Case-control study of invasive *Staphylococcus aureus* disease – host genetic susceptibility and bacterial population structure

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Faculty of Clinical Medicine

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Abstract

Staphylococcus aureus is a major pathogen associated with serious community-acquired and nosocomial disease. It is carried nasally at some time by 70% of the population, yet severe disease is relatively uncommon.

Two case-control studies were conducted in Oxford, UK and Thailand to examine bacterial and host genetic determinants of severe *S. aureus* disease. Over 800 cases and 1,600 healthy control individuals were recruited into the two studies.

The genetic population structures of *S. aureus* disease and carriage isolate populations from the Oxford study were studied and compared using phage typing, pulsed-field gel electrophoresis, and multilocus sequence typing. Natural populations of *S. aureus* have a well-defined clonal population structure, but there was no evidence for the existence of hypervirulent clones.

The presence or absence of 33 putative bacterial virulence determinants was examined. After adjusting for the effect of clonality, seven determinants (*fnbA, cna, sdrE, sej, eta, hlg* and *ica*) were significantly more common in invasive isolates; all contributed independently to virulence.

In the host genetic studies a functional single nucleotide polymorphism at amino acid position 131 of the Fc gamma receptor IIA (FcγRIIA) gene was significantly associated with severe disease. In addition an additive x additive epistatic interaction was found between this FcγRIIA polymorphism and the functional FcγRIIIB NA1/NA2 polymorphism. These significant associations were present when community-acquired disease cases were considered alone, but absent in hospital-acquired disease cases. The putatively functional polymorphisms in genes coding for mannose binding lectin and Toll-like receptor 2 were not associated with disease.

Both bacterial and host factors are important in determining the occurrence of severe *S. aureus* disease. In hospital-acquired infection it is likely that acquired host, bacterial and environmental factors predominate, lessening the importance of any host genetic component.

Attributions

There was a large amount of work involved with this project and a number of people have made major contributions, particularly in the collection of specimens and the analysis of the results presented in this thesis.

Bacterial study

The microbiology department isolated and characterised all of the *S. aureus* strains from patients with severe *S. aureus* disease in Oxford.

Phage typing was performed by Mark Ganner and Hazel Aucken at the Central Public Health Laboratory, Colindale.

In collaboration with Professor Brian Spratt, Dr. Mark Enright devised the MLST scheme for *S. aureus* which was used to examine this collection of isolates.

Dr. Ed Feil devised the BURST analysis to examine the clonal relationship of bacteria and analysed the bacterial data.

Dr. Sharon Peacock had a major intellectual and practical input into the study of adhesins and toxins in this collection of isolates. Dr. Peacock designed the primers for all of the 16 adhesins and performed a subgroup analysis on 100 strains containing 50 carriage and 50 disease causing isolates. Dr. Peacock performed PCR on all of the strains for *fbpA*, V8, *sdrC*, *sdrD* and *sdrE*. A further 5 adhesins (*spa*, *ebps*, *map/eap*, *icaA* and *fib* (*efb*)) and the agr locus (*agrI*, *agrII*, *agrIII* and *agrIV*) were examined by Anita Justice, a Research Assistant working with Dr. Nick Day and myself. A further four adhesins (coagulase, α, β and γ toxins) were evaluated phenotypically by Dr. Maria Kantzanou working in Dr. Peacocks laboratory. The results were analysed by Dr. Nick Day using Stata.

Host study

Mike Murphy and the blood transfusion teams collected an extra 10mls of blood from over 500 blood donors for this work.

Dr Alex Smarason together with the midwives collected umbilical cord blood as controls.

Thailand

Through a collaboration based in Thailand with Dr. Yupin Suputtamongkol a case-control study was also set up in Thailand.

The diagnostic microbiology laboratories in Siriraj, Khon Khan and Ubon isolated and characterised *Staphylococcus aureus* strains from patients with severe disease. In particular Dr. Amori and her colleagues collated the collection of strains in Siriraj hospital.

Dr. Suputtamongkol and her colleagues collected blood from the cases and controls. Also her laboratory assistants, Surachai, Mam and Eat helped to extract host DNA from the blood samples.

Dr. Nick Day helped to analyse the complicated statistical results in this thesis.

The author performed all other laboratory procedures and statistical analyses described in this dissertation.

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There are many people that I would like to thank who have helped to make this project possible. The first person I would like to thank enormously is Nick Day who has supported my work throughout, helped to guide and encourage me when morale was low, for his enthusiasm and help during the last few years. I would like to thank Sharon Peacock for her support and understanding of the subject and for her belief in me. I am grateful to Tony Berendt for his enthusiasm and ideas. I am grateful to Adrian Hill for his input and the wealth of knowledge that I have gained through working in his group. Thank you to Brian Spratt and Mark Enright who taught me the pros and cons of sequencing. A big thank you to Ed Feil for his support and advice. I would also like to thank all the staff in Sharon Peacock's laboratory, in particular Anita Justice who can probably set-up PCR reactions in her sleep; also to Ruth Massey both for reading my introduction and for interesting discussions. I also want to thank all members of the Hill group past and present. In particular Shelley Segal whose enthusiasm, advice and understanding was invaluable. Also other members of the group who I would like to thank particularly include Sarah Campbell whose comments on my introduction were helpful together with Patty Ramaley and Liza Bornman who were also nightly visitors to the laboratory. I would like to thank Yupin Suputtamongkol and her laboratory assistants for being so welcoming and making my trips to Thailand very enjoyable. I also wish to thank the members of the different laboratories who poured and ran numerous polyacrylamide gels or ABI 3700 runs for me including Jackie Fowler, Clarissa Pattern and Peter Field at the Wellcome Trust centre at the Churchill site and Paul Williamson at the centre for Epidemiology in South Parks Road. My final thanks go to my mother who helped to support me throughout and to my husband who has had to endure my mood swings, sleepless nights and has always been encouraging even when I spent most of my waking hours working in the laboratory.

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Abbreviations

A	adenine
ABI	Applied Biosystems
ADCC	antibody-dependent cell-mediated cytotoxicity
agr	accessory gene regulator
AIDS	acquired immunodeficiency syndrome
ANOVA	analysis of variance
bp	base pair
BSAC	British Society for Antimicrobial Chemotherapy
C	cystine
C. albicans	*Candida albicans*
CAPD	continuous ambulatory peritoneal dialysis
CAPS	cleaved amplified polymorphic sequences
CAD	community-acquired disease
cDNA	complementary DNA
CF	cystic fibrosis
CGD	Chronic Granulomatous disease
CHEF	Contour Homogenous Electrophoresis Field
CI	confidence interval
CoNS	Coagulase negative Staphylococci
CPHL	Central Public Health Laboratory
CR(N)	complement receptor (N)
CRD	carbohydrate recognising domain
CRP	C-reactive protein
Da	Dalton
DLV	double locus variant
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
dNTP	deoxynucleotide triphosphate
DZ	dizgygotic
EDTA	ethylene diamine tetra-acetic acid
EM	expectation maximisation
f	frequency
FcγR	Fc gamma receptor
FMH	functional mutant homozygotes
G	guanine
GPI	glycophosphatidylinositol
H_2O_2	hydrogen peroxide
HAD	hospital-acquired disease
Het	heterozygous
H. influenzae	*Haemophilus influenzae*

HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HWE	Hardy Weinberg equilibrium
ica	intracellular adhesion
IDDM	insulin dependent diabetes mellitus
Ig	immunoglobulin
IgG	immunoglobulin G
IL-1R	Interleukin 1 receptor
ITAM	immunonreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
IRAK	IL-1R accessory protein kinase
IVET	*in vivo* expression technology
kb	kilobase
kDa	kilodalton
LBP	lipopolysaccharide binding protein
LD	linkage disequilibrium
LDR	ligation detection reaction
LOD	logarithm of odds
LMP	low melting point agarose
LPS	lipopolysaccharide
LRR	leucine-rich repeats
MAP	mitogen-activated protein
MAPKKK	MAP kinase kinase kinases
MASP	Mannose binding lectin associated serum protease
MBL	Mannose binding lectin
$MgCl_2$	Magnesium chloride
MHC	major histocompatibility complex
MLEE	multilocus enzyme electrophoresis
MLST	multilocus sequence typing
MSCRAMM	Microbial surface component recognising adhesive matrix molecules
mut	mutant
MyD88	myeloid differentiation factor 88
MZ	monozygotic
NC	nasal carriage
NCCLS	National Committee for Clinical Laboratory Standards
NF-κB	nuclear factor-κB
NK	natural killer
N. meningitidis	*Neisseria meningitidis*
NRAMP	natural resistance associated macrophage protein
OR	odds ratio
ORF	open reading frame
p	short arm of chromosome, also probability (p=)

PAMP	pathogen associated molecular patterns
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
PGN	peptidoglycan
PHLS	Public health laboratory service
PRR	pattern recognition receptors
q	long arm of chromosome
RAPD	random amplified polymorphic DNA
REA	restriction enzyme analysis
RFLP	restriction fragment length polymorphism
ROI	reactive oxygen intermediates
RNA	ribonucleic acid
RR	relative risk
rRNA	ribosomal RNA
RTD	routine test dilution
sar	*Staphylococcus aureus* regulator
S. aureus	*Staphylococcus aureus*
sdr	SD repeat region
SLE	Systemic lupus erythematosus
SLV	single locus variant
SNP	single nucleotide polymorphism
SSSS	Staphylococcal scalded skin syndrome
ST	sequence type
STM	signature-tagged mutagenesis
T	thymine
TAMRA	N,N,N'N'-tetramethyl-6-carboxyrhibodamine
TIR	toll/IL-1R domain
TLR	toll like receptor
TRAF6	TNF receptor associated factor 6
TSST-1	toxic shock syndrome toxin
UPGMA	unweighted pair group methods with arithmetic mean
UTI	urinary tract infection
UTR	untranslated region
WG	Wegners Granulomatosis
wt	wild type
λ_R	relative risk ratio

Publications

Moore CE, Segal S, Berendt AB, Peacock SJ, Hill AVS, Day NPJ. The role of FcγRIIA and FcγRIIIB in host susceptibility to severe *Staphylococcus aureus* disease. The manuscript is currently in preparation.

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Chapter 1: Introduction

1.1 General introduction

Staphylococci are a major cause of human illness, responsible for a variety of diseases ranging from mild skin and soft tissue infections to more severe infections such as septicaemia, endocarditis and osteomyelitis (Emori and Gaynes 1993; Steinberg, Clark et al. 1996). Severe *Staphylococcus aureus* (*S. aureus*) septicaemia-associated infections in the US are associated with high rates of morbidity and mortality both within the hospital and in the community. The mortality attributable to *S. aureus* was 11.9% in one study (Weinstein, Towns et al. 1997). Staphylococci are Gram-positive cocci approximately 0.5 to 1 μM in diameter. Microscopically they form clusters resembling bunches of grapes, as cell division occurs in two planes. *S. aureus* acquired its name from the typical yellow pigmented colonial morphology on agarose culture; other microbiological characteristics include the production of coagulase and catalase and tolerance to sodium chloride. *S. aureus* produces a wide range of bacterial exoproteins, including toxins, proteases and adhesins, many of which are thought to contribute to pathogenesis (Fischetti, Novick et al. 2000). The *S. aureus* genome is approximately 2.8MB in size and consists of a circular chromosome and a variable number of plasmids (Fischetti, Novick et al. 2000). Apart from plasmids the genome contains elements suggestive of a major role for horizontal transfer of genetic material, including transposons, insertion sequences, and pathogenicity islands (Kuroda, Ohta et al. 2001, Baba, Takeuchi et al. 2002). Three *S. aureus* sequences have now been published, all methicillin resistant strains: two hospital-acquired (N315 and the vancomycin resistant Mu50)(Kuroda, Ohta et al. 2001), and one community-acquired (MW2) (Baba, Takeuchi et al. 2002). The sequencing of four other genomes is nearing completion (websites: http://www.sanger.ac.uk/Projects/S_aureus/,

http://www.tigr.org/tdb/mdb/mdbinprogress.html and http://www.genome.ou.edu/staph.html).

S. aureus has a particular propensity for rapidly developing resistance to antibiotics, often within a year or two of a drug's introduction. Clinical resistance to "Celbenem" (methicillin) developed within a year of its release as a therapeutic agent (Jenvons 1961). Resistance to methicillin has increased and in 2001, 42% of bacteraemia strains isolated in British hospitals and reported through the *S. aureus* reference laboratory, were resistant to methicillin (Centre 2002). The worrying problem is that MRSA strains are often resistant to numerous other antibiotics in addition to methicillin. There are instances of multiresistant strains also becoming intermediately (GISA/VISA) or fully (VRSA) resistant to glycopeptides such as vancomycin, currently the mainstay of treatment for multiresistant *S. aureus* infections (Hiramatsu, Hanaki et al. 1997; Hanaki, Kuwahara-Arai et al. 1998; Hiramatsu 1998; Smith, Pearson et al. 1999; Fridkin 2001). Drug resistance as a clinical problem is potentially severe in developing countries, where drug resistance is increasingly common and there are not the resources for either adequate infection control measures or effective antibiotics (Trakulsomboon, Danchaivijitr et al. 2001). Another worry is that *S. aureus* strains are resistant to newly released antibiotics such as Linezolid (Tsiodras, Gold et al. 2001).

Although there are many candidate vaccines against *S. aureus* infections (Fattom and Naso 1996; Fattom, Sarwar et al. 1996; Balaban, Goldkorn et al. 1998; McKenney, Pouliot et al. 1999; McKenney, Pouliot et al. 2000; Tollersrud, Zernichow et al. 2001) these are of unproven efficacy and are unlikely to make an impact for many years.

The carriage rate of *S. aureus* is high, with approximately 30% of the population carrying the organism in their anterior nares at any one time (Noble, Valkenburg et al. 1967). On

the other hand the incidence of *S. aureus* infection is low at approximately 20 per 100,000 per year (CDSC 2000). A critical question, one that is central to the planning of the studies described in this thesis, is why there is such a disparity between the carriage and disease rates. There are two possible explanations for this: the organisms infecting severely ill patients could be more virulent than those carried in the anterior nares of the majority of healthy carriers; or there may be specific host susceptibility factors resulting in some individuals having a higher chance of developing *S. aureus* infection independent of the virulence of the organism. It is probable that both of these scenarios coexist, though the relative importance of the two has not been determined.

This balance in the relative contributions of host and bacterial factors to disease causation varies greatly between pathogens. At one end of the scale are microorganisms capable of causing disease in nearly any host (e.g. Ebola virus and Varicella Zoster virus). At the other extreme are Coagulase negative Staphylococci (CoNS), *Candida albicans* and *Pneumocystis carinii* which are mainly opportunistic pathogens that only cause infection when host immunity is impaired. Where *S. aureus* lies along this spectrum is unclear, though as commensal organisms able to cause occasional severe disease they probably lie somewhere in the middle, between these two extremes.

Host factors can be split broadly into genetic and acquired, and both host and bacterial factors interact with the environment. Examples of acquired risk factors include diseases such as diabetes mellitus (though this in turn has a genetic component), and losing the protection of the skin by having an intravenous cannula inserted. Principle environmental factors include those determining exposure to *S. aureus*, such as living with a *S. aureus* carrier suffering from impetigo, or being admitted to hospital.

This study was designed to examine the extent to which host and bacterial factors influence the occurrence of severe *S. aureus* disease. The aim was to determine whether: i) there are particularly virulent *S. aureus* clones capable of causing disease irrespective of their human host, and ii) whether there are particular hosts more susceptible to *S. aureus* disease irrespective of the strain of *S. aureus* they encounter. To examine the importance of these factors, case-control studies were set up in Oxford and Thailand, with appropriate controls.

1.2 Epidemiology of carriage and clinical disease

1.2.1 Carriage

Up to 70% of the human population are colonised by *S. aureus* at some point in their lifetime (Kluytmans, van Belkum et al. 1997). A plethora of work studying the carriage of *S. aureus* was performed in the 1960's (Noble WC 1961; Williams 1963; Noble, Valkenburg et al. 1967; Polakoff, Richards et al. 1967), though recently it has been a relatively neglected area of study.

Babies are first colonised by *S. aureus* on their umbilical stump (Simpson, Tozer et al. 1960), and when this heals *S. aureus* can be isolated from a number of sites including the axillae and groin, though its preferred niche is the nose, in particular the anterior nares (Moss 1948; Reagan, Doebbeling et al. 1991). Eradication of *S. aureus* from the anterior nares usually leads to clearance of the organism from other areas of the body (Casewell and Hill 1986; Reagan, Doebbeling et al. 1991).

During the first year of life the rate of carriage falls with age. A study performed in our laboratory (A. Justice, personal communication) found that 40% to 50% of babies up to 8 weeks of age carried *S. aureus*, but this declined to approximately 20% in the following months. After the first year of life it has been traditional to define the carriage status of an

individual in terms of one of three groups: i) persistent carriers, ii) intermittent carriers, and iii) persistent non-carriers (Sewell, Clarridge et al. 1982). A number of swabs (at least two) need to be taken over a period of a few months to determine the carriage status of an individual (Noble, Valkenburg et al. 1967; Casewell and Hill 1986; Vandenbergh and Verbrugh 1999; VandenBergh, Yzerman et al. 1999; Nouwen, Ott et al. 2000). Persistent carriers are thought to maintain a single strain over fairly long periods of time, intermittent carriers are thought to carry different strains over time and rarely carry a single strain continuously (Williams 1963; Toshkova, Savov et al. 1997).

1.2.2 Community-acquired disease

S. aureus is a cause of a wide range of infections in the community, including skin and soft tissue infections (superficial abscesses and furuncles, and impetigo), deep abscesses, osteomyelitis, and diseases with major systemic consequences such as endocarditis and septicaemia (Hedstrom 1981; Cockerill, Hughes et al. 1997; Crossley and Archer 1997; Weinstein, Towns et al. 1997; Waldvogel and Bisno 2000). Infections commonly occur with strains carried nasally by the sufferer (White 1963; Luzar, Coles et al. 1990; von Eiff, Becker et al. 2001). Skin diseases such as impetigo may be associated with increased transmission following shedding of *S. aureus* into the environment (Toshkova, Annemuller et al. 2001).

S. aureus was found to be the most common cause of clinically significant bacteraemia in the US, at 18.9% of total bacteraemias (Weinstein, Towns et al. 1997). The mortality from community-acquired *S. aureus* bacteraemia ranges between 11 and 48% (Mortara and Bayer 1993). *S. aureus* endocarditis also has a high mortality rate, ranging from 25 to 47% (Mylonakis and Calderwood 2001). Endocarditis (usually involving infection of a heart valve) is common in severe *S. aureus* infections, 10 to 40% of community acquired

bacteraemia is associated with vegetations on one or more heart valves (Mortara and Bayer 1993). IV drug abusers are at a particularly high risk of developing endocarditis, usually of the right side of the heart (Carton, Maradona et al. 1995).

Other community-acquired infections include osteomyelitis, which occurs following seeding of bacteria from the bloodstream to the bones (Waldvogel and Bisno 2000). Osteomyelitis may occur after ineffective antimicrobial therapy of a bloodstream infection (Waldvogel and Bisno 2000). *S. aureus* can also cause severe community-acquired pneumonia, which is often associated with influenza in the elderly and carries a high rate of mortality (Lowy 1998). *S. aureus* produces a number of exotoxins, many of which contribute to pathogenesis (Fischetti, Novick et al. 2000). Examples include staphylococcal food poisoning, caused by the ingestion of food containing a Staphylococcal enterotoxin (Fischetti, Novick et al. 2000); and toxic shock syndrome, a systemic illness caused by the toxic shock syndrome toxin (TSST-1) superantigen produced by some strains of *S. aureus* and associated with the use of highly absorbent tampons by menstruating women (Fischetti, Chapman et al. 1989).

1.2.3 Hospital-acquired disease

S. aureus is responsible for many hospital-acquired infections, ranging from urinary tract infections to surgical site infections and intravenous device-related bacteraemia (Mylotte, Aeschlimann et al. 1996; Jensen, Wachmann et al. 1999). Infections are often associated with procedures that break the skin barrier, such as insertion of intravenous cannula and most surgical operations (Goldmann and Pier 1993; Anaissie, Samonis et al. 1995; Steinberg, Clark et al. 1996). Intravascular lines are widely used within hospitals and are a major risk factor for bloodstream infections (Schaberg, Culver et al. 1991). The risk of surgical site infections depend on the nature and duration of surgery, whether the patient

carries *S. aureus*, the amount of bacterial contamination of the operative area and the patients immune status (Williams, Jevons et al. 1959; Kluytmans, Mouton et al. 1995; Fischetti, Novick et al. 2000). Procedures involving prosthetic material carry the highest risk of developing infections (Calia, Wolinsky et al. 1969; Dougherty 1988; Fischetti, Novick et al. 2000; Waldvogel and Bisno 2000).

Although the majority of patients developing hospital-acquired *S. aureus* infections are infected by their own carriage strain, another route of infection within a hospital is cross-infection with increasing numbers of outbreaks involving resistant strains (Weinstein 1991; Lessing, Jordens et al. 1995; Steinberg, Clark et al. 1996; Solberg 2000). Cross-infection can occur between patients, from visitors to patients, or from healthcare worker to patient (Reboli, John et al. 1990; Schaberg, Culver et al. 1991; Lessing, Jordens et al. 1996). Strict handwashing procedures must be implemented and adhered to, this precaution is effective in reducing cross-infection (Ojajarvi 1980; Ayliffe, Babb et al. 1988).

1.3 Bacterial factors and severe disease

Many of the factors believed to be involved in *S. aureus* virulence have been well characterised, though proof of their association with disease pathogenesis is often lacking (Fischetti, Novick et al. 2000). Complex interactions occur between a person and its colonising microorganisms, though these are poorly defined. In particular there has been little work on the role of the human immune system during *S. aureus* infections. Many commensal organisms live on humans, such as alpha-haemolytic and non-haemolytic Streptococcal species, CoNS, Corynebacterium species and *S. aureus,* often living in high numbers on the surface of skin and mucous membranes (Mims, Dimmock et al. 1995). Under normal conditions these organisms do not cause infections, but colonise these

surfaces without causing inflammation. Not all organisms are commensals; for some the pathogenic process is essential for survival and transmission. For example when influenza infects a host it replicates causing disease and in the process the organism is dispersed to other hosts for survival (Mims, Dimmock et al. 1995).

S. aureus is a commensal organism which survives on the skin and mucous membranes of the host, and can act as an opportunistic pathogen causing infection after entry through a break in these barriers (Fischetti, Novick et al. 2000). After entry into the host, *S. aureus* produces a number of proteins and polysaccharides, many of which either interfere with the host defence system, or adhere to host extracellular matrix proteins (Fischetti, Novick et al. 2000).

Different organisms are known to have different propensities to cause disease. Also different strains of *S. aureus* have previously been thought of as being more likely to cause disease (such as phage type 80/81) (Shanson 1981), although this is unproven. The bacterial factors thought to increase the severity of a bacterial infection are termed virulence determinants. The virulence determinants which have been described may merely be factors which make *S. aureus* pathogenic as a species compared to less virulent organisms such as CoNS. Many determinants believed to be involved in virulence are found in almost all *S. aureus* strains. Protein A is a surface protein and was the first virulence protein characterised for *S. aureus* using isogenic mutants, (Callagen, 1994) protein A is found in nearly 100% of *S. aureus* strains and has the ability to bind the Fc portion of IgG, thus avoiding the hosts' immune system (Fischetti, Novick et al. 2000). Fibronectin and fibrinogen binding proteins promote bacterial attachment to plasma clots (Raja, Raucci et al. 1990; Moreillon, Entenza et al. 1995). Fibronectin binding protein is present in 84% of disease-causing strains (Peacock, Day et al. 2000), it is important for invasion into endothelial cells (Peacock, Foster et al. 1999). Coagulase is another

extracellular enzyme, it is omnipresent in *S. aureus* (and is used as a test to identify the organism). Coagulase binds with human prothrombin forming staphylothrombin which can convert fibrinogen to fibrin (Kawabata, Morita et al. 1985), it is also able to bind to fibrinogen (Boden and Flock 1989).

It may not solely be the presence or absence of the particular virulence determinant that causes an alteration in bacterial virulence. There may be very small differences in the base pairs or amino acids in some of the virulence factors increasing the likelihood of causing disease. Another possibility is that a variation in the different virulence factors present increases or decreases the ability of the organism to cause disease. Since the publication of the first two *S. aureus* genomes (Kuroda, Ohta et al. 2001), a community acquired MRSA strain (Baba, Takeuchi et al. 2002) and the imminent publication of 4 more strains (including 2 clinical strains collected during this work), the scope for finding and investigating potential pathogenic factors has greatly increased.

1.3.1 Population genetic structure of *S. aureus*.

Because more about the structure of bacterial populations are now known, the spread and development of bacteria can be examined over a period of time and continents. More understanding is thus gained about the pathogenesis of a variety of organisms (Gupta and Maiden 2001). The four important questions about bacterial populations that can be answered using microbial genetics are: i) the evolutionary relationship between isolates from different diseases ii) the role of horizontal transfer and recombination and iii) genetic analysis can determine whether the backbone structure of a bacterium is related to the expression of virulence factors iv) this can provide insights into the role of the host and the adaptation of a bacterium to its site of infection (Musser 1996).

To study a population of bacteria it is important to examine the right collection of strains. A background understanding of the organism and the disease (or lack of disease) are needed (Spratt and Maiden 1999). If the organism is normally found colonising its host and only occasionally causes disease, such as with *S. aureus* or *N. meningitidis*, a sample population containing both carriage and disease causing strains is appropriate (Spratt and Maiden 1999). This means that the background population can be examined across the whole spectrum. If the population as a whole is not examined the results may be severely confounded. A population might appear to be clonal if the sample has been selected only to contain disease-causing strains, as specific clones may be more adapted to causing disease (Spratt and Maiden 1999). Carriage strains could potentially be much less clonal because they are under less selection. For an understanding of clonality a good selection of the two are needed (Smith, Smith et al. 1993; Gupta and Maiden 2001).

To examine a population of bacteria, a method of typing needs to be able to separate genetically different strains. The method should not be too discriminatory otherwise it would not be possible to group related strains. The choice of method depends to an extent on the biology of the organism. For example, when an organism is known to be clonal in nature such at *M. tuberculosis* or *Yersinia pestis* (Sreevatsan, Pan et al. 1997), a relatively non-discriminatory method is likely to demonstrate a single clone of this organism. Where an organism is known to be diverse such as *Helicobacter pylori* and *N. meningitidis* (Suerbaum, Smith et al. 1998; Feil, Maiden et al. 1999; Holmes, Urwin et al. 1999; Suerbaum and Achtman 1999; Achtman and Suerbaum 2000), an over-discriminatory method may show that each strain is unique. The choice of method also depends on the epidemiological context in which it is to be employed. An epidemiological study looking to compare small differences in a group of closely related strains needs to use a very

discriminatory method. Conversely a diverse collection of strains spanning either a large period of time or a wide geographical area requires a less sensitive method.

1.3.2 Methods for determining the genetic structure of a population.

Phenotypic methods are based on differences in gene expression which can vary with different growth conditions, phase of growth, phase variation and spontaneous mutations. Phenotypic methods include biochemical profiles (such as API identification kits), lytic phage patterns (bacteriophage typing), multi locus enzyme electrophoresis (MLEE) and antibiogram testing (Schlichting, Branger et al. 1993; Tenover, Arbeit et al. 1994).

The genotypic molecular techniques available include plasmid analysis, numerous techniques based on PCR (ribotyping, restriction fragment polymorphism (RFLP), random amplified polymorphic DNA (RAPD), binary typing, pulsed field gel electrophoresis (PFGE) and multi locus sequence typing (MLST) (Collins, Smith et al. 1984; Poddar and McClelland 1991; Bannerman, Hancock et al. 1995; Maiden, Bygraves et al. 1998; Marcos, Soriano et al. 1999; van Leeuwen, Verbrugh et al. 1999; Enright, Day et al. 2000).

Phage typing was the first method to suggest the clonal nature of MRSA strains (Lacey and Grinsted 1973). Numerous studies have since supported the clonality of MRSA strains using different techniques (Musser and Kapur 1992; Kreiswirth, Kornblum et al. 1993; Salmenlinna and Vuopio-Varkila 2001; Shopsin and Kreiswirth 2001). Strains producing TSST-1 were also shown to be clonal (Musser, Schlievert et al. 1990). Just because these two small subgroups were found to be clonal, this did not mean that all *S. aureus* strains were clonal. An erroneous clonal nature could have been reported as a result of the wrong sample of strains or possibly different methods should have been applied to type the strains (both studies used MLEE which can be difficult to interpret, is subjective and is less discriminatory than other methods) (Wang, Whittam et al. 1993; Hall, Whiley et al. 1996).

Two large studies have attempted to detect whether *S. aureus* are clonal. The first study examined a large set of 2077 diverse isolates from human, ovine and bovine origin using MLEE. They found 14 different lineages, most of the strains belonged to 4 of the electrophoretic types (ET) suggesting a clonal nature (Musser, Schlievert et al. 1990). Another study examined strains from superficial infections (nasal swabs taken both in the hospital and the community). The study concluded that carriage strains and MSSA strains were similar, but MRSA differed (El-Adhami and Stewart 1997). PFGE was applied to strains collected over a period of time, and 2 stable MRSA clones was found to exist (Blanc, Struelens et al. 2001). PFGE has also been used to examine 405 invasive strains, the strains belonged to 6 major PFGE lineages (Booth, Pence et al. 2001). The evidence fits with *S. aureus* as a species having an epidemic clonal population structure.

1.3.3 Bacterial Virulence determinants

The ability to cause disease is dependent on a variety of factors, though it is often difficult to single out a specific factor causing a particular disease. Pathogenic or virulence determinants have been identified either because they code for genes causing disease or because the determinant is involved in a specific biological process (Fischetti, Novick et al. 2000). These determinants are absent in non-pathogenic organisms (Hammond 2001). Virulence determinants are often found on accessory gene products such as plasmids, transposons, prophages and pathogenicity islands (Fischetti, Novick et al. 2000). They are not essential for growth and cell division, but they are presumed to be advantageous in certain circumstances (Crossley and Archer 1997).

The *S. aureus* cell wall contains peptidoglycan, teichoic acid covalently linked to the peptidoglycan and small amounts of protein (Fischetti, Novick et al. 2000). The wall is 50% peptidoglycan and has a polysaccharide backbone made from alternating residues of

N-acetylglucosamine and N-acetylmuramic acid with peptide cross-linking the backbone and glycine rich interpeptide bridges (Espersen, Hedstrom et al. 1999). Many strains also produce microcapsules around the cell wall. Eleven cell wall capsules have been identified; only types 5 and 8 (found in 75% of invasive strains) are well characterised (Karakawa 1992; Fischetti, Novick et al. 2000).

1.3.3.1 Surface expressed proteins

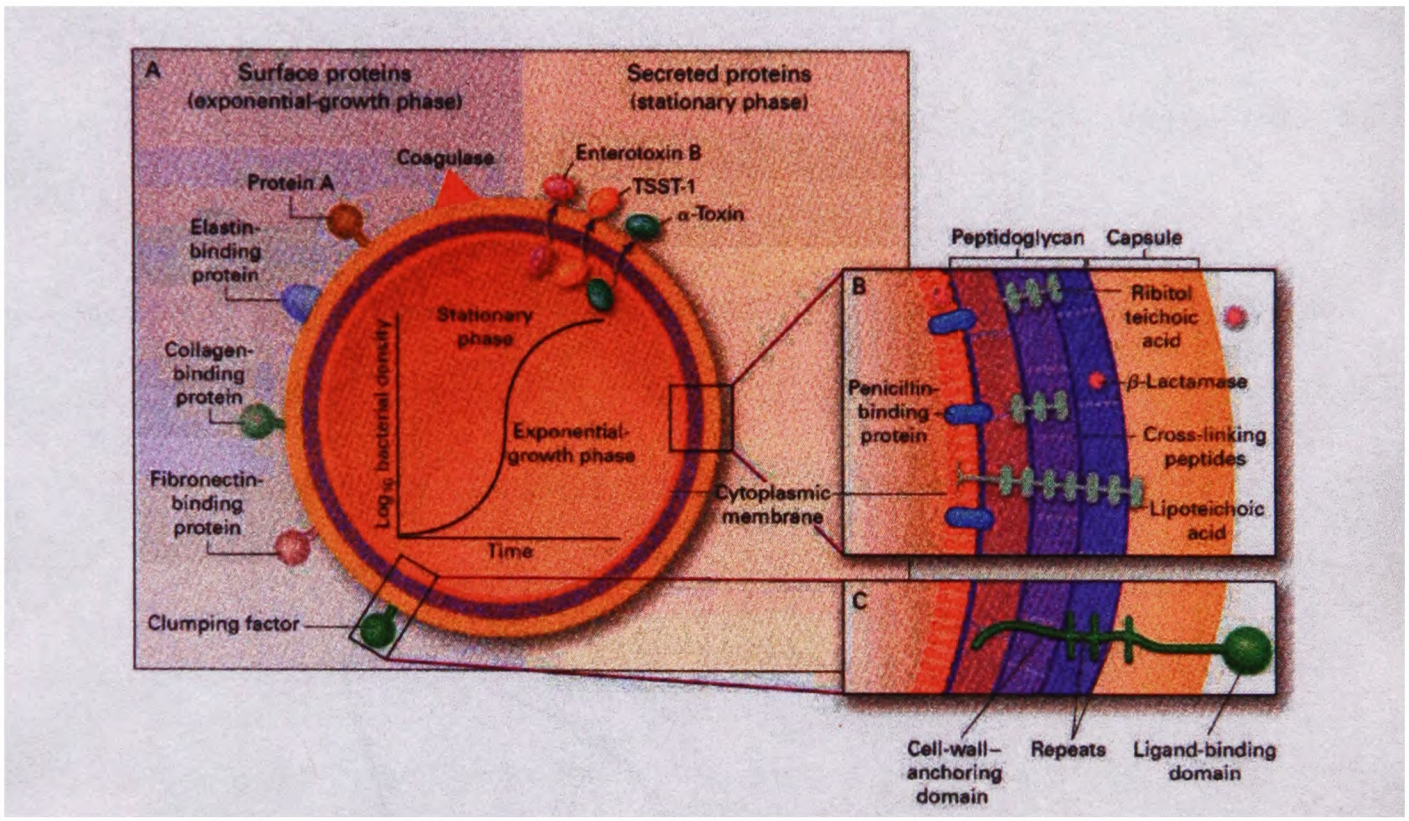


Figure 1: Structure of *S. aureus*. (Lowy 1998).

In Figure 1, panel A shows the surface proteins and secreted proteins. The synthesis of many of these proteins is dependent on the growth phase, as shown by the graph in the centre. Panel B diagrammatically shows a cross section of the cell membrane. Panel C pictures the surface proteins schematically with a wall anchoring domain, repeats and a binding domain.

Adherence of *S. aureus* to the host's extracellular matrix proteins occurs at an early stage in infection. The *S. aureus* surface proteins as a group contain a secretory signal sequence

at the N terminal; positively charged amino acids that extend into the cytoplasm; a hydrophobic membrane spanning domain and a cell-wall anchoring region at the carboxyl terminus (Crossley and Archer 1997; Fischetti, Novick et al. 2000). The C-terminus anchor is commonly a Leu-Pro-X-Thr-Gly (LPXTG) motif (Fischetti, Novick et al. 2000). The proteins are able to act as adhesins due to the ligand binding domain at the N terminus exposed on the surface of the bacterial cell. Bacteria use these proteins to adhere to the eukaryotic cell surface, extracellular matrix protein and carbohydrate structures (hence the term adhesins). These surface proteins have also been termed microbial surface components recognising adhesive matrix molecules (MSCRAMMs) (Patti, Allen et al. 1994; Patti and Hook 1994). Some of the well-known surface proteins such as fibronectin binding protein (FnbA) and the fibrinogen binding proteins (ClfA and ClfB) (Figure 1) are also thought to be putative virulence factors (Greene, McDevitt et al. 1995; Fischer, Vaudaux et al. 1996; Palma, Nozohoor et al. 1996; Peacock, Day et al. 2000; Drew, Liu et al. 2001). When isogenic mutants were compared with wild type strains using infection models, disease was less severe in the mutants (Moreillon, Entenza et al. 1995).

Most surface proteins are under the regulatory control of both *agr* and *sar* and are produced during exponential growth (Crossley and Archer 1997; Fischetti, Novick et al. 2000). Protein A binds to the Fc region of IgG, and is found bound to the cell wall and free in culture supernatant (Forsgren and Sjoquist 1966). It is possible that protein A interferes with phagocytosis of IgG-opsonised bacteria through its ability to bind to the Fc region of IgG and to von Willebrand factor (which is present at sites of endothelial damage) (Hartleib, Kohler et al. 2000). Fibronectin-binding protein facilitates binding to the extracellular matrix, which suggests an additional role in the infectious process. Fibronectin is contained in most body fluids and on many host surfaces, it is thought to facilitate the invasion of host cells and to coat implanted foreign material to which the

bacteria adhere (Vaudaux, Francois et al. 1995; Peacock, Foster et al. 1999). Most *S. aureus* strains contain two fibronectin binding genes, though one study suggested that fewer carriage strains than disease strains contain both genes (64% compared to 82%) (Peacock, Day et al. 2000). Collagen binding protein assists the bacterium to bind to collagenous material such as cartilage (Fischetti, Novick et al. 2000). Fibrinogen binding proteins mediate bacterial clumping; ClfB is produced during early logarithmic growth and ClfA throughout bacterial growth (Ni Eidhin, Perkins et al. 1998). ClfA is able to inhibit fibrinogen-dependent platelet aggregation (Yeaman, Puentes et al. 1992) and both genes are able to promote bacterial adherence to host-conditioned biomaterial (Ni Eidhin, Perkins et al. 1998). The recently identified SD repeat proteins (SdrC, SdrD, SdrE and Bbp) (Josefsson, McCrea et al. 1998) are structurally similar to ClfA and ClfB proteins, their role in virulence is not currently understood. Although not adhesin genes, products of the intracellular adhesion operon (*icaADBC*) synthesise the capsular polysaccharide (biofilm) of *S. aureus*. The co expression of *icaA* and *icaD* is required for full synthesis (Arciola, Baldassarri et al. 2001), with isogenic mutants unable to form a biofilm (Cramton, Gerke et al. 1999).

1.3.3.2 Superantigens

Many *S. aureus* toxins act as superantigens (Fischetti, Novick et al. 2000). As such they can cause polyclonal proliferation of T cells bearing specific T-cell receptor (TCR) Vβ elements (Fischetti, Novick et al. 2000). They bind specifically to major histocompatibility complex class II (MHCII) molecules on antigen presenting cells at a site distinct from the peptide binding groove, and cause the deletion of stimulated Vβ expressing T cells (Marrack and Kappler 1990; Marrack and Kappler 1990). Toxins are able to cause fever, hypotension and other diverse acute toxic shock symptoms through the release of

proinflammatory cytokines (Espersen, Hedstrom et al. 1999). The superantigen group of toxins include the enterotoxins (classified by antigenic differences, such as *sea* and *seb*) causing Staphylococcal food poisoning through ingestion of food containing the enterotoxin (Crossley and Archer 1997). Toxic shock syndrome toxin (TSST) causes both menstrual and non-menstrual toxic shock syndrome (Robbins, Kelly et al. 1989). The exfoliative toxins (*eta* and *etb*) cause Staphylococcal scalded skin syndrome (SSSS), primarily affecting children causing blisters and widespread peeling of the skin (Bailey, Lockhart et al. 1995).

1.3.3.3 Membrane active agents

S. aureus strains also produce a number of non-superantigen exotoxins. These are divided into two main groups based on their modes of action. Membrane-active agents include alpha toxin (α toxin), a cytolytic pore-forming toxin toxic to many cells including erythrocytes and platelets (Fischetti, Novick et al. 2000). Beta toxin (β toxin) causes invaginations of selected regions of the membrane of erythrocytes and leukocytes (for example monocytes) (Fischetti, Novick et al. 2000). Delta toxin (δ toxin) is thought to lyse cells by forming channels in membranes (Mellor, Thomas et al. 1988). The gamma toxin (γ toxin), leukocidin and other bicomponent toxins are a family of proteins made up of two components (the S and the F components). Gamma toxin is haemolytic and the Panton-Valentine leukocicin (PVL) can stimulate and lyse neutrophils and macrophages. The other two component toxins are mainly leukotoxic rather than haemolytic (Fischetti, Novick et al. 2000).

1.3.3.4 Extracellular proteins

S. aureus also produce a number of extracellular proteins. Coagulase binds prothrombin forming staphylothrombin (Crossley and Archer 1997). This transforms fibrinogen to fibrin and binds soluble fibrinogen forming clumps of bacteria. Staphylokinase activates plasminogen to plasmin and induces thrombolysis (Crossley and Archer 1997). Many *S. aureus* strains also produce extracellular proteolytic enzymes such as serine protease, thiol protease and metalloprotease which may be responsible for connective tissue damage (Fischetti, Novick et al. 2000). Other enzymes include the PI-phospholipase, fatty acid-modifying enzyme, hyaluronate lyase, which hydrolyses hyaluronan (part of the connective tissue) (Espersen, Hedstrom et al. 1999) and nuclease which leads to nucleic acid degradation (Fischetti, Novick et al. 2000).

Virulence determinants	Gene encoding protein	*agr* regulated	*sar* regulated
Surface protein			
Protein A	*spa*	Yes	Yes
Fibronectin-binding protein	*fnbA* and *fnbB*	Yes (↓)	Yes (↑)
Collagen-binding protein	*cna*	Yes	Yes
Fibrinogen-binding protein	*clfA* and *clfB*	Yes	Yes
SD Repeat proteins	*sdrC*, *sdrD* and *sdrE*	Yes	Yes
Intracellular adhesion	*icaA*, *icaD*, *icaB* and *icaC*		
Superantigens			
Staphylococcal enterotoxins	*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*, *sek*, *sel*, *sem*, *sen*, *seo*, *sep* and *set 1-15*	Yes	Yes
Toxic shock syndrome toxin	*tst*	Yes (↑)	Yes (↑)
Exfoliative toxins	*eta* and *etb*	Yes (↑)	No
Extracellular proteins			
Coagulase	*coa*	Yes	No
Staphylokinase	*sak*		
Serine protease	*V8/ssp*	Yes (↑)	Yes (↓)
Membrane active agents			
Alpha toxin	*hla*	Yes (↑)	Yes (↑)
Beta toxin	*hlb*	Yes (↑)	No
Delta toxin	*hld*	Yes (↑)	Yes (↑)
Gamma toxin, leukocidin and other bicomponent toxins	*hlg* and *luk-PV*	No	No

Table 1: Putative virulence determinants (Crossley and Archer 1997; Fischetti, Novick et al. 2000; Williams and Ward et al. 2000)

Table 1 contains many of the known putative virulence determinants. Where regulation by *agr* or *sar* is known the control of regulation is shown either as up regulated (↑) or down regulated (↓).

1.3.4 Regulation of virulence factor expression

The production of many *S. aureus* genes are under the control of global regulators acting in response to certain environmental signals such as heat, radiation, osmolarity, pH, oxygen tension and starvation (Chan and Foster 1998). These regulatory systems depend on quorum sensing: they sense the numbers of bacteria in the immediate surroundings and change the transcription of genes in response to the cell density and environmental conditions.

Surface proteins are produced first, these allow the organism to attach to host tissues and help to evade the hosts' immune system (Fischetti, Novick et al. 2000). The regulatory systems can sense the formation of a high concentration of *S. aureus*; at this point expression of the surface proteins decreases and expression of the exoproteins (which facilitate the survival, multiplication and spread of the organism) increases (Lowy 1998).

Surface proteins and exoproteins are termed virulence factors, they are accessory factors which are not essential for growth, and are only produced during certain phases of cell growth or under certain environmental conditions. *In vitro* the loss of each factor decreases the organisms' ability to cause disease. Many of these determinants are encoded on accessory genetic elements such as plasmids, transposons, prophages and pathogenicity islands (Fischetti, Novick et al. 2000). The continued production of the determinant is thought to be detrimental to the bacterium. The transcription of these factors are coordinated and controlled by the Staphylococcal regulatory elements, the accessory gene regulator (*agr*) and the staphylococcal accessory regulator (*sar*).

Much of the information known about the production of virulence determinants comes from *in vitro* work (Fischetti, Novick et al. 2000). New approaches such as signature-tagged mutagenesis (STM) and *in vivo* expression technology (IVET) are beginning to help us understand the factors involved *in vivo* (Mei, Nourbakhsh et al. 1997; Lowy 1998).

a) Agr

The *agr* locus is important in pathogenesis because it regulates the expression of many virulence factors in the post-exponential phase of growth. Switching *agr* on leads to down-regulation of cell surface-associated protein production, and up-regulation of secreted toxins and extracellular enzyme expression (Recsei, Kreiswirth et al. 1986; Peng, Novick et al. 1988; Lowy 1998). The use of experimental animal models such as mice (Abdelnour, Arvidson et al. 1993) and rabbits (Cheung, Eberhardt et al. 1994) have shown that *S. aureus* strains with a mutant *agr* produce an attenuated form of disease, but are not avirulent (Abdelnour, Arvidson et al. 1993; Cheung and Projan 1994; Gillaspy, Hickmon et al. 1995; Booth, Cheung et al. 1997).

The *agr* locus consists of 2 different transcriptional units; RNA II and RNAIII, controlled by the P2 and P3 promoters respectively (Novick, Projan et al. 1995). RNAIII activates expression of extracellular proteins while repressing others such as protein A and coagulase (Figure 2). Transcription of RNAIII is dependent on the activation of the *agr* genes (*agr*B, *agr*D, *agr*C and *agr*A) encoding RNAII. AgrB and AgrD generate an auto inducing peptide through quorum sensing, this peptide activates AgrC which autophosphorylates AgrA (Balaban and Novick 1995; Ji, Beavis et al. 1995; Novick, Projan et al. 1995). AgrA activates transcription of the *agr* locus inducing the down regulation of genes coding for the surface proteins and up regulating the secreted virulence factors (Balaban and Novick 1995; Ji, Beavis et al. 1995).

Four *agr* groups have been defined based on differences in their AIP structures (Mayville, Ji et al. 1999; Jarraud, Lyon et al. 2000). A given strain only activates other strains within the same *agr* group, and inhibits the *agr* response in strains belonging to other groups (Ji, Beavis et al. 1997; Novick and Muir 1999).

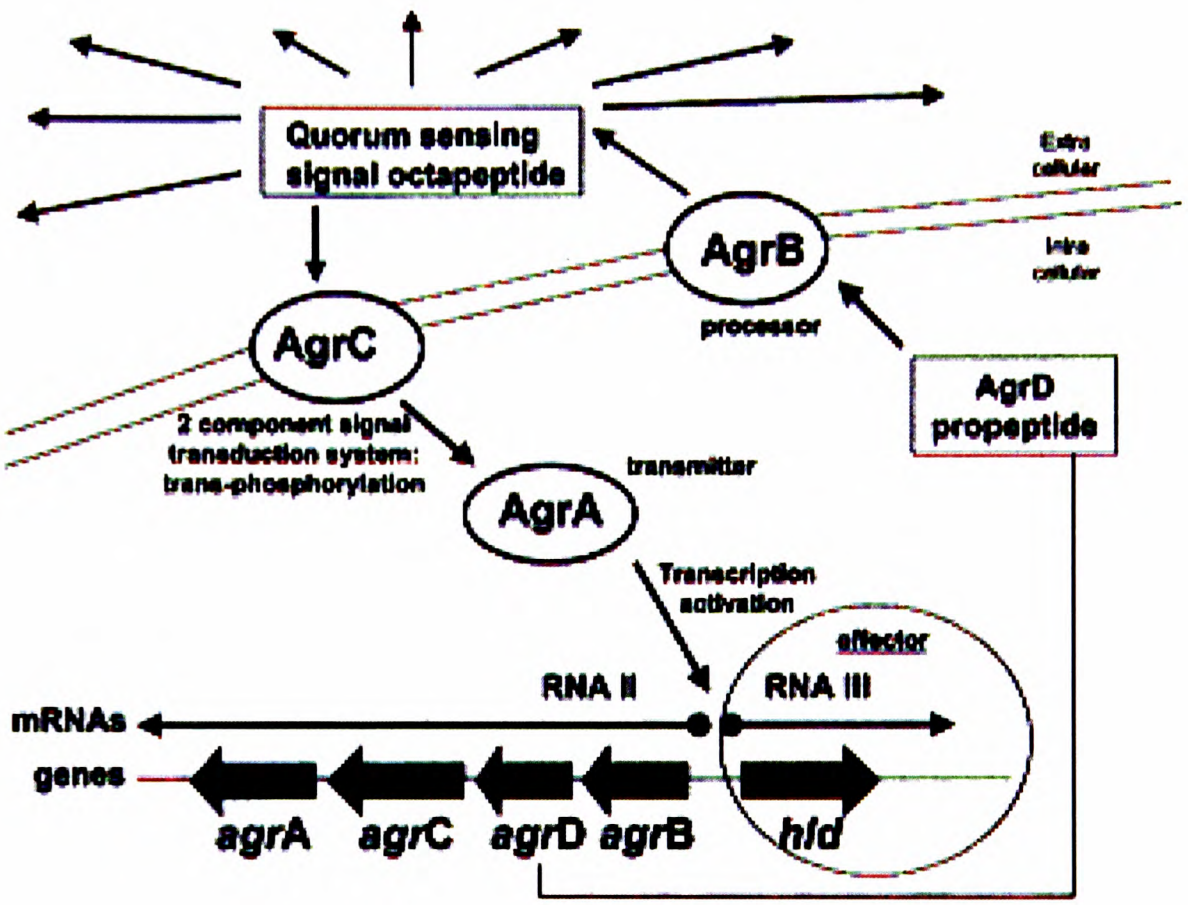


Figure 2: The agr locus and expression (van Belkum, Kools-Sijmons et al. 2002)

b) Sar

The *sar* locus is distinct from *agr* (Cheung, Koomey et al. 1992) and has been reported to have an independent affect on virulence (Cheung, Koomey et al. 1992). The *sar* locus contains three overlapping tandemly arranged promoters (Manna, Bayer et al. 1998) with a common 3' end (*sar*A, *sar*C and *sar*B) all encoded by a single gene, *sarA*. *SarA* is essential for the activated transcription of the two *agr* operons through binding to the *agr* P2 promoter (Morfeldt, Tegmark et al. 1996; Chien and Cheung 1998; Chien, Manna et al. 1998; Fischetti, Novick et al. 2000) and is essential for survival inside polymorphonuclear

leukocytes (PMN) (Gresham, Lowrance et al. 2000). *Sar* is able to independently affect the expression of some of the exoproteins directly such as α toxin, protein A and fibronectin binding protein (Chan and Foster 1998; Chien, Manna et al. 1999).

1.4 Host factors

The important events for the bacterium to cause infection are: i) entry into the host through the skin or mucous membrane barrier ii) invasion into host tissues iii) colonisation of the host tissues and iv) evasion of the host immune system. Bacteria need to possess several properties to successfully cause an infection. The bacteria must be in sufficient numbers, and they probably need to have the right combination of virulence determinants. The three eventual outcomes to severe infection are: i) the hosts' immune system kills the bacteria or ii) the patient is given antibiotics or iii) the host dies. These are detrimental to the bacteria as they would also die. In this sense, the pathogenic process appears to be detrimental to *S. aureus* compared to surviving as a commensual on the skin or mucous membrane.

There are groups of individuals who are more susceptible to *S. aureus* infections, these include people with genetic abnormalities such as Chronic Granulomatous Disease (CGD), Chediak-Higashi syndrome and Job syndrome (Crossley and Archer 1997). CGD is an autosomal X linked recessive disease leading to the absence of respiratory burst in phagocytes. The disease is caused by a mutation in any one of the 4 genes in the NADPH oxidase system which reduces phagocytosis. Catalase positive organisms most often infect these patients because they are unable to make the oxide component during phagocytosis (Fischetti, Novick et al. 2000). Patients with Chediak-Higashi syndrome suffer from recurrent pyogenic infections of the skin, soft tissue and mucous membranes because an autosomal recessive disorder results in the lack of neutrophil elastase in mature PMN (Crossley and Archer 1997). Job syndrome results in many defects in humoral immunity

though complement levels and PMN killing are both normal, patients have a propensity to suffer from recurrent severe infections involving the skin and lungs (Crossley and Archer 1997).

Other groups of susceptible hosts include patients who are immunocompromised such as people with Insulin dependent diabetes mellitus (IDDM) who often suffer from soft-tissue infections related to peripheral vascular disease and peripheral neuropathy (Crossley and Archer 1997). People with a renal impairment are more prone to *S. aureus* disease. These patients include those undergoing haemodialysis, with *S. aureus* carriers at increased risk of developing bacteraemia (Yu, Goetz et al. 1986). Patients on continuous ambulatory peritoneal dialysis (CAPD) often have fistula infections and bacteraemias with *S. aureus*. CAPD patients who persistently carry *S. aureus* are more likely to develop peritonitis or exit site infections (Sewell, Clarridge et al. 1982). Intravenous drug users are at an increased risk of acute bacterial endocarditis (Tuazon and Sheagren 1974), skin abscesses and cellulitis from needle use (Berman, Schaefler et al. 1987). Intravenous drug users also have an increased rate of carriage and are more likely to have skin infections caused by impetigo and atopic dermatitis (Aly, Shinefield et al. 1977). Other immunocompromising illnesses increase both the rate of *S. aureus* carriage and the burden of disease in these susceptible individuals. HIV and AIDS patients have a lowered immunity, they are often in hospital and have a number of puncture wounds making them more susceptible to *S. aureus* infections (Senthilkumar, Kumar et al. 2001).

1.4.1 Overview of host immune response

Host immunity to infection can broadly be categorised into 2 groups. Innate (non specific) immunity is the immediate response to infection. Innate immunity includes the action of neutrophils and macrophages. The innate immune system is built to recognise structures

on the bacterial surface as foreign, such as unmethylated CpG nucleotides, peptidoglycan (PGN), lipopolysaccharide (LPS), lipoteichoic acid (LTA) and mannose rich glycans. Adaptive immunity is a slower response taking approximately four to five days for initiation in the absence of specific memory. Adaptive immunity involves specificity and memory of different effector cells including T cells, B cells and a number of cytokines (Abbas, Lichtman et al. 2000).

A major part of the immune response involves the complement system (a group of blood borne proteins), a key component of innate immunity (Figure 3). The complement system is involved in lysis and phagocytosis of foreign particles (Frank 2001). The 3 different complementation-activation pathways include the classical pathway (involving antibodies), the alternative complement pathway and the mannose binding lectin pathway (which interacts with specific sugars on the repeating polysaccharide surface of some microorganisms then activates complement).

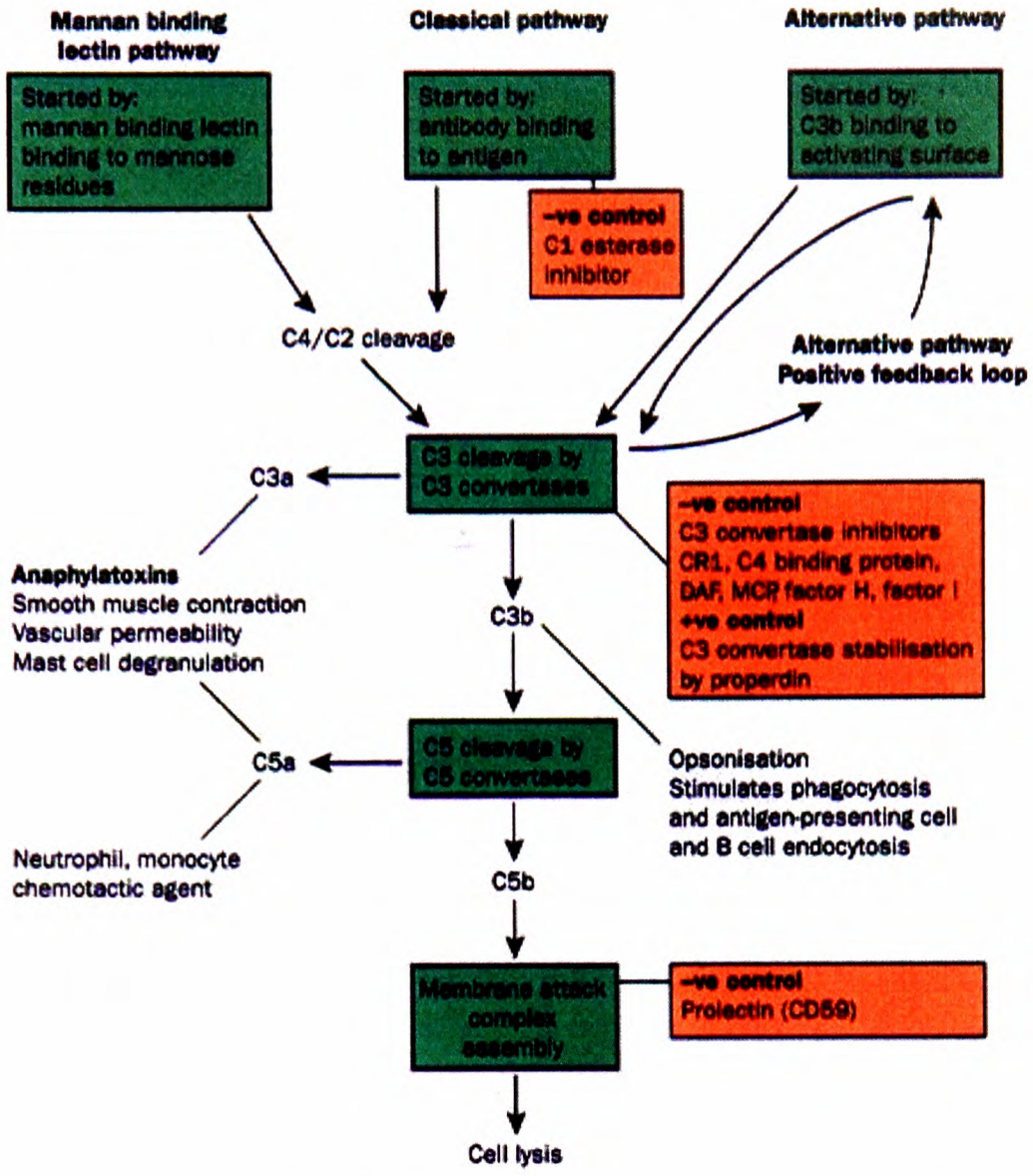


Figure 3: The complement activation pathways (Parkin and Cohen 2001).

All of the pathways in Figure 3 deposit the principal complement opsonins (C3b and iC3b fragments) of C3 onto the surface of the bacteria. When the C3 molecules are activated, an internal thioester bond in the α chain attaches and combines covalently with the surface of the microbe as C3b. Specific receptors for C3b (CR1, CD35) and iC3b (CR3, CD11b/CD18) exist on phagocytes. These receptors promote the phagocytosis of microorganisms that are coated with these opsonins.

The innate immune response recognises a number of patterns (essential for bacterial survival) found on the surface of pathogens and they are recognised as non-self by the host. The patterns are termed pathogen-associated molecular patterns (PAMPs) (Medzhitov and Janeway 1997). Different PAMP molecules are found on the surface of

different organisms, the list includes: LTA, PGN, lipopeptide/lipoprotein, LPS, lipid A, liparabinomannan (LAM) and zymosan.

The host has specific cells recognising the PAMP molecules found on pathogens. They are termed pattern-recognition receptors (PRRs) and are essential components of innate immunity. 3 classes have been described: signalling receptors, opsonic receptors and secreted proteins. Many opsonic receptors such as the FcγRs are well characterised. Also many of the secreted proteins circulating in the serum (such as MBL), have been characterised. The signalling receptors include the Toll family of receptors, which are beginning to be described and are involved in direct activation of the signalling pathways in response to PAMP recognition (Kopp and Medzhitov 1999).

The important processes involved in innate immunity to bacteria are chemotaxis, opsonisation, phagocytosis and eventual killing of the microorganism (Abbas, Lichtman et al. 2000). PMNs play a central role in this process. Cytokines such as C5a and IL-8 induce PMN to migrate, change shape then squeeze through the endothelial wall to tissues where they enter the bloodstream and travel to the site of infection (chemotaxis) (Crossley and Archer 1997). During this process of chemotaxis, microbes are constantly being covered (opsonised) with host proteins to enable the host cells to recognise the microbe (Crossley and Archer 1997).

Opsonin dependent phagocytosis is paramount in host defence against bacteria. There are many complement and IgG receptors found on the surface of PMN such as CR1, CR3 and CR4, which recognise fragments of the complement component 3 then induce opsonisation. The largest proportion of clinically infecting *S. aureus* strains are microencapsulated, they are opsonised with antibodies for the O-acetyl group of the capsular polysaccharide, with the bacteria subsequently being coated with C3 and iC3b.

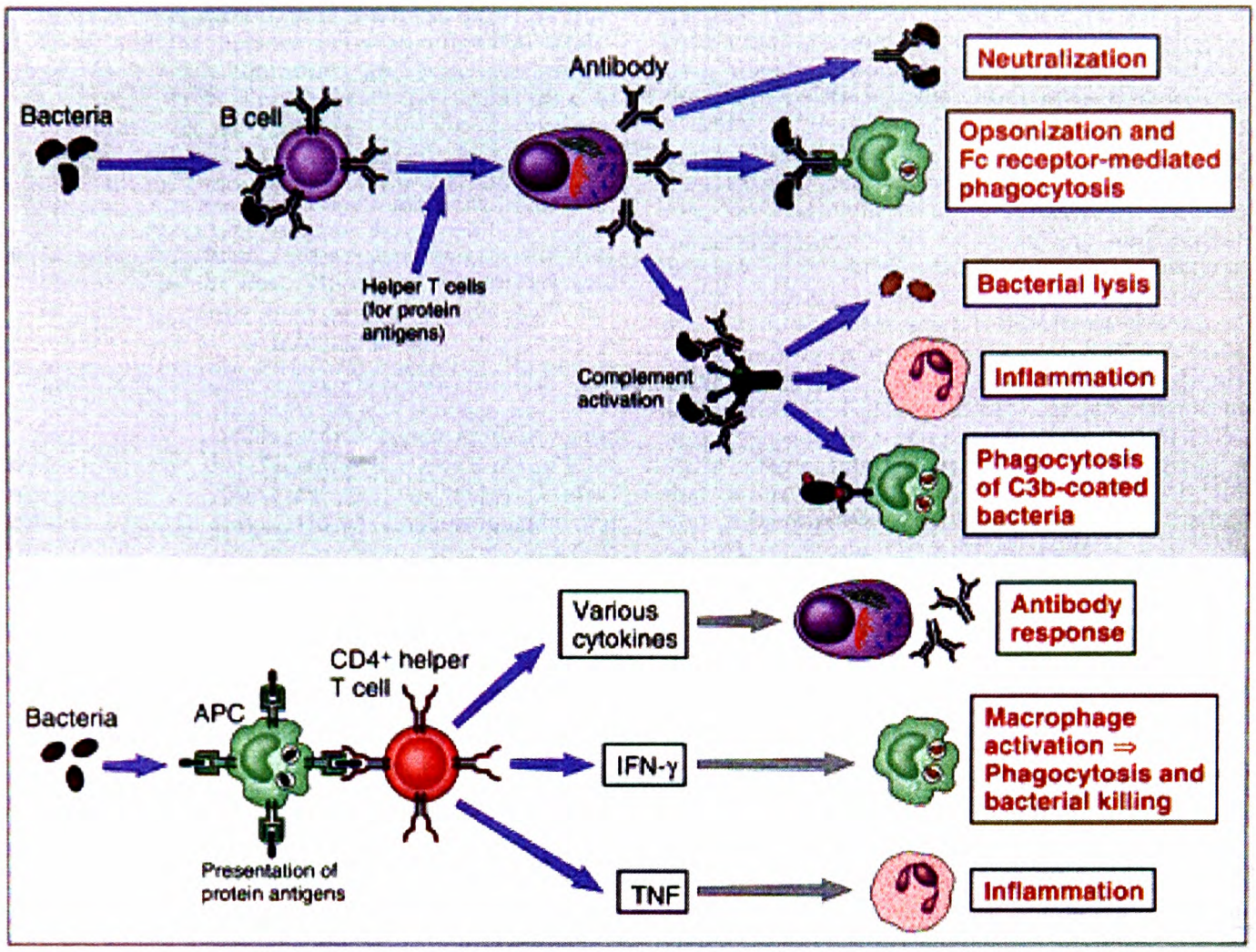


Figure 4: Phagocytosis (Abbas, Lichtman et al. 2000)

Figure 4 shows the many processes involved in phagocytosis. Phagocytes such as PMN use specific surface receptors to attach to microorganisms. The bacteria are engulfed by the phagocyte. The interactions between the opsonised bacteria and the ligands on the surface of the phagocyte induces a movement of the phagocyte cell membrane around the bacteria. The bacteria are then surrounded forming a phagosome (Crossley and Archer 1997). Granules in the cytoplasm of the phagocyte also fuse with this membrane to form a phagolysosome. Stored proteolytic enzymes are able to kill the microorganisms within this phagolysosome (Crossley and Archer 1997).

There are two pathways for killing; an oxygen dependent or independent pathway. Oxygen dependent killing involves an NADPH oxidase which converts oxygen to superoxide on ingestion of bacteria (electrons are transferred from NADPH to oxygen) (Hampton, Kettle

et al. 1996; Kaufmann, Sher et al. 2002). Superoxide reacts to produce toxic oxidants such as hydroxyl radicals and hydrogen peroxide (Hampton, Kettle et al. 1996; Kaufmann, Sher et al. 2002). Hydrogen peroxidase is converted by myeloperoxidase to hyperchlorous acid, this is an effective bactericidal oxidant. Other agents such as proteases, glucosidases and lysozymes kill independently of oxygen (Crossley and Archer 1997).

As well as the host evolving many mechanisms to kill the bacteria, *S. aureus* have also developed mechanisms to avoid the hosts' immune system. The bacteria have a thick peptidoglycan layer in the periplasmic space of the cell wall, this is able to resist the penetration of the membrane attack complex (created by complement components C5 to C9). *S. aureus* have many surface proteins which help to bind to host proteins such as fibrinogen binding proteins (fnbA and fnbB). These are able to disguise the bacteriums' antigenic surface and protect from complement. *S. aureus* has also evolved proteins which are able to bind proteins involved in the complement system such as protein A. Extracellular protein A is also able to bind the Fc portion of antistaphylococcal antibody which coats the organism with Fab fragments, this stops interactions with the Fc receptor on the surface of neutrophils. Cell wall bound protein A can also bind to the Fc of neibouring IgG molecules, thus stopping the antibodies binding to the organism (Crossley and Archer 1997).

1.4.2 Susceptibility to severe *S. aureus* disease

While the immune response is pivotal in the defence against bacterial infection, the factors leading to the occurrence of severe disease can be split into three groups:

i. Environmental. The context in which the bacteria meets the immune response can be critical. For example a patient in the hospital environment is likely to

have their skin breached by cannula or wounds, and be introduced to multiresistant organisms while receiving antibiotics.

ii. Acquired. Any illness or situation leading to an attenuation of the normal immune response will increase the risk of severe infection. Examples include poor nutrition and diabetes mellitus.

iii. Host genetic. This can take the form of major defects in the immune system such as Chronic Granulomatous disease (CGD), or more subtle variants of genes important in the immune response, both of which lead to increased susceptibility.

1.4.3 Genetic susceptibility to infectious diseases

Prior to the current study there were no reports of any host genetic susceptibility factors specifically associated with *S. aureus* disease. However, that such factors are likely to exist is suggested by: i) epidemiological evidence that genetic factors appear to be important in susceptibility to death from severe infection, and ii) the finding of specific genes conferring susceptibility to other infections such as malaria and *Mycobacterium tuberculosis* (TB) (McGuire, Hill et al. 1994; Bellamy, Ruwende et al. 1998).

1.4.3.1 Overall contribution of host genetic susceptibility to disease

Sorensen *et al.* investigated the causes of death in the biological parents and adoptive parents (at 50 years of age) of 960 Danish adoptees (Sorensen, Nielsen et al. 1988). They found the child was more than five times more likely to die from an infection by 58 years of age if the biological parent died of infection (relative risk of 5.81 (95% confidence interval (CI) 2.47 to 13.7), whereas the relative risk if the adoptive parents died of infection was 0.73 (95% CI: 0.10 to 5.36). They also found that death from cancer in the adoptive parents (but not the natural parents) was associated with an increased death rate

from cancer in the adoptees, suggesting that for this group of illnesses, environmental causes may be more important.

The role that host genetics plays in many infectious diseases is unknown. Many diseases appear to cluster in families. This is often expressed as the relative risk (λ_R), which represents the increased risk of disease in the sibling of a case compared to that of the general population (Strachan and Read 2001). If the risk within a family (λ_R) is above 1, the family members have a higher risk of contracting the disease if one of the family is ill. A λ_R above 1 could be due to host genetics, shared environment, or the interactions between the two. The λ_R in autoimmune diseases such as type 1 diabetes and multiple sclerosis has been reported to be between 15 and 20 (Risch 1987). The λ_R for most infectious diseases lies between 1 and 10 (Hill 2001). Methods used to examine the genetic effects of infectious diseases include family based studies, adoptee studies, twin studies and interpopulation (case-control) studies. The λ_R for *S. aureus* is unknown and is difficult to determine because no studies have been performed to determine host genetic factors. The only numerically feasible studies would be case-control studies rather than family based studies in *S. aureus* disease.

The most widely studied diseases are those able to cause Mendelian diseases as these diseases are rare in the population and the causative genes are likely to be found due to the strong relationship between phenotype and genotype. The Mendelian diseases to which attributable genes so far have been described include Huntington's disease, Alzheimer's disease and some forms of breast cancer (Risch and Merikangas 1996). Susceptibility loci involved in complex (or polygenic) diseases are much more difficult to examine, many different loci are probably involved and each may have either an intermediate or small effect. Also the phenotypic definition of many complex diseases are based on the decisions

of clinicians, this may cluster individuals incorrectly as many genetically different diseases may cause similar disease phenotypes.

1.4.4 Specific host genetic susceptibility factors in other infectious diseases

The human leukocyte antigens (HLA) have been examined extensively because of their importance in controlling host immune responses. Because many of the studies have been small, many results have remained inconclusive. In addition the effect of different antigens could differ between geographical areas and ethnic groups. A high rate of linkage disequilibrium (LD) also occurs across the HLA-DR genes (Cardon and Bell 2001), and because of this previous associations may be incorrect.

The HLA genes have been associated with many different diseases. HLA-B*5301 and HLA-DRB1*1302 were found independently to reduced the risk of severe malaria in children in the Gambia (Hill, Allsopp et al. 1991). Both HLA-DRB1*0101 and HLA-DRB1*1302 genes were protective in malarial disease in Kenya (Hill 1998). HLA genes have also been associated with a variety of viral infections. HLA-DRB1*1302 was a resistance gene in chronic hepatitis B in African and European populations (Thursz, Thomas et al. 1997) and was protective against hepatitis B persistence in the Gambia (McNicholl, Downer et al. 2000). HLA-B*35 has been associated with a rapid progression of HIV-1 in Caucasians (Itescu, Mathur-Wagh et al. 1992; Sahmoud, Laurian et al. 1993; Carrington, Nelson et al. 1999; Gao, Nelson et al. 2001). HLA-DR2 was found to be a risk factor for susceptibility to pulmonary TB in Indians, Russians and Indonesians and was a susceptibility gene in lepromatous leprosy in Japanese and Indians (Izumi, Sugiyama et al. 1982; Brahmajothi, Pitchappan et al. 1991; Rani, Fernandez-Vina et al. 1993; Hill 1998)

1.4.4 Evidence for host genetic susceptibility to *S. aureus* carriage

The only genetically related work performed on *S. aureus* has examined genetic susceptibility to carriage. One study examined a small set of families where members of one family did not necessarily carry the same *S. aureus* strain. The study hypothesised that host genetic susceptibility factors were involved in carriage as cross-infection would have led to carriage of identical organisms in each family (Noble, Valkenburg et al. 1967).

Two small twin studies have determined the carriage rates of *S. aureus* between monozygotic twins (MZ, identical), dizygotic twins (DZ, non-identical) and unrelated children (Hoeksma A 1963; Aly, Maibach et al. 1974). The first study found a higher concordance of carriage between MZ twins and the second was inconclusive. A genetic predisposition was also suggested in a recent case report in a single pair of MZ twins living geographically apart, as both twins suffered from the same rare skin disorder in which *S. aureus* was isolated (Douwes, Landthaler et al. 2000). Another study to suggest a genetic effect between carriage and host genetics examined HLA antigens: HLA-DR3 was associated with carriage and HLA-DR2, HLA-DR1 and HLA-Bw35 were associated with a lack of carriage (Kinsman, McKenna et al. 1983). It would be interesting to find out if these alleles have an effect on severe disease as well as carriage.

1.4.5 Practical approaches to host genetic susceptibility studies

1.4.5.1 Twin studies

One way to study the effect of host genetic susceptibility in diseases is to study twins. MZ twins share all of their genes whereas DZ twins share on average 50% of their genome. Both sets usually share a similar number of environmental factors (Jepson 1996). The underlying reason for using twins to study infectious diseases is that MZ twins should be more concordant for the disease phenotype than DZ twins if the disease has a strong

genetic basis (Strachan and Read 2001). Higher concordance rates have been recorded in MZ twins compared to DZ for infections with TB (Comstock 1978), leprosy (Fine 1981), Malaria (Jepson, Banya et al. 1995) and *Helicobacter pylori* (Malaty, Engstrand et al. 1994). Problems which arise using twins are that DZ twins still share about 50% of their genetic material.

Unfortunately a twin study of severe *S. aureus* disease is impractical, as the disease incidence is relatively low.

1.4.5.2 Linkage studies

The definition of linkage is the tendency of genes or other DNA sequences at specific loci to be inherited together as a consequence of their physical proximity on a single chromosome (Strachan and Read 2001). Linkage analysis is useful in Mendelian disorders where the whole genome can be scanned using a few hundred markers (approximately 400) searching for areas suggestive of linkage to a specific disease within a family. A fine mapping technique within the area of linkage together with candidate genes in the area is usually used (Bellamy 2000).

The presence of linkage to a marker is calculated by studying the number of recombinants and non-recombinants, thus generating a recombination fraction. If the disease gene is close to one of the markers, the inheritance pattern of that marker will be the same as the inheritance pattern of disease. The marker would be inherited more frequently by affected individuals in a family if the marker and disease loci were linked. Results must be examined with caution as this is usually the marker linked to the causative gene (not the gene itself).

Linkage studies have successfully examined rare diseases where susceptibility genes have a high impact. Complex diseases have also used linkage studies, although the disease must

have a substantial heritability. Linkage studies have been used to screen the whole genome for chromosomes segregating with disease. Diseases such as NIDDM (associated with the *CAPN10*, the calpain-10 gene) (Horikawa, Oda et al. 2000) and Crohns disease (associated with the *NOD2* gene) (Hugot, Chamaillard et al. 2001; Ogura, Bonen et al. 2001) have been studied successfully using linkage studies. One failing is that linkage studies often miss genes with a moderate effect on disease because of low power.

A linkage study would have been impractical to examine the factors involved in severe *S. aureus* disease because it would be impossible to collect a number of families where the child(ren) had a severe *S. aureus* disease.

1.4.5.3 Association Studies

Distantly related individuals are separated both by a large number of meioses and numerous random recombination events. The chromosomal segments shared by chance will be small. Individuals who inherit a mutation are also likely to inherit the same alleles at nearby marker loci. Association studies are more powerful than linkage at detecting genes which cause small to moderate effects on disease susceptibility (Risch and Merikangas 1996). Association is detected over a smaller region than linkage. This means the genes of importance may be missed using association studies with candidate genes alone being studied.

The underlying principle of association studies is that two loci on a chromosome would not normally be linked in a randomly reproducing population. If linkage is present, it suggests there may be a reason for the linkage. The term used for the association is linkage disequilibrium (LD). The DNA variants are usually close enough on the chromosome so that if recombination occurs it is infrequent. This results in the alleles occurring together more often than expected by chance, even over many generations. A founder effect (where

a small number of people in an isolated area mate together) can result in LD in a population. Normally the randomness of genetic drift constantly creates LD faster than the forces of recombination and mutation are able to decay LD (Terwilliger, Zollner et al. 1998). An allele may be selected because of a selective advantage which increases its frequency. This change could feasibly also drag along a neighbouring, neutral polymorphism (called the hitchhiker effect) (Terwilliger, Zollner et al. 1998). If population admixture occurs (where 2 genetically different populations mate), the next generation is likely to have more LD between both linked and unlinked pairs of loci. This LD would decay over generations, with previously unlinked genes separating faster than the linked ones (Terwilliger, Zollner et al. 1998).

The degree of LD is affected by the size of the population, recombination fraction, mutation rate, allele frequencies, distance apart and population history between two polymorphic sites in a stable population. Allelic associations should happen only between loci with a recombination fraction less than 1% (Strachan and Read 2001) so if allelic associations are found between two loci they are in tight linkage.

Rare disease-causing variants (<5% frequency) are likely to be specific to certain populations. The best way to examine these populations is to use a SNP and LD association approach. Common variants (>10% frequency) can also be examined using SNPs. Results from common variants should be reproducible across different ethnic groups (Risch 2000). As patterns of LD probably differ widely between ethnic groups, this can help to deduce whether the allele was causative in disease or was in LD with the causative allele.

Two similar approaches to study SNPs using LD are used. Both methods rely on the hypothesis that common genetic variants underlie susceptibility to common diseases. The

direct method tests the hypothesis that a given SNP is a causative factor in a disease by comparing SNP frequency between infected individuals and controls (Cargill, Altshuler et al. 1999; Kruglyak 1999). The indirect method types many SNPs searching for evidence of an ancestral haplotype enriched in affected individuals. The method relies on the association between disease and neutral polymorphisms (located near a risk-conferring variant). The associations may occur because of an LD between a possible susceptible locus and nearby polymorphisms. The genome is scanned systematically using a dense map of polymorphic markers looking for regions associated with disease. Although debatable, approximately 500,000 SNPs are probably needed to span the whole genome leaving a spacing of 6kb between each SNP (Kruglyak 1999).

1.4.5.4 Association studies using matched control populations

Association studies are often used to examine case–control studies. Large numbers of unrelated individuals with a particular disease are compared with ethnically matched, healthy controls. Studies search for differences in allele frequencies between the two groups. Three possible explanations for associations are: i) the locus type may predispose to disease ii) the polymorphism is in LD with the locus causing disease iii) there is a confounding factor within the choice of cases and controls. One confounding factor occurs because of the choice of ethnic groups (an extreme example are cases from one ethnic group, controls from another). This is important because evolutionary history of haplotypes and LD patterns differ between diverse ethnic groups (Cardon and Bell 2001).

The gene and its function would need to have been previously identified. The possible role of the candidate in the pathogenic process must be known to select the gene. Many genes are first characterised in knockout mice with targeted gene deletions, then the human equivalent is characterised. One such gene is *Nramp* in mice with the human equivalent,

*NRAMP*1 containing polymorphisms which have been associated with susceptibility to TB in the Gambia (Vidal, Malo et al. 1993; Bellamy, Ruwende et al. 1998).

1.4.6 Statistical considerations

Both linkage and association studies are at risk of statistical errors for different reasons. These are further explained in Appendix E3. Briefly linkage studies often employ the use of small groups and are prone to type II errors where there is a high probability of accepting the null hypothesis (thus suggesting no linkage). A true association is likely to be missed. Association studies use larger groups and are at an increased risk of type I errors where there is a probability of rejecting the null hypothesis (suggesting the presence of an association). The outcome is that a false association with infection would be reported.

Many researchers now use an approach encompassing both linkage and association to identify susceptibility genes involved in complex diseases. Linkage studies can determine an area of linkage within the genome with an association-based study then used to examine specific candidate genes within the area of linkage (Terwilliger and Weiss 1998; Bellamy 2000). Another approach to use is DNA pooling. The cases and controls are pooled together and a genome-wide association test looks for the presence of significance (Risch and Teng 1998). Two separate groups can also be used where the first group tests the significance, if this is positive a second group is used to try and replicate the first result.

1.5 Aims and outline of the study

The aim of this study was to examine some of the bacterial and host factors involved in the pathogenesis of severe *S. aureus* disease.

There were two main branches to the study, both arising from the same case-control study design:

1. The bacterial branch. This had two interrelated parts:

    i. examination of the genetic population structure of *S. aureus* in carriage and disease isolates, to identify hypervirulent clones if they exist and define the clonal background for:

    ii. a study of the relative frequencies of candidate bacterial virulence determinants in disease and carriage isolates.

2. The host branch. The patients providing the disease isolates in 1. were examined for host genetic susceptibility factors to *S. aureus* using a candidate gene approach. Control DNA was obtained from uninfected controls.

Two case-control studies were conducted; one in Oxford, UK, the second in Thailand.

Chapter 2. Study design

2.1 Oxford

2.1.1 Cases

2.1.1.1 Bacterial study

Only *S. aureus* isolates recovered from a normally sterile body site in the diagnostic microbiology laboratory were included in the study. Examples of sterile site samples were blood, cerebrospinal fluid (CSF), pleural fluid, joint or bursa fluid, an aspirate from an intracranial abscess, pus from deep abscesses and sterile site specimens such as joint prosthesis (Table 2). Strains were excluded if they were thought to be contaminants rather than true infecting strains on examination of the hospital records.

Specimen	Disease	Exclusions
Blood	Septicaemia, endocarditis	Obvious contaminants
Cerebro spinal fluid	Meningitis	
Pleural fluid	Empyema	
Joint or bursa fluid	Septic arthritis or bursitis	
Aspirate from intracranial abscess	Cerebral abscess	
Pus from deep abscess	Abdominal collections, psoas abscess, mediastinitis, pyomyositis	
Sterile site samples	Osteomyelitis, infected prosthetic material	Superficial samples, samples from sinus tract, early post operative wound breakdown

Table 2: Qualifying isolates

Six hundred and fifty patients with severe *S. aureus* disease were recruited to the study. A total of 35 patients (in the first 200) had multiple isolates from episodes of illness either separated by months or years. The relatedness of all of the multiple strains isolated from the first 200 patients enrolled in this study were examined using PFGE (the results are

shown in chapter 3). Following this, a single strain (termed the sentinel strain) was included for analysis for each patient.

After exclusions, 622 people remained in this study. The cases were further subdivided into infections acquired in the community (CAD) and those in hospital (HAD). CAD patients were defined as those admitted to hospital with an illness consistent with invasive *S. aureus* disease, and with a positive specimen within 24 hours of admission. The clinical features of the CAD cases are summarised in Table 3. Infections were termed hospital-acquired if patients had positive specimens after 24 hours of admission or if they had been admitted to hospital in the preceding 6 months. The clinical features are summarised in Table 4.

Strain information was collected from the diagnostic laboratory and any further antibiograms performed using a modified Stokes method (Appendix A1).

Disease category	**n (%)**
Osteomyelitis	21 (14)
Unexplained septicaemia	19 (12)
Bursitis	15 (10)
Deep abscess	14 (9)
Endocarditis	13 (9)
Septicaemia secondary to cellulitis	9 (6)
Primary discitis	9 (6)
Septic arthritis	9 (6)
Superficial abscess	9 (6)
Prosthetic joint infection	8 (5)
Staphylococcal pneumonia with septicaemia	5 (3)
Device related septicaemia	4 (2)
Septicaemia secondary to urinary tract infection	4 (2)
Septicaemia secondary to intravenous drug abuse	3 (2)
Empyema	1
Endophthalmitis with septicaemia	1
Infected plastic work	1
Meningitis	1
Mycotic aortic aneurysm	1
Toxic shock syndrome	1

Table 3: Clinical features of community-acquired disease cases

Disease category	n (%)
Device related septicaemia	155 (55)
Osteomyelitis secondary to metalwork	24 (85)
Prosthetic joint infection	21 (7)
Wound infection	19 (7)
Meningitis	11 (4)
Mediastinitis (post operative)	6 (2)
Unexplained septicaemia	6 (2)
Septic arthritis secondary to surgery	5 (2)
Wound infection (post internal fixation)	5 (2)
Empyema secondary to instrumentation	4 (1)
Staphylococcal pneumonia	4 (1)
Septicaemia with secondary seeding	3 (1)
Primary osteomyelitis	2 (1)
Septicaemia (post internal fixation)	2 (1)
Septicaemia secondary to UTI	2 (1)
Septicaemia secondary to cellulitis	2 (1)
Superficial abscess	2 (1)
Septic arthritis with septicaemia	1
Contaminant	1
Deep abscess	1
Endocarditis	1
Late metalwork associated infection	1
Primary osteomyelitis	1
Primary discitis	1
Prosthetic graft infection	1
Septicaemia secondary to infected graft	1
Septicaemia secondary to meningitis	1
Shunt infection with cerebral abscess	1

Table 4: Clinical features of hospital-acquired disease cases

2.1.1.2 Host study

Ethical permission (COREC number 96.229, Appendix F) was granted for collection of blood (stored in EDTA tubes) on patients whose blood was sent for routine testing to the diagnostic haematology laboratory. Blood was not available on every patient as samples were often taken before recruitment into this study with no further samples collected.

As allele frequencies can vary greatly between ethnic groups, all patients who were not Caucasians were excluded. After exclusion of patients with no blood specimen or non-

Caucasians, there was a total of 432 patients with severe *S. aureus* disease, these included 284 HAD and 148 CAD patients.

The blood was separated into a cell pellet and plasma and then frozen at –80°C. DNA was extracted from the cell pellet using the Nucleon DNA extraction kit (Scotlabs, Cambridge, UK) (Appendix C1). DNA was quantified using the Pico Green 7200 system (Appendix C2), and placed in deep well plates at a concentration of 10ng/µl. Each deep well plate contained samples from both cases and controls in random order.

2.1.2 Controls

2.1.2.1 Bacterial study

The controls used for the bacterial case-control study were *S. aureus* isolates colonising the anterior nares of healthy blood donors. The blood donors were recruited from 10 blood donor sessions spread geographically around the Oxfordshire region. All session locations were within the catchment area of the John Radcliffe Hospital (Figure 5).

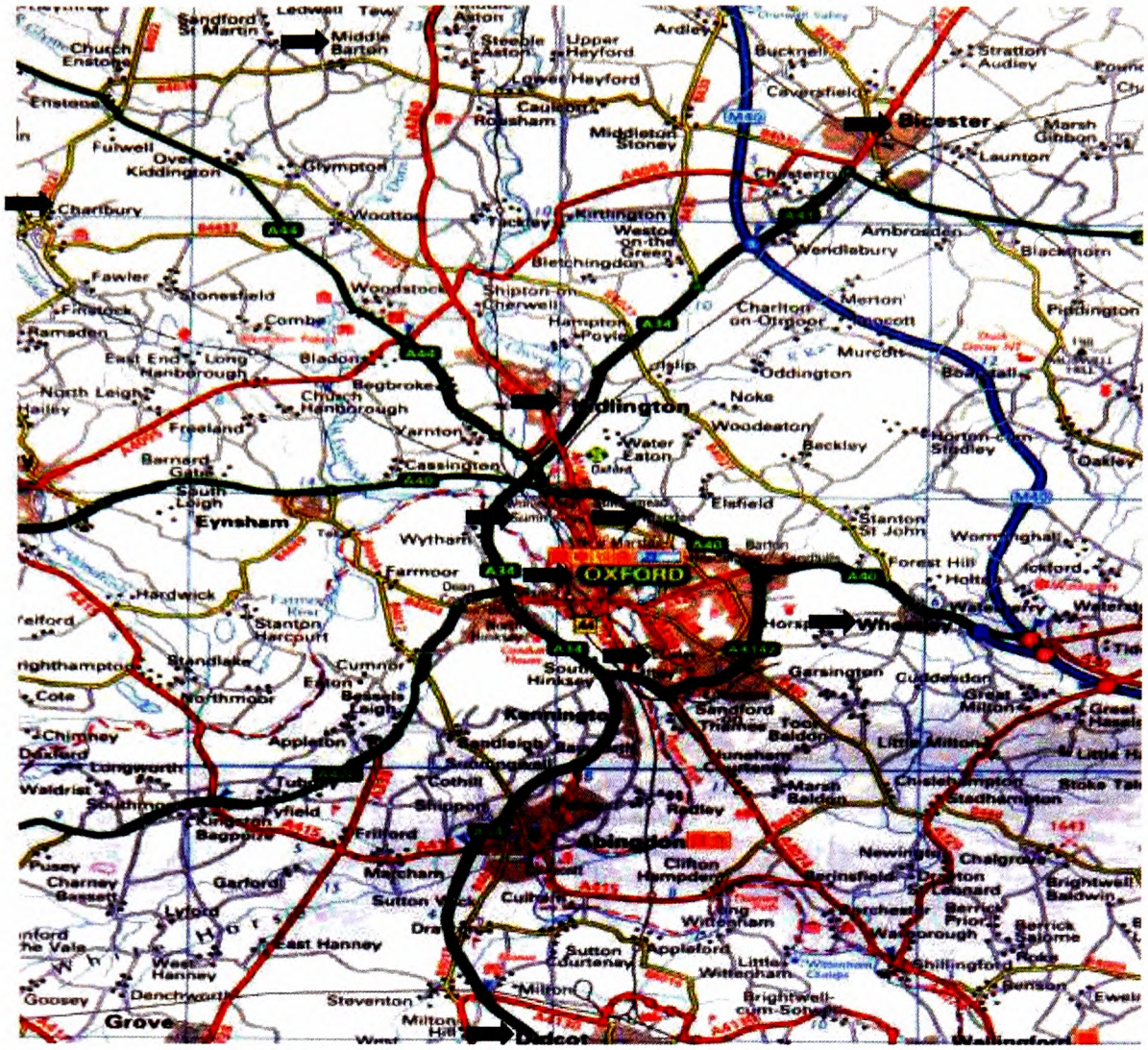


Figure 5: Map of Oxfordshire showing the hospital and blood donor clinics

Previous studies have examined swabs taken from both the left and right anterior nares. There was no evidence to suggest that both sides of the nostril are colonised with the same number of bacteria. To increase the yield of bacteria, each nostril was examined separately and the differences between nostrils were examined (Table 5).

	Right carriage	**Right no carriage**
Left carriage	134	24
Left no carriage	21	387

Table 5: Rates of nasal carriage in the blood donors

$p = 0.77$ (n=566) using McNemar's test

There was no difference in carriage between nostrils. There was also no difference in carriage between males and females (p=0.54). 568 blood donors were recruited, 185 *S. aureus* strains were isolated which gave a carriage rate of 33% from a single swab.

2.1.2.2 Host study

Two control groups of DNA were collected for this study, one from blood donors and one from umbilical cord bloods. Blood donors were potentially not representative of the normal population for Oxfordshire as they are specifically called up to donate blood according to the clinical need for specific blood types. They are also a self-selected group defined by their altruism, and although there is no evidence for a genotypic correlate of this, they may not be fully representative of the population as a whole. A potential advantage over the umbilical cord blood samples is that the carriage status of those recruited was known, at least at the time of sampling. As a cohort they may be useful in the future for defining genetic susceptibility to *S. aureus* carriage.

The blood group frequencies of the blood donors and severe *S. aureus* disease patients were examined and compared to that of the general population (Figures from Blood Transfusion Service) (Table 6). No difference was observed between the blood groups of the cases compared to the controls, both frequencies were similar to those seen in the normal population. Table 7 shows no difference in carriage occurred across the blood groups between the cases and blood donors. The blood groups were also examined for individuals who were group A positive compared to non-A (Table 8) and no difference was observed between carriage and non-carriage.

The umbilical cord blood group was collected in collaboration with Dr Alex Smarason and the team of midwives in the Delivery suite of the John Radcliffe Hospital. Cord blood was taken from the placenta after birth with precautions taken to avoid contamination of cord blood with maternal blood. A 10ml aliquot was collected in EDTA tubes, separated, and frozen at –80°C. DNA was extracted at a later date using the Nucleon II kit (Appendix C1). These had the theoretical advantage of not excluding individuals who die of *S. aureus*

disease before reaching adulthood (though in practice this is unlikely to have any major effect given the low mortality from *S. aureus* disease in this age group).

There was no information on the babies' blood groups, therefore data (including individuals Rhesus status) was collected on the mother. Cord blood is taken routinely for a Direct Coombs test from the infants of Rhesus negative mothers where haemolytic disease of the Newborn was considered a risk, there was a theoretical risk of a sampling bias either for or against these babies. The proportion of Rhesus negative individuals in the mothers of the cord blood control group was similar to the numbers seen in the general population with 84% of the population being rhesus positive and 16% being negative (Mike Murphy, BTS, personal communication) (Table 9).

Blood group	**A** n (%)	**B** n (%)	**O** n (%)	**AB** n (%)	**Total** n
Blood donors	208 (42)	47 (10)	234 (47)	5 (1)	494
Cases	180 (40)	37 (8)	214 (47)	17 (4)	448
Cord blood mothers	177 (42)	40 (9)	188 (44)	18 (4)	425
Total	565 (41)	124 (9)	636 (47)	40 (3)	1367

Table 6: ABO blood grouping for the blood donors, severe cases and cord blood

The overall Fisher's exact p value for Table 6 was 0.08. The trend towards significance was due to a shortage of ABs in the blood donor group (p=0.003 overall for AB vs non-AB). There was no difference in blood group distribution between cases and cord blood mothers (p=0.73). This bias against AB donors was one of the reasons why the cord blood controls were used for the host genetic susceptibility studies.

Status	**A** n (%)	**AB** n (%)	**B** n (%)	**O** n (%)	**Total** n (%)
No carriage	147 (43)	2 (1)	30 (9)	160 (47)	339 (68)
Carrier	61 (39)	3 (2)	18 (12)	75 (48)	157 (32)
Total	208 (42)	5 (1)	48 (10)	235 (47)	496

Table 7: Carrier vs non-carrier: ABO blood grouping for blood donors

p = 0.345 (Fisher's exact)

Status	**A** n (%)	**Not A** n (%)	**Total** n (%)
No carriage	149 (44)	190 (56)	339 (68)
Carrier	64 (41)	93 (59)	157 (32)
Total	213 (43)	283 (57)	496

Table 8: Type A positive and carriage of *S. aureus*

p=0.56 (Fisher's exact)

Rhesus	**Positive** n (%)	**Negative** n (%)	**Total**
Blood donors	336 (81)	78 (19)	414
Cases	374 (83)	74 (17)	448
Cord blood mothers	535 (83)	110 (17)	645
Total	1245 (83)	262 (17)	1507

Table 9: Rhesus status of blood donors, cases and cord blood mothers

p = 0.63 (Fisher's exact)

It was important for the host genetic studies that the cord DNA contained no contamination with maternal blood during collection. For this reason six microsatellites which were located on chromosome one were examined. A subgroup of 48 cord DNAs were first used to exclude possible contamination. The subgroup excluded contamination down to a level of 7% (using a Fishers exact test). Two further microsatellite markers have since been studied in the Oxford case-control study. For these microsatellite markers all 696 cord blood controls were examined. No samples contained more than two alleles, therefore the conclusion was that no maternal contamination occurred in the control DNA.

2.1.2.3 Database

To protect patients' and control individuals personal details, one database was set up which contained the patients' personal information such as hospital number, age, sex, ethnicity and blood group. This database can only be accessed by password and is held on a secure server. No electronic database contains the names of individuals. A second secure database contains information about the infecting or colonising strains and genotype information. In this second 'results' database, individuals are identified only by a unique study number. This was used throughout the study to anonymise all information.

2.2 Thailand

2.2.1 Cases

2.2.1.1 Bacterial study

In a collaboration with Dr Yupin Suputtamongkol a case-control study similar to one in Oxford was set up in Thailand. Ethical approval was granted by the Committee on Human Rights Related to Research Involving Human Subjects, Mahidol University, Thailand. Cases were collected from 3 areas of Thailand (Figure 6). One hospital is situated in the centre of Bangkok (Siriraj hospital) and two in the North East of Thailand (Ubon and Khon Kaen). Bacteria were isolated from sterile sites by the diagnostic microbiology laboratory based in Siriraj hospital under the direction of Dr Amori. Bacteria isolated in the North of Thailand were characterised and transported to Siriraj hospital for freezing.

Isolates were characterised as MRSA or MSSA the frozen at –20°C in Siriraj hospital. These strains were shipped back to Oxford where a full characterisation was performed on each isolate (Appendix A1). A total of 171 isolates from severe disease were collected in

Thailand. These comprised 151 isolates from Siriraj hospital, 11 from Ubon and 9 from Khon Kaen.



Figure 6: Map of Thailand showing the hospitals where the cases and controls were collected.

2.2.1.2 Host study

When patients became positive for severe *S. aureus* infection in the diagnostic microbiology laboratory, Dr Suputtamongkol and her colleagues went to the bedside of each patient. Any known HIV positive patients were excluded from this study. A 5ml aliquot of whole blood was collected into EDTA tubes for each patient. This blood was separated into red cells together with buffy coat and serum then frozen at –80°C for DNA extraction. All of the DNA was extracted using the Nucleon II kit in Bangkok and

transported back to Oxford to be examined. A total of 228 cases were collected in Thailand, 150 from Siriraj, 56 from Ubon and 22 from Khon Kaen.

2.2.2 Controls

Both the control strains and host DNA collected in Siriraj hospital were from healthy Thais being screened before working abroad. The controls from the hospitals in the North East were collected from patients attending an outpatient clinic who did not have *S. aureus* disease. A total of 307 controls were recruited. Swabs were taken both from the nose and axillae of each control to isolate any *S. aureus*. The strains were prepared then transported to Oxford where they were recharacterised and the antibiograms performed (Appendix A1). A total of 53 carriage strains were isolated which gave a carriage rate of 17%.

Chapter 3. Population structure of *S. aureus*

3.1 Introduction

S. aureus is generally thought of as a clonal organism, if mainly because of the clinical importance and newsworthiness of 'clones' of highly resistant MRSA or (potentially) VRSA causing epidemics in hospitals and occasionally in the community. Population numbers of *S. aureus* bacteria are vast, and it might be expected that the amount of variation in the population would also be vast. The reason this is not the case is the presence of selection, though this in turn raises its own questions (Maynard Smith 1995). Each time a new favourable mutation arises it would be expected to spread to fixation by selection, leading to a totally homogenous population. A combination of heterogeneity in selective pressures and horizontal transfer of genetic material between clones through recombination prevents this from occurring in most bacterial species.

An important clinically relevant question is to what degree there is heterogeneity of virulence, or ability to cause disease, within a bacterial population. If virulence is associated with particular clones, these can be identified and, potentially, therapeutic steps directed at this bacterial subgroup. This is the main reason why the examination of the population genetics of pathogens is not solely of academic interest.

3.1.1 Typing and bacterial population structure

There are at least four different reasons for typing bacteria, and the choice of typing tool depends on the aim of the exercise. The reasons are:

i. Outbreak epidemiology. In this case the method chosen needs to be highly discriminatory so as to be able to distinguish between closely related but

different strains. This is usually, but not always, carried out in a hospital setting, often when searching for a point source of infection.

ii. Clinical indication: for example distinguishing reinfection from new infection.

iii. To define the local bacterial population structure in specific clinical or epidemiological groups, for example when assessing bacterial clonality as a covariate in a host genetic susceptibility study, or searching for virulent clones.

iv. To explore the underlying genetic population structure of the bacterium – so called 'long term' epidemiology. This can for example help determine the mode of acquisition of resistance genes (Enright, Robinson et al. 2002).

With i. and ii. it is important to be able to distinguish bacteria which are epidemiologically and temporally related, and less important to place those strains in a global context. With iii. and iv. the ability to define genetic relationships on a global scale is crucial, and discriminatory power less important.

Variations in bacterial populations occur due to selection and genetic drift resulting from changes through recombination or point mutation (Feil and Spratt 2001). When bacteria reproduce asexually a mother cell produces two daughter cells with identical chromosomal DNA. This reproduction produces a clonal population which is identical to the mother cell with the variations occurring through *de novo* mutations. Because the genetic exchange is only vertical, any new mutations are also linked to the mutations which have arisen in the chromosome previously, so the chromosomal polymorphisms would be in linkage disequilibrium (LD) as the population reproduces asexually (Smith, Smith et al. 1993). A bacterium which is good at surviving and replicating in its niche would theoretically rapidly predominate in the total population. This clonal expansion leads to a reduction in the diversity of the bacterial population, with the type of population structure formed being

termed 'clonal'. The same scenario occurs on a temporary basis if particular clones are able to reproduce rapidly reducing the variation in a population (if only temporarily) (Achtman 1997), leading to an epidemic clonal population structure.

Variations in bacteria do not exclusively occur through *de novo* mutations between the mother and daughter cells. DNA can be horizontally transferred between bacteria. The methods used by bacteria for horizontal gene exchange include conjugation, transduction and transformation. The usual method of gene exchange utilised by *S. aureus* is transduction, in which DNA is transferred between one strain and another via a bacteriophage. A variety of mobile elements such as antibiotic resistance and virulence genes can be transferred between bacteria using this method (Kaper and Hacker 1999).

The *S. aureus* genome can be split broadly into two main groups: essential genes, needed for growth and cell division are consequently omnipresent; and accessory genes, the presence of which is variable between strains. The accessory genes are often encoded by accessory genetic elements such as plasmids, transposons, prophages and pathogenicity islands (Fischetti, Novick et al. 2000). Typing systems which at least partially examine variability in accessory genes (antibiograms, PFGE) are usually fairly discriminatory and potentially useful in short term epidemiological applications.

The essential genes do not differ enormously between bacteria as loss or reduction in function of a gene needed for bacterial growth or cell division would mean death for the bacterium. However these 'housekeeping' genes do contain areas of variation at the sequence level, due to point mutation and recombination, and these can be exploited for typing purposes (e.g. MLEE and MLST). Because these genes are relatively conserved across a species they are ideally suited to the examination of collections of a single species either collected over a long period of time or over a wide geographical area.

MLEE and MLST are able to determine whether a bacterial population is clonal or panmictic in nature. Panmictic populations are those where recombinational exchanges are very frequent, breaking up any LD between genes and allowing no stable clones to appear (Smith, Smith et al. 1993; Spratt and Maiden 1999). *H. pylori* and *N. meningitidis* are examples of panmictic organisms, with DNA passed easily between strains (Maiden, Bygraves et al. 1998; Suerbaum, Smith et al. 1998; Suerbaum and Achtman 1999).

The large amount of clonality previously reported within bacterial species may have occurred due to sampling bias (Smith, Smith et al. 1993; Smith, Feil et al. 2000). This bias often occurs because groups could only examine invasive strains, which are probably not representative of all clonal types. Because many organisms exist as asymptomatic commensals instead of producing disease, the disease organisms tested were probably atypical, and may not be those organisms responsible for transmission to new hosts (Feil and Spratt 2001).

There is a large body of evidence suggesting that *S. aureus* has an epidemic clonal population structure. This derives from the observed clonal spread of certain phage types through the population in the 1960s and 1970s (Nahmias and Eickhoff 1961; Rosdahl, Witte et al. 1994), studies of the spread of PFGE-defined MRSA clones and MLEE studies of natural populations of human and bovine-derived isolates (Musser, Schlievert et al. 1990; Musser and Selander 1990; Kapur, Sischo et al. 1995).

3.1.2 Study aims

The reasons for typing *S. aureus* isolates in the context of the current study was :

To look for virulent clones of *S. aureus* by comparing the population genetic structure of bacteria causing disease and those being carried asymptomatically.

To exclude the existence of one or two virulent clones infecting a substantial number of cases in the case-control study, potentially confounding or reducing the power of the host genetic susceptibility element of the study.

3.2 Overview of *S. aureus* typing methods

Phenotypic	Genotypic
Biotyping	Plasmid restriction digests
Antibiograms	Southern blotting (REA + probe) e.g. ribotyping
Capsular typing	PFGE of chromosomal DNA
Phage typing	PCR + REA of amplicon, e.g. CAPS
Electrophoretic protein typing / immunoblotting	PCR + electrophoresis of amplicon, e.g. AP-PCR, rep-PCR
MLEE	PCR + sequencing of amplicon e.g. MLST

Table 10: Phenotypic and genotypic methods for typing *S. aureus*

3.2.1 Phenotypic methods available

The phenotypic methods available to type *S. aureus* are shown in Table 10. Antibiogram testing is a useful tool for many diverse microorganisms within a routine diagnostic laboratory. The sensitivities are able to cluster organisms into groups of specific antibiogram patterns. Antibiogram testing is severely limited as a typing technique for *S. aureus* because only a few common antibiogram profiles exist. Also genetically unrelated strains can have the same susceptibility profiles. Resistance is often easily transferable between strains which favours detection of changes in the accessory genes rather than the housekeeping genes.

Phage typing is an epidemiological tool which has been in use in many reference laboratories since 1945 (Williams and Rippon 1951). The method distinguishes patterns of phage susceptibility. Organisms are sensitive to a phage if they are lysed and resistant if growth is unaffected on an agar plate. Particular patterns have been associated with food

poisoning, skin infections and epidemic MRSA strains (Parker 1983; O'Neill, Murchan et al. 2001).

Although phage typing has been in use for many years there are many associated difficulties and the method is no longer widespread. The difficulties associated with phage typing include the cost of the reagents, with 23 phages tested against each strain, the degree of expertise needed both for the execution of the method and the interpretation of results, and problems with reproducibility within and between laboratories. Problems also occur because spontaneous mutations are able to change the phage patterns. In addition, and probably most importantly, approximately 20% of isolates cannot be typed using by phage typing (Tenover, Arbeit et al. 1994; Bannerman, Hancock et al. 1995).

Because of the increased use of genetic methods, the pool of expertise for performing phage typing has decreased. There is a high degree of subjectivity in reading the plates and no concrete guidelines exist to aid the interpretation of results (Williams and Rippon 1951). Phage typing has been replaced by genetic methods in many laboratories, but remains in use as a reference tool to examine small outbreaks of MRSA strains at the *S. aureus* reference laboratory in Colindale, London (http://www.phls.co.uk.).

MLEE examines metabolic enzymes because they evolve very slowly, and are under minimal selective pressure. Enzymes are examined in cell extracts which are electrophoresed on non-denaturing starch gels. A number of enzymes are examined for each strain varying in number for different species. Approximately 11 are used for *N. meningitidis* with up to 20 for *S. aureus* (Musser and Selander 1990; Maiden, Bygraves et al. 1998). The differences in electrophoretic mobilities are thought to relate to different alleles at these loci. For a particular enzyme, bands of identical size are presumed to represent identical enzyme types, and are designated identical allele numbers. Each

electrophoretic type (ET) comprises a series of integers corresponding to these alleles (an allelic profile).

Ten to twenty enzymes are examined to ensure adequate discrimination, and as the enzymes studied are independent of each other, the method is suitable for the study of population structure. The method is labour-intensive, with up to 20 enzymes examined for each strain. It has been estimated that to change the electrophoretic mobility of an enzyme, an average of 26 nucleotides must change (Boyd, Nelson et al. 1994). This means much of the genetic information is lost in translation to the phenotype.

3.2.2 Genotypic methods available

There are numerous genotypic methods for typing *S. aureus* (Table 10). Most methods are now based on PCR technology. The methods are sub-divided into those using the whole genome and those using PCR primers specific for regions within the genome. A widely described method is arbitrary primed PCR (AP-PCR) also known as random amplified PCR (RAPD) (van Belkum, Kluytmans et al. 1995). The method incorporates a low stringency PCR amplification on the whole bacterial genome using single short primers (8 or 10 base pairs) to amplify non-specific regions of DNA. These primers are non-specific but hybridise with enough affinity at a given chromosomal locus to begin polymerisation. If 2 sites happen to be close enough on the genome and in the right orientation on opposite DNA strands, a product will be amplified (Crossley and Archer 1997). The PCR product for each strain is examined on agarose gels. Typing is based on the resulting banding patterns. This method is discriminatory and has the ability to show the maximum variation within a bacterial population (van Belkum, Kluytmans et al. 1995; Fitzgerald, Hartigan et al. 2000).

RAPD results with *S. aureus* have been variable, even using the same bacteria and primers within the same laboratory. Different primers anneal with different efficiencies resulting in a variety of band intensities; these can be very difficult to interpret. Some of the primers are less efficient than others and do not always work (van Belkum, Kluytmans et al. 1995).

3.3 Antibiogram testing

3.3.1 Introduction

Antibiograms are a standard tool in routine diagnostic microbiology laboratories for distinguishing isolates and guiding therapy. Genes determining resistance to many antibiotics are carried on mobile genetic elements (Table 11), and hence as a typing tool the antibiogram is unlikely to be of use in defining the underlying genetic population structure. Antibiotic resistance is, however, potentially related to virulence or poor clinical outcome, so it merits attention.

Resistance gene	Antimicrobial agent	Location
aac-aphD	Gentamicin	plasmid, chromosome, transposon
blaZ	Penicillin	plasmid, chromosome, transposon
ddh	Glycopeptides	chromosome
dfrA	Trimethoprim	plasmid, possibly transposon
fusA	Fusidic acid	chromosome
fusB	Fusidic acid	plasmid
mecA	Methicillin	chromosome, possibly transposon
rif	Rifampicin	chromosome
tetA(K or L)	Tetracycline	plasmid
tetA (M)	Tetracycline	chromosome
ermA	Erythromycin	chromosome, transposon
ermB	Erythromycin	plasmid, transposon
ermC	Erythromycin	plasmid
norA	Ciprofloxacin	chromosome
gyrA/B	Ciprofloxacin	chromosome
grlA/B	Ciprofloxacin	chromosome

Table 11: Resistance genes and where they are located

3.3.2 Methods

The laboratory methods are described in Appendix A1. For each clinical group the common antibiogram patterns were tabulated, and the overall numbers of antibiotics to which the bacteria were resistant was compared between groups.

3.3.3 Results

Pattern	Pen	Cip	Fus	Rif	Tet	Trim	Ery	Gent	Net	Amik	Vanc	Ox	Disease	NC	CAD	HAD
1	R	S	S	S	S	S	S	S	S	S	S	S	230 (54)	137 (74)	92 (62)	138 (50)
2	R	R	S	S	S	S	R	S	S	S	S	R	54 (12)	0	6 (4)	48 (18)
3	S	S	S	S	S	S	S	S	S	S	S	S	44 (10)	12 (6.)	20 (14)	24 (9)
4	R	S	S	S	S	R	S	S	S	S	S	S	13 (3)	1	4 (3)	9 (3)
5	R	R	S	S	S	S	S	S	S	S	S	S	13 (3)	17 (9)	4 (3)	9 (3)
6	R	R	S	S	S	S	S	S	S	S	S	R	5 (1)	0	1	4 (1)
7	R	S	S	S	S	S	R	S	S	S	S	S	5 (1)	3 (2)	3 (2)	2
8	R	R	S	S	S	S	R	S	S	R	S	R	6 (1)	0	0	6 (2)
9	R	S	R	S	R	S	S	S	S	S	S	S	3 (1)	1	1	2
10	R	S	S	S	R	S	S	S	S	S	S	S	3 (1)	1	1	2
11	R	R	S	S	S	R	R	S	S	R	S	R	4 (1)	0	0	4 (1)
12	R	R	S	S	S	R	S	S	S	S	S	S	4 (1)	2	2 (1)	2
13	R	R	S	S	S	S	R	S	S	S	S	S	1	1	1	0
14	R	R	S	S	S	R	R	R	S	S	S	R	4 (1)	0	0	4 (1)
15	R	S	R	S	S	S	S	S	S	S	S	S	2	0	2	0
16	R	S	S	S	S	S	S	S	S	R	S	S	3 (1)	0	1	2
17	R	S	S	S	S	S	S	S	S	S	S	R	3 (1)	0	1	2
18	S	S	S	S	S	R	S	S	S	S	S	S	2	0	1	1
19	R	R	S	S	S	R	R	S	S	S	S	R	2	0	0	2
20	R	S	S	S	S	R	R	S	S	S	S	S	2	0	1	1
21	R	S	S	S	S	S	R	S	S	S	S	R	1	0	0	1
22	S	S	R	S	S	S	S	S	S	S	S	S	1	0	0	1
23	S	S	S	S	R	S	S	S	S	S	S	S	1	1	1	0
24	R	R	S	S	R	S	S	S	S	S	S	R	1	0	0	1
25	R	R	S	S	R	R	R	R	R	R	S	R	1	0	0	1
26	R	R	R	R	S	S	R	S	R	S	S	R	1	0	0	1
27	R	R	S	S	S	R	R	S	R	S	S	R	1	0	0	1
28	S	R	S	S	S	S	S	S	S	S	S	S	1	2	0	1
29	R	R	S	S	S	R	S	S	S	S	S	R	1	0	0	1
30	R	R	S	S	S	R	R	R	S	R	S	R	1	0	1	0
31	R	S	S	S	R	S	R	S	S	S	S	S	1	0	1	0
32	R	S	S	S	S	S	S	S	R	S	S	S	1	0	0	1
33	S	R	S	S	S	S	R	S	S	S	S	S	1	0	1	0
34	S	S	S	S	S	S	R	S	S	S	S	S	1	1	1	0
35	S	S	S	S	S	S	S	S	R	S	S	S	1	0	1	0
36	R	R	S	S	S	S	R	R	S	S	S	R	1	0	0	1
37	R	R	R	S	S	R	R	S	S	R	S	R	1	0	0	1
38	R	R	S	S	R	R	S	S	S	S	S	S	0	1	0	0
39	R	R	S	S	R	S	S	S	S	S	S	S	0	1	0	0
40	R	R	R	S	S	S	S	S	S	S	S	S	1	1	0	1
41	R	S	R	S	S	S	R	S	S	S	S	S	0	1	0	0
42	R	S	S	R	S	S	S	S	S	S	S	S	0	1	0	0
43	R	S	S	S	R	R	S	S	S	S	S	S	0	1	0	0

Table 12: Antibiogram results for Oxford

Tables 12 and 13 contain the antibiogram results. The abbreviations are: Pen for Penicillin, Cip for Ciprofloxacin, Fus for Fusidic acid, Rif for Rifampicin, Tet for Tetracycline, Ery for Erythromycin, Gent for Gentamicin, Net for Netilmicin, Amik for Amikacin, Vanc for Vancomycin and Ox for Oxacillin.

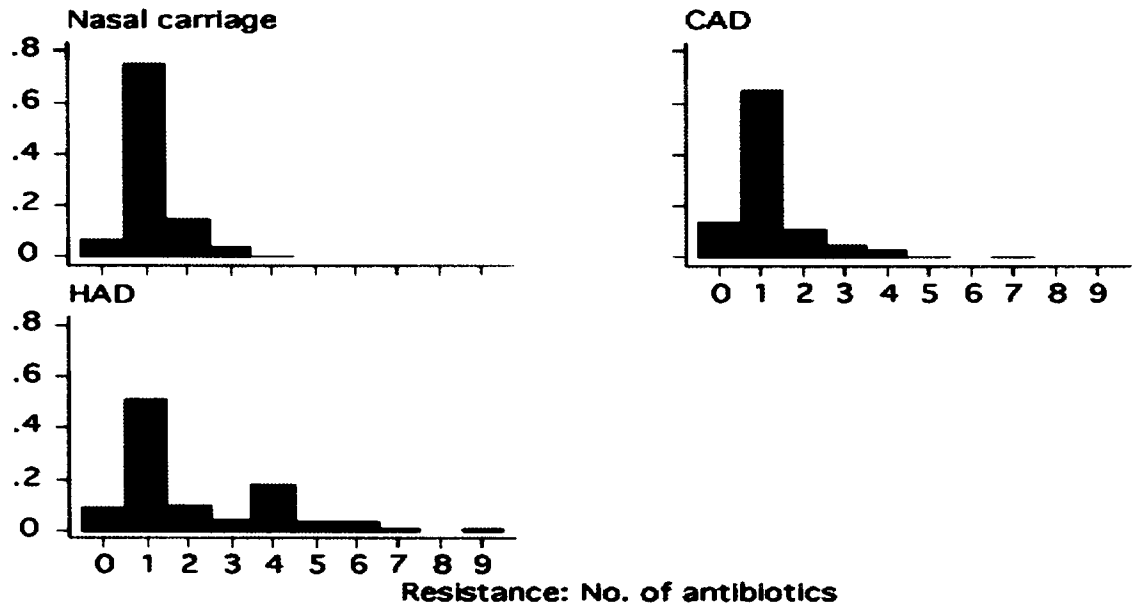


Figure 7: Number of antibiotics resistant to, histograms by clinical group in Oxford

Figure 7 shows the antibiogram results on all of the strains included in the study which were isolated in Oxford. The strains were divided into subgroups based on their place of acquisition i) NC ii) CAD and iii) HAD. The x axis depicts the numbers of antibiotics from 1 to 9 that each subgroup of strains was resistant to. The y axis depicts the percentage of strains resistant to the antibiotics with 2, 4, 6 and 8 being equivalent to 20, 40, 60 and 80 percent. The NC and CAD isolates both have similar resistance patterns whereas the HAD strains were more resistant than the other two subgroups. There was no significant difference between carriage strains and CAD strains in the number of antibiotics each strain was resistant to ($p=0.6$), suggesting that the CAD group could have been drawn randomly from the carriage population. The HAD subgroup were more resistant than either the CAD or NC subgroups ($p<0.001$ in both cases).

Pattern	Pen	Cip	Fus	Rif	Tet	Trim	Ery	Gent	Net	Amik	Vanc	Ox	Disease n (%)	Carriage n (%)
1	R	S	S	S	S	S	S	S	S	S	S	S	55 (32)	18 (34)
5	R	R	S	S	S	S	S	S	S	S	S	S	26 (15)	10 (19)
10	R	S	S	S	R	S	S	S	S	S	S	S	26 (15)	10 (19)
44	R	R	S	S	S	S	R	R	R	R	S	R	24 (14)	0
45	R	R	S	S	R	S	S	S	S	S	S	S	18 (10)	0
25	R	R	S	S	R	R	R	R	R	R	S	R	5 (3)	0
46	R	R	S	S	R	S	R	R	R	R	S	R	5 (3)	0
56	R	R	S	S	R	S	S	S	S	S	S	S	0	4 (8)
47	R	R	S	S	S	S	S	R	R	R	S	R	2	0
4	R	S	S	S	S	R	S	S	S	S	S	S	1	0
7	R	S	S	S	S	S	R	S	S	S	S	S	1	0
16	R	S	S	S	S	S	S	S	S	R	S	S	1	0
48	R	R	S	R	S	R	R	R	R	R	S	R	1	0
49	R	R	S	S	S	S	R	R	S	S	S	R	1	0
50	R	R	S	R	R	R	R	R	R	R	S	R	1	0
51	R	R	S	S	R	R	S	R	S	S	S	R	1	0
52	R	R	S	S	R	S	R	S	R	R	S	R	1	0
53	R	R	S	S	R	S	S	R	R	R	S	R	1	0
54	R	S	S	S	S	S	S	S	R	R	S	S	1	0
3	S	S	S	S	S	S	S	S	S	S	S	S	0	2 (4)
12	R	R	S	S	S	R	S	S	S	S	S	S	0	2 (4)
4	R	S	S	S	S	R	S	S	S	S	S	S	0	1 (2)
13	R	R	S	S	S	S	R	S	S	S	S	S	0	1 (2)
19	R	R	S	S	S	R	R	S	S	S	S	R	0	1 (2)
55	R	S	S	S	R	R	S	S	S	S	S	S	0	1 (2)
57	R	R	S	S	R	S	R	S	S	S	S	S	0	1 (2)
58	R	S	R	S	S	R	S	S	S	S	S	S	0	1 (2)
59	S	S	S	R	S	S	S	S	S	S	S	S	0	1 (2)

Table 13: Thailand antibiogram results

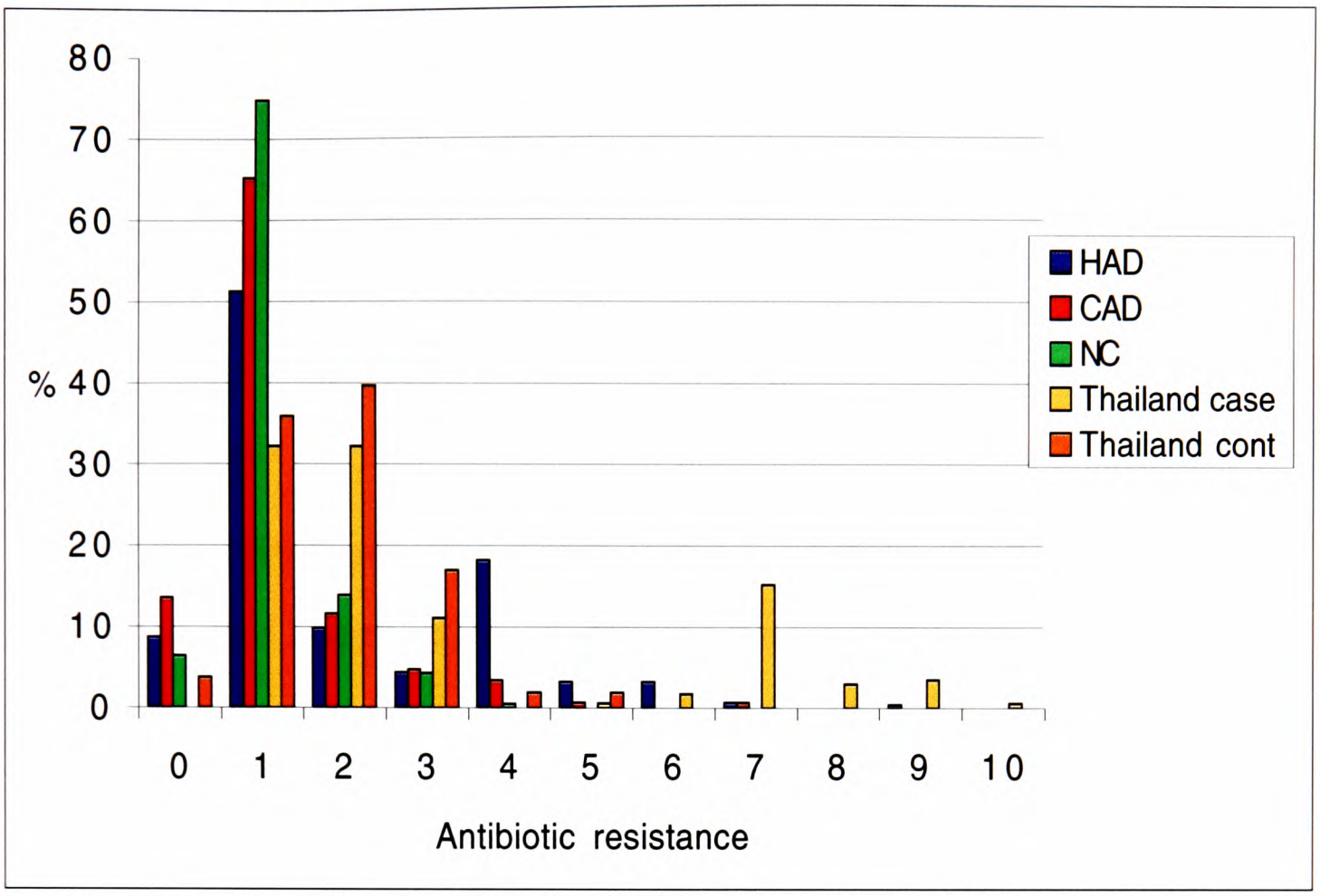


Figure 8: Number of antibiotics resistant to, by clinical group in Oxford and Thailand

Figure 8 shows the antibiogram results of all of the strains included in the study. These were subdivided into acquisition groups where i) were HAD isolates ii) were CAD isolates and iii) were asymptomatic nasal carriage isolates all from Oxford, iv) were cases from Thailand and v) were control isolates from Thailand. The x axis depicts the number of antibiotics each group was resistant to with the y axis showing the percentage of each group that was resistant. It can be seen from this figure that the cases isolated from Thailand were resistant to more antibiotics than strains isolated from Oxford. No isolates from the Thai cases were fully sensitive, but there were many which were resistant to 7, 8 or 9 antibiotics.

Tables 12 and 13 and Figure 8 all show that there was a different antibiotic susceptibility profile between the strains isolated in Oxford compared to those isolated in Thailand. The

overall picture shows that the strains isolated in Thailand were resistant to more antibiotics than those isolated in Oxford with no strains causing disease in Thailand being fully susceptible and less than 5% of carriage strains from Thailand being fully susceptible with approximately 20% of the strains causing disease in Thailand being resistant to more than 7 antibiotics.

3.3.4 Conclusions

i. Antibiogram patterns were not significantly different between CAD and nasal carriage isolates, suggesting that the former could have been drawn randomly from the latter.

ii. HAD isolates were significantly more resistant than CAD or carriage isolates.

iii. The resistance patterns differed between Oxford and Thailand.

3.4 Bacteriophage typing

3.4.1 Introduction

Phage typing is a method based on the susceptibility or resistance of *S. aureus* strains to a typing panel of phages. The susceptibility or resistance of an *S. aureus* isolate to a bacteriophage is mainly a consequence of bacterial DNA restriction mechanisms. These mechanisms act as a barrier to recombination, and hence the phage restriction profile of a bacterium is not just a typing tool, but is potentially of evolutionary importance (Barcus and Murray 1995).

3.4.2 Method

Bacteriophage typing (phage typing) was first used for epidemiological purposes to type *S. aureus* in 1942 (Fisk 1942; Fisk 1942). Wilson and Atkinson modified the method for routine use in 1945 (Wilson and Atkinson 1945). A set of 23 international phages are used to differentiate *S. aureus* strains. A lawn of freshly grown *S. aureus* is prepared with a number of different phages inoculated onto the organism. The isolate is sensitive to a phage if the organisms are lysed and resistant if the organism is able to grow in the presence of the phage (Williams and Rippon 1951).

Phage typing in this study was conducted by Mark Ganner and Dr. Hazel Aucken based at the Staphylococcal reference laboratory in Colindale, London using the following method. 27 phages were used at the routine test dilution (RTD), these comprise 23 international phages and 4 local experimental phages (see Appendix A3 for the list). The RTD used for phage typing is the highest dilution at which the phage gives confluent lysis on the propagating strain. Each typing phage has its own characteristic lytic pattern on the propagating strains. Phage typing involves growing a test strain in a broth; flooding onto a nutrient agar plate and removing the excess before drying. The Lidwell phage-typing

apparatus is used to spot the panel of phages and a negative control onto each agar plate (Norris and Ribbons 1972). After overnight incubation at 30°C, reactions are coded using a numerical system (Table 13). Reactions 1, 2 and 3 were recorded as weak with 8 and 9 recorded as strong reactions, 0 is an inhibition reaction.

Recorded result	**Growth on agar plate**
1	1-5 plaques
2	6-19 plaques
3	20-49 plaques
8	≥50 plaques
9	Confluent lysis
0	Inhibition reaction

Table 13: Phage typing reactions

Some strains remain untypable if they are permanently or temporarily resistant to all phage in the panel at RTD. These strains are tested with a concentrated phage (Appendix A3) at x100 or x1000 RTD (Williams and Rippon 1951). An inhibition reaction occurs when the phage filtrate inhibits growth of the bacteria. Plaques become apparent if the phage is diluted (Williams and Rippon 1951). For the purposes of this study an inhibition reaction was classified as lysis as the strains are susceptible to the phages.

Phage group	**Phage**
I	29 (A), 52 (B), 52A (C), 79 (D), 80 (E)
II	3A (F), 3C (G), 55 (H), 71 (I)
II M	95 (J), 81 (U)
III	6 (K), 42E (L), 47 (M), 53 (N), 54 (O), 75 (P), 77 (Q), 83A (R), 84 (S), 85 (T)
III	88 (E), 90 (F), 83C (G), 932 (H)
V	94 (V), 96 (W)

Table 14: Phage groups

Table 14 shows the groups of phages based on common clusters of single phage results. (Information provided by the PHLS laboratory at Colindale).

3.4.3 Results

There has been a suggestion that strains differ if they vary by two or more strong reactions (Norris and Ribbons 1972). No specific guidelines were available to interpret phage results as the method is not used routinely. The results were examined i) including weak reactions and ii) excluding weak reactions, to see if there was a difference in the typing of the strains (for results see Appendices A4-A6). None was observed therefore the following results include all reactions.

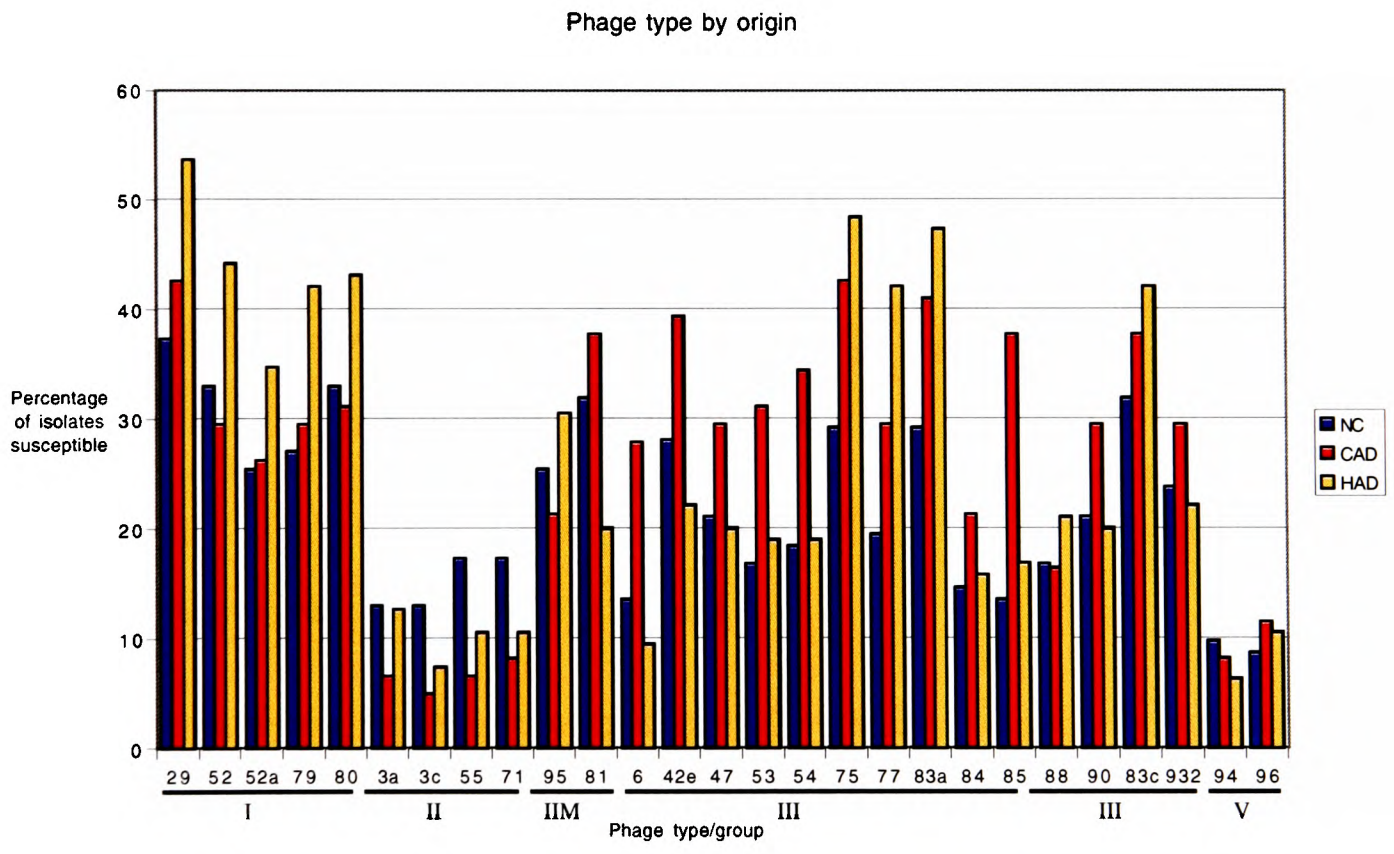


Figure 9: Percentage of each group (NC, CAD and HAD) susceptible to each typing phage

Figure 9 depicts each phage type separately, though the single phage types are arranged by phage group. There were more isolates in the HAD subgroup susceptible to the first cluster

(29, 52, 52a, 79 and 80) of phage types, compared with the NC and CAD groups. A higher percentage of NC group isolates were susceptible to the second cluster of phage types (3a, 3c, 55 and 71), and the CAD subgroup was relatively more susceptible to the third cluster of phage types (6,42e, 47, 53, 54, 84, 85, 91 and 932).

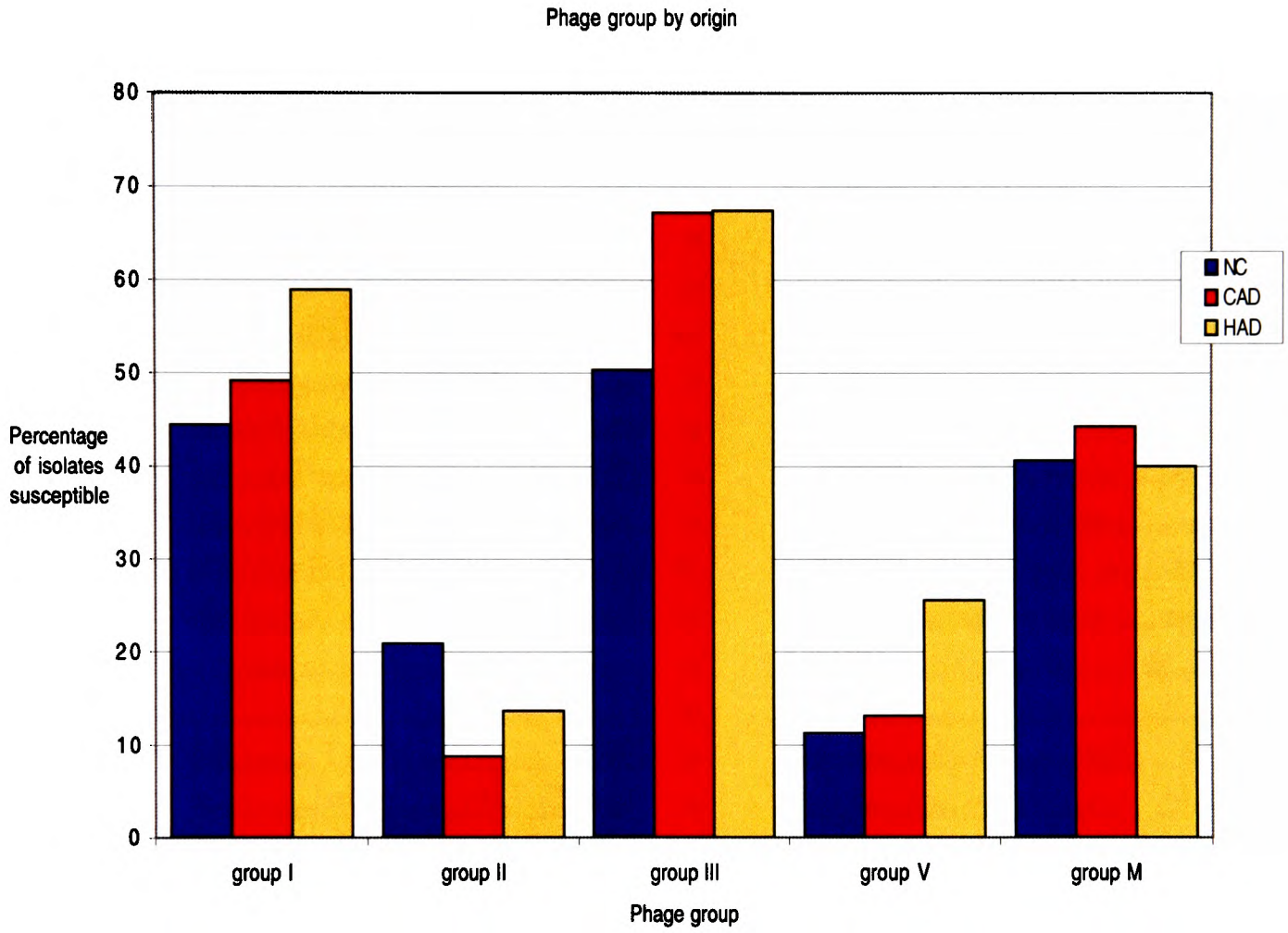


Figure 10: Phage group split by clinical group

Figure 10 shows disease isolates were significantly more likely to be susceptible to group III phages than carriage isolates (p=0.001), though this became of borderline significance once the EMRSA-16 clone was removed (p=0.02). Carriage isolates were marginally more likely to be susceptible to group II phages than disease isolates (p=0.03). There was no significant difference in group II or group III susceptibility between CAD and HAD isolates.

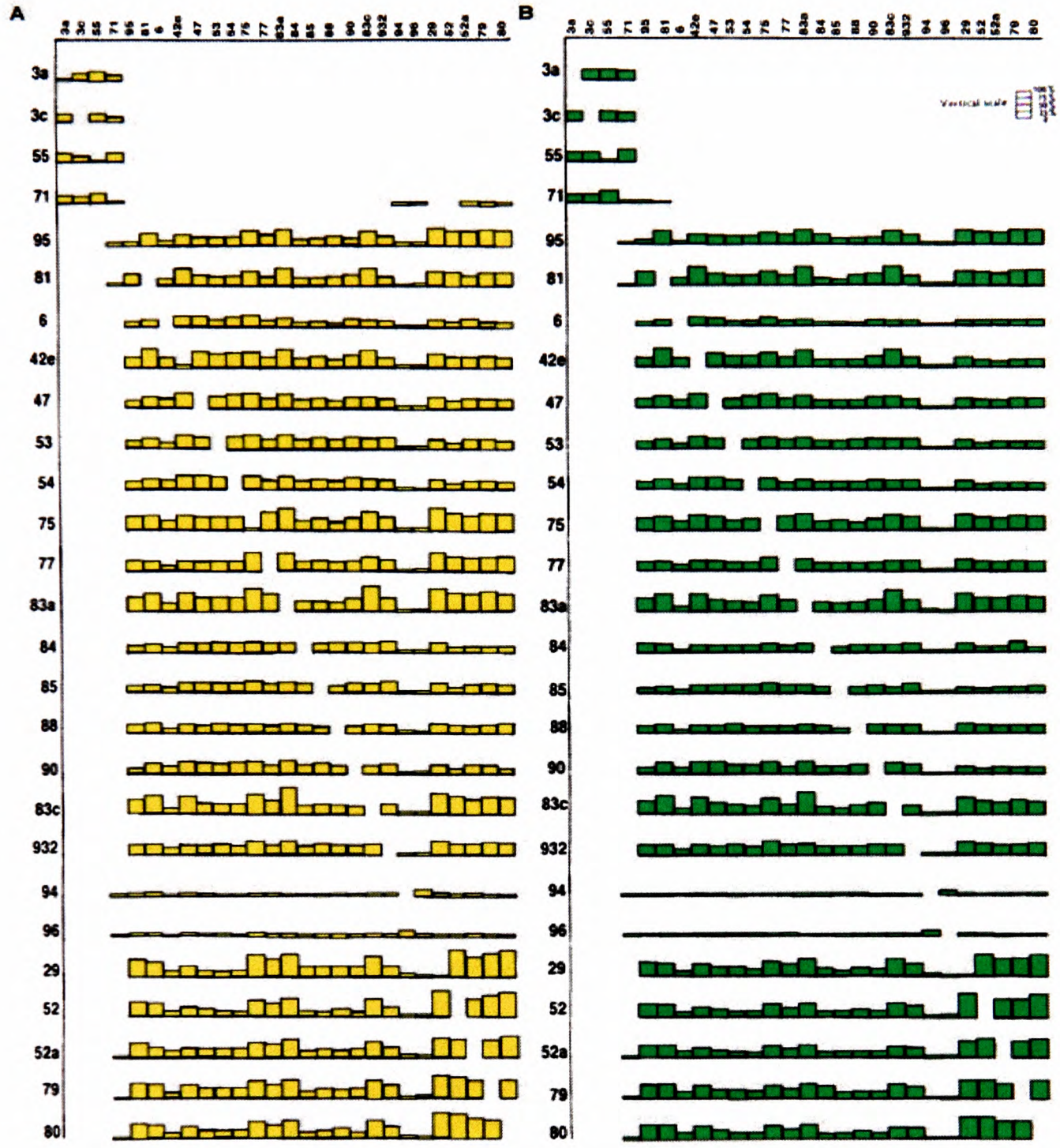


Figure 11: Block diagrams showing the interactions between phages for A. all strains and B. the nasal carriage strains

Figure 11 and 12 depict the frequency of occurrence of pairs of reactions in the phage patterns. The figures show the reactions of each phage type against each other, with the height of the bars depicting the percentage of strains. For example, phage types 3a, 3c, 55 and 77 (all phage group I) only react with other phage types from phage group I (not groups II, III or V). Also the phage types from groups II, III and V (phage types 95 to 80)

react both with phage types from the same group and with phage types from other groups (II, III and V), but not with phage group I. The pattern of reactions are similar throughout the subgroups, the only difference occurred in the HAD subgroup where more strains appeared to react with 75, 77 and 83a and 29, 52, 52a, 79 and 80. This increase was due to the group (n=22) of EMRSA-16 strains isolated within the hospital.

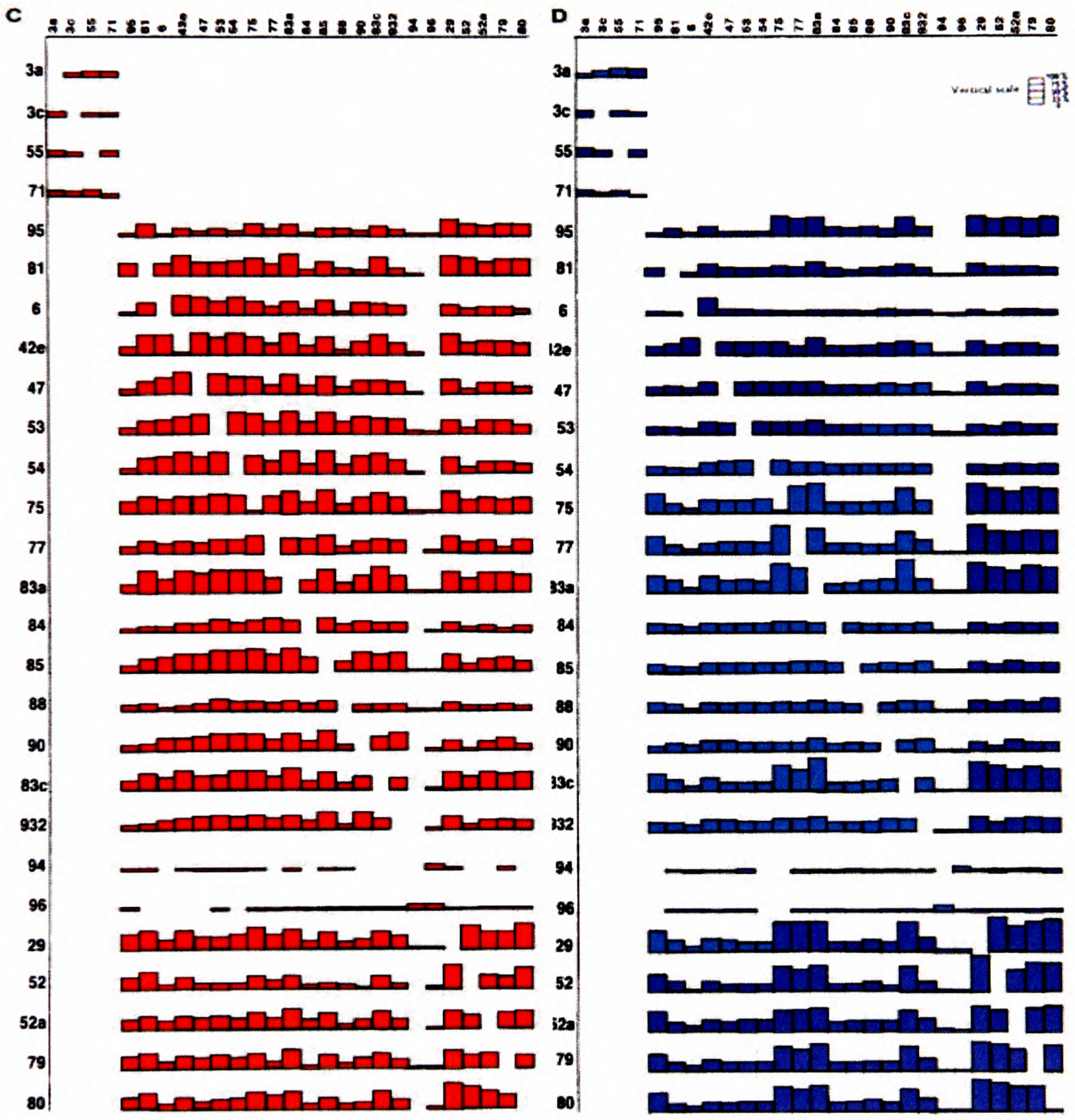


Figure 12: Block diagrams showing the interactions between phages for C. the CAD subgroup and D. the HAD subgroup

3.4.4 Conclusions

i. Phage typing was able to type 91.5% of the isolates, but interpretation of the results was difficult.

ii. Carriage isolates were marginally more likely to be susceptible to group II phages, and disease isolates to group III phages.

iii. Taking the comparison of CAD and nasal carriage isolates, there was no evidence from phage typing of specific hypervirulent clones.

Within a hospital outbreak the analysis of phage typing results is relatively straightforward as isolates of the causative strain should all have the same pattern of lysis, though it does have the disadvantage that (in the current study) 8.5% of isolates (n=29) were untypable. Phage typing cannot be used to show that two organisms are the same; it can only say that two strains are different (Williams and Rippon 1951). When looking at this diverse collection of strains taken over a two year period, the analysis was more difficult.

The scheme described by Williams and Rippon (Williams and Rippon 1951) gave some guidance for the interpretation of the phage typing results. They suggested two strong differences, such as the absence in one strain of lysis by two phages which show a strong lysis in the other, indicates that the strains are distinct.

The interpretation of inhibition reactions is not straightforward. An inhibition reaction occurs when absorption of a strong phage preparation leads to death of the Staphylococcal lawn without phage multiplication or lysis. The appearance of an inhibition reaction is influenced by the initial ratio of phage to bacteria and by the growth rate of the Staphylococcal strain (Crossley and Archer 1997). For ease of interpretation, inhibition reactions were classed as positive in this study, as it has been previously shown that plaques occur when the phage is diluted. In the current study there were no major

differences between groups when only strong reactions were used versus allowing weak reactions to be termed positive (Appendices A4-A6).

A relatively high proportion of carriage strains were in phage group II. This group is reported to include skin strains which cause impetigo, folliculitis and secondary skin diseases (Moore and Lindsay 2001). There were larger numbers of HAD in group I (phages 29, 52, 52a, 79 and 80) and there is another increase in one section of group III (75, 77, 83a and 83c). The large number of EMRSA-16 strains within the HAD group (n=22) confounded the results, and removal of these removed the excess in group III. The positive association between disease (CAD and HAD) and phage group III and the negative association with phage group II were both largely abolished by the removal of EMRSA-16 isolates ($p = 0.02$ and $p=0.07$ respectively).

Phage typing did provide some evidence that there were small differences in the bacterial populations in the three clinical groups; but these were very non-specific and covered groups of phages rather than specific phage types, and not supportive of the existence of specific particularly virulent clones of *S. aureus*.

3.5 PFGE

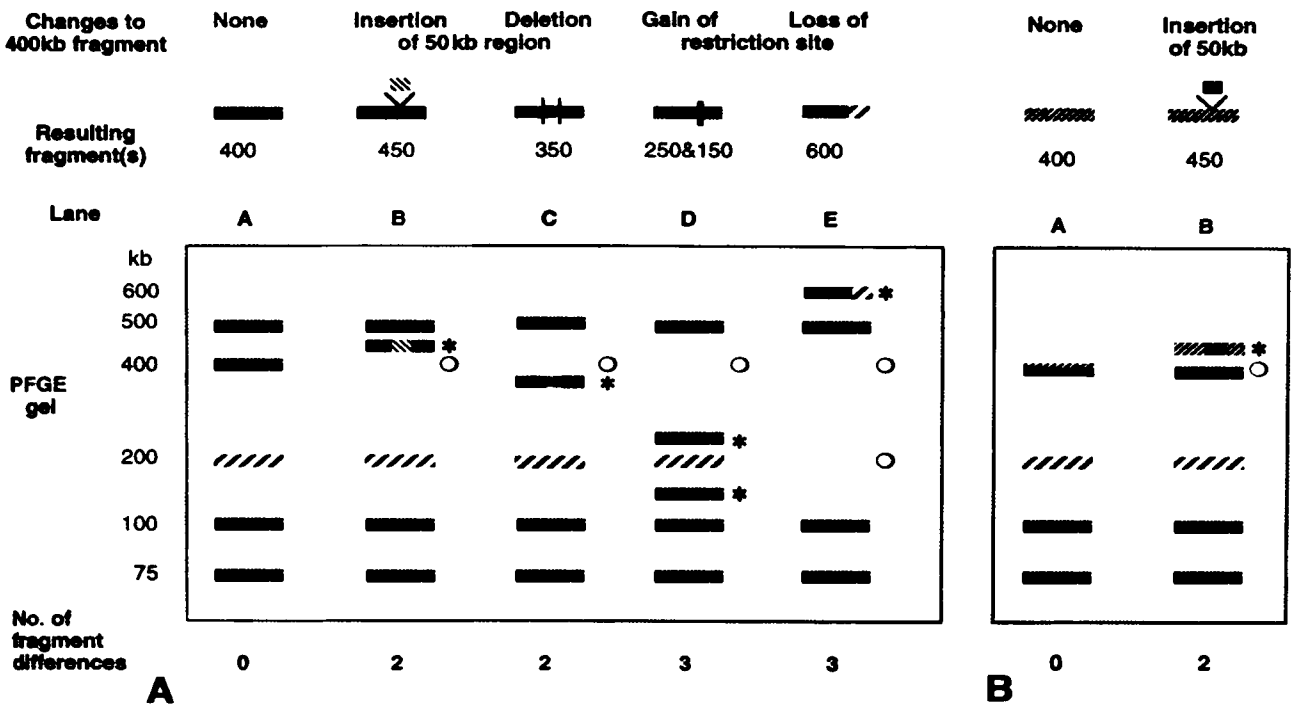
3.5.1 Introduction

PFGE enables DNA megabase-size fragments to be separated according to size, allowing chromosomal restriction fragment length polymorphisms (generated by rare cutting enzymes) to be examined. PFGE has been used both for epidemiological studies and to examine the physical map of bacterial chromosomes. A specialised electrophoresis chamber is used in which (the Contour Homogenous Electrophoresis Field (CHEF) DRIII used in the current study), an agarose gel is positioned between 3 sets of electrodes forming a hexagonal shape around the gel. Current is applied to one set of electrodes creating an electric field across the tank. The current is then switched to a second set of electrodes, changing the direction of the field. After a time it switches to the third set of electrodes shifting the field again. This pulsed electric field causes the DNA to periodically reorientate its direction of migration as it moves up the gel (Cookson, Aparicio et al. 1996; Tenover, Arbeit et al. 1997). The rate of migration through the gel is determined by the size of each DNA fragment, as smaller DNA fragments are able to reorientate faster than large ones (Goering 1993).

Many diverse organisms have been examined using a variety of different restriction enzymes. The choice of restriction enzyme will depend on the average number of specific restriction sites in a particular bacterial genome, and the required average number of resulting fragments. *Sma* I is the enzyme usually used to cut *S. aureus* as it cuts between 5'-CCC/GGG-3' sites; *S. aureus* has a low cytosine and guanine content, this enzyme cuts the genome into approximately 20 bands ranging in size from 10 to 800kb (Crossley and Archer 1997).

The nature and quality of the banding patterns resulting from electrophoresis of the restriction enzyme treated DNA depends on a number of variables, including the method of preparation of the agar-embedded DNA blocks, the switching times for reorientating the electric field, the running time of a gel and the running temperature of the machine.

Banding patterns change when point mutations create or destroy restriction sites, and with the insertion or deletion of mobile genetic elements such as plasmids (see Figure 12). Over a period of time genetic changes are likely to accumulate within a particular lineage. This is not a major problem when using PFGE to obtain epidemiological information in the context of an outbreak localised in time and geography. It becomes more of an issue when looking at strains isolated over a period of time.



(A) *Schematic diagram depicting the changes in the PFGE pattern of an isolate as a result of different classes of single genetic events. Lane A, index (outbreak) pattern; lane B, insertion of 50-kb DNA into a 400-kb fragment; lane C, deletion of 50-kb DNA from a 400-kb fragment; lane D, gain of a restriction site within the 400-kb fragment; lane E, loss of a restriction site between the 400-kb and 150-kb fragments. Open circles indicate fragments present in the index pattern and missing from the comparison isolate as a consequence of the genetic event; asterisks indicate fragments absent from the index pattern and present only after the genetic event. The number of fragment differences represents the total number of fragments present in either the index or the comparison isolate, but not in both.*

(B) *Schematic diagram depicting a change in a PFGE pattern that appears to represent the addition of an entire restriction fragment. Lane A, an index isolate for which two restriction fragments are the essentially same size (approximately 400 kb) and therefore comigrate; lane B, the comparison isolate that appears to have an entire new 450-kb fragment, but in fact the change in the pattern reflects the simple shift of one of the comigrating 400-kb fragments due to the insertion of 50-kb DNA. On reflection, it is implausible to postulate the insertion or duplication of a large DNA region that is precisely flanked by restriction sites. This example also emphasizes that comigrating fragments in different isolates need not represent the same DNA sequences.*

Figure 13: The non-linear relationship between PFGE banding patterns and underlying genetic changes (Tenover, Arbeit et al. 1995; Crossley and Archer 1997)

Figure 13 shows the non-linear relationship between DNA acquisition or loss with different banding patterns. Banding patterns are subject to the gain or loss of specific restriction sites (depending on the restriction enzyme used). For example, insertion of 50kb of DNA into an isolate with no restriction site would increase the size of a band which changes the banding pattern from the original only by 2 bands. Insertion of 50kb of DNA into an isolate with a restriction site could produce a pattern different from the original by 3 bands. Other banding differences can arise due to deletion of pieces of DNA from the isolate or due to the loss or gain of a restriction site.

A number groups have used PFGE to examine strains collected over a period of time or a widespread geographical location (Kapur, Sischo et al. 1995; Booth, Pence et al. 2001). There are difficulties however with gauging the underlying genetic differences between a pair of non-epidemiologically linked strains from a comparison of their PFGE banding patterns: there is a non-linear relationship between genetic change and restriction pattern; and because the DNA fragments are separated purely on the basis of size, bands at the same position in the gel may or may not contain the same DNA. Until recently the only guidelines for the interpretation of PFGE patterns were those published by Tenover (Tenover, Arbeit et al. 1995). These criteria relate to micro-epidemiological settings such as outbreaks, and it is unclear whether they are generalisable to the macro-epidemiological population genetic situation (see Table 15).

Category	No. of genetic differences compared with outbreak strain	Typical no. of fragment differences compared with outbreak pattern	Epidemiologic interpretation
Indistinguishable	0	0	Isolate is part of the outbreak
Closely related	1	2–3	Isolate is probably part of the outbreak
Possibly related	2	4–6	Isolate is possibly part of the outbreak
Different	≥3	≥7	Isolate is not part of the outbreak

Table 15: Interpretation of relatedness of a pair of isolates based on comparison of PFGE results (Tenover, Arbeit et al. 1995)

Booth *et al.* (Booth, Pence et al. 2001) specified criteria to group non-epidemiologically linked strains of *S. aureus* together. They considered isolates with similar banding patterns and no more than three band differences clonally related and grouped them into a *S. aureus* lineage (SAL). Isolates with no more than four band differences were considered subtypes of a given SAL, and isolates were considered sporadic if they differed by more than 4 bands.

3.5.2 PFGE methodology

The PFGE method used in the current study is a modification of that described by Bannerman (Bannerman, Hancock et al. 1995). The modification was arrived at after an extensive period of optimisation during which a number of alternative protocols for DNA block preparation and current switching times were assessed (Poddar and McClelland 1991; Prevost, Pottecher et al. 1991; Schlichting, Branger et al. 1993; Hartstein, Chetchotisakd et al. 1995; Cookson, Aparicio et al. 1996; El-Adhami, Stewart et al. 1997; Kumari, Keer et al. 1997). The resulting method was efficient, relatively quick and produced clear, reproducible results (Appendix A7).

Many of the patients entering the study with severe *S. aureus* disease had strains isolated from numerous samples and numerous body sites. During the course of the study multiple isolates were collected on a number of patients. For this reason, all strains isolated from the first 200 patients enrolled in the study between June 1997 and January 1998, including multiple isolates from the same individual, were examined by PFGE.

In the first 200 patients a total of 519 strains were collected. 79 patients had single isolates. There were a number of patients with sequential multiple isolates; 35 patients with 2 isolates; 29 with 3 isolates; 12 with 4 isolates; 9 with 5 isolates; 7 with 6 isolates; 5 people with 7 isolates; 2 people with 9 isolates and 5 people each with 8, 10, 11, 13 or 17 isolates each.

Comparison of all strains isolated from one patient produced varying results. In one patient with 9 strains isolated over the same time; 2 strains were identical to each other but differed from the other 7 by 2 bands, using the criteria described by Booth *et al.* (Booth, Pence et al. 2001) they would be considered identical. These specimens were either from CSF or the patients shunt fluid. Another patient had 5 identical isolates and one which was

different by more than 6 bands and was therefore considered different; all of these isolates were from the patient's tibia. Another patient had 3 strains where two (one from the blood and the other from part of the brain) were identical, a third taken from the spleen differed by more than 7 bands so was considered different.

There were 6 patients who had two separate episodes of severe *S. aureus* infection. One patient had 3 blood specimens, one taken a year before the other two. The first isolate differed from the other two by 6 bands and was therefore considered a different isolate. One patient had 7 strains isolated in September 1997 and a further 3 isolated 7 months later, they all had identical PFGE types.

Out of the 519 strains tested using PFGE; there were 440 multiple strains, and of these 8 strains differed from others taken from the same patient. This meant only 1.8% of the multiple strains were different. Given the large number of isolates in the database, a single sentinel sample (from the blood culture when available) was tested using PFGE (and subsequently MLST).

Blocks were also prepared from severely ill patients from Bangkok and the control strains isolated from blood donors from the Oxfordshire region. Gels were prepared with strains randomly dispersed.

3.5.3 Analysis – BioNumerics

Each gel contained a Lambda (λ) ladder in at least 3 lanes; this was identical to a reference 'gold standard' stored by the computer programme BioNumerics (Applied Maths). Each gel was digitalised and then normalised to this standard using the software. This enables banding patterns from different gels to be compared.

Banding patterns were grouped into genotypes using the method of Booth *et al* (Booth, Pence et al. 2001).

3.5.4 Results

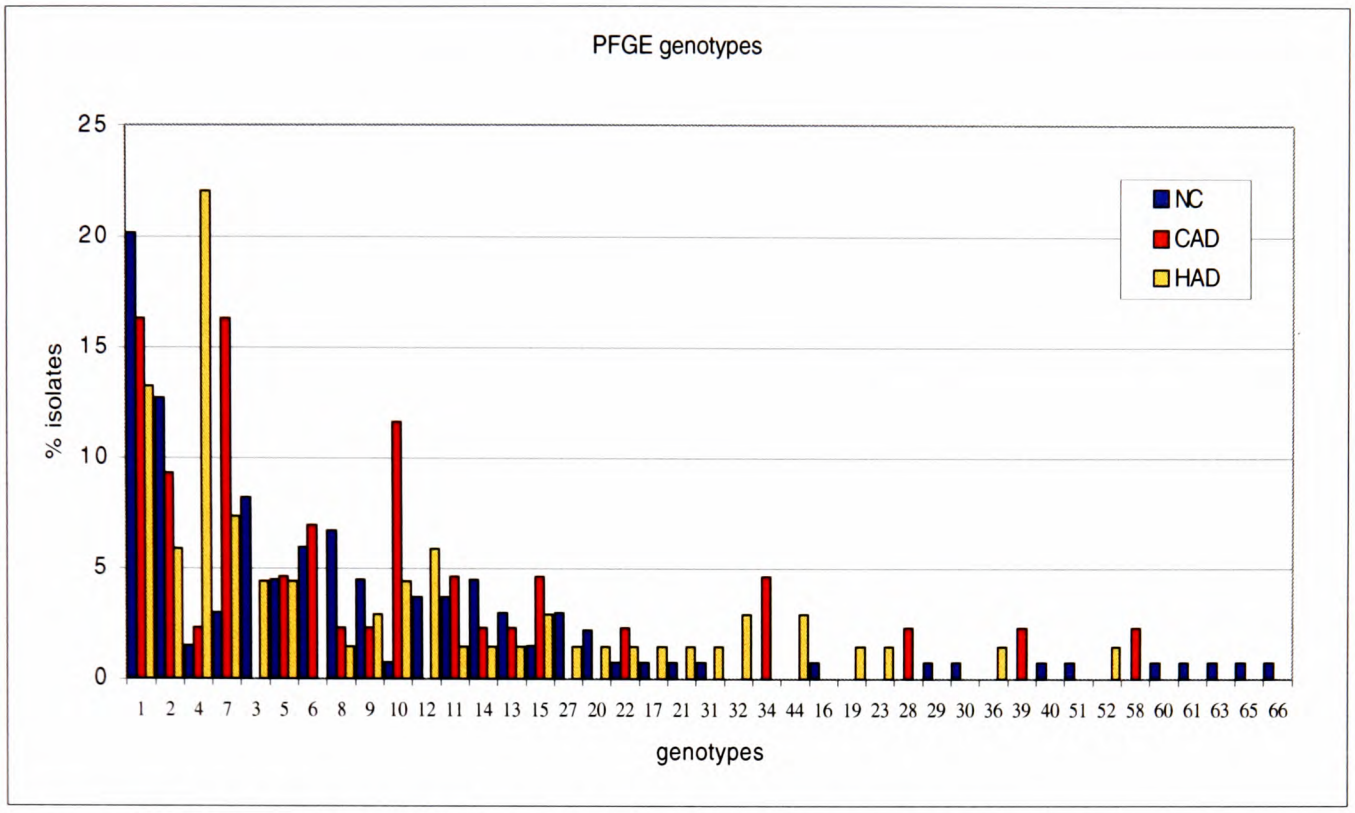


Figure 14: PFGE genotypes in the Oxford case-control study

Figure 14 shows the different genotypes assigned to the isolates from severe *S. aureus* disease. There are a wide range of PFGE genotypes (Appendix A8).

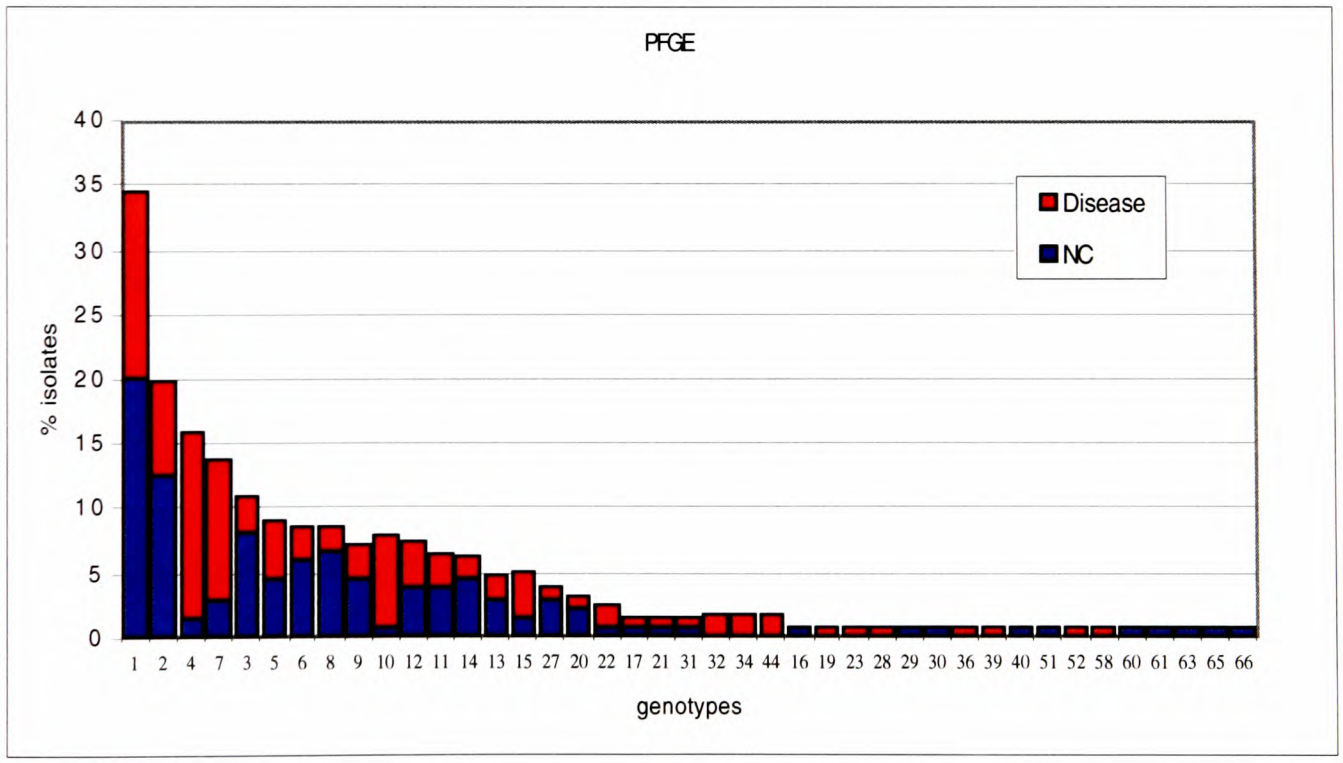


Figure 15: PFGE genotypes - disease vs carriage

Figure 15 shows overall there was no significant difference in the genotype distributions between (disease versus nasal carriage) (see Appendix A8 for numbers).

3.5.5 Conclusions

i. PFGE was a relatively rapid (3 days) genotypic method, which was able to type all the isolates in the study.

ii. The vast majority of multiple isolates from infected individuals were identical by PFGE.

iii. There were no clear differences in the PFGE genotype patterns between the clinical groups, and no evidence of hypervirulent clones.

PFGE is a method which can type all strains, but there remain many difficulties using PFGE to type hundreds of strains over a period of time. The bands produced using PFGE are unambiguous, but when the banding pattern needs to be grouped into similar patterns it becomes difficult to decide whether a strain is more like one group or another. The grouping becomes subjective, even using the criteria described by Booth *et al.* (Booth, Pence et al. 2001).

Hence, the gain or loss of multiple phages or other mobile DNA means the strain would be recorded as new instead of a variant of a known PFGE type. In this sense, 2 strains would be identical in their essential information, but would be termed different because of mobile elements. PFGE remains an excellent method to determine whether strains are members of an outbreak where there is much less subjectivity involved in interpreting the results.

Standardisation of PFGE methodology, for instance using the HARMONY criteria (Murchan 2000), would allow comparison of banding patterns between laboratories. In this way it may be possible to build a database of all known PFGE types and subtypes. Researchers would be able to compare their runs to the known types. In this way, new emerging PFGE types, possibly associated with antibiotic resistance, could then be tracked and studied.

3.6 MLST

3.6.1 Introduction

MLST was first devised in the Zoology department in Oxford and is now in use for a variety of species including *Neisseria meningitidis* (Maiden, Bygraves et al. 1998), *Streptococcus pneumoniae* (Enright and Spratt 1998), *Haemophilus influenzae* (Dr E. Feil, personal communication), *Streptococcus pyogenes* (Dr. M. Enright, personal communication,), *Campylobacter jejuni* (Dr M. Maiden, personal communication) and *S. aureus* (Enright, Day et al. 2000). Schemes are currently being devised for *Staphylococcus epidermidis* (Dr. S. Peacock, personal communication), *Burkholderia meliodosis* (Dr. B. Spratt, personal communication) and *Streptococcus agalactiae* (Dr. N. Jones, personal communication).

Through collaborative work with Prof. Spratt and Dr. Enright an MLST scheme for *S. aureus* was devised using isolates from the current case-control study. MLST is based on the same principle as MLEE, though it uses sequence directly rather than phenotype. The cytoplasmic enzymes involved in central metabolism are examined; these are more likely to be selectively neutral than surface expressed proteins, so changes occur very slowly (Spratt 1999). MLEE is an indirect approach which examines genetic variation at multiple housekeeping loci. Differences are inferred by different electophoretic mobility of the enzymes gene products on starch gels (Spratt 1999). Only a small number of the mutations influence MLEE because any amino acid change which doesn't alter the mobility of the band is not detected. In MLST nucleotide sequence is examined directly.

MLEE and MLST are generally less sensitive than PFGE, but using a number of housekeeping genes they are able to discriminate many genotypes. Strains that are ancestrally related, but have different accessory genes are identical or very similar using

MLEE or MLST. The same strains would appear different using PFGE because the accessory genes would change the banding pattern. Studies have shown that this is true for *S. enteritis* serovar *typhi* where PFGE identifies several different types (Connerton, Wain et al. 2000), MLEE shows that only 2 clones exist (Selander, Beltran et al. 1990).

MLST was devised because of limitations with MLEE. MLEE is difficult to interpret, labour intensive and the results are not unequivocal. By examining the sequence of the housekeeping genes directly the results can be compared easily between laboratories. This is impossible with many gel-based systems because of the different parameters used in different laboratories. With MLEE, the identical mobility of bands have been assumed to be identical in nucleotides. This is directly examined using MLST with the numbers of mutations and recombinations available for investigation.

One MLST database is held in a central location for each bacterial species; this database is available over the internet and has a named curator. The quality and authenticity of the results are kept high with careful interrogation of sequence data entered into the database.

The first paper to use MLST examined 107 *N. meningitidis* strains using 6 housekeeping genes (Maiden, Bygraves et al. 1998). The authors were able to differentiate more alleles using MLST compared with MLEE. MLST may also have a diagnostic role. If a specimen such as CSF or blood is taken after antimicrobial therapy, the causative organism is rarely isolated. In many instances bacterial DNA can still be found within the same sample. If a resistant or very virulent strain is known to have a certain sequence type, it may be advantageous to perform MLST on the sample directly to assist therapy. At present MLST is expensive, and would need to be made faster and cheaper for routine diagnostic use. The role of MLST in diagnostic laboratories is unclear at present, although the method has

been automated and installed in the Meningococcal reference laboratory in Scotland (Clarke 2001).

The results from MLEE and MLST both produce a series of numbers, which are compared between strains. These represent the allele numbers at each of the loci. A clustering algorithm such as the unweighted pair group method with arithmetic mean (UPGMA) can be used to examine the relationship between strains (Maiden, Bygraves et al. 1998). Because an allele with many nucleotide changes is not necessarily more distantly related to an allele which has only one base pair difference (Spratt and Maiden 1999), a strain with a housekeeping gene differing from another strain by one or more bases is given a different allele number. Importantly there is no weighting given to the number of base pair changes.

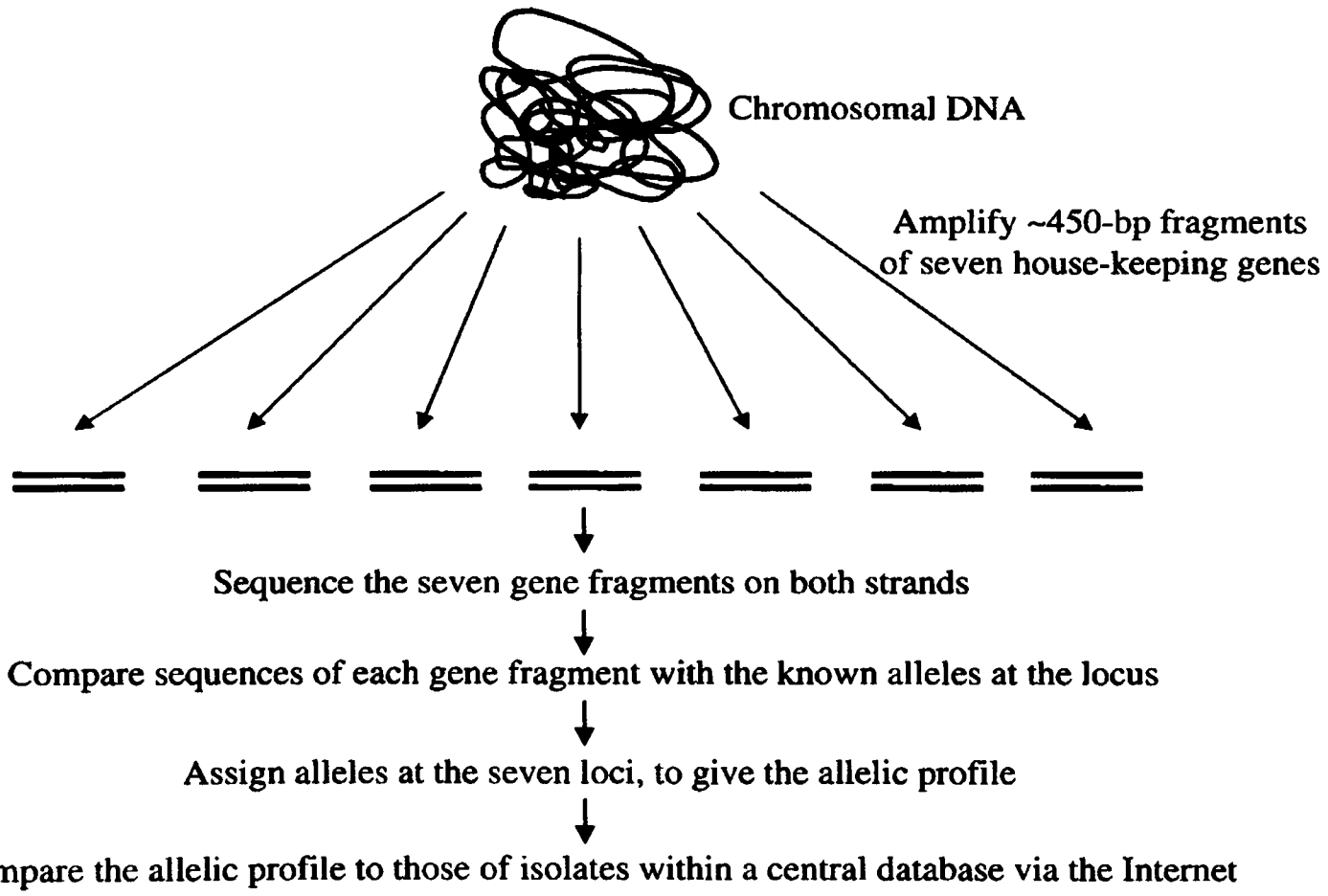


Figure 16: Overview of MLST. From Spratt, 1999 (Spratt 1999)

Figure 16 shows the method for allocating the sequence types for each bacterial isolate using MLST.

3.6.2 General method for MLST

Gene fragments for each housekeeping gene undergo PCR amplification. These are sequenced using dRhodamine labelled terminators in both directions for every strain then read using a sequencer (e.g. ABI 377 or 3700). The sequences for each housekeeping gene are compared.

The first sequence to be examined for each housekeeping gene becomes allele number 1. Each following strain is compared to the first strain (and subsequently the others in the database). Numbers are denoted sequentially if the strain is not identical to any previous allele (i.e. a new sequence). The base pair changes are not weighted, with a new allele number assigned regardless of the number of base pair changes. Each strain is assigned an allele number for each housekeeping gene. Often 7 housekeeping genes are chosen to give a good degree of discrimination. The 7 allele numbers for these loci make up the sequence type for each strain. These numbers are submitted into a central database on the Internet, which in turn provides a multilocus sequence type (ST) for each strain. These STs are then compared for relatedness using a cluster analysis method such as a UPGMA dendrogram or BURST (see Analysis 3.6.4).

3.6.3 MLST scheme for *S. aureus*

Dr. Mark Enright synthesised oligonucleotides for 14 housekeeping genes. Martin Burnham of SmithKline Beecham, UK provided the *S. aureus* genome information. The sequences of the gene products were examined using Genbank (http://www.ncbi.nlm.nih.gov/blast/blast.cgi) and were compared with the amino acid sequences of the pneumococcal protein and its homologs. These were aligned and the most variable regions were identified (Enright and Spratt 1998). The 14 primers were designed for the most conserved regions flanking these variable regions. Each pair of primers

produced a product of approximately 500 base pairs to provide an accurate sequence for approximately 450bp for each strand. See Appendix A10 for further details.

The *pyr* primers failed to synthesise a product on all 10 of the strains, so was excluded from the scheme. The other 13 primers gave results for all *S. aureus* strains. Allele numbers for each housekeeping gene were assigned using sequence output software (devised in Zoology). For each housekeeping gene, the first sequence entered into the scheme was assigned allele number 1. The sequence of the next strain was compared to the first and numbered allele 1 if it was identical or allele 2 if it differed from the first sequence. Each strain was examined and each new sequence numbered sequentially creating an allele number for each housekeeping gene in every strain.

These sequence types from the 13 genes were used to produce a dendrogram using UPGMA and compare the relatedness of the strains. Using this dendrogram and the sequence output results the most appropriate housekeeping genes were chosen. The number of alleles produced for each housekeeping gene ranged from 2 to 6 (see Figure 17). Only 2 different alleles were distinguished using *gki* so it was discarded; *gtr* distinguished 3 alleles, these were different at many bases so this was also discarded.

```
arc

  1. "1" -- allele 1
  2. "2" -- allele 2
  3. "45" -- identical to "2" ( allele 2 )
  4. "125" -- allele 3
  5. "301" -- identical to "2" ( allele 2 )
  6. "309" -- allele 4
  7. "354" -- identical to "2" ( allele 2 )
  8. "390" -- identical to "2" ( allele 2 )
  9. "543" -- identical to "2" ( allele 2 )
 10. "555" -- identical to "2" ( allele 2 )

          1122223
         84600178
         09401240
allele 1 GTTCACAT
allele 2 A.ATGTG.
allele 3 ...TGT..
allele 4 .C.....A

aro

  1. "1" -- allele 1
  2. "2" -- allele 2
  3. "45" -- identical to "2" ( allele 2 )
  4. "125" -- allele 3
  5. "301" -- identical to "2" ( allele 2 )
  6. "309" -- identical to "1" ( allele 1 )
  7. "354" -- identical to "2" ( allele 2 )
  8. "390" -- identical to "2" ( allele 2 )
  9. "543" -- identical to "2" ( allele 2 )
 10. "555" -- identical to "125" ( allele 3 )

          111234
          8035113
          9446417
allele 1 GTACACA
allele 2 A.G.GAG
allele 3 .A.TG..

gtr

  1. "1" -- allele 1
  2. "2" -- allele 2
  3. "45" -- identical to "2" ( allele 2 )
  4. "125" -- identical to "1" ( allele 1 )
  5. "301" -- identical to "2" ( allele 2 )
  6. "309" -- identical to "1" ( allele 1 )
  7. "354" -- identical to "2" ( allele 2 )
  8. "390" -- identical to "2" ( allele 2 )
  9. "543" -- allele 3
 10. "555" -- identical to "1" ( allele 1 )

                  1111111111222222223333444444
          12355890012457799001149917801245
         25768369507359238470725920678079832
allele 1 ACAAATAGTGTATTATCCATCTCTGGACTATATAA
allele 2 CTCCGCTAAACGCATC.TTATATGAAGTATCTCCG
allele 3 CTCCGCTAAACGCATCATTATATGAAGTATCTCCG

mut

  1. "1" -- allele 1
  2. "2" -- allele 2
  3. "45" -- identical to "2" ( allele 2 )
  4. "125" -- identical to "1" ( allele 1 )
  5. "301" -- identical to "2" ( allele 2 )
  6. "309" -- allele 3
  7. "354" -- identical to "2" ( allele 2 )
  8. "390" -- identical to "2" ( allele 2 )
  9. "543" -- identical to "2" ( allele 2 )
 10. "555" -- identical to "1" ( allele 1 )

          1112334
          1483491
          1401857
allele 1 TGTAGGT
allele 2 A.CGAAA
allele 3 .A.....

dfp

  1. "1" -- allele 1
  2. "2" -- allele 2
  3. "45" -- identical to "2" ( allele 2 )
  4. "125" -- allele 3
  5. "301" -- identical to "2" ( allele 2 )
  6. "309" -- identical to "1" ( allele 1 )
  7. "354" -- identical to "2" ( allele 2 )
  8. "390" -- identical to "2" ( allele 2 )
  9. "543" -- identical to "2" ( allele 2 )
 10. "555" -- identical to "125" ( allele 3 )

          111234
          8035113
          9446417
allele 1 GTACACA
allele 2 A.G.GAG
allele 3 .A.TG..

gki

  1. "1" -- allele 1
  2. "2" -- allele 2
  3. "45" -- identical to "2" ( allele 2 )
  4. "125" -- identical to "1" ( allele 1 )
  5. "301" -- identical to "2" ( allele 2 )
  6. "309" -- identical to "1" ( allele 1 )
  7. "354" -- identical to "2" ( allele 2 )
  8. "390" -- identical to "2" ( allele 2 )
  9. "543" -- identical to "2" ( allele 2 )
 10. "555" -- identical to "1" ( allele 1 )

          1234
          1770
          1922
allele 1 TTAT
allele 2 ACTA

pta

  1. "1" -- allele 1
  2. "2" -- allele 2
  3. "45" -- allele 3
  4. "125" -- allele 4
  5. "301" -- identical to "2" ( allele 2 )
  6. "309" -- allele 5
  7. "354" -- identical to "45" ( allele 3 )
  8. "390" -- allele 6
  9. "543" -- identical to "390" ( allele 6 )
 10. "555" -- identical to "125" ( allele 4 )

            12334
          28889883
          26925486
allele 1 AGCGATAA
allele 2 CA..CAG.
allele 3 .A.ACAG.
allele 4 ....CA..
allele 5 ..A..A.T
allele 6 .A..CAG.

rec

  1. "1" -- allele 1
  2. "2" -- allele 2
  3. "45" -- allele 3
  4. "125" -- identical to "1" ( allele 1 )
  5. "301" -- identical to "2" ( allele 2 )
  6. "309" -- identical to "1" ( allele 1 )
  7. "354" -- identical to "45" ( allele 3 )
  8. "390" -- identical to "2" ( allele 2 )
  9. "543" -- identical to "2" ( allele 2 )
 10. "555" -- identical to "1" ( allele 1 )

          23333
          12235
          20679
allele 1 TTTCG
allele 2 ACA.A
allele 3 ACAAA

glp

  1. "1" -- allele 1
  2. "2" -- allele 2
  3. "45" -- identical to "2" ( allele 2 )
  4. "125" -- identical to "1" ( allele 1 )
  5. "301" -- allele 3
  6. "309" -- allele 4
  7. "354" -- identical to "2" ( allele 2 )
  8. "390" -- identical to "2" ( allele 2 )
  9. "543" -- allele 5
 10. "555" -- identical to "1" ( allele 1 )

          122233
          56227917
          96476122
allele 1 TCGAGCGC
allele 2 AT....AT
allele 3 AT.T..AT
allele 4 ....AT..
allele 5 ATA...AT

gmk

  1. "1" -- allele 1
  2. "2" -- allele 2
  3. "45" -- identical to "2" ( allele 2 )
  4. "125" -- identical to "1" ( allele 1 )
  5. "301" -- identical to "2" ( allele 2 )
  6. "309" -- identical to "1" ( allele 1 )
  7. "354" -- identical to "2" ( allele 2 )
  8. "390" -- allele 3
  9. "543" -- identical to "2" ( allele 2 )
 10. "555" -- identical to "1" ( allele 1 )

          123334
          281390
          968102
allele 1 CCACGT
allele 2 TTT.AC
allele 3 TTTAAC

tpi

  1. "1" -- allele 1
  2. "2" -- allele 2
  3. "45" -- allele 3
  4. "125" -- allele 4
  5. "301" -- identical to "2" ( allele 2 )
  6. "309" -- allele 5
  7. "354" -- identical to "45" ( allele 3 )
  8. "390" -- identical to "45" ( allele 3 )
  9. "543" -- identical to "45" ( allele 3 )
 10. "555" -- identical to "125" ( allele 4 )

          112223
          560333
          917193
allele 1 TCCACA
allele 2 CTT...
allele 3 C.T...
allele 4 ...T..T
allele 5 C.TTTT

xpt

  1. "1" -- allele 1
  2. "2" -- allele 2
  3. "45" -- identical to "2" ( allele 2 )
  4. "125" -- allele 3
  5. "301" -- identical to "2" ( allele 2 )
  6. "309" -- allele 4
  7. "354" -- identical to "2" ( allele 2 )
  8. "390" -- identical to "2" ( allele 2 )
  9. "543" -- identical to "2" ( allele 2 )
 10. "555" -- identical to "125" ( allele 3 )

          11233445
          36505372
          63672242
allele 1 ACTTCCAG
allele 2 ...ACT.GA
allele 3 GT...T..
allele 4 GT......

yqi

  1. "1" -- allele 1
  2. "2" -- allele 2
  3. "45" -- identical to "2" ( allele 2 )
  4. "125" -- allele 3
  5. "301" -- identical to "2" ( allele 2 )
  6. "309" -- allele 4
  7. "354" -- identical to "2" ( allele 2 )
  8. "390" -- identical to "2" ( allele 2 )
  9. "543" -- identical to "2" ( allele 2 )
 10. "555" -- identical to "125" ( allele 3 )

          113344
          99190349
          08645507
allele 1 GTAAGCGA
allele 2 AC..ATA.
allele 3 ...GATA.
allele 4 ..T.A.AT
```

Figure 17: An example of output from 'sequence output'

The choice of housekeeping genes from the remaining 11 was arbitrary as each of them produced good sequences and could discriminate the strains equally. A dendrogram was drawn from the 13 housekeeping genes and the 7 chosen were those which produced the same dendrogram so the degree of discrimination did not differ between the 2 (Table 16 and Figures 18 and 19). The final 7 housekeeping genes, which were chosen, were *arcC*, *aroE*, *glp*, *gmk*, *pta*, *tpi* and *yquiL*. The position of these genes on the *S. aureus* genome is shown in Figure 20.

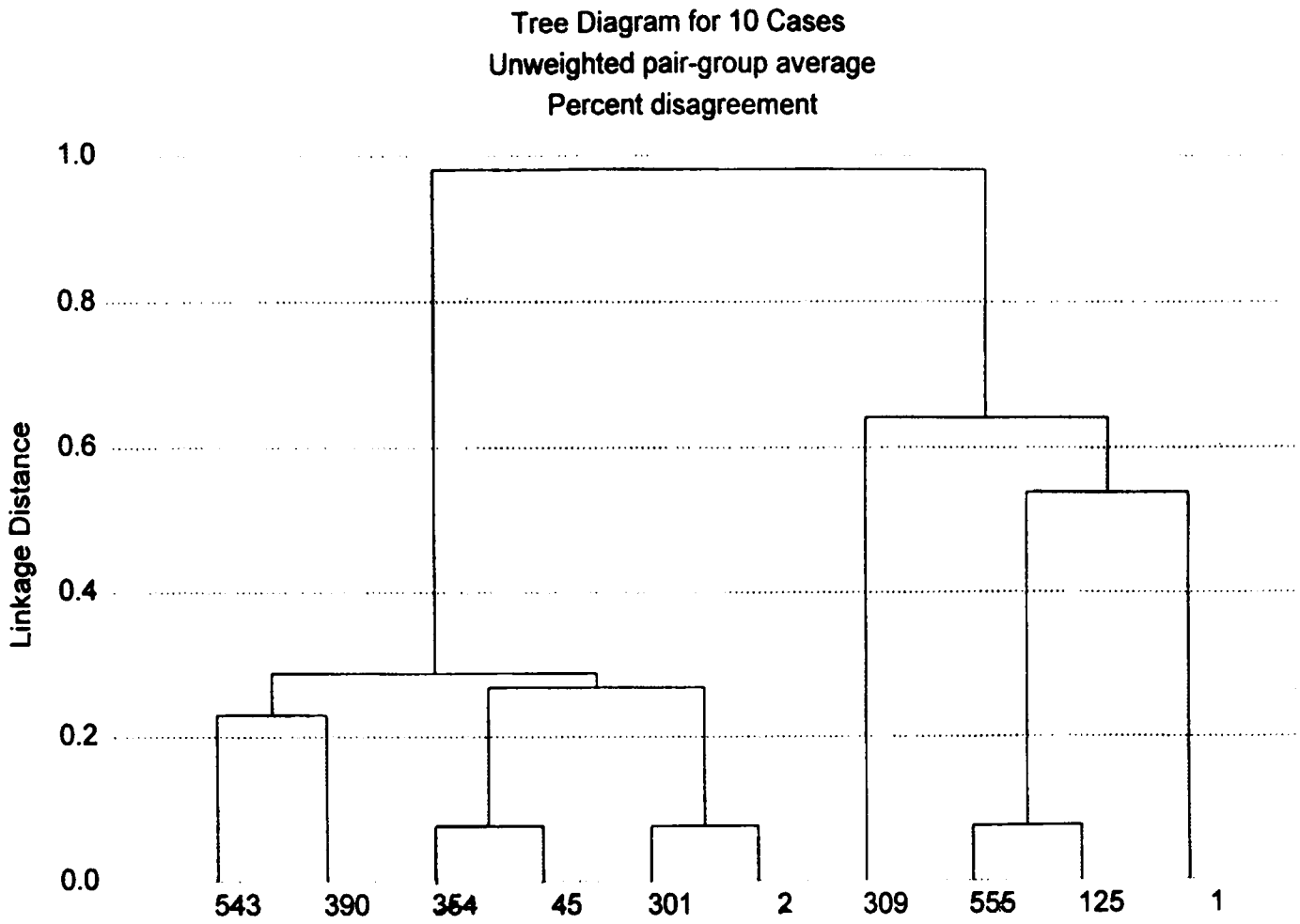


Figure 18: UPGMA dendrogram using all 13 housekeeping genes

Figure 18 shows the relatedness of the first 10 isolates using a dendrogram constructed with UPGMA. This dendrogram was produced from all 13 housekeeping genes initially tested. The x axis depicts each strain tested and the y axis depicts the differences between housekeeping genes with a linkage distance of approximately 0.1 depicting a difference in one housekeeping gene. For example, there was a difference in one housekeeping gene between strain 301 and 2 whereas two housekeeping genes differ between strains 543 and 390.

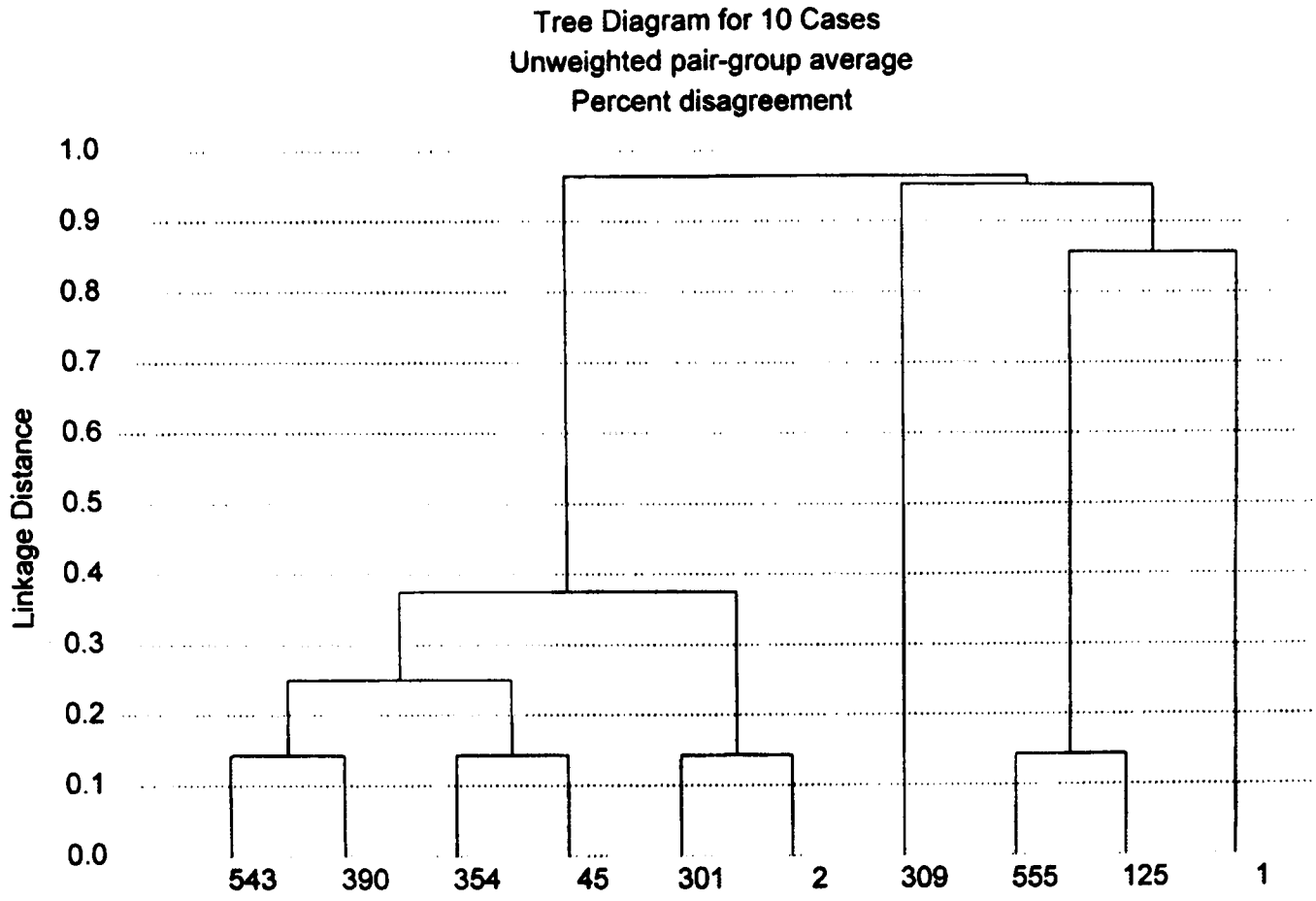


Figure 19: UPGMA dendrogram using the 7 housekeeping genes

Figure 19 shows the relatedness of the same 10 isolates as figure 18 in a dendrogram constructed using UPGMA. This figure shows the 7 housekeeping genes chosen for the MLST scheme due to the identical structure of the dendrogram to that produced using 13 housekeeping genes (figure 18). The x axis depicts the strains tested with the y axis showing the differences between housekeeping genes. One housekeeping gene was different between strains 301 and 2 whereas two housekeeping genes differed between strains 390 and 354.

Strain	*arcC*	*aroE*	*dfp*	*gki*	*glpF*	*gmk*	*gtr*	*mut*	*pta*	*rec*	*tpi*	*xpt*	*yquiL*
1	1	1	1	1	1	1	1	1	1	1	1	1	1
2	2	2	2	2	2	2	2	2	2	2	2	2	2
45	2	2	2	2	2	2	2	2	3	3	3	2	2
125	3	3	3	1	1	1	1	1	4	1	4	3	3
301	2	2	2	2	3	2	2	2	2	2	2	2	2
309	4	1	1	1	4	1	1	3	5	1	5	4	4
354	2	2	2	2	3	2	2	2	3	3	3	2	2
390	2	2	2	2	3	3	2	2	6	2	3	2	2
543	2	2	2	2	5	2	3	2	6	2	3	2	2
555	2	3	3	1	1	1	1	1	4	1	4	3	3

Table 16: The sequence types for the first 10 strains

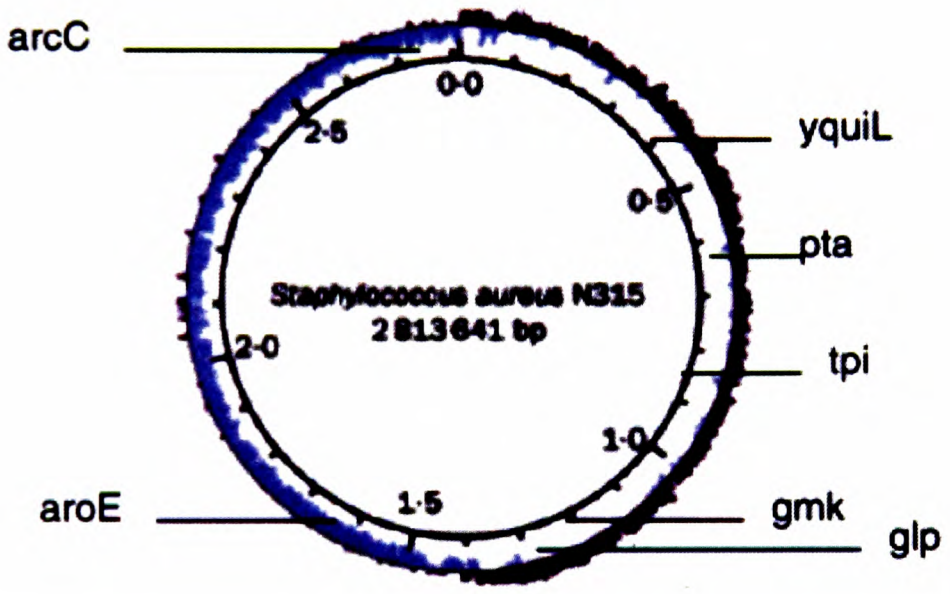


Figure 20: Distribution of the housekeeping genes around the *S. aureus* genome

The MLST scheme for *S. aureus* was validated using a larger sample size. Of 155 strains isolated from severe *S. aureus* disease; 61 were community acquired (CAD) isolates and 94 were hospital acquired (HAD). In all, 140 isolates were from blood specimens and 15 from deep pus specimens. DNA was extracted using the method described in Appendix A9 (Jordens and Pennington 1991), and the DNA passed to Dr. Mark Enright who performed MLST on the 7 housekeeping genes (Enright, Day et al. 2000).

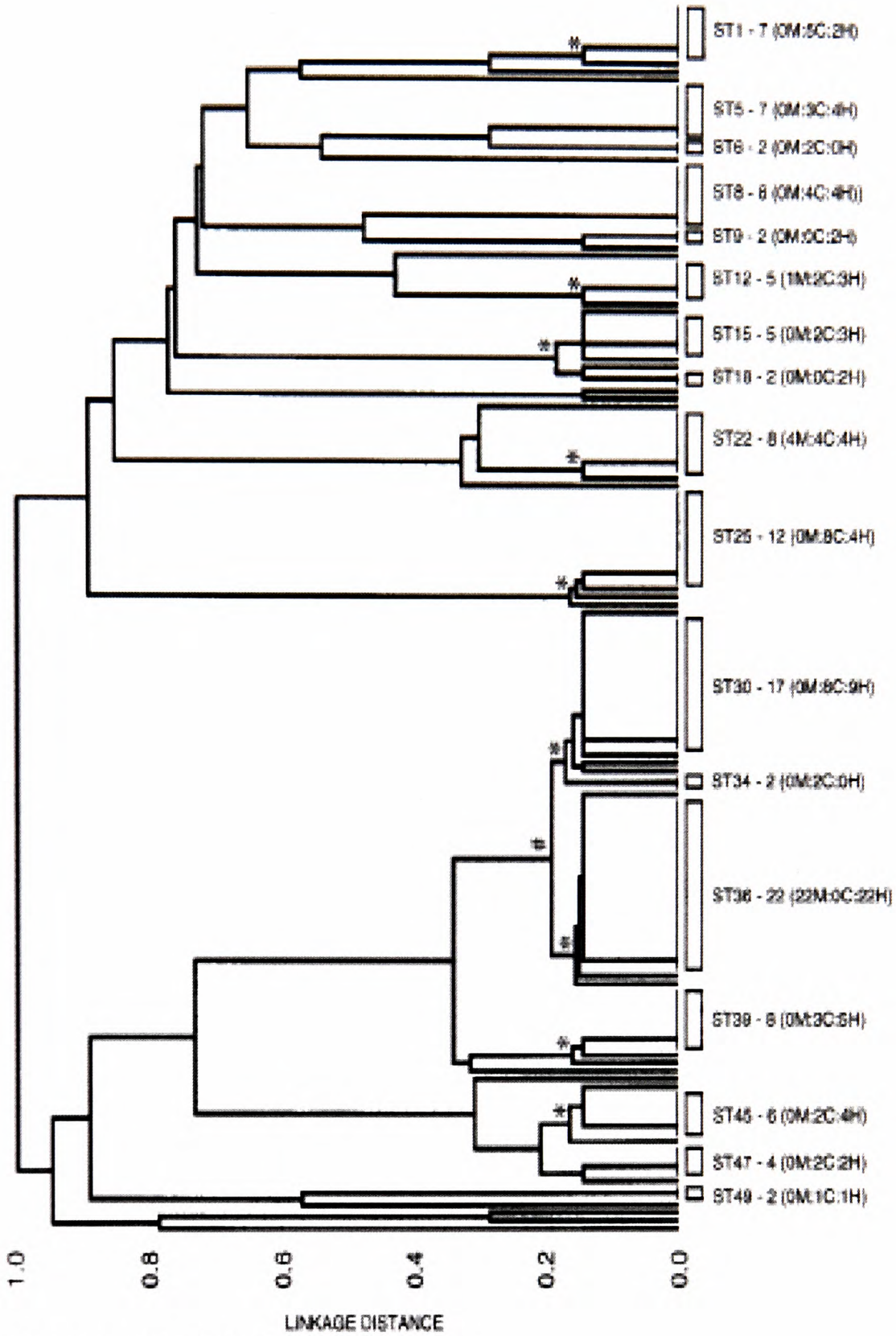


Figure 21: Dendrogram (Enright, Day et al. 2000)

Figure 21 depicts the dendrogram produced from the collection of 155 strains. Open rectangles represent many isolates with the same sequence type. The sequence types are labelled as ST and number, the number of isolates within the ST follow. Within the brackets are the number of MRSA strains within the ST (designated M); the number of isolates from community-acquired disease (designated C) followed by the number of

isolates from hospital-acquired disease (designated H). The asterixes show the nodes which define the major clonal complexes with the node showing a large cluster of closely related isolates of STs 29 to 38 shown as a hash sign.

After validation of MLST for *S. aureus*, the technique was used to examine strains isolated from asymptomatic carriers in the case-control study. The pertinent question was whether carriage strains and strains causing disease were from the same background population or whether those causing disease were different to strains carried asymptomatically.

3.6.4 Analysis

Sequence type data was analysed using either UPGMA, to build a dendrogram, or the novel analysis method BURST

3.6.4.1 UPGMA

The unweighted pair group methods with arithmetic mean (UPGMA) is a clustering method for constructing trees (dendrograms) based on relatedness of pairs of organisms. The algorithm operates on a distance matrix containing the genetic distance between all pairs of isolates. It first takes the two isolates which are most similar and produces a cluster containing these two isolates. The length of the initial branch is taken as half the distance. The closest isolate (or isolates in a cluster) to this cluster is found, and added to the tree via a branch again representing the distance from the cluster.

Trees are constructed by linking the closest pairs of isolates followed by successively more distant isolates. UPGMA assumes the same rate of evolution applies to each branch, an assumption which may not necessarily hold for bacterial strains. Different trees may also be constructed if the samples are entered into the algorithm in a different order. With many hundreds of strains it is possible to construct many thousands of trees, and a single tree may not represent the 'correct' clustering.

3.6.4.2 Based Upon Related Sequence Types (BURST)

The BURST algorithm was developed by Dr. Ed Feil and converted to web format by Man-Suen Chan (http://www.mlst.net/new/data_analysis/burst/info1.htm). BURST was devised to examine the most recent divergences in MLST dendrograms; it does not take into account relationships between the deeper roots of the dendrograms which may be distorted by recombination events. BURST examines only the very tip of a dendrogram, and no attempt is made at guessing relationships outside these closely related strains.

BURST identifies the most likely ancestral clone (the clone from which most of the variants have descended) within the clonal complexes. Instead of constructing a phylogenetic tree, which might be wrong, BURST constructs a number of circles indicating the relatedness of closely related strains to the ancestral clone. The relatedness between the circles is not analysed.

The algorithm goes through a series of steps. The strains are divided into clonal complexes. Strains are grouped together into clonal complexes if they have at least 5 housekeeping genes with identical alleles to at least one other member of the clone. The algorithm then defines the ancestral genotypes within each clonal complex. Pairwise comparisons are made between all of the allelic profiles and the ancestoral genotype is chosen as that with the highest number of genotypes differing from it by just one locus (i.e. the one with the largest number of single locus variants (SLVs)). The ancestral genotypes are not weighted by the number of each genotype present. If there are two or more genotypes defining the same number of SLVs the number of double locus variants (DLVs) are then taken into account.

The other genotypes in each complex are assigned as SLVs and DLVs of the ancestral genotype. Satellite genotypes differ by more than two loci from the ancestral genotype.

Singletons differ from all other strains by three or more loci and are not assigned as members of any clonal complex.

3.6.5 Results

The original MLST results for the Oxford case-control study contained a number of sequencing errors which led to an erroneous interpretation of the data (Day, Moore et al. 2001 and 2002). The revised results are given in Figures 22, 23 and 24, and in Appendix A11. The database contains 333 isolates as one carriage strain failed to type for a single locus using MLST. These comprise 75 different STs: 26 STs contain more than one isolate. The allele numbers and sequences for each locus in each ST can be found on the MLST website (www.mlst.net).

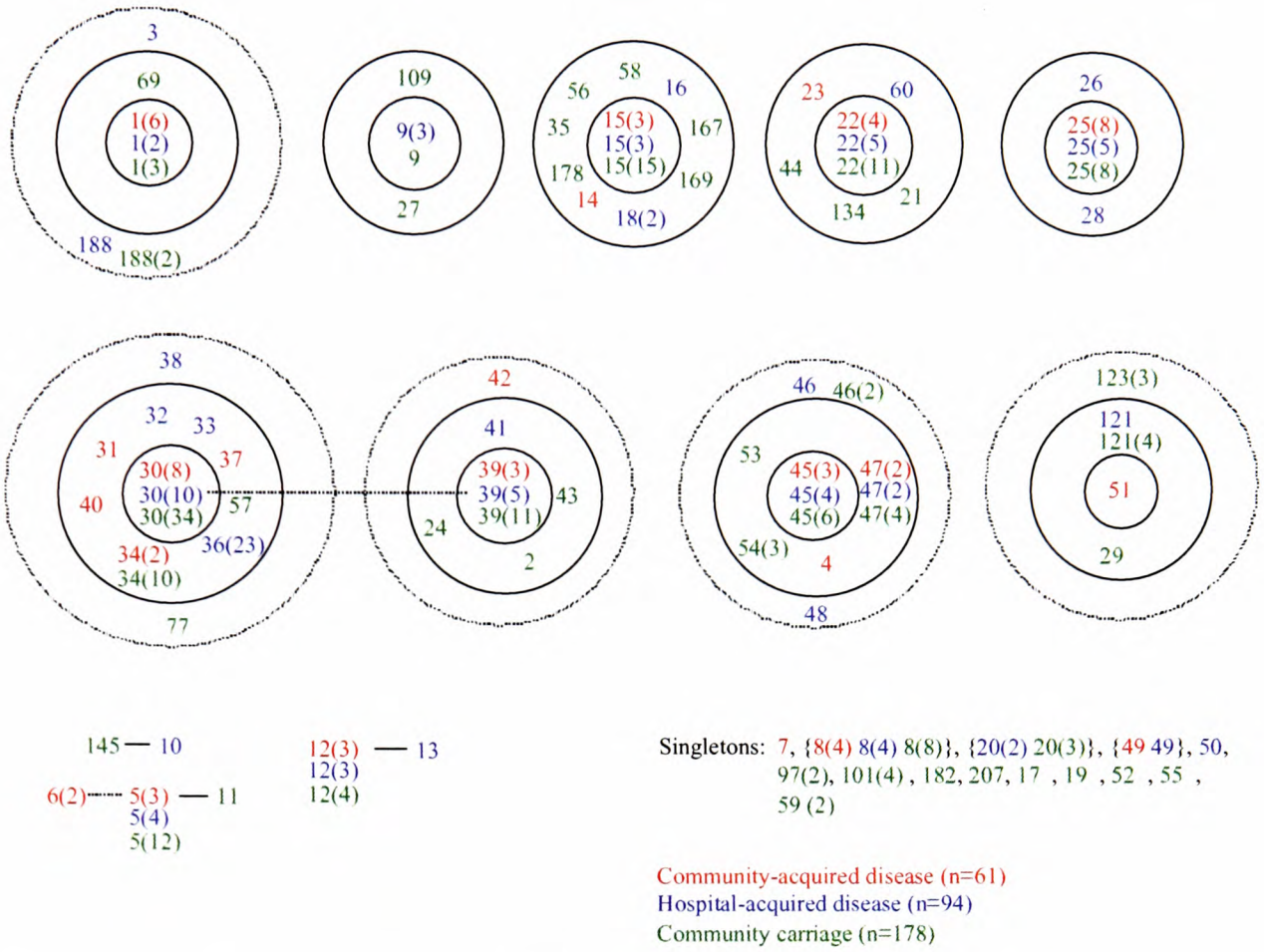


Figure 22: BURST diagram for Oxford case-control study MLST results

Clonal complexes are named after the ancestoral genotype in the centre of each complex (i.e. CC1, CC9, CC15, CC22, CC25, CC30, CC39, CC45, and CC51). Numbers denote STs. The number in brackets after the ST denotes the number of isolates in this ST if greater than one.

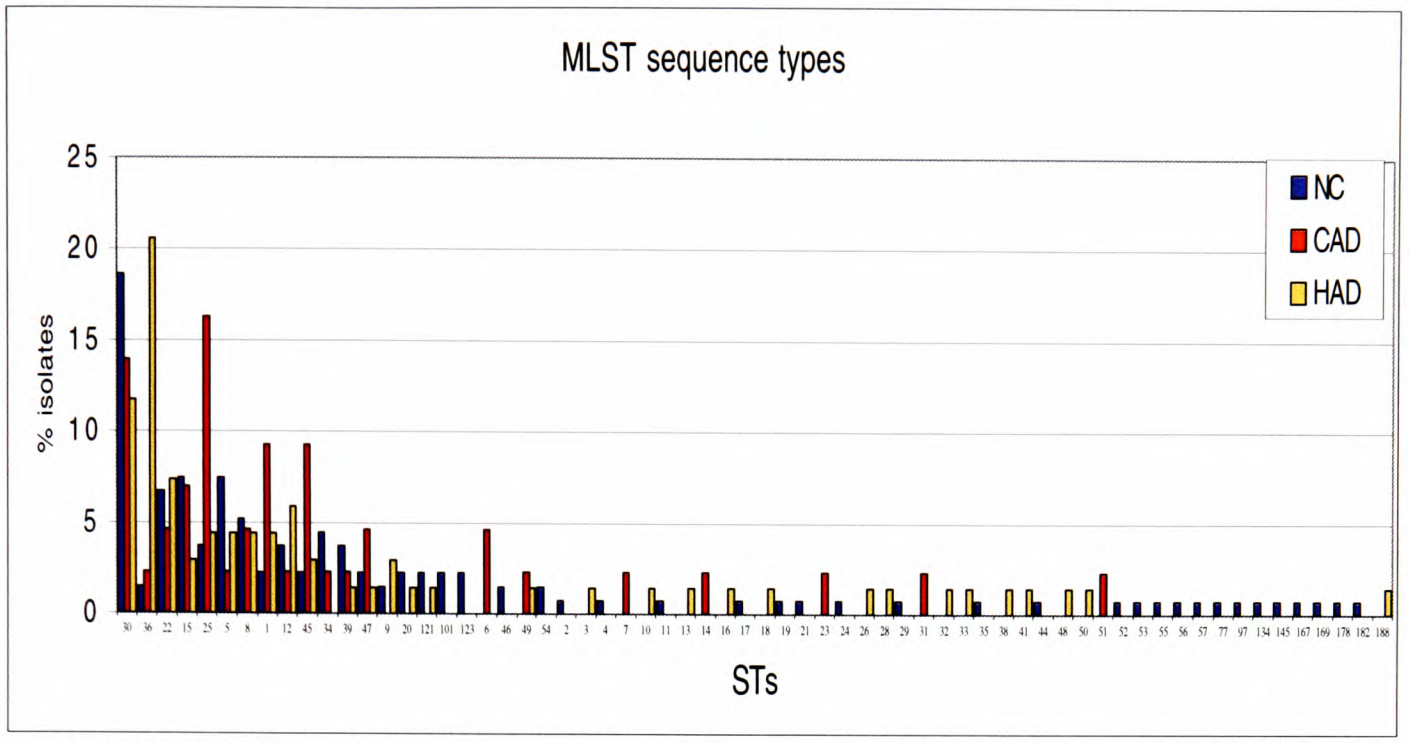


Figure 23: MLST sequence types: frequencies by clinical groups

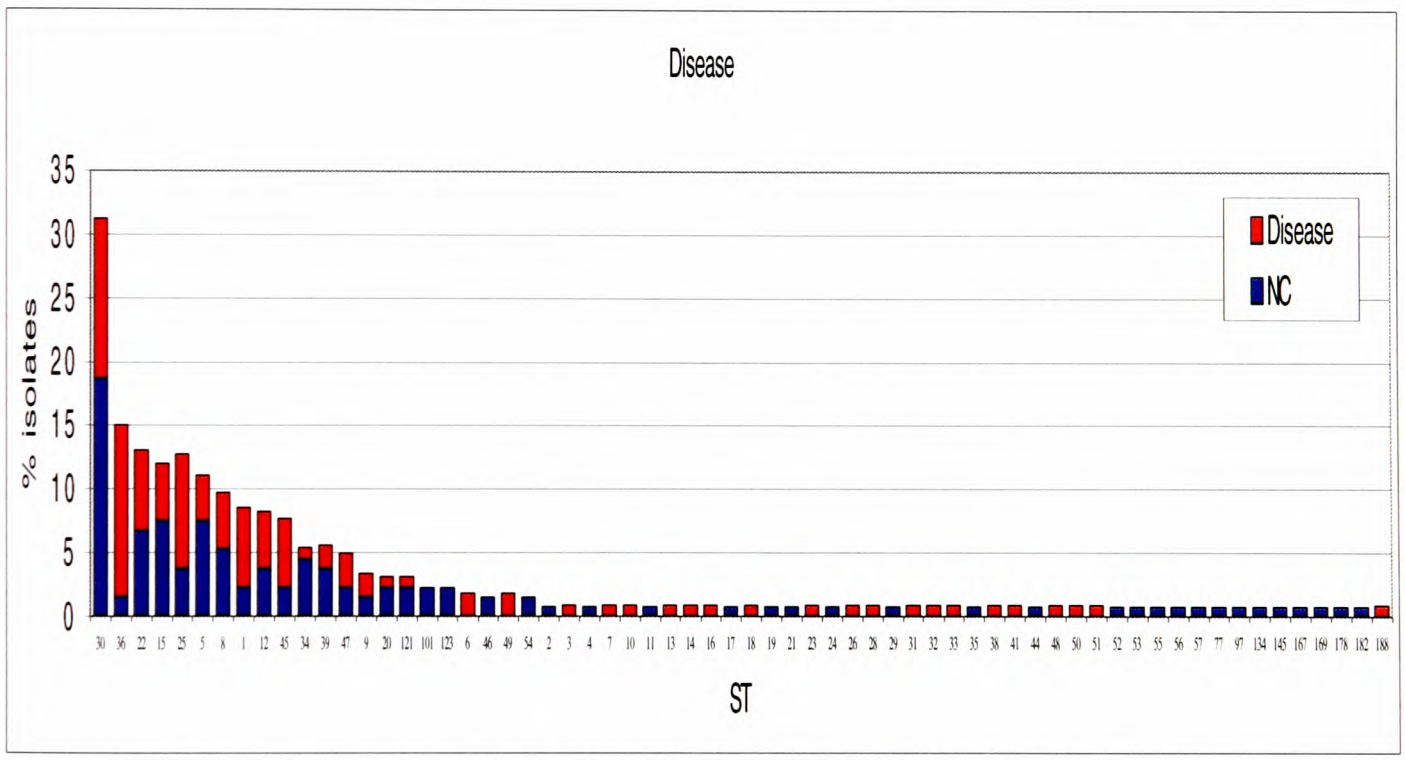


Figure 24: MLST sequence type frequencies: disease vs carriage

No significant difference in the distribution of sequence types between the cases and controls was seen (Figure 24) (p=0.31).

All the major clonal complexes contained isolates from each of the three clinical groups. Ancestral genotypes, found in the centre of the clonal complexes contained the largest number of strains. Ancestral genotypes could not be defined in all groups (the 3 groups separate from the others); these were classed as singletons for analysis. The alleles for the ancestral types were found elsewhere in the data, suggesting they occurred prior to any mutations. Any information regarding the relatedness between the different clonal complexes is not available using BURST, so the relatedness of the different clones was not examined. This has since been studied using bootstrapping methods applied to the raw sequence (Dr. E. Feil, personal communication). Using this approach it appears that most clonal complexes are much more distantly related than the clones within each clonal complex, with the exception of CC30 and CC39 which are known to be closely related.

There were no differences between the position of a strain within a clonal complex (ancestral genotype, SLV, DLV, satellite, or singleton, and whether they were disease-causing or carriage strains (Table 17)($p=0.3$). Carriage strains were equally likely to be ancestral genotypes as invasive strains. There was also no difference in position in the clonal complexes depending on whether a carriage strain was isolated from one nostril or two. Strains were equally likely to be of an ancestral genotype if they are only isolated from one nostril ($p=0.4$). There is no difference in virulence or fitness between ancestral genotypes and their variants, and none of the housekeeping genes are significantly associated with disease. This suggests they are not virulence genes, or associated with virulence genes. Common carriage clones are also common causes of disease.

Clonal position	NC n (%)	CAD n (%)	HAD n (%)
Ancestral clone	89 (50)	36 (59)	37 (39)
SLV	39 (22)	10 (16)	35 (37)
DLV	8 (4)	1 (2)	5 (5)
Singleton	43 (24)	14 (23)	17 (18)
Total	179	61	94

Table 17: Distribution within clonal complexes of isolates from the three clinical groups

4 x 3 Fishers exact test, p = 0.3

MLST data can be used to make estimates of the relative roles of point mutations and recombination in clonal diversification. Eighty four isolates differed from the ancestral type at one locus so were classed as SLVs: these SLVs consisted of 37 different STs. Comparisons were made between the SLVs and their ancestral genotypes. If a variant allele in the SLV differed by more than one nucleotide from the ancestral allele it was assigned a recombinant event on the basis that multiple point mutations in only 1 out of 7 genes is unlikely to occur. If there was only a single nucleotide difference between the SLV and the ancestral type, the variant may have arisen either through point mutation or recombination. When this occurred, the rest of the database was examined for the variant allele: if the allele occurred elsewhere in the dataset it was termed a recombinant. A point mutation is unlikely to occur by chance elsewhere in the dataset (Feil, Smith et al. 2000). This parameter is based on the numbers in the dataset. Strains in a small dataset may be wrongly assigned because of numerical restrictions.

In the Oxford dataset only 2 isolates differed by recombination, and 35 isolates differed by a point mutation. Therefore, *de novo* mutation occurs approximately 17 times more frequently than recombination in this isolate population.

3.6.6 The problems encountered with MLST

The original comparison of disease and carriage isolates using MLST generated the conclusion that carriage strains were more diverse than disease strains (Day, Moore et al. 2001). Major problems were subsequently detected with these results, retracting these conclusions (Day, Moore et al. 2002). A number of errors led to this problem or compounded it:

i. The standard operating procedures which were taught for carrying out sequencing were inadequate when applied to a technique such as MLST which is totally intolerant of sequencing errors. A single base error out of the 3,198 sequenced for each strain (0.03%) leads to an incorrect ST being assigned. The two main problems were that traces from previously sequenced alleles were used as guide templates when checking base-assignment on new samples, and incomplete complimentary strand sequences were accepted if the sequence of the first strand was of reasonable quality.

ii. A tracking error occurred during an ABI 377 sequencing run on one plate containing 47 samples being sequenced for a particular allele. This led to consensus clones being assigned as SLVs, and SLVs as DLVs, etc.

iii. Because the original interest of the collaborators was in disease isolates, these were typed first by an individual with greater experience in the techniques that myself. The carriage isolates were subsequently and separately typed. Although there were errors in the MLST results in both groups, they were more frequent in the carriage isolates (in particular the error described in ii. above occurred in this group), leading to the impression that the carriage isolates were more diverse than those causing disease.

These errors were detected after subsequently typing epidemiologically similar collections of isolates, which were found to be less diverse and more clonal. Much of the original sequencing was therefore repeated following a stricter protocol, producing the results presented in this thesis. The three problems above were addressed as follows:

i. No guide sequence was used during base-assignment checking. Sequencing was repeated if:

   a. The two strands were not complete, in agreement and of high quality.

   b. A new allele was generated

   c. A new ST was generated, even if composed of known alleles.

ii. To avoid tracking errors:

   a. A known positive control allele for each gene was included on each plate.

   b. A capillary sequencer was used where possible (e.g. ABI 3700).

   c. Lane tracking dyes were used if a gel-based sequencer was used (e.g. ABI 377).

iii. In all other work presented in this thesis, before and after the errors caused by separating cases and controls during MLST were detected, samples from cases were randomly intermixed with controls. A series of circumstances led to this rule being broken in the case of MLST, a situation accepted as at the time MLST was believed to be completely unambiguous.

3.6.7 Conclusions

i. The genetic population structures of the three clinical groups are very similar, suggesting that with the possible exception of the EMRSA-16 clone (ST36) disease isolates could have been randomly drawn from the nasal carriage isolate population. It is not possible to define hypervirulent clones of *S. aureus* on the basis of variation in 7 putatively conserved housekeeping genes.

ii. Natural populations of *S. aureus* segregate into a relatively very small number of sequence types, which themselves exist in around 10 clonal complexes of closely related strains. This supports the existence of a highly clonal population structure for *S. aureus*.

iii. Examination of the sequence of variant alleles of SLVs suggests that diversification within clonal complexes occurs by mutation rather than recombination.

Overall MLST appears to be a useful tool for the population genetic analysis for *S. aureus*. The method is discriminatory, not sensitive to mobile genetic elements as is PFGE. The method is reproducible which is generated from the many strains which have the same alleles and sequence types. MLST can also be used to estimate the extent to which alleles at different loci in a population are linked. If alleles are randomly associated, the presence of an allele at one locus is independent of alleles at other loci which suggests linkage equilibrium. If there is a high level of genetic diversity in a large population size this would predict the presence of a large number of genotypes in a population, unless the population is very clonal in nature.

Two random isolates are extremely unlikely to have identical MLST profiles (Feil and Spratt 2001) unless they are related ($p < 1$ in 10^8). The ancestral types represent clones

which are ecologically successful at the time of sampling. This may change dramatically if the area was sampled again a few years later. In this instance the variants may become the ancestoral types because of changes over time such as antibiotic use and the environment.

There are disadvantages with MLST at present. The method is expensive because it is sequence-based, is relatively labour-intensive, and is very prone to small errors in sequencing. Miscalling a single base out of over 3,000 sequenced for each isolate will lead to the isolate being given a different sequence type. For this reason it is essential that sufficient quality control measures are in place, and that the curators of MLST databases are resourced to properly scrutinise sequence traces belonging to novel alleles and STs.

3.7 Comparison of typing techniques

A number of methods were used to compare the discriminatory power and congruence of results for the 3 different methods in this epidemiological context.

3.7.1 Congruence of similarity matrices

The BioNumerics software package was used to generate similarity matrices for each method, based on comparing every pair of strains in the database. For PFGE the Dice coefficient was used to assess similarities between strains, Dice coefficient:

$$2*n/(a+b)$$

where a and b are the number of characteristics assessed for each of the two items and n is the number of characteristics both items have in common (Crossley and Archer, 1997)

For MLST and antibiograms the proportion of concordant loci or antibiotics was used. The matrices were compared using Pearson's correlation coefficient expressed as a percentage, and a secondary matrix of method-method comparisons generated. This can itself be subjected to UPGMA analysis and a dendrogram of methods produced (Figure 30 in

chapter 7 for the dendrogram including typing based on presence or absence of adhesin and toxin genes).

For the comparison of typing methods the congruence between MLST and PFGE was 59.1%, that between MLST and phage typing was 16.2% and between MLST and phage grouping 17.7%. The congruence of PFGE with phage typing was 2.5%, and with phage grouping was 14%. The congruence between phage typing and phage grouping was 44.5%, though it should be remembered that these last two measures are based on the same experiments.

3.7.2 Simpson's index of diversity

Simpson's index of diversity was used to compare the discriminatory power of MLST and PFGE, the two most widely used typing techniques for *S. aureus* (Grundmann, Hori et al. 2001). This examines the probability that any pair of strains drawn at random from the population will have different types; in a totally diverse population this will be 100%. The genetic diversity for MLST was compared to that for PFGE when the groups and subgroups were taken into account (Table 18). The non-overlapping confidence intervals indicate that PFGE is slightly, but significantly more diverse than MLST. This was as expected as MLST measures the variability in the backbone genetic structure of *S. aureus* whereas PFGE varies with changes in restriction sites or large insertions or deletions anywhere in the genome. These figures are similar to those found in community strains of *S. aureus* when MLST and PFGE were compared (H. Grundmann, personal communication).

	Overall (n=270)	Disease isolates (n=140)	Nasal carriage (n=130)
PFGE	96.4 (96.1 to 97.4)	94.9 (94.2 to 96.9)	95.9 (95.6 to 97.6)
MLST	94.4 (93.6 to 95.9)	93.9 (93.0 to 96.0)	93.1 (91.6 to 96.1)

Table 18: Simpson's Index of diversity for MLST and PFGE

It should be noted that Simpson's indices can only be compared if the sample sizes are roughly equal.

The genetic diversity of disease isolates were compared with that of controls for both MLST and PFGE (Table 18). The indices of diversity were similar between the two clinical groups for both PFGE and MLST, again suggesting that there is no major difference between the genetic population structures of disease and carriage isolates.

3.7.3 Comparing genotypes within genotypes

As a visual indicator of the degree of congruence between MLST and PFGE a histogram of the number PFGE genotypes within each MLST ST was plotted beside the number of MLST STs within each PFGE genotype (Figure 25). In addition pie charts were constructed to demonstrate the level of diversity assessed by one typing method within a single genotype measured by the other (Figures 26 and 27).

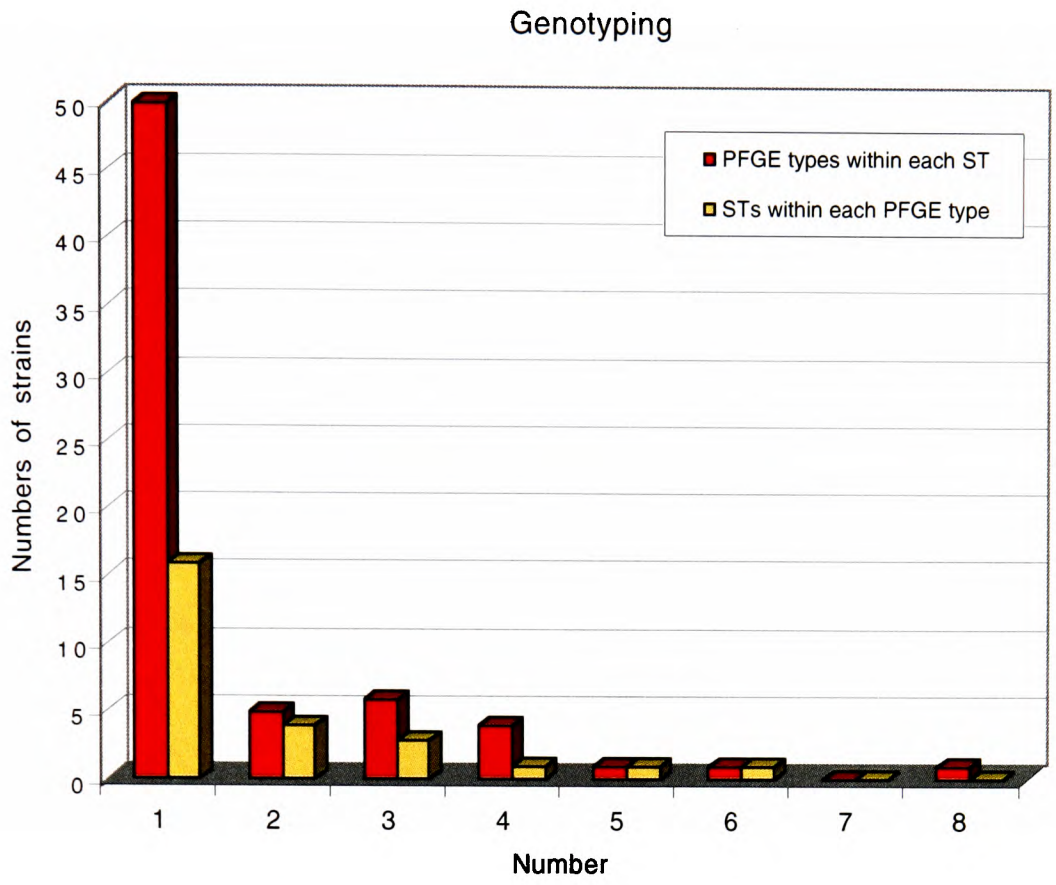


Figure 25: The number of PFGE genotypes found within MLST sequence types, and vice versa

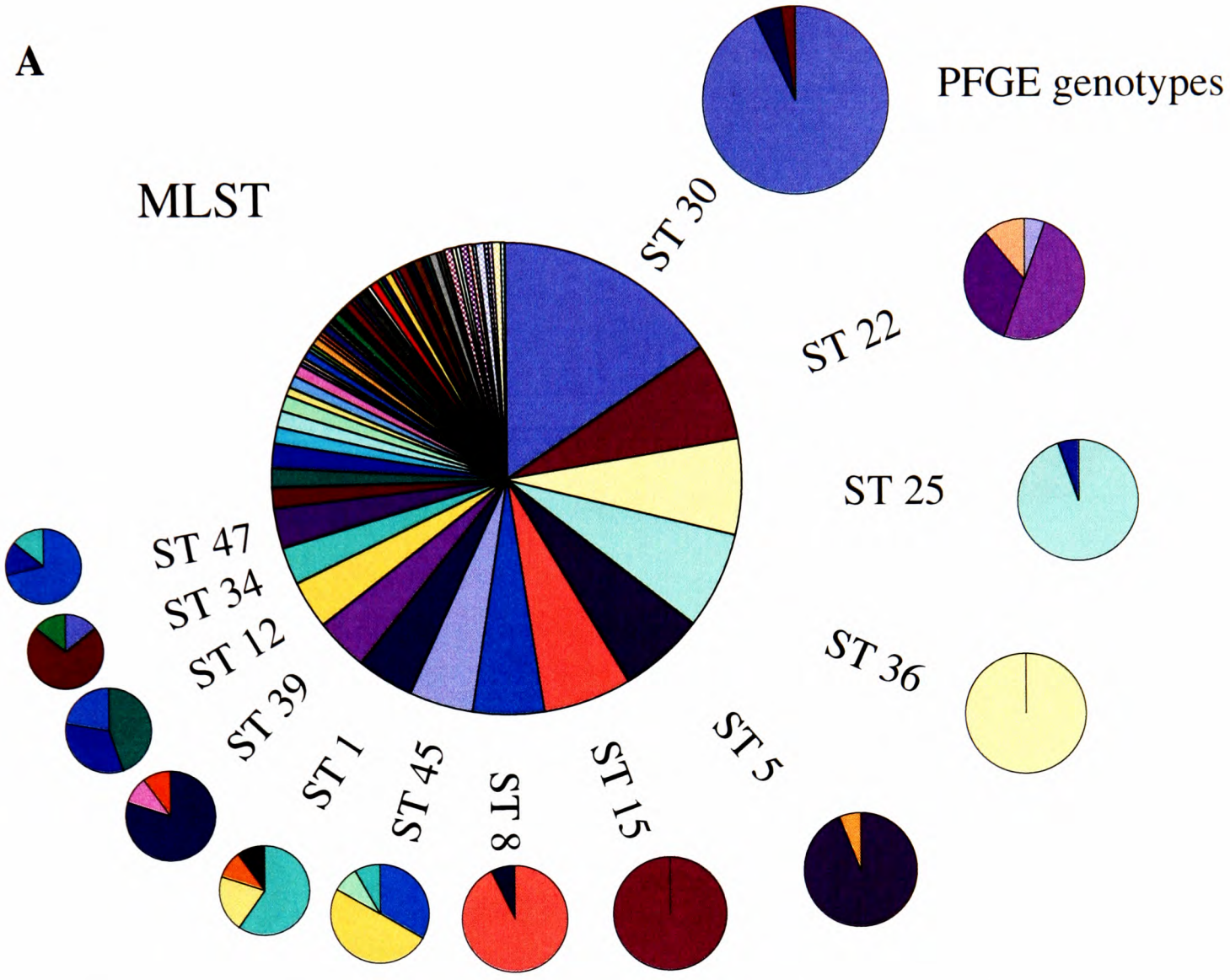


Figure 26: The relationship between MLST genotyping and PFGE genotyping

Figure 26 shows the numbers of MLST STs produced using MLST for the 245 isolates which underwent both MLST and PFGE. There are 13 MLST STs depicting PFGE genotypes containing more than one isolate. The larger pie sizes are equal to larger numbers of isolates. The smaller pie charts depict the MLST ST which contain more than one PFGE genotype where one colour is given for each PFGE genotype. Therefore, when a particular blue is shown in ST30 and ST34 it represents the same PFGE genotype (PG1).

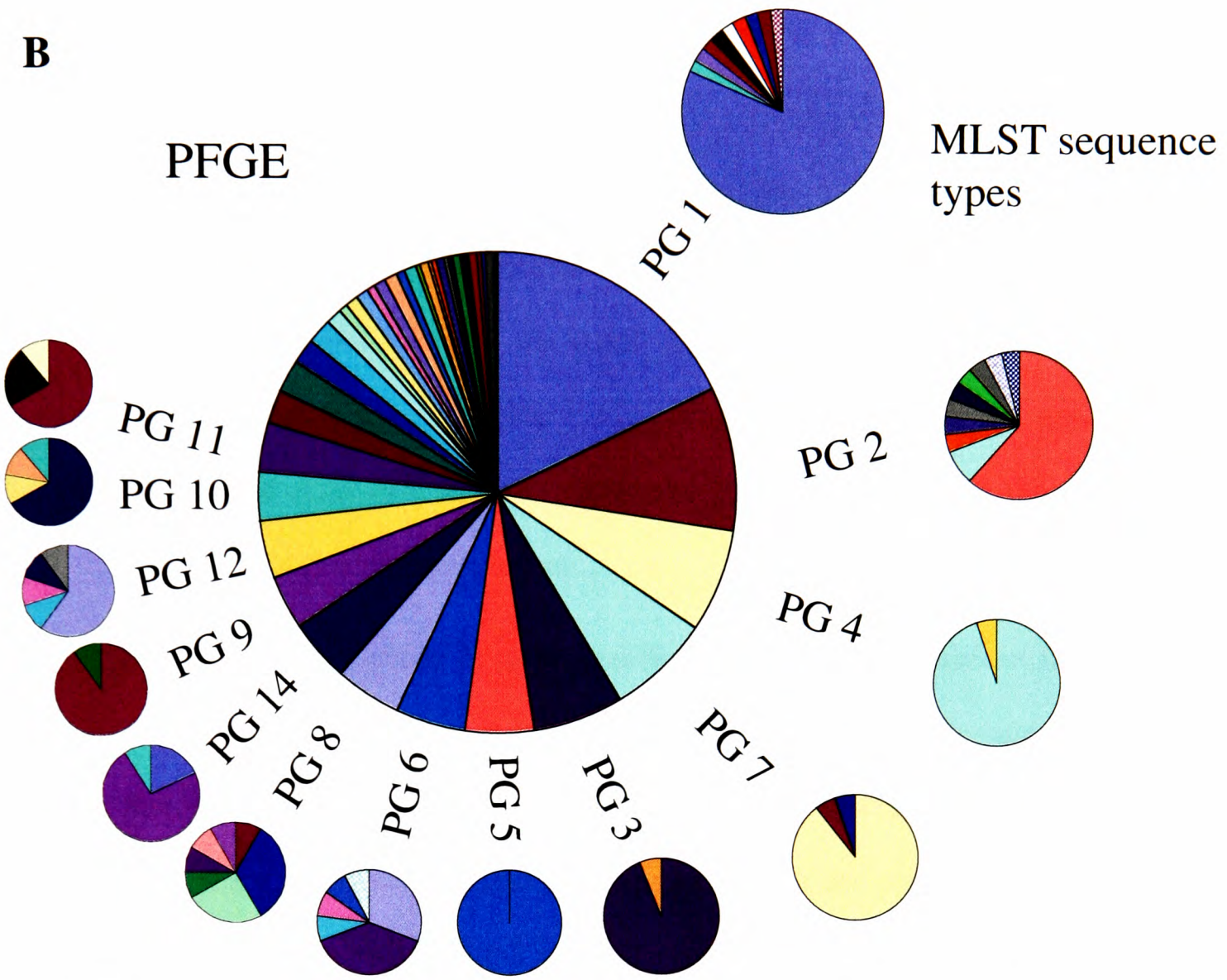


Figure 27: The relationship between PFGE genotyping and MLST genotyping

Figure 27 depicts the number of sequence types produced using PFGE on 245 isolates which underwent both PFGE and MLST typing. The larger pie chart depicts the number of PFGE genotypes produced, with the larger segments of pie depicting larger numbers of strains. The smaller pie charts around the larger one depict those PFGE genotypes which contain more than one strain and depict the different MLST STs assigned to strains with the same PFGE genotype. There are 13 small pie charts around the larger chart. The colours in the different small pie charts remain the same for specific MLST STs such that PG9 and PG11 both contain isolates defined as ST22 by MLST, depicted in purple.

3.8 Overall conclusions

i. Three different typing methods were used to examine the hypothesis that there may be hypervirulent *S. aureus* clones causing disease in the community and in hospital. Each method has different attributes, but none was able to identify major differences between the nasal carriage isolate population and the epidemiologically comparable CAD isolate population. Apart from the EMRSA-16 clone (ST 36) there was also no significant difference between the HAD and CAD isolate populations.

ii. Is it possible to conclude from this that there are no virulent clones of *S. aureus* capable of causing disease in individuals with relatively low host susceptibility factors? There is no evidence of major virulent clones that can be detected using these typing systems, but this does not exclude the possibility of virulent genotypes, particularly if the virulence determinants reside in the variable 'accessory' part of the genome.

iii. Comparisons of the typing systems demonstrated that the results of PFGE and MLST were the most congruent, with phage typing and antibiotic resistance testing yielding considerably more disparate results. Of MLST and PFGE the latter is more discriminatory that the former, and considerably less amenable to population genetic analysis

iv. Population genetic analysis using MLST showed that *S. aureus* isolates segregate into a small number of clonal complexes, within which diversification occurs predominantly through mutation rather than recombination.

Chapter 4. Putative virulence determinants

Using MLST and PFGE, no difference in underlying clonal structure was found between isolates causing HAD or CAD, and those associated with asymptomatic carriage in the community. This suggests that the 'backbone', essential structure of *S. aureus* differs little between carriage and disease. If this conclusion is sound, either there is no variation in disease occurrence attributable to bacterial factors, and *S. aureus* is purely an opportunistic pathogen; or those bacterial factors lie in the non-essential, accessory part of the genome. An obvious group of bacterial factors to consider are those termed virulence determinants. These factors are often found on accessory elements, such as plasmids or transposons, and, unless they are in LD with specific housekeeping genes, bacteria carrying them would not be identified using MLST. Conversely, bacterial genes may be incorrectly identified as associated with disease if they are in LD with a true virulence gene within an expanding clonal complex (termed hitchhiking).

Some virulence factors have been specifically associated with certain diseases. For example, TSST-1 is the cause of toxic shock syndrome (Edwin 1989); enterotoxins A and B cause staphylococcal scalded skin syndrome (Opal, Johnson-Winegar et al. 1988). Although some diseases have been associated with a single virulence determinant, it is unlikely that the majority of cases can be explained by one determinant alone. Different combinations of virulence factors may be associated with different diseases. This was suggested by work using isogenic mutant strains. Specific virulence factors such as collagen and clumping factor were removed; the strains were attenuated in virulence, but virulence was not abolished, suggesting dependence on other factors (Moreillon, Entenza et al. 1995; Hienz, Palma et al. 1996).

If these virulence factors are carried on accessory DNA, a microorganism will only continue to express the proteins while they remain beneficial to the organism. If the virulence factors become detrimental, for example in a different environment, the organism is likely to down regulate the proteins to aid survival.

To examine whether any particular virulence determinants, or combinations of virulence factors, were associated with severe *S. aureus* disease, 33 previously described bacterial virulence factors were studied in isolates from the case-control study which had already undergone MLST and PFGE. The presence or absence of genes coding for 29 of the proteins were examined using PCR, and the expression of the remaining 4 determinants were examined phenotypically. Details of the determinants examined are given in Table 19.

Bacterial determinant	Putative function	Method of detection
Adhesins		
FnBPA	Adhesin for fibronectin	PCR for *fnbA*
ClfA and ClfB	Adhesins for fibrinogen	PCR for *clfA* & *clfB*
Cna	Adhesin for collagen	PCR for *cna*
Protein A	Binds Fc domain of Ig and von Willibrand factor	PCR for *spa*
SdrC, SdrD, SdrE	Unknown; putative adhesins	PCR for *sdrC*, *sdrD*, & *sdrE*
Bbp	Adhesin for bone sialoprotein	PCR for *bbp*
EbpS	Adhesin for elastin	PCR for *ebpS*
Map/Eap	MHC class II analogue protein	PCR for *map/eap*
Toxins		
TSST-1	Exotoxin with superantigen activity	PCR for *tst*
Enterotoxins A, B, C, D, E, G, H, I, & J	Exotoxins with superantigen activity	PCR for *sea*, *seb*, etc.
Exfoliative toxins A & B	Exotoxins with superantigen activity	PCR for *eta* & *etb*
Alpha toxin	Cytolytic pore-forming toxin	lysis of rabbit erythrocytes
Beta toxin	Sphingomyelinase	hot-cold lysis of sheep erythrocytes
Delta toxin	Cytolytic toxin	Synergy of lysis between beta toxin producer and test isolate on washed sheep erythrocytes
Panton-Valentine leukocidin	Bicomponent leukocidin	PCR for *pvl*
Gamma toxin	Bicomponent leukocidin	PCR for *hlg*
Other factors		
ica locus	Polysaccharide intercellular adhesin	PCR for *icaA*
coagulase	Binds prothrombin, activating conversion of fibrinogen to fibrin	Coagulase test
Efb	Binds to fibrinogen	PCR for *efb*
V8 protease	Serine protease	PCR for *V8*
agr subgroup	Global regulator	PCR for *agr* subgroup I, II, III and IV

Table 19: The putative bacterial virulence determinants studied (Crossley and Archer 1997; Fischetti, Novick et al. 2000)

4.1.2 Methods

4.1.2.1 Toxins

The oligonucleotide primer sequences for PCR reactions for each of the toxin genes were kindly provided by Dr. Hazel Aucken from the Staphylococcal reference laboratory in Colindale, London (O'Neill, Dinerstein et al. 2000; McLauchlin, Narayanan et al. 2000). Three PCR multiplex reactions were performed on each strain. The PCR conditions and concentrations were slightly modified for optimal banding in the agarose gel. The primer mix for enterotoxins A to E contained 4pmol 16S rRNA, 15pmol *sea*, 5pmol *sed*, 15pmol *seb*, 15pmol *see*, 15pmol *sec*, this was made up to 100µl for direct use. The primer mix for enterotoxins G to J contained 4pmol 16S rRNA, 5pmol *seg*, 5pmol *seh*, 5pmol *sei* and 7pmol *sej* made up to a 100µl volume. The third set of multiplex primers contained primers for the exfoliative toxins and TSST-1 with 5pmol 16S rRNA, 10pmol *eta*, 5pmol *etb*, 7pmol *tst* which was made up to 100µl.

The PCR reactions were identical for each multiplex reaction using a 15µl reaction mixture containing approximately 50ng/µl genomic DNA, 1x PCR buffer (Gibco BRL, Life Sciences, Ca.), 1.5mM $MgCl_2$ (Gibco BRL), 1.5mM dNTP mix (Gibco BRL), 0.4µM (one of the three) multiplex PCR mix and 0.125 Units of Taq DNA polymerase (Gibco). A positive control mixture was also used in each PCR reaction. This consisted of a 100µl reaction with 4µl genomic DNA for each primer in the mixture (see Appendix B3 for positive controls). The PCR cycles used for each multiplex reaction were identical (Appendix B4). The full PCR reaction for each strain was run in a 3% agarose gel for 2 hours with a 100bp Superladder (Abgene, Surrey). The gels contained 50mg/µl ethidium bromide and were examined under UV fluorescence. The resulting bands for each multiplex PCR reaction are shown in Table 20.

Enterotoxins A to E	Band size (bp)	Enterotoxins G to J	Band size (bp)	Exfoliative toxins and TSST-1	Band size (bp)
seb	477	*sei*	465	*tst*	415
sed	319	*seh*	360	*etb*	262
sec	271	*seg*	327	16S rRNA	228
16S rRNA	228	16S rRNA	228	*eta*	119
see	178	*sej*	142		
sea	127				

Table 20: The banding sizes of the multiplex PCR reactions for the toxins

4.1.2.2 Adhesins and other proteins

The PCR reactions for these putative virulence factors were performed separately. Dr. Sharon Peacock designed the primers either using those already published (Boden Wastfelt and Flock 1995; Lina, Piemont et al. 1999) or using GenBank sequences (*map/eap*). Dr. Peacock designed the *agr* primers in or around the region within *agrD* encoding the autoinducing peptide and two separate primers for *sdrE* and *bbp*, although examination of the sequences from clinical strains 252 and 476 (http://www.sanger.ac.uk/Projects/S_aureus/) suggested they were allelic variants of the same locus (Peacock et al. In Press).

The reactions were performed in 15µl volumes: 50ng/µl of genomic DNA, 1x PCR buffer (Bioline, London), 4.8mM dNTPs (Bioline) 0.2µM primers and 0.2 U/µl Taq (Bioline). The Magnesium concentration was variable with 1.5mM $MgCl_2$ (Bioline) used for *fnbA*, *fnbB*, *cna*, *spa*, *sdrC*, *sdrD* and *efb*; 3mM $MgCl_2$ (Bioline) for *clfA*, *clfB*, *sdrE*, *bbp*, *map/eap*, *V8*, *agr*I, *agr*II, *agr*III and *agr*IV; 4 $MgCl_2$ (Bioline) for *icaA* and *ebpS*.

The candidate performed the PCR reactions on all of the toxins, *fnbA*, *fnbB*, *clfA*, *clfB*, *cna* and *spa*. Dr. S. Peacock performed the PCR reactions for *fbpA*, V8, *sdrC*, *sdrD* and *sdrE* and Mrs. A. Justice performed the PCR reactions on *spa*, *ebps*, *map/eap*, *icaA*, *fib* (*efb*) and the *agr* locus (*agr*I, *agr*II, *agr*III and *agr*IV).

Four of the toxins were examined phenotypically by Dr. Maria Kantzanou. Coagulase production was tested by emulsifying 6 colonies into 2ml of peptone buffered saline containing 200 µl of human plasma. The bottles were incubated at 37°C for 4 hours then left at room temperature over night. Each bottle was observed for the presence of a clot. The haemolysins were examined phenotypically on different agars. For α toxin, isolates were streaked onto 5% rabbit blood agar and incubated at 37°C for 18 hours. The NCTC 5655 strain was used as a positive control. Test strains were recorded as positive if haemolysis occurred. The presence of β toxin was examined on 5% sheep blood which was incubated at 37°C for 18 hours then 4°C for 30 minutes. The positive control was NCTC 7428, test strains were recorded as positive if haemolysis occurred. The presence γ toxin was identified using 3% washed sheep erythrocytes where synergy between the test isolate and a β toxin producer gave a positive result. The positive control used for γ toxin production was NCTC 9715.

4.1.2.3 Analysis

The case-control study design helps to avoid the potential confounding effect of comparing isolates from different geographical locations or time periods, and having defined the population structure of these isolates it was possible to adjust for the effects of clonality on associations between bacterial determinants and invasive disease. For example, a given determinant could become over-represented in the invasive group if it is linked to a 'true' virulence gene within a given lineage. In the primary analysis isolates were evaluated according to origin (nasal carriage isolate or invasive), and further sub-divided depending on whether the disease was hospital- or community-acquired. The second round of analysis used isolates grouped by clonal complex as defined by MLST results and analysis repeated for associations within each complex.

Contingency tables were used to compare proportions between groups. To adjust for the 'hitchhiker effect', analyses were stratified by MLST-defined clonal complex using either the Mantel-Haenszel method or conditional logistic regression. In this way the presence of particular determinant(s) were only compared between disease and carriage isolates within clonal complexes, and these separate effects summated to give an overall odds ratio. Strains that were unrelated to any other at 5 of 7 loci were arbitrarily grouped as a 'clonal complex' for the purpose of this stratification. No corrections were made for multiple comparisons, though in an attempt to recognise this potential problem statistical significance was set at p=0.01 for the purpose of interpretation and discussion. For the same reason conservative 99% confidence intervals were used throughout.

4.1.3 Results

4.1.3.1 Univariate analyses

Each virulence determinant was examined separately using univariate analysis to see if there was any difference between disease (with HAD and CAD grouped together) compared with carriage. The HAD and CAD groups were also compared to the NC strains separately; the percentage did not change, but the power decreased because of the lower numbers. Out of the 33 virulence determinants tested, 8 determinants were more common in disease compared with carriage. These determinants were the adhesins *fnbA*, *cna* and *sdrE*; the toxins *sea*, *sej*, *eta* and *hlg* and the biofilm producer *icaA* (Table 21).

After Mantel-Haenszel stratification for clonality all eight of the associated determinants remained significantly associated with disease. To exclude any bias, the largest single clone, which contained 22 exclusively hospital-acquired EMRSA-16 isolates (CC36) was removed. After removal, only 7 of the genes remained significantly associated with disease. The virulence determinant *sea* was significantly associated with the EMRSA-16

clone CC36, this was only seen in hospital acquired strains so was not associated with disease (Table 21).

The 7 genes which were significantly associated with disease were reassessed comparing the NC strains first to CAD strains then to HAD strains. When the CAD strains were compared to NC, *hlg* became non significant. This result was due purely to a lack of power because the numbers were too low. There was a difference between CAD and NC strains, but this did not reach significance.

	Unstratified analysis				Stratified analysis	
	Carriage n=178 (%)	**Invasive n=155 (%)**	**Odds ratio (99% CI)**	**p**	**Odds ratio * (99% CI)**	**p***
Adhesins						
fnbA	154 (87)	152 (98)	7.9 (1.7 to 77)	**0.0001**	12.0 (1.6 to 89)	**0.0001**
clfA	174 (98)	155 (100)		0.13		0.13
clfB	178 (100)	155 (100)		1		
cna	57 (32)	81 (52)	2.3 (1.3 to 4.3)	**0.0002**	2.0 (1.1 to 3.7)	**0.003**
spa	160 (90)	145 (94)	1.6 (0.54 to 5.4)	0.24	1.7 (0.55 to 5.5)	0.20
sdrC	178 (100)	156 (100)	-	1		
sdrD	73 (41)	74 (48)	1.3 (0.71 to 2.3)	0.27	1.9 (0.8 to 4.4)	0.05
sdrE	72 (40)	87 (56)	1.9 (1.0 to 3.4)	**0.006**	3.1 (1.4 to 6.7)	**0.0001**
bbp	76 (43)	59 (38)	0.82 (0.45 to 1.5)	0.43	0.74 (0.37 to 1.5)	0.27
ebpS	110 (62)	105 (68)	1.3 (0.70 to 2.4)	0.30	1.5 (0.65 to 3.4)	0.20
map/eap	165 (93)	149 (96)	2.0 (0.50 to 9.4)	0.24	1.7 (0.42 to 6.5)	0.34
Toxins						
tst	44 (25)	46 (30)	1.3 (0.68 to 2.6)	0.27	1.2 (046 to 2.9)	0.68
sea	30 (17)	48 (32)	2.3 (1.1 to 4.7)	**0.003**	2.6 (1.0 to 6.6)	**0.007**
seb	13 (7)	14 (9)	1.3 (0.41 to 3.9)	0.72	0.79 (0.22 to 2.8)	0.33
sec	20 (11)	15 (10)	0.84 (0.30 to 2.2)	0.72	0.85 (0.32 to 2.3)	0.66
sed	9 (5)	8 (5)	1.0 (0.24 to 4.2)	1	1.2 (0.34 to 4.4)	0.68
see	0	0	-	1		
seg	113 (64)	85 (55)	0.69 (0.38 to 1.3)	0.12	0.42 (0.2 to 0.9)	0.003
seh	18 (10)	24 (15)	1.6 (0.65 to 4.1)	0.19	1.4 (0.63 to 3.3)	0.25
sei	106 (60)	81 (52)	1 (0.40 to 1.3)	0.18	0.50 (0.24 to 1.0)	0.01
sej	12 (7)	38 (25)	4.5 (1.8 to 13)	**<0.001**	4.4 (1.7 to 11)	**<0.0001**
eta	11 (6)	34 (22)	4.2 (1.6 to 13)	**<0.001**	6.3 (2.0 to 20)	**<0.0001**
etb	6 (3)	0 (0)		0.03		0.06
pvl	3 (2)	6 (4)	2.3 (0.31 to 27)	0.31	2.7 (0.38 to 19)	0.18
hlg	157 (89)	150 (97)	3.6 (0.97 to 19)	**0.01**	4.5 (1.0 to 19)	**0.004**
α toxin	178 (100)	153 (99)		0.21		0.15
β toxin	160 (89)	122 (79)	0.45 (0.18 to 1.0)	0.02	0.43 (0.17 to 1.1)	0.01
δ toxin	144 (80)	106 (69)	0.54 (0.27 to 1.1)	0.02	0.55 (0.27 to 1.1)	0.03
Others						
ica	136 (77)	143 (92)	3.5 (1.4 to 9.8)	**<0.0001**	3.5 (1.4 to 9.1)	**0.0002**
V8	168 (94)	149 (96)	1.5 (0.34 to 7.5)	0.61	1.8 (0.42 to 7.9)	0.28
efb	105 (60)	105 (68)	1.5 (0.79 to 2.7)	0.11	1.7 (0.86 to 3.4)	0.03
Coagulase	175 (99)	155 (100)		1		0.30
agr				0.64		0.21
agr I	65 (37)	53 (34)	0.91 (0.57 to 1.5)	1	0.43 (0.15 to 1.2)	0.10
agr II	43 (24)	34 (22)	0.89 (0.51 to 1.5)	0.70	3.3 (1.0 to 11)	0.04
agr III	65 (37)	65 (42)	1.3 (0.80 to 2.0)	0.31	0.78 (0.22 to 2.8)	0.70
agr IV	5 (3)	2 (1)	0.46 (0.04 to 2.8)	0.46	0.79 (0.13 to 14)	0.79

Table 21: Association of virulence determinants with disease using univariate analysis and adjusting for clonal complexes using Mantel-Haenszel stratification

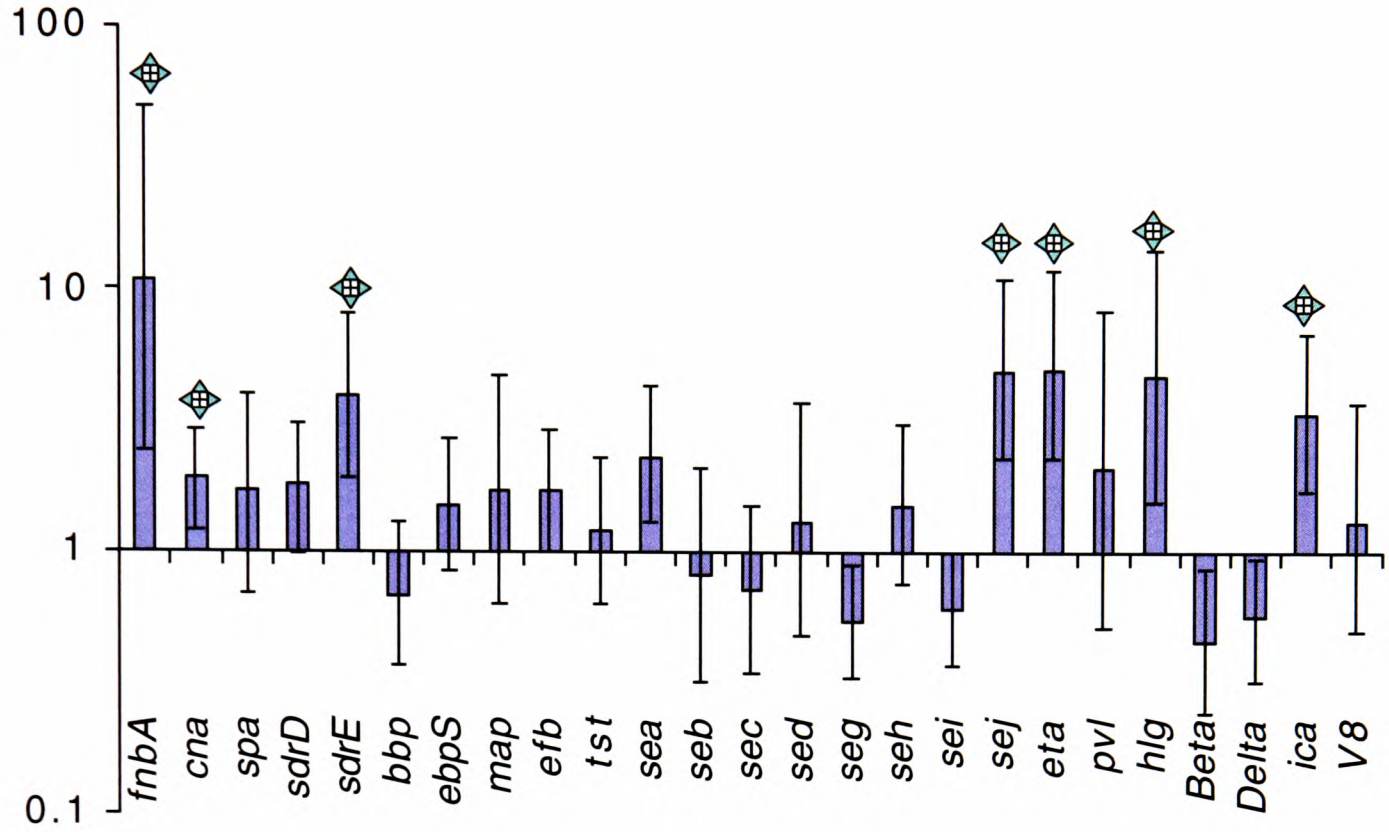


Figure 28: Presence or absence of putative virulence determinants

Figure 28 shows the odds ratios for disease, adjusted for effects of clonality by Mantel-Haenszel stratification of MLST-defined clonal complex. Error bars denote 99% confidence intervals, and a star indicates a significant association with disease ($p<0.01$). Genes that were either ubiquitous (such as *clfA* and *clfB*) or very rare/absent (*see* and *etb*) are not shown.

The genes were each examined for the presence of clonality where specific determinants might be seen linked to a specific MLST or PFGE clone. All isolates contained *clfB* and *sdrC*, but none contained *see*. These genes were therefore excluded from the calculations. Two groups emerged. The first group were associated with defined clonal complexes; these were *tst*, *eta*, *etb*, β toxin, γ toxin, *sdrD*, *sdrE*, *bbp*, *cna*, *ebpS* and *efb* ($p<0.001$ in all cases). The second group of determinants were not related to the clonal structure; these

were *fnbA*, *clfA*, *coa*, *spa*, *map*, *pvl*, *hlg*, α toxin, *icaA* and *V8* (p>0.1 in all cases). *Tst* was closely associated to clonal groups CC30 and CC39 which contain EMRSA-16 isolates. The Agr groups were found to be extremely tightly linked to the clonal structure (Table 22).

Clonal complex	*agr* I	*agr* II	*agr* III	*agr* IV	Total
CC30	2	2	**90** (96%)	0	94
CC39	0	0	**24** (100%)	0	24
CC15	0	**31** (100%)	0	0	31
CC45	**28** (93%)	0	1	1	30
CC22	**25** (100%)	0	0	0	25
CC25	**23** (100%)	0	0	0	23
CC5	3	**18** (86%)	0	0	21
CC1	3	0	**13** (81%)	0	16
CC8	**16** (100%)	0	0	0	16
CC12	0	**11** (100%)	0	0	11
CC51	1	3	1	**5** (50%)	10

Table 22: Background clonality and *agr* subgroups

The percentages in Table 22 represent the percentage of organisms within that clonal complex. The clonal complexes relate to those denoted by MLST (see Figure 21)

It was impossible to determine how close each of the determinants were to each other on the *S. aureus* genome. It is possible that many of these determinants are found in close proximity on the genome. If this is true, some of the virulence determinants could be in LD with others. This would link a determinant to disease although it is not actually causing disease.

4.1.3.2 Multivariate analyses

For this reason, all 7 genes which were independently associated with disease were included in a multivariate logistic regression analysis to examine the 7 genes. *Sej*, *fnbA*, *cna* and *sdrE* were found to be independent to each of the other genes; therefore these genes are significantly and independently associated with disease. Both *icaA* and *hlg* were

found to be in LD with *fnbA*. However each gene contributed significantly to disease on further modelling. *Eta* and *sej* were also in LD, although these genes also both contributed individually to disease. In conclusion, all of the genes were found to be independently significantly associated with disease. This does not exclude the presence of unknown genes which could be in LD with any of the candidate genes examined.

Because each of these genes were individually found to be associated with disease, it was possible that there was a cumulative effect of the numbers of these genes present. In essence; was an organism more likely to cause disease the more virulence factors it contained. On examination of this, the more virulence determinants a strain possesses, the more likely it is to cause disease (Figure 28), ($p<0.0001$). The virulence factors which are not individually associated with disease were tested for their ability to contribute to virulence when associated with other virulence factors. None of these determinants had any affect on disease.

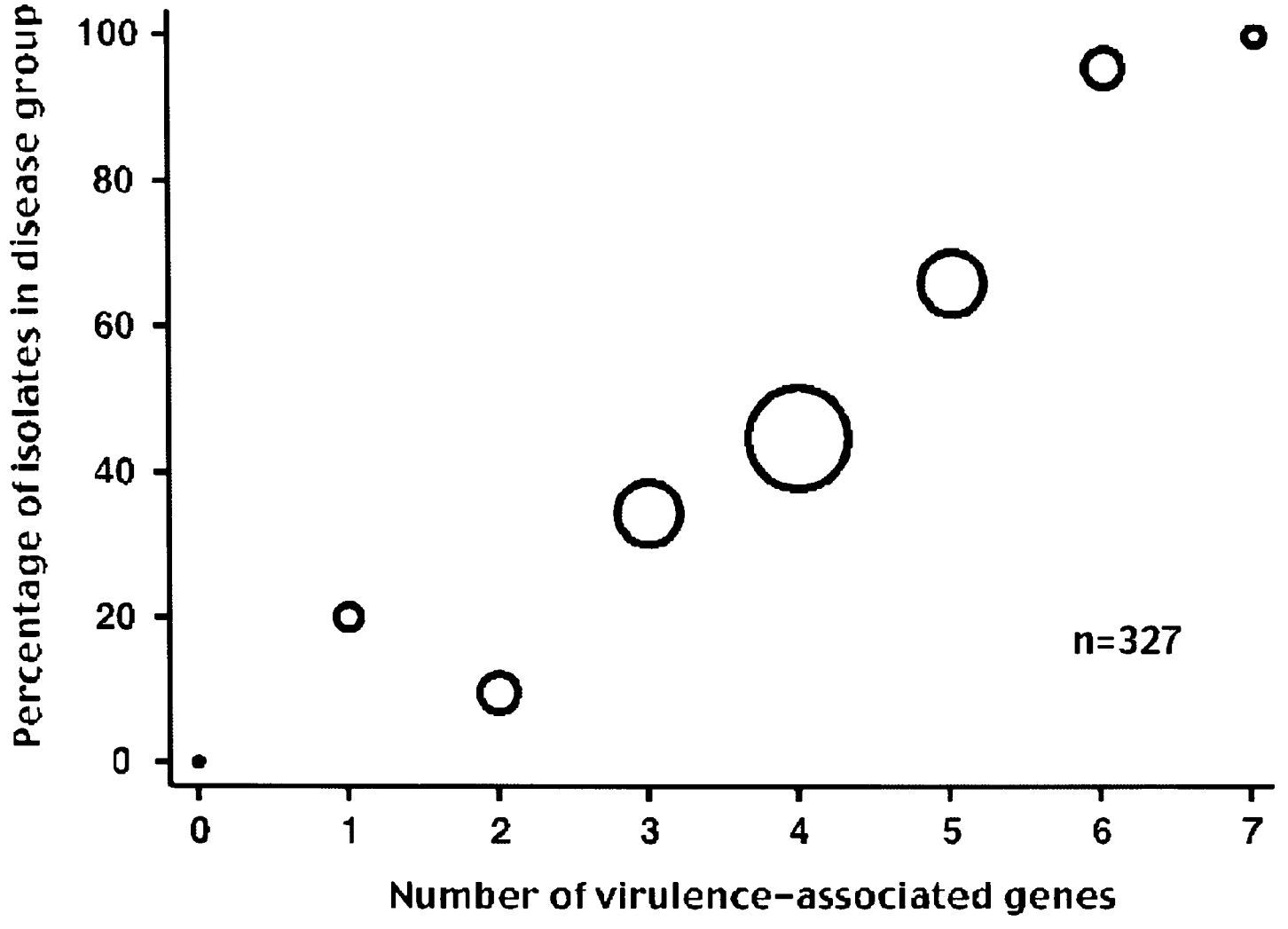


Figure 29: Association between number of virulence-associated determinants and the proportion of isolates from cases of disease (rather than carriage)

In Figure 29, 20% of isolates with one virulence-associated gene were from cases of disease and 80% were carriage strains. The area of each circle is proportional to the number of isolates with that number of determinants (going from zero genes to seven genes, n=2, 15, 21, 64, 140, 56, 22, and 7 respectively).

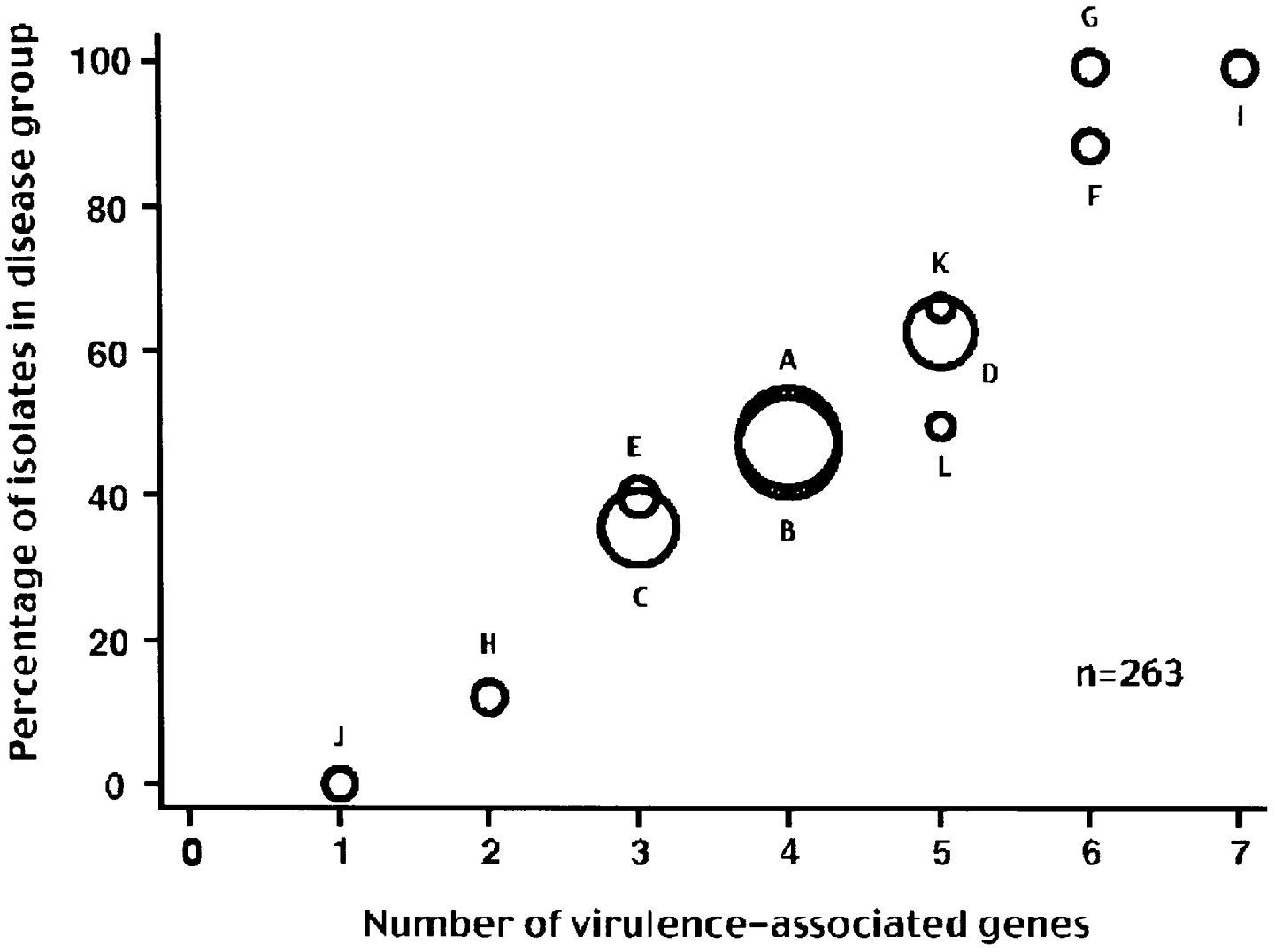


Figure 30: Association between number of virulence-associated determinants in a gene combination and the proportion of isolates with that combination from cases of disease (rather than carriage)

Each combination in Figure 30 was identified by a letter; for the actual gene complement see Table 23. The 12 commonest combinations representing 80% of isolates are shown. The area of each circle is proportional to the number of isolates in that gene combination (n for each gene combination is given in table 23).

4.1.3.3 Haplotype analyses

The results were examined to find whether any particular combinations of these genes (haplotypes) were more likely to be associated with disease. With 7 virulence determinants, there was a total of 128 possible haplotypes. Only 46 different haplotypes occurred in the study, possibly due in part to the LD which was present. Twelve of these 46 haplotypes were presented by 80.4% of the strains (table 23).

Comb.	*fnbA*	*cna*	*sdrE*	*sej*	*eta*	*hlg*	*icaA*	N	Number	% of total	Cum	Dis O	Dis P	Car O	Ca P
A	1	0	1	0	0	1	1	4	64	19.6	19.6	20.0	13.8	19.2	14.2
B	1	1	0	0	0	1	1	4	64	19.6	39.1	19.4*	11.7	18.6*	10.0
C	1	0	0	0	0	1	1	3	39	11.9	51.1	9.0	10.8	14.5	21.3
D	1	1	1	0	0	1	1	5	35	10.7	61.8	14.2	14.9	7.6	6.7
E	1	0	1	0	0	1	0	3	10	3.1	64.8	2.6	1.2	3.5	4.2
F	1	0	1	1	1	1	1	6	9	2.8	67.6	5.2*	1.3	0.6	0.1
G	1	1	0	1	1	1	1	6	8	2.4	70.0	5.2*	1.1	0.0	0.0
H	1	0	0	0	0	1	0	2	8	2.4	72.5	0.6	0.9	4.1*	6.4
I	1	1	1	1	1	1	1	7	7	2.1	74.6	4.5	1.4	0.0	0.0
J	0	0	0	0	0	1	0	1	7	2.1	76.8	0.0	0.0	4.1	0.9
K	1	0	1	1	0	1	1	5	6	1.8	78.6	2.6	4.6	1.2	1.1
L	1	0	1	0	1	1	1	5	6	1.8	80.4	1.9	3.9	1.7	0.9

Table 23: The most common haplotypes

In table 23, Comb is the letter assigned to the different combinations of virulence determinants. N is the number of virulence-associated determinants within the gene combination (of 7). Number is the number of isolates. Cum is the cumulative percent of total; Dis O is the disease observed (%); Dis P is the disease predicted (%); Car O is the carriage observed (%) and Car P is the carriage predicted (%).

The numbers were examined, then mathematically the expected percent of each haplotype (if each observation was independent) was calculated. These expected frequencies were compared with the observed frequencies to see if there was any difference in the specific

haplotypes which occurred (Table 23). There was an increased percentage of haplotype B (*fnbA*, *cna*, *hlg* and *icaA*), haplotype F (*fnbA*, *sdrE*, *sej*, *eta*, *hlg* and *icaA*) and haplotype G (*fnbA*, *cna*, *sej*, *hlg* and *icaA*) in the disease strains. There was also an increased percentage of haplotype B (*fnbA*, *cna*, *hlg* and *icaA*) and haplotype J (*hlg*) in the nasal carriage strains. These results show a larger number of haplotype B than expected in both carriage and disease. There was also an increased number of haplotypes F and G in the disease strains: each of these haplotypes contain 6 virulence genes, coincidentally there was a very low percentage of haplotype F in the carriage strains and no haplotype G in the carriage strains. There was an increased percentage of haplotype J in the carriage strains; this haplotype contained a single virulence factor, this haplotype was not observed in the disease strains.

4.1.3.4 Virulence determinants and clonality

The reason that haplotype B was so numerous in carriage and disease was because this haplotype was present in 51% of the isolates found in CC30 and CC39, it was only found at a rate of 6% in the other clonal complexes. Clonality, was not the only reason. This was seen when the EMRSA-16 strains in CC36 were examined. If the virulence determinants were associated only with clonal expansion, the haplotypes within this clone would be expected to be identical. This was not true for the 22 strains in CC36. A total of 10 different haplotypes were found, these contained a variety of virulence determinants ranging from 1 to 7 virulence genes. Examination of patterns of the virulence determinants across other clonal complexes revealed a similar pattern, suggesting that horizontal transfer of virulence genes occurs across the clonal complexes (Figure 31).

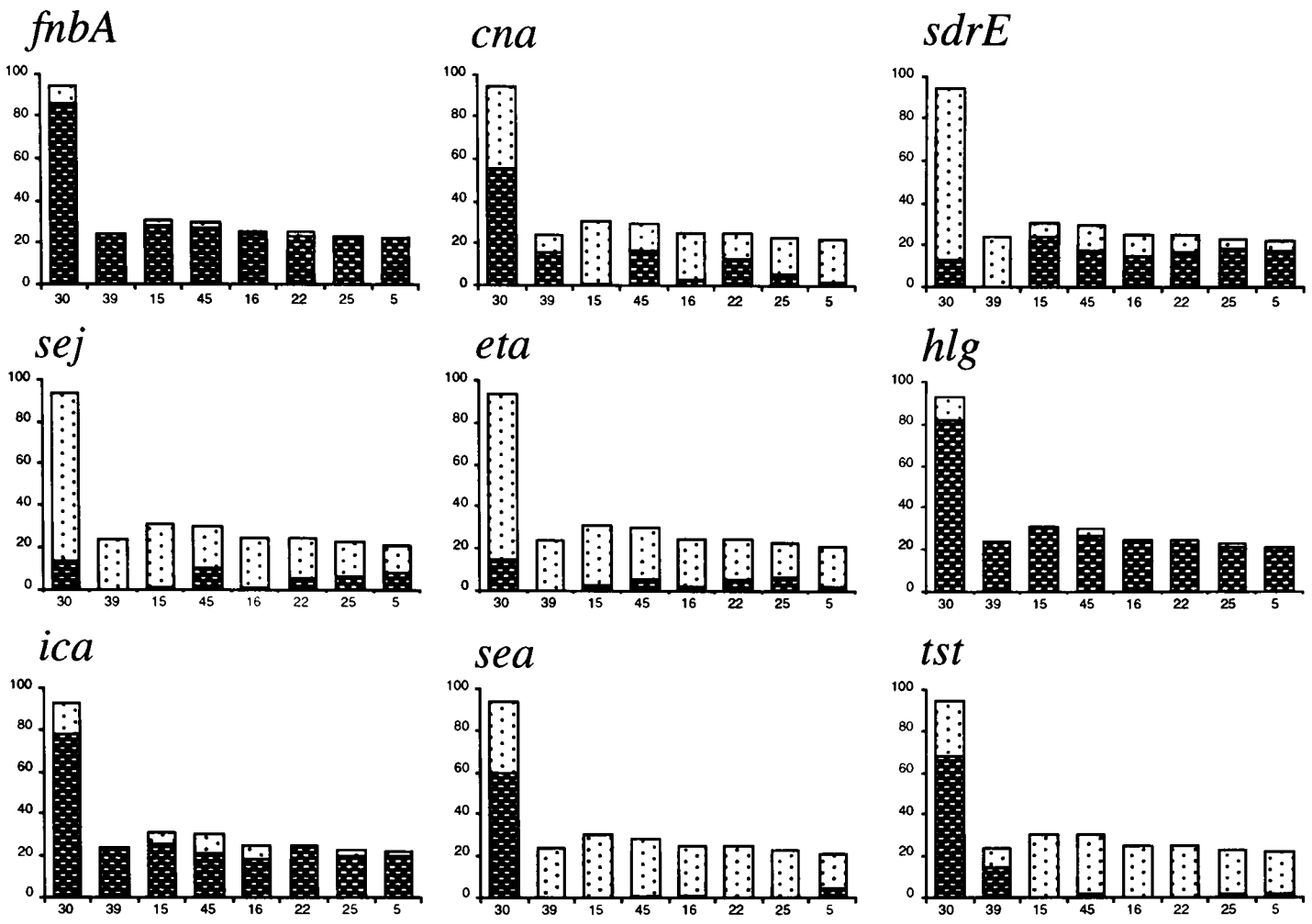


Figure 31: Distribution of genes within the 7 largest MLST-defined clonal complexes (denoted by numbers along the x axis)

In figure 31, the overall height of each bar represents the total number of isolates in the complex. The height of the dark-shaded area represents the number of isolates positive for the determinant.

4.1.3.5 Congruence of virulence genotyping with other typing techniques

As a method of assessing the overall association with clonality of the putative virulence determinants studied in this chapter, congruence with phage typing, PFGE and MLST results described in Chapter 3 was calculated. Similarity matrices were constructed for superantigen toxins, membrane toxins and adhesins subgroups, and the analysis was performed in BioNumerics (Figure 32).

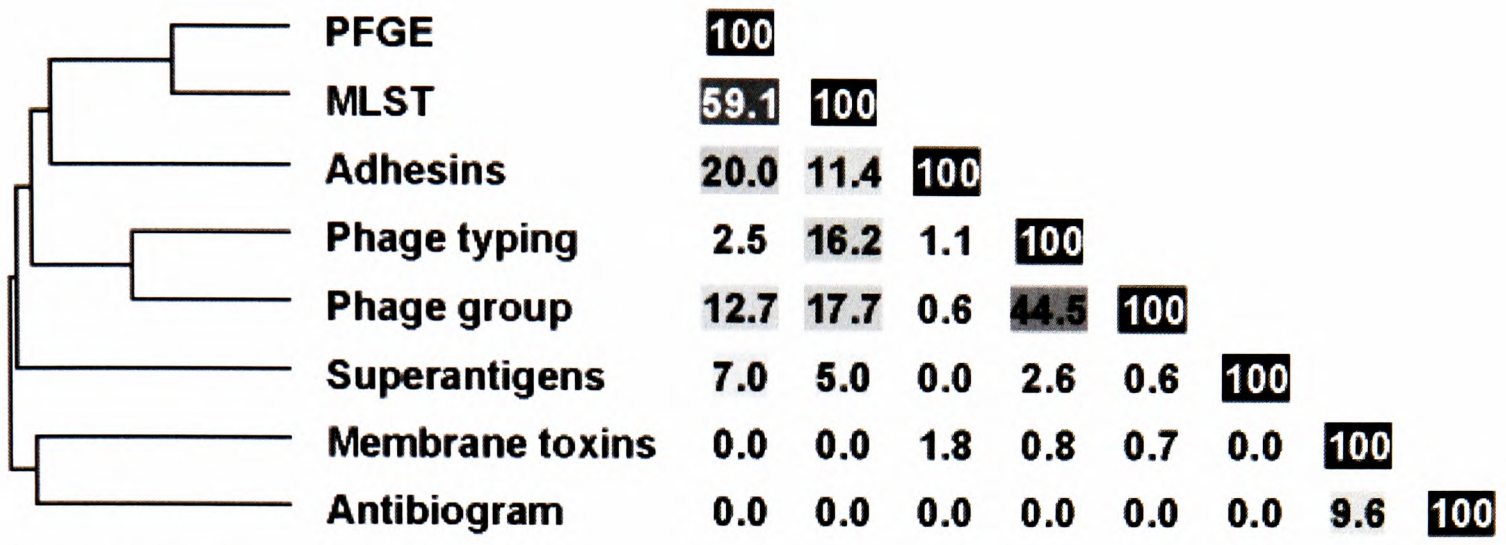


Figure 32: Congruence between all bacterial typing and genotyping methods

Figure 32 depicts all of the strains which were compared using antibiogram testing, phage testing, PFGE, MLST and were examined for the presence of toxins and adhesins. The toxins and adhesins were split according to the groups in which they were described in the introduction. Numbers in the matrix represent Pearson's correlation coefficient expressed as a percentage. The dendrogram was calculated from the congruence matrix using UPGMA.

The results demonstrate that the adhesins were the most clonally related group of virulence determinants overall. They were more congruent with PFGE than with MLST, this is consistent with PFGE covering the genome more completely than MLST. The superantigens were less congruent with the genetic typing schemes, and the membrane toxins segregated at the non-clonally related antibiogram end of the spectrum.

4.1.4 Conclusions

i. After adjusting for the effect of clonality, 7 of 33 putative virulence determinants (*fnbA, cna, sdrE, sej, eta, hlg* and *ica*) were significantly more common in invasive isolates than nasal carriage isolates. All seven factors contributed independently to virulence.

ii. No combinations of the 7 genes were either more or less likely to cause disease than others with the same number of virulence-associated genes.

iii. There was evidence of considerable horizontal transfer of genes on a background of clonality.

iv. *Agr* subtypes were distributed amongst clonal complexes in a clonal fashion, suggesting that they are very conserved and possibly very ancient features of *S. aureus* population biology.

The advantage of PCR reactions, especially when multiplexed is speed. The current disadvantage when examining putative virulence determinants in *S. aureus* is that only a few *S. aureus* sequences are available. It is known that allelic differences do occur in the virulence determinants. For example *sdrE* and *bbp* appear to be functionally different allelic variants of the same gene. *sdrE* was associated with virulence whereas *bbp* was not, though this may have been a function of the relative abundance of *sdrE*. Some of the genes such as many of the exfoliative toxins (e.g. *seb*, *sed*, *see* and *seh*) may have different allelic variants which therefore appeared negative in the reactions. Many of the reactions were performed at low annealing temperatures to decrease the specificity. It would depend how many bases changed between the primer and the gene and where the changes occurred with respect to the primer whether the primers annealed or not. This work therefore is able to suggest possible associations with 7 of the virulence determinants examined.

The results suggest that there is very little difference between the frequencies of the virulence factors found in strains causing HAD and those causing CAD. The numbers were small at 61 and 94 respectively therefore more strains would need to be examined to confirm these results. If this lack of difference between CAD and HAD isolates is upheld, it might suggest that bacterial factors are important in causing disease irrespective of the host. Many of the patients within the hospital would have implanted devices and they are likely to be immuno compromised to some extent. It would be interesting to examine this hypothesis in a group of patients who are severely immuno compromised such as HIV patients or renal dialysis patients who have indwelling catheters and are often infected with *S. aureus* strains.

This study has been largely assumption free, though the bacterial virulence factors examined were chosen because they had been previously thought to contribute to *S. aureus* virulence. This may well have increased the chance of positive associations.

The roles that the significant virulence factors (*fnbA*, *cna*, *sdrE*, *sea*, *sej*, *eta* and *hlg*) have during infections are largely unclear. It is possible for each of these factors to build a hypothesis about how their know functions may contribute to virulence, but their actual roles at present remain largely undetermined.

This study has found that seven out of the 33 virulence determinants examined are associated with disease. The effect of these genes are cumulative so that the more virulence genes (out of the 7 significant) which are present, the more likely an organism is to cause disease. This work is far from exhaustive as: i) only 33 determinants were examined and ii) the potential effects of allelic differences at each locus has not been explored. Also there are many as yet uncharacterised virulence genes present in the *S. aureus* genome; the sequencing project performed in Japan found 70 candidates for new

virulence determinants (Kuroda, Ohta et al. 2001). Microarray projects examining thousands of ORFs across hundreds of isolates are likely to be carried out in the near future.

Although a relatively large number of virulence determinants were examined, the possibility remains that the 7 determinants found to be associated with disease may not be the causative determinants. The true determinants may be unknown at present and in LD with those identified through this work. However the existence of virulent bacterial genotypes suggests that bacterial factors do play a role in determining the occurrence of severe disease.

Chapter 5. Host genetic susceptibility to severe *S. aureus* disease

5.1 Introduction

5.1.1 Study design

For two reasons a genetic case-control association study is an appropriate means of studying host genetic susceptibility to severe *S. aureus* disease. Firstly it is generally more powerful than linkage studies for finding the genetic determinants of complex disease (Risch and Merikangas 1996), and may be more powerful than family-based tests of association (Morton and Collins 1998). Secondly it is by far the most realistic approach given the relative scarcity of disease and the average patient age; twin studies, family linkage studies and family-based association studies are all impractical or logistically difficult.

Case ascertainment was carried out in the microbiology laboratory for both the Oxford and Thailand studies. Patients with severe *S. aureus* disease meeting the case definition were identified, and a blood sample obtained from which DNA was extracted. Two sets of DNA controls were collected for the Oxford study: one from the blood donors from which the bacterial carriage controls were isolated; the other umbilical cord blood from neonates born at the John Radcliffe Hospital. The cord blood DNA set was used for the Oxford host genetic susceptibility studies, as this was felt to provide the least biased and more representative sampling of the Oxfordshire population. For the Thai study control DNA was obtained from healthy Thais screened at Siriraj hospital prior to work abroad. The controls from the North East of Thailand were collected from patients attending outpatient clinics excluding individuals with *S. aureus* disease. 307 controls were collected in total.

Ethnicity is potentially a major confounder in any genetic case-control association study. As many allele frequencies differ between ethnic groups it was very important to have only one ethnic group in each study. For this reason, non-Caucasians were excluded from the Oxford study and non-ethnic Thais from the Thai study.

5.1.2 Choice of candidate genes

Candidate genes were selected on the basis *a priori* biological plausibility of a major role in protection against *S. aureus* infection, combined with previous *in vitro* or *in vivo* evidence that polymorphisms in the gene are associated with an altered response to *S. aureus*. Four genes were selected on this basis: those coding for the Fc gamma receptors IIA and IIIB, mannose binding lectin, and Toll-like receptor 2.

5.2 Fc Receptors

5.2.1 Biological importance

The Fc receptors (FcRs) are found on the surface of PMN, and play a major role in defence against bacteria (van der Pol and van de Winkel 1998). FcRs recognise and bind to the Fc portion of antibodies. Antibody-FcR complexes then act as receptors for antigen, and so are an important link between cellular and humoral immunity. Each antibody class has its own specific receptors (e.g. Fc gamma receptors (FcγRs) for IgG). FcγRs are expressed on the surface of PMNs, and bind indirectly to the microorganism as a result of receptor cooperation and binding to IgG joined to multivalent antigens or the interaction with preformed immune complexes. This triggers effector mechanisms such as phagocytosis, endocytosis, antibody-dependent cell mediated cytotoxicity, the release of inflammatory mediators and clearance of immune complexes (van de Winkel and Capel 1993; Rascu, Repp et al. 1997; Kato, Sautes-Fridman et al. 2000).

There are 3 forms of FcγR expressed on the surface of PMN: FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16). Synthesis of FcγRI is only induced on neutrophils by IFNγ (van de Winkel and Capel 1993). FcγRI is the only FcγR able to bind to monomeric IgG with high affinity (via its 3 Ig-like extracellular domains) (Daeron 1997). Both FcγRII and FcγRIII are constitutively expressed on the surface of PMN, each have 2 rather than 3 Ig-like extracellular domains; they can only bind to IgG as part of an immune complex or when many IgG molecules are bound to opsonised microorganisms (Daeron 1997).

5.2.2 FcγR isoforms

FcγRs belong to the immunoglobulin supergene family expressed on human leukocytes. Studies based on sequence redundancy, intron-exon organisation and structural organisation show that all of the FcγR genes probably originate from a single gene, with subsequent diversification arising through gene duplication, divergence and recombination. The FcγRs are divided into 3 classes (FcγRI, FcγRII and FcγRIII) based on their structure, primary amino acid sequences and ligand binding affinities (Zheng, Nibbering et al. 1994). The three separate classes comprise 9 membrane-associated and 3 soluble FcγR molecules which are encoded by 8 genes. Differences between the genes occur in the transmembrane or cytoplasmic regions (Daeron 1997) where the receptors either have 2 (FcγRII and FcγRIII) or 3 (FcγRI) Ig-like extracellular domains able to bind the Fc portion of IgG (Daeron 1997).

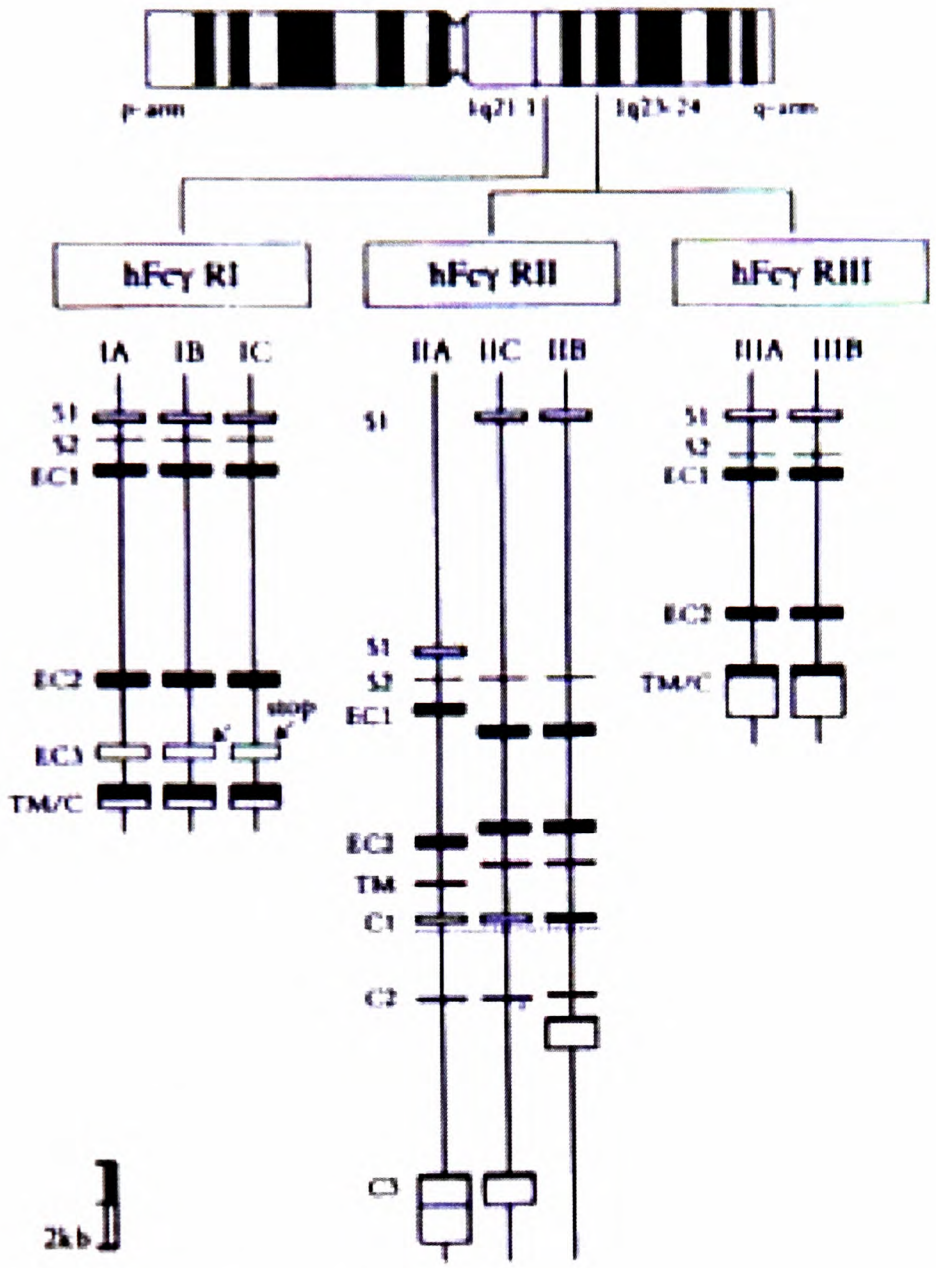


Figure 33: The FcγR genes (Ravetch and Kinet 1991)

Figure 33 shows that FcγRI and FcγRII have 3 genes each (IA, IB, IC, IIA, IIB and IIC), and FcγRIII has 2 genes (IIIA and IIIB).

All of the receptor genes colocalise on chromosome 1 (1q21-23) (Ravetch and Kinet 1991; McKenzie and Schreiber 1994; Daeron 1997). The receptors are split into 2 groups: activators such as FcγRIIA and FcγRIIIA with an immunoreceptor tyrosine-based activation motif (ITAM), and inhibitors such as FcγRIIB which have an immunoreceptor tyrosine-based inhibitory motif (ITIM).

The FcγR receptors expressed on the surface of PMN are FcγRIIA, FcγRIIC and FcγRIIIB.

5.2.3 FcγRIIA - Structure and function

There are 3 homologous FcγRII genes encoding 6 isoforms generated through alternate splicing. All of the molecules have similar extracellular regions of approximately 180 amino acids. They contain 2 Ig-like domains, a 27-29 amino acid transmembrane domain and cytoplasmic domains varying from 44 to 76 amino acids (van de Winkel and Anderson 1991).

The most widely distributed FcγRII molecule is FcγRIIA, which is a 40kDa integral membrane glycoprotein expressed on most haematopoietic cells including neutrophils, macrophages, eosinophils, myeloid cell lines, platelets and B cells (Fossati, Bucknall et al. 2001). They are low affinity IgG receptors which bind with high affinity to antibodies complexed to multivalent antigens (Unkeless 1989; Daeron 1997). FcγRIIA binds IgG1 with the highest affinity, followed in order of decreasing affinity by IgG2, IgG4 then IgG3 (Rascu, Repp et al. 1997).

FcγRIIA resembles a single chain chimera, with an intracytoplasmic domain containing one ITAM found within the receptor (Reth 1989; Daeron 1997; Cooney, Phee et al. 2001). This activation motif is composed of a twice repeated YxxL sequence (where x denotes any amino acid) flanking 12 variable residues (Daeron 1997).

ITAMs trigger one of 3 biological activities depending on the cell: activation, endocytosis and phagocytosis (Daeron 1997). When the FcγRIIA ITAM is phosphorylated it activates a set of cytoplasmic protein tyrosine kinases thus creating a potential binding site for *src* protein tyrosine kinases (Worth, Mayo-Bond et al. 1996). Another family of protein tyrosine kinases called *syk* associate and are activated with the phosphorylated ITAM. Further steps in this signalling process have yet to be clarified, but phagocytosis of

opsonised bacteria is dependent on the ITAM molecules found in FcγRIIA (Deo, Graziano et al. 1997; Rascu Repp et al. 1997; Chuang, Sassaroli et al. 2000).

All of the processes involved in phagocytosis are interlinked. Crosslinking of FcγRs leads to the breakdown of phosphoinositol (4,5)-biphosphate. This results in the activation of protein kinase C (Hamada, Aoki et al. 1993). Inositol (1,4,5)-triphosphate is formed and this increases cytosolic free calcium concentrations and forms diacylglycerol. This is an endogenous activator of protein kinase C (Zheng, Nibbering et al. 1994). Protein tyrosine kinases and phospholipase C are essential for the FcγR-stimulated oxygen-dependent intracellular killing of *S. aureus* (Zheng, Nibbering et al. 1994; Zheng, Zomerdijk et al. 1995).

5.2.4 FcγRIIIB - Structure and function

FcγRIII molecules range from 50 to 70 kDa in size. They are encoded on chromosome 1 at position 1q22 (Fridman, Bonnerot et al. 1992; van de Winkel and Capel 1993). FcγRIII are expressed on PMN, natural killer cells, T cells, eosinophils and tissue macrophages. The receptors bind weakly to monovalent IgG, but have a higher affinity for complexed IgG and are able to bind immune complexes.

Two forms of FcγIII exist: FcγRIIIA and FcγRIIIB. These are expressed in a cell-type specific way, with FcγRIIIA expressed on natural killer cells and FcγRIIIB selectively expressed on granulocytes (PMN or eosinophils) as a glycosyl phosphatidylinositol-linked (GPI) receptor (Gessner, Grussenmeyer et al. 1995). The FcγRIIIA and FcγRIIIB receptors are homologous, differing by only 9 nucleotides in their coding regions. The important difference between the 2 molecules is found at amino acid position 203; in FcγRIIIB this is serine followed by a stop codon, whereas in FcγRIIIA amino acid 203 is a phenylalanine

followed by a transmembrane domain and a 25 amino acid cytoplasmic domain (missing in FcγRIIIB) (Ory, Clark et al. 1989).

The specific action of FcγRIIIB remains unknown. The receptor may only act to enhance immune complex binding for presentation to FcγRIIA (Chuang, Sassaroli et al. 2000), this has been suggested because, unlike FcγRIIA, FcγRIIIB molecules do not have an ITAM molecule through which to signal and cause cell activation. When expressed alone on the surface of transfected Jurkat cells FcγIIIB failed to trigger any activation signal (Wirthmueller, Kurosaki et al. 1992). On the other hand, FcγRIIIB molecules are able to trigger calcium responses, degranulation and oxidative burst when aggregated on neutrophils (Unkeless, Shen et al. 1995; Chuang, Sassaroli et al. 2000).

It is possible that FcγRIIIB and FcγRIIA, both found on the surface of neutrophils, act as co-receptors. As well as the biological plausibility of this hypothesis, there is also supporting experimental evidence. Schubert *et al.* (Schubert, Hundt et al. 1992) found that crosslinking between FcγRIIIB and FcγRIIA resulted in a higher response in terms of H_2O_2 production and cytoplasmic calcium mobilisation compared to crosslinking of FcγRIIA alone. Unkeless *et al.* (Unkeless 1989) suggested that FcγRIIA and FcγRIIIB act in synergy during cell activation and that FcγRIIIB is able to use the signalling apparatus (ITAM) of FcγRIIA. Another study showed that co-crosslinking of the two receptors produced a synergistic enhancement of intra-calcium concentration over the level observed by cross-linking of FcγRIIA alone (Chuang, Sassaroli et al. 2000).

5.2.5 The polymorphisms

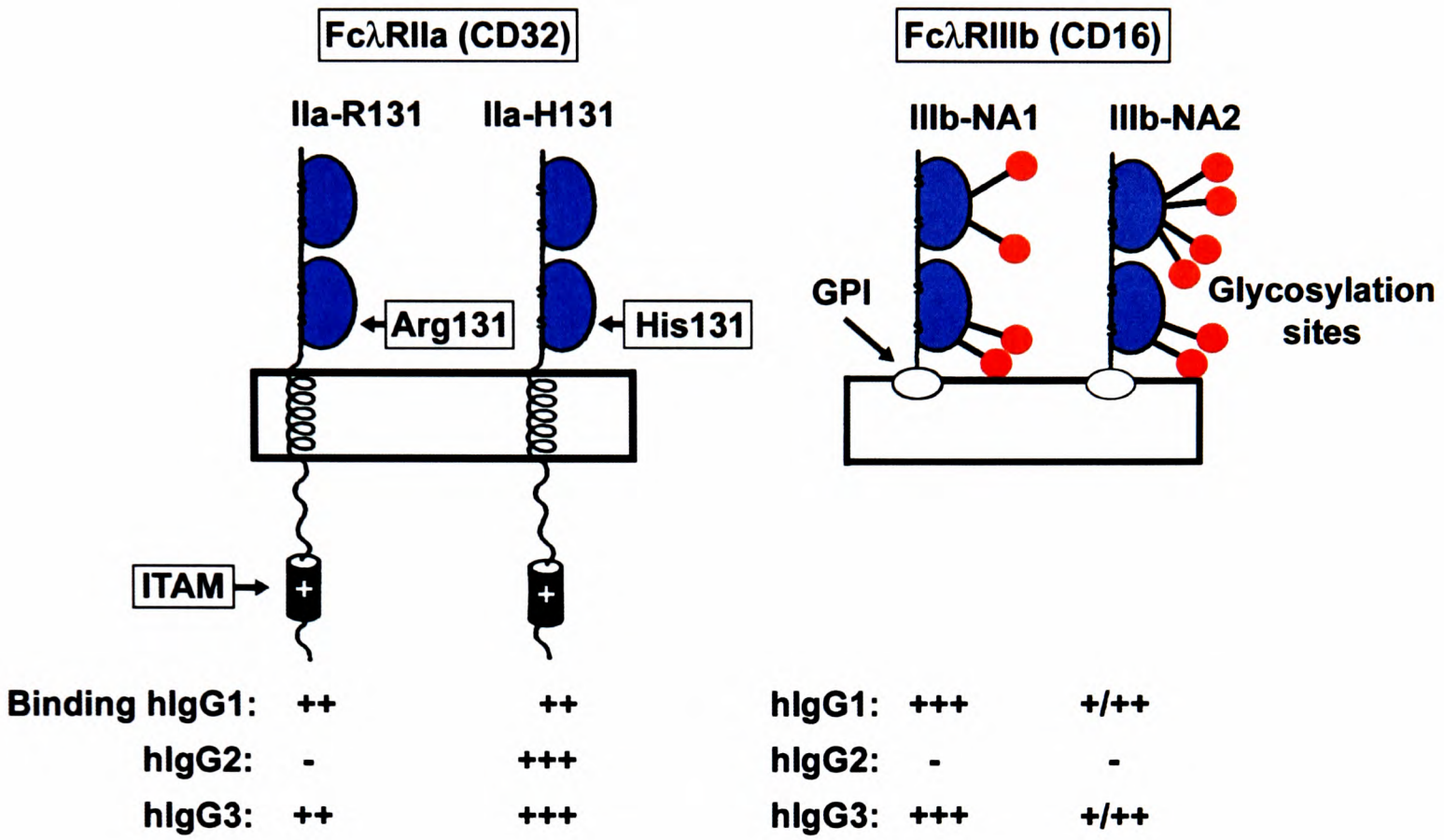


Figure 34: Diagrammatic representation of FcγRIIA and FcγRIIIB, indicating the positions of the studied polymorphisms

In figure 34; hIgG1, hIgG2, hIgG3 are human IgG1, human IgG2 and human IgG3 respectively. The alleles in the FcγRIIA polymorphism are arginine at amino acid position 131 (R131) or histidine at amino acid 131 (H131). There are 5 polymorphic sites in FcγRIIIB with two allelic forms, NA1 and NA2. These differ in numbers of glycosylation sites (shown as red circles) with 4 in NA1 and 6 in NA2. The differences in binding affinities to IgG associated with each polymorphism are demonstrated by negative and positive signs.

Both the FcγIIA and FcγRIIIB receptors have biallelic polymorphisms which affect the binding or ingestion of antigens opsonised with different IgG subclasses. Only FcγRIIA is able to bind effectively to IgG2 (Fossati, Bucknall et al. 2001). There are two bialleleic

single nucleotide polymorphisms in the gene encoding FcγRIIA, generated by base pair substitutions found at amino acid positions 27 and 131. The differential binding to IgG2 is associated with the polymorphism at position 131 (Warmerdam, van de Winkel et al. 1990). This polymorphism was first described by Tax *et al.* (Tax, Hermes et al. 1984) using anti-CD3-induced T cell proliferation assays together with murine IgG1 antibodies. Approximately 70% of Caucasians had a strong reaction with mIgG1 so were termed high responders. 30% had a weak reactivity with mIgG1 so were termed low responders (Warmerdam, van de Winkel et al. 1991; Parren, Warmerdam et al. 1992). Monoclonal antibodies were also used to effectively distinguish the two groups before the advent of sequence-based genetic techniques (van de Winkel and Capel 1993; Rascu, Repp et al. 1997). Clark *et al.* (Clark, Clarkson et al. 1989) showed that the polymorphism at amino acid position 131 was responsible for the high and low responder functional responses. The polymorphism is located in the external domain of the second IgG-like domain in FcγRIIA (van der Pol and van de Winkel 1998), and consists of a single base pair change (G to A) corresponding to a change from Arginine to Histidine. The 2 forms are termed H131 (Histidine at position 131; previously termed low responder or mutant) and R131 (Arginine at position 131; previously termed high responder or wild type) (Osborne, Chacko et al. 1994; Lehrnbecher, Foster et al. 1999). Neutrophils expressing the allotype H131 bind IgG2 and IgG3 more effectively than those expressing R131 (Warmerdam, van de Winkel et al. 1991; Parren, Warmerdam et al. 1992; Salmon, Edberg et al. 1992; Bredius, Fijen et al. 1994). A functional difference has also been observed in neutrophil phagocytosis assays: those expressing H131 homozygous receptors phagocytose IgG2-opsonised bacteria more effectively than those expressing the R131 homozygous receptors (Bredius, Fijen et al. 1994; Sanders, van de Winkel et al. 1994).

FcγRIIIB molecules express 2 allotypic variants known as Neutrophil antigens 1 and 2 (NA1 and NA2) (Ory, Clark et al. 1989; Ory, Goldstein et al. 1989; Huizinga, Kuijpers et al. 1990; Bux, Stein et al. 1997). These are also known as HNA-1a and HNA-1b respectively (Bux 2001). The biallelic polymorphism was first detected during the analysis of the alloimmune and autoimmune neutropenias and blood transfusion reactions (Lalezari and Bernard 1966; van de Winkel and Capel 1993; Rascu, Repp et al. 1997). The NA1 and NA2 polymorphisms differ by 5 amino acids (nts 141, 147, 227, 277 and 349) within exon 3. These SNPs change four of the amino acids in the membrane-distal immunoglobulin-like domain (Yamamoto, Sugita et al. 2001). The two functionally important amino acid changes are at positions 65 and 82 (nucleotide sites 227 and 349); these lead to two extra glycosylation sites in NA2 (6 compared to 4 in NA1). The resulting proteins are 65-80 kDa and 50-65 kDa in size respectively (Bux, Stein et al. 1995).

These glycosylation site differences influence the interaction between FcγRIIIB and IgG molecules. PMNs homozygous for NA1 are able to phagocytose IgG-opsonised antigens more effectively than PMNs homozygous for NA2 (Salmon, Brogle et al. 1991). The NA1 allotype demonstrates enhanced binding to IgG1 and IgG3 compared with the NA2 allotype (Bredius, Fijen et al. 1994, van Schie and Wilson 2000). Both variants of the FcγRIIIB receptor are unable to bind either IgG2 or IgG4 (Huizinga, Kuijpers et al. 1990).

5.2.6 The polymorphisms and disease

Since the first observation suggesting that the IgG2 subclass dominates the immune response to encapsulated bacteria (Siber, Schur et al. 1980), much attention has been focussed on the role of FcγRs on the pathogenesis of a variety of infectious diseases. Because of their importance in immune complex clearance FcγRs are thought to play a central role in autoimmune diseases (Duits, Bootsma et al. 1995). FcγR polymorphisms

have been studied in complement deficiencies, systemic lupus erythematosus (SLE), Wegner's Granulomatosis (WG) and Chronic Granulomatous disease (CGD), using a wide range of methodologies with conflicting and contradictory results (Trikalinos, Karassa et al. 2001).

The FcγRIIA H/R polymorphism has been shown to influence the opsonisation of *Neisseria meningitidis* serogroup B and *Streptococcus agalactiae* (Bredius, Derkx et al. 1994; Sanders, Feldman et al. 1995). Individuals who have the putatively protective H131 allele follow a relatively more benign clinical course in meningococcal and pneumococcal diseases (Platonov, Kuijper et al. 1998; Platonov, Shipulin et al. 1998; Yee, Phan et al. 2000). The NA1/NA2 polymorphism in FcγRIIIB influences the binding of PMN to IgG1-mediated phagocytosed *N. meningitidis* serogroup B (Bredius, Fijen et al. 1994).

Reports are conflicting as to whether FcγRIIA and FcγRIIIB act independently or synergistically. Some studies suggest that they act independently in terms of disease associations, with the FcγRIIA polymorphism being the most important (Salmon, Edberg et al. 1990; Sanders, van de Winkel et al. 1994). Other workers have found a significantly increased number of patients with both the putatively susceptible FcγRIIA allele (R131) and putatively susceptible FcγRIIIB (NA2) allele (Fijen, Bredius et al. 1993; Fijen, Bredius et al. 2000).

5.2.7 Importance of the polymorphisms in *S. aureus* infections

The most compelling evidence suggesting a role for both of FcγRIIA and FcγRIIIB in *S. aureus* infections was published by Bredius *et al.* (Bredius, de Vries et al. 1993; Bredius, Derkx et al. 1994; Bredius, Fijen et al. 1994). Using neutrophil cell lines they showed that PMNs with the H131/H131 homozygous genotype of FcγRIIA were able to phagocytose IgG1 or IgG2 opsonised *S. aureus* Wood strain (STAW), which has no capsule, more

efficiently than either H131/R131 heterozygous PMNs (intermediate binding) or R131/R131 homozygous PMNs (no binding) (Bredius, Fijen et al. 1994). This was consistent with previous studies in which the putatively protective H131/H131 genotype was higher in frequency in the controls compared to cases in recurrent bacterial respiratory tract infections, and in infections with encapsulated bacteria such as *Neisseria meningitidis*; conversely individuals with R131/R131 receptors were relatively more susceptible to disease (Sanders, van de Winkel et al. 1994; Fijen, Bredius et al. 2000).

The FcγRIIIB NA1/NA2 polymorphism has also been functionally associated with efficiency to phagocytose *S. aureus*. Individuals with PMNs expressing FcγRIIIB NA1/NA1 are more efficient at phagocytosing *S. aureus* opsonised with IgG1 or IgG3 than PMNs expressing NA1/NA2 or NA2/NA2 receptors (Bredius, Fijen et al. 1994).

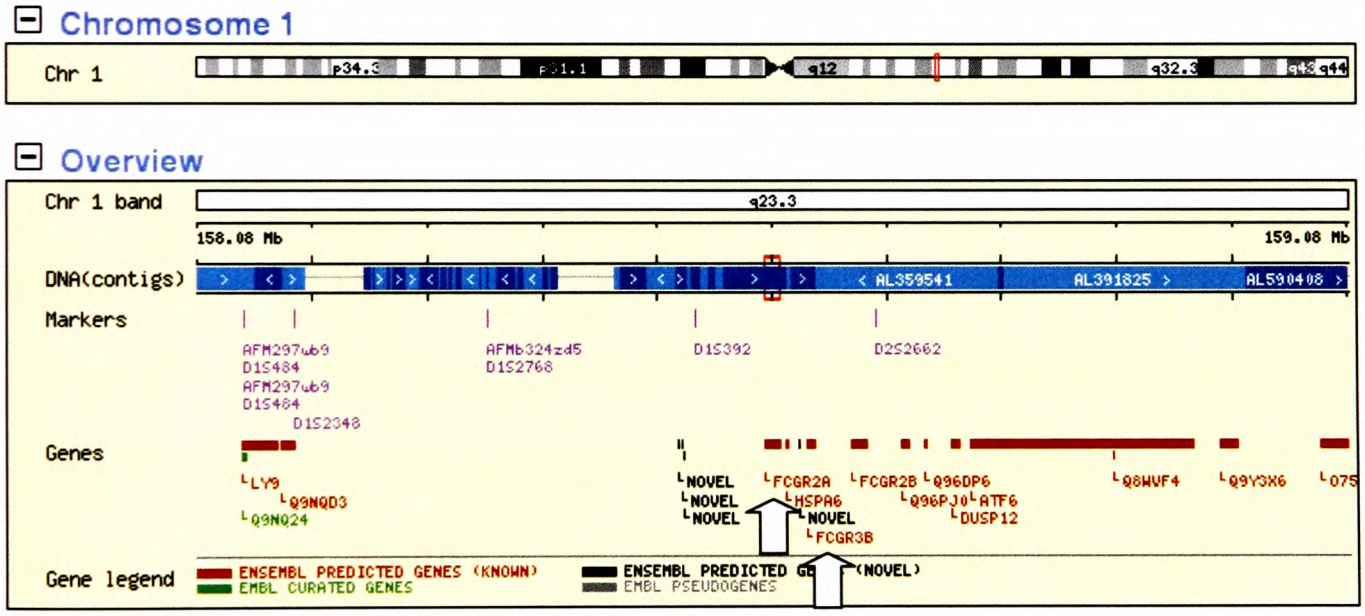


Figure 35: Chromosomal position of FcγRIIA and FcγRIIIB

Figure 35 shows both the FcγRIIA and FcγRIIIB in close proximity on chromosome 1 (within approximately 300kb on the last ENSEMBL update) raising the possibility of LD between the 2 loci. Other possible candidate genes such as CRP and CR1 are also found in close proximity on chromosome 1.

5.2.8 Methods

A slightly modified version of the allele-specific PCR described by Flesch *et al.* (Flesch, Bauer et al. 1998) was used to genotype the Oxford cases and controls and the collection from Thailand for the FcγRIIA H/R polymorphism (see Appendix D1). The method can distinguish between the homologous FcγRIIA/B/C genes as the common primer was specific for FcγRIIA, containing an area of the FcγRIIA downstream intron where the sequences between the three receptors diverge.

The reactions were performed in 25μL volumes containing 50ng of genomic DNA, 1x PCR buffer, 2mM $MgCl_2$, 0.32mM dNTPs and 1unit AmpliTaq Gold DNA polymerase (Perkin Elmer). 6pmol of the common reverse primer together with either 6pmol FcγRIIA-H or 6pmol of the FcγRIIA-R primer was used in each reaction. The internal control PCR reaction used primers specific for the surfactant gene SPA6a (SPA-1); 2pmol of the forward and 2pmol of the reverse primers were used for each reaction. See Appendix D2 for PCR conditions. The products were examined on a 1.5% agarose gel stained with 50mg/μl ethidium bromide under UV fluorescence. These primers resulted in positive controls at 442bp (SPA-1) together with an allele specific band for H or R at 253bp (FcγRIIA). Two primer sets were used for each sample, one containing the FcγRIIA-H and one containing the FcγRIIA-R set. Bands were recorded as homozygous for H, heterozygous or homozygous for R (Figure 36).

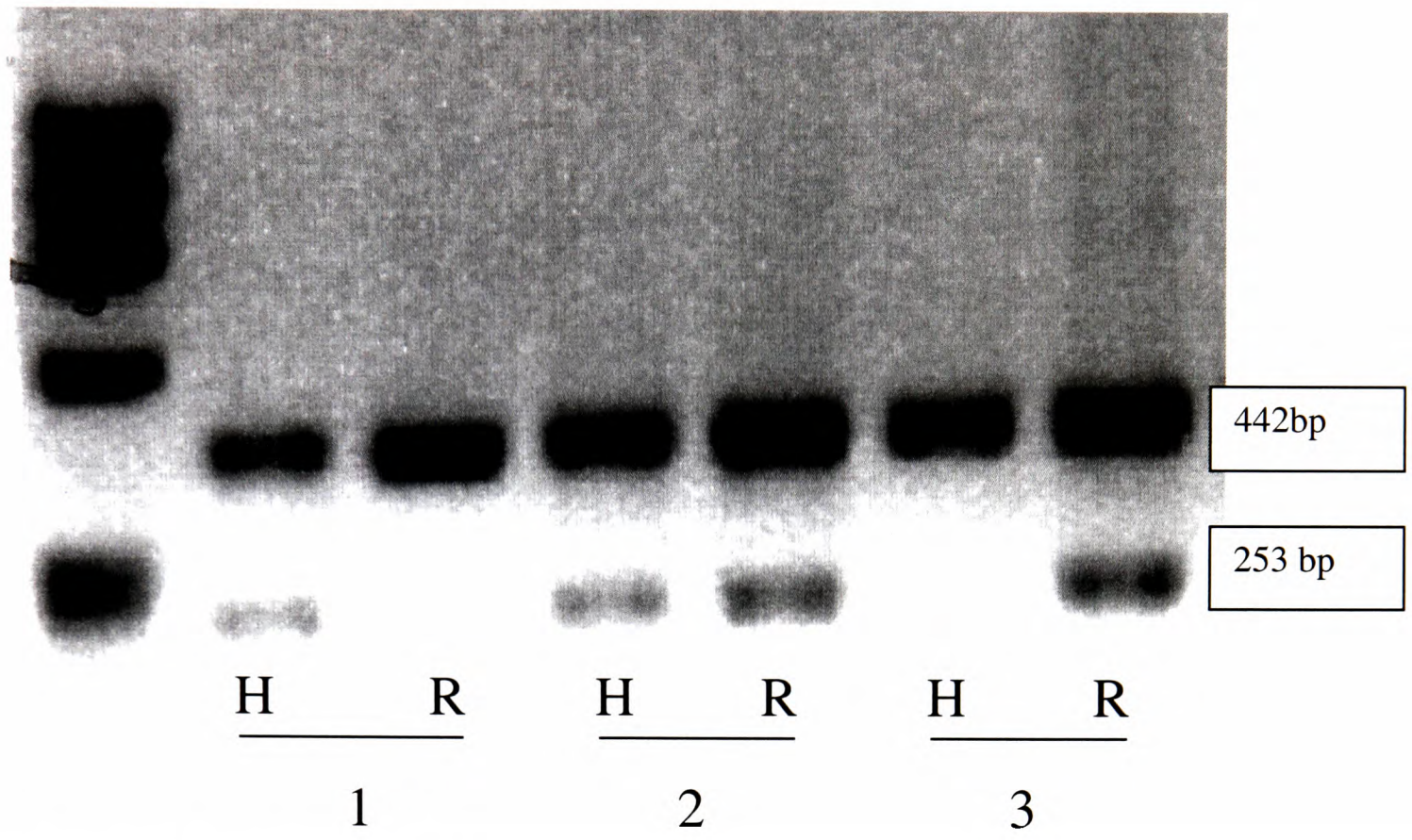


Figure 36: Allele-specific PCR

Figure 36 shows patient 1 who was positive for H in reaction one and negative for R in reaction 2 (H131/H131 homozygote). Patient 2 who was positive for both reactions (heterozygote), and patient 3 who was negative for H but positive for R in reaction 2 (R131/R131 homozygote).

The Oxford and Thai studies were also genotyped for the FcγRIIIB gene. As a high degree of homology between the FcγRIIIA and FcγRIIIB sequences exists, a restriction fragment length polymorphism (RFLP) method modified from Matsuo *et al* was used to type the DNA (Matsuo, Procter et al. 2000). This method distinguished NA1 from NA2 within FcγRIIIB specifically (Matsuo, Procter et al. 2000). Two separate PCR reactions were performed. Each 25µl PCR reaction contained 50ng of genomic DNA, 1x PCR buffer, 2mM $MgCl_2$, 0.32mM dNTPs, 6pmol forward and reverse primers (either primer set 1 or 2) and 1 unit/µl of AmpliTaq Gold DNA polymerase. See Appendix D4 for the PCR conditions.

Digestion of the PCR products from the first primer set was performed in 20µl volumes consisting of 10µl of the PCR product from primer set 1 together with 0.2 units of *Cac*8 I, 1 unit of *Xmn* I (New England Biolabs, Hutchin), 1 unit each of Buffers 2 and 3 and 1 unit BSA (NEB). The products were digested for 3 hours at 37°C, then run for 2 hours on a 3% agarose gel which was stained with 50mg/µl ethidium bromide and examined under UV fluorescence.

The PCR products from primer set 2 were digested overnight at 37°C in 20µl reactions containing 10µl PCR product, 1 unit per reaction *Hind* III (NEB) and 1 unit buffer 2. These products were examined on a 3% gel run for 1.5 hours, stained with 50mg/µl and examined under UV fluorescence. Each primer set was examined independently, the results collated, and any aberrant reactions repeated. The FcγRIIIA gene remained uncut in all of the reactions.

Digestion of the PCR products from the first primer set resulted in up to 5 bands: *Cac* 8 was able to cut when 'ACAGCG⇓TGC' was present in the PCR products representing a cut in NA2 at position 147 (NA1 is 'ACAGGGTGC'). The primers for set 1 were designed with a base pair change (T instead of A at position 232 in the reverse primer), which enhanced the specificity for *Xmn* I. *Xmn* I cut if 'GAGAAC⇓TCTTC' was present, so the NA1 allele was cut at position 227 ('GAGAGCCTCTTC' in NA2 remained uncut by *Xmn* I).

Digestion of PCR products using the second primer set resulted in up to two bands. The primer set was designed with 2 base pair changes, an A instead of a G at position 142 and an A instead of a T at position 143. Both were specific for *Hind* III which cuts at 'GGA⇓AGCTT', position 141 in NA2 ('GGGTGCTCG' in NA1 is not cut). The reverse primer of set 2 was constructed with two substitutions: a T instead of an A at position 281

and a T instead of a C at position 282 for the action of *Psp* 1406. Unfortunately *Psp* 1406 enzyme failed to cut repeatedly either alone or in combination with *Hind* III (Matsuo, Procter et al. 2000). Because the enzyme was specific for position 277 (not an extra glycosylation site) and did not alter the typing scheme this enzyme was excluded from the scheme. Only *Hind* III was used to cut the second primer set.

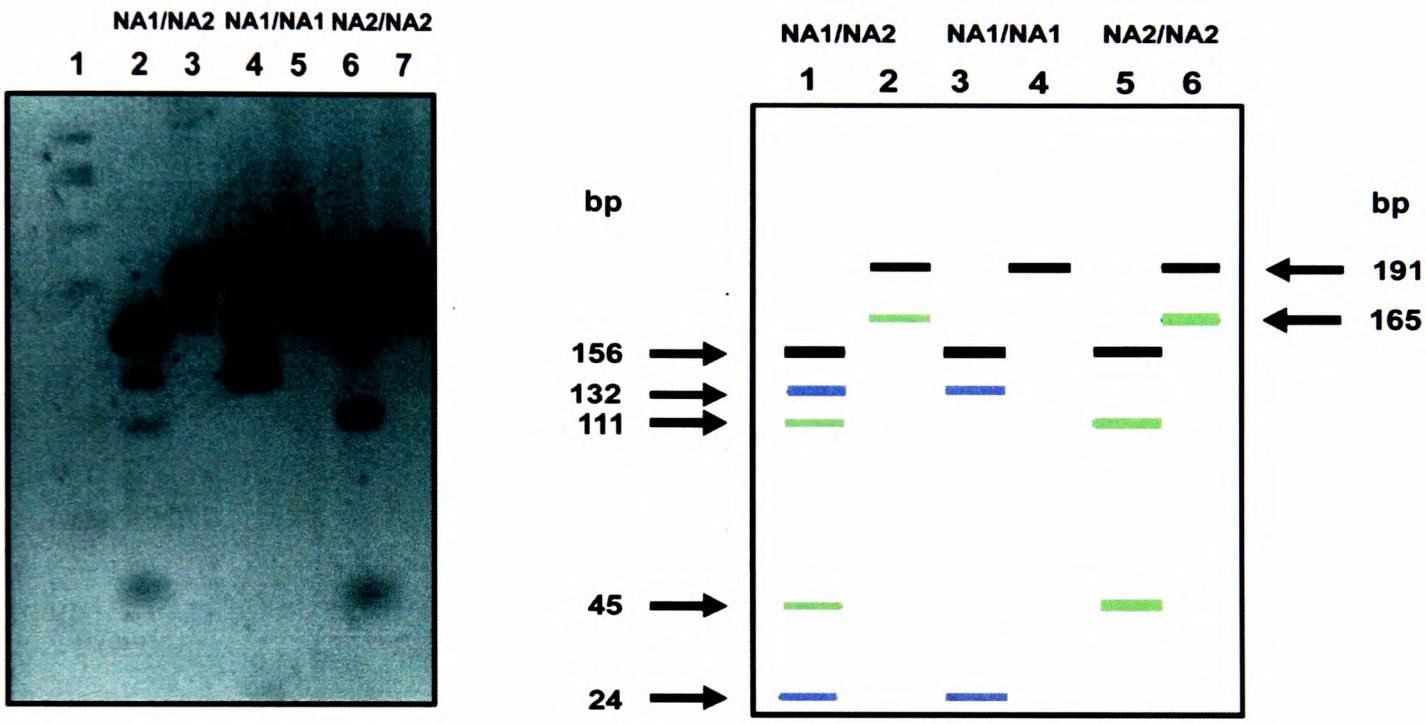


Figure 37: Digestion of PCR products for FcγRIIIB

The gel photograph and accompanying schematic diagram (Figure 37) illustrate the interpretation of the RFLP results. In the gel, the first lane contains the ϕX174 DNA-*Hae* III digest ladder (New England BioLabs). Lanes 2, 4 and 6 are digested products of primer set 1. Lanes 3, 5 and 7 are digested products of primer set 2. Lanes 2 and 3, 4 and 5, 6 and 7 represent results from 3 different individuals using primer sets 1 and 2 digesting with the respective enzymes. The first individual was a heterozygote (lanes 1 and 2 on the diagram), the second homozygous for NA1 (lanes 3 and 4 on the diagram) and the third homozygous for NA2 (lanes 5 and 6 on the diagram). The black bands at the top of each

lane was uncut DNA (at positions 156bp for primer 1 and 191bp for primer 2) which contained the FcγRIIIA.

Primer 1			**Primer 2**	
NA1/NA1(bp)	Heterozygote (NA1/NA2) (bp)	NA2/NA2 (bp)	NA1/NA1 (bp)	Heterozygote and NA2/NA2 (bp)
156	156	156	191	191
132	132			165
	111	111		26
	45	45		
24	24			

Table 24: Band sizes for the FcγRIIIB RFLP

Table 24 shows the size of the bands observed with the two primer sets for the three genotypes.

5.2.9 Genotyping results

In the primary analysis all of the cases were compared to all of the controls. Subsequent analysis examined patients with community-acquired disease and those with hospital-acquired disease separately. This subgroup analysis was specified *a priori* on the basis of likely differences in the modes of acquisition. It is possible that genetic factors play a relatively small role in disease acquired in hospital, where acquired factors (such as operations and intravenous cannula) are likely to dominate. Genetic factors may be relatively more important in community-acquired disease.

The results show that there was a significant association between the FcγIIA genotype and disease in the Oxford study (Figure 38, Tables 25 & 26). The allele conferring susceptibility is the R131 allele, which fits with the prediction from *in vitro* studies that H131 would be relatively protective. This association was much stronger for disease acquired in the community, consistent with the assumption that acquired and environmental factors may predominate in the hospital setting. The average odds ratio

(95% CI) for disease when comparing the R/R genotype with the R/H or the R/H with H/H was 0.79 (CI: 0.67 to 0.93) overall, and 0.63 (CI: 0.49 to 0.81) in the community. Tables 29 and 30 and Figure 40 show there was no association between FcγRIIIB and disease in Oxford.

In the Thailand case-control study there was no association between either FcγR gene and disease, but the allele frequencies were markedly different (Figures 39 & 41, Tables 27, 28, 31 and 32). The results for each SNP were in HWE.

5.2.9.1 Results for FcγRIIA

a) FcγRIIA results in Oxford

Genotype	Cases n (%)	Controls n (%)	Total	P value
All cases				
R/R	159 (37.1)	212 (30.6)	371 (33.1)	**0.021**
R/H	198 (46.1)	325 (46.9)	525 (46.8)	
H/H	72 (16.8)	156 (22.5)	228 (20.3)	
Total	429	693	1122	
CAD				
R/R	69 (45.7)	212 (30.6)	281 (45.7)	**0.001**
R/H	61 (40.4)	325 (46.9)	386 (40.4)	
H/H	21 (13.9)	156 (22.5)	177 (13.9)	
Total	151	693	844	
HAD				
R/R	90 (32.4)	212 (30.6)	302 (31.1)	**0.36**
R/H	137 (49.3)	325 (46.9)	386 (47.6)	
H/H	51 (18.4)	156 (22.5)	177 (21.3)	
Total	278	693	971	

Table 25: Genotyping results for FcγRIIA in Oxford

Allele frequencies	Cases n (%)	Controls n (%)	Total n (%)	Odds Ratio	95% CI	P value
All Cases				0.78	0.65 – 0.93	**0.005**
R	516 (60.1)	749 (54.0)	1265 (56.4)			
H	342 (39.9)	637 (46.0)	979 (43.6)			
Total	858	1386	2244			
CAD				0.61	0.46 – 0.80	**0.0002**
R	199 (65.9)	749 (54.0)	948 (56.2)			
H	103 (34.1)	637 (46.0)	740 (43.8)			
Total	302	1386	1688			
HAD				0.89	0.72 – 1.09	**0.25**
R	317 (57.0)	749 (54.0)	1066 (54.9)			
H	239 (43.0)	637 (46.0)	876 (45.1)			
Total	556	1386	1942			

Table 26: Allele frequencies for FcγRIIA in Oxford

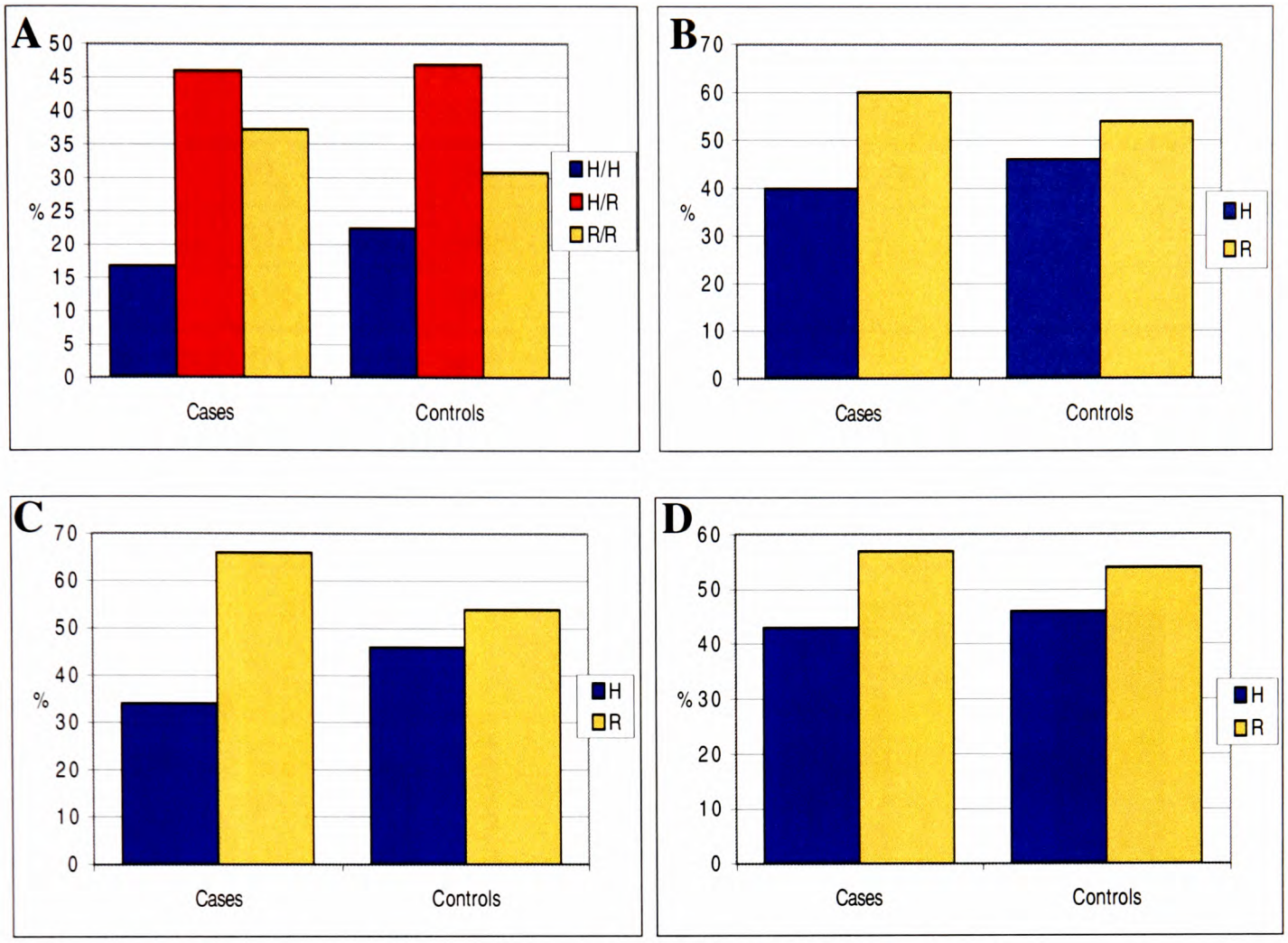


Figure 38: Graphical representation of the Oxford FcγRIIA results

Figure 38. Genotyping results for all cases and controls (p=0.021). B. Allele frequencies for all cases and controls (p=0.005). C. Allele frequencies, CAD cases subgroup (p=0.0002). D. Allele frequencies, HAD cases subgroup (p=0.25).

b) FcγRIIA results in Thailand

Genotype	Cases n (%)	Controls n (%)	Total n (%)	P value
R/R	11 (4.8)	20 (6.6)	31 (5.8)	**0.62**
R/H	99 (43.6)	124 (40.7)	223 (41.9)	
H/H	117 (51.5)	161 (52.8)	278 (52.3)	
Total	227	305	532	

Table 27: Genotyping results for FcγRIIA in Thailand

Allele frequencies	Cases n (%)	Controls n (%)	Total n (%)	Odds Ratio	95% CI	P value
R	121 (26.7)	164 (26.9)	285 (26.8)	0.99	0.73 – 1.31	**0.94**
H	333 (73.3)	446 (73.1)	779 (73.2)			
Total	454	610	1064			

Table 28: Allele frequencies for FcγRIIA in Thailand

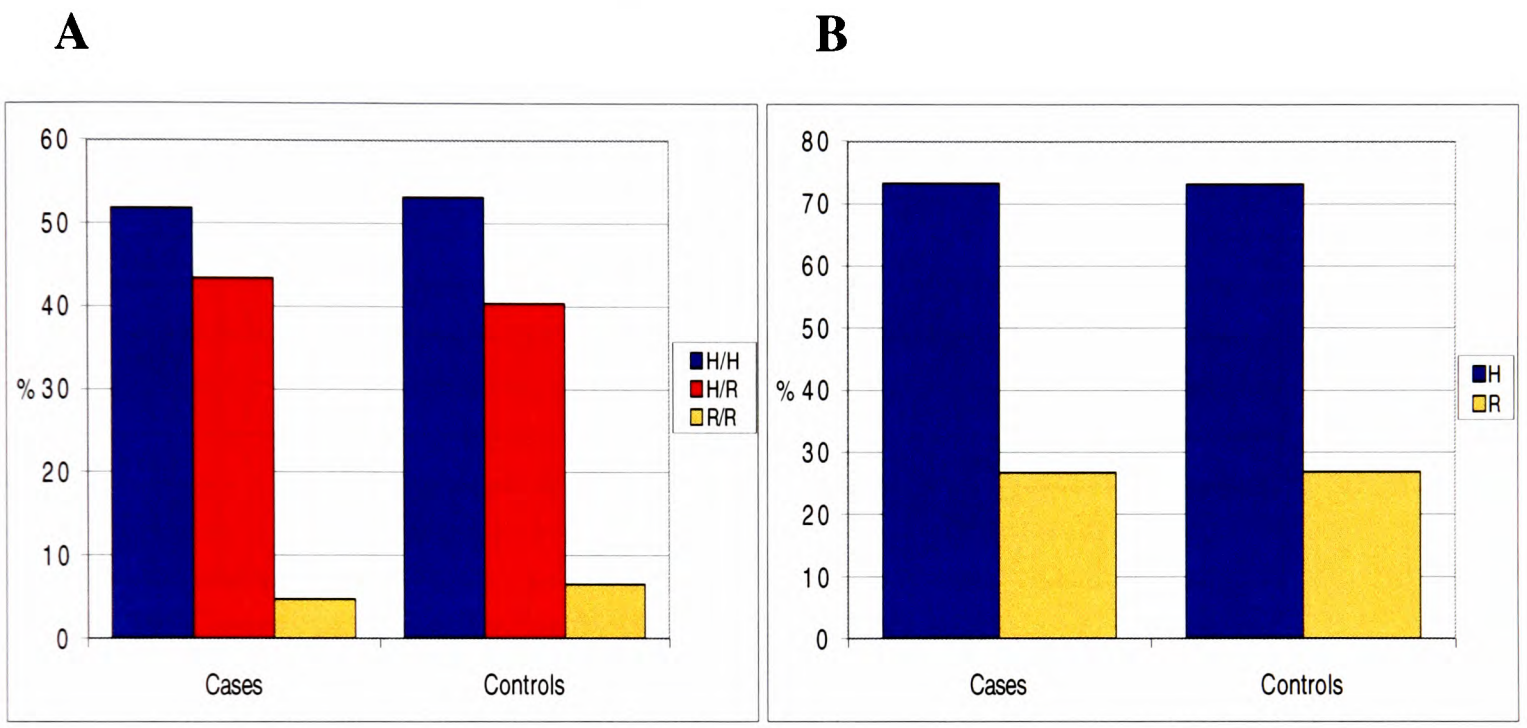


Figure 39: Graphical representation of the FcγRIIA results from Thailand

Figure 39. Genotyping results (p=0.62), and B. Allele frequencies (p=0.94).

5.2.9.2 Results for FcγRIIIB

a) FcγRIIIB results in Oxford

Genotype	Cases n (%)	Controls n (%)	Total n (%)	P value
All cases				**0.28**
NA2/NA2	181 (42.2)	280 (40.4)	461 (41.1)	
NA2/NA1	192 (44.8)	298 (43.0)	490 (43.7)	
NA1/NA1	56 (13.1)	115 (16.6)	171 (15.2)	
Total	429	693		
CAD only				**0.36**
NA2/NA2	63 (41.7)	280 (40.4)	343 (40.6)	
NA2/NA1	70 (46.4)	298 (43.0)	368 (43.6)	
NA1/NA1	18 (11.9)	115 (16.6)	133 (15.8)	
Total	151	693	844	
HAD only				**0.53**
NA2/NA2	118 (42.5)	280 (40.4)	398 (41.0)	
NA2/NA1	122 (43.9)	298 (43.0)	420 (43.3)	
NA1/NA1	38 9 (13.7)	115 (16.6)	38 (13.7)	
Total	278	693	971	

Table 29: Genotyping results for FcγRIIIB in Oxford

Table 29 shows there was no significant difference between the putatively susceptible genotype (NA2/NA2) and the putatively protective genotype (NA1/NA1) between the cases and controls in Oxford ($p = 0.28$). Three of the Oxford controls failed to type. Two controls were negative for NA and the third appeared to be NA1 using the first digestion but then cut on the second digestion suggesting a heterozygote or NA2. These were not investigated further because they were control samples.

Allele frequencies	Cases n (%)	Controls n (%)	Total n (%)	Odds Ratio	95% CI	P value
All Cases				**0.89**	**0.74 – 1.07**	**0.21**
NA2	554 (64.6)	858 (61.9)	1412 (62.9)			
NA1	304 (35.4)	528 (38.1)	832 (37.1)			
Total	858	1386				
CAD				**0.88**	**0.67 – 1.15**	**0.36**
NA2	196 (64.9)	858 (61.9)	1054 (62.4)			
NA1	106 (35.1)	528 (38.1)	634 (37.6)			
Total	302	1386				
HAD				**0.90**	**0.73 – 1.11**	**0.32**
NA2	358 (64.4)	858 (61.9)	1216 (62.6)			
NA1	198 (35.6)	528 (38.1)	726 (37.4)			
Total	556	1386				

Table 30: Allele frequencies

Table 30 shows the allele frequencies in the Oxford population separated into subgroups as before. The first group contained all cases and controls, the second the CAD and the third the HAD subgroups. There was no significant difference between the cases and controls in any of the groups (for all p = 0.21, CAD p = 0.36 and HAD p = 0.32).

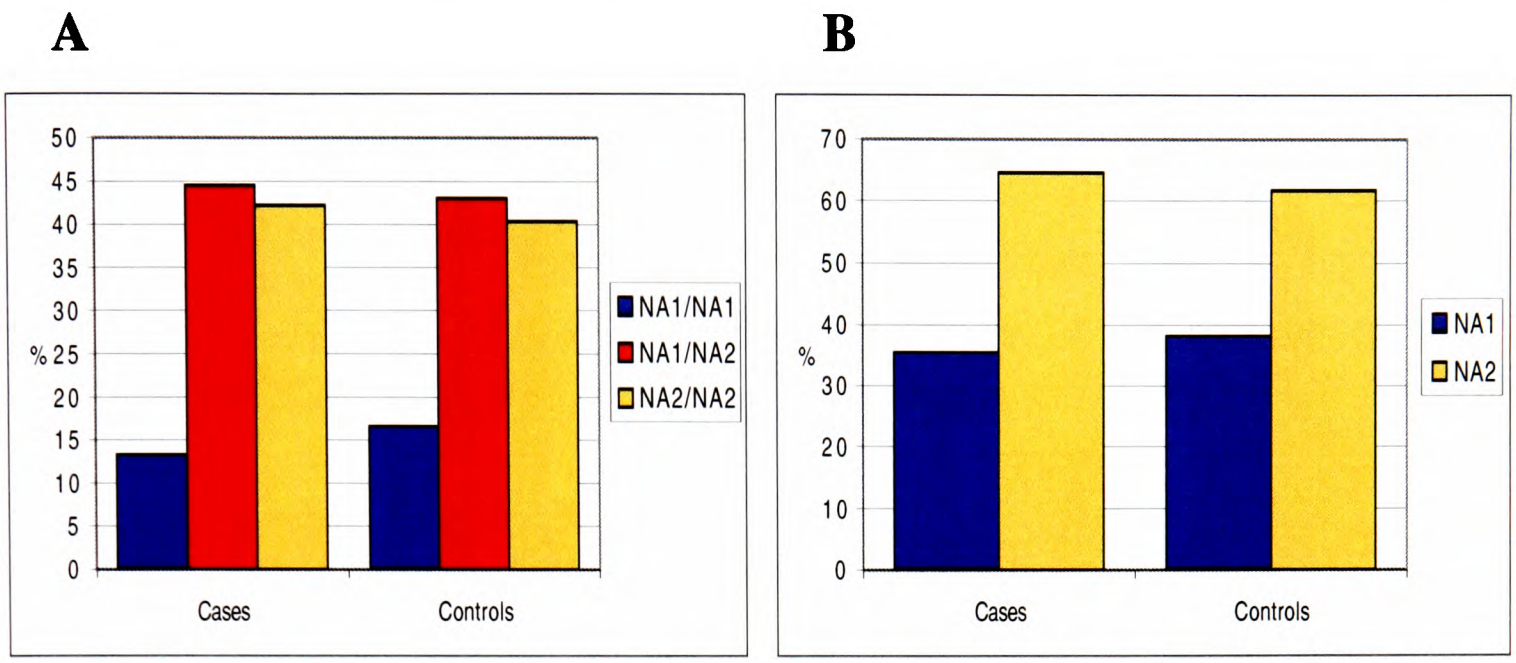


Figure 40: Graphical representation of the FcγRIIIB genotyping results from Oxford

Figure 40. Genotype results (p=0.28). B. Allele frequencies (p=0.21).

b) FcγRIIIB results in Thailand

Genotype	Cases n (%)	Controls n (%)	Total n (%)	P value
NA2/NA2	26 (11.5)	40 (13.1)	66 (12.4)	**0.801**
NA2/NA1	97 (42.7)	123 (40.3)	220 (41.4)	
NA1/NA1	104 (45.8)	142 (46.6)	246 (46.2)	
Total	227	305	532	

Table 31: Genotyping results for FcγRIIIB in Thailand

Allele frequencies	Cases n (%)	Controls n (%)	Total n (%)	Odds Ratio	95% CI	P value
NA1	305 (67.2)	407 (66.7)	712 (66.9)	**0.98**	**0.75 – 1.28**	**0.90**
NA2	149 (32.8)	203 (33.3)	352 (33.1)			
Total	454	610	1064			

Table 32: Allele frequencies for FcγRIIIB in Thailand

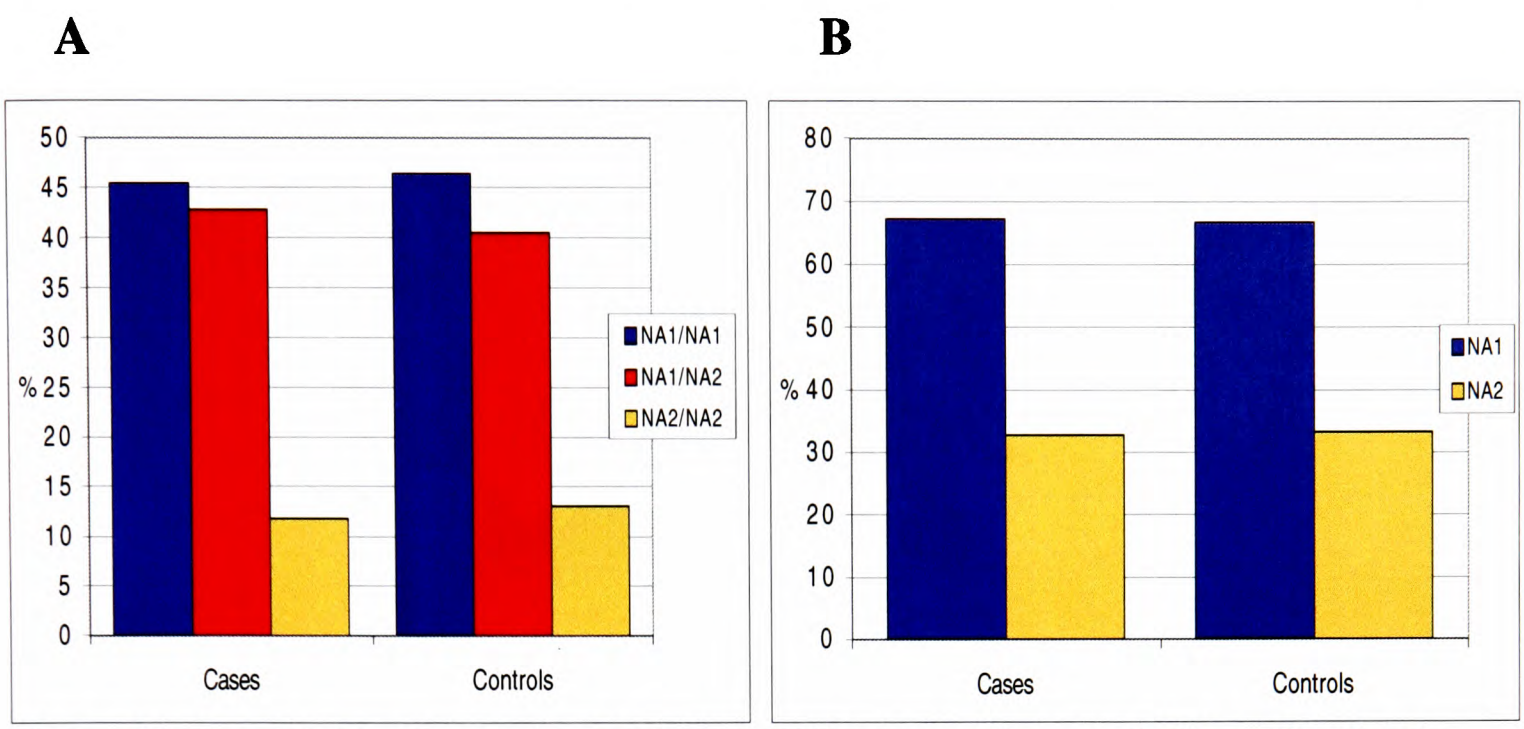


Figure 41: Graphic representation of the FcγRIIIB genotyping results from Thailand

Figure 41 shows there was no difference in genotype between the cases and controls collected in Thailand (p=0.801). Similarly there was no significant difference in the allele frequencies between the cases and controls (p=0.90).

5.2.10 Age as a potential confounder

What reasons other than differential susceptibility to disease could lead to the significant association between FcγIIA genotype and disease? The genotypes of a population of cases were compared, many of whom were elderly, with those of a very young (neonatal) control group. It is theoretically possible that stratification of FcγRIIA genotypes by age at a population level, generated by immigration of young adults of child-bearing age from elsewhere, could result in an erroneous disease association. For this reason the age distribution was examined for the case genotypes for evidence of any such stratification, none was found (p=0.29, Kruskal-Wallis test)(Figure 42). The same was true of the other genes studied.

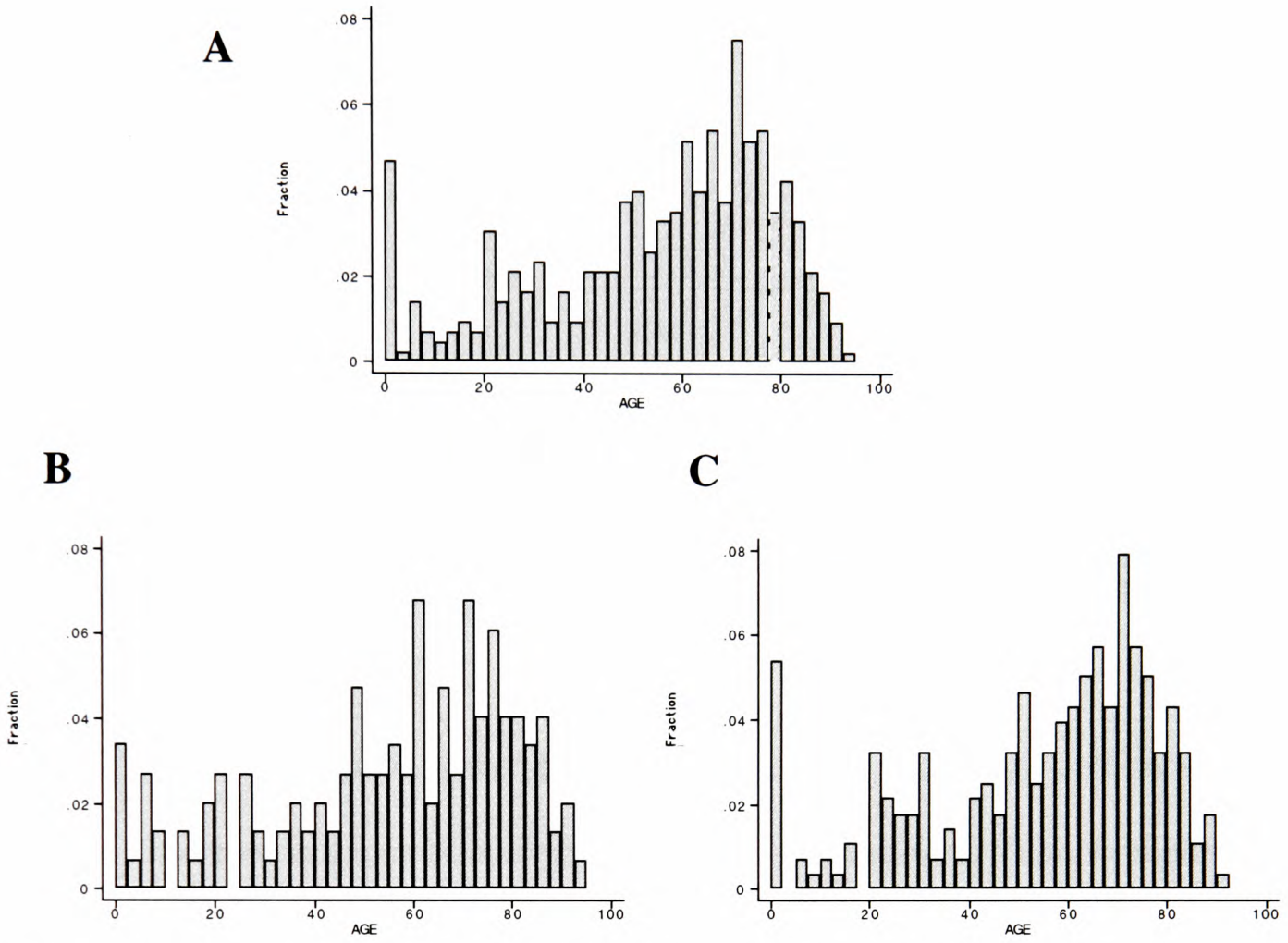


Figure 42: The age distribution for the Oxford Case-control study

Figure 42 depicts the age distribution of all of the cases with severe *S. aureus* disease. Two peaks were seen, one in the first few years of life then another about 75 years of age. The peaks at these age ranges may occur because of the decrease in host immunity and/or an increase in acquired susceptibility factors at these two extremes of age. The distribution for the CAD and HAD subgroups are shown in graphs B and C respectively. There was a peak in both groups in the first 2.5 years, though this was more pronounced in the HAD subgroup. Both groups had peaks in the later years of life.

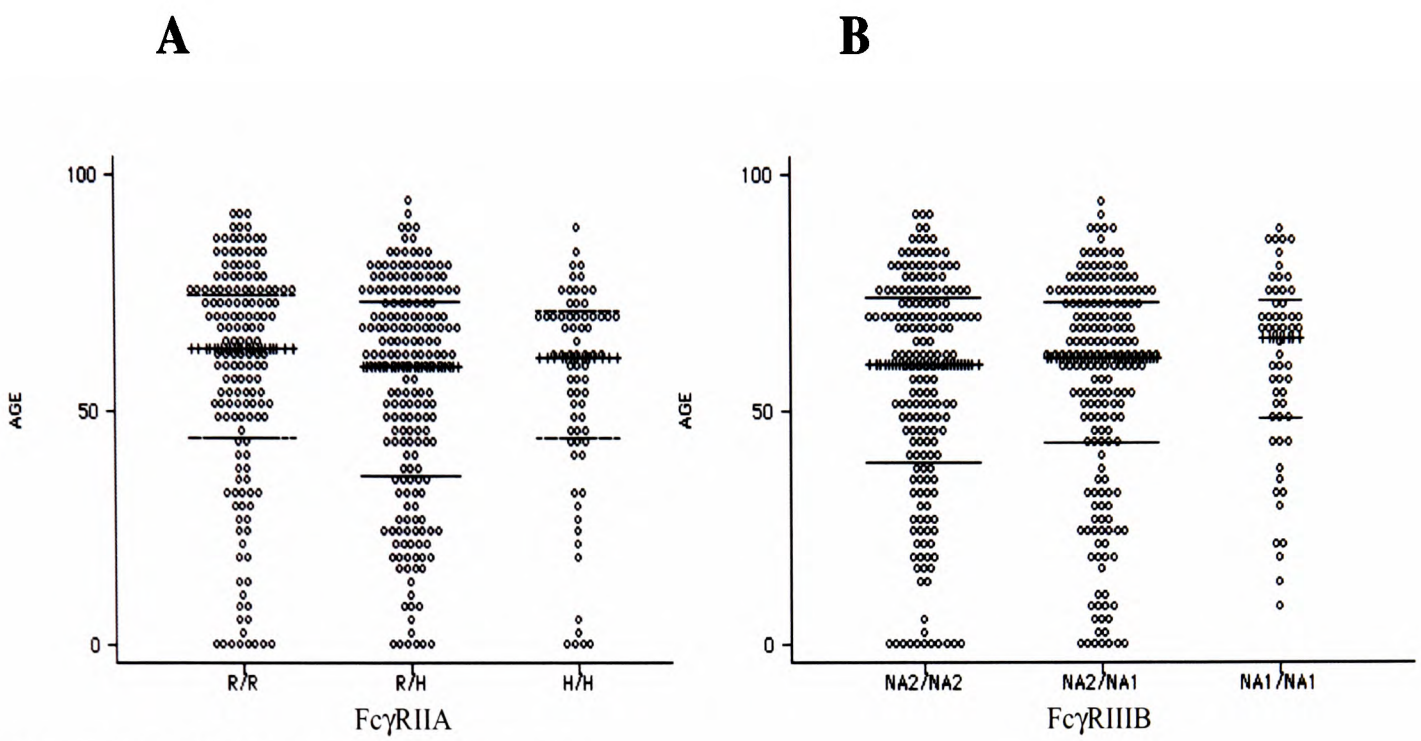


Figure 43: FcγR genotypes by age

Figure 43 depicts the FcγRIIA genotype for all of the cases against age. There was no significant difference in the median age between the three genotypes. Graph B depicts the same for FcγRIIIB for all of the cases collected in Oxford. There was no significant difference in age either across the genotypes or the disease subgroups.

5.2.11 Genotypic association between FcγRIIA and FcγRIIIB

	H/H n (%)	**H/R** n (%)	**R/R** n (%)	**Total** n (%)
Cases				
NA1/NA1	9 (12.5)	24 (12.1)	23 (14.5)	56 (13.1)
NA1/NA2	31 (43.1)	88 (44.4)	73 (45.9)	192 (44.8)
NA2/NA2	32 (44.4)	86 (43.4)	63 (39.6)	181 (42.2)
Total	72 (16.8)	198 (46.2)	159 (37.1)	**p=0.93**
Controls				
NA1/NA1	34 (21.8)	58 (17.8)	25 (11.6)	117 (16.8)
NA1/NA2	77 (49.4)	137 (42.2)	86 (40.0)	300 (43.1)
NA2/NA2	45 (28.8)	130 (40.0)	104 (48.4)	279 (40.1)
Total	156 (22.4)	325 (46.7)	215 (30.9)	**p=0.002**

Table 33: Genotypic association in the Oxford cases and controls between FcγRIIA and FcγRIIIB

Table 33 shows that there was no association between the genotypes for FcγRIIA and FcγRIIIB in the cases with an equal distribution of the putatively protective FcγRIIIB genotype (NA1/NA1) together with the FcγRIIA genotypes. Within the control group there was an association between the genotypes for FcγRIIA and those for FcγRIIIB. The putatively protective genotype for FcγRIIIB (NA1/NA1) occurred more commonly than would be expected by chance association with the protective genotype for FcγRIIA (H/H) in the controls, this decreased for the heterozygote (H/R) genotype and decreased further in the susceptible genotype (R/R). The same was true for the putatively susceptible genotype for FcγRIIIB (NA2/NA2) where the most association was seen between the susceptible FcγRIIA genotype (R/R), this decreased to the heterozygote and even further to the protective FcγRIIA genotype (H/H). This is shown graphically in Figure 44.

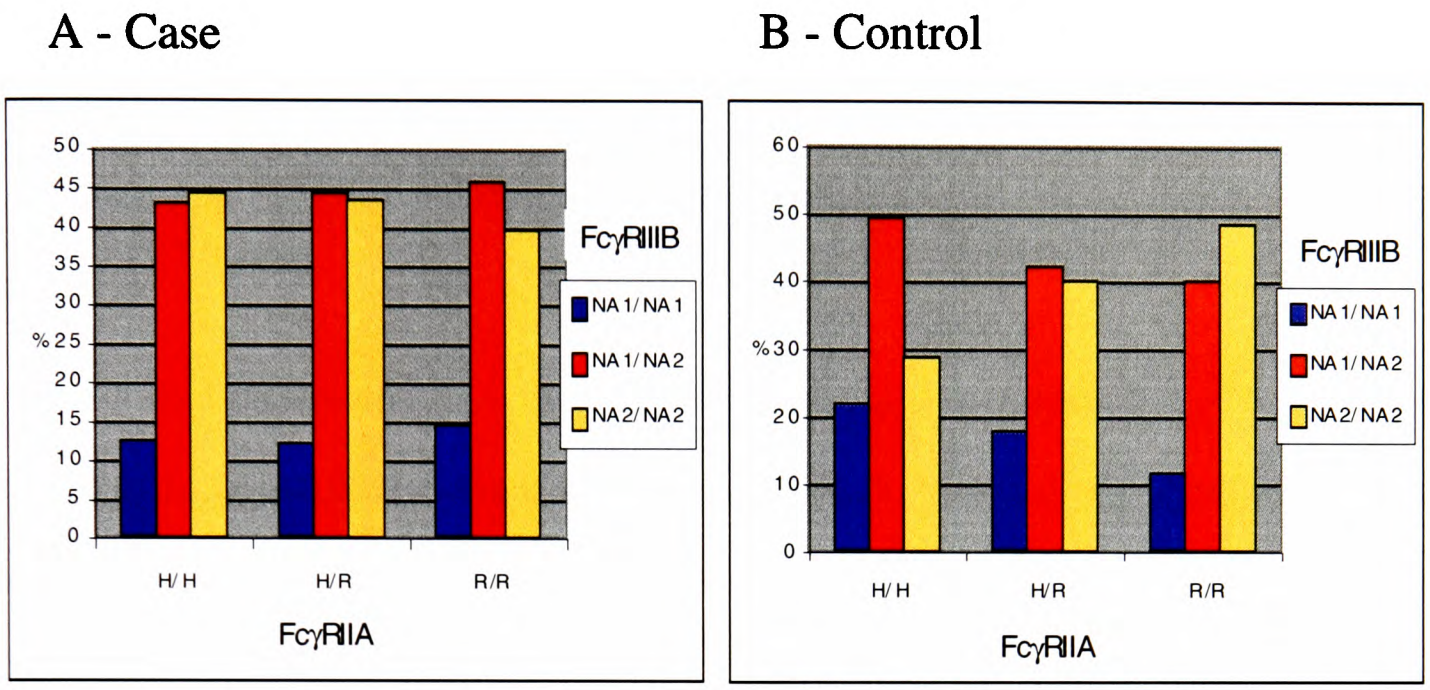


Figure 44: Graphical representation of the genotypic associations between FcγRIIA and FcγRIIIB in the Oxford study

Figure 44 shows the genotypic association between FcγRIIA and FcγRIIIB. In the cases no difference was observed across the three FcγRIIA genotypes with respect to the FcγRIIIB genotype (p=0.93). The controls showed a significant trend where the protective FcγRIIA genotype (H/H) was significantly associated with the protective haplotype of FcγRIIIB (NA1/NA1) and a trend was noted where there were less controls which had both H/H and NA2/NA2. This trend was reversed for the putatively susceptible genotypes (p=0.002).

5.2.12 Linkage Disequilibrium (LD)

Because of the close proximity of the two genes on chromosome 1 (Figure 35) it was possible that LD existed between the two loci. In the absence of family data this is impossible to derive directly as the gametic phase is unknown in case-control study genotype results. For this reason the haplotype iterative proportional fitting (‘*hapipf*’) statistical procedure in Stata (Mander 2001) was used to impute haplotype frequencies using the Expectation-Maximization (EM) algorithm, and then to compare using a log-

likelihood ratio test saturated (allowing for interactions) and unsaturated log-linear models of the haplotype data for the two loci. If the saturated model was significantly better than the unsaturated model, then LD existed. The normalised coefficient of linkage disequilibrium (D′) was calculated from the imputed haplotype frequencies (Lewontin 1974).

In the Oxford case-control study there was significant LD both overall (D′ (95% CI) 0.098 (0.072 to 0.12), p=0.002) and in the controls alone (0.17 (0.15 to 0.21), p<0.0001), but not in the cases alone (D′=0, p=0.40)). LD between the two loci was also absent in the Thai population (p=0.56).

5.2.13 Interactions between FcγRIIA and FcγRIIIB and susceptibility to disease

The phenotypic measure of susceptibility to severe *S. aureus* disease used in studies of genetic susceptibility is simply the odds for disease, calculated from the allele frequencies in the cases compared to controls. The presence of an association between the FcγRIIA and FcγRIIIB polymorphisms in the controls but not the cases, and the finding of LD between the two genes in the Oxford study, again in controls but not cases, suggests that there may be an interaction between the two genes in terms of disease susceptibility. Such an interaction would be biologically plausible in view of previous observations that the two receptors physically interact on the surface of neutrophils (Ravetch and Kinet 1991).

As an initial step the odds for disease were plotted for each of the 9 genotypes (Figure 45). This demonstrated a **qualitative** interaction between the two genotypes: the genotype for FcγRIIIB reverses the effect on disease susceptibility for the FcγRIIA genotype and *vice versa*. This is an example of an epistatic interaction.

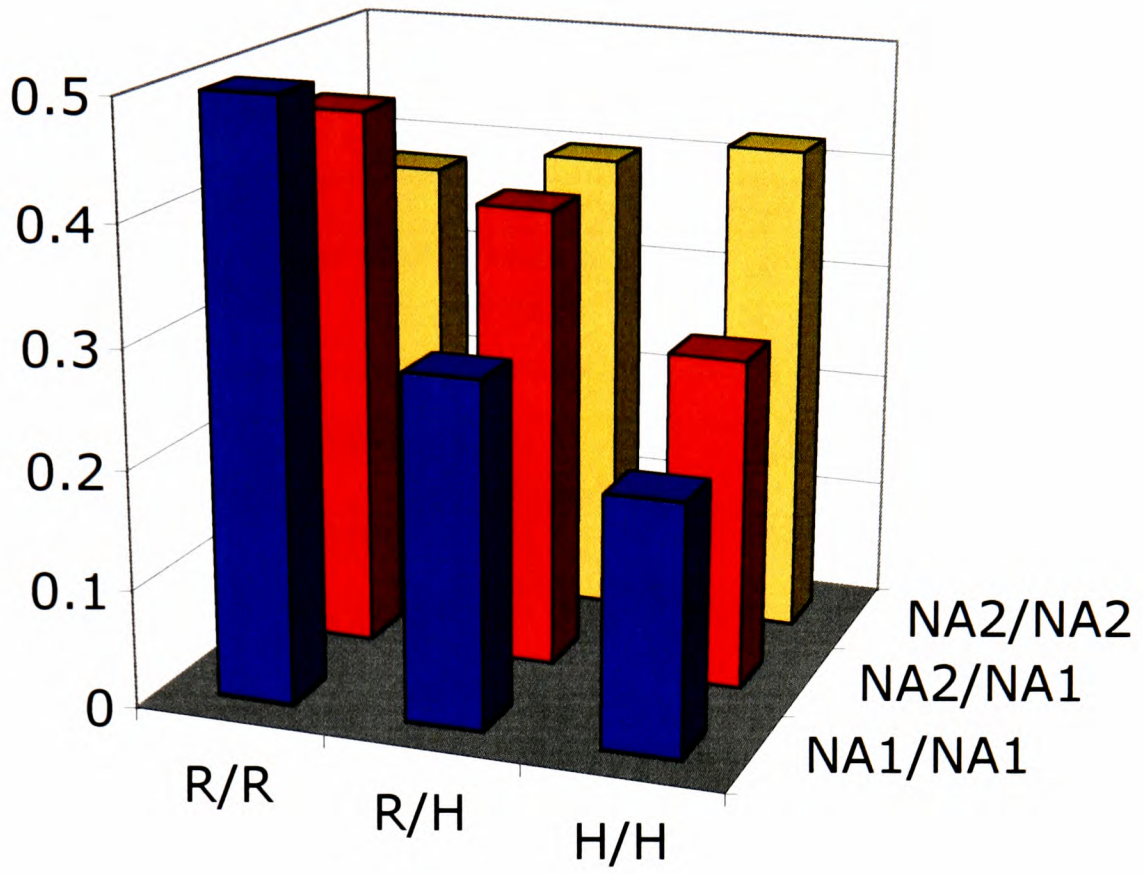


Figure 45:Odds for disease in the 9 FcγR genotypes in the Oxford study

Figure 45 depicts the qualitative epistatic interaction occurring between FcγRIIA and FcγRIIIB. The vertical axis represents odds for disease.

The interaction between the two genes was modelled using logistic regression analysis. An interaction term was generated by multiplying the inverse of database coding values for the genotypes. Although the weightings for this term are arbitrary, the direction or order is not; combinations of genotypes normally associated with susceptibility when considered alone are relatively attenuated in terms of odds for disease when considered together. The codings used in the regression were as follows:

		0	1	2
		R/R	R/H	H/H
0	NA2/NA2	4	2	0
1	NA2/NA1	2	1	0
2	NA1/NA1	0	0	0

The codings above the FcγIIA genotypes and to the left of the FcγIIIB genotypes are those used for the main effects. The 3x3 table (Table 34) represents the interaction terms.

	Saturated model		Unsaturated model		Log-likelihood test - p
	Odds Ratio for disease (95% CI)	**p value**	**Odds Ratio for disease (SE)**	**p value**	
All cases					
FcγRIIIB	0.57 (0.40 to 0.80)	**0.001**	0.92 (0.77 to 1.1)	0.32	
FcγRIIA	0.47 (0.33 to 0.67)	**0.000**	0.79 (0.67 to 0.94)	**0.008**	
Interaction term	0.66 (0.51 to 0.85)	**0.001**	–		**0.0009**
CAD only					
FcγRIIIB	0.40 (0.23 to 0.71)	**0.002**	0.93 (0.72 to 1.2)	0.59	
FcγRIIA	0.27 (0.15 to 0.48)	**<0.001**	0.63 (0.49 to 0.82)	**<0.001**	
Interaction term	0.52 (0.35 to 0.77)	**0.001**	–		**0.0007**
HAD only					
FcγRIIIB	0.65 (0.45 to 0.95)	**0.025**	0.93 (0.75 to 1.1)	0.40	
FcγRIIA	0.61 (0.40 to 0.92)	**0.018**	0.90 (0.74 to 1.1)	0.29	
Interaction term	0.73 (0.55 to 0.97)	**0.032**	–		0.03

Table 34: Logistic regression analysis including additive and interaction (epistatic) terms

In the logistic regression model containing the interaction term both FcγRIIA and FcγRIIIB were significantly associated with disease ($p < 0.001$ and $p = 0.001$ respectively) as was the interaction term ($p = 0.001$). The interaction term contributed significantly to the overall descriptive power of the model ($p = 0.0009$). This was assessed by comparing using a log-likelihood test using the saturated model including the interaction term to an unsaturated model without it. The interaction term contributed significantly to disease susceptibility both overall and in the community, but when hospital-acquired cases were

considered the effect was borderline (Table 34) and the overall descriptive power of the model weak (p=0.08).

5.2.14 Haplotype analysis and disease

5.2.14.1 Effects of LD on disease associations

The results from the Oxford case-control study indicated that FcγRIIA alone was associated with severe *S. aureus* disease whereas FcγRIIIB alone was not, that both loci are in LD, and that there was an epistatic interaction between the two loci. To examine the role of LD in these associations the EM algorithm-derived haplotypes (Table 35) were used to develop models which included both LD and disease associations. The saturated log-linear models, which included terms for interactions between the two loci and between the loci and disease, were compared using a log-likelihood test with an unsaturated model excluding the disease-association interaction term. If the saturated model was significantly 'better' than the unsaturated model, then a disease association was proved despite the LD (Mander 2001).

	R/NA1 n (%)	R/NA2 n (%)	H/NA1 n (%)	H/NA2 n (%)	p value
All					**0.004**
Case	191 (22.3)	325 (37.9)	113 (13.2)	229 (26.7)	
Control	236 (17.0)	513 (37.0)	292 (21.1)	345 (24.9)	
CAD					**0.0001**
Case	79 (26.2)	120 (39.7)	27 (8.9)	76 (25.2)	
Control	236 (17.0)	513 (37.0)	292 (21.1)	345 (24.9)	
HAD					**0.23**
Case	111 (20.0)	206 (37.1)	87 (15.6)	152 (27.3)	
Control	236 (17.0)	513 (37.0)	292 (21.1)	345 (24.9)	

Table 35: EM algorithm-generated haplotypes for the Oxford study

P values indicate the significance of the likelihood ratio test comparing models with and without the disease association interaction terms.

The putatively protective haplotype (H/NA1) was significantly lower in all the groups of cases compared to the controls. There was no significant difference in either the R/NA2 or H/NA2 between the cases and controls. There was an increased number of R/NA1 haplotypes in the cases compared to the controls.

The likelihood ratio tests showed that there was a significant disease association both overall and with community-acquired cases, but not when hospital-acquired disease was considered alone (Table 35).

5.2.14.2 Role of FcγRIIIB

In a further application of this method the role of FcγRIIIB was examined, again by comparing a saturated model to an unsaturated model of disease prediction. The saturated model includes all interactions between FcγRIIIB and FcγRIIA, FcγRIIIB and disease, and between FcγRIIA and disease. The unsaturated model assumes that FcγRIIA and disease are conditionally independent given the state of FcγRIIIB; i.e. that FcγRIIIB has no effect. FcγRIIIB was found to contribute significantly to the model (p=0.0038), in agreement with the original logistic regression modelling in which the effect on disease of FcγRIIIB became significant once the FcγRIIIB*FcγRIIA interaction term was added (Table 34).

5.2.15 Formal two locus decomposition of variance for main and epistatic effects

The FcγRIIIB and FcγRIIA genotyping results and analysis detailed above demonstrate that interactions exist between the two loci in terms of allelic association (LD), and in terms of effect on disease susceptibility. However the exact nature of these interactions is unclear, as statistically in the logistic regression model the interaction term used was fairly arbitrary, and the terms (the two main terms and the interaction term) not strictly independent. One formal method of partitioning the variance in phenotypic effect of two bi-allelic loci is to use Cockerham's genetic model (Cockerham 1954). This is an

extension to two loci of Fisher's decomposition of genotypic value (Fisher 1918), and for quantitative outcomes is based on the following linear model:

$$y = B_0 + B_{a1}x_1 + B_{d1}z_1 + B_{a2}x_2 + B_{d2}z_2 + B_{iaa}x_1x_2 + B_{iad}x_1z_2 + B_{ida}z_1x_2 + B_{idd}z_1z_2$$

Where y is a quantitative phenotype and x_i and z_i are dummy variables related to the underlying genotype at locus *i*. For example with FcγRIIA x_i would be set at 1 and z_i at –0.5 for a R/R homozygote, $x_i = 0$ and $z_i = +0.5$ respectively for a R/H heterozygote, and $x_i = -1$ and $z_i = -0.5$ for an H/H homozygote. The coefficients B_0, B_{a1}, B_{d1}, B_{a2}, and B_{d2} represent genetic parameters to be estimated corresponding to the mean effect and additive and dominance effects at loci 1 and 2. The coefficients B_{iaa}, B_{iad}, B_{ida}, and B_{idd} correspond to the interaction effects additive x additive (homozygous x homozygous), additive x dominance (homozygous x heterozygous), dominance x additive (heterozygous by homozygous) and dominance x dominance (heterozygous x heterozygous). In other words there are 4 main effects and 4 epistatic effects. These are demonstrated diagrammatically in Figure 46.

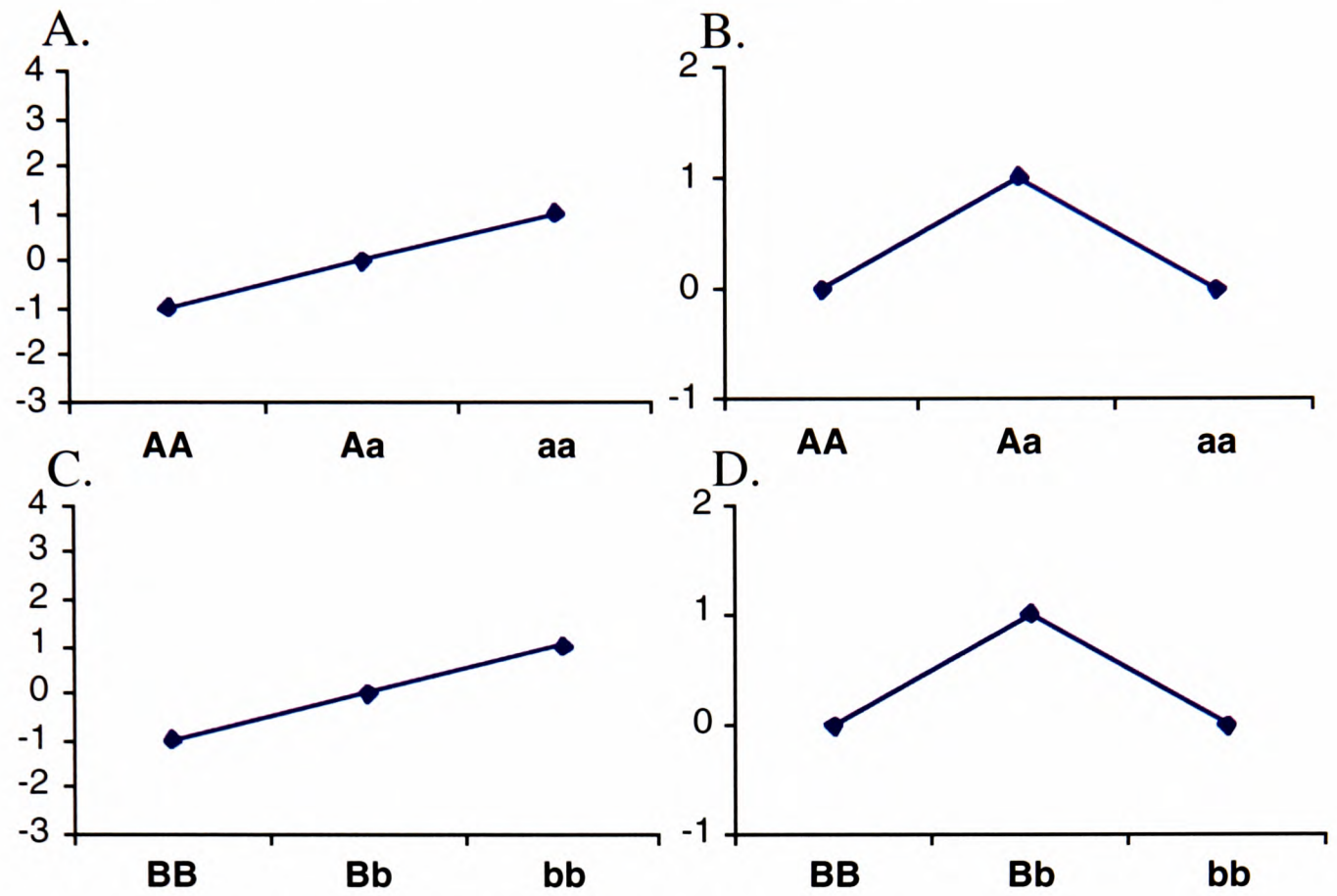


Figure 46: Main effects at two diallelic loci

Figure 46 is a diagrammatic representation. The y axis represents phenotype. A. and C: additive and dominance effects of locus 'A'. B. and D: additive and dominance effects of locus 'A'.

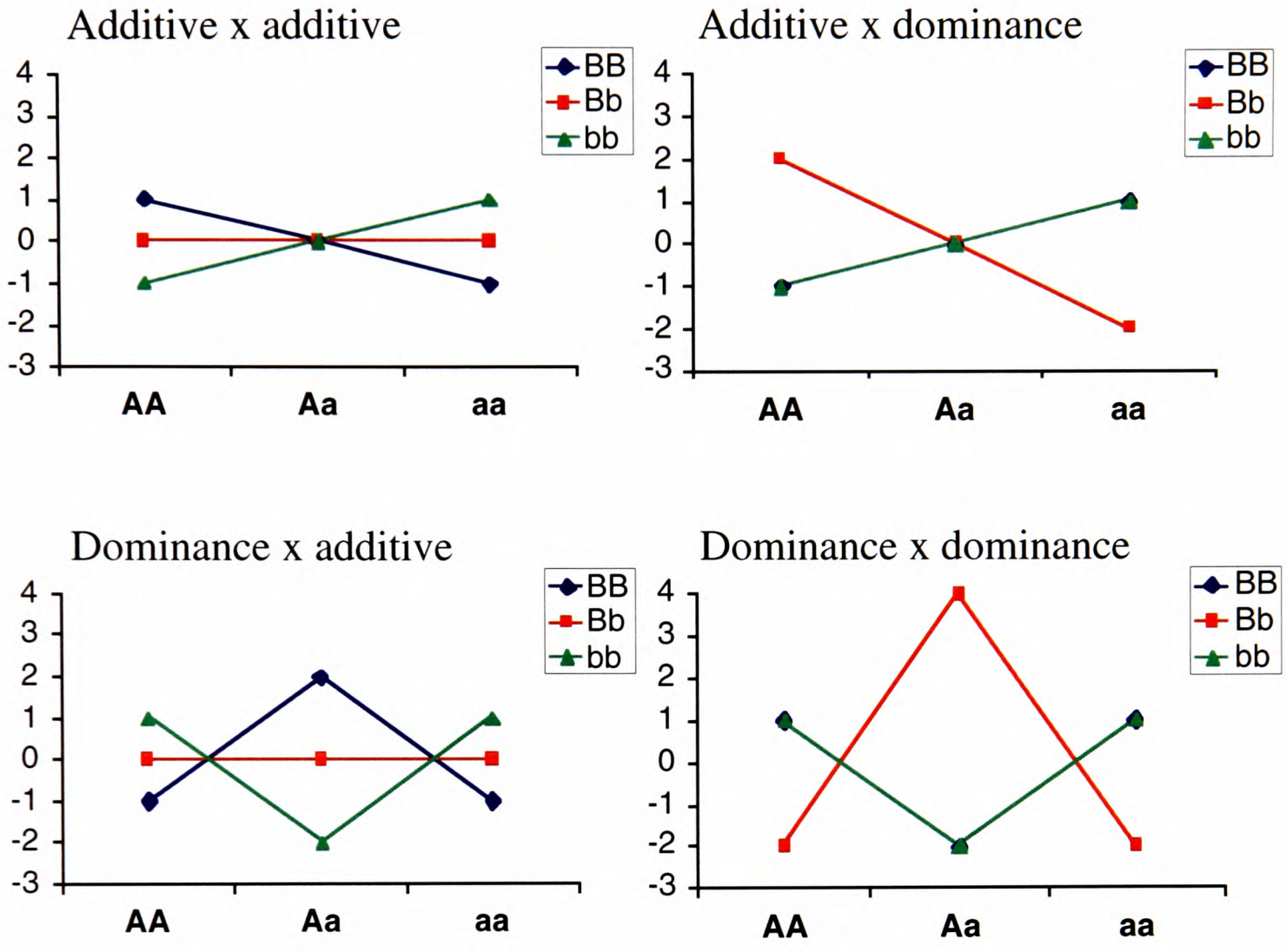


Figure 47: The four epistatic effects between two diallelic loci

Figure 47 is a diagrammatic representation. The y axis represents phenotype.

Where the phenotype is categorical, as in studies of genetic susceptibility to infectious disease, a logit transformation is applied to the odds of being diseased, giving the logistic regression equation:

$$\ln\left(\frac{p}{1-p}\right) = B_0 + B_{a1}x_1 + B_{d1}z_1 + B_{a2}x_2 + B_{d2}z_2 + B_{iaa}x_1x_2 + B_{iad}x_1z_2 + B_{ida}z_1x_2 + B_{idd}z_1z_2$$

Results of Cockerham's genetic model applied to FcγRIIA and FcγRIIIB.

Variance component	Odds ratio (95% CI)	p value
FcγRIIA additive	**0.71 (0.59 to 0.86)**	**<0.001**
FcγRIIIB additive	0.84 (0.69 to 1.0)	0.07
FcγRIIA dominance	0.99 (0.76 to 1.3)	0.95
FcγRIIIB dominance	1.1 (0.86 to 1.5)	0.41
additive x additive	**0.68 (0.52 to 0.90)**	**0.006**
FcγRIIA additive x FcγRIIIB dominance	0.90 (0.62 to 1.3)	0.58
FcγRIIA dominance x FcγRIIIB additive	0.90 (0.61 to 1.3)	0.59
dominance x dominance	1.2 (0.72 to 2.1)	0.45

Table 36: Cockerham's genetic model applied to FcγRIIA and FcγIIIB in the Oxford case-control study

In the Oxford case-control study 2 of the 8 coefficients in Cockerham's genetic study were significantly different from zero: the additive effect of FcγRIIA and the additive x additive epistatic interaction between FcγRIIA and FcγRIIIB.

The combination of an additive FcγRIIA effect and an additive x additive effect can be visualized in Figure 48, which is Figure 45 redrawn in the same style as the diagrammatic representations of the 8 terms in the full genetic model (Figures 46 & 47).

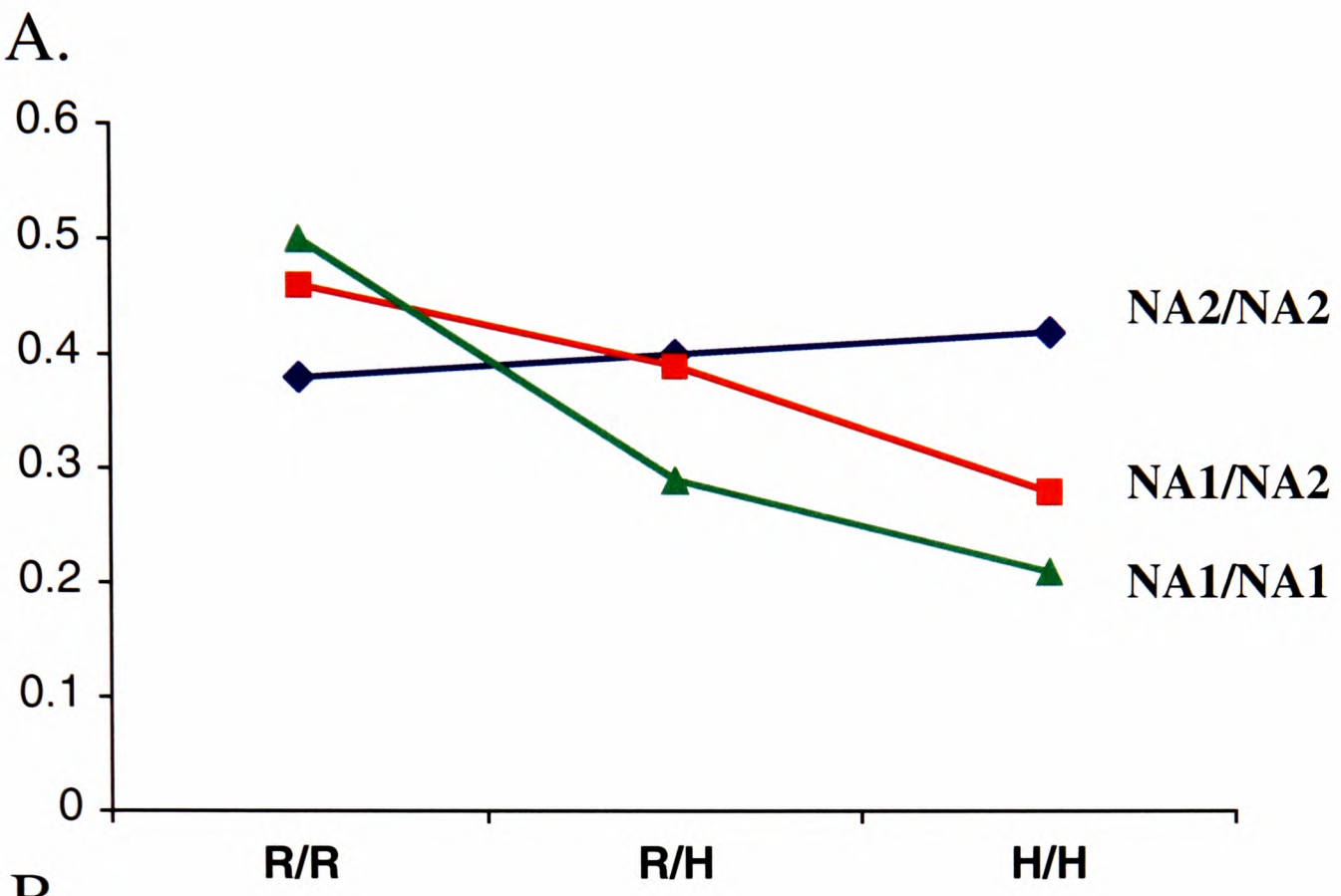


Figure 48: FcγRIIA and FcγRIIIB contributions to disease susceptibility

Figure 48 shows the epistatic interaction which occurs in the data between FcγRIIA and FcγRIIIB. The y axis depicts the odds for disease with the x axis the FcγRIIA genotype and the interactions between the FcγRIIIB genotype are shown within the graph. Figure 48A shows the interaction between all of the cases with Figure 48B showing only the

interaction in the CAD subgroup. This shows diagrammatically that the data is consistent with an additive by additive epistatic interaction (as shown statistically in table 36 and also depicted diagrammatically in Cockerham's model, Figure 47) where the homozygous genotypes (R/R and H/H) of FcγRIIA interact with the homozygous genotypes (NA1/NA1 and NA2/NA2) of FcγRIIIB. The data fits Cockerham's model when all of the cases were examined, but was even more pronounced when the CAD subgroup was examined alone.

The CAD/HAD subgroup analyses using Cockerham's genetic model confirmed the above results when CAD cases alone were considered; the odds ratio (95% CI) for the FcγRIIA additive main effect was 0.53 (CI: 0.38 to 0.75), $p<0.001$, and that for the additive x additive epistatic effect was 0.50 (CI: 0.28 to 0.88), $p=0.016$. In addition the FcγRIIIB additive main effect was of borderline significance (0.70 (CI: 0.49 to 1.0), $p=0.06$). None of the terms were significant when only HAD cases were considered.

This is consistent with the primary analysis (just of the main effects), in which there was a significant main effect of FcγRIIA. This effect has now been shown using Cockerham's model to be a mainly additive (rather than a dominance) effect. The Cockerham's model results were also consistent with the results of the haplotype analysis, which showed that despite being in LD, both genes contributed independently to disease susceptibility: the full genetic model shows that FcγRIIA has a main additive effect and an epistatic interactive effect with FcγRIIIB; and that FcγRIIIB has an epistatic interactive effect with FcγRIIA.

The result is also partially consistent with the initial logistic regression analysis. In the initial logistic regression an epistatic interaction term was found to be significant; this interaction has now been shown to be an additive x additive epistatic interaction. The initial logistic regression also showed an effect of FcγRIIIB independent of the interaction

term; although there was a trend towards a significant main effect of FcγRIIIB (an additive effect), this was not significant. There are two possible reasons for this disparity: the terms in the initial logistic regression may not be fully independent (in Cockerham's model they were designed to be so); or there could be a significant effect, but this was not detected by the Cockerham's genetic model approach because of loss of statistical power consequent on having 9 terms in the equation (instead of just 4).

5.2.16 A model of the Fcγ receptor interactions on the surface of PMN

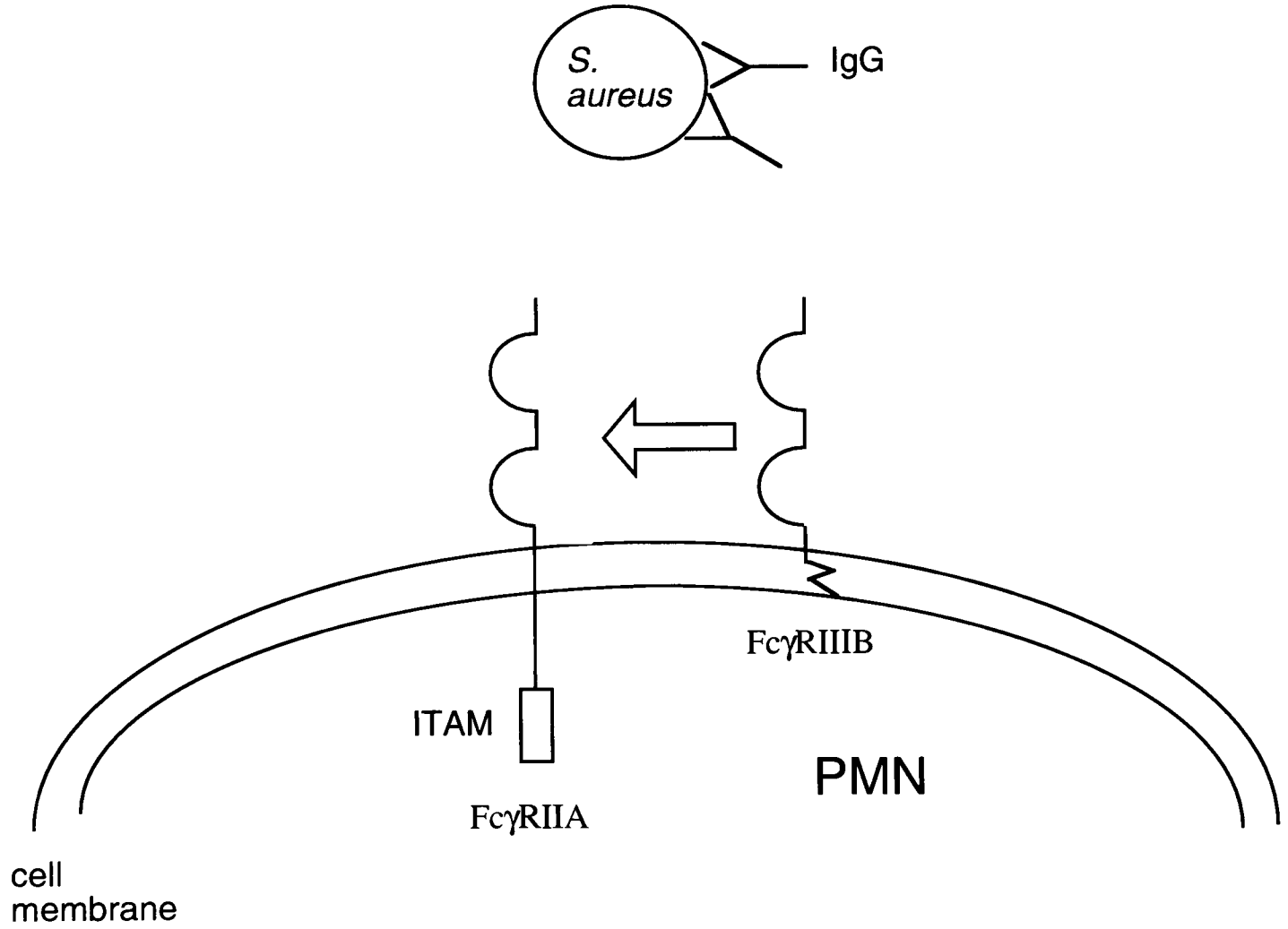


Figure 49: Interactions between the Fcγreceptors on the PMN surface

The results show that FcγRIIA alone was associated with disease whereas FcγRIIIB alone was not associated with disease, and that FcγRIIIB appears to interact with FcγRIIA. In

which case FcγRIIIB is also associated with disease. This interaction is biologically plausible as FcγRIIIB is known to have a GPI molecule which does not span the cell membrane of the PMN. A model which describes this is shown in Figure 49, the *S. aureus* molecule would be opsonised by IgG, the opsonised molecules then attach to the Fcγ receptors which are present both in the serum (with FcγRIIIB being released on neutrophil activation) and on the surface of the PMN. The FcγRIIIB molecules need to converge with the FcγRIIA molecules on the surface of the PMN as they have no cell membrane spanning domain, and are unable to send a signal into the cell. When the two Fcγ receptors physically associate, the FcγRIIIB molecule appears to be able to augment Calcium signaling by the FcγRIIA (Green et al. 1997). The ITAM molecule within FcγRIIA is then able to confer phagocytic signaling and the bacteria are phagocytosed by the PMN (Gordon et al. 1999). Although this model explains the observations made in this study of Fcγ receptors, in reality a number of other receptors (such as CR1 and CR3) also interact with the Fcγ receptors on the surface of PMN molecules to phagocytose opsonised *S. aureus*.

5.2.17 Conclusions

The FcgRIIIB and FcgRIIA genotyping results have been analysed by 4 methods: i) straightforward contingency table analysis (Fisher's exact test) of the genotypic and allotypic data; ii) logistic regression analysis of the genotypic data; iii) haplotype generation using the EM algorithm and analysis based on these; iv) decomposition of phenotypic variance applying Cockerham's genetic model, using dummy variables based on genotypic results.

Conclusions from the first three models were that:

i. The FcγRIIA H/R polymorphism was associated with susceptibility to severe *S. aureus* disease in the Oxford study, with the H allele being protective. This result was not seen in the Thai study, in which the allele frequencies were very different.

ii. There was an interaction between FcγRIIA and FcγRIIIB which significantly influenced disease susceptibility in the Oxford study.

iii. The FcγRIIIB NA1/NA2 polymorphism was independently associated with disease susceptibility in the Oxford study once the interaction in ii. was taken into account, with the NA1 allele associated with protection.

iv. All the above were much more significant when the analysis was restricted to community-acquired disease.

v. FcγRIIIB and FcγRIIA are in linkage disequilibrium in controls but not cases, and in Oxford but not in Thailand. The disease associations seen in the Oxford study are independent of this.

vi. Population stratification by age was not a confounder in this population, and had no influence on the above associations.

The analysis based on Cockerham's genetic model shed some further light on these results:

i. The effect of FcγRIIA in i. above was an additive effect with no dominance component.

ii. There was an additive x additive epistatic interaction between FcγRIIA and FcγRIIIB.

5.2.18 Discussion

Both FcγRIIA and FcγRIIIB were good candidate genes for this study, as the products of both are involved intimately in host defence against bacterial infection and both polymorphisms have been shown *in vitro* to be functional in terms of differential efficiency of phagocytosis of opsonised *S. aureus*. That these results arose from a prior hypothesis rather than as part of a 'fishing expedition' increases the chance that they are true findings rather than the result of a Type I error.

Most individuals who develop severe *S. aureus* disease in the community are previously healthy, whereas individuals who become ill with *S. aureus* disease within a hospital environment usually have predisposing factors, for example a portal of entry for the bacterium to enter the host where it can bypass many defence systems and cause disease. This would suggest that host factors would be relatively less critical in severe *S. aureus* infections acquired in the hospital compared to the community. That the significant disease associations with the FcγRIIA and FcγRIIIB polymorphisms were found only with community-acquired cases are in line with this prior hypothesis, this again lends credence to the results.

In both CAD and HAD the majority of cases occur in the over 50 year olds, presumably as a result of general decline in the competence of the immune system with age combined with an increase in comorbidity. Although only a small part of the immune system, it is worth noting that this may be due to a decrease in numbers of receptors on the surface of PMNs rather than a smaller number of neutrophils (Wenisch, Patruta et al. 2000).

There was an exaggerated difference between the two ethnically and geographically different case-control studies. The genotypes for the two FcγRs were very different between the two case-control studies, with the Oxford group containing a larger number of

putatively susceptible alleles (R in FcγRIIA and NA2 in FcγRIIIB) compared with the group from Thailand. These numbers are in agreement with previous reports of Asian and Caucasian populations (Bredius, Fijen et al. 1994; Osborne, Chacko et al. 1994; Salmon, Millard et al. 1996; Rascu, Repp et al. 1997; Flesch, Bauer et al. 1998; Dijstelbloem, Scheepers et al. 1999). Most of the cases isolated in Thailand were from hospital acquired disease rather than community acquired disease therefore in the light of the Oxford results any association of these polymorphisms with disease would be missed anyway.

There is a low incidence of disease with encapsulated organisms such as *H. influenzae* and *S. pneumoniae* in South East Asia (Osborne, Chacko et al. 1994). 70% of disease causing *S. aureus* strains have one of two microcapsules, the strains in this work have yet to be tested for microcapsules. The occurrence of disease with encapsulated organisms in Thailand might be low because the population has a much higher frequency of the protective alleles for FcγRIIA and FcγRIIIB, this difference in turn may occur because of susceptibility to another infection which is found in the tropics but not in the UK.

There was also no LD between the two receptors in the Thai case-control population whereas it was observed in Oxford. The two genes are approximately 300kb apart (from the latest ENSEMBL web page, http://www.ensembl.org/), which is a greater distance than that over which LD would normally be expected. It is possible that the epistatic interaction is maintaining LD through selection (Wolf, Brodie et al. 2000); if this is the case, this LD might be expected to decrease in successive generations as in modern times in Western populations the selective pressure from infection is negligible at a population level. The lack of LD in Thailand may be explained by the high frequencies of protective alleles, greatly reducing the importance of any epistatic component.

It is possible that these polymorphisms are not stable, and that in Western populations the protective alleles are moving towards fixation. Epistasis may be important during this journey, when allele frequencies are approximately equal. The Thai population may be further along this path, with epistasis playing a much smaller role. If this speculation is correct it is interesting to note that the reduction in infectious disease mortality may arrest the process completely.

Is there a biological rather than statistical significance to the epistatic interaction? One explanation is that it is the interaction between Fcγ receptors on the surface of neutrophils that leads to variation in the ability of the PMN to phagocytose the bacteria during infection. There are known to be ten times more FcγRIIIB receptors on the surface of neutrophils than FcγRIIA, but unlike FcγRIIA they have no intracytoplasmic domain and so by themselves cannot initiate phagocytosis. In addition to being on the surface, FcγRIIIB is also released from the neutrophil surface during activation and is found circulating free in the serum. The ITAM region of FcγRIIA is essential for phagocytic signalling and for the fusion of phagosomes with lysosomes (Tuijnman, Capel et al. 1992; Mitchell, Huang et al. 1994; Worth, Mayo-Bond et al. 2001).

It is probable that FcγRIIIB interacts with FcγRIIA on the cell surface in the presence of an opsonised bacterium, and utilises the ITAM molecule of the FcγRIIA molecule to optimally signal the initiation of phagocytosis.

It is unlikely that these two receptors act alone on the surface of neutrophils to optimally engulf and phagocytose infecting *S. aureus* strains. Other receptors are also likely to be involved. These are likely to include CR1, CR3 (the integrin $\alpha_m\beta_2$), LFA1 and CRP. Both CR1 and CR3 are found on the surface of neutrophils. CR1 promotes adhesion to particles and CR3 mediates subsequent engulfment and phagocytosis (Fallman, Andersson et al.

1993). Many interactions have been described between CR3, FcγRIIA and FcγRIIIB. Early work using phagocytosis experiments suggested that CR3 might associate with the FcγRs (Brown, Bohnsack et al. 1988). Anti-CR3 Fab fragments inhibited IgG mediated phagocytosis (Brown, Bohnsack et al. 1988). Zhou *et al.* (Zhou, Todd et al. 1993) suggested a structural association between CR3 and FcγRIIIB. They suggested the two molecules interact in a carbohydrate sensitive fashion and co-cap on the neutrophil surface. All three molecules are known to cooperate, CR3 together with FcγRIIIB stimulates the respiratory burst, and ligation of FcγRIIIB induces an increase in FcγRIIA-mediated phagocytosis in the presence of reactive oxygen species (Zhou and Brown 1994; Salmon, Brogle et al. 1995). The soluble form of FcγRIIIB also interacts with both CD11b/CD18 (CR3) and CD11c/CD18 triggering the release of IL-6 and IL-8 (Galon, Gauchat et al. 1996). Adherence studies have shown a further functional cooperation between FcγRIIA and CR3 (Annenkov, Ortlepp et al. 1996).

Other workers examining the relationship between receptors on the neutrophil surface have found that FcγRIIIB mediates phagocytic signalling when expressed in the presence of CR3 (Worth, Mayo-Bond et al. 1996). When the cytoplasmic domain of FcγRIIA was removed the neutrophil failed to phagocytose sheep erythrocytes. Phagocytosis was restored when CR3 transfectants were added to FcγRIIA molecules lacking the cytoplasmic domain. Further work by the same laboratory has shown that the cytoplasmic tail of FcγRIIA participates in phagolysosomal fusion which is distinct from the functional ITAM (Worth, Mayo-Bond et al. 2001). An FcγRIIA molecule with a mutant ITAM tail, without the interaction of CR3, fails to induce phagocytosis of IgG coated cells. Phagocytosis was restored when CR3 interacts with the receptor with the mutant ITAM tail (Worth, Mayo-Bond et al. 2001). Expression of CR3 did not affect the ability of a wild

type receptor to participate in phagolysosome fusion, although the H and R alleles were not examined in this work (Worth, Mayo-Bond et al. 2001).

Another important molecule is CRP, the gene for CRP is found, together with those for FcγRIIA and FcγRIIIB on chromosome 1. CRP is important because FcγRIIA is a major receptor for CRP on the surface of monocytes and PMN (Bharadwaj, Stein et al. 1999). The polymorphism found at position 131 is important in interactions with CRP, with the R allele (the susceptible allele) having the strongest affinity for CRP; only a minimal amount of binding occurs between CRP and the H allele (Stein, Edberg et al. 2000). Also the receptor for CRP on FcγRIIA is closely related to the site of binding for IgG. Schnackenberg *et al.* (Schnackenberg, Flesch et al. 1997) suggested that CRP may compensate for the decreased binding between encapsulated microorganisms opsonised with IgG2 and FcγRIIA which have the R allele. CRP has also been shown to produce a rise in intracellular calcium levels in PMN which have FcγRIIA receptors homozygous for R, but not for H (Schnackenberg, Flesch et al. 1997).

It is clear that many of these receptors cooperate on the neutrophils surface to endocytose and phagocytose *S. aureus*. Each receptor does not act alone to combat infection. It may be that certain haplotypes within these receptors increase susceptibility to disease or it may be that certain haplotypes are compensatory with a susceptible genotype at one receptor being compensated for by a resistant genotype in another molecule. For this reason there are a number of receptors that it would be interesting to examining further. These include CR1, CR3 and CRP. A number of SNPs of unknown function occur in CR3 (Gencik, Meller et al. 2000; Koch, Bottiger et al. 2001; Frenzel, Hampe et al. 2002). CR1 contains a well known intronic SNP affecting the copy number of the molecule, when the mutation occurs, less molecules are found on the cell surface. CRP also contains both an intronic SNP and a

microsatellite, these have both implicated in susceptibility to severe *S. pneumoniae* disease (Dr. S. Segal personal communication). The generation of haplotypes that would be possible after typing these SNPs and microsatellites might explain the difference seen in this study between these two different ethnic groups. Although no disease associations were seen in FcγRIIA or FcγRIIIB in the Thai case-control population, these polymorphisms might need to be considered in the context of the genotypes for mutations in CR1, CR3 or CRP.

In addition to examining these other polymorphisms another potentially confirmatory line of investigation might be neutrophil phagocytosis assays. It would be interesting to examine the ability of neutrophils from patients with known FcγRIIA and FcγRIIIB genotypes to phagocytose opsonised *S. aureus*, looking in particular for the main and epistatic effects seen in terms of disease susceptibility. In addition this would provide a tool with which the various interactions between receptors on the PMN surface could be elucidated.

5.3 MBL

5.3.1 Biological importance

Mannose binding ligand (MBL, synonyms: mannan or mannose binding protein) plays an important role in the opsonophagocytic process. MBL is a C-type (calcium dependent) serum lectin produced in the liver during the acute phase of the immune response (Summerfield and Taylor 1986; Ezekowitz, Kuhlman et al. 1989; Turner 1998). MBL facilitates the neutralisation of pathogenic microorganisms in an antibody-independent fashion. The lectin has two roles in innate immunity: the protein acts as a direct opsonin by specifically recognising specific carbohydrate moieties such as mannose or N-acetylglucosamine, molecules found on the surface of bacteria not present on the surface of host cells (Kuhlman, Joiner et al. 1989; Hartshorn, Sastry et al. 1993) and then delivers the opsonised microorganisms to phagocytes such as PMN, B cells and macrophages which express collectin receptors.

MBL is also able to activate complement using the lectin pathway. In the classical complement pathway, C1r and C1s form a $C1r_2C1s_2$ tetramer which binds C1q (Reid and Turner, 1994). In the lectin pathway, MBL is able to activate its own serine proteases (Mannose associated serine protease (MASP); MASP-1, MASP-2 and MASP-3) (Dahl, Thiel et al. 2001). These serine proteases are thought to be homologous to the $C1r_2C1s_2$ complex. The MASPs bind MBL independently, but may function cooperatively to fix complement (Matsushita and Fujita 1995; Wallis and Dodd 2000). MASP-2 has been shown to cleave C4 and C2 (Thiel, Vorup-Jensen et al. 1997). MASP-1 may be responsible for the direct cleavage of C3 (Matsushita and Fujita 1995). Presently the role of MASP-3 is unknown (Dahl, Thiel et al. 2001).

As a direct opsonin MBL is able to mediate the binding and phagocytosis of bacteria by PMN (Kuhlman, Joiner et al. 1989; Petersen, Thiel et al. 2001).

5.3.2 MBL - Structure and function

The MBL gene (MBL2) is 10kb and is found on chromosome 10 at position 10q11.2-21 (Sastry, Herman et al. 1989). The gene consists of 4 exons: exon 1 encodes the cysteine-rich N-terminal region and part of the collagenous region; exon 2 encodes more of the collagenous region; exon 3 encodes the neck region and exon 4 encodes the carbohydrate recognising domain (CRD). MBL exists as a multimer of an identical peptide chain with a molecular weight of 32 kDa (Turner 1996). This chain has 3 distinctive regions where the cysteine rich N-terminal region is thought to mediate inter-chain disulphide bonding. The collagenous region is composed of 18-20 Gly-Xaa-Yaa repeats and a C-terminal region of 148-155 amino acids forming the CRD (Lipscombe, Sumiya et al. 1992; Thiel, Holmskov et al. 1992). Three CRDs associate through their N-termini into a 96kDa subunit (Jack, Bidwell et al. 1997), and are in turn assembled into higher oligomers which circulate in the serum (Lipscombe, Sumiya et al. 1992), (Figure 50).

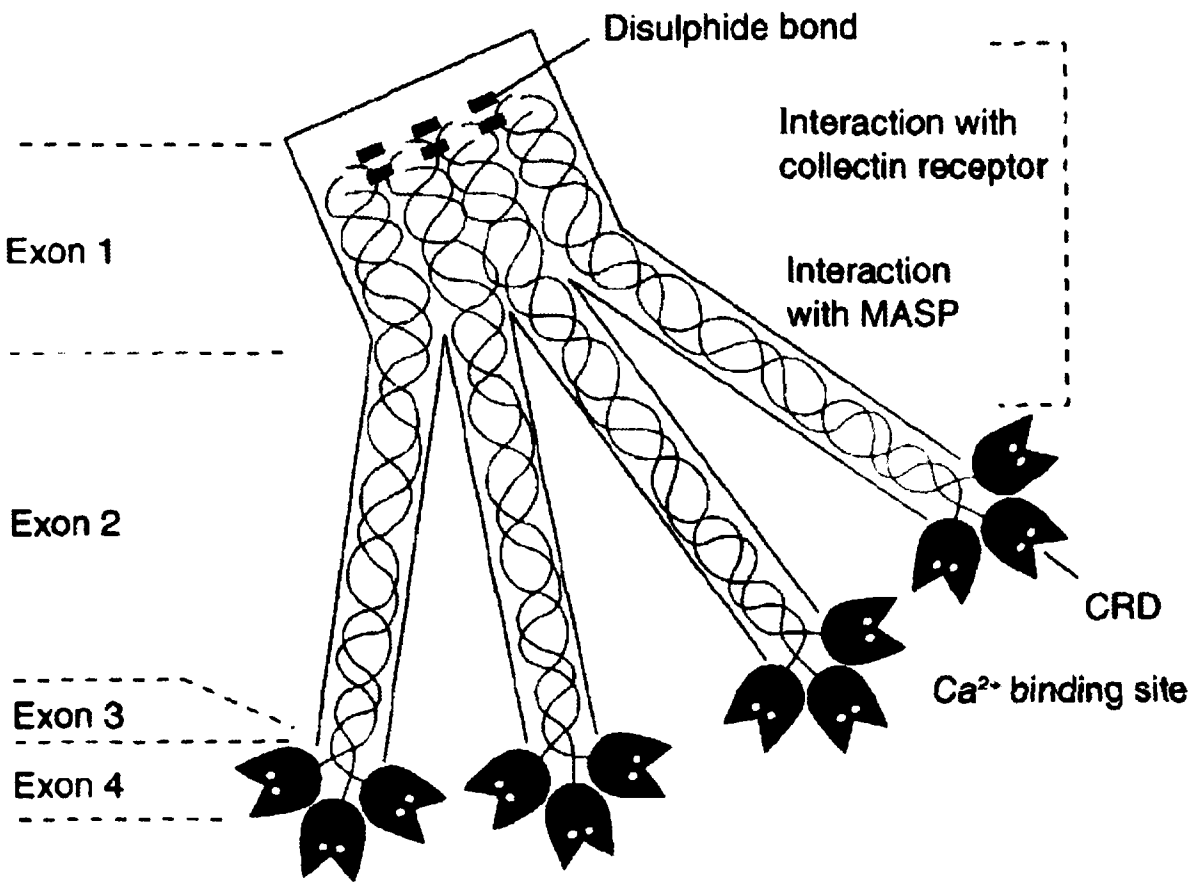


Figure 50: MBL (Sastry, Herman et al. 1989; Taylor, Brickell et al. 1989; Turner 1996)

Figure 50 depicts 4 subunits which each comprise 3 identical peptide chains of 32kDa which associate to give a disulphide linked N-terminal region. The collagenous regions are able to bind to MASP proteins with MBL-MASP complexes being able to activate the classical complement pathway. Mutations found in exon 1 are implicated in lower amounts of circulating MBL in the serum.

Low levels of MBL are associated with a common opsonic defect first reported in 1968 and found in 5% to 7% of the population (Miller, Seals et al. 1968; Super, Thiel et al. 1989). Three independent structural mutations in the CRD and 3 promoter polymorphisms have been implicated in this deficiency (Sumiya, Super et al. 1991; Lipscombe, Sumiya et al. 1992; Madsen, Garred et al. 1994). Their existence may confer some protection on the host: a decrease in the MBL-associated immune mechanisms reduces the amount of host damage caused by the release of inflammatory mediators (Lipscombe, Sumiya et al. 1992); and MBL deficiencies might protect the host against infection by intracellular parasites

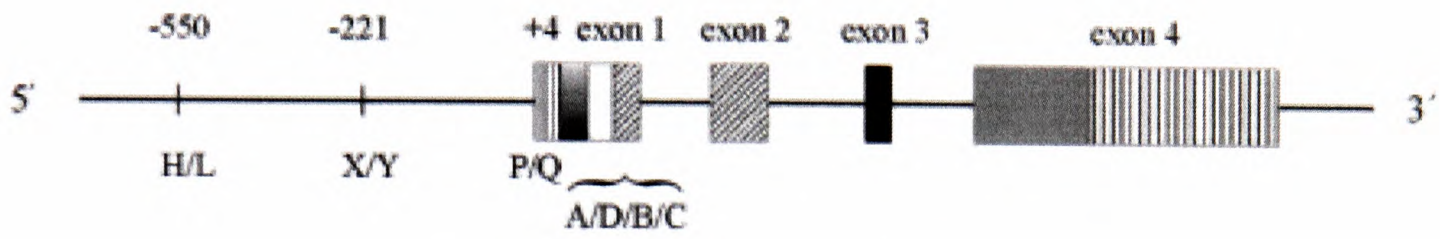
such as Mycobacteria and Leishmania which are able to exploit C3b opsonisation by using C3 receptors to invade host cells (Garred, Madsen et al. 1992; Garred, Harboe et al. 1994; Hoal-Van Helden, Epstein et al. 1999).

A decreased amount of MBL alone in the serum is thought unlikely to cause an increased susceptibility to infection. MBL deficiency is thought to become clinically relevant only when it occurs in association with another form of humoral immunodeficiency affecting opsonisation (Turner, Super et al. 1991; Aittoniemi, Baer et al. 1998).

5.3.3 Polymorphisms

There are 3 well known structural polymorphisms located in exon 1, two of which are within the CRD region. Each can lead to a decrease the level of MBL in the serum. There are also 3 well characterised polymorphisms in the promoter and the 5' UTR which also affect the amount of MBL in the serum. Other known polymorphisms include intronic mutations, other promoter polymorphisms and a 6 base pair deletion (Neonato, Lu et al. 1999; Juliger, Luckner et al. 2000). The functions and frequencies of these polymorphisms remain unknown and are likely to be in linkage with those already characterised (Neonato, Lu et al. 1999). For these reasons, the less well known polymorphisms were not typed.

Individuals homozygous for the first characterised structural mutation were found to have low levels of MBL in their serum (Sumiya, Super et al. 1991). Heterozygotes are thought to synthesise both mutant and wild type protein in equal amounts. The probability of selecting 3 normal chains to form the 96 kDa molecule from this heterozygous mixed pool would therefore be 1 in 2x2x2 (1 in 8) chance. When the serum level of individuals was examined this was found to be true (Sumiya, Super et al. 1991), a prediction which has since been upheld for the other mutations.



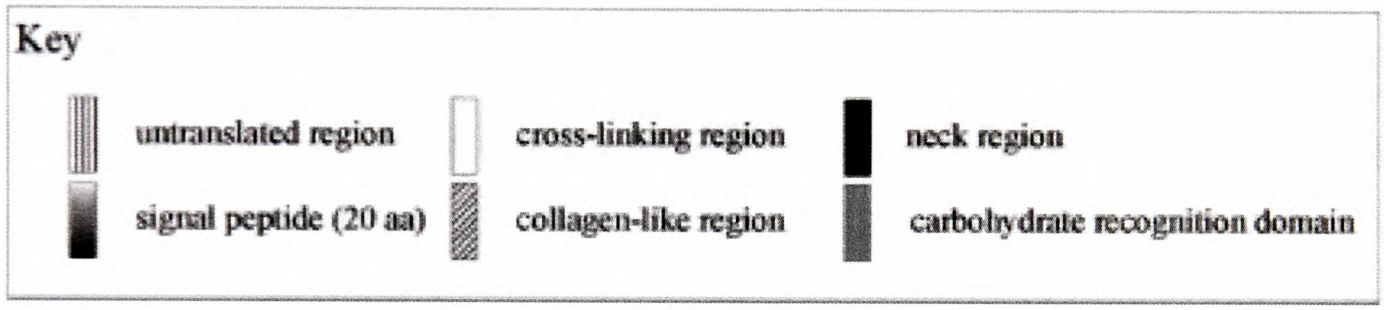


Figure 51: The polymorphisms of MBL (Petersen, Thiel et al. 2001)

Figure 51 depicts the MBL gene. The promoter polymorphisms are found at position –550 (H/L) and –221 (X/Y). The 5' UTR polymorphism is found at +4 (P/Q) and the coding polymorphisms are denoted by A (wild type), D (mutation at codon 52), B (mutation at codon 54) and C (mutation at codon 57). The boxes represent the genes which relate to the structure of MBL shown in Figure 50 with exon 1 encoding the signal peptide, cross-linking region and collagen-like region. Exon 2 also encodes the collagen-like region. Exons 3 and 4 encode the neck region of MBL with exon 4 encoding the CRD region.

6.3.3.1 Structural polymorphisms

The first SNP to be described was found at nucleotide position 230 (Sumiya, Super et al. 1991). The SNP is located at codon 54 and is termed either 54 or B. This SNP is associated with a glycine to aspartic acid amino acid substitution (GGC to GAC) at residue 34 in the mature protein (Sumiya, Super et al. 1991). The aspartic acid molecule in the mutant produces a bulkier side chain than the wild type glycine. As this mutation is found in the CRD region of MBL, the collagenous helix is distorted. This disrupts the secondary structure of MBL. This mutant protein is able to form higher order oligomers which differ from wild type oligomers (Super, Gillies et al. 1992). The subunits dissociate faster and approximately 95% of the protein is degraded (Kurata, Cheng et al. 1993; Lipscombe, Sumiya et al. 1995). The mutant protein is unable to polymerise or fix complement efficiently (Lipscombe, Sumiya et al. 1995) probably due to defects in binding to the MASP proteins (Kurata, Cheng et al. 1993; Wallis and Dodd 2000).

The concentration of MBL found in the serum of these individuals is well characterised in Caucasians. The mean level for wild type homozygote is 1,630 ng/ml, 358 ng/ml for wild type/mutant heterozygotes, and $\leq$10 ng/ml for mutant homozygotes (Lipscombe, Sumiya et al. 1992). Heterozygotes have an intermediate amount of MBL because they are able to produce both the wild type higher order oligomers and the lower order, mutant oligomers. Homozygous mutants have an undetectable amount of MBL because only the lower order oligomers, which degrade rapidly, are produced. This polymorphism has been detected in high numbers in Eurasian populations, but not African populations. The recorded gene frequencies for the 54 mutant is 0.17 in British Caucasians; 0.11 in Hong Kong Chinese; 0.13 in Danish and 0.13 in Eskimos (Garred, Madsen et al. 1992; Lipscombe, Sumiya et al. 1992). Because the mutation is relatively high in these populations and non-existent in other populations, it has been speculated that this mutation may have arisen before the

separation of ancestoral Chinese and European populations approximately 40,000 years ago (Lipscombe, Sumiya et al. 1992).

The second structural mutation is found at residue 37 in the mature protein (Lipscombe, Sumiya et al. 1992). This is the C mutation and is found at codon 57, nucleotide position 239 within the CRD region. The mutation causes a change from glycine to glutamic acid (GGA to GAA), another bulky amino acid. The resulting change in the secondary structure of MBL is analogous to the B mutation as it disrupts the sixth collagenous Gly-Xaa-Yaa repeat. This second SNP is found in high numbers in African populations, with a frequency of 0.29 in Gambians (Lipscombe, Sumiya et al. 1992) and 0.23 in Kenyans (Madsen, Garred et al. 1994), but is infrequent in Eurasian populations. The amount of serum MBL in Gambian cord blood and adults were shown to have median levels ranging from 1790 to 1915 ng/ml in homozygous wild types; 186 to 218 ng/ml in heterozygotes and $\leq$10 ng/ml in homozygous mutants (Madsen, Garred et al. 1994).

The third mutation found in the structural domain of MBL is at residue 32 in the mature protein. The mutation is termed D and is located at codon 52 (nucleotide 223). The mutation is outside the CRD region and causes a change from arginine to cysteine (GCG to GTG) (Madsen, Garred et al. 1994). This mutation differs from the previous two because the cysteine residue is no bulkier than the arginine. Additional disulphide bonds are formed in the amino terminal of the polypeptide. The structure is destabilised and the correct assembly of polypeptide chains are disrupted so a functional protein is not produced. This results in smaller oligomeric forms which are unable to activate complement efficiently (Wallis and Dodd 2000). The D mutation is found in Caucasians, Danes, Gambians and Kenyans at a low frequency of approximately 0.05; it has not been detected in Eskimos (Madsen, Garred et al. 1994; Turner 1998).

Individuals who have the wild type bases at these three structural codominant polymorphic sites are termed A; those with a mutation at codon 54 are B; a mutation at codon 57 are C, and those with a mutation at codon 52 are D. Individuals with two mutations (either homozygous at 1 position or heterozygous at 2 positions) are termed O and have undetectable amounts of MBL in their serum. People who are heterozygous for one mutation have approximately 10% of the MBL plasma concentrations found in wild type individuals. Individuals who are homozygous at one position or heterozygous at two positions have plasma concentrations <1% compared to wild type individuals (Madsen, Garred et al. 1994; Lipscombe, Sumiya et al. 1995; Turner 1996). Therefore, for many purposes individuals who have two mutant alleles (either two heterozygotes or a homozygote) are termed functional mutant homozygotes and have extremely low levels of MBL circulating in their plasma (Bellamy, Ruwende et al. 1998; Roy, Knox et al. 2002).

6.3.3.2 Promoter polymorphisms

Most occurrences of major deficiencies in serum MBL levels are attributable to the structural polymorphisms described above. However, these do not explain the large variations which occur in serum MBL between individuals of the same ethnic group. Because of these interindividual differences, Madsen *et al.* (Madsen, Garred et al. 1995) examined the MBL gene for other polymorphisms. They found two SNPs in the MBL promoter and one in the 5' UTR of exon 1 which correlated with different serum MBL levels. These promoter polymorphisms are thought to down regulate the expression of the MBL protein (Madsen, Garred et al. 1995). The promoter SNPs are found at positions –550 (the H/L polymorphism (TGT to TCT)) at -221 (the X/Y polymorphism (CCG to CGG)) and +4 (P/Q polymorphism (TCG to TTG)). The X/Y polymorphism reportedly has the largest influence on the amount of MBL transcribed (Madsen, Garred et al. 1995).

The H/L and X/Y polymorphisms have been extensively examined. The HY haplotype results in high levels of MBL in the serum; LY results in intermediate levels and LX results in low levels (Madsen, Garred et al. 1995). The LX haplotype has also been shown to have a dominant effect on the MBL levels (Madsen, Garred et al. 1995) with a change from Y to X decreasing the MBL levels more than H to L. The P/Q polymorphism has not been associated with changes in MBL concentrations independently, but may be involved in their acute phase induction (Madsen, Garred et al. 1995). The gene frequencies of the promoter haplotypes in Caucasians are approximately 0.31 for H; 0.69 for L; 0.31 for X and 0.69 for Y (Crosdale, Ollier et al. 2000). The frequency for Q is 0.24 in Caucasians and 0.48 in Africans (Madsen, Garred et al. 1995).

6.3.3.3 Haplotyping

Neither the structural polymorphisms or the promoter polymorphisms should be considered alone as both may independently have a marked effect on the amount of MBL circulating in the serum. For example, a report has shown that individuals who are homozygous for the D variant in the coding region still show an intermediate amount of MBL in their serum because this polymorphism is in LD with the high producing MBL promoter haplotype (HY) (Madsen, Garred et al. 1995). Also individuals homozygous for the B or C variants have medium levels of MBL levels due LD with the LY alleles in the promoter (Madsen, Garred et al. 1995). The low MBL producing homozygous LX haplotype together with wild type structural variants are able to down regulate the expression of MBL as effectively as two structural mutants (Madsen, Garred et al. 1995; Steffensen, Thiel et al. 2000).

The frequencies of the promoter variants differ between ethnic groups. The HY haplotype is frequent in Eskimos at 0.83. Caucasians have a frequency of 0.33 for HY, 0.39 for LY

and 0.24 for LX. Africans differ again with frequencies of 0.08 for HY, 0.23 for LY and 0.23 for LX (Madsen, Garred et al. 1995). The P variant has been shown to be present in both high and low expressing haplotypes, though the Q allele is associated with higher circulating levels of MBL in the serum of a Mozambique population (Madsen, Satz et al. 1998).

To examine the combined haplotypes of both the promoter and structural polymorphisms the *cis/trans* location of the promoter variant relative to the structural variants is important. There are a number of methods available to determine the location of the promoter variants. These include nested PCR, RFLP, sequence specific primer PCR (SSP-PCR), site directed mutagenesis, sequence specific oligonucleotide hybridisation, DNA heteroduplexes and allele specific PCR (Madsen, Garred et al. 1994; Madsen, Garred et al. 1995; Jack, Dodds et al. 1998; Madsen, Satz et al. 1998; Steffensen, Thiel et al. 2000).

Considering the six biallelic polymorphisms, there are 2^6 (64) possible MBL haplotypes. A report examined a case-control study of 203 individuals for all the possible haplotypes by performing 48 PCR reactions for each DNA and detected only 7 haplotypes (Mullighan, Marshall et al. 2000). This occurs because some of the structural polymorphisms are found in LD with the promoter polymorphisms. The four promoter haplotypes found in LD with the wild type structural polymorphism (A) are HYP, LYP, LXP and LYQ. Also, HYP is *cis* to D, LYP *cis* to B and LYQ *cis* to C (Mullighan, Marshall et al. 2000; Neth, Hann et al. 2001). The serum MBL concentration also varies with haplotype (Table 37) (Madsen, Satz et al. 1998). Serological methods are available to detect the amount of MBL in the serum but they are unable to distinguish between functional and non-functional MBL (Garred, Madsen et al. 2000). It has been suggested that only the promoter variants *cis* to a wild type coding variant contributes to genetic selection (Madsen, Garred et al. 1995).

High producing	Intermediate	Low producing	Defective	Not reported
HYPA	LYPA	LXPA	LYPB	LXQA
LYQA		LYQA	LYQC	HXPA
		HYPO	HYPD	
		(LXQO)	LYPO	
			LXPO	

Table 37: Serum MBL concentrations associated with different haplotypes (Madsen, Satz et al. 1998)

5.3.4 Polymorphisms and Disease

Since the first report suggesting the association between low serum MBL levels and an increased risk of disease (Sumiya, Super et al. 1991), there have been a number of conflicting reports. There are no clear associations between MBL deficiency and susceptibility to disease caused by specific pathogens. In many cases the results have been conflicting or equivocal due to the small sample sizes used, a marginal statistical significance, the complexity of the genotyping, a low frequency of homozygotes in the control group, ethnic heterogeneity or lack of HWE in the controls (Thomas, Foster et al. 1996; Garred, Richter et al. 1997; Summerfield, Sumiya et al. 1997; Hibberd, Sumiya et al. 1999; Tang and Kwiatkowski 1999).

MBL deficiency has been associated with recurrent infections both in infancy and in adult life (Sumiya, Super et al. 1991; Garred, Madsen et al. 1995; Summerfield, Ryder et al. 1995). Insufficiency has also been associated with an increased relative risk of a wide range of acute respiratory tract infections in children aged 6 to 17 months, with no effect observed at 18 to 23 months (Koch, Melbye et al. 2001). This observation is in agreement with the window of vulnerability hypothesis, with MBL affecting older children or adults as a disease modifying locus in an already established disease or immunodeficiency. Low levels of MBL are associated with an increased risk of TB in HIV positive patients (Garred, Richter et al. 1997) and infection in chronic Hepatitis B patients (Thomas, Foster

et al. 1996). An increased number of people with autoimmune diseases (Mullighan, Marshall et al. 2000) such as SLE (Davies, Snowden et al. 1995; Lau, Lau et al. 1996; Sullivan, Wooten et al. 1996; Davies, Teh et al. 1997), rheumatoid arthritis (Graudal, Homann et al. 1998; Garred, Madsen et al. 2000; Graudal, Madsen et al. 2000) and cystic fibrosis (Garred, Pressler et al. 1999) have been reported with MBL deficiencies.

Hibberd *et al.* (Hibberd, Sumiya et al. 1999) found the number of homozygous MBL variants was higher in patients with meningococcal disease compared to controls in a study performed both in the hospital and community. Also a study of host susceptibility factors to severe *S. pneumoniae* disease found that functional mutant homozygotes were more frequent in cases compared to controls (Roy, Knox et al. 2002).

Publication bias limits the number of published negative findings, leading to difficulty in defining the specific infections with which MBL deficiency is associated. MBL deficiency has been shown not to be associated with Malaria, Hepatitis B carriage or TB in an African population (Bellamy, Ruwende et al. 1998) or chronic Hepatitis B in a German population (Hohler, Wunschel et al. 1998).

Many researchers have suggested that infusions of MBL might be a useful treatment for patients with abnormal infections associated with a low level of MBL in the serum. To date, only two patients have received MBL infusions, this resulted in the restoration of full opsonic activity in both patients (Valdimarsson, Stefansson et al. 1998). Work with specific infections needs to be performed to examine which infections MBL infusions would be useful. Clearly a positive association between deficiency and a specific disease would be required to justify such studies.

5.3.5 Importance in Staphylococcal Infections

Because no host susceptibility studies have previously been performed in *S. aureus* infections, the closest comparison at present is work carried out on another Gram positive organism, *S. pneumoniae*. In severe *S. pneumoniae* infection, functional mutant homozygotes are more frequent in cases compared with controls (12% compared to 5%) (Roy, Knox et al. 2002).

Indirect evidence suggesting a role for MBL deficiency in susceptibility to *S. aureus* disease includes the ability of a variety of *S. aureus* strains to bind MBL. MBL binds unequivocally to different strains of *S. aureus*, *Candida albicans*, *A. fumigatus* and β haemolytic group A streptococcus (Neth, Jack et al. 2000). The same workers found C4 deposition on the surface of *S. aureus* to be proportional to MBL concentrations (Neth, Jack et al. 2000).

The presence of a capsule has been suggested to inhibit binding to MBL (van Emmerik, Kuijper et al. 1994). A comparison between a wild type *N. meningitidis* strain and a mutant with a truncated lipo-oligosaccharide (LOS) structure showed that the LOS structure, not the presence of a capsule was a major determinant for binding to MBL, although the capsule is likely to be slightly protective (Jack, Dodds et al. 1998).

The clinical syndromes associated with MBL deficiency include disorders in which *S. aureus* is likely to be the causative organism. These include dermatitis, pharyngeal abscesses, recurrent skin abscesses and recurrent otitis media (Miller, Seals et al. 1968; Soothill and Harvey 1976; Super, Thiel et al. 1989; Valdimarsson, Stefansson et al. 1998). This evidence suggests that MBL is important in protecting individuals against severe *S. aureus* disease.

5.3.6 Methods

The PCR and LDR methods used to examine the coding polymorphisms of MBL were designed and optimised by Dr. Jodene Fitness working in the Hill group laboratory, Oxford. The PCR primers were designed to include all three coding polymorphisms. The primers and conditions for the PCR are shown in Appendices D5 & D6. A 15µl reaction was performed for each sample containing 50ng of genomic DNA, 1x PCR buffer A (without $MgCl_2$), 2mM $MgCl_2$, 0.32mM dNTPs, 0.12µM of each primer and 0.2U/µl AmpliTaq Gold DNA polymerase. The products were examined on a 1.5% agarose gel stained with 50mg/µl ethidium bromide under UV fluorescence. The remaining Taq DNA polymerase activity was removed from the reaction and the common LDR probes were phosphorylated. The LDR reactions were performed separately at 73°C for the D polymorphism and 64°C for the B and C polymorphisms (see Appendix C3). The products were then pooled in a 1:1:1 reaction where 1.2µl of the pooled reaction together with 3µl of TAMRA loading dye as a size standard to control for the length of the products were run on the ABI 373 sequencer.

The PCR and LDR primers for the promoter polymorphisms were designed and optimised by Drs Suchismita Roy and Patricia Ramaley working in the Hill group laboratory, Oxford (Madsen, Garred et al. 1995). The PCR primers were designed to include the three promoter polymorphisms. The primers and conditions for the PCR are shown in Appendices D5 & D6. The 15µl PCR reactions contained 50ng of genomic DNA, 1x PCR buffer, 2mM $MgCl_2$, 0.32mM dNTPs, 0.12µM of each primer and 0.2U/µl AmpliTaq Gold DNA polymerase. The products were examined on a 1.5% agarose gel stained with 50mg/µl ethidium bromide under UV fluorescence. The Taq polymerase was removed after the PCR reaction; the common LDR probes were phosphorylated and the LDR

reaction was performed at 66°C separately for each polymorphism (Appendix C3). The products were pooled in a 1:1:1 reaction and 1.2µl of the pooled reaction together with TAMRA loading dye were run on an ABI 373 sequencer. Any results which were difficult to analyse using LDR were sequenced directly on an ABI 3700.

The P/Q results from the LDR deviated badly from HWE, an RFLP technique was subsequently developed to analyse the SNP. The primers and PCR conditions are in Appendices D5 & D6. The forward primer was designed with a base change (T to A) at the third base at the 3' end. This created a *Hind* III site because of the 'T' base in the Q allele, but not the 'C' base in the P allele. Each PCR product was examined on a 1.5% agarose gel stained with 50mg/µl ethidium bromide under UV fluorescence. Digestion was performed in a 20µl volume consisting of 10µl of the PCR product together with 1 unit per reaction of *Hind*III (New England Biolabs, Hutchin) and 1 unit of Buffer 2 (NEB). The products were digested overnight at 37°C, then run for 2 hours on a 3% agarose gel which was stained with 50mg/µl ethidium bromide and examined under UV fluorescence. The results were recorded as homozygous for P if there was a single band present at 210bp, heterozygous if 3 bands were present at 210, 193 and 17bp and homozygous for Q if two bands were present at 193 and 17bp. The results for each SNP was in HWE.

5.3.7 Results

5.3.7.1 MBL coding region polymorphism results for the Oxford case-control study

Genotype for 54	Case n (%)	Control n (%)	Genotype for 57	Case n (%)	Control n (%)	Genotype for 52	Case n (%)	Control n (%)
G/G	307 (74)	509 (75)	G/G	395 (95)	646 (95)	C/C	359 (87)	587 (87)
G/A	101 (24)	158 (23)	G/A	18 (5)	32 (5)	C/T	43 (10)	78 (12)
A/A	6 (1)	11 (2)	A/A	1 (0)	0 (0)	T/T	12 (3)	13 (2)
Total	414	678		418	678		414	678
χ^2	0.21			1.72			1.37	
P value	0.90			0.42			0.50	

Table 38: Oxford MBL coding region polymorphism genotyping results

Allele for 54	Case n (%)	Control n (%)	Allele for 57	Case n (%)	Control n (%)	Allele for 52	Case n (%)	Control n (%)
G	715 (86)	1176 (87)	G	808 (98)	1324 (98)	C	761 (92)	1252 (92)
A	113 (14)	180 (13)	A	20 (2)	32 (2)	T	67 (8)	104 (8)
Odds ratio	0.97			0.98			0.94	
Relative risk	0.98			0.99			0.96	
χ^2	0.06			0.01			0.13	
P value	0.80			0.93			0.72	

Table 39: Oxford MBL coding region polymorphism allele results

Genotype	Case n (%)	Control n (%)
Wild type (A)	252 (61)	409 (60)
Single het (A/O)	135 (32)	233 (34)
Double het/mut (O/O)	27 (7)	36 (5)
Total	414	678
χ^2	0.90	
P value	0.637	

Table 40: Oxford MBL coding region polymorphism 'Functional mutant' results

5.3.7.2 MBL promoter polymorphism results for the Oxford case-control study

Genotype for H/L	Cases n (%)	Control n (%)	Genotype for X/Y	Cases n (%)	Control n (%)	Genotype for P/Q	Cases n (%)	Control n (%)
L/L	187(46)	298 (44)	Y/Y	229 (56)	417 (62)	P/P	246 (59)	375 (55)
L/H	176 (43)	296 (44)	X/Y	160 (39)	235 (35)	P/Q	138 (33)	261 (38)
H/H	47 (12)	79 (12)	X/X	20 (5)	23 (3)	Q/Q	30 (7)	42 (6)
Total	410	673		411	675		414	678
χ^2	0.18			4.14			3.07	
P value	0.91			0.13			0.22	

Table 41: Oxford MBL promoter polymorphism genotyping results

Allele for H/L	Case n (%)	Control n (%)	Allele for X/Y	Case n (%)	Control n (%)	Allele for P/Q	Case n (%)	Control n (%)
L	550 (67)	892 (66)	Y	618 (76)	1069 (79)	P	630 (76)	1011 (75)
H	270 (33)	454 (34)	X	200 (24)	281 (21)	Q	198 (24)	345 (25)
Odds ratio	1.04			0.81			1.09	
Relative risk	1.02			0.88			1.05	
χ^2	0.15			3.90			0.64	
P value	0.70			0.048			0.42	

Table 42: Oxford MBL promoter polymorphism allele results

There were no significant associations between any of the coding region or promoter polymorphisms and disease (Tables 38 to 42), though there was a trend towards an association with the X/Y promoter polymorphism (p=0.05). The frequencies were in agreement with previous work MBL polymorphisms in Caucasian populations (Crosdale, Ollier et al. 2000; Steffensen, Thiel et al. 2000).

Haplotype	Case n (%)	Control n (%)
LYPA	95 (11.8)	118 (8.9)
LXPA	152 (18.8)	247 (18.5)
LYQA	154 (19.1)	293 (22.0)
LXQA	11 (1.4)	7 (0.5)
HYPA	200 (24.8)	365 (27.4)
HXPA	6 (0.7)	7 (0.5)
HYQA	3 (0.4)	0
HXQA	5 (0.6)	0
LYPO	97 (12.0)	168 (12.6)
LXPO	16 (2.0)	13 (1.0)
LYQO	4 (0.5)	8 (0.6)
LXQO	0	0
HYPO	42 (5.2)	78 (5.9)
HXPO	4 (0.5)	0
HYQO	3 (0.4)	0
HXQO	0	0
P value	0.63	

Table 43: EM algorithm-derived imputed haplotype for MBL polymorphisms

No significant differences were observed between imputed haplotype frequencies in the cases and controls (Table 43). Five of the possible haplotypes were not seen using these statistical methods (HYPO, HXPO, HYQO, HXQO and LYPO). Separating the haplotypes into putative high /intermediate MBL producers, low producers and MBL deficient groups, there was no association between these three groups and disease (p=0.74).

	Coding polymorphisms	Promoter polymorphisms	All 6 SNPs
All	0.46	p<0.001	p<0.001
CAD	0.81	p<0.001	p<0.001
HAD	0.19	p<0.001	p<0.001

Table 44: p values for Linkage Disequilibrium for MBL polymorphisms in the Oxford study

Table 44 shows the amount of LD which occurred i) between the coding polymorphisms ii) between the promoter polymorphisms and iii) between all six polymorphisms. The data was subdivided into all of the cases, the CAD subgroup and the HAD subgroup alone. LD

was examined using '*hapipf*' in Stata. No LD was found between the coding polymorphisms in any of the disease groups which is consistent with previous data where the B (codon 54) mutation was only found in Eurasian populations, the C mutation (codon 57) was only found in African populations and the D mutation (codon 52) was only found in low levels in any population. All of the promoter polymorphisms were in LD and LD was found between the promoter polymorphisms and the coding polymorphisms which is consistent with previous findings (Madsen, Satz et al. 1998). Subgroup analysis splitting cases into CAD and HAD revealed no differences: no associations with disease were found for the coding region and promoter polymorphisms, 'functional mutant' polymorphisms or for the imputed haplotypes (Table 44).

In the absence of any positive associations in the Oxford case-control study, genotyping of the Thai case-control study was deferred.

5.3.8 Conclusions

i. In the Oxford case-control study there were no associations between coding region or promoter polymorphisms and severe *S. aureus* disease, either overall or in the CAD and HAD subgroups.

ii. Analysis of EM algorithm-generated haplotypes showed no disease associations

iii. The promoter polymorphisms were in LD, but the coding region polymorphisms were not.

5.3.8 Discussion

Despite being an apparently excellent candidate to be an important susceptibility factor for *S. aureus* disease, and despite significant associations with infections with other pathogens such as *S. pneumoniae*, no associations were found between MBL and *S. aureus* disease. A potential limitation of the methodology used is the lack of direct *trans/cis* analysis for haplotyping. However the EM algorithm generated haplotypes with frequencies consistent with those found using direct methods in previous studies (Mullighan, Marshall et al. 2000; Steffensen, Thiel et al. 2000), and has in other settings been shown to perform very well when compared with the gold standards of direct methods (Fallin and Schork 2000; Fallin, Cohen et al. 2001).

The lack of LD between the coding polymorphisms is unsurprising as the B mutation only occurs in Eurasian populations, the C mutation only occurs in African populations and the frequency of the D mutant is very low in all populations.

This study has not addressed the issue of whether MBL is important in disease susceptibility specifically in early life, when many MBL deficiency-related infections have been reported (Summerfield, Sumiya et al. 1997). Although subgroup analysis in this age-group revealed no significant results, the number of paediatric cases in the current series is very small, and the study consequently underpowered.

5.4 Toll like receptor 2 (TLR2)

5.4.1 Biological importance

The toll receptors are ancient, conserved proteins that form an important part of the innate immune response to a variety of stimuli including bacteria, viruses and fungi. Toll was first described in *Drosophila* (dToll). Although originally described as a receptor required for dorsal-ventral patterning and polarity of the embryo during development (Anderson, Bokla et al. 1985; Anderson, Jurgens et al. 1985), it also controls the immune response to fungal infections in adult flies (Lemaitre, Nicolas et al. 1996). A human homologue to Toll was first cloned and described by Medzhitov *et al.* (Medzhitov, Preston-Hurlburt et al. 1997). Termed hToll, this was later changed to Toll-like receptor 4 (TLR4). Since the first description, a total of 10 TLRs have been discovered in the human and mouse public databases; this number is likely to increase (Akira, Takeda et al. 2001).

The Toll system in *Drosophila* is able to distinguish between different invading pathogens to trigger the appropriate response (Lemaitre, Nicolas et al. 1996). Given the number of TLRs found in the human genome, it is probable that humans also have TLRs with varying specificities for invading pathogens. Early research used over-expression studies of the proteins and concluded that TLR2 but not TLR4 was involved in recognition to LPS (found on the surface of Gram negative bacteria) (Kirschning, Wesche et al. 1998; Yang, Mark et al. 1998). This was later refuted using gene knockout experiments where the *Tlr2* knockout mice displayed the same response as wild type mice to LPS (Takeuchi, Hoshino et al. 1999). They found that *Tlr2* -/- mice responded to LPS and LTA, but not PGN, this suggested that TLR2 responds to Gram positive bacteria. *Tlr4* -/- mice responded to PGN but not LPS or LTA suggesting that TLR4 responds to Gram negative infections (Takeuchi, Hoshino et al. 1999). TLR2 is also involved in cellular responses to PGN and

LTA from Gram positive organisms (Schwandner, Dziarski et al. 1999; Yoshimura, Lien et al. 1999; Flo, Halaas et al. 2000).

Some of the TLRs are able to respond to more than one PAMP molecule. Others operate together and are able to recognise different combinations of PAMPs (Ozinsky, Underhill et al. 2000). Different combinations of TLRs may be important for host immunity. TLR2 and TLR6 are both recruited to the macrophage phagosome to recognise PGN and zymosan (Akira, Takeda et al. 2001; Hallman, Ramet et al. 2001). TLR2 functions alone or with another receptor (not TLR6) to detect bacterial lipopeptides (Akira, Takeda et al. 2001). Although the dimerisation of TLR2 is unable to induce cytokine production in a macrophage cell line, the cytoplasmic portion of TLR2 can functionally pair with TLR6 or TLR1 which results in the production of cytokines (Akira, Takeda et al. 2001).

The innate immune response is important in influencing the type of adaptive immune response through the production of immunomodulatory genes regulated by TLR activation. These genes include iNOS and IL-12 which acts as a biological adjuvant amplifying the T-cell responses to pathogens (Brightbill and Modlin 2000).

5.4.2 Structure and Function

The TLR family of genes are dispersed throughout the human genome. TLR 1 and 6 map to 4p14; TLR2 and 3 map to 4q31.3-35; TLR4 maps to 9q32-33; TLR5 to 1q33.3-42; TLR7 and 8 to Xp22 and TLR9 to 3p21.3. TLRs are found expressed on the surface of macrophages and dendritic cells. They are also found on other cells such as epithelia lining the gut and nasal passages (Brown 2001). TLR2 specifically is expressed on brain, dendritic cells, heart, liver, lung, monocytes, muscle, peripheral blood lymphocytes and importantly on PMN (Chaudhary, Ferguson et al. 1998; Rock, Hardiman et al. 1998; Muzio, Bosisio et al. 2000). TLR2 is imported to the macrophage phagosome where the

protein recognises a number of PAMPs such as LTA, lipoproteins and PGN triggering an inflammatory response (Underhill, Ozinsky et al. 1999). TLR2 and TLR6 can physically associate through their extracellular domains to recognise PGN within the macrophage (Ozinsky, Smith et al. 2000).

The TLR family are type 1 membrane proteins. They are characterised by their exracellular domain containing leucine-rich repeats (LRR). The ectodomain also contains small cysteine-rich domains; different members of the TLR family have different numbers of cysteine domains (Kopp and Medzhitov 1999). They have a signal transmembrane domain and a cytoplasmic domain responsible for signal transduction. The cytoplasmic domain is homologous to the cytoplasmic domain of the IL-1 receptor (IL-1R) family and is called the Toll/IL-1R homology (TIR) domain (Kopp and Medzhitov 1999; Hallman, Ramet et al. 2001).

Because the TLRs have only recently been described, a lot remains unknown. For example, it is not currently known whether there is a direct interaction between the bacterial products and TLRs or whether an endogenous ligand is activated on meeting the microbial ligand. *Drosphila* Toll does not bind bacterial products directly. In *Drosphila*, a protease cascade is activated which leads to extracellular cleavage of the Toll ligand Spaetzle (Levashina, Langley et al. 1999). Opposed to this, work suggests that direct actions occur in humans (Poltorak, Ricciardi-Castagnoli et al. 2000). Weak interactions between TLR2 and LPS have been demonstrated (Yang, Mark et al. 1998). On activation, the TLR family activate the NFκB pathway which in turn regulates the activity of genes producing cytokines such as TNFα and IL-1 (Brown 2001).

The TLR4 signalling pathway was the first to be described, hence it is the most extensively examined Toll receptor. Information has concentrated on the signalling pathway. TLR4 is

involved both in a myeloid differentiation factor 88 (MyD88) dependent and independent pathways (Underhill, Ozinsky et al. 1999). The MyD88 dependent pathway is the most extensively studied. TLR4 binds to LPS on the surface of Gram negative bacteria using helper molecules. LPS is complexed with LPS binding protein (LBP) on the surface of bacteria. This complex is recognised by CD14 molecules carried by macrophages (Aderem and Ulevitch 2000). It is probably a complex of LPS, LBP and CD14 that activates TLR4 (Ozinsky, Underhill et al. 2000). Another helper molecule, MD-2 also interacts with TLR4 and this is also thought to facilitate LPS binding (Schimazu, Akashi et al. 1999; da Silva Correia, Soldau et al. 2001). The MD-2 molecule is physically associated with the extracellular domain of TLR4 on the cell surface (Hallman, Ramet et al. 2001).

An adaptor protein, MyD88 is recruited upon activation of the TIR domain (Muzio, Ni et al. 1997; Wesche, Henzel et al. 1997; Medzhitov, Preston-Hurlburt et al. 1998; Muzio, Natoli et al. 1998). MyD88 recruits a serine/threonine protein kinase: IL-1R accessory protein kinase (IRAK) upon stimulation. This results in the autophosphorylation of IRAK (Muzio, Ni et al. 1997; Wesche, Henzel et al. 1997; Adachi, Kawai et al. 1998; Muzio, Natoli et al. 1998; Medzhitov and Janeway 1999; Yang, Mark et al. 1999). The phosphorylated IRAK dissociates from the receptor complex and activates TNF receptor-associated factor 6 (TRAF6) (Cao, Xiong et al. 1996). This results in the oligomerisation of TRAF6 which leads to the activation of mitogen-activated protein (MAP) kinase kinase kinases (MAPKKK) (Ninomiya-Tsuji, Kishimoto et al. 1999). This activates IκB kinases and NFκB-inducing kinase (NIK) (Song, Regnier et al. 1997; Ninomiya-Tsuji, Kishimoto et al. 1999).

In resting cells, inhibitory proteins (IκB) sequester NFκB into the cytoplasm. The activation of these IKKs frees the NFκB from the inhibitory molecules (DiDonato,

Hayakawa et al. 1997; Stancovski and Baltimore 1997; Takeuchi and Akira 2001). NFκB translocates to the nucleus where it cooperates with many other transcription factors inducing the expression of a wide variety of target genes essential for the activation of T cells (Fearon 1997; Karin and Ben-Neriah 2000). This produces many cytokines and costimulatory molecules needed to regulate the host response to invading pathogens such as TNFα and IL-12 (Cao, Xiong et al. 1996; Malinin, Boldin et al. 1997; Baeuerle 1998).

The signalling cascades in the other TLRs remain to be described. Some information about TLR2 is known, for example TLR2 also binds CD14 for immune recognition (Gupta, Kirkland et al. 1996; Dziarski, Tapping et al. 1998; Schwandner, Dziarski et al. 1999). MyD88 is also involved with TLR2 signalling (Aliprantis, Yang et al. 2000), though unlike TLR4 no MyD88 independent pathway has been described. The signal transduction molecules MyD88, IRAK, NIK and IKK are also necessary for TLR2 mediated NFκB activation in cells stimulated with micrococci and bacterial PGN (Wang, Dziarski et al. 2001).

5.4.3 Polymorphisms and disease

The two most widely described TLRs to date are TLR4 and TLR2. The TLR4 protein was found to have a mutation at position 712 changing a proline to a histidine (also termed the LPS^d domain) (Poltorak, Smirnova et al. 1998; Underhill, Ozinsky et al. 1999). The equivalent mutation in TLR2 (Pro681His) disrupts the signal transduction in response to stimulation by yeast and Gram positive bacteria including *S. aureus* (Underhill, Ozinsky et al. 1999). Also a substitution at amino acid position 677 (Arg677Trp) in the intracellular C-terminal position of TLR2 was found in patients with Lepromatous leprosy (Kang and Chae 2001).

Research performed by Lorenz *et al.* (Lorenz, Mira et al. 2000) suggested a link between a mutation and *S. aureus* disease. The group examined TLR2 for mutations in the open reading frame using a single-stranded conformation polymorphism method. They discovered three SNPs each at a frequency of 3% in healthy Caucasian blood donors. Two of the SNPs caused silent mutations where the amino acid was not altered (C to T at base 597 and T to C at base 1350). The third mutation was found at base 2251 where a G changed to an A; this resulted in an amino acid change from arginine to glutamine at residue 753, within the C-terminus of TLR2. Because the mutation is in the C-terminal it is likely to affect the signalling function of the molecule. Lorenz *et al.* (Lorenz, Mira et al. 2000) used a luciferase assay in 293 culture cells to examine the difference between a wild type TLR2 protein compared to a mutant protein, together with peptides from *Borrelia burgdorferi* (*B. burgdorferi*) and *Treponema pallidum* (*T. pallidum*). They recorded no response in the mutant cells and a 1.5 to 2 fold higher response in cells with the wild type protein. They concluded that the mutation reduces the ability to respond to bacterial peptides *in vitro*. In a case-control study comparing patients with septic shock to healthy blood donors they found the mutation to be present in 2 out of 91 patients with septic shock. When they subdivided the septic shock patients they found 22 patients had Gram positive infections: the two patients who were heterozygous for the mutant allele belonged to this subgroup. 9% of the group with Gram positive septic shock had the mutation in the TLR2 protein. Further examination revealed both of these patients were infected with Staphylococcus (although the sub-species was not named). This association may be due to chance. The sample size was small and the polymorphism was rare in the Caucasian population studied. These conclusions are intriguing in Staphylococcal infection and warrant a larger case-control investigation into the possibility of TLR2 mutations being involved in susceptibility to *S. aureus* infection.

5.4.4 Importance in *S. aureus* infections

Apart from the above, rather weak association between staphylococcal sepsis and the mutation at nucleotide position 2251 in TLR2, there are other lines of evidence to suggest an important role for TLRs in susceptibility to *S. aureus* infection.

Results with gene knockout mice suggest that TLR2 is involved in protection against PGN in Gram positive infections, with TLR4 involved in protection against LPS in Gram negative infections. Host response is presumably dependent on the pathogen. Although TLR2 fails to respond to LPS on Gram negative bacteria, TLR2 is involved in the response to LPS from Leptospira and Porphyromonas which are structurally unrelated to the LPS of Gram negative bacteria (Hirschfeld, Ma et al. 2000; Werts, Tapping et al. 2001). TLR2 also responds to lipoproteins derived from TB, *B. burgdorferi*, *T. pallidum* and *Mycobacterium fermentans* (Aliprantis, Yang et al. 1999; Brightbill, Libraty et al. 1999; Hirschfeld, Kirschning et al. 1999; Takeuchi, Kaufmann et al. 2000).

Mice deficient in TLR2 show an impaired production of TNFα, IL-6 and nitric oxide in response to a number of Gram positive bacterial cell walls. One of the proteins tested was *S. aureus* PGN (Takeuchi and Akira 2001). TLR2 deficient macrophages are hyporesponsive to *S. aureus* PGN and mycoplasmal lipopeptide. TLR4 deficient macrophages do not respond to Gram positive LTA (Takeuchi, Kaufmann et al. 2000). This suggests that TLR2 is essential for the activation of the intracellular signalling pathway elicited by PGN (Takeuchi and Akira 2001). Tissue culture work has shown that over-expression of TLR2 in 293 cells was responsive to *S. aureus*, *Bacillus subtilis* and PGN when NFκB activation was examined (Schwandner, Dziarski et al. 1999). Experiments using Chinese hamster ovary cells which expressed TLR2 (not TLR4) responded to *S. aureus* and *S. pneumoniae* in terms of NFκB DNA binding activity and the

surface CD25 expression (Yoshimura, Lien et al. 1999). Also TLR2 and MyD88 deficient mice have a higher mortality than wild type mice after inoculation with *S. aureus* (Takeuchi, Hoshino et al. 2000).

One hypothesis is that people with a diminished function in at least one TLR may be more likely to develop sepsis than their normal counterparts (Beutler and Poltorak 2001). This suggestion may explain the increased number of septic patients with Staphylococcal infection found by Lorenz *et al.* (Lorenz, Mira et al. 2000). The pathogenesis caused by shock is thought to be secondary to excessive stimulation of the host cells by different microbial constituents. The microbial molecules are potent activators of inflammatory cytokine synthesis. Production of cytokines is important in innate immunity, but their over-production is probably important in lethal septic shock.

5.4.5 Methods

An RFLP was designed to detect the coding SNP (+2251 in TLR2) described by Lorenz *et al.* (Lorenz, Mira et al. 2000). The published primers were used (Appendix D7). The PCR reaction consisted of a 15µl reaction containing 50ng genomic DNA, 1x PCR buffer, 2mM $MgCl_2$, 0.32mM dNTPs, 0.12µM of each primer and 0.2U/µl AmpliTaq Gold DNA polymerase (see Appendix D8 for PCR conditions). The products were examined on a 1.5% agarose gel stained with 50mg/µl ethidium bromide under UV fluorescence. Digestion of the products occurred overnight at 37°C in 20µl reactions containing 10µl PCR product, 2 units per reaction of *Pst* I and 2 units of NEBuffer 3. The digests were run on a 3% agarose gel for 2 hours then stained with 50mg/µl ethidium bromide under UV fluorescence. The resulting digests are shown in Table 45. Controls, known to be heterozygous or homozygous for the wild type or mutant alleles (using a sequencing

reaction), were provided by Dr. S. Segal. Any DNA shown to be heterozygous or homozygous for the mutant allele was directly sequenced for confirmation.

Wild Type homozygous	Heterozygous	Mutant homozygous
430	430	
	281	281
	149	149

Table 45: Fragment sizes (bp) produced by TLR2 +2251 RFLP

5.4.6 TLR2 genotyping results for the Oxford case-control study

Genotype	Case n (%)	Control n (%)
G/G	408 (95)	664 (95)
G/A	20 (5)	32 (5)
A/A	0	0
Total	428	696
χ^2	0.00	
P value	0.95	

Table 46: TLR2 +2251 SNP genotyping results for the Oxford case-control study

Allele	Case n (%)	Control n (%)
G	836 (98)	1360 (98)
A	20 (2)	32 (2)
Odds ratio	0.99	
χ^2	0.00	
P value	0.95	

Table 47: TLR2 +2251 SNP allele frequencies in the Oxford case-control study

The frequency of the polymorphism was consistent with that found by Lorenz *et al.* (Lorenz, Mira et al. 2000) at 4.6% (Tables 46 and 47). No homozygous mutants were observed.

The Thailand case-control study, consisting of 228 cases and 307 controls, was also typed for this SNP. The mutant allele was absent in all samples.

5.4.7 Conclusions

i. The +2251 SNP in the TLR2 gene was not associated with susceptibility to severe *S. aureus* disease, despite being previously reported as weakly associated with staphylococcal sepsis.

ii. The +2251 SNP in the TLR2 gene was absent in the Thai ethnic group.

5.4.8 Discussion

The TLR2 gene has recently been sequenced in 100 Caucasians; no polymorphism was found other than the +2251 SNP previously published by Lorenz *et al.* (Lorenz, Mira et al. 2000) (Dr. Shelly Segal, personal communication). A highly polymorphic $(CT)_nT(TG)_n$ repeat was found and typed in a case-control study of severe *S. pneumoniae* disease. One allele in the microsatellite (215bp) was found to be associated with severe *S. pneumoniae* disease. The known SNP was also typed, no association was seen with severe *S. pneumoniae* disease. Two new intronic polymorphisms have since been discovered (Dr. David Wiley, personal communication); these are currently under investigation for susceptibility in pneumococcal disease (Dr. Shelley Segal, personal communication).

The discovery of these new polymorphisms, and the apparent ubiquitous importance of the TLRs in defence against infection, suggest that the TLRs will be a rich vein for future investigations. Other TLRs of interest in *S. aureus* disease would be TLR1 and TLR6 because they may participate in the activation of macrophages for Gram positive pathogens (Hallman, Ramet et al. 2001). TLR6 is also necessary for responses to Gram positive PGN (Ozinsky, Underhill et al. 2000). Also TLR9 responds to the specific CpG found in bacteria (Hemmi, Takeuchi et al. 2000; Hallman, Ramet et al. 2001). No known polymorphisms have yet been described in these three genes but they remain possibilities for future study.

Chapter 6. Concluding Remarks

This work represents a systematic attempt to simultaneously study host genetic and bacterial factors leading to severe *S. aureus* disease. As severe *S. aureus* infection is rare, a case-control strategy was used, which enabled a large number of cases to be studied in a limited time frame. Over 600 cases were enrolled into the study in Oxford with the corresponding isolates and blood. A similar case-control study was performed in Thailand: i) to see whether the population of bacteria were similar to those found in Oxford and ii) to evaluate any positive findings in the host genetic study based in Oxford using a genetically distinct population where the incidence of disease was similar.

The first hypothesis postulated and addressed was that hypervirulent strains of *S. aureus* exist. Investigation of the population genetics of both the carriage and disease isolate populations using phage typing, PFGE and the novel sequence-based MLST revealed an essentially clonal structure which was similar in carriage and disease. The lack of identification of hypervirulent clones using any of these techniques suggested that it was unlikely that genes tightly linked to the genetic background of the organism could be involved in determining virulence potential.

In apparent contradiction to this finding a study has recently suggested that *arcC*, one of the housekeeping genes used in MLST may be involved in virulence (Dunman, Murphy et al. 2001). Using microarray expression technology it has been suggested that genes in the arginine deaminase pathway (*arc*A, *arc*B, *arc*C, *arc*D and the activatory *arc*R) are stimulated in an *agr* and a cell density-dependent manner (Dunman, Murphy et al. 2001). The researchers suggested that these might be virulence determinants as activation of this pathway in the presence of arginine allows *Bacillus licheniformis* to grow anaerobically. They hypothesised that this would be beneficial for pathogenesis in staphylococcal

infections. They also suggested the production of ammonia after utilization of arginine in the arginine deaminase would protect the bacteria from acidic environments (Dunman, Murphy et al. 2001). Work in Oxford (Dr. Ruth Massey, personal communication) has suggested that arginine deaminase is also important in anaerobic respiration in small colony variants of *S. aureus*. In the Oxford case-control study no particular *arc*C allele was more numerous in the cases compared to the controls. This gene cannot be fully discounted as a virulence determinant as there may be specific subtypes elsewhere in the *arc* gene rather than alleles in *arcC* which are associated with disease.

The finding of a largely clonal population structure in *S. aureus* is consistent with the previously published work. In particular the clonality of *S. aureus* strains causing toxic shock syndrome, and of MRSA strains has been well described (Musser, Schlievert et al. 1990; Musser and Kapur 1992; Fitzgerald, Sturdevant et al. 2001). The vast majority of *tst* containing strains and a large group of MRSA strains (EMRSA-16 or ST 36) fall within the same MLST clonal complex (CC30) in the current study. This may indicate that *tst* in particular is clonally restricted to strains with the same genetic background. MLST is proving a valuable tool for examining collections of strains isolated from diverse genetic backgrounds and has been used to characterise the spread of Methicillin resistance (Enright, Robinson et al. 2002), and to study strains collected over prolonged periods of time (Enright, Day et al. 2000).

MLST was also useful in defining the genetic background of isolates to enable a full analysis of the virulence determinant results in Chapter 4. The 7 candidate genes shown to be virulence-associated were not fully independent of the conserved housekeeping gene backbone of the organism, but were sufficiently incongruent that virulent genotypes containing these genes were not identified as hypervirulent clones in the typing studies. The technique used was limited in its ability to define the full extent of variation

associated with these loci, as only the presence or absence of genes was studied by the PCR technique employed for the majority of factors. Allelic variation at these sites may be an important determinant of disease, and warrants further investigation. With the publication of the complete genomes of three strains of *S. aureus* (N315, an MRSA strain and Mu50, a VRSA strain and MW2, a community MRSA strain) (Kuroda, Ohta et al. 2001; Baba, Takeuchi et al. 2002), it is now possible to interrogate genomic sequence for different SNPs in these genes. Also with the continued sequencing of the laboratory strains 8325 (MSSA) and COL (MRSA), the clinically isolated 252 (MRSA) and 476 (MSSA) strains (collected in this study), comparisons can be made between sequences, looking for SNPs in specific virulence determinants. With the availability of sequence data on so many isolates of *S. aureus*, including a strain causing bovine mastitis (Herron, Chakravarty et al. 2002), it would also be possible to perform genome-wide comparisons between strains as well as looking at SNPs.

A further seventy potential virulence determinants were suggested when strains N315 and Mu50 were compared (Kuroda, Ohta et al. 2001), a number which may increase further if the genome of a carriage strain was compared to that of a disease strain from a known clonal background. Because so many new virulence factors have been detected following the sequencing of *S. aureus*, the presence or absence of many more virulence factors could be determined in the Oxford collection of strains and examined for association with disease where the causative genes would be more likely to be found (rather than genes which may be in LD with the causative ones).

This work was not exhaustive; possible future work would be to examine the presence of the type 5 and type 8 capsular antigens on the surface of the bacteria. Studies suggest that 75% of disease strains contain one of these antigens, but no work has yet examined the

presence of these antigens in carriage strains. The Oxford collection of strains are ideal to examine the presence of these potentially protective antigens.

Currently there is no satisfactory animal model in which human diseases caused by *S. aureus* can be examined, though there are specific rabbit models for endocarditis and toxic shock syndrome (de Azavedo, Foster et al. 1985; Oramas-Shirey, Buchanan et al. 2001). This has meant that the function of most genes have been elucidated *in vitro* using knockout mutants and other molecular biological techniques (Phonimdaeng, O'Reilly et al. 1990; Sloane, de Azavedo et al. 1991; Greene, McDevitt et al. 1995). Putative virulence determinants and regulatory genes have been described through *in vitro* work. Recent work has suggested that one of the major regulatory genes shown to be of pivotal importance *in vitro* (*agr*) is not involved in regulation of virulence determinants *in vivo*, albeit in an animal model (Yarwood, McCormick et al. 2002). Developing *in vivo* or *ex vivo* techniques would be a valuable tool to aid the understanding of *S. aureus* pathogenesis. Future work should attempt to concentrate on the expression and regulation of genes *in vivo*, rather than in an artificial setting.

The work on the strains isolated in Thailand is ongoing. All of the strains (both cases and controls) were found to be more resistant than the strains isolated in Oxford, and the antibiogram profiles also differed from the Oxford strains. A subset of strains were examined using PFGE and MLST and were found to be distinct from the types isolated in Oxford, but the number was too small to constitute an adequate study. There are also at present insufficient control strains to compare with the cases.

The second hypothesis postulated was that certain people in the population are genetically more susceptible to *S. aureus* disease than others. This was tested in the host genetic branch of the study. Candidate genes: i) known to be involved in the host defence against bacteria and ii) possessing at least one functional polymorphism were investigated for

disease associations. The same collection of prospectively collected cases and controls as used for the bacterial branch were examined using the most likely candidate genes.

One of the first and most important immune cells attracted to an area of staphylococcal infection is the PMN. Individuals with PMN deficiencies are more susceptible to *S. aureus* infections (Fischetti, Novick et al. 2000). A number of different immune receptors are found on the surface of PMN. Two of these surface receptors, FcγRIIA and FcγRIIIB, have functional polymorphisms within their genes. Both of these receptors were examined in the Oxford case-control study. The FcγRIIA receptor has a functional polymorphism at amino acid position 131 and was independently associated with susceptibility to severe *S. aureus* disease, an association that was most significant in patients who acquired *S. aureus* disease in the community, and was absent in patients who acquired disease in hospital. Given that this finding fulfilled a prior hypothesis, it increased the probability that this FcγRIIA polymorphism was functional in *S. aureus* disease. On univariate analysis the FcγRIIIB polymorphism (known as NA1/NA2) alone was not associated with host susceptibility to *S. aureus* disease, but on multivariate analysis a strong additive x additive epistatic interaction with the FcγRIIA polymorphism was discovered. The role of epistasis in determining complex traits is controversial, as is its long term evolutionary contribution (Wolf, Brodie et al. 2000). However in this case the epistatic interaction appears plausible, as there is a biological interaction which occurs between the receptors on the surface of neutrophils, with FcγRIIIB probably using the signalling intracytoplasmic part of FcγRIIA for cytoplasmic signalling and the initiation of phagocytosis (Salmon, Brogle et al. 1991; Naziruddin, Duffy et al. 1992; Edberg and Kimberly 1994; Kocher, Siegel et al. 1997; Chuang, Sassaroli et al. 2000). The existence of LD between the two genes when they are at least 300kb apart also points to epistasis maintained by selection (Strachan and Read 2001).

Because of the positive finding with FcγRIIA and FcγRIIIB in the Oxford population, the case-control study collected in Thailand was examined for the polymorphisms in these two genes. The allele frequencies were found to be very different to those observed in Oxford, but were consistent with previously described Asian frequencies (Osborne, Chacko et al. 1994). The wild type allele of FcγRIIA (R) in the Oxford controls had an allele frequency of 54.0% compared to 26.7% in the Thai controls. The FcγRIIIB wild type gene (NA2) had an allele frequency of 61.9% in the Oxford controls compared to 35.0% in the Thai controls. Both of these alleles were putatively susceptible, therefore the Thai population had a higher proportion of putatively protective alleles compared to the Oxford population. No difference was observed in allele frequencies between the cases and controls in Thailand.

One explanation for the lack of association in Thailand is that the cases were predominantly hospital-acquired disease; few were recruited with community-acquired disease. No epistasis was observed between the two genes in either the cases or the controls. One reason for this could be the difference in allele frequencies. The presence of the putatively protective alleles were increased in this population, possibly in part due to protection against diseases such as staphylococcal infections, but also due to other infections which are encountered in the Tropics (but not in the UK), such as malaria. This suggestion is plausible, as data from a Gambian case-control study suggested that the H allele of FcγRIIA was protective against malarial disease, and the R allele was susceptible (Dr. Graham Cooke, personal communication). This suggests an interesting branch to the story between Fcγ receptors and disease. A further possibility for the difference between the two groups is that the protective alleles in the Thai population are approaching fixation due to selection, whereas the allele frequencies in the Caucasian population may be at an earlier stage in this process.

Further studies following this work would be to examine whether the host phenotype reflects the genotype. If patients have a susceptible genotype with both the FcγRIIA and FcγRIIIB does this means that phagocytosis by PMN is affected? Studies could examine the involvement of PMN in protection against *S. aureus* using phagocytic assays with PMN cell lines. Different groups could be examined such as patients with severe *S. aureus* disease, asymptomatic carriers and people who remain non-carriers, where the FcγR genotypes have been typed. A number of carefully chosen *S. aureus* strains could be examined, these could include well-characterised laboratory strains, the patients' own infecting strains and strains from specific MLST clonal types. This might help to understand whether people are clonally restricted for the bacterium (only one clone able to colonise a person with other clones being recognised and removed). This would be interesting as it would help to define the role of PMN involvement in *S. aureus* disease and carriage.

Other candidate genes examined in Oxford were the functional polymorphisms in the MBL gene which were not to be associated with disease. Also a polymorphism in the TLR2 gene, previously suggested to be associated with Staphylococcal disease was not associated with severe *S. aureus* disease in this study.

There are other receptors found on the surface of PMN which are involved in the innate immune response and may be important in host protection to *S. aureus* disease. These receptors include CR1 which has a known functional polymorphism, CR3 which contains many SNPs, and CRP which has an intronic polymorphism and a functional microsatellite. All three of these receptors are likely to interact on the surface of PMN to protect against *S. aureus* disease. CR1 and CR3 are known to interact with both FcγRIIA and FcγRIIIB. CRP is known specifically to interact with the R allele of FcγRIIA to protect against

disease. CRP has also been associated with host susceptibility in severe *S. pneumoniae* disease (Roy, Knox et al. 2002). A functional microsatellite within the TLR2 gene was also associated with severe *S. pneumoniae* disease in an Oxford based Caucasian population. It would be interesting to examine this microsatellite in *S. aureus* disease to see if the same association occurs.

Although the bacterial typing studies in Chapter 3 did not show clear-cut evidence of hypervirulent clones, the study of specific putative virulence determinants in Chapter 4 demonstrated the presence of virulent bacterial genotypes that were more likely to be associated with disease than the average carriage isolate. Similarly in Chapter 6 associations between severe disease and the FcγRIIA and FcγRIIIB polymorphisms were described, evidence that some human hosts are genetically more susceptible to severe disease. That this association occurred only in cases of community-acquired disease suggests that the role of the FcγR polymorphisms is easily swamped by acquired factors such as those leading to hospitalisation and environmental factors such as hospitalisation itself.

The role of the virulent bacterial genotype might be relatively diminished in a hospital setting, where a truly opportunistic pathogen can take advantage of host weaknesses without needing an armoury of virulence factors. The absence of any difference between HAD and CAD isolates in the presence of the 7 virulence-associated determinants is circumstantial evidence that this is not the case.

If the bacterial and host factors described in this thesis play a biologically rather than statistically significant role in severe disease causation, it is possible that the host and bacterial factors might interact rather than act independently. For example a genetically susceptible host would not require so virulent a bacterium to cause disease, and a

genetically protected individual would require a particularly virulent bacterium for the same outcome.

This is a difficult hypothesis to test in the current study, as the number of genotyped cases in which the disease-causing isolate has also been genotyped is small (n=125), and any comparison therefore underpowered (preliminary analysis reveals potentially interesting trends but no significant results). The bacterial typing needs to be extended to cover all patients typed for the FcγRs (n=429) before this question can be addressed.

To conclude, the reason for examining the factors involved in *S. aureus* disease are because asymptomatic carriage is high (up to nearly 70%), but only a very small minority of the population become severely ill with *S. aureus* disease. The reasons for this remain unknown, whether severe infection is caused alone by the bacterium or whether certain members of the population are susceptible to severe disease because of their genetic predisposition. The approach chosen to examine *S. aureus* disease was novel as both the bacterial factors and the host susceptibility factors involved in disease were examined.

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Appendices

Appendix A – Methods for *S. aureus* microbiology and molecular biology

Appendix A1 - Characterisation of isolates and antibiograms

All of the Oxford isolates were characterised for clumping factor using the tube coagulase test and deoxyriboneuclease production using standard methods or using a commercial kit measuring coagulase production (Staphaurex, Wellcome diagnostics). The laboratory results on antibiograms were recorded and any extended spectrum antibiotics performed using a modified Stokes method (BSAC guidelines). The antibiotics used to test all of the isolates were Penicillin (1μg), Ciprofloxacin (1μg), Fuscidic acid (10μg), Rifampicin (2μg), Tetracycline (10μg), Trimethoprim (1.25μg), Erythromycin (5μg), Gentamicin (10μg), Netilmicin (30μg), Amikacin (10μg), Vancomycin (30μg) and Oxacillin (1μg), all from Becton Dickinson.

Strains isolated in Thailand were stored in skimmed milk at -70°C then transported to Oxford as stab growths in LB agar. These strains were characterised using a Staphaurex , a DNAse and the above antibiograms. All characterisation was performed in Oxford.

Appendix A2 - Processing of nasal swabs

Preparation work included examining different inoculation solutions to see if they either inhibited the collection of strains or if the strains were able to grow in the inoculation fluid. The experiment was set up over different times as swabs would be sitting in a cool box at the point of collection for a period of time before plating. The different solutions that were tested were 0.1% Triton X-100, peptone water salt dilution (PWSD), saline, peptone water and tryptone soya broth. Swabs were spread onto agar plates immediately,

after 2 hours, 3 hours, 4 hours or 5 hours in the cool box and after overnight in the fridge. Three dilutions were also made from each broth to examine the counts which were recoverable after each time point. These preliminary experiments were carried out using both blood agar and mannitol salt agar (MSA) plates to examine any difference in yield. The MSA plates were counted after 48 hours incubation and after an extra 5 days at room temperature because of the increased yield after 7 days (Eriksen, Espersen et al. 1994).

The preferred method involved using PWSD from which recoverable numbers did not change over a period of time in the cool box, MSA was much easier to work with because of the ease of identification of the *S. aureus* strains. No inhibition was observed in the pilot study. Plates were read after 48 hours incubation, no difference was seen between 48 hours incubation and a further 5 days at room temperature. Also no previous work had examined whether there was any difference between nostrils, therefore one swab was used for each nostril.

Swabs were prewetted in 1ml PWSD, then rubbed over the anterior nares of one nostril using 360° x3 turns clockwise, then 360° x3 anticlockwise (Falanga, Campbell et al. 1985). The swab was broken into the broth and this was repeated for the other nostril using a new swab. The swab was stored at 4°C. The sample was vortexed and a 1 in 10 dilution prepared. This dilution was vortexed and 50µl inoculated on to an MSA plate. 50µl of the first bottle was spread onto the other half of the MSA plate. The plates were incubated at 37°C for 2 days. Yellow colonies (mannitol fermenters) were counted and recorded as suspect *S. aureus*. Six colonies from each nostril were subcultured onto a blood agar plate and incubated at 37°C for 24 hours. These colonies were characterised using catalase and Staphaurex tests. A DNAse and antibiogram testing were performed on positive cultures. Colonies were plated for purity then frozen in TSB and glycerol at –70°C.

Appendix A3 - Phage typing

Phage number	RTD	x100 and x1000 RTD
29*	*	*
52*	*	*
52A*	*	*
79	*	*
80	*	*
3A*	*	*
3C*	*	*
55	*	*
71	*	*
95	*	*
6*	*	*
42E*	*	*
47*	*	*
53*	*	*
54*	*	*
75	*	*
77	*	*
83A	*	*
84	*	*
85	*	*
81	*	*
94	*	*
96	*	*
D16	*	
47A*	*	
27	*	
33	*	
88A		*
90		*
83C		*
932		*

Table 48: List of phages used routinely at Colindale

Table 48 shows the phages used in 1946 (denoted by an asterix next to the phage number), which are still used by CPHL, Colindale.

Appendix A4 – Phage typing results, all reactions verses strong reactions alone for Nasal carriage strains

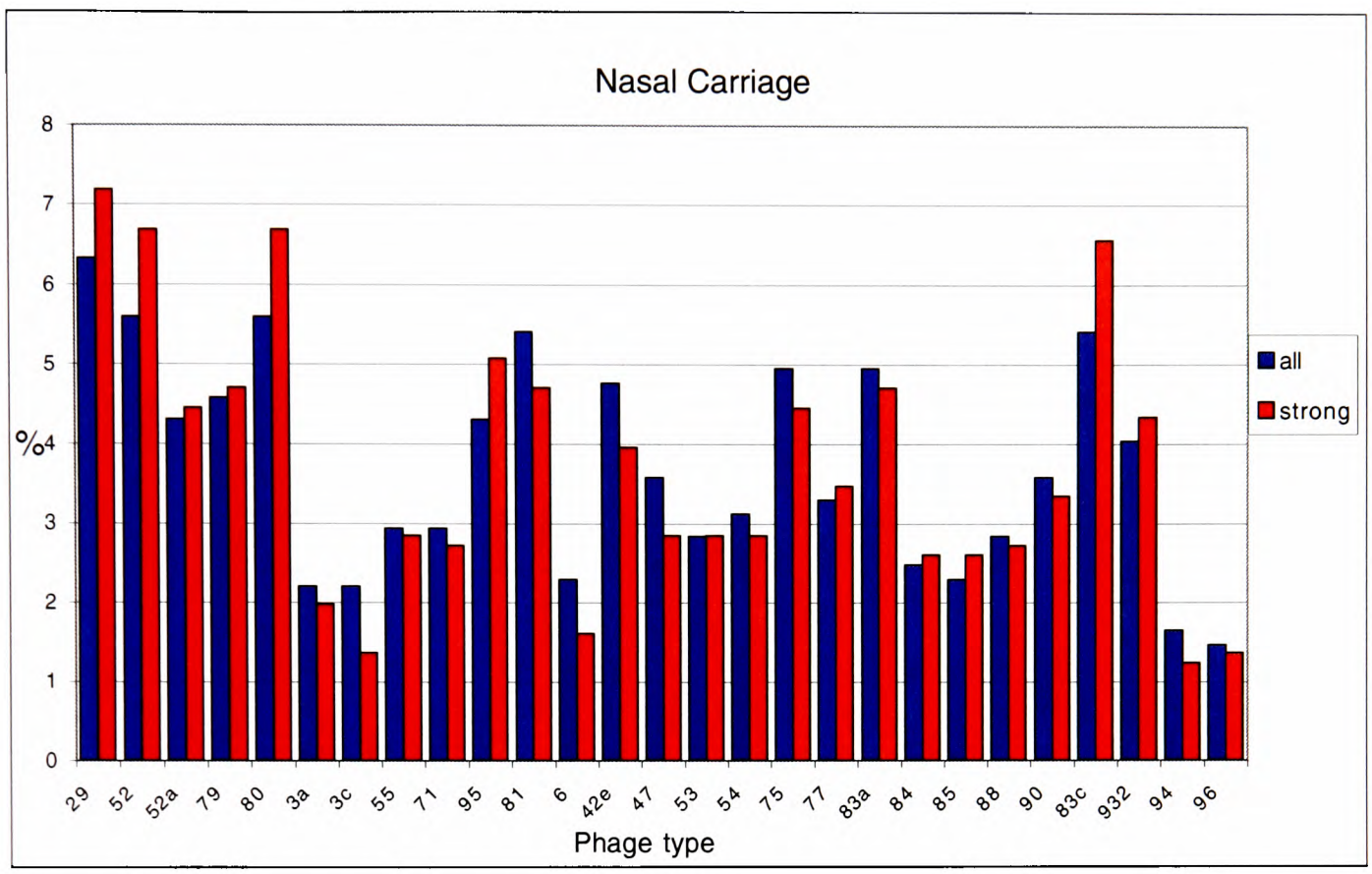


Figure 52: NC phage typing results

Figure 52 shows diagrammatically whether any difference occurred when only strong reactions were compared and when all reactions (including weak reactions) were compared for each phage type.

Appendix A5 – Phage typing results, all reactions verses strong reactions alone for strains causing severe disease in the community

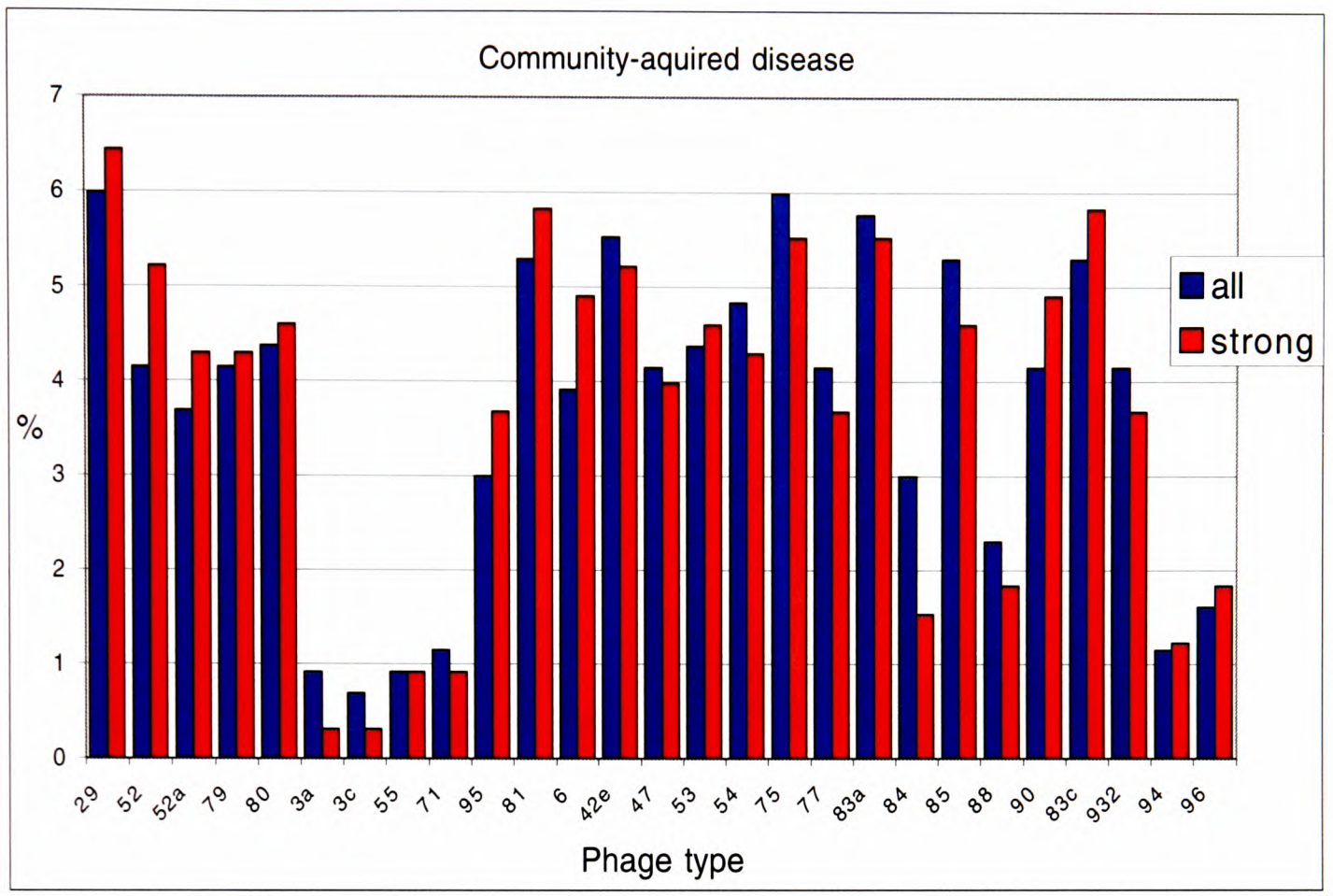


Figure 53: CAD phage typing reactions

Figure 53 shows there was also no significant difference when the reactions were compared in the CAD strains alone.

Appendix A6 – Phage typing results, all reactions verses strong reactions alone for Hospital-acquired strains

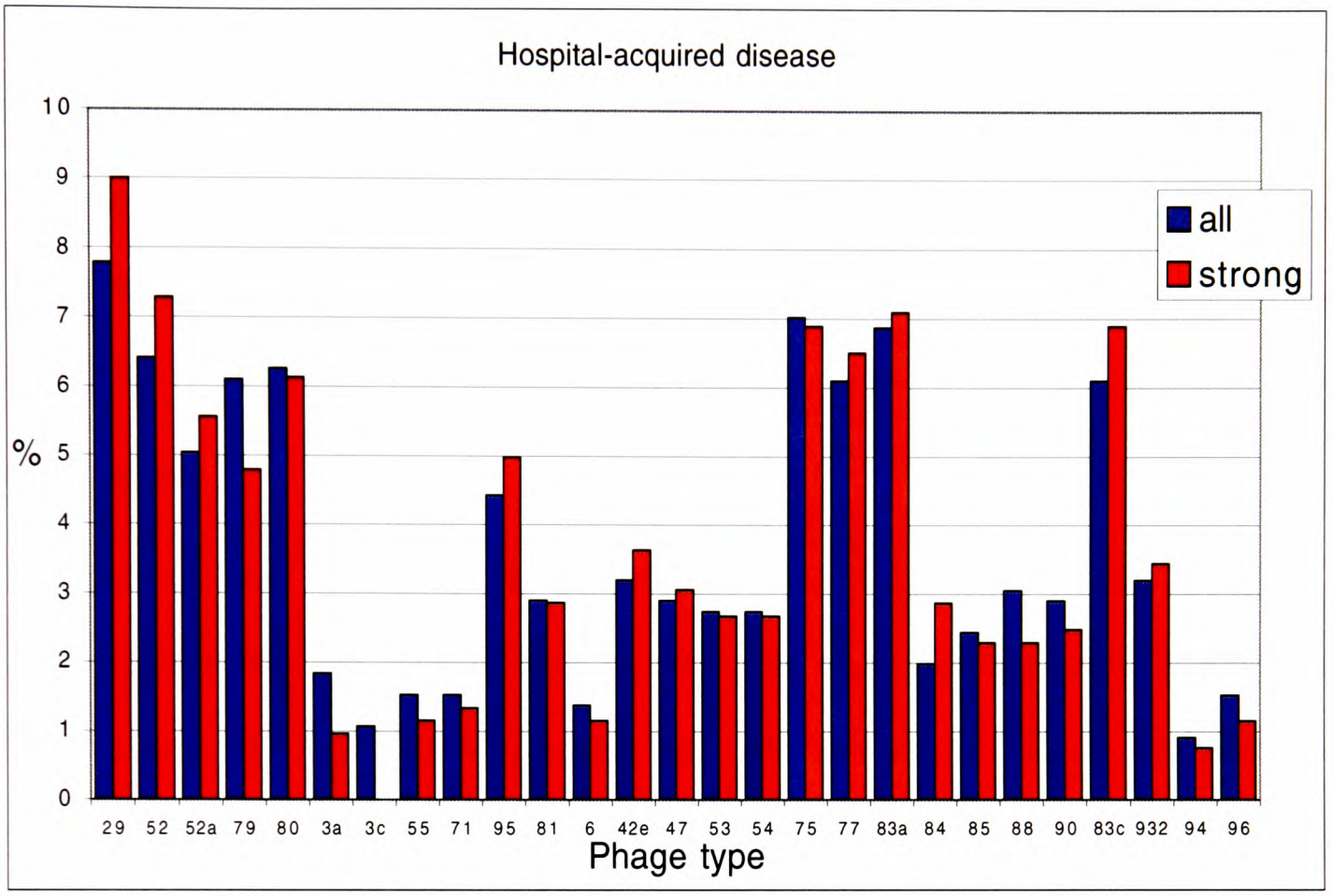


Figure 54: HAD phage typing reactions

Figure 54 shows there was no significant difference between the strong reaction in the HAD strains. The percentage was low for each phage type alone.

Appendix A7 - PFGE methodology

Block preparation

Brain Heart Infusion broths were inoculated with pure colonies of *S. aureus* then incubated at 37°C for 4 hours, then the OD was inspected (0.7 ± 0.05 at 540nm, approximately $1x10^9$ CFU/ml). 0.7ml of each broth was spun (7,000g for 2 minutes). The deposit was washed in 1ml TEN buffer (0.1M Tris Cl, 0.15M NaCl, 0.1M EDTA), resuspended and respun. The deposit was resuspended in 300µl of EC buffer (6mM Tris Cl, 1M NaCl, 0.1M EDTA, 0.5% Brij 58, 0.2% deoxycholate, 0.5% Sarkosyl), and incubated at 55°C. 2µg of

Lysostaphin (Sigma) and 300µl of 2% LMP agarose (Gibco UltraPure) were added then each strain was transferred to a plastic mould (Biorad) to solidify at room temperature.

These agarose blocks were washed in TE buffer (10mM Tris Cl, 5mM EDTA) containing 20mg/ml lysostaphin for at 37°C for 1 hour (Poddar and McClelland 1991). The solution was then replaced with buffer 1 (0.1% NaDodSO4 and 250mM EDTA) containing 1mg/ml proteinase K (Sigma) and incubated at 55°C for 1 hour. The blocks were washed 3 times at room temperature in TE buffer then stored at 4°C.

Running gels

The agarose blocks were cut in half then digested at 25°C for 2 hours using 20U *Sma 1* (New England Biolabs) together with 1% NEBuffer 4 and distilled water (Bannerman, Hancock et al. 1995). Blocks were loaded into a 1% agarose gel (Gibco UltraPure) then cemented in place with 0.08% LMP agarose. The gels were run in a CHEF DRIII in 0.5% TBE buffer at 14°C. The switching times were: block 1; 1 to 5 seconds for 12 hours, Block 2; 15 to 30 seconds for 12 hours at 6V/cm (Cookson, Aparicio et al. 1996).

Gels were stained with 10mg/ml ethidium bromide (Sigma), destained then photographed under UV light using the Gel Doc 1000 instrument (Biorad).

GelCompar

GelCompar is a computer programme designed to analyse PFGE gels (Biorad and Applied Maths, Kortrijk, Belgium). Gels were saved as TIFF files, then normalised to compare different gels. Each Bacteriophage lambda ladder (New England Biolabs) run in a gel was normalised to a gold standard λ ladder which also normalised the strains run in the gel to make comparisons between gels possible.

Appendix A8 – PFGE results

PFGE genotype	Case	Control
1	21	27
2	9	17
3	5	11
4	19	0
5	7	0
6	6	7
7	15	4
8	3	9
9	4	6
10	8	1
11	4	5
12	5	5
13	2	4
14	5	6
15	4	2
16	1	1
17	1	1
19	1	0
20	1	3
21	1	0
22	2	1
23	2	0
27	1	4
28	1	0
29	0	1
30	0	1
31	2	0
32	2	0
34	2	0
36	1	0
39	1	0
40	0	1
44	2	0
51	0	1
52	1	0
58	1	0
60	0	1
61	0	1
63	0	1
65	0	1
66	0	1

Table 49: PFGE genotypes

Appendix A9 - DNA extraction

Method 1

The method described by Jordens *et al.* (Jordens and Pennington 1991) was modified for *S. aureus*. Pure bacterial cultures were suspended in 300µl of TE8 (TE buffer pH 8.0). They were lysed in 300µl of GES containing 10µl lysostaphin (2mg/ml stock) for 30 minutes at 37°C. 250µl of cold ammonium acetate (7.5M) was added, then the tubes were placed on ice for 10 minutes. 500µl of cold chloroform:isoamylalcohol (24:1) was added then the tubes were mixed. The tubes were spun at 1250g for 20 minutes and the top aqueous phase recovered into a new eppendorf. Absolute ethanol was added to precipitate the DNA. The tubes were put on ice for 30 minutes, inverted, then centrifuged at 1300g for 20 minutes. The pellets were washed with 70% ethanol then respun for 5 minutes. The ethanol was removed then the tubes were dried. The DNA was resuspended in 50µl of TE.

Method 2

The Qiagen genomic DNA extraction method (100G) provided between 25 and 90µg DNA for each strain.

10ml of overnight culture in LB broth was prepared for each strain. The bacteria were pelleted at 3000g for 10 minutes and the supernatant was discarded. 3.5ml of Buffer 1 (50mM Tris-Cl, pH 8.0; 50mM EDTA, pH8.0; 0.5% Tween-200; 0.5% Triton X-100) containing 10mg/ml RNAse A was added and vortexed. 20mg/ml lysozyme, 20mg/ml proteinase K and 20mg/ml lysostaphin were added to each strain and incubated in a waterbath at 37°C for 30 minutes. 1.2ml of Buffer 2 (3M Guanidine HCl, 20% Tween-20) were added, the tube was inverted then incubated in a waterbath at 50°C for 30 minutes (until clear). During this step a Qiagen genomic tip was equilibrated with 4ml of QBT buffer (750mM NaCl; 50mM MOPS, pH 7.0; 15% Isopropanol; 0.15% Triton X-100)

allowing the tip to empty by gravity. The clear bacterial samples were vortexed for 10 seconds each, then poured onto the genomic tip allowing the liquid to drain then the liquid was discarded. The genomic tips were washed twice with 75ml QC buffer (1.0M NaCl; 50mM MOPS, pH 7.0; 15% Isopropanol). The liquid was again discarded. A clean tube was placed beneath each genomic tip and the DNA precipitated by the addition of 3.5ml isopropanol (room temperature). Each collection tube was inverted then centrifuged at 5000g for 15 minutes, the supernatant was removed and the pellet was transferred to a 1.5ml eppendorf tube. The pellet was washed twice with 1ml cold 70% ethanol, and respun for 10 minutes. The supernatant was removed and the pellet was air dried before resuspending the DNA in 0.5ml TE buffer (10mM TrisCl, pH 8.0; 1mM EDTA, pH 8.0) then the DNA was left to dissolve at 4°C overnight.

Appendix A10 - MLST methodology

Initial development of *S. aureus* MLST

The first 14 housekeeping genes, examined, were *recP* (transketolase), *gtr* (glucose 6 phosphate dehydrogenase), *mut S* (DNA mismatch repair protein), *aroE* (shikimate dehydrogenase), *gmk* (glycerol kinase), *glpF* (guanalate kinase), *pta* (phosphate acetyltransferase), *arcC* (carbamate kinase), *dfp* (decarboxylase), *gki* (glucose kinase), *pyr* (pyrimidine operon repressor), *xpt* (xanthine phosphoribsyltransferase), *tpi* (triosephosphate isomerase) and *yquiL* (acetyl-CoA acetyltransferase). A diverse subset of 12 staphylococcal strains were first used to find the primers which amplified the fragments consistently and gave sufficient discrimination between strains. They included 10 *S. aureus* strains and 2 *S. epidermidis* strains. The *S. aureus* strains consisted of 1 carriage strain isolated in the UK, 1 carriage strain from New Zealand (both from strains stored in the UK by Dr. S. Peacock); 2 hospital acquired MSSA; 2 hospital acquired MRSA; 2

community acquired MSSA; 1 MRSA from Bangkok and 1 MSSA from Bangkok. 8 of the 12 isolates were collected in the current study. The *S. epidermidis* isolates were clinical isolates collected in Oxford during the candidate's MSc degree.

Annealing temperatures of 55°C and 50°C were equally reproducible with a temperature of 55°C chosen for all primers. The *S. epidermidis* strains either failed to amplify or gave very low signals for all primers, so were not included in the results. The typing scheme is only applicable to strains phenotypically characterised as *S. aureus*.

PCR

PCR was used to amplify a specific target DNA sequence. Oligonucleotides were synthesised to anneal to specific sequences within the chromosomal DNA. PCR involves three stages: denaturation where the DNA is heated above 90°C to separate the two chromosomal DNA strands; primer annealing where the temperature is lowered for the oligonucleotides to anneal to the specific target in the chromosomal DNA (the temperature is dependent on the oligonucleotides and ranges between 45°C and 75°C). The last stage is extension (normally at 72°C) where the oligonucleotide primers extend with the use of *Taq* polymerase and deoxynucleotide triphosphates (dNTPs) to copy the template DNA. This cycle is repeated a number of times (from 20 to 40) depending on the reaction. This gives many million fold increase in a specific DNA sequence and provides DNA for detailed analyses (Winter, Hickey et al. 1998). The reactions were all performed on MJ Scientific PTC200 (Waltham, USA) thermal cyclers.

DNA concentration was approximated using an agarose gel then 50ng used for each PCR reaction. To this, 50ng of each primer (MWG, UK), 1 Unit of *TaqI* (Qiagen, Crawley, UK) 1xbuffer (supplied with *Taq*) and 0.2mM dNTPs (Perkin Elmer, California, USA) were added then made up to a 50µl reaction. See Appendix B2 for PCR conditions. The PCR

products were examined on a 1% gel stained with 50mg/µl ethidium bromide under UV illumination.

PCR product precipitation

The PCR product was precipitated either in the microtitre plate or in separate eppendorf tubes with 60µl PEG (20% PEG8000, 2.5M NaCl). This was mixed and spun down then left at room temperature for 1 hour. The plates or tubes were spun for 1 hour at 2750g at 4°C. The supernatant was discarded and the plates spun at 500g to remove all traces. The pellets were washed with 150µl of 70% ethanol for the microtitre plates, 600µl of 70% ethanol for each tube; then spun at 2750g for 30 minutes. The supernatants were discarded and spun briefly at 500g. The PCR products were dried, resuspended in 5µl of water then frozen at -20µl.

Sequencing

Sequencing is an automated process using fluorophores attached to dideoxynucleotides. Each sequencing reaction contains a purified PCR product, a single primer (either forward or reverse) together with the Big Dye terminator reaction mix and water. The Big Dye terminator reaction mix contains four dNTPs labelled by covalent attachment of a different coloured dye. A monitor is used to detect and record the fluorescent signal from the dNTPs as the DNA passes through. Maximum fluorescence occurs at different wavelengths for each dye. This produces an electronic record which is interpreted using computer software (Strachan and Read 2001).

Sequencing was performed using a less concentrated amount of PCR primer (12ng) together with 1µl of precipitated PCR product, 2µl BigDye fluorescent terminators and water to make 5µl. The conditions are found in Appendix B2.

Precipitation of sequenced DNA

15µl of water was added for a final volume of 20µl in each well 52µl of a mixture of Sodium acetate and ethanol (2µl 3M NaOAC, pH 4.6 and 50µl of 95% EtOH) was added to each well. The trays were secured then inverted and left at room temperature for 1 hour, then spun at 2750g for 1 hour. The supernatant was discarded and the inverted tray spun at 500g to remove all traces. 150µl of 70% ethanol was used to wash each strain then the tray was spun at 2750g for 1 hour. The supernatant was discarded and the tray spun inverted at 500g. The plates were then dried.

Running on a 377

3µl of running dye (Perkin Elmer) was added to each well and thoroughly mixed. The product was spun then denatured at 95°C for 2 minutes and the samples were run on an ABI 377 instrument.

Running on 3700

20µl of a 1:1 dilution of hi-dye formamide in distilled water was added to each well then vortexed for 2 minutes, spun then kept frozen at –20°C until run on the 3700.

Analysis of MLST bases

Sequence Navigator or DNASTAR were used to analyse all of the strains. A template strain (allele 1) was imported for each allele then the test strains were imported, compared with the template strain and the forward and reverse primers for each strain were compared. All non-corresponding bases between these two strains were examined carefully.

Simple text megafiles were produced for each primer. These megafiles were imported into the sequence output programme where the allele for each locus was assigned, then produced a sequence type (ST) for each strain.

Appendix A11 – MLST results

MLST sequence type	Case	Control
1	7	3
2	0	1
3	1	0
4	1	0
5	6	10
6	2	0
7	1	0
8	7	7
9	3	1
10	1	0
11	0	1
12	6	3
13	1	0
14	1	0
15	6	10
16	1	0
17	0	1
18	1	0
19	0	1
20	1	3
21	0	1
22	9	9
23	1	0
24	0	1
25	13	5
26	1	0
28	1	0
29	0	1
30	17	25
31	1	0
32	1	0
33	1	0
34	1	6
35	0	1
36	18	0
37	1	0
38	1	0
39	5	5
40	1	0
41	1	0
42	1	0
44	0	1
45	7	5
46	1	2
47	4	3

MLST sequence type	Case	Control
48	1	0
49	2	0
50	1	0
51	1	0
52	0	1
53	0	1
54	0	2
55	0	1
56	0	1
57	0	1
60	1	0
77	0	1
97	0	1
101	0	3
121	1	3
123	0	3
134	0	1
145	0	1
167	0	1
169	0	1
178	0	1
182	0	1
188	1	0

Table 50: MLST STs

Number	PFGE types within each ST	STs within each PFGE type
1	50	16
2	5	4
3	6	3
4	4	1
5	1	1
6	1	1
7	0	0
8	1	0

Table 51: List of PFGE genotypes within each ST and MLST STs within PFGE genotypes

Appendix B – PCR primers and conditions for *S. aureus* typing and gene detection

Appendix B1 - PCR primers for MLST

Primer Name	Primer	Sequence (5' to 3')	Product size (bp)
glucose 6 phosphate dehydrogenase	*gtrF*	TGCCTTCATTAGCCAAATCTTTA	430
	gtrR	GGTTCAGGTAAAACAACATTGTTA	
transketolase	*recPF*	CCAAATCCGATTGTTGTTTTAAC	502
	recPR	TGGTTCAGCATTATTGTATAGC	
DNA mismatch repair protein	*mutSF*	AGTACCACGTCCAATTTCATCGA	497
	mutSR	GCCATCGAATTAGAATATCAATTA	
decarboxylase	*dfpF*	GTTTATACAGATACTTTTATAGA	499
	dfpR	GTTATTGCATAGCCCATTTTTCC	
glucose kinase	*gkiF*	GTAAATGGAGCAGTAAACTTATA	495
	gkiR	TATTTCGGATTACTTGTAACAC	
pyrimidine operon repressor	*pyrF*	AGAATCGCTCACGAAATTTTGGA	466
	pyrR	ATAACTGCATTTCTTTGATCCA	
xanthine phosphorylribosyltransferase	*xptF*	AGGAGCAATTTAAAGATGAAGGG	506
	xptR	ATGGCACCTGCGTACATAGC	
carbamate kinase	*arcCF*	TTGATTCACCAGCGCGTATTGTC	456
	arcCR	AGGTATCTGCTTCAATCAGCG	
shikimate dehydrogenase	*aroEF*	ATCGGAAATCCTATTTCACATTC	456
	aroER	GGTGTTGTATTAATAACGATATC	
guanalate kinase	*gmkF*	ATCGTTTTATCGGGACCATC	429
	gmkR	TCATTAACTACAACGTAATCGTA	
glycerol kinase	*glpFF*	CTAGGAACTGCAATCTTAATCC	465
	glpFR	TGGTAAAATCGCATGTCCAATTC	
phosphate acetyltransferase	*ptaF*	GTTAAAATCGTATTACCTGAAGG	474
	ptaR	GACCCTTTTGTTGAAAAGCTTAA	
triosephosphate isomerase	*tpiF*	TCGTTCATTCTGAACGTCGTGAA	402
	tpiR	TTTGCACCTTCTAACAATTGTAC	
acetyl-CoA acetyltransferase	*yqiLF*	CAGCATACAGGACACCTATTGGC	516
	yqiLR	CGTTGAGGAATCGATACTGGAAC	

Table 52: PCR primers for MLST

Appendix B2 - PCR and Sequencing conditions for MLST

	Temperature	Time
All PCR primers:	95°C	5 minutes (denaturation)
30 cycles of:	*55°C*	*1 minute* (annealing)
	72°C	*1 minute* (extension)
	95°C	*1 minute* (denaturation)
then	72°C	5 minutes (final extension)
All Sequencing primers:		
24 cycles of:	96°C	10 seconds (denaturation)
	50°C	5 seconds (annealing)
	60°C	2 minutes (extension)

Table 53: PCR and sequencing conditions for MLST

Appendix B3 - PCR primers for the toxins and adhesins

Primers for	Primers	Sequence (5' to 3')	Positive controls	Product size (bp)
16 rRNA gene	*sa*16S*f*	GTAGGTGGCAAGCGTTATCC	8325-4	228
	*sa*16S*r*	CGCACATCAGCGTCAG		
Toxins:				
Enterotoxin A	*sea-3*	CCTTTGGAAACGGTTAAAACG	NCTC 10652	127
	sea-4	TCTGAACCTTCCCATCAAAAAC		
Enterotoxin B	*seb-1*	TCGCATCAAACTGACAAACG	NCTC 10654	477
	seb-4	GCAGGTACTCTATAAGTGCCTGC		
Enterotoxin C	*sec-3*	CTCAAGAACTAGACATAAAAGCT AGG	NCTC 10655	271
	sec-4	TCAAAATCGGATTAACATTATCC		
Enterotoxin D	*sed-3*	CTAGTTTGGTAATATCTCCTTTAA ACG	NCTC 10656	319
	sed-4	TTAATGCTATATCTTATAGGGTA AACATC		
Enterotoxin E	*see-3*	CAGTACCTATAGATAAAGTTAAA ACAAGC	ATCC 27644	178
	see-2	TAACTTACCGTGGACCCTTC		
Enterotoxin G	*segmbf*	CGTCTCCACCTGTTGAAGG	FRI 572	327
	segmbr	CCAAGTGATTGTCTATTGTCG		
Enterotoxin H	*sehmbf*	CAACTGCTGATTTAGCTCAG	ATCC 51811	360
	sehmbr	GTCGAATGAGTAATCTCTAGG		
Enterotoxin I	*seimbf*	CAACTCGAATTTTCAACAGGTAC	FRI 572	465
	seimbr	CAGGCAGTCCATCTCCTG		
Enterotoxin J	*sejmbf*	CATCAGAACTGTTGTTCCGCTAG	NCTC 10652	142
	sejmbr	CTGAATTTTACCATCAAAGGTAC		
TSST-1	*tst-3*	AAGCCCTTTGTTGCTTGCG	NCTC 11963	445
	tst-6	ATCGAACTTTGGCCCATACTTT		
Exfoliative toxin A	*eta-3*	CTAGTGCATTTGTTATTCAAGAC G	Colindale clinical strain	119
	eta-4	TGCATTGACACCATAGTACTTATT C		
Exfoliative toxin B	*etb-3*	ACGGCTATATACATTCAATTCAA TG	Colindale clinical strain	262
	etb-4	AAAGTTATTCATTTAATGCACTGT CTC		

Primers for	Primers	Sequence (5' to 3')	Positive controls	Product size (bp)
Adhesins:				
Fibronectin binding protein	*fnbAF*	CACAACCAGCAAATATAG	8325-4	1361
	fnbAR	CTGTGTGGTAATCAATGTC		
Fibronectin binding protein	*fnbBF*	TAGAAACTTCGCGAGTTG	8325-4	1167
	fnbBR	TCAAGTTCATAGGAGTAC		
Fibrinogen binding protein	*clfAF*	GTAGGTACGTTAATCGGTT	Newman	1583
	clfAR	CTCATCAGGTTGTTCAGG		
Fibrinogen binding protein	*clfBF*	TGCAAGATCAAACTGTTCCT	Newman	595
	clfBR	TCGGTCTGTAAATAAAGGTA		
Collagen binding protein	*cnaF*	AGTGGTTACTAATACTG	Phillips	Variable, depending on B rpts
	cnaR	CAGGATAGATTGGTTTA		
Protein A	*spaF*	TCGAAATAGCGTGATTTTGC	8325-4	1891
	spaR	GCACTGAGCAACAAAAGATG		
SD repeat regions	*sdrCF*	ACGACTATTAAACCAAGAAC	Newman	599
	sdrCR	GTACTTGAAATAAGCGGTTG		
	sdrDF	GGAAATAAAGTTGAAGTTTC	Newman	499
	sdrDR	ACTTTGTCATCAACTGTAAT		
	sdrEF	CCTTCAAAAGTAGACAATCA	Newman	857
	sdrER	GGTGCTACCATTTCCTAG		
Elastin binding protein	*ebpsF*	CAATCGATAGACACAAATTC	Clinical isolate 252	525
	ebpsR	CAGTTACATCATCATGTTTA		
MHC class II analogue protein	*map/eapF*	TAACATTTAATAAGAATCAA	Newman	942 to 948
	map/eapR	CCATTTACTGCAATTGT		
Polysacharide intracellular adhesin	*icaAF*	GATTATGTAATGTGCTTGGA	Clinical isolate 252	769
	icaAR	ACTACTGCTGCGTTAATAAT		

Primers for	Primers	Sequence (5' to 3')	Positive controls	Product size (bp)
Extracellular fibrinogen binding protein	*efbF*	GCGAAGGATACGGTCCAAGAGA	Clinical isolate 252	377
	efbR	CAATTCGCTCTTGTAAGACCATT		
Fibrinogen binding protein	*fbpAF*	F1-ATCGTACGATTGGGCAATTA F2-CTTGAGCAAAAAATTAAAGA	Clinical isolate 252	1 = 682 2 = 1259
	fbpAR	R1-CTAATGCCTCAGTTTTATGT R2-GTTGTATGCATTTGTTTCA		
Serine protease	*V8F*	TTGTTCTTCGAAACTT	V8	1552
	V8R	CAATAAAGCCAAAGCC		
Accessory gene regulator	*agr1F*	TATAGTACTTGTGACTTCATAAT	Clincal isolate 2	739
	agr1R	CTTGATTACGTTTATATTTCATC		
	agr2F	AACGCTTGCAGCAGTTTATTT	Isolate 19	691
	agr2R	CGAGATTATAAGTATTACAACA		
	agr3F	TATATAAATTGTGATTTTTTATTG	Isolate 252	712
	agr3R	TTCTTTAAGAGTAAATTGAGAA		
	agr4F	GTTGCTTCTTATAGTACATGTT	Isolate 3049	683
	agr4R	CTTAAAAATATAGTGATTCCAAT		

Table 54: PCR primers and positive controls for the toxins and adhesins

Appendix B4 - PCR conditions for toxins and adhesins

Primer	Temperature	Time
All toxins:	95°C	2 minutes
35 cycles of	*95°C*	*1 minute*
	58°C	*1 minute*
	72°C	*2 minutes*
then	72°C	5 minutes
Adhesins all have:		
Primary denaturation of:	94°C	2 minutes
then 30 cycles of denaturation at:	94°C	1 minute
then a specific annealing temperature and extension followed by:	72°C	10 minutes
Specific annealing temperatures:		
fnbA and *fnbB*	*50°C*	*1 minute*
	72°C	*1.30 minutes*
clfA and clfB	*45°C*	*1 minute*
	72°C	*2 minutes*
cna	*40°C*	*1 minute*
	72°C	*2 minutes*
spa	*65°C*	*1 minute*
	72°C	*2 minutes*
sdrC, sdrD and sdrE	*45°C*	*1 minute*
	72°C	*1 minutes*
ebpS	*48°C*	*1 minute*
	72°C	*1 minutes*
map/eap	*45°C*	*1 minute*
	72°C	*1.30 minutes*
icaA, agrI, agrII, agrIII and agrIV	*50°C*	*1 minute*
	72°C	*1 minutes*
efb	*65°C*	*1 minute*
	72°C	*1 minute*
fbp	*??°C*	*1 minute*
	72°C	*2 minutes*
V8	*50°C*	*1 minute*
	72°C	*2 minutes*

Table 55: PCR conditions for toxins and adhesins

Appendix C – Detailed methodology for host genetic studies

Appendix C1 – DNA extraction

A Nucleon BACC2 kit was used to extract human DNA from EDTA blood. The kit does not use phenol and chloroform, and provides large amounts of high quality DNA.

Cell preparation

3 to 10ml of blood was aliquoted into a 50 ml centrifuge tube and 4 x volume of Reagent A was added. The tubes were vortexed for 5 minutes at room temperature. The tubes were centrifuge at 1300g for 5 minutes. The supernatant was removed carefully without dislodging the white cell pellet. Another 10 ml of Reagent A was added then the tubes were vortex samples for 20 seconds. The tubes were centrifuged again and the supernatant discarded.

Cell lysis

2ml of Reagent B was added to the pellet. The tubes were vortexed to resuspend pellet. The suspensions were removed to 15ml polypropylene Falcon tubes. The tubes were agitated gently at 37°C for 15-30mins until the pellet has totally resuspended.

Deproteinisation

500µl of sodium perchlorate was added. The tubes were mixed by inverting 7 times then shaken at 37°C for 20 min. The tubes were incubated in a shaking waterbath at 65°C for 20 min.

DNA extraction

2ml of cold chloroform was added to each tube, they were mixed by inverting 7 times. 300 µl of Nucleon resin was added without mixing the phases. Samples were centrifuged

1300*g* for 3 min. Without disturbing the Nucleon silica layer, the supernatant was transferred to plastic 15ml Falcon tubes.

DNA precipitation

Two volumes of cold absolute ethanol was added. The tubes were inverted until the DNA precipitated (approx. 7 times). The tubes were centrifuged at top speed (~4000 *g*) for 5 min to pellet the DNA.

DNA washing

The supernatant was removed carefully, the pellet was washed in 1ml of 70% ethanol. The tubes were centrifuged at top speed (~4000*g*) for 5 min to pellet the DNA. The supernatant was carefully removed without disturbing the DNA. The remaining ethanol was left to evaporate by air-drying for 10 min. Pellets were resuspended in 50µl of TE and left to dissolve at 4°C before freezing.

Appendix C2 - DNA quantification using the 7200 Pico Green method

The Pico Green 7200 kit (Molecular probes, Leiden, Netherlands) was used to measure the concentration of dsDNA within samples. The method uses a fluorescent Hoechst (besbenzimide) dye which intercalates into dsDNA to emit light at approximately 520nm when excited at approximately 480nm.

Test DNAs were diluted 1:200 in TE buffer (pH 7.5) then 50µl aliquoted into a microamp plate. Standard DNA dilutions of 0, 25, 50, 100, 300, 500, 700 and 1000 ng/µl were aliquoted into positive control wells in the plate. 50µl of a 1:200 solution of picogreen was added to each well. The plate was spun at 500g to mix. The plate was then read using an ABI Prism 7200 Sequence Detector.

The results of this method were used to determine the DNA contents of each tube to make deep well plates at a concentration of 10ng/µl.

Appendix C3 – Ligation Detection Reaction (LDR)

The LDR method is a fluorescent allele specific method which is performed after PCR, and relies upon the ability of bacteriophage T4 DNA ligase to join the variable 3' ends of an allele specific probe to the 5' end of a common reverse probe next to the polymorphic site. The method has since been modified to use Taq DNA ligase (New England Biolabs, Hitchin, UK) together with fluorescently labelled probes (Sigma-Genosys, Pampsford, UK). Each allele has a different fluorescent label which can be detected using a gel based or capillary based instrument (ABI machines). The method is ideally suited for multiplexing to study PCR products containing many polymorphic sites, as they can be distinguished both fluorimetrically and by their different product sizes.

Amplification of DNA

The PCR protocol for each set of primers is performed. The PCR primers are designed to encompass all of the SNPs to be detected by LDR.

Removal of polymerase activity

The Taq polymerase activity is removed to avoid further extension of products. One volume of proteinase K (1mg/ml in 50mM EDTA) to 10 PCR product is added to each well. The plates are then heated to 37°C for 15 minutes, 55°C for 10 minutes and 90°C for 10 minutes.

Phosphorylation of common probes

Each common probe is phosphorylated in 10µl of 10xPNK buffer, 1µl of ATP (25mM), 2µl of the common probe (0.1mM), 1µl Polynucleotide kinase and water to make 100µl.

Each probe is then incubated at 37°C for 45 minutes, 100µl of proteinase K (20µg in 100µl TE) is added to each tube, then the tube was incubated at 37°C for 30 minutes and 90°C for 10 minutes.

LDR reaction

The reaction is performed on ice. 1.5µl of 10x Taq DNA ligase buffer, 0.15µl of phosphorylated common probe (1µM stock), 0.15µl of each allelic probe (1µM stock), 0.025µl Taq DNA Ligase (40U/µl stock), 1µl PCR product and water up to 15µl are used in each well. Mineral oil is overlaid then the reaction is placed onto a hot (95°C) PCR block. The block was cycled for 15 cycles of 95°C for 15 seconds followed by the specific ligation temperature for 4 minutes. When the cycling has finished the reaction is placed on ice and 3µl of 100mM EDTA is added into each well to prevent further ligase activity.

Diagrammatic illustration of LDR (Modified from Barany, 1991)

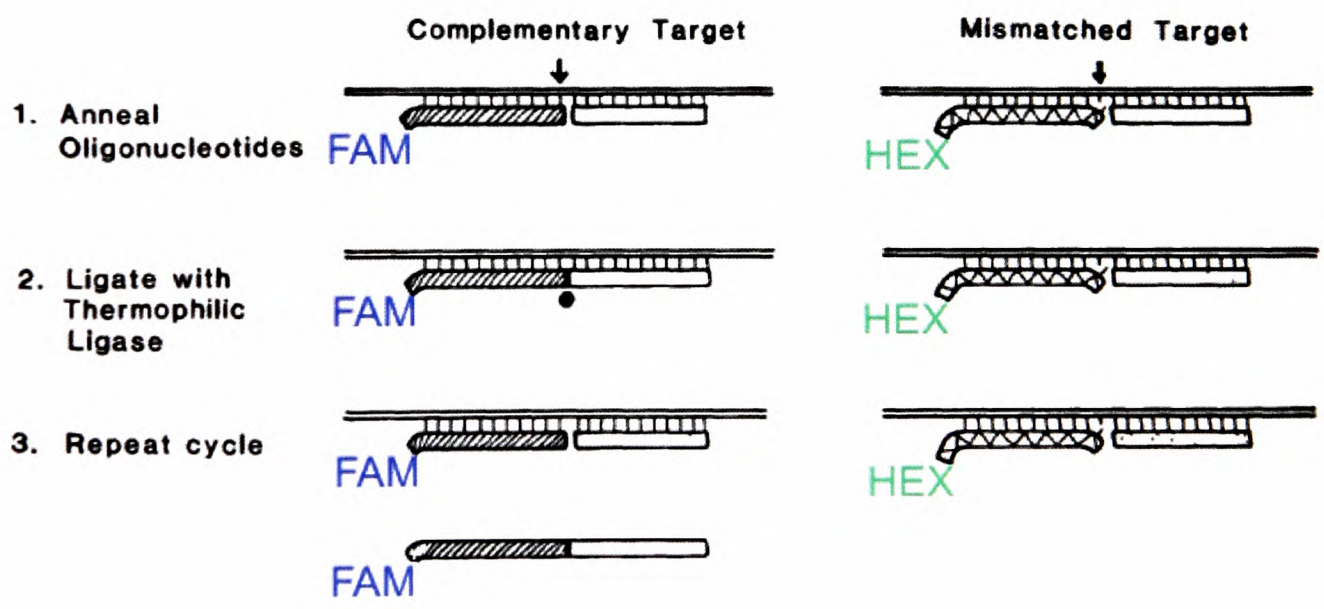


Figure 55: LDR genotyping

Running on Sequencer

Several LDR products can be pooled as long as the ligation sizes are sufficiently different to allow each peak to be determined. The products can be run equally well on an ABI 373,

377 or 3700 depending on the fluorescent dyes used. Peaks are distinguished by their size and by the fluorescent colour of the peak.

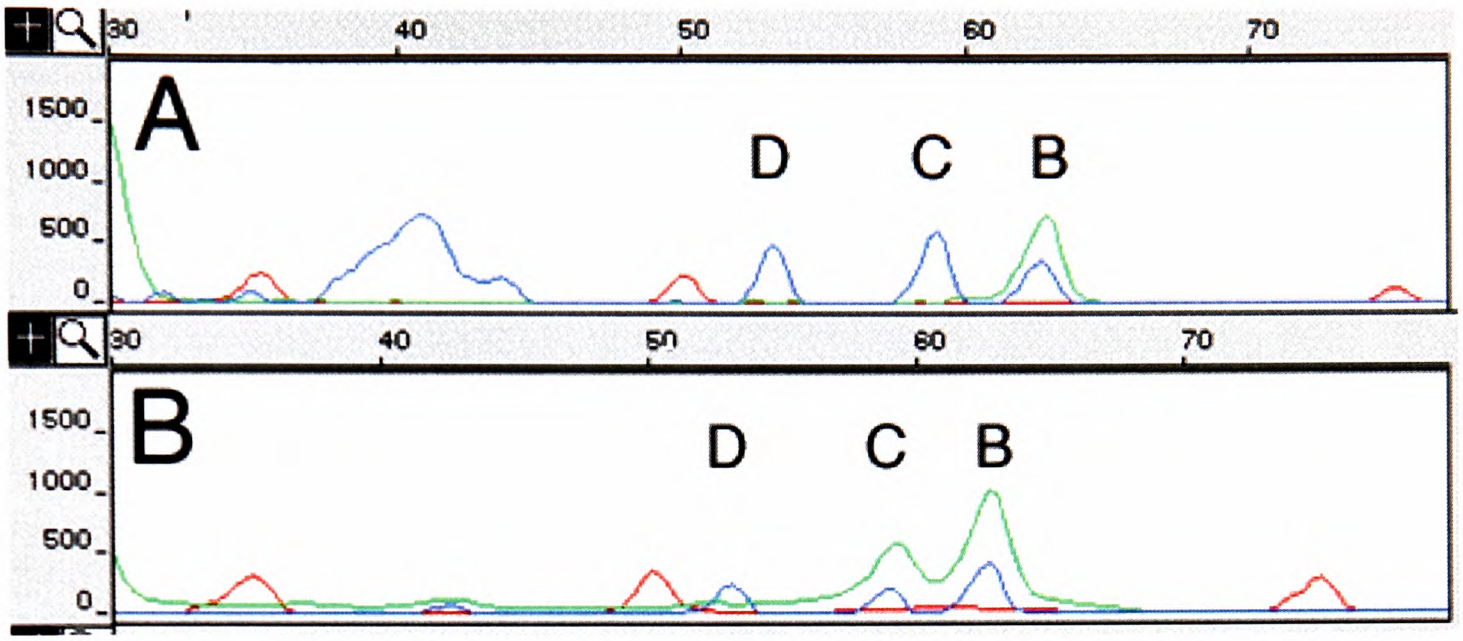


Figure 56: A sample of the LDR genotyping traces for MBL

In figure 56, the top box (A) depicts an individual homozygous for the wild type D and C, but heterozygous for B. The lower box (B) depicts an individual homozygous for the wild type D and heterozygous for B and C.

Appendix D – PCR primers and conditions for the candidate genes

Appendix D1 - FcγRIIA allele specific primers

Primer name	Sequence (5' to 3')	Size of product (bp)
CD32HF (forward)	ATCCCAGAAATTCTCCCA	253
CD32RF (forward)	ATCCCAGAAATTCTCCCG	
CD32REV (common reverse)	CAATTTTGCTGCTATGGGC	
SPA1/F (forward positive control)	ATTCCTCTGATGGGCCCTGACTCTT	442
SPA1/R (reverse positive control)	CACCCTGCTGGAACAACTACCTG	

Table 56: FcγRIIA primers for PCR

Appendix D2 - FcγRIIA PCR conditions

Primer	Temperature	Time
H Primer:	94°C	14 minutes
11 cycles of:	*94°C*	*30 seconds*
	58°C	*1 minute*
	72°C	*2 minute*
26 cycles of:	*94°C*	*30 seconds*
	55°C	*1 minute*
	72°C	*2 minutes*
then	72°C	2 minutes
R Primer:	94°C	14 minutes
11 cycles of:	*94°C*	*30 seconds*
	60°C	*1 minute*
	72°C	*2 minutes*
26 cycles of:	*94°C*	*30 seconds*
	58 °C	*1 minute*
	72°C	*2 minutes*
then	72°C	2 minutes

Table 57: FcγRIIA PCR conditions

Appendix D3 - FcγRIIIB primers

Primer name	Sequence (5' to 3')	Size of product (bp)
MatCD16-1For	ATCTCCCAAAGGCTGTGGTGTTCCTG	156
MatCD16-1Rev	AGCTCGAGGCCTGGCTTGAGAAGAGG	
MatCD16-2For	GTTCCTGGAGCCTCAATGGTACAGGAAGCT	191
MatCD16-2Rev	TTGTCTGGCACCTGTACTCTCCACTAACGT	

Table 58: FcγRIIIB PCR primers

Appendix D4- FcγRIIIB PCR conditions

Primer	Temperature	Time
Primer 1:	94°C	10 minutes
36 cycles of:	*96°C*	*1 minute*
	70°C	*1 minute*
	72°C	*1 minute*
then	72°C	2 minutes
Primer 2:	94°C	10 minutes
36 cycles of:	*96°C*	*1 minute*
	60°C	*1 minute*
	72°C	*1 minute*
then	72°C	2 minutes

Table 59: FcγRIIIB PCR primers

Appendix D5 - MBL PCR and LDR primers

Oligonucleotide	Sequence (5' to 3')	LDR temp.	Ligation/ Product length (bp)
PCR (coding):			339
MBL (forward)	GCACCCAGATTGTAGGACAGAG		
MBL (reverse)	CAGGCAGTTTCCTCTGGAAGG		
PCR (promoter):			
MBLprom-3 (forward)	GAAGCTTAGACCTATGGGGCTAG		872
MBLprom-5 (reverse)	AACTGCAGGGAAGGTTAATCGCA GTT		
LDR (coding):			
MBL 52 (wt)	**tet-** ttCGGCTTCCCAGGCAAAGATGGG C	73	52
MBL 52 (D, mt)	**fam-** AACGGCTTCCCAGGCAAAGATGG GT	73	52
MBL 52 (common)	GTGATGGCACCAAGGGAGAAAAG GGGG	73	52
MBL 54 (wt)	**tet-** aaaaaaaaCCAGGCAAAGATGGGCGT GATGG	64	62
MBL 54 (B, mt)	**fam-** aaaaaaaCCCAGGCAAAGATGGGCG TGATGA	64	62
MBL 54 (common)	CACCAAGGGAGAAAAGGGGGAA CCAGGaatt	64	62
MBL 57 (wt)	**tet-** ttatttaATGGGCGTGATGGCACCAAG GG	64	58
MBL 57 (C, mt)	**fam-** ttatttGATGGGCGTGATGGCACCAA GGA	64	58
MBL 57 (common)	AGAAAAGGGGGAACCAGGCCAA GGGaaat	64	58

Oligonucleotide	Sequence (5' to 3')	LDR temp.	Ligation/ Product length (bp)
LDR/RFLP (promoter):			
MBLprom-L (wt)	**fam-** GAAAATGCTTACCCAGGCAAGCC TGTC	66	62
MBLprom-H (mt)	**tet-** aAAAATGCTTACCCAGGCAAGCCT GTG	66	62
MBLprom-H/L (common)	TAAAACACCAAGGGGAAGCAAAC TCCAGTTAATTC	66	62
MBLprom-Y (wt)	**fam-** GGTCCCATTTGTTCTCACTGCCAC G	66	58
MBLprom-X (mt)	**tet-** GGTCCCATTTGTTCTCACTGCCAC C	66	58
MBLprom-X/Y (common)	GAAAGCATGTTTATAGTCTTCCAG CAGCAACGC	66	58
MBLprom-P (wt)	**fam-** CCAGATTGTAGGACAGAGGGCAT GCTC	66	71
MBLprom-Q (mt)	**tet-** CCAGATTGTAGGACAGAGGGCAT GCTT	66	71
MBLprom-P/Q (common)	GGTAAATATGTGTTCATTAACTGA GATTAACCTTCCCTGAGTTT	66	71
MBL RFLP			
Mbl-p/q rflpF	TTGTAGGACAGAGGGCAAGC		210
Mbl-p/q rflpR	TGGAGAGCTACAGGCAATCAC		

Table 60: MBL PCR and LDR primers

Appendix D6 - MBL PCR conditions

Primer	Temperature	Time
MBL coding:	95°C	14 minutes
40 cycles of:	*94°C*	*1.30 minute*
	58°C	*1.30 minute*
	72°C	*1.20 minute*
then	72°C	2 minutes
MBL promoter:	94°C	14 minutes
36 cycles of:	*94°C*	*30 seconds*
	65°C	*1 minute*
	72°C	*2 minutes*
then	72°C	2 minutes
MBL p/q RFLP:	94°C	14 minutes
35 cycles of:	*94°C*	*30 seconds*
	59°C	*1 minute*
	72°C	*2 minutes*
then	72°C	2 minutes

Table 61: MBL PCR conditions

Appendix D7 - TLR2 PCR primers

Primers	Sequence (5' to 3')	Size of product (bp)
TLR2-SNP For	TATGGTCCAGGAGCTGGAGA	430
TLR2-SNP Rev	TTGTCTAGTTGGGATCTTTATGTCA	

Table 62: TLR2 PCR primers

Appendix D8 - TLR2 PCR conditions

Primer	Temperature	Time
TLR2	94°C	14 minutes
39 cycles of:	*94°C*	*15 seconds*
	58°C	*30 seconds*
	72°C	*30 seconds*
then	72°C	2 minutes

Table 63: TLR2 PCR conditions

Appendix E – Statistical methods not explained in main text

Appendix E1 - Hardy-Weinberg equilibrium (HWE)

HWE incorporates Mendel's two principles: the law of segregation which describes the inheritance of a single trait at a single locus, and the law of independent random assortment which describes the joint behaviour of loci controlling two different traits (Borecki and Suarez 2001; Elston, Olson et al. 2002). Mendel's law of segregation states that during meiosis, alleles of the same gene are segregated to form gametes (Sham 1998). An individual has an equal probability of inheriting one of 2 genes from the mother and one of 2 genes from the father. Mendel's second law is the law of assortment which states that segregation of the genes for one trait is independent of the segregation of genes for other traits. During meiosis, following the exchange of genetic material by the parental chromosomes, each chromosome is randomly distributed to the offspring.

Because a population contains offspring from many different mating types, the ratios of the different genotypes in a population are weighted averages of the segregation ratios of the different mating types. The weights are the relative frequencies of the different mating types (Sham 1998). When these mating type frequencies occur through random mating, the ratios follow a mathematical model described by Hardy and Weinberg in 1908. They determined the appropriate genotype ratios for a population given their allele frequencies under a specific set of assumptions. The assumptions made are that the DNA is diploid in nature; sexual reproduction occurs; generations do not overlap; the allele frequencies are identical in males and females; panmixia occurs; the population size is large; there is little migration; there is little mutation and the alleles are selectively neutral (Sham 1998).

Consider a bialleleic locus consisting of alleles A and a, with allele frequencies denoted p and q respectively. During random mating, allele A is inherited together with allele A with

a genotype frequency of p x p (p^2). Allele a is inherited together with allele a with a genotype frequency of q^2. If A and a are inherited together the frequency of the genotype is 2 x p x q = 2pq.

Confirmation of HWE within a population it means the population is behaving in a stable and predictable manner. When HWE is upheld, the assumptions on which the equation is based are also upheld. A deviation from HWE can be suggestive of a number of factors Including non-random mating within the population, lethal genotypes within a population or preferential selection during the sampling process.

Appendix E2 - Statistical analysis of case-control samples

Two statistical analyses were employed to identify whether a chosen candidate gene was significantly associated with disease or not. The χ^2 test was used to examine the goodness of fit of the data to frequencies calculated, assuming that HWE exists. The observed and expected genotype counts were examined under HWE with a p-value determined. If the allele frequencies were low a Fishers exact test was used (the expected values that are generated by the contingency table analysis produce inflated χ^2 values). A Fishers exact test consists of evaluating the probability associated with all possible 2x2 tables with the same row and column totals as the observed data, making the assumption that the null hypothesis was true (Altman 1997).

Appendix E3 - Multiple comparisons

With more areas on the chromosome being examined either for linkage or for association, problems with multiple comparisons arise. Performing multiple comparisons can lead to an increased chance of type I (α) errors. The power of a test is the probability of rejecting the null hypothesis when it is false. Power is related to the significance level, sample size and the difference required to detect the significance. The type I (α) error is a false positive

error where the null hypothesis may be rejected when it is true. The type II (β) error is a false negative; it is where the null hypothesis is accepted when in fact it is false. This can be caused by adjusting statistically for multiple comparisons which leads to a reduction in power.

The errors are related to the study size together with the specificity and sensitivity of the test. The sensitivity of the test is the ability to detect all of the results that are truly positive. The specificity is related to the ability to eliminate all the results that are truly negative. The power of the study is related to the numbers needed in a study population and the sensitivity of the test. Enough subjects need to be recruited to correctly identify the positive results. Type II errors are associated with the power of the test. The power is the probability of rejecting the null hypothesis when it is false because insufficient subjects were collected to detect the difference. Low power (<80%) results from small sample sizes. It is related to the significance level (α), the sample size and the difference required to detect. Smaller differences are associated with lower power and larger sample sizes are needed to increase the power to acceptable levels (Evaluation 1999). This error needs to be addressed in the study design so enough subjects are collected to attain sufficient power to show any important differences.

Appendix F – Ethical permission

Oxford Radcliffe HOSPITALS

CENTRAL OXFORD RESEARCH ETHICS COMMITTEE

THE JOHN RADCLIFFE

Manor House
Headley Way, Headington
Oxford OX3 9DZ

Tel: 01865 222692/222547
Fax: 01865 222699

Our Ref. DT/CEB/96.229

16 May, 1997

Dr N Day
Senior Registrar
Centre for Tropical Medicine
NDM, Level 7
John Radcliffe Hospital

Dear Dr Day,

RE COREC: 96.229 - Investigations of host susceptibility to invasive Staphylococcus aureus infection

We have now received the letter of indemnity from the Oxford Radcliffe NHS Trust and are happy to give final approval to your study.

Best wishes.

Yours sincerely,

Dr D Talbot
Chairman
Central Oxford Research Ethics Committee

Chairman: Dr D C Talbot

The Oxford Radcliffe NHS Trust is now managing the administrative support for the Research Ethics Committees under a Service Level Agreement to Oxfordshire Health Authority

The Oxford Radcliffe Hospital
A National Health Service Trust

Centre for Tropical Medicine

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Tel: +44 1865 220970
Fax: +44 1865 220984
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7th May 1998

Dear Colleague,

Collection of Cord Blood for Study of Host Genetic Susceptibility to severe *Staphylococcus aureus* infection (COREC Study no: 96.229).

We would be grateful if you could help us with the above study. The aim is to find genetic factors that may confer susceptibility to severe invasive *S. aureus* infection, a major cause of morbidity and mortality in all age groups. The puzzle is that whereas most of us at some time or other carry *S. aureus* in our noses, severe invasive disease is relatively rare. Is this beacuse certain individuals are genetically pre-disposed? To investigate this we are setting up a case-control study. 'Cases' are patients hospitalised with a severe form of invasive *S. aureus* infection, identified through the microbiology laboratory. The control group needs to reflect the raw overall gene pool of the local population, and the best way of identifying this is by studying neonates (before life's rigors get to them) via their cord blood.

What we are asking for is 2 to 10 mLs of cord blood, taken from the cord at the same stage that you would ordinarily take cord blood gases (obviously cord gases would take priority if there was a limited amount of blood). The blood can be stored in a fridge in the special 10 mL EDTA (purple top) tubes provided, and will be collected daily. The only information we ask you to put on the tube is the date and the ethnic group of the baby. Names, hospital numbers, etc. are specifically not required. The Oxford Central Ethics Committee has said that they are happy for cord blood to be taken in this way without seeking specific parental consent, as long as the samples are anonymised.

If you have any questions or problems please contact either Alex Smarason (Bleep 1028) or Nick Day (Bleep 1264).

Thank you in advance for you help.

Nick Day

Alex Smarason

Oxford Radcliffe
NHS TRUST

CENTRAL OXFORD RESEARCH
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Our ref. DCT/JW

12 March 1998

Dr Nicholas Day
University of Oxford
Centre for Tropical Medicine
John Radcliffe Hospital
Headington
Oxford OX3 9DU

Dear Dr Day

re: COREC 96.229 - Investigations of host susceptibility to invasive Staphylococcus aureus disease

Thank you for your recent letter asking for a modification to the submitted protocol *(Protocol Amendment dated 10 March 1998)* I am happy to give you Chairman's approval for this addendum providing cases remain anonymous.

You should inform COREC of any adverse reactions. In addition, if the investigators do not follow the protocol, or have protocol changes, but fail to inform COREC, then the Ethics Committee approval will be withdrawn and will no longer be binding.

Best wishes for your continuing studies.

Yours sincerely

Dr D C Talbot
Chairman
Central Oxford Research Ethics Committee

Chairman: Dr D C Talbot

The Oxford Radcliffe NHS Trust is now managing the administrative support for the Research Ethics Committees under a Service Level Agreement to Oxfordshire Health Authority

The Oxford Radcliffe Hospital
A National Health Service Trust

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DOCUMENTARY PROOF OF ETHICAL CLEARANCE COMMITTEE ON HUMAN RIGHTS RELATED TO RESEARCH INVOLVING HUMAN SUBJECTS FACULTY OF MEDICINE SIRIRAJ HOSPITA L MAHIDOL UNIVERSITY, BANGKOK, THAILAND

TITLE OF PROJECT : A STUDY OF HOST GENETIC SUSCEPTIBILITY TO SEVERE GRAM POSTIVE INFECTION IN THAILAND

PRINCIPAL INVESTIGATOR : DR. SURAPOL SUWANAGOOL
NAME OF DEPARTMENT : DEPARTMENT OF PREVENTIVE AND SOCIAL MEDICINE
APPROVED BY COMMITTEE ON HUMAN RIGHTS RELATED TO RESEARCH INVOLVING HUMAN SUBJECTS ON 30 OCTOBER 1997

SIGNATURE OF CHAIRMAN : .....
PROF. KHUN NANTA MARANETRA
M.D.,Grad Dip Clin Sc Med, Msc Med (BKK), Thai Board Resp Med,
MD (Melb.), FRCPT, FCCP, FRACP,FRCP (Lond.) , FRCP (Glasg.) , FRCP (Edin.)

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30 OCTOBER 1997

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