Investigation of Bacterial Virulence and Host Response in Bloodstream Infections caused by Methicillin-Resistant *Staphylococcus aureus* (MRSA) and Methicillin-Susceptible *Staphylococcus aureus* (MSSA)

Sinéad McNicholas MB BCh BAO (Hons) MRCPI FRCPath
Department of Clinical Microbiology
Royal College of Surgeons in Ireland

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Research Supervisor
Dr Deirdre Fitzgerald-Hughes

Head of Department
Prof. Hilary Humphreys
Contents

Table of contents ii
Declaration x
IP Declaration xi
Acknowledgements xii
Summary xv

Chapter I: General Introduction

1.1. Staphylococcus aureus 2
    1.1.1. Methicillin resistant S. aureus 4
    1.1.2. S. aureus bloodstream infection (BSI) 6
1.2. Genotypic characteristics of S. aureus 9
    1.2.1. Genetic structure of S. aureus 9
    1.2.2. The accessory gene regulator and other regulatory systems 11
1.3. Virulence determinants of S. aureus 13
    1.3.1. Cell surface associated virulence determinants 13
        1.3.1.1. Capsule 13
        1.3.1.2. Cell wall 13
        1.3.1.3. Surface proteins 14
        1.3.1.4. Biofilm formation 17
    1.3.2. Toxins 18
        1.3.2.1. Staphylococcal enterotoxins 18
        1.3.2.2. Toxic shock syndrome toxin-1 19
        1.3.2.3. Cytolytic toxins 20
        1.3.2.4. Exfoliative toxins 21
    1.3.3. Other important virulence factors 22
        1.3.3.1. Immune evasion 22
1.3.3.2. Arginine catabolic mobile element 23
1.3.3.3. Phenol soluble modulins 24

1.4. The host innate immune response to infection 24
1.4.1. Recognition of pathogens by the innate immune system 25
1.4.2. Antimicrobial peptides 27
1.4.2.1. The defensins 28
1.4.2.2. Cathelicidins (LL-37) 30
1.4.3. The complement system 31
1.4.4. Cytokines 32
1.4.4.1. Chemokines 35
1.4.5. Phagocytosis 36

1.5. The innate immune response and S. aureus 38
1.5.1. Recognition of S. aureus by the innate immune system 39
1.5.2. Antimicrobial peptides and S. aureus 39
1.5.3. Inactivation of complement by S. aureus 41
1.5.4. Cytokines and S. aureus 42
1.5.5. Phagocytosis and S. aureus 44
1.5.5.1. Evasion of phagocytosis 44
1.5.5.2. Production of toxins that kill leucocytes 44
1.5.5.3. Inhibition of chemotaxis 45
1.5.5.4. Inhibition of phagocytosis 46
1.5.5.5. Survival within neutrophil phagosomes 46
1.5.6. Potentiation of the immune response 47

1.6. Hypothesis & aims 48
Chapter II: Materials and Methods

2.1. Study hospitals

2.1.1. Beaumont Hospital, Dublin 51

2.1.2. Mater Misericordiae University Hospital, Dublin 51

2.2. Collection of test organisms and samples 51

2.2.1. Collection of bacterial isolates 51

2.2.2. Collection of plasma samples 52

2.3. Patient data 53

2.4. Ethical approval 53

2.5. Media and growth conditions 53

2.6. Buffers and solutions 54

2.7. Sterilisation techniques 54

2.8. Bacterial molecular genetics techniques 54

2.8.1. Isolation of bacterial genomic DNA 54

2.8.1.1. Method 1 - Wizard® Genomic DNA Purification Kit 54

2.8.1.2. Method 2 – Qiagen DNeasy® Blood and Tissue Kit 55

2.8.2. Gel electrophoresis of DNA 56

2.9. spa typing 56

2.10. DNA microarray analysis 57

2.11. Cytokine analysis 59

2.11.1. Cytokine array analysis 59

2.11.1.2. Data analysis 60

2.11.2. Enzyme-linked immunosorbant assay (ELISA) 60

2.11.3. Estimation of protein levels in plasma samples 62

2.12. S. aureus killing with the antimicrobial
peptide LL-37

2.12.1. Isolates used for LL-37 killing assay 63
2.12.2. LL-37 killing assay 64
2.13. Statistical analysis 66

Chapter III: Demographics and Clinical Details of Patients with S. aureus BSI

3.1. Introduction 68
3.2. Results 68
  3.2.1. Patient demographics 68
  3.2.2. Source of S. aureus BSI 73
  3.2.3. Empiric antimicrobial treatment 76
  3.2.4. Outcomes of S. aureus BSI 77
  3.2.5. Patient factors associated with the development of complicated BSI 78
3.3. Discussion 82
3.4. Summary 91

Chapter IV: Molecular Characterisation of S. aureus Isolates Causing BSI

4.1. Introduction 93
4.2. Results 94
  4.2.1. Molecular characterisation of S. aureus isolates causing BSI according to CC, agr type, capsule type and spa type 94
  4.2.2. Virulence and antibiotic resistance gene characterisation of S. aureus isolates causing BSI 99
  4.2.3. Genetic characteristics of S. aureus isolates causing BSI according to the main CC types 102
4.2.4. Comparison of *S. aureus* isolates causing complicated and uncomplicated BSI 105

4.2.5. Comparison of MRSA and MSSA isolates causing BSI 108

4.2.6. MRSA isolates causing BSI in renal patients over a five year period 110

4.2.6.1. Genetic characterisation of MRSA isolates causing BSI in renal patients (ST, *spa* and SCCmeC types) 111

4.2.6.2. Virulence and antimicrobial resistance gene characterisation of MRSA isolates causing BSI in renal patients 113

4.3. Discussion 115

4.3.1. Molecular characterisation of *S. aureus* isolates causing BSI 115

4.3.2. Comparison of molecular characteristics of *S. aureus* isolates causing complicated and uncomplicated BSI 118

4.3.3. Comparison of molecular characteristics of MRSA and MSSA isolates causing BSI 122

4.4. Summary 125

**Chapter V: Analysis of Circulating Immune Mediators in Patients with *S. aureus* BSI**

5.1. Introduction 127

5.2. Results 129

5.2.1. Sample collection 129

5.2.2. Preliminary selection of cytokines using
pooled patient plasma and a cytokine array

5.2.3. Cytokine levels in patient samples compared to healthy control samples

5.2.4. Variations in cytokine levels over the course of the *S. aureus* BSI

5.2.5. Comparison of the cytokine response to MSSA and MRSA BSI

5.2.6. Relationship between the types of isolates causing BSI, their virulence factors and the cytokine response

5.2.7. Relationship between cytokine levels and the outcome of *S. aureus* BSI

5.2.8. Relationship between patient characteristics and the cytokine response

5.2.8.1. Cytokine levels in patients with *S. aureus* BSI receiving immunosuppressive treatment

5.2.8.2. Cytokine levels in patients with *S. aureus* BSI and diabetes mellitus

5.2.8.3. Cytokine levels in renal patients with *S. aureus* BSI

5.3. Discussion

5.3.1. IL6, RANTES, Gro-γ and leptin, patterns of induction and roles in *S. aureus* BSI

5.3.2. Antibiotic resistance, genotype and virulence of isolates causing *S. aureus* BSI and their contribution to the cytokine response

5.3.3. Relationship between the outcome of *S. aureus* BSI and the cytokine response
5.3.4. Relationship between patient characteristics and the cytokine response

5.4. Summary

Chapter VI: Susceptibility of *S. aureus* isolates causing BSI to the antimicrobial peptide LL-37

6.1. Introduction

6.2. Results

6.2.1. Selection of isolates and concentration of LL-37 used in killing assay

6.2.2. LL-37 susceptibility of *S. aureus* isolates causing complicated and uncomplicated BSI

6.2.3. LL-37 killing of MSSA isolates versus MRSA isolates causing BSI

6.2.4. Effect of clonality on susceptibility of *S. aureus* isolates to LL-37

6.2.5. Genetic factors influencing susceptibility of *S. aureus* isolates to killing by LL-37

6.3. Discussion

6.4. Summary

Chapter VII: Conclusion and Future Research

7.1. Conclusion

7.2. Future research

Chapter VIII: Bibliography

Publications
Appendices

I. Abbreviations 234

II. Methicillin susceptibility, category of infection, year of isolation and hospital of origin of the 70 S. aureus bloodstream isolates 238

III. Category of infection and year of isolation of 36 MRSA bloodstream isolates collected from renal patients 241

IV. Data collection sheet 243

V. Approval letters from Ethics Committees 250
Declaration

I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree of Doctor of Philosophy, is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research program this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

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The contents of the enclosed manuscript are confidential and should not be disclosed, or disseminated in any way, to any third party other than to staff or students of the Royal College of Surgeons in Ireland or an external examiner appointed for the purpose of reviewing the manuscript.
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Summary

*Staphylococcus aureus* (*S. aureus*) bloodstream infection (BSI) is one of the most severe manifestations of *S. aureus* infection. The outcome of *S. aureus* BSI is variable and a number of factors contribute to the clinical course of infection and the outcome. The host innate response, bacterial virulence and patient characteristics were investigated to assess their contribution to the outcome of *S. aureus* BSI. These investigations were carried out to identify modifiable factors that may be linked to an adverse outcome. The identification of host or bacterial factors that predict a poor prognosis would facilitate the stratification of patients that may benefit from more aggressive treatment or new treatment modalities.

Patient characteristics including age, co-morbidities, presence of prosthetic devices and immunosuppressive treatment were assessed in the study population to determine the impact, if any, on the outcome of *S. aureus* BSI. *S. aureus* isolates causing BSI were characterised using a DNA microarray to determine the presence or absence of 185 virulence, antimicrobial resistance and typing genes. Isolates were assigned to sequence types using data from DNA microarray analysis combined with *spa* typing. Various components of the innate immune response to *S. aureus* were also investigated to assess their role, if any, in the course of *S. aureus* BSI. Four cytokines or chemokines were identified that were differentially regulated in pooled plasma from a selection of patients with *S. aureus* BSI (complicated versus uncomplicated infections and methicillin-resistant *S. aureus* (MRSA) versus methicillin-susceptible *S. aureus* (MSSA)) and the levels of these cytokines (IL-6, RANTES, GROγ and leptin) were determined in sequential plasma samples (day 0
and 7 following diagnosis and day 14 for complicated infections) from patients with
*S. aureus* BSI taken over the course of their infection. The data was then analysed to
establish the correlation, if any, between the cytokine response and the clinical
outcome of *S. aureus* BSI. The relationship between the bactericidal activity of the
host innate defence peptide, LL-37 towards *S. aureus* isolates and the clinical
outcome of infection was also assessed.

A number of patient characteristics were associated with the development of
*S. aureus* BSI (e.g. age over 65 years, presence of a central vascular catheter and
haemodialysis). Furthermore, an increased risk of developing a complicated BSI
was associated with certain patient characteristics (e.g. persistent fever at 72h
(*p*≤0.05)). The clinical outcome of *S. aureus* BSI appeared to be independent of the
type of infecting isolate (i.e MRSA or MSSA) and virulence gene carriage. IL-6,
GROγ, RANTES and leptin levels in the plasma of patients with *S. aureus* BSI were
independent of the molecular type of infecting isolate. RANTES levels rose
significantly in all patient groups by day seven of the BSI, suggesting a role for this
chemokine in the immune response to *S. aureus*. IL-6 levels were higher in patients
with complicated BSI while leptin levels were significantly lower in patients with
complicated BSI compared to patients with uncomplicated BSI on day seven with a
similar trend identified in samples taken on the day of diagnosis of the BSI
suggesting that an attenuated leptin response may contribute to the development of a
complicated BSI. Isolates causing uncomplicated infection appeared to be more
susceptible to killing by LL-37 than isolates causing complicated infection.
Our findings strengthen the view that the response to *S. aureus* BSI is multifactorial with the clinical outcome dependent on a number of factors, with host factors playing an important role in determining the severity of infection. A number of different components of the innate immune response appear to play a key role in the clinical course of *S. aureus* BSI, and may represent prognostic indicators for the severity of the BSI or potential targets for new treatment modalities.
Chapter I
General Introduction
1.1. *Staphylococcus aureus*

*Staphylococcus aureus* (*S. aureus*) was discovered in 1880 by the Scottish surgeon Sir Alexander Ogston, who coined the name *Staphylococcus aureus*, *Staphylococcus* from the Greek expression *staphylé*, which means ‘bunch of grapes’, and *aureus* from the Latin for golden, due to the yellow-orange appearance of the colonies (1). *S. aureus* is a member of the *Staphylococcus* genus, which are Gram-positive cocci (0.5-1.5µM in diameter). *Staphylococci* occur typically in clusters, but can be seen in pairs, tetrads or short chains. *Staphylococci* are non-motile, non-spore forming, facultatively anaerobic and typically catalase positive organisms. The genus *Staphylococcus* contains at least 40 different species, half of which are found in humans. *S. aureus* is the most virulent member of the genus *Staphylococcus* and can be differentiated from other staphylococci by coagulase production, which catalyses the conversion of fibrinogen to fibrin. However, a small number of other *Staphylococci* can also produce coagulase, such as *S. pseudointermedius* and *S. hyicus* (2). *S. aureus* is both a coloniser and a pathogen of humans. It is a ubiquitous organism which colonises a variety of different body sites in humans and animals such as the anterior nares, throat, groin and axilla. The anterior nares are thought to be the most frequent carriage site with 20-30% of the human population being persistent carriers of *S. aureus* and a further 60% carrying *S. aureus* intermittently (3-5). It is unclear why some people persistently carry *S. aureus* in the anterior nares while as many as 20% are never colonised at this site. Nasal carriage is probably due to a combination of host and organism mediated factors. The ability of particular strains of *S. aureus* to adhere to the desquamated
cells of the epithelial surface of the nasal passages may be due to the presence of certain surface proteins on the organism. Clumping factor B (ClfB), iron-regulated surface determinant (Isd) proteins, \textit{S. aureus} surface protein G (SasG), SdrC and SdrD (both involved in fibrinogen binding) have all been shown to promote adhesion to squamous cells \textit{in vitro} (6). The immune status of the host is also believed to play a role in determining carriage of \textit{S. aureus}. Work by Shuter \textit{et al} (1996) suggests that the presence of mucin lining the squamous epithelium is critical for colonisation of the anterior nares as staphylococcal protein interacts with mucin carbohydrate (7). However, more recent work suggests that hosts with reduced mucin are more likely to be carriers and that mucin enhances clearance of the organism (6). In the majority of people colonised with \textit{S. aureus}, the bacterium behaves as a harmless commensal. However, colonisation with this organism significantly increases the risk of infection in the host if their defences become compromised (3).

Virulence is defined by Dorland's Medical Dictionary for Health Consumers as the degree of pathogenicity of a microorganism as indicated by the severity of disease produced and the ability to invade the tissues of the host or as the competence of any infectious agent to produce pathologic effects. \textit{S. aureus} is a virulent organism capable of causing pathologic effects in both humans and animals resulting in a range of infections from superficial to fatal. Clinical diseases caused by \textit{S. aureus} can range from more superficial skin and soft tissue infections (e.g. carbuncles, furuncles and cellulitis) to more invasive diseases such as bone and joint infections, bloodstream infections (BSI), pneumonia, infective endocarditis and the
toxin mediated toxic shock syndrome. In most cases *S. aureus* infection is endogenous in origin with the anterior nares being the most important reservoir of *S. aureus* in humans. Over the last 25 years the incidence of *S. aureus* infection has increased (8). Between 1997 and 1999 *S. aureus* was the commonest cause of BSI, skin and soft tissue infection and pneumonia in the United States, Canada, Europe, Latin America and the Western Pacific (9). The European Staphylococcal Reference Laboratory Working Group reported that between September 2006 and February 2007 there were 2,890 invasive *S. aureus* infections reported in 26 countries across Europe (10). *S. aureus* is also one of the commonest causes of healthcare associated infections (HCAIs) and in 2005 the organism was identified as the leading cause of HCAI in the United States with related costs exceeding $14 billion per year (11-13). The 2006 four country healthcare associated prevalence survey carried out in the UK and Ireland found that MRSA was the causative organism in 15.8% of all system infections (14). *S. aureus* is a cause of medical-device related infection, and can infect a variety of prosthetic devices such as central venous catheters (CVCs), prosthetic joints and implantable cardiac devices (15).

### 1.1.1. Methicillin-resistant *S. aureus*

A number of factors have contributed to the success of *S. aureus* as a pathogen (see section 1.3) and one factor that has enhanced the virulence potential of this pathogen has been the evolution of antibiotic resistance. The first strains of methicillin resistant *S. aureus* (MRSA) were identified in England in 1961, two years after the introduction of this anti-staphylococcal penicillin (16). This organism
was also found to be resistant to all other β-lactam antibiotics, such as flucloxacillin, cefuroxime and co-amoxiclav. Other methicillin-resistant strains were soon identified in other parts of the world, including other European countries, Japan, Australia and the United States (16). However, it was not until the 1980s that MRSA emerged as a nosocomial pathogen and prevalence rates increased dramatically worldwide. MRSA has been endemic in Ireland since the 1970s, is a major public health concern, results in considerable patient morbidity and mortality, and is responsible for increased healthcare costs (17). Invasive MRSA infection has been found to have a mortality rate as high as 20% in the United States (18).

The methicillin resistance gene (mecA) is carried on a mobile genetic element, the staphylococcal cassette chromosome mec (SCCmec). SCCmec consists of the mecA gene and cassette chromosome recombinase (ccr) gene complexes, which integrates into the S. aureus chromosome. Eleven different SCCmec types (SCCmec I-XI) have been identified in MRSA to date, with different combinations of mec and ccr gene complexes. The most recently discovered SCCmecXI has highly divergent features compared to all other published SCCmec types. It has been suggested that mecA and SCC were once individual genetic elements that were transferred from coagulase negative staphylococci to S. aureus (19). The mecA gene codes for an alternative penicillin binding protein (PBP) 2a, which has reduced affinity for β-lactam antibiotics and in turn facilitates cell wall synthesis when native PBPs have been inactivated by β-lactam antibiotics (20). MRSA has emerged on multiple occasions by the independent introduction of SCCmec elements into at least five distinct epidemic MSSA genetic lineages. Therefore, the majority of
healthcare-associated MRSA (HA-MRSA) strains belong to one of five genetic lineages or clonal complexes (CCs), CC5, CC8, CC22, CC30 and CC45 that share a common ancestor (16). ST22-MRSA-IV (MRSA sequence type 22, mec type IV), the predominant HA-MRSA strain circulating in Ireland and the UK at present belongs to CC22 (21).

Community-acquired MRSA (CA-MRSA), which is increasing in prevalence worldwide, is often associated with more severe infections than HA-MRSA (13). CA-MRSA is not the result of HA-MRSA strains spreading into the community but the more recent acquisition of SCCmec elements by distinct MSSA lineages e.g. ST1-MRSA-IV (CC1), ST80-MRSA-IV(CC80) and ST5-MRSA-IV(CC5) (22).

The epidemiology of MRSA is continuously evolving as exemplified by the emergence of CA-MRSA and its spread within healthcare settings. More recent changes in the epidemiology of MRSA in Europe underpins the emergence of livestock-associated MRSA (ST398-MRSA-V) (LA-MRSA) among farmers (23,24). New challenges in the management of MRSA are also constantly being encountered with increasing rates of resistance, not only to glycopeptides but to older antimicrobials such as fusidic acid and rifampicin (25-28)

1.1.2. *S. aureus* bloodstream infection

*S. aureus* bloodstream infection (BSI) is one of the most severe manifestations of *S. aureus* infection with an estimated mortality rate as high as 20% (11). Figures from the United Kingdom (UK), report 12,500 cases of *S. aureus* BSI annually with a mortality rate of 30% (29). In Ireland between 1,200 and 1,400 *S. aureus* BSIs are reported annually (European Antimicrobial Resistance Surveillance
Network (EARS-Net) data 2011, figure 1.1) with the majority of BSIs being caused by MSSA.

![Graph showing the number of S. aureus BSIs reported by participating laboratories, the total number of MRSA BSIs, and the percentage of S. aureus BSIs caused by MRSA from 1999 to quarter three 2011.](source)

**Figure 1.1. S. aureus BSI rate in Ireland 1999-quarter one 2012**
(Source [www.HPSC.ie](http://www.HPSC.ie))

EARS-net data for Ireland, 1999-quarter one 2012. The graph shows the total number of *S. aureus* BSIs reported by participating laboratories, the total number of MRSA BSIs, and the percentage of *S. aureus* BSIs caused by MRSA from 1999-quarter three 2011.

The outcome of *S. aureus* BSI is variable. Many patients have uncomplicated infections that respond to appropriate antimicrobial therapy while others develop persistent BSI (defined as persistently positive blood cultures at three days, despite appropriate antimicrobial therapy) or complicated infections with the development of metastatic foci of infection, which may prove to be fatal. The
outcome of *S. aureus* BSI is thought to be due to a combination of factors, some of which are host-mediated and some of which are organism mediated (30). A number of studies have been carried out comparing the severity of BSI caused by MSSA and MRSA (31-36). Infections caused by methicillin-resistant isolates appear to have a worse prognosis than those caused by methicillin-susceptible isolates for a variety of reasons, such as timing of the infection, patient factors and treatment factors but not due to increased virulence of the infecting MRSA strains (31-34).

The contribution of *S. aureus* from different lineages to the progress of BSI has also been investigated. Xiong *et al* (2009) found that isolates belonging to CC30 and *spa* type 16 were more likely to cause persistent BSI (37). However, Fowler *et al* (2007) found all genotypes had the potential to cause complicated BSI but a significant association with complicated BSI was found for CC5 and CC30 isolates, with *spa* types 2 and 16 also being associated with more severe disease (38).

A number of virulence factors have also been associated with the development of complicated BSI, including resistance to host antimicrobial peptides, enhanced adhesion to fibronectin and fibrinogen, and also adhesion to endothelial cells, all of which were found to be significantly associated with the development of persistent *S. aureus* BSI (37). It has been reported that MRSA BSI caused by isolates belonging to *agr* group I were associated with a higher mortality than isolates belonging to other *agr* groups (39). Peacock *et al* (2002) investigated the presence of 33 putative virulence genes in a collection of invasive and non-invasive isolates and found that *fnbA, cna, sdrE, sej, eta, hlg,* and *ica* were significantly more common in invasive isolates than non invasive isolates (40). However, a more
detailed microarray analysis of a subset of isolates from that study did not find this association (41). Host and other factors, such as increased age, community onset, the presence of intravascular devices, a higher acute physiology and chronic health evaluation (APACHE) II score, haemodialysis (HD) and diabetes mellitus (DM) play a role in the development of more complicated infections and all have been associated with the development of persistent and complicated S. aureus BSI (30-33,39,42).

Renal patients in particular are at high risk for the development of S. aureus BSI for a variety of reasons (e.g. contact with healthcare facilities, presence of central venous catheters, impaired immunity) (31,32,42-44). Recently Albur et al (2011) reported that of 38 consecutive episodes of MRSA BSI, over a third occurred in renal patients (39). Regular contact with healthcare facilities and the presence of long-term in-dwelling vascular access devices contribute to the increased rate of S. aureus BSI among this group of patients. HD has also been found to be an independent risk factor for the development of S. aureus BSI and this may be due to the impact of HD on host immunity such as uraemia-associated phagocyte dysfunction and iron overload (32).

1.2. Genotypic characteristics of S. aureus

1.2.1. Genetic structure of S. aureus

Whole genome sequencing of a number of S. aureus strains has allowed a better understanding of the genetic basis of the virulence genes carried by S. aureus (45). The sequenced S. aureus genomes range in size from 2.813 to 2.903 Mb and
consist of a single circular chromosome and in some cases a plasmid or plasmids (11). The chromosome is thought to contain between 2,592 and 2,748 genes. The genome is made up of two major components; the conserved core genome, which is present in all isolates and the accessory genome, which is unique to particular strains (figure 1.2) (11).

The core genome contains those genes that are essential for growth and survival, but also contains virulence genes such as cell surface binding proteins, toxins, exoenzymes and capsule genes. Small variations in the sequences of these core genes, such as single nucleotide polymorphisms (SNPs), diversity within genes and operons and repeat variation can result in significant phenotypic differences between strains (46). This core genome accounts for approximately 75% of the S. aureus genome (46).

Figure 1.2. Comparison of the chromosomes of six sequenced S. aureus strains, COL, Mu50, N315, MW2, MSSA476 and MRSA252 (11). The coloured bars
separating each genome represent orthologous matches. Red lines represent orthologues in the same orientation; blue lines represent orthologues in the reverse orientation. Mobile genetic elements (MGEs) are represented by coloured boxes

The accessory genome is the variable component of the *S. aureus* genome and encodes a variety of genes with non-essential functions, such as virulence genes and drug resistance genes. Most of the accessory genome comprises of mobile genetic elements (MGEs) including bacteriophages, *S. aureus* pathogenicity islands (SaPI), staphylococcal cassette chromosomes (SCC), plasmids and transposons. Virulence genes tend to be located on bacteriophages, and SaPI while resistance genes are carried on SCCs, plasmids and transposons (11). Most horizontal gene transfer in *S. aureus* is via general transduction by bacteriophages or phage conversion (11).

1.2.2. The accessory gene regulatory system and other regulatory systems of *S. aureus*

Many of the virulence genes produced by *S. aureus* are regulated by the two component regulatory system, the accessory gene regulator (*agr*). The *agr* system is involved in quorum sensing that is dependent on bacterial population density. The *agr* locus is involved in the regulation of the expression of surface adhesins during exponential growth that switches to the expression of exoproteins during post-exponential and stationary growth phases. It consists of two divergent transcriptional units, RNAII and RNAIII, which are driven by the promoters P2 and P3, respectively (figure 1.3). RNAIII is the effector molecule of the *agr* locus,
increasing the transcription of secreted virulence factors and decreasing the expression of cell surface virulence factors (47). Four different agr complexes have been identified (agr I-IV), and some studies have associated specific agr groups with more virulent strains of S. aureus (39). This may be due to the up-regulation of virulence genes by certain agr groups.

![Figure 1.3. Organisation of the agr locus. Map of the agr locus showing the major transcripts, RNAII and RNAIII (arrows) and the open reading frames (boxes) (Adapted from Morfeldt et al (1995)).](image)

Other regulatory systems affecting the expression of virulence genes have also been described in S. aureus. Transcriptional regulators, such as sae (S. aureus exoproteins), regulate gene expression of exoproteins, and is thought to act independently of agr, while arlS (autolysis-related locus sensor) regulates autolysis and finally srrAB (staphylococcal respiratory response) regulates genes expressed in growth under microaerobic conditions (48-50). Sar (staphylococcal accessory regulator) codes for a protein, SarA that positively regulates the agr complex and expression of adhesion genes. It may also affect the expression of house-keeping genes and phage-related genes (9). S. aureus also possesses one sigma factor (SigB) which is homologous to the B. subtilis SigB (σ^B). SigB plays a role in the response to environmental factors such as temperature, energy depletion and chemical stimuli (51). The sigB operon consists of four genes (rsbU, rsbV, rsbW, and sigB) and has
been shown to participate in the transcriptional regulation of sarA, principally via sigB-dependent activation of the sarA P3 promoter (51-54). It also activates expression of adhesins such as coagulase and fibronectin-binding proteins and down-regulates the production of exoproteins and toxins in the stationary phase (51,55).

1.3. Virulence determinants of *S. aureus*

*S. aureus* is a virulent organism and its ability to colonise and its transmissibility contribute to the success of this pathogen. Virulence factors enable an organism to replicate and disseminate within a host in part by subverting or eluding host defenses (56). The expression of virulence factors that have a wide variety of functions contributes to the development of severe and often fatal disease. These virulence factors allow *S. aureus* to adhere to various host tissues, evade phagocytosis and other components of the immune system, directly destroy phagocytic cells, promote iron uptake and induce symptoms of septic shock (57).

1.3.1. Cell surface associated virulence determinants

1.3.1.1. Capsule

*S. aureus* isolates possess a capsule, which is composed of polysaccharide. To date 11 serotypes have been reported but approximately 80-90% of human *S. aureus* isolates are capsular type 5 and type 8 (58,59). Polysaccharide capsules are anti-phagocytic, are immunogenic and play a key role in the pathogenesis of *S. aureus* infection (58)
1.3.1.2. Cell wall

The cell wall of *S. aureus* is composed of peptidoglycan, teichoic acids and various surface proteins. Peptidoglycan is the major component of the cell wall and consists of glycan chains made of *N*-acetylglucosamine and *N*-acetylmuramic acid disaccharide subunits linked by a β-1-4 glycosidic bond. The *N*-acetylmuramic acid moiety is linked to a highly conserved peptide stem –L-alanine-D-isoglutamine-L-lysine-D-alanine (60). Teichoic acid is composed of polyribitol-phosphate polymers cross-linked to *N*-acetylmuramic acid residues of peptidoglycan (60). Lipoteichoic acids are the plasma membrane bound form of teichoic acid. Various surface proteins, including microbial surface component recognising adhesive matrix molecules (MSCRAMMs) also form part of the cell wall (60).

The cell wall of *S. aureus* is a dynamic semi-rigid structure that has a number of functions. It plays a role in the pathogenicity of *S. aureus* both directly and indirectly, it serves as an anchor for the surface adhesins, its components are recognized by the immune system and both peptidoglycan and lipoteichoic acid can induce pro-inflammatory components of the innate immune system, such as cytokines (61-64). Alterations in the structure of the cell wall can also result in antimicrobial resistance, which in turn can increase the pathogenic potential of the organism (13).

1.3.1.3. Microbial surface component recognising adhesive matrix molecules
The ability of *S. aureus* to adhere to the host extra-cellular matrix is a key step in the pathogenesis of infection. *S. aureus* possess numerous MSCRAMMs which are proteins that contain a signal sequence directing their secretion and the LPXTG motif. The LPXTG motif is cleaved by Sortase enzymes (SrtA and SrtB) allowing the proteins to be covalently attached to peptidoglycan. Protein A, fibronectin binding protein, collagen binding protein and clumping factor A are examples of MSCRAMMs that allow the organism to adhere to a variety of host proteins such as collagen, fibrinogen and fibronectin (5,65).

Protein A, a surface-bound protein of *S. aureus* is encoded by the *spa* gene. Protein A binds immunoglobulin, inhibits phagocytosis, binds von Willebrand factor, and plays a role in adherence and induction of endovascular diseases (60,66). It is an important virulence factor and mutants that do not possess this surface protein have been shown to be less virulent (66). Other important MSCRAMMs of *S. aureus* and their main function are listed in Table 1.1.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>bbp</td>
<td>Bone sialoprotein-binding protein</td>
<td>Binds to sialoprotein</td>
</tr>
<tr>
<td>clfA</td>
<td>Clumping factor A</td>
<td>Binds to fibrinogen</td>
</tr>
<tr>
<td>clfB</td>
<td>Clumping factor B</td>
<td>Binds to fibrinogen and cytokeratin</td>
</tr>
<tr>
<td>cna</td>
<td>Collagen-binding adhesion</td>
<td>Binds to collagen</td>
</tr>
<tr>
<td>ebh</td>
<td>Cell wall associated fibronectin-binding protein</td>
<td>Binds to fibronectin</td>
</tr>
<tr>
<td>eno</td>
<td>Enolase (phosphopyruvate hydratase)</td>
<td>Catalysis of the conversion of 2-phosphoglycerate (2-PG) to phosphoenolpyruvate (PEP)</td>
</tr>
<tr>
<td>fib</td>
<td>Fibrinogen binding protein</td>
<td>Binds to fibrinogen</td>
</tr>
<tr>
<td>ebpS</td>
<td>Cell wall associated fibronectin-binding protein</td>
<td>Binds to fibronectin</td>
</tr>
<tr>
<td>fnbA</td>
<td>Fibronectin-binding protein A</td>
<td>Binds to fibronectin</td>
</tr>
<tr>
<td>fnbB</td>
<td>Fibronectin-binding protein B</td>
<td>Binds to fibronectin</td>
</tr>
<tr>
<td>map</td>
<td>Major histocompatibility complex class II analog protein</td>
<td>Binds to monomeric matrix macromolecules</td>
</tr>
<tr>
<td>sdrC</td>
<td>Serine-aspartate rich fibrinogen-binding, Bone sialoprotein-binding protein C</td>
<td>Binds to fibrinogen</td>
</tr>
<tr>
<td>vwb</td>
<td>Willebrand factor - binding protein</td>
<td>Binds to Willebrand factor</td>
</tr>
<tr>
<td>sasG</td>
<td>S. aureus surface protein G</td>
<td>Binds to nasal mucosal cells</td>
</tr>
</tbody>
</table>

The presence of the genes encoding specific surface adhesins (e.g. fibrinectin-binding protein B (FnBPB), collagen binding adhesion) has been associated with the development of certain types of invasive infections (40). Mutant strains deficient in clumping factor A have been shown to be less virulent than wild
type strains (60). Isolates producing collagen binding adhesin have been linked to bone and joint infection and keratitis (67-69).

1.3.1.4. Biofilm formation

A biofilm is an integrated community of organisms embedded in an extracellular matrix that they have produced, reactive to their environment and to each other (70). Biofilms are composed mainly of polysaccharide or protein and can form on biotic (e.g. host tissue) and abiotic surfaces (e.g. central venous catheters (CVCs)) (71). The formation of staphylococcal biofilms, in the majority of cases, is dependent on expression of the genes of the intercellular adhesion (ica) operon. The ica operon contains four genes icaADBC that encode the biosynthetic enzymes involved in the production and exportation of an extracellular polysaccharide known as polysaccharide intercellular adhesin (PIA) (71). Polymeric N-acetyl glucosamine (PNAG) is another polysaccharide adhesin produced by S. aureus which plays a role in the formation of biofilms (72). ica-independent biofilm production has also been observed and is probably due to various proteins such as accumulation-associated protein (AAP), the clumping factor A (ClfA), the staphylococcal surface proteins SasG, protein A, SasC and the biofilm-associated protein (Bap) (71,73). The fibronectin binding proteins FnBPA and FnBPB and the major autolysin (Atl) are also responsible for icaADBC-independent biofilm production (73,74). Members of the phenol soluble modulin (PSM) peptide family are also believed to play a role in biofilm production (75) Organisms contained within biofilms are more difficult to
detect and are resistant to standard concentrations of antibiotics (60). The biofilm structure also protects the bacteria within from phagocytosis and other components of the innate immune response. The importance of biofilm formation in the pathogenesis of *S. aureus* infection has not been fully elucidated. However, it plays a major role in the development of device-related infections such as those involving CVCs, prosthetic heart valves and implantable cardiac devices (76).

1.3.2. Toxins

*S. aureus* is a potent producer of toxins with a wide variety of functions. However, there is some degree of redundancy and overlap between them. Many *S. aureus* toxins have potent effects on the immune system but also have other important functions. These toxins play a key role in the pathogenesis of a number of infections.

1.3.2.1. Staphylococcal enterotoxins

The staphylococcal enterotoxins (SEs) are primarily associated with gastroenteritis. The SEs, are superantigens. Superantigens bind the major histocompatibility complex (MHC) class II protein on the surface of antigen-presenting cells and link it to T-cell receptors on the surface of T helper cells (66). This in turn can result in activation of up to 30% of the body’s T-cells and an excessive release of cytokines that can result in capillary leakage, epithelial damage and hypotension, and ultimately cause an overwhelming sepsis (60,66). To date, more than 20 SEs (including SEA, B, C, D, E, H, J, K, L, Q, R and the *egc* cluster...
SEG, I, M, N, O, U) have been identified. SEA, SEB and SEC are most frequently associated with the development of gastroenteritis. Once ingested, these enterotoxins generate visible pathological lesions in the stomach and small intestine. These consist of a hyperaemic mucosa with neutrophilic infiltrates in the epithelium and lamina propria, resulting in the rapid onset of vomiting and occasionally diarrhoea; SEs are capable of causing disease in the absence of the producing organism (77,78). SEC and SEB can also cause a non-menstruation-associated toxic shock syndrome (77). Expression of SEs is regulated by the *agr* system and they are typically found on MGEs (77). The clinical role of such a large number of enterotoxins is not clearly understood. However, the genes encoding the SEs are highly prevalent among *S. aureus* strains and the presence of certain SE genes (e.g. the *egc* cluster) may have a negative correlation with the severity of infection (78).

1.3.2.2. Toxic shock syndrome toxin-1

Toxic shock syndrome toxin-1 (TSST-1) has superantigenic activity and causes toxic shock syndrome, which is characterised by high fever, a diffuse erythematous rash, hypotension, multi organ failure and desquamation of the skin, one to two weeks after the onset of the illness and has a case fatality rate of 5% (77). Toxic shock syndrome was first identified in the 1970s among children, but a significant increase in cases was identified in the 1980s among women using tampons (79,80). The association of toxic shock syndrome with tampon use reflects the ability of the toxin to cross mucosal surfaces (77). The incidence of menstruation-associated and non-menstruation-associated toxic shock syndrome has
decreased in recent years. TSST-1 is encoded by \textit{tst} and this gene is found in many colonising and invasive strains of \textit{S. aureus} \cite{77}. However, toxic shock syndrome is relatively rare. A number of factors may predispose to the occurrence of this disease such as a lack of specific antibodies to the toxin or favourable environmental conditions for toxin production \cite{77}. 
1.3.2.3. Cytolytic toxins

Cytolytic toxins can disrupt the membrane of host cells, causing leakage of cellular contents and ultimately cell lysis (66). *S. aureus* produces a number of different cytolytic toxins, including haemolysins and leucotoxins. The haemolysins (α-haemolysin, β-haemolysin, γ-haemolysin and δ-haemolysin) are cytolytic to erythrocytes and other host cells. The haemolysins, present in most *S. aureus* isolates, are chromosomally located and regulated by the *agr* complex. Alpha-haemolysin is also dermonecrotic, neurotoxic, can lyse human platelets, monocytes and lymphocytes, and can cause influx of neutrophils and neutrophil adhesion (13,60,77). This toxin is lethal when injected into rabbits intravenously (60,77). Alpha-haemolysin is also a key virulence determinant of CA-MRSA. Gamma-haemolysin is an example of a bicomponent leucotoxin which is composed of two subunits that are secreted separately and then assembled in the membrane of leucocytes (77). There are four different bicomponent leucotoxins, γ-haemolysin, Panton-Valentine leucocidin (PVL) toxin, leucocidin E/D and leucocidin M/F-PV-like. Gamma-haemolysin, produced by the vast majority of *S. aureus* strains, is made up of two non-associated secreted proteins, the S (slow-eluting proteins) and F (fast-eluting proteins) components. Together, they are known to lyse neutrophils and macrophages as well as erythrocytes (77).

PVL is a notable bicomponent cytolytic toxin produced by *S. aureus*. PVL is encoded by *lukS/lukF-PV* genes which code for two proteins, LukS-PV and LukF-PV, that are located on a bacteriophage in 1-2% of strains (6). The main function of PVL toxin is to cause lysis of leucocytes, but it can also activate leucocytes to
produce chemokines and cytokines, such as IL-8 (13). The presence of this toxin has been associated with the development of skin abscesses and necrotizing pneumonia and it also has a strong association with CA-MRSA strains, particularly the USA300 (ST8-MRSA-IVa) clone, which is the predominant CA-MRSA clone circulating in the USA at present (81). Initially, it was believed that PVL toxin was the main virulence factor of CA-MRSA and was responsible for the severe phenotype of CA-MRSA causing infection in otherwise healthy individuals (82). However, many strains of CA-MRSA are negative for this toxin and can still cause severe disease. In recent years there have been many contradictory findings in relation to the role of PVL in disease caused by CA-MRSA (13,18,81,83-85). Some studies suggested that PVL did not contribute to the virulence of CA-MRSA at all (84). However, recent work has illustrated that PVL toxin is responsible for some of the clinical manifestations of CA-MRSA infection (86). It is now believed that a combination of virulence factors is responsible for the severe infections caused by CA-MRSA, including phenol-soluble modulins (PSMs), α-toxin and PVL (18,46,83).

1.3.2.4. Exfoliative toxins

*S. aureus* strains can produce three exfoliative toxins A, B and D. These toxins are associated with the development of staphylococcal scalded skin syndrome (SSSS) and bullous impetigo. SSSS is the generalised form of the condition and was first described in 1878 by the German physician Baron Gottfried Ritter von Rittershain (87). It typically occurs in neonates and infants less than one year of age (87). Patients present with generalized scalding of the skin with blister formation.
and systemic symptoms such as fever and lethargy. Patients usually make a full recovery and the mortality rate is less than 5% (88). Bullous impetigo is the localized form of the disease and it usually occurs due to localized spread of the toxin in a wound of individuals who have some immunity to the toxin. The exact mechanism of action of these toxins is not fully understood although it is known that they mediate their effects at the level of the strata granulosum of the epidermis (73). The genes encoding these toxins, *eta*, *etb* and *etd* are located on MGEs and the proportion of *S. aureus* strains carrying these genes is relatively low, i.e. 0-2% of isolates (88).

1.3.3. Other important virulence factors

1.3.3.1. Immune evasion

The ability of *S. aureus* to evade the host immune response contributes greatly to the virulence of this organism. Some of the important genes that contribute to *S. aureus* immune evasion are listed in Table 1.2 along with their gene product and main function. These virulence factors provide *S. aureus* with the capacity to directly inactivate components of both the innate and acquired immune response (e.g. α-haemolysin, PSMs) and evade recognition by the immune system (e.g. protein A, capsular polysaccharide). The mechanism of action of these virulence factors are described in detail in Section 1.5.
Table 1.2. Virulence factors involved in immune evasion

<table>
<thead>
<tr>
<th>Gene</th>
<th>Product</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>mprF (multiple peptide resistant factor)</td>
<td>Lysylphosphatidylglycerol (L-PG) synthase</td>
<td>Incorporates lysine into bacterial cell membrane</td>
</tr>
<tr>
<td>sak</td>
<td>Staphylokinase</td>
<td>Inactivates defensins and complement system</td>
</tr>
<tr>
<td>chp</td>
<td>Chemotaxis inhibitory protein (CHIPS)</td>
<td>Inhibits complement system, chemotaxis and phagocytosis</td>
</tr>
<tr>
<td>scn</td>
<td>Staphylococcal complement inhibitor (SCIN)</td>
<td>Inhibits complement system</td>
</tr>
<tr>
<td>aur</td>
<td>Aureolysin</td>
<td>Inactivates LL-37</td>
</tr>
<tr>
<td>eap</td>
<td>Extra-cellular adherence protein</td>
<td>Inhibits neutrophil adhesion</td>
</tr>
<tr>
<td>ssl-5</td>
<td>Staphylococcal super-antigen like protein 5</td>
<td>Inhibits neutrophil adhesion</td>
</tr>
<tr>
<td>ssl-7</td>
<td>Staphylococcal super-antigen like protein 7</td>
<td>Inhibits complement system, chemotaxis and phagocytosis</td>
</tr>
<tr>
<td>ssl-10</td>
<td>Staphylococcal super-antigen like protein 10</td>
<td>Inhibits complement system</td>
</tr>
<tr>
<td>katA</td>
<td>Catalase</td>
<td>Detoxifies hydrogen peroxide</td>
</tr>
</tbody>
</table>

1.3.3.2. Arginine catabolic mobile element

The arginine catabolic mobile element (ACME) ranges in size from 31 kb to 34 kb and is a MGE linked to SCCmecIV and is prevalent among ST8-MRSA-IVa (USA300) isolates. It is also seen in a number of other MRSA clones and was most likely acquired from S. epidermidis (89). ACME is thought to play a key role in the growth and survival of MRSA strains that contain this element (89). It consists of two main gene clusters, an arginine deiminase (arc) and an oligopeptide permease (opp) operon (13). Arginine deiminase produces ammonia and ATP and facilitates colonisation of the skin by neutralising the acidic environment of the skin (15). It
may also inhibit production of nitric oxide, which is required for neutrophil killing of *S. aureus* (13). Although ACME is not a virulence factor *per se*, it does allow MRSA to persist on human skin enhancing colonisation and transmissibility (13,89).

1.3.3.3. Phenol soluble modulins

Phenol soluble modulins are produced by MRSA but not MSSA. PSMs are thought to be virulence factors of CA-MRSA as these lineages produce more PSMs than HA-MRSA strains. In particular USA300 and USA400, the predominant CA-MRSA strains circulating in the USA at present that are associated with severe infections have been shown to have increased expression of α-type PSMs compared to HA-MRSA strains (81). PSMs are a group of protein toxins that are soluble in phenols. Alpha-type PSMs have cytolytic activity and can lyse neutrophils, erythrocytes and monocytes, while β-type PSMs lack cytolytic activity (13). All PSMs have pro-inflammatory effects such as activation and induction of chemotaxis and induction of cytokine release (13). PSMs are important virulence factors of CA-MRSA and mutants deficient in α-type PSM are not as pathogenic as wild type organisms (13). The strength of evidence supporting the importance of PSMs as a potent virulence factor of CA-MRSA is increasing particularly in light of new evidence disputing the importance of PVL toxin as a virulence factor (18,83).

1.4. The innate immune response to infection

The immune response to infection is divided into the innate and adaptive (acquired) immune responses. The adaptive immune response depends on the
recognition of specific antigens by B and T lymphocytes resulting in the formation of antibodies, which are highly specific for the stimulating antigen. The adaptive immune response is specific and has the ability to remember pathogens if they are encountered at a future time. However, it takes a number of days for the adaptive immune response to become fully active and begin pathogen eradication (63). The innate immune system is the first line of defence against infection and provides a rapid response to pathogens encountered by the human host. The importance of the innate immune response to infection was not fully appreciated in the past as it lacked specificity (61). However, recent research has improved our understanding of innate immunity and the protective power of its role in combating infection is only now being truly recognised (90).

1.4.1. Recognition of pathogens by the innate immune system

The innate immune system has the ability to recognise pathogens and to distinguish them from host cells and commensal organisms. It does this by detecting conserved microbial motifs or pathogen-associated molecular patterns (PAMPs) that are not present in host cells (64). Examples of PAMPs include lipopolysaccharide (LPS), peptidoglycan, lipoteichoic acids (LTA) and cell-wall lipoproteins. PAMPs are recognised by pattern-recognition receptors (PRRs), which are germ-line encoded recognition receptors that can be expressed by macrophages, dendritic cells, mast cells, neutrophils, eosinophils and natural killer (NK) cells (91). Recognition of PAMPs by PRRs leads to a signalling cascade that ultimately effects gene expression of immunomodulatory factors, such as cytokines and chemokines that are
capable of opsonisation, activation of complement and coagulation cascades, phagocytosis and induction of apoptosis (91). PRRs are expressed on cell surfaces, in intracellular compartments and can be secreted into the bloodstream and tissue fluids (64). A number of distinct classes of PRRs are known to exist including Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (Nod) like receptors (NLRs), C-type lectins (e.g. dectin 1), and secreted PRRs such as collectins (e.g. mannan-binding lectin), ficolins and pentraxins (e.g. C-reactive protein (CRP)) (91).

TLRs are transmembrane receptors, with an extracellular domain that contains leucine-rich repeating units and a cytoplasmic signalling domain (92). To date between 10 and 15 different TLRs have been identified in mammalian species (93). Certain microbial PAMPs will bind to specific TLRs (e.g. Gram-positive cell wall components bind mainly to TLR2, Gram-negative LPS activates TLR4, bacterial flagellin activates TLR5) (61). Intracellular signalling following attachment of a ligand to a TLR has been well described, and varies for each of the TLRs. Binding of a ligand to a TLR results in recruitment of an adaptor molecule such as MyD88, Mal (MyD88 adaptor-like, also known as TIRAP), Trif (Tir-related adaptor protein inducing interferon) or Tram (Trif-related adaptor molecule) resulting in activation of a transcription factor such as nuclear factor κB (NF-κB), MAP kinases, such as p38 and Jun amino-terminal kinase (JNK) and interferon regulatory factors (IRF)3 and IRF7 (94). Once activated these transcription factors act as promoters for a number of genes for a variety of cytokines such as TNF-α, IL-1, IL-6, IL-8 and IFNβ (64).
The NLRs are intracellular receptors that are capable of recognising bacterial and viral pathogens (91). The NLRs all consist of a similar NOD domain and a leucine rich repeat domain at the carboxy terminus, with one of three domains being found at the amino terminus, resulting in three subfamilies of NLRs (91). The NLRs have a diverse range of functions including recognition of peptidoglycan (NOD1 and NOD2), resulting in the production of pro-inflammatory cytokines and neutrophil recruitment (63).

A variety of other PRRs have recently been characterised, such as C-type lectins, which are transmembrane receptors that bind to β-glucan and play a role in antifungal defence, collectins, pentraxins and ficolins which activate acute phase proteins and the complement system resulting in opsonisation and lysis of pathogens (91).

1.4.2. Antimicrobial peptides

The skin and the mucous membranes are important mechanical barriers against infection. The epithelial cells lining these structures produce a wide variety of peptides which have both antimicrobial properties and an immunomodulatory role. Lysozyme is a bacteriolytic enzyme released by epithelial cells and neutrophil granules which can cleave peptidoglycan polymers in the bacterial cell wall and plays an important role in the host defence against Gram-positive organisms (61).

Lactoferrin is an iron binding glycoprotein, which is produced during lactation and by epithelial cells at mucosal surfaces. Lactoferrin has both antimicrobial and immunomodulatory properties. The high affinity of lactoferrin for
iron deprives organisms of essential iron resulting in bacteriostasis. Lactoferrin also has a direct bactericidal effect on some Gram-negative and Gram-positive bacteria that cannot be attributed to simple iron deprivation (95). Lactoferrin has been shown to modulate the immune response to infection by decreasing tumor necrosis factor (TNF)-α and interleukin (IL)-1β production and has both stimulatory and inhibitory effects on myelopoiesis (95).

Antimicrobial peptides are one of the major defence mechanisms against microbial infection, and play a key role in the innate immune response to infection (96,97). These peptides are found in a wide variety of animals and plants. Antimicrobial peptides found in humans are generally positively charged allowing them to interact with the negatively charged (anionic) bacterial cell surface and are known as cationic antimicrobial peptides (CAMPs) (98). A number of different CAMPs have been identified in humans such as the defensins, the cathelicidin LL-37, the thrombocidins and the histatins (99). The defensins and LL-37 are probably the most important CAMPs in humans.

1.4.2.1. The defensins

There are three types of defensins, α-, β- and θ-defensins which are all cysteine rich peptides (100). The α- and β-defensins are found in humans and differ in terms of their structure and location (100).

Six different α-defensins or human neutrophil peptides (HNPs) have been identified to date and these are found in the granules of neutrophils and in Paneth cells, which are host defence cells found in the small intestine. In neutrophils, the
defensins are found in azurophil granules that fuse with phagocytic vacuoles during the phagocytosis of micro-organisms, exposing them to high concentrations of HNPs. HNPs have antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi and viruses. HNPs also have a number of other biological functions such as degranulation of mast cells, regulation of the complement cascade, chemotaxis of T cells and dendritic cells, and inhibition of glucocorticoid production (100).

Four different human β-defensins (hBD1-4) are found in a variety of epithelial tissues, including the respiratory tract, the skin, the urogenital tract, the intestine and in monocytes and dendritic cells (100). hBD1 and hBD2 have strong antibacterial activity against Gram-negative bacteria with little or no activity against Gram-positive organisms, while hBD3 and hBD4 are active against both Gram-negative and Gram-positive organisms. Like the α-defensins, the hBDs also have other biological activities such as chemotaxis of dendritic cells, degranulation of mast cells and induction of prostaglandin production (100).

Defensins increase the permeability of the cytoplasmic membrane of microorganisms resulting in cell death (100). The positively charged antimicrobial peptide binds electrostatically to the negatively charged bacterial cell structure, e.g. lipopolysaccharide (LPS) in Gram-negative bacteria, lipoteichoic acid and teichoic acid in Gram-positive bacteria. The antimicrobial peptides cause the formation of a pore in the cell membrane causing cytoplasmic contents to leak out of the cell causing death. The production of defensins may be constitutive or it may be induced in response to bacterial products (e.g. LPS) through TLR 2 or 4 on epithelial cells or
through stimulation of epithelium by cytokines, including IL-1 or TNF-α (61). The HNPs found in neutrophil granules are produced constitutively, while those produced by paneth cells may be stimulated by the presence of microorganisms. TLRs are thought to play a key role in recognition of these stimuli and induction of defensin production (101).

1.4.2.2. Cathelicidins (LL-37)

Only one cathelicidin is found in humans, LL-37 or hCAP18 and it plays a number of important roles - protecting skin and epithelial surfaces from invading organisms, directly killing phagocytosed bacteria and immunoregulation. LL-37 is a cationic polypeptide found in a variety of cells including neutrophils, monocytes, epithelial cells, salivary glands and sweat glands (102). It has antimicrobial activity against Gram-positive organisms, Gram-negative organisms, fungi and viruses. LL-37 causes cell death by a mechanism similar to the defensins as it can also increase the permeability of the cell through the formation of pores in the cell membrane thus allowing the cytoplasmic contents to leak out of the cell. LL-37 is not only bacteriocidal, but it can bind to LPS, neutralize its endotoxin activity and can also act as a chemotactic agent for neutrophils, monocytes, T cells and mast cells. LL-37 has also been shown to play a role in wound healing and angiogenesis (103,104). Production of LL-37 is induced by bacteria, bacterial components (e.g. LPS and peptidoglycan), other CAMPs and cytokines, with TLRs acting as pattern recognition molecules that stimulate the internal cell signalling pathways (102,105).
Many organisms including *S. aureus* have shown reduced susceptibility to LL-37, allowing them to evade this important component of the innate immune system.

1.4.3. **The complement system**

The complement system has many functions and plays a role in both the innate and acquired immune response to infection. However, it is not adaptable nor does it exhibit memory like other components of the adaptive immune response. The complement system consists of at least 30 proteins found in serum or on the surface of cells (61). Activation of the complement system at the microbial surface results in the activation of a C3 convertase that cleaves C3 to form C3b, which either binds to the microbe and acts as an opsonising agent, or activates C5 and the remainder of the complement cascade (61). The complement system or cascade can be activated by one of three pathways – the classical pathway, the mannan-binding lectin (MBL) pathway or the alternative pathway.

The classical pathway is activated when an antigen binds to IgG or IgM allowing the antibody to bind to C1q resulting in the formation of C4b2a which is the classical pathway convertase which promotes cleavage of C3 into C3a and C3b. The classical pathway is the only one dependant on antibodies for activation. The MBL pathway has only recently been recognised and is similar to the classical pathway but does not involve antibodies. The binding of lectin to mannose-containing carbohydrates on the surface of microbes results in the activation of C4 resulting in the binding of C4b and C2a and the formation of C4b2a, the classical pathway convertase that cleaves C3 into C3a and C3b. The alternative pathway is
activated following hydrolysis of C3, which is then converted to C3 convertase (C3bBb), which then promotes cleavage of C3 into C3a and C3b. C3a induces production of granulocytes and can act as an anaphylotoxin causing the release of histamine from mast cells and basophils resulting in an increase in vascular dilation and permeability. C3b can opsonize pathogens facilitating phagocytosis, remove immune complexes and cause cleavage of C5 producing C5a and C5b. C5a acts as a chemotactic agent for monocytes, neutrophils and eosinophils and also increases phagocytic activity. Like C3a, C5a can also act as an anaphylotoxin. C5b remains at the cell surface and recruits and assembles C6, C7, C8 and multiple C9 molecules to assemble the membrane attack complex (MAC). The MAC can create a hole or pore in cell membranes resulting in cell lysis.

The main effector functions of the complement pathways can be summarized as follows:

- Opsonization - enhancing phagocytosis of antigens.
- Chemotaxis - attracting macrophages and neutrophils.
- Cell lysis - rupturing membranes of foreign cells.

1.4.4. Cytokines

Cytokines play a central role in the innate and adaptive immune responses to infection. Cytokines are immunomodulating agents, a heterogenous group of soluble small polypeptide or glycoprotein mediators (ranging in size from approx. 8-45kDa) that form part of a complex network that regulate the immune and inflammatory responses (61). Several cytokines have been identified to date such as
the ILs, interferons (IFNs), growth factors and the chemokines (106). Monocytes and macrophages are the main source of cytokines involved in the pathogenesis of septic shock (61). However, the majority of cells involved in the immune system (as well as other host cells e.g. epithelial cells) can both produce and respond to cytokines via specific cytokine receptors thus allowing cytokines to have a role in both the innate and adaptive immune responses (107). Cytokines often have multiple effects depending on the stimulus for production and the cell from which they are produced, and they have a variety of effects on a large number of cell types with considerable overlap and redundancy between them. They are also involved in a complex positive and negative feed-back system which allows self-regulation and regulation of the expression of other cytokines (106). Cytokines may be pro-inflammatory (e.g. IL-1, IL-4), anti-inflammatory (e.g. IL-10) or may have both pro- and anti-inflammatory functions (e.g. IL-6) (108,109).

Certain cytokines play a pivotal role in the initiation of sepsis, which is the systemic response to infection. Tumour necrosis factor-alpha (TNF-α) and IL-1 are the first two cytokines produced during a septic episode and are pro-inflammatory cytokines (61). When both of these cytokines are injected into animals they produce the same cellular effects as bacterial infection, despite the absence of microorganisms (61). TNF-α is produced predominantly by mononuclear phagocytes, but can also be produced by other cells such as neutrophils, lymphocytes, natural killer cells and endothelial cells. The major inducer of TNF-α production by monocytes is LPS, which binds to TLR4. TNF-α has potent cytotoxic effects on malignant cells, induces the development of adhesion molecules on
endothelial cells (e.g. ICAM-1, VCAM-1), which allow for the egress of white cells to sites of inflammation, activates neutrophils and induces the production of other cytokines. TNF-α is also responsible for the cachexia associated with malignancy and chronic infections and plays a major role in the pathogenesis of septic shock (61,107). TNF-α has been shown to peak early in patients with septic shock (110).

The IL-1 family consists of four different peptides – IL-1α, IL-1β, IL-1 receptor antagonist (IL-1ra) and IL-18 (107). IL-1α, IL-1β have similar functions and are pro-inflammatory cytokines, while IL-1ra acts as a cytokine antagonist and has anti-inflammatory effects (107). IL-1 is produced primarily by mononuclear phagocytes, but can also be produced by many other cell types such as endothelial cells, neutrophils and keratinocytes. Endotoxin, other cytokines and microorganisms can stimulate the production of IL-1 (61,107). IL-1 activates both B and T lymphocytes and stimulates antibody production and is also responsible for many of the clinical features associated with sepsis, such as fever, lethargy and anorexia (107). Production of acute phase proteins such as CRP and complement is induced by IL-1 interaction with hepatocytes, and IL-1, like TNF-α stimulates the production of endothelial cell adhesion molecules allowing adherence of leucocytes. TNF-α and IL-1 have many similar functions, although TNF-α does not have any effect on lymphocyte proliferation. They act synergistically as part of the immune response to infection and both can induce the production of other potent pro-inflammatory cytokines, such as IL-6 (61).

IL-17 is produced by a unique family of lymphocytes, T\textsubscript{H}17 cells and induces T-cell mediated immune responses to extracellular pathogens such as \textit{S. aureus}. IL-
17 has been shown to induce expression of a variety of cytokines and chemokines, including IL-6, IL-11, GM-CSF, CXCL8, CXCL10 and TGF-β which play a role in fibroblast activation and neutrophil recruitment (111). Increased susceptibility to *S. aureus* infection in IL-17-deficient mice suggests a key role for this cytokine in the immune response to *S. aureus* infection (111).
1.4.4.1. Chemokines

The chemokines are a group of small (8-12 kDa) cytokine-like polypeptides, whose major function is leucocyte chemotaxis (61). The chemokines are a homologous group of polypeptides that act as ligands for G protein-coupled, 7-transmembrane segment receptors (61). The chemokines can be grouped into four families based on the positioning of the N-terminal cysteine residues:

- CXC subfamily (characterized by separation of the first two cysteines by a variable amino acid)
- CC subfamily (characterized by the cysteine residues being adjacent to each other)
- C subfamily (characterized by the first and third cysteine residues being absent and retaining a single conserved residue)
- CX3C subfamily (characterized by three variable amino acids separating the N-terminal cysteine residues) (107).

To date 52 chemokines and 20 chemokine receptors have been identified, with the majority of chemokines belonging to the CXC and the CC subfamilies (111).

Inflammatory (inducible) chemokines are produced by many different cell types and are typically produced at a site of infection or inflammation. However, homeostatic (housekeeping) chemokines are produced in specific tissues or organs (112). Inflammatory chemokines are typically produced by inflamed cells following stimulation by a pro-inflammatory cytokine (e.g. IL-1) or infiltration by an infectious agent (107). Homeostatic chemokines are produced in lymphoid tissue or in non-lymphoid tissue such as the skin or mucosa (112). At least one type of
chemokine can bind to all cell types involved in the immune response. Inflammatory chemokines direct leucocytes to sites of inflammation and can induce chemotaxis of a variety of cells including neutrophils, monocytes, lymphocytes, eosinophils, fibroblasts and keratinocytes (107). The homeostatic chemokines play a role in the adaptive immune response to infection and are involved in haemopoiesis, antigen sampling and immune surveillance (112).

1.4.5. Phagocytosis

The primary cellular mechanism of the innate immune response is the phagocytosis of infecting organisms by polymorphonuclear (PMNs) cells, such as neutrophils which kill infecting organisms. Macrophages, monocytes and NK cells (lymphoid cells) are also involved in phagocytosis (92). NK cells are chiefly active against virally-infected cells. Once a bacterial infection has become established the PMNs take the following three steps:

(i) Migration to the site of infection
(ii) Recognition and phagocytosis of the infecting organism
(iii) Intracellular killing and digestion of the organism

(i) Migration to the site of infection

PMNs are attracted to the site of infection by a variety of chemoattractant molecules such as C3a, C5a, formylated peptides (secreted by bacterial cells), IL-8, GROγ and CXCL6 (18,66). Many of these molecules activate the endothelial cells
of the small vessels, slowing the PMNs, referred to as ‘slow-rolling’, and they also increase the binding avidity of the PMNs to ICAM-1 on the surface of the endothelial cells. This allows trans-endothelial migration of the PMNs allowing the PMNs to migrate from the blood to the site of infection (61).

(ii) Recognition and phagocytosis of the infecting isolate

Typically phagocytosis occurs following opsonisation of the infecting organism with IgG, C3b and occasionally IgA. Phagocytosis can also occur without opsonisation but killing is more efficient if the organism has been opsonised (61). The interaction of cell receptors with organism bound opsonins activates cytoskeletal contractile elements resulting in the formation of pseudopodia that engulf the organism (61). The organism is then contained within a phagosome which subsequently fuses with a variety of lysosomal compartments that contain microbicidal products (61).

(iii) Intracellular killing and digestion of the organism

Intracellular killing of organisms can be oxygen dependant or oxygen independent. Oxygen dependant killing depends on the enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. The oxidant products of this enzyme (produced by a process known as respiratory burst) have microbicidal activity. The importance of oxygen dependant killing is highlighted by patients with chronic granulomatous disease (CGD) who have a defect in NADPH oxidase and as a result are highly susceptible to a variety of bacterial and fungal infections,
particularly *S. aureus* infection (113). Oxygen independent killing depends on microbicidal proteins and peptides, such as lysozyme, α-defensins and LL-37, contained within the primary (azurophilic) and secondary granules that fuse with the phagosome (61). Once the PMNs have ingested and killed the infecting organisms they usually die by apoptosis, this minimises tissue damage and clears PMNs from the site of infection (61).

Neutrophil extracellular traps (NETs) are a recently identified entity composed of chromatin, histones and azurophilic granular proteins. They are produced by neutrophils and are thought to be important for ensnaring and killing bacteria and fungi (114). The origin of these NETs is not clear. However, they are believed to have significant antimicrobial activity against a variety of organisms including *S. aureus* (115).

PMNs are not only involved in the direct killing of organisms, they also play an important role in the activation of the acquired immune response. PMNs act as antigen presenting cells that transport the infecting organism to lymph nodes stimulating B cells to secrete antibodies which will enhance the immune response to infection (92).

1.5. The innate immune response and *S. aureus*

A key component of the virulence of *S. aureus* is its ability to evade, inactivate, and confuse the innate immune system. The innate immune system plays a critical role in our defence against *S. aureus* infection. By defeating this system, *S. aureus* can persist and spread within the human host and cause infection. *S. aureus*
has the potential to block every component of the innate immune response (116,117).

1.5.1. Recognition of *S. aureus* by the innate immune system

To understand how *S. aureus* interacts with the innate immune system it is important to identify which components of *S. aureus* can be recognised by the innate immune system. TLR2 has been shown to play a major role as a receptor in the innate immune response to *S. aureus* infection, and recognizes staphylococcal lipoteichoic acid (LTA) and δ-haemolysin (61,63). TLR2 deficient mice have been shown to have increased susceptibility to *S. aureus* infection (118). Staphylococcal peptidoglycan is a weak inducer of cytokine production and acts synergistically with LTA (63). It was originally thought that TLR2 also recognized peptidoglycan but it is now believed that NLR Nod2 is the innate immune receptor for this cell wall component (63). Protein A which is a virulence factor found on the surface of *S. aureus* has been found to bind to TNF-α receptor 1 (TNFR1) on airway epithelial cells eliciting the release of IL-1β, IL-4, IL-6, IL-8, IFN-γ and TNF-α (63). TLR9 is the main innate receptor for staphylococcal DNA (63). A number of other virulence factors produced by *S. aureus* are known to illicit an immune response however the receptors for these molecules are still unknown.

1.5.2. Antimicrobial peptides and *S. aureus*

*S. aureus* is highly resistant to the hydrolytic activity of lysozyme (98). This resistance is mediated by the *oatA* gene (119). Resistance is due to modification of
the cell wall by O-acetylation, the presence of cell wall teichoic acid and a high level of cross linking (120,121). This high level resistance was not seen in O-acetyltransferase mutants of *S. aureus* (66).

Many organisms, including *S. aureus* have developed resistance to CAMPs allowing them to evade this important component of the innate immune system. The ability to resist CAMPs is an important virulence factor (99). Resistance to CAMPs is mediated mostly by a net increase in the charge on microorganisms, reducing their affinity for the positively charged antimicrobial peptides (105). A number of factors have been shown to alter the net charge of *S. aureus*. The *dlt* gene encodes for an enzyme which mediates incorporation of d-alanine into teichoic acid. The incorporation of d-alanine increases the positive surface charge of the bacterium, making it less susceptible to killing by CAMPs (105,122). The gene for multiple peptide resistant factor (*mprF*) encodes for lysylphosphatidylglycerol (L-PG) synthase, an enzyme that catalyses the incorporation of lysine into phosphatidylglycerol, a major component of the bacterial cell membrane (105,122). The presence of lysine in the cell membrane increases the surface positive charge, reducing its susceptibility to killing by CAMPs (105). Organisms that have low susceptibility to CAMPs are more resistant to neutrophil killing (98). Mutant organisms, which do not possess the *dlt* and *mprF* genes, are more negatively charged and are therefore more susceptible to CAMP killing (122). Such mutants have been shown to be less virulent than *dlt* and *mprF* positive strains (122).

Changes in the net charge of the organism are not the only way *S. aureus* can resist killing by CAMPs. Staphylokinase (SAK) is a protein produced by *S. aureus*
that activates plasminogen (123). SAK also inactivates defensins and reduces the binding affinity of these peptides (98,105). PIA produced by staphylococci and involved in the formation of biofilms as previously described (Section 1.3.1.4.) reduces the ability of CAMPs to kill the bacterium (98). Other factors such as fibronectin-binding protein and the capsule may influence the activity of CAMPs (105). Aureolysin, a metalloprotease produced by S. aureus and which plays a key role in the evasion of the immune response, cleaves LL-37 at multiple sites inactivating its antimicrobial activity (66,119,124).

MRSA strains have been shown in some studies to have lower susceptibility to CAMPs than MSSA strains (105,122,125). Moreover, Ouhara et al (2008) found that MRSA was more resistant to LL-37 than MSSA. The mechanism behind this increased resistance is not clear but it was not associated with the carriage of meca (125).

1.5.3. Inactivation of complement by S. aureus

S. aureus produces a number of molecules which allow it to evade the complement system by inactivating some of its key components. Staphylococcal complement inhibitor (SCIN), encoded by scn, is probably the most effective inhibitor of the complement system produced by S. aureus (119). SCIN blocks all three complement pathways (38) and inhibits phagocytosis and production of C5a (119). SCIN stabilizes C3bBb and C4b2a on the cell surface, inhibiting further production of convertases and also prevents C3 cleavage (66). SAK, the product of the sak gene, activates plasminogen to plasmin (123). Surface bound plasmin
cleaves IgG and C3b and removes opsonic molecules from the bacterial cell surface which are needed for recognition by immune cells, resulting in impaired phagocytosis of the bacteria by neutrophils (126). Plasmin also inhibits activation of the classical pathway (126). Protein A binds the Fc portion of IgG, inhibiting phagocytosis and activation of the classical pathway through C1q (119). Extracellular fibrinogen binding molecule (Efb) blocks the classical pathway by binding C3, preventing it from binding to the cell surface and thus inhibiting opsonisation and phagocytosis (127). The chp and ssl genes encode chemotaxis inhibitory protein (CHIPs) and staphylococcal superantigen-like (SSL) proteins (e.g. SSL7, SSL10) which inhibit activation of C5a disrupting chemotaxis and phagocytosis (126).

Inhibition of various components of the complement system effectively results in decreased phagocytosis and killing of S. aureus by neutrophils – a key component of host defence against this pathogen.

1.5.4. Cytokines and S. aureus

Gram-positive infections, including those caused by S. aureus, have been shown to result in a slower and lower peak cytokine response when compared to Gram-negative infections (63). Numerous studies have shown that S. aureus is a potent stimulator of cytokine production and that these cytokines play a major role in the pathogenesis of S. aureus infection (62,63). Production of cytokines due to S. aureus infection can have both beneficial and detrimental effects for the host.
A variety of staphylococcal molecules have been shown to stimulate cytokine production. Cell wall fragments, peptidoglycan and LTA induce the production of TNF-α, IL-1β and IL-6 by macrophages and monocytes (62-64). Both peptidoglycan and lipoteichoic acid result in the production of much higher levels of IL-6 compared to TNF-α and IL-10 (63). The surface protein A causes the release of IL-1β, IL-4, IL-6, IL-8, IFN-γ and TNF-α by airway epithelial cells. The toxin, α-haemolysin has been shown to induce the production of IL-1β, IL-6, IL-8 and TNF-α and β-haemolysin causes lysis of monocytes resulting in the release of IL-1β (63). Staphylococcal DNA is capable of inducing the production of IL-6, IL-12, IFN-γ and TNF-α by binding to TLR9 (63).

Megyeri et al (2002) demonstrated the production of IL-1β, IL-6, IL-12 and IFN-α by human mononuclear cells in response to stimulation by S. aureus (128). The levels of cytokines produced following exposure to S. aureus were significantly higher when compared to the levels following exposure to S. epidermidis and S. saprophyticus in the majority of cases (128). This suggests that the immune system provides an enhanced response to a more virulent pathogen. It is also interesting to note that while all these cytokines are pro-inflammatory, IL-12 and IFN-α specifically stimulate Th1 differentiation, which increases macrophage activation enhancing elimination of S. aureus by phagocytosis.

S. aureus expresses specific cytokine receptors and can use cytokines as growth factors or as virulence factors (129-131). Although IL-1β has been shown to bind to S. aureus the exact mechanism by which this occurs has not been fully elucidated. Kanangat et al (2001) have shown that IL-1β acts as a growth factor or
plays a role in the production of a growth factor as both intra- and extracellular growth of \textit{S. aureus} is significantly enhanced in its presence (129).

Cytokines may also have the ability to up-regulate the production of virulence factors by \textit{S. aureus} particularly in the setting of persistent inflammatory conditions. It is known that organisms such as \textit{S. aureus} modulate their gene expression in response to the changing environmental conditions. Kanangat \textit{et al} (2007) reported that \textit{S. aureus} expresses significantly higher levels of MSCRAMM mRNAs and significantly lower levels of bicomponent leukotoxin mRNA when exposed to higher concentrations of IL-1β (Kanangat, 2007) (132). These findings appear to reflect the \textit{in vivo} environment of chronic inflammation, where high levels of IL-1β encourage the persistence of \textit{S. aureus} by up-regulating the production of the MSCRAMM genes thus enhancing the organism’s ability to attach to and invade cells. The mechanisms by which IL-1β alters gene expression are unclear.

\textbf{1.5.5. Phagocytosis and \textit{S. aureus}}

\textbf{1.5.5.1. Evasion of phagocytosis}

Killing of \textit{S. aureus} by PMNs is one of the most important components of the innate immune response. However, \textit{S. aureus} has developed sophisticated mechanisms to evade this component of innate immunity. \textit{S. aureus} can evade or inhibit nearly every step in the process of phagocytosis. Some of these are discussed below.

\textbf{1.5.5.2. Production of toxins that kill leucocytes}
*S. aureus* produces a number of toxins that can directly kill neutrophils (66). Alpha-toxin (α-haemolysin) is a cytolytic toxin that causes cell lysis. The bicomponent leucotoxins, such as γ-haemolysin, PVL toxin, leucocidin E/D and leucocidin M/F-PV-like also lyse leucocytes, while γ-haemolysin can also lyse erythrocytes. The production of PVL toxin by specific strains of *S. aureus* has been associated with more severe skin and soft tissue infections and pneumonia, initially suggesting that this toxin is a potent virulence factor (66). However, as previously mentioned recent work has suggested that this toxin may not be as important a virulence factor as previously thought (18,83). The α-type phenol-soluble modulins (PSMs) are peptides produced by *S. aureus* and are capable of leucocyte lysis with additional pro-inflammatory and chemotactic properties (13). PSMs may be responsible for the increased pathogenic potential of CA-MRSA as these strains tend to produce higher levels of PSMs compared to hospital acquired strains (18,83).

### 1.5.5.3. Inhibition of chemotaxis

PMNs are attracted to the site of infection by a variety of chemoattractant molecules including C5a and formylated peptides (secreted by bacterial cells) (61). The chemotaxis inhibitory protein of staphylococci (CHIPS) binds to the receptors for formylated peptides (FPR) and C5a (C5aR) on the PMN inhibiting migration of the cell to the site of infection (119). Another protein produced by *S. aureus*, extra-cellular adherence protein (Eap), binds to ICAM-1 on the surface of endothelial cells, inhibiting neutrophil adhesion and migration to the site of infection (119). SSL5, one of the staphylococcal superantigen-like proteins has been shown to inhibit
neutrophil rolling by inhibiting chemotaxis induced by chemokines and anaphylatoxins (133).

1.5.5.4. Inhibition of phagocytosis

*S. aureus* can produce a number of substances that prevent recognition of the organism by PMNs. Both capsular polysaccharide and PIA can mask the immunogenic surface molecules of *S. aureus*, inhibiting phagocytosis (66). Protein A binds to the Fc region of IgG resulting in the failure of the Fc receptor on neutrophils to recognise IgG, inhibiting phagocytosis of *S. aureus* (66). Clumping factor A (ClfA) binds fibrinogen to the surface of *S. aureus*, inhibiting opsonisation and subsequent phagocytosis (66). A number of factors inhibit the complement system (e.g. SCIN, SAK, Efb, CHIPS and SSL-7) (Section 1.5.3) and ultimately also inhibit phagocytosis as opsonisation is one of the key functions of the complement system.

1.5.5.5. Survival within neutrophil phagosomes

*S. aureus* may survive within the phagosomes of PMNs due to the production of specific enzymes and other substances such as catalase and superoxide dismutase, which are produced by all staphylococci and catalyse the detoxification of hydrogen peroxide allowing the bacteria to survive within phagosomes (18). The yellow carotenoid pigment, staphyloxanthin, of *S. aureus* scavenges oxygen free radicals within the phagosome (119). As discussed in Section 1.5.2., *S. aureus* also has the ability to inhibit or avoid many of the microbicidal proteins and peptides, such as
lysozyme and α-defensins secreted by granules into the phagosome permitting their survival (66,98,105,122,134).

It is clear that there is considerable redundancy in the mechanisms developed by *S. aureus* to evade phagocytosis, ensuring their persistence and maximising their pathogenic potential.

### 1.5.6. Potentiation of the immune response

*S. aureus* produces toxins such as TSST-1 and the SEs that have super-antigenic activity (77). Superantigens bind the MHC class II protein on the surface of antigen-presenting cells and link it to T-cell receptors on the surface of T helper cells, resulting in activation of up to 30% of the body’s T-cells and excessive release of cytokines, often resulting in overwhelming sepsis (92). As T-cells are activated, despite the absence of an antigen on an antigen-presenting cell, they do not proliferate in response to specific antigens presented in the normal fashion, resulting in decreased production of specific antibodies. The lack of specific antibodies results in an impaired immune response overall (66).

Eap is a protein produced by *S. aureus*, which prevents adhesion of neutrophils to endothelial cells (Section 1.5.5.3.) and also impairs T-cell function by binding to T-cell receptors (66). This results in a reduction in T-cell proliferation and a shift from a Th1 response to a Th2 response, which affects cell-mediated immunity. Eap also has an effect on mononuclear cells, stimulating proliferation of cells at low concentrations and apoptosis at high concentrations (66).
1.6. Hypothesis & aims

The outcome of *S. aureus* BSI is often unpredictable and the patients expected to have a poor outcome often do relatively well while those expected to do well often do poorly, and may actually succumb to their infection. The exact reasons for this are unclear. We hypothesized that host-response to *S. aureus* BSI in addition to the genetic lineage and virulence traits of the infecting isolates contribute to the clinical course of infection. In addition patient characteristics such as age, co-morbidities and other features may contribute to the clinical outcome.

Aims

The overall aim of this project was to compare MRSA and MSSA isolates causing complicated and uncomplicated *S. aureus* BSI in terms of their genotypes, virulence characteristics, susceptibility to LL-37 and level of cytokine response and the clinical characteristics of the patients with *S. aureus* BSI.

We have addressed this aim as follows:

In the first results chapter (Chapter 3) of this thesis we describe the epidemiological and clinical characteristics of patients with *S. aureus* BSI recruited to the study. We correlate these characteristics with the type of infection (complicated *versus* uncomplicated) and with the type of infecting isolate (MRSA *versus* MSSA). The aim here was to determine how these characteristics may affect the outcome of *S. aureus* BSI.
In Chapter 4, we describe the detailed genetic characterization of *S. aureus* isolates causing BSI in the recruited patients. We investigate the pattern of virulence and antimicrobial resistance genes among the isolates and we also assign *spa* types and clonal grouping to the isolates. We investigate the correlations between these factors and the outcome of BSI. We compare our findings from these patient isolates to a more genetically diverse collection of MRSA isolates from renal patients.

We then study (Chapter 5) the host immune response to *S. aureus* BSI in these patients using plasma samples obtained over the course of their infection. Based on the results of a preliminary cytokine/chemokine screen, we investigate differential levels of four cytokines in patients with complicated *versus* uncomplicated infection and in patients with MRSA *versus* MSSA BSI.

Finally in Chapter 6 we investigate the susceptibility of isolates causing complicated and uncomplicated *S. aureus* BSI to the host defence peptide LL-37.
Chapter II
Materials and Methods
2.1. Study Hospitals

2.1.1. Beaumont Hospital, Dublin

Beaumont Hospital is an 820-bed tertiary referral centre containing the national referral centre for neurosurgical patients, renal and pancreatic transplantation and cochlear implantation and it is a regional treatment centre for ear, nose and throat and gastroenterology patients. The hospital incorporates a twelve-bedded general ICU and a ten-bedded neurosurgical ICU.

2.1.2. Mater Misericordiae University Hospital, Dublin

The Mater Misericordiae University Hospital is a 570-bed tertiary referral centre with two national specialties, cardiothoracic surgery (including transplantation) and spinal injuries. Regional specialties include ophthalmology, dermatology, breast cancer screening and oncology. The hospital also provides services under a range of medical and surgical specialties, including cardiology, renal services, general and vascular surgery, urology and orthopaedics and incorporates a 17-bedded ICU.

2.2. Collection of \textit{S. aureus} isolates and patient plasma samples

2.2.1. Collection of bacterial isolates

In total, 70 isolates causing \textit{S. aureus} BSI were recovered from the patient’s blood in accordance with routine clinical practice and processed through the diagnostic laboratory in the respective hospital. \textit{S. aureus} isolates from blood cultures were prospectively collected in Beaumont Hospital from October 2008 to
February 2011 and in the Mater Misericordiae University Hospital from November 2009 to March 2010 (see Appendix II). Only clinically significant isolates, as defined by Weinstein et al (1997), were included in the study (135). Only the first S. aureus isolate from each BSI episode was collected for the purposes of the study. Isolates were obtained from the diagnostic laboratory and stored at -80 °C on Protect™ beads (Technical Service Consultants Ltd., Lancashire, UK). Blood cultures were to be repeated three days later, irrespective of the clinical status of the patient, as part of best practice to confirm sterility after the start of appropriate antibiotic treatment. Unfortunately, repeat cultures were not always taken within three days, but a repeat culture was collected from all patients in the study although at a later time point in some cases. Bloodstream isolates used in this study are listed in Appendix II.

A further collection of 36 MRSA isolates from renal patients with BSI in Beaumont Hospital over a five year period from 2005-2009 inclusive, were obtained retrospectively (Appendix III). These were used in the analysis of isolates but no blood samples were collected from these patients for other studies.

### 2.2.2. Collection of plasma samples

Blood samples (10 ml) were taken from consenting patients over the course of their infection. The first sample was obtained within three days of isolation of S. aureus in a blood culture (Sample A), a second sample was collected from all patients seven days later (Sample B) and a third sample was collected only from patients with complicated infection at day 14 (Sample C) (In the case of
uncomplicated infection, these patients had already been discharged by day 14). For the purposes of this study a complicated infection was defined as the development of infective endocarditis, persistent BSI despite at least three days of appropriate antibiotics or disseminated infection such as osteomyelitis. Blood samples were collected in Li-Heparin blood bottles (S-Monovette®, Nümbrecht, Germany), centrifuged at 5000rpm and the plasma was decanted and stored in aliquots at -80 °C until required.

2.3. Patient Data

Patient demographics, details of source of infection, antibiotic susceptibility of infecting isolates, antibiotics prescribed and outcomes of the infection were collected prospectively from patients charts, nursing notes and microbiological records and stored in a secure location without patient identifiers (see Appendix IV for data collection sheet). Similar data was collected for the renal patients and stored appropriately.

2.4. Ethical approval

Ethical approval to carry out this study was received from both Beaumont Hospital and The Mater Misericordiae University Hospital Ethics Committees (see Appendix V for approval letters).

2.5. Media and growth conditions
S. aureus isolates were routinely grown at 37 °C on brain heart infusion (BHI) (Oxoid, UK) solid media or in BHI broth. Static incubation was carried out in an incubator provided by Gallenkamp, Leicestershire, UK and if required shaking incubation was carried out in an orbital shaker also provided by Gallenkamp, Leicestershire, UK.

2.6. Buffers and solutions

Milli-Q water (Millipore Ireland, Cork, Ireland) was used for making buffers, solutions and agarose gels. Molecular Biology Reagent water was purchased from Sigma-Aldrich and was used in all PCR reactions, DNA elutions and dilutions. Tris-borate/EDTA (TBE) was used at 0.5 X concentration as a buffer for agarose gel electrophoresis. This was diluted from a 10 X stock solution supplied by Sigma Aldrich, UK.

2.7. Sterilisation techniques

All agar media and broths were autoclaved at 121 °C for 15 min. Other solutions, buffers etc. were autoclaved at 121 °C for 15 min unless heat labile in which case they were sterilised by filtration through a 0.2 µm filter (ALBET, Spain).

2.8. Bacterial molecular genetic techniques

2.8.1. Isolation of bacterial genomic DNA

Two different methods of bacterial genomic DNA extraction were used.
2.8.1.1. Method 1 - Wizard® Genomic DNA Purification Kit

Genomic DNA was isolated from staphylococcal cells using the Wizard® Genomic DNA Purification Kit (Promega, USA) according to the manufacturer’s instructions. Prior to DNA extraction, cells were pre-treated with 5-10 μL of lysostaphin (1 mg/ml) (Ambi Products Ltd, USA) in 100 μL 50 mM EDTA (Sigma-Aldrich, MO, USA) to facilitate cell lysis.

2.8.1.2. Method 2 - Qiagen DNeasy® Blood and Tissue Kit

This method was used when preparing DNA for microarray analysis which required greater concentrations of DNA. To ensure that a pure culture was used for DNA extraction, each isolate was cultured on a Columbia blood agar (CBA) (Fannin Ltd, Galway, Ireland) plate overnight at 37 °C in a static incubator (Gallenkamp, Leicestershire, UK) and an individual colony of each isolate was selected with a sterile inoculating loop, lawned onto a fresh CBA plate in a 2.5 cm² area and incubated for 18 h at 37 °C in a static incubator. Cell lysis was carried out by resuspending all the growth from this defined area in 200 μl of lysis solution and the lysis enhancer provided with the DNA microarray Staphytype Kit (Alere Technologies, Jena, Germany) and incubated for 60 min at 37 °C in a shaking water bath (Techne, Staffordshire, UK). Protein was removed by the addition of 25 μl of proteinase K and 200 μl of AL buffer (supplied with the DNeasy® Blood and Tissue kit, Qiagen, Crawley, UK), which was then vortex mixed and incubated at 70 °C for 30 min in a heating block (Techne, Staffordshire, UK). DNA extraction was carried out using the DNeasy® Blood and Tissue Kit (Qiagen, Crawley, UK) as per the
manufacturer’s instructions. DNA was eluted with 50 µl Sigma molecular biology-grade water and stored at -20 °C. The concentration of DNA in each sample was determined using a Nanodrop 8000 V2.0.0 Spectrophotometer (Thermo Scientific, USA).

2.8.2. Agarose gel electrophoresis of DNA

Electrophoresis of DNA was performed using horizontal 1% (w/v) or 2% (w/v) agarose gels containing 1 µg/ml ethidium bromide or 1 X Gel red (Promega, WI, USA). Agarose gels were prepared and electrophoresed in 0.5 X TBE buffer. A 5-10 µL sample of PCR product was mixed with 1-2 µL blue/orange loading dye (6 X) (Promega, WI, USA) and loaded into the test wells. Horizontal agarose gel tanks (Hybaid, UK) were used and electrophoresis was performed at 160 V for between 30 min. DNA bands were visualised by illumination with an ultraviolet light source (LKB Bromma, 2011 Macrovue Transilluminator) and recorded as thermal images using a gel documentation system (Stratagene Eagle Eye® II).

2.9. spa typing

spa typing was carried out by sequencing of a polymorphic 24 bp variable-number tandem repeat (VNTR) region within the 3’ end of the spa gene. spa typing was carried out according to the method described on the SeqNet website (http://www.seqnet.org). The primers used were spa113F (5’-TAAAGACGATCCTTCGGTGAGC-3’) and spa1514R (5’-CAGCAGTAGTGCCGTTTGCTT-3’). PCR reactions were carried out in a Peltier
Thermal Cycler (Viswagen Biotech, Pvt. Ltd. Kerala, India) and contained 12.5 µl of goTAQ® green master mix (Promega, WI, USA), 2 µl of template DNA, 5 pmol of forward and reverse primers, in a final volume of 25 µl sterile H2O. PCR conditions were: 80 °C for 5 min, 35 cycles of 94 °C for 45 s, 60 °C for 45 s, and 72 °C for 90 s with a final extension step for 10 min at 72 °C. Reaction mixtures were analyzed by 2% (w/v) agarose gel electrophoresis. PCR products were cleaned-up prior to sequencing using the GenElute™ PCR Clean-Up Kit (Sigma, MI, USA) as per the manufacturer’s instructions. The concentration of DNA in each sample was determined using a Nanodrop 8000 V2.0.0 Spectrophotometer (Thermo Scientific, USA). Sequencing was performed by Co-genics, Essex, UK or Source BioScience Ltd, St. James’ Hospital, Dublin. spa-sequence analysis was carried out using Ridom StaphType™ software, version 1.3 (Ridom GmbH, Würzburg, Germany). Clustering of spa types was carried out using Based Upon Repeat Patterns (BURP) analysis in the Ridom StaphType™ software version 1.3 (Ridom GmbH, Wurzburg, Germany) to assess the clonal relatedness between isolates (136).

2.10. DNA microarray analysis

The StaphyType Kit (Alere Technologies, Jena, Germany) consists of individual DNA microarrays mounted in eight-well microtitre strips which detect 334 S. aureus gene sequences which correspond to 185 genes and their alleles, including species markers, resistance genes, exotoxins, genes encoding microbial surface components recognizing adhesive matrix molecules of the host (MSCRAMMs), as well as SCCmec, capsule and agr group typing markers (137).
Genomic DNA was extracted by enzymatic lysis and using the Qiagen DNeasy kit (Qiagen, Crawley, UK) as described in Section 2.8.1.2. The DNA microarray procedures were performed according to the manufacturer’s instructions and have been previously described (137). All reagents were supplied by Alere Technologies, Jena, Germany. All target genes were amplified and biotin-16-dUTP was incorporated into the amplicons using a single primer per target and a linear primer elongation reaction in order to cover all 334 targets simultaneously. Each PCR reaction consisted of 5 µl of mastermix (4.9 µl of 2 x labelling buffer and 0.1 µl of DNA polymerase) and 5 µl of the DNA sample (at the correct concentration). PCR reactions were carried out in a Peltier Thermal Cycler (Viswagen Biotech, Pvt. Ltd. Kerala, India) with the following conditions: 96 °C for 5 min, 45 cycles of 50 °C for 20 s, 72 °C for 40 s and 96 °C for 1 min. Following PCR, products were mixed with 90 µl of C1 buffer, added to the wells of the array strip (pre-washed with C1 buffer) and incubated for 1 h at 55 °C and 550 rpm (Eppendorf thermomixer) for hybridisation to the array. Following washing (200 µl C2 buffer), horseradish-peroxidase-streptavidin conjugate solution (HRP reagent C3:buffer C4, 1/100, v/v) was added to the wells. Following incubation for 10 min at 30 °C at 550 rpm, the HRP solution was removed and wells were washed with 200 µl C5 buffer, bound HRP conjugate was stained by the addition of 100 µl of D1 reagent which contains the HRP substrate tetramethylbenzidine. The strip was incubated for 5 min at room temperature, the D1 reagent was removed, and the wells were washed briefly with distilled water and then air dried. A designated reader and software (Arraymate™, Alere Technologies, Jena, Germany) was used for analysis of DNA microarray
chips. The software interprets raw data as ‘positive’, ‘negative’ or ‘ambiguous’, using a previously described algorithm (137). The arraymate™ software can assign *S. aureus* isolates to STs and/or CCs by comparison of the DNA microarray results to those of a collection of previously characterised and MLST-typed reference strains in the Arraymate™ database (137).

2.11. Cytokine analysis

2.11.1. Cytokine array analysis

Plasma samples from three randomly selected patients (taken on day of diagnosis) from four patient groups; MRSA BSI, MSSA BSI, uncomplicated BSI and complicated BSI, were pooled for the purpose of the array. The levels of 42 different cytokines and growth factors were determined in the pooled plasma samples using the RayBio® Human Cytokine Antibody Array 3 (RayBiotech Inc., GA, USA) according to the manufacturer’s instructions. Briefly, membranes were blocked using 1 X blocking buffer at room temperature (RT) for 30 min with shaking. Following blocking, membranes were treated with 1 ml of pooled plasma in 1 X blocking buffer for 2 h at RT with shaking. Samples were removed and membranes washed for 5 min x 3 with 2 ml of 1 X wash buffer I at RT with shaking. Membranes were washed for 5 min x 2 with 2 ml of 1 X wash buffer II at RT with shaking. A solution of biotin-conjugated antibody was prepared (as per manufacturer’s instructions), and 1 ml of the solution was added to the array membranes, which were incubated for 2 h at RT with shaking. Membranes were washed sequentially with 1 X wash buffer I and 1 X wash buffer II as previously
described. Following washing, 2 ml of 1,000-fold diluted horse-radish peroxidase-conjugated streptavidin was added to each membrane and incubated overnight at 4 °C with shaking. Membranes were washed sequentially with 1 X wash buffer I and 1 X wash buffer II as previously. Signals were detected using 500 µl of a 1:1 mixture of 1 X detection buffer C and 1 X detection buffer D following incubation for 2 min at RT. Signal intensities were captured and quantified by the chemiluminescence imaging system G:BOX Chemi XL (Syngene UK, Cambridge, UK).

2.11.1.2. Data analysis

Images were captured at 18 min for analysis. Mean values for each cytokine were expressed relative to the mean positive value for each of the array membranes (6 positive control spots per membrane). Values obtained for each of the cytokines were compared for the pooled plasma from patients with MRSA versus MSSA BSI and from patients with uncomplicated versus complicated infection. A change in the cytokine levels equal to or greater than 1.4 fold between groups was considered significant. Cytokines that were significantly altered between groups were selected for investigation in plasma from all patients using ELISA.

2.11.2. Enzyme-linked immunosorbant assay (ELISA)

A sandwich ELISA was used to determine the levels of four cytokines (IL-6, GROγ, RANTES and Leptin) in all patient plasma. These cytokines were selected from cytokine array analysis of pooled plasma. These cytokines were also measured
in plasma obtained from four healthy volunteers. Antibodies and standard antigens used are listed in Table 2.1. The wells of a standard 96 well micro-titre plate were coated with 100 µl of the primary antibody diluted in Voller’s buffer (Na₂CO₃ 2.76 g/l, NaHCO₃ 1.916 g/l, pH 9.6) to the desired concentration. The plate was sealed and incubated overnight at 4 °C. Following incubation, the test cells were washed three times with phosphate-buffered saline containing 0.1% Tween 20 (PBS-Tween) emptied and tapped dry. Test wells were blocked with 200 µl of 1% bovine serum albumin (BSA) in PBS-Tween and incubated for 1 h at RT with agitation. Wells were washed, as previously described, with PBS-Tween. Standards for each of the cytokines were diluted in PBS-Tween to 2,000 pg/ml, 1,000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml and 31.25 pg/ml, and 100 µl of each was added to wells in duplicate. Plasma samples (100 µl) were also added in duplicate to wells and incubated for 2 h at RT with agitation. Wells were washed as previously described with PBS-Tween. The corresponding secondary antibody (Table 2.1) was diluted to the desired concentration with PBS-Tween and 100 µl added to the test wells. The plate was incubated for 1 h at RT with agitation and washed as before. A 1:1000 dilution of streptavidin-horseradish peroxidise (Cambridge Biosciences, Cambridge, UK) was prepared and 100 µl was added to the test wells, followed by incubation for 20 min at RT with agitation and washed as previously described. Finally, 100 µl of 2,2’-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) solution (ABTS) (Sigma-Aldrich, MO, USA) was added to each well and the plate was incubated in darkness until colour developed (20-40 min). Optical density (OD) was determined at 405 nm using a standard microplate spectrophotometer. Cytokine
concentrations were determined by reference to standard curves of absorbances versus cytokine concentrations using GraphPad Prism software 4.0 (GraphPad Software Inc, San Diego, California).

Table 2.1. Antibodies and antigens used for Sandwich ELISA.

<table>
<thead>
<tr>
<th>Cytokine/chemokine</th>
<th>Primary Antibody</th>
<th>Recombinant antigen&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Secondary Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>MAB206</td>
<td>206-IL-010</td>
<td>BAF206</td>
</tr>
<tr>
<td>GROγ</td>
<td>PP1014P1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>277-GG-010</td>
<td>PP1014B1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>RANTES</td>
<td>MAB678</td>
<td>278-RN-010</td>
<td>BAF278</td>
</tr>
<tr>
<td>Leptin</td>
<td>MAB398</td>
<td>398-MP-010</td>
<td>BAM398</td>
</tr>
</tbody>
</table>

<sup>a</sup>All recombinant antigens were from R and D Systems, Abington, UK.

<sup>b</sup>GROγ primary and secondary antibodies were from Acris Antibodies Ltd., Germany. All other antibodies were from R and D systems, Abington, UK. All secondary antibodies were biotin labelled.

2.11.3. Measurement of protein levels in plasma samples for cytokine assay

Standards were prepared with filter sterilised 2% BSA in distilled water (dH2O) (20 mg BSA in 10 ml of H2O) – 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml. A standard 96 well micro-titre plate was used for the assay, and a negative control was included (dH2O). Standards were added to the wells in duplicate. Plasma samples were diluted 1/50 with dH2O, and 5 μl of each sample was added to the wells in duplicate. 250 μl of Bradford reagent (Sigma-Aldrich, MO, USA) was added to each well and the plate was agitated for 30 s at RT. The plate was then incubated in darkness for 25-30 min at RT. Absorbance was determined at 570 nm (A<sub>570</sub>) using a microplate spectrophotometer (ThermoFisher Scientific, MA, USA).
Protein concentrations of plasma samples were determined by reference to standard curves of A\textsubscript{570} versus protein concentrations using Graphpad Prism Version 4.0.

2.12. \textit{S. aureus} killing with the antimicrobial peptide LL-37

2.12.1. Isolates used for LL-37 killing assay

The killing assay was carried out on ten isolates causing complicated and ten isolates causing uncomplicated BSI selected from the 70 study isolates (Appendix II). Those selected are listed in Table 2.2. A combination of MSSA and MSSA isolates were included in the assay. All isolates, except one causing complicated infection and one causing an uncomplicated infection, were positive for \textit{mprf}, the gene which codes for L-PG synthase, which is associated with decreased susceptibility to LL-37.
Table 2.2. Twenty isolates used in LL-37 killing assay

<table>
<thead>
<tr>
<th>Isolate No.</th>
<th>Organism</th>
<th>mprf gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSI 11</td>
<td>MRSA</td>
<td>Positive</td>
</tr>
<tr>
<td>BSI 13</td>
<td>MSSA</td>
<td>Positive</td>
</tr>
<tr>
<td>BSI 16</td>
<td>MSSA</td>
<td>Positive</td>
</tr>
<tr>
<td>BSI 22</td>
<td>MSSA</td>
<td>Positive</td>
</tr>
<tr>
<td>BSI 23</td>
<td>MSSA</td>
<td>Positive</td>
</tr>
<tr>
<td>BSI 24</td>
<td>MSSA</td>
<td>Positive</td>
</tr>
<tr>
<td>BSI 30</td>
<td>MSSA</td>
<td>Negative</td>
</tr>
<tr>
<td>BSI 32</td>
<td>MSSA</td>
<td>Positive</td>
</tr>
<tr>
<td>BSI 50</td>
<td>MSSA</td>
<td>Positive</td>
</tr>
<tr>
<td>BSI 58</td>
<td>MSSA</td>
<td>Positive</td>
</tr>
<tr>
<td>BSI 3</td>
<td>MSSA</td>
<td>Positive</td>
</tr>
<tr>
<td>BSI 7</td>
<td>MRSA</td>
<td>Positive</td>
</tr>
<tr>
<td>BSI 15</td>
<td>MSSA</td>
<td>Positive</td>
</tr>
<tr>
<td>BSI 17</td>
<td>MSSA</td>
<td>Positive</td>
</tr>
<tr>
<td>BSI 31</td>
<td>MRSA</td>
<td>Positive</td>
</tr>
<tr>
<td>BSI 33</td>
<td>MSSA</td>
<td>Negative</td>
</tr>
<tr>
<td>BSI 37</td>
<td>MSSA</td>
<td>Positive</td>
</tr>
<tr>
<td>BSI 48</td>
<td>MSSA</td>
<td>Positive</td>
</tr>
<tr>
<td>BSI 49</td>
<td>MRSA</td>
<td>Positive</td>
</tr>
<tr>
<td>BSI 53</td>
<td>MSSA</td>
<td>Positive</td>
</tr>
</tbody>
</table>

2.12.2. LL-37 killing assay

The LL-37 killing assay was adapted from a method described by Ouhara et al (2008) (125). Briefly, for selected isolates, single colonies from BHI agar were sub-cultured in 5 ml of BHI broth and incubated with shaking (Gallenkamp, Leicestershire, UK) at 37 °C overnight. The optical density (OD) of the culture was measured using a Ultrospec™ 10 Cell Density Meter (Amersham Biosciences, GE
Healthcare, USA), and the OD of the culture was adjusted to 1.0 with sterile 10 mM sodium phosphate buffer, pH 7.4 (OD of 1 was estimated to contain \(5 \times 10^9\) CFU/ml approx.). Killing assay mixtures were prepared in eppendorf tubes and contained \(5 \times 10^6\) CFU/ml \textit{S. aureus} and 2.5 µg/ml LL-37 peptide in a final volume of 100 µl 10 mM sodium phosphate buffer, pH 7.4. Control assays, where LL-37 was replaced with dH2O were also included. Assays were mixed by vortexing and incubated for 1 h at 150 rpm in a shaking incubator. Assay samples were diluted with 900 µL of 0.9% NaCl. From this dilution, 50 µL was spread onto a BHI agar plate and allowed to dry before a further incubation at 37 °C overnight. Following incubation, total plate counts were recorded. The assay was performed in duplicate and the mean of the duplicate colony counts was used to calculate the percentage killing with the following equation; \((\text{CFU control plate} - \text{CFU LL-37 plate}) ÷ \text{CFU control plate} \times 100\). All assays were performed on three separate occasions.

The assays were initially performed in triplicate using three reference strains of \textit{S. aureus}, MRSA strain COL, \textit{S. aureus} SH1000 (Kind gifts from Dr. Timothy Foster, Trinity College Dublin and Dr. Simon Foster, University of Sheffield) and \textit{S. aureus} ATCC 44300 (138,139). In preliminary assays, a range of LL-37 peptide concentrations (1.25 – 20 µg/ml) were used to determine an appropriate concentration of LL-37 that would distinguish isolates that differed in their LL-37 susceptibility.
2.13. Statistical analysis

Data processing and statistical calculations were carried out using the statistical software package GraphPad Prism Version 4.0 (GraphPad Software Inc, San Diego, California). Standard curves were generated by linear regression and unknown values were determined by reference to standard curves. The mean concentration of cytokines and mean LL-37 killing activity was compared between groups using the Mann-Whitney non-parametric test for statistical significance. Associations of single variables (eg virulence genes) with one of two groups (eg complicated versus uncomplicated infections) were evaluated in 2 x 2 contingency tables using GraphPad Software Quickcalcs (GraphPad Software Inc, San Diego, California). Relative risk (RR) was calculated using MedCalc Software version 12.1.4 (Mariakerke, Belgium) and odds ratios (OR) were calculated using an OR calculator provided by Bland et al (2000) (140). A p value ≤0.05 indicated a significant difference between the means. The following nomenclature was used to represent significant p values on graphs, * \( p \leq 0.05 \), ** \( p \leq 0.01 \), *** \( p \leq 0.0001 \) and ♦ \( p = \text{value} <0.05 \).
Chapter III
Demographics and Clinical Details of Patients with *S. aureus* BSI
3.1. Introduction

*S. aureus* is a common cause of BSI. *S. aureus* BSI occurs in patients of all ages, and in patients with and without underlying medical conditions. It can be nosocomial, health care–associated or community-acquired and can result in a variety of outcomes ranging from complete recovery to death. A study of 6,697 BSIs in the US found that *S. aureus* was the commonest pathogen, accounting for 23% of all episodes, and was more frequently associated with death than any other organism (141). In the UK about 12,500 cases of *S. aureus* BSI are reported annually with a mortality rate of 30% (29). In Ireland, approximately 1,200 *S. aureus* BSIs are reported annually (EARS-NET data). The outcome of *S. aureus* BSI is variable and a number of factors contribute to the clinical course of infection and the outcome. Some of these factors are likely to be host mediated and some are likely to be organism mediated. Certain clinical characteristics also have an impact on the outcome of *S. aureus* BSI, such as patient age, underlying co-morbidities, source of the BSI, timing of onset, and appropriateness of antimicrobial treatment (34,38,142,143).

A number of characteristics of the patients in our study population were assessed to determine the impact, if any, on the outcome of *S. aureus* BSI.

3.2. Results

3.2.1. Patient demographics

Seventy patients were prospectively recruited to the study in two tertiary referral centres. All patient characteristics are summarized in Table 3.1.
Table 3.1. Characteristics of 70 patients with *S. aureus* BSI

<table>
<thead>
<tr>
<th>Patient Characteristic</th>
<th>No. of patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>44 (62.86)</td>
</tr>
<tr>
<td>Female</td>
<td>26 (37.14)</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
</tr>
<tr>
<td>&gt;65</td>
<td>29 (41.43)</td>
</tr>
<tr>
<td>&lt;65</td>
<td>41 (58.57)</td>
</tr>
<tr>
<td><strong>Co-morbidities</strong></td>
<td></td>
</tr>
<tr>
<td>Cardiac disease</td>
<td>25 (35.71)</td>
</tr>
<tr>
<td>End stage renal failure</td>
<td>25 (35.71)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>17 (24.29)</td>
</tr>
<tr>
<td>Chronic obstructive pulmonary dis.</td>
<td>10 (14.29)</td>
</tr>
<tr>
<td>Autoimmune disease</td>
<td>6 (8.57)</td>
</tr>
<tr>
<td><strong>Onset of BSI</strong></td>
<td></td>
</tr>
<tr>
<td>Nosocomial</td>
<td>34 (48.57)</td>
</tr>
<tr>
<td>Healthcare-associated</td>
<td>28 (40)</td>
</tr>
<tr>
<td>Community-acquired</td>
<td>8 (11.43)</td>
</tr>
<tr>
<td><strong>Source of BSI</strong></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>18 (25.71)</td>
</tr>
<tr>
<td>Prosthetic device</td>
<td>35 (50)</td>
</tr>
<tr>
<td>Other</td>
<td>17 (24.29)</td>
</tr>
<tr>
<td><strong>Outcome of BSI</strong></td>
<td></td>
</tr>
<tr>
<td>Death</td>
<td>4 (5.71)</td>
</tr>
<tr>
<td>Uncomplicated BSI</td>
<td>55 (78.57)</td>
</tr>
<tr>
<td>Complicated BSI</td>
<td>15 (21.43)</td>
</tr>
<tr>
<td>Infective endocarditis</td>
<td>11 (15.71)</td>
</tr>
<tr>
<td>Persistently positive blood culture</td>
<td>2 (2.86)</td>
</tr>
<tr>
<td>Septic arthritis</td>
<td>1 (1.43)</td>
</tr>
<tr>
<td>Discitis</td>
<td>1 (1.43)</td>
</tr>
</tbody>
</table>

There was a slight preponderance of males in the study group. The median age of patients recruited to the study was 59 (range 17-84). Forty one patients
(58.57%) were less than 65 years of age and 29 patients (42.43%) were over 65 years of age. The 71-80 age-group was found to be most associated with the most S. aureus BSIs. It appears from our study group that S. aureus BSI is more common in patients over 65 years of age compared to patients under 65 years of age (29/70 versus 41/70), but this, perhaps does not take account of the age range of the general hospital population, which may not mirror the age distribution of patients with S. aureus BSI. The age range of patients with S. aureus BSI is illustrated in Figure 3.1.

![Age range of patients with S. aureus BSI.](image)

**Figure 3.1. Age range of patients with S. aureus BSI.** Range of ages of 70 patients with S. aureus BSI. The youngest patient was 17 years of age and the oldest was 84 years of age. The greatest number of S. aureus BSIs occurred in the 71-80 age-group.

Patients recruited to the study belonged to a wide variety of clinical specialties, with almost 75% of patients being medical patients. This is probably
due to the fact that a large percentage of patients with *S. aureus* BSI in our study were renal patients (27/70, 38.57%) (Figure 3.2). Among the recruited patients, there appeared to be a preponderance of renal patients who developed a *S. aureus* BSI compared to any of the other specialties.

![Figure 3.2. Specialties of patients with *S. aureus* BSI. Bars represent the distribution of 70 *S. aureus* BSI among hospital specialties](image)

A wide variety of co-morbidities were identified in the patients in our study population (Table 3.1). Fifty-three patients (75.71%) had a pre-existing medical condition. The most prevalent conditions were cardiac disease (25/70, 35.71%), end stage renal failure (ESRF) (25/70, 35.71%), diabetes mellitus (DM) (17/70, 24.29%), chronic obstructive pulmonary disease (COPD) (10/70, 14.29%) and autoimmune
disease (6/70, 8.57%). A large number of patients (56/70, 80%) recruited to the study had significant recent healthcare contact. These patients were predominantly made up of renal patients (26/70, 37.14%) who were on haemodialysis and patients (32/70, 45.71%) who had been admitted to an acute hospital in the last three months (Figure 3.3).

Figure 3.3. Recent healthcare contact of patients with S. aureus BSI. 80% of patients recruited to the study had significant recent healthcare contact, the commonest healthcare contacts recorded were haemodialysis (37.14%) and recent admission to an acute hospital (45.71%). Recent admission = admission to hospital in the last three months.

The onset of BSI can be divided into nosocomial (defined as a positive blood culture obtained from a patient who has been hospitalized for 48 h or more), healthcare–associated (defined as a positive blood culture obtained from a patient at the time of hospital admission or within 48 h of admission and who meets one of the following criteria - receives medical treatment in the home, attended a hospital or
haemodialysis within the last 30 days, was an inpatient in the last 90 days or resides in a nursing home or long-term care facility) and community-acquired (defined as a positive blood culture obtained from a patient at the time of hospital admission or within 48 h of admission and who fails to meet the criteria for health care–associated BSI) (144). The distribution of patients according to these criteria is shown in Table 3.1.

3.2.2. Source of S. aureus BSI

The source of S. aureus BSI varied within our study population. Nine different sources were identified, with approximately a quarter of patients (25.71%) having no confirmed or suspected focus of infection (Figure 3.4). Thirty-five patients (50%) had a prosthetic device identified as the source of their S. aureus BSI, these included; central venous catheters (CVC), peripheral venous catheters (PVC) or an implanted cardiac device (ICD) (Figure 3.5). The commonest source of a S. aureus BSI was an infected CVC (22/70, 31.43%). A wide variety of CVC types are used in our hospitals and the site of CVC insertion can also vary. Permacaths were the commonest CVC identified as a source of S. aureus BSI (15/22, 68.18%) (Figure 3.6). The site of placement of a CVC can also contribute to the development of infection. The majority of CVCs used in the patients described here 12/22 (54.54 %) were inserted in either jugular or subclavian neck veins (Figure 3.7). Of the 22 patients in whom a CVC was identified as the source of the S. aureus BSI, the CVC was removed within 24 h in 12 patients (54.55%), within 48 h in three patients
(13.64%), greater than 48 h in three patients (13.64%). The time to removal was not documented in the case of four patients (18.18%).

Figure 3.4. Source of *S. aureus* BSI among 70 patients. An infected CVC was identified as the commonest source of *S. aureus* BSI among our cohort of patients (22/70, 31.43%)
Figure 3.5. *Type of prosthetic device identified as the source of S. aureus BSI.* Of the 35 patients in whom a prosthetic device was identified as the source of infection, the percentage of each type of device is represented.

Figure 3.6. *Types of CVC identified as the source of S. aureus BSI.* Of the 22 patients in whom a CVC was identified as the source of infection, the percentage of specific types of CVC are represented.
Figure 3.7. Access site of CVCs associated with the development of *S. aureus* BSI. Of the 22 patients in whom a CVC was identified as the source of infection, the percentages associated with each access site are represented.

3.2.3. Empiric antimicrobial treatment

Vancomycin was the empiric antimicrobial agent used most frequently for the treatment of *S. aureus* BSI (29/70, 41.43%), followed by flucloxacillin (19/70, 27.14%). Of note, other anti-staphylococcal agents being used in patients empirically were cefuroxime, piperacillin-tazobactam, co-amoxiclav, linezolid and daptomycin. One patient was not started on antimicrobial treatment empirically (1/70, 1.43%). The empiric agents used can be seen in Figure 3.8. In total, eight patients (11.43%) were in retrospect, given an inappropriate empiric agent (i.e. on an anti-staphylococcal agent such as cefuroxime but without cover for MRSA which was subsequently isolated from the patient).
Vancomycin was the commonest empiric antibiotic among our cohort of patients (29/70, 41.43%), which is in keeping with antimicrobial policy

3.2.4. Outcomes of S. aureus BSI

Four patients (5.7%) in the study died during the follow-up period, with three of these deaths being attributed to their underlying infection, while 66 (94.29%) survived their S. aureus BSI. As previously mentioned in Chapter II, a complicated infection was defined as the development of infective endocarditis (IE) or an alternative metastatic focus of infection such as osteomyelitis or persistent BSI despite at least three days of appropriate antibiotic therapy. Fifty-five patients (78.57%) had an uncomplicated BSI, while 15 patients (21.43%) developed a complicated infection (Table 3.1). Of the three patients in whom S. aureus BSI was identified as the cause of death, only two had a confirmed complicated BSI.
There did not appear to be a greater risk of death in patients with complicated infection ($2/15$ versus $1/55$, $p=0.11$).

IE was the commonest complication recorded (73.33%) (Figure 3.9). Two patients had persistently positive blood cultures at three days despite appropriate antimicrobial therapy but neither of these patients developed a metastatic focus of infection.

![Figure 3.9. Complications of S. aureus BSI. Of the 15 patients who went on to develop a complicated infection the percentages of each type of complication are represented.](image)

### 3.2.5. Patient factors associated with the development of complicated BSI

A number of patient factors have been associated with increased mortality, persistent BSI and complicated infection in patients with *S. aureus* BSI, such as increasing age, co-morbidities, prosthetic devices and community onset of infection (29,31-33,39,42). We examined a number of patient factors to determine if they
were significantly associated with the development of a complicated BSI. These results are summarized in Table 3.2.
Table 3.2. Characteristics of patients with complicated and uncomplicated *S. aureus* BSI

<table>
<thead>
<tr>
<th>Patient characteristic</th>
<th>Patients with uncomplicated BSI n=55 (%)</th>
<th>Patients with complicated BSI n=15 (%)</th>
<th>Relative risk (95%CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex</td>
<td>39 (70.9)</td>
<td>5 (33.33)</td>
<td>-</td>
<td>0.01</td>
</tr>
<tr>
<td>Age &gt;65yrs</td>
<td>23 (41.82)</td>
<td>6 (40)</td>
<td>-</td>
<td>1.0</td>
</tr>
</tbody>
</table>

**Comorbidities**

<table>
<thead>
<tr>
<th></th>
<th>Patients with uncomplicated BSI n=55 (%)</th>
<th>Patients with complicated BSI n=15 (%)</th>
<th>Relative risk (95%CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemodialysis</td>
<td>22 (40)</td>
<td>4 (26.66)</td>
<td>0.62</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.22-1.74)</td>
<td></td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>13 (23.64)</td>
<td>4 (26.66)</td>
<td>1.13</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.41-3.1)</td>
<td></td>
</tr>
<tr>
<td>COPD</td>
<td>6 (10.9)</td>
<td>4 (6.66)</td>
<td>2.18</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.86-5.51)</td>
<td></td>
</tr>
<tr>
<td>Cardiac disease</td>
<td>19 (34.55)</td>
<td>6 (40)</td>
<td>1.2</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.48-2.98)</td>
<td></td>
</tr>
</tbody>
</table>

**Source of BSI**

<table>
<thead>
<tr>
<th></th>
<th>Patients with uncomplicated BSI n=55 (%)</th>
<th>Patients with complicated BSI n=15 (%)</th>
<th>Relative risk (95%CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prosthetic device</td>
<td>31 (56.36)</td>
<td>4 (26.66)</td>
<td>0.36</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.13-1.03)</td>
<td></td>
</tr>
<tr>
<td>CVC</td>
<td>20(36.36)</td>
<td>2(13.33)</td>
<td>0.34</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.08-1.36)</td>
<td></td>
</tr>
<tr>
<td>PVC</td>
<td>10(18.18)</td>
<td>1(6.66)</td>
<td>0.38</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.06-2.62)</td>
<td></td>
</tr>
<tr>
<td>Community-acquired</td>
<td>6(10.9)</td>
<td>2(13.33)</td>
<td>1.19</td>
<td>0.79</td>
</tr>
<tr>
<td>BSI</td>
<td></td>
<td></td>
<td>(0.33-4.35)</td>
<td></td>
</tr>
<tr>
<td>Inappropriate</td>
<td>6(10.9)</td>
<td>2(13.33)</td>
<td>1.19</td>
<td>0.79</td>
</tr>
<tr>
<td>antimicrobial therapy</td>
<td></td>
<td></td>
<td>(0.33-4.35)</td>
<td></td>
</tr>
<tr>
<td>Persistent fever at 72 h*</td>
<td>3(7.3)</td>
<td>5(38.46)</td>
<td>5.26</td>
<td>0.012</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(1.45-19.06)</td>
<td></td>
</tr>
</tbody>
</table>

COPD=Chronic obstructive pulmonary disorder, CVC=Central venous catheter, PVC=Peripheral venous catheter

*The duration of fever was only documented in 54 of the 70 patients in the study (13/15 patients with complicated BSI and 41/55 patients with uncomplicated BSI), RR was calculated based on these 54 patients
The mean age of patients who developed a complicated infection was 58 y, while the mean age of patients with an uncomplicated infection was 59 y, suggesting that age is not associated with a greater risk of complicated infection. A large number of patients in our study had multiple co-morbidities. No significant difference in the rate of complications was detected when we compared haemodialysis patients and patients not on haemodialysis (4/26 versus 11/44, \( p=0.36 \)) (RR=0.62). In our study group DM was not associated with a higher rate of complicated BSI (4/17 versus 11/53, \( p=0.81 \)) (RR=1.13), nor were the presence of a number of other co-morbidities, such as ESRF (\( p=0.19 \)) (RR=1.83), COPD (\( p=0.1 \)) (RR=2.18) or cardiac disease (\( p=0.69 \)) (RR=1.2).

In our study cohort patients with community-onset of BSI were not more likely to develop a complicated infection than patients with either health-care associated infection (2/8 versus 7/28, \( p=1.00 \)) (RR=1) or nosocomial infection (2/8 versus 6/34, \( p=0.64 \)) (RR=1.42). Eighteen patients (25.71%) had no identifiable source of BSI, but these patients were not more likely to develop a complicated infection than patients with an identifiable source of infection (6/18 versus 9/52, \( p=0.19 \)) (RR=1.93).

The risk of developing a complicated infection was not found to be significantly higher in patients in whom a prosthetic device was identified as the source of the BSI compared to patients with non-prosthetic sources of infection (4/35 versus 11/35, \( p=0.078 \)) (RR=0.36). Only two of the patients with a CVC documented as the source of their BSI developed a complicated BSI; in both cases the CVC was removed within 24 h of the positive blood culture being taken. As the
majority of prosthetic devices were PVCs or CVCs, which were removed early in
the course of the BSI (68.18% removed within 48 h of the positive blood culture),
we reviewed the risk of complication in those patients who had a more permanent
prosthesis or in those whom the time to removal of the CVC was delayed. Five of
the 35 patients (14.29%) had a more permanent prosthesis (e.g. ICD, orthopaedic
prosthesis) or delayed removal of their CVC. Only one patient went on to develop a
complicated BSI, which was not found to be significantly higher than patients with a
non-prosthetic source of BSI, or a prosthetic source that was removed early in the
course of the infection (1/5 versus 14/65, p=1.0, RR=0.93). Again, the number of
patients with a more permanent prosthesis were small, and perhaps if a larger
number of patients were included in the analysis significance may have been
attained.

Eight patients received an inappropriate antimicrobial agent, two of whom
went on to develop a complicated BSI. The occurrence of a complicated BSI was not
found to be significantly higher in patients who received an inappropriate
antimicrobial agent (p=0.79, RR=1.19). Of the 55 patients with MSSA BSI in our
study, 27 received vancomycin as empiric antibiotic therapy. Five of the 27 went on
to develop a complicated infection (5/27 versus 6/28, p=1.0) (RR=0.86). We failed
to identify an increased risk of complicated BSI in those patients with MSSA BSI
treated empirically with vancomycin.

Fifteen patients in our study population developed a complicated BSI, five of
whom had persistent fever at 72 h despite appropriate antimicrobial therapy.
However, the duration of fever was not recorded in all patients (missing data in 16
patients), and if we compare the rate of complications in those patients whom we know had prolonged fever to those whose temperature settled quickly, there is a significant association between fever persisting for more than 72 h and the development of a complicated BSI ($p \leq 0.05$).

### 3.3. Discussion

Much work has been carried out to identify patient characteristics which adversely affect the outcome of *S. aureus* BSI, in order to predict outcome and to identify those patients requiring more aggressive management.

In this study, the greatest number of *S. aureus* BSIs occurred in the 71-80 year age bracket. This likely reflects, the increased incidence of BSI among the elderly (>$65$ y), including those caused by *S. aureus* which has been previously reported (145,146). It is not surprising that *S. aureus* BSI is more common in older patients as they have more frequent contact with healthcare facilities, age-related changes of the immune system, more co-morbidities, are more likely to have prosthetic devices and are more likely to be infected with MRSA (147-149). All of these factors have been associated with a worse outcome in *S. aureus* BSI (34,38,142,143). However, in terms of the clinical course of infection, in the present study, there was no significant association between age over 65 years and the development of a complicated BSI ($p=1.0$) (RR=0.94). Furthermore, in our study 2/3 (66.66%) deaths attributed to *S. aureus* BSI occurred in patients over 65 years of age but we did not detect a significant association between an age greater than 65
years and the risk of death due to *S. aureus* BSI (*p*=0.57) (OR=2.96, CI, 0.26-34.32). A number of studies have investigated the relationship between age and the outcomes of *S. aureus* BSI (31,39,43,150,151). McClelland *et al* (1999) found that older patients had higher total (OR=2.21) and attributable (OR=2.3) mortality from *S. aureus* BSI (31). Possible explanations for this higher associated mortality were that older patients were more likely to be infected with MRSA (which has been associated with a higher mortality than MSSA BSI), use of inappropriate empiric antibiotic therapy, use of vancomycin (which is less potent than other agents), absence of fever prior to diagnosis (which may have resulted in a delay in starting appropriate antimicrobial therapy) and the presence of underlying disease which has been associated with worse outcomes in patients with *S. aureus* BSI (31,34,142,143). Of the 29 patients in our study over the age of 65, eight (27.59%) were infected with MRSA, five (17.24%) received inappropriate empiric antibiotic therapy, eight (27.59%) were apyrexial (temperature less than 37.3°C) at the time the positive blood culture was taken, 13 (44.83%) received empiric vancomycin therapy and 24 (82.76%) had at least one co-morbidity. Fowler *et al* (2003) found that advancing age was significantly associated (OR=1.01) with complicated *S. aureus* BSI (150). Albur and colleagues (2011) found that age was significantly associated (*p*=0.006) with increased mortality at one and six months. The mean age of patients who survived and who died was 66.3±14.5 years and 78.9±10.2 years respectively at 1 month (*p*≤0.006), and 63.8±15.3 years and 77.9±9.6 years respectively at 6 months (*p*≤0.004) (39). Other studies have reported that patients over 60 years (RR=1.4) (Hill *et al* (2001)) and patients over 65 years had a higher
mortality rate (OR=1.02 (Laupland et al (2003), OR=2.0 (Mylotte et al (2000)) from S. aureus BSI (15,43,151). These studies suggest that patients over 65 years should be managed more aggressively in order to prevent the development of complications and to reduce mortality, with close monitoring of vancomycin levels in patients with MRSA BSI, the early removal of prosthetic sources of BSI and the prompt investigation to identify metastatic foci of infection.

The majority of patients, 52/70 (75%) in our study belonged to a medical specialty with renal patients accounting for 38.57% of these. However, this reflects the high number of patients with ESRF attending our hospital, which is the location of the national centre for renal transplantation. The high rates of S. aureus BSI among renal patients is most likely due to a combination of factors, including HD, increased healthcare contact, increased risk of MRSA carriage, presence of CVCs and severe underlying disease. Although a high number of patients (26/70, 37.14%) in our study with S. aureus BSI were on HD, these patients did not appear to be more likely to develop a complicated BSI compared to other patients (4/26 versus 11/44, p=0.39) (RR=0.62). This reflects the clinical experience, at least anecdotally, that although S. aureus BSI is more common in patients on HD, they rarely develop overt signs of sepsis when they become bacteraemic. Often a mild increase in temperature is the only manifestation of the bacteraemia. These patients generally respond well to treatment and in our experience they rarely go on to develop complications such as IE. This seems surprising as these patients have many of the risk factors associated with the development of complicated BSI, e.g. frequent
healthcare contact, increased carriage of MRSA, presence of CVCs and severe underlying disease (31,32,42,43). Our study suggests that renal patients and patients on HD are more likely to develop \textit{S. aureus} BSI but are not more likely to develop a complicated BSI.

Only 18 (25.71\%) patients were surgical patients, with eight belonging to the specialty of neurosurgery; the national neurosurgical centre is located in Beaumont Hospital. Surgical patients were not found to have an increased risk of complicated BSI compared to patients from other specialties. A previous study found that patients on a surgical service were more likely to develop a complicated BSI ($p=0.006$), however, the authors did not suggest this as a predictor for the development of complicated BSI (150). Perhaps the presence of prosthetic material puts them at increased risk of developing a complicated infection. Eight of the surgical patients in our study had a prosthetic device identified as the source of the BSI (PVC=4, CVC=3, joint prosthesis=1). This study has highlighted a worrying trend, as all four general surgical patients, included in the study, had a PVC that was later identified as the source of the BSI. Therefore these represent potentially very preventable infections.

We identified a number of co-morbidities in our study population, such as ESRF (35.71\%), DM (24.29\%), COPD (14.29\%) and autoimmune disease (8.57\%). We failed to identify a significant association between any of these conditions and the development of complicated BSI (Table 3.1). This is in contrast to other studies, which have identified a significant association between certain underlying medical
conditions and the development of complicated BSI such as DM and chronic renal failure (15,32,33,42,43).

Of the 70 patients with *S. aureus* BSI in the study, only eight (11.43%) had true community-onset BSI reflecting the high number of patients with previous exposure to a healthcare facility. A significant association between community-onset BSI and the development of a complicated BSI was not identified in our cohort of patients (RR=1.19). This is in contrast to previous, but larger, studies that found that community-onset BSI had a significant association with the development of complicated BSI (32,143,150,152).

We studied the source of BSI as a possible contributor to the clinical course of *S. aureus BSI*. Half of the patients included in our study had a prosthetic device identified as the source. However, having a prosthetic device as the source of the BSI was not associated with an increased risk of developing a complicated BSI. Again, this is in contrast to many studies that have identified prosthetic material as a source of BSI and as a risk factor for the development of complicated *S. aureus* BSI (31,32,42,150). The reason for this remains unclear. Perhaps, in our study, it is due to the high number of CVCs and PVCs that were removed once they were identified as the potential source of sepsis in this cohort of patients. In the majority of cases, 15/22 (68.18%), CVCs were removed within two days of the positive *S. aureus* blood culture. In the case of the two patients (2.86%), who had a prosthetic device that was not easily removed (e.g. ICD), one went on to develop a complicated BSI,
Many of the studies that identified a significant association between a prosthetic device and the development of complicated *S. aureus* BSI included a much greater number of patients, i.e. 234-724 patients (31,32,42,150). Perhaps, the small number of patients in our study contributed to the failure to identify a significant association between the development of a complicated *S. aureus* BSI and the presence of a prosthetic device.

In this study, the large number of patients in whom a PVC was identified as the source of sepsis, is a significant concern. In 11/70 (15.71%) patients an infected PVC was identified as the source of the BSI. Although we did not identify a significant association between PVC as a source of BSI, and the development of a complicated BSI, one patient with a PVC as the source of their BSI did develop a complication (IE). This high level of PVC associated BSI reflects the situation in our institution where the number of *S. aureus* BSIs due to infected PVCs is on the increase (2004 – 0.7%, 2010-13.8%) (Dr Margaret Morris-Downes, Surveillance Scientist, Beaumont Hospital, personal communication). This figure is considerably higher than the national average, where in 2010 infected PVCs were the source of *S. aureus* BSI in only 7.1% of all cases (Enhanced EARS-Net data, 2010). This is a preventable risk factor and we would expect rates of PVC infection to be much lower. A PVC care bundle (a collection of interventions that should be applied to the management of PVCs) was introduced in our hospital in 2010 and should prevent most such cases.

CVCs were the most common source of BSI in our study population, with a CVC being identified as the source of BSI in 22/70 (31.43%) patients. This is a
disappointing statistic given that CVC infection is a preventable complication. However, the rate of complicated BSI was not significantly higher in patients in whom a CVC was identified as the source of BSI (RR=0.34, CI, 0.08-1.36). The high number of dialysis patients in our study population accounts, to a large extent, for the large number of patients with CVC related BSI as 17 renal patients (62.96%) had a CVC identified as the source of their BSI.

The high number of CVC BSIs is in contrast to the low number of patients (3, 4.29%) in whom an arteriovenous fistula (AVF) was identified as the source of BSI. Although we do not have any denominator data to compare the total number of patients with CVCs and AVFs in our hospital, it is possible to argue that the rate of S. aureus BSI is significantly higher in patients with CVCs compared to AVFs. AVFs have been shown previously to be associated with lower rates of BSI compared to CVCs, our study supports this finding and highlights the importance of early AVF formation in renal patients who require dialysis in order to prevent S. aureus BSI (153,154).

More patients in our study who developed S. aureus BSI had CVCs inserted in neck veins (i.e. subclavian and jugular veins) than in the femoral vein. However, Marschall et al (2008) found that the femoral access site was associated with a greater risk of insertion site infection (155). Insertion into one of the neck veins is preferable. Mermel et al found that use of the jugular insertion site was more likely to be associated with the development of an insertion site infection than the subclavian site (156). The reason for this association is not clear. In our study, the two patients with CVCs identified as the source of sepsis who went on to develop a
complicated infection had jugular permacaths as their source of sepsis, while none of the patients with femoral lines went on to develop a complicated infection. However, it is important to take into account that only small numbers are included in this analysis.

The high rate of *S. aureus* BSI associated with the use of permacaths (68.18% of BSIs caused by CVCs, 21.43% of all BSIs) was highlighted in this study. This probably reflects the high number of HD patients included in our study population (26/70, 37.14%). A permacath is an in-dwelling venous catheter that is used for venous access in renal patients on HD. The risk of insertion site infection, and subsequent BSI, should be lower with permacaths as they are tunnelled under the skin and therefore should be less likely to become colonised with skin flora and subsequently infected.

In terms of antimicrobial treatment in this group of patients, we did not identify an increased risk of complicated BSI in those patients with MSSA BSI treated empirically with vancomycin (5/27 versus 6/28, *p*=1.0) (RR=0.86). This is reassuring given the current concerns regarding the use of vancomycin as empiric therapy in *S. aureus* BSI and its decreased efficacy against MSSA (157).

Of the 15 patients infected with MRSA, four (26.67%) went on to develop a complicated BSI, of which two (13.33%) received an inappropriate empiric agent (e.g. flucloxacillin), one (6.67%) received vancomycin empirically and one (6.67%) received daptomycin as empiric therapy. Overall, there did not appear to be an
increased risk of complicated BSI in those patients treated with vancomycin empirically.

There is much debate at present regarding the use of vancomycin in the treatment of serious MRSA infection for a number of reasons. Firstly, it has poor tissue penetration, secondly, there is a lack of activity against staphylococcal biofilms and finally, it also has a slow bactericidal effect. There is also the issue of the increasing prevalence of vancomycin resistant *S. aureus* (VRSA), glycopeptide intermediate *S. aureus* (GISA), hetero-glycopeptide intermediate resistant *S. aureus* (hGISA) and the phenomenon of ‘MIC creep’, which have all raised concerns with regard to the use of vancomycin (143,158-161). The encouraging results obtained with new anti-staphylococcal agents such as daptomycin, tigecycline, dalbavancin and ceftobiprole have raised further questions about the wisdom of continuing to use vancomycin as first line treatment for severe MRSA infections. Our findings would suggest that vancomycin is a suitable agent for the treatment of MRSA BSI provided that steps are taken to ensure that the isolate remains susceptible and that optimal serum levels are attained.

There was a lack of correlation between several patient characteristics investigated here and the clinical course of infection, despite reports in the literature of significant associations. While study design in different institutions may contribute to conflicting results, the disparities highlighted suggest caution should be exercised in using patient characteristics as predictors of outcome. The only patient characteristic found to be significantly associated with development of a
complicated BSI was a persistent temperature at 72 h ($p<0.05$). This association has been seen previously and highlights the importance of closely monitoring a patient’s response to therapy, with full identification and susceptibility testing of the infecting isolate, early repeat blood cultures and finally using the appropriate investigation(s) to identify secondary foci of infection (150,152).

3.4. Summary

- Age >65 y is a known risk factor for *S. aureus* BSI and this was reflected in this study
- HD patients are a group with significant risk for *S. aureus* BSI, but this study found they were not more likely to develop a complicated *S. aureus* BSI
- CVCs as a source of *S. aureus* BSI were most common in this patient cohort but were not associated with increased risk for complicated infection
- The empiric use of vancomycin, in both MSSA and MRSA BSI was not associated with the development of a complicated BSI
- Persistent fever (>72 h) was associated with the development of a complicated BSI
Chapter IV
Molecular Characterisation of
*S. aureus* Isolates causing BSI
4.1. Introduction

*S. aureus* isolates can be classified according to their genetic lineage, clonal complex (CC) or sequence type (ST), the presence of a particular capsule type (type 1, 5 or 8) or their *agr* type (*agr* I- *agr* IV) and these characteristics may contribute to the clinical outcome of infection. Furthermore, *S. aureus* strains express a wide variety of virulence determinants which cause invasive disease. The mechanism of action of certain virulence determinants and their role in clinical infection is well understood in some instances (e.g. toxic shock syndrome and toxic shock syndrome toxin-1: staphylococcal scalded skin syndrome and the exfoliative toxins). However, in the majority of cases the mechanism of action and role of virulence determinants is not clear and the reason why infection with one strain/isolate is more likely to result in a more severe infection is unknown. It is also not known to what extent the type of infecting organism (i.e. those from different genetic lineages e.g. isolates with different capsule types) and the virulence determinants it carries, impact on the clinical course of infection.

Extensive characterisation of bacterial genes has been facilitated by the development of DNA microarray systems. The StaphyType Kit (Alere Technologies, Jena, Germany) is one such systems that detects 334 *S. aureus* gene sequences which correspond to 185 genes and their alleles, including species markers, resistance genes, exotoxins, genes encoding MSCRAMMs, as well as the SCC*mec*, capsule and *agr* group typing markers (137). Analysis of this type allows extensive virulence characterisation of *S. aureus* isolates causing BSI in addition to their assignment to a CC. *Spa* typing is a highly discriminatory typing method
which distinguishes between strains of *S. aureus* and can be used to validate the genotype (CC type) assigned by the DNA microarray. This chapter describes the use of DNA microarray and *spa* typing to characterise *S. aureus* isolates causing BSI. These data were then used to determine the relationship between the carriage of virulence genes by different *S. aureus* lineages and the clinical outcome of infection.

4.2. Results

4.2.1. Molecular characterisation of *S. aureus* isolates causing BSI according to CC, *agr* type, capsule type and *spa* type.

As previously described in Chapter III, seventy patients with *S. aureus* BSI were recruited to the study. Of these, 15 (21.43%) were infected with MRSA, 55 (78.57%) were infected with MSSA, 15 (21.43%) developed a complicated BSI and 55 (78.57%) had an uncomplicated BSI. The CC types, *spa* types, *agr* and capsule types of all isolates are summarized in Table 4.1.

Using a combination of DNA microarray analysis and *spa*-typing, 14 CC types were identified with CC22 (20/70, 28.57%), CC45 (11/70, 15.71%) and CC30 (10/70, 14.29%) being the most prevalent. Assignment of isolates to multi-locus sequence type (MLST) groups (sequence types, STs) was inferred on the basis of *spa* types and comparison of hybridisation patterns with strains of known MLST type. All 15 MRSA isolates belonged to ST22. All isolates within a given CC belonged to the same capsule type and *agr* group. These associations have been previously described (137). However, all isolates belonging to a specific *agr* type
did not belong to the same CC or have the same capsule type. *spa* typing offered further discrimination among isolates within specific CCs with 43 *spa* types identified among 14 CC types.

*S. aureus* isolates causing BSI were grouped into eight clusters using BURP analysis (Figure 4.1). *spa* types were clustered together if they contained five or more *spa* repeats and if they had a cost value ≤ 4 where the cost accounts for the number of steps of evolution between *spa* types. A low cost value indicates close evolutionary relatedness between two *spa* types. Cluster group 1 contained six *spa* types (t032 (group founder), t515, t557, t1214, t3720 and t9570) which exhibited cost values ranging from 1-4. All but one of these *spa* types was represented by CC22 isolates (t3720 – CC5 isolate). *spa* types t015 (group founder), t1510, t1574, t2784, t9854 and t9899 (all represented by CC45 isolates except t9854 which was represented by a CC22 isolate) were found in cluster group 2 with cost values ranging from 1-3. *Spa* types t002, t067, t179 (group co-founder) and t548 (group founder) (all CC5 isolates, except t067 which was represented by a CC45 isolate) belonged to cluster group 3 and exhibited cost values ranging from 1-3. Cluster 4 contained four *spa* types t012 (group founder), t019, t021 (group co-founder) and t1872 which were all represented by CC30 isolates, and exhibited cost values ranging from 1-4. *Spa* types t084, t228 (group founder), t1885 and t4579 (all CC15 isolates) were found in cluster group 5 and had a cost value ranging from 1-4. *spa* types t160 and t2133 (both represented by CC12 isolates) belonged to cluster group 6 and exhibited a cost value of 3. *spa* types t065 and t3344 were both represented by CC45 isolates and were found in cluster 7. *spa* types t008 and t304 (both CC8
isolates) were found in cluster group 8 and exhibited a cost value of 4. The following 12 spa types were defined as singletons following BURP analysis (t078, t091, t127, t159, t189, t209, t230, t891, t1149, t2643, t5276 and t9566) as they could not be clustered with any other spa type i.e. cost greater than 5 and one spa type was excluded (t026). One isolate had an unknown spa type. MRSA isolates belonged to cluster group 1 (t032, t515, t557, t1214, t9570) and 2 (t9854), with one MRSA isolate being defined as a singleton (t5276).
Table 4.1. Strain assignment following microarray analysis and spa typing of 70 S. aureus isolates causing BSI from 2008-2011

<table>
<thead>
<tr>
<th>CC (n)</th>
<th>spa type</th>
<th>agr type</th>
<th>Capsule type</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC1(1)</td>
<td>t127</td>
<td>III</td>
<td>8</td>
</tr>
<tr>
<td>CC5 (7)</td>
<td>t002(3), t548(2), t179 (1), t3720 (1)</td>
<td>II</td>
<td>5</td>
</tr>
<tr>
<td>CC7 (3)</td>
<td>t091(2), t515 (1)</td>
<td>I</td>
<td>8</td>
</tr>
<tr>
<td>CC8 (3)</td>
<td>t008(2), t304 (1)</td>
<td>I</td>
<td>5</td>
</tr>
<tr>
<td>CC9 (1)</td>
<td>t209</td>
<td>II</td>
<td>5</td>
</tr>
<tr>
<td>CC12 (2)</td>
<td>t160 (1), t2133 (1)</td>
<td>II</td>
<td>8</td>
</tr>
<tr>
<td>CC15 (8)</td>
<td>t084 (4), t159 (1), t1885 (1), t228 (1), t4579 (1)</td>
<td>II</td>
<td>8</td>
</tr>
<tr>
<td>CC22 (20)</td>
<td>t032(7), t515 (2), t557(3), t1214(3), t002 (1), t891 (1), t5276 (1), t9570 (1), t9854 (1)</td>
<td>I</td>
<td>5</td>
</tr>
<tr>
<td>CC25 (1)</td>
<td>t078</td>
<td>I</td>
<td>5</td>
</tr>
<tr>
<td>CC30 (10)</td>
<td>t012(3), t021(2), t019 (1), t026 (1), t1872 (1), t2643 (1), t9566 (1)</td>
<td>III</td>
<td>8</td>
</tr>
<tr>
<td>CC45 (11)</td>
<td>t012 (1), t015 (1), t065 (1), t067 (1), t230 (1), t1510 (1), t1574 (1), t2784 (1), t3344 (1), t9899 (1), unknown (1)</td>
<td>I</td>
<td>8</td>
</tr>
<tr>
<td>CC121 (1)</td>
<td>t159</td>
<td>IV</td>
<td>8</td>
</tr>
<tr>
<td>CC188 (1)</td>
<td>t189</td>
<td>I</td>
<td>8</td>
</tr>
<tr>
<td>CC398 (1)</td>
<td>t1149</td>
<td>I</td>
<td>5</td>
</tr>
</tbody>
</table>

*a* Where more than one spa type was identified among isolates of a given CC, the number of isolates belonging to each spa type is shown in parenthesis

*b* The fifteen MRSA isolates in the study belonged to CC22, they were further characterised by the array as belonging to ST22-MRSA-IV
Figure 4.1. BURP analysis of *spa* types of *S. aureus* isolates causing BSI from 2008-2011. 43 *spa* types were differentiated into eight cluster groups using the BURP algorithm as described in section 2.9. Group founders and co-founders (*spa* types with the second highest group founder score) are shown in blue and are determined based on the *spa* type that shares the highest sequence identity with the greatest number of *spa* types within that cluster as determined by the cost values.
The size of the dots is indicative of the number of isolates. Cost values are shown in parenthesis.

4.2.2. Virulence and antibiotic resistance gene characterisation of S. aureus isolates causing BSI.

As described above (Section 4.2.1) the isolates analysed were genetically diverse but certain virulence genes were common to all isolates even though they belonged to different CCs. The haemolysins *hlg*, *hla* (except one CC22 isolate) and *hld*, the adhesion genes *clfA/B* (clumping factor A/B), *eno* (enolase, phosphopyruvate hydratase), *fib* (fibrinogen binding protein), *ebpS* (cell wall associated fibronectin-binding protein), *FnBPA* (FnBPA), *map* (major histocompatibility complex class II analog protein) (except one CC22 isolate), *sdrC* (ser-asp rich fibrinogen-binding, bone sialoprotein-binding protein C), the biofilm genes *icaA* (intercellular adhesion protein A), *icaC* (intercellular adhesion protein C), (except one CC22 isolate), *icaD* (biofilm PIA synthesis protein D), the immune evasion genes *isaB* (immunodominant antigen B) and *isdA* (heme/transferring-binding protein), were common to all isolates tested (Table 4.2). All isolates tested were negative for the antimicrobial resistance genes *ermB* (macrolide resistance), *aacA-aphD* (aminoglycoside resistance), *aphA* (aminoglycoside resistance), *sat* (streptothricin resistance), *dfrA* (trimethoprim resistance), *tetM* (tetracycline resistance), *cat* (chloramphenicol resistance), *cfr* (lincosamides, oxazolidinone resistance), *fexA* (chloramphenicol resistance) and *qacC* (efflux pump), and the immune evasion gene *hsdS1* (site specific deoxyribonuclease subunit type 1). All isolates were negative for the ACME (arginine catabolic mobile element) locus and
one isolate carried the gene for PVL toxin (Isolate 70 - CC15, t084). This isolate was identified in a renal patient who developed S. aureus BSI secondary to an infected permacath; she responded well to treatment and did not develop severe sepsis.

The CC that carried the most resistance genes was CC5 (six isolates in total), blaZ (β-lactamase) (6/6), tet efflux (tetracycline efflux protein) (6/6) and fosB (fosfomycin resistance) (6/6), ermC (macrolide resistance) (1/6), far1 (fusidic acid) (1/6) and tet (K) (tetracycline resistance) (1/6). The fosB gene was found in CCs 5, 12, 15, 25, 30, 45 and 121. With the exception of CC45 (1/11) all isolates within any given CC were positive for this gene.

Two CCs carried no toxin genes (CC7 and CC188) and CC45 carried the largest number of toxin genes (11 in total). The gene for toxic shock toxin (tst) was identified in 9/70 isolates (CC5, CC30, CC45) but none of the patients in the study had staphylococcal toxic shock syndrome. We also identified the genes for the exfoliative toxins (etA, etB, etD) in a number of isolates (7/70, CC9, CC12, CC15, CC25, CC45, CC121 and CC398) but no patients in the study exhibited signs of bullous impetigo or staphylococcal scalded skin syndrome. The enterotoxin gene cluster egc (seg, sei, sem, sen, seo, seu) was the most prevalent gene cluster identified (50/70, 71.43%) and was seen in 7/14 CCs. In all cases, every isolate within a given CC was positive for this cluster, suggesting clonal distribution. Other enterotoxin gene clusters identified were sek/q (2/70, CC1, CC8), sed/j/r (2/70, CC5, CC8), tst/sec/l (2/70, CC45) and sec/l (19/70, CC22, CC45). In contrast to the egc-cluster, the distribution of these other gene clusters was not clonal.
The immune evasion cluster (IEC) genes, which may include *sak* (encodes staphylokinase), *scin* (encodes staphylococcal complement inhibitor), *sea* (encodes enterotoxin A), *chp* (encodes chemotaxis inhibitory protein) and *sep* (encodes enterotoxin P) or combinations of these genes (referred to as IEC variants) are important virulence factors of *S. aureus* (162). An IEC variant was detected in 68 (97.14%) isolates and the most prevalent IEC type was type B (39/70, 55.71%).

The MSCRAMM encoding genes were highly prevalent and present in each of the CCs examined. Seven of the 15 MSCRAMM genes investigated were present in all 70 isolates with the single isolate belonging to CC1 containing all 15 MSCRAMM genes (Table 4.2). In the case of the MSCRAMM genes *cna* (collagen binding adhesion), *ebh* (extra-cellular matrix binding protein), *fib* (fibrinogen binding protein) and *sasG* (*S. aureus* surface protein G), distribution appeared to be clonal with all isolates within a given CC being positive for the gene encoding the protein.

Many of the other immune evasion genes (non-IEC genes) were also common to all isolates, with only one of the nine genes (*hsdS1*) investigated being absent from all isolates and *isaB* and *isdA* were common to all isolates tested. The *mprf* gene, which codes for lysylphosphatidylglycerol (L-PG) synthase, is responsible for the incorporation of lysine into phosphatidylglycerol. This results in the cell surface being more positively charged, making it less susceptible to killing by CAMPs, such as LL-37 (105). It was found in the majority of isolates, with the exception of some isolates belonging to CC45 (8/11) and the single isolate belonging to CC398.
4.2.3. Genetic characteristics of *S. aureus* isolates causing BSI according to the main CC types

The microarray results were analyzed according to the CC types most frequently encountered in the study.

CC22 was the most prevalent clone in our collection of isolates (20/70, 28.57%). Isolates belonging to CC22 were both methicillin susceptible (n=5) and methicillin resistant (n=15), with the MRSA isolates being further characterised as belonging to ST-22-MRSA-IV. All isolates belonged to capsule type 5, *agr* group I and IEC type B. Nine different *spa* types were identified among the 20 isolates (table 4.1). Only two antimicrobial resistance genes were carried by CC22 isolates, *blaZ* (19/20) and *ermC* (12/20). All isolates in this clone carried the toxin genes *seb* and the *egc*-cluster, with 14/20 isolates also carrying *sec/l*. The *hlb* gene was truncated in all isolates within this CC. One MRSA isolate in this CC had a *hla*, *map* and *icaC* genotype while the other 19 isolates were positive for these genes. This isolate belonged to ST-22-MRSA-IV, capsule type 5, *agr* group I, and *spa* type t032.

All isolates within CC45 belonged to capsule type 8, *agr* group I and IEC type B. Ten different *spa* types and one unknown type were identified among the 11 isolates (Table 4.1). Isolates within this CC carried the antimicrobial resistance genes *blaZ* (9/11), *ermA* (2/11), *far1* (1/11), *tet efflux* (11/11) and *fosB* (1/11). The presence of the *fosB* gene has not previously been reported in this CC. These isolates carried the greatest number of toxin genes (11 in total), which included *eta* (1/11), *tst/sec/l* (2/11), *seb* (2/11), *sec/l* (5/11), the *egc*-cluster (11/11) and *seo* (1/11).
Again carriage of the *egc*-cluster appeared to be clonal. Interestingly, *sasG* was absent from all isolates within this CC.

All isolates within CC30 belonged to capsule type 8 and *agr* group III. IEC types A (5/10), B (3/10) and C (1/10) and seven different *spa* types were identified among the 10 isolates (table 4.1). The antimicrobial resistance genes *blaZ* (10/10), *ermA* (2/10), *tet efflux* (10/10) and *fosB* (10/10) were most prevalent in this CC. A variety of toxin genes were carried by isolates belonging to CC30, including *tst* (6/10), *sea* (5/10) and the *egc*-cluster (9/10). The one isolate negative for the *egc*-cluster was also negative for the IEC genes and *hsdS3*, but it was the only isolate positive for the *fnbB* gene. This isolate belonged to *spa* type t2643.
Table 4.2. Molecular characterization by a DNA microarray of 70 *S. aureus* blood stream isolates

<table>
<thead>
<tr>
<th>CC (n)</th>
<th>Antimicrobial resistance genes</th>
<th>Toxin genes</th>
<th>Haemolysins</th>
<th>IEC genes</th>
<th>MSCRAMM, adhesion &amp; biofilm genes</th>
<th>Immune evasion genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC1</td>
<td>Q6GD50, tet efflux</td>
<td>sea, seh, sek/q</td>
<td>hlb, hIII</td>
<td>sea/sak/scn (IEC type D)</td>
<td>bhp, cna, ebh, fnbB, sdrD, vwb, sasG</td>
<td>mprf, hsdS2, hsdS3, hsdSx, QFXC0</td>
</tr>
<tr>
<td>CC5</td>
<td>blaZ, ermC (1), far1 (1), tet(K) (1), tet efflux, fosB</td>
<td>tst (1), sea (1), sed/j/r (1), egc</td>
<td>hlb, hIII</td>
<td>sep/sak/chp/scn (6; IEC type F), sea/sak/sep/scn (1; IEC type D)</td>
<td>bhp, ebh, fnbB, sdrD, vwb, sasG</td>
<td>mprf, Q2YUB3(2), hsdS2, hsdS3, hsdSx</td>
</tr>
<tr>
<td>CC7</td>
<td>blaZ (2), aadD (1), tet efflux</td>
<td>hlb, hIII</td>
<td>sep/sak/chn (2; IEC type G)</td>
<td>bhp, ebh, fnbB, sdrD (2), vwb</td>
<td>mprf, Q2YUB3 (1), hsdS2 (2), hsdSx</td>
<td></td>
</tr>
<tr>
<td>CC8</td>
<td>blaZ (2), tet efflux</td>
<td>sed/j/r (1), sek/q (1), egc</td>
<td>hlb, hIII</td>
<td>sak/chp/scn (2; IEC type B), sak/scn (1; IEC type E)</td>
<td>bhp, ebh, fnbB, sdrD, vwb, sasG</td>
<td>mprf, hsdS2, hsdS3, hsdSx</td>
</tr>
<tr>
<td>CC9</td>
<td>blaZ, ermA, tet efflux</td>
<td>eta, egc</td>
<td>hlb, hIII</td>
<td>sak/chp/scn (IEC type B)</td>
<td>ehh, fnbB, sdrD, vwb</td>
<td>mprf, Q2YUB3(1), hsdS2, hsdS3, hsdSx</td>
</tr>
<tr>
<td>CC12</td>
<td>blaZ (1), tet efflux, fosB</td>
<td>ehh, hIII</td>
<td>sep/sak/chn (2; IEC type G)</td>
<td>bhp (6), ebh, fnbB, sdrD (7), vwb, sasG</td>
<td>Mprf, Q2YUB3 (1), hsdS2 (1), hsdSx</td>
<td></td>
</tr>
<tr>
<td>CC15</td>
<td>ermC (2), aadD (1), tet efflux, fosB, qacA (1)</td>
<td>eta (1), pvl (1)</td>
<td>hIII</td>
<td>chp/scn (IEC type C)</td>
<td>bhp (6), ebh, fnbB, sdrD (7), vwb, sasG</td>
<td>mprf, Q2YUB3(1), hsdS2, hsdS3, hsdSx</td>
</tr>
<tr>
<td>CC22</td>
<td>blaZ (19), ermC (12)</td>
<td>seb, sec/l (14), egc</td>
<td>hlb</td>
<td>sak/chp/scn (IEC type B)</td>
<td>bhp (18), cna, fnbB (1), sdrD (19), vwb</td>
<td>mprf, Q2YUB3 (1), hsdS2, hsdS3 (4), hsdSx (13)</td>
</tr>
<tr>
<td>CC25</td>
<td>tet efflux, fosB</td>
<td>etd, egc</td>
<td>hlb, hIII</td>
<td>sak/chp/scn (IEC type B)</td>
<td>bhp, ebh, fnbB, sdrD, vwb</td>
<td>mprf, hsdS2, hsdS3, hsdSx</td>
</tr>
<tr>
<td>CC30</td>
<td>blaZ, ermA (2), tet efflux, fosB</td>
<td>tst (6), sea (5), egc (9)</td>
<td>hlb, hIII</td>
<td>sea/sak/chp/scn (5; IEC type A), sak/chp/scn (3; IEC type B), chp/scn (1; IEC type C)</td>
<td>bhp, cna, ebh, fnbB (1), sdrD (8), vwb</td>
<td>mprf, Q2YUB3 (2), hsdS2, hsdS3 (9), hsdSx</td>
</tr>
<tr>
<td>CC45</td>
<td>blaZ (9), ermA (2), far1 (1), tet efflux, fosB</td>
<td>eta (1), tst/sec/l (2), seb (2), sec/l (5), egc, se (1), eth, sec, egc</td>
<td>hlb (2), hIII</td>
<td>sak/chp/scn (IEC type B)</td>
<td>bhp (8), cna, ebh, fnbB (10), sdrD (9), vwb (7)</td>
<td>mprf (8), Q2YUB3 (4), hsdS2, hsdS3, hsdSx (9)</td>
</tr>
<tr>
<td>CC121</td>
<td>mupR, tet efflux, fosB</td>
<td>hlb, hIII</td>
<td>sak/scn (IEC type E)</td>
<td>bhp, cna, ebh, fnbB, vwb, sdrD, vwb</td>
<td>mprf, hsdS2, hsdS3, hsdSx</td>
<td></td>
</tr>
<tr>
<td>CC188</td>
<td>blaZ, tet efflux</td>
<td>hlb, hIII</td>
<td>sak/scn (IEC type E)</td>
<td>bhp, cna, ebh, fnbB, vwb, sdrD, vwb</td>
<td>mprf, hsdS2, hsdS3, hsdSx</td>
<td></td>
</tr>
<tr>
<td>CC398</td>
<td>tet efflux</td>
<td>etd (1), sak/chp/scn (IEC type B)</td>
<td>ehh, fnbB, sdrD, vwb</td>
<td>hsdS2, hsdSx</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The number of positive isolates are indicated in parenthesis if not all isolates within a CC were positive for the gene indicated. All isolates harboured the following genes: hlg, hla (except one CC22 isolate), hld, clfA, clfB, eno, fib, ebpS, fnbA, map (except one CC22 isolate), sdrC, icaA, icaC (except one CC22 isolate), icaD, isaB, isdA.

4.2.4. Comparison of S. aureus isolates causing complicated and uncomplicated BSI

As previously described in Chapter III, 15 patients (21.43%) developed a complicated BSI. Of these isolates causing complicated BSI, there were both methicillin resistant and methicillin sensitive isolates which belonged to a variety of CCs, agr groups, capsule types and spa types (Table 4.3). No single spa type was associated with the development of a complicated BSI. In fact, all 15 complicated BSIs were caused by isolates belonging to different spa types (t012, t015, t019, t021, t032, t091, t160, t304, t515, t548, t4579, t5276, t9570, t9854, unknown). We failed to identify a significant association between any type of S. aureus isolate and the development of a complicated BSI. This lack of significance persisted even when MSSA isolates were analysed alone.
The carriage of antimicrobial resistance, toxin, IEC, MSCRAMM and immune evasion genes in *S. aureus* isolates causing complicated and uncomplicated BSI were next analysed (Table 4.4). We did not detect a significant association between isolates causing complicated BSI and carriage of any of the virulence genes. This lack of significance persisted even when we analysed MSSA isolates alone.
Table 4.4. Virulence genes associated with 70 S. aureus isolates causing complicated and uncomplicated BSI

<table>
<thead>
<tr>
<th>Virulence gene</th>
<th>Infection type</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. (%) of isolates</td>
<td>Complicated (n=15)</td>
<td>Uncomplicated (n=55)</td>
<td>Relative Risk (95% CI)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Toxin genes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>tst-1</strong></td>
<td></td>
<td>1 (6.66)</td>
<td>8 (14.55)</td>
<td>0.4841 (0.07-3.25)</td>
</tr>
<tr>
<td><strong>lukF-PV, lukS-PV</strong></td>
<td></td>
<td>0</td>
<td>1 (1.82)</td>
<td>1.13 (0.1-12.96)</td>
</tr>
<tr>
<td><strong>etA</strong></td>
<td></td>
<td>0</td>
<td>3 (5.45)</td>
<td>0.55 (0.04-7.6)</td>
</tr>
<tr>
<td><strong>etB</strong></td>
<td></td>
<td>1 (6.66)</td>
<td>1 (1.82)</td>
<td>2.43 (0.56-10.48)</td>
</tr>
<tr>
<td><strong>etD</strong></td>
<td></td>
<td>0</td>
<td>2 (3.64)</td>
<td>0.74 (0.06-9.68)</td>
</tr>
<tr>
<td><strong>sea</strong></td>
<td></td>
<td>2 (13.33)</td>
<td>5 (9.09)</td>
<td>1.38 (0.39-4.91)</td>
</tr>
<tr>
<td><strong>seb</strong></td>
<td></td>
<td>1 (6.66)</td>
<td>4 (7.27)</td>
<td>0.93 (0.15-5.69)</td>
</tr>
<tr>
<td><strong>sec</strong></td>
<td></td>
<td>7 (46.67)</td>
<td>17 (30.91)</td>
<td>1.68 (0.69-4.07)</td>
</tr>
<tr>
<td><strong>sed/j/r</strong></td>
<td></td>
<td>0</td>
<td>2 (3.64)</td>
<td>0.74 (0.057-9.68)</td>
</tr>
<tr>
<td><strong>see</strong></td>
<td></td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td><strong>seg, sei, sem, sen, seo, seu</strong></td>
<td></td>
<td>10 (66.66)</td>
<td>40 (72.73)</td>
<td>0.8 (0.31-2.05)</td>
</tr>
<tr>
<td><strong>sek/q</strong></td>
<td></td>
<td>0</td>
<td>2 (3.64)</td>
<td>0.74 (0.06-9.68)</td>
</tr>
<tr>
<td><strong>sel</strong></td>
<td></td>
<td>6 (40)</td>
<td>15 (27.27)</td>
<td>1.56 (0.63-3.82)</td>
</tr>
<tr>
<td><strong>IEC genes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>sak</strong></td>
<td></td>
<td>12 (80)</td>
<td>47 (85.45)</td>
<td>0.75 (0.25-2.22)</td>
</tr>
<tr>
<td><strong>chp</strong></td>
<td></td>
<td>12 (80)</td>
<td>47 (85.45)</td>
<td>0.75 (0.25-2.22)</td>
</tr>
<tr>
<td><strong>scn</strong></td>
<td></td>
<td>14 (93.33)</td>
<td>54 (98.18)</td>
<td>0.41 (0.1-1.78)</td>
</tr>
<tr>
<td><strong>sep</strong></td>
<td></td>
<td>3 (20)</td>
<td>7 (12.73)</td>
<td>1.5 (0.51-4.39)</td>
</tr>
<tr>
<td><strong>MSCRAMM genes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>bbp</strong></td>
<td></td>
<td>13 (86.66)</td>
<td>45 (81.82)</td>
<td>1.35 (0.35-5.2)</td>
</tr>
<tr>
<td><strong>cna</strong></td>
<td></td>
<td>10 (66.66)</td>
<td>36 (65.45)</td>
<td>1.04 (0.4-2.7)</td>
</tr>
<tr>
<td><strong>ebh</strong></td>
<td></td>
<td>11 (73.33)</td>
<td>39 (70.9)</td>
<td>1.1 (0.4-3.05)</td>
</tr>
<tr>
<td><strong>fnbB</strong></td>
<td></td>
<td>7 (46.67)</td>
<td>34 (61.82)</td>
<td>0.62 (0.25-1.5)</td>
</tr>
<tr>
<td><strong>sasG</strong></td>
<td></td>
<td>7 (46.67)</td>
<td>32 (58.18)</td>
<td>0.7 (0.28-1.7)</td>
</tr>
</tbody>
</table>
4.2.5. Comparison of MRSA and MSSA isolates causing BSI

All 15 MRSA isolates analysed belonged to ST-22-MRSA-IV, *agr* group I, capsule type 5, while the MSSA isolates analysed belonged to a variety of CCs, *agr* groups and capsule types. This strain of MRSA is the predominant strain circulating in Irish hospitals at present and is the most common cause of HA-MRSA in this country (21,163). Although all MRSA isolates belonged to ST22 we were able to further discriminate between these isolates with *spa* typing. Here, seven different *spa* types were represented among the 15 MRSA isolates (t032 (6), t1214 (2), t515 (2), t557 (2), t5276, t9570, t9854).

Of the 15 patients with MRSA BSI, four (26.67%) went on to develop a complicated BSI while 11 (73.33%) had an uncomplicated BSI. The rate of complicated BSI was not significantly higher in patients infected with MRSA (RR=1.33, CI, 0.49-3.59) (*p*=0.72). None of the patients who died were infected with MRSA.

No antimicrobial resistance, toxin, IEC, adhesin or immune evasion genes were specific to MRSA isolates and the MRSA isolates carried similar virulence genes to MSSA isolates. However, when carriage of certain antimicrobial resistance and virulence genes by MRSA and MSSA isolates were compared, a significant association between the carriage of the *sec/l* cluster (12/15 *versus* 9/55, *p*=0.0001), the *egc*-cluster (15/15 *versus* 35/55, *p*<0.005), *ermC* (10/15 *versus* 5/55, *p*=0.0001), *cna* (15/15 *versus* 31/55, *p*=0.001) and *sasG* (15/15 *versus* 24/55, *p*=0.0001) for MRSA isolates was found (table 4.5). There was also a significant association with
carriage of *ebh* (0/15 *versus* 41/55, *p*=0.0001) and *fnbB* (0/15 *versus* 41/55, *p*=0.0001) in MSSA isolates compared to MRSA isolates (Table 4.5).

### Table 4.5. Virulence genes associated with 70 MRSA and MSSA isolates causing BSI

<table>
<thead>
<tr>
<th>Virulence gene</th>
<th>MRSA (n=15)</th>
<th>MSSA (n=55)</th>
<th><em>p</em> value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Toxin genes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>tst-I</em></td>
<td>0</td>
<td>9 (16.36)</td>
<td>0.19</td>
</tr>
<tr>
<td><em>lukF-PV, lukS-PV</em></td>
<td>0</td>
<td>1 (1.82)</td>
<td>1.0</td>
</tr>
<tr>
<td><em>etA</em></td>
<td>0</td>
<td>3 (5.45)</td>
<td>1.0</td>
</tr>
<tr>
<td><em>etB</em></td>
<td>0</td>
<td>2 (3.64)</td>
<td>1.0</td>
</tr>
<tr>
<td><em>etD</em></td>
<td>0</td>
<td>2 (3.64)</td>
<td>1.0</td>
</tr>
<tr>
<td><em>sea</em></td>
<td>0</td>
<td>7 (12.73)</td>
<td>0.33</td>
</tr>
<tr>
<td><em>seb</em></td>
<td>0</td>
<td>5 (9.09)</td>
<td>0.58</td>
</tr>
<tr>
<td><em>sec</em></td>
<td>12 (80)</td>
<td>12 (21.82)</td>
<td>0.0001*</td>
</tr>
<tr>
<td><em>sed/j/r</em></td>
<td>0</td>
<td>2 (3.64)</td>
<td>1.0</td>
</tr>
<tr>
<td><em>see</em></td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td><em>seg, sei, sem, sen, seo, seu</em></td>
<td>15 (100)</td>
<td>35 (63.64)</td>
<td>0.004*</td>
</tr>
<tr>
<td><em>sek/q</em></td>
<td>0</td>
<td>2 (3.64)</td>
<td>1.0</td>
</tr>
<tr>
<td><em>sel</em></td>
<td>12 (80)</td>
<td>9 (16.36)</td>
<td>0.0001*</td>
</tr>
<tr>
<td><strong>IEC genes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>sak</em></td>
<td>15 (100)</td>
<td>44 (80)</td>
<td>0.1</td>
</tr>
<tr>
<td><em>chp</em></td>
<td>15 (100)</td>
<td>44 (80)</td>
<td>0.1</td>
</tr>
<tr>
<td><em>scn</em></td>
<td>15 (100)</td>
<td>53 (77.09)</td>
<td>1.0</td>
</tr>
<tr>
<td><em>sep</em></td>
<td>0</td>
<td>10 (18.18)</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>MSCRAMM genes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>bbp</em></td>
<td>14 (93.33)</td>
<td>44 (80)</td>
<td>0.44</td>
</tr>
<tr>
<td><em>cna</em></td>
<td>15 (100)</td>
<td>31 (56.36)</td>
<td>0.001*</td>
</tr>
<tr>
<td><em>ebh</em></td>
<td>0</td>
<td>50 (90.91)</td>
<td>0.0001*</td>
</tr>
<tr>
<td><em>fnbB</em></td>
<td>0</td>
<td>41 (74.55)</td>
<td>0.0001*</td>
</tr>
<tr>
<td><em>sasG</em></td>
<td>15 (100)</td>
<td>24 (43.64)</td>
<td>0.0001*</td>
</tr>
</tbody>
</table>

*Significant association
4.2.6. MRSA isolates causing BSI in renal patients over a five year period

The clonal nature of the MRSA isolates in this study may explain why we failed to detect any significant differences in terms of outcome of infection. In order to assess the outcomes and associations of MRSA isolates from a greater variety of ST types we carried out a retrospective review of MRSA BSI in renal patients over a five year period (2005-2009). Renal patients are at greater risk of MRSA BSI due to impaired immune function, regular contact with healthcare facilities and the presence of central venous catheters (CVCs). As previously discussed (Chapter III), in our prospective S. aureus BSI study renal patients accounted for a high proportion of patients recruited (27/70, 38.57%).

As part of this retrospective study 36 MRSA BSI isolates recovered from renal patients (19 female and 17 male) from 2005-2009 were investigated. The median age was 68 and 28 patients (78%) were on haemodialysis. The sources of BSI are listed in Table 4.6. Secondary foci of infection were identified in six (16.7%) patients and included osteomyelitis (1/36, 2.8%), IE (3/36, 8.3%) and implantable cardiac device (ICD) infection (2/36, 5.6%) (Table 4.6).
Table 4.6. Infection types found in renal patients

<table>
<thead>
<tr>
<th>Infection type</th>
<th>n=36 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Source of BSI</strong></td>
<td></td>
</tr>
<tr>
<td>Central venous catheter</td>
<td>26 (72.2)</td>
</tr>
<tr>
<td>Skin and soft tissue infection</td>
<td>2 (5.6)</td>
</tr>
<tr>
<td>Infected peripheral vascular catheter</td>
<td>2 (5.6)</td>
</tr>
<tr>
<td>Infective endocarditis</td>
<td>1 (2.8)</td>
</tr>
<tr>
<td>Surgical site infection</td>
<td>1 (2.8)</td>
</tr>
<tr>
<td>Intra-abdominal infection</td>
<td>1 (2.8)</td>
</tr>
<tr>
<td>Not identified</td>
<td>3 (8.3)</td>
</tr>
<tr>
<td><strong>Secondary foci of infection</strong></td>
<td></td>
</tr>
<tr>
<td>Osteomyelitis</td>
<td>1 (2.8)</td>
</tr>
<tr>
<td>Infective endocarditis</td>
<td>3 (8.3)</td>
</tr>
<tr>
<td>Implantable cardiac device</td>
<td>2 (5.6)</td>
</tr>
</tbody>
</table>

4.2.6.1. Genetic characterisation of MRSA isolates causing BSI in renal patients (ST, spa and SCCmec types)

The majority of renal isolates belonged to ST22-MRSA-IV (27/36, 75%), which consisted of nine spa types with t032 predominating (12/27, 44.4%) (Table 4.7). Five isolates (5/36, 13.9%) were ST5-MRSA-II and spa type t463, three (3/36, 8.3%) were ST8, spa type t190 and also harbored SCCmec IIE & ccrAB4, or a possible novel SCCmec II subtype, and one isolate belonged to ST30-MRSA-IV and spa-type t1662. All MRSA BSI isolates from renal patients recovered since 2008 belonged to ST22-MRSA-IV (reflecting the type of MRSA isolates identified in our prospective study which commenced in 2008) whereas in the three years prior to
2008, 18/27 (66.66%) belonged to ST22-MRSA-IV with the remainder consisting of several minor clones (Table 4.7).

BURP analysis identified eight spa types (t025, t032 (group founder), t515, t557, t1214, t2945, t3185, t7636) in one cluster with three spa types (t190, t1662 and t5420) being identified as singletons (Figure 4.2). All spa types in cluster 1 were represented by isolates belonging to CC22, while the singletons were represented by isolates belonging to CC8, CC22 and CC30. One spa type was excluded from the analysis (t463).

Figure 4.2. BURP analysis of spa types of S. aureus isolates causing BSI in renal patients from 2005-2009. Eight spa types were identified as belonging to one cluster group using the BURP algorithm as described in section 2.9. Group founders and co-founders (spa types with the second highest group founder score) are shown in blue and are determined based on the spa type that shares the highest sequence identity with the greatest number of spa types within that cluster as determined by the cost values. The size of the dots is indicative of the number of isolates. Cost values are shown in parenthesis.
4.2.6.2. Virulence and antimicrobial resistance gene characterisation of MRSA isolates causing BSI in renal patients

The egc-cluster was found in all renal isolates causing BSI, apart from those belonging to ST8 (33/36, 92%). The toxic-shock-toxin (tst) gene was found in all ST5-MRSA-II and ST30-MRSA-IV isolates, but the gene combination tst, sea, sed, sej and ser, was exclusive to ST5-MRSA-II isolates and this ST carried more enterotoxin genes than the other STs. The sec/sel cluster was only present in a proportion of the ST22-MRSA-IV isolates (16/27, 59.3%) but none of the other STs. An IEC variant was detected in 80% of isolates (29/36) including 22/27 (81%) ST22-MRSA-IV, 1/3 (33.3%) ST8 and all ST5-MRSA-II and ST30-MRSA-IV isolates.

MRSA BSI, with an ST22-MRSA-IV isolate, was a cause of death in one patient. In the six patients who developed a secondary focus of infection, the infecting isolates belonged to ST22-MRSA-IV (4/6, 66.6%) and ST5-MRSA-II (2/6, 33.3%). The development of a secondary focus of infection was not significantly associated with any particular ST, but the highest rate of complications was seen among ST5-MRSA-II isolates (2/5 isolates, 40%) compared to ST22-MRSA-IV (4/27 isolates, 15%). This earlier clone carried the greatest number of enterotoxin genes including sea. Interestingly, the ST5-MRSA-II isolates harboured more antimicrobial resistance genes than ST22-MRSA-IV, but the greatest number of resistance genes was detected in the ST8 isolates. The antibiotic resistant genes fosB and tet efflux were present in ST5-MRSA-II, ST8 and ST30 isolates. While nine of the MSCRAMM, adhesin and biofilm genes investigated were detected in all
isolates, only the ST22-MRSA-IV and ST30-MRSA-IV isolates harboured cna while lacking the fib and fnbB genes. The ST22-MRSA-IV isolates also had an ebh- genotype.

Table 4.7. Molecular characteristics of 36 MRSA bloodstream isolates from renal patients recovered between 2005 and 2009

<table>
<thead>
<tr>
<th>ST</th>
<th>SCCmec type (n)</th>
<th>spa type (n)</th>
<th>agr/ capsule type</th>
<th>Antimicrobial resistance genesa</th>
<th>Virulence-associated genesa</th>
<th>MSCRAMM, adhesin &amp; biofilm genesa</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST22</td>
<td>IV (27)</td>
<td>t025(1), t032(12), t515(3), t557(3), t1214(3), t2945(2), t3185(1), t5420(1), t7636(1)</td>
<td>I/5</td>
<td>erm(C) (21), lnu(A)/aacA-aphD/aadD/mupA (1)</td>
<td>seb (2), sec/l (16), egc, sak/chp/scn (22; IEC type B), ACME (1)</td>
<td>bhp (25), cna, map, sdrC, sdrD (26), sasG</td>
</tr>
<tr>
<td>ST5</td>
<td>II</td>
<td>t463</td>
<td>II/5</td>
<td>erm(A), aadD, tet efflux, fosB, merA &amp; merB (1)</td>
<td>tst, sed/j/r, egc, sea/sak/chp/scn (IEC type A)</td>
<td>bhp, ebh, fib, fnbB, map, sdrC, sdrD, sasG</td>
</tr>
<tr>
<td>ST8</td>
<td>IIE &amp; ccrAB4 (2) Novel II subtype (1)b</td>
<td>t190</td>
<td>I/5</td>
<td>erm(A), tet efflux, fosB, qacA, aacA-aphD, aadD/aphA3-sat (2), merA &amp; merB (2)</td>
<td>sea/sak/scn (IEC type D)</td>
<td>bhp, ebh, fib, fnbB (2), map (2), sdrD</td>
</tr>
<tr>
<td>ST30</td>
<td>IV</td>
<td>t1662</td>
<td>III/8</td>
<td>Q6GD50 (fusC), tet efflux, fosB</td>
<td>tst, egc, sak/chp/scn (IEC type B)</td>
<td>bhp, cna, ebh, fib, map, sdrC, sdrD</td>
</tr>
</tbody>
</table>

*The number of positive isolates are indicated in parenthesis if not all isolates within a genotype were positive for the gene indicated.*
Possible novel SCCmec II subtype identified in one ST8 MRSA isolate that yielded signals for class A mec complex, ccrAB2 but lacked signals for kdp and aadD (pUB110). All isolates harbored the beta-lactamase resistance gene blaZ and the MSCRAMM, adhesin and biofilm genes icaA, icaC & icaD, clfA & clfB, ebpS, eno, fnbA and vwb.

4.3. Discussion

4.3.1. Molecular characterisation of S. aureus isolates causing BSI

In recent years many advances have allowed the detailed assessment of the virulence potential of infecting S. aureus isolates. This has helped trace the route of spread and determine the genetic relatedness of clinical isolates. Molecular typing methods, such as the DNA microarray allow for the rapid detection of a large number of antimicrobial resistance genes, toxin encoding genes, MSCRAMM encoding genes, immune evasion genes as well as SCCmec, capsule and agr group typing markers, in turn allowing us to assign isolates to specific clonal groups based on these genotypic characteristics (137). It is possible that by correlating these findings with the clinical manifestations of infection, their impact on the clinical course of S. aureus infections can be assessed. Various different typing methods allow discrimination between strains of S. aureus, determine the genetic relatedness of isolates, monitor spread and help assess the clinical outcomes of infections caused by different strains. In this study the virulence potential and type of 70 S. aureus isolates causing BSI was assessed.

It was found that MSSA isolates belonged to a variety of clonal complexes and spa types, while MRSA isolates were highly clonal as all belonged to ST22-MRSA-IV. Isolates were further discriminated within a given CC or ST using spa
typing, indicating that *spa* typing is more discriminatory than ST typing. Burp analysis allowed us to assess the relatedness of isolates belonging to different *spa* types. The majority of MRSA isolates belonged to cluster group 1 (13/15, 65%).

There appeared to be great genetic diversity both between and within CCs. Similar to our findings, recent characterisation of other *S. aureus* isolate collections has indicated that there is a strong clonal association of virulence genes including the *egc* cluster and IEC variants (78,164). While carriage of certain virulence genes in our study appears to be clonal (e.g. *egc*-cluster), carriage of other enterotoxin genes, antimicrobial resistance genes or other immune evasion genes appear not to be (e.g. *sea, ermC* and *mprF*). Interestingly, we also detected carriage of the *fosB* gene, which encodes fosfomycin resistance, in CCs 7, 12, 15, 25, 30, 45 and 121. This is, for the most part, a chromosomal gene, but it may also be carried on a plasmid (137). Previously, this gene has been found to be specific for certain CCs but to date this gene has not been associated with CC45 or CC121 (165). The correlation between carriage of specific virulence genes and clinical outcome remains unclear because host and other factors must be considered in such an evaluation.

Although genes may be carried by an infecting isolate they may only be expressed under certain conditions in the host during invasion and the course of the infection. The extent of virulence gene expression may also contribute to the clinical outcome, but it is technically challenging to reliably determine gene expression *in vitro* that truly reflects the *in vivo* setting. Another project, undertaken in our institution using isolates collected as part of this study, measured the
transcript levels of three genes – sak, scin and hlg in patients with complicated and uncomplicated BSI using quantitative real time reverse transcription PCR (qRT-PCR). The study found that transcript levels varied from isolate to isolate and that the transcript levels of the three genes were higher, but not significantly so, in isolates from patients with complicated BSI compared to uncomplicated BSI (unpublished data). A larger study, which looked at the expression of SEB in S. aureus isolates found that toxin production varied by as much as 200-fold between strains and a 15-fold variability in serial isolates of the same clone collected from the same patient on different days (166). This suggests that gene expression, which is regulated by global regulatory systems (e.g. agr, sar, srrAB), is highly variable and dependent on a variety of in vivo factors such as iron availability, protein levels, pH, CO₂ levels, O₂ levels and perhaps many more, as yet unknown host factors (167,168).

It is well established that certain clinical manifestations of staphylococcal disease such as toxic shock syndrome, staphylococcal scalded skin syndrome and staphylococcal food poisoning are due to the expression of specific toxins by S. aureus isolates (TSST-1/SEB/SEC, exfoliative toxins A, B and D and enterotoxins A, B and C, respectively) (31-33,35,36). However, beyond these associations little is known about the exact molecular basis of invasive or systemic S. aureus disease (such as BSI). However, our study suggests that the associated genes (tst, seb, sec, etA, etB, sea, seb and sec) may be present but do not contribute to the clinical manifestation of infection in all cases.
4.3.2. Comparison of molecular characteristics of *S. aureus* isolates causing complicated and uncomplicated BSI

*S. aureus* isolates from BSI were investigated to assess if any bacterial factors were associated with the development of a complicated BSI. Previous work by Fowler *et al* (2007) identified CC5, CC30 and CC15 as having a significant association with the development of haematogenous complications (*p*=0.0025, *p*=0.0308 and *p*=0.0146 respectively) (38). Other studies have identified similar associations (37,169,170). Unfortunately, in the present study, no similar association between these CC types and complicated BSI could be identified (*p*=1.0, *p*=1.0 and *p*=0.4 respectively). However, among MRSA isolates from various CCs causing BSI over a five year period in renal patients, the highest rate of complicated BSI was caused by ST5-MRSA-II (2/5 isolates, 40%), which belongs to CC5. It is also interesting to note that within this study of renal isolates only, this clone carried the greatest number of enterotoxin genes including *sea*. This may have increased the pathogenic potential of this CC. All fifteen complicated BSIs in our study were caused by different *spa* types. The previously mentioned study by Fowler *et al* (2007) noted that *spa* types t002 and t016 were significantly associated with the development of haematogenous complications (38). Work carried out by Xiong *et al* (2009), also found an association between *spa* type t016 and the development of persistent BSI in the case of MRSA isolates (37). The four isolates in the present study belonging to t002 caused uncomplicated BSI and t016 was not detected in this group of isolates.
In the present study we could not identify a significant association between carriage of any of the virulence genes and the development of a complicated BSI. Previous studies, assessing the relationship between carriage of virulence genes and outcome of infection, have reported varied findings. Ferry and colleagues (2005) found that the prevalence of sea increased significantly with the severity of infection ($p \leq 0.001$). The same paper also found that the prevalence of egc was significantly lower in strains from patients with more severe infection ($p=0.009$) (169). These conflicting findings are surprising given that both sea and egc genes code for enterotoxins that have superantigenic activity. Staphylococcal enterotoxin A is known to be associated with gastroenteritis but the exact role of some of the more recently discovered enterotoxins has not been fully elucidated (66). The findings by Ferry et al (2005) are supported by other studies, such as that by Peacock et al (2002) where it was found that carriage of sea was significantly associated with more invasive isolates ($p<0.01$). Moreover, Fowler et al (2005) found that carriage of sea by BSI isolates was significantly associated with a greater risk of haematogenous complications. Finally, work by van Belkum et al (2006) found a significant association between colonising isolates and the egc-cluster ($p=0.0316$) (32,40,78). It would appear that the presence of sea renders the organism more invasive and virulent, while the presence of the egc-cluster has the opposite effect (78,169). However, how these genes may limit or contribute to clinical complications is difficult to establish. Possible explanations include, insertion of the MGE carrying the egc locus that causes displacement of another MGE carrying more virulent genes, or that the egc genes elicit a lower immunogenic response than
other enterotoxins (78). It is also possible that the early production of enterotoxins encoded by the \textit{egc}-cluster (genes expressed in the early exponential-growth phase, while other enterotoxins are expressed postexponentially) induces an early and mild Th2 response, which may counteract the Th1 response and limit excessive release of cytokines that lead to shock (169). Work by Dauwalder and colleagues (2009) found that SEA induces a strong Th1 response resulting in TNF-\(\alpha\) and macrophage inflammatory protein-1\(\alpha\) (MIP-1\(\alpha\)), but SEG did not (171).

It would appear that the interaction between certain enterotoxins and the immune system has a significant impact on the outcome of infection, indicating that the presence or absence of certain virulence genes may well be directly linked to the outcome of \textit{S. aureus} infection. However, we did not identify a significant association between the presence of the \textit{sea} gene and the development of a complicated BSI (RR=1.38, CI, 0.39-4.91). We did find that the \textit{egc}-cluster was more prevalent in isolates causing uncomplicated BSI (72.73\% \textit{versus} 66.66\%), but we did not identify a significant association between the presence of these genes and uncomplicated BSI.

Other virulence genes have also been shown to significantly correlate with more severe infection. Using a multiplex PCR approach \textit{fnbB, cna, sdrE, sej, eta, hlg} and the \textit{ica} operon were found to be significantly more common in invasive isolates \((p<0.006)\) (40). Certain virulence factors may also be significantly associated with the development of infection at certain sites, such as the collagen adhesin and arthritis and osteomyelitis (67,68). Isolates producing SEB have also been linked to the development of asthma, chronic rhinitis and dermatitis (166).
PVL toxin has been associated with the development of severe SSTI and necrotizing pneumonia (82,172,173). However, the role of PVL toxin as the single virulence determinant in the pathogenesis of CA-MRSA infection, is now in doubt because CA-MRSA strains have been identified that do not produce this toxin but yet still result in severe disease (13,18,81,83,174). It is likely that the success of CA-MRSA as a pathogen is due to a combination of virulence determinants such as PSMs, $\alpha$-toxin and PVL rather than a single virulence determinant alone (13). Only one MSSA isolate in our study carried the gene for PVL toxin and it was associated with an uncomplicated MSSA BSI.

A number of other studies have also failed to identify a virulence gene that is significantly associated with more invasive disease (11,32). Lindsay et al failed to identify any gene significantly associated with invasive isolates (11). Fowler et al (2005) examined patient and bacterial characteristics and found that upon multivariable analysis the only bacterial factor associated with the development of haematogenous complications in $S. aureus$ BSI was methicillin resistance (32).

It appears that there are many conflicting findings regarding the relationship between virulence gene carriage and clinical outcome. One can possibly conclude that the ability of $S. aureus$ to cause an invasive infection is not dependent on just one virulence factor. Perhaps, and as suggested by Lindsay (2009), particular combinations of virulence genes are needed to cause an invasive infection (45). Another important point to consider is that isolates causing invasive infection usually have to first colonise the host (4,175). This indicates that invasive isolates must possess factors that enable them to first colonise skin and nasal mucosa (i.e.
FnBPA, FnBPB, collagen binding adhesin, *S. aureus* surface protein G and a capsule) in addition to virulence associated factors that facilitate invasion.

It is important to remember that although we were unable to identify any single virulence factor or any combination of virulence factors significantly associated with the development of a complicated BSI, it should be noted that all isolates investigated, as part of this study, did cause a BSI. This suggests that they were all virulent and capable of causing invasive infection.

4.3.3. *Comparison of molecular characteristics of MRSA and MSSA isolates causing BSI*

It was interesting to find that despite being resistant to a wide range of antibiotics, MRSA was not associated with an increased rate of complicated BSI compared to MSSA (RR=1.33, CI, 0.49-3.59) (*p*=0.72). Others have indicated that infections caused by methicillin-resistant isolates are associated with a worse prognosis than those caused by methicillin-susceptible isolates (31-33). Fowler *et al* (2005) found that haematogenous complications were more likely to occur in patients with MRSA BSI (RR 2.09) (32). While Hawkins *et al* (2007) found that methicillin resistance was independently associated with the development of persistent *S. aureus* BSI (OR 5.22), many studies have also found higher mortality rates associated with MRSA BSI compared to MSSA BSI (33-36). As previously discussed (chapter III) these findings are due to a number of factors such as, increased age of patients, pneumonia, use of inappropriate agents early in the course of the infection and the use of less potent agents, such as vancomycin (compared
with flucloxacillin for MSSA BSI), the current treatment of choice for MRSA infections (143). Studies have also found that the insertion of the mecA element may influence the expression of certain virulence determinants such as fibrinogen or fibronectin adhesions (i.e. FnBPA, FnBPB), which may impact on the clinical outcome of S. aureus infection (176). Another potential factor, perhaps, is that MRSA infections are more likely to be healthcare associated, and occur in patients with multiple co-morbidities and prosthetic devices, factors associated with a worse clinical outcome in S. aureus BSI (32,33,39).

In this study all MRSA isolates belonged to ST22-MRSA-IV (similar to the UK strain EMRSA-15), which is the predominant strain circulating in Irish hospitals since the late 1990s. This same strain also accounted for 85% of MRSA BSI isolates in Ireland in 2009, but this has not always been the case (21,177). In Irish hospitals ST250-MRSA-I predominated in the 1970s, being replaced by ST239-MRSA-III during the 1980s, which was in turn displaced by ST8-MRSA-II in the 1990s. It is interesting to note that MRSA isolates in our five year retrospective study (2005-2009) of renal BSI isolates belonged to a number of different STs - ST5-MRSA-II, ST22-MRSA-IV, ST30-MRSA-IV and ST8 (SCCmec IIE & ccrAB4 or a possible novel SCCmec II subtype). ST22-MRSA-IV did predominate in renal isolates causing BSI, from 2008 onwards, which is when our prospective study began. In another study in our institution, between May 2007 and September 2008, 173 MRSA isolates were collected from patient (nasal or groin swabs) and environmental sources in four wards over two six week periods. Isolates underwent pulsed field gel electrophoresis (PFGE), spa typing, sequencing of the SCCmec-associated direct
repeat unit (*dru*) typing and SCC* mec* typing. One isolate representative of each *spa* type identified underwent MLST and four different STs were identified – ST22 (n=168, CC22), ST36 (n=1, CC30), ST8 (n=3, CC8) and ST87 (n=1, CC59), suggesting that although ST22-MRSA-IV did predominate amongst non-BSI isolates other ST types were circulating in the hospital at this time (21). The fact that the ST22 strain predominates and that it was the only MRSA strain to cause BSI in our study suggests that this strain may be more virulent than other colonising and environmental strains. The reason for the success of this strain as a human pathogen is not clear. It may be due to its antimicrobial resistance pattern which allows it to thrive in the hospital environment or it may be due to the combination of virulence determinants it carries which allows it to colonise and invade the host without incurring a large fitness cost. It is likely that the success of this strain is due to a variety of factors.

When we compared the carriage of virulence genes of MRSA isolates to MSSA isolates we found a significant association in MRSA isolates with carriage of the enterotoxin gene clusters *sec*/l and *egc*, the antimicrobial resistance gene *ermC* and the MSCRAMM genes, *cna* and *sasG*. However, we must interpret these results carefully given that all MRSA isolates in this study belonged to the CC22 clone and therefore carriage of some of these virulence genes could be clonal. In fact, all isolates (MRSA & MSSA) in the CC22 (n=20) clone carried the *egc*-cluster, *cna* and *sasG* genes. A comparison was made of the carriage of virulence genes by ST22 strains to non-ST22 MRSA isolates by assessing the carriage of virulence genes in MRSA isolates causing BSI in renal patients. Among the four ST types identified in
the latter study, isolates belonging to ST5 carried the greatest number of enterotoxin and MSCRAMM genes, and ST8 isolates carried the greatest number of antimicrobial resistance genes. The success of the ST22-MRSA-IV clone does not appear to be due to the presence of a greater number of virulence genes than other MRSA strains but, the reason for the success of this clone is still not clear.

Many studies have found that MRSA isolates are associated with a worse outcome in invasive *S. aureus* infection. This association is more usually attributed to the timing of the infection (community-onset *versus* healthcare associated), patient factors (e.g. age, co-morbidities, prosthetic devices), treatment factors (e.g. inappropriate or delayed initial treatment) and not to the virulence determinants of the infecting MRSA isolates. In fact there is no evidence to suggest that MRSA strains are more virulent than MSSA strains (34). This evidence is supported by the findings of the present study.

4.4. Summary

- Certain virulence genes (e.g. *egc*-cluster) are clonally distributed
- The clinical outcome of *S. aureus* BSI appears to be independent of the type of infecting isolate and virulence gene carriage
- Patients infected with MRSA are not more likely to develop a complicated BSI and MRSA isolates are not more virulent than MSSA isolates
- The ST22-MRSA-IV clone, which is the predominant MRSA clone circulating in Irish hospitals at present, does not carry more virulence genes than other MRSA clones
Chapter V
Analysis of Circulating Immune Mediators in Patients with S. aureus BSI
5.1. Introduction

Cytokines play a central role in the response to infection, as part of the innate immune system, and also, by developing adaptive immune responses. *S. aureus* is a potent stimulator of cytokine production and cytokines play a major role in *S. aureus* pathogenesis. A variety of staphylococcal molecules, such as peptidoglycan and lipoteichoic acid (LTA) have been shown to stimulate production of cytokines such as TNF-α, IL-1β, IL-6, IL-4, IL-8, IFN-γ and IL-12 (62-64). Many of the cytokines, produced in response to *S. aureus* infection, are pro-inflammatory and some specifically stimulate Th1 differentiation, increasing macrophage activation and enhancing the elimination of *S. aureus* by phagocytosis (128). However, *S. aureus* also uses cytokines as growth factors or as virulence factors (129-131). For example, IL-1β has been shown by Kanangat *et al* (2001), to enhance the growth of *S. aureus* in an *in-vitro* assay and growth was found to be mediated by the binding of IL-1β itself, or of cleavage products of this cytokine to specific receptors on the bacterial surface (129). These combined findings demonstrate the versatility of *S. aureus* in deriving benefit from the host immune responses to its presence.

Cytokines may also up-regulate the production of virulence factors by *S. aureus* (132). While ‘normal’ immune responses will result in the eradication of *S. aureus*, exaggerated responses may favour survival and enhance the virulence of the infecting organism. It is also possible that in addition to the cytokines mentioned above, other cytokines may also play a key role in the response to *S. aureus* infection, with either beneficial or detrimental effects for the host. Patient factors, such as malignancy, immunodeficiency or immunosuppressive therapy may also
influence cytokine production resulting in either an attenuated or exaggerated cytokine response, which may contribute to some of the manifestations of *S. aureus* infection.

It would appear that a measured cytokine response is beneficial for the host but a prolonged or exaggerated response may result in a worse outcome for the patient. A number of studies have been carried out to investigate the relationship between cytokine levels and the outcome of sepsis. These have shown that high levels of some cytokines (e.g. IL-6, TNF-α) are associated with a poorer prognosis and higher mortality rates (178-184). There is little in the literature describing the relationship between cytokine levels and the outcome of serious *S. aureus* infections such as BSI. The work described in this chapter was undertaken to identify cytokines or chemokines that may be differentially regulated in *S. aureus* BSI and to investigate the levels of selected cytokines in sequential plasma samples from patients with *S. aureus* BSI taken over the course of their infection (on the day of diagnosis, seven days later and in the case of patients with complicated BSI, on day 14 following diagnosis). The data was then analysed to establish the correlation, if any, between the cytokine response and the type of infecting isolate (MRSA or MSSA), the clinical outcome of *S. aureus* BSI (complicated or uncomplicated) and other patient characteristics that may contribute to the clinical outcome.
5.2. Results

5.2.1. Patient plasma samples

Plasma samples were prepared from patient blood samples collected on the day of diagnosis of the BSI (A sample), seven days later (B sample) and in the case of patients with complicated infection on day 14 (C sample). In total 61 patients were included in the cytokine analysis, 15 with MRSA BSI, 46 with MSSA BSI, 50 with uncomplicated BSI and 11 with complicated BSI. Patients were divided into two categories for analysis; according to the type of infecting isolate (MSSA versus MRSA) and the clinical outcome of infection (complicated versus uncomplicated infection).

5.2.2. Preliminary selection of cytokines using pooled patient plasma and a cytokine array

Pooled plasma from three patients, randomly selected from each of the four patient groups, MRSA BSI, MSSA BSI, uncomplicated BSI and complicated BSI, were analysed by hybridization to a cytokine array to identify cytokines that were differentially expressed between groups. Each of the arrays contained three pairs of positive controls and three pairs of negative controls along with antibodies to 42 different cytokines, and growth factors, which were present in duplicate. The position of each of the cytokine antibodies on the array is shown on the cytokine antibody array map (Figure 5.1).
Figure 5.1. Cytokine antibody array map, RayBio® Human Cytokine Antibody Array 3 (RayBiotech Inc., GA, USA), which detects the presence of 42 different cytokines and growth factors. The position of each individual cytokine can be identified by the grid markers a-1 and 1-8. Each cytokine is present in duplicate.

An image of the resulting hybridisation patterns for the pooled samples is shown in Figure 5.2.
Figure 5.2. Hybridization pattern on cytokine antibody array for pooled plasma from four patient groups. Each array was used to detect the presence of 42 different cytokines and growth factors in pooled plasma from three patients in each of the patient groups. Array A-complicated BSI, array B-uncomplicated BSI, array C-MSSA BSI, and array D-MRSA BSI. Positive (red outline) and negative controls (blue outline) are indicated by arrows on Array A. Selected cytokines are highlighted by arrows.

Comparing the relative cytokine levels between the four patient groups, using densitometry and at least a 1.4 fold difference in relative value between groups, the levels of four cytokines were differentially expressed between the groups. These were; IL-6, GRO (both GROβ and GROγ) and RANTES (Regulated...
upon Activation, Normal T-cell Expressed, and Secreted), among patients with uncomplicated and complicated BSI, and leptin among patients with MRSA and MSSA BSI (Table 5.1).

### Table 5.1. Comparison of individual cytokine levels between patient groups

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Complicated Vs Uncomplicated BSI</th>
<th>Fold-difference</th>
<th>MSSA Vs MRSA BSI</th>
<th>Fold-difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interleukin-6</td>
<td><img src="Image" alt="Image" /></td>
<td>1.4</td>
<td><img src="Image" alt="Image" /></td>
<td>1.0</td>
</tr>
<tr>
<td>Value</td>
<td>0.136</td>
<td></td>
<td>0.094</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.097</td>
<td></td>
<td>0.098</td>
<td></td>
</tr>
<tr>
<td>Gro-α</td>
<td><img src="Image" alt="Image" /></td>
<td>1.8</td>
<td><img src="Image" alt="Image" /></td>
<td>1.1</td>
</tr>
<tr>
<td>Value</td>
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<td></td>
<td>0.104</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.121</td>
<td></td>
<td>0.113</td>
<td></td>
</tr>
<tr>
<td>RANTES</td>
<td><img src="Image" alt="Image" /></td>
<td>1.7</td>
<td><img src="Image" alt="Image" /></td>
<td>1.2</td>
</tr>
<tr>
<td>Value</td>
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<td></td>
<td>0.143</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.278</td>
<td></td>
<td>0.168</td>
<td></td>
</tr>
<tr>
<td>Leptin</td>
<td><img src="Image" alt="Image" /></td>
<td>1.1</td>
<td><img src="Image" alt="Image" /></td>
<td>2.0</td>
</tr>
<tr>
<td>Value</td>
<td>0.518</td>
<td></td>
<td>0.327</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.567</td>
<td></td>
<td>0.658</td>
<td></td>
</tr>
</tbody>
</table>
5.2.3. Cytokine levels in patient samples compared to healthy control samples

The levels of the four cytokines selected from preliminary cytokine array analysis, namely IL-6, GROγ, RANTES and leptin were measured in all patient plasma samples using a sandwich ELISA. Plasma obtained from four healthy volunteers was also included for comparison. As shown in the representative scatter plot (Figure 5.3), showing, for each cytokine, the data from the day of diagnosis (A samples), there was a marked variation in the cytokine levels among patients, with the greatest variation found in the leptin levels in A samples (Figure 5.3).

![Figure 5.3. Scatter dot plot of cytokine levels in patient samples taken on the day of diagnosis (A sample) of S. aureus BSI. Each symbol represents the cytokine level in a single patient sample. Horizontal bars represent the mean cytokine concentration.](image)

As shown in Figure 5.4 (1), the mean concentration of IL-6, RANTES and GROγ was higher in the samples collected from patients on the day of diagnosis (A samples) compared to the levels detected in the samples from healthy individuals.
(represented by horizontal bars) but these differences were not statistically significant ($p=0.65$, $p=0.26$, $p=0.63$, respectively). The mean leptin level was higher in samples from healthy individuals compared to the patient samples collected on the day of diagnosis (26.80±12.55 pg/mg of protein versus 24.50±3.93 pg/mg of protein) but this difference was not statistically significant ($p=0.72$) (Figure 5.4 (1)). In subsequent patient samples, taken at day 7 and day 14 following diagnosis of S. aureus BSI, leptin and RANTES levels were higher relative to IL-6 and GROγ (Figure 5.4(2) and (3)).

![Figure 5.4](image)

Figure 5.4. Mean concentration of the four cytokines in patient samples collected on the day of diagnosis (A sample) (1), seven days after diagnosis (B sample) (2), fourteen days after diagnosis of complicated S. aureus BSI (C sample) (3). Data shown represent mean cytokine concentration ± SEM. Horizontal bars in (1) represent the mean cytokine concentrations detected in samples from healthy controls. Cytokine range in healthy controls in pg/mg protein; IL-6 (0.94-10.56), RANTES (7.37-18.05), GROγ (0.31-4.72) and leptin (0.58-56.93).
5.2.4. Variations in cytokine levels over the course of the S. aureus BSI

As shown in Figure 5.5, cytokine levels varied throughout the course of S. aureus BSI. IL-6 and GROγ levels were highest early in the course of infection, with levels decreasing considerably by day 7. For IL-6, this decrease was significant ($p<0.05$ comparing IL-6 in A samples compared to B samples). For both IL-6 and GROγ, the levels in the C samples were markedly reduced compared to those detected in the A sample and GROγ levels were found to be significantly different ($p=0.05$ comparing GROγ in A and C samples). In contrast, both RANTES and leptin levels were relatively low early in the course of the infection, with levels increasing significantly by day 7 in the case of RANTES ($p<0.0001$). Levels of both RANTES and leptin had not decreased significantly by day 14 (C sample) and furthermore RANTES levels remained higher than those detected on the day of diagnosis of the BSI (A sample).
Figure 5.5. Cytokine levels measured in A, B and C samples collected from patients with *S. aureus* BSI (n=61). IL-6 levels (1), GROγ levels (2), RANTES levels (3) and leptin levels (4). Data shown represent mean cytokine concentration ± SEM. * indicates a significant difference between IL-6 levels in A and B samples. ♦ indicates a significant difference between GROγ levels in A and C samples. *** indicates a significant difference between RANTES levels in A and B samples.

5.2.5. Comparison of the cytokine response to MSSA and MRSA BSI

*S. aureus* isolates causing BSI in this study comprised both MSSA and MRSA isolates (MSSA BSI (46 patients), MRSA BSI (15 patients)). Patients with MRSA infection often have a worse clinical outcome for a variety of reasons (e.g. delay in commencement of appropriate treatment). The cytokine response in patients with MSSA BSI and MRSA BSI was therefore compared (Figure 5.6).
In our study, IL-6 levels in both MSSA and MRSA BSI were higher early in the course of the infection. There was no statistically significant difference in IL-6 levels in patients infected with MSSA compared to MRSA at any of the time points studied. However, among patients with MSSA BSI, IL-6 levels decreased significantly from day of diagnosis to day 7 post-diagnosis ($p \leq 0.05$ comparing IL-6 in A and B samples from MSSA group). This pattern was not found in patients with BSI due to MRSA (Figure 5.6).

In our study, the GRO$\gamma$ response to infection in patients with MRSA BSI was similar to the IL-6 response, with levels being high early in the course of infection. In patients with MRSA and MSSA BSI there was no statistically significant
difference found between A and B samples for this chemokine. Interestingly, GROγ levels were significantly higher in A samples collected from patients with MRSA BSI compared to those with MSSA BSI \( (p \leq 0.05) \) but this difference was not detected in B samples (Figure 5.7).

![Graph showing GROγ levels](image)

**Figure 5.7.** GROγ levels in patients infected with MRSA and MSSA on the day of diagnosis of *S. aureus* BSI (A sample) and seven days later (B sample). Data shown represent mean cytokine concentration ± SEM. * indicates a significant difference between GROγ levels detected in patients with MRSA BSI compared to MSSA BSI on the day of diagnosis of the BSI.

In contrast to the patterns found for IL-6 in this study, RANTES levels were lower in the A samples compared to the B samples. This pattern was evident regardless of whether patients were infected with MRSA or MSSA (Figure 5.8). No statistically significant differences were identified when RANTES levels were compared in patients with MSSA and MRSA BSI. However, a significant increase
in the level of RANTES in the B samples compared to the A samples in both patient groups was found ($p=0.01$ and $p \leq 0.0001$, respectively) (Figure 5.8).

![Figure 5.8. RANTES levels in patients infected with MRSA and MSSA on the day of diagnosis of S. aureus BSI (A sample) and seven days later (B sample). ♦ indicates a significant difference between RANTES levels detected in patients with MRSA BSI on the day of diagnosis of the BSI compared with day 7. *** indicates a significant difference between RANTES levels detected in patients with MSSA BSI on the day of diagnosis of the BSI compared with day 7. Data shown represent mean cytokine concentration ± SEM](image)

The patterns of variations in leptin levels seen in patients with both MSSA and MRSA BSI were similar to that found for RANTES, with levels being low early in the course of infection and increasing by day seven (Figure 5.9). There was no statistically significant difference in the leptin levels of patients with MRSA compared to MSSA BSI over the course of their infection. Furthermore, there was
no significant difference in levels of leptin in the A and B samples in either patient group.

![Graph showing leptin levels in patients infected with MRSA and MSSA](image)

**Figure 5.9.** Leptin levels in patients infected with MRSA and MSSA on the day of diagnosis of *S. aureus* BSI (A sample) and seven days later (B sample). Data shown represent mean cytokine concentration ± SEM

### 5.2.6. Relationship between the types of isolates causing BSI, their virulence factors and the cytokine response

The cytokine responses in patients infected with different types of *S. aureus* were compared to detect if different strains of *S. aureus* elicited different cytokine responses. The mean cytokine responses measured in patients infected with *S. aureus* belonging to the predominant CC types are listed in Table 5.2. Here, RANTES and GROγ levels were significantly higher in patients infected with *S. aureus* isolates belonging to CC22 (*p*≤0.05 and *p*≤0.05, respectively). Isolates belonging to other CCs were not associated with significantly higher or lower cytokine responses.
S. aureus isolates causing BSI carry a variety of virulence determinants, such as toxin encoding genes. The relationship between a number of toxin genes and the cytokine response in patients with S. aureus BSI was assessed to determine if any significant difference in cytokine levels could be detected in patients who were positive for specific toxin encoding genes. Patients who were infected with isolates that carried the egc complex were found to have significantly higher RANTES levels compared to patients infected with S. aureus isolates with an egc− genotype \( (18.3 \pm 1.34 \text{ versus } 13.03 \pm 1.65, p \leq 0.05) \), however this may be a clonal effect. There was no statistically significant difference in the levels of IL-6, GROγ or leptin in patients

<table>
<thead>
<tr>
<th>CC type</th>
<th>IL-6</th>
<th>RANTES</th>
<th>GROγ</th>
<th>Leptin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pg/mg protein (mean ± SEM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC15 Vs Other CCs</td>
<td>3.814 ±1.36</td>
<td>13.53 ±3.58</td>
<td>4.817 ±2.72</td>
<td>26.83 ±9.53</td>
</tr>
<tr>
<td>CC22 Vs Other CCs</td>
<td>9.464 ±4.71</td>
<td>21.68 ±2.65 ( ^a )</td>
<td>5.626 ±1.6 ( ^b )</td>
<td>16.39 ±3.87</td>
</tr>
<tr>
<td>CC30 Vs Other CCs</td>
<td>3.728 ±1.44</td>
<td>14.13 ±1.78</td>
<td>2.136 ±0.81</td>
<td>36.00 ±18.83</td>
</tr>
<tr>
<td>CC45 Vs Other CCs</td>
<td>4.648 ±1.84</td>
<td>16.97 ±1.59</td>
<td>2.631 ±1.18</td>
<td>18.49 ±4.34</td>
</tr>
</tbody>
</table>

\(^a\)Mean RANTES level was significantly higher in patients infected with isolates belonging to CC22 \( (p < 0.05) \)

\(^b\)Mean GROγ level was significantly higher in patients infected with isolates belonging to CC22 \( (p < 0.05) \)
infected with isolates with an $egc^+$ genotype compared to patients infected with isolates with an $egc^-$ genotype. We failed to identify a significant difference in cytokine levels in patients infected with isolates with $tst^+$ and $sea^+$ genotypes compared to those patients infected with isolates with $tst^-$ and $sea^-$ genotypes. Only one patient was infected with an isolate that carried the genetic determinants that code for PVL toxin. In this patient the cytokine response was lower than the mean value detected in all other patients for all cytokines except leptin (IL-6 – 1.42 versus 6.0 ±1.52, RANTES – 10.92 versus 17.21 ±1.13, GROγ – 0 versus 3.49 ±0.63 and leptin 69.97 versus 23.68 ±3.92).

5.2.7. Relationship between cytokine levels and the outcome of S. aureus BSI

For the purposes of this study S. aureus BSIs were divided into uncomplicated and complicated infections. As described earlier, a complicated infection was defined as the development of infective endocarditis, persistent BSI despite at least three days of appropriate antibiotic therapy or the development of a disseminated infection such as osteomyelitis. Fifty patients (82%) had an uncomplicated infection and eleven (18%) went on to develop a complicated infection. The cytokine response in these two patient groups was compared to assess the relationship between the cytokine responses and the outcome of the BSI.

Although there was a trend toward higher IL-6 levels in patients with complicated BSI compared to those with uncomplicated BSI, this difference was not statistically significant (Figure 5.10). As seen previously in patients with MRSA and MSSA BSI (Figure 5.6), IL-6 levels were higher early in the course of the
infection with levels decreasing by day 7 in patients with complicated and uncomplicated BSI. This difference was found to be significant in the case of uncomplicated infections. IL-6 levels remained low on day 14 (complicated group).

**Figure 5.10. IL-6 levels in patients with uncomplicated and complicated BSI.** A sample - collected on the day of diagnosis of *S. aureus* BSI, B sample - collected seven days later and C sample – collected on day 14 in the case of those with complicated BSI. Data shown represent mean cytokine concentration ± SEM. * indicates a significant difference in IL-6 levels between the A sample and the B sample in patients with uncomplicated infection.

GROγ levels were higher in both the A and B samples in patients with complicated BSI compared to patients with uncomplicated BSI. However, this difference was not statistically significant (Figure 5.11). For complicated cases of BSI, by day 14, levels had dropped below the level recorded on the day of diagnosis of the BSI.
RANTES levels were significantly lower in the A samples compared to the B samples in patients with both uncomplicated and complicated BSI (figure 5.12). A similar level of response was seen in both patient groups. RANTES levels in C samples remained high and were higher than levels detected in the A samples.
Leptin levels showed a similar pattern of variation among patient groups to RANTES in that leptin levels were lower early in the course of the infection. Leptin levels in the A sample of patients, with uncomplicated BSI, were higher than the levels in patients with complicated BSI (27.25±4.62 pg/mg of protein versus 11.53±3.64 pg/mg of protein) but this difference was not statistically significant. Leptin levels were significantly higher in the B samples of patients with uncomplicated infection compared to the B samples of patients with complicated infection ($p \leq 0.05$) (figure 5.13). Leptin levels were higher in the B samples of both...
patient groups compared to the A samples although this difference was not statistically significant. Leptin levels remained high on day 14 in patients with complicated BSI.

**Figure 5.13. Leptin levels in patients with uncomplicated and complicated BSI.**
A sample - collected on the day of diagnosis of *S. aureus* BSI, B sample - collected seven days later and C sample – collected on day 14 in the case of those with complicated BSI. Data shown represent mean cytokine concentration ±SEM. *indicates that leptin levels were significantly higher in the B samples of patients with uncomplicated infection compared to those with complicated infection.

### 5.2.8. Relationship between patient characteristics and the cytokine response

A number of different patient characteristics were studied to assess their contribution, if any to the cytokine response in *S. aureus* BSI. These included immunosuppressive therapies and co-morbidities.
5.2.8.1. Cytokine levels in patients with *S. aureus* BSI receiving immunosuppressive treatment

Of the 61 patients included in the analysis, seven patients were receiving immunosuppressive treatment, which varied between patients but included steroids, interferon and azathioprine. The cytokine levels found in patients on immunosuppressive therapies are shown in Table 5.3. To show how the levels in individual patients compared to the overall mean found in all patients, the mean values in all patients are also shown for each cytokine.
Table 5.3. Cytokine levels in patients receiving immunosuppressive treatment

<table>
<thead>
<tr>
<th>Cytokine concentration in all patients</th>
<th>IL-6</th>
<th>RANTES</th>
<th>GROγ</th>
<th>Leptin</th>
</tr>
</thead>
<tbody>
<tr>
<td>pg/mg protein (mean ± SEM)</td>
<td>A°</td>
<td>B°</td>
<td>A°</td>
<td>B°</td>
</tr>
<tr>
<td>IL-6</td>
<td>5.92</td>
<td>2.73</td>
<td>17.10</td>
<td>33.03</td>
</tr>
<tr>
<td>RANTES</td>
<td>±1.49</td>
<td>±0.46</td>
<td>±1.12</td>
<td>±1.88</td>
</tr>
<tr>
<td>GROγ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leptin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Patient no. | Immunosuppressive treatment | pg/mg protein |
-------------|------------------------------|---------------|
28           | Hydrocortisone               | 2.87          |
29           | Interferon                   | 0°            |
30           | Epirubicin, cisplatin,       | 5.63          |
            | fluorouracil                 | 7.48          |
37           | Prednisolone, tacrolimus     | 4.99          |
            |                              | 9.63          |
41           | Dexamethasone                | 6.7           |
            |                              | 0.82          |
59           | Daunorubicin, vincristine,    | 2.2           |
            | prednisolone                 | 0.16          |
61           | Mesalamine, azathioprine     | 15.8          |
            |                              | 6.75          |

°Plasma sample taken on day of diagnosis of the BSI (A sample)
°Plasma sample taken on day 7 (B sample)
°Concentration was below the limit of detection of the assay

The cytokine response in patients receiving immunosuppressive therapy varied and it was difficult to observe any correlation between immunosuppression and cytokine level in response to *S. aureus* BSI. Only one patient on immunosuppressive treatment went on to develop a complicated infection (patient 37, IE). This patient had high levels of IL-6 (9.63 pg/mg of protein, mean concentration in all patients 2.73 ±0.46 pg/mg of protein) and RANTES (54.9 pg/mg of protein, mean concentration in all patients 33.03 ±1.88 pg/mg of protein) on day
seven. The leptin levels in this patient were undetectable on both the day of diagnosis and on day seven.

5.2.8.2. Cytokine levels in patients with *S. aureus* BSI and diabetes mellitus

DM is a common condition, with the prevalence of type II diabetes increasing among older patients. Patients with DM develop more frequent infections which are often prolonged or more severe than in the general population. Fourteen patients in this study had underlying DM and their cytokine response was assessed. The IL-6 response in patients with DM was significantly higher on day seven \( (p \leq 0.05) \) compared to patients without DM and there was a similar trend identified between these two groups of patients in samples taken on the day of diagnosis of the BSI \( (p=0.09) \). The cytokine response for the other three cytokines tested was higher in patients with DM compared to patients without DM on both the day of diagnosis of the BSI and on day seven. However, these differences were not statistically significant (Figure 5.14).

Three of the fourteen patients with DM went on to develop a complicated infection but the risk of developing a complicated infection was not significantly higher in the DM patients compared to patients without DM \( (3/14 \text{ versus } 8/47 \quad p=0.70) \).
Figure 5.14. Comparison of IL-6, RANTES, GROγ and leptin responses in patients with and without diabetes mellitus (DM and No DM) in (1) A samples and (2) B samples. Data shown represent mean cytokine concentration ± SEM. * Indicates a significant difference in IL-6 levels between B samples from DM patients and B samples from patients without DM.
5.2.8.3. Cytokine levels in renal patients with *S. aureus* BSI

As previously stated (Section 3.3) renal patients are at greater risk of *S. aureus* BSI due to impaired immune function, regular contact with healthcare facilities and the presence of central venous catheters (CVCs). Renal patients accounted for 40% of patients included in the whole study, the largest speciality represented. Twenty three renal patients were included, 21 of these patients were on haemodialysis, one patient was receiving plasmaphoresis for Waldenströms macroglobulinaemia and one patient had chronic renal failure and was not receiving dialysis. IL-6 and GROγ levels were significantly lower in renal patients on the day of diagnosis of the BSI ($p \leq 0.05$ and $p=0.01$, respectively) compared to other patients. RANTES levels were significantly lower in renal patients on day seven ($p \leq 0.05$) compared to other patients (Figure 5.15). Leptin levels were higher in renal patients on the day of diagnosis of the BSI and on day seven, but this difference was not statistically significant.
Figure 5.15. Comparison of IL-6, RANTES, GROγ and leptin levels in renal patients compared to patients from other specialties in (1) A samples and (2) B samples. * indicates a significant difference in IL-6 levels between A samples from renal patients and A samples from patients from other specialties. ♦ indicates a significant difference in GROγ levels between A samples from renal patients and A samples from patients from other specialties. Data shown represent mean cytokine concentration ± SEM. * Indicates a significant difference in RANTES levels
between B samples from renal patients and B samples from patients from other specialties. Data shown represent mean cytokine concentration ± SEM.

Two of the 23 renal patients went on to develop a complicated infection. However, the risk of developing a complicated infection was not found to be significantly higher or lower in this group of patients compared to patients from other specialties (2/23 versus 9/38, \( p = 0.18 \)).

5.3. Discussion

Our understanding of the roles of cytokines in innate and adaptive immunity, has improved greatly in recent years. However, the role of cytokines in the response to \textit{S. aureus} infections remains unclear and in this chapter, some aspects of the specific responses in \textit{S. aureus} BSI have been investigated.

\textit{S. aureus} is a potent stimulator of cytokine production, and cytokines play a major role in the pathogenesis of \textit{S. aureus} infection (128,132). Peptidoglycan and LTA induce the production of TNF-\( \alpha \), IL-1\( \beta \) and IL-6 by macrophages and monocytes by binding to TLR2 (62-64). The surface protein A causes the release of IL-1\( \beta \), IL-4, IL-6, IL-8, IFN-\( \gamma \) and TNF-\( \alpha \) by airway epithelial cells. \( \alpha \)-Haemolysin has been shown to induce the production of IL-1\( \beta \), IL-6, IL-8 and TNF-\( \alpha \) and \( \beta \)-haemolysin causes lysis of monocytes resulting in the release of IL-1\( \beta \) (63). Staphylococcal DNA is capable of inducing the production of IL-6, IL-12, IFN-\( \gamma \) and TNF-\( \alpha \) by binding to TLR9 (63). It is also interesting to note that while all these cytokines are pro-inflammatory, IL-12 and IFN-\( \alpha \) specifically stimulate Th\(_1\)
differentiation, which increases macrophage activation enhancing elimination of *S. aureus* by phagocytosis.

An appropriate cytokine response is likely to be beneficial to the host in overcoming serious *S. aureus* infection. However, production of too little or too much of a certain cytokine may be detrimental to the host allowing *S. aureus* to cause more serious infection. It is known that *S. aureus* can use cytokines, such as IL-1β and IL-4, as growth factors or as virulence factors, suggesting that certain cytokines may enhance the growth of *S. aureus* (129-131,185). Cytokines may also have the ability to up-regulate the production of virulence factors by *S. aureus* thereby enhancing the virulence potential of infecting *S. aureus* isolates (132). This supports the hypothesis that organisms can use immune responses to their advantage and that while ‘normal’ immune responses will result in the eradication of the infecting organism, exaggerated responses may impair the resolution of infection by favouring survival of the infecting organism. Kanangat *et al* (1999) have suggested that patients with prolonged inflammatory responses such as acute respiratory distress syndrome (ARDS) and systemic inflammatory response syndrome (SIRS), who have persistently elevated cytokine levels, such as IL-1β, TNF-α and IL-6, are more likely to succumb to infections with organisms such as *S. aureus* (130).

The cytokine response to sepsis has been well documented (108,178,186-188). However, to the best of our knowledge, the present study is the first to investigate specific cytokine responses over the course of *S. aureus* BSI. The four cytokines measured in this study were selected from a preliminary screen of 42 cytokines, based on their differential plasma levels between patient groups, on the
day of *S. aureus* BSI diagnosis. Correlations were found between the patterns and levels of these selected cytokines and the type of infection or clinical outcome. The role of IL-6 in the response to sepsis is well documented and we were therefore not surprised to find its levels varied in response to *S. aureus* BSI. However, the significance of the other three, RANTES, GROγ and leptin in *S. aureus* infection has not previously been documented. The significant variation in the levels of these cytokines in the different patient groups demonstrates the complexity of the immune response to *S. aureus*. It is likely that other cytokines also contribute to the overall response to *S. aureus* BSI.

5.3.1. IL6, RANTES, GROγ and leptin, patterns of induction and roles in *S. aureus* BSI.

IL-6 is a potent pro-inflammatory cytokine, which also has anti-inflammatory activity. A number of different factors have been shown to induce production of IL-6 including cytokines (e.g. IL-1, TNF and IFNs), bacterial products (e.g. endotoxin, peptidoglycan and lipotechoic acid), viruses (e.g. influenza, adenovirus) and trauma (62,189,190). IL-6 is the most commonly identified cytokine in non-infected post-operative patients (191). Along with IL-1 and TNF-α IL-6 acts as a pro-inflammatory cytokine and plays a central role in the induction of the immune response. IL-6 has a number of important functions, some of which are similar to those of IL-1, and indeed these two cytokines may act synergistically. IL-6 activates both B and T cells (107). It causes B cells to mature into plasma cells and begin secretion of immunoglobulin, and allows T cells to grow and differentiate
IL-6 is also an endogenous pyrogen and a potent inducer of acute phase proteins, particularly those produced by the liver (107,182,189).

Much work has been carried out to investigate the relationship between IL-6 levels and the severity and outcome of sepsis. Studies have identified IL-6 as a marker of severity during bacterial infection and have shown its positive correlation with inflammatory markers, such as CRP and APACHE II scores (108,182,188). In our study higher IL-6 levels were detected in patients with complicated infection. It has also been shown that high IL-6 levels correlate with high mortality rates, and indeed it has been suggested that IL-6 levels could be a potential marker for severity of bacterial infection (178,180-184,192).

It is known that several components of *S. aureus* can stimulate production of IL-6 (e.g. peptidoglycan, LTA, protein A and staphylococcal DNA) so it is likely that this cytokine plays a role in response to *S. aureus* infection. However, there is little data in the literature on the IL-6 response in patients infected with *S. aureus* or the impact of IL-6 on the outcome of *S. aureus* infection (62-64).

Not surprisingly, in our study IL-6 levels were higher in patients than in healthy controls, in *S. aureus* BSI, IL-6 levels were higher early in the course of the clinical infection and reduced over the course of infection. This supports the findings of previous studies looking at IL-6 levels in patients with sepsis (178,179,181-184,192). This pattern was also identified in the sub-groups of patients infected with MSSA and with uncomplicated infection. In those patients with complicated infection in whom a third plasma sample was collected on day 14, IL-6 levels had further decreased even in the presence of a complicated infection.
This suggests that the IL-6 response to serious *S. aureus* infection is similar to what has previously been reported in sepsis of any origin, with IL-6 levels rising rapidly early in the course of the infection and levels dropping rapidly even before the clinical status of the patient has improved (181,182). This reflects the role of IL-6 as a potent pro-inflammatory cytokine, released early in the course of infection, stimulating many components of the innate immune system.

Many studies have assessed cytokines as potential markers for severe sepsis. High IL-6 levels seem to consistently correlate with severe sepsis, APACHE II scores and high mortality rates (178,180-184,193). The reason for this association is unclear. It is possible that IL-6 levels are higher as the infecting organism is more virulent stimulating a more vigorous immune response, or possibly it reflects an exaggerated immune response in the host causing a more severe sepsis phenotype, which may result in increased mortality rates. A recent review, which examined the findings of 38 eligible studies, compared the sensitivity of the peripheral white cell count, CRP, erythrocyte sedimentation rate (ESR) and IL-6 levels in the diagnosis of prosthetic joint infection concluded that IL-6 levels were the most accurate marker for the diagnosis of prosthetic joint infection (192). Although not stated in the review it is likely that many of the included studies assessed prosthetic joint infections that were caused by *S. aureus*. While we found a trend towards increased plasma levels of IL-6 on the day of diagnosis of *S. aureus* BSI in patients with a complicated course of infection compared to patients with uncomplicated BSI (12.33 ±7.84 pg/mg of protein versus 4.56 ±0.71 pg/mg of protein), this increase was not statistically significant ($p$=0.84) and we conclude that IL-6 may not be a reliable
marker of severity in this group of patients. Alternatively, a larger study would be required to show a difference. However, our study suggests that IL-6 could be used as an indirect indicator of serious \textit{S. aureus} infection such as BSI in the absence of positive cultures or other results. It is also likely that IL-6 will be elevated earlier and will be a more sensitive indicator early in the course of infection than many of the more traditional markers of inflammation, such as the CRP and the ESR, as production of many of these markers is stimulated by IL-6 (107,182,189).

RANTES is a chemokine belonging to the CC subfamily (CCL5) and is an 8 kD protein secreted by T lymphocytes and epithelial cells (194,195). The main function of RANTES is to act as a chemotactic agent for a variety of white cells, including T lymphocytes, eosinophils and basophils, allowing for the recruitment of these cells to inflammatory sites (196,197). RANTES works in association with other cytokines to activate natural killer (NK) cells, it has anti-viral activity including anti-HIV activity (198,199). RANTES production is thought to be induced by TNF-\(\alpha\) and IL-1\(\alpha\), while it promotes development of IFN-\(\gamma\)-producing \(\text{T}_{\text{H1}}\) lymphocytes and increases IL-12 production. RANTES, a potent chemotactic agent, also activates eosinophils and as a result is thought to play a major role in the pathogenesis of asthma and other allergic disorders (196).

There is no published evidence to date, to the best of our knowledge, to support the role of RANTES in the immune response to \textit{S. aureus} BSI. However, our observation of a significant rise in RANTES levels in all four patient groups by
day seven of the BSI, suggests that RANTES production is stimulated by *S. aureus* and that it may have a functional role in the immune response to this pathogen.

In contrast to the pattern of decreasing IL-6 levels over the course of infection, RANTES levels increased up to day 7 following diagnosis and remained elevated in complicated cases up to 14 days post-diagnosis. This pattern persisted when isolates were divided in terms of methicillin resistance and infection type. RANTES’ main function is to act as a chemotactic agent for leucocytes allowing recruitment of these cells to inflammatory sites (196,197). RANTES production is thought to be induced by TNF-α and IL-1α, again both of which are produced in response to certain cell components of *S. aureus* (e.g. peptidoglycan, LTA) (128,132). Peak production of RANTES appears later than other cytokines (e.g. IL-6) although its production is stimulated by similar factors (i.e. IL-1 and TNF-α). It appears from the present study that once produced, RANTES levels remain high for a considerable period of time rather than returning to pre-infection levels like the other cytokines. The purpose of this persistence is not clear but it may simply reflect the ongoing response to persistent infection in patients with complicated BSI.

GROγ (growth related oncogene) is a small chemokine belonging to the CXC subfamily. It is also known as GRO3 oncogene (GRO3), CXCL3 and macrophage inflammatory protein-2-beta (MIP2b). GROγ is 90% identical in amino acid sequence to the other two GRO chemokines, GROα and GROβ, but they are the products of three distinct non-allelic human genes (200). The CXC subfamily of
chemokines are further subdivided on the basis of the presence or absence of a glutamic acid-leucine-arginine (ELR motif) amino acid sequence proximal to the CXC sequence (201). GROγ is an ELR-positive CXC chemokine. GROγ production is stimulated by inflammatory mediators such as IL-1 and TNF. It is produced by monocytes, fibroblasts, melanocytes and epithelial cells, and may be produced constitutively by certain tumour cell lines. As a chemokine, the main function of GROγ is to direct leucocytes to sites of inflammation (107). The GRO family of chemokines are also involved in arresting monocyte adhesion to endothelial cells without influencing monocyte chemotaxis (202). The ELR-positive CXC chemokines, of which GROγ is an example, are all potent inducers of angiogenesis, in both physiological and pathological settings, and neutrophil chemoattractants (201). These chemokines play a role in many diverse disease settings including malignancy, chronic inflammation, atherosclerosis and wound repair (201).

There is little evidence to support the role of GROγ in severe S. aureus infection. We identified a significant difference in GROγ levels in patients infected with MRSA compared to MSSA. The cause and clinical implications of this difference is not clear. Levels of GROγ in plasma samples rose early in the course of the infection, only decreasing slightly by day seven and then dropping significantly by day 14 compared to the day of diagnosis in those patients with complicated infection (p=0.05). Patients infected with MRSA had significantly higher GROγ levels on the day of diagnosis of the BSI compared to patients with MSSA BSI (p≤0.05).
GROγ is a chemokine whose production is stimulated by IL-1 and TNF-α, which are known to be produced following exposure to *S. aureus* (132). Therefore it is not surprising that levels are increased during *S. aureus* infection. The main function of GROγ is to direct leucocytes to sites of inflammation and act as a neutrophil chemoattractant (107). Increasing the number of leucocytes at the site of infection will hasten eradication of *S. aureus* by phagocytosis, suggesting that GROγ could play a key role in the immune response to *S. aureus* infection.

Leptin is an adipose tissue derived hormone or an adipokine. Other examples of adipokines are adiponectin and resistin (109). Leptin is a 16-kDa protein product of the *ob* gene and plays a major role in the regulation of body weight and energy intake. Leptin reduces appetite, maintains glucose homeostasis and regulates energy expenditure through its action on the hypothalamus. Elevated levels of leptin are often found in obese patients; these patients may be resistant to leptin (109).

It has long been known that leptin has many other functions including regulation of neuroendocrine, reproductive, haematopoietic and immune function. Various studies have shown that TNF-α, IL-1β, IL-6 and other cytokines can cause an increase in serum leptin concentration (203-205). Serum leptin levels are also higher in patients with cancer, severe sepsis, septic shock and SIRS (204,206). Cortisol has also been shown to increase serum leptin levels in patients (205). A number of studies have looked at the effect of endotoxin (lipopolysaccharide) on leptin levels. Serum leptin levels have been shown to increase in dogs, rats, non-
human primates and humans in response to the administration of endotoxin, however
the timing of production seems to vary between species (205,207,208).

A recent review by Sánchez-Margalet et al (2003) suggests that leptin is an
important player in the immune response and up-regulates inflammation (209). Leptin may be the signal that links the energy stores with the immune system, and
may be the cause of the immunosuppression associated with starvation, when leptin
levels are low. It has also been suggested that increased levels of leptin in obese
patients may result in an excessive immune response, and may explain why
autoimmune diseases are more common in more affluent countries where people are
more likely to be overweight (209). Leptin has been shown to stimulate the
proliferation of lymphohaematopoetic cells and to increase the phagocytic activity of
macrophages (204,205). By activating human peripheral blood monocytes leptin
causes increased production of IL-6, IL-10 and TNF-α. It also stimulates T
lymphocytes causing increased production of IL-2 and IFN-γ and leptin also inhibits
apoptosis of a number of different cell types, including T lymphocytes (209). Leptin
also causes endothelial cell activation, resulting in a severe sepsis phenotype. In fact
leptin receptors have been found on endothelial cells, leucocytes, lymphocytes,
monocytes and macrophages (210). As leptin has a central role in energy
homeostasis it is possible that leptin may be responsible for the anorexia and wasting
associated with infection (205).

The exact role played by leptin in sepsis has not been fully elucidated. Many
conflicting studies have been published, some have shown that increased levels of
leptin are associated with a better outcome, some have shown a worse outcome in
patients with high serum levels and some have shown no correlation between serum leptin levels and the clinical outcome of sepsis (109,204,205,210). Yousef et al (2010) found high levels of leptin in patients with sepsis and systemic inflammatory response syndrome (SIRS) and postulated that leptin could potentially be used as a marker in patients with sepsis (206). Bornstein et al (1998) was the first to describe leptin as a stress-related peptide and found that a high level of leptin in patients with sepsis was associated with lower mortality rates (205). A recent paper by Shapiro et al (2010) which focused on the vascular inflammatory effects of leptin has contradicted many of these findings and suggested that higher leptin levels were associated with a poorer clinical outcome for patients, and also suggested that some of the previous work carried out looking at the effect of leptin on outcome was flawed as it failed to minimise the metabolic effects of leptin (210). This paper also suggests that high levels of leptin in obese patients may explain why these patients generally have a worse outcome following sepsis (210). Although, the effect of leptin levels on clinical outcome may be in dispute, all agree that levels are elevated in patients with sepsis and septic shock.

Again there is little evidence to support the role of this cytokine in the immune response to severe S. aureus infection in the literature. Unfortunately we did not record the patient’s body mass index (BMI) at the time of data collection, so we were unable to correlate leptin levels with BMI. Here a significant decrease in leptin levels in patients with complicated S. aureus BSI, both on the day of diagnosis and on day seven was identified, suggesting that attenuated leptin levels may have
predisposed these patients to the development of complicated BSI and possibly indicating a significant role for leptin in the immune response to *S. aureus* infection.

Leptin levels followed a similar pattern to RANTES. No significant difference in leptin levels in samples collected on the day of diagnosis of the BSI and on day seven suggests that leptin levels do not increase dramatically throughout the course of the infection. It is interesting that the mean leptin level detected in samples taken on diagnosis was lower than the mean level detected in control samples and that leptin levels were higher in samples collected on day seven. These findings suggest that leptin levels increase late in the course of *S. aureus* BSI. Leptin is an important player in the immune response and has been shown to stimulate the proliferation of lymphohaematopoetic cells and to increase the phagocytic activity of macrophages, suggesting a role in the eradication of *S. aureus* (204,205). The fact that leptin levels were slow to rise in patients may reflect its natural niche in the innate immune response to infection or it may suggest that the leptin response in *S. aureus* BSI is attenuated for some reason.

In our analysis of the dynamics of the cytokine response to *S. aureus*, one limitation of this study was the lack of a true baseline cytokine level for comparison. Such a comparator would help to fully assess the magnitude of the cytokine response to *S. aureus* BSI. Many different factors, some known and some unknown will stimulate cytokine production. Furthermore, it is logistically difficult to establish a baseline level that is reliable in patients as this would require the taking of blood samples from a large patient population, a small proportion of which would then develop *S. aureus* BSI.
Patients with *S. aureus* BSI often have multiple co-morbidities and this was the case in the present study. Underlying co-morbidities such as malignancy or autoimmune disease may have also contributed to changes in cytokine levels. The variation in cytokine levels is evident even in healthy controls with no underlying illness (e.g. IL-6 levels – 10.56, 0.12, 0.94 and 2.7 pg/mg of protein). Ideally a larger sample size of healthy controls should have been recruited. Although variations that spanned two orders of magnitude were found in the cytokine levels of the controls tested, some patterns emerged when the cytokine levels in controls were compared to those recorded in patients with *S. aureus* BSI. With the exception of leptin, the mean cytokine concentration identified in samples of patients collected on the day of diagnosis of the *S. aureus* BSI was higher than the mean concentration identified in control samples, although these differences were not statistically significant. It would have been interesting to re-assay the cytokine levels in the patients when their *S. aureus* BSIs had resolved and use these values as individual baseline levels. However, this was not logistically possible in this study, as patients had been discharged from hospital at this point. It is unclear why the mean leptin level detected in control samples was higher than the mean leptin level detected in patients (26.80 ±12.55 pg/mg of protein *versus* 24.50 ±3.93 pg/mg of protein), although the difference in levels was not found to be significant (*p*=0.72). It is generally accepted that leptin levels rise in response to sepsis but some previous work has shown that low leptin levels are associated with an unfavourable prognosis (205). Perhaps the slightly attenuated leptin response in patients with *S. aureus* BSI reflects the severity of *S. aureus* BSI.
5.3.2. Antibiotic resistance, genotype and virulence of isolates causing *S. aureus* BSI and their contribution to the cytokine response

Much work has been carried out comparing the outcome of MRSA and MSSA infection with some variation in findings. Many studies have found higher mortality associated with MRSA BSI compared to MSSA BSI (34-36). Fowler *et al* (2005) found that haematogenous complications were more likely to occur in patients with MRSA BSI (RR 2.09) (32). While Hawkins *et al* (2007) found that methicillin resistance was independently associated (OR 5.22) with the development of persistent *S. aureus* BSI (33). Chang *et al* (2003) found that mortality rates due to endocarditis, caused by MRSA, were significantly higher than in patients infected with MSSA (211). Overall, it would appear that MRSA infections tend to have a worse outcome than MSSA infections, the exact reason for this discrepancy is not known. Numerous factors have been implicated in the outcome of MRSA infections particularly the efficacy of vancomycin therapy, the current treatment of choice for severe MRSA infection (143). Studies have also found that the insertion of the *meca* element may influence the expression of certain virulence determinants such as fibrinogen or fibronectin adhesions, which then may impact on the clinical outcome of *S. aureus* infection (176). Another potential factor is that MRSA infections are more likely to be healthcare associated and occur in elderly patients with multiple co-morbidities and/or prosthetic devices, which are factors associated with a worse clinical outcome in *S. aureus* BSI (32,33,39).

In this study, we found a significant difference in GROγ levels on the day of diagnosis in patients with MRSA BSI compared to MSSA BSI, (*p*<0.05). This
suggests that infection with MRSA stimulates greater GROγ production than MSSA infection early in the course of the clinical infection. The stimulus for, and the effects of this increased production are not known. The factors that are known to stimulate production of GROγ also stimulate production of other cytokines and are produced in response to elements present in both MRSA and MSSA. The effects mediated by GROγ are equally important in MRSA and MSSA infection. If mecA augments the expression of virulence factors such as fnb, as shown by Vaudaux and co-authors (1998) and certain CA-MRSA strains exhibit increased expression of virulence determinants, such as α-toxin and phenol-soluble modulins, then differential specific host responses may also result. This in turn may partially explain the increased GROγ production in response to MRSA (13,176).

As there was no significant difference identified in the levels of the other three cytokines in patients with MRSA BSI and MSSA BSI it appears that the cytokine response, for the cytokines assessed, to MSSA and MRSA BSI is similar. This is perhaps to be expected given that the components in S. aureus which are known to stimulate cytokine production are present in both MRSA and MSSA isolates.

The isolates studied belonged to a range of CCs and we assessed the relationship between the molecular type of isolate causing S. aureus BSI and the cytokine response to determine if a particular type of isolate stimulated a more vigorous cytokine response. We found that RANTES and GROγ levels were significantly higher in patients infected with S. aureus isolates belonging to CC22
(p ≤ 0.05 and p ≤ 0.05, respectively). The clinical significance of this finding is unclear. CC22 isolates are highly prevalent in the Irish healthcare setting, are a frequent cause of invasive infection and were the most prevalent isolates in this study (20/70 isolates, 28.57%). The fact that the level of the other cytokines tested was not significantly higher in response to this clone compared to the other clones investigated and the fact that isolates belonging to this clone were not found to have an association with the development of a complicated BSI suggests that the clinical impact of this finding is minimal. Isolates belonging to other CCs were not associated with significantly higher or lower cytokine responses.

We compared the cytokine response of patients infected with isolates carrying a number of different toxin encoding genes to determine if there was a significant difference in cytokine levels in patients infected with isolates positive for these genes. The only significant finding was a higher RANTES level in patients infected with isolates that were positive for the egc complex. Again the clinical significance of this finding is unclear and it may also reflect the clonal association of the egc with ST22 MRSA as mentioned earlier. The fact that the other cytokines were not significantly elevated in these patients suggests that this finding does not impact significantly on the clinical outcome of infection. Also it is important to remember that we have not measured the expression levels of these genes in the host and the fact the isolates carry the toxin encoding genes does not mean they are expressed in equal amounts at all times during the clinical course of S. aureus BSI.
5.3.3. Relationship between the outcome of *S. aureus* BSI and the cytokine response

Outcomes of *S. aureus* BSI vary greatly. Numerous factors have been identified that are known to be associated with the development of persistent BSI and haematogenous complications (32,33,39). The importance of the role of the cytokine response in the host’s immune response to and its ability to eradicate *S. aureus* is not known.

IL-6 levels were found to be higher on the day of diagnosis in patients with complicated BSI compared to patients with uncomplicated infection but this difference was not statistically significant. Our findings support previous work reporting that higher IL-6 levels are more likely to be associated with more severe bacterial infection. Previous work suggests that higher IL-6 levels are found in more severe infections and correlate well with high mortality rates (181,182,186,188). This work is the first, to our knowledge, to examine the IL-6 response in patients with *S. aureus* BSI. It appears that patients with more severe *S. aureus* infection had higher IL-6 levels detected early in the course of the clinical infection. As already stated, IL-6 has a potential role as a diagnostic indicator in sepsis and perhaps it could also be used as a diagnostic tool early in the course of *S. aureus* infections to identify patients with a poorer prognosis.

Leptin levels were significantly higher in patients with uncomplicated *S. aureus* BSI on day seven (*p*≤0.05) and a similar trend was identified on the day of diagnosis of the BSI (*p*=0.071) compared to levels detected in patients with complicated infection, indicating that patients with more severe infection had a
sustained attenuated leptin response. It would appear that leptin has both beneficial and detrimental effects for the host in the setting of sepsis. Its ability to stimulate the proliferation of lymphohaematopoietic cells and to increase the phagocytic activity of macrophages enhances the host’s ability to eradicate *S. aureus* but it can also cause endothelial cell activation, resulting in a severe sepsis phenotype (204,205). It is perhaps this combination of functions that has resulted in controversy on the relationship between leptin levels and the outcome of sepsis. Some studies have found that higher leptin levels are associated with a better outcome in patients with sepsis, while more recent work has suggested that higher levels of leptin are associated with a poorer clinical outcome (205,210). The reason for the lower level of leptin in patients with complicated *S. aureus* BSI is not clear. It may be host related or related to the infecting isolate. Many of the effects of leptin are beneficial in the host’s defence against *S. aureus* BSI, suggesting that the decreased leptin levels seen in patients with complicated infection may have contributed to the development of the complications by reducing the host’s ability to eradicate the infecting organism.

5.3.4. Relationship between patient characteristics and the cytokine response

The cytokine response may be independent of the infecting isolate and due to inherent abnormalities in the host, which may be innate or acquired. We assessed a number of different patient factors to assess their impact on the cytokine response and the outcome of infection.
Immunosuppressive therapy had a variable effect on the cytokine response in patients, with no clear patterns detected, although numbers in each group were too small to allow a robust statistical analysis of the data.

In our study patients with DM were not found to be more likely to develop a complicated infection than patients without DM (3/14 versus 8/47, \( p=0.70 \)). Hyperglycaemia, neuropathy and peripheral vascular disease put patients at increased risk of developing a number of infections such as urinary tract infection, skin and soft tissue infection and fungal infection. Alterations in the innate immune system such as impaired phagocytic activity of neutrophils have been described which may also contribute to the development of infection and type II DM is also known to have an impact on the cytokine response to infection (212,213). The only significant difference in cytokine response seen between patients with DM and patients without DM was a significantly higher IL-6 response in patients with DM on day seven (\( p \leq 0.05 \)). As mentioned previously, high IL-6 levels have been associated with more severe bacterial infection and higher mortality rates in patients with sepsis (178,180-184,193). The reason for the higher IL-6 levels in patients with DM is unclear but it did not seem to impact on the clinical outcome of infection in this patient group.

Renal patients were not found to be more likely to develop a complicated infection than patients belonging to other specialities (2/23 versus 9/38, \( p=0.18 \)). IL-6 and GRO\( \gamma \) levels were significantly lower in renal patients on the day of diagnosis of the BSI compared to patients belonging to other specialties (\( p \leq 0.05 \) and \( p=0.01 \),
respectively). RANTES levels were significantly lower in renal patients on day seven compared to other patients \((p \leq 0.05)\).

As explained earlier (see Section 3.3), renal patients are at greater risk of infection than the general population. Dialysis patients are at risk of developing \textit{S. aureus} BSI due to the presence of CVCs and frequent contact with healthcare facilities. This increased risk is reflected in the large number of renal patients included in this study (40\% of all patients included) and the presence of the national renal transplantation service in one of the hospitals. However, although these patients appear more likely to develop a \textit{S. aureus} BSI, they do not appear at increased risk of severe or complicated infection. This reflects the clinical experience, at least anecdotally – renal patients with \textit{S. aureus} BSI rarely develop overt signs of sepsis such as shock when they become bacteraemic. Often an increase in temperature is the only manifestation of the bacteraemia with the patient remaining otherwise well. Perhaps the attenuated cytokine response, in particular the significantly lower levels of IL-6, GRO\(\gamma\) and RANTES that we identified in this group is the key to this attenuated response to sepsis. Various factors, such as dialysate fluid and the dialyser membrane have been shown to activate components of the immune response such as complement and cytokine production (214-216). Some work has been undertaken looking at cytokine levels in HD patients with variable findings. Rysz et al (2006) found that IL-1, IL-6, IL-8 and TNF-\(\alpha\) levels were increased while IL-2 levels were not altered during a single HD session, while Pertosa et al (1998) found that cytokine levels did not change during HD (216,217). It would appear that any increase in cytokine levels during HD is transient and
relatively moderate (218-220). No previous study, to the best of our knowledge has found reduced levels of cytokines in HD patients.

It is also interesting to note that leptin levels were higher in these patients, but not significantly so. Although these patients do not develop any features of the sepsis phenotype, which may be detrimental to the host, and which may or may not be due to an attenuated cytokine response, the fact that their cytokine response is impaired does not appear to make them more vulnerable to the development of complicated infection. One possibility is that the higher leptin levels are protective in some way, resulting in a less severe disease phenotype and a lower rate of complicated infection. Another possibility is that because these patients have been previously exposed to *S. aureus* on numerous occasions, their antibody response is finely tuned to these pathogens and can target them specifically. This may result in a lesser role for innate or non-specific immune responses with their associated unfavourable side effects, in the eradication of the infection. This may allow patients to remain relatively ‘well’, while still overcoming the infection effectively.

### 5.4. Summary

- IL-6, GROγ, RANTES and leptin levels in patients with *S. aureus* BSI were independent of the molecular type of infecting isolate.

- IL-6 levels were higher in patients with complicated *S. aureus* BSI compared to patients with uncomplicated BSI, although this difference was not statistically significant.
• RANTES levels rose significantly in all patient groups by day seven of the BSI
• Leptin levels were statistically significantly lower in patients with complicated BSI compared to patients with uncomplicated BSI on day seven, with a similar trend identified in samples taken on the day of diagnosis of the BSI
• IL-6, GROγ and RANTES levels were significantly lower in renal patients with *S. aureus* BSI but not in those with DM
Chapter VI
Susceptibility of *S. aureus* Isolates Causing BSI to the Antimicrobial Peptide LL-37
6.1. Introduction

The innate immune system acts as the first line of defence against microorganisms, such as *S. aureus*, and cationic antimicrobial peptides are an important component of the response. Two CAMPs that have an important role in *S. aureus* killing are the α-defensins and LL-37. LL-37 is a cathelicidin, found in various cell types including neutrophils, monocytes, epithelial cells, salivary glands and sweat glands (103,104). This molecule has a number of important functions, which include; the protection of skin and epithelial surfaces from invading organisms, the direct killing of phagocytosed bacteria and immunoregulation (103,104).

A number of organisms, including *S. aureus*, have evolved mechanisms that confer reduced susceptibility to CAMPs, such as LL-37, allowing them to evade this important component of the innate immune system. The mechanisms by which *S. aureus* evades LL-37 are not fully understood, but a number of potential factors have been identified and include aureolysin activity, which inactivates LL-37, and PIA and FnBP both of which have been shown to reduce susceptibility to the host defence peptide LL-37 (98,105,124). In addition, *S. aureus* may increase its surface charge and therefore reduce susceptibility to LL-37. Examples of mechanisms for surface charge alterations include the incorporation of D-alanine into teichoic acid and the incorporation of lysine into phosphatidylglycerol, a component of the cell membrane (66,98,105,119,124). PIA production, fibronectin binding protein and the presence of a capsule influence susceptibility of *S. aureus* isolates to killing by CAMPs, although the exact mechanisms are unknown (98,105). MRSA isolates are reported
to be more resistant to killing by LL-37 than MSSA isolates, but again the mechanism of resistance is still not fully understood. It does however, not appear to be related to carriage of the mecA gene (125).

This Chapter describes an investigation of the relationship between killing of S. aureus isolates by LL-37 and the clinical outcome of infection:

- The susceptibility of MRSA and MSSA isolates causing BSI to killing by LL-37.
- The effect of strain type on susceptibility to killing by LL-37.
- The effect of some genetic characteristics (presence of specific genes, e.g. *mprF, aur, ica, fnb* and *cap* genes) on the susceptibility of isolates to killing by LL-37.

### 6.2. Results

#### 6.2.1. Selection of isolates and concentration of LL-37 used in killing assay

The selection of isolates used in the LL-37 killing assay is outlined in the Material and Methods Section (Section 2.12) and details of the selected clinical isolates are outlined in Table 6.1 below.
Table 6.1. Isolates used in LL-37 killing assay

<table>
<thead>
<tr>
<th>Isolate No.</th>
<th>Organism</th>
<th>mpf gene\textsuperscript{a}</th>
<th>aur\textsuperscript{a}</th>
<th>ica\textsuperscript{a}</th>
<th>FnBPA\textsuperscript{a}</th>
<th>Capsule type\textsuperscript{a}</th>
<th>Clonal complex (CC)\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncomplicated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>MRSA</td>
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<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>5</td>
</tr>
<tr>
<td>13</td>
<td>MSSA</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>8</td>
</tr>
<tr>
<td>16</td>
<td>MSSA</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>8</td>
</tr>
<tr>
<td>22</td>
<td>MSSA</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>5</td>
</tr>
<tr>
<td>23</td>
<td>MSSA</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>5</td>
</tr>
<tr>
<td>24</td>
<td>MSSA</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>5</td>
</tr>
<tr>
<td>30</td>
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<td>Negative</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>8</td>
</tr>
<tr>
<td>32</td>
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<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>8</td>
</tr>
<tr>
<td>50</td>
<td>MSSA</td>
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<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>5</td>
</tr>
<tr>
<td>58</td>
<td>MSSA</td>
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<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>8</td>
</tr>
<tr>
<td>Complicated</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>MSSA</td>
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<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>8</td>
</tr>
<tr>
<td>7</td>
<td>MRSA</td>
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<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>5</td>
</tr>
<tr>
<td>15</td>
<td>MSSA</td>
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<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>8</td>
</tr>
<tr>
<td>17</td>
<td>MSSA</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>5</td>
</tr>
<tr>
<td>31</td>
<td>MRSA</td>
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<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
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<td>5</td>
</tr>
<tr>
<td>33</td>
<td>MSSA</td>
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<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>8</td>
</tr>
<tr>
<td>37</td>
<td>MSSA</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>8</td>
</tr>
<tr>
<td>48</td>
<td>MSSA</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>8</td>
</tr>
<tr>
<td>49</td>
<td>MRSA</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>5</td>
</tr>
<tr>
<td>53</td>
<td>MSSA</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>5</td>
</tr>
</tbody>
</table>
\textsuperscript{a}Identified using the DNA microarray

As shown in Figure 6.1, the \textit{S. aureus} killing activity of LL-37 was concentration-dependent and furthermore, the reference isolates tested, varied in their susceptibility to LL37, with \textit{S. aureus} 44330 the most susceptible and \textit{S. aureus} COL, the least susceptible. A 2.5µg/ml concentration of LL-37 was selected as the
optimal concentration for the assay based on these results because this concentration allowed the observation of susceptibility differences between strains. At higher concentrations ($\geq 10 \, \mu g/ml$), all reference isolates were susceptible.

Figure 6.1. Killing activity of LL-37 against three *S. aureus* control strains – COL, 44300 and SH1000. *S. aureus* reference strains ($5 \times 10^6$ CFU/ml) were incubated with 0, 2.5, 5, 10 and 20 $\mu g/ml$ of LL-37 for 1 h at 37 °C in 10mM potassium phosphate buffer, pH 7.4. Data shown represent mean killing activity ± SEM from duplicate assays carried out on two separate occasions.
6.2.2. LL-37 susceptibility of \textit{S. aureus} isolates causing complicated and uncomplicated BSI

Among \textit{S. aureus} isolates causing uncomplicated BSI there was a wide range of susceptibility to killing by LL-37 under the assay conditions used (Figure 6.2.a). For example, isolate 16 was the most susceptible to killing by LL-37 with a mean killing activity of 75.76\%, while isolate 50 was not susceptible to LL-37 killing under these conditions. Isolates causing complicated BSI also showed marked variation in their susceptibility to LL-37 under the assay conditions used, ranging from 47.2\% killing activity (isolate 17) to 0\% killing activity (isolate 49) (Figure 6.2.b).

\textbf{Figure 6.2. Killing activity of LL-37 against \textit{S. aureus} isolates.} \textit{S. aureus} clinical isolates ($5 \times 10^6$ CFU/ml) causing uncomplicated BSI (a) or complicated BSI (b) were incubated with 2.5\,$\mu$g/ml LL-37 for 1 h at 37 $^\circ$C in 10mM potassium phosphate buffer, pH 7.4. Data shown represent mean killing activity $\pm$ SEM from duplicate assays carried out on three separate occasions. For comparison, the dashed line indicates the killing activity for reference isolate SH1000 under the same assay conditions.
Overall, *S. aureus* isolates causing uncomplicated BSI were more susceptible to killing by LL-37 than isolates causing complicated BSI (32.4% *versus* 25.1%) but this difference was not significant (*p*=0.52) (Figure 6.3).

![Figure 6.3. LL-37 killing of *S. aureus* isolates causing uncomplicated (n=10) versus complicated (n=10) BSI. Data shown represent mean killing activity ± SEM from 10 isolates in each group. Each isolate was assayed in duplicate on three separate occasions.](image)

6.2.3. LL-37 killing of MSSA isolates versus MRSA isolates causing BSI

As the majority of BSIs in the study were caused by MSSA (55/70, 78.57%), the majority of isolates selected for the killing assay were also MSSA (16/20, 80%). A wide variation in the susceptibility of these isolates to LL-37 was found under the assay conditions. The killing activity of LL-37 against MSSA ranged from 0% (isolate 50) to 75.76% (isolate 16). Among the four MRSA isolates selected for the LL-37 killing assay, susceptibility ranged from 0% killing activity (isolate 49) to
42.06% killing (isolate 7). Although MSSA isolates were more susceptible to killing by LL-37 than MRSA isolates (31.06% versus 19.55%) the difference was not statistically significant. ($p=0.41$) (Figure 6.4).

![Figure 6.4](image)

**Figure 6.4.** LL-37 killing of MSSA isolates causing BSI *versus* MRSA isolates causing BSI. Data shown represent mean killing activity ±SEM where $n=16$ for MSSA isolates and $n=4$ for MRSA isolated. Each isolate was assayed in duplicate on three separate occasions.
The susceptibility of MSSA isolates causing uncomplicated and complicated BSI was also compared. MSSA isolates causing uncomplicated BSI were more susceptible to killing by LL-37 than those causing complicated BSI (mean percentage killing, 35.98% versus 24.74%) but this difference was not significant ($p=0.54$) (Figure 6.5).

![Comparison of LL-37 killing of MSSA isolates causing uncomplicated (n=9) BSI versus MSSA isolates causing complicated (n=7) BSI.](image)

**Figure 6.5.** Comparison of LL-37 killing of MSSA isolates causing uncomplicated (n=9) BSI versus MSSA isolates causing complicated (n=7) BSI. Data shown represent mean killing activity ± SEM. Each isolate was assayed in duplicate on three separate occasions.

The small number of MRSA isolates included in this study precluded similar analysis of LL-37 susceptibility in uncomplicated versus complicated MRSA BSI.
6.2.4. Effect of clonality on susceptibility of *S. aureus* isolates to LL-37

*S. aureus* isolates belonging to a variety of different CCs were included in the LL-37 killing assay (Table 6.1). CC15 and CC22 were the most prevalent CCs among the collection of isolates (both accounting for 4/20 isolates). CC5 and CC30 accounted for 3/20 isolates and CC45 accounted for 2/20 isolates. Other CCs (CC8, CC12, CC20 and CC25) were only represented by a single isolate. Isolates belonging to different CCs were compared to assess their susceptibility to killing by LL-37. However, there was no significant difference in LL-37 bactericidal activity against isolates from the different CCs (Figure 6.6).

![Figure 6.6. Contribution of clonal lineage to LL-37 killing of *S. aureus* isolates causing BSI.](image)

Data shown represent mean killing activity ±SEM for isolates belonging to CC5 (n=3), CC30 (n=3), CC22 (n=4) and CC15 (n=4). Each isolate was assayed in duplicate on three separate occasions.
6.2.5. Genetic factors that may influence susceptibility of *S. aureus* isolates to killing by LL-37

Of the 20 isolates included in the LL-37 killing assay, ten belonged to capsule type 5 and ten belonged to capsule type 8. The presence of a capsule has been shown to result in reduced expression of LL-37, possibly due to masking of fibronectin-binding proteins (105). The susceptibility of isolates belonging to these two capsule types to killing by LL-37 was investigated. There was no statistically significant variation in LL-37 killing activity for isolates with capsule type 5 *versus* capsule type 8 (31.93% and 25.59 *p*=0.74) (Figure 6.7).

![Figure 6.7. Bactericidal activity of LL-37 against capsule type 5 (n=10) and capsule type 8 (n=10) *S. aureus* isolates causing BSI. Data shown represent mean killing activity ± SEM for 10 isolates where each isolate was assayed in duplicate on three separate occasions.](image-url)
Inactivation of the *mprF* gene of *S. aureus* is reported to result in increased susceptibility to LL-37 and the expression of this gene has been shown to influence susceptibility of *S. aureus* to other CAMPs (105,221). Of the 20 isolates analyzed in the LL-37 bactericidal assay two had *mprF* geneotype. The isolates that did not possess the *mprF* gene were both MSSA isolates belonging to CC45, with one causing a complicated infection and one causing an uncomplicated infection. Having an *mprF* genotype in these two isolates did not appear to increase their susceptibility to killing by LL-37 with mean killing values of 14.9% and 4.37% seen, respectively (mean percentage killing of *mprF*+ MSSA isolates was 34.13%). The isolate causing complicated infection was less susceptible to killing (4.37%) compared to that causing uncomplicated infection (14.9%)

6.3. Discussion

LL-37 is produced by many different cell types, including neutrophils, monocytes, epithelial cells, salivary glands and sweat glands (103,104). It has a number of important roles in the innate immune system such as protecting skin and epithelial surfaces from invading organisms, directly killing phagocytosed bacteria and immunoregulation (103,104). The ability of *S. aureus* to evade and inactivate this important antimicrobial peptide may contribute to its ability to cause serious infections such as BSI. *In vivo*, LL-37 is one component of a complex cascade of interactions between the infecting isolate and the innate immune system. It is well
recognised that LL-37 acts synergistically with other antimicrobial peptides such as β-defensins and lysozyme (102).

In the work described here, an *in vitro* assay was used to investigate the susceptibility of *S. aureus* isolates causing BSI to LL-37. The *in vitro* assay, unlike the determination of MIC, is carried out on cells that are not actively metabolising and in addition, may not reflect the true complexity of this interaction *in vivo*. However, the results of the killing assay indicate the potential of clinical isolates to interact with this component of innate immunity. The assay was used to investigate whether *in vitro* susceptibility to this peptide correlated with persistence or potential for complications of *S. aureus* BSI. In addition, the effect of a number of other virulence traits on susceptibility to LL-37 such as capsule type, and methicillin susceptibility were investigated.

LL-37 susceptibility among reference isolates was found to be concentration dependent and all although there were activity differentials among the reference strains at lower LL-37 concentrations they were all susceptible at higher concentrations of the peptide. There was a notably marked variation in the *in vitro* susceptibility of *S. aureus* clinical isolates to LL-37 - 0% to 75.76% (mean percentage killing 28.75%). Significant variation in killing activity was previously reported among *S. aureus* clinical isolates (122). Although not statistically significant, *S. aureus* isolates causing complicated BSI infections were less susceptible to LL-37 than isolates from uncomplicated infections. This suggests that the activity of LL-37 could potentially have an impact on the clinical outcome of some BSI caused by *S. aureus*. The study isolates included both MSSA and MRSA.
isolates. It has been reported previously that MRSA isolates are more resistant to killing by LL-37 than MSSA isolates (105,122,125). The number of MRSA isolates in this study, four, was too small to allow the comparison of LL-37 susceptibility of MRSA isolates causing complicated and uncomplicated BSI. However, when only MSSA isolates were included in the analysis (16 isolates) no statistically significant difference was found between the killing of isolates causing uncomplicated and complicated infection (p=0.54). LL-37 is only one component of the innate immune system, and other antimicrobial peptides may play a role in the defence against S. aureus infection and may act synergistically with LL-37. The α-defensins and β-defensins are produced by many of the same cells that produce LL-37 and have similar functions to LL-37 (100). Overlap and redundancy is a recurring feature of the innate immune system and this suggests that even if the infecting S. aureus isolate can evade or inactivate a single component of the system it may still be susceptible to another component with similar antimicrobial properties.

As mentioned above, MRSA isolates are reported to be more resistant to killing by LL-37 than MSSA isolates, although the exact mechanism behind this resistance is not clear (105,122,125). Ouhara found that LL-37 resistant MRSA strains had significantly higher zeta potential than LL-37-susceptible MRSA strains and that expression of the mecA gene was not necessary for LL-37 resistance in MRSA and suggested that there may be another gene that confers resistance to LL-37 (125). Although not statistically significant, we did find that MSSA isolates were more susceptible to LL-37 than MRSA isolates (mean killing 31.06% versus
Although carried out on only small numbers of *S. aureus* isolates, our results, in part, support the findings of previous studies.

The genetic background of *S. aureus* may be an important factor to consider in the pathogenesis of infection. Our own investigations (Chapter IV), and other recent reports have shown that specific virulence patterns are associated with genetic lineage or the clonal complex to which the *S. aureus* isolate belongs (21,137,165,222). Therefore, the interaction of *S. aureus* with components of innate immunity, such as LL-37, may also be related to the particular *S. aureus* clone. Previous work undertaken found that a number of CC types can be associated with the development of persistent and complicated BSI (37,38). Xiong *et al* (2009) found that isolates belonging to CC30 were more likely to cause persistent BSI, while Fowler *et al* (2007) found a significant association between CC5 and CC30 and complicated BSI (37,38). However, in our study no significant association between isolates belonging to CC5 and CC30 and the development of complicated BSI was identified (*p*=1.0, *p*=1.0, respectively). Our work did suggest that isolates belonging to CC30 were the least susceptible to killing by LL-37, but not significantly so. Perhaps resistance to LL-37 plays a significant role in allowing these organisms to gain access to the host, invade the blood stream, avoid killing, persist and cause complicated BSI.

A number of other *S. aureus* virulence determinants have been shown to affect the activity of LL-37, such as production of the metalloproteinasere aureolysin, production of PIA and the presence of fibronectin binding protein (98,105,124). We detected the presence of the genetic determinants *aur, ica* and *fnb* in all isolates
tested using the DNA microarray (Chapter IV). Therefore the absence of an appropriate control group (i.e. strains lacking these genes) precluded a robust investigation of the impact of these factors on the susceptibility of isolates to LL-37. However, the considerable variation in susceptibility to LL-37 among the isolates studied, despite their carriage of genes shown to facilitate LL-37 resistance, suggests a number of possibilities. LL-37 susceptibility may be either independent of the presence of these genes or the expression of these genes may vary among isolates depending on the growth and assay conditions. As mentioned previously, expression of virulence genes is highly dependent on the dynamic environment encountered by the organism during infection and the killing assay used here does not facilitate investigation of the effects of these factors on LL-37 susceptibility.

In addition, other factors may contribute to LL-37 susceptibility such as carriage of the mprf gene and capsule type. Two MSSA isolates included in this study had an mprF genotype and both belonged to CC45. One was associated with a complicated BSI and the other with an uncomplicated BSI. The mprF gene encodes lysylphosphatidylglycerol (L-PG) synthase that is responsible for the incorporation of lysine into phosphatidylglycerol resulting in the organism being more positively charged at the cell surface. This in turn is believed to make the cells less susceptible to killing by CAMPs, such as LL-37 (105,223). However, the absence of the mprF gene in these two isolates did not appear to increase their susceptibility to killing by LL-37, mean killing 14.9% and 4.37%, respectively (mean percentage killing of mprF+ MSSA isolates was 34.13%). These results are in conflict with previous reports of greater antimicrobial peptide susceptibility in the
absence of a functional \textit{mprF} gene (105,221). However, only two of our study isolates did not possess the \textit{mprF} gene and it is possible that they had other characteristics that made them less susceptible to LL-37. In order to assess the true impact of the presence of this gene on susceptibility to LL-37, more \textit{mprF} isolates would need to be included. The presence of a capsule has been shown to decrease susceptibility to LL-37 (105). However, no work has been carried out previously, to the best of our knowledge, to investigate the relative susceptibility of isolates with capsule types 5 and 8 (the most common capsule types among clinical isolates) to killing by LL-37 (137,165,222). We did not identify a significant difference in the susceptibility of isolates belonging to either capsule type. Perhaps, this suggests that although the capsule may contribute to LL-37 resistance, LL-37 susceptibility is independent of the capsule type.

In this study, the susceptibility of \textit{S. aureus} BSI isolates to a single LL-37 concentration (2.5\(\mu\)g/ml) was investigated and this was selected based on preliminary assays using COL, 44300 and SH1000 strains. We exposed all bacterial isolates to the same concentration of LL-37 but in the \textit{in vivo} environment it is likely that the amount of LL-37 produced varies. The LL-37 concentrations expressed \textit{in vivo} are reported to vary between 1-100 \(\mu\)g/ml and LL-37 production \textit{in vivo} is both constitutive and inducible (224). \textit{In vivo} production of LL-37 is induced by bacteria, bacterial components, other CAMPs and cytokines, with TLRs acting as pattern recognition molecules that stimulate the internal cell signalling pathways (102,105). In addition, the route of entry of the microorganism may also determine the amount of LL-37 produced. For example, it is known that epithelial cells of the skin
produce LL-37 and that it plays a key role in the innate defence against *S. aureus* isolates causing skin and soft tissue infection (122). LL-37 not only plays a role in defence against *S. aureus* infection at the level of the epithelium, it is also involved in neutrophil killing and is produced by neutrophils. It would be interesting to correlate susceptibility of isolates to LL-37 in the killing assay to the susceptibility of isolates to neutrophil killing to assess the full extent of the role of LL-37 in neutrophil killing. However, this was beyond the scope of this present study.

Our assay only assessed one role of LL-37 – its ability to kill *S. aureus*. However, LL-37 plays a significant role in immunoregulation. LL-37 chemoattracts T-cells, mononuclear cells and neutrophils, can inhibit inflammation, recruit macrophages and cause degranulation of mast cells (102). The full extent of LL-37’s involvement in the immune response to *S. aureus* infection *in vivo* is therefore difficult to determine and is beyond the scope of this current research. Given the complex roles of LL-37 in defence against infection, it is likely that its ability to up-regulate the immune response to infection by *S. aureus* may be more important in determining the clinical outcome of an infection, than the infecting isolate’s susceptibility to killing by LL-37.

### 6.4. Summary

- Isolates causing uncomplicated infection were more susceptible to killing by LL-37 than isolates causing complicated infection, but this difference was not significant
• MSSA isolates causing BSI were more susceptible to killing by LL-37 than MRSA isolates causing BSI. However, this difference was not significant

• Isolates belonging to CC30 were the least susceptible to LL-37

• No significant difference was seen in the susceptibility of isolates with capsule type 5 compared to capsule type 8
Chapter VII
Conclusion and Future Research
7.1. Conclusion

This study originated from the observation that the outcome of *S. aureus* BSI is often unpredictable. Often very ill patients with chronic underlying complicating factors recover quickly from infection whereas, for those with no underlying medical conditions, who are expected to recover well, this is often not the case. The exact reasons for this are unclear. Perhaps, chronic exposure and acute exposure to *S. aureus* may invoke differential immune responses in patients or the virulence traits of the bacterium may influence the outcome of infection in different patients. In addition, the clinical course of infection may be influenced by whether or not the infecting *S. aureus* isolate is susceptible or resistant to certain antibiotics (MSSA or MRSA). The host innate response, bacterial virulence and patient characteristic factors were investigated to assess their contribution to the outcome of *S. aureus* BSI in the hope that modifiable factors, which may be linked to an adverse outcome, could be identified. The identification of host, or bacterial factors that predict a worse prognosis would facilitate the stratification of patients that may benefit from more aggressive treatment or new treatment modalities.

Some critically ill patients were excluded from the study on the grounds that consent was unattainable or, because they succumbed to their infection before all the blood samples were collected. These difficulties meant that the cytokine response in fewer profoundly septic patients was assessed than originally hoped.

Previous work has identified a number of patient factors, such as increased age, the presence of prosthetic devices, other co-morbidities and contact with
healthcare facilities as being associated with a poor prognosis in patients with *S. aureus* BSI (34,38,142,143). Similar patient characteristics and patient associated factors were also investigated in the present study and some were also identified that would appear to influence or reflect the clinical course of *S. aureus* BSI.

In the present study, persistent fever at 72 h had a significant association with the development of a complicated BSI (*p*≤0.05). Previous studies have also identified this association (150,152). Ongoing fever suggests persistent BSI, which will increase the risk of haematogenous complications or it may reflect a persistent metastatic focus of infection that continues to ‘seed’ into the bloodstream, which is not being effectively treated with antimicrobial therapy. All patients with *S. aureus* BSI should have a follow-up blood culture taken within three days of the diagnosis of BSI, and patients who fail to respond clinically, and in whom appropriate antimicrobial treatment does not sterilise the blood, must be thoroughly investigated and aggressively managed. A recent study undertaken in our hospital reviewed the management of *S. aureus* BSI in 57 patients. A mean of three (range = 1–10) follow-up blood cultures were taken while on antibiotics to confirm blood sterility, and five (8.8%) patients did not have follow-up negative blood cultures (225). This finding suggests that appropriate follow-up in all patients with *S. aureus* BSI is not fully appreciated. This highlights the essential involvement of a microbiology, or infectious diseases service, to ensure patients are on appropriate antimicrobial therapy, to ensure they have clinically responded to treatment and that follow-up blood cultures are taken from the patient to document blood sterility.
Prosthetic devices, particularly CVC were identified as the source of a high number of \textit{S. aureus} BSI in this study (35 out of 70 cases studied with 22 of these associated with a CVC). Intravascular catheter related BSI is a leading cause of healthcare-associated BSI (accounting for 10-20\% of all nosocomial infection) and is associated with substantial morbidity and mortality (226,227). In the 2006 Prevalence Survey of Healthcare-associated Infection, intravascular catheters were significantly associated with BSI ($p<0.001$) (228). BSIs secondary to infected intravascular catheters independently increase hospital costs and length of stay and they represent potentially modifiable HCAI risk factors (229-232). Therefore, infection prevention measures are essential to ensure appropriate steps are taken during the insertion, and subsequent care of intravascular catheters. Such measures should include the education of health-care workers on catheter insertion and maintenance, the monitoring of BSI rates, adherence to hand hygiene, the use of a dedicated infusion therapy team, where possible, the use of sterile semi-permeable dressings and the prompt removal of CVCs. It is clear that BSI secondary to intravascular catheters is largely preventable, if the appropriate precautions are taken during insertion and with the subsequent care of the catheter. This study further highlights the use of intravascular devices as a risk factor for the development of \textit{S. aureus} BSI and deficiencies that exist in relation to the care of both CVCs and PVCs.

The largest group of patients in this study were renal patients (38.57\%), the majority (26/27, 96.3\%) of which were on HD. Interestingly, HD patients with \textit{S. aureus} BSI were not more likely to develop a complicated infection (RR=0.62, CI, 201
0.22-1.74), despite the fact that these patients have many risk factors for the development of complicated BSI (e.g. frequent healthcare contact, increased carriage of MRSA, presence of CVCs and severe underlying disease) (31,32,42,43). One might expect a greater rate of complicated BSI among this group, but this finding does reflect the clinical experience at least anecdotally. We have found that HD patients with *S. aureus* BSI rarely develop overt signs of sepsis when they become bacteraemic. Often an increase in temperature is the only manifestation of the bacteraemia. These patients generally respond well to treatment and in our experience rarely go on to develop complications such as IE. It is interesting that in terms of the immune response, IL-6 and GROγ levels were significantly lower in renal patients on the day of diagnosis of the BSI (\(p \leq 0.05\) and \(p = 0.01\), respectively) and RANTES levels were significantly lower in renal patients on day seven (\(p \leq 0.05\)) compared to other patients. Perhaps this attenuated cytokine response contributes to the absence of the severe sepsis phenotype (e.g. tachycardia, hypotension, septic shock), which may be detrimental to the host. It is also interesting that the impaired cytokine response did not appear to make renal patients more vulnerable to the development of complicated infection. One possibility is that because these patients have been previously exposed to *S. aureus* on numerous occasions, their humoral response may be finely tuned to these pathogens and may target them specifically, resulting in prompt eradication of the organism and better clinical outcome. Work carried out by Jacobsson *et al* (2010) showed that patients with low initial antibody levels against certain *S. aureus* antigens were associated with increased mortality and complicated BSI following invasive *S. aureus* infection, suggesting that
antibody response plays a key role in the immune response to invasive *S. aureus* infection (233). This may result in a lesser role for innate or non-specific immune responses with their associated unfavourable side effects, in the eradication of the infection. Allowing patients to remain relatively ‘well’, while still overcoming the infection effectively.

HD patients accounted for a significant proportion of all the patients with *S. aureus* BSI in our study. In these patients BSI results in considerable additional inconvenience, (admission to hospital for antibiotic therapy, unpleasant investigations, changing of CVCs and absence from work) as well as morbidity and in some cases mortality. Treatment of these infections also results in considerable economic costs to healthcare institutions (232). In this group of patients, BSI is often avoidable and many of the risk factors for the development of *S. aureus* BSI in this population are modifiable. Healthcare professionals have a responsibility to reduce rates of BSI by introducing measures to minimise the effects of these modifiable risk factors. Such measures may include early transplantation, prompt formation of AVFs, introduction of CVC care bundles or eradication of MRSA.

Vancomycin was the empiric antimicrobial agent used most frequently (41.43% of patients) in this study. Of the 55 patients infected with MSSA 27 received empiric vancomycin therapy. A significant association between the development of complicated BSI and the use of vancomycin empiric therapy in patients with MSSA was not identified in this study. This is reassuring given the current concerns with regard to the empiric use of vancomycin and it appears
appropriate to continue to use this agent initially in the treatment of *S. aureus* BSI. Ultimately, rapid confirmation of the susceptibility profile of the infecting *S. aureus* isolate is the key to early appropriate therapy rather than the use of newer agents. Newer agents, such as daptomycin should be reserved, where possible, given the ongoing rise in antimicrobial resistance. Furthermore, the implementation of molecular or other techniques (e.g. MALDI-TOF) that decrease the time to characterisation of the infecting isolate should be prioritised.

The clinical outcome of *S. aureus* BSI appears to be independent of the type of infecting isolate and virulence gene carriage. This is in contrast to a number of previous studies, which identified a significant association between certain CC types and virulence genes and the outcome of infection (37,38,40,66-68,169,170,172,173). However, in support of the findings of the present study, a number of other studies have also reported no significant association between virulence determinants and more invasive disease (11,32). Many of the previous studies that identified an association between virulence gene carriage and outcome did however, include a much larger cohort of patients (more than 300 isolates analysed) or had different outcome criteria to that used in the present study (compared colonising with invasive isolates). All isolates in our study were invasive isolates.

Invasive infections are probably opportunistic in most instances and dependent on a variety of factors, not just the pathogenic potential of the invading organism. *S. aureus* isolates must first come into contact with the host (e.g. in the hospital environment), gain entry to the host, often facilitated by the presence of prosthetic devices such as CVCs, and invade the host. Following invasion, it is
likely that the interplay between the organism and the host throughout the course of infection will determine the expression of virulence genes and the host response. Therefore, it is possible that the potential for pathogenic effects is largely dependent on the *in vivo* milieu, which is variable during the course of infection.

Very few single virulence determinants are linked to the development of severe *S. aureus* infection, with the best example being the association of TSST-1 with toxic shock syndrome. In this study we did not identify a specific virulence determinant associated with the development of complicated *S. aureus* BSI. The findings suggest that the molecular basis of invasive *S. aureus* infection is complex and dependent on a wide variety of factors, some of which are host mediated and some of which may be organism mediated.

This, to our knowledge, is the first study to investigate the systemic cytokine response to *S. aureus* BSI. As *S. aureus* BSI, by its nature, is associated with other attributable co-morbid conditions (e.g. concurrent infections, autoimmune conditions, recent surgery etc) we were unable to exclude the contribution of these conditions to the cytokine response. Another possible confounder was the timing of collection of plasma samples for cytokine analysis. The release of cytokines may occur in a pulsed fashion, with patients having fluctuating levels of cytokines in the blood at any given time. At present the optimum time for collection of plasma samples for cytokine analysis is not known.

The innate immune response to any infection consists of a complex interplay between many different components and can include immune cells, such as neutrophils and macrophages, immunomodulatory factors, such as cytokines and
chemokines, antimicrobial peptides and complement. Clearly, there is a large number of different components involved in the innate immune response, but there is also considerable overlap of function and redundancy between the different elements (61,106,234). For example, many different cytokines are produced in response to the same stimuli, will have the same functions and will in turn stimulate production of the same cytokines, CAMPs and stimulate proliferation of the same cells (106). Given these features, it would appear that a single factor alone may not determine the host’s response to an infectious agent as a deficiency in one component may be compensated for by many others. However, this is not likely to be always the case, as illustrated by the development of certain infections in patients with various different types of immunodeficiency, e.g. complement deficiency and Neisseria meningitidis infections, chronic granulomatous disease and the development of infections caused by catalase-positive organisms and fungi (113,235-237).

One of the aims of this study was to identify components of the innate immune system that play a role in the pathogenesis of serious S. aureus infection, which may act as a marker for the development of severe infection or potentially be modified in order to minimise the adverse effects of S. aureus infection in vulnerable patients. Our findings showed that IL-6 levels rose early in the course of S. aureus BSI and that these levels were higher in patients with complicated BSI. It has been shown previously that in sepsis IL-6 levels rise early in the course of infection and that higher IL-6 levels are associated with a poorer prognosis (178-184,193). Our
findings suggest that IL-6 levels could potentially be used as an early inflammatory marker in patients with suspected severe S. aureus BSI.

RANTES levels increased significantly in all patient groups with S. aureus BSI. This suggests that production of this cytokine is stimulated either directly or indirectly by S. aureus and that it plays a key role in the immune response to this infection. There is little existing evidence supporting the role of this cytokine in the immune response to S. aureus. A recent paper did show higher levels of RANTES in the muscles of mice infected with PVL expressing S. aureus isolates compared to PVL negative mutants, suggesting that particular virulence factors produced by S. aureus may stimulate the production of this cytokine (238). However, carriage of the PVL encoding genes was not a feature in the vast majority of our isolates.

Leptin is known to play a key role in the immune response to infection. However, the exact nature of this role is unclear. Some studies report that higher leptin levels are associated with a better prognosis and may be protective in patients with sepsis (205). The findings of our research support this hypothesis; leptin levels were higher in patients with uncomplicated BSI compared to those with complicated BSI. We also identified higher leptin levels in renal patients, a group that despite having many risk factors for the development of complicated BSI were not found to have an increased risk of complicated BSI compared to other patient groups. Perhaps the higher leptin levels in patients had a protective effect in some way, resulting in a more robust immune response to the S. aureus infection and decreasing the possibility of the development of secondary foci of infection. This study
suggests that patients with lower leptin levels are at greater risk of complicated BSI, and that leptin levels could be used as marker to identify patients who are more likely to develop a complicated BSI.

In conclusion, the outcome of *S. aureus* BSI is determined by the interaction of an amazingly complex organism and an even more sophisticated host with an intricate immune system. It is possible that each episode of *S. aureus* BSI is unique in terms of the sequence of events and interactions that allow it to occur or to progress to a more complicated infection. However, as a result of this and other studies, a clearer understanding of the two protagonists in this relationship is emerging. This understanding may contribute to the development of novel treatment modalities in the future including immunotherapy. In addition, the high rate of CVC and PVC-associated *S. aureus* BSI found, indicates that we need to do more to prevent the development of *S. aureus* BSI and that optimisation of the management of *S. aureus* BSI may limit the development of complicated infections. We suggest that this should be a priority for all healthcare professionals involved in the care of patients.
7.2. Future research

The work described in this thesis contributes to our understanding of the pathogenesis of *S. aureus* BSI. However a number of other studies could be undertaken to further develop our understanding of the molecular basis of *S. aureus* BSI.

These include:

- Whole genome sequencing would allow us to examine all genes carried by infecting *S. aureus* isolates. Perhaps a number of virulence genes not found on the array play a significant role in the pathogenesis of *S. aureus* BSI. Whole genome sequencing would allow us to examine the impact of all genes on the outcome of *S. aureus* BSI.

- An animal sepsis model would allow us to assess the interaction between infecting *S. aureus* isolates and particular components of the innate immune response and relate findings to the outcome of infection.

- We have found that that the clinical outcome is not influenced by the pattern of carriage of virulence genes by the infecting isolate. However, the expression of some virulence genes may contribute to pathogenic effects that cause complicated infections. Therefore, this hypothesis could be tested by measuring the transcript levels of specific genes by quantitative real time reverse transcription PCR under conditions that mimic the *in vivo*
environment. However, it is difficult to accurately correlate *in vivo* gene expression with *in vitro* expression in patient isolates.

- The novel finding that leptin levels are higher in patients with uncomplicated BSI should be further studied to determine the role of leptin regulation in *S. aureus* BSI. In particular, it would be appropriate to correlate leptin levels with BMI, to assess the relationship between BMI, leptin levels and the outcome of *S. aureus* BSI.

- It would be useful to determine how cytokine levels differ between patients with complicated and uncomplicated BSI on day 14 following diagnosis. Specifically the role of RANTES in complicated *S. aureus* BSI could be further explored by comparing levels in complicated infections and uncomplicated infections later in the course of infection (e.g. day 14). For those with uncomplicated infections, this would require following up patients who have been discharged from hospital. We were unable to obtain this plasma sample from patients recruited to the present study.

- The cytokine array approach was used here to select cytokines for further study. However other selection methods could be used to select from a wide array of cytokines that may be involved in the response to *S. aureus* BSI. For example, systems are now available that allow accurate detection of multiple specific cytokines (e.g. pro-inflammatory, Th1/Th2 response, provided by Meso Scale Discovery) simultaneously from single samples using a 96 well plate format. In addition, cytokine levels could be analysed in patients with *S. aureus* BSI in comparison to levels in age and sex matched controls.
To assess other aspects of the innate immune response to *S. aureus* BSI, host neutrophil function and the susceptibility of infecting *S. aureus* isolates to neutrophil killing could be assessed. This would involve, determining the susceptibility of clinical *S. aureus* isolates to killing by healthy neutrophils *in vitro*. Neutrophil function in patients with *S. aureus* BSI could be assessed by using patient neutrophils to kill reference *S. aureus* strains. Although of interest, these experiments would likely be logistically challenging given the need to use freshly isolated neutrophils from patients/healthy controls for the functional assay.

Since LL-37 is released from neutrophils and contributes to neutrophil killing of micro-organisms, the correlation between susceptibility of isolates to LL-37 in the killing assay and their susceptibility to neutrophil killing would further elucidate the role of LL-37 in relation to *S. aureus* BSI.

Antibody levels to key components of *S. aureus* in patients with *S. aureus* BSI should be assessed over 2-4 weeks to determine the relationship if any with the clinical outcome of infection. Previous reports have found that low initial antibody levels to various *S. aureus* antigens (e.g. teichoic acid, α-toxin, lipase, SEA, TST-1, ClfA, Efb) correlates with a poor clinical outcome (233). It would be interesting to assess antibody levels against other key *S. aureus* antigens (e.g. staphylokinase, CHIPS, SCIN, FnBP, collagen binding adhesion and PVL toxin) during the course of *S. aureus* BSI and the relationship between these levels and clinical outcome.
Chapter VIII

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219
220


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