A novel multiplex PCR-based tool of typing strains of

Staphylococcus aureus

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Abstract

Methicillin resistant *Staphylococcus aureus* (MRSA) is an important nosocomial pathogen and morbidity and mortality rates associated with this pathogen have increased markedly in recent years. The prevalence of MRSA is no longer confined to hospital patients since MRSA infections have been increasingly reported in the community. More recently, community-acquired MRSA strains have become more prevalent and their infections are no longer confined to the community but have started to replace hospital-acquired MRSA in some health care settings. MRSA strains are generally resistant to several classes of antibiotics and are therefore difficult and costly to treat. Consequently, an understanding of the epidemiological characteristics of *S. aureus* is an essential tool for the management of its infections in both the hospital and community setting. The purpose of any epidemiology study, such as the investigation of an outbreak, is to identify the potential source(s) of an infection and to monitor their dissemination. The early identification of an outbreak, making use of a rapid, precise and simple MRSA typing technique, can lead to prompt and effective precautions that avoid further spread of the infection. Pulsed-field gel electrophoresis (PFGE) is considered the gold standard for MRSA typing and has been recently supported by multi-locus sequence typing (MLST). However, technical limitations restrict the use of PFGE and MLST in the majority of routine hospital laboratories: they are time-consuming, expensive, require specific expertise and specialist equipment.

In this study a novel typing technique was developed for *S. aureus,* based on single nucleotide polymorphism (SNP) variations in and around *Sma*I-restriction sites (CCCGGG), following the analysis of eighteen *S. aureus* strains that have had their genomes sequenced. The developed *Sma*I-multiplex PCR genotyping method combines the high discriminatory power and reproducibility of PFGE, with the simplicity of a multiplex PCR-based technique that can be performed in a routine clinical laboratory. The validity of *Sma*I-multiplex PCR was carefully assessed in comparison with PFGE and MLST against many sequenced *S. aureus* strains and showed high discriminatory power and reproducibility. There was also high level of concordance in the clustering of strains analyzed by each of the techniques. The *Sma*I-multiplex PCR was ultimately evaluated against a large number of clinical *S. aureus* outbreak strains and was shown to be a useful tool for providing reliable epidemiological information for the investigation of clinical staphylococcal outbreaks. The newly developed technology is suitable for high throughput sample analysis, is relatively cheap and provides reliable and comparable genotyping data. At the same time, the *Sma*I-multiplex PCR meets most of the criteria of practical typing method: it is simple, inexpensive, highly discriminatory and does not require sophisticated equipment or expertise. Consequently, *Sma*I-multiplex PCR could be used routinely in any clinical microbiology laboratory since it relies on standard clinical laboratory apparatus (*i.e.* PCR machine and agarose gel electrophoresis). *Sma*I-multiplex PCR proved to be more discriminatory than MLST/SCC*mec* typing, but less discriminatory than PFGE. Currently the *Sma*I-multiplex PCR protocol takes between 4 to 6 hours; however, it would be possible to adapt this technology towards an automated genotyping assay using RT-multiplex PCR. This would reduce the processing time to less than 60 minutes. Since individual targets are identifiable on the basis of the size of their amplicons, the RT-PCR output could be processed directly via dedicated analytical software.

Acknowledgements

Table of contents

Table of Figures

Table of Tables

List of abbreviations

agr	Accessory gene regulator
ATM blood agar	Aztreonam blood agar
AP-PCR	arbitrarily primed polymerase chain reaction
arcC	Carbamate kinase
aroE	Shikimate dehydrogenase
bp	base pair
BHI	Brain-heart infusion
BSI	Bovine serum albumin
BSI	Blood stream infection
BT	Binary typing
CA-MRSA	Community acquired infection MRSA
CC	Critical care
CC	Clonal complex
CDC	Centers for Disease Control and Prevention
CNS	Coagulase negative staphylococci
CHEF	Contour-Clamped homogeneous electric field
ºC	Temperature
(ds)DNA	Double-stranded DNA
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
ddH2O	Double deionised water
DMSO	Dimethyl sulfoxide
EARSS	European antimicrobial surveillance system
eBURST	Electronic based upon related sequence types
EDTA	Ethylenediaminotetracetic acid
EMRSA	Epidemic Methicillin-Resistant *Staphylococcus aureus*
ENT	Ear, nose and throat
FH	Freeman Hospital
FMRSA	Freeman MRSA
FOUTB	Freeman outbreak
GPs	General practitioners

g	Gram
g	Gravitational force
GISA	Glycopeptide-intermediate resistant *S. aureus*
glpF	Glycerol kinase
gmk	Gulanylate kinase
HAI	Hospital acquired infection
HA-MRSA	Hospital acquired infection MRSA
HCWs	Health care workers
HPA	Health Protection Agency
LB	Luria Bertani medium
LMP	Low melting point agarose
LTA	Lipoteichoic acid
ICU	Intensive care unit
ITU	Intensive treatment unit
IV	Intravascular
IWG-SCC	International Working group on the Classification of Staphylococcal Cassette Chromosome Element
kb	Kilo base(1,000 bp)
M	Moles
Mb	Megabase pair
MDR	Multi-drug resistant
MEGA	Molecular evolutionary genetics analysis
MGEs	Mobile genetics elements
MLEE	Multilocus enzyme electrophoresis
MLST	Multilocus sequence typing
MLVA	Multiple locus variable number tandem repeat analysis
ml	Millilitre
MIC	Minimum inhibitory concentration
min	Minute (time)
MRCNS	Methicillin-resistant coagulase-negative staphylococci
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSCRAMM	Microbial surface components recognizing adhesive matrix Molecules
MSSA	Methicillin-Sensitive *Staphylococcus aureus*

mM	Millimole
MW	Molecular size markers
NARSA	Network on antimicrobial resistance in *Staphylococcus aureus*
NCBI	National Centre for Biotechnology Information
NCCLS	National Committee for Clinical laboratory standard
NCTC	National Collection of Type Cultures
ND	Not determined
ng	Nano grams
NGH	Newcastle General Hospital
NNISS	National Nosocomial Infection Surveillance System
NHS	National Health Service in the UK
NPT	Nose, Perineum and Throat
Nuc	Nuclease
OD_{600}	Optical density at 600 nm
OMP^{TM}	Oligonucleotide Modelling Platform software
ORFs	Open reading frames
PCR	Polymerase chain reaction
PBPs	Penicillin-binding proteins
PFGE	Pulsed-field gel electrophoresis
PF	Plasmid fingerprinting
PMSF	Phenylmethanesulfonyl fluoride
pta	Phosphate actyltransferase
PTSgs	Pyrogenic toxin superantigens
PVL	Panton-Valentin leukocidin
RAPD	Random amplified polymorphic DNA
REA	Restriction enzyme analysis
RFLP	Restriction fragment length polymorphism
REAP	Restriction endonuclease analysis of plasmid DNA
rep-PCR	Repetitive element sequence-based PCR
RLU	Regional liver unit
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
rpm	Revolutions per minute

RTI	Respiratory tract infection
RVI	Royal Victoria Infirmary
SaPI	*Staphylococcus aureus* pathogenicity island
sec	Second
SCC*mec*	Staphylococcal cassette chromosome *mec*
SCV	Small colony variants
SLV	Single locus variant
SNPs	Single nucleotide polymorphisms
ssDNA	Single-stranded DNA
SSR	Short sequences repeats
ST	Sequence type
STAR elements	*Staphylococcus aureus* repeat elements
SWI	Surgical wound infection
TBE	Tris, Boric acid, EDTA solution
TE	Tris, EDTA solution
TIFF	Tagged Image File Format
T_m	Melting temperature
tpi	Triosephoshate isomerise
TSS	Toxic shock syndrome
UPGMA	Unweighted-pair group method with arithmetic mean
UTI	Urinary tract infection
UV	Ultraviolet
U	Unit
VISA	Vancomycin-intermediate resistant *S. aureus*
VRE	Vancomycin-resistant enterococci
VRSA	Vancomycin-resistant *S. aureus*
VNTR	Variable number tandem repeat
VSSA	Vancomycin-sensitive *S. aureus*
µl	Microlitre
WGH	Walkergate Hospital
yqiL	Acetyl coenzyme A acetyltransferase

Glossary of Terms

Bacterial epidemiology   The study of the spread of a bacterial pathogen including their distribution pattern and risk factors for control of contagious diseases in populations.

Bacterial Population   A group of bacteria that inhabit a given environment.

Clonal complex   A group of bacterial isolates that show a high degree of similarity based on a defined typing or identification scheme.

Clone   Bacterial isolates that are genetically identical or very similar that are derived from a common ancestor.

Cluster analysis   Comparative analysis of data that can group a data of according to similarity.

Dendrogram   Tree diagram illustrating a cluster analysis using any chosen data sets.

Discriminatory power   The ability of any technique to differentiate effectively and operationally between individuals. Ideally, each unrelated individual is assigned as unique.

Endemic   Strain that is continuously present in giving place over a long period of time.

Epidemic   The occurrence of an infection/organism more than usual /endemic level or exceed what is expected number of an infection/organism in given human population.

Evolutionary or phylogenetic tree   A diagram showing evolutionary relationships among bacterial species based on their genetic diversity and similarity. The taxa grouped together in the tree represent taxa descended from a common ancestor taxa.

Genotype   Genetic constitution of an organism that is determined by molecular or genetic techniques

Incidence   The proportion of a defined infection that develops within a stated period. It is lower than prevalence rate

Isolate	A population of bacterial cells derived from single colony. In clinical microbiology, an isolate is defined as a population that derived from clinical specimen obtained from an individual.
Lineage	A group of isolates derived from a common ancestor that share similar characteristics.
Mutation	A change in the nucleotide sequence or arrangement of DNA. Mutation can be point mutations (one nucleotide change) or macro-mutations that include inversion, deletion and duplication.
Pathogenicity	Ability of a pathogen to cause disease.
Phenotype	Any observable characteristic or treat of a bacterial strain including its morphology, biochemical properties and behavior.
Prevalence	The total number of a defined infection in a given population at a particular period.
Sporadic	Rare or occurring at irregular intervals in time and locations, disconnected in space and time. It is the opposite of endemic and epidemic.
Technical reproducibility	The ability of a typing technique to assign the same type to an isolate tested on independent occasions.
Typing	Phenotypic and genetic analysis of bacterial isolates, that can be carried out to generate fingerprints (profiles) or database that can be used to assign an isolate to a particular group or class.
Typeability	The ability of a typing method to assign an analysed isolate to a group of class.
Virulence	The degree and properties of pathogenicity of pathogens. The pathogenic capacity of a microorganism can be determined by defining it so called virulence factors, such as toxins and enzymes.

Chapter 1

Literature Review

1.1. Introduction

Nosocomial or hospital-acquired infections (HAI) are one of the most common problems in hospitals throughout the world. It is considered one of the main reasons of morbidity and mortality amongst hospital patients, as well as the increasing costs of infection control. It is noteworthy that hospital-acquired infections are far from being a recent phenomenon, since they not only preceded the antibiotic era (1940s onwards), but have also been a recognised problem since 1847 and the pioneering work of Ignaz Semmelweis on puerperal fever (Wyklicky and Skopec, 1983). During most of the 20th century *Staphylococcus aureus* has been recognised as a major life-threatening pathogen. Although the clinical use of penicillin (1940s) initially led to a dramatic reduction in mortality from *S. aureus* infections, penicillin resistant strains soon emerged (Cookson *et al.*, 2003). Concern was not only confined to the increase mortality rate among patients, but also to the spread of skin infections amongst health-care workers. Furthermore, the ability of *S. aureus* to acquire multiple antibiotic resistances further reduced the effectiveness of chemotherapy for the treatment of staphylococcal infections. Subsequently, the development of new antibiotics, such as methicillin in 1960, led to the expectation that infections caused by this bacterium would be treatable. However, *S. aureus* rapidly developed resistance to methicillin and the first methicillin-resistant strains of *S. aureus* (MRSA) were reported in the UK in 1961. Since then, the frequency of isolation of MRSA strains has increased significantly every year worldwide (Jevons, 1961; Grundmann *et al.*, 2006).

Nowadays, MRSA is considered to be one of the most important nosocomial pathogens and the infection frequency has grown progressively both in hospitals and, more recently, in the community. It is worth noting that MRSA is no more virulent than other *S. aureus* strains, but its infections are significantly more difficult to treat due to their resistance to front-line antibiotics (Rozgonyi *et al.*, 2007). The glycopeptides have emerged as the most effective anti-MRSA agents, although the emergence of MRSA strains with reduced sensitivity to vancomycin has led to increasing concerns about the use of this antibiotic as the last resort for the treatment of MRSA infections (Hiramatsu *et al.*, 1997). Therefore, the infection control is given priority by infection control teams in hospitals as a means of limiting MRSA infections.

Early detection is crucial for the effective treatment and control of MRSA infections. There are various approaches for diagnosing MRSA, ranging from conventional methods, such as cultural characteristics and biochemical analysis, to more

advanced molecular diagnostic methods. In recent years a greater emphasis has been place on determining the source and monitoring the spread of infections. Initially this was done with phage-typing, however, in the last ten years or so, molecular epidemiology techniques have become the methods of choice because they can be performed rapidly with an extremely high level of accuracy. Pulsed-field gel electrophoresis (PFGE) is currently considered to be the gold standard for MRSA epidemiological typing (Tenover *et al.*, 1997; Weller, 2000).

1.2 *Staphylococcus aureus*:

1.2.1 History of *S. aureus*

Pasteur and Koch were the first to discover and culture *Staphylococcus* but without carrying out any detailed studies. Ogston in 1881 and Rosenbach in 1884 performed the first detailed studies on *Staphylococcus*. Ogston gave *Staphylococcus* its genus name when he saw these bacteria formed grape-like clusters in human pus. Thereafter, Rosenbach isolated *Staphylococcus aureus* in pure culture, adding its species name *aureus* (=gold). Rosenbach also reported that *S. aureus* caused some wound infections whereas *S. epidermidis* lived on the skin as a colonizer (Cookson *et al.*, 2003).

1.2.2 General description

S. aureus is a member of the *Staphylococcaceae* family (Firmicutes), Gram-positive facultative anaerobes that exhibit a coccal morphology, and are non-motile and non-sporulating. Its cells are 0.5-1.5µm in diameter and forms grape-like clusters. It is catalase and coagulase positive and some strains produce capsules. On blood agar, colonies of *S. aureus* appear golden (caused by staphyloxanthin, a membrane-bound carotenoid) surrounded by haemolytic zones (Wieland *et al.*, 1994). In addition, *S. aureus* strains secrete various extracellular virulence factors such as coagulase and enterotoxins. Despite the fact that *S. aureus* is a normal component of the microbiota of the nasal passages, skin and mucous membranes of humans and animals, it is the cause of several important diseases (Brown *et al.*, 2005; Humphreys, 2002; Bergey *et al.*, 1994; Marshall and Wilmoth, 1981).

1.2.3 Cell envelope

The cell walls of Gram-positive bacteria differ from those of Gram-negative bacteria. The *S. aureus* cell wall is a multilayered structure (20-40 nm), comprising a copolymer of peptidoglycan and teichoic acid. Peptidoglycan represents ~50% of cell wall by weight and is a polymer consisting of repeating units of sugars of 1, 4 β-linked N-acetylglucosamine and N-acetylmuramic acid. The glycan chains are cross-linked by tetrapeptide chains (L-alanine, D-glutamine, L-lysine, and D-alanine) bound to N-acetylmuramic acid and a pentaglycine bridge that links tetrapeptide chains on adjacent glycan strands. This cross-linking is catalysed by the transpeptidase (TPases) activities of penicillin banding proteins (PBPs) (Fig. 1.1) (Stapleton and Taylor, 2002; Lowy, 1998). Another major cell wall component is teichoic acid, an anionic polymer that consists of repeating alditol phosphate groups covalently linked to the muramic acid residue of peptidoglycan.

In some *S. aureus* strains the cell wall is coated with an extracellular polysaccharide capsule. More than 90% of clinical strains of *S. aureus* can produce polysaccharide capsules that are usually thin (<0.05µm) and consist of aminouronic acid sugars and fucosamine (Wright and Novick, 2003; Seaman *et al.*, 2004). Serotyping has identified 11 serotypes, with serotypes 5 and 8 being responsible for about 75% of human infections. Most MRSA strains are serotype 5 (Lowy, 1998).
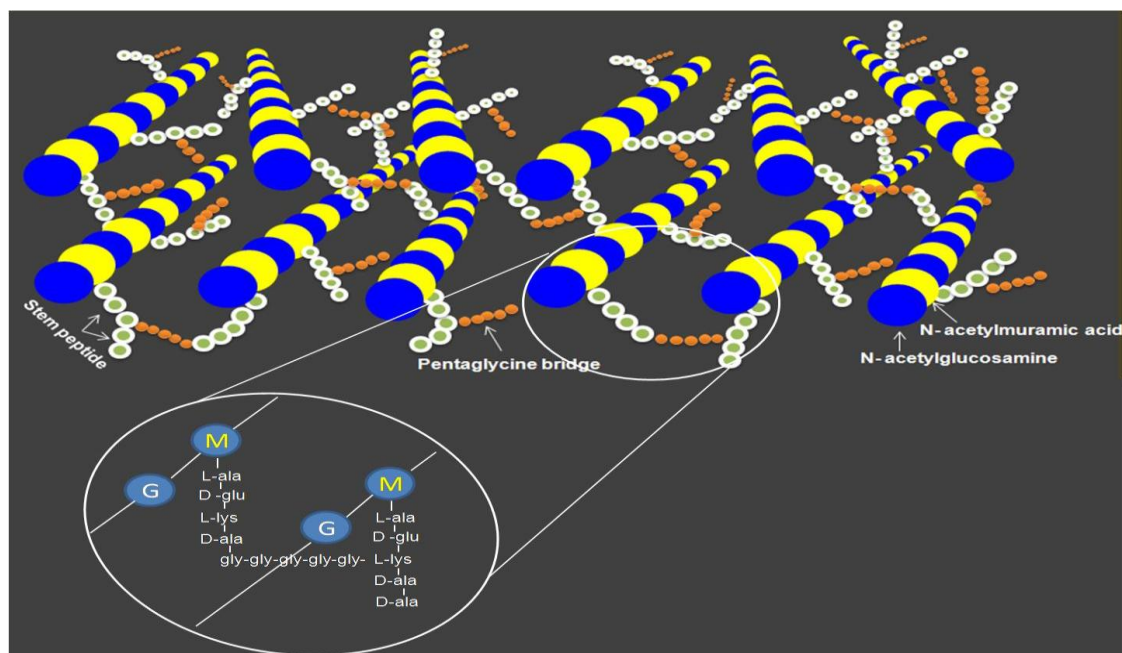
Figure 1.1:   Three-dimensional structure of *S. aureus* peptidoglycan**,** consisting of repeating units of 1, 4 β-linked N-acetylglucosamine and N-acetylmuramic acid with tetrapeptides and cross-linked by pentaglycine bridge. (adapted from Tomasz, 2006, and Todar, 2002).

1.2.4 *S. aureus* genome

The genome of *S. aureus* has been extensively studied and currently 18 staphylococcal genomes (Table.1.1) have been completely sequenced (nine are MRSA (four of which are vancomycin-intermediate resistant *S. aureus* (VISA)) and nine are MSSA) and a further 28 genomes are currently being sequenced (some of them in the finishing stage). This has provided an extraordinary glimpse into this so-called "super genome" and has led to a significant increase in our knowledge of the structure and functioning of *S. aureus*.

The staphylococcal genome is a closed circular molecule of double-stranded (ds) DNA of between 2.7 – 3.0 Mbp in length, encoding between 2509 to 2892 open reading frames (ORFs). It is composed of two domains called the core genome and the accessory genome. The core genome is inherited from the ancestors and is highly conserved in all staphylococcal species (Shittu *et al.*, 2007). The core comprises approximately 75% of *S. aureus* chromosome and it is highly conserved between strains (Lindsay and Holden, 2004). It contains all the housekeeping genes that are required for essential cell functions, such as DNA replication, proteins synthesis and core metabolism etc. The genome includes a wide variety of genes encode functions that contribute to virulence, such as toxins, exoenzymes and capsule biosynthetic cluster. The battery of virulence genes is highly strain variable (Shittu *et al.*, 2007).

The second domain, comprising of ~25% of the staphylococcal genome, is the accessory genome. The accessory genome mostly consists of mobile genetic elements (MGEs) that encode variety of non-essential components required for growth and survival. These elements include pathogenicity islands, bacteriophages, chromosomal cassettes, genomic islands, plasmids and transposons. Many of these elements encode virulence and antibiotic-resistance determinants that are transferred horizontally between strains of clinical importance (Lindsay and Holden, 2004; Shittu *et al.*, 2007). There is preliminary evidence to indicate that some of MGEs move among isolates at a high frequency whereas others move if only rarely. Although, the transfer mechanisms are not fully understood, valuable information has been obtained about how *S. aureus* causes infection from the characterisation and identification of MGEs. Several studies suggest that certain virulence and antibiotic-resistance determinants are associated with particular strains and types of infection (Lindsay and Holden, 2004).

Table 1. 1: The sequenced genomes of *S. aureus*.

Strain	Source	Type	Year	Size (bp)	ORFs	Status	GeneBank	Reference
NCTC8325	Colindale, UK	MSSA	1949	2,821,361	2892	complete	CP000253	(Gillaspy *et al.*, 2006)
COL	Colindale, UK	HA-MRSA	1961	2,809,422	2612	complete	CP000064	(Gill *et al.*, 2005)
FPR3757	Abscess, US	CA-MRSA	2006	2,872,769	2560	complete	CP000255	(Diep *et al.*, 2006)
TCH1516	Sepsis, US	CA-MRSA	2007	2,872,915	2654	complete	CP000730	(Highlander *et al.*, 2007)
MW2	Bacteremia,US	CA-MRSA	1998	2,820,462	2624	complete	CP000033	(Baba *et al.*, 2002)
MSSA476	Osteomyelitis, UK	CA-MSSA	1998	2,799,802	2571	complete	BX571857	(Holden *et al.*, 2004)
M50	Wound, Japan	HA-VISA	1997	2,878,529	2696	complete	CP000017	(Kuroda *et al.*, 2001)
Mu3	Pneumonia, Japan	HA-VISA	1997	2,880,168	2690	complete	CP000324	(Neoh *et al.*, 2008)
N315	Pharynx, Japan	HA-MRSA	1982	2,814,816	2583	complete	CP000018	(Kuroda *et al.*, 2001)
ED98	Chickens, UK	MSSA	2009	2,824,404	2661	complete	CP001781	(Lowder *et al.*, 2009)
MRSA252	Bacteremia,UK	HA-MRSA	1997	2,902,619	2650	complete	BX571856	(Holden *et al.*, 2004)
EMRSA15	Sanger, UK	HA-MRSA	2009	2,832,299	NA	complete	NA	www.Sanger.ac.uk
JH1	Blood, US	HA-VISA	2002	2,906,507	2747	complete	CP000736	(Mwangi *et al.*, 2007)
JH9	Blood, US	HA-VISA	2002	2,906,700	2697	complete	CP000703	(Mwangi *et al.*, 2007)
Newman	Osteomyelitis, UK	MSSA	1952	2,878,897	2614	complete	AP009351	(Baba *et al.*, 2008)
RF122	Bovin, Ireland	MSSA	2007	2,742,531	2509	complete	AJ938182	(Herron-Olson *et al.*,2007)
LGA251	Bovine, Sanger, UK	MSSA	2009	2,750,834	NA	complete	NA	www.Sanger.ac.uk
TW20	Bactermia, Sanger, UK	HA-MRSA	2010	3,043,210	2808	complete	FN433596	(Holden *et al.*, 2010)

NA= not available

1.2.5 Pathogenesis and virulence factors

Although one-third of mankind is asymptotically colonised by *S. aureus*, most are asymptomatic. However, under the right conditions this versatile organism is able to cause a wide range of infections (Archer, 1998). The nature of those infections depends on several factors, such as the pathogenic characteristics of the strain, host susceptibility and the route of entry into the host. In addition, *S. aureus* infections vary in seriousness and outcome from minor skin infections such as superficial lesions (furuncles, boils) and wound infections, to life-threatening infections such as septicaemia, ostemyelitis, acute endocarditis and necrotising pneumonia (Lowy, 1998; Keane, 1992)

The ability of *S. aureus* to colonise the host and the capacity of this bacterium to exchange and obtain genetic information reflect its success as a versatile pathogen. This contributes to the fact that S. *aureus* strains can express a variety of virulence factors (Table 1.2) that play key roles in their spread and proliferation in its human and animal hosts. It is noteworthy that a virulence factor can be multifunctional in pathogenesis and multiple virulence factors may play the same function (Decker, 2008; Gordon and Lowy, 2008)

The virulence factors of *S. aureus* can be divided mainly into three clusters: cell surface-associated factors including cell surface-bound proteins (the MSCRAMMs [microbial surface components recognizing adhesive matrix molecules]) and other surface-components (polysaccharide capsule & cell wall peptidoglycan); extracellular enzymes including coagulase and staphylokinase, and toxins such as haemolysins, leukocidins and toxic shock syndrome toxin (TSST) (Wright and Novick, 2003; Novick, 2006).

Most *S. aureus* strains produce a capsular polysaccharide that contributes in virulence of *S. aureus*. The capsule plays vital role in the adhesion of bacterial cells to each other and to host tissues and medical equipment. In addition, the capsule inhibits phagocytosis and restricts the ability of antibiotics to reach the bacterial cell surface. Moreover, other cell wall components (*e.g.* peptidoglycan and lipoteichoic acid) have a role in *S. aureus* pathogenicity: peptidoglycan, for example, has endotoxin activity that stimulates macrophages to release cytokines (Lowy, 1998). Lipoteichoic acid (LTA) is thought to plays an important role in septic shock and other detrimental host responses (Ferry *et al.*, 2005; Fournier and Philpott, 2005; Novick, 2006).

Staphylococcal surface proteins contribute to the spread and virulence of *S. aureus*. These proteins, which include protein A, fibronectin binding proteins, fibrinogen binding proteins and collagen binding proteins are known MSCRAMMs. These proteins perform a wide spectrum of functions and many recent studies have shown that these surface proteins play essential roles in the ability of these bacteria to colonize host tissues (Wright and Novick, 2003; Lowy.1998) by promoting adhesion to the surfaces of host cells and tissues. MSCRAMMs are covalently attached to the cell wall by sortase enzymes that recognise and cleave the Leu-Pro-X-Thr-Gly (LPXTG) motif (Schneewind *et al*., 1995; Mazmanian *et al.*, 1999). MSCRAMMs can also help the organism to evade the innate immune system and increase iron uptake (Foster, 2005). It appears that MSCRAMMs play a key role in the colonisation of prosthetic-devices and endovascular infections by, for example, assisting in the formation of biofilms. Moreover, MSCRAMM protein A is a good example of immune evasion factor that binds to the Fc portion of immunoglobulin and subsequently prevents opsonisation (Gordon and Lowy, 2008).

During infection, *S. aureus* secretes a wide variety of extracellular enzymes and toxins that contribute either directly or indirectly to pathogenesis. Most, if not all *S. aureus* strains produce haemolysins, coagulases, nucleases, protease, lipases, hyaluronidase and collegenases (van Belkum, 2007). Coagulase is produced exclusively, among the staphylococci, by almost all strains of *S. aureus* (with the exception of a few strains of *S. intermedius*). Coagulase reacts with blood-prothrombin to form a staphylothrombin complex that can convert fibrinogene to fibrin. Although, coagulase covers the bacterium with fibrin to reduce its susceptibility to host defences, its contribution to pathogenesis is not clear (Lowy, 1998; Wright and Novick, 2003). In contrast, staphylokinase disassembles fibrin by its interaction with plasminogen to form plasmin (a serine protease) that, by virtue of its ability to digests fibrin clots, allows staphylococci to spread into deep host tissue (Bokarewa *et al.*, 2006). Other enzymes secreted by *S. aureus* include proteases such as serine protease V8 and lipases that breakdown the bactericidal fatty acids produced by infected cells. Many *S. aureus* strains produce hyaluronidase that can degrade the hyaluronic acid, a component of the extracellular matrix of host tissues, and enables the bacterium to spread through host tissues. Hyaluronidase is commonly referred as the spreading factor (Hynes and Walton, 2000; Wright and Novick, 2003). The DNA and RNA of host cells are degraded by nucleases. These enzymes almost produced by all staphylococcal strains and cleave the

phosphodiester bonds of both single and double stranded DNA and RNA (Wright and Novick, 2003). Finally, *S. aureus* strains can produce β-lactamases that are responsible for the resistance of β-lactam antibiotics (Lowy, 1998).

   S. aureus produces many cytotoxins that are grouped according to their mode of action (Lowy, 1998). Haemolysins (alpha, beta, gamma and delta) are porin-like toxins that lyse a variety of host cells such as red blood cells (erythrocytes) and platelets. Another important pore-forming toxin is Panton-Valentin leukocidin (PVL) that structurally resembles alpha toxin. This cytotoxin mostly forms its pores in the outer membrane of mitochondria and kills neutrophils and macrophages. Fortunately, <5% of *S. aureus* strains produces PVL and its production is associated with cutaneous infections and, more recently, with necrotizing pneumonia (Wright and Novick, 2003, van Belkum, 2007; Decker, 2008). *S. aureus* also secretes enterotoxins (A-E, G-I), toxic shock syndrome toxin-1 (TSST-1) and exfoliative toxin (A, B). These toxins are responsible for diseases such as food poisoning, toxic shock syndrome and exfoliative dermatitis (scalded skin syndrome) (Wright and Novick, 2003). Enterotoxin and TSST represent pyrogenic toxin superantigens (PTSgs) and both play a role in derailing and over-stimulating components of the immune response (van Belkum, 2007).

   Staphylococcal virulence factors are precisely regulated by a quorum-sensing system based on the accessory gene regulator (*agr*). In order to initiate colonisation of tissue sites, MSCRAMMs are expressed during exponential growth phase whereas secreted enzymes and toxins are produced later during stationary phase so as to aid the spread of the organism (Table 1.2) (Gordon and Lowy, 2008).

Table 1.2: Extracellular proteins of *S. aureus* including virulence factors. (Adapted from Wright and Novick, 2003; Novick, 2006).

	Gene	Location	Product	Activity/Function	Timing
Superantigens	*sea*	Phage	Enterotoxin A	Food poisoning, TSS	xp
	seb	SaPI3	Enterotoxin B	Food poisoning, TSS	pxp
	sec	SaPI4	Enterotoxin C	Food poisoning, TSS	pxp
	sed	Plasmid	Enterotoxin D	Food poisoning, TSS	pxp
	eta	ETA phage	Exfoliatin A	Scalded skin syndrome	pxp
	etb	Plasmid	Exfoliatin B	Scalded skin syndrome	pxp
	tst	SaPI1,2,bov1	Toxic shock toxin-1	Toxic shock syndrome	pxp
Cytotoxins	*hla*	Chrom	α-haemolysin	Haemolysin, cytotoxin	Pxp
	hlp	Chrom	β-haemolysin	Haemolysin, cytotoxin	pxp
	hld	Chrom	δ-haemolysin	Haemolysin, cytotoxin	xp
	hlg	Chrom	γ-haemolysin	Haemolysin, cytotoxin	pxp
	lukS/F	PVL phage	P-V leukocidin	Leukocidin	pxp
Enzymes and other secreted	*SplA-F*	Chrom	Serine protease	Putative protease	pxp
	sspA	Chrom	V8protase	Spreading factor	pxp
	aur	Chrom	Metalloprotease (aureolysin)	Processing enzyme	pxp
	sspB	Chrom	Cysteine protease	Processing enzyme	
	scp	Chrom	Staphopain (protease II)	Spreading, nutrition	pxp
	geh	Chrom	Glycerol (esterhydrolase)	Spreading, nutrition	pxp
	Lip	Chrom	Lipase (butyryl esterase)	Spreading, nutrition	pxp
	fme	Chrom	Fatty acid methyl ester	Fatty acid esterification	pxp
	plc	Chrom	PI-phospholipase C	Degrades membrane lipid PI	pxp
	nuc	Chrom	Nuclease	Nutrition	pxp
	hys	Chrom	Hyaluronidase	Spreading factor	pxp
	coa	Chrom	Coagulase	Clotting, clot digestion	exp
	sak	Phage	Staphylokinase	Plasminogen activator	pxp
Surface proteins	*spa*	Chrom	Protein A	Anti-immune, ant-PMN	pxp
	cna	PT islet	Collagen binding protein	Collagen binding	pxp
	fnbA	Chrom	Fibronectin BPA	Fibronectin binding	exp
	finB	Chrom	Fibronectin BPB	Fibronectin binding	exp
	clfA	Chrom	Clumping factor A	Fibrinogen binding	exp
	clfB	Chrom	Clumping factor B	Fibrinogen binding	exp
	hlfB	Chrom	Lactoferrin BP	Lactoferrin binding	
Capsular polsaccharides	*cap5*	Chrom	Polysacch. cap.type5	Antiphagocytosis	pxp
	cap8	Chrom	Polysacch. cap.type8	Antiphagocytosis	pxp

xp = throughout exponential phase. exp = early exponential phase only. Pxp = post-exponential phase. SaPI= *Staphylococcus aureus* pathogenicity island.

1.2.6 Antibiotic resistance

Since the late 1940s, antibiotics resistance associated with hospital-acquired infections has emerged as a major problem worldwide. In this respect it is important to distinguish between bacterial strains acquired in hospitals from those acquired in the community, since the former are more likely to exhibit a greater range of antibiotic resistances than the latter (Zhang *et al.*, 2006). Penicillin resistant strains of *S. aureus* were first detected in the 1940s, shortly after this antibiotic was introduced into clinical practice (Chambers and Deleo, 2009). This resistance was due to the production of β-lactamases that inactivate the antibiotic by hydrolyzing the β-lactam ring. The main mechanism of penicillin resistance is via the *blaZ*-encodes β-lactamases (Rohrer *et al.*, 2003). The mechanism is illustrated in Figure 1.3.

Currently, 90% of clinical *S. aureus* isolates are penicillin-resistant and resistance is particularly prevalent in hospitals. Consequently, new generations of penicillins have been developed. The introduction of methicillin in the 1960s was followed rapidly by the emergence of methicillin-resistant isolates. Methicillin-resistance is not mediated by β-lactamases but by the production of an altered penicillin binding protein (PBP2a) that has a low affinity for β-lactam antibiotics (Johnson, 1998; see Section 1.3.2). The 1960s also saw the development of non-β-lactam antibiotics such as chloramphenicol, erythromycin, streptomycin and tetracycline. Although initially effective against *S. aureus,* nevertheless resistance against them developed rapidly. By 1976, resistance to kanamycin and gentamicin was reported (Fig.1.2) and, by the early of 1980s, multiresistant *S. aureus* strains were reportedly responsible for nosocomial outbreaks in many countries (Thomas and Archer, 1989).

Vancomycin and teicoplanin are glycopeptide antibiotics that have been the first choice for the treatment serious nosocomial MRSA infections for the last fifteen years. Since the emergence of vancomycin-resistant enterococci, fully vancomycin resistant strains of *S. aureus* have been expected. The first vancomycin-intermediate resistant *S. aureus* (VISA) was reported in Japan in 1996. This isolate, designated Mu50, had an MIC for vancomycin of 8μg/ml (Hiramatsu *et al*., 1997). Thereafter, clinical cases from which VISA were isolated were reported in 1997 in USA, then France and later in the UK. All these reported strains were vancomycin-intermediate *S. aureus* (VISA) and were called glycopeptide-intermediate *S. aureus* (GISA) (Cui and Hiramatsu, 2003). The National Committee for Clinical laboratory standard (NCCLS) suggested the definition for vancomycin-resistant terms to avoid any confusion. *S. aureus* strains that

have an minimum inhibitory concentration (MIC) of 4 µg/ml or below of vancomycin are defined as vancomycin-sensitive *S. aureus* (VSSA), those that have an MIC of between 8-16 µg/ml as vancomycin-intermediate *S. aureus* (VISA), and those that have an MIC of 32 µg/ml or more as vancomycin-resistant *S. aureus* (VRSA) (Srinivasan *et al.*, 2002). Although, VISA strains have been isolated, they are rare in most countries. However, 2002 witnessed the reporting of the first fully vancomycin-resistant *S. aureus* (VRSA) isolate from a renal dialysis patient in Michigan, USA (Fig.1.2) (Bartley, 2002). This strain had an MIC of >128 µg/ml and carried the vancomycin-resistance gene, *vanA* (Chang *et al.*, 2003; Aires de Sousa and Lencaster, 2004).
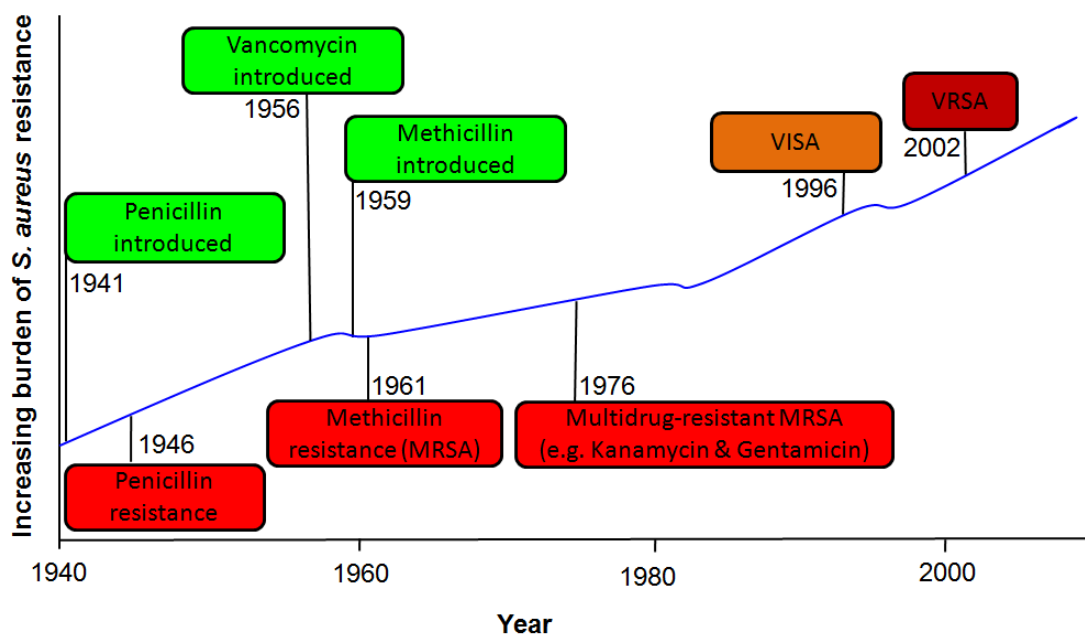
Figure 1.2: The emergence of antibiotic resistance in *S. aureus*. VRSA = vancomycin-resistant *S. aureus*. VISA = vancomycin-intermediate *S. aureus* (adapted from Chambers and Deleo, 2009).

Vancomycin acts on cell-wall peptidoglycan by binding to the carboxyl-terminal D-alanyl-D-alanine residues of peptidoglycan precursor, preventing PBPs from accessing to their natural substrate. Vancomycin has to penetrate about 20 layers of peptidoglycan to reach the cytoplasmic membrane where the transglycosylation and transpeptidation reactions of PBPs take place. In the VISA strains Mu50 and Mu3, vancomycin-resistance is associated with a thickening of the cell wall caused by excessive activation of peptidoglycan synthesis. The vancomycin molecules are trapped

by high levels of free D-alanyl-D-alanine residues due to the much-reduced levels of cross-linking (Aires de Sousa and Lencaster, 2004).

In contrast, full vancomycin-resistance in *S. aureus* (VRSA) is encoded by three determinants, namely VanA, VanB and VanD, normally associated with vancomycin-resistant enterococci (VRE). Resistance is brought about by the replacement of the native D-alanyl-D-alanine (D-Ala-D-Ala) residue of the cross-linking wall peptide with a D-alanyl-D-lactate (D-Ala-D-Lac) residue, which has a very low affinity to glycopeptides. It is noteworthy that the VanA induces high level of resistance and also confers resistance to teicoplanin. The *vanA* gene cluster is carried on a large resistance plasmid (Berger-Bachi, 2002).

1.3 Methicillin-resistant *Staphylococcus aureus* (MRSA)

1.3.1 Emerging methicillin resistance

Methicillin-resistant *S. aureus* were first discovered in the UK in 1961 by Jevons (1961). In this study, three MRSA strains were reported among 5,440 isolates and MRSA infections were initially confined to hospital patients. The first nosocomial epidemic of MRSA was reported in 1963, when an MRSA strain was isolated from an infant who was treated unsuccessfully with penicillin. The same strain was isolated from 37 children in eight wards and from one nurse. Medical centres in several European countries (*e.g.* Denmark, France and Switzerland) described outbreaks of MRSA nosocomial infections in the 1960s. The first US MRSA outbreak did not occur until 1971 (Chambers, 1988; Keane, 1992). More recently, a number of MRSA infections have been shown to be community- rather than hospital-acquired (Chambers, 1988).

In the UK, the number of MRSA infections remained limited for several years. At the beginning of the 1971, MRSA represented 10% of all *S. aureus* isolates at the general hospital in Birmingham. Interestingly by the mid-1970s, the number of reported MRSA cases declined to virtually zero, a decrease was thought to be due to a combination the use of aminoglycoside antibiotics and better infection control procedures (Griffiths *et al.*, 2004; Johnson *et al.*, 2005; Grundmann *et al.*, 2006). However, in the early 1980s, concerns about MRSA were heightened with the emergence of gentamicin-resistant MRSA in the UK, Ireland and the USA.

In 1982, an epidemic strain of multi-resistant MRSA was reported in Australia, and the same strain was discovered in London in connection with a hospital outbreak. The staphylococcal reference laboratory of the UK Public Health Laboratory Service established a numerical prefix for epidemic MRSA strains and, based on this system, 16 epidemic strains were identified in England and Wales up to 1995. Nevertheless, only three epidemic strains, UK EMRSA-3, UK EMRSA-15 and UK EMRSA-16, were still being recorded in the 1990s and EMRSA-15 and EMRSA-16 were behaviourally more dynamic. In the meantime, six epidemic MRSA strains have been recorded in some central Europe countries (Grundmann *et al.*, 2006). EMRSA15 and EMRSA16 strains have spread broadly and are associated with severe infections (Livermore, 2000). According to the Centers for Disease Control (CDC) and the National Nosocomial Infection Surveillance System (NNISS), the proportion of MRSA in US hospitals increased dramatically from 2.4% in 1975 to 29% in 1991 (Graffunder and Venezia, 2002). In the UK MRSA bacteraemias remained at 3% until 1992 and then rose considerably to reach 43% by 2002. Since then, the rate of isolation of MRSA strains has increased significantly every year worldwide (Grundmann *et al.*, 2006).

1.3.2 Mechanism of Methicillin-resistance

Methicillin-resistance in staphylococci is due to the acquisition of a large mobile DNA element (20 to 100kb in size), the so-called "Staphylococcal cassette chromosome *mec"* (SCC*mec*) (Rohrer *et al.*, 2003). Currently, eight SCC*mec* types (I to VIII) have been described in details (see Section 1.3.3).

β-lactam antibiotics destroy bacteria by inhibiting bacterial cell wall synthesis. The antibiotic, an analogue of D-alanyl-D-alanine, covalently attaches to penicillin-binding proteins (PBPs). These membrane-anchored proteins catalyse one or more of three reactions (transpeptidase, endopeptidase and carboxypeptidase) involved in cell wall synthesis. MRSA produces a modified PBP2a that can complete cell wall synthesis when the transpeptidation activities of the native PBPs are inactivated by the antibiotic (Chambers and Hackbarth, 1992). Resistance is due to the fact that PBP2a has low affinity to β-lactams antibiotics. The expression of the *mecA* gene is controlled by the products of the *mecRI* and *mecI* genes. MecRI synthesis is induced upon exposure to β-lactam antibiotics. As a result, the MecI repressor is inactivated and PBP2a produced (Figure 1.3; Lowy, 2003).
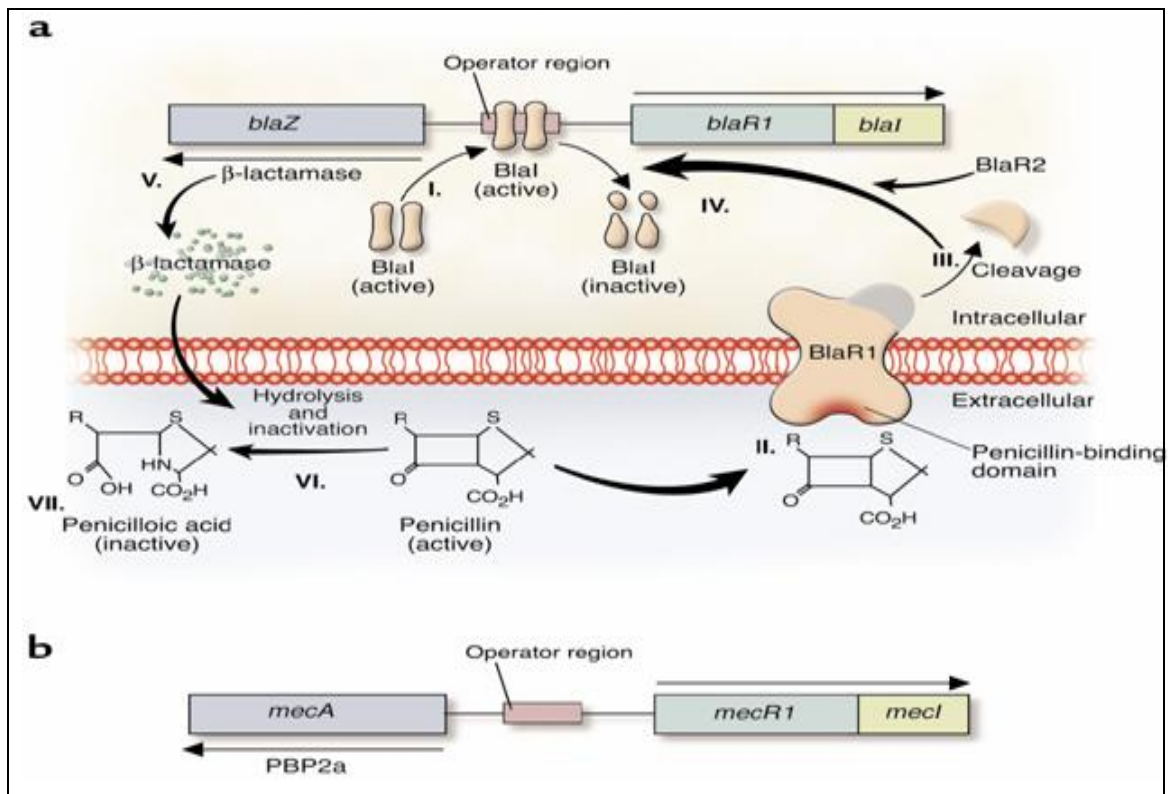
Figure 1.3: The mechanisms of β-lactam-resistance in *S. aureus*. (a) The production of β-lactamase by *blaZ* gene in the presence of penicillin, I. The operator region of *blaZ*, *blaR1* and *blaI* is bound by the DNA-binding protein BlaI that represses the transcription of *blaZ*, *blaR1* and *blaI*. II- III. The presence of penicillin stimulates the transmembrane sensor-transducer BlaR1 to inactivate the BlaI repressor. IV-V. This process results in the removal of BlaI and allowing transcription of *blaZ* to produce β-lactamases that inactivate penicillin by cleaving the β-lactam ring, (b) The mechanism of methicillin-resistance by production of PBP2a encoded by *mecA* gene. PBP2a synthesis proceeds similarly to that shown for β-lactamases. MecR1 is induced by β-lactam antibiotics and MecR1 inactivates MecI repressor allowing PBP2a synthesis (Lowy, 2003).

1.3.3 Staphylococcal cassette chromosome *mec* (SCC*mec*) elements

The emergence of MRSA is the result of the acquisition of a *mecA* gene by the chromosome of an MSSA strain. The *mecA* gene is methicillin-resistance determent that is carried on a large (21- 67 kb) mobile genetic element called the staphylococcal cassette chromosome *mec* (SCC*mec*) element. SCC*mec* integrates into the staphylococcal chromosome at a specific-site (*att* BSCC) at 3'-end of *orfX*, close to the origin of replication (de Lencaster *et al.*, 2007; Ito *et al.*, 2007). The SCC*mec* element does not resemble other mobile genetic elements such as phages or transposons, but is instead more like a pathogenicity island that lacks virulence factors (Short and Enright, 2007). The origin of SCC*mec* is still unknown but there is evidence that SCC*mec* can be

horizontally transferred between staphylococcal species, and coagulase-negative staphylococci (CNS) are a potential source for SCC*mec* (Hanssen and Sollid, 2006).

According to their genetic structure and contents, SCC*mec* elements are categorised into several types and subtypes (IWG-SCC, 2009). Almost all SCC*mec* types shared common characteristics: (1) the elements carry the *mec* gene complex; (2) They contain a cassette chromosome recombinase (*ccr*) gene complex; (3) SCC*mec* elements integrate at the same site into chromosome (near the origin chromosome of replication); (4) They are flanked by incomplete inverted and direct repeats sequences that contain integration site sequence (ISS) and located at the integration site of chromosome; those repeat sequences are recognised by *ccr* recombinases during the excision and integration of SCC*mec* (Hanssen and Sollid, 2006; Ito *et al*., 2007; IWG-SCC, 2009).

SCC*mec* elements are currently classified into various types and subtypes based on a combination of the class of the *mec* gene complex and the allotype of the *ccr* gene complex. In SCC*mec* of *S. aureus*, three classes of the *mec* gene complex have been described: class A has the intact *mec* regulon (*mec*A- *mecRI* - *mecI*) with insertion sequence IS*431mec* downstream of *mecA,* whereas classes B and C1/C2 contain *mecA* regulation genes that are truncated by insertion sequence IS*431-mecA-me*cRI-IS*1272* and IS*431- mecA- mecRI-* IS*431*, respectively (Ito *et al.,* 2003; Hanssen and Sollid, 2006; de Lencaster *et al.*, 2007; IWG-SCC, 2009). Another essential component of SCC*mec* is the *ccr* gene complex. Two genes (*ccrA* and *ccrB*) with four allotypes have been identified. Another gene, *ccrC*, has been identified in only one of the allotypes (Fig.1.4). A summary of the classification of *ccr* genes is showed in Table.1.3. The *ccr* gene complex plays a key role in the integration and excision of SCC*mec* into the conserved *orfX* locus by encoding the recombinase proteins of the invertase/resolvase family (Ito *et al*., 2003). Although the remaining regions of the SCC*mec* element carry antibiotic-resistance determents, they are called junkyard regions since they harbour the non-essential components. Three junkyard regions have been classified (J1-3) and variations in these regions are used to define the SCC*mec* subtypes (de Lencaster *et al.*, 2007).

Table 1.3:   SCC*mec* types (adapted from IWG-SCC (2009).

SCC*mec* types	*mec* gene complex	*ccr* gene complex	Size
I	B (IS*1272*- *mecA*- *mecRI*)	1 (A1B1)	34kb
II	A (IS*431*- *mecA*, *mecRI* - *mecI*)	2 (A2B2)	53-58kb
III	A (IS*431*- *mecA*, *mecRI* - *mecI*)	3 (A3B3)	67kb
IV	B (IS*431*- *mecA*- *mecRI* - IS*1272*)	2 (A2B2)	20-25kb
V	C2 (IS*431*- *mecA*- *mecRI*- IS*431*)[b]	5 (C)	28kb
VI	B (IS*431*- *mecA*- *mecRI* - IS*1272*)	4 (A4B4)	20-25kb
VII	C1 (IS*431*- *mecA*- *mecRI*- IS*431*)	5 (C)	28-30kb
VIII	A (*mecA*, *mecRI* and *mecI*)	4 (A4B4)	32kb

[b] Difference between C1 and C2, the orientation of IS*431* upstream of *mecA* is reversed in C2 *mec* gene complex.

To date, eight SCC*mec* (I − VIII; Fig. 1.4 and Table 1.3) elements have been identified according to the criteria in Table 1.3 (IWG-SCC, 2009). The sizes of SCC*mec* types I, II and III are 34kb, 53-58kb and 67kb respectively whereas type IV is 20-24kb and type V is 28kb. SCC*mec* types VI, VII and VIII are 20-25kb, 28-30kb and 32kb respectively (Ito *et al.,* 2004; Rohrer *et al.*, 2003; Oliveira *et al*., 2002; Plata *et al.*, 2009). Many antibiotic resistant genes and heavy metal resistance elements are encoded by SCC*mec* types II, III and VIII. These include integrated plasmids (pUB110 and pT181) and transposons (*Tn*554 and *IS*431). In contrast, types I, IV, V, VI and VII do not contain antibiotic resistant genes other than *mec*A (Grundmann *et al*, 2006; de Lencaster *et al*, 2007; IWG-SCC, 2009; Plata *et al*, 2009).
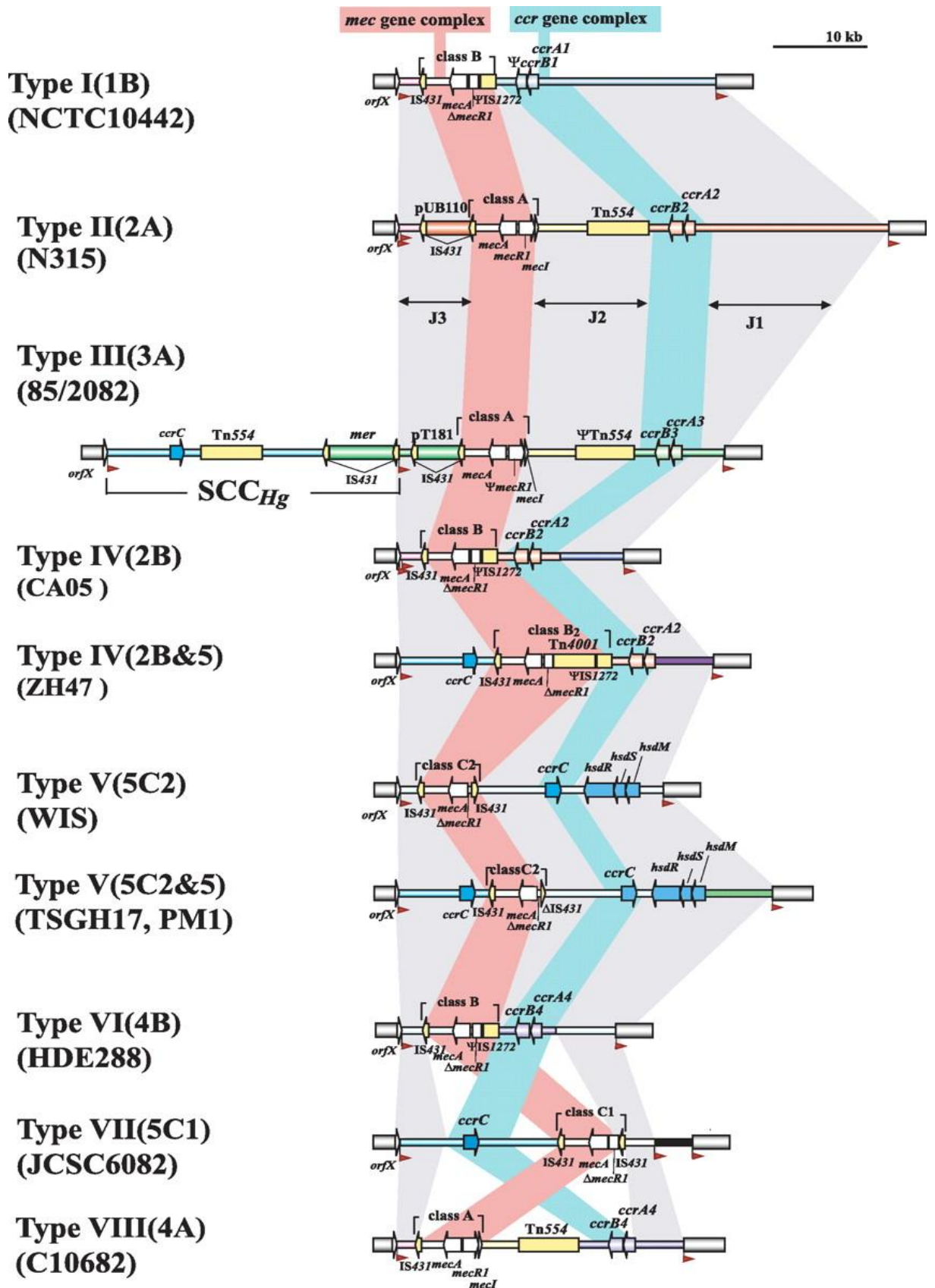
Figure 1.4: Different types of SCC*mec* verified in MRSA (IWG-SCC, 2009).

1.3.4 Epidemiology of MRSA in hospital

MRSA has become a major nosocomial pathogen and its prevalence continues to increase worldwide. MRSA infections usually result in an increase in morbidity and mortality rates among hospital patients. According to the National Nosocomial Infections Surveillance (NNIS), approximately 80,000 patients per year suffer from MRSA infections (Aires de Sousa and de Lencastre, 2004). In addition, hospital-acquired HA-MRSA strains are generally described as multi-drug resistant (MDR) MRSA because of their resistance to many classes of antibiotics such as β-lactam, chloramphenicol, erythromycin, streptomycin and tetracycline antibiotics. As a result, MRSA infections are difficult and costly to treat. Previous studies have revealed that the mortality rate associated with MRSA bacteraemia is higher than that associated with MSSA bacteraemia (Cosgrove *et al.*, 2003).

According to NNIS, between 1998 and 2002 in the USA, about 51.3% of *S. aureus* isolates from the patients in intensive care units (ICUs) were methicillin-resistant (NNIS, 2002). The prevalence of MRSA has shown variations between European countries. The European antimicrobial surveillance system (EARSS) has reported that the proportion of MRSA isolated from blood between 1999 and 2002 was 41-45% in the UK (Fig.1.5), Greece, Ireland, Malta and Italy, but less than 1% in Iceland, Denmark, Netherlands and Sweden. This disparity was not only observed between countries but also sometimes between hospitals within same country (Tiemersma *et al.*, 2004; Johnson *et al.*, 2005).
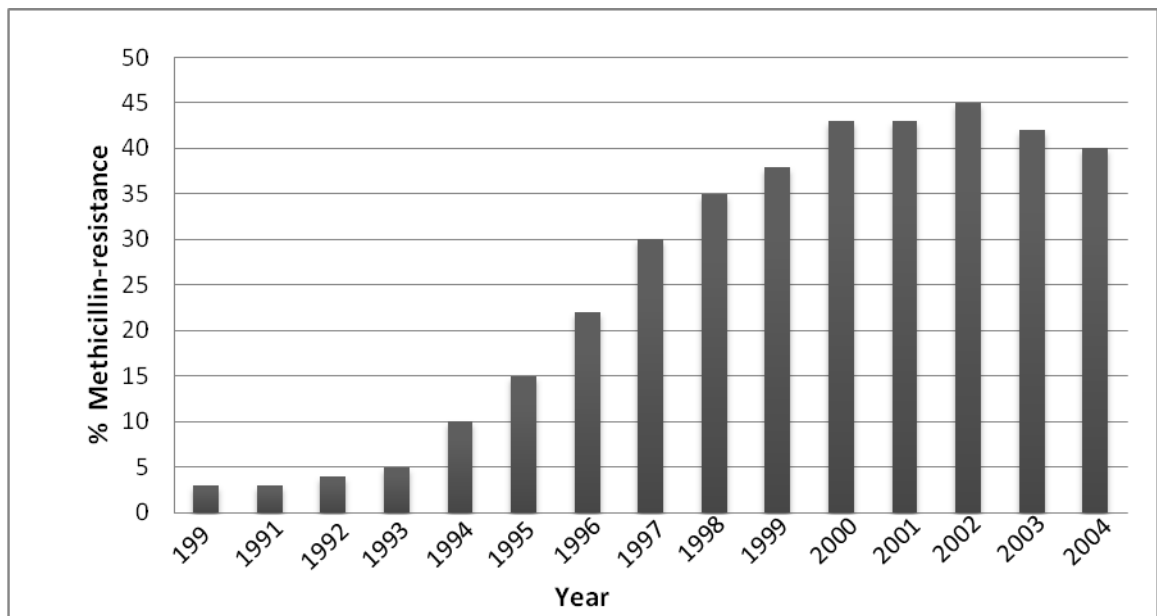
Figure 1.5: The proportion of isolates of *Staphylococcus aureus* from blood culture that are methicillin resistant, England and Wales (Johnson *et al.***,** 2005).

The most common MRSA nosocomial infections are skin and soft tissue infections, bloodstream infections, bone and joint infections, endovascular infections, osteomyelitis and meningitis. These infections vary from unit to unit. For example, MRSA nosocomial bloodstream and pneumonia infections are most common in ICUs (Chang *et al.,* 1997; Cunha, 2005).

1.3.4a Risk factors associated with nosocomial MRSA

A good knowledge of the epidemiological characteristics of MRSA is the cornerstone of establishing an effective infection control strategy. There are many risk factors associated with the emergence of MRSA nosocomial infections. Most of which can be attributed to host factors, antibiotic pressure and infection control procedures. Those risk factors are older age, male gender, previous hospitalization and exposure to antibiotics, length of hospitalization, length of stay in ICU, and underlying disease. In addition, pneumonia infections are associated with ventilator therapy, and immunosuppressive therapy in ICUs. Invasive devices play role in increasing vulnerability to nosocomial infection. Intravascular (IV) catheters are, for example, the main risk factor for MRSA bloodstream infection in ICUs (Washio *et al.*, 1997; Graffunder and Venezia, 2002). There are other factors that are linked to spread of MRSA in hospitals: transferring of patients within or between hospitals, poor communication between hospital units, difficulties in isolation MRSA patients and new epidemic MRSA strains (Vuopio-Varkila, 2007)

Those factors contribute not only to therapeutic failure and morbidity but also to increased mortality of MRSA among hospitalized patients. According to UK Government statistics, the number of deaths due to MRSA has increased dramatically from 51 in 1993 to 1,230 in 2008, although, there has been a noticeable decrease in MRSA deaths in 2008, compared with 2005, 2006 and 2007 (Fig. 1.6). It is noteworthy that colonized and infected patients and healthcare workers are the main reservoir for MRSA in hospitals. Moreover, the main road for transmission of MRSA is through the hands of health care workers (HCWs) in hospitals (Haddadin *et al.*, 2002).
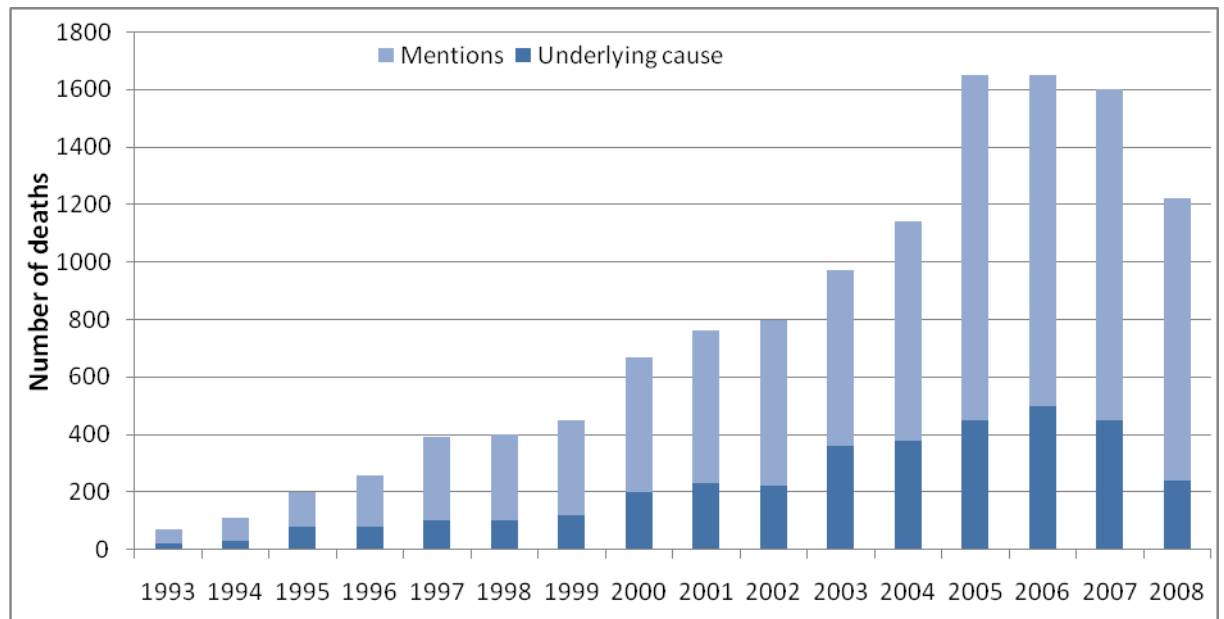
Figure 1.6: Number of death certificates mentioning methicillin resistant *Staphylococcus aureus* in England and Wales (UK Government statistics).

1.3.5 Epidemiology of MRSA in the community

Although MRSA is mainly associated with nosocomial infections, it is becoming an increasing problem in the community. In the 1993, community-acquired MRSA (CA-MRSA) was first reported in the Western Australia and by end of 1999, four deaths were reported among children due to CA-MRSA infections in USA (Aires de Sousa and de Lencastre, 2004). CA-MRSA strains differ from nosocomial strains in a number of respects. Firstly, CA-MRSA strains are sensitive to non-β-lactams antibiotics, for example clindamycin, trimethoprim/sulfamethoxazole and doxycycline. Secondly, these strains have genotypes that are distinct from those of nosocomial strains. Finally, they have different methicillin-resistance cassettes and often encode PVL (Grundmann *et al.*, 2006; Boyle-Vavra and Daum, 2007).

There is no standard definition for CA-MRSA and approximately eight classification systems have been applied to categorize community-acquired infections (Aires de Sousa and de Lencastre, 2004). The CDC defines CA-MRSA as an infection with MRSA that lacks the risk factors of HA-MRSA including: the isolation of MRSA more than 48 hours after admission, hospitalization history, recent surgery, previous isolation of MRSA and presence of an indwelling catheter or a percutaneous device at the time of culture (Fridkin *et al.*, 2005; Brasel and Weigelt, 2008). Most common CA-MRSA infections are bacteraemias, skin and soft tissue infections, septic arthritis, toxic

shock syndrome, necrotizing fasciitis and necrotizing pneumonia (Grundmann *et al.*, 2006). CA-MRSA is also associated with the production of PVL that is encoded by phage-mediated *lukS-PV* and *lukF-PV* genes. The production of this toxin is associated with severe skin and soft tissue infections (Boyle-Vavra and Daum, 2007). The CA-MRSA infections have been documented among homeless people, homosexuals, military recruits, competitive athletes, residents of community-based health-care institutions and children in day-care centres (Grundmann *et al.*, 2006). There are several risk factors attributed to the acquisition of CA-MRSA such overcrowding, high rates of skin infections, frequent use of broad-spectrum antibiotics and close contact with a person with these risk factors. It is assumed that health-care institutions are the most likely source for CA-MRSA strains since some similarities have been found between sporadic nosocomial-MRSA and CA-MRSA infections (Aires de Sousa and de Lencastre, 2004).

1.3.6 Treatment and control of MRSA

MRSA strains are resistant to most β-lactam antibiotics and several other antimicrobial agents including aminoglycosides, clindamycin, chloramphenicol fluoroquinolones and macrolides (Schmitz and Jones, 1997). The glycopeptides have emerged as the most effective agents against MRSA. However, the emergence of MRSA strains with low sensitivity to vancomycin (VISA), first reported in 1996 and thereafter VRSA in 2002, has led to an increasing concern about the use of vancomycin as the first choice for the treatment of MRSA infections (Cui and Hiramatsu, 2003).

The treatment of MRSA infections depends on the site of infection. In some cases, such as infected devices and abscess, the remove of these devices and draining of abscess are more important than antimicrobial therapy (Cunha, 2005). Recently, new guidelines for the prophylaxis and treatment of MRSA infections in the UK have been published. These guidelines recommend the use of glycopepides or linezolid as a first choice to treat MRSA pneumonia and severe skin and soft tissue infections where the risk of bacteraemia is high (Gemmell *et al.*, 2006). Linezolid is recommended for treatment of pneumonia since has showed an excellent penetration into lungs (Conte *et al.*, 2002). The same choice of glycopepides or linezolid is recommended but with longer treatment for uncomplicated bacteraemia. In bone and joint infections glycopepides can be used particularly with multiresistant MRSA and/or in combination with rifampicin or fusidic acid. Glycopeptide prophylaxis is recommended for patients

who require surgery and have a history with MRSA colonisation (Gemmell *et al.*, 2006). These recommendations take in their account not only the efficacy of the antibiotics but also their toxicity, selection of resistant bacteria and cost (Ben-David and Rubinstein, 2003)

In the UK, the control of MRSA has been given a high priority among health-care professionals (Haddadin *et al.*, 2002). Although there is, as yet, no consensus approach for the control the MRSA infections, three strategies have been proposed for this purpose. The "*Scutari strategy*" is based on the application of basic cleanliness and protective procedures. This strategy is also applicable to nursing homes and small hospitals. The "*Search and Destroy*" strategy is applicable to hospitals that do not have a major problem with MRSA but have recently experienced epidemic outbreaks. This strategy is based on isolation of all infected and colonized patients and associated attempts to eradicate MRSA from the environment. The "*SALT strategy*" (*Staphylococcus aureus* Limitation Technique) is only applicable for non-containable infections, and when resources are limited. This method is appropriate for epidemic situations in which the incidence of infection is low (Spicer, 1984; Frank, 2003)

The majority of studies have reported that the screening of infected and colonised patients, the early detection of MRSA, improved hand hygiene and the prudent use of antibiotics, are effective control methods for the MRSA infections (Frank, 2003; Hardy *et al.*, 2004; Grundmann *et al.*, 2006; Wang and Barrett, 2007). Furthermore, the most important step in controlling MRSA outbreaks is the typing of strains to distinguish between epidemic and sporadic strains (Frank, 2003) (see Section 1.4).

1.4 The typing of MRSA

The ability to type isolates of MRSA is an important tool for understanding the epidemiology of MRSA and for developing effective control measures. In the last ten years, a variety of typing methodologies have been developed for use in both clinical and epidemiological studies (Wilailuckana *et al*., 2006). In recent years traditional typing methods have been augmented with a range of molecular typing methods. These include pulsed-field gel electrophoresis (PFGE), plasmid fingerprinting (PF), arbitrarily primed polymerase chain reaction (AP-PCR), multiple locus variable number tandem repeat (VNTR) analysis (MLVA) and multilocus sequence typing (MLST). For any

typing method to be valuable it should have high discriminatory power, be reproducible, inexpensive, and easy to use and interpret (Tenover *et al*., 1997; Weller, 2000; Sabat *et al*., 2003).

1.4.1 Mechanism of genetic variability of bacterial genome

The concept behind all microbiological typing methods is that epidemiological related strains are descended from a single ancestor. Consequently, the descendents share the similar, but not necessarily identical epidemiological characteristics that are distinct from those of unrelated strains. Consequently, the analysis of such characteristics can be useful for the study of population structures and the evolution of bacterial species. The stability of such characteristics within a strain and the general diversity of species are vital factors for epidemiological typing (van Leeuwen, 2003).

The genetic diversity among strains is attributed to several types of mutational process including the accumulation of spontaneous or induced point mutations, genetic recombination and the acquisition or loss of mobile genetics elements. The latter elements, which can confer virulence factors and antibiotic resistance determents, include plasmids, bacteriophages and gene islands (Fitzgerald *et al*., 2001; van Leeuwen, 2003). Mutations are caused by three processes: mutagenic agents, including environmental factors (*e.g*. chemicals, radiation and ultraviolet light (UV)), mistakes or errors that occur spontaneously during DNA replication without exposure to any mutagenic agent and the activity of mobile genetic elements. The UV, for example, can form thymine dimers (TT) on the same strand of the DNA. The thymine dimers cannot act as a template for DNA polymerases and such dimerization therefore can be lethal by preventing of the appropriate functioning of polymerases. Some chemical mutagens such as hydroxylamine (NH_2OH) can modify cytosine to uracil that pairs with adenine instead of guanine while acridine ($C_{13}H_9N$) causes frame-shift mutations by base deletion or base addition mutations. The spontaneous mutations can be caused by DNA polymerases by mismatched base insertion or slippage errors during DNA replication resulting in changes in the base sequence (Atlas, 1997). Three types of replication errors have been characterised: (1) Single-base mispairs leading to a base substation, (2) single-base bulges leading to a single-base frameshift and (3) multiple-base mismatches leading to a sequence substitution. These changes during the DNA replication play significant role in bacterial evolution and therefore in bacterial genetic diversities (Schumann, 2006).

Although, *S. aureus* clones arise more frequently (about 15-fold) by point mutation than by recombination, Robinson and Enright (2004) suggested that large chromosomal replacements could influence the long-term evolution of this species (Feil *et al*., 2003; Robinson and Enright, 2004). The SCC*mec* cassette, for example, is a large group of mobile genetic elements whose diversity is the result of extensive recombination. The SCC*mec* contains the *mec* gene (methicillin-resistance gene) and other mobile genetic elements (*e.g.* transposons, plasmids and insertion sequences). SCC*mec* is horizontally transferred within *Staphylococcus* species, playing an important role in microbial evolution by promoting the genetic diversity (van Leeuwen, 2003).

Typing methods vary in their ability to monitor and define genetic changes, not only between species, but also between strains within a single species. This is reflected in differences in the resolving power of the various typing methods. For example, MLST is less discriminatory than PFGE for the analysis of *S. aureus* in hospital outbreaks, reflecting the fact that the the evolution of diversity over short time periods is more likely to be due the the positive selection pressure for virulence or antibiotic resistance genes based on mobile genetics elements genes, than for spontaneous mutations in housekeeping genes (Singh *et al*., 2006; See Section 1.4.3b.iii and 1.4.3d.i

1.4.2 Phenotyping methods

Phenotyping methods are used to characterize the products of gene expression in order to identify a species or to discriminate between strains. Examples of phenotyping methods include biochemical profiles, antibiogramme profiles, bacteriophages susceptibilities (phage typing), the presence of surface antigens and protein analysis. However, there are limitations in using these methods extensively for typing MRSA strains because they are influenced by growth conditions and epigenetic modifications (Tenover *et al*., 1997; van Leeuwen, 2003)

1.4.2a Phage typing

S. aureus strains (including MSSA and MRSA) can be differentiated by their susceptibility or resistance to lysis by bacteriophages (Wentworth, 1963; van Leeuwen, 2003). The concept of using bacteriophages for typing bacteria was developed in the 1940s. For decades, this technique was used as one of the principal typing method for differentiating among strains of staphylococci. Phage typing was standardized by the International Subcommittee for Typing Staphylococci. Currently, 25 standard phages

are used for typing the staphylococcal strains and they have some value for investigating MRSA isolates since they have greater discriminatory power than other phenotyping methods such as capsular typing and zymotyping. The standard phages are used at a routine test dilution (RTD) but strains that show no lysis at RTD are subsequently tested at RTD × 100 (Weller, 2000; de Gialluly *et al.*, 2003).

Although a widely used method, phage typing has important limitations. Many isolates are non-typeable by this technique (*e.g.* 20-30% of MRSA strains, in some instances this reaches 75%), its reproducibility is low and the sets of virus stock need to be maintained effectively. Phage typing is also affected by the presence of modification systems, a form of epigenetic change on the chromosomal DNA that can impact on the stability of incoming phage DNA. Consequently, phage typing has been replaced with molecular typing methods in many reference laboratories (Khalifa *et al.*, 1989; Weller, 2000; van Leeuwen, 2003).

1.4.2b Serotyping

Serotyping is the oldest phenotyping method. However, despite its value for other bacterial genera, serotyping has not been used extensively for typing of *S. aureus* strains. Serotyping is mainly aimed at revealing diversity among capsular polysaccharides and the antigenic properties of enzymes such as coagulase. There are about 11 types of *S. aureus* capsules, although between 85-90% of clinical *S. aureus* isolates belongs to just two types, serotype 5 and serotype 8. The poor discriminatory power of the serotyping technique makes it of limited value for epidemiological studies, and has generally been replaced by phage typing (Weller, 2000; Witte *et al.*, 2006).

1.4.2c Antimicrobial susceptibility patterns

The susceptibility of a bacterium to different antibiotics can be used to compare and differentiate MRSA strains. The major advantage of this technique is its cheapness and its ability to be used routinely in many clinical laboratories. However, antimicrobial susceptibility patterns (antibiogrammes) have low discriminatory power and reproducibility and are affected by environmental factors. Moreover, the acquisition or loss of resistance plasmids means that unrelated strains can give similar antibiogrammes while genetically related strains can gave very different antibiogrammes (Tenover *et al.*, 1997; Weller, 2000). In the case of MRSA, antimicrobial susceptibility patterns are not reliable as a distinctive typing technique although the antibiogramme of an epidemic or

endemic clone can be useful for monitoring and treatment purposes (Weller, 2000; van Leeuwen, 2003).

1.4.2d Multilocus enzyme electrophoresis (MLEE)

MLEE involves the extraction and analysis of key enzymes from bacterial cells by gel electrophoresis. MLEE has been used to type many bacterial species, including *S. aureus*. When MRSA isolates were examined by MLEE it was found to show good reproducibility but was only moderately discriminatory (Weller, 2000). About 12 to 20 enzymes are used, although the discriminatory power of the individual enzymes has not been fully assessed. Although MLEE correctly classified related isolates among outbreak strains, some unrelated isolates were incorrectly included. MLEE has the advantage that it is relatively easy to interpret the generated data, but is time-consuming and consequently is not recommended for use in clinical laboratories (Goh *et al.,* 1992; Weller, 2000; Tenover *et al.*, 1994). Nevertheless, MLEE can be useful for studying the diversity of bacterial populations, for example clonal relatedness and diversity among MRSA strains (Witte *et al.*, 2006).

1.4.3 Genotyping methods

A variety of typing methods have been developed for the molecular typing of *S. aureus*. For a typing method to be effective it should be inexpensive, have high discriminatory power, good reproducibility, and be easy to perform and interpret. In the last decade, several molecular techniques have been developed for the typing of bacterial strains, including arbitrarily primed polymerase chain reaction (AP-PCR), pulsed-field gel electrophoresis (PFGE), plasmid fingerprinting (PF), variable number tandem repeat analysis (VNTR) typing, multiple-locus variable-number tandem repeat analysis (MLVA), multilocus sequence typing (MLST) and, in the case of MRSA, SCC*mec* typing. These techniques have been used primarily in reference laboratories but, with passage of time, are being used in an increasing number of clinical laboratories (Sabat *et al.*, 2003; Trindade *et al.*, 2003). Molecular methods have been increasingly applied to the epidemiology of nosocomial pathogens such as MRSA (van Leeuwen, 2003). The aim of any strategy for controlling the incidence of MRSA is to develop a thorough knowledge of its epidemiology and molecular typing techniques are ideally suited for this purpose because they are not phenotype-dependent (Deurenberg *et al.*, 2007).

1.4.3a First phase molecular typing (plasmid analysis)

Analysis of the plasmid content of staphylococcal strains was introduced in the mid-70s (van Leeuwen, 2003). It was the first molecular typing technique to be used to investigate the epidemiology of MRSA. Plasmid analysis is based on the extraction of plasmids DNA from the bacterial cells and the subsequent determination of their number and sizes by gel electrophoresis. This method is easy to perform and its results simple to interpreted (Weller, 2000). However, there are limitations to its application for epidemiological studies since, as mobile extra-chromosomal DNA elements, plasmids can be lost or acquired at a relatively high frequency (Witte *et al.*, 2006). Moreover, in the case of *S. aureus,* the discriminatory power of plasmid analysis is poor (Weller, 2000; Trindade *et al.*, 2003).

The discriminatory power and reproducibility of plasmid analysis was improved by incorporating a restriction digestion step. This approach can be particularly useful for distinguishing plasmids that are genetically distinct despite having similar molecular masses. Despite some studies reporting that restriction endonuclease analysis of plasmid DNA (REAP) has more discriminatory power than PFGE, other studies have described this method as ineffective. A major problem is the lack of stability and reproducibility (Weller, 2000; Ttraindade *et al.*, 2003; van Leeuwen, 2003).

1.4.3b Second-phase molecular typing

1.4.3b.i Restriction enzyme analysis (REA)

Restriction endonucleases can digest chromosomal DNA into a large number of fragments (5-15 kb) that can be separated by conventional gel electrophoresis. Examples of the restriction endonucleases that have been used to analyse the chromosomal DNA of MRSA strains include *Pst*I, *Eco*RI and *Bgl*II (Jordens and Hall, 1988; Trindade *et al.*, 2003). The patterns can be interpreted by comparing the banding patterns from different isolates. Despite the fact that all strains of MRSA are potentially typeable by REA, the resulting large number of fragments produced can be difficult to interpret. The main problem is that fragments larger than 20kb will not be separated by conventional gel electrophoresis (Trindade *et al.*, 2003).

1.4.3b.ii Restriction fragment length polymorphism (RFLP) analysis using DNA probes (Southern hybridization).

In the mid-70s Southern hybridization became available for epidemiological studies (van Leeuwen, 2003). This method is based on the digestion of the chromosomal DNA using restriction endonucleases and the separation of the DNA fragments by gel electrophoresis. The bands are then transferred to a nylon membrane and hybridized with labelled probes that bind only to specific fragments. The resulting simplified banding pattern (Fig. 1.7) facilitates the differentiation bacterial strains (Weller, 2000; Trindade *et al.*, 2003). A modified approach is ribotyping, which is based on the presence of one or more of rRNA operons on the chromosomes of all bacterial isolates. Ribotyping uses a labelled rRNA probe to reveal the restriction fragment pattern of DNA encoding rRNA operons. Although this technique is reproducible, its use for epidemiological studies declined when it was shown to have only moderate discriminatory power (Trindade *et al.*, 2003; Tenover *et al.*, 1997). Despite this limitation, this technique remains useful for preliminary screening of large numbers of isolates (Trindade *et al.*, 2003).
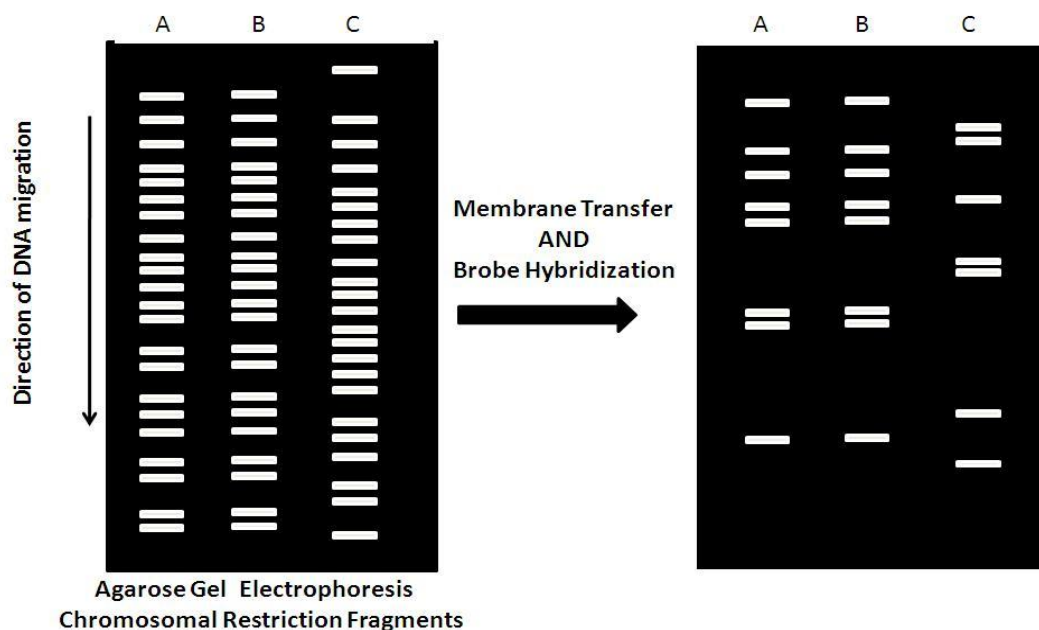
Figure 1.7: Southern hybridisation. The image on the left indicates the very large number of DNA fragments produced by restriction endonuclease. DNA fragments are blotted and the hybridized with specific nucleic probes. In the left hand image, tracks A, B and C represent independent organisms, A and B are of the same type whereas C is a different type (Tenover *et al.,* 1997).

1.4.3b. iii Pulsed-field gel electrophoresis (PFGE)

Bacterial chromosomes are generally between 2000-5000kb in length. The resulting patterns of digestion of the chromosomes can most easily be interpreted when using restriction endonucleases which generate a relatively few number of large fragments *i.e.* 10-30 fragments of between 10-800kb in length. While small DNA fragments can be separated by conventional electrophoresis this technique shows poor discriminatory power for large fragments (Weller, 2000). PFGE was introduced in 1984 by Schwarz and Cantor (1984), who used it to resolve large DNA fragments from eukaryotic organisms. This technique was subsequently adapted for use as a high resolution molecular typing system to study different bacterial species.

In conventional electrophoresis the DNA fragments migrate in a straight line from the cathode to the anode. Because large molecules adopt a hairpin-like configuration, increasing the size of a fragment has little influence on its mobility. In contrast, the PFGE apparatus consists of sets of electrodes through which the charge can be directed in a short time pulses to force the DNA molecules to re-orientate through the gel. Consequently, larger DNA fragments now migrate according to their size (Fig. 1.8) and individual fragments become clearly distinguishable (Trindade *et al.*, 2003; Tenover *et al.*, 1997; Weller, 2000).

The PFGE patterns of isolates are derived from genetic events that either change the sizes of the fragments between the restriction sites or result in the loss or gain of a restriction site. For example, a single genetic event such as a point mutation, or the insertion or deletion of DNA, and which leads to the loss or gain of restriction site, can result in difference to one to three restriction fragment while two genetic events can lead to differences in two to five restriction fragments (Stepan *et al*., 2004; Singh *et al*., 2006). Therefore, PFGE is highly discriminatory for indentifying the relatedness of isolates from short time studies (e.g. outbreak within a hospital), but it is not suitable for long-term studies of global epidemiology, which require highly discriminatory technique that are able to monitor genetic changes that accumulates slowly (Enright *et al*., 2002).

In theory, all bacterial species are typeable using PFGE despite difficulties in extracting their DNA (*e.g. C. difficile)* (Tenover *et al.*, 1997). Extra precautions are

needed during the extraction of the chromosomal DNA to ensure that it remains largely intact. Consequently the bacterial cells are embedded in low-melting point agarose during the DNA extraction steps in order to avoid the risk of mechanical breakage (Trindade *et al.*, 2003). Many studies have been performed to establish a standardised PFGE protocol. These studies have achieved success with regard to speed, reproducibility and the cost (Murchan *et al.*, 2003).

PFGE has been used for the study of MRSA and has been compared extensively with other methods. A range of restriction endonucleases has been tested but none have shown better results than *Sma*I (Trindade *et al.*, 2003; Weller, 2000). In a comparative study between PFGE and PCR-based methods, Stranden *et al.* (2003) reported that while PCR-based methods could not replace PFGE they could complement them. PFGE is therefore considered the gold standard for the typing of MRSA since it exhibits high discriminatory power and reproducibility. There are, however, some disadvantages that have impeded the wider use of PFGE: procedures are time-consuming and results are not available for several days. PFGE is also relatively costly. Despite these disadvantages, PFGE has been used extensively to investigate outbreaks of pathogens (Weller, 2000)
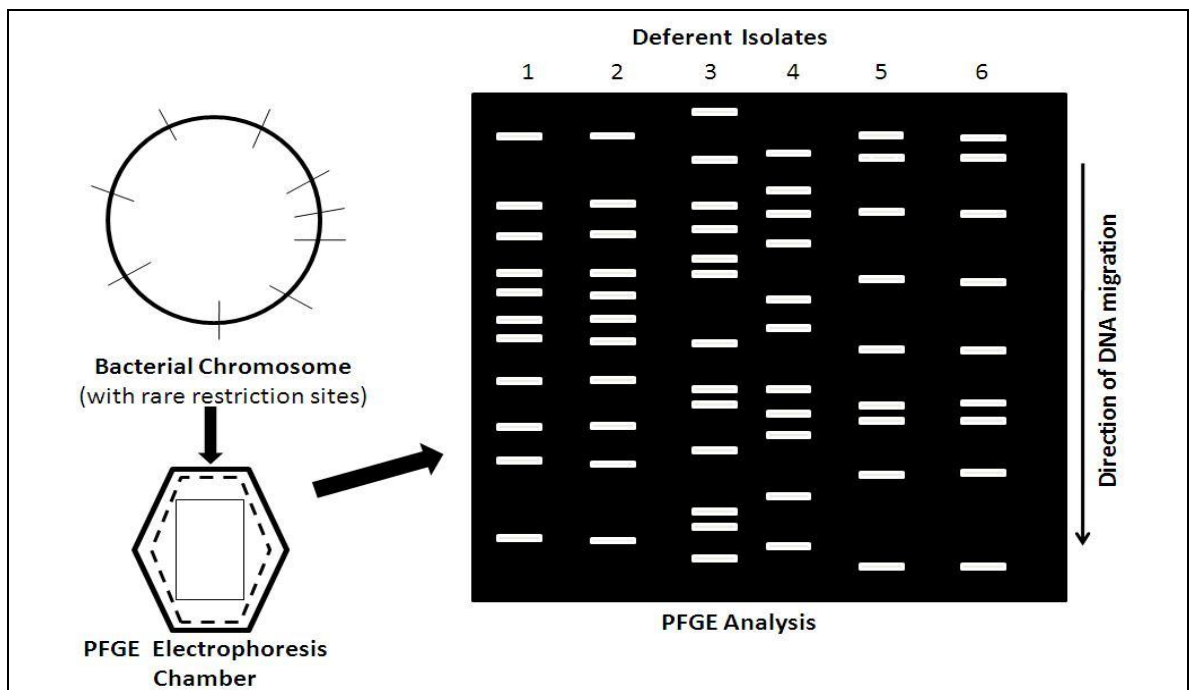
Figure 1.8: PFGE technique. DNA is digested with an infrequent cutting restriction enzyme and the resulting large fragment separated by PFGE. The image on the right shows a PFGE gel of strains showing different chromosomal DNA fragment profiles (Tenover *et al.*, 1997).

1.4.3c Third-phase molecular typing: PCR- based technique

The time-consuming and expensive nature of PFGE has necessitated the development of a variety of rapid and less expensive typing method. PCR was developed in the mid-80s and become one of the most important biological discoveries in the twentieth century. The PCR technique is based on the amplification of a specific region the target DNA (Weller, 2000; van Leeuwen, 2003).

The technique depends on cyclical repetition of three main steps: (i) the denaturation phase which take place at temperatures around 95˚C; (ii) the annealing phase in which the primer pairs bind to specific regions in the resulting ssDNA - a temperature of between 50˚C and 65˚C is used, as determined by the composition of the primers; (iii) the temperature is raised to ~70˚C for DNA synthesis. The *Taq* polymerase requires the presence of dNTPs and Mg^{2+}. In order to amplify the DNA template sufficiently for detection by ethidium bromide staining, 20 − 40 cycles are required: 30 cycles generated about 1 billion copies of the target which is optimal for the thermostable DNA polymerases used in the PCR (Tenover *et al.*, 1997; Trindade *et al.*, 2003; Harwood and Wipat, 2000).

PCR has been used for many years for the detection of pathogens in clinical samples but has more recently been adopted as a molecular typing tool. There are four main groups of PCR-based techniques that are used to differentiate among the bacterial isolates. They include arbitrarily primed PCR (AP-PCR), PCR-restriction fragment length polymorphism (PCR-RFLP), repetitive element sequence-based PCR (rep-PCR) and PCR-ribotyping. However, PCR-RFLP and PCR-ribotyping methods are no longer used for MRSA typing (Tenover *et al.*, 1997; Trindade *et al.*, 2003).

1.4.3c.i Arbitrarily primed PCR (AP-PCR)

AP-PCR is also referred to as random amplified polymorphic DNA (RAPD). This technique was introduced by Williams *et al*. (1990) and by Welsh and McClelland (1990) for the analysis of chromosomal DNA using small randomly generated primers (about 10 bases). As a result one or more DNA sequences will be amplified and be separated by gel electrophoresis. The number and size of the resulting products are used for the typing of the isolates. AP-PCR is a rapid and relatively straightforward technique. It has a good reproducibility but its discriminatory power is variable,

depending on the number of primers used and the nucleotide sequence of the target DNA (Tenover *et al.*, 1997; Trindade *et al.*, 2003; Witte *et al*, 2006). Although, AP-PCR has been successfully applied in the investigation of the outbreaks, its discriminatory power is not as good as that of PFGE. AP-PCR has been used for the typing of the MRSA strains and has shown the poor reproducibility. In addition, there is still not a standard guideline as to how to interpret the results of AP-PCR (Tenover *et al.*, 1997; Weller, 2000).

1.4.3c.ii Binary typing (BT) methods

The binary typing method is a DNA-based typing technique that is considered as alternative to MLST but depends on large genetic diversities in DNA content instead of allelic profiles. BT is a hybridization typing method that targets specific DNA sequences using a set of probes. The determination of a set of candidate probes is the most important step in the technique to ensure maximum discriminatory power (Srinivasan *et al.,* 2007). In case of *S. aureus,* van Leeuwen and his co-workers have developed a binary typing method, based on 15 DNA probes that target random amplified polymorphic DNA (RAPD). In their study, binary typing was found to be a reproducible and its discriminatory was as similar to PFGE (van Leeuwen *et al.*, 1996; van Leeuwen *et al.*, 1999). However, the typeability of binary typing was lower than PFGE in the epidemiological study of Zadoks *et al.* (2002) on bovine and human *S. aureus* isolates. They also reported that, while binary typing can used in combination with PFGE, it could not yet replace it (Zadoks *et al*., 2002).

1.4.3c.iii Repetitive element sequence-based PCR (rep-PCR)

All bacterial chromosomes have repetitive elements sequences. These elements, which are scattered throughout the chromosome, have been elucidated in detailed studies involving repetitive palindromic (REP) sequences (van Leeuwen, 2003; Stepan *et al.*, 2004). These sequences are conserved with most bacteria and can be targeted, *via* PCR, for epidemiological studies (Trindade *et al.*, 2003). This technique has been used to differentiate between various bacterial species including *S. aureus*. For typing of MRSA, rep-PCR has been used with a variety of the target sequences, such as Rep MP3, InterIS256 and *Tn*916. Although, there are not repetitive intergenic consensus primers for typing *S. aureus*, one hundred and seventy MRSA strains were studied using primer RW3A, derived from the RepMP3 sequence of *Mycoplasma* (Del Vecchio *et al.*,

1995). The results show stability with high reproducibility, even following frequent subculturing. Some intergenic repetitive regions in the coagulase (*coa*) and protein A (*spa*) genes have been proposed to improve the performance of this technique with *S. aureus* (Trindade *et al.*, 2003; Witte *et al.*, 2006). In general, Rep-PCR has good discriminatory power and its reproducibility is good compared with other methods such as RAPD and PFGE. However, the disadvantage of rep-PCR is that the DNA requires an additional purification step that increases the time taken to perform the procedure (van der Zee *et al.*, 1999; Weller, 2000; Trindade *et al.*, 2003).

1.4.3c.iv Multiple locus Variable Number Tandem repeat analysis (MLVA)

The genome of *S. aureus* has several VNTR loci and the majority of those repeats are associated with cell surface-bound proteins MSCRAMMs. VNTRs are highly variable in both the number of repeat units and by heterogeneity of the sequences amongst those units (Moser *et al.*, 2009). This variability in VNTRs might be due to slipped-strand mispairing (SSM) events that occur during DNA replication (van Belkum, 2008). This feature has been exploited to develop typing method for *S. aureus.* Initially, VNTR was based on single locus such as protein A (*spa*) typing. VNTR has been developed as a typing method, but lacks discriminatory power (Ikawaty *et al.*, 2008). The first MLVA for *S. aureus* typing was introduced by Sabat *et al.* (2003), targeting the *sdr*, *ssp*, *clfA*, *clfB* and *spa* loci using multiplex PCR. In this study, they reported that MLVA is a simple and reproducible method and its discriminatory power was comparable with other typing method including PFGE. However, the number of strains tested in this study was small (34 strains) and they concluded that MLVA may need further evaluation on large number of *S. aureus* strains. In a comparison study conducted by Malachowa and his co-workers, the discriminatory power of MVLA was lower but comparable with PFGE and they also suggested additional validation against PFGE (Malachowa *et al.*, 2005). Francois *et al*. (2005) have developed an alternative version of MLVA which included the additional loci of *finBP*, *cna* and *mecA*. Although, MVLA was rapid and showed adequate reproducibility, it was not able to distinguish HA-MRSA lineages (USA100, 200 and 500) from CA-MRSA lineages (USA300, 400, 1000 and 1100) (Tenover *et al.*, 2007). Nevertheless, evaluation of MLVA has shown it to have high discriminatory power and adequate stability. It therefore appears to be very useful for the epidemiological investigation of *S. aureus* outbreaks. In addition, as a multiplex PCR-based typing method, MVLA is easy to perform and cost effective,

meaning that it is suitable for use in clinical laboratories (Malachowa *et al.*, 2005; Luczak-Kadlubowska *et al.*, 2008). Although, MVLA could distinguish between some strains with identical PFGE profiles, further analysis is needed to ensure that MVLA is suitable for epidemiological analyses (Moser *et al.,* 2009).

1.4.3c.v PCR-restriction fragment length polymorphism (PCR-RFLP)

The products of PCR can be digested at specific sites by restriction endonucleases then separated using gel electrophoresis. The coagulase (*coa*) gene shows a high level of disparity when digested with *Alu*I. The discriminatory power of this method is moderate but can be improved by increasing the number of restriction endonucleases. Its results are similar to that obtained for MLEE, so it is useful for studying populations of microorganisms (van Leeuwen, 2003). Many MRSA strains are typeable by this technique with reproducible results even after extensive subculturing. Nevertheless, unrelated MSSA strains can exhibit the same coagulase gene type. The reproducibility of this technique is low comparing with that of PFGE (Weller, 2000). Another target for PCR-RFLP is the protein A gene (*spa*) which can be divided into three fragments using *Rsa*I (Frenay *et al*., 1994). This typing system has two major drawbacks; its reproducibility is low and the results cannot be compared directly with those obtained by other methods (Hoefnagels-Schuermans *et al*., 1997; Weller, 2000). However, Montesinos *et al*. (2002) have pointed out that typing techniques based on *coa* and *spa* genes can provide initial epidemiological information when investigating an outbreak.

1.4.3d.iv SCC*mec* typing

SCC*mec* elements carry the *mecA* gene that encodes the determinant of ß-lactam resistance. SCC*mec* elements are highly diverse in their structures and genetic composition and therefore have been classified into several types and subtypes. This technique can be carried out using either uniplex or multiplex PCRs. The classification of SCC*mec* elements by the uniplex PCR method is mainly based on combinations of primers to distinguish the allotypes of the *ccr* and *mec* gene complexes, as well as the J region (Ito *et al.*, 2001; Okuma *et al.*, 2002). This method has a number of limitations including its low discriminatory power and the number of individual PCR reactions required. Consequently, a multiplex PCR of SCC*mec* has been developed for a more discriminatory and rapid identification, using SCC*mec* components other than the *mec* and *ccr* gene complex. SCC*mec* multiplex PCR typing was introduced by Oliveira and Lencastre in 2002 when initially four SCC*mec* types (I-IV) were identified. In this

study, eight loci (A through H) within *mec* element sequences (as a control) were targeted on the basis of published SCC*mec* element sequences (Table 1.4) (Oliveira and Lencastre, 2002; Hanssen and Sollid, 2006). Ito and colleagues have developed an alternative multiplex PCR method which targeted mainly the *mec* and *ccr* gene complex (Table 1.4) (Ito *et al.*, 2001). However, the Oliveira and Lencastre technique lacks resolution in the identification of SCC*mec* type IV variants and a novel SCC*mec* type V (5C) when it omitted the core components (the *mec* and *ccr* gene complex) of SCC*mec* (Milheirico *et al.*, 2007). In addition, both methods characterized the SCC*mec* type in the same strain differently (Deurenberg *et al.*, 2007). In order to utilize real-time PCR for SCC*mec* typing, Francois *et al.* developed an assay to distinguish SCC*mec* types I-IV based on the *mec* and *ccr* gene complexes (Francois *et al.*, 2004; Deurenberg *et al.*, 2007). The RT-PCR method is rapid but does not identify any SCC*mec* subtypes. Similarly, a multiplex PCR designed to identify SCC*mec* types I – V, introduced by Boye *et al.* (2007), was unable to discriminate SCC*mec* type IV variants.

More recently, a new nomenclature for SCC*mec* types has been proposed by Chongtrakool *et al.* (2006) in an attempt to resolve the confusion in the field of SCC*mec* typing and to facilitate the identification and classification of new SCC*mec* types. This nomenclature was based on structural elements of SCC*mec*, particularly, the sequences of the *mec* gene complex (referred by letter) and *ccr* genes (referred by number) (e.g. SCC*mec* type 4B means it is defined as a class B *mec* complex and allotype 4 *ccrAB*). The application of this nomenclature is as follows: SCC*mec* type 1A (type I), type 2A (type II), type 3A (type III), type 2B (type IV) and type 5C (type V) (Chongtrakool *et al.*, 2006; Oliveira *et al.*, 2006).

The International Working group on the Classification of Staphylococcal Cassette Chromosome Element (IWG-SCC) has redefined some SCC*mec* subtypes and classified SCC*mec* into eight types I-VIII with two subtypes of types IV and V (Fig 1.4). This classification has been established according to the criteria in Table 1.3 (IWG-SCC, 2009). SCC*mec* types I, II, III and VIII carry antibiotic resistant genes other than *mec*A and are commonly found in HA-MRSA whereas other types tend not to harbour additional antibiotic resistant determents and are most common in CA-MRSA (Grundmann *et al.*, 2006; IWG-SSC, 2009).

It is worth noting that SCC*mec* typing is used in combination with MLST (ST) to study the epidemiology of MRSA clones. Nowadays, SCC*mec* typing is considered one of the most important epidemiological tools for typing MRSA strains.

Table 1.4: The major methods of SCC*mec* typing (adapted from Deurenberg *et al.*(2007) and IWG-SCC(2009)).

	Method of Ito *et al.,*		Method of Oliveira *et al.,*	IWG-SCC	
SCC*mec* types	*mec* gene complex	*ccr* gene genes	**Loci**	*mec* gene complex	*ccr* gene genes
I	B	A1/B1	A, D	B	1(A1B1)
II	A	A2/B2	B, C, D	A	2(A2B2)
III	A	A3/B3	C, E, F	A	3(A3B3)
IV	B	A2/B2	D	B	2(A2B2)
V	C	C	E	C2	5(C)
VI	-	-	-	B	4(A4B4)
VII	-	-	-	C1	5(C)
VIII	-	-	-	A	4(A4B4)

1.4.3d Forth-phase molecular typing: Sequence typing

1.4.3d.i Multilocus sequence typing (MLST)

MLST is a relatively new approach to identifying and monitoring the spread of antibiotics resistant pathogens (Enright and Spratt, 1999). This technique, which is derived from multilocus enzyme electrophoresis (MLEE), was first introduced by Maiden *et al.* (1998) to resolve certain limitations of MLEE analysis. MLST is an excellent technique for investigating MRSA clonality. MLST is based on the analysis of seven ~500-bp sequences derived from housekeeping genes. The sequences represent distinct alleles and contain sufficient information to define the isolate using an online data search tool. The MLST website, http://www.mlst.net, contains information on 21 bacterial species, including *S. aureus*. The distinct sequences of each locus are given allelic numbers, for example, a Iberian clone of MRSA has the MLST profile "3-3-1-12-4-4-16" which categorises it as sequence type ST247. Recently the nomenclature system has been expanded to include the SCC*mec* type (*e.g* MRSA-I) and the Iberian clone is now categorised as (ST247-MRSA-I) (Robinson and Enright, 2004; Deurenberg *et al.*, 2007). MLST can also be used to establish the evolutionary relationship of MRSA strains using the Electronic based upon related sequence types

(eBURST) program that groups closely related STs into co-called clonal complexes (CC).

The first application of MLST to *S. aureus* was published in 2000 by Enright *et al*. who analysed the target DNA sequences of 155 strains. The housekeeping genes used in this study were: carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol kinase (*glpF*), gulanylate kinase (*gmk*), phosphate actyltransferase (*pta*), triosephoshate isomerise (*tpi*) and acetyl coenzyme A acetyltransferase (*yqiL*). In their study, Enright and colleagues clearly demonstrated the value of MLST with respect to the analysis of MRSA and MSSA, generating results that were comparable to PFGE (Enright *et al.*, 2000). These seven housekeeping genes are thought to change slowly over time. They are also widely distributed around the genome, which decreases the impact of recent recombination events (Short and Enright, 2007). Therefore, MLST is a very useful technique for the study of evolutionary and population biology of bacterial pathogens but, is less discriminatory for the analysis of outbreaks (Cooper and Feil, 2004). Indeed, MLST provides unambiguous information and is very useful for studying the population structures of MRSA (Enright *et al.*, 2002). However, MLST technology is costly and requires specialist equipment. It is therefore not used extensively for routine epidemiological studies (Trindade *et al.*, 2003).

1.4.3d.ii Staphylococcal protein A (*spa*) sequence typing

Protein A is an important virulence factor of *S. aureus*. It has five repeating analogous α-helical domains (A, B, C, D, and E) which involve in binding the Fc portion of immunoglobulin IgG. The gene encoding Protein A (*spa*) has a hypervariable region (polymorphic X region or short sequences repeats (SSR)) that consists of 24bp repeats (variations from 21bp and 27bp have been identified) and located upstream of the region encoding the cell wall attachment sequences. This high degree of repeats is attributed to duplications, deletions and, rarely, to point mutations. The sequence of these tandem repeats in the X region has been used as alternative typing method to those currently in use. The *spa* typing approach was first introduced in 1996 as a single-locus sequence typing method by Frenay and his colleagues. Its discriminatory power lies between MLST and PFGE and has the advantages of being rapid, easy to use and interpret and has an established database (Frenay *et al.*, 1996; Shopsin *et al.*, 1999; Moodley *et al.*, 2006; Deurenberg *et al.*, 2007). Another advantage is the existence of a freely available software package for interpreting the resulting sequences. Although, the

Ridom StaphType software (Ridom GmbH, Wurzburg, Germany) is commonly used to analyse the *spa* sequences, many laboratories use the central online *spa* server (http://www.spaserver.ridom.de). This database has contributed in the widespread use of *spa* typing. To date, the *spa* database includes about 6167 *spa* types that are composed of a combination of 355 repeats from 108309 strains. Harmsen *et al.* (2003) and Koreen *et al.* (2004) have both introduced *spa* nomenclature systems. However, differences between these nomenclatures make comparisons between published *spa* data difficult (Deurenberg *et al.*, 2007). Although, *spa* typing has many advantages and is widely used as a single-locus typing technique, it is susceptible to chromosomal replacements that can falsely cluster genetically unrelated strains. Moreover, the cost of sequencing is still considered as a limitation of using *spa* typing extensively in routine clinical laboratories (Karynski *et al.*, 2008).

1.5. The study aims

This study focuses on the evaluation of existing molecular epidemiological tools to determine limitations that hinder their use as routine tools for studying the epidemiology of MRSA isolates in hospitals laboratories.

Ultimately, this study aimed to develop a novel tool to monitor the spread of MRSA that has the same or similar discriminatory power as PFGE but which can be performed in a few hours in a routine clinical laboratory.

Chapter 2

General Methods and Materials

2. General Methods and Materials
2.1 Chemicals and reagents

The chemicals and reagents that were used in this study are listed in Appendix A with their suppliers.

2.2 Preparation of buffers, media and solutions

Growth media, buffers and solutions are described in detail in Appendix A

2.3 Growth, storage of strains

2.3.1 Sources of standard staphylococci strains

The standard *S. aureus* strains and two *S. epidermidis* strains used in these studies were obtained from a variety of sources. The strains and their sources are listed in Table 2.1.

Table 2.1: Reference staphylococcal strains which were used in this study.

Strain	Description	Source
NCTC8325	Laboratory strain	NCTC
EMRSA-15(HO50960412)	UK Epidemic MRSA-15	Prof. Mark Enright
JH9	HA-VISA, USA	Prof. Herminia de Lencastre
USA300-FPR3757	CA-MRSA	NARSA
Mu50	HA-VISA, Japan	NARSA
Mu3	HA-VISA, Japan	NARSA
N315	HA-MRSA, Japan	NARSA
MRSA252	Typical UK EMRSA-16	NARSA
MSSA476	CA-MSSA, UK	NARSA
COL	Early MRSA, UK	NARSA
MW2	Typical USA CA-MRSA	NARSA
USA1000	CA-MRSA, USA	NARSA
USA1100	CA-MRSA, USA	NARSA
CA-629	CA-MRSA, USA	NARSA
S. epidermidis RP62A	CNS	NARSA
S. epidermidis ATCC12228	CNS	Prof. Jan Maarten van Dijl
NCTC6571	MSSA	Dr. John Perry
MRSA (585296) PVL +	PVL positive	Dr. John Perry
MRSA (602025) PVL-	PVL negative	Dr. John Perry

NARSA=Network on antimicrobial resistance in *Staphylococcus aureus.* CNS= Coagulase negative staphylococci.

2.3.2 Sources of clinical MRSA isolates

Newcastle upon Tyne Hospitals NHS Foundation Trust's Freeman Hospital is one of the largest NHS hospitals in the north-east of England. It has 699 inpatient beds. In addition to its own clinical samples, it receives clinical samples from General Practitioners (GPs) and from other hospitals in the Trust. It has research laboratories in addition to clinical laboratories.

2.3.3 Storage of MRSA isolates

Growth of clinical and screening samples for identification was as follows: samples were cultured on selective media followed by routine clinical identification. Confirmed MRSA strains were grown on fresh blood agar and strains were transferred to 20% of glycerol and maintained at -80 ºC for long-term storage. Each MRSA strain was allocated a unique identifier (e.g. FMRSA1 refers to the first MRSA received from the Freeman hospital; see Section 2.4). Standard strains were received on agar slopes and were subcultured on BHI agar followed by BHI broth culture and maintained in 20% glycerol at -80˚C till required.

2.3.4 Isolates data

A total of 406 MRSA isolates were collected from the Freeman Hospital during the period from 23rd of October 2006 to 25th November 2006. These isolates were isolated from both clinical and screening samples. Clinical information about the isolates was recorded in a database – patients were coded to facilitate cross-reference between samples, but no patient names were supplied.

2.4 Identification of MRSA isolates using principle approaches

The clinical samples were cultured on ATM blood agar or nalidixic acid blood agar, while screening samples were cultured on MRSA ID medium. All plates were incubated at 37˚C for 48h with the exception of nalidixic acid blood agar that was incubated anaerobically for 48h.

Green colonies on MRSA ID (Fig. 2.2) were considered to be diagnostic for MRSA and were confirmed by Slidex Staph Plus (SSP) latex reagent (bioMerieux UK). Colonies on blood agar that resembled staphylococci (Fig. 2.1) were also examined with the SSP latex test. Finally, the results were confirmed by an antibiotic susceptibility test

(Perry *et al.*, 2003). The MRSA isolates were subcultured on blood agar in order to obtain fresh pure cultures. All isolates were maintained as in Section 2.3.3 until required.
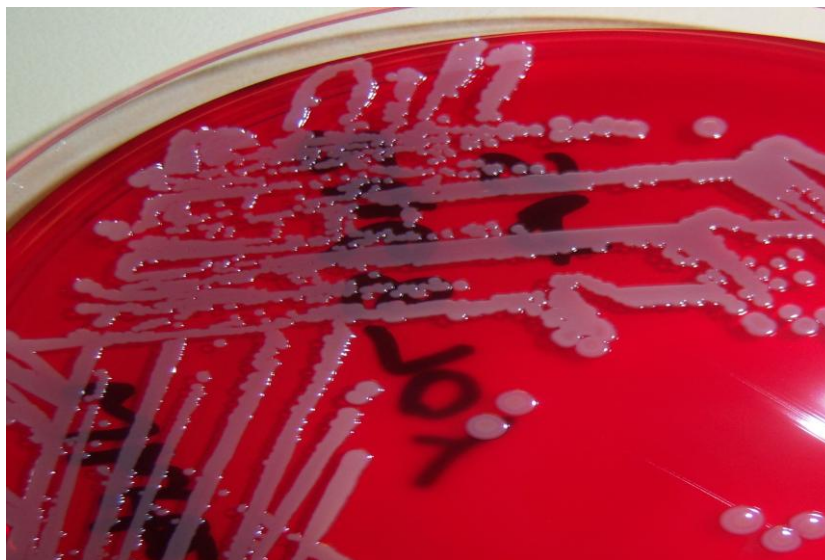
Figure 2.1: Colonies of a clinical *S. aureus* isolate on blood agar.

2.4.1 MRSA ID medium

MRSA ID (bioMerieux, La Balme Les Grottes, France) is a new chromogenic agar medium that has been developed to select and identify MRSA isolates. This medium contains a chromogenic enzyme substrate and cefoxitin. It is highly selective against non-staphylococci such as enterococci. MRSA isolates grow on this medium with green colonies due to their production of α-glucosidase (Fig. 2.2). MRSA ID has been evaluated by Perry and colleagues and shown to perform better than other chromogenic agar media (Perry *et al*., 2004).

Figure 2.2: MRSA growing with green colonies on MRSA ID medium.

2.4.2 Slidex Staph Plus (SSP) latex reagent

The Slidex Staph Plus (SSP**;** bioMerieux, La Balme Les Grottes, France) test is based on the detection of the *S. aureus*-specific clumping factor, Protein A. A single colony of the test isolate was mixed with a drop of latex reagent on slide. The test is positive if agglutination is observed (Fig. 2.3).
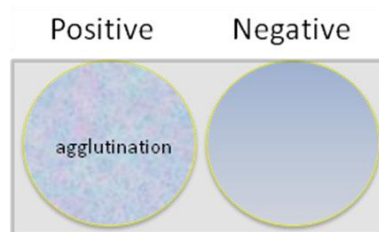
Figure 2.3: Slidex Staph Plus (SSP) latex agglutination test.

2.4.3 Disc diffusion antibiotic susceptibility test

A single colony of the test isolate was mixed thoroughly with 2ml sterile water and the suspension adjusted to 0.5 against a McFarland standard. 200µl of suspension were transferred to 2ml sterile water and the suspension spread on Columbia medium using a sterile cotton-wool swab. Antibiotic discs were placed on the dried inoculated plate that was incubated at 35-37ºC for 18-20h. MRSA isolates should be resistant to cefoxitin with an inhibition zone of diameter ≤21mm (Andrews *et al.*, 2007).

2.5 Molecular diagnostic methods

2.5.1 Bacterial growth

Bacterial isolates stored at -80˚C were thawed and 20µl cultured on brain-heart infusion (BHI) agar or nutrient agar (Appendix A) overnight at 37˚C. The following day a single colony was suspended in 10ml BHI broth and incubated overnight at 37˚C, with shaking at 200 rpm.

2.5.2 Extraction of chromosomal DNA from MRSA

2.5.2a Extraction of the DNA by lysostaphin treatment using the DNeasy kit (QIAGEN).

Six millilitres of overnight culture was transferred to four 1.5ml microcentrifuge tubes and centrifuged for 3 min at 13000*g*. The supernatants were discarded and the

pellets resuspended in 180µl lysis buffer (Appendix A) lacking lysozyme but with 10µl lysostaphin [1mg/ml]. The suspension was incubated at 37ºC for 30 min and then 25µl of proteinase K and 200 µl AL buffer were added and the mixture incubated at 56ºC for 30 min. 200 µl of ethanol (100%) was added and mixed thoroughly. The mixture was transferred into a DNeasy Mini spin column placed in a 2ml collection tube and centrifuged for 1 min at 8000g. The collection tube with flow-through was removed and replaced with a new collection tube. 500 µl of AW1buffer was added and the tube centrifuged for 1 min at 8000g and the flow-through discarded. 500 µl of AW2 buffer was added and centrifuged for 2 min at 13,000g and the flow-through again discarded. Finally, the DNeasy Mini spin column was placed in a 1.5ml microcentrifuge tube and 200µl of AE buffer was added onto DNeasy membrane, incubated 1 min at room temperature and centrifuged for 1 min at 8,000g. This step was repeated in a new 1.5ml microcentrifuge tube and the DNA stored at -20ºC.

2.5.2b Extraction of the chromosomal DNA by cell mechanical breakage using a DNeasy kit (QIAGEN)

Six millilitres of culture grown overnight in 10ml of BHI broth at 37ºC were transferred into four 1.5ml microcentrifuge tubes and centrifuged for 3 min at 13000g. The supernatants were discarded and the pellets combined by resuspending in 550µl lysis buffer lacking lysozyme. 500µl of glass beads was added in a 2ml screw cap tube and the mixture was shaken in a Mikro dismembrator (Braun, Germany) for 5 min at 2600rpm. After shaking the mixture was centrifuged for 10 min at 13000g and 180µl of supernatant transferred to 1.5ml microcentrifuge tube. 25µl of proteinase K, and 200 µl AL buffer were added to the supernatant and incubated at 56ºC for 30 min, Finally, 200 µl of ethanol (100%) was added and mixed thoroughly. The mixture was transferred to a DNeasy Mini spin column placed in a 2ml collection tube and centrifuged for 1 min at 8000g. Then final steps in Section 2.5.2a were followed to yield the DNA.

2.5.2c Rapid Extraction of chromosomal DNA using the boiling method

A colony from an overnight culture on BHI agar was transferred to 20µl of sterile milli-Q water in a microcentrifuge tube and mixed thoroughly. The cell suspension was boiled at 100ºC for 10 minutes then centrifuged at 13,000g for 5 min. 5 µl of the resulting supernatant was used in a diagnostic PCR reaction.

2.5.3 Polymerase Chain Reaction (PCR)

In order to amplify regions of the DNA the PCR technique was used. Oligonucleotide primers for amplifying specific MRSA genes were designed according to the following parameters: G/C between 50-60%, the 3'-end had a G or C and more three adjacent nucleotide repeats (*e.g.* AAAA or TTTT) were avoided. Oligonucleotide primers were obtained from MWG Biotech GmbH. Lyophilised primers were rehydrated by addition of sterile milli-Q water to a concentration of 100μM and stored at -20ºC.

PCR reactions were performed as indicated in Table 2.2, and according to the reaction conditions in Table 2.3. The sequences of primers used in this study are listed in Table 2.3

Table 2.2 : Components of PCR reaction mixture.

Components	Volume per 50 µl reaction (µl)
Milli-Q Water	25
DNA polymerase buffer (10x)	5
Forward primer (20mM)	5
Reverse primer (20mM)	5
dNTP (10mM)	1
$MgCl_2$ (25mM)	3
Template DNA (~200ng/ µl)	5
Taq polymerase (5U/µl)	1

Table 2.3: PCR reaction conditions

Genes / steps	*mecA*			*coa*			*PVL*		
	ºC	min	cycles	ºC	min	cycles	ºC	min	cycles
Denaturing	95	5	1	95	3	1	95	10	1
Denaturing	94	0.5		94	0.41		95	0.16	
Annealing	55	0.5	40	52	0.66	30	52	0.08	35
Extension	72	1		72	1		72	0.3	
Final Extension	72	5	1	72	5	1	72	5	1

ºC = temperature. min = time (minute)

2.5.3a Detection of the *mecA* methicillin-resistance gene

EMRSA-15 was used as a *mecA* positive control and *S. aureus* NCTC 6571 as a *mecA* negative control. The primer pairs designed by Murakami *et al.* (1991) were used

to amplify a 532-bp region of *mecA*. The PCR reaction was performed as in Table 2.2 according to reaction condition in Table 2.3

2.5.3b Detection of coagulase (*coa*) gene

The presence of the *coa* gene was determined using the primers of Walker *et al.* (1998). These primers were used to amplify a 830bp region of the *coa* gene according to the conditions in Tables 2.2 and 2.3. DNA from *S. aureus* NCTC 6571 was used as a *coa* positive control and *S. epidermidis* was used as a *coa* negative control.

2.5.3c Detection of the Panton-Valentin leukocidin (*lukS-PV*) gene

De novo primers were designed to target a 443bp region of the *lukS-PV* gene. The sequences of this gene were obtained from the National Centre for Biotechnology Information (NCBI) database. The PCR reaction was performed as in Table 2.2 according to reaction conditions in Table 2.3. PVL+ and PVL- MRSA strains were used as controls.

Table 2.4: Sequences of primers used in this study

Primer	Sequence (5'- 3')	Reference
mecA-for	AAAATCGATGGTAAAGGTTGGC	Murakami *et al.*, 1991
mecA-rev	AGTTCTGCAGTACCGGATTTGC	
coa-for	GAACAAAGCGGCCCATCATTA	Walker *et al.,* 1998
coa-rev	TAAGAAATATGCTCCGATTGTCG	
lukS-PV -for	GCTGCAACATTGTCGTTAGG	This study
lukS-PV -rev	CCATTACCTCCTGTTGATGG	
arcC- for	TTGATTCACCAGCGCGTATTGTC	Enright *et al.*, 2000
arcC- rev	AGGTATCTGCTTCAATCAGCG	
aroE- for	ATCGGAAATCCTATTTCACATTC	
aroE- rev	GGTGTTGTATTAATAACGATATC	
glpF- for	CTAGGAACTGCAATCTTAATCC	
glpF- rev	TGGTAAAATCGCATGTCCAATTC	
gmk- for	ATCGTTTTATCGGGACCATC	
gmk- rev	TCATTAACTACAACGTAATCGTA	
pta- for	GTTAAAATCGTATTACCTGAAGG	
pta- rev	GACCCTTTTGTTGAAAAGCTTAA	
tpi- for	TCGTTCATTCTGAACGTCGTGAA	
tpi- rev	TTTGCACCTTCTAACAATTGTAC	
yqiL- for	CAGCATACAGGACACCTATTGGC	
yqiL- rev	CGTTGAGGAATCGATACTGGAAC	

carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol kinase (*glpF*), gulanylate kinase (*gmk*), phosphate actyltransferase (*pta*), triosephoshate isomerise (*tpi*) and acetyl coenzyme A acetyltransferase (*yqiL*).

2.5.4 Agarose Gel Electrophoresis

About 5 – 10 µl of PCR product was combined with loading buffer and loaded on 0.8-1.5% agarose gel containing ethidium bromide (0.5µg/ml). Electrophoresis was carried out in 1xTBE buffer (Appendix A) at 100V for 30 to 45 min. PCR products were sized against molecular size markers (100bp-1kb) (Appendix B), their bands visualized under UV-light and documented using a gel documentation system.

2.6 Molecular typing of MRSA isolates

2.6.1 Pulsed field gel electrophoresis (PFGE)

2.6.1a Preparation of agarose-embedded bacterial DNA

Chromosomal DNA was prepared in agarose plugs using the CHEF Genomic DNA Plug Kits (Bio-Rad) according to the manufacturer's instructions excepting for the addition of lysostaphin prior to mixing the cells with low melting agarose. 2% of clean-cut agarose (Bio-Rad) was prepared and was equilibrated at 50ºC. An overnight culture in 10 ml LB was diluted and grown to an OD_{600} of between 0.8 and 1.0. Three millilitres of culture were transferred into two 1.5ml microcentrifuge tubes and centrifuged for 3 min at 13000g. The pellets were resuspended in 1ml TN buffer (Appendix A) and the suspension centrifuged at 13,000g for 3 min. The pellets were resuspended in 300µl cell suspension buffer, equilibrated at 50ºC, and 4µl of lysostaphin (1mg/ml in 100mM NaCl pH 4.5) added immediately then 300µl of 2% of clean-cut agarose. The cell suspension was mixed gently and the mixture was transferred to a CHEF plug moulds (100µl the agarose/cell suspension per mould) and were left to solidify. Solid agarose plugs were incubated for 2 h at 37ºC in 2.5ml lysis buffer containing 100µl lysozyme. The lysis buffer was removed and the plugs were rinsed with sterile deionised water. 2ml of proteinase K buffer containing 100µl proteinase K was added and incubated overnight at 50ºC. The plugs were washed four times (1 h each time) in wash buffer (1ml for each plug) at room temperature with gentle agitation. In the second wash, 60 µl of 100mM phenylmethanesulfonyl fluoride (1mM PMSF) (Appendix A) was added to the wash buffer/plugs. The plugs were stored in 1x wash buffer at 4ºC for up to six months.

2.6.1b Digestion of bacterial DNA for PFGE analysis

Prior to digestion, a single plug was washed in 1ml of 0.1x wash buffer for 1h, followed by 1 ml of 1X restriction endonuclease buffer 4 (New England Biolabs) for 1h with gentle agitation. The plug was cut in half and slices transferred to 300µl of fresh restriction buffer containing 3µl (50-60U) of the restriction endonuclease *Sma*I (CCC↓GGG) (New England BioLab). The slices were incubated overnight at 25ºC. After digestion, the slices were placed in 1ml 1x wash buffer for 30 min and were equilibrated in 0.5x TBE buffer. The plugs were melted at 65ºC for 10 minutes in a water bath and were inserted by pipette into the wells of a 21 x 14 cm 1% agarose gel (0.5x TBE buffer) for pulse-field electrophoresis.

2.6.1c Pulse-field gel electrophoresis analysis

Restriction fragments of chromosomal DNA were separated in a 1% agarose gel using a Contour-Clamped homogeneous electric field (CHEF)-DRII apparatus (Bio-Rad), at field strength of 200V for 24h with initial and final switching times of 5s and 60s respectively. The buffer temperature was kept under 14ºC. A lambda ladder (Bio-Rad) and strain NCTC8325 (Appendix B) were used as molecular size markers. After electrophoresis, the gel was stained in ethidium bromide (1µg/µl) for 30-45 min and distained in distilled water for 2 hours. The gel was photographed under UV light.

2.6.1d Data analysis of PFGE

Gel images were saved as TIFF files for analysis by fingerprinting software (BioNumerics v3.50, Applied Maths). The analysis of PFGE patterns was based on band differences and a clustering dendrogram was generated by the unweighted-pair group method with arithmetic mean (UPGMA) using the Dice similarity coefficient.

2.6.2 Multilocus sequence typing (MLST)

2.6.2a PCR amplification with MLST primers

Seven primer pairs in Table 2.4 were used to amplify internal fragments (about 500bp) of seven housekeeping genes according to Enright *et al.* (2000). The targeted genes were: carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol kinase (*glpF*), guanylate kinase (*gmk*), phosphate actyltransferase (*pta*), triosephoshate isomerise (*tpi*) and acetyl coenzyme A acetyltransferase (*yqiL*). The PCRs were performed as described as in Table 2.2 using the following reaction conditions: initial denaturation at 95ºC for 5min followed by 30 cycles of denaturation at 95ºC for 1 min,

annealing at 55ºC for 1min, extension at 72ºC for 1 min with a final extension step at 72ºC for 5 min. 5 μl of PCR reaction was run on 1% agarose gel to confirm the presence of the PCR products.

2.6.2b Purification of PCR products of MLST

2.6.2b.i Purification of PCR products using the PEG method

The PCR products were purified as described by Enright *et al.* (2000) as following: 40μl of PCR products were precipitated with 40 μl of 20% (w/v) polyethylene glycol (PEG6000) in 2.5M NaCl (Appendix A) and recovered by centrifuging for 15 min at 13,000g. The precipitate was resuspended in cold 70% ethanol and centrifuged for 10 min at 13000g. The ethanol was carefully removed and the precipitate resuspended in 10μl of 1xTE buffer (Appendix A). The PCR products were stored at -20ºC until required for sequencing.

2.6.2b.ii Purification of PCR products using QIAquick PCR purification kit

Five volumes of PB buffer were added to one volume of PCR reaction and mixed thoroughly. The entire mixture was transferred to a QIAquick spin column placed in 2ml collection tube and centrifuged for 1 min at 13,000g. The flow-through was discarded and QIAquick spin column was placed back in the same collection tube. About 0.75ml of PE wash buffer was added to the QIAquick column and centrifuged for 1 min at 13,000g. The flow-through was discarded and QIAquick column was placed back in the same collection tube. The column was centrifuged once more to remove residual ethanol. The QIAquick column was placed in a clean 1.5ml microcentrifuge tube and 30μl of elution buffer EB or ddH$_2$O (pH 7.0-8.5) was added to the centre of QIAquick column membrane and centrifuged for 1 min at 13,000g. The PCR products were stored at -20ºC until required for sequencing.

2.6.2c Sequencing and analysis of MLST data

The concentration of DNA was adjusted (see 2.6.3) and the products were sent for sequencing (MWG Biotech, Germany). Allelic profiles and sequence type (ST) were identified using the MLST database through website (http://www.mlst.net).

2.6.3 Quantification of DNA

The concentration of DNA was determined using the NanoDrop (ND-1000) system (NanoDrop Technologies). 1μl of DNA sample was pipette directly onto the

measurement surface and the 260/280 and 260/230 nm ration was calculated to evaluate the DNA purity.

2.6.4 Identification of SCC*mec* types using multiplex PCR

Multiplex PCR was carried out to determine the SCC*mec* types according to Boye *et al.* (2007) using a set of four primer pairs (Table 2.5). The multiplex PCR was performed according to the following reaction conditions: initial denaturation at 94ºC for 4 min followed by 30 cycles of 30 sec at 94ºC, 30 sec at 55ºC and 60 sec at 72ºC with final extension at 72ºC for 4 min. About 8 µl of multiplex PCR products was loaded on 1.5% agarose gel containing ethidium bromide (0.5µg/ml) and was electrophoresed in 1xTBE buffer (Appendix A) at 90V for 60 to 80 min.

Table 2. 5: Primers used in the multiplex SCC*mec* PCR and the resulting gel band patterns of SCC*mec* types I–V (Boye *et al*., 2007).

Name	Primer sequence (5'→3')	Length (bp)	I	II	III	IV	V
ccrA2-B-for	ATTGCCTTGATAATAGCCTTCT	937		X		X	
ccrA2-B-rev	TAAAGGCATCAATGCACAAACACT						
ccrC-for	CGTCTATTACAAGATGTTAAGGATAAT	518			X		X
ccrC-rev	CCTTTATAGACTGGATTATTCAAAATAT						
IS1272-for	GCCACTCATAACATATGGAA	415	X			X	
IS1272-rev	CATCCGAGTGAAACCCAAA						
mecA-IS431-for	TATACCAAACCCGACAACTAC	359					X
mecA-IS431-rev	CGGCTACAGTGATAACATCC						

Chapter 3

Analysis of clinical MRSA isolates using existing molecular typing methods

3. Analysis of clinical MRSA isolates using existing molecular typing methods

3.1 Introduction

Since the emergence of penicillin-resistant *S. aureus* strains in the 1940s (Kirby, 1944) and the subsequent emergence of methicillin-resistant *S. aureus* (MRSA) in 1961 (Jevons, 1961), MRSA has become the main concern for both patients and health care professionals. The ability of these bacteria to resist many available antibiotics, including the drug of last resort, vancomycin, has led some to predict a return to the pre-antibiotic era in relation to the treatment of infections. Since morbidity and mortality rates associated with MRSA infection have increased, high priority has been given to the control of MRSA in many healthcare settings. The National Nosocomial Infections Surveillance (NNIS) System of the Centers for Disease Control and Prevention (CDC), reports that approximately 80,000 patients/year worldwide acquire MRSA infections (Aires de Sousa and Lencastre, 2004). In the UK, mortality associated with MRSA has increased dramatically from 51 reported cases in 1993 to 1, 230 in 2008, although this in part represents improved reporting procedures.

A good understanding of the epidemiology of MRSA is the cornerstone of the control and subsequently elimination of infection. During the last century, microbiologists have introduced many epidemiological tools to differentiate between *S. aureus* strains. Initially, those tools relied on phenotypic features including phage typing and serotyping. In the last two decades, however, with the advent of advanced molecular typing methods such as PFGE, MLST and SCC*mec* typing, the epidemiological field has witnessed a rapid development (Weller, 2000; Ito *et al.*, 2001).

PFGE profiling basically relies on the digestion of whole chromosomal DNA with restriction endonucleases (*Sma*I in case of MRSA) that generate a relatively few number of large fragments. Because the generated fragments are large in size they can only be separated by pulsed-field gel electrophoresis (Schwartz and Cantor, 1984). PFGE has been used extensively for the typing of many bacterial species and it is now described as a gold standard technique for typing MRSA (Schmitz *et al.*, 1998) since it shows high discriminatory power and reproducibility. In recent years, PFGE has been supported by more rapid methods, such as multi-locus sequence typing (MLST), a sequence-based technique which provides unambiguous results. MLST relies on single

nucleotide variations (each variant is called an allele and is represented by a unique identifier) in seven housekeeping genes of *S. aureus* (Enright *et al.*, 2000).

However, there are limitations that restrict the routine use of PFGE and MLST in many hospitals' laboratories: both methods are time-consuming, expensive, require specific expertise and specialist equipment (Abele-Horn *et al.*, 2006; Sabat *et al*, 2006). In addition, the reproducibility of PFGE between laboratories is sometimes challenging (van Belkum *et al.*, 1998) and the ability of MLST to discriminate outbreak isolates is limited (Hardy *et al.*, 2006).

3.2 Aims

The aims of this part of the study were:

1. Initially, to use established genotyping typing tools to study the epidemiological characteristics of MRSA isolates isolated in local hospitals (Newcastle upon Tyne).

2. Ultimately, to evaluate the most popular molecular typing methods and determine the limitations that restrict their routine use in hospital clinical microbiology laboratories.

3.3 Results

3.3.1 Analysis of MRSA data

3.3.1a MRSA patients

MRSA strains were isolated from 286 patients throughout the period of collection. 176 (61.5%) patients were male and 110 (38.5%) female. 219 (76.5%) patients were from the Freeman Hospital while the remaining 67 patients (23.5%) were from other hospitals or primary care centres in Newcastle upon Tyne.

3.3.1a.i Patient age groups

The patient ages ranged from 11 to 100 years with a mean of patient age of 68 years. 44.5% of patients were older than 75 years while only 5.6% were under 30 years (Fig. 3.1)
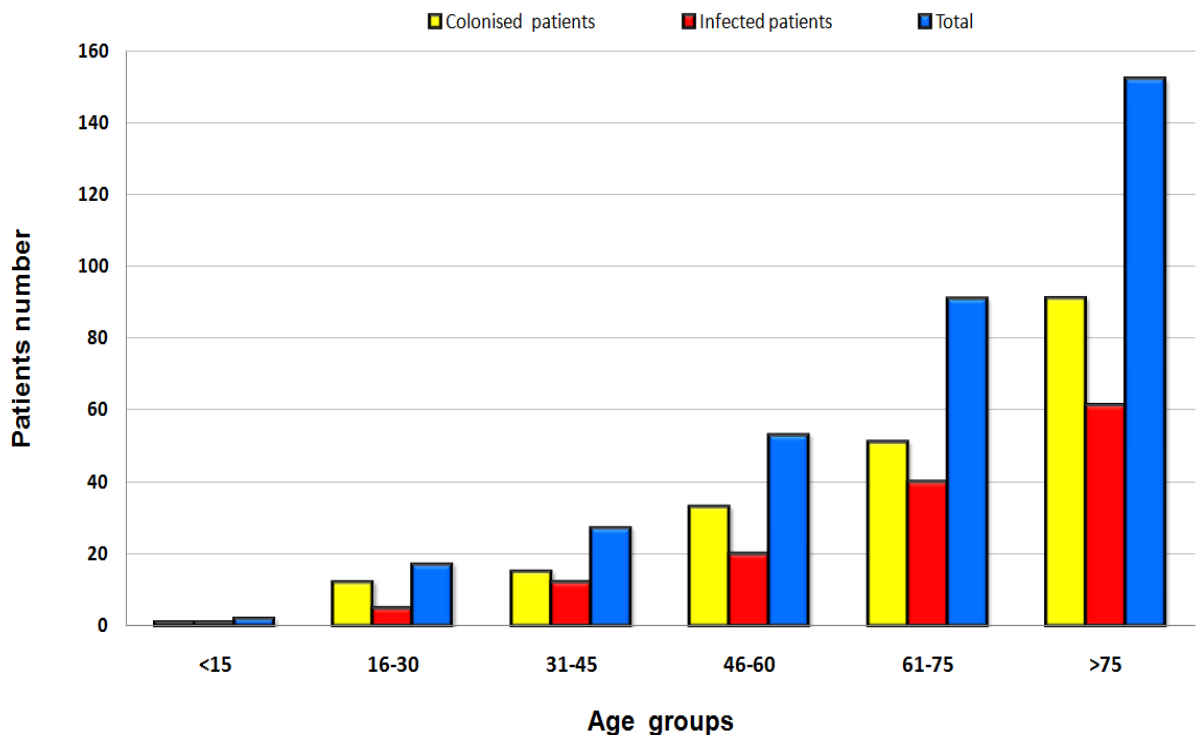
Figure 3.1: The age distribution of MRSA patients.

3.3.1a.ii Infected and colonized patients

The isolation of MRSA from colonised sites (nose, throat and perineum) of patients with no signs of infection were considered to be "colonized patients" while the isolation

of MRSA from wound, blood, urine and respiratory tract were considered to be "infected patients".

In this study, 57.7% of patients were colonised by MRSA isolates without symptoms while 42.3% were infected. Seventy percent of colonised patients were older than 60 years whereas only 7% under 30 years. Similarly, 73% of infected patients were older than 60 years while only 4% were under 30 years. These data confirm the susceptibility of older individuals to both infection and colonisation, as observed previously by others (Morgan *et al.*, 1999, 2000; Hori *et al.*, 2002; Gemmell *et al.,* 2006).

3.3.1a.iii The distribution of patients between hospital units

Fifty-nine percent of MRSA patients were in general medical (24%), urology (23%) and surgical (12%) units whereas only 1% were in orthopaedics and musculoskeletal units (Fig. 3.2). 84% of urology and 73.5% of general medical patients were colonised whereas the percentages of infected patients in these units were 16% and 26.5%, respectively. In critical care and surgical units 70% and 54% of patients, respectively, were infected (Fig. 3.2).
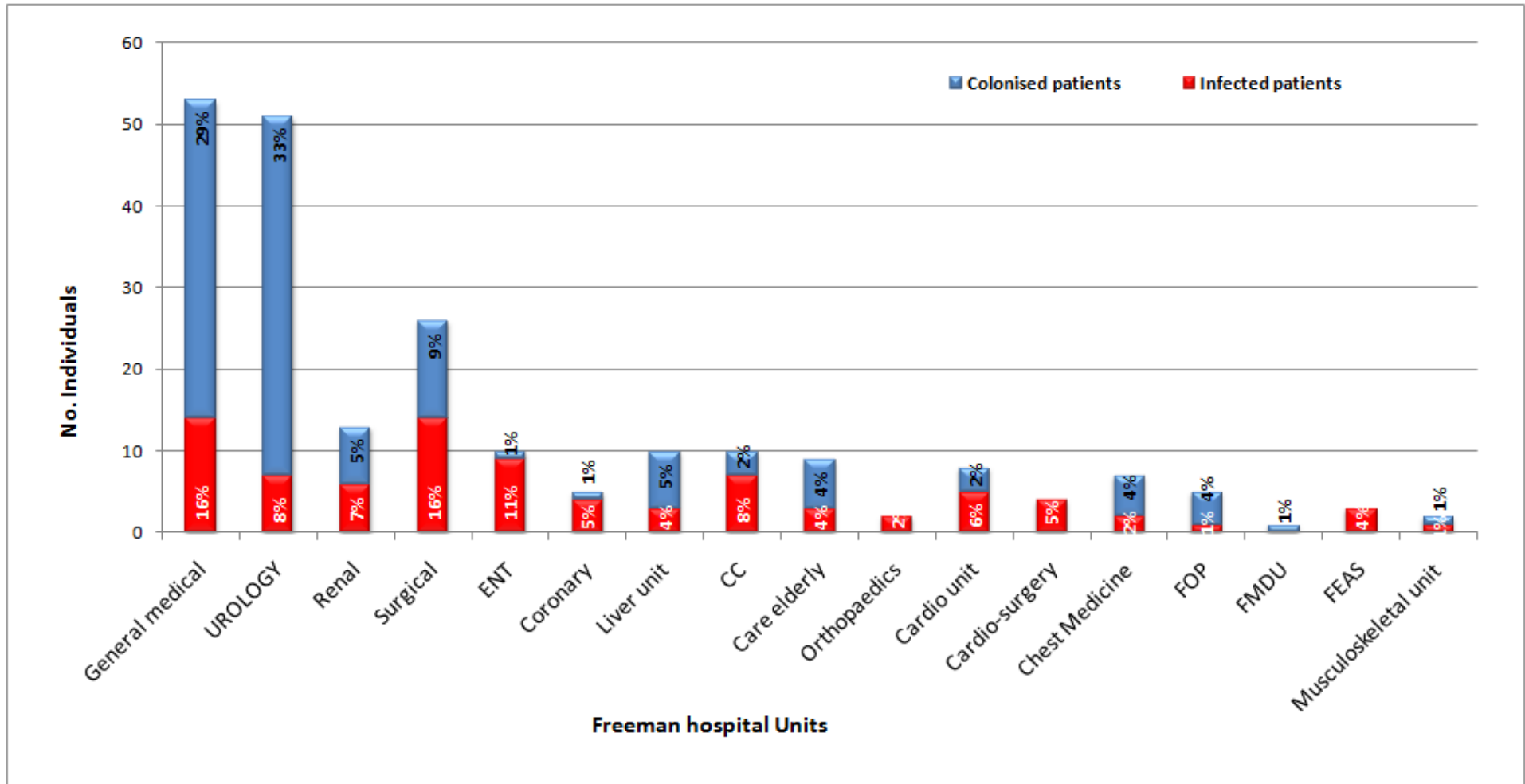
Figure 3.2: **The distribution of infected and colonised patients in units of the Freeman Hospital**. ENT: Ear, Nose & Throat Unit. CC: critical care unit. FOP: Freeman out patient. FMDU: Freeman Melville day unit. FEAS: Freeman admission suite.

3.3.1b MRSA isolates

Four hundred and six MRSA strains were isolated from clinical and screening samples. 325 (80%) strains were isolated from patients at the Freeman Hospital whilst 81 (20%) strains were isolated from patients at other hospitals or primary healthcare centre in Newcastle upon Tyne. 267 (66%) of MRSA strains were isolated from screening samples (nose, throat and perineum) while the remaining 139 (34%) were from clinical samples: 27% from wounds, 3% from blood, 3% from respiratory tract and 1% from urine samples (Fig. 3.3a). In the case of the MRSA strains isolated from patients at the Freeman Hospital, 21% were from the general medical unit, 20% from urology and 13% from surgery. Only 1% of strains were isolated from the orthopaedics and musculoskeletal units (Fig. 3.3b).
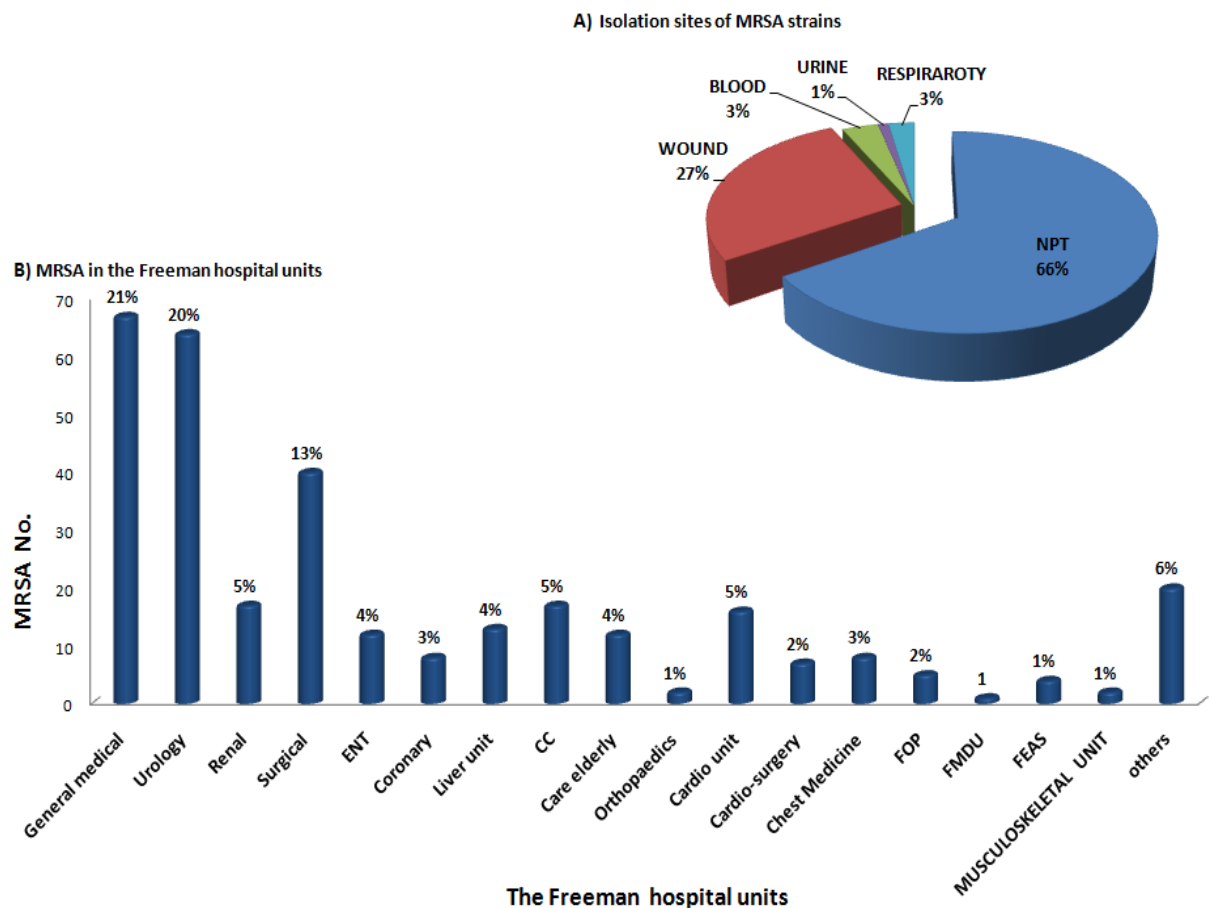
Figure 3.3:   A) The isolation sites of MRSA isolates. B) Distribution of MRSA isolates in the Freeman Hospital units. NPT: nose, perineum and throat. ENT: Ear, Nose & Throat Unit. CC: critical care unit. FOP: Freeman out patient. FMDU: Freeman Melville day unit. FEAS: Freeman admission suite.

3.3.2 Molecular diagnostic methods.

3.3.2a Extraction of chromosomal DNA for PCR analysis.

Two protocols were evaluated for the extraction of chromosomal DNA from MRSA isolates – mechanical disruption and enzymatic digestion. As shown in Figure 3.4, the quantity of DNA extracted by mechanical disruption with the Mikro dismembrator was significantly better than that extracted following digestion with lysostaphin. The concentration of DNA in the Mikro dismembrator method was between 200 to 400 ng/µl whereas the concentration was between 60 to 190 ng/µl in case of the digestion with lysostaphin. Consequently, mechanical disruption was used to extract DNA from the twenty test isolates used for the evaluation for the PCR and MLST analyses.

For diagnostic PCR, the extraction of DNA by the rapid boiling method was tested and showed excellent results. This method was applied to all isolates examined (n=76) for the diagnostic PCR detection of the gene encoding the PVL virulence factor.
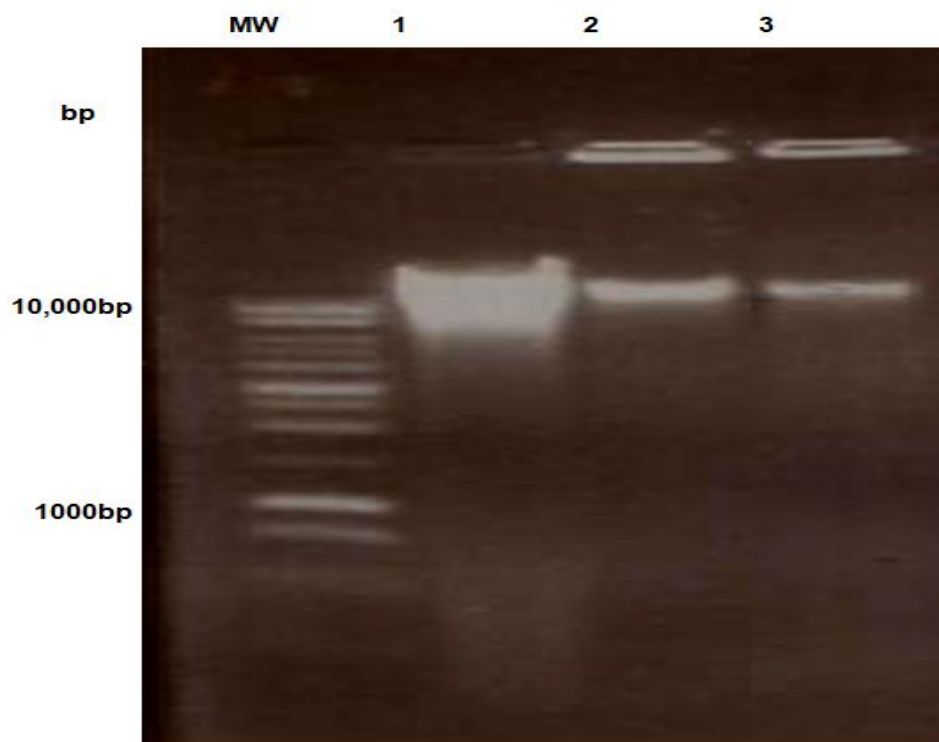
Figure 3.4:   Extraction of DNA from MRSA isolates using various protocols. Lane 1: Mikro dismembrators. Lanes 2 & 3: lysostaphin treatment. MW: 1kb DNA molecular size ladder.

3.3.2b Identification of MRSA isolates by PCR

All MRSA isolates were initially identified at the Freeman Hospital using conventional bacteriological approaches including MRSA ID media, Slidex Staph Plus (SSP) and an antibiotic susceptibility test. Thereafter, *mecA* and *coa* genes were used as targets of PCR in order to confirm the identity of MRSA isolates using various primer pairs. PCR was also used to determine the presence of genes encoding the staphylococcal virulence factor PVL.

3.3.2b.i Detection of the *mecA* gene.

PCR was used to amplify a 533bp fragment of the *mecA* gene from twenty MRSA isolates and an amplicon of the expected size was amplified from each of the isolates. The results of twelve representatives of 20 isolates are shown in Figure 3.5. The test strains were isolated from non-infected patients during routine screening (nose, throat and perineum) with the exception of MRSA3 and MRSA13, which were isolated from the wounds of patients. 70% of these patients were older than 75 years (Table 3.1).
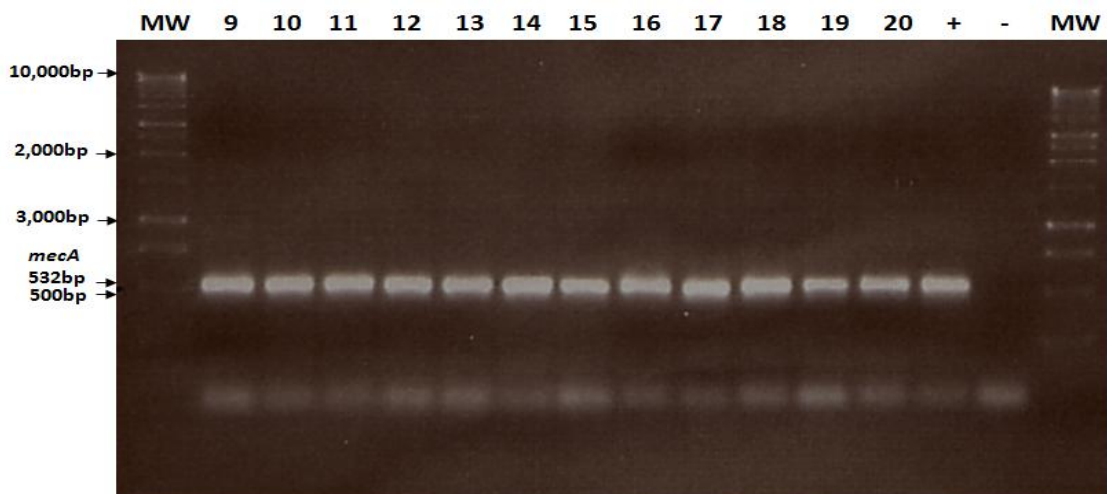
Figure 3.5:   PCR products of the *mecA* gene for MRSA from isolates 9 to 20, (+) = MRSA (585296); (-) = MSSA NCTC6571. MW =1kb DNA molecular size marker.

3.3.2b.ii Detection of *coa* gene

The same twenty MRSA isolates as above (Section 3.3.2b.i) were analysed for the presence of a 830bp fragment amplified from the *coa* gene. All of the isolates tested positive and Figure 3.6 shows twelve representative isolates. Interestingly, MRSA12 produced a shorter PCR product, which might be due to deletion in targeted region of *coa* (Figure 3.6, Table3.1).
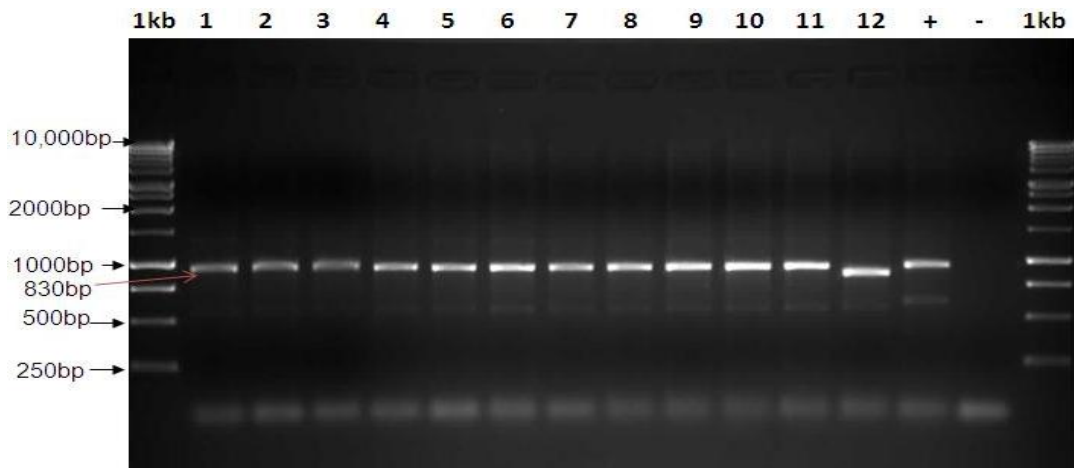
Figure 3.6:   PCR products of *coa* gene for MRSA isolates from 1 to 12. (+) = MSSA NCTC6571; (-) = *S. epidermidis* 1kb: DNA molecular size ladder.

3.3.2c Detection of the gene encoding the PVL virulence factor in MRSA isolates

Seventy-six MRSA isolates were examined for the presence of the gene encoding PVL. The primers targeted a 443bp fragment of *lukS* gene. 90% of the tested strains were isolated from screening samples whereas only 10% were from wounds. None of these isolates were PVL-positive (Fig. 3.7).
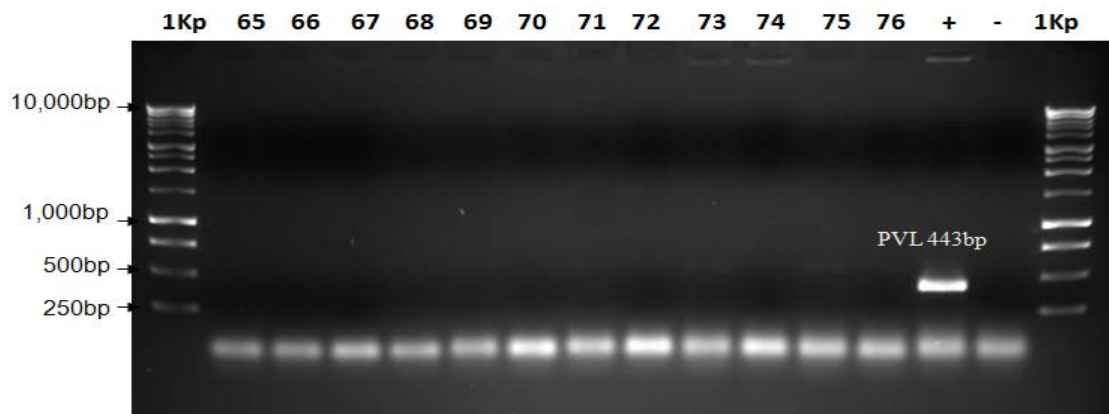
Figure 3.7:   PCR of *PVL* gene for isolates from 65 to 76. (+) = MRSA (585296) PVL (-) = MRSA (602025) PVL-. 1kb: DNA molecular size marker.

Table 3.1:   Isolation sites and PCR results of the first twenty MRSA isolates

Isolates	age of patient	sites of isolation	*coa* gene	*mecA* gene	*PVL* gene
FMRSA 1	85	NPT	+	+	-
FMRSA 2	83	NPT	+	+	-
FMRSA 3	72	Wound	+	+	-
FMRSA 4	25	NPT	+	+	-
FMRSA 5	93	NPT	+	+	-
FMRSA 6	78	NPT	+	+	-
FMRSA 7	100	NPT	+	+	-
FMRSA 8	82	NPT	+	+	-
FMRSA 9	87	NPT	+	+	-
FMRSA 10	60	NPT	+	+	-
FMRSA 11	82	NPT	+	+	-
FMRSA 12	84	NPT	+	+	-
FMRSA 13	51	Wound	+	+	-
FMRSA 14	80	NPT	+	+	-
FMRSA 15	96	NPT	+	+	-
FMRSA16	84	NPT	+	+	-
FMRSA 17	92	NPT	+	+	-
FMRSA 18	31	NPT	+	+	-
FMRSA 19	73	NPT	+	+	-
FMRSA 20	85	NPT	+	+	-

FMRSA= Freeman Hospital MRSA isolate.   + = Positive   - =Negative   NPT = (nose, throat or perineum).

3.3.3 Molecular typing of MRSA isolates

3.3.3a Pulsed-field gel electrophoresis (PFGE).

PFGE has been described as the gold standard for MRSA typing. This technique requires considerable expertise and some considerable effort was spent to optimize this technique. DNA was extracted into agarose plugs to reduce mechanical shearing of the

chromosomal DNA. *Sma*I endonuclease was used to digest the chromosomal DNA since this generated in the region of 20 fragments. Plugs were prepared for twenty MRSA isolates as indicated above (2.6.1). Initially, the DNA was digested with 20U of *Sma*I according to Wang *et al.* (2002). However, as shown in Figure 3.8, the DNA was incompletely digested and there was evidence of smearing.
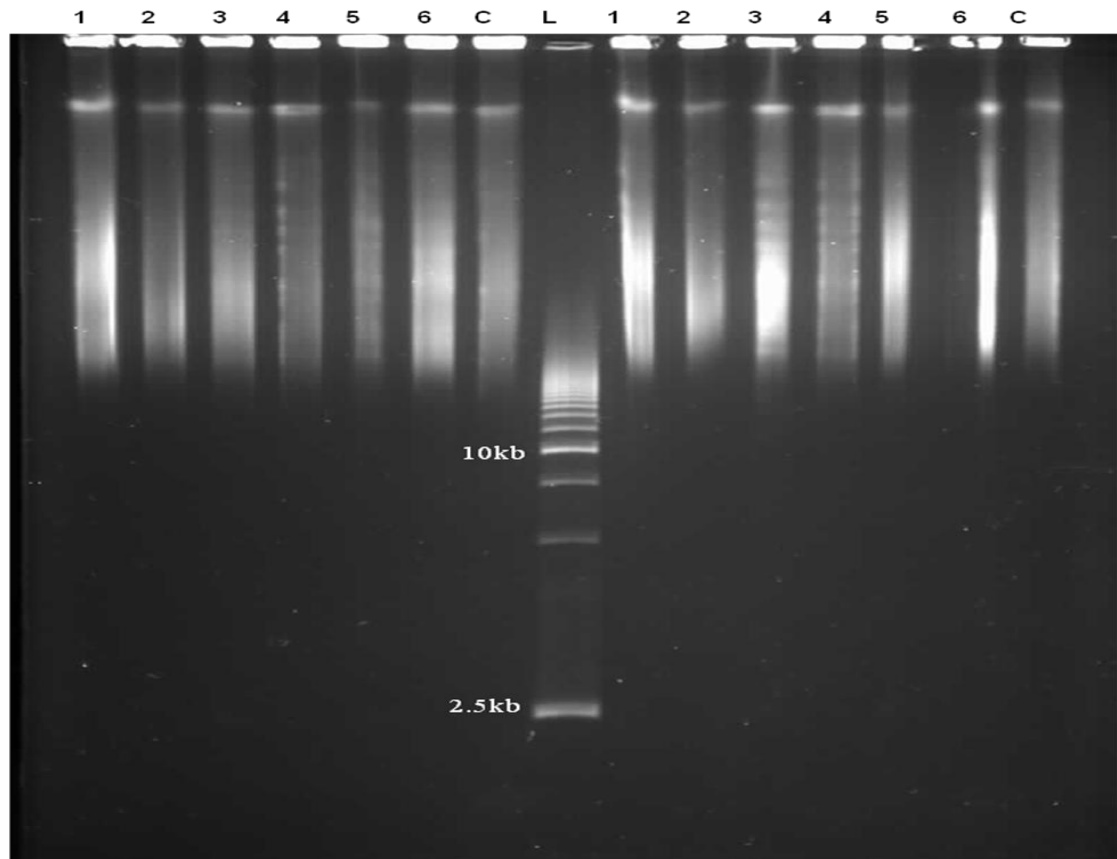
Figure 3.8:   The plugs of six MRSA isolates were digested with 20U of *Sma*I. Lanes 1 to 6: duplicate MRSA isolates. C: *S. aureus* NCTC657. L: 2.5kb molecular size marker**.**

Two plugs of a MRSA isolate were digested with different concentrations of *Sma*I (50U and 60U) and were separated under similar conditions excepting that the ramping time varied from 5s - 60s (Fig. 3.9). Sixty units of *Sma*I showed the best performance.
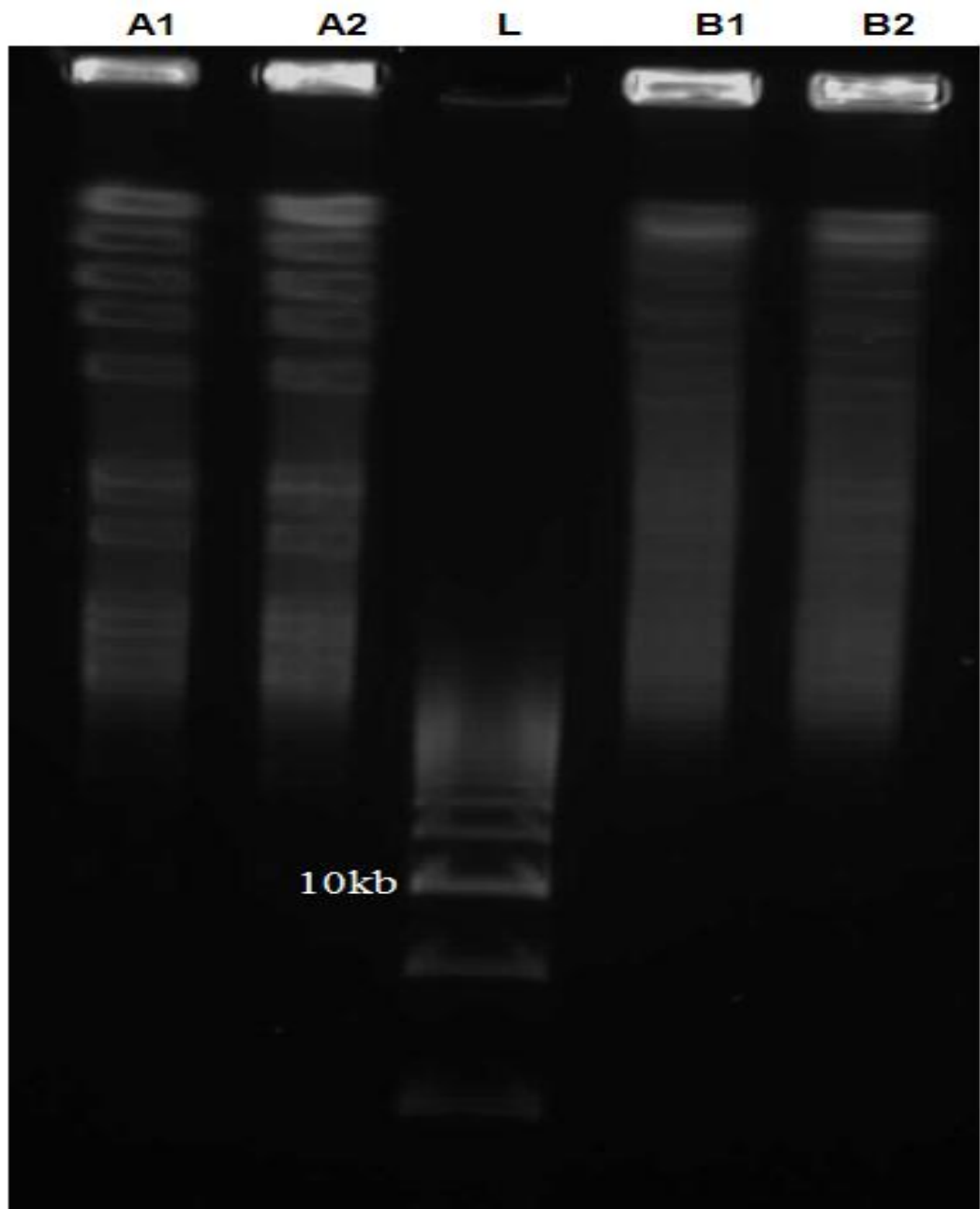
Figure 3.9:   Digestion of MRSA plugs with two concentrations of *Sma*I endonuclease. Samples A1 and A2 were digested with 60U, while samples B1 and B2 were digested with 50U. L: 2.5kb Molecular size marker.

The DNA plugs of twenty isolates were digested with 60U *Sma*I. The number of fragments produced by *Sma*I was between 10-15, with lengths varying from approximately 3kb to 700kb. These fragments were clearly distinguishable and comparable (Fig. 3.10).
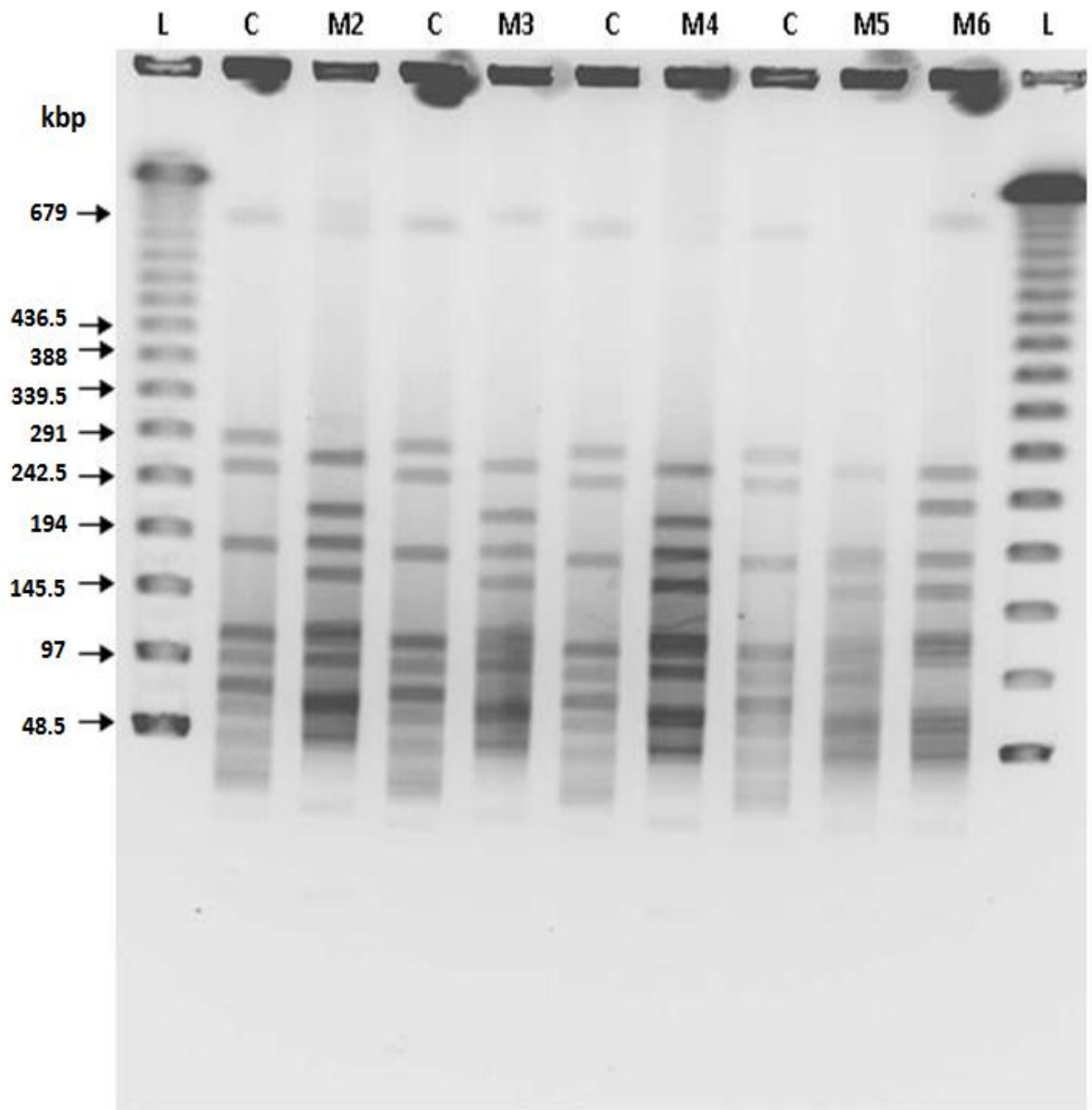
Figure 3.10:   PFGE types of *Sma*I restriction digest of MRSA isolates. C = *S. aureus* NCTC 6571 (non-MRSA). M2= FMRSA2.   M3= FMRSA3 etc.   L= Molecular size marker (lambda ladder).

The PFGE restriction fragments were interpreted according to Wang *et al.* (2002), as follows: i) strains that were identical with respect to the size and number of bands generated were deemed as the same type and were ascribed an alphabetical letter (*i.e.* A, B, C etc). ii) Strains which have up to three different bands were considered a subtype were given different numbers, (*i.e.* A2, A3 A4 etc), according to the number of different bands. iii) Strains with more than three different bands were deemed to be of a distinct type and given other letter.

The UPGMA dendrogram (Fig. 3.11) shows the *Sma*I-PFGE patterns and the genetic relationship of 20 MRSA isolates. It is clear that most of these MRSA isolates are closely related and only three PFGE types (A, B and C) were identified. Type A was predominate (n=18; 90%) while the remaining PFGE types (B, C) were only represented by one isolate each. Within Type A, eight subtypes (A1- A8) were found. Type A profiles had high similarity (more than 85%) to EMRSA-15 while Types B and C showed 80% similarity to EMRSA-15. Fifty percent of Type A isolates were isolated from the urology and general medical units at the Freeman Hospital while the remaining isolates were isolated from a variety of units at the Freeman Hospital and local healthcare centres (Table 3.3). The Type B isolate was isolated from elderly patient (87 years) in the geriatric care unit at the Freeman Hospital, while the Type C isolate was isolated from an elderly patient (96 years) at the Walkergate Hospital in Newcastle.

Strain ID	PFGE	MLST	SCCmec
NCTC6571	6571	-	MSSA
NCTC8325	8325	ST8	MSSA
FMRSA12	A3	ND	IV
FMRSA16	A1	ND	IV
FMRSA10	A1	ND	IV
FMRSA13	A1	ND	IV
FMRSA2	A1	ST22	IV
FMRSA4	A1	ND	IV
FMRSA1	A2	ST22	IV
FMRSA11	A2	ND	IV
FMRSA17	A2	ND	IV
FMRSA18	A2	ND	IV
FMRSA3	A2	ND	IV
FMRSA8	A2	ND	IV
FMRSA20	A4	ND	IV
FMRSA5	A5	ST22	IV
FMRSA14	A6	ND	IV
FMRSA19	A6	ND	IV
FMRSA7	A7	ND	IV
FMRSA6	A8	ND	IV
FMRSA15	B1	ST22	IV
FMRSA9	C1	ST22	IV

Figure 3.11:   The UPGMA dendrogram of *Sma*I-PFGE patterns based on Dice similarity coefficient, illustrating the relationships among the 20 clinical MRSA isolates. ST= sequence type. ND= not determined

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3.3.3b Multilocus sequence typing (MLST)

Initial MLST experiments with the reference strain *S. aureus* NCTC8325 correctly identified this strain as sequence type (ST) 8 (3-3-1-1-4-4-3), confirming the accuracy of our protocol. Five representative MRSA isolates (FMRSA1, 2, 5, 9 and 15) of the twenty PFGE-typed MRSA isolates (Fig. 3.11) showed that these isolates had identical sequence types, namely ST22 (7-6-1-5-8-8-6) (Fig.3.12 and Table 3.2).
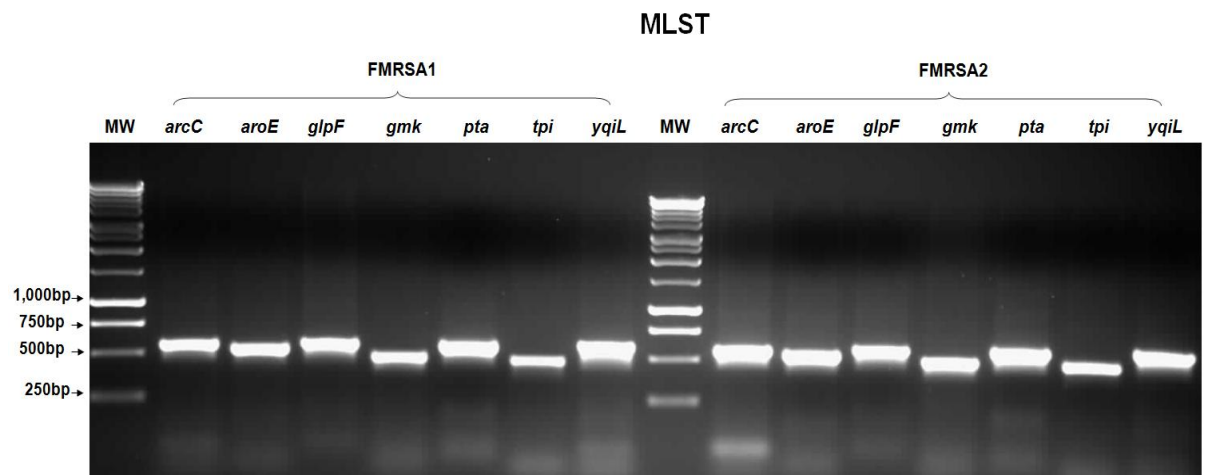
Figure 3.12:   The various fragments of the seven target housekeeping genes amplified from two clinical MRSA (FMRSA1 and FMRSA2) isolates. *arcC* : carbamate kinase; *aroE* :shikimate dehydrogenase; *glpF* : glycerol kinase; *gmk* :gulanylate kinase; *pta* : phosphate actyltransferase; *tpi* : triosephoshate isomerise; *yqiL* : acetyl coenzyme A acetyltransferase. MW =1kb DNA molecular size marker.

Table 3.2:   The sequence types and allelic profiles of the five clinical MRSA with the reference strain *S. aureus* NCTC8325.

Allele Number of MRSA strains					
Locus	NCTC8325	FMRSA1	FMRSA2	FMRSA5	FMRSA9	FMRSA15
arcC	3	7	7	7	7	7
aroE	3	6	6	6	6	6
glpF	1	1	1	1	1	1
gmk	1	5	5	5	5	5
pta	4	8	8	8	8	8
tpi	4	8	8	8	8	8
yqiL	3	6	6	6	6	6
ST	**8**	**22**	**22**	**22**	**22**	**22**

3.3.3c Staphylococcus cassette chromosome *mec* (SCC*mec*) typing

A multiplex PCR was used to identify the SCC*mec* type of twenty PFGE-typed MRSA isolates according to the method of Boye *et al*.2007. All twenty isolates were found to harbour SCC*mec*-IV and Figure 3.13 shows *SCCmec*-IV of 10 representatives MRSA isolates with control strains.
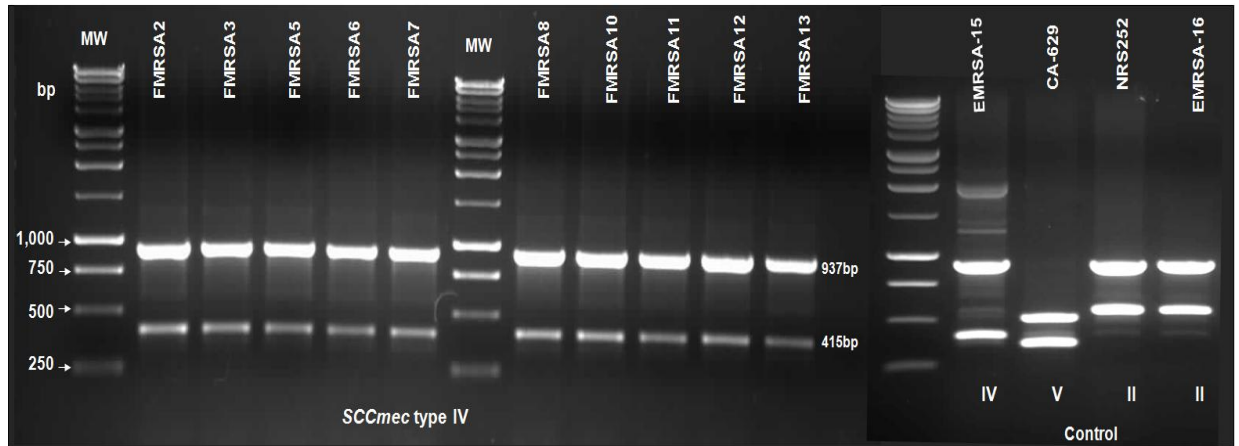
Figure 3.13: Representative multiplex PCR analyses of of 10 clinical MRSA isolates, previously typed by PFGE, to identify their SCC*mec* type. Control strains with known *SCCmec* were also analysed.

3.4 Discussion

MRSA is one of the most important nosocomial pathogens. Morbidity and mortality associated with these strains increases annually throughout the world. Analyses of the epidemiology of MRSA can assist in their control and contribute significantly to reducing the spread of MRSA infections. Typing is an effective means of aiding control since it allows epidemic and sporadic strains to be distinguished and potential sources of infection to be identified (Hryniewicz, 1999).

The Freeman Hospital is one of the largest hospitals in north-east England and was chosen as the main location of our studies. More than four hundred MRSA isolates were collected from 286 colonized and infected patients. Sixty-six percent of these isolates were isolated from screening samples (nose, perineum and throat) whereas 34% were isolated from clinical samples (wound, blood, urine and respiratory samples).

About 30% of the general population is colonised by *S. aureus* and in some cases this can reach 80% (Perla *et al.,* 2007). In this study, more than 57% percent of patients were asymptomatic carriers of MRSA whereas 43% were infected patients. The

present findings are consistent with other research indicating that the proportion of patients colonised by MRSA has increased remarkably from 4.5% in 1990 to 34.9% in 1994 at Fujiwara Memorial Hospital, Japan (Kayaba *et al.*, 1997). The MRSA colonization among hospital patients reached 46% (in Brazil) and 49% (USA) in studies conducted by Korn *et al.* (2001) and Robicsek *et al.*(2009), respectively.

Colonized and infected patients are considered to be the main reservoir of MRSA in hospitals. Increasing age is a significant risk factor associated with MRSA and the majority of previous studies have reported that colonization is highest in patients older than 75 years (Morgan *et al.*, 1999, 2000). For example, a study on patients at the Nottingham University Hospital (Hori *et al.*, 2002) found that 70% of patients colonized by MRSA are more than 75 years of age. These findings are in agreement with our observation that 73% and 70% respectively of infected and colonized patients were older than sixty years.

With regard to the clinical samples, wounds were the commonest source of MRSA, coming mainly from the general medical, urology and surgical units. This is in line with other work that has shown that MRSA is the most common wound infection pathogen (Giacometti *et al.*, 2000). In addition, general medical, urology and surgical units are the busiest units in the Freeman Hospital and consequently have the highest turnover of patients.

Carrier or colonized patients out-numbered infected patients in the general medical and urology units, but the reverse was the case in the surgical, ENT and critical care units. This might be explained by the frequent surgical interventions in surgical and ENT units as well as the increased use of medical devices and the presence of immuno-compromised patients in the critical care unit.

MRSA typing is a crucial step in controlling the spread of bacterial infections since it aids the identification of the sources of nosocomial strains and facilitates the monitoring of their distribution. The ideal typing technique should have high discriminatory power and reproducibility. Traditional phenotyping techniques for MRSA have included phage and serotyping. Although often providing useful information, they lack sufficient discriminatory power to allow them to be implemented for detailed epidemiological studies (Weller, 2000; van Leeuwen.2003).

Recently, molecular typing methods have been developed and used extensively to study the epidemiology of MRSA. Their high discriminatory power and reproducibility means that they are generally superior to phenotyping techniques. Many of molecular methods depend on PCR-based technology and, for diagnostic purposes, a number of targets have been developed (*e.g. mecA* and *coa* genes). In addition, PCR is also used to determine the presence of specific staphylococcal virulence factors such as PVL.

Twenty MRSA isolates were tested for the presence of *mecA* genes, which encode a homologue of penicillin-binding protein 2 (PBP2a), and all of the isolates were positive. Although, some methicillin-susceptible strains have non-expressed or non-functional *mecA* genes, the presence of *mec*A is considered as an indicator for the identification of MRSA. Such strains are called pre-MRSA (Lewis and Dyke, 2000; Brown *et al.,* 2005). However, because some coagulase-negative staphylococci carry *mec*A, an additional confirmatory test, involving the *coa* gene, was necessary to confirm that they were methicillin resistant *S. aureus* (Hussain *et al.*, 2000; Brown *et al.,* 2005). All of the putative MRSA isolates were confirmed by the presence of the *coa* gene.

The PVL is pore-forming exotoxin (Kaneko and Kamio, 2004) that is produced by some community-acquired MRSA (CA-MRSA) strains that can cause aggressive infections involving leukocyte destruction (Chamber, 2005). Such strains can lead to death within 72 h. The production of PVL is often associated with community-acquired skin, soft tissue and pneumonia infections. Lina and colleagues (1999) have reported that PVL production was detected in 93% of furunculosis and 85% of strains responsible for pneumonia in community-acquired *S. aureus* but rarely in hospital infection strains (Lina *et al.*, 1999). In the current study, 76 MRSA isolates were tested for the presence of the PVL-encoding gene and none were found to be PVL-positive. This result may be explained by the fact that < 5% of *S. aureus* isolates produce PVL toxin (Lina *et al.*, 1999; Holmes *et al.*, 2005).

PFGE is generally regarded as the gold standard technique for typing of bacterial species, including MRSA. It is highly discriminatory and reproducibility, but is time-consuming and expensive to carry out. In the case of MRSA, several restriction endonucleases have been evaluated but *Sma*I has been shown to give the best performance. In this study PFGE was used to type twenty clinical isolates of MRSA. All of the tested isolates were typeable by PFGE and its discriminatory power was high, with excellent reproducibility with regard to PFGE profiles of isolates but the quality of

gels was variable. Almost all of the MRSA isolates were closely related with a high percentage of similarity (Fig. 3.11). Three types (A, B and C) of *Sma*I macrorestriction profiles were distinguished with Type A predominating (Fig. 3.11). Eight subtypes were found within Type A and the majority of Type A profiles were highly similar (by more than 85%) to that of the UK epidemic strain 15 (EMRS-15). The genodiversity and prevalence of EMRSA-15 was observed in Nottingham hospitals, which showed that 80% of MRSA isolates were classical EMRSA-15, whereas genotypic variants of EMRSA-15 comprised 72% of Nottingham community-based patients (Jonas *et al.*, 2002). A recent study (Monnet *et al.*, 2004) has shown that EMRSA-15 is the most common type of MRSA in UK hospitals, with MRSA-16 a distant second. In addition, EMRSA-15 now predominates among MRSA clones in the north of the UK. In a Scottish surveillance study of 486 isolates of MRSA, conducted by Wilson and colleagues from January to December 2009, about 81% of the isolates were EMRSA-15 and only 10% EMRSA-16 (Wilson *et al.*, 2010).

Multi-locus sequence typing (MLST) is an excellent epidemiological tool for typing isolates of MRSA (Maiden *et al.*, 1998). MLST relies on the assignment of the nucleotide sequences of specific alleles and, unlike PFGE, provides unambiguous data sets (Enright and Spratt, 1999). MLST was used in the current study to analyse the sequences of internal fragments (450-500 bp) of seven housekeeping genes. Initial experiments were carried out on a reference strain, namely *S. aureus* NCTC 8325. These confirmed the accuracy of our protocol as being >99.8%. MLST analysis of representative isolates of the twenty PFGE-typed MRSA isolates shows that they were members of sequence type 22 (ST22) (Table 3.2).

SCC*mec* typing is one of the most important tools for typing MRSA and is considered to be complementary to MLST. To date, eight SCC*mec* types have been identified based on the characteristics of *mec* and *ccr* complex (IWG-SSC, 2009). Multiplex PCR was used for identification of SCC*mec* type on twenty PFGE-typed MRSA isolates and this showed that all of these isolates harboured SCC*mec* type IV. Therefore, the sequence and SCC*mec* type of the 20 PFGE-analysed MRSA isolates was ST22-SCC*mec*-IV (Table 3.3) which is consistent with them being variants of EMRSA-15.

Table 3.3:   Sources of MRSA isolates, PFGE, MLST and SCC*mec* type**.**

Isolates no.	Patient's age	Site of isolation	Unit of isolation	PFGE	MLST/SCC*mec*
FMRSA1	85	NPT	GP (out patient)	A3	ST22/IV
FMRSA 2	83	NPT	urology	A1	ST22/IV
FMRSA 3	72	Wound	urology	A2	ND/IV
FMRSA 4	25	NPT	urology	A1	ND /IV
FMRSA 5	93	NPT	general medical	A5	ST22/IV
FMRSA 6	78	NPT	GP (out patient)	A8	ND /IV
FMRSA 7	100	NPT	general medical	A7	ND /IV
FMRSA 8	82	NPT	general medical	A2	ND /IV
FMRSA 9	87	NPT	geriatric centre	C1	ST22/IV
FMRSA 10	60	NPT	FH (out-patient)	A1	ND /IV
FMRSA 11	82	NPT	urology	A2	ND /IV
FMRSA 12	84	NPT	GP (out patient)	A3	ND /IV
FMRSA 13	51	NPT	wound admission	A1	ND /IV
FMRSA 14	80	NPT	cardiothoracic	A6	ND /IV
FMRSA 15	96	NPT	WGH ward 3	B1	ST22/IV
FMRSA 16	84	NPT	RVI surgery	A1	ND /IV
FMRSA 17	92	NPT	urology	A2	ND /IV
FMRSA 18	31	NPT	GP (out patient)	A2	ND /IV
FMRSA 19	73	NPT	general medical	A6	ND /IV
FMRSA 20	85	NPT	general medical	A4	ND /IV

NPT = (nose, throat and perineum), FH = Freeman Hospital, GP = General practitioner, RVI = Royal Victoria Infirmary, WGH = Walkergate Hospital. ND= not determined

In summary, PFGE has been used extensively to investigate outbreaks of pathogens and is still considered as the gold standard technique for typing MRSA strains, despite the fact that it is time-consuming, relatively costly and the results, at best, are not available for several days. In contrast, MLST is useful for studying of *S. aureus* evolution and tracking the global spread of MRSA, but its discriminatory power is limited with respect to the investigation of local outbreaks. MLST is also time-

consuming and costly, and beyond the internal resources of most clinical laboratories, resulting in a dependence on outside agencies. Hence, the development of a new typing technique is urgently required. Such a technique should have high discriminatory power and reproducibility that is close to or exceeding that of PFGE, but should be rapid and suitable for use in all routine clinical laboratories.

Chapter 4

A novel multiplex PCR epidemiological technology for the typing of strains of *Staphylococcus aureus*

4. A novel multiplex PCR epidemiological technology for the typing of strains of *Staphylococcus aureus*

4.1 Introduction

Since its emergence in the UK more than 40 years ago, MRSA has become an important nosocomial pathogen, and morbidity and mortality rates associated with this pathogen have increased markedly in recent years worldwide. MRSA accounts for 50% or more of *S. aureus* isolates in some countries. In the UK this is between 41% - 45%, which is higher than several other European countries (*e.g.* < 3% in the Netherland and Iceland) (Aires de Sousa and de Lencastre, 2004; Johnson *et al.*, 2005). Although a consensus has yet to be reached as to the most effective means for controlling MRSA, the majority of studies indicate that the early detection of colonised and infected patients by aggressive screening and improved hand hygiene are both effective (Frank, 2003; Hardy *et al.*, 2004; Grundmann *et al.*, 2006).

A major issue is to identify the sources of MRSA infections once an outbreak has been detected and to monitor the epidemic spread of the strains involved. This requires the use of reliable typing techniques for monitoring both epidemic and sporadic outbreaks. The ability to monitor the epidemiology of an outbreak not only leads to the potential identification of its source, but can also reduce the likelihood of subsequent infections. For decades, the discrimination between strains of *S. aureus* has relied on phenotypic characters such as phage typing. However, the rapid development of molecular approaches during the last twenty years provides the opportunity to explore the unique genetic characteristics of individual strains of *S. aureus* (van Leeumen, 2003). Consequently, many molecular techniques have been introduced for studying the epidemiology of MRSA, including Pulsed-Field Gel Electrophoresis (PFGE), Multi-locus Sequence Typing (MLST), SCC*mec* type and multiple locus variable number tandem repeat (VNTR) analysis (MLVA). PFGE and MLST are the most widely used typing techniques with the former regarded as the gold standard due to its high discriminatory power and reproducibility (Enright *et al.*, 2000; Weller, 2000; Sabat *et al.*, 2003). However, technical limitations restrict the use of PFGE and MLST in the majority of routine hospital laboratories.

Currently, more than eighteen *S. aureus* genomes have been completely sequenced which allows researchers to explore in detail the genomes of this versatile pathogen. The similarities between the core genomes of *S. aureus* are very high with

between 97.7% and 99.8% nucleotide identity. The observed differences are largely due to the single nucleotide polymorphisms (SNPs) (Lindsay and Holden, 2006).

In this study, SNP variations between *S. aureus* genomes in and around *Sma*I-restriction sites (CCCGGG) were exploited to develop a genotyping technique that combines the high discriminatory power and reproducibility of PFGE, with the simplicity of a multiplex PCR-based technique that can be performed in a routine clinical laboratory**.**

4.2 Aim

The main aim of this study was to develop a novel multiplex PCR epidemiological tool to monitor the spread of MSSA and MRSA that has the same or similar discriminatory power as PFGE, but which can be performed in a few hours using technology that is available in routine clinical microbiology laboratories.

4.3 Methodology

4.3.1 Principles of a novel *Sma*I-multiplex PCR

We set out to generate a new typing system based around the output from PFGE analysis of *Sma*I (CCC ↓ GGG) macrorestriction fragments (10-30 fragments of between 10-800kb in length). However, instead of the time-consuming DNA extraction and digestion technique associated with PFGE, we devised a multiplex PCR technique based on SNPs and rapidly extracted chromosomal DNA.

4.3.2 Genomic analysis tools

The DNA sequences of the following eighteen *S. aureus* strains were obtained from the European Bioinformatics Institute (EMBL-EBI) http://www.ebi.ac.uk/genomes/ bacteria.html and The Sanger Institute http://www.sanger.ac.uk: NCTC8325, MRSA252, MSSA476, COL, RF122, JH1, JH9, N315, Mu3, Mu50, MW2, Newman, USA300FPR3757, USA300TCH1516, ED98, LGA251, TW20 and EMRSA-15. All of the sequences were analysed using a genome cutting website at http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi. The *Sma*I

restriction sites for all of the strains were mapped on to their respective chromosomes and the sizes and locations of the *Sma*I fragments determined.

4.3.3 Alignment and comparison of *Sma*I-syntenic regions

*Sma*I-syntenic regions analysis was carried out to determine the restriction patterns of each strain. Briefly, the strains with the largest number of *Sma*I restriction sites were used to develop a matrix showing the presence and absence of individual restriction sites across of the sequenced genomes. The resulting *Sma*I restriction sites were divided into two types based on their discriminatory power. Type I contains six groups of *Sma*I sites that were the most discriminatory while Type II contains four groups of *Sma*I sites with medium discriminatory properties, but which were designed to discriminate between specific strains.

4.3.4 Primer design

The ten restriction sites that were the most discriminatory between strains were identified and ten primer pairs (Table 4.1) designed according to the parameters in figure 4.1. All 10 primer pairs were designed to generate a set of uniquely sized fragments that could be both easily identified by gel electrophoresis and related to specific *Sma*I sites on the genome. The first phase primers were designed manually to for use in two multiplex PCR reactions. The second phase primers were designed using the visual OMP^TM (Oligonucleotide Modelling Platform) software (see Appendix C for the procedure of primers design using visual OMP^TM software).
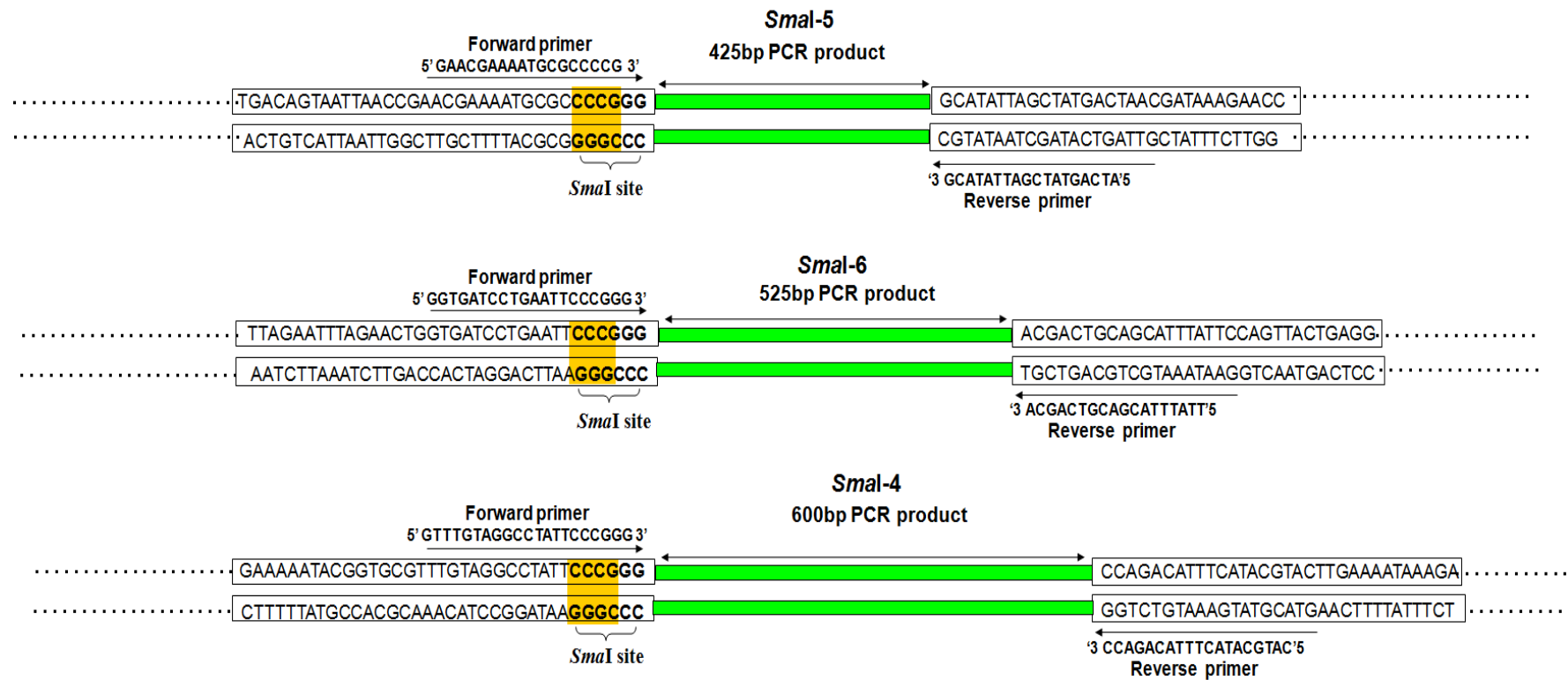
Figure 4. 1: The principle behind the design of primer pairs. The forward primer terminates at or within the *Sma*I site and the primer pairs generate a set of uniquely sized fragments that can be easily identified by gel electrophoresis.

Table 4.1: Oligonucleotide primers of a novel *Sma*I-multiplex PCR that used in this study.

	Group no.	Primer	Oligonucleotide sequences (5'-3')	GC (%)	Tm °C	Amplicon size
First phase of optimization	1	*Sma*I-1_F	ATATTTTCCAATCGCCCGGG	50	57.3	814bp
		*Sma*I-1_R	ACTTCCTAATGCTAAAAGTCCG	41	56.5	
	2	*Sma*I-2_F	TAATGCAGAAGACCCGGG	56	56.0	1100bp
		*Sma*I-2_R	CTAATGTCATGTTATCAGCACC	41	56.5	
	3	*Sma*I-3_F	ACCTGGTGATCCCGGG	64	57.6	299bp
		*Sma*I-3_R	TCAACGCTGTGCGAATCCTT	50	57.3	
	4	*Sma*I-4_F	GTTTGTAGGCCTATTCCCG	52.6	56.7	600bp
		*Sma*I-4_R	CAAGTACGTATGAAATGTCTGG	41	56.5	
	5	*Sma*I-5_F	GAAAATGCGCCCCGGG	68.8	56.9	variable
		*Sma*I-5_R	CGTTAGTCATAGCTAATATGC	38.1	54.0	
	6	*Sma*I-6_F	TGATCCTGAATTCCCGGG	55.6	56.0	525bp
		*Sma*I-6_R	GGAATAAATGCTGCAGTCGT	45	55.3	
	7	*Sma*I-7_F	TGATTTTTTTGACCCGGGGG	50	57.3	378bp
		*Sma*I-7_R	CACATCAAGTATAGATGCGT	40	53.2	
	8	*Sma*I-8_F	GGCAGTGCACCCGGG	80	58.8	930bp
		*Sma*I-8_R	GCAAACTGAGGTTGAAGGAC	50	57.3	
	9	*Sma*I-9_F	CCTTGTTTATTAGATCCCGGG	48	58.0	712bp
		*Sma*I-9_R	GCAGCGACTTGGTATGAAAAAC	45	58.5	
	10	*Sma*I-10_F	CGCGAAAAGTATTATTTCCCG	43	55.9	248bp
		*Sma*I-10_R	CAATCCAGCGTATATCCGTA	45	55.3	
	Group no.	**Primer**	**Oligonucleotide sequences (5'- 3')**	**GC (%)**	**Tm °C**	**Amplicon size**
Second phase of optimization	1	*Sma*I-1_F	TTCATATTTTCCAATCGCCCG	42.9	55.9	169bp
		*Sma*I-1 _F2	TTCATATTTTTCAATCGCCCG	38.1	54.0	
		*Sma*I-1 _R	CTATAATTCCCTATTTTGACATCTTC	30.8	56.9	
	2	*Sma*I-2 _F	GCAGGTAATGCAGAAGACCCG	57.1	61.8	263bp
		*Sma*I-2 _R	CATTAGTTCCGTATGTAATCTTATC	32	56.4	
	3	*Sma*I-3 _F	GACCTGGTGATCCCG	61.1	58.2	101bp
		*Sma*I-3 _R	GGTAATTCTGTGCCACTATTAGC	43.5	58.9	
	4	*Sma*I-4 _F	TTGTAGGCCTATTCCCG	53	58.6	124bp
		*Sma*I-4 _R	CAGTAATCTTAACTTCAATTGTGTCACC	35.7	60.7	
	5	*Sma*I-5 _ F	GAACGAAAATGCGCCCCG	61.1	58.2	variable
		*Sma*I-5 _R	AGAAAACGTATTTCTTTATCTTTTCG	26.9	55.3	
	6	*Sma*I-6 _F	GAACTGGTGATCCTGAATTCCCG	52	62.4	220bp
		*Sma*I-6 _R	GTCGAACGTGGACCAAAAGGC	57	61.8	
	7	*Sma*I-7_F	CGCTTTTGATTTTTTTGACCCG	40.9	56.5	146bp
		*Sma*I-7_R	CATCCGGAGTTTGCTCATGAC	52.4	59.8	
	8	*Sma*I-8_F	AGCAGGCAGTGCACCCG	70.6	60.0	319bp
		*Sma*I-8_R	CGATTTATGAGGTATGAAGGAAC	39.1	57.1	
	9	*Sma*I-9_F	CGGCCTTGTTTATTAGATCCCG	50	60.3	292bp
		*Sma*I-9_R	CTAAAAAGTATGTTAGGCACGTG	39.1	57.1	
	10	*Sma*I-10_F	CGCGAAAAGTATTATTTCCCG	42.9	55.9	82 bp
		*Sma*I-10_R	CTTCTTTTACTTCTCTTAACAGCG	37.5	57.6	
	mecA	*mecA*_F	GTGGAATTGGCCAATACAGGAAC	48	60.6	502bp
		*mecA*_R	GATGGCAAAGATATTCAACTAAC	35	55.3	

4.3.5 Standard staphylococci strains

In this study many well characterised *S. aureus* strains and two *S. epidermidis* strains were obtained from various sources. Sequenced *S. aureus* strains USA300-FPR3757, MSSA476, MRSA252, MW2, COL, N315, Mu3, Mu50 and sequenced *S. epidermidis* RP62A, as well as control community-MRSA strains USA1000, USA1100 and CA-629, were obtained from the network on antimicrobial resistance in *Staphylococcus aureus* (NARSA) programme, USA. EMRSA-15 (sequenced strain H050960412) was kindly provided by Prof. Mark Enright, Imperial College, London, UK. The sequenced *S. epidermidis* strain ATCC12228 was provided by Prof. Jan Maarten van Dijl, University of Groningen, the Netherlands whereas strain JH9 was provided by Prof. Herminia de Lencastre, Instituto de Tecnologia Química e Biológica, Portugal. NCTC8325 strain was obtained from the National Collection of Type Cultures (NCTC), London, UK. The PVL+ MRSA strain 585296 was provided by Dr. John Perry from the Freeman Hospital, Newcastle upon Tyne, UK.

4.3.6 DNA extraction

4.3.6a Extraction of the chromosomal DNA by cell mechanical breakage

The DNA of all strains was extracted using the cell mechanical breakage method (Section 2.5.2b).

4.3.6b Rapid Extraction of chromosomal DNA using only boiling method

 The DNA was extracted according to the boiling method (Section 2.5.2c)

4.3.6c Rapid Extraction of chromosomal DNA using boiling with lysostaphin method.

Two colonies from an overnight culture was transferred into 20µl of sterile milli-Q water contains 3µl lysostaphin [1mg/ml] in a microcentrifuge tube and mixed thoroughly. The cell suspension was incubated at 37ºC for 15 - 20 min, heated to 100ºC for 10 min and then centrifuged at 13,000g for 5 minutes. Three microlitres of the resulting supernatant were used in the multiplex PCR reaction.

4.3.7 PCR amplification

4.3.7a Uniplex PCR

The 10 pairs of primers were initially tested using conventional PCR (*i.e.* uniplex reactions) to determine their amplification specificity and discriminatory power. The reaction conditions were as follows: 5 min at 94 °C initial denaturation, followed by 30 cycles of 94°C for 1 min, 52 - 50°C for 1 min and 72°C for 1 min, with final extension at 72°C for 5 min.

4.3.7b Multiplex PCR

The optimization of multiplex PCR involved two main phases:

Phase I: All primer pairs were manually designed to be used in a multiplex PCR. Initially, three pairs of primers (*Sma*I-groups 4, 5 & 6) were tested in the same reaction conditions as those used for the uniplex PCR on DNA from three unrelated *S. aureus* strains, namely NCTC8325, EMRSA-15 and MRSA-PVL+. Multiplex PCRs were then carried out on the same set of DNA using six primer pairs (*Sma*I-groups 10, 3, 5, 6, 4 & 7), but with modifications to the reaction conditions, as outlined in Tables 4.2 and 4.3. An additional four primer pairs (*Sma*I-groups 1, 8, 9 & 2), designed to distinguish specific EMRSA strains, were tested in a multiplex PCR under the same reaction conditions as used in phase I (Table 4.2).

Phase II: In order to use all primers in a single 10x multiplex PCR reaction instead of two separate (6x and 4x) reactions, the primers were redesigned and optimised with the visual OMP^{TM} 6.0v (Oligonucleotide Modeling Platform) DNA Software in collaboration with Biogene LTD, UK. The optimization included secondary structure, homology, annealing temperature and assay conditions. The sizes of fragments generated by the new primer set were between 69bp to 406bp.

Multiplex PCR components and condition

A series of PCR reactions were carried out to identify optimal conditions for the multiplex PCR. This included varying the primer concentrations and the other components of the PCR reagents. The optimal PCR reaction conditions and components are outlined in Tables 4.2 and 4.3.

Table 4.2: PCR reaction conditions

Steps	Multiplex PCR *PhaseI*			Multiplex PCR *PhaseII*		
	ºC	min	Cycles	ºC	min	Cycles
Initial Denaturing	95	5	1	96	2	1
Denaturing	94	1		96	0.75	
Annealing	52	1	30	56	0.75	35
Extension	68	2		72	0.75	
Final Extension	68	5	1	72	2	1

Table 4.3: Components of PCR reaction mixture.

Components	Volume per 50 µl reaction (µl)		
	uniplex PCR	Multiplex PCR *PhaseI*	Multiplex PCR *PhaseII*
Milli-Q Water	28	Variable	29
DNA polymerase buffer (10x)	5	5	5
Forward primer (20mM)	5	Variable	2 µl each
Reverse primer (20mM)	5	Variable	2 µl each
BSA (100x)	-	4	4
dNTP (10mM)	1	1.5	1
MgCl (25mM)	3	4	3
Template DNA (200ng/ µl)	2	3	3
Taq polymerase (5U/µl)	1	1.5	1

4.3.8 Gel electrophoresis

For uniplex PCR and phase I multiplex PCR, the resulting amplicons were separated on 1 - 1.5% agarose (in 1x TBE with 0.5µg/ml ethidium bromide) at 90V for 50 – 130 min, then visualized using a gel documentation system.

In the phase II multiplex PCR, a higher percentage of low melting point agarose (LMP) (4% in 1xTBE) was used to resolve the smaller fragments (between 69bp to 500bp) at 110V for 3 h. The 4% gel was stained for 15 min in one litre of multiplex staining solution buffer (0.5µg/ml in 1xTBE), destained in ddH$_2$O for 15 min and then visualized with a gel documentation system.

4.3.9 PFGE

PFGE was carried out according to the protocol in Section 2.6.1 with the following modifications: 70 µl lysostaphin (1mg/ml) was added to lysis buffer and the running time increased by 2 to 26 h.

4.3.10 Data analysis of *Sma*I-multiplex PCR and PFGE

Gel images were saved as a TIFF file and analysed using the BioNumerics v3.50 (Applied Maths) software. The analysis of multiplex PCR and PFGE gels was based on band differences and a clustering dendrogram was generated by the unweighted-pair group method with arithmetic mean (UPGMA) using the Dice similarity coefficient.

4.3.11 MLST

MLST was performed according to the protocol of Enright *et al.* (2000) as described in Section 2.6.2a. PCR products for sequencing were purified using QIAquick PCR purification kit (QIAgen) according to manufacturer's instructions (see Section 2.6.2b.ii). The concentration of DNA was determined using the NanoDrop (ND-1000) system (NanoDrop Technologies) and the products were sent for sequencing (MWG Biotech, Germany). Nucleotide sequences were compared with the MLST database through website (http://www.mlst.net) for assignment of allelic profiles and sequence type (ST).

4.4 Results

4.4.1 *Sma*I-Multiplex PCR

In this study we developed a novel multiplex PCR method that can be adapted for use in any routine microbiology laboratory to monitor the relations between MSSA and MRSA strains isolated in the clinical settings. The primers were designed around *Sma*I restrictions sites so that they can be related to PFGE electropherograms.

4.4.2 Genomic analysis

All currently sequenced genomes of *S. aureus* strains were subject to a bioinformatical analysis to determine the gene neighbourhoods that contain *Sma*I restriction sites. This analysis revealed that the number of *Sma*I restriction sites varied from 24 to 29 among the sequenced *S. aureus* strains (Table 4.4).

Table 4.4:   Number of *Sma*I restriction sites among sequenced *S. aureus* strains.

	S.aureus strains	SCC*mec* type	Genome size (bp)	Number of *Sma*I-restrictions sites
MRSA	COL	I	2,809,422	27
	USA300FPR3757	IV	2,872,769	26
	USA300TCH1516	IV	2,872,915	26
	Mu50 (VISA)	II	2,878,529	25
	Mu3 (VISA)	II	2,880,168	26
	N315	II	2,814,816	25
	JH1 (VISA)	II	2,906,507	26
	JH9 (VISA)	II	2,906,700	28
	MW2	IV	2,820,462	28
	MRSA252	II	2,902,619	26
	EMRSA-15	IV	2,832,299	24
	TW20 (0582)	III	3,043,210	29
MSSA	NCTC8325	MSSA	2,821,361	25
	RF122	MSSA	2,742,531	27
	MSSA476	MSSA	2,799,802	28
	Newman	MSSA	2,878,897	26
	ED98	MSSA	2,824,404	26
	LGA251	MSSA	2,750,834	25

Using a restriction site that was invariant in all the strains, the locations of all other *Sma*I restriction sites were mapped in relation to each other (Fig 4.2).
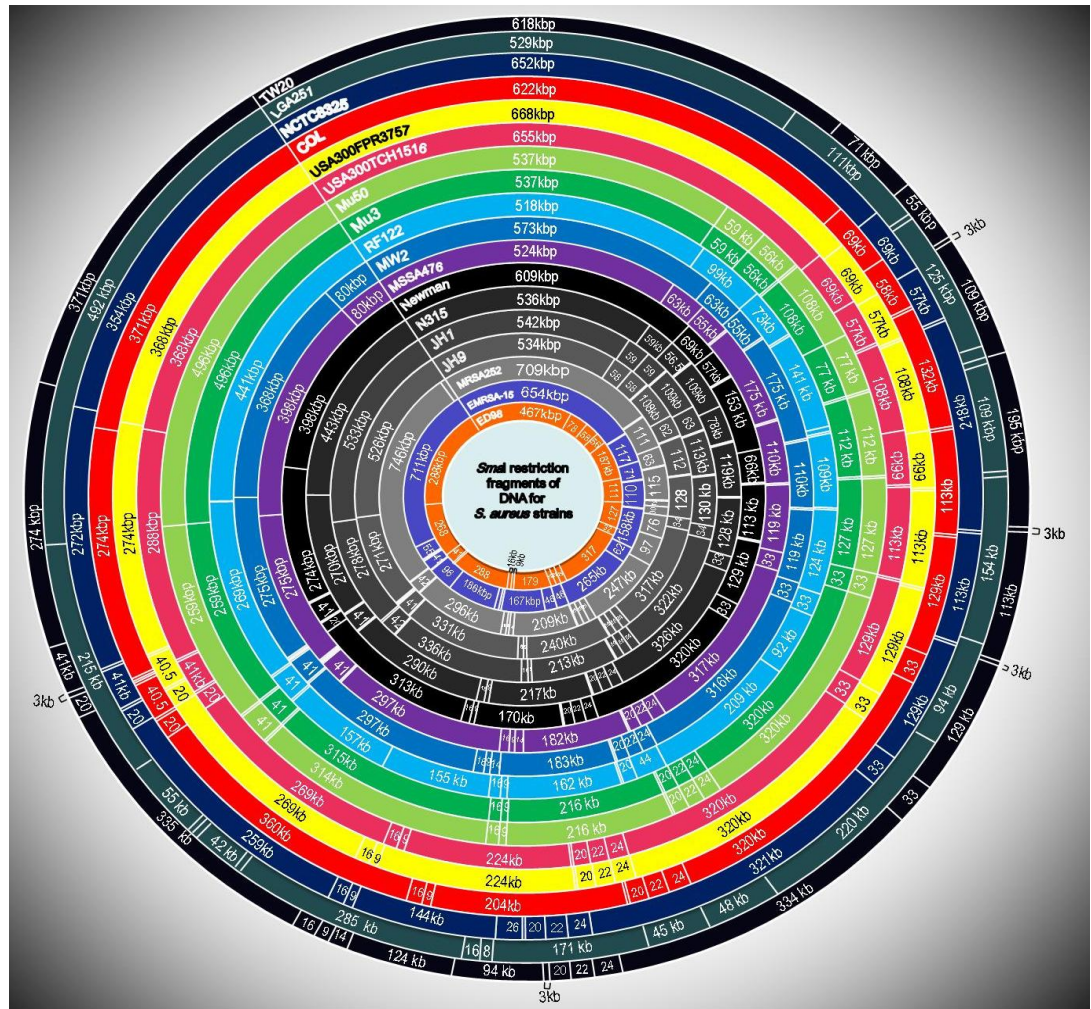
Figure 4.2: *Sma*I restriction fragments of the DNA of *S. aureus* strains

4.4.3 Alignment and comparison of *Sma*I restriction sites between strains

Alignment of the gene neighbourhoods for the syntenic regions of *Sma*I-sites showed that more than fifty *Sma*I-groups were identified within the *S. aureus* genomes. *S. aureus* has five to six copies of the rRNA operons, and each operon contains about two *Sma*I sites (Green and Vold, 1993; Wada *et al*, 1993). The gene neighbourhoods located within the ribosomal RNA (rRNA) genes were excluded due to the high similarity of the sequences within rRNA that would generate unforeseen PCR products.

A matrix of gene neighbourhoods of *Sma*I restrictions sites was constructed to show the presence (Blue) and absence (Red) of distinct restriction sites. This matrix ultimately provides a reproducible diagnostic profile for each strain. More than 30 *Sma*I groups were identified to be variable between strains and the ten most discriminatory *Sma*I groups were selected for analysis (Table 4.5).

Table 4. 5: A matrix of gene neighbourhoods of *Sma*I restrictions sites of sequenced *S. aureus* strains.

Gene neighbourhoods

No.	S. aureus strains	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
1	NCTC8325 (MSSA)																															
2	COL (First MRSA 1961)																															
3	USA300-FPR (CA-MRAS)																															
4	USA300-TCH(CA-MRSA)																															
5	JH1 (VSSA/HA-MRSA)																															
6	JH9 (VISA/HA-MRSA																															
7	Mu50 (VRSA/MRSA)																															
8	Mu3 (VRSA/MRSA)																															
9	N315 (HA-MRSA)																															
10	Newman(MSSA)																															
11	MSSA476																															
12	MW2 (CA-MRSA)																															
13	RF122 (MSSA)																															
14	ED98																															
15	LGA251																															
16	S.aureus 0582 (TW20)																															
17	MRSA252 (EMRSA-16)																															
18	EMRSA15																															
	TOTAL	4	2	8	12	13	14	11	1	2	3	16	16	17	15	16	16	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1

Present　Absent　Discriminatory primer pairs

0.06kb　0.18kb　0.24kb　0.35kb　0.41kb

Present with variable lengths

Once alignments of the gene neighbourhood were established, primers were designed. Forward primer contained the *Sma*I site at its 3' end and the reverse primer was designed from a conserved region between 100 – 412bp downstream of the target *Sma*I site (Fig. 4.1 and 4.3). Visual OMP^{TM} DNA software was used to design all primer pairs to be compatible in a single 10x multiplex PCR reaction. Visual OMP^{TM} facilitates the design of primers that conform to predetermined parameters, including the optimal length of the primers and PCR products, isothermal melting temperature, and the avoidance of primer dimers and secondary structures.
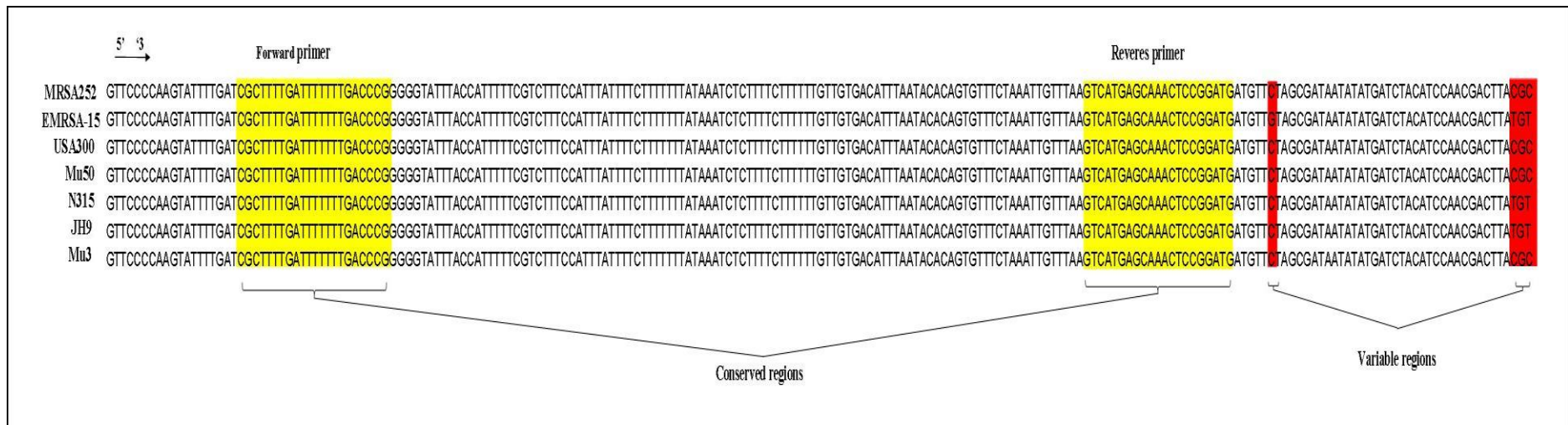
Figure 4.3: DNA sequences of the gene neighbourhood of *Sma*I-group7 for seven *S. aureus* strains. The yellow highlighted sequences are the conserved regions that were used for the design of the selected primers. The red highlighted sequences indicate variable regions that were avoided in the designed primers

4.4.4 Identified the locations of high discriminatory *Sma*I restriction sites on the *S. aureus* genome

The location of each gene neighbourhood was determined on the *S. aureus* genome and the locations of those discriminatory sites were distributed throughout the entire genome (Fig. 4.4). In addition, all PCR amplicons of *Sma*I-groups were found to be located within a single gene with the exception of *Sma*I-groups 7 and 5. The *Sma*I-group 7 amplicon was located at 5'-end and 3'-end of two genes encoding hypothetical phage proteins whereas *Sma*I-group 5 is located in an intergenic region (IGR) downstream of the dipeptidase (*pepV*) gene (Fig. 4.4). The *Sma*I-group 2 amplicon is located within a paralogous gene encoding a Ser-Asp rich fibrinogen-binding bone sialoprotein-binding protein (*sdrD*) while *Sma*I-group 9 is located on genes encoding nitrate reductase alpha chain (*narG*). The amplicons of *Sma*I-groups 3 and 8 are located within genes encoding conserved hypothetical proteins and *Sma*I-group 4 is located within the polynucleotide phosphorylase/polyadenylase gene (*pnpA*). *Sma*I-groups 1 and 6 are located within genes encoding capsular polysaccharide synthesis enzyme (*cap8J*) and serine protease (*splC*), respectively (Table 4.6 and Fig. 4.4). Once the primer pair for *Sma*I-group 5 were designed and tested, it immediately became clear that it generated fragments of different sizes: 408bp in COL and Newman, 351bp in NCTC8325, 238bp in USA300, ~ 180bp Mu50, Mu3, N315, MW2, MSSA476, and only 65bp in JH9 and JH1 (Table 4.6). The reason for this will be discussed later.
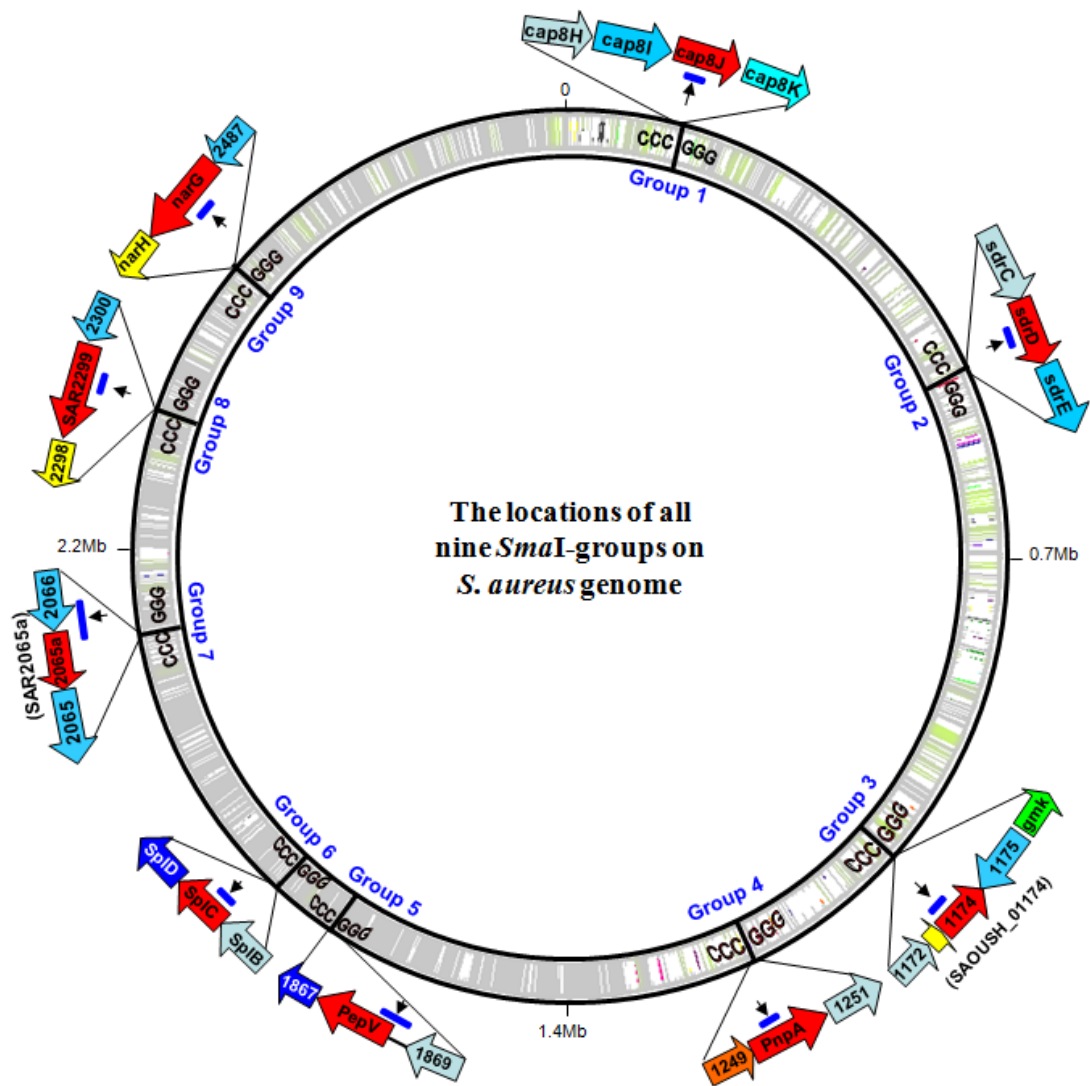
Figure 4.4: The positions and distribution of high discriminatory *Sma*I-restriction sites on the *S. aureus* genome. Blue rectangles (indicated by black arrows) refer to PCR amplicons of *Sma*I-groups. Mb= Megabase pair.

Table 4.6: The *Sma*I gene neighbourhoods and their locus tags on the *S. aureus* genomes.

*Sma*I-group no.		Strain	PCR products size (bp)	Gene name/ neighbourhood – (Locus tag)	Primer 1 (include *Sma*I) (L Coordinate)	Primer 2 (R Coordinate)
1		252	169	capsular polysaccharide synthesis enzyme (*cap8J*)- (SAR0160)	178612	178781
2		15	263	Ser-Asp rich fibrinogen/bone sialoprotein-binding protein (*SdrD*) – (SAEMRSA15_04890)	583628	583896
3		8325	101	conserved hypothetical protein – (SAOUHSC_01174)	1124335	1124436
4		252	120	Polynucleotide phosphorylase/polyadenylase (pnpA) – (SAR1250)	1310202	1310321
5	a	COL	408	241 bp at 5' of dipeptidase (*PepV*) – (SACOL1801) 272 bp at 3' of hypothetical protein	1850334	1850741
	b	8325	351	241 bp at 5' of dipeptidase (*PepV*) – (SAOUHSC_01868) 272 bp at 3' of hypothetical protein	1776375	1776726
	c	USA300	238	241 bp at 5' of dipeptidase (*PepV*) – (SAUSA300_1697) 272 bp at 3' of hypothetical protein	1872815	1873053
	d	Mu50	179	241 bp at 5' of dipeptidase (*PepV*) – (SAV1751) 139 bp at 3' of hypothetical protein	1881973	1882151
	e	JH9	65	241 bp at 5' of dipeptidase (*PepV*) – (SaurJH9_1806) 139 bp at 3' of hypothetical protein	1927295	1927359
6		8325	220	serine protease (*SplC*) – (SAOUHSC_01939)	1845160	1845380
7		252	146	79 bp at 5' of hypothetical phage protein – (SAR2065a) 117 bp at 3' of hypothetical phage protein- (SAR2066)	2150906	2151052
8		252	319	hypothetical protein	2385802	2386121
9		252	292	nitrate reductase alpha chain (*narG*)- (SAR2486)	2558309	2558601

4.4.5 Designing primers of *Sma*I restriction sites for multiplex PCR

Primer pairs groups of *Sma*I were chosen according to their discriminatory power and divided into two types. Type I consisted of *Sma*I-groups 3, 4, 5, 6, 7 & 10 and Type II of *Sma*I-groups 1, 2, 8 & 9. Type II *Sma*I-groups were particularly targeted to differentiate the UK epidemic strains EMRSA-15 and EMRSA-16.

4.4.6 Identification of validity of primer pairs in uniplex PCR

In the second stage of development, the amplification specificity and discriminatory power of primer pairs (*Sma*I-groups-4, 5 & 6) were examined by uniplex PCR on three unrelated *S. aureus* strains (NCTC8325, EMRSA-15 and MRSA-PVL+). The data showed that strains NCTC8325 and MRSA-PVL+ generated amplicons for all three primer pairs while EMRSA-15 only generated an amplicon for the group-4 primer pair. Interestingly, the length of the PCR fragment generated from the group 5 primer pair was found variable (142-bp as compared to the expected 425-bp in the case of NCTC8325) (Fig. 4.5). This observation could potentially add to its discriminatory power of the technique and the resulting fragment was designated group-5a.
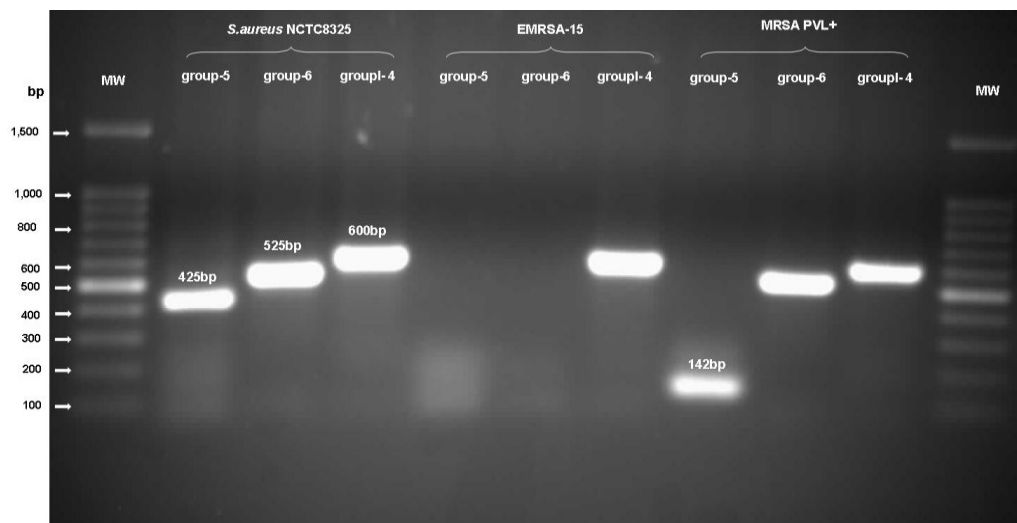
Figure 4.5: PCR amplified products generated from three primer pairs *Sma*I-group-4, 5 & 6 of *S. aureus* NCTC8325, EMRSA-15 and MRSA-PVL+. MW= molecular size marker.

The variable PCR products that were generated by *Sma*I-group 5 were due to the presence of a variable number of *Staphylococcus aureus* repeat (STAR) elements in the intergenic region between dipeptidase (*PepV*) and a hypothetical protein encoding gene (Fig.4.6)
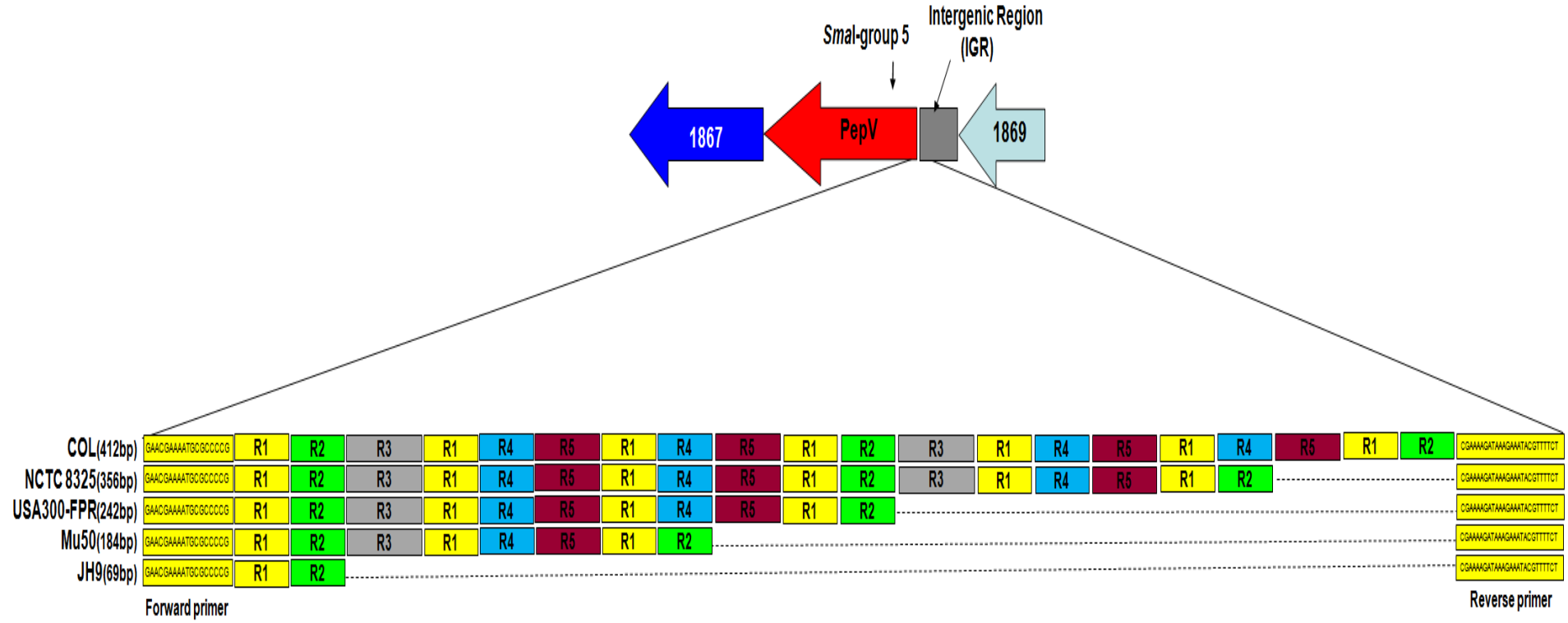
Figure 4. 6: *Sma*I-group 5 primers that amplify variable PCR products due to repeated STAR sequences in an intergenic region in different strains. R1= repeated sequence 1.

4.4.7 Multiplex PCR

4.4.7a Combined multiplex PCR against individual strains

Multiplex PCR was initially performed with the first six primer pairs of *Sma*I-groups 3, 4, 5, 6, 7 &10. In this experiment four unrelated strains (NCTC8325, EMRSA-15, EMRSA-16 and MRSA-PVL+) were typed and showed three distinct profiles, allowing two of the strains to be distinguished from each other. However, EMRSA-15 and EMRSA-16 had identical profiles. Consequently, at this stage, the first set of primers was not able to distinguish between the most predominant UK epidemic clones, namely EMRSA-15 and EMRSA-16 (Fig. 4.7).
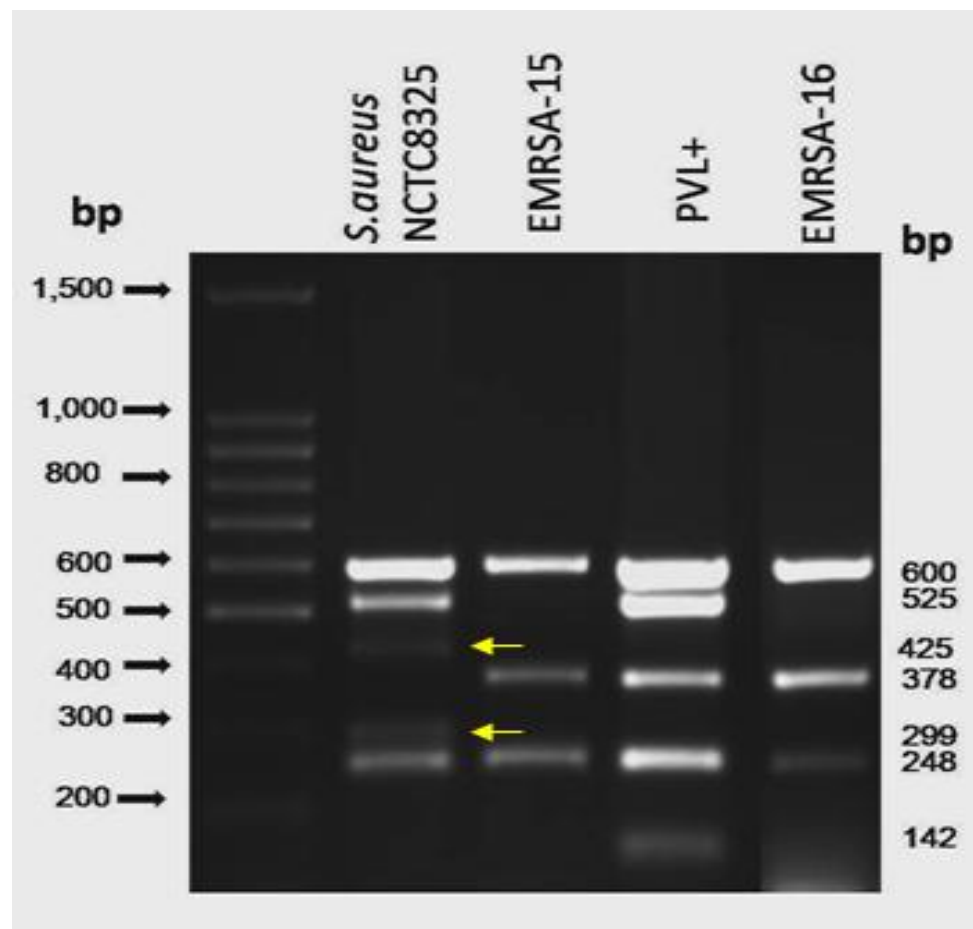
Figure 4.7:   Multiplex PCR using six primer pairs against strains (NCTC8325, EMRSA-15, EMRSA-16 and MRSA-PVL+). Strains showed different profiles except that it was not possible to distinguish between EMRSA-15 and EMRSA-16. bp = base pairs

4.4.7b Initial determination of the discriminatory power against clinical MRSA isolates

A number of clinical MRSA isolates isolated from the Freeman Hospital (Newcastle upon Tyne) were typed by multiplex PCR using all six Type I primer pairs. All of these MRSA isolates showed the same profiles as EMRSA-15 and EMRSA-16 (Fig. 4.8).
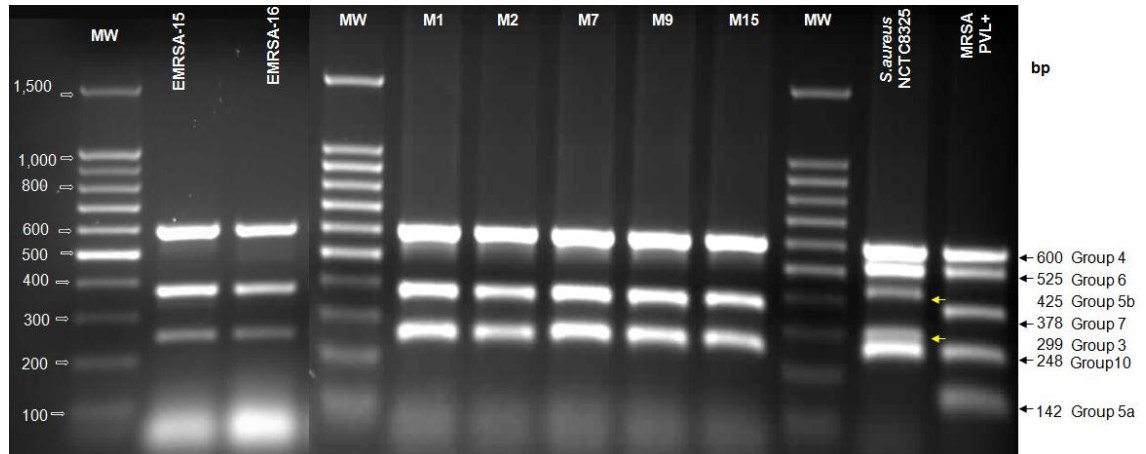
Figure 4.8:   *Sma*I profiles of five clinical isolates using multiplex PCR for all six primer pairs. M1= MRSA1. MW= molecular size marker.

In an attempt to distinguish two clones of EMRSA-15 and EMRSA-16, four new primer pairs were designed and tested using conventional PCR. Distinctive profiles were found to distinguish between these strains (Fig. 4.9).

Figure 4.9:   Evaluation of PCR primer pairs designed to differentiate between EMRSA-15 and EMRSA-16. A: Analysis by conventional PCR. B: Prospective profiles expected for a multiplex PCR typing using all 10 primer pairs. MW= molecular size marker. bp = base pairs.

The four new primer pairs were tested against EMRSA-15 and EMRSA-16 in a multiplex reaction and they distinguished clearly between these two clones (Fig. 4.10).
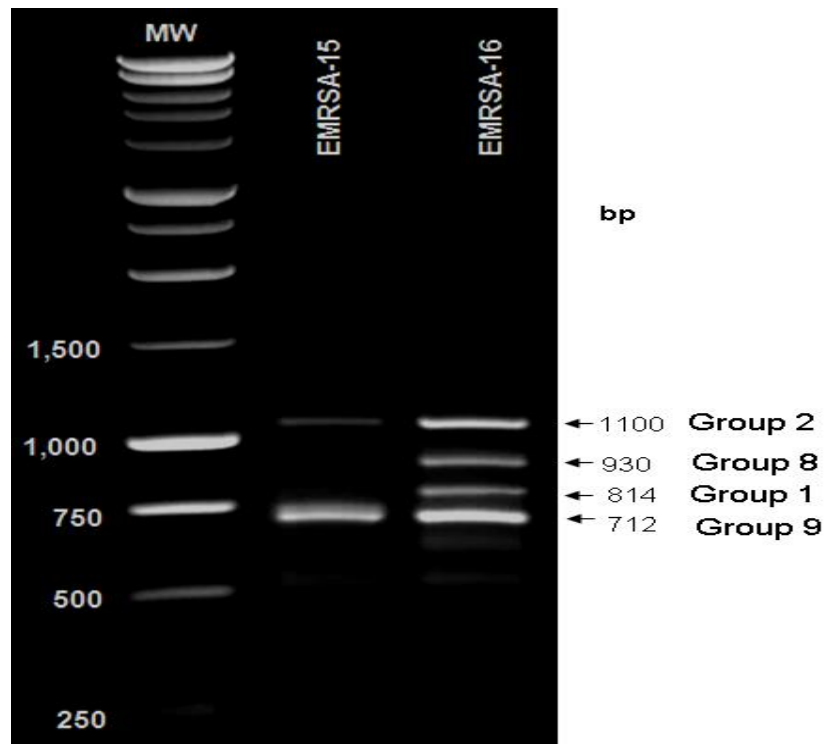
Figure 4. 10:   *Sma*I-Multiplex PCR profiles of EMRSA-15 and EMRSA-16 using four new primer pairs

4.4.7c Optimization of a novel *Sma*I-multiplex PCR typing technique

Having confirmed that the primer selection strategy was effective, the visual OMP^{TM} (Oligonucleotide Modelling Platform) software was used to redesign and optimise the primers for use in a single 10x multiplex PCR reaction instead of two (6x and 4x) multiplex PCR reactions. The sizes of PCR products ranged from ~ 69bp to 412bp and the software optimized the secondary structure, homology, annealing temperature and assay conditions.

Initially, the ten newly designed primer pairs were multiplexed with two different multiplex PCR master mixes against five unrelated *S. aureus* strains (NCTC 8325, EMRSA-15, MRSA252, NCTC 6571 and a PVL-positive MRSA). The master mix that was prepared in this study showed the best performance and the PCR amplicons were sharp and clear whereas the performance of Biogene master mix was poor and some PCR products were either weak or missing (Fig. 4.11).
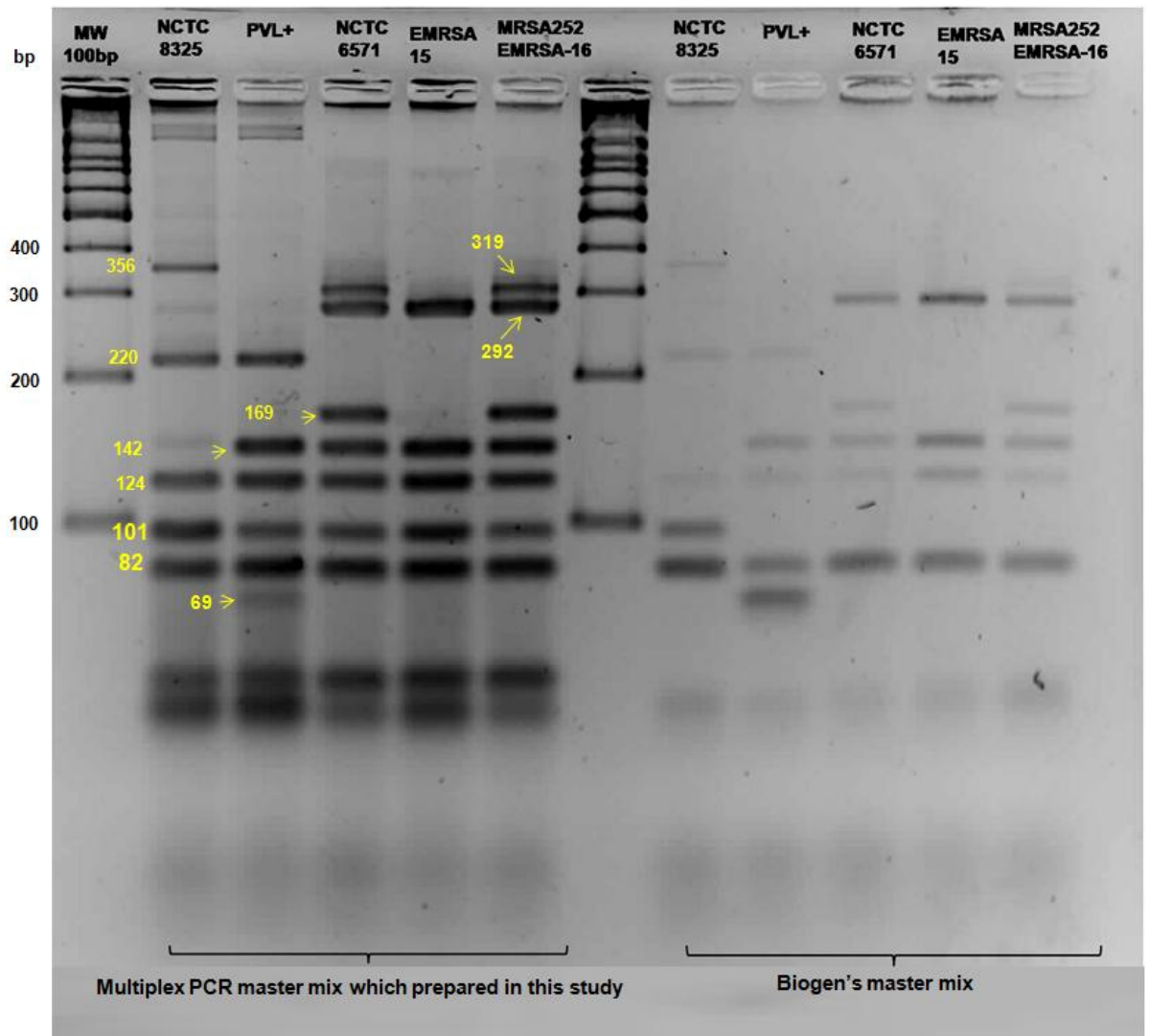
Figure 4.11: *Sma*I-multiplex PCR profile of five *S. aureus* strains (NCTC 8325, EMRSA-15, MRSA252, NCTC 6571 and a PVL-positive MRSA) using 10 new primer pairs that redesigned using OMP^{TM} (Oligonucleotide Modelling Platform) software. All primers were tested on two different multiplex PCR master mixes, one was prepared in this study (left) and another provided by Biogene LTD, UK (right).

In order to determine the optimal annealing temperature for the multiplex PCR, the ten redesigned primer pairs were tested on four *S. aureus* strains at different annealing temperatures and their performance was generally excellent. An annealing temperature in the region of 56°C to 57°C was found to be optimal. However, the primer pairs of *Sma*I-groups 4, 3 and 10 amplified PCR products for strains NCTC8325, EMRSA-15 and MRSA252, respectively, that were not predicted (Fig. 4.12).
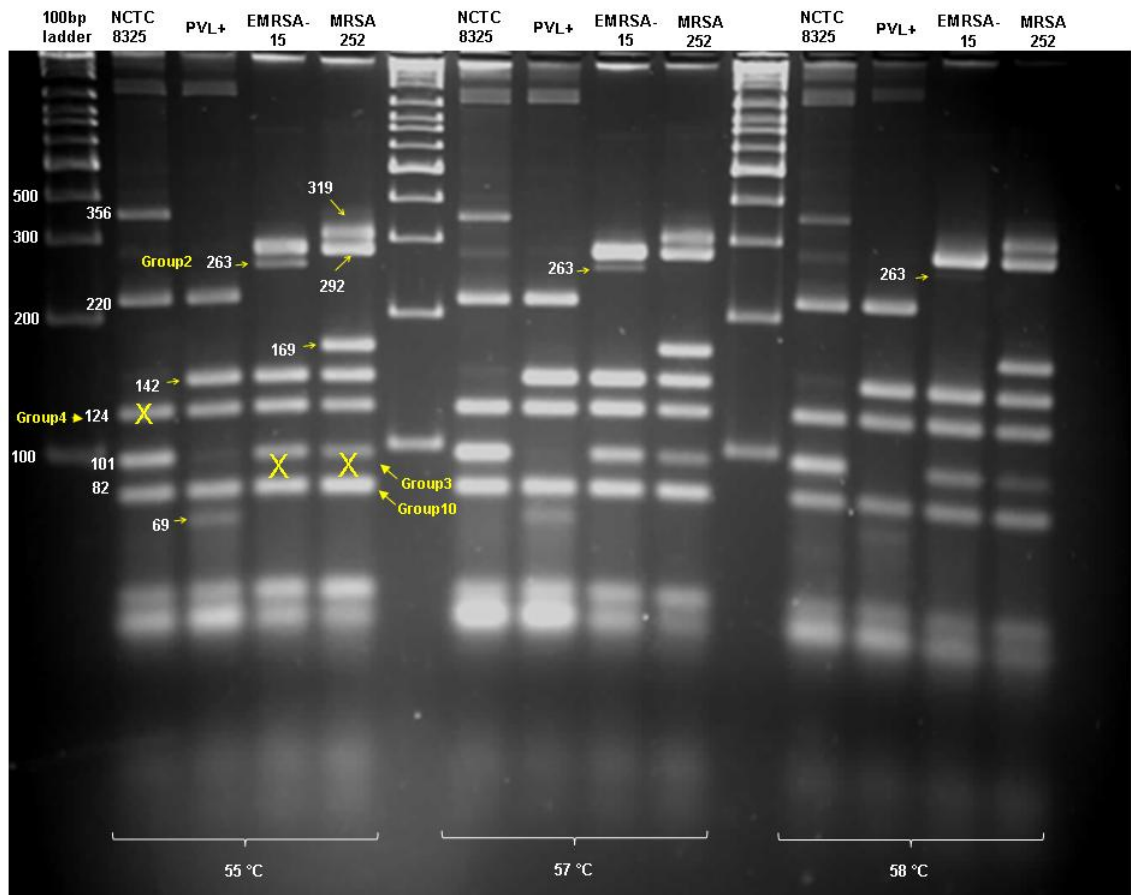
Figure 4.12:   *Sma*I-multiplex PCR profiles of four unrelated *S. aureus* strains using all ten redesigned primers in single multiplex PCR reaction at different annealing temperatures. **X** = PCR products that were not predicted by the bioinformatical analysis.

The unexpected products were due to SNPs in restriction the *Sma*I sites themselves in which the nucleotide at position 4, which is a "G", was replaced with an "A" in the case of groups 4 and 10 (CCCAGG) and "T" in the case of group 3 (CCTGGG). It was clear that, although the forward primers should not have annealed to these SNPs, the stringency was not sufficient to avoid their annealing under the condition used in the multiplex reaction. In an attempt to avoid these false positive reactions, the forward primers for groups 3, 4 and 10 were redesigned to avoid false-positive PCR amplicons by reducing their melting temperatures. The multiplex was repeated on ten of the sequenced *S. aureus* strains using all ten primer pairs, but including the new forward primers for groups 3, 4 and 10. The new primers for groups 4 (product size 124bp) and 3 (product size 101bp) produced the expected products with no false positives (Fig. 8). However, the primers from group 10 (82bp) still generated an amplicon from most of the strains, indicating that the forward primer still lacked

specificity. Significantly, each of the strains showed a distinct profile, although it was not possible to distinguish between three closely related hospital strains, namely Mu50, Mu3 and N315, and two closely related community strains, MSSA476 and MW2. In general, the specificity of the primers was excellent excepting for the group 10 primers (product size 82bp), indicated by the red arrow in Figure 4.13. This primer pair generated a false positive amplicon from strains MSSA476, MW2, Mu50, Mu3, N315, MRSA252 and EMRSA15. In addition, the PCR products of *Sma*I groups 2, 4 and 5 (variable length) were weak in some strains as indicated by yellow arrows in Figure 4.13.
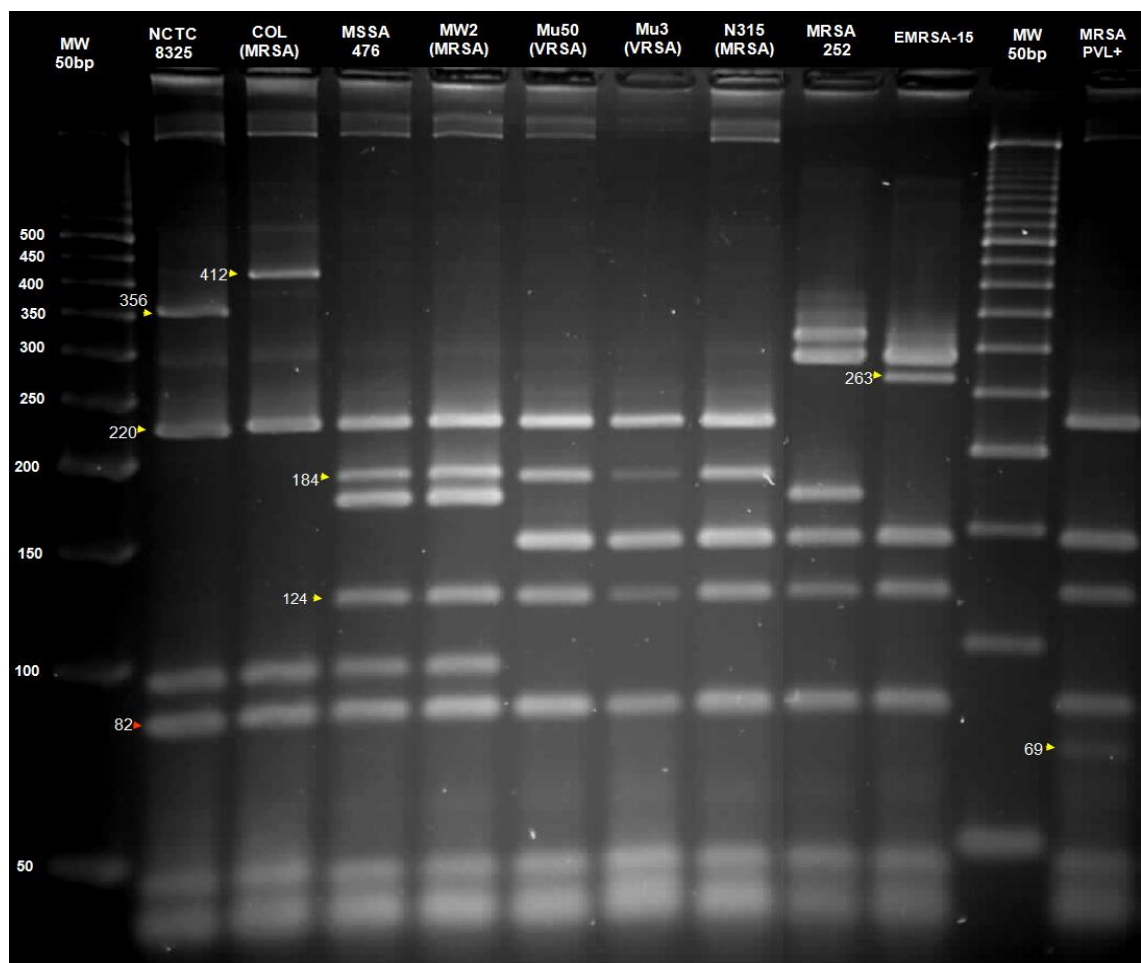
Figure 4.13:   *Sma*I multiplex PCR profiles of nine sequenced *S. aureus* strains and a PVL-positive MRSA strain with all ten redesigned primer pairs. The red arrow refers to the false positive PCR products of *Sma*I-group 10 and the yellow arrows refer to weak PCR products of *Sma*I groups 2, 4 and 5 (variable length) in some strains. MW= molecular size markers.

In order to assess the problem of *Sma*I-group10 primers, the sequences around *Sma*I-site of six strains that generated false positive results were aligned with two strains that were expected to generate amplicons. This confirmed that the only difference between these strains was the SNP *Sma*I restriction site (CCCAGG) associated with the forward primer (Fig. 4.14).

Attempts to redesign a primer to differentiate between these strains using *Sma*I-group10 were unsuccessful and consequently the *Sma*I-group-10 primer pair was replaced by a primer pair based on the methicillin-resistance gene (*mec*A). This new primer actually increased the discriminatory power of the multiplex typing technique by allowing community strains MSSA476 and MW2 to be discriminated (Fig. 4.15).

Figure 4.14: *Sma*I-group 10 sequences of two *S. aureus* (8325 and COL) with SNP in *Sma*I-site of other strains (red rectangle).
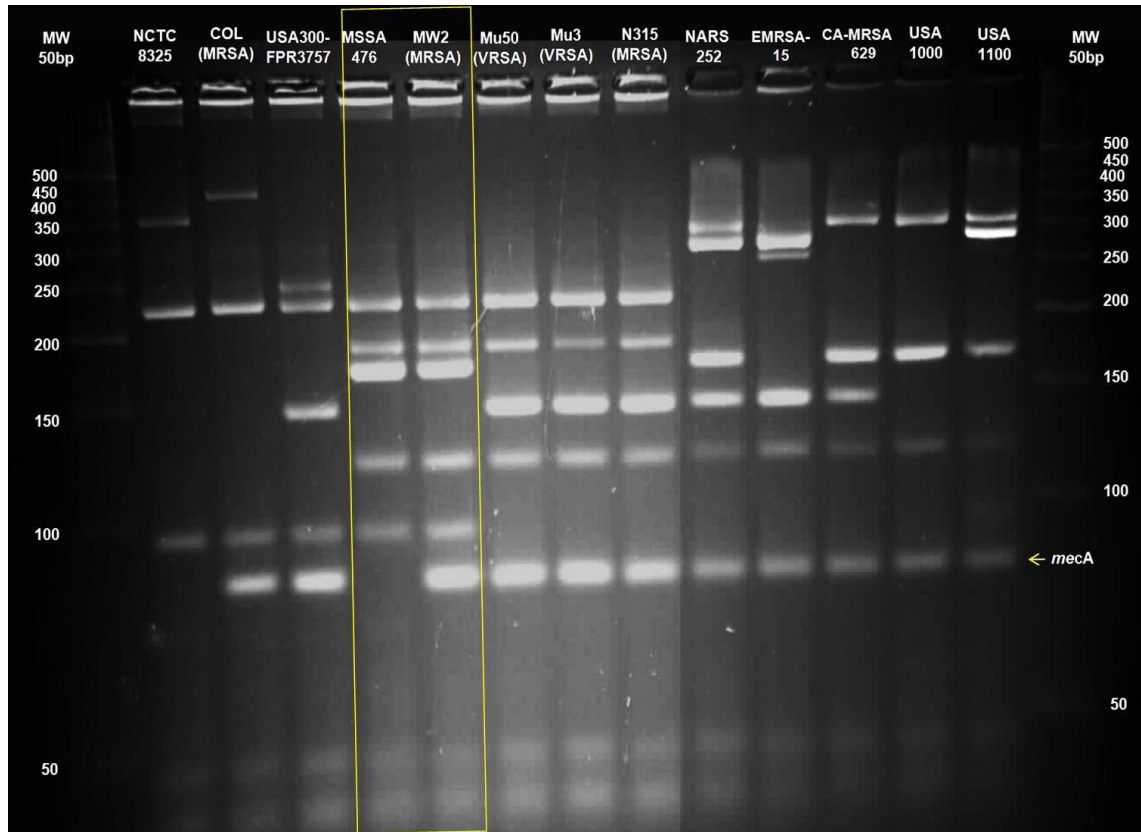
Figure 4.15:   The *Sma*I-profiles of thirteen reference *S. aureus* strains with all ten primer pairs groups after replacing the *Sma*I-group 10 primer pair with a *mec*A primer pair. The yellow box indentifies the profiles of two closely related community strains, MSSA476 and MW2, that were not previous distinguishable. MW= molecular size markers.

4.4.8 Validating the novel typing method with rapidly extracted DNA

The above studies were carried out on purified chromosomal DNA, isolated as described in Section 2.5.2b. To confirm the validity of a new method with rapidly extracted DNA, a necessary prerequisite for use in a routine clinical laboratory, four strains (NCTC 8325, EMRSA-15, MRSA252 and a PVL-positive MRSA) were tested using DNA extracted from a colony removed from an agar plate by boiling with or without a short pre-treatment with lysostaphin. As shown in Figure 4.16, the performance of the primer pairs was excellent when the DNA was extracted by pretreating the cells with lysostaphin prior to boiling. In the absence of lysostaphin pretreatment, some of the expected PCR products were either very faint or missing altogether (yellow arrows). In this experiment the specificity of the assay was assessed using two sequenced coagulase-negative staphylococci (CNS) strains (methicillin resistant *S. epidermidis* RP62A and methicillin sensitive *S. epidermidis* 12228). With

the exception of the *mecA* primer pair, the multiplex primers were shown to be specific
S. aureus since no other PCR products were generated from either of the CNS (Fig.
4.16).
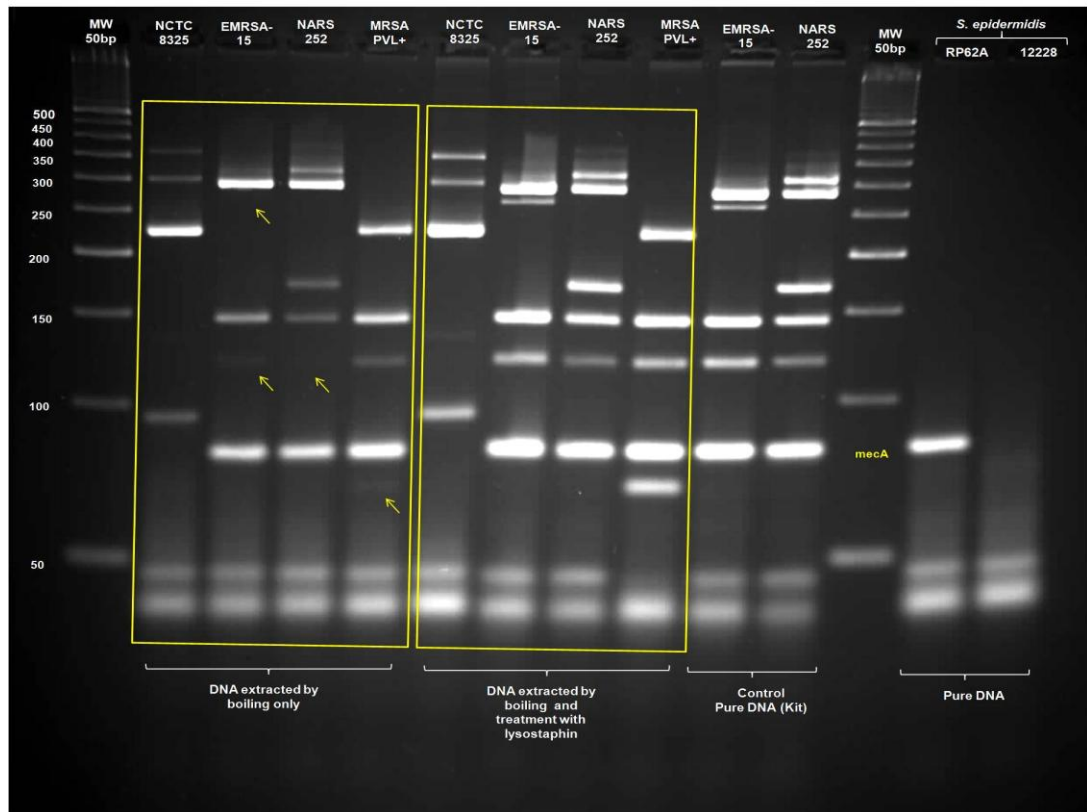
Figure 4.16:   *Sma*I-Multiplex PCR profiles of four unrelated *S. aureus* strains (NCTC 8325,
EMRSA-15, MRSA252 and a PVL-positive MRSA) using DNA extracted by
only boiling with or without lysostaphin pretreatment. The yellow arrows refer
to missed bands. On the right of the gel two sequenced *S. epidermidis* strains
were tested to determine specificity of *Sma*I-Multiplex PCR assay.

As the final optimization step, new primers for *Sma*I-groups 2, 4 and 5 (variable
lengths) were redesigned to improve their performance and amplicon band sharpness on
the gel. The *mecA* primer pair was also redesigned to generate ~500bp of *mec*A instead
of 82bp. The impact of these new primers is given in Section 4.4.9.

4.4.9 The typeability, discriminatory power and reproducibility of a novel
   *Sma*I-multiplex PCR technique

In this experiment fourteen reference strains (11 sequenced strains and 3 well
known CA-MRSA strains) were typed by the *Sma*I-multiplex PCR with all ten primer
pairs (including *mec*A). The reference strains used were: NCTC8325, MRSA252,
MSSA476, COL, JH9, N315, Mu3, Mu50, MW2, USA300FPR3757, EMRSA-15,

USA1000, USA1100 and CA-629. Chromosomal DNA was added at a final concentration of 10ng/µl and the PCR reaction conditions were the same as those used for the phase II optimization. Firstly, it can be seen (Fig. 4.17) that the performance of new primer pairs for *Sma*I-groups 2, 4 and 5 (yellow arrows) was clearly an improvement and their products were distinct and sharp in all strains.

All of the reference strains were typeable and the discriminatory power of the new method was both excellent and reproducible (more than 3 times with same profiles). The strains had distinct profiles with the exception of the closely related hospital strains Mu50, Mu3 and N315 that had identical profiles. The developed technique also distinguished between closely related community MSSA strains MSSA476 and MW2, community MRSA strains USA1000, USA1100 and CA-629 and to hospital-acquired MRSA strains EMRSA-15 and MRSA252 (EMRSA-16) (Fig. 4.17).
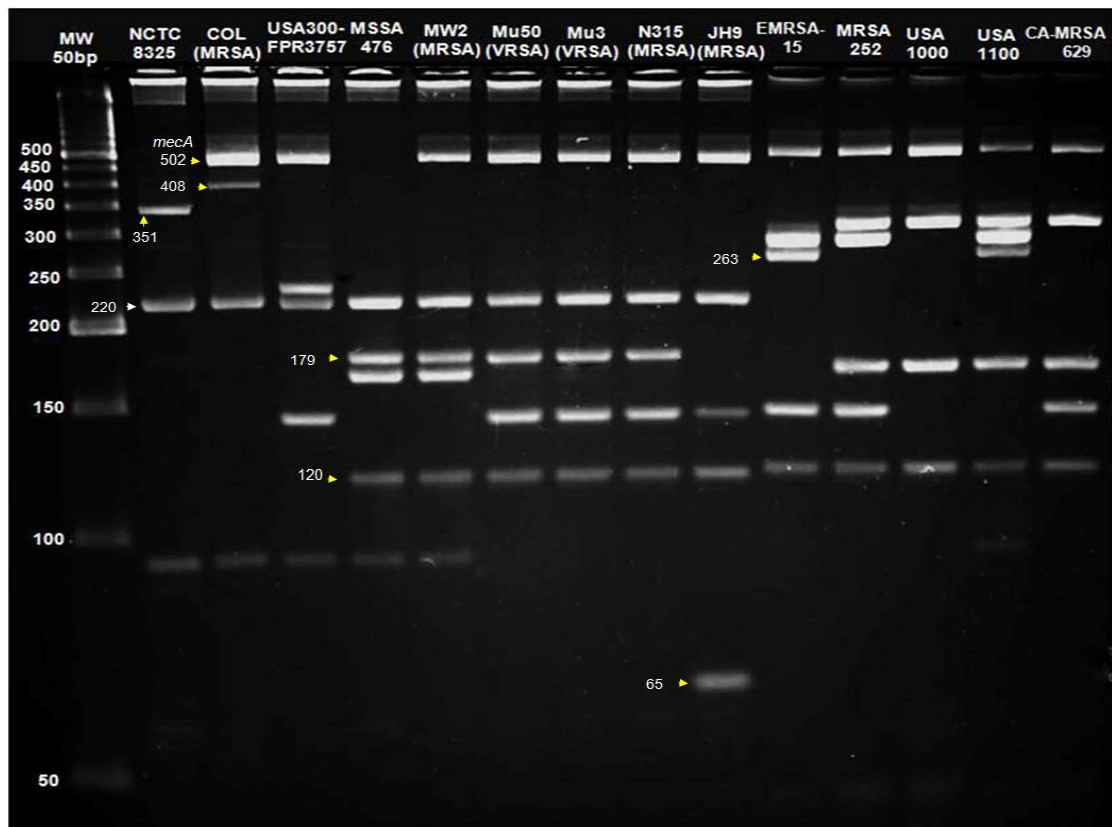
Figure 4.17:   The *Sma*I-multiplex PCR profiles of eleven reference *S. aureus* strains and three unrelated community-MRSA strains with all ten primer pairs groups as the last step of optimization of a novel typing technique. Yellow arrows refer to redesigned primers to enhance their performance. MW 50bp = 50 base pair molecular size markers. (Representative gel of 3 times repeating experiments).

4.4.10 PFGE, MLST and SCC*mec* type of reference *S. aureus* strains

PFGE was performed for all those strains typed by *Sma*I-multiplex PCR (Fig. 4.17) except strains Mu3 and CA-629. As can be seen from Figure 4.18, all strains showed distinguishable *Sma*I-PFGE patterns including closely related strains Mu50 and N315 that showed identical *Sma*I-multiplex PCR. Strains COL and USA300 showed closely related PFGE patterns and in the same situation with MSSA476 and MW2, and JH9, N315, and Mu50. EMRSA-15, MRSA252, USA1000 and USA1100 have slightly close *Sma*I-PFGE profiles (Fig. 4.18).
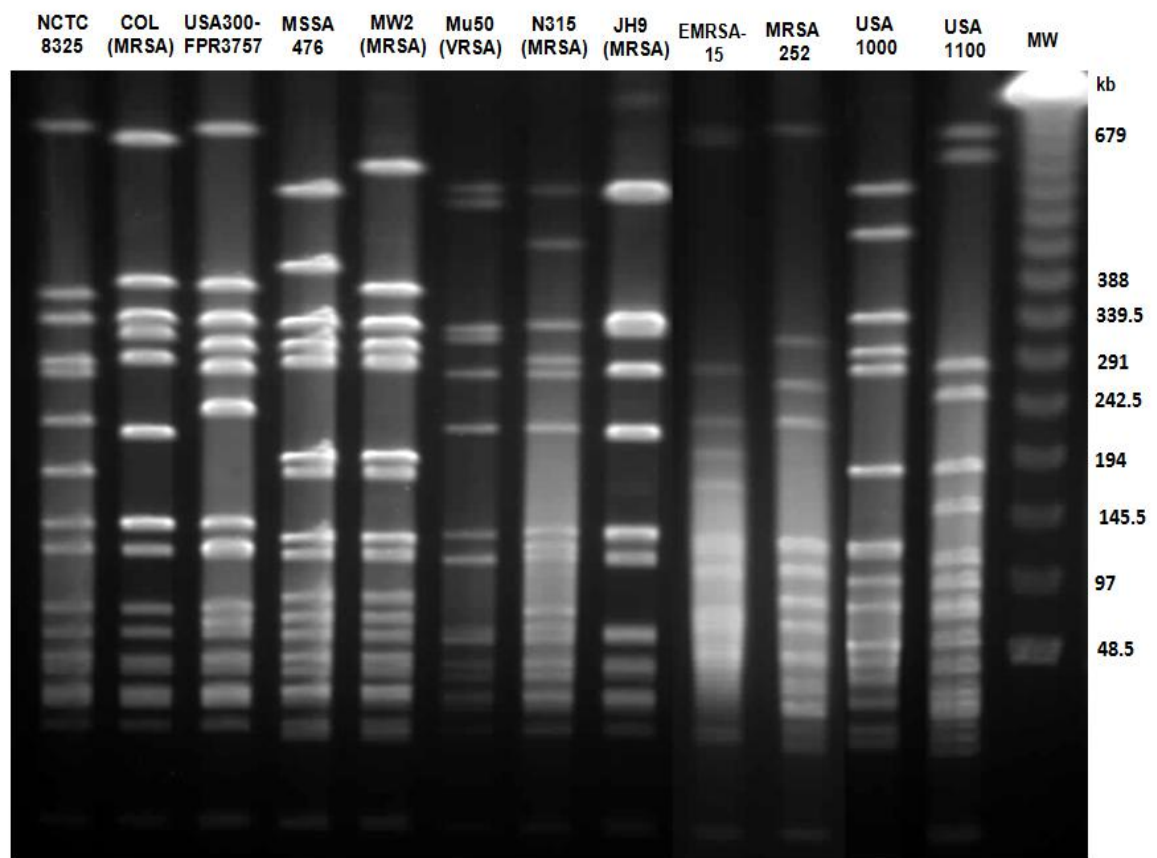
Figure 4.18: *Sma*I-PFGE patterns of ten reference *S. aureus* strains and three unrelated community-MRSA strains that were typed by *Sma*I-multiplex PCR in Figure 4.17. MW= molecular size marker (lambda ladder). Kb= kilo base.

All reference strains have known sequence types (STs) and SCC*mec* types except ST of CA-629 that was identified in this study. Nine STs were found among those fourteen *S. aureus* strains. The four strains (JH9, N315, Mu50 and Mu3) have an identical MLST type and SCC*mec* type: ST5-SCC*mec*-II. The very closely related strains MSSA476 and MW2 also have the same MLST type: ST1. Strains NCTC8325 and USA300 have ST8 whereas COL strain has ST250-SCC*mec*-I. MRSA252

(EMRSA-16) and CA-MRSA USA1100 harbour closely related STs: ST36-SCC*mec*-II and ST30-SCC*mec*-IV respectively. Also closely related ST59-SCC*mec*-IV and ST87-SCC*mec*-V were possessed by CA-MRSA USA1000 and CA-629 respectively while EMRSA-15 has ST22-SCC*mec*-IV.

4.4.11 Analysis and comparison of a novel *Sma*I-multiplex PCR results with PFGE and MLST data

In order to determine and evaluate the discriminatory power of the novel *Sma*I-multiplex PCR typing method, an analysis was carried out on 14 *S. aureus* reference strains to compare the performances of the PCR, PFGE and sequence typing. The fourteen reference strains used in the comparative study were: NCTC8325, MRSA252, MSSA476, COL, JH9, N315, Mu3, Mu50, MW2, USA300FPR3757, EMRSA-15, USA1000, USA1100 and CA-629. (Strains Mu3 and CA-629 were not used in the PFGE).

In general, the results were comparable the results for the *Sma*I-multiplex PCR, PFGE and MLST are summarised in Table 4.7. The banding patterns of *Sma*I-multiplex PCR and PFGE were analysed using the fingerprinting analysis software BioNumerics v3.50 (Applied maths) whereas the neighbour-joining tree of MLST was constructed using the allelic profiles and STs of 14 *S. aureus* reference strains.

The UPGMA dendrograms of the novel *Sma*I-multiplex PCR and PFGE profiles clustered the reference strains into the same four groups, although there were slight differences in the relationships between groups (Fig. 4.19). *Sma*I-multiplex PCR grouped NCTC8325, USA300 and COL in one contiguous cluster as PFGE (Fig. 4.19, yellow box) and each one has unique profile, though, strains NCTC8325 and USA300 shared same MLST allelic profile ST8 (3- 3- 1- 1- 4- 4- 3) and COL has close sequence type ST250 (3- 3- 1- 1- 4- 4- 16).
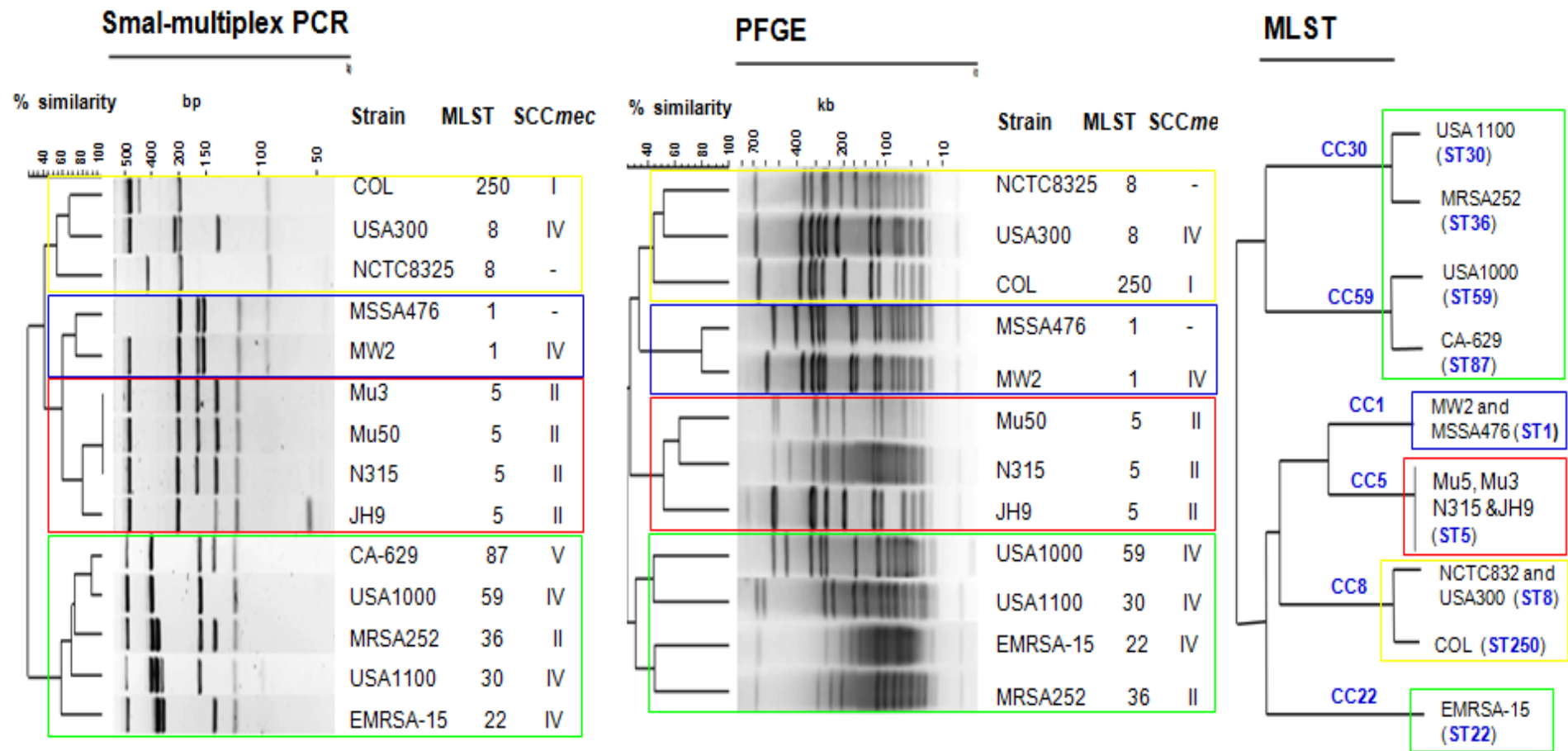
Figure 4. 19: The UPGMA dendrogram of *Sma*I-multiplex PCR and *Sma*I-PFGE patterns based on Dice similarity coefficient and the neighbour-joining tree of MLST illustrating the genetic relationship among 14 reference *S. aureus* strains. CC= clonal complex.

Two community-acquired strains, MSSA476 and MRSA-MW2, belong to the MLST allelic profile (ST1: 1-1-1-1-1-1-1), but had different *Sma*I-multiplex PCR profiles and were clustered together by *Sma*I-multiplex PCR (75% similarity) and PFGE (80% similarity) (Fig. 4.19, blue box).

Strains Mu50, Mu3 and N315 had the same MLST (ST5: 1-4-1-4-12-1-10) and *Sma*I-multiplex PCR profiles, while JH9, which was also a member of the ST5, was closely related to those strains and within same *Sma*I-multiplex PCR cluster. All four of these strains had the same SCC*mec* type, namely type II (Table 4.7). PFGE grouped Mu50, N315 and JH9 in one cluster and the similarity between Mu50 and N315 was 65% while JH9 showed 55% similarity to Mu50 and N315 (Fig. 4.19, red box). Mu3 was not analysed by PFGE.

*Sma*I-multiplex PCR clearly distinguished between the most common UK hospital epidemic strains EMRSA-15, MRSA252 (EMRSA-16) and also between USA community acquired MRSA strains CA-MRSA USA1000, USA1100 and CA-629. Those strains were grouped in single clusters by *Sma*I-multiplex PCR and PFGE, with each strain exhibiting a unique profile. CA-MRSA strains USA1000 and CA-629 have close but different *Sma*I-multiplex PCR profiles with 90% similarity and have sequence/*mecA* types ST59/SCC*mec*-IV and ST87/SCC*mec*-V, respectively. MRSA252 (EMRSA-16), with sequence/*mecA* types ST36/SCC*mec*-II, and USA1100, with have sequence/*mecA* types ST30/SCC*mec*-IV), were 85% related by *Sma*I-multiplex PCR. EMRSA-15 (ST22/SCC*mec*-IV) exhibited a unique *Sma*I-multiplex PCR profile with a 70% similarity to the MRSA252, USA1100, USA1000 and CA-629 cluster (Fig. 4.19, green box).

The results show that, in terms of discriminatory power, the order was PFGE> *Sma*I multiplex PCR>MLST. However, in terms of ease of use, the order was *Sma*I multiplex PCR>MLST>PFGE.

Table 4. 7: Typing data obtained by *Sma*I-multiplex PCR, PFGE, MLST and SCC*mec* analyses for14 reference *S. aureus* strains.

Strain	PFGE	*Sma*I- multiplex PCR type		MLST			SCC*mec* type
		*Sma*I-Type	*Sma*I-profile MecA- 1 - 2- 3- 4- 5 - 6- 7- 8- 9	ST	Allelic profile	Clonal Complex	
NCTC8325	A	1	0-0-0-1-0-1b-1-0-0-0	ST8	3- 3- 1-1- 4- 4-3	CC8	MSSA
COL	B	2	1-0-0-1-0-1a-1-0-0-0	ST250	3-3-1-1-4- 4 -16	CC8	SCC*mec*I
USA300-FPR3757	C	3	1-0-0-1-0-1c-1-1-0-0	ST8	3- 3- 1-1- 4- 4- 3	CC8	SCC*mec*IV
MSSA476	D1	4	0-1-0-1-1-1d-1-0-0-0	ST1	1-1-1-1-1-1-1	CC1	MSSA
MW2	D2	5	1-1-0-1-1-1d-1-0-0-0	ST1	1-1-1-1-1-1-1	CC1	SCC*mec*IV
Mu50	E	6	1-0-0-0-1-1d-1-0-0-0	ST5	1-4-1-4-12-1-10	CC5	SCC*mec*II
Mu3	ND	6	1-0-0-0-1-1d-1-0-0-0	ST5	1-4-1-4-12-1-10	CC5	SCC*mec*II
N315	E1	6	1-0-0-0-1-1d-1-0-0-0	ST5	1-4-1-4-12-1-10	CC5	SCC*mec*II
JH9	E2	7	1-0-0-0-1-1e-1-0-0-0	ST5	1-4-1-4-12-1-10	CC5	SCC*mec*II
EMRSA-15	F	8	1-0-1-0-1-0-1-1-0-1	ST22	7-6-1-5-8-8-6	CC22	SCC*mec*IV
MRSA252	G	9	1-1-0-0-1-0-0-1-1-1	ST36	2-2-2-2-3-3-2	CC30	SCC*mec*II
USA1100	J	10	1-1-1-0-1-0-0-0-1-1	ST30	2-2-2-2-6-3-2	CC30	SCC*mec*IV
CA-629	I	11	1-1-0-0-1-0-0-1-0-1	ST87	19-23-15-2-41-20-15	CC59	SCC*mec*V
USA1000	H	12	1-1-0-0-1-0-0-0-1-0	ST59	19-23-15-2-19-20-15	CC59	SCC*mec*IV

ST= sequence type.   CC= clonal complex.

4.5 Discussion

Since their emergence in the early 1980s, EMRSA-15 and EMRSA-16 have become the most common and prevalent hospital epidemic MRSA clones in the UK. More recently, community acquired MRSA strains have become more prevalent and their infections are no longer confined to the community but have started to replace HA-MRSA in some health care settings (D'Agata *et al.*, 2009). Understanding the transmission behaviour of those clones and distinguishing between them and their variants have emerged as major challenges for health care institutions. A wide variety of molecular techniques are currently used to type MRSA strains. These include PFGE, MLST, *spa* and SCC*mec* typing. PFGE is, by far, the most discriminatory of these, but has been supported in recent year by more rapid methods, such as multi-locus sequence typing.

Because PFGE and MLST require a high level of expertise and specialist equipment, and are technically demanding, they are usually beyond the resources of most routine clinical microbiology laboratories. In the UK, for example, potential outbreak strains are sent for typing to the Health Protection Agency (HPA) at Colindale, London, where it can take up to 4 weeks to process the samples and provide the supplying laboratory with the required epidemiology data. Hence, the development of a new typing technique is urgently required. Such a technique should have high discriminatory power and reproducibility that is similar to that of PFGE, but should be rapid and suitable for use in all routine clinical laboratories.

S. aureus is an ideal species in which to develop novel molecular epidemiological approaches. Eighteen *S. aureus* genomes have been completely sequenced and eleven are currently in progress. The availability of this large body of sequence information has allowed researchers to investigate in detail variations between the genomes of this versatile pathogen. Due to high similarity (97.7%-99.8% nucleotide identity) in core genomes of *S. aureus* strains, SNPs are one of the most effective ways of discriminating between individual strains (Lindsay and Holden, 2006).

This study was targeted at the development of a new typing tool that approaches the discriminatory power of PFGE, but is based on multiplex PCR and rapidly prepared chromosomal DNA. Similar to PFGE, this new tool is based around SNPs in *Sma*I restriction sites by incorporating discriminatory *Sma*I sites at the 3' end of the forward

primer. The presence of a PCR product should therefore reflect the presence or the absence of SNPs in specific *Sma*I restriction sites in the bacterial chromosomal DNA, with the size of the resulting products identifying the targeted *Sma*I site. The DNA sequences of eighteen *S. aureus* strains NCTC8325, MRSA252, MSSA476, COL, RF122, JH1, JH9, N315, Mu3, Mu50, MW2, Newman, USA300FPR3757, USA300TCH1516, ED98, LGA251, TW20 and EMRSA-15 were therefore used in a bioinformatical analysis to determine the number and location of their *Sma*I restriction sites. The number of *Sma*I restriction sites per genome was between 24 to 29 sites.

In the second phase of this analysis, a comparative analysis was carried out to determine the gene neighbourhoods for the syntenic regions of the *Sma*I-sites for all the sequenced strains. The gene neighbourhoods were grouped in a matrix that was used to determine their discriminatory power. Discriminatory *Sma*I-sites were chosen and their resolving power analysed in two stages. The first stage involved the evaluation of the six primer pairs with first a uniplex PCR and then multiplex PCR to differentiate between strains NCTC8325, EMRSA-15, EMRSA-16 and MRSA-PVL+. These primer pairs showed clear specificity and the test strains were, in most cases, distinguishable with unique profiles. However, EMRSA-16 and EMRSA-15 exhibited the same profile and *Sma*I-group 5 primers produced products of variable length. Subsequent analysis revealed that the latter was due to the presence of variable numbers of *Staphylococcus aureus* repeat (STAR) elements between the group 5 forward and reverse primers. STAR elements are CG-rich repeat sequences located in an intergenic region of the *S. aureus* genome (Cramton *et al.*, 2000). The presence of the STAR elements increased the discriminatory power of the *Sma*I-multiplex method.

In the third stage of development, four new *Sma*I-groups were selected and primers were designed to differentiate between the most common UK epidemic strains EMRSA-15 and EMRSA-16. As a result, both strains showed distinctive profiles and could be distinguished from each other and from other strains.

The discriminatory *Sma*I-sites were found to be located throughout the entire *S. aureus* genome (Fig. 4.4). The breadth of this distribution means that the *Sma*I-sites reflect variations of whole genome rather than relying on variations within a single gene (*e.g. spa* typing) or even seven genes (*e.g.* MLST).

Once the second optimization phase had been confirmed, in collaboration with Biogene LTD, UK, all ten primer pairs were redesigned for use in a single multiplex PCR reaction. This was done using Biogene proprietary Visual OMP^{TM} (Oligonucleotide Modeling Platform) DNA analysis software. The primer redesign and optimization included secondary structure, homology, annealing temperature and assay conditions. The ten PCR fragments were sized between ~ 69bp to 412bp.

The redesigned primers were multiplexed in a single multiplex PCR with two different multiplex PCR master mixes (one prepared in current study and the Biogene master mix) against five *S. aureus* strains (NCTC 8325, EMRSA-15, MRSA252, NCTC 6571 and a PVL-positive MRSA). Significantly, the master mix that prepared in this study showed the best performance (Fig. 4.11). Moreover, the discriminatory power and optimal annealing temperature of the redesigned primers were initially determined against four unrelated *S. aureus* strains NCTC8325, MRSA-PVL+, EMRSA-15 and MRSA252. These primers showed a clear discrimination between the strains, with an optimal annealing temperature of 56˚C. *Sma*I-groups 3, 4 and 10 generated false-positive amplicons in some strains (Fig. 4.12). In the case of groups 3 and 4, these were subsequently shown to be due to the presence of syntenic *Sma*I sites with SNPs; CCCTGG in the case of group 3 and CCCAGG in the case of group 4. SNPs were also found in their reverse primers. In the case of group 10 primers, the presence of a syntenic *Sma*I sites with a SNP was discovered; CCCAGG. Since these *Sma*I groups were highly discriminatory, and excluding them would decrease the discriminatory of a developed technique, the Visual OMP software was used in an attempt to redesign new primers for these sites. In the case of groups 3 and 4 this was successful and PCR products were amplified from the expected strains. However, it was not possible to design a primer pair for *Sma*I group 10.

The redesigned primer pairs 3 and 4 were used to replace the existing primers for these groups and a primer pair for the *mecA* gene was introduced into the multiplex reaction. Analysis of nine sequenced *S. aureus* strains gave distinct profiles for each strain with exception for hospital strains Mu50, Mu3 and N315 (Fig. 4.15).

The second advance was the development of a rapid DNA extraction protocol for the multiplex PCR. The extraction protocol takes 25 minutes and includes just two steps, the treatment of bacterial cells from one or two colonies with lysostaphin followed by boiling in water. The DNA extraction method was evaluated on four

unrelated *S. aureus* strains in comparison with DNA extracted only by boiling and purified DNA. The DNA extracted by lysostaphin pretreatment and boiling generated profiles that were as clear as those generated with purified DNA. In the case of DNA extracted by boiling alone, some of the expected PCR products were very faint or missed altogether (yellow arrows, Fig. 4.16).

The typeability, discriminatory power and reproducibility of a novel *Sma*I-multiplex PCR were validated on fourteen reference strains. Eleven of them are sequenced strains (NCTC8325, MRSA252, MSSA476, COL, JH9, N315, Mu3, Mu50, MW2, USA300FPR3757 and EMRSA-15) and three are well known community-MRSA (USA1000, USA1100 and CA-629). In general, all strains were typeable with high discriminatory power. All strains tested appeared distinguishable with distinct profile with the exception of very closely related HA-VISA strains Mu50, Mu3 and N315 that, as expected, showed the same profile (Fig. 4.17). These strains share the same sequence (ST5) and SCC*mec* (II) types and showed close a relationship in a molecular evolutionary genetics analysis (MEGA) study (Highlander *et al.*, 2007).

In order to compare the discriminatory power of a novel *Sma*I-multiplex PCR method with the most popular typing methods, all of the standard *S. aureus* strains (except Mu3 and CA-629) that were tested by *Sma*I-multiplex PCR were further subjected to PFGE analysis. Those strains have well-known MLST and SCC*mec* types. Since the multiplex is based on the similar principles to that of PFGE, the BioNumerics fingerprint analysis software was used to compare the outcomes from the *Sma*I-multiplex PCR profiles and *Sma*I-PFGE patterns. The UPGMA dendrogram of *Sma*I-multiplex PCR and PFGE, and neighbour-joining tree of MLST showed that the correlation between the three typing method was excellent (Fig. 4.19). The discriminatory power of a novel *Sma*I-multiplex PCR seemed to be between PFGE and MLST. The cluster analysis showed that the *Sma*I-multiplex PCR grouped strains into the same four clusters as PFGE and almost matched that of the MLST types as well (Fig. 4. 19). For example, the clonal complex (CC8) strains NCTC8325, USA300 and COL were grouped in one tight cluster by both *Sma*I-multiplex PCR and PFGE. Strains NCTC8325, USA300 showed different *Sma*I-multiplex PCR profiles though both have identical MLST type (ST8). In addition, the HA-MRSA COL strain showed a unique *Sma*I-multiplex PCR profile, albeit on that was relatively close to USA300. COL has

ST250, which is a single locus variant (SLV) of ST8 at the *yqiL* locus and both STs belong to same clonal complex (CC8) (Lindsay and Holden, 2006).

Interestingly, two community-acquired strains, MSSA476 and MRSA-MW2, have very close but distinguishable *Sma*I-multiplex PCR and PFGE profiles. In the case of *Sma*I-multiplex PCR, both showed identical profiles with the exception of a fragment generated from the *mec*A gene. In a comparative study, all orthologous gene pairs in the core genomes of MW2 and MSSA476 showed 99.8% of nucleotide identity (Lindsay and Holden, 2006). These strains also share an identical sequence type (ST1).

Although, HA-MRSA N315 and vancomycin-intermediate-resistant strains Mu50, Mu3 and JH9 are closely related and share same sequence type ST5 and SCC*mec*-II, *Sma*I-multiplex PCR discriminated JH9 from strains Mu50, Mu3, and N315 (Fig. 4.19, red box). This cluster of strains (Mu50, Mu3, N315 and JH9) was closely related to the MW2 and MSSA476 cluster (60% of similarity) and both clusters belong to the closely related sequence types, ST5 and ST1 respectively (Holden *et al.*, 2004).

In the UK, the hospital epidemic clones EMRSA-15 and ERSA-16 are responsible for more than 95% (60.2 and 35.4%, respectively) of *S. aureus* bacteraemia (Johnson *et al.*, 2001) and this percentage increased since 2007 to 85 and 9%, respectively (Ellington *et al.*, 2010). Moreover, CA-MRSA has been involved in hospital-acquired MRSA, and in USA, for example, CA-MRSA USA300 was responsible for 33% of hospital-acquired MRSA infections in a study conducted by Huang *et al.* (2006). This makes the distinction between the most prevalent HA-MRSA and CA-MRSA an issue in many healthcare settings.

In this study, the developed *Sma*I-multiplex PCR discriminated between the most common UK hospital epidemic strains EMRSA-15, MRSA252 (EMRSA-16, also called USA200) and also between well known USA community-acquired MRSA strains: USA1100, USA1000 and CA-629. Although, those strains were grouped in single tight cluster, each strain showed unique *Sma*I-multiplex PCR, MLST and PFGE profiles (Fig. 4.19, green box).

CA-MRSA strains, USA1000 and CA-629 showed closely related but distinguishable *Sma*I-multiplex PCR profiles and sequence types (ST59 and ST87, respectively). The ST87 is an SLV of ST59 at the *pta* locus and both clones belong to same clonal complex (CC59) (Table 4.7) (Coombs *et al.*, 2010). Similarly, MRSA252

(EMRSA-16) (ST36) and USA1100 (ST30) showed closely related *Sma*I-multiplex PCR profiles. ST36 is also an SLV of ST30 at the *pta* locus and both sequence types represent the same clonal complex (CC30) (Table 4.7). EMRSA-15 (ST22/SCC*mec*IV) was slightly divergent with a unique *Sma*I-multiplex PCR profile but it showed about 70% similarity to cluster of MRSA252, USA1100, USA1000 and CA-629 (Fig. 4.19, green box).

We proposed a simple nomenclatural system for a novel *Sma*I-multiplex PCR profiles based on the presence and absent of all nine *Sma*I-sites according to their order on the genome (*i.e. mec*A-1 to *Sma*I-9). For example, *Sma*I-multiplex PCR type 2 of the COL strain, was given the *Sma*I-profile 1-0-0-1-0-1a-1-0-0-0, with the binary numbers (1 = present, 0 = absent) referring to the presence or absence of an amplicon at a specific allele. The letter on the group 5 refers to the length of the variable PCR products generated by *Sma*I-group 5 primer pair (Table 4.7).

In conclusion, a novel PCR-based typing technique, which is relatively rapid, reproducible and discriminatory, has been developed for use in routine clinical microbiology laboratories. The technique is more discriminatory than MLST and has the potential to be as discriminatory as PFGE. It does not require expertise and uses standard clinical laboratory apparatus (PCR machine and agarose gel electrophoresis). The required DNA is extracted from lysostaphin-treated boiled colonies and the results are available within 4-6h. This technique could to be adapted for real-time multiplex PCR assay to reduce the processing time to <60 minutes and for the data to be processed automatically.

Chapter 5

Evaluation of the discriminatory power of the *Sma*I-multiplex PCR typing technique against MSSA and MRSA obtained during potential outbreaks

5. Evaluation of the discriminatory power of the *Sma*I-multiplex PCR typing technique against MSSA and MRSA obtained during potential outbreaks

5.1 Introduction

An outbreak of an infectious agent is defined as the occurrence two or more cases of infection caused by the same microorganism, or an unexpectedly large number of cases of infection that exceeds that normally observed (van Leeuwen, 2003). MRSA outbreaks occur frequently in hospitals units, with intensive care units (ICUs), neonatal units, surgical units and burn units and ICUs the most often affected. ICUs in particular, are a principal source of multi-drug resistant MRSA outbreaks (Albrich *et al.*, 1999; Embil *et al.*, 2001; Song *et al.*, 2010).

Colonised and infected individuals, whether patients or healthcare workers, are considered to be the main reservoir for MRSA, with the nose being the most common site of colonisation. Furthermore, direct contact by hand is the main route for the spread of MRSA among patients, with healthcare workers playing a key role in the dissemination of these bacteria. In addition, in many cases where an outbreak has been investigated in detail, a combination of the hospital environment and insufficient cleaning have been identified as major risk factors which contributed to the outbreak (Embil *et al.*, 2001).

During the 1960s-1970s, the incidence of MRSA outbreaks was low and due to sporadic strains. However, the prevalence of MRSA increased during the 1980s and many epidemic outbreaks were reported during this period (Hryniewicz, 1999). Most hospital outbreaks were confined to what are now recognised as HA-MRSA, however, more recently both CA-MRSA and high virulence HA-MRSA (PVL+) have been reported in some outbreaks (Taneike *et al.*, 2006). In the UK, epidemic clones EMRSA-15 and EMRSA-16 are responsible for most hospital outbreaks. Both clones have a high tendency to spread and the differentiation between these and other clones is important in monitoring this spread.

The purpose of any epidemiology study, such as the investigation of individual outbreaks, is to identify the potential source(s) of infection and to monitor dissemination. The early identification of an outbreak, making use of a rapid, precise and simple MRSA typing technique, could lead to prompt and effective precautions that

avoid the further spread of the infection. The choice of an epidemiological tool basically depends on the purpose of the analysis (short and long term) and the availability of the necessary expertise, and resources. Due to their increased accuracy, molecular typing methods (*e.g.* PFGE, MLST and SCC*mec*) have tended to replace more traditional phenotyping methods (*e.g.* phage typing) (Singh *et al*., 2006). However, both PFGE and MLST are technically demanding and expensive and they are not used routinely in most hospital clinical laboratories in the UK. Therefore, potential outbreak strains are sent to a reference laboratory (e.g. HPA, at Colindale, London) for analysis. This makes the investigation and monitoring of MRSA outbreaks difficult which, in turn limits, the effective decision making of the infection control team.

*Sma*I-multiplex PCR typing is based on single nucleotide polymorphism (SNP) in *Sma*I restriction sites, the same sites that are used in the PFGE analysis of MRSA strains. When used against sequences strains, the technique was shown to be reproducible and to exhibit good discriminatory power. It was also easy to perform and interpret, and results are available with 4-6 hours. *Sma*I-multiplex PCR was more discriminatory than MLST but less than PFGE against reference *S. aureus* strains. For the analysis of potential outbreaks, a molecular typing method should be able to differentiate between outbreak and sporadic strains. Therefore, the discriminatory power of the *Sma*I-multiplex PCR typing technique was investigated on isolates obtained from potential outbreaks in local hospitals.

5.2 Study Aims

The aim of the studies in this chapter was to evaluate the discriminatory power of the *Sma*I-multiplex PCR typing technique against MSSA and MRSA obtained during potential outbreaks in NHS Trust Hospitals in the North East of England. The resulting epidemiology data was provided to the infection control teams within 4-6 hours of receipt at the Medical School. Each analysis included known types of UK Epidemic-MRSA strains that were obtained from the Health Protection Agency (HPA).

5.3 Methods

5.3.1 Sources of strains

5.3.1a Isolates of outbreaks

Isolates from seven suspected outbreaks (in total 59 MSSA and MRSA isolates) were received over a period of four months. The strains were isolated from three major NHS Trust Hospitals in North East of England, the Freeman Hospital (FH), the Royal Victoria infirmary (RVI) and the Newcastle General Hospital (NGH). In each case the outbreaks were defined as such by clinicians, infection control teams and the microbiological laboratory. The vast majority of the strains were isolated from hospitalised patients, while a few were isolated from members of staff and the environment.

5.3.1b Health Protection Agency (HPA) strains

Twenty-four epidemic MRSA strains with known PFGE types were received from HPA to be examined by the *Sma*I-multiplex PCR typing method.

5.3.1c Other clinical MRSA isolates

See Section 2.3.2

5.3.1d *S. aureus* control strains

See Section 2.3.1

5.3.2 Bacterial growth

Bacterial isolates received on blood agar plates were tested directly by the *Sma*I-multiplex PCR technique whereas those received on agar slopes were subcultured on brain-heart infusion (BHI) agar prior to typing. In order to keep stocks for further tests, a single colony of each isolate was suspended in 10ml BHI broth and incubated overnight at 37˚C, with shaking at 200 rpm and stored in 20% of glycerol at -80˚C till required.

5.3.3 Extraction of chromosomal DNA from MRSA

5.3.3a Extraction of the DNA by lysostaphin treatment using the DNeasy kit (QIAGEN).

See Section 2.5.2a

5.3.3b Extraction of the chromosomal DNA by cell mechanical breakage using a DNeasy kit (QIAGEN)

See Section 2.5.2b

5.3.3c Rapid Extraction of chromosomal DNA using boiling with lysostaphin method.

See Section 4.3.6c

5.3.4 Molecular identification

5.3.4a Multiplex PCR diagnostic assay for detection *mecA, c*oa and PVL

A multiplex PCR diagnostic assay was designed to detect two diagnostic genes, *mecA* and *coa*, and the PVL virulence factor. The presence of the *coa* gene was used to confirm the isolate was *S. aureus* whereas the *mecA* was used to confirm the isolate was methicillin-resistant. The presence of the PVL virulence factor was used to identify leukocidin (PVL) producers.

5.3.4a.i Primer design

The sequences of all three genes were obtained from NCBI website and the primer pairs were designed according to following parameters: length 18-30-mers, %GC 35-55, T_m 50-65˚C. The design of the *coa* and *mecA* primer pairs was based on regions that were highly conserved across several versions of these genes while that of PVL primer pair included a portion of the two genes, *lukF-PV and lukS-PV,* that encode PVL. The designed also ensured that the various primer pairs generated different sizes of PCR fragments (*coa* 248bp, *mecA* 346bp, and PVL 448bp) so they could be discriminated by agarose gel electrophoresis. Oligonucleotides were obtained from MWG Biotech GmbH and rehydrated by the addition of sterile milli-Q water to a concentration of 100μM and stored at -20ºC. The sequences of primers are listed in Table 5.1.

Table 5. 1: Primers of three diagnostic genes (*mecA* and *coa* and PVL) which used in multiplex PCR diagnostic assay that designed in this study**.**

Primer	Sequences	T_m	Length of PCR product
coa-For	5'-GAAGAGAAGAAAGTTGAAGAACC- 3'	57.1˚C	248bp
coa-Rev	5'-CTTAAAGATGGTCTGTTTTGTTCC- 3'	57.6˚C	
mecA-For	5'-GCAATCGCTAAAGAACTAAGTA-3'	54.7˚C	346bp
mecA-Rev	5'-TGACACGATAGCCATCTTC-3'	54.5˚C	
lukS/F-For	5'- GGCTCAGGAGATACAAGTG-3'	56.7˚C	448bp
lukS/F-Rev	5'- GTTTCCAGCAGCTTTGAGTA-3'	55.3˚C	

T_m = melting temperature

5.3.4a.ii PCR amplification

The presence of all three genes (*coa*, *mecA* and *PVL*) was tested in a multiplex PCR against all MSSA and MRSA isolates. Each PCR reaction (50µl) contained 200 µM of dNTP; 5 µl of 10x reaction buffer; 1.5 mM of $MgCl_2$; 4 µl of 100x BSA; 0.8 µM of PVL primers; 0.4µM of *mecA*, 0.3 µM of *coa*; 1 µl of 5 U/µl Taq Polymerase (Fermentas, Germany); 3 µl of DNA template. PCR amplification was carried according to the reaction conditions: 2 min at 96˚C initial denaturation, followed by 35 cycles of 96˚C for 40sec, 56˚C for 40 sec and 72˚C for 40 sec, with a final extension at 72˚C for 2 min.

5.3.5 Agarose electrophoresis

Six microlitres samples were loaded on 1.6% agarose containing 0.5µg/µl ethiduim bromide. The gel was electrophoresed in 1x TBE buffer at 110V for 100 min. A 100bp ladder was used as a molecular size marker. DNA bands were visualised under UV light.

5.3.6 Molecular typing

5.3.6a. *Sma*I-multiplex PCR typing technique

  *Sma*I-multiplex PCR typing was carried out for all isolates according to the method in Section 4.3.7b phase II, using primers in Table 4.1. DNA was extracted by the rapid extraction method (boiling with lysostaphin) as described in Section 4.3.6c

5.3.6a.i Data analysis of *Sma*I-multiplex PCR typing

Gel images were saved as TIFF file to be analysed by the fingerprinting analysis software, BioNumerics v3.50 (Applied Maths). The analysis of *Sma*I-multiplex PCR typing was based on band differences and a clustering dendrogram was generated by the unweighted-pair group method with arithmetic mean (UPGMA), using the Dice similarity coefficient.

5.3.6b PFGE

PFGE was carried out according to the protocol in Section 2.6.1, and PFGE profiles were analysed as described in Section 4.3.10

5.3.6c MLST

MLST was performed according to the protocol in Section 2.6.2. PCR products were purified using the QIAquick PCR purification kit (QIAgen), as described in Section 2.6.2b.ii. The concentration of DNA was adjusted (see Section 2.6.3) and the products were sent for sequencing (MWG Biotech, Germany). Nucleotide sequences were compared with the MLST database through website (http://www.mlst.net) for assignment of allelic profiles and sequence type (ST).

5.3.6d SCC*mec* typing

SCC*mec* types were verified according to the method in Section 2.6.4

5.4 Results

5.4.1 Molecular epidemiology of multiple MSSA and MRSA outbreaks using *Sma*I-multiplex PCR typing.

Seven outbreaks were reported in different NHS Trust Hospitals in north east of England and about 60 MSSA and MRSA strains were isolated from these outbreaks. Seven isolates from a single outbreak were carbon dioxide (CO_2) dependent methicillin resistant *Staphylococcus aureus* (MRSA). The isolates of each outbreak were typed by *Sma*I-multiplex PCR typing and the epidemiology data was sending to the infection control office at the Freeman Hospital on the same day.

5.4.1a Outbreak A

In June 2008, we received six MRSA isolates that were isolated from different patients on the regional liver unit (RLU) at the Freeman Hospital. One of these isolates, isolated from a screening sample (nasal swab) of a liver transplant recipient, showed small poorly growing green colonies on chromogenic MRSA media (chrom ID MRSA, bioMerieux) after 24 hours aerobic incubation. This isolate repeatedly failed to grow after 24-48 hours aerobic incubation on blood agar for susceptibility testing. However, this isolate grew heavily when subcultured on blood agar and incubated for overnight at 37°C in 5% CO_2. A further five CO_2-dependent MRSA isolates were found among patients and a member of staff at the same unit. These isolates had unusual growth characteristics in that they require an increased level of carbon dioxide (5%) for growth and were also resistant to erythromycin, clindamycin, moxifloxacin and trimethoprim. A seventh case of a CO_2-dependent MRSA was reported in a patient in the same unit and sent to us later.

5.4.1a.i Molecular diagnosis

The presence of *coa*, *mecA* and PVL genes was examined by diagnostic PCR which showed that all isolates were positive for *coa* and *mecA* but negative for PVL.

5.4.1a.ii Molecular typing using *Sma*I-multiplex PCR typing.

All CO_2-dependent MRSA isolates were tested by *Sma*I-multiplex PCR typing and this revealed that the isolates were indistinguishable from EMRSA-15 (Fig.5.1).
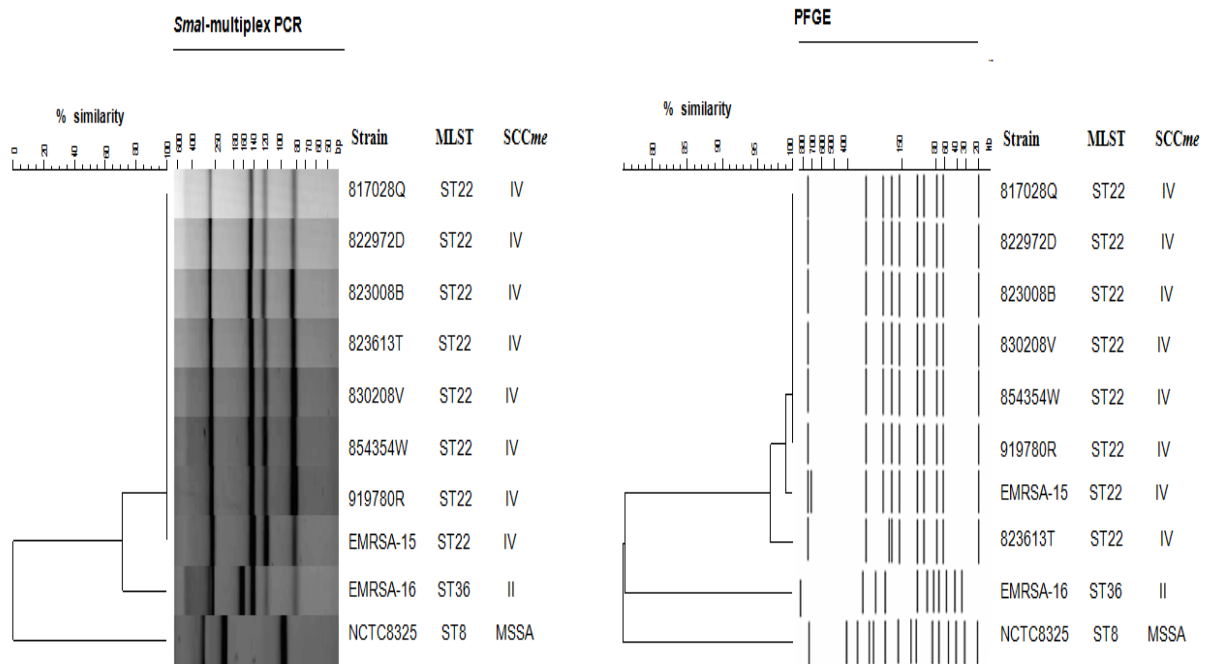
Figure 5. 1:   The UPGMA dendrogram of *Sma*I-multiplex PCR and *Sma*I-PFGE patterns based on Dice similarity coefficient, illustrating the genetic relationship among outbreak of seven CO_2-dependent MRSA isolates.

5.4.1a.ii Molecular typing using PFGE, MLST and SCC*mec* typing

All seven CO_2-dependent MRSA isolates were typed using PFGE and the data confirmed that, with exception isolate 823613, the isolates had profiles that were identical to EMRSA-15. The PFGE profile of isolate 823613 differed only with respect to one band and is therefore a variant of EMRSA-15 (Fig.5.1).

The results of MLST and SCC*mec* typing, listed in Table 5.2, revealed that all of the CO_2-dependent MRSA isolates were ST22-SCC*mec*-IV, to the same as EMRSA-15.

Table 5. 2: Molecular epidemiology of carbon dioxide (CO_2)-dependent MRSA strains isolated from a liver unit outbreak.

MRSA strains no.	*Sma*I-Multiplex PCR epidemiology	Diagnostic PCR			Multilocus sequence typing (MLST)		SCC*mec* type	PFGE
		Coa	*mecA*	*PVL*	Sequence Type	Allelic profile		
817028Q	EMRSA-15	+	+	-	ST22	7-6-1-5-8-8-6	IV	A1
822972D	EMRSA-15	+	+	-	ST22	7-6-1-5-8-8-6	IV	A1
823008B	EMRSA-15	+	+	-	ST22	7-6-1-5-8-8-6	IV	A1
823613T	EMRSA-15	+	+	-	ST22	7-6-1-5-8-8-6	IV	A2
830208V	EMRSA-15	+	+	-	ST22	7-6-1-5-8-8-6	IV	A1
854354W	EMRSA-15	+	+	-	ST22	7-6-1-5-8-8-6	IV	A1
919780R	EMRSA-15	+	+	-	ST22	7-6-1-5-8-8-6	IV	A1
EMRSA-15	EMRSA-15	+	+	-	ST22	7-6-1-5-8-8-6	IV	A1
MRSA252 (EMRSA-16)	EMRSA-16	+	+	-	ST36	2-2-2-2-3-3-2	II	EMRSA-16
S.aureus NCTC8325	NCTC8325	+	-	-	ST8	3-3-1-1-4-4-3	-	NCTC8325 type

5.4.1b Outbreak B

On 28th of August 2009, three *S. aureus* isolates from a small outbreak at the Freeman Hospital. Those isolates were isolated from post-operative wounds of three patients in the cardio surgery unit (Table 5.3).

Table 5. 3: Isolation sites and hospital units of the outbreak B isolates.

Ref. no	Isolates	Source	Site of isolation	Hospital unit
241094X	MSSA	patient	Post-operative wound	Freeman cardio surgery
243759Q	MSSA	patient	Post-operative wound	Freeman cardio surgery
2445552	MSSA	patient	Post-operative wound	Freeman cardio surgery

5.4.1b.i Molecular diagnosis

The diagnostic PCR for the presence *mecA*, *coa* and PVL confirmed that all isolates were MSSA and one isolate (243759Q) was PVL positive (Fig.5.2, labelled red).
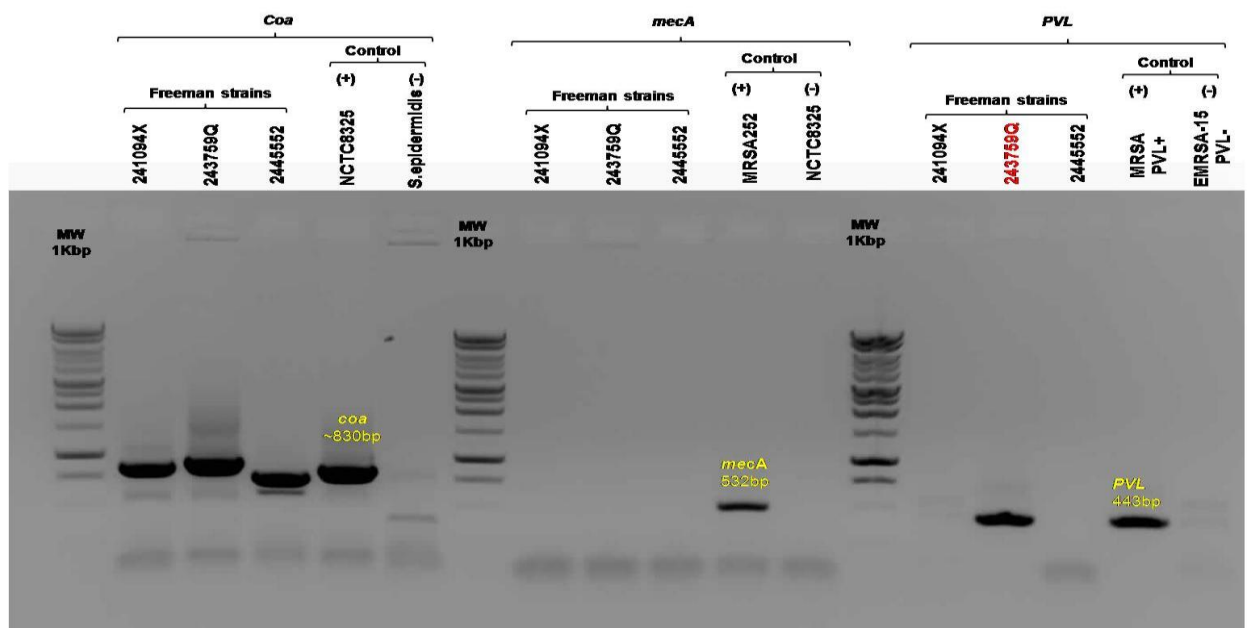
Figure 5. 2:   Diagnostic PCR for the presence of *mecA, coa* and PVL against an outbreak of three MSSA isolates**.**

5.4.1b.ii Molecular typing using a novel *Sma*I-multiplex PCR typing.

The three outbreak MSSA isolates were characterized using the *Sma*I-multiplex PCR. The results revealed that the three isolates displayed distinct but unrelated profiles (Fig. 5.3). Interestingly, MSSA isolate 2445552 exhibited a close relationship to the genotypic profile of MSSA NCTC 6571 (Oxford strain) and MRSA252 (EMRSA-16),

while the profile of MSSA isolate 243759Q was similar to that of EMRSA-15. The profile of MSSA isolate 241094X was unique and appears to be related to CA-MSSA strains.
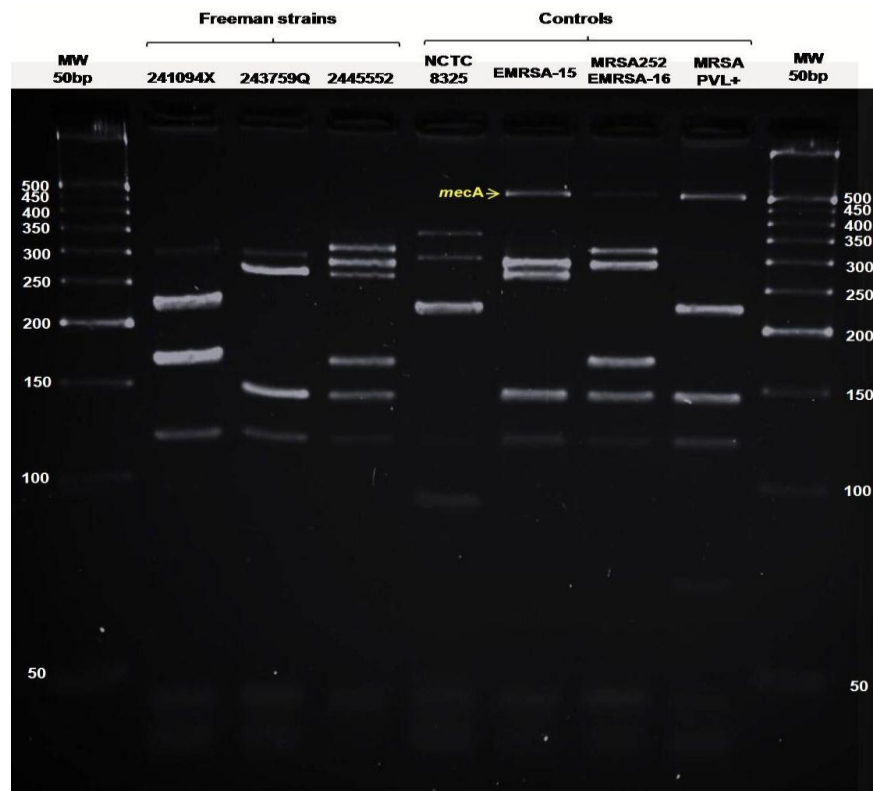
Figure 5.3: *Sma*I-multiplex PCR profiles of the outbreak B isolates.

5.4.1c Outbreak C

On 2nd of September 2009, two clusters of 17 *S. aureus* outbreak isolates were received from the Freeman Hospital. Cluster 1 consisted of eleven isolates that were isolated from the screening of patients and members of staff in a surgical ward (F5) at the Freeman Hospital. Cluster 2 contained isolates from six patients in the baby care unit of the RVI (Table 5.4). Cluster 2 isolates were also isolated from screening samples except isolate 246957K which was isolated from blood. The clinical laboratory at the Freeman Hospital identified them as MRSA isolates except for isolate 246957K, which showed a weak positive reaction in the penicillin binding protein latex test but was methicillin sensitive. The ward F5 patient from which isolate 148858X was isolated had been treated to eradicate MRSA colonization while the infant patient with isolate 063369Y had a previous history of hospitalization.

Table 5. 4:   Isolation sites and hospital units of the outbreak C isolates**.**

Ref. no	Isolates	Source	Site of isolation	Hospital unit
140842D	MRSA	Staff	Screening sample	Freeman surgical ward (F5)
148858X	MRSA	Patient	Screening sample	Freeman surgical ward (F5)
149683F	MRSA	Patient	Screening sample	Freeman surgical ward (F5)
149740A	MRSA	Staff	Screening sample	Freeman surgical ward (F5)
149941G	MRSA	Patient	Screening sample	Freeman surgical ward (F5)
155309B	MRSA	Patient	Screening sample	Freeman surgical ward (F5)
166824J	MRSA	Patient	Screening sample	Freeman surgical ward (F5)
180028B	MRSA	Patient	Screening sample	Freeman surgical ward (F5)
182548B	MRSA	Patient	Screening sample	Freeman surgical ward (F5)
182836N	MRSA	Patient	Screening sample	Freeman surgical ward (F5)
187132W	MRSA	Patient	Screening sample	Freeman surgical ward (F5)
063369Y	MRSA	Infant patient	Screening sample	RVI Baby care unit (R35)
081157C	MRSA	Infant patient	Screening sample	RVI Baby care unit (R35)
231520G	MRSA	Infant patient	Screening sample	RVI Baby care unit (R35)
234723Y	MRSA	Infant patient	Screening sample	RVI Baby care unit (R35)
237320L	MRSA	Infant patient	Screening sample	RVI Baby care unit (R35)
246957K	MSSA	Infant patient	Blood	RVI Baby care unit (R35)

RVI= Royal Victoria Infirmary

5.4.1c.i Molecular diagnosis

The presence of the *coa* gene in all the isolates was confirmed by diagnostic PCR. Outbreaks isolates were *mecA* positive with exception isolate 246957K, which was *mecA* negative. None of seventeen isolates was PVL positive.

5.4.1c.ii Molecular characterization of an outbreak C using a novel *Sma*I-multiplex PCR.

All isolates were typed by *Sma*I-multiplex PCR and 11 isolates showed a profile that was identical to that of EMRSA-15 (Fig. 5.4, labelled yellow). In addition, isolate 149941G (Fig. 5.4, labelled orange) appears to be a variant of EMRSA-15 that has not

been seen before. Isolates 148858X and 155309B (Fig. 5.4, labelled red) showed identical profiles to that of MW2 (CA-MRSA) and are therefore likely to be community associated.

Isolates 063369Y and 081157C (Fig. 5.4, labelled white), have unique profiles and were unrelated to any of the profiles we have encountered previously. However, their profiles are closer to those of community associated rather than hospital acquired MRSA strains. Isolate 246957K (Fig 5.4, labelled green) appears to be an MSSA isolate and its profile is also unique and shows no relationship with other isolates that we have analysed.
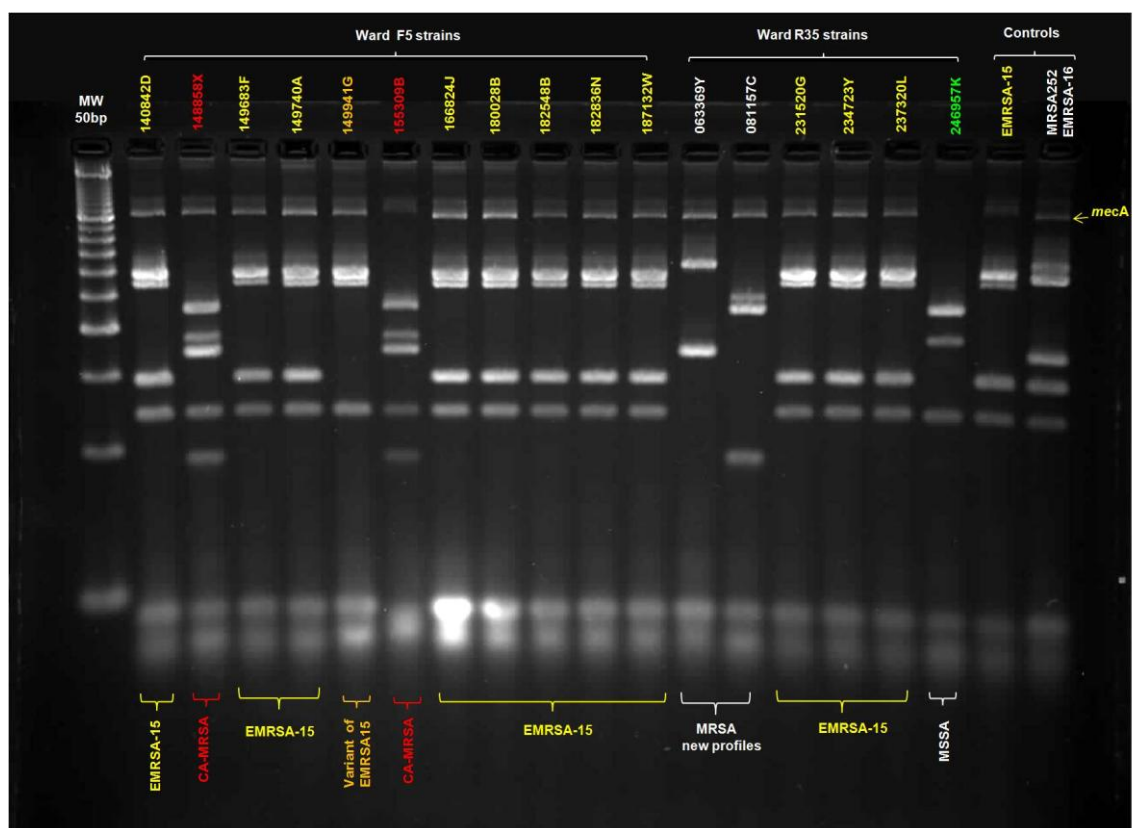
Figure 5.4:   *Sma*I-multiplex PCR profiles of the two clusters of outbreak C isolates that were isolated from wards at two local hospitals**.**

5.4.1d Outbreak D

On 14th of September 2009, thirteen MRSA isolates were isolated from an outbreak that included eight patients, three members of staff and two environment isolates. The isolates were isolated from medical ward R23 at the RVI and all were screening samples (Table 5.5). There was a concern that a member of staff (with MRSA 252348L) who works between two RVI wards, the baby care unit R35, which had outbreak C, and medical ward R23, may have been a potential source of the outbreak.

Table 5. 5: Isolation sites and hospital units of the outbreak D isolates.

Ref. no	Isolate	Source	Site of isolation	Hospital unit
248193	MRSA	Enviroment	Enviroment sample	medical ward (R23)
248188	MRSA	Enviroment	Enviroment sample	medical ward (R23)
248585	MRSA	Patient	Screening sample	medical ward (R23)
248596	MRSA	Patient	Screening sample	medical ward (R23)
248600	MRSA	Patient	Screening sample	medical ward (R23)
248602	MRSA	Patient	Screening sample	medical ward (R23)
248607	MRSA	Patient	Screening sample	medical ward (R23)
248610	MRSA	Patient	Screening sample	medical ward (R23)
248615	MRSA	Patient	Screening sample	medical ward (R23)
248618	MRSA	Patient	Screening sample	medical ward (R23)
251299	MRSA	Staff	Screening sample	medical ward (R23)
252060	MRSA	Staff	Screening sample	medical ward (R23)
252348	MRSA	Staff	Screening sample	medical wards R23 &R35

5.4.1d.i Molecular diagnostic PCR.

All isolates from outbreak D were *coa* positive and PVL negative (Fig. 5.5)
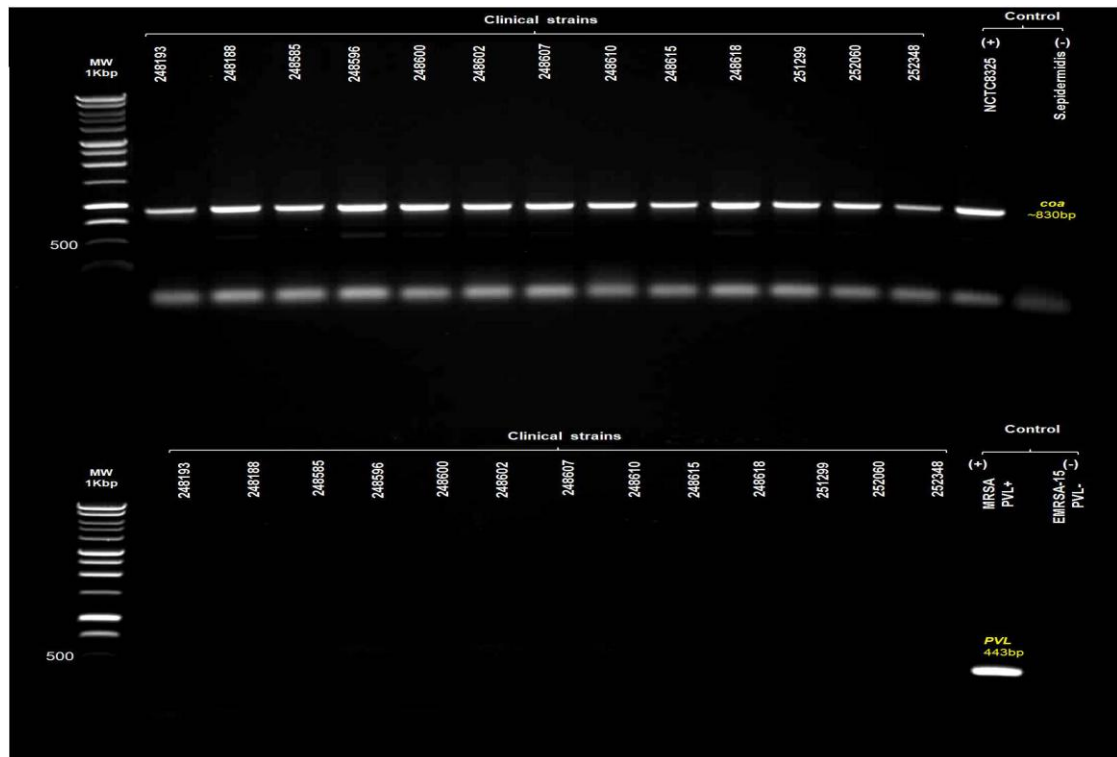
Figure 5.5:   Diagnostic PCR for the presence of *coa, mecA* and PVL in thirteen MRSA strains isolated from outbreak D.

5.4.1d.ii Molecular characterization of an outbreak using *Sma*I-multiplex PCR

The epidemiological characteristics of the MRSA isolates from outbreak D were analysed by *Sma*I-multiplex PCR, which revealed that all of the isolates showed an identical profile to that of EMRSA-15 (Fig. 5.6).
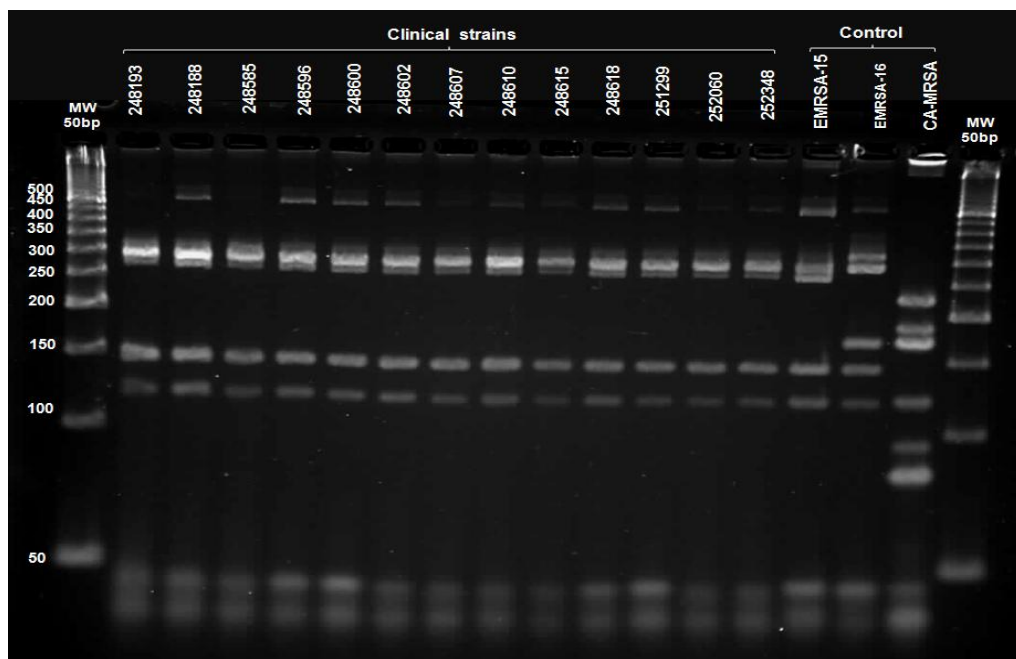
Figure 5. 6: *Sma*I-multiplex PCR profiles of 13 MRSA isolated from the outbreak D.

5.4.1e Outbreak E

On 21st of September 2009 a small outbreak of four MSSA isolates were received for epidemiological investigation. All of the isolates were isolated from child patients in ward R23 at the RVI and were isolated from infection sites (Table 5.6). The isolates were received as blood agar cultures with the exception of isolate 251510, which received as swab sample.

Table 5.6:   Isolation sites and hospital units of the outbreak E isolates.

| Ref. no	Isolate	Source	Site of isolation	Hospital unit
251510	MSSA	Child patient	Infection site	Medical ward (R23)
257076	MSSA	Child patient	Blood	Medical ward (R23
262547	MSSA	Child patient	Catheter tip or neck line	Medical ward (R23
263437	MSSA	Child patient	Infected skin	Medical ward (R23

5.4.1e.i Molecular diagnostic PCR.

Initially, an attempt was made to test isolate 251510 directly from the received swab by diagnostic PCR but no signals were detected. Consequently an overnight culture of swab was prepared and the diagnostic PCR carried out on the resulting colonies. All four isolates were *coa* positive and *mecA* negative.

5.4.1e.ii Molecular typing of an outbreak using *Sma*I-multiplex PCR.

*Sma*I-multiplex PCR profile analysis revealed that the four MSSA were distinguishable from each other and each showed unique profiles (Fig. 5.7). The profiles of isolate MSSA 262547 was more similar to HA-MSSA strains while the profiles of the remaining isolates were more similar to CA-MSSA.
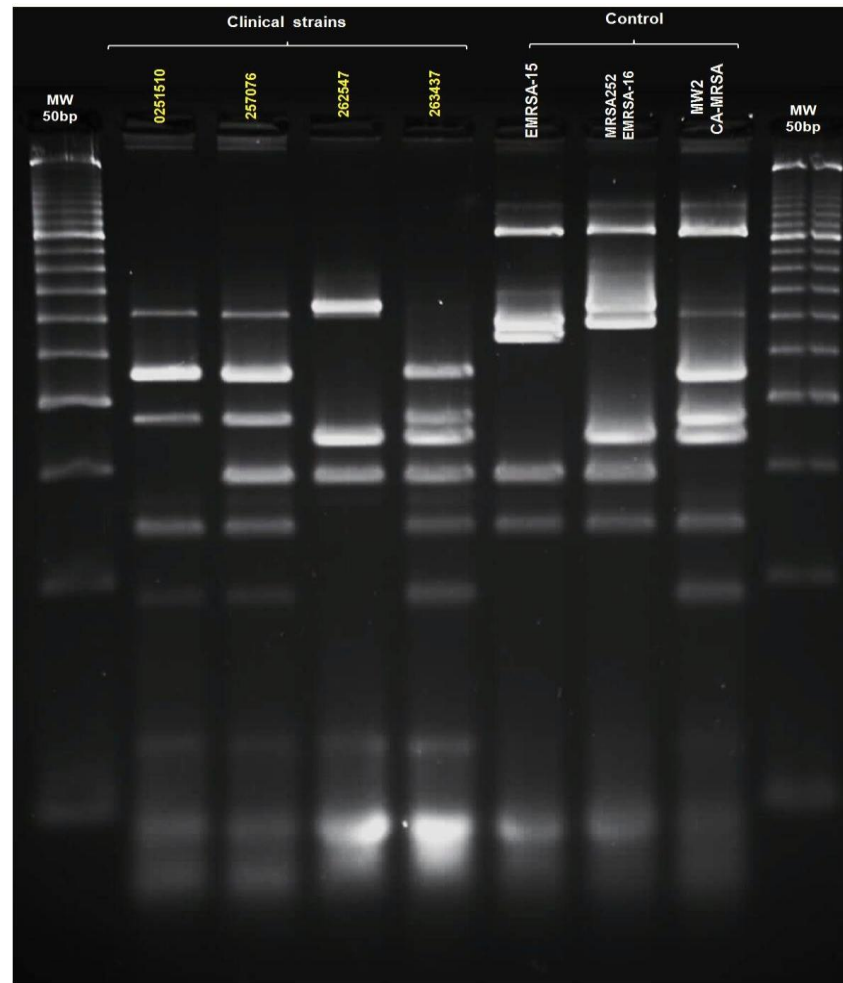
Figure 5.7:   *Sma*I-multiplex PCR profiles of outbreak E MSSA strains isolated from child patients in ward R23 of the RVI.

5.4.1f Outbreak F

On the 8th of October 2009 an outbreak of eleven *S. aureus* isolates was received for epidemiologically analysis. The isolates were isolated from the post-operative wounds of 10 patients in the cardiology ward at the Freeman Hospital. The isolates were supplied on blood agar plates and, interestingly, isolate 246870Y showed two colony morphology types; yellow and greyish-white (Fig. 5.8). Two additional MRSA isolates were also received from two patients at the Newcastle General Hospital (NGH) (Table 5.7).

Figure 5.8: MSSA isolate (246870Y) with two types of colonies, (Yellow (left) and greyish-white (right) on blood agar.

Table 5.7:   Isolation sites and hospital units of the outbreak F isolates.

Ref. no	Isolate	Source	Site of isolation	Hospital unit
246870Y	MSSA	Patient	Post-operative wound	Freeman cardio surgery
249168X	MSSA	Patient	Post-operative wound	Freeman cardio surgery
260348K	MSSA	Patient	Post-operative wound	Freeman cardio surgery
256500X	MSSA	Patient	Post-operative wound	Freeman cardio surgery
257012H	MSSA	Patient	Post-operative wound	Freeman cardio surgery
275089D	MSSA	Patient	Post-operative wound	Freeman cardio surgery
248756Y	MSSA	Patient	Post-operative wound	Freeman cardio surgery
273140Y	MSSA	Patient	Post-operative wound	Freeman cardio surgery
269537M	MSSA	Patient	Tissue	Freeman cardio surgery
277064Q	MRSA	Patient	Screening sample	NGH-Neurology
[a]275462Y	MRSA	Patient	Tissue	NGH-Orthopaedic

NGH: Newcastle General Hospital. a: MRSA isolate with an unusual antibiogram.

5.4.1f.i Molecular typing of an outbreak using *Sma*I-multiplex PCR.

Before epidemiological typing, the outbreak F isolates were tested for the presence of *coa* and *mecA*. All isolates were *coa* positive and *mecA* negative. The two

strains isolated from the NGH as MRSA isolates 277064Q and 275462Y were, as expected, *mecA* positive.

SmaI-multiplex PCR typing established that the colony variants (yellow and greyish-white) of isolate 246870Y (Fig.5.8) had identical profiles and this profile was indistinguishable from that of isolate 260348K and was smilar to that of isolate 249168X. isolates 256500X, 257012H and 275089D had identical profiles that were identical MSSA strain, NCTC6571 (Oxford strain) and very similar to EMRSA-16, indicating that they could be examples of hospital-acquired MSSA (Figs. 5.9/10). Isolates 248596Y and 273140Y each showed unique profiles that were similar in some respects to the profiles of EMRSA-15 and EMRSA-16, even though they were MSSA isolates. MSSA 269537M showed a distinct profile that was closely related to MSSA strain NCTC8325. The sole MRSA isolate, 277064Q, had a unique profile that was not related to either EMRSA15 or 16 (Fig.5.9).
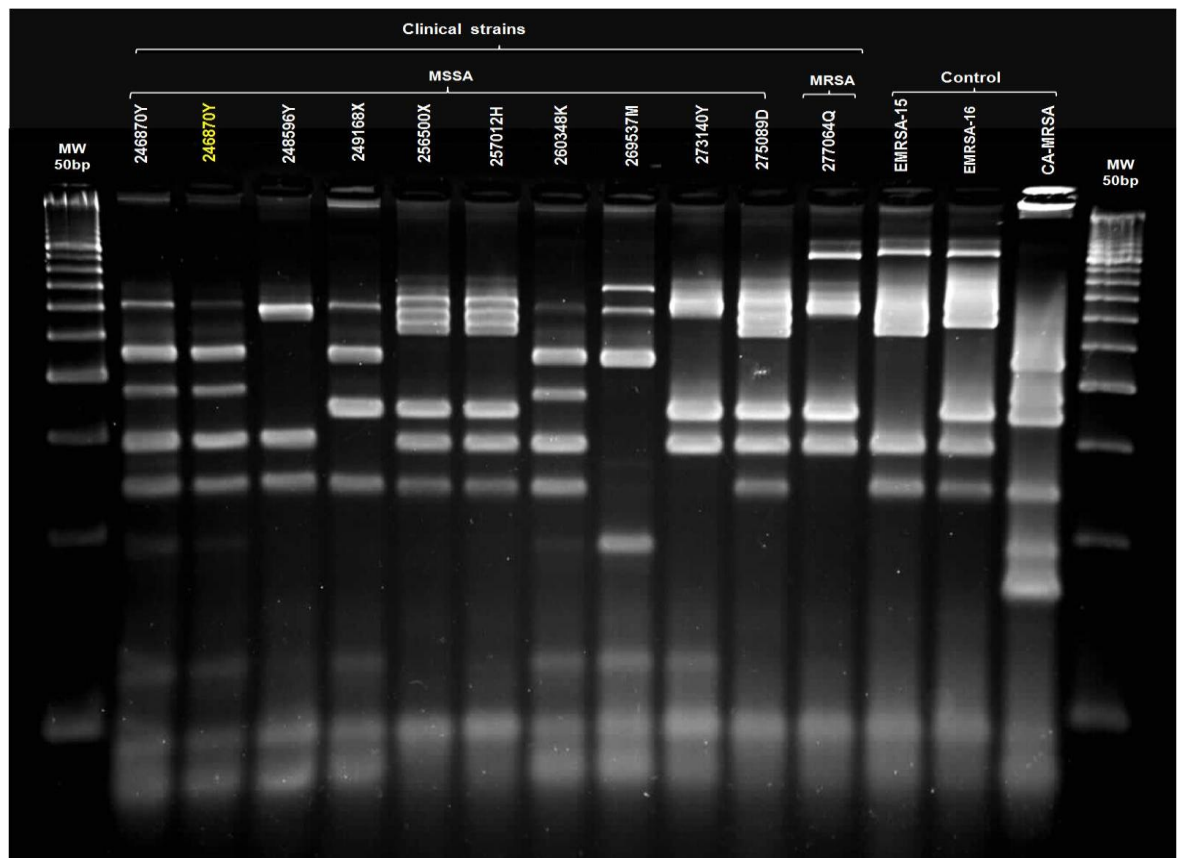
Figure 5.9:   *Sma*I-multiplex PCR profiles of 10 MSSA and one MRSA isolated from outbreak F.

5.4.1f.ii Molecular typing of an MRSA with unusual antibiogram using *Sma*I-multiplex PCR.

An MRSA isolate with an unusual antibiogram, 275462Y, was isolated at the Newcastle General Hospital from a tissue sample of a patient with chronic oestomyelitis. This isolate showed resistance to tetracycline, rifampicin, fusidic acid, erythromycin, clindamycin, trimethoprim, cloxacillin, gentamicin and moxifloxacin. This isolate was tested by *Sma*I-multiplex PCR and a showed profile that had not been seen before (Fig. 5.10, labelled yellow).

Figure 5.10:   *Sma*I-multiplex PCR profile of MRSA isolate 275462Y that showed unusual antibiogram

MLST and SCC*mec* typing showed that isolate 275462Y was ST239-SCC*mec*-III (Fig. 5.11), which is known as a Brazilian or Hungarian clone.

Figure 5.11:   SCC*mec* type-III of MRSA isolate 275462Y. Control strains with known SCC*mec* types were also included.

5.4.1g Outbreak G

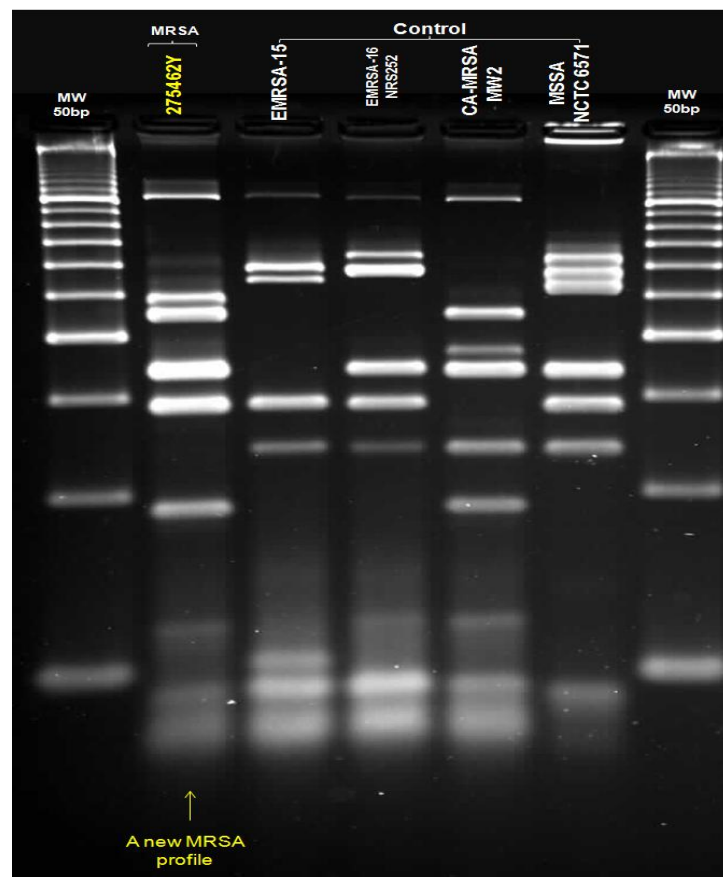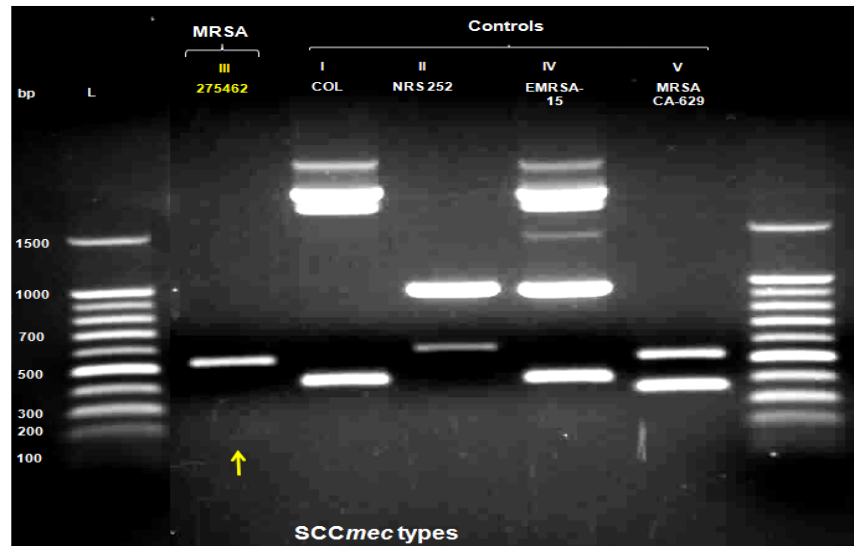On 8th of December 2009 four MSSA isolates were isolated from an outbreak in the intensive treatment unit (ITU) at Newcastle General Hospital (Table 5.8). These isolates were isolated from patients on the same ward and all isolates were recovered within the same time frame.

Table 5.8:   Isolation sites and hospital units of the outbreak G isolates.

Ref. no	Isolate	Source	Site of isolation	Hospital unit
328492	MSSA	Patient	sputum	NGH- Intensive treatment unit (ITU)
328533	MSSA	Patient	sputum	NGH- Intensive treatment unit (ITU)
328534	MSSA	Patient	sputum	NGH- Intensive treatment unit (ITU)
328914	MSSA	Patient	sputum	NGH- Intensive treatment unit (ITU)

NGH: Newcastle General Hospital

5.4.1g.i Molecular typing of an outbreak using *Sma*I-multiplex PCR.

Initially, diagnostic PCR was used to confirm the presence of *coa* in all four MSSA isolates. Subsequently, the epidemiology of the outbreak isolates was examined by *Sma*I-multiplex PCR. The results obtained from *Sma*I-multiplex PCR typing showed that the MSSA isolates were distinguishable and that each isolate showed a distinct profile (Fig. 5.12). The profile of isolate 328492 was identical to MSSA strain NCTC6571 (Oxford strain) and this profile has been seen in previous outbreaks (B &

F). Isolates 328914, 328533 and 328534 showed unique profiles that resembled CA-MSSA (Fig. 5.12).
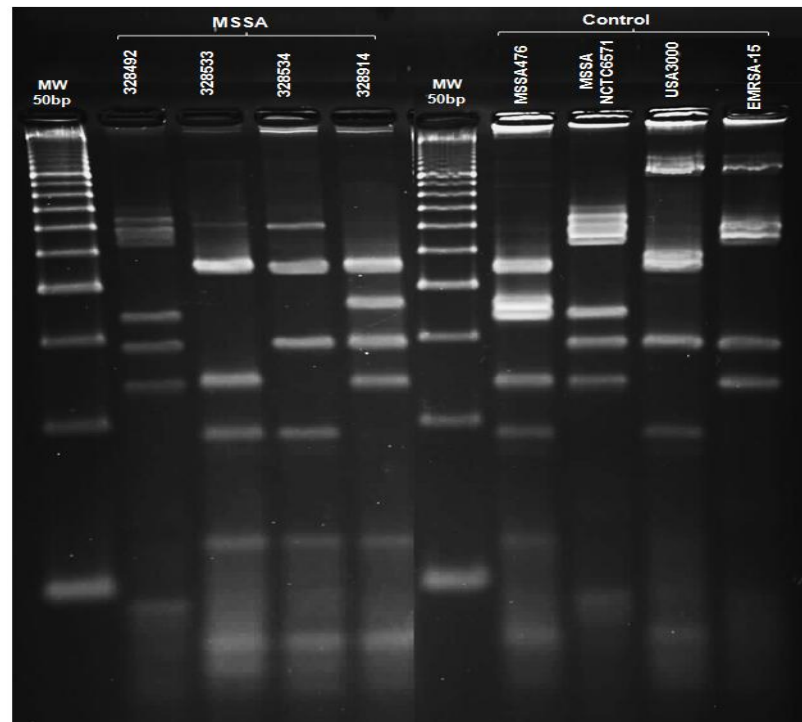
Figure 5. 12:   *Sma*I-multiplex PCR profiles of four outbreak G MSSA isolates together with control *S. aureus* strains.

5.4.2 Comparative analysis of the *Sma*I-multiplex PCR, MLST and SCC*mec* typing methods.

Nineteen representatives of *Sma*I-multiplex PCR profiles selected from the 59 MSSA and MRSA isolates that were isolated from the outbreak A-G were analysed using the fingerprint analysis software (BioNumerics v3.50, Applied Maths). The 19 representatives were analysed by MLST typing and the MRSA isolates also by SCC*mec* typing. Sixteen different sequence types were identified among the 19 *Sma*I-multiplex PCR profiles (Fig. 5.13).

Comparative analysis of the *Sma*I-multiplex PCR and MLST data by UGPMA and MLST phylogenetic tree data (Fig. 5.13) showed that the two methods were in good agreement. The ST5 MSSA isolates, which showed very similar but distinguishable *Sma*I-multiplex PCR profiles, were grouped in one cluster (Fig.5.13, green box). In the same cluster as the EMRSA-15 (ST22) variants (Fig. 5.13, red box). Isolates FOUTB11, 16 and 18, that were identified as sequence types ST47, ST45 and ST46, respectively, were clustered by *Sma*I-multiplex PCR in separate groups (Fig.5.13, blue box).

The results obtained from the present study confirm that *Sma*I-multiplex PCR typing is more discriminatory than MLST since it distinguished between several *S. aureus* that have same sequence type (ST5). In addition, *Sma*I-multiplex PCR distinguished between some variants of EMRSA-15, which shared the same sequence (ST22) and SCC*mec* (IV) types (Fig.5.13, red box).
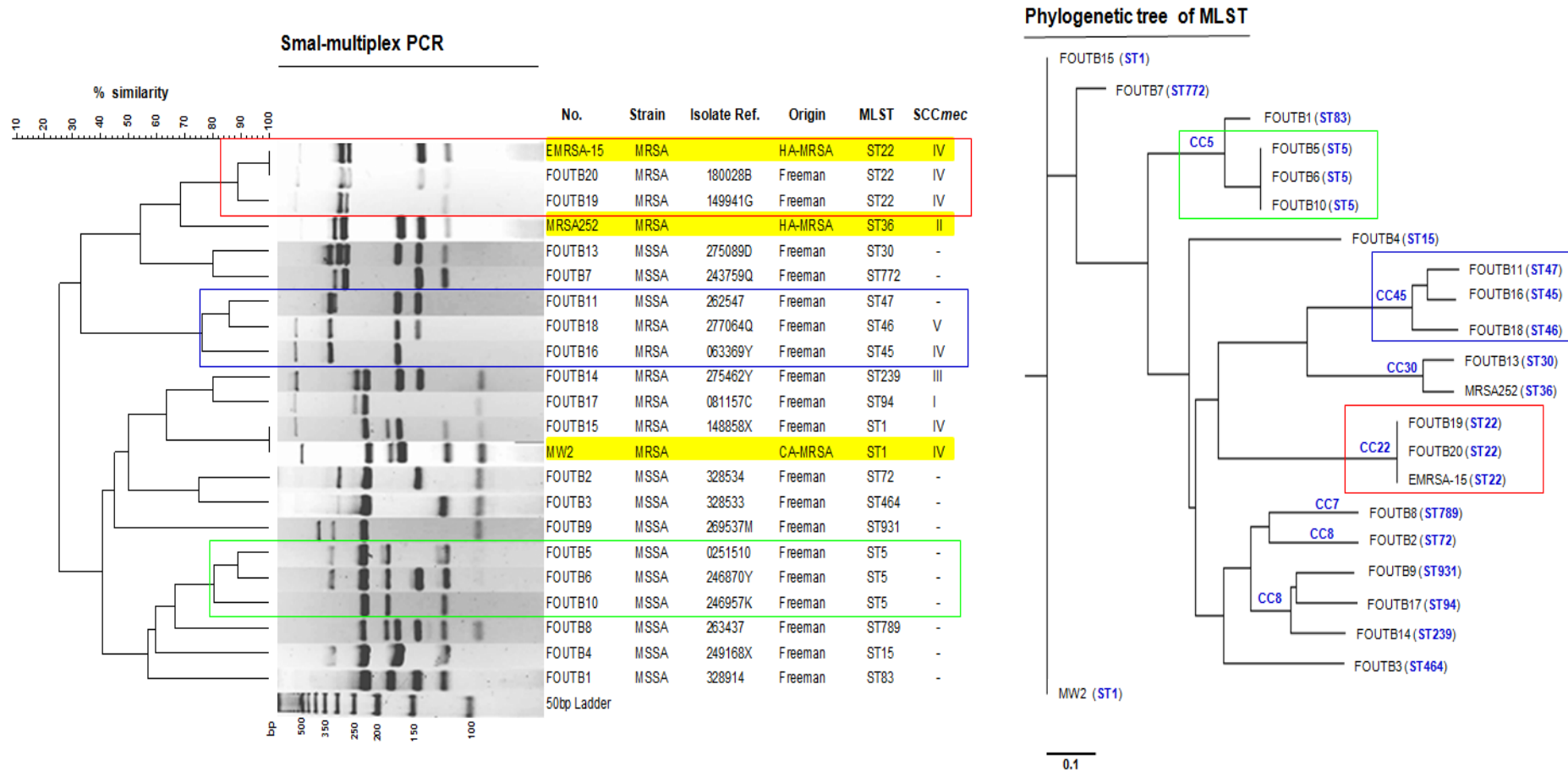
Figure 5. 13: The UPGMA dendrogram based on *Sma*I-multiplex PCR profiles and MLST phylogenetic tree showing the genetic relationship of 19 representatives of clinical MSSA and MRSA strains isolated from outbreaks. Yellow colour refers to control strains.

5.4.3   A multiplex PCR diagnostic assay for the detection *mecA*, *coa* and PVL genes

Due to huge number of *S. aureus* isolates that need to be tested for the presence of the *mecA*, *coa* and PVL genes, a multiplex PCR assay was designed for their detection using the primer pairs in Table 5.1. The resulting assay was evaluated against known clinical MSSA and MRSA outbreak isolates, in addition to some reference staphylococcal strains.

The results indicated that the performance and accuracy of assay was high (100%) and it correctly identified the genotypes of all the isolates tested, including the positive and negative controls (Fig.5.14). The expected sizes of PCR products of all three genes (*coa* 248bp, *mecA* 346bp, and PVL 448bp) were consistently observed in all the test strains.

Figure 5.14:   Multiplex diagnostic assay against MSSA and MRSA strains. CNS: coagulase negative staphylococci.

5.4.4 Evaluation of the *Sma*I-multiplex PCR against known PFGE types of UK epidemic-MRSA strains from the Health Protection Agency (HPA) PFGE collection

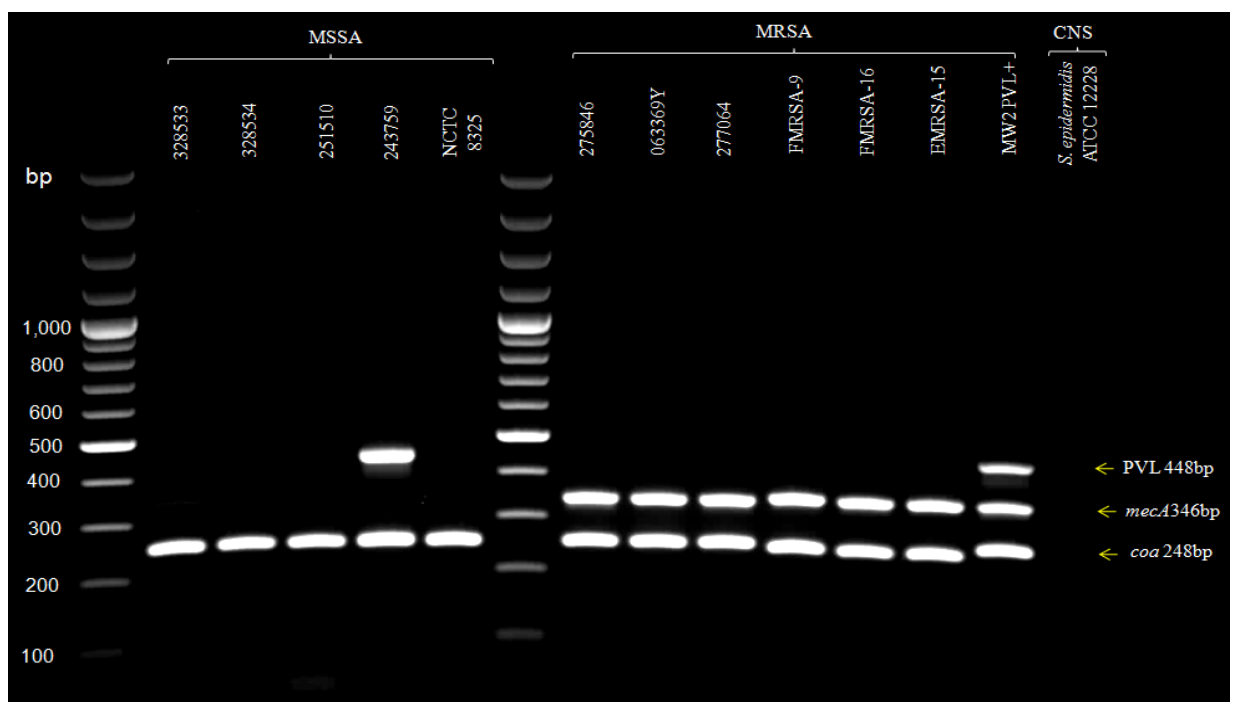On 16th of December 2009, twenty-four EMRSA strains were received from HPA. Those strains were typed by PFGE at HPA laboratories in Colindale, London and they were received without any information about their epidemiological types. In collaboration with Dr Angela Kearns and Dr Barry Cookson of the HPA, the main purpose was to evaluate the discriminatory power of the *Sma*I-multiplex typing method against characterised variants of EMRSA-15 and EMRSA-16.

All strains were received on agar slopes and overnight cultures on agar plates were prepared for the epidemiological typing. The HPA strains were analysed by *Sma*I-multiplex PCR and showed that, with one notable exception (strain ZZ0900003), all were identifiable as either EMRSA-15 or EMRSA-16, or related variants. Ten strains generated profiles that were identical to that of EMRSA-15 (Fig. 5.15, labelled yellow). Two strains, H053780592 and H0725800475, (Fig. 5.15, labelled orange) appeared to be EMRSA-15 variants since their profiles showed only one band difference from the original EMRSA-15.

Eleven strains (Fig. 5.15, labelled red) generated profiles that were related to that of the EMRSA-16. However, each has an additional band that was not observed in the EMRSA-16 profile generated from the sequenced strain MRSA252 (see controls with red arrow, Fig. 5.15). This indicates that MRSA252 may be an atypical EMRSA-16 strain. Strain ZZ0900003 (Fig.5.15, labelled dark red) generated the same bands as the other EMRSA-16 strains but generated an additional two bands (Fig.5.15, red arrows).
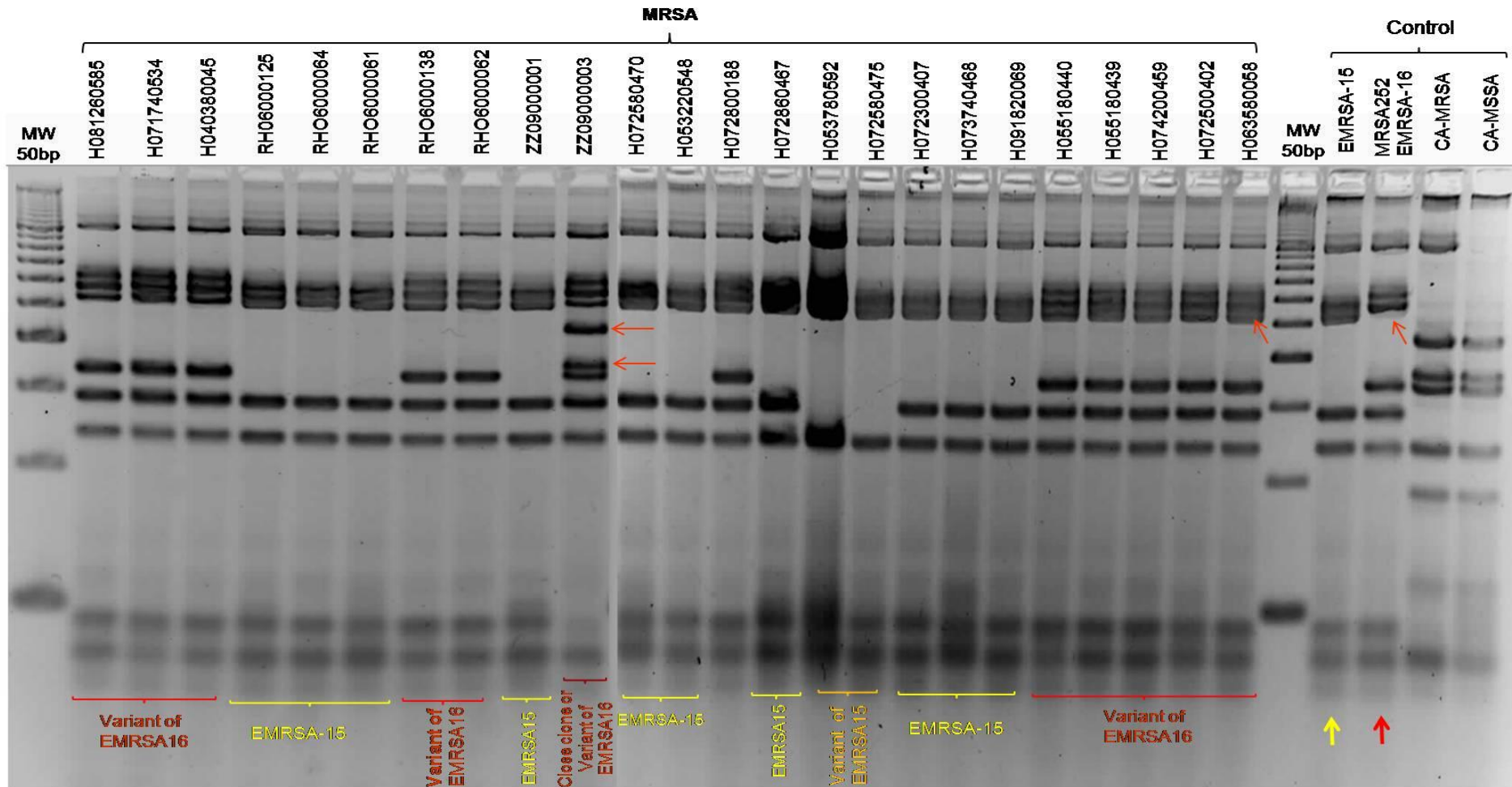
Figure 5. 15: *Sma*I-multiplex PCR against known types of UK Epidemic-MRSA strains that received from HPA. Red arrows refer to the bands that distinguish between the EMRSA-16 variants.

The *Sma*I-multiplex PCR patterns of HPA strains (Fig.5.15) were analysed using the fingerprinting analysis software BioNumerics. Figure 5.16 shows the dendrogram (Dice, UPGMA) resulting from a cluster analysis of *Sma*I-multiplex PCR patterns for the HPA strains. The strains were grouped on two main clusters, the EMRSA-15 cluster and EMRSA-16 cluster. Within the putative EMRSA-15 cluster, two *Sma*I-multiplex PCR profiles were found showing 90% similarity with the original profile of EMRSA-15. The putative EMRSA-16 strains also formed two clusters, caused by the single band difference between the HPA strains and MRSA252 (EMRSA-16) (Fig. 5.16).

The MRSA strain (RH06000062), which exhibited a similar but not identical *Sma*I-multiplex PCR pattern to EMRSA-16 (with only one band different) was analysed by MLST and SCC*mec* typing. The MLST and SCC*mec* analysis confirmed that strain RH06000062 was ST36 with SCC*mec*-II which is the sequence/SCC*mec* type of EMRSA-16. The MRSA strain ZZ06000003, with the unusual *Sma*I-multiplex PCR pattern, was ST5 with SCC*mec*-IV which is the same as the so-called "pediatric clone" (Argudin *et al.*, 2009).

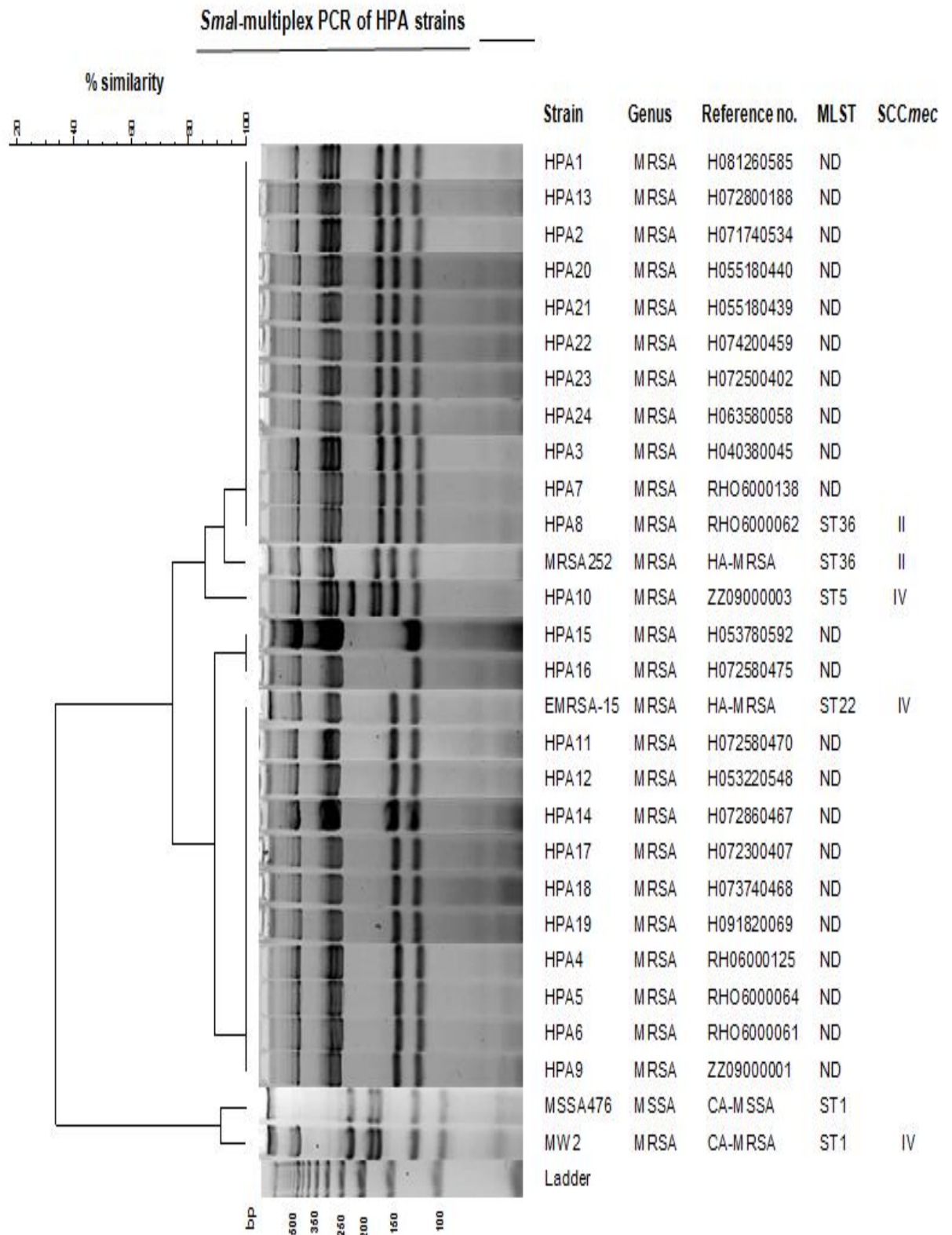| Strain	Genus	Reference no.	MLST	SCC*mec*
HPA1	MRSA	H081260585	ND	
HPA13	MRSA	H072800188	ND	
HPA2	MRSA	H071740534	ND	
HPA20	MRSA	H055180440	ND	
HPA21	MRSA	H055180439	ND	
HPA22	MRSA	H074200459	ND	
HPA23	MRSA	H072500402	ND	
HPA24	MRSA	H063580058	ND	
HPA3	MRSA	H040380045	ND	
HPA7	MRSA	RHO6000138	ND	
HPA8	MRSA	RHO6000062	ST36	II
MRSA252	MRSA	HA-MRSA	ST36	II
HPA10	MRSA	ZZ09000003	ST5	IV
HPA15	MRSA	H053780592	ND	
HPA16	MRSA	H072580475	ND	
EMRSA-15	MRSA	HA-MRSA	ST22	IV
HPA11	MRSA	H072580470	ND	
HPA12	MRSA	H053220548	ND	
HPA14	MRSA	H072860467	ND	
HPA17	MRSA	H072300407	ND	
HPA18	MRSA	H073740468	ND	
HPA19	MRSA	H091820069	ND	
HPA4	MRSA	RH06000125	ND	
HPA5	MRSA	RHO6000064	ND	
HPA6	MRSA	RHO6000061	ND	
HPA9	MRSA	ZZ09000001	ND	
MSSA476	MSSA	CA-MSSA	ST1	
MW2	MRSA	CA-MRSA	ST1	IV
Ladder				

Figure 5.16:   The UPGMA dendrogram based on *Sma*I-multiplex PCR patterns of known types of UK Epidemic-MRSA strains that were received from HPA. ND: not determined.

5.4.5 Evaluation of *Sma*I-multiplex PCR against MRSA strains isolated from blood.

Thirteen MRSA isolates, isolated from the blood of patients at the Freeman Hospital (Table 5.9), were typed by *Sma*I-multiplex PCR.

Table 5.9:   Sources of MRSA isolates that isolated from blood.

Isolate no.	Patient's age	Site of isolation	Unit of isolation
FMRSA 90	57	Blood	Freeman musculoskeletal unit
FMRSA 92	75	Blood	Freeman orthopaedics (F19)
FMRSA 93	83	Blood	Freeman surgical ward (F5)
FMRSA 94	73	Blood	Freeman ENT (F10)
FMRSA 95	74	Blood	Freeman renal unit (F4)
FMRSA 96	48	Blood	Freeman renal unit (F4)
FMRSA 97	38	Blood	Freeman surgical ward (F5)
FMRSA 142	72	Blood	Freeman chest medicine (F29)
FMRSA 166	69	Blood	Freeman liver unit (F12)
FMRSA 189	80	Blood	Freeman critical care (FCC)
FMRSA 201	78	Blood	Freeman cardio ITU (F26)
FMRSA 234	97	Blood	Freeman coronary unit (F9)
FMRSA 281	97	Blood	Freeman coronary unit (F9)

FMRSA= Freeman MRSA, ENT = ear, nose, throat, ITU= intensive treatment unit.

5.4.5a *Sma*I-multiplex PCR typing of blood isolates

Analysis of the strains isolated from blood samples by *Sma*I-multiplex PCR revealed that all but one isolate, FMRSA 166, showed identical profiles to that of EMRSA-15. FMRSA 166 generated the same profile as EMRSA-16 (Fig. 5.17).
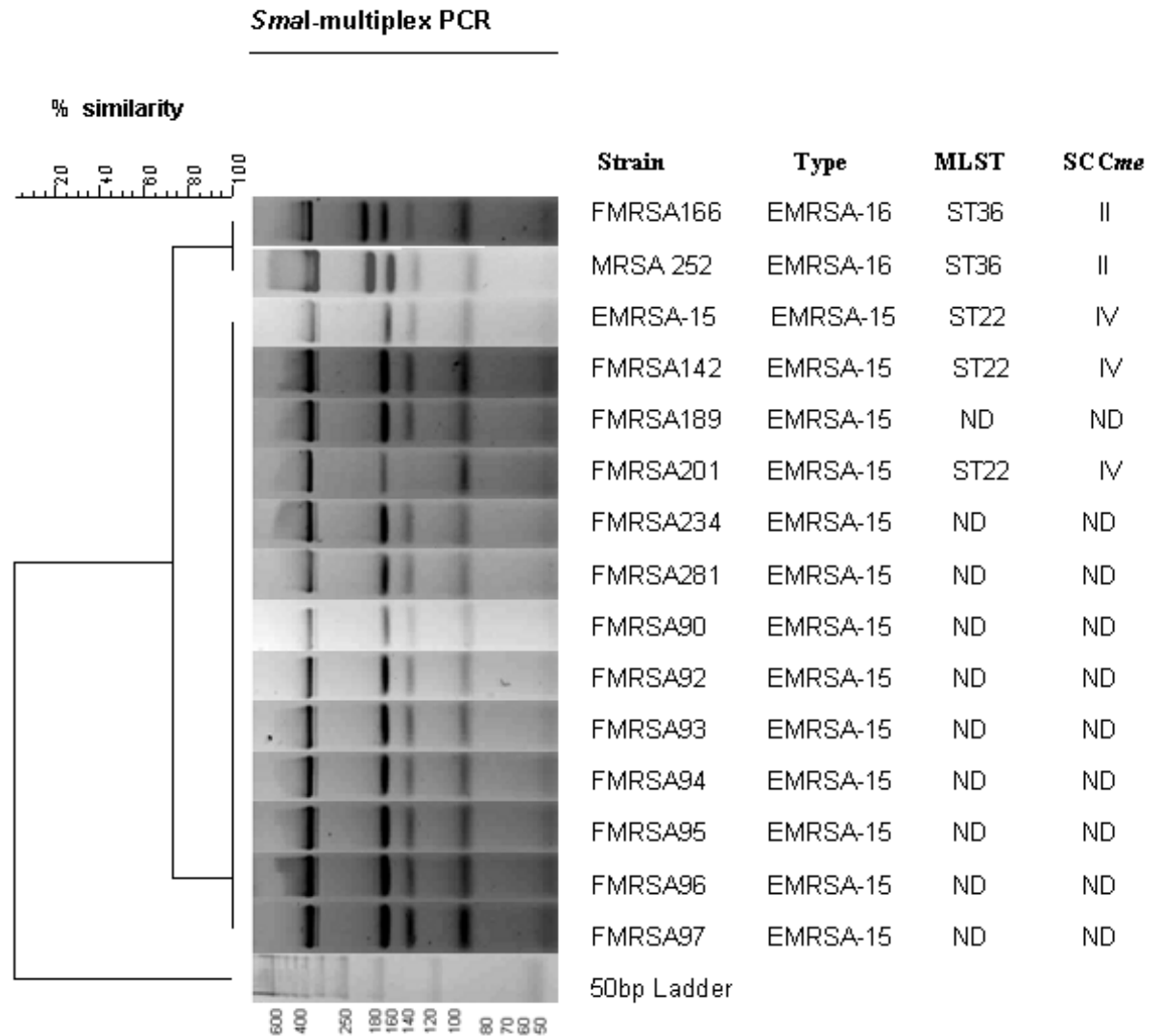
Figure 5.17:   The UPGMA dendrogram based on *Sma*I-multiplex PCR profiles of thirteen MRSA strains isolated from blood. ND: not determined

Three representative isolates (FMRSA142, 166 and 201) isolated from the blood samples were analysed by MLST and SCC*mec* typing. Both techniques confirmed that isolates FMRSA142 and 201 were ST22-SCC*mec*-IV, which is identical to EMRSA-15, whereas FMRSA166 was ST36-SCC*mec*-II, which is identical to EMRSA-16 (Fig. 5.18).
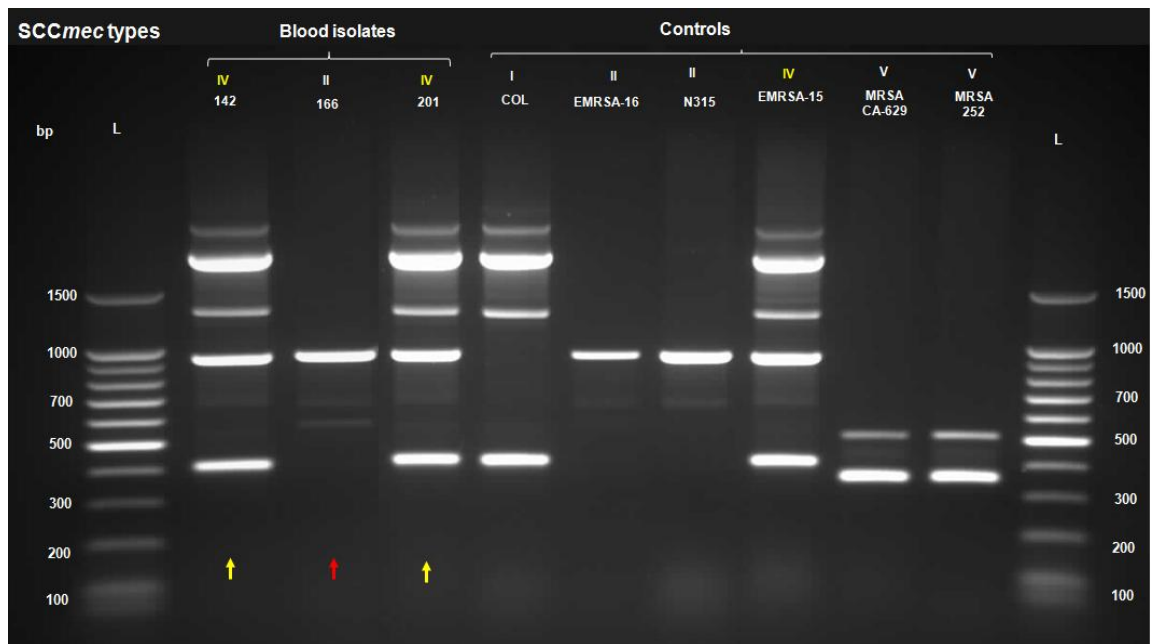
Figure 5. 18: SCC*mec* types of three representative MRSA isolates (FMRSA142, 166 and 201) that were analysed by MLST, together with various control strains

5.5 Discussion

MRSA outbreaks are frequently reported and rapid investigation, using epidemiological tools, can help the infection control team to take the correct and urgent action required to control the further spread of the outbreak. PCR-based epidemiological techniques are the most useful in terms of their speed, ease of performance and interpretation (Collier *et al*., 1996).

The *Sma*I-multiplex PCR typing technique developed in this study is a novel technological approach that is based on similar principles to that of PFGE, namely SNPs at *Sma*I recognition sites. The developed method has been tested against many sequenced *S. aureus* strains in comparison with PFGE and MLST and showed excellent discriminatory power and reproducibility. *Sma*I-multiplex PCR works with rapidly extracted chromosomal DNA and can be carried out in as little as 4-6 hours. It complies with the requirement of any typing tool, namely that its discriminatory power should be sufficient to cluster epidemiologically related strains and discriminate them from unrelated strains (Trindade *et al.*, 2003).

In the current study, we evaluated the developed *Sma*I-multiplex PCR typing technique against *S. aureus* strains isolated from putative outbreaks (59 MSSA and MRSA isolates) and blood samples (13 isolates) from local NHS hospitals. The evaluation also included MRSA strains with known PFGE types that were received

from the HPA's Colindale laboratory. The evaluation was based on several criteria: typeability, discriminatory power, stability and epidemiology concordance (Struelens, 1998).

Small colony variants (SCV) of *S. aureus* are non-pigmented, non-haemolytic pinpoint slow-growing morphological variants that are only detectable on plates after 24-72 hours incubation (von Eiff and Becker, 2003; Sifri *et al.*, 2006). Most SCVs of *S. aureus* are auxotrophs for menadione, hemin and thymidine, but rarely require CO_2. However, CO_2-dependent MRSA isolates have been isolated from nasal colonization and infection sites. It has been reported that SCVs have been associated with persistent, antibiotic resistant and relapsing infections (Sifri *et al.*, 2006; Gomez-Gonzalez *et al.*, 2010). In the present study, seven CO_2-dependent MRSA isolates were recovered from an outbreak in a regional liver transplant unit (RLU) at the Freeman Hospital (outbreak A). These isolates appeared to be SCVs since they grew very slowly after 48 hours incubation under aerobic conditions, but more rapidly after 24 hours in the presence of 5% CO_2. These isolates were confirmed to have *mecA* and *coa* genes but were PVL negative. Using the *Sma*I-multiplex PCR typing technique, all of the CO_2-dependent MRSA isolates showed profiles that were identical to that of EMRSA-15 (Fig. 5.1). This was confirmed by results obtained from PFGE, MLST and SCC*mec* typing. All isolates were ST22-SCC*mec*-IV (EMRSA-15) and had PFGE profiles that were identical to EMRSA-15 excepting for one isolate that differed from the other isolates by the presence of a single band (Fig. 5.1). This PFGE type is a known EMRSA-15 variant identified originally in the study of Hallin *et al*. (2007).

S. aureus is the most common cause of surgical wound infections (SWIs) and surgical units are, indeed, the most affected by post-operative *S. aureus* infections. In cardiac surgery units, SWIs often develop into bloodstream infections that are associated with an increase in morbidity and mortality (Carrier *et al.*, 2002). It is thought that most *S. aureus* surgical-site infections are caused by the patient's endogenous *S. aureus* flora that are usually MSSA (Kluytmans *et al.*, 1996; Trouillet *et al.*, 2005). In the current study, a small outbreak of three MSSA isolates was recovered from patients in a cardio surgery unit with post-operative wounds (outbreak B). All three isolates were confirmed by PCR to harbour the *coa* gene but were *mecA* negative. One of the isolates (243759Q) encoded the PVL determinant (Fig. 5.2). The isolates were typed using the *Sma*I-multiplex PCR technique and each isolate generated a unique *Sma*I-multiplex PCR profile (Fig. 5.3), ruling out a common source of the outbreak. Although, the *Sma*I-multiplex PCR profile of MSSA 243759Q was closely

related to that of EMRSA-15, it was shown to have a sequence type of ST772, which is a CA-MRSA and belongs to clonal complex 1 (CC1) (Strommenger *et al.*, 2008). The profile of MSSA isolate 2445552 was similar to that of MRSA-252 (EMRSA-16) but lacks the *mecA* band and has an additional band that not observed in MRSA252. Isolate 2445552 was found to be sequence type ST30, and it is thought that southwest Pacific MRSA clone (ST30-SCC*mec*-IV) has emerged by the introduction of SCC*mec*-IV into a successful ST30-MSSA clone (Enright *et al.*, 2002). MRSA ST30-SCC*mec*-IV is closely related to EMRSA-16 (ST36-SCC*mec*-IV) and both belong to the same clonal complex, namely CC30. MSSA isolate 241094X had a *Sma*I-multiplex PCR profile that was distinct and a sequence type of ST5. MSSA ST5 strains have been identified to be the predominant MSSA clone in Belgium (Hallin *et al.*, 2007) and to be prevalent in the ICUs of French hospitals (Karauzum *et al.*, 2008). Moreover, MSSA-ST5 has been isolated from both community and hospital infection and Enright reported that ST5-MSSA formed ST5-MRSA-II which led initially to ST5-VISA-II and more recently to ST5-VRSA-II (Enright, 2003).

　　S. aureus outbreak C consisted of 17 isolates in two clusters. All of these isolates encoded the *coa* and *mecA* genes excepting the blood isolate 246957K. Although, lacking *mecA,* isolate 246957K was weakly positive when tested by the penicillin binding protein latex test − possibly due to contamination of the original colony with an MRSA isolate (Sakoulas *et al.*, 2001). Molecular characterisation of this outbreak using *Sma*I-multiplex PCR typing demonstrated the discriminatory power of this technique. As EMRSA-15 is the predominant epidemic MRSA clone in the north of England (Wilson *et al.*, 2010), unsurprisingly, most of these outbreak isolates were related to this strain. Interestingly, the *Sma*I-multiplex PCR technique discriminated isolate 149941G as a variant of EMRSA-15 (Fig. 5.4, labelled orange). Two CA-MRSA isolates (148858X and 155309B) were identified among the outbreak since they showed profiles that were identical to the well-characterised CA-MRSA strain, MW2 (Fig. 5.4, labelled red). Their profiles were confirmed to have sequence/*SCCmec* types ST1-SCC*mec*-IV, which is a CA-MRSA clone. This is a clear indicator that CA-MRSA is beginning to rival epidemic HA-MRSA in UK hospitals although it is still relatively rare. In this outbreak, two unique *Sma*I-multiplex PCR profiles were found in associated with MRSA isolates 063369Y and 081157C (Fig. 5.4, labelled white). MLST typing confirmed that isolate 063369Y was ST45-SCC*mec*-IV, which is the so-called Berlin clone and most common in Belgium, Finland, Germany and Sweden (Enright *et al.*, 2002). Isolates 081157C was ST94-SCC*mec*-I. Currently, only one ST94-MSSA isolate

has been annotated in the MLST website which was isolated from a case of conjunctivitis in Cuba in 1998.

Thirteen MRSA isolates were isolated from outbreak D that included patients, members of staff and the environment within a medical ward. There was a suspicion that a specific member of staff was the source of the outbreak because he/she worked between the medical ward and the baby care unit that was involved in outbreak C. The isolates of outbreak D were sent by the infection control office to the HPA laboratories at Colindale to be analysed by PFGE. The epidemiological data obtained from *Sma*I-multiplex PCR typing indicated that the profiles of all thirteen isolates were identical to EMRSA-15 (Fig. 5.6). The *Sma*I-multiplex PCR results were confirmed by the PFGE analysis, which revealed that all isolates are EMRSA-15. However, PFGE was more discriminatory since it identified four PFGE-subtypes of EMRSA-15 within this set of isolates.

In this study, most staphylococcal outbreak strains isolated from wounds were generally caused by MSSA that are usually of endogenous origin. It is noteworthy that the highest rate of MSSA nasal colonisation was among people younger than 18 years of age whereas MRSA colonisation was more likely in older people (Kenner *et al.*, 2003). Four MSSA isolates of outbreak E were recovered from the infection sites (3 from wounds and one from blood) of four child patients. Diagnostic PCR confirmed the presence of *coa* and the absence of *mecA* among these isolates. Each isolate showed a unique *Sma*I-multiplex PCR profile indicating that they were of independent origin. The profile of MSSA isolate 262547 was similar to that of HA-MSSA (Fig.5.7) and had ST47. All nine currently annotated ST47 isolates are MSSA strains isolated in the UK. The *Sma*I-multiplex PCR profiles of the remaining three MSSA isolates indicated that they could be CA-MSSA. Isolates 251510 and 257076 were ST5 though they showed related but different *Sma*I-multiplex PCR profiles. ST5-MSSA is most common in Poland, Slovenia and the UK. MSSA 263437 showed a similar *Sma*I-multiplex PCR profile as the sequenced strain MSSA476 (CA-MSSA), but with one additional band. Isolate 263437 was ST789, originally isolated from bovine mastitis in Japan (Hata *et al.*, 2010).

The MSSA isolates from outbreak (F) were isolated from the post-operative wounds of patients in a cardio surgery unit. Isolates 246870Y and 260348K had identical profile that was similar but distinguishable to 249168X profile and their profiles are related to that of CA-MSSA strains (Fig. 5.9). Further analysis using MLST showed that MSSA 246870Y was ST5 while MSSA 249168X was ST15 - both

sequence types associated with CA-MSSA (O'Brien *et al.*, 2009). MSSA isolates 256500X, 257012H and 275089D showed the same profile, and this profile was identical to MSSA strain NCTC6571 (Oxford strain). This profile, representatives of which were also reported in previous outbreak B, was shown to be ST30. MSSA isolates 248596Y and 273140Y showed unique profiles and had sequence types ST772 and ST47, respectively (Fig. 5.9). While a unique *Sma*I-multiplex PCR profile was found with MSSA isolate 269537M, it was similar to that of MSSA strain NCTC8325, which is ST8. MSSA 269537M was found to be ST931, which is single locus variant (SLV) of ST8.

The Brazilian-Hungarian clone ST239-SCC*mec*-III is the most common clone in South East Asia (China, Pakistan, Indonesia, Singapore, Sri Lanka, Thailand, Hong Kong and India) (Cho *et al.*, 2006; Shabir *et al.*, 2010; Yao *et al.*, 2010). Although, this multidrug resistant MRSA clone has been reported in Europe, including the UK, it is not common in UK hospitals (Enright *et al.*, 2002). MRSA isolate 275462Y generated a *Sma*I-multiplex PCR profile (Fig. 5.10) that was not seen in any of the other isolates analysed in this study. When tested by MLST/SCC*mec*, isolate 275462Y was found to be ST239-SCC*mec*-III and therefore the Hungarian-Brazilian clone. Additional information about the patient revealed him/her to have been involved in a road traffic accident in Pakistan in 2005 where he/she sustained a leg injury that resulted in oestomyelitis. The patient continues to travel regularly to this country.

MRSA isolate 277064Q was typed with the outbreak F isolates. Isolate 277064Q was isolated from the screening sample of a patient at Newcastle General Hospital (NGH). This isolate showed a unique *Sma*I-multiplex PCR profile that was similar to HA-MRSA strains, although not EMRSA-15 or EMRSA-16. Isolate 277064Q was found to be ST46/SCC*mec*-V (Fig.5.9; Fig. 5.13), a clonal type that has been reported in Finland (Vainio *et al.*, 2008).

Four MSSA isolates (outbreak G) were reported in patients in the ITU at Newcastle General Hospital. Epidemiological characterization showed that each isolate had a distinct *Sma*I-multiplex PCR profile (Fig. 5.12). The profile of isolate 328492 was identical to that of MSSA NCTC6571 (Oxford strain) and this profile was seen in previous outbreak isolates (B & F). It was found to be ST30. The *Sma*I-multiplex PCR profile of MSSA isolate 328914 was similar in some respects with the profiles of two MSSA isolates (ST5 isolate 246870Y and ST15 isolate 249168X) seen in outbreak F. Isolate 328914 was ST83, a single locus variant of ST5 with a SNP in the *yqiL* allele, Both ST83 and ST5 belong to clonal complex CC5. MSSA isolates 328534 and 328533

had closely related *Sma*I-multiplex PCR profiles and were ST72 and ST464, respectively. Although, ST72 is mainly associated with community-acquired infections, it has been implicated in some hospital infections (Luedicke *et al.*, 2010). Interestingly, those isolates belong to ST72, representatives of which have been isolated from bovine milk in Japan (Hata *et al.*, 2010) and since ST464 clone associated with cattle (Andreoletti *et al.*, 2009), it is possible that isolates 328534 and 328533 might have been introduced into the ITU by carrier associated with animals.

Nineteen isolates representing all of the *Sma*I-multiplex PCR profiles in the 59 staphylococcal outbreaks isolates were analysed using the BioNumerics fingerprinting software. The 19 representatives were also subjected to MLST analysis and 16 different sequence types were identified. What is important about these data (Fig. 5.13) is that the *Sma*I-multiplex PCR typing technique accurately clustered the isolates according to the MLST data. A good example is that of isolates FOUTB11, 16 and 18, which were grouped in a cluster by *Sma*I-multiplex PCR. These isolates belong to sequences types ST47, ST45 and ST46 respectively, which are part of clonal complex 45 (Fig. 5.13, blue box).

Although, the correlation between the *Sma*I-multiplex PCR and MLST techniques were high, the former was more discriminatory. This is clearly shown in the case of the MSSA isolates in ST5, which generated three closely related but distinct *Sma*I-multiplex PCR profiles (Fig. 5.13, green box). In addition, *Sma*I-multiplex PCR could discriminate some variant clones of epidemic strains among outbreak isolates: two ST22-EMRSA-15-SCC*mec*-IV isolates showed profiles that differed by a single band (Fig. 5.13, red box).

Throughout the investigations of the outbreaks, *Sma*I-multiplex PCR typing provided valuable epidemiological information and was able to distinguish clearly between MSSA or MRSA isolates even in small outbreaks. The timely provision of the epidemiological information helped the infection control team in their investigation of these outbreaks, in some cases ruling out potential common sources of the infections.

The presence of the *coa* gene was used for identification of *S. aureus* while the *mecA* gene was used as an indicator for MRSA. In addition, PVL is associated with sever *S. aureus* infections. The detection of those genes can provide useful information about clinical isolates, particularly when detected during ongoing outbreaks. A multiplex PCR was developed for the simultaneous detection of these genes. This

diagnostic assay showed an excellent performance against known PVL-positive MSSA and MRSA strains (Fig. 5.14).

The evaluation of the *Sma*I-multiplex PCR typing against HPA MRSA strains that have known PFGE types provided the opportunity to assess the discriminatory power of this technique against variants within the same EMRSA clonal type. *Sma*I-multiplex PCR typing was able to discriminate some but not all EMRSA variants: distinguished two EMRSA-15 variants and three EMRSA-16 variants among the 24 HPA EMRSA strains. MRSA strain ZZ09000003 showed an interesting and novel *Sma*I-multiplex PCR profile: it has the same basic profile as MRSA-252 (EMRSA-16) but with three additional unique bands (Fig. 5.15). Strain ZZ09000003 also has the same basic profile of VISA strains (Mu50, Mu3 and N315) with four additional bands. According to the PFGE data, strain ZZ09000003 is a variant of EMRSA-16 but is sequence type ST5 with SCC*mec*-IV. This is the same sequence and *mecA* type as pediatric clone-USA800 (Sousa-Junior *et al*., 2009).

Further evaluation for the developed *Sma*I-multiplex PCR technology was carried out on thirteen clinical MRSA isolates. These isolates were recovered from the blood of hospitalized patients in different units at the Freeman Hospital. Approximately 90% of those patients were older than 70 years of age, which reflects the fact increasing age is a risk factor for MRSA bacteraemia (Heo *et al*., 2007). The results showed that EMRSA-15 was present in 12 of these patients, while the remaining patient was infected by an EMRSA-16 strain (Fig. 5.17). Further analysis of two representative EMRSA-15 strains and the EMRSA-16 confirmed the *Sma*I-multiplex PCR results: FMRSA142 and 201 isolates were EMRSA-15 (ST22-SCC*mec*-IV and the FMRSA166 was EMRSA-16 (ST36-SCC*mec*-II).

In conclusion, the developed *Sma*I-multiplex PCR technique showed excellent typeability and reproducibility with stability. It was more discriminatory than MLST and was easy to perform and interpret. It does not require any special expertise or equipment and can therefore be performed in any routine clinical microbiology laboratory. The rapid DNA extraction method that was developed in the current study helped to reduce the sample processing time to between 4 and 6 hours. While the *Sma*I-multiplex PCR technique did not discriminate between all variants of the main UK epidemic clones that have been identified by PFGE, it was able to distinguish some of them. The *Sma*I-multiplex PCR technique proved to be a useful tool for providing

reliable epidemiological information for the investigation of clinical staphylococcal outbreaks.

Chapter 6

General discussion

6. General discussion

There is no doubt that *S. aureus*, whether methicillin-susceptible or methicillin-resistant, will continue to be a primary cause of nosocomial infections well into the foreseeable future. Consequently, an understanding of epidemiological characteristics of *S. aureus* will continue to be essential in the management of its infections in both the hospital and community settings. The purpose of any epidemiological study is to identify relationships between isolated strains. In the investigation of an outbreak, epidemiological typing provides information about the outbreak isolates and helps to establish whether it is due to spread of a single clone. In addition, typing technique can provide essential information about population changes during a long-term epidemiological surveillance. For decades the epidemiological characterisation of *S. aureus* relied on phenotypic characteristics such as antibiograms, sensitivity to bacteriophages and serological features. In recent years, however, more advanced molecular epidemiological tools have been introduced such PFGE, MLST and SCC*mec*. Those genotyping methods are variable in their resolving power, reproducibility, and the degree of expertise and cost of the equipment required.

PFGE profiling is based on the analysis of intact bacterial DNA by specific restriction enzymes. It results in the generation of a relatively small number of large fragments that can only be separated by PFGE. This technique has been used extensively for the analysis of MRSA strains and shows a high level of discrimination. Consequently, it has become established as the gold standard for MRSA typing (Schmitz *et al.*, 1998). However, PFGE has many disadvantages for routine epidemiological typing. Those disadvantages are time-consuming nature of the procedure (4-8 days), the cost of electrophoresis equipment (>£10,000) and need for special expertise.

MLST is sequence-based genotyping method that relies on the identification of allelic variations in the sequences of seven housekeeping genes. MLST provides unambiguous data with very high reproducibility and it has been proven to be a valuable technique for tracing of the global spread of MRSA clones, particularly in long-term analyses. MLST also provides beneficial information about the evolution of MRSA clones (Enright *et al.*, 2000). SCC*mec* is a large mobile genetic element that carries the methicillin resistance determent, *mecA*. SCC*mec* elements are diverse in their structures and genetic contents. Consequently, these elements have been classified into various

types that can be used to study the epidemiology of MRSA (Oliveira and Lencastre, 2002). SCC*mec* typing can be used in combination with MLST to improve its discriminatory power. Nevertheless, MLST is currently relatively expensive, requires specialised equipment and needs highly trained personnel, or access to a commercial sequencing centre (Stepan *et al.*, 2004). In many cases, MLST is not sufficiently discriminatory for single MRSA outbreaks even when combined with SCC*mec* typing. For the reasons outlined above, neither PFGE nor MLST/SCC*mec* typing are used routinely in clinical microbiology laboratories for the analysis of ongoing outbreaks. We therefore identified the need for a discriminatory technique that was both rapid and could be applied in a routine clinical microbiology setting.

In the first step of this study, a large collection of MRSA isolates was obtained from the local NHS Trust hospitals in Newcastle upon Tyne. This collection contained more than 400 MRSA isolates, collected randomly from screening and clinical samples over a period of about two months. Chapter 3 describes the clinical and epidemiological information of these MRSA isolates. *S. aureus* has maintained a close relationship with humans and about 30 to 40% of the population carries *S. aureus* somewhere in or on their bodies (most commonly the nasal cavity) (Champer, 2001). In the current study, a high rate (57%) of MRSA carriage was found among hospital patients. Furthermore, increasing age was a clear risk factor, linked to both colonisation and infection. More than seventy percent of MRSA patients were, unsurprisingly, older than 60 years of age. For any clinical microbiology laboratory, it is important to distinguish between MRSA, MSSA and methicillin-resistant coagulase-negative staphylococci (MRCNS). For accurate identification purposes, both *coa* and *mecA* are used as markers to identify MRSA and, in this study, both genes were targeted by PCR to confirm their correct identification.

We established the PFGE, MLST and SCC*mec* typing methods in our laboratory, and evaluated their use against some of the isolates in our collection of MRSA isolates. Twenty MRSA isolates were selected for PFGE analysis and this revealed EMRSA-15 to be the predominant MRSA strain in the Newcastle upon Tyne hospitals. Three PFGE types, each variants of EMRSA-15, were identified among the 20 MRSA isolates. In conjunction with reference strains, each of the PFGE types was subjected to further analysis by MLST/SCC*mec* typing. This revealed that all of the variants were ST22/SCC*mec*-IV, again corresponding to EMRSA-15. Although a relatively small number of MRSA isolates were typed throughout these studies, the

results confirm previous reports (Jonas *et al.*, 2002; Monnet *et al.*, 2004; Wilson *et al.*, 2010) that EMRSA-15 (*i.e.* ST22/SCC*mec*IV) is the main nosocomial EMRSA clone in north-east of England. PFGE was clearly more discriminatory than MLST, particularly between variants of EMRSA-15. However, in contrast to MLST, high quality PFGE data can only be delivered following extensive technical experience in its application. Like others, we also concluded that MLST is not sufficiently discriminatory for the investigation of potential outbreak strains (Hardy *et al.*, 2006). This makes the use of both methods of limited value in routine clinical laboratories, especially, in developing countries.

Development of a novel *Sma*I-multiplex PCR typing technology

The central aim of this study was to develop a novel genotyping technology that is suitable for routine use in routine clinical hospitals laboratories. This method was designed to combine the simplicity of PCR and, ideally, the discriminatory power of PFGE. It needed to be rapid, to require low-level expertise and to employ equipment likely to be already available in clinical laboratories. Also, the generated epidemiological data should ideally show concordance with data from other epidemiological techniques. Chapter 4 describes a novel genotyping technology for MSSA and MRSA strains. The newly developed typing system is based around the *Sma*I macrorestriction fragments output from PFGE analyses. The *S. aureus* genome is AT-rich (32.8% CG) (Wong *et al.*, 2008), which accounts the selection of the GC-rich *Sma*I (CCC↓GGG) sites for macrorestriction fragment analysis. However, instead of the time-consuming DNA extraction and digestion techniques associated with PFGE, a multiplex PCR technique was devised to identify *Sma*I-based SNPs from rapidly extracted chromosomal DNA.

As a first stage in the development of a new typing method, the DNA sequences of eighteen *S. aureus* strains were analysed using a series of bioinformatic tools to determine the number and locations of their *Sma*I restriction sites. The number of *Sma*I restriction sites was, as expected, found to be between 24 to 29. The genomes of the sequenced *S. aureus* strains have between five and six ribosomal RNA (rRNA) operons and each operon usually include two *Sma*I restriction sites (Green and Vold, 1993; Wada *et al.*, 1993). In current study, the sites within the rRNA operons were excluded due to their high-sequence similarity and the likelihood that their use would result in the generation of false-positive PCR amplicons.

In the second stage in the development of the new typing method, the gene neighbourhood sequences of the syntenic regions either side of potential *Sma*I target sites were aligned. A matrix of the *Sma*I gene neighbourhoods was constructed and 10 *Sma*I-sites were chosen according to their discriminatory power. In order to approach the discriminatory power of PFGE, a set of forward primers was designed that incorporated all or part of the *Sma*I at the 3' end. The cognate reverse primers were designed to produce uniquely sized fragments using well-conserved regions. The presence of a PCR product should therefore reflect the presence or the absence of specific *Sma*I restriction sites in the bacterial chromosomal DNA, with the specific target sites being identified from the size of the amplicon. Initial evaluation of validity of new strategy included the design primers for first six target *Sma*I sites. They were tested in uniplex PCRs then multiplexed against unrelated *S. aureus* strains NCTC8325, EMRSA-15, EMRSA-16 and MRSA-PVL+. The primers showed specificity and distinguished between most of these strains, with exception of EMRSA-15 and EMRSA-16. Interestingly, the group-5 primer pairs of *Sma*I generated variable PCR fragments among test strains (Fig. 4.5), which potentially could be used to increase the discriminatory power of this technique.

In the third stage of development, the discriminatory power of the new method was improved to distinguish between the most prevalent UK clones, namely EMRSA-15 and EMRSA-16. Four new *Sma*I-group primers were designed and, when tested, clearly differentiated between epidemic strains EMRSA-15 and EMRSA-16 (Fig. 4.10). It is interesting to note that the discriminatory of *Sma*I-sites were found to be located throughout the entire *S. aureus* genome and therefore to reflect genetic variations throughout the whole genome (Fig. 4.4).

A multiplex PCR with ten primers was optimized in several phases and in the last optimization phase all primers were redesigned using the Visual OMP™ (Oligonucleotide Modeling Platform) DNA analysis software. This software optimized primer homology, secondary structure, annealing temperature and assay conditions. The first evaluation of the new 10 primer pairs was carried out in two master mixes of multiplex PCR reactions. The master mix prepared in this study showed the best overall performance (Fig. 4.11). The discriminatory power of the new primers was most clear when the annealing temperature was 56˚C. However, the primers of *Sma*I-groups 3, 4 and 10 failed to discriminate as expected between some of the strains due to the production of false-positive amplicons (Fig. 4.12). The reason for this was found to be

the presence of syntenic *Sma*I sites with SNPs that the primers could not differentiate between; CCCTGG in the case of group 3 and CCCAGG in the cases of groups 4 and 10. In an attempt to eliminate these false-positive products, the primers for those three groups were redesigned using the Visual OMP software. The new primers of groups 3 and 4 only amplified products from the expected strains with no false-positive amplicons. However, it was not possible to design a primer pair for the group-10 site as it persisted in generating false-positive amplicons. Consequently this site was excluded and replaced by a primer pair that identified the presence of the *mecA* gene in the multiplex PCR. The typeability, discriminatory power and reproducibility of the *Sma*I-multiplex PCR were investigated against fourteen reference strains (NCTC8325, MRSA252, MSSA476, COL, JH9, N315, Mu3, Mu50, MW2, USA300FPR3757, EMRSA-15, USA1000, USA1100 and CA-629). All strains showed 100% typeability by a novel method with high discriminatory power. The test strains were found to have distinct profile with the exception of very closely related HA-VISA strains Mu50, Mu3 and N315 that, as expected, showed the same *Sma*I-multiplex PCR profile (Fig. 4.17). These strains also share the same sequence (ST5) and SCC*mec* (II) types and showed a close relationship in a molecular evolutionary genetics analysis (MEGA) study (Highlander *et al.*, 2007).

The specificity of the *Sma*I-multiplex PCR technique was assessed against a large number of coagulase positive staphylococci (*i.e. S. aureus*). Consequently, when the specificity of the multiplex primers was tested against two sequenced coagulase-negative staphylococci (CNS) strains, a methicillin resistant *S. epidermidis* (RP62A) and methicillin sensitive *S. epidermidis* (12228), with the exception of the *mecA* primer pair, no other PCR products were generated. This confirmed that the nine primer pairs were specific to *S. aureus* (Fig. 4.16).

PFGE is usually reserved for reference typing, enabling microbiologist to monitor the history of an epidemic over the longer term rather than in real-time. For this purpose, the discriminatory power of the method is the most important parameter. In the current study, all fourteen of the reference strains that were typed by *Sma*I-multiplex PCR were subsequently analysed by PFGE. Moreover, the MLST and SCC*mec* types of the reference strains were already established with the exception of strain CA-629, which was, established as sequence type ST87 and SCC*mec*-V. The *Sma*I-multiplex PCR typing data were comparable with the PFGE data and was more discriminatory than MLST in distinguishing between these reference strains. The congruence between

*Sma*I-multiplex PCR, PFGE and MLST was clearly seen when the data was used to cluster the strains (Fig.4.19). *Sma*I-multiplex PCR grouped the strains into the same four clusters as PFGE and this was almost matched by MLST. Cluster 1 included laboratory strain NCTC8325, CA-MRSA USA300 and HA-MRSA COL strains with sequence types ST8 and ST250 belonging to clonal cluster CC8. The hypervirulent CA-MRSA MW2 is closely related to CA-MSSA476 and both belong to sequence type ST1 (Holden *et al.*, 2004). These strains were distinguishable by *Sma*I-multiplex PCR and PFGE and were grouped in very tight cluster (Fig. 4.19).

VISA Mu50, Mu3, JH9 and N315 are indistinguishable by MLST as ST5/SCC*mec*-II. While Mu50, Mu3 and N315 gave identical *Sma*I-multiplex PCR profiles, JH9 was distinguishable from the other strains (Fig. 4.19). However, although Mu50, N315 and JH9 showed closely related PFGE types, they were distinguishable. Data from this and the two previous clusters clearly show that *Sma*I-multiplex PCR typing is more discriminatory than MLST but less discriminatory than PFGE.

Epidemic strains EMRSA-15 and EMRSA-16 are a major cause for concern for UK hospitals and their prevalence has reached alarming proportions. More recently, the spread of CA-MRSA has been reported in some hospitals (Huang *et al.*, 2006, Kennedy and Deleo, 2009), and distinguishing between HA-MRSA and CA-MRSA has become an important issue in relation to infection control since they show differences in toxigenicity and antibiotic resistance patterns. In the presence study, *Sma*I-multiplex PCR not only distinguished between EMRSA-15 and EMRSA-16, but was also able to differentiate clearly between well known CA-MRSA strains (USA1000, USA1100 and CA-629). Moreover, the clustering of those strains on the basis of the *Sma*I-multiplex PCR profiles was in agreement with both PFGE and MLST (Fig. 4.19).

A simple nomenclatural system for a novel *Sma*I-multiplex PCR profiles was proposed based on the presence and absent of the nine *Sma*I-sites according to their order on the genome (*i.e. mecA*-1 to *Sma*I-9) (Table 4.7). For example, *Sma*I-multiplex PCR type 2 of the COL strain, was given the *Sma*I-profile 1-0-0-1-0-1a-1-0-0-0, with the binary referring to the presence or absence of an amplicon at a specific allele and the letter on the group 5 referring to the length of the variable PCR products generated by the *Sma*I-group 5 primer pair.

In summary, our studies demonstrate that the *Sma*I-multiplex PCR typing technique is a promising method for the typing both hospital-acquired and community-

acquired strains of MSSA and MRSA. In combination with the rapid DNA extraction protocol introduced in this study, the newly developed typing method can be carried out in as little as 4 hours. The technology was developed and evaluated against sequenced *S. aureus* strains and the next stage of the work involved an evaluation of the technique in real time on actual clinical outbreaks.

Evaluation of *Sma*I-multiplex PCR typing on actual clinical outbreaks and other clinical isolates

For any typing technique to be effective it needs to distinguish unambiguously between unrelated strains in the investigation of a single outbreak. Consequently, *Sma*I-multiplex PCR was evaluated on ongoing clinical outbreaks. In Chapter 5 we described the application of *Sma*I-multiplex PCR for the analysis of about 60 isolates that were recovered during outbreaks of *S. aureus* infection, a set of strains with known PFGE profiles received from HPA and a set of strains isolated from blood.

The first evaluation was against seven outbreak isolates of a CO_2-dependent MRSA that were isolated from patients and a member of staff in the regional liver transplant unit (RLU) of the Freeman Hospital. These isolates were originally classified as small colony variants (SCVs) of *S. aureus* before their CO_2-dependency was identified. The epidemiological characterization of the CO_2-dependent MRSA isolates using *Sma*I-multiplex PCR showed that they had identical profiles to EMRSA-15, and this was subsequently were confirmed by PFGE and MLST. All CO_2-dependent MRSA isolates were ST22-SCC*mec*-IV (*i.e* the EMRSA-15 type) and had PFGE profiles that were, excepting in one case, identical to EMRSA-15. The single exception differed with respect to a single band and was classified as a variant of EMRSA-15. Although, PFGE was slightly more discriminatory, the *Sma*I-multiplex PCR data was in agreement with both MLST and PFGE (Table 5.2).

The remaining 52 outbreak isolates were typed only by *Sma*I-multiplex PCR and the resulting epidemiological data was sent to the infection control office at the Freeman Hospital on the same day as the isolates were received. Nineteen *Sma*I-multiplex PCR profiles were observed among the outbreak isolates and isolates that were representatives of these profiles were subsequently analysed by MLST. This analysis confirmed that *Sma*I-multiplex PCR had a better resolving power than MLST since only 16 MLST types were found among the 19 *Sma*I-multiplex PCR profiles (Fig. 5.13). In addition, *Sma*I-multiplex PCR distinguished between isolates that have the same

sequence type (ST5) and between some variants of EMRSA-15 within the same MLST and SCC*mec* type (ST22/SCC*mec*-IV; see Fig. 5.13). The UPGMA clustering method, used to predict phylogenies from genetic comparison data, was in most cases, in good agreement with the MLST phylogenetic tree (Fig. 5.13). The observation that *Sma*I-multiplex PCR was able to identify phylogenies of clinical isolates (Chapter 5) supported the data on the reference strains (Chapter 4) in which showed a high correlation was observed between *Sma*I-multiplex PCR and MLST (Fig. 4.19).

Since multiplex PCR is quicker and more cost-effective than a uniplex PCR, a simple diagnostic multiplex PCR assay for detection the *coa*, *mecA* and PVL genes was reported in this study. The proposed diagnostic assay showed high specificity against staphylococcal isolates (Fig. 5.14) by providing an efficient and reliable identification of *S. aureus* strains. Consequently, this assay represents an important tool for the initial identification of MSSA and MRSA producers of PVL.

The HPA provides UK clinical microbiology laboratories with a microbial identification and typing data service for human pathogens. In collaboration with the HPA, twenty-four MRSA isolates with known PFGE types were received for a detailed assessment of *Sma*I-multiplex PCR typing. The HPA MRSA isolates were *Sma*I-multiplex PCR typed directly from DNA extracted by the rapid extraction protocol (25 min). The resulting *Sma*I-multiplex PCR profiles correctly distinguished between epidemic strains EMRSA-15 and EMRSA-16, but only differentiated between two variants of EMRSA-15 (out of 12) and three variants of EMRSA-16 (out of 12) (Fig. 5.15).

As the last evaluating step, thirteen MRSA blood isolates were used to determine the discriminatory power of the developed *Sma*I-multiplex PCR technology. Ninety-two percent (n=12) of the isolates showed identical *Sma*I-multiplex PCR profile to that of EMRSA-15, while the remaining isolate was identified as EMRSA-16 (Fig. 5.17). Three representatives (two EMRSA-15 isolates and one EMRSA-16) were further analysed by MLST. The MLST data confirmed the *Sma*I-multiplex PCR data which revealed that both EMRSA-15 representatives were EMRSA-15 (ST22-SCC*mec*-IV) isolates and representative of EMRSA-16 was an EMRSA-16 (ST36-SCC*mec*-II). These findings indicate the discriminatory power of a novel typing method and also reflected the dominance of the EMRSA-15 clone among MRSA bacteraemias among patients at the Freeman Hospital.

In conclusion, we described a novel *Sma*I-multiplex PCR technique for the typing both MSSA and MRSA strains. The new technology is high throughput, relatively cheap and provides reliable and comparable genotyping data. At the same time, the *Sma*I-multiplex PCR meets most of the criteria of a useful typing method: it is simple, inexpensive, highly discriminatory and does not require sophisticated equipment or expertise. Consequently, *Sma*I-multiplex PCR could be used routinely in any clinical microbiology laboratory since it relies on standard clinical laboratory apparatus (*i.e.* PCR machine and agarose gel electrophoresis). The current study demonstrates that *Sma*I-multiplex PCR typing can provide real-time information for the investigation of ongoing *S. aureus* hospital outbreaks. *Sma*I-multiplex PCR proved to be more discriminatory than MLST/SCC*mec* typing, but less discriminatory than PFGE. As previously mentioned, PFGE is more sensitive to rapid genetic changes (*e.g.* point mutation) while MLST monitors accumulated genetic changes that occurs slowly over time. The *Sma*I-multiplex PCR technique can sometimes identify single genetic events (*e.g.* changes in *Sma*I-sites), and the distribution of *Sma*I targets throughout the entire genome may enable it to discover slowly accumulating genetic changes. Moreover, the observed correlation between *Sma*I-multiplex PCR and MLST in identifying the phylogenies of clinical isolates is a good indicator for the validity of *Sma*I-multiplex PCR in study of *S. aureus* evolution.

Although, the current *Sma*I-multiplex PCR protocol takes between 4 to 6 hours, it would be possible to adapt this technology towards an automated genotyping assay using RT-multiplex PCR. This would reduce the processing time to less than 60 minutes. Since individual targets are identifiable on the basis of the size of their amplicons, the RT-PCR output could be processed directly via dedicated analytical software.

The development of the *Sma*I-multiplex PCR for studying the epidemiology of *Staphylococcus aureus* is not complete and future work needs to be aimed at improving its discriminatory power so that it is equal to or better than that of PFGE. One possible approach to achieving this is to exploit the presence of *Staphylococcus aureus* repeat (STAR) elements. The current study has already shown that the presence of STAR elements can improve the discriminatory power at the *Sma*I-group 5 site, in which we have currently identified 5 amplicon size variations (a-e).

As a preliminary to understanding whether STAR elements can be used for epidemiological analyses, a BLAST analysis was carried out to identify the number and location of STAR elements within the genomes of the currently sequences *S. aureus* strains. This analysis has revealed that the numbers of STAR elements at the different locations are highly variable (Table 6.1). The distinct feature of these elements is that they contain a signature sequence (GGGGCCCC) that can be present in many copies at a single locus, flanked by invariable sequences. The invariable sequences either side of the STAR elements could then be targeted by PCR primers to generate amplicons of various sizes.

Table 6.1:   Number of STAR elements among the sequenced *S. aureus* strains. Gray highlighted strains are closely related.

S. aureus strains	STAR element no.
RF122	57
USA300	68
COL	71
JH1	70
JH9	70
MRSA252	61
MSSA476	78
MW2	83
Mu3	75
Mu50	75
N315	76
NCTC 8325	70
Newman	76
USA300_TCH1516	69

Six loci (*uvrA*, *trxB*, *glpQ*, *acnA*, *icaC* and *atpC*) were selected to illustrate the potential of STAR elements to distinguish between closely related *S. aureus* strains. For example strains N315, Mu3 and Mu50 are difficult to distinguish using conventional molecular typing techniques since they have identical sequence and SCC*mec* types (ST5-SCC*mec*-II) and have closely related PFGE profiles (Fig. 4.19). Closely related JH1 and JH9 strains are also undistinguishable by PFGE (Sieradzki *et al*., 2003). Although the STAR element profiles of these strains would be very similar, differences within *acnA* locus would be sufficient to distinguish Mu3 from Mu50 and N315. However, STAR elements could not be used to differentiate between strains JH1 and JH9 (Table 6.2 and Appendix D). Overall, this bioinformatical analysis clearly indicates

the potential of using STAR elements for increasing the discriminatory power of *Sma*I multiplex PCR. Ultimately, the aim is to distinguish between PFGE variants of EMRSA-15 and EMRSA-16. However, until and unless the genome sequences of these variants are published, it will be necessary to determine the value of STAR elements by experimentation. It is worth exploring such a strategy since, if successful, it could considerably enhance the value of *Sma*I multiplex PCR by introducing a second-stage assay when the current technique fails to distinguish between potential outbreak strains.

Table 6.2: The numbers of STAR element repeats identified within six loci among the sequenced *S. aureus* strains. Gray highlighted strains are closely related strains that can be distinguished by the STAR elements within the *acnA* locus.

STAR elements loci / Strain	*uvrA*	*trxB*	*glpQ*	*acnA*	*icaC*	*atpC*
RF122	1	4	0	0	1	0
MW2	2	3	2	1	2	1
MSSA476	2	3	0	2	2	1
USA300	3	5	3	2	2	1
USA300TCH	3	4	3	2	2	1
MRSA252	1	3	0	0	1	4
COL	2	2	0	2	0	1
N315	3	6	1	3	1	1
Mu3	3	6	1	0	1	1
Mu50	3	6	1	3	1	1
Newman	3	6	2	0	1	1
NCTC8325	0	0	0	0	2	1
JH1	0	0	0	0	0	1
JH9	0	0	0	0	0	1

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Appendixes

Appendix A

Media, Buffers and Solutions

The chemicals and reagents which were used in this study

Chemicals	Supplier

Agarose :
    Traditional agarose
    PFGE agrose (Certified Megabase Agarose)
	PEQLAB
	Bio-Rad

Low melting point (LMP) agarose	Biogene
Trizma Base	Sigma
EDTA (Ethylene diamino tetra actic acid)	Sigma
Boric acid	Sigma
Lysostaphin	Sigma
Lysozyme	Bio-Rad
Polyethylene glycol (PEG) 6000	Sigma
Ethedium Bromide	Fluka
Phenylmethylsulfonyl fluoride (PMSF)	Sigma
Proteinase K : Extraction chromosomal DNA for PCR Extraction chromosomal DNA for PFGE *Taq* polymerase	QIAgen Bio-Rad Fermentas
dNTP	Fermentas
$MgCl_2$	Fermentas
BSA	NewEngland Biolab
Glass beads 106 microns	Sigma

- **Growth media**

Nutrient Medium	(per litre) pH 7.5 (autoclave)
'Lab_Lemco' Powder	10g
Peptone	10g
NaCl	5g
Agar	15g

Brain Heart Infusion Medium	(per litre) pH 7.0 (autoclave)
Brain Heart, Infusion from (solids)	6g
Peptic Digest of Animal Tissue	6g
Pancreatic Digest of Gelatin	14.5g
Dextrose	3g
NaCl	5g
Agar	15g

Blood Agar

ATM Blood agar was prepared (at the Freeman hospital) according to manufacturer's instructions. Columbia agar (CM331; Oxoid) was prepared and cooled to 50ºC and 5% of horse blood and 4 mg of aztreonam per litre were added. Nalidixic acid blood agar was prepared as with Columbia agar above, and then adding 5% of horse blood and 30mg of nalidixic acid per litre (Perry *et al.* 2003).

- **Buffers**

TN Buffer	(per litre) pH 7.6 (autoclave)
Tris HCl	10mM
NaCl	1mM

Tris-EDTA (TE) Buffer	(per litre) pH 7.5	(autoclave)
Tris	10mM	
EDTA	1mM	

Tris-Borate-EDTA (TBE) Buffer (5x stock)	(per litre) pH 8.0
Tris	53.9g
Boric acid	27.5g
EDTA	3.75g

Lysis Buffer (5x stock) pH 8.0	(autoclave)
Tris	25mM
EDTA	25mM
Sucrose	0.3M
dd H_2O	200ml

- **Solutions**

Glycerol Solution (70%)	(per 100ml)	(autoclave)
Glycerol (100%)	70ml	
ddH$_2$O	30ml	

Lysostaphin Solution (1mg/ml)	(filter-sterilized)
Lysostaphin	1mg
NaCl (100mM)	1ml

Sodium chloride solution (1M NaCl)	(autoclave)
NaCl	5.8g
ddH$_2$O	100ml

Polyethylene glycol (PEG) 20% (w/v) / 2.5M NaCl solution (filter-sterilized)	
Polyethylene glycol (PEG) 20% (w/v)	40% (w/v)
NaCl	5M
ddH$_2$O	50ml

Pheylmethysulfonyl fluoride (100mM PMSF)	
Pheylmethysulfonyl fluorid	0.174g
Isoprpanol (100%) (w/v)	10ml

Ethidium Bromide (EtBr) staining solution(1µg/ml) (kept in dark and cold place)	
Ethidium Bromide (10mg/ml)	100µl
TBE Buffer (0.5x stock)	1 liter

Ethidium Bromide (EtBr) staining solution (0.5µg/ml)(kept in dark and cold place)	
Ethidium Bromide (10mg/ml)	50µl
TBE Buffer (1x stock)	1 liter

Appendix B

DNA molecular size markers
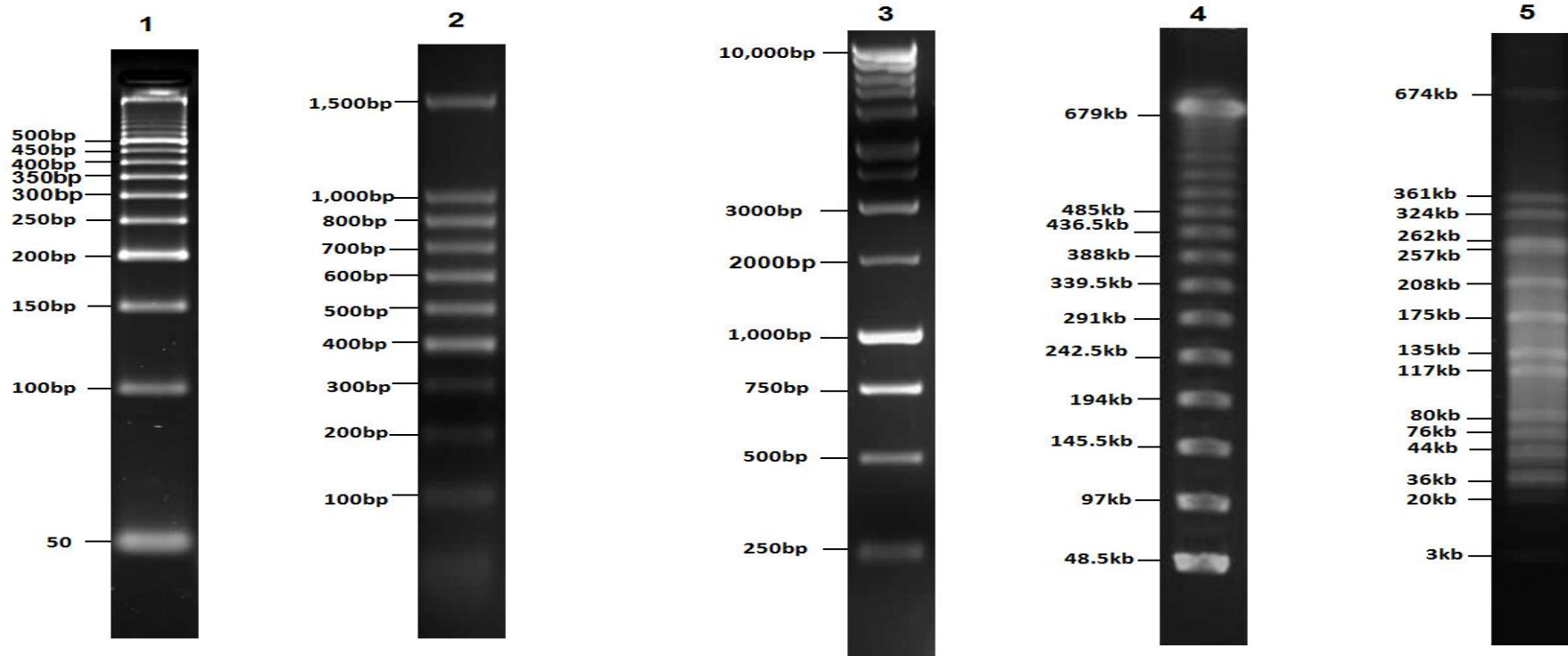
Lane 1: 50bp DNA ladder.
Lane 2: 100bp DNA ladder.
Lane 3: 1kb DNA ladder.
Lane 4: Lambda ladder, molecular size marker for PFGE.
Lane 5: NCTC8325, molecular size marker for PFGE.

Appendix C

Design of primers for multiplex PCR using visual OMP^{TM} DNA software

The second phase primers were designed using the visual OMP^{TM} (Oligonucleotide Modelling Platform) software. The OMP^{TM} allows the users to design primers and probes for multiple specific sites and optimizes the secondary structure, homology, annealing temperature of primers and assay conditions. The design of primer pairs using this software was carried out through several steps:

1- The analysis is started by selecting File/new project and experiment, and then opening a blank sequences form. This is used to enter the target sequence (*e.g.* from an NCBI genome file).
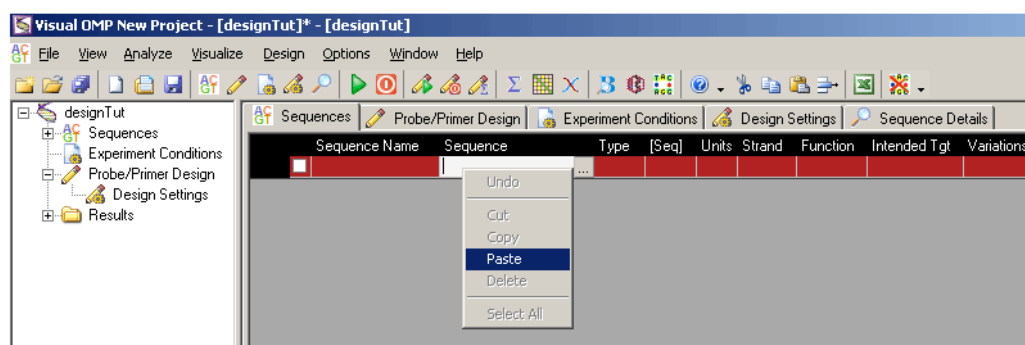
2- The experimental conditions Tab is selected and the appropriate values entered into the solution condition text boxes (the manufacture recommends running the PCR at 55°C, 50 mM NaCl, 1.5 MgCl$_2$ at pH 7.0. This step can be reiterated when the target (DNA sequence) changes.
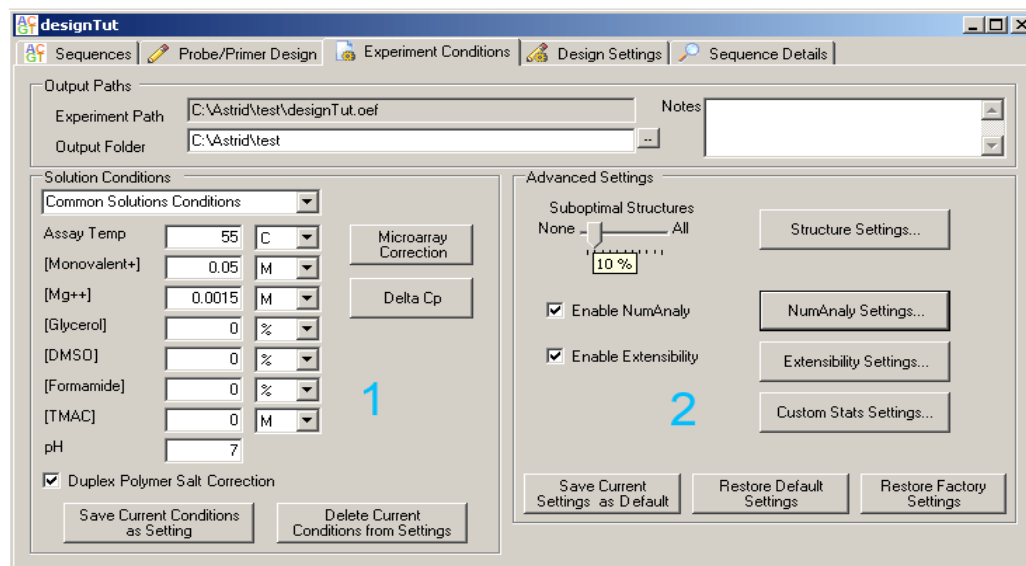
3- A simulation hybridization is run by pressing the "simulate experiment" icon on the toolbar.
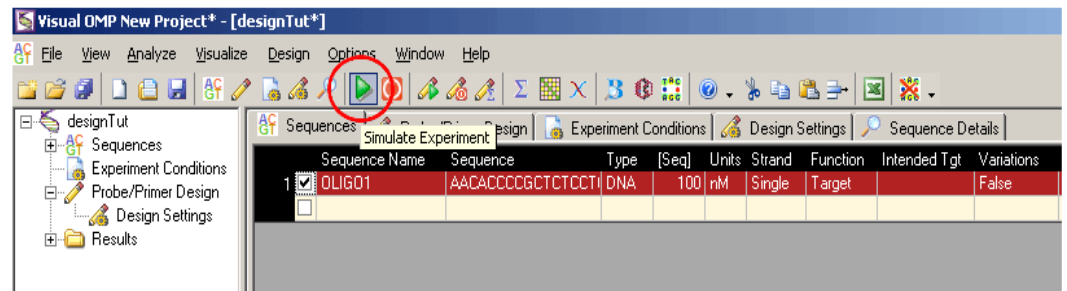
4- Select "yes" to see the results.
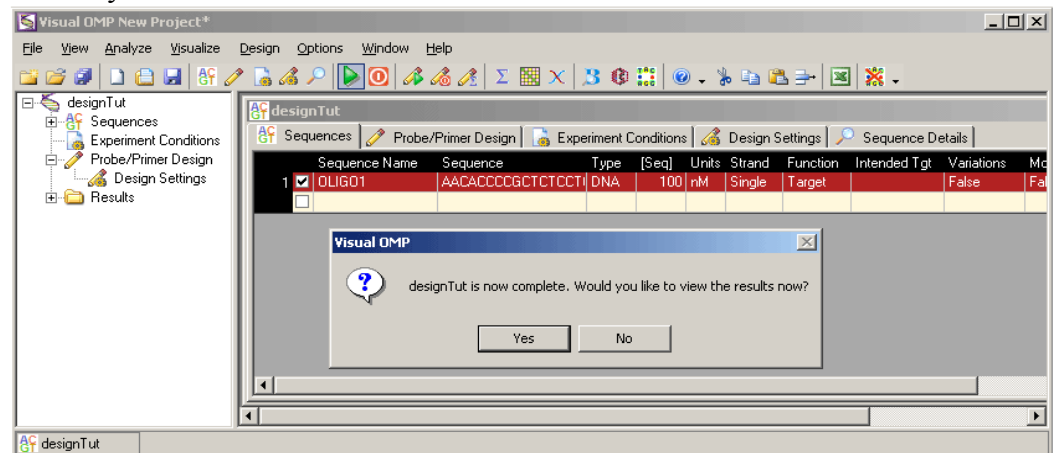
5- Clicking the "View" link (Red circle) allows the user to see the structure target with second structure.
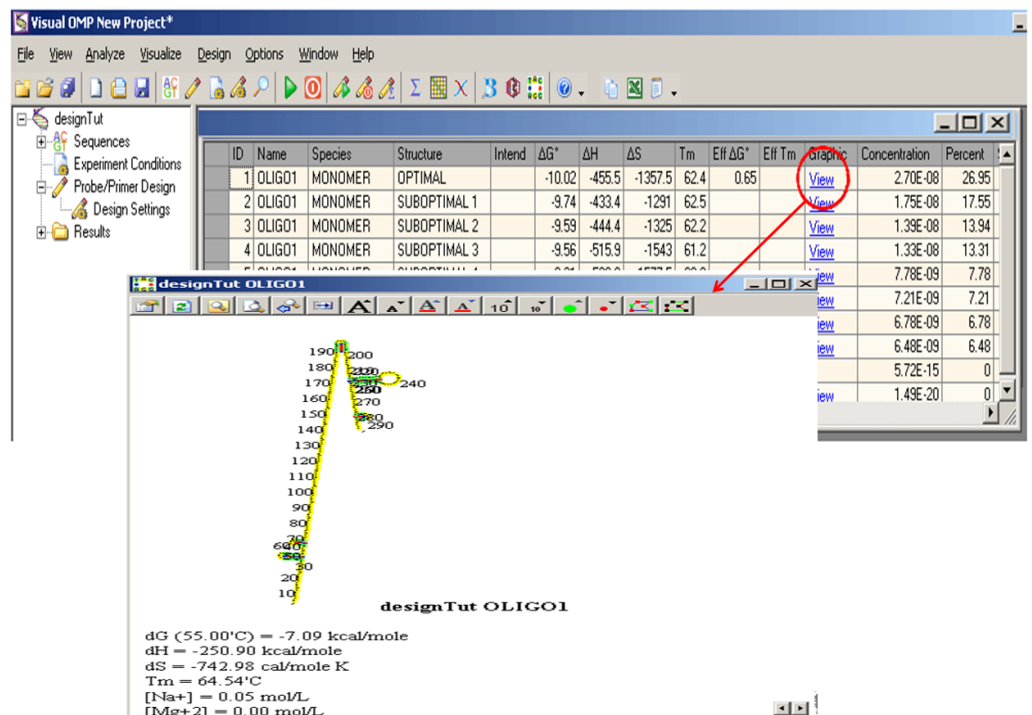
6- The "Probe/Primer Design" tab is clicked and the target name selected.
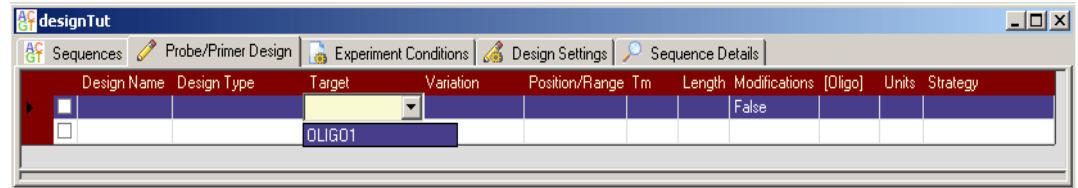
7- The range of primer (e.g. 110-160bp) is selected.
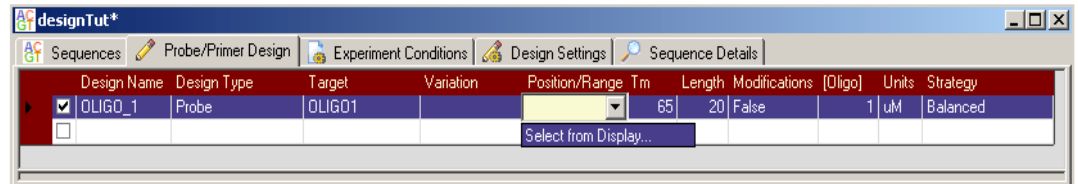
8- The characteristics of range are then seen in a dialog box.
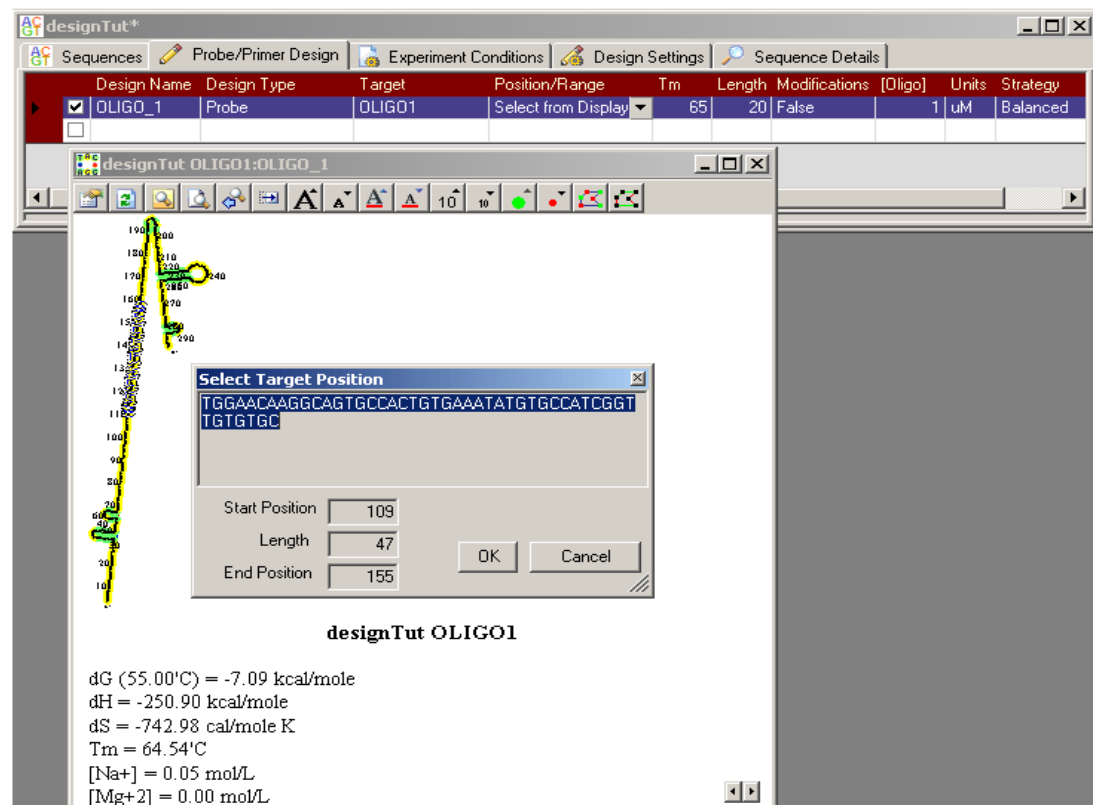
9- In case of our study, the forward primer should contain the *Sma*I site (CCCGGG) at its 3'-end and therefore the range of designs are limited at 3'-end of forward primer.

10- To design a primer pair around desired range, the primer pairs were selected from the "Design Type" column.
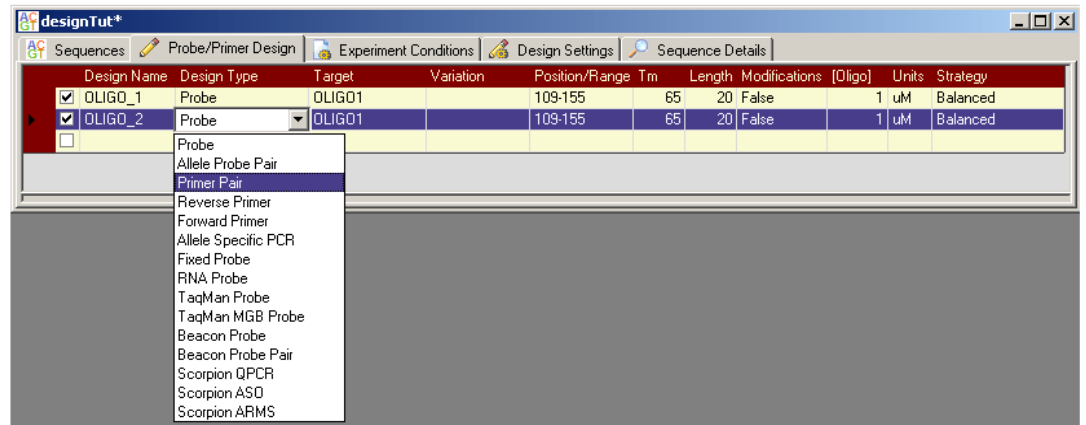
11- Selecting the "Design" tag (red circle) facilitates the design the two primers and one probe, with the software will automatically resolving all crosshybridization reactions.
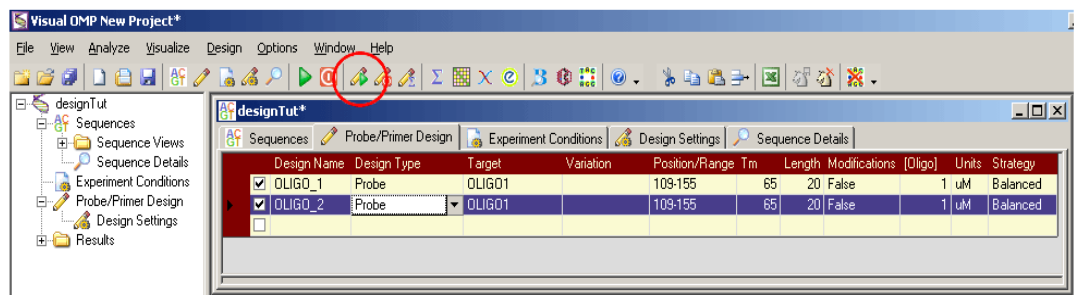
12- The software provides about 50 solutions for each set of conditions and each solution contains three oligos: two primers and one probe.

13- The required solution is selected and then added to the experiment.

14- The sequences are renamed and the target changed to double-stranded DNA.

15- The simulation is then run with to see the percentage of bound to the target (red circles).

16- In the case of the *Sma*I-group 6 target, the programme identified that the secondary structure of the target reduces the PCR efficiency to ~52% (Red circle).

17- However, increasing the primer length and adding PCR adjunct can overcome the problem and increase the PCR efficiency to 97% (Red circle). Step 2 can be reiterated if needed.

18- As the CCCGGG sequence at 3' end of forward primer is optimal for hairpin formation, removing two GG bases at 3' end increased the PCR efficiency.

19- The software also assesses the hybridization between different sequences at different concentrations and temperatures.

Appendix D
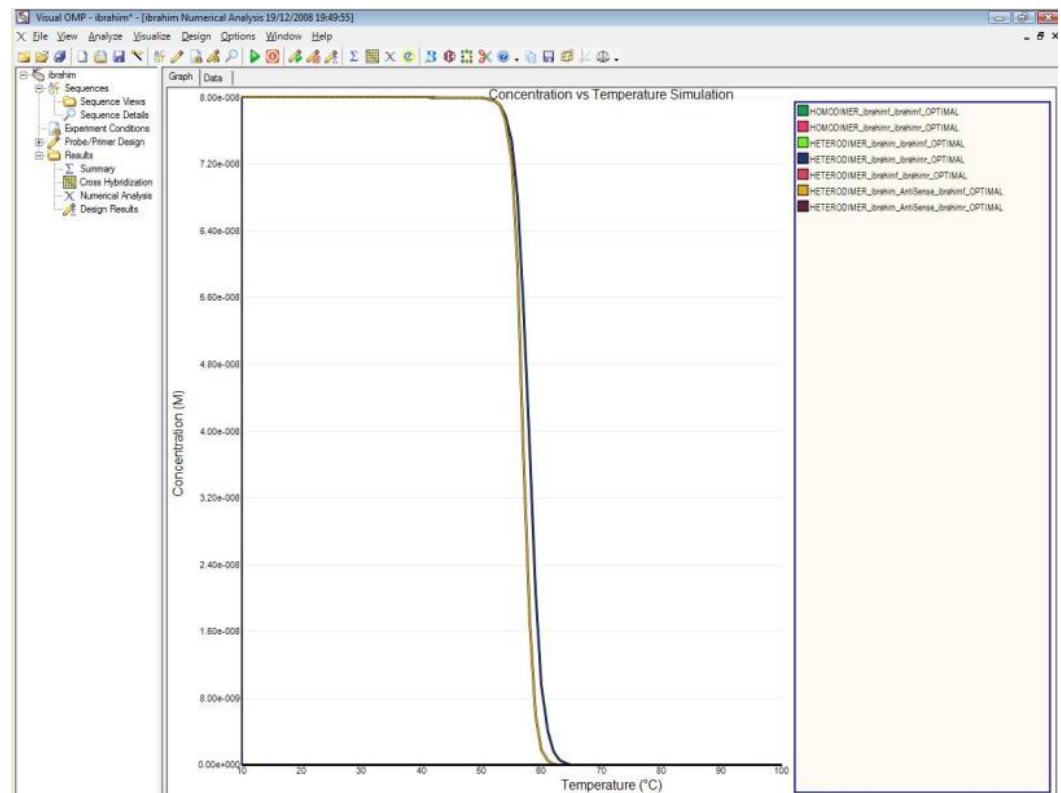
S. aureus strains	Position	Strand	Upstream Gene Name	Gene	Downstream Gene Name	Downstream Gene (same strand) Name	Downstream same strand bp	Sequence
								The STAR element within upstream of *uvrA* and downstream of *hprK* loci among *S. aureus* strains
RF122	784876	+	uvrA(+)	-/-	hprK(+)	hprK(+)	-243	aattgggaatccaatttctcTGTGTTGGGGCCCCgccaactaattcgaatttat
USA300	829079	+	uvrA(+)	-/-	hprK(+)	hprK(+)	-353	ctaaagaaaaagtatttcttTATGTTGGGGCCCCgccaacttgcattgtttgta
USA300	829134	+	uvrA(+)	-/-	hprK(+)	hprK(+)	-298	aatttcttttcgaaattcttTATGTTGGGGCCCCgccaacttgcattgtttgta
USA300	829189	+	uvrA(+)	-/-	hprK(+)	hprK(+)	-243	aatttcttttcgaaattcttTATGTTGGGGCCCCgccaactaattccaatatat
COL	850680	+	uvrA(+)	-/-	hprK(+)	hprK(+)	-298	ctaaagaaaaagtatttcttTATGTTGGGGCCCCgccaacttgcattgtttgta
COL	850735	+	uvrA(+)	-/-	hprK(+)	hprK(+)	-243	aatttcttttcgaaattcttTATGTTGGGGCCCCgccaactaattccaatatat
MRSA252	856882	+	uvrA(+)	-/-	hprK(+)	hprK(+)	-243	aattgagaatccaatttctcTGTGTTGGGGCCCCgccaactaattcgaatttat
MSSA476	812230	+	uvrA(+)	-/-	hprK(+)	hprK(+)	-298	ctaaagaaaaagtatttcttTATGTTGGGGCCCCgccaacttgcattgtttgta
MSSA476	812285	+	uvrA(+)	-/-	hprK(+)	hprK(+)	-243	aatttcttttcgaaattcttTATGTTGGGGCCCCgccaactaattccaatatat
MW2	813802	+	uvrA(+)	-/-	hprK(+)	hprK(+)	-298	ctaaagaaaaagtatttcttTATGTTGGGGCCCCgccaacttgcattgtttgta
MW2	813857	+	uvrA(+)	-/-	hprK(+)	hprK(+)	-243	aatttcttttcgaaattcttTATGTTGGGGCCCCgccaactaattccaatatat
Mu3	843714	+	uvrA(+)	-/-	hprK(+)	hprK(+)	-354	ctaaagaaaaagtatttcttTATGTTGGGGCCCAccccaacttgcattgtttgt
Mu3	843770	+	uvrA(+)	-/-	hprK(+)	hprK(+)	-298	aatttcttttcgaaattctcTGTGTTGGGGCCCCgccaacttgcattgcctgta
Mu3	843825	+	uvrA(+)	-/-	hprK(+)	hprK(+)	-243	aatttcttttcgaaattcttTATGTTGGGGCCCCgccaactaattacaatatat
Mu50	842269	+	uvrA(+)	-/-	hprK(+)	hprK(+)	-354	ctaaagaaaaagtatttcttTATGTTGGGGCCCAccccaacttgcattgtttgt
Mu50	842325	+	uvrA(+)	-/-	hprK(+)	hprK(+)	-298	aatttcttttcgaaattctcTGTGTTGGGGCCCCgccaacttgcattgcctgta
Mu50	842380	+	uvrA(+)	-/-	hprK(+)	hprK(+)	-243	aatttcttttcgaaattcttTATGTTGGGGCCCCgccaactaattacaatatat
N315	818076	+	uvrA(+)	-/-	hprK(+)	hprK(+)	-354	ctaaagaaaaagtatttcttTATGTTGGGGCCCAccccaacttgcattgtttgt
N315	818132	+	uvrA(+)	-/-	hprK(+)	hprK(+)	-298	aatttcttttcgaaattctcTGTGTTGGGGCCCCgccaacttgcattgcctgta
N315	818187	+	uvrA(+)	-/-	hprK(+)	hprK(+)	-243	aatttcttttcgaaattcttTATGTTGGGGCCCCgccaactaattacaatatat
Newman	819569	+	uvrA(+)	-/-	hprK(+)	hprK(+)	-353	ctaaagaaaaagtatttcttTATGTTGGGGCCCCgccaacttgcattgtttgta
Newman	819624	+	uvrA(+)	-/-	hprK(+)	hprK(+)	-298	aatttcttttcgaaattcttTATGTTGGGGCCCCgccaacttgcattgtttgta
Newman	819679	+	uvrA(+)	-/-	hprK(+)	hprK(+)	-243	aatttcttttcgaaattcttTATGTTGGGGCCCCgccaactaattccaatatat
TCH1516	842909	+	uvrA(+)	-/-	hprK(+)	hprK(+)	-353	ctaaagaaaaagtatttcttTATGTTGGGGCCCCgccaacttgcattgtttgta
TCH1516	842964	+	uvrA(+)	-/-	hprK(+)	hprK(+)	-298	aatttcttttcgaaattcttTATGTTGGGGCCCCgccaacttgcattgtttgta
TCH1516	843019	+	uvrA(+)	-/-	hprK(+)	hprK(+)	-243	aatttcttttcgaaattcttTATGTTGGGGCCCCgccaactaattccaatatat

The STAR element within upstream of *trxB* locus among *S. aureus* strain

S. aureus strains	Position	Strand	Upstream Gene Name	Gene	Downstream Gene Name	Downstream Gene (same strand) Name	Downstream same strand bp	Sequence
RF122	790139	+	trxB(+)	SAB0718c(-)	SAB0719(+)	SAB0719(+)	-464	gctgactttccgccagctttTGTGTTGGGGCCCCgcccccaacttgcattgtct
RF122	790198	+	trxB(+)	SAB0718c(-)	SAB0719(+)	SAB0719(+)	-405	aattgggaatccaatttctcTGTGTTGGGGCCCCgccaacttgcattgtctgta
RF122	790310	+	trxB(+)	SAB0718c(-)	SAB0719(+)	SAB0719(+)	-293	aattgggaatccaatttctcTTTGTTGGGGCCCAcaccccaactcgcattgcct
RF122	790368	+	trxB(+)	SAB0718c(-)	SAB0719(+)	SAB0719(+)	-235	aatttcttttcgaaattctcTGTGTTGGGGCCCCtgactagagttgaaaaaagc
USA300	834568	+	trxB(+)	-/-	SAUSA300_0748(+)	SAUSA300_0748(+)	-463	gctgactttccgccagcttcTGTGTTGGGGCCCCgccaacttgcacattattgt
USA300	834624	+	trxB(+)	-/-	SAUSA300_0748(+)	SAUSA300_0748(+)	-407	gctgacttttcgtcagcttcTGTGTTGGGGCCCCgccaacttgcattgtctgta
USA300	834680	+	trxB(+)	-/-	SAUSA300_0748(+)	SAUSA300_0748(+)	-351	aattgggaatccaatttctcTATGTTGGGGCCCAcaccccaactcgcattgcct
USA300	834738	+	trxB(+)	-/-	SAUSA300_0748(+)	SAUSA300_0748(+)	-293	aatttcttttcgaaattctcTGTGTTGGGGCCCAcaccccaactcgcattgcct
USA300	834796	+	trxB(+)	-/-	SAUSA300_0748(+)	SAUSA300_0748(+)	-235	aatttcttttcgaaattctcTGTGTTGGGGCCCCtgactagagttgaaaaaagc
COL	856114	+	trxB(+)	-/-	SACOL0830(+)	SACOL0830(+)	-293	gctgactttccgccagcttcTGTGTTGGGGCCCAcaccccaactcgcattgcct
COL	856172	+	trxB(+)	-/-	SACOL0830(+)	SACOL0830(+)	-235	aatttcttttcgaaattctcTGTGTTGGGGCCCCtgactagagttgaaaaaagc
MRSA252	862264	+	trxB(+)	-/-	SAR0820(+)	SAR0820(+)	-351	gctggcggaaagtcagcttcTGTGTTGGGGCCCAcccccaactcgcattgcct
MRSA252	862322	+	trxB(+)	-/-	SAR0820(+)	SAR0820(+)	-293	aatttcttttcgaaattctcTGTGTTGGGGCCCCaccccaacttgcacattatt
MRSA252	862380	+	trxB(+)	-/-	SAR0820(+)	SAR0820(+)	-235	gctgacttacgtcagcttcTTTGTTGGGGCCCCtgactataattgaaaaaagc
MSSA476	817664	+	trxB(+)	-/-	SAS0730(+)	SAS0730(+)	-348	gctgactttcgtaagcttcTGTGTTGGGGCCCCgccaacttgcattgtctgta
MSSA476	817719	+	trxB(+)	-/-	SAS0730(+)	SAS0730(+)	-293	aaattcttttcgaaattctcTGTGTTGGGGCCCAcaccccaactcgcattgcct
MSSA476	817777	+	trxB(+)	-/-	SAS0730(+)	SAS0730(+)	-235	aatttcttttcgaaattctcTGTGTTGGGGCCCCtgactagagttgaaaaaagc
MW2	819236	+	trxB(+)	-/-	MW0727(+)	MW0727(+)	-348	gctgactttcgtaagcttcTGTGTTGGGGCCCCgccaacttgcattgtctgta
MW2	819291	+	trxB(+)	-/-	MW0727(+)	MW0727(+)	-293	aaattcttttcgaaattctcTGTGTTGGGGCCCAcaccccaactcgcattgcct
MW2	819349	+	trxB(+)	-/-	MW0727(+)	MW0727(+)	-235	aatttcttttcgaaattctcTGTGTTGGGGCCCCtgactagagttgaaaaaagc
Mu3	849205	+	trxB(+)	-/-	SAHV_0762(+)	SAHV_0762(+)	-527	gctgacttatcgtaagcttcTGTGTTGGGGCCCAcaccccaactcgcattgcct
Mu3	849263	+	trxB(+)	-/-	SAHV_0762(+)	SAHV_0762(+)	-469	aatttcttttcgaaattctcTTTGTTGGGGCCCAcaccccaacttgcattgtct
Mu3	849322	+	trxB(+)	-/-	SAHV_0762(+)	SAHV_0762(+)	-410	aattggaaatccaatttctcTGTGTTGGGGCCCAcaccccaacttgcattgtct
Mu3	849381	+	trxB(+)	-/-	SAHV_0762(+)	SAHV_0762(+)	-351	aattggaaatccaatttctcTGTGTTGGGGCCCAcaccccaactcgcattgcct
Mu3	849439	+	trxB(+)	-/-	SAHV_0762(+)	SAHV_0762(+)	-293	aatttcttttcgaaattctcTGTGTTGGGGCCCAcaccccaacttgcattgtct
Mu3	849498	+	trxB(+)	-/-	SAHV_0762(+)	SAHV_0762(+)	-234	aattggaaatccaatttctcTGTGTTGGGGCCCCtgactagagttgaaaaaagc
Mu50	847760	+	trxB(+)	-/-	SAV0765(+)	SAV0765(+)	-527	gctgacttatcgtaagcttcTGTGTTGGGGCCCAcaccccaactcgcattgcct
Mu50	847818	+	trxB(+)	-/-	SAV0765(+)	SAV0765(+)	-469	aatttcttttcgaaattctcTTTGTTGGGGCCCAcaccccaacttgcattgtct
Mu50	847877	+	trxB(+)	-/-	SAV0765(+)	SAV0765(+)	-410	aattggaaatccaatttctcTGTGTTGGGGCCCAcaccccaacttgcattgtct
Mu50	847936	+	trxB(+)	-/-	SAV0765(+)	SAV0765(+)	-351	aattggaaatccaatttctcTGTGTTGGGGCCCAcaccccaactcgcattgcct
Mu50	847994	+	trxB(+)	-/-	SAV0765(+)	SAV0765(+)	-293	aatttcttttcgaaattctcTGTGTTGGGGCCCAcaccccaacttgcattgtct
Mu50	848053	+	trxB(+)	-/-	SAV0765(+)	SAV0765(+)	-234	aattggaaatccaatttctcTGTGTTGGGGCCCCtgactagagttgaaaaaagc
N315	823567	+	trxB(+)	-/-	SA0720(+)	SA0720(+)	-527	gctgacttatcgtaagcttcTGTGTTGGGGCCCAcaccccaactcgcattgcct
N315	823625	+	trxB(+)	-/-	SA0720(+)	SA0720(+)	-469	aatttcttttcgaaattctcTTTGTTGGGGCCCAcaccccaacttgcattgtct
N315	823684	+	trxB(+)	-/-	SA0720(+)	SA0720(+)	-410	aattggaaatccaatttctcTGTGTTGGGGCCCAcaccccaacttgcattgtct

N315	823743	+	trxB(+)	-/-	SA0720(+)	SA0720(+)	-351	aattggaaatccaatttctcTGTGTTGGGGCCCAcaccccaactcgcattgcct
N315	823801	+	trxB(+)	-/-	SA0720(+)	SA0720(+)	-293	aatttcttttcgaaattctcTGTGTTGGGGCCCAcaccccaacttgcattgtct
N315	823860	+	trxB(+)	-/-	SA0720(+)	SA0720(+)	-234	aattggaaatccaatttctcTGTGTTGGGGCCCCtgactagagttgaaaaaagc
Newman	825058	+	trxB(+)	-/-	NWMN_0733(+)	NWMN_0733(+)	-463	gctgactttccgccagcttcTGTGTTGGGGCCCCgccaacttgcacattattgt
Newman	825114	+	trxB(+)	-/-	NWMN_0733(+)	NWMN_0733(+)	-407	gctgactttcgtcagcttcTGTGTTGGGGCCCCgccaacttgcattgtctgta
Newman	825170	+	trxB(+)	-/-	NWMN_0733(+)	NWMN_0733(+)	-351	aattgggaatccaatttctcTATGTTGGGGCCCAcaccccaactcgcattgcct
Newman	825228	+	trxB(+)	-/-	NWMN_0733(+)	NWMN_0733(+)	-293	aatttcttttcgaaattctcTGTGTTGGGGCCCAcaccccaactcgcattgcct
Newman	825286	+	trxB(+)	-/-	NWMN_0733(+)	NWMN_0733(+)	-235	aatttcttttcgaaattctcTGTGTTGGGGCCCCtgactagagttgaaaaaagc
Newman	832378	+	trxB(+)	-/-	NWMN_0733(+)	NWMN_0733(+)	-463	gctgactttccgccagcttcTGTGTTGGGGCCCCgccaacttgcacattattgt
CH1516	848398	+	trxB1(+)	USA300HOU_0793(-)	USA300HOU_0794(+)	USA300HOU_0794(+)	-463	gctgactttccgccagcttcTGTGTTGGGGCCCCgccaacttgcacattattgt
TCH1516	848454	+	trxB1(+)	USA300HOU_0793(-)	USA300HOU_0794(+)	USA300HOU_0794(+)	-407	gctgactttcgtcagcttcTGTGTTGGGGCCCCgccaacttgcattgtctgta
TCH1516	848510	+	trxB1(+)	USA300HOU_0793(-)	USA300HOU_0794(+)	USA300HOU_0794(+)	-351	aattgggaatccaatttctcTATGTTGGGGCCCAcaccccaactcgcattgcct
_TCH1516	848568	+	trxB1(+)	USA300HOU_0793(-)	USA300HOU_0794(+)	USA300HOU_0794(+)	-293	aatttcttttcgaaattctcTGTGTTGGGGCCCAcaccccaactcgcattgcct

The STAR element within upstream of *glpQ* locus among *S. aureus* strain								
S. aureus strains	**Position**	**Strand**	**Upstream Gene Name**	**Gene**	**Downstream Gene Name**	**Downstream Gene (same strand) Name**	**Downstream same strand bp**	**Sequence**
USA300	940537	-	glpQ(-)	-/-	gudB(+)	SAUSA300_0856(-)	-6326	gctgactttccaccagcctcTGTGTTGGGGCCCCgactattttttgaaaagagcg
USA300	940593	-	glpQ(-)	-/-	gudB(+)	SAUSA300_0856(-)	-6382	gctgactttccgccagcctcTGTGTTGGGGCCCCgccaacttgcacactattgt
USA300	940707	-	glpQ(-)	-/-	gudB(+)	SAUSA300_0856(-)	-6496	gctgacttttcgccagcttcTGTGTTGGGGCCCCgccaacttgcattgtctgta
MW2	924484	-	glpQ(-)	-/-	gluD(+)	MW0835(-)	-6325	aatttcttttcgaaattctcTGTGTTGGGGCCCCgactattttttgaaaagagcg
MW2	924541	-	glpQ(-)	-/-	gluD(+)	MW0835(-)	-6382	aattggctccccaatttctcTGTGTTGGGGCCCAtccccaacttgcattgcctg
Mu3	1002300	-	glpQ(-)	-/-	gudB(+)	SAHV_0948(-)	-6325	gctgactttccaccagcctcTGTGTTGGGGCCCCgactattttttgaaaagagcg
Mu50	1000856	-	glpQ(-)	-/-	gluD(+)	SAV0953(-)	-6325	gctgactttccaccagcctcTGTGTTGGGGCCCCgactattttttgaaaagagcg
N315	924572	-	glpQ(-)	-/-	gluD(+)	SA0814(-)	-6325	gctgactttccaccagcctcTGTGTTGGGGCCCCgactattttttgaaaagagcg
Newman	917240	-	glpQ(-)	NWMN_0829(+)	gudB(+)	NWMN_0823(-)	-6382	gctgactttccgccagcctcTGTGTTGGGGCCCCgccaacttgcacactattgt
Newman	917354	-	glpQ(-)	NWMN_0829(+)	gudB(+)	NWMN_0823(-)	-6496	gctgacttttcgccagcttcTGTGTTGGGGCCCCgccaacttgcattgtctgta
TCH1516	954485	-	glpQ2(-)	-/-	gudB(+)	kapB(-)	-6326	gctgactttccaccagcctcTGTGTTGGGGCCCCgactattttttgaaaagagcg
TCH1516	954541	-	glpQ2(-)	-/-	gudB(+)	kapB(-)	-6382	gctgactttccgccagcctcTGTGTTGGGGCCCCgccaacttgcacactattgt
TCH1516	954655	-	glpQ2(-)	-/-	gudB(+)	kapB(-)	-6496	gctgacttttcgccagcttcTGTGTTGGGGCCCCgccaacttgcattgtctgta

The STAR element within upstream of *acnA* locus among *S. aureus* strain

S. aureus strains	Position	Strand	Upstream Gene Name	Gene	Downstream Gene Name	Downstream Gene (same strand) Name	Downstream same strand bp	Sequence
USA300	1366621	-	acnA(+)	-/-	opuD(+)	mscL(-)	-2608	aatttcttttcgaaattctcTGTGTTGGGGCCCCtgactagaattgaaaaaagc
USA300	1366737	-	acnA(+)	-/-	opuD(+)	mscL(-)	-2724	aattgggaatccaatttctcTTTGTTGGGGCCCAtccccaacttgcacattatt
COL	1390424	-	acnA(+)	-/-	opuD1(+)	mscL(-)	-2608	aatttcttttcgaaattctcTGTGTTGGGGCCCCtgactagaattgaaaaaagc
COL	1390540	-	acnA(+)	-/-	opuD1(+)	mscL(-)	-2724	aattgggaatccaatttctcTTTGTTGGGGCCCAtccccaacttgcacattatt
MSSA476	1379324	-	acnA(+)	-/-	SAS1288(+)	mscL(-)	-2608	aatttcttttcgaaattctcTGTGTTGGGGCCCCtgactagaattgaaaaaagc
MSSA476	1379440	-	acnA(+)	-/-	SAS1288(+)	mscL(-)	-2724	aattgggaatccaatttctcTTTGTTGGGGCCCAtccccaacttgcacattatt
MW2	1350828	-	acnA(+)	-/-	opuD(+)	mscL(-)	-2608	aatttcttttcgaaattctcTGTGTTGGGGCCCCtgactagaattgaaaaaagc
Mu50	1425051	-	acnA(+)	-/-	opuD(+)	mscL(-)	-2606	aatttcttttcgaaattctcTGTGTTGGGGCCCCtgactagaattgaaaaaagc
Mu50	1425167	-	acnA(+)	-/-	opuD(+)	mscL(-)	-2722	aattgggaatccaatttctcTTTGTTGGGGCCCAtccccaacttgcacattatt
Mu50	1425223	-	acnA(+)	-/-	opuD(+)	mscL(-)	-2778	actgactttccgccagcttcTATGTTGGGGCCCCgccaacttgcattgtctgta
N315	1348722	-	acnA(+)	-/-	opuD(+)	mscL(-)	-2606	aatttcttttcgaaattctcTGTGTTGGGGCCCCtgactagaattgaaaaaagc
N315	1348838	-	acnA(+)	-/-	opuD(+)	mscL(-)	-2722	aattgggaatccaatttctcTTTGTTGGGGCCCAtccccaacttgcacattatt
N315	1348894	-	acnA(+)	-/-	opuD(+)	mscL(-)	-2778	actgactttccgccagcttcTATGTTGGGGCCCCgccaacttgcattgtctgta
USA300TCH	1380568	-	acnA(+)	-/-	sbcC(+)	lysC1(-)	-24818	aatttcttttcgaaattctcTGTGTTGGGGCCCCtgactagaattgaaaaaagc
USA300TCH	1380684	-	acnA(+)	-/-	sbcC(+)	lysC1(-)	-24934	aattgggaatccaatttctcTTTGTTGGGGCCCAtccccaacttgcacattatt

The STAR element within upstream of *icaC* locus among *S. aureus* strain								
Organism	Position	Strand	Upstream Gene Name	Gene	Downstream Gene Name	Downstream Gene (same strand) Name	Downstream same strand bp	Sequence
RF122	2700901	+	icaC(+)	SAB2545c(-)	lip(-)	SAB2563(+)	-17147	gctgactttccgccagcttcTATGTTGGGGCCCCacaccccaactcgcattgcc
USA300	2830246	+	icaC(+)	-/-	lip(-)	SAUSA300_2620(+)	-16770	cctgactttccgccagcttcTATGTTGGGGCCCCgccaacttgcacattattgt
USA300	2830302	+	icaC(+)	-/-	lip(-)	SAUSA300_2620(+)	-16714	gctgactttccgccagcttcTTTGTTGGGGCCCCgccaacttgcattgtttgta
MRSA252	2855709	+	icaC(+)	SAR2752(+)	lip1(-)	SAR2769(+)	-16714	aattgggaatccaatttctcTTTGTTGGGGCCCCgccaacttgcattgcctgta
MSSA476	2756248	+	icaC(+)	-/-	lip1(-)	SAS2572(+)	-16769	cctgactttccgccagcttcTATGTTGGGGCCCCgccaacttgcacattattgt
MSSA476	2756304	+	icaC(+)	-/-	lip1(-)	SAS2572(+)	-16713	gctgactttccgccagcttcTTTGTTGGGGCCCCgccaacttgcattgtttgta
MW2	2776908	+	icaC(+)	-/-	lip1(-)	MW2606(+)	-16769	cctgactttccgccagcttcTATGTTGGGGCCCCgccaacttgcacattattgt
MW2	2776964	+	icaC(+)	-/-	lip1(-)	MW2606(+)	-16713	cctgactttccgccagcttcTTTGTTGGGGCCCCgccaacttgcattgtttgta
Mu3	2839484	+	icaC(+)	SAHV_2654(+)	lip(-)	SAHV_2671(+)	-16657	aatttctttcgaaattcttTTTGTTGGGGCCCCgccaagatattacttgaata
Mu50	2837845	+	icaC(+)	SAV2670(+)	lip1(-)	SAV2687(+)	-16657	aatttctttcgaaattcttTTTGTTGGGGCCCCgccaagatattacttgaata
N315	2774131	+	icaC(+)	SA2462.1(+)	lip1(-)	SA2479(+)	-16657	aatttctttcgaaattcttTTTGTTGGGGCCCCgccaagatattacttgaata
NCTC8325	2778809	+	icaC(+)	-/-	SAOUHSC_03006(-)	SAOUHSC_03006.1(+)	-2941	cctgactttccgccagcttcTATGTTGGGGCCCCgccaacttgcacattattgt
NCTC8325	2778865	+	icaC(+)	-/-	SAOUHSC_03006(-)	SAOUHSC_03006.1(+)	-2885	gctgactttccgccagcttcTTTGTTGGGGCCCCgccaacttgcattgtttgta
Newman	2836402	+	icaC(+)	-/-	lip(-)	NWMN_2585(+)	-16714	cctgactttccgccagcttcTATGTTGGGGCCCCgccaacttgcattgtttgta
TCH1516	2830392	+	icaC(+)	USA300HOU_2670(-)	lip(-)	USA300HOU_2682(+)	-10464	cctgactttccgccagcttcTATGTTGGGGCCCCgccaacttgcacattattgt
TCH1516	2830448	+	icaC(+)	USA300HOU_2670(-)	lip(-)	USA300HOU_2682(+)	-10408	gctgactttccgccagcttcTTTGTTGGGGCCCCgccaacttgcattgtttgta

The STAR element within upstream of *atpC* locus among *S. aureus* strain								
Organism	Position	Strand	Upstream Gene Name	Gene	Downstream Gene Name	Downstream Gene (same strand) Name	Downstream same strand bp	Sequence
USA300	2220287	-	atpC(-)	-/-	SAUSA300_2056(-)	SAUSA300_2056(-)	-579	ctaaagaaaaagtatttcttTATGTTGGGGCCCCgtcaactactgccaaataca
COL	2156485	-	atpC(-)	-/-	SACOL2093(-)	SACOL2093(-)	-579	ctaaagaaaaagtatttcttTATGTTGGGGCCCCgtcaactactgccaaataca
JH1	2261267	-	atpC(-)	-/-	SaurJH1_2175(-)	SaurJH1_2175(-)	-579	ttaaagaaaaagtatttcttTATGTTGGGGCCCCgtcaactactgccaaataca
JH9	2261392	-	atpC(-)	-/-	SaurJH9_2137(-)	SaurJH9_2137(-)	-579	ttaaagaaaaagtatttctt**TATGTTGGGGCCCC**gtcaactactgccaaataca
MRSA252	2258987	-	atpC(-)	-/-	SAR2189(-)	SAR2189(-)	-579	aattgggaatccaatttcttTATGTTGGGGCCCCgtcaactactgccaaatata
MRSA252	2259046	-	atpC(-)	-/-	SAR2189(-)	SAR2189(-)	-638	gctgactttccgccagctttTGTGTTGGGGCCCCgcccccaacttgcattgtct
MRSA252	2259103	-	atpC(-)	-/-	SAR2189(-)	SAR2189(-)	-695	ctaaagaaaaagtatttcttTATGTTGGGGCCCAccccaacttgcacattaatg
MRSA252	2259268	-	atpC(-)	-/-	SAR2189(-)	SAR2189(-)	-860	aattgggaatacaatttctcTTTGTTGGGGCCCCaccggcaaggttgactagaa
MSSA476	2160694	-	atpC(-)	-/-	SAS2004(-)	SAS2004(-)	-579	ctaaagaaaaagtatttctt**TATGTTGGGGCCCC**gtcaactactgccaaataca
MW2	2181589	-	atpC(-)	-/-	MW2025(-)	MW2025(-)	-579	ctaaagaaaaagtatttcttTATGTTGGGGCCCCgtcaactactgccaaataca
Mu3	2233015	-	atpC(-)	-/-	SAHV_2085(-)	SAHV_2085(-)	-579	ttaaagaaaaagtatttcttTATGTTGGGGCCCCgtcaactactgccaaataca
Mu50	2231377	-	atpC(-)	-/-	SAV2101(-)	SAV2101(-)	-579	ttaaagaaaaagtatttcttTATGTTGGGGCCCCgtcaactactgccaaataca
N315	2154945	-	atpC(-)	-/-	SA1903(-)	SA1903(-)	-579	ttaaagaaaaagtatttcttTATGTTGGGGCCCCgtcaactactgccaaataca
NCTC8325	2167209	-	atpC(-)	-/-	SAOUHSC_02339(-)	SAOUHSC_02339(-)	-214	ctaaagaaaaagtatttcttTATGTTGGGGCCCCgtcaactactgccaaataca
Newman	2224868	-	atpC(-)	-/-	NWMN_2005(-)	NWMN_2005(-)	-579	ctaaagaaaaagtatttcttTATGTTGGGGCCCCgtcaactactgccaaataca
TCH1516	2220681	-	atpC(-)	-/-	USA300HOU_2091(-)	USA300HOU_2091(-)	-579	ctaaagaaaaagtatttcttTATGTTGGGGCCCCgtcaactactgccaaataca