Surveillance of Susceptibility Profile to Antiseptics in ITU Isolates of *Staphylococcus aureus*, including MRSA

Thesis presented for the Degree of Philosophiae Doctor by

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SUMMARY

Hand hygiene is the most important measure for reducing healthcare associated infections. Previous *in-vitro* testing of alcohol hand rubs (AHR) has not reflected conditions found in practice; therefore little is known about their true efficacy.

This study aimed to discover how effective AHRs are when used in local intensive therapy Units (ITU) against *Staphylococcus aureus* clinical isolates and their mode of action.

The AHRs were unable to achieve significant bactericidal effect ($\geq$ 4 log reduction) against *S. aureus* within the time healthcare workers (HCW) took to rub AHR into their hands, particularly when tested against bacterial cells on the surface of excised skin. The AHRs had no residual effect and the mechanical action of hand rubbing only increased bacterial cell death by approximately 1 log. The AHRs demonstrated varying efficacies against the isolates and variation in susceptibility to the AHRs was also observed among the isolates. AHR damaged the cytoplasmic membrane, but this was not the cause of the bactericidal effect, nor a reason for the differences in susceptibility observed among the strains. The cell wall and/or outer layers of the bacterial cell were found to most likely be the main target of AHR, most likely due to protein denaturation, although this study was unable to confirm this hypothesis. Virulence gene expression, cell surface charge and cell surface hydrophobicity was not affected by exposure of bacterial cells to AHR and were not responsible for varying AHR susceptibility among the isolates. This study determined the quality of AHR application amongst HCWs in the ITU and the potential cross-contamination resulting from failure to perform hand hygiene before and after certain activities.

It can be concluded from this study that AHRs are unlikely to kill all microbial flora on HCWs hands. Recommendations have been made to improve hand hygiene practices and the efficacy of AHRs used in ITUs.

LIST OF ABBREVIATIONS

A	Adenine
ABR	Antibiotic Resistance
AHR	Alcohol Hand Rub
ANOVA	Analysis of Variance
ASS	Antiseptic Susceptibility
ATCC	American Type Culture Collection
BCA	Bicinchoninic acid Assay
BDMA	*n*-benzyldimethylamine
BP	length in Base Pairs
BSA	Bovine Serum Albumin
BSAC	British Society for Antimicrobial Chemotherapy
C	Cytosine
CA-MRSA	Community Acquired/Associated MRSA
CDC	Centers for Disease Control and Prevention
CFU	Colony Forming Units
CHIPS	Chemotaxis Inhibitory Protein of Staphylococci
CIP	Ciprofloxacin
CM	Cytoplasmic membrane
CN	Gentamicin
CD	Cytoplasm damage
CW	Cell wall
CYHC	CleanYourHandsCampaign
DNase	Deoxyribonuclease
dNTPs	Deoxynucleotide triphosphates
E	Erythromycin
EAP	Extracellular Adhesion Protein
EARSS	European Antimicrobial Resistance Surveillance System
EDTA	Ethylenediaminetetraacetic acid
EMRSA	Epidemic MRSA
ETA	Exfoliative Toxin A

ETB	Exfoliative Toxin B
F	Forward primer
FD	Fusidic acid
FnBP	Fibronectin Binding Protein
G	Guanine
HAA	n-Hexadecane Adherence Assay
HA-MRSA	Hospital Acquired/Associated MRSA
HCAI	Healthcare Associated Infection
HCW	Healthcare Worker
HPA	Health Protection Agency
ISA	Iso-Sensitest Agar
ITU	Intensive Therapy Unit
K+	Potassium leakage
L	Leakage
MAP	MAP = MHC class II analogous protein
MATH	Microbial Adhesion to Hydrocarbons
MD	Cytoplasmic membrane damage
MH	Morriston Hospital
MIC	Minimum Inhibitory Concentration
MLST	Multi Locus Sequence Typing
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MSSA	Methicillin-Sensitive *Staphylococcus aureus*
MUP	Mupirocin
NCBI	National Center for Biotechnology Information
NCIMB	National Collections of Industrial Marine and Food Bacteria
NCTC	National Collection of Type Cultures
NHS	National Health Service
noRT	without reverse transcriptase mix
NOSEC	National Observational Study to Evaluate the CleanYourHands Campaign
OD	Optical Density
OX	Oxacillin
PVL	Panton Valentine Leukocidin

P	Penicillin G
PBP	Penicillin Binding Protein
PBS	Phosphate Buffered Saline
PCMX	Parachlorometaxylenol
PFGE	Pulsed Field Gel Electrophoresis
pI	Isoelectric point
PMNL	Polymorphonuclear leukocytes
PNAG	Polymeric N-acetyl-glucosamine
PMSF	phenylmethanesulfonyl fluoride
QAC	Quaternary Ammonium Compounds
qPCR	Quantitative PCR
R	Reverse primer
RAPD	Random Amplification of Polymorphic DNA
RNase	Ribonuclease
r_s	Spearman's rank correlation coefficient
RT	Reverse transcription
RT-PCR	Reverse Transcription PCR
+RT	with reverse transcriptase mix
SAT	Salt Aggregation Test
SCC*mec*	Staphylococcal Cassette Chromosome *mec*
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SDW	Sterile Deionized Water
SEM	Scanning Electron Microscopy
SH	Singleton Hospital
SL	Slime layer
SOD	Superoxide dismutase
SSSS	Staphylococcal Scalded Skin Syndrome
T	Thymine
T_a	Annealing temperature
T_{BE}	Time taken to achieve a bactericidal effect
TBE	Tris-Borate-EDTA
TE	Tris-HCl EDTA

TEM	Transmission Electron Microscopy
TISA	Teicoplanin-Intermediate *S. aureus*
T_m	melting temperature
TRSA	Teicoplanin-Resistant *S. aureus*
TSA	Tryptone Soya Agar
TSB	Tryptone Soya Broth
TSC	Tryptone Sodium Chloride
TSST	Toxic Shock Syndrome Toxin
UBC	University of British Columbia
UEM	Unidentifiable Extracellular Material
UHP	Ultra High Purity
UHW	University Hospital of Wales
VA	Vancomycin
VISA	Vancomycin Intermidiate *S. aureus*
WD	Cell wall damage
WT	Cell wall thinning
XAA	Xylene Adherence Assay
ZAAPS	Zyvox Annual Appraisal of Potency and Spectrum

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PUBLICATIONS

Results from this study have been published as follows:

Cheeseman, K.E., Denyer, S.P., Hosein, I.K., Williams, G.J. & Maillard, J.-Y. (2009) Evaluation of the Bactericidal Efficacy of Three Different Alcohol Hand Rubs Against 57 Clinical Isolates of *S. aureus*. *Journal of Hospital Infection.* 72(4):319-325

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CHAPTER 1 GENERAL INTRODUCTION

1.1 *STAPHYLOCOCCUS AUREUS* IN THE HEALTHCARE ENVIRONMENT

1.1.1 Types of *S. aureus*

1.1.1.1 MSSA and MRSA

Methicillin resistant *Staphylococcus aureus* (MRSA) are now defined as *S. aureus* that are resistant to the antibiotic oxacillin, since methicillin has been superseded by oxacillin. *S. aureus* is defined as methicillin resistant if oxacillin has a minimum inhibitory concentration (MIC) breakpoint of >2 mg/L and sensitive (MSSA) if the value is ≤2 mg/L (British Society for Antimicrobial Chemotherapy (BSAC), 2006). MRSA are also resistant to all β-lactam antibiotics (Lowy, 2003).

Meta-analysis of mortality rates in *S. aureus* bacteraemia cohort studies revealed that MRSA mortality rates were much higher than those for MSSA (Cosgrove *et al.*, 2003). A retrospective study of intensive therapy unit (ITU) ventilator-associated pneumonia patients revealed that the median length of patient stay was significantly longer among those infections caused by MRSA than MSSA, despite initially receiving the appropriate antibiotic therapy (Shorr *et al.*, 2006).

1.1.1.2 HA-MRSA and CA-MRSA

MRSA infection was known strictly as a healthcare-associated infection (HCAI) or as hospital acquired MRSA (HA-MRSA) until about 15 years ago, when the organism began to cause infection among healthy members of the community who lacked risk factors for the acquisition of a HCAI (Udo *et al.*, 1993). Community associated MRSA (CA-MRSA) can cause serious illness such as severe necrotizing MRSA pneumonia (Francis *et al.*, 2005) and even death (Hunt *et al.*, 1999).

The Center for Disease Control and Prevention (CDC) Active Bacterial Core Surveillance sites that carry out CA-MRSA surveillance define CA-MRSA as an MRSA infection that does not have any of the following risk factors for HCAI: 1) a positive culture for MRSA obtained more than 48 hours after admission to a hospital;

2) prior MRSA infection or colonization; 3) hospitalization, surgery, residency in a long-term care facility, haemodialysis, or peritoneal dialysis within the past year; or 4) current indwelling percutaneous devices or catheters (Minnesota Department of Health, 2004).

CA-MRSA has found its way into hospitals and causes a significant proportion of HCAIs (Kourbatova *et al.*, 2005; Gonzalez *et al.*, 2006; Johnson *et al.*, 2006a; Seybold *et al.*, 2006), including in the ITU (Thompson *et al.*, 2009). A meta-analysis of studies found the number of MRSA infections from hospitalized patients defined as CA-MRSA was high (a total of 30.2% in 27 retrospective studies and 37.3% in 5 prospective studies), although most patients had at least one healthcare-associated risk factor and therefore exact numbers are likely to be lower (Salgado *et al.*, 2003). These figures compare with those reported by the Health Protection Agency (HPA) in UK hospitals in 2006 and 2007, where 29% of MRSA cases were detected within two days of arrival; although one quarter of these had been admitted from another hospital or nursing home (HPA, 2008).

A recent study found 7.3% of patients were carrying MRSA in the anterior nares upon arrival, 30% of which were CA-MRSA (Hidron *et al.*, 2005), indicating one route of its spread into the healthcare environment. A meta-analysis of studies found only a slightly lower prevalence of MRSA among community members (1.3%), although excluding those who had healthcare contacts reduced prevalence to 0.2% (Salgado *et al.*, 2003).

HA-MRSA can be defined as an MRSA infection where a positive culture for MRSA was obtained more than 48 hours after admission to a hospital. CA-MRSA differs from HA-MRSA in that it affects younger people, is mostly associated with skin and soft tissue infection, is more likely to be sensitive to a large number of non- β-lactam antibiotics and more likely to carry the staphylococcal cassette chromosome *mec* (SCC*mec*) IV and Panton Valentine Leukocidin (PVL) toxin (Naimi *et al.*, 2003; Piao *et al.*, 2005; Wannet *et al.*, 2005; Gonzalez *et al.*, 2006; Johnson *et al.*, 2006b; King *et al.*, 2006). However, the virulence of CA-MRSA was not found to be any

different in strains with or without PVL, indicating it is not a major virulence determinant of CA-MRSA (Saïd-Salim *et al.*, 2005; Voyich *et al.*, 2006).

1.1.1.3 PVL-positive S. aureus

PVL-positive strains of *S. aureus* can cause skin infections, tissue necrosis (Holmes *et al.*, 2005) and more severe conditions such as necrotising pneumonia. PVL is a toxin produced by *S. aureus* that is made up of two sub-units, LukS-PV and LukF-PV. These two sub-units combine to form a pore-forming heptamer on the membrane of host polymorphonuclear leukocytes (PMNL), causing lysis or apoptosis. This process leads to necrosis of host tissue, although the exact process is not clear. It is thought that either reactive oxygen species from lysed PMNLs directly cause tissue necrosis, or granule contents from lysed PMNLs initiate an inflammatory response that causes tissue necrosis (Boyle-Vavra & Daum, 2007).

The PVL gene was found to be present in MSSA as well as MRSA isolates (Swaminathan *et al.*, 2006; Bocchini *et al.*, 2007), in a total of 1.6% of *S. aureus* isolates in England and Wales (Holmes *et al.*, 2005). Its presence has been associated with more severe symptoms during infection, including a higher likelihood of bacteraemia, more severe local disease and greater systemic inflammatory response (Bocchini *et al.*, 2007).

The PVL toxin is most commonly linked with CA-MRSA (present in 77% CA-MRSA isolates), although it is infrequently isolated in HA-MRSA isolates (present in 4% HA-MRSA isolates; Naimi *et al.*, 2003). Despite this, the number of infections caused by PVL positive strains are increasing, meaning it is becoming prevalent in hospitals.

1.1.1.4 EMRSA

Epidemic MRSA (EMRSA) strains are strains of MRSA that have caused outbreaks of infection across the UK. Seventeen epidemic types of MRSA have been identified, which are known as EMRSA-1 to EMRSA-17 (Johnson *et al.*, 2005a).

EMRSA-15 and EMRSA-16 are the most significant epidemic strains and have spread widely across the UK (Johnson *et al.*, 2001).

1.1.2 Colonisation and Transmission

Studies have revealed that between 20% and 55 % of healthy adults carry *S. aureus* in the anterior nares (Kluytmans *et al.*, 1997, Vandenbergh & Verbrugh, 1999), the most common site of colonisation. Studies over time, however, have shown between 10% and 35% of healthy adults to be persistent carriers, between 20% and 75% to be intermittent carriers and between 5% and 70% to be non-carriers (Vandenbergh & Verbrugh, 1999). *S. aureus* nasal carriage is significant since it is a major risk factor for infection with the same strain (Kluytmans & Wertheim, 2005). *S. aureus* can also be found in the throat (Ringberg *et al.*, 2006) and on various skin sites, including the perineal area (Vandenbergh & Verbrugh, 1999; Larson *et al.*, 2000).

S. aureus is shed from people's skin into the environment and has been found present in the air in hospital rooms (Bauer *et al.*, 1990; Sexton *et al.*, 2006). It then settles on surfaces in the surrounding area, where it is able to survive for long periods (Schaechter *et al.*, 1999; Huang *et al.*, 2006a). *S. aureus* can also be transferred to environmental surfaces via nursing staff (Boyce *et al.*, 1997).

S. aureus transmission in the hospital environment has been demonstrated by its presence on surfaces and equipment near patients including beds, mattresses, linen, patients' gowns, tables, chairs, window ledges, patients' files, workstations, monitors, blood pressure cuffs, ledges behind beds, underneath beds, sink handles and computer keyboards (Boyce *et al.*, 1997; Bures *et al.*, 2000; Panhotra *et al.*, 2005; Hardy *et al.*, 2006; Sexton *et al.*, 2006). Oie and colleagues (2005) quantified *S. aureus* contamination in a hospital dermatological ward. They found particularly high levels of MSSA and MRSA on several surfaces. For example, MRSA counts as high as 10^4 CFU/900cm^2 were found on an immersion bathtub and MSSA counts as high as 10^5 CFU on the entire inner surface of a foot washbowl. However, presence of MRSA on these surfaces does not necessarily mean they will result in patient infection. Wilson *et al.* (2007) found that of 114 ITU patients only one developed

MRSA infection with an isolate identical to that found in their environment or on the hands of healthcare workers (HCWs). They also found that five patients became colonized with MRSA isolates during their stay that were not found in their environment beforehand.

The most common mode of transmission of microorganisms in the healthcare environment is by the HCW (Bauer *et al.*, 1990). Organisms are transferred to the HCWs either by direct contact with a patient's skin or by contact with fomites in the near vicinity of the patient (Boyce *et al.*, 1997), onto which organisms have been shed. The organism is then transmitted to another patient or another area on the same patient when the HCW touches the patient or a fomite directly in contact with a patient and subsequently touches either the same or another patient (Pittet *et al.*, 2006). Ayliffe *et al.* (1988) sampled the hands of HCWs on a general hospital ward and found 29% were carriers of *S. aureus*, with counts of up to 2.4×10^7 CFU.

1.1.3 Staphylococcal Disease

S. aureus is the most virulent species of staphylococci (Ellner & Neu, 1992). It can cause a wide range of infections, depending on the area of the body it invades. *S. aureus* causes skin infections such as scalded skin syndrome, impetigo, bullous impetigo, folliculitis, styes, furuncles (boils), carbuncles and wound infections. Bacteraemia usually occurs as a result of the spread of infection from another site into the bloodstream. More serious infections include toxic shock syndrome, endocarditis, pneumonia, empyema and osteomyelitis. Ingesting food contaminated with *S. aureus* may cause food poisoning (Murray, 1998).

The ability of *S. aureus* to cause disease is due to its combined ability to colonize and invade its host and evade the host's immune system. This ability is the result of the production of a number of 'virulence' factors and the key virulence factors are listed in Tables 1.1-1.3. *S. aureus* is able to evade the host immune system by neutralizing its net anionic surface charge with the addition of cationic molecules, thus reducing interaction with cationic antimicrobial host molecules (Fedtke *et al.* 2004).

Table 1.1: Major Virulence Factors Associated with Attachment of *S. aureus* to Its Host

Virulence Factor	Gene	Function	Reference
FnBP A/FnBP B	*fnbA/fnbB*	Bind fibronectin	Foster & Höök (1998); Peacock *et al.* (1999)
Collagen binding protein	*cna*	Binds to collagen	Gillaspy *et al.* (1998)
Clumping factor A	*clfA*	Binds to fibrinogen	McDevitt *et al.* (1997)
EAP aka MAP	*eap*	Binds to several host proteins	Palma *et al.* (1999)

EAP = Extracellular adhesion protein; FnBP = Fibronectin Binding Protein; MAP = major histocompatibility complex class II analogous protein

Table 1.2: Major Virulence Factors Associated with Invasion of *S. aureus* Into Its Host

Virulence Factor	Gene	Function	Reference
FnBP A/FnBP B	*fnbA/fnbB*	Internalization of *S. aureus* into host epithelial cells	Peacock *et al.* (1999)
Panton Valentine Leukocidin, α-toxin/ γ-haemolysin	*pvl/hla/hlg*	Destroy host cells by forming a pore in the membrane	Todar (2008); Ferreras *et al.* (1998); Lina *et al.* (1999)
Hyaluronidase	*hysA*	Breaks down hyaluronic acid in connective tissue	Farrell *et al.* (1995)
Exfoliative toxin A & B	*eta/etb*	Causes separation of the epidermal and dermal layers, allowing spread of bacteria into the dermis	Amagai *et al.* (2000)
Super antigens TSST-1, enterotoxin A,B,C,D,E,G	*tst*, *sea- see*, *seg*	Cause a massive immune response, leading to systemic toxicity and host tissue damage, facilitating their spread	Todar (2008)

FnBP = Fibronectin Binding Protein; TSST = Toxic Shock Syndrome Toxin

Table 1.3: Major Virulence Factors Associated with Evasion of Host Immune System by *S. aureus*

Virulence Factor	Gene	Function	Reference
Hyaluronidase	*hysA*	May interfere with neutrophils/macrophage function	Lin *et al.* (1994)
Capsule production	*cap5/*	Avoid phagocytosis by binding antibodies	Thakker *et al.* (1998)
	cap8		Cunnion *et al.* (2003)
Intracellular adhesion	*icaA/*	Extracellular polysaccharide adhesin PNAG is produced to form a biofilm,	O'Gara (2007)
	icaD	which protects cells from host's immune system	
Clumping factor A	*clfA*	Binds fibrinogen to the cell surface to prevent antibodies binding to it or to a PMNL, preventing phagocytosis	Higgins *et al.* (2006)
Protein A	*spa*	Binds IgG in the wrong orientation, preventing recognition by neutrophils	Foster (2005)
CHIPS	*chp*	Binds to chemo-attractants to prevent neutrophil recognition	de Hass *et al.* (1999)
EAP	*eap*	Competes with neutrophils for binding sites on endothelial cells, preventing neutrophil migration	Foster (2005)
SOD/Catalase	*sodA/*	Oxygen produced by phagocytes to kill bacterial cells is converted to H_2O_2	Clements *et al.* (1999)
	katA	by SOD, then broken down into H_20 by catalase	Sanz *et al.* (2000)

CHIPS = Chemotaxis Inhibitory Protein of Staphylococci; EAP = Extracellular Adhesion Protein; IgG = Immunoglobulin G; PMNL = Polymorphonuclear leukocyte; PNAG = polymeric N-acetyl-glucosamine; SOD = Superoxide dismutase

1.1.4 Prevalence

Most surveillance systems used to detect prevalence of *S. aureus* involve counting the number of cases of bacteraemia. This method does not reveal the true extent of *S. aureus* morbidity, since Walker *et al.* (2008) found that non-bacteraemic isolation of MRSA occurred 8 times more frequently than bacteraemic isolation, which accounted for only 8% of first clinical isolations of MRSA. Surveillance of all infection sites of MRSA using this system would lead to faster detection of changes in infection rates and may be of particular use within hospitals.

Data collected by the European Antimicrobial Resistance Surveillance System (EARSS, 2008) showed that in 2008 the proportion of *S. aureus* bacteraemia due to MRSA in European countries ranged between <1% (Norway, Sweden and the Netherlands) and >50% (Portugal and Malta). The UK showed a high proportion of MRSA in 2008 (31%), although a steady decrease was observed from 2006. MRSA proportions also decreased in many other countries, although Portugal and Switzerland showed an increase. Countries that are grouped together according to their proportions of MRSA are mostly grouped together spatially. Data from EARSS (2007) showed that cases of MRSA bacteraemia are more frequently isolated from patients on an ITU ward in UK (over 60% in 2005, but reduced to <50% in 2007).

The 2007 HPA report (HPA, 2007) shows the number of reported cases of MRSA and MSSA *S. aureus* bacteraemia in England rose significantly between 1990 and 2003, with a slight decrease between 2004 and 2006. These data are summarised in Figure 1.1. The report shows the proportion of *S. aureus* bacteraemia cases due to MRSA increased significantly over time: from <5% in 1990 to approximately 40% in 2000, with a slight decrease in 2006 to approximately 37%. The report also shows that 77% of MRSA cases occurred in adults aged over 60 and 69% in adults over 65 and that a significantly higher proportion of cases occur in males than in females. Numbers of MRSA bacteraemia cases were also shown to vary between regions, with the largest number of cases found in the London region and the lowest in the North East. The number of MRSA cases in London was considerably higher over the period of April 2001 to March 2007 compared to other regions, but the number of

cases in London began to decrease significantly in 2004. All other regions have shown a decrease in the number of MRSA bacteraemia reports beginning between 2003 and 2006.

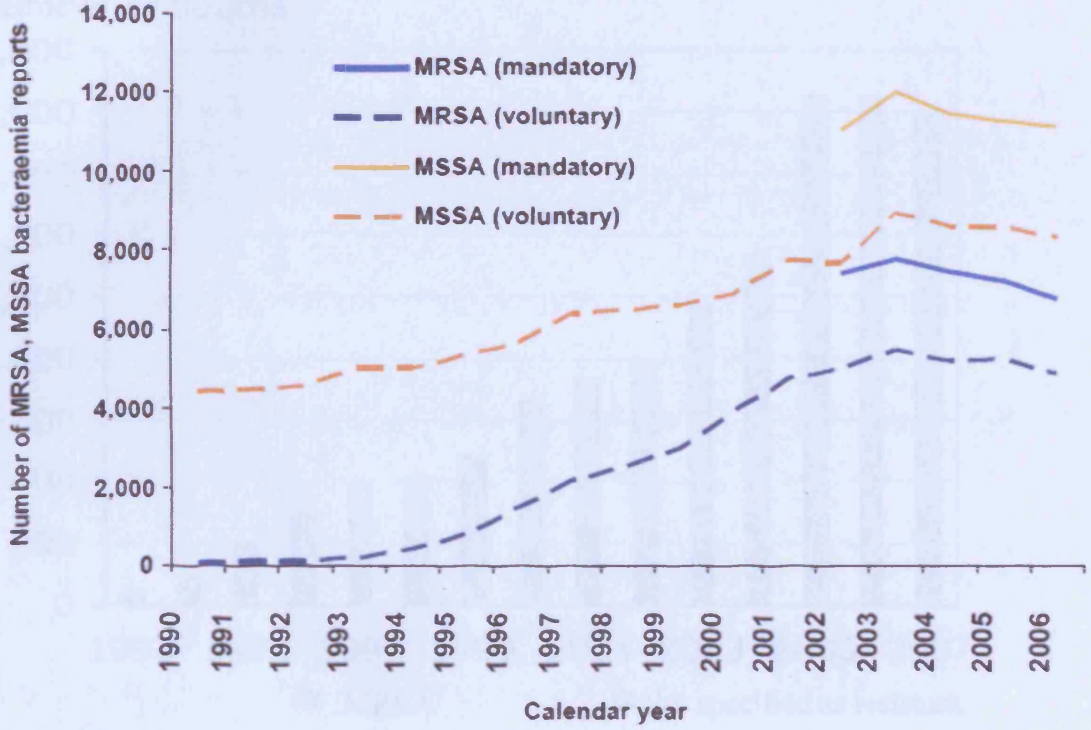

Figure 1.1: Prevalence of *S. aureus* Bacteraemia

Number of cases of *S. aureus* (MSSA and MRSA) bacteraemia reported in England via the voluntary and mandatory surveillance schemes between 1990 and 2006 (extracted from HPA 2007).

Data for *S. aureus* bacteraemia are also available for Wales (National Public Health Service for Wales, 2007). These data show that 1,039 cases of *S. aureus* bacteraemia were reported in Wales in 2005, 407 (40%) of which were MRSA, but numbers had fallen in 2006 to 1,023 cases of *S. aureus* bacteraemia, 341 (33%) of which were MRSA. The number of *S. aureus* and MRSA bacteraemia cases has remained fairly constant in Wales between April 2001 and December 2006, with a decreasing rate of MRSA.

1.1.5 Mortality

The number of deaths in England and Wales due to *S. aureus* rose between 1993 and 2005, but had decreased slightly by 2007 (National Statistics, 2008; see Figure 1.2).

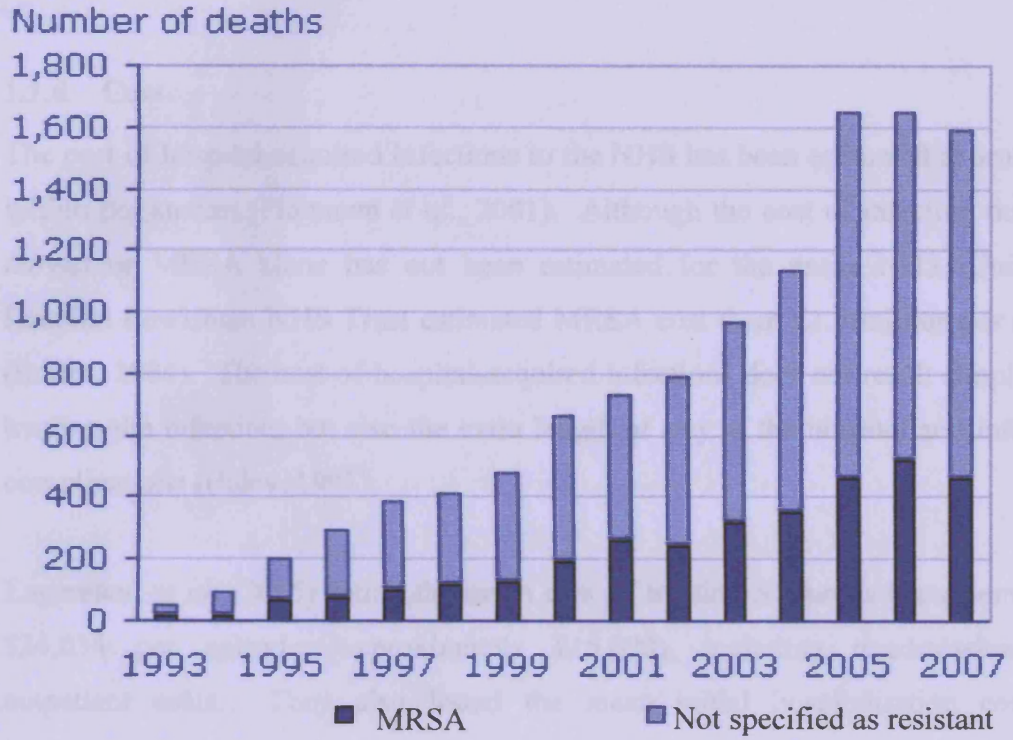

Figure 1.2: Deaths Associated with *S. aureus*

The number of death certificates mentioning MRSA and *S. aureus* not specified as resistant to methicillin in England and Wales from 1993-2007 (extracted from National Statistics, 2008).

Deaths across England and Wales due to MRSA staphylococcal septicaemia increased from 3% to 28%, staphylococcal pneumonia increased from 13% to 44% and unspecified staphylococcal infection from 19% to 83% between 1993 and 1998 (Crowcroft & Catchpole, 2002). Griffiths *et al.* (2004) revealed the number of death certificates that specifically mention MRSA also rose from 51 (12%) in 1992 to 800 (66%) in 2002, although the actual numbers may be higher because not all cases where *S. aureus* was not specified as methicillin resistant were necessarily methicillin sensitive. MRSA was found to be involved in (although not necessarily

directly responsible for) 0.07% of all deaths over the period of 1993-2002. Around double the rate of mortality was seen in males compared to females for both *S. aureus* and MRSA. A positive correlation between patient age and mortality rate was seen for both *S. aureus* and MRSA, where rates significantly increased for patients aged 65 and over.

1.1.6 Cost

The cost of hospital-acquired infections to the NHS has been estimated at around £1 billion per annum (Plowman *et al.*, 2001). Although the cost of infection due to *S. aureus* or MRSA alone has not been estimated for the entire NHS, University Hospital Lewisham NHS Trust estimated MRSA cost them £1.5 million per annum (Bourn, 2004). The cost of hospital-acquired infections does not result simply from treating the infection, but also the extra length of stay at the hospital and infectious complications (Haley, 1991).

Engemann *et al.* (2005) found the mean cost of treating *S. aureus* bacteraemia was $24,034 per episode (approximately £15,000), including re-admissions and outpatient costs. They also found the mean initial hospitalisation cost was significantly greater for patients with complicated (e.g. endocarditis, septic arthritis) versus uncomplicated *S. aureus* bacteraemia. Kanerva *et al.* (2007) found the total cost of an outbreak of MRSA was €1,821,256 (approximately £1.6 million), including isolation, microbial screening, medical treatment, administration, extra meetings, overtime, outpatient clinic for MRSA screening, screening of specimens obtained from staff members and closed beds.

Litigation costs are also a burden to the healthcare system. Claims of malpractice can be made following death or illness due to a hospital-acquired infection on the grounds that the hospital failed to adhere to acceptable standards of cleanliness and/or infection control. Guinan *et al.* (2005) studied claims of malpractice for healthcare-acquired infections in Philadelphia between 1996 and 2002 and found 29% were due to MRSA and 9% were due to MSSA. They also found that 72% of

claims were either settled out of court or withdrawn, but of those claims taken to court the success rate was 60%.

1.2 ANTIMICROBIAL ACTIVITY AND RESISTANCE

1.2.1 Antibiotics

1.2.1.1 Definitions

Antibiotics are defined as naturally occurring or synthetic organic substances that inhibit or destroy selective bacteria or other microorganisms, generally at low concentrations (McDonnell & Russell, 1999).

Resistance is defined as the relative insusceptibility of a microorganism to a particular treatment under a particular set of conditions and is usually quantified as the minimum concentration required to exert a definable effect (i.e. growth inhibition) on a population of cells (Gilbert & McBain, 2003). Antibiotic tolerance is defined as failure of an organism to respond to an appropriate, correctly dosed antibiotic *in-vivo* without an alternative explanation (Cunha *et al.*, 2008). Antibiotic resistance in *S. aureus* can be either intrinsic (inherent features of the bacterial cell that prevent antibiotic action) or acquired (horizontal transfer from another organism or by mutation of chromosomal genes). Resistance is acquired from other bacteria by conjugation, transduction and transformation (Lyon & Skurray, 1987). Conjugation is the exchange of genetic material by two bacterial cells in direct contact by means of a sex pilus, transduction is the transfer of genetic material between bacteria via a virus and transformation is the acquisition of genetic material directly from the environment after being released from another cell. Transfer of genetic material between bacteria is usually via a plasmid (Todar, 2008).

1.2.1.2 Antibiotic Use and Resistance Mechanisms

The antibiotics used to treat *S. aureus* infections, the mechanisms by which *S. aureus* is resistant to them and the frequency of resistance are described in Table 1.4. It is evident that antibiotic resistance is a major problem in the treatment of *S. aureus* and the joint working party of the BSAC, the Hospital infection Society and the Infection Control Nurses Association (Coia *et al.*, 2006) recommend the avoidance of inappropriate or excessive antibiotic therapy and prophylaxis in all healthcare settings as part of the control and prevention of MRSA in healthcare facilities.

Table 1.4: Antibiotics Available to Treat *S. aureus*

Antibiotic	Type	Mode of Activity	Resistance	Mode of Resistance	References
Penicillin	β-lactam	inhibits PBP enzymes used in peptidoglycan synthesis	Widespread	Penicillinase hydrolyses the β-lactam ring of Penicillin	Lowy (2003)
Methicillin	Modified β-lactam	As with penicillin, but is resistant to penicillinase	~40% *S. aureus* strains resistant	*mec*A gene in SCC*mec* encodes modified PBPs that cannot be bound by β-lactam antibiotics	Chambers *et al.* (1994); HPA (2006)
Vancomycin	Glycopeptide	disrupts cross-linkage of peptidoglycan layers during cell wall synthesis	Infrequent. VISA and increased MIC strains now widespread	increase in cell wall thickness traps vancomycin in the peptidoglycan layer	Murray *et al.* (1998); Cui *et al.* (2003); Appelbaum (2006); Tiwari & Sen (2006); Wang *et al.* (2006)
Teicoplanin	Glycopeptide	As for vancomycin	TISA and TRSA infrequent	As for vancomycin	Kaatz *et al.* (1990); HPA (2000); Cepeda *et al.* (2003); Tiwari & Sen (2006)
Quinupristin -dalfopristin	Streptogramin	Inhibits protein synthesis	Rare	Antibiotic is removed from the cell by efflux mechanisms	Dowzicky *et al.* (2000); Werner *et al.* (2001); Hershberger *et al.* (2004)
Linezolid	Oxazolidinones	Binds to rRNA, inhibiting protein translation	Rare. Monitored by ZAAPS	Modification of rRNA	Swaney *et al.* (1998); Ross *et al.* (2007); Arias *et al.* (2008)

MIC = Minimum Inhibitory Concentration; PBP= Penicillin Binding Protein; SCC*mec* = Staphylococcal Cassette Chromosome *mec*; TISA = Teicoplanin-Intermediate *S. aureus*; TRSA = Teicoplanin-Resistant *S. aureus*; VISA = Vancomycin Intermediate *S. aureus*; ZAAPS = Zyvox Annual Appraisal of Potency and Spectrum

1.2.2 Antiseptics

1.2.2.1 Definitions

An antiseptic is a type of biocide that destroys or inhibits the growth of microorganisms in or on living tissue (McDonnell & Russell, 1999). Biocide is the general term for a chemical agent, usually broad spectrum, which inactivates microorganisms (McDonnell & Russell, 1999). Types of biocides include disinfectants, antiseptics and preservatives (Maillard, 2005a). Resistance to antiseptics often refers to the insusceptibility of microorganisms to concentrations *in-vitro* that are above MIC level, but below the in-use concentration. Since these results rarely reflect performance in practice, true antiseptic resistance has been defined as bacterial survival following challenge with an antiseptic used at the 'in-use' concentration recommended by the manufacturer (Maillard, 2007).

1.2.2.2 General Antiseptics and Their Uses

Antiseptics are used in the healthcare environment in the form of hand washes, surgical scrubs and alcohol-based hand rubs and for pre-operative skin disinfection and topical treatment of wounds (Kampf & Kramer, 2004). Their efficacy is dependent on factors such as the presence of organic material, contact time, concentration, temperature and pH (Maillard, 2005a). The joint working party of BSAC, the Hospital Infection Society and the Infection Control Nurses Association recommend that triclosan, hexachlorophene, chlorhexidine or providone iodine alone and in combination may be suitable antiseptic agents to use in the eradication of MRSA (Coia *et al.*, 2006). The major agents used for antisepsis are described in Table 1.5.

Table 1.5: Antiseptics

Antiseptic	Class	Uses	Mode of Action	Pros/Cons
Chlorhexidine	Biguanide	Pre-operative skin disinfectant, hand scrub/rub	Cationic surfactant; damages cell wall/membrane; precipitation of proteins and nucleic acids	Broad spectrum, rapid, effective at low concentrations
Triclosan	Bisphenol	Surgical soap	Damages cytoplasmic membrane; disrupts lipid/protein/RNA synthesis	Prolonged activity; skin irritant
Providone-iodine	Iodine	Pre-operative skin disinfection, surgical scrubs	Reacts with cellular constituents	Broadest spectrum, including spores; fast-acting; skin irritation and staining
Benzalkonium chloride/cetrimide	QAC	Pre-operative skin disinfection; instant hand sanitizers	Cationic surfactant; disrupts cytoplasmic membrane	Slower activity; activity greatly affected by pH and organic material
Ethanol, isopropanol, *n*-propanol	Alcohol	Instant hand sanitizer; topical skin disinfectant	Protein denaturation/coagulation; lipid solubilization	Broad spectrum; little residual effect

QAC = Quaternary ammonium compounds. References: Russell & Day, 1993; McDonnell & Russell, 1999; Jones *et al.*, 2000; Ali *et al.*, 2001

1.2.2.3 Alcohol and Its Uses

Alcohol is the most common active ingredient of instant hand sanitizers, which are used for between-hand washing sanitization. This is due to alcohol being relatively inexpensive and non-toxic (to skin and the environment), easy to produce and colourless (Ali *et al.*, 2001). The most effective and therefore most commonly used alcohols are ethanol (a primary alcohol), isopropanol (propan-2-ol; a secondary alcohol) and *n*-propanol (propan-1-ol; a primary alcohol). They have a rapid and broad-spectrum activity (McDonnell & Russell, 1999; Ali *et al.*, 2001), but no residual effect (Ayliffe *et al.*, 1988), which is likely because they evaporate quickly. Evaporation is reduced by using the alcohol in conjunction with low concentrations of excipients that lengthen the evaporation time of the alcohol, or other biocides such as chlorhexidine that do have a residual effect. The antimicrobial activity of alcohol is the result of its low surface tension (which enables it to spread out on surfaces) and its lipid solvency (which enables it to dissolve lipids in the cytoplasmic membrane; Ali *et al.*, 2001). Alcohols have a polar hydroxyl group, which is thought to form a hydrogen bond with the ester groups of fatty acid residues in the bacterial cell membrane and cause disruption of membrane function (Franklin & Snow, 1989). Alcohol also causes denaturation and coagulation of proteins, cell lysis and disruption of cellular metabolism (McDonnell & Russell, 1999). It coagulates proteins in the cell wall and cytoplasmic membrane, where coagulation of structural proteins affects the integrity of these structures and coagulation of enzymatic proteins results in loss of cellular function (Ali *et al.*, 2001). It is most effective at high concentrations between 60% and 90% (McDonnell & Russell, 1999; Kampf & Kramer, 2004). Higher concentrations of alcohol are less effective because protein denaturation does not occur as readily in the absence of water (Ali *et al.*, 2001); lower concentrations are unsuitable because alcohol has a high concentration exponent and therefore will rapidly lose its biocidal activity when diluted (Maillard, 2005a).

1.2.2.4 Resistance to Antiseptics

Resistance to antimicrobials may occur as the result of chromosomal mutation or acquisition of resistance genes by transduction, transformation and conjugation (Lyon & Skurray, 1987). However, unlike antibiotics, antiseptics have multiple,

non-specific mechanisms of action on bacterial cells; therefore mutations in the bacterial genome are less likely to affect their activity (Jones *et al.*, 2000) and antiseptic resistance is more likely to be intrinsic or by acquisition of genes. Acquired and intrinsic factors provide a combination of ways for the bacterial cell to decrease the concentration of antiseptic to below the threshold that is harmful, either by removal from the cell or by decreasing its uptake or penetration into the cell (European Commission, 2009). These factors include a change in membrane (Maillard & Cheeseman, 2008) and cell wall permeability, use of efflux pumps (Fraise, 2002), production of exopolysaccharide (Mah & O'Toole, 2001; Campanac *et al.*, 2002), hydrophobicity (Jonsson & Wadström, 1984) and surface charge (Bruinsma *et al.*, 2006). Acquired and intrinsic factors are also responsible for reducing the lethal interaction of the antiseptic with the cell by reducing metabolic activity and damage repair (Maillard & Cheeseman, 2008) and also inactivation of the biocide may occur by enzymatic degradation (McDonnell, 2007).

Resistance to alcohol has not been demonstrated (Wille, 1976; Narui *et al.*, 2007). However, clinical isolates of *S. aureus* have been found that are resistant to a range of antiseptics. Noguchi *et al.* (1999) found 71% of MRSA isolates tested demonstrated resistance to the QAC benzethonium chloride and the biguanide chlorhexidine gluconate. Narui *et al.* (2007) found that a number of clinical MRSA isolates showed minimum bactericidal concentrations higher than the in-use concentrations of sodium hypochlorite, benzalkonium chloride, alkyl-diaminoethylglycine hydrochloride and chlorhexidine digluconate. Reduced susceptibility has been demonstrated among *S. aureus* isolates, where MICs are still below the in-use concentration for chlorhexidine (Sheng *et al.*, 2009) and triclosan (Cookson *et al.*, 1991; Al-Doori *et al.*, 2003).

Some antiseptic resistance is mediated by efflux pumps, which are able to remove a range of chemically dissimilar antiseptics from the bacterial cell (Fraise, 2002). High level antiseptic resistance is mediated by efflux pumps encoded by the genes *qac*A and *qac*B. Low-level antiseptic resistance is mediated by efflux pumps encoded by the genes, *smr* and *nor*A. The *qac*A gene is usually found on the pSK1 multi-resistant plasmid and encodes the multi-drug efflux transporter protein QacA,

which removes antiseptics such as QACs and chlorhexidine from the cell (Tennent *et al.*, 1989; Littlejohn *et al.*, 1991). The *qacB* gene is closely related to *qacA*, and is usually found on heavy metal plasmids such as pSK23 (Paulsen *et al.*, 1996). It encodes the multi-drug efflux transporter protein QacB. The *smr* gene is located on a plasmid and encodes the multi-drug efflux transporter protein Smr (Grinius *et al.*, 1992). The *norA* gene is located on the *S. aureus* chromosome and encodes a multi-drug efflux transporter protein called NorA (Neyfakh *et al.*, 1993; Ng *et al.*, 1994), which removes antiseptics from the bacterial cell (Noguchi *et al.*, 2004). Distribution of *qacA*, *qacB* and qac*C* (which is identical to *smr* gene) genes in European clinical *S. aureus* isolates was determined by Mayer *et al.* (2001). They found *qacA/B* genes present in 42% of isolates (63% of MRSA isolates, 12% of MSSA isolates) and the *qacC* gene present in 5.8% of isolates (6.4% of MRSA isolates and 5% of MSSA isolates).

Antiseptic resistance has been linked to antibiotic resistance. Akimitsu *et al.* (1999) found that MRSA mutants resistant to benzalkonium chloride showed a higher level of resistance to a number of β-lactam antibiotics, particularly oxacillin, compared to non-resistant parent strains. The most likely explanation for linked antibiotic and antiseptic resistance is that they are mediated by a common, non-specific mechanism such as cell impermeability or efflux pumps, or that mechanisms for antibiotic or antiseptic resistance are encoded in genes that are linked, such as being encoded on the same plasmid. Yamamoto *et al.* (1988) found a gentamicin-methicillin-resistant strain of *S. aureus* carrying the pSAJ1 plasmid, which carried the *qacA* gene and conferred resistance to several antibiotics and the antiseptics benzalkonium chloride, chlorhexidine and acriflavine, supporting the theory that efflux pumps may be a common mechanism for antibiotic and antiseptic removal from the cell. This may explain why Suller & Russell (1999) found that MRSA isolates showed decreased susceptibility to chlorhexidine, benzalkonium chloride and another QAC called cetylpyridinium chloride, when compared to MSSA strains.

1.3 REDUCING *S. AUREUS* COMTAMINATION IN THE HEALTHCARE ENVIRONMENT

1.3.1 Hand Hygiene

1.3.1.1 The Importance of Hand Hygiene

Since the most common mode of transmission of microorganisms is HCW's hands, hand hygiene is considered the most important measure for preventing their transmission and therefore reducing the incidence of hospital-acquired infection (Conly *et al.*, 1989; Rotter, 1997). Transient flora are defined as contaminating or non-colonizing flora, which are isolated from the skin but are not consistently present in the majority of persons and resident flora are defined as colonizing flora, which are isolated from the skin of most persons and considered permanent residents of the skin (Larson, 1995). Transient bacteria are generally attached to superficial layers of skin, whereas resident bacteria are attached to deeper layers (Langley, 2002). Transient bacteria are removed by basic hand hygiene using plain soap (Langley, 2002; Kampf & Ostermeyer, 2004), whereas resident bacteria are not (Kampf & Ostermeyer, 2004) and therefore require the use of antimicrobial agents in the form of hand washes or rubs to remove them (Kampf & Kramer, 2004; Hübner *et al.*, 2006).

1.3.1.2 Hand Hygiene Compliance

The use of alcohol hand Rubs (AHR) has resulted in a significant increase in hand hygiene compliance, mainly due to the process being much quicker and easier, although hand washing is still required for 'dirty' hands. Karabay *et al.* (2005) found hand hygiene compliance low at 15.4% among nursing staff provided only with hand washing facilities, but much higher at 72.5% among nursing staff provided with AHRs. Elsewhere, hand hygiene compliance has been found to be fairly low (42%), despite the introduction of AHRs (Johnson *et al.*, 2005b).

Hand washing and AHR compliance was found to be poorer among HCWs in ITUs than in other hospital wards (Pittet *et al.*, 1999; Karabay *et al.*, 2005). Compliance

with hand hygiene guidelines in ITUs was found to be between 28% and 51% in a review of studies conducted before the year 2000 (Pittet, 2001a). This fairly low level of hand hygiene compliance in ITUs was also observed in later studies (38.4%, Hugonnet *et al.*, 2002; 51%, Creedon, 2005 and 47%, Eldridge *et al.*, 2006), although compliance improved upon the introduction of interventions to improve hand hygiene, including the introduction of AHRs (54.5%, Hugonnet *et al.*, 2002; 83%, Creedon, 2005 and 80%, Eldridge *et al.*, 2006).

Pittet *et al.* (1999) found that hand hygiene compliance was higher among nurses than physicians, nursing assistants and other HCWs. Compliance was also found to be lowest at weekends, during procedures that carried a high risk of contamination and during high intensity of patient care.

Variation in levels of hand hygiene compliance is likely due to the difference in success of the intervention and differences within the hospitals in which the studies were conducted, such as staff-to-patient ratio. Patarakul *et al.* (2005) found hand hygiene compliance in the ITUs at one hospital to be less than 50% and questionnaires given to HCWs revealed the most common reason for non-compliance was their belief that patients' needs came first, followed by forgetfulness and skin irritation by hand hygiene products. These factors were also found to be the most common reasons for non-compliance throughout the healthcare setting and also included inaccessibility of hand hygiene products, high workload and the wearing of gloves (Pittet, 2001b).

1.3.1.3 Alcohol Hand Rubs

AHRs consist of alcohol at a concentration of typically 60-80% using at least one type of alcohol. The types of alcohols used in hand rubs are normally ethanol, isopropanol or *n*-propanol; these are used at different ratios as the active ingredient, plus different types and concentrations of emollients to soften the skin.

Results of the efficacy of AHRs in comparison to hand washing with antimicrobial soap have been mixed. Larson *et al.* (2001) found no significant difference between the CFU remaining on the hands of staff using either product type. It was also found

by Larson *et al.* (1995) that there was no significant difference between infection rates in a neonatal ITU using either product. However, Girou *et al.* (2002) found an 83% reduction in bacterial contamination of the hands of ITU staff using an AHR compared to a 58% reduction using an antimicrobial hand wash. Karabay *et al.* (2005) found bacterial contamination was reduced by 54% with the use of AHRs compared to only 27% with an antimicrobial soap. Rotter's (2001) review of hand hygiene experiments revealed that AHR was more effective at reducing numbers of bacteria than medicated soaps, which were more effective than non-medicated soaps.

Alcohol-based hand rinses have been used in the healthcare environment, but in many hospitals have been replaced by alcohol gels, which are quicker to use and therefore increase compliance and also reduce drying and irritation of the skin. However, Kramer *et al.* (2002) found that no alcohol gels tested met the criteria of EN1500 (which are that the test product should not have a significantly smaller log reduction than the reference product), whereas most of the alcohol-based hand rinses did. They also found that isopropanol at 60% v/v was more effective than ethanol at 70% v/v and is almost equivalent to ethanol at 80% v/v (*n*-propanol being the most efficacious) and therefore recommend that ethanol-based AHRs should be at a minimum concentration of 80% v/v. Rotter's (2001) review of experiments that determined the efficacy of AHRs revealed that *n*-propanol is the most effective alcohol, followed by isopropanol and then ethanol, which supports the findings by Kramer *et al.* (2002).

1.3.2 Infection Control and Prevention Programmes

Initiatives to control and prevent HCAIs initiatives include surveillance; antibiotic stewardship; screening; decolonization of nose, throat and skin; management of MRSA colonized or infected patients through patient isolation; cleaning and decontamination; minimizing patient movement and topical/systemic decontamination or prophylactic antimicrobial therapy prior to invasive procedures; ensuring there are adequate nursing staff available; hand hygiene and use of personal protective equipment (Coia *et al.*, 2006). Infection control programmes have been found to be beneficial in terms of reduction in MRSA and cost-effective, where

treating MRSA infection was far more costly than the MRSA control programme (Chaix *et al.*, 1999).

Implementation of the Health Act 2006 code of practice for the prevention of HCAIs (Department of Health, 2008) is a legal requirement throughout the NHS. This code of practice states that 'effective prevention and control of HCAIs has to be embedded into everyday practice and applied consistently by everyone. It is particularly important to have a high awareness of the possibility of HCAIs in both patients and healthcare workers to ensure early and rapid diagnosis. This should result in effective treatment and containment of the infection. Effective action relies on an accumulating body of evidence that takes account of current clinical practices. This evidence base should be used to review and inform practice. All staff should demonstrate good infection control and hygiene practice.'

This code of practice can be achieved by implementing an effective infection control programme. These are different throughout each NHS trust and several programmes have been developed to reduce the incidence of HCAIs.

The Epic Project (Pratt *et al.*, 2001) provides detailed guidelines for hospital environmental hygiene, hand hygiene, patient skin antisepsis, insertion and maintenance of indwelling medical devices such as catheters and antibiotic prophylaxis that will prevent HCAIs. The guidelines were developed as a result of the review of scientific evidence.

The low-compliance rate of hand hygiene in the healthcare environment has led to the introduction of the 'CleanYourHandsCampaign' (CYHC) throughout the NHS in 2005. This involved introduction of AHR at bedsides and ward entrances along with promotional material and patients were encouraged to ask staff if they had sanitized their hands. This campaign was initially piloted in two wards in each of six acute NHS trusts and its effectiveness was evaluated in one of the trusts by Randle *et al.* (2006). They found hand hygiene compliance rose from 32% to 63% over a six-month period and the volume of AHR consumed increased by 184%. A staff survey revealed overall positive feedback on the campaign. Similar interventions have been

carried out in many hospitals across the globe, which have all resulted in increased compliance with hand hygiene guidelines (Hugonnet *et al.*, 2002; Creedon, 2005; Johnson *et al.*, 2005b; Eldridge *et al.*, 2006). However, Gould *et al.* (2007) believe initial results may not be sustained long-term and worry this campaign is seen as a 'quick fix' to solve the problem of hospital-acquired infection and that hand hygiene has been singled out as the solution instead of an all-round robust infection-control programme.

Results of the CYHC have been determined for the 6 and 12 month period after it was first initiated by the National Observational Study to Evaluate the CYHC (NOSEC; Stone *et al.*, 2007). Results show that this campaign has resulted in a change in hand hygiene behaviour throughout the NHS in the UK, particularly the increased use of AHR without a decrease in the use of soap.

'Saving Lives' was launched in 2005 (Department of Health, 2007) with an aim to reduce infection and deliver clean and safe care and includes self-assessment, use of scorecards to illustrate compliance and high-impact interventions. This system was designed with an aim to be implemented throughout the NHS and may eventually replace the CYHC.

Pittet *et al.* (2000), McAteer *et al.* (2006) and Ebnöther *et al.* (2008) found the implementation of infection control programmes were able to reduce the rate of infection, reduce transmission of MRSA and/or increased hand hygiene compliance. Huang *et al.* (2006b) found implementation of routine MRSA surveillance rather than introducing AHR and hand hygiene campaigns resulted in a significant reduction of MRSA bacteraemia. Harrington *et al.* (2007) found a combination of the introduction of AHR containing chlorhexidine gluconate throughout a hospital, and the introduction of a hospital-wide MRSA surveillance feedback programme resulted in a reduction of MRSA bacteraemia by 39% in ITU.

1.4 AIMS AND OBJECTIVES

The aim of this project was to determine the susceptibility of *S. aureus* ITU isolates to AHRs under conditions found in practice. This was achieved with the following objectives:

- To establish the basic characteristics of the isolates used in this study, including their identity, susceptibility to antibiotics, virulence and genotype.
- To determine the hand sanitizing practices used in the ITU and the susceptibility of *S. aureus* isolates to AHR, including the effect of mechanical rubbing and residual activity on their antibacterial properties.
- To design the susceptibility tests to mimic conditions found in practice as far as possible and to extrapolate the results to *in-situ* use of AHR.
- To determine the effect of AHR on *S. aureus* by investigating their effect on the cytoplasmic membrane, cell wall, cell structural appearance, proteins and gene expression.
- To determine the properties of *S. aureus* that are linked to its susceptibility to AHR by comparing hydrophobicity, surface charge, cell size, cell aggregation and total protein content of the most- and least-susceptible isolates to AHR.

CHAPTER 2 GENERAL MATERIALS AND METHODS

2.1 GENERAL MATERIALS

All media, chemicals, materials and equipment listed in this study were purchased from Fisher Scientific (Loughborough, UK) or Sigma-Aldrich (Poole, UK), unless otherwise stated. Tryptone soya agar (TSA), tryptone soya broth (TSB) and blood agar base no. 2 were all purchased from Oxoid (Basingstoke, UK). Blood plates and slopes were prepared using blood agar base no. 2 with sheep blood defibrinated liquid (Oxoid). Tryptone sodium chloride (TSC) buffer was prepared with 0.4 g tryptone (Oxoid) and 3.4 g sodium chloride in 400 ml deionised water. All media and buffers were prepared using deionised water and autoclaved at 121 °C for 15 min according to British Pharmacopoeia 2007 recommendation (British Pharmacopoeia Commission, 2006). All plates were dried in a laminar airflow cabinet with their lids open for 5 min prior to use.

2.2 ANTISEPTICS

The antiseptics used for testing were Soft Care Med H5 alcohol gel (Johnson Diversey, Northampton, UK), Cutan gel hand sanitizer (Deb Ltd. Belper, UK) and Guest Medical AHR (Guest Medical, Edenbridge, UK). Soft Care Med H5 contains isopropyl alcohol (isopropanol) and propyl alcohol (*n*-propanol) at a total concentration of 70% w/w as the active ingredient. It also contains the emollient glycerol and is the current antiseptic used in University Hospital of Wales (UHW), Cardiff. Cutan contains ethyl alcohol (ethanol) and propyl alcohol at a total concentration of 70% w/w as the active ingredient. It also contains the emollients glycerine and panthenol and is the current antiseptic used at the bedside and by the sinks of Morriston Hospital (MH), Swansea. Guest Medical contains ethyl alcohol at a concentration of 70% v/v as the active ingredient. It also contains the emollients/skin care additives carbomer, glycerine, and vitamin E, plus isopropyl myristate to increase penetration into the skin and monopropylene glycol as a surfactant, to ease spreading of the product on the surface of the skin. It is the current antiseptic ITU nurses in MH carry around on their person. All AHRs were stored at room temperature.

2.3 BACTERIAL ISOLATES

Fifty nine hospital isolates of *S. aureus* (32 MRSA and 27 MSSA) were supplied by UHW and Singleton Hospital (SH), Swansea (see Table 2.1). These isolates originated from blood cultures taken from bacteraemia patients in the hospitals' ITUs. *S. aureus* NCIMB 9518/ATCC 6538 (National Collections of Industrial Marine and Food Bacteria; American Type Culture Collection), ATCC 49775, NCTC (National Collection of Type Cultures) 8325, NCTC 8530 and NCTC 8178 are MSSA strains. Reference strains were purchased from LGC Standards (Teddington, UK), except for ATCC 49775 and NCTC 8530, which were kindly provided by P. Lambert (Aston Universtiy) and NCTC 8178, which was kindly provided by C. Emanuel (Cardiff University).

Table 2.1: List of Hospital Isolates

Information shown is that provided by the hospital from which the samples came. UHW = University Hospital of Wales, Cardiff, SH = Singleton Hospital, Swansea. MRSA = Methicillin Resistant *S. aureus*, MSSA = Methicillin Sensitive *S. aureus*. *Found to be MSSA.

Isolate	Date Isolated	Hospital	Methicillin Resistance Profile
3,5,9,11,15,17,18,19,20,25,26,27,31, 32,37,41,42,43,45,46,48,49,50,52,55	Feb 2004-Jun 2006	UHW	MRSA
4,6,10,13,14,16,21,24,28,30,34,38,39, 44,47,51	Mar 2004-Nov 2005	UHW	MSSA
N10, K17, Q49, T4, H9	2006	UHW	MSSA
P59, O43	2006	UHW	MRSA
S1, S2, S7, S8, S10, S11,	Feb 2006-Oct 2006	SH	MSSA
S3, S4, S5*, S6, S12	Mar 2006-Nov 2006	SH	MRSA

2.4 PREPARATION OF CULTURES

2.4.1 Frozen Cultures

Upon arrival, frozen stocks of all hospital isolates of *S. aureus* were prepared in duplicate. Plastic cryovials (1.2 ml) were filled with 200 µl glycerol and autoclaved at 121 °C for 15 min. Overnight cultures were prepared according to 2.4.4 and 800 µl of each culture was added to a cryovial and stored at –80 °C.

2.4.2 Working Cultures

Each hospital isolate was inoculated onto a blood agar plate from frozen culture using a sterile swab and inoculation loop and incubated at 37 °C for 24 hours. The agar plate was checked for purity and several well-isolated colonies were inoculated onto a blood agar slope. The slope was incubated at 37 °C for 24 hours then refrigerated. Slopes were replaced every 3 months.

2.4.3 TSA Slope Culture

A loop-full of working culture was used to inoculate the surface of a TSA slope and TSA plate. These were incubated at 37 °C for 16-24 h. The TSA plate was examined for purity of the culture.

2.4.4 Overnight Broth Cultures

A loop-full of working culture (or original culture) was used to inoculate 10 ml TSB and a TSA plate. The broth was placed in a shaker incubator (Fisher Scientific) at 70 rpm, 37 °C overnight (16-18 h, unless otherwise specified) and the TSA plate incubated at 37 °C and examined for purity of culture.

2.5 PREPARATION OF A BACTERIAL SUSPENSION

2.5.1 Preparation of Bacterial Suspension from TSA slope

A TSA slope was prepared according to section 2.4.3. Slopes were washed with 5 ml TSC buffer and the suspension was centrifuged in a Mistral 1000 centrifuge (MSE, London, UK) at 2600 *g* for 15 min. The supernatant was discarded and 5 ml TSC was added to re-suspend the pellet by vortex mixing. The suspension was adjusted to the appropriate concentration according to section 2.5.3.

2.5.2 Preparation of Bacterial Suspension from TSB

An overnight broth culture was performed according to section 2.4.4 and centrifuged at 940 *g* for 10 min. The supernatant was discarded and 5 ml TSC was added to re-suspend the pellet by vortex mixing. If required, cells were washed in TSC buffer unless otherwise stated and centrifuged at 2,600 *g* for 15 min. The suspension was adjusted to the appropriate concentration according to section 2.5.3.

2.5.3 Adjusting the Concentration of a Bacterial Suspension

The optical density (OD) range at 500 nm required to achieve the desired bacterial concentration was calculated using the linear equation produced from the fitted line plot in Figure 2.1 in Section 2.7. The OD_{500} of the re-suspended pellet prepared above was measured using a spectrophotometer (Helios α spectrophotometer, Unicam, Cambridge, UK) and adjusted to within the acceptable OD range using TSC buffer. A viable count was performed according to Section 2.6 and the results of the OD and viable count were added to the fitted line plot in Figure 2.1.

2.6 PERFORMING A VIABLE COUNT OF A SUSPENSION

2.6.1 Drop Count Method

2.6.1.1 Method

Viable counts were performed to determine the concentration of a bacterial suspension and were performed using an adaptation of the Miles & Misra (1938) drop count method. A suspension was prepared according to either Section 2.5.1 or 2.5.2 and serially diluted by transferring 100 µl neat suspension into a sterile centrifuge tube containing 900 µl TSC buffer, creating a 10^{-1} dilution. After vortex mixing, 100 µl of the 10^{-1} dilution was transferred to another sterile centrifuge tube containing 900 µl TSC buffer to make a 10^{-2} dilution, with further serial dilutions to the required level. Ten µl of the appropriate dilutions were dropped in triplicate onto a TSA plate. The dilutions were then mixed using a vortex mixer and the 10 µl triplicate drops repeated on a separate TSA plate. Plates were incubated at 37°C for 24 hours and the dilutions yielding counts between 3 and 30 CFU were recorded.

2.6.1.2 Validation Method

A bacterial suspension of *S. aureus* NCIMB 9518 was prepared according to section 2.5.1 and from this ten dilution series were prepared and plated out using the drop count method in section 2.6.1.1, but on triplicate plates instead of duplicate. Statistical analysis was performed on the log data values according to section 2.8.1 to determine whether there was a significant difference between viable counts of each dilution series.

2.6.1.3 Validation Results

Statistical analysis of viable count data revealed there was no significant difference between the means of each dilution series ($P \geq 0.05$), data not shown. The drop count method was therefore demonstrated to provide reproducible results and was successfully validated.

2.6.2 Pour Plate Method

2.6.2.1 Method

The pour plate method was used to determine the concentration of a bacterial suspension. A suspension was prepared according to Section 2.5.1 or 2.5.2 and serially diluted by transferring 1 ml of neat suspension into a sterile glass bottle containing 9 ml TSC buffer, creating a 10^{-1} dilution. After vortex mixing, 1 ml of the 10^{-1} dilution was transferred to a fresh glass bottle containing 9 ml TSC buffer to make a 10^{-2} dilution, and so on down to the appropriate dilution. One ml of each appropriate dilution was transferred to duplicate Petri dishes, vortex mixing them in between the duplicate transfers. Around 20 ml molten TSA agar cooled to 45 °C was poured onto each plate and gently rotated to mix. Plates were left to set on the bench at room temperature for approximately 1 h until solidified, then inverted and incubated at 37 °C for 24 hours. The dilutions yielding counts between 30 and 300 CFU were recorded.

2.6.2.2 Validation Method

A bacterial suspension of *S. aureus* NCIMB 9518 was prepared according to section 2.5.1 and the pour plate method was performed with eight replicates according to section 2.6.2.1, except 1 in 10 serial dilutions were made by adding 350 µl bacterial solution to 3.15 ml TSC buffer and dilutions were plated out in triplicate. Statistical analysis was performed on the log data values according to section 2.8.1 to determine whether there was a significant difference between viable counts of each dilution series.

2.6.2.3 Validation Results

Statistical analysis of viable count data revealed there was no significant difference between the means of each dilution series ($P \geq 0.05$), data not shown. The pour plate method was therefore demonstrated to provide reproducible results and was successfully validated.

2.7 STANDARDISING THE CONCENTRATION OF A BACTERIAL SUSPENSION

A bacterial suspension of *S. aureus* NCIMB 9518 was prepared according to section 2.5.1; 1 in 2, 1 in 4, 1 in 5, 1 in 10, 1 in 20, 1 in 40 and 1 in 100 dilutions of the suspension were made in TSC buffer. The OD_{500} of each dilution was measured and a viable count was performed for each dilution according to 2.6.1.1. The OD_{500} of each dilution was plotted against the results of the viable count in a Fitted Line Plot graph, using Minitab (release 14 software, Minitab inc., PA, USA) and is shown in Figure 2.1. This created a linear equation that allows the optical density required to achieve the desired bacterial concentration to be determined. Subsequent OD_{500}/viable count results of each bacterial suspension performed according to 2.5 were added to Figure 2.1, data not shown.

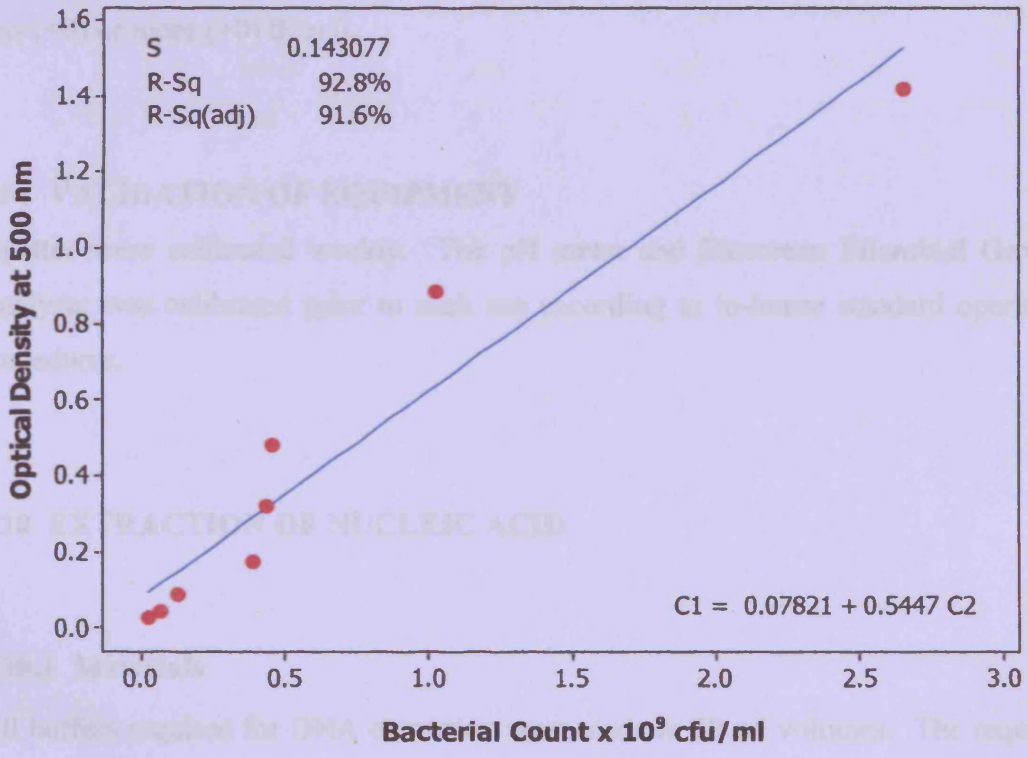

Figure 2.1: Relationship between Bacterial Count and Optical Density

Fitted Line Plot showing the relationship between bacterial count and optical density of *S. aureus* NCIMB 9518.

2.8 STATISTICAL ANALYSIS

2.8.1 One-Way ANOVA

One-way analysis of variance (ANOVA) was used at the 95% level of significance to test differences between the means of data sets. Minitab software (release 14/15 software, Minitab inc., PA, USA) was used to perform the calculations and to test the normality of distribution of the residuals and homogeneity of variances of the data. Where the assumptions of ANOVA were not met the Kruskal-Wallis test was used.

2.8.2 Paired T-Test

The paired t-test in Minitab software was performed, using a confidence level of 95.0 and a test mean of 0.00 to determine whether the mean difference was significantly less (<0) or more (>0) than 0.

2.9 VALIDATION OF EQUIPMENT

Pipettes were calibrated weekly. The pH meter and Bioscreen Microbial Growth Analyser was calibrated prior to each use according to in-house standard operating procedures.

2.10 EXTRACTION OF NUCLEIC ACID

2.10.1 Materials

All buffers required for DNA extraction were made in 50 ml volumes. The required volume of deionised water was autoclaved in a glass bottle and other materials were later added (see Table 2.2). Buffers could be kept for several weeks and were stored at room temperature. Ultrapure™ 1 M Tris-HCl pH 8.0, Ultrapure™ 0.5 M ethylenediaminetetraacetic acid (EDTA) pH 8.0 and Ultrapure™ 10% SDS were purchased from Invitrogen (Paisley, UK). RNase A in powder form was made into a 5 mg/ml working concentration in Tris-HCl EDTA (TE) buffer and boiled for 10 min

to destroy any DNase. Pronase was purchased in powder form from Roche Applied Science (Burgess Hill, UK) and prepared as a 20 mg/ml stock in DNase and RNase-free distilled water (Invitrogen). RNaseA and pronase were stored at -20 °C. Ethanol solutions were prepared in deionised water. Ethanol at 100% and 70% v/v were stored at -20 °C ready for use and ethanol at 75% v/v was stored at room temperature. Lyophilized, powdered lysozyme containing ≥40,000 units/mg protein was prepared as 3mg/ml in TE buffer that was prepared according to Table 2.2 and stored at -20 °C. Isopropyl alcohol was stored at room temperature.

Table 2.2: DNA Extraction Buffers

Buffer	Vol. Deionised Water (ml)	Vol. 1 M Tris-HCl (ml)	Vol. 0.5 M EDTA (ml)	Vol. 10% SDS (ml)	Other (µl)
TE buffer	48.5	0.5	1.0	0	0
Lysis buffer	41.5	2.5	1.0	5.0	*
Low EDTA buffer	49.5	0.5	0.01	0	5 µl RNase

* A 25 µl volume of pronase was added to the lysis buffer immediately prior to use. EDTA = Ethylenediaminetetraacetic acid; RNase = Ribonuclease; TE = Tris-HCl EDTA

2.10.2 DNA Extraction

This method was kindly provided by E. Mahenthiralingam, Cardiff University and is now published by Cheeseman *et al.* (2007). DNA was extracted from all hospital isolates and *S. aureus* NCIMB 9518 in preparation for DNA analysis. An overnight broth culture was prepared according to 2.4.4. The culture was centrifuged at 940 *g* for 10 min and the pellet was re-suspended in 100 µl TE buffer. A 2 ml screw cap plastic tube was filled 1/3 full with 0.1 mm zirconia/silica beads (BioSpec Products,

Inc., Bartlesville, OK, USA) and 500 µl of lysis buffer and 100 µl of pellet suspension were added. The tube was placed in a mini-bead beater (BioSpec Products) for 10 s at 2,500 rpm, incubated for 1-3 hours at 37°C and centrifuged in a Heraeus Biofuge Pico mini-centrifuge (DJBLabcare, Newport Pagnell, UK) at 13,000 *g* for 1 min to remove bubbles. A 200 µl volume of saturated ammonium acetate was added to the tube, which was then placed in a mini-bead beater at 2,500 rpm for 5 s and centrifuged at 13,000 *g* for 1 min. Chloroform (600 µl) was then added to the tube and placed in a mini-bead beater at 2 500 rpm for 5 s. Centrifugation at 13,000 *g* for 3 min revealed three phases. A 400 µl volume of the clear top phase was transferred to a 1.5 ml no-stick microtube (Alpha Laboratories Ltd., Eastleigh, UK) containing 1ml 100% ethanol and refrigerated for 15 min. The tube was inverted to precipitate the DNA and centrifuged at 13,000 *g* for 5 min. The ethanol was discarded and 500 µl 70% ethanol was added. The tube was centrifuged at 13,000 *g* for 1 min and the ethanol was removed with a pipette. The tube was placed for 10 min in a vacuum dryer to remove excess liquid. A 30 µl volume of Low EDTA buffer was added to the tube, which was then incubated at 37 °C for 1 h then left at room temperature overnight. Electrophoresis was performed on the extracted DNA according to 2.11 to check DNA was present and to estimate concentration. DNA was diluted 1 in 10 with DNase and RNase-free distilled water (Invitrogen) and transferred into 0.5 ml PCR tubes (Eppendorf, Cambridge, UK) and refrigerated.

2.10.3 RNA Extraction

The method was provided by Invitrogen (TRIzol LS Reagent manual). Between 4 and 6 broth cultures were prepared according to section 2.4.4, but cells were grown to the exponential growth phase (for 6 hours). Suspensions of the bacteria were adjusted to a concentration of approximately 1 x 10^7 CFU/ml in TSB according to section 2.5.3. One ml of each adjusted suspension was transferred to a plastic microcentrifuge tube flash-frozen in liquid nitrogen and centrifuged in a Heraeus Biofuge Pico mini-centrifuge at 12,000 *g*. The supernatant was discarded and the pellet was re-suspended in 1 ml TRIzol®. The mixtures were transferred to 2 ml plastic screw cap tubes containing 0.1 mm zirconia/silica beads and vortex mixed

using a horizontal vortex adaptor (Ambion, Warrington, UK) for 10 min. After 5 min 0.2 ml chloroform was added and the tubes were shaken vigorously by hand for 15 s to mix. After 3 min the tube was centrifuged at 12,000 *g* for 15 min and the top aqueous phase was transferred to a fresh tube. A 0.5 ml volume of isopropanol was added to the aqueous phase and after 10 min the mixture was centrifuged at 12,000 *g* for 10 min. The supernatant was removed and the pellet was washed with 1ml of 75% ethanol by vortex mixing and centrifugation at 7,000 *g* for 5 min. After removing the supernatant the pellets were air-dried for 10 min and one pellet was re-suspended in 20 µl HyPure™ molecular biology grade distilled water. The pellet was re-suspended and added to the second pellet, and so on until all RNA was pooled. After incubating for 10 min at 60 °C the RNA was quantified. Two µl of RNA mixture was transferred to a plastic UVette® (Eppendorf, Hamburg, Germany) containing 68 µl TE buffer prepared according to Section 2.10.1. A negative control was also set up containing 70 µl TE buffer. The concentration of RNA was measured using a BioPhotometer (Eppendorf). RNA extraction was also performed post-exposure to AHR as above, except after adjusting the concentration of the broth cultures 900 µl of each were transferred to separate microcentrifuge tubes. A volume of 100 µl AHR was added to each tube, mixed, and after a contact time of 1 h were flash frozen.

2.11 ELECTROPHORESIS OF NUCLEIC ACID

2.11.1 Materials

One sachet of Tris-Borate-EDTA (TBE) 10 x powder was dissolved in 1 L deionised water using a magnetic stirring plate (Fisher stirring hotplate, Fisher Scientific). This provided a 10 x TBE solution. One x TBE solution was prepared by adding 100 ml 10 x TBE to 900 ml deionised water. One Kb Plus DNA Ladder was purchased from Invitrogen and prepared as a 0.05 µg/ml stock for DNA analysis (50 µl ladder, 200 µl gel loading solution and 750 µl 1 x TBE) and 0.1 µg/ml stock for RNA analysis (100 µl ladder, 200 µl gel loading solution and 700 µl 1 x TBE). Stock solutions were stored in a refrigerator.

2.11.2 Preparation of Gel without Ethidium Bromide

Gels for DNA extraction were prepared using 1% agarose for routine use with 1 x TBE. Gels used for all other tests were prepared using 1.5% high resolution agarose with 1 x TBE. The appropriate volume of agarose and 1 x TBE was added to a conical flask and heat dissolved in a microwave at 950 W or on a magnetic stirring plate at 250 °C until melted and poured into a gel casting tray (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK). Trays were left to set at room temperature and refrigerated until needed.

2.11.3 Preparation of Gel with Ethidium Bromide

Gels were prepared as in section 2.11.2, except 0.005% v/v ethidium bromide was added to the molten agarose prior to pouring into the gel casting tray. Gels used for analysis of RT-PCR product were prepared with 0.001% ethidium bromide.

2.11.4 Electophoresis

Small gel casting trays were loaded into a Mini-Sub® Cell GT electrophoresis chamber (Bio-Rad Laboratories Ltd) and large gels were loaded into a Sub-Cell® GT electrophoresis chamber (Bio-Rad Laboratories Ltd), along with 1 x TBE buffer prepared according to section 2.10.1. Ten µl 1Kb Plus Ladder (prepared according to section 2.10.1) was added to the first well for a small gel and the first and last well of a large gel, plus in an additional well in the middle for large gels for random amplification of polymorphic DNA (RAPD) testing. A mixture of 2 µl gel loading solution and 5 µl of gently vortex mixed DNA sample was added to the remaining wells. Electrophoresis was performed at 90 V for routine use gels and 80V for high resolution gels using a PowerPac™ Basic power supply (Bio-Rad Laboratories Ltd). Gels performed according to section 2.11.2 were then stained with ethidium bromide by immersing in 1 x TBE containing 0.007% ethidium bromide and placing on a Grant Bio POS 300 rotating platform for 45 min at 50 rpm. Gels performed according to 2.11.3 did not require this step. Gels were then viewed under UV light using Bio-Rad Gel Doc 1000 and Bio-Rad Molecular Analyst software (Bio-Rad Laboratories Ltd.

CHAPTER 3 DETERMINING THE BASIC CHARACTERISTICS OF *STAPHYLOCOCCUS AUREUS* INTENSIVE THERAPY UNIT ISOLATES

3.1 INTRODUCTION

3.1.1 Genotypic Characterization of Isolates

The hospital isolates involved in this study (see Chapter 2, Table 2.1) were isolated from two hospitals over a period of two years. Genotyping isolates enables them to be grouped together into strains according to their genetic profile, revealing which isolates are genetically similar and allowing comparison of isolates within each hospital and between the two hospitals. Genotyping can be performed using Pulsed Field Gel Electrophoresis (PFGE), Multi Locus Sequence Typing (MLST), *spa* typing and SCC*mec* typing. PFGE uses alternating electric currents to pass through the gel, which separates the bacterial DNA into its component parts (Schwartz & Cantor, 1984). MLST categorises bacterial isolates according to the variations in sequence of gene fragments from seven housekeeping loci (Maiden *et al.*, 1998). Differences in sequence are seen among different strains and a central database stores this sequence information and allows global characterisation of isolates. *Spa* typing is used specifically in the typing of *S. aureus* and involves sequencing the short sequence repeat region of the protein A gene (*spa*; Shopsin *et al.*, 1999). SCC*mec* contains the *mec* gene complex, which includes the *mecA* gene, and the *ccr* gene complex, which includes *ccr* genes. A number of *mec* and *ccr* allotypes have been found within SCC*mec* among *S. aureus* isolates, leading to the classification of isolates according to the *mec*/*ccr* profile within the SCC*mec* (Chongtrakool *et al.*, 2006). This is known as SCC*mec* typing, although SCC*mec* classification has also been performed based on a number of loci of other regions of the complex (Oliveira & de Lencastre, 2002). Genotyping can also be performed using a technique called RAPD, which uses one or more random primers to generate banding pattern profiles (Williams *et al.*, 1990). The sequences/banding patterns in question are unique among different strains and therefore strains can be identified using these techniques.

However, factors such as time, expense, accuracy and inter-laboratory comparison need to be considered. RAPD testing often compromises between these factors because although it is not the most accurate test (Faria *et al.*, 2008) it is fast, relatively inexpensive and was found to be more discriminatory than PFGE using

one primer only and able to type all isolates tested, whereas PFGE was not (Casey *et al.*, 2007). RAPD is therefore an ideal candidate for typing the large number of isolates in this study due to the relative simplicity of testing and interpretation, although using only one primer may not be discriminatory enough.

3.1.2 Identification of Isolates

The isolates used in this study had already been identified as *S. aureus* at UHW and MH but confirmation was necessary in the event of errors during transcription of isolate information or subculture. Identification was not performed until after genotypic analysis; therefore only one isolate from each RAPD profile was tested, since isolates with the same RAPD profile were assumed to be the same strain and therefore be the same species.

3.1.3 Antibiotic Susceptibility Testing

Antibiotic resistance is a vast problem when it comes to treating hospital infections. *S. aureus* is well documented for its resistance to a wide range of antibiotics (see Table 1.4) and therefore the antibiotic susceptibility profile of the isolates is a very important piece of information, especially since antibiotic and biocide resistance is linked by common mechanisms (European Commission, 2009). Fusidic acid, erythromycin, mupirocin, gentamicin, penicillin G, vancomycin, ciprofloxacin and oxacillin are antibiotics that are currently used to screen *S. aureus* isolates in the hospital (Williams, 2006, personal communication).

3.1.4 Determining the Virulence of Isolates

Virulence is traditionally defined as the degree of pathogenicity of a microorganism, where pathogenicity refers to the ability of a microorganism to produce disease in a host organism (Todar, 2008). Virulence factors (or determinants) are products produced by microorganisms that cause disease (Smith, 1977). However, Wassenaar & Gaastra (2001) highlight the problems associated with labelling every gene involved in pathogenicity as a virulence gene, regardless of its function in the virulence process. They instead propose categories of 'true virulence genes' (which

include only genes whose products are involved in the interactions between the bacteria and host, are responsible for pathogenic damage and absent in non-pathogenic microorganisms) and 'virulence life-style genes' (genes whose products enable a microorganism to colonize the host, evade its immune system and intracellular survival). However, for the purpose of this study the term 'virulence gene/factor' will encompass Wassenaar & Gaastra's (2001) definitions of both true virulence genes and virulence life-style genes.

The 'virulence' factors chosen in this study were chosen for their role in infection, which is discussed in chapter 1 and in more detail below.

Hyaluronidase is an enzyme produced by *S. aureus* that is able to degrade hyaluronic acid in host tissue and is known to have a role in early subcutaneous infection (Makris *et al.*, 2004). Genes *cna* (encoding collagen binding protein), *hlg* (encoding γ- haemolysin), *eta* (encoding exfoliative toxin) and *fnbA* (encoding fibronectin binding protein A), plus the *ica* locus (encoding intracellular adhesion factors) were found to be significantly more common in invasive *S. aureus* isolates than isolates from healthy individuals (Peacock *et al.*, 2002). Invasive *S. aureus* isolates are more likely to contain both *fnbA* and *fnbB* (encoding fibronectin binding protein B) genes (Peacock *et al.*, 2000). Gamma haemolysin is similar to PVL, but is present in a much larger number (99%) of clinical isolates (Prevost *et al.*, 1995), and are able to lyse erythrocytes as well as phagocytes, indicating they are more significant in infection. Cap5 and cap8 are encoded by *cap5* and *cap8* gene clusters, which contain genes *cap5*A-P and *cap8*A-P, respectively and are involved in capsule production (O'Riordan & Lee, 2004). The genes encoding PNAG production, which is involved in biofilm formation, are located in the *ica* operon and only co-expression of *ica*A and *ica*D genes located in the operon were found to produce full activity (Gerke *et al.*, 1998). *S. aureus* grown in a biofilm are more resistant to antibiotics (Amorena *et al.*, 1999), biocides (Ueda & Kuwabara, 2007) and the actions of the immune system (Kropec *et al.*, 2005), thus allowing persistence of infection and survival. Exfoliative toxin A (ETA) causes bullous impetigo (localised blistering at the site of infection) and exfoliative toxin B (ETB) causes staphylococcal scalded skin syndrome (SSSS - widespread blistering) (Yamasaki *et al.*, 2005). These toxins mediate the separation

of extended areas of the epidermis below the epidermal barrier, causing blistering and allowing spread of bacteria beneath the barrier (Amagai *et al.*, 2000).

3.1.5 Aims and Objectives

The aim of this chapter was to determine the basic characteristics of the isolates used in this study, including their identification, their susceptibility to antibiotics, their virulence and also genotypic characterization.

3.2 MATERIALS AND METHODS

3.2.1 RAPD

3.2.1.1 Primers

All primers (see Tables 3.1 and 3.2) were arbitrary sequences of 10 bases in length that were chosen at random from a list provided by The University of British Columbia (Vancouver, British Columbia) with the exception of primers A to E, which were chosen because they were previously shown to produce good banding for RAPD testing using staphylococcal isolates (Marsou *et al.*, 2001, primer A and Naffa *et al.*, 2006, primers B-E). All primers were purchased from Invitrogen freeze-dried in tubes with a desalted purity and a scale of synthesis of 50 nmol. They were re-suspended in TE buffer that was prepared according to section 2.10.1, to produce a 10 μM solution and stored at -20 °C.

3.2.1.2 RAPD Method

PCR mixtures were prepared in 0.5 ml PCR tubes as shown in Table 3.3. A PCR tube was set up for each DNA sample containing 23μl of PCR mixture and 2 μl of diluted DNA prepared according to section 2.10.2. A negative control was also set up for each run with 23 μl of PCR mixture only. The PCR tubes were transferred to a Techgene Thermal Cycler PCR machine (Techne, Stone, UK) and run on the following cycle: 4 cycles of 5 min at 36 °C, 5 min at 72 °C and 5 min at 94 °C; 30

cycles of 1 min at 94 °C, 1 min at 36 °C and 2 min at 72 °C; 10 min at 72 °C. Electrophoresis was performed according to section 2.11.

3.2.1.3 Screening of Primers

Primers 208 and 272 had been previously demonstrated to show good discriminatory banding (G. Williams, personal communication) and would therefore be used for RAPD testing to test the isolates. However, it was necessary to use three primers to increase the discrimination between strains. It was therefore necessary to screen a selection of random primers (shown in Table 3.1) to find one that was able to produce good discriminatory banding for use in RAPD testing. Screening of primers was performed for isolate 30 and 45, using the RAPD method in section 3.2.1.2.

3.2.1.4 RAPD Testing of Isolates

RAPD method in section 3.2.1.2 was performed on all hospital isolates plus *S. aureus* NCIMB 9518 DNA that was extracted and diluted according to section 2.10.2. All samples were tested using primers 208, 272 and 268 (shown in bold in Tables 3.1 and 3.2). Primers 208 and 272 were used since they had previously demonstrated good discriminatory banding for *S. aureus* clinical isolates (G. Williams, personal communication) and primer 268 was found to produce the best discriminatory banding of all primers screened in section 3.2.1.3.

Table 3.1: List of RAPD Primers Part 1 of 2

Shows the 5' to 3' sequence for each primer used during RAPD testing. UBC = University of British Columbia

Primer	5' to 3' Sequence	Reference
208	ACGGCCGACC	UBC
232	CGGTGACATC	UBC
234	TCCACGGACG	UBC
235	CTGAGGCAAA	UBC
236	ATCGTACGTG	UBC
237	CGACCAGAGC	UBC
238	CTGTCCAGCA	UBC
239	CTGAAGCGGA	UBC
240	ATGTTCCAGG	UBC
241	GCCCGACGCG	UBC
242	CACTCTTTGC	UBC
243	GGGTGAACCG	UBC
244	CAGCCAACCG	UBC
245	CGCGTGCCAG	UBC
246	TATGGTCCGG	UBC
247	TACCGACGGA	UBC
248	GAGTAAGCGG	UBC
249	GCATCTACCG	UBC
250	CGACAGTCCC	UBC
251	CTTGACGGGG	UBC
252	CTGGTGATGT	UBC
253	CCGTGCAGTA	UBC
254	CGCCCCCATT	UBC
255	TTCCTCCGGA	UBC
256	TGCAGTCGAA	UBC

Table 3.2: List of RAPD Primers Part 2 of 2

Shows the 5' to 3' sequence for each primer used during RAPD testing. UBC = University of British Columbia

Primer	5' to 3' Sequence	Reference
257	CGTCACCGTT	UBC
258	CAGGATACCA	UBC
259	GGTACGTACT	UBC
260	TCTCAGCTAC	UBC
261	CTGGCGTGAC	UBC
262	CGCCCCCAGT	UBC
263	TTAGAGACGG	UBC
264	TCCACCGAGC	UBC
265	CAGCTGTTCA	UBC
266	CCACTCACCG	UBC
267	CCATCTTGTG	UBC
268	AGGCCGCTTA	UBC
270	TGCGCGCGGG	UBC
271	GCCATCAAGA	UBC
272	AGCGGGCCAA	UBC
273	AATGTCGCCA	UBC
274	GTTCCCGAGT	UBC
A	TCACGATGCA	Marsou *et al.* (2001)
B	AGGGAACGAG	Naffa *et al.* (2006)
C	CAGCACCCAC	Naffa *et al.* (2006)
D	GGGTAACGCC	Naffa *et al.* (2006)
E	GTGAATCCCCA-GGAGCTTACAT	Naffa *et al.* (2006)

Table 3.3: PCR Mixture

Solution	Volume/µl*
DNase and RNase-free Water	122.4
10x buffer (Qiagen Ltd., Crawley, UK)	30
Q buffer (Qiagen Ltd.)	60
10 mM dNTPs (Qiagen Ltd.)	6
$MgCl_2$ (Qiagen Ltd.)	18.8
Primer (as prepared in 3.2.1.1)	48
Taq Polymerase (Qiagen Ltd.)	2.4

dNTPs = Deoxynucleotide triphosphates; DNase = Deoxyribonuclease; RNase = Ribonuclease; * Volumes shown are for 12 samples

3.2.2 Identification of Isolates

One isolate belonging to each RAPD banding pattern profile after testing with all three primers (see Table 3.7) were speciated using a BBL™ Crystal™ Gram positive kit (Becton, Dickinson & Co., Oxford, UK) according to manufacturer's instructions. Identification was performed after initial genotyping, due to one of the cultures provided by MH being morphologically dissimilar to the other isolates and upon Gram staining was found not to be Gram positive cocci. It was necessary to confirm that all other isolates were *S. aureus*, but since RAPD had already separated them into strains only one of each RAPD profile needed to be identified. Results were entered into BBL™ Crystal™ MIND software (Becton, Dickinson & Co.), which compared the BBL profile results to bacteria in the database and therefore assigned a positive species identification.

3.2.3 Antibiotic Susceptibility Testing

3.2.3.1 Materials

Iso-sensitest agar (ISA) was purchased from Oxoid and columbia agar base was purchased from Becton Dickinson & Co. Antibiotic disks (fusidic acid, 10 μg/disc; erythromycin, 5 μg/disc; mupirocin, 5 μg/disc; gentamicin, 10 μg/disc; penicillin G, 1 unit/disc; vancomycin, 5 μg/disc; ciprofloxacin, 1 μg/disc and oxacillin, 1 μg/disc) were purchased from Oxoid and stored in a sealed container with a desiccant at -20 °C. Disks were removed from the freezer 15 min before use to prevent condensation.

3.2.3.2 Method

Testing was performed in duplicate for all 59 hospital isolates, including reference isolate *S. aureus* NCIMB 9518, for each antibiotic according to the BSAC method (BSAC, 2006). A bacterial suspension was prepared to a concentration of 8.14 ± 0.243 $\log_{10}$ CFU/ml according to section 2.5.1. The suspension was diluted 1 in 100 in TSC buffer and spread in 3 directions across each of two ISA plates and one Columbia agar plate with 2% NaCl using a sterile swab. Antibiotic disks were placed on the surface of the agar plate using sterile forceps. The oxacillin disk was placed on the Columbia agar plate with 2% NaCl and the remaining 7 disks (fusidic

acid, erythromycin, mupirocin, gentamicin, penicillin G, vancomycin and ciprofloxacin) were placed on two ISA plates (a maximum of 6 disks can be placed on 1 plate). ISA plates were incubated at 37 °C for 18-20 hours and Columbia agar plates with 2% NaCl were incubated at 30 °C for 24 hours. The zones of inhibition were measured for each disk and interpreted as sensitive or resistant according to the zone diameter breakpoints reported in the BSAC method (BSAC, 2006).

3.2.4 Determining the Presence/Absence of Virulence Genes among *S. aureus* Isolates

3.2.4.1 Primers

Forward and reverse primers for each virulence gene were purchased from Invitrogen freeze-dried in tubes with a desalted purity and a scale of synthesis of 50 nmol. They were re-suspended in TE buffer prepared according to section 2.10.1 at 10 μM and stored at -20 °C. Sequences were 18 to 34 bases in length and were taken from previous studies, except for the *cna* gene primer, which was self-designed (see Tables 3.4-3.5). The *cna* gene sequence from the National Center for Biotechnology Information (NCBI) CoreNucleotide Search software was entered into Primer3 software (Rozen & Skaletsky, 2000), which designed several primers. A primer was chosen from these that met the following criteria: 1) primer length was 20-25 bases in length, 2) GC content was 40-50%, 3) melting temperature was 55-75 °C. These criteria, along with a suitable primer sequence avoid formation of dimer, hairpin or secondary priming sites and mispriming.

3.2.4.2 PCR

The presence/absence of the genes described in Tables 3.4 and 3.5 was determined for all hospital isolates, plus *S. aureus* NCIMB 9518 in duplicate. This was also performed for isolates known to be positive for a specific gene(s), which served as positive controls. Positive control isolates were: *S. aureus* NCTC 8530 for the *cna* gene, *S. aureus* ATCC 49775 for the *hlgCB* gene, *S. aureus* NCTC 8325 for genes *fnbA*, *fnbB* and *hysA* and *S. aureus* NCTC 8175 for genes *hlgA*, *icaA*, *icaD* and *clfA*. No positive control isolates could be obtained for genes *cap8*, *eta* or *etb*. Where a

positive and negative result was obtained for a sample the negative result was assumed to be a test failure and this was repeated.

PCR mixtures were prepared in 0.5 ml PCR tubes as shown in Table 3.6, containing 23µl of PCR mixture and 2 µl of diluted DNA prepared according to section 2.10.2. A negative control was performed with 23 µl of PCR mixture only. PCR was performed in a Techgene Thermal Cycler PCR machine and run on the following cycle: 92°C for 2 min; 35 cycles of: 92 °C for 2 min, 50°C for 1 min, 72 °C for 2 min; 72 °C for 3 min. Electrophoresis was performed according to section 2.11.

Table 3.4: List of Virulence Gene Primers Part 1 of 2

Forward (F) and reverse (R) primer sequences for each virulence gene primer, their PCR product's length and their origin. BP = length of PCR product in base pairs.

Gene	5' to 3' Sequence	Product length (bp)	Reference
hysA	F-CTTCTCATATGACTCGTACCTATCG	242	Makris *et al.* (2004)
	R-TAGGTGTTCCAATTTCATAATCCCA		
cna	F-AAAGCGTTGCCTAGTGGAGA	196	Accession No. M81736
	R-TTTTGAGTGCCTTCCCAAAC		
fnbA	F- CATAAATTGGGAGCAGCATCA	127	Otsuka *et al.* (2006)
	R- ATCAGCAGCTGAATTCCCATT		
fnbB	F- GTAACAGCTAATGGTCGAATTGATACT	524	Otsuka *et al.* (2006)
	R- CAAGTTCGATAGGAGTACTATGTTC		
hlgA	F-ATGATTAAAAATAAAATATTAACAGCAACT	350	Bronner *et al.* (2000)
	R-ATCAACATTAGAGTCTTTTCGTTTT		
Hlg	F- AGCTCTCGAACAACATATTATA	940	Bronner *et al.* (2000)
CB	R- CTGCAGCTTTAAGCACTAAAG		

Table 3.5: List of Virulence Gene Primers Part 2 of 2

Forward (F) and reverse (R) primer sequences for each virulence gene primer, their PCR product's length and their origin. BP = length of PCR product in base pairs.

Gene	5' to 3' Sequence	Product length (bp)	Reference
cap5	F-GTCAAAGATTATGTGATGCTACTGAG	361	Verdier *et al.* (2007)
	R-ACTTCGAATATAAACTTGAATCAATGTTATACAG		
cap8	F-GCCTTATGTTAGGTGATAAACC	173	Verdier *et al.* (2007)
	R-GGAAAAACACTATCATAGCAGG		
icaA	F-ACACTTGCTGGCGCAGTCAA	187	Grinholc *et al.* (2007)
	R-TCTGGAACCAACATCCAACA		
icaD	F-ATGGTCAAGCCCAGACAGAG	197	Grinholc *et al.* (2007)
	R-AGTATTTTCAATGTTTAAAGCAA		
clfA	F-GGCTTCAGTGCTTGTAGG	1042	Kalorey *et al.* (2007)
	R-TTTTCAGGGTCAATATAAGC		
eta	F- GCAGGTGTTGATTTAGCATT	93	Mehrotra *et al.* (2000)
	R - AGATGTCCCTATTTTTGCTG		
etb	F- ACAAGCAAAAGAATACAGCG	226	Mehrotra *et al.* (2000)
	R - GTTTTTGGCTGCTTCTCTTG		

Table 3.6: PCR Mixture

Solution	Volume/µl*
DNase and RNase-free Water	122.4
10x buffer (Qiagen Ltd., Crawley, UK)	30
Q buffer (Qiagen Ltd.)	60
10 mM dNTPs (Qiagen Ltd.)	6
$MgCl_2$ (Qiagen Ltd.)	18.8
Forward Primer (as prepared in 3.2.4.1)	24
Reverse Primer (as prepared in 3.2.4.1)	24
Taq Polymerase (Qiagen Ltd.)	2.4

dNTPs = Deoxynucleotide Triphosphates; DNase = Deoxyribonuclease; RNase = Ribonuclease; * Volumes shown are for 12 samples

3.3 RESULTS

3.3.1 RAPD

3.3.1.1 Screening of Primers

Most of the primers screened showed either no banding or poor discriminatory banding. Primers 261, 265 and particularly 268 showed good discriminatory banding (see Figure 3.1). Primer 268 was therefore used for RAPD testing of isolates in Section 3.2.1.2.

3.3.1.2 RAPD Testing of Isolates

Primers 208 (Figure 3.2) and 272 (Figure 3.3) produced good discriminatory banding. Primer 268 showed good discriminatory banding when used to test all samples (Figure 3.4). Banding was found on the negative control of some RAPD tests although it was faint and did not correspond to any banding found for any of the isolates in the same run. This banding was therefore ignored.

Banding patterns for each isolate were cross-referenced against each other and isolates were grouped together according to their banding pattern profile for each primer individually. Isolates with an identical banding profile for primers 208, 272 and 268 collectively were then grouped together in Table 3.7. Each profile pattern was numbered, the first identified was named number one and subsequent profiles identified were assigned consecutive numbers.

Twenty four distinct banding pattern profiles were seen among the isolates using primer 208, 18 using primer 272 and 20 using primer 268. Thirty nine overall distinct banding pattern profiles, and therefore 39 different genotypes, were seen among the isolates (see Table 3.7). MRSA isolates fell into 17 groups, whereas MSSA isolates fell into 25 groups, meaning there are 17 MRSA and 25 MSSA strains among all isolates. Three of the groups had both MSSA and MRSA isolates in them.

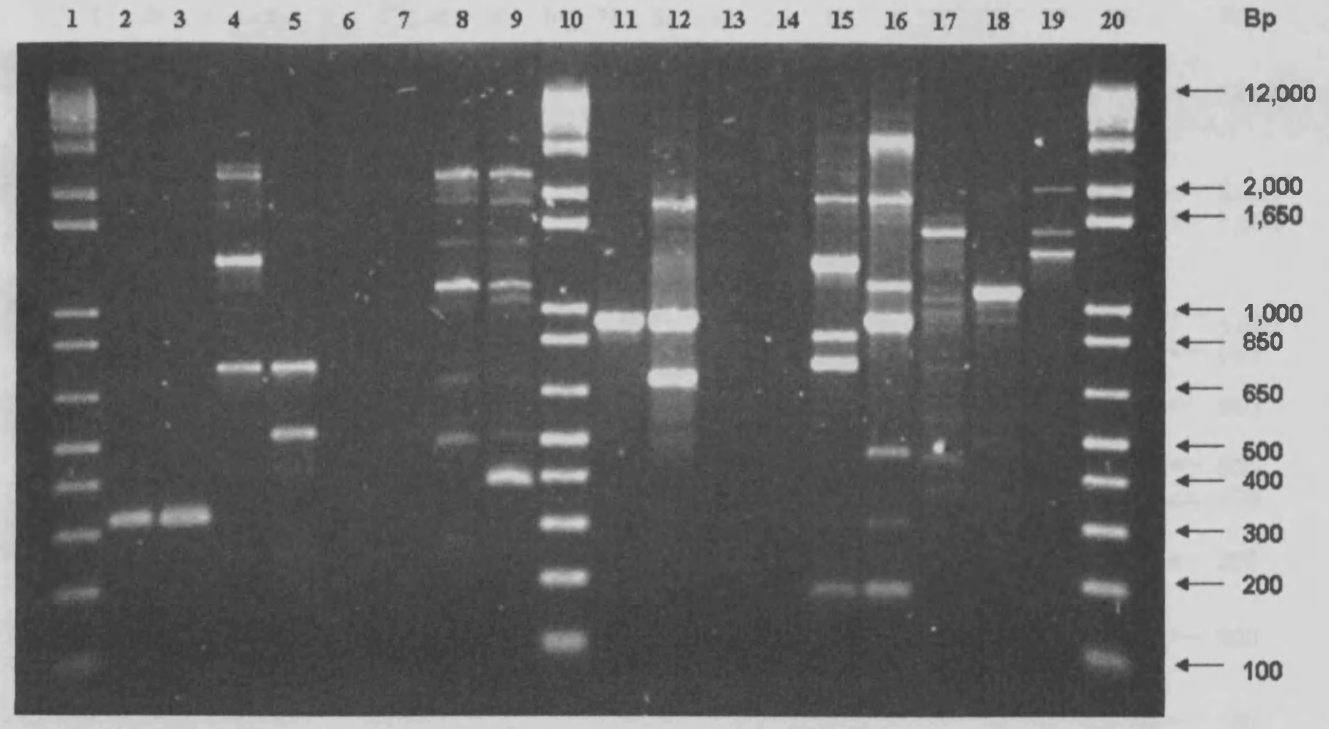

Figure 3.1: Example of Screening of Primers

RAPD testing of isolates 30 and 45 using primers 260, 261, 264-268, 270 and 271. BP = length in base pairs

Well	Contents
1	1Kb Plus ladder
2	Isolate 30, primer 260
3	Isolate 45, primer 260
4	Isolate 30, primer 261
5	Isolate 45, primer 261
6	Isolate 30, primer 264
7	Isolate 45, primer 264
8	Isolate 30, primer 265
9	Isolate 45, primer 265
10	IKb Plus Ladder
11	Isolate 30, primer 266
12	Isolate 45, primer 266
13	Isolate 30, primer 267
14	Isolate 45, primer 267
15	Isolate 30, primer 268
16	Isolate 45, primer 268
17	Isolate 30, primer 270
18	Isolate 45, primer 270
19	Isolate 30, primer 271
20	IKb Plus Ladder

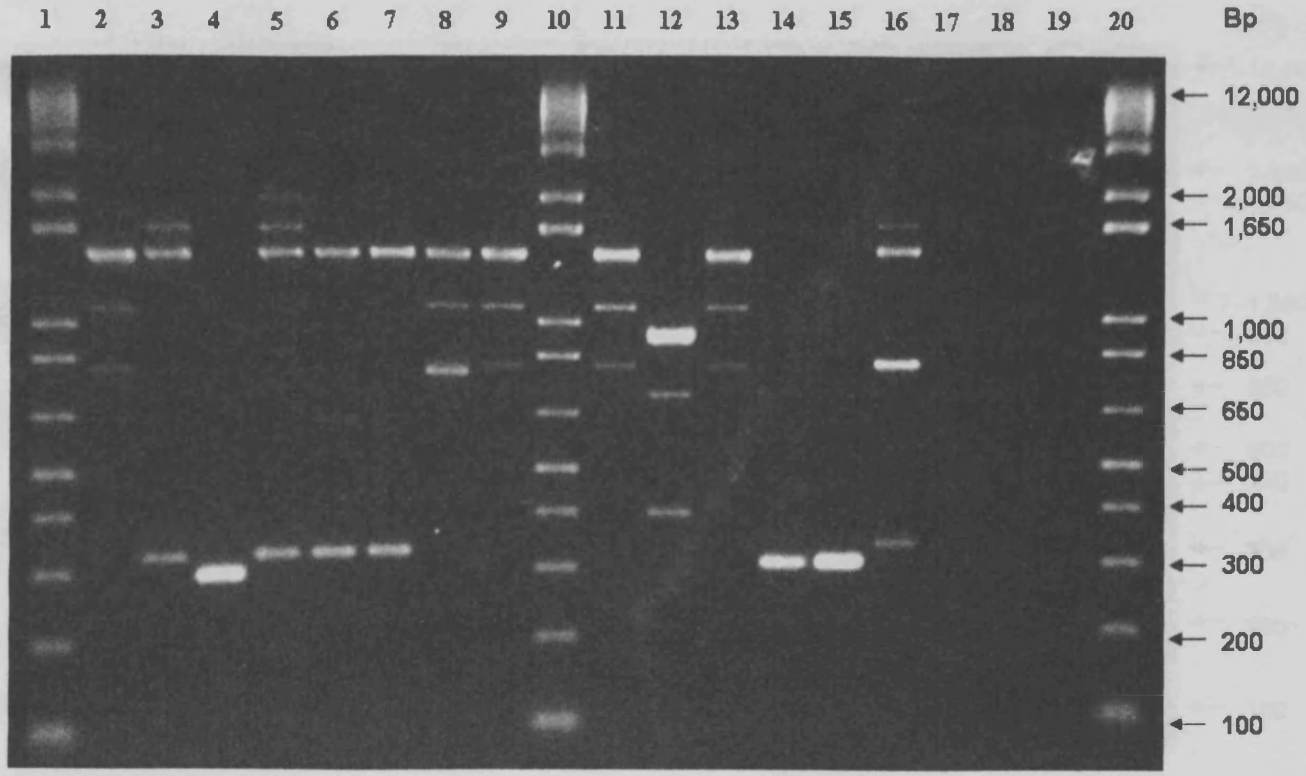

Figure 3.2: Example of RAPD Testing Using Primer 208

RAPD testing of multiple isolates using primer 208. BP = length in base pairs

Well	Contents
1	1Kb Plus ladder
2	Isolate 3, primer 208
3	Isolate 4, primer 208
4	Isolate 5, primer 208
5	Isolate 6, primer 208
6	Isolate 9, primer 208
7	Isolate 10, primer 208
8	Isolate 11, primer 208
9	Isolate 13, primer 208
10	IKb Plus Ladder
11	Isolate 15, primer 208
12	Isolate 16, primer 208
13	Isolate 17, primer 208
14	Isolate 18, primer 208
15	Isolate 20, primer 208
16	Isolate 21, primer 208
17	Negative control
18	No sample
19	No sample
20	1 Kb Plus Ladder

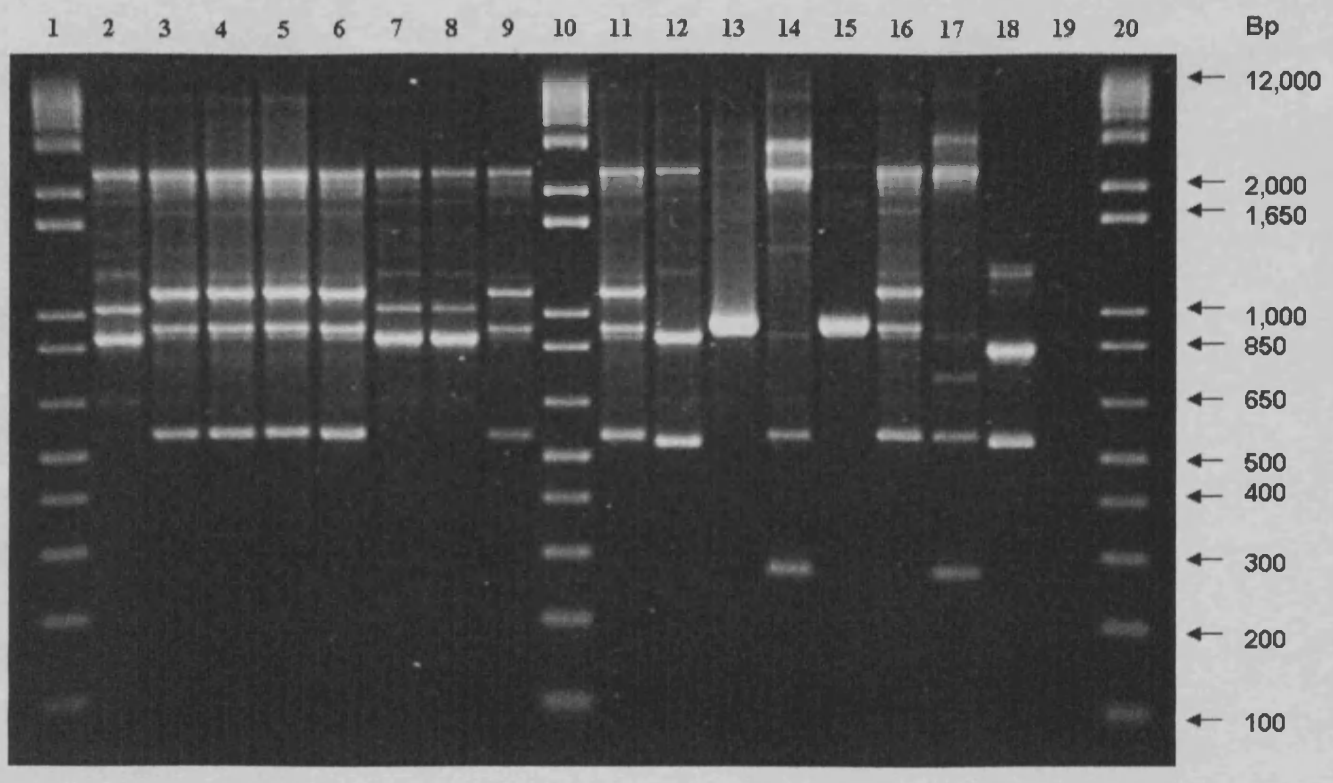

Figure 3.3: Example of RAPD Testing Using Primer 272

RAPD testing of multiple isolates using primer 272. BP = length in base pairs

Well	Contents
1	1Kb Plus ladder
2	Isolate 41, primer 272
3	Isolate42, primer 272
4	Isolate 43, primer 272
5	Isolate 45, primer 272
6	Isolate 46, primer 272
7	Isolate 48, primer 272
8	Isolate 49, primer 272
9	Isolate 50, primer 272
10	IKb Plus Ladder
11	Isolate 52, primer 272
12	Isolate 55, primer 272
13	Isolate 4, primer 272
14	Isolate 6, primer 272
15	Isolate 10, primer 272
16	Isolate 13, primer 272
17	Isolate 14, primer 272
18	Isolate 16, primer 272
19	Negative control
20	1 Kb Plus Ladder

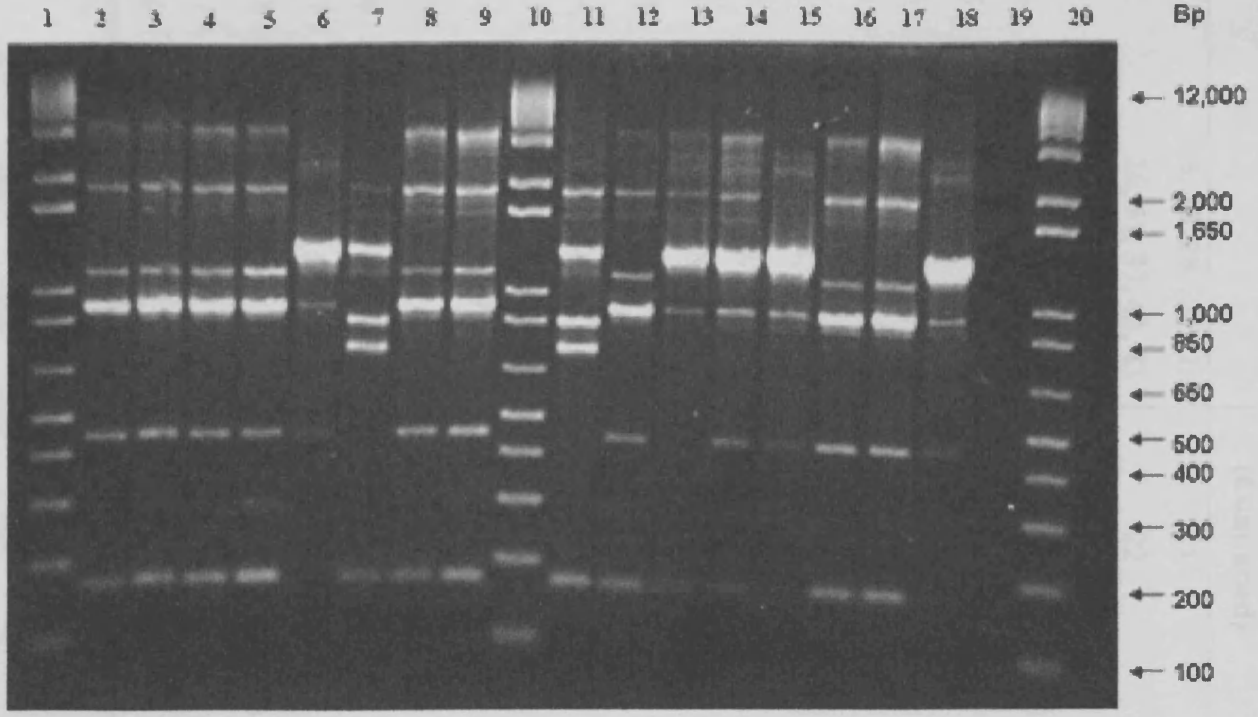

Figure 3.4: Example of RAPD Testing Using Primer 268

RAPD testing of multiple isolates using primer 268. BP = length in base pairs

Well	Contents
1	1Kb Plus ladder
2	Isolate 14, primer 268
3	Isolate 19, primer 268
4	Isolate 26, primer 268
5	Isolate 27, primer 268
6	Isolate 28, primer 268
7	Isolate 30, primer 268
8	Isolate 31, primer 268
9	Isolate 32, primer 268
10	IKb Plus Ladder
11	Isolate 34, primer 268
12	Isolate 37, primer 268
13	Isolate 38, primer 268
14	Isolate 39, primer 268
15	Isolate 41, primer 268
16	Isolate 42, primer 268
17	Isolate 43, primer 268
18	Isolate 44, primer 268
19	Negative control
20	1 Kb Plus Ladder

Table 3.7: RAPD Banding Pattern Profiles Obtained for Primers 208, 272 & 268 combined

Isolates are grouped together according to their banding pattern profiles, as determined during RAPD testing for primers 208, 272 and 268 combined. Each group is given a strain number. Isolates shown in bold are MRSA, all other samples are MSSA.

Banding Pattern Profile/Strain	Isolate	Banding Pattern Profile/Strain (Continued)	Isolate
1	**5, 18, 20,** 28, **41,**	21	**S12**
2	**26, 31, 37, 42, 43**	22	**45**
3	**19, 32,**	23	51
4	**27, 46, 50**	24	38
5	S8, H9	25	44
6	S7, S10	26	24
7	**11, 17**	27	14
8	13, **15**	28	Q49
9	30, 34	29	K17
10	**S3, S4, S6**	30	S11
11	**48, 49**	31	6
12	T4, N10	32	4
13	**9**, 10	33	39
14	NCIMB 9518	34	16
15	**O43**	35	**25**
16	S1	36	21
17	**55**	37	47
18	**P59**	38	S2
19	3	39	**52**
20	S5		

3.3.2 Identification of Isolates

All 39 RAPD strains plus *S. aureus* NCIMB 9518 were identified as *S. aure*us using the BBL™ Crystal™ system. Therefore the identity of all 59 hospital strains were confirmed as *S. aureus*.

3.3.3 Antibiotic Susceptibility Testing

Antibiotic resistance profiles for isolates are shown in Table 3.8. Identical resistance profiles were found for duplicate tests of each isolate. There were 10 different patterns of resistance profiles among the isolates. Susceptibility testing for all isolates agreed with the MRSA/MSSA profile provided by the hospital, except for S5. Isolate S5 is documented as MRSA, but was found to be susceptible to methicillin, which may be a documentation error from the hospital. Isolate S5 was therefore treated as MSSA. Five hospital isolates had an identical antibiotic susceptibility profile to the reference isolate *S. aureus* NCIMB 9518.

The percentage of isolates from this study showing resistance to each antibiotic was calculated and is shown in Table 3.9. No isolates showed resistance to vancomycin, whereas isolates were most commonly resistant to penicillin G. More MRSA isolates were found to be resistant to the antibiotics than MSSA isolates. MRSA isolates showed resistance to a wider range of antibiotics, with at least 1 MRSA isolate showing resistance to each antibiotic tested, with the exception of vancomycin. MSSA isolates, however, only showed resistance to fusidic acid, penicillin G and ciprofloxacin. All MRSA isolates were also found to be resistant to penicillin G and ciprofloxacin, whereas a lower percentage of MSSA isolates showed resistance to them, particularly to ciprofloxacin. The percentage of isolates from this study showing resistance to each antibiotic was compared to those found by Hope *et al.* (2008; Table 3.9), who screened *S. aureus* isolates from bacteraemia patients in the UK and Ireland between 2001 and 2006. The findings of their study can be compared to those from this study, despite their use of a different method (the BSAC agar dilution method) to determine the antibiotic susceptibility. Table 3.9 reveals data from this study to be similar to those found by Hope and colleagues, indicating that isolates from UHW and MH are fairly representative of those found throughout

the UK in terms of antibiotic susceptibility and therefore subsequent results from this study can be more confidently extrapolated to the whole of the UK. Rates of MRSA found in isolates used in this study were much higher (53%) than in isolates reported to the HPA in England during the same period of isolation (37-38%; HPA, 2007). This is likely to be due to the fact that the isolates used in this study originated from ITUs, in which the EARSS surveillance found MRSA rates to be as high as 60% during the same period of isolation (EARSS, 2007).

Table 3.8: Antibiotic Susceptibility Profile of *S. aureus* Isolates

FD = fusidic acid, E = Erythromycin, MUP = Mupirocin, CN = Gentamicin, P = Penicillin G, VA = Vancomycin, CIP = Ciprofloxacin, OX = Oxacillin. S = sensitive, R = resistant, according to BSAC interpretation. MRSA isolates are shown in bold

Isolate	Antibiotic susceptibility profile							
	FD	**E**	**MUP**	**CN**	**P**	**VA**	**CIP**	**OX**
6, 10, 13, 16, 21, 28, 30, 44, N10, K17, Q49, T4, S1, S2, S7, S10, S11	S	S	S	S	R	S	S	S
14	R	S	S	S	R	S	S	S
3, 15, 17, 19, 25, 26, 31, 43, 45, 46, 50, 55, P59, S6, S12	S	R	S	S	R	S	R	R
5, 18	S	R	R	R	R	S	R	R
9, 11, 27, 32, 37, 42, 52, O43, S3, S4	S	S	S	S	R	S	R	R
20	R	R	R	R	R	S	R	R
4	S	S	S	S	S	S	R	S
38, 51, H9, S5	S	S	S	S	R	S	R	S
41, 48, 49	S	R	R	S	R	S	R	R
24, 34, 39, 47, NCIMB 9518, S8	S	S	S	S	S	S	S	S

Table 3.9 Percentage of *S. aureus* Isolates Resistant to Antibiotics Comparison of Antibiotic Susceptibility in *S. aureus* Isolates from Hope *et al.* 2008 and this Study

The % MRSA and MSSA isolates resistant to a number of antibiotics found in this study and by Hope *et al.* (2008).

Antibiotic	% MRSA resistant isolates		% MSSA resistant isolates	
	This study n = 31	Hope *et al.* (2008) n = 163	This study n = 29	Hope *et al.* (2008) n = 835
Fusidic Acid	3.23	9.3	3.45	8.6
Erythromycin	67.74	81.7	0	28
Mupirocin	19.35	Not tested	0	Not tested
Gentamicin	9.68	9.0	0	2.5
Penicillin G	100	99.8	75.86	82.4
Vancomycin	0	0	0	0
Ciprofloxacin	100	95.9	17.24	9.1
Oxacillin	100	99.8	0	0

3.3.4 Determining the Presence/Absence of Virulence Genes among *S. aureus* Isolates

Results of the screening isolates for presence of virulence genes are shown in Table 3.10. All isolates showed bands of identical size for each virulence gene and bands were confirmed as being the correct gene by checking the size of the band against that described in the literature (provided in Tables 3.4 and 3.5) or in the case of *cna* using CoreNucleotide Search software. Ten virulence gene profiles were seen among the isolates. All positive control isolates tested positive for the appropriate virulence gene.

The percentage of isolates carrying each virulence gene is shown in Table 3.11. No isolates possessed the *eta* or *etb* gene, whereas all isolates possessed genes *hysA*, *HlgCB*, *icaA*, *icaD* and *clfA*. All other genes were present in a varying high percentage of isolates.

Table 3.10: Virulence Gene Profiles of *S. aureus* Isolates

+ = gene present, - = gene absent

Isolates	*hysA*	*cna*	*fnbA*	*fnbB*	*hlgA*	*hlgCB*	*cap5*	*cap8*	*icaA*	*icaD*	*clfA*	*eta*	*etb*
3	+	+	-	+	+	+	+	-	+	+	+	-	-
4,5,6,9,10,11,13,14,15,16,17, 18,19,20,21,24,25,26,27,37,47	+	+	+	+	+	+	+	+	+	+	+	-	-
28,30,34,41,48,49	+	+	+	+	+	+	-	+	+	+	+	-	-
38	+	-	+	+	-	+	+	-	+	+	+	-	-
39	+	-	+	+	+	+	+	-	+	+	+	-	-
44	+	-	+	+	+	+	-	+	+	+	+	-	-
31,32,42,43,45,46,50,51,52	+	+	+	+	+	+	+	-	+	+	+	-	-
55,P59,O43,H9,S2,S3,S4, S5,S6,S8,S10,S11,S12	+	+	+	-	+	+	+	+	+	+	+	-	-
N10,K17,Q49,NCIMB 9518	+	-	+	+	+	+	+	+	+	+	+	-	-
T4,S1,S7	+	-	+	-	+	+	+	+	+	+	+	-	-

Table 3.11: Percentage of *S. aureus* Isolates Carrying Virulence Genes

Percentage of hospital isolates plus *S. aureus* NCIMB 9518 positive for each virulence gene.

Virulence Gene	% Isolates Carrying Gene (n=60)
HysA	100
cna	83
fnbA	98
fnbB	72
hlgA	98
hlgCB	100
cap5	88
cap8	80
icaA	100
icaD	100
clfA	100
eta	0
etb	0

3.4 DISCUSSION

3.4.1 RAPD

RAPD was able to group *S. aureus* isolates that were indistinguishable following typing with three primers as a single strain. The observation that MSSA isolates could be divided into more RAPD types than MRSA isolates (section 3.3.1.2) indicates that there was more variability within MSSA isolates and reflects the fact that MSSA has a much broader lineage compared to MRSA, since MRSA has evolved more recently (Deurenberg & Stobberingh, 2008). The observation that three of the RAPD groups consisted of both MRSA and MSSA isolates indicates that the three primers used did not complement part of the sequence of the gene that encodes methicillin resistance (*mec*A gene).

The observation that three primers provided better discrimination between isolates' genotypic profiles led to the formation of a larger number of RAPD types (strains) than either one or two primers. Greater discrimination using multiple primers was also found by Madadgar *et al.* (2008). Multiple primers may be more beneficial, since being able to distinguish between isolates more accurately may then enable better tracking of *S. aureus* isolates in the hospital environment.

The primers used for the RAPD method are random and therefore arbitrary and therefore not suitable for inter-laboratory comparison of strains. RAPD is relatively quick and easy to perform in comparison to other genotypic methods and therefore may be useful for in-house reference only.

3.4.2 Antibiotic Susceptibility Testing

Data showing the percentage of *S. aureus* isolates resistant to the antibiotics are shown in Table 3.9. These data reveal that a low percentage of the isolates were resistance to mupirocin, fusidic acid and gentamicin and none were resistant to vancomycin indicating that current ITU isolates can be treated easily with a number of therapy options. This means that teicoplanin and new antibiotics such as linezolid and quinupristin-dalfopristin may not be required for regular use for some time and

the predicted explosion of untreatable *S. aureus* infections appears to be further in the future.

3.4.3 Determining the Presence/Absence of Virulence Genes among *S. aureus* Isolates

Data showing the percentage of *S. aureus* isolates carrying each virulence gene are shown in Table 3.11. No isolates possessed the *eta* or *etb* gene, which means none are capable of causing SSSS or bullous impetigo. All isolates possessed genes *hysA*, *hlgCB*, *icaA*, *icaD* and *clfA*; all other genes were present in a high percentage of isolates.

Mehrotra *et al.* (2000) also found no *eta* or *etb* genes among isolates from nasal swabs of healthy individuals, clinical isolates and surveillance cultures. The percentage of isolates positive for *clfA* is very similar to those found in *S. aureus* clinical isolates by Tristan *et al.* (2003); however, a much higher percentage of isolates were positive for *cna*, *fnbA* and *fnbB* than those found by Tristan and colleagues. This could be explained by the fact that the clinical isolates tested by Tristan and colleagues were from a mixture of nasal swabs from asymptomatic carriers and specimens from patients with endocarditis and osteomyelitis/arthritis. They found a significant difference in the percentage of isolates positive for these genes depending on which sample type the isolates originated, which may explain the differences seen between this study and those by Tristan and colleagues. Wiśniewska *et al.* (2008), however, found a similar percentage of *S. aureus* clinical isolates positive for genes *fnbA*, *fnbB* and *clfA*, although they found a much lower percentage positive for the *cna* gene. The isolates they tested were from a wide range of different infection sites, including bloodstream, but they found no correlation between origin of specimen and presence of the genes. Peacock *et al.* (2002) found a similar percentage of *S. aureus* invasive clinical isolates positive for *fnbA*, *hlgCB*, *clfA*, *icaA* and *etb*; however, they found a much lower percentage of isolates positive for the *cna* gene and a much higher percentage of isolates positive for the *eta* gene. Verdier *et al.* (2007) found a much lower percentage of *S. aureus* bacteraemia isolates contained *cap5* and especially *cap8* genes than was found in this

study. They also found *cap5* and *cap8* genes present in a much lower percentage of *S. aureus* isolates from other infection sites. The reason for such large differences in the presence of the *cap5* and *cap8* genes between these studies is not clear. Although isolates studied by Verdier and colleagues were not from the UK, neither were the isolates studied by Wiśniewska *et al.* (2008) and Tristan *et al.* (2003) that were shown to have virulence genes in similar numbers.

No positive control isolate could be obtained for genes *eta*, *etb* and *cap8*, therefore they could not be used in this study to confirm the accuracy of the primers under the conditions of this study. However, the primers used in this study for genes *eta* and *etb* were previously validated with a positive control in Mehrotra *et al.* (2000) and primers for *cap8* gene were used previously by Verdier *et al.* (2007).

3.4.4 Summary

The RAPD, antibiotic susceptibility and virulence gene profiles of the isolates provides important information for characterisation and tracking of these isolates and provides important information regarding their potential ability to cause disease. Possible relationships between genotypic and phenotypic characteristics studied in this chapter are discussed in Chapter 7, with RAPD and antibiotic susceptibility profiles directly compared in Table 7.1. The genetic profile of isolates in this study cannot be compared to those of other isolates in the UK or worldwide due to characterization of most isolates being carried out using PFGE or MLST. The antibiotic susceptibility and virulence gene profiles of the isolates in this study are comparable to those of other *S. aureus* isolates in the UK and worldwide and are similar to those found in other studies, particular with regards to antibiotic susceptibility. Knowledge of the characteristics of these isolates is important, since these characteristics may provide insight as to how they are able to infect, how they spread throughout the hospital and how they can be treated. Differences found between the isolates in future testing, such as antiseptic susceptibility may also be explained by differences in their genotypic, antibiotic and virulence gene profiles.

CHAPTER 4 DETERMINING THE EFFICACY OF ALCOHOL HAND RUBS AGAINST *STAPHYLOCOCCUS AUREUS* ISOLATES

4.1 INTRODUCTION

4.1.1 Hand Sanitizing Practices in Intensive Therapy Units

It is important to determine whether the current hand sanitizing practices are effective, or whether they lead to potential spread of *S. aureus*, particularly to the patient, thus increasing the risk of nosocomial infection. It is also important to discover the mechanisms of potential spread of *S. aureus* in the ITU. Developing an understanding of how and when AHRs are used in practice will enable the development of a method for testing AHRs against conditions found in practice. Antiseptic susceptibility testing can therefore be designed and interpreted according to the results of the ITU observations with respect to those contact times used in practice.

Studies have shown increased compliance of HCWs with hand hygiene using AHRs compared to hand washing (Girou *et al.*, 2002; Karabay *et al.*, 2005) and after hygiene compliance interventions (Hugonnet *et al.*, 2002). The effect of the use of AHRs has also been well studied with regards to the subsequent reduction in HCAIs such as MRSA (Johnson *et al.*, 2005b; Harrington *et al.*, 2007). These studies either observe compliance rates based on the number of applications of AHR compared to the total number of hand hygiene opportunities (Girou *et al.*, 2002; Hugonnet *et al.*, 2002; Karabay *et al.*, 2005; Johnson *et al.*, 2005b; Patarakul *et al.*, 2005), and/or the volume of AHR depleted from stocks (Johnson *et al.*, 2005b; Harrington *et al.*, 2007).

The quality of hand hygiene has been less well studied. Studies by Larson *et al.* (2001) and Girou *et al.* (2002) included observation of the length of time taken by HCWs from ITUs to rub AHR into their hands, although the HCWs were volunteers and were aware of the nature of observations and provided with instructions on how to use the AHR. Larson *et al.* (2001) also studied whether AHR was rubbed in correctly, although these observations were not recorded individually, but grouped as failure to perform the hygiene procedure correctly along with re-contamination of hands. The use of gloves has also been previously studied by Girou *et al.* (2002).

4.1.2 Methods Currently Used for Testing the Efficacy of Alcohol Hand Rubs

The AHRs used in UHW and MH are assumed by hospital staff, patients and relatives to be effective at killing organisms likely to be found on the hands of HCWs (such as *S. aureus*). However, since manufacturers have not tested the AHRs against clinical isolates it is not clear whether they are effective in practice. Furthermore, some isolates may be less susceptible to AHRs than others due to differences in cell structure or composition. It is therefore important to establish the antiseptic susceptibility profile of a large number of ITU isolates to the AHRs to which they are exposed, thus determining the efficacy of the AHR *in-situ*.

The main test used by manufacturers to test AHRs is the hygienic hand disinfection test EN1500 (European Standard EN1500, 1997), which involves inoculating hands with the non-pathogenic strain *Escherichia coli* K17. Fingertips are sampled before and after application of the test product according to manufacturer's instructions. The method is repeated with a standard reference product, 60% w/v propan-2-ol, and $\log_{10}$ reduction of bacterial cells must not be significantly less for the test AHR than that found by the standard reference product. AHRs may alternatively be tested using European Standard EN12791 (2005), which uses a similar method as EN1500, except the reference product is 60% propan-1-ol. These standard methods all test the AHR under more or less practical conditions, but for ethical reasons cannot incorporate use of pathogenic species that can be found on the hands of HCWs.

Some manufacturers also test their AHRs using a standard suspension test such as (European Standard EN1276, 1997) or EN12054 (European Standard prEN12054, 1995), which measure the $\log_{10}$ reduction of a small number of standard bacterial strains, including *S. aureus,* in a suspension. EN1276 is designed to test the bactericidal activity of chemical disinfectants and antiseptics used in food, domestic and industrial areas and a $\log_{10}$ reduction of ≥ 5 must be achieved within 5 min. EN12054 is designed to test the bactericidal activity of hygienic and surgical handrub and handwash products and a $\log_{10}$ reduction of ≥ 4.52 for a hygienic hand rub is required within 1 min. Bacterial cells are significantly less susceptible to biocide treatment when they are attached to a surface, compared to a suspension (Lindsay and von Holy, 1999); therefore results obtained using suspension tests

cannot predict whether the product is effective at killing bacteria on the surface of hands. Thus although suspension tests provide additional efficacy results to those provided by EN1500, they are unable to demonstrate the product's efficacy under conditions found in practice.

4.1.3 Alternative Tests for Testing the Efficacy of Alcohol Hand Rubs

Carrier tests are not used by AHR manufacturers to test their products, but they most accurately replicate conditions of AHR use found in practice. This is because carrier testing determines the efficacy of antibacterial products against bacteria dried on a surface. The stainless steel disk carrier test EN13697 (European Standard EN13697, 2001) is an appropriate carrier test method for the screening of a large number of isolates. The criteria for this test is to achieve a $\geq$ 4 log reduction within 5 min.

The efficacy of AHRs has been tested *in-vivo* on hands against resident flora (Marchetti *et al.*, 2003; Kampf & Kapella, 2003; Suchomel *et al.*, 2009a; Suchomel *et al.*, 2009b) and non-pathogenic organisms (Kampf *et al.*, 2003; Kampf, 2008). The efficacy of AHRs has been tested on standard strains of pathogenic organisms (Kampf *et al.*, 2003; Marchetti *et al.*, 2003) and also against clinical isolates, including a small number of *S. aureus* isolates (Kampf & Hollingsworth, 2003; Kampf & Hollingsworth, 2008; Hall *et al.*, 2009) using a suspension test method. The efficacy of AHR against clinical isolates has not previously been tested using a carrier test, nor has the difference been tested between the susceptibility of a large number of *S. aureus* isolates to AHRs.

The stainless steel disk carrier test EN13697 (European Standard EN13697, 2001) was chosen as the basis for testing the efficacy of AHRs against *S. aureus* isolates and a range of contact times were chosen to incorporate the contact times used in practice, as shown in Table 4.1. Dirty conditions only were chosen to reflect the high level of organic material likely to be found on the hands of HCWs. The presence of organic material decreases the efficacy of a biocide by inhibiting the interaction between the biocide and bacteria either by quenching the biocide or protecting the bacteria (Maillard, 2005b). It is important to use clinical strains of

bacteria, rather than one standard strain, in order to give a more accurate prediction as to how effective the AHR is against isolates found in practice, especially since it has been previously demonstrated that clinical and reference strains have varied susceptibilities (Kampf & Hollingsworth, 2008).

The Bioscreen method is a high throughput method suitable for enumeration of surviving cells from the carrier test. It is particularly relevant to this study, which required testing all fifty nine hospital isolates against three AHRs and with three replicates. However, Lambert & van der Ouderaa (1999) found a discrepancy between $\log_{10}$ reductions using the Bioscreen and plate count methods after exposure to biocide, where the Bioscreen $\log_{10}$ reductions were higher. This is almost certainly due to the fact that cells which have undergone sub-lethal damage by a biocide require time to repair themselves before they can grow. Sub-lethally damaged bacteria will have a longer lag phase than healthy cells and will therefore take longer to achieve the OD required for analysis, so it will appear as if fewer cells had survived AHR exposure than actually had, resulting in an overestimation of $\log_{10}$ reduction. The plate count method, however, allows for delays in growth due to cell damage because the incubation time is longer and colony size is not taken into account when counting. Therefore, if the $\log_{10}$ reduction in bacterial number following exposure to AHR when using the Bioscreen method is significantly higher than using the plate count method this will imply that the AHR has sub-lethally damaged the cells. A comparison of these two methods of enumeration will determine firstly whether the Bioscreen estimates comparable $\log_{10}$ reduction values and secondly whether bacterial cells that survived exposure to AHRs are sub-lethally damaged.

In-vitro tests such as the stainless steel carrier test, particularly using the Bioscreen to enumerate recovery, are appropriate for large-scale screening of isolates, but they are not truly representative of conditions of AHR exposure in practice. This is because they do not take into consideration the effect the surface of skin will have on the test organism or the AHR. *In-vivo* tests are not appropriate for testing of pathogens such as *S. aureus* due to ethical reasons, and therefore an *ex-vivo* skin test is needed. *Ex-vivo* testing involves using excised skin as a surface. Skin is not readily available in

large quantities and therefore inappropriate for large-scale screening of isolates, but is crucial to validate the $\log_{10}$ reduction values obtained for stainless steel carrier testing using a small number of isolates. Human skin is difficult to obtain due to the necessity of ethical approval and therefore animal skin can be used as an alternative. Pig skin is the most appropriate animal skin to use, due to the similarities in structure (Montagna & Yun, 1964), lipid composition (Gray & Yardley, 1975) and permeability (Bhatti *et al.*, 1988; Dick & Scott, 1992) to human skin. The fact that pigs are commercially reared and slaughtered provides a readily available source of skin. The use of pig skin as a model surface for testing antiseptic hand washes has been successful and $\log_{10}$ reductions have been similar to those found *in-vivo* (McDonnell *et al.*, 1999). An *ex-vivo* method for testing the antimicrobial efficacy of antiseptic agents on skin surfaces was developed by Maillard *et al.* (1998).

4.1.4 Aims and Objectives

The aim of this chapter was to determine the hand sanitizing practices used in the ITU and the susceptibility of *S. aureus* isolates to AHRs, as well as to determine the antibacterial behaviour of AHR in practice, such as the effect of mechanical rubbing and residual activity. The aim was to design susceptibility tests to simulate conditions found in practice and to extrapolate the results to *in-situ* use of AHRs.

4.2 MATERIALS AND METHODS

4.2.1 Observation of Hand Sanitizing Practices in ITU

Hand sanitizing practices were observed on the ITU ward on two separate occasions at UHW and on one occasion at MH, with the full approval of the senior nurse in charge. This involved standing at several locations in the ward and observing doctors, nursing staff and visitors as they sanitized their hands and touched objects, materials and patients. Observations included whether or not staff washed their hands or used AHRs, the quality of hand sanitization with AHRs, whether or not gloves were worn and the incidences of potential cross-contamination that occurred as a result of the failure of staff to perform hand sanitizing between touching a

patient/object. A healthcare observation period began upon the approach of a HCW to the vicinity of the patient, or if a HCW was already present when observations were due to commence then observation would not start until the current healthcare procedure taking place had ended. A healthcare observation period ended when a healthcare procedure ended.

4.2.2 Stainless Steel Carrier Testing

4.2.2.1 Materials

Stainless steel disks used were 2 cm diameter, with a grade 2B finish and were purchased from Goodfellows Cambridge Ltd. (Huntingdon, UK). Between each use the disks were autoclaved, rinsed in water and placed in 5% (v/v) Decon®90 (Decon Laboratories Limited, Hove, UK) in deionised water for 60 min, rinsed with deionised water, dried in a glass drier and autoclaved to sterilize.

Bovine serum albumin (BSA) was prepared weekly from powder purchased from Acros Organics (Geel, Belgium) and dissolved in the appropriate volume of deionised water. BSA was stored under refrigerated conditions and the required volume was filter sterilized using a 0.45 μm syringe filter (Fisher Scientific) daily prior to use. Dirty conditions are represented by the presence of 3 g/L BSA (European Standard EN13697, 2001) and is therefore required at a concentration of 6 g/L BSA to achieve dirty conditions with a 1:1 ratio of BSA and bacterial suspension.

4.2.2.2 Validation of Neutralizer

This test was performed under dirty conditions for Soft Care Med H5, Cutan and Guest Medical AHRs and was a modified version of the neutralization validation in EN13697 (European Standard EN13697, 2001). A bacterial suspension of *S. aureus* NCIMB 9518 was prepared to a concentration of 8.29 ± 0.34 $\log_{10}$ CFU/ml according to section 2.5.1. One ml 6 g/L sterile BSA prepared according to section 4.2.2.1 and 1 ml bacterial suspension was mixed together and 20 μl was inoculated onto each of two stainless steel disks. Disks were incubated at 37 °C in a Petri dish with the lid

partially opened for approximately 1 h until visibly dry. A 6-well culture plate (Corning, NY, USA) was set up with two wells filled with 9.9 ml TSC buffer. One hundred µl TSC buffer was added to the first well, which served as a control and 100 µl AHR was added to the other, which served as a test. After 5 min neutralization time, one disk was placed in each of the two wells, inocula-side up. Bacteria were removed from the surface of each disk by flushing 5 ml TSC buffer from the well across the surface of the disk with a pipette 15 times. A viable count was performed according to section 2.6.2.1. This procedure was performed 5 times for each AHR, using a fresh inoculum for each replicate.

The recovery of cells (neutralizer toxicity) and the $\log_{10}$ reduction in number of cells (neutralizer efficacy) was calculated using the following equations, respectively:

1) N – NC and 2) NC – NT

Where N = $\log_{10}$ number of CFU/20 µl in original BSA/suspension, NC = $\log_{10}$ number of CFU recovered per disk for the control, NT = $\log_{10}$ number of CFU recovered per disk for the test. Results were interpreted according to European Standard (EN13697, 2001) guidelines.

4.2.2.3 Carrier Testing of Standard Strain Using the Plate Count Method

This test was performed under dirty conditions for Soft Care Med H5, Cutan and Guest Medical AHRs and was based on the method in EN13697 (European Standard EN13697, 2001). This test was performed on reference NCIMB 9518 only in order to perform a comparison between the plate count method of recovery and Bioscreen method of recovery. A bacterial suspension was prepared to a concentration of 8.60 ± 0.08 $\log_{10}$ CFU/ml and inoculated onto stainless steel disks according to 4.2.2.2. A 100 µl volume of AHR was added to the surface of the first disk, entirely covering the inoculum spot. After a contact time of 10 s the disk was transferred inoculum-side down in a flat-bottomed 100 ml glass bottle 4-5 cm in diameter (Fisher Scientific) containing 10 ml TSC buffer and 5 g 3 mm glass beads (Sigma Aldrich). After 5 min neutralization time the bottle was placed on a Grant Bio POS 300

rotating platform (Patterson Scientific, Luton, UK) at 150 rpm for 1 min to remove cells and a viable count was performed according to 2.6.2.1. A control was performed on the remaining disk as described above, using 100 µl TSC buffer. The procedure was repeated with contact times of 30 s, 1 min, 2 min and 5 min and the entire procedure for all contact times was repeated a further four times using a fresh inoculum.

The % recovery was calculated using the following equation, where the inocula was divided by 2 to account for subsequent dilution of the inocula with BSA and divided by 50 to account for only 20 µl being inoculated onto the disk:

$$\% \text{ Recovery} = \frac{100}{(\text{CFU/ml inocula} \div 2 \div 50)} \times \text{CFU/disk}$$

Log_{10} reductions were calculated for each time point for each AHR using the following equation, where Nc is the log_{10} number of CFU recovered per disk for the control and Nd is the log_{10} number of CFU recovered per disk for the test:

Log_{10} reduction = Nc – Nd

An Nc-Nd value of ≥ 4 log_{10} order demonstrates a target bactericidal effect. Due to the limit of detection of this method, which is 1 cfu/ml, if a value of 0 cfu was obtained on duplicate counts it was recorded as (<) 0.5 cfu. Statistical analysis was performed on the percentage recovery values to validate the recovery method, and to compare the log_{10} reduction values at each time point for each AHR. Statistical analysis was performed according to section 2.8.1.

4.2.2.4 Determining the Viable Count of Bioscreen Growth from Optical Density Value

A bacterial suspension of *S. aureus* NCIMB 9518 was prepared to a concentration of approximately 10^9 cfu/ml according to section 2.5.1. This was serially diluted to 10^{-8} and a viable count of the undiluted to 10^{-8} dilutions was performed according to section 2.6.2.1. The dilutions were also enumerated using the Bioscreen method,

where 50 µl of each dilution was placed in 5 wells each of a 100-well honeycomb plate (Thermo Electron Corporation, Basingstoke, UK) containing 350 µl TSB. A negative control (blank) was performed by adding 50 µl TSC buffer in 5 wells of the honeycomb containing 350 µl TSB. The honeycomb plate was incubated in a Bioscreen C Microbial Growth Analyser (Oy Growth Curves Ab Ltd., Helsinki, Finland) at 37°C for 14 hours with OD readings taken automatically every 15 min using a wideband filter with a wavelength of 420-580 nm. The plate was shaken for 10 s before each $OD_{420\text{-}580}$ reading. This process was repeated a further nine times, using a fresh culture for each replicate to produce CFU/ml and $OD_{420\text{-}580}$ readings for ten dilution series. The $\log_{10}$ CFU/ml of each dilution in each dilution series was plotted against the time taken to reach an $OD_{420\text{-}580}$ of 0.2 in a fitted line plot, using Minitab. This created a linear equation that allows the $\log_{10}$ CFU/ml to be calculated of any suspension according to the time taken to reach an $OD_{420\text{-}580}$ of 0.2. An $OD_{420\text{-}580}$ of 0.2 was chosen because the growth curves revealed that this OD showed the best discrimination between each dilution.

4.2.2.5 Carrier Testing of Standard Strain Using the Bioscreen Method

A direct comparison with the plate count method of recovery was performed to ensure the Bioscreen method was able to accurately predict the true bactericidal effect of AHR. This test was performed on a reference strain NCIMB 9518 under dirty conditions for Soft Care Med H5, Cutan and Guest Medical AHRs and was based on the method in 4.2.2.3. A bacterial suspension of *S. aureus* NCIMB 9518 was prepared according to section 2.5.1 and 4 ml of 6 g/l sterile BSA prepared according to section 4.2.2.1 was mixed with 4 ml bacterial suspension to achieve a final concentration of 8.34 ± 0.07 $\log_{10}$ CFU/ml. Bacterial suspensions were tested according to section 4.2.2.3, except that after cells were removed from the rotating platform a viable count of each test and control solution was performed according to the Bioscreen method of enumeration described in section 4.2.2.4. The mean of each test/control $OD_{420\text{-}580}$ reading was calculated at each 15 min reading interval and the overall mean OD value of the blank was subtracted from each mean test and control mean value to provide mean minus blank values. Testing was performed in triplicate.

Log_{10} CFU/ml values were calculated from mean minus blank OD values using the linear equation from the Fitted Line Plot (Figure 4.4) produced according to section 4.2.2.4. These log_{10} values were converted to inverse log_{10} values and multiplied by 10.1 to obtain cfu/disk values. Due to the limit of detection being 4 CFU/ml for this method, if a value of 0 CFU was obtained in each of the 5 wells they were recorded as (<) 4 CFU/ml. The test log_{10} CFU/disk values were subtracted from control log_{10} CFU/disk values to obtain log_{10} reduction values/disk. Statistical analysis was performed on log_{10} reduction values according to section 2.8.1 to compare the log_{10} reduction values at each time point for each AHR.

4.2.2.6 Comparison of the Carrier Test plate Count and Bioscreen Methods for the Standard Strain

Log_{10} reduction values from the carrier test Bioscreen method performed in section 4.2.2.5 at each time point for Soft Care Med H5, Cutan and Guest Medical AHRs were statistically compared against the respective carrier test plate count values performed in section 4.2.2.3, according to section 2.8.1 to determine whether there was a significant difference between them.

4.2.2.7 Carrier Testing of Isolates Using the Bioscreen Method

All 59 hospital isolates were tested against Soft Care, Cutan and Guest Medical AHRs in triplicate according to section 4.2.2.5. However, data were not adjusted to compensate for the limit of detection, the reasons for which are discussed in section 4.3.2.5. Bacterial suspensions used were prepared to a concentration of 8.55 ± 0.14 log_{10} CFU/ml.

4.2.2.8 Determining the Log_{10} Reduction of Isolates 44 and S1 Using Guest Medical AHR with the Carrier Test Plate Count Method

Carrier testing using the plate count method was performed in triplicate for isolates 44 and S1 using Guest Medical AHR only, according to section 4.2.2.3. This was performed in order to determine the true log_{10} reduction values of these two isolates, since they were used in further testing.

4.2.3 *Ex-Vivo* Carrier Testing

4.2.3.1 Materials

Pig ears were kindly provided by Dr Charles Heard (Cardiff University) following collection from a local slaughterhouse. Ears were frozen on the day of collection and thawed on the day of preparation of skin samples. Glass diffusion cells were purchased from J.B. & D.W Jones (Loughborough, UK).

4.2.3.2 Preparation of Skin

Ears were thawed under a laminar air flow cabinet, during which time they were placed in a container to prevent drying. After thawing they were washed thoroughly in sterile deionised water (SDW) to remove residues of blood and dirt. The ears were shaved using an electric razor and rinsed in fresh SDW, after which the skin was separated from the ear using a scalpel blade, taking care to remove excess subcutaneous fat and cartilage. The skin was cut into approximately 2 cm^2 pieces using a scalpel blade and was frozen in sterile Petri dishes until needed. Prior to testing, skin was removed from frozen storage and left to thaw in a closed Petri dish for a few minutes. The skin pieces were decontaminated by immersion in 85% v/v ethanol for 30 s and were transferred to a sterile Petri dish. The top surface of the skin was gently rubbed using alcohol-coated gloved fingertips to physically dislodge resident flora and finally the skin was immersed in 85% ethanol for a further 10 s. Immediately after removal from ethanol the skin was immersed in SDW for 5 min to neutralize the effect of the alcohol and was finally transferred to a sterile Petri dish.

4.2.3.3 Assembly of the Diffusion Cell

The lips of the donor and receptor compartments of the diffusion cells were coated with a thin layer of high vacuum grease and autoclaved to sterilise. The set-up of diffusion cells for testing was carried out aseptically in a laminar air flow cabinet and is shown in Figure 4.1. The recipient compartment of each sterile diffusion cell was fixed to the surface of a tray and 1 ml TSC buffer was added to keep the dermis of the skin moist. A piece of skin that had been prepared according to section 4.2.3.2 was placed face up on top of each recipient compartment and left for the surface to dry in the laminar air flow cabinet for 20 min. The donor compartment of each

diffusion cell was then placed on top of each piece of skin and fixed in place using a pinch clamp. To evaluate the decontamination process existing bacterial flora were removed from the surface by adding 1 ml TSC buffer to the donor compartment and flushed across the surface 15 times using a pipette. This was enumerated according to section 2.6.1.1. This piece of skin was also used for testing, but the TSC buffer was discarded and the skin dried in the laminar air flow cabinet for 10 min prior to inoculation.

4.2.3.4 Determining the Contact Time Required by an AHR to Achieve a Bactericidal Effect against S. aureus Isolates Using the Ex-Vivo Carrier Test

This test was based on that described by Maillard *et al.* (1998). Testing was carried out in a laminar air flow cabinet and was performed under dirty conditions with Soft Care Med H5, Cutan and Guest Medical AHR for isolates 44, *S. aureus* NCIMB 9518 and S1 in triplicate. Skin pieces were prepared according to section 4.2.3.2 and skin was set up in diffusion cells according to section 4.2.3.3. An equal volume of bacterial suspension (prepared according to section 2.5.1) and 6 g/L sterile BSA (prepared according to 4.2.2.1) was mixed to achieve a final concentration of 9.24 ± 0.11 $\log_{10}$ CFU/ml. A 20µl volume was added to the skin surface via the donor compartment. This was left to dry for approximately 20 min, until the inoculum spot was visibly dry. A volume of 100 µl AHR was added to the skin surface via the donor compartment, entirely covering the inoculum spot.

After the appropriate contact time 900 µl TSC buffer was added to the donor compartment and was flushed across the skin surface 15 times using a pipette to remove bacteria from the surface of the skin. Surviving bacteria were enumerated according to section 2.6.1.1. A control was also performed for each time point, adding 100 µl TSC buffer to the skin surface.

The % recovery was calculated using the following equation:

$$\% \text{ Recovery} = \frac{100}{(\text{Inocula} \div 50)} \times \text{CFU/skin surface}$$

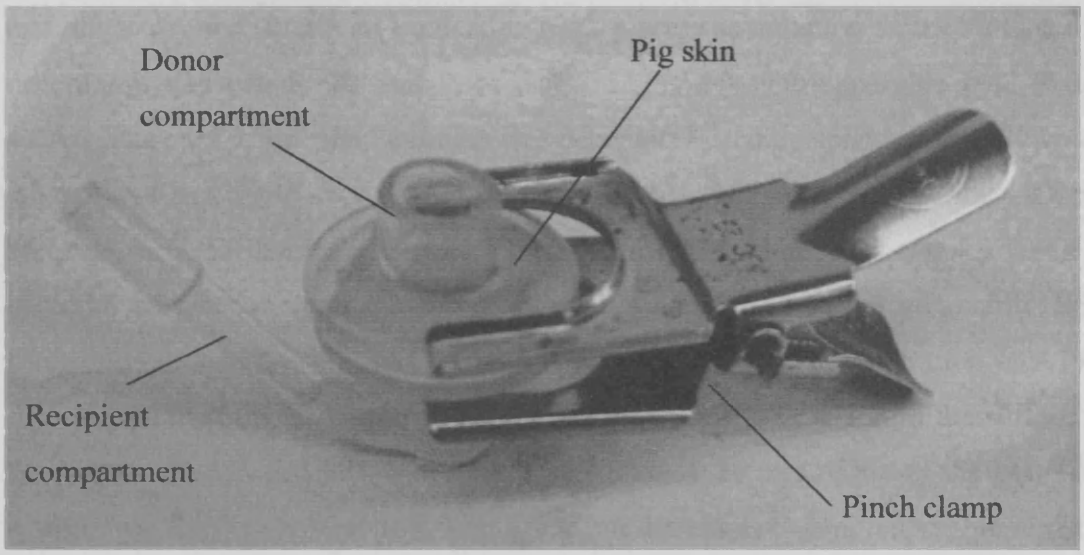

Figure 4.1: An Assembled Diffusion Cell for *Ex-Vivo* Testing

Statistical analysis was performed on percentage recovery values to validate the recovery method and on $\log_{10}$ reduction values to compare the $\log_{10}$ reduction values at each time point for each AHR and for each time point for each isolate. Statistical analysis was performed according to section 2.8.1.

4.2.3.5 Determining the Effect of Mechanical Rubbing Using the Ex-Vivo Test

This test was adapted from the method described by Messager *et al.* (2004). Testing was carried out in a laminar air flow cabinet and was performed under dirty conditions only for Soft Care Med H5, Cutan and Guest Medical AHR for *S. aureus* NCIMB 9518 in triplicate. Skin pieces were prepared according to section 4.2.3.2 and left to dry for 10 min on each side. Each piece was mounted on the surface of a decontaminated plastic 40 mm Petri dish lid (Fisher) with superglue (see Figure 4.2A). The surface of each Petri dish was decontaminated by spraying with 70 % v/v ethanol and leaving to dry. Two pieces of mounted skin were required for each run and one piece for evaluating the decontamination procedure. An equal volume of bacterial suspension (prepared according to section 2.5.1) and 6 g/L sterile BSA (prepared according to 4.2.2.1) was mixed to achieve a final concentration of 9.19 ± 0.07 $\log_{10}$ CFU/ml. A 20µl volume of each suspension was added to the surface of two pieces of skin and left to dry for approximately 20 min. One of the two Petri dishes was fixed to a steel rod with a 3.5 cm attachment using superglue and the other Petri dish was fixed to a polystyrene block. The block was placed on a balance and the rod was attached to a drill (IKA Labortechnik, Staufen, Germany) and adjusted so the two pieces of skin were aligned and were pressed together with a weight of 100 ± 8 g (see Figure 4.2 B & C). The drill scaffold was lifted and 100 µl AHR was added to the surface of the bottom piece of skin. Immediately the scaffold was lowered to achieve the previously adjusted weight and the rod was rotated at 150 rpm for 10 s, after which both Petri dishes were de-mounted and placed skin-side down in one 90 mm sterile Petri dish containing 5 ml TSC buffer. After 5 min neutralization time each 40 mm Petri dish was held at a 90 degree angle over the 90 mm Petri dish and the surface of the skin was flushed 30 times with TSC buffer using a pipette to remove bacteria from the surface of the skin. A control was performed using 100 µl TSC buffer. One mounted piece of skin was also added to a Petri dish containing 5 ml TSC buffer and the surface was flushed. This served to

evaluate the efficiency of the decontamination procedure. The contents of each Petri dish were enumerated according to section 2.6.1.1.

The % recovery was calculated using the following equation:

$$\% \text{ Recovery} = \frac{100}{(\text{CFU/ml of inocula} \div 25)} \times \text{CFU/pair of skin surfaces}$$

Statistical analysis was performed on percentage recovery values to validate the recovery method and on $\log_{10}$ reduction values to compare the $\log_{10}$ reduction values for each AHR. Statistical analysis was performed according to section 2.8.1.

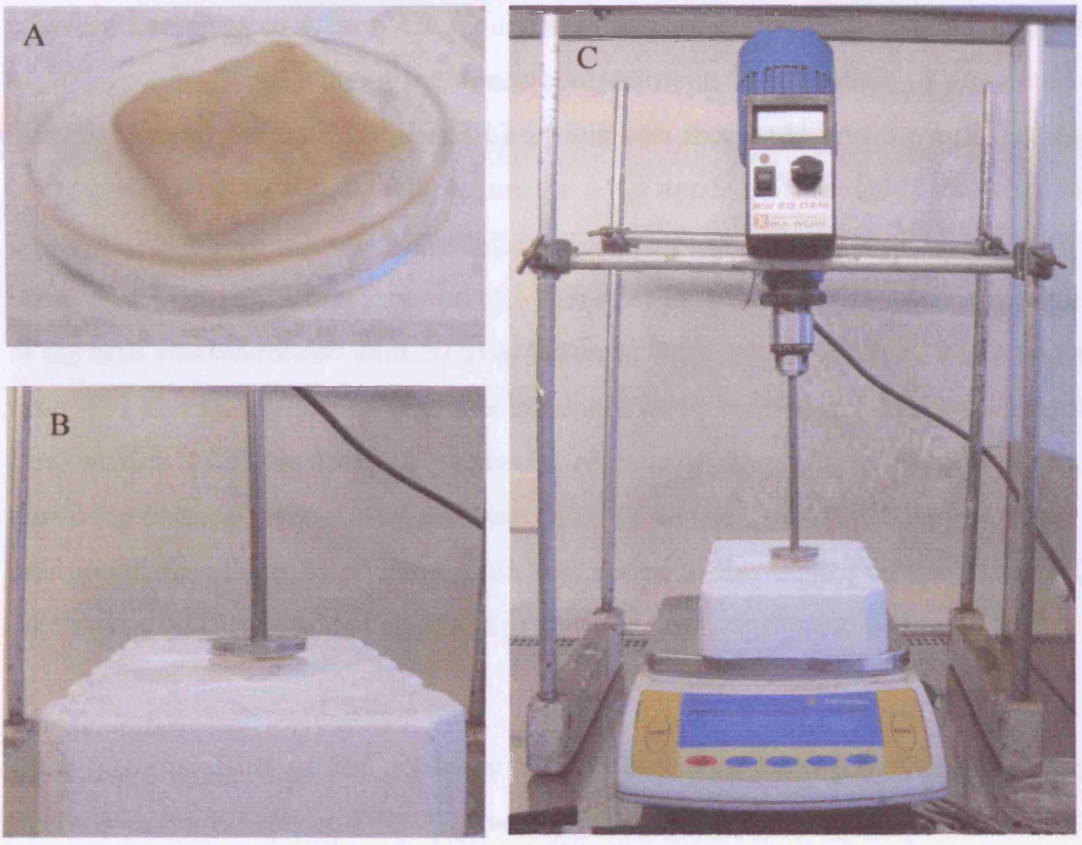

Figure 4.2: ***Ex-Vivo*** **Test Using Mechanical Rubbing**

Picture A shows a piece of skin mounted on a Petri dish. Picture B shows a close-up of the two pieces of skin pressed together. Picture C shows the *ex-vivo* test using mechanical rubbing.

4.2.3.6 Determining the Residual Activity of AHR Using the Ex-vivo Test

This test was carried out in a laminar air flow cabinet and was performed under dirty conditions only for Soft Care Med H5, Cutan and Guest Medical AHR for *S. aureus* NCIMB 9518 in triplicate. An equal volume of bacterial suspension (prepared according to section 2.5.1) and 6 g/L sterile BSA (prepared according to 4.2.2.1) was mixed to achieve a final concentration of 8.30 ± 0.13 $\log_{10}$ CFU/ml. Skin pieces were prepared according to section 4.2.3.2 and skin was set-up in diffusion cells according to section 4.2.3.3 but without the donor compartment. The surface of one piece of skin was inoculated with 20 µl AHR and this was massaged into the entire surface for 15 s using a glass rod, by which time the AHR appeared thoroughly absorbed into the skin. The donor cell was immediately placed on top of the skin and held together using a pinch clamp. After a period of 1 min after inoculation with AHR, the surface of the skin was inoculated with 20 µl of bacterial suspension. After a 20 min contact time, 480 µl TSC buffer was added to the donor compartment and flushed across the skin surface 15 times using a pipette to remove bacteria from the skin surface. Surviving bacteria were enumerated according to section 2.6.1.1. A control was also performed, for which 20 µl suspension was added to the surface of the skin for 20 min, but no AHR was added prior to this step.

4.2.4 Comparison of the Efficacy of AHRs Using Stainless Steel and Skin Surface Carrier Tests and *Ex-Vivo* with Mechanical Rubbing

The *ex-vivo* carrier test was performed under dirty conditions for Soft Care Med H5, Cutan and Guest Medical AHR for *S. aureus* NCIMB 9518 in triplicate and using a contact time of 10 s, according to section 4.2.3.4. The results of this test, plus the results of stainless steel plate count carrier testing from section 4.2.2.3 and *ex-vivo* with mechanical rubbing from section 4.2.3.5 for *S. aureus* NCIMB 9518 with 10 s contact time were compared statistically according to section 2.8.1.

4.3 RESULTS

4.3.1 Observation of Hand Sanitizing Practices in ITU

Hand sanitizing observations were made of 54 separate healthcare observation periods in UHW ITU during two visits and of 56 separate healthcare observation periods in MH ITU during one visit. A summary of these observations is shown in Table 4.1. Results of these observations are also published (Cheeseman *et al.*, 2009).

Only one visitor was observed during the visits, all other observations were of HCWs. No patient-to-patient contact via staff hands was observed in either hospital. Potential staff-to-object and staff-to-patient cross-contamination usually occurred when a member of staff touched an object/patient without gloves or without first sanitizing their hands, but also when a member of staff touched their own skin/hair after hand sanitizing or putting on gloves then touched an object/patient, thereby potentially transferring any microorganisms that they may have picked up from their skin/hair to the object/patient. Potential object-to-object cross-contamination occurred when a member of staff touched an object and then touched another object, thereby potentially transferring any microorganisms that may have been picked up from the first object to the second object. Potential object-to-patient cross-contamination occurred when a member of staff touched an object then touched a patient, thereby potentially transferring any microorganisms that may have been picked up from the object to the patient. Potential patient-to-object cross-contamination occurred when a member of staff touched a patient then touched an object, thereby potentially transferring any microorganisms that may have been picked up from the patient to the object. These incidences of potential cross-contamination could be avoided either by sanitizing hands or by applying/changing gloves either prior to, or in-between, these actions.

Table 4.1: Hand Sanitizing Observations

UHW = University Hospital of Wales, MH = Morriston Hospital.

Observation	UHW	MH
% healthcare observations during which staff washed hands	28	20
% healthcare observations during which staff used AHR	30	9
Mean number of pumps of AHR used	1.3	1.0
Mean time taken (s) to rub in AHR	11	15
% of AHR uses where the required areas of hands were cleaned	75	80
% healthcare observations during which staff wore gloves	40	34
Of those observations where HCWs wore no gloves, % observations where HCWs used AHR	41	14
% observations where HCWs wore no gloves and used no AHR, but washed hands	17	19
% observations where HCWs used no hand protection/sanitization	19	46
% healthcare observations resulting in potential staff-to-object cross-contamination	30	59
% healthcare observations resulting in potential staff to patient cross-contamination	4	0
% healthcare observations resulting in potential object-to-object cross-contamination	70	88
% healthcare observations resulting in potential object-to-patient cross-contamination	20	9
% healthcare observations resulting in potential patient-to-object cross-contamination	17	9

4.3.2 Stainless Steel Carrier Testing

4.3.2.1 Validation of Neutralizer

The mean recovery rate and $\log_{10}$ reduction values of *S. aureus* NCIMB 9518 after contact with Soft Care Med H5, Cutan and Guest Medical AHRs for the carrier test neutralizer validation are shown in Table 4.2. According to European Standard EN13697 (2001), the neutralizer is valid if N-NC is not greater than 2 $\log_{10}$ orders and if NC-NT is not greater than ± 0.30. Data in Table 4.2 therefore demonstrate that the neutralizer was effective at neutralizing the bactericidal activity of Soft Care Med H5, Cutan and Guest Medical AHRs under dirty conditions, but was not toxic, at a 1 in 100 dilution.

4.3.2.2 Carrier Testing of Standard Strain Using the Plate Count Method

Percentage recovery of *S. aureus* NCIMB 9518 using the carrier test plate count method was 52.01±14.04 and the differences in recovery were not found to be significant ($P \geq 0.05$). Log_{10} reduction values of *S. aureus* NCIMB 9518 for the carrier test plate count method using Soft Care Med H5, Cutan and Guest Medical AHRs are shown in Figure 4.3. Exposure to Soft Care Med H5 resulted in a ≥4 $\log_{10}$ reduction average, and therefore bactericidal effect, within 10 s. Exposure to Cutan and Guest Medical, however, only showed a ≥4 $\log_{10}$ reduction average, and therefore bactericidal effect, following a contact time of 5 min.

Soft Care Med H5 had a higher $\log_{10}$ reduction at contact times of 10 s, 30 s, 1 min and 2 min compared to Cutan and Guest Medical AHRs ($P < 0.05$); therefore it was significantly more efficacious against *S. aureus* NCIMB 9518 than Cutan and Guest Medical AHRs.

Table 4.2: Carrier Test Neutralizer Validation Results

Shows the mean recovery rate (N-NC) and $\log_{10}$ reduction (NC-NT) ± standard deviation (SD) of *S. aureus* NCIMB 9518 after exposure to Soft Care Med H5, Cutan and Guest Medical AHRs for the validation of neutralizer test. N = $\log_{10}$ number of CFU/20 µl in original BSA/suspension; NC = $\log_{10}$ number of CFU recovered per disk for the control; NT = $\log_{10}$ number of CFU recovered per disk for the test

AHR	N-NC	NC-NT
Soft Care Med H5	0.08 ± 0.15	0.06 ± 0.08
Cutan	0.58 ± 0.06	-0.05 ± 0.03
Guest Medical	0.34 ± 0.05	0.02 ± 0.04

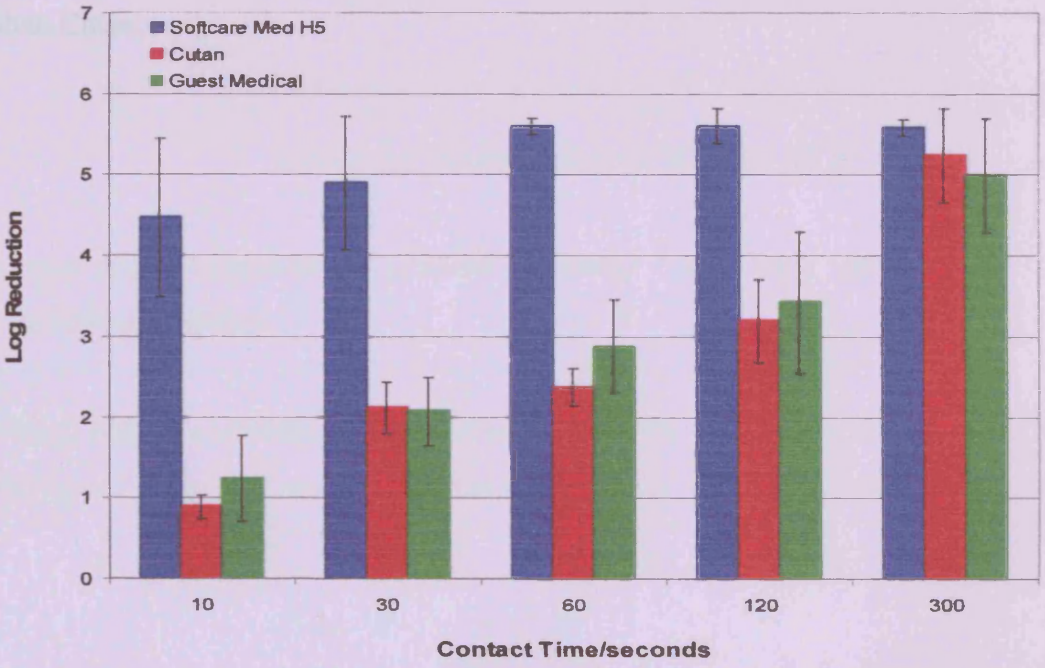

Figure 4.3: Results of Carrier Testing of *S. aureus* NCIMB 9518 Using Plate Count Method

Mean $\log_{10}$ reduction ± SD of *S. aureus* NCIMB 9518 after exposure to Soft Care Med H5, Cutan and Guest Medical AHRs for the carrier test plate count method.

4.3.2.3 Determining Viable Count of Bioscreen Growth from Optical Density Value

The fitted line plot of viable count versus OD is shown in Figure 4.4.

4.3.2.4 Carrier Testing of Standard Strain Using the Bioscreen Method

Log_{10} reduction values of *S. aureus* NCIMB 9518 for the carrier test Bioscreen method using Soft Care Med H5, Cutan and Guest Medical AHRs are shown in Figure 4.5. After exposure to Soft Care Med H5 and Guest Medical AHR a ≥ 4 log_{10} reduction average, and therefore a bactericidal effect, was observed within 30 s. Exposure to Cutan, however, resulted in a ≥ 4 log_{10} reduction average being observed by a contact time of 5 min.

Soft Care Med H5 and Guest Medical had a higher log_{10} reduction at contact times 10 s, 30 s, 1 min and 2 min compared to Cutan ($P < 0.05$). Therefore, similarly to plate count enumeration results (Figure 4.3), Bioscreen enumeration resulted in Soft Care Med H5 AHR appearing more efficacious than Cutan AHR, although contrary to plate count enumeration results, Guest Medical appeared to be more efficacious than Cutan.

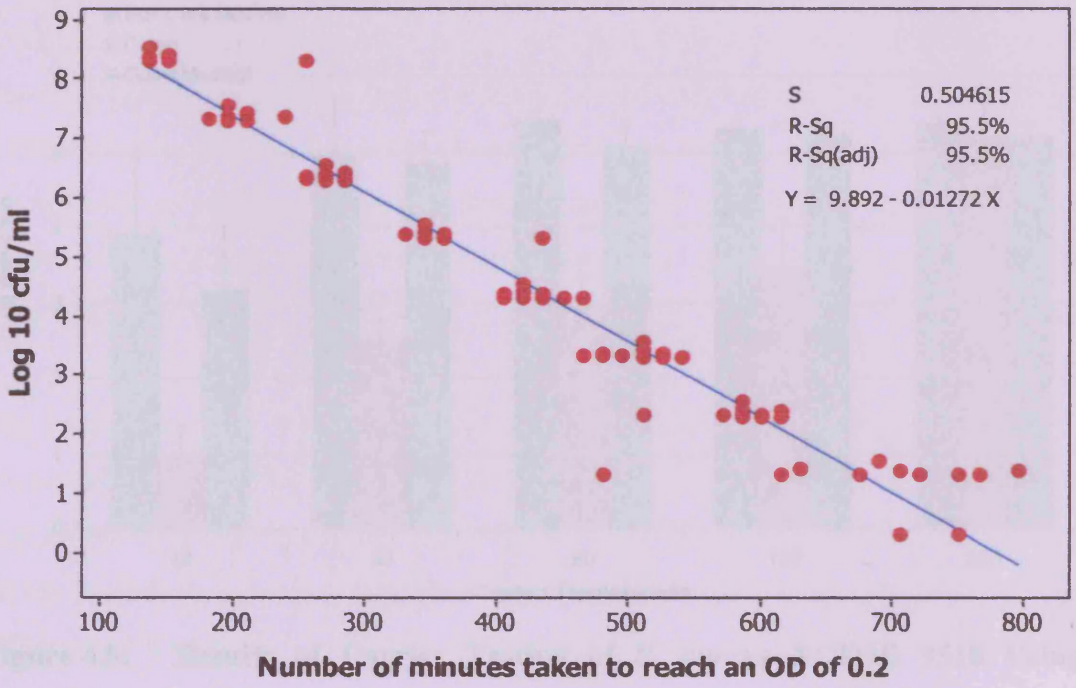

Figure 4.4: Relationship between Bacterial Count and Optical Density of Bioscreen Readings

Fitted line plot showing the relationship between $\log_{10}$ bacterial count/ml of *S. aureus* NCIMB 9518 and OD of Bioscreen readings. The linear equation is shown.

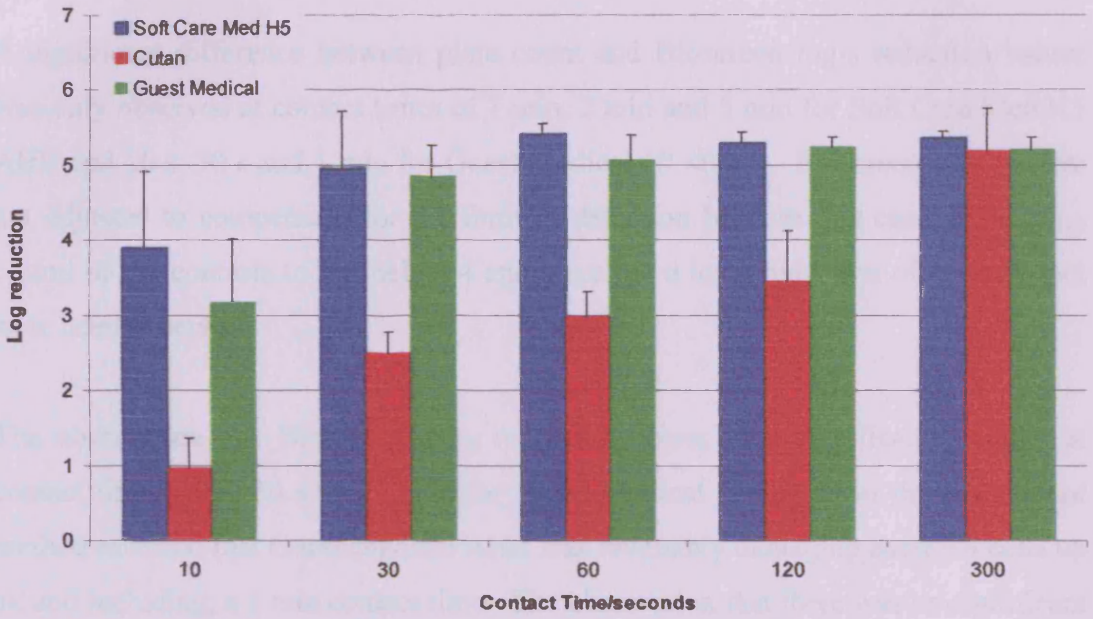

Figure 4.5: Results of Carrier Testing of *S. aureus* NCIMB 9518 Using Bioscreen Method

Mean $\log_{10}$ reduction ± SD of *S. aureus* NCIMB 9518 after contact with Soft Care Med H5, Cutan and Guest Medical AHRs for the carrier test Bioscreen method of enumeration.

4.3.2.5 Comparison of Carrier Testing of Standard Strain Using Plate Count and Bioscreen Methods

Log_{10} reductions of the carrier test plate count and Bioscreen test methods that were performed in sections 4.2.2.3 and 4.2.2.4, respectively are shown in Figure 4.6, which provides a comparison between values from both methods of enumeration for Soft Care Med H5, Cutan and Guest Medical AHRs. A comparison of these methods is published in Cheeseman *et al.* (2009).

A significant difference between plate count and Bioscreen log_{10} reduction values was only observed at contact times of 1 min, 2 min and 5 min for Soft Care Med H5 AHR and 10 s, 30 s and 1 min for Guest Medical (P <0.05). Bioscreen results were not adjusted to compensate for the limit of detection because this caused the log_{10} cfu/ml of the controls to fall below 4 and therefore a log_{10} reduction of 4 would not have been observed.

The observation that Bioscreen log_{10} reduction values were significantly higher at contact times 10 s, 30 s and 1 min for Guest Medical compared to the plate count method revealed that Guest Medical AHR was reversibly damaging bacterial cells up to, and including, a 1 min contact time. The observation that there was no significant difference between log_{10} reduction values of Bioscreen and plate count methods for Guest Medical AHR at contact times 2 min and 5 min revealed that bacterial cells were not irreversibly damaged until a contact time of 2 min. Therefore Bioscreen results for Guest Medical AHR should be interpreted with caution and plate count results for NCIMB 9518 in Figure 4.6 indicate log_{10} reduction values found during subsequent Bioscreen testing using Guest Medical may more closely reflect those seen for Cutan AHR.

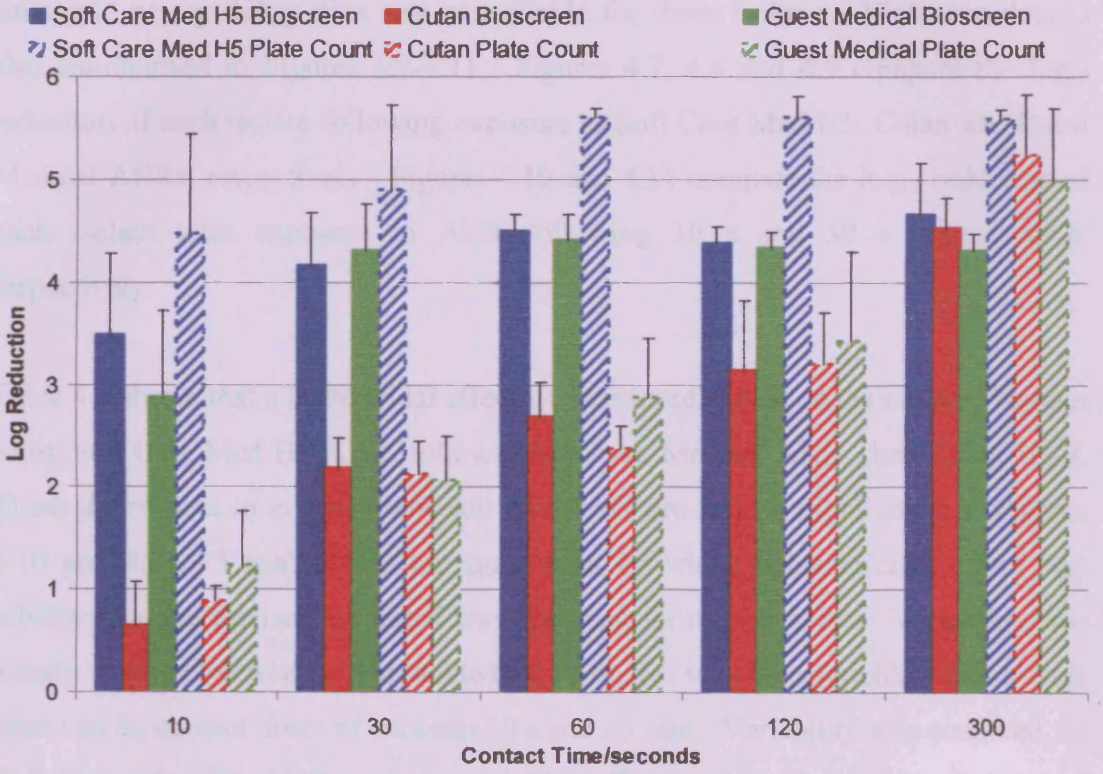

Figure 4.6: Comparison of Carrier Testing of *S. aureus* NCIMB 9518 Using Plate Count and Bioscreen

Mean $\log_{10}$ reduction ± SD of *S. aureus* NCIMB 9518 after contact with Soft Care Med H5, Cutan and Guest Medical AHRs for the carrier test using Bioscreen and plate count methods of enumeration.

4.3.2.6 Carrier Testing of Isolates Using the Bioscreen

The mean contact time by which each AHR had a bactericidal effect (≥4 $\log_{10}$ reduction) for each hospital isolate plus *S. aureus* NCIMB 9518 is shown in Table 4.3 and is published in Cheeseman *et al.* (2009). Isolates 16 and 20, however, did not grow sufficiently for control wells to demonstrate a 4 $\log_{10}$ growth during Bioscreen testing, meaning a 4 $\log_{10}$ reduction could not be detected and therefore antiseptic susceptibility data was unavailable for these isolates. Bioscreen data is also summarised in Figures 4.7-4.11. Figures 4.7, 4.8 and 4.9 compare the $\log_{10}$ reduction of each isolate following exposure to Soft Care Med H5, Cutan and Guest Medical AHRs, respectively. Figures 4.10 and 4.11 compare the $\log_{10}$ reduction of each isolate after exposure to AHR following 10 s and 30 s contact time, respectively.

Table 4.3 shows that a bactericidal effect was achieved fastest on the hospital isolates using Soft Care Med H5 AHR, followed by Guest Medical AHR, then Cutan AHR. These differences in efficacy between the AHRs are further highlighted in Figures 4.10 and 4.11. Variability in the time point by which a bactericidal effect was achieved among the isolates tested was observed for all the AHRs. Variability was mostly widely observed for Guest Medical AHR, for which bactericidal effects were observed by contact times of between 10 s and >5 min. Variability was observed for Soft Care Med H5 AHR, with bactericidal effects observed by contact times of between 10 s and 1 min, although this was achieved by a contact time of 30 s for the majority of isolates. There was very little variability for Cutan AHR, which achieved a bactericidal effect against most isolates by a contact time of 5 min, with the exception of 8 isolates by 2 min and one isolate by >5 min. Variability between strains at contact times of 10 and 30 s, which are contact times closest to those found in practice, is further illustrated in Figures 4.7-4.9, which also illustrate the change in $\log_{10}$ reduction between these time points.

By a 10 s contact time Soft Care Med H5 and Guest Medical AHRs were only able to exert a bactericidal effect on one isolate (<2% total isolates) and Cutan AHR was not able to exert a bactericidal effect on any isolates. Within a 30 s contact time Soft Care Med H5 and Guest Medical AHRs were able to exert a bactericidal effect on 50

(86%) and 25 (42%) isolates, respectively, whereas Cutan AHR was still unable to exert a bactericidal effect on any isolates.

The mean $\log_{10}$ reduction among isolates after 10 s exposure to Soft Care Med H5, Cutan and Guest Medical AHRs is 2.67±0.68, 0.696±1.74 and 1.96±3.59, respectively. The mean $\log_{10}$ reduction among isolates after 30 s exposure to Soft Care Med H5, Cutan and Guest Medical AHRs is 4.58±0.52, 1.74±0.53 and 3.60±1.11, respectively.

Table 4.3: Results of Carrier Testing of Isolates Using Bioscreen

Time point by which a bactericidal effect is observed for Soft Care Med H5, Cutan and Guest Medical AHRs against each isolate using the carrier test Bioscreen method. MRSA isolates are shown in bold. *Isolates 16 and 20 are not included.

Isolate*	Time Point by which a bactericidal effect was observed (mean ≥4 log_{10} reduction)		
	Soft Care Med H5	Cutan	Guest Medical
44	10 s	5 min	30 s
27	30 s	5 min	10 s
37, 41, 42, 43, 46, 49, 50, S4, S6	30 s	2 min	30 s
3, 5, 10, 13, **17, 19, 25, 26, 31, 32**, 39, **45**, S11, NCIMB 9518	30 s	5 min	30 s
11, 14, **15, 18**, 21, 24, 38, 47, **48, 52, O43, P59**, N10, **S3**	30 s	5 min	1 min
34, **55**, T4, Q49, K17, H9, S8	30 s	5 min	2 min
9, 30, S7, **S12**	30 s	5 min	5 min
S5	30 s	>5 min	2 min
4, 6	1 min	5 min	1 min
51	1 min	5min	2 min
28, S2, S10	1 min	5 min	5 min
S1	1 min	5 min	>5 min

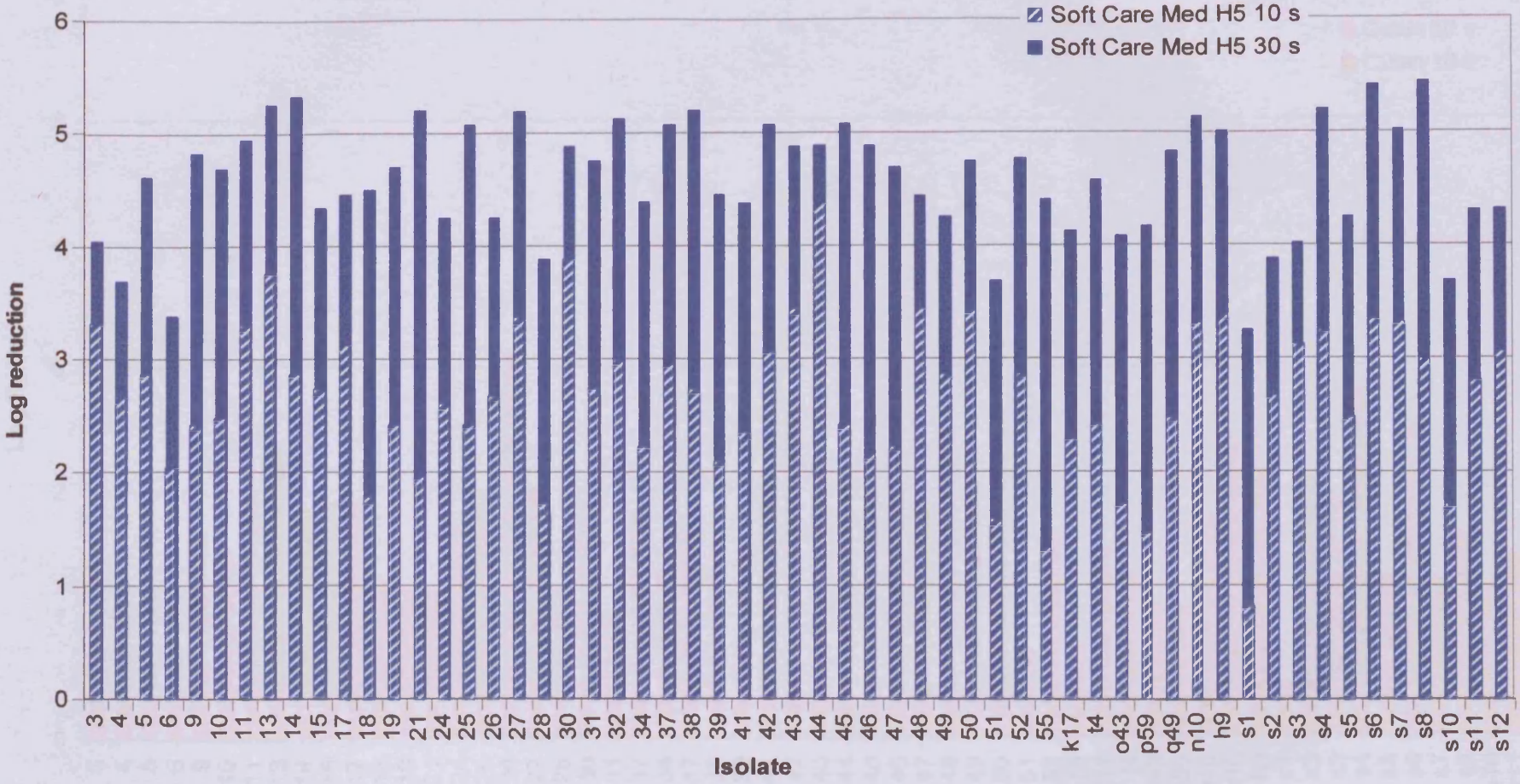

Figure 4.7: Mean Log_{10} Reduction of *S. aureus* Isolates With Soft Care Med H5 AHR at 10 s and 30 s Contact Times Using the Bioscreen

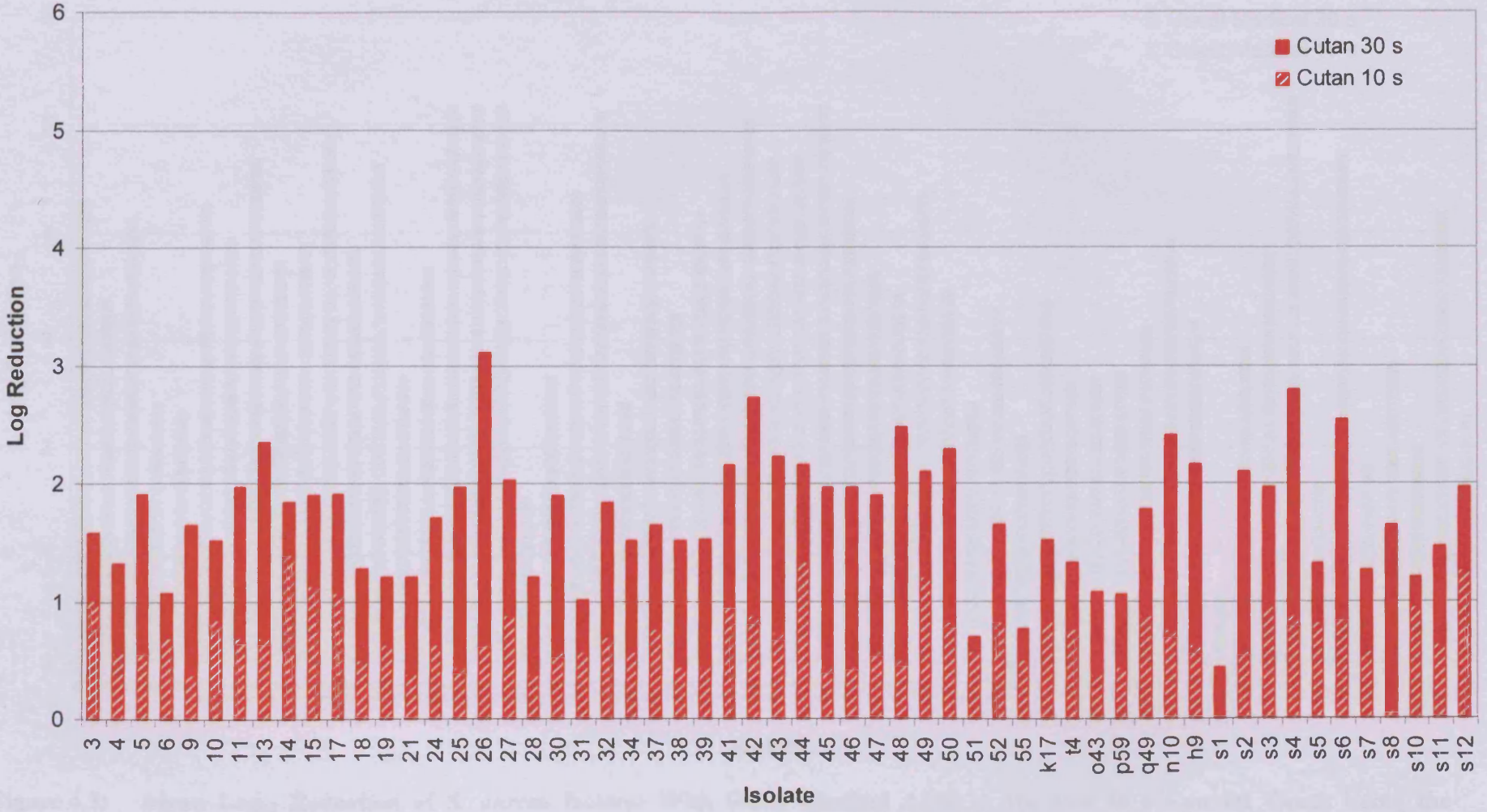

Figure 4.8: Mean Log_{10} Reduction of *S. aureus* Isolates With Cutan AHR at 10s and 30 s Contact Times Using the Bioscreen

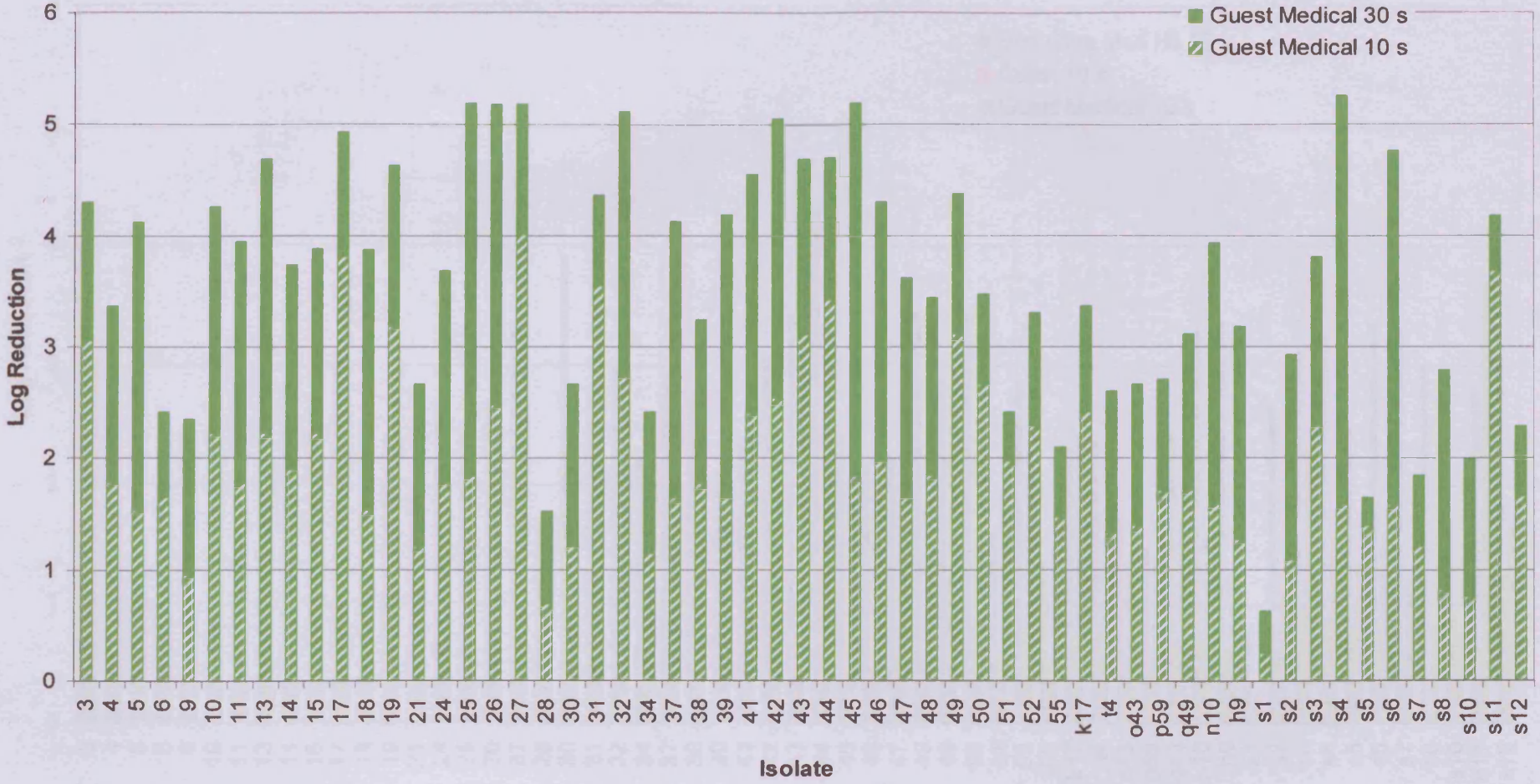

Figure 4.9: Mean Log_{10} Reduction of *S. aureus* Isolates With Guest Medical AHR at 10s and 30 s Contact Times Using the Bioscreen

Figure 4.10: Mean Log_{10} Reduction of *S. aureus* Isolates After 10 s Exposure to AHRs Using the Carrier Test Bioscreen Method

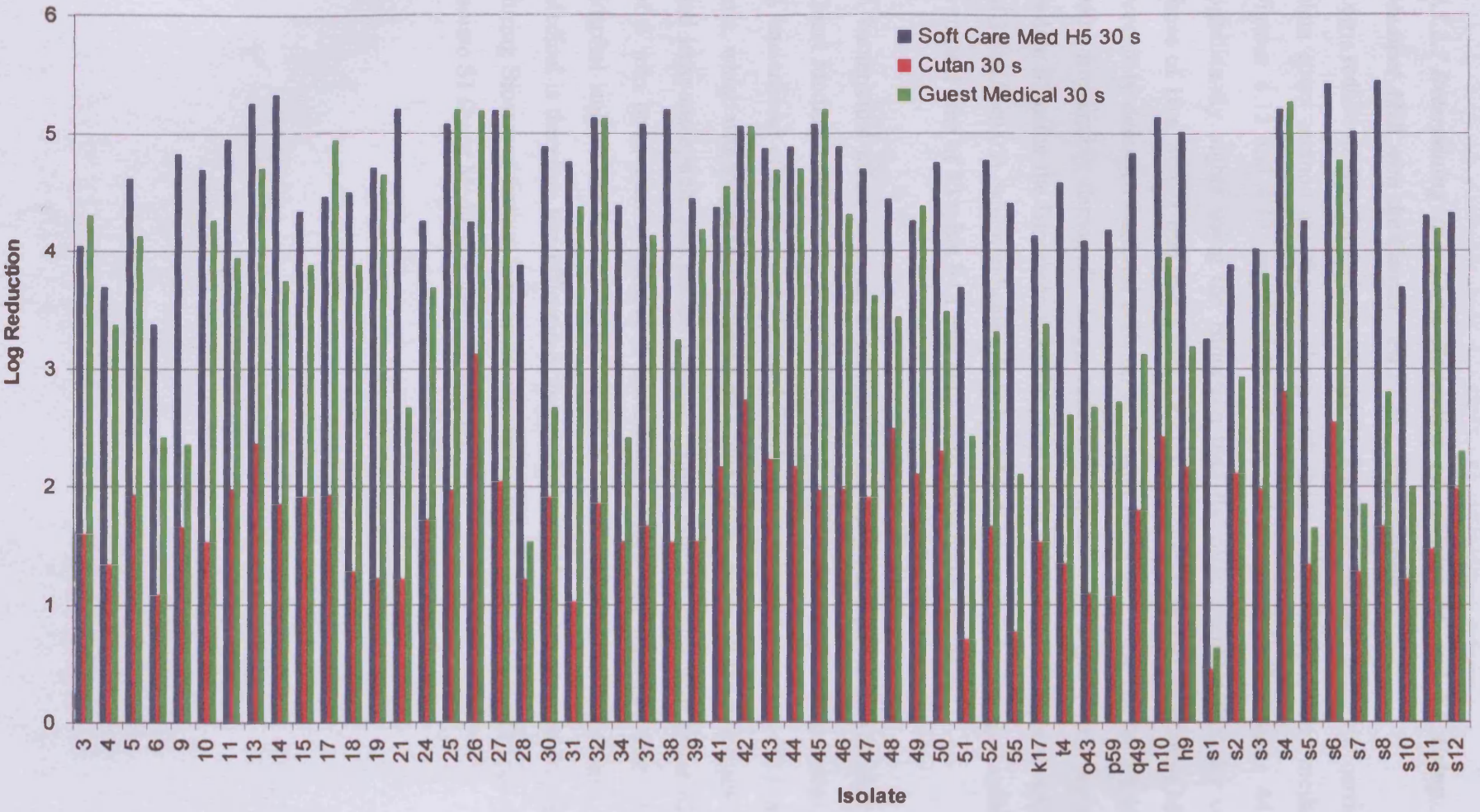

Figure 4.11: Mean Log_{10} Reduction of *S. aureus* Isolates After 30 s Exposure to AHRs Using the Carrier Test Bioscreen Method

4.3.2.7 Determining the Log_{10} Reduction of Isolates 44 and S1 Using Guest Medical AHR with the Carrier Test Plate Count Method

Log_{10} reduction data for isolates 44 and S1 for Guest Medical using the carrier test plate count method are shown, along with those using the Bioscreen method, in Figures 4.12 and 4.13, respectively. Log_{10} reduction values for isolate 44 were significantly higher using the Bioscreen than the plate count method for contact times of 10 s, 30 s, 1 min and 2 min, (P <0.05), which indicates that Guest Medical reversibly damaged bacterial cells up to contact times of 2 min, but by 5 min cells were irreversibly damaged. Log_{10} reduction values were significantly higher for isolate S1 using the Bioscreen than the plate count method following a contact time of 30 s (P <0.05), which indicates that Guest Medical reversibly damaged cells up to a contact time of 30 s, but by 1 min cells were irreversibly damaged.

A bactericidal effect was not observed for the most-susceptible isolate (44) using Guest Medical until 5 min, which is the same contact time required by Cutan AHR. A bactericidal effect was not observed for the least-susceptible isolate (S1) until >5 min, which was longer than required by Cutan AHR. Since all three isolates (most- and least-susceptible and standard strain) require contact times with Guest Medical of 5 min or greater to achieve a bactericidal effect, this indicates that all other hospital isolates are also likely to require a 5 min contact time or greater. Guest Medical is therefore less efficacious than Soft Care and, contrary to results obtained during Bioscreen testing, is no more efficacious than Cutan AHR (and in the case of isolate S1 Guest Medical is less efficacious).

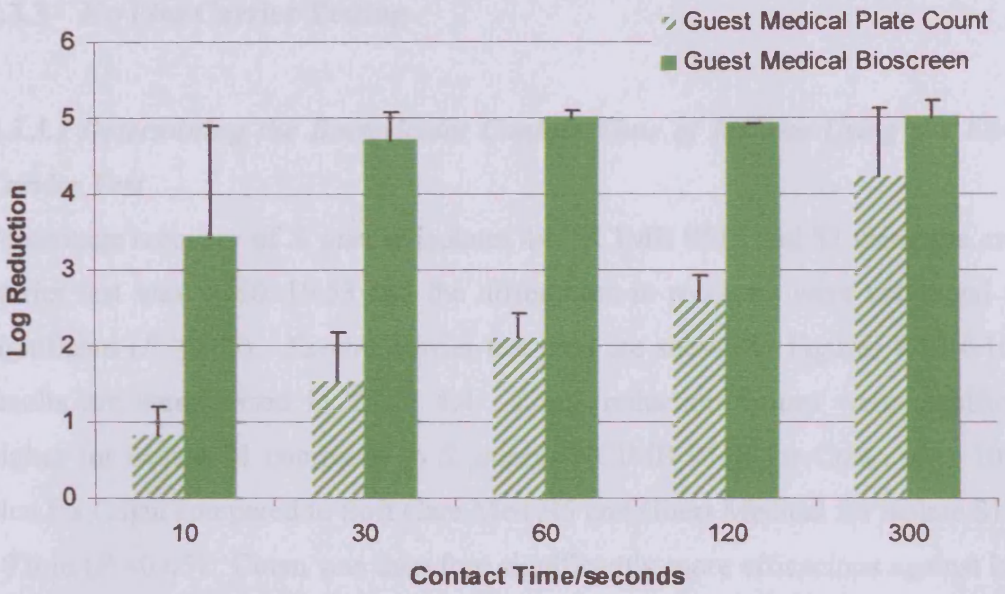

Figure 4.12: Log_{10} Reduction of Isolate 44 Using Guest Medical AHR with the Carrier Test Plate Count Method

Mean log_{10} reduction ± SD of isolate 44 after contact with Guest Medical AHR for the carrier test using the plate count method of enumeration.

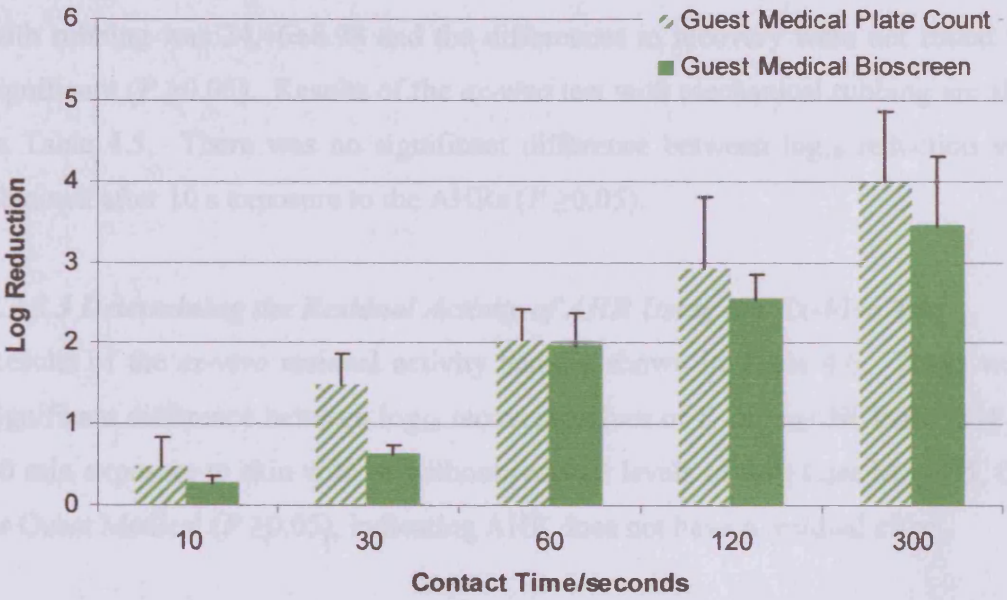

Figure 4.13: Log_{10} Reduction of Isolate S1 Using Guest Medical with the Carrier Test Plate Count Method

Mean log_{10} reduction ± SD of isolate S1 after contact with Guest Medical AHR for the carrier test using the plate count method of enumeration.

4.3.3 *Ex-Vivo* Carrier Testing

4.3.3.1 Determining the Bactericidal Contact Time of Isolates Using the Ex-Vivo Carrier Test

Percentage recovery of *S. aureus* isolates 44, NCIMB 9518 and S1 using the *ex-vivo* carrier test was 99.60±19.53 and the differences in recovery were not found to be significant ($P \geq 0.05$). *Ex-vivo* carrier test data are shown in Figures 4.14-4.16 and results are summarised in Table 4.4. Log_{10} reduction values were significantly higher for isolate S1 compared to *S. aureus* NCIMB 9518 for Cutan after 10 min, plus for Cutan compared to Soft Care Med H5 and Guest Medical for isolate S1 after 10 min ($P < 0.05$). Cutan was therefore significantly more efficacious against isolate S1 after 10 min compared to Soft Care Med H5 and Guest Medical and also that isolate S1 was more susceptible to Cutan after a contact time of 10 min than isolate 9518. Apart from these observations there was no significant difference between the efficacies of the AHRs using the *ex-vivo* carrier test.

4.3.3.2 Determining the Effect of Mechanical Rubbing Using the Ex-Vivo Test

Percentage recovery of *S. aureus* isolate NCIMB 9518 using the *ex-vivo* carrier test with rubbing was 24.46±8.98 and the differences in recovery were not found to be significant ($P \geq 0.05$). Results of the *ex-vivo* test with mechanical rubbing are shown in Table 4.5. There was no significant difference between log_{10} reduction values obtained after 10 s exposure to the AHRs ($P \geq 0.05$).

4.3.3.3 Determining the Residual Activity of AHR Using the Ex-Vivo Test

Results of the *ex-vivo* residual activity test are shown in Table 4.6. There was no significant difference between log_{10} recovery values of *S. aureus* NCIMB 9518 after 20 min exposure to skin with or without residual levels of Soft Care Med H5, Cutan or Guest Medical ($P \geq 0.05$), indicating AHR does not have a residual effect.

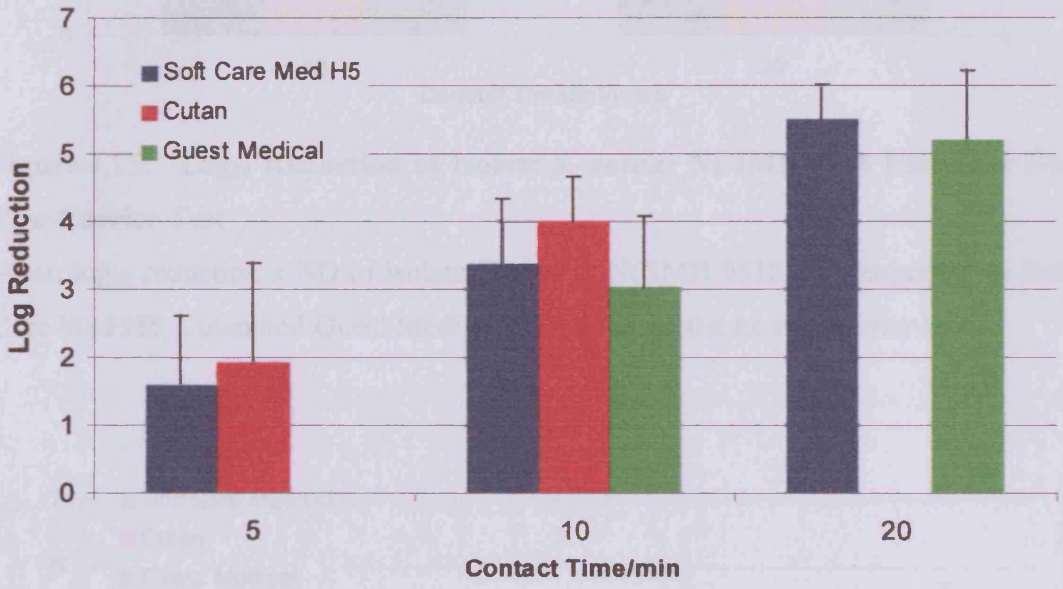

Figure 4.14: Log_{10} Reduction of Isolate 44 Using the *Ex-Vivo* Carrier Test

Mean log_{10} reduction ± SD of isolate 44 after exposure to Soft Care Med H5, Cutan and Guest Medical isolates using the *ex-vivo* carrier test. Experiment was not performed for 5 min exposure to Guest Medical or 20 min exposure to Cutan AHR

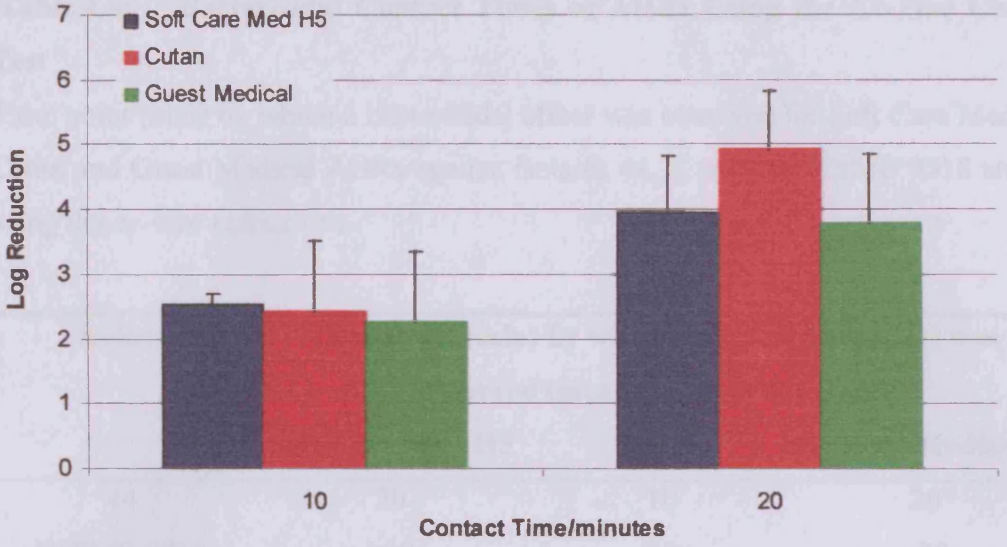

Figure 4.15: Log_{10} Reduction of Isolate *S. aureus* NCIMB 9518 Using the *Ex-Vivo* Carrier Test

Mean log_{10} reduction ± SD of isolate *S. aureus* NCIMB 9518 after exposure to Soft Care Med H5, Cutan and Guest Medical isolates using the *ex-vivo* carrier test

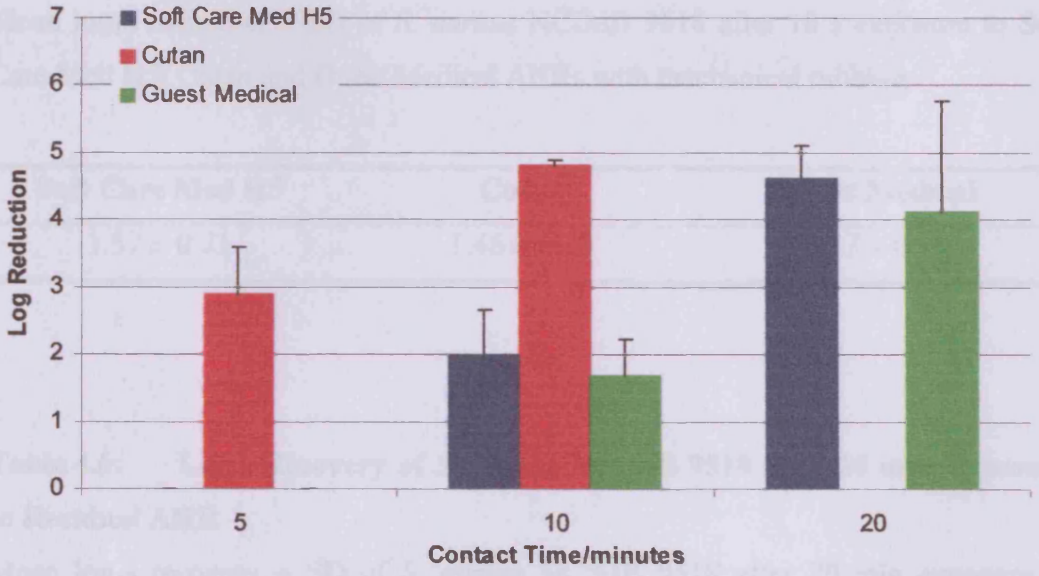

Figure 4.16: Log_{10} Reduction of Isolate S1 Using the *Ex-Vivo* Carrier Test

Mean log_{10} reduction ± SD of isolate S1 after exposure to Soft Care Med H5, Cutan and Guest Medical isolates using the *ex-vivo* carrier test. Experiment was not performed for 5 min exposure to Soft Care Med H5 or Guest Medical, or 20 min exposure to Cutan

Table 4.4: Bactericidal Contact Times of AHRs Using the *Ex-Vivo* Carrier Test

Time point (min) by which a bactericidal effect was observed for Soft Care Med H5, Cutan and Guest Medical AHRs against isolates 44, *S. aureus* NCIMB 9518 and S1 using the *ex-vivo* carrier test.

Isolate	Time Point (min) by which a bactericidal effect was observed (mean ≥4 log_{10} reduction)		
	Soft Care Med H5	Cutan	Guest Medical
44	20	10	20
NCIMB 9518	>20	20	>20
S1	20	10	20

Table 4.5: Log_{10} Reduction of *S. aureus* NCIMB 9518 after 10 s Exposure to AHR with Mechanical Rubbing

Mean log_{10} reduction ± SD of *S. aureus* NCIMB 9518 after 10 s exposure to Soft Care Med H5, Cutan and Guest Medical AHRs with mechanical rubbing

Soft Care Med H5	Cutan	Guest Medical
1.37 ± 0.21	1.46 ± 0.20	1.47 ± 0.19

Table 4.6: Log_{10} Recovery of *S. aureus* NCIMB 9518 after 20 min Exposure to Residual AHR

Mean log_{10} recovery ± SD of *S. aureus* NCIMB 9518 after 20 min exposure to residual Soft Care Med H5, Cutan and Guest Medical AHRs

Control	Soft Care Med H5	Cutan	Guest Medical
7.00 ± 0.023	**7.01 ± 0.031**	**7.10 ± 0.099**	**6.97 ± 0.06**

4.3.3.4 Comparison of the Efficacy of AHRs Using Stainless Steel and Ex-Vivo Carrier Tests and the Ex-Vivo Test with Mechanical Rubbing

Results of the *ex-vivo* carrier test after a 10 s contact time are shown in Figure 4.17. There was no significant difference between $\log_{10}$ reduction values for the *ex-vivo* carrier test after a 10 s contact time for Soft Care Med H5, Cutan and Guest Medical AHRs ($P \geq 0.05$).

The average $\log_{10}$ number of cells per surface was 6.31±0.11 for the stainless steel carrier test, 6.07±0.07 for the *ex-vivo* carrier test and 6.45±0.19 for the *ex-vivo* test with mechanical rubbing and therefore the results of these tests are directly comparable.

A comparison of the $\log_{10}$ reduction values of *S. aureus* NCIMB 9518 after 10 s exposure to AHRs using the stainless steel carrier test, the *ex-vivo* carrier test and the *ex-vivo* carrier test with mechanical rubbing is shown in Figure 4.17. Log_{10} reductions were higher for the stainless steel carrier test and *ex-vivo* carrier test with rubbing compared to the *ex-vivo* carrier test for Soft Care Med H5, Cutan and Guest Medical AHRs ($P < 0.05$). Log_{10} reductions were higher for the stainless steel carrier test compared to the *ex-vivo* test with rubbing for Soft Care ($P < 0.05$), but higher for the *ex-vivo* test with rubbing compared to the stainless steel carrier test for Cutan ($P < 0.05$).

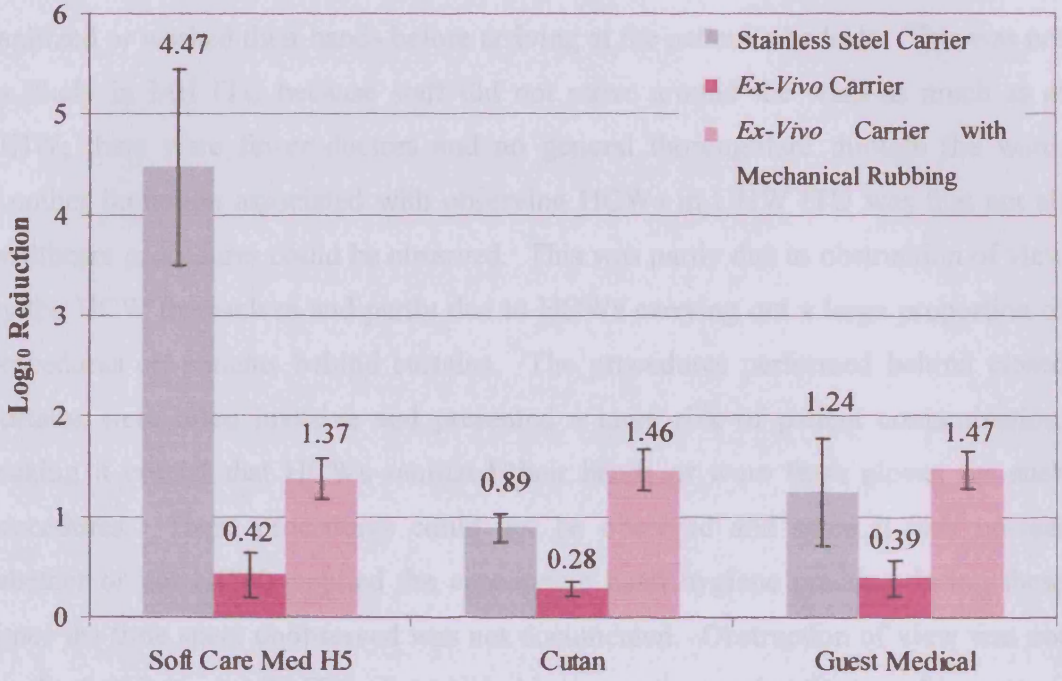

Figure 4.17: Log_{10} Reduction of Isolate *S. aureus* NCIMB 9518 after 10 s Contact Time for Stainless Steel Carrier, *Ex-Vivo* Carrier and *Ex-Vivo* Carrier with Mechanical Rubbing Tests

Mean log_{10} reduction ± SD of isolate *S. aureus* NCIMB 9518 after 10 s contact time with Soft Care Med H5, Cutan and Guest Medical AHRs using the stainless steel carrier test, the *ex-vivo* carrier test and the *ex-vivo* carrier test with mechanical rubbing. Mean log_{10} reduction value labels are provided.

4.4 DISCUSSION

4.4.1 Observation of Hand Sanitizing Practices in ITU

Observations of hand sanitizing procedures in UHW and MH ITUs are summarized in Table 4.1. Observing HCWs in UHW ITU had limitations because staff may have sanitized or washed their hands before arriving at the patient's bedside. This was not as likely in MH ITU because staff did not move around the ward as much as at UHW; there were fewer doctors and no general thoroughfare through the ward. Another limitation associated with observing HCWs in UHW ITU was that not all healthcare procedures could be observed. This was partly due to obstruction of view by the HCW themselves and partly due to HCWs carrying out a large proportion of procedures on patients behind curtains. The procedures performed behind closed curtains were often invasive and presented a large risk of patient contamination, making it crucial that HCWs sanitized their hands or wore fresh gloves for such procedures. These procedures could not be observed and since it was unclear whether or not HCWs applied the appropriate hand hygiene practice during these times the time spent unobserved was not documented. Obstruction of view was not much of an issue at MH ITU. Potential object-to-patient and patient-to-object cross-contamination may therefore be more frequent.

The observed low incidence of use of AHR may have been due to the large proportion of staff wearing gloves. It has been documented that poor adherence to hand hygiene practices is partly due to the belief that wearing gloves removes the need for hand sanitizing (Boyce & Pittet, 2002). However, gloves used in the ITUs of UHW and MH were not sterile, meaning that even if no other surface is touched by a HCW in between wearing gloves and touching the patient, there is still a possibility of introducing microorganisms originating from the gloves onto patients and surfaces. There was still a large percentage of staff (particularly from UHW) that did not use any sanitization or gloves, although this rarely occurred prior to touching patients at UHW and never at MH, only prior to touching objects. A much higher percentage of ITU HCWs were found to wear gloves in the study by Girou *et al.* (2002), compared to HCWs in this study (see Table 4.7). Karabay *et al.* (2005),

however, found a much lower percentage (29% among HCWs using hand wash and 41% among HCWs using AHR) of HCWs both from ITU and non-ITU wards using gloves, which is similar to results found by this study.

The volume of AHR used was also low. The Hand Decontamination Policy at UHW (Hosein, 2004) states that the amount of AHR used should be that specified by the manufacturer, which is 2-3 pumps in the case of Soft Care Med H5 AHR (used at UHW). The number of pumps required is not stated by the manufacturers of Cutan or Guest Medical AHR (used at MH), although Guest Medical AHR states to use a thumbnail amount. The actual number of pumps observed at UHW was less than is specified for Soft Care Med H5, which may be partly due to the fact that the required number of pumps is not written on the Soft Care Med H5 bottle. Kampf (2008) studied the difference in efficacy of varying volumes of four different AHRs when applied to hands contaminated with *Serratia marcescens* and rubbed in until hands were completely dry. He found that a volume of AHR found to sufficiently cover both hands (2.4 ml) was only able to provide $\log_{10}$ reductions of 1.90-2.79 whereas a volume of 3.6 ml provided significantly higher $\log_{10}$ reductions (2.53-3.04). These findings indicate that use of smaller volumes of AHR may have a major impact on its efficacy.

No minimum time is specified in the Hand Decontamination Policy for UHW staff or in the manufacturer's use instructions for massaging the gel into the hands for Soft Care Med H5 or Guest Medical AHRs. However, it states on the Cutan bottle to massage AHR into hands for >30 s, which is twice as long as staff at MH took to rub AHR into their hands. It states on the Guest Medical bottle that AHR should be rubbed into hands until dry, which takes around 25 s of continual massaging until hands are completely dry after using one pump. The average length of time observed by MH staff was much less than this, although there were on occasions a length of time after sanitization before the HCW began a procedure or touched a surface. The Hand Decontamination Policy advises that the process is complete when hands are completely dry, but does not specify that hands should be continually massaged during this time period. A previous study that included observation of AHR contact times in ITUs showed similar contact times to this study (Larson *et al.*, 2001),

whereas another study showed much longer contact times (Girou *et al.*, 2002), as shown in Table 4.7.

Table 4.7 also shows that a similar high percentage of ITU staff in this study rubbed the AHR into their hands correctly, compared to those in the study by Larson *et al.* (2001).

Hand washing was infrequently observed, but this may meet the instructions laid down in the hand decontamination policy that if hands are physically clean then AHR can be used instead of soap. AHR cannot be used on gloves; therefore object-to-object cross-contamination is unavoidable. However, object-to-patient and patient-to-object cross-contamination can easily be avoided by a change of gloves immediately prior to touching a patient. The Hand Decontamination Policy also states that hand decontamination should occur before and after each patient contact, which was not observed in some situations.

Hand sanitization in the form of hand washing, especially use of AHR, was lower at MH compared to UHW. A similar percentage of staff massaged the AHR into all areas of their hands at both hospitals, although staff at MH did this for, on average, a slightly longer period of time. However, it must be remembered that these percentages were taken from only 5 observations where AHR was used, compared to 30 at UHW, making a true comparison difficult. A similar percentage of staff wore gloves at both hospitals, although a much larger percentage used no form of sanitization or protection during procedures at MH. This therefore led to a higher percentage of potential staff-to-object and object-to-object cross-contamination at MH compared to UHW, although the lower percentage of potential staff to patient, object to patient and patient to object cross-contamination observed at MH was likely due to staff there touching the patients less than at UHW.

Table 4.7: Comparison of Hand Sanitizing Observations from this Study and Previous Studies

A comparison of hand sanitizing observations found in this study and studies by Larson *et al.* (2001) and Girou *et al.* (2002). N/A = not applicable, UHW = University Hospital of Wales, MH = Morriston Hospital

Observation	This Study		Larson *et al.*	Girou *et al.*
	UHW	MH	(2001)	(2002)
Number of healthcare observations	54	56	26	114
% activities where gloves were worn	40	34	n/a	85
Mean AHR contact time (s)	11	15	13	30 (median)
% users applied AHR correctly to hands	75	80	92	N/A

The Epic Project guidelines (Pratt *et al.*, 2001) state that 'hands must be decontaminated immediately before each and every episode of direct patient contact/care and after any activity or contact that potentially results in hands becoming contaminated'. Since infection-causing bacteria are found in the hospital environment then touching a surface could, according to the guidelines, result in hands becoming contaminated and therefore hands should be decontaminated after touching anything. If these guidelines were implemented in UHW and MH ITUs the percentage of patient-to-object, object-to-patient and object-object potential cross-contamination would be dramatically reduced.

4.4.2 Stainless Steel Carrier Testing

Results of the carrier test plate count and Bioscreen methods for *S. aureus* NCIMB 9518 are compared in Figure 4.6. An explanation for why Soft Care Med H5 AHR $\log_{10}$ reductions using the plate count method were significantly higher than the Bioscreen results is not clear. The discrepancy between carrier test Bioscreen and plate count $\log_{10}$ reductions can be explained by Lambert and van der Ouderaa's (1999) theory that cells are reversibly damaged, but this means that Bioscreen results for Guest Medical against the hospital isolates must be interpreted with caution because they too are likely to be overestimated. This also means that in addition to cells killed during exposure, Guest Medical is sub-lethally damaging a number of those surviving; therefore although these may not be able to replicate on the surface of HCWs hands, they may still be able to cause infection if they are able to enter the body of a patient. The Bioscreen method is a high-throughput method and therefore extremely beneficial for the testing of a large number of samples. It is necessary, however, to validate the method using the plate count method to pre-determine whether the biocide in question is capable of causing reversible damage.

Plate count and Bioscreen results for isolate 44 and S1 are compared in Figures 4.12 and 4.13. Isolate 44 (the most-susceptible isolate) and S1 (the least-susceptible isolate) were also found to be reversibly damaged by Guest Medical AHR and this is most likely the case for all isolates.

The results of carrier testing of isolates using the Bioscreen method are summarized in Table 4.3. Bioscreen data for screening of isolates were not adjusted to compensate for the limit of detection because test samples where there was 0 CFU in 5 wells may have actually contained 0 CFU/ml. Compensating for limit of detection for Bioscreen results seriously affected the results, where a 4 $\log_{10}$ control, and therefore a bactericidal effect, was not always achieved. The contact times taken to achieve a bactericidal effect shown in Table 4.3 are therefore not adjusted to compensate for the limit of detection.

Differences in efficacy observed between the AHRs are apparent in Table 4.3 and are further highlighted in Figures 4.10 and 4.11. True Guest Medical $\log_{10}$ reductions are those provided by the plate count methods and are shown in Figures 4.3, 4.12 and 4.13 for isolates *S. aureus* NCIMB 9518, 44 and S1, respectively. The differences in efficacy observed between Soft Care Med H5, Cutan and Guest Medical AHRs observed may be partially due to the type of alcohol used in the products. Propyl alcohol is the most efficacious, followed by isopropyl alcohol and finally ethanol (Rotter, 2001) and this has also been demonstrated to be the case on pig skin (Bush *et al.*, 1986). This explains why Soft Care Med H5 (a combination of propyl and isopropyl alcohols) was more efficacious than Cutan (a combination of ethanol and propyl alcohol) and why Guest Medical (consisting of ethanol) was the least efficacious when considering the true bactericidal effects achieved by the plate count carrier test. The reduced efficacy observed for Guest Medical may also be partially due to the lower concentration of alcohol in Guest Medical compared to Soft Care Med H5 and Cutan. This is because although alcohol was present at 70% for all the AHRs, Guest Medical AHR was 70% v/v, which is equivalent to only 62.6% w/w (Ali *et al.*, 2001). Since alcohol has a high concentration exponent, this slightly lower concentration may have a large impact on efficacy. The difference in efficacy of the AHRs may also be explained by differences in the concentration exponent of their alcoholic constituents. Concentration exponents of aliphatic alcohols (which, among others, include propanol, isopropanol and ethanol) range between 6.0 and 12.7 (Denyer *et al.*, 1987), therefore even at the same concentration they may have different efficacies. The fact that Cutan AHR performed less well than Soft Care Med H5 could also be explained by the very high viscosity of Cutan, which may

reduce its antimicrobial activity due to its restricted ability to access indentations on a surface and its potentially reduced access to the surface.

Variability was observed between the isolates and this is shown in Table 4.3 and also highlighted in Figures 4.7-4.9. It is unclear yet why AHRs would show variability in efficacy between hospital isolates and why some AHRs resulted in more variability than others. It is likely to be due to some isolates possessing some factor that enables them to endure longer periods of exposure to AHRs without being killed, or in the case of Guest Medical AHR without being irreversibly damaged. Isolate 44 is most susceptible and isolate S1 is least susceptible to the AHRs. These organisms must therefore possess some differences in genotype or phenotype that result in the AHRs exerting a bactericidal effect in such different time frames. This will be investigated further.

Carrier test results in Table 4.3 show that Soft Care Med H5 AHR met the criteria of EN13697 (European Standard EN13697, 2001; $\geq$4 log reduction within 5 min) for all isolates, whereas Cutan and Guest Medical AHRs met the criteria for all but one isolate. However, the contact time EN13697 allows to reach a $\geq$ 4 log reduction is not achieved in practice, due to the rapid evaporation time of the AHR. The criteria should therefore be met within the in-use time (11-15 s, Table 4.1) in order to achieve this reduction in practice.

Carrier test results obtained for the hospital isolates plus reference isolate shown in Table 4.3 do not reflect those obtained by Kampf and Hollingsworth (2008). They tested *S. aureus* NCIMB 9518, plus a vancomycin-intermediate and a methicillin-resistant *S. aureus* hospital strain against an 85% w/w ethanol AHR using a suspension test for a contact time of 15 s. They found that all three *S. aureus* strains showed a >5 $\log_{10}$ reduction (bactericidal). However, since bacteria are easier to kill in a suspension than when dried on a surface these results cannot be directly compared and these $\log_{10}$ reductions are therefore unlikely to occur on the hands of healthcare workers after 15 s. They also did not add organic material and therefore the conditions did not reflect those found on a HCWs hand and may have led to a higher $\log_{10}$ reduction than would have been found under dirty conditions. Finally

the AHR used was at a much higher concentration than those used in practice against the isolates in this study.

4.4.3 *Ex-Vivo* Carrier Testing

Ex-vivo carrier testing was carried out using thawed skin samples. Ears were frozen, then thawed to excise skin and re-frozen until the day of use. Ears were frozen upon arrival due to the sporadic and unpredictable supply of skin, making it impossible to excise and use fresh skin on the same day. Skin was re-frozen after excision, rather than used on the day of excision due to the fact that excision of skin took a long period of time and that some samples would have been wasted. A study by Messager *et al.* (2003) found that fresh and freshly-thawed excised human skin was morphologically similar, with no apparent damage except that some parts of the stratum corneum of the freshly-thawed skin showed slight fragmentation. Messager and colleagues (2003) also found significant levels of lactate dehydrogenase, a marker for skin viability, in both fresh and freshly-thawed skin, although levels in thawed skin were higher. They found that freshly-thawed skin contained a lower concentration of oxygen, a marker for metabolic activity, than fresh skin samples, although there was no significant difference in their pH. Most importantly, Messager (2002) found there was no significant difference in the antibacterial activity of a range of antiseptics to *S. aureus* applied on either fresh or freshly-thawed skin.

Results of *ex-vivo* carrier testing are shown in Figures 4.14-4.16 and summarised in Table 4.4. The reason Cutan was the most efficacious AHR using the *ex-vivo* test and not Soft Care Med H5 is unclear, since Soft Care Med H5 was the most efficacious AHR using the stainless steel carrier test and the combination of alcohols it contains are the most effective. The most likely explanation is due to the high viscosity of Cutan AHR when compared to Soft Care and Guest Medical, which is likely to have reduced the evaporation time of the alcohol and since the *ex-vivo* carrier test involved long contact times, differences in evaporation rate of alcohol between AHRs is likely to have a large effect on bactericidal contact times. The reason isolate S1 was more susceptible to Cutan is also not clear, since Table 4.3

demonstrates that isolate S1 was as susceptible to Cutan as isolate NCIMB 9518 during stainless steel carrier testing.

Comparison of stainless steel carrier testing results in Table 4.3 and *ex-vivo* carrier testing in Table 4.4 also revealed that a much longer contact time was required to achieve a bactericidal effect using the *ex-vivo* carrier test compared to the stainless steel carrier test for isolates 44, NCIMB 9518 and S1. This is likely to be a combination of the fact that bacteria are able to 'hide' in crevices in the skin, where they would not be as exposed to the AHR, and the fact that skin acts as a partitioning phase so that some alcohol is lost into the skin layers. Biochemical properties of the skin may also affect the AHR or bacteria in some way. This theory is supported by the observation that the *ex-vivo* carrier test with rubbing was more effective than the *ex-vivo* carrier test without rubbing, since rubbing facilitates penetration of AHR into the crevices. Variation between AHRs and isolates found in Table 4.3 are not reflected in carrier test ex-vivo results in Figures 4.14-4.16. The reason there were not larger differences in variation could be due to the long time required to achieve a bactericidal effect using the *ex-vivo* test, making differences in the efficacy of AHRs and susceptibility to isolates less pronounced.

Results of the *ex-vivo* residual activity test are shown in Table 4.6. HCWs took between on average 11-15 s to rub AHR into their hands and during the residual activity test 15 sec of rubbing caused all AHR to be absorbed by the pig skin, with no residual effect observed. This indicates that the $\log_{10}$ reduction values obtained for stainless steel (Table 4.3) and *ex-vivo* testing (Figures 4.14-4.16) by contact times longer than 10 s are unlikely to be achieved in practice. Wade & Casewell (1991) also found that alcohol, in the form of 60% v/v isopropanol, had no residual activity against species of *Enterobacter in-vivo*. Similarly, Lowbury *et al.* (1974) found 70% v/v ethanol had no residual activity against skin inoculated with *S. aureus in-vivo*, although they found that wearing gloves for 3 h after application of the alcohol resulted in a further reduction in bacterial numbers. Lilly *et al.* (1979), however, were able to demonstrate the residual activity of 70% w/v ethanol after 3 h against *S. aureus in-vitro*, although the additional decrease in bacterial numbers observed during the 3 h period may have been attributed to the temperature at which the dried

suspensions were held. The alcohol used in these studies, however, was not in gel form.

4.4.4 Comparison of the Efficacy of AHRs Using Stainless Steel and *Ex-Vivo* Carrier Tests and the *Ex-Vivo* Test with Mechanical Rubbing

Results of the *ex-vivo* carrier test for *S. aureus* NCIMB 9518 at a 10 s contact time are shown in Figure 4.17. The fact that no difference was observed between $\log_{10}$ reduction values after 10 s using Soft Care Med H5, Cutan and Guest Medical supports similar findings in Figure 4.15 for longer contact times.

The *ex-vivo* carrier test method showed the best recovery rate of cells (data shown in section 4.3.3.1). Recovery of cells from the *ex-vivo* carrier test with mechanical rubbing was very low in comparison (data shown in section 4.3.3.2) and this difference is most likely to be due to the bacteria being more deeply embedded in the skin crevices and therefore harder to recover with flushing.

The stainless steel carrier test also showed less recovery than the *ex-vivo* carrier test (data shown in section 4.3.2.2), although the recovery rate was much higher than achieved for the *ex-vivo* carrier test with mechanical rubbing. The reason for this is likely due to the drying step of the bacteria onto the stainless steel disk, which was achieved by incubation of the bacteria at 37°C, compared to air-drying at room temperature for the *ex-vivo* carrier test. It may also be due to the increased length of time of the drying process for the stainless steel disks, which was approximately 1 hour, compared to approximately 20 min for the *ex-vivo* carrier test. Reduced recovery cannot be explained by the action of the glass beads killing some cells during the recovery step because recovery of NCIMB 9518 from stainless steel disks was performed using the flushing technique (in section 4.2.2.2) and similar recovery rates were achieved as those using glass beads (46.25±20.10 for carrier testing with flushing, compared to 52.83±13.91 for carrier test with glass beads).

Messager *et al.* (2004) found that mechanical rubbing resulted in a $\log_{10}$ reduction of between 0.73 and 2.49 higher than without rubbing for the *ex-vivo* test. Although they used a slightly different contact time and different antiseptics, their results were

comparable to those figures obtained by comparison of $\log_{10}$ reduction values of *ex-vivo* carrier testing and *ex-vivo* testing with mechanical rubbing from Figure 4.17, which were of between 0.95 and 1.18 $\log_{10}$ reduction higher. The reason the AHRs were more efficacious in the presence of mechanical rubbing is most likely due to the mechanical action, which caused cells to be removed from the surface of the skin into the AHR as a suspension, making them easier to kill. Some cells may also have been removed from skin crevices and therefore become more exposed to AHR. However, the reason a larger difference was not seen in $\log_{10}$ reduction with mechanical rubbing compared to no mechanical rubbing is likely due to the mechanical action causing some bacteria to be forced into the crevices and therefore not exposed to the AHR, plus also the fact that the contact time allowed only a maximum of 10 s for the re-suspended bacteria to be killed and since there was only a small volume of AHR, the increased efficacy of the AHRs due to being in a suspension would not be as great.

4.4.5 Summary

HCWs in two local ITUs were found to apply AHR for a contact time of between 11 and 15 s. Stainless steel carrier testing of clinical isolates of *S. aureus* from these ITUs was performed and revealed that by a contact time of 10 s Soft Care Med H5 and Guest Medical AHRs were only able to achieve a bactericidal effect on 1 isolate and Cutan was unable to achieve a bactericidal effect on any isolates. None of the AHRs were able to achieve a mean $\log_{10}$ reduction of ≥ 4 against the sixty isolates as a whole after 10 s; only Soft Care Med H5 was able to after a contact time of 30 s.

Comparison of plate count and Bioscreen methods of enumeration of the stainless steel carrier test revealed that Guest Medical $\log_{10}$ reductions are overestimated for the Bioscreen method due to sub-lethal damage to the isolates. This means firstly that bacterial cells which have not been killed upon exposure to Guest Medical may be damaged, but they may still be able to cause infection if they enter a patient's body, and secondly that Bioscreen $\log_{10}$ reductions may not be true $\log_{10}$ reductions. Bioscreen $\log_{10}$ reductions for the most- and least-susceptible isolates were confirmed to be overestimated by comparison with the plate count method of

enumeration, which found Soft Care to be the most-efficacious, followed by Cutan, then finally Guest Medical. Differences in efficacy between the AHRs are likely to be due to differences in formulation. It is recommended that if the Bioscreen is used to enumerate efficacy testing it should first be compared with the plate count method to determine whether the biocide being tested causes sub-lethal damage. If this is the case it may be beneficial to perform the plate count method alongside the Bioscreen so that accurate bactericidal data are obtained from the plate count results, while sub-lethal damage data can be collected from the Bioscreen.

Ex-vivo testing revealed that a bactericidal effect took much longer to be achieved on a skin surface than a stainless steel surface, with bactericidal times of between 10 and >20 min seen among the AHRs. A larger bactericidal effect was seen for the AHRs when mechanical rubbing was used. The AHRs were found to have no residual activity and therefore data from this section indicates that application of AHR by an ITU HCW is unlikely to result in a bactericidal effect. It is therefore likely that although hand sanitizers meet the criteria of standard methods such as the hygienic hand disinfection test EN1500 and suspension test EN1276, these performances may not be indicative of their efficacy in practice.

Since this study showed that AHRs are unable to achieve a bactericidal effect under conditions found in practice it is important to either increase the efficacy of the AHR or to change the conditions in which the AHR is used in practice. Firstly, the type of alcohol could be changed to a more efficacious combination. The concentration of alcohol could also be increased to find the optimal ratio with water. The most important change to the conditions in practice is by the application of a larger volume of AHR (as found by Kampf, 2008) and also by increasing the contact time on the hands of HCWs. Increasing the volume of AHR used will most likely increase the time taken by HCWs to rub it into their hands, since they are unlikely to leave their hands sticky.

HCWs did not always apply hand hygiene prior to, and during healthcare procedures, which could potentially result in cross-contamination between the HCW, the patient and the immediate surroundings. Hand washing and/or use of AHR is sufficient for

activities not involving the patient and re-application of AHR is not necessary in between touching different objects, so the observed object-to-object potential cross-contamination is unavoidable. However, patient-to-object and object-to-patient potential cross contamination can be easily avoided by application/removal of gloves immediately prior/post touching the patient. Staff-to-patient and staff-to-object cross-contamination can also be easily avoided by ensuring that hand washing or AHR application is performed at regular intervals and immediately after staff touch their hair, forearms or clothing.

CHAPTER 5 THE EFFECT OF ALCOHOL HAND RUB ON *STAPHYLOCOCCUS AUREUS*

5.1 INTRODUCTION

5.1.1 The Cytoplasmic Membrane as a Target for Alcohol Hand Rub

The bacterial cytoplasmic membrane is a very important structure composed of lipid and protein and its integrity can therefore be compromised by any agent that is known to react with these components. The cytoplasmic membrane is also very accessible, since the only barrier between the cytoplasmic membrane and the environment is the cell wall and outer layers such as the capsule and slime layer. This makes it relatively easy for biocides to gain access to the membrane and is therefore a common site of action of biocides (Denyer & Stewart, 1998). Alcohol has a direct effect on the lipids and enzymes of the cytoplasmic membrane and indirectly impairs membrane biosynthesis (Denyer & Stewart, 1998; Kalathenos & Russell, 2003). The components and functions of the cytoplasmic membrane that are targets for disruption/damage by alcohol are summarised in Table 5.1.

Damage to the cytoplasmic membrane includes changes in permeability, leakage of intracellular material, osmotic lysis and inhibition of metabolic activity associated with the membrane (Denyer & Hugo, 1991). When a membrane is damaged rapid release of potassium ions (K^+) occurs first and is therefore a marker for membrane damage. Release of larger cell constituents follows, including purines, pyrimidines, pentoses, inorganic phosphates and amino acids (Denyer & Hugo, 1991), which is initially rapid, followed by a slower, gradual release that is thought to be the result of autolysis. Autolysis is brought about when damage to the membrane causes disruption of intracellular homeostasis and involves activation of latent ribonucleases that break down RNA and cause leakage of nucleotides and nucleosides (Lambert & Smith, 1976; Denyer & Hugo, 1991; Denyer & Stewart, 1998). Autolytic enzymes such as murein hydrolase may also be activated, which damage the cell wall and eventually lead to cell death (Rice & Bayles, 2003).

Table 5.1: Components and Functions of the Bacterial Cytoplasmic Membrane That May Be Targeted by Alcohol

Description of the main components of the bacterial cytoplasmic membrane damaged by alcohols and the main functions of the bacterial cytoplasmic membrane that alcohols are able to disrupt. References: (Stanier *et al.*, 1986; Kalathenos & Russell, 2003; Winn *et al.*, 2005).

Component	Function
Phospholipid	Structural
Permease	Active transport of nutrients and essential ions into the cell
Biosynthetic enzymes	Mediate synthesis of membrane lipids and cell wall constituents such as peptidoglycan, teichoic acid, polysaccharide and lipopolysaccharide
Electron transport enzymes	Form the electron transport chain, which carries out redox reactions that release energy in the form of ATP as part of oxidative phosphorylation
Membrane potential	Creates energy
Barrier structure	Retains useful compounds such as metabolites and excludes harmful external compounds

Membrane damage can be detected by a number of assays. These are mainly assays that can detect leakage of intracellular materials including K^+, 260 nm-absorbing materials (purines and pyrimidines), pentose, inorganic phosphate, amino acids, proteins and the enzyme β-galactosidase (Denyer & Hugo, 1991; O'Neill *et al.*, 2004). Membrane damage can also be detected by loss of membrane impermeability. This can be demonstrated by a change in ion gradients across the membrane, lysis of protoplasts as a result of influx of previously impermeable ions and water, loss of salt tolerance, penetration of dye into the cytoplasm as measured by fluorescence spectrophotometry (for example using the BacLight assay; O'Neill *et al.*, 2004) and lysis of whole bacterial cells in a hypotonic solution as a measurement of severe membrane damage (Denyer & Hugo, 1991). Structural damage to the cytoplasmic membrane can also be detected by electron microscopy (Kim *et al.*, 2007), although this is a qualitative rather than quantitative approach.

The effect of membrane-active biocides on the leakage of K^+ ions has been previously studied by a number of researchers, using flame photometry with an atomic absorption spectrophotometer (Heipieper *et al.*, 1991; Codling *et al.*, 2003; Walsh *et al.*, 2003), inductively coupled plasma spectrometry (Johnston *et al.*, 2003) and a K^+ selective electrode (Inoue *et al.*, 2004).

5.1.2 The Cell Wall as a Target for AHRs or Barrier to Prevent Their Penetration

In Chapter 4 the time taken for AHRs to have a bactericidal effect on *S. aureus* cells was determined. Since protoplasts are bacterial cells without a cell wall, comparison of the rate of lysis of bacterial cells and protoplast cells will determine whether the cell wall is the primary target of AHR (Xiong *et al.*, 2005). It will also determine whether the cell wall and/or outer layers act as a barrier to AHR penetration. If the rate of protoplast lysis is the same, or similar, as the bactericidal rate found in Chapter 4 then the membrane is the primary target. If lysis of protoplasts is much slower than lysis of bacterial cells, or non-existent, the membrane is not the primary target, but the cell wall or outer layers, since they are the components that are missing from protoplasts (Xiong *et al.*, 2005). However, if lysis of protoplasts is

much quicker than lysis of bacterial cells, then the cell wall or outer layers must act as a barrier to prevent penetration of AHR.

Lysis of protoplasts by biocides has been measured quantitatively by a decrease in OD_{420} (Koo *et al.*, 1997) and culturing on agar containing 20% sucrose (Xiong *et al.*, 2005). Lysis can also be quantified using phase contrast microscopy.

5.1.3 The Effect of AHR on Cell Structural Appearance

Transmission electron microscopy (TEM) enables the physical appearance of the intracellular structure of bacterial cells to be observed (Holt & Beveridge, 1982). TEM results can provide physical evidence of cellular damage, such as cell wall and membrane damage (deQueiroz & Day, 2007). Intracellular damage, or lack thereof, seen during TEM studies may therefore provide suggestions as to the mode of activity of a biocide and point to an area for further study. TEM may also be able to show the variation in the extent and nature of bacterial cell damage resulting from differences in AHR susceptibility among isolates by comparison of their intracellular damage at a range of contact times.

Alcohol is known to damage proteins and lipids and cause cell lysis (McDonnell & Russell, 1999; Ali *et al.*, 2001). Alcohol is therefore likely to cause damage to the cytoplasmic membrane, cell wall, cytoplasm and capsule. Alcohol is known to be active against the cytoplasmic membrane (Franklin & Snow, 1989); therefore cytoplasmic membrane damage should be observed.

The effect of alcohol on the structural appearance of bacterial cells has not previously been studied, although the effect of a range of other biocides has been well studied (Kim *et al.*, 2007; Huang *et al.*, 2008; Oulé *et al.*, 2008). Structural damage to *S. aureus* cells after exposure to antibiotics has been demonstrated using TEM (Thomas *et al.*, 1999).

5.1.4 The Effect of AHR Protein Denaturation

Alcohol is known for its ability to denature proteins (McDonnell & Russell, 1999). Protein denaturation is the loss of a protein's function as a result of structural damage and can be irreversible. Denaturation affects the weak bonds that determine the 3-D structure of the protein (the secondary and tertiary structures), which include hydrogen bonds, salt bridges, disulfide bonds and hydrophobic interactions (Tanford, 1968). Peptide bonds are much stronger and denaturation reactions are therefore unable to break them, resulting in the protein's primary structure remaining intact. Alcohol denatures proteins by breaking side chain intramolecular hydrogen bonds, which are involved in maintaining the tertiary structure of proteins. Hydrogen bonds are then formed between the new alcohol molecule and the protein side chains (Ophardt, 2003), as shown in Figure 5.1.

The carboxyl and amino groups of proteins have different charges, which mean that proteins containing different groups will have different net surface charges (Xia, 2007). The charge of these groups is pH-dependent and they will therefore have different charges at different pH values. Protein solubility is dependent on net surface charge because proteins with a net positive or negative surface charge are more likely to interact with water than other proteins, making them soluble (Rabilloud, 2002). However, at a pH where the net charge is zero (known as the isoelectic point, or pI) proteins are more likely to interact with other proteins and therefore are at their most insoluble, due to the potential of aggregation. The pI of proteins is between pH 3 and pH 12, but for most proteins it is between 4 and 7 (Eidhammer *et al.*, 2008).

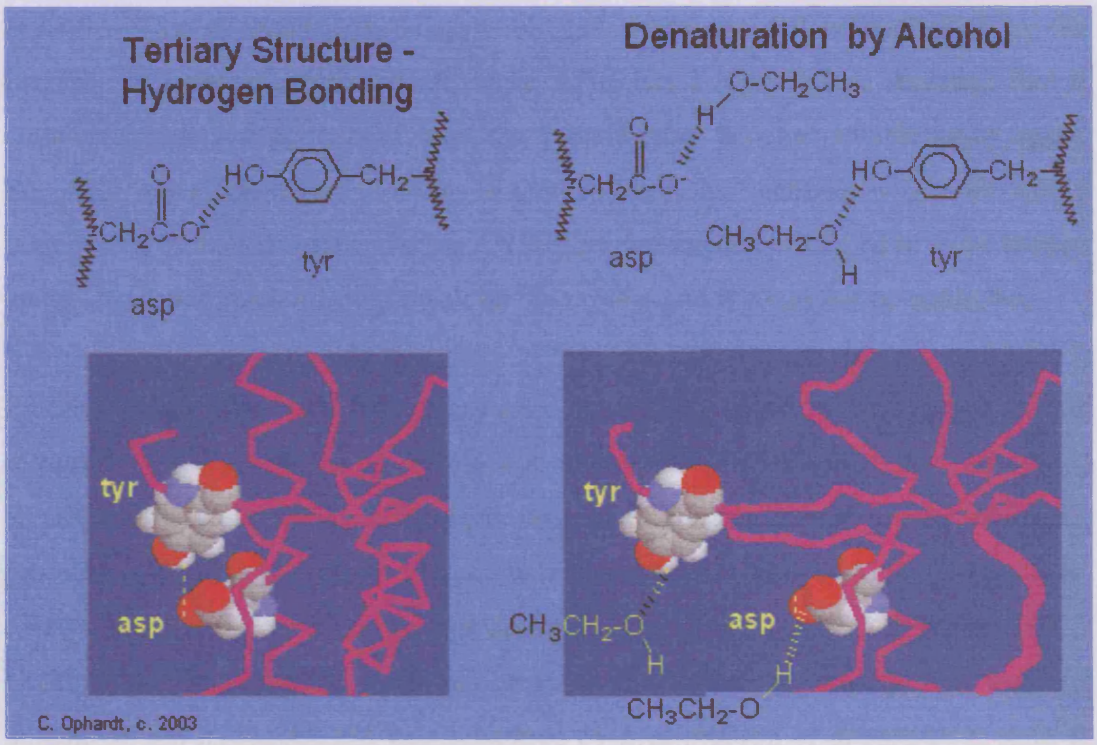

Figure 5.1: Protein Denaturation by Alcohol

Shows disruption of the tertiary structure of proteins by alcohol and the subsequent formation of a new alcohol molecule with the protein side chains. Taken from Ophardt (2003).

Denaturation results in the inner hydrophobic core of the protein being exposed to the hydrophilic environment, which often results in protein aggregating and becoming insoluble (Vojdani, 1996); therefore at the pI a protein in denatured form will be less soluble than in its native form. Protein denaturation can therefore be measured by filtering the solution to remove the aggregated denatured protein and measuring the change in total protein content (Suzuki & Suzuki, 1962) or measuring the change in OD_{280}. These methods would not be appropriate for this study for two reasons. Firstly, changes in OD_{280} could not be determined without washing the bacteria in water to remove AHR, since AHR has a high OD_{280}, meaning that if denaturation is not permanent then the protein may become soluble once again. Secondly, since this study is looking at total cell protein, treatment with AHR would be required on whole cells and after AHR exposure the cell would have to be broken up by sonication, which would break up the protein and it could not be quantified.

Protein denaturation has been previously measured indirectly by comparing the nitrogen content of denatured protein and native protein (Krueger & Nichols, 1935; Hendrix & Dennis, 1938). Nitrogen content is slightly lower among denatured protein, compared to native protein, which is due to the loss of nitrogen-rich compounds and the addition of water during denaturation (Hendrix & Dennis, 1938). Hendrix & Dennis performed this test on individual protein species and the denatured and native protein was separated by precipitation of denatured protein at the isoelectric point. This test would not be appropriate for the purposes of this study, since the aim was to determine the quantity of total cellular protein that is denatured after bacterial cells are exposed to AHR. Bacterial proteins are composed of a number of different species, which would not all have the same isoelectric point and therefore not all denatured protein would be precipitated.

5.1.5 The Effect of AHR on Expression of Virulence Genes

The presence/absence of virulence genes determined in Chapter 3 is not a true indication of the virulence genes used by the isolates under normal growth conditions or upon exposure to AHR, since few genes are consistently expressed and most are only switched on when required (Brown, 2006).

Expression of specific genes can be determined by quantification of RNA, since RNA is transcribed from DNA as part of the process of generating the gene product. RT-PCR involves reverse-transcribing cellular RNA into DNA using reverse transcriptase, followed by amplification of the DNA product using PCR (Brown, 2006). It can be used to measure gene expression comparatively, where the concentration of DNA can be given as a ratio of that of a co-amplified internal consistently expressed housekeeping gene. Quantitative real-time PCR (qPCR) involves quantification of DNA product after each amplification cycle, using fluorescent dyes or probes and comparing results to those of known concentrations of DNA (VanGuilder *et al.*, 2008). Expression of genes can also be performed using microarrays, which detect up- or down-regulation of all genes within the genome (Schena *et al.*, 1995).

Alcohol has previously been shown to alter expression of bacterial genes. The bacterium *Acinetobacter baumannii* is able to metabolise low concentrations of ethanol and the presence of ≤1% AHR has been found to stimulate the secretion of extracellular protein that is possibly involved in increased pathogenicity (Edwards *et al.*, 2007). The presence of low concentrations of ethanol, isopropanol and *n*-propanol (≤6%) was found to increase production of biofilm in a number of clinical isolates of *Staphylococcus epidermidis* (Knobloch *et al.*, 2002).

5.1.6 Aims and Objectives

The aim of this chapter was to determine the effect of AHR on *S. aureus* by investigating the effect of AHR on the cytoplasmic membrane, the cell wall, cell structural appearance, proteins and gene expression.

5.1.7 Hypotheses

5.1.7.1 The Cell Wall as a Target for AHR or Barrier to Prevent Its Penetration

In Chapter 4 it was determined that isolate 44 was the most susceptible and isolate S1 was the least susceptible to AHRs. A likely explanation as to why these isolates have varying susceptibilities is that there are differences in the composition and structure of the cell wall or outer layers between the isolates, which differentially slow the penetration of AHR into the cell. It can be determined whether the variation in susceptibility of these isolates is due to differences in cell wall or outer layer penetration by subjecting isolate 44 and S1 protoplasts to AHR. If they are lysed as rapidly as each other there must be differences in the cell wall or outer layer of these isolates that caused the variation in susceptibility observed in Chapter 4. However, if they are lysed in the same order as they were found to be killed in Chapter 4 it may be that there must be differences in the cytoplasmic membrane or intracellular contents.

5.1.7.2 The Effect of AHR on Expression of Virulence Genes

One hypothesis is that some or all of the virulence genes are up-regulated (more greatly expressed) upon exposure to AHR, meaning that any bacterial cells that survive on the surface of HCWs hands may be more virulent. Another hypothesis is that some or all of the virulence genes will be down-regulated (less expressed) upon exposure to AHR due to the bacteria requiring up-regulation of genes to repair cellular damage.

5.2 MATERIALS AND METHODS

5.2.1 Determining the Effect of AHR on Leakage of Potassium Ions

The method used was based on that used by Walsh *et al.* (2003) and was performed in triplicate for *S. aureus* isolates 44, NCIMB 9518 and S1 with Soft Care Med H5, Cutan and Guest Medical AHRs. The isolates were streaked onto 3 TSA plates and incubated at 37 °C for 18-24 h. Cells were harvested with a sterile cotton swab into 5

ml ultra-high purity (UHP) water and centrifuged at 2,600 *g* for 15 min. Cells were washed twice in UHP water and re-suspended in UHP water to a concentration of 9.90 ± 0.26 $\log_{10}$ CFU/ml according to section 2.5.3.

Positive controls were performed to determine the total K^+ within the cells, where 3 ml of the bacterial suspension was transferred to a fresh vessel and placed in a water bath for 20 min at 80 °C to allow complete breakdown of the bacterial cells. The sample was diluted 1 in 10 in UHP water and passed through a 0.2 μm syringe filter into a plastic universal.

For the test samples 10 ml of the bacterial suspension was transferred to a fresh vessel and centrifuged at 2,600 *g* for 15 min. The supernatant was discarded and the pellet was re-suspended in 10 ml AHR. After time points of 10s, 30s, 1 min, 2 min, 5 min and 10 min 1 ml of suspension was transferred to 9 ml UHP water and passed through a 0.45 μm syringe filter, followed by a 0.2 μm syringe filter into a plastic universal. When this procedure was performed for Cutan AHR the suspension was transferred to 9 ml UHP water containing 3% w/v NaCl to reduce the viscosity of the AHR sufficiently to enable passage through the filters.

Negative controls were performed to determine the leakage of K^+ from cells under the testing conditions. This was performed according to the method for the test samples, except the pellet was re-suspended in UHP water instead of AHR.

The K^+ content of each AHR was also determined. A 1 ml volume of each AHR was added to 9 ml UHP water (UHP water containing 3 % w/v NaCl for Cutan AHR) and passed through a 0.45 syringe filter followed by a 0.2 μm syringe filter into a plastic universal.

The K^+ content of each sample was determined by a technician using an atomic absorption spectrophotometer (Instrumentation Laboratory, Barcelona, Spain). All K^+ samples were provided in ppm and the mean K^+ value in each AHR plus in the positive control were combined to give the maximum (100%) K^+ possible in the

sample. K^+ leakage was calculated as a percentage of the maximum K^+ for the test and negative control samples.

5.2.2 Determining the Role of the Cell Wall as a Barrier to AHR Penetration

5.2.2.1 Materials

Hypertonic buffer consisted of 20% w/v sucrose, 0.05 M Tris-HCl and 0.15 M NaCl, pH 7.6. This was prepared in deionised water and filter sterilized when needed. Lysostaphin was prepared as a 1 mg/ml stock in hypertonic buffer and stored at -20 °C until needed. DNase I was prepared as a 0.5 mg/ml stock in hypertonic buffer and stored at -20 °C until needed.

5.2.2.2 Generation of Protoplasts

This method was based on that by Xiong *et al.* (2005) and was performed for *S. aureus* 44, NCIMB 9518 and S1. Cultures were prepared according to section 2.4.3. The cells were washed from the surface with PBS and pooled together for centrifugation at 2,600 *g* for 15 min. The pellet was re-suspended in hypertonic buffer. A 340 µl volume of lysostaphin stock (final concentration of 68 µg/ml) and 320 µl of DNase I stock (final concentration of 32 µg/ml) was added to the suspension, which was incubated at 37 °C for approximately 4.5 h to allow digestion of the cell wall. Protoplasts were collected by centrifugation at 6,000 *g* for 15 min and re-suspended in fresh digestion buffer. A 10 µl volume was observed under a light microscope at 100 x magnification to confirm the presence of undamaged protoplasts.

5.2.2.3 Determining the Rapidity of Protoplast damage by AHR

A qualitative test was performed with isolate NCIMB 9518 and Soft Care, Cutan and Guest Medical AHRs to determine the effect of AHR over time. This preliminary test was necessary to determine after which contact time to perform quantitative analysis. A 10 µl volume of protoplast suspension prepared according to 5.2.2.2 was added to 990 µl of neat AHR. After gentle mixing with a pipette a 10 µl volume was observed under an Olympus BX50 light microscope (Olympus, Southend-on-Sea,

UK) at 100 x magnification. The entire slide was observed to determine the density of protoplast cells and their integrity, plus presence of debris, for a total of 20 min. This was also performed with a positive control and water control, where 10 µl of protoplast suspension was added to 990 µl of hypertonic buffer and SDW, respectively.

5.2.2.4 Determining the Effect of AHRs on Protoplasts

This method was performed with isolate NCIMB 9518 and Soft Care, Cutan and Guest Medical AHRs to determine whether there is any difference between the effect of each AHR on *S. aureus* protoplasts. Protoplast suspensions prepared according to 5.2.2.2 were adjusted to a concentration so that a 1 in 100 dilution resulted in between approximately 10 and 30 protoplasts per field of vision under a light microscope at 100 x magnification. A 10 µl volume of protoplast suspension was added to 990 µl of either AHR or neat digestion buffer (control). After 20 min a 10 µl volume was observed under a microscope at 100 x magnification and the number of protoplasts in 10 fields of vision was counted. A water control was also performed, where 10 µl of protoplast suspension was added to 990 µl of SDW, to show the effect of a hypotonic solution on protoplasts. Each test was performed in triplicate. Statistical analysis was performed according to 2.8.1 to determine whether there was a significant difference between the total number of protoplasts in 10 fields of vision in the control solution and the AHR and water solutions.

5.2.2.5 Determining the susceptibility of S. aureus Protoplasts to AHR

This method was performed according to 5.2.2.4 with isolates 44 and S1, using Guest Medical AHR only, to determine whether the protoplasts of *S. aureus* isolates shown to be more susceptible to the bactericidal effects of AHR are also more susceptible to AHR. Statistical analysis was performed according to 2.8.1 to determine whether there was a significant difference between the total number of protoplasts in 10 fields of vision in the control solution and the AHR for each isolate.

5.2.3 Determining the Effect of AHR on Bacterial Cell Structural Appearance

5.2.3.1 Materials

All TEM chemicals were prepared with double distilled water. Glutaraldehyde, araldite, dodecyl succinic anhydride and *n*-benzyldimethylamine (BDMA) were purchased from Agar Scientific Ltd (Stansted, UK). Solutions of 1% osmium tetroxide and 2% glutaraldehyde were prepared in 0.1 M Sorensen buffer. Sorensen buffer consisted of 0.2 M monobasic sodium phosphate and 0.2 M dibasic sodium phosphate.

5.2.3.2 Exposure of Isolates to AHR

This procedure was performed for Guest Medical AHR only. Bacterial suspensions of *S. aureus* isolates 44, NCIMB 9518 and S1 were prepared to concentrations of 8.32, 8.32 and 8.31 $\log_{10}$ CFU/ml, respectively, according to section 2.5.1. Four 5 ml volumes of each suspension were transferred to separate centrifuge tubes and centrifuged at 2,600 *g* for 15 min. For pre-exposure testing a pellet was re-suspended in 9 ml TSC buffer. For post-exposure testing three pellets were re-suspended in 1 ml Guest Medical AHR for either 10 s, 1 min or 5 min. After the appropriate contact time 9 ml TSC buffer was added to the post-exposure tubes and left for 5 min neutralization time. All suspensions were washed in TSC buffer, re-suspended in 750 µl TSC buffer and transferred to a microcentrifuge tube.

5.2.3.3 Preparation of Sample

Suspensions prepared according to 5.2.3.2 were washed in 2% glutaraldehyde and left for 1 h in 2% glutaraldehyde to fix. The samples were washed twice in 0.1 M Sorensen buffer and left for 1 h in 1% osmium tetroxide. The pellets were washed three times in Sorensen buffer and the pellet was embedded in 6% agar. The pellets were left for 10 min in progressively increasing concentrations of ethanol to dehydrate the agar for 10 min. The ethanol concentrations used were 50%, 70%, 80%, 90% and 4 steps at 100%. The pellet was then washed in propylene oxide for 10 min and left in resin (15 g araldite CY212, 15 g Dodecyl succinic anhydride and 0.45 g BDMA) overnight at room temperature on a rotating platform. Pellets were removed from the resin, transferred to plastic moulds containing fresh resin and

incubated for 48 h at 60 °C to solidify. Sections were cut from three separate areas of each sample using a Reichert-Jung Ultracut microtome (Wien, Austria) and placed onto separate copper grids. Samples were stained with 2% uranyl acetate for 10 min and Reynolds lead citrate for 5 min, rinsing twice in double distilled water after each step.

5.2.3.4 Analysis of Samples Using Transmission Electron Microscopy

Samples were analysed using an EM 280 transmission electron microscope (Philips, Croydon, UK) at 10,000, 40,000, 50,000 and 63,000 x magnification. At least 10 random fields of vision were observed for each of the three grids for each sample and representative images were taken at 10,000 x magnification from one of the grids. Images taken at 40,000, 50,000 and 63,000 x magnification were taken to highlight particular cellular morphologies and were not necessarily representative.

5.2.4 Determining the Effect of AHR on Expression of Virulence Genes

5.2.4.1 Materials

All RT-PCR reagents were purchased from Promega (Southampton, UK), with the exception of dNTPs, which were purchased from Qiagen Ltd.

5.2.4.2 Reverse Transcription-PCR

This procedure was performed in duplicate for *S. aureus* isolates 44, NCIMB 9518 and S1. RNA was extracted pre-exposure and post-exposure to Soft Care Med H5, Cutan and Guest Medical AHRs according to section 2.10.3. An RNA/primer mix was prepared for each RNA sample on ice in a pre-chilled 0.5 ml PCR tube, as described in Table 5.2. The tubes were placed in a Techgene Thermal Cycler PCR machine for the following cycle: 70 °C for 5 min, 4 °C for 5 min and transferred immediately to ice.

Reverse transcription (RT) was performed for each RNA/primer sample, including a mixture with reverse transcriptase mix (+RT), a mixture without reverse transcriptase (noRT, to ensure the absence of DNA) and a mixture with all the RT components but

no RNA (water control, to ensure the components of the mixture contain no contaminants). The RT, noRT and water controls were prepared in pre-chilled 0.5 ml PCR tubes in volumes sufficient for three RNA samples (Table 5.3). Tubes were kept on ice. A 6.5 µl volume of each of the three RNA samples was transferred to pre-chilled 0.5 ml PCR tubes containing 19.5 µl of the +RT mix and to pre-chilled PCR tubes containing 19.5 µl of the noRT mix. One further pre-chilled PCR tube was set up containing 19.5 µl of the water control mix with 6.5 µl of nuclease-free water. Tubes were placed in a Techgene Thermal Cycler PCR machine and run on the following cycle: 25 °C for 5 min, 42 °C for 60 min, 70 °C for 15 min and stored at -20 °C until needed.

PCR was performed on the RT samples to amplify the DNA products. PCR mixture (in 23µl volumes; prepared according to Table 3.6, Chapter 3) was added to 0.5 ml PCR tubes containing 2 µl of each RT sample. A negative control was performed with 23 µl of PCR mixture only. PCR was performed for each RT sample with the primers for each virulence gene that was determined to be present in each isolate in section 3.2.4, plus a housekeeping gene (see Table 5.4). The *GlyA* gene is a housekeeping gene that encodes serine/glycine hydroxymethlyl transferase A, which is involved in amino acid catabolism (Theis *et al.*, 2007). This gene is likely to be expressed at a consistent level pre- and post-exposure to AHR and was therefore used in this study as a control gene. Samples were transferred to a Techgene Thermal Cycler PCR machine and run on the following cycle: 94 °C for 3 min; 35 cycles of: 94 °C for 1 min, the optimum annealing temperature (T_a) specific for the primer pair (see Table 5.4) for 1 min, 72 °C for 2 min; 72 °C for 3 min. The T_a of each forward and reverse primer was calculated as follows:

$T_a = T_m - 5$ $\quad$ $T_m = 2\ °C \times (A+T) + 4\ °C \times (C+G)$

Table 5.2: RNA/Primer Mix

Volume of RNA/primer mix required for RT-PCR of one RNA sample

Component	Volume
Random primer	1.5 µl
RNA	3 µg
Nuclease-free water	To a final volume of 15 µl

Table 5.3: Reverse Transcription Mix

Volume of each mixture required to perform RT on three RNA samples. RT mixtures included a mixture with reverse transcriptase (+RT), a mixture with no reverse transcriptase (noRT) and a mixture with no RNA (water control)

Component	+RT (µl)	noRT (µl)	Water Control (µl)
Nuclease-free water	28.6	33.3	22.9
5 x reaction buffer	18.8	18.8	15
25 mM $MgCl_2$	11.3	11.3	9
10 mM dNTP	4.69	4.69	3.75
RNasin	2.35	2.35	1.88
Reverse transcriptase	4.69	0	3.75

T_m = melting temperature of the primer, which was provided by Invitrogen. Adenine (A), Thymine (T), Cytosine (C) and Guanine (G) are the nucleotides in the primer sequences. The T_a of each primer pair was determined, which was the same as for each forward and reverse primer if the T_a values were the same. Where the T_a was different for the forward and reverse primers the temperature belonging to the shortest primer was chosen and if the primers were the same length the highest temperature was chosen. The T_a must be between 50-68 °C; therefore if the calculated T_a fell out of this range it was adjusted to fall within it. Primer sequences for virulence genes are provided in Tables 3.4 and 3.5 (Chapter 3) and those for the housekeeping gene are provided in Table 5.5.

Electrophoresis was performed according to section 2.11. Where banding was found to be inconsistent between the two replicates then the procedure was carried out a third time, with a fresh RNA extraction. If the third replicate result matched that of either of the two previous replicates then that result was taken to be the true result.

Table 5.4: Genes Amplified for PCR

Genes found to be present (+) in *S. aureus* isolates 44, NCIMB 9518 and S1 during screening in section 3.2.4 (Chapter 3) plus a housekeeping gene. The optimum annealing temperature (T_a) is provided for each forward (F) and reverse (R) primer pair. * = housekeeping gene

Gene	Isolate 44	Isolate NCIMB 9518	Isolate S1	Optimum T_a of F and R Primers (°C)
hysA	+	+	+	67
fnbA	+	+	+	55
fnbB	+	+	-	65
hlgA	+	+	+	59
hlgCB	+	+	+	55
cap5	-	+	+	67
cap8	+	+	+	57
icaA	+	+	+	57
icaD	+	+	+	57
clfA	+	+	+	51
*glyA**	+	+	+	50

Table 5.5: Housekeeping Gene Primers

Details of forward (F) and reverse (R) primer sequences for the housekeeping gene *glyA*, their product's length and their origin.

Gene	5' to 3' Primer Sequence	Product Length (bp)	Reference
glyA	F-CTACAAACTCACAGCCAC R-GTATCGGAAGCGGTTATG	98	Theis *et al.* (2007)

5.3 RESULTS

5.3.1 Determining the Effect of AHR on Leakage of Potassium Ions

Data showing the mean % K^+ leakage for *S. aureus* isolates 44, NCIMB 9518 and S1 after exposure to Soft Care Med H5, Cutan and Guest Medical AHRs are provided in Table 5.6 and are summarised in Figure 5.2. Leakage of K^+ was highest from cells of isolate 44, followed by NCIMB 9518 and was lowest from isolate S1 after exposure to Cutan. However, leakage of K^+ was highest from cells of isolate NCIMB 9518 and of a similarly lower percentage from isolates 44 and S1 after exposure to Soft Care Med H5 and Guest Medical. Overall, leakage of K^+ from the isolates was highest after exposure to Cutan, and of a similarly lower percentage after exposure to Soft Care Med H5 and Guest Medical.

It is difficult to compare K^+ leakage from the different isolates using the different AHRs due to the large SD that occurred under most conditions (Table 5.6). The mean K^+ leakage had a tendency to increase sharply by a contact time of 10 s, reach a peak by either 30 s or 1 min and decrease. By a contact time of 10 min the percentage of K^+ leakage had levelled off, with the exception of isolate 44 after exposure to Cutan and isolates NCIMB 9418 and S1 after exposure to Soft Care Med H5, which was still increasing, plus isolate NCIMB 9518 after exposure to Guest Medical, which was still decreasing.

The effect of each AHR on K^+ leakage was compared with its bactericidal effect to determine whether change in membrane permeability provides a major contribution to cell death. Table 5.7 compares K^+ leakage with bactericidal activity and shows that K^+ leakage had reached a peak prior to a bactericidal effect being achieved for all isolates under all conditions ($\geq$ 4 log reduction), with the exception of isolate 44 after exposure to Soft Care Med H5. Table 5.7 also shows that K^+ leakage was below 50% under most conditions by the time a bactericidal effect was achieved. This indicates that membrane damage was not a major contributor to the bactericidal activity of AHR.

Table 5.6: Percentage of K+ Leakage from *S. aureus* Isolates After Exposure to AHR

Mean percentage leakage ± SD of K^+ from *S. aureus* isolates 44, NCIMB 9518 and S1 after exposure to Soft Care Med H5, Cutan and Guest Medical AHRs.

Contact Time	Isolate 44			Isolate NCIMB 9518			Isolate S1		
	Soft Care Med H5	Cutan	Guest Medical	Soft Care Med H5	Cutan	Guest Medical	Soft Care Med H5	Cutan	Guest Medical
10 s	19.65 ±2.48	58.99±8.46	19.26±2.28	23.58±16.70	33.18±12.67	31.08±5.87	20.08±4.97	4.51±1.80	18.93±9.62
30 s	21.15±2.19	64.81±19.07	19.78±3.10	26.82±19.08	62.72±14.91	37.61±9.94	20.62±4.48	4.61±0.82	18.88±7.39
1 min	22.94±1.35	69.51±24.30	20.85±1.45	30.10±19.82	51.53±9.78	33.48±6.94	23.30±7.66	5.55±0.50	23.16±10.33
2 min	25.48±2.66	71.58±35.09	19.99±2.51	27.13±15.93	55.92±7.00	32.78±6.73	21.69±3.08	4.29±1.51	19.68±13.01
5 min	27.15±1.24	72.13±30.63	25.70±5.21	28.23±19.91	54.39±7.03	56.03±20.45	15.87±8.35	5.79±2.37	24.59±13.03
10 min	26.17±1.83	75.48±36.06	23.38±2.43	34.88±28.99	53.63±7.93	32.25±2.97	33.08±19.91	5.76±1.73	23.50±9.86

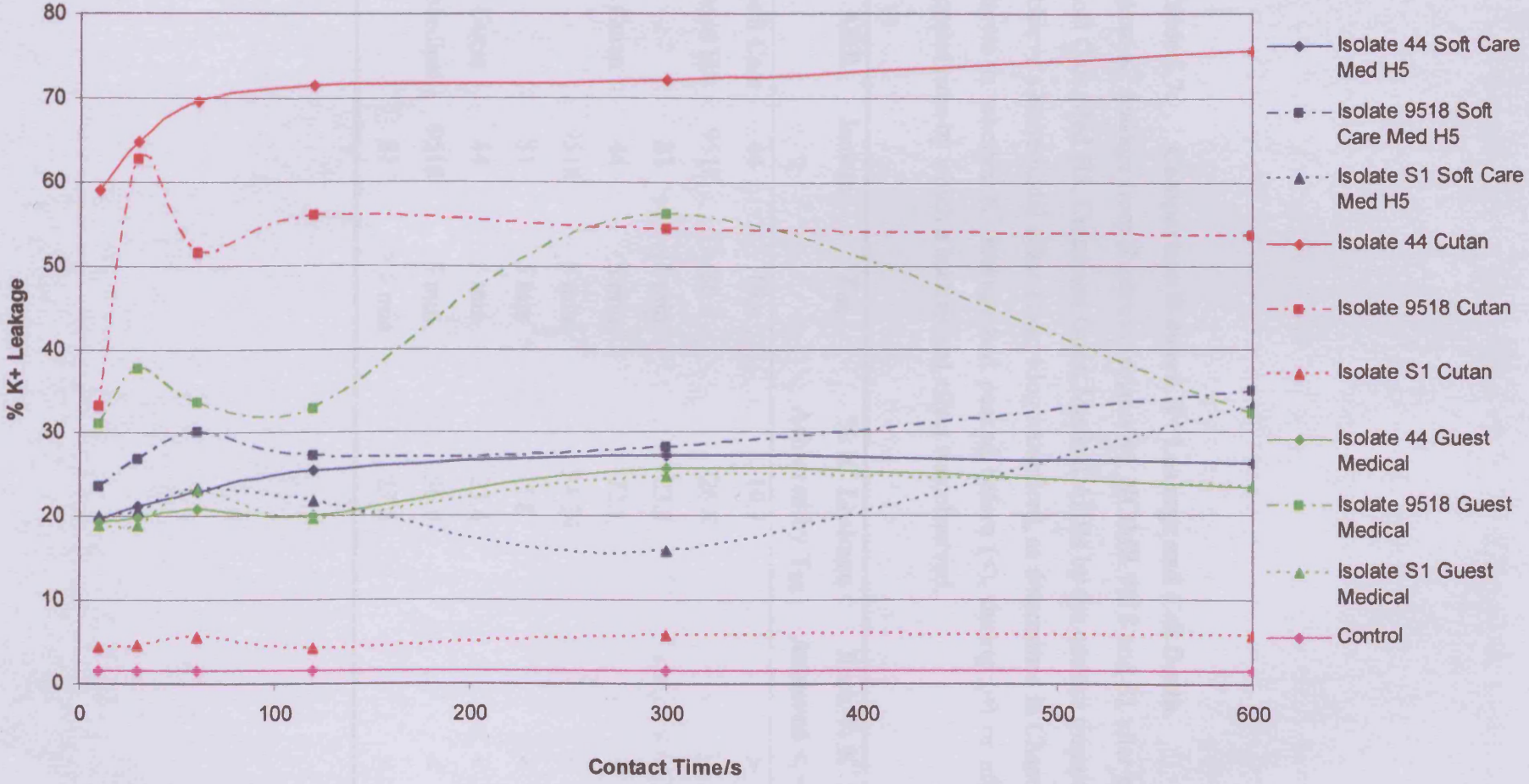

Figure 5.2: Percentage K+ Leakage from *S. aureus* Isolates After Exposure to AHR

Mean percentage K^+ leakage from *S. aureus* isolates 44, NCIMB 9518 and S1 after exposure to Soft Care Med H5, Cutan and Guest Medical

Table 5.7: Comparison Between K+ Leakage and Cell Death

Mean K^+ leakage from *S. aureus* isolates 44, NCIMB 9518 and S1 after exposure to Soft Care Med H5, Cutan and Guest Medical AHRs by the contact times required to achieve a bactericidal effect (T_{BE}; 4 log reduction), as determined in Chapter 4. Also shown is whether K^+ leakage had peaked before (<), during (=) or after (>) the contact time by which a bactericidal effect was observed.

AHR	Isolate	T_{BE}	% K^+ Leakage Achieved by T_{BE}	Peak % K^+ Leakage Achieved <, = or > T_{BE}
Soft Care	44	10 s	19.7	>
Med H5	9518	30 s	26.8	<
	S1	1 min	23.3	<
Cutan	44	5 min	72.1	<
	9518	5 min	54.34	<
	S1	5 min	5.8	<
Guest	44	5 min	23.4	<
Medical	9518	5 min	56.0	<
	S1	> 5 min	23.5	<

5.3.2 Determining the Role of the Cell Wall as a Barrier to AHR Penetration

5.3.2.1 Determining the Rapidity of Protoplast damage by AHR

Figure 5.3A shows an image of protoplasts prior to treatment with AHR. During the first 5 min of exposure of protoplast cells to Soft Care Med H5, Cutan and Guest Medical AHR there was no observable difference in the density of the suspension or the appearance of cells. After a contact time of 5 min a very small number of protoplasts (only a few on the entire slide) had a change in appearance, where the edge (Figure 5.3C) and/or surface (Figure 5.3D) had become damaged. At a contact time of 10 min a larger number of protoplasts had the damaged appearance of those in Figure 5.3C and 5.3D, plus a small number of cells had lost their integrity and the intracellular contents had leaked out (Figure 5.3E). The number of damaged and broken cells had increased by a contact time of 20 min and 25 min, but still the vast majority of cells appeared healthy and undamaged, like those in Figure 5.3A. Protoplasts in the water control lysed very quickly, whereby the number of cells present reduced at a fast rate and there was a large volume of debris present. By a contact time of only 5 min a large portion of cells present appeared damaged or lysed and a large proportion of the remaining cells had swollen to a very large size (Figure 5.3B), due to the hypotonicity of the water. By 10 min most of the cells had lysed and by 20 min a very small number were left intact.

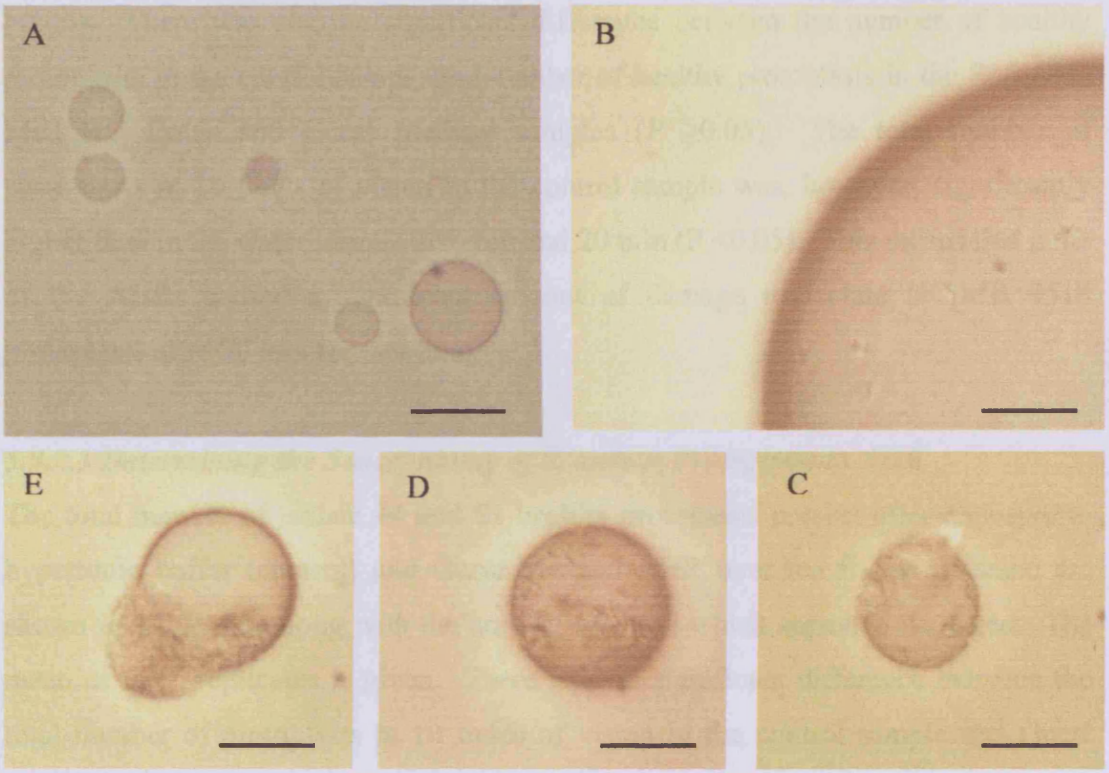

Figure 5.3: Protoplast Appearance pre- and post- Exposure to AHR

Light microscope images of *S. aureus* NCIMB 9518 protoplasts. Scale bar = 120 μm. A = pre-treatment, B = water control, C & D = following 5 min exposure to Guest Medical AHR, E = following 10 min exposure to Guest Medical AHR.

5.3.2.2 Determining the Effect of AHRs on Protoplasts

The total number of NCIMB 9518 healthy protoplasts present after exposure to hypertonic buffer (control), Soft Care Med H5, Cutan, Guest Medical and water over ten fields of vision are shown in Table 5.8, along with the number of which appeared damaged. The mean of three replicates is given. There was no significant difference between the total number of protoplasts in 10 fields of vision in the control sample and Soft Care Med H5, Cutan or Guest Medical samples for isolate NCIMB 9518 ($P \geq 0.05$). There was also no significant difference between the number of healthy protoplasts in the control sample and number of healthy protoplasts in the Soft Care Med H5, Cutan and Guest Medical samples ($P \geq 0.05$). The total number of protoplasts in 10 fields of vision in the control sample was, however, significantly higher than in the water sample at 5 min and 20 min ($P < 0.05$). This means that none of the AHRs caused a significant amount of damage to isolate NCIMB 9518 protoplasts after 20 min contact time.

5.3.2.3 Determining the Susceptibility of S. aureus Protoplasts to AHR

The total number of isolate 44 and S1 healthy protoplasts present after exposure to hypertonic buffer (control) and Guest Medical AHR over ten fields of vision are shown in Table 5.9, along with the number of those which appeared damaged. The mean of three replicates is given. There was no significant difference between the total number of protoplasts in 10 fields of vision in the control sample and Guest Medical samples for either isolate 44 or isolate S1 ($P \geq 0.05$). There was also no significant difference between the number of protoplasts in the control sample and number of healthy protoplasts in the Guest Medical samples for either isolate 44 or isolate S1 ($P \geq 0.05$). This means that Guest Medical AHR did not cause significant damage to either of the isolates after 20 min contact time.

Table 5.8: Number of *S. aureus* NCIMB 9518 Protoplasts Pre- and Post-Exposure to AHRs

The mean of three replicates is shown of the total number of healthy and damaged protoplasts of *S. aureus* NCIMB 9518 over ten fields of vision at 100 x magnification ± SD for the control (in hypertonic buffer) and after exposure to AHR and water. * Presence of cell debris.

Condition	Mean Number of Healthy Protoplasts	Mean Number of Damaged Protoplasts
Control	204.33 ± 12.42	0
Soft Care Med H5	196.33 ± 34.93	2.67 ± 1.53
Cutan	190.00 ± 19.00	3.00 ± 1.00
Guest Medical	185.67 ± 39.27	1.33 ± 1.53
Water 5 min	21.33 ± 5.03	0*
Water 20 min	9.33 ± 2.52	0*

Table 5.9: Number of Protoplasts Pre- and Post- Exposure to AHR

The mean of three replicates is shown of the total number of healthy and damaged protoplasts of *S. aureus* isolates 44 and S1 over ten fields of vision at 100 x magnification ± SD after exposure to a hypertonic buffer (control) and Guest Medical AHR.

Condition	Mean Number of Protoplasts	Mean Number of Damaged Protoplasts
Isolate 44 Control	184.67 ± 34.53	0
Isolate S1 Control	216.67 ± 13.05	0
Isolate 44 Guest Medical	185.00 ± 16.37	4.00 ± 2.65
Isolate S1 Guest Medical	242.00 ± 32.23	2.33 ± 1.53

5.3.3 Determining the Effect of AHR on Bacterial Cell Structure

Transmission Electron micrographs of *S. aureus* isolate 44, NCIMB 9518 and S1 are shown pre- and post-treatment with Guest Medical AHR in Figures 5.4 to 5.12. An unidentifiable extracellular material (UEM) was observed in all organisms pre- and post-exposure to AHR. This was in relatively small quantities pre-exposure and for isolate S1 post-exposure to AHR. Slightly larger quantities were observed for isolate NCIMB 9518 after 1 min and 5 min exposure to AHR, whereas quantities were greatly increased for isolate 44 after 1 min and 5 min exposure. This UEM is likely to be material from the bacterial surface that has come away from the bacterial cell wall, possibly during EM preparation, which may come away more easily when damaged.

There was no observable difference in the appearance of cells after 10 s exposure of isolate 44 to Guest Medical AHR (pictures not shown), compared to those pre-treatment (Figure 5.4). However, after 1 min exposure (Figure 5.5) areas of the cell wall of a large proportion of cells had become thicker and fuzzy. This may have been due to the cell wall being damaged, possibly by protein denaturation. The membrane of a large proportion of cells had also been damaged, which was indicated by a lack of integrity. Figure 5.5b also shows that after 1 min exposure the intracellular contents had leaked from one cell, but this was not a common occurrence. After 5 min exposure (Figure 5.6) a larger proportion of cells demonstrated the same cell wall and membrane damage seen in Figure 5.5, plus a small number of cells showed a change in the appearance of their cytoplasm, probably the result of coagulation of the cytoplasm by alcohol.

S. aureus NCIMB 9518 cells appeared to be less healthy pre-treatment (Figure 5.7) than isolate 44, due to the appearance of some membrane damage that may be the result of TEM preparation. There was no observable difference in the appearance of bacterial cells after 10 s exposure of isolate NCIMB 9518 to Guest Medical AHR (pictures not shown). However, by 1 min exposure (Figure 5.8) a number of cells had a large quantity of UEM that was either attached to the cell or forming a circle around the cell. It was different in appearance to, and in addition to, the UEM that was found in all the samples. Figure 5.8 also shows cell wall and cytoplasmic

membrane damage had occurred by 1 min exposure, although this was not as extensive as in isolate 44. By 5 min (Figure 5.9) cell wall and cell membrane damage occurred in a large proportion of cells, although not as commonly as in isolate 44. After 5 min leakage of extracellular material was also observed, although this was not common.

Isolate S1 pre-treatment cells appeared healthy (Figure 5.10). Unlike isolates 44 and NCIMB 9518, isolate S1 showed membrane and cell wall damage in a number of cells after 10 s exposure to Guest Medical AHR (Figure 5.11). This was observed in a larger proportion of cells after a 1 min contact time (images not shown) and after 5 min contact time damage was more extensive, including the presence of ghost cells (Figure 5.12).

Guest Medical therefore had the greatest effect on the physical appearance of cells of isolate 44 followed by isolate S1, with the least effect on cells from isolate NCIMB 9518.

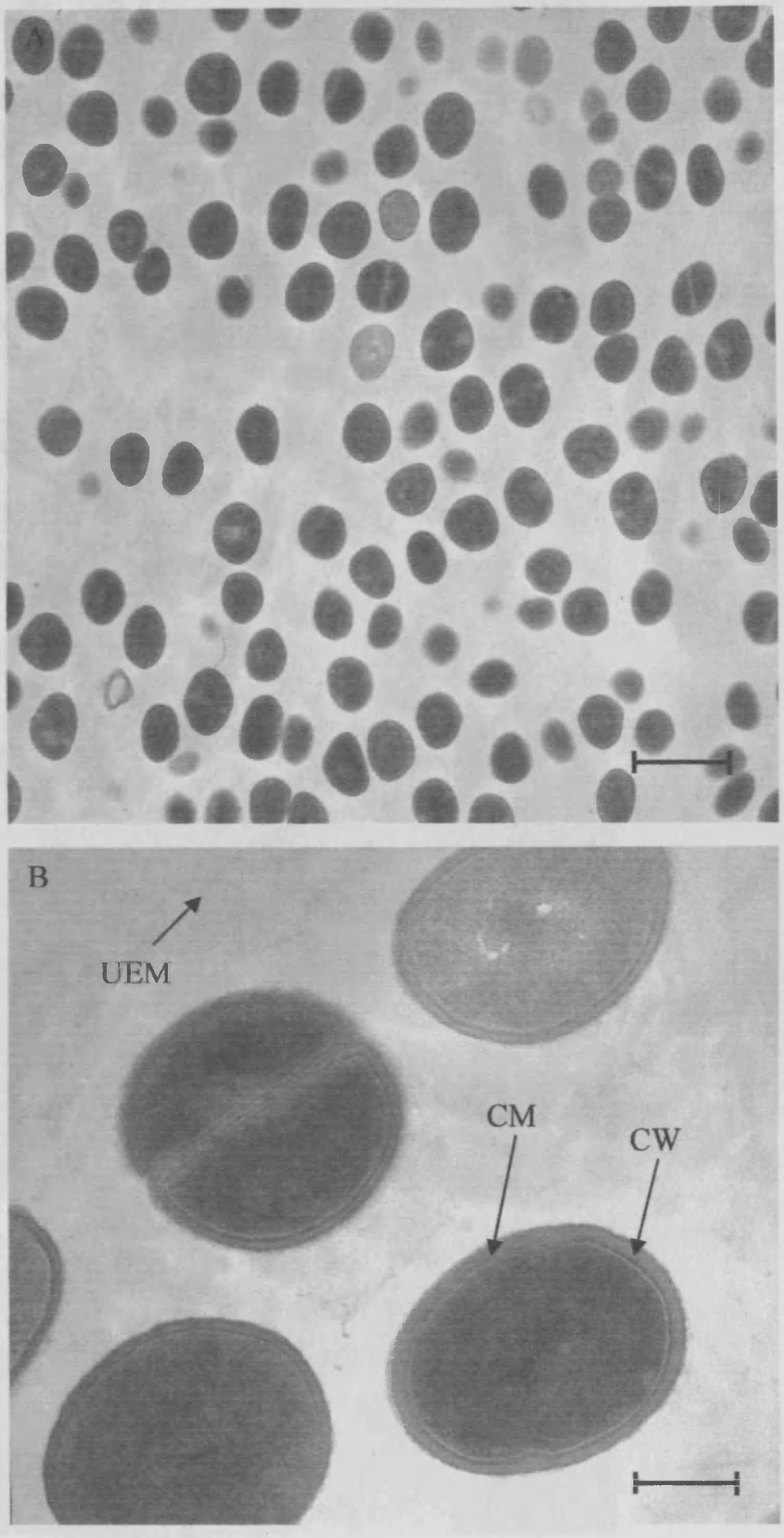

Figure 5.4: TEM image of *S. aureus* isolate 44 pre-treatment

A = 10,000 x magnification, scale bar = 950 nm, B = 50,000 x magnification, scale bar = 187 nm. CM = cytoplasmic membrane, CW = cell wall; UEM = unidentifiable extracellular material

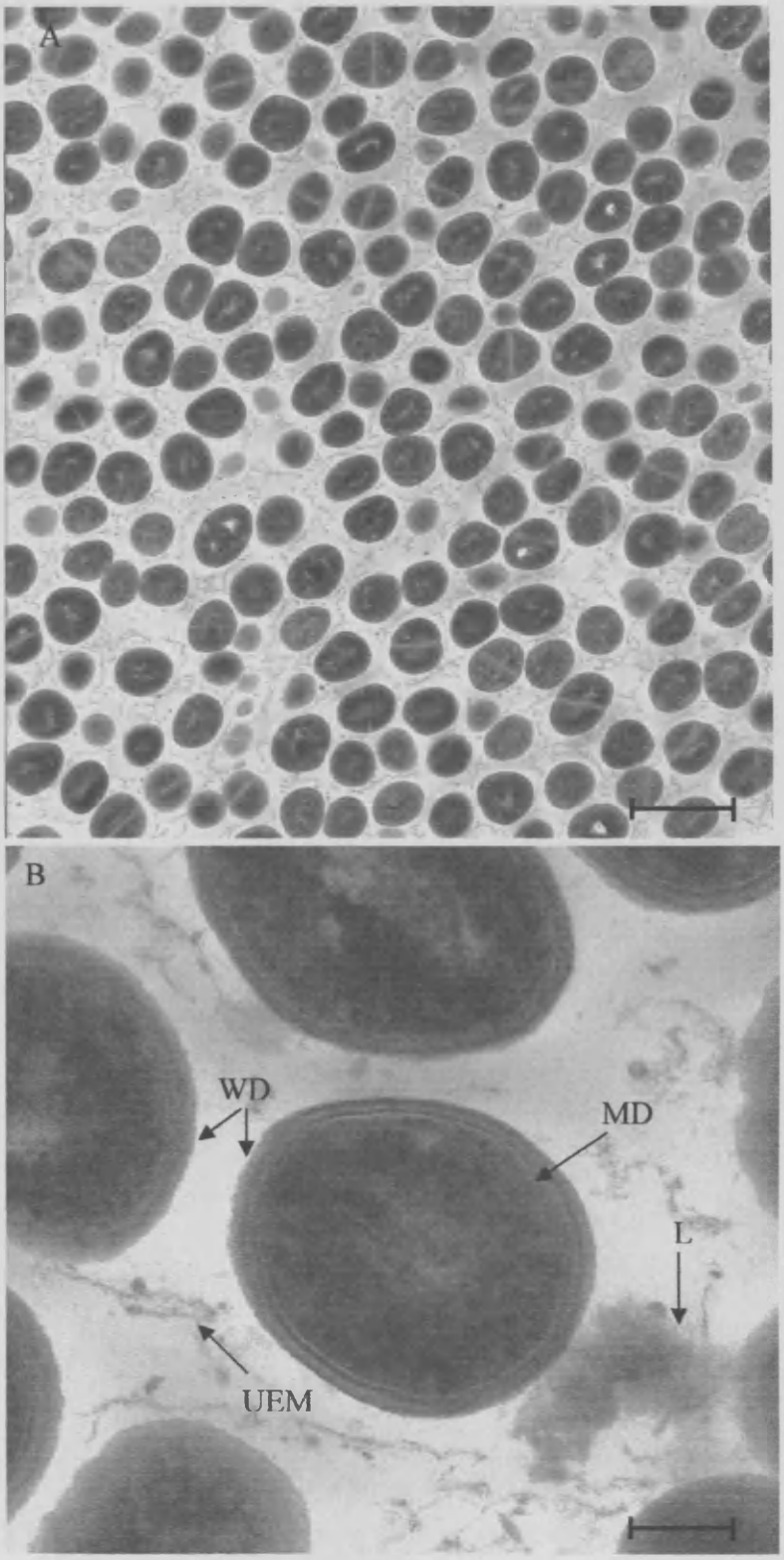

Figure 5.5: TEM image of *S. aureus* isolate 44 after 1 min exposure to Guest Medical AHR

A = 10,000 x magnification, scale bar = 950 nm, B = 63,000 x magnification, scale bar = 157 nm. L = leakage of intracellular material, MD = cytoplasmic membrane damage, WD = cell wall damage, UEM = unidentifiable extracellular material

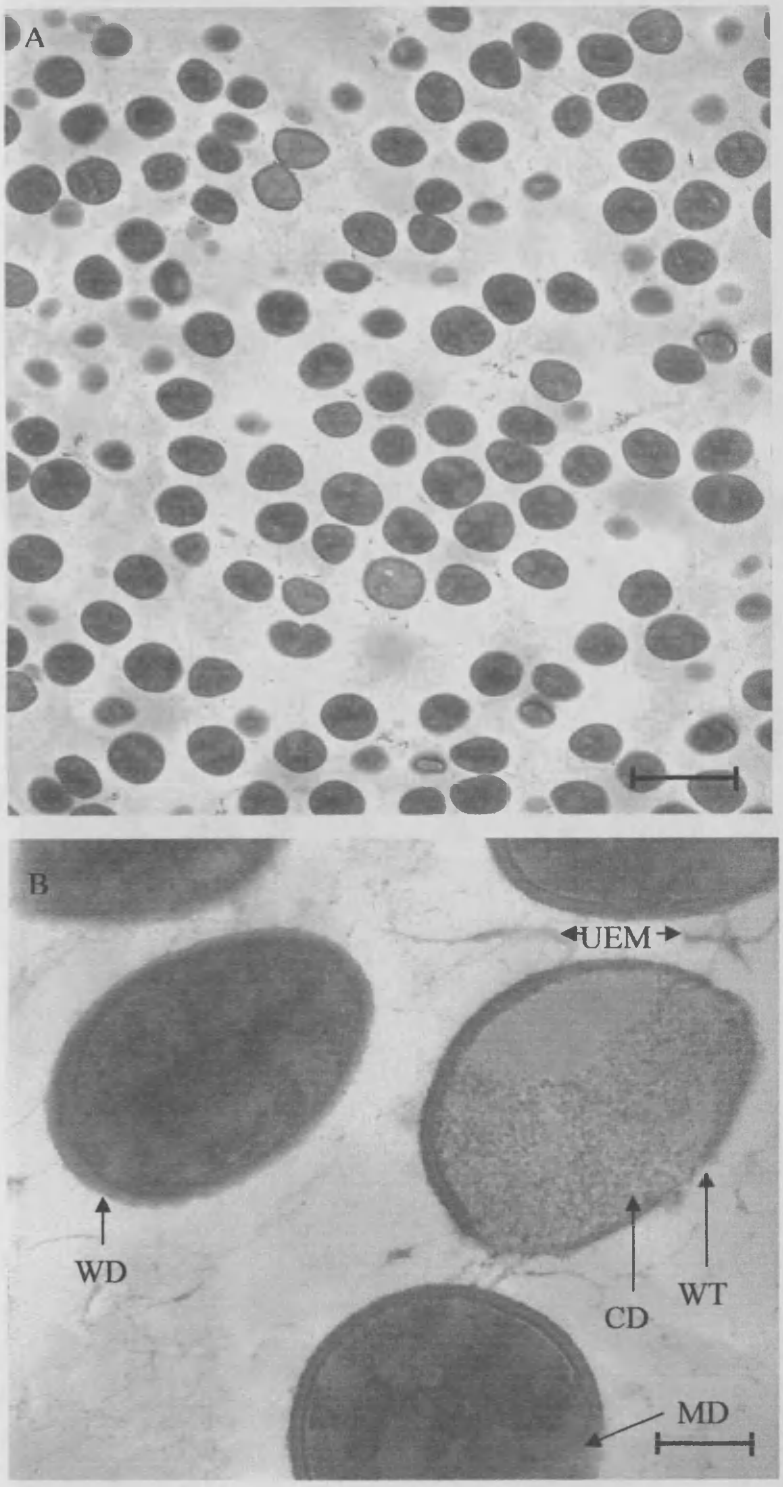

Figure 5.6: TEM image of *S. aureus* isolate 44 after 5 min exposure to Guest Medical AHR

A = 10,000 x magnification, scale bar = 950 nm, B = 50,000 x magnification, scale bar = 187 nm. CD = cytoplasm damage, WD = cell wall damage, MD = cytoplasmic membrane damage, UEM = unidentifiable extracellular material, WT = cell wall thinning.

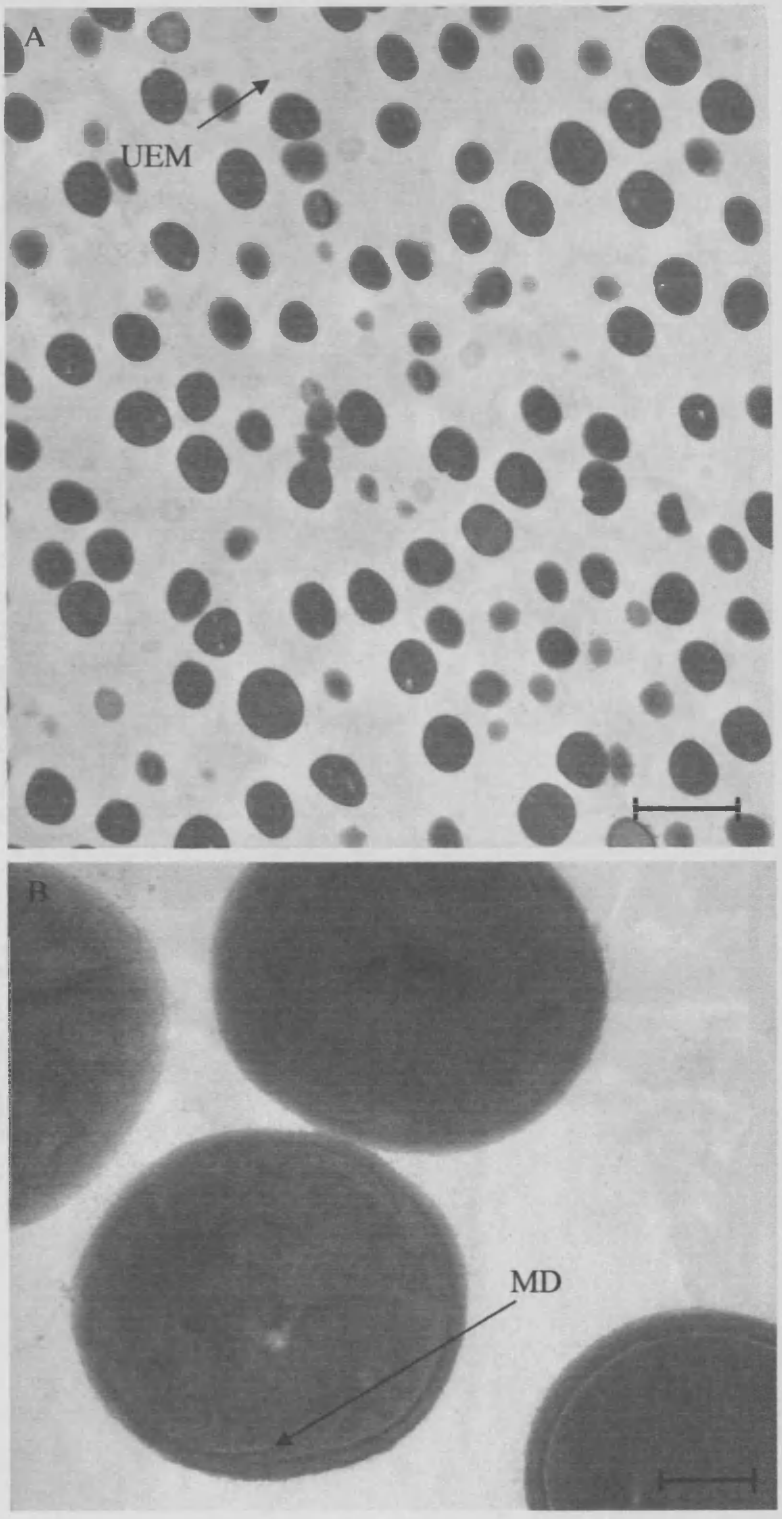

Figure 5.7: TEM image of *S. aureus* isolate NCIMB 9518 pre-treatment

A = 10,000 x magnification, scale bar = 950 nm, B = 63,000 x magnification, scale bar = 157 nm. MD = membrane damage. UEM = unidentifiable extracellular material.

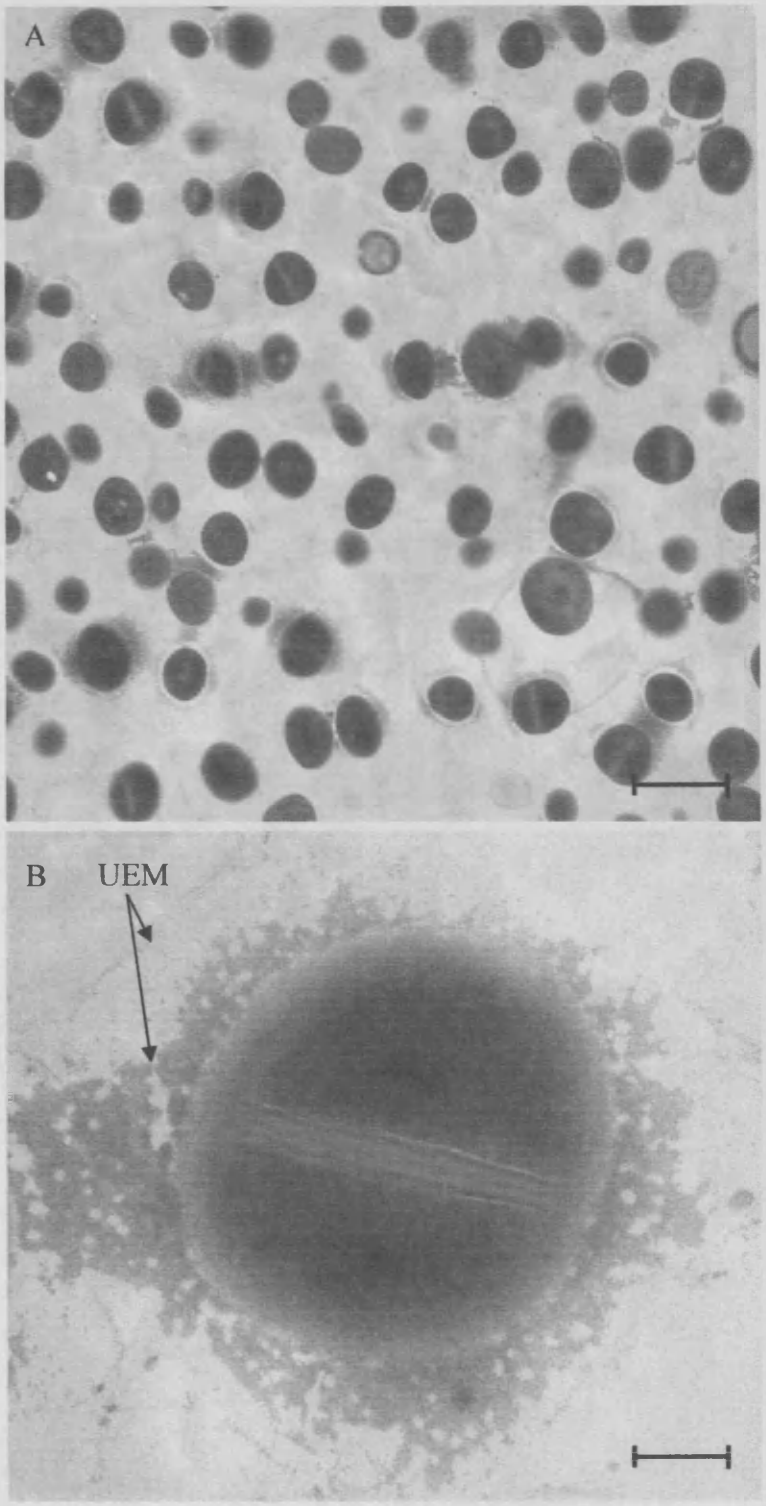

Figure 5.8: TEM image of *S. aureus* isolate NCIMB 9518 after 1 min exposure to Guest Medical AHR

A = 10,000 x magnification, scale bar = 950 nm, B = 63,000 x magnification, scale bar = 157 nm. UEM = unidentifiable extracellular material.

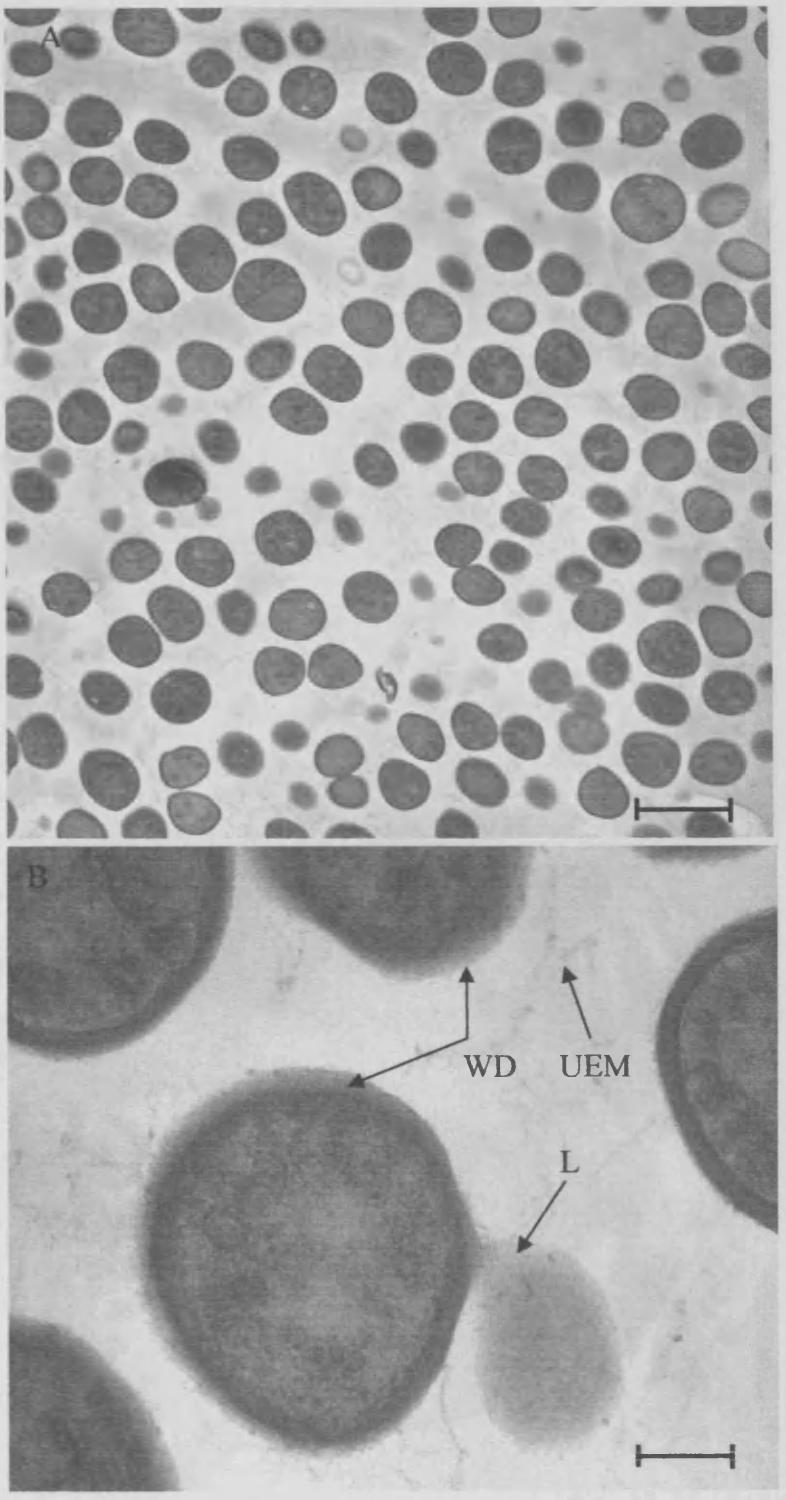

Figure 5.9: TEM image of *S. aureus* isolate NCIMB 9518 after 5 min exposure to Guest Medical AHR

A = 10,000 x magnification, scale bar = 950 nm, B = 63,000 x magnification, scale bar = 157 nm. WD = cell wall damage, L = leakage of intracellular material, UEM = unidentifiable extracellular material

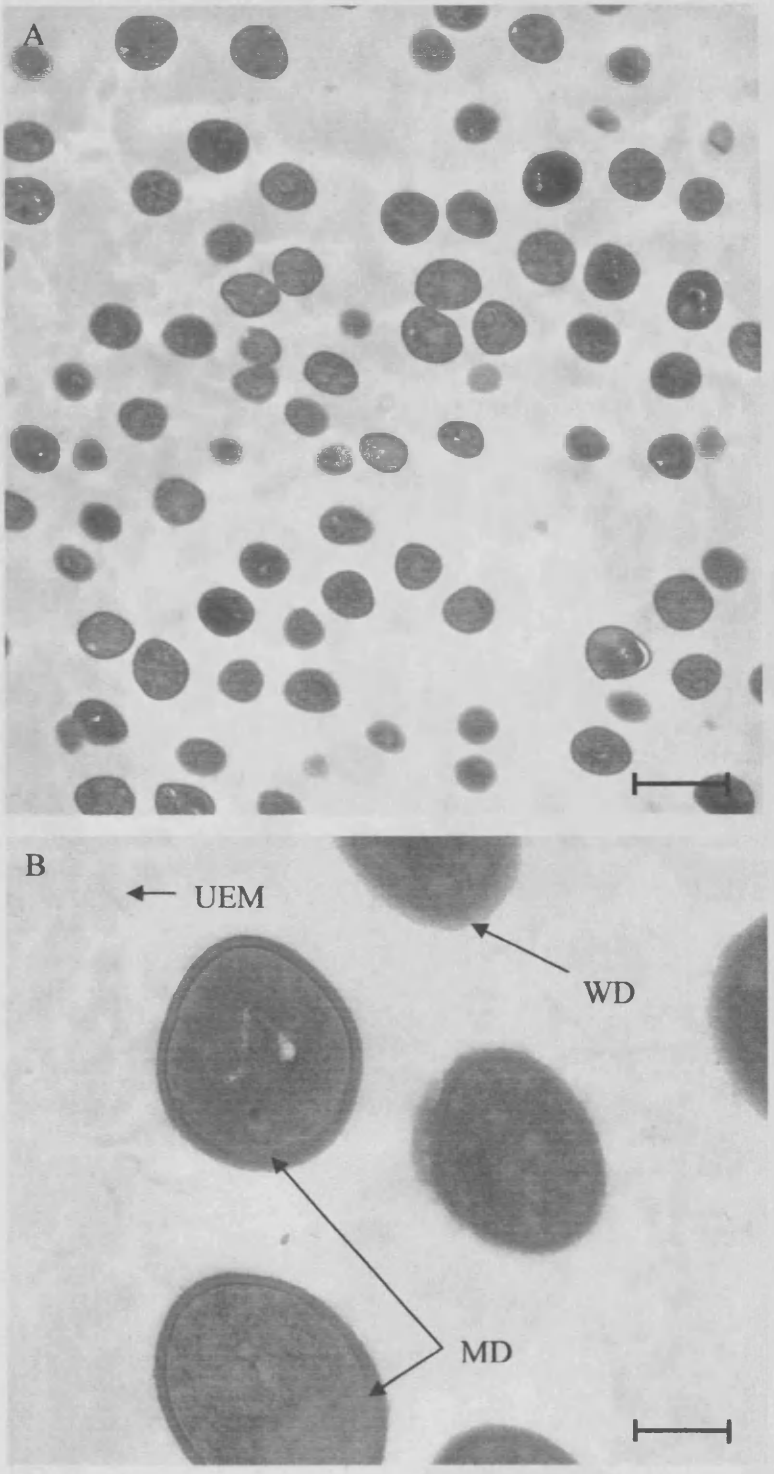

Figure 5.11: TEM image of *S. aureus* isolate S1 after 10 s exposure to Guest Medical AHR

A = 10,000 x magnification, scale bar = 950 nm, B = 40,000 x magnification, scale bar = 130 nm. MD = cytoplasmic membrane damage, WD = cell wall damage. UEM = unidentifiable extracellular material.

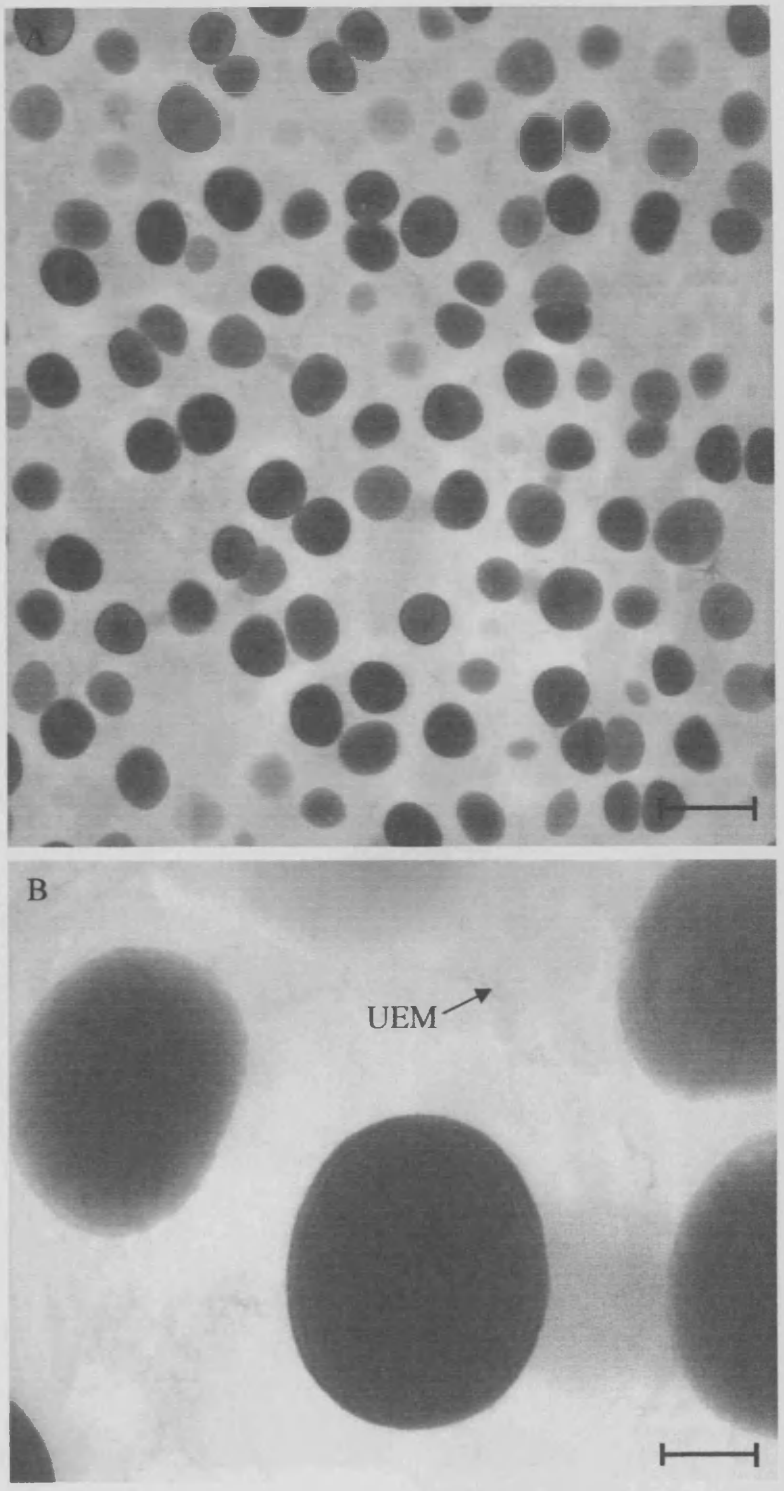

Figure 5.10: TEM image of *S. aureus* isolate S1 pre-treatment

A = 10,000 x magnification, scale bar = 950 nm, B = 50,000 x magnification, scale bar = 187 nm. UEM = unidentifiable extracellular material.

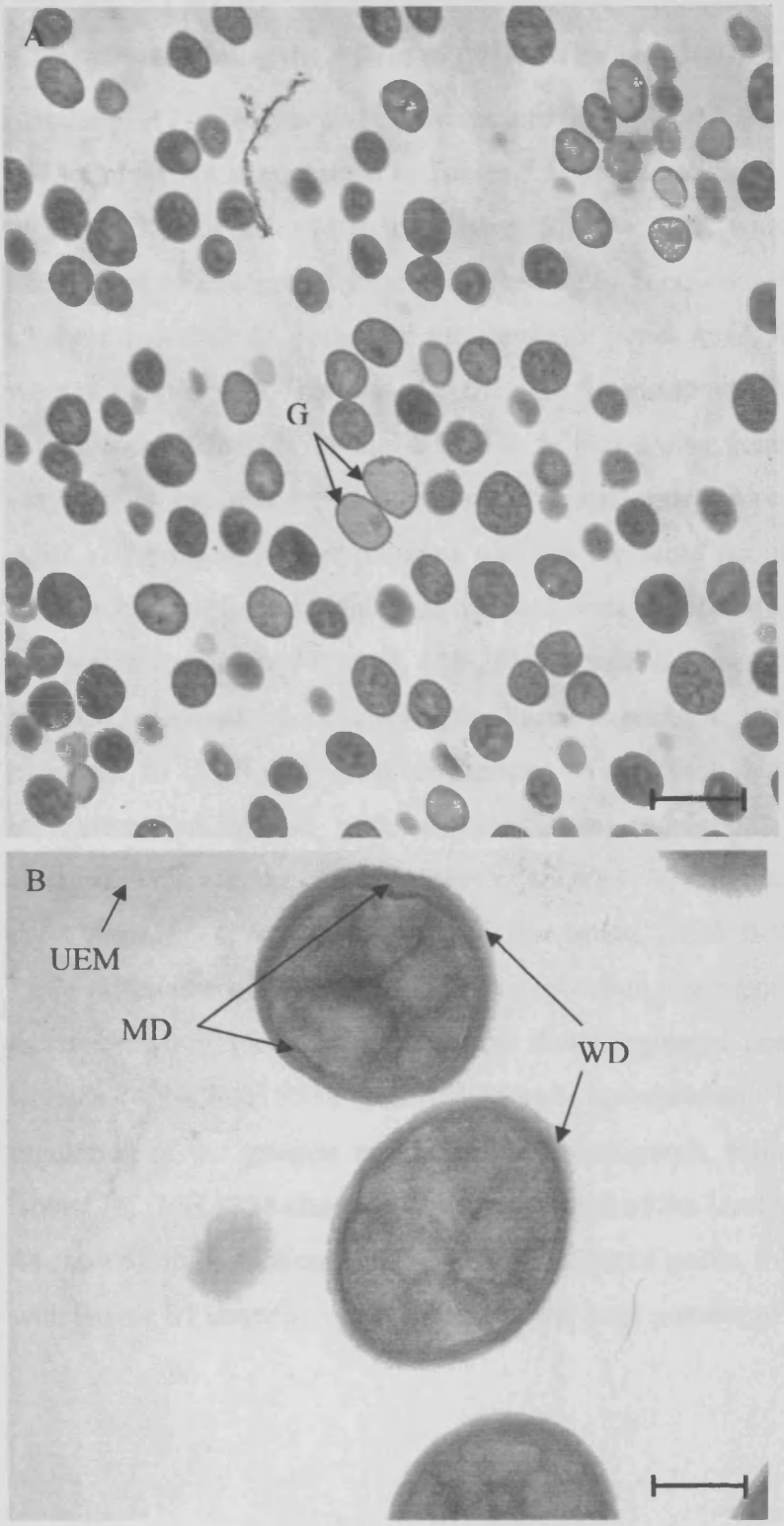

Figure 5.12: TEM image of *S. aureus* isolate S1 after 5 min exposure to Guest Medical AHR

A = 10,000 x magnification, scale bar = 950 nm, B = 50,000 x magnification, scale bar = 187 nm. G = ghost cell, MD = membrane damage, WD = cell wall damage. UEM = unidentifiable extracellular material.

5.3.4 Determining the Effect of AHR on Expression of Virulence Genes

Results of RT-PCR pre- and post-exposure to AHR for *S. aureus* isolates 44, NCIMB 9518 and S1 are summarised in Tables 5.10, 5.11 and 5.12, respectively. Examples of RT-PCR gels are shown in Figures 5.13 to 5.15, which also demonstrate the assignment of expression strength according to band intensity. As demonstrated in Chapter 3, isolate 44 possessed the virulence genes *hysA*, *fnbA* and *fnbB*, but these were not expressed. This was also the case for virulence genes *hysA*, *cap5* and *fnbA* for isolates NCIMB 9518 and S1. The housekeeping gene *glyA* was, as expected, very highly expressed and its expression was constant pre- and post-exposure to AHR. The strength of expression was not the same for all genes pre-exposure to AHR in both replicates. This was the case even after a third replicate for genes *cap8* and *hlgCB* for isolate 44; *cap8*, *hlgA*, *hlgCB* and *icaA* for isolate NCIMB 9518; *hlgA* and *clfA* for isolate S1. The strength of gene expression was often not the same post-exposure to AHR for both replicates. This often led to replicates showing inconsistent change in gene expression (up-regulation, down-regulation or no change). This was the case for genes *cap8*, *hlgA*, *hlgCB*, *icaA* and *clfA* for isolate 44; *cap8*, *fnbB*, *hlgA*, *icaA*, *icaD* and *clfA* for isolate NCIMB 9518; *clfA* for isolate S1. Little difference was observed in gene expression pre-exposure to AHR between the isolates. More virulence genes were down-regulated post-exposure to AHR for isolates 44, NCIMB 9518 and S1 than were up-regulated. Isolate S1 showed down-regulation of the greatest number of virulence genes, followed by isolate 44, with isolate NCIMB 9518 showing down-regulation of the least number of genes. Isolate 44 showed up-regulation of the greatest number of genes, followed by NCIMB 9518, with isolate S1 showing up-regulation of the least number of genes.

Table 5.10: RT-PCR Results for *S. aureus* Isolate 44

Results of expression of a range of virulence genes plus a housekeeping gene in *S. aureus* isolate 44 pre- and post-exposure to Soft Care Med H5, Cutan and Guest Medical AHRs, using RT-PCR. Genes are documented as being very highly expressed (++++), highly expressed (+++), moderately expressed (++), slightly expressed (+), or not expressed (-). Post-exposure results shown in red are more greatly expressed, those in black are expressed equally and those shown in blue are less expressed than pre-exposure. * Performed in triplicate. ** Housekeeping gene.

Gene	Pre-Exposure	Post-Soft Care Med H5	Post-Cutan	Post-Guest Medical
hysA	—	—	—	—
	—	—	—	—
*cap8**	+++	+++	+++	+++
	++	—	+++	+++
	++++	++++	++++	+++
fnbA	—	—	—	—
	—	—	—	—
fnbB	—	—	—	—
	—	—	—	—
*hlgA**	+++	++	++	++
	—	++	++	++
	—	—	—	+
*hlgCB**	++	—	+	+
	++++	++++	++++	++++
	+	+++	+++	—
*icaA**	++			
	+++	+++		
	+++			
icaD	+++			
	+++			
*clfA**	++		++	
	++++	++++	++++	++++
	++++	++++		
*glyA***	++++	++++	++++	++++
	++++	++++	++++	++++

Table 5.11: RT-PCR Results for *S. aureus* Isolate NCIMB 9518

Results of expression of a range of virulence genes plus a housekeeping gene in *S. aureus* isolate NCIMB 9518 pre- and post-exposure to Soft Care Med H5, Cutan and Guest Medical AHRs, using RT-PCR. Genes are documented as being very highly expressed (++++), highly expressed (+++), moderately expressed (++), slightly expressed (+), or not expressed (-). Post-exposure results shown in red are more greatly expressed, those in black are expressed equally and those shown in blue are less expressed than pre-exposure. * Performed in triplicate. ** Housekeeping gene.

Gene	Pre-Exposure	Post-Soft Care Med H5	Post-Cutan	Post-Guest Medical
hysA	—	—	—	—
	—	—	—	—
cap5	—	—	—	—
	—	—	—	—
*cap8**	+++	+	—	—
	++	++	++	++
	+	+	++	+
fnbA	—	—	—	—
	—	—	—	—
*fnbB**	—	—	—	—
	++	+	—	—
	—	—	—	—
*hlgA**	++	+	++	—
	+++	++	++	++
	—	—	—	—
*hlgCB**	++	—	—	—
	—	—	—	—
	+	+	+	+
*icaA**	+	+		+
	+++			
	++		++	
*icaD**	+++	+++	+++	+++
	++			
	+++		+++	+++
clfA*	++++		++++	
	++++	++++	++++	++++
	—	—	++	++
glyA**	++++	++++	++++	++++
	++++	++++	++++	++++

Table 5.12: RT-PCR Results for *S. aureus* Isolate S1

Results of expression of a range of virulence genes plus a housekeeping gene in *S. aureus* isolate S1 pre- and post-exposure to Soft Care Med H5, Cutan and Guest Medical AHRs, using RT-PCR. Genes are documented as being very highly expressed (++++), highly expressed (+++), moderately expressed (++), slightly expressed (+), or not expressed (-). Post-exposure results shown in red are more greatly expressed, those in black are expressed equally and those shown in blue are less expressed than pre-exposure. * Performed in triplicate. ** Housekeeping gene.

Gene	Pre-Exposure	Post-Soft Care Med H5	Post-Cutan	Post-Guest Medical
hysA	—	—	—	—
	—	—	—	—
cap5	—	—	—	—
	—	—	—	—
*cap8**	+++	+++	+++	+++
	++++	+++	+++	+++
	++++	+	+++	+++
fnbA	—	—	—	—
	—	—	—	—
*hlgA**	++	+	+	+
	+++	—	+	+
	—	—	—	—
*hlgCB**	+++	+	++	+
	++++	+++	+++	+++
	++++	+	++++	+++
icaA	++	-	+	-
	++	+	+	+
*icaD**	+++	-+	—	-+
	++	—	—	+
	+++	+++	+++	+++
*clfA**	—	+++	+++	+-
	++++	++++	++++	++++
	+++	+++	+++	+
*glyA***	++++	++++	++++	++++
	++++	++++	++++	++++

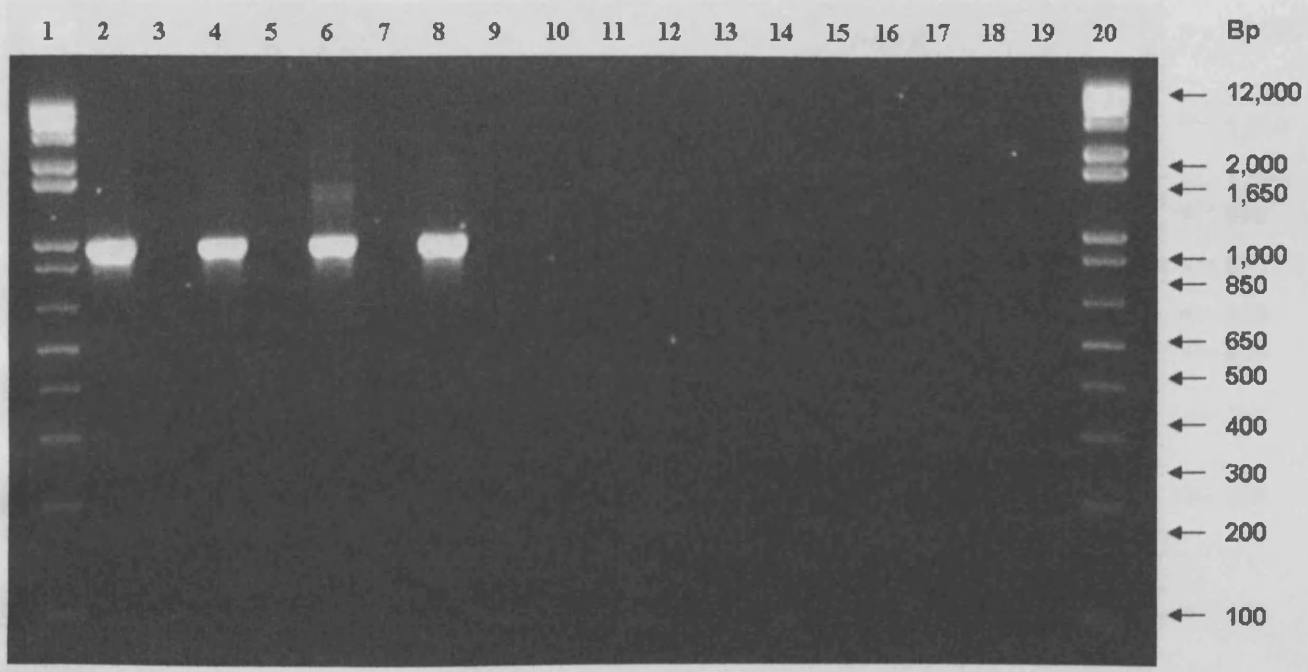

Figure 5.13: Example of Gene Expression in *S. aureus* Isolate 44

The intensity of bands in lanes 2, 4, 6 and 8 were interpreted as greatly expressed (++++). All other lanes showed no expression (-). BP = length in base pairs.

Well	Contents
1	1Kb Plus ladder
2	*clfA* pre (+RT)
3	*clfA* pre (noRT)
4	*clfA* Soft Care (+RT)
5	*clfA* Soft Care (noRT)
6	*clfA* Cutan (+RT)
7	*clfA* Cutan (noRT)
8	*clfA* Guest Med (+RT)
9	*clfA* Guest Med (noRT)
10	*fnbB* pre (+RT)
11	*fnbB* pre (noRT)
12	*fnbB* Soft Care (+RT)
13	*fnbB* Soft Care (noRT)
14	*fnbB* Cutan (+RT)
15	*fnbB* Cutan (noRT)
16	*fnbB* Guest Med (+RT)
17	*fnbB* Guest Med (noRT)
18	*clfA* negative control
19	*fnbB* negative control
20	1 Kb Plus Ladder

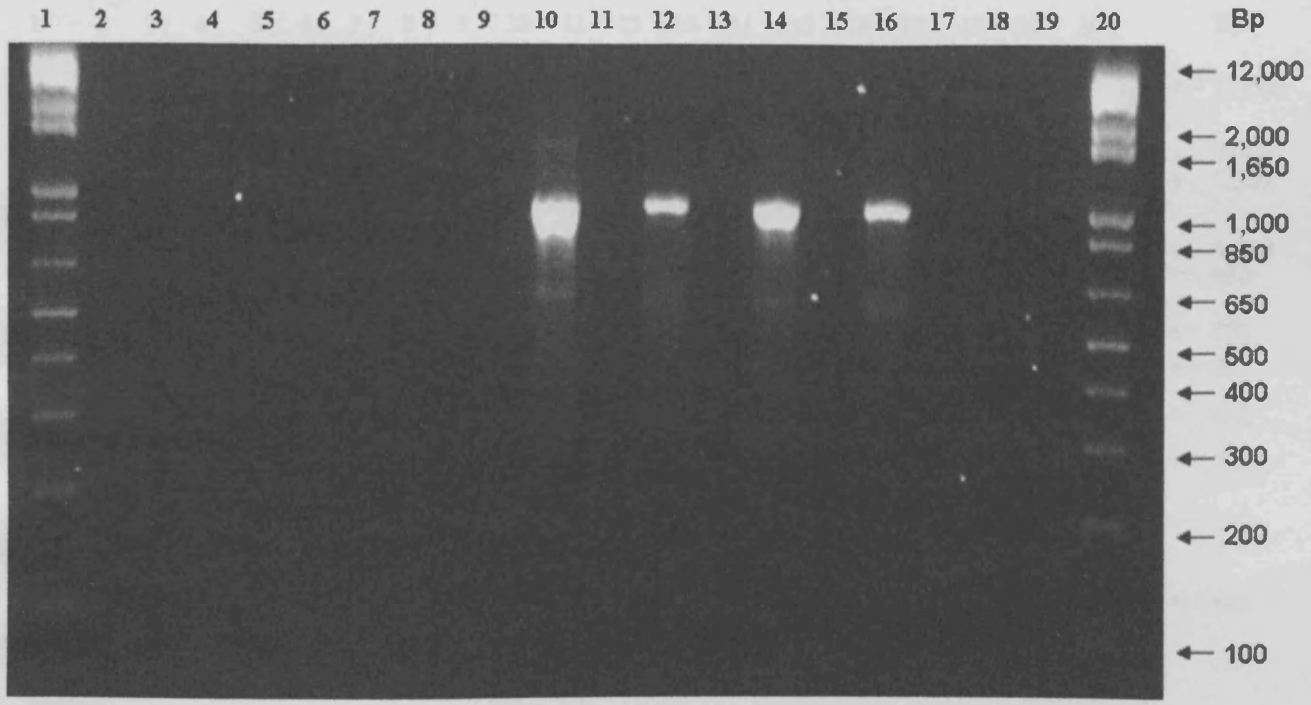

Figure 5.14: Example of Gene Expression in *S. aureus* Isolate NCIMB 9518

The intensity of the bands in lanes 10 and 14 were interpreted as greatly expressed (++++), whereas bands in lanes 12 and 16 were interpreted as highly expressed (+++). All other lanes showed no gene expression (-). BP = length in base pairs

Well	Contents
1	1Kb Plus ladder
2	*fnbB* pre (+RT)
3	*fnbB* pre (noRT)
4	*fnbB* Soft Care (+RT)
5	*fnbB* Soft Care (noRT)
6	*fnbB* Cutan (+RT)
7	*fnbB* Cutan (noRT)
8	*fnbB* Guest Med (+RT)
9	*fnbB* Guest Med (noRT)
10	*clfA* pre (+RT)
11	*clfA* pre (noRT)
12	*clfA* Soft Care (+RT)
13	*clfA* Soft Care (noRT)
14	*clfA* Cutan (+RT)
15	*clfA* Cutan (noRT)
16	*clfA* Guest Med (+RT)
17	*fnbB* Guest Med (noRT)
18	*fnbB* water control
19	*clfA* negative control
20	1 Kb Plus Ladder

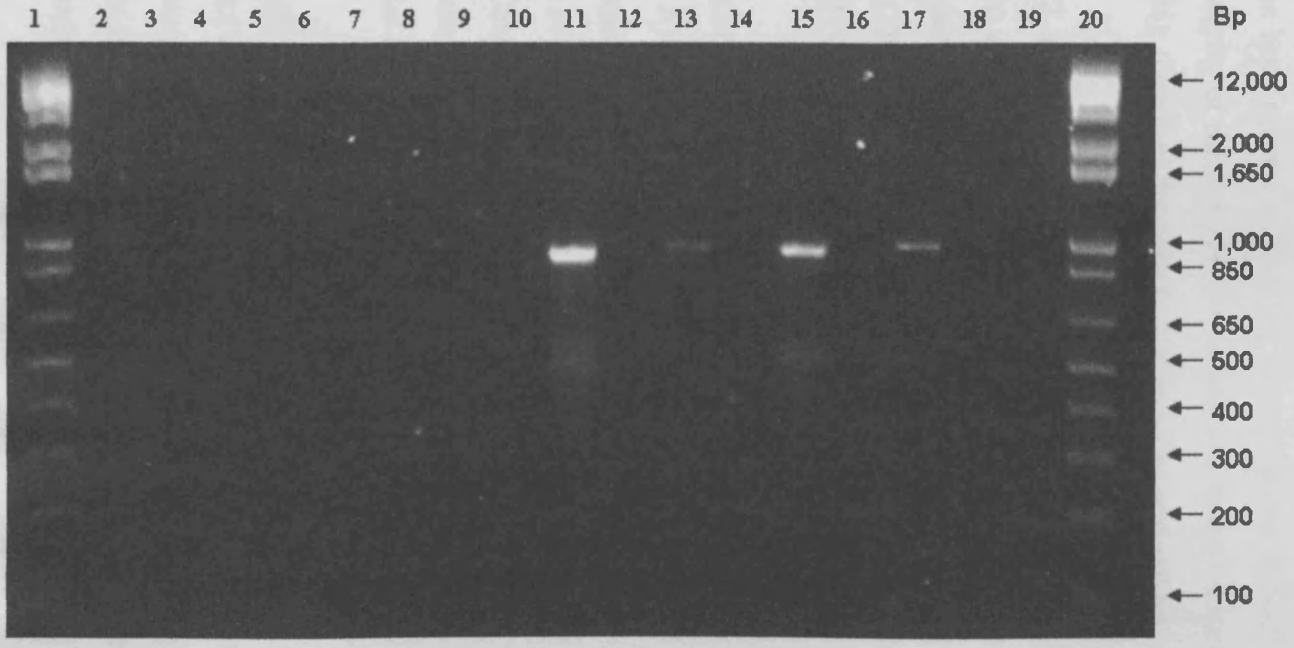

Figure 5.15: Example of Gene Expression in *S. aureus* Isolate S1

The intensity of the bands in lanes 11, 13, 15 and 17 were interpreted as highly expressed (+++), slightly expressed (+), moderately expressed (++) and slightly expressed, respectively. All other lanes showed no gene expression (-). BP = length in base pairs.

Well	Contents
1	1Kb Plus ladder
2	*fnbA* pre (+RT)
3	*fnbA* pre (noRT)
4	*fnbA* Soft Care (+RT)
5	*fnbA* Soft Care (noRT)
6	*fnbA* Cutan (+RT)
7	*fnbA* Cutan (noRT)
8	*fnbA* Guest Med (+RT)
9	*fnbA* Guest Med (noRT)
10	*hlgCB* pre (+RT)
11	*hlgCB* pre (noRT)
12	*hlgCB* Soft Care (+RT)
13	*hlgCB* Soft Care (noRT)
14	*hlgCB* Cutan (+RT)
15	*hlgCB* Cutan (noRT)
16	*hlgCB* Guest Med (+RT)
17	*hlgCB* Guest Med (noRT)
18	*hlgCB* negative control
19	No sample
20	1 Kb Plus Ladder

5.4 DISCUSSION

5.4.1 The Cytoplasmic Membrane as a Target for Alcohol Hand Rub

All AHRs were too viscous to pass undiluted through the 0.45 and 0.2 μm filters, therefore diluting 1 in 10 in UHP water was sufficient to allow passage of Soft Care Med H5 and Guest Medical through the filters. However, Cutan was extremely viscous, which is due to the ingredient C10-30 alkyl acrylate crosspolymer, which is a thickener commonly used in clear gels that makes the product easier to pump and handle. The crosspolymer was broken up by diluting Cutan in 3% w/v NaCl. *S. aureus* has been proven to have an osmotolerance to NaCl, with no apparent effect on the integrity of the cytoplasmic membrane below concentrations of 5% (Hajmeer *et al.*, 2006); therefore the presence of NaCl should not have affected the test.

Figure 5.2 shows the K^+ leakage curve for most isolates under most conditions featured an early peak in % leakage, followed by a reduction in leakage and in some cases a later, more gradual increase. Similar shaped curves have been found for release of 260 nm material from *S. aureus* after exposure to biocide (Denyer & Hugo, 1991).

The order of leakage of K^+ amongst isolates 44, NCIMB 9518 and S1 after exposure to Cutan AHR fits in with results of stainless steel carrier testing from Chapter 4, which found that a bactericidal effect was achieved quicker for isolate 44, followed by isolate NCIMB 9518, and slowest for isolate S1. This was not, however, the case for Soft Care Med H5 and Guest Medical AHR. It is unclear why Cutan caused the greatest membrane damage when Cutan was found to take longest to achieve a bactericidal effect in Chapter 4.

K^+ leakage was studied for longer contact times than those used to achieve a bactericidal effect in Chapter 4. If membrane damage was the primary mechanism of activity more K^+ leakage would be expected by the bactericidal contact times than those seen for most samples. Furthermore the bacteria used to test K^+ leakage were in a suspension, rather than on a surface and therefore AHR would be expected to

cause damage to the bacteria quicker than was found in Chapter 4. The inoculum size for the stainless steel carrier test, however, was much smaller than that used for the K^+ leakage test, a bactericidal effect ($\geq$ 4 log reduction) would mean a much lower number of cells killed than for the K^+ leakage test.

With the exception of isolate 44 after exposure to Soft Care Med H5, all isolates had leaked out as much K^+ from their cells as they would do in the entire 10 min period prior to the contact time by which a bactericidal effect was achieved in Chapter 4. This suggests that the damage caused to the cytoplasmic membrane that caused K^+ leakage may eventually have led to cell death. However, since a large portion of cells were able to survive for some period of time after this leakage occurs, the damage that led to leakage of K^+ may not have been sufficient to cause cell death; therefore other factors are likely to have caused cell death.

K^+ leakage was virtually non-existent from isolate S1 after exposure to Cutan and was relatively low for Guest Medical and Soft Care Med H5 for all isolates within the bactericidal contact times, and even up to 10 min, indicating that membrane damage may not be severe enough to cause a bactericidal effect under these conditions.

The fact that not all AHRs were able to cause >50% of cellular K^+ to leak from one or more of the isolates either within the contact time required to achieve a bactericidal effect (as shown in Table 5.7) or within the full 10 min contact time tested (as shown in Table 5.6) indicates that the major cause of bactericidal activity of AHR is not membrane damage. This theory is supported by findings by Johnston *et al.* (2003) that a membrane-active biocide was able to cause leakage of the total cellular K^+ pool within 1 min. Inoue *et al.* (2004) tested three terpene alcohols (compounds derived from plants that are composed of alcohol with a terpene hydrocarbon) at various concentrations for membrane activity against *S. aureus*. Two of the alcohols caused very rapid leakage of K^+, where the maximum K^+ loss was achieved almost immediately. The third alcohol caused much slower leakage, where maximum K^+ loss was achieved by between 1 and >15 min contact time. It is worth noting that a bactericidal effect ($\geq$4 $\log_{10}$ reduction) was achieved for the

terpene alcohols, which were used at double the maximum concentration used for K^+ leakage testing, by between 1-6 h. K^+ leakage was therefore relatively rapid for all terpene alcohols. The study by Inoue *et al.* (2004) further supports the theory that the mode of action of Soft Care, Cutan and Guest Medical is not primarily the cytoplasmic membrane because the membrane-active agents tested caused maximum K^+ loss at a much earlier time point than a bactericidal effect was achieved, whereas the AHRs tested in this study did not.

5.4.2 The Cell Wall as a Target for AHR or Barrier to Prevent Its Penetration

Since section 5.3.1 revealed the cytoplasmic membrane may not be the main cause of bactericidal effect, the cell wall was the most likely alternative. Differences in the susceptibility of isolates to AHR observed in Chapter 4 may therefore be due to differences in either AHR activity against the cell wall or differences in penetration of AHR into the cell. This was investigated by subjecting cells without a cell-wall (protoplasts) to AHR, which has not previously been performed. None of the AHRs caused significant damage to protoplasts after even a contact time much longer than that necessary to achieve a bactericidal effect on whole cells. This firstly confirmed results from section 5.3.1, that damage to the cytoplasmic membrane and intracellular contents is not the main mode of activity of AHR. This secondly indicated that the differences in efficacy observed between the AHRs against whole cells in Chapter 4 is likely to be due to differences in the cell wall or outer layers, such as the capsule or slime layer (Xiong *et al.*, 2005). There was no significant damage to protoplasts of either the most- or least-susceptible strains following exposure to AHR, indicating that differences in susceptibility observed among the isolates in Chapter 4 was not due to differences in the cytoplasmic membrane or intracellular contents, rather differences in the cell wall or outer layers (Xiong *et al.*, 2005), since these are the components missing. Gilby & Few (1960) found that varying concentrations of ethanol were able to lyse large numbers of *Micrococcus lysodeikticus* protoplasts by a contact time of 20 min. It is unclear why similar outcomes were not observed in this study.

5.4.3 The Effect of AHR on Cell Structural Appearance

As explained in Table 5.1 alcohols are known to damage lipid and proteins. However, it is not known what parts of the cell AHR damages and the effect on the physical appearance of the cell. This was determined using electron microscopy. A bactericidal effect ($\geq$4 $\log_{10}$ reduction) was observed for isolates 44 and NCIMB 9518 within 30 s exposure to Guest Medical AHR. Physical damage was seen after 1 min exposure (30 s exposure was not performed), although not in as many cells as those that had been shown to be killed in Chapter 4. This indicates that damage sufficient to achieve a bactericidal effect must occur at earlier contact times and in a larger number of cells and this damage may not overtly alter the physical appearance of cells. In contrary to this observation, damage to isolate S1 was physically apparent at a contact time as early as 10 s and extensive at 5 min, despite a >5min contact time being required to achieve a bactericidal effect using Guest Medical AHR in Chapter 4. This suggests that isolate S1 may survive at least some of the damage that results in a change of physical appearance. The fact that damage to the appearance of bacterial cells of isolate S1 was so rapid when it was the least-susceptible to being killed indicates that the damage seen at the earlier contact times is sub-lethal. Bacterial cells of isolate 44 were the most severely damaged, which supports the fact that isolate 44 was the most easily killed. This also indicates that some bacterial cell damage observed contributed to the bactericidal effect.

Damage to the bacterial cytoplasm observed in *S. aureus* cells (Figure 5.6) is likely to be due to coagulation of protein (McDonnell & Russell, 1999). The identity of the material surrounding isolate 44 after 1 min (Figure 5.5) and 5 min (Figure 5.6) exposure to Guest Medical AHR and to a lesser extent isolate NCIMB 9518 after 1 min (Figure 5.8) and 5 min (Figure 5.9) exposure to Guest Medical AHR is not clear. It is present in all samples, although not in such great quantity. It is most likely to be material that has come away from the bacterial surface during preparation and the greater quantities may be the result of increased damage to the cell surface, which has caused the material to come away easier. This theory is likely, since a large quantity of the material was present post-exposure for isolate 44, the most susceptible isolate to Guest Medical AHR, whereas only a small increase in the amount of material was found post-exposure for isolate NCIMB 9518 and no

increase for isolate S1, the least-susceptible isolate. Cuputy & Costerton (1982) found that preparation of thin sections for EM caused glycocalyx from the surface of *S. aureus* cells to be torn away. Jones *et al.* (1969) found material in their EM samples identical in appearance to the UEM found in this study coming away from the cell surface of bacterial cells and present throughout the sample. They identified it as polysaccharide-like. Jones *et al.* (1969) also found that the polysaccharide material was separated from the cell surface in some samples by a clear area of no polysaccharide, which was also found in EM samples of this study. They are unsure as to the reason for the clear area immediately surrounding the cell surface, but hypothesize it may be an artefact of dehydration or due to polysaccharide production ceasing or formation of a protective microenvironment around the cell. As mentioned in section 5.1.5 small concentrations of AHR has been found to stimulate the secretion of extracellular protein in *Acinetobacter baumannii* (Edwards *et al.*, 2007). Another theory as to the identity of the UEM is that since *S. aureus* is also able to metabolise ethanol (Artzatbanov & Petrov, 1990) it may stimulate secretion of extracellular protein, which may form whole, or part of, the UEM found in this study. The denser UEM found in Figure 5.8 was not similar in appearance to that found by Jones *et al.* (1969), but it is likely that the material has maintained its position during preparation. The polysaccharide found in Figure 5.8 may be a form of glycocalyx, such as a slime layer. The role of slime layers in biocide tolerance is not well known, but they at least provide a physical barrier to penetration or absorb the biocide (McDonnell, 2007). It is not clear why this material was surrounding cells following 1 min exposure to AHR but not following 5 min exposure. It could be that the material has been broken down by the alcohol, which is possible if this material consists of protein or lipid.

5.4.4 The Effect of AHR on Protein Denaturation

It was shown in section 5.3.2 that the cell wall or outer layers are likely to be the main target for AHR and are likely to be responsible for the difference in susceptibility seen among the isolates. Differences in susceptibility may be due to reduced penetration or increased repair of cell wall. If this is the case the main mode of action of AHR on the cell wall is most likely due to denaturation of proteins, since

alcohol is known to denature proteins (McDonnell & Russell, 1999). Quantification of protein was unachievable, however, as outlined in section 5.1.4. It was therefore unknown whether differences between the susceptibility of isolates was due to differences in penetration of the AHR into the cell or whether is was due to differences in susceptibility of the cell wall to damage, most likely due to protein denaturation. Uptake studies are possible for biocides, which may rule out/implicate differences in penetration. However, such studies involve mixing bacterial cells with a known volume of biocide and after separation by either centrifugation or filtration either the quantity of biocide remaining in solution or the quantity taken into the cell are quantified (Salt & Wiseman, 1991). These methods are not possible to perform with AHRs, since their high viscosity does not permit separation without dilution, thus not allowing easy quantification of concentration.

5.4.5 The Effect of AHR on Expression of Virulence Genes

The reason some isolates are less susceptible to AHR than others may be due to reduced penetration or due to increased expression of genes that may aid survival against AHR, such as those that encode biofilm production. If virulence genes are more expressed after exposure to AHR and AHR is not effective at killing *S. aureus* isolates in practice, then exposure to AHR in the healthcare environment may result in isolates becoming more virulent. There was a trend towards down-regulation of a number of the genes, indicating that these are not considered necessary for the cell during exposure to AHR and that more useful genes that aid survival are likely to be up-regulated. These results are supported by Bore *et al.* (2007), who found no up-regulation of virulence genes upon exposure to harmful conditions.

RT-PCR results were inconsistent and cannot therefore be relied upon to provide an accurate representation of gene expression. The use of qPCR is more accurate and would therefore provide a more accurate gene expression analysis (VanGuilder *et al.*, 2008). The virulence genes chosen, however, do not reflect genes that are likely to be used for repair or survival (with the exception of *icaA* and *icaD*, which encode production of biofilm); therefore microarray analysis would more useful, since it

would identify all genes that are up- or down-regulated upon AHR exposure (Schena *et al.*, 1995).

S. aureus has previously been found to respond to acidification to pH 4.5 by up-regulation of genes involved in acid defence, along with other stress response genes, down-regulation of genes involved with cellular growth, but no virulence gene up-regulation (Bore *et al.*, 2007). Such genes may be similarly affected by exposure to AHR, along with currently unknown genes that may help *S. aureus* survive exposure.

5.4.6 Summary

This chapter demonstrated that although AHR had an effect on the cytoplasmic membrane (as demonstrated by the K^+ leakage assay and TEM), it is most likely not the main target of AHR. It was demonstrated that the cell wall or outer layer of the cell are targeted by AHR and this was likely to be due to protein denaturation. It was also demonstrated that whilst damage to the cell affected its physical appearance this damage was not necessarily correlated to a bactericidal effect. Furthermore, bactericidal activity may occur prior to damage being observable. Not all virulence genes were expressed under pre- AHR exposure conditions. Although exposure to AHR caused a change in expression of some virulence genes, with a tendency towards down-regulation, these results were inconsistent due to variability in the test results.

CHAPTER 6 PROPERITES OF *STAPHYLOCOCCUS AUREUS* LINKED TO ALCOHOL HAND RUB SUSCEPTIBILITY

6.1 INTRODUCTION

6.1.1 Bacterial Adhesion

Bacteria are able to adhere to living and inanimate surfaces. Adhesion to a surface protects bacteria from the environment and adhesion to skin assists entry into the host. Bacterial adhesion to surfaces is mediated by non-specific physical interactions and specific ligand-receptor interactions (Dalton & March, 1998). Non-motile bacteria such as *S. aureus* come into contact with a surface via gravity, convection or Brownian motion. The first phase of adhesion to a surface is the reversible, non-specific adhesion of bacteria to the surface using weak bonds such as van der Waal's forces, electrostatic interactions and hydrophobic interactions (Fletcher, 1996; Figure 6.1). This attachment is reversible, since shear force will remove cells from the surface.

The second phase of adhesion to a surface involves specific interactions (Figure 6.1), which consist of ligand-receptor interactions that enable *S. aureus* to selectively bind to host cells. These are described in detail in Table 1.1, Chapter 1.

6.1.2 Cell Surface Hydrophobicity

Cell surface hydrophobicity refers to the degree to which a cell is repelled from water, where hydrophobic cells would be repelled by water away from the aqueous phase onto a surface (Rosenberg & Doyle, 1990). Hydrophobic interactions are one of the mechanisms of bacterial cell adhesion (Gorman, 1991; Fletcher, 1996). The bacterial surface consists of a number of moieties that promote (hydrophobins) and reduce (hydrophilins) hydrophobicity. Differences between bacterial cell surface hydrophobicities are the result of differences in concentrations, distribution and configuration of these moieties (Rosenberg & Doyle, 1990). One such hydrophobin is protein A. Jonsson & Wadström (1984) found that presence of protein A on the surface of *S. aureus* cells contributed to high levels of hydrophobicity among clinical isolates.

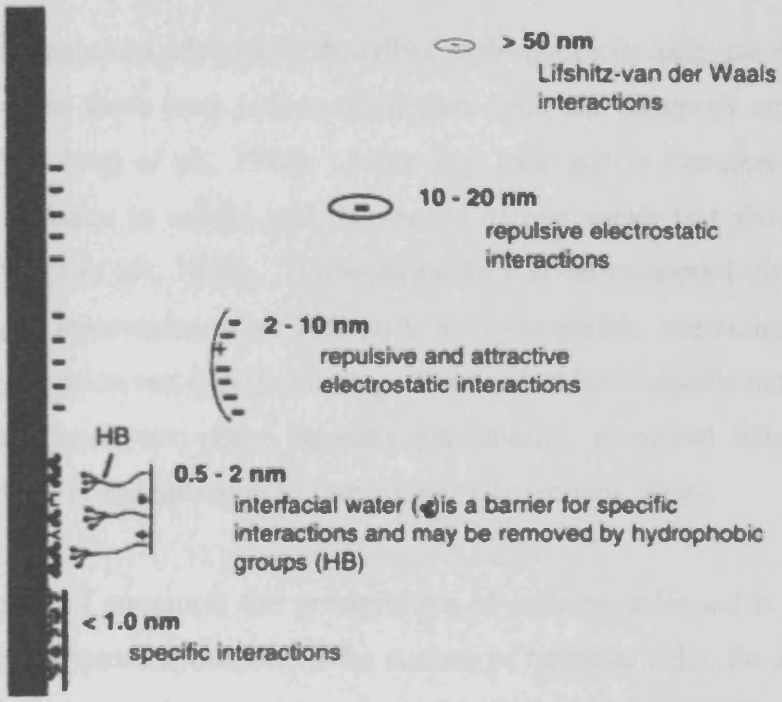

Figure 6.1: Forces Involved in Bacterial Adhesion to Surfaces

Diagram illustrating the forces involved in the interaction of a bacterium with a surface at various distances. Reproduced from Fletcher (1996).

Bacteria that have more hydrophobic surfaces are able to auto-aggregate (Jonsson & Wadström, 1984) and adhere more easily to human skin (Rosenberg *et al.*, 1981) and invasive devices (Pascual *et al.*, 1986; Boujaafar *et al.*, 1990). Bacteria that are more hydrophobic than phagocytes are able to resist phagocytosis (Cunningham *et al.*, 1975). Absolom (1988) found that *S. aureus* was the most hydrophobic species of a number of microorganisms tested and therefore was not phagocytosed by neutrophils as readily as other species. Hydrophobic bacteria are therefore more virulent (Ljungh *et al.*, 1985).

No single test adequately describes hydrophobicity because experimental conditions used in these tests influence/interfere with the observed outcome to some degree (Rosenberg *et al.*, 1980). More than one test is therefore necessary to increase confidence in results and the results of one single test should not be relied upon (Dillon *et al.*, 1986). Hydrophobicity can be measured directly by water contact angle measurements or indirectly by hydrophobic interaction chromatography, salt aggregation test (SAT), binding of individual hydrophobic molecules such as anionic surfactants, two-phase aqueous partitioning, microbial adhesion to hydrocarbons (MATH) and adhesion to polystyrene (Rosenberg, 2006).

The SAT measures the precipitation of cells by salts and is based on the principle that the more hydrophobic the surface of bacterial cells, the lower the concentration of salt required to aggregate them (Lindahl *et al.*, 1981). The hexadecane adherence assay (HAA) is a MATH test that measures the adherence of bacterial cells to the hydrocarbon *n*-hexadecane, whereby more adherent cells are more hydrophobic. However, hexadecane is highly negatively charged due to ion adsorption and therefore HAA test results are also likely to reflect the electrical surface charge of cells to a small degree (Geertsema-Doornbusch *et al.*, 1993). The xylene adherence assay (XAA) measures the adherence of bacterial cells to the hydrocarbon xylene in the same manner as for the HAA (Galliani *et al.*, 1994).

6.1.3 Surface Charge

The surface of a bacterial cell has a net negative charge due to the phosphate groups of teichoic acid, carboxylates of peptidoglycan, acidic polypeptides and polysaccharides, plus polysaccharides of the glycocalyx (Somasundaran, 2006). This charge at the cell surface creates an electrostatic field surrounding the cell and is therefore balanced by positively charged ions in the immediate vicinity of the cell surface, which form an electrical double layer (Figure 6.2). The potential arising from the negatively charged surface decreases exponentially with distance from its surface, meaning that as a bacterium moves through a suspension medium it will carry a thin layer of the medium with it, including some positively charged ions (Finean *et al.*, 1978). The potential at the surface of shear, or slipping plane (where the layer moves past the bulk solution) is called the zeta potential and is therefore a few mV lower than the true surface potential. The zeta potential of particles is measured by applying an electric field to the particle dispersion. Particles within the dispersion with a zeta potential will migrate toward the electrode with an opposite charge to themselves. The velocity at which this occurs depends on the magnitude of their zeta potential.

Surface charge is a critical property that influences bacterial adhesion to surfaces because it determines the electrostatic interaction between bacterial cells and surfaces (Luo *et al.*, 2005). The more negatively charged a bacterium, the more effective it is at attaching to surfaces (Dickson & Koohmaraie, 1989). The negative charge of bacterial cells also means that cationic antimicrobial peptides produced by epithelial cells and neutrophils can easily bind to the bacterial membrane and lyse the cell (Sahl *et al.*, 2005). *S. aureus* is able to modify its surface charge by addition of positively charged groups to components of the cell wall and cytoplasmic membrane, thereby neutralizing the negative charge and leaving *S. aureus* resistant to these, and other, host defences (Kraus *et al.*, 2008). Bacterial surface charge has also been found to be influenced by quorum sensing, which can lead to aggregation and production of biofilm (Eboigbodin *et al.*, 2006).

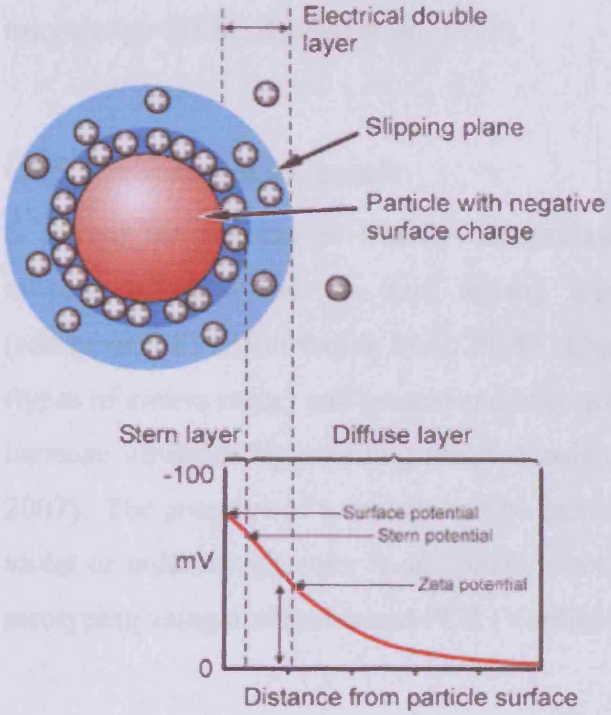

Figure 6.2: Schematic Representation of Zeta Potential

Representation of the electrostatic phenomena that occur in the immediate vicinity of a negatively charged particle such as a bacterial cell. Reproduced from Malvern Instruments Ltd., Malvern, UK.

6.1.4 Cell Size and Arrangement

Isolates of the same species are known to vary in size. *S. aureus* has been shown to increase cell size in response to the presence of harmful agents in the environment, to reduce the relative area of the cell surface and therefore reduce the chance of damage (Raju *et al.*, 2007). This may be the result of the presence of surface structures, such as a capsule or slime layer, or a thicker cell wall. Increased cell size may, therefore result in the cells being less susceptible to AHR exposure. Bacterial cell size is usually measured using an electronic particle size counter or scanning electron microscopy (SEM; Russell *et al.*, 1973).

6.1.5 Presence of a Capsule

S. aureus isolates can be heavily encapsulated (serotype 1 and 2, which produce mucoid colonies and are rare among clinical isolates) or microencapsulated (serotypes 3-8; O'Riordan & Lee., 2004). Capules consist of hexosaminuronic acids (types of amino sugar) and protein and may act as a barrier to biocide penetration and increase virulence by avoiding phagocytosis (O'Riordan & Lee, 2004, McDonnell, 2007). The presence of a capsule can be determined by negative staining with crystal violet or india ink (Brooks *et al.*, 2007), electron microscopy (Arizono *et al.*, 1991), serotyping using antibodies and PCR (Verdier *et al.*, 2007).

6.1.6 Protein Content of *S. aureus*

Chapter 5 concluded that the major effect of AHR is likely to be protein denaturation. Protein is present in the cytoplasm, the cytoplasmic membrane and the cell wall of *S. aureus*, as well as in the outer S-layer, capsule and sometimes the slime layer (McDonnell, 2007). The total protein content is usually determined by the Bradford method (Bradford, 1976), Lowry method (Lowry *et al.*, 1951) and bicinchoninic acid assay (BCA; Smith *et al.*, 1985) methods, which are all colourimetric assays that measure a change in absorbance as a result of a colour change in the reagent. The BCA method is often the assay of choice since it is quicker to perform than the Lowry method and more accurate than the Bradford method (Berges *et al.*, 1992).

6.1.7 Aims and Objectives

The aim of this chapter was to determine the properties of *S. aureus*, including possible changes in those properties following AHR exposure that are linked to its susceptibility to AHR. The properties investigated were those that may affect susceptibility to AHR, including surface properties such as hydrophobicity and surface charge, cell size, cell arrangement and total protein content of the most- and least-susceptible isolates to AHR.

6.2 MATERIAL AND METHODS

6.2.1 Determining the Sub-Lethal Concentration of AHRs

6.2.1.1 Validation of PBS as a Neutralizer for AHR at a 1 in 9 Dilution

A bacterial suspension of *S. aureus* NCIMB 9518 was prepared to a concentration of 8.61 ± 0.11 $\log_{10}$ CFU/ml according to section 2.5.1. One ml of AHR was added to 8 ml phosphate buffered saline (PBS) and vortex mixed, then left for 5 min to allow neutralization. One ml of bacterial suspension was added and vortex mixed, then after 5 min a viable count was performed according to section 2.6.1.1. A control was also performed, where 1 ml bacterial suspension was added to 9 ml PBS. This procedure was performed 5 times for Soft Care Med H5, Cutan and Guest Medical AHRs, using a fresh bacterial culture for each replicate. Statistical analysis was performed on the $\log_{10}$ duplicate counts according to section 2.8.1 to determine whether there was a significant difference between the test and control counts.

6.2.1.2 Validation of TSB as a Neutralizer for AHR at a 1 in 9 Dilution

The test was performed five times for Soft Care Med H5, Cutan and Guest Medical AHRs according to the neutralizer validation of PBS in section 6.2.1.1, except the test and controls were performed with TSB instead of PBS. Suspensions used were prepared to a concentration of 8.57 ± 0.09 $\log_{10}$ CFU/ml.

6.2.1.3 Determining the Sub-Lethal Concentration of AHRs

A bacterial suspension of *S. aureus* NCIMB 9518 was prepared to a concentration of 8.53 ± 0.11 $\log_{10}$ CFU/ml according to section 2.5.1. AHR was mixed with TSB at concentrations of 1 in 10, 1 in 12 and 1 in 14 and left for 5 min. Ten wells of a Bioscreen honeycomb plate were filled with 350 µl of each AHR concentration. A 50 µl volume of suspension was added to the first 5 wells for each AHR concentration. A fifty µl volume of TSC buffer was added to the remaining 5 wells of each AHR concentration, which acted as negative controls (blanks). A further ten wells were filled with 350 µl TSB. Fifty µl of suspension was added to the first five wells, which acted as positive controls. Fifty µl TSC buffer was added to the remaining five wells to serve as negative controls (blanks). The honeycomb plate was incubated in a Bioscreen microbial growth analyser at 37 °C for 14 hours with an OD reading taken every 15 min using a wideband filter with a wavelength of 420-580 nm. The plate was set to shake for 10 s before each $OD_{420\text{-}580}$ reading. The mean of each test and positive control $OD_{420\text{-}580}$ reading was calculated at each 15 min reading interval and the overall mean value of each blank was subtracted from the appropriate test and positive control mean values to provide a true $OD_{420\text{-}580}$ value. The mean-blank results were used to create a graph and the resulting growth curves were analysed.

6.2.2 Determining the Cell Surface Hydrophobicity of *S. aureus* Isolates

6.2.2.1 Materials

Sodium phosphate (Acros Organics) was used to make sodium phosphate buffer to a concentration of 0.002 M in deionised water and was adjusted to pH 6.8 using ammonium hydroxide (Acros Organics). Ammonium sulphate was prepared to a concentration 4 M in 0.002 M sodium phosphate buffer.

6.2.2.2 Salt Aggregation Test

This test was originally described by Lindahl *et al.* (1981). An overnight broth culture was prepared according to 2.4.4, and centrifuged at 940 *g* for 10 min. The pellet was washed twice with 0.002 M sodium phosphate buffer and re-suspended in

0.002 M sodium phosphate buffer to a concentration of 9.70 $\log_{10}$ CFU/ml according to section 2.5.3. A range of different concentrations of ammonium sulphate ('salt') were prepared in 0.002 M sodium phosphate buffer. These included dilutions of 4.0 M to 0.2 M (differing by 0.2 M per dilution) and dilutions of 0.2 M to 0.02 M (differing by 0.02 M per dilution). Twenty five µl of each 'salt' dilution was added to a glass depression slide (Fisher Scientific) and 25 µl of bacterial suspension was added. A negative control was also performed with 25 µl 0.002 M sodium phosphate buffer and 25 µl suspension. The mixtures were placed on a Grant Bio POS 300 rotating platform at 120 rpm for 2 min. Mixtures were observed manually for changes in visual appearance. A positive result was signified by optimal aggregation, which consisted of a clear solution with white aggregates approximately 1-10 mm in diameter. This test was performed with *S. aureus* isolates 44, 27, 37, NCIMB 9518, 52, 34, S12, S5, 6, 51, 28 and S1.

6.2.2.3 Hexadecane Adherence Assay

This test method was based on those described by Rosenberg *et al.* (1980) and Greene *et al.* (1992). A 24 h broth culture was prepared according to 2.4.4 and centrifuged at 940 *g* for 10 min. The pellet was washed twice with PBS and re-suspended in PBS to an OD_{540} of 0.54 ± 0.03. Eight hundred µl *n*-hexadecane was added to a round-bottomed glass test tube (Fisher Scientific) containing 3 ml of the bacterial suspension. The test tube was incubated for 10 min at 30°C and following 1.5 min of vortex mixing, was left for 15 min for bacterial cells to adhere to the hexadecane. Finally, 1 ml of the aqueous (bottom) layer was extracted using a Pasteur pipette and the OD_{540} was measured. This test was performed in triplicate for *S. aureus* isolates 44, 27, 37, NCIMB 9518, 52, 34, S12, S5, 6, 51, 28 and S1. The % adherence for each replicate was calculated using the following equation:

$$\% \text{ adherence} = \left[\frac{OD_{initial} - OD_{+hexadecane}}{OD_{initial}} \right] \times 100$$

The method used to detect adherence post-exposure to AHR was similar to that described above, except after washing twice in PBS cells were re-suspended in 5 ml of a 1 in 10 dilution of AHR in PBS and left for a contact time of 10 min. Cells were

centrifuged at 2600 *g* for 15 min, washed twice in PBS and re-suspended in PBS to an OD_{540} of 0.54 ± 0.03. This test was performed in triplicate for *S. aureus* isolates 44, 27, 37, NCIMB 9518, 52, 34, S12, S5, 6, 51, 28 and S1 using Soft Care Med H5, Cutan and Guest Medical AHRs.

Statistical analysis was performed according to section 2.8.1 to determine whether % adherence values varied among the isolates. Statistical analysis was also performed according to section 2.8.2 to determine whether % adherence values were different pre- and post-exposure.

6.2.2.4 Xylene Adherence Assay

This test method was based on those described by Rosenberg *et al.* (1980) and Galliani *et al.* (1994). A 24 h broth culture was prepared according to section 2.4.4 and centrifuged at 940 *g* for 10 min. The pellet was washed twice with PBS and re-suspended in PBS to an OD_{540} of 1.05 ± 0.03. A volume of 250 µl *p*-xylene (Acros Organics) was added to a round-bottomed glass test tube containing 3 ml of the bacterial suspension. The tube was incubated for 10 min at 30°C and following 1.5 min of vortex mixing, was left for 15 min for bacterial cells to adhere to the *p*-xylene. Finally, 1 ml of the aqueous (bottom) layer was extracted using a Pasteur pipette and the OD_{540} was measured. This test was performed in triplicate for *S. aureus* isolates 44, 27, 37, NCIMB 9518, 52, 34, S12, S5, 6, 51, 28 and S1 and the % adherence for each replicate was calculated using the following equation:

$$\% \text{ adherence} = \left[\frac{OD_{initial} - OD_{+\,p\text{-xylene}}}{OD_{initial}} \right] \times 100$$

The % adherence post-exposure to AHR was a modified form of the above method, where after washing twice in PBS cells were re-suspended in 5 ml of a 1 in 10 dilution of AHR in PBS and left for a contact time of 10 min. Cells were centrifuged at 2600 *g* for 15 min, washed twice in PBS and re-suspended in PBS to an OD_{540} of 1.05 ± 0.03. This test was performed in triplicate for *S. aureus* isolates 44, 27, 37, NCIMB 9518, 52, 34, S12, S5, 6, 51, 28 and S1 using Soft Care Med H5, Cutan and Guest Medical AHRs. Statistical analysis was performed as in section 6.2.2.3.

6.2.3 Determining the Surface Charge of *S. aureus* Isolates

This test was based on the method in Matz & Jürgens (2001). It was performed in triplicate for *S. aureus* isolates 44, 27, 37, NCIMB 9518, 52, 34, S12, S5, 6, 51, 28 and S1 pre- and post-exposure to Soft Care Med H5, Cutan and Guest Medical AHRs. For pre-exposure testing a 24 h broth culture was prepared according to section 2.4.4. Suspensions were prepared in 0.0001 M KCl buffer according to section 2.5.2, with two washing steps. Suspensions were adjusted to achieve a concentration of 6.21 ± 0.14 $\log_{10}$ cfu/ml and enumerated according to section 2.6.1. Two ml of each suspension was analysed using a Zetasizer 2000 (Malvern Instruments Ltd., Malvern, UK), which took five measurements of the zeta potential (mV). For post-exposure testing washed suspensions were centrifuged at 2600 g and the pellet was re-suspended in 5 ml AHR diluted 1 in 10 in 0.0001 M KCl buffer for a contact time of 10 min. Suspensions were washed twice in 0.0001 M KCl buffer and adjusted, enumerated and analysed as above.

Statistical analysis was performed according to section 2.8.1 to determine whether zeta potential values varied among the isolates. Statistical analysis was also performed according to section 2.8.2 to determine whether zeta potential values were different pre- and post-exposure.

6.2.4 Determining Cell Size, Arrangement and Surface Properties of *S. aureus* Using Microscopy

6.2.4.1 Materials

All SEM chemicals were prepared with double distilled water. Cacodylate buffer consisted of 300 mM Tris-HCl, 1 M sodium cacodylate and 10 mM cobalt chloride.

6.2.4.2 Preparation of Sample

S. aureus isolates 44, NCIMB 9518 and S1 were incubated in broth according to section 2.4.4 and 750 µl of culture was transferred to a microcentrifuge tube and centrifuged at 2,200 *g* for 5 min. The pellets were washed in 2% glutaraldehyde in 0.1 M Sorensen buffer and left for 1 h in 2% glutaraldehyde to fix. The samples

were washed twice in 0.1 M cacodylate buffer and left for 1 h in enough 1% osmium tetroxide in cacodylate buffer to cover the pellet. The pellets were washed three times in cacodylate buffer, re-suspended and placed in purpose-made plastic filtration capsules containing 13 mm 0.2 μm cellulose acetate filters (Sartorius, Epsom, UK). The filtration capsules were centrifuged at 2,200 *g* for 5 min and the samples were progressively dried by adding increasing concentrations of ethanol to the filtration capsule for 5 min, which were 50%, 70%, 90% and three steps of 100%. Samples were dried in a critical point drier (Samdri 780, Maryland, USA), then removed from the filtration capsules and attached with double-sided tape to brass SEM specimen carriers. The specimens were coated with gold using a gold sputter coater (EMScope, UK).

6.2.4.3 Analysis of Samples Using Scanning Electron Microscopy

Samples prepared according to section 6.2.4.2 were examined at 1,000 x and 10,000 x magnification using an XL20 scanning electron microscope (Philips, Croydon, UK). Each sample was examined at 10,000 x magnification to observe cell size. Ten fields of vision were observed at this magnification and five representative images were taken.

6.2.4.4 Measuring the Size of S. aureus Cells Using SEM Images

The size of *S. aureus* cells was determined by measuring the diameter of 15 random bacterial cells per field of vision. The actual diameter was calculated using the following equation:

$$\text{Actual diameter of cell (μm)} = \frac{\text{scale bar size (μm)}}{\text{length of scale bar (mm)}} \times \text{observed diameter of cell (mm)}$$

The mean diameter plus SD of bacterial cells for each of the five fields of vision for each isolate was calculated to determine the overall mean diameter of bacterial cells for each isolate. Statistical analysis was performed on the mean diameter of cells from each field of vision according to section 2.8.1 to determine whether there was a significant difference between bacterial cell diameter of the three isolates.

6.2.4.5 Determining the Presence/Absence of Capsules Surrounding S. aureus Cells

This method was performed for *S. aureus* isolates 44, NCIMB 9518 and S1. Cultures were prepared according to section 2.4.3 and washed from the surface with SDW. Cells were air dried onto a microscope slide and stained with crystal violet (Pro-Lab Diagnostics, Neston, UK) for 8 min. The crystal violet was washed from the surface of the slide with 20% w/v copper sulphate. Cells were observed under a light microscope at 100 x magnification and capsules were observed as a light coloured halo surrounding the cell surface.

6.2.5 Determining the Total Protein Content of *S. aureus* Cells

6.2.5.1 The Bicinchoninic Acid Protein Assay

The BCA method was performed according to the method provided by Sigma-Aldrich. Overnight broth cultures were performed for *S. aureus* isolates 44, NCIMB 9518 and S1 according to section 2.4.4. Cultures were centrifuged at 2,600 *g* for 15 min, washed in SDW and finally re-suspended in SDW. The concentration of culture used during testing was determined by measuring the dry weight of an equal volume of the suspension used for the BCA assay. This was achieved by centrifuging a 1 ml volume of suspension and drying the pellet at 105 °C in a heating block (Techne) for 6 hours. A further 1 ml volume of each suspension was diluted with 1.5 ml SDW and 100 µl 100 mM phenylmethanesulfonyl fluoride (PMSF) prepared in ethanol was added. Each suspension was sonicated on ice for three sets of 60 s, with 30 s intervals between each sonication. After this process samples were kept on ice. Each suspension was centrifuged at 4 °C at 2,500 *g* for 15 min and the supernatant was retained for further testing. One hundred µl volumes of each suspension were added to 2 ml working reagent (stock prepared as 100 ml BCA and 2 ml 4 % w/v copper sulphate pentahydrate). One hundred µl volumes of BSA at a range of known concentrations between 0 and 1000 µg/ml were also added to 2 ml working reagent and all reactions were incubated at 37 °C for 30 min. The OD_{562} of all reactions was measured. Tests were performed in triplicate.

6.2.5.2 Calculating the Total Protein Content

A fitted line plot of BSA protein concentration (μg/ml) against OD_{562} was created, allowing the protein concentration of each sample in the reaction tubes to be determined using the linear equation (Figure 6.9). The concentration of protein in the original 1 ml suspension was determined by multiplying the concentration of protein in the reaction tube.

6.3 RESULTS

6.3.1 Determining the Sub-Lethal Concentration of AHRs

6.3.1.1 Validation of PBS as a Neutralizer for AHR at a 1 in 9 Dilution

PBS was used to dilute AHR to a sub-lethal concentration during hydrophobicity testing in section 6.2.2; therefore its ability to quench the bactericidal effect of AHR was validated. Results are shown in Table 6.1. There was no significant difference between test and control counts for Soft Care Med H5, Cutan and Guest Medical ($P \geq 0.05$), indicating that PBS was effective at neutralizing the bactericidal activity of the AHRs at a 1 in 9 dilution.

6.3.1.2 Validation of TSB as a Neutralizer for AHR at a 1 in 9 Dilution

TSB was used to dilute AHR to a sub-lethal concentration during the test to determine the sub-lethal activity of AHRs in section 6.2.1.3; therefore its ability to quench the bactericidal effect of AHR was validated. Results are shown in Table 6.2. There was no significant difference between test and control counts for Soft Care Med H5, Cutan and Guest Medical ($P \geq 0.05$), indicating that TSB was effective at neutralizing the bactericidal activity of the AHRs at a 1 in 9 dilution.

Table 6.1: PBS Neutralization Validation Results

Mean ± SD log10 cfu/ml of *S. aureus* NCIMB 9518 after exposure to Soft Care Med H5, Cutan and Guest Medical AHRs for the validation of PBS neutralizer test.

AHR	Log_{10} Control CFU/ml	Log_{10} Test CFU/ml
Soft Care Med H5	7.86 ± 0.05	7.89 ± 0.09
Cutan	7.80 ± 0.11	7.82 ± 0.06
Guest Medical	7.65 ± 0.28	7.68 ± 0.35

Table 6.2: TSB Neutralization Validation Results

Mean ± SD log10 cfu/ml of *S. aureus* NCIMB 9518 after exposure to Soft Care Med H5, Cutan and Guest Medical AHRs for the validation of TSB neutralizer test.

AHR	Log_{10} Control CFU/ml	Log_{10} Test CFU/ml
Soft Care Med H5	7.72 ± 0.10	7.69 ± 0.10
Cutan	7.70 ± 0.10	7.66 ± 0.10
Guest Medical	7.68 ± 0.07	7.77 ± 0.08

6.3.1.3 Determining the Sub-Lethal Concentration of AHRs

Growth curves of *S. aureus* NCIMB 9518 in the presence of sub-lethal concentrations of AHR are shown in Figure 6.3. The 1 in 10 dilution grew slower than the 1 in 12, which grew slower than the 1 in 14 dilution, for all AHRs. Soft Care Med H5 reduced bacterial growth the most, followed by Guest Medical, and Cutan reduced bacterial growth the least. Cutan and Guest Medical AHRs showed a clear increase in bacterial number over time at dilutions 1 in 10, 1 in 12 and 1 in 14, whereas Soft Care Med H5 AHR did not. No dilutions of any AHR showed growth of *S. aureus* as rapid as that of the control. A 1 in 10 dilution of AHR produced the strongest sub-lethal activity against *S. aureus* NCIMB 9518 and therefore this dilution of AHR will be used during subsequent testing in this chapter to achieve a sub-lethal effect on *S. aureus* isolates.

6.3.2 Determining the Cell Surface Hydrophobicity of *S. aureus* Isolates

6.3.2.1 Salt Aggregation Test

Results of the SAT are shown in Table 6.3. No optimal aggregation was observed for any of the isolates at any concentration. A few small aggregates were observed for isolates 28, 34 and 51 at a concentration of 4.0 M, but this did not comply with the definition of 'true aggregation' according to Lindahl *et al.* (1981) and was therefore regarded as a negative result. Since no end point was observed for this test it was aborted after 1 run of pre-exposure to AHR.

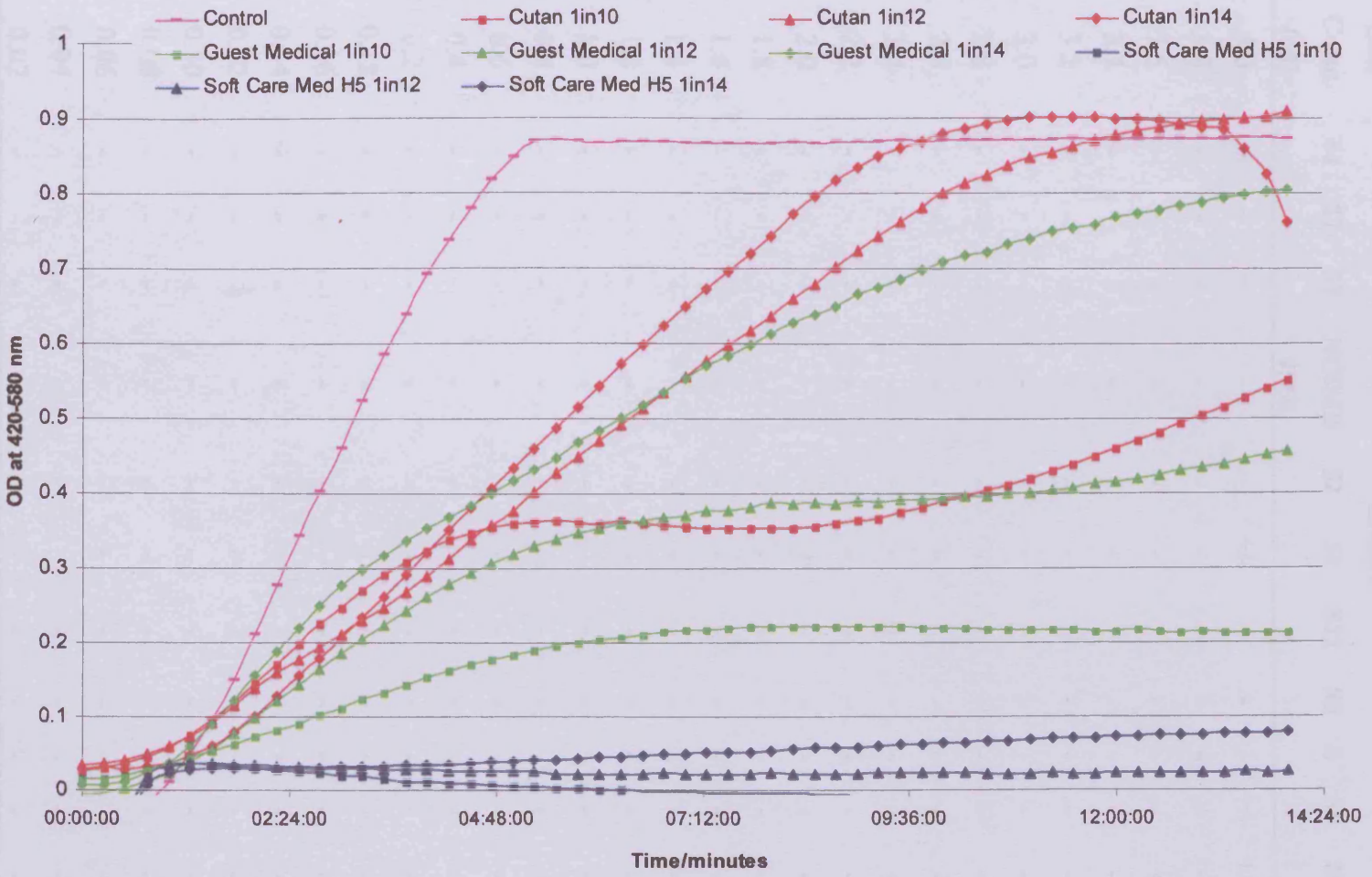

Figure 6.3: Sub-Lethal of 1in 10, 1 in 12 and 1 in 14 Diluted AHRs

Growth of *S. aureus* NCIMB 9518 in the presence of Soft Care, Cutan and Guest Medical AHRs diluted 1 in 10, 1 in 12 and 1 in 14

Table 6.3: Results of Salt Aggregation Test

Presence or absence of aggregation for *S. aureus* NCIMB 9518 plus 11 hospital isolates in the presence of varying concentrations of 'salt'. +/- = few or no aggregates, classed as a negative result, - = no aggregation.

Salt Conc. (M)	Isolate											
	44	27	37	NCIMB 9518	52	34	S12	S5	6	51	28	S1
4.0	-	-	-	-	-	+/-	-	-	-	+/-	+/-	-
3.8	-	-	-	-	-	-	-	-	-	-	-	-
3.6	-	-	-	-	-	-	-	-	-	-	-	-
3.4	-	-	-	-	-	-	-	-	-	-	-	-
3.2	-	-	-	-	-	-	-	-	-	-	-	-
3.0	-	-	-	-	-	-	-	-	-	-	-	-
2.8	-	-	-	-	-	-	-	-	-	-	-	-
2.6	-	-	-	-	-	-	-	-	-	-	-	-
2.4	-	-	-	-	-	-	-	-	-	-	-	-
2.2	-	-	-	-	-	-	-	-	-	-	-	-
2.0	-	-	-	-	-	-	-	-	-	-	-	-
1.8	-	-	-	-	-	-	-	-	-	-	-	-
1.6	-	-	-	-	-	-	-	-	-	-	-	-
1.4	-	-	-	-	-	-	-	-	-	-	-	-
1.2	-	-	-	-	-	-	-	-	-	-	-	-
1.0	-	-	-	-	-	-	-	-	-	-	-	-
0.8	-	-	-	-	-	-	-	-	-	-	-	-
0.6	-	-	-	-	-	-	-	-	-	-	-	-
0.4	-	-	-	-	-	-	-	-	-	-	-	-
0.2	-	-	-	-	-	-	-	-	-	-	-	-
0.18	-	-	-	-	-	-	-	-	-	-	-	-
0.16	-	-	-	-	-	-	-	-	-	-	-	-
0.14	-	-	-	-	-	-	-	-	-	-	-	-
0.12	-	-	-	-	-	-	-	-	-	-	-	-
0.10	-	-	-	-	-	-	-	-	-	-	-	-
0.08	-	-	-	-	-	-	-	-	-	-	-	-
0.06	-	-	-	-	-	-	-	-	-	-	-	-
0.04	-	-	-	-	-	-	-	-	-	-	-	-
0.02	-	-	-	-	-	-	-	-	-	-	-	-

6.3.2.2 Hexadecane Adherence Assay

The mean % adherence values for isolates pre- and post-AHR exposure are shown in Figure 6.4. Adherence was considered to be positive if % adherence was >75%, intermediate if % adherence was 25-75% and negative if % adherence was <25%. Isolates with a positive adherence were hydrophobic, those showing intermediate adherence were of intermediate hydrophilicity/hydrophobicity and those isolates with a negative adherence were hydrophilic. A total of 67% of the isolates were hydrophobic and 33% were of intermediate hydrophobicity/hydrophilicity pre-exposure to AHR.

The % adherence values of the 12 isolates were not significantly different from each other under either pre- or post-AHR exposure conditions ($P \geq 0.05$), except after exposure to Cutan ($P < 0.05$). There was also no significant difference between the % adherence values pre-and post-exposure to AHR for most isolates ($P \geq 0.05$). However, the % adherence values were significantly higher after exposure to Soft Care Med H5 for isolates 27, 37, S5 and 28, after exposure to Cutan for isolates S5 and 28 and after exposure to Guest Medical for isolate S5 ($P < 0.05$). The % adherence values were significantly lower after exposure to Cutan for isolates NCIMB 9518 and 34 ($P < 0.05$). This means that exposure to Soft Care Med H5 caused cells to become significantly more hydrophobic for isolates 27, 37, S5 and 28; exposure to Cutan caused cells to become significantly more hydrophobic for isolates S5 and 28, but caused cells to become significantly less hydrophobic for isolates NCIMB 9518 and 34; exposure to Guest Medical caused cells to become significantly more hydrophobic for isolate S5.

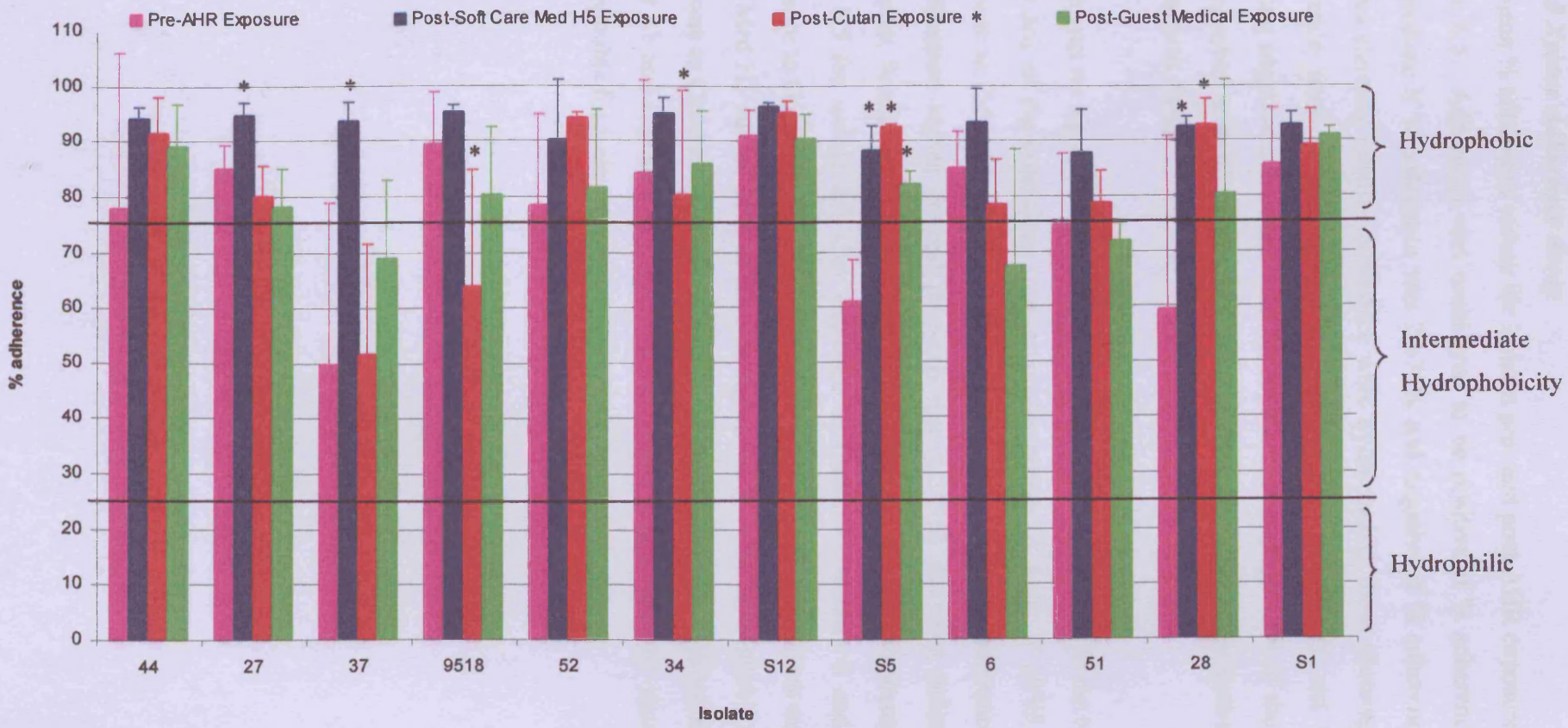

Figure 6.4: Results of the Hexadecane Adherence Assay

Mean percentage adherence +/- SD of *S. aureus* NCIMB 9518 plus 11 hospital isolates pre- and post- exposure to Soft Care Med H5, Cutan and Guest Medical AHRs for the Hexadecane Adherence Assay. * indicates where a value was significantly different.

6.3.2.3 Xylene Adherence Assay

The mean % adherence values for isolates pre- and post-AHR exposure are shown in Figure 6.5. Adherence was considered to be positive if % adherence was >75%, intermediate if % adherence was 25-75% and negative if % adherence was <25%. Isolates showing positive adherence were hydrophobic, those showing intermediate adherence were of intermediate hydrophilicity/hydrophobicity and those isolates showing negative adherence were hydrophilic. A total of 33% of the isolates were hydrophobic and 67% were of intermediate hydrophobicity/hydrophilicity pre-exposure to AHR.

There was no significant difference between the % adherence of the twelve isolates under any of the conditions (pre- or post-exposure to AHR; $P \geq 0.05$), except after exposure to Cutan ($P < 0.05$). There was also no significant difference between the % adherence values pre-and post-exposure to AHR for most isolates ($P \geq 0.05$). However, % adherence values were significantly higher after exposure to Soft Care Med H5 for isolate S1, after exposure to Cutan for isolates 6 and S1 and after exposure to Guest Medical for isolate 6 ($P < 0.05$). This means that exposure to Soft Care Med H5 caused cells to become significantly more hydrophobic for isolate S1, exposure to Cutan caused cells to become significantly more hydrophobic for isolates 6 and S1 and exposure to Guest Medical caused cells to become significantly more hydrophobic for isolate 6.

Figure 6.5: Results of the Xylene Adherence Assay

Mean percentage adherence +/- SD of *S. aureus* NCIMB 9518 plus 11 hospital isolates pre- and post- exposure to Soft Care Med H5, Cutan and Guest Medical AHRs for the Xylene Adherence Assay. * indicates where a value was significantly different.

6.3.2.4 Comparison of HAA and XAA

The HAA and XAA hydrophobicity tests produced different % adherences for *S. aureus* isolates 44, 27, 37, NCIMB 9518, 52, 34, S12, S5, 6, 51, 28, S1 (Figures 6.4 and 6.5, respectively), which resulted in some of the isolates being categorised as being of different hydrophobicities. This was partly due to the % adherence values being near the boundary of hydrophobic and intermediate categories, partly due to the large variability between replicates and partly due to actual differences between the tests. A comparison of the hydrophobicity of the isolates using the HAA and XAA is shown in Table 6.4.

Statistical comparison of HAA and XAA % adherence results pre- and post-exposure to AHR was also performed, according to section 2.8.1. There was no significant difference between the two assays ($P \geq 0.05$), except isolate S5 had a significantly higher % adherence after exposure to Cutan using the HAA compared to using the XAA ($P < 0.05$). This is in spite of the fact that less isolates were categorised as hydrophobic and % adherence appeared lower for some isolates for the XAA compared to the HAA.

The isolates were ranked from 1-12 according to the highest (1) to the lowest (12) mean % adherence values for the HAA and the XAA hydrophobicity tests pre- and post-exposure to each AHR (see Table 6.5). The isolates were found to not be ranked in the same order for the HAA and XAA for pre- or post-exposure conditions.

The correlation co-efficient was determined for HAA and XAA to determine whether they were positively correlated. Scatterplots were created of the mean % adherence values for each of the isolates for the HAA and XAA, one plot for each condition (pre- and post- exposure to AHR). Distribution of data for each scatterplot was confirmed as satisfactory for using the Spearman's rank correlation coefficient. The Spearman's rank correlation coefficients (r_s) are shown in Table 6.6. The r_s values were lower than the critical value (minimum value required for a significant result, which was determined from a standard table of values according to the number of pairs of data points) for each condition; therefore there was no correlation between the HAA and XAA pre- or post-exposure to AHR ($P < 0.05$).

Table 6.4: Comparison of Hydrophobicity of *S. aureus* Isolates Using the HAA and XAA

Hydrophobicity of *S. aureus* isolates pre- and post-exposure to Soft Care Med H5, Cutan and Guest Medical using the HAA and XAA hydrophobicity tests. + = hydrophobic (adherence of >75%), +/- = intermediate hydrophobicity/hydrophilicity (adherence of 25-75%). Isolates are listed from the most-susceptible to AHR (isolate 44) to the least-susceptible (isolate S1). HAA = hexadecane adherence assay, XAA = xylene adherence assay.

Isolate	Pre-exposure		Post-Soft Care Med H5		Post-Cutan		Post-Guest Medical	
	HAA	XAA	HAA	XAA	HAA	XAA	HAA	XAA
44	+	+	+	+/-	+	+	+	+
27	+/-	+/-	+	+	+/-	+/-	+/-	+
37	+	+/-	+	+	+/-	+/-	+	+
NCIMB 9518	+	+/-	+	+	+	+/-	+	+/-
52	+	+	+	+	+	+	+	+
34	+	+	+	+	+	+	+	+
S12	+/-	+	+	+	+	+	+	+/-
S5	+	+/-	+	+	+	+	+/-	+
6	+	+/-	+	+	+	+	+	+/-
51	+/-	+/-	+	+	+	+	+/-	+/-
28	+/-	+/-	+	+	+	+	+	+
S1	+	+/-	+	+	+	+	+	+

Table 6.5: Rank of *S. aureus* Isolates According to % Adherence Using HAA and XAA

Isolates are listed from the most-susceptible to AHR (isolate 44) to the least-susceptible (isolate S1). Isolates were ranked from 1-12 according to the highest (1) to the lowest (12) mean % adherence values for the HAA and the XAA pre- and post-exposure to Soft Care Med H5, Cutan and Guest Medical AHR. HAA = Hexadecane Adherence Assay, XAA = Xylene Adherence Assay.

Isolate	Ranked % Adherence Values for HAA versus XAA							
	Pre-Exposure		Post-Soft Care Med H5		Post-Cutan		Post-Guest Medical	
	HAA	XAA	HAA	XAA	HAA	XAA	HAA	XAA
44	5	10	8	1	8	10	10	12
27	1	2	7	11	1	3	2	5
37	11	1	11	2	2	2	6	6
NCIMB 9518	6	6	3	3	1	1	7	4
52	7	9	10	7	6	9	9	7
34	12	12	12	12	12	8	11	10
S12	3	11	2	10	9	7	8	2
S5	8	3	6	4	3	6	1	8
6	9	5	9	9	5	4	4	3
51	4	4	1	5	4	5	3	1
28	2	7	4	6	10	11	5	9
S1	10	8	5	8	7	12	12	11

Table 6.6: Correlation between HAA and XAA

Spearman's rank correlation coefficients of the ranked % adherence values for the HAA versus XAA pre- and post- exposure to Soft Care Med H5, Cutan and Guest Medical. The critical value was based on the number pairs of data points used to calculate the correlation coefficient. HAA = Hexadecane Adherence Assay, XAA = Xylene Adherence Assay, r_s = Spearman's rank correlation coefficient, - = no correlation.

	Ranked % Adherence Values for HAA versus XAA			
	Pre-Exposure	Post-Soft Care Med H5	Post-Cutan	Post-Guest Medical
r_s	0.08	0.12	0.39	0.53
Critical Value	0.59	0.59	0.59	0.59
Correlation	-	-	-	-

6.3.3 Determining the Surface Charge of *S. aureus* Isolates

The results of surface charge analysis are shown in Figure 6.6. Data were interpreted according to manufacturer's instructions (Malvern Instruments), where <-30 mV or >+30 mV is classed as a high zeta potential and >-30 to <+30 is classed as a low zeta potential; therefore out of a total of 48 samples (12 bacterial strains under 4 conditions) 19 isolates had high zeta potentials and 29 isolates had low zeta potentials. There was a significant difference in the zeta potential values between the 12 isolates pre-exposure and post-exposure to Soft Care Med H5 (P <0.05), but no significant difference in the zeta potential values between the isolates post-exposure to Cutan and Guest Medical ($P \geq 0.05$). There was no significant difference between zeta potential values pre- and post-exposure to AHR for any isolates ($P \geq 0.05$), except isolate 28 had a significantly lower (less negative) and isolate S5 had a significantly higher (more negative) zeta potential after exposure to Soft Care Med H5, isolate 37 had a significantly higher zeta potential after exposure to Cutan and isolate S5 had a significantly higher zeta potential after exposure to Guest Medical (P <0.05).

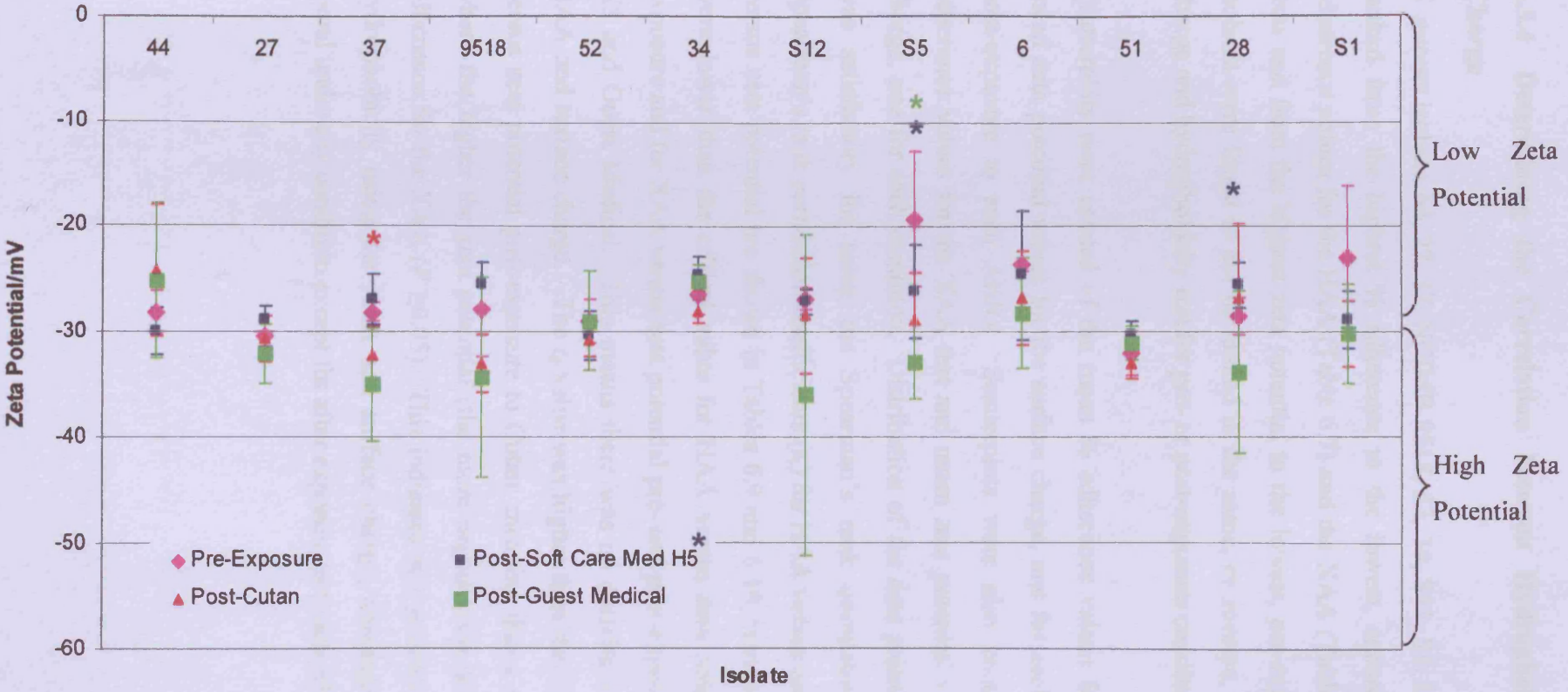

Figure 6.6: Results of Surface Charge Analysis of *S. aureus* Pre- and Post- Exposure to AHR

Mean zeta potential ± SD of *S. aureus* NCIMB 9518 plus 11 hospital isolates pre- and post-exposure to Soft Care Med H5, Cutan and Guest Medical AHRs. * indicates where a value was significantly different and their colour indicates to which condition they refer.

6.3.4 Determining the Correlation between Hydrophobicity and Surface Charge

S. aureus isolates 44, 27, 37, NCIMB 9518, 52, 34, S12, S5, 6, 51, 28 and S1 were ranked from the highest % adherence to the lowest, according to the mean % adherence values for the HAA (Table 6.7) and the XAA (Table 6.8) hydrophobicity tests and from the highest zeta potential to the lowest, pre-exposure to AHR. The isolates were found to not be ranked in the same, or reverse, order for the surface charge and hydrophobicity test for pre- or post-exposure conditions.

Scatterplots were created of the mean % adherence values for the HAA test and mean zeta potential values for the surface charge, one for each condition (pre- and post-exposure to each AHR). Scatterplots were also created of the mean % adherence values for the XAA test and mean zeta potential values for the surface charge, one for each condition. Distribution of the data points for each scatterplot was satisfactory for using the Spearman's rank correlation coefficient. The Spearman's rank correlation coefficients (r_s) for HAA versus zeta potential and XAA versus zeta potential are shown in Tables 6.9 and 6.10, respectively. The r_s values were lower than the critical value for HAA versus zeta potential pre- and post-exposure and for XAA versus zeta potential pre- and post-exposure to Soft Care Med H5 and Guest Medical. This means there was no correlation (P <0.05) between HAA and surface charge. The r_s value was higher than the critical value for XAA versus zeta potential post-exposure to Cutan, meaning that a correlation may exist where the higher the zeta potential (the more negative the mV) the higher the % adherence for the XAA ($P \geq 0.05$). This indicates at a possible association between hydrophobicity using the XAA and surface charge, although no association was found under any condition except for after exposure to Cutan AHR.

Table 6.7: Rank of *S. aureus* Isolates According to % Adherence Using HAA and Zeta Potential

Isolates are listed from the most-susceptible to AHR (isolate 44) to the least-susceptible (isolate S1). Isolates were ranked from the highest % adherence to the lowest, according to the mean % adherence values for the HAA and from the highest zeta potential to the lowest, pre- and post- exposure to Soft Care Med H5, Cutan and Guest Medical.

Isolate	Ranked % Adherence Values for HAA versus Zeta Potential							
	Pre-Exposure		Post-Soft Care Med H5		Post-Cutan		Post-Guest Medical	
	HAA	Zeta	HAA	Zeta	HAA	Zeta	HAA	Zeta
44	5	5	8	3	8	12	10	11
27	1	6	7	7	1	3	2	2
37	11	7	11	9	2	2	6	3
NCIMB 9518	6	3	3	1	11	4	7	9
52	7	9	10	12	6	9	9	12
34	12	8	12	6	12	8	11	1
S12	3	12	2	8	9	7	8	5
S5	8	10	6	11	3	10	1	10
6	9	2	9	5	5	5	4	6
51	4	1	1	2	4	1	3	7
28	2	4	4	10	10	11	5	4
S1	10	11	5	4	7	6	12	8

Table 6.8: Rank of *S. aureus* Isolates According to % Adherence Using XAA and Zeta Potential

Isolates are listed from the most-susceptible to AHR (isolate 44) to the least-susceptible (isolate S1). Isolates were ranked from the highest % adherence to the lowest according to the mean % adherence values for the XAA and from the highest zeta potential to the lowest, pre- and post- exposure to Soft Care Med H5, Cutan and Guest Medical.

Isolate	Ranked % Adherence Values for XAA versus Zeta Potential							
	Pre-Exposure		Post-Soft Care Med H5		Post-Cutan		Post-Guest Medical	
	XAA	Zeta	XAA	Zeta	XAA	Zeta	XAA	Zeta
44	10	5	1	3	10	12	12	11
27	2	6	11	7	3	3	5	2
37	1	7	2	9	2	2	6	3
NCIMB 9518	6	3	3	1	1	4	4	9
52	9	9	7	12	9	9	7	12
34	12	8	12	6	8	8	10	1
S12	11	12	10	8	7	7	2	5
S5	3	10	4	11	6	10	8	10
6	5	2	9	5	4	5	3	6
51	4	1	5	2	5	1	1	7
28	7	4	6	10	11	11	9	4
S1	8	11	8	4	12	6	11	8

Table 6.9: Correlation between HAA and Surface Charge

Spearman's rank correlation coefficients of the ranked % adherence values for the HAA versus zeta potential values for surface charge pre- and post- exposure to Soft Care Med H5, Cutan and Guest Medical. The critical value was based on the number of pairs of data points used to calculate the correlation coefficient. HAA = Hexadecane Adherence Assay, r_s = Spearman's rank correlation coefficient, - = no correlation.

	Ranked % Adherence Values for HAA versus Zeta Potential			
	Pre-Exposure	**Post-Soft Care Med H5**	**Post-Cutan**	**Post-Guest Medical**
r_s	0.24	0.34	0.45	0.13
Critical Value	0.59	0.59	0.59	0.59
Correlation	-	-	-	-

Table 6.10: Correlation between XAA and Surface Charge

Spearman's rank correlation coefficients of the ranked % adherence values for the XAA versus zeta potential values for surface charge pre- and post- exposure to Soft Care Med H5, Cutan and Guest Medical. The critical value was based on the number of pairs of data points used to calculate the correlation coefficient. XAA = Xylene Adherence Assay, r_s = Spearman's rank correlation coefficient, + = correlation, - = no correlation.

	Ranked % Adherence Values for HAA versus Zeta Potential			
	Pre-Exposure	**Post-Soft Care Med H5**	**Post-Cutan**	**Post-Guest Medical**
r_s	0.34	0.15	0.71	0.15
Critical Value	0.59	0.59	0.59	0.59
Correlation	-	-	+	-

6.3.5 Determining the Correlation between Bacterial Adhesion and Susceptibility to AHR

S. aureus isolates 44, 27, 37, NCIMB 9518, 52, 34, S12, S5, 6, 51, 28 and S1 were ranked from the highest mean log reduction after 10 s exposure to AHR using the carrier test Bioscreen method to the lowest, using data from Figure 4.10 in Chapter 4 (see Table 6.11).

Scatterplots were created of the mean log reductions of each isolate versus either the mean % adherence values for the HAA, the mean % adherence values for the XAA or the mean zeta potential values. Comparison was performed post- exposure to Soft Care Med H5, Cutan and Guest Medical. Distribution of the data points for each scatterplot was satisfactory for using the Spearman's rank correlation coefficient. The Spearman's rank correlation coefficients (r_s) for log reduction versus HAA, XAA and zeta potential are shown in Table 6.12. The r_s values were lower than the critical value and therefore there was no correlation between bacterial adhesion and exposure to AHR ($P < 0.05$).

Table 6.11: Rank of *S. aureus* Isolates According to % Log Reduction after 10 s Exposure to AHR

Isolates are listed from the overall most-susceptible to AHR (isolate 44) to the overall least-susceptible (isolate S1). Isolates were ranked from the lowest log reduction after 10 s to the highest, after exposure to Soft Care Med H5, Cutan and Guest Medical.

Isolate	Ranked Mean log Reduction after 10 s exposure to AHR		
	Soft Care Med H5	Cutan	Guest Medical
44	12	12	11
27	11	10	12
37	9	7	6.5
NCIMB 9518	2	3	3
52	8	8.5	10
34	6	4.5	4
S12	10	11	8
S5	7	8.5	5
6	5	6	6.5
51	3	4.5	9
28	4	2	4
S1	1	1	1

Table 6.12: Correlation between Bacterial Susceptibility to AHR and Adhesion

Spearman's rank correlation coefficients of the ranked mean % adherence values for the HAA and XAA and the ranked mean zeta potential values versus the ranked mean log reduction after 10 s exposure to AHR. AHRs included Soft Care Med H5, Cutan and Guest Medical. The critical value was based on the number of pairs of data points used to calculate the correlation coefficient. HAA = Hexadecane Adherence Assay, XAA = Xylene Adherence Assay, r_s = Spearman's rank correlation coefficient, - = no correlation.

	Ranked Mean Log Reduction after 10 s Exposure to AHR Versus Post-Exposure to Adhesion Tests								
	Soft Care Med H5			**Cutan**			**Guest Medical**		
	HAA	**XAA**	**Zeta**	**HAA**	**XAA**	**Zeta**	**HAA**	**XAA**	**Zeta**
r_s	0.38	-0.01	0.37	-0.35	-0.13	0.21	-0.25	-0.28	0.12
Critical Value	0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.59
Correlation	-	-	-	-	-	-	-	-	-

6.3.6 Determining Cell Size, Arrangement and Surface Properties of *S. aureus* Using Microscopy

6.3.6.1 Cell Size and Arrangement of S. aureus Cells

SEM images of S. aureus cells at 10,000 x magnification are shown in Figure 6.7. Differences were observed between the arrangement of *S. aureus* cells among the isolates. Figure 6.7 shows that bacterial cells belonging to isolate S1 (Figure 6.7c) are arranged in large groups, whereas bacterial cells belonging to isolate NCIMB 9518 (Figure 6.7b) are arranged in smaller groups. Isolate 44 was arranged in smaller groups, but there were less bacteria in the specimen (Figure 6.7a).

The mean bacterial cell diameter was 0.76 ± 0.03 μm, 0.70 ± 0.01 μm and 0.74 ± 0.02 μm for *S. aureus* isolates 44, NCIMB 9518 and S1, respectively. Statistical analysis revealed that isolates 44 and S1 were significantly larger than isolate NCIMB 9518 ($P < 0.05$), but there was no significant difference between the diameter of isolates 44 and S1 ($P \geq 0.05$).

6.3.6.3 Determining the Presence/Absence of Capsules Surrounding S. aureus Cells

Images of capsule staining are shown in Figure 6.8. Capsules were found to be present for isolates 44 (Figure 6.8a), isolate NCIMB 9518 (Figure 6.8b) and S1 (Figure 6.8c).

6.3.7 Determining the Total Protein Content of *S. aureus* Cells

Figure 6.9 shows the fitted line plot of BSA concentrations against OD_{562}. The calculated concentration of protein in each test suspension is shown in Table 6.13. There was no significant difference between the protein concentration of isolates 44, NCIMB 9518 and S1 ($P \geq 0.05$). There was no significant difference between the dry weight of the bacterial samples ($P \geq 0.05$).

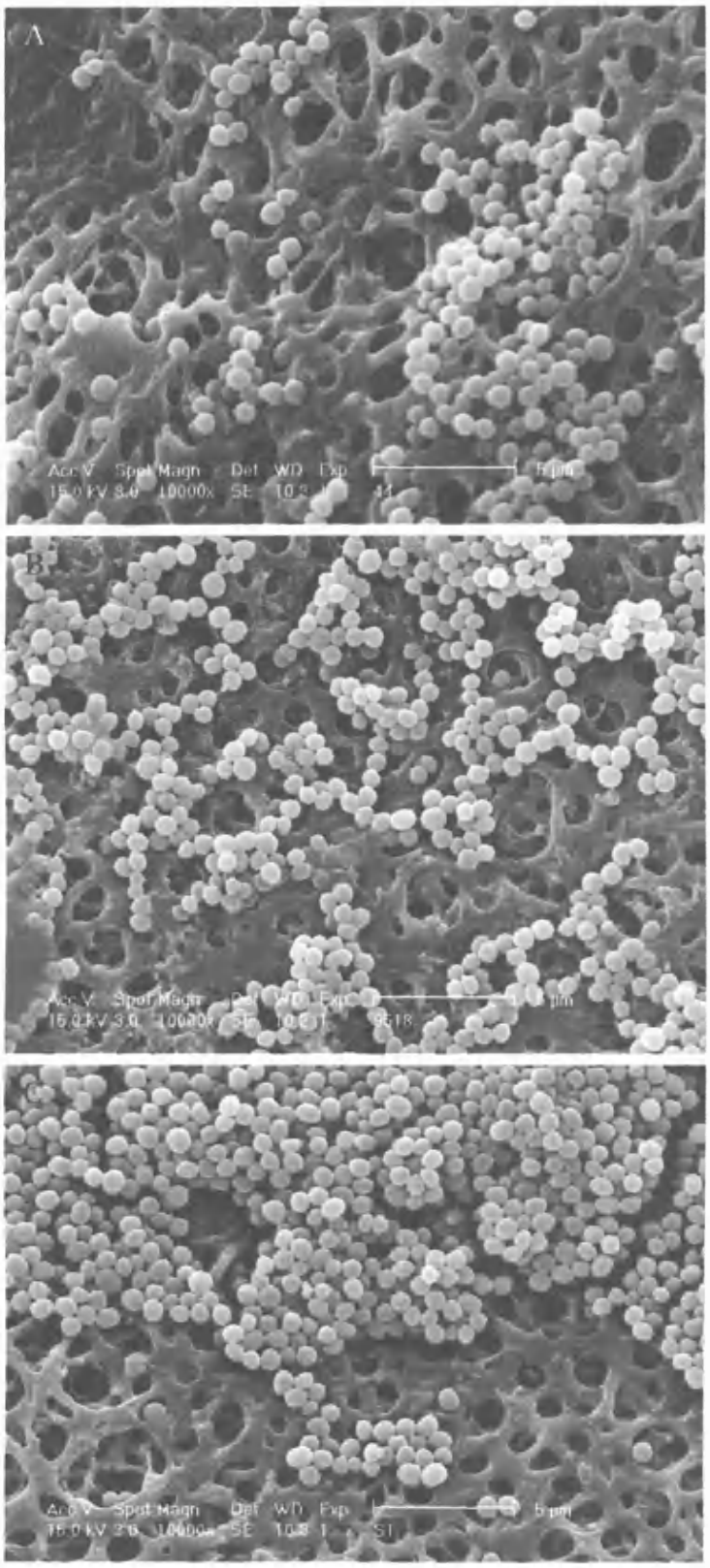

Figure 6.7: Size of *S. aureus* Cells

Shows *S. aureus* cells at 10,000 x magnification using SEM imaging. A = isolate 44, B = NCIMB 9518, C = isolate S1.

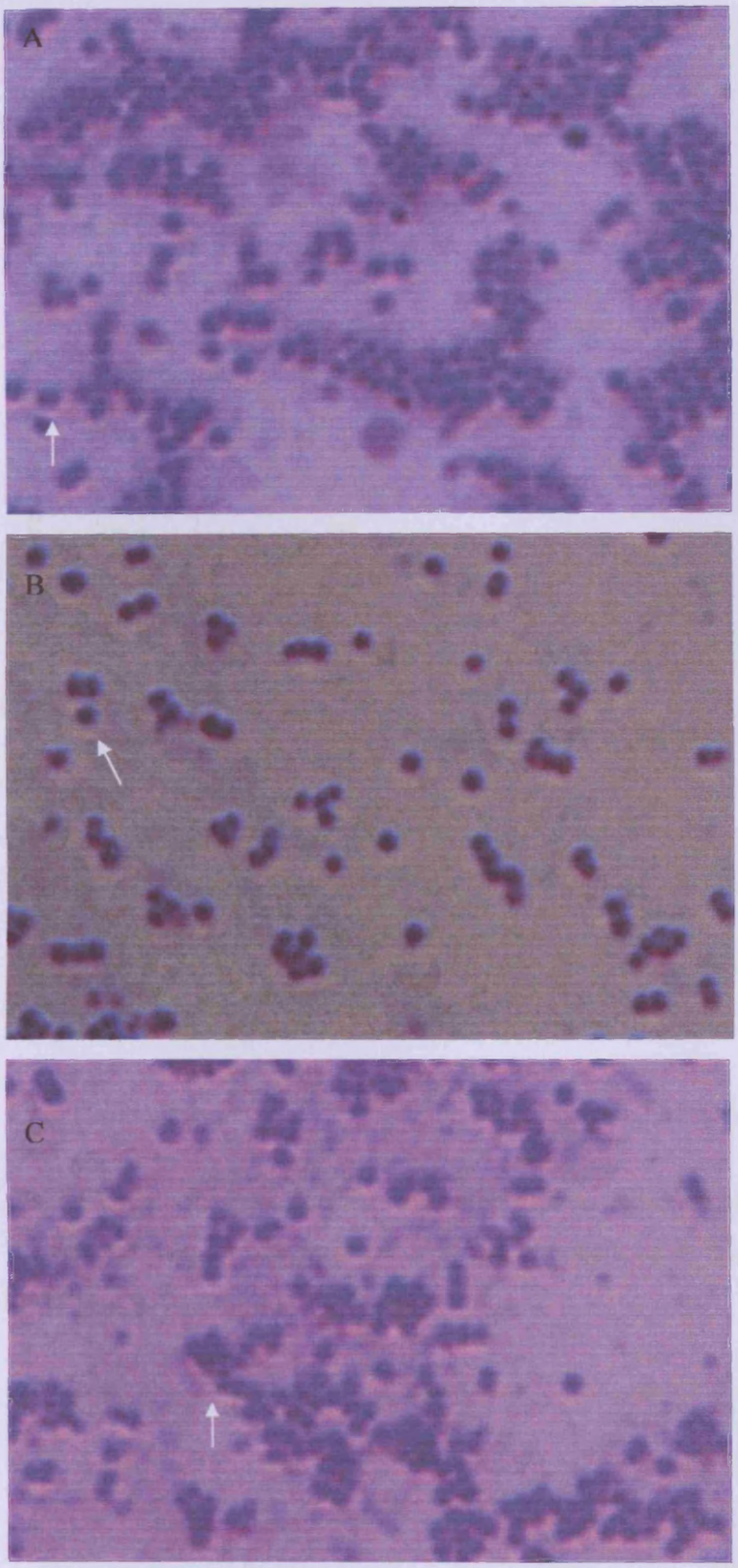

Figure 6.8: Capsule Staining of *S. aureus* Isolates

A = isolate 44, B = NCIMB 9518, C = S1. Arrows show presence of a capsule

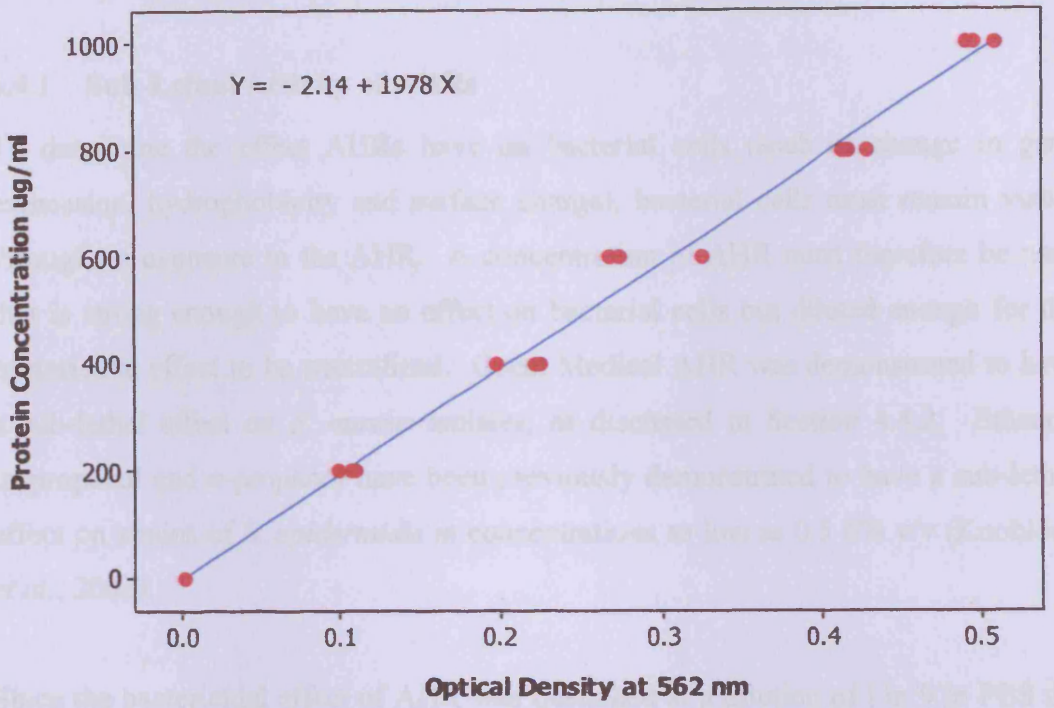

Figure 6.9: Fitted Line Plot of Protein Concentration vs Optical Density

The OD_{562} of known concentrations of BSA were plotted to provide a calculation for determining the protein concentration of test samples based on their OD_{562}.

Table 6.13: Concentration of Protein in *S. aureus* Isolates

Shows the mean ± SD total protein concentration of *S. aureus* isolates 44, NCIMB 9518 and S1 per 1 ml test sample.

Isolate	Mean Protein Concentration (mg/1 ml sample)
44	10.96 ± 2.07
9518	10.74 ± 2.24
S1	13.75 ± 1.08

6.4 DISCUSSION

6.4.1 Sub-Lethal Activity of AHRs

To determine the effect AHRs have on bacterial cells (such as change in gene expression, hydrophobicity and surface charge), bacterial cells must remain viable throughout exposure to the AHR. A concentration of AHR must therefore be used that is strong enough to have an effect on bacterial cells but diluted enough for the bactericidal effect to be neutralized. Guest Medical AHR was demonstrated to have a sub-lethal effect on *S. aureus* isolates, as discussed in Section 4.4.2. Ethanol, isopropanol and *n*-propanol have been previously demonstrated to have a sub-lethal effect on strains of *S. epidermidis* at concentrations as low as 0.5-6% v/v (Knobloch *et al.*, 2002).

Since the bactericidal effect of AHR was quenched at a dilution of 1 in 9 in PBS the assumption was that any other buffer would neutralize the AHR at a dilution of 1 in 9, due to the high concentration exponent of alcohols. The efficacy of 0.0001 M KCl as a neutralizer, which was used during surface charge testing, was therefore not validated. However, it was felt necessary to check the ability of TSB to quench the bactericidal effect of AHR at a dilution of 1 in 9, due to the greater quantity of ingredients it contains.

6.4.2 Hydrophobicity Testing and Its Limitations

The SAT has several limitations. Firstly, the SAT is fairly lengthy and is therefore not ideal for testing a large number of samples. Secondly, the test is qualitative and therefore only able to rank isolates according to their hydrophobicity and compare the hydrophobicity pre- and post- exposure to various conditions, rather than provide an actual value. Thirdly, electrostatic interactions are more likely to influence the results than other hydrophobicity tests (Rosenberg & Doyle, 1990). The SAT did not work on *S. aureus* isolates tested in this study, which was also the case for Dillon *et al.* (1986) and Emanuel (2008, personal communication). The SAT has, however, been previously successful in determining the cell surface hydrophobicity of *S. aureus* (Ljungh *et al.*, 1985; Rozgonyi *et al.*, 1985).

The HAA method had to be modified from that described in Greene *et al.* (1992) to include a washing step after exposure of cells to AHR. This is because the AHR precipitated upon exposure to hexadecane, resulting in a white appearance that caused the $OD_{+hexadecane}$ to be much higher than $OD_{initial}$. Washing was also performed using the XAA, since it was assumed that the same precipitation would occur with any hydrocarbon. Standardizing the two methods also allowed for a more reliable comparison of results. Washing cells was not found to prevent a significant change in hydrophobicity after exposure of cells to a biocide, even after washing cells three times (Camacho *et al.*, 2007).

Most isolates were hydrophobic and some were of intermediate hydrophobicity/hydrophilicity pre-exposure to AHR, which supports similar findings in a review of studies by Wadström, (1990), which revealed that most strains were hydrophobic.

Standard deviations for the % adherence obtained using the HAA and XAA were often very high; therefore interpretation of statistical data and forming conclusions derived from results should be done with caution. The lack of observed statistically significant increase in hydrophobicity post-AHR exposure for most isolates could be due to the fact that the hydrophobicity of some of the isolates was initially very high and also due to the large variation in replicate results. On the other hand, the statistical significance observed in some of the isolates under certain conditions may have proved non-significant if performed on other occasions, due to high variability. However, performing both tests and detecting a significant difference between the assays only for one isolate under one condition provides additional strength to an otherwise unreliable assay. High standard deviations may also be responsible for the fact that although there was a tendency towards lower % adherence values for the XAA compared to the HAA this was not statistically significant under most conditions.

The hydrophobicity of the isolates varied depending on whether the HAA or XAA was used; therefore the isolates were ranked differently according to their % adherence for these two tests. Dickson & Koohmaraie (1989) ranked a range of

bacterial species according to their hydrophobicity, as determined by the HAA and XAA. They also found that while some bacteria were ranked the same, or similar, for both tests other isolates were ranked very differently. Dillon *et al.* (1986) tested two strains of *S. aureus* using HAA and XAA and found to be similar, especially for one of the isolates.

6.4.3 Link between Hydrophobicity and Surface Charge

Tables 6.7 and 6.8 show that very few isolates were ranked the same, or similar, according to their surface charge and hydrophobicity; therefore no association was seen between hydrophobicity and surface charge in this study. Dickson & Koohmaraie (1989) also ranked bacteria according to their hydrophobicity as determined by the HAA and XAA and their surface charge. They also found that very few isolates were ranked the same or similar according to hydrophobicity and surface charge and there was therefore no association between hydrophobicity and surface charge. Matz & Jürgens (2001) also found no association between cell surface hydrophobicity and surface charge. Theoretically an inverse correlation should exist between hydrophobicity and surface charge, where the higher the cell surface hydrophobicity, the lower the surface charge. This is because surface charge increases the likelihood of polar interactions with water molecules, which results in more charged surface groups and therefore a lower hydrophobicity (Rosenberg & Doyle, 1990). Rosenberg & Doyle (1990) review a number of studies that compared hydrophobicity and surface charge, which either found no correlation or an inverse correlation.

6.4.4 The Effect of AHR Exposure on Non-Specific Adhesion in *S. aureus*

The effect of exposure of *S. aureus* to AHR on non-specific adhesion was investigated with respect to hydrophobicity using the HAA and XAA and surface charge (zeta potential). The results are summarised in Table 6.14. Although there was mostly an increase in hydrophobicity and surface charge after exposure to AHR, this was not often significant and some isolates under some conditions showed a decrease that was sometimes significant. These results indicate there is no strong effect of AHR exposure on the non-specific adhesion of bacteria, especially since

there is no apparent pattern among the isolates or the AHRs. Differences in adhesion of bacteria as a result of hydrophobicity and surface charge reflect differences in cell surface molecular structure, composition and orientation (Rosenberg & Doyle, 1990; Somasundaran, 2006). Since there were no real differences in hydrophobicity using the HAA and XAA or surface charge testing the zeta potential it therefore appears that there may not be differences in bacterial cell surface properties between isolates 44, NCIMB 9518 and S1 or pre- and post exposure to AHR. Further testing could be performed, however, using tests that have less variability, since it is important to determine whether *S. aureus* isolates that are able to survive exposure are more or less virulent than before.

Gorman (1991) found that change in the hydrophobicity of isolates from a number of bacterial species was not the same after exposure to chlorhexidine, providone iodine and taurolidine. He found that the change in hydrophobicity was not even the same among different hydrophobicity tests, although isolates tended to show a decrease in hydrophobicity. Jones *et al.* (1996) found a significant reduction in the number of *Pseudomonas aeruginosa* cells able to attach to plastic surfaces with incorporated biocides than without and cell surface hydrophobicity was lower. Bruinsma *et al.* (2001) found that after damaging *P. aeruginosa* cells by either sonication or exposure to a lens care solution they were less hydrophobic, which was likely due to damage caused to the cell surface. Bruinsma *et al.* (2006) found that *P. aeruginosa* isolates resistant to a QAC-based lens care solution had a less negative surface charge than susceptible strains, although there was no difference between the hydrophobicity of sensitive and resistant strains. They conclude that electrostatic repulsion between cationic surface molecules and QAC obstructs entry of biocide into the cell. The fact that exposure to AHR did not increase surface charge remarkably in this study indicates that entry of alcohol into the bacterial cell may not be impeded by the bacteria itself.

Table 6.14: The effect of Exposure of *S. aureus* to AHR on Non-Specific Adhesion

Indicates differences in hydrophobicity (% adherence using HAA and XAA) and surface charge (zeta potential) after exposure to Soft Care Med H5, Cutan and Guest Medical AHRs. * indicates a significant difference. HAA = Hexadecane Adherence Assay, XAA = Xylene Adherence Assay, ↑ = increase, ↓ = decrease

Isolates	Post-Exposure to Soft Care Med H5			Post-Exposure to Cutan			Post-Exposure to Guest Medical		
	HAA	XAA	Zeta	HAA	XAA	Zeta	HAA	XAA	Zeta
44	↑	↓	↑	↑	↑	↓	↑	↑	↓
27	↑*	↑	↓	↓	↑	↑	↓	↑	↑
37	↑*	↑	↓	↑	↑	↑*	↑	↑	↑
NCIMB 9518	↑	↑	↓	↓*	↑	↑	↓	↑	↑
52	↑	↑	↑	↑	↑	↑	↑	↑	↓
34	↑	↑	↓	↓*	↑	↑	↑	↑	↓
S12	↑	↑	↑	↓	↓	↑	↓	↑	↑
S5	↑*	↑	↑*	↑*	↓	↑	↑*	↓	↑*
6	↑	↑	↑	↓	↑*	↑	↓	↑*	↑
51	↑	↑	↓	↑	↑	↑	↓	↓	↓
28	↑*	↑	↓*	↑*	↑	↓	↑	↑	↑
S1	↑	↑*	↑	↑	↑*	↑	↑	↑	↑

6.4.5 Cell Size and Arrangement

It has previously been observed that environmental and clinical isolates may be larger than reference strains of the same species (Martin, personal communication), which supports the findings of this study. Cell size (or rather the differences in structure between different cell sizes) is unlikely to have an impact on the susceptibility to AHR, especially since the most- and least-susceptible isolates were the same size.

6.4.6 Capsule Formation

Isolates 44, NCIMB 9518 and S1 did not have mucoid colonies and therefore are not heavily encapsulated. They all possessed a capsule, as determined by microscopy and are therefore all microencapsulated.

6.4.7 Summary

The *S. aureus* isolates used in this study were either hydrophobic or of intermediate hydrophobicity and of both high and low zeta potential. No link was demonstrated between non-specific adhesion of *S. aureus* (by way of their hydrophobicity and surface charge) and their susceptibility to AHR. There was mostly no difference in the hydrophobicity or surface charge of the isolates post-exposure to AHR and certainly no trend in the direction of the exceptions. It therefore appears as though surface interactions do not change after exposure to AHR.

There was also no observable link between cell size and presence of capsule and their susceptibility to AHR, although cells of isolates less susceptible to AHR clumped together in larger clusters than those more-susceptible. It is likely, therefore that differences in susceptibility to AHR are related to the structure and composition of the bacterial cell wall and/or outer layers, since those were determined to be the main target for AHR in Chapter 5.

CHAPTER 7 GENERAL DISCUSSION

7.1 THE POTENTIAL LINK BETWEEN GENOTYPIC AND PHENOTYPIC TRAITS OF *S. AUREUS* ISOLATES

There were 10 different antibiotic resistance profiles for all hospital isolates (Table 3.8, Chapter 3), compared to 38 different RAPD banding patterns (Table 3.7, Chapter 3). This indicated that more than one genotypically identical group of isolates produced an identical antibiotic resistance (ABR) profile. However, some isolates that appeared to be of the same strain because they had identical banding profiles for all primers tested showed different ABR profiles. Genotypic and phenotypic profiles did not therefore match (Cheeseman *et al.*, 2007). This was also found by Norazah *et al.* (2009) and Uma Karthika *et al.* (2009). Variability within a strain may be the result of the loss/gain of ABR genes, but the reason variability was observed was most likely due to the small portion of the genome sampled by RAPD, which is unlikely to coincide with an ABR gene. Phenotypic results may not match genotypic results even if the primers were for ABR genes. This is because ABR genes may not be switched on in some cases, despite the gene being present, or the genes may be expressed in varying degrees (Depardieu *et al.*, 2007).

There were 12 antiseptic susceptibility (ASS) profiles for all hospital isolates (Table 4.3, Chapter 4), compared to the 38 RAPD banding patterns. More than one genotypically identical group of isolates showed the same ASS profile. However, some isolates with the same RAPD profile showed different ASS profiles. It is unclear what mechanisms are responsible for differences in ASS profiles. However, if reduced susceptibility is due to genetic differences between isolates the genes encoding them are unlikely to be included in the small portion of the genome transcribed by the primer.

The relationship between genotypic RAPD profiles and ABR/ASS profile is shown in Table 7.1. It shows that there was only one incidence where 3 isolates shared the same RAPD and ABR profiles. It also shows that there were six incidences where 2 isolates shared the same RAPD and ABR profiles, three incidences where 2 isolates shared the same RAPD and ASS profiles, plus only 1 incidence where 2 isolates shared the same RAPD, ABR and ASS profiles. Since there were 60 isolates used in this study some similarities would be expected to occur by chance. It is unlikely,

therefore, that there is a link between their genotypic grouping using RAPD and the phenotypic traits determined in this study among the isolates. Isolates found to be the same strain according to RAPD testing should not therefore be assumed to share the same phenotypic characteristics.

Table 7.1: The Relationship between Genotypic and Phenotypic Profiles of *S. aureus* Isolates

Previous chapters determined that there were 39, 10 and 12 RAPD, antibiotic resistance (ABR) and antiseptic susceptibility (ASS) profiles among the isolates, respectively. The isolates in this table are therefore assigned the numbers 1 to 39, 1 to 10 and 1 to 12 according to their RAPD, ABR and ASS profiles, respectively. The numbers are assigned arbitrarily to all isolates with the same profile; therefore all isolates within the same column with the same number showed the same profile. The numbers assigned to isolates should not be compared between columns, since this does not demonstrate a relationship. Rather, isolates assigned the same number in one column and also assigned the same number in a second column (but not necessarily the same number as the first column) show a relationship. For example, isolates 10 and 13 have been assigned the same ABR profile number (1) and have also been assigned the same ASS profile number (4). This means that in the case of both ABR and ASS, isolates 10 and 13 have the same profiles. The numbers have been colour-coded to aid comparison. All ABR and ASS profiles have been colour-coded, plus all but one of the RAPD profiles assigned to multiple bacteria.

Table 7.1:

Isolate	Profile Number			Isolate	Profile Number		
	RAPD	ABR	ASS		RAPD	ABR	ASS
3	19	3	4	43	2	3	3
4	32	7	9	44	25	1	1
5	1	4	4	45	22	3	4
6	31	1	9	46	4	3	3
9	13	5	7	47	37	10	5
10	13	1	4	48	11	9	5
11	7	5	5	49	11	9	3
13	8	1	4	50	4	3	3
14	27	2	5	51	23	8	10
15	8	3	5	52	39	5	5
16	34	1	NA	55	17	3	6
17	7	3	4	N10	12	1	5
18	1	4	5	K17	29	1	6
19	3	3	4	P59	18	3	5
20	1	6	NA	O43	15	5	5
21	36	1	5	Q49	28	1	6
24	26	10	5	T4	12	1	6
25	35	3	4	H9	5	8	6
26	2	3	4	S1	16	1	12
27	4	5	2	S2	38	1	11
28	1	1	11	S3	10	5	5
30	9	1	7	S4	10	5	3
31	2	3	4	S5	20	8	8
32	3	5	4	S6	10	3	3
34	9	10	6	S7	6	1	7
37	2	5	3	S8	5	10	6
38	24	8	5	S10	6	1	11
39	33	10	4	S11	30	1	4
41	1	9	3	S12	21	3	7
42	2	5	3	NCIMB 9518	14	10	4

7.2 THE POTENTIAL LINK BETWEEN SUSCEPTIBILITY TO AHR AND OTHER PHENOTYPIC TRAITS OF *S. AUREUS* ISOLATES

MRSA and MSSA isolates appeared to be evenly distributed among the antiseptic susceptibility profiles (Table 4.3, Chapter 4). This means MRSA did not have reduced susceptibility to AHR compared to MSSA, unlike with other antiseptics (Suller & Russell, 1999). This may mean that reduced susceptibility to AHR and other antiseptics is not mediated by the same mechanisms. Table 7.1 shows that the same ABR and ASS profiles were shared between 2 isolates on 7 occasions, 3 isolates on 4 occasions, 4 isolates on 2 occasions and 7 isolates on 1 occasion. Therefore although there were a number of situations where isolates shared both ABR and ASS profiles, this usually occurred between small numbers of isolates and does not necessarily indicate a relationship between ABR and ASS. This is because the similarities observed are likely due to there only being a small number of profiles to which the isolates could belong. It is therefore unlikely that a relationship exists between ABR and ASS, which may not be mediated by the same mechanisms.

This study found no link between non-specific adhesion of *S. aureus* (by way of their hydrophobicity and surface charge) and their susceptibility to AHR (section 6.3.5). The 12 isolates tested for non-specific adhesion were also tested for the presence/absence of the adhesion genes *cna*, *fnbA*, *fnbB* and *clfA* in section 3.3.4. Table 7.2 shows the adhesion gene profile of the 12 isolates in relation to their ABS profile. Six of the 12 isolates possessed all of the adhesion genes, five of the remaining six isolates did not possess one adhesion gene and one isolate did not possess two adhesion genes. There was also no apparent difference between the adhesion gene profiles of isolate 44 (the most-susceptible isolate to AHR) and isolate S1 (the least-susceptible isolate to AHR), except that isolate S1 did not have the gene *fnbB*, which encodes adhesion to fibronectin on host tissue. There does not appear, therefore, to be an association between non-specific adhesion and susceptibility to AHR.

Table 7.2: Adhesion Gene Profiles of *S. aureus* Isolates

Demonstrates presence (+) or absence (-) of adhesion genes in *S. aureus* reference strain NCIMB 9518 plus 11 hospital isolates. Isolates are listed from the most-susceptible to AHR (isolate 44) to the least-susceptible (isolate S1).

Isolates	*fnbA*	*fnbB*	*cna*	*clfA*
44	+	+	-	+
27	+	+	+	+
37	+	+	+	+
NCIMB 9518	+	+	-	+
52	+	+	+	+
34	+	+	+	+
S12	+	-	+	+
S5	+	-	+	+
6	+	+	+	+
51	+	+	-	+
28	+	+	+	+
S1	+	-	-	+

The surface charge of bacteria was found to be associated with susceptibility to cationic compounds, where more negatively charged bacteria were more susceptible to cationic compounds (Bruinsma *et al.*, 2006). This was due to electrostatic repulsion between the cationic compound and the less negatively charged bacterial surface reducing interaction with, and therefore gaining entry to, the bacterial cell. AHR is not a cationic compound and surface charge of the bacteria cannot reduce AHR interaction with bacterial cells. The differences observed in susceptibility of the isolates to AHR is therefore not likely to be linked to their ability to adhere to surfaces, or to each other and is likely due to some other factor.

7.3 HOW EFFECTIVE IS AHR AGAINST *S. AUREUS?*

Soft Care Med H5 was found to be the most efficacious AHR against *S. aureus* using the stainless steel carrier test (Table 4.3, Chapter 4). Cutan was found to be the most effective using the *ex-vivo* carrier test (Table 4.4, Chapter 4), which was likely due to its high viscosity decreasing evaporation time of the alcohol and increasing the efficacy over the 20 min contact time. In practice, however, the AHR is rubbed in and evaporates quickly, making Soft Care Med H5 the most efficacious AHR in practice. This is due to its formulation, since Soft Care Med H5 contains alcohols that are most efficacious (Rotter *et al.*, 2001).

Soft Care Med H5 AHR met the criteria of EN13697 (European Standard EN13697, 2001), since it was able to achieve a ≥ 4 $\log_{10}$ reduction within 5 min. However, Cutan and Guest Medical did not, since a ≥ 4 $\log_{10}$ reduction was not achieved for all isolates within 5 min (only 16% and 86% of isolates, respectively). This indicates that some AHRs are not of an acceptable standard according to the European Committee for Standardization. AHRs have also failed to meet the criteria of EN1500 (European Standard EN1500, 1997; Kramer *et al.*, 2002). These standard methods do not, however, take into consideration the contact time of AHR on the hands of HCWs in practice, which is due to the rapid evaporation of AHR and the lack of residual activity (section 4.3.3.3). It was found that only Soft Care Med H5 and Guest Medical were able to achieve a ≥ 4 log reduction for only one isolate (Table 4.3, Chapter 4) within the contact times used in practice (11-15 s, Table 4.1, Chapter 4) using the stainless steel carrier test. It was found that AHR was even less

effective at reducing the number of viable *S. aureus* cells on the surface of skin, with no AHR able to meet the criteria of EN13697 (Table 4.4, Chapter 4). It does not therefore appear that AHRs are effective at achieving an acceptable reduction of *S. aureus* cells from the hands of HCW by contact times set by the European Committee for Standardization, or those used in practice.

The bactericidal efficacy of alcohol is caused by its ability to damage proteins and lipids within bacterial cells (Ali *et al.*, 2001) and is membrane active (Franklin & Snow, 1989; Ali *et al.*, 2001). The AHRs tested in this study disrupted the integrity of the cytoplasmic membrane of *S. aureus* cells (section 5.3.3), causing intracellular leakage of K^+ (Figure 5.2, Chapter 5). Membrane damage was, however, unlikely to contribute to the bactericidal activity of the AHRs (section 5.3.2.3). AHR also damaged the intracellular contents of *S. aureus* cells of the most-susceptible isolate to AHR, resulting in a change in the appearance of the bacterial cytoplasm (section 5.3.3) that is likely due to coagulation of protein (McDonnell & Russell, 1999). However, this damage to the bacterial cytoplasm was not observable until after a bactericidal effect was achieved and was not observed in ≥ 4 $\log_{10}$ cells, indicating that intracellular damage may not be the cause of the bactericidal effect. Exposure to AHR caused an increase in the appearance of UEM (section 5.3.3), which is likely to be polysaccharide that has come away from the bacterial cell surface as a result of damage (Jones *et al.*, 1969). It is therefore unclear, from this study and previous investigations, the exact cause(s) of the bactericidal activity of AHR against *S. aureus*.

7.4 HOW USEFUL IS AHR IN THE INTENSIVE THERAPY UNIT?

AHRs are the most efficacious instant hand sanitizers (Ayliffe *et al.*, 1988) and the use of AHR versus hand washing is known to increase hand hygiene compliance in the ITU (Hugonnet *et al.*, 2002; Creedon, 2005 and Eldridge *et al.*, 2006). AHR has also been found to be as effective as, or more effective than, hand washing at reducing bacterial numbers on the hands of HCWs (Larson *et al.*, 2001; Rotter, 2001; Girou *et al.*, 2002; Karabay *et al.*, 2005) and preventing HAI (Larson *et al.*, 1995). This is why AHR is used in the ITU and throughout the healthcare setting.

AHR is not, however, appropriate for use over gloves and since direct patient contact by HCWs involves the use of gloves, AHR does not have a direct role in the prevention of potential object-to-patient cross-contamination (Table 4.1, Chapter 4). This may partially explain why AHR was so infrequently used, particularly in MH (Table 4.1, Chapter 4). Use of AHR may, however, reduce potential contamination of objects by the hands of HCWs that may subsequently be transferred from the object to the patient via gloved hands.

AHR is also not effective at killing all microorganisms that are found in the healthcare environment. Most notable is that AHR is unable to reduce numbers of *Clostridium difficile* on the hands of HCWs (Oughton *et al.*, 2009). This is not limited to AHR, since alcohol, chlorhexidine, iodophors, parachlorometaxylenol (PCMX) and triclosan are not reliably sporicidal against *Clostridium spp* (Boyce & Pittet, 2002). AHR may not therefore be the most useful agent on a busy ward that is subject, or especially prone, to *Clostridium difficile* outbreak.

AHR is also not the ideal agent for use by HCWs whose religious beliefs do not permit them the consumption of alcohol, since their hand hygiene compliance may be low. Allegranzi *et al.* (2009) found that although a number of religions prohibit the consumption of alcohol, AHR is usually accepted in a medicinal capacity since it is used for optimal patient care delivery. Despite this they rationalize that some HCWs may avoid the use of AHR to prevent inhalation or absorption through the skin; therefore compliance may be reduced.

AHR also does not have any residual activity (section 4.3.3.3) and therefore cannot eliminate transient organisms that are picked up by the HCW in between applications. Hospital policy directs HCWs to sanitize their hands before wearing sterile gloves, before and after each patient contact, after any potential microbial contamination and after touching clinical equipment (Hosein, 2004). Hand hygiene should therefore be performed after very short intervals so that elimination of transient organisms will be achieved with repeat hygiene procedures, rather than through residual effect. Not all directions within the hospital hand decontamination policy were adhered to at all times, however, and as a result hand hygiene was not

performed as frequently as desired, allowing transient organisms to potentially be transferred via HCWs hands (Table 4.1, Chapter 4). The use of a hand hygiene agent with an effective immediate and persistent antimicrobial activity may therefore be more effective for ITU HCWs, such as an AHR containing a low percentage of antiseptic with a persistent antimicrobial effect (Gaonkar *et al.*, 2005; Shintre *et al.*, 2006).

7.5 CONCLUSION

Despite AHR comprising a large part of the hand hygiene regimens in the healthcare environment very little is known about its efficacy against conditions found in practice, such clinical strains attached to a surface. Very little is also known of their effect on bacterial cells and the causes of differences in susceptibility among isolates. This study aimed to discover how effective AHRs are in practice against *S. aureus*, the nature of their bactericidal effect on *S. aureus* cells and the cause of differences in susceptibility among *S. aureus* isolates. It was found that Soft Care Med H5, Cutan and Guest Medical AHRs were unable to achieve a bactericidal effect within the time HCWs took to rub AHR into their hands, which was particularly apparent when AHR was tested against bacterial cells on the surface of skin. AHR was found to have no residual effect and the mechanical action of hand rubbing was not found to increase bacterial cell death by very much. It is therefore unlikely that AHRs kill all microbial flora on HCWs hands in practice, although only *S. aureus* has been tested and such a large number of bacterial cells as used during carrier testing is not likely to be present in such a concentrated fashion.

As previously stated in the literature, AHR was found to damage the cytoplasmic membrane, which was demonstrated using K^+ leakage and TEM testing. This was not, however, found be the cause of the bactericidal effect or the differences in susceptibility among strains, as demonstrated with *S. aureus* protoplasts. Protoplast testing found it was the cell wall or outer layers that were implicated in the bactericidal effect, which is most likely to be protein denaturation. Protein denaturation could not be confirmed as the primary cause of the bactericidal effect due to lack of a suitable protocol. Further work is therefore necessary to determine the main mode of action of AHR using a quantitative method.

The presence/absence and expression of the selected virulence genes, hydrophobicity and surface charge appeared to provide no contribution to susceptibility of *S. aureus* to AHR. Further work is therefore necessary to find the cause of the difference in susceptibility to AHR.

S. aureus isolates in the hospital environment that have survived exposure to AHR did not appear to be more adherent to surfaces in terms of their hydrophobicity or surface charge and did not up-regulate expression of virulence genes that are not involved in increasing their survival, but down-regulated them in some instances. It is therefore not likely that *S. aureus* isolates that are exposed to AHR but not killed become more virulent.

AHRs have a number of disadvantages that make them less useful to HCWs in ITUs, and other hospital wards, than they could potentially be. Hand santitizing regimens currently used by ITU staff in local hospitals do not maximize prevention of transmission of microorganisms or HCAI. Improvements could be made to the AHR and hand sanitizing regimens to increase their usefulness and efficacy.

7.6 RECOMMENDATIONS

The author of this study proposes several recommendations:

It is recommended that future efficacy testing of instant hand sanitizing agents are performed using the stainless steel carrier test according to EN13697, but using clinical strains of organisms likely to be found in the hospital environment. It is also recommended that an *ex-vivo* test be performed where possible, since it reflects more accurately the conditions found in practice.

It is recommended that improvements are made to AHRs to increase their efficacy and usefulness and to correct their disadvantages. The advantages and disadvantages of AHRs are shown in Table 7.3, along with recommendations for improvement. It is recommended that the efficacy of AHR is increased by increasing the volume of AHR used per application. Since compliance is a problem amongst HCWs it is recommended that manufacturers alter the AHR dispensers so that one pump expels

a larger volume of AHR, rather than asking HCWs to use another pump, which may be too large a volume. It is recommended that the efficacy of AHR is also increased by developing the most efficacious combination of alcohol type and concentration. Until this has been completed it is recommended that the most efficacious AHR from this study (Soft Care Med H5) replace Cutan and Guest Medical AHRs and be used in conjunction with hand washing. It is also recommended that a non-alcoholic instant hand sanitizer be made available that is also sporicidal, particularly against *C. difficile*. This hand sanitizer can be provided for HCWs whose religion prohibits use of AHR so they will feel more comfortable in the workplace and may show increased hand hygiene compliance. This sanitizer can also be used in wards during an outbreak of *C. difficile*.

It is recommended that improvements are made to the hand hygiene regimens used in ITUs to minimize the spread of *S. aureus* and other microorganisms and potentially reduce HCAI. The best practice for hand sanitizing in ITUs is shown in Table 7.4, including improvements that can be made to achieve this. Firstly, HCWs should be re-trained to ensure that they put on gloves immediately prior to touching the patient to avoid the possibility of contaminating the patient with microorganisms picked up on the gloves from the environment. Secondly, HCWs should be informed that hand washing and/or use of AHR is sufficient for activities not involving the patient and that re-application of AHR is not necessary in between touching different objects. Thirdly, HCWs should be re-trained to ensure they apply AHR at regular intervals and immediately after touching their hair, forearms or clothing. This should change the low frequency of AHR use observed in the ITUs, particularly in MH (Table 4.1, Chapter 4).

Table 7.3: The Ideal Properties of Instant Hand Sanitizers

Shows the advantageous properties of an instant hand sanitizer and whether AHRs demonstrate these properties to excellent levels (++), acceptable levels (+) or unacceptable levels (x). Potential improvements are also shown, where realistically achievable.

Ideal Properties	Level demonstrated in AHR	Alcohol Hand Rub Improvements
Rapid activity	x	Increase volume dispensed with each pump and alter alcohol type/concentration
Broad activity	+	Add sporicidal agent
Residual activity	x	Add an active agent with residual activity
Sporicidal	x	Add a sporicidal agent
Gentle on skin	+	Provide alternative hand sanitizer for affected HCWs
Cost	++	Not applicable
Stain-resistance	++	Not applicable
Religious acceptance	+	Provide alternative hand sanitizer for affected HCWs

Table 7.4: Best Practice for Hand Sanitization in ITUs

Shows the best practice for hand sanitization in ITUs and whether HCWs observed in this study were observed to achieve this (Table 4.1, Chapter 4) to excellent levels (++), acceptable levels (+) or unacceptable levels (x). Potential improvements are also shown, where realistically achievable.

Best Practice for Hand Sanitization in ITUs	Achieved?	Improvements
Wash hands only if physically dirty/after patient contact	++	Not applicable
Use AHR frequently	x	Retrain staff
Apply a generous amount of AHR to hands	x	Retrain staff
Apply AHR to all areas of the hand	+	Retrain staff
Rub into hands for at least 1 min	x	Retrain staff
Apply AHR before glove application	x	Retrain staff
Apply gloves immediately prior to patient contact	x	Retrain staff
Remove gloves immediately after patient contact	+	Retrain staff
Apply AHR if own hair, skin or clothing is touched	x	Retrain staff

7.7 FUTURE WORK

Although Soft Care Med H5 was found to be the most efficacious AHR further work should be carried out to determine whether any other AHRs currently available are even more efficacious. Work should also be carried out to potentially develop the most efficacious combination of alcohol type and concentration to ensure AHRs used in the healthcare environment are as efficacious as they can be.

Further work should be performed to determine the effect of AHR on other major HCAIs such as *Streptococcus pneumoniae*, *Klebsiella spp*, *Enterococcus spp*, coagulase-negative staphylococci and *Escherichia coli* (HPA, 2008). Further work should also be performed to determine whether AHR is effective against rare but serious nosocomial pathogens such as *Mycoplasma* and *Ureaplasma spp.* Mycoplasma and Ureaplasma can be found in the urethra (Zenilman, 2007); therefore insertion of catheters and use of bed pans may facilitate the introduction of these organisms into the environment and thus their spread via the hands of HCWs.

This study was unable to determine the reason why *S. aureus* isolates showed differences in susceptibility to AHR. Further testing should therefore be performed to determine the genotypic/phenotypic differences in the isolates that cause differences in susceptibility. One hypothesis is that since protein denaturation is a mode of bactericidal activity (and likely the major mode of activity), differences in susceptibility of isolates to protein denaturation may be the reason for differences in bactericidal susceptibility. This hypothesis can be tested by comparison of the protein profiles of the most- and least-susceptible *S. aureus* isolates using two-dimensional gel electrophoresis, which separates proteins according to their isoelectric point and molecular weight (Gravel, 2002). If there are any differences in the presence/absence or proportion of particular protein species between these isolates this may be responsible for reduced (or increased) susceptibility to protein denaturation, decreased penetration through the cell wall or increased cell repair. Proteins of interest could be isolated from the gel and sequenced to discover their identity and function. This technique could not be performed post-exposure to AHR, since denatured proteins may be destroyed by the method used to break apart the cells to extract protein. This may, however, form a crude determination of the

protein undamaged during alcohol exposure and pre- and post- AHR exposure protein profiles could be performed and compared between isolates.

Further work should be performed to determine the effect of AHR on gene expression and identify the gene(s) responsible for causing bacteria to be less susceptible to AHR. This would need to be performed using microarray, since this method allows expression of all genes within the bacterial genome to be analysed (Schena *et al.*, 1995). The genome of isolates can be compared to determine the differences in gene expression between isolates with different susceptibility to AHR and compare an individual isolate pre- and post-exposure to AHR.

Currently manufactured alcohol-free instant hand sanitizers that are sporicidal should be searched for (or developed specifically) and tested according to conditions in practice, particularly with respect to their efficacy against *C. difficile*.

CHAPTER 8 REFERENCES

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