strains, the MVLF and MVLA methods resolved seven and six subtypes respectively and they were able to identify the distinct EMRSA-15 subtypes that caused the outbreaks with both assays (Holmes et al., 2010). Since the MVLA and MVLF techniques are more rapid than PFGE and the MVLF method was found to be comparable to PFGE, Holmes *et al.* (2010) recommended this method for hospital outbreak investigations.

In this study, the 137 MRSA clinical isolates were from wound swabs and 94.9% (130/ 137) were collectively EMRSA-15 and EMRSA-16 strains. This suggests that the molecular epidemiology of MRSA isolates from wound infections is dominated by these two clones. These results concur with the results of surveillance studies in England and Wales which have shown that HA-MRSA infections in these regions are extensively caused by the EMRSA-15 and EMRSA-16 clones (Boakes et al., 2010, Ellington et al., 2010c, Khandavilli et al., 2009, Johnson et al., 2005). Studies by Shore *et al.* (2010) and Goering *et al.* (2008) have shown that these clones are also highly prevalent in Ireland and Scotland and have outcompeted former dominant strains. The majority (122/137, 89.1%) of the isolates belonged to the EMRSA-15 clone and less to the EMRSA-16 clone (8/137, 5.8%). These proportions were comparable to the results by Rollason *et al.* (2008) for MRSA isolates from skin and soft tissue infections of non-hospitalised patients in the West Midlands region. They found that EMRSA-15 accounted for 87% of the isolates whereas EMRSA-16 accounted for 8% (Rollason et al., 2008). Furthermore, Ellington *et al.* (2010) investigated UK bacteraemia isolates from the year(s) 2001 to 2007 and found that EMRSA-15 and EMRSA-16 consistently accounted for 95% of the MRSA isolates. However the proportions of EMRSA-16 declined from 21.4% in 2001 to 9% in 2007, whereas the proportion of EMRSA-15 accounted for 85% of MRSA in 2007. They postulated that the decline of EMRSA-16 preceded a subsequent decline in cases of MRSA bacteraemia (Ellington et al., 2010c).

The *spa* typing assay is a widely used *S. aureus* typing tool and is variably comparable to PFGE (Grundmann et al., 2010, Petersson et al., 2009, Faria et al., 2008, Golding et al., 2008, Mellmann et al., 2008, Strommenger et al., 2008, Strommenger et al., 2006, Harmsen et al., 2003, Oliveira et al., 2001, Shopsin et al., 1999). In this study forty nine isolates were selected for *spa* typing, thirty-five of which depicted various EMRSA-15 PFGE patterns. The most prevalent *spa* type of the EMRSA-15 strains was t032, which is characteristic of the EMRSA-15 clone and the other *spa* types exhibited by the EMRSA-15 strains were close variants of this *spa* type (t022, t379, t879, t910, t4185 and t4424) (http://spaserver2.ridom.de/spatypes.shtml) (Grundmann et al., 2010, Khandavilli et al., 2009, Soliman et al., 2009). The t012 and t018 *spa* types which are characteristic of the EMRSA-16 clone were depicted by the eight isolates with EMRSA-16 related patterns. This indicated that within the selected clinical isolates the *spa* typing method successfully characterised EMRSA-15 and EMRSA-16 strains. However one isolate was not typeable with the *spa* typing method because amplification of the *spa* target sequence was not successful. This may be due to either mutations in the primer binding sites or the possible lack of this gene in this particular isolate (Baum et al., 2009, Strommenger et al., 2008). Furthermore strains that contain *spa* sequences comprising a few repeats i.e. $\leq$3 repeats cannot be included in the cluster analysis of *spa* types, so to a certain extent these are also non-typeable. These cases are rare but indicate a limitation of the typeability capacity of the *spa* typing method in *S. aureus*.

In comparison to PFGE, *spa* typing was found to be much less discriminatory for the EMRSA-15 strains (n=35) i.e. thirteen PFGE patterns versus seven *spa* types. In addition a specific *spa* type was not confined to a particular PFGE pattern which implied that *spa* types were not consistent predictors of PFGE patterns. Nonetheless, there was congruence in the clustering of strains into PFGE groups (isolates with $\leq$6 band difference) and *spa*-BURP clusters. The EMRSA-15 strains and EMRSA-16 strains were demarcated into distinct clusters with both methods and the strains in the groups were largely the same. However one EMRSA-15 strain exhibited the t4185 *spa* type which was defined as a singleton. In comparison to the *spa* types of the other EMRSA-15 clinical strains (t022, t032, t379, t879, t910, t4185 and t4424) the t4185 *spa* type has deletions of several consecutive repeats (r31-r29-r17-r31-r29-r17-r25) and possibly this was an underlying cause of this *spa* type being defined as a singleton by *spa*-BURP cluster software. These results were comparable to those of several studies

which have demonstrated that although *spa* typing tends to be slightly less discriminatory than PFGE the *spa* clusters are highly concordant with PFGE groups (isolates exhibiting patters with ≤6 band difference). In addition *spa*-BURP clusters are highly concordant with MLST clonal complexes (Shore et al., 2010, Petersson et al., 2009, Vindel et al., 2009, Faria et al., 2008, Golding et al., 2008, Mellmann et al., 2008, Strommenger et al., 2008, Cookson et al., 2007, Strommenger et al., 2006, Harmsen et al., 2003, Oliveira et al., 2001, Shopsin et al., 1999). One study included 1681 *S. aureus* clinical isolates and the discriminatory capacity of a multi-locus VNTR analysis (MVLA) method involving eight loci was compared to *spa* typing and PFGE (Schouls et al., 2009). The *spa* typing and MVLA methods are based on similar principles which involve the amplification and DNA sequencing or fragment size analysis of loci containing polymorphic repeat regions. Except in *spa* typing only one VNTR locus is analysed whereas with MVLA several VNTR loci are analysed. In the study by Schouls *et al.* (2009) the *S. aureus* clinical isolates (n=1682) had been submitted to a national institute for public health in the Netherlands from 2005 to 2007. All of the isolates were typeable with the MVLA method and a total of 511 MVLA types were represented in the 1681 isolates, these were then clustered into eleven MVLA complexes. Diversity indices were determined to evaluate the discriminatory power of the MVLA method, *spa* typing and PFGE, which showed that the MVLA method was comparable to PFGE whereas *spa* typing was less discriminatory than both of the methods (Schouls et al., 2009). On a grouping level there was congruence between most of the *spa*-clusters and the eleven MVLA complexes except, in two cases whereby two of the MVLA complexes were included in one *spa*-cluster. This demonstrated that at a grouping level the *spa* typing method is still less discriminatory than MVLA. In addition, there was strong agreement between the MLST clonal complexes and MVLA complexes which indicates that the MVLA method may be a suitable typing tool for long-term evolutionary studies of *S. aureus* (Schouls et al., 2009). The inclusion of several VNTR loci in MVLA methods, as opposed to one locus in *spa* typing, also reduces the probability of strains being un-typeable. In another study diverse *S. aureus* clinical isolates (n=198) collected from hospitals in the 1980s from nineteen countries of Europe, Latin America and USA were evaluated with *spa* typing, PFGE and MLST assays (Faria et al., 2008). Statistical tests were applied in order to quantify the levels of diversity and congruence between molecular techniques for the collection as a whole and also for the MRSA (n=116) and MSSA (n=82) separately. Even though *spa* type(s)

could predict PFGE type(s) with 92% probability for the MSSA, the probability was markedly reduced to 40% for the MRSA (Faria et al., 2008). PFGE typing was found to be more discriminatory than *spa* typing for the collection as a whole and for the MRSA but with the MSSA isolates PFGE and *spa* typing had comparable discriminatory power (Faria et al., 2008). In a further study a collection of 98 *S. aureus* isolates termed the Harmony collection had been collected from eleven European countries in the 1980s and these isolates were analysed with *spa* typing, MLST and PFGE (Cookson et al., 2007). The collection of isolates had been previously used in the EU Harmony Project which involved the standardisation of the PFGE protocol for European countries (Cookson et al., 2007). The *spa* typing method had a similar discriminatory capacity as PFGE and the groupings of strains with the three methods exhibited a high level of concordance (Cookson et al., 2007). In the study by Hallin *et al.* (2007) *S. aureus* strains (n=217) were selected from the Belgian staphylococci reference laboratory. The collection included representative strains of the major Belgian HA-MRSA clones and some sporadic strains with diverse PFGE patterns. These were selected from national surveillance programmes which had occurred from 1992 to 2003 (Hallin et al., 2007). In contrast to the afore-mentioned studies, the *spa* typing method had slightly more discriminatory power than PFGE.

The molecular characterisation of isolates in this study and the results of the afore-mentioned studies suggest that *spa* typing is generally less discriminatory than PFGE particularly for MRSA strains. However, in terms of practicality this method is advantageous because it is easier to perform than the intricate PFGE protocol: also the universal nomenclature of *spa* types and the on-line database allows an accessible source of epidemiological data world-wide. Since the clustering of *spa* types is highly concordant with MLST clonal complexes this indicates that this method has the added advantage of potentially being an adequate typing tool for long-term epidemiology investigations. Both PFGE and *spa* typing can be affected by inter-genomic recombination events but these events are rare in *S. aureus*. However they are more effectual in *spa* typing because a large chromosomal replacement during a recombination event may include the *spa* locus (Hallin et al., 2007, Koreen et al., 2004). This results in strains of the same clonal lineage depicting unrelated *spa* types. For instance Robinson and Enright (2004) demonstrated that the ST239-MRSA-II/ Brazilian clone evolved by the homologous recombination of a 557-kb fragment from the ST30

chromosome into the ST8 genetic background (Robinson and Enright, 2004). Thus approximately 20% of the ST239 chromosome is identical to the ST30 genetic background and 80% is identical to the ST8 genetic background. Interestingly with the MLST method ST239 strains are clustered into the CC8 clonal complex which harbours the ST8-MRSA-IV/ USA300 clone however, with *spa* typing these strains typically exhibit the t037 *spa* type which is affiliated to the CC30 clonal complex which harbours the ST30-MRSA-IV/ Southwest Pacific clone (Strommenger et al., 2006). These discrepancies can be resolved by *spa* typing being performed in combination with an additional genotyping typing assay (Golding et al., 2008, Hallin et al., 2007, Koreen et al., 2004). Although MLST is the optimal assay for the definition of MRSA lineages or clones, it may be financially impractical in high volume studies. The combination of *spa*-BURP typing and PFGE has been shown to be less expensive and similarly effective.

Approximately 92% of the isolates in this study carried SCC*mec* type IV elements, these included all of the EMRSA-15 strains (n=122) and four diverse strains. This indicates that although the SCC*mec* type IV element was extensively found in strains with EMRSA-15 genetic background it was not restricted to this genetic background. This element is the smallest (21-24 kb) of the currently defined SCC*mec* types (I to VIII- 28 to 67kb) and its small size is thought to award it the advantage of high transmissibility within different genetic backgrounds of staphylococci (Deurenberg and Stobberringh, 2008, Hanssen and Sollid, 2006, Ito et al., 2004). In contrast the SCC*mec* type II element was less prevalent and found in only eight isolates in the present study. The carriage of larger SCC*mec* elements (II and III) is hypothesized to come at a fitness cost to MRSA which may include a lower growth rate and this may contribute to the higher prevalence of MRSAIV strains in the community and their increasing prevalence in hospital environments (Deurenberg and Stobberringh, 2008, de Sousa and de Lencastre, 2003, Okuma et al., 2002). Similarly the carriage of the SCC*mec* type IV element may also confer these selective advantages to the EMRSA-15 clone and contribute to its higher prevalence in the UK in comparison to EMRSA-16. For three of the 137 MRSA isolates in this study, the characterisation of the SCC*mec* elements was more ambiguous. Isolates 7 and 8 had an element that was putatively identified as SCC*mec* type V due to the lack of detection of a sequence upstream of the *hsdM, S, R* genes in the J1 region of SCC*mec* type V. Whereas for isolate 151, although the *ccrC*

gene was identified, the *mec* class was not confirmed. These results were not enough to unambiguously identify the element as a SCC*mec* type V since the *ccrC* gene has been found in other elements e.g. the composite element ($SCC_{Mercury}$/SCC*mec*III) in the MRSA 85/ 2085 strain and in MSSA isolates by Shore *et al.* (2008) (Shore et al., 2008, Chongtrakool et al., 2006). Chongtrakool *et al.* (2006) carried out a larger study comprising 615 strains from 11 Asian countries and similar to the present study they used primers which were specific for the different *ccr* types and *mec* classes in MRSA. The majority of their isolates carried SCC*mec* type III (3A) and II (2A) elements but they also had five isolates that were non-typeable. Three had type *ccrA2B2* but an unidentifiable *mec* class whereas two had *ccr* genes which were non-typeable. Interestingly they also found that one isolate with a type II (2A) element and fifteen with type 2B (IV) elements also harboured *ccrC* but they were not able to verify that the gene was actually located in the SCC*mec* elements. This indicates that despite the development of comprehensive typing assays their accuracy remains dependent the current published SCC*mec* sequences. However the discovery of these has not been exhausted and novel elements may arise from the recombination of currently defined elements.

Attempts to sub-type the 126 clinical isolates that harboured the SCC*mec* type IV element were largely unsuccessful even though the MRSAIVa, IVb and IVc reference strains were successfully sub-typed with both of the PCR methods that were employed in this study (Kondo et al., 2007, Milheirico et al., 2007a). Out of the 126 clinical isolates that were investigated 122 were EMRSA-15 strains whereas the remaining four were strains that had unique PFGE patterns and *spa* types. Interestingly, there was a clear disparity in the sub-typing results, as only the four unique clinical strains were successfully sub-typed whereas all of the EMRSA-15 strains remained non-typeable, including the laboratory EMRSA-15 reference strain. The two sub-typing assays that were used in this study comprised primers that were specific for sequences in the J1-regions of the published SCC*mec* type IV elements. The Milheirico *et al.* (2007a) assay was specifically included because the HAR22 strain, a prototypic EMRSA-15 strain was included in the development of the method. This strain had not produced any fragments with PCR methods for the amplification of the J1-region of SCC*mec* types IVa to IVg elements. So they sequenced the SCC*mec* element harboured by the strain and compared it to published type IV sequences. It had almost 100% homology in the

mec, *ccr* and J2 and J3-regions, however, the J1-region was different to other type IV elements. This region was homologous to the J1-region of the PL72 strain, a SCC*mec* sporadic variant which was originally isolated from a Polish hospital (Milheirico et al., 2007a). Consequently they regarded the element as a novel type IV sub-type and defined it as IVh. In their study five EMRSA-15 strains from Portugal and one ST254-MRSA strain from Greece contained the novel IVh sub-type. In this study, the magnitude of EMRSA-15 strains that were non-typeable with this method is surprising but it is not altogether incomprehensible. The EMRSA-15 strain that was used by Milherico et al., 2007a could not be sub-typed with a comprehensive set of primers that were specific for J1-regions in a diverse array of type IV subtypes i.e. IVa, IVb, IVc, IVd, IVE, IVF and IVg. This implies that there is a certain novelty in the J1-region of EMRSA-15 strains and perhaps more heterogeneity in other EMRSA-15 strains. The prototype strain that they used had a J1-region that was homologous to a sporadic Polish strain, so it is not unlikely that the J1-region may be rare or restricted to a subset of EMRSA-15 strains in specific geographical locations. Possibly the EMRSA-15 strains from other geographical regions including the strains from the epidemiological setting of this study may have a different J1-region in their SCC*mec* type IV elements. Certainly the identification of novel SCC*mec* subtypes has not been exhausted and this is shown by the discoveries of MRSA isolates with non-typeable elements in other studies (Berglund et al., 2009, Hanssen and Sollid, 2007, Rossney et al., 2007).

In this study the arginine catabolic mobile element (ACME) element was detected in two EMRSA-15 strains suggesting that it is rare in wound MRSA isolates from the epidemiological setting of this study. However, the ACME element is frequently present in coagulase-negative staphylococci such as *S. epidermidis* and *S. haemolyticus* and these are considered to be the reservoir for these elements (Miragaia et al., 2009, Pi et al., 2009). In *S. aureus*, ACME is consistently harboured by methicillin resistant USA300 strains depicting the USA300-0114 PFGE pattern. It is speculated that it has a contributory role in bacterial colonisation of the human skin and mucosal surfaces (Miragaia et al., 2009, Diep et al., 2006). It was initially identified by Diep *et al.* (2006) in a multidrug resistant USA300 FPR3757 strain and it was presumed that it was restricted to the USA300-0114 genetic background. The EMRSA-15 clone belongs to the ST22-MRSA-IV lineage whereas ACME positive USA300 strains belong to the ST8-MRSA-IVa lineage. Thus the detection of ACME in this study, presents the first

documented detection of ACME in specifically the EMRSA-15 genetic background. It has also been detected in other lineages. For instance Ellington *et al.* (2008) investigated its prevalence in MRSA (n=203) of diverse genetic backgrounds in England and Wales and detected ACME in seventeen strains. Most of the strains belonged to the ST8-MRSA-IVa (USA300) lineage, several were ST1-MRSA-IVa strains and one MRSA was a ST97-MRSA-V strain. This also showed a relatively low prevalence of this element in UK MRSA isolates (Ellington et al., 2008). Earlier Goering *et al.* (2007) had investigated 214 *S. aureus* isolates from strain collections of the Centres for Disease Control and Prevention in the USA. They had detected ACME in all 82 ST8-MRSA-IVa strains, six ST5/ USA100 strains and also in two MSSA strains. The MSSA belonged to the ST8 lineage and depicted a PFGE pattern that was closely related (1 band difference) to the USA300-0114 pattern. They hypothesized that these were previously methicillin resistant USA300 strains which had undergone a precise excision of SCC*mec*IVa. Later, Diep *et al.* (2008) performed molecular analysis of ACME elements from diverse genetic backgrounds, i.e. ST8-MRSA-IVa, ST5-MRSA-II and ST59-MRSA-IVa and showed that these were distinct ACME allotypes. The ACME element in the USA300/ ST8-MRSA-IVa strains harbours two gene clusters, *arc* (encodes arginine deiminase pathway) and *opp3* (encodes oligopeptide permease) which are homologues of genetic elements linked with pathogenesis in group A *Streptococcus* species. However the ACME elements in the ST5 and ST59 strains harboured the *arc* cluster but were deficient of the *opp3* cluster (Diep et al., 2008b).

In this study the ACME element was detected in only two of the 122 EMRSA-15 strains and these isolates carried non-subtypeable SCC*mec* type IV elements. The eight EMRSA-16 clinical strains containing SCC*mec* type II elements and the seven diverse MRSA isolates with SCC*mec* type IV or V elements did not contain the ACME element. Since 89.1% of the MRSA isolates in this study were characterised as EMRSA-15 strains this suggests that the prevalence of the ACME element was low in the EMRSA-15 genetic background. It seems like the ACME element is almost exclusively present in the USA300 clone and rarely present in other genetic backgrounds (Diep et al., 2008b, Goering et al., 2007). However it is possible that this element is frequently transferred to diverse *S. aureus* genetic backgrounds but remains largely unnoticed because these genotypes have minimal selective advantage in the respective epidemiological settings and thus remain less prevalent than the USA300

clone. Furthermore the SCC*mec* type IVa element is typically carried by USA300 strains and the ACME element is located adjacent to this element in the USA300 chromosome. In rare cases it has been identified in isolates harbouring SCC*mec* type V or SCC*mec* type II elements (Diep et al., 2008b, Goering et al., 2007). It is likely that the infrequent detection of ACME in this study was because the SCC*mec* type IVa element was not carried by most of the MRSA isolates of this study. Since ACME is located adjacent to SCC*mec* it is hypothesized that it is reliant on the recombinase genes of this element for integration and excision from the *S. aureus* chromosome. It is possible that the SCC*mec* type IVa element may contribute to an enhanced stabilisation of ACME in the USA300 genetic background in comparison to other types of SCC*mec* elements in other MRSA strains.

In the UK the early 1990's saw the emergence of the EMRSA-15 and EMRSA-16 strains (Johnson et al., 2005) which were very successful at spreading between hospitals in England. Almost twenty years later these two clones continue to be the dominant MRSA strains in England and Wales (Ellington et al., 2009). The PFGE and *spa* typing results of this study undoubtedly concur with this phenomenon and illustrate a sustained predominance of the EMRSA-15 and EMRSA-16 strains in the molecular epidemiology of wound isolates from Southwest Wales. This implies that these two strains have some selective epidemiological advantage in this area and population, over other HA-MRSA clones i.e. Archaic clone, ST250-MRSA-I; the Brazilian/ Hungarian clone, ST8-MRSA-III; the Iberian clone, ST247-MRSA-I; the New York / Japan clone ST5-MRSA-II and the Paediatric clone, ST5-MRSA-IV (Deurenberg and Stobberringh, 2008).

In conclusion, in this study 137 MRSA isolates were characterised as EMRSA-15 (n=122), EMRSA-16 (n=8) and unique MRSA strains (n=7). Most of the isolates (n=126) contained SCC*mec* type IV whereas fewer isolates harboured the SCC*mec* type II element (n=8). Two isolates harboured elements which were affiliated to the SCC*mec* type V type element and one isolate had an element which was non-typeable. The accurate and rapid identification of MRSA isolates is vital for infection control and the performance of diagnostic tests that are able detect diverse MRSA strains with a high level of accuracy is essential.

Chapter Five

Optimisation of three SCC*mec-orfX* PCRs

5.0 Introduction

The *mecA* PCR is the gold standard assay for detection of methicillin resistance and duplex assays detecting *mecA* and a *S. aureus* specific gene e.g. *nuc*, *femA*, *femB* or *Sa442* have been described for the identification of MRSA from clinical isolates (Costa et al., 2005, Francois et al., 2003, Jonas et al., 2002, Reischl et al., 2000b, Vannuffel et al., 1995). However these assays are limited as MRSA detection cannot be conclusively inferred from clinical specimens harbouring diverse staphylococci i.e. methicillin-resistant coagulase-negative staphylococci and methicillin-susceptible *S. aureus* (MSSA) because these could generate false positive results.

The *mecA* gene is located on the SCC*mec* element which comprises characteristic genetic features: the *mec* and *ccr* complexes and regions outside of these, termed J1, J2 and J3. In the *S. aureus* chromosome, SCC*mec* integrates specifically at *att*B, a site located at the 3' end of *orfX*. The chromosomal extremities at the left and right junctions are termed *att*B-L and *att*B-R, whereas the SCC*mec* extremities are termed the *att*SCC-L and *att*SCC-R (see Figure 5.1). The junction of the right extremity of SCC*mec* and the *S. aureus* specific *orfX* locus, provides a surrogate marker for MRSA detection (Cuny and Witte, 2005, Hagen et al., 2005, Huletsky et al., 2004). However there are diverse SCC*mec* types I-VIII and the sequences downstream of *mecA* differ by varying degrees (Zhang et al., 2009, Berglund et al., 2008, Oliveira et al., 2006, Ito et al., 2004, Ito et al., 2003, Ma et al., 2002). In SCC*mec* type I, II, IVa, IVb and IVc, the sequence is highly similar but it is different and longer in SCC*mec* type III (Huletsky et al., 2004, Oliveira and de Lencastre, 2002) (see Figure 5.2).

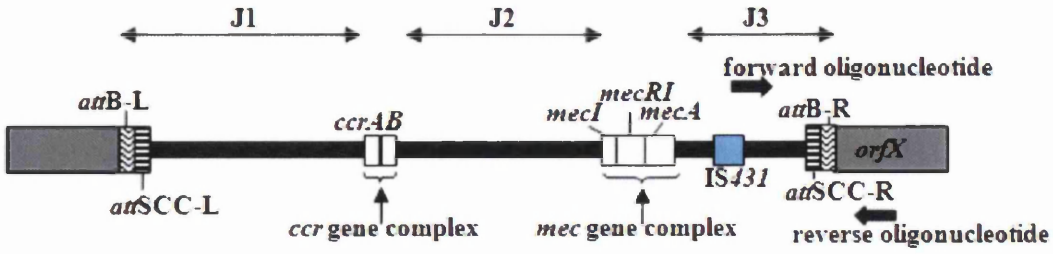


Figure 5.1. Schematic diagram of a SCC*mec* element showing general locations of oligonucleotides for SCC*mec-orfX* amplification (Katayama et al., 2000)

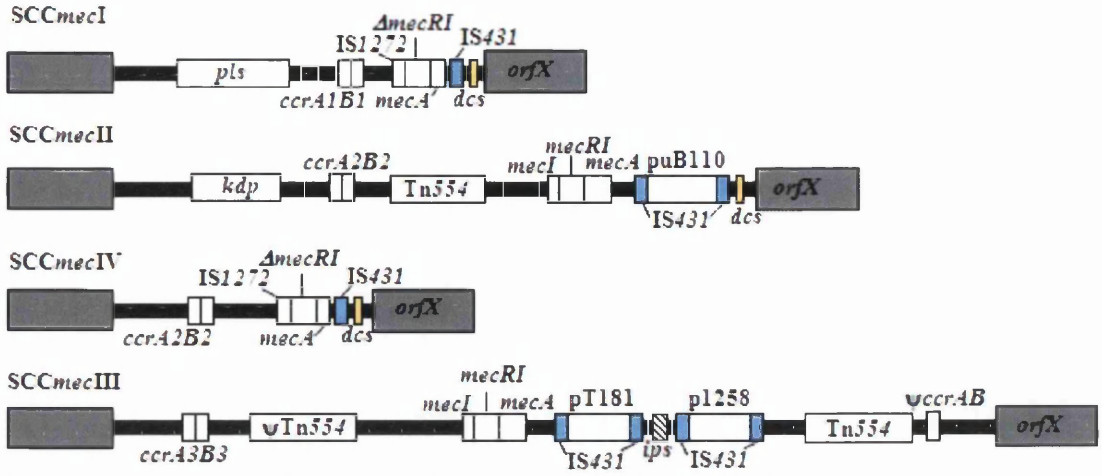


Figure 5.2. Schematic diagrams of SCC*mec* types I-IV (Deurenberg et al., 2007)

This necessitates that SCC*mec-orfX* amplification methods demonstrate the capacity to detect diverse SCC*mec* types. In 2004 the concept of MRSA detection by amplification of the SCC*mec-orfX* junction was published by Huletsky *et al.* (2004), subsequently two additional studies were published by Hagen *et al.* (2005) and Cuny and Witte (2005) and these were advocated for use in routine clinical testing. The Huletsky *et al.* (2004) and Hagen *et al.* (2005) methods are multiplex systems, comprising one forward oligonucleotide for the detection of SCC*mec* type I, II, IVa, IVb, IVc, an additional one for SCC*mec* type III and one reverse nucleotide in *orfX*. Three additional forward oligonucleotides were designed in the Huletsky *et al.* (2004) study, for the detection of eleven MRSA strains which were initially undetected. The Cuny and Witte (2005) method employs only one forward oligonucleotide and a reverse oligonucleotide in *orfX* for the detection of SCC*mec* type I to V elements.

The objective of this study was to compare the three SCC*mec-orfX* amplification methods developed by Cuny and Witte (2005), Hagen *et al.* (2005) and Huletsky *et al.* (2004) for the detection of MRSA in a collection of 561 *S. aureus* isolates. They are referred to as: PCR-A for the Huletsky *et al.* (2004) method, PCR-B for the Hagen *et al.* (2005) method and PCR-C for the Cuny and Witte (2005) method. The methods were optimised with reference strains prior to comparative analysis.

5.1 Results

5.1.1 Effects of the modified PCR annealing temperatures on the detection of reference strains and clinical isolates

The bacterial reference strains employed in this study were: NCTC10442 (SCC*mec* type I/ accession number AB033763); N315 (SCC*mec* type II/ accession number D86934) and 85/2082 (SCC*mec* type III/ accession number AB037671), as described by Ito *et al.* (2001). MRSA strains: CA05 (SCC*mec* type IVa/ accession number AB063172) and JCSC1978 or 8/6-3P (SCC*mec* type IVb/ accession number AB063173) as described by Ma *et al.* (2002). MR108 or 81/108 (SCC*mec* type IVc/ accession number AB096217) as described by Ito *et al.* (2003) and WIS or JCSC3624 (SCC*mec* type V/ accession number AB121219) as described by Ito *et al.* (2004). The Oxford MSSA reference strain and two clinical MSSA isolates were also included as negative controls.

Initially the annealing temperatures were maintained at those stated in the original published methods: 60°C for PCR-A, 56°C for PCR-B and PCR-C. At these conditions MRSAI to IVa,b,c strains were successfully amplified with the PCR-A and PCR-B methods and the MSSA isolates were not amplified (see Table 5.1). Thus the initial conditions were retained for these two methods. Similarly with the PCR-C method, MRSAI to IVa,b,c strains were also amplified and no MSSA were amplified. The MRSAV strain (SCC*mec*V/AB121219/WIS) had also been included in the original method and was successfully amplified. Even though the same strain was employed in this study amplification was not achieved on repeated attempts at 56°C and 55°C (see Table 5.1). Amplification was only achieved when the annealing temperature was reduced to 54°C and the total number of PCR cycles was extended from 45 to 50 cycles. However the average C_t value (C_t=38) of the MRSAV strain was considerably higher than average C_t value (C_t=28) of the MRSAI to IVa,b,c strains. At these conditions the MSSA isolates were also amplified (average C_t value of 40) (see Figure 5.3), so for subsequent PCR-C runs an annealing temperature of 55°C was maintained and the Oxford MSSA reference strain, included as the negative control, also served as a cut-off for possible amplifications with high C_t values. Therefore if the Oxford MSSA strain was amplified, all isolates that were amplified at C_t values equal to or greater than the C_t value of the Oxford strain were considered to have negative results i.e. not MRSA. If the Oxford strain was not amplified, all amplified isolates were considered to have positive results i.e. were considered MRSA.

Table 5.1. Ct values of MRSA reference strains and MSSA isolates with *mecA*, $femA_{SA}$, PCR-A, PCR-B and PCR-C assays

Strain	$femA_{SA}$	*mecA*	PCR-A (60°C)	PCR-B (56°C)	PCR-C (55°C)
	Av. $C_t \pm$ SD	Av. $C_t \pm$ SD	Av. $C_t \pm$ SD	Av. $C_t \pm$ SD	Av. $C_t \pm$ SD
MRSAI	19.9 ± 0.0	21.5 ± 0.1	22.2 ± 0.5	27.8 ± 0.1	30.3 ± 0.1
MRSAII	22.3 ± 0.4	21.5 ± 0.1	25.6 ± 0.1	34.0 ± 0.5	30.3 ± 0.6
MRSAIII	21.6 ± 0.0	22.1 ± 0.1	25.7 ± 0.0	34.9 ± 0.2	30.4 ± 0.4
MRSAIVa	22.1 ± 0.1	23.8 ± 0.2	27.0 ± 0.3	32.0 ± 0.0	29.1 ± 0.1
MRSAIVb	34.9 ± 0.4	22.4 ± 0.1	25.7 ± 0.4	31.8 ± 0.8	28.6 ± 0.3
MRSAIVc	17.1 ± 0.2	17.6 ± 0.5	19.4 ± 0.2	23.6 ± 0.4	23.3 ± 0.1
MRSAV	22.9 ± 0.4	22.7 ± 0.1	--	--	--
Oxford MSSA	21.6 ± 0.4	--	--	--	--
MSSA 1	21.3 ± 0.2	--	--	--	--
MSSA 2	21.4 ± 0.1	--	--	--	--

Key: Oxf. Oxford; C_t-cycle threshold; Av.-average; SD-standard deviation; --not amplified; Av. and SD values calculated from duplicate results

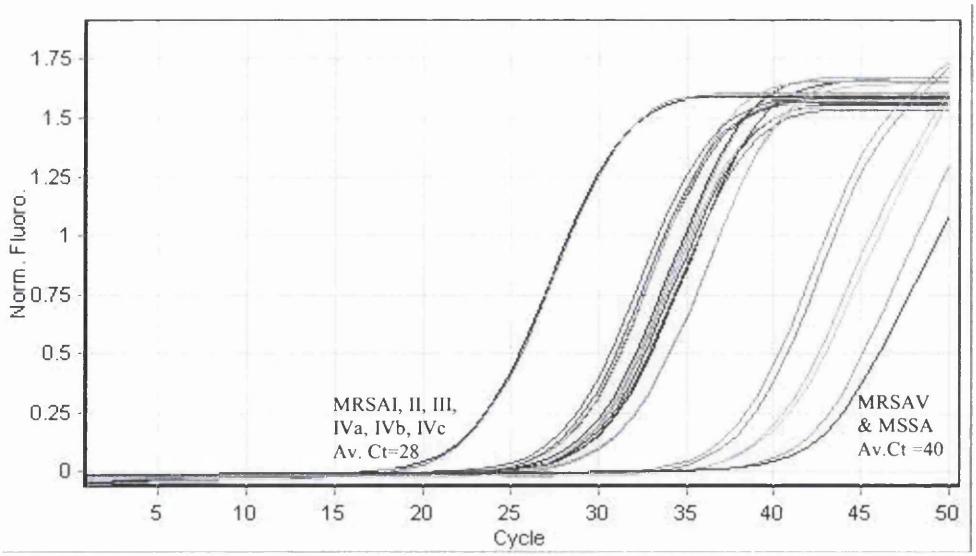


Figure 5.3. Amplification curves of MRSAI, II, III, IVa, IVb, IVc verses MRSAV and MSSA with PCR-C at annealing temperature of 54°C

5.1.2 Alignment of oligonucleotides on SCC*mec* sequences

To determine the probable cause of the late amplification of the MRSAV WIS strain and the MSSA isolates, a search of the location of the PCR-C oligonucleotides on published SCC*mec* sequences was performed with a National Centre for Biotechnology Information (NCBI) BLAST search tool. The forward oligonucleotide (5'-TATGATATGCTTCTCC-3') aligned precisely to SCC*mec* type II and IV sequences but with SCC*mec* type I and III there was one mismatch and with SCC*mec*V, there were two mismatches (see Table 5.2). In AB033763/SCC*mec*I, the thymidine to adenine (T→A) substitution at position nine from the 3' end, presents an internal T:T mismatch, whereas with AB037671/SCC*mec* type III the thymidine to cytosine (T→C) substitution presents an internal T:G mismatch. In SCC*mec*V sequences the two T→C substitutions, present two internal T:G mismatches. Evidently the forward PCR-C oligonucletide also aligned to two MSSA sequences: AP009351/ *S.aureus* Newman and AJ938182/ *S. aureus* RF122, with sequences identical to the SCC*mec* type V sequences (see Table 5.2).

Table 5.2. PCR-C forward oligonucleotide on SCC*mec* type I to V sequences

Accession number and SCC*mec* type	**Sequence**	**Position**
PCR-C forward oligonucleotide	TATGATA**T**GC**T**TCTCC	--
D86934 (SCC*mec*II)/ N315	TATGATATGCTTCTCC	57641 - 57656
AB063172 (SCC*mec*IVa)/ CA05	TATGATATGCTTCTCC	25210 - 25225
AB063173 (SCC*mec*IVb)/ JCSC1978	TATGATATGCTTCTCC	21207 - 21222
AB033763 (SCC*mec*I)/ NCTC10442	TATGATA**A**GCTTCTCC	38855 - 38870
AB037671 (SCC*mec*III)	TATGATA**C**GCTTCTCC	67782 - 67797
AB121219 (SCC*mec*V)/ WIS	TATGATA**C**GC**C**TCTCC	892 - 877
AB478780 (SCC*mec*V)/ JCSC 5952	TATGATA**C**GC**C**TCTCC	42073 - 42058
AP009351 (*S. aureus* Newman)	TATGATA**C**GC**C**TCTCC	34392 - 34377
AJ938182 (*S. aureus* RF122)	TATGATA**C**GC**C**TCTCC	34166 - 34151

A comparison of the location of the oligonucleotides of all three SCC*mec-orfX* assays on the SCC*mec* type I to IVa sequences was also conducted. Within the sequences of SCC*mec* type I, II and IV, the forward oligonucleotides of PCR-A and PCR-B are located in close proximity and they overlap in the sequence of SCC*mec* type III (see Figure 5.4). These oligonucleotides are also located upstream of the SCC*mec-orfX* junction, in contrast to the PCR-C forward oligonucleotide which is located at the SCC*mec-orfX* junction, partially overlapping into *orfX*.

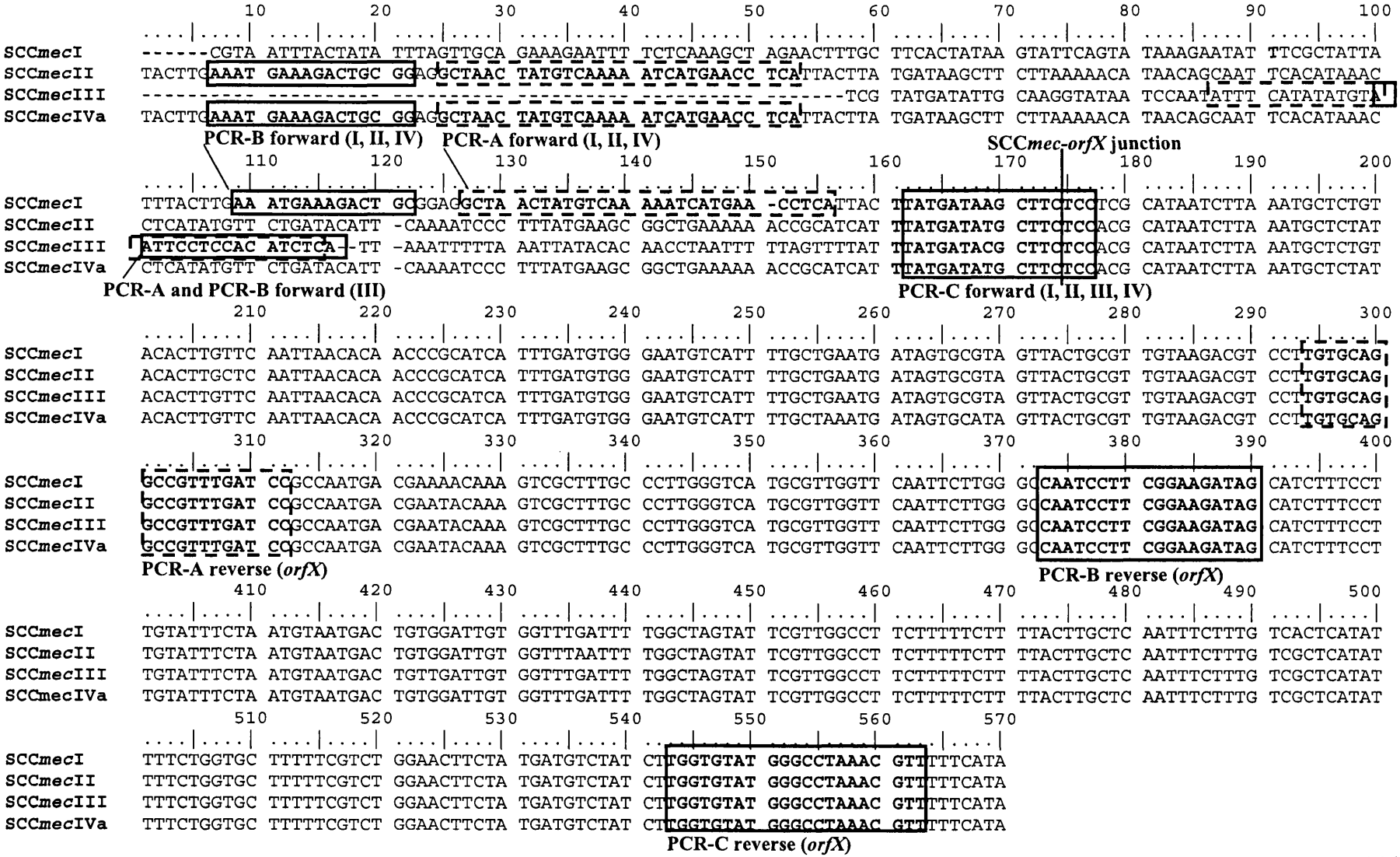


Figure 5.4. Location of the oligonucleotides of PCR-A, PCR-B and PCRC on SCC*mec* type I to IVa sequences (adapted from Hagen *et al.* 2005). Black boxes, location of PCR-B and PCR-C oligonucleotides; dashed black boxes, location of PCR-A oligonucleotides.

The MRSAV WIS reference strain was not amplified with the PCR-A and PCR-B methods. In order to verify that this was not due to possible suboptimal test conditions, the location of the forward and reverse oligonucleotides of these methods on the SCC*mec* type V/AB121219 sequence of the MRSAV WIS strain was investigated with the NCBI BLAST search tool. Since the PCR-A and PCR-B methods are both multiplex PCRs, all of the forward primers were investigated and none of these aligned to the SCC*mec* type V/ AB121219 sequence. However the reverse primer did align to a location in *orfX* (see Table 5.3). Nevertheless since none of the forward primers aligned to the sequence, the amplification of a PCR product was not feasible. This demonstrated that the oligonucleotides of the PCR-A and PCR-B methods were not specific for the amplification of the MRSAV WIS SCC*mec* type V sequence. On the other hand the oligonucleotides of the PCR-A and PCR-B methods did align to two other SCC*mec* type V sequences (see Table 5.3):

i) SCC*mec* type V (5C1)/ AB373032, carried by the JCSC6082 strain

ii) SCC*mec* type V/ AB478780, carried by the JCSC5952 strain.

However on these sequences both the forward and reverse primers were located in *orfX*. Therefore because the forward primer was located in *orfX* and not at the right extremity of SCC*mec*, a PCR product from these strains would be non-specific as it would be an *orfX* sequence and not the required SCC*mec-orfX* sequence (see Table 5.3).

Table 5.3. PCR-A and PCR-B oligonucleotides on SCC*mec* type V sequences

Strain	Accession number/ SCC*mec* type	Oligonucleotide	Position	Locus
MRSAV WIS	AB121219/SCC*mec* type V (5C2)	PCR-A DM11 (reverse)	742-760	*orfX*
		PCR-B DM5 (reverse)	664-681	
JCSC6082[a]	AB373032/ SCC*mec* type V (5C1)	PCR-A DM7 (forward)	681-653	*orfX*
		DM11 (reverse)	459-477	
		PCR-B DM4 (forward)	668-652	*orfX*
		DM5 (reverse)	381-398	
JCSC5952[b]	AB478780/ SCC*mec* type V	PCR-A DM7 (forward)	857-829	*orfX*
		DM11 (reverse)	635-653	
		PCR-B DM4 (forward)	844-828	*orfX*
		DM5 (reverse)	557-574	

[a] JCSC6082 MRSA strain described by Berglund *et al.*, 2008
[b] JCSC5952 MRSA strain (Japanese CA-MRSA) sequence submitted from unpublished work.

5.1.3 PCR efficiency and detection limit of the *mecA*, *femA*$_{SA}$ and *SCCmec-orfX* assays

The efficiency of the PCR assays was determined by generating standard curves from serial dilutions (neat to 10^{-5}) of a MRSAI suspension and applying the slope of the curves to the reaction efficiency formula: $[-1 + 10^{(-1/\text{slope})}] \times 100$. Values of 90-110% (slope -3.1 to -3.6) illustrate a doubling of the PCR product at each cycle and a slope of -3.322 indicates 100% efficiency. The MRSAI standard curves of the *mecA* and *femA*$_{SA}$ PCRs had slopes of: -3.8 (81.5%) and -3.1 (110%) respectively (see Figure 5.5a and b). The slopes of PCR-A, PCR-B and PCR-C methods were: -3.75 (84.79%), -3.76 (84.48%) and -3.54 (91.64%) respectively, indicating that the *femA* and PCR-C assays had the highest efficiency (see Figure 5.6a, 5.5b and 5.5c). Colony counts showed that the neat suspension of the MRSAI strain had 1.23×10^8 CFU/ml and amplification of MRSAI was achieved up to the 10^{-5} dilution for the three SCC*mec-orfX* methods. Since 2 µl was used as the template for the PCRs, this corresponded to a detection limit of 2.5 CFU/reaction for the three methods.

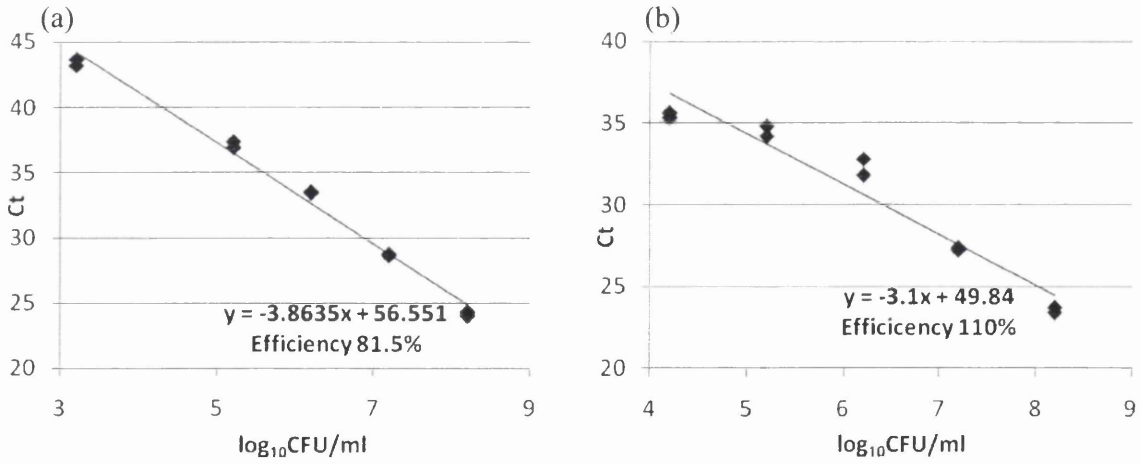
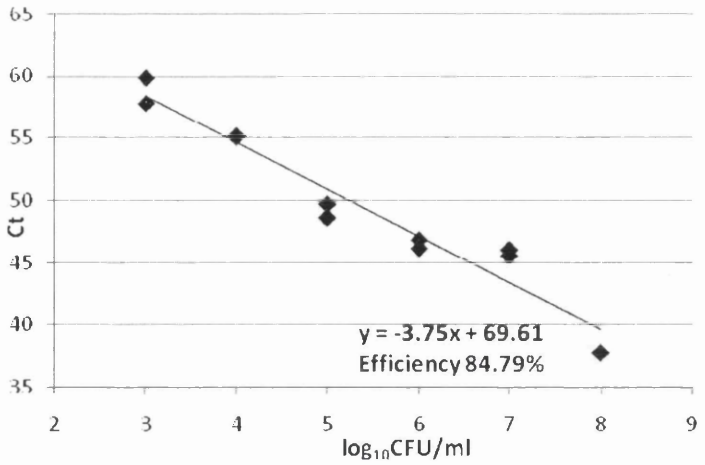
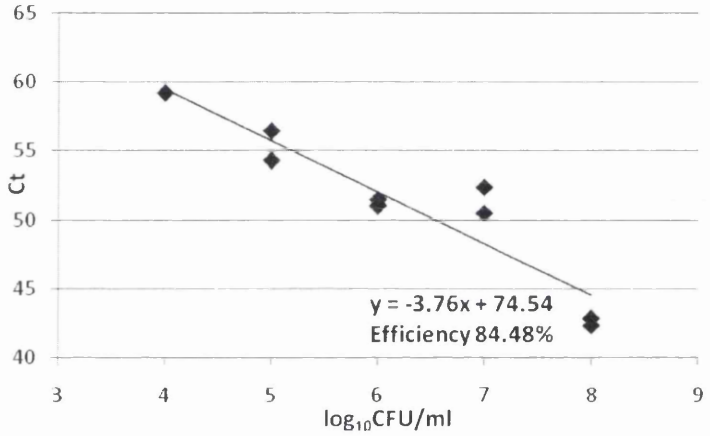


Figure 5.5. Standard curve of MRSAI with the *mecA* and *femA*$_{SA}$ PCR assays

(a)



(b)



(c)

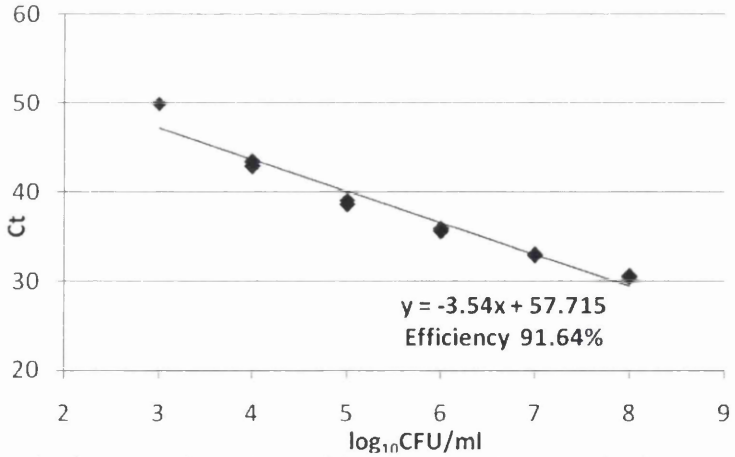


Figure 5.6. Standard curve of MRSAI with PCR-A, PCR-B and PCR-C

5.2 Discussion

The three SCC*mec-orfX* assays PCR-A, PCR-B and PCR-C, successfully detected MRSAI, II, III, IVa,b,c reference strains, which was comparable to the results attained in the original assays (Cuny and Witte, 2005, Hagen et al., 2005, Huletsky et al., 2004). The MRSAV WIS strain had also been detected in the original study of PCR-C but in this study the initial attempts to amplify this strain were unsuccessful. At a lower annealing temperature the MRSA WIS strain was detected but with the simultaneous amplification of the MSSA isolates, suggesting that the observed amplification was due to non specific amplification.

This was further investigated by examining the alignment of the forward PCR-C oligonucleotide to published SCC*mec* sequences which revealed two T:G mismatches within the SCC*mec* type V (AB121219 and AB478780) sequences. Mismatches can hinder efficient binding of the oligonuceotide to the template and certain mismatches at the 3' end can deter the polymerase from adding nucleotides to the terminus. Notably A:G (purine: purine) and C:C (pyrimidine:pyrimidine) mismatches at the 3'end, can have up to a 100-fold reduction on the PCR product yield (Kwok et al., 1990). However in this study, the type and internal location of the T:G mismatches (and also the T:T mismatch observed in SCC*mec* type I) have minimal adverse effect on the amplification of the target sequence (Kwok et al., 1990). Nevertheless it is probable that the combination of the initial higher annealing temperatures (56°C and 55°C) and the mismatches on the SCC*mec* type V sequence was initially a deterrent for robust amplification. As the conditions were made less stringent by lowering the annealing temperature (54°C), amplification of the SCC*mec* type V target sequence was achievable. However the PCR-C forward oligonucleotide also aligns to published MSSA sequences: AP009351/ *S.aureus* Newman and AJ938182/ *S. aureus* RF122, indicating that the PCR-C method could also result in the amplification of these MSSA strains. Similarly it is highly likely that the detection of MSSA isolates in this study was due to the amplification of homologous PCR-C target sequences in these isolates. The presence of the PCR-C target sequences in MSSA strains casts some uncertainty on the suitability of this assay for the exclusive detection of MRSA strains in clinical specimens. Furthermore in this study the template DNA was maintained at a relatively standard concentration i.e. 2-3 colonies of the isolates were consistently used in

preparation of the template DNA. Therefore in the PCR-C assay the late Ct values could be discerned as positive or negative results based on the Ct value of the negative control. However in clinical specimens the concentration of the target DNA is variable and it may be difficult to make a clear distinction between the results.

The MRSAV WIS reference strain was also not detected with the PCR-A and PCR-B methods. An evaluation of the location of the oligonucleotides on the SCC*mec* type V/ AB121219 sequence demonstrated that none of the forward oligonucleotides of these two methods aligned to this sequence and thus amplification was not feasible. However there was some indication that non-specific amplification of a sequence in *orfX* was possible with two other MRSAV strains, the JCSC6082 strain and the JCSC5952 strain. The sequence of the latter strain was submitted to GenBank from unpublished work and the strain seems to be a Japanese CA-MRSA strain whereas, the former strain was originally isolated in Sweden, a country with a very low prevalence rate of MRSA (<1%) (Berglund et al., 2008). Evidently these MRSAV strains appear to be rare so the likely occurrence of their detection amongst UK clinical strains is low. The JCSC6082 strain harbours a variant SCC*mec* type V element and the clinical history of this strain is interesting. It was originally isolated in Sweden in 2002 from the subcutaneous abdominal wall abscess of a previously healthy 42-year-old woman (Berglund et al., 2008). The abscess was initially considered to be a rare community-acquired infection but the patient had undergone abdominal surgery 24 years earlier and within the present abscess cavity remnants of a suture were found (Berglund et al., 2008). Therefore it was suspected that the JCSC6082 strain had possibly persisted in dormancy, embedded in a biofilm for more than 20 years and was only identified due to the occurrence of the abscess. This unusual strain was found to contain a SCC*mec* element with the *ccrC* gene complex (type 5) and a class C1 *mec* gene complex (Berglund et al., 2008). This was a unique combination because even though the *ccrC* gene is also present in the SCC*mec* type V element of the MRSAV WIS strain, the class C1 *mec* complex differs from class C2 *mec* complex which is present in the MRSAV WIS strain. In the class C1 *mec* complex the orientation of IS*431* inserted downstream of *mecA* is in the opposite orientation to that found in class C2 *mec* complex. Since the class C1 *mec* had only been previously identified in methicillin-resistant *Staphylococcus haemolyticus* strain SH631 (Katayama et al., 2001), this was the first description of a class C1 *mec* complex in an MRSA strain (Berglund et al., 2008).

A comparison of the locations of the oligonucleotides of the three PCR-A, PCR-B and PCR-C assays on published SCC*mec* type I to IV sequences showed that the oligonucleotides of PCR-A and PCR-B were in close proximity. This suggests that these assays could have similar diagnostic capacity. Also the PCR-A and PCR-B oligonucleotides are located further upstream of the SCC*mec-orfX* junction than the forward oligonucleotide of the PCR-C assay. In fact the PCR-C forward oligonucleotide is located right at the SCC*mec-orfX* junction and partially overlaps into *orfX*. Seemingly the oligonucleotides of PCR-A and PCR-B are better located for the amplification of a sequence comprising both the right extremity of SCC*mec* and a region in *orfX*, whereas the location of the PCR-C forward oligonucleotide could potentially enable the amplification of a sequence in *orfX* alone, causing the amplification of both MRSA and MSSA isolates.

In this study, the initial results indicated that all three SCC*mec-orfX* assays detected MRSAI, II, III, IVa, IVb and IVc strains. However, the amplification of MSSA isolates, the presence of mismatches and the position of the forward oligonucleotide in published SCC*mec* sequences, questions the adequacy of PCR-C for robust detection of MRSA. However a more comprehensive evaluation of the diagnostic performance of the PCR-A, PCR-B and PCR-C SCC*mec-orfX* methods was performed with a larger collection of *S. aureus* isolates (n=561) and the results are presented and discussed in chapter 6.

Chapter Six

The diagnostic performance of three SCC*mec-orfX* amplification methods

6.0 Introduction

MRSA is endemic in hospitals in the UK and the reduction of MRSA infections is a priority in this country. The UK Health Protection Agency (HPA) voluntary reporting surveillance data of England, Wales and Northern Ireland, has shown a reduction in the prevalence of MRSA over the past decade. In 1999 MRSA accounted for approximately 37.4% of *S. aureus* bacteraemia however by 2008 this rate had reduced to 24.4%. Mandatory surveillance data has shown that the rate of MRSA bacteraemia reports has also declined over the past year i.e. for April 2009 to March 2010 the rate was 3.7/ 100,000 population, a reduction from 5.7/ 100,000 population in the previous year. Furthermore the recent quarterly report showed that in April to June 2010 the rate further decreased to 3.3/ 100,000 population (http://www.hpa.org.uk/web/HPAwebFile/HPAweb_C/1284473407318).

Individuals who are colonised with MRSA are at greater risk of contracting infections and transmitting MRSA, so it is recommended that patients are screened on or before hospital admission (Jeyaratnam et al., 2008b). MRSA screening is performed on clinical swabs including nasal, axilla, groin and skin break swabs and it is essential that implemented diagnostic tests are accurate, and desirable that they should be rapid, as it is postulated that rapid detection will lead to faster implementation of infection control procedures and possibly a reduction in MRSA transmission. However conventional laboratory protocols are lengthy i.e. 24 to 72 hours, as they typically involve culture-based assays i.e. enrichment broths, selective media and chromogenic media. In contrast, PCR tests have shorter execution periods (2-4 hours) and thus they could be ideal screening tests (Snyder et al., 2010, Jeyaratnam et al., 2008b, Paule et al., 2007, van Hal et al., 2007).

The PCR-A, PCR-B and PCR-C SCC*mec-orfX* amplification methods discussed in chapter 4, could be suitable for MRSA screening as they are rapid (less than two hours) and they detect diverse MRSA. However a comprehensive evaluation of the diagnostic performance of the tests has to be undertaken prior to their implementation. In this study the three methods were evaluated with pure cultures of *S. aureus* isolates.

To hasten the PCR set up stage, crude DNA extracts (supernatant of a heated bacterial suspension made from 2-3 colonies suspended in sterile distilled H_2O) were used as the template DNA in all the assays.

The definition of positive and negative results was based on the results of chapter 4. With PCR-A and PCR-B the negative control had been consistently undetected therefore all isolates that generated an amplification signal were regarded as MRSA and those that did not generate an amplification signal were regarded as MSSA. Since with the PCR-C method there was possible late amplification of MSSA isolates, the Ct value of the negative control was used as the cut off for negative results. Therefore only isolates with Ct values less that the Ct value of the negative control were regarded as MRSA whereas those with values greater than Ct value of the negative control were regarded as MSSA.

The objective of this study was to compare the diagnostic performance of the PCR-A, PCR-B and PCR-C methods by determining the sensitivity, specificity and predictive values with a collection of 561 *S. aureus* isolates. The false negative and false positive results were further investigated to determine the probable causes of these results.

6.1 Results

6.1.1 The diagnostic performance of the SCC*mec-orfX* assays

Five hundred and sixty one *S. aureus* isolates were screened with the reference method (*mecA*, $femA_{SA}$ and $femA_{SE}$ PCRs) and the $femA_{SA}$ gene was detected in all the isolates whereas $femA_{SE}$ was not detected in any isolates. The *mecA* gene was detected in 137 isolates and undetected in 424 isolates, therefore out of the 561 *S. aureus* isolates, 24.4% were MRSA and 75.6% were MSSA. After the exclusion of replicate isolates from consecutive visits (occurring within 3 months), 118 (22.7%) MRSA and 401 (77.3%) MSSA were retained for the comparison of the PCR-A, PCR-B and PCR-C methods. The average Ct values of the MRSA (n=118) for the $femA_{SA}$ and *mecA* PCRs were: 19.3 and 21.1 respectively.

Out of the 118 MRSA, 114 (96.6%) isolates were amplified with the PCR-A method and of the 401 MSSA isolates 394 (98.3%) were not amplified. The values for the PCR-B method were identical to those of the PCR-A method. With the PCR-C method, out of the 118 MRSA, 113 (95.8%) were amplified and of the 401 MSSA isolates 392 (97.8%) were not amplified (see Table 6.1). The average Ct values of the MRSA (n=118) for the PCR-A, PCR-B and PCR-C assays were: 26.4, 28.9 and 30.5 respectively.

Table 6.1. The number of *mecA* positive and *mecA* negative *S. aureus* isolates detected with the three SCC*mec-orfX* assays.

	***mecA* PCR**	
SCC*mec-orfX* PCR	**Positive (n=118)**	**Negative (n=401)**
PCR-A	114 (96.6%)	394 (98.3%)
PCR-B	114 (96.6%)	394 (98.3%)
PCR-C	113 (95.8%)	392 (97.8%)

Table 6.2. Average and median Ct values of the MRSA (n=118) for the PCR assays

PCR	**Ct values**	
	Av. Ct + SD	**Median**
$femA_{SA}$	19.3 ± 2.9	19.8
mecA	21.1 ± 3.5	21.6
PCR-A	26.4 ± 5.7	25.7
PCR-B	28.9 ± 5.2	28.9
PCR-C	30.5 ± 5.3	30.0

Ct- cycle threshold; Av.-Average; SD- standard deviation

Even though the results of the three SCC*mec-orfX* assays were largely comparable to the *mecA* PCR, there were apparent discrepancies. Four *mecA* positive *S. aureus* isolates: 17, 18, 19 and 20 were not amplified with the PCR-A and PCR-B methods. Similarly, with the PCR-C method, four *mecA* positive *S. aureus*: 21, 22, 18, and 7 were not amplified and one additional isolate (isolate 8) had a high Ct value of 44.6 which was regarded as a negative result based on the Ct value (42.6) of the negative control in that particular run. Isolate 18 was not amplified by all three SCC*mec-orfX* PCRs (see Table 6.3). Although the false negative isolates were undetected with the SCC*mec-orfX* assays, their $femA_{SA}$ and *mecA* PCR Ct values were comparable to the average Ct value of the respective tests i.e. 19.3 and 21.1 (see Table 6.2 and 6.3).

In addition to the false negatives, there were *mecA* negative isolates which were amplified with the three SCC*mec-orfX* assays. Seven isolates: 23, 24, 25, 26, 27, 28 and 16 were amplified with PCR-A and PCR-B. With the PCR-C nine *mecA* negative isolates were amplified: 29, 30, 31, 32, 33, 34, 35, 36 and 16 (isolate 16 was amplified by all three assays). Isolates 32, 34 and 36 had higher Ct values (41.8, 44.8 and 42.9 respectively) than the other isolates and based on the Ct value (41.3) of the negative control, they had negative results (see Table 6.4). Nevertheless they were retained for the further investigations to determine the cause of the amplification of these clinical isolates.

Table 6.3. Ct values of the *mecA* positive and SCC*mec-orfX* negative *S. aureus* isolates with the *mecA*, $femA_{SA}$ PCR and the PCR-A, PCR-B and PCR-C methods

Isolate	*mecA*	$femA_{SA}$	PCR-A	PCR-B	PCR-C
17	21.6	22.4	--	--	25.6
18*	19.8	18.53	--	--	--
19	21.4	18.2	--	--	29.6
20	22.4	20.2	--	--	27.8
21	16.7	21.7	20.6	30.0	--
22	18.8	23.0	21.8	28.9	--
7	25.6	17.6	25.7	24.8	--
8	15.3	14.1	23.8	30.9	44.6[a]

-- not amplified

*Isolate 18 not amplified in all three assays

[a] Isolate 8 was negative with PCR-C because Ct value 44.6 > Ct value (42.4) of the detected negative control in run.

Table 6.4. Ct values of the *mecA* negative and SCC*mec-orfX* positive *S. aureus* isolates with the *mecA*, $femA_{SA}$ PCR and the PCR-A, PCR-B and PCR-C methods

Isolate	*mecA*	*femA*	PCR-A	PCR-B	PCR-C
23	--	24.4	18.6	17.2	--
24	--	19.7	19.6	29.5	--
25	--	21.9	24.6	32.5	--
26	--	22.7	28.3	27.4	--
27	--	13.3	14.6	29.2	--
28	--	25.1	26.0	29.9	--
16*	--	19.9	25.1	31.4	28.1
29	--	15.3	--	--	27.5
30	--	19.8	--	--	28.9
31	--	15.1	--	--	33.6
32	--	19.0	--	--	41.8[a]
33	--	18.8	--	--	33.9
34	--	19.7	--	--	44.8[a]
35	--	16.5	--	--	42.9[a]
36	--	14.9	--	--	32.5

*Isolate 16, amplified in all three assays; -- not amplified

[a]Isolates 32, 34 and 35 negative because Ct values > Ct value (41.3) of detected negative control in run.

Table 6.5. Sensitivity, specificity, PPV and NPV values of SCC*mec-orfX* PCRs of 118 MRSA and 401 MSSA isolates

Performance	PCR-A	PCR-B	PCR-C
% Sensitivity (N)	96.6 (114)	96.6 (114)	95.8 (113)
% Specificity (N)	98.3 (394)	98.3 (394)	97.8 (392)
% PPV[a]	94.2	94.2	92.6
% NPV[b]	99.0	99.0	98.7

%Sensitivity: [SCC*mec-orfX* positives / *mecA* positives] x100

% Specifiticity: [SCC*mec-orfX* negatives / *mecA* positives] x100

%PPV[a]= [True positives/ (True positives + False positives)] x100

%NPV[b]= [True negatives/ (True negatives + False negatives)] x100

The sensitivity results of the PCR-A, PCR-B and PCR-C methods were: 96.6%, 96.6%, 95.8% and the specificity results were: 98.3%, 98.3% and 97.8% respectively. The positive predictive values (PPV) were 94.2%, 94.2% and 92.6% respectively and the negative predictive values (NPV) were: 99.0%, 99.0%, and 98.7% respectively. Therefore the PCR-A and PCR-B methods had slightly higher diagnostic performance results than the PCR-C method (see Table 6.5).

6.1.2 Investigating the *mecA* positive and SCC*mec-orfX* negative isolates

6.1.2.1 Application of purified genomic DNA and effect on Ct values

The SCC*mec-orfX* false negative isolates were further investigated to determine the probable causes of the discrepant results. The initial measure undertaken was to ascertain if the lack of amplification was due to PCR inhibitors in the crude DNA extracts.

Fresh pure cultures were prepared from a single colony and purified genomic DNA was attained with an extraction and purification kit (QIAamp genomic DNAMini Prep, Qiagen) and a 1µl aliquot of the genomic DNA was used (in triplicate) in the repeated *mecA*, $femA_{SA}$ PCRs and the SCC*mec-orfX* assays. Two MRSA reference strains: MRSAII (MRSA252), MRSAIVb (8/6-3P), the MSSA Oxford reference strain and three clinical MRSA (37, 38 and 39) which had previously been successfully amplified, were included in the repeated runs. With the PCR-A and PCR-B methods the four false negative isolates were amplified however there was a distinct disparity between the high Ct values of the false negative isolates (ranging within 33.9 – 36.0 cycles) and those of the reference strains (ranging within 14.9 - 16.9 cycles) (see Figure 6.1 and Table 6.6). In contrast, the Ct values with the *mecA* and $femA_{SA}$ PCRs were low and comparable to those of the MRSA reference and MRSA clinical strains (see Table 6.6).

The presence of PCR inhibitors in the crude extracts was an unlikely cause of the initial lack of amplification because crude extracts were also used with *mecA* and $femA_{SA}$ PCRs and the false negative isolates were amplified at Ct values comparable to those of the MRSA reference and MRSA clinical strains (see Table 6.3). The subsequent amplification of the false negative isolates suggests that the initial lack of amplification was due to a low concentration of DNA in the crude extracts and the use of a higher concentration of DNA resulted in the amplification of the isolates. However the concentration of the extracted genomic DNA was not measured before it was used in the repeated assays, therefore the disparity of the Ct values may have been due to higher concentrations of genomic DNA in the PCR reactions of the MRSA reference strain and lower concentrations in the reactions of the false negative isolates.

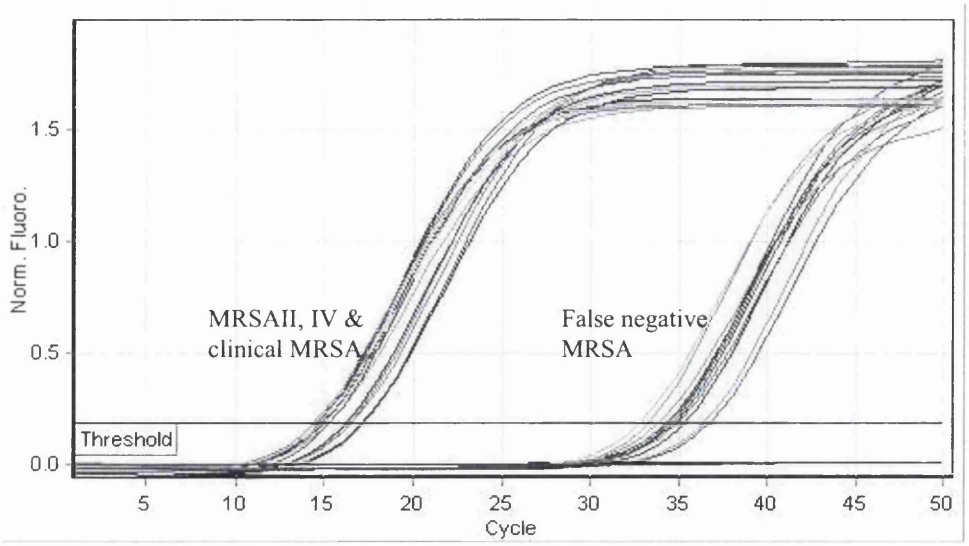


Figure 6.1. PCR-A amplification curves of MRSA reference strains, clinical MRSA and false negative MRSA isolates

Table 6.6. Ct values of MRSA reference and clinical strains and the PCR-A and PCR-B false negative isolates with use of purified genomic DNA in assays

Isolate	*mecA* Av. Ct ± SD	*femA*$_{SA}$ Av. Ct ± SD	PCR-A Av. Ct ± SD	PCR-B Av. Ct ± SD
MRSA II	11.4 ± 0.1	9.3 ± 0.2	14.9 ± 0.2	16.3 ± 0.4
MRSA IVb	12.4 ± 0.4	8.9 ± 0.2	15.5 ± 0.1	16.5 ± 0.3
Oxford MSSA	--	11.0 ± 0.1	--	--
37 (clinical MRSA)	13.1 ± 0.3	10.3 ± 0.2	15.6 ± 0.8	17.0 ± 1.3
38 (clinical MRSA)	13.9 ± 0.3	10.9 ± 0.2	16.9 ± 0.5	18.0 ± 0.2
39 (clinical MRSA)	13.0 ± 0.5	11.5 ± 0.1	16.4 ± 1.3	17.4 ± 0.4
False negative isolates				
17	12.8 ± 0.1	10.7 ± 0.3	33.9 ± 1.0	34.6 ± 0.6
18	12.2 ± 0.2	10.4 ± 0.1	34.9 ± 0.8	35.8 ± 0.7
19	12.0 ± 0.2	9.9 ± 0.1	36.0 ± 1.0	36.4 ± 0.4
20	13.1 ± 0.3	9.9 ± 0.3	34.8 ± 0.2	35.1 ± 0.5

Key: Av.-average; SD-standard deviation; -- not amplified

6.1.2.2 The application of standard concentrations of genomic DNA and the effect on Ct values

To investigate if disparity of the Ct values of the MRSA reference strains and false negative isolates was due to differences in the template DNA concentrations, a standard concentration of crude and purified genomic DNA was used in the assays. For the crude templates, bacterial suspensions were prepared at a standard optical density of 0.3 at 600 nm and 2 µl of the supernatant was used (in triplicate) in the repeat PCR-A and PCR-B assays. For the purified genomic DNA, a concentration of 20 ng/µl was maintained for all the isolates and 1 µl was used (in triplicate) in the repeated PCRs.

With the standardised crude template, the MRSA reference and MRSA clinical strains were amplified but the false negative isolates were not amplified. The standard use of 20 ng/ µl DNA in the assays once again resulted in the false negative isolates amplified at late Ct values in comparison to the MRSA reference and MRSA clinical strains. As previously observed with the *mecA* and $femA_{SA}$ PCRs, the Ct values of the false negative isolates were comparable to those of the MRSA reference and MRSA clinical strains (see Table 6.7).

This indicated that the divergent results were not due to variable concentrations of the DNA template however they may have been due to the polymorphism of the SCC*mec* right extremity region in MRSA strains and resultant differences in the target sequences.

Table 6.7. Ct values of MRSA reference strains, three clinical MRSA and four false negative isolates with use of 20ng/µl of DNA template

Isolate	$femA_{SA}$ Av. Ct ± SD	*mecA* Av. Ct ± SD	PCR-A Av. Ct ± SD	PCR-B Av. Ct ± SD
MRSA II	10.8 ± 0.0	14.5 ± 0.7	14.6 ± 0.2	16.3 ± 0.5
MRSA IVb	10.6 ± 0.0	14.0 ± 0.1	15.4 ± 0.2	16.9 ± 0.9
Oxford MSSA	11.0 ± 0.1	--	--	--
37 (clinical MRSA)	11.6 ± 0.1	13.9 ± 0.3	15.1 ± 0.4	16.4 ± 0.1
38 (clinical MRSA)	11.4 ± 0.2	13.5 ± 0.2	14.9 ± 0.2	16.7 ± 0.5
39 (clinical MRSA)	11.2 ± 0.1	13.6 ± 0.2	15.1 ± 0.2	16.4 ± 0.4
False negatives				
17	11.4 ± 0.0	13.6 ± 0.2	34.7 ± 0.8	35.9 ± 0.5
18	11.4 ± 0.1	13.6 ± 0.2	36.6 ± 0.8	37.7 ± 0.6
19	11.2 ± 0.0	13.6 ±0.2	37.2 ± 0.3	38.7 ± 0.7
20	11.1 ± 0.1	13.4 ± 0.2	35.4 ± 0.4	37.3 ± 0.9

Key: Av.-average; SD-standard deviation; --not amplified

6.1.2.3 Analysis of the sizes and melting temperatures of the PCR-A and PCR-B amplification fragments

To determine if there were differences in the sizes the PCR fragments of the MRSA reference strains and the false negative isolates (17, 18, 19 and 20), the fragments were resolved in a 2% agarose gel. The PCR-A fragment size of the four false negative isolates was approximately 278 bp and with PCR-B, approximately 400 bp. These sizes were comparable to those of the MRSAII and MRSAIVb reference strains with both methods (see Figure 6.2a and 6.2b).

A melt analysis of the PCR fragments showed that the melting temperatures (T_m) of the fragments of the MRSAII and MRSAIVb reference strains were 80.7°C and 80.5°C with PCR-A and 83.2°C and 83.3°C with PCR-B. In the original PCR-B study i.e. by Hagen *et al.* (2005), PCR fragments with T_m values within a ±0.8 range of the positive control T_m were considered to be specific amplification products. Similarly in this study the T_m values of the PCR fragments of the false negative isolates were within the ±0.8 range of the MRSAII and MRSAIVb reference strains in both methods (see Figure 6.3).

These results showed that the PCR fragment sizes of the false negative isolates were comparable to those of the MRSAII and MRSAIVb reference strains. Furthermore the similar T_m values suggested that the sequences of the PCR fragments of the false negative isolates were possibly homologous to those of the MRSAII and MRSAIV reference strains.

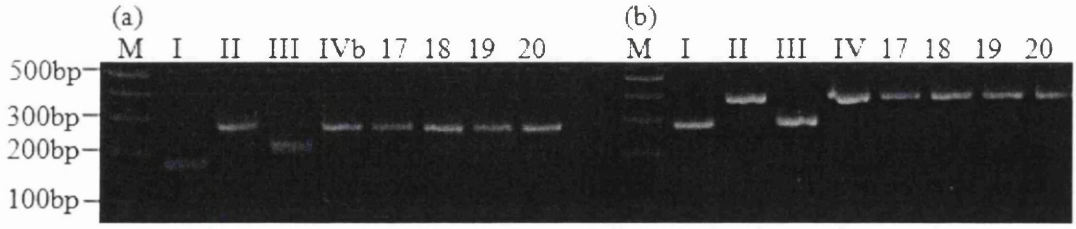


Figure 6.2. PCR fragments of the MRSA reference strains and the false negative isolates with PCR-A (a) and PCR-B (b). M, 100bp molecular marker; I, MRSAI; II, MRSAII; III, MRSAIII; IV, MRSAIVb; 17- 20, isolates 17, 18, 19 and 20.

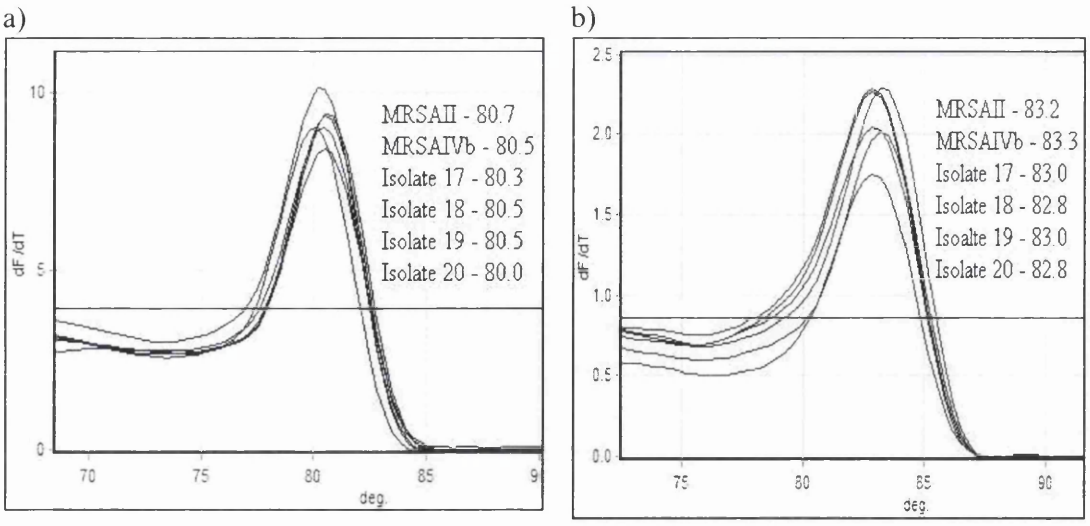


Figure 6.3. Melt curves of the PCR fragments of the MRSA reference strains and the false negative isolates of PCR-A (a) and PCR-B (b).

6.1.2.4 Analysis of nucleotide sequences of the false negative isolates

The PCR fragments of the PCR-A and PCR-B false negative isolates were sequenced (forward and reverse sequences) and aligned to the sequence of the MRSAIVb reference strain. This was performed with a new pair of primers (NB1 and NB2) which flanked those of PCR-A and PCR-B in the SCC*mec-orfX* region. This also enabled the assessment of the primer binding sites of the assays for possible primer:template mismatches which may have hindered optimal annealing of the primers and caused a reduction in the PCR product yield. The MRSAIVb reference strain and three additional clinical MRSA isolates (1, 40 and 41), were also included in the NB1-NB2 PCR. With this PCR the four false negative isolates were still amplified at higher Ct values (34.6 to 36.6) in comparison to the MRSA reference and MRSA clinical strains (14.7 to 15.9) (see Table 6.8 and Figure 6.4).

The alignment of the fragment sequences showed that the false negative isolates only had five nucleotide differences at random locations and there were no nucleotide differences within the primer binding sites of the PCR-A and PCR-B methods. This showed that the target sequences of the false negative isolates were homologous to the target sequence of the MRSAIVb reference strain. Therefore the differences in the Ct values were not due to variable target sequences or primer: template mismatches.

Table 6.8. Ct values of MRSA reference strains, three clinical MRSA and four false negative isolates with NB1 and NB2 PCR.

NB1 - NB2 PCR	
Isolate	**Av. ± SD**
MRSAIVb	14.7 ± 0.5
1 (clinical MRSA)	16.1 ± 0.2
40 (clinical MRSA)	15.9 ± 0.1
41 (clinical MRSA)	15.9 ± 0.1
17	34.6 ± 0.1
18	35.7 ± 0.3
19	36.4 ± 0.4
20	35.4 ± 0.4

Key: Av.-average; SD-standard deviation

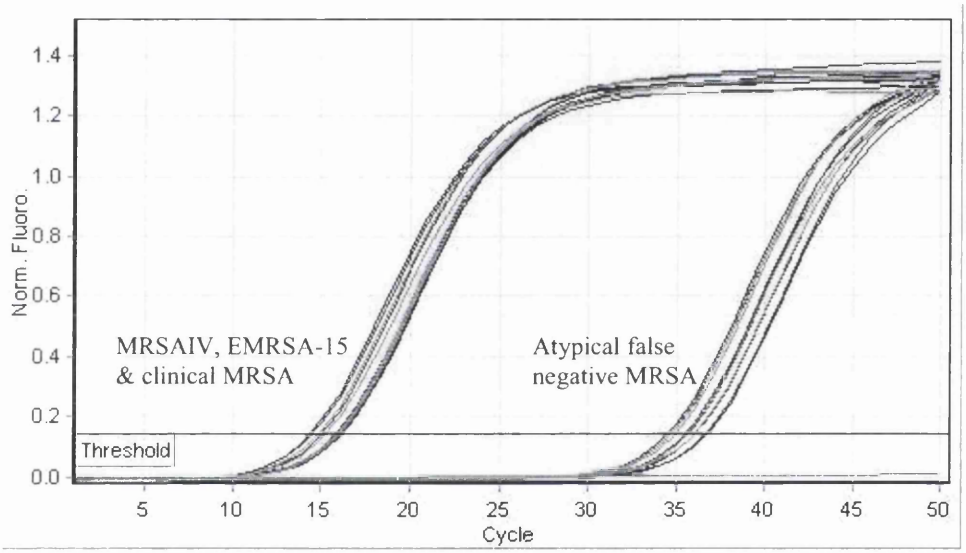


Figure 6.4. NB1 and NB2 PCR amplification curves of MRSAIV, EMRSA-15, clinical MRSA and false negative isolates

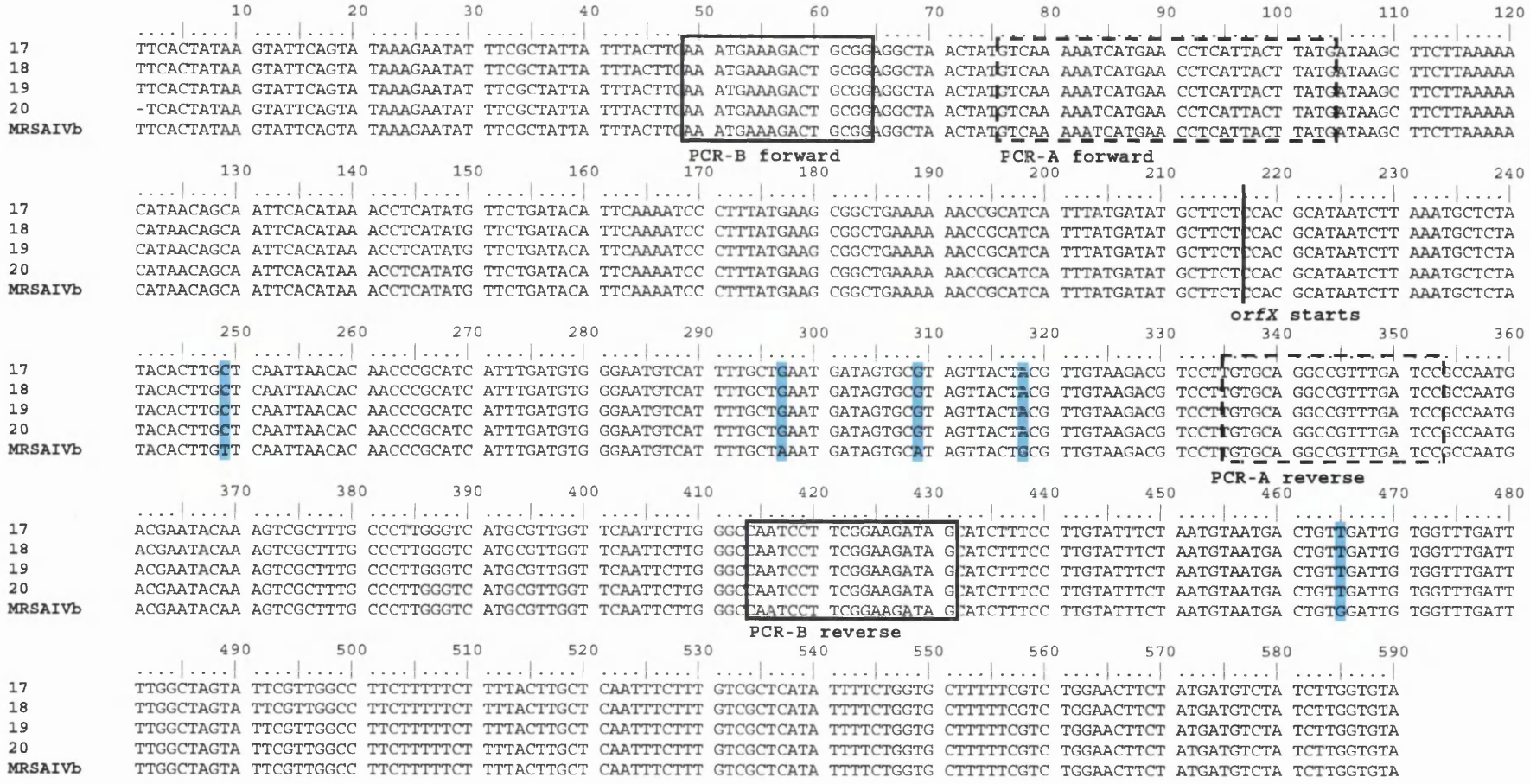


Figure 6.5. Alignment of nucleotide sequences of PCR fragments of the false negative isolates and the MRSAIVb reference strain (Hagen et al., 2005). Solid black boxes, positions of the PCR-B forward and reverse primers; dashed black boxes, positions of the PCR-A forward and reverse primers; blue areas nucleotides differences.

The results of the previous investigations indicated that the disparity of the Ct values was not due to: PCR inhibitors, different PCR fragment sizes, variable target sequences or primer: template mismatches. However a hypothetical cause of the atypical results could be a SCC*mec-orfX* associated deletion in the false negative isolates. The deletion could be inclusive of the binding sites of the forward primers of PCR-A and PCR-B and may occur frequently, resulting in the majority of cells containing the deletion and only a minority of cells carrying the complete SCC*mec-orfX* junction (see Figure 6.6). A low prevalence of the complete target sequences in the false negative isolates would result in later detection, represented by late Ct values with the PCR-A and PCR-B methods.

The frequency of the complete SCC*mec-orfX* junction may be postulated from the Ct values of the *femA*$_{SA}$, *mecA* PCRs and PCR-A (see Table 6.9). The *femA*$_{SA}$ gene is located in the *S. aureus* chromosome and thus represents a genome equivalent. A *femA*$_{SA}$ standard curve (Ct versus $\log_{10}$ CFU / ml) was generated with a 10-fold dilution series of the MRSAI reference strain (presented in chapter 4) and under optimal conditions there would be 10-fold increase of genome equivalents every 3.3 cycles. With the MRSAIVb reference strain and four false negative isolates the *femA*$_{SA}$ and *mecA* PCR Ct values were comparable, indicating that the *mecA* gene was detected in an equal number of genome equivalents in both the MRSAIVb strain and the false negative isolates (see Table 6.9).

However with the PCR-A there was a 20.1 cycle difference between the Ct value of the MRSAIVb strain (Ct 14.7) and the Ct value of the false negative isolate, 17 (Ct 34.7) (see Table 6.9). There was a similar difference in the remaining false negative isolates. With the assumption that 3.3 cycles represent one $\log_{10}$ CFU / ml difference, or a 10-fold difference of genome equivalents, 20.1 cycles would be equivalent to six $\log_{10}$ CFU/ ml difference or 10^6 genome equivalents. Therefore it could be estimated that with the false negative isolates the PCR-A SCC*mec-orfX* target sequence was detected in as few as 10^{-6} genome equivalents. This estimated prevalence of a complete SCC*mec-orfX* junction in the false negative isolates could explain the late amplification of these isolates in comparison to the MRSA reference strains with PCR-A and PCR-B assays.

Table 6.9. Ct values of MRSAIVb and the false negative isolates with the *femA*$_{SA}$, *mecA* and PCR-A

Isolate	Ct values			
	femA$_{SA}$ PCR	*mecA* PCR	PCR-A	Cycle difference
MRSAIVb	10.8	14.0	14.6	
Isolate 17	11.4	13.6	34.7	20.1
Isolate 18	11.4	13.6	36.6	22.0
Isolate 19	11.2	13.6	37.2	22.6
Isolate 20	11.1	13.4	35.4	20.8

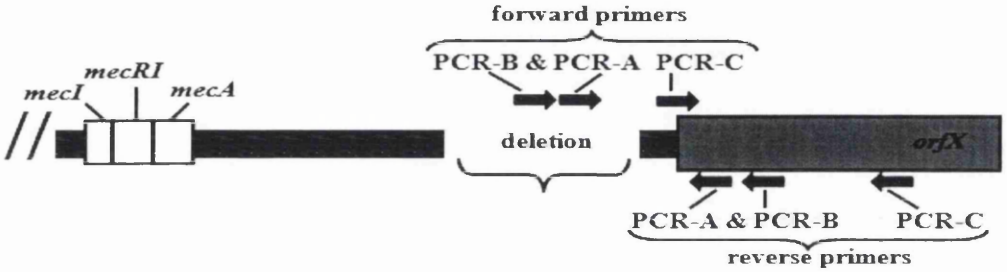


Figure 6.6. Schematic diagram of proposed deletion inclusive of PCR-A and PCR-B forward primer positions at the right extremity of SCC*mec*

6.1.2.5 The false negative isolates of the PCR-C method

Five *mecA* positive *S. aureus* isolates (isolates 21, 22, 18, 7 and 8) were not amplified with the PCR-C method. To determine the cause of these results the steps performed for the false negative isolates of the PCR-A and PCR-B methods were undertaken. To ascertain if PCR inhibitors were present in the crude DNA templates, purified genomic DNA attained with an extraction and purification kit was used alternatively (QIAamp genomic DNAMini Prep, Qiagen). An aliquot (1 µl) of the purified genomic DNA of the false negative isolates was used (in triplicate) in the repeated *mecA*, $femA_{SA}$, PCR-C assays and the MRSAII and IVb reference strains and three MRSA clinical strains which had previously amplified successfully were also included in the repeated tests.

With the use of purified genomic DNA in the repeated PCR-C assay, regrettably the MSSA reference strain (negative control) was amplified at much earlier cycles i.e. average Ct value, 25.5 (see Table 6.10). This value was greater than the Ct values of the MRSAII (20.3), MRSAIVb (20.7) reference strains and MRSA clinical strains (20.9, 20.6 and 21.2 respectively). Two of the five false negative isolates (isolate 22 and isolate 18) were amplified at low Ct values i.e. 20.9 and Ct 21.1 which were lower than the value of the negative control and comparable to the values of the MRSA reference and MRSA clinical strains however the remaining three isolates: 21, 7 and 8, were amplified at considerably higher Ct values i.e. 34.1, 37.1 and 38.8 respectively (see Table 6.10). With the *mecA* and $femA_{SA}$ PCRs the Ct values of all the false negative isolates were comparable to those of the MRSA reference and MRSA clinical strains.

Apart from the apparent amplification of the MSSA reference strain, the results of the false negative isolates in the repeated PCR-C assay were reminiscent of the results of the PCR-A and PCR-B false negatives. There was a disparity between the lower Ct values of the MRSA reference strains, MRSA clinical strains and isolates 22 and 18 versus the higher Ct values of isolates 21, 7 and 8 (see Figure 6.7). Therefore the basis of this phenomenon was thought to be similar to the proposed cause of the PCR-A and PCR-B false negative Ct results.

Table 6.10. Ct values of reference strains, clinical MRSA strains and PCR-C false negative isolates with use of purified genomic DNA as template in assay

Isolate	*mecA*	*femA*$_{SA}$	PCR-C
	Av. Ct ± SD	Av. Ct ± SD	Av. Ct ± SD
MRSA II	11.4 ± 0.1	9.3 ± 0.2	20.3 ± 0.2
MRSA IVb	12.4 ± 0.4	8.9 ± 0.2	20.7 ± 0.3
Oxford MSSA	--	11.0 ± 0.1	25.5 ± 0.1
37 (clinical MRSA)	13.1 ± 0.3	10.3 ± 0.2	20.9 ± 0.6
38 (clinical MRSA)	13.9 ± 0.3	10.9 ± 0.2	20.6 ± 0.2
39 (clinical MRSA)	13.0 ± 0.5	11.5 ± 0.1	21.2 ± 0.5
False negatives			
21	11.6 ± 0.2	9.8 ± 0.1	34.1 ± 0.2
22	13.0 ± 0.2	11.4 ± 0.2	20.9 ± 0.8
18	12.2 ± 0.2	10.4 ± 0.1	21.1± 0.4
7	14.9 ± 0.3	9.0 ± 0.3	37.1 ± 0.6
8	13.1 ± 0.1	11.0 ± 0.3	38.8 ± 0.4

Key: Av.- average; SD-standard deviation;--not amplified

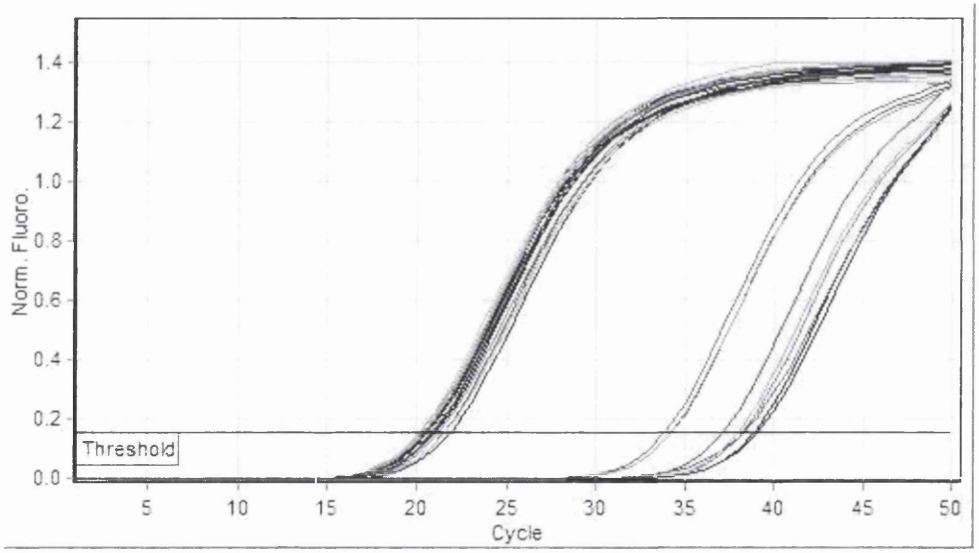


Figure 6.7. PCR-C amplification curves of MRSA reference strains, clinical MRSA and false negative MRSA isolates

6.2 Investigating the *mecA* negative and SCC*mec-orfX* positive isolates

6.2.1 Application of purified genomic DNA and effect on Ct values

The PCR-A and PCR-B assays had seven false positives and the PCR-C assay had nine, one isolate (isolate 16) was a false positive in all three assays. To ascertain if these results were due to contamination of the initial MSSA cultures with MRSA, fresh cultures of the MSSA isolates were sub cultured from a single colony. Genomic DNA was obtained from the cultures with an extraction and purification kit (QIAamp genomic DNAMini Prep, Qiagen) and the *mecA*, $femA_{SA}$ and SCC*mec-orfX* PCRs were repeated with 1µl (in triplicate) of the genomic DNA. The MRSAII, MRSAIVb reference strains and two clinical MRSA (37 and 39) were included in the repeat runs.

All of the PCR-A and PCR-B false positive isolates were amplified at Ct values which were comparable to the MRSA reference and MRSA clinical strains (see Table 6.11). These results illustrated that although these false positive isolates were *mecA* negative they amplified with similar efficiency to the MRSA strains (see Figure 6.8). With the PCR-C method, disappointingly the MSSA reference strain once again amplified at low cycles i.e. average Ct, 23.0, which was comparable to the Ct values of the MRSA reference strains (see Table 6.12). Unlike the Ct values of the PCR-A and PCR-B false positives, the Ct values of the PCR-C false positive isolates were more variable. Isolates 29, 30, 31, 33 and 16 had Ct values which were comparable to the MRSA reference strains but the Ct values of isolates 32, 34 and 36 were considerably higher (>30).

Table 6.11. Ct values of the PCR-A and PCR-B false positive isolates when purified template was used for the SCC*mec-orfX* PCRs

Isolate	$femA_{SA}$ Av. Ct + SD	PCR-A Av. Ct + SD	PCR-B Av. Ct + SD
MRSAII	9.3 ± 0.2	14.3 ± 0.3	18.7 ± 1.5
MRSAIVb	9.2 ± 0.1	14.1 ± 0.3	19.2 ± 0.5
Oxford MSSA	11.0 ± 0.1	--	--
37 (clinical MRSA)	10.8 ± 0.2	15.1 ± 0.4	19.6 ± 0.9
39 (clinical MRSA)	11.5 ± 0.2	15.4 ± 0.5	20.0 ± 0.7
23	13.5 ± 0.4	13.5 ± 0.6	23.7 ± 0.7
24	12.5 ± 0.1	12.7 ± 0.6	23.0 ± 0.4
25	12.3 ± 0.3	12.4 ± 0.5	22.0 ± 0.6
26	12.9 ± 0.2	12.9 ± 0.5	22.7 ± 1.4
27	12.5 ± 0.0	12.1 ± 1.0	22.5 ± 0.6
28	12.9 ± 0.9	12.9 ± 1.0	22.1 ± 0.6
16	10.6 ± 0.2	14.9 ± 0.5	18.8 ± 0.4

Key: Av.-average; SD-standard deviation; -- not amplified;

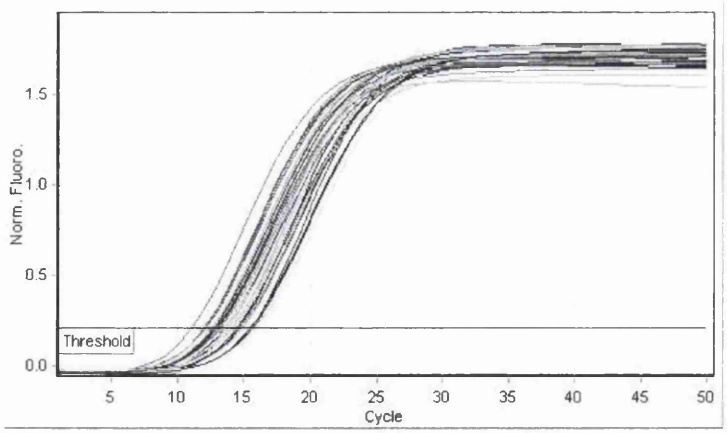


Figure 6.8. PCR-A amplification curves of MRSAII, MRSAIVb, clinical MRSA and PCR-A false positive isolates.

Table 6.12. Ct values of PCR-C false positive isolates when purified template was used for the SCC*mec-orfX* PCRs

Isolate	***femA*$_{SA}$** **Av. Ct + SD**	**PCR-C** **Av. Ct + SD**
MRSAII	9.3 ± 0.2	19.6 ± 0.4
MRSAIVb	9.2 ± 0.1	17.2 ± 0.3
*Oxford MSSA	11.0 ± 0.1	23.0 ± 0.4
37 (clinical MRSA)	10.8 ± 0.2	21.0 ± 0.2
39 (clinical MRSA)	11.5 ± 0.2	21.8 ± 0.3
29	10.6 ± 0.5	18.6 ± 0.1
30	11.4 ± 0.2	18.6 ± 0.3
31	11.1 ± 0.3	23.0 ± 0.2
32	10.9 ± 0.1	32.8 ± 0.4
33	10.1 ± 0.5	22.9 ± 0.2
34	10.3 ± 0.3	32.2 ± 0.1
35	9.9 ± 0.5	25.9 ± 0.5
36	9.9 ± 0.1	32.0 ± 0.5
16	10.6 ± 0.2	18.2 ± 0.7

*Oxford MSSA reference strain amplified with PCR-C assay

The amplification of the PCR-A and PCR-B false positive isolates with purified genomic DNA extracted from verified pure cultures indicated that the initial amplification of the isolates was credible and the SCC*mec-orfX* target sequences were probably present in these isolates. Unfortunately with the PCR-C method the use of purified genomic DNA resulted in robust amplification of the Oxford strain. Even though this is a MSSA reference strain, it was implausible to use it as a cut off for false positive results and justifiably distinguish the true positive and false positive results.

6.2.2 Analysis of the PCR fragment sizes of the false positive isolates

To ascertain if there were any differences in the PCR fragment sizes of the false positives and the MRSA reference strains, the fragments were resolved in a 2% agarose gel. Six of the seven (23, 24, 25, 26, 27 and 28) PCR-A false positives had smaller fragments (approximately 176 bp) which were comparable to the MRSA I fragment (see Figure 6.9a). Isolate 16 which was detected by all three SCC*mec-orfX* assays had a larger PCR fragment (approximately 278 bp) which was comparable to the MRSA II and MRSA IVb fragments. With PCR-B the same six false positives had a smaller fragment comparable to the MRSA I and MRSA III fragments i.e. 300 bp and 310 bp respectively (see Figure 6.9b). Whereas isolate 16 had a larger fragment which was comparable to the MRSAII and MRSAIV fragments (400 bp).

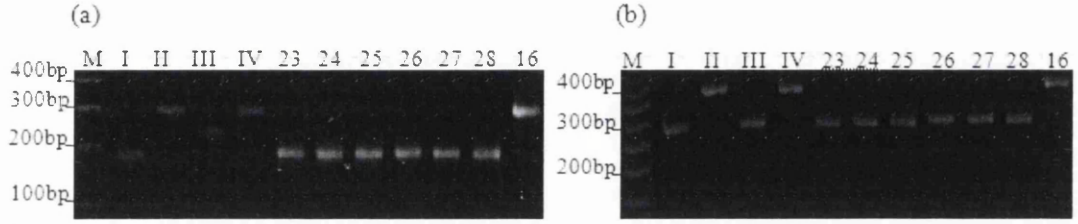


Figure 6.9. PCR fragments of the MRSA reference strains and the false positive isolates with PCR-A (a) and PCR-B (b). M, 100bp molecular marker; I, MRSAI; II, MRSAII; III, MRSAIII; IV, MRSAIV; 23-16, isolates 23, 24, 25, 26, 27, 28 and 16.

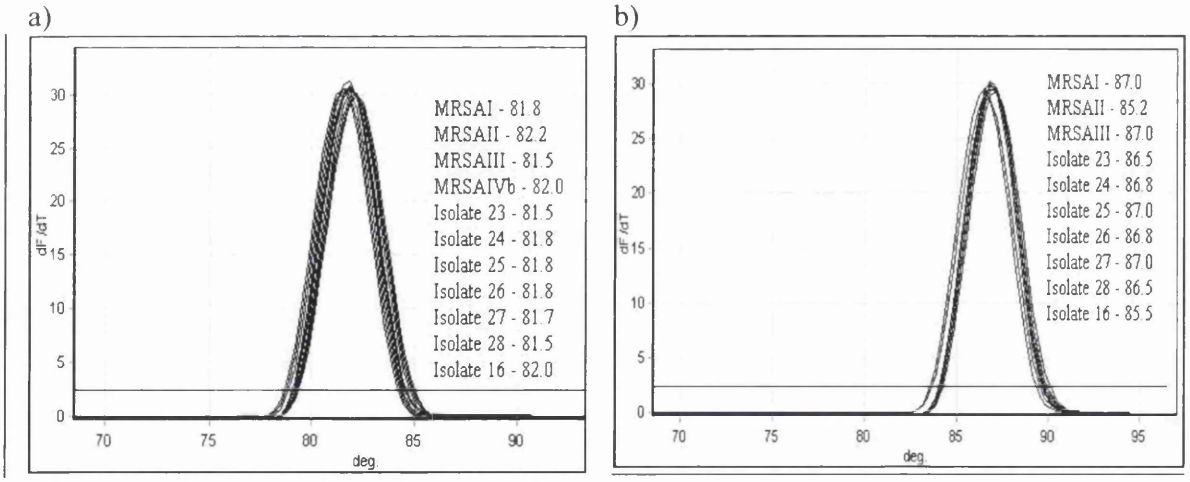


Figure 6.10. Melt curves of the PCR fragments of MRSA reference strain and the false positive isolates of PCR-A (a) and PCR-B

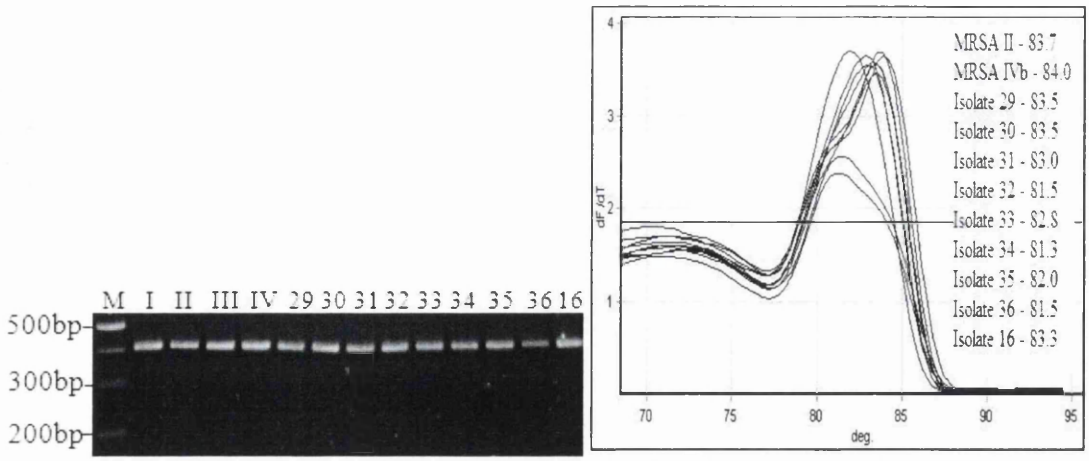


Figure 6.11. PCR fragments and melt curves of the MRSA reference strains and the false positive isolates with PCR-C. M, 100bp molecular marker; I, MRSAI; II, MRSAII; III, MRSAIII; IV, MRSAIVb; 29-16, isolates 29, 30, 31, 32, 33, 34, 35, 36 and 16.

The fragments of all nine PCR-C false positives (29, 30, 31, 32, 33, 34, 35, 36 and 16) were of the same size i.e. approximately 400 bp, and were comparable to the fragments of MRSA I, II, III and IV reference strains (see Figure 6.10).

Melt curve analyses of the fragments were performed to ascertain if the false positive isolates had melting temperatures (T_m) which were comparable to the MRSA reference strains. The six PCR-A false positive isolates which had smaller PCR fragments comparable to the MRSA I fragment also had T_m values (81.5 to 81.8°C) which were within the ±0.8 range of this reference strain (81.8°C) (see Figure 5.10). Similarly the same isolates with the PCR-B assay had T_m values (86.5 to 87.0°C) within the ±0.8 range of the MRSAI and MRSA III T_m values (87.0°C). Isolate 16 which had a larger PCR fragment with both assays, exhibited T_m values (82.0°C and 85.5°C) which were comparable to the T_m values of the larger sized fragments of the MRSA II and IVb reference strains in the PCR-A and PCR-B assays (see Figure 6.10). Since the values of the PCR-A and PCR-B false positive isolates were within the ±0.8°C range of the respective MRSA reference strains, this strongly suggested that they were specific PCR products of the two methods. Conversely, the nine PCR-C false positive isolates were of equal size but the T_m values were more diverse and five isolates (32, 33, 34, 35 and 36) had T_m values ranging from 81.3 to 82.0°C, these were below the ±0.8°C range of the MRSAII and IVb T_m values (83.7 and 84.0°C respectively), implying that these may have been non-specific amplification products (see Figure 6.11).

6.2.3 Comparison of nucleotide sequences of the false positive isolates to published SCC*mec* sequences

The PCR fragments of the false positive isolates of the PCR-A and PCR-C assays were sequenced and aligned i.e. both the forward and reverse strands (the false positive isolates of the PCR-A and PCR-B methods were identical, so only the fragments of the PCR-A assay were sequenced). Since the PCR-A false positive fragments were comparable to the fragments of MRSAI and MRSAII, the sequences were aligned with the SCC*mec* type I (NCTC10442) and SCC*mec* type II (N315) sequences.

Six of the seven PCR-A false positives which had the smaller PCR fragments (similar to the MRSAI fragment) had identical sequences and they were homologous to the SCC*mec* type I sequence, with only four nucleotide differences (see Figure 6.12). The remaining PCR-A false positive (isolate 16) which had the larger fragment size (similar to the fragment sizes of MRSAII and MRSAIV) had a sequence which was homologous to the SCC*mec* type II sequence, with only three nucleotide differences (see Figure 6.12). Surprisingly even though the T_m values of the PCR fragments of the nine PCR-C false positive isolates were diverse, the sequences of these isolates were extensively homologous to the SCC*mec* type IVb sequence, with some nucleotide differences at random locations in the respective sequences (see Figure 6.13).

The homology between the sequences of the PCR-A and PCR-C false positive isolates and the SCC*mec* type I, II and IVb sequences suggests that the PCR fragments of the false positives were not generated by non specific amplification but were due to the amplification of SCC*mec-orfX* associated sequences within these MSSA isolates.

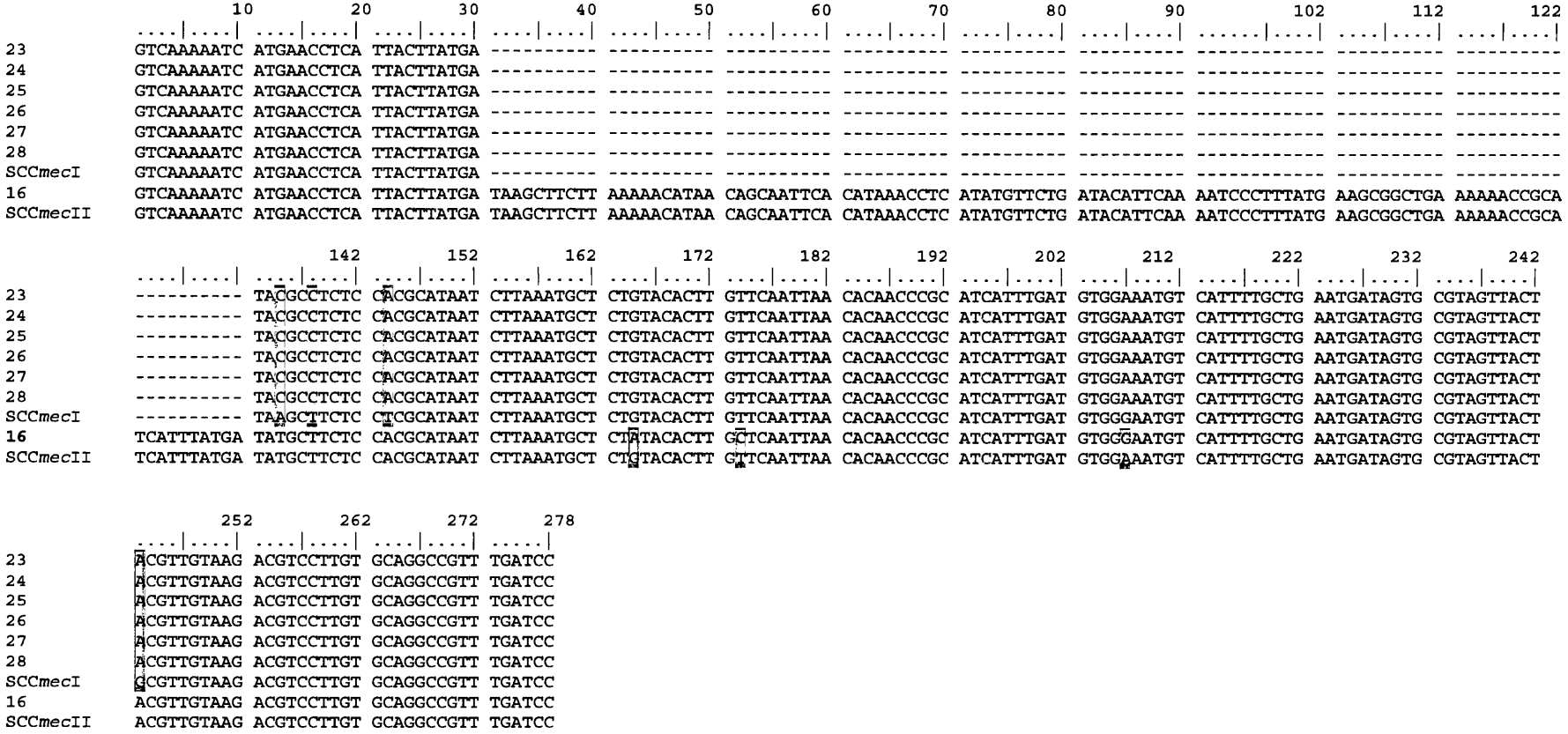


Figure 6.12. Alignment of sequences of the PCR-A false positive fragments and SCC*mec* type I (NCTC10442) and SCC*mec* type II (N315) sequences. Blue areas- nucleotide differences.

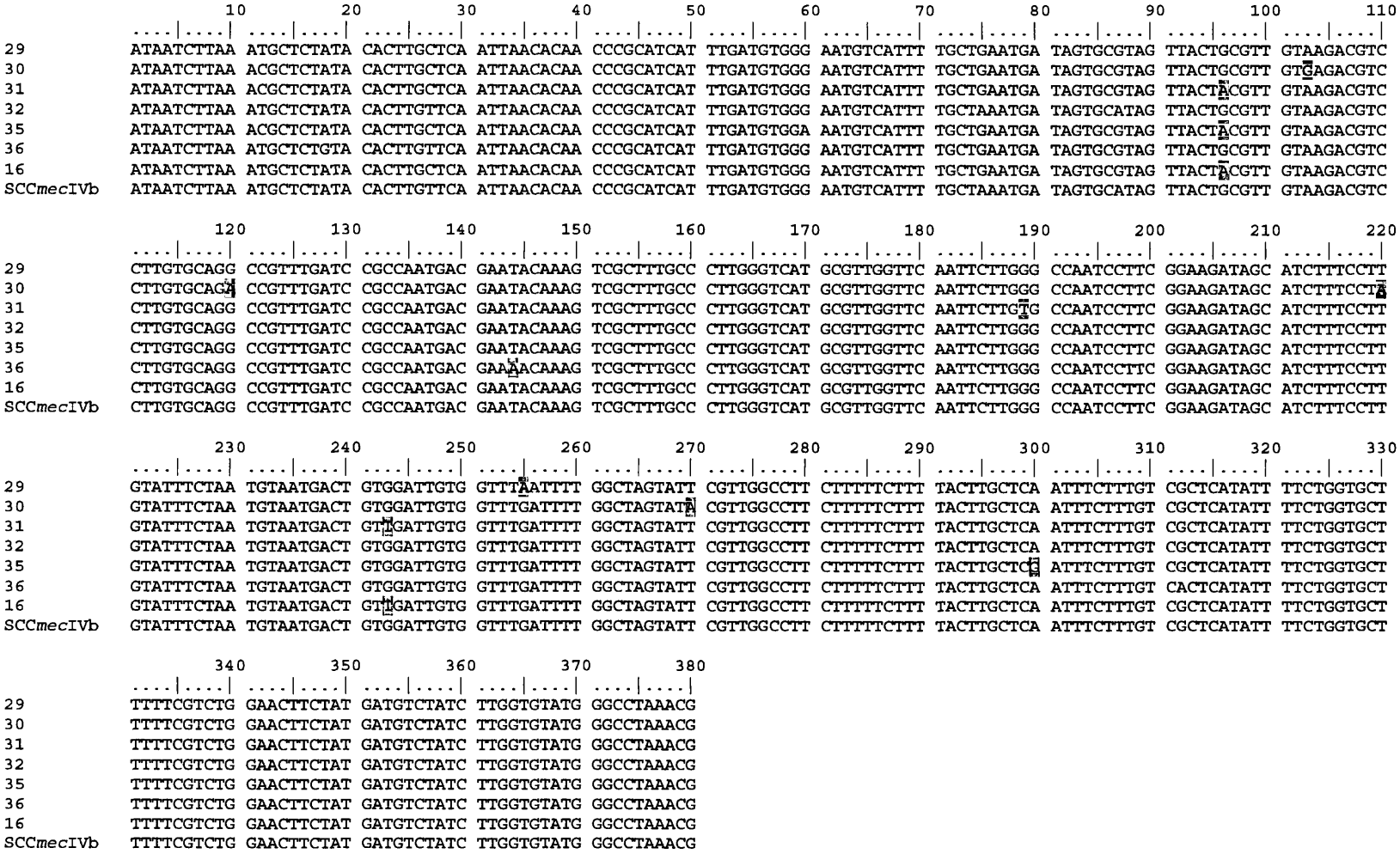


Figure 6.13. Alignment of sequences of seven of the PCR-C false positive fragments and the SCC*mec* type IVb sequence. Blue areas- nucleotide differences

6.3 Discussion

6.3.1 Comparison of PCR-A, PCR-B and PCR-C to the original studies

The PCR-A, PCR-B and PCR-C SCC*mec-orfX* amplification methods had high accuracy (sensitivity ≥95%) in the detection of *mecA* positive *S. aureus* in a collection of 118 (22.7%) MRSA and 401 (77.3%) MSSA isolates. The original PCR-A method was developed by Huletsky *et al.* (2004) as a real-time PCR with the Cepheid Smart Cycler and they evaluated a much larger collection of isolates comprising 1657 MRSA and 569 MSSA isolates. A comparison of the results of the present study and the original study shows that Huletsky *et al.* (2004) attained higher sensitivity (98.7% versus 96.6%) and PPV results (98.5% versus 94.2%) but lower specificity (95.4% versus 98.3%) and NPV results (96.3% versus 99.0%) (see Table 6.13, 1st and 2nd column). Even though Huletsky *et al.* (2004) had a higher PPV result, the number of false positives (n=26, 4.6%) was significantly higher (p=0.02) than in this study (n=7, 1.7%), indicating that in the original study more MSSA isolates were misidentified as MRSA. The NPV result of the present study exceeded the result attained by Huletsky *et al.* (2004) and this was most likely due to the lower MRSA prevalence rate in this study (22.7% versus 74.4%). The results of the present and original studies illustrate that the PCR-A SCC*mec-orfX* assay has high accuracy for MRSA detection in clinical isolates.

In a later study by Huletsky *et al.* (2005) the SCC*mec-orfX* assay was performed on 331 nasal swabs of which 81 (24.5%) were MRSA positive by the culture method. The sensitivity, specificity, PPV and NPV results were 100%, 98.4%, 95.3% and 100% respectively, demonstrating particularly high sensitivity and NPV results and suggesting that the PCR-A SCC*mec-orfX* method retains high accuracy for MRSA detection in both clinical isolates and nasal swabs (see Table 6.13, 3rd column).

Table 6.13. Performance results of the PCR-A method in the present study and in the Huletsky *et al.* (2004, 2005) studies

	Present study *S. aureus* **isolates**	**Huletsky *et al.* (2004)** *S. aureus* **isolates**	**Huletsky *et al.* (2005) Nasal swabs**
Sensitivity	96.6 %	98.7 %	100%
Specificity	98.3 %	95.4 %	98.4%
PPV	94.2 %	*98.5%	95.3%
NPV	99.0 %	*96.3%	100%

*Predictive values calculated from sensitivity, specificity, false negative, and false positive results of Huletsky *et al.* (2004)

The PCR-B method was originally developed by Hagen *et al.* (2005) as a real-time PCR with the Roche LightCycler system and they evaluated 256 clinical isolates comprising 124 MRSA isolates, similar to the number (n=118) evaluated in this study but a lower number of MSSA isolates (n=53 versus n=401). They also included 47 methicillin resistant-coagulase negative staphylococci (MR-CoNS) and 32 methicillin susceptible-coagulase negative staphylococci (MS-CoNS). The Hagen *et al.* (2005) study had higher sensitivity (98% versus 96.6%), specificity (100% versus 98.3%) and PPV results (100% versus 94.2%) but the NPV results were comparable in both studies i.e. 98% versus 99.0% (see Table 6.14, 1st and 2nd column). Their specificity and PPV results were faultless indicating the absence of false positive results but this was most likely due to the low number of MSSA isolates tested in the study. The overall results of the present and original studies indicated a high accuracy of the PCR-B SCC*mec-orfX* assay for MRSA detection in clinical isolates. Hagen *et al.* (2005) also performed the assay on 60 nasal swabs of which 29 (48.5%) were MRSA positive by a culture method. Even though the specificity and PPV results remained high, the decline in the sensitivity and NPV results, 93% and 94% respectively, indicated a lower accuracy of the assay for MRSA detection in nasal swabs and a higher likelihood of false negative results with these clinical specimens (see Table 6.14, 3rd column).

A comparison of the original results of the Huletsky *et al.* (2004) study to those of the Hagen *et al.* (2005) study shows that the respective SCC*mec-orfX* assays had comparable sensitivity results with clinical isolates i.e. 98.7% and 98% respectively (see Table 6.13 and 6.14) and similarly in this study, the PCR-A and PCR-B methods had identical results. However with nasal swabs, the Huletsky *et al.* (2005) method (PCR-A) had higher sensitivity (100% versus 93%) and NPV results (100% versus 94%) than the Hagen *et al.* (2005) method (PCR-B), suggesting that with nasal swabs the PCR-A method is potentially more accurate than the PCR-B method (see Table 6.13 and 6.14).

Table 6.14. Performance results of the PCR-B method in the present study and in the Hagen *et al.* (2005) study

	Present study *S. aureus* **isolates**	**Hagen *et al.* (2005)** *S. aureus* **isolates**	**Hagen *et al.* (2005) Nasal swabs**
Sensitivity	96.6 %	98%	93%
Specificity	98.3 %	100 %	100%
PPV	94.2 %	100%	100%
NPV	99.0 %	98%	94%

In this study the PCR-C method had the lowest diagnostic performance out of all three SCC*mec-orfX* assays. The original method was developed by Cuny and Witte (2005) as a conventional PCR and the fragments were resolved in an agarose gel. The positive results were defined by the presence of a band (approximately 400bp) and negative results verified by the absence of the band. In this study the method was modified into a real-time PCR on the Corbett Rotor-Gene 6000 platform. Cuny and Witte (2005) had performed the assay on 330 staphylococci isolates: 100 MRSA, 100 MSSA, 50 MR-*S. epidermidis*, 30 MS-*S. epidermidis*, 30 MR-*S. haemolyticus*, 10 MR-*S. hominis* and 10 MS-*S. hominis* and they had perfect sensitivity and specificity results (100%). However the lower sensitivity (95.8%), specificity (97.8%), PPV (92.8%) and NPV results (98.7%) of PCR-C in the present study, suggest that in a larger collection of isolates this method does generate false negative and false positive results (see Table 6.15).

In this study the PCR-C method had the lowest PPV result in comparison to the PCR-A and PCR-B methods (92.6% versus 94.2% and 94.2% respectively), indicating a higher likelihood of false positive results with this method. This was not unexpected as the late amplification of MSSA isolates was observed in previous results (presented in chapter 4). The likely cause of these results is the design and location of the PCR-C forward primer, the sequence of the primer is homologous to two published MSSA sequences and it overlaps into the *orfX* sequence (discussed in chapter 4). This concern has been highlighted by Kolman *et al.* (2010), they performed the Cuny and Witte (2005) assay on 150 staphylococci isolates: 50 MRSA, 50 MSSA, 25 MR-CoNs and 25 MS-CoNs and attained even lower specificity and PPV results i.e. 86% and 77.8% respectively, they subsequently excluded the assay from further evaluations (Kolman et al., 2010).

Table 6.15. Results of the PCR-C method in the present study and results of the Cuny and Witte (2005) study

	Present study *S. aureus* **isolates**	**Cuny and Witte (2005)** *S. aureus* **isolates**
Sensitivity	95.8 %	100 %
Specificity	97.8 %	100 %
PPV	92.6 %	100%
NPV	98.7 %	100 %

In the present study the template concentration per reaction was kept relatively constant by the consistent use of two to three *S. aureus* colonies in the preparation of the crude DNA extracts. Based on the evaluation of the Ct values of the amplified isolates, the discrimination of the positive and negative results was possible. However with the use of purified genomic DNA a higher concentration of DNA was being added to the PCR reactions and this resulted in the robust amplification of both MRSA and MSSA strains at similar low Ct values, therefore it was difficult to validly distinguish the true positive results from the false positive results with the PCR-C assay. These observed results question the suitability of this assay as an MRSA diagnostic test in routine clinical analysis. Furthermore in clinical specimens the bacterial load is variable and consequently the target sequence concentration may be low, this could also make the discrimination of positive and negative results problematic with this assay. However, the original method by Cuny and Witte (2005) was implemented as a commercial MRSA screening test for nasal swabs, indicating that under certain defined reaction conditions undoubtedly this method is a suitable MRSA screening test (Hain LifeScience).

6.3.2 The diagnostic performance of SCC*mec-orfX* assays on clinical swabs

Out of the three SCC*mec-orfX* assays evaluated the PCR-A and PCR-B methods had the best diagnostic performance. However, the high performance is not guaranteed in routine MRSA screening swabs due to the lower prevalence rate of MRSA in these clinical specimens. Studies by Maudsley *et al.* (2004), Grundmann *et al.* (2002) and Abadu *et al.* (2001) conducted in London, Nottingham and Birmingham respectively indicate that in the UK the prevalence of nasal carriage of MRSA in the community is very low i.e. 0.78%, 0.83% and 1.5% (Maudsley et al., 2004, Grundmann et al., 2002, Abadu et al., 2001). Lower prevalence rates will affect the predictive values of the SCC*mec-orfX* assays i.e. with decreasing prevalence the PPV decreases and the NPV increases, thus increasing the likelihood of false positive results (Akobeng, 2006).

The original PCR-A method was the first SCC*mec-orfX* PCR to be approved as a commercial assay for MRSA detection in nasal swabs (BD GeneOhm MRSA test). This is a qualitative in vitro real-time PCR for direct detection of MRSA in nasal swabs. It involves an initial lysis step, during which the nasal swab is placed directly into the kit lysis buffer, then an aliquot of the lysate is transferred to a tube containing a volume of

the kit PCR master mix and the reaction mix is then placed into the designated themocycler i.e. the Cepheid SmartCycler: the PCR takes less than 2 hours. The amplified target sequences are detected with hybridisation probes labelled with fluorophores, the amplification, detection and interpretation of the results is done automatically by the SmartCycler software. Other commercial real-time PCR tests for MRSA detection in nasal swabs include the Xpert MRSA (Cepheid) and the LightCycler MRSA Advanced test (Roche), these also utilise specific kit reagents and require the use of designated thermocyclers (www.cepheid.com/tests-and-reagents/) (http://molecular.roche.com). Another assay is the GenoQuick MRSA test (Hain LifeScience). Although this is an amplification method, it is not a real-time PCR assay, rather it involves the use of a DNA strip coated with specific complementary probes to detect the amplified MRSA target sequence and the bound amplified sequence is then made visible in a colorimetric reaction (www.hain-lifescience.de/). Further tests include GenoType MRSA (Hain LifeScience) for MRSA detection in bacterial cultures, the BD GeneOhm StaphSR for the detection of MSSA and MRSA in blood cultures and the Xpert MRSA/SA SSTI and Xpert MRSA/SA BC tests (Cepheid) for MRSA detection in skin and soft tissue infections swabs and blood cultures respectively.

Several studies have investigated the BD GeneOhm MRSA test (previously termed the IDI-MRSA) and other commercial SCC*mec-orfX* tests on various screening swabs. Those in which the tests were performed directly on nasal swabs with variably low MRSA prevalence rates, have a similar trend of results: relatively high (>90%) sensitivity and specificity values but disappointingly low PPV values, in certain studies the values dropped below 80% (Snyder et al., 2010, Park et al., 2009, de San et al., 2007, Paule et al., 2007, Zhang et al., 2007, Bishop et al., 2006, Warren et al., 2004) (see Table 6.16). In a study by Park *et al.* (2009) the overall performance of the BD GeneOhm MRSA test was notably lower than in the present study (sensitivity 85.8%, specificity 77.5%, PPV 72.8% and NPV 93.5%). In the study by Wolk *et al.* (2009) the Cepheid Xpert MRSA test was performed on nasal swabs and despite a MRSA prevalence rate of 44%, the results of that study also showed a low PPV result for the assay. Nevertheless in most of the studies which used nasal swabs, the NPV (94%-100%) results were exceptionally high and comparable to the values attained in this study with clinical isolates (see Table 6.16).

Table 6.16. The sensitivity, specificity and predictive results of SCC*mec-orfX* methods in different studies

Results in nasal swabs							
Reference	**Specimen no.**	**Tests evaluated**	**% Sens.**	**% Spec.**	**% PPV**	**% NPV**	**Comments**
(Peterson et al., 2010)	1402 nasal swabs Patients in nursing homes, intensive care, dialysis units and medical staff	Roche Light cycler MRSA test BD GeneOhm MRSA	92.2 93.2	98.9 94.2	94.0 75.0	98.5 98.7	MRSA prevalence in swabs , 13.3%. Light cycler and BD GeneOhm tests have comparable sensitivity and NP BD GeneOhm has lower specificity PPV
(Snyder et al., 2010)	627 nasal swabs of patients in intensive care	BD GeneOhm MRSA	100	96.7	70.3	100	MRSA prevalence in swabs ,7.2%. Although sensitivity and NPV result perfect PPV very low
(Park et al., 2009)	295 nasal swabs of patients in intensive care	BD GeneOhm MRSA	85.8	77.5	72.8	93.5	MRSA prevalence in swabs high, 44 however performance values much lower than in this study
(Wolk et al., 2009)	1077 nasal swabs Hospital, nursing home patients	Cepheid GeneXpert MRSA	94.3	93.2	73.0	98.8	MRSA prevalence in swabs, 5.2- 44. PPV much lower than in this study
(Boyce and Havill, 2008)	286 nasal swabs Hospital patients	BD GeneOhm MRSA	100	98.6	95.8	100	MRSA prevalence in swabs , 22%. Performance values comparable to t study
(Paule et al., 2007)	403 nasal swabs	BD GeneOhm with kit lysis step BD GeneOhm with in house lysis	98.0 97.9	96.0 95.2	77.9 74.6	99.7 99.7	MRSA prevalence in swabs, 12.4%. Sensitivity, specificity and NPV comparable to this study, however P results much lower

Key: No.-number; Sens.- sensitivity; Spec.-specificity; PPV-positive predictive value; NPV-negative predictive value

Table 6.16. contd. The sensitivity, specificity and predictive values of SCC*mec-orfX* methods in different studies

Results in nasal swabs							
Reference	**Specimen no.**	**Tests evaluated**	**% Sens.**	**% Spec.**	**% PPV**	**% NPV**	**Comments**
(Warren et al., 2004)	Nasal swab-288 From admitted patients >3days with prior MRSA colonisation	IDI- MRSA (BD GeneOhm)	91.7	93.5	82.5	97.1	MRSA prevalence in swabs, 25.0%. Although NPV similar to this study other values lower.

Results in nasal swabs versus swabs from other sites							
Reference	**Specimen no.**	**Tests evaluated**	**% Sens.**	**% Spec.**	**% PPV**	**% NPV**	**Comments**
(Lucke et al., 2010)	1595 swabs (nasal, groin, wound, axilla throat, rectum,vagina and miscellaneous	BD GeneOhm MRSA					
		All swabs	84.3	99.2	88.4	98.9	MRSA prevalence in all swabs, 6.5%. Specificity high in all swabs comparable to this study. Sensitivity reduced except for wounds. PPV generally reduced for all swabs.
		Nasal swabs	85.0	99.5	91.9	99.1	
		Groin swabs	85.7	99.3	88.2	99.2	
		Wound swabs	95.2	96.9	83.3	99.2	
		Other swabs	78.3	99.2	87.5	95.9	
(de San et al., 2007)	Nasal swab-522 Throat swab-212 Perineum swab-206 Wound swab-60 from patients in intensive care	IDI- MRSA (BD Gene Ohm)					
		All swabs	81.0	97.0	75.0	97.0	MRSA prevalence in all swabs, 10% IDI-MRSA test had better diagnostic performance with nasal swabs, except low PPV. Had high specificit and NPV values, comparable to this study
		Nasal swabs	90.6	97.1	67.4	99.4	
		Non nasal swabs	76.5	96.8	80.0	96.1	

Key: No.-number; Sens.- sensitivity; Spec.-specificity; PPV-positive predictive value; NPV-negative predictive value

Table 6.16. contd. The sensitivity, specificity and predictive values of SCC*mec-orfX* methods in different studies

Results in nasal swabs versus swabs from other sites

Reference	Specimen no.	Tests evaluated	% Sens.	% Spec.	% PPV	% NPV	Comments
(van Hal et al., 2007)	Nasal swab-101	IDI- MRSA (BD Gene Ohm)					MRSA prevalence in swabs:
	Axilla swab-52	All swabs	90.0	96.0	93.0	94.0	Nasal swabs, 52.4%
	Groin swab-52	Nasal swabs only	94.0	94.0	94.0	94.0	Axillaswabs,15.4%
	High risk admitted	Groin and axilla swabs	80.0	97.0	85.0	90.0	Groin swabs, 32.7%
	patients, colonised	Genotype MRSA Direct					IDI-MRSA better than Genotype fo
	or infected with	All swabs	80.0	97.0	85.0	90.0	detection of MRSA from all swabs.
	MRSA	Nasal swabs only	70.0	96.0	95.0	73.0	However for both tests, best
		Groin and axilla swabs	68.0	96.0	85.0	90.0	performance in nasal swabs. Result
							of both tests lower than in this stud
(Zhang et al., 2007)	2127 swabs: nasal,	IDI- MRSA (version 2)					MRSA prevalence in all swabs, 8.5
	rectal, and other	Nasal swabs only	93.9	96.9	64.6	99.6	IDI- MRSA (version 3) marketed i
	sites	Rectal swabs only	96.6	96.4	58.3	99.8	2006. Both version 2 and 3 have hi
		Other swabs	100	93.0	53.0	100	sensitivity and NPV results. Howe
		IDI- MRSA (version 3)					PPV results still low in both earlier
		Nasal swabs only	100	96.8	69.2	100	and later versions of test
		Rectal swabs only	93.1	95.4	61.4	99.4	
		Other swabs	100	95.0	60.0	100	
(Bishop et al., 2006)	Nasal and groin	IDI- MRSA (BD Gene Ohm)					MRSA prevalence in swabs:
	swabs-192	Nasal and groin swabs	88.0	91.6	61.1	98.1	Nasal swabs, 5.7%
	Adults of >18 years	Nasal swabs only	90.0	91.7	56.3	98.8	Groin swabs, 3.1%
	attending hospital	Groin swabs only	83.3	90.2	46.9	98.1	NPV high but PPVs extremely low
							Test performed better for nasal swa

Key: No.-number; Sens.- sensitivity; Spec.-specificity; PPV-positive predictive value; NPV-negative predictive value

Table 6.16. contd. The sensitivity, specificity and predictive values of SCC*mec-orfX* methods in different studies

Results in pooled swabs Reference	Specimen no.	Tests evaluated	% Sens.	% Spec.	% PPV	% NPV	Comments
(Nulens et al., 2010)	500 patients nasal and throat swabs	BD GeneOhm Staph SR Pooled swabs	62.5	99.0	50.0	99.4	MRSA prevalence very low, 1.6%. Sensitivity and PPV results very low
		Cepheid GeneXpert MRSA Pooled swabs	62.5	97.1	31.3	99.4	
(Wassenburg et al., 2010)	1764 nasal, throat, perineum swabs	BD GeneOhm MRSA Pooled swabs	85.2	96.5	44.2	99.5	MRSA prevalence low, 3.3%. Sensitivity and PPV results very low
		Cepheid GeneXpert MRSA Pooled swabs	75.0	94.5	33.3	99.1	
(Jeyaratnam et al., 2008a)	201 pooled nasal, axilla, groin swabs 32 throat, rectum, Perineum, wounds	IDI- MRSA (BD Gene Ohm) Pooled nasal, axilla, groin	84.8	95.1	94.4	86.6	MRSA prevalence high in total nasal axilla and groin swabs, 49.3%, but results lower than in the present study
		Other sites	94.1	80.0	84.2	92.3	
(Desjardins et al., 2006)	287 pooled nasal and rectal swabs.	IDI- MRSA (BD Gene Ohm) Pooled nasal and rectal	96.0	96.0	90.0	98.0	MRSA prevalence in total nasal and rectal swabs, 24.7%. Although P PV result lower than in this study, other performance results were comparabl

Key: No.-number; Sens.- sensitivity; Spec.-specificity; PPV-positive predictive value; NPV-negative predictive value

Although the BD GeneOhm MRSA test is approved for nasal swabs, several studies have investigated the performance of this assay on non-nasal swabs. The results of some studies indicate that the sensitivity of the IDI-MRSA and the BD GeneOhm MRSA tests is higher with nasal swabs than with swabs from other anatomical sites (de San et al., 2007, van Hal et al., 2007, Bishop et al., 2006) (see Table 6.16). Conversely, Zhang *et al.* (2007) attained comparable high sensitivity values (>90%) for both rectal and nasal swabs with an earlier and newer version of the IDI-MRSA test. However the PPV results with the non-nasal swabs were very low for both versions of the test (58.3% and 61.4%) (see Table 6.16). Overall these results suggest that with non-nasal swabs the diagnostic performance of SCC*mec-orfX* assays may further decline and false positives may be more frequent.

The pooling of screening specimens could reduce the time required for testing and the performance of SCC*mec-orfX* assays on pooled specimens e.g. nasal swabs, axilla, perineum, rectal swabs, has been investigated in some studies. Nulens *et al.* (2010), Wassenberg *et al.* (2010) and Jeyaratnam *et al.* (2010) attained lower sensitivity results and the PPV results of the Nulens *et al.* (2010) and Wassenberg *et al.* (2010) studies were particularly low (50.0% and 44.2%) (see Table 6.16) but in both studies the NPV values of the assays were remarkably high (99%). Also in these studies the BD GeneOhm test was compared to the Cepheid GeneXpert test and although both tests had poor sensitivity and PPV results, the BD GeneOhm tests had slightly better results with the pooled specimens (see Table 6.16). These results indicate that as with non-nasal swabs, the accuracy of SCC*mec-orfX* assays may decrease with pooled clinical specimens also and an increase in the rate of false positive results is likely, thus these assays perform more optimally with nasal swabs.

6.3.3 SCC*mec-orfX* false negative and false positive results

In this study false negative results were attained with the PCR-A, PCR-B and PCR-C SCC*mec-orfX* assays. Initially these were thought to be due to PCR inhibitors in the crude templates however the same isolates were successfully amplified using the crude templates in the *mecA* and $femA_{SA}$ PCR assays. If inhibitors were present these would have also hindered amplification in all the PCR assays. Subsequent amplification with the SCC*mec-orfX* assays when purified genomic DNA was used suggested that the initial lack of amplification was probably due to lower concentrations of target DNA in

the crude extracts. Several possible causes of the divergent Ct values of the false negative isolates and the MRSA reference strains with the PCR-A and PCR-B methods were investigated but a conclusive explanation was not achieved. In fact the sequences of these false negative MRSA were homologous to the MRSAIVb reference strain. A proposed cause of the unusual results was a deletion within the SCC*mec* right extremity region which was inclusive of the binding sites of the PCR-A and PCR-B forward primers. This is not implausible because the SCC*mec* right extremity sequence is adjacent to *orfX*, the site for the integration and excision of the SCC*mec* elements. It is postulated that the excision of the elements reduces the fitness cost incurred by their carriage, so it is possible that under high antibiotic pressure since the excision of an entire element may be detrimental, only certain regions may be deleted and the proposed deletion may have arisen under such circumstances. Perhaps in the false negative isolates there was over-expression of the recombinase genes which mediate the excision of the SCC*mec* element and this may have caused the frequent deletion of the regions inclusive of the PCR-A and PCR-B target sequences.

However the location and extent of the proposed deletion was not established in this study and several strategies could be implemented to further explore this hypothesis. The structural composition of the SCC*mec* elements contained in the false negative isolates could be investigated by DNA hybridisation microarray analysis. A microarray could be developed which comprises oligonucleotide sequences that are representative of all the open reading frames (*orfs*) of currently sequenced SCC*mec* types. The analysis of the false negative isolates would enable a comprehensive characterisation of the SCC*mec* elements and also it would allow the identification of *orfs* that are absent or not homologous to currently defined SCC*mec* types. A whole genome microarray has already been developed based on the genomes of seven *S. aureus* strains belonging to different clonal lineages (Lindsay et al., 2006, Witney et al., 2005). This assay could be used to determine the genomic differences between the MRSA reference strains and the false negative isolates. The target sequences of the SCC*mec-orfX* amplification methods employed in this study are located in the J3-region (*mecA* to *orfX*) of SCC*mec* type I to V elements. The proposed deletion could be investigated by sequencing this region in the SCC*mec* elements of the false negative isolates. This would require the optimisation and implementation of long range PCR(s) and DNA sequence analysis of the PCR fragments.

In addition to the false negative results, the SCC*mec-orfX* assays also generated false positive results. Seven MSSA isolates were amplified with PCR-A and PCR-B and nine MSSA isolates were amplified with PCR-C. False positive results can arise from the amplification of non specific products however this was ruled out because the sequences of the false positive PCR fragments aligned to published SCC*mec* type I, II and IVb sequences, indicating that SCC*mec-orfX* sequences were present in these isolates even though the *mecA* gene was absent. The presence of the SCC*mec-orfX* junction in the absence of *mecA* in *S. aureus* isolates has been described in several studies (Noto et al., 2008a, Donnio et al., 2005, Wada et al., 1991). Donnio *et al.* (2005) described the presence of a SCC*mec* associated remnant, *IS*431::pUB110::*IS*431::*dcs* in tobramycin resistant MSSA strains collected from various French hospitals from 1992 to 2002. They postulated that the fragment resulted from the partial excision of possibly SCC*mec*IA, II or IVA elements as these carry the pUB*110* plasmid which has *aadD,* the gene encoding tobramycin resistance (Donnio et al., 2005). The detection of SCC*mec-orfX* in MSSA can also be caused by the carriage of non-*mecA* containing SCC elements i.e. SCC_{capl} and SCC_{476} present in certain MSSA strains. The presence of SCC*mec*-associated DNA, possibly resulting from an excised SCC*mec* or from an integrated non *mecA* SCC element was subsequently investigated and the results are reported and discussed in chapter 7.

Several factors contribute to the excision of SCC*mec* from the chromosome including long-term storage or long-term cultivation in the absence of antibiotic selective pressure (van Griethuysen et al., 2005, Deplano et al., 2000, Lawrence et al., 1996). As such van Griethuysen *et al.* (2005) recommended the addition of oxacillin to cryopreservatives to enable stabilisation of *mecA*. In this study the *S. aureus* isolates were collected as 24 to 48 hour cultures which were grown on Iso-Sensitest plates and colonies were picked from these plates for the PCR tests or isolates were then subcultured (24 hours) onto CBA plates for repeat testing. Even though these media are antibiotic free the incubation times were not extensive, so these conditions present a low risk for SCC*mec* excision.

SCC*mec-orfX* assays are based on the detection of SCC*mec* right extremity sequences of currently published SCC*mec* elements. However, the identification of novel elements has not been exhausted, thus current assays may prove less optimal for the detection of future MRSA strains with novel SCC*mec* elements. In addition, the presence of different MRSA clones or the varying prevalence rates of respective MRSA clones in various geographical locations may affect the performance of SCC*mec-orfX* assays. This was observed by Bartels *et al.* (2009) who evaluated the BD GeneOhm MRSA assay against a diverse collection of MRSA which were mainly from Copenhagen, Denmark (Bartels et al., 2009). Fifteen percent of MRSA isolates gave false negative results and subsequent engagement of the authors with collaborators from BD GeneOhm enabled assessment of the sequences of these isolates. This revealed that they contained SCC*mec* right extremity types which could not be detected by the assay. Ninety five percent of the undetectable isolates belonged to the ST8-MRSA-IVa clone (*spa* type t024) which was common in Copenhagen at the time of the study.

The reduction of MRSA infections is a priority in the UK and the rapid but accurate detection of MRSA in screening clinical specimens is an integral part of infection control. The results of this study indicate that the SCC*mec-orfX* PCR assays, particularly the PCR-A and PCR-B methods have high sensitivity and NPV results and therefore they could be attractive tests for MRSA screening programmes. However this would require careful consideration of local MRSA prevalence rates as these will affect the diagnostic predictive values of the tests. Also the diverse molecular epidemiology of MRSA strains in various geographical locations necessitates the inclusion of local MRSA strains in the validation process. Nevertheless in spite of the high sensitivity results of the SCC*mec-orfX* assays it was evident that certain MRSA strains were undetected and several MSSA were amplified with these assays. Even though the exact cause of these results was not verified in this study, the genotypic characteristics of the atypical MRSA and MSSA strains were investigated with molecular typing methods and the results are presented in the proceeding chapter.

Chapter Seven

Molecular characterisation of the SCC*mec-orfX* false negative and false positive *S. aureus* isolates

7.0 Background

Previously 137 MRSA isolates were characterised by PFGE, *spa* typing and SCC*mec* typing and 89.1% (n=122) were EMRSA-15 strains, 5.8% (n=8) were EMRSA-16 strains and 5.1% (n=7) were diverse MRSA strains (results presented in chapter 4) (see Table 7.1). The SCC*mec* typing results demonstrated that out of the 137 MRSA isolates 92% (n=126) contained SCC*mec* type IV elements, thus were defined as MRSAIV strains and 5.8% (n=8) contained SCC*mec* type II elements, thus were defined as MRSAII strains. Of the remaining three isolates, two had elements which were provisionally defined as SCC*mec* type V elements and one isolate had an element which was not typeable (results presented in chapter 4).

Three SCC*mec-orfX* amplification methods: PCR-A, PCR-B and PCR-C had been evaluated with MRSA reference strains and demonstrated the capacity to amplify MRSAI to MRSAIV reference strains. However when these were performed on 118 of the 137 MRSA isolates and 401 MSSA isolates, several MRSA and MSSA isolates generated false negative and false positive results (results presented in chapter 6). Four MRSA isolates: 17, 18, 19 and 20 were not amplified with the PCR-A and PCR-B assays and five MRSA isolates: 21, 22, 18, 7 and 8 were not amplified with the PCR-C assay (isolate 18 was not amplified in all three assays). Whereas seven MSSA isolates were amplified with the PCR-A and PCR-B assays: 23, 24, 25, 26, 27, 28 and 16 and nine MSSA isolates were amplified with the PCR-C assay: 29, 30, 31, 32, 33, 34, 35, 36 and 16 (isolate 16 was amplified in all three assays).

Table 7.1. Genotypic characteristics of 137 MRSA clinical isolates as determined by PFGE, *spa* typing and SCC*mec* typing (refer to section 4.1.1 and 4.1.4 in chapter 4)

Clone	MRSA (n=137)
EMRSA-15	122 (89.1%)
EMRSA-16	8 (5.8%)
Unique	7 (5.1%)

SCC*mec* type	MRSA (n=137)
IV	126 (92.0%)
II	8 (5.8%)
V*	2 (1.5%)
Non-typeable	1 (0.7%)

*SCC*mec* type V designation was provisional

Therefore the objective of this study was to examine the genotypic characteristics of the MRSA isolates which were not amplified with the PCR-A, PCR-B and PCR-C methods. In addition the genotypic characteristics of the false positive MSSA isolates were investigated with PFGE and *spa* typing. The SCC*mec* typing had been previously performed with the Milheirico *et al.* (2007b) and the MPCR-1 and MPCR-2 typing methods (Kondo et al., 2007) (see section 4.02 in chapter 4). In this study these methods were also performed on the false positive MSSA isolates to investigate if they contained SCC*mec* associated loci (see Table 7.2 and 7.3).

Table 7.2. Expected PCR fragment sizes of the Milheirico *et al.* (2007b) method for SCC*mec* types I to V

Loci	Size(bp)	SCC*mec*I	SCC*mec*II	SCC*mec*III	SCC*mec*IV	SCC*mec*V
pls	495bp	+				
ccrC	449bp					+
III- J3	414bp			+		
V- J1	377bp					+
dcs	342bp	+	+		+	
ccrB2	311bp		+		+	
kdp	284bp		+			
III- J1	243bp			+		
mecI	209bp		+	+		
mecA	162bp	+	+	+	+	+
No. of fragments		3	5	4	3	3

Table 7.3. Expected PCR fragment sizes of *ccr* and *mec* loci of SCC*mec* type I to VI elements with MPCR-1 and MPCR-2 assays

Loci MPCR-1	Size(bp)	SCC*mec*I (1B)	SCC*mec*II (2A)	SCC*mec*III (3A)	SCC*mec*IV (2B)	SCC*mec*V (5C)
mecA	286bp	+	+	+	+	+
ccrA1B1	695bp	+				
ccrA2B2	937bp		+		+	
ccrA3B3	1791bp			+		
ccrA4B4	1287bp				(+)	
ccrC	518bp					+
MPCR-2						
mecA-mecI	1963bp		+	+		
mecA*-IS*1272	2827bp	+			+	
mecA*-IS*431	804bp					+

(+) SCC*mec*VI which contains *ccrAB4* has been described which is structurally similar to SCC*mec*IV

7.1 Results

7.1.1 Molecular characterisation of the false negative isolates

7.1.1.1 Pulsed field gel electrophoresis

Pulsed field gel electrophoresis was performed on the four MRSA isolates: 17, 18, 19 and 20 which were not amplified with the PCR-A and PCR-B assays and the five isolates: 21, 22, 18, 7 and 8 which were not amplified with the PCR-C assay (isolate 18 was not amplified in all three assays).

The PFGE patterns of the four PCR-A and PCR-B false negative isolates were either identical or differed by a single band (see identical patterns of 17, 18 and 19, 20 in Figure 7.1a). In comparison to the PFGE pattern of the EMRSA-15 reference strain, isolates 17 and 18 had a 5-band difference and isolates 19 and 20 had a 6-band difference (see Figure 7.1a). Since these isolates depicted ≤6 band differences to the EMRSA-15 reference strain this indicated that they were related to this strain, according to the Tenover *et al.* (1995) criteria. The PFGE patterns of isolates 17 and 18 were termed EMRSA-15.2 and those of isolates 19 and 20 were termed EMRSA-15.2i.

The PFGE patterns of the PCR-C false negative isolates (21, 22, 18, 7 and 8) were diverse. Isolate 21 had a 3-band difference to the PFGE pattern of the EMRSA-16 reference strain whereas isolate 22 had a 3-band difference to the PFGE pattern of the EMRSA-15 reference strain, indicating that these isolates were closely related to the respective reference strains (see Figure 7.1b). The PFGE patterns of isolate 21 and 22 were termed EMRSA-16ii and EMRSA-15.1i respectively (isolate 18 was related to the EMRSA-15 reference strain). The remaining two PCR-C false negative isolates (7 and 8) had PFGE patterns which were dissimilar to the other isolates and the EMRSA-15 and EMRSA-16 reference strains, indicating that they were unrelated to all of the strains (see Figure 7.1b).

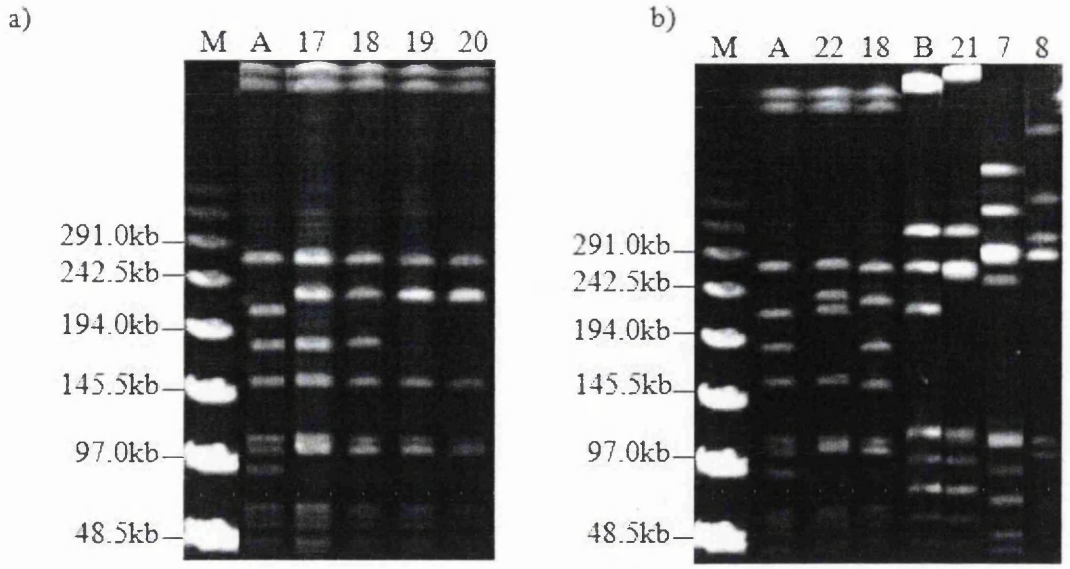


Figure 7.1. PFGE patterns of the false negative isolates (compiled from different gels). **a.** M, lambda DNA Marker; A, EMRSA-15 reference strain; PCR-A and PCR-B isolates 17, 18, 19 and 20. **b.** M, lambda DNA Marker; A, EMRSA-15 reference strain; PCR-C isolates 22 and 18; B, EMRSA-16; PCR-C isolates 21, 7 and 8.

7.1.1.2 *spa* typing of false negative isolates

Isolates 17, 18 and 20 exhibited *spa* type t4424 whereas 19 depicted *spa* type t032. These *spa* types are closely related albeit the presence of repeat r31 in t4424 in contrast to r23 in t032. The r31 repeat sequence (AAAGAAGA**T**GGCAACAAACCTGGC) differs from the r23 sequence (AAAGAAGA**C**GGCAACAAACCTGGC) by one nucleotide at position 9. The *spa* types of the four PCR-A and PCR-B false negative isolates were also closely related to the *spa* type of the EMRSA-15 reference strain (t025), indicating that these were EMRSA-15 strains, as previously ascertained by PFGE (see Table 7.6). In contrast, the *spa* types of the PCR-C false negative isolates (21, 22, 7, 8 and 18) were more diverse: t018, t032, t437, t1778 and t4424 respectively. The *spa* types of isolate 22 (t032) and 18 (t4424) were also closely related to the *spa* type of the EMRSA-15 reference strain whereas the *spa* type of isolate 21 (t018) was identical to the *spa* type of the EMRSA-16 reference strain. The unique *spa* types of isolates 7 and 8 showed that these isolates were unrelated to the other isolates and the reference strains. Therefore both the PFGE and *spa* typing results revealed that the four PCR-A and PCR-B false negative isolates were EMRSA-15 strains whereas the PCR-C isolates were a mixture of EMRSA-15 and EMRSA-16 strains and diverse MRSA strains.

7.1.1.3 SCC*mec* typing of false negative isolates

The four PCR-A and PCR-B false negative isolates (17, 18, 19 and 20) and the EMRSA-15 and EMRSA-16 reference strains were successfully typed with the Milheirico *et al.* (2007b) assay. The four isolates and the EMRSA-15 reference strain exhibited all the SCC*mec* type IV fragments (*mecA*, 162 bp; *ccrB2*, 311 bp and *dcs*, 342 bp) and were defined as MRSAIV strains (see Table 7.4). The five PCR-C isolates (18, 21, 22, 7 and 8) were more diverse, only isolate 21 exhibited all of the SCC*mec* type II fragments like the EMRSA-16 reference strain, whereas isolate 22 (and 18) depicted the SCC*mec* type IV fragments, thus these isolates were defined as MRSA II and MRSA IV strains. Isolates 7 and 8 exhibited only two of the three SCC*mec* type V fragments: *ccrC* (449 bp) and *mecA* (162 bp) whereas the SCC*mec* type V J1 fragment was not amplified, so they were provisionally defined as MRSAV strains (refer to section 4.1.4). With the MPCR-1 and MPCR-2 assays the isolates exhibited the expected SCC*mec* type V fragments: *ccrC* (518 bp) and *mecA* (286 bp) with MPCR-1 and *mecA*-IS*431* (804bp) with MPCR-2. However due to the absence of the SCC*mec* type V J1-fragment, these isolates could not be conclusively regarded as MRSAV strains (see Table 7.4 and 7.5).

Table 7.4. SCC*mec* typing results of PCR-A, PCR-B and PCR-C false negative isolates with the Milheirico *et al.* (2007b) method

	SCC*mec* types I to V loci										
	mecA	***mecI***	**III J1**	***kdp***	***ccrB2***	***dcs***	**V- J1**	**III J3**	***ccrC***	***pls***	**Type**
	162bp	209bp	243bp	284bp	311bp	342bp	377bp	414bp	449bp	495bp	
PCR-A and PCR-B false negative isolates											
17	+				+	+					IV
18*	+				+	+					IV
19	+				+	+					IV
20	+				+	+					IV
PCR-C false negative isolates											
21	+	+		+	+	+					II
22	+				+	+					IV
7	+						none		+		V?
8	+						none		+		V?

* Isolate 18, was a false negative for all three assays; none- expected product not amplified

Table 7.5. Results of isolate 7 and 8 with MPCR-1 and MPCR-2 assays

	mecA	***ccrA1B1***	***ccrA2B2***	***ccrA3B3***	***ccrA4B4***	***ccrC***
	286bp	695bp	937bp	1791bp	1287bp	518bp
Isolate 7 and 8	+					+
	mecA-mecI		***mecA*-IS*1272***		***mecA*-IS*431***	
	1963bp		2827bp		804bp	
Isolate 7 and 8					+	

Table 7.6. Genotypic and phenotypic characteristics of the PCR-A, PCR-B and PCR-C false negative isolates

Isolate	PFGE Pattern	*spa* typing type	Repeat	SCC*mec* type	Antibiotic resistance
PCR-A and PCR-B false negative isolates					
17	EMRSA-15.2	t4424	26-23-**31**-13-23-31-29-17-31-29-17-25-17-25-16-28	SCC*mec* type IV	Ox, Cip, Ery, FA
18*	EMRSA-15.2	t4424	26-23-**31**-13-23-31-29-17-31-29-17-25-17-25-16-28	SCC*mec* type IV	Ox, Cip, Ery, FA
19	EMRSA-15.2i	t032	26-23-**23**-13-23-31-29-17-31-29-17-25-17-25-16-28	SCC*mec* type IV	Ox, Cip, Ery, FA
20	EMRSA-15.2i	t4424	26-23-**31**-13-23-31-29-17-31-29-17-25-17-25-16-28	SCC*mec* type IV	Ox, Cip, Ery, FA
EMRSA-15 ref. strain		t025	26-23-**23**-13-23- -29-17-31-29-17-25-17-25-16-28	SCC*mec* type IV	Ox, Cip
EMRSA-16 ref. strain		t018	15-12-16-02-16-02-25-17-24-24-24	SCC*mec* type II	Ox, Cip
PCR-C false negative isolates					
21	EMRSA-16ii	t018	15-12-16-02-16-02-25-17-24-24-24	SCC*mec* type II	Ox, Cip
22	EMRSA-15.1i	t032	26-23-**23**-13-23-31-29-17-31-29-17-25-17-25-16-28	SCC*mec* type IV	Ox, Cip, Ery
7	Unique	t437	04-20-17-20-17-25-34	SCC*mec* type V*	Ox –S, Clin, Ery, Tet, Trim,
8	Unique	t1778	26-23-21-17-13-34-16-34-33-13	SCC*mec* type V*	Ox, Cip, Ery, FA, Trim

*Isolate 18, false negative of all three PCR-A, PCR-B and PCR-C assays. Ref.- reference Ox.-oxacillin, Cip.-ciprofloxacin, Ery.-erythromycin, FA-fusidic acid, Tet.-tetracycline, Trim.-trimethoprim. Ox-S. Oxacillin susceptible; SCC*mec* type V*- designation of SCC*mec* type provisional

7.1.1.4 Antibiotic susceptibility testing of the false negative isolates

Antibiotic susceptibility testing was performed with the BD Phoenix on all the false negative isolates and the EMRSA-15 and EMRSA-16 reference strains. All four PCR-A and PCR-B isolates exhibited resistance to the same non β-lactam antibiotics: ciprofloxacin, erythromycin, and fusidic acid (see Table 7.6). Even though these isolates were determined to be EMRSA-15 strains by PFGE and *spa* typing, their multiple antibiotic resistance profile was in contrast to the EMRSA-15 reference strain which was largely susceptible to non β-lactam antibiotics i.e. depicting resistance to ciprofloxacin only. The five PCR-C isolates (21, 22, 7, 8 and 18) had variable resistance to non β-lactam antibiotics i.e. ciprofloxacin (n=4), erythromycin (n=4), fusidic acid (n=2), trimethoprim (n=2) and tetracycline (n=1). Isolates 21 and 22 were determined to be EMRSA-16 and EMRSA-15 strains by PFGE and *spa* typing and exhibited narrow resistance profiles similar to the reference strains i.e. resistance to ciprofloxacin (and erythromycin). Interestingly, isolate 7 and 8 which had unique PFGE and *spa* type were resistant to more non β-lactam antibiotics than the other isolates. Notably isolate 7 was an unusual strain because it was *mecA* positive by PCR but was oxacillin susceptible and was resistant to clindamycin, erythromycin, tetracycline and trimethoprim. Isolate 8 had resistance to ciprofloxacin, erythromycin, fusidic acid and trimethoprim (see Table 7.6).

7.1.2 Molecular characterisation of the false positive isolates

7.1.2.1 Pulsed field gel electrophoresis

Seven MSSA isolates were amplified with the PCR-A and PCR-B assays i.e. 23, 24, 25, 26, 27, 28 and 16 and nine MSSA isolates were amplified with the PCR-C assay i.e. 29, 30, 31, 32, 33, 34, 35, 36 and 16 (isolate 16 was amplified in all three assays). The PFGE patterns of five (isolates 24, 25, 26, 27 and 28) of the seven PCR-A and PCR-B false positive isolates had some degree of similarity i.e. a ≤6 band difference, indicating that these isolates were possibly related (see Figure 7.2a). The PFGE patterns of isolates 24, 25, 26, 27, and 28 were termed PFGE-1.i, PFGE-1.ii, PFGE-1.iii, PFGE-1.iv and PFGE-1.v respectively. In contrast, only two (isolates 31 and 33) of the seven PCR-C false positive isolates exhibited patterns which were identical (termed PFGE-3) whereas the remaining five isolates had diverse patterns (>7 band difference), indicating that only two of the PCR-C false positive isolates were related and the majority of the isolates were unrelated (see Figure 7.2b).

The PFGE patterns of the false positive isolates were different to PFGE patterns of the EMRSA-15 and EMRSA-16 reference strains, except for isolate 16 which had ≤3 band difference to the EMRSA-15 reference strain pattern, illustrating that this MSSA isolate was closely related to the EMRSA-15 reference strain and the PFGE pattern of isolate 16 was termed EMRSA-15.1.4 (see Figure 7.1c).

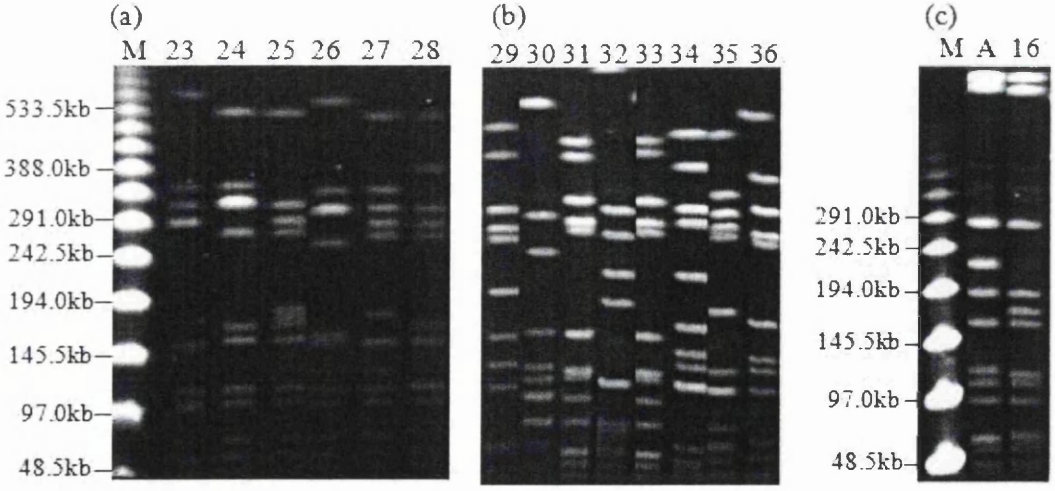


Figure 7.2. PFGE patterns of the false positive isolates (compiled from different gels). **a.** M, lambda DNA Marker; PCR-A and PCR-B isolates 23, 24, 25, 26, 27, and 28. **b.** M, lambda DNA Marker; PCR-C isolates 29, 30, 31, 32, 33, 34, 35 and 36. **c.** M, lambda DNA Marker; A, EMRSA-15 reference strain; isolate 16.

7.1.2.2 *spa* typing of false positive isolates

The *spa* types of the false positive isolates also revealed that the PCR-A and PCR-B isolates were genetically related in contrast to the more diverse PCR-C isolates. The five PCR-A and PCR-B false positive isolates (24, 25, 26, 27 and 28) that had exhibited PFGE patterns with some degree of similarity also had identical *spa* types or closely related types. Isolates 25, 26 and 28 had *spa* type t127 whereas 24 and 27 had *spa* types t1381 and t559 which were related variants of t127 (see Table 7.7). The t1381 *spa* type differs from t127 by the presence of the terminal r34 sequence (AAAGAAGACAACAA**A**AAACCTGGT) which differs from the r13 sequence (AAAGAAGACAACAA**C**AAACCTGGT) by one nucleotide at position 15. Whereas t559 differs from t127 by three deleted repeat sequences (r16-r34-r33). Only the two PCR-C isolates (31 and 33) which had identical PFGE patterns had related *spa* types i.e. t216 and t471. These differed by the presence of repeat r20 in t216 versus r02 in t471. The r20 repeat sequence (AAAGAAGACAACAA**C**AAACCTGGC) differs from the r02 sequence (AAAGAAGACAACAA**A**AAACCTGGC) by one nucleotide at position 15.

Table 7.7 Genotypic characteristics of false positive isolates by PFGE and *spa* tying

PCR-A and PCR-B false positive isolates			
Isolate	**PFGE pattern**	***spa* type**	***spa* repeat**
23	PFGE-2	t1778	26-23-21-17-13-34-16-34-33-13
24	PFGE-1.i	t1381	07-23-21-16-34-33-**34**
25	PFGE-1.ii	t127	07-23-21-16-34-33-**13**
26	PFGE-1.iii	t127	07-23-21-16-34-33-**13**
27	PFGE-1.iv	t559	07-23-21- - - -13
28	PFGE-1.v	t127	07-23-21-16-34-33-13
16*	EMRSA-15.1.4	t022	26-23-13-23-31-29-17-31-29-17-25-17-25-16-28
PCR-C false positive isolates			
Isolate	**PFGE pattern**	***spa* type**	***spa* repeat**
31	PFGE-3	t216	04-**20**-17-20-17-31-16-34
33	PFGE-3	t471	04-**02**-17-20-17-31-16-34
29	Unique	t311	26-23-17-34-20-17-12-17-16
30	Unique	t089	04-33-31-12-16-34-16-12-33-34
32	Unique	t917	09-02-16-34-13-13-17-34-16-34
34	Unique	t587	14-12-23-02-02-34-34
35	Unique	t091	07-23-21-17-34-12-23-02-12-23
36	Unique	t008	11-19-12-21-17-34-24-34-22-25

* Isolate 16, false positive of all three PCR-A, PCR-B and PCR-C assays

7.1.2.3 SCC*mec* typing of false positive isolates

The amplification of MSSA isolates with the PCR-A, PCR-B and PCR-C SCC*mec-orfX* assays indicated the presence of the right SCC*mec*-chromosome junction, *attR*. Despite the lack of *mecA*, the detection of *attR* in these isolates suggested the possible presence of SCC elements (elements lacking *mecA*) or remnant SCC*mec* elements resulting from an imprecise excision. The Milheirico *et al.* (2007b), MPCR-1 (*ccr* complex) and MPCR-2 (*mec* complex) typing assays were performed to detect any SCC*mec* associated loci in the isolates. Only four (isolates 16, 29, 30 and 35) yielded PCR fragments and the *mecA* fragment was not exhibited in any of the isolates. With the Milheirico *et al.* (2007b) assay, only isolate 16 yielded a single 342 bp fragment corresponding to the expected size of the *dcs* fragment which is present in SCC*mec* types I, II and IV, no further fragments were attained with the other typing assays (see Figure 7.3a). Isolates 29, 30 and 35 (PCR-C false positive isolates) yielded a single fragment with the MPCR-1 assay but none with the Milheirico *et al.* (2007) and MPCR-2 assays:

i) Isolate 29 - 1 287 bp fragment (*ccrAB4*), see 29 in Figure 7.3b.

ii) Isolate 30 - 695 bp fragment (*ccrAB1*), see I and 30 in Figure 7.3b.

iii) Isolate 35 - 518 bp fragment (*ccrC*), see V and 35 in Figure 7.3b.

To verify that the fragments were not non-specific amplification products resulting from the multiplex PCR procedures, the assays were repeated independently using single pairs of primers for *ccrAB1*, *ccrAB4*, *ccrC* and *dcs* respectively. MRSA reference strains containing SCC*mec* types I (NCTC10442), IVb (JCSC1978) and V (WIS) were also included as positive controls for the amplification of *ccrAB1*, *dcs* and *ccrC* respectively. An MRSA strain containing a SCC*mec* type VI which harbours *ccrAB4* was not available, thus the expected size (1 287 bp) of the *ccrAB4* fragment was used for verification. Isolates 16, 29, 30 and 35 yielded the same results as initially observed and it was concluded that the PCR fragments were due to specific amplification of existing SCC*mec* associated sequences harboured in these isolates:

i) 342 bp fragment (*dcs*) - see IVb and 16 in Figure 7.4

ii) 1 287 bp fragment (*ccrAB4*) - see 29 in Figure 7.4

iii) 695 bp fragment (*ccrAB1*) – see I and 30 in Figure 7.4

iv) 518 bp fragment (*ccrC*) – see V and 35 in Figure 7.4

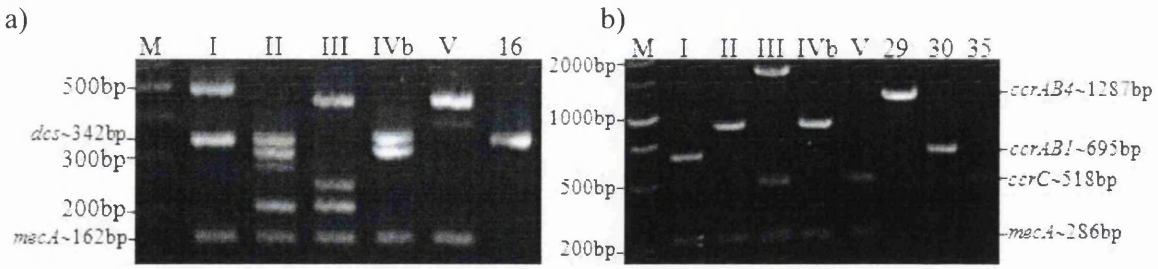


Figure 7.3. PCR fragments of the SCC*mec* typing assays in MRSA reference strains and false positive isolates. (a) Milheirico *et al.* (2007) fragments. M, 100bp molecular marker; I, II, III, IVb, V- MRSAI, MRSAII, MRSAIII, MRSAIVb, MRSAV; 16, isolate 16. (b) MPCR-1 fragments M, 1kb molecular marker; I, II, III, IVb, V- MRSAI, MRSAII, MRSAIII, MRSAIVb, MRSAV; isolates 29, 30, 35.

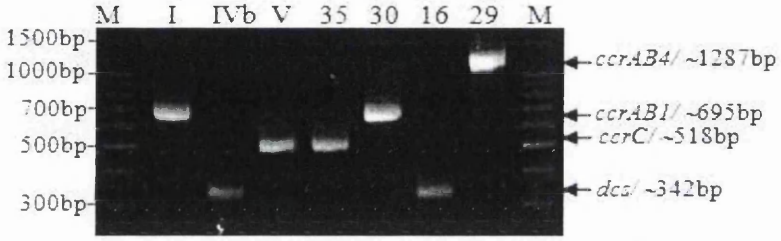


Figure 7.4. PCR fragments of the MPCR-1 assay in MRSA reference strains and false positive isolates. M, 100bp molecular marker; I, IVb, V- MRSAI, MRSAIVb and MRSAV; Isolates 35, 30, 16 and 29.

7.1.2.4 Investigation of SCC*mec* associated DNA in false positive isolates

SCC*mec* elements integrate at *att*B which is located at the 3' terminus of *orfX*. This site comprises a conserved 15 bp sequence (TTATGATACGCCTC) which is duplicated at the other end of the element during insertion, thus generating direct repeats which flank the left and right ends (see Figure 7.6). Degenerate inverted repeats are also formed at the left and right SCC*mec* boundaries, thus the left and right chromosome-SCC*mec* junctions are characterised by direct (*att*B-L and *att*B-R) and inverted repeats and are referred to as *att*L and *att*R respectively (see Figure 7.5 and 7.6). When the element is excised the *att*B site is reconstituted in the chromosome and the ends of the SCC*mec* element combine forming a circular version of the element (Wang and Archer, 2010, Ito et al., 2004, Ito et al., 2001).

The further detection of *ccrAB1, ccrAB4, ccrC* and *dcs* in the four MSSA isolates, implied the possible presence of SCC elements (elements lacking *mecA*) or remnant SCC*mec* elements and this was investigated by undertaking efforts to amplify the sequence from the left to the right SCC*mec*-chromosome junctions (see figure 7.5). Three respective forward primers located upstream of *att*B-L in MRSAI, II, IVa, IVb, IVc strains and a single reverse primer (cR2) located in *orfX* (Jansen *et al.* 2006, Katayama *et al.* 2000) were employed for this study and the MRSAI, II, MRSAIVb and MRSAIVc reference strains were also included. A long range high fidelity PCR kit (Roche) was used, the fragments were resolved in a 1% agarose gel and then sequenced.

i) Forward primer, cL1 (*att*B-L of MRSAI, II and IVb) and reverse primer cR2

ii) Forward primer, E3 (*att*B-L of MRSAIVc) and reverse primer cR2

iii) Forward primer, E4 (*att*B-L of MRSAIVa) and reverse primer cR2

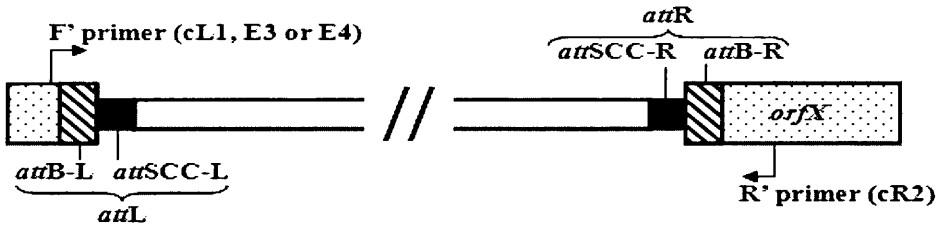


Figure 7.5. A schematic diagram of the left (*att*L) and right (*att*R) SCC*mec*-chromosome junctions and the locations of the forward and reverse primers (Katayama *et al.*, 2000).

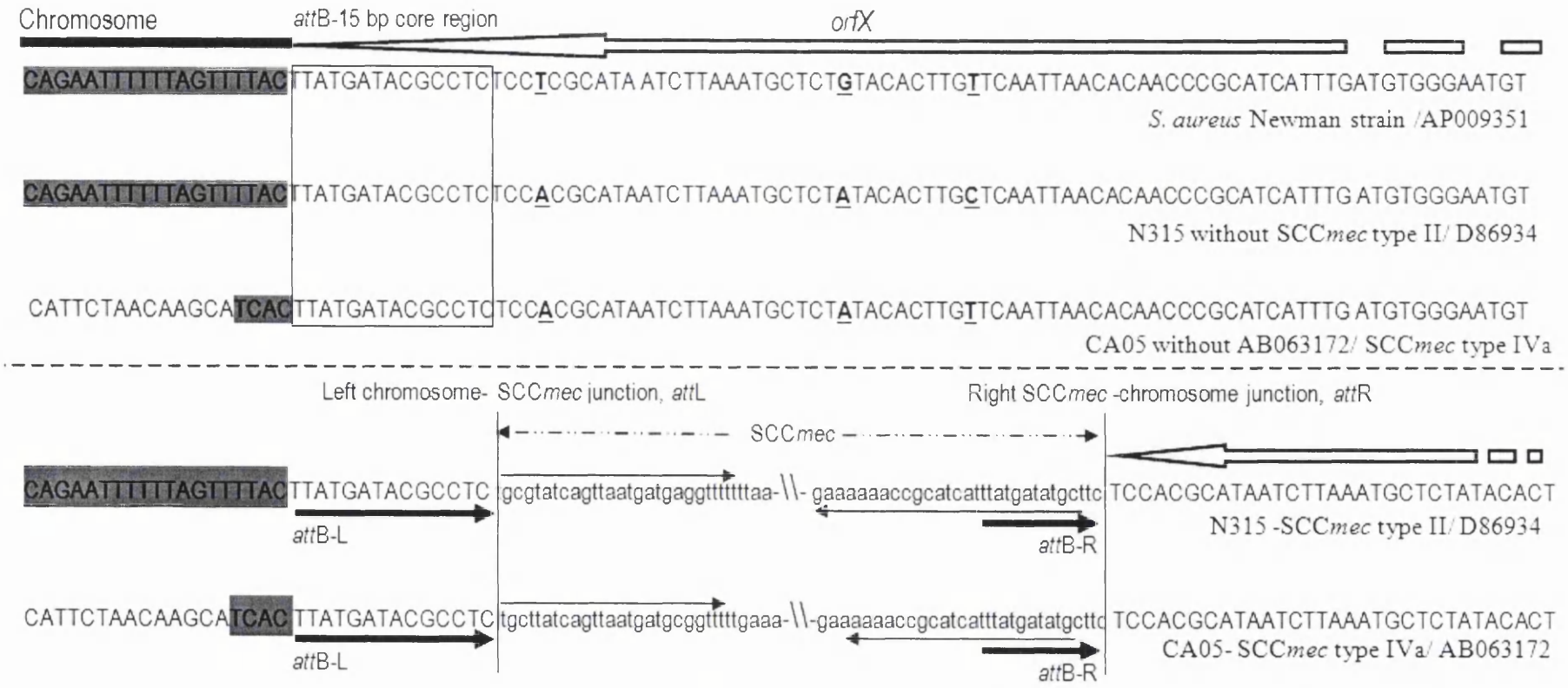


Figure 7.6. Nucleotide alignment of *att*B and surrounding sequences in *S. aureus* strains prior and post integration of SCC*mec* (Noto *et al.*, 2008). Higher case letters are chromosome sequences and lower case letters are SCC*mec* sequences. Box- *att*B core sequence, grey-conserved regions upstream of *att*B. ➡ Direct repeat at left and right chromosome-SCC*mec* junctions, ⟶ inverted repeats at left and right chromosome-SCC*mec* junctions

Regrettably the attempts to amplify the sequence spanning from *att*L to *att*R were largely unsuccessful and no PCR fragments were obtained with the PCR-A and PCR-B false positive isolates and the MRSA reference strains (including isolates 16, 29, 30 and 35 which had previously yielded the *dcs*, *ccrAB4, ccrAB1* and *ccrC* fragments). Three of the nine PCR-C isolates (32, 34 and 36) yielded relatively small fragments:

i) Isolate 32: approximately 600 bp fragment with E4 and cR2 primers

ii) Isolates 34, 36: approximately 550 bp fragment with cL1 and cR2 primers

The complete SCC*mec* type I to V elements are considerably large (ca. 20-60 kb) thus the failed amplification of a PCR fragment in the MRSAI, II, IVb and IVc reference strains was plausible as the PCR assay employed in this study had an amplification limit of 10 kb. Therefore it is also likely that in the false positive isolates, in which no fragment was attained, a SCC element or remnant SCC*mec* may have been present but it was larger than 10kb and thus the PCR conditions were inadequate for successful amplification of the entire sequence.

The small fragments of isolate 32, 34 and 36 (600 bp, 550 bp and 550 bp respectively), implied that either the *att*B site did not contain SCC*mec* associated sequences or that the existent sequences were short. The amplified fragments were sequenced and the sequences were compared to published ones in Genbank with the NCBI BLAST programme. Those of isolates 34 and 36 (549 nucleotides) were highly similar albeit five nucleotide differences and they were homologous (99%) to a region (nucleotides 33685 to 34232) in the *S. aureus* Newman DNA/ AB009351 (see Figure 7.7). This region comprised *att*B and the sequences upstream and downstream of this site. Notably the *S. aureus* Newman strain does not harbour SCC*mec* or SCC elements and so this also indicated that isolates 34 and 36 did not contain SCC*mec* remnant sequences or SCC elements. However the sequences of isolate 34 and 36 were also homologous (99%) to two regions in the NCTC10442, N315, and JCSC1978 MRSA strains which harbour AB033763/ SCC*mec* type I, D86934/ SCC*mec* type II and AB063173/ SCC*mec* type IVb, respectively. Furthermore the sequence of isolate 32 (597 nucleotides) was homologous to two regions in the CA05 and WIS MRSA strains (99% and 96% homology respectively) which carry the AB063172/ SCC*mec* type IVa and AB121219/ SCC*mec* type V elements (see Figure 7.8).

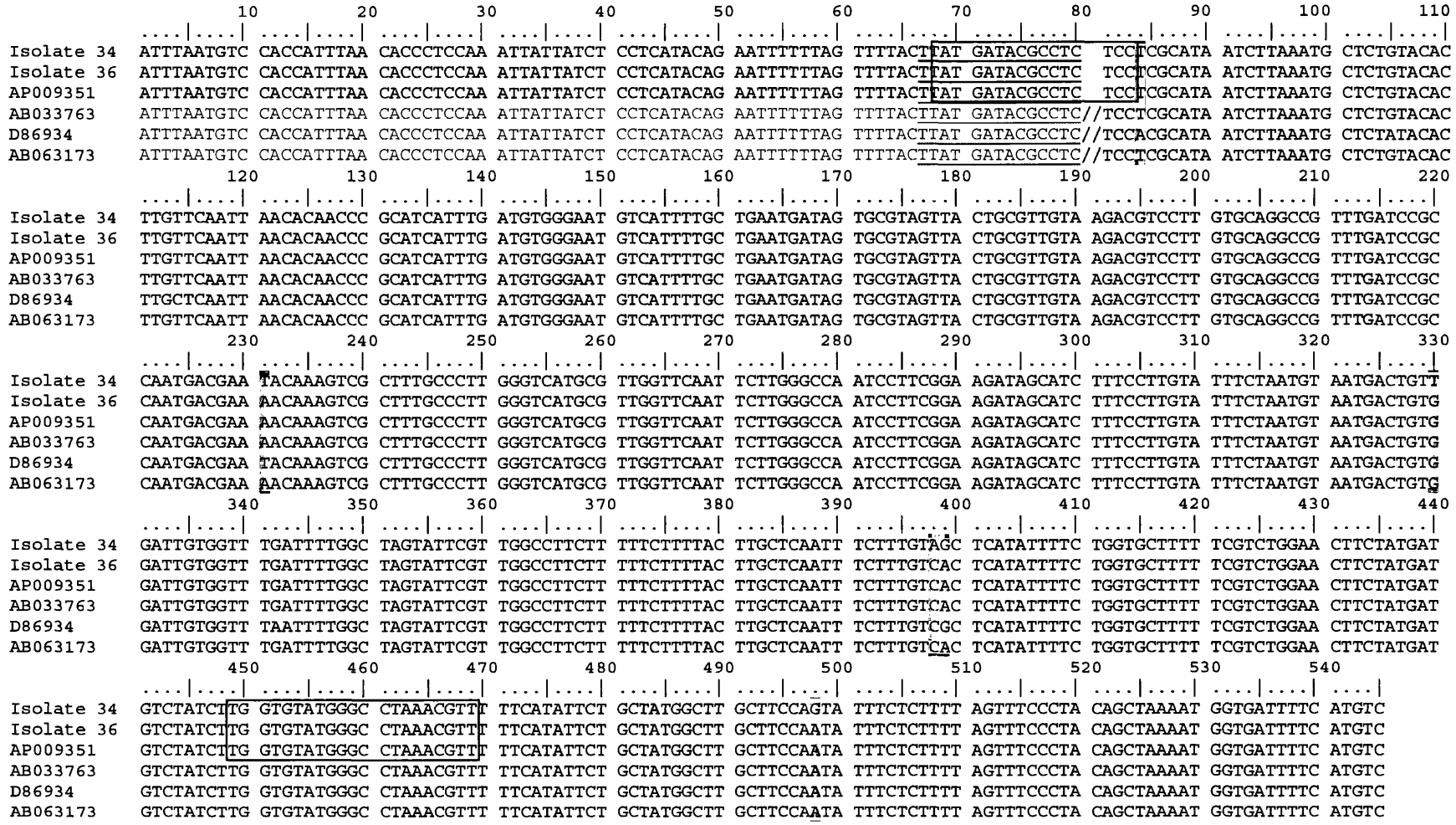


Figure 7.7. The alignment of DNA sequences of isolates 34, 36, AP009351/ *S. aureus* Newman DNA, AB0033763/SCC*mec* type I, D86934/ SCC*mec* type II and AB063173/ SCC*mec* type IVb. Underlined-*att*B; //-position of SCC*mec* in MRSA strains; red-sequence upstream of left SCC*mec*-chromosome junction; black- sequence downstream of right SCC*mec*-chromosome junction; blue-nucleotide differences; box-PCR-C forward and reverse primers.

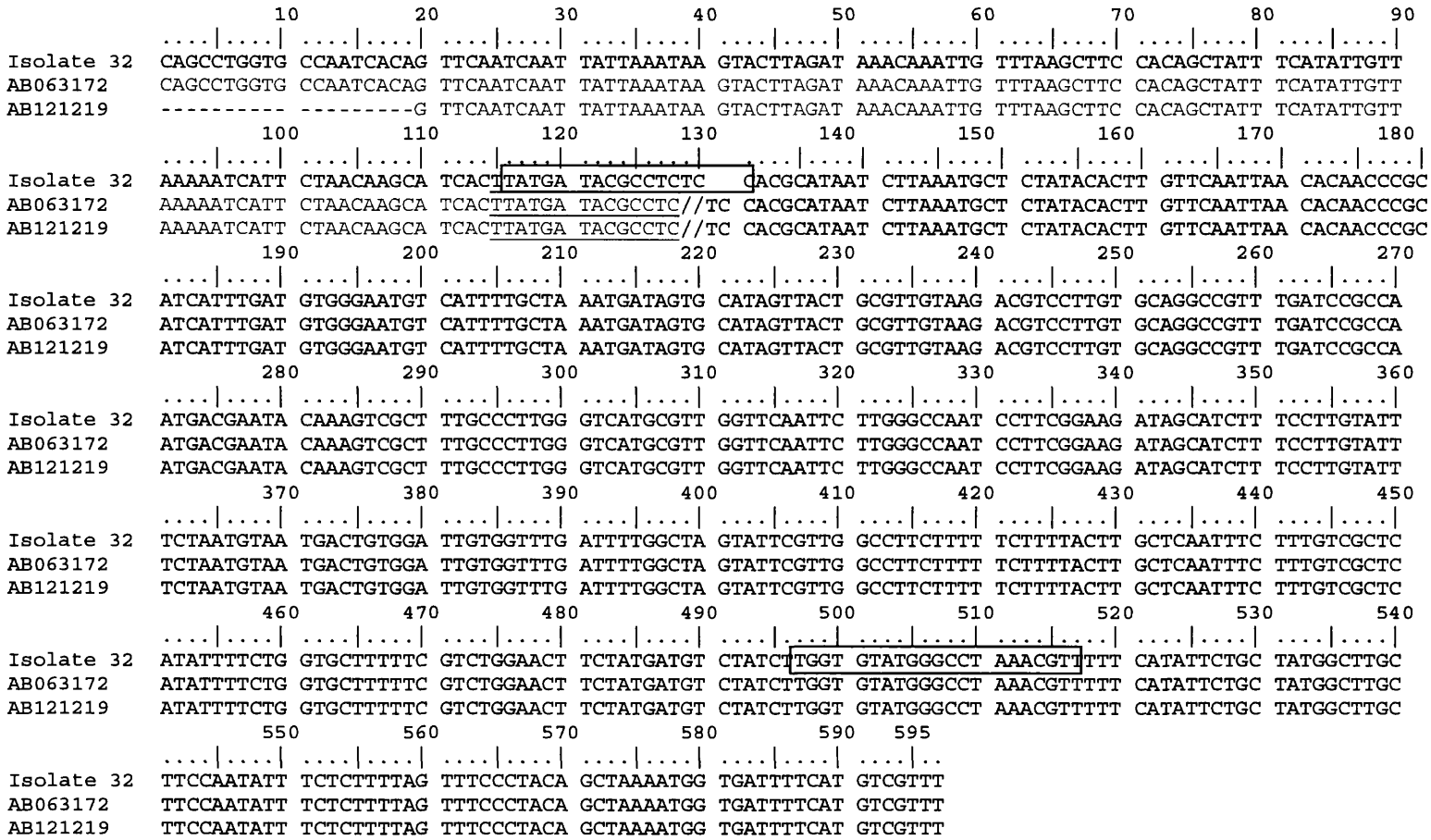


Figure 7.8. The alignment of DNA sequences of isolate 32, AB063172/ SCC*mec* type IVa and AB121219/ SCC*mec* type V. --- no sequence available; Underlined- core sequence of *att*B; //- position of SCC*mec* in MRSA strains; red-sequence upstream of left SCC*mec*-chromosome junction; black-sequence downstream of right SCC*mec*-chromosome junction; blue-nucleotide differences; box-PCR-C forward and reverse primers.

The sequences of isolates 32, 34 and 36 were homologous to sequences outside of the SCC*mec* elements which did not include the terminal inverted repeats or other characteristic SCC*mec* loci which are internally located in the elements e.g. recombinase genes (*ccrAB* or *ccrC*). For instance in the AB063172/ SCC*mec* type IVa sequence, the first 129 nucleotides of the isolate 32 sequence aligned to a region upstream of the left chromosome-SCC*mec* junction, extending up to the end of *att*B-L and nucleotides 130 to 597 aligned to a region downstream of the right SCC*mec*-chromosome junction (see CA05/AB063172/ SCC*mec* type IVa in Figure 7.9). In SCC*mec* type V, nucleotides 20 to 128 of isolate 32 aligned to a sequence upstream of the left chromosome-SCC*mec* junction, extending up to the end of *att*B-L and nucleotides 129 to 597 aligned to the sequence downstream of the right SCC*mec*-chromosome junction (see WIS/AB121219/ SCC*mec* type V in Figure 7.9). Similar results were observed with isolates 34 and 36, the sequences aligned to regions exclusively outside of the left and right junctions of SCC*mec* type I, II and IVb elements (see AB033763/ SCC*mec* type I and D86934/ SCC*mec* type II in figure 6A.12, since the sequences of isolate 34 and 36 aligned to identical positions, only isolate 34 is shown in Figure 7.9).

Even though *att*B-L was detected, the lack of detection of inverted repeats indicated that the left and right boundaries of SCC*mec* were not present in isolate 32, 34 and 36. These results indicate that the sequences of isolates 32, 34 and 36 (597 nt, 545 nt and 545 nt respectively) do not harbour remnant SCC*mec* or SCC elements rather they comprise an unoccupied *att*B site and the sequences upstream and downstream of this site. The sequences of isolates 34 and 36 are homologous to the *att*B site and adjacent sequences of the *S. aureus* Newman MSSA strain. The alignment of the sequences to regions outside of the SCC*mec* elements in the MRSA strains (NCTC10442, N315, CA05, JCSC1978 and WIS) suggests that isolates 32, 34 and 36 contain *att*B and adjacent sequences which are also homologous to the *att*B and adjacent sequences of certain *S. aureus* strains which subsequently acquired SCC*mec* types I (AB033763), II (D86934), IVa (AB063172), IVb (AB063173) and V (AB121219) elements (see Figure 7.6).

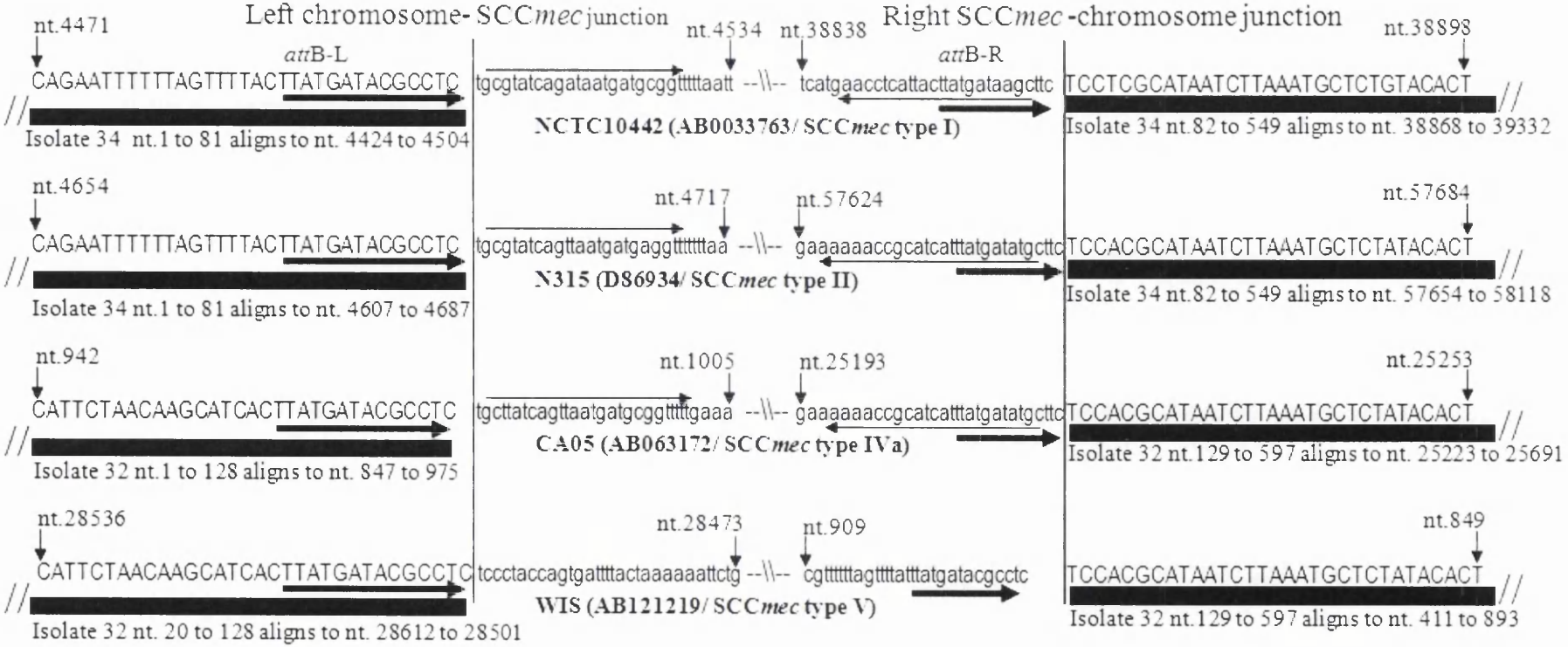


Figure 7.9. The nucleotide sequences at left (*att*L) and right (*att*R) chromosome-SCC*mec* junctions of SCC*mec* type I/ AB033763, SCC*mec* type II/ D86934, SCC*mec* type IVa/ AB063172 and SCC*mec* type V/ AB121219. Higher case letters are chromosome sequences and lower case letters are SCC*mec* sequences. ➞ Direct repeat at left and right junctions, ⟶ degenerate inverted repeats at left and right junctions, ▬ sequences of isolates 32 and 34 aligned at left and right junctions, numbers indicating the nucleotide positions at both boundaries.

The lack of detection of any remnant SCC*mec* sequences in isolates 32, 34 and 36 is seemingly contradictory to initial results which showed that these isolates were amplified with the PCR-C SCC*mec-orfX* assay, which is based on the detection of the right SCC*mec*-chromosome junction. It was previously shown (presented in chapter 5) that the PCR-C forward primer had up to two mismatches to enable the detection of SCC*mec* types I, III and V however consequently there were identical regions in two published MSSA strains i.e. the *S. aureus* Newman and *S. aureus* RF122 strains (see Table 7.8). This indicated that this method could potentially amplify both MRSA and MSSA strains. Evidently the target sequences for the PCR-C primers were also present in isolates 32, 34 and 36 demonstrating that the amplification of these clinical MSSA isolates was not due to the presence of a remnant SCC*mec-orfX* junction but rather chromosomal sequences which contain the PCR-C primer binding sites (see Figure 7.7, 7.8 and Table 7.8).

Table 7.8. PCR-C forward primer binding site in sequences of SCC*mec* type I, III, V and MSSA strains

Accession number / SCC*mec* type	**Sequence**	**Position**
PCR-C forward oligonucleotide	TATGATA**T**GC**T**TCTCC	--
AB033763 (SCC*mec*I)/ NCTC10442	TATGATA**A**GCTTCTCC	38855 - 38870
AB037671 (SCC*mec*III)	TATGATA**C**GCTTCTCC	67782 - 67797
AB121219 (SCC*mec*V)/ WIS	TATGATA**C**GC**C**TCTCC	892 - 877
AP009351 (*S. aureus* Newman)	TATGATA**C**GC**C**TCTCC	34392 - 34377
AJ938182 (*S. aureus* RF122)	TATGATA**C**GC**C**TCTCC	34166 - 34151
Isolate 32 (clinical isolate, this study)	TATGATA**C**GC**C**TCTCC	116 -131 (figure 7.8)
Isolate 34 (clinical isolate, this study)	TATGATA**C**GC**C**TCTCC	68 - 83 (figure 7.7)
Isolate 36 (clinical isolate, this study)	TATGATA**C**GC**C**TCTCC	68 - 83 (figure 7.7)

7.1.2.5 Antibiotic susceptibility testing of the false positive isolates

Antibiotic susceptibility testing was performed with the BD Phoenix on all the PCR-A, PCR-B and PCR-C false positive isolates and collectively the isolates were resistant to penicillin and all except one were resistant to trimethoprim (see Table 7.7). Only three isolates (23, 26 and 29) exhibited resistance to ≥3 classes of non β-lactam antibiotics: isolate 23 was resistant to ciprofloxacin, erythromycin, fusidic acid and trimethoprim; isolate 26 was resistant to ciprofloxacin, fusidic acid, tetracycline and trimethoprim; isolate 29 was resistant to ciprofloxacin, erythromycin, and trimethoprim. The remaining isolates had narrow resistance profiles and only a few were resistant to erythromycin (n=2), ciprofloxacin (n=1), fusidic acid (n=1) and gentamicin (n=1).

The clinical history of the patients from whom the fifteen isolates were identified was investigated in order to ascertain if MRSA isolates had been previously or later reported from the same patients. The information was retrieved from the Public Health Wales (PHW) Microbiology ABM Laboratory data management system (see Table 7.9). In thirteen of the fifteen cases there were no previous or later reports of MRSA from the patients and in six of these cases, there seemed to be no prior or later submissions of wound specimens to the PHW microbiology lab. However in two cases i.e. isolate 16 and 25, MRSA had been reported in the PHW microbiology data management system. In the case of isolate 16, it had been reported as an MRSA and there was considerable history (ten months) of intermittent reports of MRSA from the patient, including about four months prior and two months after isolate 16 was reported. In the case of isolate 25, an MRSA isolate was reported from the patient 5 months after isolate 25 was reported.

Table 7.9. Genotypic and phenotypic characteristics of false positive isolates and the bacteria identified prior or post the false positive isolates

PCR-A and PCR-B false positive isolates

Isolate	Genotypic characteristics			Antibiotic resistance	Reported results by PHW Microbiology ABM Laboratory		
	SCC*mec* DNA	PFGE	*spa*		Report date	Associated infection	Bacteria reported
					16/01/2007	Wound discharge	**MRSA**
					23/03/2007	Leg ulcer	**MRSA**
					13/04/2007	Leg ulcer	**MRSA**
16*	*dcs*	EMRSA-15.1.4	t022	Pen, Cip	13/08/2007*	Discharging ulcer	**MRSA,** Anaerobes, Corynebactrium
					30/10/2007	Discharging ulcer	**MRSA**
23	none	PFGE-2	t1778	Pen, Cip, Ery, FA, Trim	25/04/2007	Wound on right big toe	MSSA, Anaerobes No MRSA reported previously or later
24	none	PFGE-1.i	t1381	Pen, Trim	13/05/ 2007	Wound swab	MSSA No MRSA reported previously or later
25	none	PFGE-1.ii	t127	Pen, Trim	20/05/2007	Left ankle wound not healing	MSSA
					08/10/2007	Left ankle swab	**MRSA**
26	none	PFGE-1.iii	t127	Pen, Cip, FA, Tet, Trim	25/07/2007	Necrotic lesions on lower limbs	MSSA No MRSA reported previously or later
27	none	PFGE-1.iv	t559	Pen, FA, Trim	30/11/2007	Discharging pin site	MSSA No MRSA reported previously or later
28	none	PFGE-1.v	t127	Pen, Trim	31/11/2007	Infected catheter site	MSSA, *S. epidermidis,* Coliforms No MRSA reported previously or later

*Isolate 16- false positive of PCR-A, PCR-B and PCR-C; Pen.-penicillin, Cip.-ciprofloxacin, Ery.-erythromycin, FA-fusidic acid, Tet-tetracycline, Trim-trimethoprim, Gent-gentamicin, *S. viridians- Streptococcus viridians*

Table 7.9. Genotypic and phenotypic characteristics of false positive isolates and the bacteria identified prior or post the false positive isolates

PCR-C false positive isolates

Isolate	Genotypic characteristics			Antibiotic resistance	Reported results by PHW Microbiology ABM Laboratory		
	SCC*mec* DNA	PFGE	*spa*		Report date	Associated infection	Bacteria reported
29	*ccrAB4*	Unique	t311	Pen, Cip, Ery, Trim	16/05/2007	Wound infection	MSSA No further results
30	*ccrAB1*	Unique	t089	Pen, Trim	26/01/2007	Shoulder wound post trauma	MSSA, *S. viridians* No further results
31	none	PFGE-3	t216	Pen, Ery, Trim	18/07/2007	Infected eczema, right buttock	MSSA No further results
32	none	Unique	t917	Pen, Trim	24/07/2007	Leg amputation	MSSA No further results
33	none	PFGE-3	t471	Pen, Trim	25/07/2007	Inflamed wound	MSSA No further results
34	none	Unique	t587	Pen, Ery, Trim	25/07/2007	Chemical burn, right foot	MSSA, Acinetobacter, *S. viridians* No MRSA reported previously of later
35	*ccrC*	Unique	t091	Pen, Gent, Trim	03/08/2007	Inflamed wound with exudate	MSSA, *S. epidermidis,* Corynebactrium No further results
36	none	Unique	t008	Pen	11/07/2007	Eye infection	MSSA No MRSA reported previously of later

Pen.-penicillin, Cip.-ciprofloxacin, Ery.-erythromycin, FA-fusidic acid, Tet-tetracycline, Trim-trimethoprim, Gent-gentamicin, *S. viridians- Streptococcus viridians*

7.2 Discussion

The SCC*mec-orfX* junction presents an ideal genetic marker for methicillin resistance in *S. aureus* and the amplification of this junction signifies detection of MRSA. However, eight MRSA and fifteen MSSA isolates had false negative and false positive results with PCR-A, PCR-B and PCR-C SCC*mec-orfX* amplification assays and this provided an opportunity to determine the genotypic characteristics of these atypical clinical isolates by performing PFGE, *spa* tying and SCC*mec* typing assays.

Both PFGE and *spa* typing revealed that five of the eight false negative MRSA isolates (isolate 17, 18, 19, 20 and 22) were EMRSA-15 strains which harboured the SCC*mec* type IV element whereas isolate 21 was an EMRSA-16 strain which contained the SCC*mec* type II element. EMRSA-15 and EMRSA-16 are dominant hospital acquired MRSA clones in the UK therefore these results imply that despite the capacity of the PCR-A, PCR-B and PCR-C methods to detect diverse MRSA strains, certain local endemic strains may be undetected with these assays (Rollason et al., 2008, Johnson et al., 2005). The two remaining isolates (7 and 8) were PCR-C false negative isolates which were unrelated to EMRSA-15 and EMRSA-16 reference strains. These isolates contained SCC*mec* elements that seemed to have some similarity to the SCC*mec* type V element harboured in the MRSAV WIS reference strain (refer to section, 4.1.4 in chapter 4). Previously it had been observed the PCR-C assay did not robustly amplify the MRSAV WIS strain (results presented in chapter 5) thus it is likely that isolates 7 and 8 were not adequately amplified due to the carriage of an SCC*mec* element which is similar to the one contained in the MRSAV WIS strain.

On the other hand with the PCR-A and PCR-B methods isolates 7 and 8 were successfully amplified even though the MRSAV WIS strain had not been amplified with both assays. A previous examination of the primer sequences of these methods demonstrated that they were not specific for the SCC*mec* type V/ AB121219 element contained in this strain (refer to section 5.1.1, chapter 5). Therefore this further suggests that the SCC*mec* elements harboured in isolates 7 and 8 may be similar but not identical to the SCC*mec* type V element of the MRSAV WIS strain.

SCC*mec*-associated loci i.e. *dcs, ccrAB1, ccrAB4* and *ccrC*, were detected in four (16, 29, 30 and 35) of the fifteen false positive MSSA isolates. The detection of SCC*mec* associated DNA in MSSA isolates has also been described in several other studies (Bartels et al., 2009, Shore et al., 2008, Donnio et al., 2007, Rupp et al., 2006, Donnio et al., 2005, Corkill et al., 2004, Huletsky et al., 2004). Although the *dcs* locus (downstream constant segment) was detected in isolate 16, it is typically located downstream of *mecA* and closely mapped to *orfX* in SCC*mec* types I, II and IV (Oliveira and de Lencastre, 2002, Oliveira et al., 2000). The detection of *dcs* in MSSA strains has also been described by Shore *et al.* (2008), Donnio *et al.* (2007, 2005) and Corkill *et al.* (2004). Since the MSSA in these studies had genetic backgrounds which were indistinguishable to prevalent local HA-MRSA strains, the authors concluded that the MSSA had emanated from the local HA-MRSA strains. Similarly in this study, the PFGE pattern and *spa* type of isolate 16 (EMRSA-15.1.4 and *spa* type t022) indicated that it was closely related to the EMRSA-15 reference strain. Therefore it is likely that isolate 16 resulted from the imprecise excision of a SCC*mec* type IV element from a local EMRSA-15 strain and consequently a fragment containing the *dcs* locus remained in this isolate. Since *dcs* is typically just upstream of *orfX*, it is plausible that the remnant fragment constituted the right SCC*mec-orfX* junction and resulted in the detection of isolate 16 by the PCR-A, PCR-B and PCR-C assays. In the studies by Shore *et al.* (2008) and Donnio *et al.* (2007) they were able to confirm that the *dcs* locus was located in SCC*mec* fragments in the MSSA isolates with various PCR and DNA sequencing experiments. Disappointingly in this study attempts to amplify possibly existent SCC*mec* remnants were unsuccessful and thus the actual location of *dcs* and the other detected loci i.e. *ccrAB1, ccrAB4* and *ccrC* was not established.

The integration and excision of SCC*mec* type I to V elements is mediated by site-specific recombinases which are encoded by *ccr* gene complexes located within SCC*mec*. Non-*mecA* SCC elements contained in certain *S. aureus* strains e.g. SCC*cap*1, SCC_{476} also contain recombinase genes and it is postulated that these genes have the same role in these elements (Hanssen and Sollid, 2006). The *ccrAB1* locus which was detected in isolate 30, is typically located in SCC*mec* type I elements carried by MRSA I strains. However, Corkill *et al.* (2004) detected *ccrAB1* in MSSA clinical isolates which were fusidic acid-resistant and these had been isolated from patients with bacteraemia, initially caused by fusidic acid-resistant MRSA. They ascertained

indistinguishable genetic backgrounds of the MSSA and MRSA and concluded that the MSSA emanated from the MRSA. It is tempting to draw similar implications in this study and assume that the *ccrAB1* in isolate 30 resulted from a previous excision of a SCC*mec* type I element from a MRSA I strain or from the presence of a non *mecA* SCC element. However the location of this locus with respect to SCC*mec-orfX* right junction could not be established and thus these propositions cannot be verified.

The detection of *ccrAB4* in isolate 29 was performed with oligonucleotides by Kondo *et al.* (2007) based on the *ccrAB4* sequence of the HDE288 strain. This is a prototypic strain of a dominant paediatric MRSA clone in Portugal which was subsequently re-defined as MRSA VI due to the novelty of the recombinase genes harboured in the SCC*mec* element of this strain (Oliveira et al., 2006). However, subsequently Shore *et al.* (2008) also identified the *ccrAB4* gene in several MSSA isolates. The gene was located in chromosomal regions outside of residual fragments of SCC*mec* type II variants and it was 100% homologous to *ccrAB4* harboured in SCC-CI, a composite island in the *S. epidermidis* ATCC 12228 strain. Another *ccrAB4* allotype has been identified in MRSA clinical isolates containing the SCC*mec*N1 element, identified in intravenous drug users in Switzerland (Ender et al., 2007). These studies indicate that there are various *ccrAB4* allotypes in diverse staphylococci genetic backgrounds and they are not restricted to intact or residual SCC*mec* elements. Thus the detection of *ccrAB4* in isolate 29 does not necessarily imply that this isolate emanated from a MRSA strain because it is possible that *ccrAB4* is innate to the chromosome of this isolate.

A single MSSA (isolate 35) isolate yielded a PCR product for *ccrC* which is typically located in the SCC*mec* type V element. The detection of *ccrC* in MSSA has been described by Chlebowicz *et al.* (2010). They identified isogenic MRSA and MSSA isolates of the ST398 lineage (a veterinary clone) in a mother and child who suffered from pneumonia and an umbilicus infection, respectively. Interestingly the generation of the MSSA isolate was not from the excision of the SCC*mec* but rather from the loss of the *mecA* gene complex during recombination of two *ccrC* allotypes within the SCC*mec* type V element of the MRSA strain (Chlebowicz et al., 2010). In this study no other SCC*mec* type V loci were detected in the isolate 35, thus it is unlikely that a significant fragment of the SCC*mec* type V element was present in this isolate.

Five of the false positive MSSA isolates in this study had similar PFGE patterns (termed PFGE-1.i, PFGE-1.ii, PFGE-1.iii, PFGE-1.iv and PFGE-1.vi) and *spa* types which were close variants of *spa* type t127. However collectively the fifteen false positive isolates were genetically diverse and were also unrelated to the local HA-MRSA clones i.e. EMRSA-15 and EMRSA-16. This variability and the detection of the different SCC*mec* associated loci i.e. *dcs*, *ccrAB1*, *ccrAB4*, *ccrC*, could suggest that independent excision events in diverse MRSA genetic backgrounds may have occurred and yielded these MSSA isolates. However there is greater diversity of SCC*mec* loci i.e. *ccr* allotypes in coagulase-negative staphylococci (CoNS) and these species are thought to harbour the SCC*mec* reservoir. Even though the specific mechanism of transfer between strains is not well understood, it is postulated that *S. aureus* acquire SCC*mec* and SCC like elements from CoNS species (Garza-Gonzalez et al., 2010, Ruppe et al., 2009, Hanssen and Sollid, 2007). So it is also likely that the MSSA isolates in this study could have acquired the SCC*mec* associated loci from CoNS strains and not necessarily from MRSA.

Several attempts to determine the extent of presumed SCC*mec* remnants in the false positive MSSA isolates were largely unsuccessful and only three isolates yielded PCR products which were actually the unoccupied *att*B site and adjacent sequences to this site. It is possible that for the remaining twelve false positive isolates the anticipated remnant SCC*mec* sequence exceeded the 10 kb amplification limit of the assay and were not adequately amplified. However it is also possible that the failure of amplification was due to non specificity of the forward oligonucleotides. The ones used in this study were designed from sequences in the N315/ MRSA II, CA05/ MRSA IVa, JCSC1978/ MRSA IVb and MR108/ MRSAIVc strains. However Noto *et al.* (2008) examined the *att*B site and adjacent sequences in 42 diverse MSSA isolates and several MRSA including COL/ MRSAI and MRSA252/ MRSA II and they found that the sequences upstream of the *att*B are heterogeneous in *S. aureus* srains (Noto et al., 2008b). Therefore it is likely that the target sequences of the forward oligonucleotides were not present in the twelve false positive MSSA isolates of this study.

MSSA originating from multi-antibiotic resistant MRSA have been shown to retain the initial resistance to non β-lactam antibiotics (Shore et al., 2008, Donnio et al., 2005, Corkill et al., 2004). Donnio *et al.* (2005) verified that prevalent tobramycin resistant MSSA strains in French hospitals had originated from a dominant tobramycin resistant MRSA clone. Similarly Shore *et al.* (2008) and Corkill *et al.* (2004) also showed that MSSA emanating from gentamicin and fusidic acid resistant clinical MRSA strains, retained these resistance phenotypes after the SCC*mec* excision. Ellington *et al.* (2010) have shown that the dominant UK HA-MRSA strains: EMRSA-15 and EMRSA-16, have a narrow resistance profile and similarly in this study the EMRSA-15 and EMRSA-16 reference strains were largely susceptible to non β-lactam antibiotics, except to ciprofloxacin. Most of the false positive MSSA isolates were also susceptible to non β-lactam antibiotics except for two isolates which were resistant to >3 non β-lactam classes of antibiotics. Nevertheless because the genotypes of most of the isolates were unrelated (except isolate 16) to the EMRSA-15 and EMRSA-16 reference strains this strongly suggests that they did not originate from these UK HA-MRSA strains. In addition a comparison of the *spa* types of these isolates to those of the UK EMRSA-1 to 14 strains (see Table 4.6b in chapter 4) demonstrated that they were unrelated, suggesting that they did not originate from these HA-MRSA strains either. Thirteen of the fifteen false positive isolates were from patients without previous or later reports of MRSA isolates and this also supports the notion that these MSSA isolates probably did not emanate from MRSA strains.

However isolate 16 is particularly interesting because like the EMRSA-15 reference strain this MSSA isolate was resistant to ciprofloxacin and the additional consideration of the genotypic characteristics i.e. detection of the SCC*mec-orfX* right junction and *dcs*, the PFGE pattern and t022 *spa* type, persuasively imply that isolate 16 was previously an EMRSA-15 strain which subsequently underwent partial excision of the SCC*mec* type IV element. For the purposes of this study *S. aureus* cultures were collected from the PHW Microbiology ABM Laboratory on Iso-Sensitest media which had been used for susceptibility testing. Therefore the collection of the cultures may have occured 24 to 48 hours after completion of the tests. It is possible that at the time of collection from the patient and testing at the microbiology laboratory, isolate 16 contained the complete element but at some time point leading up to the *mecA* and

SCC*mec-orfX* PCR testing in this study, excision of the element occurred. It would have been possible to verify this by genotyping both the original cultures and the cryopreserved stocks subsequently prepared in this study. However this was not possible because the original Iso-Sensitest cultures were not retained and the cultures prepared from both of the stocks of isolate 16 i.e. cryopreservation beads and glycerol stocks, were *mecA* negative.

Despite the robust capacity of the PCR-A, PCR-B and PCR-C SCC*mec-orfX* assays for MRSA detection, a total of six EMRSA-15 and EMRSA-16 strains were undetected with these assays and two MRSA V strains were specifically undetected with the PCR-C assay, indicating that if these assays are implemented as routine MRSA screening assays certain local endemic strains may remain undetected. However this may be an infrequent occurrence because the prevalence of false negative results in this study was relatively low (3.4% to 4.2%, presented in chapter 5). The fifteen MSSA isolates which were detected with the three SCC*mec-orfX* assays were extensively genetically diverse, indicating that a specific MSSA genetic background was not the underlying cause of the false positive results. However these results do highlight that the amplification of the SCC*mec-orfX* junction does not provide absolute verification of MRSA presence.

Chapter Eight

Molecular epidemiology of Panton-Valentine Leukocidin-positive *S. aureus* isolates

8.0 Introduction

The pathogenicity of *S. aureus* is affiliated to its expression of diverse virulence factors. These have various functions which enable strains to colonise host tissues and evade innate host defence mechanisms (DeLeo et al., 2009, Foster, 2005, Rooijakkers et al., 2005). Recognition and phagocytosis by neutrophils and other phagocytes are critical defence strategies against invading microorganisms. However, *S. aureus* strains express toxins and phenol soluble modulin peptides which form pores in the cytoplasmic membrane of targeted immune cells and cause subsequent lysis of these cells (Foster, 2005, Voyich et al., 2005). The alpha toxin is the archetype of a family of pore forming beta-barrel toxins that cause lysis of different cell types including monocytes, platelets and erythrocytes (DeLeo et al., 2009, Foster, 2005, Valeva et al., 1997). Other members of this family include bi-component toxins e.g. gamma toxin, leukocidin D/ E, the Panton Valentine-Leukocidin (PVL) and a PVL-like leukocidin M/ F (Foster, 2005). The PVL toxin mainly causes the lysis of neutrophils and tissue necrosis. However α-toxin and γ-toxin are present in virtually all *S. aureus* strains whereas PVL is only expressed in 2 to 3% of strains (Loffler et al., 2010, Otto, 2010, Clark, 2008). Nevertheless certain *S. aureus* strains which carry PVL genes have caused extensive morbidity. For instance in the 1950's and 1960's the PVL-positive phage type 80/ 81 strain was highly prevalent in the UK and caused severe infections in both the community and hospitals (Ellington et al., 2010a). The recent emergence of CA-MRSA strains and the high prevalence of certain clones e.g. the USA300 clone in the USA is a public health concern. Several studies have demonstrated that the leading CA-MRSA clones have certain common genetic features e.g. carriage of SCC*mec* type IV or V elements and PVL genes. Furthermore certain clones such as USA300 and MW2 have been implicated in fatal infections like necrotising pneumonia and this has prompted the suggestion that PVL may have a role in the virulence of CA-MRSA strains (Ito et al., 2008, Hageman et al., 2006, Soderquist et al., 2006, CDC, 1999).

In the UK it has been demonstrated that the prevalence of PVL-positive *S. aureus* strains is relatively low at 1.6% - 4.9% and that these strains are polyclonal (Ellington et al., 2010b, Ellington et al., 2009, Otter et al., 2009, Holmes et al., 2005). However this information has been primarily obtained from investigations on isolates submitted to the UK Staphylococcus Reference Unit in Colindale. Since reference laboratories primarily receive selected isolates the results may not be a true representation of the epidemiology of PVL-positive *S. aureus* strains in the UK community. So in order to attain a more comprehensive insight it would be informative to also investigate the molecular epidemiology of unselected clinical isolates which are submitted to diagnostic laboratories as these may be more representative of the epidemiology in the community.

Therefore the objective of this study was to determine the prevalence of PVL genes in consecutive wound swab *S. aureus* isolates (n=519) from the PHW Microbiology ABM Laboratory (PHW-ABM). Genotyping of the isolates was conducted with the following molecular typing methods: PFGE, *spa* typing, SCC*mec* typing and detection of the ACME element by PCR. Furthermore the molecular epidemiology of these PVL-positive isolates was compared to a second collection of PVL-positive *S. aureus* isolates (n=61) from a reference laboratory, the Specialist Antimicrobial Chemotherapy Unit (SACU) in Cardiff.

8.1 Results

8.1.1 Prevalence of PVL genes in PHW-ABM *S. aureus* isolates

The prevalence of PVL genes in 519 *S. aureus* isolates (MRSA n=118 and MSSA n=401) was investigated by real-time PCR and these were detected in a total of nineteen isolates (3.7%). Out of the 118 MRSA isolates two (1.7%) were PVL-positive in comparison to seventeen (4.2%) of the 401 MSSA (see Table 8.1). However there was no significant difference between the prevalence of PVL in MRSA and MSSA isolates (p>0.05; two-tailed Fishers exact test).

Table 8.1 Prevalence of PVL genes in clinical MSSA and MRSA

Isolate	PVL-positive	PVL-negative	Total
MRSA	2 (1.7%)*	116 (98.3%)	118
MSSA	17 (4.2%)*	384 (95.8%)	401
Total	19 (3.7%)	500 (96.3%)	519

*(p>0.05; two-tailed Fishers exact test)

8.1.2. Analysis of patient demographics and clinical infections associated with PVL-positive *S. aureus* isolates

Demographic data of the *S. aureus* isolates (n=519) was retrieved from the PHW Microbiology ABM Laboratory data management system and the gender or age data was not available in four cases. More than half (51.4%; 265/515) of the *S. aureus* isolates were identified in individuals who were more ≥ 61 years of age (see Table 8.2). Whereas 21.2% (109/515) were identified from individuals within 41 to 60 years; 16.3% (84/515) were from individuals of 21 to 40 years and the least number of isolates (11.1%; 57/515) were identified from individuals who were ≤20 years of age (see Table 8.2). There were significantly more PVL-positive *S. aureus* (11.9%; 10/84) identified in the young adult age-group (21 to 40 years) than from all of the individuals (2.1%; 8/374) who were ≥41 years of age (p=0.0003; two-tailed Fishers exact test). This indicated that PVL-positive *S. aureus* were more associated with young adults than with older patients.

Table 8.2. Patient demographics of PVL-positive and negative *S. aureus* (n=519). MRSA (n=118) and MSSA (n=401)

Age (years)	Gender	Pathogen					
		PVL-positive		PVL-negative		PVL-pos / neg	PVL-pos No. (%)
		MRSA	MSSA	MRSA	MSSA		
61+	Male	1	2	39	79		
	Female	0	0	40	104	3/ 262	
41 to 60	Male	0	1	10	41		
	Female	0	4	13	40	5/ 104	8 (2.1%)*
21 to 40	Male	0	5	7	38		
	Female	1	4	3	26	10/ 74	10 (11.9%)*
0 to 20	Male	0	0	1	31		
	Female	0	0	2	23	0 / 57	0 (0.0%)
[a]TOTAL		2	16	115	382	18 / 497	18

*(p=0.0004; two-tailed Fishers exact test); [a]Gender or age data not available: PVL-positive MSSA (n=1); PVL-negative MRSA (n=1); PVL-negative MSSA (n=2)

Information on the clinical infections associated with the *S. aureus* isolates was retrieved from the specimen forms that were attached to the clinical samples, However, in 226 cases the information was not specific. Based on the available information more isolates (n=140) were identified from mild purulent skin infections e.g. abscesses, ulcers and boils, than from device-related/ surgical infections (n=72) and other infections (n=81). A statistically significantly higher number of PVL-positive *S. aureus* isolates were associated with mild purulent skin infections (8.6%, 12/140) than all of the other types of infections (1.8%; 7/379) (p=0.0008; two-tailed Fishers exact test) (see Table 8.3).

An additional collection of sixty-one PVL-positive *S. aureus* isolates was obtained from the Specialist Antimicrobial Chemotherapy Unit (SACU). Based on the available information a significantly higher number of isolates were associated with purulent skin infections, particularly abscesses (44.3%; 27/61) as compared to the other infections (p=0.006; two-tailed Fishers exact test) (see Table 8.4). This was comparable to the clinical data associated with the PVL-positive *S. aureus* isolates from the PHW Microbiology ABM Laboratory (PHW-ABM).

Table 8.3. Clinical infections associated with the PVL-positive *S. aureus* from PHW-ABM

Infection	PVL positive	PVL negative	Total
Abscess, ulcer, boil, folliculitis cysts and nodule	12 (8.6%)*	128 (91.4%)	140
Other infections			
Medical device related & surgical related	1	71	72
Screening swab (eye, groin) and other (i.e. infected nail, ear)	2	79	81
Not specified- wound swabs (arm, leg, abdominal, burn)	4	222	226
	7 (1.8%)*	372 (98.2%)	379
Total	19	500	519

*(p=0.0008; two-tailed Fishers exact test)

Table 8.4. Clinical infections associated with the PVL-positive *S. aureus* from SACU

Infection	No. (%)
Abscesses	14 (23.0%)
Boils	8 (13.1%)
Cellulitis	5 (8.2%)
Subtotal	**27 (44.3%)***
Other infections	
Necrosis	2 (3.3%)
Ear discharge, pacemaker, fever, otalgia, screen	7 (11.5%)
Wound swab (site unknown)	3 (4.9%)
Subtotal	**12 (19.7%)***
Clinical details not available	22 (36.0%)
Total	61

*(p=0.006; two-tailed Fishers exact test)

8.2 Molecular characterisation of the PVL-positive *S. aureus*

A comparison of the molecular epidemiology of the nineteen PVL-positive isolates from PHW Microbiology ABM Laboratory (PHW-ABM) and the SACU PVL-positive isolates (n=61) was conducted by performing the following assays: *mecA* PCR, PFGE, *spa* typing, SCC*mec* typing and detection of the arginine catabolic mobile element (ACME) by PCR. There was a significantly higher prevalence of *mecA*-positive *S. aureus* (57.4%; 35/61) in the SACU isolates in comparison to PHW-ABM *mecA*-positive *S. aureus* isolates (10.5%, 2/19) (p=0.0004; two-tailed Fishers exact test) (see Table 8.5).

Table 8.5. PVL-positive MRSA and MSSA in SACU (n=61) and PHW-ABM (n=19) isolates

	PHW-ABM	SACU	
***MRSA**	2 (10.5%)	35 (57.4%)	37
MSSA	17 (89.5%)	26 (42.6%)	43
Total	19	61	80

*(p=0.0004; two-tailed Fishers exact test)

8.2.1. Pulsed Field Gel Electrophoresis of the PVL-positive MRSA

PFGE was successfully performed on the PVL-positive MRSA (n=37) isolates (PHW-ABM, n=2; SACU, n=35) and thirteen patterns were resolved. Isolates with patterns that had ≤6 band differences were defined as clones, in accordance with the Tenover *et al.* (1995) criteria. The majority (n=16, 43.2%) of the 37 MRSA isolates exhibited the A1 pattern and one isolate had the A1i pattern which had 2-band differences to the former (see Figure 8.1). The A1 pattern was identical to the PFGE pattern (USA300-0114) of two USA300 reference strains (FPR3857 and SF8300 strains), signifying that 16 (43.2%) of the 37 PVL-positive MRSA were USA300 strains. The patterns termed B1, C1 and D1 were displayed by five (13.5%; 5/37), four (10.8%; 4/37) and two (5.4%; 2/37) isolates respectively (see Figure 8.1).

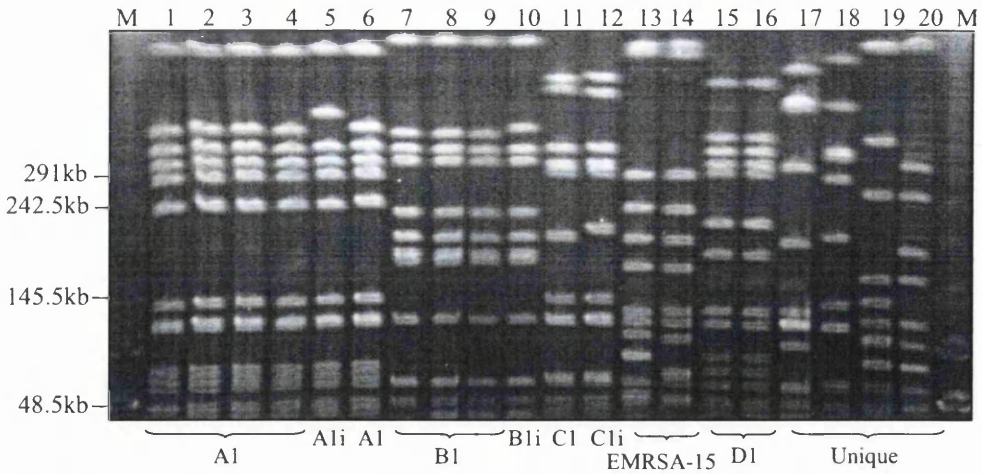


Figure 8.1. PFGE patterns of twenty SACU PVL-positive MRSA. Lane M, lamda DNA marker; lane 1 to 6, A1 and A1i; lane 7 to 10, B1 and B1i; lane 11 and 12, C1 and C1i; lane 13 and 14, EMRSA-15a and EMRSA-15b; lane 15 and 16, D1; lanes 17 to 20 unique.

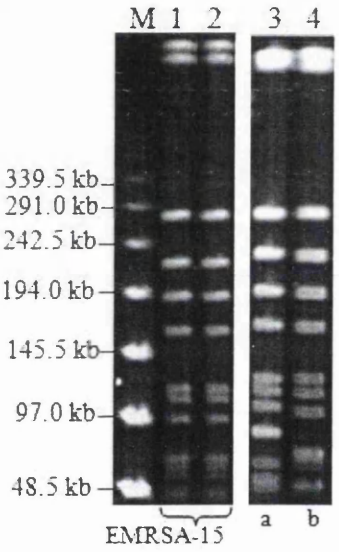


Figure 8.2. PFGE patterns of EMRSA-15 reference strain and two SACU PVL-positive MRSA with similar patterns (different gels). Lane M, lamda DNA marker; lane 1 and 2, EMRSA-15 reference strain; lane 3, EMRSA-15a pattern; lane 4, EMRSA-15b pattern.

Two isolates exhibited patterns which were similar to the PFGE pattern of the EMRSA-15 reference strain (see Figure 8.2). However these had >7 band difference to the pattern of the EMRSA-15 reference strain, so the isolates were provisionally affiliated to this clone and the patterns were termed EMRSA-15a and EMRSA-15b (see Figure 8.2). The remaining eight (21.6%) isolates had unique PFGE patterns. In summary, five clones were evident in the PVL-positive MRSA (n=37), the USA300 clone was the most prevalent (43.2%), followed by the B1 (13.5%) and C1 (10.8%) clones, whereas the D1 and EMRSA-15 clones were less prevalent. Notably these clones were exclusively present in SACU isolates (n=35) as the two PHW-ABMU PVL-positive MRSA had unique patterns.

8.2.2. *spa* typing of the PVL-positive MRSA

The PVL-positive MRSA (n=37) isolates were successfully typed with the *spa* typing method except for one isolate in which amplification of the targeted sequence was not successful. Eighteen *spa* types were exhibited by the 36 PVL-positive MRSA and the prevalent *spa* types were: t008 (n=12); t044 (n=4); t002 (n=3) and t127 (n=2) (see Table 8.6). There were also *spa* types which were evidently closely related to the former types albeit single nucleotide differences or the deletion or insertion of *spa* repeats such as, t024, t1578, t1624 and t2743 are close variants of *spa* type t008.

Therefore within the PVL-positive MRSA isolates (n=36): 17 (47.2%) exhibited *spa* type t008 and closely related variants; five (13.9%) had *spa* type t044 and a closely related variant (t4725); four (11.1%) had *spa* type t002 and a closely related variant (t311) and two (5.6%) isolates depicted t127. The *spa* types t005 and t223 were also closely related, differing by the absence of one repeat unit (r17) in t223.

Correlation between the *spa* typing results and the PFGE patterns was evident and sixteen isolates which exhibited the t008 *spa* type or close variants (t024, t1578, t1624, t2743) displayed the USA300-0114 PFGE pattern (see Table 8.6). The five isolates with the t044 and t4724 *spa* types exhibited the B1 PFGE pattern and the three isolates with the t002 *spa* type all had the C1 PFGE pattern. Interestingly, the isolate which was not typeable with the *spa* typing method exhibited a PFGE pattern (C1.1) which had a ≤ 6 band difference to the C1 pattern (see Table 8.6). The two isolates depicting *spa* type t127 exhibited the D1 pattern and the isolates with the t005 and t223 *spa* types depicted the EMRSA-15a and EMRSA-15b patterns. However, there were two discrepancies between PFGE and *spa* typing. One isolate with the t008 *spa* type had a pattern that was markedly different (>7 band difference) to the USA300 PFGE pattern (see Table 8.6). Also one isolate with the t311 *spa* type, a close variant of t002 had a PFGE profile that was unrelated to the C1 PFGE pattern (see Table 8.6).

Table 8.6. Genotypic characteristics of MRSA from SACU and ABMU

***spa* type**	***spa* repeat alignment**	**Number (%)**	**PFGE**	**SCC*mec***	**ACME**	**Location**
t008	11-19-12-21-17-34-24-34-22-25	11	A1	IVa	Pos	SACU
t024	11- -12-21-17-34-24-34-22-25	1	A1	IVa	Pos	SACU
t1578	11-19-12-21-17-34-24-34-**17**	2	A1	IVa	Pos	SACU
t1624	11- -12-21-17- -24-34-22-25	1	A1	IVa	Pos	SACU
t2743	11-19-12-21-17-34-24-34-**23**-25	1	A1	IVa	Pos	SACU
Subtotal		**16 (44.4 %)**				
t008	11-19-12-21-17-34-24-34-22-25	1 (2.8%)	Unique	IVc	Neg	SACU
t008	11-19-12-21-17-34-24-34-22-25	--	A1	IVa	Pos	FPR3857 strain
t008	11-19-12-21-17-34-24-34-22-25	--	A1	IVa	Pos	SF8300 strain
t044	07-23-12-34-34-33-34	4	B1	IVc	Neg	SACU
t4725	07-23-12-**13**-34-33-34	1	B1	IVc	Neg	SACU
Subtotal		**5 (13.9%)**				
t002	26-23-17-34-17-20-17-12-17-16	3	C1	IVc	Neg	SACU
t311	26-23-17-34- -20-17-12-17-16	1	Unique	IVa	Neg	SACU
Subtotal		**4 (11.1%)**				
t127	07-23-21-16-34-33-13	2 (5.6%)	D1	IVa	Neg	SACU
t005	26-23-13-23-31-05-17-25-17-25-16-28	1	EMRSA-15a	IVc	Neg	SACU
t223	26-23-13-23- -05-17-25-17-25-16-28	1	EMRSA-15b	IVa	Neg	SACU
Subtotal		**2 (5.6%)**				
t026	08-16-34	1	Unique	IVa	Neg	SACU
t202	11-17-23-17-17-16-16-25	1	Unique	IVa	Neg	SACU
t275	15-12-16-02-25-17-24-24	1	Unique	IV*nt*	Neg	SACU
t6187(new)	03-34-16-12-33-34	1	Unique	IVa	Neg	SACU
t019	08-16-02-16-02-25-17-24	1	Unique	IVd	Neg	PHW-ABM
t437	04-20-17-20-17-25-34	1	Unique	V	Neg	PHW-ABM
Subtotal		**6 (16.6%)**				
Total		**36**				
nt		1	C1.1	IV*nt*	Neg	SACU

nt-not typeable, new – new *spa* type identified from this study

8.2.3. SCC*mec* typing of the PVL-positive MRSA

The SCC*mec* typing was successfully performed on all of the 37 PVL-positive MRSA isolates and the majority (n=36) of the isolates carried the SCC*mec* type IV element. One isolate contained a SCC*mec* element that was putatively regarded as a type V (as previously discussed in chapter 4) (see Table 8.6). The sub-typing of the isolates (n=36) with the SCC*mec* type IV elements was also performed and they contained: type SCC*mec* type IVa (n=25), IVc (n=10) and IVd (n=1), two isolates contained type IV elements which were not sub-typeable with this method (see Table 8.6).

All of the sixteen isolates with the USA300 PFGE pattern and *spa* type t008 and close variant *spa* types, t024, t1578, t1624, t2743 contained SCC*mec* type IVa (see Table 8.6). The one isolate with the t008 *spa* type and a unique PFGE pattern harboured SCC*mec* type IVc. The five isolates with the B1 PFGE pattern and *spa* type t044/ t4725 also contained SCC*mec* type IVc (see Table 8.6). Similarly the three isolates with the C1 PFGE pattern and the t002 *spa* type also had and SCC*mec* type IVc but the isolate with t311 *spa* type (a closely related variant of t002) and a unique PFGE pattern contained SCC*mec* type IVa (see Table 8.6). The two isolates depicting the D1 PFGE pattern and *spa* type t127 both contained SCC*mec* type IVa. The two isolates that had the EMRSA-15a and b harboured different SCC*mec* type IV subtypes i.e. type IVa and IVc.

8.2.4. Detection of the arginine catabolic mobile genetic element

The eighty PVL-positive *S. aureus* (MRSA n=37, MSSA n=43) were screened for the arginine catabolic mobile element (ACME) by amplification of the *arcA* gene of ACME (*arcA*-ACME) with PCR. The ACME element was only detected in the sixteen of the 80 PVL-positive *S. aureus* isolates, which exhibited the USA300 PFGE pattern, the t008 *spa* type (or closely related t024, t1578, t1624 and t2743 *spa* types) and the SCC*mec* type IVa element (see Table 8.6). The two USA300 reference strains (FPR3857 and SF8300 strains) were also *arcA*-ACME positive.

8.2.5. Pulsed Field Gel Electrophoresis of the PVL-positive MSSA

With the 43 PVL-positive MSSA isolates (SACU n=26; PHW-ABMU, n=17) eighteen PFGE patterns were resolved. These included patterns that were related (≤6 band difference) to those exhibited by the MRSA isolates: A1 (A1.1i and A1.1ii), C1 (C1.1i and C1.1ii) and D1 (D1.1i and D1.1ii) respectively. Other patterns that were evident were termed F1 to J1, EMRSA-15c, EMRSA-15d and there were also unique patterns (see Table 8.7). The most prevalent pattern was H1 (and several related patterns) which were exhibited by ten (23.3%; 10/43) of the isolates. Four (9.3%; 4/43) isolates had patterns (A1.1i and A1.1ii) that were related to the PFGE pattern of the USA300 strains (see Table 8.7).

8.2.6. *spa* typing of the PVL-positive MSSA

A diverse array of *spa* types (n=30) were exhibited by the 43 PVL-positive MSSA and there was no particular *spa* type which was clearly predominant over other types. The largest groups contained four MSSA isolates which exhibited the t159 and t314 *spa* types respectively and three MSSA isolates depicted the t021 and t211 *spa* types respectively (see Table 8.7).

8.3. *spa*-BURP analysis of the PVL-positive *S. aureus* isolates (n=80)

The genetic relatedness of the eighty PVL-positive *S. aureus* isolates was investigated with the *spa*-BURP analysis using the Ridom StaphType version 1.5.2.1 software. The *spa* types that were shorter than five repeats were excluded and *spa* types were placed in clusters if costs were ≤ 4 (see Figure 8.3 and 8.4)

Table 8.7. Genotypic characteristics of the SACU and PHW-ABM MSSA isolates (n=43)

***spa* type**	***spa* repeat**	**No.**	**PFGE**	**Location**
t159	14-44-13-12-17-17-23-18-17	4	H1 & H1.ii	ABMU & SACU
t162	14-44-12-17-17-23-18-17	1	H1.i	SACU
t645	14-44-13-12-17-23-18-17	1	H1.ii	ABMU
t1425	14-44-13-12-17-17	1	H1	SACU
t3204	14-44-13-12-17-17-23-23-18-17	1	H1.i	SACU
t5448	14-44-13-12-17-17-22-18-17	2	H1.i	SACU
Subtotal		**10 (23.3%)**		
t314	08-17-23-18-17	4 (9.3%)	J1 & J1.i	ABMU & SACU
t021	15-12-16-02-16-02-25-17-24	3	G1	ABMU
t2271	15-12-16-16-02-25-17-24-24	2	G1.1	SACU
t6186 (new)	15-12-16-02-16-02-24-24-24-17	2	I1	SACU
Subtotal		**7 (16.2%)**		
t019	08-16-02-16-02-25-17-24	1	F1	SACU
t138	08-16-02-25-17-24	1	F1	SACU
Subtotal		**2 (4.7%)**		
t211	11-19-12-12-21-17-34-24-34-22-25	3	A1.1ii	SACU
t1635	11-19-12- 21-17-34-25	1	A1.1i	SACU
Subtotal		**4 (9.3%)**		
t002	26-23-17-34-17-20-17-12-17-16	1	C1.1ii	ABMU
t105	26-23-17-34-17-20-17-17-16	1	C1.1i	SACU
Subtotal		**2 (4.7%)**		
t005	26-23-13-23-31-05-17-25-17-25-16-28	1	EMRSA-15d	ABMU
t891	26-23-13-23-31-05-17-25-17-25-28	1	EMRSA-15c	ABMU
t1869	26-23-34-23-31-05-17-25-17-25-16-28	1	EMRSA-15c	SACU
Subtotal		**3 (7.0%)**		
t345	26-23-13-21-17-34-34-33-34	1	Unique	SACU
t4363(new)	26-23-13-21-17-34-34-24-33-34	1	Unique	ABMU
Subtotal		**2 (4.7%)**		
***spa* type**	***spa* repeat**		**PFGE**	
t084	07-23-12-34-34-12-12-23-02-12-23	1	Unique	ABMU
t160	07-23-21-24-33-22-17	1	D1.1i	SACU
t177	26-23-21-16-34-33-13	1	D1.1ii	SACU
t189	07-23-12-21-17-34	1	Unique	ABMU
t903	26-22-19-17-17-20-17-12	1	Unique	ABMU
t917	09-02-16-34-13-13-17-34-16-34	1	Unique	ABMU
t2393	07-12-21-17-13-13-13-34-33-34	1	Unique	SACU
t3011	08-23-16-13-17-34-16-34	1	Unique	ABMU
t4791	07-56-16-33-31-57-21-12	1	Unique	ABMU
Subtotal		**9 (20.8%)**		
Total		**43**		

The performance of *spa*-BURP cluster analysis on the 80 PVL-positive *S. aureus* isolates demonstrated that eight *spa* clusters were evident, whereby CC002, CC005, CC008, CC159, CC275 were so termed to reflect the postulated ancestral strain of the cluster e.g. *spa* type t002 for CC002 (see Figure 8.3 and 8.4). The remaining clusters were defined as cluster 6, cluster 7 and cluster 8 as no ancestral strain was postulated by the software. Thirteen *spa* types were regarded as singletons which indicated that they were unrelated to the other PVL-positive *S. aureus* isolates i.e. t084, t160, t189, t202, t314, t437, t903, t917, t1635, t2393, t3011, t4791, t6187. The t026 *spa* type which was exhibited by one MRSA isolate was excluded from the cluster analysis as it had less than five repeats.

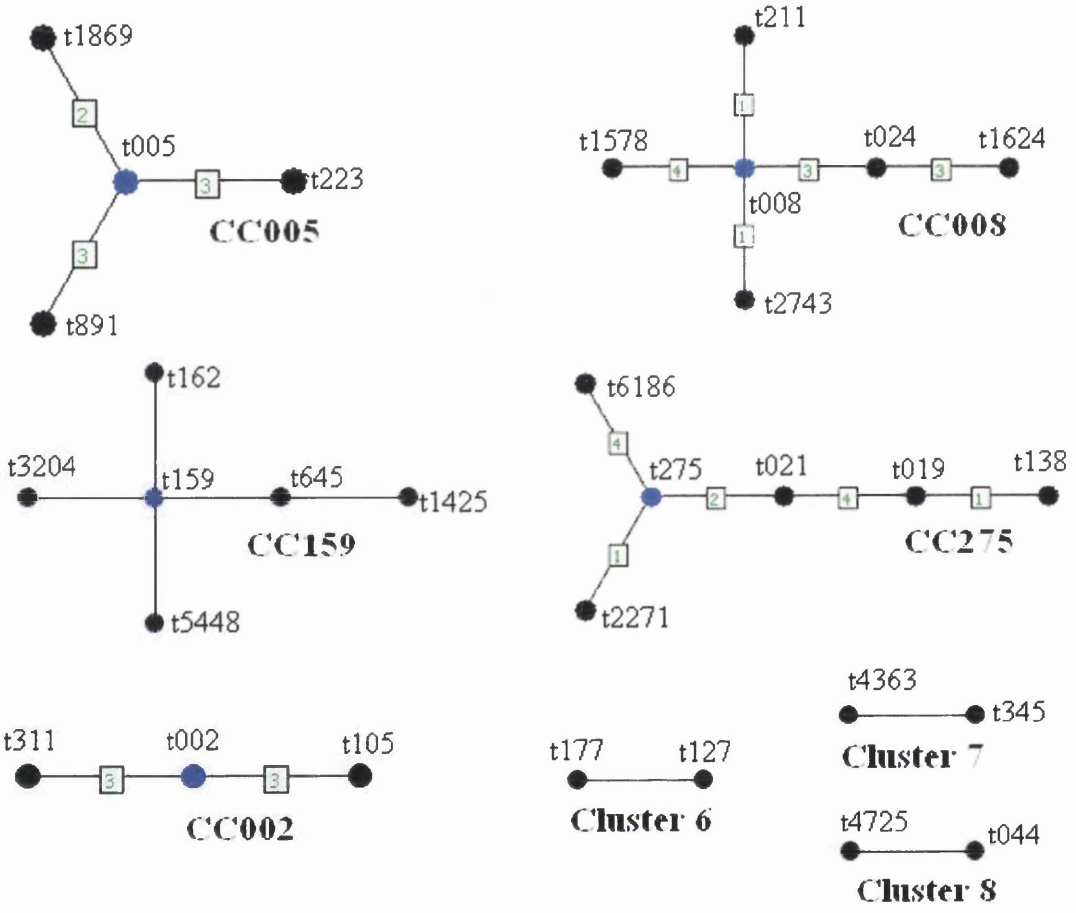


Figure 8.3. Minimum spanning trees generated with Ridom StaphType software. Circles represent respective *spa* types and the central circle depicts the *spa* type defined as the founder of the cluster i.e. t005 for *spa*-CC005. Numerical values on branches represent the similarity (distance) between the respective *spa* types and the founder or between the *spa* types where there is no founder.

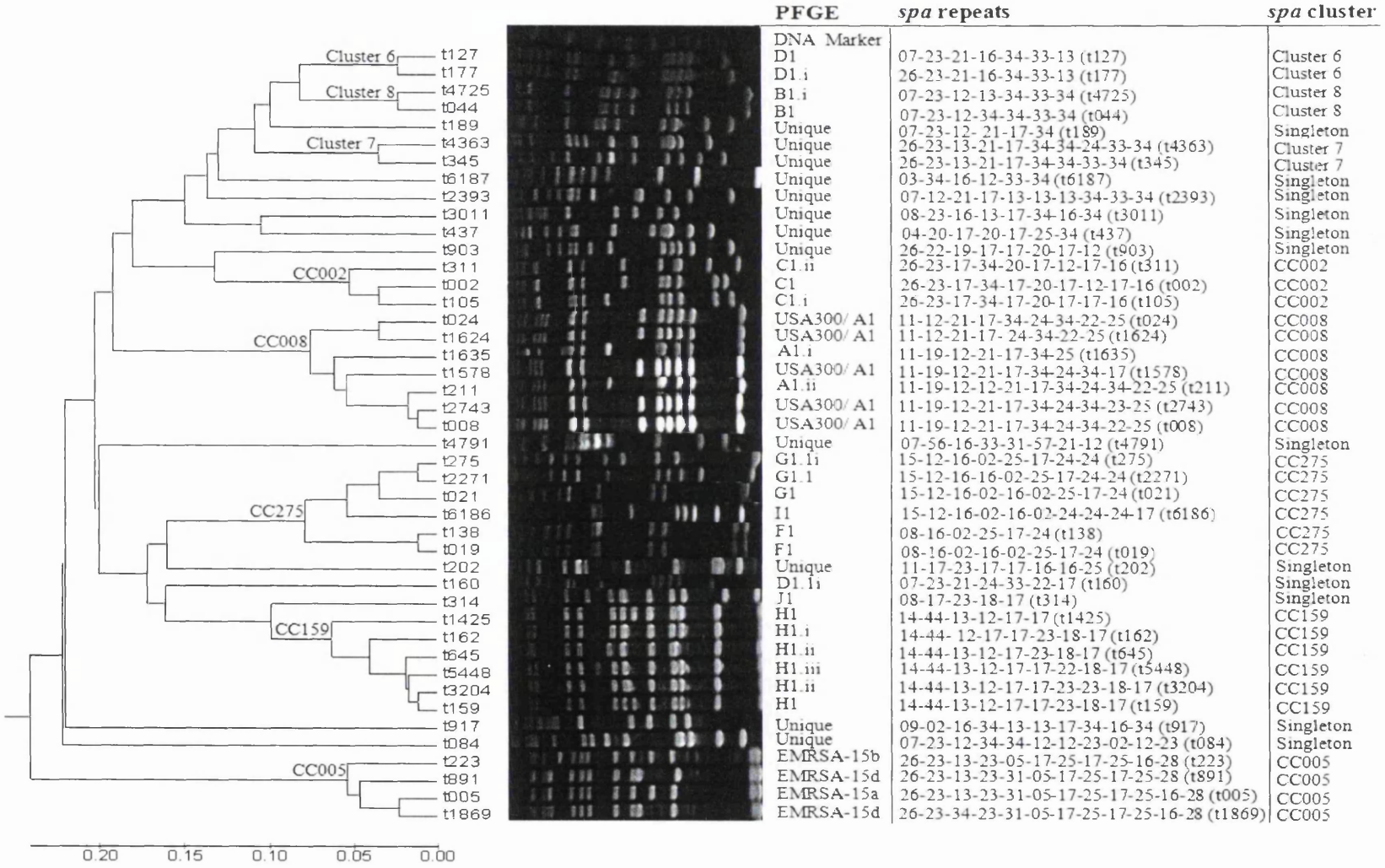


Figure 8.4. UPGMA dendrogram illustrating the relatedness of *spa* types of PVL-positive *S. aureus* and corresponding PFGE patterns, *spa* repeats and the *spa*-BURP clusters.

8.4 Genotypes and antibiotic resistance profiles of PVL-positive *S. aureus*

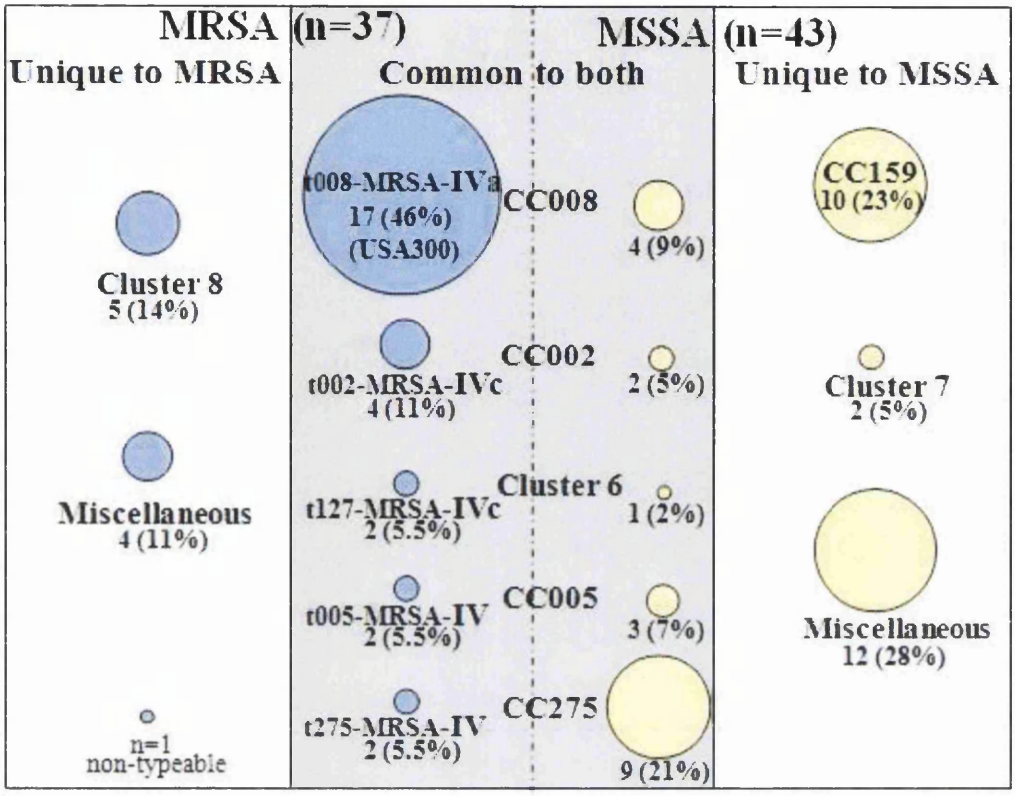


Figure 8.5. Prevalence of PVL-positive MRSA and MSSA isolates within *spa*-BURP clusters (Blue-MRSA, yellow-MSSA).

The *spa*-BURP cluster analysis demonstrated the genetic relatedness of the isolates but the MRSA (n=37) and MSSA (n=43) were distributed within the clusters in different proportions (see Figure 8.5). Cluster 7 and CC159 contained only PVL-positive MSSA whereas cluster 8 comprised only PVL-positive MRSA strains. The genotypic characteristics and the antibiotic resistance profiles of the PVL-positive MRSA and MSSA strains within each cluster were further examined.

8.4.1 Genotypes and antibiotic resistance profiles of PVL-positive MRSA (n=37)

8.4.1.1 Cluster CC008

The t008 and related *spa* types were grouped into *spa* cluster CC008 which comprised both MSSA and MRSA (see Figure 8.5). This was the most prominent cluster of the PVL-positive MRSA (n=37) comprising seventeen (46.0%; 17/37) MRSA isolates (see Table 8.8). The core genotype expressed by the sixteen USA300 strains was t008-(or closely related *spa* types)-MRSA-IVa. Antibiotic susceptibility testing was performed with the BD Phoenix and the majority of the strains were resistant to trimethoprim (n=12) and erythromycin (n=11). Eight were resistant to tobramycin and a few were resistant to ciprofloxacin (n=3) and co-trimoxazole (n=2). The remaining MRSA isolate depicted the t008 *spa* type but had a unique PFGE pattern, was ACME negative, carried a SCC*mec* type IVc element and was only resistant to trimethoprim.

8.4.1.2 Cluster 8

Cluster 8 exclusively contained MRSA and it comprised five (13.5%; 5/37) isolates with *spa* types t044 and t4725, they had the t044/ t4725-MRSA-IVc genotype and had the same B1 PFGE pattern. All five MRSA strains were resistant to tobramycin and trimethoprim but had variable resistance to tetracycline (n=4) and fusidic acid (n=3) (see Table 8.8).

8.4.1.3 Cluster 6

Two (5.4%; 2/37) MRSA and one MSSA (depicting *spa* type t177) were contained within cluster 6 and the two MRSA isolates exhibited the t127-MRSA-IVa genotype and the D1 PFGE pattern (see Figure 8.5). Both of the MRSA isolates were resistant to fusidic acid and trimethoprim but only one was resistant to tobramycin (see Table 8.8).

Table 8.8. Genotypic characteristics and antibiotic resistance of PVL-positive MRSA (n=37)

N. (%)	Antibiotic resistance(n)	Cluster	*spa* type(n)	*spa* repeat	PFGE	SCC*mec*	ACME
17(46.0%)	Ox, Trim(12), Ery(11),Tob(8), Cip(3), Tmp-Sx(2)	CC008	t008 (11)	11-19-12-21-17-34-24-34-22-25	USA300	IVa	Pos
			t024 (1)	11-12-21-17-34-24-34-22-25	USA300	IVa	Pos
			t1578 (2)	11-19-12-21-17-34-24-34-17	USA300	IVa	Pos
			t1624 (1)	11-12-21-17-24-34-22-25	USA300	IVa	Pos
			t2743 (1)	11-19-12-21-17-34-24-34-23-25	USA300	IVa	Pos
	Ox, Trim		t008 (1)	11-19-12-21-17-34-24-34-22-25	Unique	IVc	Neg
5 (13.5%)	Ox, Tob, Trim, Tet(4), FA(3)	Cluster 8	t044 (4)	07-23-12-34-34-33-34	B1-Europe	IVc	Neg
			t4725 (1)	07-23-12-13-34-33-34	B1-Europe	IVc	Neg
4 (10.8%)	Ox, Trim(2), Tet(1), Tmp-Sx(1)	CC002	t002 (3)	26-23-17-34-17-20-17-12-17-16	C1	IVc	Neg
			t311 (1)	26-23-17-34-20-17-12-17-16	C1.ii	IVa	
1 (2.7%)	Ox, Ery, Clin^{I}, FA, Tob	Neg	Neg	Neg	C1.1	IVnt	Neg
2 (5.4%)	Ox, FA, Trim, Tob (1)	Cluster 6	t127	07-23-21-16-34-33-13	D1	IVc	Neg
2 (5.4%)	Ox, Tob, Trim, Ery(1), Clin^{I}(1), Gent(1), Tmp-Sx	CC005	t005 (1)	26-23-13-23-31-05-17-25-17-25-16-28	EMRSA-15a	IVc	Neg
			t223 (1)	26-23-13-23-05-17-25-17-25-16-28	EMRSA-15b	IVa	
2 (5.4%)	Ox, FA(1)	CC275	t019	08-16-02-16-02-25-17-24	F1.i	IVd	Neg
			t275	15-12-16-02-25-17-24-24	G1.i	IV*nt*	
4 (10.8%)	Ox, Trim(2), Tob(2)	Singleton	t026	08-16-34	Unique	IVa	Neg
			t202	11-17-23-17-17-16-16-25	Unique	IVa	
			t6187	03-34-16-12-33-34	Unique	IVa	
	Ery, Clin^{C}, Tet		t437	04-20-17-20-17-25-34	Unique	V	

Key: Ox-oxacillin; Cip-ciprofloxacin; Ery-erythromycin; Clin^{I}-inducible clindamycin; Clin^{C}-constitutive clindamycin; FA-fusidic acid; GM-gentamicin; Tob-tobramycin, Tet-tetracycline; Trim-trimethoprim; Tmp-Sx-co-trimoxazole

8.4.1.4 Cluster CC002

The CC002 cluster comprised *spa* types depicted by both MSSA and MRSA and a total of four (10.8%; 4/37) MRSA isolates were contained in this cluster (see Figure 8.5). Three isolates had identical t002-MRSA-IVc genotypes, the C1 PFGE pattern and were susceptible to all classes of non β-lactam antibiotics, except for one isolate which was resistant to trimethoprim. The other MRSA isolate had *spa* type t311 which is highly similar to t002 but it had a unique PFGE pattern and harboured the SCC*mec* type IVa element. Unlike the former three isolates it had multiple antibiotic resistances i.e. tetracycline, trimethoprim and co-trimoxazole (see Table 8.8).

8.4.1.5 Cluster CC005

Cluster CC005 comprised *spa* types exhibited by both MRSA and MSSA and the two (5.4%; 2/37) MRSA displaying t005-MRSA-IVc and t223-MRSA-IVa genotypes were included in this cluster (see Figure 8.5). They exhibited the EMRSA-15a and EMRSA-15b PFGE patterns respectively and both isolates were resistant to tobramycin, trimethoprim and co-trimoxazole. However the t223-MRSA-IVa isolate was also resistant to gentamicin whereas the t005-MRSA-IVc isolate was also resistant to erythromycin and clindamycin (inducible) (see Table 8.8).

8.4.1.6 Cluster CC275

Two (5.4%; 2/37) MRSA strains with t019-MRSA-IVd and t275-MRSA-IV*nt* genotypes were contained in cluster CC275 which comprised both MRSA and MSSA (see Figure 8.5). However the t019 *spa* type appeared to be remotely related to the t275 *spa* type, the putative ancestral *spa* type of the cluster. It has a single nucleotide substitution in the first repeat: r08 versus r15 in *spa* type t275 and exhibits several deletions and insertions of repeats. Both of the MRSA isolates were largely susceptible to non β-lactam antibiotics with only the t275-MRSA-IV*nt* isolate being resistant to fusidic acid (see Table 8.8).

8.4.1.7 Miscellaneous

Four (13.5%; 4/37) MRSA had diverse *spa* types (t026, t202, t437 and t6187) and PFGE patterns. Only the t437-MRSA carried a SCC*mec* type V element whereas the other three isolates harboured SCC*mec* type IVa elements. These diverse isolates also had different antibiotic resistance profiles (see Table 8.8).

8.4.2 Genotypes and antibiotic resistance profiles of PVL-positive MSSA (n=43)

8.4.2.1 Miscellaneous

Unlike the MRSA isolates which comprised a relatively low prevalence of isolate with diverse *spa* types (10.8%; 4/37), twelve (27.9%; 12/43) MSSA isolates were not included in any of the *spa* clusters and they exhibited the following diverse *spa* types: t084, t160, t189, t314 (n=4), t903, t917, t2393, t3011, and t4791. They showed variable resistance to trimethoprim (n=5), tetracycline (n=4), erythromycin (n=2), clindamycin (inducible) (n=2), co-trimoxazole (n=2), tobramycin (n=1), fusidic acid (n=1) (see Table 8.9).

8.4.2.2 Cluster CC159

The CC159 cluster only comprised ten MSSA isolates (23.3%; 10/43) (see Figure 8.5) and these were characterised by the t159 *spa* type and closely related variants (t162, t645 t1425, t3204 and t5448) and similar H1, H1.i or H1.ii PFGE patterns. They were extensively resistant to trimethoprim (n=8) but had variable resistance to erythromycin (n=4) and clindamycin (inducible) (n=4). A few isolates were resistant to tobramycin (n=3), tetracycline (n=2) and co-trimoxazole (n=2) (see Table 8.9).

8.4.2.3 Cluster CC275

Cluster CC275 extensively comprised MSSA isolates (20.9%; 9/43) and only two MRSA isolates (see Figure 8.5). The MSSA exhibited the t021 or related *spa* types (t019, t138, t2271 and t6186) and the F1, G1, G1.ii and I1 PFGE patterns. Two of the MSSA with the t019 and t138 *spa* types and had identical F1 patterns which were remotely related (7 band difference) to the G1 pattern. The nine MSSA were extensively resistant to trimethoprim (n=5) but had variable resistance to tobramycin (n=2), ciprofloxacin (n=1), tetracycline (n=1), gentamicin (n=1), fusidic acid (n=1) and co-trimoxazole (n=1) (see Table 8.9).

Table 8.9. Genotypic characteristics and antibiotic resistance of PVL-positive MSSA (n=43)

N. (%)	Antibiotic resistance(n)	Cluster	*spa* type (n)	*spa* repeat	PFGE
10 (23.3%)	PenG, Trim(8), Ery(4), Clind[I](4), Tob(3), Tet(2), Tmp-Sx(2)	CC159	t159 (4)	14-44-13-12-17-17-23-18-17	H1 & H1.ii
			t162 (1)	14-44-12-17-17-23-18-17	H1.i
			t645 (1)	14-44-13-12-17-23-18-17	H1.ii
			t1425 (1)	14-44-13-12-17-17	H1
			t3204 (1)	14-44-13-12-17-17-23-23-18-17	H1.i
			t5448 (2)	14-44-13-12-17-17-22-18-17	H1.i
9 (20.9%)	PenG, Trim(5), Tob(2), Cip(1), FA(1), Gent(1), Tet(1), Tmp-Sx(1)	CC275	t021 (3)	15-12-16-02-16-02-25-17-24	G1
			t019 (1)	08-16-02-16-02-25-17-24	F1
			t138 (1)	08-16-02-25-17-24	F1
			t2271 (2)	15-12-16-16-02-25-17-24-24	G1.ii
			t6186 (2)	15-12-16-02-16-02-24-24-24-17	I1
4 (9.3%)	PenG, Ery(3), Trim(1)	CC008	t211 (3)	11-19-12-12-21-17-34-24-34-22-25	A1.ii
			t1635 (1)	11-19-12-21-17-34-25	A1.i
3 (7.0%)	PenG, Tob, Gent(2),Trim(2), Tmp-Sx (2)	CC005	t005 (1)	26-23-13-23-31-05-17-25-17-25-16-28	EMRSA-15d
			t891 (1)	26-23-13-23-31-05-17-25-17-25-28	EMRSA-15c
			t1869 (1)	26-23-34-23-31-05-17-25-17-25-16-28	EMRSA-15c
2 (4.6%)	PenG, Trim, Tob(1)	CC002	t002 (1)	26-23-17-34-17-20-17-12-17-16	C1.iii
			t105 (1)	26-23-17-34-17-20-17-17-16	C1.i
2 (4.6%)	PenG, Trim(1), Tmp-Sx(1)	Cluster 7	t4363 (1)	26-23-13-21-17-34-34-24-33-34	Unique
			t345 (1)	26-23-13-21-17-34-34-33-34	Unique
1 (2.3%)	PenG, FA, Trim	Cluster 6	t177 (1)	26-23-21-16-34-33-13	D1.i

Table 8.9 contd. Genotypic characteristics and antibiotic resistance of PVL-positive MSSA (n=43)

N. (%)	Antibiotic resistance(n)	Cluster	*spa* type (n)	*spa* repeat	PFGE
12 (27.9%)	PenG, Trim(5), Tet(4), Ery(2), Clind^{I}(2), Tmp-Sx(2), FA(1), Tob(1).	Singleton	t084 (1)	07-23-12-34-34-12-12-23-02-12-23	Unique
			t160 (1)	07-23-21-24-33-22-17	D1.ii
			t189 (1)	07-23-12-21-17-34	Unique
			t314 (4)	08-17-23-18-17	J1 & J1.i
			t903 (1)	26-22-19-17-17-20-17-12	Unique
			t917 (1)	09-02-16-34-13-13-17-34-16-34	Unique
			t2393 (1)	07-12-21-17-13-13-13-34-33-34	Unique
			t3011 (1)	08-23-16-13-17-34-16-34	Unique
			t4791 (1)	07-56-16-33-31-57-21-12	Unique

Key: Ox-oxacillin; Cip-ciprofloxacin; Ery-erythromycin; Clin^{I}-inducible clindamycin; Clin^{C}-constitutive clindamycin; FA-fusidic acid; GM-gentamicin; Tob-tobramycin, Tet-tetracycline; Trim-trimethoprim; Tmp-Sx-co-trimoxazole

8.4.2.4 Cluster CC008

In contrast to the MRSA where a considerable number (46.0%; 17/37) of isolates belonged to the CC008 cluster, only four (9.3%; 4/43) MSSA belonged to this cluster (see Figure 8.5). Three MSSA with *spa* type t211 and one isolate with *spa* type t1635 had similar PFGE patterns (A1.i and A1.ii) which were related (≤6 band difference) to the USA300 pattern. However, in contrast to the t008-MRSA-IVa isolates, these MSSA were largely susceptible to non β-lactam antibiotics, three were resistant to erythromycin (n=3) and only one was also resistant to trimethoprim (see Table 8.9).

8.4.2.5 Cluster CC005

Cluster CC005 comprised three (7.0%; 3/43) MSSA isolates with the t005, t891, t1869 *spa* types and the EMRSA-15c PFGE pattern. The isolates with the t005 and t891 *spa* types were resistant to gentamicin, tobramycin, trimethoprim and co-trimoxazole, whereas the MSSA depicting t1869 was only resistant to tobramycin (see Table 8.9).

8.4.2.6 Cluster CC002

Cluster CC002 comprised two (4.7%; 2/43) MSSA exhibiting *spa* types t002 and t105 and the C1.1ii and C1.1i PFGE patterns. These were largely susceptible to non β-lactam antibiotics, both having resistance to trimethoprim and one also resistance to tobramycin (see Table 8.9).

8.4.2.7 Cluster 7

Cluster 7 exclusively comprised two (4.7%; 2/43) MSSA isolates with the t345 and t4363 *spa* types. Even though these isolates had related *spa* types i.e. t4363 differed from t345 by one inserted repeat, they had unrelated PFGE patterns. They also had different resistance; the t345-MSSA isolate was resistant to trimethoprim and co-trimoxazole whereas the t4363-MSSA isolate was susceptible to all non β-lactam antibiotics (see Table 8.9).

8.4.2.8 Cluster 6

Only one (2.3%; 1/43) MSSA isolate was contained in cluster 6 which also comprised two MRSA with the t127-MRSA-IVa genotype. The one MSSA isolate had the t177 *spa* type which was highly similar to t127 albeit one nucleotide difference in the first repeat. It had a D1.1ii PFGE pattern which was similar (≤6 band difference) to the D1 pattern of the t127-MRSA-IVa isolates. Furthermore the t127-MRSA-IVa isolates and the t177-MSSA were similarly resistant to fusidic acid and trimethoprim (see Table 8.9).

8.5 A comparison of the genotypes of the PVL-positive *S. aureus* isolates from PHW-ABM versus the PVL-positive *S. aureus* isolates from SACU

The eighty PVL-positive *S. aureus* isolates were composed of two collections: unselected consecutive isolates (n=19) from PHW-ABM and isolates (n=61) referred to SACU from microbiology laboratories across Wales. The molecular epidemiology of these isolates was compared by assessing the *spa*-clusters represented in both collections. The PHW-ABM isolates were distributed into five of the eight clusters i.e. CC002 (n=1), CC005 (n=2), CC159 (n=5), CC275 (n=4) and cluster 7 (n=1) and six isolates had diverse genotypes. The SACU isolates were distributed into all of the eight clusters and CC008 was the most prominent (see Table 8.10 and Figure 8.6). This indicated that both collections of PVL-positive *S. aureus* isolates were polyclonal. Five clusters: CC002, CC005, CC159, CC275 and cluster 7 were common to both collections of isolates. Notably the CC008 genotype was significantly more prevalent in the SACU isolates (p=0.002), whereas the prevalence differences of the other genotypes between the two strain collections were not statistically significant (see Table 8.10 see Figure 8.6).

Table 8.10. Distribution of PHW-ABM (n=19) and SACU (n=60) PVL-positive *S. aureus* in the eight *spa*-BURP clusters

	PHW-ABM			SACU			
Cluster	**Total**	**MRSA**	**MSSA**	**Total**	**MRSA**	**MSSA**	**[a]*p*-value**
CC008	0			21 (35.0%)	17	4	p=0.002
Cluster 6	0			3 (5.0)	2	1	p=1.000
Cluster 8	0			5 (8.3%)	5	0	p=0.332
CC002	1 (5.2 %)	0	1	5 (8.3%)	4	1	p=1.000
CC005	2 (10.5%)	0	2	3 (5.0%)	2	1	p=0.588
CC159	5 (26.3%)	0	5	9 (15.0%)	0	9	p=0.302
CC275	4 (21.1%)	1	3	7 (11.7%)	1	6	p=0.205
Cluster 7	1 (5.3%)	0	1	1 (1.8%)	0	1	p=0.421
Misc.	6 (31.6%)	1	5	6 (10.0%)	3	3	p=0.086
Total	19	2	17	60*	34	26	

[a]*p*-values by two-tailed Fishers exact test; *one MRSA not typeable with *spa* typing; Misc-miscellaneous*

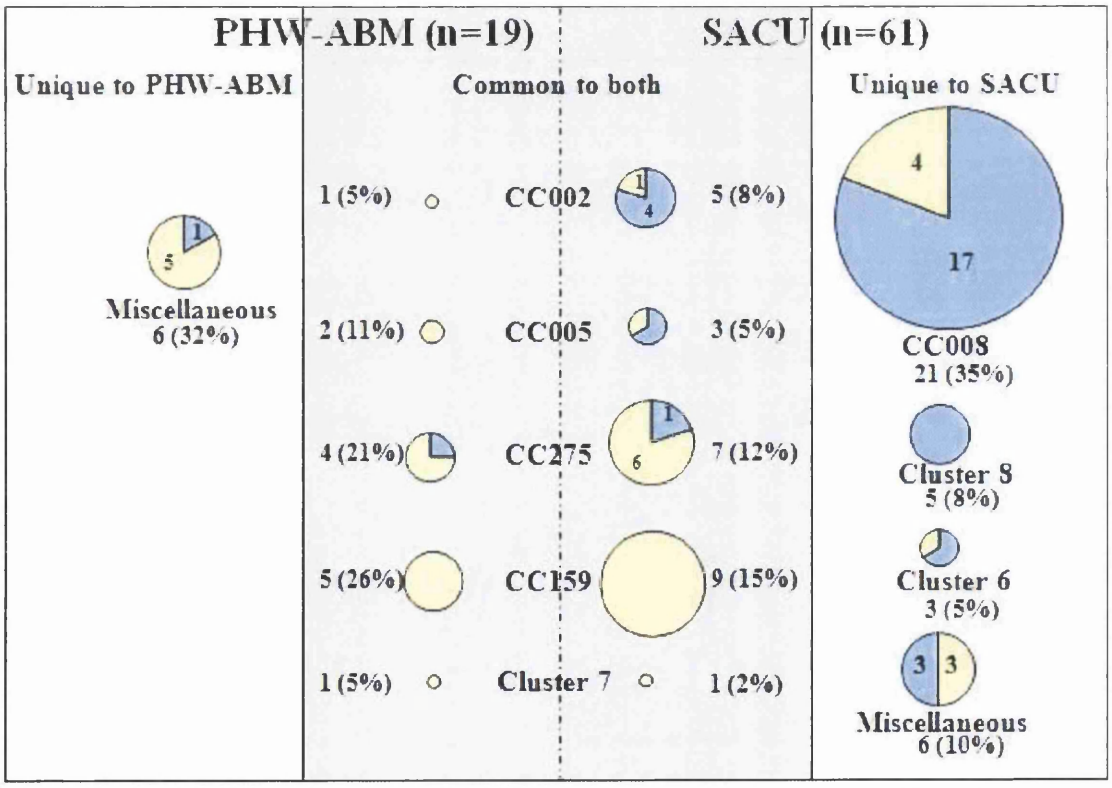


Figure 8.6. A comparison of clonal complexes according to *spa*-BURP analysis of PVL-positive *S. aureus* from (n=61) and PHW-ABM (n=19) (Blue-MRSA, yellow-MSSA).

8.6. Susceptibility testing of the PVL-positive *S. aureus* isolates

Susceptibility testing was performed with the BD Phoenix on the PVL-positive *S. aureus* isolates (n=80) and the majority (n=51, 63.8%) were resistant to trimethoprim (see Table 8.11). There was extensive resistance to tobramycin (n=29, 36.3%), erythromycin (n=22, 27.5%) and variable resistance to tetracycline and co-trimoxazole (n=14, 17.5%) respectively. There was low resistance to fusidic acid (n=10, 12.5%), gentamicin (n=4, 5.0%) and ciprofloxacin (n=4, 5.0%). The dissociation test (D-test) was performed on the erythromycin resistant isolates (n=22) according to the BSAC version 9.1, March 2010 methods for antimicrobial susceptibility testing. This revealed that eight isolates (36.3%) had inducible resistance to clindamycin and one had constitutive resistance to clindamycin (see Table 8.11). Apart from the evident statistically significant difference in the prevalence of methicillin resistance between the PHW-ABM and SACU isolates, most differences in resistance to non β-lactam antibiotics were not statistically significant. There was a statistically significant higher number of PHW-ABM isolates which were resistant to tetracycline (42.1% versus 9.8%) and gentamicin (15.8% versus 1.6%) ($p<0.05$: two-tailed Fishers exact test) (see Table 8.11).

Table 8.11. Antibiotic resistance of PVL-positive *S. aureus* (n=80)

Antibiotic	All (n=80)	SACU (n=61)	PHW-ABM (n=19)
Oxacillin	45.0% (36)	57.4% (35)	5.3% (1)*
Gentamicin	5.0% (4)	1.6% (1)	15.8% (3)*
Tobramycin	36.3% (29)	41.0% (25)	21.1% (4)
Erythromycin	27.5% (22)	29.5% (18)	21.1% (4)
Clindamycin^{C}	1.3% (1)	0.0% (0)	5.3% (1)
Clindamycin^{I}	10.0% (8)	8.2% (5)	15.7% (3)
Tetracycline	17.5% (14)	9.8% (6)	42.1% (8)*
Trimethoprim	63.8% (51)	67.2% (41)	52.6% (10)
Co-trimoxazole	17.5% (14)	14.8% (9)	26.3% (5)
Ciprofloxacin	5.0% (4)	4.9% (3)	5.3% (1)
Fusidic acid	12.5% (10)	14.8% (9)	5.3% (1)

*$p<0.05$; two-tailed Fishers exact test; Clindamycin^{C}-constitutive resistance; Clindamycin^{I}- inducible resistance.

8.7. Discussion

In this study 519 consecutive unselected wound swab *S. aureus* isolates were collected from the PHW Microbiology ABM Laboratory from April 2007 to September 2007. By real-time PCR, PVL genes were detected in 3.7% (n=19) of these isolates This prevalence rate was comparable to the results of a previous investigation on *S. aureus* isolates (n=515) which had been submitted to the UK *Staphylococcus* Reference Unit (SRU) in 2002 (Holmes et al., 2005). In the latter study a subset of every tenth isolate received was tested for PVL genes by PCR and a prevalence rate of 1.8% was determined. A second collection of 470 isolates was used to determine the distribution of PVL genes in different types of staphylococcal infections and 23 (4.9%) were PVL-positive. The majority of these isolates (n=15) ($p<0.00005$) were associated with skin and soft tissue infections (SSTIs): abscesses (n=7), cellulitis (n=3), skin lesions (n=3) and boils. The remaining isolates were from cases of pneumonia, burn infections, bacteraemia and scalded skin syndrome. PVL was not detected in the isolates from deep-seated infections e.g. endocarditis, osteomyelitis (Holmes et al., 2005). This was similar to the present study whereby a significantly ($p=0.0008$) higher number of the PVL-positive isolates were associated with purulent skin infections as opposed to other types of wound infections (see section 8.1.2). However, in the present study the clinical information was not very specific in several cases and so the classifications of infection types for the isolates may have been less robust than in the study by Holmes *et al.* (2005).

In a later study the prevalence of PVL-positive strains in consecutive bacteraemia isolates which were submitted to the UK Staphylococcus Reference Unit in 2005 was investigated and only four of the 244 isolates (1.6%) harboured PVL genes (Ellington et al., 2007). This suggests that PVL-positive *S. aureus* are not a significant cause of bacteraemia in the UK. In a recent study the prevalence rate of PVL was investigated in 390 consecutive unselected *S. aureus* strains isolated from clinical specimens submitted to the Royal Free Hampstead NHS Trust in North London (Shallcross et al., 2009). In the overall collection 12.6% (n=49) of the isolates harboured PVL genes however, a higher prevalence rate (22.6%) was determined in strains isolated from SSTIs and in wound infection isolates the rate was 9.2%. In addition the PVL prevalence rate in the wound infection isolates (9.2%) was considerably higher than the prevalence rate (3.9%) determined in the wound swab isolates of the present study.

However similar to the present study the PVL-positive *S. aureus* isolates (n=49) from the study by Shallcross *et al.*, 2009, were predominantly MSSA isolates (n=44) whereas only five of the isolates were MRSA (Shallcross et al., 2009). From quantitative statistical analysis of their clinical data they determined that certain factors were independently associated with PVL including: increased duration of hospital stay, male gender (which they attributed to increased body hair which may predispose to infection) and SSTIs. However, it is debatable if these factors are truly specific to PVL-positive strains since they appear to be characteristic features of *S. aureus* infections in general.

In another study DNA microarrays were used to characterise 155 *S. aureus* strains isolated from nasal swabs of asymptomatic carriers from Saxony Germany (Monecke et al., 2009). The microarrays covered 332 target sequences corresponding to distinct genes, including virulence genes, antimicrobial resistance markers, superantigens, exotoxins, SCC*mec*, capsule and *agr* group typing markers. Within the 155 *S. aureus* isolates PVL was detected in one MSSA isolate (0.65%) and *mecA* was detected in three isolates (1.94%), indicating that the prevalence of methicillin resistance and PVL is low in *S. aureus* strains from asymptomatic carriers of Saxony, Germany (Monecke et al., 2009). However in an earlier study carried out in the same geographical region 30% of 100 *S. aureus* strains isolated from SSTIs including abscesses and chronic soft tissue infections harboured PVL genes. This indicated a greater association of PVL with *S. aureus* strains isolated from SSTIs rather than asymptomatic nasal carriage (Monecke et al., 2009, Monecke et al., 2007). A comparison of the PVL prevalence rate in *S. aureus* strains isolated from nasal swabs of healthy individuals and strains isolated from infections was also undertaken in a study conducted in Rotterdam, the Netherlands (Melles et al., 2006). In a collection of 1033 *S. aureus* strains isolated from nasal swabs of healthy children (<19 years) and adults (>55yeas) the *mecA* gene had not been identified in any of the isolates, which is indicative of the low MRSA prevalence rate in the Netherlands (Melles et al., 2006). Eight hundred and ninety two isolates selected from the same collection were used to evaluate the prevalence of PVL in carriage and invasive isolates (146 *S. aureus* strains isolated from blood cultures). Five (1.6%) PVL-positive *S. aureus* strains were identified in the carriage group and three (2.1%) blood-culture *S. aureus* isolates carried PVL genes. However a significantly higher ($p<0.0001$) prevalence rate of PVL (38.9%; 7/18) was reported in 18 strains isolated from SSTIs such as abscesses (Melles et al., 2006).

This was comparable to prevalence rates reported in an earlier study conducted in Strasbourg, France (Prevost et al., 1995). In the latter study low PVL prevalence rates were reported in 31 nasal carriage *S. aureus* isolates (0%) and 69 blood culture isolates (1.4%) but a higher PVL prevalence rate (23.2%) was reported in 246 *S. aureus* strains which had been isolated from cutaneous infections such as abscesses, furunculosis, folliculitis and paronychia (Prevost et al., 1995)

In summary the results of the present study and afore-mentioned studies indicate that the prevalence rate of PVL-positive *S. aureus* is relatively low in the UK and these strains are largely methicillin susceptible (Shallcross et al., 2009, Holmes et al., 2005). Furthermore the PVL-positive *S. aureus* strains are extensively associated with purulent SSTIs such as abscesses in contrast to invasive infections such as bacteraemia (Ellington et al., 2010a, Shallcross et al., 2009, Monecke et al., 2009, Melles et al., 2006, Prevost et al., 1995). This is markedly different to the situation in the USA where it has been reported that the prevalence of the USA300 clone, a PVL-positive CA-MRSA is accounting for 59% (range 15% to 74%) of SSTIs among patients seeking treatment at accident and emergency departments across USA cities (Edelsberg et al., 2009).

In this study it was evident that PVL-positive MRSA isolates (n=37) were polyclonal and by *spa*-BURP analysis belonged to eight clusters: CC002, CC005, CC008, CC159, CC275, cluster 6, 7 and 8. The majority of the MRSA isolates (n=16, 43.2%) belonged to the USA300 clone which constituted the CC008 *spa* cluster. Other prominent clusters were cluster 8 (n=5, 13.5%) and CC002 (n=4, 10.8%) which comprised strains exhibiting the t044-MRSA-IV and t002-MRSA-IV genotypes. Previous studies and the epidemiological information associated with *spa* types in the on-line Ridom SpaServer (http://spaserver2.ridom.de/spatypes.shtml) have demonstrated that these genotypes are characteristic of the European (ST80-MRSA-IV) and USA800 (ST5-MRSA-IV) clones respectively (Deurenberg and Stobberringh, 2008, Larsen et al., 2008, Strommenger et al., 2006). The less prominent clusters were cluster 6, CC005 and CC275 which were characterised by strains with the following core genotypes: t127-MRSA-IV; t005-MRSA-IV and t019-MRSA-IV. The latter are typically exhibited by strains in the ST1, ST22 and ST30 lineages which harbour the USA400 (ST1-MRSA-IV), EMRSA-15/Barnim (ST22-MRSA-IV) and Southwest Pacific clones (ST30-MRSA-IV)

respectively (Deurenberg and Stobberringh, 2008). Therefore these results indicate that in addition to the USA300 clone, representative strains of other established CA-MRSA clones such as the European, USA800, USA400 and the South-West Pacific clones were present.

Other studies have also illustrated the polyclonal epidemiology of PVL-positive MRSA isolates in the UK (Ellington et al., 2010b, Ellington et al., 2009, Otter et al., 2009, Holmes et al., 2005). These studies include an investigation of 275 PVL-positive MRSA isolates from England and Wales which were submitted to the SRU from 2005 to 2006. The isolates belonged to eight MLST clonal complexes: CC1, CC5, CC8, CC22, CC30, CC59, CC80 and CC88 and the majority belonged to CC80 (32.0%), CC8 (25.5%) and CC30 (17.8%) which were characterised by the following genotypes: t044-MRSA-IVc, t008-MRSA-IVa and t019-MRSA-IV typical of the European, USA300 and South-West Pacific clones (Ellington et al., 2009). This indicated that in contrast to the present study, strains related to the European clone were predominant (32% versus 13.5% in this study) whereas USA300 strains were less prevalent (25.5% versus 43.2% in this study). In another study the molecular epidemiology of 76 PVL-positive MRSA isolates with multiple antibiotic resistance, submitted to the SRU from 2005 to 2008 was investigated (Ellington et al., 2010b). These isolates belonged to six MLST clonal complexes: CC1, CC8, CC22, CC59, CC80 and CC5 however, unlike the previous study the CC80 (14.5%) and CC8 (9.2%) lineages were less prevalent and the majority of isolates belonged to CC1 (n=29, 38.2%). Interestingly, the latter lineage was characterised by strains exhibiting *spa* type t657 and the SCC*mec* type VII element. It was established that most of those strains were associated with travel to the Bengal Bay, mainland India or family links to these regions (Ellington et al., 2010b). Otter *et al.* (2009) compared PVL-positive MRSA isolates (n=71) from a hospital in England and a hospital in New Haven Connecticut, USA (n=43). They observed marked differences in the molecular epidemiology of the two collections. The UK isolates displayed more genetic diversity and belonged to several *spa*-BURP clusters affiliated to the following MLST lineages: ST1, ST5, ST8, ST22, ST59, ST80, ST88 and ST97. The majority belonged to the ST1 lineage (n=27, 38%) and only 5.6% of the isolates belonged to the ST8 lineage which contained strains with the USA300 PFGE pattern and *spa* type t008. In contrast, only two lineages, ST8 and ST59 were represented in the USA isolates and 65.1% of the strains belonged the ST8 lineage (Otter et al., 2009).

Collectively these results demonstrate the polyclonal epidemiology of the PVL-positive MRSA isolates in the UK. Certain lineages were commonly identified across the respective studies, including the ST80-MRSA-IV (European clone), ST1-MRSA-IV (Midwest/USA400), ST5-MRSA-IV (USA800) and ST8-MRSA-IV (USA300) lineages. However in contrast to the present study the USA300 strains were not predominant in the previous studies (Ellington et al., 2010b, Ellington et al., 2009, Otter et al., 2009, Holmes et al., 2005). The differences in the prevalence rates may be due to a variety of factors which affect the endemicity, transmissibility and dissemination of strains such as genetic background of the strains, patient demographics (including age, gender, ethnicity, socio-economic factors), travel history and environmental factors (Otter et al., 2009). It is possible that the unusual higher rate of USA300 strains in the present study was associated with travel to the USA where these strains are highly prevalent. However, this could not be verified due to limited patient history information.

Twenty-eight percent of the PVL-positive MSSA isolates in this study (n=43) had diverse genotypes which were not affiliated to any of the *spa*-BURP clusters and the remaining isolates belonged to seven of the eight clusters. This indicated the MSSA strains were more genetically diverse than the MRSA isolates. The CC159 (23%; 10/43) and CC275 (21%; 9/43) *spa*-BURP clusters were the predominant genotypes in the MSSA isolates. The former was characterised by strains exhibiting the t159 *spa* types (and closely related variants). This genotype was absent in the MRSA and suggesting that although it may be a successful PVL-positive MSSA lineage it remains unreceptive to SCC*mec* elements in this local epidemiological setting. The *spa* type t159 is associated with the ST121 lineage which has been extensively associated with MSSA rather than MRSA in several studies in different countries like the UK, Denmark, China, Cambodia and Thailand (Chheng et al., 2009, Gomes et al., 2006, Holmes et al., 2005) (http://spaserver2.ridom.de/spatypes.shtml). The CC275 genotype was characterised by t019, t021 and t138 *spa* types which are associated with the ST30 lineage which harbours the Southwest Pacific CA-MRSA clone (ST30-MRSA-IV) (http://spaserver2.ridom.de/spatypes.shtml). Therefore these results indicated that the majority of the PVL-positive MSSA isolates in this study were affiliated to the ST121 and ST30 lineages. In a recent study the molecular epidemiology of two collections of PVL-positive *S. aureus* strains from two different geographical regions was compared by microarray analysis (Monecke et al., 2007). One collection comprised 30 PVL-

positive *S. aureus* strains which had been identified from 100 consecutive isolates received at a university hospital in Dresden, Saxony, Germany (Monecke et al., 2007). These were predominantly MSSA strains (MSSA n=27; MRSA n=3) and had been isolated from SSTIs including abscesses, chronic soft-tissue infection, complicated acne, furunculosis and paronychia. The second collection comprised 18 PVL positive MSSA strains isolated from abscesses which had been submitted to the SRU in the UK, over a 2-year period (2004 to 2006) (Monecke et al., 2007). Even though the collections were from two different countries, in both collections the majority of the isolates belonged the ST121, ST30 and ST22 clonal lineages (Monecke et al., 2007). This was comparable to the present study whereby the CC159 and CC275 *spa* clusters, associated with the ST121 and ST30 lineages respectively, were predominant in the PVL-positive MSSA isolates. Since the UK strains in the former study had been collected from 2004 to 2006 and the isolates in the present study were collected from April 2007 to September 2007, this suggests that the ST121 and ST30 lineages remained predominant in UK PVL-positive MSSA strains from 2004 to 2007.

In the present study the CC005 *spa*-cluster genotype was present to the MSSA (7.0%; 3/43) and MRSA (5.5%; 2/37) isolates but was less prevalent in both sets of isolates. These PVL-positive strains exhibited the t005, t223 *spa* types (and related variants t891 t1869) and PFGE patterns that were similar to the EMRSA-15 reference strain. The t005 *spa* type is affiliated to the ST22 lineage which harbours the EMRSA-15/Barnim clone (t032-MRSA-IV) a predominant PVL-negative HA-MRSA clone in the UK. It is postulated that the PVL-negative t032 strains and the PVL-positive t005 strains evolved independently from the ancestral ST22-MSSA lineage (Boakes et al., 2010). Initially the t032-MRSA-IV HA-MRSA clone evolved from a PVL-negative ST22-MSSA ancestral strain and then the t005-MRSA-IV clone evolved separately after the ancestral strain acquired PVL genes (Boakes et al., 2010). Though the t005-MRSA-IV and t032-MRSA-IV genotypes are closely related, the latter genotype is evidently advantaged in UK hospital settings but apparently remains unreceptive to bacteriophages which carry PVL genes, whereas the t005-MSSA/ MRSA genotype seems to provide a more selective advantage in community settings (Boakes et al., 2010).

In the UK several studies have been performed on PVL-positive *S. aureus* isolates that have been submitted to reference laboratories (Boakes et al., 2010, Ellington et al.,

2010b, Ellington et al., 2010a, Ellington et al., 2009, Ellington et al., 2007). However these isolates may not sufficiently represent the epidemiology of PVL-positive strains of a geographical region as they are often selected based on various underlying factors and this may influence the selection of particular genotypes. On the other hand other studies have investigated the prevalence and molecular epidemiology of PVL-positive isolates submitted to routine clinical laboratories for diagnostic purposes (Otter et al., 2009, Rollason et al., 2008, Rossney et al., 2007). In the present study in order to achieve a more comprehensive view of the molecular epidemiology of PVL-positive isolates a direct comparison of the unselected PHW-ABMU isolates (n=19) and isolates referred to SACU (n=61) was performed. Interestingly even though both collections of isolates were from Wales, they were markedly different and this was initially indicated by the high prevalence rate of methicillin resistance (57.4%; 35/61) in the SACU isolates in contrast to the PHW-ABM isolates which were essentially methicillin susceptible (89.5%; 17/19). These results are interesting because some of the world-wide established CA-MRSA clones are PVL-positive and so it may be presumed that PVL-positive strains are predominantly methicillin resistant. Certainly solely based on the results of the SACU isolates this also appears to be the case, however, the results of the unselected isolates contradict this and imply that PVL-positive isolates in Southwest Wales are predominantly MSSA strains. The PHW-ABM and SACU isolates were both polyclonal but the existing clones differed in their composition and prevalence rates. A distinct difference was the predominance of USA300 strains (26.2%) in the SACU isolates (n=61) and yet these strains were completely absent in the unselected PHW-ABM isolates. Similarly cluster 8 (8%) and cluster 6 (5%) which were associated with the European and USA400 clones were present in the SACU isolates but absent from the unselected PHW-ABM isolates. Actually 32% (6/19) of PHW-ABM isolates had *spa* types which were not associated with any of the *spa*-BURP clusters indicating that these were from unrelated genetic backgrounds. The remaining thirteen isolates (68%; 13/19) were represented by five different *spa*-BURP clusters which were also present in the SACU isolates. These results indicate that despite the high prevalence of established CA-MRSA strains, particularly the USA300 clone in the SACU isolates, these were absent in unselected isolates from PHW-ABMU. Accordingly this may also imply that these clones are infrequent in the PVL-positive *S. aureus* strains of the Southwest Wales region.

In this study the majority of the PVL-positive *S. aureus* isolates were resistant to trimethoprim (63.8%; 51/80) and a variable number of isolates were resistant to tobramycin (36.3%; 29/80) and erythromycin (27.5%; 22/80). Furthermore 10% percent of the isolates had inducible clindamycin resistance and one had constitutive resistance. Several isolates were also resistant to tetracycline (17.5%; 14/80) and co-trimoxazole (17.5%; 14/80). However antibiotic resistance profiles could not be specifically affiliated to particular clones in this study. For instance resistance to fusidic acid, tetracycline and neomycin has been previously identified as the typical resistance profile for the European clone (ST80-MRSA-IV) (Ellington et al., 2009). However in this study this profile was not restricted to the t044-MRSA-IV isolates and strains in other clusters e.g. CC002, cluster 6 and CC275 also exhibited resistance to fusidic acid, tobramycin and tetracycline. These results support the growing concern that multiple antibiotic resistance is increasingly exhibited by PVL-positive CA-MRSA strains (Higuchi et al., 2010, Ellington et al., 2010b, Coombs et al., 2006).

The UK Health Protection Agency has recommended susceptibility to ciprofloxacin as a putative marker for CA-MRSA (Ellington et al., 2010a, Ellington et al., 2010b). In this study ciprofloxacin resistance was detected in 5% (n=4) of the eighty isolates; these included three USA300 strains (SACU isolates) and one MSSA strain (PHW-ABM isolate) which depicted the t021-MSSA genotype. Since the leading CA-MRSA clones are PVL-positive, this suggests that ciprofloxacin susceptibility is still a relatively good marker for PVL-positive MRSA. However Ellington *et al.* (2010b) observed ciprofloxacin resistance in PVL-positive MRSA strains belonging to several clones in the UK i.e. ST1, ST5, ST80, ST22 and ST8. Consequently they suggested that the utility of ciprofloxacin susceptibility as a marker is diminishing. Markers based on antibiotic resistance or susceptibility may be disingenuous because the acquisition of resistance by bacteria is dynamic especially under a selective environments.

The UK Health Protection Agency (HPA) 2008 report - 'Guidance on the diagnosis and management of PVL-associated *Staphylococcus aureus* infections in England, 2nd edition' - recommends the administration of clindamycin for the treatment of moderate SSTIs caused by PVL-positive MSSA. For PVL-positive MRSA the combination of rifampicin with either doxycycline, fusidic acid, trimethoprim or clindamycin is recommended. The results of this study showed a high prevalence of trimethoprim

resistance thus suggesting that this drug may be largely ineffective for the treatment of SSTIs caused by PVL-positive *S. aureus* in Wales. The observed variable resistance to tetracycline (17.5%; 14/80) suggests that the utility of this class of antibiotics i.e. doxycline may also be diminishing. Clindamycin is recommended for severe infections by PVL-positive MSSA e.g. deep seated infections and osteomyelitis and has also been shown to have some effectiveness in necrotising pneumonia (in combination with vancomycin). In this study 10% of the isolates expressed inducible clindamycin resistance and one had constitutive resistance, which suggests the possible ineffectiveness of this drug in some cases of severe infections. The low resistance to fusidic acid, ciprofloxacin, gentamicin and complete susceptibility of all isolates to rifampicin, vancomycin, daptomycin, mupirocin and linezolid indicates that these drugs remain effective against PVL-positive *S. aureus* infections in Wales.

In conclusion, this study has been informative as it has enabled the determination of the prevalence of PVL-positive strains in Southwest Wales and furthermore granting some insight into the molecular epidemiology of these strains in Wales. The results were comparable with previous findings in that they indicated that the prevalence rate of PVL-positive *S. aureus* remains low (3.9%). However contrary to an observed high prevalence rate of methicillin resistance in isolates that were submitted to the SACU reference laboratory, the results of the unselected isolates from PHW Microbiology ABM Laboratory suggest that PVL-positive *S. aureus* strains in Southwest Wales are largely represented by genetically diverse MSSA strains.

Chapter Nine

Identification of *Staphylococcus aureus* isolates by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry

9.0 Introduction

S. aureus is a significant pathogen which typically causes various skin and soft tissue infections e.g. abscesses and boils, but can also cause severe infections like bacteraemia and necrotising pneumonia. Of particular concern are MRSA strains which are associated with increased morbidity and are prevalent in hospital settings in many countries world-wide. The implementation of infection control measures is essential for the prevention of dissemination of MRSA strains and rapid diagnosis is an integral part of infection control. A plethora of phenotypic and molecular diagnostic methods have been developed for the identification of *S. aureus* e.g. conventional culture-based phenotypic tests, PCR and sequencing-based methods. In addition mass spectrometry has been widely used for the analysis of micro-organisms and the characterisation of proteins and peptides. In recent years several studies have demonstrated the capacity of matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry for the identification of bacterial isolates to species level e.g. staphylococci, enterobacteria, non-fermentative gram negative rods, listeria, pneumococci and anaerobes (Szabados et al., 2010b, Dubois et al., 2009, Nagy et al., 2009, Rajakaruna et al., 2009, Seng et al., 2009, Barbuddhe et al., 2008, Grosse-Herrenthey et al., 2008, Williamson et al., 2008, Carbonnelle et al., 2007).

The flexibility, speed and accuracy of the MALDI-TOF MS assay are key factors in its increasing popularity in routine clinical analysis. Either intact bacteria (bacterial colonies) or bacterial extracts may be used in the simple set-up procedure which involves an initial co-crystallization step of the sample with a radiation-absorbing matrix followed by exposure to laser radiation. The resulting ions are directed into a flight tube and the time taken by respective ions to arrive at the detector is measured and converted to a mass to charge ratio (m/z) (Marvin et al., 2003). This yields a spectrum consisting of a series of protein peaks recorded in a mass-to-charge ratio of 2,000 to 20,000 m/z (Dubois et al., 2009, Bittar et al., 2009, Carbonnelle et al., 2007, Jackson et al., 2005, Keys et al., 2004).

The discriminatory capacity of the MALDI-TOF method at a subspecies level and its application as a typing technique is a current area of interest and recently Wolters *et al.* (2010) demonstrated its potential to discriminate five MRSA lineages e.g. CC5, CC8, CC22, CC30 and CC45. Other studies have demonstrated its potential as a typing tool for EMRSA-15 and EMRSA-16 strains, *Salmonella enterica* and *Streptococcus* species (Wolters et al., 2010, Lartigue et al., 2009, Dieckmann et al., 2008, Walker et al., 2002, Edwards-Jones et al., 2000). This method has also been applied for the discrimination of antibiotic resistant and susceptible strains and the detection of virulence factors in bacteria e.g. Panton Valentine-Leukocidin (PVL) in *S. aureus* (Bittar et al., 2009, Majcherczyk et al., 2006, Du et al., 2002, Walker et al., 2002, Edwards-Jones et al., 2000). However this particular application remains debatable as other studies have questioned the stability and reproducibility of previously identified mass ion peaks which were recommended as biomarkers for antibiotic resistance or virulence factors (Szabados et al., 2010a, Rajakaruna et al., 2009).

In this study *S. aureus* clinical isolates (n=137) had previously been determined as MRSA by using *mecA* and $femA_{SA}$ real-time PCR methods (results presented in chapter 3). The isolates were further characterised by PFGE, *spa* typing and SCC*mec* typing and the following strains were identified: EMRSA-15 (n=122), EMRSA-16 (n=8) and unique MRSA strains (n=7) (results presented in chapter 4). By *spa*-BURP analysis 37 PVL-positive MRSA isolates were shown to belong to eight genotypes including the USA300 clone and the t044-MRSA-IV genotype which has been affiliated with the European clone. The objective of this study was to explore the MALDI Biotyper 2.0 System (Bruker Daltonics) with bacterial extracts of *S. aureus* isolates (n=137), to determine the diagnostic sensitivity of the method and to investigate the potential of this method for the discrimination of MRSA strains.

9.1 Results

9.1.1 Identification of *S. aureus* isolates with MALDI-TOF MS method

The generated mass spectra profiles of the respective *S. aureus* isolates (n=137) were automatically analysed by the MALDI Biotyper 2.0 software (Bruker Daltonik, Bremen, Germany). The spectra comprised mass ion peaks of varying intensity (see y-axis in Figure 9.1) over a broad range of 2000 to 20000 m/z (see x-axis in figure 9.1). The assay proved reproducible as shown by high intensity peaks repeatedly present in six replicates of the respective extracts (see Figure 9.1).

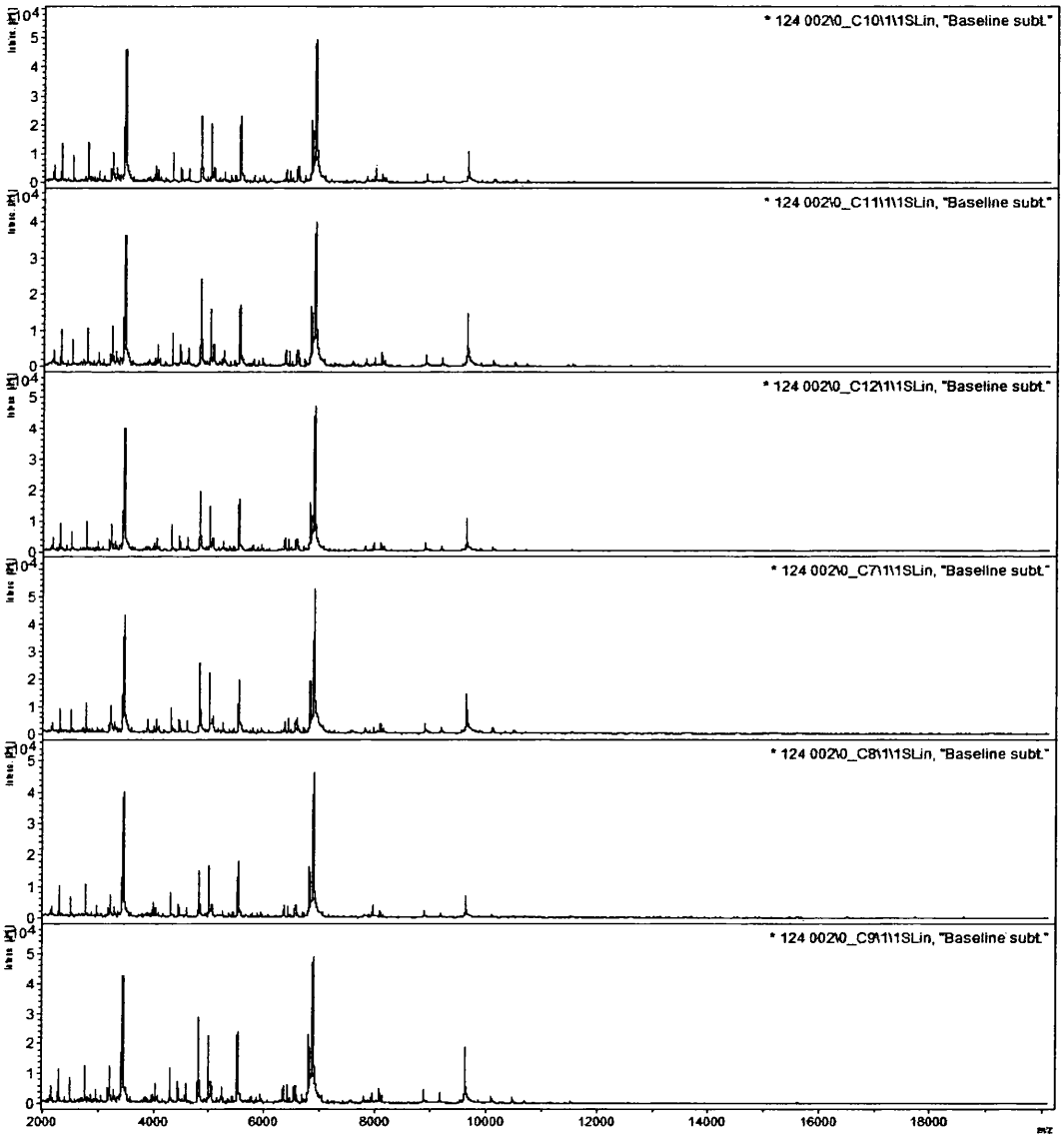


Figure 9.1. Mass spectra of six replicates of a bacterial extract of a single *S. aureus* isolate

The mass ion peaks were automatically evaluated against reference spectra profiles in the MALDI Biotyper 2.0 database and log scores were generated based on the classification of results into four categories: a log score of ≥2.3 indicates a highly probable species identification, a log score of ≤2.299 and ≥2.0 indicates secure genus identification, probable species identification, a log score value of ≤1.999 and ≥1.7 indicates probable genus identification, and a log score of ≤1.699 indicates no reliable identification. The isolates (n=137) had been previously identified as S. *aureus* with the *femA*$_{SA}$ real-time PCR and with the MALDI-TOF method all of the isolates were correctly identified at species level, illustrating that the assay had 100% diagnostic sensitivity. The mean log score value was 2.47 (standard deviation 0.04), the median log score value was 2.48, the lowest and highest log score values were 2.29 and 2.56 respectively. The mass spectra profile of a randomly selected *S. aureus* clinical isolate (isolate 39) was compared to that of the *S. epidermidis* strain 1457 and by visual comparison there were clear differences between the profiles of the two isolates (see Figure 9.2). Within the range of 2000 to approximately 7000 m/z, there was a higher peak density of mass ions in the spectra of the *S. aureus* isolate in contrast to the *S. epidermidis* isolate.

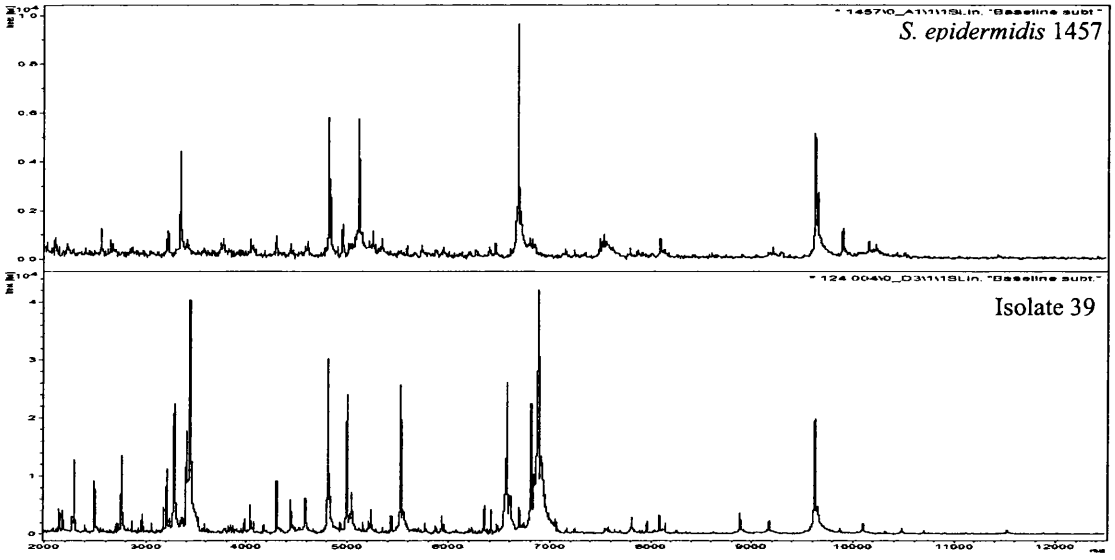


Figure 9.2. Raw mass spectra of *S. epidermidis* isolate, 1457 (top) and *S. aureus* isolate, 39 (bottom).

Further evaluation of the proportion of mutual mass ion peaks versus unique mass ion peaks between the two isolates was carried out by assessing a graphical representation generated by the software, as seen in Figure 9.3.a. The green, yellow and red colour coding enables identification of mass ion peaks which do or do not match between the compared spectra. A comparison of the peaks of the *S. aureus* isolate 39 and the *S. epidermidis* strain 1457 revealed that the majority of peaks of the *S. aureus* were not present in the *S. epidermidis* strain (all the peaks of the *S. epidermidis* strain 1457 are depicted in blue and unmatched peaks are depicted in red, see Figure 9.3a). However a few peaks were coded in yellow which depicted mass ions that were mutual to both the *S. aureus* and *S. epidermidis* strains, an indication of the presence of staphylococcal genus-specific mass ions (see Figure 9.3a). A subsequent comparison of the peaks of two *S. aureus* strains: isolate 39 (an EMRSA-15 clinical isolate) and the EMRSA-15 reference strain showed that a majority of mass ion peaks were shared by both isolates (peaks depicted in green, see Figure 9.3b) and a few peaks were unique to the each strain (peaks depicted in red, see Figure 9.3b).

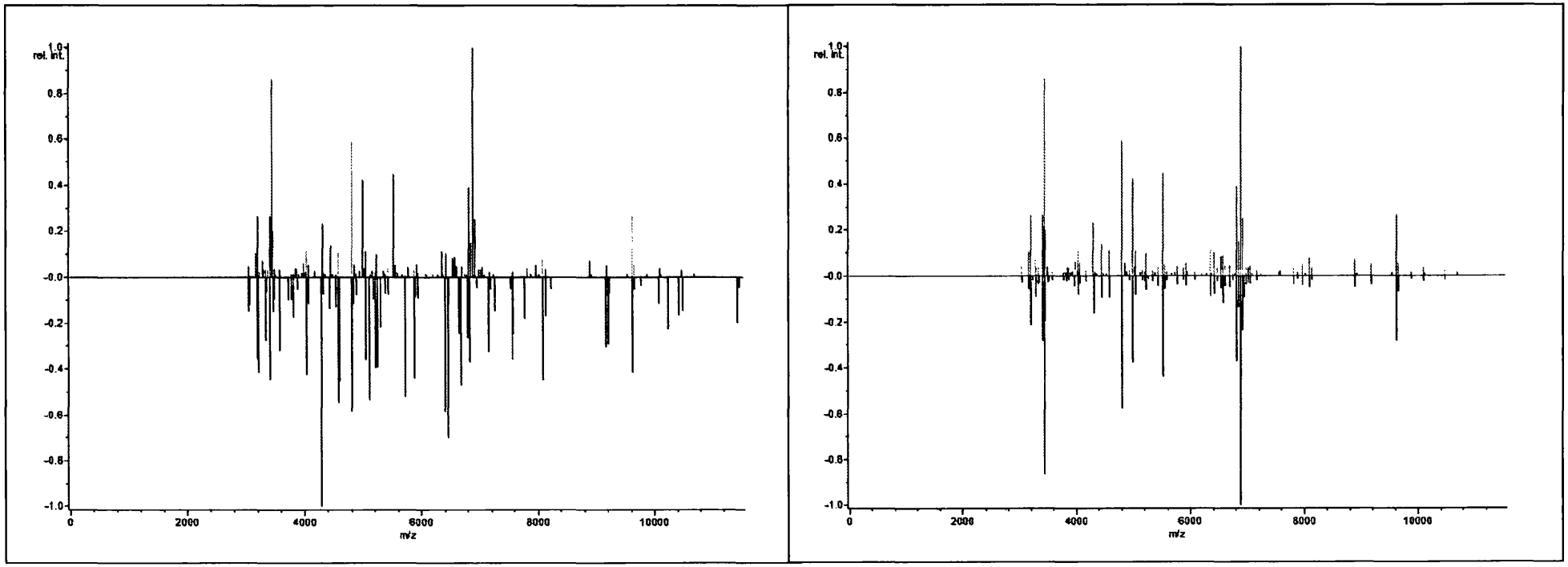


Figure 9.3a. A comparison of the mass ion peaks of a *S. aureus* clinical isolate 39 (top) and the *S. epidermidis* strain 1457 (bottom). **b.** A comparison of the mass ion peaks of the *S. aureus* 39 (top) and the EMRSA-15 reference strain (bottom). Green-mass ion peaks with good match, yellow-mass ion peaks with moderate match, red- mass ion peaks that do not match. Blue- all mass ion peaks of isolate.

9.2 Investigating the potential of MALDI-TOF to discriminate EMRSA-15 and EMRSA-16 strains

A comparison of the mass spectra of the EMRSA-15 reference strain and two clinical EMRSA-15 strains (isolate 39 and 40) and the EMRSA-16 reference strain and two clinical EMRSA-16 strains (isolate 21 and 145) was conducted. There were two high intensity peaks (approximately 2000m/z and 7000m/z) which were noticeably present in the spectra of the reference and clinical strains, thus representing two common *S. aureus* species-specific mass ions (see Figure 9.4a). However the profiles of the EMRSA-16 reference and clinical isolates 21 and 145 had a higher peak density within the range of 2000 to approximately 7000 m/z, illustrating the presence of mass ions which were specific to these particular strains but absent in the EMRSA-15 strains (see lower three rows in Figure 9.4a).

According to the Tenover *et al.* 1995 criteria, isolates with PFGE patterns that are indistinguishable or that have a ≤ 3 band difference are considered closely related and belong to a single clone, whereas those with >6 band difference are unrelated. As seen in Figure 9.4b isolates 39 and 40 had PFGE patterns with a ≤ 3 band difference strains to the EMRSA-15 reference strain, signifying that they were strains of this clone. Similarly the mass spectra of these two clinical isolates were very similar to the EMRSA-15 reference strain (see first three rows in Figure 9.4a). Likewise by PFGE the clinical isolates 21 and 145 had patterns which signified that they belonged to the EMRSA-16 clone and similarly they had mass spectra profiles which were comparable to the EMRSA-16 reference strain. These results suggested that the MALDI-TOF method has the capacity to generate *S. aureus* strain-specific mass ions. Similarly, isolates which were designated as EMRSA-15 and EMRSA-16 strains by PFGE, exhibited profiles with the MALDI Biotyper 2.0 system which were highly similar to the EMRSA-15 and EMRSA-16 reference strains, respectively. This illustrated that there was congruence between the two methods and indicated the possibility of applying the MALDI-TOF Biotyper 2.0 system as a typing assay for *S. aureus*.

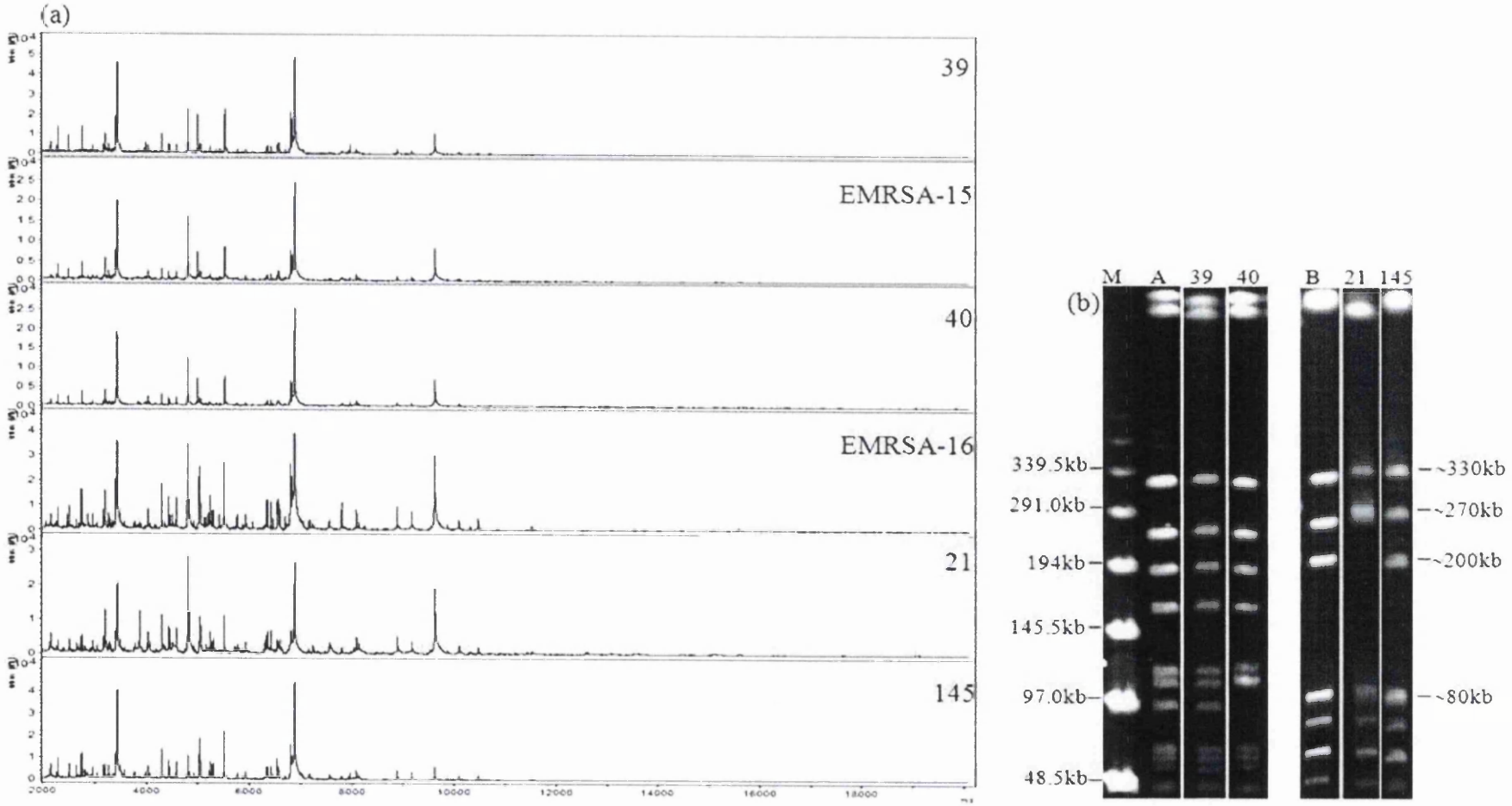


Figure 9.4a. Raw mass spectra EMRSA-15, EMRSA-16 reference strains and *S. aureus* clinical isolates (39, 40, 21 and 145). **b.** PFGE patterns of EMRSA-15, EMRSA-16 reference strains and clinical isolates (39, 40, 21 and 145). Lane M, lambda DNA marker; A, EMRSA-15 reference strain; isolate 39; isolate 40; B, EMRSA-16 reference strain; isolate 20; isolate 145.

9.3 The potential of MALDI-TOF method as a typing tool for *S. aureus* isolates

To investigate the potential typing capacity of the MALDI-TOF assay the following *S. aureus* clinical isolates belonging to four different clones as previously determined by PFGE and *spa* typing analysis were randomly selected:

- EMRSA-15 clone: clinical strains (n=14) termed EM15a to EM15n
- EMRSA16 clone: clinical strains (n=7) termed EM16a to EM16g
- USA300 clone: clinical strains (n=7) termed USA300a to USA300g
- t044-MRSA-IV clinical strains (n=3) termed EUa to EUc

In this study the bacterial extracts were spotted on the target plate six times, so each isolate was run in six replicates. Using the MALDI-TOF Biotyper 2.0 software the six replicate mass spectra of each respective isolate were compiled into a single main spectra profile (MSP) for each. Cluster analysis with the MALDI Biotyper software was then performed on the MSPs of the isolates. The EMRSA-15 and EMRSA-16 reference strains were also included in the analysis. Initially the analysis was performed on six EMRSA-15 (EM15a to EM15f) and six EMRSA-16 (EM16a to EM16f) strains and these were distributed separately into two distinct clusters in the generated dendrogram (see Figure 9.5a). Therefore the clusters defined by the MALDI Biotyper 2.0 system were congruent with EMRSA-15 and EMRSA-16 clones as defined by PFGE and *spa* typing, indicating that this method has the potential to distinguish two *S. aureus* clones.

Six USA300 strains (USA300a to USA300f) were then assessed together with the previous EMRSA-15 and EMRSA-16 strains. In the dendrogram two clusters were evident at a distance level of 500 (arbitrarily selected). The six USA300 strains were designated into one cluster but two EMRSA-15 strains (EM15c and EM15f) were also included in this cluster (see Figure 9.5b). The remaining four EMRSA-15 strains and all six EMRSA-16 strains were designated into the second cluster. At a distance level of 400 three clusters were evident, one cluster contained only EMRSA-15 strains (EM15a, d and e) but there were discrepancies in the other two clusters. One cluster contained all of the EMRSA-16 strains and one EMRSA-15 (EM15b) strain whereas the other comprised all of the USA300 strains and two EMRSA-15 strains (EM15c and f) (see Figure 9.5b). Therefore with a larger collection of strains in which three clones were represented, cluster analysis by the MALDI Biotyper 2.0 software had less correlation to PFGE.

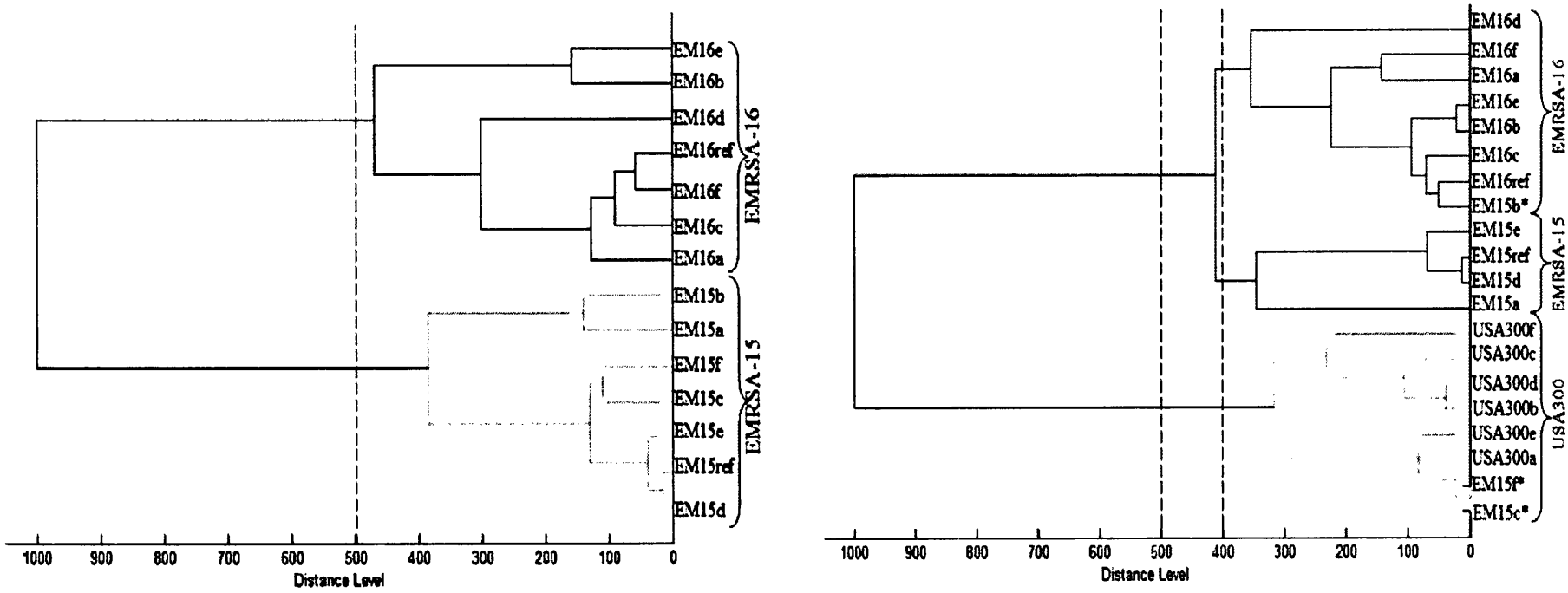


Figure 9.5. Dendrogram by MALDI Biotyper 2.0 software illustrating cluster analysis of strains belonging to different *S. aureus* clones **a.** EMRSA-15 and EMRSA-16 strains. **b.** *S. aureus* strains belonging to the EMRSA-15, EMRSA-16 and USA300 clones. Vertical lines indicate the similarity cut-off value chosen arbitrarily to indicate clusters

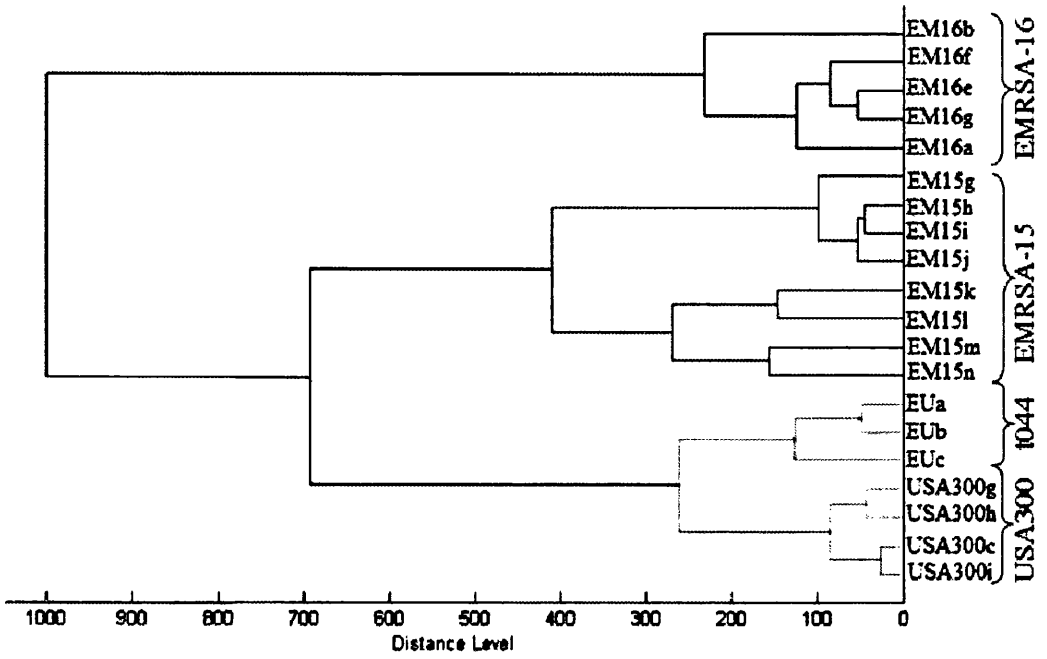


Figure 9.6. Dendrogram by MALDI Biotyper 2.0 software illustrating cluster analysis of *S. aureus* strains belonging to the EMRSA-15, EMRSA-16, USA300 clones and the t044-MRSA-IVgenotype.

Cluster analysis with the MALDI Biotyper 2.0 software was also performed on the raw spectra of strains belonging to four clones: EMRSA-15, EMRSA-16, USA300 and strains with the t044-MRSA-IV genotype which is associated with the European clone. In the generated dendrogram there was evident discrimination of the strains according to the four clones and the EMRSA-16 and EMRSA-15 strains were designated into separate clusters. However at a cut-off distance level of 500 (arbitrarily selected) the USA300 and the t044-MRSA-IV strains constituted a single cluster. Interestingly the EMRSA-15 and EMRSA-16 strains are predominant UK HA-MRSA strains whereas, the USA300 strains and the t044-MRSA-IV strains are PVL-positive strains which are predominantly associated with community-acquired infections. This suggested that the MALDI Biotyper 2.0 software had the potential to discriminate four MRSA clones and the defined clusters were largely concordant with the clones as defined by PFGE and *spa* typing (see Figure 9.6).

9.4 Discussion

In this study the MALDI Biotyper 2.0 System was applied to bacterial extracts of 137 *S. aureus* clinical isolates and all of the isolates were correctly identified at species level, demonstrating 100% diagnostic sensitivity of the assay. These results are comparable to previous studies in which collections of *S. aureus* and coagulase-negative staphylococci were evaluated and identified with excellent accuracy (Szabados et al., 2010b, Dubois et al., 2009, Carbonnelle et al., 2007). However lower diagnostic sensitivity values have been attained in some recent studies including one study which used MALDI-TOF for the analysis of 95 *S. aureus* isolates and the MicrobeLynx software for the processing of the data (Rajakaruna et al., 2009). The isolates were initially analysed with a 2005 reference database and four isolates were misidentified as *Streptococcus pyogenes*, *S. haemolyticus*, *S. epidermidis* and *S. warneri*. In a subsequent analysis using an updated 2006 reference database containing additional *S. aureus* reference spectra, the previously misidentified isolates were correctly identified as *S. aureus* isolates. In another study the MALDI Biotyper 2.0 System was used on a collection of diverse bacterial species (n=1660) including anaerobic bacteria and 95.4% of the isolates were correctly identified (Seng et al., 2009). Similar to the afore-mentioned study, 2.8% of the isolates had been misidentified due to the lack of representative spectra in the reference database. This illustrates that despite the advantageous speed of the MALDI-TOF assay, the diagnostic performance is dependent on an accurate and comprehensive reference database because strains which lack representative spectra will not be identified.

The typing of *S. aureus* has revealed existing HA-MRSA and CA-MRSA clonal lineages which are endemic in certain geographic regions or have disseminated internationally. Currently molecular typing techniques are largely employed for the characterisation of isolates e.g. pulsed field gel electrophoresis (PFGE), multi locus sequence typing and *spa* typing. PFGE is the gold standard because of its excellent discriminatory power in short-term epidemiology but this technique is laborious and inter-laboratory comparison of data is problematic mainly due to methodological variations. Therefore the development of alternative discriminatory typing assays with an added advantage of speed is desirable. In this study the MALDI Biotyper 2.0 System demonstrated the potential to discriminate *S. aureus* strains. A visual comparison of the mass spectra profiles of the EMRSA-15 (n=3) and EMRSA-16 strains (n=3) revealed

mass-ion peaks which were specific to the respective strains. The application of cluster analysis with the MALDI Biotyper 2.0 software to a modest collection of strains (n=14) resulted in the clear demarcation of EMRSA-15 and EMRSA-16 strains into two separate clusters. This indicated that the two MRSA clones could be distinguished with this technique however, subsequent analysis of three or four clones was less precise.

When strains belonging to three clones: EMRSA-15 (n=6), EMRSA-16 (n=6) and USA300 (n=6) were analysed two clusters were apparent but one comprised a mixture of EMRSA-15 (n=2) and all of the EMRSA-16 strains, whereas the second contained two EMRSA-15 and all of the USA300 strains. This implied that increased strain diversity and a higher number of strains affected the discriminatory capacity of the method and it exhibited reduced correlation to PFGE typing. When the raw spectra of strains belonging to four clones were evaluated there was an encouraging indication of the discrimination of the four clones. The EMRSA-15, EMRSA-16 strains were demarcated into separate clusters however the USA300 and t0440-MRSA-IV strains were demarcated into a single cluster. Notably the strains of the latter clones were segregated within the single cluster indicating that to some extent the strains were distinguished by the software. Nevertheless their demarcation into a single cluster indicated that the software recognised a degree of relatedness and this implied that there were mass ion peaks which were common to both of the MRSA clones. Interestingly in contrast to the EMRSA-15 and EMRSA-16 strains, the USA300 and t0440-MRSA-IV strains harboured genes for Panton-Valentin Leukocidin and they are regarded as CA-MRSA clones whereas the former are HA-MRSA clones. It is possible that the mass ion peak for the PVL toxin may have been one of the distinguishing markers between the afore-mentioned HA-MRSA and CA-MRSA clones however, this was not verified in this study. In this study the cluster analysis was done on MSPs which had been compiled from replicate raw mass spectra profiles. The mass ion peaks were reproducible across the replicate spectra however, these were not normalised to minimise any spectra: spectra variations. The latter may have caused slight shifts in the sizes of the mass ion peaks and it is possible that these may have contributed to the ambiguity of the results.

Recently a more succinct discrimination of five major HA-MRSA genotypes was demonstrated with the MALDI-TOF assay (Wolters et al., 2010). The raw mass spectra profiles were normalised with the FlexAnalysis software and analysed with the MALDI BioTyper 2.0 Software. Twenty-five MRSA clinical isolates which were previously identified as belonging to the CC5, CC8, CC22, CC30 and CC45 genotypes were analysed and although there were many mass ion peaks which were commonly present in all of the genotypes, thirteen mass ion peaks between the 2500 to 7734 m/z range distinguished the five genotypes (Wolters et al., 2010). Based on the presence or absence of the thirteen mass peaks 60 additional MRSA isolates could be classified into fifteen MALDI-TOF types. Subsequent cluster analysis of the peak profiles of the fifteen MALDI-TOF types was largely concordant to the five HA-MRSA genotypes except for four isolates which belonged to ST80, ST15, ST88 and ST1 clonal lineages which were demarcated into a cluster affiliated to the CC5 HA-MRSA genotype (Wolters et al., 2010). This study successfully demonstrated a preliminary typing application of the MALDI-TOF technique which is robust and rapid for the discrimination of five MRSA genotypes.

The rapid detection of methicillin resistance in *S. aureus* isolates is essential in routine clinical diagnosis and several studies have identified mass ion peaks which could be used as potential methicillin resistance markers with the MALDI-TOF method. Edwards-Jones *et al.* (2000) applied the MALDI-TOF assay to MRSA (n=7) and MSSA (n=7) strains and were able to discriminate the MRSA from MSSA by visual comparison of the spectra. In a follow-up study they employed the SPSS for Windows version 8.0 statistics package to distinguish EMRSA-15 and EMRSA-16 strains based on the peak sizes of mass ions generated with a MALDI-TOF system (Walker et al., 2002, Edwards-Jones et al., 2000). Similar results were also attained by Du *et al.* (2002) and they demonstrated that the MALDI-TOF and the use of the MicrobeLynx software could distinguish MRSA and MSSA strains. They performed a cluster analysis of the mass spectra of 76 *S. aureus* clinical isolates and in the generated dendrogram three clusters were evident, two of which largely contained MRSA and one containing only MSSA strains. Nevertheless five false positives (MSSA demarcated in an MRSA cluster) were observed in their study. However in a recent study which also used the MALDI-TOF and the MicrobeLynx software, attempts to identify mass ion peaks which

could discriminate MRSA and MSSA strains in 134 *S. aureus* isolates was unsuccessful (Rajakaruna et al., 2009).

In another study involving 81 *S. aureus* isolates two peaks at 4448 *m/z* and 5302 *m/z* were recommended as potential markers for the presence of the PVL toxin (Bittar et al., 2009). However in a later study the two recommended peaks were detected in 104 MRSA isolates irrespective of the presence of PVL (Szabados et al., 2010a). Consequently it has been emphasized that prior to the recommendation of certain peaks as biomarkers the stability and reproducibility of the peaks needs to be verified because they may become less precise as more wild-type isolates are evaluated (Rajakaruna et al., 2009). In support of this assertion Rajakaruna *et al.* (2009) demonstrated that the mass spectra profiles of the same isolates grown on different media can be different. This was demonstrated in the case of an *S. aureus* isolate grown on Columbia blood agar (CBA) and Chocolate agar (CHOC). Although the composition of CBA and CHOC media is similar except for the presence of lysed blood in the latter, the mass spectra profiles of the *S. aureus* isolate exhibited striking differences (Rajakaruna et al., 2009). This served to show that different growth culture conditions can affect the expression of proteins and consequently the mass spectra profiles. The reproducibility of results with the MALDI-TOF method is important and efforts have been undertaken to standardise the different stages constituting the preparatory and analysis protocols, including bacterial growth conditions, extraction procedures, the concentration of the matrix solution and the MALDI settings (Jackson *et al.* 2005). This is essential as the performance of a standardised method across different studies could better facilitate the identification of stable peaks which can be applied in the typing of strains or used as the markers for the identification of diverse antibiotic or virulence factors in *S. aureus*.

The results of this study indicate that the MALDI Biotyper 2.0 system is a robust method for the identification of *S. aureus* and these results are consistent with the findings of previous studies (Szabados et al., 2010b, Dubois et al., 2009, Carbonnelle et al., 2007). There was an encouraging indication of the typing potential of this assay and a preliminary capacity to discriminate MRSA clones, however a more comprehensive study involving a larger collection of isolates and more scrupulous processing of the data is required for the verification of these results and possibly the development of a *S. aureus* typing method with excellent discriminatory power.

10.0 Conclusion

MRSA are a leading cause of hospital acquired infections (HAIs) in the UK and in many countries world-wide. In clinical diagnosis the accurate identification of MRSA is vital because the misidentification of MRSA as MSSA could have adverse clinical, therapeutic, and emotional effects on the patient and increased financial repercussions on the healthcare system. Therefore it is essential that implemented diagnostic tests have excellent diagnostic sensitivity, specificity and are rapid, as this could reduce the misidentification of MRSA, facilitate faster implementation of infection control procedures thereby possibly reducing MRSA transmission, and lead to earlier implementation of definite antimicrobial therapy with improved outcomes. Individuals who are colonised with MRSA are at greater risk of contracting infections and transmitting MRSA, so it is recommended that the screening of patients occurs before or during hospital admission. Screening is performed primarily on nasal swabs and also on swabs from different anatomical sites i.e. axilla, groin and skin breaks.

The Public Health Wales (PHW) Microbiology ABM Swansea laboratory is the diagnostic laboratory for Abertawe-Bro Morgannwg University Health Board with three major acute hospitals (approximately 1570 beds) serving a population of approximately 350000. ABM University Health Board has multiple secondary and tertiary services including haematology with regional bone-marrow transplant centre, neonatal ICU sub-regional centre, cardiac surgery, and the Welsh Centre for Burns and Plastic Surgery. In this high volume clinical laboratory, methicillin susceptibility testing is based on conventional phenotypic methods including the 1μg oxacillin disc diffusion assay and the BD Phoenix automated microbiology system which incorporates both oxacillin and cefoxitin for methicillin susceptibility testing. However these laboratory protocols are lengthy i.e. 24 to 72 hours, whereas PCR tests have shorter execution times (2 hours) and are generally more sensitive. Therefore the implementation of a PCR assay would facilitate the reduction of the turn-around times of results and provide higher accuracy in the detection of methicillin resistance in *S. aureus*. In light of the priority to reduce MRSA in hospital-acquired infections in the UK, the rationale of this research was to evaluate PCR assays which could be potentially implemented as MRSA screening tests in the PHW Microbiology ABM Laboratory in lieu of the currently used phenotypic assays in order to facilitate the reduction of turn-around times and increase the accuracy of MRSA identification from clinical specimens. However despite the accuracy and

speed of a candidate PCR assay, clinical laboratories are constrained within financial and technical limitations. Therefore in addition to an investigation of the diagnostic performance of a candidate assay, the financial and technical implications of implementing a recommended assay would also require meticulous review.

The term methicillin resistant specifically refers to strains that carry the *mecA* gene whereas methicillin susceptible refers to strains that do not carry this gene. Accordingly the detection of *mecA* by real-time PCR is the gold standard as this provides unequivocal identification of methicillin resistance, whereas the detection of resistance to oxacillin and/ or cefoxitin is a surrogate method for the detection of *mecA* which is based on the interpretation of zone diameters or MIC results which are within ranges established for methicillin resistance. These assays are based on the assumption that the transcription of *mecA* is strongly induced by these antibiotics and the expression of PBP2a directly results in high MICs or narrow (or absent) inhibition zones representing methicillin resistance. However there are multiple factors which are involved in the regulation of *mecA* expression and these may result in some MRSA strains exhibiting susceptible phenotypes in vitro. In addition non-*mecA* mediated oxacillin resistance i.e. mutations in the binding site of native PBPs or the hyperproduction of penicillinase can cause borderline oxacillin resistance phenotypes in *mecA*-negative *S. aureus* strains (BORSA). Therefore in contrast to the direct detection of the *mecA* gene by PCR, oxacillin or cefoxitin susceptibility tests are not unequivocal methods for the detection of *mecA*-mediated methicillin resistance, as they can result in the misidentification of *mecA*-positive strains as methicillin susceptible or *mecA*-negative strains as methicillin resistant. This hypothesis was examined in chapter 3, by investigating sixteen discrepancies between the *mecA* real-time PCR results and the reported results of the PHW Microbiology ABM Laboratory for 137 *mecA*-positive and 424 *mecA*-negative wound swab *S. aureus* isolates.

Out of the sixteen discrepancies, twelve of the 137 *mecA*-positive *S. aureus* isolates had been reported as MSSA and one of the 424 *mecA*-negative *S. aureus* had been reported as an MRSA. On repeating the 1µg oxacillin disc diffusion assay and antibiotic susceptibility testing with the BD Phoenix, eleven of the *mecA*-positive *S. aureus* were clearly resistant to oxacillin but interestingly three *mecA*-positive *S. aureus* isolates exhibited methicillin susceptible phenotypes and one isolate had a borderline methicillin

resistant phenotype. Evidently these cases illustrated the misidentification of MRSA as MSSA and ambiguous results with the antibiotic susceptibility assays. The *mecA*-negative *S. aureus* isolate displayed methicillin susceptibility with the oxacillin disc diffusion test and the BD Phoenix but exhibited borderline methicillin resistance with the cefoxitin disc diffusion test. This indicated that the *mecA*-negative isolate was a possible BORSA strain which was subsequently misidentified as a MRSA and this represented the misidentification of an MSSA as an MRSA with antibiotic susceptibility assays.

An assumption of this study was that the collection of 561 clinical isolates for real-time PCR testing had initially been confirmed as *S. aureus* with the identification tests used in the PHW Microbiology ABM Laboratory i.e. an agglutination slide test for clumping factor or protein A and the DNase test. Furthermore the subsequent detection of the $femA_{S.\ aureus}$ gene by real-time PCR confirmed that all of the 561 isolates were *S. aureus*. However the audit of the PHW Microbiology ABM Laboratory reported results showed that in three of the sixteen discrepant cases, *S. aureus* had not been reported but rather *S. epidermidis* were reported in two cases and in the third case a *Streptococcus* species, respectively. The reporting of *S. epidermidis* or *Streptococcus* species may have been due to errors made during the entering of the results into the computerised data system. Alternatively *S. aureus* isolates may have indeed been misidentified with the initial phenotypic assays as it is possible for the morphology of *S. aureus* colonies to be misidentified as *S. epidermidis* or *Streptococcus* species on certain selective media like Columbia Colistin Nalidixic Acid media. Furthermore there are rare *S. aureus* strains that do not express protein A and they would exhibit negative results with protein A agglutination slide tests resulting in them being misidentified as coagulase-negative staphylococci (CoNS). False-negative results are also possible due to the expression of capsular polysaccharides by certain MRSA strains which mask clumping factor and protein A. Even though the reason for the reporting of *S. epidermidis* or *Streptococcus* species was not conclusively determined, these cases provided further examples of the possible misidentification of *S. aureus* isolates with assays that are based on the detection of phenotypic markers rather than the direct detection of the *S. aureus* specific genetic determinants with PCR assays.

A limitation of this study was that the initial phenotypic tests conducted at the PHW Microbiology ABM Laboratory and the PCR assays were not done concurrently. Therefore the initial cultures on the selective media, the results of the identification tests and the oxacillin disc diffusion tests could not be immediately re-evaluated. As a result the analysis of the initial phenotypic results was based on an audit of the final reported results. Nevertheless, this study highlighted the limitations of exclusively using phenotypic susceptibility tests for the detection of *mecA*-mediated methicillin resistance in *S. aureus*. By repeating the oxacillin, cefoxitin and BD Phoenix susceptibility tests, it was evident that there were several MRSA strains and one MSSA isolate with borderline methicillin resistance. These represent atypical strains which could be misidentified with oxacillin and cefoxitin susceptibility tests. Therefore the molecular detection of the *mecA* gene by PCR is the optimal method for the conclusive detection of methicillin resistance in *S. aureus* and the implementation of this assay in the PHW Microbiology ABM Laboratory could facilitate a reduction in the misidentification of *mecA*-positive or *mecA*-negative *S. aureus* isolates.

Duplex PCR assays detecting *mecA* and a *S. aureus* specific gene e.g. *nuc, femA, femB* or *Sa442* have been described for the identification of MRSA from clinical isolates. However *mecA* is not exclusively carried by MRSA, it is also carried by methicillin resistant-CoNS (MR-CoNS) species. Since CoNS species such as *S. epidermidis, S. haemolyticus* are common contaminants of non-sterile clinical specimens (e.g. screening swabs and wound swabs) these could generate false positive results with the duplex PCR assays. Therefore these PCRs are limited to pure clinical isolates or specimens from normally sterile sites such as blood cultures. The PHW Microbiology ABM Laboratory receives a variety of clinical specimens from both sterile and non-sterile sites therefore the performance of a PCR assay that provides conclusive identification of MRSA from all specimen types is essential. The *mecA* gene is located on the SCC*mec* element which integrates at a *S. aureus* specific locus termed *orfX* within the chromosome. Theoretically the amplification of the junction of the SCC*mec* right extremity and *orfX* provides an ideal marker for MRSA in both clinical isolates and non-sterile clinical specimens. MRSA detection by the amplification of the SCC*mec-orfX* junction was a method initially published by Huletsky *et al.* (2004) and subsequently two additional methods were published by Hagen *et al.* (2005) and Cuny and Witte (2005). These three SCC*mec-orfX* PCR methods represented candidate tests

that could be implemented in the PHW Microbiology ABM Laboratory as in-house MRSA screening tests which could be performed on both clinical isolates and non-sterile clinical specimens. Subsequent to the initial publication of the three SCC*mec-orfX* PCR methods, a commercial kit termed the BD GeneOhm MRSA test based on the Huletsky *et al.* (2004) method was approved for MRSA diagnosis in nasal swabs. For the purposes of this study, a direct evaluation of the diagnostic performance of the BD GeneOhm MRSA test on a collection of nasal swabs was considered, however commercial tests are financially costly in a high volume laboratory, whereas an in-house MRSA screening test is more economically sustainable. Therefore the evaluation of the three original SCC*mec-orfX* methods as candidate in-house MRSA screening tests was pursued in this study.

The disadvantage of the SCC*mec-orfX* junction as a surrogate methicillin resistance marker is that unlike the *mecA* gene which is highly conserved in MRSA strains, there are diverse SCC*mec* types (I to VIII) and there is polymorphism at the SCC*mec* right extremity sequence which forms the junction with *orfX*. For instance the SCC*mec* types I, II, IV have similar right extremity sequences whereas SCC*mec* type III and V have different sequences. Even though this can be circumvented by the development of multiple forward primers which are specific to multiple SCC*mec* elements, the failure to detect novel SCC*mec* elements remains a possibility. For instance, when the three SCC*mec-orfX* assays were published in 2004 and 2005, the forward primers were specific for target sequences in the right extremity of published SCC*mec* types I to V sequences. However SCC*mec* types VI, VII and VIII were later described and the discovery of further novel SCC*mec* elements has not been exhausted, consequently former SCC*mec-orfX* assays would be obsolete. Therefore this presents the underlying problem of possible false negative results with some MRSA strains. In addition several SCC elements lacking *mecA* have been described in certain *S. aureus* strains e.g. SCC_{476}, SCC*cap1*, SCC*Hg*, SCC*fur*. These elements have characteristic features of SCC*mec* elements i.e. carriage of a *ccr* gene complex, integration at *orfX* and flanking direct repeats. Therefore the amplification of the junction of the right extremity of these elements and *orfX* could generate false positive results with SCC*mec-orfX* PCR assays. Furthermore the imprecise excision of a SCC*mec* element may result in the deletion of *mecA* and other structural components of the element but a remnant of the SCC*mec*-

orfX junction may remain. This may also generate false positive results with a *mecA*-negative *S. aureus* strains which harbour these remnants.

Therefore although SCC*mec-orfX* PCR assays are theoretically ideal assays for the identification of MRSA, they can generate false negative results with *mecA*-positive *S. aureus* strains harbouring novel SCC*mec* elements and false positive results with *mecA*-negative *S. aureus* strains harbouring remnant SCC*mec-orfX* sequences. This hypothesis was investigated in chapter five and six by performing three SCC*mec-orfX* assays re-termed the PCR-A, PCR-B and PCR-C methods on 118 (22.7%) MRSA and 401 (77.3%) MSSA clinical isolates. The *mecA* PCR was used as the reference method and the diagnostic performance of the PCR-A, PCR-B and PCR-C methods was evaluated by determining the sensitivity, specificity and predictive values. The MRSA isolates with false negative results were investigated for novel SCC*mec* elements and the MSSA isolates with false positive results were investigated for remnant SCC*mec-orfX* sequences, SCC*mec* associated DNA or possible SCC elements.

In comparison to the *mecA* PCR the sensitivity results of the PCR-A, PCR-B and PCR-C methods were: 96.6%, 96.6%, 95.8% and the specificity results were: 98.3%, 98.3% and 97.8% respectively. The positive predictive values (PPV) were 94.2%, 94.2% and 92.6% respectively and the negative predictive values (NPV) were: 99.0%, 99.0%, and 98.7% respectively. Out of the three SCC*mec-orfX* assays the PCR-A and PCR-B methods had the best diagnostic performance. Nevertheless, the high performance is not guaranteed in routine screening swabs due to the lower prevalence rate of MRSA in these clinical specimens. Studies by Maudsley *et al.* (2004), Grundmann *et al.* (2002) and Abadu *et al.* (2001) conducted in London, Nottingham and Birmingham respectively indicate that in the UK the prevalence of nasal carriage of MRSA in the community is very low i.e. 0.78%, 0.83% and 1.5%. Lower prevalence rates would affect the predictive values of the SCC*mec-orfX* assays because with decreasing prevalence rates even though the NPV increases, the PPV decreases, increasing the probability of false positive results. Furthermore in this study 2 to 3 colonies of the isolates were consistently used in preparation of the template DNA therefore the template DNA was maintained at a relatively standard concentration. However in clinical specimens the concentration of the target DNA is variable and lower concentrations at the detection limit could reduce the sensitivity of the assays when

performed on clinical specimens. Therefore despite the excellent diagnostic performance of the PCR-A and PCR-B methods on clinical isolates, further studies are necessary in order to conclusively advocate either of the methods as ideal MRSA screening tests for the PHW Microbiology ABM Laboratory. An important future study would be an evaluation of the diagnostic performance of these methods on a collection of nasal swabs. In addition a comparative analysis of the diagnostic performance and the financial costs (reagents, consumables and training of technical staff) of these methods versus the commercial BD GeneOhm MRSA test on the collection of nasal swabs would also be valuable. The BD GeneOhm MRSA test has only been approved for nasal swabs, however MRSA screening often includes swabs from other anatomical sites, therefore it would also be valuable to evaluate these methods versus the BD GeneOhm MRSA test on a collection of diverse screening swabs either tested separately or pooled.

Four (3.4%) MRSA isolates generated false negative results with the PCR-A, PCR-B assays and five (4.2%) were false negatives with PCR-C. Seven (1.7%) MSSA isolates generated false positive results with the PCR-A, PCR-B assays whereas nine (2.2%) were false positive with PCR-C. Molecular typing of the MRSA isolates with PFGE and *spa* typing demonstrated that the PCR-A and PCR-B false negative isolates were genetically related to the EMRSA-15 HA-MRSA clone. This indicated the false negative isolates were local endemic strains which were misidentified with the PCR-A and PCR-B SCC*mec-orfX* assays. Although the prevalence of the false negative strains was low, the misidentification of these EMRSA-15 subtypes could lead to selection of these clones and they would become prevalent through failure to implement infection control measures. This is a further factor to be considered prior to the possible implementation of either method as MRSA screening tests in lieu of currently performed phenotypic tests in the PHW Microbiology ABM Laboratory.

The reason for the lack of amplification of the false negative MRSA was proposed to be a frequent deletion of a sequence in the SCC*mec-orfX* junction which included the primer binding sites of the SCC*mec-orfX* assays. Alternatively a possible inversion of the right extremity sequence of SCC*mec* may have also caused the lack of amplification of the SCC*mec-orfX* sequence. However these proposals could not be verified within the duration of this study. In the future this hypothesis could be investigated by performing a Southern blot hybridisation assay following *Sma*I-PFGE, with a probe

spanning from *mecA* to *orfX* which could be designed from the SCC*mec* type IV sequence of the published EMRSA-15 genome. The false negative isolates, an EMRSA-15 reference strain and several previously successfully amplified clinical EMRSA-15 strains could be included to ascertain if the size of the *Sma*I fragment containing the SCC*mec* element varies between the false negative isolates. Alternatively, a long-range PCR for the amplification of a fragment spanning from *mecA* to *orfX* could be performed. Then the *mecA-orfX* PCR fragment could be sequenced and compared with the nucleotide sequences of published SCC*mec* sequences in order to locate the position of the proposed deletion or other mutations in the sequence of the false negative isolates.

The typing of *S. aureus* strains facilitates the determination of the local epidemiology of strains, investigations of outbreaks, the transmission of clones and the evolutionary history of this bacterial species. Multi Locus Sequence Typing (MLST) has been fundamental to the current understanding of the clonal structure of *S. aureus*, revealing that it comprises ten dominant human lineages (CC1, CC5, CC8, CC12, CC15, CC22, CC25, CC30, CC45 and CC51) and additional minor lineages (Lindsay, 2010, Feil et al., 2003, Enright et al., 2002, Enright et al., 2000). These are referred to by their clonal complex (CC) numbers which are defined by the clustering of the sequence types (ST) with an electronic algorithm which is Based Upon Related Sequence Types (e-BURST). MRSA clones are described by their STs and the SCC*mec* elements i.e. ST22-MRSA-IV (EMRSA-15), ST8-MRSA-IV (USA300 clone). Furthermore their names may also give reference to the geographical regions where they are or were highly prevalent, their pulsed field gel electrophoresis (PFGE) pattern or where they have caused the most severe infections e.g. Midwest clone/ ST1-MRSA-IV, European clone/ST80-MRSA-IV. Interestingly even though there are ten dominant human *S. aureus* clonal lineages, a select number of lineages harbour established HA-MRSA clones (CC5, CC8, CC22, CC36 and CC45) which have become the leading cause of MRSA infections worldwide. The major HA-MRSA clones which belong to these lineages include the: Archaic (ST250-MRSA-I/ CC8); Brazilian (ST239-MRSA-III/ CC8); Hungarian (ST239-MRSA-III/ CC8); Iberian/ EMRSA-5 (ST247-MRSA-I/ CC8); New York/ Japan (ST5-MRSA-II/ CC5); Paediatric/ USA800 (ST5-MRSA-IV/ CC5); Southern Germany (ST228-MRSA-I/ CC5); EMRSA-2/ USA500 (ST8-MRSA-IV/ CC8); EMRSA-3 (ST5-MRSA-I/ CC5); EMRSA-15 (ST22-MRSA-IV/ CC22) and EMRSA-16/ USA200

(ST36-MRSA-II/ CC36). Apart from MLST, the clonal lineages can be determined with alternative techniques such as DNA microarray analysis and *spa* typing. DNA microarray analysis is a hybridisation method which enables the investigation of the expression of diverse genes and the genome structure of tested isolates. Whole genome DNA microarrays have been shown to be comparable with MLST for the determination of clonal lineages (Lindsay et al., 2006). Similarly with *spa* typing, the clustering of *spa* types as determined by the BURP algorithm has also been shown to be comparable to MLST clonal lineages. The PFGE technique is highly discriminatory and has been traditionally used for the typing of *S. aureus* strains. However it rapidly indexes genetic variation so even though it is excellent for outbreak investigations and local epidemiological studies it is not suitable for long-term epidemiology investigations. Even though MLST is the ideal technique for the characterisation of the respective *S. aureus* clonal lineages it involves multi-locus PCRs and DNA sequencing which is financially costly to conduct on a large collection of strains. Similarly DNA microarray analysis is a robust technique for the determination of clonal lineages and it generates a wealth of data of gene prevalences, however in this study the equipment and expertise for PFGE typing, *spa* typing and SCC*mec* typing were available in-house and so these techniques were performed for the typing of the MRSA isolates.

Interestingly amongst the diversity of HA-MRSA clones world-wide, in the UK HA-MRSA infections are extensively caused by the EMRSA-15 and EMRSA-16 clones (Boakes et al., 2010, Ellington et al., 2010c, Khandavilli et al., 2009, Johnson et al., 2005). These clones have consistently accounted for approximately 95% of consecutive nosocomial bacteraemia MRSA isolates collected from 2001 to 2007 for a BSAC Bacteraemia Surveillance programme (Ellington et al., 2010c, Johnson et al., 2005, Moore and Lindsay, 2002). In addition studies by Shore *et al.* (2010) and Goering *et al.* (2008) in Ireland and Scotland have demonstrated that these clones have outcompeted former dominant strains and are currently the predominant HA-MRSA strains in these regions. In this study the 561 *S. aureus* wound isolates (MRSA, n=137; MSSA, n=424) were collected from April 2007 to September 2007 from the PHW Microbiology ABM Laboratory. Since the EMRSA-15 and EMRSA-16 clones have consistently constituted 95% of bacteraemia MRSA isolates in the UK from 2001 to 2007, accordingly these clones would similarly constitute a significant proportion of MRSA wound isolates from the Southwest Wales region within the same period. This hypothesis was

investigated in chapter 4, by typing of the 137 MRSA wound swab isolates which had been collected from April 2007 to September 2007 with the PFGE, *spa* typing and SCC*mec* typing methods.

Out of the 137 MRSA isolates, 122 (89.1%) MRSA isolates were determined to be EMRSA-15 strains, eight (5.8%) were determined to be EMRSA-16 strains and seven (5.1%) were diverse MRSA strains. Therefore the EMRSA-15 and EMRSA-16 strains collectively accounted for 94.9% (130/ 137) of the wound swab isolates, illustrating that the molecular epidemiology of MRSA wound isolates is indeed dominated by these two clones. The majority (89.1%) of the MRSA isolates belonged to the EMRSA-15 clone and less to the EMRSA-16 clone (5.8%). These proportions were comparable to the results by Rollason *et al.* (2008) for MRSA isolates from skin and soft tissue infections of non-hospitalised patients in the West Midlands region. They found that EMRSA-15 accounted for 87% of the isolates whereas EMRSA-16 accounted for 8% (Rollason et al., 2008). The results of this study concur with previous studies which indicate that the EMRSA-15 and EMRSA-16 clones have some selective advantage over other HA-MRSA clones, which enables them to be more successful at surviving, colonising and spreading in the hospitals within the UK (Moore and Lindsay, 2002). This is thought to be due to a combination of the virulence factors expressed by the EMRSA-15 and EMRSA-16 clones and host factors (Moore and Lindsay, 2002). An investigation of the factors(s) facilitating the dominance of the EMRSA-15 and EMRSA-16 in the UK would require a comprehensive study investigating a wide range of diverse virulence factors expressed by these clones in comparison with other HA-MRSA clones and an evaluation of host factors which may also have a role in the dominance of these clones. The genome of the MRSA252/ EMRSA-16 strain (accession number BX571856) has been published and comparative genomics of EMRSA-15 is currently underway (http://www.sanger.ac.uk/resources/downloads/bacteria/staphylococcus-aureus.html) (Holden et al., 2004). Therefore a comparative analysis of their genomes with other published HA-MRSA genomes would also grant insight into the success of these clones in the UK.

In the 1990s, community associated-MRSA (CA-MRSA) emerged in patients without links to health-care units or hospitals; these have increased in prevalence worldwide especially in the USA. However a select number of CA-MRSA clones belonging to

only five major clonal lineages (CC1, CC8, CC30, CC59 and CC80) are the predominant causes of community-associated MRSA infections world-wide (DeLeo et al., 2010, Lindsay, 2010). Generally CA-MRSA are associated with skin and soft tissue infections (SSTIs) especially abscesses. However in 1997 to 1999 a Midwestern strain /MW2 with a pulsed field type termed USA400 (ST1-MRSA-IV/ CC1) was implicated in four paediatric deaths in Minnesota and North Dakota. In addition the USA300 clone (ST8-MRSA-IV/ CC8) has been increasingly implicated in community-acquired necrotising pneumonia, indicating that CA-MRSA strains can cause severe invasive infections (Hidron et al., 2009, Francis et al., 2005).

The prevalence of CA-MRSA clones varies world-wide, for instance in the USA initially both USA400 and USA300 were the dominant CA-MRSA clones. However the USA300 clone has become the predominant cause of SSTIs in the community and is increasingly a cause of hospital-associated infections (DeLeo et al., 2010). In contrast to the USA, in Europe CA-MRSA infections are less prevalent and are characterised by clonal heterogeneity (Otter and French, 2010). They have been reported in most European countries, including the Netherlands and Nordic countries, which have low rates of HA-MRSA (Otter and French, 2010). The most common CA-MRSA strain is the European clone (ST80-MRSA-IV/ CC80) but reports of USA300 are increasing. In isolates from Norway, collected from 1995 to 2003 from outpatients without any links to healthcare facilities, both the USA300 and the European clone were found to be prevalent (Hanssen et al., 2005). Similarly in Denmark, the epidemiology of CA-MRSA was investigated on a nationwide scale from 1993 to 2004. The isolates were from SSTIs in patients outside of hospitals and they extensively belonged to the European clone (Larsen et al., 2008). Other established CA-MRSA include the Southwest Pacific/ Oceania clone (ST30-MRSA-IV/ CC30) which is postulated to be a descendant of the penicillin-resistant 80/81 MSSA clone which was highly prevalent in the 1950's and 1960's. It is prevalent in Australia and has also been reported in Europe and South America (DeLeo et al., 2010). The Taiwan clone (ST59-MRSA-V_T/ CC59) is the predominant CA-MRSA clone in Taiwan and has also been reported in Singapore and Malaysia (DeLeo et al., 2010). Other CA-MRSA clones have arisen in Europe, notably the ST398-MRSA-V pig-associated clone in the Netherlands and Denmark (Otter and French, 2010). The detection of Panton-Valentine Leukocidin (PVL) in USA300 and an overwhelming majority of the established CA-MRSA clones led to the speculation that

PVL has a significant role in CA-MRSA virulence. Even though the direct role of PVL is debatable, it is frequently used as a provisional epidemiological marker for CA-MRSA (Voyich et al., 2005, Vandenesch et al., 2003). In addition CA-MRSA are also characterised by the carriage of the SCC*mec* type IV or V elements.

Investigations of the molecular epidemiology of CA-MRSA in the UK, specifically within the epidemiological setting of this study, Southwest Wales, is essential to monitor the transmission of clones and to guide new control initiatives to prevent these strains from becoming endemic like the HA-MRSA in the UK. The implications of published epidemiological studies seem to suggest that community-associated infections are primarily caused by PVL-positive CA-MRSA but this is not a universal trend. In the UK, even though the PVL-negative EMRSA-15 and EMRSA-16 clones are a leading cause of hospital-acquired infections, MRSA are not a prominent cause of community-acquired infections. The prevalence of PVL-positive *S. aureus* strains is relatively low, 1.6% - 4.9%, these strains are mainly MSSA and they are polyclonal (Ellington et al., 2010b, Ellington et al., 2009, Otter et al., 2009, Holmes et al., 2005). In addition PVL-positive *S. aureus* strains are not specifically restricted to community-associated infections, they have also been implicated in sporadic hospital-associated outbreaks. For instance a PVL-positive MSSA strain was implicated in an outbreak in the maternity unit of Derriford hospital, Plymouth, in 2003 (Dyer, 2007). Ten mothers and babies were infected and four developed extensive abscesses which needed surgery. In September 2006, the PVL-positive ST30-MRSA-IV clone was implicated in an outbreak which affected eleven healthcare workers and patients and resulted in the death of a nurse and a patient at the University Hospital of North Staffordshire, Stoke-on-Trent (Orendi et al., 2010). In December 2006 at the Norfolk and Norwich University NHS Trust in East Anglia, a PVL-positive MSSA strain was isolated from six babies in a neonatal unit and caused the death of one premature baby (Dyer, 2007).

However published prevalence data and the genetic characterisation of PVL-positive *S. aureus* isolates is primarily from investigations carried out on isolates submitted to the UK Staphylococcus Reference Unit. Since reference laboratories primarily receive selected isolates this may create a bias for certain clones over others and this may not be a true representation of the prevalence and epidemiology of PVL-positive *S. aureus* strains in the UK. In order to attain a comprehensive insight it would be informative to

investigate the prevalence of PVL genes in unselected consecutive clinical *S. aureus* isolates which are submitted to diagnostic laboratories. This hypothesis was investigated in chapter 8, by determining the prevalence of PVL genes by real-time PCR in unselected consecutive wound swab *S. aureus* isolates (n=519) from the PHW Microbiology ABM Laboratory (PHW-ABM). Furthermore the molecular epidemiology of PVL-positive isolates which have been submitted to a reference laboratory could be different to unselected PVL-positive isolates from a diagnostic laboratory. This was investigated in chapter 8, by comparing the molecular epidemiology of a collection of PVL-positive *S. aureus* isolates (n=61) from the Specialist Antimicrobial Chemotherapy Unit (SACU) in Cardiff which receives isolates from microbiology laboratories across Wales and the molecular epidemiology of the collection of PVL-positive *S. aureus* isolates (n=19) from the PHW-ABM laboratory. Genotyping of the isolates was conducted by PFGE, *spa* typing, SCC*mec* typing and detection of the ACME element by PCR. The determination of *S. aureus* clonal lineages was determined by clustering *spa* types with the BURP algorithm.

By real-time PCR, PVL genes were detected in 3.7% (n=19) of the 519 consecutive unselected wound swab *S. aureus* isolates, only two of the nineteen PVL-positive *S. aureus* were MRSA. This was comparable to the prevalence rate (1.8% to 4.9%) of PVL genes in *S. aureus* isolates (n=515) which had been submitted to the UK Staphylococcus Reference Unit (Holmes et al., 2005). The PVL-positive *S. aureus* (n=19) from the PHW-ABM laboratory were distributed into five *spa*-BURP clusters CC002 (n=1), CC005 (n=2), CC159 (n=5), CC275 (n=4) and cluster 7 (n=1), associated with the ST5, ST22, ST121 and ST30 lineages and six isolates had diverse genotypes. This demonstrated that in the region of Southwest Wales, PVL-positive *S. aureus* are not highly prevalent and they are constituted extensively by polyclonal MSSA and not MRSA. The polyclonal nature of the PVL-positive *S. aureus* was similar to the heterogeneity of CA-MRSA strains in mainland Europe but markedly different to the situation in the USA where it has been reported that the USA300 clone, accounts for 59% (range 15% to 74%) of SSTIs among patients seeking treatment at accident and emergency departments across USA cities (Edelsberg et al., 2009).

The molecular epidemiology of the unselected consecutive isolates (n=19) from the PHW-ABM laboratory and SACU isolates (n=61) showed interesting differences. Even

though both collections of isolates were from Wales, they were markedly different and this was initially indicated by the high prevalence rate of methicillin resistance (57.4%; 35/61) in the SACU isolates in contrast to the PHW-ABM isolates which were essentially methicillin susceptible (89.5%; 17/19). These results are interesting because since the leading CA-MRSA clones are extensively PVL-positive, it may be presumed that PVL-positive strains are predominantly methicillin resistant. Certainly solely based on the results of the SACU isolates this appeared to be the case, however the results of the unselected isolates contradict this and imply that PVL-positive isolates in Southwest Wales are predominantly methicillin susceptible. Both collections of PHW-ABM and SACU PVL-positive *S. aureus* isolates were polyclonal; however the proportions of isolates which belonged to the various *S. aureus* clonal lineages in both collections were variable. A distinct difference was the predominance of USA300 strains (26.2%) in the SACU isolates (n=61) and yet these strains were completely absent in the unselected PHW-ABM isolates. Similarly cluster 8 (8%) and cluster 6 (5%) which were associated with the European and USA400 clones were present in the SACU isolates but absent from the unselected PHW-ABM isolates. Actually 32% (6/19) of PHW-ABM isolates had *spa* types which were not associated with any of the *spa*-BURP clusters indicating that these were from unrelated genetic backgrounds. These results indicate that despite the presence of established CA-MRSA strains, particularly the USA300 clone in the SACU isolates, these were absent in unselected isolates from PHW-ABMU. The European clone which is regarded as the prevalent CA-MRSA clone in certain countries in mainland Europe, was also notably absent from the PHW-ABM isolates. Accordingly this may imply that these clones are infrequent in the PVL-positive *S. aureus* strains of the Southwest Wales region. This suggests that the molecular epidemiology of PVL-positive *S. aureus* in the Southwest Wales region is somewhat unique in comparison to mainland Europe and the USA.

This study contributed insights into the molecular epidemiology and prevalence of PVL-positive strains in Southwest Wales. The results were comparable with previous findings in that they indicated that the prevalence rate of PVL-positive *S. aureus* remains low (3.9%). However contrary to an observed high prevalence rate of methicillin resistance in isolates that were submitted to the SACU reference laboratory, the results of the unselected isolates from PHW Microbiology ABM Laboratory suggest that PVL-positive *S. aureus* strains in Southwest Wales are largely represented by

genetically diverse MSSA strains. Differences in the molecular epidemiology and prevalence rates of HA-MRSA clones and PVL-positive *S. aureus* across different regions world-wide may be due to a variety of factors which affect the endemicity, transmissibility and dissemination of strains. These may include the genetic background of the strains, the virulence genes and accessory genes harboured by strains, patient demographics (including age, gender, ethnicity, socio-economic factors), host factors, travel history and environmental factors (Otter et al., 2009). For instance it is possible that the unusual higher rate of USA300 strains in the present study in the SACU isolates was associated with travel to the USA where these strains are highly prevalent. However, this could not be verified due to limited patient history information. Nevertheless continual investigations of the prevalence rates of HA-MRSA and CA-MRSA strains are important for the strategic planning of infection control measures, in order to reduce the incidence and transmission of these strains. Furthermore comprehensive genetic characterisation of these strains is valuable for the further understanding of the epidemiology of these strains. This will facilitate the identification of virulence factors which may be targeted for effective therapeutic strategies.

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