Application of whole-genome sequencing to understand transmission of healthcare-associated *Staphylococcus aureus*

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Abstract

Staphylococcus aureus is a leading cause of healthcare associated infection. Efforts to reduce the burden of *S. aureus* infections in healthcare settings have targeted patient carriage and preventing person-to-person transmission. These measures have only been partially successful. Our understanding of *S. aureus* transmission is limited by low discrimination of currently available typing techniques. The high resolution offered by whole-genome sequencing (WGS) has the potential to overcome these limitations. Work undertaken in this thesis exploits recent advances in WGS to understand *S. aureus* transmission in healthcare settings and in so doing establish the potential for WGS to replace current typing systems in infection control practice.

The first study compares conventional approaches and WGS to investigate an outbreak of Methicillin Resistant *S. aureus* (MRSA) blood stream infections in a single UK hospital. By WGS, isolates within the EMRSA-16 lineage (one of the two dominant nosocomial MRSA lineages) showed strikingly low genetic diversity demonstrating the emergence of a clonal variant. This clonal variant, indistinguishable from the ancestral strain by conventional typing, accounted for 89% of EMRSA-16 bacteraemia isolates at the outbreak hospital from 2006 and was associated with greater neutrophilia ($p<0.001$) compared with infection caused by other strains. Investigation of local and national *S. aureus* collections revealed the presence of isolates highly related to the variant in other hospitals across England, suggesting spread across large geographical areas. This represents the first report of a clonal variant being associated with an outbreak and provides novel insight into the epidemiology and population structures within dominant *S. aureus* lineages.

The second study investigated the role of colonised patients as the source of new *S. aureus* acquisition in a hospital setting. Over 14 months all patients admitted to an adult intensive care unit were assessed for carriage, acquisition and their role in transmission. Among 680 patients where two or more serial samples were available only 44 acquisitions were observed. Isolates were available for genetic analysis from 37 acquisitions and among these only 7 (18.9%) could be explained by patient-to-patient transmission. WGS disproved 3 transmission events indicated by conventional methods (*spa*-typing combined with overlapping patient stay) and also revealed 4 transmission and 2 acquisition events. Furthermore, WGS has the

resolution to differentiate carriage strains within and between hosts allowing thresholds to be established to interpret the relationship of transmission pairs. This represents the first systematic evaluation of nosocomial patient-to-patient transmission of *S. aureus* using WGS. These results challenge previous assumptions about the role of patients in *S. aureus* transmission routes in hospital settings.

The third study was conducted to assess whether staff carriage or environmental contamination accounted for the gap observed in nosocomial *S. aureus* transmission. To investigate this, patient screening was extended and coupled with staff screening and extensive environmental sampling. Carriage rates were higher in staff than patients (57.6% vs. 20.6%, $p<0.01$). Using *spa*-typing to identify potential routes of acquisition it was possible to infer a staff or environment source for 48/88 (54%) evaluable patient acquisitions including six ward-based outbreaks. Of note 13% of staff were found to carry *S. aureus* in their throat but not their nose and in 6 instances this was implicated in patient acquisition. Despite screening over 95% of patients and staff, and extensive environmental sampling, a donor could not be identified for a quarter of patient acquisitions according to *spa*-typing. WGS data were not available at the time of writing this thesis and are needed to fully understand the extent of staff and environmental sources in patient acquisition. Nevertheless these data indicate an important role for staff and environmental contamination in *S. aureus* acquisition and also suggest that the role of additional groups (visitors, other healthcare workers) should now be evaluated.

The data presented contribute towards improving our understanding of nosocomial *S. aureus* transmission. WGS allows rapid identification of newly emerging strains enabling targeted investigation of transmission routes and, in turn, facilitates the development of effective infection control measures. Furthermore WGS permits detailed characterisation of transmission routes to optimise infection control strategies and manage outbreaks. These will support the translation of WGS as a tool for the assessment of *S. aureus* transmission from research into clinical practice.

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Author declaration

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to these or any other university for a degree, and does not incorporate any material already submitted for a degree.

Signed:

Dated: 15th May 2014

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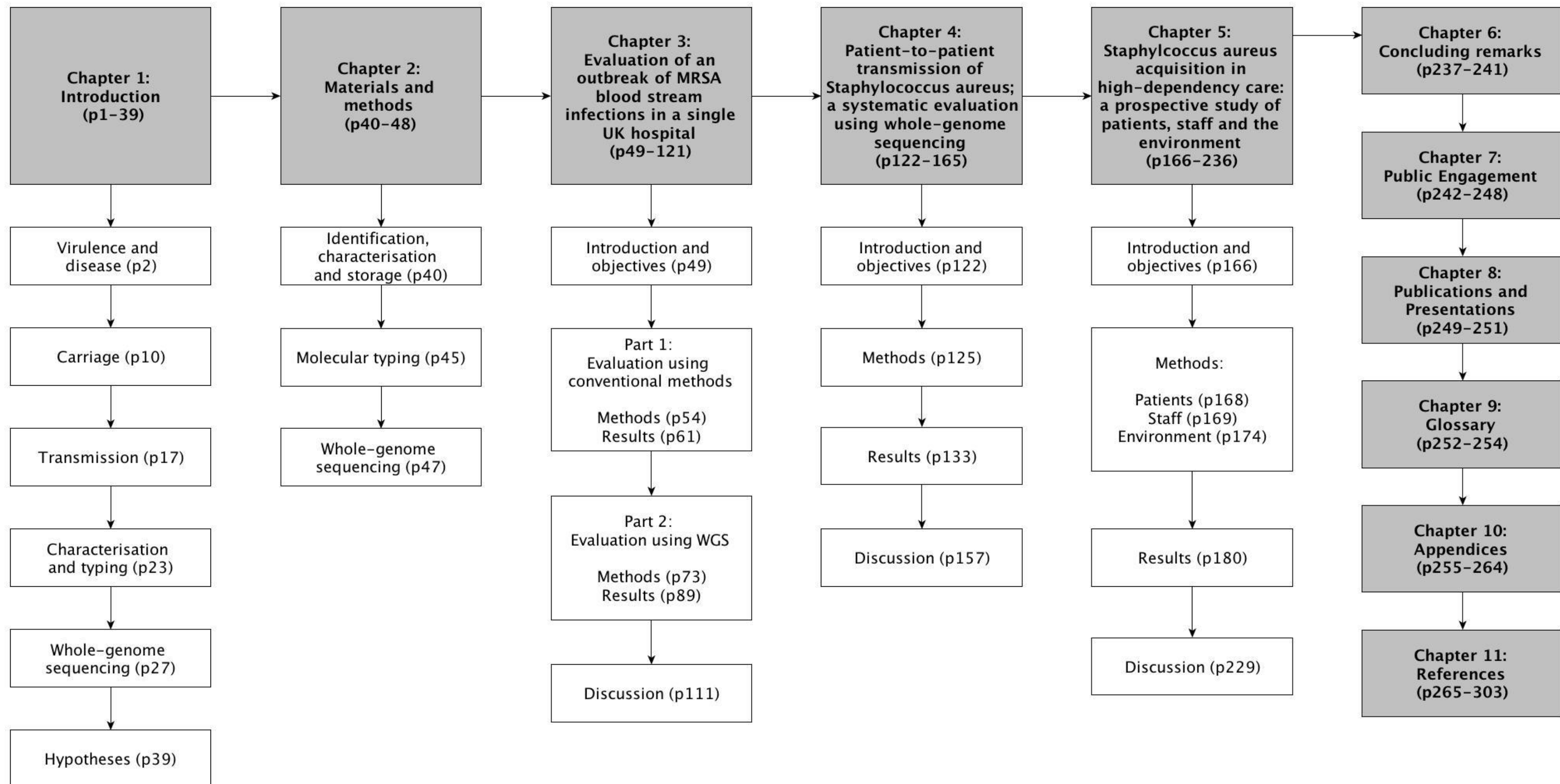

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Abbreviations

bp	Base pair
BGM	Blood gas machine
BLAST	Basic Local Alignment Search Tool
BSAC	British Society for Antimicrobial Chemotherapy
BSUH	Brighton and Sussex University Hospital NHS Trust
BURP	Based Upon Random Pattern
CBA	Columbia blood agar
CC	Clonal complex
CI	Confidence interval
CRF	Case report file
CRP	C-reactive protein
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotidetriphosphates
EDTA	Ethylenediaminetetraacetic acid
EMRSA	Epidemic Methicillin Resistant *Staphylococcus aureus*
g	Gram
GP	General Practitioner
HCl	Hydrochloric acid
HDU	High Dependency Unit
HIV	Human immunodeficiency virus
HPA	Health Protection Agency

H_2O	Water
ICU	Intensive Care Unit
IQR	Interquartile range
IST	Iso-sensitest agar
IV	Intravenous
IVDU	Intravenous drug user
Kb	kilobase
K_2HPO_4	Dipotassium phosphate
L	Litre
MALDI-TOF	Matrix Assisted Laser Desorption/Ionisation – Time of Flight
mg	Milligram
$MgCl_2$	Magnesium chloride
MIC	Minimum inhibitory concentration
MICE	Minimum inhibitory concentration evaluator
ml	Millilitre
MLST	Multi-locus sequence type
mM	Millimolar
MMM	Modernising Medical Microbiology
MRSA	Methicillin Resistant *Staphylococcus aureus*
MSSA	Methicillin Sensitive *Staphylococcus aureus*
NaCl	Sodium chloride
NHS	National Health Service
OR	Odds ratio

OUH	Oxford University Hospitals
PBP	Penicillin binding protein
PCR	Polymerase Chain Reaction
PFGE	Pulsed-field gel electrophoresis
PHE	Public Health England
PVL	Panton-Valentine Leukocidin
RM-typing	Restriction-modification typing
RR	Relative risk
RSCH	Royal Sussex County Hospital, Brighton, UK
s	Second
SCC	Staphylococcal chromosomal cassette
SD	Standard deviation
sea	Staphylococcal enterotoxin A
sej	Staphylococcal enterotoxin J
SIBP	Specific identification of biomarker proteins
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variant
SSSS	Staphylococcal scalded skin syndrome
ST	Sequence type
t-	*spa*-type
TE	Tris ethylenediaminetetraacetic acid
Tris	2-amino-2-hydroxymethyl-propane-1,3-diol
TSS	Toxic shock syndrome

tst	Toxic shock syndrome toxin
UCLH	University College London Hospitals
UK	United Kingdom
UKCIRG	United Kingdom Clinical Infection Research Group
UKCRC	United Kingdom Clinical Research Collaboration
UPC	Unique participant code
WCC	White cell count
WGS	Whole-genome sequencing
WTCHG	Wellcome Trust Centre for Human Genetics
µg	Microgram
µl	Microlitre
µM	Micromolar

1 Introduction

At the time of writing the genus *Staphylococcus* comprises 47 species and 24 sub-species.[1] Whilst the majority of staphylococci have low pathogenic potential *Staphylococcus aureus* is a major pathogen in humans. Alexander Ogston first drew attention to '*staphylococcus'* as an agent of postsurgical sepsis more than 100 years ago.[2] In the pre-antibiotic era invasive *S. aureus* infection had a mortality in excess of 80%.[3] Despite diagnostic and therapeutic advances *S. aureus* remains a leading cause of nosocomial blood stream infections worldwide and mortality rates remain high at 30%.[4,5] In addition, management of infection is hampered by the emergence of antibiotic resistant strains.[6,7] Consequently it is important to differentiate *S. aureus* from other staphylococci.

Staphylococci are spherical, non-motile, non-spore forming bacteria appearing singly, in pairs or in grape-like clusters on Gram stain. Growth is optimal at 30-37°C on a wide variety of culture media in both aerobic and micro-aerophilic conditions. Colonies on solid media are round, smooth and vary from white to yellow in colour. Identification of *S. aureus* in routine laboratory practice involves determining the presence of: (i) catalase (bubbles in the presence of hydrogen peroxide due to enzymatic conversion to oxygen and water), (ii) coagulase (coagulation of rabbit plasma following enzymatic conversion of fibrinogen to fibrin), (iii) clumping factor or Protein A (commercial latex agglutination assays for these virulence adhesins).[8] Recently introduced chromogenic agar allows identification through colour changes in response to phosphatase activity present in *S. aureus*. Furthermore, due to the halotolerant nature of *S. aureus* (growth in the absence as well as presence of high salt concentrations) salt broth (5-7% NaCl) can be used for selective culture. Molecular methods based on detection of *S. aureus* specific genes such as nuclease (*nuc*), coagulase (*coa*) and protein A (*spa*) are now being used to detect the presence of *S. aureus* from clinical samples.[9] Mass spectrometry (matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF)) is increasingly being used for rapid organism identification in cultured isolates through molecular mass profiling of species-specific proteins.[10]

1.1 Virulence

1.1.1 Genome and phylogeny

The circular genome of *S. aureus* is composed of about 2.8 million nucleotides and is about one thousand times smaller than the human genome (c.3 billion nucleotides).[11] Most of the genome is composed of genes present in all strains (the core genome) that encode proteins involved in fundamental functions such as cellular metabolism, growth and replication. About 10% of the genome consists of sets of genes that vary between different lineages and is designated the 'core variable genome'.[12] Between 10% and 20% of the genome consists of 'mobile genetic elements' (MGE); regions that are gained and lost by organisms at high frequencies (lateral gene transfer) and which often encode virulence factors and resistance genes. Many virulence and resistance genes are located on *S. aureus* Pathogenicity Islands (SaPI), 15-70kb MGE frequently acquired from other organisms.[13]

S. aureus has a markedly clonal population structure.[14-17] Most disease-causing isolates belong to a small number of lineages or clonal complexes. Indeed most of the strains that colonise humans belong to one of ten dominant lineages.[12] Several nomenclatures exist and can cause confusion. The naming of the most dominant lineages are set out in Table 1.1. Within lineages, differences in the core genome occur as a result of point mutation and to a lesser extent through recombination events.[14] The lineage population structure of *S. aureus* has been explained by the presence of lineage-specific variations in enzymic restriction modification systems that limit acquisition of DNA.[18] In recent decades, two epidemic lineages, designated EMRSA-15 and EMRSA-16 (originally defined by phage typing patterns), became the dominant healthcare-associated strains in the UK. There is geographical variation in the prevalence of different strains across Europe.[19] Different strains appear to emerge and decline in waves[20] but the driving forces behind such processes remain unclear.

Table 1.1 Common international *Staphylococcus aureus* strains and their associated nomenclature according to different typing schemes; Multi-Locus Sequence Type (MLST), *spa*-type (*spa*), UK epidemic MRSA (EMRSA), pulsed-field gel electrophoresis (PFGE) and Staphylococcal Cassette Chromosome (SCC) *mec* typing.

Clonal Complex	MLST	*spa*[1]	UK Epidemic MRSA	PFGE	SCC*mec*	Common Names
CC1	ST1	t128		USA400	IV	Canadian MRSA-7
	ST772	t657			V	Bengal Bay clone, WA MRSA-60
CC5	ST5	t001	EMRSA-3	USA100	II	New York-Japan Clone, Rhine-Hesse Epidemic Strain, Canadian MRSA-2, Irish AR07.3/07.4/AR11
	ST228	t041			I	South German Epidemic Strain, Italian Clone, Spanish PFGE types E6/9/15/17/18
CC8	ST8	t008		USA300	IV	WA MRSA-12, Canadian MRSA-10, Spanish PFGE A
	ST8	t190	EMRSA-2, -6, -12, -13, -14		IV	Lyon Clone, Irish-1/-2
	ST239[2]	t037	EMRSA-1, -4, -7, -9, -11		III	Czech/Vienna/Hungarian/Portuguese/Brazilian Clone, AUS-EMRSA-2/-3, Irish phenotype III, Irish AR01/09/15/23, Canadian MRSA-3/-6
	ST247	t051	EMRSA-5, -8, -17		I	North German Epidemic Strain, Rome Clone, Irish AR22, Irish New02, Iberian Clone, Spanish PFGE E1
	ST250	t194	EMRSA-8		I	Early/ancestral MRSA, Irish AR02, Irish phenotype 1/2
	ST254	t009	EMRSA-10		Atypical	Hannover Epidemic Strain
CC22	ST22	t032	EMRSA-15		IV	Irish AR06, Barnim Epidemic Strain, Canadian MRSA- 8, Spanish PFGE type E13
CC30	ST30	t019		USA1100	IV	Southwest Pacific Clone, West Samoan Phage Pattern Clone
	ST36	t018	EMRSA-16	USA200	II	Irish AR7.0/07.2, Canadian MRSA-4, Spanish PFGE E12
CC45	ST45	t004		USA600	II	Canadian MRSA-1
CC59	ST59	t437			V	South-East Asia Clone, Taiwan Clone
CC80	ST80	t044			IV	European (community acquired MRSA) clone
CC93	ST93	t202			IV	Queensland Clone

[1] Depicts the most frequently reported *spa*-type; divergent *spa*-types are not included in table (www.spa.ridom.de)
[2] Allocated to CC8 but contains recombination [CC8 (80%) and CC30 (20%)]

1.1.2 Virulence determinants

A variety of determinants contribute to the virulence of *S. aureus*. Virulence factors include peptidoglycans in cell wall, capsule production, and expression of surface proteins, toxins and enzymes.[21] Typical strains of *S. aureus* carry multiple virulence factors. These factors can be important individually but also there is evidence of co-ordination of virulence through multiple factors.[22,23] Little is known about their precise interactions but gene expression is thought to be regulated by global regulator genes.[21] Putative candidate genes have been epidemiologically linked to distinct clinical phenotypes that may suggest roles in the pathogenesis. Virulence factors can be categorised into three categories (Table 1.2). Firstly, adhesins (such as Protein A, fibronectin-binding proteins, clumping factor) allow colonisation through adhesion and evasion of the immune system. Secondly, an array of variably present toxins are associated with *S. aureus*. Some may result in distinct clinical phenotypes; enterotoxins cause gastroenteritis, exfoliative toxins cause scalded skin syndrome and toxic shock syndrome toxins results in toxic shock syndrome (severe sepsis). The precise advantage conferred by these toxins is not understood. Others such as the haemolysins and leukocidins play a clearer role in pathogenesis. Thirdly, secreted enzymes contribute towards pathogenesis by promoting tissue destruction and spread of bacteria.

S. aureus encodes several leukocidin toxins and among these there has been recent interest in Panton Valentine Leukocidin (PVL) as a virulence factor in community infections but its precise role in disease pathogenesis remains controversial. PVL is encoded by two genes (*lukS-PV* and *lukF-PV*) present on a mobile phage which may be present in both MSSA and MRSA strains. Expression results in formation of a cytotoxin that creates pores in host cell membranes causing tissue necrosis and neutrophilic destruction. In contrast to other highly prevalent leukocidins the epidemiology of PVL is variable. Universally present amongst community-acquired MRSA (CA-MRSA) two PVL-expressing clones (USA300/400) have successfully spread throughout North American communities. Commonly associated with aggressive skin and soft tissue infections in young healthy individuals CA-MRSA also causes severe bone and joint infections, necrotising pneumonia, and sepsis. In Europe PVL-related disease is uncommon and more frequently associated with MSSA.

1.1.3 Antibiotic resistance

The development of resistance to antibiotics has contributed to the on-going burden of *S. aureus* infections. This is thought to be the result of selective pressure from antibiotic usage, particularly in hospitals. Resistance has been reported to all antibiotic classes but the most notable are to β-lactams (such as penicillin and methicillin) and glycopeptides (vancomycin).

Penicillin resistance is mediated by penicillinases, inducible enzymes that hydrolyses penicillin and stops its inhibitory action on bacterial cell-wall assembly. Their emergence rapidly followed the introduction of penicillin in the 1940s and currently penicillin resistance is found in up to 80% of hospital and community *S. aureus* isolates.[24]

Methicillin resistant *Staphylococcus aureus* (MRSA) strains evolved through acquisition of *mecA* gene on Staphylococcal Chromosomal Cassette (SCC), transferred on a mobile genetic element. The *mecA* gene encodes penicillin binding protein 2A (PBP2A) possessing a low affinity to methicillin and most other β-lactam antibiotics.[25] Currently, 6 types of SCC*mec* have been identified and appear to be lineage-specific, for example type II is associated with EMRSA-16 and type IV is associated with EMRSA-15.[26] Hence, MRSA strains may be classified and typed according to the composition of SCC*mec*.[27]

Hospital-associated (HA-) MRSA strains presumably arose due to increased antibiotic pressures, providing one explanation why HA-MRSA is typically multi-drug resistant and confined to only few clones. For example, in the UK HA-MRSA was caused by two clones (EMRSA-15 and EMRSA-16). Infections caused by HA-MRSA typically occur in those with preceding healthcare admission or those with indwelling devices, mostly causing blood stream infections. MRSA is now prevalent in healthcare institutes worldwide.[28] This is a concern as HA-MRSA infections are associated with longer patient stays, increased mortality and higher financial burden.[6,29] More recently MRSA has been found outside hospital settings. Community-associated (CA-) MRSA is a frequent cause of aggressive skin and soft tissue infections, particularly in young people with no preceding hospital contact.[30] In contrast to hospital-associated strains CA-MRSA exhibit less drug resistance, with the exception of the USA300 strain that spread throughout northern America.[13,30]

Increasing rates of β-lactam resistance have resulted in increased use of other classes of antibiotics, most notably glycopeptides. Glycopeptides (including vancomycin and teicoplanin) are also cell-wall active drugs but inhibit peptidoglycan elongation by binding to the D-alanyl-D-alanine tail and preventing addition of amino acids.[31] Vancomycin and teicoplanin both possess a common heptapeptide backbone core but differ by the number of side chains present.[32] They are currently first line treatment in MRSA, and MSSA infection in patients with anaphylactic penicillin allergy.

Glycopeptide susceptibility is currently assessed by determining the lowest antibiotic concentration required to inhibit organism growth, the minimum inhibitory concentration (MIC). Vancomycin and teicoplanin susceptible *S. aureus* strains have an MIC <4mg/L.[33] In recent years reduced susceptibility to vancomycin has been reported in three groups of isolates; (i) vancomycin resistant *S. aureus* (VRSA; MIC ≥16mg/L), (ii) vancomycin intermediate *S. aureus* (VISA; MIC 4-8mg/L), and (iii) heterogenous vancomycin intermediate *S. aureus* (hVISA; MIC <4mg/L with subpopulations growing at higher MICs).[34-36] Reduced susceptibility to vancomycin is usually associated with reduced teicoplanin susceptibility.[37] Whilst authors have reported treatment failures and increased mortality rates associated with infections caused by isolates with higher vancomycin MICs[38,39] these relationships have not been universally observed.[40] Currently the prevalence of these isolates is low but with increasing MRSA burden and subsequent vancomycin use this is expected to change. A number of mechanisms for glycopeptide resistance have been identified, including transposon acquisition from *Enterococcus faecalis*.[41]

As vancomycin susceptibility diminishes, and resistance follows, alternative therapeutic agents are urgently needed. In 2000 the Food and Drug Administration approved linezolid for treatment of complicated *S. aureus* skin and soft tissue infections and *S. aureus* pneumonia. In 2006 daptomycin was approved by the European Medical Agency for the treatment of *S. aureus* bacteraemia and right-sided infective endocarditis. A number of other alternative antimicrobial agents (tigecycline, telavancin) are currently being evaluated[42,43], and novel agents (dalbavancin, oritavancin, ceftobiprole, ceftaroline) are undergoing clinical trials.[43] Older agents such as rifampicin and tetracyclines remain active against many strains of MRSA and may be used, particularly in combination regimens.

Table 1.2 Principal *Staphylococcus aureus* virulence factors and their pathogenic function

Virulence Factor	Gene	Function	Reference
Adhesins			
Protein A	*spa*	Cell wall component that binds antibodies and inhibits phagocytosis	Gomez *et al.* (2006)[44] Hartleib *et al.* (2000)[45]
Fibronectin-binding protein	*fnbA-B*	Fibronectin adhesion promoting binding to mucosal cells	Greene *et al.* (1995)[46]
Clumping Factor	*clfA-B*	Fibrinogen adhesion promoting binding to mucosal cells	McDevitt *et al.* (1994)[47] Ni Eidhin *et al.* (1998)[48]
Collagen-binding protein	*cna*	Collagen adhesion	Patti *et al.* (1992)[49]
Sdr Family	*sdrC,D,E*	Putative adhesive factors with unknown function	Josefsson *et al.* (1998)[50]
Intracellular adhesion (ica)	*icaA-C*	Biofilm formation	Cramton *et al.* (1999)[51]
Cytotoxins			
Haemolysins (α,β,γ,δ)	*hla,hlb, hld, hlg*	Damage to cell membrane of red blood cells results in cell lysis	Bhakdi *et al.* (1991)[52] Arbuthnott (1982)[53]
Panton-Valentine leukocidin (PVL)	*lukS-PV, lukF-PV*	Pore formation in leucocytes results in cell lysis	Tristan *et al.* (2007)[54]
Leukocidin E-D	*LukED*	Lysis of leucocytes	Reyes-Robles *et al.* (2013)[55]
Enterotoxins (A-O)	*Sea - seo*	Exotoxin causing superantigenic activity in intestinal walls leading to vomiting and secretory diarrhoea	Bohach *et al.* (1990)[56]
Exfoliative toxins (A-B)	*eta, etb*	Protease activity causes blistering and desquamation leading to scalded skin syndrome in children.	Tristan *et al.* (2007)[54]
Toxic shock syndrome toxins	*tst-1*	Superantigenic hyperactivation leading to morbilliform rash and septic shock	
Enzymes			
Coagulase	*coa*	Surface-bound enzyme promoting clot digestion	Cheng *et al.* (2010)[57]
Nuclease	*nuc*	Combating host immune response	Berends *et al.* (2010)[58]
Hyaluronidase	*hys*	Tissue invasion and spreading factor	Cheung *et al.* (2002)[59]
Staphylokinase	*sak*	Dissolves fibrin and evasion of host defences	
V8 protease	*ssp*	Tissue invasion	

1.2 Disease

Staphylococci frequently colonise the skin and mucosal surfaces of humans and animals.[13] Many species in the genus seldom appear as human pathogens outside the context of immunosuppression and implanted prosthetic material. Among the staphylococci that cause disease in humans *S. aureus* is preeminent because of its large range of virulence factors and the diverse spectrum of disease. Other species occasionally cause disease in humans; *S. saprophyticus* causes urinary infections in young women, *S. lugdunensis* is a rare cause of endocarditis. Several species are animal pathogens and rare causes of zoonotic disease, most notably *S. intermedius* which is a pathogen of dogs.[13]

The progression of *S. aureus* in a patient from harmless commensal to invasive pathogen is multifactorial. Its capacity to adhere to connective tissue permits frequent colonisation. A diverse collection of virulence factors contribute towards host invasion, metastatic spread in the blood and the establishment of deep foci of infection. Host factors that are common in hospitalised patients also predispose towards infection, such as loss of protective skin barrier and underlying co-morbidities. These, coupled with the ability of *S. aureus* to acquire resistance to commonly used antibiotics, make it a highly successful nosocomial pathogen.

1.2.1 Clinical spectrum of disease

S. aureus is a leading cause of hospital and community acquired infection worldwide.[60,61] The first step in the pathogenesis of *S. aureus* is colonisation. Acutely, disease can occur locally at the site of colonisation or at remote sites via toxin production. Pyogenic infections of the skin (such as folliculitis, furuncles and carbuncles) and soft tissues (cellulitis, erysipelas) are common.[21] Severe soft tissue infections such as pyomyositis and necrotising fasciitis are rare. Spread of disease from the primary site can occur, with extension occurring through either contiguous extension or dissemination in the blood. The presence of bacteria in the blood (bacteraemia) is commonly associated with secondary site infections (such as infective endocarditis and discitis) and results in significant morbidity and mortality.

Genes encoding protein exotoxins, mostly carried on mobile genetic elements, are present in a proportion of *S. aureus* strains and are associated with particular

virulence phenotypes. For example, the exfoliative toxins A and B act on the epidermis resulting in staphylococcal scalded skin syndrome (SSSS). Originally described by Baron Ritter von Rittershain in 1878 SSSS can cause a range of clinical presentations from localised blistering to generalised scalding.[13] A separate important class of toxins are the superantigens. These trigger polyclonal T-cell activation resulting in a massive uncoordinated release of cytokines and an overwhelming inflammatory reaction.[62] *S. aureus* can produce two groups of superantigens; toxic shock syndrome (TSS) toxin-1 and enterotoxins. TSS was originally described in association with retained highly absorbent tampons, where alterations in environmental conditions permitted toxin expression that led to fulminant sepsis.[63] Non-menstrual TSS is rare but can occur in any patient colonised with a toxigenic strain. Enterotoxins represent a diverse group of 15 heat-stable superantigens (staphylococcal enterotoxin A-O) that cause severe vomiting and diarrhoea 2-4 hours after ingestion.

1.2.2 Risk factors

As for almost any bacterial pathogen, *S. aureus* carriage precedes disease. Our understanding of this relates to three bodies of work. Firstly, infections occur more commonly in carriers than non-carriers.[64] Whilst persistent carriers are at higher risk of invasive disease than intermittent or non-carriers, mortality risk following invasive disease is higher in patients newly acquiring *S. aureus*.[65] Secondly, strains causing infection are usually the same strain type as the colonising strain (their own strain).[66] Thirdly, transient eradication reduces risk of subsequent infection.[67-69]

Additional factors have been repeatedly associated with increased risk of *S. aureus* infection including: (i) demographic factors such as male gender and extremes of age; (ii) underlying conditions such as human immunodeficiency virus (HIV), malignancy, diabetes, rheumatoid arthritis, heart disease; (iii) medical care such as renal replacement therapy (haemodialysis and peritoneal dialysis); (iv) social factors such as intravenous drug use and alcohol abuse.[13,70-72]

1.3 Carriage

1.3.1 General population carriage

Over one third of the general population are reported to carry *S. aureus* at any one time.[73] Whilst the anterior nares are the most common site of carriage, *S. aureus* can also be isolated from extra-nasal sites including axillae, pharynx, groin, perineum and rectum.[74-78] Longitudinal studies have demonstrated three carrier states; *persistent* colonisation with the same strain (20%), *intermittent* colonisation with varying strains for short periods of time (60%), and *non-carriers* (20%).[73,79,80] These studies suggest that up to 80% of the general population may become colonised with *S. aureus* at some point in their lives. The distinction between different carrier profiles may be important as persistent carriers are at increased risk of developing infection.[81] Different carriage phenotypes might also influence potential for *S. aureus* transmission.

The asymptomatic carrier state is considered to be important in the development, persistence and spread of antibiotic resistant strains.[73] The ability to resist traditional antibiotics has given rise to increasing prevalence of MRSA carriage.[82] Carriage rates vary across age, gender and ethnic groups.[74,79,83-85] Carriage rates are higher in patients with (i) underlying co-morbidities, such as diabetes mellitus, recurrent skin infections and/or skin disease, end-stage liver disease, HIV, intra-venous drug use and those requiring renal replacement therapy[72,86-92], and (ii) healthcare exposure including previous antibiotic exposure, prolonged hospital stay, surgery, intensive care unit (ICU) admission, nursing home resident and previous contact with MRSA colonised or infected persons.[93,94]

Whilst many studies assume that individuals are colonized with a single strain, colonisation of >1 genotype is also well described. Bloemendaal *et al.* (2009) evaluated multiple genotype carriage in patients across 6 European ICUs and identified polyclonal colonization in 8% patients.[95] Furthermore Cespedes *et al.* (2005) revealed polyclonal nasal colonization in 6.6% of HIV positive patients and former drug users using mathematical modelling analysis.[96] Conversely, Mongkolrattathai *et al.* (2011) evaluated *S. aureus* carriage in 41 children and identified ~30% samples cultured >1 genotype.[97] Discrepancies in rates may result

from differences in sampling frames, preceding antibiotic exposure or methodologies.

1.3.2 Host-pathogen relationship

S. aureus colonisation appears to involve a dynamic interaction between bacterium and host. Numerous bacterial factors have been found to contribute towards carriage. Strains that are successful at colonising adhere to the host through the expression of various tissue-adherence factors, such as cell wall-associated microbial surface component recognising adhesive matrix molecules (MSCRAMMS), clumping factor and fibronectin-binding proteins.[98,99] Once attached strains express immune evasion molecules. Some target immunoglobulins (Protein A) or complement (chemotaxis inhibitory proteins (CHIPS) and staphylococcal complement inhibitor (SCIN)) and others counteract the effects of antimicrobial substances such as lysozyme and defensins (cell-wall teichoic acid, staphylokinase). Furthermore, during colonisation there is down-regulation of virulence genes and toxin production.[100] These responses are co-ordinated by bacterial regulatory components such as the *agr* locus.

Colonising *S. aureus* strains share their environment with other commensal organisms, commonly *Corynebacterium* spp., *Propionibacterium* spp. and other *Staphylococcus* spp.. There is evidence to suggest that interactions between these organisms influence *S. aureus* carriage. Frank *et al.* (2010) suggested that in non-*S. aureus* colonised adults *S. epidermidis* is the predominant colonising organism, yet when *S. aureus* is carried it dominates the nasal microbiota.[101] Furthermore, Uehara *et al.* (2000) reported complete eradication of *S. aureus* carriage in patients artificially inoculated with strains of *Corynebacterium* species.[102] The mechanisms behind these interactions remain unclear.

Despite its successful adaptation to colonising humans carriage profiling has indicated that 20% of the population never carry *S. aureus,* suggesting host factors also influence colonisation. In contrast to our understanding of bacterial factors known to be associated with colonisation little is known about the role of the host. Two host factors appear to be important; anatomy and immunity.

Three lines of evidence support the concept that anatomical factors influence *S. aureus* carriage. First *S. aureus* preferentially colonises the vestibulum nasi, the most

anterior part of the nasal vestibule void of ciliated epithelia.[103] Shuter *et al.* (1996) observed that *S. aureus* poorly adhere to ciliated epithelia (cells found throughout the rest of the upper respiratory tract) suggesting preferential adherence to particular cell types.[103] Secondly, nasal mucosal cells from known *S. aureus* carriers have higher affinity for *S. aureus* adherence compared with known non-carriers.[104] Thirdly, variations in bacteriocidal activity of nasal secretions effect *S. aureus* carriage.[105]

Host immunity also appears to influence carriage. This is suggested by (i) the strong association between patients with impaired immune systems (HIV, chronic diseases) and higher *S. aureus* colonisation rates[72,86-92] and (ii) the association between persistent carriage and mutations in various elements of the innate immune system including the glucocorticoid receptor[106], serine protease C1 inhibitor[107], mannose-binding lectin genes[108] and interleukin-4.[109] Adaptive immunity also appears important as frequent exposure to *S. aureus* leaves an immunological antibody memory, although the mechanism of antibody induction by colonisation has not established. Ehrenkranz (1966) revealed that previously colonised patients who were re-inoculated with the same strain following decolonisation have shorter carriage duration than those inoculated with a different strain, suggesting an immune-mediated role.[110] Harrison *et al.* (2009) suggest that colonisation alone is not enough to stimulate an adaptive memory response and proposed mechanisms include transient bacteraemia, minor infections or absorption of toxins across the mucosa.[111] Most adults and children have varying degrees of *S. aureus* antibodies irrespective of whether they are colonised or not. Maternal transfer of *S. aureus* antibodies to an infant does not protect the child from colonisation during infancy, nor does the development of the child's own antibodies.[112,113]

Despite the high prevalence of *S. aureus* antibodies (of varying classes) it remains unclear whether they prevent colonisation or disease. Population-based studies on host genetic predisposition to *S. aureus* carriage are conflicting. Nobel *et al.* (1967) suggested a familial predisposition to carriage due to observations of significant excess of *S. aureus* carriers in families of carriers.[114] Conversely, persistent carriage patterns are not concordant between genetically highly related individuals (same-sex siblings or twin pairs).[98,115] Furthermore the role of protective immunity is unclear in those patients who get recurrent *S. aureus* infections (i.e. boils and abscesses).

An understanding of host immune responses to carriage (and disease) provides the possibility of passive and active immunization strategies. Passive immunization using monoclonal antibodies has the potential to treat disease (in combination with conventional treatments) or prevent infection in at-risk patients. Animal model studies have supported observations in human studies that various immunological constituents influence *S. aureus* colonisation and disease.[116-118] Furthermore murine vaccination models have provided promising results indicating that certain vaccines epitopes provide protective responses against *S. aureus* disease.[119,120] Together these studies have provided a platform for the evaluation of human *S. aureus* immunisation. To date there have been six human passive immunization studies (3 involving treatment of infection and 3 aimed at disease prevention) evaluating monoclonal antibodies to various *S. aureus*-specific epitopes (including clumping factor A and lipoteicoic acid).[121] All have either failed to show significant responses or data have not been published. Active immunisation using vaccines has the potential to prevent colonisation and disease development. To date there have been two registered Phase III trials evaluating the effect of active vaccination on rates of *S. aureus* bacteraemia in patients at risk of developing disease. Both have failed to show efficacy.[121,122] One trial (unpublished) evaluating a novel vaccine (targeting haemoglobin receptor, *isdB*) in patients undergoing cardiothoracic surgery was prematurely stopped due to excess of deaths related to multi-organ failure.[121] The reason for these studies failing to show efficacy remains unclear but is likely to reflect differences in immune responses between animals and humans, and the complexity of the human-pathogen relationship.[123]

1.3.3 Carriage among healthcare workers

Most investigations of *S. aureus* carriage in healthcare workers, particularly MRSA, are in the context of outbreaks. Albrich *et al.* (2008) reviewed available studies of *S. aureus* carriage in healthcare workers. In 127 studies the average carriage rate of MRSA was 4.6% (range 0-59%, CI 1-8.2%).[124] Whilst fewer studies (n=41) were available on MSSA carriage, an average carriage rate of 23.7% (range 0-40%, 95% CI 10.7-36.7) was observed.

Whilst nasal carriage appears to be the dominant reservoir in hospital staff some studies have reported high rates of colonisation at other anatomical sites. Nilsson *et*

al. (2006) observed significantly higher rates of throat carriage compared to nasal carriage (54% vs. 36%, p=0.023) in 87 orthopaedic healthcare workers.[78] Furthermore hand carriage has also been observed, although this is usually temporary and dependant on compliance with hand hygiene.[124]

Duration of carriage can vary from days, months, to years.[124-126] In addition to the carriage profiles described in the general population an additional carrier state of *transient* carriage has been described in healthcare workers.[125,127] Staff become colonised during a shift and are culture negative the following day. Cookson *et al.* (1989) reported 46% of nursing staff become transiently colonised in their nose or on their hands during a work shift.[125] Healthcare worker colonisation rates vary depending on the patient group cared for and ward specialty. Particularly high rates have been observed in older patients and in general wards.[124] In institutions where MRSA prevalence is high staff are more likely to be MRSA colonised compared with lower prevalence settings.[128]

Whilst few studies have specifically evaluated risk factors associated with MSSA carriage in hospital staff, they appear largely to mirror those identified in the general population. Risk factors association with MRSA carriage in healthcare workers are summarised in Table 1.3. In addition staff members may themselves be suffering from staphylococcal infection.[129,130] Albrich *et al.* (2008) reported that 48/942 (5.1%) surveyed healthcare workers had symptomatic MRSA infections, the most common involving skin and soft tissue and upper respiratory tract.[124]

Reported rates of colonisation vary dramatically between healthcare professions.[124,127,131-133] Nursing staff and physicians hold higher rates of *S. aureus* colonisation compared with other healthcare workers. It has been speculated that different degrees of carriage is related to differences in patient contact[125,134] but others have questioned this.[135] Reasons for possible differences in carriage between healthcare worker groups remain undetermined.

1.3.4 Environmental contamination

The environment becomes contaminated when *S. aureus* is shed from colonised individuals. This may occur via the shedding of dead skin cells.[136] Some individuals may contaminate the environment through droplet spread during the course of upper respiratory tract infection.[137] *S. aureus* can survive in the environment despite exposure to ranges of temperature and humidity.[138] It can persist in the environment for months to years[76,139]. Dominguez *et al.* (1994) showed environmental persistence of the same strain type for 5 years during an outbreak in a Spanish hospital.[140]

In the hospital environment inanimate objects readily become colonised including mattresses, hospital curtains, keyboards, door handles, pens, stethoscopes, tourniquets, beds and telephones.[141-148] In the community the home environment has been recognised as a reservoir[149-152] *S. aureus* has been cultured from toilet handles, doorknobs, and sinks.[153] Outbreaks of disease have been observed in the community. In particular outbreaks related to sports players have been associated with shared razors and towels.[154-156] Nguyen *et al.* (2005) reported an outbreak of severe skin and soft tissue infections in a college football team where sharing bars of soap was associated with developing infection.[156]

Table 1.3 Risk factors associated with methicillin resistant *Staphylococcus aureus* (MRSA) carriage in healthcare workers

Risk Factor	Reference
Medical	
Skin conditions (such as eczema, psoriasis)	Bartzokas *et al.* (1984)[157] Berthelot *et al.* (2003)[158] Blok *et al.* (2003)[132]
Respiratory conditions (such as sinusitis, rhinititis, cystic fibrosis)	Boyce *et al.* (1993)[159] Sheretz *et al.* (1996)[160] Downey *et al.* (2005)[161]
Recent urinary tract infection	Cookson *et al.* (1989)[125]
Chronic otitis externa	Bertin *et al.* (2006)[130]
Recent antibiotic use	Cookson *et al.* (1989)[125]
Work	
Long duration of service	Eveillard *et al.* (2004)[162]
High hospital MRSA prevalence (endemic)	Blok *et al.* (2003)[132]
Close patient contact	Cox *et al.* (1997)[163] Cookson *et al.* (1989)[125]
Poor infection control practices	Cespedes *et al.* (2002)[134]
High work load	Price *et al.* (1980)[164]
Work abroad	Blok *et al.* (2003)[132]
Social	
Household MRSA contamination	Allen *et al.* (1997)[165] Kniehl *et al.* (2005)[166]

1.4 Transmission and acquisition in healthcare settings

Healthcare workers, patients and the environment have been implicated in transmission and subsequent patient acquisition of *S. aureus* in hospitals. Yet the specific routes of transmission from donor to recipient are incompletely understood. It is likely that acquisitions occur via different transmission pathways. Possible nosocomial transmission pathways are displayed in Figure 1.1.

Healthcare workers may contribute directly or indirectly to *S. aureus* transmission. First, staff may transmit directly to patients via contaminated hands.[167] Whilst the nose is thought to be the main reservoir of hand contamination extra-nasal colonisation and sub-clinical skin infections may have a role.[158] Hand contamination may be transient, implicating healthcare workers as an indirect transmission route. Transient carriage has been frequently associated with transmission events.[125,168] Secondly, healthcare workers shed *S. aureus* through airborne dispersal (coughing) or shedding skin cells. Whilst these result in direct transfer to patients they also result in environmental contamination.

Several studies have implicated healthcare workers' involvement in transmission events and outbreaks.[125,126,132,163,165,169,170] Vonberg *et al.* (2006) reviewed 191 MRSA outbreaks and found asymptomatic staff members were likely to be the source in only 1.3% cases.[171] In comparison, Albrich *et al.* (2008) identified 27/106 (25.5%) outbreaks where there was both molecular and epidemiological evidence to implicate healthcare worker involvement.[124] Variations of results are likely to reflect the diverse settings and typing methods used. Further support of staff involvement comes from high rates of patient acquisition observed during periods of overcrowding and understaffing, presumably as increased workloads result in poor infection control practices.[172,173] This is reinforced as transient hand carriage has been associated with transmission events.[127,132] Higher rates of transmission have been found in high dependency settings, thought to be due to greater antibiotic use, increased patient susceptibility and increased healthcare worker contact with patients.[172,174]

Patient acquisition can arise as a result of transmission from another patient.[175] Whilst direct transmission is plausible between ambulant patients direct contact

between patients is infrequent in hospitals, particularly in high dependency settings. It seems more likely that transmission occurs either via indirect transfer on the contaminated hands of staff or environmental contamination. The latter is important as patients frequently colonise their surrounding ward environment.[176]

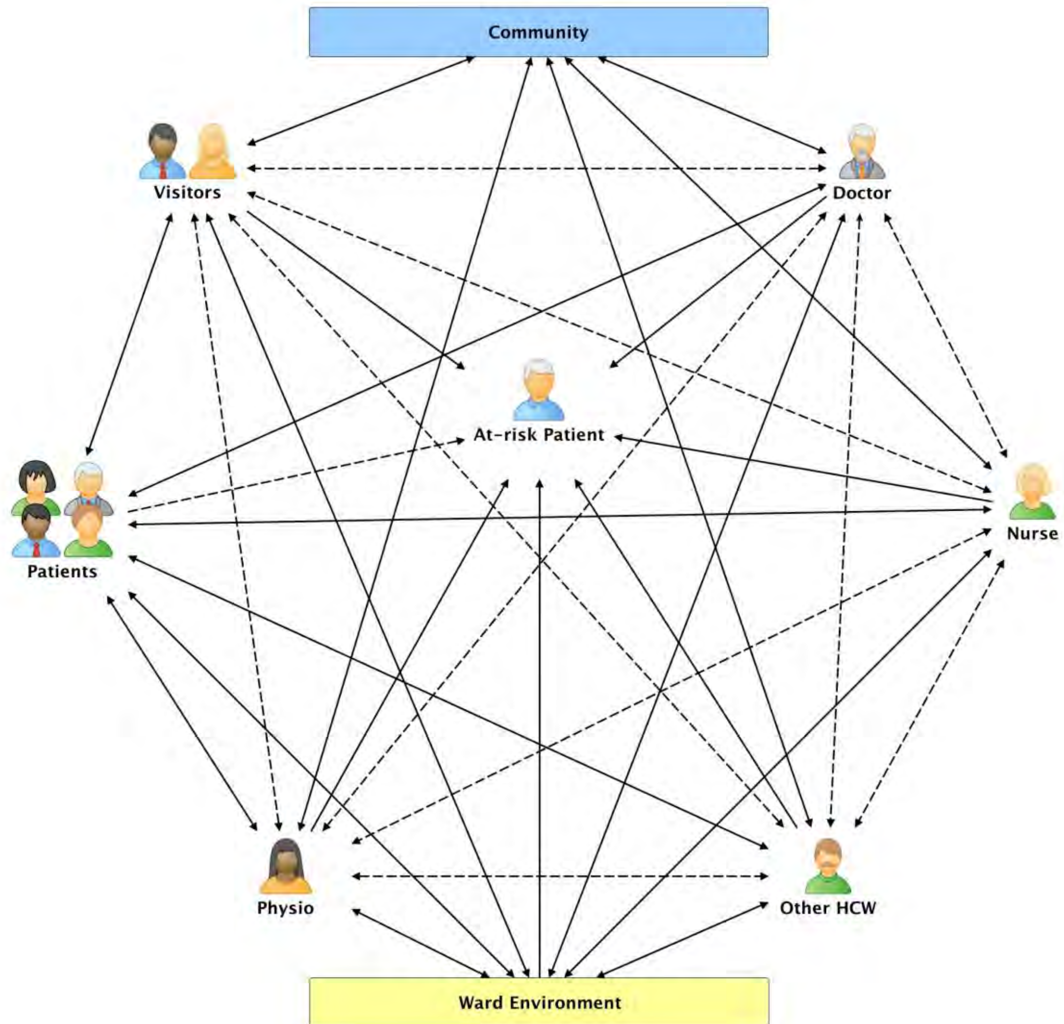
It has been suggested that some colonised individuals may act as 'super-spreaders', resulting in large numbers of acquisitions (i.e. outbreaks).[177] Whilst the mechanism of this remains unclear differences in rates of shedding have been identified. For example, men shed more often than women.[178-181] Furthermore site of carriage may influence shedding. Throat colonised individuals with upper respiratory tract infections may more readily contaminate their environment, through coughing and subsequent airborne disperals.[129,137,160] To my knowledge there are no data specifically evaluating differences in *S. aureus* transmission between different types of healthcare workers.

Patient acquisition can also occur indirectly via staff becoming transiently colonised from environmental isolates. Boyce *et al.* (1997) shows that nearly half of nurses entering rooms of MRSA-colonised patients cultured the patients' strains from gloved hands and aprons despite no direct patient contact.[182] In the same study healthcare worker acquisition rate increased to 65% in those with direct patient contact. The contribution of the environment to transmission is thought to depend on the frequency and level of contamination, and organism viability in the environment.[183]

As members of the general population carry *S. aureus* hospital visitors may also play a role in transmission to patients, either directly or indirectly through environmental contamination. As far as I am aware no studies have specifically looked at this.

While there are no reasons to think that routes of MRSA and MSSA transmission might be different there is evidence indicating that MRSA may be more transmissible. Vriens *et al.* (2002) compared the rates of spread of MSSA and MRSA.[184] They observed that whilst MRSA spread easily within their ICU setting no MSSA transmission was identified between patients and healthcare workers. One explanation for this observation may relate to antibiotic pressures that provide a selective survival advantage over MSSA.[184]

Figure 1.1 Transmission routes of *Staphylococcus aureus* in healthcare settings. Probable (complete lines) and plausible (dotted lines) transmission routes are depicted. Directionality is inferred by arrows.



1.4.1 Infection control measures

Despite the evidence cited above, major areas of uncertainty remain. Firstly, the precise routes of transmission from colonised patient to other patients are unclear. Secondly, the contribution of colonised healthcare workers to transmission is incompletely understood. Thirdly, our understanding the role environmental contamination plays in nosocomial *S. aureus* transmission is unclear. Fourthly, as most studies have focused on MRSA the contribution of MSSA to outbreaks and disease acquisition is under appreciated.

The lack of a sound evidence base describing *S. aureus* transmission hampers our ability to guide effective intervention. Consequently efforts to control the burden and spread of *S. aureus*, particularly MRSA, are based predominantly on expert opinion. Thirty years ago, Spicer (1984) described three approaches to control MRSA; (i) simple hygiene measures and barrier nursing (Scutari Strategies; named after the hospital in which Florence Nightingale worked in the Crimean War), (ii) actively identifying and isolating colonised patients and environmental eradication (search and destroy), and (iii) isolation of non-containable infections (*S. aureus* limitation techniques (SALT)).[185] Currently infection control policies vary dramatically between countries and broadly reflect the background prevalence of MRSA. In countries where MRSA prevalence is low `search and destroy` methods have been adopted.[186,187] In higher prevalence settings MRSA surveillance policies are employed with targeted interventions to suppress rates of MRSA to a manageable level.[188,189] Individual countries provide their own national advice on MRSA control. Hand hygiene is thought to be the most important measure for preventing healthcare associated infections and spread of pathogens in hospitals.[190] General recommendations to manage MRSA transmission include: (i) active MRSA surveillance, (ii) isolation, cohorting and barrier nursing of MRSA colonised or infected patients with dedicated equipment, (iii) decolonisation of MRSA colonised patients, (iv) antimicrobial stewardship, and (v) environmental decontamination.[186-189]

Despite guidance on managing patients and the environment there is a paucity of direct guidance on managing healthcare workers especially amongst high prevalence countries. This includes whether to test for MRSA colonisation in healthcare

workers and if colonised how best to manage them. Table 1.4 compares international guidelines for screening healthcare workers in endemic and non-endemic countries. International guidelines vary dramatically, which most likely reflects a lack of understanding regarding the role of the hospital staff in nosocomial transmission of *S. aureus*. In turn, this lack of understanding reflects the limitations of conventional typing techniques to discriminate highly related isolates to inform on individual routes of transmission.

Table 1.4 Comparison of healthcare worker screening policies for different countries

	Norway	Denmark	Sweden	Finland	Iceland	Netherlands	W. Australia	New Zealand	Germany	UK	Scotland	Ireland	Canada	USA	
MRSA Prevalence[191-196]	**LOW**						**MODERATE**		**HIGH**						
	7	2.08%	1.02%	1.95%	0%	0.97%	3%	9%	18.44%	27.82%		26.8%	27%	40-59%	
Guidelines	SSAC (2004)[186]					WIP (2009)[187] SWAB (2007)[197]	DOH (2005)[198]	MOH (2002)[199]	GMS (2009)[200]	Coia *et al* (2006)[189]	SISS (2006)[201]	SARI (2006)[202]	PIDAC (2010)[203]	Calfee *et al* (2008)[188]	Siegel *et al* (2007)[204]
Pre-Employment Screening	Yes*	Yes*	Yes*	Yes*	Yes*	Yes	Yes	Yes	-	No	Yes†	No	-	-	-
Routine Screening	Yes†	Yes†	Yes†	Yes†	Yes†	Yes†	No	No	No	No	No	No	No	No	No
Outbreak Screening	Yes†	Yes	Yes	Yes†	Yes	Yes	-	Yes	Yes	Yes†	Yes	Yes	Yes	Yes	Yes
Symptomatic Screening	Yes	Yes	Yes	Yes	Yes	Yes	-	-	-	Yes	Yes	-	Yes	-	-
Screening after MRSA contact	Yes†	Yes†	Yes†	No	Yes†	Yes	-	-	Yes†	No	No	No	Yes	No	-
Exclusion from work	Yes	Yes	Yes	Yes	Yes	Yes	Yes†	Yes†	Yes†	Yes†	Yes†	Yes†	Yes†	-	-
Eradication Failure	-	-	-	-	-	Specialist opinion	-	Specialist opinion	-	Deployment	Deployment	-	-	-	-

*if worked abroad. †in certain situations. Not mentioned (-)

1.5 *Staphylococcus aureus* characterisation and typing

The ability to distinguish between strains (typing) has applications with both short-term (such as outbreak identification and determination of transmission pathways) and long-term (understanding phylogeny) relevance. Traditionally phenotypic and molecular methods (genotyping) have been used to type *S. aureus*.

1.5.1 Conventional typing methods

Phenotypic typing methods that exploit variations in observable strain characteristics such as antibiotic susceptibility pattern, phage-typing profile, and serotype have been used to characterise *S. aureus.* In reality in most hospitals the possibility of transmission is tested at the most basic level by comparing antibiotic resistance panels. Whilst dominant strains frequently have identical antimicrobial susceptibility patterns strains with unique susceptibility profiles can provide surrogate markers of strain type. For example, ciprofloxacin sensitivity has been used to distinguish healthcare-associated from community-acquired MRSA strains.[205] Whilst antibiotic profiles are relatively inexpensive they lack discriminatory power compared to molecular methods.[206,207] Among the most widely used molecular typing methods are staphylococcal protein A (*spa*)-typing, multi-locus sequence typing (MLST), and pulsed field gel electrophoresis (PFGE).

1.5.1.1 *spa*-typing

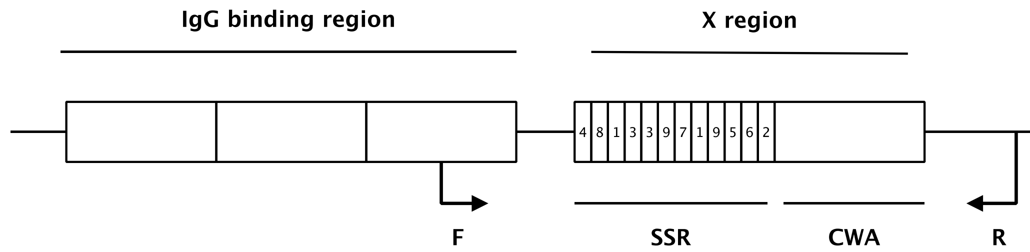
spa-typing is a single gene locus typing method that exploits a highly variable X region within the Protein A gene (*spa*) coding for a cell-surface virulence factor.[208] The X region consists of a cell wall attachment sequence and a short sequence repeat (SSR) region (Figure 1.2). The SSR region contains varying numbers of 21-30 base pair (bp) repeats which are subject to point mutations, duplication and deletion in sufficient variation to distinguish between *S. aureus* strains. Following sequencing the SSR region each unique repeat is allocated a distinct numerical code using RidomStaphType software[209], the sequence of which forms the *spa*-type (t-).

A critical limitation of *spa*-typing is that the *spa*-type does not indicate genetic relatedness so that two *spa* types may be genetically very close (such as t001 and t002) or very distinct (such as t003 and t004) (http://spa.ridom.de/spatypes.shtml). To address this *spa*-types can be grouped into clonally related clusters using Based

Upon Repeat Pattern (BURP) analysis, inferring evolutionary relationships between strains. A 'cost' (designated by Ridom developers) is assigned to gain and loss of repeats, in addition to point mutations, and *spa*-types differing by cost ≤ 4 are clustered as clonally related isolates.[210] For example, cost 1 is allocated for two *spa*-types differing due to a repeat duplication or loss, and cost 2 is allocated for two *spa*-types that differ by a single point mutation.

This rapid and affordable typing tool can determine strain lineages with comparable (or greater) resolution to other conventional molecular methods.[211] *spa*-typing has increasingly been used to characterise *S. aureus* and is currently the typing method employed by the UK Staphylococcal Reference Unit (www.hpa.org.uk). But *spa*-typing has three main limitations. Firstly, it cannot differentiate between closely related isolates within the same lineage. Secondly, a single base pair change within a repeat can change the *spa*-type and it has thus been a concern that two isolates of different *spa*-types may in fact be highly genetically related. Whilst BURP analysis aims to account for this it is not widely available. Thirdly, as a single locus typing method there are concerns that the *spa* gene, and evolutionary relationship inferred from *spa*-type, are not representative of the entire genome.

Figure 1.2 A diagrammatic representation of the Protein A (*spa*) gene including the short sequencing repeat (SRR) region and the cell wall attachment (CWA) region. The numbers in the SSR represent the numerical allocations to each unique sequence repeat. The locations of forward (F) and reverse (R) primer attachment are marked. Adapted from Shopsin *et al.* (1999)[208]



1.5.1.2 Multi-locus sequence typing (MLST)

MLST is based on sequence variation in multiple housekeeping genes.[212,213] Originally designed for *Neisseria meningitidis* MLST has since been developed for a host of bacterial species, including *S. aureus*.[213] MLST classifies strains into groups (sequence types (ST)) that reflect phylogeny, allowing the study of population structure and evolutionary history.[14,214,215] Conserved housekeeping genes evolve slowly compared to other areas of the bacterial genome and, hence, MLST provides insight into distant evolutionary events. Different MLST sequence types can be grouped into clonal complexes (CC) on the basis that they share some of the seven (or more) loci.[216] Concordance between MLST and *spa*-typing is high.[217] It is used internationally through an accessible database (http://saureus.mlst.net/) being useful for global epidemiology but due to the number of PCR and sequencing reactions MLST is time consuming and can only be performed in limited settings.

1.5.1.3 Pulsed-field gel electrophoresis (PFGE)

In PFGE enzymes are used to cleave DNA into fragments of different sizes which form strain-specific patterns when separated by gel electrophoresis.[218] Isolates with the same profile are considered the same. Compared with MLST and *spa*-typing, PFGE is relatively good at resolving differences between strains and many authors have promoted it as a tool for local outbreak investigation.[219,220] PFGE has been extensively used for outbreak investigations of MRSA in understanding the epidemiology of both endemic and epidemic strains. Until recently it has been the gold standard for short term MRSA epidemiological characterisation.[220] PFGE appears to be less useful than other genotyping techniques in long-term epidemiological surveillance or the study of phylogenetic relationships presumably due to the limited genetic variation within shorter time-frames.[221] Despite only simple equipment required the main limitations of PFGE include the requirement of skilled operators and lack of transferable data. Interpreting criteria have since been standardised and computer software has been developed to compare results between laboratories, but there has been limited success in establishing national databases of MRSA PFGE profiles.[220,222]

1.5.2 Limitations of conventional typing methods

Currently used molecular methods rely on variations in single or multiple genes to allocate strains to lineages. The short sequences used represent only a fraction of the whole genome (c. 2.8 million nucleotides).[11] Subsequently they lack the resolution to discriminate between isolates that are highly genetically related. For example, when two isolates are indistinguishable it does not mean that they are identical. This is challenging given the highly clonal population structure of *S. aureus* and is a significant weakness in studies analysing transmission events and outbreaks. Furthermore, partial sequencing techniques cannot reveal the fine genetic detail that accumulates between *S. aureus* strains as they diversify over time. Even when used in combination, conventional methods are limited in their potential to distinguish between isolates within a major lineage.

1.6 Whole-genome sequencing

Whole-genome sequencing (WGS) refers to the construction of the complete nucleotide sequence of a genome. It is now possible to obtain highly discriminatory typing data in potentially near-to-real time using WGS and determine with greater certainty than has previously been possible, the true genetic relationships between strains of *S. aureus* isolated from different individuals at different points in time. WGS has the resolution to discriminate down to single nucleotide differences and, hence, it is possible to determine when sequences actually are identical and if not, to state exactly by how much that they differ. This allows inferences to be made about the relationships between strains not just in terms of global population structure but also in terms of local patterns of transmission. At the same time WGS can provide information on the genetic basis of phenotypic characteristics, including virulence. With recent advances in sequencing technology the speed and cost-efficiency of WGS is becoming comparable to conventional typing techniques.[223,224] Hence WGS has the potential to provide a universal test that can facilitate outbreak investigation, enable the detection of emerging strains, and predict their clinical importance. Table 1.5 depicts the differences in cost, practicalities and achievable resolutions of conventional typing techniques and WGS.

Table 1.5 Comparison of *Staphylococcus aureus* typing techniques. Pulsed-field gel electrophoresis (PFGE), multi-locus sequence typing (MLST), spa (*spa*-typing), whole-genome sequencing (WGS).

Technique	Set up cost	Cost per isolate	Current Availability	Time to results	Data Analysis	Data Transferability	Common Nomenclature	Level of Resolution
PFGE	Low	£4-7	Local and reference labs	2-3 days	Minimal	Limited	USA Type (USA-)	Lineage
MLST	High	£20	Research and reference labs	Days	Moderate	Yes – widely	Sequence Type (ST-)	Lineage
spa	High	£3-5	Local and reference labs	24 hours	Moderate	Yes - widely	*spa*-type (t-)	Lineage
WGS	High	~£100	Research labs	Real-time[3]	High	*Being addressed*	*To be determined*	Base pair

[3] Third generation sequencing platforms

1.6.1 Whole-genome sequencing platforms

In 1977, Frederick Sanger developed a method that revealed the first complete genome sequence of a virus.[225] This 'first generation sequencing' used capillary-electrophoresis methods to sequence DNA fragments, a process which was expensive and slow.[226] The terms 'second generation' or 'next-generation' sequencing (NGS) refer to methods that parallelize the sequencing process, thus dramatically lowering costs and increasing capacity. NGS first became available in 2004 and a number of different sequencing platforms are currently commercially available.[227] These include the Analyser IIx and HiSeq2000 Platforms (Illumina, USA), 454 genome sequencer (Roche Applied Science, Switzerland), SOLiD platform (Life Technologies, Applied Biosystems, USA) as well as the first bench-top machines such as the MiSeq (Illumina, USA) and IonTorrent platforms (Life Technologies, USA) which are anticipated to be among the first platforms to be routinely implemented for clinical applications.

A major milestone has been the development of technology to allow sequencing of genomes to be performed in real-time. This is termed 'third generation sequencing' (TGS).[226] Instead of fragmenting and reconstructing read sequences, TGS platforms allow direct observation of the DNA polymerase enzyme while it constructs a strand of DNA. By not having to pause between individual base identification, and by taking advantage of the enzyme's speed in adding nucleotide bases to the growing chain, the throughput is greatly increased and the reads are dramatically longer.[228] This lowers costs and aids read assembly. Currently available TGS technology have limitations; error rates are high at 5% and the output data are differently formatted to those yielded by Sanger and NGS.[226] These issues are being addressed but for the time being second-generation machines are the platforms of choice. Table 1.6 shows a comparison of different sequencing technologies.

Table 1.6 Comparison of whole-genome sequencing technologies

	Sequencing Technology Platform		
	First Generation	Second Generation	Third Generation
Resolution	Average of multiple DNA copies	Average of multiple DNA copies	Single molecule
Read length generated	800-1000bp	<400bp	1000-10,000bp
Financial cost per base	High	Low	Moderate
Financial cost per run	Low	High	Low
Sample preparation	Moderate	Complex	Variable
Time to result	Hours	Days	Minutes to Hours

1.6.2 Sequencing process

The various commercially available sequencing platforms employ similar sequencing processes and to illustrate these Illumina sequencing has been used an example as this technology has been used in the thesis. Illumina sequencing requires prior preparation of a library of DNA fragments of approximately 200bp lengths that then undergo two PCR-based reactions to ensure similar lengths and suitability for adaptor ligation. Following adaptor ligation to either end of the DNA fragments one adaptor binds to the surface of a flow cell. Subsequently the unattached adaptor binds to a complementary adaptor on the flow cell surface, forming a bridge. Each bridge is amplified simultaneously using bridge PCR, resulting in double stranded fragments. These are then denatured and the processes are successively repeated until clusters of DNA fragments are formed on the flow cell. Four fluorescently labelled reversible chain terminators (corresponding to nucleotides A,T,C,G) are then added that bind to the relevant terminal base in each fragment within a cluster. Following light excitation the emitted fluorescence of each cluster is captured and the first base is identified. The sequencing cycle is repeated to determine each base in each fragment cluster in turn, revealing the DNA sequences of each cluster.

1.6.3 Sequence assembly

Sequencing machines generate thousands of small DNA sequences, called reads, which can be between 40 and 1000 base pairs in length depending on the technique used. Each read represents the sequence of a small fraction of the genome. The reads overlap so that each position of the genome is included in several reads. This number is called 'the coverage'. To be useful, the reads need to be assembled into the whole-genome sequence. This can be done in one of two ways; either by comparison with a previously sequenced 'reference' strain (mapping-based assembly), or by piecing the reads together on the basis of their overlaps (*de novo* assembly).[229,230] Mapping-based assembly produces results that are easier to interpret, but are dependent upon the choice of reference genome, so that sequences not present in the reference genome remain unmapped. In principle, *de novo* assembly can recover the whole genome, but in practice it returns several assembled regions (called contigs) whose further assembly tends to remain unresolved. In either case, longer read lengths and higher coverage make assembly easier and more accurate.[231] Storing the reads for a single *S. aureus* genome requires approximately 1GB of storage space; roughly

equivalent to 10 music albums in MP3 format. The term high-throughput sequencing is used to refer to newer sequencing technologies that can generate sequence data faster and more economically than previous platforms. Consequently, sequencing platforms are rated according to their throughput speeds, setup and processing costs and length of reads produced.[227] A medium sized sequencing facility can process thousands of bacterial isolates in a year. New platforms (Oxford Nanopore and Ion Proton) offer the promise of projected cost reductions to the order of $1,000 for a whole human genome, sequenced in 15 minutes to two hours. Such performance would translate into sequencing costs for bacteria of approximately $1 per whole genome.

1.6.4 Analysis

The first complete *S. aureus* genome was published in 2001.[11] As recently as 2008 only 12 complete genomic sequences of *S. aureus* were available in the public domain.[232] At the time of writing (September 2013) there are 408 fully annotated *S. aureus* genomes publically available in the National Centre for Biotechnology Information (NCBI) Reference Sequences (RefSeq) database.[233] The first few genomes of *S. aureus* to be sequenced were fully annotated with the position of each gene and probable function stated when known. The wealth of information contained in these annotations makes these genomes suitable for use as references. With higher sequencing throughput now achievable, much larger numbers of genomes can be sequenced and the emphasis has shifted from annotation to data analysis. The depth of analysis required depends on the question being asked. For example, full annotations as described above are not necessary for determining the relatedness of isolates in transmission and outbreak investigations. In such situations comparisons are required between strains in terms of single nucleotide variants (SNVs) and insertion or deletion events.

1.6.4.1 Evolutionary rate

As a bacterial population evolves and diversifies from a common ancestor its constituent members accumulate differences in their genome sequences. By comparing sequence data it is possible to infer phylogenetic relationships. A key requirement when interpreting such data is to be able to estimate the rate at which mutations accumulate during the evolution of a genome, the so-called ‘molecular

clock'. With knowledge of the molecular clock, it is possible directly to convert a number of differences between two genomes into the length of evolutionary time that has elapsed since they last shared a common ancestor. This is an area where there is still some uncertainty. The molecular clock may not tick at a constant speed in different settings (environment, commensal and in blood for example), in different hosts or even across lineages. Over long time periods selection-pressure may lead to a lower measured mutation rate. However, existing measures of mutation rate are remarkably consistent. Harris *et al.* (2010) used a linear regression between isolation dates and root-to-tip distances in a phylogenetic reconstruction to estimate an average rate of 3.3×10^{-6} mutations per site per year in *S. aureus*.[234] Over the whole genome, this represents an average of 9.2 mutations per genome per year. A similar rate was estimated by Young *et al.* (2012)[235] for carriage isolates sampled serially from the same patients using the Bayesian phylogenetic method BEAST.[236] Estimated mutation rates vary between 2.0-3.4 $\times 10^{-6}$ mutations per site per year, equating to 5.6-9.5 mutations/year over the whole genome or around one SNV difference every 5-10 weeks.[234,235,237,238] This rate is high enough to envision the application of WGS to the investigation of patient-to-patient transmission.

We are still learning how to interpret the significance of SNV differences between isolates. Wide genetic divergence between isolates from different patients reliably excludes the possibility of recent transmission, whilst there can be reasonable confidence that pairs of genetically identical or highly related isolates indicate recent transmission. However, Golubchik *et al*. (2013) have detected genetically divergent strain subpopulations (up to 40 SNV differences) in single patients.[239] Another potential confounding factor relates to calibration of the molecular clock as there may be rapid microevolution during invasive disease.[235]

1.6.5 Application of whole-genome sequencing into clinical practice

As WGS becomes cheaper and faster it may displace current technologies in several areas of clinical practice.

1.6.5.1 Infection control in hospitals

WGS can be used to track transmission within a hospital and to assess the importance of patients, healthcare workers, visitors and the hospital environment as potential sources or vectors. With an understanding of the rate of *S. aureus* evolution single nucleotide variants (SNVs) can be used like a molecular clock to measure the length of time passed since two isolates shared a common ancestor and make inferences about short-term transmission. Interpretation must take into account the possibility of recombination events that result in large genetic differences (>100bp) between closely related strains. Bioinformatic software is used to detect recombination events.

Outbreaks are generally detected through recognition of increasing numbers or clustering of cases caused by a particular strain type.[240] Through prospective analysis the resolution offered by WGS makes it possible to recognise newly emerging strains, even when they belong to prevalent lineages of *S. aureus*. Harris *et al*. (2010) demonstrated the presence of a set of genetically related isolates (≤14 SNVs) occurring amongst members of the same lineage (ST239) prevalent in a Thai hospital.[234] Hence, WGS can be used to reveal transmission networks that might remain undetected using conventional methods.[241] WGS can also link genetically-related isolates of differing phenotype. Eyre *et al*. (2012) investigated a suspected outbreak involving heteroresistant MRSA and showed that despite differences in antibiotic susceptibility, all isolates were genetically highly related (0-3 SNVs).[242] The authors identified differences in penicillin and tetracycline susceptibility that were shown by *in silico* interrogation of whole genome data to be due to presence or absence of mobile genetic elements bearing *blaZ* or *tetK* genes.

1.6.5.2 Epidemiological surveillance, population structure and biology

WGS offers the prospect of a high-resolution typing method for surveillance of *S. aureus* epidemiology locally, regionally and globally.[234,243] Coupled with clinical epidemiology data this would allow precise tracking and monitoring of strains, provide early recognition of emerging pathogenic clones and measure success of

interventions to limit transmission. Early identification of newly emerging strains could allow prompt implementation of targeted infection control resource.

WGS is starting to provide insight into the genetic basis behind the emergence and persistence of successful strains. WGS has been used to investigate the molecular basis of emergence of CA-MRSA, including the USA300 clonal strain as an agent of skin and soft tissue infection. Kennedy *et al.* (2008) compared 10 USA300 genomes and identified low genetic diversity suggesting recent emergence. Conversely, Carpaij *et al.* (2011) observed considerable genetic variation between 14 sequenced USA300 isolates retrieved from one centre over one month, suggesting continuous evolution was occurring rather than persistence of a single clone.[244] Furthermore, Vogel *et al.* (2012) used WGS to investigate the short-term evolution and persistence of the South German clone (ST228, *spa* t041) in a Swiss tertiary centre[245], revealing low genetic diversity between 8 isolate genomes. Subtle genetic variations, such as replacement and loss of plasmids, were identified as contributing towards the local evolution of the clone. The effect of these genetic variations on virulence and spread requires further investigation.

1.6.5.3 Understanding and predicting virulence

Genome-wide association studies have the potential to uncover the genetic basis of severe disease. The sequencing and annotation of the first two complete *S. aureus* genomes resulted in the discovery of 70 novel candidate virulence factors.[11] Baba *et al*. (2002) interrogated a genome from a strain causing septic arthritis and fatal septicaemia in a child. The authors identified 19 toxin genes present in the strain genome absent in reference genomes, suggesting putative factors associated with disease.[246] McAdam *et al.* (2011) used WGS to compare three sequential *S. aureus* isolates from a persistently colonised cystic fibrosis patient.[247] The authors identified point mutations affecting antibiotic resistance, growth and global regulation of virulence factors that provide insight into the genetic basis of adaption during chronic infection. Such studies cast light on the relationship between genotype and clinical phenotype. Köser *et al*. (2012) showed that *in silico* interrogation of the *S. aureus* genome for known virulence determinants could be used as an alternative to conventional PCR-based methods.[241] Results can be generated rapidly using bench-top platforms and offer the advantage of providing information on toxin genes,

antibiotic resistance determinants as well as a measure of relatedness to other isolates from a single test.[223]

1.6.5.4 Understanding and predicting antimicrobial resistance

Knowledge of the genetic basis of resistance will allow the development of WGS-based approaches to antibiotic resistance testing and will potentially aid drug discovery. It has been shown that *in silico* interrogation of WGS data can reliably predict antibiotic susceptibility phenotype.[241,242] A study using a panel of 18 genes associated with resistance to 10 commonly used anti-staphylococcal agents yielded overall sensitivity and specificity of greater than 95%.[248] These results are comparable with those obtained by phenotypic methods such as Vitek[249] and are within acceptable limits set by the US Food and Drug Administration for marketing approval of new susceptibility testing methods.[250] The sensitivity can be increased by the addition of further genes to the panel at no extra cost, and previously sequenced isolates can be screened almost instantly for newly recognised resistance genes.

It is unlikely that *in silico* prediction can entirely replace culture since the presence or absence of a gene does not always correlate with susceptibility. However, as data accumulate and a comprehensive database of resistance genes is developed, the confidence with which resistance can be predicted will increase. Further investigation is required to determine to what extent we will be able to rely on genotype alone.

1.6.6 Hurdles to adoption of whole-genome sequencing in clinical practice

Some obstacles need to be overcome before WGS can be translated from the research arena into the routine laboratory. Currently available platforms generate multiple short read lengths that must be mapped to a reference genome or assembled *de novo* by matching overlapping sequences. At present, considerable bioinformatic expertise is required in order to assemble a whole genome sequence from these fragments. Mapping based assembly tends to leave gaps when sequences present in test isolates fail to match reference genome sequences and *de novo* methods tend to generate incompletely resolved results. Hence, analysis and interpretation requires a high level of bioinformatic input to generate information that is comprehensible to the infection specialist. This situation will be made easier by sequencers that produce

longer read lengths. Furthermore, the development of software solutions that allow semi-automated sequence assembly, analysis and presentation of results is being developed to ameliorate these difficulties. An example of this is the Genomespace initiative (www.genomespace.org), a web-based environment to support genomic analysis by providing bioinformatics tools through an easy-to-use Web interface.

Another issue is the possibility of laboratory cross-contamination when handling multiple isolates from suspected outbreaks. Hence, development of WGS as an infection control tool will require carefully controlled and validated methods for sampling, culture and analysis. Also the scale of information technology infrastructure required to store and exchange the large quantities of data generated by WGS cannot be underestimated.

Current approaches to WGS have inherent limitations. At present, using high-throughput sequencing machines it takes several weeks to generate a DNA sequence due to complex sample preparation, large numbers of scanning/washing cycles and reliance on PCR amplification of DNA templates. Furthermore the reads produced are relatively short which can make genome assembly challenging. At the time of writing it is important to realise that the limitations of mapping-based and *de novo* assembly techniques for short read sequences mean that the term "whole-genome sequence" actually refers to the 80-90% of the actual entire genome that is rendered visible by those techniques. The introduction of new platforms that generate much longer reads will make visible the remaining `concealed` sequences to routine analysis.

1.6.7 Experience with other pathogens

WGS has been applied to several other major pathogens in order to improve our understanding of their epidemiology. He *et al.* (2010) used WGS to demonstrate that the strains of *Clostridium difficile* associated with disease emerged from multiple lineages.[251] This contradicted the received wisdom that bacterial pathogens arise from a single lineage as a result of gaining genetic properties through gene transfer or mutation. Didelot *et al.* (2012) used WGS to investigate cases of *C. difficile* infection that appeared to be linked according to conventional typing results.[252] The resolution provided by WGS made it possible to `rule out` transmission for the majority of cases, thereby challenging the assumption that *C. difficile* infection is

usually acquired as a result of direct transmission from symptomatic patients. WGS has already been applied during the investigation of some high-profile outbreaks. The Asian origins of the Haitian outbreak of cholera that killed several thousands in 2010-2011 were determined through WGS of the outbreak strain of *Vibrio cholera*.[253] In 2003 WGS was used to characterise the SARS virus, showing it to be a previously unrecognised coronavirus.[254] In 2011 sequencing technology was used to characterise a Shiga-toxin producing *Escherichia coli* O104:H4 strain that affected nearly 4000 people and caused 47 deaths in Europe and North America. Sequencing rapidly confirmed clonal expansion from a common ancestor and allowed identification of virulence factors.[255] Gardy *et al.* (2011) used WGS to evaluate a Canadian outbreak of *Mycobacterium tuberculosis*.[256] Outbreak isolates were indistinguishable by conventional genetic fingerprinting methods (MIRU-VNTR) and furthermore, epidemiological information suggested a probable point source. WGS of isolates revealed that two distinct lineages were circulating simultaneously. Evolutionary analysis using historical isolates from the region showed that both lineages were present long before the dual outbreak was recognised. WGS was able to `rule in` four historical tuberculosis cases that had not previously been recognised as part of the outbreak. Hence, WGS provides a tool for optimising contact tracing resources.

1.7 Summary

WGS has the potential to transform our knowledge of *S. aureus.* Its greatest impact may be in our understanding of transmission. WGS has the potential to allow proper characterisation of transmission routes to optimise infection control strategies and manage outbreaks. With a clearer understanding of the genetic basis of virulence WGS-based surveillance could permit early determination of emerging virulent strains (and new virulence genes) locally and globally. Characterising genetic diversity over time will provide new insights into *S. aureus* population structures, allowing us to understand the genetic basis of epidemics and strain success. Additionally, rapid prediction of resistance could improve clinical outcome by permitting earlier effective management. If WGS is to be adopted into routine microbiology laboratory practice it is necessary to establish its performance in the clinical environment and in comparison with existing methodologies.

1.8 Hypotheses

The work presented in this thesis tested three hypotheses concerning *S. aureus* transmission within healthcare settings:

Hypothesis 1. Changes in clinical epidemiology of nosocomial *S. aureus* infections may be associated with the emergence of a novel clone within a dominant *S. aureus* lineage.

This was tested by studying an outbreak of MRSA blood stream infections in a single UK hospital using both conventional typing methods and the higher resolution offered by WGS.

Hypothesis 2. Colonised patients are commonly the source of new *S. aureus* acquisitions among hospitalised patients.

This was tested by conducting a prospective study on an adult intensive care unit evaluating patient-to-patient transmission in a non-outbreak setting using conventional approaches (*spa*-typing and epidemiological data) and WGS.

Hypothesis 3. Colonised healthcare workers are commonly the source of new *S. aureus* acquisitions among hospitalised patients.

This was tested by coupling the on-going patient surveillance study with a prospective, observational study assessing *S. aureus* carriage in staff and the environment in an adult high-dependency unit.

2 Materials and Methods

2.1 Materials

Tris-EDTA (TE) Buffer (x1)	1ml Tris-HCl (1M; pH8.0), 0.2ml EDTA (0.5M), 98.8ml H_2O
1.5% Tryptone soya agar	17g/L Tryptone, 3g/L Soytone, 2.5g/L Glucose, 5g/L NaCl, 2.5g/L K_2HPO_4, 1L H_2O, 15g/L agar

2.2 *Staphylococcus aureus* culture and identification

2.2.1 Culture of bacteraemia isolates

Archived bacteraemia isolates preceding 2008 from Brighton and Sussex University Hospital (BSUH) were retrospectively retrieved from Microbank™ storage beads (Pro-Lab Diagnostics, Cheshire, UK) archived in routine laboratory freezers at minus 80°C. All bacteraemia isolates identified by the routine microbiology laboratory at BSUH from October 2008 were collected prospectively. Isolates from regional and national hospitals, including those received from Pubic Health England, were transported on ambient agar slopes. All bacteraemia isolates were grown on Columbia blood agar (CBA) (Oxoid, Basingstoke, UK) at 35°C overnight.

2.2.2 Culture of carriage isolates

As part of routine clinical practice all patients admitted to the high dependency settings at BSUH undergo MRSA screening, consisting of swabbing multiple anatomical sites (nose and perineum and usually another site). Routine MRSA swabs are inoculated directly onto chromogenic Brilliance MRSA agar (Oxoid, Basingstoke, UK) and cultured overnight at 35°C. Brilliance MRSA agar causes *S. aureus* to grow as dark blue colonies for rapid identification. During the study period screening swabs were also cultured for MSSA by directly plating the swab on Columbia CAP Selective agar (Oxoid, Basingstoke, UK). CAP agar is selective for staphylococci and streptococci species. All swabs were plated onto CAP agar before Brilliance agar as the latter contains products that inhibit growth of MSSA. All

isolates were stored on ambient agar slopes, a sealable tube containing 1.5% Tryptone soya agar.

All staff and environmental swabs underwent broth enrichment prior to agar culture. Broth enrichment improves the sensitivity of *S. aureus* detection.[257,258] Swab tips were placed in 7.5% salt broth (Oxoid, Basingstoke, UK) overnight at 35°C. The salt broth was selective for *S. aureus* growth, as many other bacteria do not grow in these high salt concentrations. Following this, a 5µl loop of broth was inoculated onto half a plate of SaSelect chromogenic agar (Oxoid, Basingstoke, UK). SaSelect chromogenic agar causes *S. aureus* to grow with a characteristic denim blue colour.

In Chapter 5 intensive screening was performed to assess whether culture methods affected observed carriage rates. A sub-set of patient swabs underwent broth enrichment prior to agar culture as described above.

2.2.3 Identification

2.2.3.1 Staph Xtra latex kit

PROLEX™ Staph Xtra latex kit (Pro-Lab Diagnostics, Cheshire, UK) is a rapid agglutination test that detects the presence of clumping factor and protein A found on the surface of *S. aureus*. Bacterial colonies are emulsified in Staph latex reagents (and negative control) for 10-20 seconds. The reagent contains particles coated with human immunoglobulin G and fibrinogen. The presence of *S. aureus* causes a protein-protein interaction between clumping factor and protein A with immunoglobulin G and fibrinogen. Agglutination of the latex in the positive reagent alone indicates the presence of *S. aureus*.

2.2.3.2 Matrix-assisted laser desorption/ionisation - time of flight (MALDI-TOF)

Microflex™ series Matrix-assisted laser desorption/ionisation - time of flight (MALDI-TOF) (Bruker Daltonics, Coventry, UK) employs mass spectrometry to characterise organisms by protein fingerprinting. A colony of bacteria is smeared thinly onto a steel target plate. Then 0.5µl matrix solution is added and left to dry in air; the matrix solution causes bacterial cell lysis. The plate is introduced into the mass spectrometer under vacuum. The plate surface containing the sample is exposed to short wave laser pulses that cause the matrix to ionise. This in turn causes the sample molecules to ionise through proton transfer from the matrix. The energised

ions of the sample accelerate towards a detector at the other end of a flight tube. Small ions reach the detector before larger ones. The time for ions to reach the detector provides a protein profile of the sample. The ions are then identified by the spectrometer as peptide fragments according to their molecular weight. Comparison with reference profiles on MALDI database allows determination of characteristic profile spectra. Results are expressed using standard scores based on algorithms for the specific identification of biomarker proteins (SIBP). An SIBP score greater than 2 is highly accurate for genus and species identification. Isolates with an SIBP >2 were identified as *S. aureus*.

Cultures that were positive on chromogenic agar, Staph Latex and MALDI-TOF were considered to be *S. aureus*.

2.3 Determination of antibiotic susceptibility

2.3.1 Disc diffusion testing

S. aureus were tested for β-lactam (including methicillin) resistance by growing isolates overnight on Iso-Sensitest agar (IST) (Oxoid, Basingstoke, UK) at 35°C with a cefoxitin disc (Oxoid, Basingstoke, UK) containing 10μg applied to the surface of the agar. Susceptibility to cefoxitin is used as a surrogate marker of methicillin (and β-lactam) susceptibility.[259] Full antibiotic susceptibility profiles were tested in parallel by growing *S. aureus* in the same conditions in the presence of antibiotic impregnated discs placed on the surface of the agar plate. Susceptibility was determined by assessing growth around the discs (Table 2.1) and interpreted using British Society of Antimicrobial Chemotherapy (BSAC) guidelines.[33]

Table 2.1 Antibiotic disc concentrations used to assess antibiotic susceptibility

Antibiotic	Concentration (μg)
Penicillin	1
Erythromycin	5
Gentamicin	10
Clindamycin	2
Vancomycin	5
Fusidic Acid	10
Ciprofloxacin	1
Tetracycline	10
Linezolid	10
Rifampicin	2
Mupiricin	20
Teicoplanin	30

2.3.2 Minimum inhibitory concentration (MIC) evaluation

For isolates where methicillin susceptibility was unclear the minimum inhibitory concentration (MIC) was determined. The MIC is the lowest concentration of an antibiotic that will inhibit an organism's growth. MICs were determined using a minimum inhibitory concentration evaluator (MICE) (Etest®, Oxoid, Basingstoke, UK). MICE are plastic strips containing labelled gradient methicillin concentrations. Two to three bacterial colonies were emulsified in peptone water (Oxoid, Basingstoke, UK) and spread evenly onto Columbia 2% salt (Oxoid, Basingstoke, UK) agar using a swab. The agar was dried in air for 10 minutes and the MICE strip applied. Cultures were incubated at 30°C overnight. Following this the MIC was read at the point where growth inhibition meets the strip. Oxacillin (a β-lactam) MIC was used as a surrogate marker of methicillin (and β-lactam). Resistance was indicated by oxacillin MIC $>$ 2mg/L; susceptibility was determined with an MIC of $\leq$ 2mg/L.

2.4 Long term storage of bacterial cultures

All bacteria were stored as glycerol stocks. 5µl loop of fresh cultures were emulsified in 400µl 0.85% Saline (Oxoid, Basingstoke, UK). 10µl of emulsified saline solution was added to 50µl of 1x Tris-EDTA buffer and 200µl 45% glycerol (ThermoFisher Scientific, Cheshire, UK). Two stocks were made for each isolate; a master and working stock that were stored separately. Isolates were archived using matrix 2D barcoded storage tubes (ThermoFisher Scientific, Cheshire, UK) and stored at -80°C.

2.5 DNA extraction

Bacterial DNA was extracted by two different methods. Crude DNA extraction was used for *spa*-typing. Homogenisation was used for whole-genome sequencing as the sequencing platform (Illumina) required higher concentrations of pure DNA than can be provided by crude DNA extracts.

2.5.1 Crude chromosomal DNA extract

A 5µl loop sweep from SaSelect agar was emulsified in 400µl 0.85% Saline (Oxoid, Basingstoke, UK). 10µl of the emulsified bacterial solution was then added to 50µl Tris-EDTA buffer and heated to 99.9^{o}C for 10 minutes to lyse the cells. The solution was centrifuged at 13,200 rpm for 2 minutes and the supernatant, containing the bacterial DNA, was extracted and stored at -20°C.

2.5.2 Homogenisation and purification extract

Cultures were incubated overnight on CBA at 35°C. Each isolates was grown on a separate agar plate to avoid contamination. According to the manufacturer's protocol for gram positive bacteria cultures were lysed using a FastPrep homogeniser (MP Biomedicals, Illkirch, France) with Matrix B beads, with two 40 second homogenisations at a setting of 6.0m/s on the FastPrep instrument. This technique employs a high-speed bench-top homogeniser to lyse cells through multi-directional beating of beads against the organisms. The released DNA was purified from the cell lysate using QuickGene DNA blood kits (Autogen, USA), washed with 99% ethanol and eluted into DNAse free water. Quickgene technology uses pressurised filtration through porous membranes to isolate genomic DNA. NanoDrop spectrophotometer (Labtech International, East Sussex, UK) was used to determine DNA concentrations. The whole-genome sequencing platform used in this project (Illumina) required 20ng/µL of DNA per sample. DNA quantification was verified and adjusted using PicoGreen dsDNA Assay Kit (Invitrogen, Paisley, UK). PicoGreen is a highly sensitive florescent nucleic acid stain that selectively binds to double-stranded DNA. Bound PicoGreen fluoresces under UV light (unbound dye does not fluoresce). Nucleic acid concentrations were determined by comparing sample fluorescence to a standard curve.

2.6 Molecular Typing

2.6.1 *spa*-typing

The X region of the *spa* gene was amplified from crude DNA extractions by PCR with primers 109F (5'-AGACGATCCTTCGGTGAGC-3') and 1517R (5'-GCTTTTGCAATGTCATTTACTG-3') (Invitrogen, Paisley, UK). PCR reactions contained a final volume of 10µl containing 2µl template DNA, 0.1µl 25mM dNTPs

(Fermentas Life Sciences, Germany), 0.1µl 0.5U/µl GoFlexi Taq DNA polymerase (Promega, Southampton, UK), 1µl 10 x Buffer (Promega, Southampton, UK), 1µl 25mM $MgCl_2$ (Promega, Southampton, UK) and 0.5µl of each 0.5µM primer. Reactions were cycled as follows: 94°C for 2 minutes; 35 cycles of [94°C for 30 seconds, 50°C for 30 seconds, 72°C for 1 minute]; final extension at 72°C for 5 minutes. PCR products were purified using Agencourt AMPure XP beads (Beckman Coulter, London, UK). PCR products were sequenced using PCR primers and BigDye v3.1 chemistry (Applied Biosystems, USA) and cycled as follows: 96°C for 10 seconds; 30 cycles of [50°C for 5 seconds, 60°C for 2 minutes]. Sequencing products were purified using Agencourt CleanSEQ beads (Beckman Coulter, London, UK). PCR products sequencing analysis was performed on an ABI 3730 DNA Analyzer (Applied Biosystems, USA). *spa*-types were determined using Ridom StaphType (Ridom GmbH, Germany).

2.6.2 *spa*-grouping

Isolates of different *spa*-types can be highly related as small genetic variations within the *spa* gene change the *spa*-type. Groups of related *spa*-types can be identified using automated Based Upon Repeat Pattern (BURP) algorithm analysis.[210] In brief, clonal relatedness of *S. aureus* isolates can be measured from *spa* repeat regions. Evolutionary steps between two different *spa*-types are termed 'cost'. These steps can be insertion or deletion of repeats, or base pair changes within repeats. The program designers (Ridom Bioinformatics, http://www.spaserver.ridom.de) have weighted these evolutionary steps based on likelihood of occurrence. For example, duplications of repeats have been assigned a cost of 1 as they are common whereas base pair changes within repeats are perceived as less common and have been assigned a cost of 2. BURP algorithms calculate cost differences between *spa*-types and allocate strains into related *spa*-groups.

Mellman *et al.* (2007) evaluated BURP to infer the clonal relatedness of 400 *S. aureus* isolates of the most prevalent *spa*-types.[210] BURP results were compared against grouping strategies developed from multi-locus sequence typing (MLST) data, which are currently the perceived gold standard in determining isolates relatedness based on 7 conserved housekeeping genes (see Section 1.5.1.2). The authors identified two optimal conditions to achieve high rates of concordance

(95.6%) between BURP and MLST groups. Firstly, isolates with *spa* genes possessing <5 repeats in the sequence should be excluded from BURP analysis as their sequences are too short to make meaningful inferences; this parameter excludes no more than 10% of isolates.[210] Secondly, *spa*-groups should include isolates with a maximum cost difference of 4 as inclusion of isolates with greater cost dramatically reduces concordance of group allocation to <60%. These parameters were used in this study. Isolates allocated to the same *spa*-group were presumed clonally related.

2.7 Whole-genome sequencing

Bacterial libraries were produced for sequencing from 1.25µg of sample DNA. Whole genome sequencing was undertaken at the Wellcome Trust Centre for Human Genetics (http://www.well.ox.ac.uk). In Chapter 3 two sequencing platforms were used; Illumina Genome Analyser IIx machine and Illumina HiSeq2000. In Chapter 4 only the Illumina HiSeq2000 sequencing platform was used. The output was multiple overlapping DNA fragments (or reads) that vary in length. The data were processed using a pipeline developed specifically for bacterial sequence data (www.modmedmicro.ac.uk). Reads were mapped to clonal complex-specific references (MRSA252[1] and EMRSA15[2] respectively), using Stampy.[260] Single nucleotide variants (SNVs) were identified across all mapped non-repetitive sites using SAMtools.[261] Two filters were used to improve the confidence in calling a site; (i) a consensus of >75% across all reads was required to call a SNV, and (ii) a minimum of 5 reads (with at least one in each direction) was required at each site.

2.7.1 Pairwise difference matrices

To assess the diversity between isolates pairwise distance matrices were created. The matrices were generated using a python script, written by Dr Madeleine Cule (University of Oxford), and calculate the number of called SNV sites between each pair of isolates. These did not including uncalled positions.

2.7.2 Maximum likelihood trees

The relatedness of sequenced isolates was evaluated by creating maximum likelihood trees from mapped whole genomes. A maximum likelihood tree is a statistical model of estimating the phylogeny of organisms. Trees were created using a python script

written by Dr David Eyre (University of Oxford) using PhyML[262], a program that estimates maximum likelihood phylogenies from nucleotides sequences. In brief, the script compares variant sites between genomes (identified as part of the distance matrix script) to estimate the most likely points of evolutionary divergence between sequences.

2.7.3 Phylogenetic relationships

Maximum likelihood trees infer phylogeny based on SNV differences. Phylogenetic relationships can also be estimated based on diversity over time, allowing estimates of time since divergence from recent common ancestor. This was performed using ClonalFrame[263], a programme that infers the micro-evolutionary history of isolates using sampling times to place dates on the phylogeny. Using SNV differences to establish genetic relatedness and time since strain divergence can be overestimated in the presence of recombination. An advantage of ClonalFrame over other phylogenetic methods is that it accounts for bacterial horizontal gene transfer. ClonalFrame was used to determine phylogenetic and temporal relationships of isolates in Chapter 3.

2.7.4 Replicates

The ability to detect relevant SNV is highly dependant on accurate, reproducible sequencing platforms. Multiple replicates of a *S. aureus* reference stain (MRSA 252[264]) have been sequenced and assembled, using the specifically developed bioinformatics pipeline. Previous work undertaken by our collaborating group (Modernising Medical Microbiology (MMM)) has estimated the false positive rate (where a spurious variant is detected) for our pipeline to be 2.5×10^{-9} per nucleotide (or 0.0075 per genome).[239,252] As part of this work 10 *S. aureus* isolates were replicated and SNV differences compared; 0 SNV differences were observed between replicate pairs. Consequently, the likelihood of incorrect variant calling through sequencing error is small using our bioinformatic pipeline.

3 Evaluation of an outbreak of MRSA blood stream infections in a single UK hospital

3.1 Introduction

In 2004 Brighton and Sussex University Hospital NHS Trust (BSUH), a large teaching hospital in southern England, experienced an abrupt increase in rates of MRSA blood stream infections (or bacteraemia) that persisted until 2007 (Figure 3.1). Rates of MRSA bacteraemia reported to the Health Protection Agency (now Public Health England) mandatory reporting scheme demonstrated that BSUH experienced an outbreak between 2004-2007 during which the peak rate of MRSA bacteraemia was markedly higher and later than that seen in comparable trusts (Figure 3.1). Doctors treating patients with *S. aureus* bacteraemia at Brighton reported that secondary site infections and mortality seemed more common than previously observed.

Rising rates of hospital infection are often attributed to poor infection control, however outbreaks of MRSA infection may also be caused by the importation of new strains.[241,265,266] In theory clonal variants arising within epidemic MRSA strains could, like new lineages, contribute to changes in infection rate or severity. This has not been demonstrated to date. Conventional approaches to typing *S. aureus* (such as typing of the *spa* gene or multi-locus sequence typing (MLST)) are too poorly discriminatory to detect such variation within epidemic MRSA clones. Whole-genome sequencing (WGS) has the potential to detect sub-clones within dominant lineages of MRSA and to reveal the genetic basis for clonal expansion of virulence.[224,234,243,256,266]

3.2 Objectives

The overarching objective of this study was to evaluate an outbreak of MRSA blood stream infections in a single UK hospital. The study was conducted in two parts. The first part used conventional approaches to investigate three hypotheses. First, changes in patient characteristics (such as demographics and co-morbidities)

contributed towards the MRSA outbreak. To test this patient data were collected from time points before and during the outbreak. Secondly, the observed excess of complicated disease and mortality was associated with sub-optimal clinical management. To test this detailed clinical data were collected on a subset of 100 *S. aureus* bacteraemias during the outbreak. Thirdly, the outbreak resulted from the emergence of a novel clone. To test this isolates were retrieved from three collections before and during the outbreak and evaluated using conventional typing methods. Differences in patient factors and clinical management did not explain the excess of MRSA bacteraemia. Conventional methods failed to identify the emergence of a new clone but did suggest changes in MRSA clones dominant in the hospital.

The second part of this chapter presents a further evaluation of the outbreak using whole-genome sequencing (WGS) to test the hypothesis that the outbreak was caused by the emergence of a novel clonal variant within a dominant lineage. This work identified the emergence of a novel clonal variant from within a dominant MRSA lineage that coincided with the outbreak. This observation was expanded through:

1. characterisation of the clinical phenotype of the novel clonal variant
2. determination of genomic events which may account for the emergence of the variant
3. evaluation of the presence of isolates highly related to the variant in neighbouring hospitals and nationally in the UK.

Figure 3.1a MRSA bacteraemia rates at Brighton and Sussex University Hospital (BSUH) trust (red), Oxford University Hospitals (OUH) (blue) and six other hospitals in South East England (black) as reported to the Health Protection Agency mandatory reporting scheme for England and Wales (www.hpa.org.uk). The rate at Brighton rose from 21.4 episodes per 100,000 bed days (national average of 17.1) in 2001 to a peak of 47.1 in 2005, taking the hospital from 41st to 1st highest out of 167 hospitals in England and Wales.

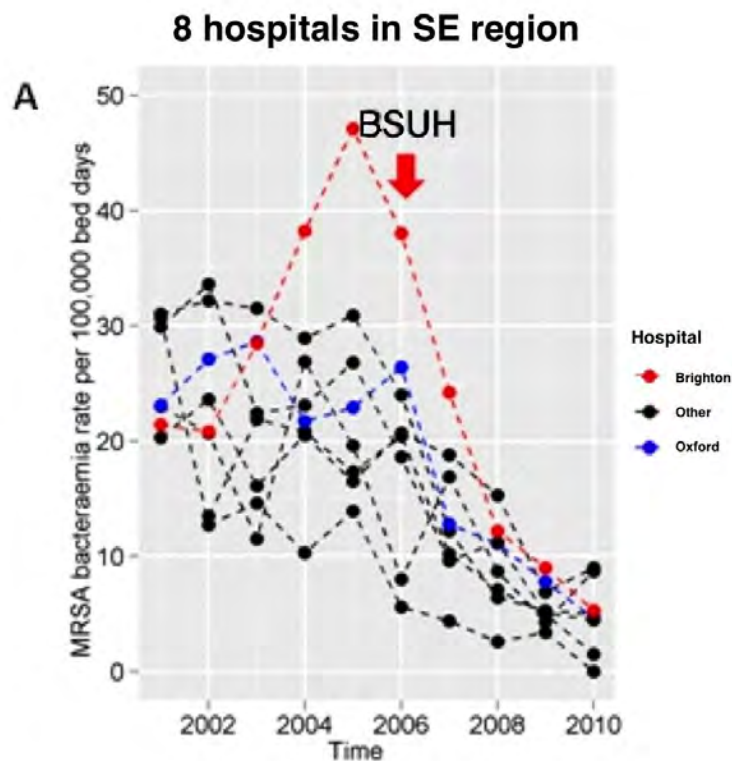
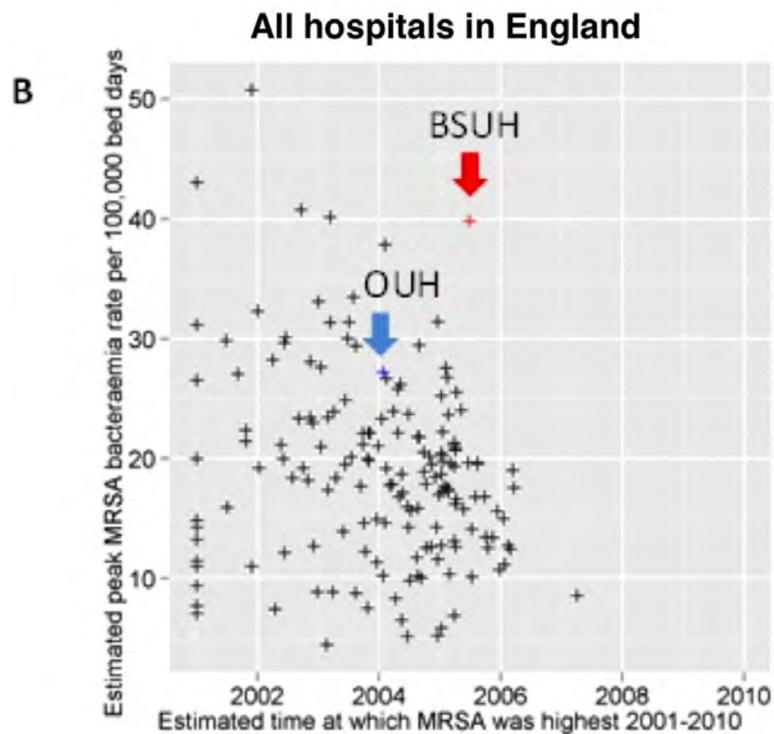


Figure 3.1b Estimated date and magnitude of MRSA bacteraemia rates for all hospitals in England and Wales between 2001 and 2011 derived from Mandatory Reporting Scheme data and bed occupancy (KH03 Statistics) from NHS England. The peak MRSA bacteraemia rate at Brighton and Sussex University Hospital NHS Trust (BSUH) (red) was higher and later than Oxford University Hospitals (OUH) (blue) and almost all other hospitals.



3.3 Attributions

MRSA bacteraemia trajectories in Figure 3.1 were created by Dr David Wyllie (HPA, Oxford). A sub-set of Brighton isolates were previously typed using multi-locus sequence typing (MLST) as part of unpublished research. Brighton and Oxford isolates evaluated in part 1 of this chapter were whole-genome sequenced by Dr Ruth Miller (University of Oxford). Velvet assembly of SCC*mec* genes were performed by Dr Elizabeth Batty (University of Oxford). Phylogenetic analysis was performed by Dr Xavier Didelot (University of Oxford). Genomic innovation analysis was performed by Dr Rory Bowden (Wellcome Trust Centre for Human Genomics, Oxford). Molecular signature assay primers were designed and validated by Dr Antonia Votintseva (University of Oxford). Multiple logistic regression analysis was performed by Professor Timothy Peto (University of Oxford). National isolates retrieved from the Staphylococcal reference unit had been previously *spa*-typed by the Health Protection Agency. All other work presented was performed by James Price.

Part 1. Investigation of MRSA bacteraemia outbreak using conventional approaches

3.4 Methods

3.4.1 Setting

Brighton and Sussex University Hospitals (BSUH) is an 890-bedded general and teaching hospital on the south coast of England. BSUH is a tertiary referral centre for renal, HIV, cardiothoracic, neurology, and neurosurgery services. Rates of MRSA bacteraemia reported to the Health Protection Agency mandatory reporting scheme demonstrate that BSUH experienced an outbreak between 2004-2007 during which the peak rate of MRSA bacteraemia was markedly higher and later than that seen in comparable trusts (Figure 3.1a) and nationally across all trusts (Figure 3.1b)

3.4.2 Patient characteristics

To assess changes in patients' characteristics (demographics and co-morbidities) associated with the outbreak clinical data were collected on 273 patients' bacteraemia episodes. Historical data were pragmatically gathered from all retrospectively available case notes and laboratory data systems, including 57 episodes in 1999-2001 and 116 from 2004. Prospective data were gathered for 100 consecutive episodes that occurred between July 2006 and May 2007. Data collected included: patient demographics (age, gender); timing of bacteraemia (community acquired and community onset); ward location of patient at time of bacteraemia; co-morbidities (diabetes mellitus, intra-venous drug use, haemodialysis); and social factors (smoking).

S. aureus bacteraemia was considered to be community-associated using the United States Centers for Disease Control and Prevention (CDC) definition (diagnosis <48hours of admission, no previous hospital admission with twelve months, no previous MRSA positive cultures, and no long-term indwelling devices).[267] Community-onset disease was defined as *S. aureus* bacteraemia diagnosed within 48hours of admission but not fulfilling the criteria for community-acquired disease. All bacteraemia diagnosed after 48hours of admission was defined as hospital-onset.

3.4.3 Clinical management

During the excess of *S. aureus* disease at BSUH doctors treating the patients reported higher rates of secondary site infections, disease relapse and mortality than previously observed. To confirm these observations and evaluate whether sub-optimal clinical management factors were associated detailed clinical data were collected on a sub-set of 100 *S. aureus* bacteraemia episodes from 2006-07. This sampling time frame of this patient sub-set was determined by the tenure of the author running the study. Data collected included: identification and removal of focus; time to effective treatment; treatment duration; and outcome. Outcome was assessed by the development of complicated disease and all-cause 30-day mortality. Complicated disease was defined as the development of either a secondary deep focus of infection (on the basis of clinical or microbiological evidence) or relapse (a second episode of *S. aureus* bacteraemia within three months of the first episode). Initial antibiotic management was defined as having been "effective" if the patient received parenteral therapy with a β-lactam antibiotic or glycopeptide (depending on sensitivity or β-lactam allergy) either alone or in combination with other agents.

3.4.4 Microbial factors

In order to determine whether the emergence of a novel strain was associated with the MRSA outbreak *S. aureus* isolates were retrieved from frozen archives at BSUH. In total 229 *S. aureus* blood culture isolates (113 MRSA) were retrieved: 78 from 1999-2001, 54 from 2004, and 97 from 2006-07. Whilst extensive efforts were made to retrieve all historical bacteraemia isolates to represent a continuous collection from 1999 the interrupted time frames (1999-2001 and 2004) represent pragmatic collection of all available archived isolates. Whilst this chapter details an evaluation of an MRSA bacteraemia outbreak MSSA isolates were also collected and analysed for comparison. All isolates were identified and had methicillin susceptibilities determined as previously described (Section 2.2). Biochemical data at the time of blood culture were collected retrospectively.

S. aureus isolates were typed using two conventional methods available at the time; Multi-Locus Sequence Typing (MLST)[212] and Restriction-Modification (RM-) typing[268]. As described in Section 1.5.1.2, MLST assigns strains to a sequence type (ST), and subsequently lineage, according to variations in 7 sequenced housekeeping

genes.[212] MLST was performed on 1999-2001 and 2004 isolates as part of a previous unpublished study. MLST was unavailable for the 2006-2007 isolates so an alternative typing method was employed. RM-typing assigns *S. aureus* isolates into six of the ten major *S. aureus* lineages; clonal complexes (CC) 1, 5, 8, 22 (corresponding to ST22, predominantly EMRSA-15), 30 (corresponding to ST30 and 36, predominantly EMRSA-16), and 45 (corresponding to ST45, 4 and 54).[14,268] RM-typing exploits the close relationship between variations in the two *hsdS* genes which are specific to dominant *S. aureus* lineages.[18] Combinations of eight primers are used in three PCR reactions to produce products of different sizes (Table 3.1).[268] PCR reactions contained a final volume of 10µl containing 1ul of template DNA, 0.2µl 10mM dNTPs (Fermentas Life Sciences, Germany), 0.1µl 1U/µl GoFlexi Taq DNA polymerase (Promega, Southampton, UK), 2µl 5 x Go Taq Buffer (Promega, Southampton, UK), 0.8µl 2.5mM $MgCl_2$ (Promega, Southampton, UK), 3.1µl H_2O and 1µl of each Primer (at a concentration of 5µM). Reactions were cycled as follows: 94°C for 5 minutes; 35 cycles of [94°C for 30 seconds, 55°C for 30 seconds, 72°C for 2 minute]; final extension at 72°C for 2 minutes.[268] Positive controls were retrieved from an archived collection. PCR products were visualised by electrophoresis on 2.5% agarose gel.

Of 229 isolates available 190 were successfully allocated to a clonal complex using MLST and RM-typing; MLST data were unavailable on 21 isolates from 1999-2001 and 18 isolates from 2004. All 190 typed isolates were allocated into their corresponding lineage (or clonal complex).

To provide a consistent typing method across all 229 isolates a third typing method was used, *spa*-typing, As described in Section 1.5.1, *spa*-typing is a molecular typing method allocating strain to a *spa*-type (from which lineage can be inferred) based on the Protein A (*spa*) gene.[208] All 229 isolates were successfully *spa*-typed and were allocated into their respective lineages.

Table 3.1 Primers used in Restriction-Modification typing. Adapted from Cockfield *et al.* (2007)[268]

Product Name	Primer Name	Sequence (5`-3`)	Amplification Size (bp)
RM 1	AF	AGGGTTTGAAGGCGAATGGG	203 (CC30) 990 (CC22)
	AR30	CAACAGAATAATTTTTTAGTTC	
	AR22	TCAGAGCTCAACAATGATGC	
RM 2	AF	AGGGTTTGAAGGCGAATGGG	722 (CC45) 1037 (CC1)
	AR45	GGAGCATTATCTGGTGTTTTCC	
	AR1	GGGTTGCTCCTTGCATCATA	
RM 3	BF	CCCAAAGGTGGAAGTGAAAA	680 (CC8) 1071 (CC5)
	BR8	CCAGTTGCACCATAGTAAGGGTA	
	BR5	TCGTCCGACTTTTGAAGATTG	

3.4.4.1 Genotyping

Movement of novel genetic components into strains on mobile genetic elements can contribute towards *S. aureus* causing disease.[269] This has been observed in a variety of putative virulence genes, in particular Panton-Valentine Leukocidin (PVL), Toxic Shock Syndrome Toxin (*tst-1*), and enterotoxins.[269] Isolates were investigated to determine whether acquisition of putative virulence factors were associated with the excess of MRSA bacteraemia outbreak. Four genes were chosen due to previous associations with invasive disease and their presence on mobile genetic elements, including PVL, *tst-1*, and staphylococcal enterotoxins A and J (*sea*, *sej*).[56,269-272] Evaluations were undertaken using PCR assays using published primers (Table 3.2).

PCR reactions contained a final volume of 10µl containing 1ul of template DNA, 0.2µl 10mM dNTPs (Fermentas Life Sciences, Germany), 0.1µl 1U/µl GoFlexi Taq DNA polymerase (Promega, Southampton, UK), 2µl 10 x Go Taq Buffer (Promega, Southampton, UK), 0.8µl 2.0mM $MgCl_2$ (Promega, Southampton, UK), 3.9µl H_2O and 1µl of each Primer (at a concentration of 5µM). Reactions were cycled as follows: 95°C for 5 minutes; 25 cycles of [95°C for 30 seconds, 65°C for 30 seconds, 72°C for 1 minute]; final extension at 72°C for 5 minutes. PCR reactions using *tst* primers had an annealing temperature of 60°C. Positive controls were retrieved from an archived collection. PCR products were visualised by electrophoresis on 1% agarose gel.

Table 3.2 Primers for putative virulence factor genotyping

Gene	Primer Name	Sequence (5`-3`)	Amplification Size (bp)	Reference
PVL	PVL-1	ATCATTAGGTAAAATGTCTGGACATGATCCA	433	Jarraud *et al* (2002)[273]
	NPVL-2	GCATCAASTGTATTGGATAGCAAAAGC		
tst-1	TST-3	AAGCCCTTTGTTGCTTGCG	445	Becker *et al* (1998)[274]
	TST-6	ATCGAACTTTGGCCCATACTTT		
sea	SEA-A1	AAAGTCCCGATCAATTTATGGCTA	234	Tsen *et al* (1992)[275]
	SEA-A2	GTAATTAACCGAAGGTTCTGTAGA		
sej	mpSEJ-1	TAACCTCAGACATATATACTTCTTTAACG	300	Jarraud *et al* (2002)[273]
	mpSEJ-2	AGTATCATAAAGTTGATTGTTTTCATGCAG		

3.5 Statistical analysis

Data were analysed using SPSS (version 20.0.0, IBM®, USA). Associations between complicated disease and the variables described were assessed using the Fisher's exact test for proportions and the Mann-Whitney U test for non-parametrically distributed continuous variables. For categorical variables the relative risk (RR) and 95% confidence intervals (CI) were calculated. Probability values ≤0.05 were considered significant.

3.6 Ethical approval

All isolates collected for these epidemiological studies were an evaluation of service and were covered by Statutory Instrument Regulations 2002 No. 1438, section (iii) 'Communicable disease and other risks to public health (Health Service Control of Patient Information)' of section 60 of the Health and Social Care Act and therefore did not require research ethics committee approval.[276]

Isolate storage and data collection was approved in Brighton by the BSUH Research and Development office as a service evaluation, involving anonymized data from patient records and not requiring formal ethical review.

3.7 Results

As part of an investigation to understand the emergence of an MRSA bacteraemia outbreak comparison of patient characteristics, clinical management, and microbial factors were undertaken over three time frames at BSUH. For comparison all episodes of *S. aureus* bacteraemia (MSSA and MRSA) were evaluated during the study.

3.7.1 Patient characteristics

In order to evaluate whether changes in patient populations could have contributed towards the outbreak clinical data on patients' characteristics (demographics and co-morbidities) were collected retrospectively on 57 *S. aureus* bacteraemia cases from 1999-2001, 116 in 2004, and 100 prospectively collected patients in 2006-2007 (Table 3.3). Good quality basic demographic and microbiological data were available on the great majority of cases but full case notes were available from archives for only 22/57 (38.6%) from 1999-2001 and 73/116 (62.9%) from 2004. All cases notes were available from 2006-2007.

Clinical characteristics including patient demographics and co-morbidities were comparable across all time frames suggesting that there were no significant differences in patient factors associated with the outbreak. Very few community-acquired *S. aureus* bacteraemias were observed and 67-77.3% disease was hospital-onset. Rates of complicated disease among evaluable patients were comparable across the series although lower rates were found in the 1999-2001 series (2/22 (9.1%)) but this did not reach statistical significance (p=0.19). Whilst this gives an insight the data sets were incomplete for 1999-2001 and 2004 studies.

Table 3.3 Comparison of clinical characteristics of *Staphylococcus aureus* bacteraemia cases identified retrospectively from 1999-2001 and 2004, and prospectively from 2006-07. Location data were available for 47, 112 and 100 cases, respectively. Completely evaluable case notes were available for 22, 73 and 100 cases, respectively. Missing data is depicted by a dash (-).

Clinical Characteristics	1999-2001 n=57	2004 n=116	2006-2007 n=100	Significance (p)
Age median (IQR)	63.1 (53-78)	68.9 (52-80)	65.5 (IQR 46-78)	0.9
Male gender	38 (66.7%)	81 (69.8%)	59 (59%)	0.24
Location				
Admission unit	8/47 (17%)	18/112 (16.1%)	27 (27%)	0.58
ICU	8/47 (17%)	19/112 (16.9%)	11 (11%)	
Renal unit	8/47 (17%)	26/112 (23.2%)	19 (19%)	
Medical wards	17/47 (36.2%)	32/112 (28.6%)	30 (30%)	
Surgical wards	6/47 (12.8%)	17/112 (15.2%)	13 (13%)	
Comorbidities				
Diabetes mellitus	2/22 (9%)	20/73 (27.4%)	20 (20%)	0.16
Intra-venous drug use	0/22 (0%)	7/73 (9.6%)	7 (7%)	0.31
Smoker	6/22 (27.3%)	19/73 (26%)	22 (22%)	0.78
Excess alcohol	-	-	11 (11%)	-
HIV	-	-	0	-
Haemodialysis	-	-	17 (17%)	-
Community-acquired	2/22 (9.1%)	4/73 (5.5%)	7 (7%)	0.82
Community-onset	5/22 (22.7%)	21/73 (28.7%)	33 (33%)	0.6
Complicated disease	2/22 (9.1%)	20/73 (27.4%)	27 (27%)	0.19

3.7.2 Clinical management

To evaluate the observed excess of complicated disease and mortality during the outbreak detailed clinical data were collected on 100 patients with *S. aureus* bacteraemia from 2006-07, representing a time during the outbreak (Table 3.4). All patients with *S. aureus* bacteraemia (MSSA and MRSA) identified during the study period were included to provide a comparison of clinical management and outcome. This 100 patient subset was determined by the tenure of the author conducting the study.

Overall 27% developed complicated disease; 24 secondary site infections and 6 relapses. Furthermore 38/97 (39.2%) died within 30 days of diagnosis. The risk of death in patients with complicated disease was higher (RR=1.29, 95% CI 0.99-1.7, p=0.057).

Clinical management of *S. aureus* bacteraemia at BSUH was evaluated to assess whether sub-optimal practices contributed to these findings. In 77/100 (77%) cases a primary source of bacteraemia was apparent; 41/77 (53.2%) had their primary focus removed within 72 hours. Although the presence or absence of a source did not correlate with complicated disease, removal of an identified focus within 72 hours was associated with a lower risk of complicated disease than when a focus was removed beyond this time or not removed at all (RR=0.71, 95% CI 0.57-0.9, p=0.006).

32% of patients had effective antibiotic therapy delayed for >48 hours after the start of symptoms, with significantly higher rates in bacteraemias caused by MRSA strains (40.4% vs. 10.4%, p=0.001). Bacteraemic patients who were symptomatic for >48 hours before effective antibiotic therapy was started were at a higher risk of complicated disease (RR=2.1, 95% CI 1.22-3.61, p=0.015). 66% patients received at least 2 weeks treatment. There was no relationship between duration of effective intravenous therapy <2 weeks and complicated disease (RR=1.07, 95% CI 0.84-1.37, p=0.64). An interesting observation was that higher all-cause mortality rates were observed with MRSA bacteraemia (56% vs. 21.3%, p<0.001).

Table 3.4 Clinical management and outcome of 100 *Staphylococcus aureus* bacteraemias evaluated in Brighton between 2006-07

Clinical Management	Total n=100	MRSA N=52	MSSA N=48	Significance (p)
Management factors				
Primary focus identified	77 (77%)	39 (75%)	38 (79.2%)	0.62
Primary focus removed <72 hours	41 (41%)	29/77 (37.7%)	24/77 (31.2%)	0.29
Initiation of effective antibiotics ≥48hours	32 (32%)	21(40.4%)	5 (10.4%)	**0.001**
Duration of IV treatment > 2 weeks	66 (66%)	31 (59.6%)	35 (73%)	0.16
Complicated Disease				
Total	27[§] (27%)	15 (30%)	12 (25%)	0.67
Secondary site infection	24 (24%)	13 (25%)	11 (23%)	0.81
Relapse	6 (6%)	3 (5.8%)	3 (6.3%)	0.94
Outcome				
Mortality (all cause at 30 days)	38/97[**] (39.2%)	28/50 (56%)	10/47 (21.3%)	**<0.001**

[§] 3 patients, with both secondary site infection and relapse
[**] 3 patients were lost to follow up at 30 days, including 2 with complicated disease

3.7.3 Microbial analysis

Lineage allocation was performed by RM-typing and MLST on 190 Brighton *S. aureus* bacteraemia isolates available at the time; including 57/78 isolates from the 1999-2001 series, 36/54 from the 2004 series, and 97/97 from 2006-2007 series (Table 3.5). No significant changes in the lineages of *S. aureus* causing bacteraemia occurred between 1999 and 2007, although the proportion of MRSA bacteraemias caused by CC30 (EMRSA-16) increased progressively from 36.6% to 54%, while the contribution of CC22 (EMRSA-15) decreased from 50% to 38%.

To provide a consistent typing method across all isolates *spa*-typing was performed on all 229 isolates available from the three time series (78 from 1999-2001, 54 from 2004 and 97 from 2006-2007) including 190 isolates that underwent previous RM-typing and 39 additionally retrieved isolates (Table 3.6). Of note repeat methicillin susceptibility testing performed at the time identified concordant results on all isolates except one MRSA isolate from 2006-2007 previously identified as methicillin susceptible. Methicillin resistance was confirmed using minimum inhibitory evaluation (MIC) evaluation as described in Section 2.3. Whilst MRSA isolates were conserved over two *spa*-types dominant in UK hospitals MSSA isolates comprised a diverse collection of *spa*-types. Results reveal similar trends to those observed with RM-typing and MLST; MRSA bacteraemia isolates at BSUH showed a trend to increased rates of EMRSA-16 (33.3% to 54.9%) compared to reducing rates of EMRSA-15 (31.1% to 27.4%).

There were 9 (4%) discrepancies in lineage allocation between the three typing methods all involving MRSA isolates. In each case one method allocated a strain into either EMRSA-15 or EMRSA-16 whereas at least one other method suggested a different lineage.

Table 3.5 Lineages determined by Multi-Locus Sequence Typing (MLST) and Restriction Modification (RM) typing of 190 *Staphylococcus aureus* bacteraemia isolates in Brighton from 1999-2001, 2004 and 2006-2007. % given in parenthesis.

Clonal Complex	MSSA 1999-2001	MSSA 2004	MSSA 2006-2007	MRSA 1999-2001	MRSA 2004	MRSA 2006-2007
CC22	2 (7.4)	2 (13.3)	4 (8.5)	15 (50)	10 (47.6)	19 (38)
CC30	7 (25.9)	3(20)	10 (21.3)	11 (36.6)	8 (38.1)	27 (54)
CC45	6 (22.2)	3 (20)	11 (23.4)	0	0	0
CC1	1 (3.7)	1 (6.7)	6 (12.8)	0	0	4 (8)
CC8	1 (3.7)	0	5 (10.6)	2 (6.7)	0	0
CC5	3 (11.1)	0	4 (8.5)	2 (6.7)	0	0
Other	7* (25.9)	6† (40)	7^(14.9)	0	3** (14.3)	0
Total	27	15	47	30	21	50

*comprising MLSTs 6, 12(x2), 15, 123(x2), and 188. †comprising MLSTs 12,15,20,97,101,123. **comprising MLST 12(x3)

Table 3.6 Lineages determined by *spa*-typing of 229 *Staphylococcus aureus* bacteraemia isolates in Brighton from 1999-2001, 2004 and 2006-2007. % given in parenthesis.

spa-type	MSSA 1999-2001	MSSA 2004	MSSA 2006-2007	MRSA 1999-2001	MRSA 2004	MRSA 2006-2007
EMRSA-15 (t032, CC22)	0	3 (8.1)	0	14 (31.1)	8 (47)	14 (27.4)
EMRSA-16 (t018, CC30)	0	8 (21.6)	1 (2.2)	15 (33.3)	6 (35.3)	28 (54.9)
Other	33 (100)	26 (70.3)	45 (97.8)	16 (35.6)	3 (17.7)	9 (17.7)
Total	33	37	46	45	17	51^

^includes isolates previously identified as MSSA

3.7.4 Virulence factor genotyping

To assess the role of putative virulence factors associated with excess MRSA bacteraemia available isolates were evaluated for the presence of four toxins (PVL, *tst*, *sea*, *sej*) using PCR-based genotyping. Toxin genotyping was performed on 188 isolates including 52 from 1999-2001, 39 from 2004 and 97 from 2006-07 series (Table 3.7). Figure 3.2 shows the gel electrophoresis results from *sea* genotyping.

Toxin genotyping demonstrated that 3/188 (1.6%) were PVL positive and other gene frequencies were: *tst* 83/188 (44.1%), *sea* 66/188 (35%), and *sej* 4/188 (2.1%). No differences were observed in the frequency of these toxins between the three time-series. There was no relationship between toxin genotype and complicated disease or mortality at three months. However, 71% of CC30 strains were *tst* positive and all four *sej* positive isolates were of CC5 (Table 3.8).

Table 3.7 Distribution of toxin genes identified in *Staphylococcus aureus* isolates across three time series (n=188). Toxin genes included Panton-Valentine Leukocidin (PVL), toxic shock syndrome toxin (*tst*) and Staphylococcal enterotoxin A and J (*sea* and *sej*, respectively). % given in parenthesis. In total 48/188 isolates contained >1 toxin gene, including 12/52 from 1999-2001(*), 10/39 from 2004 (**) and 26/97 from 2006-07(†)

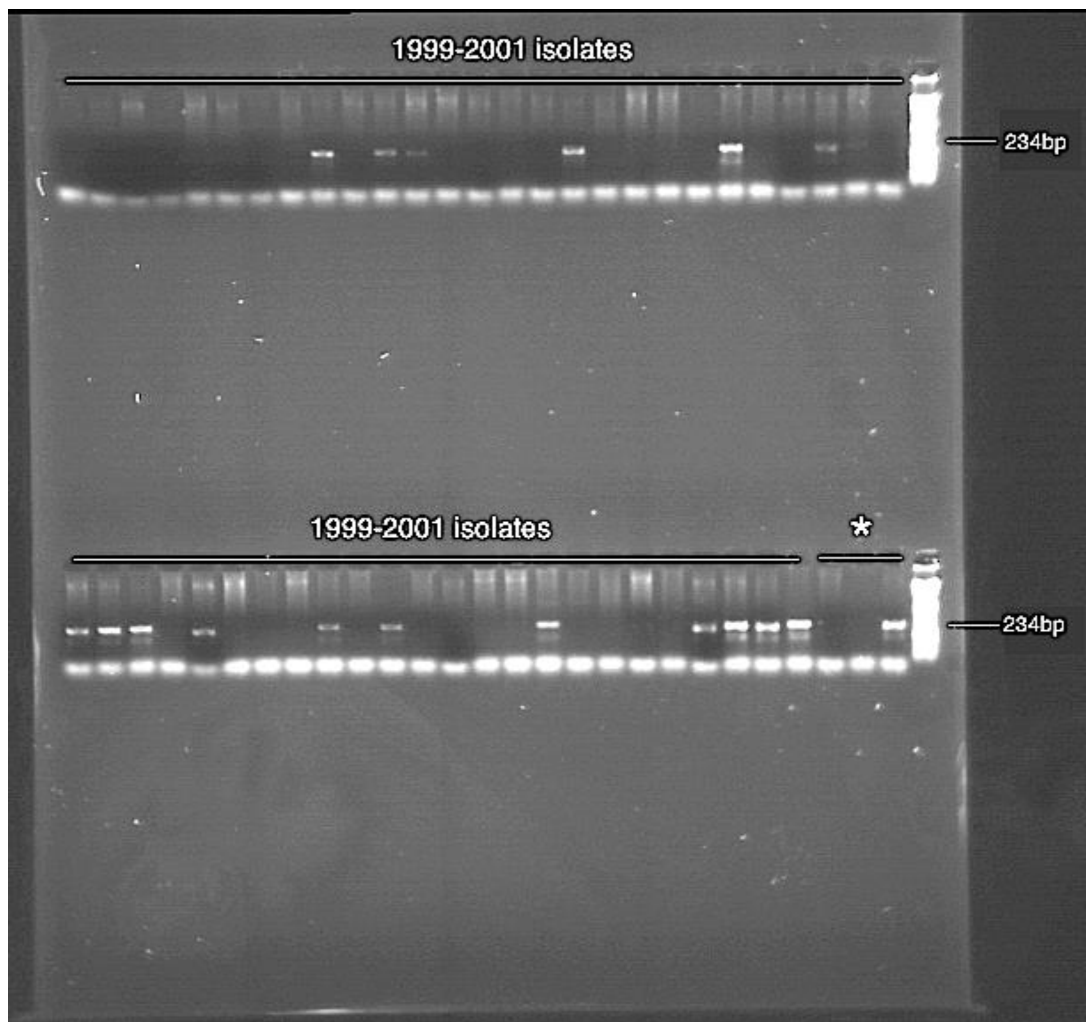
Gene	Isolate collections		
	1999-2001 n=52*	2004 n=39**	2006-07 n=97†
PVL	0	0	3 (3.1)
tst	22 (42.3)	26 (66.7)	33 (34)
sea	17 (32.7)	15 (38.5)	34 (35)
sej	2 (3.8)	0	2 (2)

Table 3.8 Distribution of toxin genes within the six dominant *Staphylococcus aureus* lineages, or clonal complex (CC). Toxin genes included Panton-Valentine Leukocidin (PVL), toxic shock syndrome toxin (*tst*) and Staphylococcal enterotoxin A and J (*sea* and *sej*, respectively). % given in parenthesis.

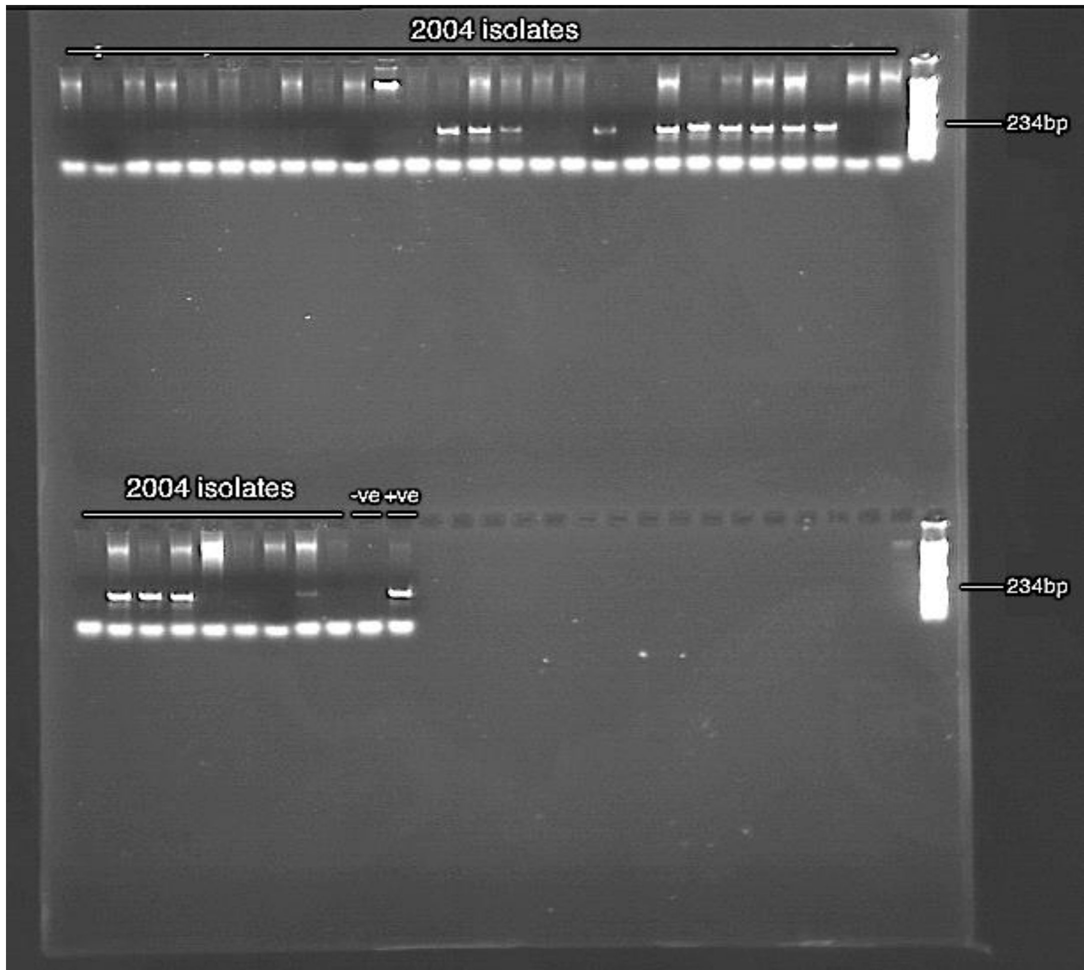
Gene	Clonal Complex							
	CC30	CC22	CC45	CC1	CC8	CC5	Other	Total
PVL	0	1 (33.3)	0	0	0	0	2 (66.6)	3
tst	59 (71.1)	8 (9.6)	2 (2.4)	1 (1.2)	1 (1.2)	3 (3.6)	9 (10.8)	83
sea	42 (63.6)	1 (1.5)	1 (1.5)	6 (9.1)	6 (9.1)	4 (6.1)	6 (9.1)	66
sej	0	0	0	0	0	4 (100)	0	4

Figure 3.2 Gel electrophoresis results of Staphylococcal enterotoxin A (*sea*) genotyping. 188 *S. aureus* isolates were tested on four agarose gels (A-D) including 52 from 1999-2001 and 39 from 2004 (including three isolates labelled with * on gel A), and 97 from 2006-07. Reference strains known to contain and not contain the *sea* gene were used as positive (+ve) and negative (-ve) controls respectively. 66 test isolates contained the gene product (234 bp).

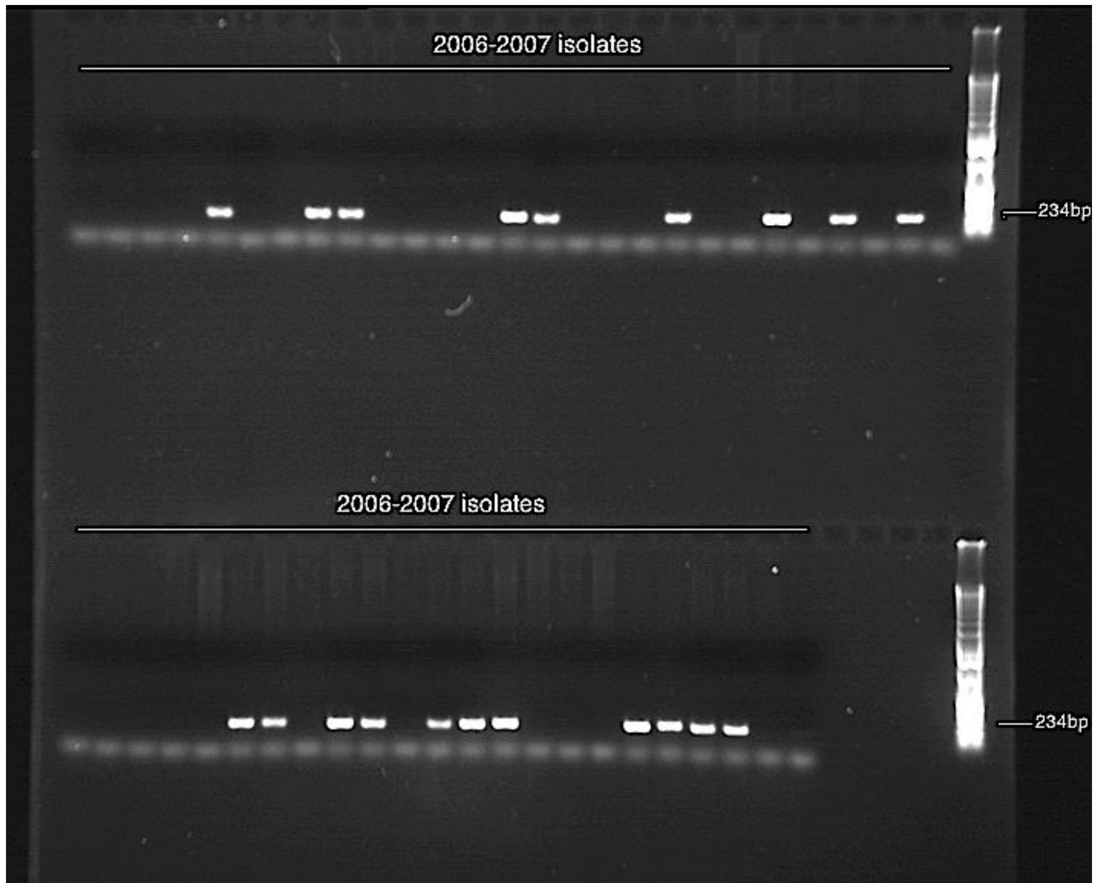
A



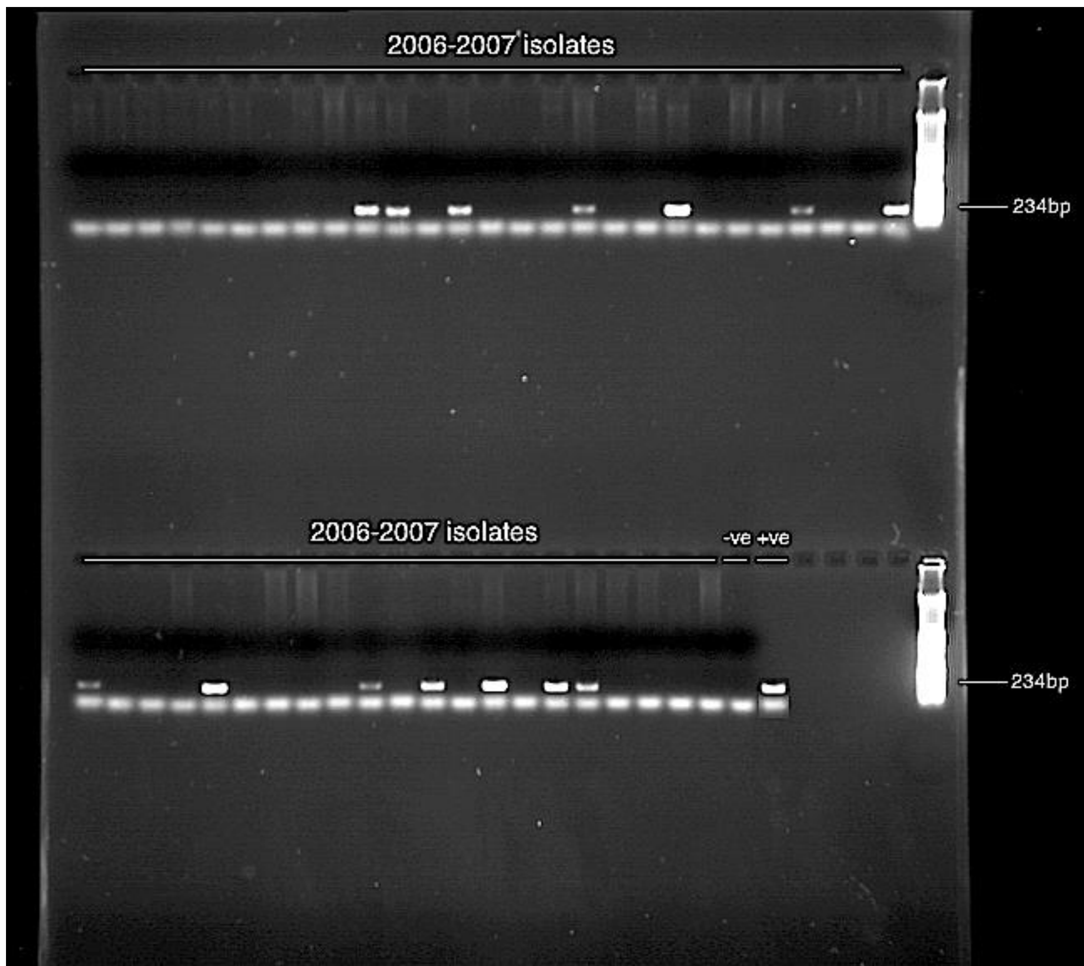
B



C



D



Part 2. Investigation of the MRSA bacteraemia outbreak using whole-genome sequencing

Conventional approaches failed to identify changes in patient characteristics or clinical management associated with the outbreak. Traditional typing methods failed to show the emergence of a novel strain associated with increased MRSA bacteraemia in Brighton. However, trends in dominant MRSA lineages in Brighton suggested an increase in EMRSA-16 and a decrease in EMRSA-15. This was in contrast to trends observed nationally where EMRSA-16 was decreasing.[277] This suggested the possibility of the emergence of a clone *within* a dominant lineage that was invisible by limited resolution of conventional typing methods. The second part of the study presents further investigation of the MRSA bacteraemia outbreak using WGS.

3.8 Methods

3.8.1 Whole-genome sequencing (1)

To evaluate the MRSA outbreak further a subset of MRSA strains from before and during the outbreak were investigated using WGS. These represented bacteraemia strains from the collection used in Part 1 of this chapter; 30 from 1999-2001 and 40 from 2006-07. Isolates corresponding to the two dominant MRSA lineages in UK hospitals (EMRSA-15 and EMRSA-16) were chosen for WGS due to trends observed. In total, 70 isolates were selected and successfully sequenced; 32 EMRSA-15 isolates (16 from 1999-2001 and 16 from 2006-07) and 38 EMRSA-16 isolates (14 from 1999-2001 and 24 from 2006-07). EMRSA-15 isolates corresponded to CC22 or *spa*-type t032 and EMRSA-16 isolates corresponded to CC30 or *spa*-type t018.

For comparison a collection of carriage and bacteraemia isolates over a comparable time period were retrieved from Oxford University Hospitals (OUH), a 1600-bedded general and teaching hospital in southern England whose MRSA bacteraemia rates were close to the national average across the study period (Figure 3.1). These included MRSA bacteraemia isolates collected as part of previous work in Oxford in 1996-1998 (n=28) and 2003-2007 (n=192).[270,278,279] Each sample set comprised

single isolates from consecutive episodes of bacteraemia. All Oxford isolates (n=220) had previously undergone characterisation by MLST and allocated into their respective lineage. A total of 93 isolates allocated to EMRSA-15 (4 from 1997-98 and 40 from 2003-07) and EMRSA-16 (24 from 1997-98 and 25 from 2003-07) were selected for WGS.

Overall 143 isolates (70 from BSUH and 93 from OUH) were selected for WGS and had paired-end, indexed libraries produced as previously described (Section 2.7). This study spanned developments in software and hardware used for sequencing and consequently two Illumina sequencing platforms were used; 51 samples were sequenced on the Genome Analyser IIx (GAII) and 92 samples were sequenced on the HiSeq2000. Quail *et al.* (2012) compared sequence data generated by different next-generation sequencing platforms and showed that GAII and HiSeq2000 have highly comparable accuracy for single nucleotide variant (SNV) identification in *S. aureus*.[280] These data suggest that it is acceptable to compare results using both methods. Reads from EMRSA-15 and EMRSA-16 samples were mapped to clonal complex-specific references (CC22 and CC30 respectively[264]), using Stampy[260]. Samtools was used to call and filter variants as previously described.[261]

As described previously, the alignment of reads using mapping-based assembly results in a small proportion of reads being left unmapped. This was due to genetic variation present in the test genomes that were absent from the reference genome. By selecting closely matching reference strains the proportion of unmapped reads is reduced. Typically up to 95% of reads generated are mapped. In this study a median of 97.2% and 97.9% of the respective reference genomes were covered by reads suggesting a close match between the reference genomes and our isolates. A summary of the genomic data is presented in Table 3.9, revealing that the genomic data obtained was good quality and suitable for evaluation. Maximum likelihood trees were constructed as described in Section 2.7.

Table 3.9 Summary of statistics for sequencing and mapping EMRSA-15 and EMRSA-16 isolates from Brighton and Oxford

	Lineage	Median (IQR)	Maximum	Minimum
Reads (n)	**EMRSA-15**	3.49×10^6 ($2.79 – 5.13 \times 10^6$)	22.55×10^6	2.16×10^6
	EMRSA-16	3.57×10^6 ($2.81 – 5.26 \times 10^6$)	24.97×10^6	1.32×10^6
Reads mapped (n)	**EMRSA-15**	3.41×10^6 ($2.72 – 4.94 \times 10^6$)	22.02×10^6	2.0×10^6
	EMRSA-16	3.47×10^6 ($2.75 – 5.12 \times 10^6$)	24.82×10^6	1.26×10^6
Reads mapped (%)	**EMRSA-15**	97.9 (97.5–98.0)	99.5	89.0
	EMRSA-16	97.2 (96.2–98.2)	99.6	86.2
Read depth	**EMRSA-15**	72 (31–96)	253	21
	EMRSA-16	70 (31–95)	251	14

3.8.2 Confirmation of lineage allocation

Lineage allocation by conventional methods (MLST, RM-typing, *spa*-typing) was discrepant between techniques in 9 isolates. As a result lineage membership was confirmed using SCC*mec* evaluation. As described in Section 1.1.3 SCC*mec* analysis is an effective method to determine strain lineages as dominant lineages contain specific SCC*mec* types due to their conserved nature within lineages (Table 1.1). EMRSA-15 isolates contain SCC*mec* type IV and EMRSA-16 isolates contain SCC*mec* II. SCC*mec* types were identified by comparison of Velvet assemblies[230] with a panel of type-representative SCC*mec* sequences obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank/). In brief, the Velvet assembler combines reads to make sets of overlapping DNA to represent a consensus region of DNA (a contig). In terms of SCC*mec* analysis, as the SCC*mec* gene (>2000bp) is longer than individual reads (<400bp) creating a contig spanning the entire gene allows accurate comparison with known sequences.

3.8.3 Phylogenetic relationships

In order to assess the phylogenetic relationships of Brighton and Oxford isolates the evolutionary history of each lineage was reconstructed using ClonalFrame.[263] In brief, this program identifies the clonal relationships between sequence data. By incorporating culture date and rates of genetic evolution (molecular clock) accurate inferences can be made on the time since isolates shared a common ancestor. An advantage of using ClonalFrame is that it is designed to account for recombination events. This is important as using SNV differences to establish genetic relatedness and time since strain divergence can be overestimated in the presence of recombination.

3.8.4 Genomic innovations

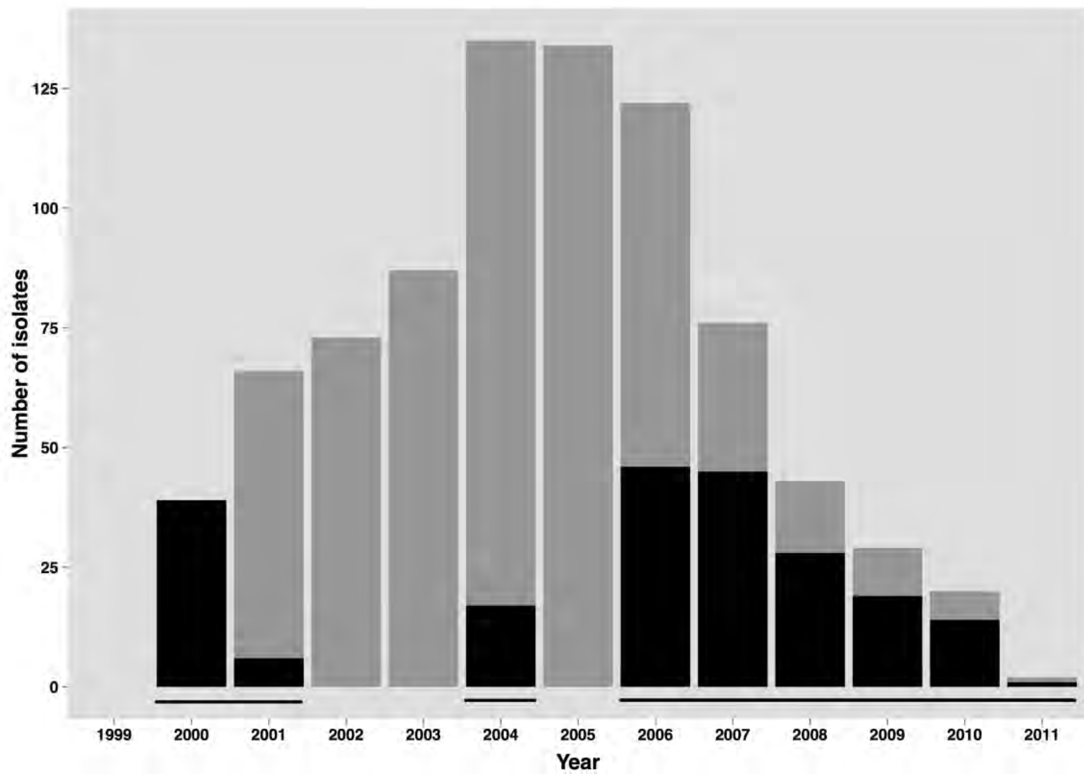
Following identification of a novel clonal variant within EMRSA-16 genomic comparisons were undertaken to assess putative genomic events associated with a selective advantage to cause disease excess. Point mutations and recombination events were identified and those unique to the EMRSA-16 variant strains were assessed further. Genes associated with genetic insertions were identified by mapping to a library of unique *S. aureus* sequences obtained from GenBank.

3.8.5 Evaluating the contribution of EMRSA-16 variant to the outbreak

To evaluate the contribution of the EMRSA-16 variant to blood stream infections in Brighton additional bacteraemia isolates from BSUH were retrieved from previously unavailable archives. They included 25 *S. aureus* isolates (13 MRSA) from early 2006 and 261 (89 MRSA) from late 2007-2011 representing time periods during and after the MRSA outbreak. Basic demographic and biochemical data were collected retrospectively for these isolates. All isolates additionally retrieved from Brighton archives underwent *spa*-typing and were thus allocated to appropriate lineages. Isolates of the same *spa*-type (t018) as the EMRSA-16 variant were identified for further assessment.

In total 515 isolates (215 MRSA) from BSUH were available from 1999-2011; 229 isolates collected in Part 1 of this chapter and 286 additionally retrieved. The aim was to retrieve as complete as possible a collection of bacteraemia isolates from before, during and after the outbreak. The number of MRSA isolates retrieved from BSUH was compared with MRSA bacteraemia rates reported by the trust to the Health Protection Agency mandatory surveillance (Figure 3.3); MSSA bacteraemia surveillance data are only available from 2011 and hence could not be compared. Whilst a large number of bacteraemia isolates were not retrieved during this time there is no reason to think that those retrieved were in any way a biased sample of the total.

Figure 3.3 Brighton MRSA bacteraemia isolates (n=215) retrieved (black bars) compared to the total number reported to the Health Protection Agency (HPA) during the same time period (grey bars). The black lines below the bars indicate the timeframes of isolate collections. HPA data are unavailable for 1999-2000.



3.8.5.1 Molecular signature assay

In order to determine whether newly retrieved (and previously un-sequenced) MRSA t018 isolates were EMRSA-16 variants a molecular signature assay was developed. This PCR-based assay provided a fast and inexpensive identification tool without the need to undertake WGS. The assay targeted five unique single nucleotide polymorphisms (SNPs) on the three branches ancestral to EMRSA-16 variant isolates (Table 3.10). Five pairs of primers were designed to amplify regions containing the SNPs. The assay was validated with previously sequenced EMRSA-16 variant isolates as positive controls and EMRSA-16 non-variant and EMRSA-15 isolates as negative controls. Isolates were identified as the variant if the genotype observed was concordant to the variant at all five SNPs.

PCR reactions contained a final volume of 10µl and contained 0.1µl 0.25mM dNTPs (Fermentas Life Sciences, Germany), 0.1µl 0.5U/µl GoFlexi Taq DNA polymerase (Promega, Southampton, UK), 1µl 10 x Buffer (Promega, Southampton, UK), 1µl 25mM $MgCl_2$ (Promega, Southampton, UK) and 0.5µl of each 0.5µM primer. Reactions were cycled as follows: 94°C for 2 minutes; 35 cycles of [94°C for 30 seconds, 52°C for 30 seconds, 72°C for 1 minute]; final extension at 72°C for 5 minutes. PCR products were purified using Agencourt AMPure XP beads (Beckman Coulter, London, UK). PCR products were sequenced using PCR primers and BigDye v3.1 chemistry (Applied Biosystems, USA) and cycled as follows: 96°C for 10 seconds; 30 cycles of [52°C for 5 seconds, 60°C for 2 minutes]. Sequencing products were purified using Agencourt CleanSEQ beads (Beckman Coulter, London, UK). PCR products sequencing analysis was performed on an ABI 3730 DNA Analyzer (Applied Biosystems, USA).

13 MSSA t018 isolates were identified from bacteraemia samples from the Brighton collection, including 8 from 2004 representing a time when rates of MRSA bacteraemia began to rise. To investigate the possibility of MSSA ancestors of the EMRSA-16 variant these 13 MSSA t018s were also evaluated using the molecular signature assay.

Table 3.10 Five single nucleotide polymorphisms (SNP) unique to the EMRSA-16 variant and primer sequences used for the molecular signature assay

SNP Name	SNP position on genome	Primer name	Reference base	EMRSA-16 variant base	Primer (5'-3')
S1	1138701	113-Br1-F	A	G	CGCATGGCTAGCAGTTGTAA
		113-Br1-R			ACACGTGTACGTTTAAAGCC
S2	486162	486-Br2-F	C	T	TGATAAACTCAGGCGCTACC
		486-Br2-R			ACCTTGCCCTAGAACGGTTA
S3	1497289	149a-Br2-F	A	T	TGGTCAACGTATGAAGCTCC
		149a-Br2-R			CCTTGGCACCTACCAAATGA
S4	542018	542-Br3-F	C	T	AAGTTGAACGTATGCAAGCG
		542-Br3-R			TCAGCTGTGTCTTCCCCATA
S5	770373	770-Br3-F	G	A	ACCTGAACGACTGAAACCAG
		770-Br3-R			TAACGGAATTTGCCCCTGTT

3.8.5.2 Determining methicillin susceptibility

Methicillin susceptibility of MSSA t018 isolates was confirmed using two methods. Firstly, through determining minimum inhibitory concentrations to oxacillin as described in Section 2.3. Secondly, by determining the presence of *mecA*, the gene conferring β–lactam resistance in Staphylococci. This was performed using two multiplex PCR assays (Table 3.11).

Multiplex 1 included one pair of primers to confirm the presence of bacterial DNA (16S) and two primer pairs to amplify different sized *mecA* gene products (mecA1 and mecA2).[281,282] Positive and negative controls were retrieved from a collection of whole-genome sequenced isolates where their genome had been interrogated for the presence or absence of *mecA*. PCR reactions contained a final volume of 25µl containing 5ul of template DNA, 0.25µl 25mM dNTPs (Fermentas Life Sciences, Germany), 0.25µl 1U/µl GoFlexi Taq DNA polymerase (Promega, Southampton, UK), 2.5µl 10 x Buffer (Promega, Southampton, UK), 1µl 2.5mM $MgCl_2$ (Promega, Southampton, UK), 15.75µl H_2O and 0.042µl of each Primer (at a concentration of 130µg/mL). Reactions were cycled as follows: 94°C for 5 minutes; 30 cycles of [94°C for 30 seconds, 52°C for 30 seconds, 72°C for 1 minute]; final extension at 72°C for 10 minutes. PCR products were assessed by electrophoresis on 1% agarose gel.

A second multiplex PCR assay was developed based on a published *mecA* multiplex PCR protocol.[283] This included three primer pairs; 16S2 to confirm DNA, *nuc* to confirm species using primers specific for *S. aureus* nuclease gene, and mecA3 to assess presence of *mecA*. PCR reactions and cycle conditions were the same as Multiplex 1 except the annealing temperature was 55°C.

Table 3.11 Primers for multiplex PCR assays used to identify the presence of *mecA* gene in 13 methicillin susceptible *S. aureus* isolates

Multiplex	Product Name	Primer Name	Sequence (5`-3`)	Amplification Size (bp)	Reference
1	16S	Staph 756F	AACTCTGTTATTAGGGAAGAACA	756	McClure *et al* (2006)[281]
		Staph 750R	CCACCTTCCTCCGGTTTGTCACC		
	mecA (1)	MecA1F	GTAGAAATGACTGAACGTCCGATAA	310	
		MecA2R	CCAATTCCACATTGTTTCGGTCTAA		
	mecA (2)	MecA147-F	GTGAAGATATACCAAGTGATT	147	Zhang *et al* (2005)[282]
		MecA147-R	ATGCGCTATAGATTGAAAGGAT		
2	16S2	16S-1	GTGCCAGCAGCCGCGGTAA	886	National Food Institute[283]
		16S-2	AGACCCGGGAACGTATTCAC		
	nuc	Nuc-1	TCAGCAAATGCATCACAAACAG	255	
		Nuc-2	CGTAAATGCACTTGCTTCAGG		
	mecA3	mecA-1	GGGATCATAGCGTCATTATTC	527	
		mecA-2	AACGATTGTGACACGATAGCC		

3.8.6 Clinical profile

Patient data were retrieved as described in part 1 of this chapter. In addition, associated routine laboratory clinical data were collected (haemoglobin, neutrophil count, total white cell count, creatinine, urea, C-reactive protein and albumin) at the time of bacteraemia between the EMRSA-16 clonal variant and all other episodes of MRSA bacteraemia (including EMRSA-15 and other MRSA strains) at Brighton during the study period.

3.8.7 Evaluating the geographical spread of EMRSA-16 variant

In order to evaluate the uniqueness of the EMRSA-16 variant to Brighton, bacteraemia isolates from neighbouring hospitals (termed regional) were retrieved to assess spread to hospitals closely located to Brighton. All available *S. aureus* bacteraemia isolates were retrieved from three regional hospitals that geographically surround Brighton; Worthing hospital, Western Sussex Hospitals NHS trust (n=179) is located 12 miles west, Crawley Hospital, Surrey and Sussex NHS Trust (n=166) is located 23 miles north, and Conquest Hospital, East Sussex Healthcare NHS Trust, Hastings (n=26) is located 40 miles east (Figure 3.4). All isolates were from blood cultures following the outbreak period (2007-2011).

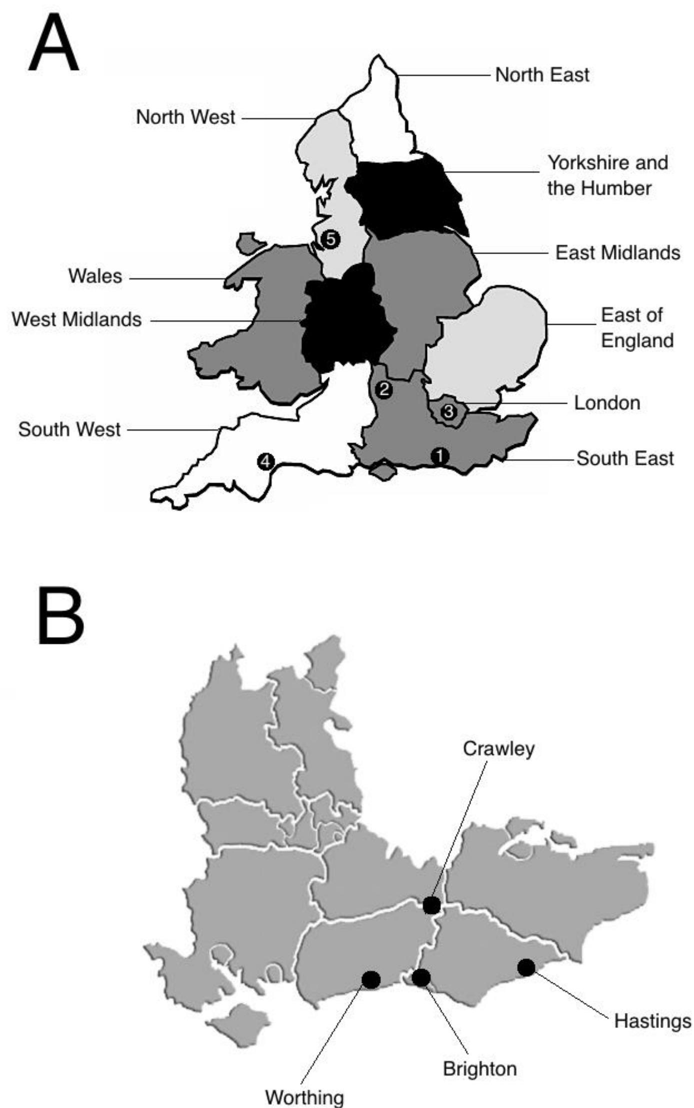
To develop a national context for the population structure of EMRSA-16 isolates were retrieved from hospitals geographically separate from Brighton. Through collaboration with UK Clinical Infection Research Group (UKCIRG; http://www.ukcirg.co.uk) isolates from a well-described *S. aureus* collection were retrieved for evaluation. These included invasive *S. aureus* isolates from five hospitals involving four geographically distinct regions within England following the outbreak period (2008-2011); Plymouth Hospitals NHS Trust and Royal Devon and Exeter NHS Foundation Trust, Exeter (South East), Oxford University Hospitals (Southern), University College London Hospitals (London) and Royal Liverpool University Hospital, Liverpool (North west) (Figure 3.4).

In addition 45 isolates were retrieved from the Staphylococcal Reference Unit (Public Health England (PHE)). These represented a collection of bacteraemia, clinical and carriage strains that had been previously been identified as EMRSA-16 as part of national surveillance following the outbreak period (2009-2012). Isolates

were selected by Professor Angela Kearns (PHE) to represent samples from 9 UK regions (Figure 3.4); no samples were selected from Brighton.

All isolates from regional and national collections were *spa*-typed to identify isolates of the same *spa*-type (t018) as the EMRSA-16 variant for WGS analysis.

Figure 3.4 Geographical locations of Brighton, regional and national hospitals where isolates were retrieved: (A) defines the geographical boundaries of the nine Health Protection Agency regions in England and the location of the five UKCIRG centres including (1) Brighton and regional hospitals (enlarged in B), (2) Oxford University Hospitals, (3) University College London Hospitals, (4) Devon and Exeter, and (5) Liverpool; (B) illustrates the South East region depicting the location of Brighton and neighbouring hospitals (Worthing, Crawley and Hastings).



3.8.8 Whole-genome sequencing (2)

In total 173 t018 isolates were identified from Brighton, regional hospitals and from national collections. As described in Section 3.8.1, 38 MRSA t018 (EMRSA-16) isolates from BSUH had previously been whole-genome sequenced. To allow comparison additionally retrieved t018 isolates that were previously un-sequenced underwent WGS. This was undertaken for three reasons: (i) to determine genetic relationships between EMRSA-16 variants identified through the molecular signature assay, (ii) to evaluate the t018s from regional and national collections for isolates highly related to EMRSA-16 variant, and (iii) to assess whether MSSA t018 isolates identified in Brighton found to contain the 5 SNPs pertaining to the EMRSA-16 variant were MSSA ancestors to the variant.

In total 135 previously un-sequenced isolates were prepared for WGS including 67 from Brighton, 9 from regional hospitals, and 59 from national collections. WGS was performed on a single platform (Illumina HiSeq2000) using previously described protocols for bacterial library preparation and bioinformatics processing[242] in which SNVs were identified by mapping to a CC30-specific reference (MRSA252[264]). 133 genomes were successfully assembled and two isolates failed library preparation; one from Brighton and one from the HPA collection. A median of 93.9% of the reference genomes were covered by reads suggesting a close match between the reference genomes and our isolates. Table 3.12 displays a summary of the sequencing data revealing good quality sequences suitable for further analysis. Genomic data from these isolates were evaluated with data from the 38 previously sequenced EMRSA-16 isolates. Maximum likelihood trees were constructed as described in Section 2.7.

Table 3.12 Summary of statistics for 133 successfully sequenced isolates from Brighton, regional hospitals and national collections

	Median (IQR)	Maximum	Minimum
Reads (n)	1.93 $x10^6$ (1.57 – 2.29 $x10^6$)	4.16 $x10^6$	0.92 $x10^6$
Reads mapped (n)	1.89 $x10^6$ (1.56 – 2.23 $x10^6$)	4.10 $x10^6$	0.90 $x10^6$
Reads mapped (%)	98.5 (96.6–98.6)	99.8	84.7
Read depth	100 (81–118)	214	48

3.9 Statistical analysis

Data were analysed using SPSS (version 20.0.0, IBM®, USA) or Stata (version 13, StataCorp®, USA). Associations between clonal variant and the variables described were assessed using the Fisher's exact test for proportions, chi-squared for categorical variables, t-test for parametrically distributed continuous variables and the Mann-Whitney U test for non-parametrically distributed continuous variables. Biochemical variables significantly associated with the clonal variant in the univariate analysis were analysed in a multivariate logistic regression model to adjust for patient location. Probability values ≤ 0.05 were considered significant.

A phylogenetic reconstruction programme (ClonalFrame[263]) was used to determine 95% credible intervals to estimate the time to recent common ancestor between isolates. Credible intervals are analogous to confidence intervals used in frequentist statistics.

3.10 Ethical approval

As described in part 1 of this chapter all isolates collected for these epidemiological studies were an evaluation of service and were covered by Statutory Instrument Regulations 2002 No. 1438.[276] Isolate storage and data collection was approved in Brighton by the BSUH Research and Development office as a service evaluation, involving anonymised data from patient records and not requiring formal ethical review. Oxford 1997–1998 isolates were collected from the routine microbiology laboratory and stored without personally identifying information. Isolates from UKCIRG collection were reviewed by the UK National Research Ethics Service and were deemed an evaluation of service, not requiring review by ethics committee. On request the Staphylococcal reference unit provided anonymised isolates for comparison, not requiring formal ethics review.

3.11 Results

3.11.1 Evaluation of MRSA outbreak using whole-genome sequencing

To assess whether the MRSA bacteraemia outbreak observed in Brighton was associated with emergence of a novel clone within a lineage the higher resolution offered by WGS was used. Available isolates from CC22 (including EMRSA-15) and CC30 (including EMRSA-16) from before and after the Brighton outbreak were evaluated. In total 81 Brighton isolates were prepared for sequencing including 38 CC22 (18 from 1999-2001 and 20 from 2006-07) and 43 CC30 (15 from 1999-2001 and 28 from 2006-07). 11 failed library preparation (6 from CC22 and 5 from CC30) leaving 70 available for analysis. All CC22 isolates were identified as either MLST ST22 or *spa*-type t032 corresponding to EMRSA-15 and all CC30 isolates were MLST ST36 or *spa*-type t018 corresponding to EMRSA-16.

As a control group Brighton isolates were compared to a set of MRSA carriage and bacteraemia isolates from Oxford University Hospitals (OUH), where rates of *S. aureus* bacteraemia were comparable with those observed nationally. 93 MRSA isolates from OUH were successfully sequenced including 44 from CC22 (all MLST ST22 corresponding to EMRSA-15) and 49 from CC30 (all MLST ST36 corresponding to EMRSA-16).

As a result of discrepancies in lineage allocation between the typing methods all MRSA isolates of CC22 and CC30 from Brighton and Oxford had their membership of the respective lineage confirmed using WGS. As described previously MRSA strains may be classified and typed according to composition of SCC*mec*.[26,27] Subsequently all isolates had the SCC*mec* characterised through WGS interrogation. All CC22 isolates carried type IV SCC*mec* characteristic of EMRSA-15, and all but one CC30 isolate carried SCC*mec* II characteristic of EMRSA-16. The remaining CC30 isolate was identified as a probable community-associated MRSA since it carried SCC*mec* V, was genetically distinct and was ciprofloxacin sensitive (all other EMRSA-16 isolates were resistant); it was excluded from phylogenetic analysis.

To investigate the diversity of isolates from Brighton pairwise SNV differences were compared between strains of the same lineage (Figure 3.5); 703 EMRSA-16 pairs and 496 EMRSA-15 pairs were generated for comparison. EMRSA-15 isolates

clustered together with a single peak showing a median SNV difference of 93 (IQR 78-107), suggesting a conserved population structure. Conversely, whilst EMRSA-16 isolates had a median SNV difference of 85 (IQR 23-336) four distinct peaks were observed. Whilst recombination events may affect the diversity between isolates an alternative explanation is the presence of a diverse population structure. Peaks in the pairwise distance distributions may correspond to clusters of highly related isolates but sampling bias can affect these distributions. Consequently these data need to be interpreted alongside phylogenetic estimations to identify clusters.

To investigate this further maximum likelihood trees were created to estimate the diversity and infer phylogenetic relationships between isolates (Figure 3.6). The relationship of EMRSA-15 isolates reveals a star-shaped topology with branches of comparable length, suggesting strain divergence from a common ancestor. In comparison, the shape of the EMRSA-16 maximum likelihood trees is very different. Firstly, there is a cluster of highly related isolates suggesting recent divergence. Secondly, the longer branch lengths in other areas of the tree suggest distinct groups of isolates with much larger genetic divergence. Whilst this may represent the true genetic diversity between isolates an alternative explanation is the presence of recombination. Maximum likelihood trees calculate genetic diversity based on SNV differences between isolates. From this time since divergence can be inferred based on knowledge of the natural rate of evolution (molecular clock). The incorporation of large sections of DNA into the genome (recombination) increases the SNV differences between two isolates. Maximum likelihood methods calculate the diversity between isolates based on number of variant sites and fail to identify recombination events. This can result in long branch lengths and an overestimation of time since divergence. To address this a manual inspection was made of the genome positions of the variant sites. Whilst the SNVs appeared to be dispersed throughout the genomes this did not rule out recombination.

Figure 3.5 Histogram of the pairwise genome-wide diversity of EMRSA-15 (n=32) and EMRSA-16 (n=38) isolates from Brighton

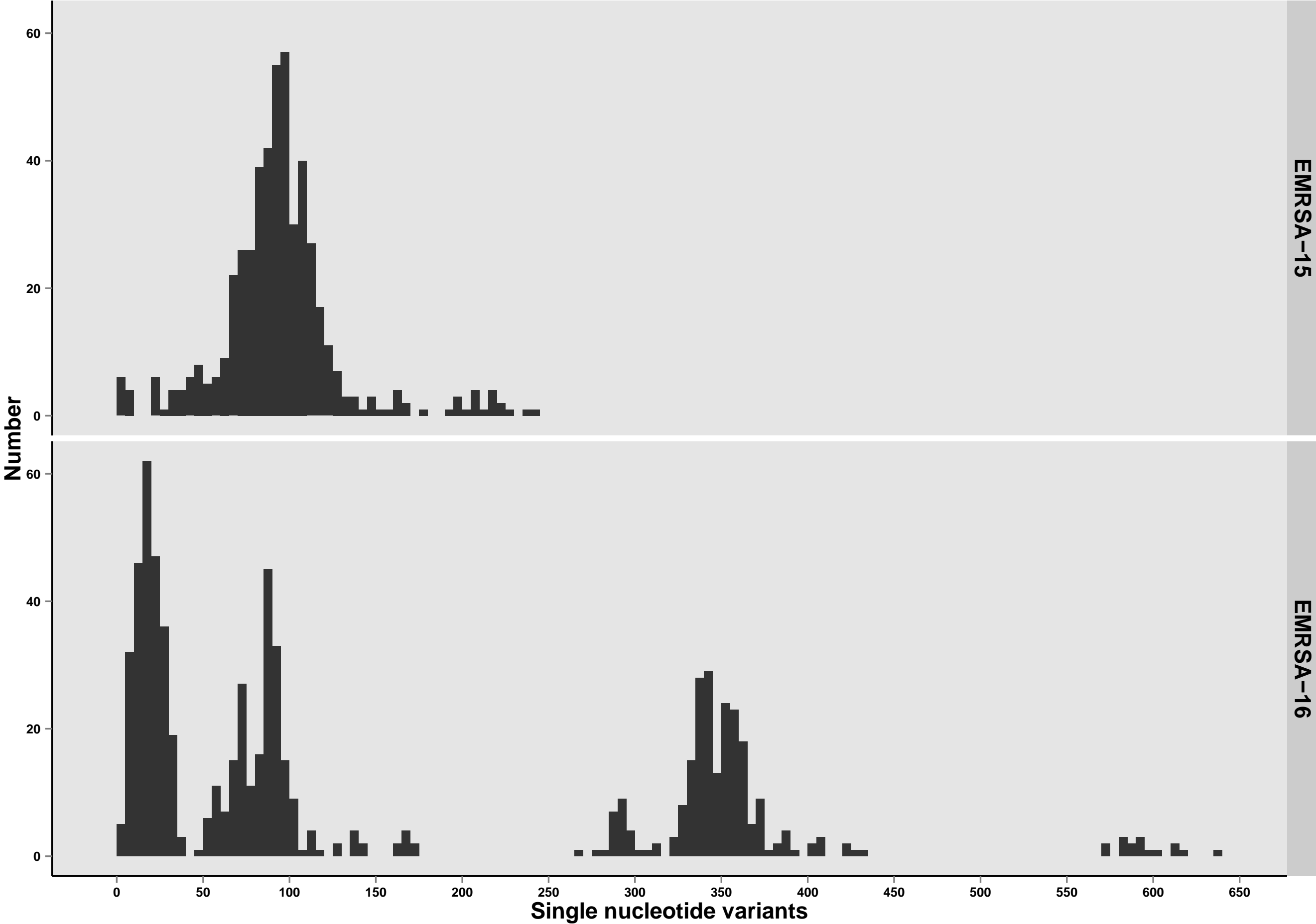
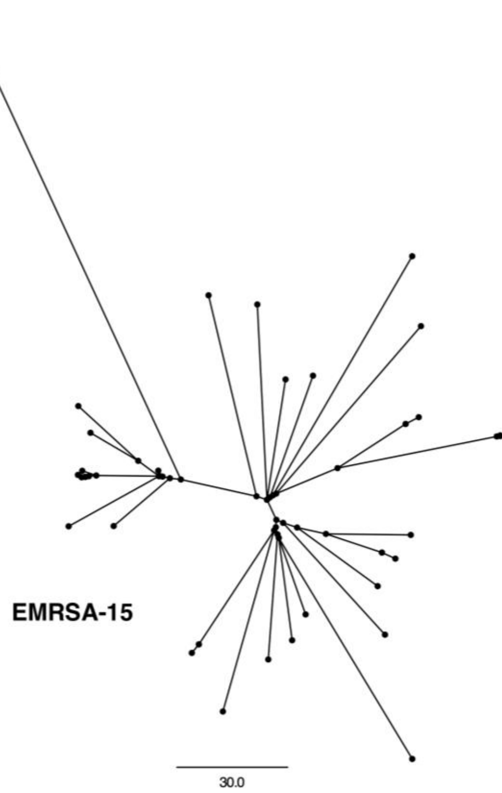
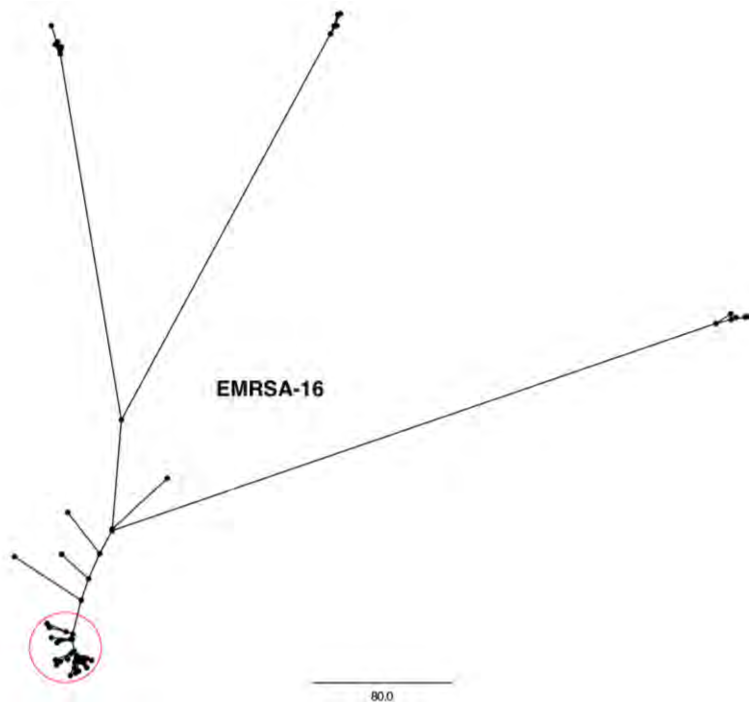


Figure 3.6 Maximum likelihood trees of EMRSA-15 and EMRSA-16 isolates from Brighton. Distal nodes (black) represent isolates. Scales of single nucleotide variants (SNV) are given per tree. The red circle represents a cluster of highly related isolates.





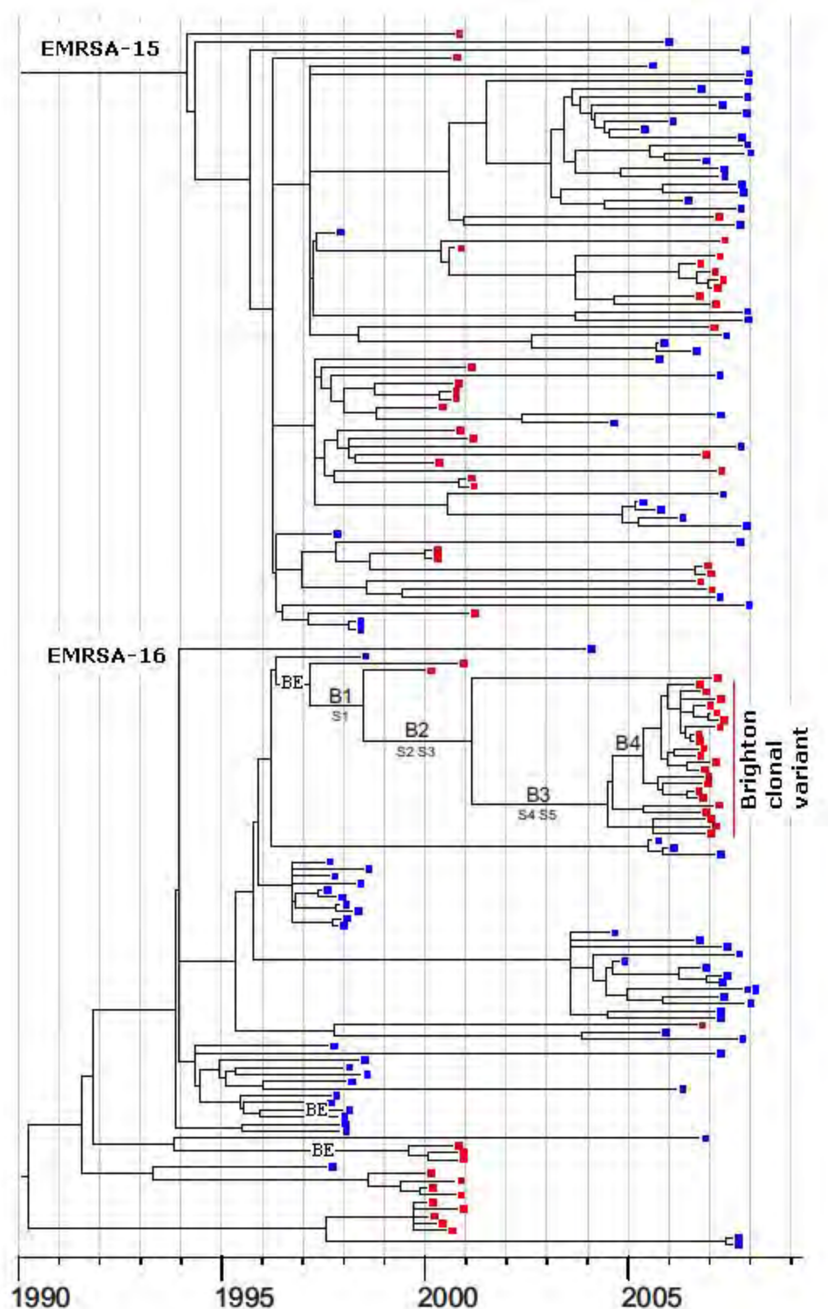
To evaluate this further, the evolutionary relationships within EMRSA-15 and EMRSA-16 isolates were reconstructed using sample dates to inform temporal relationships (Figure 3.7). For comparison the control collection of sequenced isolates from Oxford (OUH) were used. Phylogenetic reconstruction was performed using ClonalFrame. As described in Section 2.7 this tool identifies the clonal relationship between isolates and estimates positions of recombination events.

For EMRSA-15 isolates the most recent common ancestor in the sample was estimated to have occurred in 1994 (95% credible interval May 1993–October 1994) although EMRSA-15 was first described in English hospitals in 1991.[284,285] EMRSA-15 showed no dominant local clones and few clusters with recent common ancestors (<3 years).

The most recent common ancestor of EMRSA-16 strains in the sample was estimated to have occurred in 1990 (95% credible interval March 1989–February 1991), coinciding closely with the reported 1991 emergence of EMRSA-16 in the UK.[286] There was strong clustering of isolates in the EMRSA-16 tree with respect to sampling time and location. In Brighton the largest EMRSA-16 genetic cluster in 1999-2001 involved 5/14 (36%) isolates compared to 22/24 (92%) from 2006-2007. In contrast, the largest EMRSA-16 genetic clusters in Oxford were 11/23 (48%) in 1996-1998 versus 13/25 (52%) in 2003-2007.

The EMRSA-16 isolates from Brighton 2006-07 period showed strikingly low molecular and lineage diversity, with the most recent common ancestor of the 22 EMRSA-16 genomes forming a single tight genetic cluster (termed the EMRSA-16 "variant") estimated to have existed in 2004 (credible interval February 2004–January 2005), coinciding with the rise in MRSA bacteraemias in Brighton. The genome of each Brighton EMRSA-16 clonal variant differed from its nearest neighbour by a median of 7 (range 0-22) SNV. Such close genomic relationships are consistent with recent transmission and clonal expansion, i.e. rapid, recent emergence.[234]

Figure 3.7 Phylogenetic tree for EMRSA-15 and EMRSA-16 isolates analysed from Brighton (red) and Oxford (blue). The x-axis indicates date, where branch tips are the date of sampling and branches join at the estimated dates of their presumed common ancestors. The EMRSA-16 tree is annotated to show branches labelled B1-B3 which are ancestral to all outbreak isolates and include SNVs S1-S5 used in the molecular signature assay to identify clonal variants. In addition, a 5.3kb recombinational replacement was mapped to branch B1 and two separate insertions of 20kb of highly-similar non-reference sequences are indicated by BE.



3.11.2 Contribution of the EMRSA-16 variant to the outbreak

To explore the contribution of the variant to the outbreak 286 additional *S. aureus* bacteraemia isolates (102 MRSA) from BSUH were retrieved; 25 from early 2006 and 261 from 2007-2011. All additional isolates were *spa*-typed and 55 MRSA t018s were identified. A molecular signature assay was designed to allowed rapid assessment of MRSA t018 isolates to determine if they corresponded to the EMRSA-16 variant without having to use WGS. All 55 newly identified t018s underwent evaluation using the molecular signature assay; 44 possessed the 5 SNPs pertaining to the EMRSA-16 variant.

In total 215 Brighton MRSA strains were retrieved between 1999-2011 and of these 93 MRSA t018 were identified and evaluated for EMRSA-16 variant; 38 through WGS and 55 additionally retrieved using the molecular signature assay. 66/93 were identified as the EMRSA-16 variant (22 by WGS and 44 by molecular signature assay) (Table 3.13). Among the isolates from 2000-01, 0/15 of EMRSA-16 strains were the variant; in 2004 2/6 (33%) were the variant; while after 2006, 64/72 (89%) EMRSA-16 strains represented the clonal variant.

Table 3.13 MRSA bacteraemia isolates from Brighton evaluated for the presence of EMRSA-16 variant using whole-genome sequencing (WGS) and molecular signature assay (MSA)

Cohort	Total	EMRSA-16	WGS	MSA	EMRSA-variant*
1999-2001	45	15	14	1	0
2004	17	6	0	6	2
2006-2011	153	72	24	48	64
Total	215	93	38	55	66

*Identified by either WGS or MSA

3.11.3 Clinical phenotype of EMRSA-16 variant

Clinical data retrieved from 215 cases of MRSA bacteraemia between 1999-2011 were evaluated in order to understand whether the EMRSA-16 variant corresponded to a particular clinical phenotype. MRSA bacteraemias caused by EMRSA-16 variant occurred in patients of all ages and throughout the hospital (Figure 3.8). There was no difference in age or sex of cases (Table 3.14). However, the proportion of MRSA bacteraemias caused by it differed between units with a greater proportion of clonal variant MRSA bacteraemias in intensive care (19/33 MRSA bacteraemias) and a lower proportion on the renal unit (7/46) ($p<0.001$). To evaluate this further the time from ICU admission to positive blood culture was used to infer whether bacteraemia was acquired before (<48hours) or during (>48hours) ICU admission; 16/19 EMRSA-16 variant ICU bacteraemias were identified >48hours from ICU admission.

After adjustment for patient location, MRSA bacteraemia episodes caused by the variant were associated with a significantly higher total white cell count (difference +4.0 (1.7-6.3 x10^9/l; $p<0.001$) and neutrophil count (+3.9 (1.7-6.0x10^9/l); $p<0.001$) than episodes caused by non-variant strains (non-variant EMRSA-16, EMRSA-15 and other strains) with 40% of variant cases having neutrophil counts above 15 x 10^9/l versus only 14% in other strains (Table 3.15). Platelet count was also higher in episodes caused by the variant (difference +62 (15-108 x 10^9/l); $p=0.009$). There was no significant difference in other laboratory values including C-reactive protein.

Figure 3.8 Contribution of EMRSA-16 clonal variant to cases of MRSA bacteraemia in Brighton. 190 cases of MRSA bacteraemia where location was known; EMRSA-variant (red) and non-variant (black). MRSA isolates reported to the Health Protection Agency that were not captured in the study are depicted in white.

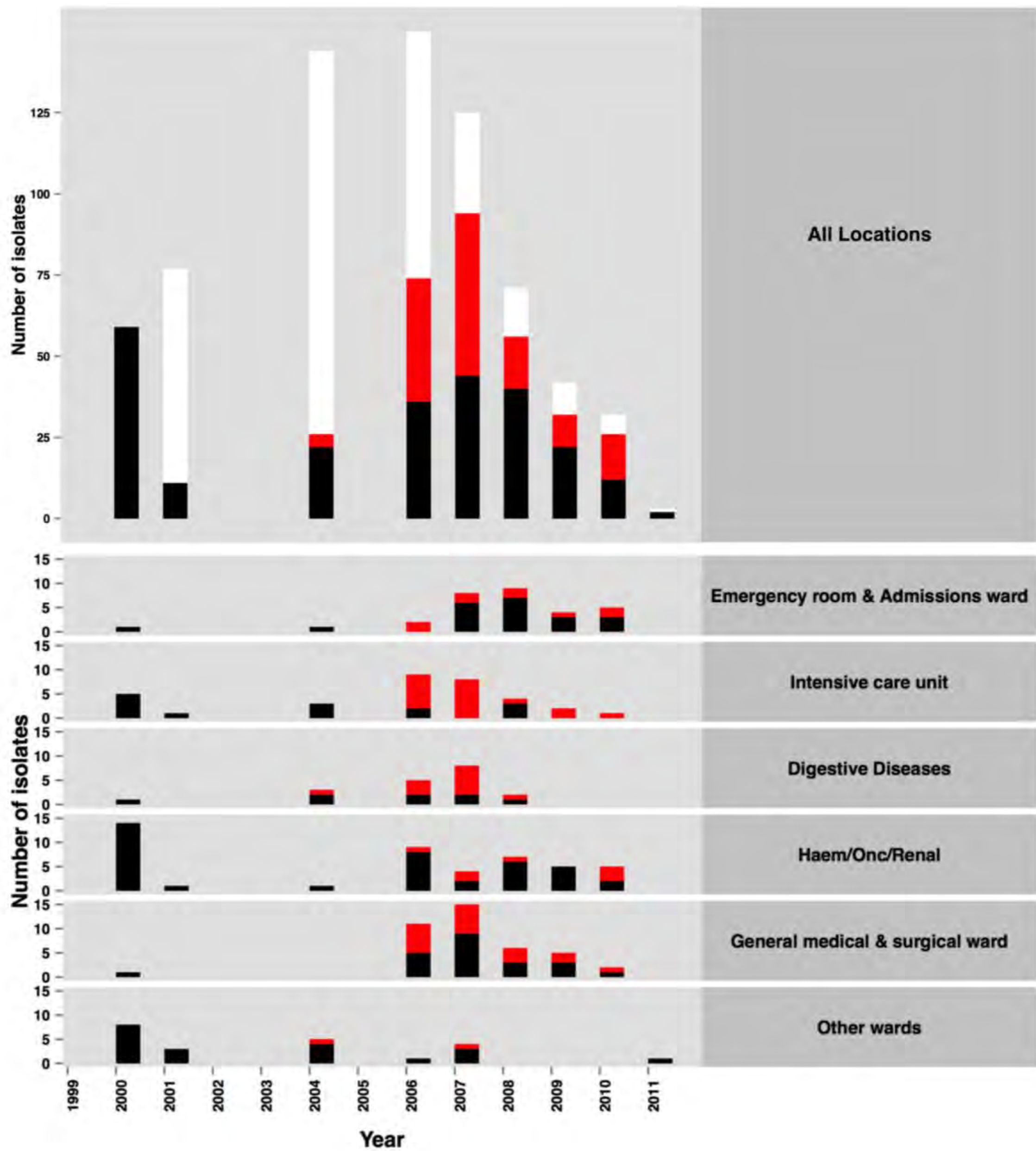


Table 3.14 Demographic and location data for MRSA bacteraemia episodes from which strains were available for typing in Brighton between 1999-2011. P-values calculated by chi-squared test (sex), Fisher's exact test (location) or t-test (age). SD = standard deviation. Whilst the EMRSA-16 variant was identified throughout the hospital there was a significant difference in its ward distribution. During the study period 19/33 (58%) MRSA bacteraemia on the intensive care unit were caused by the EMRSA-16 variant compared to 7/46 (15.2%) on haematology, oncology and renal units (p<0.001).

	Clonal variant MRSA (n=66)	Non-variant MRSA (n=149)	p-value
Demographics	n=65	n=139	
Male, n (%)	39 (60%)	96 (69%)	0.2
Age, mean (SD)	68.1 (17.3)	65.3 (16.8)	0.27
Location, n (%)	n=66	n=124	
Emergency Room / Admissions ward	9 (14%)	21 (17%)	
Intensive Care Unit	19 (29%)	14 (11%)	
Haematology / Oncology / Renal wards	7 (11%)	39 (31%)	
Digestive Diseases ward	11 (17%)	8 (6%)	
Other medical/surgical wards	18 (27%)	22 (17%)	
Other wards	2 (3%)	20 (16%)	

Table 3.15 Laboratory data for 215 episodes of MRSA bacteraemia from which strains were available for typing in Brighton between 2000-2011. *The relationship between the biochemical marker and the clonal variant was adjusted for patient location. †2-tailed correlation. ^multiple logistic regression. SD=standard deviation, CI=confidence interval.

	Clonal variant MRSA		Non-clonal variant MRSA		Univariate significance†	Adjusted difference and significance*	
	n	mean (SD)	n	mean (SD)	p	Difference (95% CI)	p value^
Creatinine (uM)	65	132 (137)	107	224 (266)	**0.003**	-20 (-67.5 - 27.0)	0.40
C-Reactive Protein (mM)	50	168 (112)	81	169 (103)	0.96	-	-
Albumin (mM)	51	25.9 (6.9)	82	29.6 (6.8)	**0.004**	-1.2 (-3.4 – 1.1)	0.32
Urea (mM)	65	11.5 (6.9)	107	12.9 (10.4)	0.34	-	-
Potassium (mM)	65	4.1 (0.7))	107	4.2 (0.7)	0.47	-	-
Sodium (mM)	65	138.3 (7.0)	107	137.6 (5.8)	0.50	-	-
Haemoglobin (g/dl)	65	10.3 (1.9)	106	10.2 (1.6)	0.97	-	-
White Cell Count (x10^9/l)	65	16.6 (9.3)	106	12.2 (5.5)	**<0.001**	4.0 (1.7-6.3)	**<0.001**
Neutrophils (x10^9/l)	65	14.4 (8.6)	106	10.2 (5.4)	**<0.001**	3.9 (1.7-6.0)	**<0.001**
Platelets (x10^9/l)	65	307 (177).	106	250 (108)	**0.01**	62 (15-108)	**0.009**

3.11.4 Genomic innovations associated with EMRSA-16 clonal variant

In order to evaluate a genetic basis for the emergence of the EMRSA-16 variant genomes of the EMRSA-16 isolates that underwent WGS (as described in Section 3.11.1) were interrogated. Genomic events (such as mutations, homologous recombinations, gain or loss of gene content) on branches of the phylogeny ancestral to the variant (B1, B2, B3 in Figure 3.7) were sought that might explain its emergence via a selectively favoured change in phenotype. 25 point mutations (21 non-synonymous including 11 non-synonymous substitutions on B3, the branch immediately ancestral to the clonal expansion) were identified across the three branches. Comparisons with known gene sequences showed that two non-synonymous changes occurred in *ebh*, a gene encoding a very large cell wall-associated fibronectin-binding protein consistent with patterns recently reported among EMRSA-16 strains in which this gene was hypothesised to have an effect on virulence.[243,287] A large (5.3kb) homologous recombination event was detected on B2 (Figure 3.7) which shows high similarity to regions of a family of bacteriophages (phi Sa3) in the *S. aureus* reference strain (MRSA252[264]) upstream of the regions encoding the Panton-Valentine Leukocidin (PVL) gene. In addition 3.2kb of unique sequences (termed 'BE' in Figure 3.7) were identified, variants of which had also inserted on other parts of the EMRSA-16 tree. BE comprises part of a large (~20kb) fragment of a phage (phiNM4-like) whose position could not be uniquely determined using short-read data.

3.11.5 Methicillin susceptible t018s

While MRSA isolates of *spa*-type t018 are highly prevalent disease-causing strains in UK hospitals (corresponding to EMRSA-16) MSSA t018s are uncommon causes of *S. aureus* bacteraemia.[288] It is notable therefore that 13 MSSA t018s were identified from bacteraemia samples in Brighton; 8 from 2004, 1 from 2006, 1 from 2007, 2 from 2009 and 1 from 2011. As 8 of these isolates were retrieved prior to the outbreak in 2004 it was plausible that they may represent the MSSA ancestors to the EMRSA-16 variant. 7/13 possessed all 5 SNPs including 6 from 2004 and 1 from 2010.

To confirm that these 7 isolates were MSSA strains, methicillin susceptibility was assessed in two ways. Firstly, the minimum inhibitory concentration (MIC) to oxacillin was determined for each isolate. Oxacillin (a β-lactam) is frequently used as a surrogate marker of methicillin (and β-lactam) susceptibility. According to current guidelines *S. aureus* isolates with oxacillin MIC ≤2mg/L are susceptible to methicillin.[33] All isolates were susceptible with oxacillin MIC ranging between 0.12-0.5mg/L (Table 3.16). Secondly, isolates were assessed for the presence of *mecA* gene using 3 primer sets. As described in Section, 1.1.3 methicillin resistance in *S. aureus* is conferred by the *mecA* gene. Two separate multiplex methods were used involving three sets of *mecA* primers; Multiplex 1 contained two *mecA* primer sets and Multiplex 2 contained one. Results from multiplex 1 and 2 were concordant. Interestingly 2/7 isolates possessed the *mecA* despite being phenotypically susceptible.

Table 3.16 Evaluation of oxacillin minimum inhibitory concentrations (MIC) and presence of *mecA* gene in 7 MSSA t018 isolates possessing the 5 single nucleotide polymorphisms pertaining to the EMRSA-16 variant

Year Collected	Isolate Number	*spa*-type	Oxacillin MIC (mg/L)	Multiplex 1		Multiplex 2
				mecA1	mecA2	mecA3
2004	1	t018	0.5	-	-	-
	2	t018	0.25	+	+	+
	3	t018	0.5	-	-	-
	4	t018	0.5	-	-	-
	5	t018	0.12	-	-	-
	6	t018	0.12	+	+	+
2010	7	t018	0.5	-	-	-

3.11.6 Spread of EMRSA-16 variant

To understand whether the EMRSA-16 variant was unique to Brighton collections of bacteraemia isolates were retrieved from three neighbouring hospitals (regional) and hospitals in separate geographical locations (national). All isolates were *spa*-typed and those of *spa*-type t018 (corresponding to the variant) were evaluated further.

371 *S. aureus* bacteraemia isolates (72 MRSA) were retrieved from regional hospital collections (Table 3.17); 9 were MRSA t018. 8/9 were evaluated by the molecular signature assay and 3 isolates (all from Worthing hospital) possessed the 5 SNPs corresponding to the EMRSA-16 variant.

966 *S. aureus* isolates were retrieved from 5 hospitals across England (Table 3.18) including two hospitals (Devon and Exeter) from the same trust in South West England. Following *spa*-typing 13 t018 isolates (9 MRSA) were identified from 3 hospitals; collections from Exeter and Liverpool did not contain any t018 strains.

45 t018 isolates (39 MRSA) were retrieved from the Staphylococcal reference unit (Public Health England) to represent isolates from 9 UK regions (Figure 3.18). These included 7 bacteraemia isolates, 15 clinical specimens and 23 carriage isolates.

Isolates from national and Staphylococcal reference unit collections were retrieved following molecular signature assay evaluations and were subsequently not investigated using this assay.

Table 3.17 Summaries of *Staphylococcus aureus* bacteraemia isolates retrieved from regional hospitals; (A) Worthing, (B) Crawley, and (C) Hastings. EMRSA-16 variants were identified by molecular signature assay

A

	Worthing n=179							
Date	**Retrieved**			***spa*-typed**	**t018**		**EMRSA-16 variant**	
	Total n=179	**MRSA** n=37	**MSSA** n=142	n=178	**MRSA** n=6	**MSSA** n=0	**MRSA** n=3	**MSSA** n=0
2007	0	0	0	-	-	-	-	-
2008	33	5	28	32	0	0	-	-
2009	70	20	50	70	2	0	0	-
2010	48	10	38	48	4	0	3	-
2011	7	0	7	7	0	0	-	-
Unknown	21	2	19	21	0	0	-	-

B

	Crawley n=166							
Date	**Retrieved**			***spa*-typed**	**t018**		**EMRSA-16 variant**	
	Total n=166	**MRSA** n=29	**MSSA** n=137	n=165	**MRSA** n=3	**MSSA** n=0	**MRSA** n=0	**MSSA** n=0
2007	10	1	9	10	0	0	-	-
2008	26	3	23	26	1	0	0	-
2009	45	10	35	45	1	0	0	-
2010	79	14	65	78	1	0	0[††]	-
2011	6	1	5	6	0	0	-	-
Unknown	0	0	0	-	-	-	-	-

C

	Hastings n=26							
Date	**Retrieved**			***spa*-typed**	**t018**		**EMRSA-16 variant**	
	Total n=26	**MRSA** n=6	**MSSA** n=20	n=26	**MRSA** n=0	**MSSA** n=0	**MRSA** n=0	**MRSA** n=0
2007	0	0	0	-	-	-	-	-
2008	0	0	0	-	-	-	-	-
2009	0	0	0	-	-	-	-	-
2010	23	6	17	23	0	0	-	-
2011	3	0	3	3	-	0	-	-
Unknown	0	0	0	-	-	-	-	-

[††] Not evaluated by molecular signature assay

Table 3.18 Summary of national isolates retrieved from five English hospitals including Oxford, University College London Hospital (UCLH), Plymouth, Exeter, and Liverpool and a collection from Public Health England (PHE)

	National Isolates								
Collection	Total isolates	Bacteraemia	Source Clinical	Carriage	Organism MRSA	MSSA	*spa*-typed	t018 MRSA	MSSA
Oxford (2008-2011)	567	453	114	0	100	467	562	3	0
UCLH (2008-2011)	169	169	0	0	40	129	156	6	3
Plymouth (2008-2011)	85	85	0	0	10	75	75	0	1
Exeter (2008-2011)	98	98	0	0	12	86	98	0	0
Liverpool (2008-2011)	47	47	0	0	12	35	46	0	0
PHE (2009-2012)	45	7	15	23	39	6	45	39	6
TOTAL	**1011**	**859**	**129**	**23**	**213**	**798**	**982**	**48**	**10**

3.11.6.1 Evaluation of regional and national t018s by whole-genome sequencing

All available t018 isolates from Brighton, regional and national collections underwent WGS to assess whether the higher resolution offered could inform the presence of strains highly related to the EMRSA-16 variant. In total 173 t018s were prepared for sequencing; 93 MRSA t018s isolates from Brighton (as described in Section 3.11.2), 13 MSSA t018 isolates identified in Brighton (as described in Section 3.11.5), 9 regional MRSA t018s and 58 national t018s isolates (including 10 MSSA). WGS data were available on 171 isolates as two failed library preparation.

SNV differences were determined through pairwise comparison. Of 171 isolates 14,535 pairwise comparisons were assessed; the median SNV difference was 165 SNVs (IQR 77-522). Figure 3.9 is a histogram of pairwise SNV differences. The pairwise difference distribution revealed four peaks including one showing highly divergent pairs (>10,000 SNV differences) and one showing highly related pairs (<100 SNV differences). The multi-modal pattern was similar to results observed in Figure 3.5 and suggests a diverse population structure within this lineage.

To investigate the relationship of these isolates further a maximum likelihood tree was created (Figure 3.10). The topology was strikingly similar to the EMRSA-16 maximum likelihood tree in Figure 3.6 with two dominant features. Firstly, four distinct long branches were observed emanating out from a central cluster representing groups of highly divergent isolates. This suggests that the natural population structure of EMRSA-16 comprises genetically divergent strains. As recombination events could also explain the observed diversity a manual inspection of variant sites was performed to identify areas of consecutive SNV differences suggestive of recombination. Variant sites appeared distributed throughout the genome.

Secondly, a single cluster of highly related isolates was observed in the centre of the tree (red box in Figure 3.10). This included 65 isolates that exhibited low genetic diversity with a median SNV difference of 28 (IQR 20-38) between isolate genomes. The maximum genetic diversity between isolates across the cluster was 100 SNV. The cluster included 57 isolates from Brighton (including 20 previously identified as EMRSA-16 variant by WGS and 37 isolates from 2004-1010), 4 from regional hospitals (3 Worthing and 1 Crawley) and 4 from national hospitals (1 UCLH, 2

south west HPA region, 1 northwest HPA region). These include isolates from 2004-2011, representing a timeframe spanning the rise and fall of the MRSA bacteraemia outbreak in Brighton. All isolates within this cluster were methicillin resistant except for four that were retrieved from Brighton in 2004.

These data suggest that EMRSA-16 has a highly diverse population structure and within this a cluster of highly related isolates was observed. This cluster included isolates previously identified as the Brighton EMRSA-16 variant. WGS also identified isolates from Brighton from before and following the outbreak that were highly related to the variant, including 4 methicillin susceptible strains that preceded the outbreak. In addition isolates from hospitals neighbouring Brighton and from trusts across England were also identified as being genetically highly related to the variant.

Figure 3.9 Diversity of 171 t018 *S. aureus* isolates from Brighton, regional and national collections. Single nucleotide variants are shown on a logarithmic scale.

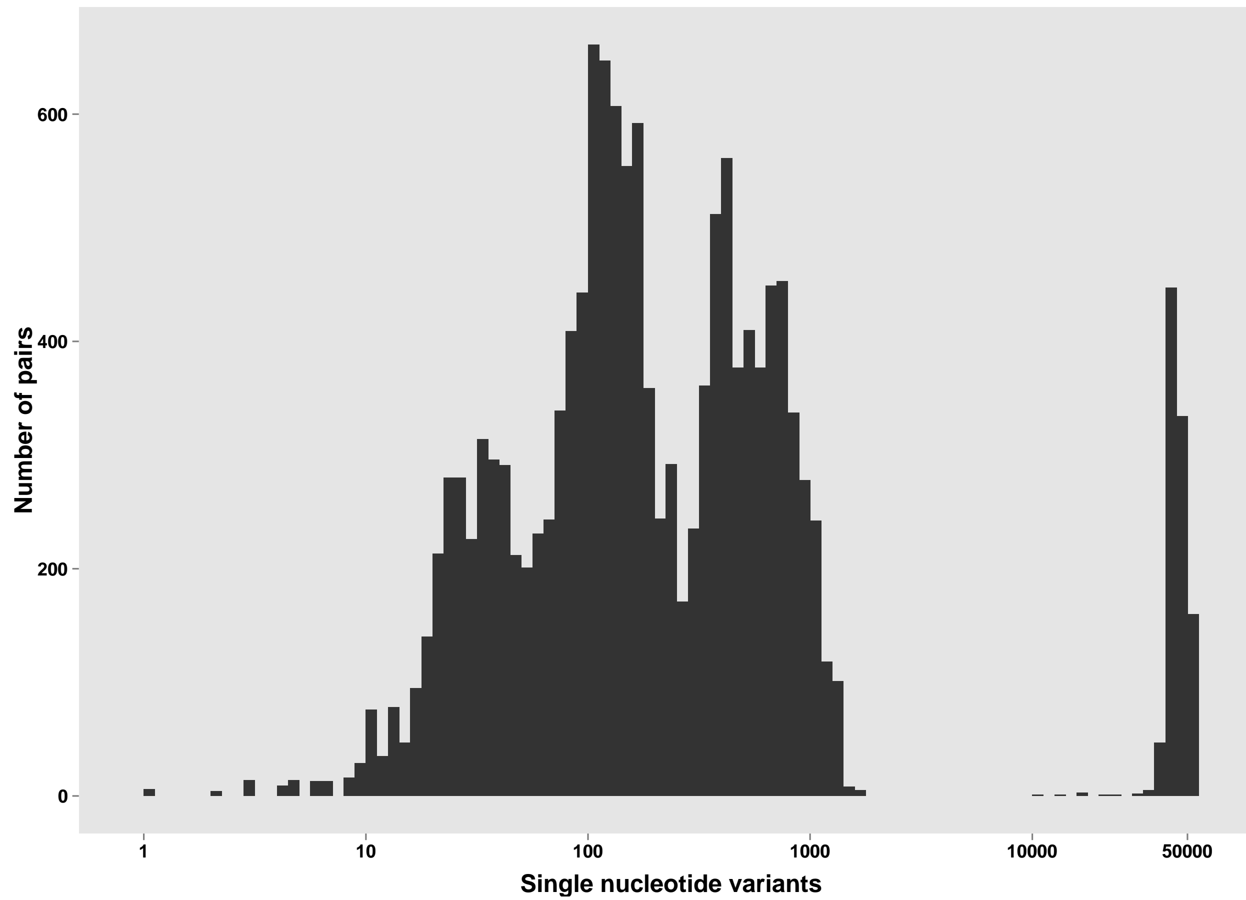
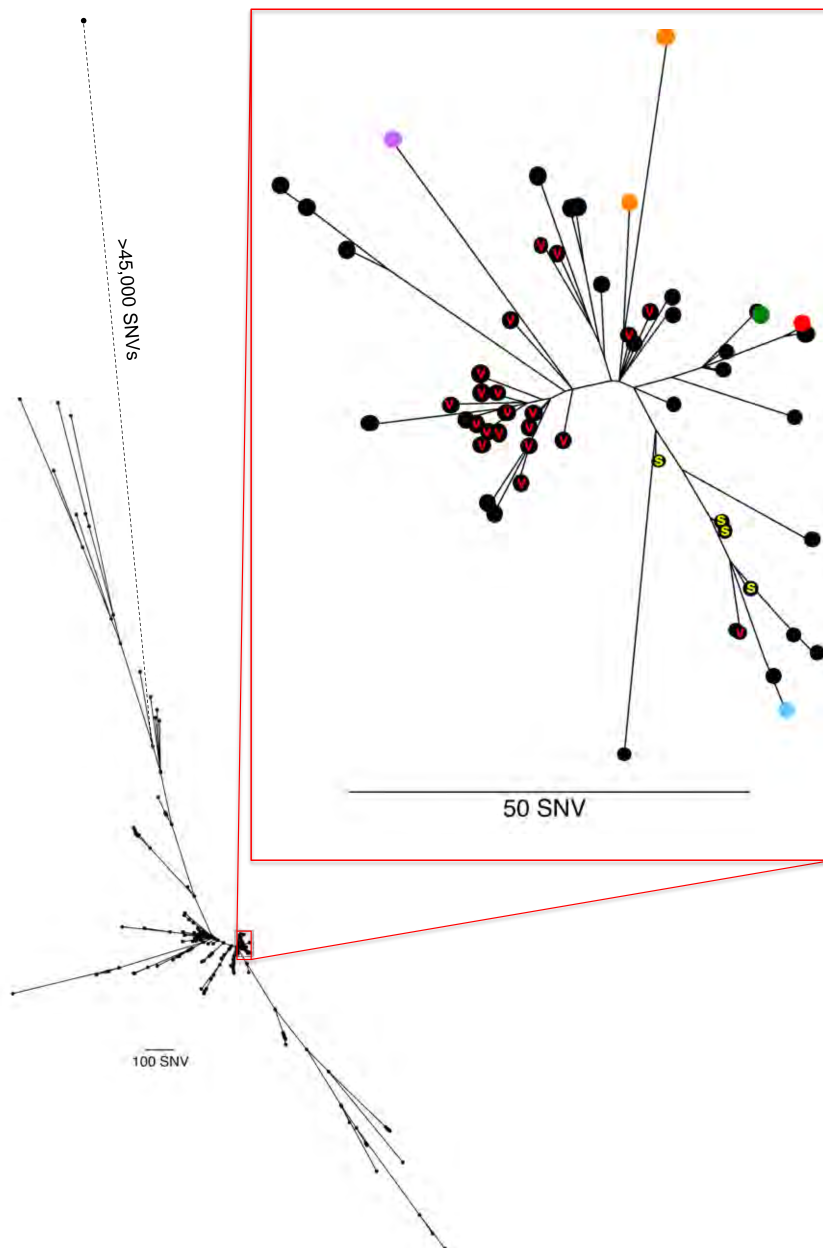


Figure 3.10 Maximum likelihood tree of 171 t018 isolates from Brighton, regional and national collections. Nodes depict isolates and edges represent single nucleotide variants between nodes; solid black edges are to scale and dotted edges are scaled down to include highly divergent isolates. A cluster of 65 highly related isolates is enlarged in the red box. Nodes are coloured by location; Brighton (●), Worthing (●), Crawley (●), UCLH (●), South West (●), North West region (●). Brighton isolates previously identified as EMRSA-16 variant are annotated (V). All isolates are MRSA except four methicillin susceptible strains (S).



3.12 Discussion

Nosocomial infection rates, particularly those for MRSA bacteraemia, are regarded as key indicators of infection control effectiveness.[289,290] Not only do nosocomial infections have significant impact on patient outcomes, but high rates may also adversely affect reputation, leading to loss of revenue, and further declines in quality.[291] The emergence of novel pathogen strains can dramatically influence disease epidemiology.[265,266] A challenge with clonal organisms such as *S. aureus* is that conventional methods may fail to detect the emergence of novel variants within major lineages, although it is plausible that such variants could influence rates and severity of disease. Advances in microbial WGS make it possible for the first time to detect variants of epidemic MRSA strains and assess their impact.[266]

In Brighton, rates of MRSA bacteraemia increased dramatically between 2004-07. In order to investigate this data were retrieved on patient characteristics and clinical management that failed to identify an explanation for the outbreak. To assess putative microbial factors bacteraemia isolates were retrieved from before and after the outbreak. Comparison of isolates using conventional typing methods failed to identify bacteriological factors associated with the outbreak, although changes in lineages associated with disease was suggested. To investigate this further isolates were compared using WGS. The higher resolution offered by WGS revealed the emergence of a clone within a lineage dominant in UK hospitals (EMRSA-16) that corresponded to the outbreak. Clinical and biochemical data were collected to evaluate whether the clonal variant displayed a distinct phenotype. In response to these data additional isolates were retrieved from regional and national hospitals to evaluate the uniqueness of the clonal variant to Brighton.

There were three key findings from this work. Firstly, WGS had the resolution to reveal the emergence of a novel clone within a dominant lineage (EMRSA-16 variant) that corresponded to the Brighton MRSA bacteraemia outbreak. Secondly, the EMRSA-16 variant caused bacteraemia throughout the hospital (particularly the ICU) and was associated with a heightened white cell response. Thirdly, WGS identified isolates highly related to the EMRSA-16 variant present in regional and national hospitals following the outbreak.

3.12.1 Part 1: Evaluation of outbreak using conventional methods

The first part of the study evaluated the MRSA outbreak using three conventional approaches. Firstly, as described in Section 1.2.2 certain patient groups are at higher risk of developing *S. aureus* bacteraemia including those with underlying conditions such as HIV, malignancy, diabetes, rheumatoid arthritis, heart disease and patients receiving renal replacement therapy.[13,70-72] To determine whether changes in patient populations contributed towards the outbreak patient characteristics were evaluated from three time points before and during the outbreak. Patients acquiring *S. aureus* bacteraemia across these three time frames showed comparable characteristics suggesting that changes in patient groups were not associated with the observed rise in MRSA infections. This was supported by the fact that no new clinical services were set up prior to the outbreak that could perceivably increase the proportion of patients at risk of *S. aureus* infection (e.g. haemodialysis unit, diabetes clinics).

Secondly, during the outbreak it was observed by doctors caring for patients with *S. aureus* bacteraemia that rates of secondary site infections and mortality were higher than previously experienced within the same trust. To evaluate these observations detailed clinical information was prospectively collected on a sub-set of 100 patients. Results showed that during the outbreak rates of complicated disease (27%) and 30-day mortality (39%) were higher than reports from other studies.[5,292] Thwaites *et al.* (2012) undertook a detailed evaluation of *S. aureus* bacteraemia in twelve hospitals including eight in the UK and four in South East Asia.[293] The authors showed rates of metastatic disease to be 11% and an overall morality rate during admission of 24%. In response an evaluation was undertaken to assess whether sub-optimal clinical management accounted for these observations.

The importance of patient and management factors in determining outcome of *S. aureus* bacteraemia is well established.[294,295] In this study the development of complicated disease was associated with delay in focus removal. Previous studies have shown that failure to remove the focus of *S. aureus* infection is an independent risk factor for treatment failure.[42,296,297] Furthermore receipt of effective antibiotics > 48hours after onset of symptoms was associated with the development of complicated disease. This finding supports Lodise *et al*. (2003) who showed that

delayed effective antibiotic therapy was an independent predictor of infection-related mortality in 167 patients with hospital-acquired *S. aureus* bacteraemia.[298]

Current guidance recommends a minimum of two weeks therapy based on few studies suggesting secondary complication rates are low following 10-14 days treatment.[299-301] Interestingly in this study duration of therapy <2 weeks did not affect outcome. This contrasts with previous studies finding that intravenous therapy for <2 weeks is associated with metastatic infections and relapse.[42] While our sample size is too small to exclude the possibility of adverse outcome being associated with short course treatment our findings are in keeping with other work suggesting that shorter courses may be effective.[296]

Complicated disease and 30 days all-cause mortality were higher at BSUH during the outbreak compared to rates of complicated disease (up to 11%) and mortality (up to 30%) observed in other studies.[5,293] Whilst some clinical management factors were associated with poor outcome the data suggest that clinical practice in Brighton appears broadly within the range of practice in other UK hospitals.[293] In addition bacteraemia caused by methicillin resistant strains was associated with significantly higher 30-day mortality rates; similar observations have been found in other studies.[5,292,302]

To evaluate whether the excess of disease was associated with the emergence of a novel strain bacteraemia isolates were retrieved from before and during the outbreak. Three conventional typing methods were used to type strains and allocate them into corresponding lineages. Whilst discrepancies in lineage allocation were observed in 9 isolates (4%) a study by Hallin *et al.* (2007) showed that 12% of strains typed using *spa*-typing and MLST resulted in discordance of the assigned type.[303] Conventional methods failed to detect a new strain and showed that the nationally prevalent EMRSA-15 and EMRSA-16 strains caused 92% of bacteraemias.[304] However, a curious finding was that although no new lineage appeared EMRSA-16 dominated in Brighton at a time when EMRSA-16 rates were falling nationally in relation to EMRSA-15.[277] Although failure in infection control was possible, it seemed unlikely since it would have most probably resulted in an increase in all bacterial strains.

Strain acquisition of novel virulence factors has the potential to increase the virulence of an organism.[269] However, while a wide range of bacterial toxin and virulence factors are variably present in *S. aureus*[305] the relationship between strain variation and outcome of *S. aureus* infection is not clear. Peacock *et al.* (2002) reported an association between seven variable toxin and adhesion genes and invasive disease[270], while Lindsay *et al.* (2006) found no association between putative virulence genes and invasive disease using a microarray approach.[12] To assess the possibility of newly acquired virulence factors causing excess disease in Brighton isolates retrieved at the time of the outbreak were assessed for the presence of four virulence factors transferrable on mobile genetic elements. Adjunctive genotyping failed to identify associated putative virulence factors, with prevalence of PVL (1.6%), *tst-1* (43%), *sea* (35%), and *sej* (2.1%). The presence of *tst* gene was strongly related to CC30 (EMRSA-16), which is consistent with previous studies.[12,270,306] However in contrast to the findings of Holtfreter *et al.* (2007) who identified 93.3% of *sej* gene positive bacteraemic strains in CC8 and 6.7% in CC45, in BSUH isolates *sej* was found only in isolates of CC5.[306] These findings uphold their hypothesis that superantigen genes have a strong association with clonal complex, and the most likely explanation for the lineage-specific differences relates to geographical variation. But changes in genotype did not explain the outbreak.

In looking at genotype this study combined robust prospectively gathered data with the less complete retrospectively gathered data set. While not being ideal this had allowed us to more confidently exclude a major role for bacterial genotype in determining outcome and is unlikely to have introduced any significant element of bias.

3.12.2 Part 2: Evaluation of outbreak using whole-genome sequencing

To evaluate bacteriological factors in more detail the higher resolution offered by WGS was applied to investigate the three-year hospital outbreak of MRSA bacteraemia. WGS alone demonstrated the emergence of a clonal variant of an epidemic MRSA lineage (EMRSA-16) that was undetectable using conventional genotyping. The appearance of the variant coincided with the onset of the outbreak in 2004, and accounted for virtually all EMRSA-16 isolates by the peak of the

outbreak. MRSA bacteraemia rates in Brighton began to decline in 2007 (Figure 3.1), possibly as a result of intensive infection control measures, although no specific additional intervention was made.

The variant caused bacteraemias in patients of all ages and throughout the hospital, although it was most prevalent in the intensive care unit. Bacteraemias caused by the variant were associated with higher neutrophil counts than other MRSA bacteraemias. There are several possible explanations for this since the neutrophil response to an infection depends on the immune status of the patient and the source of the infection. However, it is notable that neutrophil count has recently been reported as a biomarker for detecting the emergence of severe *Clostridium difficile*.[307] In this study a limitation of determining a clinical phenotype is that only partial data were available. Furthermore, due to the relatively low numbers of isolates comparisons were made between EMRSA-16 variants and all other isolates, including isolates of different lineages. Virulence factor repertoires are conserved within lineages but vary between lineages[12]. Consequently, it is likely that clinical phenotypes will differ between lineages. To date there is no published literature comparing clinical phenotype of EMRSA-15 and EMRSA-16. Hsu *et al.* (2007) compared all-cause mortality and rates of complicated disease in patients with *S. aureus* bacteraemia caused by two endemic strains; EMRSA-15 (ST22) and ST239.[308] Whilst the authors failed to show a difference between strain type and outcome their study was limited by a small sample size and strain identification methods. Further data collection is required to validate differences in clinical phenotype within lineages.

Suboptimal infection control practice in Brighton might have led to the emergence of the variant and the founder effect could be evoked to explain the expansion of a quasi-randomly selected strain that was not typical of the ancestral population. Although poor infection control practice may have contributed to the outbreak, it is unlikely that this would preferentially favour a particular strain. Hence, this cannot be the sole explanation for the observed expansion of a single clone. Furthermore, it seems unlikely that a single MRSA lineage would have expanded across the entire hospital by chance, outcompeting other prevalent MRSA lineages. In contrast, a lapse in infection control specific to a single unit is not compatible with the presence of the variant across the hospital. Staff were not screened for MRSA during the

period of the outbreak, so the possibility of one or more healthcare workers distributing the variant cannot be excluded. Further studies of the role of healthcare workers in nosocomial transmission of *S. aureus* are warranted.

One explanation for the emergence of the variant is that one or more genomic innovations may have conferred a selective advantage leading to the clonal expansion. Although these data do not definitively demonstrate that the clonal variant caused the outbreak, the counter explanation that it was a feature of the outbreak also indicates a transmission advantage of the variation within it. Relatively small fitness advantages may have a substantial impact on transmission, might be specific to a local environment and could relate to several aspects of bacterial ecology, for example, the reported interaction between microbial genetics and infection control practice in the selection of chlorhexidine resistance in MRSA.[309,310] Other studies have demonstrated the impact of new clones on the epidemiology of *S. aureus* infection within specific hospital units.[241,266] As WGS enters clinical practice we will come to understand how often new clonal variants emerge within healthcare settings and how often this is associated with clinically significant changes in disease phenotype or transmission. The failure to identify a specific genetic explanation for the emergence of the variant is consistent with reports describing the difficulty of establishing a causal link retrospectively.[235,311] The potential relationship between mutations in *ebh* or the recombination events and higher neutrophil counts in bacteraemia caused by the clonal variant requires further investigation.

Whilst t018 strains (corresponding to EMRSA-16) frequently cause MRSA bacteraemia in the UK, invasive infections caused by MSSA t018 are less commonly observed.[288] In light of the emergence of an MRSA clonal variant of *spa*-type t018 the identification of 13 MSSA t018s causing bacteraemia prompted further assessment. Methicillin susceptibility was confirmed by two methods; (i) establishing oxacillin MIC and (ii) evaluating the presence of the *mecA* gene. Whilst all isolates were phenotypically susceptible two strains possessed the *mecA* gene. Plausible explanations include the down regulation of the *mecA* gene following long-term frozen storage, or the presence of a heterogenous population of strains containing those positive and negative for *mecA*. It appears that long-term storage can affect the *mecA* gene. van Griethuysen *et al.* (2004) reported that 14.4% of MRSA isolates lost their *mecA* during storage at -80°C over a two year period.[312]

Evaluation using the molecular signature assay showed that 7 MSSA t018s possessed the 5 SNPs pertaining to the EMRSA-16 variant, including 6 from a time when the variant was thought to arise (c.2004). It is important to note that the molecular signature assay was designed and validated for MRSA strains. As the SNPs were not located in, or surrounding, the SCC*mec* gene there is no reason that this would affect its use with methicillin susceptible strain. A plausible explanation is that these isolates represent methicillin susceptible ancestors of the MRSA variant. Furthermore this is supported by maximum likelihood estimates showing four MSSA isolates highly related to the previously identified variants. To evaluate this further phylogenetic analysis is required to compare genetic and temporal relationships. If these methicillin susceptible isolates correspond to ancestral branches to the MRSA variant then future work would include genomic interrogation to understand whether the acquisition of SCC*mec* had an impact of driving the emergence of the clonal variant.

To assess the exclusivity of the EMRSA-16 variant to Brighton an evaluation of a large collection of isolates from regional and national hospitals was undertaken. WGS revealed a cluster of 65 isolates, including 20 isolates previously identified as EMRSA-16 variant and 45 highly related isolates present in Brighton and hospitals across England following the outbreak. As regional and national collections were retrieved following the Brighton outbreak it remains unclear whether the presence of highly related isolates across the country represents the natural phylogeny of the variant in the UK or if this signifies spread from Brighton. As Brighton is a regional tertiary referral centre it is plausible that the presence of highly related isolates in regional hospitals resulted from spread from shared patient populations, for example renal dialysis patients. Clinical data were unavailable from regional and national hospitals. The presence of related strains in a London hospital (UCLH) and hospitals in the south west and north west of England remain less clear. *S. aureus* transmission over large geographical distances has previously been observed. Harris *et al.* (2010) used WGS to identify intercontinental spread of *S. aureus*, revealing a two year outbreak in a London hospital likely resulted from a single transmission event from Thailand.[234]

The time frame from Brighton outbreak (2004-2007) and isolate collections from regional (2007-2011) and national (2008-2012) collections ranges from 3-8 years.

According to the current rates of *S. aureus* evolution (~1 SNV every 5 weeks[234,235,237,238]) natural strain evolution would account for diversity (up to 83 SNVs) observed between strains. The maximum diversity across isolates in the cluster was 100 SNVs. Whilst some isolates exhibited greater diversity than might be expected as part of natural evolution it is important to note that the current molecular clock is calibrated for carriage strains; these isolates are bacteraemia samples. It remains unclear whether the clock ticks faster in disease states and further work is needed to characterise this. Consequently transmission from Brighton to other hospitals remains a plausible explanation for the geographical distribution of these related strains. The method of transmission remains unclear although one could speculate spread occurred via patients, healthcare workers or hospital visitors.

It could be hypothesised that the EMRSA-16 variant had an intrinsic ability to spread throughout BSUH, causing the outbreak of disease observed between 2004-2007. If this were the case then one might expect the same to happen if the same strain entered another hospital; this was not observed. Rates of bacteraemia in hospitals where strains highly related to the variant were identified did not have the same increase in bacteraemia rates as observed in Brighton (according to HPA data). One explanation could be that genomic events selected against a transmissible phenotype, although this is unlikely to have occurred across all isolates in hospitals outside of Brighton. Conversely there may have been something unique to Brighton that permitted rapid spread within a single hospital, such as a single visitor or healthcare worker acting as a 'super-shedder'.

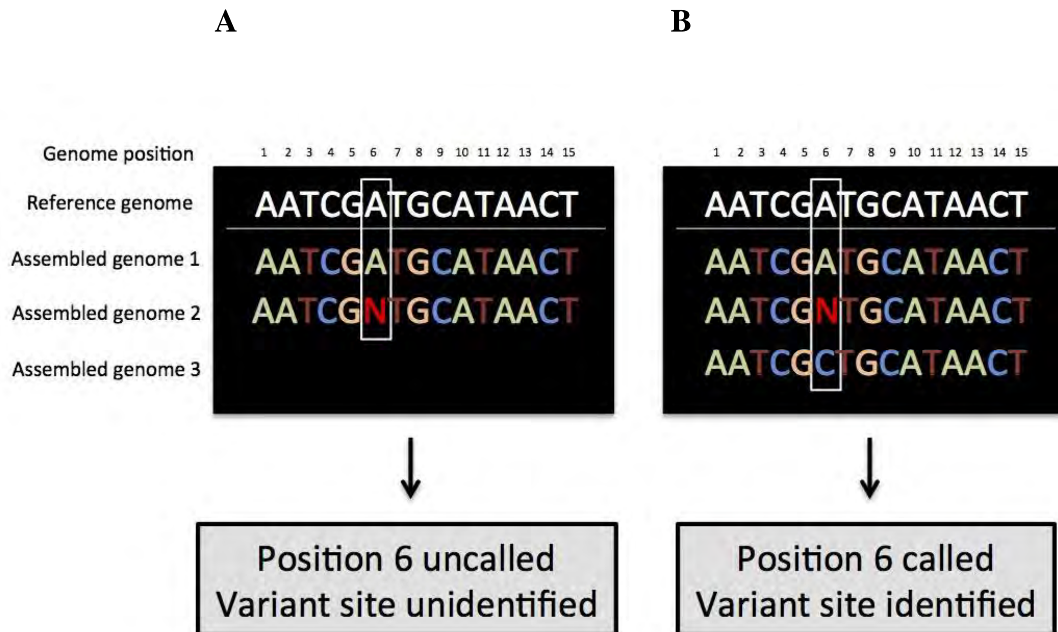
The highly related cluster of 65 t018 isolates contained 20/22 previously identified EMRSA-16 variant isolates; the remaining two lay on separate branches of the tree. This was unexpected as previous work revealed that isolates corresponding to the EMRSA-16 variant were highly related (<22 SNVs). The most likely explanation relates to the variant calling methods used. The ability to identify variant sites between genomes is dependent on the number of sites called (or visible) and subsequently available for comparison. An uncalled site in one genome cannot be compared to the same (called) site in another genome (Figure 3.11a). When this occurs these sites are assumed by the variant-calling program to be the same and, hence, if these are truly variant sites this diversity will be missed. Comparison of

additional genomes increases the likelihood of a variant (if present) being called at a particular site and, hence, the true diversity is revealed (Figure 3.11b).

3.13 Future work

Methicillin susceptible t018 strains retrieved from 2004 were highly related to the EMRSA-16 variant. Currently it remains unclear whether they represent methicillin susceptible ancestors to the clonal variant. Phylogenetic analysis will form part of future work to address this by evaluating genomic coupled with temporal relationships. Furthermore there is the potential to assess the relative virulence of the EMRSA-16 variant through use of *in vitro* and *in vivo* models.

Figure 3.11 A diagrammatic representation of variant calling. (A) shows that at a given site an uncalled base in one genome cannot be compared with a called base in the same site in another genome, hence potential variation is missed. (B) shows that additional genomes may reveal diversity by increasing called variants at a given site.



3.14 Conclusion

In this chapter I have shown that:

- WGS revealed the emergence of a clonal variant within a dominant lineage that corresponded with an MRSA outbreak and was invisible by conventional typing methods (main objective). This observation confirms our primary hypothesis.
- The EMRSA-16 variant was present throughout the hospital in Brighton (particular the ICU) and was associated with higher white cell count host response (objective 1)
- Isolates highly related to the EMRSA-16 variant were present in Brighton (before and after the outbreak) and in other hospitals across the country (objective 2)

Microbial WGS is providing insights into the national and international spread of MRSA lineages.[234,237,243] This technology is entering clinical practice and has the potential to be applied rapidly enough to influence patient care.[234,241] This study demonstrates the emergence of a clonal variant within a nosocomial epidemic MRSA lineage associated with an outbreak. If, at the time of the outbreak, WGS had been used for routine surveillance, the novel variant would have been recognised, allowing targeted investigation of transmission routes and sources and the initiation of evidence-based control measures. This study demonstrates the potential of WGS to improve understanding of the epidemiology of MRSA within hospitals.

4 Patient-to-patient transmission of *Staphylococcus aureus*; a systematic evaluation using whole-genome sequencing

4.1 Introduction

Efforts to reduce *S. aureus* infections focus on preventing patient-to-patient transmission through screening, decolonisation and infection control measures.[189,313] In England for example, screening to identify patients colonised with Methicillin Resistant *S. aureus* (MRSA) has been mandatory since 2006. Nevertheless, patients still acquire *S. aureus* in hospital and rates of infection by methicillin sensitive (MSSA) strains have fallen much less than MRSA strains.[314,315]

spa-typing is commonly employed by reference laboratories to investigate epidemiological trends of *S. aureus* infections and evaluate putative nosocomial transmission networks. The population structure of *S. aureus* in hospitals is dominated by strains of few dominant lineages which account for the majority of infection.[12] Whilst *spa*-typing allocates strains into relevant lineages it lacks the resolution necessary to discriminate within these lineages and hence fails to reveal detailed patterns of transmission.[224,316] Furthermore single base pair changes within the *spa* gene result in allocation to different *spa*-types, despite the strains being highly related.[211] Failure to identify highly related isolates can plausibly result in transmission donors being missed. Although not widely available, clusters of related *spa*-types (*spa*-group) can be determined using a computer programme that infers the relatedness between *spa*-types by allocating a 'cost' based on the type of diversity between the two *spa*-types.[210] The application of this in interpreting transmission events remains unclear.

Whole-genome sequencing (WGS) can reveal the genetic relatedness of isolates at a far greater resolution than conventional techniques, at a cost that will soon be similar and in comparable processing times. WGS can determine if two isolates are truly identical and if not by exactly how much they differ. In terms of transmission when donor and recipient genomes are genetically indistinguishable you can be confident that a transmission event has occurred. Conversely wide genetic variations support no recent transmission. In cases where putative donor and recipient genomes have

subtle genetic differences a clear understanding of the evolutionary rate and within host diversity is needed to determine whether a transmission event has occurred. Firstly, the evolutionary rate of *S. aureus* allows the likelihood of short-term transmission to be inferred. *S. aureus* clones evolve primarily through point mutation, accumulating single-nucleotide variants (SNV)s over time.[14] Recent estimates put the rate of mutation between 2.0-3.4 $x10^{-6}$ mutations per site per year.[234,235,237,238] This equates to 5.6-9.5 mutations/year over the whole genome or around one SNV difference every 5-10 weeks. These suggest that there is very little evolutionary diversity in direct short-term transmission events. Secondly, an understanding of the diversity within a host provides a threshold to rule out putative donors. Golubchik *et al.* (2013) reported that the maximum diversity within a colonized individual is 40 SNVs, suggesting that donor and recipient isolates could differ by up to 40 SNVs and still be involved within the same transmission event.[239] Whilst this provides a threshold for current research these data require validation. WGS has the potential to 'rule in' and 'rule out' transmission events between otherwise indistinguishable isolates. WGS has been applied to the investigation of MRSA outbreaks[241,317] and Eyre *et al*. (2012) have described the practical application of bench-top sequencers in near-to-real time outbreak investigation.[242]

To date the contribution of different hospital sources to *S. aureus* transmission in a non-outbreak setting has not been evaluated using WGS. This chapter describes the first systematic evaluation of nosocomial patient-to-patient transmission of *S. aureus* using WGS. Screening samples from unselected patients admitted to an adult intensive care unit (ICU) were analysed to investigate: 1) the role of colonised patients as the source of identified new *S. aureus* acquisitions; 2) the diversity of *S. aureus* within and between hosts, and 3) the reliability of conventional approaches to identifying patient-to-patient transmission.

4.2 Objectives

The main objectives of the work described in this chapter were to:

1) determine the rate of *S. aureus* acquisition in patients admitted to the ICU
2) investigate the role of colonised patients as a common source of *S. aureus* acquisitions on the ICU

In achieving these objectives the study also set out to:

3) establish the ability of whole-genome sequencing to differentiate carriage strains of *S. aureus* by evaluating within-host and between-host diversity
4) establish the reliability of conventional approaches to detecting transmission compared with whole-genome sequencing

This chapter will firstly outline the methods used, including *spa*-typing and WGS. After this acquisition will be determined using a conventional method approach, *spa*-typing and epidemiological data. Then the additional high-resolution provided by WGS will be used to test conventional methods to inform within-host diversity, diversity between hosts, and the dynamics of patient-to-patient transmission. Isolates relating to a potential MRSA ward-based outbreak will be investigated in further detail using WGS.

4.3 Attributions

Culture, identification and archiving of isolates was performed by Kevin Cole (Research Biomedical Scientist). Dr David Wyllie (PHE, Oxford) wrote the core R scripts for admission maps. Evaluations of read mapping using BLAST analysis was performed by Dr Daniel Wilson (University of Oxford). All other work presented in this chapter was undertaken by James Price.

4.4 Methods

4.4.1 Patients and setting

Brighton and Sussex University Hospital (BSUH) is an acute teaching hospital on the south coast of England. The study was conducted on the hospital's 16 bed, adult medical and surgical ICU between 1st January 2010 and 28th February 2011 (14 months). The ICU consists of one 5-bedded area, one 4-bedded area, 3 double side rooms, and 1 single side room. During this time, clinical practice was to screen all patients admitted to ICU for MRSA at admission and weekly thereafter. During the study period retrieved routine screens were cultured for all *S. aureus* strains. Admission screens are taken within 24hours of admission to ICU. Screens consisted of a single nasal swab and in most cases swabs from the perineum with additional samples sent in some patients of groin, sputum, urine and from wounds. From May 2010 patients were also screened at time of discharge. All patients received antibacterial skin washes (Chlorhexidine) while on the ICU and patients identified as carrying MRSA received adjunctive antibacterial nasal ointment (mupirocin).

Anonymised demographic details and hospital stay data were collected from patient records. Maps of patient ICU admissions were created using a data visualisation package (R ggplot2 v0.9.0).

4.4.2 Definitions

4.4.2.1 Acquisition

Acquisitions were determined using two methods. Firstly, *S. aureus* acquisition was defined as either (i) culture-negative admission screen, then *S. aureus* cultured from a follow-up sample or (ii) any screen positive for *S. aureus*, then cultured a different strain (by *spa*-type, methicillin sensitivity or a SNV difference of >40 that being the maximum reported within host diversity[239]) on a follow-up sample.

4.4.2.2 Acquisition rates

Acquisition rates were determined by evaluating the 'time at risk' of acquisition in the 'at-risk' population. The 'at-risk' population was any patient with >1 screen taken. 'Time at risk' was defined as length of time from first to final swab,

representing the time period an acquisition could be identified. In those who acquired *S. aureus* the at-risk time was from first swab to day of first acquisition.

4.4.2.3 Patient-to-patient transmission

Likely patient-to-patient transmission was defined as the acquisition of a *S. aureus* strain whose genotype and methicillin susceptibility matched those of a strain cultured previously from a colonised patient with overlapping ICU stay.

For likely patient-to-patient transmission identified using conventional typing methods matching genotyping was defined as isolates of the same *spa*-type. For patient-to-patient transmission identified using WGS a matching genotype was defined as isolates with <40 SNVs different in their genomes.[239]

4.4.3 Identification and typing

All available isolates from all swabs from all sites from all patients underwent culture, identification, *spa*-typing and WGS.

4.4.3.1 Microbiology

All screening swabs were cultured for *S. aureus* by inoculation onto chromogenic MRSAselect™ (Bio-Rad, Redmond, USA) and Columbia CNA (Oxoid Ltd, Basingstoke, UK) agar plates. These were incubated at 35-37°C in air for 18 hours. Presumptive *S. aureus* were confirmed with PROLEX™ Staph Xtra Latex Kit (Pro-Lab Diagnostics, Cheshire, UK) and Microflex™ series MALDI-TOF (Bruker Daltonics). Antibiotic susceptibilities were determined by disk diffusion.[33]

4.4.3.2 *spa*-typing

spa-typing was performed according to published criteria.[208] As described in Section 2.6, the X region of the *spa* gene was amplified by PCR using published primers and PCR products were purified using Agencourt AMPure XP beads (Beckman Coulter, London, UK). Following sequencing products were purified using Agencourt CleanSEQ beads (Beckman Coulter, London, UK). Sequencing was performed on an ABI 3730 DNA Analyzer (Applied Biosystems, USA). *spa*-types were determined using Ridom StaphType (Ridom GmbH, Germany).

4.4.3.2.1 spa-grouping

As described in Section 2.6 one limitation of *spa*-typing is that isolates can be genetically highly-related but be allocated into different *spa*-types. Consequently *spa*-type can miss putative donors involved in transmission events. In order to provide an understanding of potential donors that may be missed by using *spa*-type alone, clusters of related *spa*-types (*spa*-groups) were identified using Based Upon Repeat Pattern (BURP) algorithms.[210] As described in Section 2.6 this method allocates a value (cost) denoting the genetic relatedness between different *spa*-types. Isolates that have highly related *spa* genes (cost ≤4) are clustered into *spa*-groups and isolates within the same *spa*-group were presumed to be clonally related.

4.4.3.3 Whole-genome sequencing

Cultures were incubated overnight on individual Columbia Blood Agar (Oxoid, Basingstoke, UK). DNA was purified from a 5µl loop sweep of culture growth using QuickGene DNA tissue kits (Autogen, USA) according to manufacturer's instructions.

Bacterial libraries were produced for sequencing at the Wellcome Trust Centre for Human Genetics, Oxford, using Illumina HiSeq2000. As described in Section 2.7, the output (multiple overlapping DNA fragments (or reads) of varying length) was processed using a pipeline developed specifically for bacterial sequence data. To measure the similarity of genome sequences full sets of paired reads were assembled by mapping to a CC30 specific reference MRSA252[264] using Stampy.[260] SNVs were identified across all mapped non-repetitive sites using SAMtools[261] requiring a consensus of at least 75% across all reads and a minimum depth of at least 5 reads.

All available isolates (n=276) underwent WGS and one isolate failed WGS library preparation. As discussed in Section 1.6.6 current sequencing platforms assemble 'near-complete' genomes where between 80-90% of the actual genome is called and visible for analysis.[224] A summary of these data is presented in Table 4.1 but briefly in this study, of the 275 successfully sequenced genomes, a mean of 87% of the MRSA252 reference genome was called. Using mapping-based assembly it is common that not all reads get mapped to the reference genome.[223] This is because genomic elements present in the test strain and absent in the reference genome will not be assembled. The problem can be minimised by using a closely related

reference. In this study a common reference strain (correlating with CC30) was used resulting in a mean of 95% of all reads generated mapped to the reference. Following assembly the confidence to call a base is dependent on the number of reads aligned to that nucleotide site (read depth). The assembly pipeline used in this study requires a minimum read depth of 5 to call a base; a mean read depth of 126 was achieved providing high confidence with base calls.

One isolate had only 13.23% reads mapped to the *S. aureus* reference strain (at an average read depth of 13.3). Following manual inspection the mapped reads spanned the reference genome creating a usable sequence. In order to investigate this unusually high proportion of unmapped reads they were evaluated using a Basic Local Alignment Search Tool (BLAST). In brief, BLAST is an automated computer program that finds regions of similarity between sequences. This technique allows comparison of the unmapped reads with multiple reference genomes at once. Hence it is possible to determine if they were present in a genome of a different organism. BLAST evaluation showed that most unmapped reads aligned to areas within a *Candida tropicalis* reference genome. This was unexpected and suggests probable fungal contamination either during the DNA extraction or library preparation process. This highlighted two points; firstly that it is important to minimise contamination during sequencing preparation, and secondly current genome assembly methods can accurately identify genomic fragments unrelated to the mapping reference. The few remaining unmapped reads may represent diversity in the test *S. aureus* genome that was not present in the *S. aureus* reference genome.

4.4.3.3.1 Determining relatedness

As described in Section 2.7 the relatedness of sequenced isolates was evaluated by determining pairwise SNV differences between all sequenced samples. Estimates of the phylogenetic relationship of isolates were estimated by creating maximum likelihood trees from mapped whole genomes using PhyML.[262]

4.4.3.3.2 Determining diversity

In order to evaluate the ability of WGS to differentiate between carriage strains within and between colonised hosts isolate diversity was assessed.

To validate our selection of 40 SNVs as the threshold to differentiate acquisition from within host diversity we measured the within host diversity of *S. aureus* seen in

the study participants. The diversity present within individuals over time ***within*** sites was assessed by measuring the maximum pairwise genetic differences between isolates obtained from the same individual from the same site (e.g. nose or groin) excluding acquisition isolates. The diversity present within individuals ***between*** sites was assessed by measuring the maximum pairwise genetic differences between isolates obtained from the same individual from different sites at the same time.

To understand the use of WGS to identify transmission events the natural diversity of *S. aureus* carriage strains between individuals was evaluated. This was assessed by measuring the minimum pairwise genetic diversity between isolates obtained within 24 hours of admission to the ICU and any previous admission isolates.

Table 4.1 Sequencing statistics summary for 275 successfully sequenced and mapped isolates

	Median (IQR)	Maximum	Minimum
Reads (n)	3.74 x10^6 (3.43–4.10 x10^6)	5.95 x10^6	2.62 x10^6
Reads mapped (n)	3.56 x10^6 (3.27–3.94 x10^6)	5.85 x10^6	3.87 x10^6
Reads mapped (%)	95.3 (94.2-96.7)	99.4	13.23
Read depth	122.7 (112.6-135.6)	201.7	13.3

4.4.4 Ethics

To achieve the study objectives access was required to data and samples that were already being collected as part of routine clinical practice. Samples and data were only sought that were collected as part of routine clinical management. There was no contact between the research team and patients, and individual patient management was not affected by the study. As a result the study was conducted without individual patient consent under the Statutory Instrument (SI) 1438/Section 60 of the Health and Social Care Act. SI 1438 permits the processing of confidential patient information without consent for specific purposes by persons employed or engaged for the purposes of the health service provided that the processing has been approved by a research ethics committee, and providing that approval has been obtained from the National Information Governance Board.

This work was conducted under the approvals in place for the UKCRC Modernising Medical Microbiology work titled 'Integrating Strain Typing & Database Technologies in Research Service' study which has approval from the Berkshire Research Ethics Committee (Reference 10/H0505/83) and the National Information Governance Board Ethics and Confidentiality Committee (Reference ECC 8-05 (e)/2010) (available at www.nigb.nhs.uk/s251/registerapp). Furthermore all work undertaken through UKCRC modernising medical microbiology has been approved by the Health Protection Agency's National Information Governance Board (NIGB).

Favourable regional ethics committee opinion applied to NHS sites with R&D approval and management permission. Subsequently local R&D approval was obtained from Clinical Investigation and Research Unit, Brighton and Sussex University Hospital NHS Trust (BSUH). Furthermore permission to undertaken the study was sought and gained from the Chief Executive at BSUH (Mr Duncan Selbie, 22nd March 2011).

4.4.5 Statistical analysis

All data were analysed using R (version 2.15.3, RStudio, USA) or SPSS (version 20, IBM®, UK). To describe continuous data, medians and IQR were used as they are not skewed by extreme variables. Rates of carriage were compared using Chi-squared tests. Acquisition rates were analysed using Poisson regression and exact two-sided Poisson tests. Time to event analysis was performed Kaplan-Meier survival times and log rank test. *P* values of ≤ 0.05 were considered statistically significant.

4.5 Results

The study included 1181 ICU admissions (comprising 14 patients already on the unit on 1 January 2010 and 1167 subsequent admissions) involving 1065 patients. At the study end, 12 patients had not been discharged from the ICU. All patients were evaluated as potential donors and for *S. aureus* acquisition. Median age at admission was 64 years (IQR 49-76), 688 (59%) were male and the median length of stay on the unit was 2.6 days (IQR 1.0-5.6).

4.5.1 Carriage at admission

A first screen for *S. aureus* carriage was conducted within 24 hours of admission to ICU in 1109 patients (93.9% of admissions). 1104 (99.5%) had both nasal and extra-nasal swabs taken. 185 patients (16.7%) were found to carry *S. aureus*, 59 (5.3%) MRSA (Figure 4.1). A further 27 patients had a first screen performed more than 24 hours after ICU admission. Of patients culturing *S. aureus* at admission there was no significant difference in carriage rates in patients admitted directly to ICU from hospital admission (≤24hours) and those with >24hours in hospital wards prior to ICU admission (20.9% vs. 18.3%, p=0.48).

4.5.1.1 Serial screening

680 patients had 2 or more samples taken for *S. aureus* carriage during their ICU stay and could be assessed for acquisition (Figure 4.1). Table 4.2 compares the number of screens taken for each admission with the length of admission. The number of screens ranges from 0-19 (median 2; IQR 1-2). Of 1181 admissions 1136 (96.2%) received at least one screen. Of the 45 (3.8%) patients who did not receive a screen during their ICU admission 32 (71%) were admitted to ICU for ≤ 1day. 449/1136 (39.5%) had only one screen despite 15 (3.3%) having an ICU admission > one week.

Figure 4.1 Sampling of patients involved in the study. Including nasal and extra-nasal samples and serial samples *S. aureus* was isolated 329 times as follows [1]206 isolates, [2]8 isolates, [3]115 isolates. *Includes one patient who changed strains twice. ^Includes one patient who experienced two strain changes after acquiring *S. aureus*.

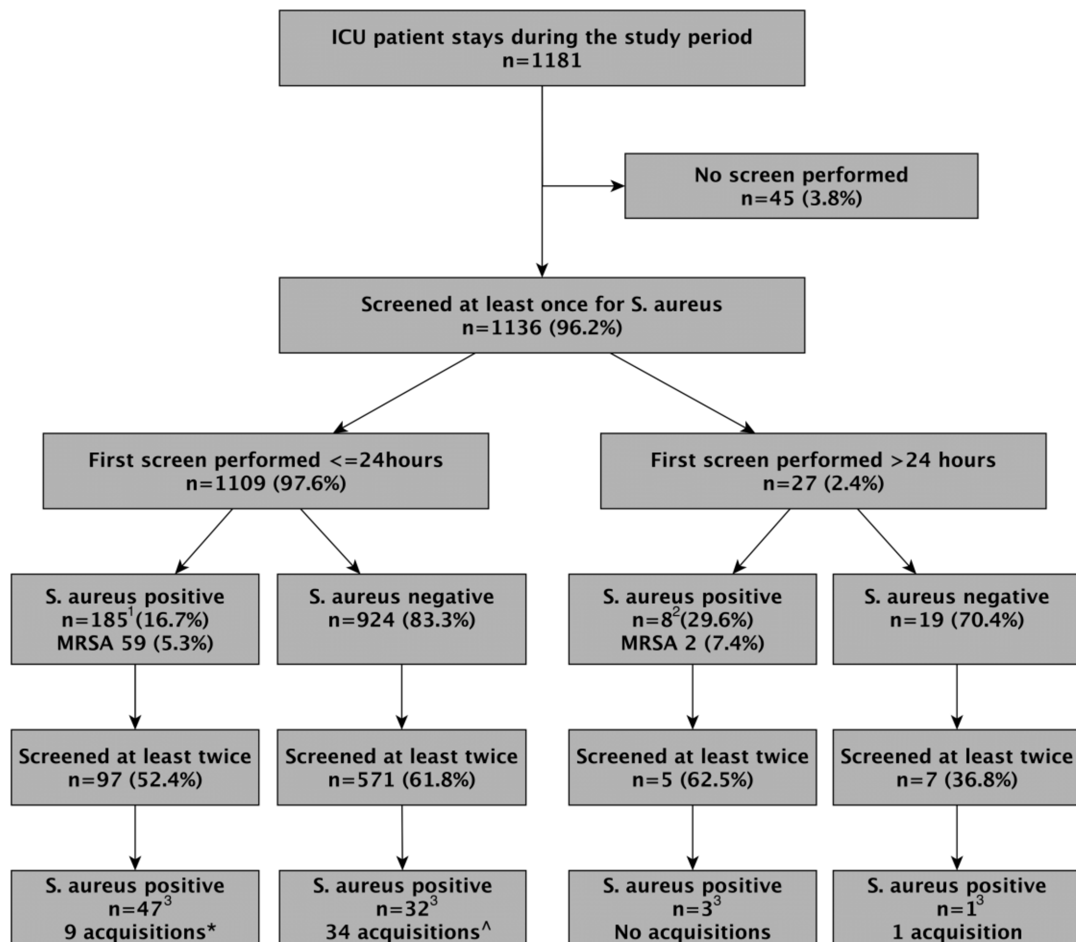


Table 4.2 Routine screens for *Staphylococcus aureus* collected per admission compared with the length of admission time to the intensive care unit

		Screens Collected per admission (n)												Total
		0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**10**	**17**	**19**	
Admission Time (days)	**1**	32	126	49	6	0	1	0	0	0	0	0	0	214
	2	8	121	78	15	1	0	0	0	0	0	0	0	223
	3	3	95	57	23	2	1	0	0	0	0	0	0	181
	4	1	37	52	18	0	0	0	0	0	0	0	0	108
	5	1	26	49	9	3	0	0	0	0	0	0	0	88
	6	0	25	30	18	6	1	0	0	0	0	0	0	80
	7	0	4	18	18	3	0	0	0	0	0	0	0	43
	8-10	0	8	56	24	6	1	0	0	0	0	0	0	95
	11-14	0	6	15	24	8	1	1	0	0	0	0	0	55
	15-21	0	0	5	20	9	5	2	0	0	0	0	0	41
	22-28	0	0	1	2	8	7	3	0	0	0	0	0	21
	29-49	0	1	1	0	2	9	5	9	1	0	0	0	28
	≥50	0	0	0	0	0	0	0	0	0	2	1	1	4
Total		45	449	411	177	48	26	11	9	1	2	1	1	1181

4.5.2 Typing of isolates

Including all samples taken from all patients, *S. aureus* was cultured from 329 samples. Isolates from 53 samples could not be retrieved from storage and 1 failed *spa*-typing and WGS leaving 275 isolates (Table 4.3). Mupirocin susceptibility was tested in all isolates and no mupirocin resistance was identified.

4.5.2.1 *spa*-typing

spa-types were obtained for 275 (96%) isolates. Although 97 unique *spa*-types were identified, certain *spa*-types predominated especially amongst MRSA where three (t032 (38.2%), t018 (19.1%) and t012 (10.3%)) accounted for 66% of all MRSA isolates. MSSA *spa*-types were more diverse with only two (t084 (6.8%) and t012 (5.8%)) comprising more than 5% of the total (Figure 4.2).

4.5.2.2 *spa*-grouping

To assess the relatedness of different *spa*-types *spa*-groups were determined using BURP analysis. 13 *spa*-groups were identified; the largest contained 57 isolates from 12 *spa*-types. 20 *spa*-types were not allocated to a group (singletons) as their sequences differed by a cost >4 compared to all other *spa*-types. 10 were excluded because their *spa* sequences were <5 repeats (Table 4.4).

Table 4.3 Patients screened and swabs processed during the study period. *1 isolate failed preparation for *spa*-typing and whole-genome sequencing

Number of screens	Carriage phenotype	Total Admissions	Number of positive swabs	Number of isolates yielded	Isolates lost
1	Negative	365	0	0	-
	Positive	91	111	89	22
>1	Negative to Negative	545	0	0	-
	Negative to Positive	33	44	38	6
	Positive to Negative	52	55	46	9
	Positive to Positive				
	same *spa*-type	44	102	89	13
	different *spa*-type	7 (6 patients)	17	14*	3
		1136	329	276*	53

Figure 4.2 Distribution of *spa*-types among isolates identified in the study. Number of isolates of each *spa*-type is given and proportion of pie denotes methicillin resistant (red) and methicillin sensitive (blue) *S. aureus*.

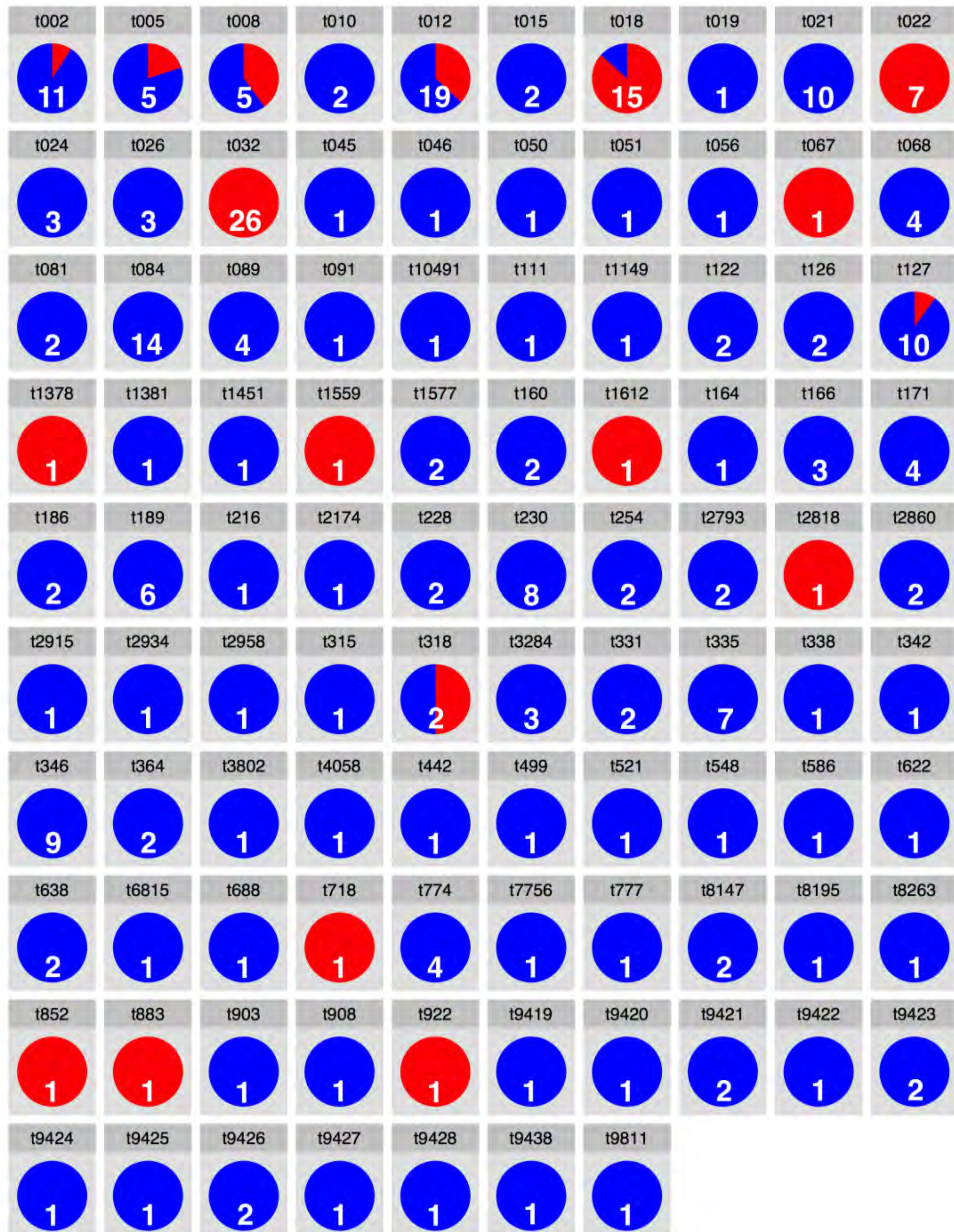


Table 4.4 *spa*-groups of clonally related *spa*-types allocated by Based Upon Repeat Pattern (BURP) analysis. The number of isolates and *spa*-types assigned to each group. Singletons and excluded isolates shown.

***spa*-group**	**Isolates (%)**	***spa*-types (%)**
Cluster 1	57 (20.7)	12 (12.4)
Cluster 2	43 (15.6)	10 (10.3)
Cluster 3	44 (16)	9 (9.3)
Cluster 4	20 (7.3)	9 (9.3)
Cluster 5	8 (2.9)	6 (6.2)
Cluster 6	14 (5.1)	5 (5.2)
Cluster 7	12 (4.4)	3 (3.1)
Cluster 8	4 (1.5)	3 (3.1)
Cluster 9	3 (1.1)	2 (2.1)
Cluster 10	9 (3.3)	2 (2.1)
Cluster 11	6 (2.2)	2 (2.1)
Cluster 12	7 (2.5)	2 (2.1)
Cluster 13	4 (1.5)	2 (2.1)
Singletons	31 (11.3)	20 (20.6)
Excluded	13 (4.7)	10 (10.3)

4.5.3 Acquisition

680 patients had two or more samples taken for *S. aureus* carriage during their ICU stay. These identified 44 acquisitions in 41 patients as follows (Figure 4.3). Thirty-three patients who were negative for *S. aureus* carriage on first screen yielded *S. aureus* from a later screen (patients 1-33 in Figure 4.3). Patient 33 subsequently acquired 2 new *spa*-types sequentially; hence 35 acquisitions in this group. Eight patients colonised with *S. aureus* on admission swab acquired a strain of a different genotype (patients 34-41 in Figure 4.3). Patient 35 acquired 2 strains of differing genotypes; hence 9 acquisitions in this group.

Two acquisitions were detected solely by WGS. The first (patient 40) involved a patient who was colonised with *spa*-type t012 at admission who subsequently acquired a genetically highly distinct (41,518 SNVs) strain but was also *spa*-type t012. The second (patient 41) was a patient colonised with MRSA t032 at admission who acquired a genetically distinct isolate (222 SNVs) of the same *spa*-type two days later.

4.5.3.1 Length of stay

Length of ICU stay in all patients serially screened for *S. aureus* (n=680) was compared between those who acquired *S. aureus* during their admission (n=41) and those who did not acquire *S. aureus* (n=639). Patients acquiring *S. aureus* (median 6.8 days (IQR 4.1-18.8)) were found to have significantly longer ICU admissions (Mann Whitney U test, p=0.004) compared to those patients who did not acquire (median 4.7 days (IQR 2.3 – 8.6)).

4.5.3.2 Time to acquisition

In order to understand whether time spent in ICU affected rate of acquisition date of swabs were used to infer time to acquisition. The overall time from ICU admission to acquisition ranged from 1–54 days with a median 4 days (IQR 2–9). The median time to acquisition was the same (4 days) irrespective of methicillin susceptibility and type of acquisition (new vs. strain change).

The proportion of patients acquiring *S. aureus* at differing time intervals was assessed. 4/44 (9%) acquisitions occur within 48 hours from admission, 30/44 (68.2%) within 7 days, 38/44 (86.4%) within 14 days, and 42/44 (95.5%) within 28 days (Figure 4.4).

Figure 4.3 Sampling histories of 41 patients who acquired *Staphylococcus aureus*. For each patient negative samples (white), methicillin sensitive *S. aureus* positive samples (blue) and methicillin resistant *S. aureus* positive samples (red) are shown. Acquisitions are ordered by type; patients 1-33 were negative to positive status, 34-41 change in genotype. *spa*-types are given for 37 isolates that were available and are therefore missing for acquisitions 1,3,5,9,20,33,34. Patients experienced one acquisition except patient 33 who experienced three acquisitions and patient 35 who experienced two acquisitions. Patient-to-patient transmissions are identified as *transmissions identified by conventional criteria (*spa*-typing and overlapping stay) and †whole-genome sequencing (WGS).

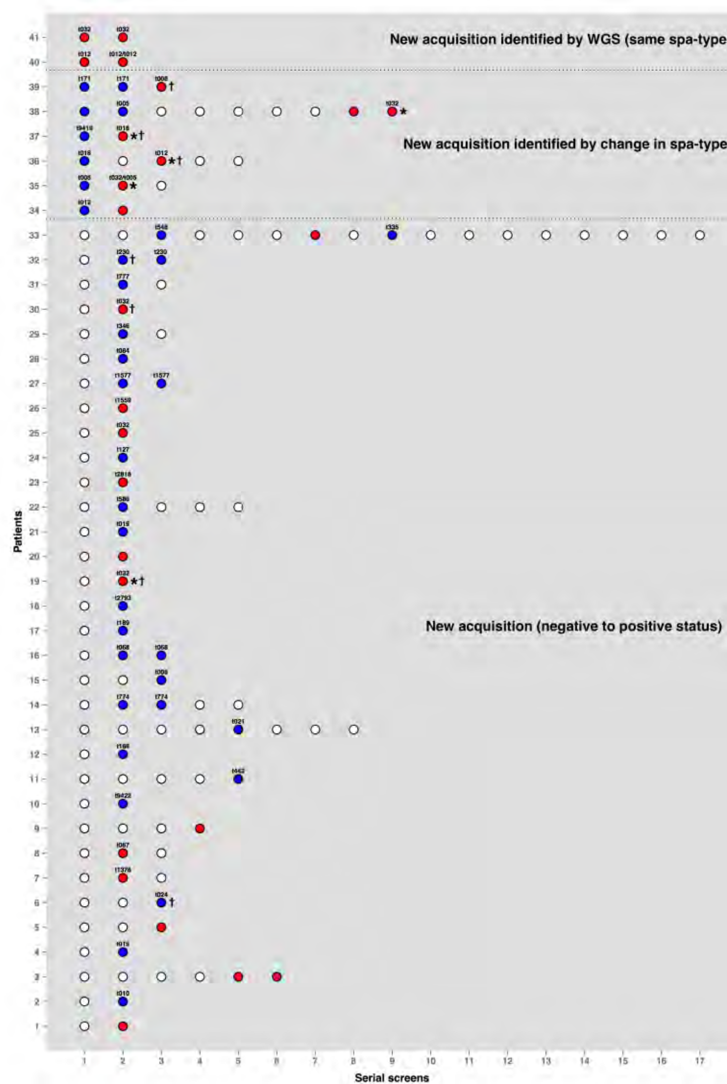
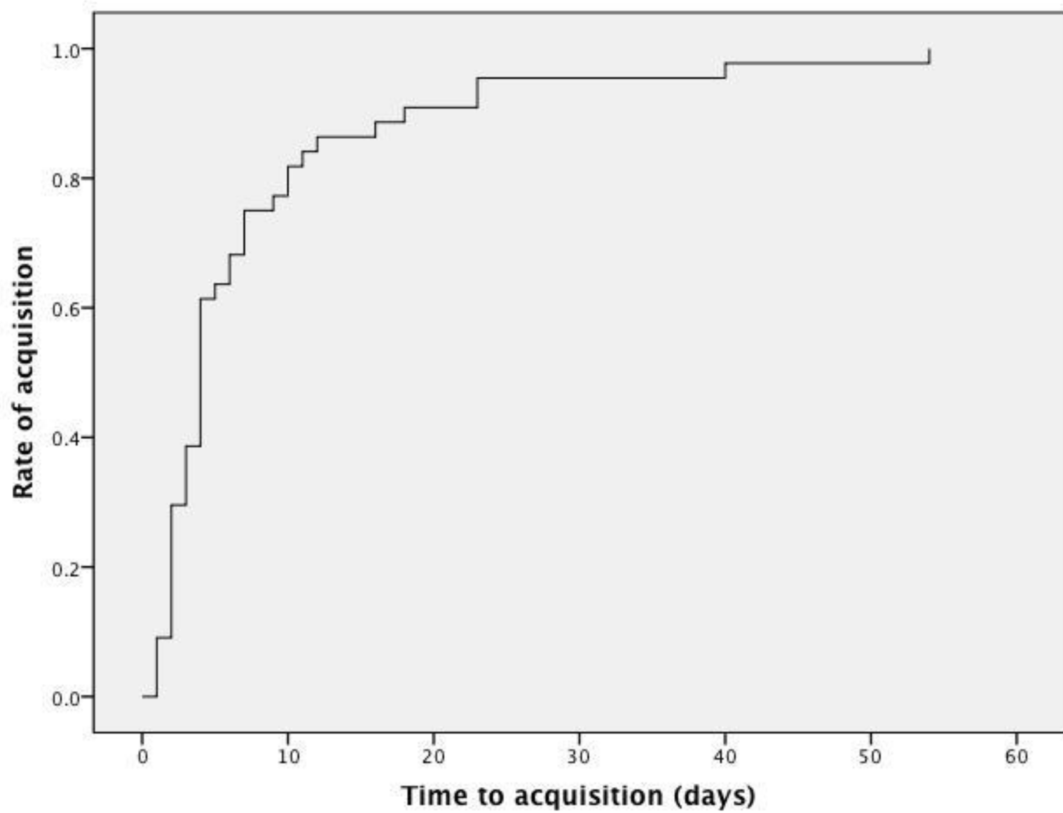


Figure 4.4 Kaplan-Meier curve depicting time to acquisition identified in the intensive care unit (ICU) between January 2010 and February 2011. Time to acquisition (days) was calculated from date of ICU admission to date of screen yielding acquired *S. aureus* isolate. Rate of acquisition denotes the cumulative acquisitions observed (44 acquisitions in 41 patients).



4.5.3.3 Acquisition between ICU admissions

Of 1181 ICU admissions 116 patients were readmitted during the study period; 6 patients colonised with *S. aureus* on both sets of admission screens. One re-admitted patient cultured *spa*-discordant MSSA isolates (t8263 & t2958) from admission swabs taken 70 days apart, suggesting a change of strains between admissions. BURP analysis was used to measure the relatedness of these isolates and revealed that they are highly related (cost 1); their *spa* genes differed by a single base pair change within a single repeat (Table 4.5). This suggests that the isolates recently diverged and most likely represents within host evolution of the same strain rather than new acquisition occurring outside of the ICU. WGS revealed the isolate genomes differed by 33 SNVs which is in keeping with the maximum diversity within a colonised host.[239]

Table 4.5 Putative *Staphylococcus aureus* acquisitions between admissions to the intensive care unit (ICU). One patient who was readmitted to ICU had serial admission swabs yielding MSSA of discordant *spa*-types. Analysis of *spa*-type repeat patterns reveals a single repeat difference (grey box). Comparisons of the discordant repeat sequences reveal a single base pair difference (underlined). The evolutionary distance (cost) and single nucleotide variants (SNV) between the *spa*-type pair are displayed

Admission	*spa*-type	*spa* repeat pattern	Discordant repeat sequence	Cost	SNV
1	t8263	r35 r17 r34 r17 r20 r17 r12 r17	AAAGAAGACGGCAACAAGCCTGGT	1	33
2	t2958	r35 r17 r34 r17 r20 r17 r12 r16	AAAGAAGACGGCAACAA<u>A</u>CCTGGT		

4.5.3.4 Acquisition rate

Of 1136 admissions 680 had at least one further screen. The acquisition rate was calculated for each primary acquisition (n=41) based on time at-risk of acquisition (Table 4.6). The overall rate of *S. aureus* acquisition rate was 9.9 per 1,000 patient days (95% CI 7.1–13.4) and rates according to isolate methicillin susceptibility were comparable (20 vs. 21 per 1,000 patient days).

The at-risk time definition makes an assumption that patients who underwent an acquisition event were no longer at risk of further acquisition. To test these assumptions acquisition rates were calculated using time from first swab until last swab in all patients including acquisitions (4186 patient days) revealing a non-significant reduction in rates to 9.8 per 1,000 patient days (95% CI 7.0–13.3).

Table 4.6 Acquisition rates of *Staphylococcus aureus* during the study period. Acquisition rates were calculated using Poisson regression.

	Acquisitions (% of at-risk admissions, 95%CI) (n=680)	Acquisition rate per 1,000 patient days (95% CI) (patient days = 4157)
Overall	41 (5.9%, 4.1–7.8)	9.9 (7.1–13.4)
MRSA	20 (2.8%, 1.6–4)	4.8 (2.9–7.4)
MSSA	21 (3.1%, 1.9–4.4)	5.1 (3.1–7.7)

4.5.4 *S. aureus* transmission assessed by *spa*-typing and overlapping patient stay

As seven acquisition isolates were among those that could not be retrieved for typing, 37/44 acquisition events could be evaluated. Of these, five (14%) met conventional criteria for patient-to-patient transmission based on overlapping stay with a patient carrying the same *spa*-type. All five involved predominant MRSA strains; patient number 37 (MRSA t018), patient number 36 (MRSA t012) and patient number 19, 35, 38 (MRSA t032) (Figure 4.3). All three t032 MRSA strains occurred within three weeks of each other, suggesting a possible outbreak. Two other patients on the ICU at this time were found to carry MRSA t032; the first was patient 41 who newly acquired their strain two days before patient 35, and the second culture-positive on their first screen suggesting a possible donor.

4.5.4.1 Acquisitions defined by *spa*-group

It is plausible that some patient donors may have been missed as they carried highly related strains of different *spa*-types. To investigate this patient-to-patient transmission criteria were broadened to include donors with overlapping admission time colonised with an isolate of the same *spa*-group. A further 15 putative patient donors were identified, increasing rates of patient-to-patient transmission to 42.9%. Hence, nearly 60% patient acquisitions could not be attributed to donation from another patient even when accounting for related *spa*-types.

4.5.5 *S. aureus* diversity assessed by whole-genome sequencing

In order to assess the use of WGS to determine acquisitions and transmission events within-host and between host diversity was assessed. Firstly to internally validate previously published work on within host diversity that transmission thresholds were based on, and secondly to evaluate the diversity of strains carried between hosts.

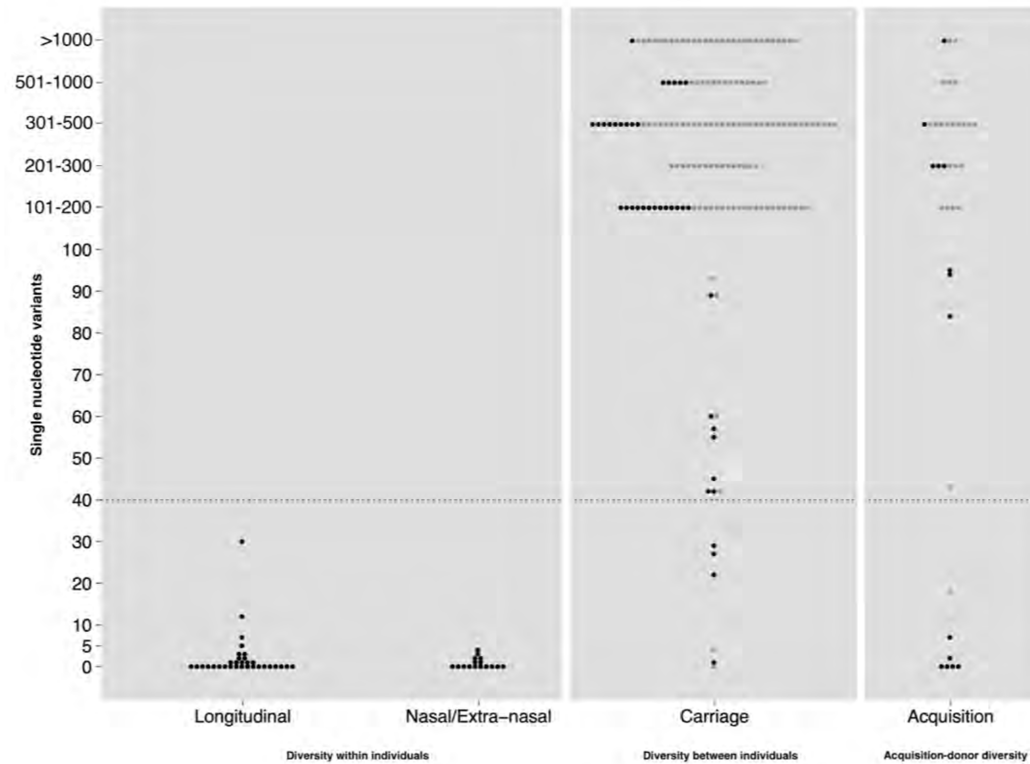
Among the 275 whole-genomes analysed, the diversity seen within and between hosts was markedly different (Figure 4.5). Within-host diversity, excluding acquisition isolates, was minimal either longitudinally over time or cross-sectionally between nasal and extra-nasal samples. Among 48 evaluable isolates (32 available to assess longitudinal diversity and 16 to assess between-site diversity) 44 were within

4 SNVs of the most distant within host isolate and all were within 40 SNVs, the maximum within host diversity observed by Golubchik *et al*.[239]

Among 160 admission isolates of *S. aureus* where between host diversity could be determined, 143 were >100 SNVs distant from the most similar isolate carried by another patient and for 154/160 the minimum SNV difference was >40.

In order to evaluate the use of WGS to determine transmission events the minimum diversity between acquisition isolates and carriage isolates from another patient (identified prior to acquisition) was evaluated. Evaluable acquisition isolates (n=37) showed a bimodal distribution of minimum SNV differences compared to other *S. aureus* isolates sequenced with 26/37 being >100 SNVs from the most closely related isolate sequenced in the study, 7 being <40 SNVs and 5 being <4 SNVs.

Figure 4.5 Diversity of *Staphylococcus aureus* isolates by whole-genome sequencing. Pairwise genetic distances in single nucleotide variants (SNV) of: (a) maximum genetic diversity within individuals over time (Longitudinal) and between anatomical sites (Nasal/Extra-nasal), (b) minimum genetic diversity between individuals from isolates cultured within 24 hours of ICU admission (Carriage), (c) minimum genetic diversity between acquisition isolates and carriage stains (Acquisition). Each dot represents a pair of isolates and is coloured according to whether the pair has concordant (black) or discordant *spa*-types (grey).



4.5.6 Transmission of *S. aureus* assessed by whole-genome sequencing

WGS data allowed scrutiny of the five instances of patient-to-patient transmission identified by *spa*-typing and overlapping ICU stay. The MRSA t018 isolate from patient 37 (Patient G in Figure 4.6) was genetically indistinguishable (0 SNVs) from the putative donor (Patient F in Figure 4.6) confirming patient-to-patient transmission.

The MRSA t012 isolate from patient 36 (Patient I in Figure 4.6) differed by 1,100 SNVs from the putative donor isolate (Patient M in Figure 4.6) disproving patient-to-patient transmission. However the most closely related isolate to this was an MRSA t018 carried by a patient on the unit at the same time that differed by 18 SNVs (Patient A1 in Figure 4.6).

The 'outbreak' identified by conventional criteria in which patients 19, 35, 38 and 41 acquired MRSA t032 is illustrated in Figure 4.7 showing the ICU stays of all 17 patients from whom MRSA t032 was isolated in the study period (assigned letters A to Q). MRSA t032 isolates in the 'outbreak' period from patients J and L/19 were highly related (2 SNVs) confirming patient-to-patient transmission while isolates from the other three acquisitions (patients H/35, I/41 and K/38) were genetically distinct from both potential donors (J) (102-354 SNVs) and from each other (102-337 SNVs) disproving transmission (Figure 4.8).

Figure 4.6 Maximum likelihood tree of MRSA t018 (Patients A-H) and MRSA t012 (Patients I-M) isolates. The branch length reflects single nucleotide variants identified between isolates (annotated). Nodes have been coloured according to isolate type; red = acquisition isolate, yellow = carriage isolate, black = hypothetical. Isolates sharing circles are genetically indistinguishable. 21 isolates were available from 13 patients including one pair of genetically identical isolates from patient F and G/Pt37, 4 patients with two genetically identical isolates (*), and three genetically identical isolates from patient A (†).

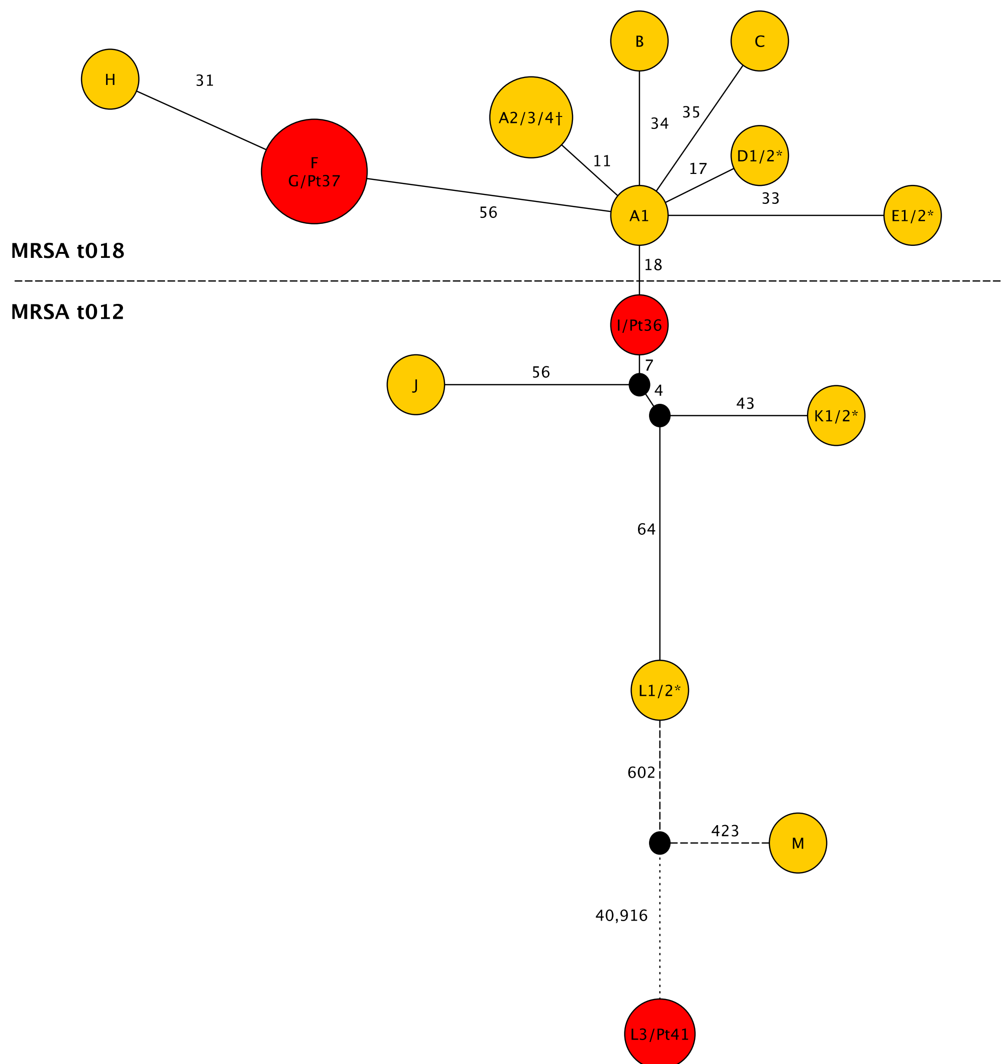


Figure 4.7 ICU stays of all 17 patients from whom MRSA t032 was isolated. Patients are labelled A-Q with patients H, I, K and L corresponding to patients 35, 41, 38 and 19 in figure 4.3. Each acquisition had at least one preceding screen that was culture-negative or yielded an isolate of a different *spa*-type. The red box highlights a putative 'outbreak' suggested by conventional criteria (same *spa*-type and overlapping stay).

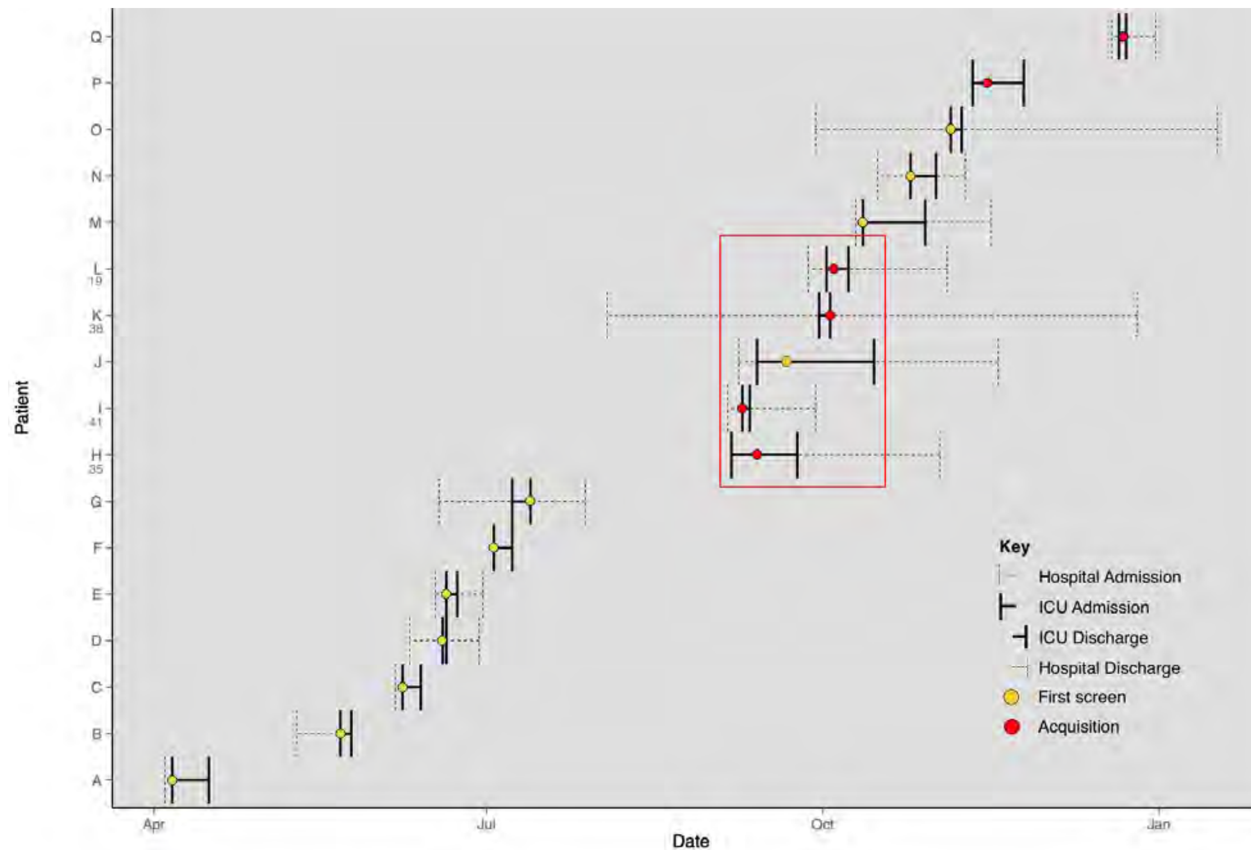
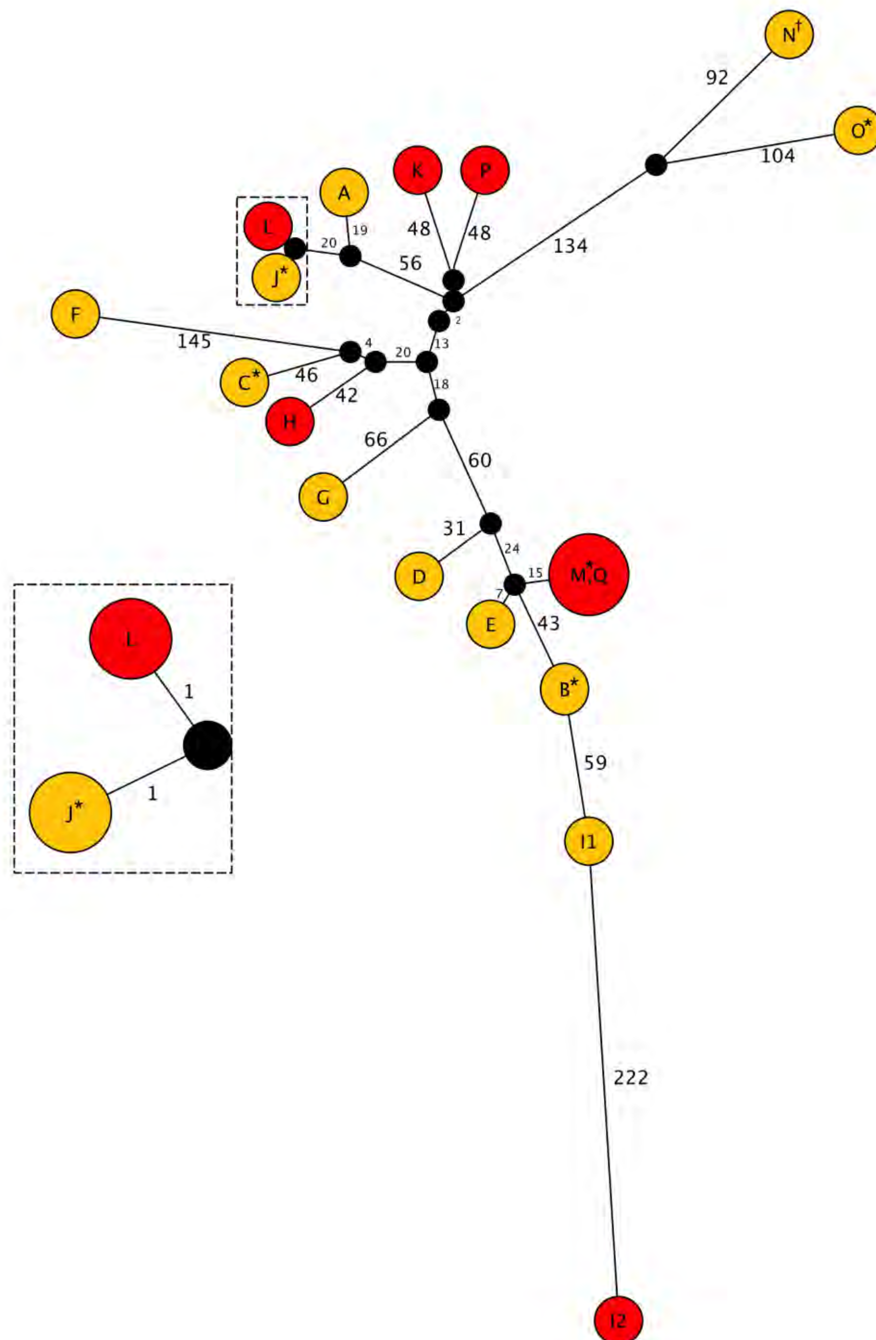


Figure 4.8 Maximum likelihood tree of MRSA t032 isolates (Patients A-Q). The branch length reflects single nucleotide variants identified between isolates (annotated). Nodes have been coloured according to isolate type; red = acquisition isolate, yellow = carriage isolate, black = hypothetical node. Isolates sharing circles are genetically indistinguishable and touching nodes differ by 1 SNV. 26 isolates were available from 17 patients; one isolate from 10 patients, two isolates from six patients (* and I1/2 from patient I) and four isolates from one patient (†).



4.5.6.1 WGS reveals transmission undetected by conventional criteria

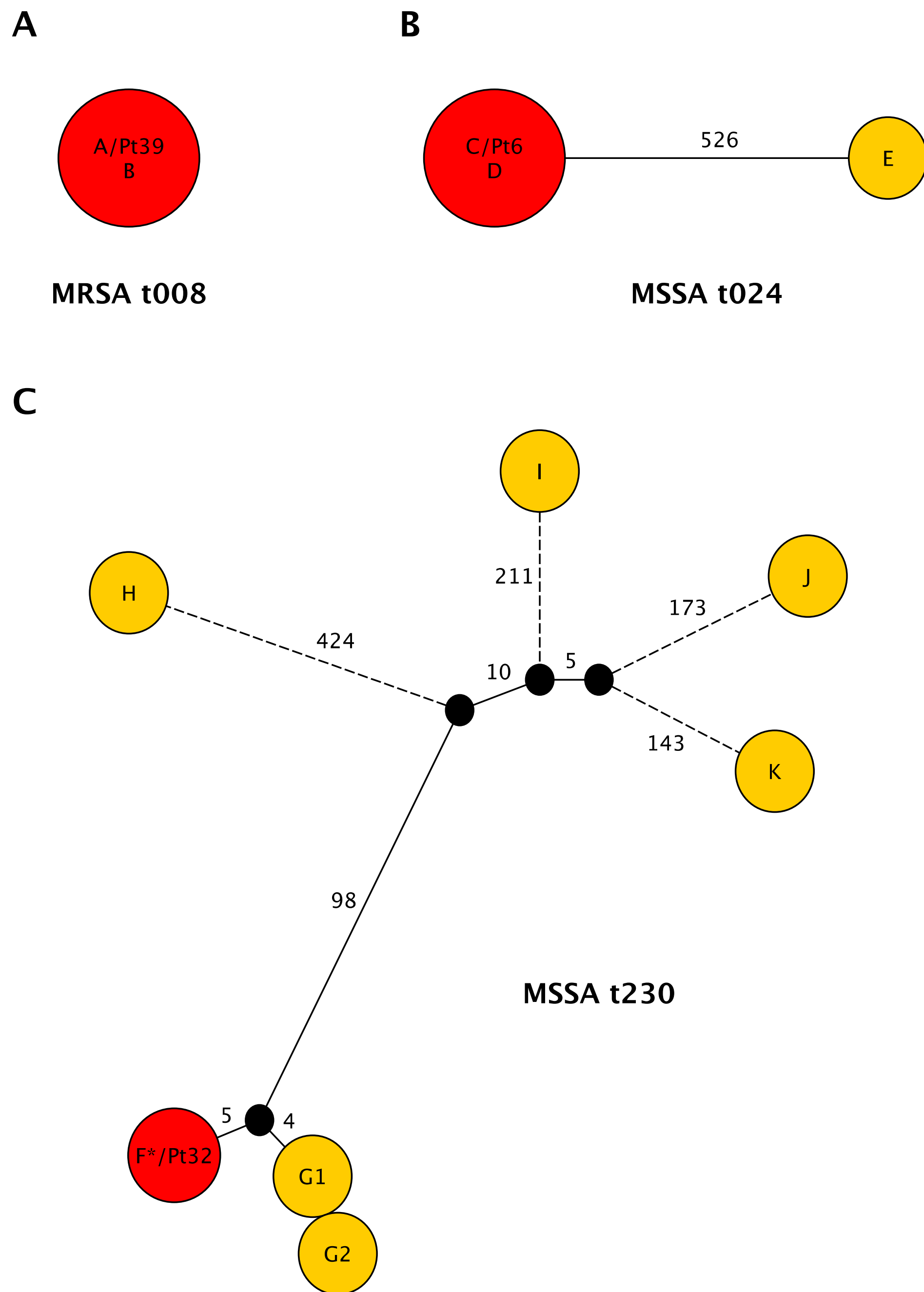
Additional transmission events were identified solely from WGS. Two patients acquired MRSA t032 during the study period without sharing time on the ICU with any other patient known to be carrying this strain. These are patients 25 and 30 being patients P and Q in Figure 4.7. Analysis of the genetic relatedness of all MRSA t032 isolates from the study (Figure 4.8) demonstrated that while patient P/25's isolate was 96 SNVs different from its nearest neighbour, the isolate from patient Q/30 was genetically indistinguishable (0 SNVs) from that carried by patient M who was discharged 7 weeks earlier.

There were two other instances where patients acquired strains that were genetically indistinguishable (0 SNVs) from isolates identifed earlier on in the ICU despite the patient admissions being 18 and 34 days apart respectively; one involved MRSA t008 (patient 39 in Figure 4.3, patient A in Figure 4.9a), the other MSSA t024 (patient 6 in Figure 4.3, patient C in Figure 4.9b).

One further acquisition involved highly related pairs of isolates. The MSSA t230 strain from patient 32 in Figure 4.3 (patient F in Figure 4.9c) differed by 9 SNVs from an isolate of the same *spa*-type colonising a patient (G1 in Figure 4.9c) discharged from the ICU 107 days previously.

In total, therefore, WGS discounted three transmissions indicated by conventional criteria, confirmed two and identified five additional transmissions. These comprised four where isolates were genetically indistinguishable (3 with 0 SNVs) or closely related (9 SNVs) despite patients not having shared time on ICU, and one where *spa*-types were discrepant despite only differing by 18 SNVs and patients having shared time on ICU.

Figure 4.9 Maximum likelihood tree of (A) MRSA t008 (Patients A-B), (B) MSSA t024 (Patients C-E) and (C) MSSA t230 (Patients F-K). The branch length reflects single nucleotide variants identified between isolates (annotated). Nodes have been coloured according to isolate type; red = acquisition isolate, yellow = carriage isolate, black = hypothetical node. Isolates sharing circles are genetically indistinguishable and touching nodes differ by 1 SNV. Two genetically indistinguishable isolates from patient F (*).



4.5.6.2 WGS reveals new acquisitions undetected by conventional methods

As described in Section 4.5.3, WGS identified two additional acquisitions involving patients colonised on admission who acquired genetically diverse isolates of the same *spa*-type during their ICU stay. The first (patient 40 on Figure 4.3) yielded MRSA t012 (L1 on Figure 4.6) on admission swab and two isolates of the same *spa*-type (L2 and 3 on Figure 4.6) from follow-up swabs taken two days later. Whilst L1 and L2 were genetically indistinguishable (0 SNVs) these isolates were highly diverse from L3 (41,518 SNVs) inferring new acquisition of the same *spa*-type. The second (patient 41 on Figure 4.3) yielded MRSA t032 on admission and follow-up swabs that were genetically diverse (222 SNVs) from each other (I1 and I2 in Figure 4.8) revealing a new acquisition.

4.5.6.3 WGS excludes acquisitions identified by conventional criteria

Of the remaining evaluable acquisitions WGS had the resolution to rule out two acquisitions determined by the *spa*-type and epidemiological definitions. One patient was admitted to the ICU colonised with MSSA t005 and subsequently cultured MRSA t005 7 days later. By our definition this would constitute strain change but this seems unlikely. To investigate this genomic comparison were made revealing that both strains were genetically indistinguishable (0 SNVs). Oxacillin minimum inhibitory concentration (as surrogate marker of penicillinase-stable penicillins such as methicillin) were determined to re-assess susceptibilities; discordant susceptibilities (0.25 and >256µg/mL) were confirmed. Isolate were interrogated using Basic Local Alignment Search Tool (BLAST), as described in Section 4.4.3.3, for the presence of the *mecA* gene using publically available gene sequences (data accessible at NCBI database[233], Holden et al (2004)[264] Gene ID: 2861157). *mecA* was not present in either isolate suggesting a non-*mecA* mediated methicillin resistance such as hyper-beta-lactamase producer.[318]

A second patient cultured MSSA t774 from a nasal swab following a negative admission screen 4 days before, suggesting a new acquisition. Interrogation of epidemiological data revealed that this patient was previously admitted to the ICU (22 days between ICU admissions) colonised (nose) with MSSA t774. Comparison of genomes revealed 0 SNVs suggesting either false negative nasal admission swab or missed extra-nasal carriage on subsequent ICU admission.

4.6 Discussion

Efforts to prevent nosocomial *S. aureus* infection focus on preventing patient-to-patient transmission through MRSA screening, decolonisation, isolation of colonised patients and hand hygiene.[189,313] The reduction in MRSA infections which has occurred in the United Kingdom has been attributed to these interventions but rates of nosocomial infections generally, and of MSSA infections specifically, have fallen much less.[319,320] It is not clear whether patients continue to acquire *S. aureus* infection because prevention of patient-to-patient transmission is only partially effective or because other routes of transmission play an important part. To answer this question this study aimed to determine acquisition rates in patients within an ICU setting and assess the role of other ward patients as donors in these acquisitions. In addition the use of WGS in determining transmission and acquisition events was evaluated by (i) assessing the ability to differentiate carriage strains within and between hosts, and (ii) comparing results with conventional approaches (*spa*-typing and epidemiological data) used to currently assess transmission. Whilst previous studies have demonstrated the value of WGS for investigation of *S. aureus* outbreaks[241,321] this study is the first to explore the role of patients in acquisition of *S. aureus* in an endemic non-outbreak setting.

This study has four key findings. Firstly, patient acquisition of *S. aureus* is infrequently observed in our ICU (9.9 per 1,000 patient days). Secondly, a minority of *S. aureus* acquisitions result from patient to patient transmission. Thirdly, WGS can accurately differentiate within and between host isolates permitting thresholds to be developed to determine transmission events. Fourthly, WGS has the resolution to confirm, refute and reveal previously undetected transmission and acquisition events compared to conventional *spa*-typing methods.

4.6.1 Patient carriage

Previous studies have reported rates of *S. aureus* carriage of between 14% and 53% among hospitalised patients, with MRSA accounting for 5-30%[73,322] and even higher rates (18-46%) in high-dependency settings.[323] The rate of carriage at ICU admission in this study (17%) is thus relatively low. One plausible reason for this is prior receipt of antibiotics. As this study utilised information collected as part of routine clinical practice data on patient medications was not retrieved. To indirectly address

this colonisation rates for patients admitted to ICU ≤24hours of hospital admission (likely direct admission to ICU with presumed low rates of prior antibiotics) and >24hours (likely admission from hospital ward with presumed higher rates of antibiotic use) were reviewed and no statistically difference was identified. It is also possible that more sensitive sampling methods might have detected higher rates of carriage. However, due to enhanced infection control efforts it is also likely that our study patients were subject to more anti-staphylococcal decolonisation measures than patients recruited to studies among non-critical care patients or prior to the introduction of MRSA screening and decolonisation.

4.6.2 Diversity

To understand the genetic relationship of isolates involved in transmission event the genetic diversity of *S. aureus* carriage within and between hosts was determined in a non-outbreak setting using WGS. Over short periods of time the majority of isolates within a colonized host exhibited low diversity. This is consistent with the rate of evolutionary divergence of *S. aureus.*[239] This study was not designed to evaluate confounding factors, such as recent antibiotic use, but a recent study suggests that this does not have a significant effect.[239]

Within-host diversity evaluation revealed two previously undetected acquisitions where two patients yielded isolates of the same *spa*-type on serial swabs that were genetically diverse. Whilst the first involved highly diverse isolates (41,518 SNVs) the second pair differed by 222 SNVs. Whilst an alternative explanation for the latter is natural within host diversity, this is unlikely as this degree of diversity would take a minimum of 21.3 years according to current estimate of molecular clock.

Evaluation of diversity between colonized patients revealed that most strains differed by at least 100 SNVs. This complements recent work revealing an average diversity of isolates of the same (396 SNVs) and between (22,738 SNVs) lineages.[239] The authors identified a minimum diversity of 44 SNVs. In this study there were a minority of carriage isolates that exhibited low genetic diversity including 13/150 (8.7%) exhibiting <44 SNVs; 2 (1.3%) with 0 SNVs which may represent unobserved transmissions preceding ICU admission. These data provide a framework for interpreting the genetic relationship between isolates in a transmission pair between different persons. By establishing thresholds of diversity

within and between persons it becomes possible to interpret the relatedness of a putative transmission pair in terms of the likelihood of a short term transmission occurring.

4.6.3 Acquisitions

Amongst 680 patient stays evaluated with repeated sampling only 44 acquisitions were identified, indicating acquisition is uncommon among ICU patients in our hospital. Conventional investigations of transmission combine epidemiological information with typing of isolates. This study shows that use of a conventional approach to investigate transmission (*spa*-typing and epidemiological association) falsely suggests transmission links between patients but also fails to identify transmission links particularly where patient stays do not overlap and transmission might have occurred indirectly such as via healthcare workers or environmental contamination.

The overall rate of acquisition observed in this study (6.5%) is relatively low. Bloemendaal *et al.* (2009) assessed *S. aureus* acquisition in six European ICUs, occurring in 14.2% of serially screened patients over three months.[323] Lower proportions of acquisition observed in this study may underestimate the true rate but may also reflect implementation of enhanced attention to hospital infection control.

When compared to time at risk acquisition rates observed in this study (9.9 per 1,000 patient days) were comparable to those observed in other European ICUs[323,324] suggesting our study is representative of clinical practice. Interestingly overall acquisition rates of MRSA and MSSA were comparable despite enhanced infection control policies (nasal mupirocin) targeting patients colonized with MRSA. The reason for this is unclear but may reflect only partial effectiveness of additional MRSA infection control measures, failure to target all routes of transmission or that MRSA strains are intrinsically more transmissible.[184] To calculate acquisition rates time from first to last swab was used to infer total time at risk as this represented the time period evaluable. Time from first swab until discharge could have been used instead, although calculating acquisition rates using these parameters did not significantly affect results. Time to secondary acquisitions were excluded from the analyses as the numbers were small and it remains unclear whether a patient acquiring *S. aureus* remains at equal risk of newly acquiring subsequent strains.

One might expect that longer ICU admissions are associated with higher rates of *S. aureus* acquisition. Whilst this study was not specifically designed to assess this assumption admission data and swab dates were used as surrogate markers. In turn three lines of evidence from the study support this hypothesis. First, the overall median time to acquisition was 4 days (IQR 2-8.5), being longer than the median admission times in all other patients (2.6 days, IQR 1-5.6). Of note no differences in time to acquisition were observed according to methicillin susceptibility and type of acquisition. Secondly, serially screened patients acquiring *S. aureus* had significantly longer ICU admissions compared to those who did not acquire. Thirdly, few acquisitions (9%) occurred within 48 hours of admission, with two thirds occurring within one week and 95% occurred within one month. These data are limited by small numbers. Furthermore, time to acquisition was determined using the date the acquisition swab was taken yet the true time of acquisition could have occurred any time from the previous swab. Further work is required to validate these findings and determine whether the increased risk of acquisition over time is linear or not.

Previous studies almost universally employ a single definition of acquisition as culture-positive following culture-negative screen.[323,325] This would miss colonised patients undergoing an acquisition of a different genotype. By applying dual definitions in this study it was possible to identify an additional 9 acquisitions, including two patients who underwent multiple acquisitions during their ICU stay. It is interesting that despite the broader definition the acquisition rate observed remained lower than previously documented in the literature.

Despite this the proportion of acquisitions in our study involving MRSA strains (47.6%) was higher than anticipated. Previous studies, in hospitals with comparable MRSA carriage rates, have identified MRSA acquisition rates in ICU patients ranging from 1.7-31.1%.[323,326-328] This may support work suggesting that the MRSA strains involved are more transmissible than MSSA[184] but could also relate to patient mix, antibiotic prescribing practice or infection control practices.

4.6.4 Transmissions

One of the primary findings of the study is that patient-to-patient transmission accounts for only a small proportion of new acquisitions, refuting our primary hypothesis that patients are a frequent source of acquisition. This cannot be

explained by low sensitivity of the sampling method unless patients with false-negative screening results make a disproportionately high contribution to transmission. Prior to the introduction of intensive hand-hygiene efforts, patient-to-patient transmission of *S. aureus* may have been much more common. In this ICU, which is likely to be typical of such units in other high-income countries, data indicate that patient-to-patient transmission is rare and suggests that other sources should be sought, such as among healthcare workers and the environment.

Another key finding in the study is that WGS has the resolution to characterize three temporally-related acquisitions thought to be patient-to-patient transmissions suggesting a ward-based MRSA outbreak. This ability to exclude such outbreaks in real-time could have dramatic implications in terms of infection control response. The study also revealed that WGS has the resolution to identify previously undetermined transmissions. These findings are based on applying what is known about *S. aureus* mutation rates to determine whether isolates share a common ancestor compatible with patient-to-patient transmission. This is possible because previous studies have measured mutation rates at 5-10 SNVs per year.[234,235,237,238] In each instance where patient-to-patient transmission was suggested by *spa*-typing and overlapping ICU stay, it has been possible to exclude transmission because of SNV differences of 10^2-10^3 or to confirm transmission has occurred because of SNV differences of 0-2. Conversely, the finding of two instances where isolates were genetically indistinguishable despite lack of overlapping ICU stay indicates that recent transmission has occurred. These results highlight the potential for WGS technology to revolutionise our understanding of transmission and outbreak management. By characterising transmission routes infection control methods can be efficiently targeted potentially saving both financial and labour costs.

To avoid underestimating the frequency of patient-to-patient transmission we used a limit of >40 SNV differences to exclude recent transmission given that SNV differences of up to 40 may be detected within an individual.[239] This threshold is supported by other work suggesting within-host diversity is low (<10 SNVs).[266] The within host diversity observed in this study indicates this was a reasonable assumption since among acquisition isolates 5 had SNV differences of <4, 7 <20 and only 1 was close to our cut-off of 40.

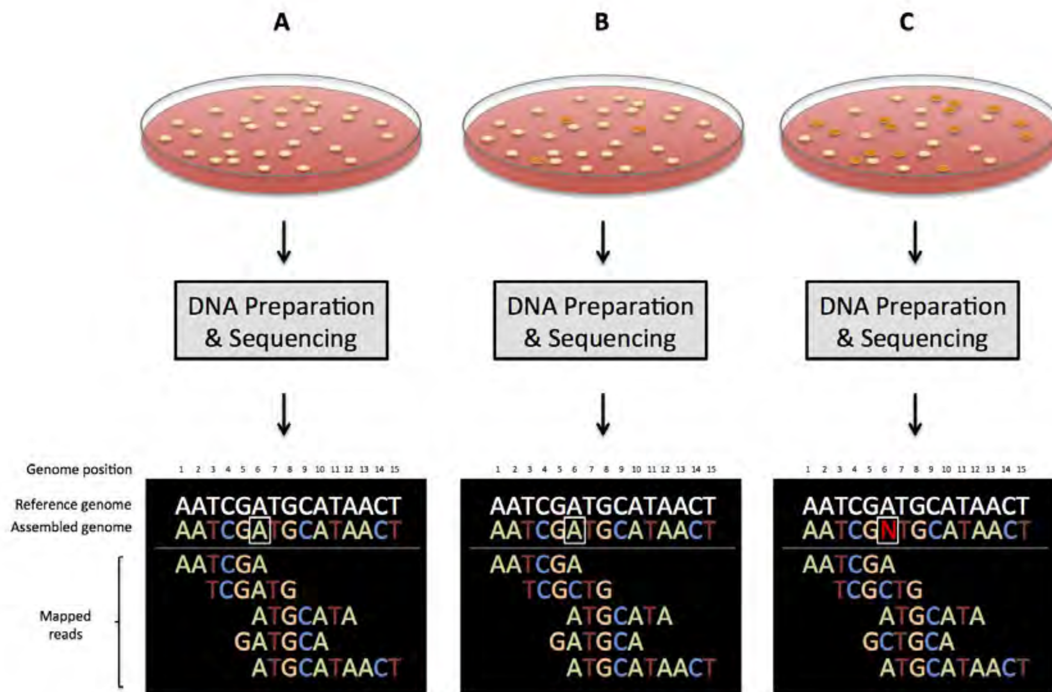
A major strength of the study is that 94% of patients were sampled within 24 hours of admission. Thus there can be confidence that the principal conclusion, that patients are not the major source of *S. aureus* acquisition on the ICU, is robust. These findings do not undermine the importance of infection control practices to prevent patient-to patient spread of *S. aureus*. Rather they are likely to be evidence of their efficacy.

The limitations of this study are a potential lack of sensitivity of swab-culture, the lack of follow-up swabs on a proportion of patients, and a failure to retrieve all cultured isolates. These may have lead to an underestimation of rates of carriage and acquisition. Conversely, some acquisition events may have been wrongly identified due to false-negative admission swabs leading to a possible over-estimation of acquisitions. Routine clinical practice is to inoculate agar plates with the swabs directly. Studies suggest that direct plating is less sensitive than including a broth enrichment step prior to culture on solid media.[329] As the swabs were collected as part of routine clinical practice additional enrichment steps were not available. This is to be addressed in future work.

The need to accurately determining the number of SNVs between isolate genomes is essential to interpret transmission events accurately. Consequently a robust method of accurately assembling genomes and nucleotide variant calling is needed. In this study genome assembly was undertaken using a pipeline developed by collaborators at UKCRC MMM. It is designed to assemble genomes for isolates with a single genotype (haploid model), typically generated by sequencing single colonies from a sample. In this study multiple colonies (sweep) were used to generate a genome. As recent work suggests that a host carries sub-populations differing by up to 40 SNVs[239] it is likely that a nasal swab will yield this variation. Culturing this swab and creating a sequence from a 'sweep', rather that an individual colony, will generate reads that incorporate these (up to) 40 variant sites. When aligned to a reference genome there will be heterogeneity in the reads at these sites. Depending on the assembly filters used these sites are likely not to be called, unless there is dominance of a particular variant site. Figure 4.10 provides a visual representation of potential outcomes from sequencing single genotypes and sweeps. As a result an assembled genome created from a sweep could remove some, if not all, of the within-host diversity within a sample. This could be advantageous in terms of

understanding transmission events. Instead of having to account for within-host diversity this method could remove it. Subsequently SNV differences would represent only 'outside-host' genetic diversity between isolates. Further work is required to evaluate the pipeline to interpret multiple genotypes.

Figure 4.10 Theoretical interpretations of genome reconstruction using sweeps in cultures with varying proportions of sub-populations (under an assumption that base calling requires >75% concordance at heterogenous sites). Three scenarios are depicted: (A) colonies with single genotype, (B) sub-populations with a dominant genotype, (C) sub-populations with no dominant genotype. Nucleotide bases (A, T, C, G) and uncalled bases (N) are labelled.



4.6.5 Future work

Only a minority of patient acquisitions can be explained by patient donors so other reservoirs of transmission need to be investigated. In the next chapter I describe in detail a follow-on study to determine the role of staff members and the environment in *S. aureus* transmission. Furthermore, validation of results from this study is needed in other hospital wards and in ICU in other hospital trusts.

4.6.6 Conclusion

In this chapter I have showed that:

- 6% patients acquired *S. aureus* on our ICU at a rate of 9.9 per 1,000 bed days (Objective 1)
- Patients account for only 18.9% of acquisitions in other patients within the ICU setting at our hospital (Objective 2). This observation refutes our primary hypothesis that patients are a common source of acquisition in other patients.
- Whole-genome sequence has the resolution to differentiate carriage strains by discriminating the minimal (<10 SNV) diversity within host and the diversity of isolates (>100 SNVs) between hosts (Objective 3)
- Conventional typing methods lack the resolution to discriminate highly related isolates resulting in falsely making transmission links and falsely excluding transmission links between patients (Objective 4)

Efforts to reduce the burden of *S. aureus* should acknowledge that other routes are now responsible for the majority of nosocomial transmission. WGS has the potential to redefine our understanding of *S. aureus* transmission as well as to replace conventional epidemiological methods as a routine tool.

5 *Staphylococcus aureus* acquisition in high-dependency care: a prospective study of patients, staff and the environment

5.1 Introduction

By testing routinely gathered samples taken for MRSA surveillance swabs for *S. aureus* we have been able to demonstrate on-going acquisition of *S. aureus* by patients that cannot, in the majority of cases, be explained by importation of *S. aureus* to the intensive care unit (ICU) by other patients. This begs the question; from where are patients acquiring *S. aureus*? Plausible sources include staff members, in particular those providing direct bedside care, contamination of the environment, visitors and the food chain. Understanding the contribution of these sources to nosocomial transmission and patient acquisition could reveal novel effective strategies to reduce the incidence of *S. aureus* infection. If strategies achieved an impact similar to that of policies focused on patient-to-patient transmission this could save over 4000 lives and £17.7 million in the NHS alone.

Staff members have long been considered potential sources for *S. aureus*.[126,130,132,160,330-332] Vonberg *et al.* (2006) conducted a systematic evaluation of 191 reported MRSA outbreaks and concluded that healthcare workers were the likely source in only 1.6% cases.[171] In comparison Albrich *et al.* (2008) identified 27/106 (25.5%) outbreaks where there was molecular and epidemiological evidence to suggest staff involvement.[124] The diversity of results reflects the variety of settings (different clinical areas, mostly outbreaks) and the low-resolution of typing methods used to evaluate transmission pathways. We know from recent studies using whole-genome sequencing (WGS) that conventional typing methods can both over- and under-estimate individuals involvement in outbreaks.[241,242] To date only one study has used WGS to assess the role of healthcare workers in *S. aureus* transmission. Harris *et al.* (2012) employed WGS to evaluate a recurrent neonatal MRSA outbreak where staff were screened during an outbreak. WGS indicated a single healthcare worker was colonised with a strain highly related to the outbreak isolate and the authors concluded that the staff member was involved in the outbreak based on phylogenetic analysis.[266] Others have challenged this interpretation of the results on

the basis that the authors made assumptions on within-host variation and diversity of acquired isolates that may not be reliable.[333] Furthermore, since this study was conducted in an outbreak it is unclear whether staff members are implicated in endemic transmission.

Screening staff to inform transmission involves significant ethical and logistic issues. There may be serious implications for healthcare workers of being identified as a carrier, particularly of antibiotic resistant strains. The best approach to management of colonised staff members is unclear. These issues are reflected in the widely varying policies which exist in different health care systems (Table 1.4). To allow meaningful evaluation of transmission a high proportion of staff need to be assessed to reduce the possibility of missed carriers. Additionally, screening should not impact on work commitments.

To assess the role of healthcare workers and environmental contamination in patient acquisition of *S. aureus* we extended the screening of ICU admissions described in the previous chapter for an additional 14 months and simultaneously undertook: (i) staff screening, initially involving nursing staff and then medical and physiotherapy staff, and (ii) extensive environmental sampling.

The overarching aim of this work was to establish whether staff carriage or environmental contamination account for *S. aureus* acquisition not explained by patient-to-patient transmission. In achieving this aim I fulfilled several subordinate aims:

1) to assess frequency of *S. aureus* carriage among staff members
2) to determine risk factors for *S. aureus* carriage
3) to determine whether staff may carry *S. aureus* transiently after a shift of duty
4) to assess the performance of a single nasal swab against more extensive sampling in detecting *S. aureus* carriage

At the time of writing the WGS analysis is incomplete and therefore this chapter relates to *spa*-typing results.

5.2 Attributions

Recruitment of staff participants, swabbing and questionnaire collection was performed by Andrew Bexley (study research nurse). Culture and identification of isolates was performed by Kevin Cole (study biomedical scientist). Environmental screening and air sampling was undertaken by Kevin Cole and Andrew Bexley. Poisson modelling of rates used to evaluate intensive sampling data was performed by Professor Sarah Walker (Oxford Biomedical Research Centre, Oxford). All other work presented in this chapter was undertaken by James Price.

5.3 Methods

5.3.1 Study design

The project was a prospective observational non-interventional study in a high-dependency setting. This work was developed and designed through a series of consultations with staff. The study was conducted between 31st October 2011 and 23rd December 2012.

5.3.2 Setting

This study was conducted in the adult ICU and high-dependency unit (HDU) at Brighton and Sussex University Hospital (BSUH). As described previously, the adult ICU is a 16-bedded unit consisting of one 5-bedded area, one 4-bedded area, 3-double side rooms, and 1 single side room. The adult HDU (opened in December 2010) is located two floors below ICU and consists of a 12-bedded unit with two 4-bedded areas, one 2-bedded areas, and 2 single side rooms. The 2-bedded area was opened in April 2011, 7 months into the study period.

5.3.3 Patient sampling

Patient sampling was a continuation of that described in Chapter 4 but extended to include; (i) patients admitted to the adult HDU in addition to the ICU, representing all adult high-dependency areas at BSUH, and (ii) clinical samples (such as wound swabs and blood cultures) culturing *S. aureus* from 1st March 2012.

Anonymised patient details, hospital stay data and ICU/HDU bed-stay data were collected from patient records. Infection control policy in adult high-dependency settings at BSUH was the same as previously described in Chapter 4. Records of all ICU and HDU routine patients screens were retrieved from the pathology database at BSUH and comparison with retrieved isolates allowed determination of any isolates that were missed.

5.3.3.1 Ethics

As a continuation of previous work patient sampling was conducted under the approvals in place for the Modernising Medical Microbiology work titled 'Integrating Strain Typing & Database Technologies in Research Service' study which has approval from the Berkshire Research Ethics Committee (Reference 10/H0505/83) and the National Information Governance Board Ethics and Confidentiality Committee (Reference ECC 8-05 (e)/2010) (available at www.nigb.nhs.uk/s251/registerapp).

5.3.4 Healthcare worker sampling

The study recruited healthcare workers with direct patient contact working in the adult ICU and HDU.

5.3.4.1 Nursing staff

Nursing staff were recruited during the entire study period. During the study a total of 208 nurses were employed to work in adult high-dependency settings at BSUH. This included temporary (usually bank) staff intermittently employed on the units and includes a small cohort of regular high-dependency trained nurses. Up to 21 nursing staff work on the ICU/HDU per shift depending on the number of patients. Shifts are typically 12.5 hours long (07:30-20:00 and 19:30-08:00) and allocation to work on ICU or HDU is determined on a shift-by-shift basis.

5.3.4.2 Doctors

Doctors were recruited during the final eight months of the study (16th April 2012 - 23rd December 2012). In total 42 doctors worked on the ICU/HDU during the study. 10 consultants regularly worked on the ICU/HDU and were typically present for periods of 7 days (4 days in ICU then 3 days in HDU) on a five-weekly rotational

basis. There were 8 junior doctors (Registrars and Senior House Officers) working in the units who rotate on a four-monthly basis.

5.3.4.3 Physiotherapists

Physiotherapists were also recruited during the final eight months of the study (16th April 2012 -23rd December 2012). In total 9 physiotherapists worked on the ICU/HDU during the study. High-dependency physiotherapy services include a permanent senior physiotherapist (band 7) and four junior physiotherapists (bands 5 and 6) who rotate on a four-monthly basis.

5.3.4.4 Ethics

As this study involved anonymised staff screening and collection of personal data ethical approval was sought. A favourable opinion was received from the South East Coast (Surrey) National Research and Ethics Committee (REC reference: 11/LO/1451) on condition of local management permissions. Research and Development approval was subsequently obtained from the Clinical Investigation and Research Unit at BSUH.

A major ethics amendment was obtained in April 2012 to allow:

(i) other staff (including doctors and physiotherapists) to be recruited to the study
(ii) serial throat carriage screens to be performed

5.3.4.5 Assessment and follow-up

To be included in the study a participant must be a member of the ICU/HDU staff (over the age of 18) with direct patient contact and provide written informed consent to participate in the study. Staff with no direct patient contact or refusal to consent to participate in the study were not recruited.

Each member of staff received a written invitation to make an appointment with a member of the research team (study doctor or research nurse) and to go through the consent process face-to-face. Consent was a two-part process. The first part was consent for the main study, assessing *S. aureus* nasal carriage. All subjects taking part in the main study consented to:

1. Serial anonymised single nasal swabs performed every 4 weeks (±1 week)
2. Serial anonymised questionnaires completed at the same time as nasal swabs every 4 weeks (±1 week)

The second part was separate consent for three sub-studies as they involved more frequent and more widespread swabs to be taken to assess duration and extent of carriage. The sub-studies were to assess: (i) transient carriage of *S. aureus* during a shift, (ii) cross-sectional extra-nasal carriage, and (iii) longitudinal throat carriage. All subjects taking part in the sub-studies consented to:

1. nasal swabs taken before and after shifts over two-working days
2. additional swabs taken from the throat, axillae, groins and any broken skin (e.g. cuts, eczema, psoriasis) at the time the study entry samples are taken.
3. six-monthly throat screens

The project was designed as a study and sub-study to allow subjects to enter the main study but opt out of the sub-studies requiring more frequent and extensive sampling. This was chosen as the ability to meet the objectives depends on being able to recruit a very high proportion staff to the main study.

Throughout the study regular screening 'clinics' were conducted, timetabled to fit in with start and end of shifts. Staff willing to consent to the study were seen in these clinics to go through the participant information sheet (Appendix 1), be given time to consider and, if necessary, discuss with friends, family and colleagues. Staff wishing to participate then signed the consent form. During the study any new employees or bank nurses who fulfilled the study eligibility criteria were invited to join the study, and sub-studies, in the same way.

5.3.4.6 Entry into the main study

Having given consent, participants were allocated a unique participant code (UPC) to unlink the subject information and samples from the individual participant. The ward matron (and delegates) held the UPC conversion key on a trust computer with password protection. If any participant misplaced their UPC they could retrieve it from the ward matron (or delegate). The conversion key was not known to members of the research team ensuring complete anonymity for staff. Following completion of

the study the ward matron destroyed the UPC conversion to ensure non-reversible identification of participant data.

5.3.4.7 Screening and questionnaires

Following consent *S. aureus* screening was performed and the participant was asked to complete a structured questionnaire.

5.3.4.7.1 Main study

The research nurse used a single swab to screen each participant for *S. aureus* carriage. A swab was placed in both nostrils (anterior nares) and rotated 5 times. The participant was asked to label the swab with their corresponding UPC, and then place the swab in a dedicated sample collection box. The swabs were then processed in the dedicated research area of the microbiology laboratory at BSUH. From month 6 single throat swabs were also taken (every 6 months) at the same time as the nasal swab.

Each participant then completed a questionnaire and labelled it with their UPC (Appendix 2). Pseudo-anonymised data were collected on basic participant demographics, co-morbidities, and predisposing risk factors for *S. aureus* carriage. The questionnaire included questions to establish: (i) known chronic illness (including diabetes mellitus, renal dialysis, skin conditions), (ii) medication history (including antibiotics, chemotherapy, corticosteroids), (iii) social history (including home situation, smoker, living abroad), (iv) previous employment history (including working abroad), (v) recent hospital inpatient or outpatient episodes, (vi) recent GP appointments, (vii) recent surgery (including vascular access/urinary catheter insertion), (viii) known MRSA colonisation or contact. The questionnaire was completed in private and deposited in a dedicated ‘ballot box’.

5.3.4.7.2 Sub-studies

As part of the sub-study evaluating transient carriage, subjects had three additional nasal screens taken over two consecutive workdays; one before and after a shift on day 1 and one before the start of their shift on day 2. These nasal swabs were anonymised with the participant’s UPC.

As part of the sub-study evaluating extra-nasal carriage participants had additional swabs taken at the time that the entry-to-study nasal swabs were taken. The

additional samples were from the throat, axillae, groins and any areas of broken skin. Nasal and throat swabs were taken by the study nurse whilst groin and axillae swabs were taken by the participant. All samples were labelled using the participant's UPC.

5.3.4.8 Follow-up procedure

Each participant was reviewed every four weeks (± 1week) in one of the study 'clinics'. The follow-up procedure involved a follow-up single nasal swab being taken. In addition a follow-up throat swab was taken at six monthly intervals. These swabs were processed anonymously in the same way as the initial screen.

In order to determine acquired or continuing *S. aureus* risk factors for *S. aureus* carriage each participant completed a further questionnaire at the time of subsequent swabs (Appendix 3). These questionnaires including information on: (i) new diagnoses of chronic disease, (ii) recent antibiotic use, and (iii) any changes in social circumstances. These were anonymously completed in the same format as the enrolment questionnaire.

5.3.4.9 Data recording

Personal identification details were not held with the study records. Confirmation of written consent and the date of consent were recorded; the consent forms were not held with the patient study records. All data collected during the study were recorded on individual case report files (CRFs). A CRF was completed for each consenting participant and recorded the UPC. Data transferred to the study database were linked to the CRF by the UPC. Study databases were securely stored on a password protected and encrypted computer only accessible by the study team.

5.3.4.10 Loss to follow-up/withdrawal

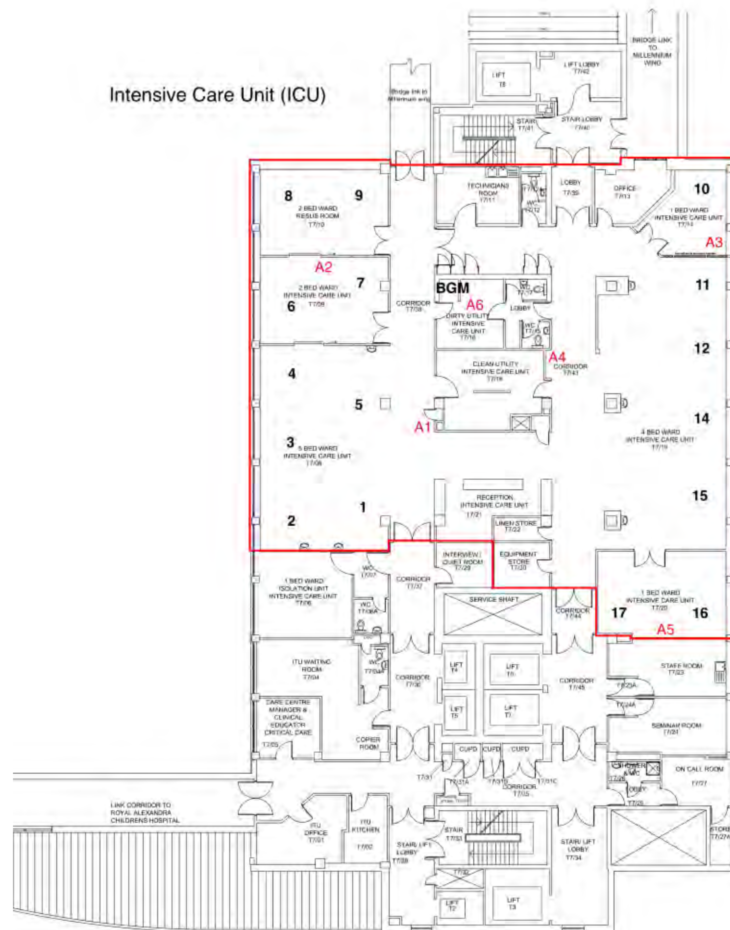
Following four-weekly screening any participant unscreened was deemed 'missed' and could re-join the study at the next screening clinic. If a participant wished to withdraw from the study they were free to do so without reason, and a withdrawal form was completed to document this. All data and culture results collected prior to the withdrawal date would still be included in the analysis unless the participant requested to have the information removed. If any member of staff recruited to the study left the trust their data was processed up to the point of leaving.

5.3.5 Environmental sampling

To evaluate environmental *S. aureus* environmental screening was undertaken on a four-weekly basis during the study period. Serial air sampling was performed at 10 locations on ICU and HDU (Figure 5.1) using an airIDEAL® (Biomerieux, France) air sampler. In brief, viable organisms in the air were retrieved through active controlled intake of air over a selective media (SAselect agar). The air sampler operates with air intake of 100L/min and impact speed of <20m/sec as recommended by the International Organisation for Standardisation (Cleanrooms & Associated Controlled Environments–Biocontamination Control standard ISO/DIS 14698-1, 1998).

Air sampling was coupled with serial screening of each bed-space on ICU and HDU (Figure 5.2). Of the 28 bed spaces 26 were screened throughout the study period and 2 (both HDU) were opened and assessed from April 2012. Each screen consisted of 5 swabs representing areas of frequent staff contact (monitor button, wipe-clean keyboard, disposable curtain) and those less frequently touched by staff (floor behind bed, underside of bed). Swabs were also taken of the communal blood gas machine located in a central ICU utility room.

Figure 5.1 Floor plans of the intensive care unit and high-dependency unit depicting bed spaces (black number 1-29), blood gas machine (BGM), sites of air sampling (red A1-11), and ward boundaries (red line). There is no bed 13.



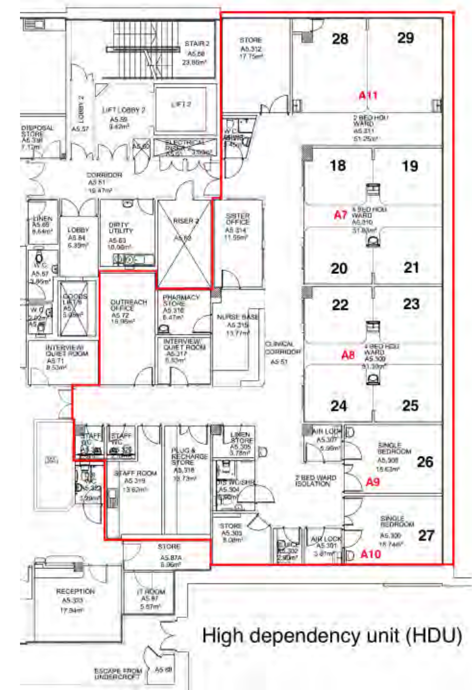


Figure 5.2 A photograph of a bed space in the intensive care unit. The environmental swabbing sites are labelled.

5.3.6 Definitions

5.3.6.1 Staff carriage profiles

VandenBergh *et al.* (1999) developed a carrier index to determine carriage profiles based on the number of swabs yielding *S. aureus* divided by the total number of swabs performed for that person.[80] In this chapter I have determined carriage profiles using an adapted version of their carrier index:

$$\frac{\text{Number of genotype-matching } \textit{S. aureus} \text{ positive serial samples}}{\text{Number of serial samples taken}}$$

The original index used ranges of carrier index values to determine different carrier profiles. I have adapted these definitions as follows; *persistent carriers* were defined as participants with a carrier index of 1, *intermittent* carriers were defined as carrier index $0 < x < 1$, and *non-carriers* were defined as participants with a carrier index of 0.

5.3.6.2 Transient carriage

Cookson *et al.* (1989) defined transient carriage as nasal or finger carriage detected immediately after a shift but lost by the next day.[125] In this study I have adapted this definition to account for carrier status before a shift starts. I have defined transient carriage as culture-negative swab pre-work on day 1 followed by culture-positive swab post-shift on day 1, then culture-negative swab pre-shift on day 2.

Furthermore to account for transient carriage in participants already carrying *S. aureus* I have used a second definition. Any participant yielding *S. aureus* of the same *spa*-type from all three swabs who also yielded an isolate of a different *spa*-type from post-shift swab on day 2 only was considered to undergo transient carriage.

5.3.6.3 Mixed carriage

Mixed carriage was defined as culture of ≥2 *S. aureus* of different genotypes from the same screen.

5.3.6.4 Acquisition

S. aureus acquisition in patients was defined as either (i) admission screen (or clinical sample taken on day of admission) that was culture-negative for *S. aureus*, then *S. aureus* was cultured from a follow-up sample or (ii) any screen (including clinical swab) culture-positive for *S. aureus*, then cultured a different genotype on a follow-up sample.

The same definitions were applied to determine acquisition in staff who received serial screens. While staff screens were taken less frequently than patients (at 4-weekly intervals) this provided insight into acquisitions in healthcare workers.

5.3.6.5 Acquisition rates

Acquisition rates were determined by evaluating the 'time at risk' of acquisition in the 'at-risk' population. The 'at-risk' population was any patient with >1 screen taken. 'Time at risk' was defined as length of time from first to last swab, and in those who acquired *S. aureus* the at-risk time was from first swab to day of first acquisition.

5.3.6.6 Outbreak

Public Health England (previously Health Protection Agency) defines an outbreak as an incident in which two or more people experiencing a similar illness are linked in time or place.[334] In this study I adapted this version and defined a *S. aureus* outbreak as ≥2 acquisitions of the same *spa*-type and methicillin susceptibility occurring within 30 days.

5.3.6.7 Transmission

Two definitions of transmission were used:

(i) Likely staff-to-patient transmission was defined as patient acquisition of a *S. aureus* strain of the same *spa*-type cultured from a staff member who was working in the ICU/HDU during the patient's admission and before the acquisition.

(ii) Likely patient-to-patient transmission, as previously described, was defined as the acquisition of a *S. aureus* strain whose *spa*-type matched those of a strain cultured previously from a colonised patient with overlapping ICU stay.

5.3.7 Identification and typing

All available isolates underwent culture, identification, *spa*-typing, *spa*-grouping and WGS preparation as previously described in Chapter 2.

5.3.8 Statistical considerations

All data were analysed using R (version 2.15.3, RStudio, USA), SPSS (version 20, IBM®, UK) or Stata (version 13, StataCorp®, USA). To describe continuous data, medians and IQR were used as they are not skewed by extreme variables. Chi-squared tests were used to compare rates of carriage. To evaluate the effect of intensive sampling on swab culture rates t-tests were used to compare means. Culture-positive rates and incident rate ratios were calculated using a Poisson model for rate. To investigate factors affecting staff carriage logistic regression analysis was used to calculate odds ratios and correct for the possible confounding factors. Acquisition rates were analysed using Poisson regression and exact two-sided Poisson tests. Time to event analysis was performed using Kaplan-Meier curves and log rank test. P values of ≤ 0.05 were considered statistically significant.

5.4 Results

5.4.1 On-going patient surveillance

Between 31st October 2011 and 23rd December 2012 there were 1933 ICU/HDU admissions (involving 1760 patients) including 1889 complete admissions, 20 who were already admitted to the ICU/HDU at the start of the study, and 24 who remained on the ICU/HDU at the end of the study. All admissions were considered potential donors and were assessed for acquisitions. Of the 1933 admissions 1164 (60.2%) were male, the median age at admission was 65.5 years (IQR 48.8–76.6) and the median length of stay was 3 days (IQR 1.6–6).

5.4.1.1 Screening and swabs

During the study period 3728 screens were taken as part of routine clinical practice. From March 2012 (month 5 of study) *S. aureus* isolates from clinical specimens were included and 144 clinical samples cultured *S. aureus*.

Of 1933 admissions 1854 (96%) were screened at least once for *S. aureus*; 1784 (92.3%) first screen taken ≤24hours of admission. The number of screens range from 1-32 (median 2; IQR 1–2). Of the 79 (4%) that did not receive a screen during their ICU admission 50 (63%) were admitted to ICU for ≤24hours (Table 5.1).

Table 5.1 Summary of the number of screens retrieved per patient admission and the length of admission to the intensive care unit

		Screens retrieved per admission (n)																Total
		0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**13**	**15**	**17**	**32**	
Admission Time (days)	**1**	50	260	125	5	0	0	0	1	0	0	0	0	0	0	0	0	441
	2	10	167	150	23	1	0	0	0	0	0	0	0	0	0	0	0	351
	3	8	107	127	34	4	0	0	0	0	0	0	0	0	0	0	0	280
	4	3	56	79	36	5	1	0	0	0	0	0	0	0	0	0	0	180
	5	2	27	62	30	3	0	0	0	0	0	0	0	0	0	0	0	124
	6	1	19	50	41	9	0	0	0	0	0	0	1	0	0	0	0	121
	7	2	15	41	35	1	2	0	0	0	0	0	0	0	0	0	0	96
	8–14	3	22	73	85	24	8	3	0	0	0	0	0	0	0	0	0	218
	15–21	0	3	3	23	17	13	2	1	1	1	0	0	0	0	0	0	64
	22–28	0	0	0	5	9	3	3	3	0	0	0	1	0	0	0	0	24
	29–49	0	0	0	1	1	7	8	3	1	2	1	2	1	1	0	0	28
	≥50	0	0	0	0	0	0	1	1	0	0	2	0	0	0	1	1	6
Total		79	676	710	318	74	34	17	9	2	3	3	4	1	1	1	1	1933

5.4.1.2 Carriage among patients at admission

A first screen for *S. aureus* carriage was conducted within 24 hours of admission to ICU or HDU in 1784 patients (92.3% admissions). 370 (20.7%) patients were found to carry *S. aureus* (38 MRSA (2.1%)). A further 70 patients had a first screen performed more than 24 hours after admission; 12 culturing *S. aureus* (1 MRSA) (Figure 5.3). In the preceding chapter the *S. aureus* carriage rate at admission was 16.7% (5.3% MRSA).

Of the 1854 screened admissions 382 (20.6%) cultured *S. aureus* on their first screen (39 MRSA (2%)). A total of 430 isolates were retrieved; 412 isolates from routine swabs and 18 from clinical specimens (Table 5.2). 370 colonised patients were identified by routine screening swabs and 12 patients were identified as carriers as *S. aureus* was cultured from clinical isolates and screen swabs were culture-negative. Comparison of pathology database results identified 14 additional isolates that were missed (all MRSA) including 8 from routine screens and 6 from clinical samples.

Patients admitted to ICU ≤24hours following admission to hospital were twice as likely to culture *S. aureus* on their first screen compared with patients admitted to ICU following >24hours in a hospital ward (32.5% vs. 16.2%, $p<0.001$). In the preceding chapter no significant difference was identified.

5.4.1.3 Serial screens

Of the 1854 screened admissions 1125 (60.7%) had more than one screen during their ICU admission; this is comparable to the serial screening rates observed in the preceding chapter (59.9%). 207 patients cultured *S. aureus* on serial screening and a total of 332 isolates were retrieved (Table 5.2). Following review of the pathology database 70 additional isolates were missed including 11 from routine screens and 59 from clinical samples.

Clinical isolates were only collected from month 6 of the study therefore all identified isolates prior to then were not retrieved. In total there were 143 culture-positive clinical screens during the study period of which 78 (54.5%) isolates were retrieved.

Figure 5.3 Flowchart of swabbing in patients admitted to the intensive care unit and high-dependency unit during the study. 762 isolates were retrieved in total comprising [1]417, [2]13, [3]225, [4]105, and [5]2 isolates.

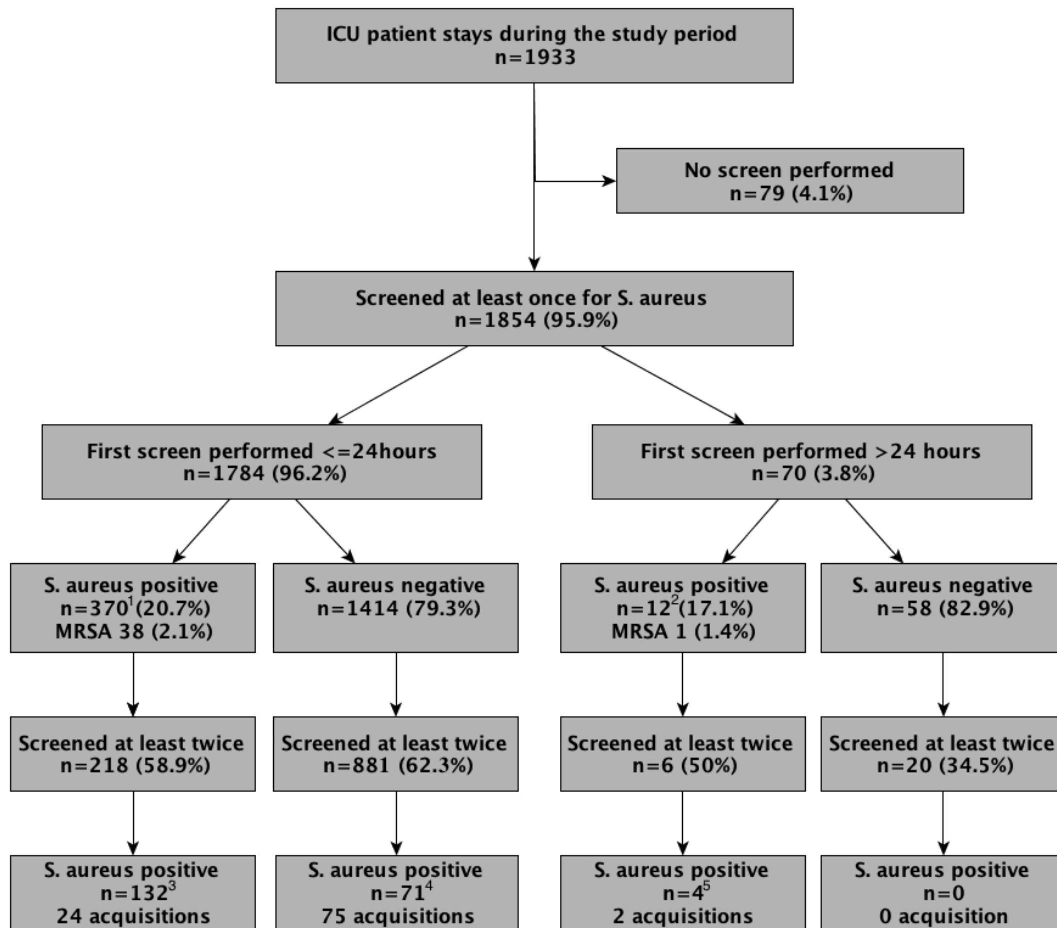


Table 5.2 Isolates retrieved from routine screening and clinical samples from patients during their admission to the intensive care unit and high-dependency unit. Missed isolates were identified by comparing the number of retrieved isolates with those identified on the Trust's pathology database.

	Overall	Admission				Serial			
		Total	MRSA	MSSA	Missed	Total	MRSA	MSSA	Missed
Screen	684	412	43	369	8	272*	26	246	11
Clinical	78	18	2	16	6	60	5	55	59
TOTAL	762	430	45	383	14	332	31	303	70

*270 swabs were culture positive with 2 swabs culturing 2 isolates of different genotypes

5.4.1.4 Carriage profiles

Of 1854 screened patients 1125 were serially screened and carriage profiles could be evaluated; 830 were culture-negative on all screens, 88 were culture-positive on admission and became culture-negative on second screen, 112 cultured *S. aureus* of the same genotype on at least two screens, 24 changed strain genotype (acquisition), and 71 became culture-positive following a negative admission screen (acquisition) (Table 5.3)

Of the 112 patients culturing the same genotype on >1 screen, 8 had at least one culture-negative screen between culture-positive screens suggesting either intermittent carriage or false negative results.

Table 5.3 Summary of patient carriage profiles observed and isolates retrieved during the study

Number of screens	Carriage profile	Total Admissions	Number of positive swabs	Number of isolates yielded	Isolates lost
1	Negative	571	0	0	-
	Positive	158	192	183	9
>1	Negative to negative	830	0	0	-
	Negative to positive	71	119	105	12
	Positive to negative	88	109	98	8
	Positive to positive				
	- same genotype	112	*323	291	42
	- different genotype	24	101	85	13
	Total	1854	844	762	84

*two swabs cultured 2 isolates of different genotypes

5.4.1.4.1 Mixed colonisation

Of 382 patients colonised at admission 7 could not be assessed for mixed carriage; 6 cultured isolates that were not retrieved and 1 failed *spa*-typing. Of 375 evaluable admissions 7 (2%) yielded >1 genotype from swabs taken at the same time point (Table 5.4).

To account for the relatedness of *spa*-types the BURP algorithm (as described in Section 2.6.2) was used to measure the relatedness of *spa*-types (cost) and allocate closely related strains into *spa*-groups (*spa*-types with cost difference ≤4). 5 patients (1.3%) carried ≥2 isolates that did not belong to the same *spa*-group (cost differences of 12-24) suggesting genetically distinct isolates. The remaining two patients carried *spa*-types that were related (cost 1) and were from the same *spa*-group. The first (patient 3 in Table 5.4) was colonised with t084 and t085, related *spa*-types differing by a mid-sequence repeat duplication. The second (patient 5 in Table 5.4) was colonised with t022 and t1616 that differed by a mid-sequence repeat resulting from a single base pair change.

Table 5.4 Carriage characteristics in seven patients identified with mixed colonisation during the study. Based Upon Repeat Pattern (BURP) algorithms[210] were used to measure the relatedness of *spa*-types (cost). Culture results of swabs are displayed and represent either: (i) isolation of methicillin sensitive (MSSA) and resistant (MRSA) *S. aureus* and *spa*-type (t-), (ii) no growth (Negative) or (iii) swab not taken (-).

Patient	Swab site					Cost
	Nose	Perineum	Urine	Wound	Clinical	
1	MSSA t223	Negative	-	-	MSSA t1664	24
2	Negative	Negative	MSSA t065/t2963	-	-	14
3	MSSA t084	MSSA t085	-	-	-	1
4	MSSA t084	MSSA t209	-	-	-	12
5	MRSA t022	MRSA t1616	-	MRSA t022/t1616	-	1
6	MSSA t1183	MSSA t065	MSSA t065	-	-	20
7	MSSA t166	Negative	MSSA t760	-	-	21

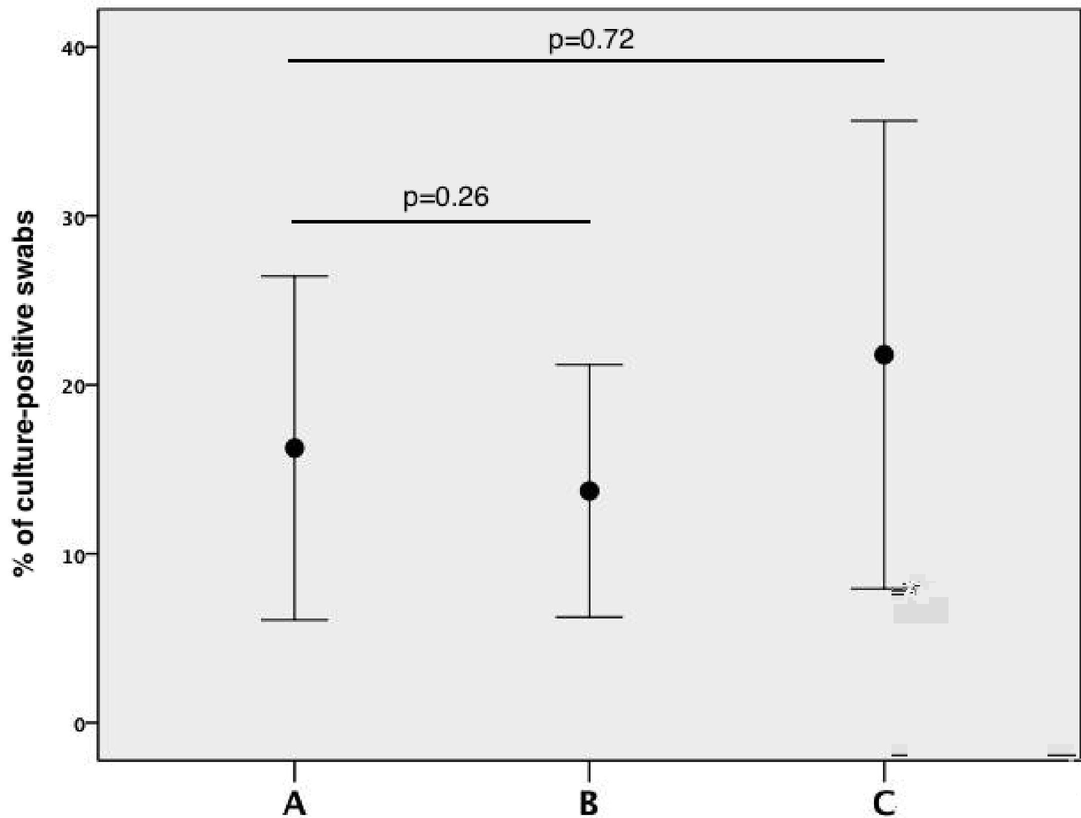
5.4.1.5 Intensive sampling

To address the possibility that use of nasal and perineal swabs could fail to identify a significant proportion of *S. aureus* carriers, the impact of two alternative sampling methods were evaluated in a sub-set of patients. Firstly, to evaluate whether nursing staff influence culture results the research nurse performed patient screens taken on Mondays for 5 consecutive weeks between 1st October and 4th November 2012. Ward staff took admission screens taken at other times during the week. Secondly, to understand whether routine culture methods (direct agar plate inoculation) influence results all routine swabs underwent adjunctive broth enrichment over a 4-week period (3rd-23rd December 2012).

As different methods were used on different days I evaluated differences in the proportion of positive swabs on days when the different method were used and mean culture-positive swab rates were determined (Figure 5.4). Mean rates of positive swabs during weeks when ward nurses sampled patients (16.3% (SD ±10.2)) were comparable (t-test p=0.26) to rates observed during research nurse sampling (13.7% (SD ±7.5)). While there was a trend to higher culture-positive swabs during weeks when broth enrichment was used (mean 21.8% (SD ±13.8)) compared to routine screening using direct plating methods (16.3% (SD ±10.2)) these findings were non-significant (t-test, p=0.72).

To assess these associations further a Poisson regression model was used. This model uses the average rates of culture-positive swabs during routine screening to determine the degree of spread around the average (Poisson distribution). This, in turn, was used to predict whether culture-positive rates observed during intensive sampling were outside the expected spread. Incident rate ratios (IRR) were used to measure the relative differences in culture-positive swab rates between the methods. There was no evidence that the research nurse significantly impacted the rate of positivity (IRR 0.84, 95% CI 0.5-1.5, p=0.54), but there is a trend towards enrichment leading to a 42% higher relative positive rate (IRR 1.42 95%, CI 0.82-2.46, p=0.22), where the confidence intervals are compatible with being up to 2.46-fold higher.

Figure 5.4 Patient carriage rates of *Staphylococcus aureus* observed during the intensive sampling sub-study. Three sampling methods were compared; (A) routine method of ward nurse sampling and plating swab direct to agar plate, (B) research nurse performing swabs and (C) broth enrichment. Plotted are mean carriage rates (black dot) and error bars represent one standard deviation. T-tests were used to compare mean carriage rates.



5.4.2 Healthcare workers carriage

In total 208 healthcare workers were approached to enter the study and 198 (95.2%) consented. These included 149/157 (95%) nurses, 40/42 (95.2%) doctors and 9/9 (100%) physiotherapists. 10 staff declined to enter the study including 8 nurses; 4/128 (3.1%) female and 4/29 (13.8%) male (p=0.06). Table 5.5 provides a summary of the staff screening during the study period.

5.4.2.1 Description of healthcare workers

There was a preponderance of females aged <40 years old, particularly in nursing staff and physiotherapists (Table 5.6). Whilst the prevalence of co-morbidities was low, 12.6% suffered from skin disorders (e.g. eczema, psoriasis). Within the two years preceding the study 32.8% of staff had sought medical care and 18 staff members underwent an in-patient hospital admission including 15 requiring surgery. A higher proportion of nurses and physiotherapists sought GP consult compared with doctors (38.9% vs. 44.4% vs. 7.5%, p<0.01). In the two years preceding the study 47% had received antibiotic treatment and 8.1% received corticosteroid therapy. Few healthcare workers had worked in nursing homes, residential care or for the prison service. Foreign travel was common with 177/198 (89.4%) travelling abroad within the last two years. Nursing staff were significantly more likely to be current smokers than doctors and physiotherapist (22.8% vs. 5% vs. 11.1%, p=0.005). Most staff (90.8%) do not live alone; nurses and doctors were more likely to live with partners and physiotherapists were more likely to live with flatmates. 4.6% of participants reported having previously been found to be colonised with MRSA. 87.9% of participants reported that the had actively cared for a patient known to be colonised with MRSA.

Table 5.5 Summary of staff recruited and evaluated during the study. Rates of nasal colonisation are displayed per 4-weekly cycle. % in parenthesis.

	Four weekly cycle														
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Cumulative staff	104	111	115	117	126	129	155	168	174	177	179	189	201	208	208
Refused consent	8	0	0	0	0	0	2	0	0	0	0	0	0	0	0
Cumulative recruited	96 (91)	103 (92)	107 (92)	109 (92)	118 (93)	121 (93)	145 (94)	158 (94)	164 (94)	167 (94)	169 (94)	179 (95)	191 (95)	198 (95)	198 (95)
Participant loss*	0	1	1	0	0	2	0	5	1	4	9	4	7	1	5
Cumulative staff in study	96	102	105	107	116	117	141	149	154	153	146	152	157	163	158
Staff evaluated^	96 (100)	100 (98)	100 (95)	103 (96)	105 (91)	112 (96)	139 (99)	140 (94)	139 (90)	140 (92)	121 (83)	137 (90)	134 (85)	140 (86)	128 (81)
Nasal colonisation	**35 (36)**	**37 (37)**	**37 (37)**	**38 (37)**	**40 (38)**	**33 (30)**	**50 (36)**	**52 (37)**	**50 (36)**	**51 (36)**	**48 (40)**	**52 (38)**	**50 (37)**	**49 (35)**	**42 (33)**
MSSA	29 (30)	30 (30)	30 (30)	30 (29)	33 (31)	28 (25)	44 (32)	45 (32)	43 (31)	44 (31)	42 (35)	47 (34)	45 (33)	45 (32)	36 (28)
MRSA	5 (5)	6 (6)	7 (7)	8 (8)	7 (7)	5 (5)	6 (4)	7 (5)	7 (5)	7 (5)	6 (5)	5 (4)	4 (3)	4 (3)	6 (5)
Both	1 (1)	1 (1)	0	0	0	0	0	0	0	0	0	0	1 (1)	0	0
Uncolonised	61 (64)	63 (63)	63 (63)	65 (63)	65 (62)	79 (71)	89 (64)	88 (63)	89 (64)	89 (64)	73 (60)	85 (62)	84 (63)	91 (65)	86 (67)

* ICU/HDU employment terminated during study period. ^ nasally swabbed and completed questionnaire

Table 5.6 Comparison of staff characteristics at entry to the study. % in parenthesis.

	Healthcare worker group				
	Total n=198	Nurse n=149	Doctor n=40	Physiotherapist n=9	P value
Age Range					
20-29	57 (28.8)	40 (26.8)	9 (22.5)	8 (88.9)	
30-39	89 (44.9)	71 (47.7)	17 (42.5)	1 (11.1)	**0.017**
40-49	39 (19.7)	28 (18.8)	11 (27.5)	0	
50-59	12 (6.1)	9 (6)	3 (7.5)	0	
60+	1 (0.5)	1 (0.7)	0	0	
Gender					
Male	51 (25.8)	25 (16.8)	24 (60)	2 (22.2)	**<0.01**
Female	147 (74.2)	124 (83.2)	16 (40)	7 (77.8)	
Medical Care (within 2 years)					
GP	65 (32.8)	58 (38.9)	3 (7.5)	4 (44.4)	**<0.01**
Hospital Outpatient	33 (16.7)	25(16.8)	7 (17.5)	1 (11.1)	0.98
Hospital Inpatient	18 (9.1)	16 (10.7)	2 (5)	0	0.33
Co-morbidities					
Diabetes	0	0	0	0	-
Eczema	19 (9.6)	14 (9.4)	3 (7.5)	2 (22.2)	0.39
Psoriasis	6 (3)	5 (3.4)	1 (2.5)	0	0.83
Malignancy	3 (1.5)	3 (2)	0	0	0.61
Chronic Renal Failure	0	0	0	0	-
Treatment (within 2 years)					
Surgery	15 (7.6)	12 (8.1)	3 (7.5)	0	0.68
Haemodialysis	0	0	0	0	-
Chemotherapy	1 (0.5)	1 (0.7)	0	0	0.85
Antibiotics	93 (47)	75 (50.3)	14 (35)	4 (44.4)	0.22
Corticosteroids	16 (8.1)	12 (8.1)	3 (7.5)	1 (11.1)	0.94
Social*					
Lives alone (no children)	22 (11.3)	15 (10.3)	7 (17.5)	0	
Lives alone (with children)	2 (1)	2 (1.4)	0	0	
Lives with partner (no children)	64 (32.8)	50 (34.2)	13 (32.5)	1 (11.1)	**0.025**
Lives with partner (children)	53 (27.2)	40 (27.4)	13 (32.5)	0	
Lives with flatmates (no children)	46 (23.6)	32 (21.9)	7 (17.5)	7 (77.8)	
Lives with flatmates (children)	2 (1)	2 (1.4)	0	0	
Lives with partner &flatmates	6 (3.1)	5 (3.4)	0	1 (11.1)	
Smoking^					
Never smoked	109 (55.6)	71 (48.3)	30 (75)	8 (88.9)	**0.005**
Ex-smoker	50 (25.5)	42 (28.6)	8 (20)	0	
Current smoker	37 (18.9)	34 (23.1)	2 (5)	1 (11.1)	
Travel abroad (within 2 years)					
Europe	143 (72.2)	102 (68.5)	32 (80)	9 (100)	0.06
North America & Canada	37 (18.7)	29 (19.5)	7 (17.5)	1 (11.1)	0.8
South America	15 (7.6)	12 (8.1)	2 (5)	1 (11.1)	0.75
South East Asia	51 (25.8)	34 (22.8)	15 (37.5)	2 (22.2)	0.16
Africa	32 (16.2)	24 (16.1)	7 (17.5)	1 (11.1)	0.89
Australasia	18 (9.1)	13 (8.7)	5 (12.5)	0	0.48
Employment (within 2 years)					
Residential Home	5 (2.5)	5 (3.4)	0	0	0.43
Nursing Home	7 (3.5)	7 (4.7)	0	0	0.3
Prison Service	1 (0.5)	1 (0.7)	0	0	0.85
Microbiology					
Known MRSA colonised*	9 (4.6)	6 (4.1)	3 (7.5)	0	0.53
Known MRSA contact	174 (87.9)	132 (88.6)	34 (85)	8 (88.9)	0.82

* available from 146 nurses (total 195 staff) ^available from 147 nurses (total 196 staff).

5.4.2.1.1 Follow up of staff during study

Of 198 recruited participants 7 received only one swab and questionnaire (1 nurse, 3 doctors, 3 physiotherapists) as they left the ICU in the month following recruitment. No participants withdrew from the study. Hence 191 healthcare workers could be followed up. The proportion of staff that completed follow-up questionnaires and were screened per 4-weekly cycle ranged from 81-100% (Table 5.5). Reasons for missed screens and questionnaires included long-term ward leave (annual, sickness, compassionate, maternity/paternity) and bank staff without shifts.

Serial questionnaires were completed to assess whether staff were diagnosed with new medical conditions or medical care or medical treatment during the study (Table 5.7). None were newly diagnosed with diabetes mellitus, eczema, psoriasis, cancer or chronic renal failure during the study. Of 191 participants who underwent >1 questionnaire 84 (42.4%) received medical care at least once during the study period including 61 who sought care by their general practitioner (GP), 35 who received outpatient care, 7 who received inpatient care and 6 requiring surgery. Nurses were significantly more likely to undergo GP consult compared to doctors and physiotherapists (39% vs. 5.6% vs. 0%, $p<0.01$). 55 healthcare workers received at least one course of antibiotics during the study and of these 53 (96.4%) were nurses ($p<0.01$). Overall nurses were significantly more likely to received medical care or treatment than doctors and physiotherapist (57.4% vs. 27.8% vs. 28.6%, $p=0.003$). Interestingly 11 healthcare workers reported a course of antibiotics in a month where they did not receive any medical care (including GP, outpatients, inpatient or surgery).

Table 5.7 Medical care and treatment received by different healthcare worker groups during the study. Some participants received >1 episode of medical care.

	Total n=191	Healthcare worker Nurse n=148	Doctor n=36	Physiotherapist n=7	Significance (p)
≥1 episode of medical care or treatment received	97 (50.8%)	85 (57.4%)	10 (27.8%)	2 (28.6%)	**0.003**
Medical Care during study					
GP	61 (31.9%)	59 (39.9%)	2 (5.6%)	0	**$<$0.01**
Hospital Outpatient	35 (18.3%)	31 (20.9%)	4 (11.1%)	0	0.17
Hospital Inpatient	7 (3.7%)	7 (4.7%)	0	0	0.35
Surgery	6 (3.1%)	5 (3.4%)	1 (2.8%)	0	0.9
Treatment during study					
Surgery	6 (3.1%)	5 (3.4%)	1 (2.8%)	0	0.87
Chemotherapy	0	0	0	0	-
Antibiotics	55 (28.8%)	53 (35.8%)	0	2 (28.6%)	**0.01**
Corticosteroids	21 (11%)	18 (12.2%)	2 (5.6%)	1 (14.3%)	0.5

5.4.2.2 Staff carriage of *S. aureus*

Of 198 staff screened 114 (57.6%) yielded *S. aureus* from nasal swabs at least once during the study period (5% MRSA). The mean prevalence of *S. aureus* colonisation per 4-weekly cycle was 36.2% (MRSA 5.2%) (Table 5.5). There was no significant difference between rates of carriage at study entry and staff groups (59.7% vs. 50% vs. 55.6%, p=0.62). Furthermore whilst the proportion of strains according to methicillin susceptibility were not significantly different (p=0.62) across healthcare worker groups MRSA was not cultured from any doctor's swab (Table 5.8). Staff carriage rates were significantly higher than those observed in patients (57.6% vs. 20.6%, Chi-squared p<0.01)

5.4.2.2.1 Carriage profiles

191 staff members had >1 screen. Carriage status could be assessed in 190 as one staff members isolate failed *spa*-typing. The carrier index was determined for each serially screened staff member (Table 5.8). Of 190 staff 82 (43.2%) healthcare workers had a carrier index of 0 (culture negative on all screens) and 29 (15.3%) had a carrier index of 1 (culturing same genotype on all screens). The remaining 79 (41.5%) had a carrier index between 0-1 (intermittent carriers); 8 culture-positive on all screens with >1 genotype and 71 intermittently culture-positive (52 with same genotype and 19 >1 genotype). There was no significant difference (Chi-squared test, p=0.14) in carriage profiles across staff groups.

Table 5.8 Comparison of *Staphylococcus aureus* nasal carriage and carriage profiles in healthcare workers identified during the study. CI = carrier index. % in parenthesis.

	Healthcare worker				
	Total n=198	**Nurse n=149**	**Doctor n=40**	**Physiotherapist n=9**	**Significance**
Carriage organism					
MSSA	104 (52.5)	80 (53.7)	20 (50)	4 (44.4)	p=0.62
MRSA	8 (4.1)	7 (4.7)	0	1 (11.1)	
MSSA and MRSA	2 (1)	2 (1.3)	0	0	
Uncolonised	84 (42.4)	60 (40.3)	20 (50)	4 (44.4)	
Serially screened*	**n=190**	**n=147**	**n=36**	**n=7**	
Carriage profile					
Persistent non-carriers (CI 0)	82 (43.2)	60 (40.8)	19 (52.8)	3 (42.8)	p=0.14
Persistent carriers (CI 1)	29 (15.2)	19 (12.9)	8 (22.2)	2 (28.6)	
Intermittently carriers (CI 0-1)	79 (41.6)	68 (46.3)	9 (25)	2 (28.6)	

* and successfully *spa*-typed.

5.4.2.2.2 Factors affecting carriage at entry to study

The strength of association between risk factors and *S. aureus* carriage at entry to study was determined using odds ratios (OR) in a univariate analysis (Table 5.9). In those with a significant association ($p<0.05$) multivariate analysis was performed to adjust for confounding factors with univariate associations of $p<0.1$.

114 staff members were colonised on entry screen. The only factor significantly associated with increased risk of carriage was employment in nursing homes. Seven participants worked in a nursing home and they were 11 times more likely (OR 11.1, 95% CI 1.3-94.1, p=0.03) to be colonised with *S. aureus* on entry to the study. This association remained significant (OR 11.3, 95% CI 1.1-113, p=0.04) following multivariate analysis.

Furthermore on univariate analysis there was a trend to increased risk of *S. aureus* carriage in staff who had received outpatient medical care in the preceding two years prior to the study (OR 2.1, 95% CI 0.97–4.4, p=0.056) and in the 5 staff members who worked in residential homes (OR 7.2, 95% CI 0.8-65.6, p=0.08).

Table 5.9 Univariate and multivariate analysis of risk factors associated with *Staphylococcus aureus* carriage in healthcare workers at admission. Significance values were calculated using logistic regression analysis.

				Univariate Analysis			Multivariate Analysis		
Risk Factor	**Total n=198**	**Carriers n=73**	**Non-carriers n=125**	**Odds Ratio**	**95% CI**	**p-value**	**Odds Ratio**	**95% CI**	**p-value**
Male	51	21	30	1.3	0.7–2.5	0.46			
Recent GP	65	23	42	0.9	0.5–1.7	0.76			
Recent outpatient	33	17	16	2.1	0.97–4.4	0.056			
Recent inpatient	18	9	9	1.8	0.7–4.8	0.23			
Recent surgery	15	7	8	1.6	0.5–4.5	0.4			
Eczema	19	8	11	1.3	0.5–3.3	0.62			
Psoriasis	6	3	3	1.7	0.3–8.9	0.5			
Recent antibiotics	93	35	58	1.1	0.6–1.9	0.83			
Recent steroids	16	6	10	1	0.4–3.0	0.95			
Lives alone	22	5	17	0.45	0.2–1.3	0.13			
Lives with partner	123	52	71	1.8	0.95–3.3	0.07			
Lives with flatmates	54	19	35	0.9	0.5–1.7	0.7			
Lives with children	57	20	37	0.9	0.5–1.7	0.7			
Current smoker	37	12	25	0.8	0.4–1.7	0.56			
Recent travel to Europe	143	48	95	0.6	0.3–1.1	0.12			
Recent travel to South America	15	9	6	2.8	0.95–8.2	0.053			
Recent travel to Africa	32	12	20	1.0	0.5–2.3	0.94			
Recent travel to North America	37	9	28	0.5	0.2–1.1	0.08			
Recent travel to South East Asia	51	21	30	1.3	0.7–2.5	0.5			
Recent travel to Australasia	18	8	10	1.4	0.5–3.8	0.48			
Residential Home	5	4	1	7.2	0.8–65.6	0.08			
Nursing Home	**7**	**6**	**1**	**11.1**	**1.3–94.1**	**0.03**	**11.3**	**1.1-113**	**0.04**
Known MRSA carriage	9	3	6	0.8	0.2–3.4	0.8			
Known MRSA contact	174	62	112	0.7	0.3–1.5	0.3			

5.4.2.2.3 Factors affecting staff acquisition during the study

47/191 serially screened healthcare workers were found to acquire *S. aureus* during the main study; 39 went from negative to positive and 8 colonised staff changed strain type. Since serial swabs were taken at 4 weekly intervals, new acquisitions could have occurred at any point during this time. Whilst this study wasn't specifically designed to characterise staff acquisitions I have compared data retrieved from serial staff questionnaires to provide some insight into factors that may be associated with acquisition. In those participants who acquired *S. aureus* their serial questionnaires were evaluated to identify if they received any medical care prior to the acquisition.

Strength of association between recent medical care and *S. aureus* acquisition was determined using odds ratios (OR) through univariate analysis (Table 5.10). In those with a significant association ($p<0.05$) multivariate analysis was performed to adjust for putative confounding factors with univariate associations of $p<0.1$.

In the univariate analysis recent inpatient stay was associated with 4.8 times increased risk of acquisition (OR 4.8 95% CI 1-22.3, $p=0.046$) and a recent course of antibiotics was associated with 2.3 times increased risk of acquisition (OR 2.3, 1.2-4.7, $p=0.018$). Following multivariate analysis only recent antibiotic treatment remained significantly associated with acquisition of *S. aureus* (OR 2.0, 95% CI 1.0-2.0, $p=0.05$).

Table 5.10 Risk of acquiring *Staphylococcus aureus* in healthcare workers during the study. Staff who were serially screened were evaluated. Univariate analysis was used to identify medical and treatment factors associated with *S. aureus* acquisition. Multivariate analysis was performed on those found to be significant to account for confounding factors. Significance was determined using logistic regression analysis.

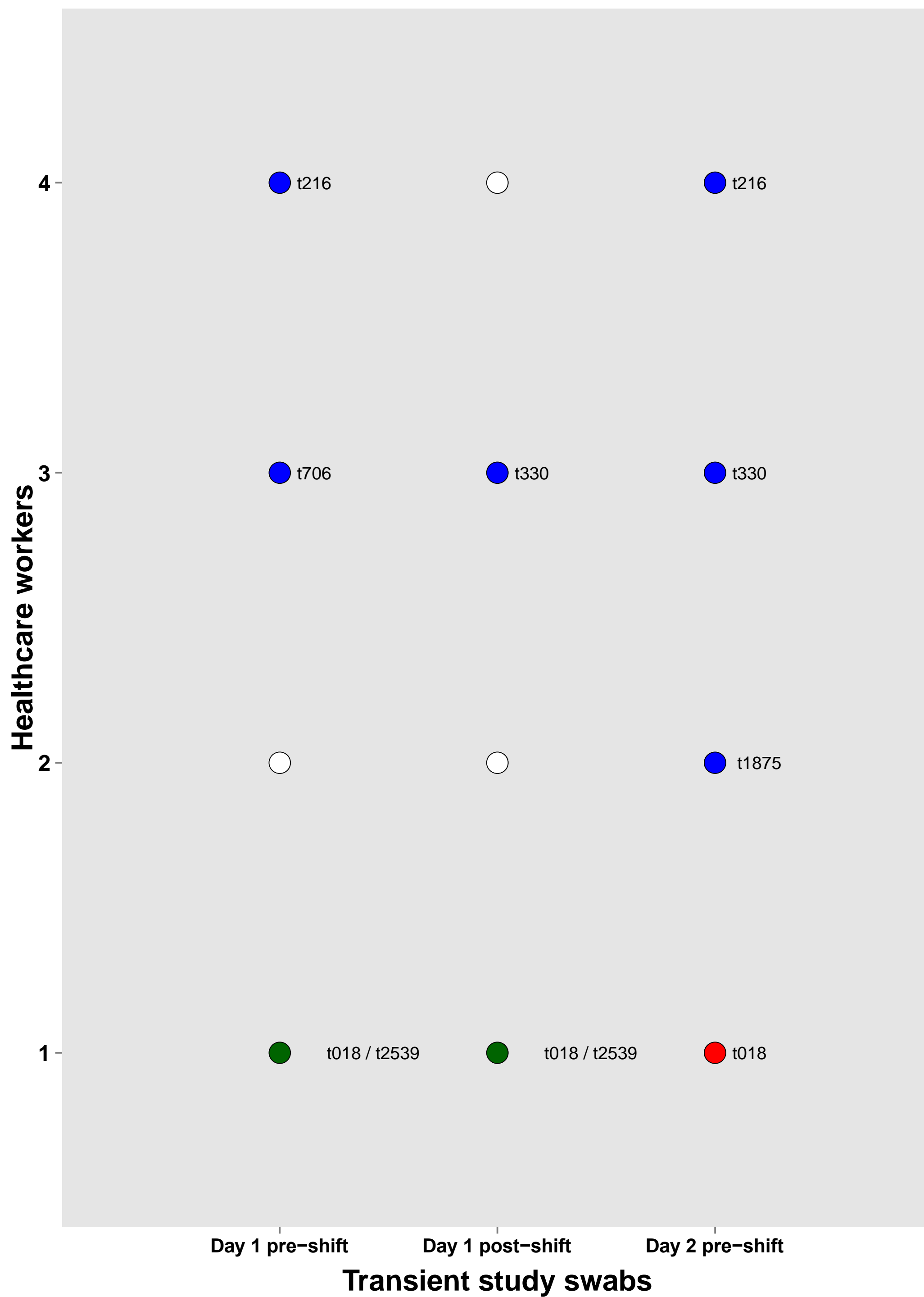
Medical care	n	Univariate Analysis			Multivariate Analysis		
		Odds Ratio	95% CI	p	Odds Ratio	95% CI	p
GP consult	18/61	1.67	0.8-3.4	0.15			
Outpatient appointment	11/35	1.7	0.8-3.8	0.2			
Inpatient stay	4/7	**4.8**	**1.0-22.3**	**0.046**	3.2	0.65-15.9	0.15
Surgery	2/6	1.7	0.3-9.6	0.55			
Antibiotic treatment	20/55	**2.3**	**1.2-4.7**	**0.018**	**2.0**	**1.0-2.0**	**0.05**
Steroid treatment	8/21	1.8	0.7-4.8	0.24			

5.4.2.3 Transient colonisation sub-study

Transient carriage following duty shifts has been observed in healthcare workers and is suggested as a means of transmission to patients.[125] In order to evaluate this 103 nurses underwent nasal screening pre- and post-shift on day 1 and pre-shift on day 2.

Transient carriage was not identified in any staff member. 64 (62%) were persistently culture-negative from all three screens and 35 (34%) persistently cultured the same genotype from all screens. Swabs taken pre- and post-shift from 4 staff members showed discrepant results but none were consistent with my definition of transient acquisition during a shift (Figure 5.5). The first patient had mixed carriage (MRSA t018 and MSSA t2539) on day 1 and cultured a single genotype (MRSA t018) on day 2. The second was culture-negative on both day 1 screens and cultured MSSA t1875 pre-shift on day 2. The third cultured MSSA t706 pre-shift on day one, then cultured MSSA t330 from screens post-shift on day 1 and pre-shift day 2. The relatedness of these *spa*-types was assessed using BURP algorithms, as described in Section 2.6.2. This revealed that they are clonally related, differing by a single repeat duplication within the *spa* gene. The fourth cultured MSSA t216 pre-shift day 1, was culture-negative post-shift day 1, and then culture-positive with MSSA t216 on day 2.

Figure 5.5 Evaluations of four healthcare workers assessed for transient carriage revealing intermittent carriage over the course of 24 hours. Screen culture results are displayed as dots; MSSA (blue), MRSA (red), both MSSA & MRSA (green), and culture negative (white). *spa*-types are annotated to right of screen.



5.4.2.4 Extra-nasal carriage sub-study

In order to evaluate the sensitivity of a nasal swab to detect *S. aureus* carriage multiple site carriage was assessed by simultaneously screening staff's nose, throat, axilla, groin and wounds. 122 nurses were assessed in this way and of these 45 (36.9%) yielded *S. aureus* from nasal swabs (including 35 culturing *S. aureus* from at least 1 other site) and 19 (15.6%) who were culture-positive from extra-nasal swabs alone (including 16 from throat swabs) (Figure 5.6).

Nasal screening alone detected only 70.3% of colonised participants. To derive performance measures for nasal screening alone as a measure of *S. aureus* carriage it was assumed that all true positives are identified through the multisite screening (Table 5.11). Using these assumptions nasal screening alone has a sensitivity of 77%. Combining nose and throat screen culture results increased the *S. aureus* carriage detection rate to 95.3% (identifying 61/64 carriers) and improves sensitivity to 95.5% (Table 5.11). The remaining 3 colonised patients were only detected through groin swabbing.

No demographics, co-morbidities or pre-study medical care (including preceding antibiotic and corticosteroid therapy) were significantly associated with extra-nasal carriage status.

Figure 5.6 Multisite carriage profiles of 64 nurses colonised with *Staphylococcus aureus*. Culture results are shown as dots; culture negative (white), MSSA (blue), MRSA (red), both MRSA & MSSA (green). *spa*-types are annotated to right of swab.

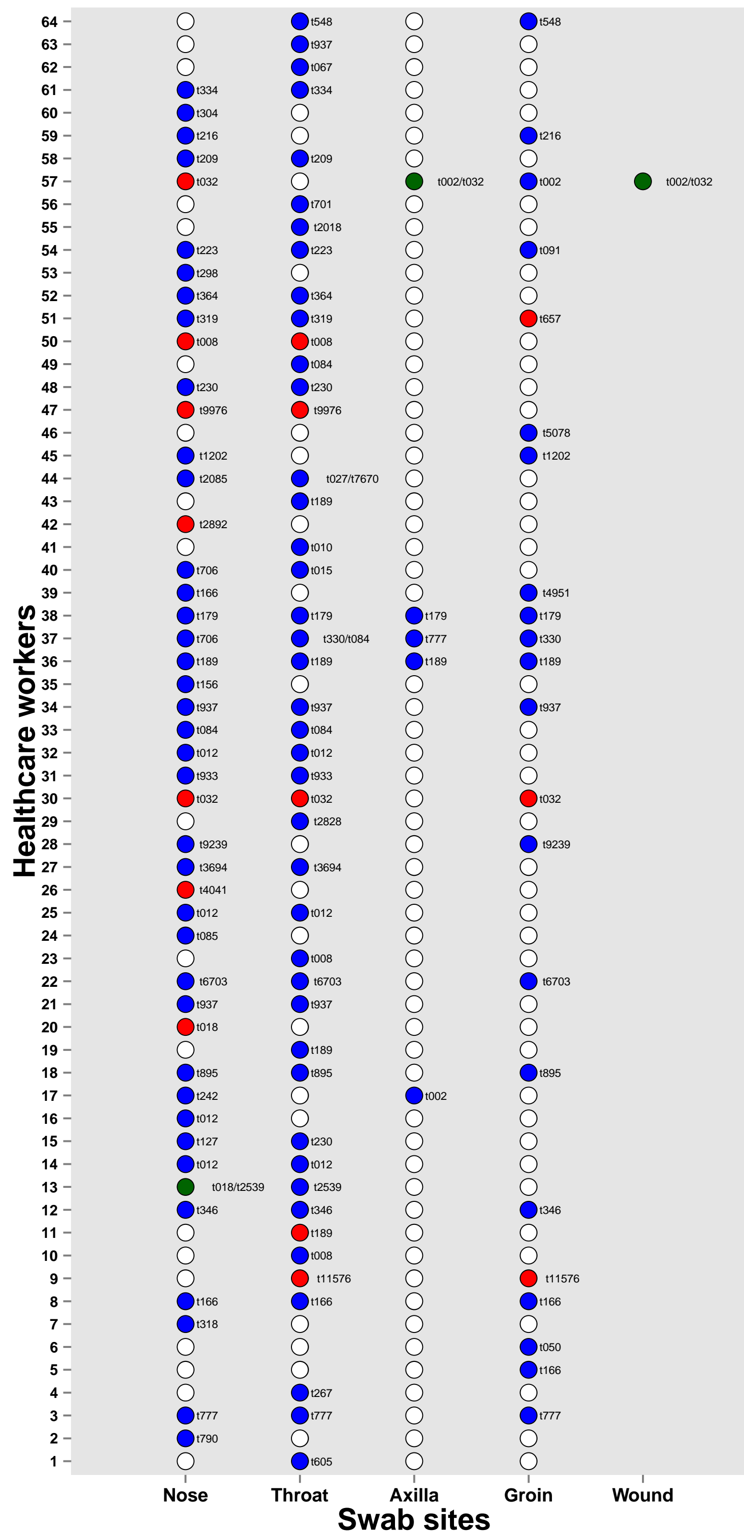


Table 5.11 Measuring the performance of nasal screening alone versus nose and throat screening to detect carriage of *Staphylococcus aureus* in healthcare workers. Of 64 colonised staff 45 were culture-positive from nasal swabs, 16 were culture-positive from throat swabs alone, and 3 from groin alone.

	Nasal alone		Nose and Throat screen	
	Probability (%)	**95% CI (%)**	**Probability (%)**	**95% CI (%)**
Sensitivity	77.1	66.6–85.6	95.5	87.5–99
Specificity	100	93.8–100	100	93.8–100
Positive predictive value	100	94.3–100	100	94.3–100
Negative predictive value	75.3	64.2–84.4	95.1	86.3–98.9

5.4.2.5 Throat colonisation study

An unexpected observation from the extra-nasal sub-study was that 25% (16/64) staff carriers were culture-positive in their throat alone. To confirm that these findings were an accurate reflection of colonisation rates in staff working on our ICU I extended part of the extra-nasal sub-study by performing paired nose and throat swabs at two further time points taken at six monthly intervals. Additional anatomical sites were not swabbed.

Table 5.12 presents the culture results from serial nose and throat swabs taken at three time points during the study. Carriage rates were comparable across the three time points (Chi squared p=0.86) suggesting that initial observations were an accurate reflection of carriage rates.

Table 5.12 Culture results from staff members who received nose and throat swabs during the throat carriage sub-study. Data represents nose and throat culture results from the extra-nasal sub-study taken (Month 0) and from serial nose and throat screens taken at six (Month 6) and 12 (Month 12) monthly intervals.

	Serial Nose and Throat Swabs			
	Month 0	**Month 6**	**Month 12**	**Significance**
Total screened	122	163	158	
Total culture-positive*	61 (50%)	72 (44.2%)	82 (51.9%)	
Nose alone	16 (26.2%)	20 (27.8%)	23 (28%)	p=0.86
Throat alone	16 (26.2%)	17 (23.6%)	23 (28%)	
Nose and Throat	29 (47.6%)	35 (48.6%)	36 (43.9%)	
Culture-negative	61 (50%)	91 (55.8%)	76 (48.1%)	

*by nose or throat swab.

5.4.3 Environment

A total of 2154 environmental swabs were taken from the 28 bed spaces screened during the study period. All bed spaces were screened 15 times except two in HDU that were screened only 9 times as they opened in month 6 of the study. Each bed space screen involved 5 swabs (bed, curtain, floor, keyboard, monitor (Figure 5.2)) except 3 (bed space 10, 26 and 27) that did not have a curtain so only had four swabs. One bed space (bed space 9) was not screened on month 13. Nine individual swabs from different bed spaces could not be taken for practical reasons. The blood gas machine was swabbed 15 times. Air sampling was undertaken from 11 sites (6 ICU and 5 HDU); initially 10 with an additional site in HDU following opening of two beds in month 6. One air-sampling screen in month 7 (ICU) could not be taken due to practical reasons.

5.4.3.1 Presence of *S. aureus* in the environment

At each screening time point 2-11 bed spaces were positive with at least 1 swab; all but 2 of the 28 spaces was positive at least once in the study (Table 5.13). Air sampling was culture positive at every screening time point (Table 5.14). Overall 7.2% of environmental samples taken yielded *S. aureus*. Of 2154 screens taken 173 *S. aureus* isolates (20 MRSA) were cultured; 68 (39.3%) from air samples and 105 (60.7%) from environmental swabs. Almost all positive environmental swabs were from the floor or curtains (83.8%) and together with air sampling made up 156/173 (90.2%) isolates. The median number of isolates cultured from each bed space per month was 4 (IQR 2–6). 20 MRSA isolates were identified from environmental screening. There was no significant difference (Chi squared p=0.4) in the proportion of MRSA isolates cultured from air sampling (10/68 (14.7%)) and bed space screening (10/105 (9.5%)). Isolates obtained from the same bed space at the same time point were frequently of different *spa*-types. Among 15 instances where more than one bed-space swab was positive >1 *spa*-type was identified in 7 (47%). The maximum number of isolates of the same genotype cultured from a bed space during a monthly screen was 3; bed 1 cultured MSSA t177 from curtain, floor and keyboard on month 14. Similarly over time different *spa*-types were identified from the same bed-space. The same *spa*-type identified from the same bed space for >2 months only once and this was an MSSA t015 identified from the floor of bed-space 11 from 3 consecutive floor swabs.

Table 5.13 Distribution of *Staphylococcus aureus* isolates cultured according to screening site around bed space in the intensive care unit (ICU) and high-dependency unit (HDU). There is no bed space 13 in ICU. Curtain swabbing was not available during six screens (NA).

Bed space site	Organism	Bed Space																												Total
		ICU																HDU												
		1	2	3	4	5	6	7	8	9	10	11	12	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	
Bed	MRSA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	MSSA	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	2	1	0	0	0	1	0	2	0	0	0	1	0	9
Curtain	MRSA	0	0	0	0	0	0	1	0	1	NA	0	0	0	0	0	1	0	0	1	0	0	0	0	0	NA	NA	0	0	4
	MSSA	2	0	2	0	2	2	0	2	1	NA	0	0	1	0	1	0	0	1	1	0	1	0	0	0	NA	NA	0	0	16
Floor	MRSA	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	5
	MSSA	4	4	4	1	5	4	4	1	1	1	4	2	3	3	2	3	1	1	3	2	3	1	1	0	1	0	3	1	63
Keyboard	MRSA	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
	MSSA	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	3
Monitor	MRSA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	MSSA	0	0	0	0	0	0	0	0	0	0	1	0	0	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	4
Total		8	4	6	1	7	6	6	4	4	2	6	2	4	4	6	7	2	3	5	3	5	1	3	0	1	0	4	1	105

Table 5.14 Distribution of *Staphylococcus aureus* isolates cultured from environmental sampling in intensive care unit (ICU) and high-dependency unit (HDU) during the study.

Location	Organism	Month															Total
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
ICU Air sampling	MRSA	4	1	0	0	0	0	1	0	0	0	0	0	0	0	0	6
	MSSA	0	0	2	2	2	0	4	5	3	2	2	0	3	8	1	34
ICU Bed space	MRSA	3	0	0	0	0	1	2	0	0	1	0	0	0	0	1	8
	MSSA	2	1	1	3	9	1	5	2	2	5	6	10	8	10	4	69
HDU Air sampling	MRSA	0	0	0	0	3	1	0	0	0	0	0	0	0	0	0	4
	MSSA	0	1	2	0	4	1	0	3	3	0	5	2	1	0	2	24
HDU Bed space	MRSA	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	2
	MSSA	0	1	3	0	1	0	1	3	1	0	5	4	1	1	5	26
Blood Gas Machine	MRSA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	MSSA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total		9	4	8	5	19	4	13	13	9	9	18	16	13	19	14	173

5.4.4 Comparison of patient, staff and environmental isolates

All isolates from patients, staff and the environment had antibiotic susceptibilities determined (including mupirocin) and were *spa*-typed to allow investigation of their role in transmission. The epidemiology of carriage isolates across patients, staff and environment was remarkably similar; MSSA isolates showed diversity over a large number of *spa*-types and MRSA strains were conserved to only a few (Figure 5.7). No mupirocin resistance was identified.

In total 762 isolates were retrieved from patients during the study including 684 from swabs and 78 from clinical specimens. All isolates underwent *spa*-typing and *spa*-types were available on 757; 5 isolates repeatedly failed. 214 *spa*-types were identified. MSSA strains (n=683) showed diversity across 208 *spa*-types, with only two *spa*-types representing over 5% of isolates; t012 (6.1%) and t216 (5.5%). MRSA strains (n=74) were conserved to 18 *spa*-types; the three largest being t032 (32.4%, corresponding to EMRSA-15, CC22), t018 (10.8%, corresponding to EMRSA-16, CC30), and t012 (9.5%).

In total 939 isolates (106 MRSA) were retrieved from staff including 672 from the main nose screening study, 77 from transient carriage sub-study, 78 from the extra-nasal sub-study and 112 from the longitudinal throat carriage sub-study. Of 939 isolates 938 were successfully *spa*-typed; one MSSA isolate repeatedly failed *spa*-typing. 118 different *spa*-types were identified in total. Whilst MSSA isolates showed diversity across 107 *spa*-types MRSA isolates were conserved to 12 *spa*-types; the three largest being t032 (30.2%), t018 (22.6%) and t008 (15.1% isolates, corresponding to CC8).

A total of 173 isolates (20 MRSA) were retrieved from environmental samples including 68 from air sampling and 105 from swabs. All isolates were successfully *spa*-typed. 57 *spa*-types were identified. MSSA isolates showed diversity over 52 *spa*-types. MRSA isolates were conserved over 9 *spa*-types with 70% associated with 3 *spa*-types; t032 (30%), t018 (20%), t2892 (20%).

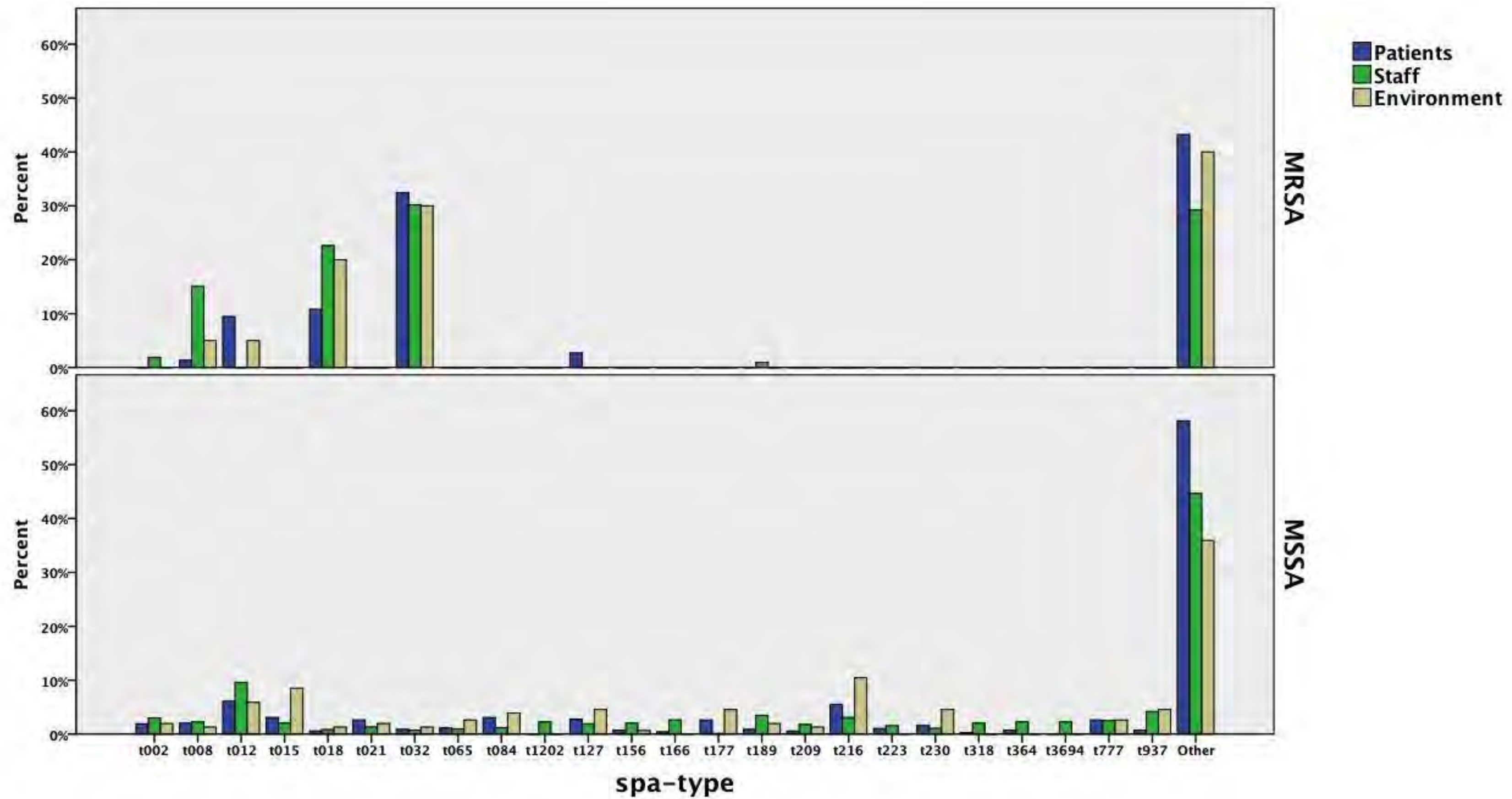
5.4.4.1 Determining the relatedness of strains by *spa*-grouping

The contribution of patient, staff and environment to acquisitions was based on *spa*-type. As described previously some *spa*-types are highly related and subsequently

there is concern that using *spa*-type alone may miss highly related strains of a different *spa*-type. Subsequently *spa*-grouping was performed to identify those isolates that were highly related. As described in Section 2.6.2 Based Upon Repeat Pattern (BURP) analysis can be used to measure the relatedness of *spa*-types by allocating cost difference based on variations within the *spa* gene. Optimal parameters (cost ≤4 and exclusion of strains with <5 repeats within the *spa* gene) have been determined to identify related strains based on concordance with Multi-Locus Sequence Typing (MLST).[210] Isolates within a *spa*-group were presumed to be clonally related.

All patients, staff and environmental isolates successfully *spa*-typed were analysed using BURP algorithms to measure their relatedness and subsequent allocation into *spa*-groups. In total 18 *spa*-groups were identified, the largest containing 406 strains and 38 different *spa*-types. This implies that there are larger numbers of highly related isolates involving different *spa*-types across patient, staff and environmental samples.

Figure 5.7 Proportion of *spa*-types according to methicillin susceptibility identified from patients (blue), staff (green) and the environment (cream) during the study. *spa*-types representing <1% of the total for each category were grouped together (Other).



5.4.5 Patient acquisitions

During the 14 month study period 1125/1854 (60.7%) sampled admissions had at least 1 follow up screen and thus could be evaluated for acquisition. 101 acquisitions (21 MRSA) in 95 patients were identified comprising 72 instances for culture-positive following negative screen (Patients 1-71 in Figure 5.8) and 29 changes in genotype (Patients 71-95 in Figure 5.8). Overall this corresponds to an acquisition rate of 9%. This is higher than the acquisition rate of 6.5% measured in the preceding chapter. However, while 81/101 (80.2%) acquisitions were identified through routine clinical screening swabs 20 were identified through clinical specimens including 16 sputum samples, 3 blood cultures and 1 bronchoalveolar lavage. Such clinical specimens were not used to assess acquisition in the work described in the preceding chapter.

4 patients had > 1 acquisition event. The first (Patient 70 in Figure 5.8) acquired two MSSA strains of differing genotypes (t223 and t382, cost 8) following 2 culture-negative screens. The second (Patient 71 in Figure 5.8), who spent 43 days in ICU, underwent four acquisitions; newly acquiring MSSA t491 on day 3, changed to MSSA t1875 5 days later, changed to MSSA t346 30 days later, and then changing again to MSSA t015 1 day later. The third and fourth (Patients 72–73 in Figure 5.8) underwent two genotype changes.

Of 101 acquisitions 13 were not evaluable by *spa*-typing (9/72 new and 3/28 strain changes) due to 12 un-retrieved acquisition isolates (Patients 2, 11-13, 16-17, 26, 35, 64, 75, 77, 94) and 1 strain failing *spa*-typing (Patient 49).

In order to determine whether related *spa*-types could account for acquisitions where a change in genotype was observed the relatedness between isolates was measured using BURP analysis. Of the 25 evaluable acquisitions associated with changes in genotype 2 (8%) involved isolates that were clonally highly related (cost ≤1). The first (Patient 73 in Figure 5.8) involved a change from MRSA t032 to MSSA t032 (cost 0) and whilst *spa*-cost alone suggests clonally relatedness the isolates have discordant methicillin susceptibility patterns. The second (Patient 79 in Figure 5.8) yielded MSSA t11572 on admission and, following a negative screen, yielded MSSA t11573. These novel *spa*-types are highly related (cost 1) suggesting within host diversity rather than novel acquisition.

Figure 5.8 Culture results of screens from 95 patients acquiring *Staphylococcus aureus* on the high dependency care units. Each circle represents a screen; culture negative (white), methicillin sensitive *S. aureus* (blue), methicillin resistant *S. aureus* (red). *spa*-types are presented above screen where available.

5.4.5.1 Time to acquisition

In order to evaluate whether time spent in ICU/HDU affected rate of acquisition the date of swabs was used to infer times to acquisition. Across all patients the median ward admission time was 3 days (IQR 1.6-6). The overall time from admission to acquisition ranged from 1–60 days with a median time of 4 days (IQR 2–7). This is comparable to acquisition times observed in the preceding chapter (median 4 days, IQR 2-8.5). There was no significant difference in time to acquisition according to strain methicillin susceptibility and type of acquisition (Table 5.15).

5.4.5.2 Acquisitions between ICU admissions

During the study period there were 173 episodes of re-admission involving 136 patients. Of these 136 re-admitted patients 11 were culture-negative during their first admission and were subsequently culture positive on readmission screen, suggesting acquisition between ICU admissions. The mean time between these admissions of these patients was 65.4 days (range 4–170 days). In addition, one patient underwent strain change between ICU admissions; culturing MSSA t216 on primary admission and re-admission 218 days later yielding a clonally unrelated (MSSA t267, cost 16) isolate according to BURP analysis. One patient had 14 re-admissions during the study period where MSSA t382 was cultured during 4 admissions. During the additional 10 admissions the patient was culture-negative, although only single screens were taken during these admissions.

5.4.5.3 Acquisition rate

Acquisition rates were estimated in two ways. Firstly, the rate was calculated using the time at-risk of acquisition for each primary acquisition (n=95) (Table 5.16). Of the 1125 serially screened patients the total at-risk time was 6428 patient days giving an acquisition rate of 14.8 per 1,000 patient days (95% CI 12-18). The rate was significantly higher for MSSA than MRSA (11.8 vs. 3.0 per 1,000 bed days, $p<0.001$). In the preceding chapter the acquisition rate was 9.9 per 1,000 patient days and there was no significant difference according to methicillin susceptibility. Secondly, the acquisition rate was calculated using time from first swab until discharge (7901 patient days) in all patients (including acquisitions). This approach gave a slightly lower estimate of rate at 12 per 1,000 patient days (95% CI 9.7–14.7) and MSSA rates remained significantly higher than MRSA (9.6 vs. 2.4 per 1,000 bed days, $p<0.001$).

Table 5.15 Comparison of time to acquisition according to (i) methicillin susceptibility and (ii) type of acquisition

	Time to acquisition (days)		Log-Rank
	Mean (95% CI)	Median (95% CI)	(Mantel-Cox)
MRSA (n=20)	7 (4.6–9.5)	3 (2.3–3.7)	p=0.193
MSSA (n=81)	8.8 (5.8–11.9)	7 (1–13)	
New acquisition (n=72)	7 (4.7–9.4)	3 (2.2–3.8)	p=0.483
Strain change (n=29)	8.3 (4.4–12.3)	4 (2.5–5.5)	

Table 5.16 Rates of *Staphylococcus aureus* acquisition identified in patients during their admission to the intensive care unit and high-dependency unit. Acquisition rates were calculated using Poisson regression.

	Acquisitions (% of at-risk admissions, 95%CI) (n=1125)	Acquisition rate per 1,000 patient days (95% CI) (patient days = 6428)	Significance (p)
Overall	95 (8.4%, 6.8–10)	14.8 (12–18.1)	
MRSA	19 (1.7%, 1–2.5)	3.0 (1.8–4.6)	**p<0.001**
MSSA	76 (6.8%, 5.3–8.3)	11.8 (9.3–14.8)	

5.4.6 Patient acquisitions explained from other patients

Of the 88 acquisitions evaluable by *spa*-typing and epidemiological data 21 (23.9%) met the criteria of patient-to-patient transmission according to *spa*-type and overlapping stay (4 MRSA). To assess whether highly related *spa*-types could account for patient-to-patient transmission the criteria were broadened to include any patient isolate from the same *spa*-group as the acquisition isolate. This increased the number of potential patient-to-patient transmissions to 40 (45.5%). In the preceding chapter 14% met the criteria of patient-to-patient transmission, increasing to 43% when *spa*-group was used.

Of 21 patient-to-patient transmissions identified by *spa*-type and overlapping stay, four involved isolates where the same *spa*-type was not observed in any healthcare worker or environmental sample preceding the acquisition. This included an outbreak of MSSA t190 involving two patients with overlapping admissions acquiring the same genotype on the same day; this genotype was not identified in any staff or environmental sampling. These would support the conclusion that patient-to-patient transmissions occurred.

In addition to these 21 there were seven acquisitions where a matching genotype was only observed in patients admitted to the unit prior to acquisition. This suggests patient-to-patient transmission via an intermediate vector may have occurred between patients lacking temporal relationships.

5.4.7 Patient acquisitions explained by staff

The contribution of staff members towards patient acquisitions was evaluated. Results of healthcare worker sampling were compared with patient acquisitions and environmental sampling to infer putative staff-to-patient transmission. This revealed that in 44/88 acquisitions, staff members could be implicated as a source on the basis that the same *spa*-type had been found in a staff member working in the department at the time of the patient acquisition. Whilst it was possible to determine whether a healthcare worker worked on the ward at the same time as a patient data were unavailable to show direct physical contact between staff and patients. Of these 44 instances where staff to patient transmission could have occurred 16 were part of 6 outbreaks and the remainder were single acquisition events.

5.4.7.1 Healthcare workers involved in outbreaks

Six outbreaks identified during the study involved acquisition of a *spa*-type also carried by a staff member. These are described below.

Outbreak 1: Twelve patients cultured MSSA t015 during the study (patients A-L in Figure 5.9). An outbreak was observed involving 6 of these patients who acquired MSSA t015 during their ICU stay; 4 of these acquisitions occurred over a 30-day period (Patients G, I, J, K). The ICU stays of these 4 patients overlapped with each other and with the stay of another patient who was colonised with MSSA t015 at ICU admission (Patient H). Also in keeping with the possibility of an outbreak was the fact that 4 patients acquiring MSSA t015 occupied adjacent beds (11-15) in a 4-bedded area of the ICU (Figure 5.1). MSSA t015 was cultured from 5 staff members including (i) a nurse who was persistently colonised with the strain during the outbreak, (ii) a nurse who yielded MSSA t015 from a throat swab preceding the outbreak (not during outbreak), (iii) a doctor who was not working on the unit at the time of the outbreak, and (iv) two staff members (nurse and physiotherapist) who cultured MSSA t015 during the outbreak following >5 preceding culture-negative swabs suggesting new acquisition. MSSA t015 was first identified in the environment 2 weeks preceding the outbreak (swabs and air sampling) and was persistently cultured from the floor of bed space 11 on 3 consecutive monthly screens. A further acquisition (Patent L) occurred 39 days later with no overlapping patient stay.

Outbreak 2 & 3: Thirteen patients cultured MSSA t127 and 5 acquired this genotype during their admission. Two outbreaks were observed involving patients colonised with MSSA t127. The first involved three patients with overlapping stays of whom two acquired MSSA t127 in May 2012 over 10 days; the first patient was in bed 5 of the ICU and the second in bed 20 of the HDU. This genotype was last detected in a patient discharged 67 days previously. During their admissions 2 nurses (both with ≥2 preceding negative screens) became culture-positive for MSSA t127, suggesting new acquisition. The same *spa*-type was also identified in HDU environmental screens from bed spaces and air sampling surrounding bed 20 at the same time. The second outbreak involved three patients who cultured MSSA t127 in December 2012, seven months after the previous outbreak. Two of these patients acquired isolates of this genotype over 12 days although their admissions did not overlap. MSSA t127 was last detected in one patient discharged 49 days previously. One nurse working on the ward at the time of the outbreak was colonised with a strain of the same genotype. MSSA t127 was not cultured from the environment during this outbreak and was last detected in environment sampling in June 2012.

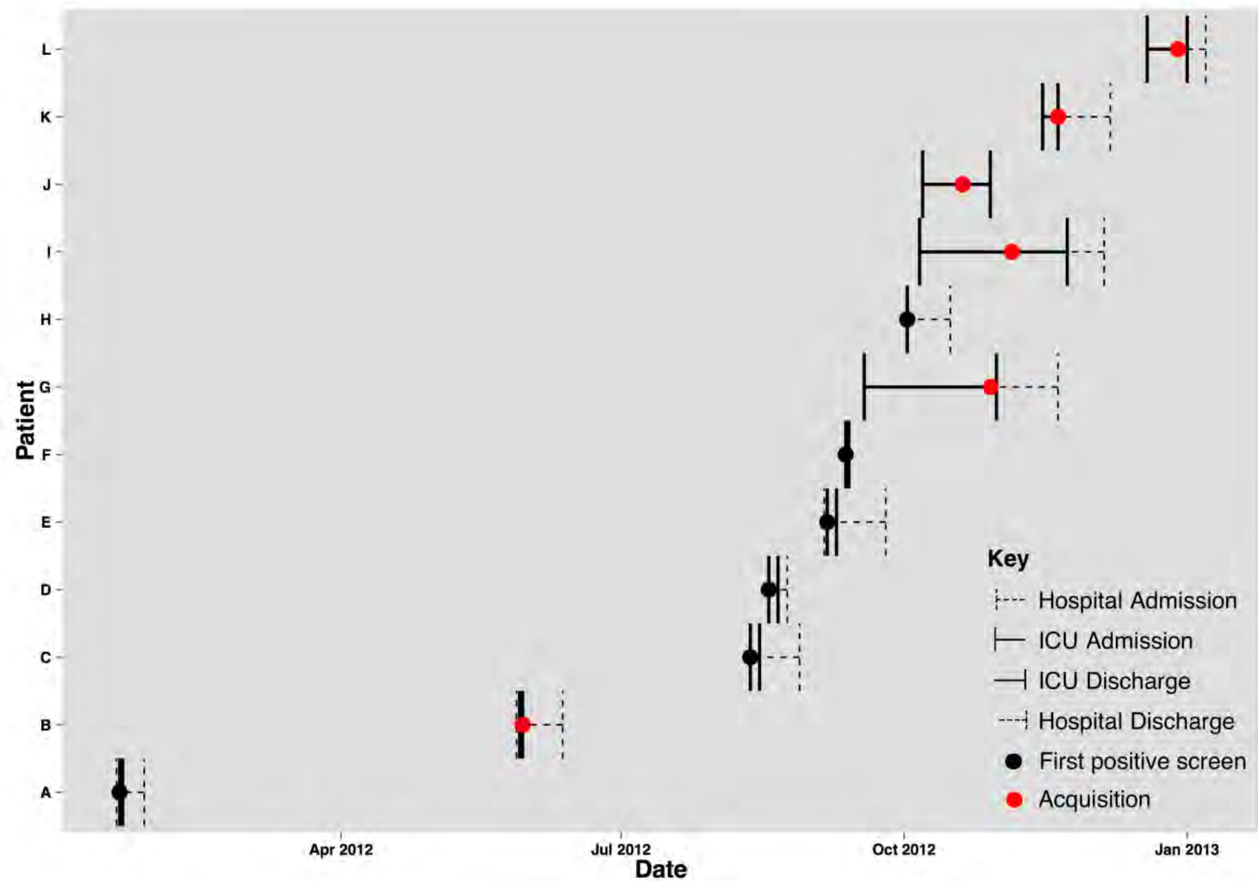
Outbreak 4: Eighteen patients cultured MRSA t032 during the study and 6 acquired their strain during admission. An outbreak was observed involving three patients of whom two acquired this genotype over 7 days in March 2012. Whilst their admissions did not overlap with each other one acquisition did overlap with another colonised patient. This genotype was last detected in a patient 34 days previously. Three staff members working on the ward at the time of the acquisition were colonised with a strain of the same genotype. MRSA t032 was cultured from air sampling at the time of the outbreak and was last detected in the environment in November 2011.

Outbreak 5: Four patients cultured MSSA t148 during the study. An outbreak was observed involving two of these patients with overlapping ICU admissions who both acquired their strains over two days in December 2012. This genotype was last detected in a patient discharged 69 days previously. At the time of the outbreak one nurse working on the ward was colonised with the same genotype. Whilst ICU environmental screens taken at the time were culture-negative for MSSA t148 this genotype was cultured from a bay in HDU from where one of the patients had

recently been transferred. This was the only time this genotype was identified in the environment.

Outbreak 6: Six patients cultured MSSA t267 during the study. An outbreak was observed involving two of these patients with overlapping admissions who acquired this genotype over 3 days in March 2012. This genotype was last cultured from a patient who also acquired it 34 days previously. No environmental carriage of MSSA t267 was identified. A single nurse working in the department during all three of these acquisitions was found to have throat colonisation with the same genotype.

Figure 5.9 Hospital and intensive care unit (ICU) admissions of 12 patients (A–L) culturing MSSA t015. 6 acquisitions were identified and 4 occurred over a short period of time (G, I-K). Each acquisition had at least one preceding screen that was culture-negative or yielded an isolate of a different *spa*-type.



5.4.7.2 Healthcare worker involvement in acquisition events

In addition to the outbreaks described above there were 28 acquisitions of *spa*-types that were also carried by a staff member. To illustrate these eight are described below.

Vignette 1: One patient acquired MSSA t005 in June 2012, a genotype that had not been cultured from another patient or the environment in the preceding four months. A doctor who began work in the department two weeks before the acquisition was colonised with MSSA t005.

Vignette 2: One patient acquired MSSA t037 in October 2012. The last patient known to be carrying this *spa*-type was discharged 99 days earlier and no environmental samples revealed this *spa*-type. A doctor was colonised with MSSA t037 at the same time as the acquisition.

Vignette 3: One patient acquired MSSA t189 in December 2011. No isolate of this *spa*-type had previously been found in patients or the environment. A nurse working on the ICU at the time of the acquisition had been found to carry this *spa*-type in their throat on a sample taken 47 days earlier.

Vignette 4: One patient acquired MSSA t777 in January 2012. This *spa*-type was not found in any other patient or environmental samples preceding the acquisition. At the time of acquisition an isolate of the same *spa*-type was cultured from a nose swab from a nurse working on the unit.

Vignette 5: One patient acquired MSSA t937 in February 2012. No other patients or environmental swabs yielded the same *spa*-type prior to the acquisition. At the time of the acquisition three nurses were colonised with the genotype including one becoming culture-positive following 4 negative cultures suggesting new acquisition.

Vignette 6: One patient acquired MSSA t032 during his ICU admission (bed 10) in November 2012. No other patients cultured this *spa*-type. MSSA t032 was cultured from ICU environmental swabs in a separate side room (bed 16 & 17) 15 days before the acquisition. At the time of the acquisition a nurse cultured the *spa*-type from a nose swab.

Vignette 7: One patient acquired MSSA t346 in October 2012. This *spa*-type was not cultured from any other patient or environmental screen. At the time of the acquisition MSSA t346 was cultured from two nurses including one newly culturing *S. aureus* following ten culture-negative nose swabs.

Vignette 8: One patient acquired MSSA t304 during their HDU admission in May 2012. This *spa*-type was not cultured from any other patient during the study period. At the time of the acquisition MSSA t304 was cultured from the floor sampling in ICU and from nasal swabs from two healthcare workers.

5.4.7.3 Value of throat screening

Given that *S. aureus* carriage was identified in the throat alone for 27 healthcare workers there is a possibility that throat carriage could contribute to patient acquisition. Two examples have already been described above; the outbreak of MSSA t267 and vignette 3. Additionally there are three acquisition events which illustrate that this may be a real possibility.

One patient acquired MSSA t091 in June 2012. This genotype was last cultured from another patient in May 2012 (30 days previously) and had never been cultured from environmental samples. On the day of the acquisition the same genotype was cultured from a throat screen in a nurse having previously been culture-negative on six preceding monthly nose screens and culturing a different genotype from throat screen 6 months beforehand.

One patient acquired MSSA t230 in March 2012. The same genotype had not been cultured from another patient since December 2011. At the time of the acquisition environmental screening cultured MSSA t230 for the first time. A nurse working in the unit at the time of the acquisition cultured this genotype from their most recent throat screens taken in December 2011.

One patient acquired MSSA t065 (in bed space 3) in July 2012. No other patient had had this genotype isolated for >5 months. Whilst this genotype had not been cultured from the environment preceding the acquisition it was grown from the air and floor (bed space 5) on the screen taken 20 days later. However a nurse working on the ward had MSSA t065 identified in a nose and throat swab taken in May 2012 having

previously been culture negative at both sites. Interestingly prior to the acquisition she had seen her GP and received a course of antibiotics. An explanation could be that the staff member suffered from (and was subsequently treated for) a respiratory tract infection, a condition commonly associated with coughing. Working on the ICU with a symptomatic respiratory infection could have resulted in dispersal (and hence transmission) of an isolate colonising her throat.

5.4.8 Patient acquisitions and associated environmental contamination

Environmental contamination around the bed space of a patient acquiring *S. aureus* was frequently detected. In addition to previously described vignettes there are four further scenarios observed where the environment was associated with patient acquisition.

Firstly, a patient on HDU (bed space 21) acquired MSSA t056 in September 2012 at a time when this genotype had not been cultured from another patient for >6 months, or any staff member screen during the study. Environmental screens taken during the 15 days after the acquisition cultured MSSA t056 from samples taken in the same HDU bay as the patient. Environmental screens taken four weeks later did not culture this genotype.

Secondly, a patient acquired MSSA t177 in November 2012 when no other patient or healthcare worker cultured the same genotype before the acquisition. After an initial admission to ICU the patient was transferred to HDU where environmental screening cultured, for the first time, MSSA t177 from swabs and air samples.

Thirdly, a patient acquired MRSA t012 in November 2011 at a time when no other patient or healthcare worker had cultured the same genotype. At the time of the acquisition MRSA t012 was identified on air sampling in the ward of the patient. This was the only time this genotype was detected in the environment during the study.

Fourthly, a patient acquired MSSA t382 in August 2012. This genotype had not been detected in a patient for 26 days. No healthcare worker cultured MSSA t382 during the study. At the time of the acquisition this genotype was detected in an air sample

in the same ward as the patient. This was the only time it was detected in environmental sampling.

5.4.9 Unexplained patient acquisitions

25 (28.4%) acquisitions did not have a matching *spa*-type donor identified in patients, healthcare workers or environmental samples. In order to assess whether related *spa*-types could account for these unmatched acquisitions BURP analysis was used to identify related strains. Using *spa*-groups to identify donors reduced the number of acquisitions without potential donors to 8 (9.1%).

5.5 Discussion

The work described in this chapter attempts to determine whether acquisitions of *S. aureus* among patients on ICU that are not explained by patient-to-patient transmission may be explained by acquisition from staff or the environment. By intensive staff screening and environmental sampling, and using *spa*-typing to infer relatedness, the study shows that a considerable proportion (54.5%) of acquisitions may relate to staff or environmental sources.

In assessing staff carriage of *S. aureus* I have made three other important observations:

1. carriage rates of *S. aureus* are higher among staff than patients
2. working in a nursing home was a dominant risk factor for *S. aureus* carriage by staff
3. a significant proportion of staff (13%) carry *S. aureus* in the throat but not the nose and in six instances (including an outbreak) this was potentially implicated in patient acquisition of *S. aureus*.

5.5.1 Patient carriage

Carriage rates in patients were determined as part of an on-going collection of patient sampling, continuing from work described in Chapter 4. Patient demographics, length of stay on the unit, and proportion of patients screened were comparable between this and the previous study. But there were some important differences observed. *S. aureus* carriage rates in this study were higher than previously identified (20.7% vs. 16.5%, p<0.001). There were no significant differences in patient cohorts or any changes to infection control policies observed to account for this observation. One difference in this study was that clinical samples were retrieved in addition to screening swabs. Interestingly only 12/382 colonised patients cultured *S. aureus* from clinical specimens whilst their screening swabs were culture-negative. These patients only contributed towards a small increase in the observed carriage rate from 20% (370/1854) to 20.6% (382/1854). This suggests that additional clinical sampling did not account in full for the differences observed. Conversely, in this study MRSA carriage at admission was significantly lower than rates previously observed (5% vs. 2%, p<0.001), which is likely to reflect a

background of falling rates and enhanced MRSA infection control policies. Furthermore patients who spent <24 hours in hospital prior to coming into ICU had significantly higher rates of carriage than those who had admission to other wards prior to ICU admission. This suggests that carriage is influenced by infection control measures or preceding antibiotics received prior to coming to ICU.

Whilst rates of carriage in patients were higher than previously observed in our hospital they remain lower than those reported elsewhere.[73] To investigate whether this observation was influenced by users performing the sampling or culture methods used intensive sampling was undertaken. The intensive screening was performed instead of routine methods so whilst results cannot be directly compared these provide useful insight into possible differences in techniques. There was no evidence to suggest that a research nurse performing the swabs impacts on the rate of positivity. A trend was observed for broth enrichment to be associated with a 42% higher relative positivity rate, but the data were also compatible with no effect ($p=0.22$). These data suggest that positive rates using standard methods might be lower, but this is of borderline significance and given the variations observed in rates at different time points it is also possible that this is a result of random variations.

5.5.2 Staff carriage

Healthcare workers have been implicated in a number of reported outbreaks.[124,171] These studies commonly use conventional typing methods coupled with epidemiological data to evaluate transmission. Whist providing insights into the role of staff many of these studies are limited by incomplete sampling frames or retrospective analysis. In order to implement effective infection control measures to control acquisition of *S. aureus* a clear understanding of transmission routes is essential.

The prevalence of *S. aureus* carriage in staff members per month (29.5–39.7%) was comparable to rates published in the literature.[124] There was no significant difference in carriage rates across healthcare worker groups. In this study carriage rates were significantly higher in staff than patients (57.6% vs. 20.6%, $p<0.01$), which is in keeping with decolonisation regimens and antibiotics having an effect on carriage status. Interestingly despite this observation the diversity of *spa*-types colonising

healthcare workers was less than in patients. This suggests that there are variations in strains carried by staff and patients.

A striking observation was that carriage rates in healthcare workers were underestimated by nose swabbing alone. In this study we found that nasal carriage alone was only 77% sensitive in detecting *S. aureus* carriage. 13% staff cultured *S. aureus* from throat swabs alone and by coupling nose and throat screens the sensitivity was increased to 95.3%. Few studies have evaluated screening methods in healthcare workers but this is consistent with previous reports evaluating patients. Struelens *et al.* (2009) showed that nasal screening alone for MRSA has a sensitivity of over 80%, increasing to 95% when additional sites are concurrently screened.[335] Mertz *et al.* (2007) compared nasal and throat screening in 2966 patients and found that 12.8% cultured *S. aureus* from throat swabs alone, suggesting that throat swabs increase sensitivity by 25.7%.[336] Whilst similar results have been found in other patient studies[337,338] others have failed to show the benefit of adjunctive throat screening.[339] Throat carriage is likely to represent an under-appreciated reservoir in hospital staff and reflects recommendations that suggest dual nose and throat swabs when staff screening is necessary.[124] Throat colonisation also appears to play an important role in transmission. There were six scenarios where a putative staff donor was only identified through throat swabs, including an outbreak of MSSA. Failure to perform throat screens on patients as part of routine clinical practice is likely to result in a failure to identify a cohort of colonised putative donors. This also illustrates a potential weakness in identifying patient donors as throat swabs are not performed as part of routine *S. aureus* screening in our trust.

In this study there was a lack of evidence for transient carriage of *S. aureus* in healthcare workers. This is contrary to Cookson *et al.* (1989) who identified transient carriage in 46% staff[125] and other authors who have reported transient carriage associated with patient-to-patient transmission.[125,168,340] One explanation for the difference may relate to variations in methodologies used. In this study serial nasal screens were taken to detect transient carriage whereas other studies have used both nasal and hand swabs. Whilst these may account in part for differences observed, the Cookson study identified transient carriage through hand screening in only a minority (7.7%) of cases.[125]

Staff members who worked in nursing homes were found to be significantly more likely to carry *S. aureus* carriage at the time of entry to the study. While *S. aureus* carriage is common in care home residents[341,342] it remains unclear why contact with other healthcare settings would increase risk of *S. aureus* carriage as these staff work in a healthcare setting on a daily basis. Furthermore, the large confidence intervals observed reflect the small number of staff within these groups. Consequently, further work would be required to validate these findings.

Acquisition of *S. aureus* by healthcare workers in our ICU was observed during the study. We found that recent antibiotic use was significantly associated with acquisition. To my knowledge no previous study has evaluated the effect of antibiotics on staff acquisition of *S. aureus*. In relation to patients other authors have observed this association previously.[73,328,343] A plausible reason for this is that antibiotics alter the composition of colonising organisms permitting colonisation with other organisms including *S. aureus*. Whilst these data provide insight into healthcare worker carriage dynamics and effects of medical care on acquisition the timing of the events is variable and a detailed intensive study would be required to fully understand these associations.

5.5.3 Patient acquisitions

101 acquisitions were identified in 95 patients during the study period, corresponding to an acquisition rate of 14.8 per 1,000 patient days. This acquisition rate was significantly higher that those observed in the previous study (14.8 vs. 9.9 per 1,000 bed days; p=0.035). Whilst a true increase in rates is plausible it is more likely that these differences are related to the inclusion of clinical isolates. In this study 20 acquisitions were identified from clinical samples and if these were excluded the increase in acquisition rates between studies was non-significant (9.9 vs. 11.7 per 1,000 bed days, p=0.45). Acquisitions could be attributed to transmission from another patient in only a minority of cases (23.9%). These results, based on *spa*-type and epidemiological data, are comparable with those observed in Chapter 4 (13.5% vs. 23.9%, p=0.29).

A striking observation was that staff-to-patient transmission was common. There were 44 instances (50%) where healthcare workers were implicated as putative donors, including six previously undetected outbreaks. Most of the literature relating

to the role of healthcare workers in transmission to patients is based on MRSA, particularly in the setting of outbreaks. Ward staff are reported to be associated with 25.5% of MRSA acquisitions[124] and 6% outbreaks.[171] Conversely Blok *et al.* (2003) evaluated outbreaks of MRSA in a Dutch hospital over 10 years and reported that healthcare workers were involved in 76% of MRSA outbreaks.[132] WGS data were unavailable at the time of writing this thesis but based on work undertaken in Chapter 4 the higher resolution offered by sequencing technology is likely to exclude many of these putative transmissions and also identify previously undetermined transmission events and acquisitions.

S. aureus was infrequently recovered (7%) from the environment in this study. Whilst this suggests that current cleaning methods are effective it is also likely that the sensitivity of environmental screening is low as only a limited number of sites could be screened. Multiple strains were found in nearly half (47%) of bed-spaces with >1 culture positive swab and these varied over time suggesting transient contamination by patients, staff or visitors. Interestingly at the time of acquisitions environmental contamination with the same genotype was commonly observed. This is likely to reflect shedding from a donor (staff, patient, other) or from the patient newly acquiring it.

A key finding is that 28% of evaluable acquisitions in this study did not have a matching *spa*-type donor and, hence, a putative route of transmission failed to be established. This is despite screening over 95% staff members, patients and extensively sampling of the environment. One explanation is that unscreened staff and patients contributed towards acquisitions although as relatively few staff (n=10) and patients (n=79) were unscreened they would have to contribute towards a large number of acquisitions. Furthermore, this study was designed to include healthcare workers with direct patient contact. It is plausible that other ward staff members (such as porters, radiology staff, pharmacists) may also play a role in transmission. In addition visitors may represent a group of putative donors who were not assessed in this study.

As highly related isolates can be allocated to different *spa*-types due to subtle genetic variations within the *spa* gene (repeat duplications, point mutations) it is plausible that some patient, staff and environmental donors may be missed using *spa*-type

alone. To evaluate this all isolates were clustered into *spa*-groups of highly related *spa*-types; this was determined using BURP algorithm. This method increased the number of putative donors (patients, staff and environment) to >90%. From work using WGS undertaken in the preceding chapter only one acquisition was associated with a *spa*-discrepant donor. Whilst it is unlikely that *spa*-grouping will increase the number of true missed donors in short-term transmission events further evaluation using WGS is required.

5.5.4 Strengths of the study

A major strength of this study is that healthcare recruitment rates were high (>95%) and no staff member withdrew from the study. This provides confidence in the interpretations that have been made regarding the role of ward staff. Furthermore 95% of patients were sampled for *S. aureus* during their admission providing assurance that patients still contribute towards a minority of ICU acquisitions.

5.5.5 Limitations

This study had some limitations. First, WGS data were not available at the time of analysis. Whilst putative transmission events were made using conventional approaches (*spa*-typing and overlapping stay) results of work employing WGS undertaken in Chapter 4 would suggest that only a minority would be confirmed as true transmissions, and additional transmissions (and acquisitions) are likely to be identified using sequencing data that are invisible by traditional methods.

Second, single nasal swabs were used to identify *S. aureus* carriage in healthcare workers. I have shown that nasal swabbing fails to identify 30% *S. aureus* carriers. Consequently some carriers may have been falsely identified as culture-negative which would result in missed patient and staff carriers. By combining nose and throat screens in healthcare workers the sensitivity of detecting *S. aureus* carriage increased to 95%. Further work is required to evaluate whether similar results would be found in patients. If so, this would strongly support the need to undertake adjunctive throat sampling in routine clinical practice.

Third, doctors and physiotherapists were not evaluated during the first 6 months of the study that likely resulted in missed donors during this time. However it is important to note that *spa*-matching donors were not identified during times when all

staff groups were enrolled in the study, so this may not account for all uncharacterised acquisitions. Furthermore up to 19% of recruited healthcare workers missed monthly screening, commonly due to sickness or leave. Whilst it is plausible that these healthcare workers represent unidentified donors their absence from the hospital suggests that they are unlikely to contribute towards transmission.

Fourth, other healthcare workers (e.g. pharmacists, radiographers, porters) and ward visitors were not screened during the study. Whilst these groups are less likely to have regular direct contact with ICU/HDU patients (compared with nurses, doctors and physiotherapists) they may represent reservoirs of *S. aureus* contributing towards transmission that were missed in this study.

Fifth, host factors influencing colonisation were not investigated in this study. Various anatomical and immunological factors (including antibody responses) have been found to effect *S. aureus* carriage (see Section 1.3.2). It is plausible that these factors also influence (at least in part) transmission and acquisition. Large association studies (epidemiological and genomic) would be required to answer these questions.

5.6 Future work

A future aim of this study is to evaluate the role of patients, staff and the environment using WGS. All available isolates from these groups have been prepared for sequencing but genome data were unavailable to include in this chapter. On-going work will focus on evaluating acquisitions findings made through conventional typing methods using the higher resolution of WGS.

Furthermore this study was undertaken in a single high-dependency setting. To validate our findings and assess their generalizability to other hospital wards this study needs to be expanded to other ward areas.

5.7 Conclusions

In this chapter I have shown that:

- Patients account for only a minority (24%) of *S. aureus* acquisitions on our ICU and HDU (main objective)
- Staff and environment are frequently (54.5%) implicated in acquisitions on our ICU and HDU (main objective). This confirms our primary hypothesis.
- Healthcare workers in our ICU and HDU are commonly colonised with *S. aureus* (objective 1)
- Working in a nursing home was a dominant risk factor associated with *S. aureus* carriage in our healthcare workers (objective 2)
- Transient carriage was not observed in our staff (objective 3)
- Single nasal swabs are 77% sensitive at detecting *S. aureus* carriage (objective 4)
- Throat carriage is an important reservoir for *S. aureus* carriage and acquisition.

Despite infection control measures in hospitals primarily targeting patients *S. aureus* acquisition continues to occur. Conventional approaches used in this study have confirmed that patients infrequently contribute towards acquisitions and revealed that staff and the environment are frequently implicated as donors. It is likely that WGS will characterise these putative associations further. Together these findings have the potential to guide and optimise infection control policy.

6 Concluding remarks

When the Modernising Medical Microbiology (MMM) programme of research started in 2008 it was uncertain at the time if the promise offered by WGS would be realised because of doubts about the capacities of the technology. Over the course of this project WGS has exceeded expectations. The technology moved faster than expected with novel high throughput platforms becoming increasingly available (Illumina HiSeq 2500) as well as bench-top sequencers (MiSeq) generating sequence data in clinically relevant time-frames.[344] Furthermore the costs have fallen and are beginning to approach those of conventional typing methods. WGS has been shown to be applicable to the investigation of a range of disparate organisms including *Mycobacterium tuberculosis*[345,346] and *Clostridium difficile*.[242,347]

In the field of *S. aureus* research other groups have made important contributions. Work undertaken at the Wellcome Trust Sanger Institute in Cambridge has shown that WGS can be used to map *S. aureus* transmission routes over both large geographical areas and within the context of a hospital outbreak investigation, and that it provides the resolution to implicate individuals (including healthcare workers) in the persistence of such outbreaks.[234,241,266] The mutation rate of *S. aureus* has been measured and appears to be fairly constant across different experimental settings.[234,235,237,238] This has provided a framework for interpreting the significance of *S. aureus* in short-term transmission.

This thesis describes three bodies of work that utilised recent advances in WGS to evaluate three specific research questions. The first study evaluated whether the emergence of novel clones within dominant MRSA lineages can be associated with changes in the clinical epidemiology of nosocomial infections. This study comprised a reinvestigation of an apparent outbreak of MRSA bacteraemia which involved EMRSA-16. This demonstrated the emergence of a clonal variant within EMRSA-16 occurring at the time of the outbreak. The variant was present throughout the hospital (particularly the ICU) and was associated with higher white cell responses. Evaluation of a large collection of regional and national isolates using WGS identified isolates that were highly related to the clonal variant and that were present in hospitals throughout England following the outbreak. A limitation of this study is its dependence on archived isolates that resulted in interrupted sampling time frames.

Although this makes it hard to plot the time course of the outbreak, there is no reason to think this introduces any bias to the observation and the use of time-scaled phylogenies means that it does not undermine the central observation. Most importantly these findings demonstrate the ability of WGS to identify the emergence and spread of novel clones within dominant lineages. This means that WGS could be applied in real-time to detect the emergence and persistence of novel clones within endemic S. *aureus* lineages. In turn this could provide early warning of novel strain emergence and allows links to be made between genotype and clinical phenotype.

The second study explored the role of patients colonised with *S. aureus* as common sources of new acquisitions by other patients on the intensive care unit. Preventing patient-to-patient transmission has been a cornerstone of infection control practice particularly in relation to MRSA. It should be noted that my study was conducted in a unit where stringent infection control measures to prevent transmission have been in place for a considerable time. Hence, these findings may not be applicable to some other settings. Nevertheless, my crucial observation that patient-to-patient transmission accounts for a minority of acquisitions demands a reappraisal of approaches to reduce the burden of *S. aureus* and demonstrates that further reductions are likely to require a careful assessment of other sources. During this study it was also possible to demonstrate the ability of WGS both to link patient carriage isolates that were not considered to be part of a transmission chain and exclude transmission suspected by *spa*-typing and epidemiological data. It is clear that WGS applied in real time could reliably identify when transmission has occurred, allowing prompt interventions and providing a reliable measure of infection control efficacy.

The final study extended these observations to attempt to undertake what I believe to be the most comprehensive study of *S. aureus* transmission conducted to date. This study was designed to address whether staff carriage or environmental contamination accounted for the gap observed in nosocomial *S. aureus* transmission. A striking finding was that staff and environmental isolates were frequently implicated in patient acquisitions, including six previously unidentified outbreaks. A major strength of this study was the high recruitment rates of staff and patients and extensive environmental sampling, which provide confidence in assumptions regarding their involvement. The data presented represented work based on *spa*-

typing and epidemiological data, and analysis of WGS data will be required in order to make confident conclusions about the role of staff and the environment. Currently the role of healthcare workers in *S. aureus* transmission is a controversial and under-evaluated area. Taken together these data will aid a clearer understanding of the contribution of staff carriage and environmental contamination to patient acquisition that, in turn, will aid optimisation of the management of colonised healthcare workers and infection control practices.

6.1 Novel findings

The work undertaken in this thesis has revealed six novel findings:

1. Variations within dominant *S. aureus* lineages (as exemplified by a clonal expansion of a variant within EMRSA-16) may be associated with changes in clinical epidemiology undetected by conventional typing approaches.
2. Acquisition of *S. aureus* is infrequent in patients admitted to our high-dependency units.
3. Patients rarely account for *S. aureus* transmission in our high-dependency units.
4. Healthcare workers and the environment are frequently associated with *S. aureus* transmission in our high-dependency units according to conventional approaches (*spa*-typing and epidemiological data).
5. The contribution of MSSA towards transmission and acquisition is greater than MRSA, which is likely to reflect the higher prevalence rates.
6. Throat colonisation is as prevalent as nasal carriage in healthcare workers.

6.2 Recommendations

Various infection prevention and control measures have been instigated in UK hospitals over the last decade with the aim of reducing *S. aureus* disease, particularly MRSA. Based on the results of this thesis the following recommendations are made:

1. *Use of whole-genome sequencing in disease outbreaks of un-determined aetiology*.

   An outbreak of MRSA blood stream infections over three years was found to be associated with the emergence of a clonal expansion within a dominant

lineage using WGS alone. The ability to detect invasive disease caused by highly related organisms (as early as the second case) holds the potential to instigate early infection control measures (e.g. screening) and employ prospective epidemiological surveillance measures (either through WGS or using WGS to develop simple molecular signature assays). It is feasible that WGS will become more widely available in the near future. At this time hospital trusts should consider employing WGS in disease outbreak situations.

2. *Continue current infection control policy in high-dependency areas*.

   Currently used infection control measures are effective at maintaining low patient carriage rates of *S. aureus* (particularly MRSA) in our high-dependency units. Furthermore rates of patient acquisition on our units are lower than rates reported in the literature. It remains unclear whether these low rates relate to all or some of the measures used. Interventional studies are required to answer this. Data from this thesis supports continuing current infection control measures including chlorhexidine washes (with adjunctive nasal mupirocin in MRSA colonised patients), hand hygiene and environmental cleaning.

3. *Enhanced staff infection control measures*.

   Conventional approaches used in this study to evaluate the role of healthcare workers suggest that up to 50% of patient acquisitions are potentially attributable to staff. As the observed 'staff-associated' acquisitions involve different *S. aureus* strains (according to *spa*-type and methicillin susceptibility) they are unlikely to represent transmission from a single 'super-spreader', hence routine staff screening is unnecessary. It is likely that staff involvement in transmission is secondary to lapses in infection control measures. Hence, these data support a recommendation to enhance staff infection control measures (e.g. hand hygiene). It is important to note that these results are based on conventional measures and likely to change when evaluated using WGS.

4. *Consider screening for methicillin-susceptible S. aureus*.

   MSSA outbreaks on our high-dependency units were more commonly observed than those caused by MRSA, which is likely to reflect higher prevalence rates. These outbreaks would be undetected by routine screening selectively culturing for MRSA. Further work is required to evaluate the incidence of MSSA disease on the unit, but national data suggests that whilst invasive MRSA disease is declining rates of MSSA disease remains high.[314] Data from this thesis would support consideration of routine MSSA screening to identify outbreaks and permit instigation of appropriate infection control measures.

5. *Consider throat screening in patients*.

   Rates of throat carriage identified in staff members were comparable to nasal carriage. It is plausible that throat carriage is an undetermined *S. aureus* reservoir in patients, although further work would be required to confirm this. Currently throat screening is not performed as part of routine clinical practice. Data from this thesis supports consideration of adjunctive throat screening in patients as part of routine clinical practice to detect *S. aureus* carriage.

7 Public engagement

The work in this thesis has been undertaken in collaboration with the UK Clinical Research Collaboration Modernising Medical Microbiology (UKCRC MMM) consortium. Anna Dumitriu is the Leverhulme Artist in Residence for the UKCRC MMM and as part of her residency, shadows researchers in this consortium that work with *S. aureus*, *M. tuberculosis*, Norovirus and *C. difficile*. The aim of her work is to communicate the MMM consortium aims and research to the general public through art, particularly the application of novel sequencing technologies to inform infection prevention and control.

My collaboration with Anna began in 2010 and aimed to provide a platform for members of the public (and scientific community) to question their own knowledge about methicillin resistant *S. aureus* (MRSA), develop a clearer appreciation of how MRSA and humans interact, and obtain an informed understanding about how healthcare services are working towards ways to better understand and control MRSA. We have worked together to tell the story of MRSA through a textile work, focusing on diagnostic challenges and antimicrobial resistance. We created an "MRSA quilt" as a visual representation of MRSA and the effects of antibiotics on the organism (http://annadumitriu.tumblr.com/ModMedMicro). It was made from pieces of calico impregnated with antibiotics and natural substances perceived to have antimicrobial properties (such as turmeric, saffron, cloves, green tea and garlic). The material was placed on the chromogenic agar and MRSA inoculated on top. The samples were incubated overnight and then autoclaved to ensure sterility. Colour changes from the chromogenic agar, representing organism growth, were absorbed into the material and patterns were created from interactions with antimicrobial substances (Figure 7.1). The quilt was made from 135 samples over which Anna embroidered intricate patterns representing bacterial communication networks (Figure 7.2).

Our work featured in an article in Wired UK magazine published in June 2013, profiling Anna and her collaborative work with UKCRC MMM (www.wired.co.uk). The MRSA quilt was also chosen to appear in the peer-review journal 'Proceedings of the National Academy of Sciences of the United States of America (*Prot Natl Acad Sci USA* 2012; 109**:** 12; 4337-4708) to illustrate published work undertaken by

UKCRC MMM collaborators (Figure 7.2). To date the MRSA quilt has been publically exhibited at: The Linen Rooms in Lisburn, Northern Ireland (Figure 7.3); The Jesse and Marion Art Gallery, at the Rockefeller Art Center State University of New York, USA (Figure 7.4); the Victoria and Albert Museum, London; the Cambridge Science Centre as part of the Festival of Ideas. The piece has provided a unique discussion point, inviting members of the public to talk about MRSA; their concerns, personal experiences and questions.

Anna and myself have also attended public galleries and run interactive microbiological exhibits to provide platforms to engage with the public on MRSA. For example, through nose swabbing demonstrations we have been able to discuss the concept of nasal colonisation and how the scientific community is undertaking research to control MRSA transmission in hospitals (http://annadumitriu.tumblr.com/InfectiveTextiles). The `Hypersymbiont Enhancement Salon` provided alternative and engaging platform to discuss bacterial colonisation of humans (http://www.wellcomecollection.org/whats-on/events/the-hypersymbiont-salon.aspx). Anna has had the opportunity to present our work at a number of other public engagement events (http://flic.kr/s/aHsjAbbRkW).

In December 2012 I was invited to present a summary of my research at Mutamorphosis, an international art conference held in the National Theatre in Prague, Czech Republic (http://mutamorphosis.org/2012/). This meeting aims to unify the arts and sciences and address a wide range of challenging scientific concepts, attracting an eclectic mix of people from varying backgrounds. I presented the concept of MRSA as a human coloniser and pathogen, its transmissibility in healthcare settings, and how medical research is working to improve infection control practices through evaluation of novel technologies. A theme that became apparent from discussions at the conference was that there is considerable uncertainty about how antibiotic resistance emerges and the potential impact this could have on society. To address we have worked with other scientific researchers to illustrate how bacteria acquire resistance mechanisms to improve public understanding about the complexities of bacterial genetics and the potential consequences of antibiotic overuse generating antimicrobial resistance. Recently we have created a textile piece depicting growth of vancomycin resistance *S. aureus* (VRSA) strains in the presence of various chemical and natural antimicrobials

substances (Figure 7.5). This ‘VRSA’ quilt was made in the same way as previously described. The aim is to provide a talking point about the on-going emergence of drug resistance. The final piece was displayed in the Victoria and Albert Museum, London, in September 2013.

Figure 7.1 Original calico samples used to make the 'MRSA quilt'. Each sample shows the susceptibility of methicillin resistant *Staphylococcus aureus* (MRSA) to various antibiotic agent and naturally occurring substances

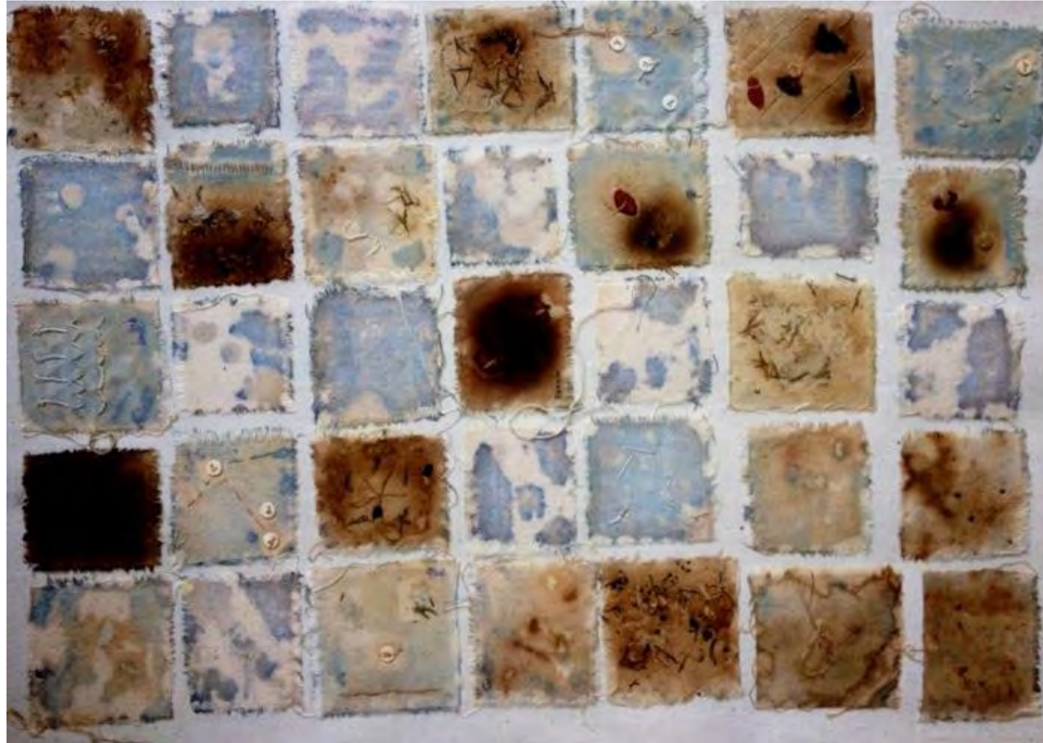


Figure 7.2 Publication of the 'MRSA quilt' in a peer-review journal. The image is a close-up of the completed quilt and shows the patterns created from bacteria-antibiotic interactions and the detailed embroidery.

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In This Issue

Staph population genetics may reveal pathogenesis

Whole genome sequencing has helped researchers understand how viral pathogens evolve within their hosts. Compared with viruses, however, bacteria have larger genomes that replicate more faithfully and tend to conceal genetic variations that reflect population dynamics and functional adaptation within the host. Bernadette Young et al. (pp. 4550–4555) used whole genome sequencing to examine methicillin-resistant *Staphylococcus aureus* (MRSA), the bacteria responsible for a deadly, antibiotic-resistant hospital-acquired infection. The authors report that just eight mutations may underlie the transition from bacteria carried in an asymptomatic population to the bugs responsible for a fatal bloodstream illness. From a large cohort of subjects with nasally carried asymptomatic methicillin-sensitive *S. aureus* (MSSA), the authors charted the evolution of the bacterial population in an elderly patient who developed an *S. aureus* bloodstream infection 15 months after joining the study. When compared with two asymptomatic carriers, the authors found dynamic populations of staphylococci, harboring relatively few genetic variations that evolved measurably through time. Notably, the authors report, half of the mutations that distinguish the asymptomatic from bloodstream bacteria produce stop codons that truncate proteins prematurely and likely contribute to pathogenesis. The authors conclude that high-throughput sequencing may help researchers characterize bacterial genetic variation and evolution within the host. — T.J.

Image courtesy of Anna Dumitriu (Artist-in-Residence, Modernising Medical Microbiology Consortium).

The MRSA Quilt, a textile stained with bacteria grown on chromogenic agar, then autoclaved.

Hematopoietic stem cell precursors originate in yolk sac

Blood cells are so extensively mobile that researchers have been unable to pinpoint the developmental origin of the adult hematopoietic system. Yosuke Tanaka et al. (pp. 4515–4520) explored the longstanding question of whether nascent hematopoietic populations emerge from a single location during a discrete ontogenic event or from multiple ontogenic sources during an extended developmental period. The authors examined the nascent hematopoietic system in mice, focusing on the role of Runx1, a key transcription factor that regulates the development of adult hematopoietic system. The study revealed that blood progenitors and adult-type hematopoietic stem cells (HSCs) originate predominantly in the extraembryonic mesoderm of the yolk sac. The authors designed a mouse "embryo-rescue system" in which Runx1 was reactivated in Runx1-knockout conceptuses. Trials with the reactivation system, which the authors claim closely recapitulates the process of de novo hematopoiesis, revealed that Runx1 rescues the production of HSC precursors in the proximal region of the early yolk sac. These nascent cells, the authors report, then acquire the adult-type HSC phenotype later in gestation. The findings demonstrate that HSC production depends critically on Runx1 and pinpoints the cells' ontogenic source within the extraembryonic mesoderm, according to the authors. — T.J.

www.pnas.org/cgi/doi/10.1073/iti1212109

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Figure 7.3 The 'MRSA quilt' on display at the Linen Rooms in Lisburn, Northern Ireland

Figure 7.4 Display of the 'MRSA quilt' at the Rockefeller Arts Center in New York, USA

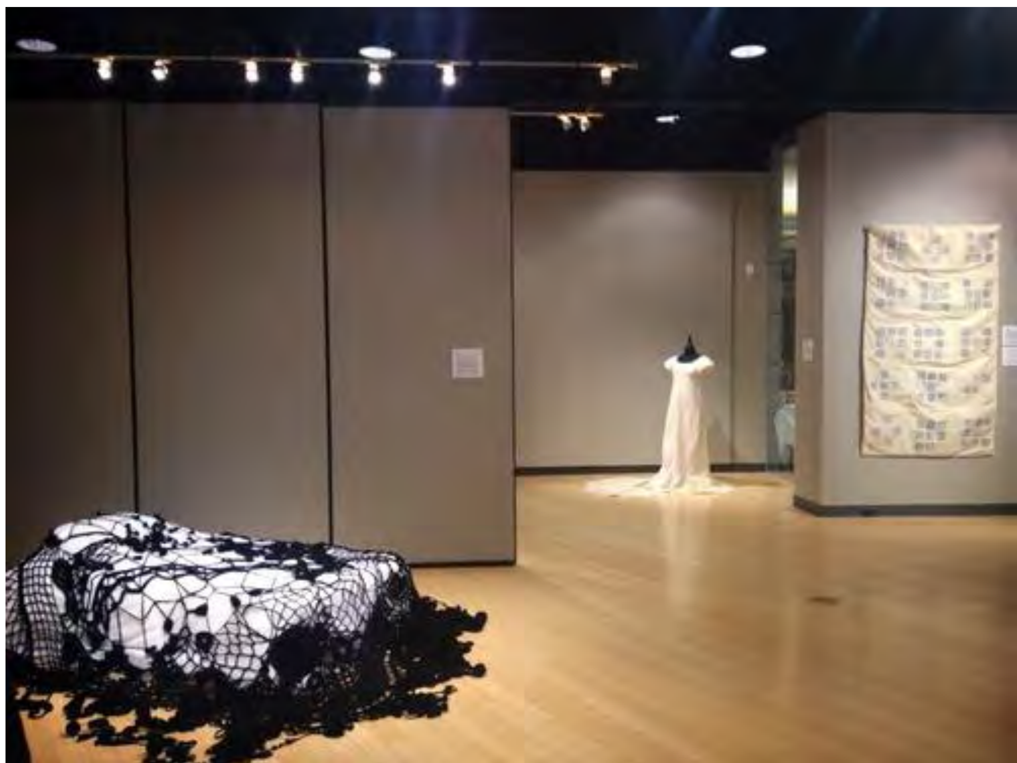


Figure 7.5 The 'VRSA quilt' depicting growth of vancomycin resistant *Staphylococcus aureus* (VRSA) in the presence of various antimicrobial agents and natural substances

8 Publications and presentations

8.1 Publications

Gordon NC, **Price JR**, Cole K, Everitt R, Morgan M, Finney J, Kearns AM, Pichon B, Wilson D, Llewelyn M, Paul J, Crook DW, Walker AS, Golubchik T. Independent validation of *in silico* prediction of *Staphylococcus aureus* antimicrobial susceptibility from whole-genome sequencing. *Journal of Clinical Microbiology* 2014 doi: 10.1128/JCM.03117-13

Price JR, Golubchik T, Cole K, Wilson DJ, Crook DW, Thwaites GE, Bowden R, Walker AS, Peto TEA, Paul J, Llewelyn M. Whole-Genome Sequencing Shows That Patient-to-Patient Transmission Rarely Accounts for Acquisition of *Staphylococcus aureus* in an Intensive Care Unit. *Clinical Infectious Diseases* 2014; **58**(5): 609-18

Miller R, **Price J**, Batty E, Didelot X, Wyllie D, Golubchik T, Crook DW, Paul J, Peto TEA, Wilson DJ, Cule M, Ip C, Day NPJ, Moore CE, Llewelyn MJ, Bowden R. Healthcare-associated outbreak of meticillin-resistant *Staphylococcus aureus* bacteraemia: role of a cryptic variant of an epidemic clone. *Journal of Hospital Infection* 2013; **86**(2); 83-9

Price JR, Gordon NC, Crook DW, Llewelyn MJ, Paul J. The usefulness of whole genome sequencing in *Staphylococcus aureus* infection. *Clinical Microbiology and Infection* 2013; **19**(9): 784-9

Price JR, Didelot X, Crook DW, Llewelyn MJ, Paul J. Whole genome sequencing in the prevention and control of *Staphylococcus aureus* infection. *Journal of Hospital Infection* 2013; **83**(1): 14-21

Price J, Baker G, Heath I, Walker-Bone K, Cubbon M, Curtis S, Enright M, Lindsay J, Paul J, Llewelyn M. Clinical and microbiological determinants of outcome in *Staphylococcus aureus* bacteraemia. International Journal of Microbiology Volume 2010 (2010), Article ID 654858

8.2 Oral presentations

Whole-genome sequencing shows that patient-to-patient transmission rarely accounts for hospital acquisition of *Staphylococcus aureus*. Society of General Microbiology Annual Conference, University of Sussex, Brighton (4th September 2013)

Staphylococcus aureus; where is it coming from? Public Health England, Colindale, London (30th May 2013)

The power of whole-genome sequencing in improving understanding of *Staphylococcus. aureus* infections. European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), Invited Speaker, Berlin, Germany (27-30th April 2013)

Man, Machine and MRSA. Mutamorphogenesis, Prague, Czech Republic (7th December 2012)

Where is MRSA coming from? Healthcare workers, patients or environment. Modernising Medical Microbiology workshop, Oxford, UK (3rd December 2012)

The application of whole-genome sequencing in the evolution and diversity of *Staphylococcus aureus*. Health Protection Agency Annual Conference, Warwick, UK (13-14th September 2011)

8.3 Poster presentations

The application of whole-genome sequencing in understanding the role of patients in the nosocomial transmission of *Staphylococcus aureus*. IDweek 2012, San Diego, USA (16-20th October 2012)

Work in progress on mapping routes of *Staphylococcus aureus* transmission in a healthcare setting. Health Protection Agency Annual Conference, Warwick, UK (11-12th September 2012)

Understanding to role of patients in the transmission of *Staphylococcus aureus* in a health care setting. Federation of Infection Societies Annual Conference, Manchester, UK (15-18th November 2011)

8.4 Abstracts

Gordon NC, Golubchik T, **Price J**, Walker AS, Peto T, Crook D. Genotypic prediction of anti-microbial susceptibility in *Staphylococcus aureus* (2013) – *presented by NC Gordon at British Infection Association 16th Annual Scientific Meeting on 16th May 2013*

Miller R, Golubchik T, Wilson D, Didelot X, **Price J**, Paul J, Llewelyn M, Crook D, Bowden R. Diversity of two Methicillin resistant *Staphylococcus aureus* (MRSA) Clonal Complexes (CCs) over space and time (2011) – *presented by R Miller at the American Society of Microbiology in January 2011*

Miller R, Golubchik T, Wilson D, Farr H, Paul J, Llewelyn M, **Price J**, Moore C, Walker AS, Peto T, Crook D, Bowden R. Diversity of *Staphylococcus aureus* Clonal Complexes (CCs) over space and time (2010) - *presented by R Bowden at Infectious Diseases Genomics and Global Health Conference, Hinxton, UK (Sep 12-15, 2010) and presented by R Miller at 8th International Conference of Molecular Epidemiology and Evolutionary Genetics of Infectious Diseases in November 2010*

9 Glossary

Bacteraemia

The presence of bacteria in the blood stream

Carriage

The presence of a potentially pathogenic organism without a host inflammatory response identified at a single time-point (cf. colonisation)

Clone

Bacterial isolates that have identical genotypes due to common ancestry

Clonal complex

A group of bacteria showing a high degree of genetic similarity that is conventionally based on near-identical multi-locus sequences types

Colonisation

The presence of a potentially pathogenic organism without a host inflammatory response identified over a period of time (cf. carriage)

Contig

A set of overlapping DNA segments that together represent a consensus region of DNA

Credible interval

Interval estimation used in Bayesian statistics, analogous with confidence intervals used in frequency estimation

Epidemic

The sudden presence of an organism above the level expected for a particular setting (unexpectedly high incidence)

Genome

The complete genetic information of an organism

Genotype

The genetic composition of an organism

Isolate

A population of bacterial cells in pure culture derived for a single colony

Lineage

Groups of isolates sharing characteristics due to common descent

Maximum Likelihood Tree

A statistical model of estimating the phylogeny of organisms

Node

A point on phylogenetic tree that infers divergence from a recent common ancestor of descendant strains

Nosocomial

Originating in hospital, or hospital-acquired

Outbreak

Increase in frequency of individual, or combination of different, strains causing disease with a local setting

Phenotype

Observable characteristics of an organism

Phylogenetic Tree

A visual representation of the hypothetical evolutionary relationships between organisms

Read

DNA fragment generated during whole-genome sequencing

Single nucleotide polymorphism (SNP)

Nucleotide variation at a given genetic locus found to be frequent within a population (cf. SNV)

Single nucleotide variant (SNV)

Nucleotide variation at a given genetic locus where the frequency in the population is minimal or unknown (cf. SNP)

Strain

The descendants of a single isolate in pure culture

Type

Allocation of bacteria to a named type according to conventional typing methods

Typing

Phenotypic and/or genetic analysis of isolates below the level of species/sub-species

Virulence

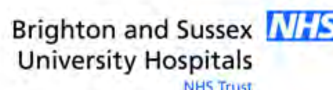
The property of an organism that determines the production of disease

Whole-genome sequencing

Sequencing the complete, or near-complete, genetic composition of an organism at one point in time

10 Appendices

10.1 Appendix 1: Participant information sheet



Department of Microbiology, RSCH, Brighton, BN2 5BE
Tel: +44 (0)1273 696955, Fax: +44 (0)1273 690175
Website: www.modmedmicro.ac.uk

Study title: Carriage of *Staphylococcus aureus* amongst hospital staff
Ethics committee number: 11/LO/1451
Chief Investigator: Dr. Martin Llewelyn

Participant Information Sheet

You are being invited to take part in a research study. Before you decide to take part it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish.

Please feel free to ask us if there is anything that is not clear or if you would like more information. Thank you for considering taking part in this study.

---

1. **What is the purpose of the study?**

In healthy people the bacterial germ *Staphylococcus aureus* often lives harmlessly in the nose. However, people who are ill in hospital are at risk of developing severe *Staph aureus* infections and so hospital staff work very hard to try and stop patients catching *Staph aureus*, particularly methicillin-resistant strains (MRSA) while in hospital. As a result of these efforts rates of *Staph aureus* and MRSA infection in hospitals have fallen considerably. Unfortunately though patients do still catch *Staph aureus* infection in hospital, particularly in settings like the intensive care unit (ICU). It is likely that some of these infections come from the environment and other patients, but it is also likely that some come from the hospital staff. New techniques for distinguishing different *Staph aureus* strains, in particular *'whole genome sequencing'* (WGS) make it possible for the first time to establish accurately how closely related are *Staph aureus* strains from different people.

The purposes of this study are to help us understand whether carriage of *Staph aureus* by staff might contribute to patients catching *Staph aureus* and to determine whether WGS provides useful information about *Staph aureus* transmission in hospital. We have two other studies underway; one looking at patient to patient transmission and another will look at *Staph aureus* in the ward environment.

By taking swabs from the staff working in different units of the hospital we will be able to understand how many carry *Staph aureus*, where on the body it is carried, and for how long.

Whenever we find *Staph aureus* in a staff member's sample we will type it with the new WGS approach. By combining this information with typing information from patient and environmental samples we will be able to determine whether carriage of *Staph aureus* by staff may account for patient-acquisitions of *Staph aureus* during the study period. By typing the *Staph aureus* strains we find by both traditional typing methods and the new WGS approach we will be able to determine whether WGS provides a significant amount of additional information.

2. Why have I been chosen?

You have been invited to take part in the study because you work on a ward where the study is taking place and have clinical contact with patients. We are approaching all members of staff who have regular patient contact and hope that as many as possible will take part so we can get a complete picture of *Staph aureus* carriage among the staff.

3. Do I have to take part?

It is up to you to decide whether or not to take part. This information sheet is to help you make this decision.

If you are interested in taking part you will be asked to make an appointment to meet one of the study doctors or nurses at one of our 'Study Clinics'. We are holding these regularly on the ward to fit in with shift patterns. The study doctor or nurse will then discuss the study with you and answer any questions you have.

The study involves have a nasal swab and completing a brief questionnaire, every 4 weeks, for a maximum of two years. In addition a single throat swab will be taken at the time of the first nose swab, and repeated every six months.

You will be free to go away and consider whether to take part in the study for up to 72 hours. If you do decide to participate you will be asked to sign a consent form. You are free to refuse to take part without giving any reason. This decision will not be disclosed to other members of staff. If you do decide to take part you can withdraw at any time without giving a reason and again without any member of staff being aware of this decision.

4. What will happen to me if I take part?

A research doctor / nurse will see you in a study clinic timed to fit in just before you start a shift. At your first visit you will be allocated a unique personal code. You will keep this code and use it on all samples and questionnaires used in the study in place of your name to ensure anonymity. No one in the research team will know which participant has which code. A list linking participant names to codes will be kept by your ward matron only in case you forget your code.

If you have consented to the study this will involve:

1. an anonymised single nasal swab every 4 weeks (+/- 1 week)
2. an anonymised questionnaire completed at same time as the nasal swab every 4 weeks (+/- 1 week)
3. an anonymised throat swab performed at baseline and every 6 months at the same time as the nose screen.
4. Contact by the research nurse for the purpose of making study visit appointments through emails sent to your trust email account.

Nasal and throat swabbing for *Staph aureus* involves having a small cotton bud inserted into the front of your nose or into the back of your mouth. It is painless and lasts a couple of seconds. It is the same swab that is routinely performed on patients. We will ask you to write you own code onto the swab(s) and swab transport packet(s), seal the swab in the transport packet, then put it into the `*swab box*` (see **Figure 1**). Then you will be asked to complete a short anonymised questionnaire about yourself. This will be completed by yourself in private and your name will not be on the questionnaire. We will ask you

to put your own code on the questionnaires, fill it out, and put it into the `*questionnaire box*` (see **Figure 2**). The time to complete the screen and questionnaire will be between 3-5 minutes.

At your first visit to the study clinic, or by email afterwards, the study doctor or nurse will arrange your next appointment for four weeks' time. This will happen at every visit. Each visit to the study clinic will be immediately before you start a shift so it should not require you to arrive more than 15 minutes early for work once each month. At each visit to the study clinic the study doctor / nurse will take a nasal swab which will be labelled with your UPC and be asked to fill out a short questionnaire again labelled with your UPC. This will take 5-10 minutes. An additional throat swab will be taken with the first nose swab and repeated every 6 months.

5. What do I have to do to take part?

If you are interested in taking part, tell the doctor or nurse who has given you this information or contact a member of the study team bellow. They will arrange a time for you to come to the study clinic to discuss the study further, sign a consent form and start taking part.

6. What does carrying *Staphylococcus aureus* mean for me?

Healthy people carry *Staph aureus* without any risk to their health. Even so, because people might worry about being identified as a carrier the staff members involved in designing the study have recommended that all testing is anonymous. Therefore we will not know whether or not any participant is a carrier of *Staph aureus*. If you are worried about *Staph aureus* we advise that you discuss this with occupational health or your own doctor.

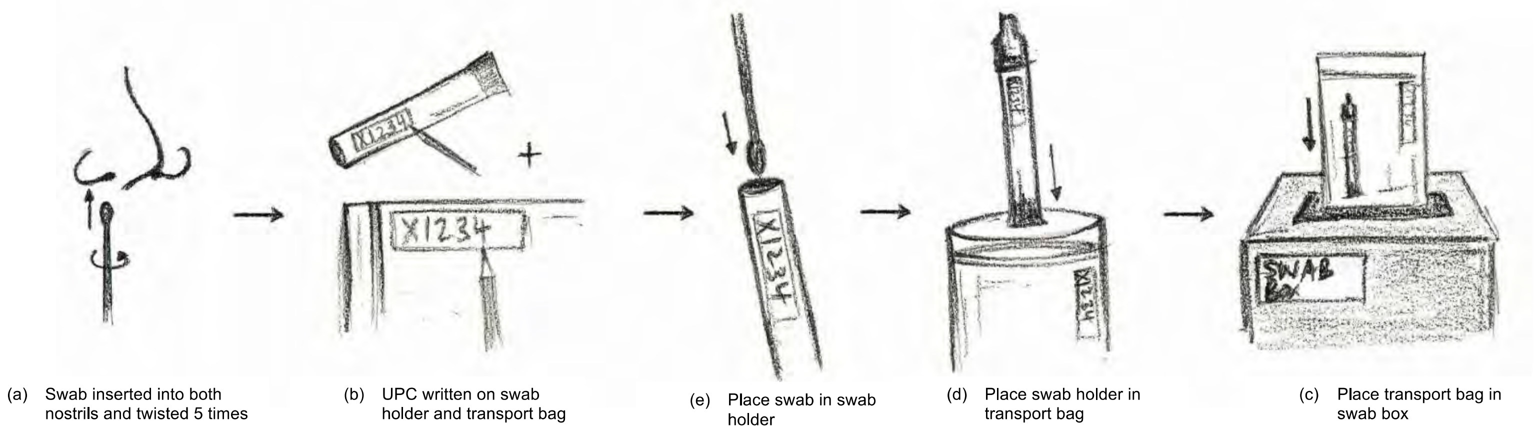
7. What are the possible disadvantages and risks of taking part?

There is a very small risk of injury from nasal swabbing if the cotton wool swab is pushed very far up the nose. To minimise the chances of injury the swab will be performed by a trained nurse.

8. What are the possible benefits of taking part?

There are no direct benefits to you from taking part in this study. We hope that by participating you will help us find out more about how patients and healthcare workers acquire *Staphylococcus aureus*. By understanding this we may be able to put into place more effective infection control policies which might benefit patients in the future.

Figure 1. Diagram of nasal screening



(a) Swab inserted into both nostrils and twisted 5 times

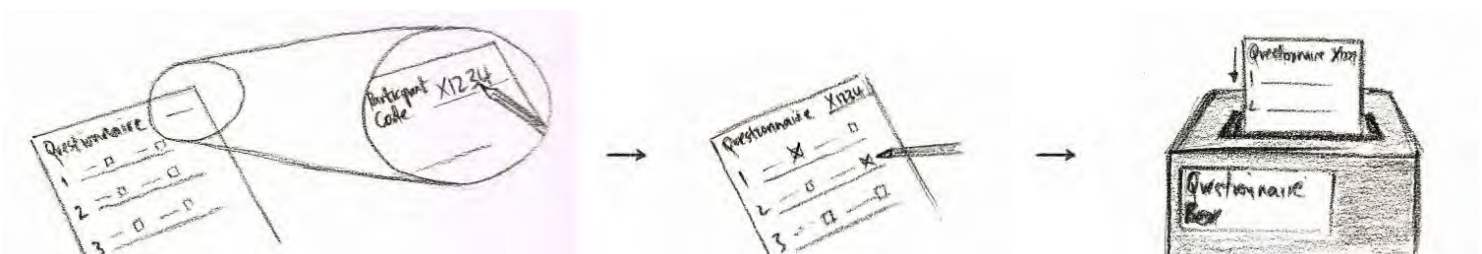
(b) UPC written on swab holder and transport bag

(e) Place swab in swab holder

(d) Place swab holder in transport bag

(c) Place transport bag in swab box

Figure 2. Diagram of completing the anonymised questionnaire



9. Will I be paid to take part?
Participants will receive no payment for taking part in the study but we do appreciate that people who do complete follow up will have made a significant investment of time over the year.

10. What will happen if I don't want to carry on with the study?
If you wish to withdraw from the study we would like to use your data and samples up to your withdrawal but will not ask for any more. However, you can request that data or samples that have already been collected not be used. A decision to withdraw at any time, or a decision not to take part, will not affect your employment status.

11. What if there is a problem or something goes wrong?
If you are harmed by taking part in this research project due to someone's negligence, then you may have grounds for a legal action but you may have to pay your legal costs. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms are available to you.

12. Will my taking part in this study be kept confidential?
Yes, all information collected about you during the course of the research will be kept strictly confidential. Your name will not be used on study forms. Only the Unique Participant Code (UPC) will be used. This will be linked to your name only on a list kept by your ward matron to allow them to remind you of your code should you forget it. This list will be destroyed once the study is completed and will not be accessible to the research team at any stage during the study. Your name will not appear on any scientific reports or publications written as a result of this study. Authorised personnel from the Trust Research and Development may also look at your research records for purposes of audit.

13. What will happen to the samples and information I give?
The samples of *Staphylococcus aureus* and samples of its bacterial DNA will be stored indefinitely in the Clinical Investigation and Research Unit, Brighton, and at the Nuffield Department of Medicine Laboratories in Oxford and will only be used for studies of *Staphylococcus aureus*. The answers to the questions in the questionnaire are confidential, they will be kept securely and your name will not be in the same place as the answers you gave.

14. What happens when the research study stops and what happens to the results?
At the end of the study the results will be made available to all healthcare professionals (through publication in medical journals). The references to these journals will be available on the study website http://www.modmedmicro.ac.uk or alternatively you can email or write to us at the addresses at the top of this leaflet and we will send you a summary of the results of the study.

15. Who is organising and funding the research?
The study is a collaboration between Brighton and Sussex University Hospital NHS Trust and the Nuffield Department of Medicine at the Oxford Radcliffe Hospital, Oxford. The research is being funded by the UK CRC Modernising Medical Microbiology through Wellcome Trust and Medical Research Council grants.

16. Who has reviewed the study?

This study was developed by the study team and reviewed by colleagues in the departments of infectious diseases and microbiology at Brighton and Oxford. It was also part of the peer-reviewed Modernising Medical Microbiology programme of work funded by the Wellcome Trust and Medical Research Council through the UK Clinical Research Consortium. It has also been reviewed by an independent group of people called a multi-centre Research Ethics Committee as well as external scientific experts have reviewed the study to protect your safety, rights, wellbeing and dignity.

17. Contact for further information

Your research nurse is the best person to ask for further information:

Research Nurse: Drew Bexley

Email: andrew.bexley@bsuh.nhs.uk

Mobile: 07432 321044

Or you may contact other members of the study team:

Dr. James Price
Infectious Diseases & Microbiology SpR
Tel: 01273 696955 Ext. 7516

Dr. Martin Llewelyn
Infectious Diseases Consultant (Study Lead)
Tel: 01273 231447

For further information about the Modernising Medical Microbiology Project please see www.modmedmicro.ac.uk

You should keep a copy of this information sheet and you will be given a copy of the informed consent form.

10.2 Appendix 2: Baseline questionnaire

Participant Code ____________

Baseline Questionnaire

Demographic Data

Date of interview / baseline swab — *Day* | *Month* | *Year*

Age Range (yrs) <20 ☐ 20-29 ☐ 30-39 ☐ 40-49 ☐ 50-59 ☐ ≥60 ☐

Gender — Male ☐ Female ☐

Job — Nursing care ☐ Physiotherapist ☐ Doctor ☐

Ward — ICU/HDU ☐ Cardiothoracics ☐ Renal ☐

Other ☐ If other, please specify________________________

Medical Background

1. Have you personally needed any medical care in the past two years?

   No ☐ Yes ☐

   If yes, what medical care was needed?

   GP ☐ Hospital – Outpatient ☐

   Hospital – Inpatient ☐

2. Have you ever been diagnosed with any of the following conditions?

   Diabetes Mellitus ☐ Cancer ☐

   Eczema ☐ Chronic Renal Failure ☐

   Psoriasis ☐

3. Have you undergone any of the following types of care or procedures in the two years?

   Surgery No ☐ Yes ☐ Not Known ☐

   Renal Dialysis No ☐ Yes ☐ Not Known ☐

   Chemotherapy No ☐ Yes ☐ Not Known ☐

4. Have you received any courses of antibiotics in past two years?

   No ☐ Yes ☐

Baseline Questionnaire
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Participant Code ____________

5. Have you taken any courses of steroids in past two years?

   No ☐ Yes ☐

Social Information

6. Who lives with you at home? (*please tick more than one if appropriate*)

Alone	☐	Flatmate(s)	☐
Partner	☐	Children (aged 0-16years)	☐

7. Are you, or have you ever been, a smoker?

   Never smoked ☐ Ex-smoker ☐ Current ☐

8. Have you travelled abroad in the last two years?

   No ☐ Yes ☐

   If yes, where did you travel?

Europe	☐	North America & Canada	☐
South America	☐	South East Asia	☐
Africa	☐	Australasia	☐

Work Information

9. In the last two years have you worked in any of the following?

Residential Home	No ☐	Yes ☐
Nursing Home	No ☐	Yes ☐
Prison Service	No ☐	Yes ☐

10. Have you ever knowingly been colonised with MRSA?

    No ☐ Yes ☐

11. Have you been in contact with anyone known to be colonised with MRSA?

    No ☐ Yes ☐

Thank you for completing this questionnaire

10.3 Appendix 3: Follow-up questionnaire

Participant Code________________

Follow-up Questionnaire

Demographic Data

Date of interview / follow-up swab — *Day* ☐☐ *Month* ☐☐ *Year* ☐☐

Age Range (yrs) <20 ☐ 20-29 ☐ 30-39 ☐ 40-49 ☐ 50-59 ☐ ≥60 ☐

Gender Male ☐ Female ☐

Job Nursing care ☐ Physiotherapist ☐ Doctor ☐

Ward ICU/HDU ☐ Cardiothoracics ☐ Renal ☐

Other ☐ If other, please specify________________________

Medical Background

1. Have you personally needed any medical care in your last study screen?

   No ☐ Yes ☐

   If yes, what medical care was needed?

   GP ☐ Hospital – Outpatient ☐

   Hospital – Inpatient ☐

2. Have you been diagnosed with any of the following conditions since your last study screen?

   Diabetes Mellitus ☐ Cancer ☐

   Eczema ☐ Chronic Renal Failure ☐

   Psoriasis ☐

3. Have you undergone any of the following types of care or procedures since your last study screen?

   Surgery No ☐ Yes ☐ Not Known ☐

   Renal Dialysis No ☐ Yes ☐ Not Known ☐

   Chemotherapy No ☐ Yes ☐ Not Known ☐

Follow-up Questionnaire
Version 2.0 28/02/2012 REC Ref. No. 11/LO/1451

Participant Code_______________

4. Have you received any courses of antibiotics since your last study screen?

   No ☐ Yes ☐

5. Have you taken any courses of steroids since your last study screen?

   No ☐ Yes ☐

Social Information

6. Have you taken up smoking since your last study screen?

   No ☐ Yes ☐

7. Have you travelled abroad since your last study screen?

   No ☐ Yes ☐

   If yes, where did you travel?

Europe	☐	North America & Canada	☐
South America	☐	South East Asia	☐
Africa	☐	Australasia	☐

Work Information

8. Have you worked in any of the following since your last study screen?

Residential Home	No ☐	Yes ☐
Nursing Home	No ☐	Yes ☐
Prison Service	No ☐	Yes ☐

9. Have you been in contact with anyone known to be colonised with MRSA since your last study screen?

   No ☐ Yes ☐

Thank you for completing this questionnaire

11 References

1. Euzeby J. List of prokaryotic names with standing in nomenclature. 2013. http://www.bacterio.net/index.html (accessed 27th August 2013).

2. Ogston A. Micrococcus Poisoning. *J Anat Physiol* 1882; **16**: 526-67.

3. Skinner D, Keefer C. Significance of bacteremia caused by Staphylococcus aureus. *Arch Intern Med* 1941; **68**: 851-75.

4. Shorr AF, Tabak YP, Killian AD, Gupta V, Liu LZ, Kollef MH. Healthcare-associated bloodstream infection: A distinct entity? Insights from a large U.S. database. *Crit Care Med* 2006; **34**: 2588-95.

5. Wyllie DH, Crook DW, Peto TE. Mortality after Staphylococcus aureus bacteraemia in two hospitals in Oxfordshire, 1997-2003: cohort study. *Br Med Bull* 2006; **333**: 281.

6. Cosgrove SE, Sakoulas G, Perencevich EN, Schwaber MJ, Karchmer AW, Carmeli Y. Comparison of mortality associated with methicillin-resistant and methicillin-susceptible Staphylococcus aureus bacteremia: a meta-analysis. *Clin Infect Dis* 2003; **36**: 53-9.

7. Lodise TP, McKinnon PS, Levine DP, Rybak MJ. Impact of empirical-therapy selection on outcomes of intravenous drug users with infective endocarditis caused by methicillin-susceptible Staphylococcus aureus. *Antimicrob Agents Chemother* 2007; **51**: 3731-3.

8. Health Protection Agency. UK Standards for Microbiology Investigations: Identification of Staphylococcus species, Micrococcus species and Rothia species. In: Health Protection Agency, editor. Bacteriology - identification. 2nd Edition ed; 2011. p. 1-19.

9. Ekkelenkamp M, Rooijakkers S, Bonten MJ. Staphylococci and micrococci. In: Cohen J, Opal S, Powderly W, eds. Infectious Diseases,. Third Edition ed. Maryland, USA: Mosby Elsevier; 2010: 1632-44.

10. Hillenkamp F, Karas M, Beavis RC, Chait BT. Matrix-assisted laser desorption/ionization mass spectrometry of biopolymers. *Anal Chem* 1991; **63**: 1193A-203A.

11. Kuroda M, Ohta T, Uchiyama I, et al. Whole genome sequencing of meticillin-resistant Staphylococcus aureus. *Lancet* 2001; **357**: 1225-40.

12. Lindsay JA, Moore CE, Day NP, et al. Microarrays reveal that each of the ten dominant lineages of Staphylococcus aureus has a unique combination of surface-associated and regulatory genes. *J Bacteriol* 2006; **188**: 669-76.

13. Que Y-A, Moreillon P. Staphylococcus aureus (Including Staphylococcal Toxic Shock). In: Mandell G, Bennett J, Dolin R, eds. Principles and Practice of Infectious Diseases. 7th Edition ed: Churchill Livingstone; 2010: 2543-78.

14. Feil EJ, Cooper JE, Grundmann H, et al. How clonal is Staphylococcus aureus? *J Bacteriol* 2003; **185**: 3307-16.

15. Vos M, Didelot X. A comparison of homologous recombination rates in bacteria and archaea. *ISME Journal* 2009; **3**: 199-208.

16. Pérez-Losada M, Browne EB, Madsen A, Wirth T, Viscidi RP, Crandall KA. Population genetics of microbial pathogens estimated from multilocus sequence typing (MLST) data. *Infect Genet Evol* 2006; **6**: 97-112.

17. Hanage WP, Fraser C, Spratt BG. The impact of homologous recombination on the generation of diversity in bacteria. *J Theor Biol* 2006; **239**: 210-9.

18. Waldron DE, Lindsay JA. Sau1: a novel lineage-specific type I restriction-modification system that blocks horizontal gene transfer into Staphylococcus aureus and between S. aureus isolates of different lineages. *J Bacteriol* 2006; **188**: 5578-85.

19. Grundmann H, Aanensen DM, van den Wijngaard CC, et al. Geographic distribution of Staphylococcus aureus causing invasive infections in Europe: a molecular-epidemiological analysis. *PLoS Med* 2010; **7**(1): e1000215.

20. Wyllie D, Paul J, Crook D. Waves of trouble: MRSA strain dynamics and assessment of the impact of infection control. *J Antimicrob Chemother* 2011; **66**: 2685-8.

21. Lowy FD. Medical progress - Staphylococcus aureus infections. *N Engl J Med* 1998; **339**: 520-32.

22. Abdelnour A, Arvidson S, Bremell T, Ryden C, Tarkowski A. The accessory gene regulator (agr) controls Staphylococcus aureus virulence in a murine arthritis model. *Infect Immun* 1993; **61**(9): 3879-85.

23. Cheung AL, Eberhardt KJ, Chung E, et al. Diminished virulence of a sar-/agr- mutant of Staphylococcus aureus in the rabbit model of endocarditis. *J Clin Invest* 1994; **94**(5): 1815-22.

24. Appelbaum PC. Microbiology of antibiotic resistance in Staphylococcus aureus. *Clin Infect Dis* 2007; **45 Suppl 3**: S165-70.

25. Chambers HF, Deleo FR. Waves of resistance: Staphylococcus aureus in the antibiotic era. *Nat Rev Microbiol* 2009; **7**: 629-41.

26. International Working Group on the Staphylococcal Cassette Chromosome elements. Classification of Staphylococcal Cassette Chromosome mec (SCCmec): Guidleines for Reporting Novel SCCmec Elements. *Antimicrob Agents Chemother* 2011; **53**(12): 4961-67.

27. Chongtrakool P, Ito T, Ma XX, et al. Staphylococcal Cassette Chromosome mec (SCCmec) Typing of Methicillin-Resistant Staphylococcus aureus Strains Isolated in 11 Asian Countries: a Proposal for a New Nomenclature for SCCmec Elements. *Antimicrob Agents Chemother* 2006; **50**: 1001-12.

28. Diekema DJ, Pfaller MA, Schmitz FJ, et al. Survey of infections due to Staphylococcus species: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe, and the Western Pacific region for the SENTRY Antimicrobial Surveillanc. *Clin Infect Dis* 2001; **32 Suppl 2**: S114-32.

29. Cosgrove SE, Qi Y, Kaye KS, Harbarth S, Karchmer AW, Carmeli Y. The impact of methicillin resistance in Staphylococcus aureus bacteremia on patient outcomes: mortality, length of stay, and hospital charges. *Infect Control Hosp Epidemiol* 2005; **26**: 166-74.

30. Deleo FR, Otto M, Kreiswirth BN, Chambers HF. Community-associated meticillin-resistant Staphylococcus aureus. *Lancet* 2010; **375**: 1557-68.

31. Greenwood D, Finch R, Davey P, Wilcox M. Antimicrobial Chemotherapy. Oxford, UK: Oxford University Press; 2007.

32. Van Bambeke F. Glycopeptides in clinical development: pharmacological profile and clinical perspectives. *Curr Opin Pharmacol* 2004; **4**(5): 471-8.

33. Andrews J, on behalf of the BSAC Working Party on Susceptibility Testing. BSAC standardized disc susceptibility testing method (version 8). *J Antimicrob Chemother* 2009; **64**: 454-89.

34. Hiramatsu K, Hanaki H, Ino T, Yabuta K, Oguri T, Tenover FC. Methicillin-resistant Staphylococcus aureus clinical strain with reduced vancomycin susceptibility. *J Antimicrob Chemother* 1997; **40**(1): 135-6.

35. Sieradzki K, Roberts RB, Haber SW, Tomasz A. The development of vancomycin resistance in a patient with methicillin-resistant Staphylococcus aureus infection. *N Engl J Med* 1999; **340**(7): 517-23.

36. Tenover FC, Biddle JW, Lancaster MV. Increasing resistance to vancomycin and other glycopeptides in Staphylococcus aureus. *Emerg Infect Dis* 2001; **7**(2): 327-32.

37. Liu C, Chambers HF. Staphylococcus aureus with heterogeneous resistance to vancomycin: epidemiology, clinical significance, and critical assessment of diagnostic methods. *Antimicrob Agents Chemother* 2003; **47**(10): 3040-5.

38. Lodise TP, Graves J, Evans A, et al. Relationship between vancomycin MIC and failure among patients with methicillin-resistant Staphylococcus aureus bacteremia treated with vancomycin. *Antimicrob Agents Chemother* 2008; **52**(9): 3315-20.

39. Soriano A, Marco F, Martinez JA, et al. Influence of vancomycin minimum inhibitory concentration on the treatment of methicillin-resistant Staphylococcus aureus bacteremia. *Clin Infect Dis* 2008; **46**(2): 193-200.

40. Price J, Atkinson S, Llewelyn M, Paul J. Paradoxical relationship between the clinical outcome of Staphylococcus aureus bacteremia and the minimum inhibitory concentration of vancomycin. *Clin Infect Dis* 2009; **48**: 997-8.

41. Kos VN, Desjardins CA, Griggs A, et al. Comparative genomics of vancomycin-resistant Staphylococcus aureus strains and their positions within the clade most commonly associated with Methicillin-Resistant S. aureus hospital-acquired infection in the United States. *mBio* 2012; **3**: e00112-12.

42. Thwaites GE, Edgeworth JD, Gkrania-Klotsas E, et al. Clinical management of Staphylococcus aureus bacteraemia. *Lancet Infect Dis* 2011; **20**: 208-22.

43. Rasmussen RV, Fowler VG, Skov R, Bruun NE. Future challenges and treatment of Staphylococcus aureus bacteremia with emphasis on MRSA. *Future Microbiol* 2011; **6**: 43-56.

44. Gómez MI, O'Seaghdha M, Magargee M, Foster TJ, Prince AS. Staphylococcus aureus protein A activates TNFR1 signaling through conserved IgG binding domains. *J Biol Chem* 2006; **281**: 20190-6.

45. Hartleib J, Köhler N, Dickinson RB, et al. Protein A is the von Willebrand factor binding protein on Staphylococcus aureus. *Blood* 2000; **96**: 2149-56.

46. Greene C, McDevitt D, Francois P, Vaudaux PE, Lew DP, Foster TJ. Adhesion properties of mutants of Staphylococcus aureus defective in fibronectin-binding proteins and studies on the expression of fnb genes. *Mol Microbiol* 1995; **17**: 1143-52.

47. McDevitt D, Francois P, Vaudaux P, Foster TJ. Molecular characterization of the clumping factor (fibrinogen receptor) of Staphylococcus aureus. *Mol Microbiol* 1994; **11**: 237-48.

48. Ní Eidhin D, Perkins S, Francois P, Vaudaux P, Höök M, Foster TJ. Clumping factor B (ClfB), a new surface-located fibrinogen-binding adhesin of Staphylococcus aureus. *Mol Microbiol* 1998; **30**: 245-57.

49. Patti JM, Jonsson H, Guss B, et al. Molecular characterization and expression of a gene encoding a Staphylococcus aureus collagen adhesin. *J Biol Chem* 1992; **267**: 4766-72.

50. Josefsson E, McCrea KW, Ní Eidhin D, et al. Three new members of the serine-aspartate repeat protein multigene family of Staphylococcus aureus. *Microbiology* 1998; **144**: 3387-95.

51. Cramton SE, Gerke C, Schnell NF, Nichols WW, Götz F. The Intercellular Adhesion (ica) Locus Is Present in Staphylococcus aureus and Is Required for Biofilm Formation. *Infect Immun* 1999; **67**: 5427-33.

52. Bhakdi S, Tranum-Jensen J. Alpha-toxin of Staphylococcus aureus. *Microbiol Rev* 1991; **55**: 733-51.

53. Arbuthnott JP. Bacterial cytolysins (membrane-damaging toxins). In: Cohen P, van Heyningen S, editors. Molecular actions of toxins and viruses. Amsterdam, The Netherlands: Elsevier Biomedical Press; 1982. p. 107-29.

54. Tristan A, Ferry T, Durand G, et al. Virulence determinants in community and hospital meticillin-resistant Staphylococcus aureus. *J Hosp Infect* 2007; **65 Suppl 2**: 105-9.

55. Reyes-Robles T, Alonzo F, 3rd, Kozhaya L, Lacy DB, Unutmaz D, Torres VJ. Staphylococcus aureus leukotoxin ED targets the chemokine receptors CXCR1 and CXCR2 to kill leukocytes and promote infection. *Cell Host Microbe* 2013; **14**(4): 453-9.

56. Bohach GA, Fast DJ, Nelson RD, Schlievert PM. Staphylococcal and streptococcal pyrogenic toxins involved in toxic shock syndrome and related illnesses. *Crit Rev Microbiol* 1990; **17**: 251-72.

57. Cheng AG, McAdow M, Kim HK, Bae T, Missiakas DM, Schneewind O. Contribution of coagulases towards Staphylococcus aureus disease and protective immunity. *PLoS Pathog* 2010; **6**(8): e1001036.

58. Berends ET, Horswill AR, Haste NM, Monestier M, Nizet V, von Kockritz-Blickwede M. Nuclease expression by Staphylococcus aureus facilitates escape from neutrophil extracellular traps. *J Innate Immun* 2010; **2**(6): 576-86.

59. Cheung AL, Projan SJ, Gresham H. The Genomic Aspect of Virulence, Sepsis, and Resistance to Killing Mechanisms in Staphylococcus aureus. *Curr Infect Dis Rep* 2002; **4**(5): 400-10.

60. Wisplinghoff H, Bischoff T, Tallent SM, Seifert H, Wenzel RP, Edmond MB. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. *Clin Infect Dis* 2004; **39**: 309-17.

61. Fluit AC, Jones ME, Schmitz FJ, Acar J, Gupta R, Verhoef J. Antimicrobial susceptibility and frequency of occurrence of clinical blood isolates in Europe from the SENTRY antimicrobial surveillance program, 1997 and 1998. *Clin Infect Dis* 2000; **30**: 454-60.

62. Proft T, Fraser JD. Bacterial superantigens. *Clin Exp Immunol* 2003; **133**: 299-306.

63. Shands KN, Schmid GP, Dan BB, et al. Toxic-shock syndrome in menstruating women: association with tampon use and Staphylococcus aureus and clinical features in 52 cases. *N Engl J Med* 1980; **303**: 1436-42.

64. Robicsek A, Suseno M, Beaumont JL, Thomson Jr. RB, Peterson LR. Prediction of methicillin-resistant Staphylococcus aureus involvement in disease sites by concomitant nasal sampling. *J Clin Microbiol* 2008; **46**: 588-92.

65. Wertheim HF, Vos MC, Ott A, et al. Risk and outcome of nosocomial Staphylococcus aureus bacteraemia in nasal carriers versus non-carriers. *Lancet* 2004; **364**: 703-5.

66. von Eiff C, Becker K, Machka K, Stammer H, Peters G. Nasal carriage as a source of Staphylococcus aureus bacteremia. *N Engl J Med* 2001; **344**: 11-6.

67. Harbarth S, Martin Y, Rohner P, Henry N, Auckenthaler R, Pittet D. Effect of delayed infection control measures on a hospital outbreak of methicillin-resistant Staphylococcus aureus. *J Hosp Infect* 2000; **46**: 43-9.

68. Boelaert JR, Van Landuyt HW, Godard CA, et al. Nasal mupirocin ointment decreases the incidence of Staphylococcus aureus bacteraemias in haemodialysis patients. *Nephrol Dial Transplant* 1993; **8**: 235-9.

69. Kluytmans J. Reduction of surgical site infections in major surgery by elimination of nasal carriage of Staphylococcus aureus. *J Hosp Infect* 1998; **40 Suppl B**: S25-9.

70. Laupland KB, Church DL, Mucenski M, Sutherland LR, Davies HD. Population-based study of the epidemiology of and the risk factors for invasive Staphylococcus aureus infections. *J Infect Dis* 2003; **187**: 1452-9.

71. Jacobsson G, Dashti S, Wahlberg T, Andersson R. The epidemiology of and risk factors for invasive Staphylococcus aureus infections in western Sweden. *Scand J Infect Dis* 2007; **39**: 6-13.

72. Nguyen MH, Kauffman CA, Goodman RP, et al. Nasal carriage of and infection with Staphylococcus aureus in HIV-infected patients. *Ann Intern Med* 1999; **130**: 221-5.

73. Kluytmans J, van Belkum A, Verbrugh H. Nasal carriage of Staphylococcus aureus: epidemiology, underlying mechanisms, and associated risks. *Clin Microbiol Rev* 1997; **10**: 505-20.

74. Williams RE. Healthy carriage of Staphylococcus aureus: its prevalence and importance. *Bacteriol Rev* 1963; **27**: 56-71.

75. Peacock SJ, de Silva I, Lowy FD. What determines nasal carriage of Staphylococcus aureus? *Trends Microbiol* 2001; **9**: 605-10.

76. Wertheim HF, Melles DC, Vos MC, et al. The role of nasal carriage in Staphylococcus aureus infections. *Lancet Infect Dis* 2005; **5**: 751-62.

77. Squier C, Rihs JD, Risa KJ, et al. Staphylococcus aureus rectal carriage and its association with infections in patients in a surgical intensive care unit and a liver transplant unit. *Infect Control Hosp Epidemiol* 2002; **23**: 495-501.

78. Nilsson P, Ripa T. Staphylococcus aureus throat colonization is more frequent than colonization in the anterior nares. *J Clin Microbiol* 2006; **44**: 3334-9.

79. Eriksen NH, Espersen F, Rosdahl VT, Jensen K. Carriage of Staphylococcus aureus among 104 healthy persons during a 19-month period. *Epidemiol Infect* 1995; **115**: 51-60.

80. VandenBergh MF, Yzerman EP, van Belkum A, Boelens HA, Sijmons M, Verbrugh HA. Follow-up of Staphylococcus aureus nasal carriage after 8 years: redefining the persistent carrier state. *J Clin Microbiol* 1999; **37**: 3133-40.

81. Nouwen JL, Fieren MW, Snijders S, Verbrugh HA, van Belkum A. Persistent (not intermittent) nasal carriage of Staphylococcus aureus is the determinant of CPD-related infections. *Kidney Int* 2005; **67**: 1084-92.

82. Gorwitz RJ, Kruszon-Moran D, McAllister SK, et al. Changes in the prevalence of nasal colonization with Staphylococcus aureus in the United States, 2001-2004. *J Infect Dis* 2008; **197**: 1226-34.

83. Cole AM, Tahk S, Oren A, et al. Determinants of Staphylococcus aureus nasal carriage. *Clin Diagn Lab Immunol* 2001; **8**: 1064-9.

84. Peacock SJ, Justice A, Griffiths D, et al. Determinants of acquisition and carriage of Staphylococcus aureus in infancy. *J Clin Microbiol* 2003; **41**: 5718-25.

85. Parnaby RM, O'Dwyer G, Monsey HA, Shafi MS. Carriage of Staphylococcus aureus in the elderly. *J Hosp Infect* 1996; **33**: 201-6.

86. Lipsky BA, Pecoraro RE, Chen MS, Koepsell TD. Factors affecting staphylococcal colonization among NIDDM outpatients. *Diabetes Care* 1987; **10**: 483-6.

87. Williams JV, Vowels B, Honig P, Leyden JJ. Staphylococcus aureus isolation from the lesions, the hands, and anterior nares of patients with atopic dermatitis. *J Emerg Med* 1999; **17**: 207-11.

88. Steele RW. Recurrent staphylococcal infection in families. *Arch Dermatol* 1980; **116**: 189-90.

89. Hoeger PH, Lenz W, Boutonnier A, Fournier JM. Staphylococcal skin colonization in children with atopic dermatitis: prevalence, persistence, and transmission of toxigenic and nontoxigenic strains. *J Infect Dis* 1992; **165**: 1064-8.

90. Kirmani N, Tuazon CU, Murray HW, Parrish AE, Sheagren JN. Staphylococcus aureus carriage rate of patients receiving long-term hemodialysis. *Arch Intern Med* 1978; **138**: 1657-9.

91. Luzar MA, Coles GA, Faller B, et al. Staphylococcus aureus nasal carriage and infection in patients on continuous ambulatory peritoneal dialysis. *N Engl J Med* 1990; **322**: 505-9.

92. Chapoutot C, Pageaux GP, Perrigault PF, et al. Staphylococcus aureus nasal carriage in 104 cirrhotic and control patients. A prospective study. *J Hepatol* 1999; **30**: 249-53.

93. Calfee DP, Durbin LJ, Germanson TP, Toney DM, Smith EB, Farr BM. Spread of methicillin-resistant Staphylococcus aureus (MRSA) among household contacts of individuals with nosocomially acquired MRSA. *Infect Control Hosp Epidemiol* 2003; **24**: 422-6.

94. Lucet JC, Chevret S, Durand-Zaleski I, Chastang C, Regnier B. Prevalence and risk factors for carriage of methicillin-resistant Staphylococcus aureus at admission to the intensive care unit: results of a multicenter study. *Arch Intern Med* 2003; **163**: 181-8.

95. Bloemendaal ALA, Fluit AC, Jansen WMT, et al. Colonization with Multiple Staphylococcus aureus Strains among Patients in European Intensive Care Units. *Infect Control Hosp Epidemiol* 2009; **30**: 918-20.

96. Cespedes C, Said-Salim B, Miller M, et al. The clonality of Staphylococcus aureus nasal carriage. *J Infect Dis* 2005; **191**(3): 444-52.

97. Mongkolrattanothai K, Gray BM, Mankin P, et al. Simultaneous carriage of multiple genotypes of Staphylococcus aureus in children. *J Med Microbiol* 2011; **60**: 317-22.

98. Andersen PS, Pedersen JK, Fode P, et al. Influence of host genetics and environment on nasal carriage of staphylococcus aureus in danish middle-aged and elderly twins. *J Infect Dis* 2012; **206**(8): 1178-84.

99. O'Brien LM, Walsh EJ, Massey RC, Peacock SJ, Foster TJ. Staphylococcus aureus clumping factor B (ClfB) promotes adherence to human type I cytokeratin 10: implications for nasal colonization. *Cell Microbiol* 2002; **4**(11): 759-70.

100. Burian M, Wolz C, Goerke C. Regulatory adaptation of Staphylococcus aureus during nasal colonization of humans. *PLoS One* 2010; **5**(4): e10040.

101. Frank DN, Feazel LM, Bessesen MT, Price CS, Janoff EN, Pace NR. The human nasal microbiota and Staphylococcus aureus carriage. *PLoS One* 2010; **5**(5): e10598.

102. Uehara Y, Nakama H, Agematsu K, et al. Bacterial interference among nasal inhabitants: eradication of Staphylococcus aureus from nasal cavities by artificial implantation of Corynebacterium sp. *J Hosp Infect* 2000; **44**: 127-33.

103. Shuter J, Hatcher VB, Lowy FD. Staphylococcus aureus binding to human nasal mucin. *Infect Immun* 1996; **64**(1): 310-8.

104. Aly R, Shinefield HI, Strauss WG, Maibach HI. Bacterial adherence to nasal mucosal cells. *Infect Immun* 1977; **17**(3): 546-9.

105. Cole AM, Dewan P, Ganz T. Innate antimicrobial activity of nasal secretions. *Infect Immun* 1999; **67**: 3267-75.

106. van den Akker EL, Nouwen JL, Melles DC, et al. Staphylococcus aureus nasal carriage is associated with glucocorticoid receptor gene polymorphisms. *J Infect Dis* 2006; **194**(6): 814-8.

107. Emonts M, de Jongh CE, Houwing-Duistermaat JJ, et al. Association between nasal carriage of Staphylococcus aureus and the human complement cascade activator serine protease C1 inhibitor (C1INH) valine vs. methionine polymorphism at amino acid position 480. *FEMS Immunol Med Microbiol* 2007; **50**(3): 330-2.

108. van Belkum A, Emonts M, Wertheim H, et al. The role of human innate immune factors in nasal colonization by Staphylococcus aureus. *Microbes Infect* 2007; **9**(12-13): 1471-7.

109. Emonts M, Uitterlinden AG, Nouwen JL, et al. Host polymorphisms in interleukin 4, complement factor H, and C-reactive protein associated with nasal carriage of Staphylococcus aureus and occurrence of boils. *J Infect Dis* 2008; **197**(9): 1244-53.

110. Ehrenkranz NJ. Nasal rejection of experimentally inoculated Staphylococcus aureus: evidence for an immune reaction in man. *J Immunol* 1966; **96**: 509-17.

111. Harrison CJ. Innate immunity as a key element in host defense against methicillin resistant Staphylococcus aureus. *Minerva Pediatr* 2009; **61**(5): 503-14.

112. Prevaes SM, van Wamel WJ, de Vogel CP, et al. Nasopharyngeal colonization elicits antibody responses to staphylococcal and pneumococcal proteins that are not associated with a reduced risk of subsequent carriage. *Infect Immun* 2012; **80**(6): 2186-93.

113. Verkaik NJ, Lebon A, de Vogel CP, et al. Induction of antibodies by Staphylococcus aureus nasal colonization in young children. *Clin Microbiol Infect* 2010; **16**(8): 1312-7.

114. Noble WC, Valkenburg HA, Wolters CH. Carriage of Staphylococcus aureus in random samples of a normal population. *J Hyg (Lond)* 1967; **65**(4): 567-73.

115. Roghmann MC, Johnson JK, Stine OC, et al. Persistent Staphylococcus aureus colonization is not a strongly heritable trait in Amish families. *PLoS One* 2011; **6**(2): e17368.

116. Fournier B. The function of TLR2 during staphylococcal diseases. *Front Cell Infect Microbiol* 2012; **2**: 167.

117. Narita K, Hu DL, Mori F, Wakabayashi K, Iwakura Y, Nakane A. Role of interleukin-17A in cell-mediated protection against Staphylococcus aureus infection in mice immunized with the fibrinogen-binding domain of clumping factor A. *Infect Immun* 2010; **78**(10): 4234-42.

118. Spellberg B, Ibrahim AS, Yeaman MR, et al. The antifungal vaccine derived from the recombinant N terminus of Als3p protects mice against the bacterium Staphylococcus aureus. *Infect Immun* 2008; **76**(10): 4574-80.

119. Frank KM, Zhou T, Moreno-Vinasco L, Hollett B, Garcia JG, Bubeck Wardenburg J. Host response signature to Staphylococcus aureus alpha-hemolysin implicates pulmonary Th17 response. *Infect Immun* 2012; **80**(9): 3161-9.

120. Pancari G, Fan H, Smith S, et al. Characterization of the mechanism of protection mediated by CS-D7, a monoclonal antibody to Staphylococcus aureus iron regulated surface determinant B (IsdB). *Front Cell Infect Microbiol* 2012; **2**: 36.

121. Fowler VG, Jr., Proctor RA. Where Does a Staphylococcus aureus Vaccine Stand? *Clin Microbiol Infect* 2014; doi: 10.1111/1469-0691

122. Shinefield H, Black S, Fattom A, et al. Use of a Staphylococcus aureus conjugate vaccine in patients receiving hemodialysis. *N Engl J Med* 2002; **346**(7): 491-6.

123. Pirofski LA. Why antibodies disobey the Hippocratic Oath and end up doing harm: a new clue. *J Clin Invest* 2010; **120**(9): 3099-102.

124. Albrich WC, Harbarth S. Health-care workers: source, vector, or victim of MRSA? *Lancet Infect Dis* 2008; **8**: 289-301.

125. Cookson B, Peters B, Webster M, Phillips I, Rahman M, Noble W. Staff carriage of epidemic methicillin-resistant Staphylococcus aureus. *J Clin Microbiol* 1989; **27**: 1471-6.

126. Lessing MP, Jordens JZ, Bowler IC. When should healthcare workers be screened for methicillin-resistant Staphylococcus aureus? *J Hosp Infect* 1996; **34**: 205-10.

127. Scarnato F, Mallaret MR, Croize J, et al. Incidence and prevalence of methicillin-resistant Staphylococcus aureus nasal carriage among healthcare workers in geriatric departments: relevance to preventive measures. *Infect Control Hosp Epidemiol* 2003; **24**: 456-8.

128. Hawkins G, Stewart S, Blatchford O, Reilly J. Should healthcare workers be screened routinely for meticillin-resistant Staphylococcus aureus? A review of the evidence. *J Hosp Infect* 2011; **77**(4): 285-9.

129. Bassetti S, Bischoff WE, Walter M, et al. Dispersal of Staphylococcus aureus into the air associated with a rhinovirus infection. *Infect Control Hosp Epidemiol* 2005; **26**: 196-203.

130. Bertin ML, Vinski J, Schmitt S, et al. Outbreak of methicillin-resistant Staphylococcus aureus colonization and infection in a neonatal intensive care unit epidemiologically linked to a healthcare worker with chronic otitis. *Infect Control Hosp Epidemiol* 2006; **27**: 581-5.

131. Akhtar N. Staphylococcal Nasal Carriage of Health Care Workers. *J Coll Physicians Surg Pak* 2010; **20**: 439-43.

132. Blok HE, Troelstra A, Kamp-Hopmans TE, et al. Role of healthcare workers in outbreaks of methicillin-resistant Staphylococcus aureus: a 10-year evaluation from a Dutch university hospital. *Infect Control Hosp Epidemiol* 2003; **24**: 679-85.

133. Wall EC, Ghazy A, Bulley S, Ahmad F, Aali A, Lynn W. Medical staff at a London district hospital are not chronic vectors of MRSA or Clostridium difficile. *J Infect* 2009; **58**: 469-71.

134. Cespedes C, Miller M, Quagliarello B, Vavagiakis P, Klein RS, Lowy FD. Differences between Staphylococcus aureus isolates from medical and nonmedical hospital personnel. *J Clin Microbiol* 2002; **40**: 2594-7.

135. Creamer E, Dorrian S, Dolan A, et al. When are the hands of healthcare workers positive for methicillin-resistant Staphylococcus aureus? *J Hosp Infect* 2010; **75**: 107-11.

136. Dancer SJ. Importance of the environment in meticillin-resistant Staphylococcus aureus acquisition: the case for hospital cleaning. *Lancet Infect Dis* 2008; **8**: 101-13.

137. Sherertz RJ, Bassetti S, Bassetti-Wyss B. "Cloud" health-care workers. *Emerg Infect Dis* 2001; **7**: 241-4.

138. Colbeck JC. Environmental aspects of staphylococcal infections acquired in hospitals. I. The hospital environment--its place in the hospital Staphylococcus infections problem. *Am J Public Health Nations Health* 1960; **50**: 468-73.

139. Wagenvoort JH, Sluijsmans W, Penders RJ. Better environmental survival of outbreak vs. sporadic MRSA isolates. *J Hosp Infect* 2000; **45**: 231-4.

140. Dominguez MA, de Lencastre H, Linares J, Tomasz A. Spread and maintenance of a dominant methicillin-resistant Staphylococcus aureus (MRSA) clone during an outbreak of MRSA disease in a Spanish hospital. *J Clin Microbiol* 1994; **32**: 2081-7.

141. Klakus J, Vaughan NL, Boswell TC. Meticillin-resistant Staphylococcus aureus contamination of hospital curtains. *J Hosp Infect* 2008; **68**: 189-90.

142. Ndawula EM, Brown L. Mattresses as reservoirs of epidemic methicillin-resistant Staphylococcus aureus. *Lancet* 1991; **337**: 488.

143. Banerjee D, Fraise A, Chana K. Writing pens are an unlikely vector of cross-infection with methicillin resistant Staphylococcus aureus (MRSA). *J Hosp Infect* 1999; **43**(1): 73-5.

144. Berman DS, Schaefler S, Simberkoff MS, Rahal JJ. Tourniquets and nosocomial methicillin-resistant Staphylococcus aureus infections. *N Engl J Med* 1986; **315**(8): 514-5.

145. Bures S, Fishbain JT, Uyehara CF, Parker JM, Berg BW. Computer keyboards and faucet handles as reservoirs of nosocomial pathogens in the intensive care unit. *Am J Infect Control* 2000; **28**(6): 465-71.

146. Ciragil P, Gul M, Aral M. Bacterial contamination of computers and telephones in a university hospital in Turkey. *J Hosp Infect* 2006; **62**(2): 247-8.

147. Cohen HA, Amir J, Matalon A, Mayan R, Beni S, Barzilai A. Stethoscopes and otoscopes--a potential vector of infection? *Fam Pract* 1997; **14**(6): 446-9.

148. Oie S, Hosokawa I, Kamiya A. Contamination of room door handles by methicillin-sensitive/methicillin-resistant Staphylococcus aureus. *J Hosp Infect* 2002; **51**(2): 140-3.

149. Busato CR, Carneiro Leao MT, Gabardo J. Staphylococcus aureus Nasopharyngeal Carriage Rates and Antimicrobial Susceptibility Patterns Among Health Care Workers and Their Household Contacts. *Braz J Infect Dis* 1998; **2**(2): 78-84.

150. Huijsdens XW, van Santen-Verheuvel MG, Spalburg E, et al. Multiple cases of familial transmission of community-acquired methicillin-resistant Staphylococcus aureus. *J Clin Microbiol* 2006; **44**(8): 2994-6.

151. L'Heriteau F, Lucet JC, Scanvic A, Bouvet E. Community-acquired methicillin-resistant Staphylococcus aureus and familial transmission. *J Am Med Assoc* 1999; **282**(11): 1038-9.

152. Wagenvoort JH, Toenbreker HM, Nurmohamed A, Davies BI. Transmission of methicillin-resistant staphylococcus aureus within a household. *Eur J Clin Microbiol Infect Dis* 1997; **16**(5): 399-400.

153. Ojima M, Toshima Y, Koya E, et al. Hygiene measures considering actual distributions of microorganisms in Japanese households. *J Appl Microbiol* 2002; **93**(5): 800-9.

154. Begier EM, Frenette K, Barrett NL, et al. A high-morbidity outbreak of methicillin-resistant Staphylococcus aureus among players on a college football team, facilitated by cosmetic body shaving and turf burns. *Clin Infect Dis* 2004; **39**(10): 1446-53.

155. Kazakova SV, Hageman JC, Matava M, et al. A clone of methicillin-resistant Staphylococcus aureus among professional football players. *N Engl J Med* 2005; **352**(5): 468-75.

156. Nguyen DM, Mascola L, Brancoft E. Recurring methicillin-resistant Staphylococcus aureus infections in a football team. *Emerg Infect Dis* 2005; **11**(4): 526-32.

157. Bartzokas CA, Paton JH, Gibson MF, Graham F, McLoughlin GA, Croton RS. Control and eradication of methicillin-resistant Staphylococcus aureus on a surgical unit. *N Engl J Med* 1984; **311**: 1422-5.

158. Berthelot P, Grattard F, Fascia P, et al. Implication of a healthcare worker with chronic skin disease in the transmission of an epidemic strain of methicillin-resistant Staphylococcus aureus in a pediatric intensive care unit. *Infect Control Hosp Epidemiol* 2003; **24**: 299-300.

159. Boyce JM, Opal SM, Potter-Bynoe G, Medeiros AA. Spread of methicillin-resistant Staphylococcus aureus in a hospital after exposure to a health care worker with chronic sinusitis. *Clin Infect Dis* 1993; **17**: 496-504.

160. Sherertz RJ, Reagan DR, Hampton KD, et al. A cloud adult: the Staphylococcus aureus-virus interaction revisited. *Ann Intern Med* 1996; **124**: 539-47.

161. Downey DG, Kidd TJ, Coulter C, Bell SC. MRSA eradication in a health care worker with cystic fibrosis; re-emergence or re-infection? *J Cyst Fibros* 2005; **4**: 205-7.

162. Eveillard M, Martin Y, Hidri N, Boussougant Y, Joly-Guillou ML. Carriage of methicillin-resistant Staphylococcus aureus among hospital employees: prevalence, duration, and transmission to households. *Infect Control Hosp Epidemiol* 2004; **25**: 114-20.

163. Cox RA, Conquest C. Strategies for the management of healthcare staff colonized with epidemic methicillin-resistant Staphylococcus aureus. *J Hosp Infect* 1997; **35**: 117-27.

164. Price EH, Brain A, Dickson JA. An outbreak of infection with a gentamicin and methicillin-resistant Staphylococcus aureus in a neonatal unit. *J Hosp Infect* 1980; **1**: 221-8.

165. Allen KD, Anson JJ, Parsons LA, Frost NG. Staff carriage of methicillin-resistant Staphylococcus aureus (EMRSA 15) and the home environment: a case report. *J Hosp Infect* 1997; **35**: 307-11.

166. Kniehl E, Becker A, Forster DH. Bed, bath and beyond: pitfalls in prompt eradication of methicillin-resistant Staphylococcus aureus carrier status in healthcare workers. *J Hosp Infect* 2005; **59**: 180-7.

167. Pittet D, Allegranzi B, Sax H, et al. Evidence-based model for hand transmission during patient care and the role of improved practices. *Lancet Infect Dis* 2006; **6**: 641-52.

168. Cooper BS, Stone SP, Kibbler CC, et al. Isolation measures in the hospital management of methicillin resistant Staphylococcus aureus (XMSA): Systematic review of the literature. *Br Med J* 2004; **329**: 533.

169. Cookson BD. Methicillin-resistant Staphylococcus aureus in the community: new battlefronts, or are the battles lost? *Infect Control Hosp Epidemiol* 2000; **21**: 398-403.

170. Hancox R, Cummins A, Kelsey MC. An outbreak of EMRSA 2 associated with long-term colonization of medical staff. *J Hosp Infect* 1992; **22**: 170-2.

171. Vonberg RP, Stamm-Balderjahn S, Hansen S, et al. How often do asymptomatic healthcare workers cause methicillin-resistant Staphylococcus aureus outbreaks? A systematic evaluation. *Infect Control Hosp Epidemiol* 2006; **27**: 1123-7.

172. Grundmann H, Hori S, Winter B, Tami A, Austin DJ. Risk factors for the transmission of methicillin-resistant Staphylococcus aureus in an adult intensive care unit: fitting a model to the data. *J Infect Dis* 2002; **185**: 481-8.

173. Andersen BM, Lindemann R, Bergh K, et al. Spread of methicillin-resistant Staphylococcus aureus in a neonatal intensive unit associated with understaffing, overcrowding and mixing of patients. *J Hosp Infect* 2002; **50**: 18-24.

174. Bootsma MC, Diekmann O, Bonten MJ. Controlling methicillin-resistant Staphylococcus aureus: quantifying the effects of interventions and rapid diagnostic testing. *Proc Natl Acad Sci U S A* 2006; **103**: 5620-5.

175. Herwaldt LA, Boyken LD, Coffman S, Hochstetler L, Flanigan MJ. Sources of Staphylococcus aureus for patients on continuous ambulatory peritoneal dialysis. *Perit Dial Int* 2003; **23**: 237-41.

176. Wilson AP, Hayman S, Whitehouse T, et al. Importance of the environment for patient acquisition of methicillin-resistant Staphylococcus aureus in the intensive care unit: a baseline study. *Crit Care Med* 2007; **35**: 2275-9.

177. Austin DJ, Anderson RM. Transmission dynamics of epidemic methicillin-resistant Staphylococcus aureus and vancomycin-resistant enterococci in England and Wales. *J Infect Dis* 1999; **179**: 883-91.

178. Hill J, Howell A, Blowers R. Effect of clothing on dispersal of Staphylococcus aureus by males and females. *Lancet* 1974; **2**: 1131-3.

179. Ayliffe GA, Babb JR, Collings BJ. Letter: Dispersal of Staphylococcus aureus. *Lancet* 1974; **2**: 1573.

180. Bethune DW, Blowers R, Parker M, Pask EA. Dispersal of Staphylococcus Aureus by Patients and Surgical Staff. *Lancet* 1965; **1**: 480-3.

181. McLure HA, Mannam M, Talboys CA, Azadian BS, Yentis SM. The effect of facial hair and sex on the dispersal of bacteria below a masked subject. *Anaesthesia* 2000; **55**: 173-6.

182. Boyce JM, Potter-Bynoe G, Chenevert C, King T. Environmental contamination due to methicillin-resistant Staphylococcus aureus: possible infection control implications. *Infect Control Hosp Epidemiol* 1997; **18**: 622-7.

183. Boyce JM. Environmental contamination makes an important contribution to hospital infection. *J Hosp Infect* 2007; **65 Suppl 2**: 50-4.

184. Vriens MR, Fluit AC, Troelstra A, Verhoef J, van der Werken C. Is methicillin-resistant Staphylococcus aureus more contagious than methicillin-susceptible S. aureus in a surgical intensive care unit? *Infect Control Hosp Epidemiol* 2002; **23**: 491-4.

185. Spicer WJ. Three strategies in the control of staphylococci including methicillin-resistant Staphylococcus aureus. *J Hosp Infect* 1984; **5 Suppl A**: 45-9.

186. Nordic Working Party. The First Report of the SSAC Nordic Working Party on MRSA. 2004. p. 1-21.

187. Dutch Workingparty Infection Prevention. Policy for Methicillin-resistant Staphylcoccus aureus. The Netherlands; 2009. p. 1-22.

188. Calfee DP, Salgado CD, Classen D, et al. Strategies to prevent transmission of methicillin-resistant Staphylococcus aureus in acute care hospitals. *Infect Control Hosp Epidemiol* 2008; **29 Suppl 1**: S62-80.

189. Coia JE, Duckworth GJ, Edwards DI, et al. Guidelines for the control and prevention of meticillin-resistant Staphylococcus aureus (MRSA) in healthcare facilities. *J Hosp Infect* 2006; **63 Suppl 1**: S1-44.

190. Boyce JM, Pittet D, Healthcare Infection Control Practices Advisory Committee; HICPAC/SHEA/APIC/IDSA Hand Hygiene Task Force. Guideline for

Hand Hygiene in Health-Care Settings. Recommendations of the Healthcare Infection Control Practices Advisory Committee and the HICPAC/SHEA/APIC/IDSA Hand Hygiene Task Force. *Am J Infect Control* 2002; **30**(8): S1-45.

191. Boyce JM, Cookson B, Christiansen K, et al. Meticillin-resistant Staphylococcus aureus. *Lancet Infect Dis* 2005; **5**: 653-63.

192. Chambers HF. The changing epidemiology of Staphylococcus aureus? *Emerg Infect Dis* 2001; **7**: 178-82.

193. European Centre for Disease Prevention and Control. Annual report of the European Antimicrobial Resistance Surveillance Network (EARS-Net). 2009. p. 208.

194. New Zealand Public Health Surveillence. Antimicrobial resistance data from hospitals and community laboratories. 2009. p. 1-71.

195. Siegel J, Jackson M, Chiarello L, The Healthcare Infection Control Practices Advisory Committee CDC. Management of Multidrug-Resistant Organisms in Healthcare Settings. *Am J Infect Control* 2007; **35**: S165-93.

196. Zhanel GG, DeCorby M, Adam H, et al. Prevalence of antimicrobial-resistant pathogens in Canadian hospitals: results of the Canadian Ward Surveillance Study (CANWARD 2008). *Antimicrob Agents Chemother* 2010; **54**: 4684-93.

197. Dutch Working Party on Antibiotic Policy. Optimalization of the antibiotic policy in the Netherlands XI: SWAB Guidelines for the Treatment of MRSA Carriage. The Netherlands; 2007. p. 1-22.

198. Department of Heath Government of Western Australia. Control of Methicillin-Resistant Staphylococcus aureus (MRSA) and Epidemic MRSA (EMRSA) in Hospitals. In: Department of Health, editor.; 2005. p. 1-15.

199. New Zealand Ministry of Health. Guidelines for the Control of Methicillin-resistant Staphylococcus aureus in New Zealand. 2002. p. 1-71.

200. Commission for Hospital Hygiene and Infection Prevention at the Robert Koch Institute. Recommendations for the prevention and control of methicillin-resistant Staphylococcus aureus (MRSA) in hospitals and other healthcare facilities. Germany; 2009. p. 1-6.

201. Scottish Infection Standards and Strategy Group. Guidance for the hospital management of meticillin-resistant Staphylococcus aureus. Royal College of Physicians of Edinburgh; 2006. p. 1-56.

202. A Strategy for the Control of Antimicrobial Resistance in Ireland (SARI). The Control and Prevention of MRSA in Hospitals and in the Community. Ireland; 2006. p. 1-41.

203. Provincial Infectious Diseases Advisory Committee (PIDAC). Screening, Testing and Surveillence for Antibiotic-Resistant Organisms (AROs). In: Ministry of Health and Long-Term Care, editor.; 2010. p. 1-71.

204. Siegel JD, Rhinehart E, Jackson M, Chiarello L. Management of multidrug-resistant organisms in health care settings, 2006. *Am J Infect Control* 2007; **35**: S165-93.

205. Health Protection Agency. UK Standards for Microbiology Investigations: Investigation of Specimens for Screening for MRSA. London, UK: Health Protection Agency; 2012. p. 1-27.

206. Rossney AS, Coleman DC, Keane CT. Antibiogram-resistogram typing scheme for methicillin-resistant Staphylococcus aureus. *J Med Microbiol* 1994; **41**: 430-40.

207. Weller TM. Methicillin-resistant Staphylococcus aureus typing methods: which should be the international standard? *J Hosp Infect* 2000; **44**: 160-72.

208. Shopsin B, Gomez M, Montgomery SO, et al. Evaluation of protein A gene polymorphic region DNA sequencing for typing of Staphylococcus aureus strains. *J Clin Microbiol* 1999; **37**: 3556-63.

209. Ridom GmbH. Ridom Spa Server Database. 2013. http://spa.ridom.de (accessed 25th August 2013).

210. Mellmann A, Weniger T, Berssenbrügge C, et al. Based Upon Repeat Pattern (BURP): an algorithm to characterize the long-term evolution of Staphylococcus aureus populations based on spa polymorphisms. *BMC Microbiol* 2007; **7**: 98.

211. Strommenger B, Kettlitz C, Weniger T, Harmsen D, Friedrich AW, Witte W. Assignment of Staphylococcus isolates to groups by spa typing, SmaI macrorestriction analysis, and multilocus sequence typing. *J Clin Microbiol* 2006; **44**: 2533-40.

212. Enright MC, Day NP, Davies CE, Peacock SJ, Spratt BG. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of Staphylococcus aureus. *J Clin Microbiol* 2000; **38**: 1008-15.

213. Maiden MC, Bygraves JA, Feil E, et al. Multilocus sequence typing: A portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc Natl Acad Sci U S A* 1998; **95**: 3140-5.

214. Enright MC, Robinson DA, Randle G, Feil EJ, Grundmann H, Spratt BG. The evolutionary history of methicillin-resistant Staphylococcus aureus (MRSA). *Proc Natl Acad Sci U S A* 2002; **99**: 7687-92.

215. Robinson DA, Enright MC. Multilocus sequence typing and the evolution of methicillin-resistant Staphylococcus aureus. *Clin Microbiol Infect* 2004; **10**: 92-7.

216. Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. *J Bacteriol* 2004; **186**: 1518-30.

217. Strommenger B, Braulke C, Heuck D, et al. spa Typing of Staphylococcus aureus as a Frontline Tool in Epidemiological Typing. *J Clin Microbiol* 2008; **46**: 574-81.

218. Ichiyama S, Ohta M, Shimokata K, Kato N, Takeuchi J. Genomic DNA fingerprinting by pulsed-field gel electrophoresis as an epidemiological marker for study of nosocomial infections caused by methicillin-resistant Staphylococcus aureus. *J Clin Microbiol* 1991; **29**: 2690-5.

219. Faria NA, Carrico JA, Oliveira DC, Ramirez M, de Lencastre H. Analysis of typing methods for epidemiological surveillance of both methicillin-resistant and methicillin-susceptible Staphylococcus aureus strains. *J Clin Microbiol* 2008; **46**: 136-44.

220. McDougal LK, Steward CD, Killgore GE, Chaitram JM, McAllister SK, Tenover FC. Pulsed-Field Gel Electrophoresis Typing of Oxacillin-Resistant Staphylococcus aureus Isolates from the United States: Establishing a National Database. *J Clin Microbiol* 2003; **41**: 5113-20.

221. Melles DC, Van Leeuwen WB, Snijders SV, et al. Comparison of multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), and amplified fragment length polymorphism (AFLP) for genetic typing of Staphylococcus aureus. *J Microbiol Methods* 2007; **69**: 371-5.

222. Cookson B, HARMONY participants. HARMONY--the International Union of Microbiology Societies' European Staphylococcal Typing Network. *Euro Surveill* 2008; **13**(19): pii: 18860.

223. Didelot X, Bowden R, Wilson DJ, Peto TE, Crook DW. Transforming clinical microbiology with bacterial genome sequencing. *Nat Rev Genet* 2012; **13**: 601-12.

224. Price J, Didelot X, Crook DW, Llewelyn MJ, Paul J. Whole genome sequencing in the prevention and control of Staphylococcus aureus infection. *J Hosp Infect* 2013; **83**: 14-21.

225. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A* 1992; **24**: 5463-7.

226. Schadt EE, Turner S, Kasarskis A. A window into third-generation sequencing. *Hum Mol Genet* 2010; **19**: R227-40.

227. Metzker ML. Sequencing technologies — the next generation. *Nat Rev Genet* 2010; **11**: 31-46.

228. Eid J, Fehr A, Gray J, et al. Real-time DNA sequencing from single polymerase molecules. *Science* 2009; **323**: 133-8.

229. Nielsen R, Paul JS, Albrechtsen A, Song YS. Genotype and SNP calling from next-generation sequencing data. *Nat Rev Genet* 2011; **12**: 443-51.

230. Zerbino DRAEB. Velvet: algorithms for de novo short red assembly using de Bruijn graphs. *Genome Res* 2008; **18**: 821-9.

231. Schatz MC, Delcher AL, Salzberg SL. Assembly of large genomes using second-generation sequencing. *Genome Res* 2010; **20**: 1165-73.

232. Holden MTG, Lindsay JA. Whole Genomes: Sequence, Microarray and Systems Biology. In: Lindsay J, editor. Staphylococcus: molecular genetics. 1st edn ed: Norfolk, UK: Caister Academic Press; 2008. p. 1-28.

233. Pruitt KD, Tatusova T, Brown GR, Maglott DR. NCBI Reference Sequences (RefSeq): current status, new features and genome annotation policy. *Nucleic Acids Res* 2012; **40**: D130-D5.

234. Harris SR, Feil EJ, Holden MT, et al. Evolution of MRSA during hospital transmission and intercontinental spread. *Science* 2010; **327**: 469-74.

235. Young BC, Golubchik T, Batty EM, et al. Evolutionary dynamics of Staphylococcus aureus during progression from carriage to disease. *Proc Natl Acad Sci U S A* 2012; **109**: 4550-5.

236. Drummond AJ, Rambaut A. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol Biol* 2007; **7**: 214.

237. Nübel U, Dordel J, Kurt K, et al. A Timescale for Evolution, Population Expansion, and Spatial Spread of an Emerging Clone of Methicillin-Resistant Staphylococcus aureus. *PLoS Pathog* 2010; **6**: 12.

238. Smyth DS, McDougal LK, Gran FW, et al. Population Structure of a Hybrid Clonal Group of Methicillin-Resistant Staphylococcus aureus, ST239-MRSA-III. *PLoS One* 2010; **5**: 10.

239. Golubchik T, Batty E, Miller R, et al. Within-Host Evolution of Staphylococcus aureus during Asymptomatic Carriage. *PLoS One* 2013; **8**: e61319.

240. Wenzel RP, Reagan DR, Bertino JSJ, Baron EJ, Arias K, Bertino Jr. JS. Methicillin-resistant Staphylococcus aureus outbreak: a consensus panel's definition and management guidelines. *Am J Infect Control* 1998; **26**: 102-10.

241. Köser CU, Holden MT, Ellington MJ, et al. Rapid whole-genome sequencing for investigation of a neonatal MRSA outbreak. *N Engl J Med* 2012; **366**: 2267-75.

242. Eyre DW, Golubchik T, Gordon NC, et al. A pilot study of rapid benchtop sequencing of Staphylococcus aureus and Clostridium difficile for outbreak detection and surveillance. *BMJ open* 2012; **2**: e001124.

243. McAdam PR, Templeton KE, Edwards GF, et al. Molecular tracing of the emergence, adaptation, and transmission of hospital-associated methicillin-resistant Staphylococcus aureus. *Proc Natl Acad Sci U S A* 2012; **109**: 9107-12.

244. Carpaij N, Willems RJ, Rice TW, et al. Genetic variation in spatio-temporal confined USA300 community-associated MRSA isolates: a shift from clonal dispersion to genetic evolution? *PLoS One* 2011; **6**: 6.

245. Vogel V, Falquet L, Calderon-Copete SP, Basset P, Blanc DS. Short term evolution of a highly transmissible methicillin-resistant Staphylococcus aureus clone (ST228) in a tertiary care hospital. *PLoS One* 2012; **7**: e38969.

246. Baba T, Takeuchi F, Kuroda M, et al. Genome and virulence determinants of high virulence community-acquired MRSA. *Lancet* 2002; **359**: 1819-27.

247. McAdam PR, Holmes A, Templeton KE, Fitzgerald JR. Adaptive evolution of Staphylococcus aureus during chronic endobronchial infection of a cystic fibrosis patient. *PLoS One* 2011; **6**: e24301.

248. Gordon NC, Price JR, Cole K, et al. Independent validation of *in silico* prediction of *Staphylococcus aureus* antimicrobial susceptibility from whole-genome sequencing. *Journal of Clinical Microbiology* 2014 doi: 10.1128/JCM.03117-13

249. Nonhoff C, Rottiers S, Struelens MJ. Evaluation of the Vitek 2 system for identification and antimicrobial susceptibility testing of Staphylococcal spp. *Clin Microbiol Infect* 2005; **11**: 150-3.

250. US Food and Drug Administration. Class II special controls guidance document: antimicrobial susceptibility test (AST) systems. 2007. http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm080564.htm (accessed 25th August 2013).

251. He M, Sebaihia M, Lawley TD, et al. Evolutionary dynamics of Clostridium difficile over short and long time scales. *Proc Natl Acad Sci U S A* 2010; **107**: 7527-32.

252. Didelot X, Eyre DW, Cule M, et al. Microevolutionary analysis of Clostridium difficile genomes to investigate transmission. *Genome Biol* 2012; **13**: R118.

253. Chin C-S, Sorenson J, Harris JB, et al. The origin of the Haitian cholera outbreak strain. *N Engl J Med* 2011; **364**: 33-42.

254. Marra MA, Jones SJ, Astell CR, et al. The Genome sequence of the SARS-associated coronavirus. *Science* 2003; **300**: 1399-404.

255. Cheung MK, Li L, Nong W, Kwan HS. EscherichiaColiO104:H4@2011 German Escherichia coli O104:H4 outbreak: whole-genome phylogeny without alignment. *BMC Res Notes* 2011; **4**: 533.

256. Gardy JL, Johnston JC, Ho Sui SJ, et al. Whole-genome sequencing and social-network analysis of a tuberculosis outbreak. *N Engl J Med* 2011; **364**: 730-9.

257. Safdar N, Narans L, Gordon B, Maki DG. Comparison of culture screening methods for detection of nasal carriage of methicillin-resistant Staphylococcus aureus: a prospective study comparing 32 methods. *J Clin Microbiol* 2003; **41**(7): 3163-6.

258. Stoakes L, Reyes R, Daniel J, et al. Prospective comparison of a new chromogenic medium, MRSASelect, to CHROMagar MRSA and mannitol-salt medium supplemented with oxacillin or cefoxitin for detection of methicillin-resistant Staphylococcus aureus. *J Clin Microbiol* 2006; **44**(2): 637-9.

259. Fernandes CJ, Fernandes LA, Collignon P. Cefoxitin resistance as a surrogate marker for the detection of methicillin-resistant Staphylococcus aureus. *J Antimicrob Chemother* 2005; **55**: 506-10.

260. Lunter G, Goodson M. Stampy: A statistical algorithm for sensitive and fast mapping of Illumina sequence reads. *Genome Res* 2010; **21**: 936-9.

261. Li H, Handsaker B, Wysoker A, et al. The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 2009; **25**: 2078-9.

262. Guindon S, Gascuel O. A simple, fast and accurate method to estimate large phyogenies by maximum-likelihood. *Syst Biol* 2003; **52**: 696-704.

263. Didelot X, Falush D. Inference of bacterial microevolution using multilocus sequence data. *Genetics* 2007; **175**: 1251-66.

264. Holden MT, Feil EJ, Lindsay JA, et al. Complete genomes of two clinical Staphylococcus aureus strains: Evidence for the rapid evolution of virulence and drug resistance. *Proc Natl Acad Sci U S A* 2004; **101**: 9786-91.

265. Edgeworth JD, Yadegarfar G, Pathak S, et al. An outbreak in an intensive care unit of a strain of methicillin-resistant Staphylococcus aureus sequence type 239 associated with an increased rate of vascular access device-related bacteremia. *Clin Infect Dis* 2007; **44**: 493-501.

266. Harris SR, Cartwright EJP, Torok ME, et al. Whole-genome sequencing for analysis of an outbreak of meticillin-resistant Staphylococcus aureus: a descriptive study. *Lancet Infect Dis* 2012; **13**: 130-6.

267. Klevens RM, Morrison MA, Nadle J, et al. Invasive methicillin-resistant Staphylococcus aureus infections in the United States. *JAMA* 2007; **298**(15): 1763-71.

268. Cockfield JD, Pathak S, Edgeworth JD, Lindsay JA. Rapid determination of hospital-acquired meticillin-resistant Staphylococcus aureus lineages. *J Med Microbiol* 2007; **56**: 614-9.

269. Lindsay J. S. aureus Evolution: Lineages and Mobile Genetic Elements (MGEs). In: Lindsay J, ed. Staphylococcus molecular genetics. Norfolk, UK: Caister Academic Press; 2008: 45-70.

270. Peacock SJ, Moore CE, Justice A, et al. Virulent Combinations of Adhesin and Toxin Genes in Natural Populations of Staphylococcus aureus. *Society* 2002; **70**: 4987-96.

271. Shallcross L, Fragaszy E, Johnson A, Hayward A. The role of the Panton-Valentine leucocidin toxin in staphylococcal disease: a systematic review and meta-analysis. *Lancet Infect Dis* 2013; **13**: 43-54.

272. Fowler VG, Justice A, Moore C, et al. Risk factors for hematogenous complications of intravascular catheter-associated Staphylococcus aureus bacteremia. *Clin Infect Dis* 2005; **40**: 695-703.

273. Jarraud S, Mougel C, Thioulouse J, et al. Relationships between Staphylococcus aureus genetic background, virulence factors, agr groups (alleles), and human disease. *Infect Immun* 2002; **70**(2): 631-41.

274. Becker K, Roth R, Peters G. Rapid and specific detection of toxigenic Staphylococcus aureus: use of two multiplex PCR enzyme immunoassays for amplification and hybridization of staphylococcal enterotoxin genes, exfoliative toxin genes, and toxic shock syndrome toxin 1 gene. *J Clin Microbiol* 1998; **36**(9): 2548-53.

275. Tsen HY, Chen TR. Use of the polymerase chain reaction for specific detection of type A, D and E enterotoxigenic Staphylococcus aureus in foods. *Appl Microbiol Biotechnol* 1992; **37**(5): 685-90.

276. Her Majesty's Stationary Office Regulations 2002 (HMSO). The Health Service (Control of Patient Information) No. 1438. 2002. p. 1-7.

277. Ellington MJ, Hope R, Livermore DM, et al. Decline of EMRSA-16 amongst methicillin-resistant Staphylococcus aureus causing bacteraemias in the UK between 2001 and 2007. *J Antimicrob Chemother* 2010; **65**: 446-8.

278. Miller M, Cook HA, Furuya EY, et al. Staphylococcus aureus in the community: colonization versus infection. *PLoS One* 2009; **4**: e6708.

279. Miller R, Esmail H, Peto T, Walker S, Crook D, Wyllie D. Is MRSA admission bacteraemia community-acquired? A case control study. *J Infect* 2008; **56**: 163-70.

280. Quail MA, Smith M, Coupland P, et al. A tale of three next generation sequencing platforms: comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq sequencers. *BMC Genomics* 2012; **13**: 341.

281. McClure J-A, Conly JM, Lau V, et al. Novel Multiplex PCR Assay for Detection of the Staphylococcal Virulence Marker Panton-Valentine Leukocidin Genes and Simultaneous Discrimination of Methicillin-Susceptible from -Resistant Staphylococci. *J Clin Microbiol* 2006; **44**: 1141-4.

282. Zhang K, Mcclure J-a, Elsayed S, Louie T, Conly JM. Novel Multiplex PCR Assay for Characterization and Concomitant Subtyping of Staphylococcal Cassette Chromosome mec Types I to V in Methicillin-Resistant Staphylococcus aureus. *J Clin Microbiol* 2005; **43**(10): 5026-33.

283. National Food Institute. Protocol: Multiplex PCR for the detection of the mecA gene. 2008; 2008. p. 1-15.

284. Marples RR, Speller DC, Cookson BD. Prevalence of mupiricin resistance in Staphylococcus aureus. *J Hosp Infect* 1995; **29**: 153-5.

285. Richardson JF, Reith S. Characterization of a strain of methicillin-resistant Staphylococcus aureus (EMRSA-15) by conventional and molecular methods. *J Hosp Infect* 1993; **25**: 45-52.

286. Cox RA, Conquest C, Mallaghan C, Marples RR. A major outbreak of methicillin-resistant Staphylococcus aureus caused by a new phage-type (EMRSA-16). *J Hosp Infect* 1995; **29**: 87-106.

287. Clarke SR, Harris LG, Richards RG, Foster SJ. Analysis of Ebh, a 1.1-Megadalton Cell Wall-Associated Fibronectin-Binding Protein of Staphylococcus aureus. *Infect Immun* 2002; **70**: 6680-7.

288. Pichon B, Hill R, Blackburn R, et al. Molecular epidemiology of Staphylococcus aureus bacteraemia in the UK - 2011. 22nd European Congress of Clinical Microbiology and Infectious Diseases (ECCMID): Poster 268; 2012.

289. Johnson A, Davies J, Guy R, et al. Mandatory surveillance of methicillin-resistant Staphylococcus aureus (MRSA) bacteraemia in England: the first 10 years. *J Antimicrob Chemother* 2012; **67**: 802-9.

290. US Department of Health and Human Services. National Action Plan to Prevent Healthcare-Associated Infections: Roadmap to Elimination, 2013.

291. Donaldson L. PL CMO (2003)4: Surveillance of Healthcare Associated Infections. CMO Report. In: Department of Health, editor.; 2003. p. 1-4.

292. Turnidge JD, Kotsanas D, Munckhof W, et al. Staphylococcus aureus bacteraemia: a major cause of mortality in Australia and New Zealand. *Med J Aust* 2009; **191**(7): 368-73.

293. Thwaites GE, United Kingdom Clinical Infection Research Group (UKCIRG). The Management of Staphylococcus aureus Bacteremia in the United Kingdom and Vietnam: A Multi-Centre Evaluation. *PLoS One* 2010; **5**: e14170.

294. Fowler VG, Olsen MK, Corey GR, et al. Clinical identifiers of complicated Staphylococcus aureus bacteremia. *Arch Intern Med* 2003; **163**: 2066-72.

295. Ringberg H, Thoren A, Lilja B. Metastatic complications of Staphylococcus aureus septicemia. To seek is to find. *Infection* 2000; **28**(3): 132-6.

296. Fowler VG, Jr., Sanders LL, Sexton DJ, et al. Outcome of Staphylococcus aureus bacteremia according to compliance with recommendations of infectious diseases specialists: experience with 244 patients. *Clin Infect Dis* 1998; **27**(3): 478-86.

297. Jensen AG, Wachmann CH, Espersen F, Scheibel J, Skinhoj P, Frimodt-Moller N. Treatment and outcome of Staphylococcus aureus bacteremia: a prospective study of 278 cases. *Arch Intern Med* 2002; **162**(1): 25-32.

298. Lodise TP, McKinnon PS, Swiderski L, Rybak MJ. Outcomes analysis of delayed antibiotic treatment for hospital-acquired Staphylococcus aureus bacteremia. *Clin Infect Dis* 2003; **36**: 1418-23.

299. Ehni WF, Reller LB. Short-course therapy for catheter-associated Staphylococcus aureus bacteremia. *Arch Intern Med* 1989; **149**(3): 533-6.

300. Iannini PB, Crossley K. Therapy of Staphylococcus aureus bacteremia associated with a removable focus of infection. *Ann Intern Med* 1976; **84**(5): 558-60.

301. Raad, II, Sabbagh MF. Optimal duration of therapy for catheter-related Staphylococcus aureus bacteremia: a study of 55 cases and review. *Clin Infect Dis* 1992; **14**(1): 75-82.

302. Melzer M, Eykyn SJ, Gransden WR, Chinn S. Is methicillin-resistant Staphylococcus aureus more virulent than methicillin-susceptible S. aureus? A comparative cohort study of British patients with nosocomial infection and bacteremia. *Clin Infect Dis* 2003; **37**(11): 1453-60.

303. Hallin M, Deplano A, Denis O, De Mendonca R, De Ryck R, Struelens MJ. Validation of pulsed-field gel electrophoresis and spa typing for long-term, nationwide epidemiological surveillance studies of Staphylococcus aureus infections. *J Clin Microbiol* 2007; **45**(1): 127-33.

304. Price J, Baker G, Heath I, et al. Clinical and Microbiological Determinants of Outcome in Staphylococcus aureus Bacteraemia. *Int J Microbiol* 2010; **2010**: 654858.

305. Gordon RJ, Lowy FD. Pathogenesis of methicillin-resistant Staphylococcus aureus infection. *Clin Infect Dis* 2008; **46 Suppl 5**: S350-9.

306. Holtfreter S, Grumann D, Schmudde M, et al. Clonal distribution of superantigen genes in clinical Staphylococcus aureus isolates. *J Clin Microbiol* 2007; **45**: 2669-80.

307. Schlackow I, Walker AS, Dingle K, et al. Surveillance of Infection Severity: A Registry Study of Laboratory Diagnosed Clostridium difficile. *PLoS Med* 2012; **9**: e1001279.

308. Hsu LY, Loomba-Chlebicka N, Koh TH, Kang ML, Tan BH, Tambyah PA. EMRSA-15 Bacteremia is not Associated with a Worse Outcome Compared with

Bacteremia Caused by Multidrug-Resistant MRSA. *Int J Biomed Sci* 2007; **3**(2): 97-103.

309. Cooper BS, Medley GF, Stone SP, et al. Methicillin-resistant Staphylococcus aureus in hospitals and the community: stealth dynamics and control catastrophes. *Proc Natl Acad Sci U S A* 2004; **101**: 10223-8.

310. Batra R, Cooper BS, Whiteley C, Patel AK, Wyncoll D, Edgeworth JD. Efficacy and limitation of a chlorhexidine-based decolonization strategy in preventing transmission of methicillin-resistant Staphylococcus aureus in an intensive care unit. *Clin Infect Dis* 2010; **50**: 210-7.

311. Lieberman TD, Michel J-B, Aingaran M, et al. Parallel bacterial evolution within multiple patients identifies candidate pathogenicity genes. *Nat Genet* 2011; **43**: 1275-80.

312. van Griethuysen A, van Loo I, van Belkum A, et al. Loss of the mecA gene during storage of methicillin-resistant Staphylococcus aureus strains. *J Clin Microbiol* 2005; **43**(3): 1361-5.

313. Department of Health. Clean, safe care: Reducing infections and saving lives. In: Department of Health, editor. First ed: Crown; 2008. p. 1-40.

314. Health Protection Agency. Voluntary reporting of Staphylococcus aureus bacteraemia in England, Wales and Northern Ireland, 2009. Health Protection Agency; 2010. p. 1-10.

315. Pearson A, Chronias A, Murray M. Voluntary and mandatory surveillance for methicillin-resistant Staphylococcus aureus (MRSA) and methicillin-susceptible S. aureus (MSSA) bacteraemia in England. *J Antimicrob Chemother* 2009; **64 Suppl 1**: i11-i7.

316. Price J, Gordon NC, Crook D, Llewelyn M, Paul J. The usefulness of whole genome sequencing in the management of Staphylococcus aureus infections. *Clin Microbiol Infect* 2012; **19**(9): 784-9.

317. Harris SR, Clarke I, Seth-Smith HMB, et al. Whole-genome analysis of diverse Chlamydia trachomatis strains identifies phylogenetic relationships masked by current clinical typing. *Nat Genet* 2012; **44**: 413-9.

318. McDougal LK, Thornsberry C. The role of beta-lactamase in staphylococcal resistance to penicillinase-resistant penicillins and cephalosporins. *J Clin Microbiol* 1986; **23**: 832-9.

319. Health Protection Agency. English National Point Prevalence Survey on Healthcare-associated Infections and Antimicrobial Use, 2011: Preliminary data. Health Protection Agency: London; 2012. p. 1-140.

320. Stone SP, Fuller C, Savage J, et al. Evaluation of the national Cleanyourhands campaign to reduce Staphylococcus aureus bacteraemia and Clostridium difficile infection in hospitals in England and Wales by improved hand hygiene: four year, prospective, ecological, interrupted time series study. *Br Med J* 2012; **344**.

321. Nübel U, Nachtnebel M, Falkenhorst G, et al. MRSA transmission on a neonatal intensive care unit: epidemiological and genome-based phylogenetic analyses. *PLoS One* 2013; **8**: e54898.

322. Dulon M, Haamann F, Peters C, Schablon A, Nienhaus A. MRSA prevalence in european healthcare settings: a review. *BMC Infect Dis* 2011; **11**: 138.

323. Bloemendaal ALA, Fluit AC, Jansen WMT, et al. Acquisition and cross-transmission of Staphylococcus aureus in European intensive care units. *Infect Control Hosp Epidemiol* 2009; **30**: 117-24.

324. Williamson S, Walker AS, Knox K, et al. Comparison of Staphylococcus aureus acquisition and transmission rates in three wards using spa-typing. *Infectious Diseases Society of America (ISDA) Annual Conference* 2012; **Poster 401**.

325. Cheng VCC, Chan JFW, Lau EHY, et al. Studying the transmission dynamics of meticillin-resistant Staphylococcus aureus in Hong Kong using spa typing. *J Hosp Infect* 2011; **79**: 302-8.

326. Dancer SJ, Coyne M, Speekenbrink A, Samavedam S, Kennedy J, Wallace PGM. MRSA acquisition in an intensive care unit. *Am J Infect Control* 2006; **34**: 10-7.

327. Fishbain JT, Lee JC, Nguyen HD, et al. Nosocomial transmission of methicillin-resistant Staphylococcus aureus: a blinded study to establish baseline acquisition rates. *Infect Control Hosp Epidemiol* 2003; **24**: 415-21.

328. Marshall C, Harrington G, Wolfe R, Fairley CK, Wesselingh S, Spelman D. Acquisition of methicillin-resistant Staphylococcus aureus in a large intensive care unit. *Infect Control Hosp Epidemiol* 2003; **24**: 322-6.

329. Brown DFJ, Edwards DI, Hawkey PM, et al. Guidelines for the laboratory diagnosis and susceptibility testing of methicillin-resistant Staphylococcus aureus (MRSA). *J Antimicrob Chemother* 2005; **56**: 1000-18.

330. Coombs GW, Van Gessel H, Pearson JC, Godsell M-R, O'Brien FG, Christiansen KJ. Controlling a multicenter outbreak involving the New York/Japan methicillin-resistant Staphylococcus aureus clone. *Infect Control Hosp Epidemiol* 2007; **28**: 845-52.

331. Grundmann H, Aires-de-Sousa M, Boyce J, Tiemersma E. Emergence and resurgence of meticillin-resistant Staphylococcus aureus as a public-health threat. *Lancet* 2006; **368**: 874-85.

332. Wang JT, Chang SC, Ko WJ, et al. A hospital-acquired outbreak of methicillin-resistant Staphylococcus aureus infection initiated by a surgeon carrier. *J Hosp Infect* 2001; **47**: 104-9.

333. Loman N. Whole-genome sequencing for MRSA epidemiology: Transmission and "clouds of variation". *Pathogens: Genes and Genomes* 2012.

http://pathogenomics.bham.ac.uk/blog/2012/11/whole-genome-sequencing-mrsa-epidemiology-clouds-of-variation (accessed 27th August 2013).

334. Hird C, Lighton L, McEvoy M, Morgan D. The Communicable Disease Outbreak Plan: Operational Guidance. Health Protection Agency, 2012.

335. Struelens MJ, Hawkey PM, French GL, Witte W, Tacconelli E. Laboratory tools and strategies for methicillin-resistant Staphylococcus aureus screening, surveillance and typing: state of the art and unmet needs. *Clin Microbiol Infect* 2009; **15**: 112-9.

336. Mertz D, Frei R, Jaussi B, et al. Throat swabs are necessary to reliably detect carriers of Staphylococcus aureus. *Clin Infect Dis* 2007; **45**: 475-7.

337. Bignardi GE, Lowes S. MRSA screening: throat swabs are better than nose swabs. *J Hosp Infect* 2009; **71**: 373-4.

338. Ringberg H, Cathrine Petersson A, Walder M, Hugo Johansson PJ. The throat: an important site for MRSA colonization. *Scand J Infect Dis* 2006; **38**: 888-93.

339. Harbarth S, Schrenzel J, Renzi G, Akakpo C, Ricou B. Is Throat Screening Necessary To Detect Methicillin-Resistant Staphylococcus aureus Colonization in Patients upon Admission to an Intensive Care Unit? *J Clin Microbiol* 2007; **45**: 1072-3.

340. Solberg CO. Spread of Staphylococcus aureus in hospitals: causes and prevention. *Scand J Infect Dis* 2000; **32**: 587-95.

341. Baldwin NS, Gilpin DF, Hughes CM, et al. Prevalence of methicillin-resistant Staphylococcus aureus colonization in residents and staff in nursing homes in Northern Ireland. *J Am Geriatr Soc* 2009; **57**: 620-6.

342. Mody L, Kauffman CA, Donabedian S, Zervos M, Bradley SF. Epidemiology of Staphylococcus aureus colonization in nursing home residents. *Clin Infect Dis* 2008; **46**: 1368-73.

343. Muller A, Thouverez M, Talon D, Bertrand X. Contribution of antibiotic pressure in the acquisition of methicillin-resistant Staphylococcus aureus (MRSA) in a university hospital. *Pathol Biol* 2003; **51**(8-9): 454-9.

344. Eyre DW, Golubchik T, Gordon NC, et al. A pilot study of rapid benchtop sequencing of Staphylococcus aureus and Clostridium difficile for outbreak detection and surveillance. *BMJ open* 2012; **2**(3).

345. Walker TM, Monk P, Smith EG, Peto TE. Contact investigations for outbreaks of Mycobacterium tuberculosis: advances through whole genome sequencing. *Clin Microbiol Infect* 2013; **19**(9): 796-802.

346. Walker TM, Ip CL, Harrell RH, et al. Whole-genome sequencing to delineate Mycobacterium tuberculosis outbreaks: a retrospective observational study. *Lancet Infect Dis* 2013; **13**(2): 137-46.

347. Eyre DW, Cule ML, Wilson DJ, et al. Diverse sources of C. difficile infection identified on whole-genome sequencing. *N Engl J Med* 2013; **369**(13): 1195-205.