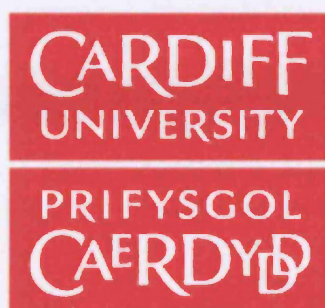


# **A Molecular and Cellular Characterisation of *Staphylococcus aureus* in Chronic Wounds**

**Thesis submitted in fulfilment of the requirements of the degree of**

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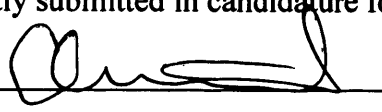
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## Summary

Chronic skin wounds (CW) represent a significant world health problem including reservoirs of multi-drug resistant bacterial biofilms. The precise role of bacteria in the aetiology of chronic inflammation and non-healing remains unclear. This study characterised MRSA from human CW and investigated how they inhibit wound healing, modulate immunological responses and resist treatment in comparison to control MRSA from asymptomatic nasal carriers (NC).

Routine cultural analysis of 150 chronic wounds revealed 50% were colonised with *S. aureus*, of which 22.6% were MRSA. Multi-locus sequence typing identified two new sequence types and demonstrated that wound MRSA represented two clonal complexes (22 and 30) with almost 90% identified as hospital-acquired EMRSA-15. MRSA isolated from CW and NC were characterised for virulence factor (VF) expression and modulation of the innate immune system. Presence and expression of MRSA VFs indicated an association with sequence type. Greater expression of colonisation- (*cna*) and degradation- associated (*hysA*) VFs was evident in the wound MRSA, suggesting that modulation of virulence is important for non-healing. The ability of conventional biocides (iodine, silver, and potassium permanganate) to treat chronic wound bacteria, using the carrier-test method, revealed iodine as the only effective biocide.

*In vitro* stimulation of the ability of the MRSA to induce innate immunity showed that CW-MRSA exhibited decreased TLR, cytokine and complement responses compared with NC-MRSA (IL-8, TNF $\alpha$ , complement activation;  $P < 0.05$ ). Moreover, biofilm-induced reductions in immunogenicity were observed compared with planktonic growth in monocyte and complement assays ( $P < 0.05$ ). Scratch wound assays indicated that MRSA failed to inhibit keratinocyte migration ( $P > 0.05$ ), although bacterial growth conditions (biofilm vs. planktonic) significantly affected the observed cellular migration ( $P < 0.05$ ).

Virulence factor production and ability to modulate/evade the host innate immune response are important potential mechanisms by which MRSA are able to colonise chronic wounds. These studies provide important new insights into the role of MRSA in delayed dermal healing.

## Abbreviations

$\beta$	Beta
$\phi$	Bacteriophage
(F)	Forward
(R)	Reverse
$\Delta$	Deletion (part of a gene)
1X	One times concentration
5' – 3'	5 prime to 3 prime – direction of a primer sequence
A <sub>260</sub>	Absorbance at 260 nm
<i>agr</i>	Accessory gene regulator locus
ANOVA	Analysis of variance
APC	Antigen presenting cells
AR	Antibiogram-resistogram
ATCC	American Type Culture Collection
AU	Arbitrary units per millilitre
BA	Blood agar
BAA	Bile aesculin agar
BHI	Brain heart infusion broth
bp	Base pair
BS	British Standard
BSA	Bovine serum albumin
CAA	Cyclohexane adherence assay
CA-MRSA	Community-acquired meticillin-resistant <i>Staphylococcus aureus</i>
CC	Clonal complex
<i>ccr</i>	Cassette chromosome recombinase gene complex
CD	Cluster of differentiation
CDC	Centre for Disease Control and Prevention
CFU	Colony forming units
CFU/ml	Colony forming units per millilitre
CHIPS	Chemotaxis inhibitory protein of staphylococci
CLSM	Confocal laser scanning microscopy
CLW	Chronic leg wound
CNS	Coagulase negative staphylococci

CpG	Unmethylated cytosine-guanosine
CSW	Chronic surgical wound
CVLU	Chronic venous leg ulcer
CW	Chronic wound
d	Days
DAMP	Damage associated molecular patterns
DC	Dendritic cell
DEPC	Diethylpyrocarbonate
DFU	Diabetic foot ulcer
DGGE	Denaturing gradient gel electrophoresis
dH <sub>2</sub> O	Distilled sterile water
DLV	Double locus variant
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dsRNA	Double stranded ribonucleic acid
EARS-Net	European Antimicrobial Resistance Surveillance Network
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
<i>egc</i>	Enterotoxin gene cluster
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EMRSA	Epidemic meticillin-resistant <i>Staphylococcus aureus</i>
EN	European Standard
<i>entA</i>	Enterotoxin A
EPS	Extracellular polysaccharide substance
EtOH	Ethanol
FACS	Fluorescence activated cell sorting
FAD	Flavin adenine dinucleotide
FAME	Fatty acid modifying enzyme
FCS	Foetal calf serum
FDVH	Federated Dublin Voluntary Hospitals
FGF-2	Fibroblast growth factor
FSC	Forward scatter
FSL-1	Fibroblast stimulating lipopeptide-1.

h	Hour(s)
H <sub>0</sub>	Null hypothesis
HAA	Hexadecane adherence assay
HA-MRSA	Hospital acquired-MRSA
HBSS	Hanks buffered salt solution
Hlb	Beta-toxin/haemolysin
Hlg	Gamma-toxin/haemolysin
HPA	Health Protection Agency
hVISA	Heteroresistant vancomycin-intermediate <i>Staphylococcus aureus</i>
<i>hysA</i>	Hyaluronate lyase gene
<i>ica</i>	Intercellular adhesion
ICU	Intensive Care unit
IKK	Inhibitor of NF-κB kinase
IL	Interleukin
IRAK	Il-1 receptor associated kinase
IRF	Interferon (INF) regulatory factor,
ITU	Intensive Therapy unit
IκB	Inhibitor of nuclear factor kappa B
J	Joining or junkyard region
kb	Kilobase
L agar	Luria-Bertani agar
L broth	Luria-Bertani broth
LAL	<i>Limulus</i> amebocyte lysate
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
LTA	Lipoteichoic acid
mAb	Monoclonal antibody
MAL	Myeloid adapter (MyD88) like protein
MAP	Mitogen activating protein
MAPK	Mitogen activated protein kinase
MASP	Mannose-binding lectin associated serine protease
MBC	Minimum bactericidal concentrations
MBL	Mannose binding lectin
MD-2	Myeloid differentiation protein 2

MDR	Multi-drug resistant
<i>mec</i>	Meticillin resistance gene complex
Meth <sup>R</sup>	Meticillin resistant
MFI	Median fluorescence intensity
MIC	Minimum inhibitory concentration
min	Minute(s)
MLEE	Multilocus enzyme electrophoresis
MLST	Multilocus sequence typing
MMP	Matrix metalloproteinases
Mo	Monocytes
MØ	Macrophages
MRSA	Meticillin-resistant <i>Staphylococcus aureus</i>
MSSA	Meticillin sensitive <i>Staphylococcus aureus</i>
MTT	Methylthiazolyldiphenyl-tetrazolium bromide
MVC	Mean viable count
mw	Molecular weight
MyD88	Myeloid differentiation factor 88
n/a	Not applicable
NC	Nasal carrier
NCIMB	National Collection of Industrial, Food and Marine Bacteria
NCTC	National Collection of Type Cultures
ND	Not determined
NEMO	NF-κB essential modulator (or IKKγ),
NF-κB	Nuclear factor κB
NOD	Nucleotide binding and oligomerization domain
NPHLS	National public health laboratory service
O/N	Overnight
OD	Optical density
OD <sub>420-580</sub>	Optical density at 420 to 580 nm
ORF	Open reading frame
Oxa <sup>R</sup>	Oxacillin resistant
P	Measure of probability, with a value ranging from zero to one/ statistical significance
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
PAMP	Pathogen associated molecular patterns

PBP	Penicillin binding protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PFGE	Pulsed-field gel electrophoresis
PGN	Peptidoglycan
pH	Power of hydrogen concentration
PIA	Polysaccharide intercellular adhesion
PMN	Polymorphonuclear leucocytes
pmol/μl	Pico mole/microlitre
PRR	Pattern recognition receptors
PU	Pressure ulcer
PVL	Panton-Valentine leucocidin
PVP	Polyvinylpyrrolidone
PVP-I	Povidone - Iodine
QAC	Quaternary ammonium compound
<i>qacABCD</i>	Quaternary ammonium compound resistance genes
r.p.m.	Revolution per minute
RANTES	Regulated upon activation normal T cell expressed and secreted
RAPD	Random amplified polymorphic DNA
RBC	Red blood cells
RHE	Reconstituted human epidermis
RIP	Receptor interacting protein,
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT	Reverse transcriptase
s	Seconds
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
SACU	Specialist Antimicrobial Chemotherapy Unit
Sak	Staphylokinase
<i>sar</i>	Staphylococcal accessory regulator
SAT	Salt aggregation test
SBA	Sheep blood agar
SCC	Staphylococcal chromosomal cassette

SCC <i>mec</i>	Staphylococcal chromosomal cassette <i>mec</i>
SCIN	Staphylococcal complement inhibitor
SDS	Sodium dodecyl sulphate
SE	Staphylococcal enterotoxins
SEM	Scanning electron microscopy
slv	Single locus variant
Sp.	Species
<i>spa</i>	Staphylococcal protein A
SS	Stainless steel
SSC buffer	Saline-sodium citrate buffer
SSC	Side scatter
ssRNA	Single stranded ribonucleic acid
SSSI	Staphylococcal scalded skin infection
SSSS	Staphylococcal scalded skin syndrome
ST	Sequence type
TAK-1	TGF (transforming growth factor)- $\beta$ -activated kinase 1
TANK	TRAF family member associated NF- $\kappa$ B activator
TBE	Tris-borate EDTA
TBK	TANK binding kinase,
TCC	Terminal complement complex
TCD	Trinity college, Dublin
TEM	Transmission electron microscopy
TGF $\beta$	Transforming growth factor
TIMP	Tissue inhibitor metalloproteinase
TIR	Toll-interleukin 1 receptor domain
TIRAP	TIR domain containing adaptor protein
TLR	Toll-like receptors
TRAF	Tumor necrosis factor receptor associated factor
TRAM	TRIF-related adapter molecule
TRIF	TIR-domain containing adaptor inducing IFN- $\beta$
TSA	Tryptone soy agar
TSB	Tryptone soy broth
TSC	Tryptone sodium chloride
TSS	Toxic shock syndrome (clinical disease)
TSST-1	Toxic shock syndrome toxin - 1

<i>tst</i>	Toxic shock toxin gene
U	Unit
UK	United Kingdom
UPGAMA	Unweighted pair group method using arithmetic averages
USA	United States
UV	Ultra violet
V	Volts
v/v	% volume in a volume
VEGF	Vascular endothelial growth factor
VISA	Vancomycin-intermediate <i>Staphylococcus aureus</i>
VLU	Venous leg ulcer
VRE	Vancomycin resistant enterococci
VRSA	Vancomycin-resistant <i>Staphylococcus aureus</i>
w/v	% weight in a volume
WHRU	Wound healing research unit
<i>x g</i>	Gravity
XAA	Xylene adherence assay
X-gal	5-bromo-4-chloro-indoyl-b-D-galactoside



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# **Chapter 1:**

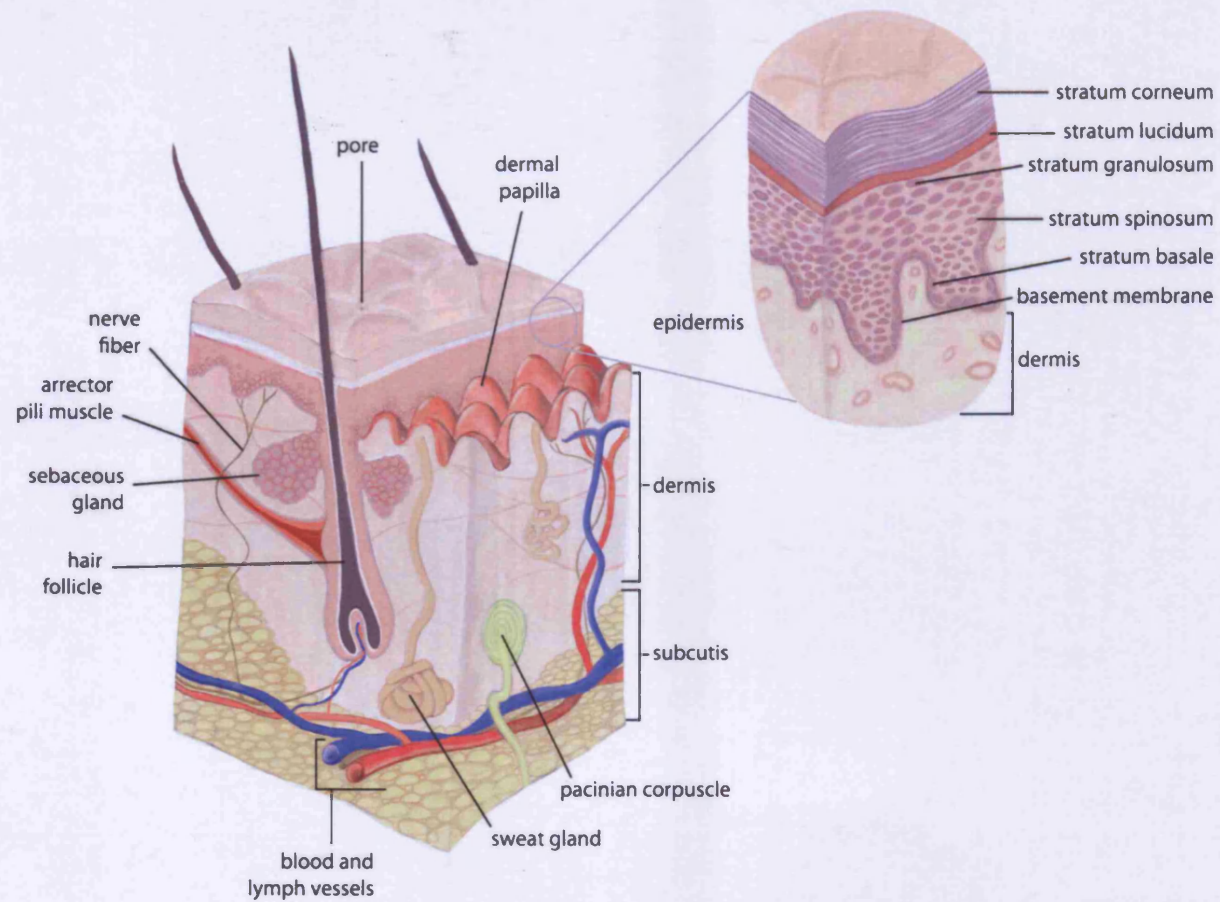
## **General introduction**

## 1.1 Introduction

In health the skin is colonised by the resident microflora, a diverse collection of microorganisms, including bacteria, archaea, fungi, viruses and mites, all of which form part of the human microbiome (Kong, 2011). The human microbiome project has been initiated to investigate all human habitats including skin, oral cavity and gastro-intestinal tract, in part to determine the role of bacteria in health and disease (Grice, 2008, Hsiao and Fraser-Liggett, 2009, Wade, 2010). These studies have shown that factors such as age, moisture and body site affect the microbial composition (Carlisle and Morowitz, 2011, Grice and Segre, 2011). It has been estimated that the normal flora consists of  $10^{14}$  microbes (Tlaskalová-Hogenová et al., 2004) with approximately  $10^6$  bacteria/cm<sup>2</sup> on the skin, based on punch biopsy sampling (Grice, 2008).

One of the principal functions of the skin is as a barrier to prevent the ingress of colonising bacteria and potential pathogens to the internal milieu, thus forming an integral part of the innate immune system (Paige, 2005). Following injury, disruption of the normal barrier of human skin (Figure 1.1) induces a complex series of inter-related but overlapping events, which constitute normal wound healing i.e. haemostasis, inflammation, re-epithelialisation, angiogenesis, granulation tissue formation, wound contraction, scar formation and tissue remodelling (Blakytny and Jude, 2006). The events involve both resident (keratinocytes, fibroblasts) and migratory cells e.g. neutrophils (PMNL) and monocyte/macrophages (Mo/MØ). These processes are coordinated by the expression of cytokines, autocrine and paracrine growth factors, released at the site of injury, and cues from the extracellular matrix (ECM; Xu and Clark, 1996, Gill and Parks, 2008, Vorotnikova et al., 2010). Interruption in this process can lead to wound breakdown and failed or delayed healing.





**Figure 1.1** Diagrammatic representation of skin structure. Courtesy of Miss R. Bowen, Illustration Department, Cardiff University.

## 1.2 The skin microbiome

Not only does the skin provide an innate immunological barrier to the ingress of pathogens it harbours complex microbiological communities, which is affected by environmental factors such as temperature, light, humidity, e.g. host factors such as gender, immune status, genotype and cosmetic use (Gao et al., 2007). The ecology of the skin forms a significant part of the human microbiome.

As part of the human microbiome project to determine whether a core microbiota in healthy humans exist and further recent molecular studies, the skin microbiome has been observed to be diverse and topographically distinct (Grice, 2009). Whereby local factors e.g. anatomy, lipid content, pH, sweat and sebum play a significant role in microbial colonisation. A major challenge for the human microbiome project is to ascertain an accurate study sample (Grice, 2008), studies by Gao et al., (2007) indicate that cultivation methods of the human skin significantly underestimate bacterial diversity.

*Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes* have been observed to be the typical colonising phyla of human skin (Grice, 2008, Grice, 2009). Characterisation of the skin microbiota may provide insight into the development of dermatological disease, indicated by the predilection of microorganisms for specific skin sites (Grice, 2009). Gao et al., (2007) presented evidence that the skin microbiota is a frequently fluctuating dynamic system; this diversity was also reflected by a study by Grice et al., (2009) observing 205 genera on 10 healthy humans over 20 sites. *Propionibacteria* spp. and *Staphylococci* spp. were predominant in sebaceous sites, whilst *Corynebacteria* spp. was dominant in moist sites, although *Staphylococci* spp. were also present. Furthermore the skin microbiome not only is vastly different to the gut microbiota, which primarily are of the *Firmicutes* and *Bacteroidetes* division, but also indicates a low level of interpersonal variation unlike the gut (Grice, 2008).

## **1.3 Normal wound healing**

### **1.3.1 Inflammation**

Following injury, inflammatory cells are attracted to the site of injury by chemotactic factors such as platelet-derived growth factor (PDGF), and transforming growth factor- $\beta$  (TGF- $\beta$ ), damaged ECM and complement fractions. In the acute phase of inflammation PMNL predominate and act to remove damaged host tissue and kill invading pathogens via phagocytosis, free-radical generation and enzymatic degradation. Following this (>24 h) Mo are recruited to the site of injury and release an array of cytokines e.g. tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), chemokines e.g. interleukin-8 (IL-8) and growth factors e.g. TGF- $\beta$  and fibroblast growth factor (FGF). These cytokines stimulate cellular proliferation and migration in the resident wound cells and, in the case of fibroblasts, ECM synthesis in the adjacent dermis (Cotton, 1998, Sorrell et al., 2008).

### **1.3.2 Proliferation**

During this phase, cellular proliferation and contraction occurs to close the defect. Fibroblasts are involved in the secretion of ECM and matrix remodelling via matrix metalloproteinases (MMPs), whilst endothelial cells and pericytes are involved in angiogenesis. In the epidermis, PDGF synergistically induces cellular proliferation in the basal layer, with epidermal growth factor (EGF) produced from keratinocytes and somatomedins (insulin-like growth factor; IGF-1 and IGF-2). In the dermis, myofibroblasts migrate in response to PDGF and TGF- $\beta$ , the secreted collagen and fibronectin aids migration of epithelial cells (Parsons, 1998, Jones et al., 2004). Wound closure is induced immediately upon wounding; keratinocytes in the adjacent epithelia or hair follicles within the wound proliferate and migrate across the provisional wound matrix. Following wound closure, remodelling occurs over many months (up to 1 year following initial injury) mediated principally by fibroblasts and myofibroblasts.

The rapidity of wound closure and re-epithelialisation is evolutionarily important to minimise the risk of infection supervening (Ferguson, 1993, Moulin et al., 2000). In wounds which heal with primary intention (with the wound edges approximated) this process is complete within 24 hours. In more extensive wounds however, where the edges are not approximated, the wound bed may be exposed to the external environment and potential colonisation/invasion of bacteria for many days (Bowler, 2003).

#### **1.4 Chronic wounds**

Chronic, non-healing skin wounds are a major cause of disability and distress occurring principally in the extremities of elderly individuals. They present in 3 typical forms: pressure ulcers (PU), diabetic foot ulcers (DFU) and venous ulcers, and are defined as chronic if they fail to show a 40% reduction in area in a 2 – 4 week period following optimal therapy (Siddiqui and Bernstein, 2010). Such wounds annually account for the loss of >2 million workdays in the US (McGuckin et al., 2002); with an associated estimated cost of \$13 to \$15 billion (Siddiqui and Bernstein, 2010).

Interestingly, the majority of non-healing wounds will occur in individuals/tissue exhibiting impaired immunological responsiveness (diabetic ulcers), inadequate perfusion (venous leg ulcers) and chronic trauma (pressure ulcers). The wounds, therefore, occur in populations with increased risk of bacterial carriage. Indeed, whilst the precise aetiology of these wounds is uncertain, all chronic wounds harbour bacteria (Davies et al., 2004, Davies et al., 2007). Moreover, a number of the changes which characterise the wounds at the gene, protein and cellular level may be directly or indirectly related to bacteria present within the wound bed (Wall et al., 2002, Stephens et al., 2003). Workers have demonstrated that the histological features of chronic, ineffectual inflammation may be mediated by alterations in fibroblast chemokine profiles and immunosuppressive effects of local anaerobic bacteria, and that the elevated levels of proteases in the

wounds may be induced by bacterial activation of local immune responses (Wall et al., 2002, Wall et al., 2008). The observed degradation of the ECM may also be mediated by bacterial proteolytic activity, as might the defective re-epithelialisation and angiogenesis which is observed within the wound bed (Stephens et al., 2003). Workers have suggested that the changes in cellular senescence observed in the wound may directly influence healing. Fibroblasts cultured from chronic wounds exhibit reduced population doublings and early senescence compared with matched normal dermal fibroblasts (Mendez et al., 1998, Wall et al., 2008). Along with this, decreased cytokine production facilitates dysfunction in immune, ECM and keratinocyte regulation all of which are significant for healing (Rodero and Khosrotehrani, 2010).

### **1.5 The microflora of skin wounds**

Skin wounds provide an ideal environment for microbial colonisation and proliferation, and these wounds harbour both aerobic and anaerobic bacterial populations (Bowler et al., 2001, Davies et al., 2007, Dowd, 2008). The bacterial species isolated from individual wounds are dependent upon wound site and typically derived from the local resident population (Thomson and Smith, 1994). A systematic review (Bowler, 1998) demonstrated that *S. aureus* is the most frequently isolated organism from skin wounds, being commonly associated with *P. aeruginosa* in both chronic skin and particularly burn wounds. Similar findings have been observed in prospective studies of infected surgical wounds, demonstrating that *S. aureus* was the most frequently encountered wound isolate followed by; *P. aeruginosa*, *E. coli*, *S. epidermidis* and *Enterococcus faecalis* (Giacometti et al., 2000). Perera et al., (2005) attempted to categorise the distribution of normal and pathogenic skin flora (Table 1.1).

Past characterisation of chronic wound bacteria has however, relied upon culture techniques alone, which have many limitations. Culture-based methodologies

**Table 1.1** Distribution of normal and pathogenic cutaneous flora (aerobic and facultative anaerobes)

Normal flora		Common pathogens	Uncommon pathogens
Phylum	Species		
<i>Proteobacteria</i>	<i>Acinetobacter</i> spp.	<i>Candida</i> spp.	<i>Acinetobacter</i> spp.
	<i>Comamonas</i> spp.	<i>Corynebacterium</i> spp.	<i>Enterobacter</i> spp.
	<i>Delftia</i> spp.	<i>Enterococcus</i> spp.	Group B, C, G streptococci
	<i>Enterobacter</i> spp.	<i>Escherichia coli</i>	<i>Klebsiella</i> spp.
	<i>Haemophilus</i> spp.	Group A <i>Streptococcus</i>	<i>Pseudomonas mallei</i>
	<i>Halomonas</i> spp.	<i>Morganella morganii</i>	<i>Serratia marcescens</i>
	<i>Janthinobacterium</i> spp.	<i>Proteus mirabilis</i>	<i>Streptococcus faecalis</i>
	<i>Klebsiella</i> spp.	<i>Proteus vulgaris</i>	
	<i>Methylobacterium</i> spp.	<i>Pseudomonas aeruginosa</i>	
	<i>Neisseria</i> spp.	<i>Staphylococcus aureus</i>	
	<i>Pasteurella</i> spp.	<i>Staphylococcus epidermidis</i>	
	<i>Pseudomonas</i> spp.	<i>Shigella</i> spp.	
	<i>Serratia</i> spp.	<i>Serratia</i> spp.	
	<i>Sphingobacterium</i> spp.	<i>Stenotrophomonas</i> spp.	
	<i>Stenotrobacteria</i> spp.		
<i>Firmicutes</i>	<i>Clostridium</i> spp.	Diabetic wounds	<i>Rhodopseudomonas</i> spp.
	<i>Enterococcus</i> spp.		
	<i>Lactobacillus</i> spp.		
	<i>Staphylococcus</i> spp.		
	<i>Streptococcus</i> spp.		

(Adapted from Perera and Hay, 2005, Sra et al., 2005, Gao et al., 2007, Dowd et al., 2008, Grice, 2008, Grice, 2009, Price et al., 2009, Gontcharova et al., 2010, Grice, 2010, Grice and Segre, 2011)

**Table 1.1 continued** Distribution of normal and pathogenic cutaneous flora (aerobic and facultative anaerobes)

Normal flora		Common pathogens	Uncommon pathogens
Phylum	Species		
<i>Bacteroidetes</i>	<i>Acidobacteri</i> spp.		
	<i>Chryseobacterium</i> spp.		
	<i>Cyanobacteria</i> spp.		
	<i>Sphingobacterium</i> spp.		
<i>Actinobacteria</i>	<i>Actinomyces</i> spp.		
	<i>Brevibacterium</i> spp.		
	<i>Corynebacterium</i> spp.		
	<i>Goronia</i> spp.		
	<i>Kocuria</i> spp.		
	<i>Microbactium</i> spp.		
	<i>Micrococcus</i> spp.		
	<i>Mycobacterium</i> spp.		
	<i>Rhodococcus</i> spp.		
	<i>Rothia</i> spp.		
Fungal	<i>Cryptococcus</i> spp.		
	<i>Debaryomyces</i> spp.		
	<i>Malassezia</i> spp.		

(Adapted from Perera and Hay, 2005, Sra et al., 2005, Gao et al., 2007, Dowd et al., 2008, Grice, 2008, Grice, 2009, Price et al., 2009, Gontcharova et al., 2010, Grice, 2010, Grice and Segre, 2011)

**Table 1.1 continued** Distribution of normal and pathogenic cutaneous flora (anaerobes)

Normal flora		Common pathogens	Uncommon pathogens
Phylum	Species		
<i>Proteobacteria</i>		<i>Anaerococcus</i> spp. <i>Finegoldia</i> spp. <i>Helcococcus</i> spp. <i>Parvimonas</i> spp. <i>Peptoniphilus</i> spp.	<i>Bacteroides fragilis</i> <i>Clostridium perfringens</i>
<i>Firmicutes</i>	<i>Anaerococcus</i> spp. <i>Clostridium</i> spp. <i>Gemella</i> spp. <i>Veillonella</i> spp.	Diabetic wound <i>Bacteroides</i> spp. <i>Clostridium</i> spp. <i>Peptostreptococcus</i> spp. <i>Prevotella</i> spp. <i>Veillonella</i> spp.	
<i>Bacteroidetes</i>	<i>Porphyromonas</i> spp. <i>Prevotella</i> spp.		
<i>Actinobacteria</i>	<i>Propionibacterium acnes</i> <i>Propionibacterium avidum</i>		

(Adapted from Perera and Hay, 2005, Sra et al., 2005, Gao et al., 2007, Dowd et al., 2008, Grice, 2008, Grice, 2009, Price et al., 2009, Gontcharova et al., 2010, Grice, 2010, Grice and Segre, 2011)



rely upon the growth of bacteria in an artificial environment, which preferentially nurtures rapidly growing organisms. Hence, it has been estimated that only 1% of bacteria have been cultured (Wade et al., 1997, Dowd, 2008), with many human pathogens, particularly anaerobes being uncultivable e.g. 50% oral microflora (Wade et al., 2005). The last two decades has seen the advent of molecular methods to identify pathogens associated with disease (Dymock et al., 1996, Davies et al., 2001, Dowd, 2008), e.g. 16S rRNA random fragment length polymorphism (RFLP), denaturing gradient gel electrophoresis (DGGE) and more recently high-throughput sequencing methods such as pyrosequencing and whole-genome shotgun sequencing. A limitation of such DNA sequencing methods is the inability to discriminate between live and dead bacteria (Grice, 2008). Functional genomic studies e.g. metagenomics, including metabolomic investigations, have led to a greater understanding of bacterial ecology and function in health and disease as observed in gut microbiota (Preidis and Versalovic, 2009, Yang et al., 2009). These methods have been used in comparative analysis of sampling methods and reveal that although bacterial numbers present were significantly different using swab and biopsy techniques, similar bacterial communities were identified (Grice, 2008).

Longitudinal studies of the skin microbiota have shown stable bacterial skin colonisation over time, although this was dependant on sample site, and indicated that healthy individuals demonstrate little inter-person variation (Grice, 2008, Grice, 2009). Conversely in leg ulcers, biofilm bacterial colonisation was highly diverse between patients. Pressure (62%) and diabetic (30%) leg ulcers were likely to harbour anaerobes, whilst venous leg ulcers had much fewer numbers (1.6%; Dowd, 2008). Murine models have shown longitudinal shifts in bacterial presence with non-healing wounds (Grice et al., 2010).

Within wounds bacterial presence has been artificially and arbitrarily categorised into: “contamination”, “colonisation”, “critical-colonisation” and “infection” (Edwards and Harding, 2004); where “critical colonisation” describes the level of colonisation that begins to adversely affect wound healing. Infection has

been defined as *“the product of the entrance, growth, metabolic activities, and resultant pathophysiologic effects of microorganisms in the tissues of the patient”* (Robson, 1997). The cardinal signs of infection are: ‘dolor’ (pain), ‘tumor’ (swelling), ‘calor’ (heat), ‘rubor’ (erythema) and ‘loss of function’. Other signs indicative of infection are suppuration and in terms of chronic skin wounds i.e. ulceration, an increase in size and depth of the wound. However, clinical assessment of chronic wounds has proven to be variable and of low reliability (Lorentzen and Gottrup, 2006). The transition from critical colonisation to infection is not simply related to bacterial density but reflects changes in host susceptibility, increased microbial numbers, and alteration in bacterial virulence. It is evident that the deep tissues of all non-healing wounds harbour bacteria, whether infected or not (Cooper et al., 2009). Attempts to correlate the outcome of wound healing with individual bacteria and bacterial density have been made for over 40 years, with varying outcomes.

### **1.5.1 Microbiology and the healing of chronic wounds**

Although there is a dense microflora associated with chronic wounds, they have relatively low rates of infection <5% (Bowler et al., 2001). Some authors hypothesise that the density of the microorganisms is critical to healing, whilst others argue that the presence of specific pathogens is of primary importance. A third argument is that organisms themselves play a minimal role (Eriksson et al., 1984, Bowler et al., 2001). The mean number of species isolated from a chronic wound has been noted to be between 1.6 and 5.8 (Hansson et al., 1995a, Howell-Jones et al., 2005, Cooper et al., 2009). In terms of bacterial load, workers have suggested that the risk of infection is associated with bacterial numbers in the tissue  $>10^6$  CFU/g (Ferguson, 1993) however; this is based on traumatic wound studies.

Many studies have investigated the relationship between bacterial quantification and non-healing. The debate over the significance of microbial load originated in 1964, where Bendy et al., (1964) reported that healing in decubitus

ulcers only progressed when the bacterial load was  $<10^6$  CFU/ml, whilst Robson et al., (1999) postulated that bacterial presence  $>10^5$  CFU/g tissue, based on biopsies, was indicative of infection risk and non-healing. Further to this, healing in acute wounds has been associated with bacterial loads  $<10^5$  CFU/g tissue (Bowler, 1998). Whist Breidenbach and Trager et al., (1995) demonstrated that a critical load of  $>10^4$  CFU/g tissue was needed to cause an infection in extremity wounds. Thus, much debate regarding quantitative bacteriology and non-healing has occurred. However, due to the variety of techniques utilised in these studies it is difficult to ascertain whether bacterial load, was more important than species present, e.g.  $\beta$ -haemolytic streptococci counts associated with non-healing are estimated to be  $<10^4$  CFU/g (Heggors, 1994, Thomson and Smith, 1994, Edwards and Harding, 2004). More significantly, many researchers have found a limited use of the  $10^5$  CFU/g rule in chronic wounds due to their complex aetiology and pathogenesis compared with acute wounds (Bowler, 2003) whereby  $>10^6$  CFU/ml bacteria have been associated with non-infected chronic wounds (Hill et al., 2003, Davies et al., 2007, Cooper et al., 2009).

The presence of multiple species within a wound appears to be of more importance. Trengove et al., (1996) found that healing was inversely associated with  $\geq 4$  bacterial species isolated from venous ulcers. These findings have been further corroborated by other researchers (Davies et al., 2007, Cooper et al., 2009), and it would appear that bacterial diversity is more likely to be of significance in healing than the number of bacteria isolated, particularly in chronic wounds.

### **1.5.2 Disease association with individual bacteria**

A number of studies have identified potential pathogens associated with the chronic wound environment. Cultural analysis of wounds via swab and biopsy techniques have indicated *S. aureus*, *P. aeruginosa* and  $\beta$ -haemolytic streptococci to be the most frequent isolates from delayed healing and infected wounds (Bowler et al., 2001, Davies et al., 2007, Cooper et al., 2009). Specific studies of particular

wound types e.g. DFU, observed that *S. aureus* was the most prevalent isolate along with *S. epidermidis*, *Streptococcus* spp. *P. aeruginosa*, *Enterococcus* spp. and coliforms (Armstrong et al., 1995a). Interestingly, although often overlooked, detailed microbiological analysis of DFU infections revealed that >90% of wounds contained anaerobes e.g. *Peptostreptococcus*, *Bacteroides* and *Prevotella* sp. (Gerding, 1995).

Studies employing molecular techniques have characterised the microflora of chronic wounds. Molecular based techniques have been applied to characterise both the number (DGGE) and via sequencing, the identity of uncultivable organisms within chronic wounds (Hill et al., 2002, Davies et al., 2004, Dowd, 2008, Melendez et al., 2010). DGGE studies having demonstrated a greater number of species (band profiles) in non-healing wounds than those identified by culture techniques alone e.g. *Pseudomonas* sp. were present in all samples, even though it was not always cultured (Davies et al., 2004). Sequencing studies by Hill et al., (2003), demonstrated for the first time the increased diversity of the wound microflora compared to purely cultural analysis, and recent molecular studies employing high-throughput sequencing screening (Dowd, 2008, Price, 2009, Wolcott et al., 2009) have demonstrated similar substantially greater diversity in the bacterial communities of the wound. Wolcott et al., (2009) identified a total of 62 genera in 40 wounds using pyrosequencing. Also, these methods have identified the relative stability of the microflora of the uninjured skin microbiome (Grice, 2009). The use of imaging techniques have provided insight into the topographical arrangements of bacteria within wounds (Bjarnsholt et al., 2008, Kirketerp-Moller et al., 2008, Malic et al., 2009). In almost all studies, be they cultural or molecular, the importance of and prevalence of *S. aureus* in the wound environment has been stressed (Martin et al., 2009, Siddiqui and Bernstein, 2010, Grice and Segre, 2011, Kong, 2011).

## 1.6 *Staphylococcus aureus*

Staphylococci are Gram and catalase-positive cocci that are frequently isolated from skin infections. They are approximately 0.5 to 1.5  $\mu\text{m}$  in diameter and form grape-like clusters under light microscopy. They are non-motile and non-spore forming facultative bacteria. The genus consists of  $\geq 30$  species with 17 sub-species (Bannerman, 2003, Massey et al., 2006), and the genome ranges from 2000 to 3000 kilobases (kb) in size, and contain a variable number of mobile genetic elements (Bannerman, 2003, Witte et al., 2006, Tristan et al., 2007).

*S. aureus* is the most prevalent member of the staphylococcal family and is responsible for a diverse range of human diseases; from minor soft-tissue to life-threatening systemic infections (Perera and Hay, 2005, Petkovsek et al., 2009). *S. aureus* is part of the resident flora in the anterior nares of approximately 30% of the population, whilst up to 60% of the population show a transient colonization of the nares, axilla, perineum or vagina (Breuer et al., 2002, Aires de Sousa and Lencastre, 2004, Miller and Diep, 2008). Colonisation with *S. aureus* is increased in immunosuppressed individuals (e.g. diabetic and renal patients), disorders of the skin and hospitalisation (Breuer et al., 2002, Reich-Schupke et al., 2010, Schechter-Perkins et al., 2011). The mode of spread has been demonstrated to follow a regular pattern; colonisation, in-particular of the nares, by *S. aureus* leads to hand carriage and from the hands, the organisms frequently spread to other body areas (Tenover and Gorwitz, 2006).

Following the introduction of penicillin in the 1940s, the ability of *S. aureus* to resist treatment by the production of penicillinase was evident (Boyle-Vavra and Daum, 2007), and in 1959 the first semi-synthetic penicillin, methicillin was introduced (now named meticillin) to combat this problem (Livermore, 2000a, Zhang et al., 2005). Whilst meticillin and flucloxacillin have been the mainstay of treatment for *S. aureus* infections for the last 50 years, within 2 years of its development meticillin resistant *S. aureus* (MRSA) clinical isolates were observed (Shanson, 1981, Ayliffe et al., 1998) and are now a major nosocomial pathogen

(Oztoprak et al., 2006, Niederman, 2009). The cost of hospital-acquired infection is estimated to be £1 billion per year (Plowman et al., 2001) with MRSA accounting for 10% of hospital-acquired infections (Higgins et al., 2010)

### **1.7 Antimicrobial resistance in chronic wound patients**

The emergence of antibiotic resistance and multi-drug resistant (MDR) organisms as a major world health problem, with its associated morbidity and mortality, has been inexorable (Colsky et al., 1998, Gould, 2008, French, 2010, Jean and Hsueh, 2011, Lo et al., 2011). Whilst consideration is most commonly given to antibiotic prescription, it is important to also recognise that antibiotic resistance may be acquired due to community environmental acquisition (Hosein et al., 2002, Moellering, 2006). Due to the difficulties in characterising infection (Section 1.5) patients with chronic wounds are at increased risk of (often inappropriately) receiving antibiotics (Howell-Jones et al., 2005). Price et al., (2009) showed that the use of antibiotics in wound patients significantly affected bacterial presence with an increase in pseudomonadaceae, corynebacteriaceae and oxalobacteraceae. Numerous studies have demonstrated the increased prevalence of antibiotic-resistant and MDR organisms within the microflora of chronic wounds (Perera and Hay, 2005, Stanaway et al., 2007, Jappe et al., 2008). Interestingly, the first two cases of vancomycin resistant *S. aureus* in the US were isolated from chronic wound patients (Prevention, 2002a, Prevention, 2002b)

#### **1.7.1 Mechanisms of resistance**

Resistance falls into one of three mechanistic classes; firstly, prevention of the accumulation of an antimicrobial within the bacterial cells usually by either dedicated or general efflux pumps frequently seen with biocide resistance e.g. quaternary ammonium compounds (QAC). Secondly, an alteration of the molecular target; thirdly, inactivation of the substance (Perera and Hay, 2005). From a genetic point of view, resistance is achieved by mutation or acquisition. The most adaptable

mechanism of antibiotic resistance is by the exchange of genetic material, as observed by the acquisition of the staphylococcal cassette chromosome *mec* in *S. aureus* species. Horizontal transfer of genes by bacteriophage and plasmids is a common route for acquisition of antibiotic resistance (Perera and Hay, 2005, Kong, 2011). The chronic wound environment is conducive to the transfer of genetic resistance, due to the diversity of the microflora present and the persistent use of antibiotics to which these bacteria are exposed (Colsky et al., 1998)

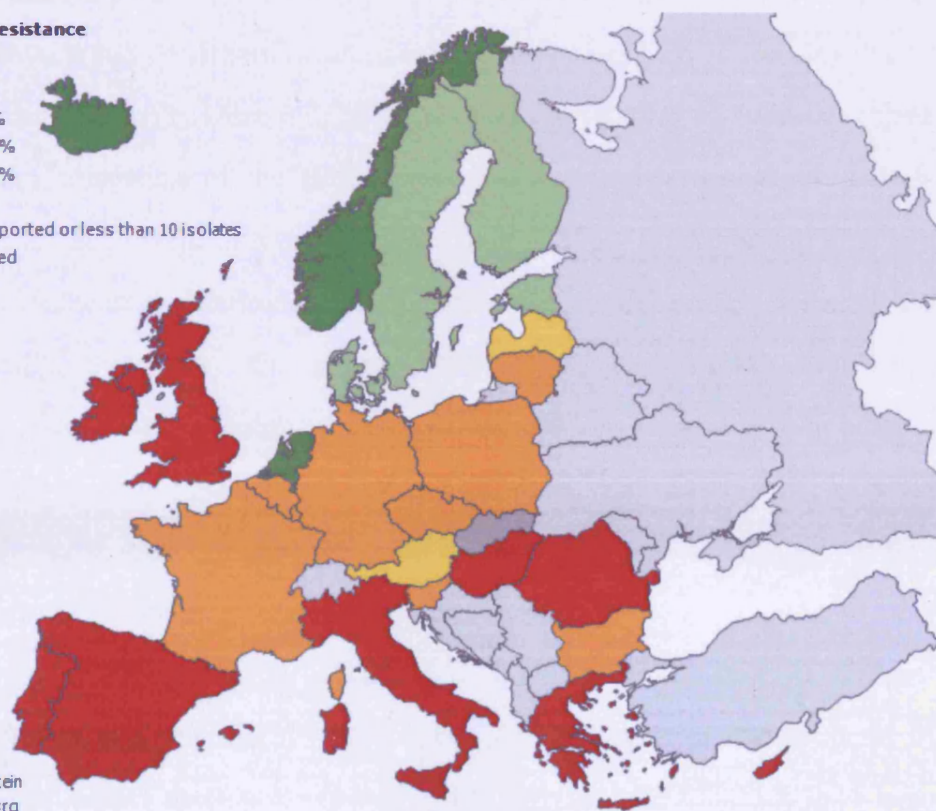
### 1.7.2 MRSA resistance

The term MRSA is misleading because these microbes are resistant to a number of antibiotics. It is thought that 36% of *S. aureus* isolates in the UK are resistant to meticillin (Woodford and Livermore, 2009). Meticillin resistance is associated with the *mecA* gene which encodes a 76 kilo Dalton (kDa) low-affinity penicillin binding protein (PBP2) named PBP2a, which has a low affinity for all  $\beta$ -lactam antibiotics e.g. penicillin. PBP2a is a transpeptidase that is assisted by the transglycosidase domain of the native PBP2 of *S. aureus* and this takes over the function of cell wall biosynthesis in the presence of  $\beta$ -lactam antibiotics (Aires de Sousa and Lencastre, 2004). The *mecA* gene is a 2.1 kb exogenous DNA fragment that is carried on the mobile genetic element, the staphylococcal cassette chromosome *mec* (SCC*mec*), which inserts at site-specific locations on the staphylococcal chromosome, and is acquired via horizontal gene transfer (Enright and Spratt, 1999, Enright et al., 2000a, Enright et al., 2002, Enright, 2003, Hanssen et al., 2004, Enright, 2006, Piette and Verschraegen, 2009). Due to the acquisition of plasmids and transposons, MRSA has successfully acquired resistance to multiple antibiotics including quinolones, and more recently reports of glycopeptide (vancomycin) resistance in Japan, US and Europe (Wootton et al., 2001, Strommenger et al., 2006a). Figure 1.2 shows the distribution of MRSA within Europe during 2009.

**Percentage resistance**

- < 1%
- 1 to < 5%
- 5 to < 10%
- 10 to < 25%
- 25 to < 50%
- ≥ 50%
- No data reported or less than 10 isolates
- Not included

- Liechtenstein
- Luxembourg
- Malta



(C) ECDC/Dundes/TESSy

**Figure 1.2** Proportion of MRSA bacteraemias within Europe in 2009 (reproduced with permission from <http://ecdc.europa.eu/en/activities/surveillance/EARS-Net/database>)



### 1.7.2.1 Staphylococcal cassette chromosome (SCC*mec*)

The staphylococcal cassette chromosome (SCC*mec*) is a mobile genetic element approximately 20-70 kb in size, containing the *mec* region which encodes for meticillin resistance (Zhang et al., 2005). There are 8 types (I to VIII) of SCC*mec* to date (IWG-SCC, 2009, Basset et al., 2010, Ghaznavi-Rad et al., 2010) which are further sub-classified, with novel variants found on a frequent basis. SCC*mec* elements typically share four characteristics: firstly, they carry the *mec* gene complex (*mec*) consisting of the meticillin resistance determinant *mecA* and its regulatory genes and insertion sequences *mecI* and *mecRI*. It is this region where antimicrobial resistance determinants associated with transposable elements are found; secondly, they carry the cassette chromosome recombinase (*ccr*) gene complex responsible for the mobility of the element; thirdly, present at both ends, is a characteristic directly-repeated nucleotide sequence; and finally, all cassettes integrate at the same chromosomal site (*attB<sub>SCC</sub>*) into the 3' end of an open reading frame (ORF), *orfX* (Shore et al., 2005, Kondo et al., 2007).

The *mec* gene complex, for which 5 have been described, (A to D; C has 2 sub-divisions) is composed of IS431*mec*, *mecA* and an intact or truncated set of regulatory genes *mecR1* and *mecI*. The *ccr* gene complex, five of which have been described, consists of the *ccr* genes *ccrA* and *ccrB* in combination (*ccrAB*) or *ccrC* alone (Shore et al., 2005, IWG-SCC, 2009). The rest of the SCC*mec* element previously described as the junkyard rejoin due to its content of non-essential genes, currently referred to as the joining region (J) for which 3 regions have been described. The SCC*mec* type is defined by the *mec* gene complex, the *ccr* gene complex and the J region. Figure 1.3 shows a schematic diagram of SCC*mec* (IWG-SCC, 2009).

Risk factors for the acquisition of MRSA include: previous hospitalisation, intravascular lines, pressure ulcers, underlying disease and recent antibiotics (Maslow et al., 1995, Ayliffe et al., 1998). Some recent studies have also shown that household pets may be additional reservoirs for community-acquired (CA) MRSA

**Type I(1B)**  
(NCTC10442)

**Type II(2A)**  
(N315)

**Type III(3A)**  
(85/2082)

**Type IV(2B)**  
(CA05)

**Type IV(2B&5)**  
(ZH47)

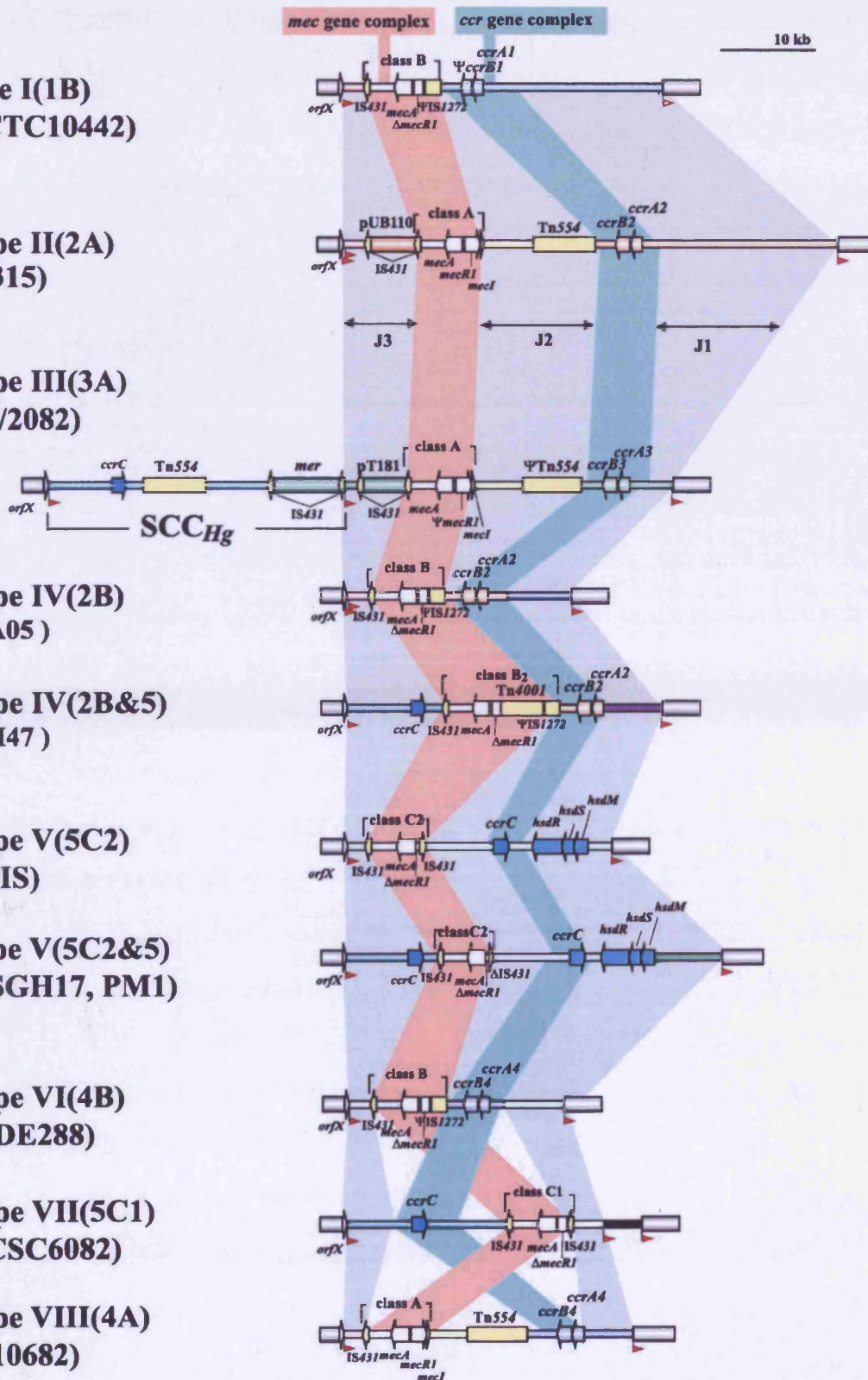
**Type V(5C2)**  
(WIS)

**Type V(5C2&5)**  
(TSGH17, PM1)

**Type VI(4B)**  
(HDE288)

**Type VII(5C1)**  
(JCSC6082)

**Type VIII(4A)**  
(C10682)



**Figure 1.3** Schematic diagram of all *SCC<sub>mec</sub>*, types I to VIII. (Reproduced with permission from International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements, 2009).

(Moellering, 2006). Due to the rise in both CA-MRSA and patient colonisation, occasionally requiring hospitalisation, there has been a blurring of the distinction between hospital-acquired (HA) MRSA and CA-MRSA, and this represents a major problem in infection control. The main circulating epidemic strains in UK hospitals are EMRSA-3, EMRSA-15 and EMRSA-16.

### **1.8 Chronic wounds and MRSA**

Patients with skin lesions are particularly susceptible to long-term carriage of MRSA as this provides a niche which bacteria can colonise (Reich-Schupke et al., 2010). As these chronic skin wounds may lead to hospitalisation, they therefore expose others who are vulnerable to this pathogen, perpetuating the cross infection cycle (Halcon and Milkus, 2004). In a prospective population study of skin and soft tissue infections caused by MRSA, 75% of infections were associated with CA-MRSA in comparison to only 37% HA-MRSA (Naimi et al., 2003). *S. aureus* and MRSA have been implicated as major colonisers of chronic wounds (Jappe et al., 2008), with studies implicating nasal carriage as a major route of transmission to the wound (Stanaway et al., 2007).

Genetic studies have identified 5 major lineages of MRSA (clonal complexes) associated with HA-MRSA. These clonal complexes (CCs) are: CC5, CC8, CC22, CC30 and CC45 (Tristan et al., 2007). They have arisen from the successful acquisition of the SCCmec element by *S. aureus*. Clones of these lineages are responsible for most of the HA-MRSA globally (Robinson and Enright, 2003, Deurenberg and Stobberingh, 2008). Due to the significant increase in CA-MRSA, the need to track this pathogen is important in our understanding of its ancestry, development and clonal expansion.

### **1.9 Typing of MRSA**

A number of epidemiological typing methods have been used to describe MRSA including: antimicrobial susceptibility testing, genomic restriction fragment

length polymorphism analysis using pulsed-field gel electrophoresis (PFGE), DNA hybridisation, phage typing, multilocus enzyme electrophoresis, genotypic and sequence-based typing methods. These are used to understand the spread of the organism and cross-infection control.

### **1.9.1 Genetic based typing methods**

#### **1.9.1.1 Pulsed field gel electrophoresis (PFGE)**

Pulsed Field Gel Electrophoresis was considered the “gold standard” typing method for the investigation of outbreaks, until recently. This method is based on the distribution of restriction endonuclease cleavage sites on the bacterial genome and is reflected by fragment length. Large lengths of uncharacterised DNA are resolved by PFGE and agarose gels. Strain differences relate to genetic events e.g. point mutations, which change the endonuclease restriction site, i.e. insertions, deletions, inversions or transpositions. It is a highly discriminatory method and has added to the knowledge of epidemiology of many pathogenic bacteria in nosocomial outbreak settings e.g. MRSA, vancomycin resistant enterococci (VRE) and *E. coli*. Such fragment pattern changes appear to accumulate rapidly, which is important in the epidemiology of bacterial studies during outbreaks (Enright and Spratt, 1999, Enright et al., 2000b). This rapid variation in fragment patterns within the same clone however, makes it difficult to determine (with 100% accuracy) ancestral lineage using this method (Enright et al., 2000a, Enright et al., 2000b). PFGE has excellent discriminatory power; however it is a difficult method to standardise between laboratories (Shopsin et al., 1999).

#### **1.9.1.2 Random amplified polymorphic DNA (RAPD)**

Random amplified polymorphic DNA is a PCR based method for the typing of MRSA and other bacteria. RAPD utilises single, short primers of arbitrarily chosen (8-10) nucleotide sequences which are employed in comparatively low annealing temperature PCRs, and allow random amplification of the genomic DNA.

These primers recognise sequences only partly complementary or homologous to them. The products are separated on agarose gels producing a fingerprint for the strain in question, which can be compared with others to identify polymorphisms. This method has been used to study the diversity of plant, animal and bacterial species, and requires no previous information of the genomic structure. This method shows similar disadvantages to PFGE in terms of inter-laboratory and strain variability (Witte et al., 2006).

#### **1.9.1.3 Multilocus sequence typing (MLST)**

MLST, is based on nucleotide sequencing and is believed to represent the “gold standard” for MRSA typing (Urwin and Maiden, 2003) allowing unambiguous characterisation of bacterial strains via the existing databases (Enright and Spratt, 1999). Enright was the first to describe the use of MLST in the typing of MRSA (Enright and Spratt, 1999). This method compares internal fragment size (402 – 516 bp) of 7 housekeeping genes (Enright et al., 2002) avoiding parts of the genome that are rapidly evolving due to strong selective pressures, e.g. antibiotic and immunological factors (Urwin and Maiden, 2003) and thus are stable over time. The genes used are: carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol kinase (*glp*), guanylate kinase (*gmk*), phosphatase acetyltransferase (*pta*), triphosphate isomerase (*tpi*), and acetyl coenzyme A acetyltransferase (*yqiI*), which are highly conserved (Urwin and Maiden, 2003). Each fragment of the 7 housekeeping gene sequences are assigned a distinct allele number and each isolate is defined by the alleles at each of the seven housekeeping loci i.e. the allelic profile. This corresponds to a sequence type (ST), enabling the researcher to distinguish between billions of different allelic profiles (Enright et al., 2000a). Accumulation of nucleotide changes is relatively slow and the allelic profile is sufficiently stable over time for the method to be ideal for global epidemiological purpose (Enright et al., 2000a). MLST therefore allows international comparison of isolates, whilst recording the emergence of antibiotic-resistant bacteria.

#### **1.9.1.4 Staphylococcal cassette chromosome *mec* (SCC*mec*) typing**

The acquisition of meticillin resistance by *S. aureus* is via the transfer of the mobile genetic element the staphylococcal cassette chromosome (SCC) which carries the resistance gene *mecA* (Katayama et al., 2000). The SCC*mec* is typed via multiplex PCR technology by defining the *mec* gene complex, the cassette chromosome recombinase (*ccr*) gene complex, and in the case of a class IV SCC*mec*, the joining region (Chapter 1, Section 1.7.2.1; Kondo et al., 2007, IWG-SCC, 2009). Long-term epidemiological studies of MRSA and its evolution have shown that complete characterisation of MRSA lineage requires identification of the core *mecA* gene.

The proposed nomenclature is that MLST is reported with the SCC*mec* type (Urwin and Maiden, 2003). Epidemiological studies have shown that CA-MRSA contain SCC*mec* IV and V, whilst HA-MRSA strains tend to harbour the larger SCC*mec* elements I, II, III (Zhang et al., 2005). The typing of the SCC*mec* has increased the precision of strain typing and epidemiological evolution (Ito and Hiramatsu, 1998).

Combination of MLST and SCC*mec* in the study of MRSA has provided three important insights. Firstly, meticillin resistance has emerged in multiple phylogenetically distinct lineages. Secondly, meticillin resistance has emerged on multiple occasions within a given phylogenetic lineage; and finally, MRSA disease is caused by a relatively small number of pandemic clones (Robinson and Enright, 2003).

#### **1.9.1.5 Staphylococcal protein A (*spa*) typing**

Further sequence-based methods have now been developed. Staphylococcal protein A (*spa*) typing is based on the polymorphic region of the protein A gene which consists of direct repeats (Malachowa et al., 2005, Strommenger et al., 2006b). This is of particular interest in the hospital setting because it is a rapid

method and offers high isolate resolution (Shopsin et al., 2000, Harmsen et al., 2003).

### **1.10 Microbial virulence**

A virulence factor can be defined as a substance, which when purified and introduced into a host, produces a pathogenic reaction (Projan and Novick, 1997). Bacterial-host interactions depend on several factors, including the efficiency of the host defense mechanism, bacterial growth rate, and production of virulence factors. Analysis of virulence genes, either by allelic replacement, insertion or inactivation of particular genes, has given some evidence to the role of virulence factors in disease.

It has been postulated that successful MRSA clones have acquired virulence factors to compensate for the loss of fitness associated with antibiotic resistance (Tristan et al., 2007). Such virulence factors produced by the bacteria can be cell-associated or extracellular and contribute to the evasion of the host defence system. These virulence factors enable the organism to attack the local cellular and structural elements of diverse tissues and organs, while the surface components enable the bacteria to adhere to the tissue components to resist phagocytosis and other host defences. Bacteria regulate their virulence factors so that they are used only at a time and stage appropriate to their disease progression e.g. once the infection has become systemic, there is a down-regulation of factors required to adhere and colonise the host tissues (Camara et al., 2002).

### **1.11 Quorum sensing and the accessory gene regulator (*agr*)**

The expression of virulence factors and indeed biofilm phenotypic changes are globally regulated via quorum sensing. This complex regulatory network is arranged around a two component regulatory system, one of which is the accessory gene regulator locus (*agr*). Via the production of an auto-inducing peptide pheromone, quorum sensing interacts with the *agr* to sense cell density and allows for cell-to-cell interactions to aid bacterial infiltration of the host and development of

communities (Novick and Muir, 1999, Camara et al., 2002, Siddiqui and Bernstein, 2010) The *agr* system coordinates the production of virulence factors during the different phases of the cell cycle, e.g. it down-regulates the production of cell-wall associated proteins and up-regulates secreted proteins at late to stationary growth phase *in vitro* (Ji et al., 1995, Mullarky et al., 2001, Camara et al., 2002, Bjarnsholt et al., 2008). This density-dependant regulation of virulence factors becomes particularly important in biofilm formation (Iwatsuki et al., 2006).

#### **1.11.1 Accessory gene regulator (*agr*) specificity**

The accessory gene regulator has been shown to regulate the synthesis of many virulence factors during bacterial growth (Mullarky et al., 2001). The *agr* is conserved throughout the staphylococcal family. There are 4 (I-IV) specific *agr* groups in *S. aureus*. Mutual inhibition exists between separate *agr* groups where the *agrD*-derived peptide pheromone from strains of one group can cross-activate an *agr* of the same group to produce a response e.g. an up-regulation of virulence factors in other strains of the same group. It can also inhibit the *agr* of members of other groups (Camara et al., 2002). Many studies have identified this as a potential for new anti-infective therapy (Finch et al., 1998, Hentzer and Givskov, 2003, Rasmussen and Givskov, 2006, Clatworthy et al., 2007, Raina et al., 2009).

The *agr* type has been linked with disease processes e.g. toxic shock syndrome (TSS) and CA-MRSA have been linked with *agr* III, and staphylococcal scalded skin syndrome (SSSS) has been linked with *agr* IV, whilst enterotoxin mediated disease with *agr* I and II (Ji et al., 1995, Jarraud et al., 2000, Jarraud et al., 2002, Vandenesch et al., 2003, Robinson et al., 2005). *agr* type has also been linked to the severity of disease; *agr* I has been associated with invasive infections such as bacteraemia and *agr* III associated with non-invasive infections (Ben Ayed et al., 2006).



## 1.12 Virulence factors

Sotto et al., (2008) looked at the virulence potential of *S. aureus* in DFU. They found that the presence of 5 virulence genes (*cap8*, *sea*, *sei*, *lukE* and *hlgv*) could distinguish between non-infected and infected wounds and also predict healing outcome. The Health Protection Agency (HPA) frequently examines the toxin genes present in referred staphylococci associated with disease.

### 1.12.1 Pyrogenic toxin superantigens

Pyrogenic toxin superantigens classically include the staphylococcal enterotoxin type A to E and toxic shock syndrome toxin-1 (TSST-1). Most are regulated by the *agr* and staphylococcal accessory regulator (*sar*) loci. Depending on the toxin, the gene may be harboured on a mobile genetic element such as a plasmid, or bacteriophage, or on pathogenicity islands. Toxic shock syndrome (TSS) is an acute illness, and the patient classically presents with hypotension, fever, rash and desquamation of hands and feet during convalescence, with the involvement of at least three organ systems and may be categorised as menstrual or non-menstrual (Bohach, 2006). *S. aureus* is known to elicit a host immune response via pathogen associated molecular patterns (PAMPs) e.g. lipoteichoic acid (LTA) and capsular polysaccharide both induce the production of IL-8 by Mo, and endothelial cells (DeLeo et al., 2009). The production of this chemokine allows the transmigration of neutrophils to the site of infection. Virulence factors e.g. TSST-1, enterotoxin A (SEA) and enterotoxin B (SEB) can also elicit an IL-8 response. Superantigens interact with the immune system to cause polyclonal proliferation of T-cells and bind specifically to the major histocompatibility complex (MHC) class II, which results in the deletion of  $\beta$ -expressing T-cells. It has been proposed that *S. aureus* benefits from the immunosuppression induced by pyrogenic toxin superantigens (Bohach, 2006).

### **1.12.2 Exfoliative (epidermolytic) toxins**

Exfoliative toxins have been conclusively implicated in SSSS, which is characterised by the formation of bullae or skin blisters. The lesions are characterised by the separation of the stratum granulosum cells, causing intradermal skin peeling leading to desmosome degeneration. The structural gene *eta*, responsible for the expression of this virulence factor, is generally located on the chromosome and is sometimes harboured on a phage, while *etb* and *etd* are located on plasmids and pathogenicity islands (Bohach, 2006); all of which are associated with severe skin reactions.

### **1.12.3 Leucotoxins**

Leucotoxins are a family of toxins encoded by several genetic loci, and are able to cause cellular lysis of host leucocytes and erythrocytes. Staphylococci have many leucotoxins e.g.  $\gamma$ -haemolysin and Panton-Valentine leucocidin (PVL). They contain two synergistically active proteins: one S component and one F component, designated according to their mobility in ion exchange chromatography as “slow” or “fast”. Synergistic functions involve sequential binding of the F and S components.

#### **1.12.3.1 Panton-Valentine leucocidin (PVL)**

PVL is a pore-forming cytotoxin that targets human and rabbit mononuclear and polymorphonuclear cells (Holmes et al., 2005). When injected intradermally into rabbits, PVL induces severe inflammatory lesions, leading to capillary dilation, chemotaxis, polymorphonuclear karyorrhexis and skin necrosis (Holmes et al., 2005). Exposure of neutrophils to PVL *in vitro* leads to swelling and rounding of the cells and their nuclei, followed by degranulation and nuclear rupture, leading to cell lysis. It is thought to be linked to specific human *S. aureus* infections such as primary skin and soft tissue disease and severe necrotising pneumonia, where the mortality rate is about 75% (Holmes et al., 2005, Health Protection Agency et al., 2006a Tristan et al., 2007, Ellington et al., 2009). Dufour et al., (2002) and Van der

Mee-Marquet et al., (2004) found that PVL producing *S. aureus* strains could also cause chronic cutaneous infections.

PVL is coded by *lukS-PV* and *lukF-PV* genes, previously used as a marker of CA-MRSA (Vandenesch et al., 2003, Holmes et al., 2005) and was associated with disease in healthy individuals. PVL has been reported to be produced by 2-15% of all *S. aureus* (Lina et al., 1999, Holmes et al., 2005, Wannet et al., 2005, Ellington et al., 2007), although this low number may be a function of under-reporting. The significance of PVL has been investigated in the clinical setting following outbreaks of skin and soft tissue infection and clusters of death from necrotising pneumonia in atypical patients. Often the presence of the PVL gene (*lukF-PV* & *lukS-PV*) has been investigated by routine testing in combination with superantigen toxins by PCR. Many researchers have used PCR to investigate disease processes associated with PVL-negative and PVL-positive strains, whilst others have purified this protein and introduced it to animal models to investigate its potency with conflicting results (Voyich et al., 2006, Dumitrescu et al., 2007, Ellington et al., 2007, Labandeira-Rey et al., 2007).

#### **1.12.3.2 Gamma haemolysin (*hlg*)**

$\gamma$ -haemolysin is thought to be produced by >99% of staphylococci. The composition and activity of Hlg is associated with 3 proteins. HlgA and HlgC belong to the S class and HlgB belongs to the F class, thus forming two pairs of units (Prevost et al., 1995a, Prevost et al., 1995b, Lina et al., 1999). Animal studies (rabbits), have shown that Hlg is inflammatory but not necrotic (Konig et al., 1995, Konig et al., 1997) and that  $\alpha$ - and  $\beta$ -haemolysins are able to induce caspase-1 (Munoz-Planillo et al., 2009).

#### **1.12.4 Extracellular enzymes**

*S. aureus* produces a large number of extracellular enzymes which may be important virulence factors. They can degrade organic macromolecular tissue

constituents to promote the spread of the bacterium in host tissues e.g. hyaluronate lyase and lipases, which break down hyaluronic acid and fatty tissue respectively (Arvidson, 2006).

#### **1.12.4.1 Glycerol ester hydrolase (*geh*)**

Many lipases suppress immune function (Lee and Iandolo, 1986, Rollof et al., 1988, Izdebska-Szymona et al., 1992, Rollof et al., 1992, Staffan, 2006). Staphylococcal lipase with fatty acid modifying enzyme (FAME) is able to interact with bactericidal lipids to enhance survival within an abscess (Kapral et al., 1992, Lowe et al., 1998). Previously, lipase activity has been tested in mouse models with abscess formation and chromogenic plate assays (Burlak et al., 2007, Gould et al., 2009). Currently, lipase producing *S. aureus* strains are strongly linked to invasive disease and phagocytosis interference (Rollof et al., 1992, Rosenstein and Gotz, 2000).

#### **1.12.4.2 Hyaluronate lyase (*hysA*)**

Linker et al., (1956) postulated that hyaluronate lyase from *S. aureus* worked by  $\beta$ -elimination of the uronic acid unit within hyaluronic acid, and is thought to be present in >90% of *S. aureus* (Choudhuri and Chakrabarty, 1969). Murine models have shown that wild-type *S. aureus* with *hysA* were able to affect wound healing and associated subcutaneous infections when compared with *hysA*-negative strains (Makris et al., 2004).

#### **1.12.5 Attachment and collagen binding protein (*cna*)**

In a study by Holderbaum et al., (1987) only 43% of clinical *S. aureus* isolates were able to bind collagen, and it was concluded that *cna* was not a constitutive element for all *S. aureus* strains. They postulated that strains able to bind collagen were more likely to be adherent in tissue beds and associated with invasive disease, than those with uncomplicated bacteraemia (without other

infections). A study by Xu et al., (2004) observed that *S. aureus* with a higher affinity for collagen possessed a greater virulence potential. However, expression of *cna* was not enough to produce pathogenic *staphylococci* sp. in a septic arthritis model.

### **1.13 The immune system**

The immune system is crucial to the survival of man, and is essential in protection against invading organisms and tumour cells. The innate immune system represents the “first line” of defence, with the adaptive immune system providing specific cytotoxicity and immunological memory to the immune system (Lien and Ingalls, 2002, Fournier and Philpott, 2005).

#### **1.13.1 The innate immune system**

The innate immune response forms the early host response to infection which is present in all individuals (Lien et al., 1999, Fournier and Philpott, 2005, Thorgersen et al., 2009). The cells involved instigate the removal of bacteria and modulate the adaptive immune responses (Lien et al., 1999, Miyake, 2003). The major cellular components of the innate immune system are the PMNL, Mo and dendritic cells (DC). These cells are able to identify microbes via pathogen associated molecular patterns (PAMP) and host damage via damage associated molecular patterns (DAMP) by pattern recognition receptors (PRR), the most extensively studied of these being the Toll-like receptors (TLR).

##### **1.13.1.1 Toll-like receptors**

TLR were originally identified in *Drosophila* associated with the dorsoventral pattern in developing embryos of fruit flies (Takeda and Akira, 2004, Mollen et al., 2006). Since their discovery it has been found that these receptors form an essential part of the mammalian immune system. Flies with mutated Toll-receptors have been found to be more susceptible to infections from fungi and

bacteria (Lemaitre et al., 1996, Lien and Ingalls, 2002, Fournier and Philpott, 2005). TLR are expressed on immunocompetent cells such as PMNL, Mo, MØ, microglia, DC, B-cells, T-cells, natural killer (NK)-cells, mast cells, astrocytes, epithelial cells and a number of endothelial cells (Hornef et al., 2002, Zhang and Schluesener, 2006, Morris et al., 2009, Pukstad et al., 2010). TLRs are able to identify bacteria, virus, fungi and protozoa and to date, 13 mammalian TLRs have been identified, of which 10 have been reported active in humans (Table 1.2; Konat et al., 2006, Etokebe et al., 2010, Pukstad et al., 2010, Yamamoto and Takeda, 2010). Dendritic cells and Mo express the greatest number of TLR (Baiyee et al., 2006). Ligand recognition via TLR at sites of skin wounding stimulates the release of cytokines and pro-inflammatory cytokines, as a result of nuclear factor  $\kappa$ B (NF- $\kappa$ B) translocation to the nucleus. This signalling pathway is a complex concert of adapter molecules which activate NF- $\kappa$ B. Figure 1.4 is a schematic diagram of TLR signalling pathways.

### **1.13.2 The role of the immune system in wound healing**

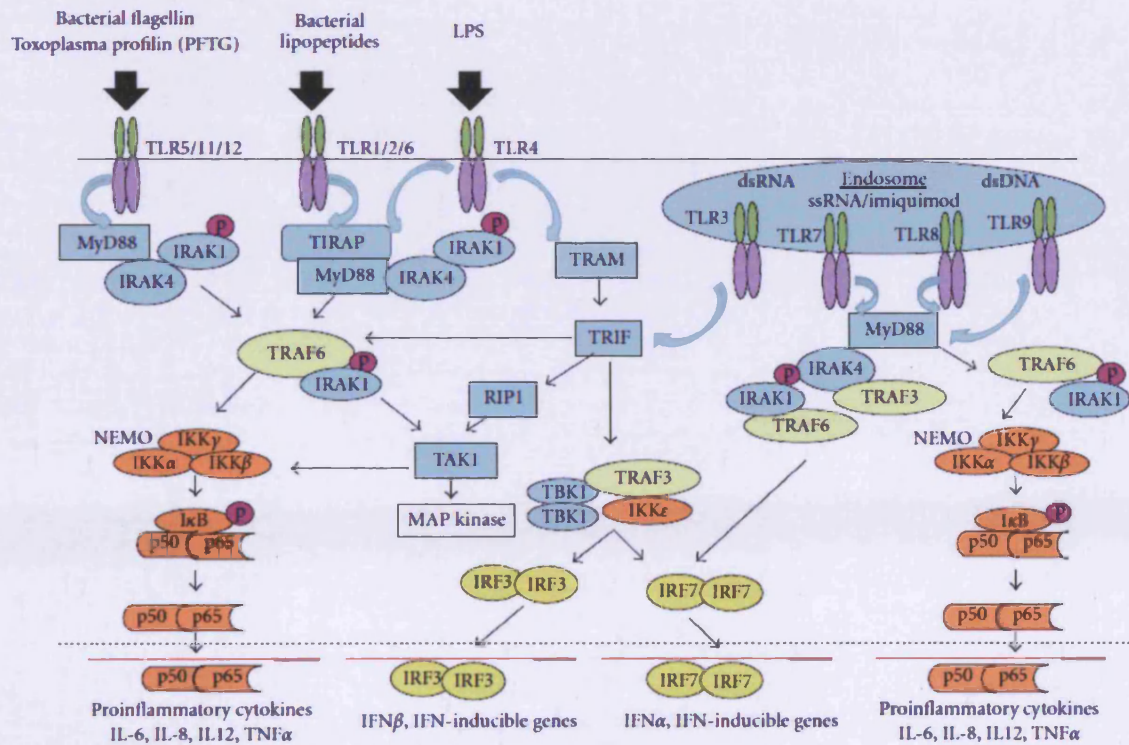
Following wounding there is an up-regulation of pro-inflammatory cytokines and chemokines to activate the resident population of inflammatory cells and stimulate recruitment of inflammatory cells from the circulatory system via the up-regulation of adhesion molecules such as integrins and selectins (Jones et al., 2004). The importance of the immune system in mediating response to injury is evident in experimentally (chemical) immunosuppressed animals and knock-out studies (Grabig et al., 2006, Mirza et al., 2009, Ueda et al., 2010). Significantly, it is also evident in the context of chronic wounds in diabetic animals and patients (Jiwa, 1997, Currie et al., 1998, Harding et al., 2002, Oncul et al., 2007).

PMNL and Mo migrate into the wound; effecting the removal of invading pathogens and damaged ECM via phagocytosis following TLR recognition (Chapter 4). The subsequent release of cytokines stimulates cellular migration of resident fibroblasts and keratinocytes to repair the wound and close any breach in the surface barrier, which intact normal skin represents (Rodero and Khosrotehrani, 2010).

**Table 1.2** Human Toll- like receptors (TLR)

TLR	Cellular compartment	Identify	Ligand	Heterodimer formation
TLR1	Extracellular	<i>Borrelia burgdorferi</i>	Pam <sub>3</sub> CysSK <sub>4</sub> Protein A	TLR2
TLR2	Extracellular	Gram positive bacteria  Fungi  <i>Neisseria</i>  Spirochetes	LTA PGN Lipoprotein Zymosan  Lipoarabinomannan Porins Glycolipids	TLR2
TLR6	Extracellular	Mycobacterium	LTA Lipopeptide	TLR2
TLR3	Intracellular	Virus	dsRNA	
TLR4	Extracellular	Gram negative bacteria  Host cell  Gram positive bacteria	LPS  HA Fibrinogen Fibronectin Heparan sulfate Lipoteichoic acids	
TLR5	Extracellular	Bacteria	Flagellin	
TLR7	Intracellular	Virus	ssRNA	
TLR8	Intracellular	Virus	G-rich oligonucleotides	
TLR9	Intracellular	Unmethylated bacterial DNA	CpG	
TLR10	Extracellular	Unknown	Unknown	TLR1 & TLR2

CpG, deoxycytidyl-deoxyguanosine; dsRNA, double stranded ribonucleic acid; HA, hyaluronic acid; LPS, lipopolysaccharide; LTA, lipoteichoic acid; Pam<sub>3</sub>CysSK<sub>4</sub>; N-palmitoyl-S-dipalmitoylglycerol-Cys-Ser-Lys; PGN, peptidoglycan; ssRNA, single stranded ribonucleic acid; TLR, toll-like receptor.



**Figure 1.4** TLR signalling pathway and downstream mediators.

dsDNA, double stranded deoxyribonucleic acid; dsRNA, double stranded ribonucleic acid; IκB, inhibitor of nuclear factor kappa B; IKK, IκBα kinase complex; IRAK, interleukin-1 receptor associated kinase; IRF, interferon (INF) regulatory factor; LPS, lipopolysaccharide; MAP, mitogen activating protein; MyD88, myeloid differentiation factor 88; NEMO, NF-κB essential modulator (or IKKγ); NF-κB, nuclear factor – kappa B; RIP, receptor interacting protein; ssRNA, single stranded ribonucleic acid; TAK, TGF β- activated kinase; TANK, TRAF family member associated NF-κB activator; TBK, TANK binding kinase; TIR, toll-IL-1 receptor; TIRAP, TIR domain-containing adaptor molecule; TLR, toll-like receptor; TRAF, tumour necrosis factor receptor (TNFR) associated factor; TRAM, TRIF-related adaptor molecule; TRIF, TIR-domain-containing adapter-inducing interferon-β. (Reproduced with permission Zhu and Mohan, 2010).



The removal of antigenic stimuli by these processes result in resolution of the acute immune response and the wound is healed. These processes are disordered in chronic wounds (Falanga, 2005, Kirketerp-Moller et al., 2008, Mirza et al., 2009).

Chronic wounds are characterised by the protracted elevation of chronic inflammatory cells from Mo and lymphocyte lineages, the wounds being “arrested” in the inflammatory phase of the healing cycle (Schultz et al., 2003, Kirketerp-Moller et al., 2008, Pukstad et al., 2010). The continuing influx of inflammatory cells and their potent inflammatory mediators e.g. matrix metalloproteinases (MMPs), cytokines/chemokines and free-oxygen radicals play a significant role in mediating further tissue damage and immune responses in a negative-feedback loop. A further example is the generation of low molecular weight (mw) ECM degradation products perpetuate immune response (Termeer et al., 2000, Taylor et al., 2004). The important role of the immune system in impaired healing has been highlighted in a recent study by Pukstad et al., (2010) using wound fluid from chronic and acute wounds. The authors observed that alterations in wound cytokine profiles (e.g. IL-8, IL-1 $\alpha$ , IL-1 $\beta$ , TNF $\alpha$ ) were related to healing and wound chronicity. Moreover, these authors demonstrated that healing correlated with a decreased TLR-stimulating ability of the wound fluid, suggesting a microbial role.

#### **1.14 Biofilms and chronic wounds**

Whilst studies of wound bacteria and their activity and response to treatment have been carried out in planktonic systems, increasingly attention has been directed to the bacterial biofilm of the chronic wound (Mertz, 2003, Davis et al., 2008, Charles et al., 2009). Biofilms have been described as ‘*a microbially-derived sessile community characterised by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription*’ (Donlan and Costerton, 2002).

Biofilms have been estimated to be associated with 65% of nosocomial infections (Percival and Bowler, 2004), and are associated with many chronic disease processes e.g. dental caries, cystic fibrosis, periodontal disease and urinary tract infections (Davis et al., 2008, Burmølle et al., 2010). Many of the chronic wound bacteria produce biofilms, including *S. aureus* (Evans et al., 1998, Elasri et al., 2002, Gilbert et al., 2002, Kropec et al., 2005, Toledo-Arana et al., 2005, Davis et al., 2008, Percival et al., 2008, Hill et al., 2010, Percival et al., 2011a). Clear evidence for the presence of biofilms in chronic wounds has been established. Confocal laser scanning microscopy (CLSM) of wound biopsies (*in vivo*) and reconstituted human epidermis (RHE) models have observed the presence of bacteria and biofilms (Bjarnsholt et al., 2008, Malic et al., 2009). James et al., (2008) suggested that polymicrobial biofilm formation was more evident in chronic rather than acute wounds. The exact role that the biofilm itself plays in wound healing has yet to be elucidated.

A number of methods have been employed to study the ability of *S. aureus* to form a biofilm. These have included PCR-based methods for the identification of the *ica* operon, as well as more qualitative methods such as tube-based assays, tissue culture plate assays, and the use of Congo red agar (Knobloch et al., 2002). Many of these tests have resulted in conflicting results *in vitro* and thus it has been suggested that the expression of *ica* is environmentally regulated (Knobloch et al., 2002, Beenken et al., 2004, Cafiso et al., 2007, O'Neill et al., 2007), with laboratory manipulation of media having a significant impact (Fitzpatrick et al., 2005, O'Neill et al., 2007). Within *S. aureus*, the conserved *ica* operon is responsible for the production of polysaccharide intercellular adhesion (PIA), which is important in the formation of a biofilm (Cramton et al., 1999, Cafiso et al., 2004, Fluckiger et al., 2005). Moreover, studies have shown that *S. aureus* isolates associated with clinical infection, such as prosthetic joint infections, bacteraemia and catheter-related disease showed the presence of the *ica* locus (Cramton et al., 1999, Arciola et al., 2001,

Fowler et al., 2001, Knobloch et al., 2002, Martin-Lopez et al., 2002, Fluckiger et al., 2005).

### **1.15 Bacterial evasion of the immune system**

In parallel with the possession of antibiotic resistance, the acquisition of specific immune evasion tactics allow these wound bacteria to subvert the host immune response (Rooijackers et al., 2005). The immune system not only comprises physical barriers to the entrance of bacteria, and cells to remove invading pathogens, but is complemented by host production of many molecules e.g. soluble opsonising factors, antibodies and antimicrobial peptides. Bacteria have overcome many of these soluble molecules by the production of degradative peptides (Chapter 3 & 4). Bacteria are also able to avoid phagocytosis by the production of capsules and toxins which can cause host cell lysis (Hornef et al., 2002).

An interesting method by which bacteria evade recognition is by steric alterations in PAMPs, e.g. *Salmonella* lipopolysaccharide (LPS) and *Yersinia pestis*, which has a tetra-acetylated LPS that does not activate TLR4 (Hornef et al., 2002, Comer et al., 2010). *Y. pestis* has an ability to delay/suppress the immune system, as indicated by the moderate collection of PMNL following infection, whilst TNF $\alpha$  and  $\gamma$ -interferon (IFN $\gamma$ ) are found only in the advanced stages of infection (Comer et al., 2010). The ability of *S. aureus* to become internalised via fibronectin bridges and to replicate intracellularly, can lead to cellular apoptosis or chronic infection, which again is a mechanism for protection from the immune system and antimicrobial therapy (Jevon et al., 1999, Kahl et al., 2000, Foster, 2005, Keiji et al., 2006). The ability of *S. aureus* to colonise skin and the anterior nares reflects its relative resistance to lysozyme, which is secreted to control colonisation (Bera et al., 2005, Foster, 2005, Fournier and Philpott, 2005, Herbert et al., 2007). Fibrinogen-binding protein, found on the surface of many *S. aureus* and the production of staphylococcal complement inhibitor (SCIN) can lead to the prevention of complement factor C3 deposition by blocking C3 convertase complexes (C4bC2a; classic/lectin pathway,

C3bBb; alternate pathway). This can result in decreased phagocytosis and inhibition of the complement cascade (Lee et al., 2004, Foster, 2005, van Belkum, 2006, Nizet, 2007). *S. aureus* is also able to cleave immunoglobulins IgG, IgM and IgA by the action of serine proteases, thus escaping opsonization and clearance via phagocytosis.

Biofilms are associated with persistent infections and delayed wound healing (Mah and O'Toole, 2001, Percival et al., 2008, Martin et al., 2009). There are a number of human diseases associated with chronic bacterial colonisation in which the biofilm state is believed to resist the action of the immune system and contribute to the chronicity of the disease e.g. chronic leg ulcers, periodontal disease, cystic fibrosis (Mori et al., 2003, Davis et al., 2008, Burmølle et al., 2010). The physical biofilm phenotype allows colonising bacteria to protect themselves from antimicrobial agents and host responses by providing protection against phagocytosis, complement and free-oxygen radicals (Johnson et al., 1986, Davis et al., 2008, Percival et al., 2008, Martin et al., 2009). Moreover, the secretion of various virulence factors by pathogens within biofilms e.g. leucocidins and proteases may induce apoptosis of host immunological effector cells (Kahl et al., 2000, Horne et al., 2002, Kirketerp-Møller et al., 2008, DeLeo et al., 2009, Comer et al., 2010).

### **1.16 Biocides and chronic wounds**

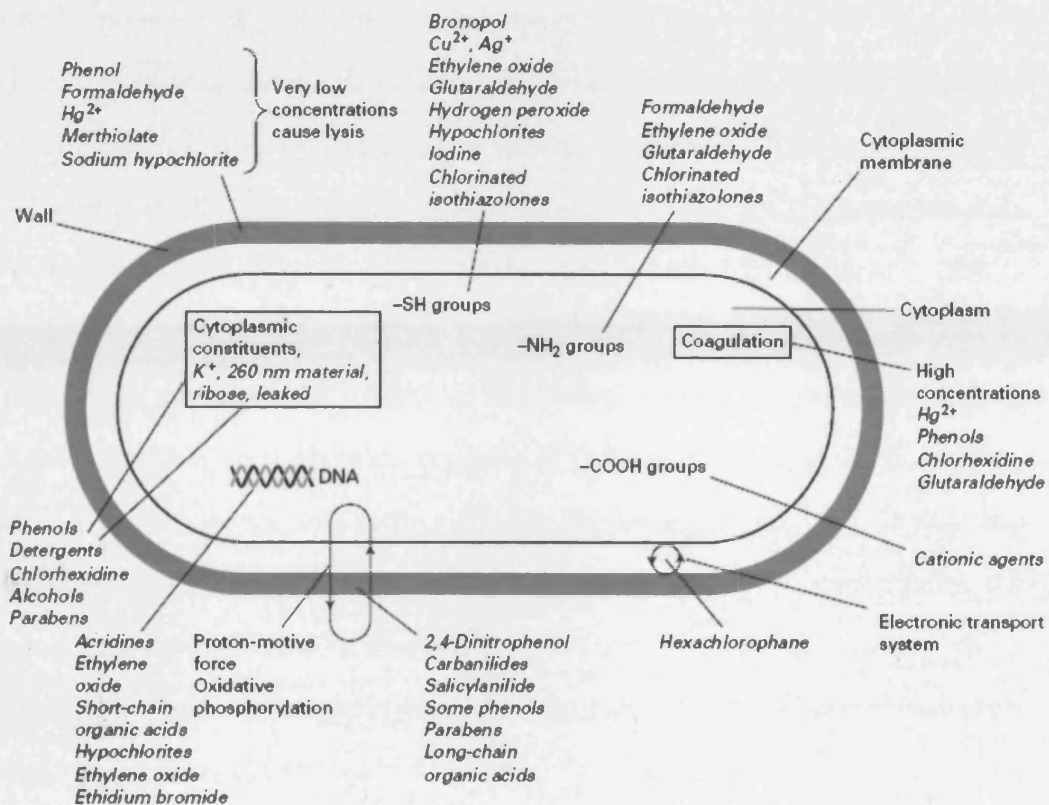
Biocides can be broadly described as “*chemical agents that inactivate microorganisms*” (McDonnell and Russell, 1999). Due to the increasing incidence of antimicrobial resistance, the use of biocides to control bacterial colonisation within the chronic wound bed is gradually being more advocated (O'Meara et al., 2010). In particular, the potential for biocides to treat the biofilms present within wounds has attracted special attention. Biocides used in chronic wounds have been employed in many forms e.g. iodine compounds, silver compounds, chlorhexidine, potassium permanganate, hydrogen peroxide, honey and benzalkonium chloride (Fonder et al., 2008, Atiyeh et al., 2009, BNF, 2010). Hill et al., (2010)

demonstrated the efficacious ability of iodine over that of silver with mature biofilms. In contrast to antibiotics, biocides have many modes of activation and target a number of bacterial structures (Figure 1.5).

#### **1.16.1 Biocide resistance**

Concerns have been raised regarding biocide resistance or tolerance in recent years. Bacteria are able to use intrinsic resistance mechanisms such as alterations in cell wall thickness, efflux-pump systems, alterations in surface porins, biofilm production and hydrophobicity to decrease their sensitivity to biocides (McDonnell and Russell, 1999, Russell, 2001, Russell, 2003, Maillard, 2007). Moreover, the effectiveness of biocides may be reduced in the proteinaceous wound environment (Russell, 1995, McDonnell and Russell, 1999, Khan and Naqvi, 2006, Narui et al., 2007). Specific acquired resistances have also been identified, and the acquisition of these via genetic mutations or transfer of plasmids and transposons has been previously studied e.g. quaternary ammonium compound (QAC) resistance genes (*qacABCD*), silver-resistance genes (*silE*, *silS*, and *silP*) and the biocide resistance gene *sepA* found in *S. aureus* (McDonnell and Russell, 1999, Russell, 2001, Drosou et al., 2003, Russell, 2003, Maillard, 2007, Narui et al., 2007, Loh et al., 2009).

Biofilms play a significant role in resistance to biocide treatments (Hill et al., 2010); the biofilm itself poses a diffusion barrier to the actions of biocides and antimicrobials and also the ionic charge of the biofilm may be significant to the entrance of biocides (Percival et al., 2011a). Coupling the effects of decreased uptake of the antimicrobial into the biofilm is the likely reduced bacterial growth rate and metabolic activity, leading to suboptimal biocide activities (Cloete, 2003, Buckingham-Meyer et al., 2007, Høiby et al., 2010).



**Figure 1.5** Biocide action sites in a bacterial cell. (Reproduced with permission Denyer and Russell, 2007)

### **1.16.2 Biocides used in chronic wound treatments**

Povidone-iodine (PVP-I) is now routinely used in surgical scrubs, ointments and dressings (Drosou et al., 2003, Cooper, 2004). Recent formulations include cadexomer iodine; a controlled-release system. Many studies have shown the efficacy of PVP-I to decrease bacterial load and its rapidity of action (Berkelman et al., 1982, McLure and Gordon, 1992, Brown et al., 1995, Khan and Naqvi, 2006, Durani and Leaper, 2008). A systematic review by Vermeulen et al., (2010) showed that iodine did not have deleterious effects on wound healing and was as effective as other biocide agents, although inferior to topical antibiotics. The exact mode of action of iodine is unknown, but it is thought to affect protein structure, amino acids and to react with nucleotides (Cooper, 2004). The bactericidal effect of iodine is dependent on the concentration of PVP-I, as this affects the free iodine available in the wound. The concentration of free iodine follows a bell shaped curve starting from a 10% solution and increases reaching a maximum value at 0.1% strength solution and then decreases with further dilution (Berkelman et al., 1982, Durani and Leaper, 2008). The development of resistance to iodine is rare, as this requires the alteration of cellular proteins (Khan and Naqvi, 2006, Durani and Leaper, 2008). Whilst PVP-I is sensitive to organic matter, it is still able to reduce bacterial numbers in a wound (Lawrence, 1998).

The use of silver in wounds has been well documented; specifically in burns (Drosou et al., 2003, Cooper, 2004). Silver sulfadiazine and silver nitrate were amongst the more common applications although, because of skin discolouration and the advent of dressings containing nanocrystalline silver, there has been a decline in their use (Leaper, 2006, Walker et al., 2006). Ionic silver binds to negatively charged proteins and nucleic acids, thus affecting the viability of the cell. The effects are dependent upon the physical structure of the silver nano-particles used, reflecting differences in cellular uptake (Ruparelia et al., 2008). Silver ions are thought to interact with thiol-groups, carboxylates, phosphates, hydroxyls and amines (Feng et al., 2000, Cooper, 2004, Woo et al., 2008). Although silver exerts a broad-spectrum

antimicrobial effect, resistance is sporadic and rare. Silver resistance is linked to mobile genetic elements such as plasmids, which also confer resistance to other heavy metals. Resistance to silver was first reported in *Salmonella* sp. in the mid 70s (Gupta et al., 1999). Interestingly, *silE* was the only gene of three possible silver resistance genes found in an MRSA study, although this did not confer *in vitro* resistance to silver (Loh et al., 2009).

### 1.17 Aims

It is well documented that MRSA and *S. aureus* are present in chronic wounds, however, their role and relationship within the non-healing of these wounds or most effective treatment has yet to be determined. MRSA is classically a MDR bacterium, thus, it is difficult to treat without the use of potent systemic antibiotics, which poses inherent risks to the patient. Chronic wound treatment practices are not consistent with many being ineffective. Biocides are frequently employed as a treatment modality, yet there is little evidence of their benefit in the treatment of chronic wound MRSA. The aims of this study are to further our understanding of the interaction between MRSA and the resident host cell populations in the pathogenesis of non-healing chronic skin wounds by the study of produced virulence factors. These studies also aim to further our understanding of the role of MRSA and *S. aureus* in the chronicity of non-healing skin wounds and to examine their interaction with host-mediated immunological responses and their resistance to treatment by the study of frequently prescribed antibiotics and biocides. To this end, this study is expected to answer; if MRSA is present in a wound, what is its role; is it associated with the non-healing phenotype and what is the best treatment choice?

The specific aims of this study are to:

1. Investigate bacterial resistance and genetic diversity of chronic wound MRSA isolates using cultural and molecular techniques.



2. Investigate the effectiveness (at a cellular and structural level) of conventional biocide treatments against chronic wound bacteria.
3. Characterise, at a molecular level, the expression of virulence factors by chronic wound MRSA isolates in comparison to MRSA isolates colonising healthy individuals.
4. Investigate the interaction between wound MRSA isolates and other frequently identified colonisers from chronic non-healing wounds and the host resident immunological cells by characterising the effect of bacteria and their metabolites on Toll-Like Receptors (TLRs) and keratinocyte function in a series of cellular and immunological assays.

**Chapter 2:**

**Characterisation of the antibiotic  
resistance profiles of chronic  
wound staphylococci and biocide  
efficacy on chronic wound MRSA**

## **2.1 Introduction**

### **2.1.1 Chronic wounds**

Chronic wounds are wounds which fail to heal within the normal time frame. They involve a complex relationship between bacteria, host immune response and underlying co-morbidities (Pukstad et al., 2010, Percival et al., 2011a). They encompass a spectrum of diseases which are typically classified as pressure, venous and diabetic ulcers (Fonder et al., 2008), and arise as a result of a breakdown in the normal healing processes (Bjarnsholt et al., 2008).

Chronic wounds are classically known to be polymicrobial in nature, with colonisation arising from external environments, or local reservoirs such as surrounding skin, the gastro-intestinal tract and oral mucosa (Bowler, 2003). The role of micro-organisms in non-healing wounds has been extensively studied. However, due to variation in study approach e.g. microbial collection, culture and identification technique and patient demographics, it is difficult to elicit the exact relationship that bacteria have within the wound healing process, and to identify a single causative organism (Davies et al., 2001, Howell-Jones et al., 2005, Melendez et al., 2010). This is most likely to be a multi-factorial process involving many organisms.

### **2.1.2 Chronic wound microbiology**

The role of microorganisms have been extensively investigated in many disease states, most notably in the case of oral disease e.g. periodontitis and peri-implantitis, and chronic lung disease e.g. cystic fibrosis (Listgarten and Lai, 1999, Davies and Bilton, 2009). There exists considerable evidence, which both directly and indirectly link microorganisms to the chronicity of disease processes. The presence of any organism in a wound may be due to contamination, colonisation or infection (Ueda et al., 2010). Bacterial colonisation alone is unlikely to mediate non-healing, and it is difficult to differentiate which bacteria have a detrimental effect on healing, particularly if synergistic effects are taken into account in the clinically non-

infected wound (Trengove et al., 1996, Davies et al., 2007). *Pseudomonas aeruginosa* has been used as a predictor of non-healing, because wounds harbouring this organism are frequently larger than wounds without (Kirketerp-Moller et al., 2008). The transition to infection, with bacterial invasion and invoked host response in patients with chronic skin wounds poses the risk of sepsis and subsequent bacteraemia (Ueda et al., 2010).

Molecular based techniques have changed our understanding of the ecology of microbes in wounds e.g. presence of anaerobes, biofilm formation and diversity (Davies et al., 2004, Dowd et al., 2008, Wolcott et al., 2009). Many investigators have tried to decipher the critical mass of bacteria in these wounds because studies have indicated that either the number of bacteria (Robson et al., 1999) or species present are important in non-healing (Davies et al., 2001, Davies et al., 2007; Chapter 1, Section 1.5). Not only has increased bacterial diversity and population complexity been characterised using molecular techniques, but studies also suggest that a single bacterial species is unlikely to be solely responsible for the non-healing phenotype. These facts suggest that, consortia of genotypically distinct bacteria symbiotically produce a pathogenic community in these wounds (Trengove et al., 1996, Davies et al., 2007, Dowd et al., 2008).

Within sites of persistent diseases bacterial populations are often highly organised and may exist as communities in biofilms (Martin et al., 2009) which may significantly contribute to the non-healing phenotype of chronic wounds. Recent work by Hill et al., (2010) identified *P. aeruginosa* and *S. aureus*, as common wound isolates. They have a tendency to co-aggregate, indicating their potential involvement in the initiation of biofilm formation *in vivo*. This biofilm state also makes it difficult to recover and culture bacteria (Kirketerp-Moller et al., 2008).

Wound cultures can be obtained in many ways e.g. superficial wound swabs, deep swabs, tissue biopsy, curetting and aspiration. Superficial swabs are most frequently employed as they pose no significant discomfort to patients, and are less technically demanding, but the results obtained are highly reliant upon the efficiency

of the individual carrying out the task (Verran et al., 2010a). In clinical practice, routine microbiological culture swabs typically identify easy to culture key organisms such as *S. aureus* and *P. aeruginosa*, with corresponding antimicrobial therapy often directed only at these organisms (Howell-Jones et al., 2006). However, cultural analysis of anaerobes from the wound environment, which are known to play a role in non-healing, is poor (Wall et al., 2002, Price, 2009). Studies by Stephens et al., (2003) have also shown that bacterial supernatants from anaerobic chronic wound organisms e.g. peptostreptococci sp. have an ability to impair keratinocyte cellular responses.

#### **2.1.2.1 Bacteria associated with chronic wounds**

Chronic wounds are colonized by a diverse array of organisms, however, appear clinically non-infected. Many studies have demonstrated that the organisms frequently isolated are; *S. aureus*, coagulase negative staphylococci (CNS; e.g. *S. epidermidis*), *P. aeruginosa*, *Proteus* sp., *Enterococcus* sp., *Streptococcus* sp., *Clostridium*, *Peptoniphilus*, *Bacteroides* sp. (Sapico et al., 1986, Gerding, 1995, Johnson et al., 1995, Hill et al., 2003, Wolcott et al., 2009).

#### **2.1.3 Antibiotic resistance**

Antibiotic resistance is a vital public health issue. Due to the distress that non-healing wounds pose to the patient, and probable lack of understanding of their aetiology, antibiotic prescriptions are a common feature of their treatment. Despite GP guidelines,  $\geq 65\%$  of patients suffering with chronic wounds receive at least one course of systemic antibiotics over the period of a year (Howell-Jones et al., 2005). Moreover, it is evident that flucloxacillin, co-amoxiclav and ciprofloxacin were significantly prescribed more frequently for chronic wound patients than age-matched non-wound patients (Howell-Jones et al., 2006). Furthermore, transfer of antibiotic resistance can result from cross-contamination of wounds by patients, inanimate objects, health care personnel, and also other risk factors including;

antibiotic use, prior hospitalisation or long term institutionalisation (Livermore, 2000a, Livermore, 2000b, Howell-Jones et al., 2005).

Many studies have identified antibiotic resistant organisms in chronic wounds e.g. meticillin resistant *Staphylococcus aureus* (MRSA) has been shown to be present in 30 to 50% of all leg ulcers (Colsky et al., 1998, Tentolouris et al., 1999). Unsurprisingly, ciprofloxacin resistant *P. aeruginosa* was found to be present in a third of leg ulcers, which is concerning because this is a common antibiotic used for treatment of these wounds (Colsky et al., 1998, Howell-Jones et al., 2005). Price et al., (2009) observed in a group of patients suffering with chronic wounds that antibiotic use altered the bacterial community structure by reducing streptococci present and increasing the presence of pseudomonads. Due to their polymicrobial nature, the chronic wound environment makes a perfect niche for the transfer of antibiotic resistance (Howell-Jones et al., 2005).

Recently using an *in vivo* wound biofilm model, Hill et al (2010) showed that mixed *Pseudomonas* and *Staphylococcus* biofilms were unaffected by two common chronic wound antibacterial agents, ciprofloxacin and flucloxacillin, even when used at concentrations equivalent to twice that of the therapeutic dose, and at 5 or 15 times the minimum inhibitory concentration (MIC) of planktonically grown *S. aureus* or *P. aeruginosa* respectively. Therefore, within chronic wounds, empirical antibiotic prescription, long-term antibiotic use and patient non-compliance, indicates that further antibiotic resistance is likely to arise in this vulnerable patient group (Armstrong et al., 1995b).

Increase in antibiotic resistance incidence has led to surveillance by the European Antimicrobial Resistance Surveillance Network (EARS-Net) and the need for more stringent antibiotic susceptibility testing and cross-infection procedures. Antibiotic susceptibility is tested using a variety of methods, such as the disc diffusion assay, broth dilution and agar dilution methods, or using automated systems e.g. BD Phoenix™ (Moran et al., 1988, Andrews, 2009).

### **2.1.3.1 Meticillin Resistant *Staphylococcus aureus* (MRSA)**

Meticillin resistant *Staphylococcus aureus* was first discovered in the 1960s (O'Neill et al., 2001), and has become a popular political topic, with many hospitals graded on the number of hospital acquired MRSA infection. The presence of a chronic wound is an associated risk factor for MRSA carriage (Stanaway et al., 2007, Reich-Schupke et al., 2010). Meticillin resistance is tested with the use of cefoxitin, oxacillin or meticillin and is associated with a chromosomal genetic determinant, *mecA* (Chapter 1, Section 1.7.2).

### **2.1.4 Biocides and wounds**

Whilst many topical agents are employed in chronic wounds to promote healing and decrease infection, the advent of antibiotic resistance e.g. MRSA and lack of new formulations has seen a resurgence of interest in the use of biocides (Cooper, 2004, Lipsky and Hoey, 2009). Whilst chronic wounds are clinically non-infected almost all harbour multi-resistant bacteria (Hill et al., 2010). These bacteria may impair wound healing either directly or indirectly (White et al., 2001, Drosou et al., 2003, White et al., 2006b).

In terms of their use in medical practice, biocides have been categorised as antiseptics and disinfectants. In the treatment of wound bacteria, biocides have a number of advantages; they target all bacteria including MRSA and are associated with a lower level of resistance development and hypersensitivity (McDonnell and Russell, 1999, Drosou et al., 2003, Niedner, 1997). Some authors suggest that biocides encourage cutaneous healing (White et al., 2001, Harding, 2008).

#### **2.1.4.1 Povidone-Iodine**

Iodine has been used in the prevention of infection and treatment of wounds for more than a century (Cooper, 2004). The use of iodine fell out of vogue following problems with molecular iodine, such as tissue staining and sensitisation (Berkelman et al., 1982, Durani and Leaper, 2008). Since 1949 iodine use has

increased following the development of iodophores (Cooper, 2004), such as polyvinylpyrrolidone (PVP) which is used as a carrier, forming povidone-iodine (PVP-I). PVP has an affinity for cell membranes (Durani and Leaper, 2008) and interestingly PVP-I has been shown to be highly effective against chronic wound biofilms (Hill et al., 2010). Whilst conflicting evidence regarding the efficacy of PVP-I exists, much of this is due to variation in concentration, exposure time, organic substance presence and diversity of test procedures e.g. animal models, minimum inhibitory concentration (MIC) and suspension tests. Furthermore, iodine preparation and test strains may significantly affect the outcome of iodine activity (Drosou et al., 2003, Cooper, 2004, Khan and Naqvi, 2006, Durani and Leaper, 2008). PVP-I has been demonstrated to be almost 100% effective at removing MRSA colony forming units (CFU) on artificially contaminated healthcare workers hands (Guilhermetti et al., 2001).

#### **2.1.4.2 Silver compounds**

Silver is extensively utilised in wound treatments in 3 forms; silver salts (silver nitrate), compounds (silver sulfadiazine) and nanocrystalline silver (silver dressings e.g. Acticoat<sup>TM</sup>; Fong and Wood, 2006, White and Cutting, 2006a). Silver exerts its antimicrobial activity by disrupting the function of bacterial cell membranes, decreasing bacterial metabolism and binding to DNA and RNA preventing division (Leaper, 2006). Silver is effective against a broad range of microbes (Feng et al., 2000, Woo et al., 2008) and some animal studies have suggested that silver compounds may promote wound healing (Geronemus et al., 1979, Lansdown et al., 1997). The antimicrobial effect of silver is dependant on the achieved bacterial intracellular concentrations (Cooper, 2004).

#### **2.1.4.3 Potassium permanganate**

Potassium permanganate use has declined with the advent of silver and iodine wound dressings, and due to its associated skin staining, but it is still



employed in the management of chronic wounds, dermatitis, psoriasis and eczema to reduce bacterial load (Breuer et al., 2002, Harding, 2008). Gauze dressings soaked in potassium permanganate have been shown to reduce the bacterial count of venous leg ulcers (VLU; Hansson and Faergemann, 1995b).

Potassium permanganate is a strong oxidative agent which is the basis of its antimicrobial activity (McDonnell and Russell, 1999). It is extensively employed in aquaculture (Darwish et al., 2008) and water purification; despite its effectiveness in other models it has received little attention in chronic wounds.

#### **2.1.5 Biocides and their cellular effects**

The mechanisms by which biocides exert their cidal effects is undetermined but imaging techniques such as transmission electron microscopy (TEM; Hobot et al., 2008) and scanning electron microscopy (SEM; Wale et al., 1989) have been employed extensively to visualise disruption of bacterial cell walls and cellular contents as an indication of cell damage. TEM has previously been utilised to study the effects of various wound dressings on bacteria. Such studies have indicated cellular disruption by silver ions on common wound isolates (Feng et al., 2000, Hobot et al., 2008).

#### **2.1.6 Biocide testing**

Numerous methods have been described to test the antimicrobial efficacy of biocides, including: disc diffusion, MIC, minimum bactericidal concentration (MBC), suspension and carrier tests. A lack of consistency in the reported literature reflects variation in test conditions e.g. temperature, organic matter, concentration, contact times and preparation (McDonnell and Russell, 1999, Koburger et al., 2010). European and British standards have established standardised methods for biocide testing. Simple conventional tests such as the MIC, MBC and disc diffusion assays assess the sensitivity of the microorganism. They do not however, test the cidal vs. static properties of a biocide. In this respect, the “carrier test” method distinguishes

the “cidal” vs. “static” properties of the biocide by incorporating a biocide neutralisation step (Lambert, 2001, Johnston et al., 2002). Quenching agents employed to neutralise the biocide prevent over-estimation of the activity of a biocide for any given exposure time (Johnston et al., 2002). *In vitro* and *in vivo* many biocides are inactivated by the presence of organic substances which are omnipresent in the wound environment. The British/European standards therefore also describe the use of “dirty” conditions, by the addition of bovine serum albumin (BSA) to the experiments.

Quantifying a bacterial response to biocide exposure may be performed using manual or automated methods. The “drop count” method first described by Miles and Misra (Miles et al., 1938) is less labour-intensive than “pour plate” methods (Herigstad et al., 2001). Automated systems, such as the Bioscreen Microbial Growth Analyser, measure the optical density (OD) of bacterial suspensions over a given time and CFU can be deduced (Lambert et al., 1998, Johnston et al., 2002).

### **2.1.7 Hydrophobicity and biocides**

Cell surface hydrophobicity is important in bacterial adherence, colonisation of surfaces and their consequent pathogenicity (Rosenberg et al., 1983, Das et al., 2001, Kustos et al., 2003, Koudhi et al., 2010). Surface hydrophobicity reflects the presence of protein or protein-associated molecules on the cell surface (Reifsteck et al., 1987). In the case of staphylococci, protein A is believed to be of particular importance to the hydrophobic nature of the cell (Miørner et al., 1982). A variety of cellular and electrophysiological methods exist to test the hydrophobicity of bacterial cells, e.g. salt aggregation test (SAT), microbial adhesion to hydrocarbons (MATH), zeta potential and polystyrene affinity measurement (Rosenberg, 1981, van Loosdrecht et al., 1987, Denyer et al., 1993, Geertsema-Doornbusch et al., 1993). Growth and environmental conditions may affect the analysis of hydrophobicity. Bacteria grown on blood-containing culture media may express greater hydrophobicity, whilst some tryptone soy broth (TSB) cultures may lower

hydrophobicity (Pembrey et al., 1999, Das et al., 2001, Das and Kapoor, 2004). Hydrophobicity can affect the action of biocide diffusion, which is dependent both on the hydrophobic nature of the bacteria tested and the antimicrobial used (Russell, 1995, McDonnell and Russell, 1999).

## **2.2 Aims**

The aims of this study were to determine the antibiotic susceptibility profiles for aerobic bacteria isolated from a group of chronic wound patients, specifically;

1. To phenotypically characterise isolated aerobic Gram-positive chronic wound bacteria by gram-stain, and identification of catalase and coagulase factor.
2. To determine the antibiotic susceptibility profiles of the staphylococcal isolates using disc diffusion assays and molecular techniques.
3. To determine meticillin resistance carriage amongst bacteria isolated from chronic wound patients.

Due to the identification of multiple multi-drug resistant bacteria present in the wounds of these patients, further investigation into the treatment modalities to eradicate these bacteria was thought to be of significant importance. In these studies, three common biocides were investigated to determine the most efficacious for the treatment of chronic wound MRSA (characterised in Chapter 3) using a combination of cellular and structural analyses. The specific aims were to:

4. Characterise the biocide effectiveness of povidone-iodine, potassium permanganate and silver nitrate against MRSA isolated from chronic wound patients using the carrier test method, enumerated by employing the drop count and Bioscreen analysis.
5. Determine if MRSA strain type or previous biocide treatment is associated with biocide resistance.
6. Investigate the structural effects of iodine exposure using SEM and TEM.

## 2.3 Materials and Methods

### 2.3.1 Collection of wound bacterial isolates

The isolates utilised in this study were collected as part of a routine audit by Dr R. S. Howell-Jones (2007) at the specialist Wound Healing Research Unit, Cardiff University with permission from the local clinical governance committee (Appendix I). The out-patients that participated in the audit had been referred for assessment, diagnosis and treatment of non-healing skin wounds. The audit was undertaken over a 10-week period in May - July 2005 and included all patients attending the clinics with a variety of non-healing wounds (leg ulcers, diabetic foot ulcers and surgical wounds). Data on antibiotic prescription from general practice and hospital notes, and patient's medical history and wound data were recorded contemporaneously.

Microbiological samples (n = 151 patients) were taken by nurses present at the specialist clinic by irrigating the test wound with saline prior to swabbing the entire wound with an Aimes and Charcoal cotton-tipped swab (Sterilin, UK). If more than one wound was present, the largest was sampled. Swabs were then transported to the Cardiff School of Dentistry Microbiological Laboratory and processed within 2 hours. Bacteria were identified following plating onto four different agars. Growth media used included; blood agar (BA; LabM 15, Bury, UK) supplemented with 5% defibrinated sheep blood (Oxoid) and selective media; mannitol-salt agar (MSA; Oxoid, Basingstoke, UK), *Pseudomonas* agar (PA; LabM) supplemented with 200 mg/l cefrimide and 15 mg/l of nalidixic acid; and bile aesculin agar (BAA, Oxoid) supplemented with 6 µg/l vancomycin to facilitate selection of *Staphylococcus*, *Pseudomonas*, and *Enterococcus* respectively. Following incubation (37°C, 48 h) *Pseudomonas aeruginosa*, *Staphylococcus* sp. and vancomycin resistant *enterococci* (VRE) were identified using Gram stain, catalase, oxidase, coagulase tests and *Streptococcus* antigen grouping (Howell-Jones, 2007). Isolates and swabs were stored at -80°C in microbank vials containing

cryopreservative fluid and porous beads (Pro-Lab diagnostics, UK) and were used in the following experiments.

### **2.3.2 Culture of bacterial isolates**

Samples previously identified as *S. aureus* and CNS (Section 2.3.1) were recovered from the microbank vials aseptically and incubated overnight on BA (LabM 15, Bury, UK) supplemented with 5% sheep defibrinated blood at 37°C. Frozen swabs recorded as “no growth” or “no growth of staphylococci” were also recovered from the microbank vials, plated on MSA and BA and incubated overnight (O/N) at 37°C. Plates which failed to produce any growth were re-incubated for a further 24 h at 37°C. Individual colonies from MSA were transferred to BA and incubated (37°C, O/N) for identification. Single colonies from each plate were then transferred to new individual microbank vials and stored at -80°C for future use.

### **2.3.3 Identification of isolates**

#### **2.3.3.1 Classification of cell wall using Gram stain**

The Gram stain is a commonly used technique to identify eubacteria according to cell wall type; Gram-positive bacteria stain purple due to crystal violet in the peptidoglycan layer, whilst Gram-negative bacteria are pink due to carbol-fuchsin counterstain.

A single colony of bacteria from the BA was smeared onto a glass slide in distilled sterile water, allowed to air-dry and then heat-fixed briefly. Slides were flooded with 1% (v/v) crystal violet for 2 min. Following brief washing with water, the slide was flooded with 3% (v/v) Lugol's iodine for 2 min. Slides were decolourised with 100% acetone for 2 s, rinsed with distilled water, flooded with 1% (v/v) carbol-fuchsin (counterstain) for 2 min, washed with distilled water, blotted and air-dried (all reagents were supplied by Pro lab diagnostics, Cheshire, United Kingdom). Samples were viewed using light microscopy (Carl Zeiss Ltd., UK)

using immersion oil at x40 and x100 magnification (Figure 2.1), cell colour, shape and morphology were recorded.

#### **2.3.3.2 Coagulase test to identify *Staphylococcus aureus***

An accepted identification characteristic of *S. aureus* is its ability to produce coagulase (clumping factor), differentiating *S. aureus* from CNS. The coagulase test was performed using a Staphylase test kit (Oxoid, Basingstoke, UK) according to the manufacturer's instructions. Briefly, test and control reagents were shaken vigorously before use; two-three identical colonies of each test bacteria were smeared into a drop of distilled water on each of the two test circles. The test suspensions were then mixed and both circles were observed for agglutination. A positive test result (positive *S. aureus* identification) was indicated by clumping of the test cells in the test reagent solution and no clumping in the control reagent (Figure 2.2).

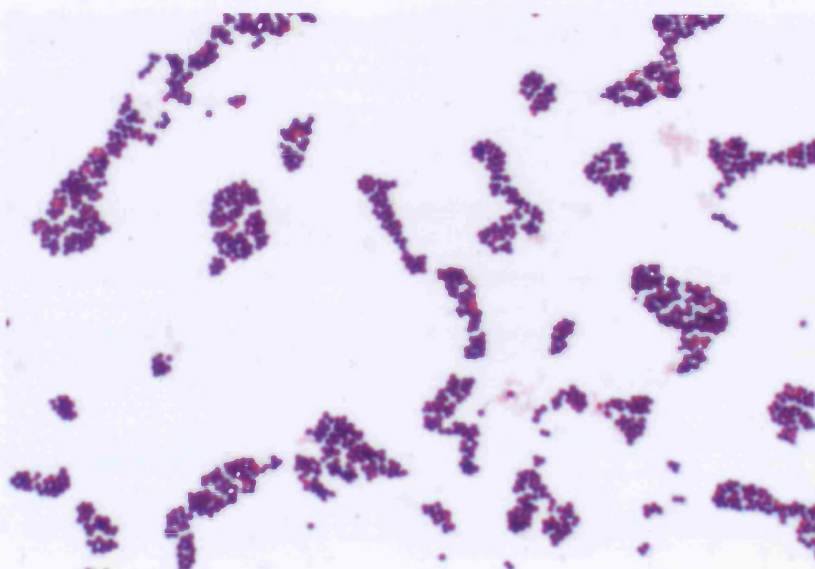
#### **2.3.3.3 Catalase test**

The catalase test employs hydrogen peroxide to identify the presence of catalase enzyme and enables differentiation of staphylococci from streptococci with the aid of the Gram stain. A single test colony was transferred to a glass slide and 1 drop of 3% (v/v) hydrogen peroxide (Oxoid) was added to the smear. The development of foaming was indicative of a positive catalase reaction.

### **2.3.4 Antibiotic susceptibility testing**

#### **2.3.4.1 Testing of meticillin resistance**

Initial identification of meticillin resistance was performed using a meticillin strip (Mast Diagnostics, Bootle, UK). A single colony from bacterial cultures grown O/N on BA was streaked onto iso-sensitest<sup>TM</sup> agar (ISA; Oxoid) in a horizontal line. A meticillin strip was aseptically placed perpendicular to the culture streak and the plate incubated (30°C, 24 h). Positive (MRSA; NCTC 12493) and negative



**Figure 2.1** Gram stain of *Staphylococcus aureus* isolate.



**Figure 2.2** Staphylase test to identify *Staphylococcus aureus*. Circles 1 and 4, *S. aureus* positive control (NCTC 6571); Circles 2 and 5, negative control (NCTC 11047); Circles 3 and 6, false positive using a wound isolate.

(meticillin sensitive *S. aureus*, MSSA; NCTC 6571) control strains were employed for each test plate (Figure 2.3).

#### **2.3.4.2 Susceptibility testing – disc diffusion assay**

The disc diffusion assay was performed as per British Society for Antimicrobial Chemotherapy guidelines (BSAC, 2007).

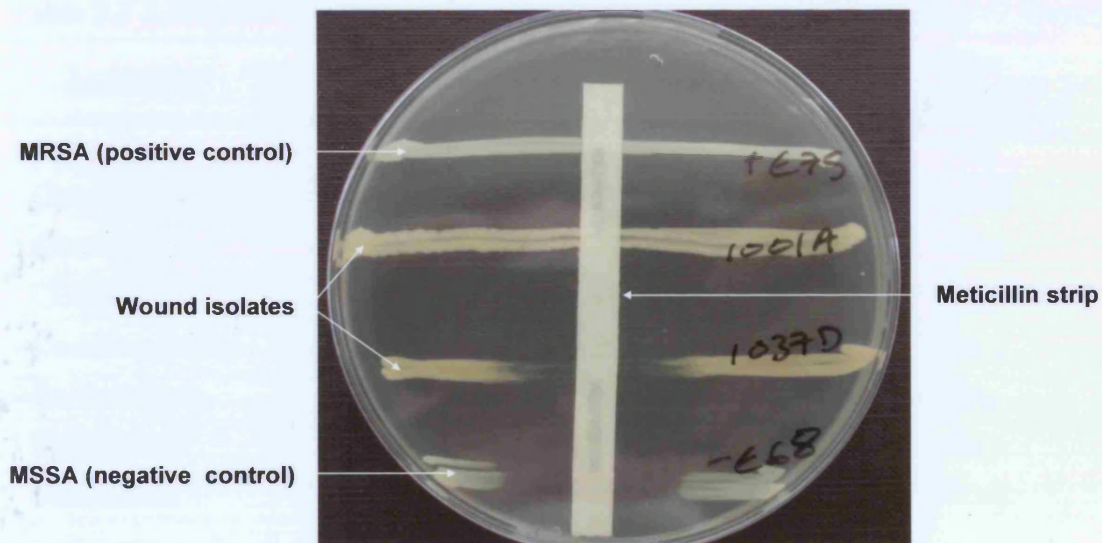
##### **2.3.4.2.1 Preparation of inoculum**

The inoculum was prepared by direct colony suspension method. Colonies from pure O/N (37°C) growth on BA were re-suspended in 5 ml of sterile distilled water to an optical density (OD) equivalent to that of 0.5 McFarland standard (BSAC, 2007). The suspensions were used to inoculate the test agar plate within 15 mins of preparation. Mueller-Hinton (MH) and Columbia agar (CA) supplemented with 2% (w/v) NaCl were used as test agar for oxacillin and meticillin, while isosensitest<sup>TM</sup> agar was used to test other antibiotics.

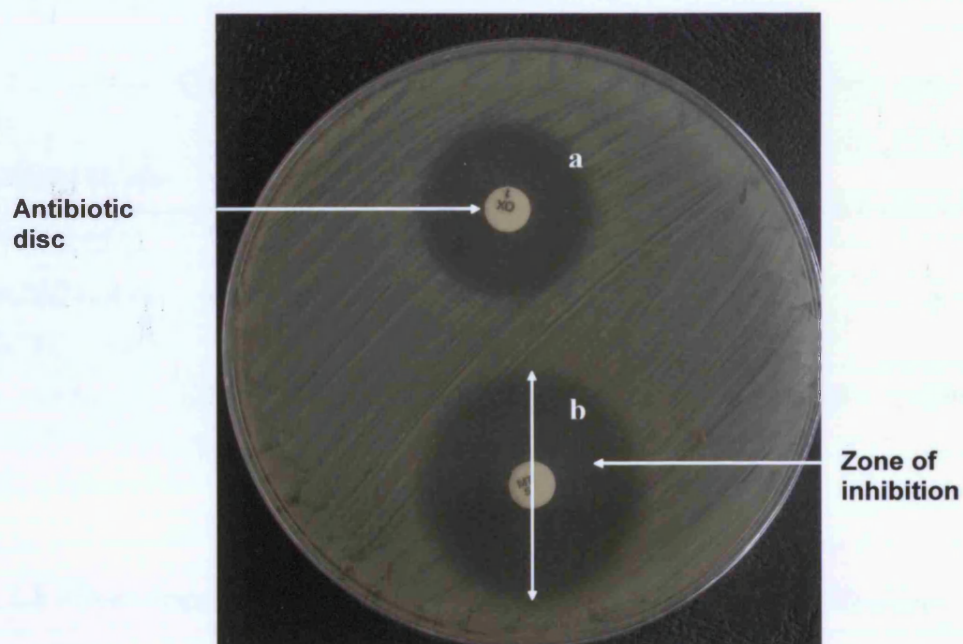
##### **2.3.4.2.2 Inoculation of agar plate**

Sterile cotton wool swabs were dipped into bacterial suspensions (Section 2.3.4.2.1) and excess liquid removed on the side of the container. The inoculum was then spread over the surface of the plate in three directions. Plates were allowed to dry at room temperature (RT). Antibiotic discs (Table 2.1; Mast Diagnostics, Bootle, UK) were aseptically placed onto the inoculated agar plate within 15 min of inoculation. A maximum of 6 discs were applied to each plate. The agar plates were stacked ( $\leq 3$  plates high) within 15 min of the application of discs, and incubated for 18 - 20 h at 35°C, (30°C; 24 h when testing oxacillin and meticillin). Reference strains used as controls are listed in Table 2.2.





**Figure 2.3** Meticillin resistance testing using a meticillin strip.



**Figure 2.4** Antibiotic susceptibility testing using a disc diffusion assay. Zones of inhibition for meticillin susceptible *S. aureus* (NCTC 6571); a) oxacillin disc (1 µg); b) meticillin disc (5 µg).

**Table 2.1** Antibiotic discs and concentrations used for susceptibility testing

Antibiotic	Disc content	Antibiotic	Disc content
Cefoxitin	10 µg	Oxacillin	1µg
Chloramphenicol	10µg	Penicillin	1 unit
Ciprofloxacin	1µg	Quinupristin/Dalfopristin	15µg
Clindamycin	2µg	Rifampicin	2µg
Co-trimoxazole	25µg	Tetracycline	10µg
Erythromycin	5µg	Trimethoprim	5µg
Fusidic acid	10µg	Vancomycin	5µg
Gentamicin	10µg	Sulfamethoxazole	25 µg
Meticillin	5µg	Fosphomycin glucose 6 phosphate	30 µg
Mupirocin	5µg		

**Table 2.2** National Collection of Type Cultures (NCTC) reference strains used as controls

Control strain	Isolate information
NCTC 6571	MSSA
NCTC 12493	MRSA
NCTC 11047	<i>Staphylococcus epidermidis</i> Tc <sup>R</sup>

MSSA, meticillin sensitive *Staphylococcus aureus*; MRSA, meticillin resistant *Staphylococcus aureus*; Tc<sup>R</sup>, tetracycline resistant.

**Table 2.3** Primer sequence for *mecA* gene PCR amplification and identification

Primer name	Sequence 5' to 3'	Amplicon size (bp)	Reference
MR1 (F)	GTGGAATTGGCCAATACAGG	1.339 kb	Tokue et al., (1992)
MR2 (R)	TGAGTTCTGCAGTACCGGAT		

bp, base pair.

#### **2.3.4.2.3 Zone of inhibition measurement**

Plates were examined under direct light to determine colony growth in the zone of inhibition and the zone measured (Figure 2.4). The measurement was then compared to zone breakpoints found in the BSAC guidelines for staphylococci (BSAC, 2007).

#### **2.3.4.3 Identification of meticillin resistance gene (*mecA*)**

##### **2.3.4.3.1 DNA preparation for *mecA* identification**

An O/N culture of test organisms was prepared in 5ml Tryptone soya broth (TSB; Oxoid) and incubated at 37°C. One ml of culture was centrifuged (9500 x g, 5 min) and the supernatant removed. The bacterial pellet was washed twice with 1 ml lysis buffer (50 mM Tris-HCl, pH 8.0; 5 mM EDTA, pH 8.0; 50 mM NaCl), and re-centrifuged (9500 x g, 5 min), then re-suspended in 1 ml lysis buffer with 20 µl lysostaphin (1 mg/ml; Sigma Aldrich, UK), and 5 µl RNase A (5 mg/ml; Sigma Aldrich, UK) added. The lysis mix was incubated (37°C, 30 min) with agitation. The lysate was centrifuged (9500 x g, 10 min) and the supernatant used as the DNA template for PCR amplification (Araj et al., 1999).

##### **2.3.4.3.2 *mecA* identification by PCR**

Bacterial DNA template was prepared as previously described (Section 2.3.4.3.1). Twenty-five µl PCR reactions were performed in thin-walled PCR tubes (all reagents were supplied by Promega, UK unless otherwise stated). A mixture of 1 µl of DNA template, 200 µM (each) deoxynucleoside triphosphates (dNTPs), 1 µM (each) of forward and reverse primers (Table 2.3; MWG-biotech, Ebersberg, Germany), 2.5 U *Taq* DNA polymerase, 2.5 µl of 10X buffer, 1.5mM MgCl<sub>2</sub> and 16.8 µl of nuclease-free water was amplified in a thermal cycler. PCR reactions were performed with an initial denaturation step (94°C, 10 s), followed by 25 cycles; denaturation (94°C, 30 s), annealing (55°C, 30 s), elongation (72°C, 2 min); then a

final extension step (72°C, 5 min). Ten µl of PCR product was combined with 2 µl of loading dye and loaded onto a 1% agarose gel (Fisher Scientific Ltd) with ethidium bromide incorporated (0.5 µg/ml). The product was separated by electrophoresis alongside a 100 bp molecular weight standard in 1X Tris-borate-EDTA (TBE; 0.1 M Tris Base; 0.09 M Boric Acid; 1 mM EDTA, pH 8.0) running buffer at 70 V for 1 h. The resultant gel was visualised under UV light (Gel Doc™, Bio-Rad®) and a 1.3 kb product was indicative of the *mecA* gene and therefore positive for meticillin resistance (Tokue et al., 1992).

### **2.3.5 Biocide testing**

#### **2.3.5.1 Growth of bacterial isolates**

Seven MRSA isolates from chronic wound patients were used, to represent appropriate examples of sequence types (ST), wound origin (chronic leg wound, chronic surgical wound) and previous biocide exposure. In addition, a control MRSA (ITU2) isolated from an intensive therapy unit patient (ITU) with bacteraemia kindly provided by Dr R. Howe (Specialist Antimicrobial Chemotherapy Unit; SACU, University Hospital Wales, Cardiff). A meticillin sensitive *Staphylococcus aureus* (MSSA) NCIMB 9518/ATCC 6538 described by British and European (BS EN) Standard 13697: 2001 was used as a control strain. Table 2.4 shows the isolates employed to test the biocides. In these experiments, strains were grown overnight (O/N) on tryptone soy agar (TSA) plates at 37°C. Biocide testing was performed using prepared 10 ml TSA slopes. A representative colony of each strain was used to inoculate a bacterial slope which was incubated O/N at 37°C.

**Table 2.4** Isolates employed in biocide testing

Isolate	Isolate information	<sup>a</sup> ST	<sup>b</sup> Pulse type
ATCC 6538	Control strain (MSSA)	ND	ND
1004	Chronic Leg Wound	22	A
1011	Chronic Surgical wound	30	Ci
1021	Chronic Leg Wound	36	Cii
1106	Chronic Surgical wound	22	B
1108	Chronic Surgical wound	970	A
2018	Chronic Surgical wound	22	A
2124	Chronic Surgical wound	22	A
ITU2	ITU isolate	1	ND

<sup>a</sup>Sequence type, defined by MLST; <sup>b</sup>pulse type, as defined by PFGE (Chapter 3);  
 ND, not determined; MSSA, methicillin *Staphylococcus aureus*.

**Table 2.5** Tested biocide concentrations and neutralising agent

Biocide	Working biocide concentration (w/v)	Neutralising agent
Povidone-iodine	1%	Lecithin (3 g/l)
		Polysorbate 80 (30 g/l)
Potassium permanganate	0.05%	Sodium thiosulphate (5 g/l)
Silver nitrate	0.45%	Sodium thiosulphate (5 g/l)

### 2.3.5.2 Experimental materials

The “carrier test” was performed using 1.5 mm thick, 20 mm diameter grade 2B finish stainless steel (SS) discs (Goodfellows Cambridge Ltd, Huntington, UK). Each disc was marked with a permanent marker to allow identification of the test side. Following use, the discs were autoclaved and rinsed with water then placed in 5% (v/v) Decon<sup>®</sup>90 (Decon Laboratories Ltd, Hove, UK) in de-ionized water for 60 mins, then rinsed with de-ionized water and allowed to dry. Discs were then sterilised (121°C, 15 min) in a sealed Duran<sup>®</sup> bottle until use. Sterile tryptone sodium chloride (TSC) solution (tryptone 1 g/l; NaCl 8.5 g/l) was used as a diluent for each test procedure and was freshly prepared every 3 d.

PVP-I, silver nitrate and potassium permanganate (Sigma-Aldrich Ltd, Dorset, UK) were tested for their efficacy against wound MRSA. Each test biocide and neutraliser (Table 2.5) was freshly prepared every 3 d and sterilised (121°C, 15 min), then stored at 4°C and kept in darkness until use. Bovine Serum Albumin (BSA) solution was freshly prepared every 3 d with 60 g/l BSA (Acros Organics, Geel, Belgium) in de-ionized water and kept at 4°C. The required volume was filter-sterilised using a 0.2 µm filter prior to use. A final BSA concentration of 3g/l was used in each test procedure as described in BS EN 13697; 2001.

Sterilised 100 ml Duran<sup>®</sup> bottles containing 5 g of 3 mm borosilicate glass beads (Sigma-Aldrich) were used to retrieve bacteria from the SS discs in each biocide test.

Terminology used in this Chapter to describe biocide test conditions are as follows: working bacterial suspension, obtained suspension following washing an inoculated TSA slope with TSC, and used to inoculate SS disc subsequent to the addition of BSA; test biocide, biocide under investigation i.e. PVP-I, silver nitrate, potassium permanganate; control biocide, sterile distilled water; test solution, neutralised test biocide; control solution, neutralised control biocide; test suspension, bacteria isolated from SS discs treated with test biocide and neutralised; control

suspension, bacteria isolated from SS discs treated with control biocide and neutralised.

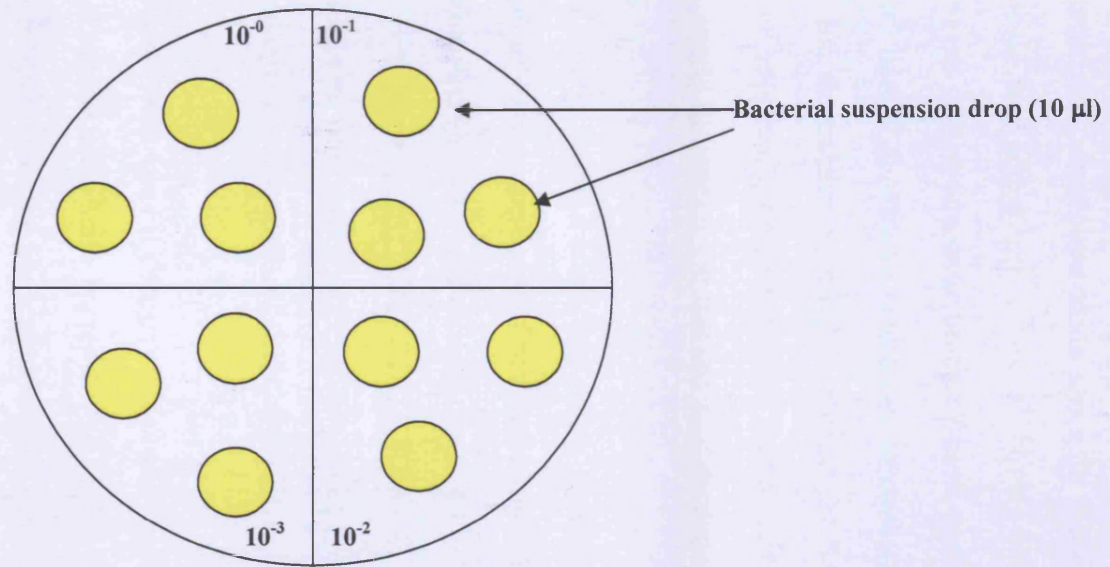
#### **2.3.5.3 Validation of drop count**

The drop count method was performed using an adaptation of the method described by Miles and Misra (1938). A working bacterial suspension of *S. aureus* ATCC 6538 was prepared by harvesting the bacterial growth on an O/N TSA slope with 5 ml of sterile TSC. The suspension was centrifuged ( $2000 \times g$ , 10 min) and the supernatant discarded. The bacterial pellet was washed with 5 ml of TSC (x2) and re-suspended in 5 ml TSC. One ml of the working bacterial suspension was diluted in 4 ml of TSC and cell suspension corrected to approximately  $10^9$  CFU/ml cells using an OD<sub>500nm</sub>. BSA (final concentration 3 g/l) was added to the test solution and 10-fold serial dilutions of the working bacterial suspension were performed (x10). Ten µl of each dilution were pipetted in triplicate onto triplicate agar plates (Figure 2.5), allowed to air-dry and then incubated (37°C, 24 h).

The dilution series yielding counts  $3 < n < 45$  CFU was used to determine the average number of colonies per plate dilution. Counts were expressed as CFU/ml (Herigstad et al., 2001). Intra-experimental variations were determined using a one-way analysis of variance (ANOVA) with a 95% confidence interval for log<sub>10</sub> transformed data in Minitab® (Release 14 software, Minitab® Inc, USA; Appendix III.i).

#### **2.3.5.4 Validation of neutraliser efficacy**

Validation of the neutralising agents was performed to confirm its ability to quench the biocides (Table 2.5) without detrimental effect on the test bacteria. Tests were performed using “dirty conditions” and the validation methods in BS EN 13697: 2001. Working bacterial suspensions of *S. aureus* ATCC 6538 were prepared (Section 2.3.5.3) the resultant bacterial pellet was re-suspended in 2 ml TSC and then 1.5 ml of the working bacterial suspension was combined with 0.5 ml



**Figure 2.5** Representation of the drop count method.



of BSA (final concentration 3 g/l). Six SS discs were transferred aseptically to a sterile Petri dish and inoculated with 20 µl of the bacterial/BSA suspension (unmarked side), these were incubated (37°C, 25 min) to dry. Ten ml of the neutralising agent was pipetted into each of 6 sterile Duran® (100 ml) bottles containing 5g of 3 mm glass beads. Once the inoculated disc had dried, 100 µl of the test biocide was added into 3 Duran® bottles containing neutraliser solution (test solution) and 100 µl of control biocide (sterile distilled water) was added to the other 3 Duran® bottles containing neutraliser solution (control solution). The biocide or control and neutraliser were left in contact for 5 min. Single inoculated SS discs were then aseptically transferred to a Duran® bottle containing the test solution or control solution, leaving the marked side facing up. These were then placed on a rotating platform (Grant Bio POS 300, Patterson Scientific, Luton UK) for 1 min at 150 rpm to re-suspend the bacterial cells. Viable counts were performed on 10-fold serial dilutions (in triplicate) from the initial working bacterial suspension, test suspension and control suspension as described in Section 2.3.5.3 and the drop count method performed (in triplicate). The CFU/ml for the working bacterial suspension, test suspension and control suspension were calculated. Validation of the neutraliser was based on the equations and rules of BS EN 13697: 2001, shown in Table 2.6.

#### **2.3.5.5 Bioscreen optical density values and viable count relationship**

To correlate viable bacterial counts for each serial dilution with observed optical density (OD), the Bioscreen Microbial Growth Analyser (Oy Growth Curves Ab Ltd, Finland) was employed. A working bacterial suspension of *S. aureus* ATCC 6538 was prepared and serially diluted ( $10^{-8}$ ) in triplicate, CFU/ml was recorded for each dilution (Section 2.3.5.3). Using a 100-well Thermo Labsystems Honeycomb 2 plate (Thermo Electron Corporation, UK), 350 µl of TSB and 50 µl of each bacterial serial dilution (neat to  $10^{-8}$ ) was added to 5 replicate wells. Control wells contained 350 µl TSB and 50 µl of TSC. Plates were incubated (37°C, 14 h) in the Bioscreen and growth recorded at OD<sub>420-580</sub> using a wideband filter, at 15 min intervals with

**Table 2.6** Equations used to validate neutralisers and biocides tested and the rules applied to each test (BS EN 13697: 2001)

Test	Equation		Rules
Recovery test	Recovery (%)	$\frac{\text{MVC control suspension (CFU/disc)}}{\text{MVC working suspension (CFU/20 } \mu\text{l)}} \times 100$	
Neutraliser toxicity	Log <sub>10</sub> of CFU working suspension (CFU/disc)	- Log <sub>10</sub> of CFU neutraliser solution (CFU/disc)	If <2, the neutraliser is not toxic
Neutraliser efficacy	Log <sub>10</sub> CFU of neutralised control solution (CFU/disc)	- Log <sub>10</sub> CFU of neutralised biocide solution (CFU/disc)	If <0.3, the neutraliser is effective
Biocide efficacy	Log <sub>10</sub> CFU of control suspension (CFU/disc)	- Log <sub>10</sub> CFU of test suspension (CFU/disc)	If > log <sub>10</sub> 3 reduction, the biocide is effective

MVC, mean viable count; CFU, colony forming units.

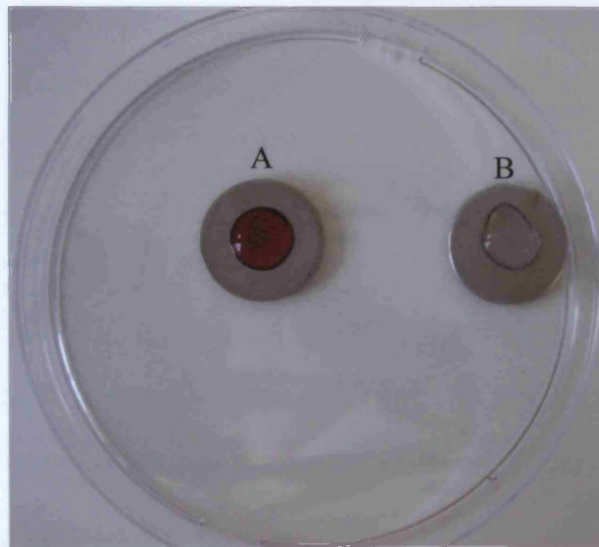
shaking (10 s) prior to each reading (ten replicates). The  $\log_{10}$  CFU/ml for each dilution from the plate count was plotted against the time taken to reach an  $OD_{420-580}$  of 0.2 as this showed the greatest distinction between the dilutions. A “fitted line” plot was produced using Minitab<sup>®</sup> and the resulting linear equation used to determine  $\log_{10}$  CFU/ml for subsequent analyses based on the time to reach an  $OD_{420-580}$  of 0.2 (Appendix III.ii).

#### **2.3.5.6 Biocide efficacy using the carrier test method**

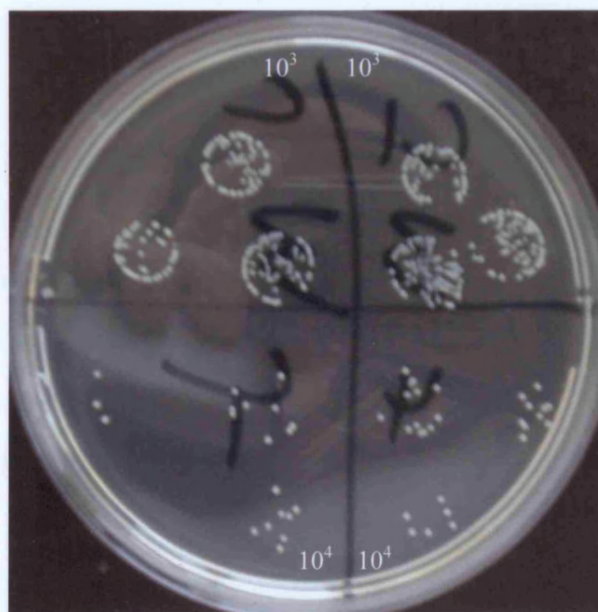
Biocides (Table 2.5) were tested using the carrier test method described in BS EN 13697: 2001 modified with  $\log_{10}$  reductions for efficacy based on BS EN 1499: 1997. Briefly bacteria were cultured and working bacterial suspensions supplemented with BSA (Sections 2.3.5.1 and 2.3.5.4). SS discs were inoculated as previously described (Section 2.3.5.4) and CFU/ml calculated for serial dilutions of the prepared working bacterial suspensions (Sections 2.3.5.3 and 2.3.5.4). Ten ml of the appropriate neutralising agent (Table 2.5) was transferred into Duran<sup>®</sup> bottles containing glass beads. Then 100  $\mu$ l of test biocide (Table 2.5) was added to the dried inoculated test SS disc, and 100  $\mu$ l of sterile water (control) was added to the control SS discs (Figure 2.6). The SS discs were left at room temperature for the test contact times (1, 15 and 30 min). Each SS disc were then aseptically transferred to an individual Duran<sup>®</sup> bottle containing the neutralising agent with test side downwards. The bottles were placed onto a rotator platform (150 rpm, 1 min) then the discs and solution left in contact for a further 4 min. The Duran<sup>®</sup> bottles were then vortexed for 30 s and samples taken for drop count serial dilution (100  $\mu$ l), and Bioscreen analysis (50  $\mu$ l). Each biocide and contact time was performed in triplicate using freshly prepared bacterial culture on 3 separate days.

#### **2.3.5.7 Drop count method**

Ten-fold serial dilutions (neat to  $10^8$ ) of the working bacterial suspension, test suspension and control suspension were performed in triplicate using 100  $\mu$ l of



**Figure 2.6** Stainless steel discs during carrier test experiment. A) biocide potassium permanganate and bacteria; B) sterile water and bacteria.



**Figure 2.7** TSA plate following drop count method and 24 h incubation.

the suspension and drop counts performed (Section 2.3.5.3; Figure 2.7). The CFU/ml was calculated for the working bacterial suspension, test suspension and control suspension. Recovery and biocidal efficacy was determined using the defined equations (Table 2.6). Plate readings of 0 CFU were set as 1 CFU/ml due to the limits of the assay (BS EN 1499: 1997).

#### **2.3.5.8 Bioscreen method**

A volume of 350  $\mu$ l tryptone soya broth (TSB; Oxoid) was aseptically added to each well of the honeycomb plate. To this, 50  $\mu$ l of serially diluted working bacterial suspension or test suspension or control suspension or neutraliser or a further 50  $\mu$ l of TSB was added in 5 replicate wells (Appendix III.iii). Plates were incubated in the Bioscreen (37°C, 14 h; Section 2.3.5.5) and mean CFU/ml calculated for the test suspension, control suspension and working bacterial suspension (serial dilution). Using the fitted line plot (Section 2.3.5.5) the  $\log_{10}$  CFU/ml values were calculated and  $\log_{10}$  reduction calculated (Table 2.6).

Statistical analysis was performed to determine differences in drop count and Bioscreen methods using the  $\log_{10}$  values for all investigated biocides and contact times using GraphPad InStat3<sup>®</sup> (GraphPad Software Inc, California, USA). A one-way ANOVA with a 95% level of significance between the means of the data sets was performed, with a Tukey post-test, to compare biocides. A paired T-test was used with confidence intervals of 95% to test if the difference in the mean of Bioscreen and drop count methods were significant.

#### **2.3.5.9 Hydrophobicity determination using salt aggregation testing (SAT)**

Salt aggregation testing for hydrophobicity (Lindahl et al., 1981) is based on the precipitation (“salting out”) of cells from a suspension. Bacteria were grown O/N on TSA slopes (Section 2.3.5.1) the slopes were washed with 0.002 M PBS (pH 6.8) and bacterial suspension centrifuged (2000  $\times$  g, 5 min). The supernatant was discarded and bacterial pellet washed ( $\times$ 3) in 5 ml PBS before the final bacterial

pellet was re-suspended in 2 ml PBS and the OD<sub>660nm</sub> adjusted to 1 to yield  $3 \times 10^9$  CFU/ml. Varying molarities of ammonium sulphate (0.2 to 4 M) were prepared in 0.002 M PBS (pH 6.8) and 25 µl of solution was pipetted into 24-well microtitre plate (Greiner Bio-One, UK). To each well, 10 µl of bacterial suspension was added and the plate was placed on a rotating platform (80 rpm, 2 min, RT). Visualisation of aggregation was facilitated by reading against a black background.

#### **2.3.5.10 Hydrophobicity determination using microbial adherence to hydrocarbons (MATH) testing**

Microbial adhesion to hydrocarbons (MATH) was employed to study the hydrophobicity of *S. aureus* (Rosenberg et al., 1980, Greene et al., 1992, Galliani et al., 1994, Das et al., 2001), to determine whether surface charge influences susceptibility to iodine, and to determine if PVP-I can change bacterial surface hydrophobicity. Bacterial slopes were prepared (Section 2.3.5.1) and incubated (37°C, 24 h). The bacterial slopes were washed with 5 ml of sterile PBS (Oxoid) and centrifuged (2000 x g, 10 min), washed (x2) with 5 ml of PBS and centrifuged (2000 x g, 10 min) then re-suspended in 5 ml of PBS. The OD<sub>540nm</sub> was corrected with PBS to an OD of 0.5 for testing with n-hexadecane (hexadecane adherence assay; HAA) and cyclohexane (cyclohexane adherence assay; CAA; Greene et al., 1992) and to an OD<sub>540nm</sub> of 1 for testing with p-xylene (xylene adherence assay; XAA; Galliani et al., 1994, Das et al., 2001). These tests employed disposable glass culture tubes 16 x 150 mm (Fisher Scientific UK Ltd, Loughborough, UK). Three ml of bacterial suspension was transferred to each tube, (2 sets of tubes were used for each hydrocarbon to compare iodine treated and control solutions for increasing quantities of hydrocarbon). To one set of tubes, 0.5 ml of PBS was added and to the second set of tubes 0.5 ml of a “non-lethal” concentration of stock 0.1% PVP-I was added. After 1 min, the OD<sub>540nm</sub> for the control (PBS/iodine) was recorded. To the remaining test tubes, the test hydrocarbon was added in increasing volumes; n-hexadecane or cyclohexane 0.4 to 1.6 ml (0.2 ml increments), p-xylene 250 µl to

1750 µl (250 µl increments). Tubes were sealed with parafilm® (Fisher Scientific) and vortexed (30 s) and left to phase separate for 30 min and 1 h. After separation, 1 ml of the aqueous phase was transferred to disposable cuvettes (Fisher Scientific). The OD<sub>540nm</sub> of each solution was recorded, corrected against PBS or PBS with 0.01% PVP-I controls. Tests were performed in triplicate. Readings were plotted on a line graph and relative hydrophobicity calculated using the following equation and rules (Greene et al., 1992). A paired T-test was performed using GraphPad InStat3® to determine if there was a significant change in hydrophobicity following PVP-I treatment.

$$\% \text{ adherence} = \frac{A_{540} (\text{initial OD}) - A_{540} (\text{final OD})}{A_{540} (\text{initial OD})} \times 100$$

Initial OD = A<sub>540</sub> of bacterial suspension without adding hexadecane

Final OD = A<sub>540</sub> of bacterial suspension on adding hexadecane

Rules for grading hydrophobicity: strong positive if >75%; intermediate if 25 to 75%; negative if <25%.

### **2.3.5.11 Effect of PVP-I on the cellular structure of MRSA using scanning electron microscopy (SEM)**

#### **2.3.5.11.1 Preparation of SEM sample**

Shaking overnight cultures of test MRSA were grown in TSB at 37°C for 24 h. Purity plates were performed on blood agar to confirm purity of sample. Cultures were diluted 1:500 and 2 ml was pipetted directly onto a Nucleopore 0.2 µm track-etched polycarbonate/ester membrane disc (Whatman® Ltd, GE Healthcare, UK) in 24-well tissue culture plate (Corning Life Sciences, Amsterdam). The tissue culture lid was sealed with parafilm® and the plates incubated (37°C, 24 h). Three wells were inoculated per test isolate.

These experiments were performed at RT. TSB was aspirated from each well and 200 µl PVP-I (0.1%; 1%), or sterile water (control) added to individual wells. One minute later, 1 ml of sodium thiosulphate was added to “quench” the biocide (determined as described in Section 2.3.5.4), and allowed to stand for 5 min. The quenched biocide was aspirated and 1 ml of 2.5% (v/v) glutaraldehyde added for 1 h. The glutaraldehyde was removed and each well washed (x3) with 1 ml of distilled sterile water. Membranes were covered with 1 ml of distilled sterile water and stored O/N at -80°C, and then placed O/N in a high vacuum freeze drier (Modulyo-Edwards, Edwards High Vacuum International, UK).

#### **2.3.5.11.2 Specimen Preparation for SEM**

Sample processing and imaging was performed with the help of Mrs W. Rowe, Cardiff University. Briefly, nucleopore membranes were mounted onto specimen stubs and placed into an Argon vacuum specimen chamber. Specimens were sputter-coated with a 7-10 nm layer of gold, using a K675 sputter coater (Quorum Emitech Ltd. UK). Samples were viewed using an EBT1 scanning electron microscope (SEM Tech Ltd, Derbyshire, UK). Representative images were recorded at 7 different sites in each sample.

#### **2.3.5.12 Effect of PVP-I on the internal structure of MRSA using transmission electron microscopy (TEM)**

##### **2.3.5.12.1 Broth culture preparation for 1% PVP-I testing**

Overnight cultures (MRSA isolate 1106) were prepared in 10 ml TSB, and used to prepare a 1:500 bacterial dilution in three 50 ml conical plastic flasks (Corning B.V. Life Sciences, Amsterdam) which were incubated in a shaking incubator (Innova 4330, New Brunswick Scientific, Cambridge, UK) at 37°C until mid-log phase. Purity plates were used to ensure no sample contamination. A final concentration of 1% PVP-I was added to two flasks and left at RT (1 and 30 min) before fixing. The third flask was used as a control. Then, 25% glutaraldehyde in



0.1 M Sorensen's phosphate buffer (0.1M  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ ; 0.3M  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , pH7.2; stock sol. 25% glutaraldehyde; TAAB, UK, No. G004) was added to each flask to give a final concentration of 2.5% v/v. The flasks were fixed O/N at RT.

#### **2.3.5.12.2 TSC bacterial culture preparation for 1% PVP-I testing**

To investigate the effect of PVP-I on MRSA under "contact test" conditions, bacterial cultures (MRSA isolate 1106) were prepared (Section 2.3.5.12.1). All bacterial cultures were centrifuged ( $13000 \times g$ , 10 min), supernatants discarded, the bacterial pellet washed in TSC (50 ml) and re-centrifuged ( $13000 \times g$ , 10 min). The resultant bacterial pellet was re-suspended in either TSC (50 ml) containing 3 g/l BSA ( $n = 3$  flask) or TSC (50 ml; control). PVP-I (1%) was added to 2 flasks containing the bacterial suspension in TSC and BSA and left at RT for 1 and 30 min before fixing (Section 2.3.5.12.1). The third flask containing the bacterial suspension in TSC with 3 g/l BSA was used as a second control.

#### **2.3.5.12.3 Bacterial harvesting and preparation of bacterial samples for TEM**

Samples were processed and imaged by Dr J. Hobot, Cardiff University. Bacteria were isolated from the fixation solution by filtration (0.45  $\mu\text{m}$  Whatman cellulose nitrate filters) and carefully removed from the surface of the filter using a spatula and re-suspended in molten ( $37^\circ\text{C}$ ) low melting point agarose 2% (w/v; Sigma-Aldrich) prepared in TSB containing 3 drops of blue beads (Immobilised Cibacron Blue F3Ga; Pierce, USA). The agarose was allowed to solidify, and then sectioned (approx.  $<1\text{mm}^3$ ) and placed in ethanol (50%) for processing. Samples were fully dehydrated (Table 2.7). Agar blocks were then placed into a 'size 0' vol 0.68 ml gelatine capsule to which  $0^\circ\text{C}$  LR White resin (Hard Grade; London Resin Co, London, UK), containing accelerator was added. This was allowed to polymerise at  $0^\circ\text{C}$  for 24 h, followed by 2 h at  $50^\circ\text{C}$ .

**Table 2.7** Dehydration steps for the preparation of agarose slices for TEM imaging

Dehydration step	Time required
50% ethanol	10 min
70% ethanol	10 min
90% ethanol	10 min
100% ethanol	60 min
1:1 LR White:100% ethanol	30 min
1:0 LR White	20 min
1:0 LR White	20 min
1:0 LR White	20 min
1:0 LR White	20 min

LR, London resin white acrylic resin

#### **2.3.5.12.4 TEM imaging**

The samples were cut into ultra thin sections using a LKB III Ultra-microtome. Sections were stained with aqueous uranyl acetate (4% w/v; 5 min), washed with water, and counterstained with Millonig's lead acetate for 30 s. Sections were viewed using a Philips/FEI CM12, (80 kV) and images recorded (SIS MegaView III digital camera).

### **2.4 Results**

#### **2.4.1 Demographic data**

The initial audit cohort of patients collected by Dr R. S. Howell-Jones (Howell-Jones, 2007) included 81 (54%) female and 69 (46%) male patients, and observed a wide age range (from 24 to 93 years). The number of wounds per patient ranged from 1 to 8 wounds, with a wound duration period between 3 months and 42 years. The aetiology of wounds represented a full spectrum, with the majority of patients (51%) having chronic venous leg ulcers. Chronic surgical wounds represented 28% of patients and foot ulcers represented 13.3% of patients, whilst 7.3% of patients were categorised as having miscellaneous chronic wounds e.g. perineal sinus, pressure ulcers.

#### **2.4.2 Identification of isolates**

Cultural analysis of swab samples in this thesis found that 75 (50%) patients were colonised with *S. aureus*. Of these 58 (77.3%) isolates were meticillin and oxacillin sensitive and 17 (22.6%) isolates were found to be MRSA. Three of the patients found to be colonised by MRSA were positive on two sampling occasions; two patients were colonised with MRSA on the second visit but not the first. Four patients identified as having a wound colonised with MRSA at the first visit were not found to be MRSA positive at the second sampling. MRSA was isolated in; 4 (20%)

patients with foot ulcers, 5 (6.5%) patients with leg ulcers, 1 (9%) miscellaneous wound and 7 (16.6%) surgical wounds.

CNS were harboured by 57 (38%) patients, of which 36 (63.2%) were sensitive to meticillin, and 17 (29.8%) were meticillin resistant. Interestingly, 4 (7%) patients were colonised with CNS that were meticillin sensitive but oxacillin resistant.

### **2.4.3 Antibiotic susceptibility testing**

#### **2.4.3.1 Disc diffusion assay**

The antibiotic susceptibilities of all *S. aureus* and CNS isolated from chronic wounds using the disc diffusion assay are shown in Table 2.8. All isolated MRSA were resistant to meticillin, oxacillin, cefoxitin, ciprofloxacin and penicillin. However, there was low resistance to tetracycline, chloramphenicol, and fosfomycin glucose-6-phosphate and no vancomycin resistant strains in this group of MRSA isolates. A number of the isolates however exhibited multi-resistance. Seven (41.2%) of the isolated MRSA indicated resistance to beta-lactams and a further 5 groups of antibiotics (Table 2.9). No resistance to meticillin, oxacillin, cefoxitin or vancomycin was identified amongst the MSSA isolates (Table 2.8). The majority of MSSA were resistant to penicillin, trimethoprim, co-trimoxazole, rifampicin and ciprofloxacin.

All isolated meticillin resistant CNS were resistant to meticillin, oxacillin, cefoxitin, ciprofloxacin and penicillin. There were no vancomycin resistant strains. In spite of this, a number of the isolates were multi-resistant, with 6 (35.3%) meticillin resistant CNS indicating resistance to beta-lactams and a further 5 groups of antibiotics (Table 2.10). Oxacillin resistant, meticillin sensitive, coagulase negative staphylococci were also resistant to penicillin. None showed resistance to meticillin, cefoxitin, tetracycline, chloramphenicol or vancomycin, although there was mixed antibiotic resistance to the other antibiotics tested. Of the isolated meticillin and oxacillin sensitive CNS, none were resistant to meticillin, oxacillin,

**Table 2.8** Antibiotic resistance of chronic wound staphylococci

Antibiotic	Number of bacterial isolates with resistance				
	MRSA (n=17)	MSSA (n=58)	CNS met <sup>R</sup> (n=17)	CNS oxa <sup>R</sup> (n=4)	CNS (n=36)
Meticillin	17 (100%)	0 (0%)	17 (100%)	0 (0%)	0 (0%)
Oxacillin	17 (100%)	0 (0%)	17 (100%)	4 (100%)	0 (0%)
Vancomycin	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Penicillin	17 (100%)	50 (86.2%)	17 (100%)	4 (100%)	31 (86.1%)
Clindamycin	14 (82.4%)	19 (32.8%)	10 (58.8%)	4 (100%)	16 (44.4%)
Gentamicin	7 (41.2%)	10 (17.2%)	9 (52.9%)	1 (25%)	4 (11.1%)
Fusidic acid	14 (82.4%)	36 (62%)	14 (82.4%)	4 (100%)	29 (80.6%)
Erythromycin	9 (53%)	4 (6.9%)	14 (82.4%)	4 (100%)	17 (47.2%)
Trimethoprim	15 (88.3%)	48 (82.8%)	16 (94.1%)	3 (75%)	30 (83.3%)
Co-trimoxazole	6 (35.3%)	45 (77.6%)	16 (94.1%)	3 (75%)	34 (94.4%)
Tetracycline	1 (5.9%)	4 (6.8%)	3 (17.6%)	0 (0%)	1 (2.8%)
Chloramphenicol	1 (5.9%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Fosphomycin glucose-6-phosphate	2 (11.8%)	1 (1.7%)	2 (11.8%)	2 (50%)	11 (30.6%)
Cefoxitin	17 (100%)	0 (0%)	17 (100%)	0 (0%)	0 (0%)
Rifampicin	13 (76.5%)	46 (79.3%)	8 (47%)	2 (50%)	10 (27.8%)
Ciprofloxacin	17 (100%)	41 (70.7%)	17 (100%)	4 (100%)	19 (52.8%)
Mupirocin	6 (35.3%)	4 (6.9%)	6 (35.3%)	2 (50%)	6 (16.7%)
Quinupristin/Dalfopristin	4 (23.5%)	1 (1.7%)	5 (29.4%)	0 (0%)	0 (0%)

MRSA, meticillin resistant *Staphylococcus aureus*; MSSA, meticillin sensitive *Staphylococcus aureus*; CNS met<sup>R</sup>, meticillin resistant coagulase negative staphylococci; CNS oxa<sup>R</sup>, oxacillin resistant coagulase negative staphylococci; CNS, coagulase negative staphylococci.

**Table 2.9** Antibiotic resistance profiles of wound MRSA isolates

Isolate	Antibiotic																	
	MET	OXA	PEN	CEF	TMP	SMX	GEN	ERY	VAN	TET	CIP	SYN	CLIN	FUS	CHL	MUP	RIF	FOS
1001	R	R	R	R	R	R	R				R		R	R		R	R	
1004	R	R	R	R	R			R			R		R	R		R	R	
1009	R	R	R	R	R	R		R			R		R			R	R	
1011	R	R	R	R	R	R	R				R		R	R				
1021	R	R	R	R	R	R	R	R			R		R	R				R
1044	R	R	R	R	R			R			R	R	R					
1047	R	R	R	R	R			R			R	R		R	R		R	R
1051	R	R	R	R	R	R	R				R	R	R	R		R	R	
1057	R	R	R	R	S		R			R	R		R	R			R	
1067	R	R	R	R	R	R		R			R		R	S				
1103	R	R	R	R	R						R		R	R			R	
1106	R	R	R	R							R			R			R	
1108	R	R	R	R	R						R			R			R	
1141	R	R	R	R	R			R			R	R	R	R		R	R	
1150	R	R	R	R	R		R	R			R		R	R		R	R	
2018	R	R	R	R	R		R	R			R		R	R			R	
2124	R	R	R	R	R						R		R	R			R	

MET, meticillin; OXA, oxacillin; PEN, penicillin; CEF, cefoxitin; TMP, trimethoprim; SMX, sulfamethoxazole; GEN, gentamicin; ERY, erythromycin; VAN, vancomycin; TET, tetracycline; CIP, ciprofloxacin; SYN, quinupristin/dalfopristin; CLI, clindamycin; FUS, fusidic acid; CHL, chloramphenicol; MUP, mupirocin; RIF, rifampicin; FOS, fosfomycin glucose-6-phosphate.

**Table 2.10** Antibiotic resistance profiles of wound meticillin resistant CNS isolates

Isolate	Antibiotic																	
	MET	OXA	PEN	CEF	TMP	SMX	GEN	ERY	VAN	TET	CIP	SYN	CLIN	FUS	CHL	MUP	RIF	FOS
1015	R	R	R	R	R	R		R			R			R		R		
1038	R	R	R	R	R	R	R	R			R		R	R			R	
1040	R	R	R	R	R	R	R	R		R	R			R		R		
1056	R	R	R	R	R	R	R				R		R	R		R	R	
1057	R	R	R	R	R		R				R		R	R			R	
1064	R	R	R	R		R	R	R			R		R	R			R	
1067	R	R	R	R	R	R		R			R		R	R			R	
1084	R	R	R	R	R	R		R			R			R				R
1088	R	R	R	R	R	R		R			R		R	R			R	
1090	R	R	R	R	R	R		R			R			R				
1096	R	R	R	R	R	R	R	R			R							
1112	R	R	R	R	R	R	R				R			R				
1142	R	R	R	R	R	R	R	R			R		R			R		
1143	R	R	R	R	R	R	R	R			R			R		R		R
1147	R	R	R	R	R	R		R			R		R	R		R		
2023	R	R	R	R	R	R		R		R	R		R	R			R	
2072	R	R	R	R	R	R		R		R	R		R					

MET, meticillin; OXA, oxacillin; PEN, penicillin; CEF, cefoxitin; TMP, trimethoprim; SMX, sulfamethoxazole; GEN, gentamicin; ERY, erythromycin; VAN, vancomycin; TET, tetracycline; CIP, ciprofloxacin; SYN, quinupristin/dalfopristin; CLI, clindamycin; FUS, fusidic acid; CHL, chloramphenicol; MUP, mupirocin; RIF, rifampicin; FOS, fosfomycin glucose-6-phosphate.

cefoxitin, chloramphenicol, or vancomycin. However, almost all were resistant to penicillin, and approximately 53% were resistant to ciprofloxacin.

#### **2.4.3.2 *mecA* gene identification by PCR**

PCR of the *mecA* gene was used to identify the presence of meticillin resistance in isolates that exhibited oxacillin, meticillin and cefoxitin resistance. All CNS and *S. aureus* sensitive to meticillin and oxacillin were also tested.

The *mecA* PCR demonstrated that all isolates deemed MRSA or meticillin resistant CNS from susceptibility testing were positive for the *mecA* gene. Of those isolates classified as meticillin sensitive (including oxacillin resistant CNS) none were positive for the *mecA* gene (Table 2.11). Figures 2.8 and 2.9 demonstrate agarose gel separation of the *mecA* PCR products for *S. aureus* and CNS wound isolates.

#### **2.4.4 Effectiveness of biocides using carrier test methodology**

##### **2.4.4.1 Validation of drop count method**

Validation of the drop count method (Appendix III.i) demonstrated there was no significant intra-researcher or method variance between the replicates ( $P > 0.05$ ).

##### **2.4.4.2 Validation of neutraliser efficacy**

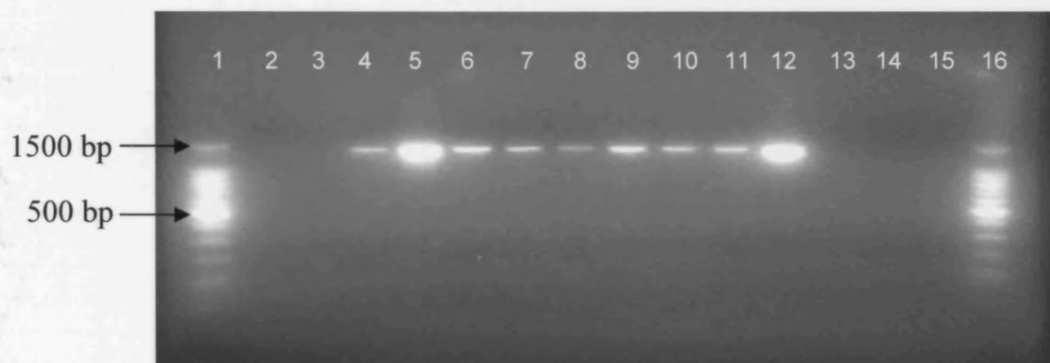
Table 2.12 shows the mean CFU/ml for the working bacterial suspension, test solution (test biocide), control solution (control biocide), plus efficacy of each neutralising agent and its toxicity values. All neutralising agents were found to have  $< 2 \log_{10}$  difference, indicating that none were toxic to *S. aureus*. All neutralisers were effective at quenching the known biocide, indicated by values  $\leq 0.3$  difference between control and test solution viable bacterial counts.



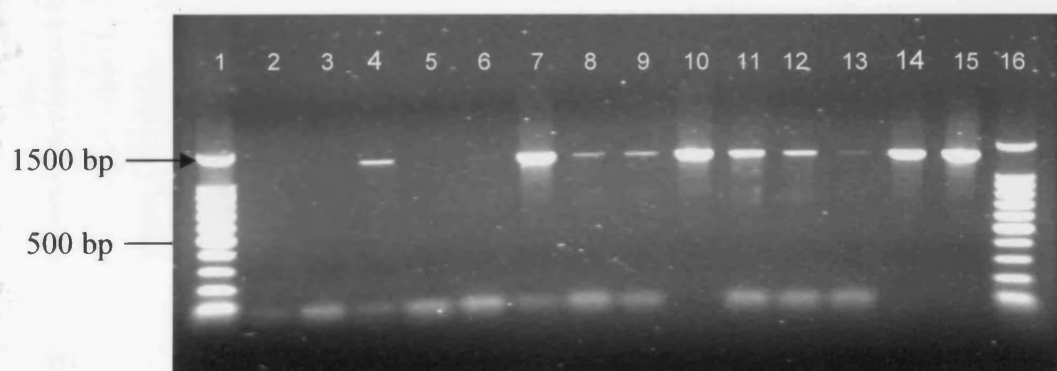
**Table 2.11** Number of isolates with *mecA* as identified by PCR

Isolate	Number of tested isolates	Number of isolates with <i>mecA</i> present
MRSA	17	17 (100%)
MSSA	58	0 (0%)
CNS met <sup>R</sup>	17	17 (100%)
CNS oxa <sup>R</sup>	4	0 (0%)
CNS	36	0 (0%)

MRSA, meticillin resistant *Staphylococcus aureus*; MSSA, meticillin sensitive *Staphylococcus aureus*; CNS met<sup>R</sup>, meticillin resistant coagulase negative staphylococcus; CNS oxa<sup>R</sup>, oxacillin resistant coagulase negative staphylococcus, meticillin sensitive; CNS, coagulase negative staphylococcus.



**Figure 2.8** Amplified *mecA* PCR product (1339 bp) for *Staphylococcus aureus* isolates. Lanes 1 and 16, 100 bp molecular weight standard (Promega); lane 2 and 3, negative controls, H<sub>2</sub>O and NCTC 6571; lane 4, positive MRSA control NCTC 12493; lanes 5 to 12, wound MRSA isolates; lanes 13 to 15, wound MSSA isolates.



**Figure 2.9** Amplified *mecA* PCR product (1339 bp) for coagulase negative staphylococci isolates. Lanes 1 and 16, 100 bp molecular weight standard (Promega); lane 2 and 3, negative controls, H<sub>2</sub>O and NCTC 11047; lane 4, positive MRSA control NCTC 12493; lane 5, wound meticillin sensitive CNS; lanes 6 to 15, wound meticillin resistant CNS.

**Table 2.12** Validation of the neutraliser for each test biocide in terms of toxicity and efficacy

<b>Biocide</b>	<b>Neutraliser</b>	<b>Mean CFU/ml working suspension</b>	<b>Mean CFU/ml biocide</b>	<b>Mean CFU/ml control</b>	<b>Mean neutraliser toxicity (log<sub>10</sub>)</b>	<b>Mean neutraliser efficacy (log<sub>10</sub>)</b>
Silver nitrate	Sodium thiosulphate	9.09	8.37	8.64	0.45 ± 0.09	0.25 ± 0.02
Povidone- iodine	Lecithin and polysorbate	9.05	8.68	8.82	0.23 ± 0.15	0.14 ± 0.11
Potassium permanganate	Sodium thiosulphate	8.3	7.37	7.38	0.95 ± 0.03	0.03 ± 0.02
Povidone- iodine	Sodium thiosulphate	9.70	8.65	8.67	1.03 ± 0.04	0.09 ± 0.02

#### **2.4.4.3 Biocide efficacy using the carrier test and drop count enumerating method**

The exposure times required to achieve a 3 log<sub>10</sub> bacterial reduction for each biocide and test bacteria are outlined in Table 2.13. No isolates achieved the required bacterial log reduction with any biocide using a 1 min exposure time.

Of the isolates exposed to PVP-I, 88.9% achieved the target bacterial growth reduction with a 30 min exposure, with 55.6% of isolates reaching the target log<sub>10</sub> reduction within 15 min of exposure. Like PVP-I, 88.9% of isolates achieved a 3 log<sub>10</sub> reduction with a 30 min contact time with potassium permanganate. In comparison, of the isolates exposed to silver nitrate only 55.6% of isolates reached the target bacterial reduction with a 30 min contact time.

#### **2.4.4.4 Biocide efficacy using the carrier test and Bioscreen enumerating method**

The required contact time to achieve a 3 log<sub>10</sub> reduction in bacterial growth following exposure to each biocide is shown in Table 2.13. Marked variation between biocide and strain was evident. The onset of a biocidal effect was evident with PVP-I

with one third demonstrating the 3 log<sub>10</sub> reduction in one minute. Furthermore, the majority of isolates (78%) achieved the target bacterial reductions in ≤15 min contact with PVP-I. Of the two isolates that did not achieved this, isolate 1108 fell just short (2.9 at 15 min). One strain (ATCC 6538) remained resistant to the action of PVP-I at 30 min.

Isolates exposed to silver nitrate and potassium permanganate failed to achieved the required 3 log<sub>10</sub> reduction with 1 min contact times using the Bioscreen method. For silver nitrate and potassium permanganate longer contact times (≥30 min) were necessary to achieve an effective biocidal effect in all strains save 1021 with silver nitrate. Wound MRSA isolate (1106) was tolerant to the biocidal action of silver nitrate over the duration of the experiment.

**Table 2.13** Biocide contact times (min) necessary to achieve a 3 log<sub>10</sub> (CFU/ml) reduction in bacterial growth using the drop count and Bioscreen method

Isolate	Drop count			Bioscreen		
	Povidone-iodine	Silver nitrate	Potassium permanganate	Povidone-iodine	Silver nitrate	Potassium permanganate
ATCC 6538	15	30	30	<sup>a</sup> 2.5 at 30	30	<sup>a</sup> 2.9 at 30
1004	15	N/A	N/A	1	30	30
1011	30	N/A	30	<sup>a</sup> 2.5 at 1	<sup>a</sup> 2.8 at 30	30
1021	15	30	30	15	15	30
1106	<sup>a</sup> 2.7 at 30	N/A	30	1	N/A	30
1108	30	N/A	30	<sup>a</sup> 2.9 at 15	30	30
2018	30	30	30	15	30	30
2124	15	30	30	<sup>a</sup> 2.8 at 1	30	30
ITU 2	15	30	30	1	30	30

N/A, not applicable, did not reach a 3 log<sub>10</sub> reduction; <sup>a</sup>n at x, n log<sub>10</sub> reduction with x min contact time.

#### **2.4.4.5 Comparison of contact times to achieve 3 log<sub>10</sub> bacterial growth reductions using the Bioscreen and Drop count methods**

Inter-experimental variation was clearly evident between the Bioscreen and drop count methods, although similar trends were observed. Contact times necessary to reach the required log<sub>10</sub> reduction, were generally higher for the drop count method than for the Bioscreen (Table 2.13). This is illustrated in the data for PVP-I where the contact times necessary for the required biocidal effect were less with the Bioscreen in 77.7% of isolates tested. The biocidal effect (log<sub>10</sub> reductions) generally increased with contact time for all biocides. However, the rapidity of this was greater with PVP-I. It was observed that the drop count method had increased log<sub>10</sub> reductions at contact times  $\geq 15$  min with PVP-I when compared with the Bioscreen method. Similar patterns were observed for isolates treated with potassium permanganate at contact times  $\geq 30$  min, and were observed in 40% of isolates treated with silver nitrate at contact times  $\geq 30$  min.

Statistical analysis of the log<sub>10</sub> results comparing the drop count and Bioscreen methods are shown in Table 2.14. The table demonstrates significant differences ( $P < 0.05$ ) in the results using the two enumerating methods obtained for PVP-I, silver nitrate, and potassium permanganate, for the 3 contact times. A large number of isolates treated with PVP-I showed a significant statistical difference ( $P < 0.05$ ) when comparing log<sub>10</sub> reductions using the Bioscreen and drop count method, with the drop count accounting for greater log<sub>10</sub> reductions ( $\geq 15$  min). In contrast, all of the obtained statistical differences ( $P < 0.05$ ) in log<sub>10</sub> reductions associated with enumerating methods for potassium permanganate and silver nitrate were associated with increased Bioscreen reductions.

**Table 2.14** Statistical differences ( $P<0.05$ ) in  $\log_{10}$  reduction for Bioscreen and Drop count method

Isolate	Silver nitrate			Povidone-Iodine			Potassium permanganate		
	1 min	15 min	30 min	1 min	15 min	30 min	1 min	15 min	30 min
ATCC 6538				B**	D**	D**			
1004				B**		D*		B**	
1011						D***		B*	
1021				B***	D*	D***			
1106									
1108		B*						B**	
2018						D*			
2124	B*		B**	B**	D*	D**		B*	
ITU 2	B*			B**		D**			

Significant difference between Bioscreen and drop count for each isolate at the contact times; B, greatest  $\log_{10}$  reduction observed with Bioscreen method; D, greatest  $\log_{10}$  reduction observed with Drop count method; \*, significant  $P\leq 0.05$ ; \*\*, very significant  $P\leq 0.01$ ; \*\*\*, highly significant  $P\leq 0.001$

#### **2.4.5 Hydrophobicity testing**

Hydrophobicity testing was performed on isolates exposed to PVP-I, because this was the most efficacious biocide. The aim was to determine if the biocide altered the hydrophobicity of the cells, indicating causality for the decreased magnitude of alterations in log<sub>10</sub> reductions with increased contact times.

##### **2.4.5.1 Hydrophobicity testing using Salt aggregation test**

This method did not show aggregation of any MRSA isolates in this study (Results not shown) at any of the concentrations previously described by Lindahl (1981).

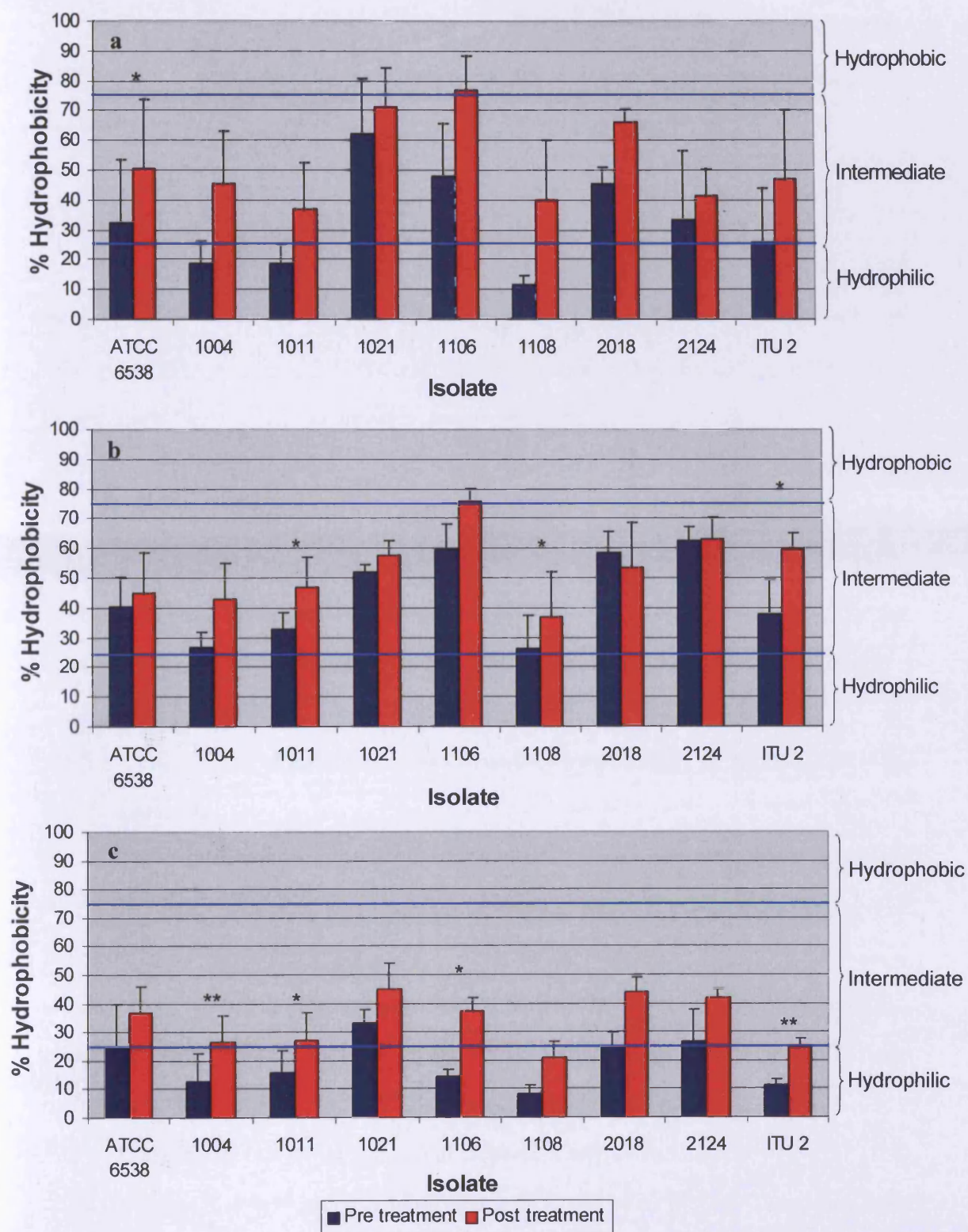
##### **2.4.5.2 Hydrophobicity testing using the n-hexadecane adherence assay (HAA)**

The changes in percentage (%) adherence of isolates following exposure to PVP-I using 0.8 ml n-hexadecane is shown in Figure 2.10a. In these studies there was a wide variation in strain hydrophobicity. Following PVP-I exposure, there was an increase in % adherence of the cells tested. Prior to treatment, 33.3% of the isolates were classified as hydrophilic and 66.6% as intermediately hydrophobic. However, following 1 min treatment with PVP-I, 88.8% were classified as intermediately hydrophobic and 11.1% as hydrophobic. A statistical significant difference ( $P < 0.05$ ) in hydrophobicity between pre- and post-treatment of *S. aureus* isolate ATCC 6538 was achieved.

##### **2.4.5.3 Hydrophobicity testing using the cyclohexane adherence assay (CAA)**

The changes in % adherence of isolates following exposure to PVP-I using 0.8 ml cyclohexane is shown in Figure 2.10b. Like the HAA assay, inter-strain hydrophobic variability was observed and following PVP-I treatment cell adherence increased. Prior to treatment with PVP-I, all of the isolates were classified intermediately hydrophobic. Following treatment, 88.9% were classified as intermediate and only 11.1% as hydrophobic. Statistical differences ( $P < 0.05$ ) in





**Figure 2.10** Changes in mean adherence/hydrophobicity of MRSA isolates following exposure to povidone-iodine. a) n-Hexadecane; b) Cyclohexane; c) p-Xylene.

pre- and post-treatment hydrophobicities for isolates 1011, 1108 and ITU2 were observed. An increase in hydrophobicity for isolates 1021, 1004, 1106 post-treatment was also observed but this was not statistically significant ( $P>0.05$ ).

#### **2.4.5.4 Hydrophobicity testing using the p-xylene adherence assay (XAA)**

Changes in % adherence of isolates following exposure to PVP-I using 0.25 ml p-xylene is shown in Figure 2.10c. Again from these data it was observed that following the exposure of PVP-I there was an increase in % adherence of cells. Prior to treatment 66.6% of the isolates were classified as hydrophilic and 33.3% as intermediately hydrophobic. Following treatment 77.8% were classified as intermediate hydrophobic and 22.2% as hydrophilic. A statistical difference was achieved for pre- and post-treatment hydrophobicity ( $P<0.05$ ) of isolates 1011, 1004, 1106 and ITU 2. Hydrophobic changes post PVP-I exposure were also observed for isolates 1021, 2018, 1108. However, this was not statistically significant ( $P>0.05$ ).

#### **2.4.5.5 Comparison of adherence assays and hydrophobicity of MRSA following iodine exposure**

Table 2.15 shows comparison of changes in hydrophobicity post-exposure to PVP-I for all isolates in all three adherence assays. Each isolate exhibited increased hydrophobicity following exposure to PVP-I, except for isolate 2018 using the CAA assay. Both HAA and XAA placed 44.4% of isolates into a different hydrophobic category post PVP-I exposure. Only 11.1% of isolates were placed into a different hydrophobic category following PVP-I exposure using the cyclohexane assay.

Contrasting results for HAA and XAA pre- and post-exposure to PVP-I were observed with 33.3% of isolates placed in a different hydrophobic category (Table 2.15). Similar hydrophobicity results were observed pre-treatment using HAA and CAA, 33.3% were categorised as different hydrophobic natures, yet post-exposure all isolates were similarly typed. Greater disparity of isolate hydrophobicity categorisation was observed using XAA and CAA pre-and post-exposure to PVP-I.

**Table 2.15** Comparison of hydrophobicity changes associated with the adherence assays post-exposure to povidone-iodine

Isolate	Adherence assay		
	n-Hexadecane	p-Xylene	Cyclohexane
ATCC 6538	↑ *	↑	↑
1004	↑	↑ **	↑
1011	↑	↑ *	↑ *
1021	↑	↑	↑
1106	↑	↑ *	↑
1108	↑	↑	↑ *
2018	↑	↑	↓
2124	↑	↑	↑
ITU 2	↑	↑ **	↑ *

↑, increase in % adherence; ↓, decrease in % adherence; \*statistical significant difference (P<0.05) pre- and post-exposure to PVP-I % adherence; \*\*statistical significant difference (P<0.01) pre- and post-exposure to PVP-I % adherence.

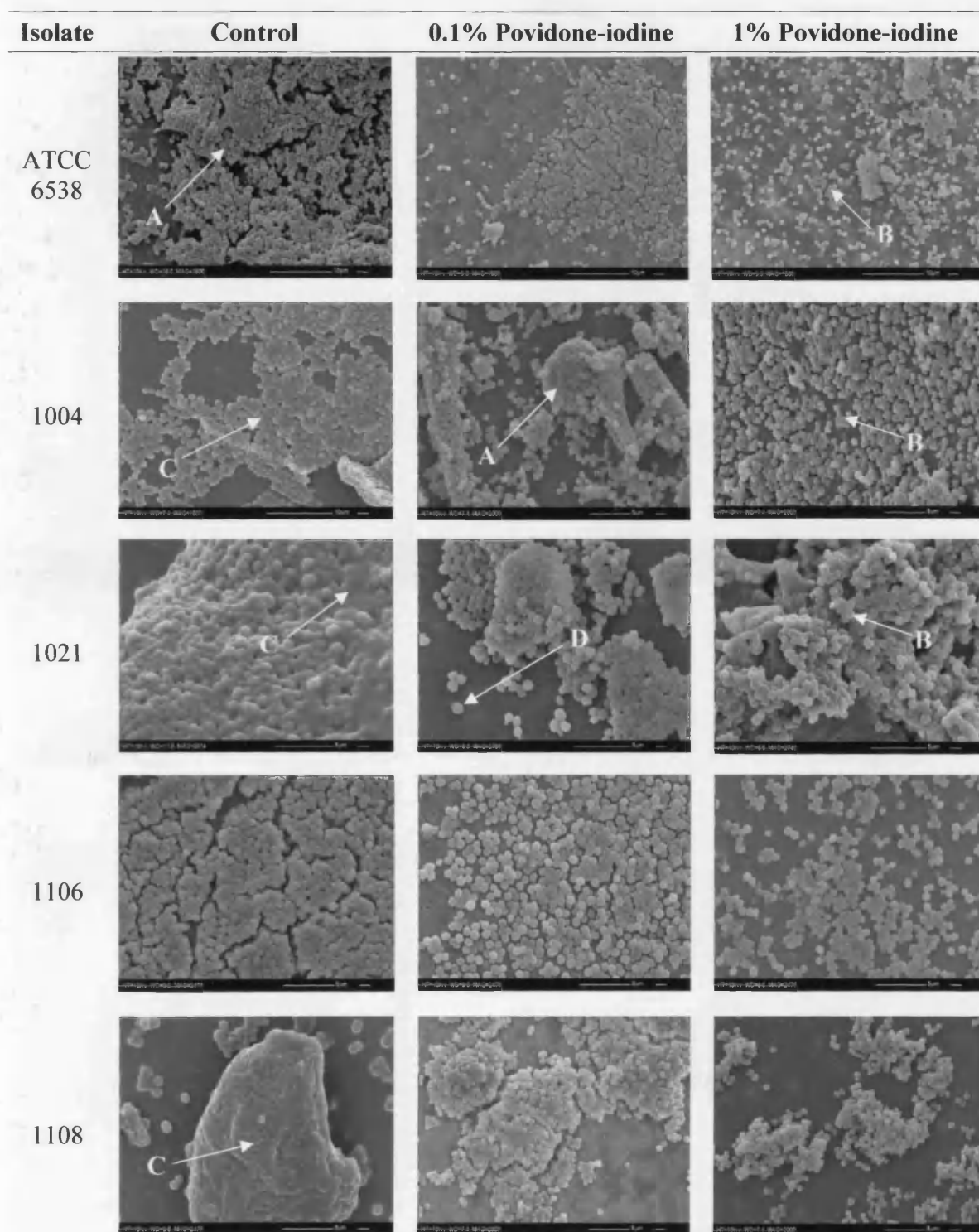
#### **2.4.6 Effect of PVP-I on the cellular structure of MRSA**

SEM images of the *S. aureus* isolates following 1 min treatment with 0.1 % and 1 % PVP-I, the most efficacious of the 3 biocides tested in this study, are shown in Figure 2.11. The control samples show multiple cell layers and varying levels of bacterial cell coverage with a strain dependant extracellular polysaccharide substance (EPS; glycocalyx). Dose-dependent effects were observed following application of PVP-I. In all samples, there was a decrease in matrix covering the bacteria using 0.1% PVP-I compared with the control, with the decrease more apparent with 1% PVP-I. No cellular conformational changes were observed following treatment with PVP-I.

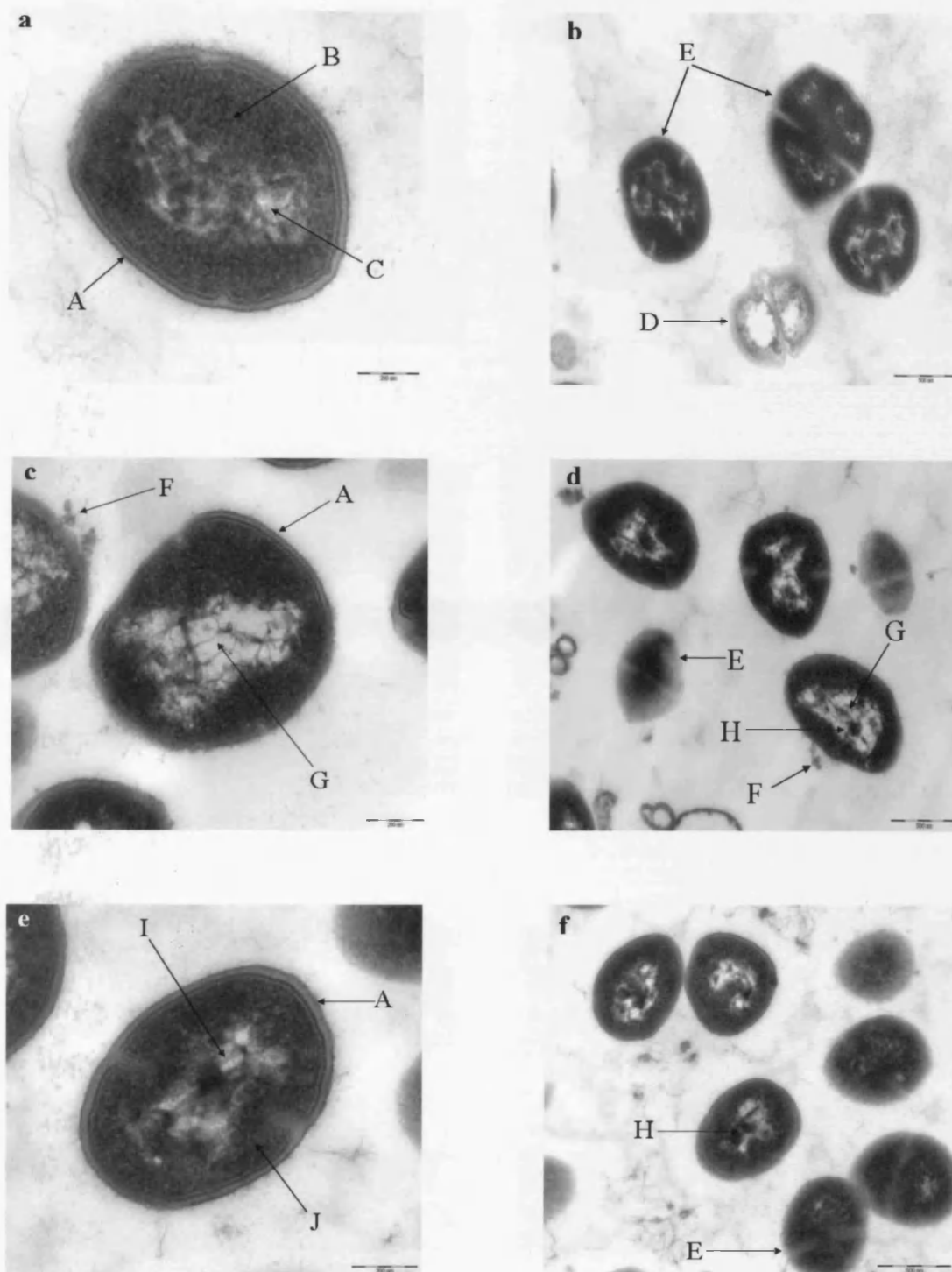
#### **2.4.7 Effect of 1% PVP-I on internal cellular structures of MRSA using transmission electron microscopy (TEM)**

The effects of PVP-I exposure on the internal structure of MRSA isolate 1106 is shown in Figures 2.12 and 2.13. This isolate was chosen as it was the wound isolate most tolerant to PVP-I and showed the greatest hydrophobic change. Bacterial cells in broth culture exhibited normal organisation of the nuclear material and cell wall (Figures 2.12a and b). Bacterial cells treated with PVP-I in broth showed an electron dense cell membrane, with movement of the cellular contents from the membrane and partitioning of the cytoplasm. Loss of cellular material was also observed in broth bacteria treated with PVP-I (Figures 2.12c and d), alongside time-dependent reconfiguration and rearrangement of the nucleoid material (Figures 2.12e and f).

As biocides may be affected and neutralised by proteins and organic matter, TEM was repeated in TSC and BSA to confirm if images from TSB broth with PVP-I could be replicated. Artifactual differences including “collapse” and DNA aggregation consistent with centrifugal forces, prior to re-suspension of bacteria were evident (Figures 2.13a and b).

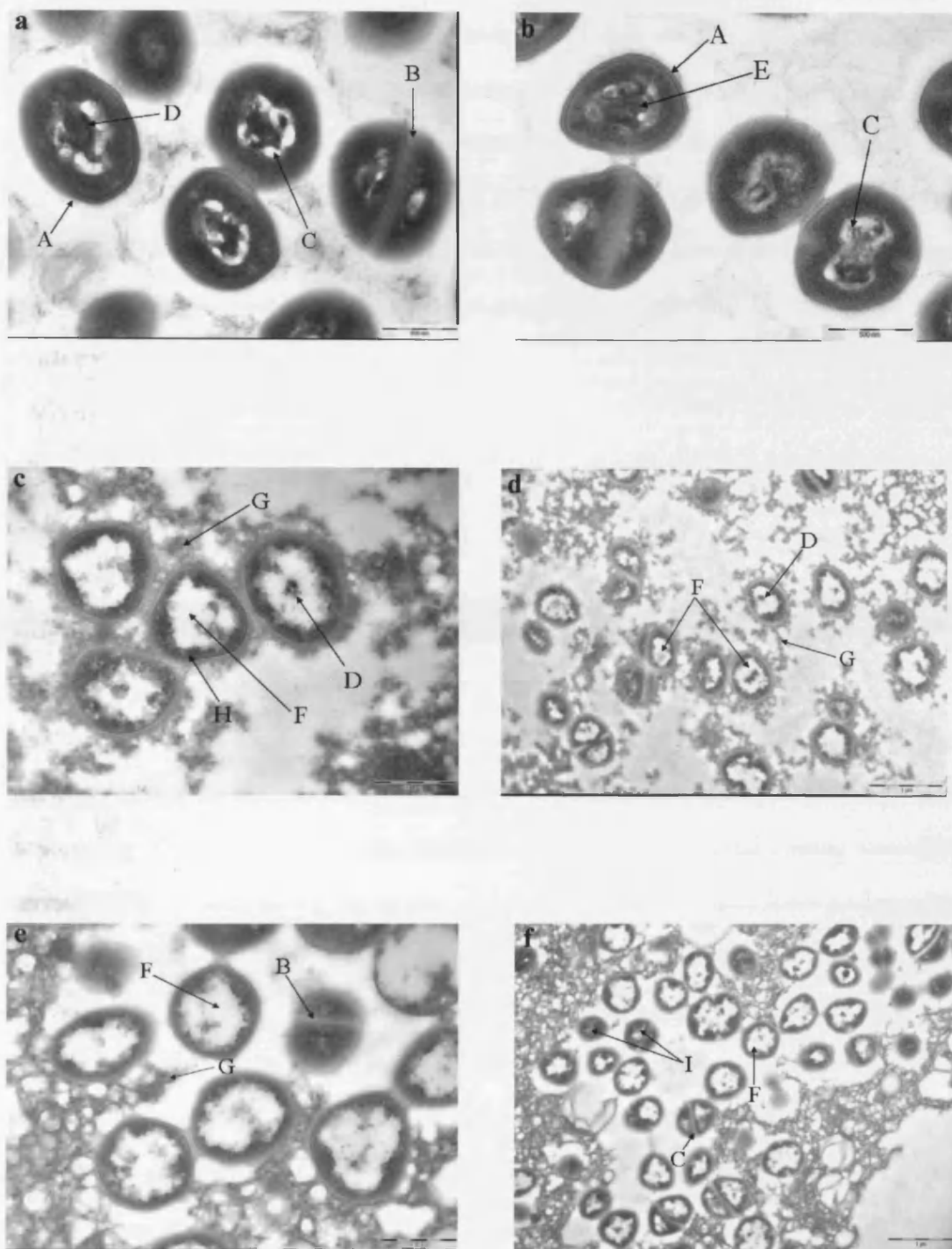


**Figure 2.11** Scanning electron micrographs of *Staphylococcus aureus* cells treated with povidone-iodine. A), multiple cell biofilm; B), loss of extracellular polysaccharide substance (glycocalyx); C), extracellular polysaccharide substance (glycocalyx); D), single *Staphylococcus aureus* cell.



**Figure 2.12** Transmission electron micrographs of MRSA isolate 1106 under TSB broth conditions. a, b) Control; c, d) 1 min PVP-I; e, f) 30 min PVP-I; a, c, e, x 100,000 magnification; b, d, f, x 40,000 magnification; A), cell wall; B), ribosome; C), nucleoid and nuclear contents; D), ghost cells; E), dividing bacterial cell; F), cellular leakage; G), nucleoid collapse; H), DNA aggregation; I), nucleoid recovery; J), cytoplasm.





**Figure 2.13** Transmission electron micrographs of MRSA isolate 1106 under tryptone sodium chloride (TSC) conditions. a) Control TSC; b) Control TSC & BSA; c) 1 min PVP-I, TSC & BSA; d) 1 min PVP-I, TSC & BSA; e) 30 min PVP-I, TSC & BSA; f) 30 min PVP-I, TSC & BSA. a, b, c, e), x 40,000 magnification; d, f), x 20,000 magnification; A), cell wall; B), dividing cell; C), nucleoid collapse associated with centrifugal forces; D), DNA aggregation; E), nucleoid; F), severe collapse of nucleoid; G), loss of cellular material; H), cytoplasm; I), nucleoid recovery.

In 1% PVP-I, bacterial cells showed collapse of the nucleoid structure, aggregation of DNA with a finger like projection arrangement, and significant loss of cellular material in bacteria suspended in TSC and BSA (Figures 2.13c-f). Recovery of the bacterial cells in TSC with BSA was not evident unlike that observed with TSB broth cultures and is likely to be associated with the initial severity of damage seen at one minute exposure, and may also be associated with initial centrifugal force alterations.

## **2.5 Discussion**

### **2.5.1 Antibiotic resistance**

A total of 150 patients were included in this study, (one was rejected on the basis of insufficient data). Of the 150 patients, 39 had repeat sampling over the 10 week period. Detailed microbiological investigation of these wounds demonstrated that *Staphylococcus aureus* was isolated from 50% of patients in this study, of which the majority 77.3% were meticillin sensitive, with 22.6% of isolates being meticillin resistant. This relates to 11.3% of the total cohort. Many studies have investigated the isolation of *S. aureus* in chronic wounds; for example Bowler and Davies (1999) employed samples from infected and non-infected wounds and found that 33% of wounds carried *S. aureus*. A prospective study by Davies et al. (2007) found a 34.8% surface carriage and 25.8% deep tissue carriage (via biopsy) of *S. aureus* in non-infected wounds. In contrast, this study shows greater carriage of *S. aureus* than both previous studies. This may indeed be the case in relation to Davies et al. (2004) because some of the patients identified in this study showed signs of infection.

Surveillance of pathogens has identified that 30-50% of the population show transient colonisation of *S. aureus* (Tenover and Gorwitz, 2006) and that certain groups show greater tendencies to colonisation. The nature of these wounds may explain the greater carriage of *S. aureus* by this particular patient group, as patients with skin lesions are known to be more susceptible to long-term carriage of MRSA



(Halcon and Milkus, 2004, Birnie et al., 2008, Haslund et al., 2009, Kim et al., 2009) and also improved isolation and culture techniques may have been employed.

The carriage rate of MRSA was relatively low (11.3%), and accounted for 22.6% of the total number of staphylococci isolated. This is half of the national average percentage of MRSA associated with bacteraemia and invasive disease, purported to be 40% prior to 2008 (Ayliffe et al., 1998, Tiemersma et al., 2004, Coia et al., 2006, Health Protection Agency., 2006b). There are obvious differences in the pathology and bacterial roles in chronic skin wounds compared to MRSA bacteraemia, which may partly explain the differences in isolation rates. However, one would expect carriage rates to be significantly greater than in the general population, especially when looking at risk factors for colonisation of MRSA; antibiotic usage, hospitalisation and diabetes which are prevalent in these patients. The results observed reflected recent (2009) MRSA resistance rates of 28%, (ECDC, 2005 - 2011). Possible reasons for lower observed rates of MRSA isolation in this study may be associated with; microbial sampling methods (and culture techniques), or a low number of MRSA colony forming units (CFU) present in the wound or possibly better infection-control measures used by the wound clinic.

CNS is found as a normal skin commensal, and the exact role of the organism in wounds has yet to be established; it may only be present as a coloniser. Importantly, however, CNS has been demonstrated to play a role in the transmission of resistance; MSSA acquisition of the *SCCmec* by horizontal transfer from methicillin resistant CNS has been demonstrated (Hanssen and Ericson Sollid, 2006). Significantly, CNS is also increasingly implicated in biofilm-related persistent infection and associated diseases e.g. catheter related disease (Marques de Mattos et al., 2003, Piette and Verschraegen, 2009, Hellmark et al., 2010). The pathogenicity of this particular microorganism may therefore have been previously underestimated, in the chronic wound biofilm.

A higher percentage of MRSA was isolated from patients with foot ulcers and surgical wounds (20% and 16.6% respectively) when compared to leg ulcers.

The increased prevalence of carriage in these wounds in comparison to leg ulcers (6.5%) and miscellaneous wounds (9%) may be associated with wound aetiology. Surgical wounds are associated with increased hospitalisation, whilst patients with foot ulcers have underlying wound aetiologies and a greater tendency to be diabetic, a further risk factor for colonisation by this pathogen (Tenover and Gorwitz, 2006, Stanaway et al., 2007, Schechter-Perkins et al., 2011).

Antibiotic susceptibility testing using the disc diffusion assay demonstrated multiple resistances among the MRSA isolates. All of the MRSA isolates were resistant to meticillin, oxacillin, penicillin and ceftazidime as would be expected due to the presence of PBP2a used in cell wall formation. In addition, unsurprisingly, all of the isolates were resistant to ciprofloxacin, a common antibiotic prescribed for the treatment of chronic wound *Pseudomonas* sp. infections and is frequently prescribed in these patients (Howell-Jones et al., 2006). Reassuringly, no vancomycin resistant isolates were identified. Only one isolate was resistant to tetracycline and chloramphenicol and may reflect the infrequent use of these antibiotics to treat staphylococcal and soft tissue chronic wound infections, and as a consequence a lack of selective pressure. This finding is further supported by Strommenger et al. (2003) who postulated that 'old' antibiotics should now be included in susceptibility testing due to decreased resistance frequencies. The observed number of MRSA (35%) isolates identified as resistant to mupirocin, (the current preferred mode of treatment for MRSA colonisation) was concerning and highlights the importance of susceptibility testing in chronic wound patients.

Antibiotic susceptibility testing amongst the MSSA isolates proved that none were resistant to meticillin, oxacillin or ceftazidime which is as would be expected as these organisms did not harbour the *mecA* gene. No isolates were resistant to chloramphenicol or vancomycin and only 4 of the isolates were resistant to tetracycline, erythromycin and mupirocin. The resistance to penicillin by *S. aureus* isolates in this study is similar to those previously reported (Ayliffe et al., 1998, Deurenberg et al., 2007), where approximately 80% of the MSSA were resistant to

penicillin, (associated with penicillinase production by the organism). Four CNS isolates showed resistance to oxacillin but not to meticillin or ceftiofur, even with prolonged incubation. Due to the ability of CNS to frequently indicate a decreased susceptibility to meticillin via disc and broth susceptibility testing methods, the identification of the *mecA* gene is imperative in this circumstance. Therefore, for correct classification, the determinant of genetic resistance mechanisms must be proved. In these four isolates, no genetic resistance (*mecA*) was observed and, therefore, the isolates were classified as hyper-producers of penicillinase (Brown, 2001, Buchan and Ledwith, 2010).

Identification of the *mecA* gene by PCR is a simple and specific method to identify meticillin resistance, and proved to be 100% effective in this study, with no false positives produced. Hence overall, *mecA* PCR identification of meticillin resistance is likely to be more accurate than the disc diffusion assay. Particularly as there is risk of significant inter-researcher variance, predominantly in the preparation of the inoculum (0.5 McFarland standard turbidity) and in reading the zones of inhibition with the disc diffusion assay.

This study has demonstrated that non-healing skin wounds harbour multiple staphylococci spp. many of which show multi-drug resistance (MDR) which may play a crucial role in their wound aetiology. These MDR organisms moreover, are able to transfer pathogenicity and resistance via horizontal gene transfer, and are of utmost importance in terms of cross infection policies and hazards to health. These patients represent important reservoirs of cross-infection due to hospitalisation, communal residences and interactions with healthcare professionals. It is therefore important to continue to screen patients with chronic wounds for the inevitable increase in antibiotic resistance that is expected based on current prescribing practices.

Further cultural analysis of wound patients should include the culture of normal skin and other areas of colonisation e.g. nose, to determine if chronic wound microbes are simply wound colonisers or more worryingly, the patient is a persistent

reservoir for infection. Determining a comprehensive bacterial molecular and resistance profile for chronic wound patients over time may provide insights into possible risk factors for the development of these debilitating wounds, and act as an aid to treatment and future prevention.

### **2.5.2 Biocide efficacy**

These antibiotic resistance studies observed a significant number of MDR bacteria, which are present in these chronic wounds. The use of systemic antibiotics should be limited to the systemically unwell patient, yet there is a need to control the bacterial burden in these wounds.

These studies investigated the antimicrobial efficacy of three clinically utilised biocides, based upon iodine, silver and potassium permanganate, used in the treatment of chronic wounds to decrease bacterial colonisation. To date, this is the first time the efficacies of these biocides have been investigated using the carrier test method against MRSA. The methods utilised, and the test conditions employed were based upon published standards, but varied in their ease of use and reliability. The carrier test was labour-intensive, requiring the use of large volumes of analysis compared with MIC and broth dilution methods. However, the test at least resembled the wound environment with direct surface contact and incorporation of the effect of organic matter in the test system, unlike disc diffusion and MIC assays.

This experiment negated the effects that broths may have (e.g. neutralisation by proteins) on the tested biocides. In nature, bacteria exist as biofilms and the carrier test method is more representative of this than traditional MIC tests (particularly broth dilution testing). It is important to note that the growth conditions of the bacteria during biocide testing may affect the results (Johnston et al., 2002, Hill et al., 2010). The carrier test method investigated the effect of a biocide on bacteria in multiple layers and examined the effectiveness of a biocide to kill bacteria at specific exposure times due to the use of neutraliser which can rapidly

and specifically quench biocidal activity (Lambert et al., 1998, Johnston et al., 2002, Johnston et al., 2003).

The drop count method was able to quantify the biocidal effects of each antimicrobial agent, obviating the need for multiple plate testing (as in the pour plate method) and hence was a more convenient means of enumerating bacterial growth. In contrast, Bioscreen analysis allowed higher sample throughput when compared with the drop count method. In these experiments, PVP-I was clearly the most effective biocide studied, adequately reducing bacterial growth within comparatively short contact times (<15 min). Potassium permanganate required increased exposure times to induce the required biocidal effect ( $\geq 30$  min). Silver nitrate was less effective than either PVP-I or potassium permanganate, only achieving the required 3 log<sub>10</sub> reduction in <50% of the isolates within a 30 min contact time. Despite these observations, and previous studies questioning usefulness of silver, there is increasing interest and widespread use of silver-containing medicaments. Previous studies have shown that extended contact times (>2 h) are necessary to demonstrate silver biocidal activity (Woo et al., 2008). The data in this study shows that silver products have a limited effect against MRSA in wounds, a finding suggested recently by other authors (Hill et al., 2010).

The techniques utilised demonstrated considerable variation in the time dependant log<sub>10</sub> reduction induced by the biocides. The variation observed here between the Bioscreen and drop count methods is well described (Lambert and van der Ouderaa, 1999, Cheeseman et al., 2009). The Bioscreen method is known to overestimate log<sub>10</sub> reduction with respect to the drop counts. In these experiments PVP-I achieved a 3 log<sub>10</sub> reduction with the Bioscreen within 1 min, whilst with the drop count method was more commonly 15 min. Inter-assay variation is likely to be related to the Bioscreen method only accounting for intact healthy cells and not damaged (but viable) cells. The repair time needed for such cells to start proliferating is known as the lag phase (Lambert et al., 1998, Lambert and van der Ouderaa, 1999) and describes the ability of bacteria to recover following sub-lethal injury

(Cheeseman et al., 2009). To overcome the discrepancies in quantification, selective counting of large colonies on the plate could have been used (Lambert and van der Ouderaa, 1999) assuming that all small colonies have arisen from injured bacterial cells taking longer to grow. The demonstration here that Bioscreen  $\log_{10}$  reductions were increased in comparison to the drop count reflects this sub-lethal damage to cells (Lambert et al., 1998). The exposure time for iodine to achieve the target  $\log_{10}$  reduction with the Bioscreen was far quicker than for the other biocides (89% in 15 min), with the Bioscreen and drop count methods demonstrating similar exposure times (>30 min) to achieve a 3  $\log_{10}$  reduction using potassium permanganate and silver nitrate.

Increased PVP-I contact times (>15 min) resulted in greater  $\log_{10}$  reductions for the drop count method over the Bioscreen that were statistically significant ( $P<0.05$ ), indicating that irreversible damage had been achieved. In contrast, for silver nitrate 5 isolates showed increased  $\log_{10}$  reductions with the Bioscreen compared to the drop count analysis, indicating non-lethal damage was occurring using this biocide at the 30 min contact time, from which the cells were able to recover.

From these analyses PVP-I appeared the most efficacious biocide. Clinically, iodine is usually employed at maximum concentrations of 10% (w/v; Durani and Leaper, 2008). This data demonstrates a rapid ( $\geq 1$  min) and pronounced biocidal activity of PVP-I using a 1% (w/v) solution. Interestingly, the dissociation of “free iodine” from PVP-I is known to increase with dilution, which shows a corresponding increase in bactericidal effect. It is conceivable from these data that a 1% solution may be as effective as the surgical scrub application (Berkelman et al., 1982, Brown et al., 1995, Durani and Leaper, 2008).

This study raises further concerns over the effectiveness of silver compounds for the treatment of MRSA. In these studies, bacteria inoculated onto a flat dry surface exhibited minimal bacterial reduction despite prolonged contact times with silver. It is therefore conceivable that this poor efficiency is repeated in the *in vivo*

situation when utilising silver-impregnated dressings. For effective activity against bacteria in the wound environment, the biocide must penetrate the tissues, and any associated bacterial biofilms, to exert its bacteriocidal effect; a far more complex interaction than that replicated in these studies which showed silver to have poor biocide efficacy. *In vivo* it has been suggested that  $\geq 20$  ppm of silver needs to be released into the wound bed for effective bacterial killing (Wright et al., 1998, Edwards-Jones, 2006). Evidence exists that nanocrystalline silver formulations, e.g. Acticoat<sup>®</sup>, are more effective due to an ability to increase the bioavailability of silver ions in solution (approximately  $\geq 3$  times the concentration of bound silver; Dunn and Edwards-Jones, 2004, Fong et al., 2005, Edwards-Jones, 2006, Newman et al., 2006, Woo et al., 2008). Silver containing dressings have also been developed to adsorb bacteria, facilitating increased contact times and the removal of wound exudates, which may neutralise the silver. In this respect, the use of nanocrystalline silver with increased contact times may be informative. Necessary contact times for silver ( $\geq 7$  h) have been attributed to the Gram-positive cell wall (Newman et al., 2006), whilst Hill et al., (2010) observed that silver dressings were not effective against 7 day old biofilms. It is conceivable that the slow activation of silver nitrate in this study is associated with the release of silver ions into solution or the formation of chemical complexes (reducing silver ion availability). This chemical complexing is clinically avoided by repeated application of the agent ( $\leq 12$  times a day; Dunn and Edwards-Jones, 2004).

Interestingly, whilst PVP-I showed large initial  $\log_{10}$  reductions (with short contact times) using the Bioscreen and drop count methods, longer contact times did not result in  $\log_{10}$  reductions of such magnitude, as previously described (McLure and Gordon, 1992, Koburger et al., 2010). It is likely that these represent binding of iodine with the organic load (Khan and Naqvi, 2006) which may be both concentration and temperature dependant (Traore et al., 1996). Increases in  $\log_{10}$  reductions using the drop count method with increased contact time (i.e. no marked increase with the Bioscreen method observed) may indicate that the severity of the

cellular damage induced by PVP-I was irreversible. The reduction in effect of prolonged activity was a marked feature of the chronic wound isolate 1106, and was thought to be associated with the isolates ability to recover following chemical insult; perhaps due to specific cell surface characteristics e.g. hydrophobicity. These findings indicate a need for regular dressing changes to ensure complete effectiveness of iodine as a wound antimicrobial. Recently, attempts have been made to produce “smart dressings” to aid clinicians in the identification of pathogens within wounds, to complement their clinical presentation (Jenkins and Young, 2010).

In this relatively small number of isolates, it was possible to examine previous patient biocide exposure and compare with the carrier test results (see Appendix III.iv). These experiments demonstrated that prior treatment with iodine and potassium permanganate did not increase the contact time necessary to achieve the required  $\log_{10}$  reduction. An interesting finding was that 2/3 of the patients in this study received  $\geq 2$  applications of silver treatment. Two MRSA strains previously exposed to silver did not achieve the required  $\log_{10}$  reduction; but neither did MRSA strains not previously treated with silver. The small number of patient isolates, and the limited data on previous treatment for the patients in this study make it difficult to definitively state whether biocide tolerance/resilience were associated with previous exposure. The small sample size of this study precludes deductions regarding the relationship between ST and biocide sensitivity, although previous studies have failed to reveal a relationship between phage variants and antibiograms with antiseptic susceptibility (McLure and Gordon, 1992).

There are inherent problems in the carrier test method, such as potential inter-researcher/inter-experiment differences characterised by the wide range of standard deviations obtained. This test does not adequately represent the dynamic wound environment, and in particular the representation of quenching effects of the skin and plasma proteins which are far more pronounced *in vivo* (Verran, 2002, Cheeseman et al., 2009). Like many disinfectant tests, the relevance of the laboratory to the clinical setting is also not clear (Fong et al., 2005, Verran, 2010b), making



interpretation of the results more difficult and indicating that further clinical studies are required to fully understand the fundamentals of biocides currently in medical use.

Microbial adhesion to hydrocarbon (MATH) studies showed that exposure to PVP-I induced changes in percentage adherence or hydrophobicity of MRSA. It is interesting that isolate 1106 (showing greatest tolerance to PVP-I) became hydrophobic whilst all other isolates remained intermediately hydrophobic. These changes in hydrophobicity could be a way for bacteria to mitigate biocide attacks. Hydrophobicity of the isolate potentially prevented penetration of iodine (a hydrophile) into the cell. It is known that hydrophobicity of bacteria may modulate biocide tolerance (Maillard, 2007) as has been described in *Pseudomonas aeruginosa* (Russell, 1995).

MATH hydrophobicity testing was performed using 3 hydrocarbons at different volumes, decrease in absorbance of the aqueous phase of the bacterial suspension being used to measure hydrophobicity (Rosenberg, 2006). Hence, adhesion to the liquid hydrocarbon is associated with the hydrophobic nature of the cellular surface (Rosenberg et al., 1980, Rosenberg et al., 1983). Adherence increased with increasing test volumes (Rosenberg, 2006) which is likely associated with the greater number of hydrocarbon droplets to adhere to (van Loosdrecht et al., 1987). Greene et al., (1992) observed that the volume of hydrocarbon may be important in hydrophobicity testing. In this study, the ideal hydrocarbon test volume was 0.8 ml (n-hexadecane, cyclohexane) and 0.25 ml (p-xylene), both previously identified (Greene et al., 1992, Galliani et al., 1994). Hence, the inter-strain and hydrocarbon variation may reflect the ability of the organism to bind to the phase-separating medium (Das et al., 2001) which can be affected by both the growth medium and centrifugation (Pembrey et al., 1999, Das and Kapoor, 2004). The bacteria in this study were grown on TSA to maintain consistency between experiments, and strains were generally classified as intermediately hydrophobic,

unlike a study by Kouidhi et al., (2010), which classified most *S. aureus* strains (grown on blood agar containing 4% NaCl) as hydrophilic using n-hexadecane.

n-hexadecane (aliphatic) and cyclohexane (cyclo-aliphatic) treatment produced similar hydrophobic profiles, whereas p-xylene treatment resulted in decreased adherence of cells, which may be a feature of the ability of aromatic hydrocarbons like p-xylene to cause bacterial lysis (Pembrey et al., 1999). n-hexadecane testing was biased towards greater hydrophobicity; this may be associated with its chemical structure and lower water solubility.

Structural studies were employed to investigate the effect of PVP-I (the most active biocide) on the cell surface of the MRSA isolates. SEM analysis demonstrated the effective action of PVP-I on the presence of EPS. In these studies, PVP-I ( $\leq 1\%$  w/v) exhibited a concentration dependant loss of EPS. The loss of EPS and disruption of bacterial adhesion reflected the increased killing observed in the carrier test (drop count) assay with increased contact times; bacterial exposure to biocide surface area increasing with time. Studies have demonstrated that effective concentrations of biocides vary in 3-dimensional structures, allowing cell survival in the centre of a biofilm (Brown et al., 1995, Oie et al., 1996, Buckingham-Meyer et al., 2007, Maillard, 2007). These structural effects in the SEM studies were observed within a 1 min contact time, and it is anticipated that increased contact would result in more antimicrobial effects.

The intracellular effects of exposure to the effective biocide (1%) PVP-I was studied using two contact times employing TEM. Wound isolate 1106 was used as this was the most recalcitrant of the isolates. Bacteria treated with PVP-I appeared to exhibit a difference to the control (broth and TSC cultures). PVP-I treated cells exhibited coagulation of nuclear material and partitioning of the cytoplasm, a feature seen by Schreier et al., (1997) reflecting the likely denaturation of cellular proteins (Cooper, 2007). Evidence of cytoplasmic recovery (reorganisation) was observed for experiments conducted in the nutrient rich broth  $\geq 30$  min PVP-I exposure. This finding has been attributed to the organic load (proteins and extracts) of the broth

effectively neutralising the iodine (Koburger et al., 2010) and to the cells innate capacity to recover following sub-lethal stresses. This corresponds to the limited decrease in CFU/ml with greater contact times that were observed during the carrier tests. These findings may potentially reflect either efflux-pump activity or hydrophobic changes, decreasing the amount of intracellular iodine. This notion is supported by studies demonstrating no biocidal activity with increased contact times (McLure and Gordon, 1992, Koburger et al., 2010).

Interestingly, bacteria grown in broth and then re-suspended in TSC/BSA displayed specific artifact (centrifugal-related) changes in the nucleoid region, prior to biocide treatment. It is important to note that the damage observed is not simply a result of exposure to PVP-I in this experiment. Following biocide treatment however, significant changes were evident; gross cell lysis; loss of cellular content as a result of pore formation, leading to empty cells and accumulation of extracellular debris were evident from even 1 min PVP-I exposure. Few cells were able to recover from this in the 30 min period of the experiment.

Topical antiseptics still have a significant role in the control of wound bacteria. In these experiments using chronic wound MRSA, iodine proved to be an effective antimicrobial, whilst silver and potassium permanganate were not. As only a single concentration of each biocide was tested in these studies, it would be prudent to perform dose response curves for each biocide examined using these test conditions as future work.

Further investigation is required to determine how bacteria recover following biocide insult to ascertain if this is as a result of intrinsic or acquired resistance patterns. For example, biocide resistant mobile genetic elements may exist in the wound environment, much like antibiotic resistance. Investigation of other silver biocides would also be interesting e.g. nanocrystalline dressings, which may prove to be more effective than the silver compound used here. Further research is also necessary to determine if tolerance is associated with ST of the MRSA or previous biocide exposure.

**Chapter 3:**  
**Investigation of MRSA virulence**  
**factors**

## **Chapter 3 Part I: MRSA typing and virulence factor profiles of chronic wound and asymptomatic nasal carrier MRSA**

### **3.1 Introduction**

Meticillin resistant *Staphylococcus aureus* (MRSA) poses a worldwide nosocomial problem, which resulted in greater than 2000 deaths in 2005 in the UK alone ([www.statistics.gov.uk/pdftdir/deaths0207.pdf](http://www.statistics.gov.uk/pdftdir/deaths0207.pdf); accessed 04/05/10). The global nature of MRSA has led to attempts to track the pathogen, identify genotypic diversity and descriptive epidemiological studies. In this time, a number of laboratory methods for the identification of MRSA have been developed, revealing that epidemic MRSA (EMRSA) 15 and 16 are most commonly identified strains in UK hospitals (Holden et al., 2004, Ellington et al., 2010).

MRSA is frequently identified as an acute wound isolate, and can be associated with colonisation of the healthy host. The role of MRSA in chronic wound healing is however unclear. Moreover, it is known that MRSA produce a range of virulence factors which facilitate survival and enhance pathogenicity. A number of these factors are attributed to specific disease processes e.g. toxic shock and staphylococcal scalded skin infections (SSSI; Jarraud et al., 1999, Peacock et al., 2002). Such virulence factors may clearly play specific roles in modulating the non-healing of chronic wounds.

#### **3.1.1 Typing MRSA isolates**

In outbreak settings of MRSA, typing may facilitate bacterial identification, and identify “clusters” of patients or those at risk; leading to implementation of prevention strategies. Due to the emergence of MRSA, further acquisition of resistance genes and associated healthcare risks, active monitoring is performed to aid cross infection programmes e.g. the European Antimicrobial Resistance Surveillance Network (EARS-Net) provides European reference data on antimicrobial resistance and search and destroy strategies (Higgins et al., 2010).

Numerous typing methods are employed, with the most contemporary being sequence based (Chapter 1, Section 1.9)

### **3.1.2 Chronic wound carriage of MRSA**

Chronic wounds are known to be polymicrobial. Both *S. aureus* and coagulase-negative staphylococci (CNS) were found to be the predominant organisms isolated from both prospective (Davies et al., 2007) and retrospective (Tentolouris et al., 1999) studies. A recent study by Yates et al., (2009) in diabetic foot ulcers, found that patients were twice as likely to harbour MRSA in their wounds if they had received in-patient care, and that MRSA carriage was significantly higher in chronic vs. acute wounds. Whilst MRSA have been described in numerous microbiological studies of chronic wounds, little is known about their role in non-infected wounds.

### **3.1.3 Colonisation and nasal carriage of MRSA**

Little is understood about the risk factors for nasal carriage of *S. aureus*. However, there are three lines of evidence to show a link between *S. aureus* carriage and staphylococcal infection (Peacock et al., 2001). Many studies link *S. aureus* colonisation of the nares with disease. There is less data however, on the carriage of MRSA, and there is significant discrepancy in the literature on this. The prevalence of MRSA has increased, and that this has been associated with the development of community-acquired (CA) MRSA (Miller and Diep, 2008). Surprisingly, many of the outbreaks associated with CA-MRSA have been in the young and healthy e.g. school children, sport teams, and institutionalised individuals (Fergie and Purcell, 2001, Begier et al., 2004, Zinderman et al., 2004, Lee et al., 2005).

Colonisation by MRSA frequently results in skin and soft tissue infections (Elston and Barlow, 2009). Transmission and dissemination of this pathogen is chiefly associated with colonisation of the normal skin flora, and intimate contact, more so than associated with nasal colonisation alone (Miller and Diep, 2008). The

normal colonising area for MRSA and *S. aureus* is the anterior nares. However, a study by French et al., (2009) demonstrated <80% of MRSA-positive individuals were identified by nasal swabbing alone, suggesting that in screening this should be supplemented with additional swabs from the throat and perineum.

Difficulties in providing actual carriage rates for MRSA have arisen due to the number of studies performed in countries which have high MRSA rates (e.g. Portugal, Malta, and Cyprus). In addition, many studies make no distinction between MRSA and methicillin sensitive *S. aureus* (MSSA). This is compounded by isolation and technical problems, e.g. the previous use of media supplemented with ciprofloxacin which selects against CA-MRSA carriage. Such techniques have led to under-reporting, as CA-MRSA is ciprofloxacin sensitive; hospital-acquired (HA) MRSA is resistant. In the US, there has been a >15 fold rise in CA-MRSA related disease between 1996 and 2004 (Miller and Diep, 2008). The most endemic US strain is USA300 (CA-MRSA), which is Panton-Valentine leucocidin (PVL) positive. Whilst MRSA, and in particular HA-MRSA have been reported since 1959, CA-MRSA has only been recognised since 1980 (Elston and Barlow, 2009). However, authors cannot agree on this due to terminology confusion in the literature.

The carriage rate of MRSA in the UK is approximately 6.5% based on hospital studies (Gopal Rao et al., 2007, Jeyaratnam et al., 2008). Of this, <1% is associated with CA-MRSA (Elston and Barlow, 2009), although this is likely to be an underestimate and is dependant on the population studied. Prevalence rates of CA-MRSA are primarily based on rates of infection. Kearns et al., (2004) reported an unusual single clone of MRSA associated with an intravenous drug abuser. This was believed to be the first CA-MRSA strain in the UK; and has been prevalent in the UK since 2001 (Holmes et al., 2005, Health Protection Agency, 2005). CA-MRSA is frequently associated with a type IV SCC*mec* and clonal complexes ST30, ST80 and ST8 (de Lencastre et al., 2007). The HPA has confirmed that the proportion of CA-MRSA is increasing including those identified within hospitals;

specifically clone USA300 described in the US (Klevens et al., 2006, Maree et al., 2007).

### **3.1.4 Virulence factors**

*S. aureus* may present in a variety of disease states, varying both in symptoms and severity. It is believed there are over 40 virulence factors associated with *S. aureus*, involved in colonisation, nutrition and dissemination (Argudin et al., 2009, Tubby et al., 2009). The expression of many surface and secreted proteins (including enzymes) facilitate survival. Wassenaar and Gaastra (2001) categorised virulence genes into three distinct roles; true virulence genes, virulence-associated genes and virulence life-style genes.

#### **3.1.4.1 Previous virulence testing**

Research of *S. aureus* virulence factors has focused primarily on MSSA, with attempts to correlate virulence factor production and specific disease processes e.g. endocarditis, acute soft tissue infections and osteomyelitis, using principally characterisation of enterotoxins, and superantigen presence. Recently, reports of PVL in healthy individuals have led to increased research interest in this area. Previous laboratory investigations have relied upon the identification of virulence genes. However, few studies have attempted to investigate their expression. More recently quantitative RT-PCR and microarray technology have attempted to address this issue. To date, most studies have limited testing to the expression of enterotoxins, exfoliative toxins and PVL.

Analysis of virulence genes either by allelic replacement, insertion or inactivation provides some evidence to the role of virulence factor activity. Results however, are difficult to interpret due to the presence of multiple virulence factors (Wassenaar and Gaastra, 2001). Additionally, the loss of one virulence factor does not necessarily make the organism less pathogenic.



Early studies on virulence factors employed enzyme-linked immunosorbent assay (ELISA), radioimmunoassay or commercial kits such as the semi-quantitative reversed passive latex agglutination toxin detection kits (Johnson et al., 1991b). These methods are limited by their variable sensitivity and are highly dependent on adequate gene expression and protein formation. Low toxin-producing strains or cross-reactive antigens can frequently provide false results (Johnson et al., 1991b, Klotz et al., 2003, Varshney et al., 2009).

#### **3.1.4.2 Accessory gene regulator and disease**

The regulation of virulence expression is tightly coordinated by the accessory gene regulator (*agr*). This quorum sensing mechanism is able to globally regulate transcription associated with cell-density, growth phase and stage of infection (Cheung et al., 2004, Korem et al., 2010). A study of the virulence potential of *S. aureus* in diabetic foot ulcers showed that MRSA and *agr* I were more likely to be associated with infection and poor outcome for healing (Sotto et al., 2008).

#### **3.1.4.3 Staphylococcal Toxins**

Staphylococcal enterotoxins (SE) genes, of which many have been identified i.e. *sea* to *seu* (Argudin et al., 2009) are frequently responsible for toxin-mediated disease. Staphylococcal toxic shock syndrome toxin (TSST-1) and SE are potent T-cell activators which lead to the release of pro-inflammatory cytokines (Argudin et al., 2009). Studies have attempted to correlate the presence of various SEs with the outcome of *S. aureus* infections, although this is still unclear (Varshney et al., 2009).

#### **3.1.4.4 Collagen binding protein (*cna*)**

Collagen binding protein has been described as a virulence factor in septic arthritis, osteomyelitis, endocarditis and keratitis. CNA has also been associated with invasive pathological disorders (Switalski et al., 1993, Gillaspay et al., 1997, Rhem et

al., 2000, Elasri et al., 2002, Peacock et al., 2002, Nashev et al., 2004, Zong et al., 2005) suggesting collagen binding is an advantage for pathogenesis.

#### **3.1.4.5 Glycerol ester hydrolase (*geh*)**

Fatty acids and lipids can act to disrupt bacterial membranes; as such many staphylococci are thought to be lipolytic (Gould et al., 2009). *geh* encodes a lipase enzyme, which hydrolyses long-chain triacylglycerols, water-soluble triacylglycerols and Tweens® (Arvidson and Tegmark, 2001, Arvidson, 2006), which may additionally facilitate the acquisition of bacterial nutrients.

#### **3.1.4.6 Leucotoxins**

Leucotoxins e.g. PVL and haemolysins (e.g.  $\alpha$ ,  $\beta$ ,  $\gamma$ ), are believed to be involved in host defence evasion. They are able to lyse polymorphonuclear leucocytes (PMNL) and erythrocytes in the case of haemolysin, via the formation of pores in the cell membranes, leading to cell death.

##### **3.1.4.6.1 Panton-Valentine leucocidin (PVL)**

Panton-Valentine leucocidin (PVL) is a two component toxin composed of proteins LukS-PVL and LukF-PVL. Researchers have investigated the PVL protein by purifying and injecting it intradermally in animal models. This demonstrated its ability to induce a severe inflammatory lesion (Ward and Turner, 1980). The genes for these proteins are carried by a temperate bacteriophage, which integrates its DNA into the *Staphylococcus aureus* chromosome. Eight lysogenic bacteriophages are known to carry the *lukS* and *lukF* genes (Kaneko et al., 1998, Wirtz et al., 2010). Induction of these bacteriophage has been purported to be initiated by  $\beta$ -lactams, ciprofloxacin, trimethoprim and mitomycin C (Lindsay, 2009, Wirtz et al., 2009).

Since 2006 there has been an explosion in the number of UK studies involving the PVL virulence factor amongst *S. aureus* species, particularly methicillin resistant *S. aureus* (MRSA) following the deaths of a nurse and patient in a West

Midlands hospital. PVL has commanded global notoriety due to its ability to affect otherwise young and healthy individuals. Infections caused by strains with the gene for the toxin are prevalent in close communities or clusters, such as children's playgrounds, and sports teams. Presenting with a range of symptoms from minor skin infections to necrotising pneumonia which has a mortality rate of 75% (Dumitrescu et al., 2007). As a result, diagnostic laboratories have been inundated with requests from clinicians for testing of this virulence factor.

#### **3.1.4.6.2 Community-acquired MRSA and Panton-Valentine leucocidin**

Originally it was thought that PVL was solely associated with MRSA in the community setting and as such it was used as a marker for community-acquired (CA) MRSA (Vandenesch et al., 2003, Boyle-Vavra and Daum, 2007). Typically PVL has been associated with MRSA sequence types (ST) 1, 30 and 80 which are found within the community setting (Holmes et al., 2005, Takizawa et al., 2005) and contain the *SCCmec* IV element. The presence of PVL genes within the CA-MRSA genome has been suggested as the cause of the enhanced virulence of these strains (Lindsay, 2009). However, more recent studies have found that PVL cannot be used as a sole marker for CA-MRSA, because the gene has also alarmingly appeared in ST22 i.e. EMRSA-15 (Rossney et al., 2007).

#### **3.1.4.7 Hyaluronate lyase (*hysA*)**

Hyaluronic acid forms a major component of the extracellular matrix (ECM) (Farrell et al., 1995), and bacterial extracellular hyaluronidase (encoded by *hysA*) , cleaves hyaluronan, and has been implicated in the spread of *Streptococcus pyogenes* (Hynes et al., 2000).

#### **3.1.4.8 Biofilm (*ica*)**

Biofilm formation has been implicated in the failure of prosthetic implants and infections associated with indwelling devices; *S. aureus* being frequently

associated with these infections (Zimmerli and Ochsner, 2003, Fluckiger et al., 2005). Clinical isolates associated with biofilm-associated disease show the presence of the intercellular adhesion (*ica*) locus (Fluckiger et al., 2005).

### **3.1.5 Chronic wound healing and potential virulence factors**

A number of the staphylococcal virulence factors described above may play obvious roles in the failure of cell migration, defective ECM and persistence of MRSA in chronic wounds. In this study the presence and expression of these virulence factors was systematically investigated in MRSA isolated from chronic wounds and contrasted with asymptomatic nasal carriers. It is possible that the MRSA clonal type and virulence gene expression may contribute to the chronicity of the chronic wound lesion.

## **3.2 Aims**

The aims of this study were to:

1. Investigate the clonal types of MRSA isolated from chronic wounds compared to nasal carriage using RAPD, PFGE, MLST and *SCCmec*.
2. Determine the difference in the presence of virulence genes in MRSA isolated from chronic wounds compared to nasal carriage.
3. Compare expression of virulence factors of MRSA isolated from chronic wounds compared with nasal carriage to determine if they play a role in chronic wounds.

## **3.3 Materials and Methods**

### **3.3.1 Culture of bacterial isolates**

Isolation of chronic wound MRSA (n = 17) is described in Chapter 2 Section 2.3.1 and 2.3.2. Nasal MRSA isolates were obtained from 7 asymptomatic nasal

carriers following routine nasal screening by the National Public Health Services (NPHS), Cardiff. Isolates were cultured on blood agar (BA; LabM) supplemented with 5% (v/v) defibrinated sheep blood (Oxoid) overnight (O/N) at 37°C.

### **3.3.2 MRSA Typing**

#### **3.3.2.1 DNA preparation for Random Amplified Polymorphic DNA (RAPD) analysis**

Overnight cultures of the test organism were grown at 37°C in 5 ml tryptone soya broth (TSB; Oxoid). One ml of culture was centrifuged (9500  $\times$  g, 5 min), and the supernatant removed. Following washing (x2) with 1 ml lysis buffer (50 mM Tris- HCl, pH 8.0; 5 mM EDTA, pH 8.0; 50 mM NaCl), the pellet was re-suspended in 1 ml of lysis buffer containing 0.02 mg/ml lysostaphin (Sigma), and 0.025 mg/ml RNaseA (Sigma). Following incubation (37°C, 30 min) with agitation, the lysate was centrifuged (9500  $\times$  g, 10 min). The supernatant was used as a RAPD DNA template following quantification (Nano-drop; Nano-Vue, GE Healthcare) and dilution (5 ng/ $\mu$ l) with nuclease free water.

##### **3.3.2.1.1 Random amplified polymorphic DNA (RAPD)**

Bacterial DNA templates were prepared (Section 3.3.2.1). All PCR reagents were supplied by Promega, Southampton, UK unless otherwise stated. A 50  $\mu$ l PCR reaction was performed in thin-walled PCR tubes; consisting of 2  $\mu$ l of DNA template; 0.2 mM (dGTP, dCTP, dATP, dTTP; each) deoxynucleoside triphosphates (dNTPs); 50  $\mu$ M of primers 1, 7 and E2 (Table 3.1; MWG-Biotech) in 3 individual PCR reactions; 5 U *Taq* DNA polymerase; 10  $\mu$ l of 10X buffer; 2.5 mM MgCl<sub>2</sub> and 6.6  $\mu$ l of nuclease-free water was amplified in a thermal cycler using conditions described in Table 3.2. Thirty-five  $\mu$ l of the PCR product was combined with 8  $\mu$ l of loading dye and separated by electrophoresis using a 1.5% (w/v) of a 1:1 ratio agarose (Fisher Scientific UK Ltd, Leicestershire, UK) and NuSieve GTG Agarose

**Table 3.1** Primer sequences employed in typing analysis

Typing method	Target gene	Primer name	Sequence 5' to 3'	Amplicon size (bp)	Reference
RAPD		1	GGTTGGGTGAGAATTGCACG		(van Belkum et al., 1995)
		7	GTGGATGCGA		
		ERIC2 (E2)	AAGTAAGTGACTGGGGTGAGCG		
MLST	Carbamate kinase	<i>arcC</i> (F)	TTGATTCAACGAGCGCGTATTGTC	456	(Enright et al., 2000a)
		<i>arcC</i> (R)	AGGTATCTGCTTCAATCAGCG		
	Shikimate dehydrogenase	<i>aroE</i> (F)	ATCGGAAATCCTATTTACATTTC	456	
		<i>aroE</i> (R)	GGTGTTGTATTAATAACGATATC		
	Glycerol kinase	<i>glpF</i> (F)	CTAGGAAGTCAATCTTAATCC	465	
		<i>glpF</i> (R)	TGGTAAAATCGCATGTCCAATTC		
	Guanylate kinase	<i>gmk</i> (F)	ATCGTTTTATCGGGACCATC	429	
		<i>gmk</i> (R)	TCATTAACCTACAACGTAATCGTA		
	Phosphate transferase	<i>pta</i> (F)	GTTAAAATCGTATTACCTGAAGG	474	
		<i>pta</i> (R)	GACCCTTTTGTTGAAAAGCTTAA		
	Triphosphate isomerase	<i>tpi</i> (F)	TCGTTCATTCTGAACGTCGTGAA	402	
		<i>tpi</i> (R)	TTTGCACCTTCTAACAATTGTAC		
	Acetyl coenzyme A acetyltransferase	<i>yqiL</i> (F)	CTGCATACAGGACACCTATTGGC	516	
		<i>yqiL</i> (R)	CGTTGAGGAATCGATACTGGAAC		
<i>mec</i>	Class A <i>mec</i>	mI6	CATAACTTCCCATTCTGCAGATG	1963	(Kondo et al., 2007)
	Class B <i>mec</i>	IS7	ATGCTTAATGATAGCATCCGAATG	2827	
	Class C <i>mec</i>	IS2(iS-2)	TGAGGTTATTTCAGATATTTTCGATGT	804	
		mA7 (R)	ATATACCAAACCCGACAACCTACA		

RAPD, random amplified polymorphic DNA; MLST, multilocus sequence typing; *mec*, meticillin resistance gene; (F), forward primer; (R), reverse primer; bp, base pair.

**Table 3.1 continued** Primer sequences employed in typing analysis

Typing method	Target gene	Primer name	Sequence 5' to 3'	Amplicon size (bp)	Reference
<i>ccr</i>	<i>mec</i>	mA1 (F)	TGCTATCCACCCTCAAACAGG	286	(Kondo et al., 2007)
		mA2 (R)	AACGTTGTAACCACCCCAAGA		
	<i>ccr1</i>	α1	AACCTATATCATCAATCAGTACGT	695	
	<i>ccr2</i>	α2	TAAAGGCATCAATGCACAAACACT	937	
	<i>ccr3</i>	α3	AGCTCAAAAGCAAGCAATAGAAT	1791	
		βc (R)	ATTGCCTTGATAATAGCCITCT		
	<i>ccr4</i>	α4.2 (F)	GTATCAATGCACCAGAACTT	1287	
		β4.2 (R)	TTGCGACTCTCTTGCGGTTT		
	<i>ccrC</i>	γ (R)	CCTTTATAGACTGGATTATTCAAAATAT	518	
		γ (F)	CGTCTATTACAAGATGTTAAGGATAA		

*ccr*, cassette chromosome recombinase gene; (F), forward primer; (R), reverse primer; bp, base pair; *mec*, meticillin resistance gene.

**Table 3.1 continued** Primer sequences employed in typing analysis

Typing method	Gene	Primer name	Sequence 5' to 3'	Amplicon size (bp)	Reference
<b>J type</b>	<i>ccrB2</i> (internal positive control)	<i>ccrB2</i> (F) <i>ccrB2</i> (R)	CGAACGTAATAACATTGTCTG TTGGCWATTTTACGATAGCC	203	(Milheirico et al., 2007)
	Type IVa	J IVa (F) J IVa (R)	ATAAGAGATCGAACAGAAGC TGAAGAAATCATGCCTATCG	278	
	Type IVb and IVF	J IVb (F) J IVb (R)	TTGCTCATTTCAGTCTTACC TTACTTCAGCTGCATTAAGC	336	
	Type IVc and IVE	J IVc (F) J IVc (R)	CCATTGCAAATTTCTCTTCC ATAGATTCTACTGCAAGTCC	483	
	Type IVd	J IVd (F) J IVd (R)	TCTCGACTGTTTGCAATAGG CAATCATCTAGTTGGATACG	575	
	Type IVg	J IVg (F) J IVg (R)	TGATAGTCAAAGTATGGTGG GAATAATGCAAAGTGGAACG	792	
	Type IVh	J IVh (F) J IVh (R)	TTCCTCGTTTTTTCTGAACG CAAACACTGATCTTGTGTCG	663	

J, joining; (F), forward primer; (R), reverse primer; bp, base pair; *ccr*, cassette chromosome recombinase gene.



**Table 3.2** PCR and electrophoresis conditions

PCR reaction	Thermocycler conditions							Electrophoresis			
	Activation step	Initial Denature	Cycles	Denature	Annealing	Elongation	Final Extension	Agar %	Ethidium bromide	Voltage	Time
	(°C - min)	(°C - min)		(°C - min)	(°C - min)	(°C - min)	(°C - min)	(w/v)	(µg/ml)	(v)	(h)
RAPD		94 - 4	35	94 - 1	25 - 1	74 - 2	74 - 10	1.5	0.5	40	12
MLST		95 - 3	30	95 - 1	55 - 1	72 - 1	72 - 10	1	0.5	70	1
<i>ccr</i> type		94 - 2	30	94 - 2	57 - 1	72 - 2	72 - 2	2	0.5	70	2
<i>mec</i> type		94 - 2	30	94 - 2	60 - 1	72 - 2	72 - 2	2	0.5	70	2
J type		94 - 4	35	94 - 2	48 - 0.5	72 - 2	72 - 4	2	0.5	70	2
<i>agr</i> type		94 - 3	30	94 - 1	55 - 1	72 - 1	72 - 3	2	0.5	105	4
enterotoxin		95 - 2	28	95 - 1	55 - 1	72 - 2	72 - 5	2.5	0.5	120	2
'in-house' virulence factor		94 - 1	30	94 - 0.5	55 - 0.5	72 - 1	72 - 4	1	0.5	70	1
<b>RT-PCR reaction</b>											
enterotoxin	50 - 30	95 - 15	35	94 - 0.5	55 - 0.5	72 - 1	72 - 10	2.5	0.5	120	2
'in-house' virulence factor	50 - 30	95 - 15	35	94 - 0.5	55 - 0.5	72 - 1	72 - 10	1	0.5	100	1

(Cambrex IEP, Wiesbaden, Germany) gel using parameters described in Table 3.2, with 100 and 500 bp molecular weight standard (Fermentas, York, UK) in a 1X Tris-Borate-EDTA (TBE; 0.1 M Tris Base; 0.09 M Boric Acid; 0.1 mM EDTA) running buffer. Gels were visualised using a UV transilluminator GelDoc<sup>®</sup> (Bio-Rad<sup>®</sup>, Hertfordshire, UK) and analysis of the resulting RAPD fingerprint was performed using the software package Quantity One<sup>®</sup> (Bio-Rad<sup>®</sup>) to produce an Unweighted Pair Group Method with Arithmetic Mean (UPGMA) to study phylogeny.

### **3.3.2.2 Pulsed field gel electrophoresis (PFGE)**

Bacterial suspensions were prepared by inoculating 3 ml of brain heart infusion broth (BHI; Oxoid) with an individual colony from test isolates. Following incubation (37°C, 16-18 h), 150 µl of suspension was transferred into sterile microfuge tubes (Fisher Scientific) and centrifuged (18000 x g, 2 min) and the pellet re-suspended in 150 µl cell suspension buffer (10 mM Tris – HCl, pH 7.2; 20 mM NaCl; 50 mM EDTA).

PFGE plugs were prepared by adding 0.06 mg/ml of lysostaphin to each bacterial suspension, and incubating at room temperature (RT, 30 min). Following this, 150 µl of 20% (w/v) low melting point agarose (Bio-Rad<sup>®</sup>) made with 1X TBE buffer was added, gently mixed and used to overfill the plug mould (Bio-Rad<sup>®</sup>). The plugs were allowed to set (RT, 20 min) and then extruded into a 24-well tissue culture plate (Greiner Bio-One Ltd, Stroudwater, UK) containing 500 µl lysis buffer (10 mM Tris- HCl, pH 7.2; 50 mM NaCl; 50 mM EDTA; 2% w/v, Deoxycholate; 0.5% w/v, Sarkosyl, Sigma) and incubated (37°C, 1 h). The lysis buffer was then removed and 500 µl of proteinase K (PK) buffer (250 mM EDTA, pH 9.0; 1% Sarkosyl), and 2.73 mg/ml of proteinase K (Sigma) added and incubated (50°C, 30 min). The PK buffer was removed and the samples washed (x3) in 1.4 ml wash buffer (10 mM Tris-HCl, pH 7.6; 0.1 mM EDTA) for 30 min each time at RT. The plugs were then stored at 4°C in wash buffer until required.

Each PFGE plug was cut into 3 segments, with the middle section placed into a 96-well microtiter plate for digestion with *Sma*I (Invitrogen Ltd, Paisley, UK). Restriction digests were prepared by diluting (1:10) the buffer React<sup>®</sup>4 (supplied with the *Sma*I enzyme; Invitrogen) with sterile distilled water, adding 300 µl to each plug. These were incubated (25°C, 10 min), the buffer removed and 150 µl of diluted React<sup>®</sup>4 restriction buffer and 25 U (2.5 µl) of *Sma*I added to each sample well and incubated (25°C, 2 h).

A pulsed field gel was created by using a 1% (w/v) agarose gel (Fisher Scientific) prepared with 120 ml 1X TBE buffer and 0.5 µg/ml ethidium bromide. The digested plugs were assembled onto the comb of the pulsed field mould, along with a 2 mm slice of the molecular weight standard PFG Lambda ladder (New England Biolabs Ltd, Hertfordshire, UK) and were affixed using melted agarose. The comb with the attached plugs was inserted into the setting mould.

The pulsed field gel was placed in the PFGE tank (CHEF-DR<sup>®</sup>-III; Bio-Rad<sup>®</sup>). Electrophoresis was performed in 0.5X TBE buffer at 6 V/cm with the following settings; 120 included angle, initial switch time of 5.3 min and a final switch time of 34.9 min, run time 18 h, buffer temperature 14°C (Mulvey et al., 2001). The gel was stained for 20 – 60 min in 200 ml water containing 1 µg/ml of ethidium bromide, visualised and recorded using GelDoc<sup>™</sup> (Bio-Rad<sup>®</sup>). Analysis of the PFGE fragment fingerprint was assessed using the set of standard criteria in Table 3.3.

### **3.3.2.3 Multilocus sequence typing (MLST)**

#### **3.3.2.3.1 DNA preparation using the Qiagen<sup>®</sup> DNeasy<sup>®</sup> kit**

Test MRSA were grown on tryptone soy agar (TSA; Oxoid) O/N at 37°C. An individual colony from each purity plate was streaked in a 2.5 x 2.5 cm patch on half of a TSA plate and incubated (37°C, O/N). Cells harvested from the TSA plates were added to 250 µl of lysis buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH

**Table 3.3** Criteria for interpreting PFGE patterns

<b>Category</b>	<b>No. of genetic differences compared with outbreak strain</b>	<b>Typical no. of fragment differences compared with outbreak pattern</b>	<b>Epidemiologic interpretation</b>
Indistinguishable	0	0	Isolate is part of the outbreak
Closely related	1	2-3	Isolate is probably part of the outbreak
Possibly related	2	4-6	Isolate is possibly part of the outbreak
Different	$\geq 3$	$\geq 7$	Isolate is not part of the outbreak

(Tenover et al., 1995)

8.0) containing 0.05 mg/ml lysostaphin, and 20 mg/ml lysozyme. Samples were vortexed for 30 s and incubated (37°C, 3 h), vortexing at 30 min intervals. Then 25 µl of the supplied proK and 200 µl of buffer AL were added; samples were vortexed and incubated (70°C, 30 min). Following incubation, 200 µl ice cold 95% (v/v) ethanol (EtOH) was added and each sample inverted (x4). The total content of each tube was pipetted into individual DNeasy<sup>®</sup> minicolumns nested in 2 ml collection tubes and then centrifuged (9500 x g, 1 min). The collection tube was discarded and the centrifuge minicolumn was placed in a new 2 ml collection tube, and 500 µl AW1 buffer (Qiagen<sup>®</sup> kit) added. Following centrifugation (11500 x g, 1 min), the centrifuge minicolumn was placed in a new 2 ml collection tube and 500 µl of AW2 buffer (Qiagen<sup>®</sup> kit) added. Samples were centrifuged (18600 x g, 3 min). The collection tube was discarded and the centrifuge minicolumn placed in a sterile 1.5 ml microfuge tube. Then 100 µl AE buffer (Qiagen<sup>®</sup> kit) was added directly onto the membrane of each sample column and left for 5 min at RT. Samples were centrifuged (11500 x g, 1 min). The eluent was used as template DNA for MLST. Quality assurance of DNA samples was performed using 5 µl of each sample run on a 0.8% (w/v) agarose gels containing ethidium bromide (0.5 µg/ml) and visualised (Section 3.3.2.1.1).

#### **3.3.2.3.2 PCR for multilocus sequence typing (MLST)**

MLST typing was performed as described previously (Enright et al., 2000a). For each isolate, internal DNA fragments of seven housekeeping genes were amplified in individual PCR reactions using the MLST primer pairs (Table 3.1). The PCR reaction was carried out in 96-well plates with a total reaction volume of 50 µl containing 1 µl of chromosomal DNA, 200 µM of forward and reverse primers, 1U of *Taq* DNA polymerase, 5 µl of 10X buffer, 2.5 mM MgCl<sub>2</sub> and 0.2 mM dNTPs. PCR reactions were performed using the conditions described in Table 3.2 The PCR

products were separated by electrophoresis and compared with a 100 bp molecular weight standard (Promega) in 1x TBE running buffer (Table 3.2; Section 3.3.2.1.1).

#### **3.3.2.3.3 Purification of PCR products using the QIAquick® 96 PCR purification kit: Method 1**

PCR products were purified using the QIAquick® 96 PCR Purification Kit (Qiagen®) as per the manufacturer's instructions. PCR products (45 µl) of each isolate were transferred to a provided 96-well plate containing 3X volume of buffer PM (Qiagen® QIAquick® kit). The liquid phase was removed using a QIAvac vacuum pump (Qiagen®). The wells were washed with 900 µl of the supplied buffer PE and the buffer was removed using the pump. This step was repeated, and a vacuum was then applied at maximum for 10 min to dry the membrane of each column. The nozzle of the QIAquick® plate was "blotted" onto paper towels. The QIAquick® waste tray was then replaced with the supplied blue collection microtube rack upon which the 96-well microplate was placed. The final elution step was performed by adding 60 µl of the supplied buffer EB directly to the centre of each well and applying a vacuum for a further 5 min. Purified PCR products (2 µl) were separated on a 0.8% (w/v) agarose gel as previously described (Section 3.3.2.3.1) for quality assurance.

#### **3.3.2.3.4 Purification of PCR products using ExoSAP-IT®: Method 2**

PCR products were purified using ExoSAP-IT® solution (GE Healthcare, Buckinghamshire, UK). ExoSAP-IT® solution contains exonuclease I and shrimp alkaline phosphatase. The perceived advantages are; no loss of sample, less chance of contamination and fewer steps. The ExoSAP-IT® solution was maintained on ice during the procedure; 15 µl of PCR product with 6 µl of ExoSAP-IT® solution were mixed and incubated (37°C, 15 min) to degrade any unincorporated nucleotides and primers. The samples were then incubated (80°C, 15 min) to inactivate ExoSAP-IT® enzymes. The samples were used directly for sequencing. Purified PCR

products (2 µl) were separated on a 0.8% (w/v) agarose gel as previously described (Section 3.3.2.3.1) for quality assurance.

### **3.3.2.3.5 Sequencing of PCR products and MLST analysis**

PCR products were sequenced using forward and reverse MLST PCR primers (Table 3.1). Sequencing was performed by either Lark, Co Genetics, Essex using an ABI 3730xl sequencer (Applied Biosystems, USA) or at the DNA Sequencing Core facility, Molecular Biology Unit, School of Biosciences, Cardiff University using an ABI 3130xl sequencer (Applied Biosystems, USA). For each isolate, the alleles at each of the seven housekeeping loci were identified by comparing the sequences obtained from the test isolates with sequences held in the MLST database (<http://saureus.mlst.net>). Allelic profiles and sequence types (ST) for each isolate was determined using this database.

### **3.3.2.4 SCC<sub>mec</sub> typing**

#### **3.3.2.4.1 *ccr* gene complex typing**

Bacterial DNA template was prepared with the Qiagen DNeasy® kit as previously described (Section 3.3.2.3.1). A 50 µl reaction was performed for each isolate in a 96-well plate. The *ccr* gene complex and internal *mec* control gene were identified using a multiplex PCR reaction using 10 primer sequences (6 primer pairs; Table 3.1). A PCR reaction mixture of 1 µl DNA template, 26.35 µl nuclease-free water, 0.1 µM each primer, 2.5 U of *Taq* DNA polymerase, 10 µl of 10X buffer, 3.2 mM MgCl<sub>2</sub> and 200 µM (each) dNTPs, was amplified in a thermal cycler (Table 3.2). The amplified product was separated by electrophoresis and compared with a 100 bp molecular weight standard (Promega) in a 1X TBE running buffer using the parameters described in Table 3.2. The resulting gel was visualised (Section 3.3.2.1.1) using the *mec* gene as an internal control. The *ccr* gene complex was assigned by size of PCR product (Table 3.1).

#### 3.3.2.4.2 *mec* class typing

A bacterial DNA template was prepared using the Qiagen DNeasy® kit (Section 3.3.2.3.1). For each isolate a 50 µl reaction was performed in a 96-well plate where the *mec* gene complex was identified using a multiplex PCR of 4 primer sequences (3 primer pairs, Table 3.1). A PCR reaction mixture of 1 µl DNA template, 31.75 µl nuclease-free water, 0.1 µM each primer, 2.5 U of *Taq* DNA polymerase, 10 µl of 10X buffer, 2 mM MgCl<sub>2</sub> and 200 µM (each) dNTPs, was amplified in a thermal cycler (Table 3.2). The amplified products were compared with a 100 bp and 1 kb molecular weight standard (Promega; Table 3.2). The *mec* gene complex was assigned by the size of PCR product (Table 3.1)

#### 3.3.2.4.3 Joining (J) region typing for sub-classification of SCC*mec* IV variants

A bacterial DNA template was prepared using the Qiagen DNeasy® kit. A 25 µl reaction was performed in a 96-well plate to determine the SCC*mec* IV subtype by determining the J type using a multiplex PCR of 7 primer pairs (Table 3.1). A PCR reaction mixture of 1 µl DNA template, 10.25 µl nuclease-free water, 1.25 U of *Taq* DNA polymerase, 10 µl of 10X buffer, 1.5 mM MgCl<sub>2</sub> and 40 µM dNTPs, 0.2 µM of primers J IVa, J IVb; 0.4 µM of primers *ccrB2* (F), J IVc; 0.8 µM of primers *ccrB2* (R), J IVd; 0.9 µM of primers J IVg and 1.8 µM of primers J IVh was amplified in a thermal cycler (Table 3.2). The amplified products were compared with a 100 bp molecular weight standard (Thermo Scientific, Northumberland, UK) and 50 bp molecular weight standard (Fisher Scientific) with a 1X TBE running buffer (Table 3.2). The resulting gel was visualised (Section 3.3.2.1.1), J type was assigned by size of PCR product (Table 3.1).

#### 3.3.2.4.4 Assignment of SCC*mec* type

The assignment of each SCC*mec* type is based on the combination of the *ccr* allotype and *mec* class, followed by the J type, if SCC*mec* IV is present. Table 3.4



**Table 3.4** Assignment of SCC*mec* type based on *ccr* and *mec* complex

SCC <i>mec</i> type	<i>ccr</i> allotype	<i>mec</i> class	<sup>a</sup> J type
I	1	B	n/a
II	2	A	n/a
III	3	A	n/a
IV	2	B	a to j
V	5	C2	n/a
VI	4	B	n/a
VII	5	C1	n/a
VIII	4	A	n/a

n/a – not applicable; <sup>a</sup>Joining region SCC*mec* subtype.

**Table 3.5** Primer sequences used for *agr* typing

Primer name	Sequence 5' to 3'	Amplicon size (bp)
pan- <i>agr</i> (F)	ATGCACATGGTGCACATGC	
<i>agr</i> I (R)	GTCACAAGTACTATAAGCTGCGAT	440 bp
<i>agr</i> II (R)	GTATTACTAATTGAAAAGTGCCATAGC	572 bp
<i>agr</i> III (R)	CTGTTGAAAAAGTCAACTAAAAGCTC	406 bp
<i>agr</i> IV (R)	CGATAATGCCGTAATACCCG	588 bp

assigns SCC*mec* I to VIII (Zhang et al., 2005, Kondo et al., 2007, Milheirico et al., 2007, Chen et al., 2009, IWG-SCC, 2009).

### 3.3.3 Virulence profiles

#### 3.3.3.1 Accessory gene regulator typing (*agr*)

Bacterial DNA templates were prepared using the Qiagen DNeasy<sup>®</sup> kit (Section 3.3.2.3.1). A 50 µl reaction was performed in a 96-well plate. The *agr* type was determined by using one forward primer and 4 reverse primers in a multiplex PCR reaction (Table 3.5). A PCR reaction mixture of 1 µl DNA template, 13.8 µl nuclease-free water, 20 µM each primer, 2.5 U of *Taq* DNA polymerase, 5 µl of 10X buffer, 2 mM MgCl<sub>2</sub> and 350 µM dNTPs was amplified in a thermal cycler (Table 3.2). The amplified product was diluted 1:4 with nuclease-free water and separated by electrophoresis with a 50 bp molecular weight standard (Fisher Scientific) in a 1X TBE running buffer (Table 3.2). The resulting gel was visualised (Section 3.3.2.1.1) and *agr* type assigned by PCR product (Table 3.5; Shopsin et al., 2003, Ben Ayed et al., 2006).

#### 3.3.3.2 Multiplex PCR detection of Staphylococcal toxin genes

Bacterial DNA templates were prepared using the Qiagen DNeasy<sup>®</sup> kit (Section 3.3.2.3.1) and diluted 1:10 in nuclease free water. Holmes et al., (2005) described a method for detecting multiple toxin genes in staphylococci. Using Holmes' method, adapted by the Health Protection Agency (HPA), London, the presence of genes encoding 14 toxins was determined by the use of four multiplex PCR reactions (Table 3.6). All multiplex reactions consisted of primers for 16S rRNA gene as an internal control, and reaction 4 also utilised internal control *mecA* primers.

A primer mastermix was prepared for each reaction. The final concentration for multiplex reaction 1 (enterotoxin A to E) was 1 µM for 16S rRNA primers and 4.8 µM for *sea*, *seb*, *sec*, *sed*, *see* primers; for multiplex reaction 2 (enterotoxin G to J)

**Table 3.6 PCR primer sequences for multiplex toxin screening of MRSA**

Multiplex reaction	Primer name	Sequence 5'to 3'	Toxin name	Amplicon size (bp)	Reference
1	<i>sea-3</i> (F)	CCTTTGGAAACGGTTAAAACG	Enterotoxin A	127	(Becker et al., 1998)
	<i>sea-4</i> (R)	TCTGAACCTTCCCATCAAAAAC			
	<i>seb-1</i> (F)	TCGCATCAAACGACAAACG	Enterotoxin B	477	
	<i>seb-4</i> (R)	GCAGGTACTCTATAAGTGCCTGC			
	<i>sec-3</i> (F)	CTCAAGAACTAGACATAAAAGCTAGG	Enterotoxin C	271	
	<i>sec-4</i> (R)	TCAAAATCGGATTAACATTATCC			
	<i>sed-3</i> (F)	CTAGTTTGGTAATATCTCCTTTAAACG	Enterotoxin D	319	
	<i>sed-4</i> (R)	TTAATGCTATATCTTATAGGGTAAACATC			
2	<i>see-3</i> (F)	CAGTACCTATAGATAAAGTTAAAACAAGC	Enterotoxin E	178	(Monday and Bohach, 1999)
	<i>see-2</i> (R)	TAACTTACCGTGGACCCTTC			
	<i>seg</i> (F)	CGTCTCCACCTGTTGAAGG	Enterotoxin G	327	
	<i>seg</i> (R)	CCAAGTGATTGTCTATTGTCG			
	<i>seh</i> (F)	CAACTGCTGATTTAGCTCAG	Enterotoxin H	360	
	<i>seh</i> (R)	GTCGAATGAGTAATCTCTAGG			
	<i>sei</i> (F)	CAACTCGAATTTTCAACAGGTAC	Enterotoxin I	465	
	<i>sei</i> (R)	CAGGCAGTCCATCTCCTG			
	<i>sej</i> (F)	CATCAGAACTGTTGTTCCGCTAG	Enterotoxin J	142	
	<i>sej</i> (R)	CTGAATTTTACCATCAAAGGTAC			

**Table 3.6 continued** PCR primer sequences for multiplex toxin screening of MRSA

Multiplex reaction	Primer name	Sequence 5'to 3'	Toxin name	Amplicon size (bp)	Reference	
3	<i>tst-3</i> (F)	AAGCCCTTTGTTGCTTGCG	Toxic shock syndrome toxin	445	(Becker et al., 1998)	
	<i>tst-6</i> (R)	ATCGAACTTTGGCCCATACTTT				
	<i>eta-3</i> (F)	CTAGTGCATTTGTTATTCAAGACG	Exfoliative toxin A	119		
	<i>eta-4</i> (R)	TGCATTGACACCATAGTACTTATTC				
	<i>etb-3</i> (F)	ACGGCTATATACATTCAATTCAATG	Exfoliative toxin B	262		
	<i>etb-4</i> (R)	AAAGTTATTCATTTAATGCACTGTCTC				
4	<i>etd-1</i> (F)	AACTATCATGTATCAAGG	Exfoliative toxin D	376	(Yamaguchi et al., 2002)	
	<i>etd-2</i> (R)	CAGAATTTCCCGACTCAG				
	PVL-1 (F)	ATCATTAGGTAAAATGTCTGGACATGATCCA	Panton Valentine Leucocidin	433		
	PVL-2 (R)	GCATCAASTGTATTGGATAGCAAAAGC				
	<i>mecA</i> -P4 (F)	TCCAGATTACAACCTTCACCAGG	<i>mecA</i> gene	162		(Oliveira and de Lencastre, 2002)
	<i>mecA</i> -P7 (R)	CCACTTCATATCTTGTAACG				
1 – 4	16S rRNA (F)	GTAGGTGGCAAGCGTTATCC	16S rRNA gene	228	(Monday and Bohach, 1999)	
	16S rRNA (R)	CGCACATCAGCGTCAG				

1  $\mu$ M of 16S rRNA primers and 2.7  $\mu$ M for *seg*, *seh*, *sei*, and *sej* primers; for multiplex reaction 3 (exfoliatin toxin A and B and TSST-1) 1  $\mu$ M for 16S rRNA primers and 2.7  $\mu$ M for *eta*, *etb*, and *tst*, primers; for multiplex reaction 4 (exfoliative toxin D and PVL) 0.9  $\mu$ M for 16S rRNA primers, 4.5  $\mu$ M for *mecA* primers and 14.5  $\mu$ M for *etd* and PVL primers.

For each isolate, a 50  $\mu$ l reaction was performed in a 96-well plate. A PCR reaction mixture of 2  $\mu$ l DNA template, 31.75  $\mu$ l nuclease-free water, 2.5 mM dNTPs, 5  $\mu$ l of 10X buffer, 3 mM MgCl<sub>2</sub>, 1.25 U *Taq* DNA polymerase and 1  $\mu$ l of the corresponding reaction primer mix was amplified in a thermal cycler (Table 3.2). The amplified products (10  $\mu$ l) were separated by electrophoresis with a 100 bp (Thermo Scientific) and 50 bp (Fisher) molecular weight standard with 0.5X TBE running buffer (Table 3.2). The resulting gel was visualised (Section 3.3.2.1.1), and genes for toxin presence were identified by PCR product size (Table 3.6).

### 3.3.3.3 PCR detection of staphylococcal virulence factors

Many of the virulence factors produced by staphylococci may have an impact on the healing of wounds. A number of these were identified and investigated. Using the nucleotide search on the National Centre for Biotechnology Information (NCBI) website (<http://www.ncbi.nlm.nih.gov>; accessed 10/4/08), sequences for the identified virulence factors were obtained and primers designed using Primer3<sup>®</sup> (<http://primer3.sourceforge.net/>; accessed 11/4/08; Table 3.7). DNA was isolated using a Qiagen DNeasy<sup>®</sup> kit (Section 3.3.2.3.1). A 25  $\mu$ l reaction for each virulence factor was performed in a 96-well plate. A PCR reaction mixture of 1  $\mu$ l DNA template, 12.5  $\mu$ l ABgene<sup>®</sup> master mix (Thermo Scientific) 9.5  $\mu$ l of nuclease-free water, and 0.4  $\mu$ M of each primer was amplified in a thermal cycler (Table 3.2). The amplified products were separated by electrophoresis using a 100 bp molecular weight standard (Thermo Scientific) with a 0.5X TBE running buffer (Table 3.2; Section 3.3.2.1.1), and virulence genes identified (Table 3.7).

**Table 3.7** Primer sequences for the detection of virulence factors

Gene	Virulence factor	Primers (5'-3')	Amplicon size (bp)	<sup>a</sup> Housekeeping gene	<sup>b</sup> Accession No/Reference
<i>cna</i>	Collagen-binding protein	CAGCAGTTAGATTTAAGCAC CCTTGTGGAATTGTTACATC	161	<i>yqil</i>	AB266877
<i>hlgA</i>	Gamma-haemolysin	GCAAGGCTTCATTAGCTCTAGA ATATTATAGCCTAATTTCTGAC	189	<i>yqil</i>	L0055
<i>hlgB</i>	Gamma-haemolysin	GGTACTGCTAATGCTGAAGG GTACTAAAGTATCTTTATCAT	172	<i>yqil</i>	L0055
<i>hlgC</i>	Gamma-haemolysin	ATCTGTGAGCTTACTTGCC GCTAATGAATCCTTGCATC	226	<i>yqil</i>	L0055
<i>geh</i>	Lipase (glycerol ester hydrolase)	CGACAAGGTTCTAAACAGT TGTAAGTTGAAGTTGGTGCTG	207	<i>yqil</i>	NC009641
<i>hysA</i>	Hyaluronate lyase	TCAAATCGCTGTGGCTGATA CCGCTCCTGACCACAAGTAT	209	<i>yqil</i>	NC00964
<i>luk-PV-1</i> <i>luk-PV-2</i>	Panton-Valentine Leukocidin	ATCATTAGGTAAAATGTCTGGACATGATCCA GCATCAASTGTATTGGATAGCAAAAGC	433	<i>yqil</i>	(Lina et al., 1999)
<i>icaA</i>	Intercellular adhesion protein (biofilm)	TGGCTGTATTAAGCGAAGTC CCTCTGTCTGGGCTTGACC	669	<i>pta</i>	(Knobloch et al., 2002)

<sup>a</sup> Table 3.1 shows the amplicon size of each housekeeping gene (MLST); <sup>b</sup> Accession number of the sequences used for primer design; bp, base pair

### **3.3.3.4 Expression of virulence factors and toxins**

#### **3.3.3.4.1 Preparation of RNA using an RNeasy® Qiagen Kit**

One bacterial colony from TSA was used to inoculate 10 ml of TSB, and incubated at 37°C using agitation until log phase, optical density (OD) of 0.7 at  $A_{600}$  (6 to 8 h approximately). Bacterial cultures were maintained on ice until use. For RNA preparation, 4.5 ml of bacterial culture was centrifuged ( $16000 \times g$ , 4°C, 6 min). The pellet re-suspended and washed in 3 ml of diethyl pyrocarbonate water (DEPC, Nuclease-free water: DEPC 1:2000) then centrifuged ( $16000 \times g$ , 4°C, 6 min). The pellet was re-suspended in 100  $\mu$ l of TE buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA), to which 6.06 mg/ml lysozyme (Sigma) and 0.12 mg/ml lysostaphin (Sigma) was added and incubated (RT, 90 min).

The RNA extraction was performed using an RNeasy® Kit (Qiagen), as per the manufacturer's instructions. To the bacterial/enzymatic suspension 350  $\mu$ l of the supplied Buffer RLT (containing 10  $\mu$ l/ml of  $\beta$ -mercaptoethanol) was added and vortexed (30 s), then 350  $\mu$ l of 70% (v/v) EtOH added and mixed gently by pipetting. The resulting liquid and precipitate was transferred to an RNeasy® column. The column was centrifuged ( $9500 \times g$ , 15 s) and collection tube discarded. The column was placed in a new collection tube and 350  $\mu$ l of the supplied RW1 buffer added. Following centrifugation ( $9500 \times g$ , 15 s) the column was placed in a new collection tube and DNase digestion performed by adding 80  $\mu$ l of the supplied DNase1 incubation mix (10  $\mu$ l of DNase1 stock solution: 70  $\mu$ l of buffer RDD) directly onto the membrane and incubating (RT, 15 min). Then 350  $\mu$ l of the supplied buffer RW1 was added to the column and centrifuged ( $9500 \times g$ , 15 s) the column placed in a new collection tube and 500  $\mu$ l of buffer RPE (containing 4 volumes of 100% ethanol) was added. Following centrifugation ( $9500 \times g$ , 15 s) the column was placed in a new collection tube and centrifuged to dry the membrane ( $16000 \times g$ ; 1 min) then placed into a clean 1.5 ml microfuge tube. RNase free water (50  $\mu$ l) was added directly onto the membrane and allowed to stand (1 min) followed

by centrifugation (9500  $\times$  g, 1 min) the final elution step was repeated using the RNA elute.

#### **3.3.3.4.2 RNA quantification and standardisation**

Prepared RNA was quantified using a Nano-drop system (Nano-View, GE Healthcare). RNA was standardised by diluting (330 ng/ $\mu$ l) with DEPC water prior to toxin and virulence factor expression investigation.

#### **3.3.3.4.3 Expression of virulence factors using reverse transcription (RT) PCR**

The isolates confirmed by PCR to harbour genes for the tested virulence factors (Section 3.3.3.2 & 3.3.3.3) were investigated further using RT-PCR. Expression of the individual virulence factors was investigated, followed by expression in a multiplex reaction with an internal control (MLST housekeeping gene) to attempt to semi-quantitatively assess levels of expression.

For each positive isolate, a 25  $\mu$ l reaction was performed using OneStep RT-PCR kit (Qiagen®). A PCR mixture of 5  $\mu$ l of 5X Qiagen® OneStep RT-PCR buffer, 400  $\mu$ M dNTPs, 1  $\mu$ l of Qiagen® OneStep Reverse Transcriptase (RT) enzyme mix, 0.6  $\mu$ M final concentration of primer, 1  $\mu$ l of standardised RNA template (330 ng/ $\mu$ l) and 15  $\mu$ l of RNase free water was amplified in a thermal cycler (Table 3.2). Ten  $\mu$ l of product was separated with a 100 bp (Thermo Scientific) molecular weight standard in 0.5X TBE running buffer (Table 3.2). Products were visualised using UV light and expression confirmed by correct band size (Table 3.7). To compare expression levels for each virulence factor, a multiplex PCR reaction was used with a suitable control (MLST housekeeping gene; Table 3.7). A 25  $\mu$ l reaction mixture as described for the singleplex 'in-house' virulence factor using the OneStep RT-PCR kit (Qiagen®) was performed in a thermal cycler (Table 3.2). The amplified products were separated using the parameters in Table 3.2. The products were visualised under UV light using GelDoc™ software (Bio-Rad®). Band intensity was assessed



and visually assigned a numerical value I to III in order of increasing intensity (Figure 3.10).

#### **3.3.3.4.4 Expression of toxins using reverse transcription (RT)-PCR**

Expression of toxins was completed using the four multiplex reactions (Table 3.6). A 25 µl reaction was performed using OneStep RT-PCR kit (Qiagen®) as per the manufacturer's guidelines. A PCR mixture of 5 µl of 5X Qiagen® OneStep RT-PCR buffer, 400 mM of dNTPs (each), 1 µl of OneStep RT enzyme mix, 2 µl of toxin primer mix (Section 3.3.3.2), 1 µl of diluted RNA template and 16 µl of RNase free water was amplified in a thermal cycler (Table 3.2). Electrophoresis was used to separate 10 µl of the amplified product with a 100 bp (Thermo Scientific) and 50 bp (Fisher Scientific) molecular weight standards in 0.5X TBE running buffer (Table 3.2). The products were visualised and assigned intensities (Section 3.3.3.4.3).

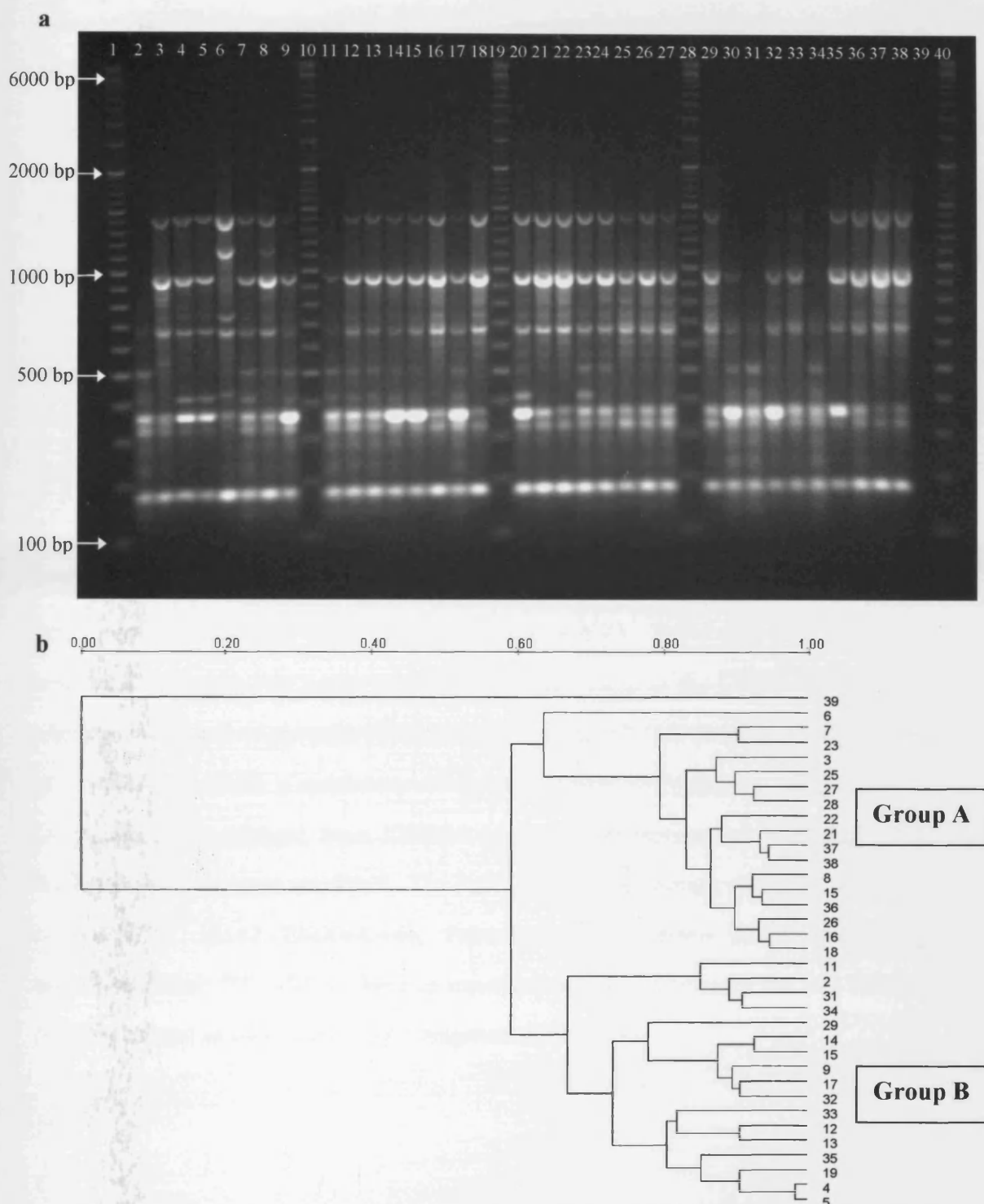
### **3.4 Results**

#### **3.4.1 Genetic typing of MRSA**

##### **3.4.1.1 Random Amplified Polymorphic DNA (RAPD) of MRSA wound isolates**

RAPD fingerprints and analysis using Quantity One® Bio-RAD for each MRSA isolate using RAPD primers 7, E2 and 1 is demonstrated in Figures 3.1 and Appendix IV.i. Unweighted Pair Group Arithmetic Method Analysis (UPGMA) was used to identify relatedness between MRSA isolates and to produce phylogenetic trees for each primer.

In these experiments it was evident that each primer used to determine the phylogeny of the isolates produced different results. For example, primer 7 separated the collected MRSA isolates into 2 major clusters termed A and B (Figure 3.1). In each cluster there were 9 and 8 isolates respectively. Both these groups were similar at 0.60. Primer E2 grouped the wound MRSA isolates into 2 clusters



**Figure 3.1 (a)** Agarose gel showing RAPD fingerprint using Primer 7; **(b)** Phylogenetic tree using UPGMA analysis of Primer 7 fingerprints produced by Quantity One<sup>®</sup> software. Lane numbers match those denoted on the phylogenetic tree. Lanes 1, 10, 19, 28 and 40, 100 bp and 500 bp molecular weight standard (Fermentas; Figure 3.1a); lane 2, MRSA wound isolate; lane 3, EMRSA 15; lane 4, EMRSA 16; lane 5, NCTC 13143; lane 6, NCTC 6571; lane 7, NCTC 12493; lanes 8, 9, 11 to 18, 20 to 27, 29, 31 & 32, MRSA wound isolates; lanes 30 and 33 to 37, CNS, MSSA wound isolates; lane 39, negative H<sub>2</sub>O control.

termed A and B whilst cluster C contained only the control isolates. Cluster A and B contained 5 and 12 isolates respectively; both were similar at 0.60 indicating that the isolates were similar in all groups. Primer E2 grouped duplicate test isolates and indicated high similarity indices (~0.85). Primer 1 separated the majority of isolates into a single group termed C; with only 6 isolates in group B and 1 in group A. Primer 1 showed similarities between these groups at 0.70, indicating similarity between isolates. It was clear from this analysis that none of the primers had 100% matching of group types.

#### **3.4.1.2 Pulsed Field Gel Electrophoresis (PFGE) of MRSA wound isolates**

The analysis of the PFGE macrorestriction profile (Table 3.8) separated the MRSA wound isolates into three major pulse-types; A, B and C, with pulse type C being further subdivided into two subgroups (i and ii). Pulse-type A (Figure 3.2) contained seven (41.2%) isolates and was an exact match to the EMRSA-15 control, whilst pulse-type B (Appendix IV.ii) contained eight (47.1%) isolates closely related to EMRSA 15 (with a maximum of three fragment differences). Pulse-type C (Appendix IV.ii) differed from EMRSA-15 and other isolates by 7 bands, which indicated that they were unrelated. The latter group had a similar fragment pattern to control NCTC 13143 (EMRSA-16). Pulse-type C was further subdivided into Ci and Cii as isolate 1011 did not have an exact match of band patterns but was deemed possibly related as there were only 5 fragment differences.



**Figure 3.2** Pulse-type A fragment restriction digests from PFGE of MRSA wound isolates. Lanes 1 and 14, PFG lambda ladder (standard; New England Biolabs; NEB); lane 2, EMRSA-15 control isolate; lanes 3 to 5, Intensive Therapy Unit (ITU) MRSA control isolate (provided by Dr Robin Howe); lanes 6 to 13, MRSA wound isolates.

**Table 3.8** Distribution of pulse-types associated with fingerprint analysis of MRSA wound isolates using PFGE

	Pulse Types			
	A	B	Ci	Cii
<b>Number of isolates</b>	7 (41.2%)	8 (47.1%)	1(5.9%)	1 (5.9%)

#### 3.4.1.3 Multilocus Sequence Typing (MLST) of MRSA isolates

MLST differentiated the wound MRSA isolates into four sequence types (ST; Table 3.9). ST22 was the most common, representing 82.4% of the MRSA isolates, frequently associated with the hospital (HA-MRSA) setting in the UK. ST30, ST36 and ST970 represented a single isolate in each group. ST970 was associated with patient 1108 and represents a novel sequence type due to a single locus variant (slv) of ST22 at the *aroE* allele (i.e. shikimate dehydrogenase). Amongst the 17 MRSA wound isolates, two clonal complexes or ancestral lines were identified (CC22 and CC30).

In contrast to the MRSA wound isolates, the nasal isolates exhibited a greater variation of ST. This diversity is typical in CA-MRSA (Table 3.10). Isolate S07 150 was associated with a novel ST, 1509 and is related to ST 1 via a slv of the *pta* allele (i.e. phosphate transferase).

#### 3.4.1.4 *ccr* gene complex typing by PCR

Results of the analysis of the *ccr* gene complex for the isolated MRSA are shown in Tables 3.9 & 3.10. All (100%) of the chronic wound MRSA isolates in this study were shown to harbour a type 2 *ccr*, which is a combination of *ccrB2* and *ccrA2* elements (Zhang et al., 2005). Of the nasal isolates studied, 71.4% represented a type 2 *ccr*, with 14.3% representing type 1 (*ccrB1-ccrA1*) and type 5 *ccr* (*ccrC*). Figure 3.3 shows separation of the multiplex PCR products for the *ccr* gene complex typing.

#### 3.4.1.5 *mec* gene complex typing by PCR

Analysis of wound MRSA *mec* gene complex demonstrated that 16 (94.1%) MRSA isolates in the study harboured Class B *mec* gene complex, composed of IS431-*mecA-mecR1*-IS1272 (Zhang et al., 2005). One (5.9%) isolate harboured the Class A *mec* element composed of IS431-*mecA-mecR1-mecI* (Zhang et al., 2005).

**Table 3.9** Chronic wound MRSA isolate typing

No. of isolates	Pulse type	<sup>a</sup> PFGE synonym	<sup>b</sup> <i>agr</i> group	Sequence Type	Clonal Complex	Allelic Profile	<i>ccr</i> type	<i>mec</i> class	SCC <i>mec</i> type	<sup>c</sup> J type
6 (35.3%)	A	EMRSA-15	I	22	22	7.6.1.5.8.8.6	2	B	IV	h
8 (47.1%)	B	EMRSA-15	I	22	22	7.6.1.5.8.8.6	2	B	IV	h
1 (5.9%)	A	EMRSA-15	I	<sup>d</sup> 970	22	7.159.1.5.8.8.6	2	B	IV	h
1 (5.9%)	Cii		III	30	30	2.2.2.2.6.3.2	2	B	IV	c & E
1 (5.9%)	Ci	EMRSA-16	III	36	30	2.2.2.2.3.3.2	2	A	II	n/a

EMRSA, epidemic MRSA; n/a, not applicable; <sup>a</sup> PFGE synonym based on similarities in finger print patterns to EMRSA-15 and EMRSA-16; <sup>b</sup> accessory gene regulator; <sup>c</sup> joining region; <sup>d</sup> novel ST assigned by MLST database curator.

**Table 3.10** Typing of asymptomatic nasal carrier MRSA isolates

No. of isolates	<sup>a</sup> <i>agr</i> group	Sequence Type	Clonal Complex	Allelic Profile	<i>ccr</i> type	<i>mec</i> class	SCC <i>mec</i> type	<sup>b</sup> J type
2 (28.6%)	III	1	1	1.1.1.1.1.1.1	2	B	IV	a
1 (14.3%)	II	5	5	1.4.1.4.12.1.10	2	B	IV	a
1 (14.3%)	III	34	30/39	8.2.2.2.6.3.2	1	B	I	n/a
1 (14.3%)	III	45	45	10.14.8.6.10.3.2	2	B	IV	a
1 (14.3%)	IV	121	51	6.5.6.2.7.14.5	5	C	V	
1 (14.3%)	III	<sup>c</sup> 1509	1	1.1.1.1.169.1.1	2	B	IV	a

n/a, not applicable; <sup>a</sup>accessory gene regulator; <sup>b</sup>joining region; <sup>c</sup>Novel ST assigned by the MLST database curator.



**Figure 3.3** PCR products for *ccr* gene complex typing. The smallest PCR amplicon (286 bp) is the internal *mec* control. Lanes 1 and 20, 100 bp molecular weight standard (Promega); lanes 2 to 13, MRSA wound isolates; lane 14, control isolate PHII.43.2 for type *ccr* 1 (695 bp); lane 15, control isolate CA05 type *ccr* 2 (937 bp); lane 16, control isolate E0898 type *ccr*3 (1791 bp) and type *ccr*C (518 bp); lane 17, control isolate M001.005.2 type *ccr* 4 (1287 bp); lane 18, Irish clinical isolate; lane 19, negative H<sub>2</sub>O control.



**Figure 3.4** PCR products for *mec* class type. Lanes 1 and 19, molecular weight standard 100 bp and 1 kb respectively (Promega); lanes 2 to 12, MRSA wound isolates; lane 13, control isolate CA05 for Class B *mec* type (2827 bp); lane 14, control isolate E0898 for Class A *mec* (1963 bp); lane 15, control isolate WIS for Class C *mec* (804 bp); lane 16, negative H<sub>2</sub>O control; lane 17, negative control NCTC 6571; lane 18, positive control NCTC 12493.



Of the nasal isolates, 85.7% harboured a Class B *mec*, one (14.3%) isolate associated with a Class C *mec* complex composed of IS431-*mecA*- $\Delta$ -*mecR1*-IS431 (Zhang et al., 2005) which was first described by Ito et al., (2004). Figure 3.4 shows the separation of the PCR products and determination of the *mec* class.

#### **3.4.1.6 Combined SCC*mec* typing**

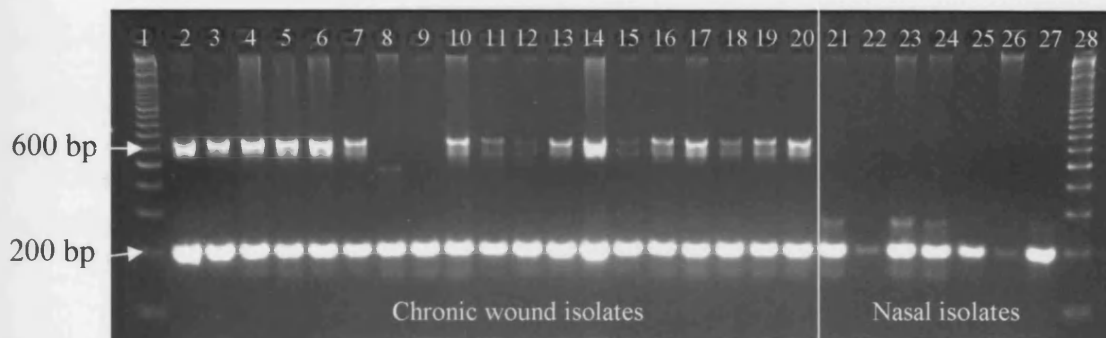
All (100%) chronic wound MRSA isolates harboured type 2 *ccr*, of which 16 isolates (94.1%) carried the Class B *mec* gene complex, and one isolate (5.9%) contained the Class A *mec* element. The combination of these elements designates the SCC*mec* type, for which the majority, 16 (94.1%) of MRSA wound isolates, were type IV, with one patient harbouring the SCC*mec* II element. Of the nasal isolates 85.7% harboured Class B *mec* and one isolate (14.3%) was associated with a Class C *mec* complex and 71.4% of isolates had a type 2 *ccr* and 14.3% were type 1 and 5 *ccr*. Hence, the majority of nasal isolates (71.4%) were classified as SCC*mec* IV, whilst the remainder (14.3%) were SCC*mec* I and V.

#### **3.4.1.7 Joining (J) region typing for classification of SCC*mec* IV variants**

All wound (100%) isolates associated with a ST22-MRSA-IV had an 'h' J region. The one wound isolate identified as ST30-MRSA-IV was sub-typed as IVc & IVE J region. In contrast, all (100%) of the nasal isolates with a class IV SCC*mec* were subtyped as J type 'a'. Figure 3.5 shows the separation of the multiplex reaction for typing of the J region. Tables 3.9 & 3.10 show the distribution of the J typing amongst the MRSA isolates tested.

#### **3.4.1.8 Epidemiology of isolates**

Based on their sequence types, the epidemiology of the MRSA isolates identified amongst the patient group with wounds showed two clonal complexes (CC) or ancestry lines CC22 and CC30. The majority of patients (94.1%) harboured the SCC*mec* element IV, with one patient harbouring the SCC*mec* II element. The



**Figure 3.5** PCR products to determine the joining rejoin for MRSA isolates. Lanes 1 and 28, molecular weight standard 100 bp and 500 bp ladder (Fermentas); lanes 2 to 20, MRSA wound isolates; lane 9, positive control *ccr*, negative for a J region, corresponding to a ST and *SCCmec* of ST36-MRSA-II; lanes 21 to 27, asymptomatic nasal MRSA isolates.



**Figure 3.6** Agarose gel showing the separation of PCR amplified products used to determine the *agr* locus. Lanes 1 and 36, 50 bp molecular weight standard (exACTGene<sup>®</sup>, Fisher); lane 2, negative H<sub>2</sub>O control; lanes 3 and 4, 0.5 and 1 µl DNA respectively of control isolate RN4850; lane 4, control *agr* IV locus (588 bp); lane 5, control isolates TY114 showing *agr* locus III (406 bp); lane 6, control isolate MRSA 252 showing *agr* locus I (440 bp); lane 7, control isolate NCTC 8178 showing *agr* locus III (406 bp); lanes 8 and 9, control isolates Mu50 and NCTC 13130 showing *agr* locus II (572 bp); lane 10; control isolate HT2000132 showing *agr* locus III (406 bp); lanes 11 to 27, MRSA wound isolate; lanes 28 to 35, nasal MRSA isolate.

epidemiological classification for this group of wound MRSA isolates was therefore; 14 (82.4%) ST22-MRSA-IVh, 1 (5.9%) ST30-MRSA-IVc & IVE, 1 (5.9%) ST36-MRSA-II and 1 (5.9%) ST970-MRSA-IVh. In contrast, a greater number of ancestral lines were identified for nasal isolates with 42.9% of isolates associated with a CC1, and the remaining isolates representing CC5, 30/39, 45 and 51. The majority of the nasal isolates harboured SCC*mec* IV (71.4%).

### 3.4.2 Accessory gene regulator (*agr*) typing of MRSA isolates

PCR amplification was used to identify the *agr* locus. Two *agr* types were identified amongst the wound isolates; 15 (88.2%) were associated with *agr* I, and 2 (11.8%) were associated with *agr* III (Table 3.9). Further analysis of the ST and *agr* locus type identified all ST22 & ST970 (CC22) as *agr* I, and ST30 and ST36 (CC30) as *agr* III. *agr* typing of the nasal carrier isolates showed that five (71.4%) were classified as *agr* III and one isolate each (14.3%) as *agr* II or *agr* IV (Table 3.10). Figure 3.6 shows separation of the amplified product used to classify the *agr* types.

### 3.4.3 Virulence factor analysis

Virulence factor profiles for nasal isolates and chronic wound MRSA isolates are shown in Table 3.11. The table shows the percentage of isolates with the presence and expression of the genes studied; whilst also indicating the level of expression intensity.

#### 3.4.3.1 Enterotoxin gene analysis

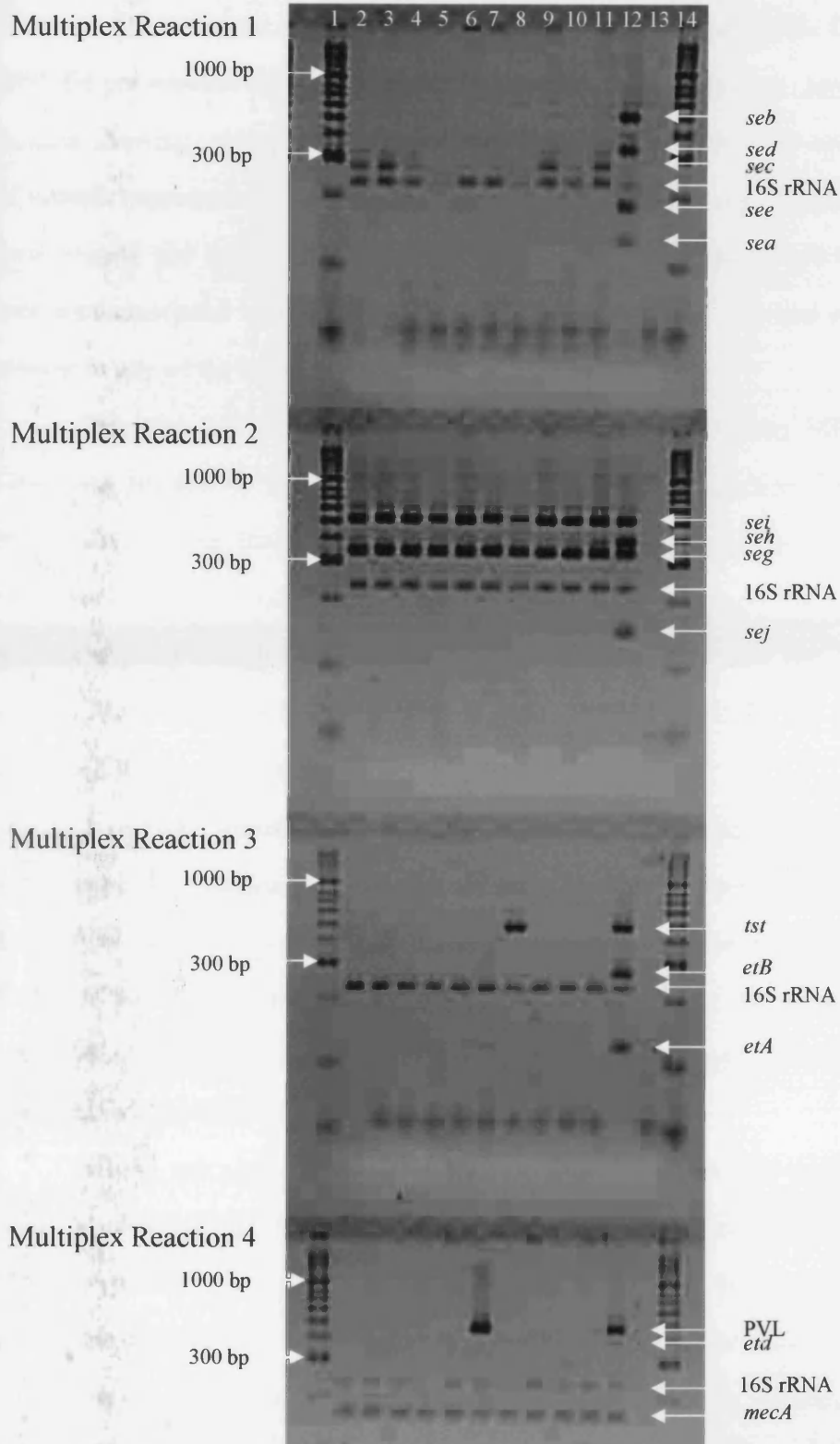
Holmes' multiplex toxin screen (Holmes et al., 2005) was an efficient method to determine the presence and expression of toxins (Table 3.11 & Figure 3.7). This demonstrated diversity in toxin profile gene presence, and variable levels of gene expression in the MRSA isolates.

Several toxin genes (*sea*, *sed*, *sej*, *etA* and *etB*) were only found to be present in the nasal MRSA isolates, with their presence ranging from 14.3% to 71.4% of

**Table 3.11** Virulence factor profiles of MRSA isolates

Virulence factor	Gene present in wound isolate (n=17)	Expression level (%)				Gene present in nasal isolate (n=7)	Expression level (%)			
		0	I	II	III		0	I	II	III
<i>seb</i>	0					0				
<i>see</i>	0					0				
<i>etD</i>	0					0				
<i>etA</i>	0					1				1 (100)
<i>etB</i>	0					1				1 (100)
<i>sed</i>	0					2		1 (50)	1 (50)	
<i>sej</i>	0					2		1 (50)		1 (50)
<i>sea</i>	0					5	1 (20)	2 (40)		2 (40)
PVL	1				1 (100)	0				
<i>tst</i>	1				1 (100)	3			1 (33)	2 (67)
<i>seh</i>	1	1 (100)				5	1 (20)	1 (20)		3 (60)
<i>sec</i>	13			2 (15)	11 (85)	3		1 (33)	1 (33)	1 (33)
<i>cna</i>	14			1 (7)	13 (93)	7		4 (57)	2 (29)	1 (14)
<i>sei</i>	17	1 (6)		4 (23)	12 (71)	4	1 (25)	2 (50)	1 (25)	
<i>seg</i>	17	1 (6)			16 (94)	4		1 (25)		1 (75)
<i>geh</i>	17				17 (100)	7				7 (100)
<i>hlgA</i>	17		1 (6)	3 (18)	13 (76)	7				7 (100)
<i>hlgB</i>	17		2 (12)	5 (29)	10 (59)	7	1 (14)		2 (29)	4 (57)
<i>hlgC</i>	17				17 (100)	7				7 (100)
<i>hysA</i>	17		1 (6)		16 (94)	7	2 (29)	4 (57)	1 (14)	
<i>icaA</i>	17				17 (100)	7				7 (100)

■ Greatest presence of virulence gene comparing wound and nasal groups; ■ Greatest gene expression comparing wound and nasal groups



**Figure 3.7** Typical agarose gel showing PCR products from the four Holmes' multiplex reactions; Lanes 1 and 14, molecular weight standard (Hyperladder II; Bioline); lane 12, positive control; lane 13, negative control; lanes 2 to 11, MRSA wound isolates; lanes 2 to 6 and 9 to 11, ST22-MRSA-IVh; lane 7, ST30-MRSA-IVc & IVE; lane 8, ST36-MRSA-II.

isolates. For toxin *sea*, 40% of isolates expressed the gene as grade III or I, whilst 20% did not express this gene despite its presence being detected. Moreover, those isolates showing grade III expression were associated with type III *agr*. Only 50% of isolates expressed *sed* and *sej*, as grade II and III respectively. Toxins *etA* and *etB* were present and both highly expressed (grade III) by the same single isolate; which was also associated with a type IV *agr*. Three genes (*seb*, *see* and *etD*) were not present in any of the isolates.

Presence and expression levels of genes varied between MRSA sources. Genes *sec*, *seg* and *sei* were found to be present in a high proportion (76.5% to 100% respectively) of the wound MRSA isolates. Conversely, approximately 50% of the nasal MRSA isolates harboured these genes. A high proportion (84.6%) of wound isolates showed a high (grade III) level of expression for toxin *sec*, for which all isolates were associated with an *agr* I type. Only 33.3% of the nasal isolates expressed the *sec* toxin as grade III.

Toxin *seg* showed comparable high levels of expression; (94.1% and 75% with a grade III expression for wound and nasal isolates respectively). Similarly *sei*, like *sec* and *seg*, showed a predominant gene presence in the wound isolates, with 70.6% of the wound isolates having grade III expression of the *sei* toxin. MRSA isolates associated with the *sei* toxin were classed as type I *agr*. In contrast, none of the nasal isolates showed high expression of the *sei* gene.

Whilst *seh* and *tst* were found to be present in both the nasal and wound MRSA isolate groups, they were more prevalent in the nasal isolates (71.4% and 42.9% respectively); both genes were only found in one (5.9%) wound isolate. The toxin gene *seh* was expressed highly (grade III) in 60% of the nasal isolates and all of these were associated with an *agr* III type. The remaining isolates showed low (grade I) or no expression at all. Only one wound isolate carried the *seh* gene, although this was not expressed. PVL was present in only one isolate, associated with a chronic wound and was highly expressed (grade III).

### 3.4.3.2 'In-house' virulence factor analysis

Additional virulence factors, which could possibly play a role in the non-healing phenotype of a chronic wound, were tested. Again, MRSA isolates from wound and nasal carriers showed the presence of the majority of these virulence factors, however, there was variation in the expression in the two MRSA groups (Table 3.11). The use of control strains demonstrated that the 'in-house' PCR primers were appropriate to identify the presence and expression of the genes under investigation.

The genes for the virulence factors *geh*, *hlgA*, *hlgB*, *hlgC*, *hysA* and *icaA* were present in all isolates, with *geh*, *hlgC* and *icaA* classed as grade III expression for all wound and nasal isolates. Figure 3.8 shows the separation of multiplex RT-PCR products for *icaA* and internal control *pta*.

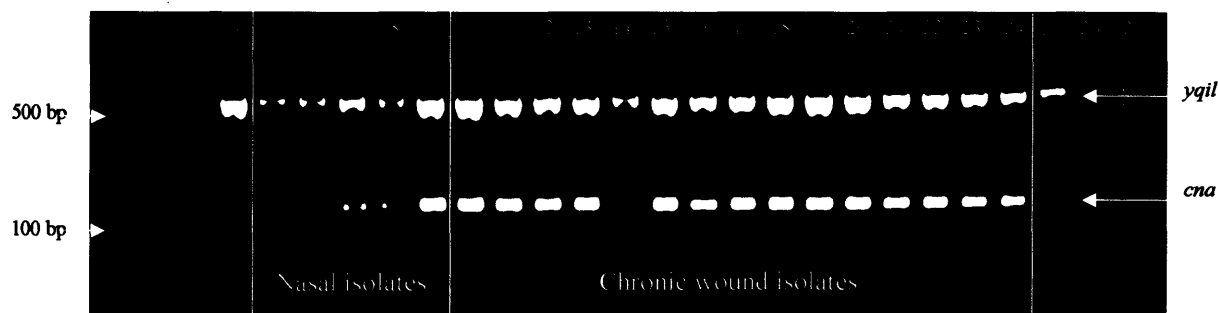
Conversely, collagen binding protein (*cna*) was present in all of the nasal isolates but only 82.4% of wound isolates harboured the gene. Nonetheless, the expression profile was the opposite. A high proportion (92.9%) of all the wound isolates were classed as having grade III expression, with the remaining having grade II expression for the *cna* gene. The majority (57.1%) of the nasal isolates were found to have low expression of the *cna* gene, and only a small number (14.3%) found to have grade III expression for *cna*. Figure 3.9 shows the separation of multiplex RT-PCR products for *cna* and internal control *yqil*.

The *hysA* gene was present in 100% of wound and nasal isolates. In spite of this, the expression of the *hysA* showed considerable variability. Similar to that of *cna*, the majority of the wound isolates (94.1%) were classed as having a high (grade III) expression. The nasal isolates in contrast were found to have a low expression of *hysA*, with 57.1% being classed as grade I and approximately 30% not expressing this virulence factor at all.

Figures 3.10 and 3.11 show separation of singleplex and multiplex RT-PCR electrophoretic gels for the *hysA* and housekeeping gene respectively. When the RT-



**Figure 3.8** Separation of RT-PCR products for *yqil* (516 bp) and *icaA* (669 bp). Lanes 1 and 27, 100 bp molecular weight standard (Thermo); lanes 2 and 3, positive control isolates PJ141 and PJ156 respectively for the *icaA* operon; lanes 4 to 17, wound MRSA isolates; lanes 18 to 24, nasal carrier MRSA isolates; lane 25, negative H<sub>2</sub>O control; lane 26, blank.

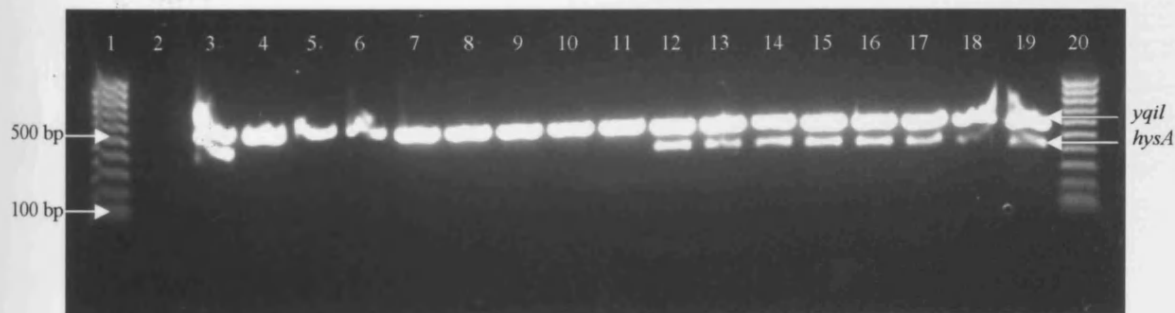


**Figure 3.9** Separation of RT-PCR products for *yqil* (516 bp) and *cna* (161 bp). Lanes 1 and 27, 100 bp molecular weight standard (Thermo); lane 2, nasal carrier MRSA isolate; lane 3, *cna* negative control isolate Mu50; lane 4, positive control isolate MRSA 252; lane 5 to 9, nasal carrier MRSA isolate; lane 10 to 24, wound MRSA isolates, lane 25, *cna* negative control isolate NCTC 8178; lane 26, negative H<sub>2</sub>O control.





**Figure 3.10** Separation of RT-PCR products for *hysA* (438 bp). Lanes 1 and 28, 100 bp molecular weight standard (Thermo); lane 2 negative H<sub>2</sub>O control; lane 3 positive control isolate MRSA 252; lane 4, positive control NCTC 8178; lanes 5 to 15, 22 to 24 and 26 to 27, wound MRSA isolates; lanes 16 to 21 and 24, nasal carrier MRSA isolates. Lane 5, represents grade III expression; lane 18, represents grade II expression and lane 20, represents grade I expression.



**Figure 3.11** Separation of RT-PCR products for *yqil* (516 bp) and *hysA* (438 bp). Lanes 1 and 20, 100 bp molecular weight standard (Thermo); lane 2, and 4, negative controls H<sub>2</sub>O, NCTC 8325 respectively; lane 3 and 5, positive control isolates NCTC 8178, MRSA 252 respectively; lanes 6 to 11, nasal MRSA isolates; lanes 12 to 19, wound MRSA isolates; lanes 5, 6, 7 and 8 correspond to lanes 3, 25, 20 and 21 in Figure 3.10 respectively, classed as grade I expression; Lanes 9, 10 and 11 correspond to lanes 16, 19 and 17 in Figure 3.10 respectively, classed as a grade 0 expression; lanes 12 to 19 correspond to lanes 5 to 12 in Figure 3.10 respectively, classed as grade III expression.

PCR was performed in a multiplex fashion, faint bands (equivalent to grade I expression in the singleplex PCR) were not produced suggesting a possible risk for under-reporting of this gene (Figure 3.11). Those isolates classed as grade III expression however were still distinct. This phenomenon may have resulted from exhaustion of dNTPs or *Taq* polymerase enzyme in the reaction mixture leading to preferential competition with the housekeeping gene. PVL or *lukS/lukF* was only found in one (5.9%) of all the wound patient isolates. Nevertheless, this was expressed as a grade III level and found to be associated with ST30-MRSA-IVa & IVE. None of the nasal isolates harbored or expressed this virulence factor.

#### **3.4.3.3 Comparison of virulence factor profiles for chronic leg and chronic surgical wound MRSA isolates**

Isolates in this study were obtained from wounds representing chronic leg wounds (CLW) and chronic surgical wounds (CSW) as their primary aetiology. Table 3.12 shows the comparison of virulence gene presence and expression between the two wound types.

Whilst the patterns for presence of the virulence genes *sec*, *seg*, *sei*, *geh*, *hlgA*, *hlgB*, *hlgC*, *hysA* and *icaA* were similar irrespective of wound source, variation in gene expression was observed. All the isolates in both the CLW and CSW groups were classed as having a grade III expression for the genes *hlgC*, *icaA* and *geh*. Of those isolates found to have the genes *sec* or *seg* present, all of the CLW's were classed as having grade III expression, whilst between 66.7% and 87.5% of CSW's were classed as a grade III expression respectively.

The expression of *hysA*, *hlgA*, *cna*, and *hlgB* in all (100%) CSW isolates was high (grade III). This expression intensity was not observed in the CLW group for the same genes. The *tst* gene was only harboured by a CLW isolate and was associated with an ST36 MRSA. PVL was present in one CSW isolate and associated with ST30.

**Table 3.12** Expression levels of virulence factors present in chronic leg wound, chronic surgical wound and nasal MRSA isolates

Virulence factor	Gene present <sup>a</sup> CLW MRSA (n=9)	Expression level <sup>a</sup> CLW MRSA (%)				Gene present <sup>b</sup> CSW MRSA (n=8)	Expression level <sup>b</sup> CSW MRSA (%)				Gene present nasal MRSA (n=7)	Expression level nasal MRSA (%)			
		0	I	II	III		0	I	II	III		0	I	II	III
<i>seb</i>	0					0					0				
<i>see</i>	0					0					0				
<i>etD</i>	0					0					0				
<i>etA</i>	0					0					1				1 (100)
<i>etB</i>	0					0					1				1 (100)
<i>sed</i>	0					0					2	1 (50)		1 (50)	
<i>sej</i>	0					0					2	1 (50)			1 (50)
<i>sea</i>	0					0					5	1 (20)	2 (40)		2 (40)
PVL	0					1				1 (100)	0				
<i>tst</i>	1				1 (100)	0					3			1 (33)	2 (67)
<i>seh</i>	1	1 (100)				0					5	1 (20)	1 (20)		3 (60)
<i>sec</i>	7				7 (100)	6			2 (33)	4 (67)	3		1 (33)	1 (33)	1 (33)
<i>cna</i>	7			1 (14)	6 (86)	7				7 (100)	7		4 (57)	2 (29)	1 (14)
<i>seg</i>	9				9 (100)	8	1 (13)			7 (88)	4		1 (25)		1 (75)
<i>sei</i>	9			3 (33)	6 (67)	8	1 (13)	1 (13)		6 (75)	4	1 (25)	2 (50)	1 (25)	
<i>hlgB</i>	9		2 (22)	3 (33)	4 (44)	8		2 (25)		6 (75)	7	1 (14)		2 (29)	4 (57)
<i>hlgA</i>	9		1 (11)	3 (33)	5 (56)	8				8 (100)	7				7 (100)
<i>hysA</i>	9		1 (11)		8 (89)	8				8 (100)	7	2 (29)	4 (57)	1 (14)	
<i>hlgC</i>	9				9 (100)	8				8 (100)	7				7 (100)
<i>geh</i>	9				9 (100)	8				8 (100)	7				7 (100)
<i>icaA</i>	9				9 (100)	8				8 (100)	7				7 (100)

■ Greatest presence of virulence gene comparing wound and nasal groups; ■ Greatest gene expression comparing wound and nasal groups;

<sup>a</sup>Chronic leg wound; <sup>b</sup>Chronic surgical wound.

The following parameters were further correlated with virulence factor presence and associated wound type: relative pain, inflammatory and clinical score based on clinical wound presentation. In this respect, no correlation between wound types, virulence factor profile or any clinical parameters were found.

### **3.5 Discussion**

These studies demonstrated the comparative benefits of the currently available methods for genetic typing of MRSA. RAPD was technically, relatively simple in comparison to the other typing methods described here. The three primers used in this study separated the isolates into two or three clusters with varying results; none of the primers placed the isolates into the same cluster of relatedness. Inter-primer and inter-cluster variance made interpretation of the results ambiguous. Interestingly, Primer 1 placed the majority of isolates in one group which correlated with the more complex techniques. However, the three primers highly related each cluster.

RAPD is a recognised method for rapid initial identification of relatedness in outbreaks of nosocomial isolates (Witte et al., 2006), yet, this study demonstrated that multiple variables affected the distinction of groups e.g. thermo cycler used (results not shown) and primer design. This is supported by similar studies, which demonstrated similarly contrasting results obtained in individual laboratories (personal communication with Dr A. Shore; Witte et al., 2006). In these studies, the analysis of band patterns was difficult to perform and essentially arbitrary, a finding which may explain the significant grouping differences. These discrepancies have been predominantly attributed to the detection of small/faint bands, accuracy of the software package, and UV exposure of the original gel.

In contrast to RAPD, PFGE was highly discriminatory, although associated with significant cost and time implications. These studies demonstrated that the use of the criteria described by Tenover et al., (1995) to interpret patterns was both

simple and reproducible. The PFGE method identified the isolates into three main groups or pulse-types and was able to relate the isolates to their clonal ancestry. Whilst PFGE has its place in an outbreak setting, variances in switch times and inter-laboratory equipment may produce varying patterns of fragments between laboratories.

Multilocus sequence typing is described as the “gold standard” of MRSA typing (Urwin and Maiden, 2003). This method was simple and the least ambiguous of the typing methods employed. Methodological complexity and cost were, however, inherent. Comparing PFGE and MLST data; it is important to note that those isolates classified as ST22 via MLST were represented by PFGE pulse types A and B. For this discriminatory reason and cost implications, many hospital microbiological laboratories continue to use PFGE in an outbreak situation; as point mutations may not be identified using MLST if the genetic mutation has occurred outside the house-keeping gene, causing less accurate resolution of the isolates (Shopsin et al., 1999, Grundmann et al., 2002, Montesinos et al., 2002, Harmsen et al., 2003).

MLST divided wound isolates into four sequence types. The two clonal types associated with the wound patient cohort were, ST22 representing MRSA clone EMRSA-15 and ST36 representing EMRSA-16. These are the two most common hospital-acquired pandemic clones circulating in the UK. In this study population, it is impossible to determine whether these isolates were acquired due to hospitalisation, outpatient attendance, or long-term community residences, which are risk factors for colonisation in this elderly population. Whilst healthcare providers are blamed for the spread of MRSA (due to lack of hygiene), no consideration of the possible role that patients who harbour these isolates attending out-patient clinics and hospitals, interacting with other vulnerable patients may play (Halcon and Milkus, 2004). A study of skin and soft tissue infections associated with MRSA identified that 75% were associated with CA-MRSA compared to 37% of HA-MRSA (Tenover and Gorwitz, 2006). These proportions were not seen in this group

of isolates, presumably because these are chronic wounds and hence more likely to be associated with hospital contact.

The study of the *SCCmec* element by Oliviera et al., (2002) showed evidence that MRSA clones have arisen more than once by the acquisition of different *SCCmec* elements (Urwin and Maiden, 2003); MLST alone has now been described as not being discriminatory enough for detailed epidemiological typing (Witte et al., 2006). All of the wound MRSA isolates were identified as harbouring type 2 *ccr* and having either a Class B *mec* or Class A *mec* gene complex. These *SCCmec* types are typical for EMRSA-15 and EMRSA-16 respectively. When ST36 (i.e. EMRSA-16) evolved from ST30 it exchanged its *SCCmec* element from a IV to II, examples of which are observed here in patients 1011 (ST30 *SCCmec* IV) and 1021 (ST36 *SCCmec* II; Robinson and Enright, 2003).

The wound MRSA isolates in this study were associated with two ancestor clonal complexes CC22 and CC30, whilst the nasal MRSA isolates were associated with a more diverse range of clonal complexes. This was expected due to the likelihood of harbouring CA-MRSA in the asymptomatic nasal carriers. This pattern is typical of CA-MRSA and HA-MRSA, with CA-MRSA being more diverse due to the frequent acquisition and loss of the *SCCmec* element (Holden et al., 2004, Collery et al., 2008, Diep and Otto, 2008, Argudin et al., 2009).

The *SCCmec* type of nasal isolates was typically associated with a type IV *mec* element (71.4%); asymptomatic carrier status is more likely to be associated with CA-MRSA, for which this *SCCmec* element is common. Isolates S07 229 and S07 191 were classified as ST5-MRSA-IV and ST45-MRSA-IV respectively which represent the 'paediatric' and 'Berlin' clone of MRSA (Enright et al., 2002). The type IV *mec* element is smaller and believed to be more mobile, facilitating transfer between staphylococcal sp. and of less 'fitness cost' (Lee et al., 2007, Diep and Otto, 2008, Zakour et al., 2008, Elston and Barlow, 2009).

EMRSA-15 or ST22 predominated in the wound group of patients (82.4%) and was classed as HA-MRSA (as is ST36 or EMRSA-16). The greater proportion

of ST22 seen in this cohort of patients may be associated with a transition of ST in the hospital setting. Indeed this has been noted previously; ST36 or EMRSA-16 is now becoming less prevalent in the hospital setting (O'Neill et al., 2001, Murchan et al., 2004, Ellington et al., 2010). This may be related to the acquisition of the smaller *SCCmec* IV, or in this patient cohort associated with the wound environment.

Use of the *SCCmec* element as a typing tool is evolving, with additional genetic components being used in the typing of MRSA. It is now common to distinguish the J region of isolates with *SCCmec* IV. This has arisen as there are a number of ST's, with specific reference to community isolates, that contain the *SCCmec* type IV. The J region sits outside of the *ccr/mec* gene complex on the *SCCmec* element. A large proportion (88.2%) of the wound group isolates were associated with an 'h' type J region. This is a similar finding to other studies (Milheirico et al., 2007, IWG-SCC, 2009). All nasal isolates with a *SCCmec* IV had an 'a' type J region.

MRSA is continuously in the press and continues to cause problems in the medical world. With the advent of increased CA-MRSA carriage rates, MRSA associated infections are likely to increase. There has also become a blurring of the boundaries regarding CA-MRSA and HA-MRSA. This is, in part, associated with insufficient clarity of definitions in previous publications, plus some ST associated CA-MRSA isolates are now becoming resident in the hospital setting e.g. USA300. In the long-term, it is likely, therefore, that typing methods in hospital settings will become increasingly sequence-based. In future studies, the use of more recent typing methods e.g. single-nucleotide polymorphisms may provide greater discrimination between isolates and improve disease and virulence factor correlation (Harris et al., 2010).

Due to the low numbers of wound isolates with MRSA in this study and an uneven distribution of the STs, it was impossible to describe correlation with wound severity/chronicity and ST. The accessory gene regulator (*agr*) type has been linked

to disease (Robinson et al., 2005) with *agr* types I and II linked with enterotoxin mediated disease and *agr* types III and IV associated with non-invasive and exfoliating disease (Jarraud et al., 2002). Further analysis of the ST and *agr* type identified all ST 22 & 970 (CC 22) as *agr* I and finally ST30 and ST36 (CC30) as *agr* III, in keeping with previous studies (Rossney et al., 2007). It is interesting that the majority of wounds (88%) were associated with ST22 and *agr* I in keeping with the invasive nature of the disease process. Furthermore, it is remarkable that the majority of the isolates obtained from asymptomatic nasal carriers were associated with an *agr* III, which is typically associated with non-invasive disease (Jarraud et al., 2002, Holtfreter et al., 2007).

This observational study aimed for the first time to identify potential virulence factors associated with disease in wound patients and asymptomatic nasal carriers. The virulence factors tested were chosen based on their potential advantage within the host tissues i.e. colonisation of the host (*cna*, *icaA*; Holderbaum et al., 1987, Arciola et al., 2001), survival and evasion of the host immune system (*geh*, *hlgA*, *hlgB*, *hlgC*, *icaA*, PVL; Dalla Serra et al., 2005, Holmes et al., 2005, Kropec et al., 2005), and importance in tissue destruction (*geh*, and *hysA*; Makris et al., 2004, Burlak et al., 2007).

Virulence factor screening of enterotoxins and PVL has become more common in reference laboratories (Monday and Bohach, 1999, Holmes et al., 2005), yet little is known of the expression of other virulence factors produced by staphylococci in human diseases states, where they may play a role in disease progression/chronicity e.g. chronic wounds.

In this study, the expression of a number of enterotoxins (i.e. *sec*, *seg*, and *sei*) were significantly greater in the wound isolates associated with *agr* I type (data not shown). This resembled previous studies (Jarraud et al., 2002), where the genes *seg* and *sei* were found grouped in the enterotoxin gene cluster (*egc*) on a genomic island  $\nu$ SA $\beta$  (Lindsay and Holden, 2006, Tristan et al., 2007, Sauer et al., 2008, Argudin et al., 2009, Varshney et al., 2009). Enterotoxin-mediated disease is usually



associated with gastro-intestinal (GI) bacteria, causing lysis and affecting epithelial permeability of the intestinal wall (Johnson et al., 1991b). It is possible that these enterotoxins may play a role in the damage of skin epithelial cells and therefore a link to epithelial disruption and non-healing of these wounds.

As previously described, only isolates associated with *agr* III expressed *etA*, *etB* and *tst*, (Jarraud et al., 2002, Argudin et al., 2009), with *etA* and *etB* present only and expressed by the same isolates. Co-expression of *tst* with *sea* has previously been described (Johnson and Yu, 1991a, Nashev et al., 2004, Tristan et al., 2007, Argudin et al., 2009) and is often related to EMRSA-16 or ST36 (Murchan et al., 2004) as in this study.

Biofilm production is an aid to bacterial colonisation, but also provides a barrier to host defences, antibiotics, and may help with genetic transfer between the various species that form the biofilm (Gilbert et al., 2002, Peacock et al., 2002, Diep and Otto, 2008, Varshney et al., 2009). Biofilms cause major problems in the health care setting in terms of colonisation of medical devices such as catheters and titanium implants (Fürst et al., 2007, Salvi et al., 2008). Unsurprisingly, both wound and nasal isolates harboured and expressed *icaA*. Clinical strains of *S. aureus* commonly harbour the *icaA* operon (Cramton et al., 1999, Fluckiger et al., 2005, Otto, 2008). A recent study by Kouidhi et al., (2010) found that only 60% of *S. aureus* from the oral cavity harboured the *icaA* operon were able to produce a slime layer. Whilst genetic testing is valid, gene expression alone however, does not necessarily reflect biofilm synthesis. This may be interesting to investigate further, comparing the two distinct groups (wound vs. nasal). It is important to remember however, that a large number of environmental factors (e.g. shear forces, oxygen concentration and bacterial species) regulate biofilm establishment in the *in vivo* environment, all of which are difficult to reproduce *in vitro*.

The ability of an organism to bind to extracellular matrix (ECM) may play a role in adhesion, colonisation and persistence. In this study, whilst the presence of *cna* was predominant in the nasal isolates, the expression of *cna* in wound isolates

was significantly greater; a finding which may relate to exposure of the wound bacterium to collagen in the wound bed. The level of *cna* gene presence reported here is greater than reported in other studies, which have shown that 50% of isolates carried the gene and that *cna* is not constitutively present (Holderbaum et al., 1987, Deurenberg et al., 2007). A possible reason for the greater predominance of *cna* in this study may be due to the large numbers of ST22 isolates. Several studies show that *S. aureus* harbouring the *cna* gene are associated with increased pathogenicity (Holderbaum et al., 1987, Patti et al., 1994, Wassenaar and Gaastra, 2001). In support of this, *cna* has been reported to be less important in the adherence of bacteria in “collagen-free” environments, but more important for persistence when there is exposed collagen as would be the case in the wound environment (Patti et al., 1994). Confirming these results, other studies show a greater association of *cna* with disease than colonisation (Peacock et al., 2002, Nashev et al., 2004). In comparing the chronic surgical and leg wound isolates, a difference was observed in the level of *cna* expression. All the surgical wound isolates expressed this gene at a high level; a finding which may reflect the lack of environmental advantage. Rather, *cna* may confer further resilience in an environment rich in ECM e.g. collagen in the exposed wound bed.

$\gamma$ -haemolysin is a complex protein made of a number of subunits. It is able to lyse erythrocytes and leucocytes, leading to evasion of the host and aid bacterial colonisation. Whilst the expression of these genes was similar in wound and nasal isolates, a significant difference in expression was observed when comparing chronic leg and surgical wounds. In this case, all surgical wound isolates harboured all 3 genes (*hlgA*, *hlgB* and *hlgC*), all with high levels of expression (grade II and III). In comparison, chronic leg isolates expressed *hlgA* and *hlgB* proportionally less. Although PVL may play a role in destruction of the host immune response, it was only found in a single surgical wound. Interestingly, PVL subunits may unite with components of the *hlg* family to produce a functional protein for leucocytosis (Cooney et al., 1993, Lina et al., 1999, Dalla Serra et al., 2005).

Hyaluronate lyase (*hysA*) is able to degrade hyaluronic acid (HA) which is a major component of the ECM in the skin and consequently in wounds. It has been suggested that degradation of HA in tissue facilitates bacterial invasion of the tissues (McClean, 1943). HA degradation and the generation of low mw HA fragments stimulate the immune response via Toll-like receptor (TLR) mediated mechanisms (Chapter 4). The degradation of this important ECM component may contribute to prolonged inflammation and a non-healing phenotype. In keeping with this, a significant difference in expression of *hysA* was observed between wound (94.1%) and nasal (0%) isolates at grade III expression. Moreover wound isolates from surgical and leg wounds exhibited high (grade III) expression of *hysA*, (100% and 88.9% respectively), suggesting a potential role in wound colonisation and persistence of MRSA.

Overall this study has demonstrated clear differences in the expression of virulence factors by MRSA isolates, which are dependant on their colonising environment. This may indicate that the bacteria present and their secretory factors have an important role in the non-healing phenotype of chronic wounds, as observed for the expression of *cna*, and *hysA* genes when comparing the wound vs. asymptomatic carrier.

The results of this study should, however, not be overestimated. It is a relatively small study group (24 patients) and the relationships merely observational. Areas where the data are less easy to interpret include the finding that 6 STs were present in the nasal carrier group; whereas within the chronic wound group ST22 was predominant, thus it is not possible to say if these isolates express these factors based on environment or due to its strain genetic background.

Retrospectively, a more satisfactory study design would be to include patient matched samples including both wound and non-wound sites e.g. nose, skin (undamaged), for comparison with asymptomatic carriers. In these studies RT-PCR was employed to study the expression of virulence factors of interest. Whilst it is a useful screening tool, the categorisation of expression by this technique, although

employed in previous studies, is by no means definitive. Further analysis with Northern blotting or quantitative RT-PCR (qRT-PCR) to identify real-time expression of the virulence factors would be needed to confirm these data quantitatively. Further investigation into other virulence factors such as proteases may also prove valuable. The use of gene array technology would be a valuable screening tool followed by proteomic and metabolomic studies to study bacterial physiology as a whole.

A consideration in the interpretation of these results and other studies is that the expression of virulence factors in this study was performed using bacteria grown in a planktonic environment. It is known that bacterial gene/protein expression is influenced by a variety of factors (e.g. matrix, O<sub>2</sub> stress, biofilm formation and growth states) and it is impossible to exactly model these environmental features. As biofilms characterise human wounds, studies of these bacteria in a biofilm environment may be useful.

The study shows a potential role for virulence factor expression in wound-associated MRSA on non-healing. Virulence factor expression was generally widespread amongst the MRSA isolates. Notionally, the moist, nutrient-rich bed of the wound is an ideal environment for the exchange of genetic resistance, pathogenicity islands and mobile virulence factors. Therefore, further study of virulence factors, to decipher their effect on wound healing is warranted. This may lead to the development of new therapeutics to control virulence factor production, to potentially aid the healing of wounds and with that, associated increases in patient care.

## **Chapter 3 part II: Problems associated with PVL detection**

Whilst investigating the virulence profiles of MRSA isolated from chronic wounds and asymptomatic nasal carriers (Chapter 3, part I) it was observed that a number of the wound isolates associated with ST22-MRSA-IVh (EMRSA-15) appeared to be borderline positive for PVL. Using PCR primers designed by Lina et al., (1999) faint bands were sporadically observed on agarose gels, whilst negative controls were clearly negative and positive controls clearly positive. It was thought unlikely that so many ST22 (EMRSA-15) from such a small study group were likely to harbour the PVL gene based on previous reports (Vandenesch et al., 2003, Rossney et al., 2007). However, due to this banding inconsistency, it was difficult to assign a virulence profile for these particular isolates. Interestingly, following personal communication, this phenomenon had previously been experienced by Dr M. Wootton of the Specialist Antimicrobial Chemotherapy Unit (SACU), Cardiff, Wales and Dr A. Shore, Trinity College, Dublin; however the reason for this had not been investigated.

### **3.6 Aims**

The aim of this chapter was:

1. To further investigate the cause of inconsistent ambiguous band profiles experienced with the clinical wound isolates when investigating the presence of the PVL gene.
2. To determine the presence of PVL in chronic wound MRSA isolates using PCR-based techniques (using three different DNA preparations), sequence-based identification, Southern hybridisation and DNA microarray techniques to describe their true virulence profile.

### **3.7 Materials and Methods**

#### **3.7.1 Bacteria and growth media**

All chronic wound MRSA isolates (n = 17) were cultured as previously described (Chapter 2, Section 2.3.2). In addition sixteen MRSA bacterial isolates positive and negative for PVL carriage were acquired from Dr M. Wootton, SACU, Cardiff, for blind testing alongside the chronic wound isolates. Each isolate was cultured on blood agar (BA; LabM) supplemented with 5% defibrinated sheep blood.

#### **3.7.2 DNA preparation using Qiagen DNeasy® kit and 3 h lysis (Qiagen® 3 h)**

The isolation of DNA from the chronic wound MRSA, PVL control MRSA strains and nasal MRSA carrier isolates has been previously described (Section 3.3.2.3.1).

#### **3.7.3 DNA preparation using microLYSIS® kit**

This method is employed by the Health Protection Agency (HPA), London. Freshly subcultured bacteria were grown O/N on nutrient agar (Oxoid) at 37°C. One colony of the test isolate was re-suspended in 20 µl of microLYSIS® solution (Microzone Ltd, West Sussex, UK) and placed in a thermocycler to isolate DNA using the following parameters; 65°C for 5 min, 96°C for 2 min, 65°C for 4 min, 96°C for 1 min, 65°C for 1 min and 96°C for 30 s; hold at 20°C until use.

#### **3.7.4 DNA preparation using Qiagen DNeasy® kit and 30 min lysis (Qiagen® 30 min)**

This method is employed by SACU. Bacteria were grown overnight on TSA plates at 37°C. Half a loop of bacterial growth was added to 200 µl of distilled water in microfuge tubes and mixed by pipetting. The bacterial suspensions were centrifuged (7500 x g, 10 min). The supernatant was removed and re-suspended in 180 µl of lysis buffer (20 mM Tris-HCl, pH 8.0; 2 mM sodium EDTA pH 8.0; 1.2% Triton® X100; 20 mg/ml lysozyme), then 10 µl of 1 mg/ml of lysostaphin was added

to each bacterial suspension and mixed gently by pipetting. The suspension was incubated (37°C, 30 min), then 25 µl of proteinase K and 200 µl buffer AL (Qiagen® kit) was added and the suspension vortexed for 5 s, followed by incubation (56°C, 30 min). Following incubation, 200 µl ethanol (96%; v/v) was added and mixed by pipetting and again vortexed for 5 s. The suspension was then transferred to a DNeasy® mini spin column, and centrifuged (8000 x g, 1 min). The flow-through was discarded, 500 µl of buffer AW1 (Qiagen® kit) added and the column was centrifuged (8000 x g, 1 min). The flow-through and collection tube was once again discarded, 500 µl buffer AW2 (Qiagen® kit) added and the column was centrifuged (18600 x g, 3 min) to dry the DNeasy membrane. The column was placed in a sterile microfuge tube and 100 µl of buffer AE (Qiagen® kit) was pipetted directly onto the DNeasy® membrane and incubated for 1 min at RT. The column was subsequently centrifuged (8000 x g, 1 min) to elute the DNA.

### **3.7.5 Identification of PVL using Lina PVL PCR primers**

Identification of PVL in both chronic wound and SACU control PVL MRSA isolates was performed using primers designed by Lina et al (1999), Table 3.13. DNA was isolated from wound MRSA using two DNA preparation methods; Qiagen® 3 h and Qiagen® 30 min DNA preparations (Section 3.7.2 & 3.7.4), and DNA for the SACU control PVL MRSA isolates using Qiagen® 3 h method only (Section 3.7.1 & 3.7.2). PCR was performed as previously described (Section 3.3.3.3).

### **3.7.6 Identification of PVL using Fey PVL PCR primers**

DNA was extracted using the Qiagen® 3 h method (Section 3.7.2). A 25 µl PCR reaction was performed using the Fey PVL PCR primers (Table 3.1.3; Fey et al., 2003). The PCR reaction mixture consisted of 1 µl of DNA template, 5 µl of 10X buffer, 3 mM MgCl<sub>2</sub>, 0.4 µM of each primer, 1.25 U of *Taq* DNA polymerase,

**Table 3.13** PCR primers used to amplify Panton-Valentine leucocidin (PVL) gene

Primer name	Virulence factor	Primers (5' to 3')	Amplicon size (bp)	Reference
<i>luk-PV-1</i> <i>luk-PV-2</i>	PVL	ATCATTAGGTAAAATGTCTGGACATGATCCA GCATCAASTGTATTGGATAGCAAAAGC	433	Lina et al., (1999)
<i>lukS-PV</i> <i>lukF-PV</i>	PVL	GGCCTTTCCAATACAATATTGG CCCAATCAACTTCATAAATTG	1554	Fey et al., (2003)

PVL, Panton-Valentine Leucocidin; bp, base pair.

**Table 3.14** PCR primers used in reaction 4 of multiplex toxin screen (Holmes et al., 2005)

Primer name	Virulence factor/ Gene	Primers (5' to 3')	Amplimer size (bp)	Reference
<i>etd-1</i> <i>etd-2</i>	<i>etd</i>	AACTATCATGTATCAAGG CAGAATTTCCCGACTCAG	376	Yamaguchi et al., (2002)
<i>luk-PV-1</i> <i>luk-PV-2</i>	PVL	ATCATTAGGTAAAATGTCTGGACATGATCCA GCATCAASTGTATTGGATAGCAAAAGC	433	Lina et al., (1999)
<i>mecA-P4</i> <i>mecA-P7</i>	<i>mecA</i>	TCCAGATTACAACCTTCACCAGG CCACTTCATATCTTGTAACG	162	Oliveira and de Lencastre (2002)
<i>sal6S</i>	16S rRNA	GTAGGTGGCAAGCGTTATCC CGCACATCAGCGTCAG	228	Monday and Bohach (1999)

*etd*, exfoliative toxin D; PVL, Panton-Valentine Leucocidin; *mecA*, meticillin resistance determinant; rRNA, ribosomal ribonucleic acid; bp, base pair.



200  $\mu$ M (each) deoxynucleoside triphosphates (dNTPs) and 11.25  $\mu$ l of nuclease free water, all reagents were obtained from Promega, UK. The PCR reactions were performed with an initial denaturation step (94°C, 5 min), followed by 35 cycles (denaturation, 94°C, 45 s; annealing, 55°C, 45 s; extension, 72°C, 105 s), followed by a final extension step (72°C, 10 min). The products were separated using electrophoresis alongside Hyperladder III molecular weight standard (Bioline Ltd, London, UK) in a 2% agarose gel containing ethidium bromide (0.5  $\mu$ g/ml) for 1 h at 120 V.

### **3.7.7 Identification of PVL using a multiplex toxin screen**

PVL presence in the chronic wound MRSA was identified using Holmes et al., (2005) multiplex PCR toxin screen reaction 4 (Section 3.3.3.2; Table 3.14) using 1:10 diluted Qiagen<sup>®</sup> 3 h (Section 3.7.2) and microLYSIS<sup>®</sup> prepared DNA (Section 3.7.3).

### **3.7.8 Identification of PVL using Southern blotting and DNA hybridisation**

Following multiple PCR amplification attempts to correctly identify the PVL positive chronic wound isolates using the Qiagen<sup>®</sup> 3 h DNA preparation and Lina PVL PCR primers (Section 3.7.2 & 3.7.5). Southern hybridisation was performed on an agarose gel of separated PVL PCR products. The gel was photographed (GelDoc<sup>™</sup>, Bio-Rad<sup>®</sup>) and then placed in denaturing solution (0.5 M NaOH; 1.5 M NaCl) for 30 min at RT with continuous agitation. The gel was then soaked at RT for 30 min in a neutralising solution (0.5 M Tris-HCl, pH 7.5; 1.5 M NaCl). A nylon membrane (Hybond<sup>™</sup>, GE Healthcare, Amersham) was cut to the size of the gel and soaked in 2X saline-sodium citrate buffer (SSC; 3 M NaCl; 0.3 M Na<sub>3</sub>C<sub>6</sub>H<sub>5</sub>O<sub>7</sub>, pH 7.0) for 5 min. The neutralised gel was blotted using a wick of Whatman 3MM paper (Whatman<sup>®</sup> Ltd, GE Healthcare, UK) with each end submerged in 20X SSC. The pre-soaked nylon membrane was placed over the gel. A sheet of 3MM Whatman filter paper previously soaked in 20X SSC was placed over the nylon

membrane followed by 3 sheets of dry 3MM Whatman filter paper and 5 sheets of absorbent blotting paper (Quickdraw, Amersham). Several layers of paper towels were placed on the blotting stack. A Perspex plate and weight were finally placed on the filters O/N.

The nylon membrane was then removed and DNA fixed using UV light for 4 min. The nylon membrane was placed into a glass hybridisation tube (Hybaid) and 20 ml of pre-hybridisation solution was added (6X SSC, 0.1%, w/v, polyvinylpyrrolidone; 0.1%, w/v, ficoll; 0.1%, w/v, bovine serum albumin, BSA; 0.5%, w/v, sodium-dodecyl sulphate, SDS; 10mg/ml herring DNA) and incubated (65°C, 5 h).

A probe for the identification of PVL was prepared by extracting the positive control (NCTC 6571) PVL band from an agarose gel followed by purification using QIAquick® Gel Extraction Kit. Briefly, the agarose gel band was placed in a microfuge tube, and 300 µl of buffer QG (supplied) was added and incubated (RT, 15 min), to which 100 µl isopropanol was added. The sample was applied to a QIAquick® spin column and centrifuged (17900 x g, 1 min) the flow through was discarded and 500 µl of buffer QG added to the membrane and re-centrifuged (17900 x g, 1 min) the membrane was washed with 750 µl buffer PE (supplied) for 2 min, then centrifuged (17900 x g, 1 min) the flow through was discarded and column re-centrifuged (17900 x g, 1 min). The column was placed in a clean microfuge tube 50 µl of nuclease-free water was added directly to the membrane and incubated (RT, 1 min) followed by centrifugation (17900 x g, 1 min) to elute the DNA. The resulting flow through (extracted band) was labelled with <sup>32</sup>P dCTP random primer extension (Prime-It II Random Primer Labelling Kit, Stratagene, USA).

Twenty-one µl of the DNA was combined with 10 µl of random primers (Stratagene kit). The sample was placed in a boiling waterbath for 5 min, then 10 µl of buffer dCTP (Stratagene kit), 2.5 µl of <sup>32</sup>P and 1 µl Klenow was added to the sample and placed in a lead jar (37°C, 15 min). Removal of unused nucleotides was

performed using a G50 Sephadex column (Nick column, Pharmacia). After putting the sample onto the column it was washed with 320 µl of buffer (0.1 M Tris-HCl, pH 7.5). The column was then placed over a new collecting tube to collect the probe by washing the column with 430 µl of buffer (0.1 M Tris-HCl, pH 7.5). The collected solution was placed in a boiling waterbath for 6 min to denature the DNA. This probe was immediately added to the hybridisation tube containing the nylon membrane and allowed to incubate (65°C, O/N).

Following hybridisation, the probe was removed and stringency washes performed; the nylon membrane was washed in 300 ml of 2X SSC containing 0.1% (w/v) SDS (65°C, 15 min), followed by 0.1X SSC containing 0.1% (w/v) SDS (65°C, 15 min). The membrane was blotted with 3MM Whatman paper and wrapped in clingfilm. Using an intensifying screen and Amersham Hyperfilm<sup>TM</sup>-MP (GE Healthcare, UK) an autoradiographic image was recorded (4 h exposure).

### **3.7.9 Sequencing of PVL products**

A number of PCR reactions for the chronic wound MRSA resulted in faint bands of the correct size for PVL (433 bp) using the Lina PVL PCR primers. In contrast, the positive controls using these primers had brightly defined bands and the negative controls (H<sub>2</sub>O and Mu50) showed no bands at all. The Fey PVL PCR primers also produced some faint bands for the chronic wound MRSA isolates. Again the negative controls produced no bands and the positive controls produced bright, defined bands of the correct size (1554 bp). The PCR products for chronic wound isolates exhibiting faint indiscriminate bands, and PCR products for positive and negative controls and a selection of positive SACU PVL controls were sequenced. The PCR products were purified using ExoSAP-IT<sup>®</sup> (Section 3.3.2.3.4), and sequenced as described (Section 3.3.2.3.5) using the appropriate primer set (Table 3.13). The sequences were analysed using Basic Local Alignment Search Tool (BLAST; <http://blast.ncbi.nlm.nih.gov>).

### **3.7.10 PVL identification by DNA microarray**

DNA microarray analysis was performed by Dr A. Shore, School of Dental Science, Trinity College, Dublin on three isolates from the chronic wound patients exhibiting faint PCR bands (1009, 1057, 2018), chronic wound isolate 1011 and positive control NCTC 6571 (showing an unequivocal positive signal) using the StaphType kit (Alere, Jena, Germany).

## **3.8 Results**

### **3.8.1 Identification of PVL with Lina PVL PCR primers (Qiagen® 3 h)**

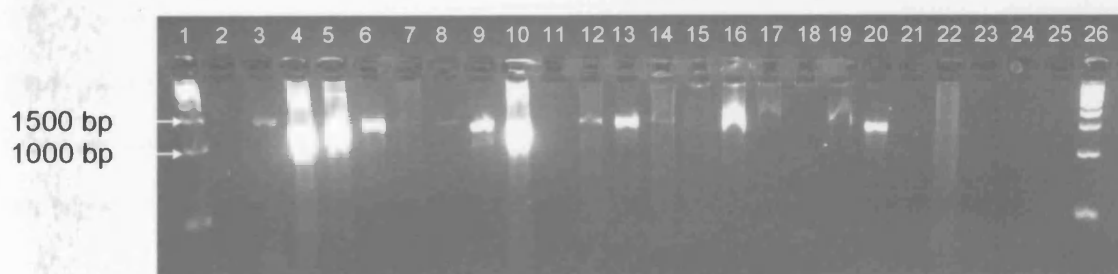
Using this method, no nasal isolate was found to harbour PVL genes. Figure 3.12 demonstrates a representative agarose gel of PCR products produced by DNA from chronic wound isolates using Lina PVL PCR and the Qiagen® 3 h DNA preparation method. The gel shows that whilst the PCR products were of the correct size (433 bp) a range of band intensities was produced. The positive control isolates (NCTC 6571) was significantly positive, and the negative control isolates (H<sub>2</sub>O and Mu50) showed no band at all. However, there were faint bands for a number of samples (e.g. lanes 4, 5, 6, 7, 15, 22). Lane 4 contained isolate (NCTC 8178, Newman stain) previously used in Lina et al., (1999) as a negative control. Indeed, 47% of the chronic wound isolates showed ambiguous faint bands which were not consistent for each PCR reaction.

### **3.8.2 Identification of PVL using Fey PVL PCR primers and Qiagen® 3 h DNA preparation**

Again the positive controls and chronic wound isolate 1011 exhibited intense bands for the PVL product (1554 bp), whilst 65% of the chronic wound isolates and NCTC 8178 exhibited faint bands. The negative controls Mu50 and H<sub>2</sub>O exhibited no bands. Figure 3.13 shows an agarose gel with the separated PCR products using the Fey PVL PCR primers.



**Figure 3.12** Agarose gel showing separation of Lina PVL PCR products. Lanes 1 and 28, 100 bp molecular weight standard (Thermo Scientific); lane 2, negative H<sub>2</sub>O control; lane 3, positive control, NCTC 6571 (MSSA); lane 4, negative control, NCTC 8178; lanes 5 to 7, chronic wound MRSA isolates; lane 8, clinical isolate, ITU 3; lane 9, negative control NCTC 13143 (EMRSA-16); lane 10, chronic wound isolate; lane 11, chronic wound MRSA isolate; lane 12, control isolate NCTC 13130 (EMRSA-03); lane 13, positive control isolate NCTC 6571 (MSSA; SACU); lanes 14 to 19, chronic wound MRSA isolates; lane 20, control isolate NCTC 12493 (MRSA); lane 21, chronic wound MRSA isolate; lane 22, clinical isolate ITU 4; lanes 23 to 24, chronic wound MRSA isolate; lane 25, clinical isolate ITU1; lane 26, chronic wound MRSA isolate; lane 27, negative control Mu50.



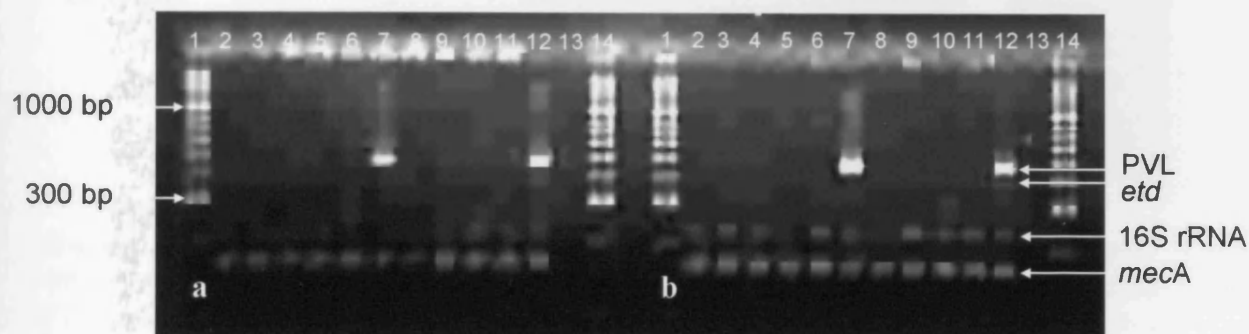
**Figure 3.13** Agarose gel showing the separation of Fey PVL PCR products. Lanes 1 and 26, molecular weight standard Hyperladder III (Bioline Ltd); lane 2, negative H<sub>2</sub>O control; lane 3, negative control isolate NCTC 8178; lane 4, positive control isolates NCTC 6571; lane 5, positive control isolates HT2000.132; lanes 6 to 9, chronic wound MRSA isolates; lane 10, chronic wound isolate 1011 (ST30-MRSA-IVc & E); lanes 11 to 22, chronic wound MRSA isolates; lane 23, negative control isolate Mu50; lanes 24 and 25, blank.

### **3.8.3 Identification of PVL with Holmes' multiplex toxin reaction 4**

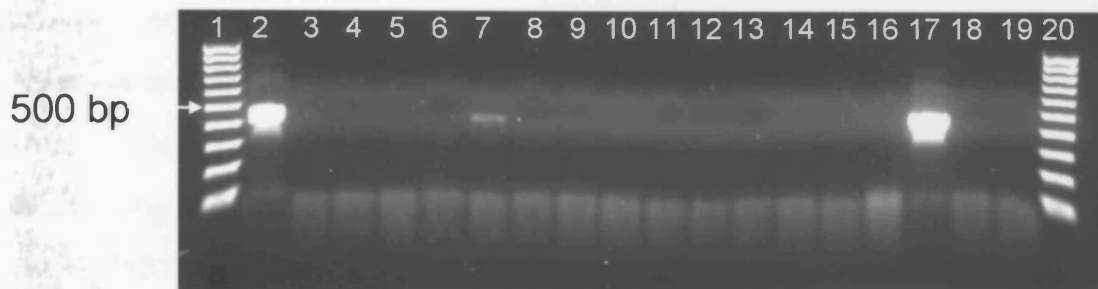
PCR products using both the microLYSIS<sup>®</sup> DNA and 1:10 diluted Qiagen<sup>®</sup> 3 h DNA preparation in conjunction with the Holmes' multiplex toxin reaction 4 resulted in only one chronic wound MRSA isolate (1011; ST30-SCC*mec* IVc & E) demonstrating positivity for PVL. None of the isolates with previous ambiguous bands were positive for PVL using this PCR method with either of the DNA preparations (Figure 3.14).

### **3.8.4 Comparison of PVL identification with Lina PVL PCR primers using Qiagen<sup>®</sup> 30 min and Qiagen<sup>®</sup> 3 h DNA preparations**

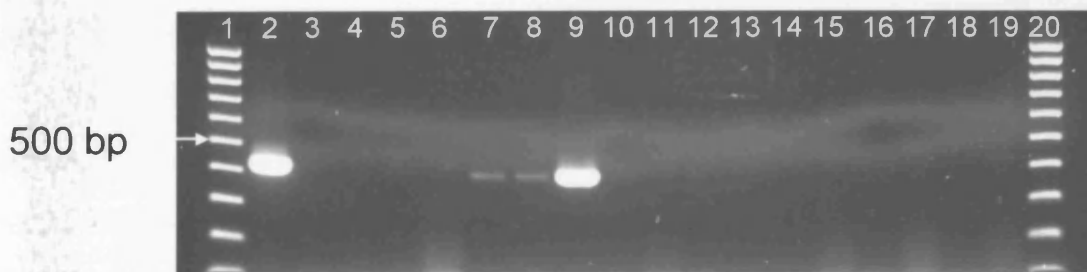
Figures 3.15 and 3.16 show a comparison of PVL identification with Lina PVL PCR primers using DNA prepared using Qiagen<sup>®</sup> 30 min (Section 3.7.4) or Qiagen<sup>®</sup> 3 h (Section 3.7.2). All the nasal isolates were negative for PVL using both DNA extraction methods. Positive controls and chronic wound isolate 1011 (ST30-MRSA-IVc & E) were positive for PVL with distinct unequivocally bright bands. Negative controls showed no bands with either method. The Qiagen<sup>®</sup> 30 min DNA preparation showed that a single chronic wound isolate (6.25%) had a faint ambiguous band, whilst the Qiagen<sup>®</sup> 3 h DNA preparation produced four (25%) chronic wound isolates with faint ambiguous bands. The isolates exhibiting these ambiguous bands differed for both DNA preparation methods used.



**Figure 3.14** Agarose gel showing the separation of PCR products for a) microLYSIS<sup>®</sup> DNA and b) 1:10 diluted Qiagen 3 h DNA preparation performed with Holmes' reaction 4 (i.e. PVL, *etd*, 16S rRNA and *mecA*). Lanes 1 and 14, molecular weight standard Hyperladder II (Bioline Ltd, London); lanes 2 to 5, wound isolates, ST22-MRSA-IVh; lane 6, wound isolate, ST30-MRSA-IVc & IVE; lane 7, wound isolate, ST36-MRSA-II; lanes 8 to 11, wound isolates, ST22-MRSA-IVh; lane 12, PVL positive control NCTC 13300, *etd* positive control clinical isolate; lane 13, negative H<sub>2</sub>O control, all controls were kindly provided by the HPA.



**Figure 3.15** Separation of PVL products using Lina PVL primers and the Qiagen<sup>®</sup> 30 min DNA preparation. Lanes 1 and 20, 100 bp molecular weight standard (Thermo Scientific); lane 2, positive control NCTC 6571; lane 3, negative control Mu50; lanes 4 to 6, chronic wound MRSA isolates; lane 7, faint positive band chronic wound isolate 1108 (ST 970-MRSA-IVh; CC22); lanes 8 to 10, nasal MRSA isolates; lane 11, chronic wound MRSA isolate; lanes 12 to 14, nasal MRSA isolates; lanes 15 to 16, chronic wound MRSA isolate; lane 17, chronic wound MRSA isolate 1011 (ST30-MRSA-IVc & E); lane 18 to 19, chronic wound MRSA isolates.



**Figure 3.16** Separation of PVL products using Lina PVL primers and Qiagen<sup>®</sup> 3 h DNA preparation. Lanes 1 and 20, 100 bp molecular weight standard (Thermo Scientific); lane 2, positive control NCTC 6571; lane 3, negative control Mu50; lanes 4 to 6, chronic wound MRSA isolates; lane 7, faint ambiguous band, chronic wound MRSA isolate 1141 (ST22-MRSA-IVh; CC22); lane 8, faint ambiguous band, chronic wound MRSA isolate 1051 (ST22-MRSA-IVh; CC22); lane 9, intense PVL band, chronic wound MRSA isolate 1011 (ST30-MRSA-IVc & E); lanes 10 to 19, chronic wound MRSA isolates.



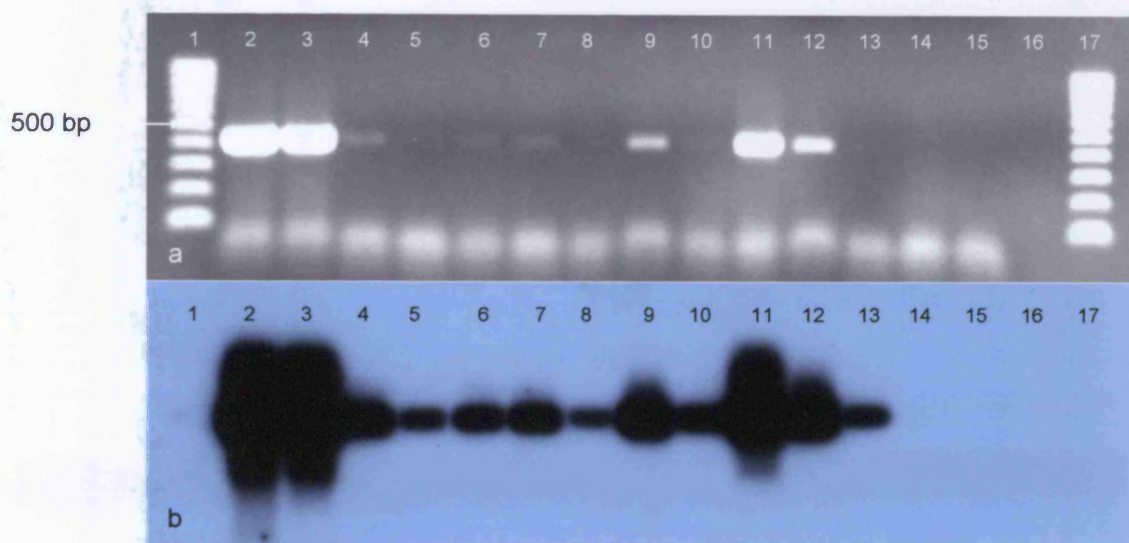
### **3.8.5 Identification of PVL using Southern hybridisation**

PCR products which resulted in ambiguous faint PCR bands like those in Figure 3.12 were separated on an agarose gel alongside appropriate positive and negative controls (Figure 3.17a). Southern transfer confirmed that faint bands present on an agarose gel were positive for PVL (Figure 3.17b). However, positive control isolate (NCTC 6571, lane 2) showed a more intense band on the autoradiograph than those isolates with the ambiguous faint PCR bands (chronic wound MRSA isolates). Lanes 4 to 7, 9, 10 and 12 of chronic wound MRSA isolates all contained indistinct faint bands on the agarose gel, but showed positive banding on the autoradiograph. Negative controls (lanes 15 and 16) had no bands on the agarose gel or the autoradiograph as did a nasal MRSA isolate (lane 14) previously shown to be PVL negative.

### **3.8.6 Sequencing of PVL PCR products**

Sequences obtained from the PCR products of both the Lina and Fey PVL PCR primers were analysed using an alignment program ([www.ebi.ac.uk/Tools/emboss/align](http://www.ebi.ac.uk/Tools/emboss/align)) and a Basic Local Alignment Search Tool (<http://blast.ncbi.nlm.nih.gov>; Tables 3.15 - 3.18; Appendix V).

Forty-two products using the Lina PVL PCR primers were sequenced. Included were positive (5%) and negative controls (5%) for PVL; PVL positive SACU isolates (26%); chronic wound isolates identified by hybridisation (12%); a positive chronic wound isolate (2%); chronic wound isolates with ambiguous PCR results (48%) and one PVL negative nasal MRSA isolate (2%). Of this selection 20 (48%) produced a readable sequence (Table 3.15), of which 15 isolates were able to provide a consensus sequence (sequenced in both directions), two of which were associated with chronic wound isolates (Table 3.16). Five isolates produced only a good reverse sequence and were related to the chronic wound MRSA isolates.



**Figure 3.17** Southern transfer of Lina PVL PCR products a) Agarose gel following the separation of the PVL PCR products (433 bp) prior to Southern hybridisation; b) Autoradiographic image of the agarose gel Figure 3.17a. Lanes 1 and 17, 100 bp molecular weight standard (Thermo Scientific); lane 2, positive control NCTC 6571; lanes 3 to 13, chronic wound MRSA; lane 14, nasal MRSA isolate; lane 15, negative control Mu50; lane 16, negative H<sub>2</sub>O control.

**Table 3.15** Sequence analysis using Lina PVL PCR primers showing closest sequence match

Isolate	Isolate type	Forward sequence				Reverse sequence			
		Sequence length (bp)	Homology with	Homology (%)	Accession No. of closest match	Sequence length (bp)	Homology with	Homology (%)	Accession No. of closest match
H <sub>2</sub> O	Negative	NP				NP			
MU50	Negative	NP				NP			
NCTC 6571	Positive	362	<i>lukS-PV</i> <i>lukF-PV</i>	100	AB532026	367	<i>lukS-PV</i> <i>lukF-PV</i>	100	AB532026
HT2000.132	Positive	367	<i>lukS-PV</i> <i>lukF-PV</i>	100	GU360978	368	<i>lukS-PV</i> <i>lukF-PV</i>	100	AB532026
554	SACU	351	<i>lukS-PV</i> <i>lukF-PV</i>	100	AB532026	364	<i>lukS-PV</i> <i>lukF-PV</i>	99	AB532026
597	SACU	365	<i>lukS-PV</i> <i>lukF-PV</i>	100	AB532027	336	<i>lukS-PV</i> <i>lukF-PV</i>	100	AB532027
5121	SACU	349	<i>lukS-PV</i> <i>lukF-PV</i>	100	AB532026	365	<i>lukS-PV</i> <i>lukF-PV</i>	99	AB532026
5122	SACU	297	<i>lukS-PV</i> <i>lukF-PV</i>	100	GU360978	35	<i>lukS-PV</i> <i>lukF-PV</i>	100	AB532027
5126	SACU	358	<i>lukS-PV</i> <i>lukF-PV</i>	100	GU360978	354	<i>lukS-PV</i> <i>lukF-PV</i>	100	AB532026

NP, did not prime; SACU, Specialist Antimicrobial Chemotherapy Unit isolates; bp, base pair; Positive, positive control; Negative, negative control; *lukS-PV*, *lukF-PV*, *Staphylococcus aureus* Panton-Valentine leucocidin genes.

**Table 3.15 continued** Sequence analysis using Lina PVL PCR primers showing closest sequence match

Isolate	Isolate type	Forward sequence				Reverse sequence			
		Sequence length (bp)	Homology with	Homology (%)	Accession No. of closest match	Sequence length (bp)	Homology with	Homology (%)	Accession No. of closest match
5127	SACU	334	<i>lukS-PV</i> <i>lukF-PV</i>	100	AB532026	363	<i>lukS-PV</i> <i>lukF-PV</i>	100	AB532026
5131	SACU	334	<i>lukS-PV</i> <i>lukF-PV</i>	100	AB532026	372	<i>lukS-PV</i> <i>lukF-PV</i>	99	AB532026
5145	SACU	356	<i>lukS-PV</i> <i>lukF-PV</i>	100	AB532026	366	<i>lukS-PV</i> <i>lukF-PV</i>	99	AB532026
5151	SACU	355	<i>lukS-PV</i> <i>lukF-PV</i>	100	GU360978	356	<i>lukS-PV</i> <i>lukF-PV</i>	96	AB532027
5155	SACU	348	<i>lukS-PV</i> <i>lukF-PV</i>	100	AB532026	345	<i>lukS-PV</i> <i>lukF-PV</i>	100	AB532027
5160	SACU	360	<i>lukS-PV</i> <i>lukF-PV</i>	100	GU360978	344	<i>lukS-PV</i> <i>lukF-PV</i>	100	AB532026
S07146	Nasal	NP				NP			
1011	Chronic	365	<i>lukS-PV</i> <i>lukF-PV</i>	100	GU360978	365	<i>lukS-PV</i> <i>lukF-PV</i>	100	AB532026
1057	Chronic	320	<i>lukS-PV</i> <i>lukF-PV</i>	100	AY508231	361	Φ2958 PVL	99	AP009363

NP, did not prime; SACU, Specialist Antimicrobial Chemotherapy Unit isolates; bp, base pair; *lukS-PV*, *lukF-PV*, *Staphylococcus aureus* Panton-Valentine leucocidin genes; Φ2958 PVL, *Staphylococcus aureus* bacteriophage Φ2958 PVL, proviral DNA; chronic, chronic wound MRSA isolate; nasal, nasal MRSA isolate.

**Table 3.15 continued** Sequence analysis using Lina PVL PCR primers showing closest sequence match

Isolate	Isolate type	Forward sequence				Reverse sequence			
		Sequence length (bp)	Homology with	Homology (%)	Accession No. of closest match	Sequence length (bp)	Homology with	Homology (%)	Accession No. of closest match
1047	Chronic	NP				298	Φ2958 PVL	100	AP009363
1047	Chronic	NP				375	Φ2958 PVL	98	AP009363
1106	Chronic	NP				220	Φ2958 PVL	97	AP009363
1108	Chronic	NP				375	Φ2958 PVL	99	AP009363
2018	Chronic	Noise				296	Φ2958 PVL	96	AP009363

NP, did not prime; SACU, Specialist Antimicrobial Chemotherapy Unit isolates; bp, base pair; Φ2958 PVL, *Staphylococcus aureus* bacteriophage Φ2958 PVL, proviral DNA; chronic, chronic wound MRSA isolate.

**Table 3.16** Consensus sequence identification using Lina PVL PCR primers showing closest sequence match

Isolate	Isolate Type	Consensus sequence			
		Sequence length (bp)	Homology with	Homology %	Accession No. of closest match
NCTC 6571	Positive control	357	<i>lukS-PV</i> <i>lukF-PV</i>	100	AB532026
HT2000.132	Positive control	322	<i>lukS-PV</i> <i>lukF-PV</i>	100	AB532026
554	SACU	310	<i>lukS-PV</i> <i>lukF-PV</i>	99	AB532026
597	SACU	324	<i>lukS-PV</i> <i>lukF-PV</i>	100	AB532027
5121	SACU	310	<i>lukS-PV</i> <i>lukF-PV</i>	99	AB532026
5122	SACU	66	<i>lukS-PV</i> <i>lukF-PV</i>	100	AB532027
5126	SACU	283	<i>lukS-PV</i> <i>lukF-PV</i>	100	AB532026
5127	SACU	295	<i>lukS-PV</i> <i>lukF-PV</i>	100	AB532026
5131	SACU	303	<i>lukS-PV</i> <i>lukF-PV</i>	100	AB532026

SACU, Specialist Antimicrobial Chemotherapy Unit isolates; bp, base pair; Positive, positive control; *lukS-PV*, *lukF-PV*, *Staphylococcus aureus* Panton-Valentine leucocidin genes.

**Table 3.16 continued** Consensus sequence identification using Lina PVL PCR primers showing closest sequence match

Isolate	Isolate Type	Consensus sequence			
		Sequence length (bp)	Homology with	Homology %	Accession No. of closest match
5145	SACU	314	<i>lukS-PV</i> <i>lukF-PV</i>	100	AB532026
5151	SACU	280	<i>lukS-PV</i> <i>lukF-PV</i>	100	AB532026
5155	SACU	283	<i>lukS-PV</i> <i>lukF-PV</i>	100	AB532026
5160	SACU	286	<i>lukS-PV</i> <i>lukF-PV</i>	100	AB532026
1011	Chronic wound	312	<i>lukS-PV</i> <i>lukF-PV</i>	100	AB532026
1057	Chronic wound	252	Φ2958PVL	100	AP009363

SACU, Specialist Antimicrobial Chemotherapy Unit isolates; bp, base pair; *lukS-PV*, *lukF-PV*, *Staphylococcus aureus* Panton-Valentine leucocidin genes; Φ2958 PVL, *Staphylococcus aureus* bacteriophage Φ2958 PVL, proviral DNA; chronic, chronic wound MRSA isolate.

The two positive control isolates NCTC 6571 and HT2000.132 returned a consensus sequence showing 100% homology to the PVL genes. Both negative controls as expected did not prime (Table 3.15). All SACU isolates, which had shown intense bands, returned consensus sequences with >99% homology to PVL genes. One chronic wound isolate (1011) showed a 100% homology to the PVL gene. One further chronic wound isolate associated with an ambiguous PCR band, returned a consensus sequence that showed 100% homology to  $\Phi$ 2958 PVL a bacteriophage known to encode the PVL locus.

Five (21%) of the remaining chronic wound isolates associated with ambiguous bands resulted in a readable reverse sequences (but the forward sequence did not prime). These sequences were associated with >96% homology with  $\Phi$ 2958 PVL a bacteriophage known to harbour the PVL locus.

Thirteen PCR products were sequenced using Fey PVL PCR primers, of which two were positive controls (showing intense PCR bands) and one negative control (NCTC 8178), which had shown faint ambiguous bands. The remaining 10 were chronic wound MRSA isolates, one of which had shown an intense PCR band (1011), whilst the others showed ambiguous bands (Table 3.17). Only 46% of the isolates returned both forward and reverse sequences for consensus sequence analysis (Table 3.18). Two of these were associated with the positive control isolates. Isolate NCTC 6571 showed a 100% homology to PVL genes, whilst the consensus sequence of HT2000.132 only showed a 85% homology to PVL genes despite 99% homology with individual sequences. Four chronic wound isolates returned a consensus sequence, one of which (1011) was 100% homologous to the PVL genes. Of the three remaining, one was 100% homologous to  $\Phi$ PVL-CN125 a bacteriophage known to harbour the PVL locus, and the two other were >87% homologous to *S. aureus* leuF-P83, a gene associated with the F component of leucocidin R. Five other chronic wound isolates also showed >95% homology to the *S. aureus* leuF-P83, but only by the reverse sequence as the forward did not prime. The isolates associated with bacteriophage PVL and leucocidin R sequence



homology all indicated faint ambiguous bands when testing for PVL by PCR. No isolate associated with faint PCR bands resulted in a 100% homologous sequence for PVL with either the Lina or Fey PVL PCR primers.

### **3.8.7 Identification of PVL using microarray analysis**

This method proved that there was only one truly PVL positive isolate from amongst the chronic wound isolates, isolate 1011 (ST30-MRSA-IVc & E). This isolate was continuously represented by an intense PCR and autoradiograph band when tested using the Lina and Fey PVL PCR primers. All isolates with faint bands were negative for PVL using microarray analysis.

**Table 3.17** Sequence analysis using Fey PVL PCR primers showing the closest match

Isolate	Isolate type	Forward sequence				Reverse sequence			
		Sequence length (bp)	Homology with	Homology (%)	Accession No. of closest match	Sequence length (bp)	Homology with	Homology (%)	Accession No. of closest match
NCTC 8178	Negative	NP				NP			
NCTC 6571	Positive	888	<i>lukS-PV</i> <i>lukF-PV</i>	99	AB532026	908	<i>lukS-PV</i> <i>lukF-PV</i>	99	AB532026
HT2000.132	Positive	732	<i>lukS-PV</i> <i>lukF-PV</i>	99	EU518769	890	<i>lukS-PV</i> <i>lukF-PV</i>	99	EU518764
1011	Chronic	852	<i>lukS-PV</i> <i>lukF-PV</i>	100	AB532026	885	<i>lukS-PV</i> <i>lukF-PV</i>	100	AB532024
1021	Chronic	532	<i>lukS-PV</i> <i>lukF-PV</i>	100	AB532027	794	ΦPVL-CN125	100	FJ713816
1067	Chronic	572	Leucocidin subunit S	98	EF672356	758	<i>leuF-P83</i>	98	X64389
1106	Chronic	795	<i>leuF-P83</i>	96	X64389	841	<i>leuF-P83</i>	97	X64389
1004	Chronic	NP				775	<i>leuF-P83</i>	98	X64389

NP, did not prime; bp, base pair; Positive, positive control; Negative, negative control; *lukS-PV*, *lukF-PV*, *Staphylococcus aureus* Panton-Valentine Leucocidin genes; ΦPVL-CN125, *Staphylococcus* phage ΦPVL-CN125; Chronic, chronic wound MRSA isolate; *LeuF-P83*, *Staphylococcus aureus leuF-P83* gene for F component of leucocidin R; leucocidin subunit S gene, *Staphylococcus aureus* leucocidin subunit S gene.

**Table 3.17 continued** Sequence analysis using Fey PVL PCR primers showing the closest match

Isolate	Isolate type	Forward sequence				Reverse sequence			
		Sequence (bp)	Homology with	Homology (%)	Accession No. of closest match	Sequence (bp)	Homology (%)	Homology with	Accession No. of closest match
1044	Chronic	NP				665	<i>leuF-P83</i>	97	X64389
1047	Chronic	NP				301	<i>leuF-P83</i>	95	X64389
1103	Chronic	NP				225	<i>leuF-P83</i>	93	X64389
2124	Chronic	NP				567	<i>leuF-P83</i>	97	X64389

NP, did not prime; bp, base pair; chronic, chronic wound MRSA isolate; *leuF-P83*, *Staphylococcus aureus leuF-P83* gene for F component of leucocidin R.

**Table 3.18** Consensus sequence analysis using Fey PVL PCR primers showing the closest match

Isolate	Isolate type	Consensus sequence			
		Sequence (bp)	Homology with	Homology %	Accession No. of closest match
NCTC 6571	Positive	335	<i>lukS-PV</i> <i>lukF-PV</i>	100	AB532027
HT2000.132	Positive	997	<i>lukS-PV</i> <i>lukF-PV</i>	85	EU518768
1011	Chronic	227	<i>lukS-PV</i> <i>lukF-PV</i>	100	AB532027
1021	Chronic	193	ΦPVL-CN125	100	FJ713816
1106	Chronic	201	<i>leuF-P83</i>	98	X64389
1067	Chronic	780	<i>leuF-P83</i>	87	X64389

Positive, positive control; *lukS-PV*, *lukF-PV*, *Staphylococcus aureus* Pantón-Valentine leucocidin genes; ΦPVL-CN125, *Staphylococcus* phage ΦPVL-CN125; chronic, chronic wound MRSA isolate; *LeuF-P83*, *Staphylococcus aureus leuF-P83* gene for F component of leucocidin R.

### 3.9 Discussion

The accurate identification of PVL is of paramount importance in understanding the epidemiological evolution and treatment of disease by *S. aureus*. The identification of faint bands associated with the Lina PVL PCR primers and ambiguous products associated with the Fey PVL PCR primers in this study highlights the potential for false reporting, or worse, under reporting of the presence of such a potentially harmful virulence factor.

The results obtained with the Lina PVL PCR primers were very sensitive to the DNA preparation method used. DNA isolated with long lysis steps (>3 h) produced a number of faint ambiguous bands of the expected product size (433 bp), although true positive isolates produced distinct bright bands and were unequivocally PVL positive. A reduction in the number of ambiguous bands was seen with DNA prepared using the Qiagen® kit but with a shorter lysis step (30 min). However, again only the true positive isolates had distinct bright bands. Using a microLYSIS® kit or a 1:10 dilution of Qiagen® 3 h DNA preparation, it was observed that true PVL positive isolates had intense bright bands and there were no ambiguous faint bands.

Sequencing of the PCR products with intense PVL bands showed that they had 100% homology with the PVL gene, regardless of whether Fey or Lina PVL PCR primers were used. The faint bands occasionally associated with the Lina PVL PCR primers using the highly concentrated DNA (Qiagen® 3 h) highlighted also with Southern hybridisation did not show PVL homology with sequencing. The sequencing of the chronic wound PCR products using the Lina PVL PCR primers resulted in priming only of the reverse sequence, and was homologous to bacteriophage  $\Phi$ PVL. These bacteriophages are involved in the original genetic transfer of this virulence factor to *S. aureus*.

Sequencing of the Fey PVL PCR products indicated that truly positive isolates with strong positive PCR bands were homologous with PVL. The moderately strong bands were associated with wound isolates using these primers

and could easily be inadvertently treated as PVL positive. Sequencing of these products indicated again homology with bacteriophage  $\Phi$ PVL but more commonly *leuF-P83* gene for the F component of leucocidin R. Leucocidin R has only a 43% homology with PVL. However, as the Fey PVL PCR primers had a 100% homology with a priming site in the leucocidin R gene, it is therefore unsurprising that leucocidin R was amplified. Due to the high homology of the other leucocidins and leucotoxins associated with *S. aureus* to PVL, is it difficult to prevent this type of non-specific primer attachment. An attempt to design new PVL primers, in this study was unsuccessful due to the high number of homologous binding sites within the *S. aureus* leucocidin family. Interestingly, NCTC 8178 (Newman strain), used by Lina as a negative control, demonstrated faint ambiguous bands when using concentrated DNA (Qiagen® 3 h), this product, however, did not result in a sequence. The sequence analysis of NCTC 8178 ([www.ncbi.nlm.nih.gov/nuccore/NC\\_009641](http://www.ncbi.nlm.nih.gov/nuccore/NC_009641); accessed 24/4/11) demonstrated that bacteriophage PVL  $\Phi$ NM3 was present, and possibly priming during PVL PCR.

From the investigations performed using this selection of isolates presenting with faint bands with PVL PCR, it is hypothesized that during DNA preparations using long lysis extraction methods (and thereby producing highly concentrated DNA) that DNA from the extra-chromosomal bacteriophage genetic elements associated with the *Staphylococcus* host is isolated, and that these temperate phage ( $\Phi$ PVL) harbour PVL genes. Temperate bacteriophages play an important role in *S. aureus* pathogenicity through their horizontal gene transfer of virulence factors such as PVL. It is clear that this bacteriophage DNA was only isolated due to protocols containing long lysis steps. Hence, to ensure the prevention of these ambiguous PCR results and misinterpretation, either dilution of target DNA or less rigorous DNA isolation methods should be employed such as the DNA release methods, e.g. the microLYSIS®.

**Chapter 4:**

**Characterisation of the innate  
immunological responses to  
chronic wound bacteria *in vitro***

## **4.1 Introduction**

The immune system is a collection of cellular and humoral components enabling the discrimination of self and non-self and is essential for protection against invading pathogens. The immune system can be sub-divided into the innate and adaptive systems (Lien and Ingalls, 2002, Fournier and Philpott, 2005).

### **4.1.1 The innate immune system**

The innate immune system is our first line non-specific response to microbial invasion, and constitutes the preventive and immediate response of the host (Lien et al., 1999, Fournier and Philpott, 2005, Thorgersen et al., 2009). Innate immunity is composed of anatomic barriers (e.g. skin, ciliated epithelia), secretory molecules (e.g. antimicrobial peptides) and cellular response elements (e.g. neutrophils, PMNL; monocytes, Mo; macrophages, MØ; dendritic cells, DC) recruited to the site of injury (Froy, 2005, Mollen et al., 2006, Nizet, 2007).

#### **4.1.1.1 Toll-like receptors**

Toll-like receptors (TLR) play a pivotal role in the recognition of invading pathogens and the regulation of the innate immune response. TLRs are type I transmembrane signalling pathogen recognition receptors (PRR), with an extracellular domain made up of leucine-rich repeats (LRR) and a Toll/IL-1 receptor (TIR) cytoplasmic domain (Lien et al., 1999, Mori et al., 2003, Jin and Lee, 2008). Thirteen mammalian TLRs have been identified to date (Etokebe et al., 2010, Gonçalves et al., 2011, Michailidis et al., 2011). Each TLR forms an essential part of the innate immune system by identifying specific conserved patterns on pathogens known as pathogen associated molecular patterns (PAMP). TLRs are expressed on both “classical” immunocompetent cells (PMNL, Mo, B and T-cells) and resident cell populations in the dermis e.g. keratinocytes and endothelial cells (Morris et al., 2009, Pukstad et al., 2010).



TLRs are activated by the binding of ligands to the LRR domain which then induces a complicated series of intracellular signalling events via the TIR domain and its adapter molecules. For example myeloid differentiation factor 88 (MyD88) activates translocation of nuclear factor  $\kappa$ B (NF- $\kappa$ B), which induces transcription of cytokines and chemokines such as tumour necrosis factor- $\alpha$  (TNF $\alpha$ ), interleukin (IL)-1 $\beta$ , IL-8 and IL-6 (Takeuchi et al., 2000, Hume et al., 2001, Zhang and Schluesener, 2006, Morris et al., 2009) which in turn initiate immune responses at the site of injury (Mori et al., 2003, Møller et al., 2005, Baiyee et al., 2006).

#### **4.1.1.2 TLR and pathogen associated molecular patterns**

Individual TLRs recognise specific PAMPs, which are usually structures essential to the invading organism or ligands. For example, TLR4, in complex with myeloid differentiation protein 2 (MD-2) and cluster of differentiation (CD) 14, recognises lipopolysaccharide (LPS) from Gram-negative bacteria (Miyake, 2003, Adib-Conquy et al., 2006, Mollen et al., 2006, Jin and Lee, 2008). In contrast, TLR2 recognises Gram-positive bacteria via signalling induced by ligands such as peptidoglycan (PGN), lipopeptides, lipoproteins and lipoteichoic acid (LTA; (Shaykhiev et al., 2008). TLR2 is also able to form heterodimers with other TLRs e.g. TLR1 and TLR6 enabling the identification of further ligands/pathogens e.g. zymosan (yeast particles). TLR5 is responsible for the identification of flagellin (a principal component of bacterial flagella), and TLR9, an intracellular receptor signalling unmethylated cytosine-guanosine (CpG) dinucleotide-rich DNA released from bacteria or endogenous cells. Mammalian DNA has a less stimulatory effect via TLR9 due to decreased CpG expression and extensive (80%) methylation of cytosine (Weiner et al., 1997, Wooldridge et al., 1997, Lien and Ingalls, 2002, Konat et al., 2006, Baiyee et al., 2006, Morris et al., 2009). Studies of TLR knock-outs have broadened our knowledge of their role in infection. Many studies on TLR-deficient mice have shown that clearance of an invading pathogen is reduced, and that intranasal carriage is increased in these mutants (Takeuchi et al., 2000,

Gonzalez-Zorn et al., 2005, Hoebe et al., 2005). It has also been observed that genetic polymorphism in ligand-binding regions of TLR5, (found in mice and humans) may lead to hypersusceptibility to pneumonic legionellosis (Morris et al., 2009). TLRs identify not only invading pathogens, but are able to distinguish host tissues damaged and/or degraded by the process of injury (Mollen et al., 2006, Zhang and Schluesener, 2006). For example, low molecular weight (mw) hyaluronan products (4-16 oligosaccharides in length) may signal through TLR4 following tissue injury, to activate DC and endothelial cells via the NF- $\kappa$ B pathway (Termeer et al., 2000, Taylor et al., 2004).

#### **4.1.2 Wound bacteria and inflammation**

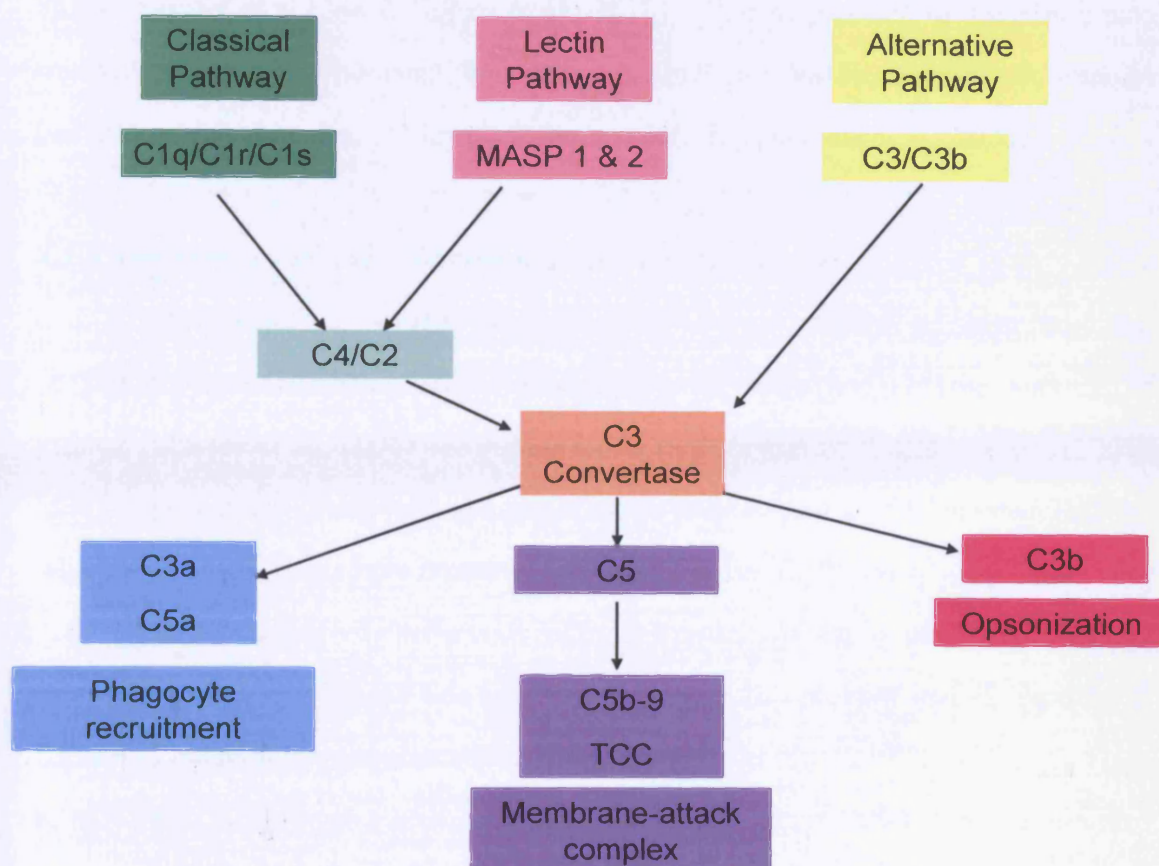
Whilst the precise molecular pathogenesis of chronic wounds is unclear, the sustained inflammatory response and persistence of bacteria are likely to play an important role in non-healing. To date, whilst no individual bacterial species has been identified as the cause of chronic wound persistence (Chapter 1, Section 1.5.1), the presence of  $\geq 4$  bacterial sp. may have a detrimental effect on healing (Davies et al., 2007). Following a break in epithelial integrity, TLRs are employed by immunocompetent cells to prevent bacterial ingress. TLR activation by exogenous ligands have been reported to promote wound healing via TLR2 and TLR4 (Pukstad et al., 2010). The absence of TLR4 may play a significant role in normal healing, as shown in wound repair models. TLR4-deficient mice show early reduction in TNF $\alpha$ , with increased collagen production, leading to increased wound strength (Bettinger et al., 1994, Mollen et al., 2006). The problematic nature of bacterial wound colonisation, like other chronic diseases, is the continuous influx of inflammatory cells and their release of active molecules (e.g. cytokines, free oxygen radicals and proteolytic enzymes) into the wound bed mediating increased inflammation and further tissue damage (Kirketerp-Moller et al., 2008).

### 4.1.3 Complement

Complement forms an important fluid-phase of the innate immune response and has three major activating pathways; the classical, alternate and lectin pathways. Complement is a “cascade” of proteins and proteolytic fragments, derived by enzymatic cleavage, to form the potent terminal complement complex (TCC; C5b-9) which leads to cellular lysis (the membrane-attack complex). Complement also regulates further immune responses via cytokine production and opsonization via C3b and C5a (Kirschfink and Mollnes, 2003, Sprong et al., 2004, Foster, 2005, Keiji et al., 2006, Thorgersen et al., 2009). Figure 4.1 is a schematic overview of the main components and effector actions of complement. Deficiencies in the complement system frequently lead to increased risk of infection (Kirschfink and Mollnes, 2003, Hellerud et al., 2008). Peptidoglycan from *S. aureus* is able to directly induce the alternate pathway, whilst the mannose-binding lectin (MBL) pathway is induced by peptidoglycan (PGN) and lipoteichoic acid (LTA) and some forms of LPS (Roosjakkars et al., 2005).

### 4.1.4 Chronic stimulation and evasion of the host immune response

An active/effective immune system plays a pivotal role in defence against invading pathogens and in inducing the reparative processes of wound healing. The persistence of bacteria within wounds has been shown to play an important and often unrecognised role in the chronic inflammation observed in chronic, non-healing wounds (Mirza et al., 2009). A number of human diseases are characterised by chronic bacterial colonisation and disrupted host/bacterial interaction e.g. periodontal disease and chronic lung diseases such as cystic fibrosis (Mori et al., 2003). In their evasion of the immune system, bacteria such as MRSA, may utilise a number of different mechanisms, e.g. the production of virulence factors (e.g. leucocidins and proteases; Chapter 3) which may lead to leucocyte apoptosis (Kahl et al., 2000, Hornef et al., 2002, Kirketerp-Moller et al., 2008, DeLeo et al., 2009) and alteration of bacterial phenotype.



**Figure 4.1** Schematic overview of complement components and effector actions. MASP; mannose-binding lectin associated serine protease, TCC; terminal complement complex (Adapted from Murphy et al., 2008).

Furthermore, biofilm formation protects bacteria from antimicrobials and host responses by inhibiting phagocytosis, complement and free-oxygen radicals (Johnson et al., 1986, Davis et al., 2008, Percival et al., 2008, Martin et al., 2009, Percival et al., 2011a). Bacteria may also evade innate recognition via steric alterations of PAMPs, e.g. *Salmonella* LPS is tetra-acetylated and does not trigger TLR4 (Hornef et al., 2002, Comer et al., 2010). The acquisition of specific innate immune evasion mechanisms by bacteria, which are easily transferred, enables bacterial survival at sites of chronic inflammation (Rooijackers et al., 2005).

#### **4.1.5 *Staphylococcus aureus* evasion of the immune system**

*Staphylococcus aureus* is responsible for a variety of hospital- and community-based illnesses and is associated with acute and chronic patterns of disease (Massey et al., 2006). *S. aureus* exists as a commensal bacterium on the skin and mucous membranes, and possesses both passive and active mechanisms by which it can modify the host immune response (Keiji et al., 2006).

The production of chemotaxis inhibitory protein of staphylococci (CHIPS) by *S. aureus* interferes with the C5a and formyl peptide receptors of PMNL, leading to decreased chemotaxis and migration of immune cells to the site of infection (de Haas et al., 2004, Keiji et al., 2006, Nizet, 2007, DeLeo et al., 2009). *S. aureus* also produces a number of cytolytic leukotoxins, including Panton-Valentine leucocidin (PVL), and haemolysins, which are able to lyse recruited PMNL (Chapter 3). As well as these leucotoxins, virulence factors such as proteases (e.g. aureolysin) assist resistance to host antimicrobial peptides and PMNL phagosomes, thus preventing lysis (Foster, 2005, DeLeo et al., 2009). The polysaccharide capsule and anti-opsonic proteins on the surface of *S. aureus* prevent the deposition of antibodies and complement formation and resist phagocytosis by opsonization (Nizet, 2007). Bacterial charge also affects microbial susceptibility to host antimicrobial peptides e.g. lipocalin. *S. aureus* modifies its membrane charge by substituting the principal lipid membrane phosphatidylglycerol with lysine and adding D-alanine to teichoic

acid; effectively reducing the net negative-charge (Hornef et al., 2002). *S. aureus* is also able to cleave immunoglobulins IgG, IgM and IgA by the action of serine proteases, to escape opsonization and phagocytosis. Superantigen virulence factors, e.g. Toxic shock syndrome toxin (TSST-1) and the exfoliative enterotoxins, can over-stimulate T-cells (DeLeo et al., 2009) which may “overwhelm” the host system leading to severe disease and tissue destruction. The production of a biofilm is a further important mechanism by which *S. aureus* can resist immune system recognition and PMNL mediated lysis (DeLeo et al., 2009, Percival et al., 2011a).

#### **4.1.6 *Pseudomonas aeruginosa* evasion of immune system**

*Pseudomonas aeruginosa* is similarly associated with chronic disease states such as non-healing wounds and lung disease e.g. cystic fibrosis and bronchiectasis (Morris et al., 2009). *P. aeruginosa* is extensively associated with biofilm formation in non-healing chronic wounds (Madsen et al., 1996, Kirketerp-Moller et al., 2008). The production of such biofilms facilitates defences against the host immune system and evasion of antimicrobial therapy, and like *S. aureus*, produces a number of virulence factors (e.g. leukocidin toxin and rhamnolipid B) to limit the host immune response (Kirketerp-Moller et al., 2008). During lung infection with *P. aeruginosa*, host-induced apoptosis reduces the release of pro-inflammatory cytokines, and correspondingly reduces leukocyte infiltration, leading to increased *P. aeruginosa* survival.

#### **4.1.7 Chronic inflammation in wound healing**

Previous studies have investigated the presence of bacteria in chronic wounds, identifying individual species, microbial diversity and bacterial load as possible important factors (Andersen et al., 2007, Percival et al., 2011a; Chapter 1, Section 1.5.1). The complex relationship between bacteria and the local immune response in the chronic wound has surprisingly received little attention. It is thought that a chronic wound is static and dominated by a prolonged inflammatory phase

which is attributed to the presence of the bacterial microflora (Pukstad et al., 2010). This process is highly dynamic with continuous influx of inflammatory cells, e.g. PMNL, and their release of vaso-active and lytic molecules such as cytokines, free-oxygen radicals and enzymes into the wound environment, mediating further tissue damage, in a potentially destructive “positive feedback loop” leading to further recruitment of inflammatory cells to the detriment of the healing process (Kirketerp-Moller et al., 2008).

Pukstad et al., (2010) characterised the potential role of bacterial modulation of the immune system using wound fluid samples isolated from healing and non-healing wounds. Cytokine analysis of non-healing wound fluid demonstrated increased levels of pro-inflammatory cytokines with increased wound duration (e.g. IL-1 $\beta$ ). The angiogenic chemokine IL-8 was associated with healing wounds, and was observed to decrease with time in non-healing wound fluid. Lipocalin-2, an important antibacterial peptide, decreased in wound fluid collected from healing wounds over an 8 week period, whilst non-healing wounds showed that this protein was continuously produced, signifying that a bacterial component was present at the wound site. TLR2 and TLR4 stimulation via non-healing and healing wound fluid also varied with time. Non-healing wound fluid continuously stimulated TLR2 and TLR4, and was proposed to reflect evidence of the microbial bioburden in the chronic wound environment.

#### **4.1.8 Modelling the effect of bacteria and the innate immune system in human wound healing**

##### **4.1.8.1 NF- $\kappa$ B luciferase assay**

Previous studies of TLR regulation in disease as a result of bacteria have employed immunohistochemistry (Mori et al., 2003). Gene expression and cellular events are now commonly studied using the aid of genetic reporter systems. Firefly luciferase is frequently utilized in reporter assays, as it does not require post-translational processing, and it has a high quantum efficiency of light production,

making it very sensitive. Light is produced following luciferin oxidation by firefly luciferase to produce oxyluciferin. Dual luciferase reporter assays are often employed, whereby a second reporter (control e.g. *Renilla*) is driven by a constitutively expressed promoter. This second reporter normalises experimental results as it overcomes transfection efficacy and cytotoxicity between cells and wells (Adib-Conquy et al., 2006). Following stimulation of TLR by an appropriate ligand, NF- $\kappa$ B is translocated to the cell nucleus to induce cytokine translation. The NF- $\kappa$ B luciferase reporter assay utilises Elam luciferase to determine quantification of NF- $\kappa$ B intracellular activity upon TLR stimulation (Lien et al., 1999, Latz et al., 2002, Sandor et al., 2003).

#### **4.1.8.2 Whole blood model**

The *in vitro* whole blood model using lepirudin (refludan) has been used to investigate complement activation associated with *E. coli* (Mollnes et al., 2002). Unlike isolated cell-based assays, the whole blood model is a more physiological approach to simulate *in vivo* conditions, as it uses human blood to mimic the complex inflammatory processes that occur (Mollnes et al., 2002, Kirschfink and Mollnes, 2003). The receptor CD11b is expressed by activated Mo and granulocytes during complement activation (Thorgersen et al., 2009) and can be analysed by fluorescence activated cell sorting (FACS). The terminal complement complex (TCC) ELISA assay (Mollnes et al., 1985b) represents a reliable method for the detection of C5b-C9 activation (terminal pathway) rather than quantification of individual complement activation products e.g. C5a, C3 which have relatively short half-lives (Mollnes et al., 1993, Kirschfink and Mollnes, 2003, Mollnes et al., 2007).

#### **4.1.8.3 “Scratch” wound Assay**

It has been demonstrated that an important component of the innate immune system is cellular barrier formation and TLRs have been demonstrated to play an important role in re-epithelialisation and keratinocyte migration.



The “scratch” assay is a simple *in vitro* cellular wound healing model which utilises a monolayer of cells to study cellular wound closure following experimental (physical or chemical) wounding (Lampugnani, 1999, Liang et al., 2007). Whilst the model employs single cell types e.g. keratinocytes or fibroblasts, it is devoid of extracellular matrix (ECM) components and inflammatory molecules. These factors notwithstanding, it enables determination of the direct effect of stimuli (such as bacterial supernatants) on cell re-population and wound closure (Stephens et al., 2003).

#### **4.2 Aims:**

*S. aureus* and *P. aeruginosa* have been extensively implicated in the aetiology of chronic wounds, and their critical colonisation is intimately linked to non-healing. Yet, little investigation of the immunogenic roles of these pathogenic wound bacteria or the comparison to asymptomatic colonisers has been performed. The aim of this study was to differentiate changes in immune stimulation via TLR, cytokine production and complement activation by MRSA isolated from chronic wounds and to compare them to an asymptomatic nasal carrier control group. In this way, it was hoped to determine, not only if wound MRSA exhibit immunomodulatory properties which aid colonisation of chronic wounds, but also to determine their potential role in impairing healing. These studies also compared bacteria from planktonic and biofilm conditions, to determine if the bacterial growth environment plays a further role in colonisation and non-healing of wounds. The use of wound assays was employed to determine the role of wound and nasal carrier MRSA on wound healing and to further demonstrate the role of bacterial growth environments in the modulation of wound healing.

The aims of this study are to:

1. Investigate and compare the immunostimulatory properties of MRSA isolated from asymptomatic carriers and chronic wound sites and investigate the immunomodulatory properties of *S. aureus* and *P. aeruginosa* from biofilm and planktonic growth conditions.
2. Investigate the direct effect of MRSA bacterial supernatants isolated from asymptomatic carriers and chronic wounds sites and *S. aureus* and *P. aeruginosa* from biofilm and planktonic growth conditions on healing using a 'scratch wound assay'.

### **4.3 Materials and Methods**

#### **4.3.1 Culture of bacterial isolates**

Bacteria from chronic leg wounds, chronic surgical wounds and asymptomatic nasal carriers were isolated and cultured as previously described (Chapter 3, Section 3.3.1). Clinical control isolates included: chronic venous leg ulcer (CVLU) *Staphylococcus aureus*, FC2 (Davies et al., 2004); *Pseudomonas aeruginosa*, NCTC 10662; and mucoid producing strain *P. aeruginosa*, ATCC 39324. Pure cultures for the chosen bacteria in Table 4.1 were established on blood agar (BA; LabM) supplemented with 5% (v/v) sheep defibrinated blood (Sigma-Aldrich) at 37°C overnight (O/N).

#### **4.3.2 Production of bacterial supernatants**

The media used to produce bacterial supernatants were tryptone soya broth (TSB; Oxoid), brain heart infusion broth (BHI; Oxoid), and Dulbecco's modified eagle medium with 4 g/l D-glucose, L-glutamine, (no additional sodium pyruvate or phenol red; DMEM; Invitrogen) supplemented with 10% (v/v) foetal calf serum (FCS; Invitrogen). Working cultures used to produce bacterial growth curves and supernatants were prepared by inoculating a single bacterial colony into 10 ml of

**Table 4.1** Bacterial isolates used to compare immunostimulation

Isolate	Number of isolates	Isolate type	<sup>a</sup> Supernatant growth condition (number of supernatants)	
			Planktonic	Biofilm
Chronic surgical wound	4	MRSA	4	0
Chronic leg wound	4	MRSA	4	0
Asymptomatic nasal carrier	4	MRSA	4	0
FC2	1	<i>Staphylococcus aureus</i> (control isolate)	1	1
NCTC 10662	1	<i>Pseudomonas aeruginosa</i> (control isolate)	1	1
ATCC 39324	1	Mucoid <i>Pseudomonas aeruginosa</i> (control isolate)	1	0

MRSA, Methicillin Resistant *Staphylococcus aureus*; <sup>a</sup> growth condition of produced supernatant.

corresponding sterile liquid growth media and incubated (37°C O/N) on a roller (50 rpm).

#### **4.3.2.1 Growth curves to determine stationary phase**

Growth curves were determined for each isolate in all 3 growth media (Section 4.3.2). Working cultures of bacteria were produced (Section 4.3.2), diluted (1:50 dilution, DMEM; 1:500 dilution, broth), and 1 ml transferred to 3 wells of a 24-well cell culture plate (Costar®, Corning®, Sigma-Aldrich). This was sealed with DuraSeal™ (Sigma-Aldrich, UK) to prevent evaporation. Absorbance was measured hourly at 620 nm using a FLUOstar OPTIMA plate reader (BMG Labtech GmbH, Germany) over a 48 h period at 37°C with agitation prior to measurement. The growth curves were plotted from the recorded absorbance (with the growth media employed as a control) and stationary phase identified.

#### **4.3.2.2 Supernatant preparation**

Supernatants were prepared in a 250 ml conical flasks by inoculating 100 ml of growth media with a 1:50 or 1:500 (DMEM or broth respectively) dilution of the prepared working culture (Section 4.3.2). Cultures were incubated at 37°C until stationary phase was attained in a shaking incubator at 160 rpm for planktonically grown cultures or stationary for 48 h for biofilm growth conditions.

The final absorbances of the bacterial cultures were then tested using the FLUOstar OPTIMA at an absorbance of 620 nm ( $A_{620}$ ) to confirm stationary phase. Purity checks were performed on both the working and supernatant cultures and CFU/ml determined for the stationary phase supernatant cultures (Chapter 2, Section 2.3.5.3). Following centrifugation (13000  $\times$  g, 4°C, 10 min) the supernatants were filter-sterilised (0.2  $\mu$ m filter; IWAKI glass Co. Ltd, Tokyo, Japan) and stored at -80°C. Samples were re-filtered prior to use.

#### **4.3.2.3 Determination of protein content**

Protein content of each supernatant was determined using a BCA<sup>TM</sup> Protein assay KIT in 96-well plates (Pierce Protein Research Products, Thermo scientific, UK; Greiner Bio-one Ltd, UK), according to the manufacture's instructions.

#### **4.3.3 Heat killed bacteria**

Working cultures of bacteria were prepared in 20 ml TSB (Section 4.3.2) and diluted (1:500) in 20 ml TSB. Bacteria were incubated on a rotating platform (37°C, 220 rpm) until stationary growth phase was achieved (Section 4.3.2.1). Cultures were centrifuged (2000  $\times$  g, 10 min) washed (x2) in PBS, and then re-suspended in 10 ml PBS. The CFU/ml was determined using the drop count method (Chapter 2, Section 2.3.5.3) on luria-bertani (LB) agar (1%, w/v tryptone; 0.5%, w/v yeast extract; 1%, w/v NaCl; 2% w/v agar bacteriological; pH 7.5; Oxoid). Bacteria were heat killed (68°C, 2 h) confirmed by inoculating 20  $\mu$ l of heat killed bacterial suspension on LB agar and incubating (37°C, 48 h) in triplicate, bacteria were stored at -80°C until use.

#### **4.3.4 Toll-like Receptor (TLR) stimulation; Luciferase assay**

The luciferase reporter assay was utilised to study TLR activation as previously described by Hellerud et al., (2008).

##### **4.3.4.1 Growth of human embryonic kidney (HEK) 293E cells**

Human Embryonic Kidney (HEK) 293E cells were grown in monolayer in a T75 flask (Corning life sciences, Sigma-Aldrich Norway AS) containing HEK 293E cell culture medium (DMEM, Invitrogen; supplemented with 0.68 mM L-glutamine, Sigma-Aldrich; 10  $\mu$ g/ml ciprofloxacin, Cellgro<sup>®</sup>, Mediatech, Inc. USA and 10% FCS, Invitrogen) in a humid incubator (37°C, 8% CO<sub>2</sub>) until approximately 80% confluent. Cells were trypsinised by removing the cell culture media and washing with pre-warmed PBS (Invitrogen; x2). Trypsin-EDTA (3 ml) was added to the flask. When the cells detached, the Trypsin-EDTA was inactivated by the addition of

10 ml of HEK 293E cell culture medium and the resultant cell suspension centrifuged (350 x g, 7 min) and re-suspended in 4 ml of HEK 293E cell culture media. The cells were split (1:8) with luciferase media (DMEM; Euroclone, Milano, Italy), supplemented with 0.68 mM L-glutamine; 10 µg/ml ciprofloxacin and 10% FCS, and 100 µl of cell suspension used to seed a 96-well cell culture plate (Corning life sciences, Sigma-Aldrich, Norway). The remaining cells were maintained as a stock in a T75 flask containing HEK 293E cell culture media.

#### **4.3.4.2 Transfection of HEK 293E**

HEK 293E cells were incubated in 96-well plates until 70 - 80% confluent and media changed prior to transfection. A 100 µl volume of luciferase media was added to each well. A total volume of transfection mixture (Appendix VI) was prepared in a 50 ml centrifuge tube. To each well a 25 µl total volume of transfection mixture was added. The transfection mixture (per well) was prepared by combining 24.7 µl of plain DMEM (Euroclone, Milano, Italy) and 0.3 µl of GeneJuice® (Novagen, Merck, Dramstadt, Germany) vortexed and incubated for 5 min at RT. To this, a total of 100 ng plasmid DNA was added. The plasmids used for transfection were as follows; NF-κB-dependant luciferase reporter plasmid, pELAM-luc (Chow et al., 1999); pcDNA3 expressing human CD14; pEFBOS expressing human MD2; pcDNA3 expressing TLR4; pRK7 expressing human TLR2 (Hellerud et al., 2008); pcDNA3 expressing TLR5 and pRL-TK expressing *Renilla*; the total dosage being corrected by the addition of pcDNA3. The transfection mixture (Appendix VI) was incubated (RT, 15 min) then 25 µl added to each corresponding test well. The cells were incubated for a further 24 h in a humid incubator (37°C, 8% CO<sub>2</sub>) until 70 - 90% confluent.

#### **4.3.4.3 Preparation of supernatants and controls for stimulation of TLR transfected cells**

The supernatants prepared in Section 4.3.2.2 were recovered, filter-sterilized and serially diluted 1:5 in round bottom 96-well cell culture plates using HEK 293E cell culture medium (Section 4.3.4.1). Each test dilution (31 µl) was pipetted into the corresponding transfected test wells. *S. aureus* supernatants were used to stimulate TLR2 and TLR negative (pcDNA3) transfected cells and *P. aeruginosa* supernatants were used to stimulate TLR4, TLR5 and TLR negative (pcDNA3) transfected cells. TLR ligands were used as positive and negative controls for transfection and luciferase activity. Fibroblast stimulating lipopeptide-1 (FSL-1; EMC Microcollections, Germany; 20 ng/ml) was used to stimulate TLR negative and TLR2 transfected cells and utilised as a negative control for TLR4 transfected cells. Lipopolysaccharide (LPS; Ultrapure, Invitrogen; 50 ng/ml) was used to stimulate TLR negative and TLR4 transfected cells and as a negative control for TLR2 transfected cells. The LPS ligand was placed in an ultrasonic bath prior to dilution to disrupt any micelle formation. Flagellin (InvivoGen, San Diego, California, USA; 1 ng/ml, 10 ng/ml and 100 ng/ml) was used to stimulate TLR negative and TLR5 transfected cells. Flagellin was also used as a control for the TLR2/4 and TLR negative transfected cells at 10 ng/ml working concentration. Plain DMEM and supernatant growth media were also used as controls for background activity and cytotoxicity. The cells were incubated and stimulated for 20 h (37°C, 8% CO<sub>2</sub>).

#### **4.3.4.4 Luciferase activity**

Prior to recording luciferase activity, cells were visualised using light microscopy for morphological changes or evidence of cytotoxicity following stimulation. The cell media/supernatant was aseptically removed and 50 µl of passive-cell culture lysis reagent (Promega) was added to each well to obtain the cytoplasmic extract. Plates were then sealed in aluminium foil and placed on an orbital shaker for 30 min (50 rpm). Luciferase substrate was reconstituted with the

provided buffer (Promega) and *Renilla* substrate (Sigma-Aldrich) diluted to 1 mg/ml with sterile PBS. Following lysis of the stimulated HEK 293E cells, 10 µl of the cell-lysate was transferred to two white, opaque 96-well microplates (OptiPlate<sup>TM</sup>-96, Perkin Elmer). Luciferase substrate (25 µl) was then added to the cell lysate and the signal recorded by a Victor3<sup>TM</sup> plate reader using Wallac 1420 software (Perkin Elmer, Norway). Luminescence was measured following 2 s shaking, for 0.1 s per well. The luciferase activity was determined by normalising the Elam-luc signal against the *Renilla* signal to control for variation in transfection, cell number and viability. The TLR activity was calculated relative to the TLR-negative cells (pcDNA3).

#### **4.3.5 Monocyte assay to determine IL-8, TNF- $\alpha$ and IL-1 $\beta$ production following stimulation with bacterial supernatants**

##### **4.3.5.1 Isolation of monocytes from buffycoat**

Buffycoat A+ venous blood was obtained from the blood bank at St Olav's Hospital, Trondheim, Norway (following approval of the Local Regional Ethical Committee, Appendix I). Following dilution (1:5) with preheated (37°C) Dulbecco's phosphate buffered saline (D-PBS; Invitrogen), 35 µl aliquots were pipetted into 50 ml centrifuge tubes and 10 ml of pre-heated Lymphoprep<sup>TM</sup> added (Nycomed Pharma AS, Norway) followed by centrifugation (690  $\times$  g, 25 min). The mononuclear cell layer was removed and distributed between two new centrifuge tubes which were then centrifuged (840  $\times$  g, 10 min). The resultant cell pellet was re-suspended in 1 ml of preheated Hanks buffered salt solution (HBSS; Invitrogen) and 19 ml of HBSS added. Samples were centrifuged (345  $\times$  g, 8 min) and the washing process repeated (x2). The resulting cellular pellets were combined in one 50 ml centrifuge tube and 20 ml of HBSS added. Cell number was determined using 20 µl of the mononuclear cell/HBSS solution diluted in 10 ml of Isoton water containing 2 drops of Zaptoglobin (Beckman Coulter, Norway), to lyse erythrocytes, and the white blood cells were counted using an automated cell counter (Beckman Coulter, Norway).



Cells were re-suspended in plain Roswell park memorial institute media (RPMI; Invitrogen), and diluted to a concentration of  $4 \times 10^6$  cells/ml in Mo growth media (RPMI; Invitrogen, supplemented with gentamicin 40 µg/ml, 0.01% glutamine and 2% human A+ serum). A 96-well cell culture plate was seeded with 100 µl of cell suspension and following incubation (37°C, 5% CO<sub>2</sub>, 90 min), wells were viewed using a Zeiss microscope to demonstrate Mo adherence. Wells were washed with preheated 130 µl HBSS (x4) to remove non-adherent cells. Then 160 µl of RPMI growth medium supplemented with 6.25% A+ human serum, gentamicin 40 µg/ml, and 0.01% glutamine was added to each well.

#### **4.3.5.2 Monocyte stimulation with bacterial supernatants**

Bacterial supernatants and growth media controls were filter-sterilised (0.2 µm) prior to use and serially-diluted (1:5) using plain RPMI in round bottom 96-well cell culture plates. Then 40 µl of supernatant was used to stimulate Mo cells. Ligands LPS and FSL-1 (10 ng/ml), Adenosine triphosphate (ATP; 5 mM) and Mo RPMI growth media supplemented with 2% A+ serum growth medium were used as controls. All controls were used in primed and non-primed Mo condition for the activation and analysis of IL-1β. Priming was performed using LPS (final concentration 50 pg/ml). Stimulated cells were incubated (37°C, 5% CO<sub>2</sub>, 5 h) and 100 µl of supernatant was recovered to estimate IL-1β and TNFα production. After 16 h, cell supernatants were recovered for IL-8 analysis. All Mo supernatants were stored at -20°C until required.

#### **4.3.5.3 MTT assay following monocyte stimulation**

Following supernatant collection for cytokine analysis, an MTT assay was performed to investigate cytotoxicity. After removal of cellular supernatants 100 µl Mo RPMI growth medium containing 50 ng/ml 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, (MTT formazan; Sigma-Aldrich) was added to

each well. Following incubation (37°C, 5% CO<sub>2</sub>, 4 h) cells were visualised microscopically for crystal formation. The MTT solution was removed and 150 µl of 100% isopropanol containing 0.25% (v/v) ammonia added, and the plate shaken (30 min) to allow cellular lysis. Absorbance was measured at 570 nm, using a Victor3<sup>TM</sup> plate reader (Perkin Elmer, Norway).

#### **4.3.5.4 Enzyme-linked immunosorbent assay (ELISA) analysis of IL-8, TNFα and IL-1β production by monocytes**

Monocyte cytokine production was assayed using commercial ELISA kits for IL-8 and TNFα (R&D systems, Abington, UK) and IL-1β (BD biosciences, San Diego, USA). Supernatants collected from Mo cultures (Section 4.3.5.2) were diluted using the reagent diluents (Table 4.2). Half-area flat-bottom 96-well ELISA plates (Corning life sciences, Norway) were coated with 25 µl of capture antibody diluted to 4 µg/ml with PBS (Table 4.2). Plates were sealed with an adhesive film (Greiner bio-one, MedProbe, Norway) and incubated O/N at RT. The plates were washed three times (0.05% Tween 20 in PBS; Sigma-Aldrich) using a plate washer (UltrawashPlus, Thermolabsystems), and “blocked” with 100 µl of blocking agent (1% BSA, v/v; PBS; Sigma-Aldrich) for IL-8 and reagent diluents for IL-1β and TNFα (Table 4.2) and incubated at RT for 1 h. The plates were then washed (x3) with wash buffer and blotted by inverting onto blotting paper. Diluted Mo supernatant (25 µl) and standard (diluted with reagent diluents; Table 4.2) were added, the plates sealed with adhesive film and incubated (37°C, 1 h). Following washing (x3 with wash buffer) and blotting, 25 µl of detection antibody (Table 4.2) was added to each well. Following incubation (37°C, 1 h), washing (x3) with wash buffer and blotting, 25 µl of Streptavidin horse-radish peroxidase conjugate (Streptavidin-HRP; 1:200; R&D Systems) was added to each well and the plate incubated in the dark for 20 min at RT. The plates were washed again (x3), blotted dry, and 25 µl of tetramethylbenzidine (TMB) substrate (BioLegend, Nordic BioSite

**Table 4.2** Concentration of antibody and reagents for ELISA analysis of IL-8, TNF $\alpha$  and IL-1 $\beta$ 

<sup>a</sup> ELISA	Supernatant dilution	Reagent diluent	Capture antibody	Standard	Detection antibody
IL-8	1:100	0.1% BSA (v/v), 0.05% Tween <sup>®</sup> 20 (v/v), PBS	<sup>b</sup> 4 $\mu$ g/ml	0 to 4000 pg/ml	<sup>c</sup> 20 ng/ml
TNF $\alpha$	1:20	1% BSA (v/v), PBS	<sup>c</sup> 4 $\mu$ g/ml	0 to 250 pg/ml	<sup>f</sup> 300 ng/ml
IL-1 $\beta$	1:20	1% BSA (v/v), PBS	<sup>d</sup> 4 $\mu$ g/ml	0 to 1000 pg/ml	<sup>g</sup> 75 ng/ml

<sup>a</sup>ELISA as per manufacturer's instruction; <sup>b</sup>anti-human IL-8 monoclonal antibody (mAb); <sup>c</sup> anti-human TNF $\alpha$  mAb; <sup>d</sup>anti-human IL-1 $\beta$  mAb; <sup>e</sup>biotinylated anti-human IL-8; <sup>f</sup> biotinylated anti-human TNF $\alpha$ ; <sup>g</sup>biotinylated anti-human IL-1 $\beta$ .

AS) added to each well and incubated at RT for 15 to 20 min in darkness. The reaction was stopped using stop solution (2NH<sub>2</sub>.SO<sub>4</sub>, 25 µl). The optical density of each well was determined using a Victor3<sup>TM</sup> microplate reader at an absorbance of 450 nm and corrected by reading the absorbance at 570 nm. The concentration of cytokine production was calculated based on the standard values.

#### **4.3.6 Whole blood model and complement activation**

Refludan (50 µg/ml; Pharmion, Norway) anti-coagulated venous whole blood was obtained from a healthy volunteer using sterile 4.5 ml tubes (Nunc A/S, Thermo Scientific, Denmark). Following venepuncture, 375 µl of blood was aliquoted equally into sterile 1.8 ml polypropylene tubes (Nunc, Thermo Scientific, Denmark) containing 150 µl of bacterial supernatant in increasing (1:5) dilutions and maintained at 37°C. Control tubes contained 150 µl of PBS, 150 µl zymosan (20 µg/ml; Sigma-Aldrich) and 150 µl LPS (0.10 µg/ml; Ultrapure, Invitrogen). Control (PBS/blood; 50 µl) was sampled immediately (4 min incubation) and added to a 96-well plate containing 50 µl of 1% v/v paraformaldehyde (PFA; BD Falcon, Norway) and used as a control for FACS analysis. The remaining control/PBS was added to 9.5 µl EDTA (10.0 mM final concentration) to arrest complement activation and used as a negative control, Time 0 (T0). After 15 min of incubation at 37°C (T15), 50 µl of each bacterial supernatant/blood and control/blood samples were transferred to 96-well (PCR) plate containing 50 µl of 1% v/v PFA, and incubated (37°C, 4 min), then kept at RT and used for FACS analysis.

Following 30 min (T30) of incubation of the initial experimental samples, 100 µl of bacterial supernatant/blood and control/blood sample were transferred to a Nunc tube containing 2 µl EDTA (10.0 mM final concentration; Sigma-Aldrich) to arrest complement activation and were stored on ice. Samples were centrifuged (2000 x g, 15 min, 4°C) plasma collected and stored at -80°C for IL-8 and terminal complement complex (TCC) analysis. Following 120 min of incubation (T120) at

37°C, the remaining bacterial supernatant/blood and control/blood sample reaction was arrested with 3 µl EDTA (10.0 mM final concentration) and tubes centrifuged (2000 x g, 15 min, 4°C) plasma collected and stored at -80°C for IL-8 analysis.

#### **4.3.6.1 Fluorescence-activated cell sorting (FACS) analysis**

FACS analysis was performed by Mrs L. Ryan NTNU, Trondheim, Norway on the T0 and T15 samples (Section 4.3.6) to analyse CD11b expression on Mo and granulocytes, indicating complement activation. Fluorescently labelled antibodies specific for CD11b-PE (phycoerythrin), CD14-FITC (fluorescein isothiocyanate) and CD14-PE (BD Biosciences, Norway), and LDS-751 (Sigma-Aldrich, Norway) were used. FITC-mouse IgG2b-k was used as a negative control. Following fixation of samples in PFA (Section 4.3.6), 25 µl of the fixed whole blood sample was added to 15 µl of antibody solution (FITC-control, 8.3 µg/ml; CD14-PE, 8.3 µg/ml; and LDS-751, 5.3 µg/ml; or CD14-FITC, 8.3 µg/ml; CD11b-PE, 8.3 µg/ml; and LDS-751, 5.3 µg/ml). Samples were incubated for 15 min in darkness at RT. The stained samples were transferred to a plastic test tube and 450 µl of PBS added. The sample was then analysed using a fluorescence-activated cell sorter (BD Vantage, BD PharMingen) setting the threshold for the nuclear dye LDS-751 to separate leukocytes and erythrocytes. Granulocytes and Mo were gated on forward scatter (FSC) and side scatter (SSC) dotplots using CD14, and used to analyse the cellular population expressing CD11b with regards to median fluorescence intensity (MFI).

#### **4.3.6.2 Terminal complement complex (TCC) analysis and IL-8 production for the whole blood model**

IL-8 analysis was performed using an ELISA based assay as in Section 4.3.5.4 using T120 whole blood samples from Section 4.3.6. The serum collected was diluted (serum derived following incubation with 1:5; 1:25 and 1:125 bacterial supernatants were diluted 1:10; 1:3 and 1:2 respectively) using diluting reagent. TCC analysis was performed by Mrs L. Ryan NTNU, Trondheim, Norway. A 96-

well ELISA plate (Nunc, Thermo scientific, Denmark) was coated using 50 µl of aE11 95A antibody (a gift from Professor T. E Mollnes of Nordland Central Hospital, Bodø, Norway; Mollnes et al., 1985a) and incubated (4°C, O/N). The plate was washed with PBS containing 0.05% Tween<sup>®</sup> 20 (wash buffer). A standard curve was prepared using zymosan-activated serum diluted with PBS; 0.2% Tween<sup>®</sup> 20 and 10 mM EDTA to a maximum concentration of 200 AU/ml. The standard and samples (50 µl) were added to the plate and incubated (RT, 1 h). The plate was washed (x3), and detection antibody (50 µl) biotinylated mAb 9C4 (anti-C6; Hogasen et al., 1995) added and incubated (37°C, 45 min), followed by washing with wash buffer (x3). Then Streptavidin-HRP (R&D Systems, Norway) was diluted 1:200 with reagent diluents and 50 µl pipetted into each well. Plates were incubated (37°C, 30 min) in darkness, washed (x3) with wash buffer and blotted dry. Then 50 µl of TMB substrate solution was added to each well. The reaction was stopped (2NH<sub>2</sub>.SO<sub>4</sub>, 50 µl) after 30 min and the plate absorbance measured at 450 nm and corrected by reading the absorbance at 570 nm.

#### **4.3.7 Detection of LPS in bacterial supernatants**

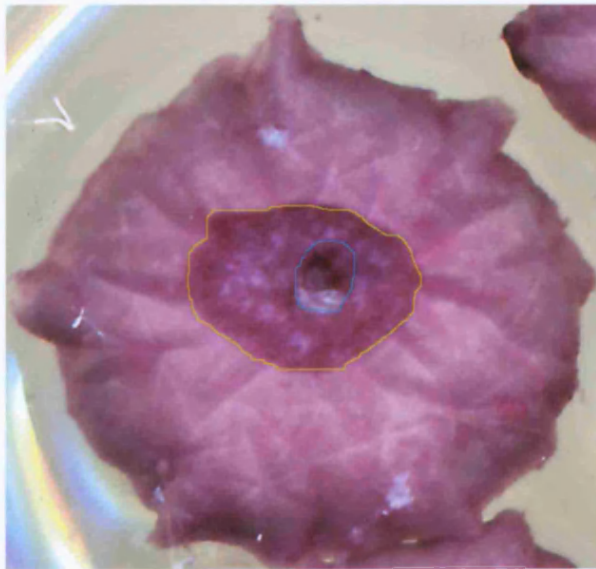
LPS levels of *P. aeruginosa* supernatants were determined with the help of Dr Ø. Rognstad, NTNU, Trondheim, Norway, using the chromogenic *Limulus* amoebocyte lysate (LAL) assay (Cambrex, Walkersville, Maryland, USA). Bacterial supernatants were diluted 1:1000; 1:3000; 1:9000 with sterile water (Invitrogen). Using a pre-warmed microplate (37°C), 50 µl of standard (0.1 to 1 EU/ml) and test sample were dispensed into duplicate wells, LAL reagent water (50 µl) was used as a negative control. To each well, 50 µl LAL was added and incubated (37°C, 10 min) then 100 µl of pre-warmed (37°C) substrate solution was added. Following incubation (37°C, 6 min) 100 µl of stop solution (25%, v/v, glacial acetic acid in H<sub>2</sub>O) was added. Optical density was recorded at 405 nm using a Victor3<sup>™</sup> microplate reader, and LPS concentrations calculated based on standard values.

#### **4.3.8 *In vitro* human wound healing model**

Discarded human abdominal skin was acquired following abdominoplasty, from St Olav's hospital, Trondheim, Norway (following approval of the Local Ethical Committee from the Plastic Surgery Department at St Olav's Hospital, Trondheim, Norway; Appendix I). Using an aseptic technique in a laminar flow cabinet, the skin was cleansed with 70% alcohol and underlying adipose tissue removed. A 6 mm punch biopsy into the connective tissue (approximately 5 mm) was created for the wound explants and within this, a 3 mm punch biopsy wound was prepared into the epidermis (Figure 4.2). The wounded explants were incubated (37°C, 8% CO<sub>2</sub>, O/N) in DMEM supplemented with 10% FCS, gentamicin 10 µg/ml and amphotericin B 0.25 µg/ml (Cascade biologics Inc, Portland, USA). Bacterial supernatants were filter-sterilised (0.2 µm) and diluted (1:10, 1:50 and 1:250) with DMEM supplemented with 10% FCS, gentamicin 10 µg/ml and amphotericin B 0.25 µg/ml prior to use. The wounds were placed in 6-well tissue culture plates (Corning Life Sciences, Norway). To each well, 1 ml of bacterial supernatant/media or growth media (control) per wound was added, incubated (37°C, 8% CO<sub>2</sub>) for 8 d, and media replenished every 48 h (Jansson et al., 1996). The media was removed and 1 ml per wound of supplemented DMEM (10% FCS; 10 µg/ml ciprofloxacin; 0.68 mM glutamine and 0.5 mg/ml MTT) added. Following incubation (37°C, 8% CO<sub>2</sub>, 4 h), wounds were washed and re-suspended in 2 ml of PBS. Using a Cannon office scanner, the inverted wounds were imaged and analysed using ImageJ software (ImageJ 1.43v; <http://rsb.info.nih.gov/ij/>) to determine wound healing as a percentage of re-epithelialisation (Figure 4.3).



**Figure 4.2** Wound preparation of human abdominal skin used for *in vitro* wound model. a) Punch biopsy; b) Excision of wound from underlying connective tissue.



**Figure 4.3** Skin wound explant following MTT staining. Outline of initial wound (yellow); wound following re-epithelialisation (blue).



#### **4.3.9 The effect of bacterial supernatants on wound healing using the “scratch” wound assay**

These studies employed the spontaneously immortalized, epidermal keratinocytes, Human adult low Calcium Temperature (HaCaT) cell line (Boukamp et al., 1988) to study wound healing based on a monolayer “Scratch” wound assay (Lampugnani, 1999, Stephens et al., 2003).

##### **4.3.9.1 Culture of keratinocyte (HaCaT) cells**

Cells were cultured in T175 flasks in keratinocyte media (Flavin adenine dinucleotide; FAD) containing supplemented high glucose DMEM (Invitrogen; 22.5% v/v Ham F-12 nutrient media, Invitrogen; 10% v/v FCS; 0.089 mM adenine; 10 ng/ml epidermal growth factor; 0.08 µg/ml cholera toxin; 400 ng/ml hydrocortisone; 5 ng/ml insulin, Sigma-Aldrich; 100 U/ml penicillin G; 100 µg/ml streptomycin sulphate, and 0.25 µg/ml amphotericin B, Invitrogen) at 37°C, 5% CO<sub>2</sub> in a humid environment until 70 - 80% confluent.

##### **4.3.9.2 Culturing HaCaT cells for scratch wound assay**

T175 flasks were washed with pre-warmed (37°C) PBS and cells trypsinised by adding 3 ml of 0.05% trypsin/EDTA (Invitrogen) and incubating at 37°C for 2 min until the cells had detached. Cells were suspended in 10 ml of FAD media, centrifuged (180 x g, 5 min) and re-suspended in 4 ml of FAD. Viable cells number was determined using a Casy<sup>®</sup> cell counter model DT (Schärfe System GmbH, Germany);  $1.5 \times 10^5$  cells/well seeded into 24-well plates (BD Falcon<sup>™</sup>, New Jersey, USA) and incubated (37°C, 5% CO<sub>2</sub>, 36 h) until confluent.

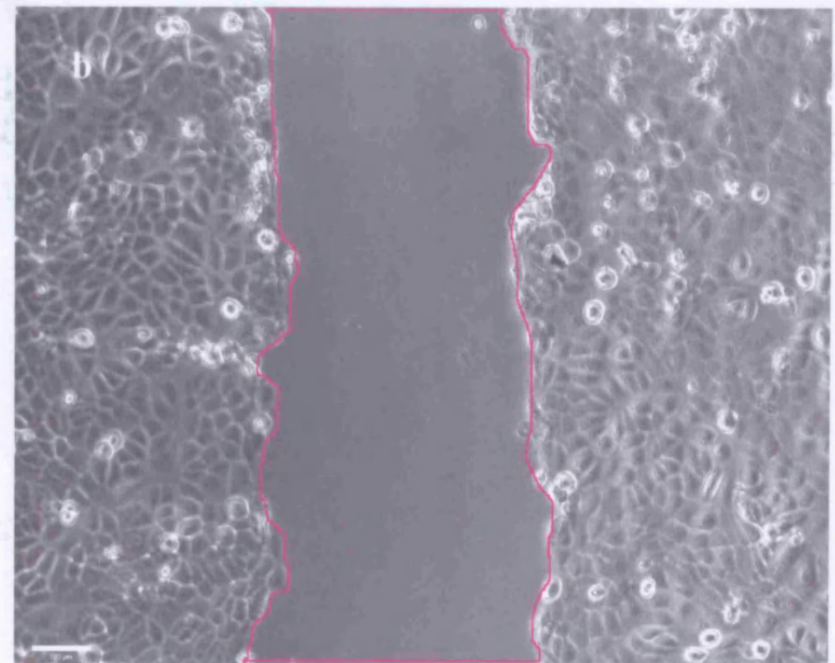
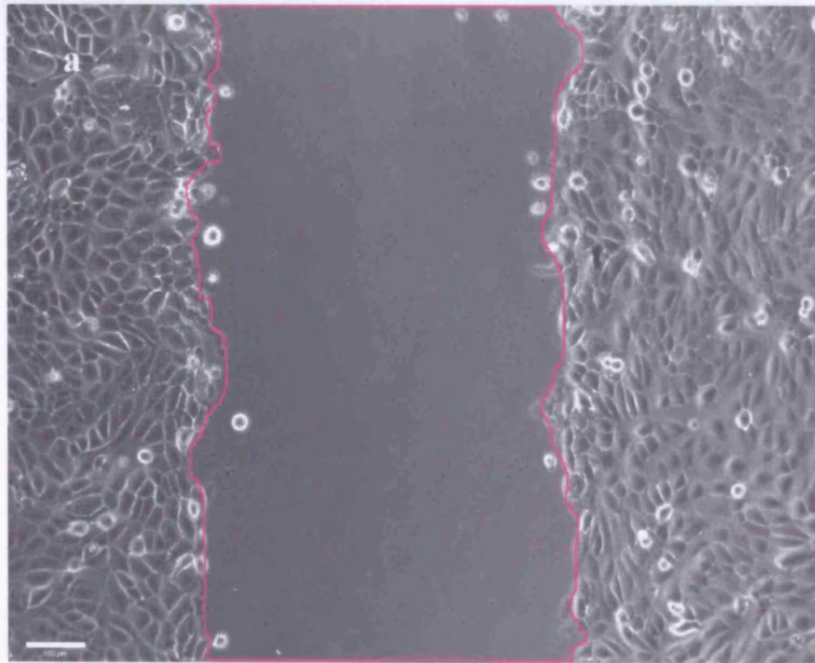
##### **4.3.9.3 “Scratch” wound assay**

The DMEM prepared supernatants (Section 4.3.2.2) were filter-sterilised (0.2 µm filter), and serially diluted (1:10) with pre-warmed (37°C) FAD media. A linear wound was created using a 200 µl sterile filter tip in the centre of each well. The

media was removed and the wells washed with PBS (x2) and diluted bacterial supernatants (1.5 ml) added to duplicate wells. Scratch wounds incubated in FAD media alone were used as controls. Experiments were repeated in triplicate and cell migration was visualised over a 24 h period using a time-lapse microscope (Zeiss Axiovert 200M) attached to a Hamamatsu digital camera using Openlab 5.0.2<sup>®</sup> software (Improvision, Perkin Elmer) under culture conditions (37°C, 5% CO<sub>2</sub>). Digital images were captured at 20 min intervals and sequences recorded as QuickTime<sup>®</sup> (Apple Computer Inc, USA) movie files.

#### **4.3.9.4 Image analysis**

The wound area and rate (speed) of closure of each scratch wound was estimated for all supernatants at dilutions 1:10 and 1:31250 at times 0, 6, 12 and 24 h. Using OpenLab<sup>®</sup> software (Open Lab, Florence, Italy), Quicktime<sup>®</sup> images were exported for analysis using ImageJ software (ImageJ 1.43v; <http://rsb.info.nih.gov/ij/>). Wound outline and pixel area was recorded for each time point (Figure 4.4). The velocity of wound area reduction was used to deduce speed of wound closure (cell migration).



**Figure 4.4** Images of control (FAD media) “scratch” wound from time lapse outlining wound space for analysis. a) 0 h; b) 6 h (scale bar represents 100  $\mu\text{m}$ ).

#### **4.3.10 Statistics**

All statistical analysis was performed using GraphPad prism3<sup>®</sup> (GraphPad software Inc, California USA) and Minitab<sup>®</sup> (Release 14 software, Minitab<sup>®</sup> Ltd, Coventry, UK). Statistical analysis of NF- $\kappa$ B values for the luciferase reporter assay, Mo cytokine production and “scratch” wound assay (speed of wound closure and wound area reduction) were performed using a Kruskal-Wallis test to compare individual and grouped isolates. Where this was significant ( $P < 0.05$ ) a Dunn post-test was performed. A Mann-Whitney test was performed for the luciferase, Mo cytokine production and “scratch” wound assay to compare planktonic vs. biofilm produced supernatants (*P. aeruginosa* and *S. aureus*).

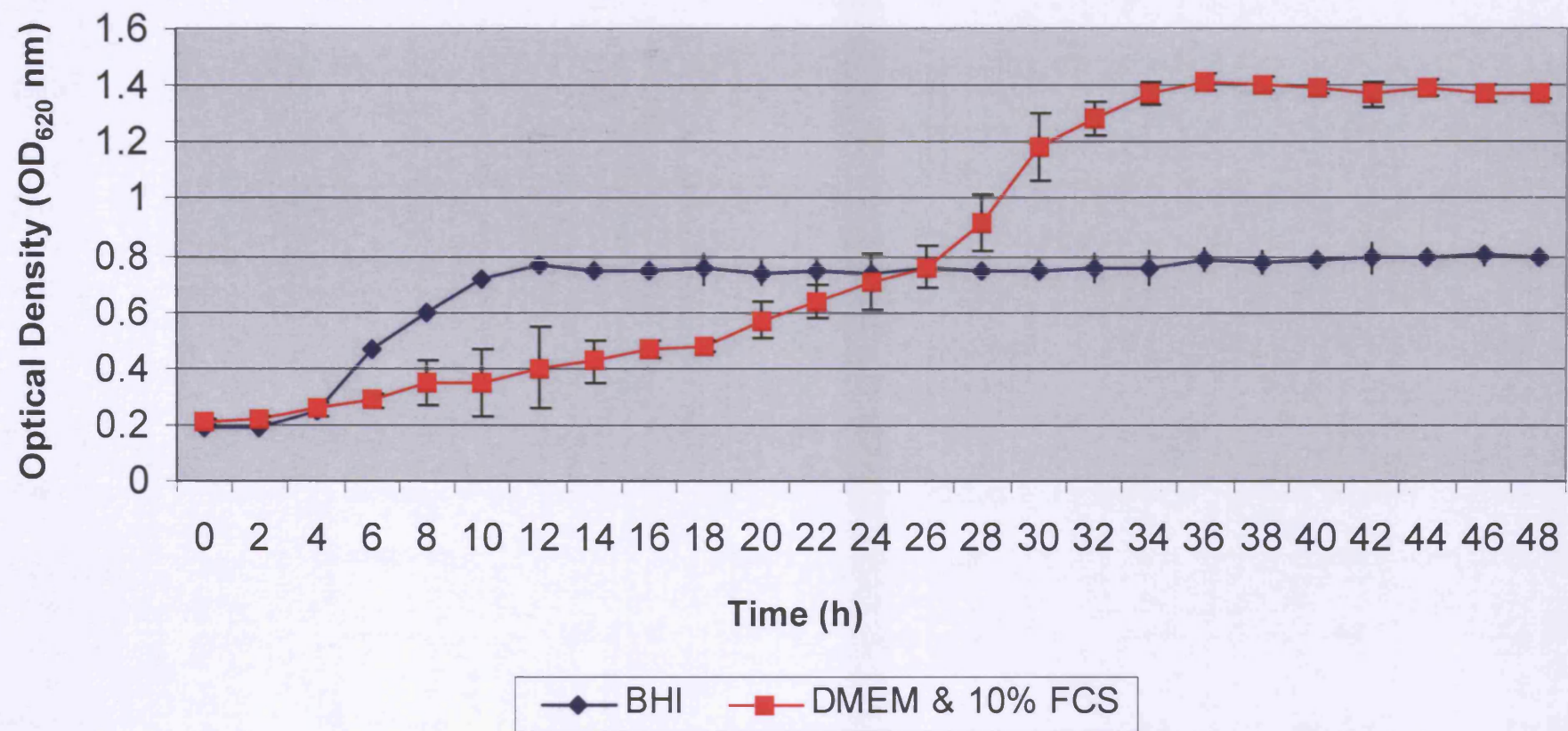
### **4.4 Results**

#### **4.4.1 Growth curve determination**

The growth curves of all studied bacterial isolates were initially performed in DMEM. There was however, limited bacterial growth and DMEM supplemented with 10% FCS was subsequently employed. Figure 4.5 shows the typical growth curve achieved with bacteria grown in DMEM supplemented with 10% FCS, delaying stationary phase to approximately 36 h. In contrast, isolates grown in conventional media typically attained stationary phase within 10 h.

#### **4.4.2 Protein content analysis**

The protein content of the bacterial supernatants derived using the BCA assay and CFU/ml for each growth medium is shown in Table 4.3. It was evident from this that bacteria were able to survive and grow in DMEM supplemented with 10% FCS based upon achieved CFU/ml.



**Figure 4.5** 48 h growth curve for MRSA isolate (1011) in DMEM supplemented with 10% FCS and BHI.

**Table 4.3** Protein content and colony forming units derived from growth media to produce supernatants

Isolate	Origin	<sup>a</sup> Growth condition	<sup>b</sup> TSB		<sup>b</sup> BHI		<sup>b</sup> DMEM & 10%FCS	
			Protein content (µg/ml)	Mean CFU	Protein content (µg/ml)	Mean CFU	Protein content (µg/ml)	Mean CFU
Control media			1089.712		1230.13		637.32	
MRSA	Nasal	Planktonic	606.5 ± 49.7	2.5 x 10 <sup>7</sup>	927 ± 218.5	3.1 x 10 <sup>7</sup>	561.1 ± 151.4	3 x10 <sup>6</sup>
MRSA	CSW	Planktonic	557.9 ± 25.1	1.9 x 10 <sup>7</sup>	933.7 ± 85.2	2.3 x 10 <sup>7</sup>	500 ± 178.8	2.8 x 10 <sup>6</sup>
MRSA	CLW	Planktonic	613 ± 59	2.3 x 10 <sup>7</sup>	868.5 ± 125.1	3 x 10 <sup>7</sup>	569.5 ± 135	3.3 x10 <sup>6</sup>
<i>S. aureus</i>	FC2	Planktonic	643.2	3.5 x 10 <sup>7</sup>	737.86	4.67 x 10 <sup>7</sup>	738.2	1.9 x 10 <sup>6</sup>
<i>S. aureus</i>	FC2	Biofilm	691.5	1.4 x 10 <sup>7</sup>	858.56	1.63 x 10 <sup>7</sup>	538.46	1.4 x 10 <sup>6</sup>
<i>P. aeruginosa</i>	NCTC 10662	Planktonic	537.6	4.5 x 10 <sup>8</sup>	n/a	n/a	607.2	2.1 x 10 <sup>8</sup>
<i>P. aeruginosa</i>	NCTC 10662	Biofilm	663.8	3.2 x 10 <sup>8</sup>	n/a	n/a	568.5	1.8 x 10 <sup>8</sup>
<i>P. aeruginosa</i> (mucoid)	ATCC 39324	Planktonic	652.3 ± 106.7	4 x 10 <sup>8</sup>	n/a	n/a	407.9 ± 20	1.2 x 10 <sup>8</sup>

CSW, chronic surgical wound; CLW, chronic leg wound; TSB, tryptone soy broth; BHI, brain heart infusion broth; DMEM & 10% FCS, DMEM supplemented with 10% FCS; CFU, colony forming units; n/a, not applicable; FC2, clinical (CVLU) isolate (Davies et al., 2004);

<sup>a</sup>growth condition in which supernatant produced; <sup>b</sup>media used to produce supernatant.

### **4.4.3 Luciferase reporter assay (Toll-like receptor stimulation)**

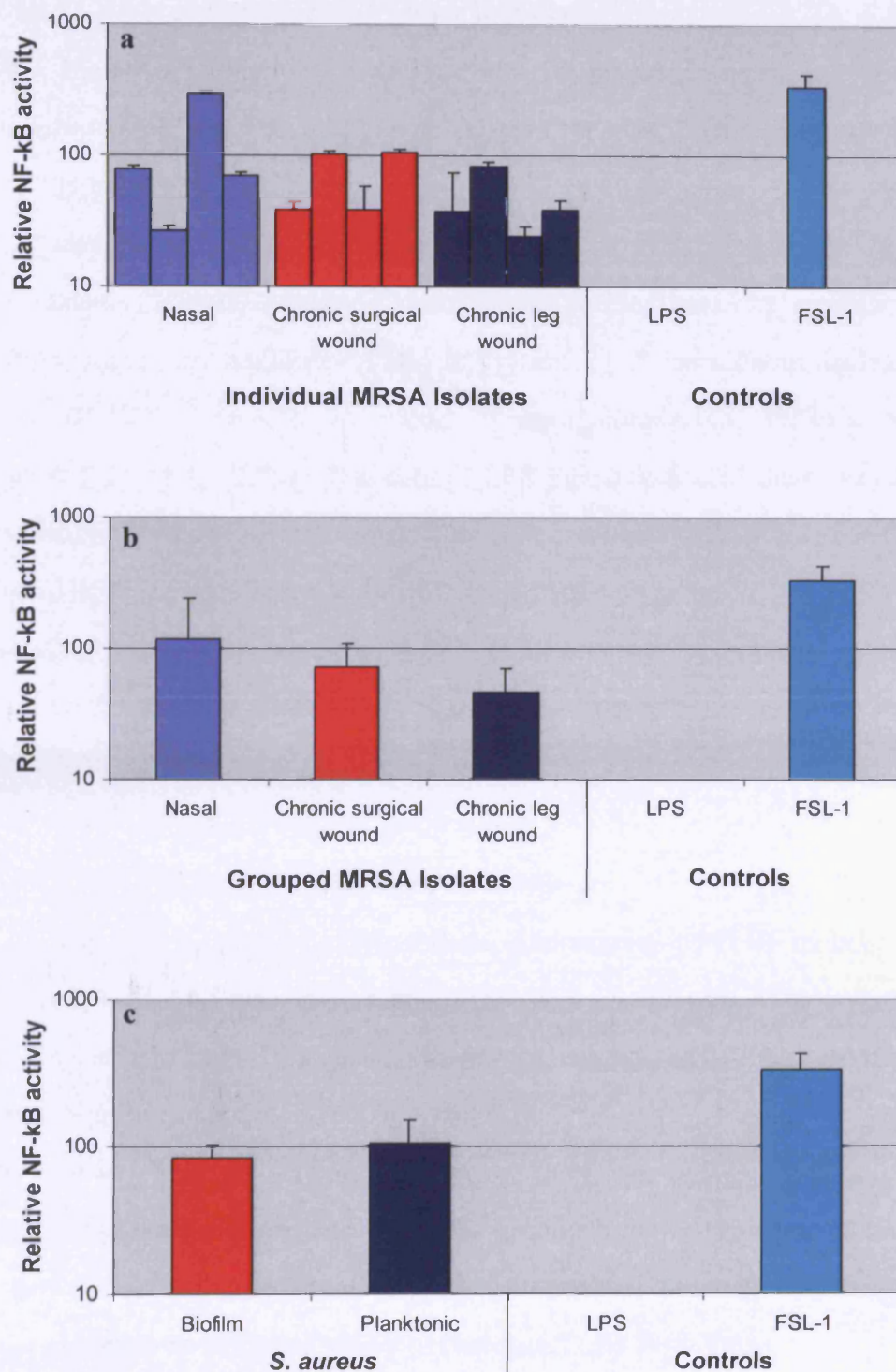
#### **4.4.3.1 TLR2 response to MRSA supernatants**

NF- $\kappa$ B activity induction relative to *Renilla* luciferase and TLR negative cells, following stimulation of TLR2 transfected HEK 293E cells with 1:25 diluted DMEM asymptomatic nasal carrier, chronic leg wound (CLW) and chronic surgical wound (CSW) MRSA supernatants is shown in Figure 4.6. Variation in NF- $\kappa$ B activation by different MRSA isolates was observed. Figure 4.6a shows activation for individual MRSA isolates (n = 4 per wound group). Figure 4.6b shows mean NF- $\kappa$ B activation for MRSA grouped isolates. TLR2 stimulation was relatively low compared with the positive control FSL-1. Nasal MRSA isolates stimulated TLR2 more than either group of chronic wound MRSA isolates (Figure 4.6b) although this was not significant ( $P>0.05$ ). In these experiments, the high activation of NF- $\kappa$ B by FSL-1 (positive control) indicated that transfection was successful. Also, no significant activation of NF- $\kappa$ B by LPS negative control indicated that activation was not due to TLR4.

#### **4.4.3.2 TLR2 response to bacterial biofilm and planktonic supernatants**

NF- $\kappa$ B activity induction relative to *Renilla* luciferase and TLR negative cells following stimulation of TLR2 transfected HEK 293E cells with biofilm and planktonic *S. aureus* (FC2) supernatants is shown in Figure 4.6c. No significant difference ( $P>0.05$ ) in TLR2 stimulation was achieved for *S. aureus* supernatants prepared under different growth conditions.





**Figure 4.6** NF- $\kappa$ B activity relative to *Renilla* luciferase and TLR negative cells following stimulation of TLR2 transfected HEK 293E cells with 1:25 diluted DMEM bacterial supernatants. a) Individual MRSA isolates; b) Grouped MRSA i.e. nasal, chronic surgical or chronic leg wound; c) *S. aureus* (FC2) supernatant produced under biofilm or planktonic growth conditions. Error bars denote standard deviation of the mean. LPS, lipopolysaccharide; FSL-1, fibroblast stimulating lipopeptide-1.

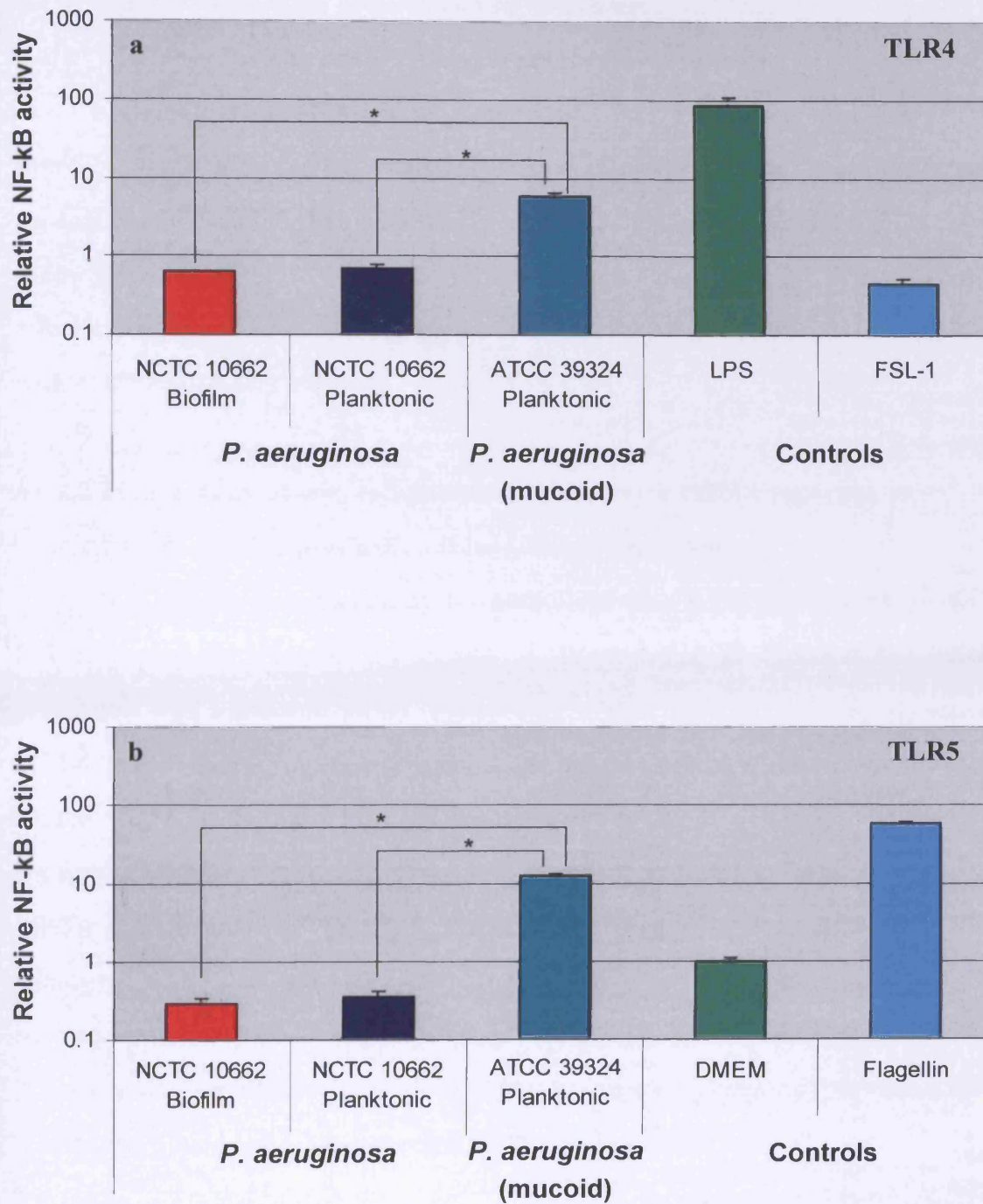


#### 4.4.3.3 TLR4 response to DMEM supernatants

The NF- $\kappa$ B activity induction relative to *Renilla* luciferase and TLR negative cells following stimulation of TLR4 transfected HEK 293E cells is shown in Figure 4.7a. In these experiments there was no significant difference in TLR4 stimulation by *P. aeruginosa* (NCTC 10662) when grown in either planktonic or biofilm environments ( $P>0.05$ ) for any dilution of the DMEM bacterial supernatants. In addition, there was negligible TLR4 activation by *P. aeruginosa* isolate NCTC 10662 and DMEM control. In contrast, *P. aeruginosa* ATCC 39324 significantly stimulated TLR4 ( $P<0.05$ ). The control LPS ligand indicated there was adequate transfection of the HEK 293E cells. Ligand FSL-1 indicated negligible background NF- $\kappa$ B via TLR2 stimulation and that the transfection was specific to TLR4 ligands. Interestingly, the LAL analysis of the pseudomonad experimental supernatants demonstrated that at dilutions  $<1:10,000$ , concentrations of LPS exceeded the standard curve ( $>900$  ng/ml; results not shown).

#### 4.4.3.4 TLR5 response to DMEM supernatants

The relative NF- $\kappa$ B activity following stimulation of TLR5 transfected HEK 293E cells with DMEM supernatants is shown in Figure 4.7b. The control ligand flagellin indicated that the transfection was successful, whilst the DMEM negative control signified little background stimulation of cells. Again, no significant difference in TLR5 stimulation was observed between NCTC 10662 biofilm and planktonic growth environments. In these experiments, whilst isolate NCTC 10662 (*P. aeruginosa*) failed to stimulate TLR5, the mucoid pseudomonad strain ATCC 39324 exhibited an increased ability to stimulate TLR5 ( $P<0.05$ ).



**Figure 4.7** Relative NF-κB activity following stimulation of transfected HEK 293E cells with *P. aeruginosa* DMEM bacterial supernatants. a) TLR4, 1:25 dilution; b) TLR5, 1:125 dilution. Error bars denote standard deviation of the mean; \* $P < 0.05$ ; LPS, lipopolysaccharide; FSL-1, fibroblast stimulating lipopeptide-1.

#### **4.4.4 Monocyte activation by bacterial supernatants**

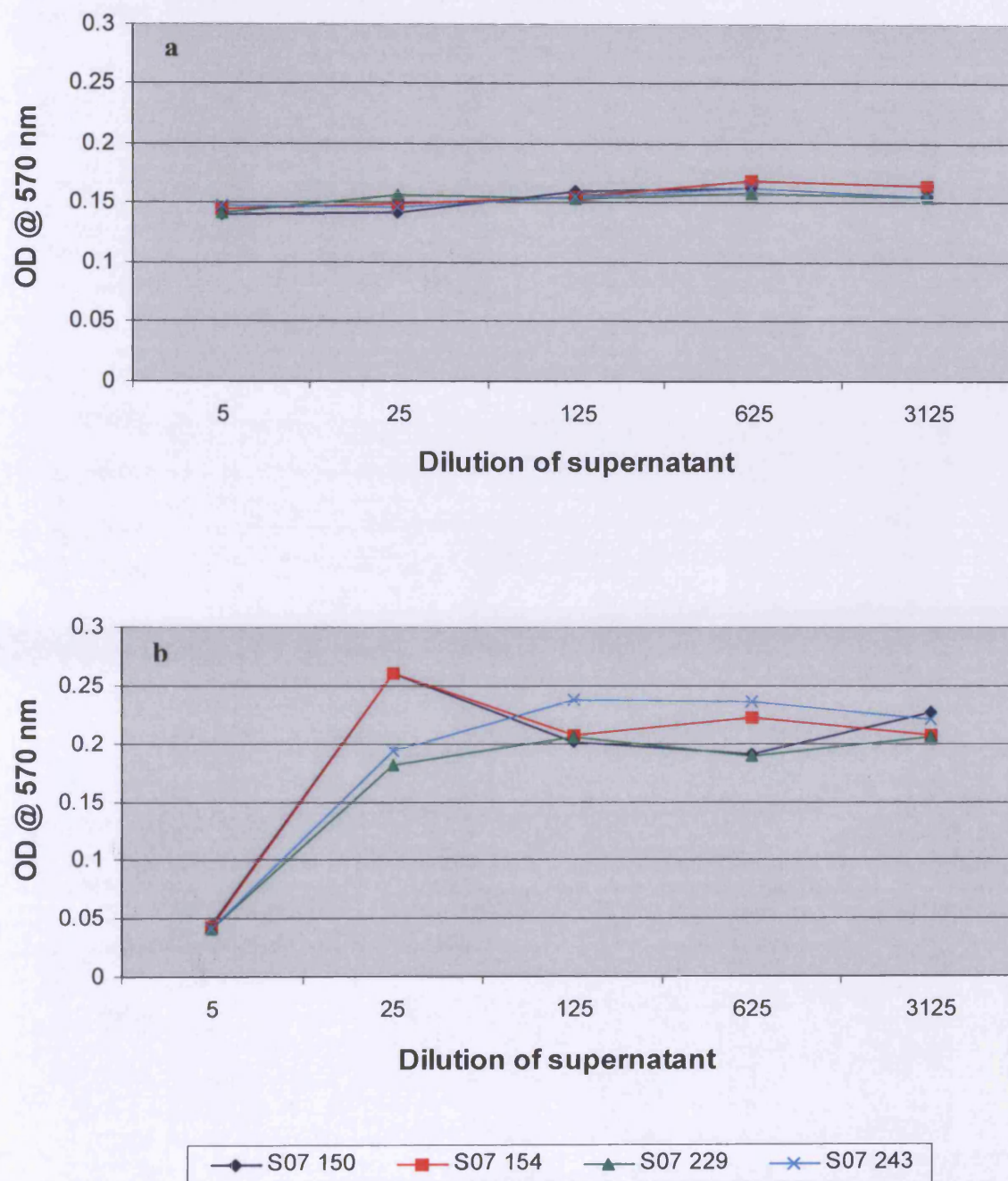
##### **4.4.4.1 MTT cytotoxicity assay following monocyte stimulation**

Cytotoxicity of all bacterial supernatants was determined using an MTT assay. Figure 4.8 shows a typical dose-response curve for nasal bacterial supernatants. The MTT assay indicated a minimal effect on the viability of the cells at low dilutions of the DMEM supernatants (Figure 4.8a). However, the cytotoxic effect was exacerbated in BHI supernatants (Figure 4.8b) necessitating normalisation with >1:25 supernatant dilutions.

##### **4.4.4.2 Monocyte cytokine production stimulated with MRSA supernatants**

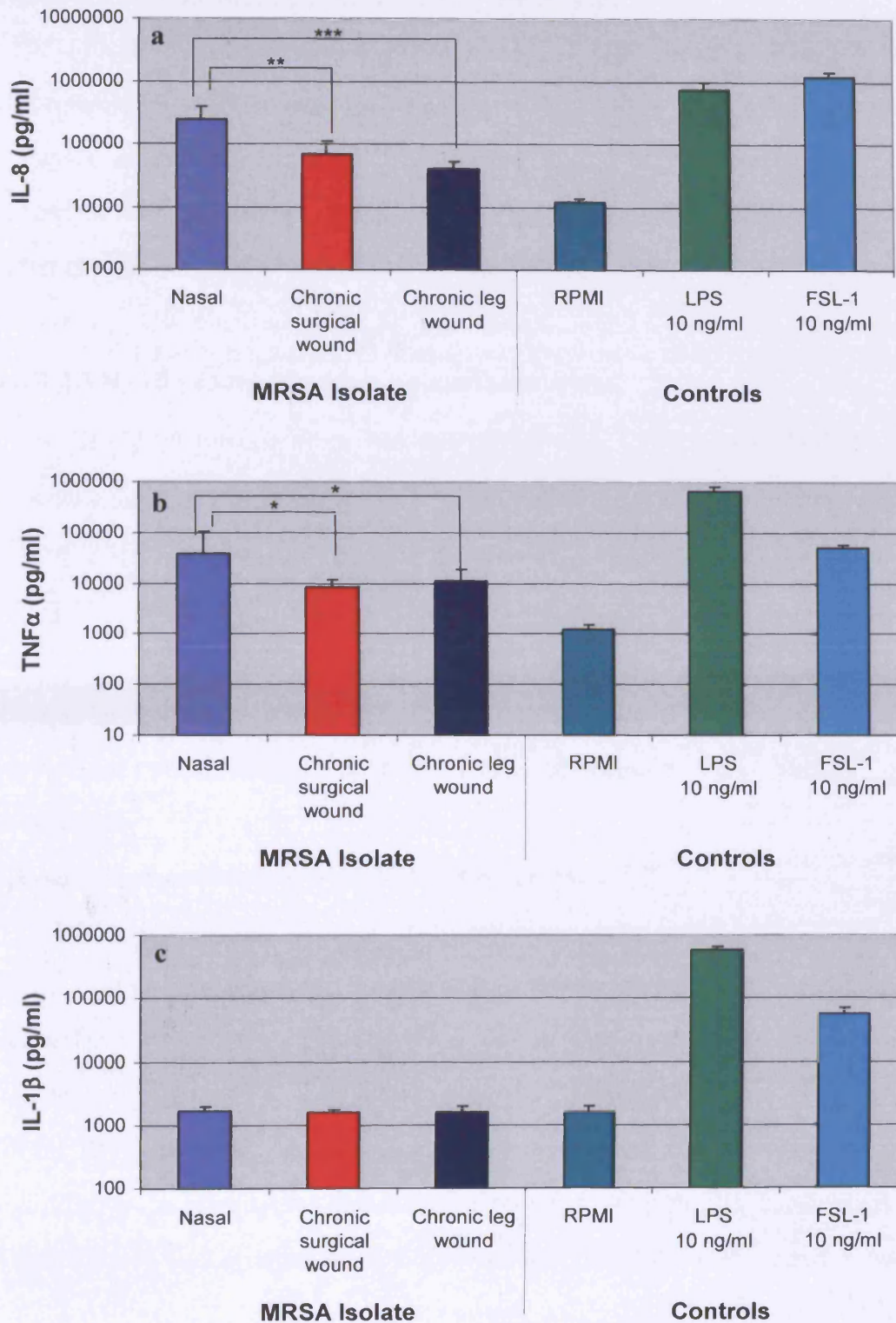
###### **4.4.4.2.1 IL-8 cytokine production from isolated monocytes**

IL-8 cytokine production by Mo stimulated with 1:125 dilutions of DMEM MRSA supernatants studied by ELISA is demonstrated in Figure 4.9a. IL-8 production was stimulated by all dilutions of MRSA supernatants. Nasal carrier MRSA induced significantly increased production of IL-8 compared to the chronic wound MRSA in these experiments, whilst results for chronic surgical and chronic leg wound MRSA were similar ( $P>0.05$ ). This trend was evident for all dilutions of MRSA supernatants (1:5 to 1:625, results not shown). IL-8 production by Mo stimulated with (1:25 and 1:125 diluted) nasal and chronic surgical wound MRSA supernatants was statistically significant ( $P<0.01$ ). A statistically significant difference was also observed for Mo IL-8 production when stimulated with nasal and chronic leg wound MRSA (supernatant dilution 1:5 to 1:125;  $P<0.01$ ).



**Figure 4.8** Dose response MTT monocyte cytotoxicity assay following exposure to nasal MRSA supernatants. a) DMEM; b) BHI.





**Figure 4.9** Monocyte cytokine production following stimulation with MRSA bacterial supernatants. a) IL-8, 1:125 dilution; b) TNFα, 1:25 dilution; and c) IL-1β, 1:125 dilution. Error bars denote standard deviation of the mean. \*P<0.05; \*\*P<0.01; \*\*\*P<0.001; LPS, lipopolysaccharide; FSL-1, fibroblast stimulating lipopeptide-1; RPMI, Roswell Park Memorial Institute medium.

#### **4.4.4.2.2 TNF $\alpha$ production from isolated monocytes**

TNF $\alpha$  production from Mo stimulated with 1:25 dilutions of MRSA DMEM supernatants is demonstrated in Figure 4.9b. Nasal isolates again exhibited an increased ability to stimulate TNF $\alpha$  as with IL-8 ( $P < 0.05$ ) compared with both groups of chronic wound MRSA. There was no statistical significance between either chronic surgical or chronic leg wound MRSA isolates ( $P > 0.05$ ).

#### **4.4.4.2.3 IL-1 $\beta$ production from isolated monocytes**

IL-1 $\beta$  production from Mo stimulated with 1:125 diluted DMEM MRSA supernatants is shown in Figure 4.9c. There was no significant difference between MRSA groups in their ability to stimulate IL-1 $\beta$  production ( $P > 0.05$ ), and IL-1 $\beta$  levels were similar to the RPMI control.

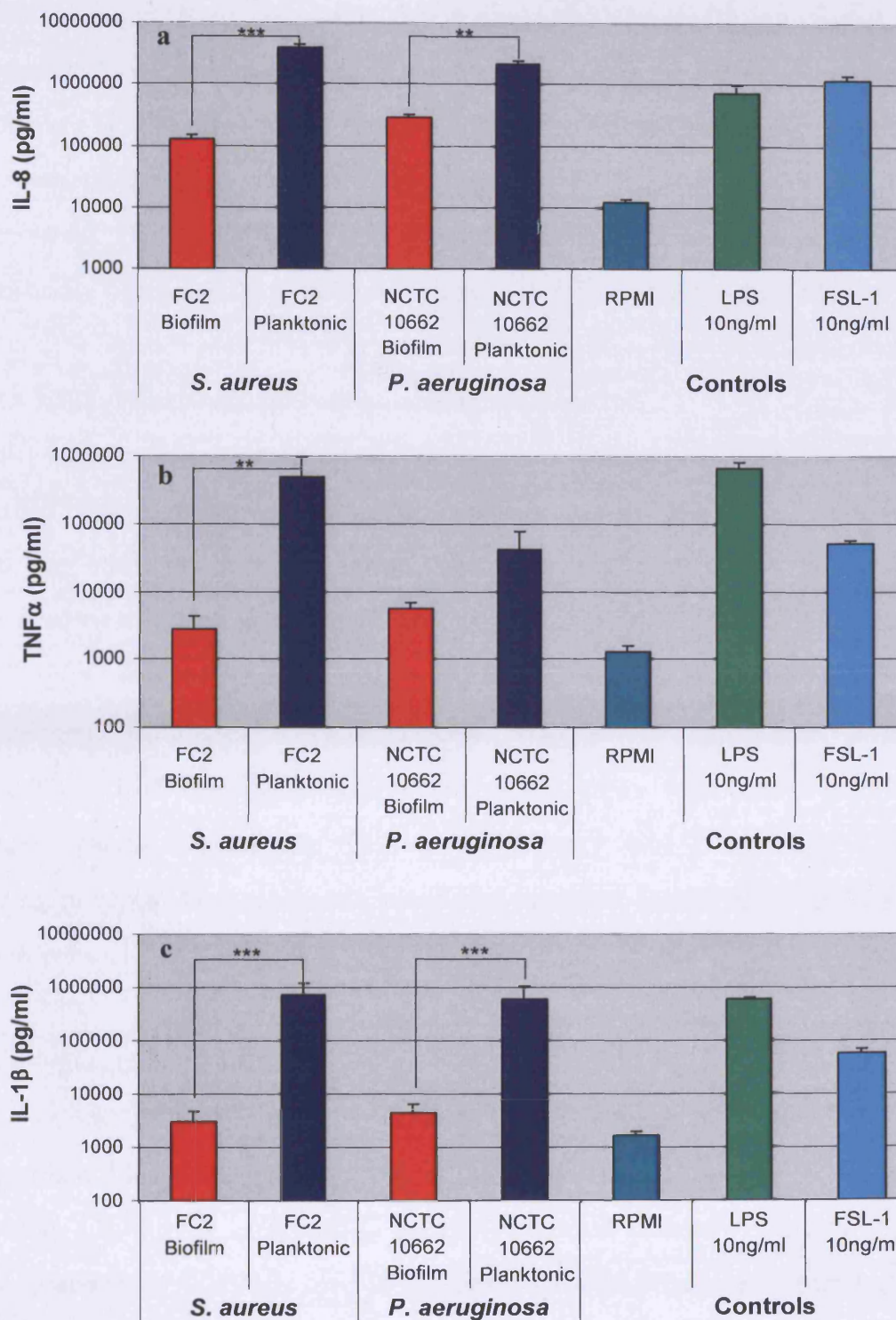
#### **4.4.4.3 Monocyte cytokine production stimulated with *S. aureus* and *P. aeruginosa* supernatants derived from planktonic and biofilm growth conditions**

##### **4.4.4.3.1 IL-8 production from isolated monocytes**

Monocyte production of IL-8 following stimulation with 1:125 diluted DMEM bacterial supernatants from biofilm and planktonic growth conditions for *S. aureus* and *P. aeruginosa* is shown in Figure 4.10a. All bacterial supernatants irrespective of growth condition stimulated IL-8 release above the values observed for the RPMI controls. In these experiments, planktonic growth conditions induced significantly increased IL-8 production compared with biofilm cultures ( $P < 0.01$ ). This increased IL-8 stimulation was observed for both *P. aeruginosa* and *S. aureus*.

##### **4.4.4.3.2 TNF $\alpha$ production from isolated monocytes**

The production of TNF $\alpha$  from stimulated Mo using 1:125 diluted DMEM supernatants produced in biofilm and planktonically grown *S. aureus* and *P. aeruginosa* are shown in Figure 4.10b. In these experiments, all supernatants



**Figure 4.10** Monocyte cytokine production following stimulation with *S. aureus* and *P. aeruginosa* biofilm and planktonic supernatants. a) IL-8, 1:125 dilution; b) TNFα, 1:125 dilution; and c) IL-1β, 1:25 dilution. Error bars denote standard deviation of the mean. \*\*P<0.01; \*\*\*P<0.001; LPS, lipopolysaccharide; FSL-1, fibroblast stimulating lipopeptide-1; RPMI, Roswell Park Memorial Institute medium.

regardless of growth condition were able to stimulate TNF $\alpha$  production. Again, both planktonic grown supernatants indicated a greater stimulation of TNF $\alpha$  than their equivalent biofilm supernatant. This trend was observed at all supernatant dilutions for both isolates, and was statically significant for *S. aureus* 1:25 and 1:125 supernatant dilutions ( $P < 0.01$ ,  $P < 0.05$  respectively). However, this was only statistically significant for *P. aeruginosa* using the 1:5 supernatant dilution ( $P < 0.01$ ).

#### **4.4.4.3.3 IL-1 $\beta$ production from isolated monocytes**

Monocyte production of IL-1 $\beta$  following stimulation with 1:25 diluted DMEM planktonic and biofilm produced supernatants are shown in Figure 4.10c. Like the other cytokine experiments, again the planktonic grown supernatants exhibited an increased stimulatory effect, with minimal levels of IL-1 $\beta$  stimulation by biofilm produced supernatants. Planktonically derived *S. aureus* supernatants significantly stimulated IL-1 $\beta$  production using  $\leq 1:125$  supernatant dilutions ( $P < 0.05$ ). IL-1 $\beta$  stimulation by planktonic *P. aeruginosa* supernatants using 1:25 dilutions yielded a statistically significant difference ( $P < 0.001$ ). Increased dilutions of biofilm supernatants resulted in stimulation similar to the RPMI control (results not shown).

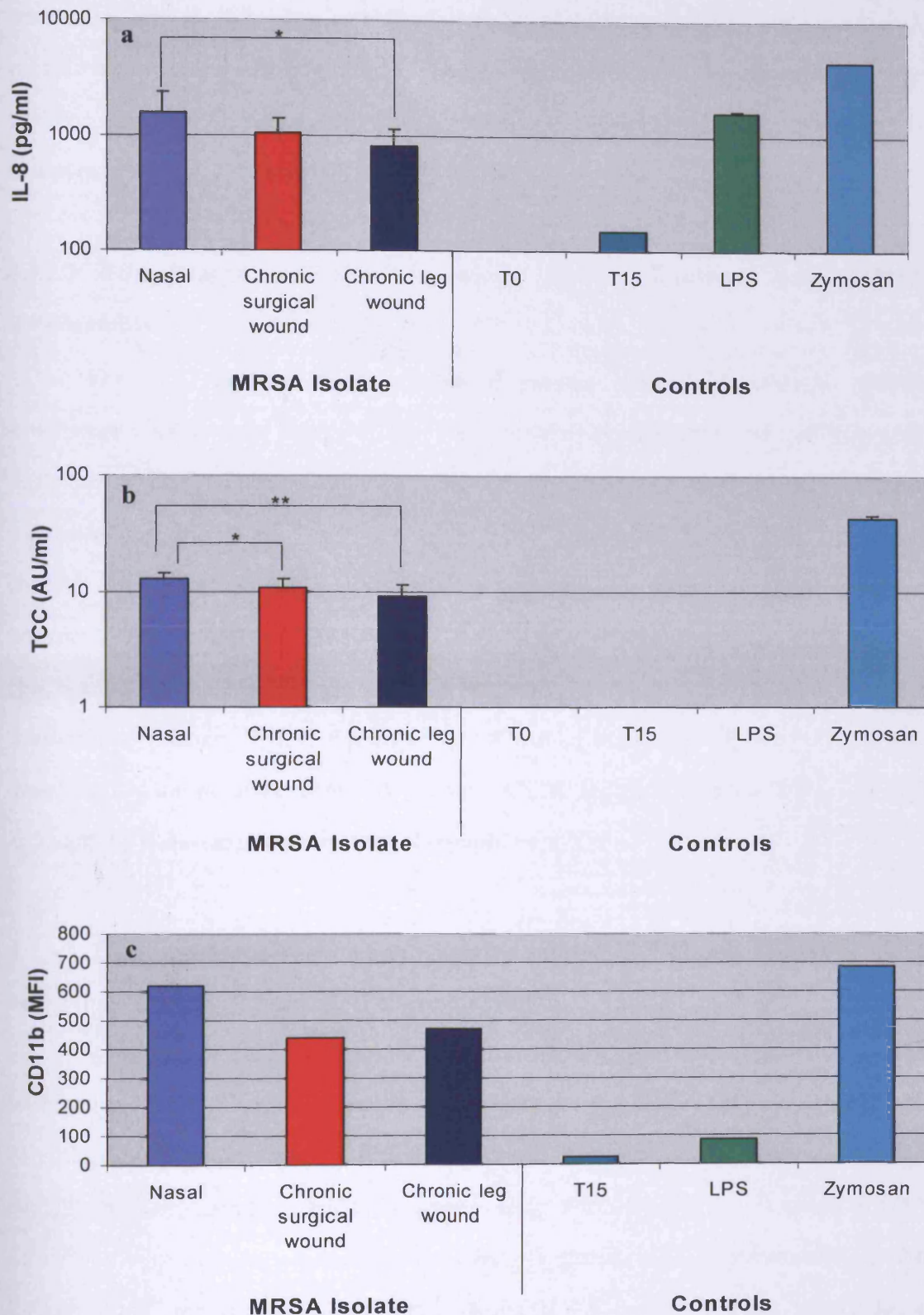
#### **4.4.5 Whole blood model**

Complement activation by the bacterial supernatants was determined using the “whole blood” model. The production of IL-8, and Terminal Complement Complex (TCC) was determined in the EDTA arrested plasma of activated blood. The formation of TCC was used to assess the ability of bacterial supernatants to stimulate complement activation

##### **4.4.5.1 IL-8 production in whole blood stimulated with MRSA supernatants**

Whole blood production of IL-8 following stimulation with 1:5 dilutions of DMEM MRSA supernatants is demonstrated in Figure 4.11a. In these assays, IL-8





**Figure 4.11** Whole blood stimulation with DMEM MRSA supernatants. a) IL-8, 1:5 dilution; b) Terminal Complement Complex (TCC), 1:25 dilution; c) Mean Fluorescence Intensity (MFI) of CD11b stained granulocytes, 1:25 dilution. Error bars denote standard deviation of the mean. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; LPS, lipopolysaccharide; T0, 0 min; T15, 15 min.

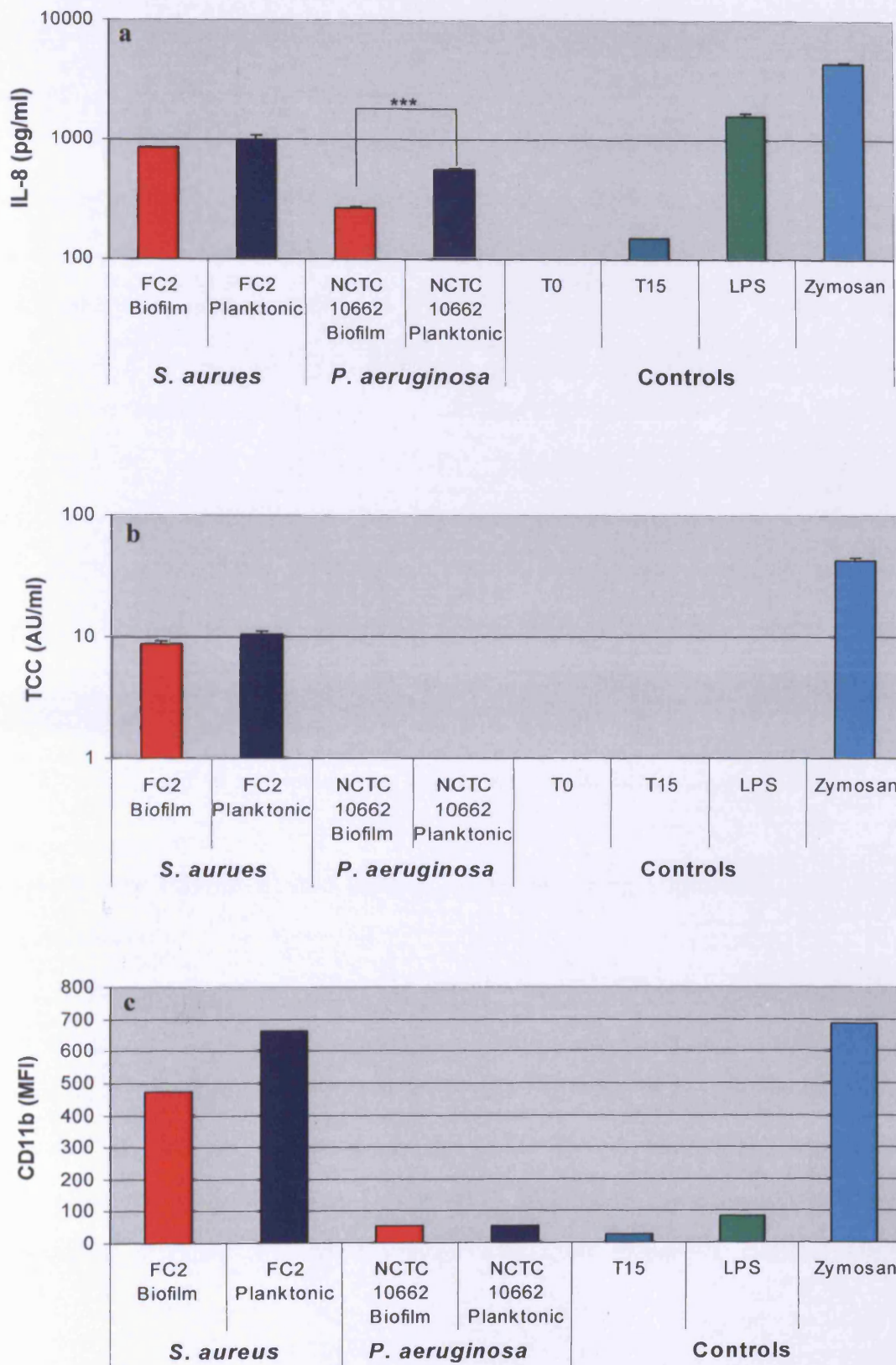
production was significantly increased with nasal MRSA isolates, compared with chronic leg wound MRSA ( $P<0.05$ ). There was however, no significant difference between the wound isolates ( $P>0.05$ ). The trends observed in this assay were similar to that experienced in the stimulated Mo assay.

#### **4.4.5.2 Complement activation in whole blood stimulated with MRSA supernatants**

The TCC derived from stimulated plasma with 1:25 DMEM MRSA supernatants is shown in Figure 4.11b. Nasal isolates significantly induced increased formation of TCC. This was evident using nasal and chronic surgical wound MRSA supernatant dilutions 1:25 and 1:125 ( $P<0.05$ ) and 1:25 dilutions of nasal and chronic leg wound isolates ( $P<0.05$ ). No significant difference was observed between the wound group MRSA isolates ( $P>0.05$ ). These data were supported by FACS analysis of CD11b expression on Mo and granulocytes indicated by median fluorescence intensity (MFI; Figure 4.11c). A strong activation of complement was observed for the positive control zymosan, whilst negative controls PBS (T0 and T15) and LPS showed no activation of complement.

#### **4.4.5.3 IL-8 production in whole blood stimulated with *S. aureus* and *P. aeruginosa* planktonic and biofilm supernatants**

Whole blood IL-8 production following stimulation with 1:5 diluted DMEM supernatants from *S. aureus* and *P. aeruginosa* grown in biofilm and planktonic conditions is shown in Figure 4.12a. In this assay, not only were species differences evident in their response, but also growth state was important. Biofilm growth conditions were associated with a significant decrease in IL-8 production in the “whole blood” model for *P. aeruginosa* ( $P<0.001$ ). *S. aureus* biofilm supernatants also resulted in decreased IL-8 production, however, this was only significant using the 1:125 supernatant dilution ( $P<0.01$ ).



**Figure 4.12** Mean whole blood stimulation with *S. aureus* and *P. aeruginosa* biofilm and planktonic DMEM supernatants. a) IL-8, 1:5 dilution; b) Terminal Complement Complex (TCC), 1:25 dilution; c) Mean Fluorescence Intensity (MFI) of CD11b stained granulocytes, 1:25 dilution. Error bars denote standard deviation of the mean. \*\*\* $P < 0.001$ ; LPS, lipopolysaccharide; T0, 0 min; T15, 15 min.

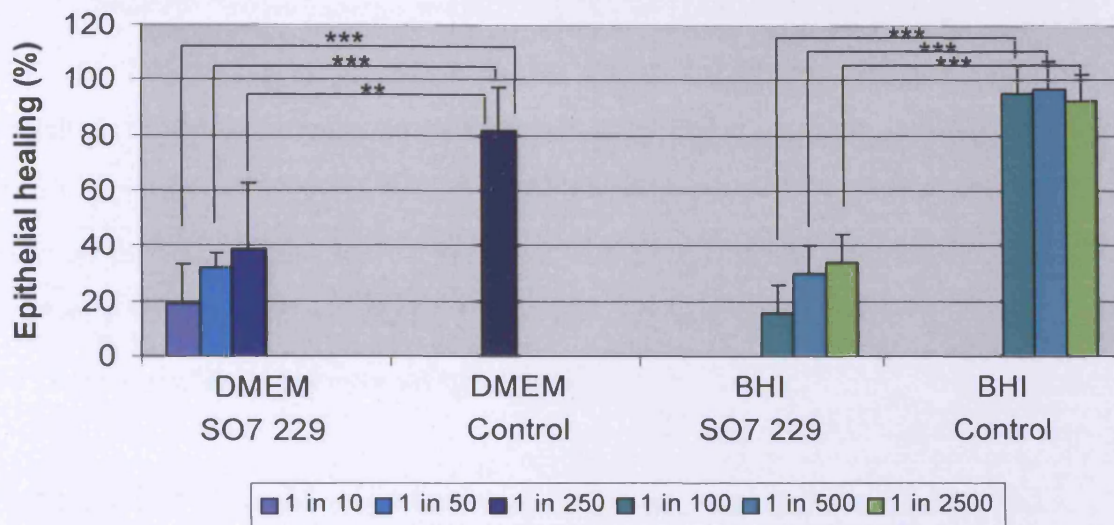
#### **4.4.5.4 Complement activation in whole blood stimulated with *S. aureus* and *P. aeruginosa* planktonic and biofilm supernatants**

TCC activation by 1:25 diluted *S. aureus* and *P. aeruginosa* supernatants produced in biofilm and planktonic conditions are shown in Figure 4.12b. Whilst obvious differences were evident between the two species in their ability to stimulate complement, *S. aureus* stimulated TCC in both planktonic and biofilm growth conditions, with no significant difference observed ( $P>0.05$ ). In this model, *P. aeruginosa* exhibited little ability to induce TCC activation. These data were supported by FACS analysis of CD11b expression on granulocytes and Mo, indicated by the MFI (Figure 4.12c). The induction of CD11b observed in this assay was significantly greater for *S. aureus* than *P. aeruginosa*. Indeed *P. aeruginosa* results in the TCC and CD11b assays were comparable to the PBS control (T15). It was observed that the planktonic grown *S. aureus* stimulated the CD11b greater than biofilm produced supernatants at all supernatant dilutions, and the MFI levels for planktonically grown *S. aureus* were comparable to the control zymosan.

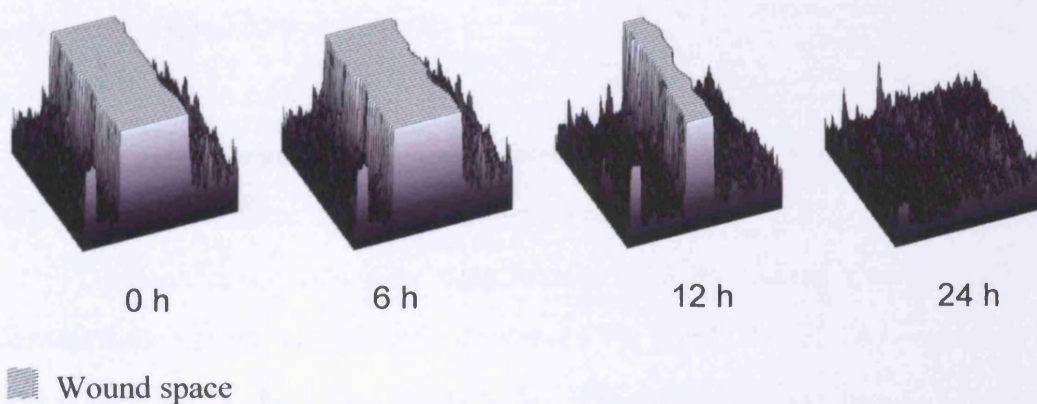
#### **4.4.6 *In vitro* human wound healing model following stimulation with MRSA supernatants**

The percentage of epithelial healing observed following incubation of skin explants in DMEM and BHI grown nasal MRSA supernatants is demonstrated in Figure 4.13. Wound re-epithelialisation was estimated following staining of the wounds with MTT. In this assay, it was observed that MRSA supernatants, irrespective of growth media, significantly influenced re-epithelialisation at all supernatant dilutions studied compared with their respective controls DMEM ( $P<0.005$ ) and BHI ( $P<0.001$ ).





**Figure 4.13** Percentage epithelial healing of the *in vitro* human skin wound explants following incubation with nasal MRSA (SO7 229) bacterial supernatants. Error bars denote standard deviation of the mean. BHI, Brain heart infusion broth; DMEM, Dulbecco's Modified Eagle Medium; \*\*P<0.01; \*\*\*P<0.001.



**Figure 4.14** Topographic representation of wound space closure over a 24 h period following incubation with FAD media control.

#### **4.4.7 “Scratch” wound analysis**

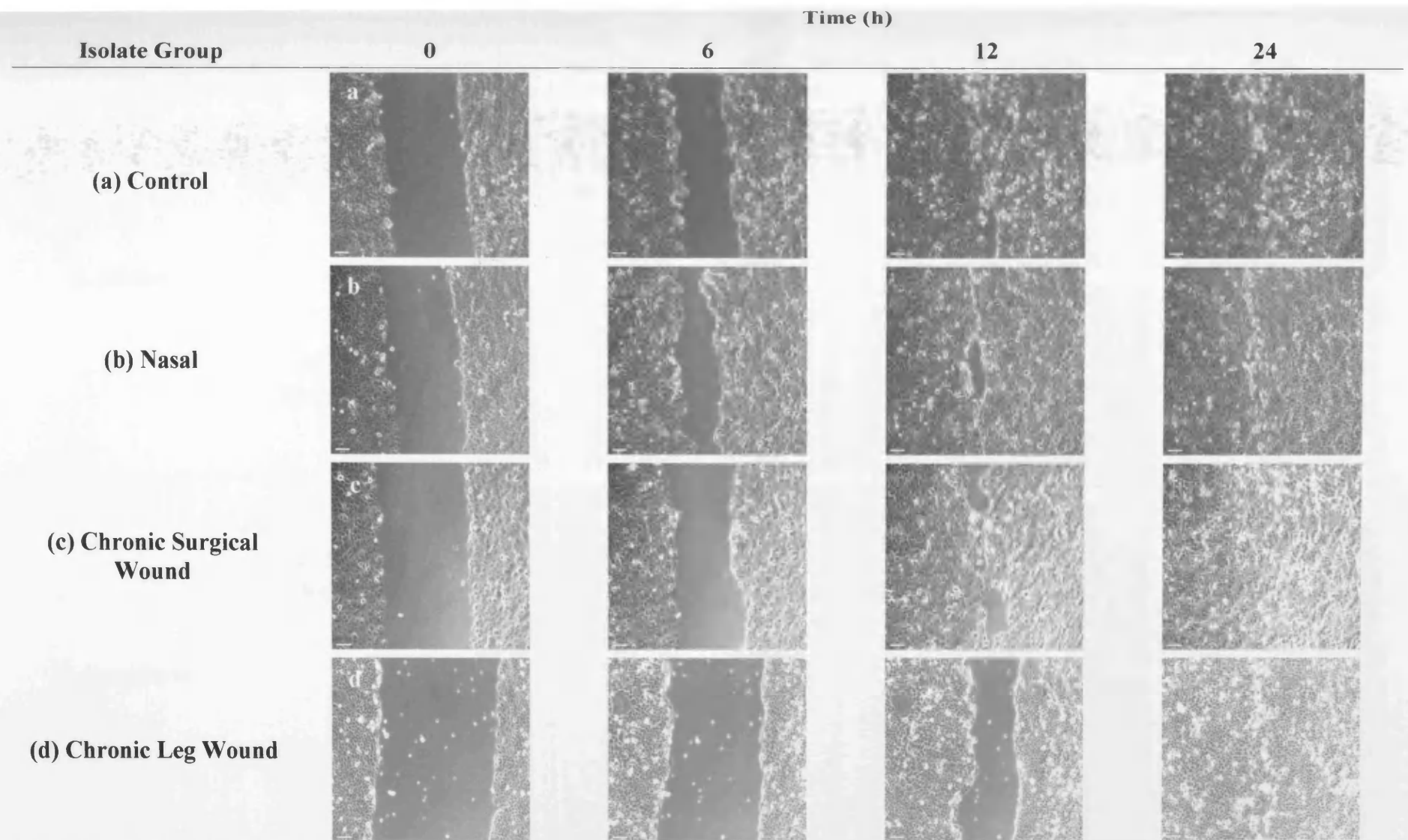
“Scratch” wound assays employing HaCaT cells were used to quantify the effect of bacterial DMEM supernatants on speed of cellular migration and percentage closure of an *in vitro* wound. A topographical representation of a control treated “scratch” wound for 0, 6, 12 and 24 h is shown in Figure 4.14. It was observed that the wound space decreased with time with all supernatants. Figure 4.15 and 4.16 demonstrate images of “scratch” wound frozen time frames for 0, 6, 12 and 24 h incubation with bacterial supernatants.

##### **4.4.7.1 Wound area reduction following incubation with MRSA supernatants**

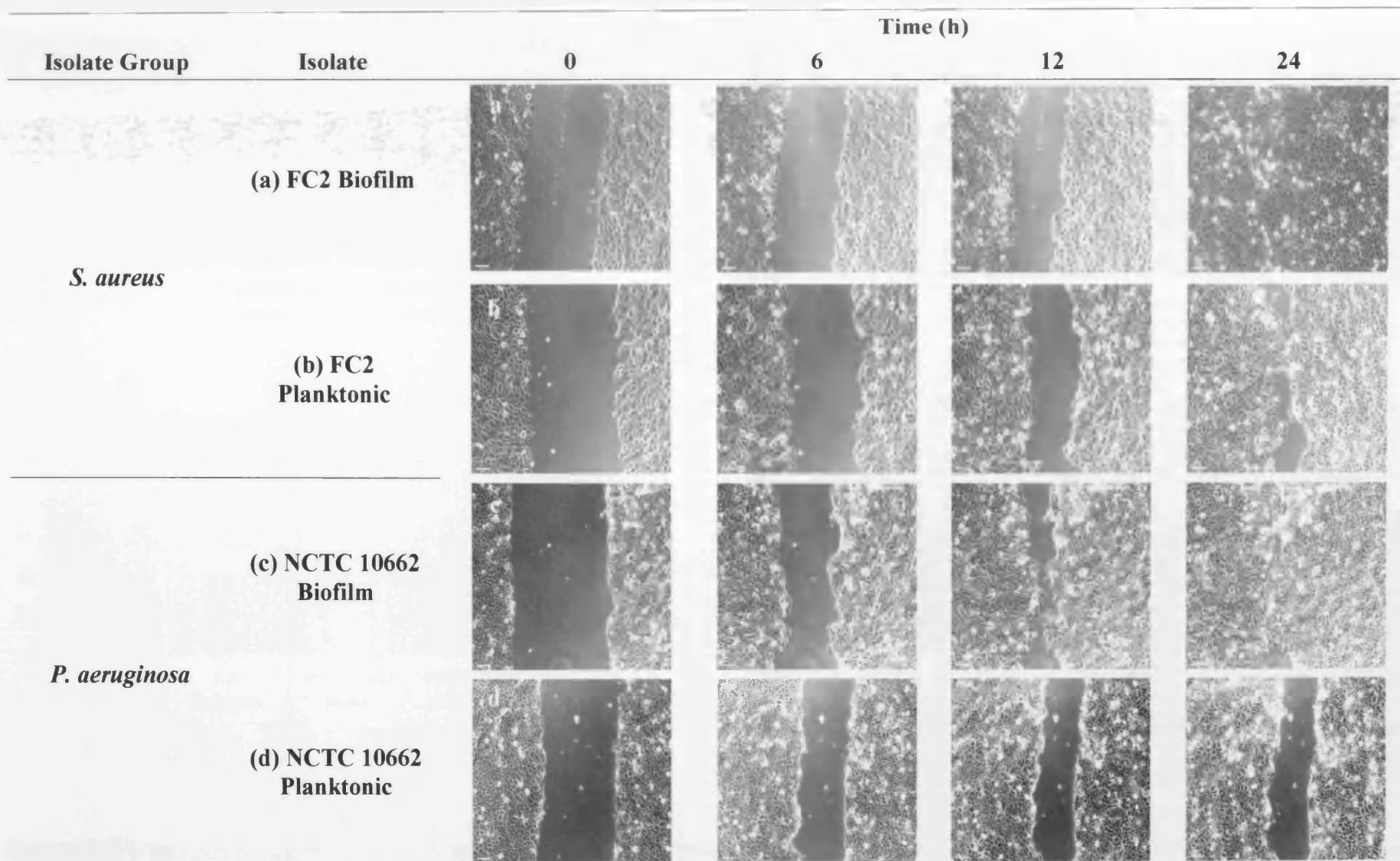
Percentage wound area reduction of the initial “scratch” wound was determined over a 24 h period. Figure 4.17a and b represents the area reduction for 12 and 24 h wounds treated with MRSA supernatants. In these experiments, no difference in wound area reduction was observed for MRSA isolated from nasal carriers or wound patients. However, a significant difference in percentage wound closure was observed at 24 h between the control media (FAD) and MRSA supernatants ( $P<0.05$ ).

##### **4.4.7.2 Wound area reduction following incubation with *S. aureus* and *P. aeruginosa* planktonic and biofilm supernatants**

Growth conditions also significantly affected wound closure. Planktonic derived supernatants significantly decreased the ability of cellular migration, which was evident at 12 and 24 h (Figure 4.16). Percentage wound area reduction for 12 and 24 h incubation of the “scratch wound” assay treated with biofilm and planktonic *P. aeruginosa* and *S. aureus* supernatants are shown in Figure 4.17c and d. In these experiments, a significant difference in percentage wound area reduction was observed for *P. aeruginosa* supernatants at times greater than 12 h ( $P<0.05$ ). A significant difference in wound area reduction was also observed for times greater than 12 h for both planktonic supernatants and control media ( $P<0.01$ ).

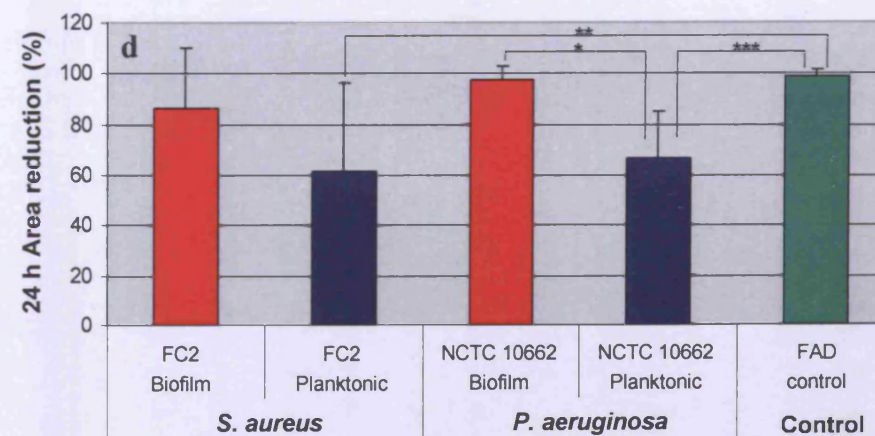
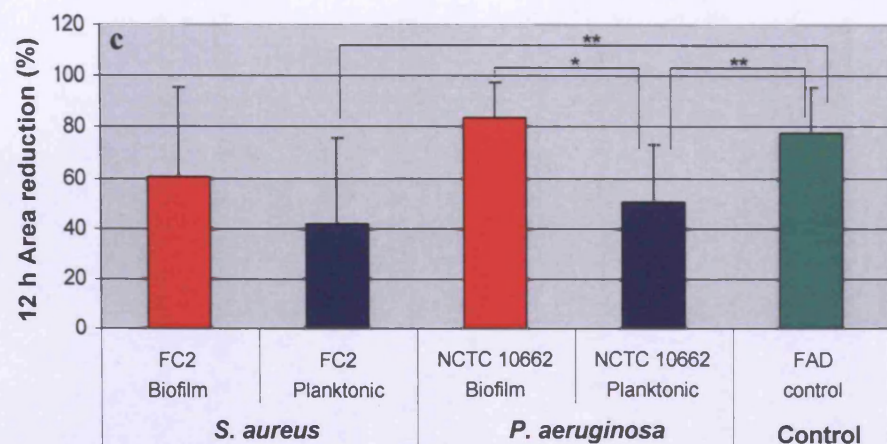
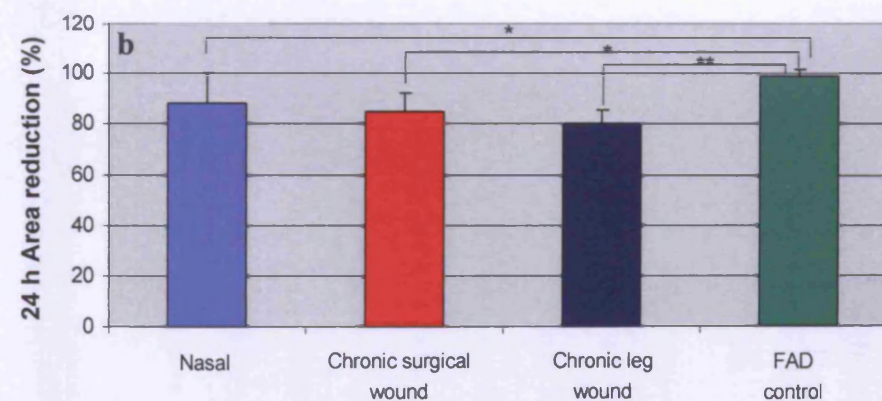
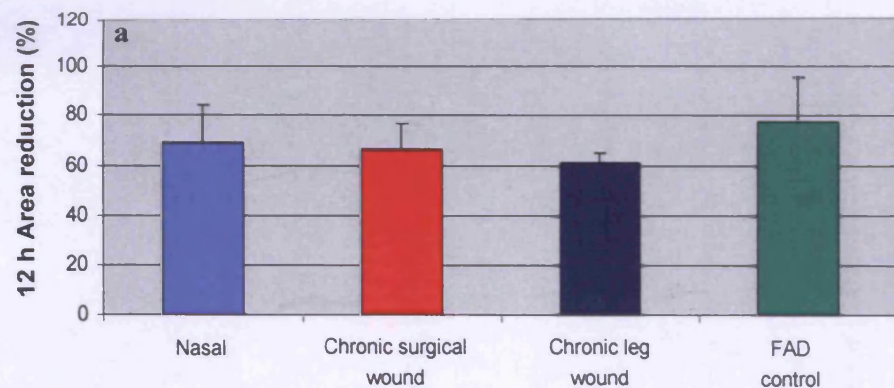


**Figure 4.15** HaCaT cell migration at 0, 6, 12 and 24 h time points in response to bacterial supernatants. a) Control media, FAD; b) nasal MRSA, S07 154; c) chronic surgical wound MRSA, 2018; d) chronic leg wound MRSA, 1011. Scale bar represents 100  $\mu$ m.



**Figure 4.16** HaCaT cell migration at 0, 6, 12 and 24 h time points in response to *S. aureus* and *P. aeruginosa* bacterial supernatants. a) and c) biofilm; b) and d) planktonic. Scale bar represents 100  $\mu\text{m}$ .





**Figure 4.17** Mean percentage wound area reduction following incubation with DMEM bacterial supernatants 1:10. a) MRSA supernatant, 12 h; b) MRSA supernatant, 24 h; c) *S. aureus* and *P. aeruginosa* biofilm and planktonic, 12 h; d) *S. aureus* and *P. aeruginosa* biofilm and planktonic, 24 h. Error bars denote standard deviation of the mean. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

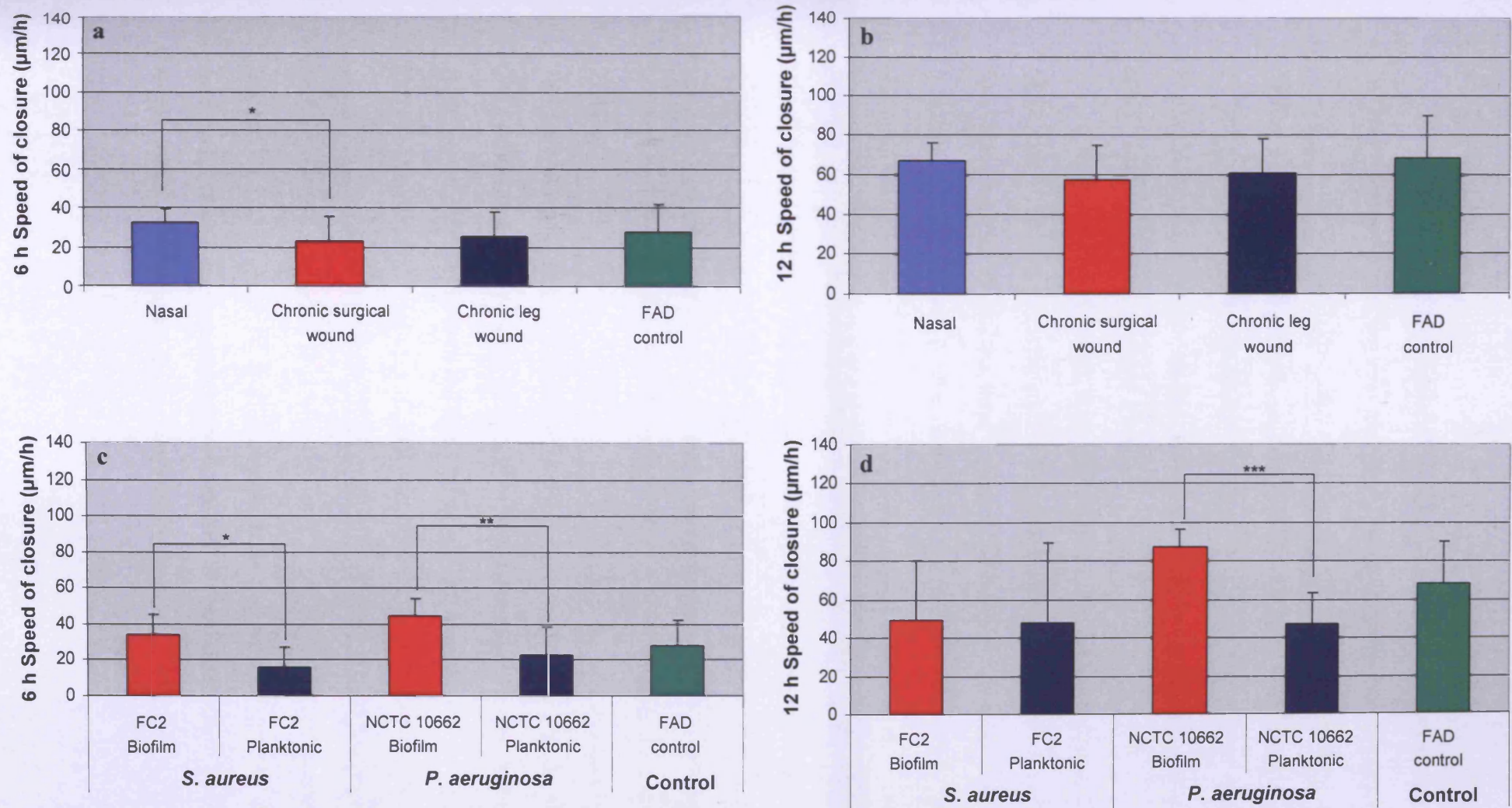
No significant difference was noted between biofilm produced supernatants and control media over the 24 h period ( $P>0.05$ ).

#### **4.4.7.3 Speed of wound closure following incubation with MRSA supernatants**

The speed of wound closure (cell migration) for 1:10 diluted DMEM MRSA supernatants at 6 and 12 h are shown in Figure 4.18a and b. A significant difference in speed of wound closure was observed with 1:10 diluted supernatants at 6 h time point for nasal carrier and chronic surgical wound MRSA ( $P<0.05$ ). No significant difference was observed when comparing nasal carrier and chronic leg wound MRSA isolates, or between the two wound groups. However, there was a trend that chronic surgical wound MRSA wounds healed more slowly than the nasal carrier and chronic leg wound MRSA groups. No significant differences in speed of wound closure were observed for increased dilutions of supernatant ( $P>0.05$ ).

#### **4.4.7.4 Speed of wound closure following incubation with *S. aureus* and *P. aeruginosa* planktonic and biofilm supernatants**

The speed of wound closure (cell migration) following incubation with 1:10 DMEM *S. aureus* (FC2) and *P. aeruginosa* (NCTC 10662) biofilm and planktonic supernatants is shown in Figure 4.18c and d. Biofilm supernatants were observed to produce a greater speed of closure than wounds treated with planktonic supernatants. Prolonged exposure to *S. aureus* supernatants (24 h) in both biofilm and planktonic growth conditions did not show detrimental effects on the rate of wound closure. A significant difference in the speed of wound closure was observed for planktonic and biofilm *S. aureus* supernatant treated scratch wounds at 6 h ( $P<0.01$ ) although no significant difference was observed with prolonged treatment times ( $\geq 12$  h;  $P>0.05$ ). The growth condition of *P. aeruginosa* significantly affected the speed of wound closure at  $\geq 6$  h ( $P<0.01$ ). Increased dilutions of bacterial supernatants did not affect the speed of wound closure significantly ( $P>0.05$ ; results not shown).



**Figure 4.18** Mean speed of wound closure (HaCaT cell migration) following incubation with DMEM bacterial supernatants 1:10. a) MRSA supernatant, 6 h, b) MRSA supernatant, 12 h; c) *S. aureus* and *P. aeruginosa* biofilm and planktonic, 6 h; d) *S. aureus* and *P. aeruginosa* biofilm and planktonic, 12 h. Error bars denote standard deviation of the mean. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

## 4.5 Discussion

These studies investigated the immunostimulatory properties of commonly isolated chronic wound bacteria and the effect of biofilm growth conditions on their role in wound healing. Bacterial supernatants of MRSA from asymptomatic nasal carriers were compared with those from chronic wounds. The effect of *S. aureus* and *P. aeruginosa* growth conditions (biofilm and planktonic) was also studied in immunological response assays and in a relatively simple model of wound repopulation i.e. scratch wound assay.

In these experiments, bacterial supernatants were produced in two broths (TSB and BHI) and supplemented cell culture media (DMEM with 10% v/v FCS). The proliferation of the bacteria varied as expected, according to nutrient and substrate levels in the media, witnessed by delayed stationary phase growth measurements ( $\log_{10}$  CFU/ml) and protein content. Despite this, the CFU/ml of bacteria was similar to that observed clinically (Bowler et al., 2001). In these experiments, bacterial supernatants were produced in supplemented DMEM to overcome cytotoxicity issues identified in preliminary studies associated with concentrated broth supernatants and background TLR and cytokine activation (personal communication Dr J. Stenvik). Thus cytotoxic issues observed in the MTT assay may have been associated with broth components and increased concentration of bacterial metabolites. The use of supplemented cell culture media to produce the supernatants allowed biological variation in experimental analysis to be attributed to bacterial products and not to the supernatant growth media.

There was inter-experimental variation in protein content for each of the growth media used. The BCA<sup>TM</sup> assay employed to determine the protein content in each of the growth media demonstrated that it was not possible to discern the exact amount of protein derived from bacteria from that already present in the growth media. Therefore, the concentration of protein in each supernatant may be attributed to growth media or/and solely bacterial metabolism.



The Luciferase reporter assay was utilised to study TLR activation. Intra-species variation was observed for TLR2 stimulation by nasal isolates, which may reflect the greater variation in the sequence type (ST) of these MRSA. It is likely that differences in the ST of nasal isolates are associated with the route of colonisation. Nasal carriage can frequently be transient, but is also more likely to be associated with community-acquired (CA) MRSA for which there is greater variation in the ST populations. Less variation in chronic wound (CW) MRSA TLR stimulation was observed and these isolates were associated with a more homogenous ST; the hospital-acquired ST (ST22) being predominant amongst this group (Chapter 3). Two possible reasons exist for this. Patients are more likely to have been hospitalised or institutionalised e.g. in a nursing home, or these STs preferentially colonise such chronic wounds and survive (Chapter 3). Importantly, the variation in stimulation did not reflect variation in protein content or CFU/ml of bacteria in the supernatants, in that the nasal MRSA with the greatest TLR2 stimulation had the lowest CFU/ml. It is likely therefore, that this result reflects increased “shedding” or generation of a more stimulatory ligand e.g. LTA or PGN.

The greatest stimulation of TLR2 was observed by nasal MRSA isolates although this was not statistically significant. Broth supernatants and heat-killed MRSA produced similar results, represented by nasal MRSA providing increased TLR2 stimulation (results not shown). An interesting finding was that  $\geq 10^8$  CFU/ml heat killed nasal isolates induced greater activation of TLR2 than both chronic surgical and leg wound MRSA. This finding may be explained by either a structural component (e.g. LTA or PGN) being more abundant in the nasal isolates, or that the wound MRSA exhibit steric alterations in key ligands e.g. alanylation of LTA (Travassos et al., 2004, Fournier and Philpott, 2005). This variation in TLR stimulation may indicate that wound MRSA have adapted to allow chronic colonisation in the absence of significant (effective) inflammation (Mirza et al., 2009, Pukstad et al., 2010). In these experiments, TLR2 stimulation in biofilm and planktonic growth environments exhibited marginal differences in NF- $\kappa$ B activation

via TLR2, as would be expected since the PAMP should be identical, due to the use of the same isolate in both growth conditions.

Lipopolysaccharide (LPS) is major constituent of Gram-negative bacterial cell walls (e.g. *P. aeruginosa*) and known to be a potent stimulator of TLR4, as observed by the control LPS in the Luciferase assay. In these studies, *P. aeruginosa* isolate NCTC 10662 (a clinical strain able to form both biofilm and mucoid phenotypes; [www.hpacultures.org.uk/collections/nctc](http://www.hpacultures.org.uk/collections/nctc); accessed 17/1/11) did not elicit a TLR4 response, despite significant levels of LPS endotoxin being present in the bacterial supernatant as assessed by the LAL assay. The low NF- $\kappa$ B activation may be associated with structural differences in LPS isoforms, specifically the lipid A component and O-antigen side chain, associated with acylation such as penta, hexa or hepta acylated LPS (Currie et al., 2003, Pier, 2007). Such conformational changes have been observed in *Y. pestis* organisms during plague infection (Comer et al., 2010). Previous investigations in cystic fibrosis patients have shown that *P. aeruginosa* change their phenotype to become more mucoid, which is often associated with changes in LPS structure (Rocchetta et al., 1999) and  $Mg^{2+}$  levels (Pier, 2007). This conformational change is a possible mechanism by which these bacteria may alter PAMPs to evade the immune response and decrease TLR4 recognition *in vivo*. Further investigation is warranted to confirm this mechanism. There was no difference in TLR4 stimulation by planktonic and biofilm grown *P. aeruginosa* as expected. As the planktonic form did not stimulate TLR4, it is unlikely that the biofilm form would, based on potential ligand structural differences. Evasion of the immune system was also witnessed in the TLR5 study, which is associated with bacterial flagella. Little activation of TLR5 was observed with isolate NCTC 10662 and this may be related to the loss of flagella from this bacterium which is often observed in mucoid forming bacteria and represents a mechanism to evade host immune responses (Garrett et al., 1999, Jensen et al., 2010).

Cytokine induction following bacterial invasion is a pivotal component of both the innate and acquired immune response in humans, and these responses are essential in modulating repair following injury. In these studies, Mo cytokine expression by nasal MRSA isolates exhibited increased stimulatory ability compared with MRSA from wounds (which themselves were similar). These differences in the immunostimulatory properties of the two groups (i.e. carrier vs. disease) were reflected in the TLR2 studies. Decreased immunological responsiveness in the wound environment may facilitate increased bacterial survival and chronicity of inflammation. This is in keeping with previous studies of *S. aureus* from sites of chronic inflammation which have been associated with reduced induction of TNF $\alpha$ , IL-8 or IL-1 $\beta$  (Sladek et al., 2005). Differences in PAMP (e.g. LTA, PGN and capsular polysaccharide) are known to elicit varying levels of IL-8 production from Mo and may also be associated with these findings (DeLeo et al., 2009). The observed variations following MRSA stimulation may be associated with the ST and secreted virulence factors (Chapter 3). Increased TLR and cytokine stimulation by nasal isolates may be associated with expression of specific virulence factors e.g. SEA and TSST-1, released from the nasal carrier MRSA strains in culture or infection (DeLeo et al., 2009). A reduced production of these pro-inflammatory cytokines (TNF $\alpha$ , IL-1 $\beta$  and IL-8) would result in reduced cellular migration and proliferation of inflammatory cells at the site of infection (Hornef et al., 2002) allowing uncontrolled bacterial proliferation at the wound site leading to further tissue destruction by released factors such as matrix metalloproteinases (MMP).

Numerous studies have demonstrated that increased wound closure has been associated with microbial factors that induce IL-1 $\beta$ , TNF $\alpha$  and IL-8 (Zhang and Schluesener, 2006, Kirketerp-Moller et al., 2008, Shaykhiev et al., 2008). These cytokines and bacterial products signalling via TLR2, TLR4 and TLR5 are thought to play a role in the migration and proliferation of epithelial cells and stimulation of epithelial wound repair (Zhang and Schluesener, 2006, Shaykhiev et al., 2008, Pukstad et al., 2010). It is interesting therefore, that bacterial cells isolated from

chronic non-healing wounds are associated with decreased cytokine stimulation. The increased immunostimulatory properties expressed by nasal MRSA are reflected in the lack of symptoms in these patients. In this situation, there is an effective immunological response, which quickly removes the MRSA via the innate immune system, unlike the chronic wound MRSA which appear to have adapted to evade the host immune system leading to a reduced functional immune response.

In contrast to the Mo cytokine release experiments, the “whole blood” model is a complex assay, allowing stimulation of the complement system in a physiological environment, and is the closest *in vitro* representative of the host response. As in the TLR and Mo cytokine assays, nasal MRSA exhibited a markedly higher stimulation of complement than the wound isolates. Increased levels of IL-8 production were again observed for the nasal isolates, as was confirmed with heat-killed bacteria and broth supernatants. This again stressed the ability of the chronic wound MRSA to evade the immune system, which is likely to occur through production of altered PAMPs. Heat-killed bacteria indicated that nasal isolates simulated whole blood to produce greater IL-8 levels than wound isolates, indicating a potential structural difference in the bacterial cell e.g. LTA alanylation or PGN formation (Fournier and Philpott, 2005). Quantification of the terminal complement complex (TCC) also suggested that nasal MRSA isolates were more potent immune stimulators than wound isolates; a finding mirrored by CD11b expression on granulocytes and Mo, a complement-dependent receptor studied using FACS analysis (Thorgersen et al., 2009). Heat-killed bacteria demonstrated similar complement activation between MRSA groups, suggesting the observed differences were likely to be associated with soluble bacterial products, rather than with bacterial surface components. *S. aureus* is known to produce factors that modify complement activation e.g. staphylococcal complement inhibitor (SCIN) and chemotaxis inhibitory protein of staphylococci (CHIPS) which may mediate the variation observed in TCC and CD11b activation between the nasal and wound MRSA isolates (Nizet, 2007, DeLeo et al., 2009).



Cytokine stimulation via bacterial supernatants produced under different growth conditions for both *S. aureus* and *P. aeruginosa* indicated that planktonically grown supernatants had an increased ability to stimulate Mo IL-8, TNF $\alpha$  and IL-1 $\beta$  production. The bacterial biofilm environment within the wound is believed to provide specific protection (obstructing recognition by the immune system) from immunological responses (Kirketerp-Moller et al., 2008, Percival et al., 2008, Martin et al., 2009). This concept is supported by the data in this study, which demonstrated reduced stimulation by the biofilm supernatants. Within the biofilm, not only do phenotypic changes in the bacterial population present support reduced immune stimulation properties, but the physical dynamics of the biofilm may also play a role, such as in containment of the bacterial products within the extracellular polysaccharide substance (EPS) and contact with the wound. Apart from the biofilm EPS providing a physical barrier to the immune system, immunomodulating molecules released (e.g. PGN, LTA, and LPS) may be less stimulatory. Compounding this is the continuous production of bacterial degradative proteins encased and released from the biofilm matrix.

In the studies of biofilm vs. planktonic growth conditions, a major limitation of the study design is that, whilst the immune properties of released microbial products in the culture media could be determined, the potential effect of the biofilm structure itself in these assays was unknown. Bacteria in biofilms express differences in genotype, phenotype, physiology and biochemistry (Davis et al., 2008). Whilst important changes occur during the growth of bacterial biofilms, it is uncertain whether the potential down-regulation of growth and protein synthesis in these studies was due to density-dependent quorum sensing or just reduced bacterial growth rate both of which are known to occur in biofilm environments (Kirketerp-Moller et al., 2008). Simple protein estimation was performed utilising the BCA assay, however this was unable to characterise proteins encased in the biofilm (EPS). It was unclear whether the planktonic supernatants are more immunostimulatory simply due to non-specific trapping of the biofilm-produced proteins and/or secreted

immunomodulatory ligands, or is directly related to down-regulation of metabolism and transcription in the biofilm environment. In this respect, further investigation of the functional and potential immune stimulation of the structure and contents of wound biofilms is required, and would clearly be an interesting area of further research. However, this would be challenging without altering the biofilm structure itself. IL-8 production in the whole blood model for both *P. aeruginosa* and *S. aureus* indicated again like previous experiments, that the planktonically grown bacteria stimulated the resident immune cells greater than those from the biofilm. Moreover, variability of IL-8 production by *P. aeruginosa* from both the biofilm and planktonic growth environments was greater than for *S. aureus*.

Complement activation analysis of *P. aeruginosa* revealed minimal expression of CD11b and formation of TCC; a finding similar to that for the control LPS. A previous study by Brekke et al., (2008) when investigating complement regulation of *E. coli*, and Sprong et al., (2004) when investigating *N. meningitidis*, also found almost complete inhibition of the inflammatory response by LPS. Whilst differences in response to LPS activity in the whole blood model could be rationalised by the formation of micelle structures, this is unlikely in the present study because LPS within the supernatant clearly stimulated IL-8 production. Complement activation by the heat-killed bacteria also indicated the role of other cell wall components in complement activation. Sprong et al., (2004), suggested that the outer membrane proteins porin and opacity protein induce the mannose-binding lectin pathway in *N. meningitidis*. The minimal activation of complement by the soluble LPS could simply be associated with a low concentration or LPS structure of this *P. aeruginosa* (Brekke et al., 2008). Planktonic *S. aureus* supernatants stimulated increased complement activation which was reflected in the increased TCC, monocyte and granulocyte CD11b expression. These data further support the idea that bacteria in biofilms are able to evade the immune system by dampening its response, via the decrease or alteration in production of antigenic molecules or by their entrapment in the matrix itself.

The *in vitro* human wound healing model employing MTT staining to determine healing made analysis simple, less time-consuming and less technique-sensitive than the original cryosectioned haematoxylin-eosin (HE) stained cross-sectional studies previously used to characterise the kinetics of re-epithelialisation (Jansson et al., 1996). This model represents more closely the intricate process of skin healing than mono-layer cultures, although it does not contain all the elements of the functional immune response. The use of mounted skin equivalent systems would avoid the contraction associated with the healing in this model, a current major limitation. Both DMEM- and broth- produced MRSA supernatants reduced epithelial migration compared with the matched control, indicating that bacterial products/metabolites impaired healing.

In contrast to the immunological assays, the monolayer “scratch wound” assay was a simple model in that it measured only one component of wound healing i.e. cellular repopulation. Therefore, it could be used to directly study the effect of bacterial supernatants on cellular migration. Moreover, the epithelial responses in this model are known to be modulated via the action of cytokines and the expression of TLRs on the keratinocytes themselves. Stephens et al., (2003) demonstrated how anaerobic bacterial supernatants inhibited wound closure by both fibroblasts and keratinocytes using the “scratch wound” assay. Two parameters were studied using this model; rate of cellular migration (i.e. speed of wound closure), and percentage healing (i.e. % wound space closed). These were used to compare supernatants derived from nasal MRSA isolates with chronic wound MRSA isolates, and also the effect of bacterial growth conditions. In these experiments, nasal and wound isolate variances were evident in the initial 6 h following wounding. Interestingly, the speed of wound closure for wounds treated with nasal MRSA supernatants was similar to that of the control. The accelerated scratch wound closure with *S. aureus* noted here has previously been observed (Shaykhiev et al., 2008) the author found that PGN significantly increased wound repair. In these experiments, MRSA

supernatants of different colonisation origins appeared to have little effect on the migration of keratinocytes following prolonged exposure.

Comparison of wound healing associated with specific growth conditions (biofilm and planktonic) for the 2 common chronic wound pathogens *P. aeruginosa* and *S. aureus* was also performed. Planktonic supernatants inhibited wound closure more than the biofilm supernatants. As with the immunological studies, this may be associated with the protein content of the supernatants, (i.e. the trapping of bacterial products or due to decreased formation and cellular turnover in the biofilm environment). Moreover, in this study the *P. aeruginosa* biofilm speed of wound closure was quicker than the control. Shaykhiev et al., (2008) found that microbial factors inducing wound closure, cellular migration and proliferation, induced IL-1 $\beta$ , TNF $\alpha$ , IL-6 and IL-8 in epithelial cells, and may potentially be the cause of this increased speed of closure in the bacterial-stimulated wounds.

There are a number of limitations to this assay, a major one being its oversimplified nature. Human epidermal keratinocytes in this system are able to secrete cytokines and chemokines such as IL-8, IL-6, TNF $\alpha$  and IL-1 $\beta$  following exposure to Gram-positive and Gram-negative bacteria via the expression of TLR2 and TLR4 (Froy, 2005, Shirakata, 2010). The production and accumulation of such products in this *in vitro* model may have affected cellular migration. It has also been postulated that bacterial ligands can induce the production of MMP in the wound environment, which would have a profound effect on cell migration and wound healing (Stephens et al., 2003). The method used in this study was unable to replicate the many products produced *in vivo* by cellular populations, nor the dynamic physiology of the wound environment (Fong et al., 2010). Also the use of HaCaT cells used for the scratch wound assay does not reflect the phenotype of the wound keratinocyte, which may behave differently to bacterial supernatants under similar conditions.

Biofilms in the chronic wound environment are associated with delayed healing (Kirketerp-Moller et al., 2008, Percival et al., 2008, Kirker et al., 2009,

Schierle et al., 2009). These scratch wound data do not wholly support this theory. It is clear that the biofilm supernatants contained a lot less protein than the other supernatants, suggesting that some of the bacterial biomass and biofilm-related products may have been trapped in the biofilm matrix itself, rather than being released into the supernatant. This may certainly be the case for *P. aeruginosa* biofilm treated scratch wounds, whereby LPS is trapped in the biofilm matrix. This theory is supported by Loryman and Mansbridge (2008) who found that keratinocyte migration inhibition was LPS dose dependent. Biofilms represent highly structured communities with co-ordinated metabolic activities which are known to differ considerably in their gene expression from matched planktonically grown cells. In all, this indicates that the chronic wound environment and the role of bacteria is a highly complex relationship. The effect of the structure (architecture) of a biofilm on cellular migration is also not evident from these experiments. Kirker et al., (2009) demonstrated a significant reduction in cell migration with both biofilm and planktonic *S. aureus* conditioned media. The migration of cells in co-culture with *S. aureus* biofilms showed initial increases in wound size. Such differences between this study and Kirker's could be a result of the bacterial cell number, i.e. Kirker used  $3 \log_{10}$  greater bacterial cell numbers than in this present study.

These studies have demonstrated that bacteria are able to adapt to their environment to facilitate survival and colonisation, and is reflected in the *in vivo* non-infected wound. Adaptation is likely to occur via alteration in excreted products and surface factors (PAMP). The development of a biofilm is a clear way in which these bacteria are able to evade the host, thus making them more recalcitrant to eradication, not only by host immune system, but also antibiotics and other antimicrobials. This delayed response, or lack of a significant response, by the immune system to the bacteria and biofilms may result in continued exposure to bacterial stimuli which results in maximal host activation (Hume et al., 2001). This increased or prolonged exposure to pro-inflammatory cytokines and MMPs results in tissue destruction via host mediation, providing further nutrients for the bacteria

present (Zhang and Schluesener, 2006, Martin et al., 2009). The origin of MRSA appeared to have little effect on cellular migration. However, the specific bacterial growth conditions i.e. biofilm or planktonic, proved to be important.

This study employed a series of *in vitro* experiments to understand immune modulation by bacteria from different colonising environments and growth conditions. Further investigation is warranted to understand the intricacies of colonisation during wound healing, and the role of MRSA. Initial immunostimulatory experiments using the same ST MRSA isolated from wound and nasal cavities of chronic wound patients and nares of asymptomatic patients needs to be investigated to understand the significance of MRSA ST. This will further determine if the immunomodulatory responses are localised to the disease/wound or associated only with specific bacteria. Further investigation to determine if the bacteria are able to induce immunosuppressive cytokines e.g. IL-10, which decreases TNF $\alpha$  (Hornef et al., 2002) would also be pertinent. Attempts to address this on other models have employed array technologies such as gene expression, and more recently, cytokine arrays systems (using wound fluid samples; Pukstad et al., 2010).

There were a number of limitations in this study; firstly (as in many previous studies) the immune response was studied in isolation, whereas systemic disease, co-morbidity and tissue perfusion would modulate immune function *in vivo*. The use of immunocompetent cells also makes direct comparison to the wound environment difficult. Secondly, cells were used in isolation, unlike the dynamic wound environment.

Importantly, bacterial biofilms for which many authors have tried to establish a working biofilm model resembling the wound colonisation and evolution of species (Hill et al., 2010) need to be employed to study the immune response in these assays, e.g the use of a constant depth film fermenter or microtiter plate assays to produce a biofilm and its conditioned media. A limitation of the growth condition experiments was that biofilms were produced as mono-species. Chronic wounds are often polymicrobial and recent evidence indicates that wound biofilms show

aggregates of single species in a polymicrobial environment (Burmølle et al., 2010). Therefore, testing of wound related biofilms produced in a polymicrobial environment and mixed-species biofilms (i.e. *S. aureus* and *P. aeruginosa*) may be of considerably more relevance. The age of the biofilm may also be of importance. Kirker et al., (2009) studied keratinocyte migration inhibition using >72 h biofilms in co-culture finding that cell migration was significantly impaired. Further studies of the biofilm physical structure and its impact on cellular migration and proliferation would be interesting. Finally, structural and cellular component examination of *P. aeruginosa* and *S. aureus* to determine potential alterations in ligands, which lead to reduced immune stimulation, would be pertinent.

Whilst there are a number of limitations to these experiments in their direct relationship to the chronic wound environment, the data generated highlights potential mechanisms bacteria use to effectively evade the host immune response.

# **Chapter 5:**

## **General Discussion**



## 5.1 General discussion

This study has attempted to further understanding of the relationship between bacteria found within chronic non-healing skin wounds and the observed failure of the normal dermal repair processes. These studies focussed on *Staphylococcus aureus* and meticillin resistant *Staphylococcus aureus* (MRSA) utilising a sample population of patients (with differing wound aetiologies) from a previous antimicrobial audit undertaken in the Wound Healing Research Unit at this hospital (Howell-Jones, 2007). Whilst *S. aureus*, including MRSA, was frequently isolated in this population (75 of 150 patients) the rate of MRSA in this patient cohort was lower than in previous studies or reported in contemporaneous MRSA UK surveillance rates (ECDC, 2005 - 2011). This study however, showed that these chronic wound patients harboured multi-drug resistant (MDR) organisms which pose a significant health and cross-infection problem (Chapter 2). Utilising conventional cultural techniques to isolate organisms from this population of chronic non-healing skin wounds was valid, although to further understand the complexity of the relationship of bacterial communities and non-healing phenotypes, molecular methods should be employed. The use of high-throughput sequencing techniques e.g. pyrosequencing (Dowd et al., 2008, Wolcott et al., 2010) would aid the identification of bacterial species present, particularly of the uncultivable population, much like the human and oral microbiome project (Grice, 2008, Dewhirst et al., 2010). A greater understanding of the microbiome of chronic diseases such as chronic skin wounds, may inform the development of novel therapeutic strategies, but the adoption of such techniques was beyond the scope of this project.

Current clinical practice of the Wound healing research unit (WHRU) does not routinely screen for MRSA in the general population attending these clinics. At present only 1 microbiological sample per 20 patients is undertaken, for wounds unresponsive to empirical treatment. Following personal communication with Dr M. Heginbotham, of the antimicrobial surveillance unit for Wales, regarding current levels of MRSA in patients attending the WHRU; identified 40% (7 MRSA

identified in 17 *Staphylococcus aureus* isolates) of the *Staphylococcus aureus* isolates from the WHRU were classified as MRSA during 2010. Whilst it is difficult to compare these numbers with data from the patient cohort in this study due to the likely bias of sample, it is a clear indication that further investigation and surveillance of chronic wound patients who harbour MDR organisms is required.

Many of the wounds harboured coagulase negative staphylococci (CNS), which have become an important human pathogen; biofilms being of particular relevance where this species is concerned (Watts et al., 1990, Piette and Verschraegen, 2009). Again the direct role of CNS in the chronic wound environment has not been elucidated. Whilst it may be relatively innocuous, it is far more likely to be involved in some synergistic role with other pathogens e.g. in biofilm formation and transfer of antibiotic resistance (Hanssen and Ericson Sollid, 2006, Hellmark et al., 2010).

In the attempt to type MRSA, sequence-based typing methods were more conclusive than the other methods used (Chapter 3). Interestingly, in this study the majority of wound MRSA were classified as sequence type (ST) 22, also known as EMRSA-15, frequently associated with hospital-acquired (HA) MRSA. In contrast to this, nasal MRSA STs were more diverse, a pattern typical of community-acquired (CA) MRSA. Whilst it is impossible from this study to determine the route by which these bacteria were acquired, it is remarkable that 88.2% of MRSA were associated with ST 22; possibly reflecting the wound environment that these bacteria inhabit. Such a study would require longitudinal data to identify bacterial succession within the wounds. However, such a study would be fraught with difficulty notwithstanding patients' age and underlying co-morbidity in this elderly population, who are at increased risk of antibiotic treatment and resistance development (Howell-Jones et al., 2006).

MRSA are known to express a range of virulence factors to aid survival and these may induce host damage and potentiate disease (Van der Mee-Marquet et al., 2004, Argudin et al., 2009). This is the first study in the known literature to compare

in detail MRSA virulence factor expression in chronic wound and asymptomatic nasal carrier patients (Chapter 3). The presence of enterotoxin genes appeared to be more specific to ST than wound type/origin. This has been previously documented in other disease processes, and is likely because enterotoxin genes are frequently associated with the presence of pathogenicity islands (Bohach, 2006, Varshney et al., 2009). However, variation in wound and nasal carrier MRSA isolates was observed for expression of several chosen virulence factors specifically *cna* and *hysA* which are associated with colonisation and tissue destruction. Interestingly, hyaluronate lyase (HysA) may degrade hyaluronic acid (HA) to generate low molecular weight HA oligomers. These may potentially induce the innate immune system via TLR4 recognition (Termeer et al., 2002, Taylor et al., 2004) and possibly linked to chronic stimulation of the immune process leading to further tissue damage. A major limitation of this study could be perceived to be the small sample size and a lack of similar sequence types between the 2 diverse groups of patients to draw clear comparisons.

Variation in virulence expression was observed when comparing wound aetiology i.e. leg wound vs. surgical wound. Again the differences seen were associated with colonisation (*cna*), tissue degradation (*hysA*), and host evasion (*hlg*, PVL) with all these virulence factors more highly expressed in surgical than leg wounds. One can hypothesize that this is potentially associated with the underlying aetiology of the wound; based upon decreased vascular perfusion associated with VLUs, resulting in reduced immune infiltrate, therefore leading to less pressure on the colonising bacteria to survive. It must be remembered however, that the original study population was 157 patients. This population allowed us to make fascinating comparisons between the nasal, chronic leg and surgical wound groups. Future investigations would require an expanded virulence factor repertoire and intra-patient control samples (e.g. wound vs. normal skin vs. nose) to identify firstly whether MRSA were colonising the entire host or just present in the wound, and secondly whether expression of virulence factors is altered according to their

environment. Much larger patient studies are required and should include acute wounds (healing and infected) and nasal carriers with ideally similar ST distributions. In this aim, the latter would, in particular, be difficult to achieve, particularly as the diversity of STs in nasal carriers is so broad. Gene array studies (Saunders et al., 2004) and quantitative RT-PCR would be methods of choice due to their high levels of discrimination. Ideally testing should be performed immediately on isolates to mimic *in situ* expression, and virulence factor expression should be studied in relation to the biofilm growth state. In an attempt to obtain insight into bacterial physiology within the respective environments, metabolomic studies of the isolates may also potentially be employed (Yang et al., 2011).

Whilst the immune system plays a pivotal role in the delayed healing of chronic wounds, surprisingly little research has been conducted on the role of immune regulation by chronic wound bacteria. These are the first studies in the known literature comparing the immune modulation potential of MRSA isolated from chronic wounds with asymptomatic carriers (Chapter 4). These studies clearly indicated that bacteria isolated from nasal carriers were more immunogenic than those from either chronic leg or surgical wounds. This may reflect alterations in pathogen associated molecular patterns (PAMP) or potentially ST. Within the wounds, this altered immune modulation *in vivo* could theoretically be associated with a reduced bacterial clearance, leading to prolonged tissue damage mediated by the host and bacteria.

An important point to remember with these studies however, is the variation in ST of the MRSA utilised. To conclusively determine that the variations seen with these bacteria are significant, it would be necessary to employ a much larger group of nasal carriers (preferentially with similar STs, which would be difficult to obtain; *vide supra*). Nevertheless, these data, give valuable insight into the potential importance of immunogenicity and immunological recognition in determining outcome following MRSA colonisation of body surfaces. Comparative studies in

these symptomatic and asymptomatic hosts suggest how this pathogen may potentially evade the host immune response.

As previously suggested, biofilm formation is a method of immune evasion (Percival et al., 2011a). These studies used two common wound organisms (*S. aureus* and *P. aeruginosa*) revealed that planktonic-derived supernatants were more immunogenic than biofilm-derived supernatants in these *in vitro* assay systems. This may be associated with the biofilm structure itself acting as a diffusion barrier to secreted products, or reducing the passage of immunogenic molecules through the extracellular polymeric substance (EPS) of the biofilm. The data observed for planktonic vs. biofilm conditions demonstrate another potential mechanism by which bacteria within biofilms may evade host defenses.

The scratch wound assay employed in these studies failed to elicit an association between MRSA origin and healing over the 24 hour period of this assay. However, the initial delayed healing response to chronic wound MRSA supernatants may indicate a relationship between early MRSA wound colonisation and delayed healing in medically compromised patients. The data observed for planktonic vs. biofilm conditions demonstrated delayed healing, particularly for planktonic *P. aeruginosa*. These data give insight into the importance of bacterial growth conditions in the wound environment and indicate that biofilm trapping of bacterial metabolites may lead to localised effects.

A significant limitation of such studies is the use of immunocompetent cells employed for these assay systems, in contrast to the often immunocompromised effector cells of elderly patients and diseased sites. Whilst further studies with isolated immune cells from chronic wound patients would be attractive, caution must be applied to the artificial environment that the laboratory process introduces in their isolation.

The treatment of non-infected chronic wounds poses many quandaries. Biocide use is more routinely used for the control of bacterial bio-burdens (Fonder et al., 2008, Atiyeh et al., 2009, Lipsky and Hoey, 2009, O'Meara et al., 2010).

Furthermore, there are still limitations on the translation of biocide laboratory testing to the bedside (Thomas and McCubbin, 2003, Fong et al., 2005, Maillard and Denyer, 2006, Cooper, 2007, Verran, 2010b). The biocide testing method employed in these studies utilised bacterial arrangements more representative of those found in the wound when compared to the traditional suspension tests, i.e. bacteria were in multiple layers and on a surface. This study implicitly observed that iodine was the most effective biocide to reduce MRSA CFU/ml (Chapter 2) and is supported by other studies in biofilm environments (Hill et al., 2010). Whilst iodine is an “old” and cheap remedy, it unfortunately receives little attention (BNF, 2010). In contrast to this, silver has gained considerable popularity for the treatment of wounds and is relatively expensive (BNF, 2010) its prominence in the literature being related to marketing and reports of decreased matrix metalloproteinase (MMP) activity properties (Lansdown et al., 1997, Chopra, 2007). There is however, little clinical evidence of its benefit or *in vivo* activity (Vermeulen et al., 2007).

Silver ions are taken up by bacteria by either non-specific mechanisms using cell membrane transporters, or by controlled substrate-specific transporters. The silver ions then interact with thiol groups affecting cellular respiration and binding to nucleic acids; possibly affecting bacterial DNA transcription (Maillard and Denyer, 2006). However, these studies like others indicated the limited biocidal ability of silver, which may be associated with resistance determinants such as *silE* (Loh et al., 2009) or lack of uptake by the bacterial cell itself. For silver to exert its effect on a cell, it must first be able to diffuse across the cell membrane. Chapter 3 showed that MRSA isolates expressed *icaA* a gene associated with biofilm formation, which could affect the cellular uptake and activity of silver. Previous studies have observed poor silver activity in the presence of bacterial biofilms (Percival et al., 2007, Hill et al., 2010). The type of silver compound used in these studies (namely AgNO<sub>3</sub>) may explain its limited biocidal effect based on a decreased ability to release silver ions.

A study of the more commercial nanocrystalline silver and other silver compounds would in retrospect have been useful as it is the clinical treatment

received by most of the study patients. It is believed that the nanocrystals produce a large surface area, enabling a continuous release of silver ions (Strohal et al., 2005) from non-ionic silver. Thus, it is believed to be less affected by organic matter and chloride ions and is able to interact more intimately with bacterial membranes (Ruparelia et al., 2008).

Future studies employing the carrier method using an *ex vivo* model such as porcine skin may be more relevant to human wounds. This could then be followed by a wound model with bacteria grown in a biofilm environment and exposed to the biocide for varying contact times employing image analyses e.g. confocal laser scanning microscopy (CLSM) and live-dead staining. This study adopted conventional techniques i.e. monoculture. The testing of multi-species bacterial communities may afford greater insight and be more relevant to the *in vivo* wound environment (Hill et al., 2010, Percival et al., 2011a, Percival et al., 2011b). Furthermore, clinical testing of biocides could be performed *in vivo*, with wound swabs sampled prior to and after application of the biocide, and with growth effects determined from bacterial growth counts, and live-dead staining of swab or biopsy samples. In addition, used dressings (e.g. iodine and silver-containing) incorporating biocides could themselves be studied directly to observe bactericidal effects using cultural and image analysis. In this study, no correlation with ST or previous biocide use was observed. However, the limited patient data and small numbers of STs available to test these theories indicate that further research in this field is warranted.

## 5.2 Conclusions

This study has provided an insight into the roles of bacteria that in their attempts to colonise, invade and survive in the human wound site, may modulate wound healing processes. These studies have characterised MRSA and have shown that in addition to the potential problem of antibiotic resistance, this organism possesses the ability to modulate the activity of the extracellular matrix, immune cells and resident cells such as keratinocytes. Whilst these bacteria are unlikely to be

the sole cause of a chronic wound failing to heal and are likely to be present as “colonisers”, in these actions they may effectively provide an environment in which other genera may proliferate and survive. The ability of these organisms to potentiate disease is modulated by their growth conditions, with the biofilm state potentially modifying a number of these responses. Whilst the implementation of silver-containing products to treat chronic skin wounds is rising inexorably (Chopra, 2007, Beam, 2009) the study has clearly shown that iodine, an often-overlooked biocide, may have considerable future utility in the treatment of these wounds and MRSA.

### 5.3 Future studies

Future studies to understand the epidemiology of MRSA in chronic wounds might include prospective longitudinal studies of patients at risk of chronic wounds, with intra-patient controls (nares, skin and wounds) using high-throughput sequencing and metagenomic methods. Such a study would facilitate our understanding of virulence factor expression, and could be linked to array-based studies to compare virulence factor expression in patient and wound-matched MRSA with qRT-PCR. Increased understanding of the role of the innate immune system in chronic wound healing is essential. The data obtained indirectly by Pukstad et al., (2010) and in this thesis (*in vitro*) should be substantiated by prospective examination of immune responses using cytokine array, immunocytochemistry and/or imaging techniques. Further investigation of MRSA activity and biocide treatment may usefully examine the acquisition of biocide tolerance, in particular its association with MRSA ST and previous exposure, which would require a prospective study.



# **Appendix I:**

## **Ethics**

19 December 2007

Rebecca Howell-Jones  
Department of Oral Surgery  
Dental School  
Cardiff University

Dear Rebecca

Thank you for your recent enquiry seeking clarification of matters relating to the audit that you undertook with us in the Directorate of Wound Healing as part of your PhD work.

I am writing to confirm that work was in-part generated by myself asking the question "Do we prescribe excessive amounts of antibiotics which would increase the risk of patients attending my clinics developing resistant organisms and how much impact does general practitioner prescribing have on the pattern of bacteria present and bacterial resistance in patients who attend my clinics?".

I can confirm that the ideas were discussed and the final plan was agreed in a departmental audit meeting in Spring 2005 and the results of the study were presented to us in autumn 2005.

The access to records that are held by my clinic was not an issue as this was an agreed departmental audit. The obtaining of information regarding the same patients from their general practitioner to determine whether additional courses of antibiotics had been prescribed for those patients were, in my opinion, an extension and a legitimate question to ensure validity of the audit findings. While it would have been possible to have requested this information from the patient's GP as part of their routine care I made the decision as Clinical Director to ask patients for consent to ensure there was clarity of purpose in requesting this information.

We did complete the audit cycle as a result of the information generated by the project and I have reduced further my prescription of oral antibiotics for patients seen in my clinics.

I trust this clarifies the position. If you or anyone else wishes to discuss this matter with me further I would be delighted to do so.

Yours sincerely



**Keith Harding**  
**Clinical Director of Wound Healing Cardiff & Vale NHS Trust**  
**Head, Department of Wound Healing**  
**Professor of Rehabilitation (Wound Healing)**

**Private and Confidential**

[Headed paper]

*Date 2005*

Dear,

**Subject: Antibiotic resistance audit in patients with wounds**

We are currently undertaking an audit, of the patients we treat at our wound healing clinics in Cardiff, with which we would be very grateful for your support.

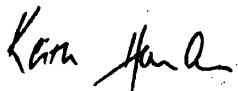
As part of the audit, we are investigating our prescription of antibiotics and the levels of antibiotic resistant organisms in patients' wounds. We are also interested in looking at those factors which predispose patients to having such organisms when they attend at our clinics.

To be able to place our prescribing for wound infection in the context of antibiotics that patients may have received for other conditions, we require information about patients' previous exposure to commonly known risk factors. Therefore, we would be most grateful if you would assist us with our enquiry by completing the attached single-sided form for the following patient(s):

Name; Date of birth; Patient's postcode

We understand that it takes time to find this information and to complete the forms and, we are able to provide payment of £3 per patient, on return of the information.

Thank you in advance for your co-operation with this interesting and relevant investigation. If you have any queries, feel free to contact Rebecca Howell-Jones on (029) 2074 4252.



Yours sincerely  
Prof. Keith G. Harding  
Clinical Director of Wound Healing  
Head of University Department of Surgery  
Professor of Rehabilitation Medicine (Wound Healing)

imap://imap.ansatt.ntnu.no:143/fetch%3EUID%3E.INBOX.DMF...

Fra: Regional komite for medisinsk og helsefaglig forskningsetikk REK midt

Til:  
Asbjørn Magne Nilsen  
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Dokumentreferanse: 2009/2245-2  
Dokumentdato: 05.01.2010

**HUMANT BLOD OG CELLER FRA BLOD TIL BASALFORSKNING OG SERUM TIL CELLEDYRNING. INFORMASJON OM  
VEDTAK**

Med hjemmel i lov om behandling av etikk og redelighet i forskning § 4 og helseforskningsloven § 10 har Regional komité for medisinsk og helsefaglig forskningsetikk, Midt-Norge vurdert prosjektet i sitt møte 4. desember 2009 med følgende vilkår og vurdering:

**Vedtak:**

"Komiteen godkjenner oppretting av generell forskningsbiobank: Humant blod og celler fra blod til basalforskning og serum til celledyrking. Ansvarshavende person er førsteamanuensis Asbjørn Magne Nilsen og forskningsansvarlig er NTNU, Det medisinske fakultet."

NTNU  
Norges teknisk-naturvitenskapelige  
Universitet

Det medisinske fakultet  
Regional komite for medisinsk og  
helsefaglig forskningsetikk  
Helseregion Midt-Norge



Dr. scient. Jørgen Stenvik

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Medisinsk teknisk forskningssenter  
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Besøksadr: ISM, Røde Kors 3 etg.  
St.Olavs Hospital

Vår dato:  
11.02.2008

Vår ref.:  
4.2008.217

Deres dato:

Deres ref.:

#### Bruk av human overskuddshud i studier av alginat-effekter på sårtilheling.

Med hjemmel i lov om behandling av etikk og redelighet i forskning § 4 har Regional komité for medisinsk og helsefaglig forskningsetikk, Midt-Norge vurdert prosjektet i sitt møte 25. januar 2008 med følgende vilkår og vurdering:

Hensikten med studien er ved å undersøke om overskuddshud fra bryst- og magereduserende kirurgi kan brukes i en in vitro ("i glass") hudmodell for å undersøke hvordan ulike alginater påvirker sårhelingsprosessen. En slik modell med hel human hud vil være et viktig supplement til andre metoder som blir brukt i prosjektet. I tillegg ønsker vi å isolere og bruke hudceller (keratinocytter) fra overskuddshuden, for å sammenlikne disse med keratinocytter som kjøpes kommersielt. Hypotesen er at alginater har gunstige effekter på tilheling av sår i en hudmodell ved å aktivere celler og beskytte celler mot bakterielle toksiner.

Hudbiopsier med små sår vil lages fra overskuddshud fra frivillige donorer. Huden dyrkes i laboratoriet in vitro. Effekten av ulike alginater på sårheling vil undersøkes ved ulike betingelser. En vil undersøke om alginater bedrer tilhelingen av sår ved nærvær og fravær av bakterier og bakterielle komponenter. Relevante bakterier for kroniske sår vil brukes. Stimulering av hudceller og induksjon av bioaktive komponenter som er viktige for sårtilheling (eks. vekstfaktorer, cytokiner, og hyaluronsyre) i hud og hudceller (keratinocytter) isolert fra hud vil også undersøkes. Forsøkene vil pågå så snart som mulig og vil vare ut FUGE-prosjektets varighet (ut 2009). Hud fra frivillige donorer som gjennomgår bryst- eller magereduserende inngrep vil brukes. Forespørsel om egnede pasienter vil gjøres ved kontakt med plastiske kirurger/klinikker i Trondheim. Det vil brukes hud fra friske personer av begge kjønn i alder 18-60 år. Hud fra 4-5 personer vil brukes for å teste og standardisere forsøksbetingelsene. Det vil undersøkes sår i minimum triplete for hver parameter, og det vil være nødvendig med minst tre replikate forsøk (donorer) for å bekrefte de funn man kommer fram til. Da flere alginater i kombinasjon med ulike bakterier/bakteriekomponenter skal brukes under ulike konsentrasjoner, kan et anslag på 25 personer være nødvendig. Dette ansees også som realistisk å gjennomføre sett i forhold til prosjektets varighet.

Komiteen har følgende merknader til prosjektet:

Postadresse  
Medisinsk teknisk forskningssenter  
7489 Trondheim  
dmf-post@medisin.ntnu.no

Besøksadresse  
Medisinsk teknisk forskningssenter  
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Telefaks +47 73 59 88 65  
Org. nr. 974 767 880

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4.2008.217

- Komiteen viser til prosjektprotokollen. Komiteen har ingen merknader til målsetting eller plan for gjennomføring, men stiller spørsmål om nødvendigheten ved å ha materialet personidentifiserbart. Om dette ikke trengs bør materialet oppbevares anonymt; dersom man må ha identifiserbarhet bør materialet avidentifiseres og identifikasjonsnøkkelen oppbevares hos den klinisk ansvarlige.
- Det skal tas ut biologisk materiale i prosjektet. I henhold til Biobankloven må det søkes spesielt om dette, jf. § 4 i loven, og dette skal vurderes av Regional komité for medisinsk forskningsetikk før det meldes til Sosial- og helsedirektoratet. Komiteen viser til skjema for melding av forskningsbiobank og har ingen merknader til de prøver og analyser det er gjort greie for. Komiteen har ingen innvendinger til at denne forskningsbiobanken blir etablert slik det er gjort greie for.
- Sekretariatet vil oversende biobankmeldingen til direktoratet.
- Komiteen viser til informasjonsskrivet og ber om at dette gjennomgås og komiteen viser til vedlagt veiledning for utforming av slike informasjonsskriv. Komiteen ber om å få tilsendt revidert informasjonsskriv. Komiteens leder får fullmakt til å vurdere det reviderte skrivet, og denne godkjenning må være klar før prosjektet settes i gang.

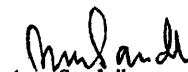
Komiteen ber om å få tilsendt artikkel/rapport når studien er fullført.


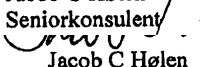
**Vedtak:**

**"Komiteen godkjenner at prosjektet gjennomføres med de merknader som er gitt."**

Vedtaket kan påklages og klagefristen er tre uker fra mottagelsen av dette brev, jf. fvl. §§ 28 og 29. Klageinstans er Den nasjonale forskningsetiske komité for medisin og helsefag (NEM), men en eventuell klage skal rettes til REK Midt-Norge. Avgjørelsen i NEM er endelig. Det følger av fvl. § 18 at en part har rett til å gjøre seg kjent med sakens dokumenter, med mindre annet følger av de unntak loven oppstiller i §§ 18 og 19.

Med hilsen

  
Arne Sandvik  
Professor  
Leder i komiteen

  
Jacob C Hølen  
Seniorkonsulent  
  
Jacob C Hølen  
Seniorkonsulent

NTNU  
Norges teknisk-naturvitenskapelige  
Universitet

Det medisinske fakultet  
Regional komite for medisinsk og  
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Vår dato:  
15.08.2008

Vår ref.:  
4.2008.217

Deres dato:  
11.08.2008

Deres ref.:

**Bruk av human overskuddshud i studier av alginateffekter på sårtilheling.**

Viser til revidert informasjonsskriv og tar dette til etterretning uten ytterligere merknader.

Med hilsen

  
Arne Sandvik  
Professor  
Leder i komiteen

  
Arild Hals  
Seniorrådgiver

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## **Appendix II:**

### **Control isolates**



**Table II.i Control isolates used**

Isolate name	Other name	Isolate type	Control	Chapter	Reference/Accession No.	Acknowledgement
NCTC 6571	Oxford <i>Staphylococcus aureus</i>	<i>Staphylococcus aureus</i>	<i>mecA</i> negative	2		
			PVL	3		
NCTC 12493		MRSA	<i>mecA</i>	2		
NCTC 11047/ ATCC14990	Meticillin resistant CNS	<i>Staphylococcus epidermidis</i> Tc <sup>R</sup>	<i>mecA</i> Tetracycline resistance	2		
ATCC 6538/ NCIMB 9518		<i>Staphylococcus aureus</i>	Biocide testing	2	BS EN 13697:2001	
ITU 1	Clinical ITU strain	MRSA	MRSA typing	3		Dr R. Howe, SACU
ITU2	Clinical ITU strain	MRSA	MRSA typing	3		Dr R. Howe, SACU
			Biocide testing	2		
ITU 3	Clinical ITU strain	MRSA	MRSA typing	3		Dr R. Howe, SACU
ITU 4	Clinical ITU strain	MRSA	MRSA typing	3		Dr R. Howe, SACU
EMRSA 15	MRSA	MRSA	PFGE	3		Dr R. Howe, SACU

ATCC, American type culture collection; CNS, coagulase negative staphylococci; EMRSA, epidemic MRSA; ITU, Intensive therapy unit; MRSA; meticillin resistant *Staphylococcus aureus*; NCTC, National collection of type cultures; PVL, Panton-Valentine leucocidin; PFGE, Pulsed field gel electrophoresis; SACU, Specialist Antimicrobial Chemotherapy Unit; Tc<sup>R</sup>, tetracycline resistant.

**Table II.1 continued Control isolates used**

Isolate name	Other name	Isolate type	Control	Chapter	Reference/Accession No.	Acknowledgement
EMRSA 16	MRSA	MRSA	PFGE	3		Dr R. Howe, SACU
NCTC 13142	EMRSA-15	MRSA	<i>agr</i> I J type h SCC <i>mec</i> IV	3	(Gomes et al., 2005)	
NCTC13130	EMRSA-3	MRSA	<i>agr</i> II PFGE	3	(Howe et al., 2004)	
NCTC 13143	EMRSA-16	MRSA	<i>agr</i> III PFGE	3	(Gomes et al., 2005)	
RN4850		MRSA	<i>agr</i> IV <i>etA/etB</i>	3	(Jarraud et al., 2000, Holmes et al., 2005, Rossney et al., 2007)	Professor R. Novick, New York School of Medicine, USA
E0898		MRSA	Class A <i>mec</i> <i>ccr3/ccrC</i> SCC <i>mec</i> III	3	(Shore et al., 2008)	Dr A. Shore, EARS-Net, TCD

*agr*, accessory gene regulator; *ccr*, cassette chromosome recombinase; EARS-Net, European Antimicrobial Resistance Surveillance Network; EMRSA, epidemic MRSA; J type, joining region of SCC*mec*; *mec*, meticillin resistant determinant; MRSA; meticillin resistant *Staphylococcus aureus*; NCTC, National collection of type cultures; PFGE, Pulsed field gel electrophoresis; SCC*mec*, staphylococcal cassette chromosome *mec*; TCD, Trinity College, Dublin; USA, United States of America.

**Table H.1 continued Control isolates used**

Isolate name	Other name	Isolate type	Control	Chapter	Reference/Accession No.	Acknowledgement
M00.005.2		MRSA	Class B <i>mec</i> <i>ccr4</i> SCC <i>mec</i> VI	3	(Shore et al., 2008)	Dr A. Shore, TCD National Reference Laboratory, St James Hospital, Ireland
CA05		MRSA	Class B <i>mec</i> <i>ccr2</i>	3	(Ma et al., 2002, Milheirico et al., 2007, Chen et al., 2009)	
WIS		MRSA	Class C <i>mec</i> <i>ccrC</i> SCC <i>mec</i> V	3	(Ito et al., 2004, Chen et al., 2009)	Professor T. Ito, Juntendo University, Japan
PH11 43.2		MRSA	<i>ccr1</i>	3	(Shore et al., 2005)	Dr A. Shore, TCD
FRI913	<i>Staphylococcus aureus</i>		<i>sea/sec/see/tst</i>	3	(Monday and Bohach, 1999, Smyth et al., 2005)	Professor C. Smyth, TCD
NCTC 10652	<i>Staphylococcus aureus</i>		<i>sea/sej</i> <i>agrI</i>	3	(Holmes et al., 2005)	Professor A. Kearns, HPA

*agr*, accessory gene regulator; *ccr*, cassette chromosome recombinase; HPA, Health protection agency MRSA; meticillin resistant *Staphylococcus aureus*; NCTC, National collection of type cultures; SCC*mec*, staphylococcal cassette chromosome *mec*; *se*, staphylococcal enterotoxin; *mec*, meticillin resistant determinant; TCD, Trinity College, Dublin.

**Table II.i continued Control isolates used**

Isolate name	Other name	Isolate type	Control	Chapter	Reference/Accession No.	Acknowledgement
NY19		<i>Staphylococcus aureus</i>	<i>seb</i>	3	(Smyth et al., 2005, Rossney et al., 2007)	Professor C. Smyth, TCD
NCTC 10654		<i>Staphylococcus aureus</i>	<i>seb</i>	3	(Holmes et al., 2005)	Professor A. Kearns, HPA
NCTC 10655		<i>Staphylococcus aureus</i>	<i>sec</i>	3	(Holmes et al., 2005)	Professor A. Kearns, HPA
NCTC 10656		<i>Staphylococcus aureus</i>	<i>sed</i> <i>agrII</i>	3	(Holmes et al., 2005)	Professor A. Kearns, HPA
FRI472		<i>Staphylococcus aureus</i>	<i>sed/sei/seg/sej</i>	3	(Monday and Bohach, 1999, Smyth et al., 2005)	Professor C. Smyth, TCD
ATCC 27644		<i>Thermobifida alba</i>	<i>see</i>	3	(Holmes et al., 2005)	Professor A. Kearns, HPA
FRI572		<i>Staphylococcus aureus</i>	<i>seg/sei</i>	3	(Monday and Bohach, 1999)	Professor A. Kearns, HPA
ATCC 51811		<i>Staphylococcus aureus</i>	<i>seh</i>	3		Professor A. Kearns, HPA
FRI569		<i>Staphylococcus aureus</i>	<i>seh</i>	3	(Monday and Bohach, 1999, Smyth et al., 2005)	Professor C. Smyth, TCD

*agr*, accessory gene regulator; ATCC, American type culture collection; HPA, Health protection agency; MRSA; NCTC, National collection of type cultures; *se*, staphylococcal enterotoxin; TCD, Trinity College, Dublin.

**Table II.i continued Control isolates used**

Isolate name	Other name	Isolate type	Control	Chapter	Reference/Accession No.	Acknowledgement
TY114		<i>Staphylococcus aureus</i>	<i>etD</i> <i>agrIII</i>	3	(Yamaguchi et al., 2002, Holmes et al., 2005, Rossney et al., 2007)	Professor M. Sugai, Hiroshima University, Japan.
NCTC 11963		<i>Staphylococcus aureus</i>	<i>tst</i> <i>agrIII</i>	3	(Holmes et al., 2005)	Professor A. Kearns, HPA
NCTC 8178	Newman	MRSA	PVL/ <i>cna</i> negative <i>hysA/geh/hlgA/hlgB/hlgC/icaA/etA</i> positive <i>agrIII</i>	3	(Lina et al., 1999)  NC_009641	
Mu50	LLA	hVISA	PVL/ <i>cna</i> negative <i>hysA/geh/hlgA/hlgB/hlgC/icaA/etA</i> positive <i>agrII</i>	3	(Kuroda et al., 2001)  NC_002758	Dr M. Wootton, SACU

*agr*, accessory gene regulator; *cna*, collagen binding gene; *et*, exfoliative toxin; *geh*, glycerol ester hydrolase; *hlg*, gamma-haemolysin; *hysA*, hyaluronate lyase gene; *icaA*, intercellular adhesin gene; HPA, Health protection agency; MRSA; meticillin resistant *Staphylococcus aureus*; NCTC, National collection of type cultures; PVL, Panton-Valentine leucocidin; SACU, Specialist Antimicrobial Chemotherapy Unit; *tst*, toxic shock toxin gene; VISA, vancomycin intermediate *Staphylococcus aureus*.

**Table II.1 continued Control isolates used**

Isolate name	Other name	Isolate type	Control	Chapter	Reference/Accession No.	Acknowledgement
MRSA 252		MRSA	PVL negative <i>geh/cna/hysA</i> precursor <i>/hlgA/hlgB/hlgC/icaA</i> positive <i>agrI</i>	3	(Holden et al., 2004)  NC_002952	Professor T. Foster, Dublin
HT2000 132		MRSA	PVL  <i>agrIII</i>	3	(Rossney et al., 2007)	Jerome Etienne Staphylococcal reference lab, Lyon France
NCTC 13300		<i>Staphylococcus aureus</i>	PVL			Professor A. Kearns, HPA
NCTC 8325		<i>Staphylococcus aureus</i>	<i>cna/hysA/geh/hlgA/hlgB/hlgC/icaA</i> negative <i>agrI</i>	3	(Jarraud et al., 2000, Rossney et al., 2007) NC_007795	
PJ141		<i>Staphylococcus aureus</i>	<i>icaA</i>	3	(Malic, 2008)	Dr S. Malic, CU
PJ156		<i>Staphylococcus aureus</i>	<i>icaA</i>	3	(Malic, 2008)	Dr S. Malic, CU

*agr*, accessory gene regulator; *cna*, collagen binding gene; CU, Cardiff University *geh*, glycerol ester hydrolase; *hlg*, gamma-haemolysin; *hysA*, hyaluronate lyase gene; *icaA*, intercellular adhesin gene; MRSA; meticillin resistant *Staphylococcus aureus*; NCTC, National collection of type cultures; PVL, Panton-Valentine leucocidin.

**Table H.1 continued Control isolates used**

Isolate name	Other name	Isolate type	Control	Chapter	Reference/Accession No.	Acknowledgement
FC2	CVLU	<i>Staphylococcus aureus</i>	Clinical MSSA immunostimulation (Planktonic/Biofilm)	4	(Davies et al., 2004)	Dr K.E. Hill, CU
NCTC 10662		<i>Pseudomonas aeruginosa</i>	Clinical strain <i>P. aeruginosa</i> immunostimulation (Planktonic/Biofilm)	4		
ATCC 19142		Mucoid <i>Pseudomonas aeruginosa</i>	Mucoid strain <i>P. aeruginosa</i> immunostimulation	4	(Linker and Jones, 1964)	
ATCC 39324		Mucoid <i>Pseudomonas aeruginosa</i>	Mucoid <i>P. aeruginosa</i> immunostimulation	4	(Pier et al., 1983)	

ATCC, American type culture collection; CU, Cardiff University; CVLU, chronic venous leg ulcer; MSSA; meticillin sensitive *Staphylococcus aureus*; NCIMB, National Collection of Industrial, food and Marine Bacteria; NCTC, National collection of type cultures.

## **Appendix III:**

## **Biocide testing**



### **Appendix III.i Validation of the drop count method**

The results from the validation of the drop count method are shown in Table III.i. Values represent the 10 repeats of the drop count method recorded from the  $10^6$  serial dilutions. The first column represents the mean CFU/ml for each triplicate per plate, while the second column shows the mean transformed CFU/ml to log data per plate. The validation indicated that there was no significant intra-researcher variance between the replicates or method.

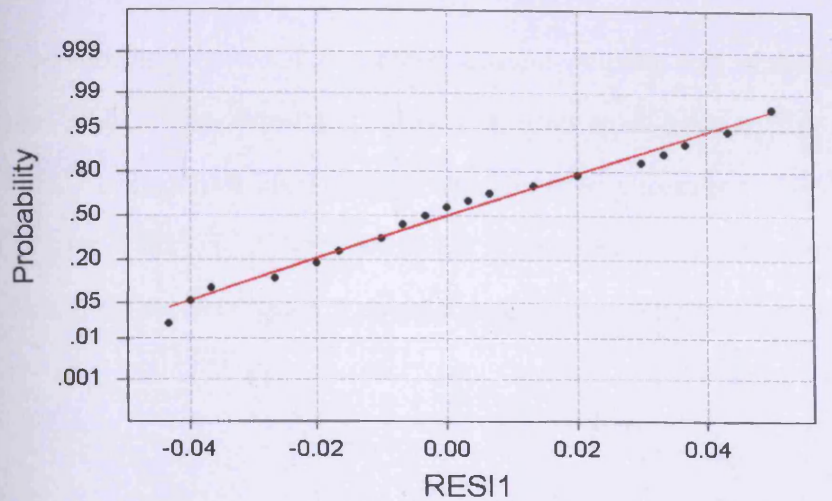
Normality testing and distribution of variance was performed using the mean  $\log_{10}$  values per plate and ANOVA in Minitab®. A P-value of 0.588 was achieved using the Anderson-Darling test indicating the residuals were normally distributed, thus satisfying this assumption for the parametric test. Figure III.i shows the normality plot for the  $\log_{10}$  CFU/ml. A test for equal variances was performed as shown in Figure III.ii, resulting in a. Bartlett test, P-value = 0.453 and Levene test, P = 0.806 at a 95% confidence interval, indicating the variances were equal, satisfying the second criteria for a one-way ANOVA test.

No significant difference ( $P > 0.05$ ) between the mean values of the validation plates and CFU/ml was achieved using ANOVA. The drop count method therefore was a valid test of bacterial growth following biocide testing.

**Table III.i** Drop count validation results of each plate and average triplicate

Mean CFU/ml per 10 <sup>6</sup> dilution	Mean log <sub>10</sub> CFU/ml per 10 <sup>6</sup> dilution	Plate series	Plate number
8.33	8.92	1	1
8.67	8.94	1	2
8.33	8.92	1	3
8.00	8.90	2	1
7.33	8.87	2	2
6.67	8.82	2	3
7.67	8.88	3	1
8.33	8.92	3	2
7.33	8.87	3	3
7.67	8.88	4	1
8.33	8.92	4	2
9.00	8.95	4	3
7.67	8.88	5	1
8.33	8.92	5	2
8	8.9	5	3
7.67	8.88	6	1
7.33	8.87	6	2
7.33	8.87	6	3
7.67	8.88	7	1
7.00	8.85	7	2
7.00	8.85	7	3
8.67	8.94	8	1
7.67	8.88	8	2
7.33	8.87	8	3
8.33	8.92	9	1
8.00	8.90	9	2
7.67	8.88	9	3
8.67	8.94	10	1
7.00	8.85	10	2
7.67	8.88	10	3

### Normal Probability Plot

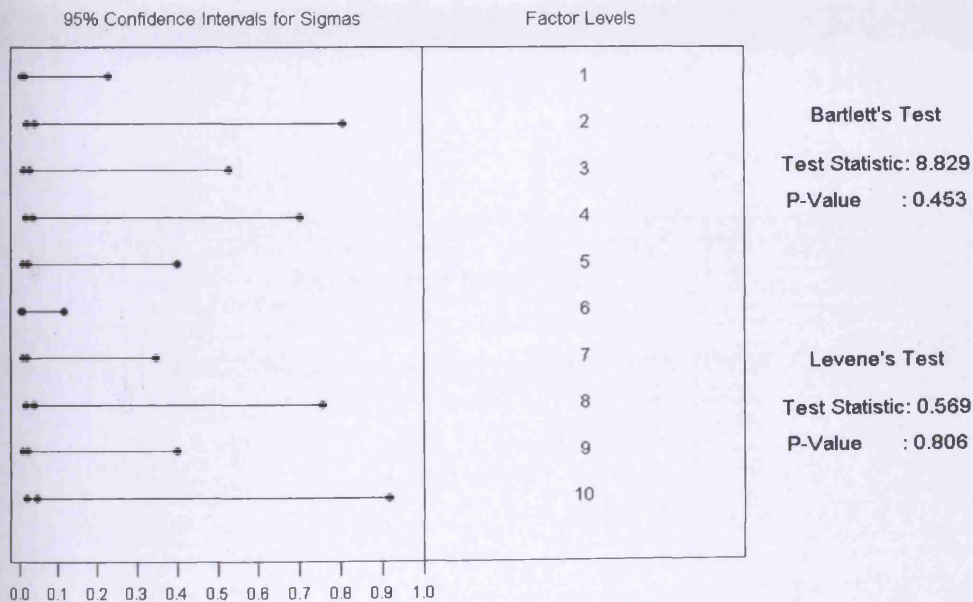


Average: 0.0000000  
StDev: 0.0240211  
N: 30

Anderson-Darling Normality Test  
A-Squared: 0.290  
P-Value: 0.588

**Figure III.i** Normality plot for the  $\log_{10}$  mean CFU/ml for each validation plate.

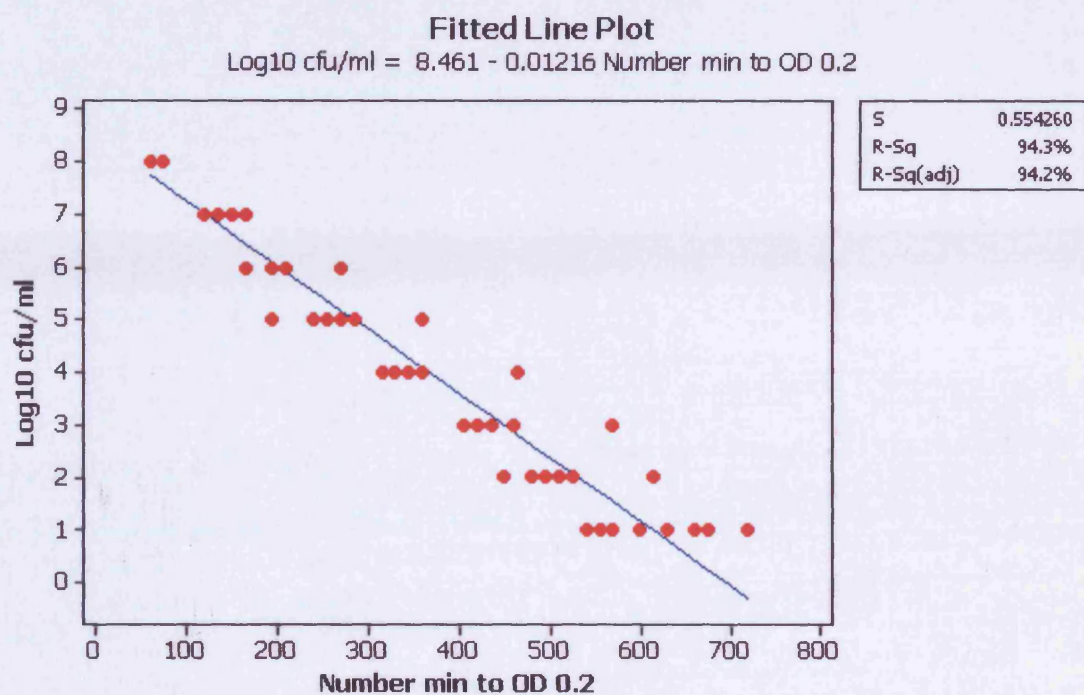
### Test for Equal Variances for response



**Figure III.ii** A chart of equal variances for validation plates indicating the  $p$ -values for Levene and Bartlett's test.

### Appendix III.ii Relationship between Bioscreen and optical density

The relationship between Bioscreen optical density and viable counts is shown in Figure III.iii. The fitted line plot was used to determine the viable count ( $\log_{10}$  CFU/ml) of bacterial growth from the recorded Bioscreen OD. The equation  $\log_{10}$  CFU/ml =  $8.461 - 0.01216(t)$  (where  $t$  is the number of min to reach OD 0.2) was used to determine bacterial growth using the Bioscreen enumeration method.



**Figure III.iii** Relationship of Bioscreen OD to viable count.



### Appendix III.iii Bioscreen honeycomb plate layout

	A	B	C	D	E	F	G	H	I	J
1	-8	-8	-8	-8	Blank	Control a	Control c	Control e	Test b	Test d
2	-7	-7	-7	-7	Blank	Control a	Control c	Control e	Test b	Test d
3	-6	-6	-6	-6	Blank	Control a	Control c	Control e	Test b	Test d
4	-5	-5	-5	-5	Blank	Control a	Control c	Control e	Test b	Test d
5	-4	-4	-4	-4	Blank	Control a	Control c	Control e	Test b	Test d
6	-3	-3	-3	-3	Neutraliser	Control b	Control d	Test a	Test c	Test e
7	-2	-2	-2	-2	Neutraliser	Control b	Control d	Test a	Test c	Test e
8	-1	-1	-1	-1	Neutraliser	Control b	Control d	Test a	Test c	Test e
9	-8	-7	-6	-5	Neutraliser	Control b	Control d	Test a	Test c	Test e
10	-4	-3	-2	-1	Neutraliser	Control b	Control d	Test a	Test c	Test e

**Figure III.iv** Organisation of honeycomb plate. -1 to -8, serial dilutions of the working solutions ( $10^{-1}$  to  $10^{-8}$ ); Blank, Tryptone soy broth (TSB) media control; Neutraliser, TSB with experimental neutraliser; Control, bacteria employing sterile water as a biocide (experimental control); Test, bacteria under test condition employing the investigated biocide (experimental test); a,b,c,d,e, individual test strains.

# Appendix III.iv Patient biocide treatment and experimental biocide test contact times

**Table III.ii** Relationship of contact time (min) necessary for 3 log<sub>10</sub> bacterial reductions based on drop count enumeration and previous known clinical history of patient biocide exposure

Biocide Treatment of Patient Wound Over a 90 Day Period						
Isolate	Iodine	Povidone-iodine	Silver	Silver nitrate	Potassium	Potassium permanganate
1004	<sup>a</sup> 3	15	<sup>a</sup> 4	N/A	0	N/A
1011	0	30	0	N/A	0	30
1021	0	15	0	30	<sup>a</sup> 3	30
1106	0	30	<sup>a</sup> 2	N/A	0	30
1108	<sup>a</sup> 1	30	0	N/A	0	30
2018	<sup>a</sup> 2	30	2	30	0	30
2124	<sup>a</sup> 1	15	0	30	0	30

N/A, not applicable, did not reach a 3 log<sub>10</sub> bacterial growth reduction; 0, no biocide treatment in 90 d period before MRSA isolation;

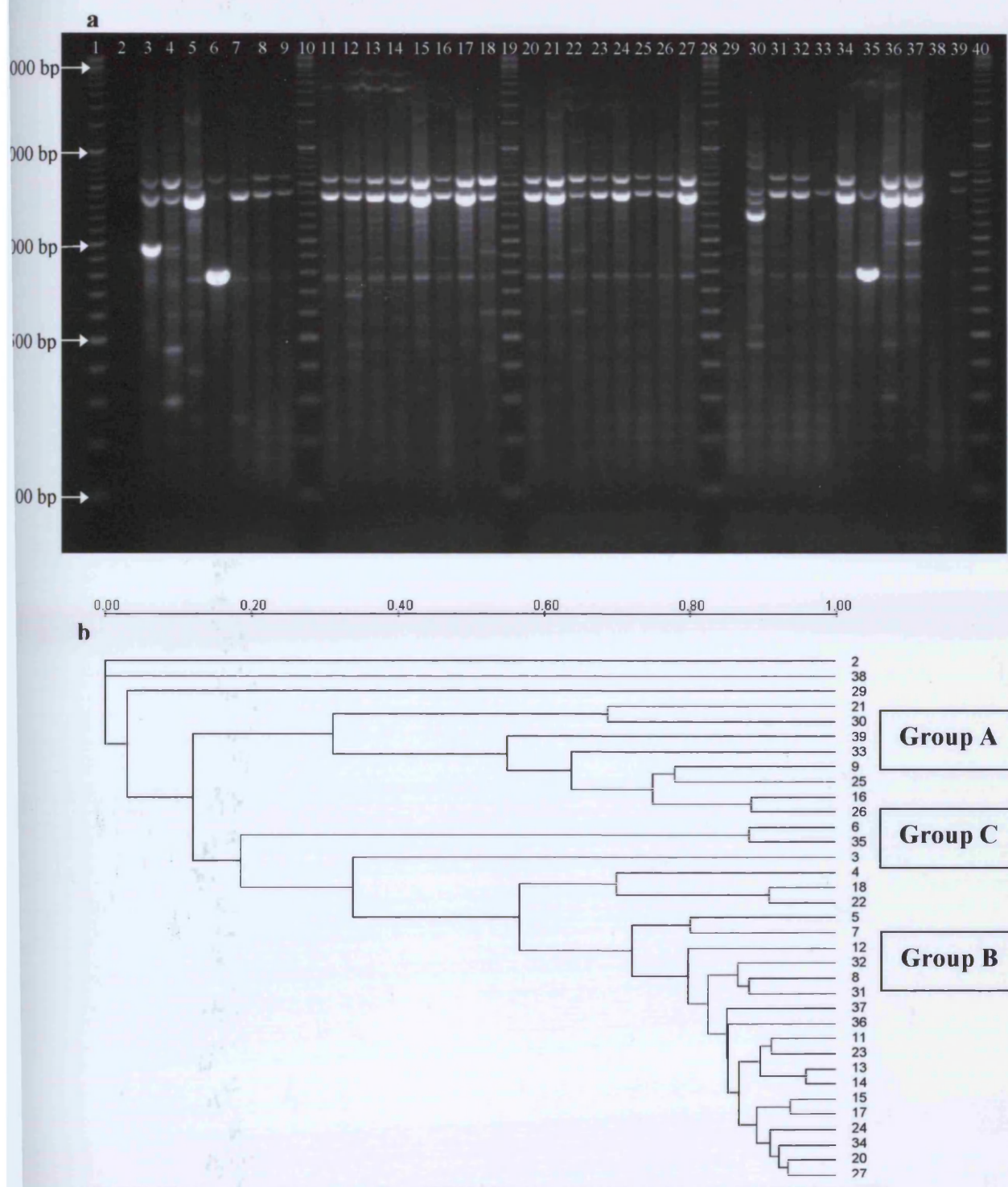
<sup>a</sup>reported number of biocide exposures in a 90 day period.

## **Appendix IV:**

### **MRSA typing**

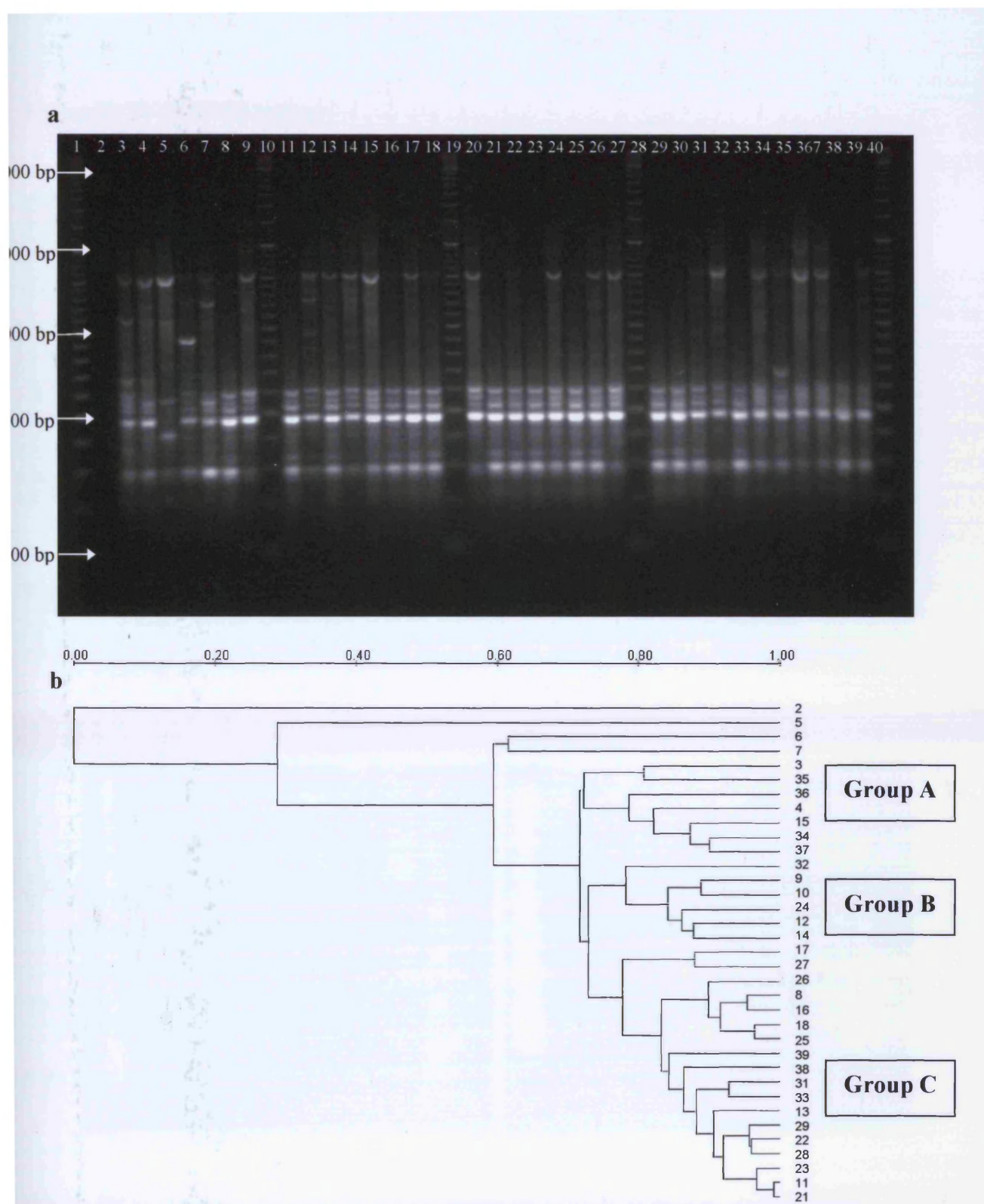


## Appendix IV.i RAPD typing



**Figure IV.i (a)** Agarose gel showing RAPD fingerprint using Primer E2. **(b)** Phylogenetic tree using UPGAMA analysis by Quantity One software to identify similarity patterns in fingerprints produced by primer E2. Lane numbers match those denoted on the phylogenetic tree. Lanes 1, 10, 19, 28 and 40, 100 bp and 500 bp molecular weight markers (Fermentas). Lane 2 and 38, negative H<sub>2</sub>O control; lanes 8, 9, 11 to 18, 20 to 27, 29, 31, 32 and 39, MRSA isolates; lanes 3 to 7, EMRSA 15, EMRSA 16, NCTC 13143, NCTC 6571 and NCTC 12493 control isolates respectively; lanes 30 and 33 to 37, CNS, MSSA and MRSA controls.



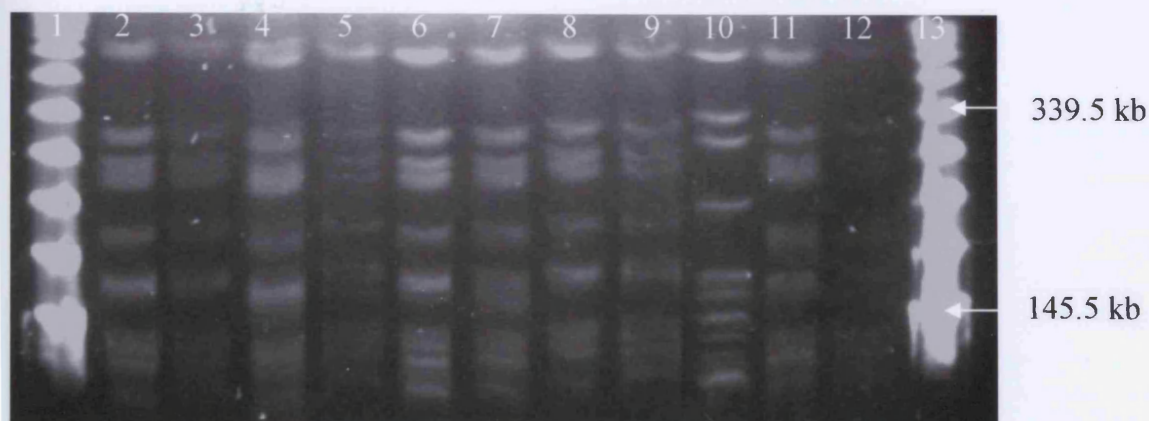


**Figure IV.ii (a)** Agarose gel showing RAPD fingerprint using Primer 1. **(b)** Phylogenetic tree UPGAMA analysis by Quantity One software to identify similarity patterns in fingerprints produced by Primer 1. Lane numbers match those denoted on the phylogenetic tree. Lanes 1, 10, 19, 28, and 40, 100 bp and 500 bp molecular weight markers (Fermentas). Lane 2, negative H<sub>2</sub>O control; lanes 8, 9, 11 to 18, 20 to 27, 29, 31, 32 and 39, MRSA isolates; lanes 3 to 7, EMRSA 15, EMRSA 16, NCTC 13143, NCTC 6571 and NCTC 12493 control isolates respectively; lane 30 and 33 to 37, CNS, MSSA and MRSA controls.

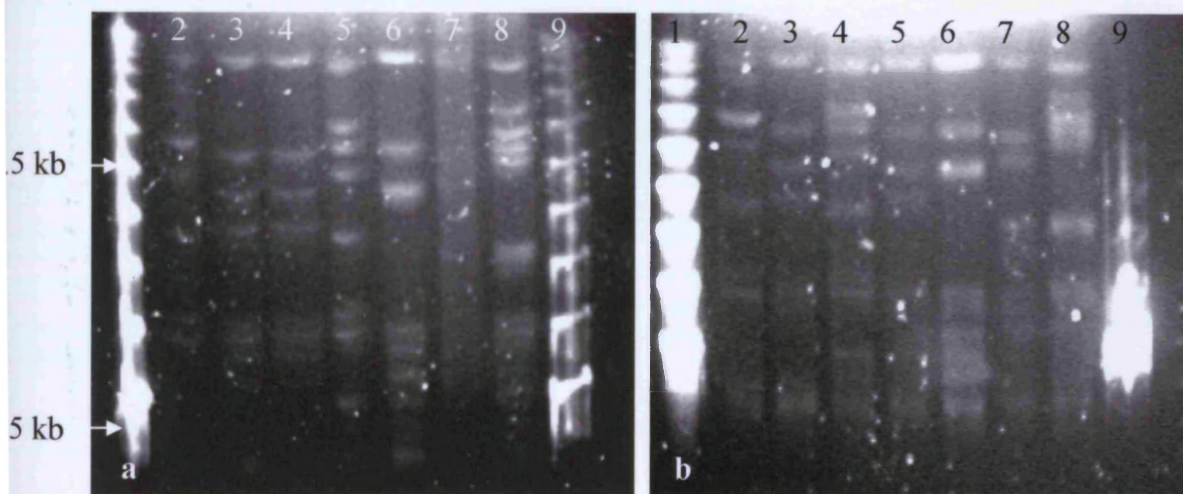
# **Appendix V:**

## **PVL alignment sequences**

## Appendix IV.ii PFGE typing



**Figure IV.iii** Agarose gel of pulse-type B fragment restriction digests. Lanes 1 and 13, PFG lambda ladder molecular weight markers (NEB); lanes 2 to 9, MRSA wound isolates; lane 10, control isolate NCTC 6571; lanes 11 and 12, MRSA isolates.



**Figure IV.iv** Agarose gel of pulse-type C fragment restriction digests. Lanes 1 and 9, PFG lambda ladder molecular weight markers (NEB). a) Lanes 3, 4 and 7, MRSA isolates; lane 2, control isolates NCTC 12493; lane 5, control isolate NCTC 13130 (EMRSA 3); lane 6, control isolate NCTC 13143 (EMRSA 16); lane 8, ICU MRSA control; b) lanes 3, 5, and 7, MRSA isolates.

## Appendix V.i Alignment using Lina PVL PCR primer sequences

Control isolate NCTC 6571 consensus sequence alignment with accession number AB532026. Sequence alignments with *Staphylococcus aureus* strain C5 Pantone-Valentine leucocidin S start codon.

1	ATATAGTCAAAATCCGAGAGACTATTTTGTGCCAGACAATGAATTACCCC	50
633	atatagtcaaaatccgagagactatTTTgtgccagacaatgaattacccc	682
51	CATTAGTACACAGTGGTTTCAATCCTTCATTTATTGCAACTGTTTCTCAT	100
683	cattagtacacagtggTTTcaatccttcatttattgcaactgTTTctcat	732
101	GAAAAAGGCTCAGGAGATACAAGTGAATTTGAAATAACGTATGGCAGAAA	150
733	gaaaaaggctcaggagatacaagtgaatttgaaataacgtatggcagaaa	782
151	TATGGATGTTACTCATGCTACTAGAAGAACAACACACTATGGCAATAGTT	200
783	tatggatgTTactcatgctactagaagaacaacacactatggcaatagtt	832
201	ATTTAGAAGGATCTAGAATACACAACGCATTTGTAAACAGAAATTACACA	250
833	atttagaaggatctagaatacacaacgcatttgtaaacagaaattacaca	882
251	GTTAAATATGAAGTGAAGTGGAAACTCATGAAATTAAAGTGAAAGGACA	300
883	gttaaatatgaagtgaactggaaaactcatgaaattaaagtgaaaggaca	932
301	TAATTGATATGAAAAAATAGTCA	324
933	taattgatatgaaaaaatagtca	956

Control isolate HT2000 132 consensus sequence alignment with accession number AB532026. Sequence alignments with *Staphylococcus aureus* strain C5 Pantón-Valentine leucocidin S start codon.

1	TGGACATGATCCAAATTTATTTGTTGGATATAAACCATATAGTCAAATC	50
597	TGGACATGATCCAAATTTATTTGTTGGATATAAACCATATAGTCAAATC	646
51	CGAGAGACTATTTTGTGCCAGACAATGAATTACCCCCATTAGTACACAGT	100
647	CGAGAGACTATTTTGTGCCAGACAATGAATTACCCCCATTAGTACACAGT	696
101	GGTTTCAATCCTTCATTTATTGCAACTGTTTCTCATGAAAAAGGCTCAGG	150
697	GGTTTCAATCCTTCATTTATTGCAACTGTTTCTCATGAAAAAGGCTCAGG	746
151	AGATACAAGTGAATTTGAAATAACGTATGGCAGAAATATGGATGTTACTC	200
747	AGATACAAGTGAATTTGAAATAACGTATGGCAGAAATATGGATGTTACTC	796
201	ATGCTACTAGAAGAACAACACACTATGGCAATAGTTATTTAGAAGGATCT	250
797	ATGCTACTAGAAGAACAACACACTATGGCAATAGTTATTTAGAAGGATCT	846
251	AGAATACACAACGCATTTGTAAACAGAAATTACACAGTTAAATATGAAGT	300
847	AGAATACACAACGCATTTGTAAACAGAAATTACACAGTTAAATATGAAGT	896
301	GAACTGGAAAACATCATGAAATTAAAGTGAAAGGACATAATTGATATGAAA	350
897	GAACTGGAAAACATCATGAAATTAAAGTGAAAGGACATAATTGATATGAAA	946
351	AAAATAGTCAAATCATCA	368
947	AAAATAGTCAAATCATCA	964

Blind MRSA isolate 5155 consensus sequence alignment with accession number AB532026. Sequence alignments with *Staphylococcus aureus* strain C5 Panton-Valentine leucocidin S start codon.

1	GACTATTTTGTTCAGACAATGAATTACCCCATTTAGTACACAGTGGTTT	50
652	gactatTTTgtgccagacaatgaattacCCCATTTAGTACACAGTGGTTT	701
51	CAATCCTTCATTTATTGCAACTGTTTCTCATGAAAAAGGCTCAGGAGATA	100
702	caatccttcatttattgcaactgtttctcatgaaaaaggctcaggagata	751
101	CAAGTGAATTTGAAATAACGTATGGCAGAAATATGGATGTTACTCATGCT	150
752	caagtgaatttgaaataacgtatggcagaaatattggatgttactcatgct	801
151	ACTAGAAGAACAACACACTATGGCAATAGTTATTTAGAAGGATCTAGAAT	200
802	actagaagaacaacacactatggcaatagttatttagaaggatctagaat	851
201	ACACAACGCATTTGTAAACAGAAATTACACAGTTAAATATGAAGTGAAC	250
852	acacaacgcatttgtaaacagaaattacacagttaaatattgaagtgaact	901
251	GGAAAACTCATGAAATTAAAGTGAAAGGACATA	283
902	ggaaaactcatgaaattaaagtgaaggacata	934



Blind MRSA isolate 554 consensus sequence alignment with accession number AB532026. Sequence alignments with *Staphylococcus aureus* strain C5 Pantone-Valentine leucocidin S start codon.

1	GAGAGACTATTTTGGTGCCAGACAATGAATTACCCCCATTAGTACACAGT	50
648	gagagactatTTT-gtgccagacaatgaattacccccattagTACACAGT	696
51	GGTTTCAATCCTTCATTTATTGCAACTGTTTCTCATGAAAAAGGCTCAGG	100
697	ggTTTcaatccttcattttattgcaactgTTTctcatgaaaaaggctcagg	746
101	AGATACAAGTGAATTTGAAATAACGTATGGCAGAAATATGGATGTTACTC	150
747	agatacaagtgaatttgaaataacgtatggcagaaatattggatgttactc	796
151	ATGCTACTAGAAGAACAACACACTATGGCAATAGTTATTTAGAAGGATCT	200
797	atgctactagaagaacaacacactatggcaatagttatttagaaggatct	846
201	AGAATACACAACGCATTTGTAAACAGAAATTACACAGTTAAATATGAAGT	250
847	agaatacacaacgcattttgtaaacagaaattacacagttaaatatgaagt	896
251	GAACTGGAAAACATCATGAAATTAAAGTGAAAGGACATAATTGATATGAAA	300
897	gaactggaaaactcatgaaattaaagtgaaggacataattgatatgaaa	946
301	AAAATAGTCA	310
947	aaaatagtca	956

Blind MRSA isolate 597 consensus sequence alignment with accession number AB532026. Sequence alignments with *Staphylococcus aureus* strain C5 Panton-Valentine leucocidin S start codon.

1	ATATAGTCAAAATCCGAGAGACTATTTTGTTCAGACAATGAATTACCCC	50
633	atatagtc aaaatccgagagactat tttgttcagacaatgaattacccc	682
51	CATTAGTACACAGTGGTTTCAATCCTTCATTTATTGCAACTGTTTCTCAT	100
683	cattagtacacagtggtttcaatccttcatttattgcaactgtttctcat	732
101	GAAAAAGGCTCAGGAGATACAAGTGAATTTGAAATAACGTATGGCAGAAA	150
733	gaaaaggctcaggagatacaagtgaatttgaaataacgtatggcagaaa	782
151	TATGGATGTTACTCATGCTACTAGAAGAACAACACACTATGGCAATAGTT	200
783	tatggatgttactcatgctactagaagaacaacacactatggcaatagtt	832
201	ATTTAGAAGGATCTAGAATACACAACGCATTTGTAAACAGAAATTACACA	250
833	atttagaaggatctagaatacacaacgcatttgtaaacagaaattacaca	882
251	GTTAAATATGAAGTGAAGTGGAAAACATGAAATTAAAGTGAAAGGACA	300
883	gttaaataatgaagtgaactggaaaactcatgaaattaaagtgaaaggaca	932
301	TAATTGATATGAAAAAATAGTCA	324
933	taattgatatgaaaaa atagtca	956



Wound MRSA isolate 1011 consensus sequence alignment with accession number AB532026. Sequence alignments with *Staphylococcus aureus* strain C5 Pantone-Valentine leucocidin S start codon.

1	TCCGAGAGACTATTTTGTGCCAGACAATGAATTACCCCCATTAGTACACA	50
645	tccgagagactatTTTgtgccagacaatgaattacCCCCattagTAcAcA	694
51	GTGGTTTCAATCCTTCATTTATTGCAACTGTTTCTCATGAAAAAGGCTCA	100
695	gtggtttcaatccttcatttattgcaactgtttctcatgaaaaaggctca	744
101	GGAGATACAAGTGAATTTGAAATAACGTATGGCAGAAATATGGATGTTAC	150
745	ggagatacaagtgaatttgaaataacgtatggcagaaatatggatgttac	794
151	TCATGCTACTAGAAGAACAACACACTATGGCAATAGTTATTTAGAAGGAT	200
795	tcatgctactagaagaacaacacactatggcaatagttatttagaaggat	844
201	CTAGAATACACAACGCATTTGTAAACAGAAATTACACAGTTAAATATGAA	250
845	ctagaatacacaacgcatttgtaaacagaaattacacagttaaatatgaa	894
251	GTGAACTGGAAAACATCATGAAATTAAAGTGAAAGGACATAATTGATATGA	300
895	gtgaaactggaaaactcatgaaattaaagtgaaaggacataattgatatga	944
301	AAAAAATAGTCA	312
945	aaaaaatagtca	956

Wound MRSA wound isolate 1057 consensus sequence alignment with accession number AP009363. Sequence alignment with the *Staphylococcus* phage  $\Phi$ 2958PVL precursor of the Panton-Valentine leukocidin S chain open reading frame JP059, for the *LukS-PV* gene.

```

1 ACACAGTGGTTTCAATCCTTCATTTATTGCAACTGTTTCTCATGAAAAAG      50
  |||
45484 acacagtggtttcaatccttcatttattgcaactgtttctcatgaaaaag    45533

51 GCTCAGGAGATACAAGTGAATTTGAAATAACGTATGGCAGAAATATGGAT      100
  |||
45534 gctcaggagatacaagtgaatttgaaataacgtatggcagaaatatggat    45583

101 GTTACTCATGCTACTAGAGAACAACACACTATGGCAATAGTTATTTAGA      150
  |||
45584 gttactcatgctactagaagaacaacacactatggcaatagttatttaga    45633

151 AGGATCTAGAATACACAACGCATTTGTAAACAGAAATTACACAGTTAAAT      200
  |||
45634 aggatctagaatacacacacgcatttgtaaacagaaattacacagttaaat    45683

201 ATGAAGTGAAGTGGAAAACATGAAATTAAAGTGAAAGGACATAATTGA      250
  |||
45684 atgaagtgaactggaaaactcatgaaattaaagtgaaaggacataattga    45733

251 TA      252
  ||
45734 ta      45735

```

Wound MRSA wound isolate 1108 reverse sequence alignment with accession number AP009363. Sequence alignment with the *Staphylococcus* phage  $\Phi$ 2958PVL precursor of the Panton-Valentine leukocidin S chain open reading frame JP059, for the *LukS-PV* gene.

```

      1 CATTAGGTAAAATGTCTGGACATGATCCAAATTTATTTGTTGGATATAAA      50
      ||||||||||||||||||||||||||||||||||||||||||||||||||||
45375 cattaggtaaaatgtctggacatgatccaaatTTATTtGTTGGATATAAA 45424

      51 CCATATAGTCAAAATCCGAGAGACTATTTTGTGCCAGACAATGAATTACC      100
      ||||||||||||||||||||||||||||||||||||||||||||||||||||
45425 ccatatagtcaaaatccgagagactatTTTtGTGCCAGACAATGAATTACC 45474

     101 CCCATTAGTACACAGTGGTTTCAATCCTTCATTTATTGCAACTGTTTCTC      150
      ||||||||||||||||||||||||||||||||||||||||||||||||||||
45475 cccattagtacacagtggTTTtCAATCCTTCATTTATTGCAACTGTTTCTC 45524

     151 ATGAAAAAGGCTCAGGAGATACAAGTGAATTTGAAATAACGTATGGCAGA      200
      ||||||||||||||||||||||||||||||||||||||||||||||||||||
45525 atgaaaaaggctcaggagatacaagtgaatTTGAAATAACGTATGGCAGA 45574

     201 AATATGGATGTTACTCATGCTACTAGAAGAACAACACACTATGGCAATAG      250
      ||||||||||||||||||||||||||||||||||||||||||||||||||||
45575 aatatggatgTTACTCATGCTACTAGAAGAACAACACACTATGGCAATAG 45624

     251 TTATTTAGAAGGATCTAGAATACACAACGCATTTGTAAACAGAAATTACA      300
      ||||||||||||||||||||||||||||||||||||||||||||||||||||
45625 ttatttagaaggatctagaatacacaaacgcattTTGTAAACAGAAATTACA 45674

     301 CAGTTAAATATGAAGTGAAGTGGAAAACACAGGAAATTAAAGTGAAGGA      350
      |||||||||||||||||||||||||.|||.|||||||||||||||||||||
45675 cagttaaatatgaagtgaactggaaaactcatgaaattaaagtgaagga 45724

     351 CATAATTGATA-GAAAAAATAGTCA      375
      ||||||||||||| |||||||||||||||
45725 cataattgatatgaaaaaaatagtca 45750

```

Wound MRSA wound isolate 1047 reverse sequence alignment with accession number AP009363. Sequence alignment with the *Staphylococcus* phage  $\Phi$ 2958PVL precursor of the Panton-Valentine leukocidin S chain open reading frame JP059, for the *LukS-PV* gene.

```

      1 ATCATTAGGTAAAAATGTCTGGACATGATCCAAATTTATTTGTTGGATATA      50
      ||||||||||||||||||||||||||||||||||||||||||||||||||||
45373 atcattaggtaaaaatgtctggacatgatccaaatTTATTTGTTGGATATA 45422

      51 AACCATATAGTCAAAATCCGAGAGACTATTTTGTGCCAGACAATGAATTA      100
      ||||||||||||||||||||||||||||||||||||||||||||||||||||
45423 aaccatatagtcaaaatccgagagactatTTTGTGCCAGACAATGAATTA 45472

     101 CCCCCATTAGTACACAGTGGTTTCAATCCTTCATTTATTGCAACTGTTTC      150
      ||||||||||||||||||||||||||||||||||||||||||||||||||||
45473 cccccattagtacacagtggTTTCAATCCTTCATTTATTGCAACTGTTTC 45522

     151 TCATGAAAAAGGCTCAGGAGATACAAGTGAATTTGAAATAACGTATGGCA      200
      ||||||||||||||||||||||||||||||||||||||||||||||||||||
45523 tcatgaaaaaggctcaggagatacaagtgaatTTGAAATAACGTATGGCA 45572

     201 GAAATATGGATGTTTACTCATGCTACTAGAAGAACAACACACTATGGCAAT      250
      ||||||||||||||||||||||||||||||||||||||||||||||||||||
45573 gaaatatggatgTTTACTCATGCTACTAGAAGAACAACACACTATGGCAAT 45622

     251 AGTTATTTAGAAAGGATCTAGAATACACAACGCATTTGTAAACAGAAAT      298
      ||||||||||||||||||||||||||||||||||||||||||||||||||||
45623 agttatTTAGAAAGGATCTAGAATACACAACGCATTTGTAAACAGAAAT 45670

```

## Appendix V.ii Alignment using Fey PVL PCR primer sequences

Control isolate NCTC 6571 consensus sequence alignment with accession number AB532027. Sequence alignments with *Staphylococcus aureus* strain NN47 Panton-Valentine leucocidin S and F start codons.

1	CTCATGAAATTAAAGTGAAAGGACATAATTGATATGAAAAAATAGTCAA	50
908	ctcatgaaattaaagtgaaaggacataattgatatgaaaaaatagtcaa	957
51	ATCATCAGTTGTTACATCAATTGCATTGCTTTTGCTATCCAATACAGTTG	100
958	atcatcagttggttacatcaattgcattgcttttgctatccaatacagttg	1007
101	ATGCAGCTCAACATATCACACCTGTAAGTGAGAAAAAGGTTGATGATAAA	150
1008	atgcagctcaacatatcacacctgtaagtgaagaaaaagggttgatgataaa	1057
151	ATTACTTTGTACAAAACAACGCAACATCAGATTCCGATAAGTTAAAAAT	200
1058	attactttgtacaaaacaactgcaacatcagattccgataagttaaaaat	1107
201	TTCTCAGATTTTAACTTTTAATTTTATTAAAGATAAAAGTTATGATAAAG	250
1108	ttctcagatTTTAACTTTTAATTTTATTAAAGATAAAAGTTATGATAAAG	1157
251	ATACATTAATACTCAAAGCTGCTGGAAACATTTATTCTGGCTATACAAAG	300
1158	atacatTAATACTCAAAGCTGCTGGAAACATTTATTCTGGCTATACAAAG	1207
301	CCAAATCCAAAAGACACTATTAGTTCTCAATTTTA	335
1208	ccaaatccaaaagacactattagttctcaatttta	1242

Wound MRSA wound isolate 1011 consensus sequence alignment with accession number AB532027. Sequence alignments with *Staphylococcus aureus* strain NN47 Pantone-Valentine leucocidin S and F start codons.

```

1  CATAATTGATATGAAAAAATAGTCAAATCATCAGTTGTTACATCAATTG      50
  ||||||||||||||||||||||||||||||||||||||||||||||||||||
931 CATAATTGATATGAAAAAATAGTCAAATCATCAGTTGTTACATCAATTG      980

51  CATTGCTTTTGCTATCCAATACAGTTGATGCAGCTCAACATATCACACCT     100
  ||||||||||||||||||||||||||||||||||||||||||||||||||||
981 CATTGCTTTTGCTATCCAATACAGTTGATGCAGCTCAACATATCACACCT    1030

101  GTAAGTGAGAAAAAGGTTGATGATAAAATTACTTTGTACAAAACAACTGC     150
  ||||||||||||||||||||||||||||||||||||||||||||||||||||
1031 GTAAGTGAGAAAAAGGTTGATGATAAAATTACTTTGTACAAAACAACTGC    1080

151  AACATCAGATTCCGATAAGTTAAAAATTTCTCAGATTTTAACTTTTAATT     200
  ||||||||||||||||||||||||||||||||||||||||||||||||||||
1081 AACATCAGATTCCGATAAGTTAAAAATTTCTCAGATTTTAACTTTTAATT    1130

201  TTATTAAAGATAAAAAGTTATGATAAAGATACATTAATACTCAAAGCTGCT     250
  ||||||||||||||||||||||||||||||||||||||||||||||||||||
1131 TTATTAAAGATAAAAAGTTATGATAAAGATACATTAATACTCAAAGCTGCT    1180

251  GGAAACATTTATTCTGGCTATACAAAG      277
  ||||||||||||||||||||||||||||
1181 GGAAACATTTATTCTGGCTATACAAAG     1207

```

Wound MRSA wound isolate 1021 consensus sequence alignment with  
 accession number FJ713816. Sequence alignment with the *Staphylococcus* phage  
 ΦPVL-CN125 precursor for Panton-Valentine leucocidin F chain.

1	CAGCTCAACATATCACACCTGTAAGTGAGAAAAAGGTTGATGATAAAATT	50
42674	cagctcaacatatcacacctgtaagtgagaaaaaggttgatgataaaatt	42723
51	ACTTTGTACAAAACAACGCAACATCAGATTCCGATAAGTTAAAAATTTC	100
42724	actttgtacaaaacaactgcaacatcagattccgataagttaaaaatttc	42773
101	TCAGATTTTAACTTTTAATTTTATTAAAGATAAAAGTTATGATAAAGATA	150
42774	tcagattttaacttttaattttattaaagataaaagttatgataaagata	42823
151	CATTAATACTCAAAGCTGCTGGAAACATTTATTCTGGCTATAC	193
42824	cattaataactcaaagctgctggaaacatttattctggctatac	42866

Wound MRSA wound isolate 1106 consensus sequence alignment with accession number X64389. Sequence alignment with the *Staphylococcus aureus* leuF-P83 gene for F component of leucocidin R.

```

      1 TAGTCAAATCATCCGTTGCTACATCTATGGCATTATTATTACTTTCTGGT      50
      |||||||||||||||||||.|||||||||||||||||||||.||
1186 tagtcaaatcatccgttgctacgtctatggcattattattacttttcta      1235

      51 ACTGCTAATGCTGAAGGCAAAATCACACCAGTTAGCGTCAAAAAAGTAGA      100
      |||||.||||||||||||||||||||||||||||||||||||||
1236 actgcaaatgctgaaggcaaatcacaccagttagcgtcaaaaaagtaga      1285

     101 TGATAAAGTTACTTTTATACAAAACCTACAGCTACAGCAGATTCAGATAAAT      150
      ||||||||||||||||||||||||||||||||||||||||||||
1286 tgataaagttactttatacaaaactacagctacagcagattcagataaat      1335

     151 TCAAAATTTTACAGATTTTAAACATTTAATTTTCATCAAAGATAAAAGTTAT      200
      |||||||||||||||||||.||||||||||||||||||||||
1336 tcaaaatttcacagatTTTaaacattcaatttcacaaagataaaagt      1385

201 G      201
   |
1386 g      1386

```



Wound MRSA wound isolate 1067 consensus sequence alignment with  
accession number X64389. Sequence alignment with the *Staphylococcus aureus*  
leuF-P83 gene coding the F component of leucocidin R.

```

1  ACTCGGTGGTAATGGATCCTTTATACAAAACCTACAGCTACAGCAGATTCA      50
|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.
1280 agtagatgataa-agttactttatacaaaaactacagctacagcagattca      1328

51  GATAAATTCAAATTTTCACAGATTTTAACATTTAATTTTCATCAAAGATAA      100
|||||||||||||||||.|||||||||||||||||.|||||||||||||||||.
1329 gataaattcaaaatttcacagattttaacattcaatttcacaaagataa      1378

101 AAGTTATGATAAAGATACTTTAGTACTTAAAGCTGCAGGTAATATTAAC      150
|||||||||||||||||.|||||||||||||||||.|||||||||||||||||.
1379 aagttatgataaagataacttttagtgcttaaagctgcaggtaatattaact      1428

151 CAGGTTATGAAAGACCAAACCTAAAGACTATGACTTTTCAAAAAGTGTT      200
|||||||||||||||||.|||||||||||||.|||||||||||||.
1429 caggttatgaaagaccaaatacctaaagactacgacttttcaaaaa---t      1474

201 ATATTGGGGTGTCAAAGCGAATTCATTCGTCCTGCATCAGGTCAAAGT      250
||||||||| ||| |||.|||.|||.|||.|||.|||.|||.|||.
1475 atattggggtg-caa---aatacaat-gtgtct--atcagttcacaa-t      1515

251 CAGCATTGATAGTGATTCGGTTAATCGTAGGATACAATGCACCTAAAAA      300
| |||.||||||||||||| |||.|||.|||.|||||||||||||
1516 c-----taatgattcggttaat-gtagtagactatgcacctaataa      1555

301 TCAAAATGAAGAATTTCAAGTGCAAAACACTTTAGGATACACATTTGGTG      350
|||||||||||||||||.|||||||||||||.|||.|||||||||
1556 tcaaaatgaagaatttcaagttcaaaacactttaggctatacatttggtg      1605

351 GTGACATCAGTATTACCACCTCTAATGGTTTATCTGCAAAGTGGATTAA      400
||||||||| ||| |||.||||||||| |||.|||.|||.
1606 gtgacatcagtat-----ctctaattggtttatctg---gcggacttaa      1645

401 CGGAAACACTGCATTTATCTGAAACAATTAACACAAACAAGAAAGTTAC      450
||||||||| |||.||||||||| |||.|||.|||||||||
1646 cggaaacactgc-tttttctgaaacaattaattataaacaagaaagttac      1694

451 AGAACAACCTTTAAGTCGCAACACGAGCGAATTTGAAAAATGTCGGTTGGG      500
|||||||.||||||||| |||.|||.|||||||||
1695 agaacaacattaagtcgcaaca---caaattataaaaatgtcggttggg      1740

501 GTGTAGAAGCACATAAAATTATGAATAATGGTTGGGGCCCCTCATGGCCA      550
||||||||| |||.||||||||| |||.|||.
1741 gtgtagaagcacataaaattatgaataatggttgggg-accttatgg---      1786

551 TTAAAAGATAGCTTCCATCCAACACATTATGGCAACAGTTA--TCTTCTT      598
|..||||||||| |||.|||. |||.|||. |||.
1787 --acgagatagcttccaccaaca---tatggtaa---tgaactcttct      1828

599 AGCTGGTAGACAAAGCAGTTCATA--ATG--CATTTAAAAACTTCATAGC      644
|||||||.||||||||| |||. ||| |||.
1829 agctggcagacaaagcagtgcatagctggcc-----aaaacttcatagc      1873

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645	GCAACACCAAATGCCATTATTATCTATGAAGTAACTTCAATCCAGAATTT	694
1874	gcaacaccaaagccattattatcta-gaagtaacttcaatccagaattt	1922
695	TTAAGCGTACTATCACACAGACAAGATGGCGCTAAAAAATCTAAAATTAC	744
1923	ttaagcgtactatcacacagacaagatggcgctaaaaaatctaaaattac	1972
745	AGTACACTTATCAACGTGAAATGGATTTATACCAAATTAGTCAAATCATC	794
1973	agta-acttatcaacgtgaaatggatttataccaatt-----	2009
795	CGTTG	799
2010	cgttg	2014



## **Appendix VI:**

# **TLR transfection**

**Table VI.i** Transfection mixture and concentration per well (ng/ $\mu$ l; 25 $\mu$ l well)

<sup>a</sup> Transfection mix	Transfected plasmid content (ng/ $\mu$ l)									GeneJuice ( $\mu$ l)
	<sup>f</sup> Elam-luc	<sup>g</sup> Ren-luc	CD14	MD2	TLR4	TLR2	TLR5	pCDNA3	DMEM ( $\mu$ l)	
<sup>b</sup> TLR-neg	21.6	11.8						66.8	24.7	0.3
<sup>c</sup> TLR2	21.6	11.8				22.2		44.5	24.7	0.3
<sup>d</sup> TLR4/MD2/CD14	21.6	11.8	25.3	22.0	23.1			0	24.7	0.3
<sup>e</sup> TLR5	21.6	11.8					22.2	44.5	24.7	0.3

<sup>a</sup>Contents of media added to each well for transfection; <sup>b</sup>Transfection mixture containing no TLR, used as control; <sup>c</sup>TLR2 plasmid transfection mixture; <sup>d</sup>TLR4 plasmid transfection mixture; <sup>e</sup>TLR5 plasmid transfection mixture; <sup>f</sup>Elam-luciferase plasmid; <sup>g</sup>renilla luciferase plasmid.

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