SENSITIVITY OF *STAPHYLOCOCCUS EPIDERMIDIS* TO CHLORHEXIDINE AND ASSOCIATED RESISTANCE PROPERTIES

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Summary

Staphylococcus epidermidis are common Gram-positive bacteria and are responsible for a number of life-threatening nosocomial infections. Treatment of *S. epidermidis* infection is problematic because the organism is usually resistant to many antibiotics. The high degree of resistance of this organism to a range of antibiotics and disinfectants is widely known.

The aims of this thesis were to investigate and evaluate the susceptibility of isolates of *S. epidermidis* from various infections to chlorhexidine (CHX) and to other disinfectants such as benzalkonium chloride (BKC), triclosan (TLN) and povidone - iodine (PI). In addition, the mechanisms of resistance of *S. epidermidis* to chlorhexidine (the original isolates and strains adapted to chlorhexidine by serial passage) were examined and co-resistance to clinically relevant antibiotics investigated.

In 3 of the 11 *S. epidermidis* strains passsged in increasing concentrations of chlorhexidine, resistance to the disinfectant arose (16-fold). These strains were examined further, each showing stable chlorhexidine resistance. Co-resistance to other disinfectants such as BKC, TLN and PI and changes in cell surface hydrophobicity were observed. Increases in resistance were accompanied by an increase in the proportion of neutral lipids and phospholipids in the cell membrane. This increase was most marked in diphosphatidylglycerol. These observations suggest that some strains of *S. epidermidis* can become resistant to chlorhexidine and related disinfectants/antiseptics by continual exposure. The mechanisms of resistance appear to be related to changes in membrane lipid compositions.

Key words: *Staphylococcus epidermidis*; disinfectant/chlorhexidine resistance; serial passage.

For

my parents, my husband and my daughters

Donya and Delsa

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ABBREVIATIONS

APCI-MS	atmospheric pressure chemical ionisation mass spectrometry
aa	amino acid
AAP	adhesion-associated protein
API	Analytical Profile Index
ATCC	American Type Culture Collection
BHI	brain heart infusion
BHIA	brain heart infusion agar
BKC	benzalkonium chloride
CAPD	continuous ambulatory peritoneal dialysis
CAPS	3[cyclohexylamino] -propane sulphonic acid
cfu	colony forming unit
CHEF	contour-clamped homogeneous electric field
CoNS	Coagulase-Negative Staphylococci
CVC	central venous catheter
DMF	N, N-dimethylforniamide
DMSO	dimethyl sulphoxide
EDTA	ethylenediaminetetraacetic acid
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
ES	EDTA, sarcosyl
ESP	EDTA, sarcosyl, proteinase K
FAME	fatty acid modifying enzyme
FPLC	fast protein liquid chromatography
FAMEs	fatty acid methyl esters
GC	gas chromatography
LPS	lipopolysaccharide
H	hour
HH	Hope Hospital, Satford
HHB	Heartlands Hospital, Birmingham
IE	infective endocarditis
IVET	*in vivo* expression technology
kb	kilo-base pairs

kDa	kilodattons
LB	Luria-Bertani
MBC/MLC	minimum bactericidal concentration minimum lethal concentration
MGDA	methylglycinediacetic acid
MIC	minimum inhibitory concentration min
Mr	relative molecular mass
MSCRAMM	microbial surface components recognising adhesion matrix molecules
Mw	molecular weight
na	nucleic acid
NCTC	National Collection of Type Cultures
NET-100	Tris-buffered saline EDTA
NVE	native valve endocarditis
NZY	NZ amine- yeast extract agar
OD	optical density
OR	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDH	pyruvate dehydrogenase complex
pfu	plaque-forming unit
PI	isoetectric point
PIA	polysaccharide intercellular adhesin
PS/A	polysaccharide/adhesin
PVDF	polyvinyldifluoridine
PVE	prosthetic valve endocarditis
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
PMN	polymorphonucteocyte
QAC	quaternary ammonium compound
QEH	Queen Elizabeth Hospital, Biniingham
RAPD	random amplified polymorphic DNA
RND	resistance-nodulation-division
ROH	Royal Orthopaedic HospitaL Birmingham
rpm	revolutions per minute

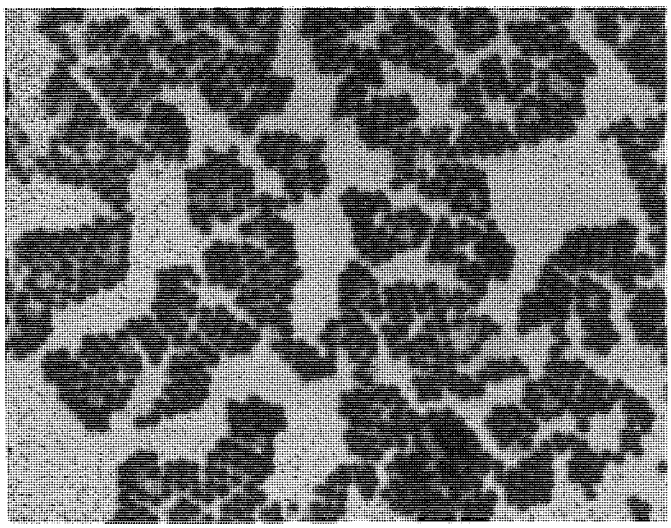
SAA	slime-associated antigen
sarcosyl	N-lauroyl-sarcosine
SDS	sodium dodecyl sulphate
SM	Tris-buffered saline, magnesium sulphate, gelatin
SSP	staphylococcal surface protein
TLC	thin layer chromatography
Tris	tris(hydroxymethyl)aminomethane
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TBS	Tris-buffered saline
TBST	Tris-buffered saline and Tween 20
TE	Tris EDTA
TEMED	N, N, N'N'-tetramethyl-ethylenediamine
TSA	tryptone soya agar
TSB	tryptone soya broth
UV	ultra violet
W/V	weight by volume
X-gal	5-brom-4-chloro-3-indolyi-p-D- galactopyranoside

Chapter 1: Introduction

1.1. Staphylococci

During the early 1880s, Sir Alexander Ogston, a Scottish surgeon, convincingly showed that a cluster-forming bacterium was the cause of certain abscesses in humans and that the disease could be reproduced in mice by the injection of the purulent material (Ogston, 1880). In recognition of its appearance microscopically, he called the microbe *Staphylococcus* (Figure1. 1), derived from the Greek *staphyle* (bunch of grapes) and *kokkus* (berry).

Figure 1. 1: Gram stain of Staphylococci



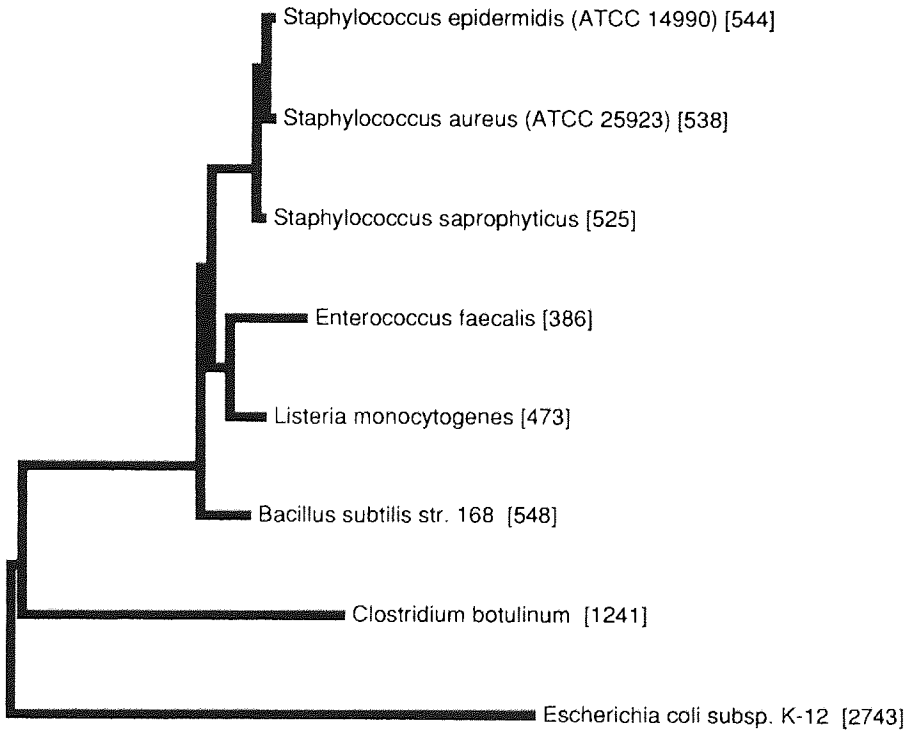
Staphylococci are Gram-positive spherical bacteria that occur in microscopic clusters resembling grapes. Bacteriological culture of the nose and skin of normal humans invariably yields staphylococci. In (1884), Rosenbach described the two-pigmented colony types of staphylococci and proposed the appropriate nomenclature: *Staphylococcus aureus* (yellow) and *Staphylococcus albus* (white). The latter species is now named *Staphylococcus "epidermidis"*. *Staphylococcus aureus* and *Staphylococcus "epidermidis"* are the most significant in their interactions with humans. Table 1.1 lists the essential properties of *S. epidermidis*.

Table 1.1: Major distinguishing features of *S. epidermidis* (Crowe, 2000)

1.2 Taxonomy and Phylogeny of Staphylococci

Determination of 16S rRNA sequences has allowed the construction of a universal phylogenetic tree and has led to the recognition of major bacterial phylogenetic groups or phyla (Woese CR, 1987). The genus *Staphylococcus* is a low G + C species of the Gram-positive bacteria group Staphylococci are closely related to members of the *Bacillus* genus, it has been suggested that "Staphylococcus" can be considered a morphologically degenerate form of *Bacillus* (Stackebrandt and Woese, 1981) (Fig.1.2).

Figure.1.2: Phylogeny of the *Staphylococcus* genus based on 16S r RNA sequences.



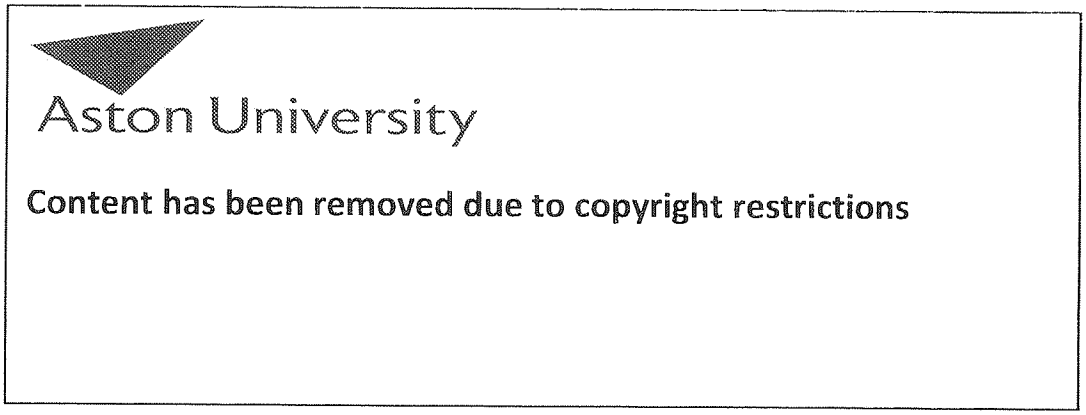
1.3 Coagulase-negative staphylococci (CoNS)

Staphylococci are facultative anaerobes that grow by aerobic respiration or by fermentation that yields principally lactic acid. The bacteria are catalase-positive and oxidase-negative (Todar, 1998). Staphylococci have been, and continue to be, a major cause of human disease, especially in the hospital setting, where they cause upward of one million serious infections per year. Staphylococci are also among the most common causes of bacterial infections in the community, accounting for a large majority of simple skin infections. Staphylococci are also responsible for a significant number of serious infections in the normal population, such as endocarditis, osteomyelitis, and septic shock (Projan, 1997).

The staphylococci that cause disease in humans and animals are not inherently pathogenic organisms (unlike the influenza virus, for example, which must cause disease to propagate). For every illness caused by a staphylococcus, many more individuals are benignly colonized with no sign of disease (Projan, 1999).

Staphylococcus epidermidis is a ubiquitous inhabitant of human skin and mucous membranes on the human cutaneus surface, comprising 65% to 90% of all recovered staphylococci (Archer, 1990) (Figure1.3). *S. epidermidis* may be haemolytic or nonhaemolytic, it requires biotin for growth and its lack of coagulase activity makes it different from *S. aureus*. *S. epidermidis* has a glycerol wall teichoic acid and has no protein A (Puppme *et al.*, 1994). *S. epidermidis* is one of the most common contaminants of samples sent to the diagnostic laboratory (Foster *et al.*, 1996).

Figure 1.3: Distribution of staphylococci on human skin (Kloos, 1996).



Most diseases caused by *S. epidermidis* involved infection of an implanted device such as a heart valve, hip prosthesis, pacemaker, vascular graft, or intravenous catheter. *S. epidermidis* also causes urinary tract infection (UTI) in elderly hospitalised men and causes native valve endocarditis in intravenous drug users and also causes peritonitis in patients receiving continuous ambulatory peritoneal dialysis (CAPD) (Foster *et al.*, 1996). Infections caused by *S. epidermidis* are often persistent and relapsing. Treatment of *S. epidermidis* infection is problematic because the organism is usually resistant to many drugs (Ruppme *et al.*, 1994).

1.3.1 Epidemiology of coagulase-negative staphylococci(CoNS)

The evaluation of epidemiological studies of CoNS is notoriously difficult. Traditional typing methods such as biotyping, antibiogram and bacteriophage typing have low discrimination. Strains of *S. epidermidis* exhibit too few distinct properties for phenotypic traditional methodology to have any significant discriminatory power (Toldos *et al.*, 1997; Geary *et al.*, 1997; Hedin, 1996). Molecular methods such as restriction endonuclease analysis of genomic DNA, ribotyping, plasmid analysis, DNA-DNA hybridization and randomly amplified polymorphic DNA (RAPD) analysis have provided valuable tools for the evaluation of the relatedness of staphylococcal isolates (Olive and Bean, 1999; Kluytmans *et al.*, 1998). Of these techniques, PFGE is currently considered to be the "gold standard" method for the epidemiological study of CoNS (Lang, 2000; Olive and Bean, 1999; Bannerman *et al.*, 1995).

1.3.1.1 Epidemiology of community-acquired infections

The major community-acquired infections due to CoNS are those associated with chronic indwelling catheters, such as intraperitoneal catheters in patients receiving CAPD, tunneled central venous catheters in patients with haematologic diseases or AIDS, (Fichtenbaum *et al.*, 1995) and ventriculoperitoneal catheters in patients with hydrocephalus. The other major community acquired infections due to CoNS are those associated with prosthetic joints, (Powers *et al.*, 1990) prosthetic cardiac valves, pacemakers, and vascular grafts. Coagulase -negative staphylococcus infections of vascular and other indwelling catheters may be secondary to contamination at the time of the procedure but are more often assumed to be acquired subsequent to initial placement. Infections of CoNS associated with implanted prostheses are more likely to be traced to contamination at the initial surgical procedure (Arber *et al.*, 1994).

CoNS and *S. aureus* are the most common causes of peritonitis associated with CAPD. When CoNS have been speciated, most infections are due to *S. epidermidis*

(Von Graevenitz and Amsterdam, 1992). Early studies seeking the source of CoNS responsible for peritonitis concluded that the source of infection also was organisms carried on the skin around the catheter site (Crossley and Kent, 1997).

With increasing use of semipermanent intravenous catheters, such as Hickman catheters, and implantable infusion ports for therapy in patients with cancer, AIDS, short bowel syndrome, and a variety of other illnesses, increased numbers of infections of these devices have been re ported. Coagulase- negative staphylococcus constitute the largest group of organisms causing intravascular catheter-related infections (Graham *et al.,* 1991 and Rannem *et al.,*1986) and most reviews of hospital-acquired CoNS bacteremias have found intravascular catheters to be the major source of the bacteremia. Most of these infections are due to *S. epidermidis,* many of which are resistant to most antibiotics; generally, these infections are caused by hospital strains that either have colonized the patient or have been transmitted by health care workers accessing these devices (Crossley and Kent, 1997).

1.3.2 Modes of transmission

Until recently, methods for differentiating one strain of *S. epidermidis* from another were not available and, as a result, establishing modes of transmission of these organisms has been difficult. Direct contact transmission by cardiac surgeons whose hands were contaminated with an epidemic strain has been documented on several occasions (Boyce *et al.,* 1990).

Airborne transmission has been suggested as another means by which CoNS are transmitted, based on the fact that air adjacent to the operative field may contain bacteria, including CoNS (Duhaime *et al.,* 1991). Reducing the number of airborne bacteria in the operative field by using ultra clean air handling systems and surgical isolator systems has been shown to reduce infection rates following implant surgery (Crossley and Kent, 1997).

It is possible that indirect transmission of CoNS by fomites such as clothing of personnel or contaminated medical equipment may occur, but appropriate studies to address this issue have not been done (Hedin and Hambraeus, 1991).

1.4 Coagulase-negative staphylococci as pathogens

In the 1960s, several groups recognized the pathogenic potential of CoNS. Their important role in infection became widely accepted until the 1970s (Kloos and Bannerman, 1994). The incidence of CoNS associated nosocomial bacteremia in the United States rose from 9% to 27 % (Lang, 2000; Peters *et al.*, 1995).

In addition to the mortality and morbidity associated with these infections, it is estimated that CoNS nosocomial bacteraemia has a mortality rate of 21% (Edmond *et al.*, 1999).

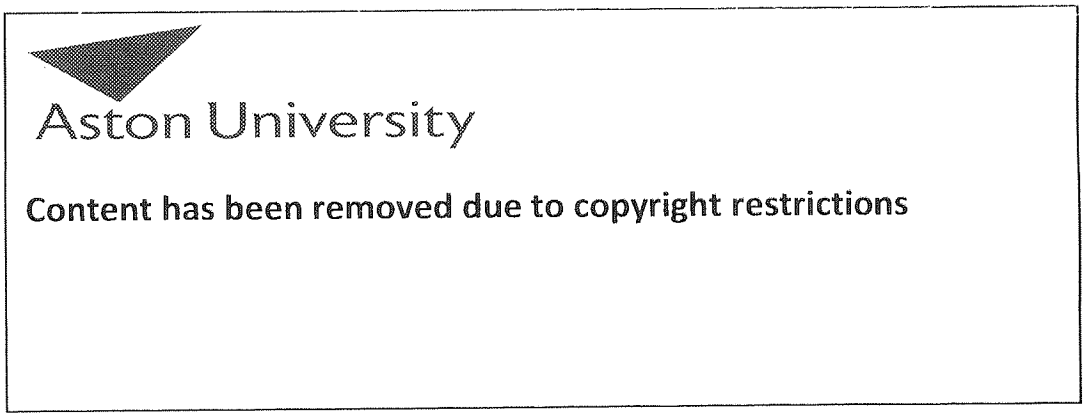
S. epidermidis is by far the most frequently isolated species of the CoNS group from native valve endocarditis (NVE) (Kessler *et al.*, 1998). *S. epidermidis* is the most common cause of prosthetic valve endocarditis (PVE) among other CoNS (Lang, 2000; Leung *et al.*, 1999; Vandenesch *et al.*, 1993).

1.5 Staphylococcal virulence factors

Although CoNS are less virulent than *S. aureus*, they are capable of expressing a number of potential virulence factors (Lang, 2000) (Table 1.2). Amongst these virulence factors are adherence receptors for host extra cellular matrix proteins, such as fibronectin and collagen, which are collectively termed MSCRAMMs, an acronym for microbial surface components recognizing adhesive matrix molecule (Pattie *et al.*, 1994).

Table 1.2. Possible virulence factors of CoNS (Lang, 2000).

Table 1: 2 continued

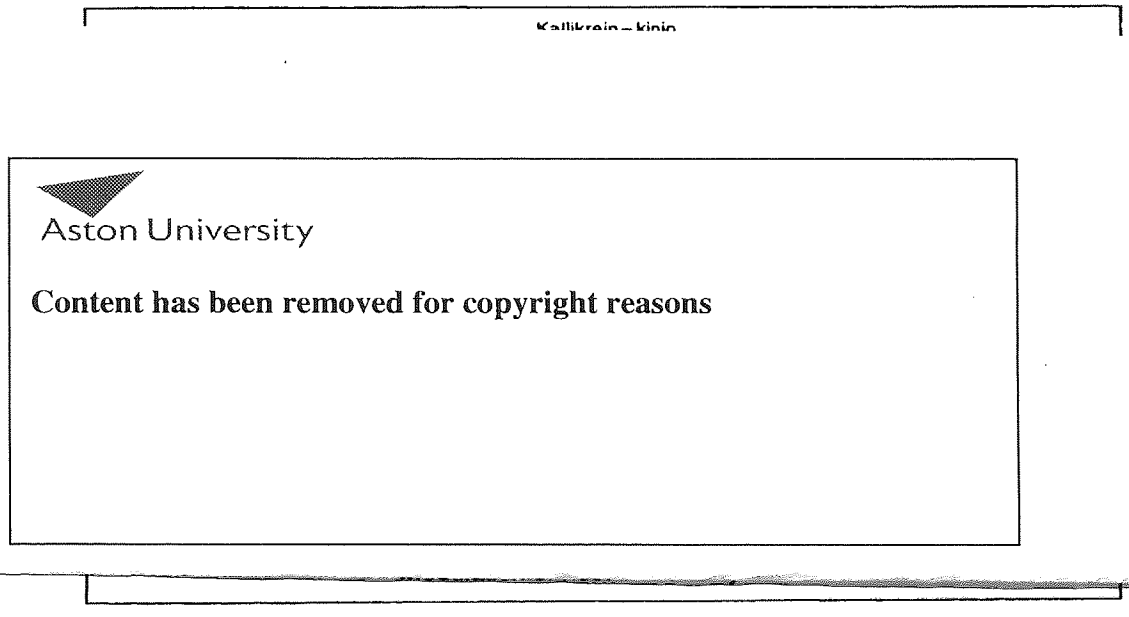
Factors potentially associated with	
	
	Gemmell, 1987

Although factors are placed within distinct groupings, determinants are not necessarily limited to a single pathogenic role. For example the extracellular polysaccharide slime substance is a proposed adherence factor but evidence also suggests that it is capable of impeding phagocytosis and promoting antimicrobial resistance (Christensen, Baldassarri and Simpson, 1994).

S. aureus and *S. epidermidis* have been shown to secrete lipases (glycerol ester hydrolase) and esterases (lipolytic activity on water-soluble substrates), which express no substrate specificity, during the exponential phase of the growth cycle (Vuong *et al.*, 2000., Farrel *et al.*, 1993). The physiological roles of these enzymes remain unknown but, like iron scavenging mechanisms, lipase may have both nutritional and virulence functions (Lang, 2000). Lipase is produced by *S. epidermidis* in a very similar fashion (Farrel *et al.*, 1993). The ability to delay production of extra cellular factors may be beneficial to the organism in colonising the skin (Chamberlin, 1999).

Proteinase activity in *S. epidermidis* has been studied by Potempa *et al.* (1988). Evidence suggests that the *S epidermidis* elastase, like the S. *aureus* equivalent, is a cysteine proteinase enzyme (Sloot *et al.*, 1992). Proteinases are central to survival, multiplication and pathogenicity of the bacterium within the host (Lang, 2000; Finalay *et al.*, 1997). These enzymes mediate tissue degradation, attenuation of the host defense mechanisms and modulation of the immune system (Travis *et al.*, 1995). The various functions of proteinase are summarized in (figure1.4).

Figure 1.4: Multiple functions of bacterial proteinase in infection (Travis *et al.*, 1995).



Fatty acid modifying enzyme (FAME) is an extracellular enzyme that inactivates bactericidal fatty acids by esterifying them to cholesterol. Inactivation of these fatty acids may allow *S. epidermidis* to live for long periods of time on the skin (Chamberlin, 1999). FAME activity is not produced in significant amounts by *S. epidermidis* until early in the stationary phase (Chamberlain and Brueggemann, 1997).

The expression of virulence determinants is stimulated and regulated by the conditions encountered in the host, such as nutrient starvation, stress, pH, oxygen tension and iron availability (Mekalanos, 1992). The first serodiagnostic test for staphylococcal infection detected anti-α-toxin antibody (anti-staphylolysin) (Easmon, 1990). Cellular antigens including the wall teichoic acid have been investigated for their use in a routine serodiagnostic test for staphylococcal infections (Crowder *et al.*, 1972). Eventually the commercial kit, ENDO STAPH, was produced based on teichoic acid antibody (Wheat *et al.*, 1984). Despite favourable

reports, the kit failed to become widely employed. Attention then moved from cellular to extracellular antigens and the development of tests to distinguish between serious staphylococcal infection and septicaemia (Espersen *et al.*, 1986).

An enzyme-linked immunosorbent assay (ELISA) based on the detection of anti-lipid S (a secreted short chain form of lipoteichoic acid) antibody has been developed (Lambert *et al.*, 2000; Elliott *et al.*, 2000).

1.6 Vaccination against Staphylococcus epidermidis and other CoNS

Vaccines against *S. epidermidis* have encompassed a variety of substances, most of which remain poorly characterized. Yoshida and Ichiman and colleagues (1981) in Japan reported that *S. epidermidis* expressed three distinct capsular polysaccharide antigens that gave raise to protective immunity. However, with the advent of better techniques for speciation of coagulase-negative staphylococci, it was determined that only one of their three strains was actually *S. epidermidis*. Reports of the chemical composite of the purified capsular materials were not definitive (Kojima *et al.*,1990).

The major focus in developing vaccines for *S. epidermidis* over the past decade has been to identify antigens in the extracellular slime and action that could elicit protective immunity. Numerous studies indicated that slime producing CoNS were more frequently encountered among clinical isolates than among commensal isolates from the skin (Espersen *et al.*, 1986).

To date there has been only one human trial of a vaccine designed specifically to prevent *S. epidermidis* infections (Dimkovic *et al.*, 1993). These workers used a un-characterized, formalin-killed mixture of whole *S. epidermidis* cells to subcutaneously vaccinate 10 patients on chronic peritoneal dialysis once weekly for 6 weeks. Increases of antibody in serum and peritoneal fluid to whole bacterial cells were reported, and these workers claimed that the 10 vaccinated patients had only one episode of *S. epidermidis* peritonitis per 93 patient months, compared with one

episode per 26.5 patient months in 10 unvaccinated controls (Crossely and Kent, 1997). Recently Staph Vax has been tested on patients receiving renal dialysis. Staph Vax consist of the capsular polysaccharide 5 and 8 conjugated to carrier protein derived from the *P. aeruginosa* exotoxin A. The vaccination efficacy is of 57%. The surface polysaccharide PNSG, which is expressed by both *S. epidermidis* an *S. aureus* as new vaccine is promising as well (Anon. 2002).

Hussain *et al* (1991) and (1993) claim that teichoic acid constitutes 80 percent of slime, and protein the remaining 20%. Tojo *et al* (1988) also found teichoic acid and protein to be major components of CoNS slime, and Arvanitis *et al* (1994) described a small (90 kDa) sulfated polysaccharide in the slime layer of *S. epidermidis*. Recently, Mack *et al* (1994) and (1996) found a hexosamine-rich polysaccharide in slime that appears to mediate accumulation of bacterial cells into biofilms. However, the major component of slime is a polysaccharide adhesin (PS/A) initially described by Tojo *et al* (1988) as a polysaccharide component of slime that mediates bacterial cell attachment to plastic and is also the organism's capsule.

1.7 Pathogenicity and biology of S. epidermidis

CoNS form a group of more than 30 defined species. They share many basic biological properties with the much smaller group of coagulase-positive staphylococci typified by the predominant species *S. aureus*. However, CoNS show substantial differences to *S. aureus*, especially with respect to their ecology and their pathogenic potential. The normal habitat of CoNS is skin and mucous membranes of humans and animals (Fischetti *et al.*, 2000). Diagnostically, CoNS are separated from *S. aureus* by their inability to produce free coagulase. They can further be differentiated on the basis of their novobiocin-susceptibility: novobiocin resistant CoNS resemble the *Staphylococcus saprophyticus* group, and novobiocin – susceptible CoNS mainly resemble the *S. epidermidis* group. By far the most frequently isolated species, from normal flora as well as from clinical specimens, is *S. epidermidis* (Heilmann *et al.*, 1997).

CoNS, particularly *S. epidermidis*, are the most frequently isolated microorganisms in the clinical microbiology laboratory. The vast majority of infections attributed to CoNS are nosocomial infections (Ziebuhr *et al.*, 1997). *S aureus* causes a broad variety of pyogenic infections as well as toxin-mediated diseases in the normal host, and novobiocin-resistant CoNS, particularly *S. saprophyticus*, are found in urinary tract infections. In contrast, *S. epidermidis* rarely causes pyogenic infections in the normal host, except for natural valve endocarditis, and there is little to suggest any role in toxin-mediated disease. However, when the host is compromised, *S. epidermidis* may even be the predominant cause of infection (Von Eiff *et al.*, 1998).

One such group is intravenous drug (heroin) abusers who develop right-sided endocarditis. In this group *S. epidermidis* is the main cause of repeated bacteremia with high inoculates due to unsterile injection procedures. A second group is immunocomprised patients: *S. epidermidis* is the leading cause of septicemia, with the onset later than 48 h in premature newborns. Patients with aplasia (neutropenia)

after cytostatic and/or immunosuppressive therapy are also highly susceptible to *S. epidermidis* septicemia owing to the low number of functioning polymorphonuclear neutrophils (PMNs). The sources of infection in all these patients are skin and mucous membranes, and the port of entry is very often an intravascular catheter (Fischetti *et al.*, 2000). A third group, probably the most important, is patients with foreign bodies such as indwelling catheters or implanted polymer devices of various materials (e.g. polyethylene, polyurethane, silicon rubber), increasingly used in diagnostic or therapeutic procedures. Infection is the major complication associated with the use of such devices, and overall, CoNS, mainly *S. epidermidis*, are the most frequently isolated microorganisms in these infections. Depending on the kind of device and its insertion site, different infection syndromes generate a variety of clinical presentations (Von Eiff *et al.*, 1998).

The clinical picture of *S. epidermidis* infections markedly differs from that of *S. aureus* infections. Normally, there are no fulminant signs of infection, and clinical course is more sub- acute or even chronic. Accordingly, making the diagnosis of *S. epidermidis* infection is often difficult. Therapy is especially problematic in foreign body (polymer)-associated infections: despite the use of appropriate antibiotics with proven *in vitro* efficacy and despite a generally functioning host response, it is often not possible to eradicate the focus on the infected device, thus, removal of the device and subsequent renewal become necessary (Von Eiff *et al.*,1998).

Therefore, *S. epidermidis* infection contrasts with *S. aureus* infection in that *S. epidermidis* requires an especially predisposed host. Only in such a circumstance can *S. epidermidis* change from a commensal or saprophytic organism in the human cutaneus or mucocutaneus ecosystem to a pathogen (Fischetti *et al.*, 2000).

1.8 Microbial colonization

1.8.1Biofilm formation

Biofilm formation is thought to result from the concerted action of primary attachment to a specific surface and accumulation in multilayered cell clusters (Heilmann *et al.*, 1996). Formation of these sessile communities and their inherent resistance to antimicrobial agents are at the root of many persistent and chronic bacterial infections. In biofilms, resistance seems to depend on multi-cellular strategies (Dunne, 2002)

The extracellular mucoid substance secreted by CoNS was first described by Bayston *et al* (1972). This polysaccharide secreted substance is commonly referred to as "slime" (Christensen *et al.*, 1982). Although the term "slime" remains in common use, it is more appropriate to refer to the material as biofilm (Lang, 2000; Hussain *et al.*, 1993).

An extracellular polysaccharide adhesin represents a key virulence determinant in *S. epidermidis* and is required for biofilm formation. Production of this adhesin, which is encoded by the *ica* operon, is subject to phase variable regulation (ON OFF switching). The most important step in the pathogenesis of *S. epidermidis* foreign body-associated infectious diseases is the colonization of the polymer surface by forming multilayered cell clusters, which are embedded in an amorphous extra cellular material (Conlon *et al.*, 2002). Infection of the polymer likely occurs by inoculation with a few bacteria from the patient's skin or mucous membranes during implantation of the device (O'Gara and Humphreys, 2001). The colonizing bacteria together with the extra cellular material, which is mainly, composed of cell wall teichoic acids and host products are referred to as biofilm (Hussain *et al.*, 1993).

Biofilm formation proceeds in two steps: rapid attachment of the bacteria to the surface is followed by a prolonged accumulation phase that involves cell proliferation and expression of a polysaccharide intercellular adhesion (PIA). The molecular mechanisms involved in biofilm formation, are summarized in (Fig 1.5).

Figure: 1.5 Different phases of *S. epidermidis* biofilm formation on a prosthetic polymer device and bacterial factors involved. SSP-1/SSP-2, staphylococcal surface proteins; AtLE, autolysin; PS/A, polysaccharide/adhesion; Fbe, fibrinogen-binding protein; PIA, polysaccharide intercellular adhesion; AAP, accumulation-associated protein (Fischetti *et al*., 2000)

Biofilm formation in *S. epidermidis* is dependent upon the *ica* operon-encoded polysaccharide intercellular adhesion, which is subject to phase-variable and environmental regulation (Fey *et al.*, 1998). The *ica* operon encodes PIA, a linear β 1, 6-linked glucosaminoglycan involved in cell accumulation (Gerke *et al.*, 1998). Isolates that carry the *ica*, PIA counterparts (Heilmann *et al.*,1996). The regulation of this operon is complex and may involve three or more other gene loci (Gerke *et al.*, 1998). Some evidence demonstrates that PIA expression and biofilm formation are essential virulence factors (Rupp *et al.*, 1999a; Rupp *et al.*, 1999b). The *ica* gene cluster, which contains all the genes necessary for production of polysaccharide adhesin, was identified by transposon mutagenesis to isolate mutant *S. epidermidis*

strains deficient in biofilm formation. The *ica* locus contains an operon, *icaADBC* which appears to contain the structural genes required for PIA synthesis (Figure1.6) (O'Gara and Humphreys, 2001).

Figure: 1.6 Genetic Organisation of the ica gene cluster from *S. epidermidis* (O'Gara and Humphreys, 2001).

1.8.2 Microbial Attachment

Bacterial attachment to a surface can be divided into several distinct phases, including primary and reversible adhesion, secondary and irreversible adhesion, and biofilm formation. Each of these phases is ultimately controlled by expression of one or more gene products. The biofilm protects its inhabitants from predators, dehydration, biocides, and other environment extremes while regulating population growth and diversity through primitive cell signals (Dunne, 2002).

Initial microbial adherence to biomaterials depends on the cell surface characteristics of the bacteria and on the polymer material. Factors involved include physicochemical forces such as charge, van der Waal's forces, and hydrophobic interactions. Cell surface hydrophobicity and initial adherence have been attributed to bacterial surface-associated proteins. With the aid of monoclonal antibodies that efficiently block adherence, the antigenically related staphylococcal surface proteins SSP-1 and SSP-2 (280 kDa and 250KDa, respectively) have been identified as fimbrialike structure involved in *S. epidermidis* 354 adherence to polystyrene (Veenstra *et al.*, 1996). Recently, the gene encoding the surface-associated autolysin AtlE of *S. epidermidis* 0-47, which mediates primary attachment of bacterial cells to a polymer surface, has been cloned and sequenced (Heilmann *et al.*, 1997).

Aside from proteins, a polysaccharide structure, capsular polysaccharide/adhesin (PS/A) has been associated with initial adherence and slime production (Muller *et al.*, 1993). PS/A was originally reported by Tojo *et al.* (1988). Recently it has been identified as sharing the β 1,6-linked glucosamine backbone described in PIA. Furthermore, both PIA and PS/A are encoded by the *ica* operon and immunization with PS/A results in protection against infection (McKenny *et al.*, 1998).

1.8.2.1 Microbial interaction with extra cellular matrix proteins

Direct interaction between bacteria and naked polymer surfaces plays a crucial role in the early stages of the adherence process in vitro and probably *in vivo*. Additional factors may be important in later stages of adherence in vivo, because implanted material rapidly becomes coated with plasma and extra cellular matrix proteins such as fibronectin, fibrinogen, vitronectin, thrombospondin, and von Willebrand factor (Rupp, 1996). Some of these factors could serve as specific receptors for colonizing bacteria. Indeed, adherence of clinical coagulase-positive and negative staphylococcal isolates is significantly promoted by surface-bound fibronectin in comparison with surface bound albumin (Allison, 1993). Adherence of all *S. aureus* strains tested is markedly promoted by immobilized fibrinogen, while adherence of *S. epidermidis* strains to immobilized fibrinogen was found to vary significantly among different strains (Nilsson *et al.*, 1998).

Few data on host factor-binding proteins of CoNS are available. Recently a fibrinogen-binding protein (Fbe) in *S. epidermidis* was cloned and sequenced. Sequence comparison revealed that the 119/kDa Fbe showed similarity to the cell wall-bound fibrinogen receptor (clumping factor, ClfA) of *S. aureus*, which has been shown to contribute to virulence (Nilsson *et al.*, 1998).

Other bacterial products associated with slime and biofilm formation include:

* An immunogenic, slime-associated 20kDa polysaccharide (Karamanos *et al.* ,1997).

* Associate-adhesion protein (AAP), a 140kDa extra cellular protein associated with the accumulation stage of biofilm formation (Hussain *et al.*, 1997).

* Staphylococcal surface proteins (SSP-1and SSP-2) may be responsible to form a linear fimbrial structure to initiate irreversible cell binding to the polymer surface (Timmerman *et al.*, 1991; Veenstra *et al.*,1996).

* Wall-derived teichoic acid (Hussain *et al.*, 1992).

* AtlE, an autolysin that mediates the initial attachment of the cells to a polymer surface (Heilmann *et al.*, 1997).

Vitronectin-binding activity was also found for the autolysin AtlE from *S. epidermidis*. There is preliminary evidence for an in vivo role of AtlE: in a central venous catheter-associated infection model (Heilmann *et al.*, 1997).

1.8.3 Accumulation process

After succeeding in primary attachment to the polymer surface, bacteria proliferate and accumulate in multilayered cell clusters, which require intracellular adhesion. Transposon mutants that are not able to accumulate in multi layered cell clusters lack a specific polysaccharide antigen referred to as polysaccharide intercellular adhesion (PIA). Purification and structural analysis of PIA revealed that it consists of a major polysaccharide I ($\rangle$ 80%) and a minor polysaccharide II ($\langle$ 20%). Polysaccharide I is a linear β-1, 6-linked glucosaminoglycan mainly composed of at least 130 2-deoxy-2-amino-D-glucopyranosyl residues, of which 80 to 85% are N-acetylated. Polysaccharide II is structurally related to polysaccharide I but has a lower content of non-N-acetylated glucosaminyl residues and contains a small amount of phosphate and ester-linked succinyl residues. Thus, PIA represents a so far unique structure (Mark *et al.*, 1996).

The genes (*ica*ABC) that mediate cell clustering and PIA synthesis in *S. epidermidis* have been cloned and sequenced (Heilmann *et al.*, 1996). The haemagglutinin of *S. epidermidis* has been demonstrated to be associated with biofilm production and to be a polysaccharide structure. A study investigating the pathogenic properties of strains obtained from polymer associated septicaemic disease compared with saprophytic skin and mucosal isolates demonstrated a strong correlation of biofilm formation and presence of the *ica* gene cluster essentially associated with disease isolates (Ziebuhr *et al.*, 1997).

Formerly, another antigenic marker of slime production and accumulation designated as slime-associated antigen (SAA) was defined (Baldassarri *et al.*, 1996).

Another factor seems to be essential for accumulation and biofilm formation in *S. epidermidis*. A 140-kDa extra cellular protein AAP (accumulation-associated protein), missing in the accumulation-negative mutant M7 was shown to be essential

for accumulative growth in certain *S. epidermidis* strains on polymer surfaces (Hussain *et al.*, 1997).

1.8.4 Extracellular toxins and enzymes

Staphylococci, in particular *S. aureus*, have developed multiple mechanisms, including production of a variety of extra cellular protein and enzymes, such as protein A, lipases, protease, esterases, phospholipases, fatty acid modifying enzymes, as well as production of hemolysins, and toxins with superantigenic properties such as entro toxins, exfoliative toxins, and toxic shock syndrome toxin-1 (TSST-1) (Heilmann *et al.*, 1996). Additionally, protease may play a role in proteolytic inactivation of host defense mechanisms such as antibodies and platelet microbicidal proteins as well as in destruction of tissue proteins causing increased invasiveness (Fischetti *et al.*, 2000).

In *S. epidermidis*, an extra cellular metalloprotease with elastase activity has been detected, and the gene has been cloned and sequenced (Teufel *et al.*, 1993). Previously, an elastase from *S. epidermidis* that degrades human secretory immunoglobulin A, immunoglobulin M, serum albumin, fibrinogen, and fibronectin has been identified as a cysteine protease and thus assumed to be a virulence factor (Sloot *et al.*, 1992). An extracellular serine protease is involved in epidermin processing (Geissler *et al.*, 1996).

The genes of two lipases from *S. epidermidis* exhibiting a high degree of similarity (97.8% identical amino acids) have been cloned and sequenced, and they have been proposed to be involved in skin colonization (Farrell *et al.*, 1993 and Simons *et al.*, 1998).

The expression and characterization of fatty acid modifying enzymes in *S. epidermidis* have been described by Chamberlain *et al.*, 1997.

In contrast to *S. aureus*, which produces all of the above-mentioned toxins in a strain dependent manner, *S. epidermidis* is much less toxigenic. *S. epidermidis* can produce delta toxin, which differs from *S. aureus* delta toxin in only three amino acids (McKevitt *et al.*, 1990). The delta toxin is encoded by *hld,* which is a component of the regulatory *agr* system and acts by formation of pores in the membrane, leading to the lysis of erythrocytes and other mammalian cells. Reports on unusual *S. epidermidis* strains producing enterotoxin C or TSST-1 are controversial (Marin *et al.*, 1992).

1.8.5 Host Defense Factors

Septicemia is a serious consequence of *S. epidermidis* polymer associated infection. In the pathophysiology of inflammatory events in septicaemia, the production of cytokines such as tumur necrosis factor alpha (TNFα), interleukin 1β (IL-1β) and IL-6 is thought to play a major role. Peptidoglycan and teichoic acid, cell wall components purified from an *S. epidermidis* strain, stimulate human monocytes to release TNF-α, IL-1, and IL-6 in a concentration dependent manner (Mattsson *et al.*,1994). Another feature of extracellular products of *S. epidermidis* is the interference with several neutrophil functions (Johnson *et al.*, 1986).

Extracellular slime produced by *S. epidermidis* has also been shown to reduce the blastogenic response of human peripheral mononuclear cells to T cell mitogens (phytohemagglutinin and streptococcal blastogen A) in a dose dependent manner (Gray *et al.*, 1998).

1.8.6 Acquisition of iron inside the host

As do all bacteria, the staphylococci require iron for their growth. However, the free iron concentration (10^{-18} M) in the extracellular body fluids, owing to the presence of high affinity iron binding glycoproteins such as transferrin and lactoferrin, is much too low to support staphylococcal growth (Fischetti *et al.*, 2000).

Both *S. epidermidis* and *S. aureus* express a number of iron repressible cell wall and cytoplasmic membrane associated proteins when isolated during infection in human as well as when grown *in vivo* in laboratory animal infections (Modun *et al.*, 1998a). These include a 42-kDa cell wall protein that functions as a receptor for human transferrin (Mudun *et al.*, 1998b) and a 32-kDa cytoplasmic membrane associated lipoprotein (Cockayne *et al.*, 1998). Cloning and sequencing of the DNA region encoding the 32-kDa cytoplasmic membrane associated lipoprotein of *S. epidermidis* revealed that the corresponding gene (*sit*C) is part of a translationally coupled, iron regulated operon (*sit*ABC) that encodes an ABC-type transporter. It is speculated that this novel ABC transporter is involved in either siderophore or transferring mediated iron uptake in *S. epidermidis* (Cockayne *et al.*, 1998).

1.9 Virulence Factors Regulation

1.9.1 Fur-like Protein (fur)

The low availability of iron in the host is the reason that many pathogenic bacteria use low iron concentrations as a signal to active certain virulence factors, including toxins, adhesions, and invasins. The corresponding genes and genes involved in the biosynthesis and transport of siderophores are often regulated by Fur (ferric uptake regulator), which binds, only in the presence of iron, to a consensus sequence termed the Fur box located within the promoter region of the target genes. When iron levels are low, Fur does not bind, and genes are transcribed. A gene (*fur*) for a Fur-like protein has been identified in *S. epidermidis* (Heidrich *et al.*, 1998). Although the *S. epidermidis* Fur protein is unable to complement an *Escherichia coli fur* mutant, the Fur protein of *E.coli* binds to the Fur box of the *S. epidermidis fur* promoter region (Heidrich *et al.*, 1998).

1. 9.2 Diphtheria toxin repressor (DtxR)

In Gram-positive bacteria an alternative iron dependent repressor is DtxR, which was first identified as a repressor of diphtheria toxin synthesis in *Corynebacterium diphtheriae* (Fischetti *et al.*, 2000).

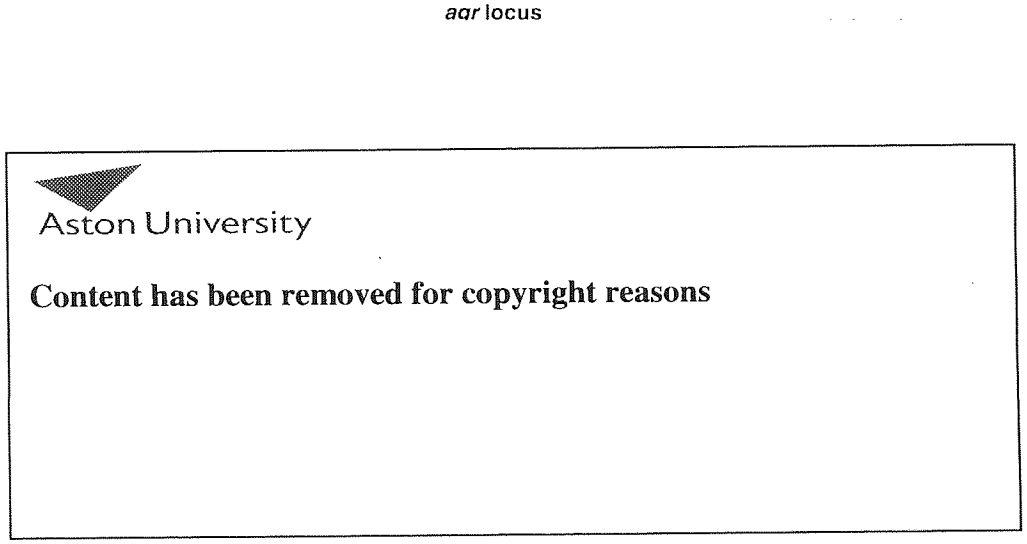
Most recently, a DtxR homologue, designated SirR (staphylococcal iron regulator repressor), was identified in *S.epidermidis* by sequence analysis of the DNA region located upstream of the *sit*ABC operon (Hill *et al.*, 1998). DNA mobility shift assays confirmed that SirR binds to the Sir box only in the presence of metal ions such as Fe^{2+} and Mn^{2+}. Southern hybridization experiments revealed that there are at least five Sir boxes in the *S. epidermidis* genome (Hill *et al.*, 1998 and Fischetti *et al.*, 2000).

1.9.3 The Accessory Gene Regulator (agr)

In 1998 an *agr* homolog of *S. epidermid s* was been identified and sequenced (Otto *et al.*, 1998 and Van Wamel *et al.*, 1998). This global regulatory quorum sensing system has been shown to be central in controlling the synthesis of exocellular and cell surface associated proteins in staphylococci (Vuong, Gotz and Otto, 2000). The *agr* locus has two promoters (P2 and P3), which generate the transcripts RNAII and RNAIII, respectively The RNAII transcript encodes four proteins: AgrA, AgrB, AgrC and AgrD (Tegmark,Morfeldt and Arvidson,1998 ;Lang, 2000) (Fig1.7).

Figure: 1.7 The Staphylococcal *agr* and *sar* loci.

The agr promoters P2 and P3 generate the RNAII and RNAIII transcripts, respectively.RNAIII encodes *AgrA ,B, C, and D*. An autoinducer is formed by the processing of AgrD by AgrB. This autoinducer binds to the AgrC two component regulatory system and, in conjunction with SarA, activates transcription of the P2 and P3 promoters (Projan and Novick, 1996).



DNA sequence analysis revealed a pronounced similarity between the *S. epidermidis* and *S. aureus agr* system. The extra cellular signaling molecule produced by a typical *S. epidermidis* strain is a cyclic octapeptide (DSVCASYF) that is encoded by *agr*D and contains a thiolester linkage between the central cysteine and the C terminal carboxyl group (Otto *et al.*, 1998). The AgrD proteins of *S.epidermidis* and *S.aureus* show evident similarity in the region located C terminal of the signaling peptides, suggesting that this region represents a structural element important for the modifying reaction probably mediated by AgrB (Tegmark *et al.*, 1998). The *S. epidermidis* histidine kinase AgrC shares 50.5% identical amino acids with the *S. aureus* AgrC, with pronounced similarity in the C

terminal protein and low similarity in the N terminal portion (Otto *et al.*, 1998). The *S. epidermidis* RNAIII has the ability to completely repress transcription of protein A and the ability to activate transcription of the alpha toxin (*hla)* and serine protease (*ssp*) genes in an RNAIII deficient *S. aureus* mutant. However, the stimulatory effect was reduced compared with that of *S. aureus* RNAIII (Van wamel *et al.*, 1998: vandernesch *et al.*, 1993b; Janzon *et al.*, 1989). Construction and analysis of *S. epidermidis S. aureus* RNAIII hybrids showed that both the 5' and 3' halves of the RNA molecule are important for the regulatory function (Tegmark *et al.*, 1998).

1.9.4 The staphylococcus accessory regulator (sar) locus

Another global regulator, *sar* in *S. aureus* controls exoprotein synthesis by modulating the expression of *agr. This* locus has been identified, cloned and sequenced in *S. epidermidis* as well (Fluckiger *et al.*, 1998). It was revealed that the SarA protein of *S. epidermidis* is nearly identical (84%) to SarA of *S. aureus*. In contrast the *sarA* flanking DNA sequence shows only 50% identity between both strains, and the two smaller open reading frames are absent in *S. epidermidis* (Fluckiger, Wolz and Cheung, 1998; Heinrichs, Bayer and Cheung, 1996). Moreover, functional analysis confirmed that the *S. epidermidis sar* homologue was able to restore alpha-toxin production in an *S. aureus* mutant (Fluckiger *et al.*, 1998). Because most of the typical virulence determinants of *S. aureus* are missing in *S. epidermidis*, it has to be clarified which genes are under the control of *agr* in *S. epidermidis* and other CoNS. Possible candidates include genes encoding protease, delta-toxin, lipases, autolysin, fibrinogen-binding protein, AAP, PS/A, and PIA production (Vuong, *et al.*, 2000).

1.9.5 Bacteriocins in S. epidermidis

Production of bacteriocins called lantibiotics is another biological property that distinguishes *S. epidermidis* from *S. aureus*. Their production may play a substantial

role in bacterial interference on skin and mucous membranes and thus create an ecological niche for *S. epidermidis*. Lantibiotics are antibiotic peptides that contain the rare thioether amino acids lanthionine and/or methyllanthionine. Type A lantibiotics act by generating pores in the cytoplasmic membrane. Other Gram-positive bacteria such as *Bacillus subtilis* and lactobacilli also produce lantibiotics. Among those produced by *S. epidermidis* are the well-characterized epidermin, Pep5, epilancin K7 and epicidin 280 (Geissler *et al.*, 1996, Kupke *et al.*, 1995 and Kupke *et al.*, 1996).

1.10 Antiseptic (Disinfectant)

1.10.1 Introduction

At present, nosocomial infections including those caused by methicillin-resistant *S. aureus* pose a serious problem in many medical institutions. Disinfection of the hands and environment is one of the most important countermeasures to control nosocomial infections. In choosing disinfectants for these uses, comparative bactericidal effects of various disinfectants against bacteria isolated from clinical isolates should be taken into consideration (Schito, 2002).

Despite a century of applying antiseptic principles to the practice of surgery and several decades of using prophylactics, the association between staphylococci and postoperative wound infections remains strong. Various estimates suggest that between 325,000 (Mayhall, 1993), 520,000 (Haley *et al.*, 1985), and 920,000 (Wenzel, 1992) of the 23 million patients who undergo surgery annually in the United States develop a wound infection. Staphylococci are the most common species recovered. Schaberg *et al.*, (1991) reported that CoNS ranked third among aerobic bacteria associated with wound infections. Crossley and Kent (1997) reviewed the applicability of the germ theory to surgery and the association between staphylococci and postoperative infection. They further supported the view that hand washing and chlorinated lime soaks reduce the risk of puerperal fever following childbirth. These events provided the basis for the slow but eventual widespread acceptance of antisepsis as the major preventive measure against surgical infection.

Antimicrobial agents are chemical substances that can either kill or inhibit the growth of microorganisms. Such substances may be natural products or synthetic chemicals, they are of undoubted value for the treatment of infection, and in well-defined circumstances they may be used for the prevention of infection. Agents that kill microorganisms are termed *cidal* (e.g. bactericidal), whereas those that do not kill but merely inhibit growth are referred to as *static* agents (e.g. bacteriostatic) (Allison *et al.*, 2001).

Antibacterial agents can be classified in one of three ways: (1) whether they are bactericidal or bacteriostatic; (2) by the level of selective toxicity at the target site and (3) by chemical structure. Classification on the basis of the latter alone is not of practical value, since antibacterial agents include a wide range of different chemical structures. Rather, a combination of target site and chemical structure provides a useful working classification (Allison *et al.*, 2001).

Biocides are used widely in modern society. Their use varies from the justifiable to the excessive. At one end of the spectrum, disinfectants are used in healthcare environments to render contaminated surfaces safe and to prepare skin prior to surgical operations (Fraise, 2002). 'Disinfectant' describes products intended for use in the presence of dirt and dense bacterial populations, for example in the cleaning of animal quarters or drains. The term biocide includes disinfectants, antiseptics and preservatives. It does not include antibiotics, which, tend to be categorized separately. In recent years there has been a trend towards use of biocides in the home environment (Fraise, 2002).

'Biocide' is used more particularly for preservatives that prevent bacterial and fungal attack on wood, paper, textile and all kinds of organic material. 'Antiseptic' is a term usually reserved for a substance that can safely be applied to the skin with the aim of reducing the chances of infection by killing the surface bacteria (Nicolletti *et al.*, 1992). These authors also reported that the requirements for a compound having disinfectant or antiseptic action differ markedly from those needed in a systemic antibacterial. Many compounds successfully used against bacterial infections do not actually kill bacteria, but only prevent their multiplication; most are inactive against non-growing bacteria. Nicolletti *et al.*, (1992) have stated that many compounds are bacteriostatic at low concentrations and bactericidal at higher concentrations, and the effect may also depend on the conditions of culture. However, for antiseptics and disinfectants a bactericidal effect is required under all normal conditions of application. Such compounds must be able to kill bacteria whether they are growing

or resting and must be able to deal with most of the common bacteria likely to be found in the environment.

The word antiseptic has acquired the special meaning of an antimicrobial agent, which is sufficiently free from injurious effects to be applied to surfaces of the body or to exposed tissues, though not suitable for treatment of infection by systemic administration (Russell *et al.*, 1993). Antiseptics and disinfectants play important roles in microbial control in the hospital. Selection of antiseptics and disinfectants should be based on knowledge of their characteristics. Antiseptics should exert a sustained effect against microorganisms without causing tissue damage (Boothe, 1998). Disinfecting of tissue involves the killing and removal of transient organisms from the skin, whereas surgical hand disinfection involves the killing and removal of the resident flora (Hedin *et al.*, 1993 and Russell *et al.*, 1993). Biocidal agents tend to have multiple target sites in the bacterial cell (Russell *et al.*, 1997). Although there might be a primary or major effect on the cell, other 'secondary' effects contribute to the overall lethal effect, thereby explaining how uptake into the bacterial cell, bacteriostasis and bacterial cell death arise (Denyer 1996, Russell *et al.*, 1999 and Bloomfield *et al.*, 2000). Although there are differences between the actions of these two classes of compound they show many common features. A review of the most reliable evidence suggests the following generalizations (Nicolletti *et al.*, 1992).

Antiseptics bind readily to bacteria; the amount adsorbed increasing with an increasing concentration in solution. The most important site of adsorption is the cytoplasmic membrane. The extent of killing of the bacteria is governed by three principal factors (McDonnell and Russell, 2000).

a) Biocide concentration, a major factor in biocidal activity
b) Nature of the organism.
c) Time of contact

The lowest concentration of the antiseptic that causes death of the bacteria also brings about leakage of cytoplasmic constituents of low molecular weight. The most immediate effect is loss of potassium ions (Russell, 2002). The necessary characteristic of antiseptics is bactericidal action, but there is often a low and rather narrow concentration range in which their effect is bacteriostatic. In the presence of higher concentrations of antiseptic and after prolonged treatment, the compound usually penetrates the cell and brings about extensive ill-defined damage to cell components (McDonnell and Russell, 1999).

The cell wall plays a vital role in bacterial growth and survival in hostile environments. The role of the cell wall as a barrier to the penetration of toxic molecules differs considerably between Gram-positive bacteria, Gram-negative bacteria and mycobacteria (Lambert, 2002).

The cytoplasmic membrane is often considered as the major target site for biocides. Damage to the membrane can take several forms: (i) physical disruption of the membrane; (ii) dissipation of the proton motive force (PMF) and (iii) inhibition of membrane-associated enzyme activity. Bacterial cytoplasm contains the chromosome, ribosomes and various enzymes with different functions. These cell components are probably not primary target sites, since a biocide has to penetrate within the cell to reach the cytoplasmic constituents (Maillard, 2002).

1.10.2 Cationic antiseptics

Cationic classification covers a number of compounds, including quaternary ammonium compounds (QACs) and bisbiguanides. Their common features are the presence of strongly basic groups attached to a fairly large lipophilic region. Although antiseptic action is found quite widely in compounds having these characteristics the degree of activity is sharply dependent on structure within any particular group (Nicolletti *et al.*, 1992).

1.10.3 Bacterial Resistance to Antiseptics and Disinfectants

Biocide resistance among Gram-positive cocci is being increasingly recognized. Possible mechanisms for such resistance include efflux, altered target sites and cell wall changes (Fraise, 2002). A number of scientists have expressed concern that biocide use may also be a contributory factor in the development of antibiotic resistance (Stickler and Thomas 1980; McMuny *et al.*, 1998; Russell *et al.*, 1998, 1999; Levy, 1998 and Bloomfield *et al.*, 2000). Gram-negative bacteria are not always more resistant to biocides than Gram-positive bacteria. For example, chlorine is more active against *P. aeruginosa* and *Proteus mirabilis* than against *Staphylococcus aureus* (Russell *et al.*, 1999; Russell, 2002).

Resistance is generally acknowledged as the insusceptibility of a bacterium to a test material (eg, antibiotic, antiseptic, and preservative). The definition of antiseptic resistance varies greatly throughout the literature and is often based on *in vitro* test results rather than clinical or in vivo data (Russell *et al.*, 1996). According to Rhonda (1999) a resistant isolate has at least a two-fold increase in MIC. It is generally believed that bacteria rarely become resistant to biocides. It has been suggested that this may be related to the multiple modes of action of most biocides (Russell, 1998). Biocides might act on microbial cells by damaging the bacterial cell membrane and by interfering with cell wall synthesis. Both actions result in a loss of cell constituents. There may also be damage inflicted upon essential amino acids and enzymes. Typical cell constituents that may be lost through damage of cell membranes or cell walls include purines, pyrirmidines, amino acids and electrolytes (Fraise, 2002).

In recent years, considerable progress has been made in understanding more fully the responses of different types of bacteria to antibacterial agents (Russell, 1997). McDonnell and Russell (1999) have reported that resistance can be either a natural property of an organism (intrinsic) or acquired by mutation or acquisition of plasmids (self-replicating, extra-chromosomal DNA) or transposons (chromosomal or plasmid integrating, transmissible DNA cassettes). Intrinsic resistance is

demonstrated by Gram-negative bacteria, bacterial spores, mycobacteria, and, under certain conditions, staphylococci (Table 1. 3).

Table: 1.3 Intrinsic resistance mechanisms in bacteria to antiseptics and disinfectants (McDonnell and Russell, 1999).

According to McDonnell and Russell (1999) acquired, plasmid-mediated resistance is most widely associated with mercury compounds and other metallic salts. In recent years, acquired resistance to certain other types of biocides has been observed, notably in staphylococci.

The critical measurements in determining antiseptic acting against resistant strains in the clinical environment have been considered by Day and Russell (1993). Others have demonstrated the necessity to define antiseptic resistance on the basis of in vivo methods. Lyon *et al* (1987), Cohen and Tartasky (1991) Levy (1992), Russell *et al* (1993) and Jones (1999) have published extensive reviews of microbial drug resistance.

Resistance to antiseptics, disinfectants and preservatives has been less extensively studied than antibiotic resistance (Russell, 1996). An early conclusion by Russell

(1985) was that plasmid-meditated resistance to inorganic mercury compounds and organomercurials, but not to other biocidal agents, was often found. Since then, the responses of bacteria to antiseptics and disinfectants have been more widely investigated, but have posed several unanswered questions. As with antibiotics, bacterial resistance to antiseptics and disinfectants may be acquired, but is more commonly intrinsic (Russell, 1996; Russell *et al.*, 1999).

Staphylococci are generally considered to be sensitive to antiseptics and disinfectants. However, staphylococci have been isolated which show a degree of resistance to certain types of agent, a finding of potential clinical importance (Russell *et al.*,1986).

1.10.3.1 Intrinsic bacterial resistance mechanisms

For an antiseptic or disinfectant molecule to reach its target site, the outer layers of a cell must be crossed. The nature and composition of these layers depend on the organism type and may act as a permeability barrier, in which there may be a reduced uptake. Alternatively but less commonly, constitutively synthesized enzymes may bring about degradation of a compound (Russell, 1997). Intrinsic (innate) resistance is thus a natural, chromosomally controlled property of a bacterial cell that enables it to circumvent the action of an antiseptic or disinfectant. Gram-negative bacteria tend to be more resistant than Gram-positive organisms, such as staphylococci (Bloomfield, 1992; McDonnell and Russell, 1999).

1.10.3.2 Acquired bacterial resistance mechanisms

The study, carried out by McDonnell *et al* (1999), suggests that with antibiotics and other chemotherapeutic drugs, acquired resistance to antiseptics and disinfectants can arise by either mutation or the acquisition of genetic material in the form of plasmids or transposons. It is important to note that "resistance" as a term can often be used loosely, and in many cases must be interpreted with some prudence. This is

particularly true with MIC analysis. Unlike antibiotics, "resistance," or an increase in the MIC of a biocide, does not necessarily correlate with therapeutic failure.

1.10.3.3 Plasmids and bacterial resistance to antiseptics and disinfectants

Russell (1985) examined the role of plasmids in encoding resistance (or increased tolerance) to antiseptics and disinfectants. Chopra (1991) further considered that plasmid-mediated antibiotic resistance in Gram-positive bacteria is well- established as a significant clinical problem. A detailed review by Russell (1997) showed that at present staphylococci are the only bacteria that have been studied in any detail. The genetic basis of reduced biocide susceptibility in staphylococci is plasmid-mediated system. Russell (1999) concluded that, apart from certain specific examples such as silver, other metals and organomercurials, plasmids were not normally responsible for the elevated levels of antiseptic or disinfectant resistance associated with certain species or strains.

Plasmid-mediated antiseptic resistance is classified as follows:

a) Plasmid-mediated antiseptic and disinfectant resistance in Gram-negative bacteria: Occasional reports have examined the possible role of plasmids in the resistance of Gram-negative bacteria to antiseptics and disinfectants (McDonnell and Russell, 1999).

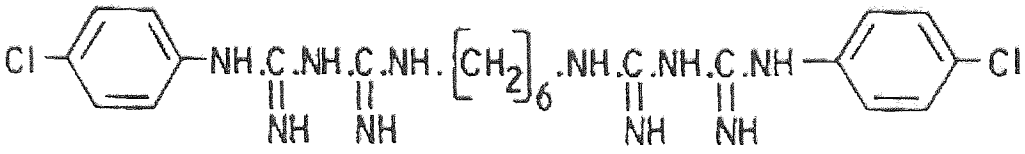
b) Plasmid-mediated antiseptic and disinfectant resistance in staphylococci: Methicillin-resistant *S. aureus* (MRSA) strains are a major cause of sepsis in hospitals throughout the world, although not all strains have increased virulence. Many can be referred to as "epidemic" MRSA because of the ease with which they can spread (McClure and Gordon, 1992). Patients at particularly high risk are those who are debilitated or immunocompromised or who have open sores (Russell, 1999).

Staphylococci are the only bacteria in which the genetic aspects of plasmid-mediated antiseptic and disinfectant resistance mechanisms have been described. In *S. aureus*, these mechanisms are encoded by at least three separate multidrug resistance determinants (Sasatsu *et al.*, 1995). Increased antiseptic MICs have been reported to be widespread among MRSA strains and to be specified by two gene families (*qacAB* and *qac CD*) of determinants (Leelaporn *et al.*, 1996). The *qacAB* family of genes encodes proton-dependant export proteins that develop significant homology to other energy-dependent transporters such as the tetracycline transporters found in various strains of tetracycline-resistant bacteria (Russell *et al.*, 1991, 1999).

1.11 Chlorhexidine, properties, uses and action

Chlorhexidine is an antimicrobial agent first synthesized at Imperial Chemical Industries in 1954 in a research program to produce compounds related to the antimalarial, proguanil. The chlorhexidine molecule, a bisbiguanide, is symmetric. A hexamethylene chain links two biguanide groups to each of which a *p* chlorophenyl radical is bound (Hugo and Russell, 1998 and Russell *et al.*, 2002).

Figure: 1.8 Chemical structure of Chlorhexidine



Chlorhexidine

Chlorhexidine base is not readily soluble in water therefore the freely soluble salts, acetate, gluconate and hydrochloride, are used in formulation. Chlorhexidine exhibits the greatest antibacterial activity at pH 7-8 where it exists exclusively as a di-cation. The cationic nature of the compound results in activity being reduced by anionic compounds including soap and many anions due to the formation of insoluble salts. Anions to be wary of include bicarbonate, borate, carbonate, chloride, citrate and phosphate with due attention being paid to the presence of hard water. Deionized or distilled water should preferably be used for dilution purposes. Reduction in activity will also occur in the presence of blood, pus and other organic matter (Hugo and Russell, 1998).

Chlorhexidine is available as the acetate (diacetate), hydrochloride and gluconate salts. These are stable in solution and can be autoclaved although small amounts of chloroaniline are released (Goodall *et al.*, 1968). Activity is also reduced or abolished by phospholipids (a factor of significance in neutralizing chlorhexidine activity during the performance of biocidal tests) and by organic matter including serum (Hugo *et al.*, 1992 and Russell *et al.*, 1993).

Chlorhexidine has widespread use, in particular as an antiseptic. It has significant antibacterial activity though Gram-negative bacteria are less sensitive than Gram-positives. A concentration of 1:2 000 000 prevents growth of, for example, *S. aureus* whereas a 1:50000 dilutions prevents growth of *P. aeruginosa.* Chlorhexidine is ineffective at ambient temperatures against bacterial spores and *M. tuberculosis.* A limited antifungal activity has been demonstrated which unfortunately restricts its use as a general preservative. Skin sensitivity has occasionally been reported, although, in general, chlorhexidine is well tolerated and non-toxic when applied to skin or mucous membranes and is an important preoperative antiseptic (Hugo and Russell, 1998).

Chlorhexidine is an important medical, dental and pharmaceutical antiseptic, disinfectant and preservative (Table1.7) (Hugo *et al.,* 1992; Scott *et al.*, 1992; Russell *et al.*, 1986 and Russell *et al.* 1990). It is bactericidal and fungicidal, but does not kill bacterial spores or mycobacteria (Russell *et al.*, 1993).

Table: 1.4 Activity of chlorhexidine towards different organisms (McDonnell and Russell, 1999).

Enveloped viruses	Most [illegible]

Jones (1997) has reported that after 20 years of use by the dental profession, chlorhexidine is recognized as the gold standard against plaques and gingivitis. Chlorhexidine formulations are also employed for surgical hand disinfection in procedures for pre-operative disinfection on surgeons' hands (McDonnell and Russell, 1999).

Chlorhexidine is much less surface active than cetrimide and has little detergent action. However, it acts against a wide range of bacteria at concentrations between 10 and 50 μg/ml. Its toxicity is low and it has so little irritancy that it can be used on the most sensitive mucosal surfaces. During recent years it has found an application in oral hygiene (Nicolletti *et al.*, 1992).

The mode of action of chlorhexidine is particularly well documented. The biguanide is membrane active, it initially causes a high rate of leakage of intracellular components but, at higher concentrations, it causes coaggulation of the cytosol (Hugo and Longworth, 1964, Daltrey and Hugo, 1974; Fitzgerald *et al.* 1989). However, there might not be an obvious relationship between the amount of cell constituents released and the decrease in cell viability (Tattawasart *et al.*, 1999 and Maillard, 2002).

High concentrations of chlorhexidine cause coagulation of intracellular constituents. As a result, the cytoplasm becomes congealed, with a consequent reduction in leakageof cytoplasmic constituents Longworth (1971), so that there is a biphasic effect on membrane permeability. An initial high rate of leakage is reduced at higher biocide concentrations because of the coagulation of the cytosol (Russell *et al.*, 1999).

Chlorhexidine was claimed by Harold *et al* (1969) to be an inhibitor of both membrane-bound and soluble ATPase as well as of K^+ uptake in *Enterococcus faecalis*. However, only high biguanide concentrations inhibit membrane-bound ATPase (Chopra, 1991), which suggests that the enzyme is not a primary target for

chlorhexidine action. Although chlorhexidine collapses the membrane potential, it is membrane disruption rather than ATPase inactivation that is associated with its lethal effects (Barrett *et al.*, 1991, Larson, 1996 ; McDonnell *et al.*, 1999).

Chlorhexidine is not sporicidal but it has little effect on the germination of bacterial spores (Russell, 1977) but inhibits outgrowth (Russell *et al.* 1985 and 1999). Mycobacteria are generally highly resistant to chlorhexidine (Russell *et al.*, 1996, 1999).

Despite the advantages of chlorhexidine, its activity is pH dependent and is greatly reduced in the presence of organic matter (Russell *et al.*, 1996).

1.11.1 Bacterial resistance to CHX

Russell *et al* (1986) have reported that bacterial resistance to antibiotics and other chemotherapeutic agents is a phenomenon that has been known for many years. This has resulted in extensive laboratory investigations of the mechanisms of resistance and in clinical expertise at overcoming the problem. Bacterial resistance is usually the result of drug exclusion, tolerance, drug inactivation or modification, alteration of a target enzyme, or alteration of a target-binding site.

According to Russell (1997) plasmid-mediated resistance to organic cationic agents, which are important biocides, has been described for chlorhexidine and quaternary ammonium compounds (and also for the less important acridines, diamidines and ethidium bromide) in antibiotic-resistant *S. epidermidis* strains.

Prince *et al* (1978) reported that resistance to chlorhexidine could be induced in some organisms but not in others. Fitzgerald *et al* (1992) were unable to develop stable chlorhexidine resistance in *E. coli* or *S. aureus*. Similar observations were made by Denyer *et al* (1995), who worked with MRSA and other strains of *S. aureus* by McDonnell *et al* (1997), McDonnell and Russell (1999).

According to Yasuda *et al* (1997), opportunistic infections caused by Gram-negative rods (GNR) (e.g. *Proteus mirabilis* and *Providencia sp.*) conventionally regarded as organisms with low or no pathogenicity, and intractable infections caused by various resistant organisms pose a great problem now. With chlorhexidine, 1 strain each of both species was not killed within 10 min of exposure at a concentration of 0.2%. They also found that the time required for chlorhexidine to kill strains of these species of bacteria was longer for resistant strains than for sensitive strains. In particular, strains, which were proven to be highly resistant to chlorhexidine, showed also a high degree of resistance to various antibacterial agents.

Kunisada *et al* (1997) determined the bacterial activity of commonly used antiseptic especially chlorhexidine against clinical isolates. In the case of chlorhexidine gluconate (CHG), residual bacteria were observed in most species. Next, acquisition of resistance to antiseptics was examined, and it was found that remarkable increases in MICs were seen for CHG.

Nicolleti *et al* (1992) demonstrated that chlorhexidine engendered the greatest level of resistance in all their tested strains. Resistance to chlorhexidine was least with *Candida albicans* and greatest with the Gram-negative bacilli.

Walker *et al* (1985) reported that chlorhexidine resistance is a complex phenomenon, which is difficult to evaluate. If *in vitro* tests are to be used to evaluate the clinical relevance of reduced sensitivity to chlorhexidine, they must mimic the in-use conditions as closely as possible. Some bacterial species like, *Proteus* and *Providencia,* are naturally resistant to chlorhexidine (Russell and Chopra, 1990 and Russell *et al.,* 1993).

1.11.2 Resistance of staphylococci to CHX

Russell *et al* (1993) reported the possible relationship between chlorhexidine susceptibility and resistance in staphylococcus. Many studies have examined the plasmind-associated *qac* genes in staphylococci. At least five of these genes (*qacA-E*) are known, with *A* and *B*, and *C* and *D* sharing homology. In addition *qacA* encodes resistance to chlorhexidine and is often carried on a penicillinase plasmid. Resistance has usually been measured in terms of MIC increases, which in resistant strains are 2-8 fold higher than in sensitive strains. Antibiotic-sensitive *S. aureus* and CoNS are usually antiseptic-sensitive, whereas strains for which the MICs of antiseptics indicate intermediate or high resistance are also more resistant to a wide range of antibiotics (Reverdy, 1992 ; Russell *et al.*,1999).

According to Jones (1999) each of the *qac* genes has a slightly different specificity. *qacA* confers resistance to intercalating dyes (e.g. ethidium bromide), quaternary ammonium chlorides, and divalent cations (e.g. chlorhexidine), *qacB* confers a low-level or no resistance to divalent cations, *qacC/D* are specific for resistance to quaternary ammonium compounds and some dyes, and *qacE* is similar to the multidrug resistance of *qacA*.

Russell *et al* (1999) investigated whether the presence of a *qac* gene selects for antibiotic-resistant bacteria. It is unlikely that resistance to such agents will be a clinical problem. The *qacA* gene encodes a membrane protein, which is involved in an antiproton system whereby, drugs and low concentrations of biocides are exported and protons (H^+) are taken up. At high disinfectant concentrations, severe membrane damage occurs and the amount exposed is very low and insufficient to prevent cell death. Investigation of the effects of high and low residual concentrations of QACs and chlorhexidine on membrane perturbation in sensitive and multi resistant *S. aureus* strains would provide some instructive information about membrane damage, loss of viability and efflux.

Al Masaudi *et al* (1991) and Russell *et al* (1993) have reviewed the genetic basis of antimicrobial resistance. Chlorhexidine resistance in *S. aureus* has been transferred to *E. coli* by recombinant plasmids (Yamamoto *et al.,* 1988) and in *Streptococcus. sanguis* by transformation (Westergren *et al.,* 1980). Plasmid containing strains of Gram-negative bacteria do not appear to be more resistant to chlorhexidine than isogenic strains lacking the plasmid; in some instances they may even be rather more sensitive. Chlorhexidine reduces plasmid transfer in *S. aureus* (Briand *et al.,* 1986).

Thus, development of resistance to biocides such as chlorhexidine, which have a multiplicity of cellular target sites, would appear to require a similar evolutionary process. By analogy, it is logical to expect to see only small increases in resistance which is not necessarily stable, and not to envisage major changes. These low increases in resistance might be associated with alterations in the cellular surface (Fitzgerald *et al.,* 1992).

The major cause of resistance to antimicrobials is production of inactivating or modifying enzymes (e.g. beta-lactamases and aminoglycoside modifying enzymes). With the introduction of antibiotics, which are resistant to this kind of inactivation, the pattern of resistance is changing. Consequently we are facing increasing reports of resistance due to changes in the targets themselves, and to exclusion of the antimicrobial (Lambert, 2002).

With an increase in the isolation of methicillin-resistant *S. aureus* (MRSA), taking preventive measures against nosocomial infections has attracted more and more attention. As a result, antiseptics are used extensively in clinical practice. However, it is known that some of the strains of MRSA, *Serratia marcescens*, *Pseudomonas aeruginosa* and *S. epidermidis*, cause opportunistic infections (Kunisada *et al.,* 1997).

Nosocomial infections occur more often in compromised hosts. With the rapid progress achieved in medicine in recent years, even patients with serious diseases can be treated. As a result, the incidence of nosocomial infections has increased sharply recently, nosocomial infections caused by MRSA now pose a major problem. Among pathogenic organisms, which cause nosocomial infections, those which are resistant to antiseptics have been reported by many workers (Furuta et *al.*, 1992, McLure and Kunisada *et al.*, 1997).

According to Russell *et al* (1993) MRSA strains are no less sensitive to the lethal effects of chlorhexidine than methicillin sensitive *S. aureus* (MSSA). Cookson *et al* (1991) demonstrated that two gentamicin-resistant MRSA isolates without plasmid were more sensitive to chlorhexidine than several MSSA strains. In contrast, seven distinct MRSA isolates with plasmids encoding resistance to gentamicin had higher (MICs).Oie *et al* (1996) investigated the handling and use of antiseptics and disinfectants. They concluded that it is necessary to check microbial contamination of diluted benzalkonium chloride and diluted chlorhexidine gluconate that are in use. Such diluted products are not recommended as antiseptics.

Cookson and Bolton (1991) reported a moderate increase of resistance to chlorhexidine in antibiotic-resistant *S. aureus*. Hedin *et al* (1993) reported the same results in *S. epidermidis*. According to Hedin *et al* (1993) strains of *S. epidermidis* resistant to one or more of the antibiotics used for selective culture (subsequently referred to as resistant *S. epidermidis*) were found in 89% of their tested samples.

A more likely explanation by Brown et *al* (1989) about the increased resistance of *S. epidermidis* to chlorhexidine *in vitro* is that they colonised lower layers of the skin and were not reached by disinfecting agents. Hedin *et al* (1993) also reported the persistence of strains on the skin. Staphylococcal resistance to antiseptics has been reported throughout the world. Al-Masudi *et al* (1991) and Jones (1999) reported low-level resistance to antiseptics (benzalkonium chloride, chlorhexidine, and

hexamidine di-isethionate) in 36.7% of a collection of 392 strains representing 26 *Staphylococcus* species.

Chopra (1992) investigated the genetic mechanisms responsible for the resistance to antiseptics and disinfectants in clinical isolates of CoNS. Several authors such as Al-Masudi *et al* (1991), Chopra (1992) and Jones (1999) have concluded that the antiseptic resistance genes evolved before the introduction and use of topical antimicrobial products and biocides. Sasatsu *et al* (1994) have reported that the gene encoding for increased MIC levels for antiseptics (e.g. *ebr*, which has now been found to be identical to *qacC/D*) has been found in sensitive and resistant strains of staphylococci. Jones (1999) concluded that the normal function of the *ebr* gene is to remove toxic substances from the bacterial cells.

In conclusion, there is an urgent need to investigate more fully the nature of the inhibitory and lethal effects of chlorhexidine in a range of microorganisms. Possible multiple target sites and concentration-dependent effects would form an important aspect of such research, which would also provide a better understanding of intrinsic and acquired bacterial resistance mechanisms.

1.12 Aims and objectives of this study

Chlorhexidine is an important medical, dental and pharmaceutical antiseptic, which is widely used in the healthcare environment as an active antimicrobial component of a number of common and surgical scrub and skin disinfectant solutions. Several authors have studied chlorhexidine activity against many Gram-positive and Gram-negative bacteria. It is bactericidal and fungicidal and is recognized as the gold standard against plaques and gingivitis. Coagulase-negative staphylococci, particularly *S. epidermidis*, are the most frequently isolated microorganisms in the clinical microbiology laboratory. The vast majority of infections attributed to CoNS are nosocomial. In this group *S. epidermidis* is the main cause of repeated bacteremia and infection.

The aims of this thesis were to investigate the development of resistance of clinical strains of *S. epidermidis* to chlorhexidine by serial passage and investigate any cross-resistance to other disinfectants such as benzalkonium chloride (BKC), triclosan (TLN) and povidone-iodine (PI) and clinically relevant antibiotics. In addition the mechanisms of resistance to chlorhexidine by *S. epidermidis* original strains and those strains showing adaptive resistance were examined. The thesis is presented in the following sections: each experimental chapter preceded by a description of the methods employed:

***Chapter1 (Introduction)*:**
A review of the role of *S. epidermidis* in a clinical setting and a brief review of chlorhexidine and its action upon the *S. epidermidis* cytoplasmic membrane.

***Chapter 2*:** Selection of clinical isolates of CoNS obtained from a range of significant infections: CAPD-related peritonitis, CVC associated septicemia and osteomyelitis related to internally fixed bone fractures and hip joints.

***Chapter 3*:** Sensitivity of *S. epidermidis* isolates to chlorhexidine.

***Chapter 4*:** Investigation of the lipid composition of *S. epidermidis*.

***Chapter5*:** Generation of resistance to chlorhexidine by serial passage of *S. epidermidis* cells

***Chapter 6*:** Investigation of the mechanism of resistance to chlorhexidine.

***Chapter7*:** Investigation of membrane damage induced by chlorhexidine in *S. epidermidis*.

***Chapter 8*:** Conclusions.

Chapter 2 Diagnosis of bacterial strains, materials and methods

2.1 Introduction

This chapter aims to identify *S. epidermidis* from other CoNS among clinical isolates by standard laboratory tests and biotyping using the Analytical Profile Index (API STAPH). Due to different sources of the strains, expression of potential virulence factors of CoNS was also investigated.

2.2 Bacterial strains

2.2.1Coagulase-negative staphylococci (CoNS)

A total of 94 CoNS isolates were obtained from 76 patients with clinical presentations of infection: CVC-associated septicaemia; CAPD-related peritonitis; osteomyelitis related to prosthetic hip joints and internally fixed bone fractures (Table 2.1).

Table: 2.1 Origin of CoNS included in the study

Type of infection	*Medical centre*	*Number of patients*	*Total number*
CVC	QEH	15	27
CAPD	QEH	24	25
Fixed bone fracture (NU)	HH	17	17
Prosthetic hip	ROH	20	25
Total		76	94

QEH, Clinical Microbiology Laboratory Birmingham UK, Queen Elizabeth Hospital (QEH), ROH, Royal Orthopaedic Hospital, Birmingham; HH, Hope Hospital, Salford. CVC, isolates from central venous catheters;CAPD,isolates from patients with peritonitis associated with continuous ambulatory peritoneal dialysis; fixed bone fractures, isolates from infected bone with internal metal fixation; prosthetic hip, isolates from infected prosthetic hip joints.

2.2.2 Identification of staphylococci

When there is a possibility of staphylococcal infection, isolation and identification of *S. aureus* is of clinical importance. These virulent strains can be differentiated from other staphylococci (e.g *S. epidermidis* and *S. saprophyticus*) and identified by a variety of laboratory tests, some of which are illustrated in (Table 2: 2).

Table 2:2 Laboratory Tests for Differentiation of Staphylococcal Species

Test	*S. aureus*	*S. epidermidis*	*S. saprophyticus*
Growth in Mannitol Salt Agar	+	+	+
Fermentation	+	–	–
Colonial pigmentation	Golden/yellow	White	White
Coagulase	+	–	–
DNase	+	–	–
Haemolysis	Generally beta	–	–
Novobiocin Sensitivity	Sensitive	Sensitive	Resistant

All reagents used in this study were standard laboratory grade chemicals purchased from Sigma Chemical Company, USA, except where stated.

All growth media components were purchased from Oxoid Ltd, UK, except where stated otherwise.

2.3 Diagnosis and speciation of CoNS

The clinical isolates examined in this study were biotyped using the API STAPH (bioMerieux, France), following the manufacturer's instructions, and accordingly assigned to a species. A number of the API profiles required additional tests.

Figure 2:1 Procedure of API STAPH test.

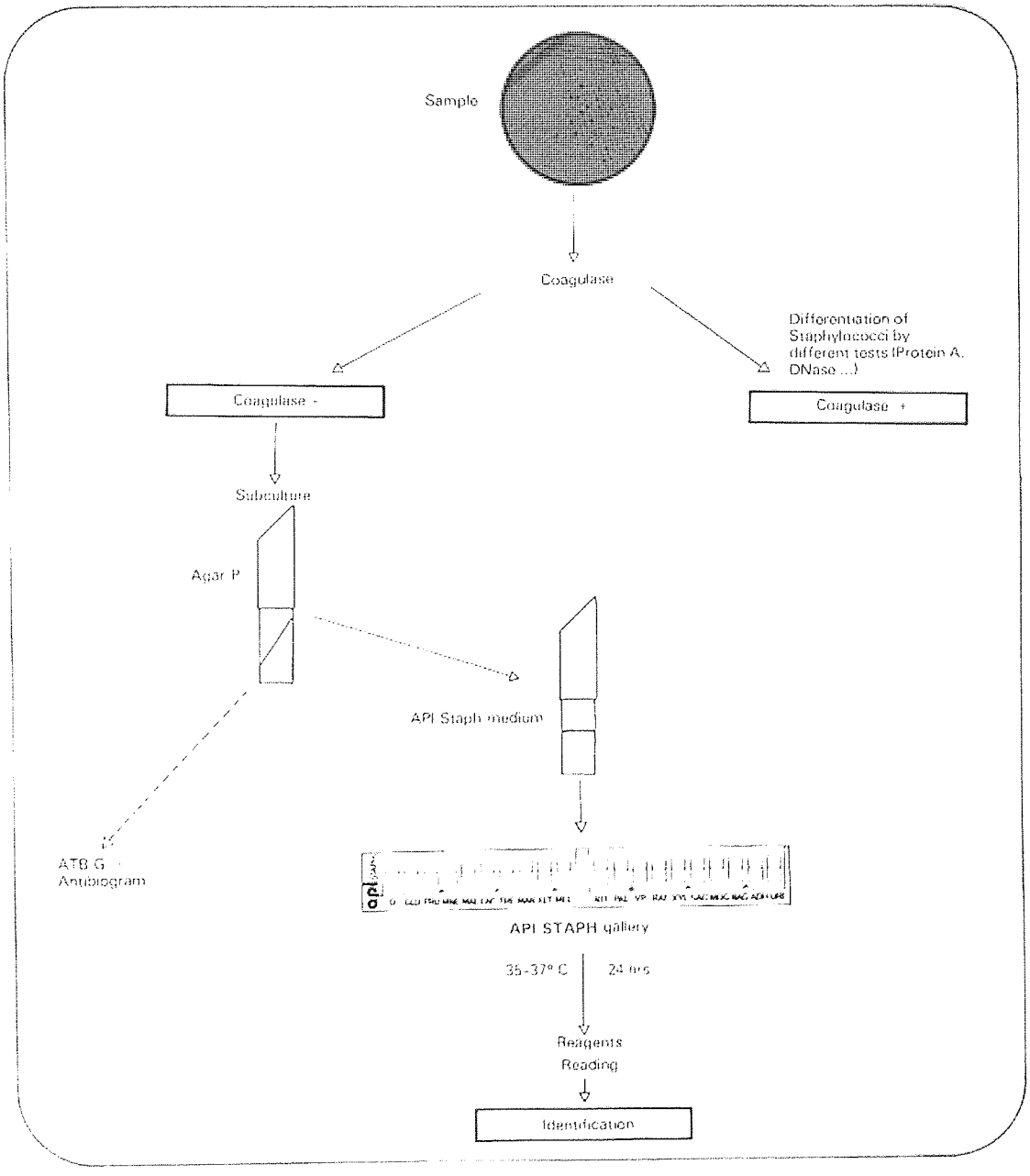
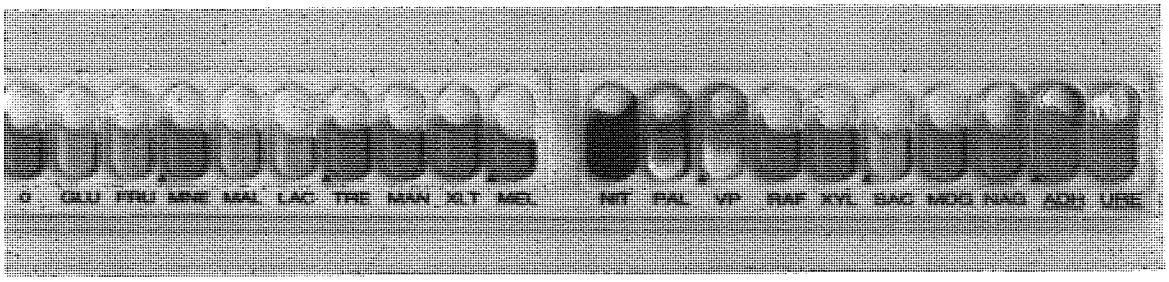


Figure 2:2 Identification of *S. epidermidis* by API STAPH method.



Other laboratory tests for differentiation of staphylococcal species were performed as follows:

2.3.1 Growth on mannitol salt agar

This medium is selective for salt-tolerant organisms such as staphylococci. Differentiation among the staphylococci is predicated by their ability to ferment mannitol. Following incubation, mannitol- fermenting organisms, typically *S. aureus* strains, exhibit a yellow halo surrounding their growth, and non- fermenting strains do not.

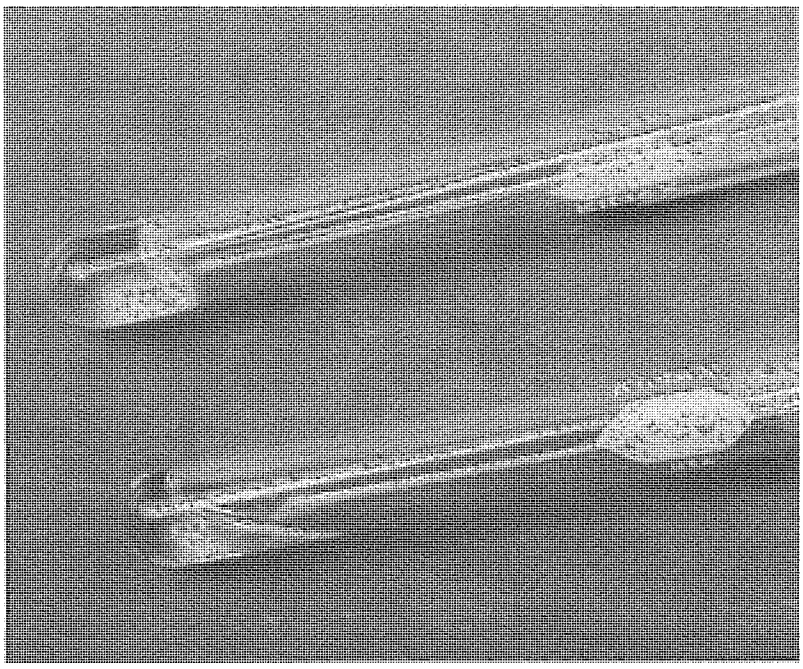
2.3.2 Gram's stain

Morphology and Gram's reaction were evaluated by the standard microbiological Gram's stain.

2.3.3 Coagulase tube test

Production of coagulase is indicative of *S. aureus*. The enzyme acts within host tissues to convert fibrinogen to fibrin. In the coagulase tube test for bound and free coagulase, a suspension of the test organism in citrated plasma prepared and the inoculated plasma then periodically examined for fibrin formation, or coagulation. Clot formation within 4 hours is interpreted as a positive result and indicative of a virulent *S. aureus* strain. The absence of coagulation after 24 hours of incubation is a negative result, indicative of an avirulent strain. *S. epidermidis* NCTC 11047 and a fresh broth-plasma were included as negative controls. *S. aureus* NCTC 6571 was used as positive control.

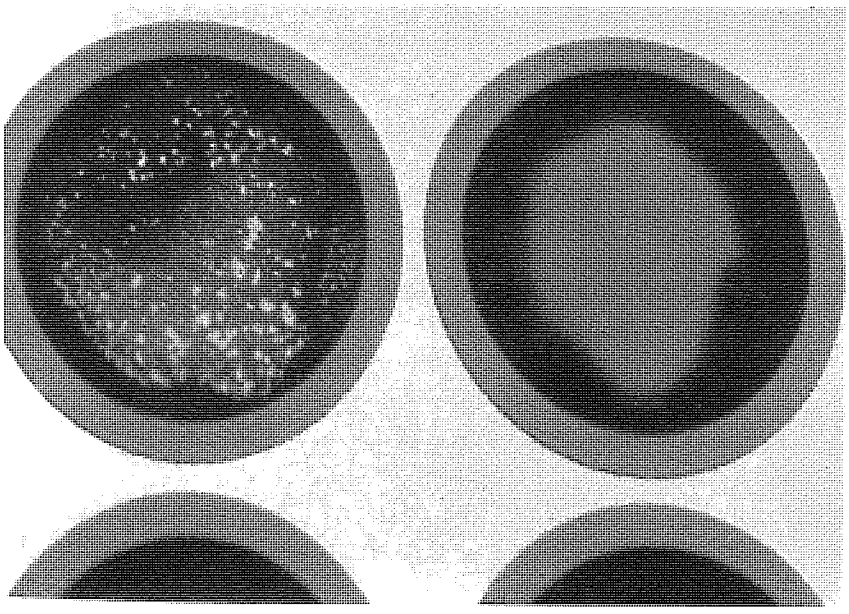
Figure: 2:3 Coagulase tube test. Top tube shows a positive reaction and bottom tube show a negative reaction.



2.3.4 Agglutination test

This test is a rapid agglutination test for the identification of staphylococci that possess clumping factor, protein A (cell surface antigens) and/or surface antigens characteristic of *Staphylococcus aureus.* Clumping factor is a cell-associated substance that binds plasma fibrinogen, causing agglutination of the organisms by binding them together with aggregated fibrinogen, With the exception of *Staphylococcus lugdunensis*, which is clumping factor positive but not coagulase positive, those organisms that produce clumping factor also elaborate the coagulase enzyme and can be identified presumptively as *S. aureus.* Not all *S. aureus* strains produce clumping factor (Baron *et al*, 1994). The test was performed by using the Staphaurex plus kit (ABBOTT Murex) following the manufacturer's procedure. *S. aureus* ATCC 25923 and *S .epidermidis* ATCC 12228 were used as reference strains (Figure 2:4).

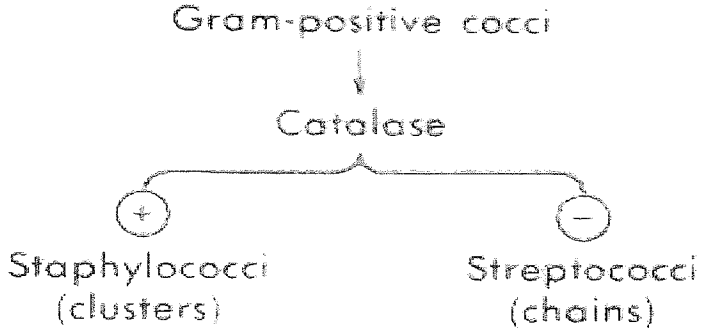
Figure 2:4 Agglutination system (Staphaurex,Murex) for identification of *S. aureus* based on presence of protein A or clumping factor on bacterial surfaces. Agglutination indicates a positive reaction.



2.3.5 Catalase Test

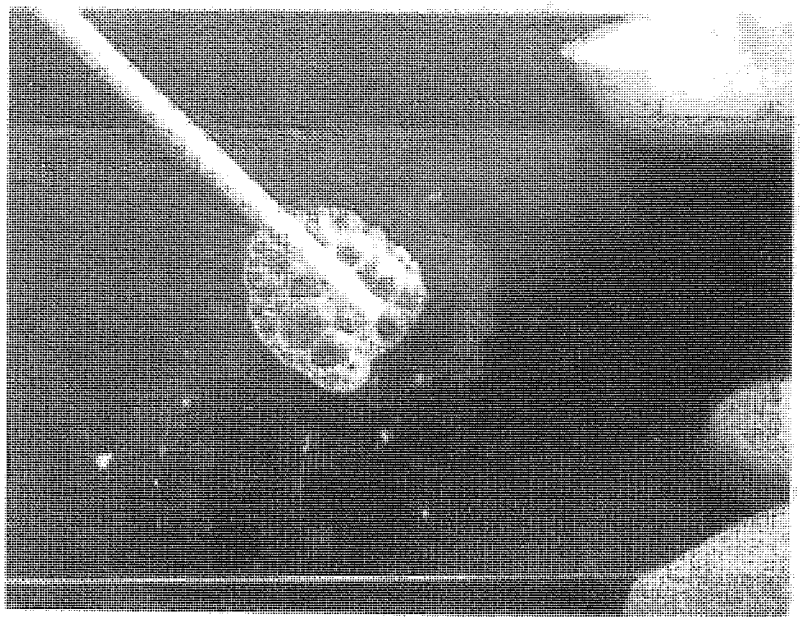
Some microorganisms have the ability to degrade hydrogen peroxide by producing the enzyme catalase. During aerobic respiration, microorganisms produce hydrogen peroxide and, in some cases, an extremely toxic superoxide. Accumulation of these substances will result in death of the organism unless they can be enzymatically-degraded. Organisms capable of producing catalase rapidly degrade hydrogen peroxide as illustrated. Gram-positive cocci are differentiated with the catalase test (Figure 2:5) (Cappuccino and Sherman, 2001).

Figure 2: 5 Differentiation of Gram positive cocci with the catalase test



Fifty µl of 3 %(v/v) hydrogen peroxide (H_2O_2) was used as the substrate. Catalase production was determined by adding the substrate to incubated bacterial colony on a glass slide. Bubbles of free oxygen indicated the positive chemical reaction. The absence of bubble formation has a negative- catalase test (Figure 2:6).

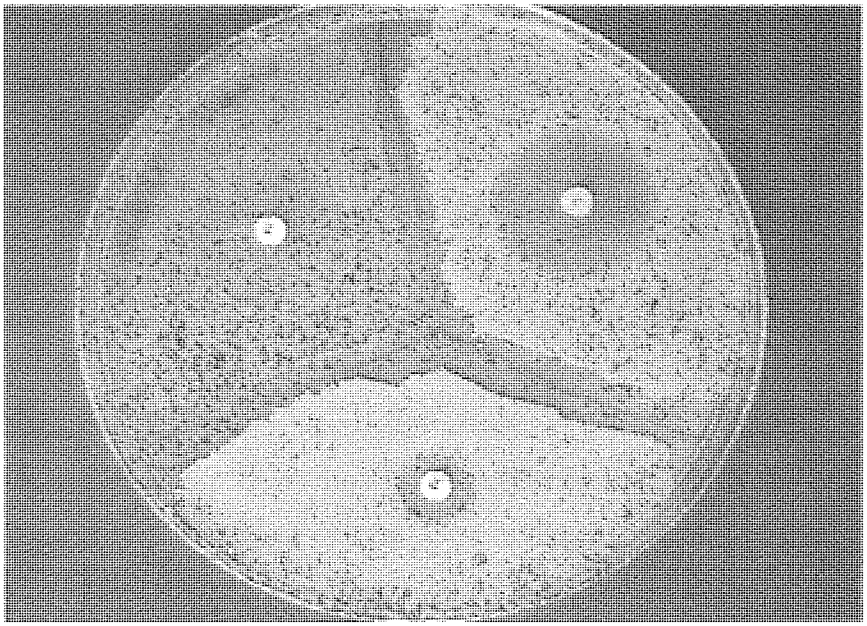
Figure 2:6 Demonstration of catalase test.



2.3.6 Novobiocin sensitivity test

This test was performed to distinguish between *S. epidermidis* and *S. saprophyticus*. A bacterial suspension of the test strain was inoculated onto a Mueller-Hinton agar plate and a 5µg novobiocin disc (Oxoid, UK) was positioned on the surface of the agar. Following incubation at 37° C for 18 hours, the sensitivity of an organism to the antibiotic was determined by the Kirby –Bauer method. The method is described in the antibiogram section (Figure 2:7).

Figure 2:7 Novobiocin sensitivity test: Top left: *S. aureus top right, S. epidermidis* –sensitive. Bottom: *S. saprophyticus*.



2.3.7 β-glucosidase assay

Phosphate buffer [0.067M Na_2HPO_4, 0.067M KH_2PO_4, pH 8.0] was supplemented with 0.1% (w/v) β-glucopyranoside. The solution was filter sterilized and dispensed into 0.5ml aliquots. Aliquots of the β-glucopyranoside substrate were inoculated directly with three to five colonies taken from a brain-heart infusion agar (BHIA) plate of the test strain, and incubated at 37°C for 18h. A yellow coloration of the initially colourless substrate indicated β-glucosidase activity (Isenberg, 1992; Holt *et al.,* 1994, Lang, 2000, Cappuccino and Sherman, 2001). Clinical isolates were used as negative (*S. lugdunensis*) and positive (*S. capitis*) controls.

2.4 Potential virulence factors of CoNS

2.4.1 Introduction

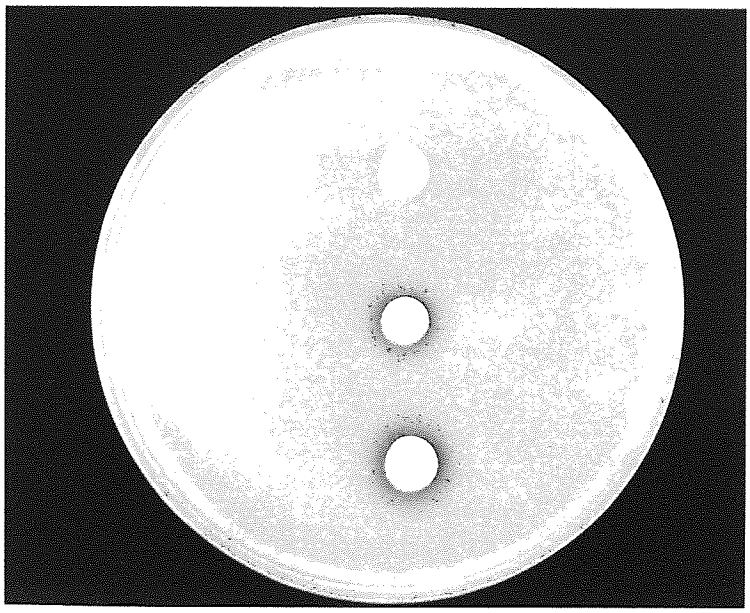
Strains of *S. aureus* produce a variety of metabolic end products, some of which may play roles in the organisms' pathogenicity. Included among these are coagulase, which causes clot formation; leukocidin, which lyses white blood cells; haemolysins, which are active against red blood cells; and enterotoxin, which is responsible for a type of gastroenteritis. Additional metabolites of a nontoxic nature are DNase, lipase, gelatinase, and the fibrinolysin staphylokinase. In contrast, CoNS elaborate few of these factors. Thus although CoNS are the cause of most of the nosocomial infections in hospitals it remains unclear what virulence factors allow these usually avirulent microorganisms to cause infection.

The bacterial isolates were cultivated in 20ml TSB at 37°C for 18h with aeration at 200rpm. Bacterial cells and culture supernatant were prepared as required for each assay. One-quarter of a BHIA plate was inoculated with each strain to confirm the purity of the bacterial culture. Every isolate was screened by duplicate or triplicate assays performed on separate occasions.

2.4.2 Deoxyribonuclease (DNase) test

Generally, CoNS produce the hydrolytic enzyme DNase. The test organism was grown on an agar medium containing DNA (Oxoid). Following incubation at 37°C for 18h, DNase activity was determined by the addition of 1M HCl to the surface of a DNase agar plate. A zone of clearing around the bacterial colonies indicated DNase activity (Figure 2:8). *S. aureus* NCTC 6571 was included as a positive control and *S. epidermidis* NCTC 11047 as a negative control (Figure 2.8).

Figure 2:8 DNase test by the DNase agar method. A zone of hydrolysed DNA around the bacterial colonies indicated DnNase activity. Top *S. epidermidis* negative control. Middle DNase producing clinical CoNS strain. Bottom *S. aureus* positive control.



2.4.3 Urease Test

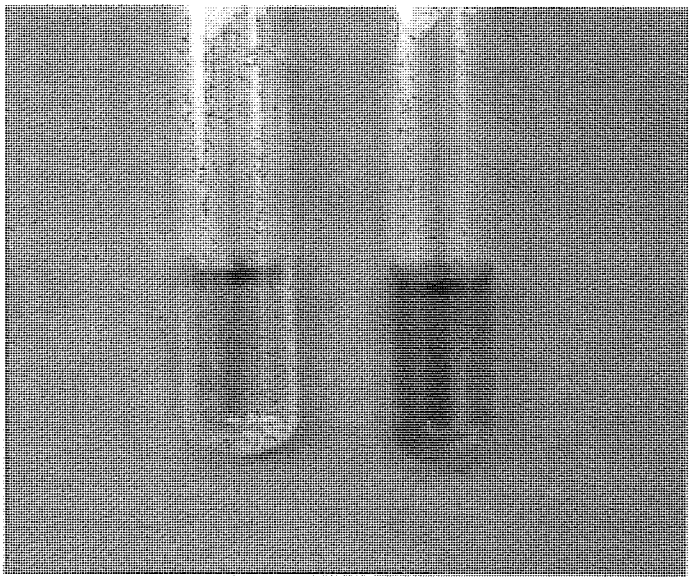
Hydrolysis of urea by the enzyme urease releases the end product ammonia, the alkalinity of which causes the indicator phenol red to change from yellow to red include (Figure 2:9). The broth method employs buffers that control the pH change and speed the reaction. Urea broth was prepared as follows:

Yeast extract monopotassium	0.1g
phosphate	0.091g
Disodium phosphate	0.095g
Urea	20g
Phenol red	0.01g
Distilled water	1000ml

Ingredients were mixed together and store in refrigerator in small glass screw-capped tubes in aliquots of 0.5 ml. A tube of broth inoculated with heavy suspension of the organism to

be tested was incubated at 35° C and observed at 15, 30 and 60 minutes and up to 4 hours for a change in colour to pink or red.

Figure 2: 9 Urease Test. Left tube shows a negative reaction and the right tube indicates a positive reaction.



2.4.4 Urease activity

Urease activity has previously been demonstrated in isolates of *S. aureus, S. epidermidis, S. xylosus* and *S. saprophyticus* (Mobley, Island and Hausinger, 1995; Schafer and Kaltwasser, 1994).

2.4.5 Proteinase activity

Culture supernatant recovered from each strain was assessed for proteinase activity by a skimmed milk agar plate method. A zone of clearing (extending up to 5mm from the edge of the well) around the well indicated non-specific proteinase activity.

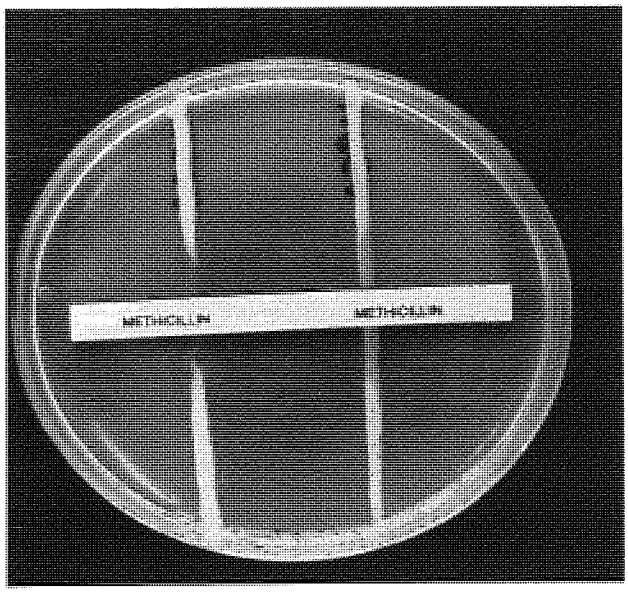
2.4.6 Lipase/esterase activity

Lipase/esterase activity of the culture supernatant was assessed by a nonspecific method - glycerol tributyrate agar assays (Lang, 2000). A zone of clearing around the wells of the glycerol tributyrate agar plate (extending up to 5mm from the well edge) indicated lipase and/or esterase activity.

2.4.7 Methicillin sensitivity test

Sensitivity to methicillin was assessed for each strain. As recommended by the manufacturer, each prepared bacterial strain was line inoculated onto a salt agar plate [Columbia agar supplemented with 2% (w/v) NaCl (Oxoid)]. Each plate was inoculated with one test strain and a positive control (a clinical strain of MRSA) and a negative control strain (*S. aureus* NCTC 6571). A methicillin strip (25μg) was placed onto the plate perpendicular to the line inoculations and incubated at 30°C for 18h.

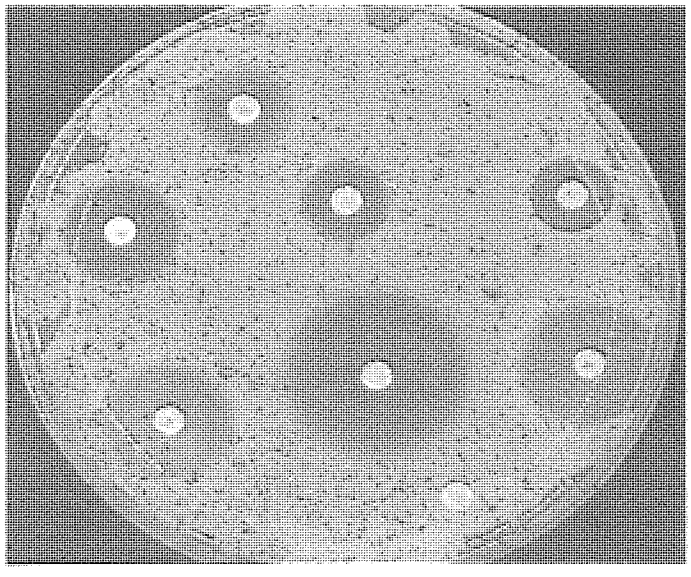
Figure 2: 10 Methicillin sensitivity test. Right sensitive strain, left resistant strain



2.4.8 Antibiograms

The sensitivity of each bacterial strain against a panel of antimicrobial agents was assessed by the standardized filter-paper disc-agar diffusion procedure, known as the Kirby-Bauer method (Cappuccino and Sherman, 2001). This method allows the rapid determination of the efficacy of a drug by measuring diameter of the zone of inhibition that results from diffusion of the agent in to the medium surrounding the disc. In this procedure, filter-paper discs of uniform size were impregnated with specified concentrations of different antibiotics and then placed on the surface of a Mueller –Hinton agar plate that had been seeded with the organism to be tested. Following incubation, the plates were examined for the presence of growth inhibition, which was indicated by a clear zone surrounding each disc. A measurement of the diameter of the zone of inhibition in millimeters was made, and its size was compared to that contained in a standard chart (Figure 2:11).

Figure 2:11 Antibiograms by the Kirby-Bauer antibiotic susceptibility test.



Sensitivity of each bacterial strain was measured by disc diffusion method as well (Scott, 1989). A bacterial suspension of the control strain *S. aureus* NCTC 6571, prepared by diluting an 18h culture 1:100 with sterile TSB, was inoculated onto the outer 1cm ring of

a Mueller-Hinton agar plate using a spiral plater. A suspension of the test strain, similarly prepared was inoculated onto the remaining section of the plate. The antibiotic discs (penicillin 1 unit, erythromycin 5μg, gentamicin 10μg, vancomycin 5μg, fusidic acid 10μg, ciprofloxacin 1μg, all manufactured by Oxoid) were placed on the interface between the control and the test strain, equidistantly separated. The plate was incubated at 37°C for18h.

2.5 Results

2.5.1 Diagnosis and speciation of CoNS by API STAPH test

A few bacterial strains gave either a low discrimination profile or a profile that was not recognised by the API database (version 3.1). Isolates that repeatedly failed to identify were confirmed to be tube-coagulase- negative, catalase+ positive, Gram-positive cocci. These strains were referred to as CoNS.

2.5.2 Antibiogram

Each strain was tested for sensitivity to seven antibiotics by the disc diffusion method. The zone of inhibition in mm of the test strain around the antimicrobial disc was compared to that of the control strain. Test strain zones that were larger or equal to those of the control strain indicated sensitivity, whereas growth up to the disc or zones smaller than those of the control denoted resistance to the antibiotic.

The antibiograms result (Table 2:3) showed that generally the catheter-related sepsis strains were sensitive to fewer of the antibiotics than the osteomyelitis strains. This probably reflects the origin of the pathogens; nosocomial-acquired microorganisms tend to be more resistant to antimicrobials than community-acquired infections. There were no distinct antibiograms associated with a particular infection type (Table 2: 3).

Table 2:3 Lists the antibiotic sensitivity of the strains CVC, CAPD, ROH, NU and EN.

CoNS	Meth	Pen	Ery	Cip	Gent	Fus	Van
CVC1	S	R	S	S	S	S	S
CVC7	R	R	R	R	R	R	S
CVC9	R	R	R	R	R	S	S
CVC11	S	R	R	S	S	S	S
CVC 12	R	R	S	S	R	S	S
CVC16	R	R	R	R	R	S	S
CVC17	S	R	R	R	R	R	S
CVC19	S	R	R	R	R	R	S
CVC20	R	R	R	R	R	S	S
CAPD1	R	R	R	S	S	S	S
CAPD2	R	R	R	R	R	R	S
CAPD3	S	S	S	S	S	S	S
CAPD4	R	R	S	S	S	S	S
CAPD5	R	R	R	R	R	S	S
CAPD6	S	R	S	S	S	S	S
CAPD7	S	R	S	S	S	R	S
CAPD8	R	R	R	S	R	S	S
CAPD9	S	R	S	S	S	R	S
CAPD10	R	R	R	R	R	R	S
CAPDIIb	R	R	R	R	R	R	S
CAPD13	R	R	R	S	S	S	S
CAPD14	R	R	R	R	R	S	S
CAPD15	R	R	S	S	S	R	S
CAPD18	R	R	R	R	S	S	S
CAPD19	S	R	R	R	S	S	S
CAPD20	S	R	R	R	R	S	S
CAPD21	S	R	S	S	S	S	S
CAPD23	R	R	S	S	S	S	S
CAPD25	R	R	R	R	R	R	S
ROH1	R	R	S	S	R	S	S
ROH2	S	R	S	S	S	S	S
ROH3	S	S	S	S	S	S	S
ROH4	S	R	R	S	S	R	S
ROH5	S	R	S	R	S	S	S
ROH6	S	R	S	S	S	S	S
ROH8	R	R	R	S	S	R	S
ROH10	S	R	S	S	S	S	S

The inhibition zones were measured in mm.

Table 2:3 Continued

CoNS	Meth	Pen	Ery	Cip	Gent	Fus	Van
R0H11	R	R	R	R	R	S	S
ROH13	R	R	R	R	R	S	S
ROH14	R	R	R	R	R	S	S
BONE6	R	R	R	S	S	S	S
NU1	R	S	S	S	S	S	S
NU3	R	R	R	S	S	S	S
NU7	R	R	R	S	S	S	S
NU11	R	R	S	S	R	S	S
NU24	R	R	R	S	S	S	S
NU27	S	S	S	S	S	S	S
NU104	R	R	R	S	S	S	S
NU159	S	S	S	S	S	S	S
NU160	S	R	R	S	S	S	S
NU168	R	R	R	S	S	S	S
NU257	S	S	S	S	S	S	S
NU5436	R	R	R	S	S	S	S
NU5438	R	R	S	S	S	S	S
EN10	R	R	S	R	R	R	S
EN13	S	R	S	S	S	S	S
EN19	S	S	S	S	S	S	S
EN23	S	R	R	R	R	R	S
EN27	S	S	S	S	S	S	S
EN32	R	R	S	S	R	S	S
EN33	R	R	R	S	S	S	S
NCTC 11047	S	R	S	S	S	S	S

meth, methicillin; pen, penicillin; ery, erythromycin; cip, ciprofloxacin; gent, gentamicin; fus, fusidic acid; van, vancomycin.

The CoNS are colour coded to indicate the infection type from which the isolates were recovered;
CVC-related sepsis
CAPD-associated peritonitis
Internally fixed bone fracture-associated osteomyelitis
Prosthetic hip-related osteomyelitis
Endocarditis
NCTC type strain
The inhibition zones were measured in mm.

2.5.3 Virulence factors tests

Virulence factor expression for the *S. epidermidis* clinical isolates is shown in Table 2:4. Only a small minority of the strains investigated (five out of sixty eight) produced an observable level of DNase. The failure of the majority of strains to hydrolyse DNA suggests that the activity of this enzyme had little if any pathogenic function in the infections investigated. The majority of CoNS strains screened in this study produced a urease enzyme. Most of the strains associated with catheter-related sepsis secreted a proteinase enzyme, whereas substantially fewer isolates recovered from the other infections exhibited enzymic activity. Lipase and esterase activity was observed in at least three-quarters of the strains in a detectable level of secretion.

Table 2:4 Lists the production of potential virulence factors by clinical strain isolate of *S. epidermidis*.

Strain of isolate	Species	Nonspecific proteinase	Proteinase -elastase (elastin agar)*	Lipase	Esterase	Slime-congo red agar	Dnase **	Haemolysis (Sheep red blood cells) ***	Urease ****
CVC1	*S. epidermidis*	+	+ 5	+	3 +	-	-	-	++
CVC10	*S. epidermidis*	+	+/-	+	5 +	+	-	-	+
CVC9	*S. epidermidis*	+	+/-	+	2 +	+	-	-	+
CVC 11	*S. epidermidis*	+	+ 11	+	5 +	-	-	-	++
CVC12	*S. epidermidis*	-	-	+	-	-	-	-	+
CVC16	*S. epidermidis*	+	-	+	3 +	+	-	-	+++
CVC17	*S. epidermidis*	+	-	+	3 +	+	-	-	+
CVC19	*S. epidermidis*	+	+ 8	+	3 +	+	-	-	++
CVC20	*S. epidermidis*	+	-	+	+/-	-	-	-	+
PAL1	*S. epidermidis*	+	+/-	+	7 +	+	-	-	++
PAL2	*S. epidermidis*	+	-	+	7 +	+	-	-	+
PALS	*S. epidermidis*	-	-	-	-	-	-	-	++
PAL4	*S. epidermidis*	+	+ 21	+	5 +	+	-	-	++
PALS	*S. epidermidis*	+	.	+	5 +	-	-	-	++
023	*S. epidermidis*	+	+ 11	+	3 +	-	-	-	-
9865	*S. epidermidis*	+	+ 21	+	3 +	+	-	-	+
CAPD1	*S. epidermidis*	+	-	+	2 +	-	-	-	+
CAPD2	*S. epidermidis*	-	-	+	11 +	-	-	-	-
CAPD3	*S. epidermidis*	+	+/-	+	2 +	-	-	-	++
CAPD4	*S. epidermidis*	+	+ 11	+	2 +	-	-	-	+++
CAPD5	*S. epidermidis*	+	+ 21	+	7 +	+	-	-	++
CAPD6	*S. epidermidis*	+	-	+	2 +	+	-	-	+
CAPD7	*S. epidermidis*	+	+/-	+	1 +	-	-	-	+
CAPD8	*S. epidermidis*	+	+ 3	+	2 +	-	-	-	+++
CAPD9	*S. epidermidis*	+	-	+	2 +	-	-	-	+

Table 2:4 Continued

Strain of isolate	Species	Non-specific proteinase	Proteinase -elastase (elastin agar)	Lipase	Esterase	Slime-congo red agar	DNase	Haemolysis (Sheep red blood cells)	Urease
CAPD10	*S. epidermidis*	+	-	+	5 +	-	-	-	+
CAPD11b	*S. epidermidis*	+	-	+	5 +	-	-	-	+
CAPD13	*S. epidermidis*	+	+/-	+	1 +	+	-	-	+
CAPD14	*S. epidermidis*	+	+/-	+	5 +	-	-	-	+++
CAPD15	*S. epidermidis*	+	+/-	+	3 +	-	-	-	-
CAPD18	*S. epidermidis*	+	-	+	3 +	-	-	-	-
CAPD19	*S. epidermidis*	+	+/-	+	5 +	+	-	-	+
CAPD20	*S. epidermidis*	+	+ 5	+	5 +	+	-	-	+
CAPD21	*S. epidermidis*	+	+/-	+	5 +	+	-	-	+++
CAPD23	*S. epidermidis*	-	+/-	+	3 +	-	-	-	+++
CAPD25	*S. epidermidis*	+	+/-	+	3 +	+	-	-	+
NU1	*S. epidermidis*	+	+ 7	+	3 +	+	-	-	++
NU3	*S. epidermidis*	+	+/-	+	5 +	-	-	-	-
NU7	*S. epidermidis*	-	-	+	3 +	-	-	-	-
NU11	*S. epidermidis*	-	-	+	+/-	-	-	-	+
NU24	*S. epidermidis*	+	-	+	11 +	-	-	-	+
NU27	*S. epidermidis*	+	+/-	+	11 +	-	-	-	++
NU104	*S. epidermidis*	-	-	+	3 +	+	-	-	++
NU159	*S. epidermidis*	-	+/-	+	+/-	+/-	-	-	++
NU160	*S. epidermidis*	+	+ 21	+	7 +	+/-	-	-	++
NU168	*S. epidermidis*	-	-	+	7 +	+	-	-	-
NU257	*S. epidermidis*	+	+ 21	+	5 +	-	-	-	-

Table 2:4 Continued

Strain of isolate	Species	Nonspecific proteinase	Non-specific lipase/ esterase	Lipase	Esterase	Slime-congo red agar	DNase	Haemolysis (Sheep red blood cells)	Urease
NU5436	*S. epidermidis*	+	+	+	5 +	-	-	-	-
NU5438	*S. epidermidis*	-	+	-	7 +	-	-	-	+
BONE6	*S. epidermidis*	+	+	+	2 +	+	-	-	++
ROH1	*S. epidermidis*	+	+	+	3 +	-	-	-	-
ROH1	*S. epidermidis*	+	+	+	3 +	-	-	-	-
ROH3	*S. epidermidis*	+	-	+	3 +	-	-	-	-
ROH5	*S. epidermidis*	+	+	+	5 +	+	-	-	++
ROH6	*S. epidermidis*	-	+	+	3 +	-	-	-	-
ROH8	*S. epidermidis*	-	+	+	11 +	-	-	-	++
ROH10	*S, epidermidis*	-	+	+	3 +	-	-	-	+
ROH11	*S. epidermidis*	+	+	+	5 +	-	-	-	+
ROH13	*S. epidermidis*	+	+	+	2 +	+	-	-	+++
ROH14	*S. epidermidis*	+	+	+	5 +	-	-	-	+

Table 2:4 Continued.

Strain of isolate	Species	Non-specific proteinase	Proteinase -elastase (elastin agar)	Lipase	Esterase	Slime-congo red agar	DNase	Haemolysis (Sheep red blood cells)	Urease
EN10	*S. epidermidis*	+	-	+	2 +	+	-	-	+
EN13	*S. epidermidis*	+	-	+	5 +	-	-	-	-
EN19	*S. epidermidis*	+	-	+	7 +	-	-	-	++
EN23	*S. epidermidis*	+	-	+	7 +	+	-	-	+
EN27	*S. epidermidis*	+	-	+	11 +	-	-	-	-
EN32	*S. epidermidis*	-	-	+	-	+	-	-	-
EN33	*S. epidermidis*	-	-	+	3 +	+	-	-	+
NCTC 11047	*S. epidermidis*	-	-	+	3 +	+	-	-	0

The specimen is co lour coded to indicate the infection from which the isolate was recovered;
CVC- (cental venus catheter) related sepsis
CAPD-associated peritonitis
Internally fixed bone fracture-associated osteomyelitis (NU)
Prosthetic hip-related osteomyelitis (ROH)
Endocarditis (EN)
NCTC type strain

Explanation of the table:
* The "proteinase-elastase (elastin agar)" and the "esterase" results indicate the minimum incubation time required (days) for the strain to produce a positive result, whilst a +/-.
symbol indicates that both negative and positive results were obtained for successive assays.
** The "DNase" result is recorded as negative (-), reduced zone of nuclease activity (+), comparable zone size (++), and increased zone size (+++) in comparison to the *S. aureus*.
Oxford NCTC 6571 control strain.
*** "Haemolysis" of sheep erythrocytes is recorded as no haemolysis (-), or the reciprocal of the dilution at which haemolysis was observed.
**** "Urease" activity is presented as no activity observed (-), weak band (+), clear band (++), or strong band observed (+++).

Table2.5 Biotyping of CoNS from CAPD infection using API STAPH

SPECIMEN	PATIENT ID	API PROFILE	API EVALUATION	API ID
CAPD1	G874514	6604112	Good ID 93.5%	*S. epidermidis*
CAPD2	G688015	6704110	Good ID 80.0%	*S. epidermidis*
CAPD3	G451436	6706112	Good ID 98.5%	*S. epidermidis*
CAPD4	G573549	6604113	Good ID 97.2%	*S. epidermidis*
CAPD5	V166406	6306112	Good ID 93.3%	*S. epidermidis*
CAPD6	G861813	660113	Good ID 97.9%	*S. epidermidis*
CAPD7	V075044	670110	Good ID 94.3%	*S. epidermidis*
CAPD8	V183441	6706112	Good ID 98.5%	*S. epidermidis*
CAPD9	V174691	670110	Good ID 94.3%	*S. epidermidis*
CAPD10	G649552	6704152	Good ID 92.4%	*S. epidermidis*
CAPD11a	G865543	610411	Good ID 97.5%	*S. capitis*
CAPD11b	G865543	6704112	Good ID 98.6%	*S. epidermidis*
CAPD12	G546345	6716150	Acceptable ID 88.7%	*S. lugdunensis*
CAPD13	V169737	6606110	Acceptable ID 89.5%	*S. epidermidis*
CAPD14	G769994	6604112	Good ID 93.5%	*S. epidermidis*
CAPD15	G667658	6606111	Good ID93.0 %	*S. epidermidis*
CAPD16	G507221	6606112	Low discrimination	*S. epi/hominis*
CAPD17	S523945	6736110	Acceptable ID 89.8%	*S. sciuri*
CAPD18	G693294	6606110	Acceptable ID 89.5%	*S. epidermidis*
CAPD19	V078481	6606110	Acceptable ID 89.5%	*S. epidermidis*
CAPD20	G862270	6606110	Acceptable ID 89.5%	*S. epidermidis*
CAPD21	G367533	6606110	Acceptable ID 89.5%	*S. epidermidis*
CAPD22	G750726	610600(4)	Excellent ID 99.9%	*Mic. Varians/ roseus*
CAPD23	G897915	6306112	Good ID 93.3%	*S. epidermidis*
CAPD24	G842657	6436153	Good ID 96.7%	*S. simulans*

Table 2:6 Biotyping of CoNS from CVC sepsis using API STAPH

SPECIMEN	SPECIMEN ID	SPECIMEN	API PROFILE	API EVALUATION	API ID
CVC1	1401881	Blood culture	6606113	Good ID 97.9%	*S. epidermidis*
CVC2	1401838	Fistula	6606113	Good ID 97.9%	*S. epidermidis*
CVC3	1401843	Fistula	6606113	Good ID 97.9%	*S. epidermidis*
CVC4	1401842	Blood culture	6606113	Good ID 97.9%	*S. epidermidis*
CVC5	1401916-1	Blood culture	6606111	Good ID 93.0%	*S. epidermidis*
CVC6	1401916-2	Blood culture	6606113	Good ID 97.9%	*S. epidermidis*
CVC7	1403523	Hickman catheter	6704112	Good ID 98.6%	*S. epidermidis*
CVC8	1403539	Blood culture	6704112	Good ID 98.6%	*S. epidermidis*
CVC9	1402454	Blood culture	6606113	Good ID 97.9%	*S. epidermidis*
CVC10	1403934	Blood culture	2716152	Good ID 93.4%	*S. lugdunensis*
CVC11	1403990	Blood culture	6606113	Good ID 97.9%	*S. epidermidis*
CVC12	1405751	Blood culture	6606113	Good ID 97.9%	*S. epidermidis*
CVC13	1405752	Blood culture	6606113	Good ID 97.9%	*S. epidermidis*
CVC14	1405793	Blood culture	6606113	Good ID 97.9%	*S. epidermidis*
CVC15	1405704	Blood culture	6606113	Good ID 97.9%	*S. epidermidis*
CVC16	6009981	CVC tip	6706113	Very good ID 99.1%	*S. epidermidis*
CVC17	1405786	Blood culture	6606113	Good ID 97.9%	*S. epidermidis*
CVC18	1407678	Blood culture	6606113	Good ID 97.9%	*S. epidermidis*
CVC19	1406678	Blood culture	6704152	Good ID 92.4%	*S. epidermidis*
CVC20	1407605	Blood culture	6606113	Good ID 97.9%	*S. epidermidis*
PAL1	NCIMB 40896	-	6706113	Very Good ID 99.1%	*S. epidermidis*
PAL2	NCIMB 20946	-	6606112	Low discrimination 93.	*S. epi/ hominis*
PAL3	NCIMB 40946	-	6206112		*S. epidermidis*
PAL4	NCIMB 40948	-	6706113	Very good ID 99.1%	*S. epidermidis*
PAL5	NCIMB 40947	-	6706112	Good ID 98.5%	*S. epidermidis*

Table 2:7 Biotyping of CoNS from non union fracture infections using API STAPH

SPECIMEN	SITE	API PROFILE	API EVALUATION	API ID
NU1	Soft tissue	6604113	Good ID 97.2%	*S. epidermidis*
NU3	Soft tissue	6606110	Acceptable ID 89.5%	*S. epidermidis*
NU7	Bone	6604113	Acceptable ID 89.5%	*S. epidermidis*
NU11	Soft tissue	6604113	Good ID 93.5%	*S. epidermidis*
NU24	Soft tissue	6606110	Acceptable ID 89.5%	*S. epidermidis*
NU27	Bone	6606110	Acceptable ID 89.5%	*S. epidermidis*
NU33	Soft tissue	6604113	No profile	*CNS*
NU104	Soft tissue	6606110	Acceptable ID 89.5%	*S. epidermidis*
NU142	Bone	6216113	Low discrimination 64.3%	*S. hominis*
NU159	Soft tissue	6604113	Good ID 92.0%	*S. epidermidis*
NU160	Soft tissue	6606110	Acceptable ID 89.5%	*S. epidermidis*
NU168	Bone	6606110	Acceptable ID 89.5%	*S. epidermidis*
NU257	Bone	6606110	Acceptable ID 89.5%	*S. epidermidis*
NU291	Commensal	6636172	No profile	*CNS*
NU294	Commensal	6206113	Acceptable ID 89.3%	*S. epidermidis*
NU296	Bone	6636153	Low discrimination 12.4%	*S. simulans*
NU339	Commesal	6206112	Low discrimination 77.3%	*S. epidermidis*
NU355a	Commensal	61/206111/3	Low discrimination	*CNS*
NU355b	Commensal	6306111	Low discrimination 49.5%	*S. epidermidis*
NU355c	Commensal	6716152	Low discrimination	*S. xylosus or S. lug*
NU357	Commensal	6716152	Low discrimination	*S. xylosus or S. lug*
NU779	Bone	6606112	Low discrimination 93.7%	*S. epidermidis*
NU5436	Bone	6706110	Good ID 94.3%	*S. epidermidis*
NU5438	Bone	670110	Good ID 94.3	*S. epidermidis*

In table2:5 all organisms were isolated from the CAPD fluid of case clinically identified as CAPD peritonitis.

In table 2.6 all organisms were isolated from patients with clinically identified CVC infections.

In table 2.7 all organisms were isolated from patients with clinically identified infection of Non-union fractures.

2.6 Additional bacterial strains

Staphylococcus: the type strains, *S. epidermidis* NCTC 11047; *S. aureus* NCTC 6571 (Oxford strain); *S. aureus* ATCC 12598 (Cowan I); S. *epidermidis* ATCC 35984 (RP62A) were obtained from Microbiology Research Group Culture Collection Aston University, Birmingham.

2.7 Maintenance and culture of bacterial strains

All bacterial strains were maintained on either Columbia agar base supplemented with 5% (v/v) defibrinated horse blood or Brain Heart Infusion Agar (BHIA) and sub-cultured every four to six weeks. The strains were cultivated in Luria-Bertani (LB) broth [1% (w/v) NaCl, 1% (w/v) tryptone, 0.5% (w/v) yeast extract, pH 7.0],Triptic Soya Broth(TSB) or Brain Heart Infusion Broth (BHIB) at 37°C for 18h with aeration at 200rpm. All isolates were stored at -70°C in TSB supplemented with 10% (v/v) glycerol.

All isolates of *S. epidermidis* identified in this section were stored for further investigation.

Chapter 3 Sensitivity of S. epidermidis to chlorhexidine

3.1 Introduction

Disinfection of the hands and environment is one of the most important countermeasures to control nosocomial infections. CoNS, mainly *S.epidermidis*, are the most frequently isolated microorganisms in these infections.

Chlorhexidine is an important medical, dental and pharmaceutical antiseptic, disinfectant and preservative and is widely used in the healthcare environment as a surgical scrub and skin disinfectant.

The focus of the work in this chapter was to evaluate the antimicrobial activity of chlorhexidine against the clinical isolates of *S. epidermidis* identified in chapter 2.

3.2 Chlorhexidine (CHX)

Chlorhexidine (CHX) dihydrochloride (1, 1'-hexamethylenebis [5-(p--chlorophenyl) biguanide]) and chlorhexidine digluconate (1,1'-hexamethylenebis[5-(p-chlorophenyl) biguanide) were obtained from sigma. Solutions were filter sterilised (Gelman Acrodisc 32, 0.2 μm pore size) (Cellulose Nitrate).

3.3 Materials and Methods

3.3.1 Minimum inhibitory concentrations (MICs)

The minimum inhibitory concentrations (MICs) is defined as the lowest concentration of an anti-microbial that will inhibit the visible growth of a microorganism after overnight incubation, and the minimum bactericidal concentration (MBCs) is the lowest concentration of antimicrobial that will prevent the growth of an organism after subculture on to antibiotic-free media (Andrews, 2002).

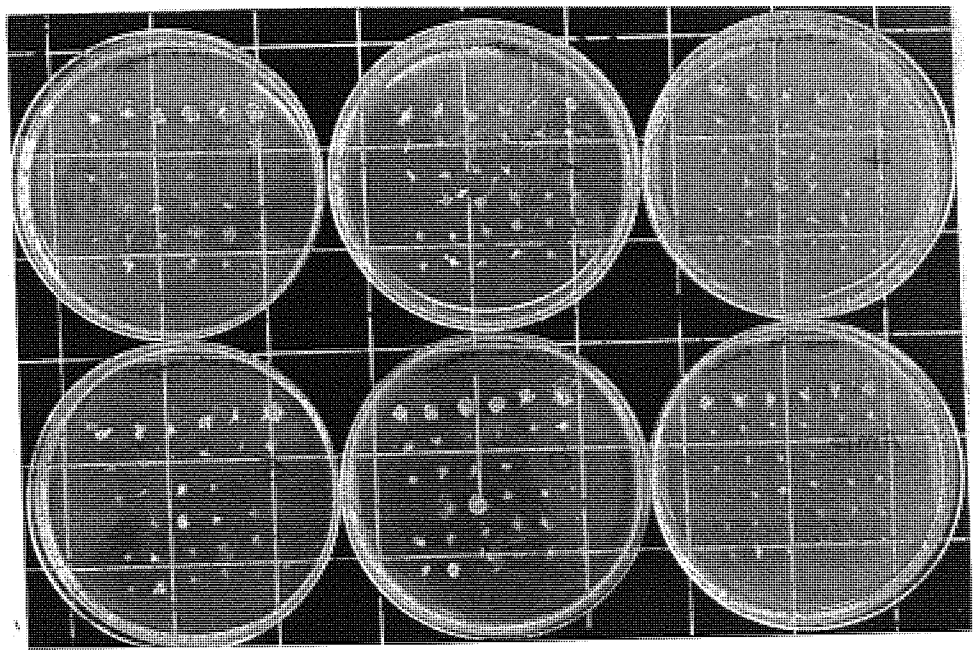
3.3.2 Determination of MIC values by the broth micro dilution method

This method was carried out in accordance with NCCLS standards (2001). An overnight culture of each organism was grown on plates of MHA at 37°C. A loopful of the organism was suspended in sterile saline and the optical density was adjusted to 1.0 at 470nm. This gave a concentration of approximately 10^8 cfu/ml. This suspension was then diluted 1:100 in sterile MHB to give a cell density of 10^6 cfu/ml. Sterile U–bottomed micro titre plates (Fisher,UK) were used for the MIC tests. A volume of 50µl of sterile MHB was dispensed into each well on the plate. The solution of chlorhexidine was prepared at 2.56mg/ml in sterile 10 mM SPB, and diluted 1:10 in sterile MHB to give a concentration of 256µg/ml. A volume of 50µl was dispensed into the wells of the first column, and mixed thoroughly. A 50µl aliquot was then removed from the wells in the first column and dispensed into the second column. This process was repeated across the wells of the plate. The concentration of chlorhexidine now ranged from 128µg/ml in the first column of wells to 0.06µg/ml in the twelfth well. A volume of 50µl of the test organism was then inoculated into each well of the micro titre plate. The final antibiotic concentration ranged from 64µg/ml to 0.03µg/ml. Each well was inoculated with 105cfu/ml. The micro titre plates were sealed with adhesive plastic film and incubated for 24 hours at 37°C. Growth of the test organism was seen as a "button" of cells at the bottom of the well.

3.3.3 Agar dilution method for the determination of MIC values to chlorhexidine

MHA was prepared, sterilized and tempered in a water bath to 45°C. The required test organisms were grown overnight at 37°C on MHA plates. A loopful of each organism was removed from the plate and suspended in sterile saline to give an optical density of 1.0 at 470_{nm}. Each organism was then diluted 1:100 in sterile saline to give a cell concentration of 10^6cfu/ml. Chlorhexidine was prepared in 10mM SPB. An aliquot of 100µl of the test biocide at the required concentration was dispensed into a 30ml sterile plastic universal container. A volume of 24.9ml of molten MHA was added to the universal and mixed thoroughly. The molten agar was immediately poured into a sterile petri dish and allowed to cool and set, prior to storage at 4°C until required. The concentration range of the test antibiotics was 64-0.03µg/ml. Aliquots of 150µl of each test organism were dispensed into 36 wells of a sterile microtitre plate. A 36-pronged multipoint inoculator was sterilised with 70% v/v ethanol and used to inoculate the surface of each agar plate. The multi-point inoculator delivered a volume of 10-20µl, thus the final inoculum was in the order of 10^5cfu/ml. The inoculated plates were incubated for twenty-four hours at 37°C, and then examined for growth.

Figure 3:1 Multi-point inoculation method



3.3.4 The Quantitative Suspension Test

The suspension tests were performed to assess the bactericidal efficacy of the chlorhexidine. An overnight culture was centrifuged to remove traces of the culture medium and the pellet resuspended in LB to give ($\approx 10^9$ cfu/ml, OD 470nm=1.0). 0.1ml of the suspension was transferred to 9.9ml of chlorhexidine solution. After appropriate contact times (1min, 3min and 6 minutes) 1ml of the suspension ($\approx 10^7$ cfu/ml) was added to 9ml of a neutralising solution (2% lecithin and 5% Tween80 Sigma) to neutralise the chlorhexidine. After appropriate serial dilution in PBS and plating on Nutrient Agar (NA), counts of colony forming units (cfu) were performed as outlined by Suller *et al.* (1999). Independent experiments were carried out to confirm that the inactivation stage did not result in reduction in the viability of treated cells. Control experiments were carried out in parallel omitting chlorhexidine to determine the number of organisms present.

3.4 Increased Resistance:

In order to select *S. epidermidis* strains with increased resistance to CHX the following techniques were used:

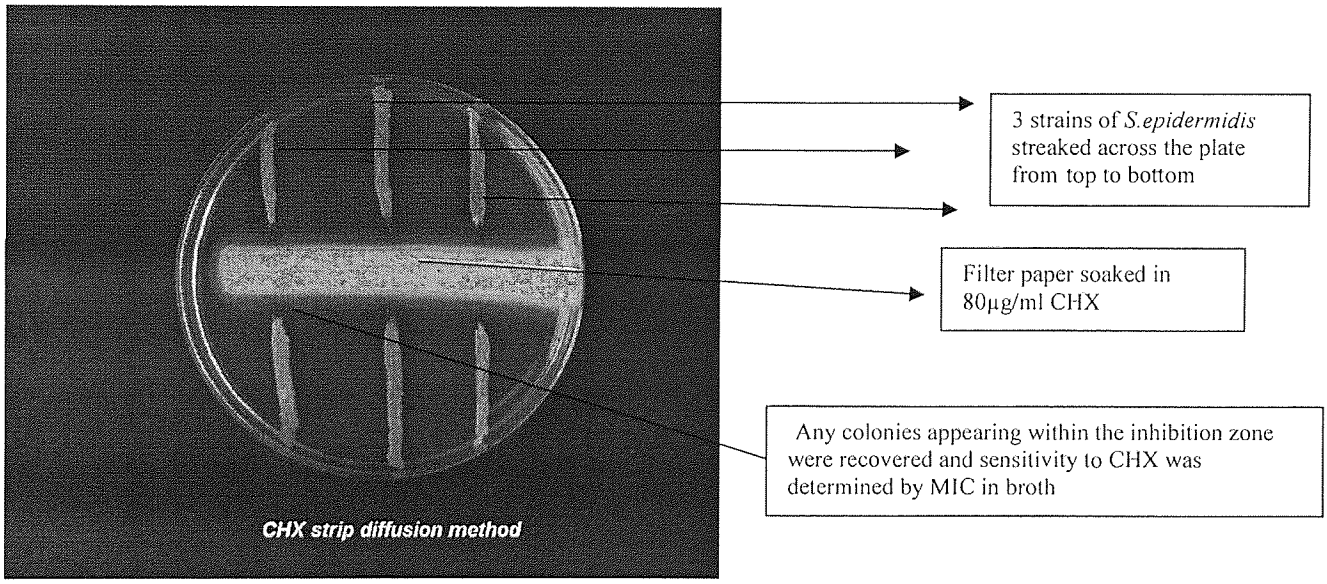
3.4.1 Step-wise broth method

An overnight drop of culture was diluted 10-fold in PBS and used to inoculate a series of tubes containing a range of chlorhexidine concentrations. The tubes were incubated at 37°C for 24 hours. The organisms growing in the highest concentration of chlorhexidine were used to inoculate a further series of chlorhexidine- containing tubes. The method was repeated several times in an attempt to raise the MIC value.

3.4.2 Strip diffusion test

Nutrient agar plates were inoculated with overnight cultures of *S. epidermidis* in single streaks across the plate. Filter paper strips pre-soaked in chlorhexidine solution (80μg/ml) were placed on to the surface of the agar at right angles to the inoculation streaks. The plates were incubated at 37°C for 24 hours. Any colonies which grew in the inhibition zone around the strip, were sub cultured overnight in nutrient broth and tested for elevated MIC values (Figure 3:2).

Figure 3:2 Chlorhexidine Strip Diffusion Test



3.5 Results

The MIC values of chlorhexidine for all *S. epidermidis* strains are shown in Table 3:1. The MICs of CHX for some of these strains showed up to 16-fold increase compared with the other strains.

These resistant strains to CHX in comparison with other tested strains were chosen for further investigation.

Table 3:1 MIC values of *S. epidermidis* isolates to chlorhexidine based on NCCLS standards (2001) method.

Strain	MICμg/ml	Strain	MICμg/ml	Strain	MICμg/ml
CVC1	2	CAPD19	2	NU3	2
CVC2	1	CAPD20	2	NU7	4
CVC9	16	CAPD21	2	NU11	2
CVC10	1	CAPD22	4	NU24	4
CVC11	2	CAPD23	0.5	NU27	8
CVC12	2	CAPD24	4	NU33	8
CVC16	8	CAPD25	1	NU104	4
CVC17	4	ROH1	2	NU142	8
CVC19	1	ROH2	2	NU159	2
CVC20	1	ROH3	4	NU160	16
CAPD1	4	ROH4	2	NU168	4
CAPD2	8	ROH5	8	NU257	8
CAPD3	4	ROH6	16	NU296	2
CAPD4	2	ROH7	1	NU779	16
CAPD5	4	ROH8	2	NU5436	2
CAPD6	1	ROH9	16	NU5438	16
CAPD7	4	ROH10	2	EN1	2
CAPD8	4	ROH11	4	EN4	4
CAPD9	1	ROH12	16	EN5	1
CAPD10	1	ROH13	2	EN7	2
CAPD11a	8	ROH14	2	EN8	2
CAPD11b	8	ROH15	2	EN9	16
CAPD12	1	BONE1	8	EN10	2
CAPD13	4	BONE2	4	EN13	4
CAPD14	4	BONE3	2	EN19	2
CAPD15	8	BONE5	1	EN23	1
CAPD16	2	BONE6	8	EN27	1
CAPD17	4	BONE10	2	EN32	2
CAPD18	2	NU1	2	EN33	2
ATCC35984	1	NCTC11047	1	EN34	1

3.6 Discussion

Microorganisms can adapt to a variety of environmental chemical and physical conditions. It is therefore not surprising that several authors and researchers have reported resistance to extensively used antiseptics and disinfectants. It has been reported that some acquired mechanisms (in particular with heavy-metal resistance) are clinically significant, but in most cases the results have been speculative. Elevated MICs of 16 μg/ml were found for some of the clinical strains of *S. epidermidis* in this study. (e.g. CVC9, ROH6, ROH9, NU160, NU779, NU5438 and EN9). Some of these were also resistant to several of the antibiotic tested (e.g. CVC9 resistant to 5/7 antibiotics) whereas others were generally sensitive (e.g.ROH6 resistant to 1/7 antibiotics). No clear relationship was apparent between resistance to chlorhexidine and antibiotics in the group of isolates as a whole. More tests and research were performed in the subsequent next chapters to investigate the mechanism of resistance in clinical strains of *S. epidermidis* to chlorhexidine.

Chapter 4 Lipid composition of S. epidernidis

4.1Aims

This chapter reviews the role of lipids in the staphylococcal cell membrane and the possible connection between lipid composition and sensitivity to chlorhexidine. The total cellular lipids of *S. epidermidis* strains were extracted and analysed. Lipid compositions of chlorhexidine sensitive and resistant strains were compared.

4.2 Introduction

The term 'lipid' covers an extremely diverse range of molecular species; thus unlike proteins, carbohydrates and nucleic acids, it is not possible to provide a single chemical definition beyond the very simplest. Lipids are classed as being sparingly soluble in water but readily soluble in organic solvents such as chloroform, hydrocarbons, alcohols, ethers and esters. Structures of the more common lipids can be loosely divided into two categories: (i) those structures based on long-chain fatty acids or their immediate derivatives such as alkanes (and some alkenes) and fatty alcohols; and (ii) structures derived from an isoprene unit and which are loosely known as the terpenoid lipids (Gunstone and Herslof, 2000).

Lipids, as broadly defined, would include a few materials which cannot be placed in either of the above categories: such lipids include the polyesters of bacteria (e.g. poly-β-hydroxybutyrate) as well as some of the cyclodepsipeptides which achieve lipid status by virtue of their solubility characteristics rather than any particular structural features (Ratledge and Wilkinson, 1988, Christie, 2003).

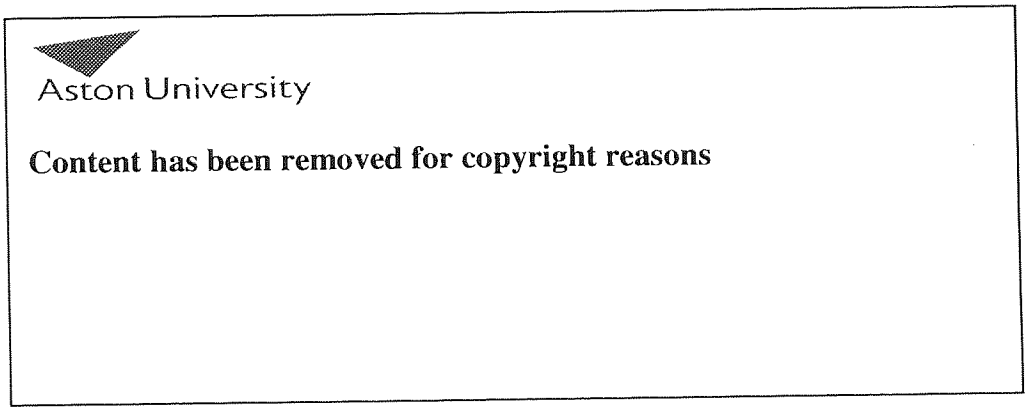
4.2.1Function and Diversity

Lipids form the building blocks of all biomembranes. Variation in the head group and in acyl chain composition results in the presence of hundreds of different lipid species. This diversity suggests that lipids do more than just form a hydrophobic barrier between the inside and outside (Voorst and Kruijff, 2000). Lipids may be relatively simple molecules, as for example the fatty acids themselves, or more complex and contain

phospho or sulpho groups, amino acids, peptides and their derivatives, sugars and even oligosaccharides. Some may contain several of these polar groups, e.g. the glycerophospho-sulpholipids found in certain bacteria (Christie, 2003).

The diversity of lipids signifies a diversity of function. Lipids can act as storage materials in microbial cells, where the lipids typically occur in the form of triacylglycerols in eukaryotic cells and as poly-β-hydroxybutyrate in certain prokaryotes. Lipids are also responsible for the structure of cell membranes (Figure 4:1) where the lipids mainly occur as the amphiphilic (glycero-) phospholipids. Besides these well-known roles, lipids carry out many other functions (Christie, 2003).

Figure 4:1. Simplified phospholipid bilayer structure of the membrane of most cells. Membrane-associated proteins are indicated (Hugo and Russell, 1998).



Lipids, besides their universal role in the structure of membranes also participate in the organization of bacterial cell envelopes, as components of the lipoteichoic acids associated with the cytoplasmic membrane of Gram-positive bacteria (Figure.4: 2)

Figure 4:2. Generalised structure of the cell envelope of a Gram-positive bacterium. Cytoplasmic membrane consists of protein and phospholipids biolayer (Hammond, Lambert and Rycroft, 1984).

4.2.2 Variability

Microorganisms contain a wide range of lipids. In addition, the amounts may vary from the cell mass. The lipid composition may vary not only in amount but also in type, according to how the microorganism is grown, and at what stage during growth the microorganism is taken for analysis (Ratledge and Wilkinson, 1988). Thus it is important to standardize growth conditions to eliminate, as far as possible the innate variations in lipid composition, which can arise from changing growth conditions. Aeration, pH and also the rate of growth of the cells must be considered (Christie, 2003). Also consideration of the methods used for lipid extraction and storage is important to prevent unwanted changes arising before analysis takes place.

4.2.3 Extraction

Microbial cell lipids can be readily removed from cells by simple extraction following mechanical, chemical or enzymatic disruption of the cells or after a hydrolytic step. Methods for disrupting cells have included treatment with dilute acids or alkalis, though such methods will hydrolyse many lipids and so are only useful for isolation of fatty acids or if the 'total' lipid content is being determined. Mechanical disruption of cells is more frequently advocated than chemical attack, as this does not knowingly degrade the lipids.

Recommended procedures have included the passage of cells through hydraulic presses, freeze-drying, ultrasonics or simple mechanical grinding either alone, or with either solvents or grinding agent present (Vance and Vance, 1996). Extraction with chloroform-methanol (2:1, v/v) is the most generally employed method for isolation of polar lipids (Bligh and Dyer, 1959; Ames, 1968).

4.2.4Analysis

Techniques for lipid analysis are as extensive and varied as lipid chemistry itself. Several valuable compilations of methods for the fractionation, analysis and structural characterization of lipids are available. Thin-layer chromatography (TLC), gas chromatography (GC) and gas chromatography allied to mass spectrometry (GCMS) are the most common techniques (Christie, 2003).

4.2.5 Thin Layer Chromatography (TLC)

TLC is widely used for analysis of polar lipids as it is rapid, uses little of the compound and is inexpensive (Kunisada *et al,* 1987 and Mulkhamedova and Glushenkova, 2000).

4.3 Fatty acids

Fatty acids are of the widest distribution in all living cells and rarely found naturally in the free form (Table 4:1). They are mainly found with carbon chain lengths between C_{14} and C_{20}, though both longer and shorter chains are not unusual. Fatty acids may be fully saturated and may be linear, branched, or contain alicyclic rings. Unsaturated fatty acids occur as frequently as the saturated ones: they may contain several double bonds, though 1 or 2 is the most usual.

Table 4:1. Examples of fatty acid found in bacterial cells

n: Straight-chain acid $CH_3–(CH_2)_{14}–COOH$	n-hexadecanoic acid ($C_{16:0}$) (Palmitic acid)
i: Iso-branched acid $CH_3–CH(CH_3)–(CH_2)_{12}–COOH$	14-methyl pentadecanoic acid (i–$C_{16:0}$)
a: Anteiso-branched acid $CH_3–CH_2–CH(CH_3)–(CH_2)_{12}–COOH$	12-Methyl tetradecanoic acid (a–$C_{15:0}$)
n:1: Monounsaturated acid $CH_3–(CH_2)_5–CH=CH–(CH_2)_9–COOH$	11–Octadecenoic acid (Δ^{11}–$C_{18:1}$) (*cis*–Vaccenic acid)
Δ: Cyclopropane acid $CH_3–(CH_2)_5–CH(CH_2)–CH–(CH_2)_9–COOH$	11, 12–Methyleneoctadecanoic acid ($\Delta C_{19:0}$) Lactobacillic acid
10-Methyl branched acid $CH_3–(CH_2)_7–CH(CH_3)–CH_2–(CH_2)_7–COOH$	10–Methyloctadecanoic acid (10–Me $C_{19:0}$) (Tuberculostearic acid)
ω–Cyclohexyl acid $CH_2(CH_2–CH_2)CH_2(CH_2–CH_2)CH–(CH_2)_{10}–COOH$	ω–Cyclohexylundecanoic acid
3-Hydroxy acid $CH_3–(CH_2)_{10}–CH(OH)–CH_2–COOH$	3-Hydroxytetradecanoic acid (3–OH $C_{14:0}$)
2–Hydroxy acid $CH_3–(CH_2)_9–CH(OH)–COOH$	2-Hydroxydodecanoic acid (2–OH $C_{12:0}$)

Table 4:2. The most commonly occurring, saturated, straight-chain fatty acids

Systematic name	**Trivial name**	**Shorthand designation**
Decanoic acid	capric acid	10:0
Undecanoic acid		11:0
Dodecanoic acid	Lauric acid	12:0
Tridecanoic acid		13:0
Tetradecanoic acid	myristic acid	14:0
Pentadecanoic acid		15:0
Hexadecanoic acid	palmitic acid	16:0
Heptadecanoic acid	margaric acid	17:0
Octadecanoic acid	stearic acid	18:0
Nonadecanoic acid		19:0
Icosanoic acid	arachidic	20:0
Docpsanoic acid	behenic	22:0
Tetracosanoi cacid	lignoceric acid	24:0
Hexacosanoic acid	cerotic acid	26:0
Octacosanoic acid	montanic acid	28:0
Triacontanoic acid	melissic acid	30:0
Dotriacontanoic acid	lacceroic acid	32:0

4.4 Polar Lipids

Phospholipids form an essential component of the cell membrane and are related to the integrity and permeability properties of the membrane. Phospholipids show a distinctive amphipathic characteristic because they possess not only a hydrophobic region but also a hydrophilic region in the molecule (Kunisada *et al.*, 1987 and Mulkhamedova and Glushenkova, 2000). The method for lipid extraction of Bligh and Dyer (1959) is still the most generally employed method.

4.4.1 Glycerophospholipids

The simplest phospholipid is phosphatidic acid (PA), which is 1,2-diacyl-sn-glycero-3-phosphate (Figure4:3). Esterification by a variety of mono- and polyhydroxy compounds gives rise to a family of phosphodiesters (often, but misleadingly, referred to as phosphatides), which are the structurally important microbial lipids (Vance, 2002). The most common glycerophospholipids of this class are shown in Table 4:3.

Table4:3. Phospholipids found in bacterial cells.

Phosphatidylcholine	**PC**
Phosphatidic acid	**PA**
Phosphatidylethanolamine	**PE**
Phosphatidylserine	**PS**
Phosphatidylinositol	**PI**
Phosphatidylinositolmannosides	**PIMs**
Phosphatidylglycerol	**PG**
Diphosphatidylglycerol (cardiolipin)	**DPG**
Lysylphosphatidylglycerol	**LDG**

Figure 4:3 The structure of some phospholipids. A, the structure of phosphatidic acid. H* of this structure is replaced by grouping B-D to give the following phospholipids: B, phosphatidyle ethanolamine: C, phosphatidylglycerol; D, diphosphatidylglycerol (cardiolipin). RA.COO and RB.COO are fatty acid residues (Hugo and Russell, 1998).

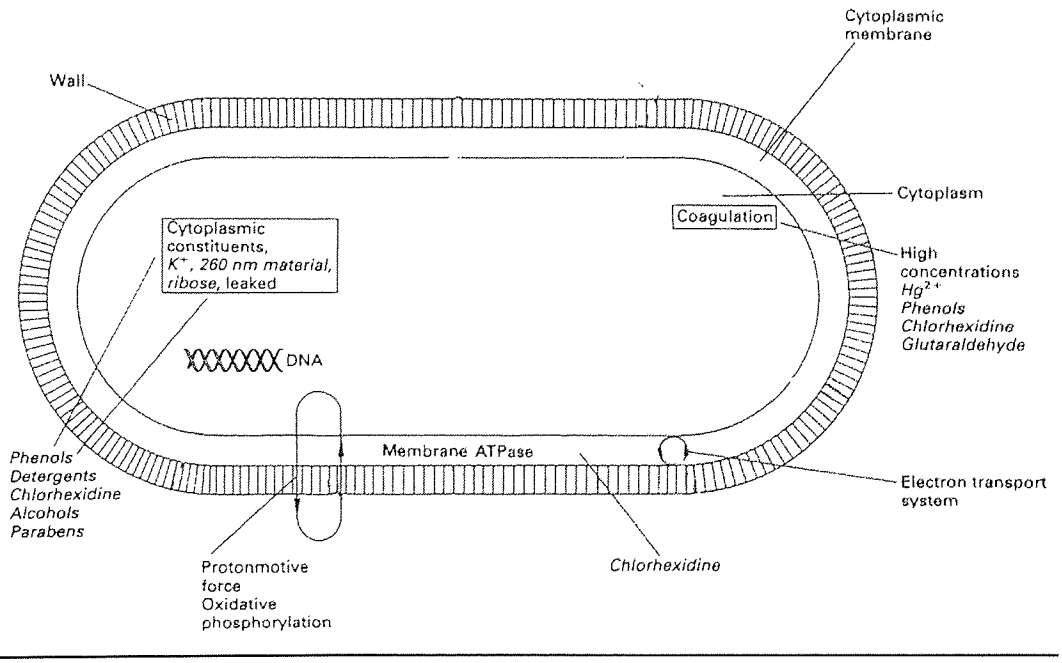
4.5 Bacterial cell wall

The bacterial cell wall plays a vital role in bacterial growth and survival in hostile environments. The cell wall supports the cytoplasmic membrane against the high internal osmotic pressure and controls the cell shape. Mediation of adhesion to surfaces and other cells and involvement in the export of cellular products are other functions of the cell

wall. The role of the cell wall as a barrier to the penetration of toxic molecules differs considerably between Gram-positive bacteria and Gram-negative bacteria. The outer membrane of Gram-negative bacteria poses a significant barrier to the penetration of small hydrophilic molecules, restricting their rate of penetration and excluding larger molecules.

Chlorhexidine acts on the cytoplasmic membrane and inhibits the membrane ATPase and could thus inhibit anaerobic processes. Treatment of bacterial cells with appropriate concentrations of chlorhexidine causes a leakage of a group of characteristic chemical species (Figure 4:4). The potassium ion, being a small entity, is the first substance to appear when the cytoplasmic membrane is damaged. High concentrations of chlorhexidine will coagulate the cytoplasm and in fact it is this kind of reaction, which give rise to the epithet 'general protoplasmic poison' (Hugo and Russell 1998, Russell, 2002).

Figure 4:4. Site of action of chlorhexidine and other antimicrobials on the bacterial cell (Adapted from Hugo and Russell 1998).



4.6 Gram-positive cocci

The cell wall of staphylococci is composed essentially of peptidoglycan and teichoic acids. Substances of high molecular weight can traverse the wall, a ready explanation for the sensitivity of these organisms to most biocides (Russell, 1995). However, the plasticity of the bacterial cell envelope is well known and the growth rate and any growth-limiting nutrient will affect the physiological state of the cells (Nikaido and Vaara, 1985). The thickness and degree of cross-linking of peptidoglycan may be modified and hence the sensitivity of the cells to antibacterial agents (Russell and Day, 1996). Likewise 'fattened' cells of *S. aureus*, which have been trained in the laboratory to contain much higher levels of cell wall lipid than normal cells, are less sensitive to higher phenols (Russell, Hugo and Ayliffe, 1998). Normally, staphylococci contain little or no cell wall lipid and consequently the lipid-enriched cells represent physiologically adapted cells, which present an intrinsic resistance to certain biocidal agents (Hugo and Russell, 1998).

4.6.1 Staphylococcal Lipids

Most lipid data on this genus have been obtained from studies of *S. aureus* and, to a lesser extent, *S. epidermidis.*

4.6.2 Fatty acids

In staphylococci most species contain a fair proportion (15-35%) of straight chain, even-carbon acids; branched-chain acids (iso and anteiso) are preponderant in general, with C_{15} acids the most prominent. In *S. aureus,* a-15:0 is the most abundant acid, but in other species the ratio of a-15:0 to i-15:0 varies considerably. The proportions of unsaturated acids are low or nil. A 12:3 acid has been reported in the phospholipids of some methicillin-resistant strains of *S. aureus* (Nahaie *et al.,* 1984). Another notable feature of staphylococcal fatty acids is the presence of 20:0, which reaches substantial levels in many species, and 22:0, which can be a minor but possibly diagnostic component (Ratledge and Wilkinson, 1988).

4.6.3 Polar lipids

The cellular lipid content of *S. aureus* is typically 5-7% of the dry cells (Rogers *et al,* 1980) but the efficiency of extraction can depend markedly on the method used. Thus, mechanical disintegration of the cells or prior treatment with lysostaphin greatly improves the recovery of DPG, in particular, from *S. aureus*. Phospholipids typically constitute about 65-80% of the total cellular lipids, and consist almost entirely of PG and related lipids (Table 4:4). The lysyl ester of PG may be a major lipid only in *S. aureus* (Nahaie *et al,* 1984). Although the vast majority of studies of *S. aureus* point to the absence of PE, it has been reported to comprise up to 7% of the total phospholipid (Vance, 2002). Other minor phospholipids suggested are PC and PS, both in *S. aureus,* and PA in *S. epidermidis* (Collins, 1985). Phosphatidylglucose has been described as a minor lipid in *S. aureus,* but other studies indicate that this lipid is really a glycerophospho derivative of β-gentiobiosyldiacylglycerol in both *S. aureus* and *S. epidermidis.* In *S. epidermidis,* this lipid accounted for up to 15% of the polar lipids (Ratledge and Wilkinson, 1988).

Glycolipids typically comprise 10-20% of the total cellular lipids in *S. aureus* and about 25% in a halotolerant strain of *S. epidermidis* (Hamilton and Hamilton, 1987).

4.6.4 Apolar lipids

These lipids contribute 5% to 20% of the cellular lipids in *S. aureus,* and up to 20% in *S. epidermidis*. Staphylococci vary in their isoprenoid quinones. *S. aureus* strains usually have MK-8 as the major menaquinone but may also contain a substantial or comparable amount of MK-7. *S. epidermidis* like *S. aureus* contains mainly exclusively MK-7, α-tocopherol-quinone, quinol, squalene and polyprenols. Other apolar lipids reported for staphylococci are diacylglycerols, which are the major apolar lipids in halotolerant *S. epidermidis,* free fatty acids, and even triacylglycerols and steroids (Ratledge and Wilkinson, 1988).

Table 4:4. Phospholipid composition of *Staphylococcus* species (Ratledge and Wilkinson, 1988).

4.7 Materials and methods

4.7.1 Extraction of whole cell lipids

Lipids were extracted from whole cells of chlorhexidine-resistant and sensitive strains of *S. epidermidis* using methods based on those of Bligh and Dyer (1959) and Ames (1968). Cells from overnight cultures of each strain grown in MHB for 18h at 37°C on an orbital shaker were recovered by centrifugation at 10,000 x g for 10-minutes and re-suspended in 30ml of water. Chloroform and methanol were mixed with the bacterial suspension in the ratio of 1 part chloroform, 2 parts methanol and 0.8 parts bacterial suspension. The suspension was mixed by shaking vigorously in a separating funnel and left to stand overnight at room temperature. Chloroform and water were then added to the suspension in the proportions 1 part water, 1 part chloroform and 1 part suspension. This was mixed and allowed to separate into organic (lower) and aqueous (upper) phases. The lower phase containing extracted cellular lipids was carefully recovered and the solvent removed by rotary evaporation. A small residual amount of water was then removed by freeze-drying. The total amount of cellular lipid extracted from each strain was determined by weighing (Table 4:5). The extracted lipids were then dissolved in a 1ml of methanol: chloroform (2:1) and stored at -20°C in a glass vial with a foil lined caps.

4.7.2 Phospholipid extraction

Phospholipids were recovered from the whole cell lipids extracted from cells grown in 1 L of MHB. The total extracted lipids were dissolved in 1 ml chloroform: methanol (2:1 v/v), 20ml diethyl ether was added and left overnight at -10°C. After centrifugation at 5,000 x g for 10 min the supernatant was recovered, evaporated to dryness and the lipid residue dissolved in 1 ml chloroform: methanol (2:1 v/v). To precipitate the phospholipids 20 ml acetone was added and stored overnight at -10°C. Phospholipids were recovered by centrifugation (5,000 x g 10 min), dried in a vacuum desiccator and weighed.

The remaining acetone supernatant was evaporated, dried and weighed. This fraction contained neutral lipids (glycolipids and diglycerides).

4.7.3 Separation of lipids by Thin Layer Chromatography (TLC)

Spots of approximately 10 μg of each lipid solution were applied to silica-coated aluminum chromatography plates (20 x 20cm, layer thickness 200μm, particle size 2-25μm, pore size 60Å) (Aldrich) using a micro capillary tube. In addition, samples of authentic reference phospholipids (phosphatidylethanolamine, PE; phosphatidylglycerol, PG; diphosphatidylglycerol, DPG; phosphatidylcholine, PC; phosphatidylserine, PS; each obtained from Sigma) were spotted at similar concentrations. The plate was allowed to dry fully at room temperature. The freshly-prepared mobile phase (chloroform:methanol:water, 65:25:4 by volume) was allowed to equilibrate in a glass chromatography tank, sealed with a glass lid for at least an hour. The dry plate was placed with the glass tank and allowed to develop until the solvent front had progressed nearly to the top of the plate. For some analyses a two-dimensional solvent system was employed to ensure maximum separation of lipids. A single sample of lipid extract was applied to the plate, which was developed with chloroform:methanol:water (65:25:4 by volume) in the first dimension and chloroform: acetic acid: methanol : water (80:18:12:5 by volume) in the second dimension.

4.7.4 Visualisation of lipids

A number of spray reagents were used to detect lipids after separation by TLC. Ninhydrin in ethanol (Sigma) 0.2% v/v followed by heating to 110°C for 15-minutes was used for detection of amino-containing phospholipids (e.g. PE and PS). The amino-containing phospholipids were visible as reddish-purple spots. Molybdenum blue reagent was used for detection of lipids containing phosphate esters (e.g.PE, PG, DPG, PC and PS). Plates were sprayed with a solution of 1.3% w/v molybdenum oxides in 4.2M H_2SO_4 (Sigma). Phospholipids rapidly appeared as blue spots on a white background. As the background of the plates also stains blue after further exposure (10 mins), the plates were photographed immediately using a UVP scanner. The image

visualised on the scanner was saved to disk and this was used in the Phoretix gel analysis program to determine the proportions of each lipid in the cell samples. Those plates sprayed with ninhydrin were not used for determining proportions of lipids, but were used to confirm the presence of phosphotidylethanolamine and to putatively identify its lyso (partially deacylated) form. Two other reagents, α-naphthol (Sigma) and phosphomolybdic acid (Sigma) were employed for detecting glycolipids, steroids, lactones, phenolic compounds and unsaturated fatty acids (Figures 4:5, 4:6, 4:7 and 4:8).

Table 4:5 Comparisons of phospholipids and neutral lipids/fatty acids in 4 selected CHX sensitive (S) and 4 selected CHX resistant isolates (R) of *S. epidermidis* (See Table 3:1 for MIC). Results for resistant strain are shaded.

Sample	Total lipid % dry wet of cell	% FA+NL to total lipid	% PL to total lipid
NU 160 R	14.1	25	75
NU 159 S	8.1	25	75
CVC 9 R	12.63	25	75
CVC 1 S	11.26	29	71
CVC16 R	12.63	27	73
CVC10 S	7.69	25	75
CADP 20 R	15.29	27	72
CAPD 12 S	6.41	24	76

Key:

NL= Neutral Lipid FA=Fatty Acid PL=Phospholipid

4.8 Thin Layer Chromatography Figures

In Figures 4:5-4:7 phospholipids from selected strains were separated on silica gel plates in Chloroform:methanol:water (65:25:4).

Figure 4:5 Ninhydrine reagent spray.

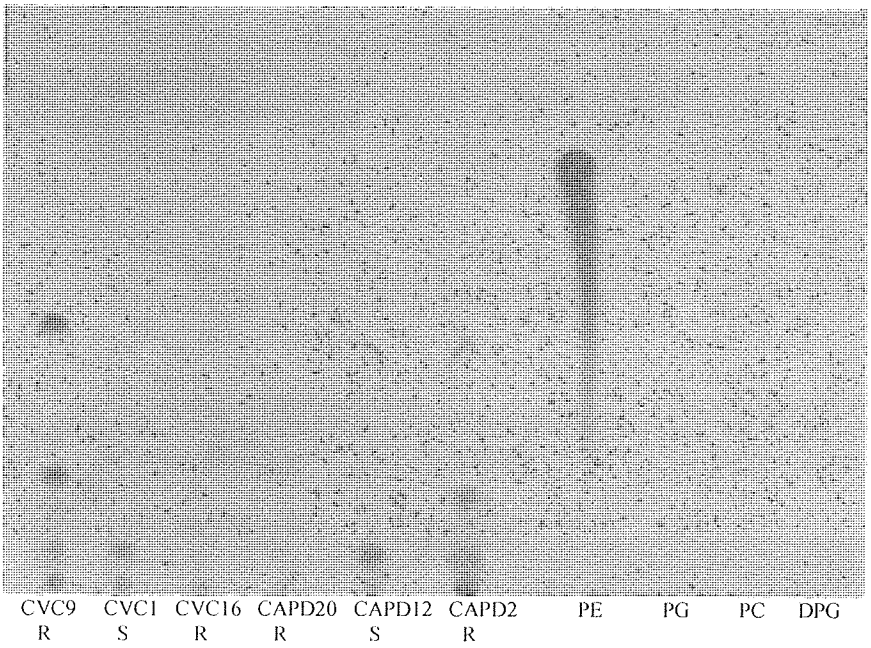


Figure 4:6 Molybdenum blue reagent spray.

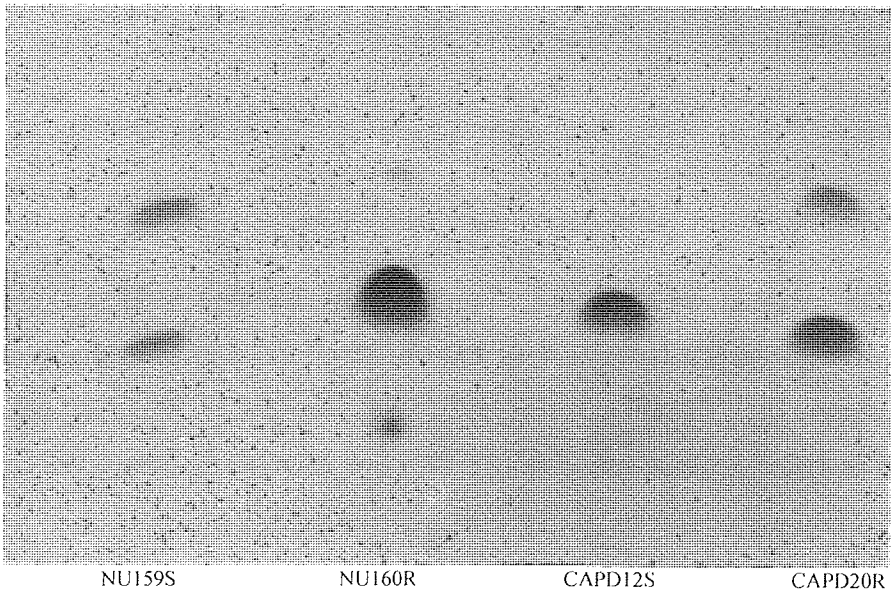
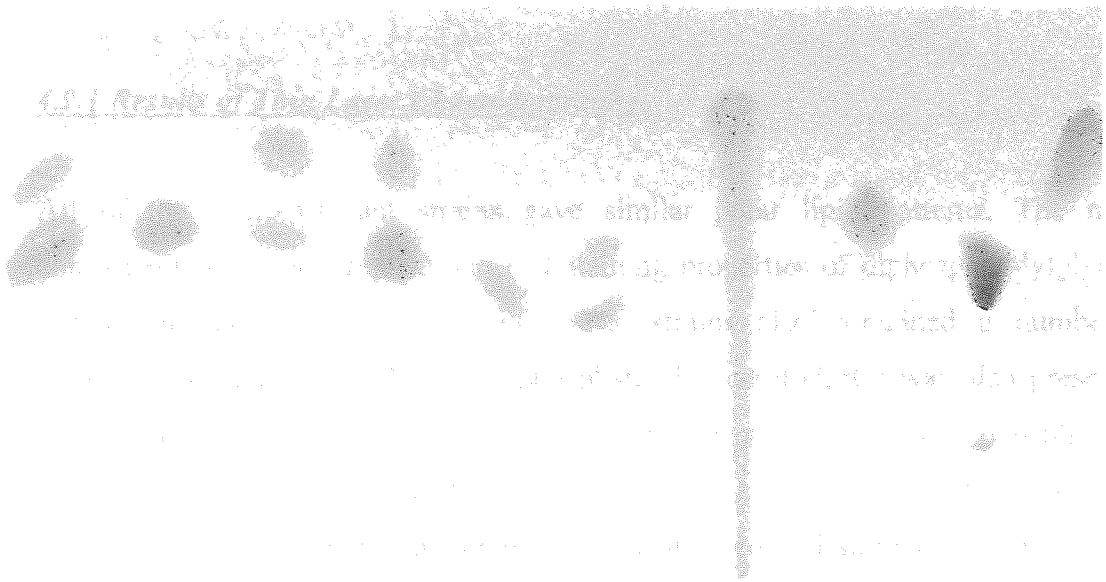


Figure 4:7 Molybdeneum blue reagent spray.



Key: PE= Phosphatidylethanolamine PG=Phosphatidylglycerol

PC=Phosphatidylc DPG=Diphosphatidylglycerol

Bacterial Strains	Diphosphatidylglycerol DPG		Phosphatidylglycerol PG		Other Phospholipids (PC, PE and unknown)	
⇓	RPA	%	RPA	%	RPA	%
CVC1S	9612.5	14.16	52315.3	77.11	5912.31	8.71
CVC9R	13203.4	13.99	73211.9	77.68	7921.41	8.39
CVC16R	13206	13.71	79120.3	82.13	4012.5	4.16
CVC20R	9312.8	9.82	83240.2	87.74	2314.2	2.44
CAPD12S	7416.6	15.76	37421.6	79.53	2214.5	4.70
CAPD20R	12466.49	12.31	84101.5	80.04	4712.5	4.65

Table 4.6 Data collated from Phoretix 1D software analysis of figure 4.7 and identification of phospholipids present in 4 selected CHX– resistant *S. epidermidis* strains (CVC9R, CVC16R, CVC20R and CAPD20R) in comparison with two sensitive strains (CAPD12S and CVC1S) as controls. PRA refers to the relative peak area corresponding to the density of colour of the separated phospholipids as determined by the phoretix 1D software. PC and PE refer to phosphatidylcholine and phosphatidylethanolamine respectively.

4.9 Results

4.9.1 Results of Thin Layer Chromatography

All of the CHX-resistant strains gave similar polar lipid patterns. The major components chromatographed and had staining properties of diphosphatidylglycerol (DPG) and phosphatidylglycerol(PG). The strains also contained a number of unknown phospholipids (P). Lyso- phosphatidylglycerol (LPG) was also present in some strains (e.g.NU160R in figure 4:7). Comparison of the stained phospholipids from resistant and control clinical strains of *S. epidermidis* showed that resistant strains contained more phospholipids than sensitive control strains. However pattern of individual phospholipids did not show a consistent difference between resistant and control strains. Some of the sensitive strains contained only PG (e.g.CVC1 and CAPD12 in Figure 4:7) whereas another sensitive strain contained equal contents of PG and DPG (NU159S in Fig 4:6).

4.9.2 Results of total lipid extraction

As discussed in table 4:5 the weight of total lipid in CHX- resistant clinical strains was more than in control CHX- sensitive strains of *S. epidermidis*. In strains such as NU160, PAL4 and CAPD12 the weight of total lipid was nearly twice that in sensitive control strains. The amount of phospholipids in these strains was also about twice that in sensitive control strains. The same results were obtained by comparing the weight of neutral lipid/ fatty acids in both resistant and control strains.

4.10 Discussion and concluding comments

Staphylococcal resistance to antiseptics has been reported throughout the world. Al-Masudi *et al* (1991) and Jones (1999) reported low-level resistance to antiseptics (benzalkonium chloride, chlorhexidine, and hexamidine di-isethionate) in 36.7% of a collection of 392 strains representing 26 *Staphylococcus* species.

Cookson and Bolton (1991) reported a moderate increase of resistance to CHX in antibiotic-resistant *S. aureus*. Hedin *et al* (1993) reported the same results in *S.* epidermidis. According to their research strains of *S. epidermidis* resistant to one or more of the antibiotics used for selective culture (subsequently referred to as resistant *S. epidermidis*) were found in 89% of their tested samples. A more likely explanation for the increased resistance of *S. epidermidis* to CHX in vitro reported by Brown *et al* (1989) is that they colonised lower layers of the skin that were not reached by disinfecting agents.

Jones *et al* (1999) have studied *Staphylococcus* plasmids, finding 5 distinct genes identified as *qac* A-E, which specify resistance to antibiotics and antiseptics. Although the *qac* genes confer a variable level of resistance, the *qac* mechanism is a cellular efflux-mediated system that limits the final uptake of the antiseptic or antibiotic.

Several authors such as Al-Masudi *et al* (1991), Chopra (1992) and Jones (1999) have found that the antiseptic resistance genes evolved before the introduction and use of topical antimicrobial products and biocides.

Sasatsu *et al* (1994) found the gene encoding for increased MIC levels for antiseptics (e.g. *ebr*, to be identical to *qac* C/D) in sensitive and resistant strains of staphylococci. Jones (1999) concluded that the normal function of the *ebr* gene is to remove toxic substances from the bacterial cells.

It has also been reported that some acquired mechanisms (in particular with heavy - metal resistance) have been shown to be clinically significant, but in most cases the results have been speculative.

Although significant progress has been made with bacterial investigations, a greater understanding of these mechanisms is clearly lacking for other infectious agents. Increased MICs have been confirmed, in particular for Staphylococci. Future goals of research and practical work should be to better understand and define the molecular pathogenesis of these resistant strains. More work to identify the extra extracted lipids and their possible function in resistance is needed to prevent the emergence of resistance to existing drugs.

Results in this chapter show that the weight of total lipids in CHX-resistant strains is more than in the CHX- sensitive strains. The increased lipid content might be related to the CHX-resistance either by blocking penetration of CHX through the bacterial cell wall or by protecting the cytoplasmic membrane.

Chapter 5 Generation of resistance to CHX by serial passage of S. epidermidis cells

5.1 Introduction

5.1.1 Aims

Several studies have claimed that biocides can select for antibiotic resistance, e.g. resistance to cationic compounds (quaternary ammonium compounds and CHX) (Stickler *et al.,* 1983 and Tattawasart *et al.,* 1999) and to phenolics (pine oil disinfectant, triclosan) (Moken *et al.*, 1997 and Chuenchen *et al.*, 2001). Workers have selected resistant organisms by growing bacterial strains such as *Pseudomonas* and *Staphylococcus* species in increasing concentrations of disinfectants (Nagaev *et al.*, 2001; Reynols, 2000; Davies and Maillard, 2001; Jones *et al.,* 1989; Russell *et al.,* 1998; Mechin *et al.,* 1999). However, few comparisons have been made between laboratory-passaged strains and those isolated from the environment.

This work examines whether both clinical and laboratory strains of *S. epidermidis* can be adapted to resist the action of CHX by serial passage. In addition, any co-resistance of such adapted strains to a range of other disinfectants and antibiotics was determined.

5.1.2 Serial passage

Disinfectant and antibiotic resistant strains of *S. aureus* and *S. epidermidis* have been recognised for many years (Kim *et al* 2003, Rehm, 2002; Russell 2002; Livermore, 2000; Strausbaugh 1999; Suller and Russell 1999; Raad *et al.,* 1998; Paulsen *et al.,* 1997 and Littlejohn *et al.*, 1992). The examination of such strains requires a means of selecting for resistant cells within a population. There are two main methods used to achieve this. A population of cells can be challenged with the MIC or a higher concentration of antimicrobial and resistant cells isolated by plating onto agar containing the same antimicrobial. Alternatively populations of cells can be grown in increasing concentrations of an antimicrobial to select for cells with increased resistance to the agent. This method is commonly known as serial passage.

5.1.3 Determination of culture purity by pulsed field gel electrophoresis and other molecular methods

It is essential when studying disinfectant resistant strains derived by serial passage to determine that contamination has not occurred during the selection process. When examining each strain for alterations in phenotype due to adaptation it is vital to ensure that changes are not the result of such contamination. Contamination by other bacterial species such as skin carried staphylococci or micrococci are readily identifiable by examining the colonial morphology of suspect cells on an agar plate. However, when dealing with different strains of the same organism, other means are required.

In the past two decades new techniques have been used to both identify the strains and to discover how related strains are to each other. At present there are two main methods for both determination of relatedness of isolates and perhaps more importantly in a clinical sense, the determination of sources of hospital acquired infections. The first is the randomly amplified polymorphic DNA (RAPD) fingerprinting method (Kersulyte *et al.,* 1995; Renders *et al.,* 1996). This involves the sample genomic DNA being used as a template for a polymerase chain reaction (PCR) based upon primers of arbitrarily chosen sequence. The DNA template need not be of a high molecular weight, of high purity, or of a double stranded nature. In addition only a few nanograms of DNA are required and, while one or two primers are generally sufficient for distinguishing unrelated strains, if more sensitivity is required then more primers can be used, thus utilising a larger portion of the genomic template.

A second technique that has been compared favourably with RAPD is that of pulsed field gel electrophoresis (PFGE) (Kersulyte *et al.,* 1995; Renders *et al.,* 1996). In this procedure the genome of an organism is cut with a selected restriction enzyme and the pattern of DNA fragments compared with that produced by the same enzyme treatment upon another isolate. This method utilises restriction enzymes that have cutting sites that occur infrequently in the genome of the organism to be investigated, so that the number of fragments produced can be visualised relatively easily. The choice of enzyme is usually based upon %GC content of the genome.

The work of Tenover *et al.,* (1995) provides a set of criteria by which isolates can be compared upon the similarity of their band profile. These criteria are most appropriate if PFGE resolves at least 10 distinct fragments since the discrimination drops if fewer fragments are compared. This technique has been primarily used to examine the epidemiological spread of disease, where isolates from separate cases can be compared and genetic relatedness determined.

PFGE and RAPD are widely used to discriminate between strains (Ratnaningsih *et al.,* 1990; Schmidt *et al.,* 1996). The techniques have been used to check the culture purity of strains of *P. aeruginosa* that had been passaged in disinfectant rich media for up to 6 weeks. However, on some occasions they have been unsuccessful in discriminating between clinically related and epidemiologically unrelated isolates(Rautlein and Hanninen, 1999).

5.1.4 Stability of CHX resistance

The process of growing bacteria in increasing concentrations of an antimicrobial has been shown to result in bacteria more resistant to the agent (Jones *et al.,* 1989; Russell *et al.,* 1998; Mechin *et al.,* 1999). The mechanism behind this resistance could be one of two possibilities. The presence of such a selection pressure can select for those organisms in a population that, by spontaneous mutation, have a higher resistance to the antimicrobial and gradually these organisms will become the dominant phenotype by a process of natural selection. The second possibility is that the organisms already possess the means for resistance but the mechanism would place them at a disadvantage in an environment without the antimicrobial. For example, the overproduction of a membrane-stabilising protein that protects against the antimicrobial, but requiring significant energy expenditure for the organism, and is only "switched on" in the presence of the antimicrobial. In order to determine which of the two mechanisms is responsible for changes in resistance, cells can be grown in antimicrobial- free media to determine if the absence of selective pressure removes the resistance phenotype form the population of cells.

5.1.5 Cross-resistance to disinfectants and antibiotics

Resistance to one antimicrobial can often confer resistance to others, either structurally related (for example β-lactamases confer resistance to a range of β-lactam antibiotics), or not linked by structure at all. For example methicillin resistance is widespread and most methicillin-resistant strains also exhibit multiple resistances to other antibiotics. A plasmid associated with vancomycin resistance has been detected in the enterococci (Todar, 2002). This plasmid can be transferred to *S. aureus* in the laboratory, and has recently occurred naturally (Sievert, *et al.*, 2002).

Therefore, by determining whether the organisms resistant to CHX also exhibit resistance to other agents it is possible to determine what type of resistance mechanisms may be involved.

5.2 Materials and Methods

5.2.1 Strains of S. epidermidis

Eleven *S. epidermidis* strains were used in the generation of CHX resistant strains. Nine out of eleven were clinical isolates of *S. epidermidis* and the other two strains were from culture collections. These strains and their origins are listed in table 5:1.

Table 5:1 Strains of *S. epidermidis* used in the production of CHX resistant organisms.

Strain	Source and date
CVC1	QEH (central venous catheter sepsis 1997)*
CVC2	QEH (central venous catheter sepsis 1997) *
CAPD12	QEH (central venous catheter sepsis 1997) *
CAPD20	QEH (central venous catheter sepsis 1997) *
CAPD23	QEH (central venous catheter sepsis 1997) *
CAPD25	QEH (central venous catheter sepsis 1997) *
PAL1	QEH (central venous catheter sepsis 1997)*
NU11	ROH (osteomyelitis) 1997*
NU159	ROH (osteomyelitis) 1997*
ATCC 35984	-
NCTC 11047	-

*QEH, Queen Elizabeth Hospital, Edgbaston, Birmingham, UK. *ROH, Royal Orthopaedic Hospital, Northfield Birmingham, UK. Strains were kindly supplied by Dr S.Lang.

Organisms were maintained as frozen cultures at -70°C for up to 36-months and on agar plates for no more than 1-month. Unless otherwise stated, all overnight cultures were passaged from a plate culture.

5.2.2 Minimum inhibitory concentration (MIC) determination

The MICs of all antimicrobial agents used were determined by the broth dilution method using serial-fold dilutions of each agent. Approximately 1 x 10^6 bacteria in stationary

culture were inoculated into test tubes of nutrient broth (Oxoid) containing various concentrations of each agent. The bacteria were grown as static broth cultures for 16-18 hours at 37°C. The MIC was determined as the lowest concentration of agent that inhibited visual growth of the organism in broth.

5.2.3 Minimum bactericidal concentration (MBC) determination

The MBC of antimicrobial agents was determined by plating one loopful of culture from those tubes containing no visible growth onto plates of nutrient agar. These were incubated at 37°C for 18-hours. The MBC was determined as the lowest concentration of agent that prevented subsequent growth on the agar plates.

5.2.4 Adaptation of strains to CHX

Strains were inoculated into static cultures of nutrient broth containing 0.5µg/ml CHX, a concentration that was 50% lower than that of the lowest MIC of the strains. The cultures were grown for 16-hours at 37°C in a static environment after which 100µl of each culture was transferred to a fresh tube of nutrient broth containing the same concentration of CHX. This was repeated every day for 5 days. On the sixth day, the MIC of each strain was determined. If the strain did not produce an MIC of at least double that of the solution it was grown in, it was discarded. Of those remaining strains, a sample was taken from each and frozen at -70°C in nutrient broth containing the same concentration of CHX and 10% v/v glycerol. Using bacteria from each strain that grew at double the original concentration of CHX (0.5µg/ml), a new set of 6 passages was begun at 1µg/ml CHX.

This process was repeated, producing strains passaged at CHX concentrations of 1, 2, 4, 8, 16, 32 and 64 (µg/ml).

5.2.5 Determination of culture purity

5.2.5.1 Pulsed field gel electrophoresis

5.2.5.2.1 Preparation of agarose blocks for pulsed field gel electrophoresis

Cells from an overnight culture were centrifuged at 13,500 rpm for 10-minutes in an Eppendorf microcentrifuge, and the pellet resuspended to a minimum concentration of 20mg/ml wet weight in 1ml NET-100 (0.1M NaCl, 0.1M EDTA pH 8.0, 0.01M Tris-HCl pH 8.0). The cells were vortexed until they were an even suspension, then recentrifuged at 13,500 rpm for 10-minutes and again resuspended in 1ml NET-100 to 20mg/ml wet weight. A stock solution of 0.9% w/v chromosomal grade agarose in NET-100 was prepared, autoclaved for 5 minutes at 15psi and kept molten at 60°C. The bacterial suspension was kept warmed at 60°C and 0.5ml samples mixed with 0.5ml of molten agarose. The agarose and cell mixture was briefly vortexed and transferred into a 9mm Perspex mould (Biorad) maintained at 4°C on ice. The mould was left on ice for at least 20 minutes to allow the agarose and cell mixture to solidify fully. Solid agarose blocks were carefully removed from the mould and transferred to 5ml plastic bijou bottles containing 3ml lysis solution (6mM Tris-HCl pH 7.6, 1M NaCl, 0.1M EDTA pH 8.0, 0.5% w/v Sarkosyl, 1mg/ml molecular biology grade lysozyme). The bottles were sealed and rolled on a horizontal tube roller for 24 hours at 37°C to ensure complete cell lysis. After 24-hours the lysis solution was replaced with 3ml ESP solution (0.5M EDTA pH9.0, 1% w/v Sarkosyl, 1.5mg/ml proteinase K). The bottles were resealed and statically incubated for 48-hours at 50°C to allow full degradation of remaining cellular debris. Blocks were then stored in fresh ESP at 4°C for further treatment.

5.2.5.2.2 Pre- digestion treatment

Prior to restriction enzyme digestion of the genomic DNA, the ESP solution was replaced with 3ml of TE (10mM Tris pH 8.0, 1mM EDTA pH 8.0). The bottles were rolled for 2 hours at room temperature. The TE was replaced with fresh TE and the bottles rolled for further 2 hours at room

temperature. Finally, the TE was removed and the blocks washed 3 times for 20 minute each, at room temperature, with fresh TE. Washed blocks were then stored in a further 3ml of TE at 4°C until required.

5.2.5.2.3 DNA digestion

A washed block was placed on a piece of Parafilm and excess TE blotted with filter paper. A slice, (approximately 1mm x 1mm x 9mm) was cut from the block using a microscope slide glass cover slip. The slice was transferred to a sterile 1.5ml micro- centrifuge tube, with the remainder of the block returned to the bottle of TE and stored again at 4°C. The slice was then bathed with 200µl of the buffer appropriate to the enzyme used. The buffer had been diluted with sterile ddH_2O to the manufacturer's specifications and was incubated with the agarose slice on ice for 15 minutes. The buffer was replaced with a mixture of 160µl of fresh buffer and 40µl of restriction enzyme, *SmaI* (cutting at 5'-TTTAAA-'3). This was thoroughly mixed, stored on ice for 15 minutes, and then incubated at 37°C for 20-hours to allow full digestion of the DNA within the block.

5.2.5.2.4 Electrophoresis of genomic fragments

A stock solution of 10xTBE (1.0M Tris, 0.89M boric acid, 0.02M EDTA pH 8.0) was prepared in advance. Melting 1.2g of molecular biology grade agarose in 100ml 0.5xTBE using an 850W microwave oven at full power produced 100ml of molten agarose. Once the agarose had cooled to approximately 50°C, a gel was cast onto a supporting plate using the BioRad CHEF DR-III casting mould. A levelled platform was used to produce a uniform, flat gel.

The CHEF DR-III electrophoresis cell was prepared by filling with 2L of freshly made 0.5xTBE which was pre-cooled by setting the apparatus pump to maximum (100), and the cooling unit to 10°C.

The restriction enzyme solution was carefully removed and replaced with 200µl of ES (0.5M EDTA pH 9.0, 0.1% w/v Sarkosyl). The agarose slice was then incubated at 50°C for 15 minutes to quench the activity of the restriction enzyme. The ES was replaced by 1.0ml TE and left at room temperature for 15 minutes prior to loading into the gel. The slices of agarose were loaded one into each lane of the gel. Each slice sealed into place with a coating of molten 0.5% w/v molecular biology grade agarose in 0.5xTBE. The final lane on each gel was loaded with a 2mm thick slice of DNA size standard, commercially produced for PFGE (BioRad Lambda ladder containing concatamers of c1857 Sam7 providing DNA bands in increasing units of 48.5Kb up to approximately 1000kb).

Once the sealing agarose had solidified, the electrophoresis cell buffer flow rate was reduced to 70% of maximum. This gave a flow of approximately IL/minute. The gel was transferred from the mould to the locating bracket within the electrophoresis cell. In this position the gel was now submerged in cooled 0.5xTBE. The lid to the cell was placed in position and the gel left to equilibrate to the temperature of the running buffer for 5-minutes. The DNA fragments were then separated electrophoretically using running conditions that were specific to each enzyme. *SmaI* required an initial pulse time of 2 seconds, a final pulse time of 25-seconds, a total run time of 22-hours, at a temperature of 10°C, with electric field intensity of 6 V/cm and at an electrode angle of 120°.

Once the run time had elapsed the gel was carefully removed from the electrophoresis cell, submerged in 500ml of ethidium bromide staining solution (5µg/ml in 500ml ddH$_2$O), and gently agitated to allow visualisation of DNA bands. After 60 minutes the gel was carefully removed from the staining solution and destained by submerging in 1L of ddH$_2$O for up to 2 hours.

Banding patterns were visualised by placing the gel on an ultraviolet transilluminator and the image captured using UVP software. Those strains that showed no change in banding pattern after passaging in CHX for a number of weeks were examined for their resistance to other antimicrobials.

5.2.5.2.5 Standard stock solution for pulsed field gel electrophoresis

1) NET-100

0.1 M NaCl, 0.1 M EDTA in 0.01 M Tris-HCl pH 8.0.

100 ml was prepared by mixing 20 ml 0.5M EDTA pH 8.0 + 1.0ml Tris-HCl pH 8.0 + 0.58g NaCl, diluting to 100 ml with distilled water and autoclaving.

2) ESP

0.5 M EDTA pH 9.0 was prepared (1.5 mg/ml proteinase K and 1%w/v sarcosyl was added prior to use).

3) ES

0.5 M EDTA pH 9.0 was used without proteinase K.

4) Chromosomal grade agarose 0.9%

50 ml NET mixed with 0.45g chromosomal agarose (Bio Rad) autoclaved for 5 minutes and used freshly or melted in microwave as required.

5) Lysis solution

6mM Tris-HCl(pH7.6)+ 1M NaCl+100mM EDTA pH 8.0. 100 ml was prepared by mixing 0.6ml Tris HCl pH 7.6(1M stock) + 20ml 0.5M EDTA pH8.0 + 5.8g NaCl diluting to 100ml with distilled water and autoclaved. Prior to use 0.5 g/ml sacrosyl and 1mg/ml lysozyme was added.

5.2.6 Determination of resistance properties

5.2.6.1 Preparation of antimicrobial agents

Solutions of antimicrobial agents were prepared as stated in table 5.2 and sterilized by membrane filtration using 0.2μm cellulose acetate pore filters.

Table 5:2 Details of preparation of antimicrobial agents and storage prior to use.

Antimicrobial agent	Preparation	Storage
Chlorhexidine (CHX)	Water	4°C
Benzalkonium chloride (BKC)	Water	4°C
Triclosan (TLN)	Water	4°C
Cefuroxime sodium (Zinacef)	Water	4°C
Cefotaxime (Claforan)	Water	4°C
Vancomycin	Industrial Methylated Spirits (IMS)	Made fresh
Fucidin	Water	4°C
Cetrimide (CET)	Water	4°C
Norfloxacin	Dimethyl Sulphoxide (DMSO)	4°C
Trimethoprim	Dimethyl Sulphoxide (DMSO)	4°C
Erythromycin	Industrial Methylated Spirits (IMS)	Made fresh
Gentamicin (Cidomycin)	Water	4°C
Ceftazidime (CEF)	Water	Made fresh
Ciprofloxacin (CIP)	Water	4°C
Chloramphenicol (CPCL)	Industrial Methylated Spirits (IMS)	Made fresh
Flucloxacillin (Floxapen)	Water	Made fresh

Antimicrobial agents were prepared and stored according to protocols from Journal of Antimicrobial Chemotherapy (1991, Supl.Vol.27).

The MBC for each antibacterial agent was determined for agents, which precipitated at higher concentrations, rendering an MIC impossible to visualise. These MBC values were used instead of MIC values where appropriate.

5.2.6.2 Stability of CHX resistance

To determine the stability of CHX resistance the most resistant passaged cells derived from strains NU11, NU159 and CAPD12 (all passage 7) were grown in CHX-free broth for the same number of passages used to generate the resistance. This was 5x7 days passage for three strains. After each 5 day period the MIC for CHX was determined for each strain in the manner described in section 5.2.2.

5.3 Results

5.3.1 Adaptation of S.epidermidis to CHX

As strains were passaged in increasing concentrations of CHX, their MIC values were determined. The results are shown in table 5.3.

Table 5.3 MICs of *S. epidermidis* strains before and after serial passage.

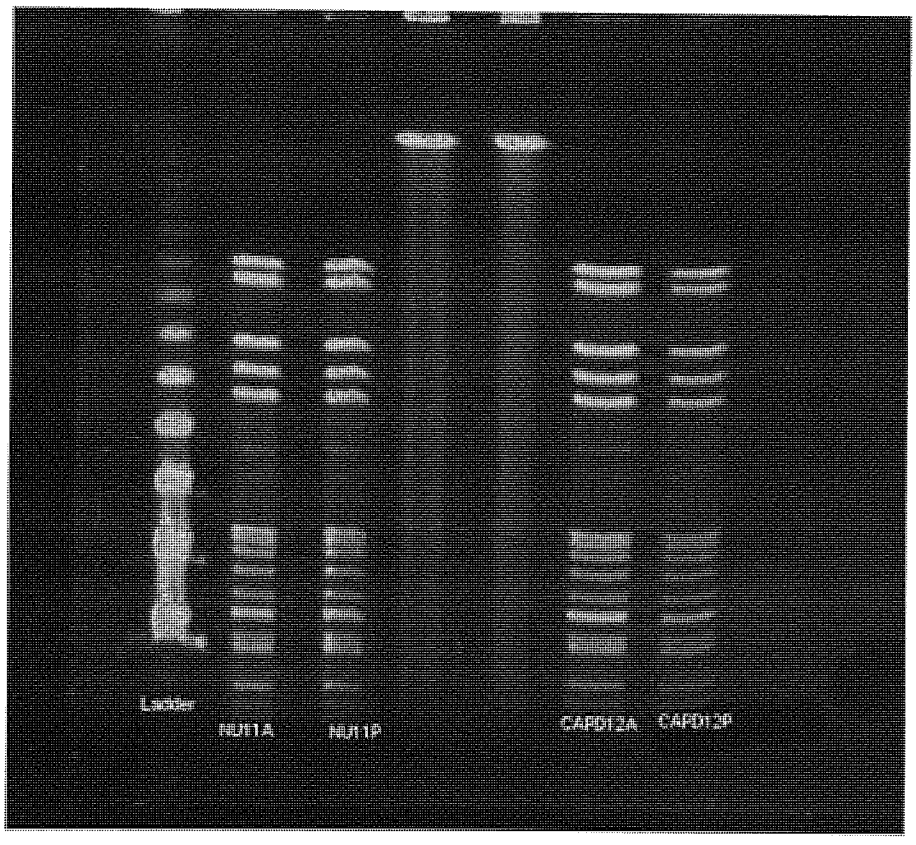
Strains	Parent Before serial passage in CHX	Adapted After serial passage in CHX
CVC1	2	4
CVC2	1	2
CAPD12	1	32
CAPD20	2	8
CAPD23	0.5	1
CAPD25	1	2
ROH1	2	4
NU11	2	64
NU159	2	64
ATCC35984	1	2
NCTC11047	1	2

The results in table 5.3 show that the susceptibilities of three strains of *S. epidermidis* to chlorhexidine (CHX) were decreased 16-fold by serial passage in the presence of increasing concentrations of CHX (highlighted by shading). However, other strains became non-viable. For example, CAPD20 became non-viable at 8 μg/ml of CHX after showing a slight increase in resistance. Other strains did not show an increase in MIC to CHX after seven passages. No increase in MIC was noted in the culture collection strains after being treated with CHX.

5.3.2 Pulse field gel electrophoresis

In order to check the purity of the cultures undergoing multiple passages three adapted strains were examined by PFGE. Figure 5.1 shows the PFGE DNA macro restriction patterns of the adapted strains together with the pattern of the parent strain.

Figure 5.1 PFGE banding patterns of NU11; NU159 and CAPD12 adapted cells to determine purity of passaged cultures.



Ladder NU11A NU11P NU159A NU159P CAPD12A CAPD12P

There was no visible alteration in the banding patterns of the digested chromosomal DNA of NU11 and CAPD12 parent (P) and adapted (A) cells. The image of PFGE was a confirmation that no contamination had occurred in the process of adaptation, thus supporting the view that alteration in resistance was due to adaptive changes (phenotypic or mutational) in the NU11, NU159 and CAPD12 cells themselves. Unfortunately no clear patterns were obtained for the NU159 parent and adapted strains.

5.3.3 Co-resistance to other antimicrobial agents

Strains NU11, NU159 and CAPD12 were examined to determine if the passage in CHX altered their resistance to a range of the antimicrobial agents including disinfectants and antibiotics. The MIC values for NU11, NU159 and CAPD12 are shown in table 5:4, CHX –adapted strains that showed marked changes in sensitivity to other agents are highlighted.

Table 5.4 MIC values (μg/ml) for parent (P) and adapted (A) strains passaged in CHX.

Antibiotics & Biocides	*NU11P*	*NU11A*	*NU159P*	*NU159A*	*CAPD12P*	*CAPD12A*
Chlorhexidine	2	**64**	2	**64**	1	**32**
Benzalkonium chloride	4	**16**	8	8	2	**8**
Triclosan	1	**4**	0.125	0.125	0.125	0.125
Povidone Iodine	8192	**16384**	4096	**8192**	8192	8192
Cefotaxime	>128	>128	8	**>128**	32	**8**
Ciprofloxacin	64	**32**	32	**16**	32	32
Trimethoprim	128	128	128	128	128	**64**
Chloramphenicol	16	**64**	16	**64**	64	64
Vancomycin	4	**8**	8	8	4	**8**
Fucidin	32	**16**	16	**2**	16	**2**
Ceftazidime	32	32	8	8	8	8
Erythromycin	>128	>128	>128	>128	>128	>128
Gentamicin	>128	>128	32	**16**	32	**64**
Flucloxacillin	>128	>128	>128	>128	>128	>128

Changes in sensitivity for each pair of strains are highlighted in Figures 5:2-5:4.

Figure 5.2 Fold increase in resistance to biocides in strain NU11P and its CHX passaged (adapted) strain NU11A.

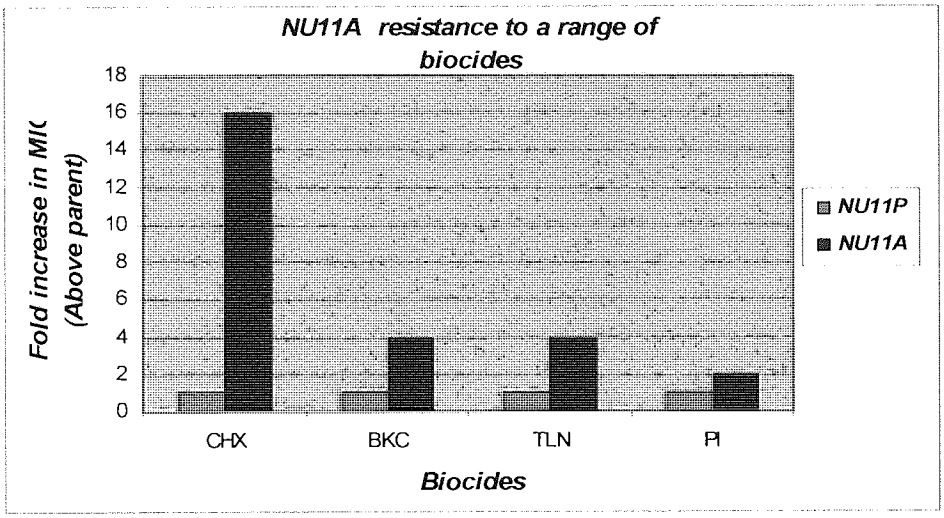


Figure 5.3 Fold increase in resistance to biocides in strain NU159 P and its CHX passaged (adapted) strain, NU159A.

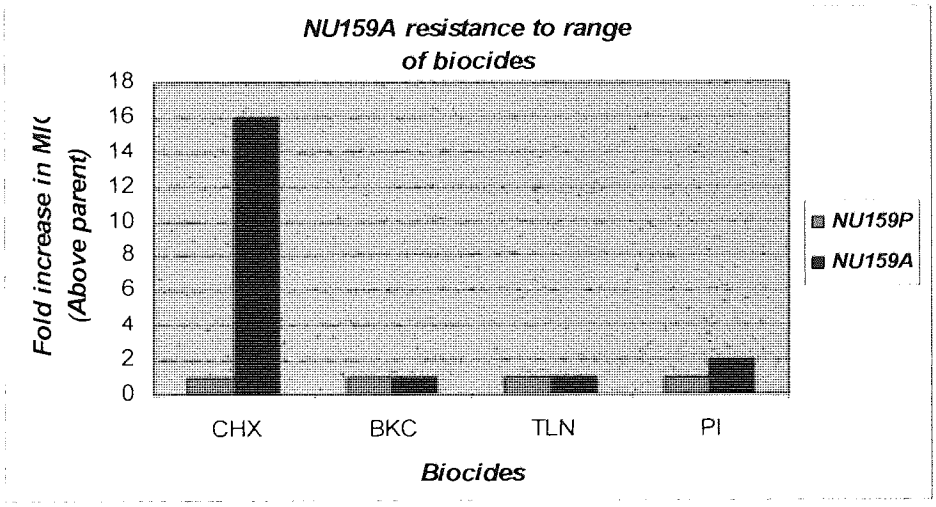
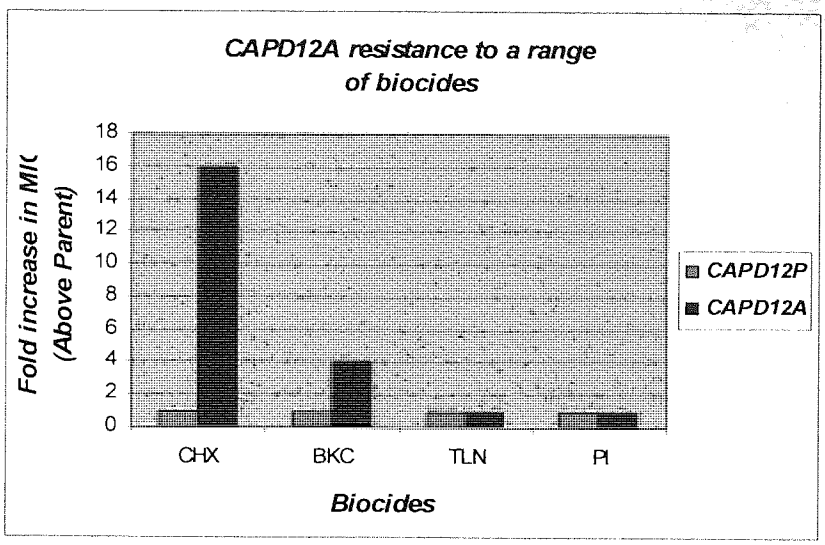


Figure5.4 Fold increase in resistance to biocides in strain CAPD12P and its CHX passaged (adapted) strain, CAPD12A.



Key: CHX=Chlorhexidine PI=Povidone Iodine

BKC=Benzalkonium chloride TLN=Triclosan

As described before the susceptibility of three adapted strains NU11, NU159 and CAPD12 was decreased 16-fold to CHX.

NU11 and CAPD12 adapted cells to CHX both showed an increase in resistance to benzalkonium chloride (BKC), while NU159 showed no changes in susceptibility to this agent. NU11 and NU159 both showed an increase in resistance to povidone iodine (PI). NU11 was the only adapted strain that showed an increase in resistance to triclosan (TLN).

There was a large increase (16-fold) in the MIC of cefotaxime between the WT parental and adapted strains of NU159, while CAPD12 showed a decrease (4-fold) in MIC to this antibiotic.

The NU11 and NU159 adapted strains both showed a slight (2-fold) decrease in the MIC for ciprofloxacin. CAPD12 adapted cells showed a slight (2-fold) decrease in the MIC for trimethoprim.

The NU11 and NU159 adapted cells both showed a 4-fold increase in MIC for chloramphenicol, while no change in MIC to this antibiotic was detected in the CAPD12 CHX-adapted strains compared to the parent cells.

Susceptibility to vancomycin was decreased (2-fold) in NU11 and CAPD12 adapted cells.

All three adapted strains; NU11 (2-fold), NU159 (8-fold) and CAPD12 (8-fold) showed a decrease in the MIC to fucidin.

Adapted strains of NU159 showed a 2-fold decrease in the MIC to gentamicin, while there was a slight (2-fold) increase between the CAPD12 parent and adapted strains.

There were no MIC changes between parental and adapted strains of NU11, NU159 and CAPD12 to the antibiotics, flucloxacillin, erythromycin and ceftazidime.

5.3.4 Sensitivity test to commonly used Antibiotics

Clinical sensitivity tests with antibiotics including gentamicin, fusidic acid, vancomycin, erythromycin, penicillin G and ciprofloxacin were carried out for the CHX- adapted strains, which showed no increased antibiotic resistance (Table5:5).Using this method no changes were noted in sensitivity between the pair of parental and CHX-adapted strains.

Table5: 5 Antibiotic sensitivity tests for Adapted and Parental strains.

Strains	penicillin G	gentamicin	vancomycin	erythromycin	fusidic acid	ciprofloxacin
NU11 P	R	R	S	R	S	R
NU11 A	R	R	S	R	S	R
NU159 P	R	R	S	R	S	R
NU159 A	R	R	S	R	S	R
CAPD12 P	R	R	S	R	S	S
CAPD12 A	R	R	S	R	S	S

Key: P= Parent strain (wild type) A= CHX adapted strain R=Resistant S=Sensitive

5.3.5 Stability of CHX resistance

The MICs for CHX of the adapted cells NU11, NU159 and CAPD12 were recorded after multiple passages in disinfectant- free broth (Table 5:6).

Table 5.6 Stability of resistance to CHX in passaged cells of NU11, NU159 and CAPD12

Strain	MIC (μg/ml) of CHX after passage in CHX- free media for a number of days				
	0 days	10 days	15 days	20 days	30 days
NU11 A	64	64	64	64	64
NU159 A	64	64	64	64	64
CAPD12 A	32	32	32	32	32

The three strains showed stability of resistance to CHX in their adapted cells even when grown in CHX-free media.

5.4 Discussion

5.4.1 Adaptation to CHX

In accordance with previous research (Stickler *et al.*, 1983; Joynson *et al.*, 1999; Mechin *et al,* 1999) it was possible to generate resistance to BKC and CHX by serial passage of *S. epidermidis* in increasing concentrations of disinfectant. Many strains showed an increase in resistance to BKC and CHX when exposed to a concentration of the disinfectant 4-fold lower than the MIC. Two strains became non-viable after a low number of passages, indicating an inability to adapt to the disinfectant. The remaining strains showed an ability to grow in CHX concentrations of 0.025% w/v, a higher concentration than that in appropriately diluted disinfectants. The most resistant of these cells had an MIC of more than 0.05% w/v CHX. This is a similar concentration to that of *P. aeruginosa* to BKC in the work of Joynson *et al* (1999), and far higher than that reached by cells of the ATCC15442 *P. aeruginosa* strain used in the work of Mechin *et al* (1999), although the fold-increase in resistance in all cases was comparable (Loughlin, 2001).

In this study the susceptibility of three out of eleven clinical *S. epidermidis* strains exposed to CHX by serial passage was decreased 16-fold. These adapted strains were NU11, NU159 and CAPD12. In addition, the CHX resistant phenotype was found to be stable in all three NU11, NU159 and CAPD12 strains in disinfectant-free media over a period equal to that required generating the phenotype.

Several previous investigations showed that bacterial tolerance can result from single or repeated exposure to sub-lethal concentrations of biocide and this type of exposure is likely to take place *in situ* raising the possibility of the development of bacterial tolerance in such situations (Lear *et al.*, 2002).

In other studies investigating bacterial adaptation to adverse conditions (e.g. extreme pHs), it was suggested that secreted extracellular induction components (EICs) are involved in inducing resistance responses by "warning" other bacteria of impending, stress situations. These "alarmones" may be involved in resistance to other inhibitory chemicals (McBain and Gilbert, 2001 and Rowbury *et al.*, 1998).

Davies and Maillard (2001) conducted a preliminary study to assess the release of "alarmones" from bacterial cultures after a treatment with sub-minimum inhibitory concentrations of biocides. In this investigation, cultures of *E. coli* and *S. aureus* were exposed to chlorhexidine diacetate and /or triclosan. Results of this preliminary experiment confirmed that "alarmones" might be involved in the emergence of tolerant bacteria to biocides. These observation support findings that "alarmones" produced by bacteria enduring stress are not only capable of inducing resistance in non-exposed bacteria, but also help protect the organism producing them against the stress involved (Rowbury *et al.*, 1998). In addition, this study showed that the possible" alarmones" produced by *S. aureus* in response to exposure to CHX were heat stable. The possibility of inducing low-level biocide resistance in bacteria with the involvement of "alarmones" is interesting and needs to be pursued further. It is possible that such "alarmones" trigger an efflux-type response in Gram-positive bacteria (Davies and Maillard, 2001). The chemical nature of "alarmones" remains to be established.

Russell (2000) suggested that bacterial resistance was not necessarily the result of a single mechanism, but could arise from the combined contributions of several mechanisms. The induction of "alarmones" by bacteria in response to biocide may be a significant part of the bacterial resistance arsenal and needs further investigation.

5.4.2 Cross-resistance

Cross-resistance between disinfectants and antibiotics in bacteria caused to become resistant by exposure to disinfectants is an area of research of growing interest. The disquieting thought that the hospital environment, already known to generate resistant bacteria due to use of

antibiotics, may be harbouring bacteria that disinfectant use has made resistant has been examined in work by Russell *et al.,* (1998 and 2002).

5.4.3 Cross-resistance to other disinfectants

Biguanides such as CHX and alexidine have a membrane-active bactericidal action. Resistance to such compounds has been thought to involve changes in the membranes surrounding bacteria and resistance to CHX would logically confer resistance to other membrane active agents (Russell, 2002). In the case of *S. epidermidis* adapted strains NU11, NU159 and CAPD12 this has been shown to be true. NU11 showed co-resistance to BKC, TLN and PI while NU159 and CAPD12 showed resistance to PI and BKC respectively. In the results obtained by Loughlin (2001) neither of the *P. aeruginosa* strains adapted to BKC showed any resistance to the bisbiguanide chlorhexidine or the phenolic disinfectant, triclosan. However, it appears more and more that cross-resistance to other disinfectants is a strain-specific phenomenon and caution must be taken not to generalise results to cover whole species.

5.4.4 Cross- resistance to antibiotics

The emergence of bacterial resistance to antibiotics is increasingly being considered in the context of its source, effects on individual patients and on hospital practice. Bacteria may adopt strategies to enhance the impermeability to antibiotics and biocides alluded to above, including the following: energy-dependent efflux pumps that can remove therapeutic levels of antibiotics and low (but probably not "in-use") concentrations of biocides; deletion of porins; changes in envelope composition; altered target sites; overproduction of target; antibiotic and possibly biocide metabolism in the periplasmic space; and altered membrane fluidity (Russell 2002).

According to Reverdy *et al* (1992) and Russell *et al* (1999) antibiotic- sensitive *S. aureus* and other staphylococci are usually antiseptic-sensitive, whereas strains for which the MICs of antiseptics indicate intermediate or high resistance are also more resistant to a wide range of antibiotics.

It has been shown that resistance to the biocide BKC is closely linked to oxacillin resistance in *S. aureus*. BKC-resistant mutants of methicillin-resistant *S. aureus* (MRSA) had oxacillin MICs as high as 512 mg/L, compared with 16 mg/L for the parent strain and 0.3 mg/L for methicillin-susceptible *S. aureus* (MSSA) (Fraise, 2002 and Arias *et al.*, 2003).

The potential for biocide-selected cross-resistance to clinically important antibiotics is the subject of much discussion in the literature (Russell *et al.*, 1999; Levy 2000; Russell 2000 and Schweizer, 2001). Reports that biocide resistant (i.e. efflux) genes do not predominate in MRSA or methicillin susceptible *S. aureus* (MSSA) (Bamber and Neal, 1999; Suller and Russell, 1999) and that biocides such as triclosan are effective at killing clinical MRSA isolates (Webster 1992; Webster *et al.*, 1994; Zafar *et al.*, 1995) suggest that, clinically at least, biocide- antibiotic cross-resistance is not a problem in *S. aureus*. This is supported by observations that *S. aureus* triclosan resistance in the laboratory does not create antibiotic resistance (Suller and Russell 2000; Poole, 2002).

Akimitsu *et al* (1999) showed that increase in resistance of MRSA to β-lactams is caused by mutations conferring resistance to BKC. It is usual to examine co-resistance to other disinfectants such as BKC when examining resistance attributed to growth in biguanide compounds such as chlorhexidine, but usually the interest in antibiotic resistance in such cases is limited to the membrane active of agents (Joynson *et al.*, 1999). The work of Yamamoto *et al* (1988) confirmed that antiseptic and antibiotic resistance in *S. aureus* is related to the presence of plasmids that confer chlorhexidine and acrinole resistance. Links between disinfectant adaptation and resistance to antibiotics not regarded as membrane-active are plentiful, if largely explained by the general mechanism of "reduced permeability". With some antibiotics such as novobiocin and erythromycin this is likely to be true, as the outer membrane of Gram-negative bacteria poses a considerable barrier for them (Russell, 2002). The work of Russell *et al.* (1998) showed chlorhexidine adapted *P. stutzeri* cells were more resistant to ampicillin and erythromycin. They observed increases in resistance to both cetylpyridinium chloride and to gentamicin, although the highest increases in resistance to these compounds did not occur in the same strains. This implies a general

alteration in permeability to these agents, as it is unlikely that separate resistance mechanisms would have been acquired in the process of adaptation.

In *E. coli,* resistance to pine oil is associated with co-resistance to the antibiotics, ampicillin and chloramphenicol (Moken *et al.,* 1997), although this is thought to be linked to the AcrAB efflux system present in *E. coli* and shows no co-resistance to phenolic disinfectants or QACs. Low- level exposure to triclosan can easily be used to select for *FabI* enoyl reductase mutants of *E.coli* that are less susceptible to triclosan (Gilbert, McBain and Bloomfield, 2002).

In this study the CAPD12 strain adapted to CHX by serial passage showed a slight (2-fold) increase in MIC for vancomycin and gentamicin. There was a 2-fold increase in the MIC for vancomycin and a 4-fold increase in the MIC for chloramphenicol in NU11 cells adapted to CHX. NU159 adapted cells showed a 16-fold increase in MIC to cefotaxime and a 4-fold to chloramphenicol.

5.5 Conclusions

Microorganisms vary in their sensitivity to the action of chemical agents. Some organisms, either because of their resistance to disinfection or because of their significance in cross-infection or nosocomial infections, merit attention. It is possible to generate stable adaptive resistance to the disinfectant, which was discussed before. In this study generation of adaptive strains to CHX was examined by serial passage in clinical *S. epidermidis* strains, which confirmed this possibility. The laboratory strains of *S. epidermidis* tested in this work did not show any changes in MIC after serial passage to CHX. Co-resistance was observed to other disinfectants such as BKC, TLN and PI, although such changes differ from strain to strains.

Chapter 6: Investigating mechanisms of resistance to CHX

6.1 Introduction

6.1.1 Aims

Having determined that *S. epidermidis* strains NU11, NU159 and CAPD12 became resistant to CHX and other antimicrobials following passage in CHX, the aim was to characterise any changes in the strains that might be linked to their resistance properties.

6.1.2 Efflux of biocides

The importance of efflux pumps in bacterial antibiotic resistance is now widely recognized (Webber and Piddock, 2003). Efflux of disinfectants and antibiotics is commonly seen as a mechanism of resistance in *S. aureus* (Brown and Skurray, 2001, Mitchell *et al.*, 1998), CoNS (Leelaporn *et al.*, 1994) and in Gram-negative bacteria such as *P. aeruginosa* (Nikaido *et al.*, 1998). The action of certain efflux pumps can be retarded by the addition of the alkaloid reserpine and has been used in the past to determine whether such pumps have a role in bacterial resistance (Gill *et al.*, 1999). *S. aureus* has demonstrated a predilection to develop resistance to a wide range of antimicrobial compounds, most probably in response to the selective pressures incurred in the clinical environment. These agents include antibiotics such as aminoglycosides, ß-lactams, chloramphenicol, tetracycline and an extensive range of toxic organic cationic chemicals, which are commonly used as antiseptics and disinfectants (Brown and Skurray, 2001).

6.1.3 Membrane proteins

Three plasmid-encoded determinants, *qacA, qacB* and *smr*, specify membrane proteins, which mediate resistance to numerous structurally dissimilar antimicrobial compounds via active transport, and have hence been termed multidrug resistance systems (Leelaporn *et al.*, 1994). Substrates of the QacA, QacB and Smr multidrug export proteins are monovalent cationic chemicals, such as quaternary ammonium compounds (QACs) and

dyes (e.g. acriflavine and ethidium). In addition, *qacA* confers resistance to divalent cationic compounds, such as the diamidines (e.g., propamidine) and the biguanidines (e.g. CHX) (Paulsen *et al.*, 1996; Mitchell *et al.*, 1999, Brown and Skurray, 2001).

6.1.4 Membrane Lipids

Alteration of the membrane lipids and fatty acids of bacterial adapted by to growth in antimicrobial agents has been reported for a number of organism and agents (Peschel *et al.*, 2001, Moore *et al.*, 1984, Conrad and Galanos, 1989 and Guerin-Mechin *et al.*, 1999). However, this work aims to examine alterations in the cellular lipidsof adapted strains of *S. epidermidis* to CHX. Phospholipids are an essential part of membranes. Their composition affects the structure and permeability of membranes and changes have been observed in the amount and type of phospholipids present in organisms subjected to antimicrobial agents (Anderes *et al.*, 1971; Conrad and Gilleland, 1981; Gilleland and Conrad, 1982; Champlin *et al.*, 1983; Moore *et al.*, 1984; Gilleland *et al.*, 1984 and Peschel *et al.*, 2001). Isolation of phospholipids from cells is based upon the methods of Bligh and Dyer (1959). Specific lipids are identified by thin layer chromatography where the mobility of each phospholipid component of the bacteria sample is compared with those of pure phospholipid samples. Visualisation of phospholipids is achieved by spraying the plate containing the lipids with molybdenum blue reagent (Dittmer & Lester, 1964). Further characterisation can be achieved by spraying plates with ninhydrin and heating for a short time. This identifies samples containing free amino groups such as phosphatidylethanolamine and phosphatidylserine. This also allows detection of lyso (partially deacylated) forms of phospholipids containing free amino acids. Fatty acids can be extracted from membranes after hydrolysis and methylation in alkaline methanol. They are separated as the methyl esters by gas chromatography and identified by comparison of retention time standards.

6.1.5 Cell surface hydrophobicity

At the bacterial cell surface there are a number of nonspecific forces involved in the interaction between the cell and the surrounding environment. This environment can include: the cell surface of other microorganisms, host cells, antimicrobial agents, non-organic surfaces, or more simply the media that supports the growth of the organism. One force that is important in the ability of an organism to interact with the environment is hydrophobicity. The measurement of hydrophobicity has been discussed in several reports (Rosenberg and Doyle, 1990; Van der Mei *et al.*, 1991, Peschel *et al.*, 2001). Hydrophobicity reflects the inability of water to accommodate non-polar chemical species. In liquid water, a hydrogen bond network extends throughout the liquid. In the case of polar or charged species, it is possible for the molecules to interact with the network by either donating or accepting hydrogen bonds from the water. Non-polar molecules, however, cannot interact so easily with the network as they have no groups to donate or accept hydrogen bonds. This results in the network rearranging itself around the non-polar molecule rather than interacting directly with it. This "lack of attraction" between a molecule and water forms the basis of the term "hydrophobic", whereas such charged, polar compounds that form hydrogen bonds easily with water are described as "hydrophilic" or "water loving". The cell surfaces of bacteria are neither completely hydrophobic nor hydrophilic, but are made up of species contributing to both these characteristics, species known as hydrophobins, or hydrophilins respectively (James, 1991).

6.1.5.1 Measurement of hydrophobicity by microbial adhesion to hydrocarbon(MATH)

The outer surface of microbial cells contains a variety of chemical compounds which may be involved in the attachment of cells to surfaces. Hydrophobic hydrophilic interactions play an important role in attachment, leading to the development of the concept of cell surface hydrophobicity as a measure of the tendency of a cell to attach to a surface. One of the most popular tests of this tendency is the microbial adhesion to hydrocarbons (MATH assay) (Pembrey *et al.*, 1999).

There are a number of methods used to determine hydrophobicity and recently they have been examined to determine the effect each has upon the cells examined (Pembrey *et al.*, 1999). The MATH assay measures the proportion of cells in a suspension that, when mixed with a hydrocarbon, partitions into the hydrophobic phase. The higher the proportion of cells that partition into the hydrophobic phase, the more hydrophobic the cells are considered to be. While the reproducibility of this assay has been seen to be dependent upon the pH of the suspension used and there is evidence that cell surfaces are disrupted by the use of hydrocarbons (Pembery *et al.*, 1999), it is still a common assay used to examine alterations of hydrophobicity in a range of organisms. These include Gram-positive and Gram-negative bacteria, protozoa and fungi (Van der Mei *et al.*, 1997; Smith *et al.*, 1998 and Jana *et al.*, 2000).

6.1.5.2 Cell surface hydrophobicity of CoNS

The cell surface of both *S. aureus* and various CoNS has a high negative net surface charge related to polymers such as ribitol and glycerol teichoic acids (Mamo *et al.*, 1987, Doyle and Rosenberg, 1990). However, it is not known whether lipoteichoic acid, a key polymer in membrane staphylococcal lipid cell metabolism, can be transported to the cell surface and expose its fatty acids. Lipoteichoic acid has been proposed as a surface amphiphile in hydrophobic characteristics of staphylococci (Koch *et al.*, 1984). The surface hydrophobicity common to *S. aureus* as well as strains of certain CoNS species seems to involve protease and heat-sensitive surface structures, not surface amphiphiles (Doyle and Rosenberg 1990).

The cell surface hydrophobicity of *S. epidermidis* strains was examined. Studies have confirmed that strains of *S. epidermidis* isolated from various human infections fall into two groups: strains expressing lower cell surface hydrophobicity when grown in sugar-supplemented trypticase soy broth (group I strains) and strains not much affected by growth in sugar-supplemented media (group II) (Doyle and Rosenberg1990 and Peschel *et al.*, 2001).

6.1.6 Effect on the cell surface and CHX uptake or binding

The cell membrane, as well as providing a dynamic link between metabolism and transport serves to maintaining the pool of metabolites within it (Lambert, 2002). Before an antimicrobial agent can exert its effect on a cell it must combine with that cell. This process often follows the pattern of an adsorption isotherm. Clearly, factors that affect the state of the cell surface, as the pH of the cell's environment must do, must affect, to some extent, the adsorption process (Hugo and Russell 1998). Some biocides e.g. glutaraldehyde and cationic agents, as well as cationic antibiotics have marked effects on bacterial surface. However, in understanding how antibiotics and biocides act it is important to know how they are taken up into bacterial cells as well as to understand the ensuing effects. Antibiotics and biocides readily penetrate the staphylococcal cell wall; although glycopeptide antibiotic resistance in *S. aureus* has been associated not only with peptidoglycan changes but also with thickened cell walls that limit access of vancomycin to its target site (Russell, 2002).

CHX acts upon the cytoplasmic membrane causing both membrane disorganisation and damage. It has action on general membrane permeability. Treatment of bacterial cells with appropriate concentrations of CHX causes a leakage of a group of characteristic chemical species (Hugo and Russell, 1998). The potassium ion, being a small entity, is the first substance to appear when the cytoplasmic membrane is damaged. Aminoacids, purines, pyrimidines and pentoses are examples of other substances that will leak from treated cells (Hugo and Russell, 1998 and Russell, 2002). In high concentration CHX will coagulate the cytoplasm resulting in reduced leakage.

6.1.7 Susceptibility of adapted strains to other disinfectant

6.1.7.1 Quaternary ammonium compounds (QAC)

The quaternary ammonium disinfectant benzalkonium chloride (BKC) is widely used as the active antimicrobial component of a number of common disinfectant solutions. It has strong surface-active properties and attacks the inner (cytoplasmic) membrane. It also damages the outer membrane and thus is believed to mediate its own uptake into the cells (Scott and Gorman, 1998 and Hugo and Russell, 1998). Salton (1968) proposed the following sequences of events with microorganisms exposed to cationic agents: (i) adsorption and penetration of the agent into the cell wall; (ii) reaction with the cytoplasmic membrane (lipid or proteion) followed by membrane disorganization; (iii) leakage of intracellular low-molecular –weight material; (iv) degradation of proteins and nucleic acids; and (v) wall lysis caused by autolytic enzymes. There is thus a loss of structural organization and integrity of the cytoplasmic membrane in bacteria, together with other damaging effects to the bacterial cell (Reverdy *et al.*, 1997).

6.1.7.2 Halogen-Releasing Agents

6.1.7.2.1 Povidone-Iodine

Povidone-iodine has a wide spectrum of antimicrobial activity. Gram-negative and Gram-positive organisms and bacterial spores (on extended exposure) are all susceptible. The active agent is the elemental iodine molecule, I_2. As elemental iodine is only slightly soluble in water, iodine ions are required for aqueous solutions such as aqueous iodine solution, Lugol's solution containing 5% iodine in 10% potassium iodine solution. Iodine has action on membrane enzymes, which contain –SH, groups (Hugo, 1998). The antimicrobial action of iodine is rapid, even at low concentrations, but the exact mode of action is unknown. Iodine rapidly penetrates into microorganisms and attacks key groups of proteins (in particular the sulphur-containing amino acids cysteine and methionine, nucleotides, and fatty acids (Dychdala, 1991), which culminate in cell death (Kemp, 1998).

6.1.7.3 Bisphenols

6.1.7.3 .1 Triclosan

Triclosan (2, 4, 4'-2'-hydroxydiphenyl ether; irgasan DP 300) exhibits particular activity against Gram-positive bacteria (McDonnell and Russell, 1999). It is used as an antimicrobial agent in hygiene products, plastics and kitchenware, and for treating methicillin-resistant *S. aureus* (MRSA) outbreaks (Suller and Russell, 2000). At concentrations used for disinfections the primary effects of triclosan are on the cytoplasmic membrane. Studies with an *E. coli* triclosan-resistant mutant for which the triclosan MIC was 10-fold greater than that for a wild-type strain showed no significant differences in total envelope protein profiles but did show significant differences in envelope fatty acids (Barkvoll and Rolla, 1994). Minor changes in fatty acid profiles were recently found in both *E. coli* and *S. aureus* strains for which the triclosan MICs were elevated; however, the MBCs were not affected, suggesting, as for other phenols, that the cumulative effects on multiple targets contribute to the bactericidal activity (Mc Donnell *et al.*,1997, McDonnell and Russell, 1999).

6.2 Materials and Methods

6.2.1 MIC determination for CHX with addition of resrpine

The MICs for three adapted strains were determined for CHX in the manner described in section 4.2.2.

6.2.2 Fatty acid extraction

All glassware and caps were prepared by washing in acetic acid followed by membrane material were transferred to 10ml Pyrex glass hydrolysis tubes and mixed, by vortexing, with 1ml of 3.8M NAOH in 50% v/v aqueous methanol. The tubes were sealed with Teflon lined caps, and incubated at 100° C for 5-minutes. Tubes were then revortexed and incubated for further 25 minutes at 100° C in the same manner as before. Following this tubes were removed from heat and left to cool to room temperature. 6ml of a (1:1) solution of 6M HCL and methanol was added and the unsealed tubes heated at 80°C for 10 minutes to form the methyl esters of the free fatty acids that had been released from lipids in the bacterial membranes by the alkaline hydrolysis. The samples were then removed from the heat at left to cool to room temperature. A volume of 1 ml of (1:1, v/v) hexane/diethyl ether mixture was added and repeatedly inverted for 10 minutes. This allowed partitioning of the fatty acid methyl esters (FAMEs) from the aqueous phase to the organic phase and samples were allowed to stand for 10 minutes to allow the two phases to separate. The upper organic layer was removed carefully by glass pipette and mixed with 3ml of 0.3M NaOH in a sealable glass tube. The mixture was then repeatedly inverted for 10 minutes to allow extraction of any unwanted residues from the organic to the aqueous phase. The upper organic phase was then transferred to a 2 ml glass tube with a foil-lined cap. The organic solvent was evaporated by the passage of compressed nitrogen gas into each tube at room temperature. The completed preparations were dissolved in 1 ml hexane sealed and stored at -20°C until analysed.

6.2.3 Chromatographic analysis of fatty acid methyl easter profiles

A volume of 1µl of each sample was loaded onto a Hewlett-Packard HP-1 capillary column on a Unicam 610 series Gas Chromatograph (GC). Conditions used are shown in table 6.1. In addition a second column (Supelco Omegawax 320) was used to confirm the reproducibility of this technique and to aid identification of the integrated and the data analyzed using the Unicam software package. Fatty acids were identified by comparing the retention times of the sample peaks with those of a standard mixture of known fatty acids (CPTM Mix Supelco), prepared according to manufacturer's instructions. An Omega wax column was used; four standard bacterial fatty acid mixes were used to identify sample fatty acids.

Table 6.1 Conditions used in gas chromatography of fatty acid samples

Conditions	Used in column HP1	Used in column Omega wax
Sample split	1:50	1:50
Mobile gas phase	Helium	Helium
Column length (m) x column diameter (mm)	25x0.32	30 x 0.32
Film thickness	0.17 µm	0.25 µm
Linear velocity (hexane) cm/sec	20.8	35.78
Initial temperature	150°C	200°C
Programme rate	4°C/min	4°C/min
Means of peak detection	Flame ionization detector	Flame ionization detector.
Standard used	Bacterial acid methyl esters CP ™ Mix (Supelco)	RM-1 1084, NHI-F 1092, GLC-60, GLC-100.

6.2.4 Identification of unknown peaks

To identify those peaks that did not correspond with any FAME present in the standards the GC separation was repeated using a Hewlett Packard 5989B Quadrapole mass

spectrophotometer to identify peaks by electron impact mass spectrometry. Conditions for the HP column were as detailed in table (6.1) with the exception of the flow rate being controlled by a constant pressure sensor. The source temperature was 260°C (max 375°C) and a quad temperature of 100°C (max 150°C). The mass:charge ratio range scanned was between 95 and 320, a full ×50 higher than the largest fatty acid likely to be present.

6.2.5 Whole cell lipids

6.2.5.1 Preparation of whole cell lipids

A volume of 500 ml of overnight culture from each strain was centrifuged at 9630 g for 10 minutes and resuspended in 30ml of dd$H_2$0. Chloroform and methanol were mixed with the bacterial suspension in the ratio of 1 part chloroform, 2 parts methanol and 0.8 parts bacterial suspension. This solution was mixed, covered with aluminum foil and left to stand overnight at room temperature. Chloroform and water were added to the solution in the proportions of 1 part water, 1 part chloroform and 1 part solution. This was mixed and allowed to separate into organic and aqueous phases. The lower phase was carefully removed and dried using a rotary evaporator. The lipid residue was dissolved in a 1ml solution of 2 parts methanol, 1 part chloroform and stored at -20°C in a glass vial with a foil-lined cap.

6.2.5.2 Separation of whole cell lipids

The lipids were separated by thin layer chromatography. Spots of approximately 10µl of each lipid solution were applied onto a silica coated aluminum chromatography plate (20 x 20cm, layer thickness 200µm, particle size 2-25(m, pore size 60Å, Sigma) using a micro capillary tube. In addition, samples of authentic common phospholipids (Sigma) were spotted at similar concentrations. The plate was allowed to dry fully at room temperature. The mobile phase (25 parts methanol, 65 parts chloroform and 4 parts water) was left to equilibrate in the glass chromatography tank at room temperature,

sealed with a glass lid for at least an hour. This allowed the atmosphere within to saturate with mobile phase vapour.

The dry plate was placed with the glass tank, the mobile phase settling below the spotted samples. The tank was sealed until the solvent front had progressed nearly to the top of the plate.

6.2.5.3 Visualisation of lipids

Two methods of visualization were used. For lipids containing amino groups, plates were sprayed with 0.2% v/v ninhydrin in ethanol and dry heated to 110°C for up to 15 minutes. Phospholipids were visualised by spraying with molybdenum blue spray, a solution of 1.3% w/v molybdenum oxide in 4.2M H2SO4 (Sigma), onto the dry plates. Once the plates had been developed an image of them was recorded using a UVP scanner. The image visualised on the scanner was saved to disk and this was used in the Phoretix gel analysis program to determine the proportions of each lipid in the cell samples. Those plates sprayed with ninhydrin were not used for determining proportions of lipids, but were used to confirm the presence of phosphotidylethanolamine its lyso form.

Glycerides were visualised by exposure to iodine vapour. Once the plates had been developed an image of them was recorded using a UVP scanner and digital camera. The image visualised on the scanner was saved to disk. Three glycerides 1-monooleoyl-rac-glycerol (C18: 1, [C15]-9), diolein (C18: 1, [cis]-9) as mixed isomers (99%) and triolein (C18: 1, [cis]-9 (1, 2, 3-tri [cis-9-octadecenoyl] glycerol)(95%) were used as standard (Sigma).

6.2.6 Hydrophobicity assay

Cells from overnight cultures were centrifuged at 10,000x g for 5 minutes and resuspended in PUM buffer (2.22% w/v K2HP04, 0.726% w/v KH2PO4, 0.18% cells w/v urea, 0.02% w/v MgSO4). Cells were re-centrifuged and resuspended in PUM buffer to a

final optical density of 0.5 at 470nm. Samples of 1.25ml were taken in triplicate and transferred to acid washed test tubes. A volume of 200µl of hexadecane was added and left at room temperature for 10 minutes. Each tube was vortexed for 45 seconds and left to stand for further 15 minutes at room temperature. The upper layer containing hexadecane and any cells that had partitioned from aqueous to organic phase, was carefully removed by pipetting. The tubes were then incubated at 5°C for 15 minutes. While still at 5°C the hardened crust of residual hexadecane was removed from the top of each tube using a wire loop. The tubes were allowed to remain at room temperature for 15 minutes after which the optical density of the remaining solution was measured at 470nm and compared to that of cells left untreated by hexadecane.

PUM Buffer

Di-potassium hydrogen phosphate (K2HP04)	11.1g
Potassium di-hydrogen phosphate (KH2P04)	3.63g
Urea	0.90g
Magnesium Sulphate (Mg2P04)	0.1g
Distilled water	500ml

6.3 Results

6.3.1 MIC determination for CHX with addition of reserpine

The addition of reserpine to media containing CHX used in an MIC experiment had no significant effect upon the MIC and MBC when compared to a parallel experiment without reserpine. This was the case for all three adapted strains of NU11, NU159 and CAPD12. The data are recorded in tables 6.2, 6.3 and 6.4.

Table 6. 2 Effect of reserpine on the MIC (μg/ml) and MBC (μg/ml) of CHX to adapted cells of NU11.

Strain	MIC (no reserpine)	MBC (no reserpine)	MIC (with reserpine)	MBC (with reserpine)
NU11P	2	4	2	4
NU11A	32	32	32	32

Table 6.3 Effect of reserpine on the MIC (μg/ml) and MBC (μg/ml) of CHX to adapted cells of NU159.

Strain	MIC (no reserpine)	MBC (no reserpine)	MIC (with reserpine)	MBC (with reserpine)
NU159P	2	4	2	4
NU159A	32	32	32	32

Table 6. 4 Effect of reserpine on the MIC (μg/ml) and MLC (μg/ml) of CHX to adapted cells of CAPD12.

Strain	MIC (no reserpine)	MIC (no reserpine)	MIC (with reserpine)	MIC (with reserpine)
CAPD12P	2	2	2	2
CAPD12A	32	32	32	32

6.3.2 Fatty acids of membranes of adapted strains

The proportion of each fatty acid was determined by examining the area of peaks produced by gas chromatography. Proportions were calculated from peaks between a retention time of 3 minutes and approximately 25 minutes. Figure 6.1 shows the profile of the FAME standard trace while figure 6.2, 6.3 and 6.4 show examples of profiles from the strains used in the standard are listed in table 6.5 and those identified in figures 6.2, 6.3 and 6.4 are shown in table 6.6.

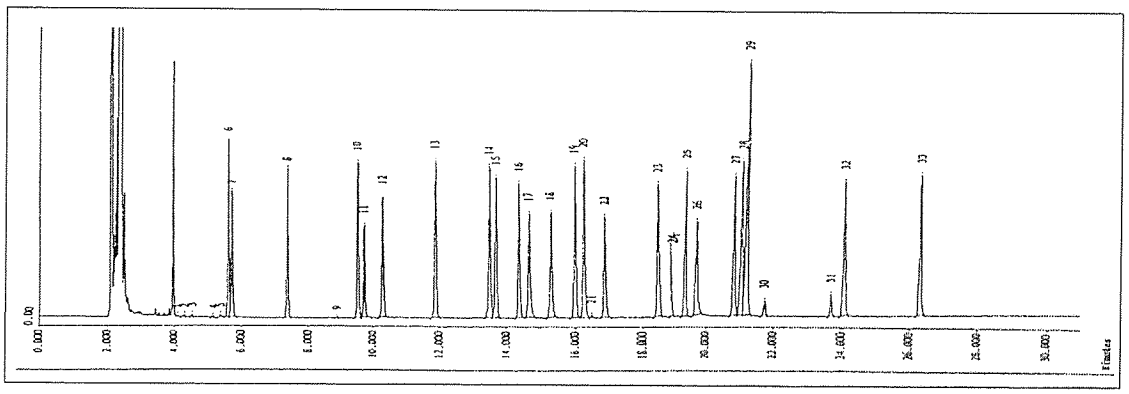


Figure 6:1 Gas Chromatography trace of Standard Bacterial FAME CP^{TM} Mix (Supelco). The X-axis indicates retention time in minutes and the Y-axis indicates relative concentrations of each fatty acid methyl ester determine by flame ionisation detection.

Table 6.5 List of fatty acid methyl esters used in the standard shown in figure 6.1.

Peak	Fatty acid methyl ester	Peak	Fatty acid methyl ester
1	False peak	18	**3-OH 14:0** 3- hydroxytetradecanoate
2	False peak	19	**i-16:0** 14-methylpentadecanoate
3	False peak	20	**16:1** cis-9-hexadecenoate
4	False peak	21	False peak
5	False peak	22	**16:0** hexadecanoate
6	**11:0** undecanoate	23	**i-17:0** 15-methylhexadecanoate
7	**2-OH 10:0** 2-hydroxydecanoate	24	**17:O**Δcis-9,10-methyleneheaxadecanoate
8	**12:0** dodecanoate	25	**17:0** heptadecanoate
9	False peak	26	**2-OH 16:0** 2-hydroxyhexadecanoate
10	**13:0** tridecanoate	27	**18:2** cis-9,12octadecadienoate
11	**2-OH 12:0** 2-hydroxydodecanoate	28	**18:1** cis-9 octadecenoate
12	**3-OH 12:0** 3-hydroxy dodecanoate	29	**18:l***trans-9octadecenoate & cis-11 octadecenoate
13	**14:0** tetradecanoate	30	**18:0** octadecanoate
14	**i-15:0** 13 -methyltetradecanoate	31	**19:O**Δ cis-9, 10methyleneoctadecanoate
15	**a-15:0** 12-methyltetradecanoate	32	**19:0** nonadecanoate
16	**15:0** pentadecanoate	33	**20:0** eicosanoate
17	**2-OH 14:0** 2-hydroxytetradecanoate		

Figure 6:2 Sample trace showing methyl esters of fatty acids present in whole cells of CAPD12P (red) and CAPD12A (black). The X-axis indicates retention time in minutes and the Y-axis indicates relative concentrations of each fatty acid methyl ester.

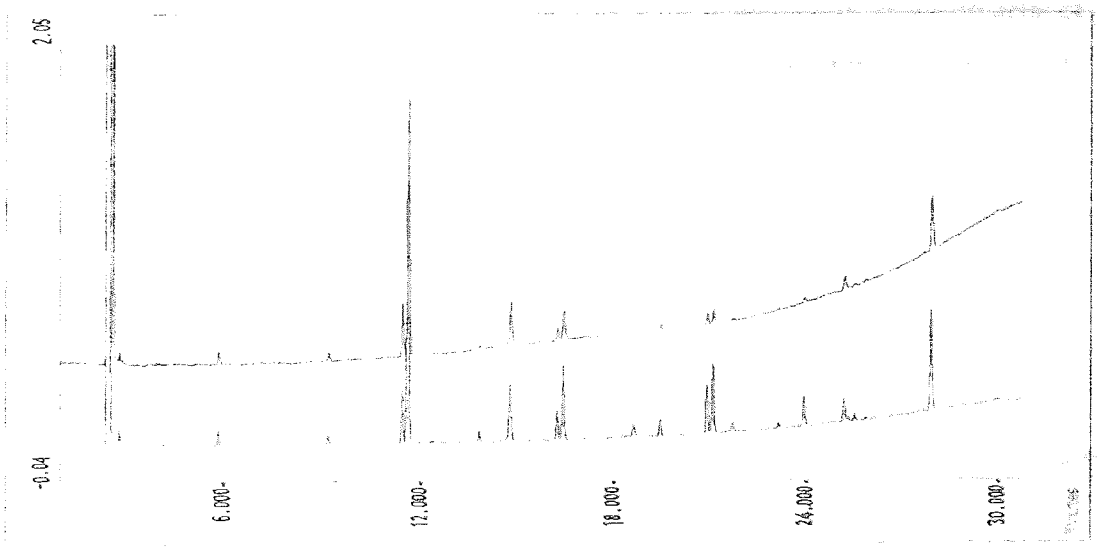


Table 6.6 List of fatty acids identified from gas chromatography trace in figure 6:2.

Peak number	Fatty acid methyl ester	Peak	Fatty acid methyl ester
1	**2-OH 12:0** 2--hydroxydodecanoate hydroxydodecanoate	8	**17:O**Δcis-9, 10-methyleneheaxadecanoate
2	**14:0** tetradecanoate		
3	**i-15:0** 13 -methyltetradecanoate		
4	**i-16:0** 14-methylpentadecanoate		
5	**16:1** cis-9-hexadecenoate		
6	**16:0** hexadecanoate		
7	**i-17:0** 15-methylhexadecanoate		

There appears to be no significant change in the proportion of fatty acids in whole cells of *S. epidermidis* strains NU11A, NU159A and CAPD12A as they became adapted to CHX.

Figure 6:3 Sample trace showing methyl esters of fatty acids present in whole cells of NU11P (red) and NU11A (black). The X-axis indicates retention time in minutes and the Y-axis indicates relative concentrations of each fatty acid methyl ester.

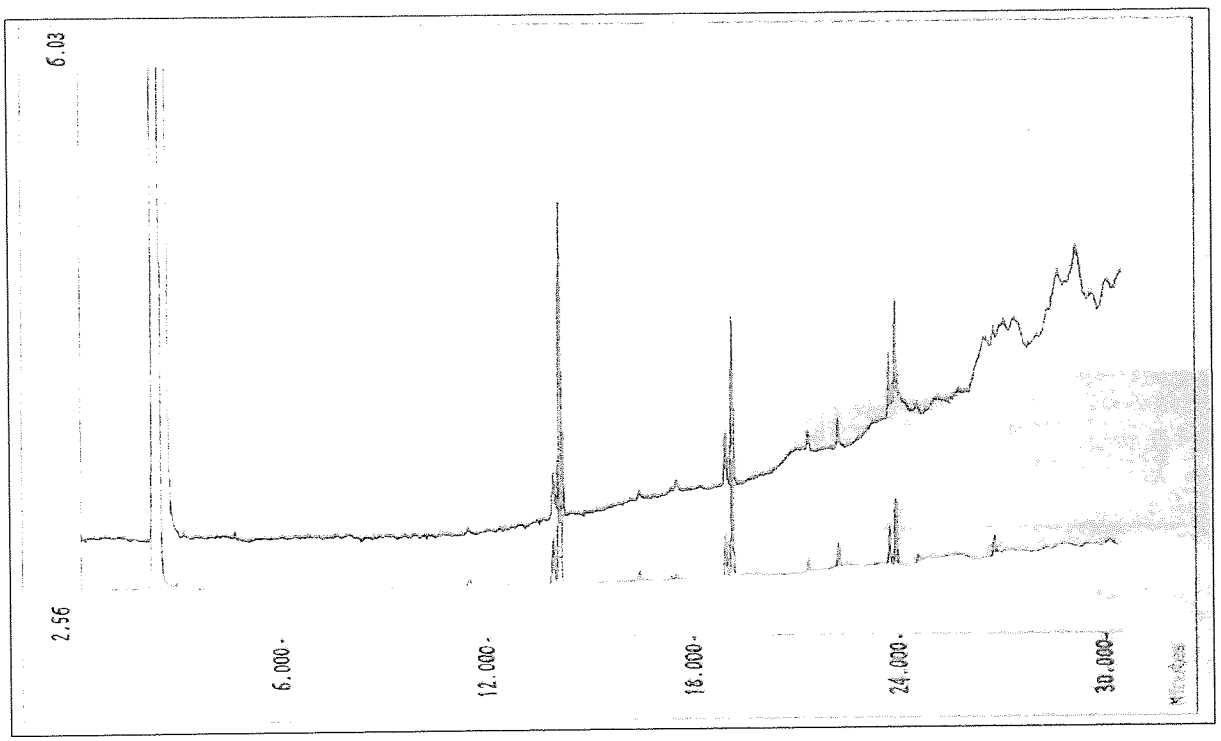


Figure 6.4 Sample trace showing methyl esters of fatty acids present in whole cells of NU159P (black), NU159A (red), NU11P (yellow) and NU11A (blue). The X-axis indicates retention time in minutes and the Y-axis indicates relative concentrations of each fatty acid methyl ester.

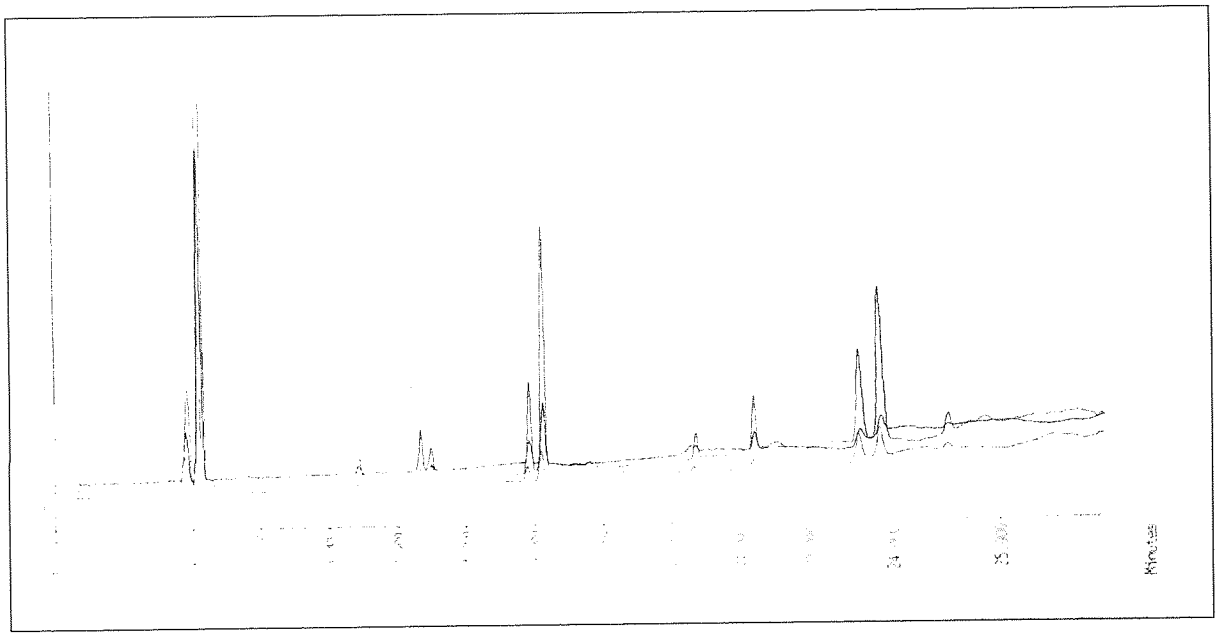


Table 6.7 Comparisons of phospholipids and neutral lipids/fatty acids in three parental and their adapted strains to CHX by serial passage. Results for adapted strains are shaded.

Strains	Total Lipid % dry wet of cell	% FA+NL to total lipid	% PL to total lipid
NU 11P	7.66	26	74
NU 11A	12.68	24	76
NU 159P	8.31	23	77
NU 159A	14.27	22	78
CAPD 12P	6.39	24	76
CAPD 12A	15.39	27	73

Key:

P=Parent A=Adapted PL=Phospholipid NL=Neutral Lipid FA=Fatty Acid

6.3.3 Whole cell lipids

The TLC plates were photographed (Figures.6: 5- 6:8) and scanned for use with the Phoretix software to calculate the identity of each lipid resolved and the proportion of each in the organism. The raw data and proportions calculated from the photographs are shown in table 6:7.

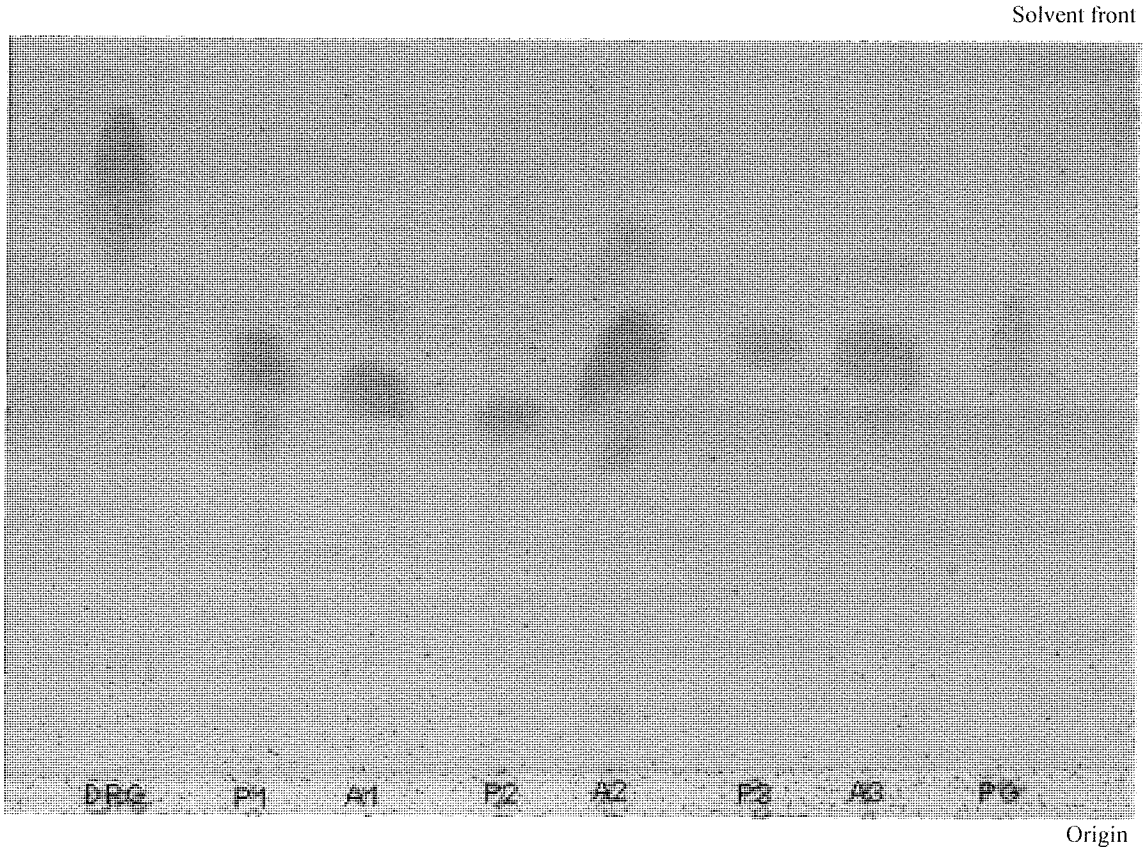


Figure 6.5 Comparison of whole cell phospholipids (parent and adapted) cells of *S. epidermidis* strains NU11, NU159 and CAPD12 visualised with molybdenum blue and ninhydrin. P1, P2 and P3 refer to NU11, NU159 and CAPD12 parent strains respectively. A1, A2 and A3 refer to NU11, NU159 and CAP12, which are the corresponding adapted strains after serial passage. DPG refers to diphosphatidylglycerol and PG refers to phosphatidylglycerol.

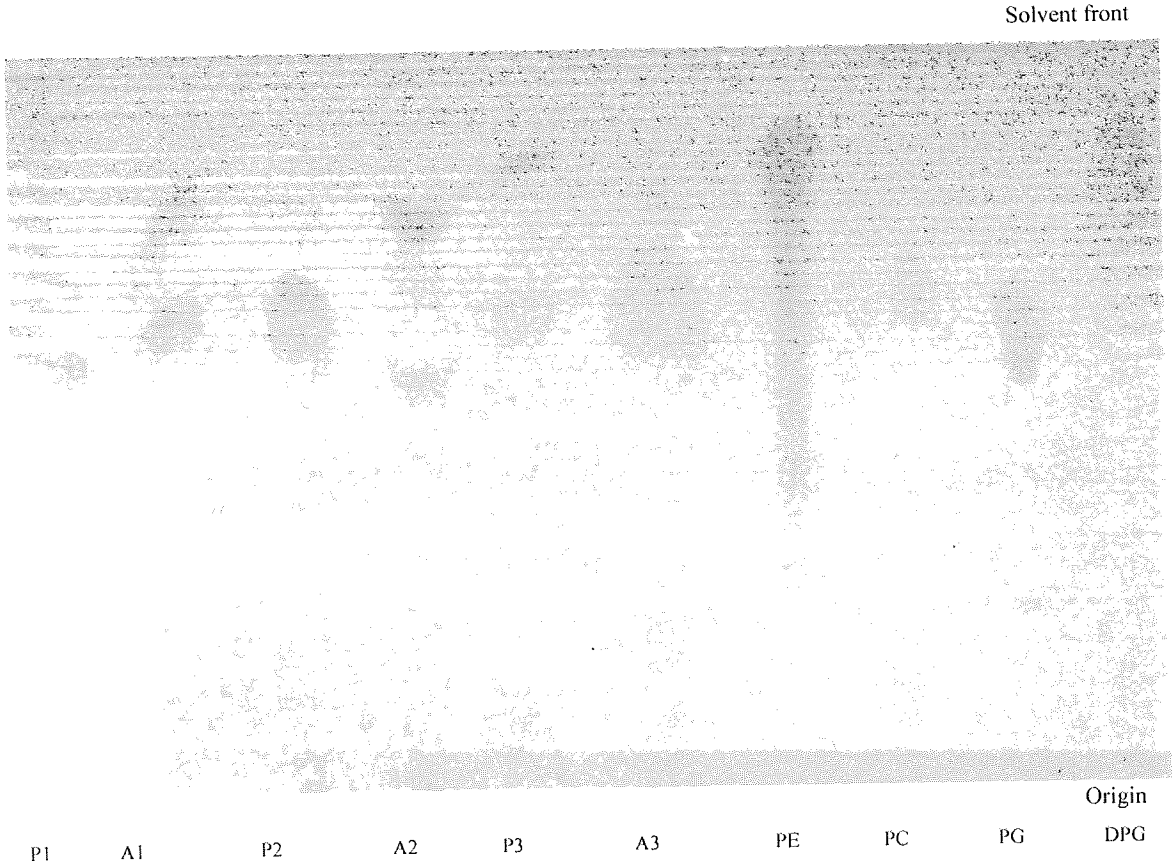


Figure 6.6 Whole cell phospholipids (parent and adapted) cells of *S. epidermidis* strains NU11, NU159 and CAPD12 visualised with molybdenum blue and ninhydrin. P1, P2 and P3 refer to NU11, NU159 and CAPD12 parent strains respectively. A1, A2 and A3 refer to NU11, NU159 and CAP12 respectively, which are the respective adapted strains after serial passage. DPG refers to diphosphatidylglycerol, PG refers to phosphatidylglycerol, and PC and PE refer to phosphatidylcholine and phosphatidylethanolamine respectively.

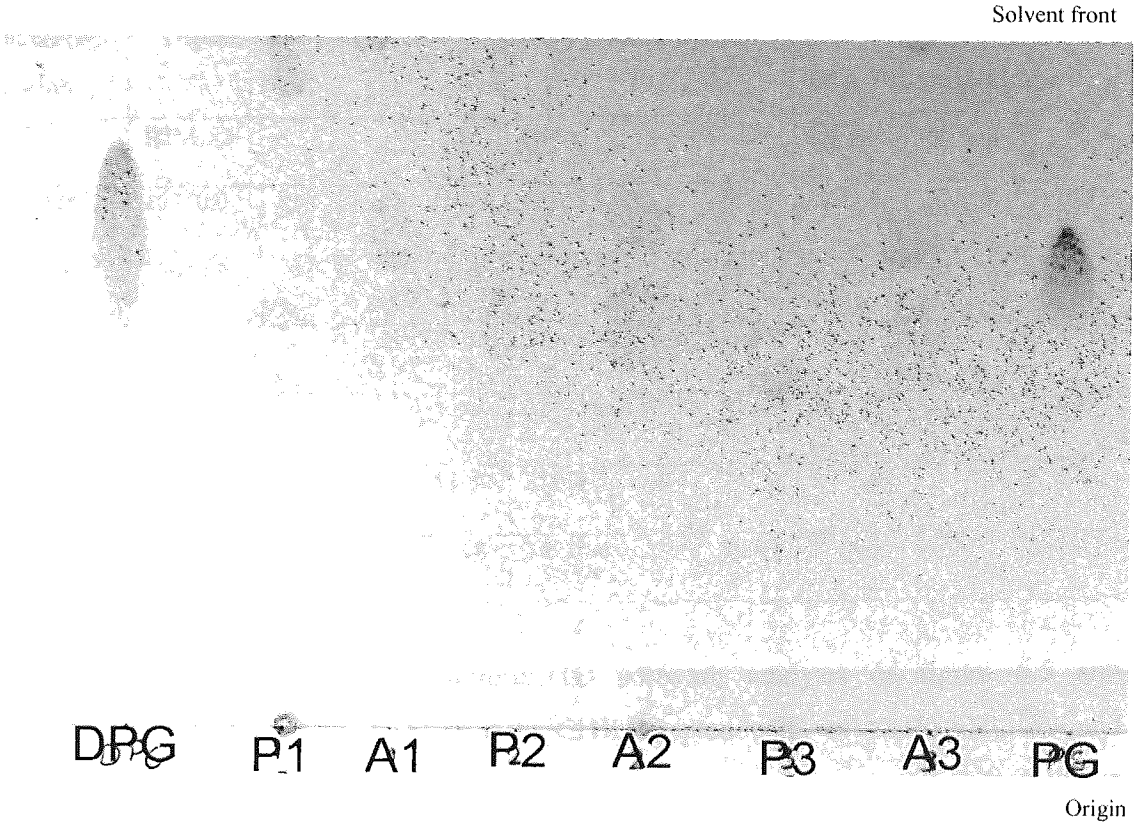


Figure 6.7 Comparison of whole cell phospholipids (parent and adapted cells) of *S. epidermidis* strains NU11, NU159 and CAPD12 visualised with molybdenum blue. P1, P2 and P3 refer to NU11, NU159 and CAPD12 parent strains respectively. A1, A2 and A3 refer to NU11, NU159 and CAP12, which are the adapted strains after serial passage. DPG refers to diphosphatidylglycerol and PG refers to phosphatidylglycerol.

Bacterial Strains	Diphosphatidylglycerol DPG		Phosphatidylglycerol PG		Other Phospholipids (PC, PE and unknown)	
⇓	RPA	%	RPA	%	RPA	%
NU11P	10710.5	16.09	49115.6	73.79	6731.45	10.11
NU11A	14614.7	14.71	76130.4	76.63	8592.12	8.64
NU159P	8012.3	19.50	31461.7	76.57	1614	3.92
NU159A	12412.53	12.49	82140.9	82.66	4809.4	4.84
CAPD12P	7130.4	15.82	35911.3	79.70	2013.4	4.46
CAPD12A	8431.9	8.68	86230.4	88.83	2406.1	2.47

Table 6.8 Data collated from Phoretix 1D software analysis of figure 6.5 and identification of phospholipids present in CHX adapted cells of NU11, NU159 and CAPD12 and their parents. RPA refers to the relative peak area corresponding to the density of colour of the separated phospholipids as determined by the Phoretix 1D software. PC and PE refer to phosphatidylcholine and phosphatidylethanolamine respectively.

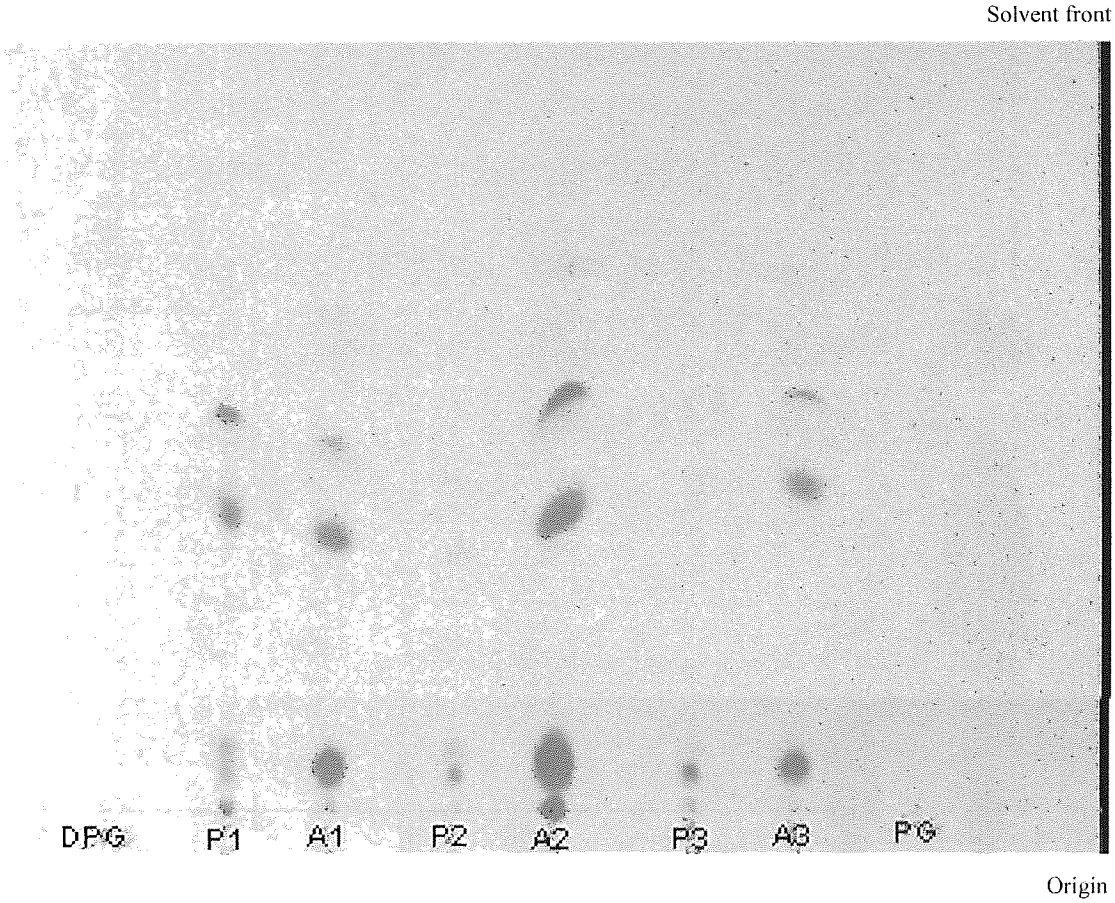


Figure 6.8 Comparison of whole cell phospholipids of parent and adapted cells of *S. epidermidis* strains NU11, NU159 and CAPD12 visualised with ninhydrin spray.
P1, P2 and P3 refer to NU11, NU159 and CAPD12 parent strains respectively. A1, A2 and A3 refer to NU11, NU159 and CAP12 respectively, which are the adapted strains after serial passage. DPG refers to diphosphatidylglycerol and PG refers to phosphatidylglycerol.

In all three strains there appeared to be an increased in the amount of phosphatidylglycerol in each of the adapted strains compared with the parent strains.

No other trends of alteration in phospholipid proportions were observed in NU11 and CAPD12. There appears to be an alteration in the phospholipid proportions of NU159

adapted strains to CHX. An extra band related to phosphatidylcholine was detected on the TLC plate sprayed with molybdenum blue reagent for this strain.

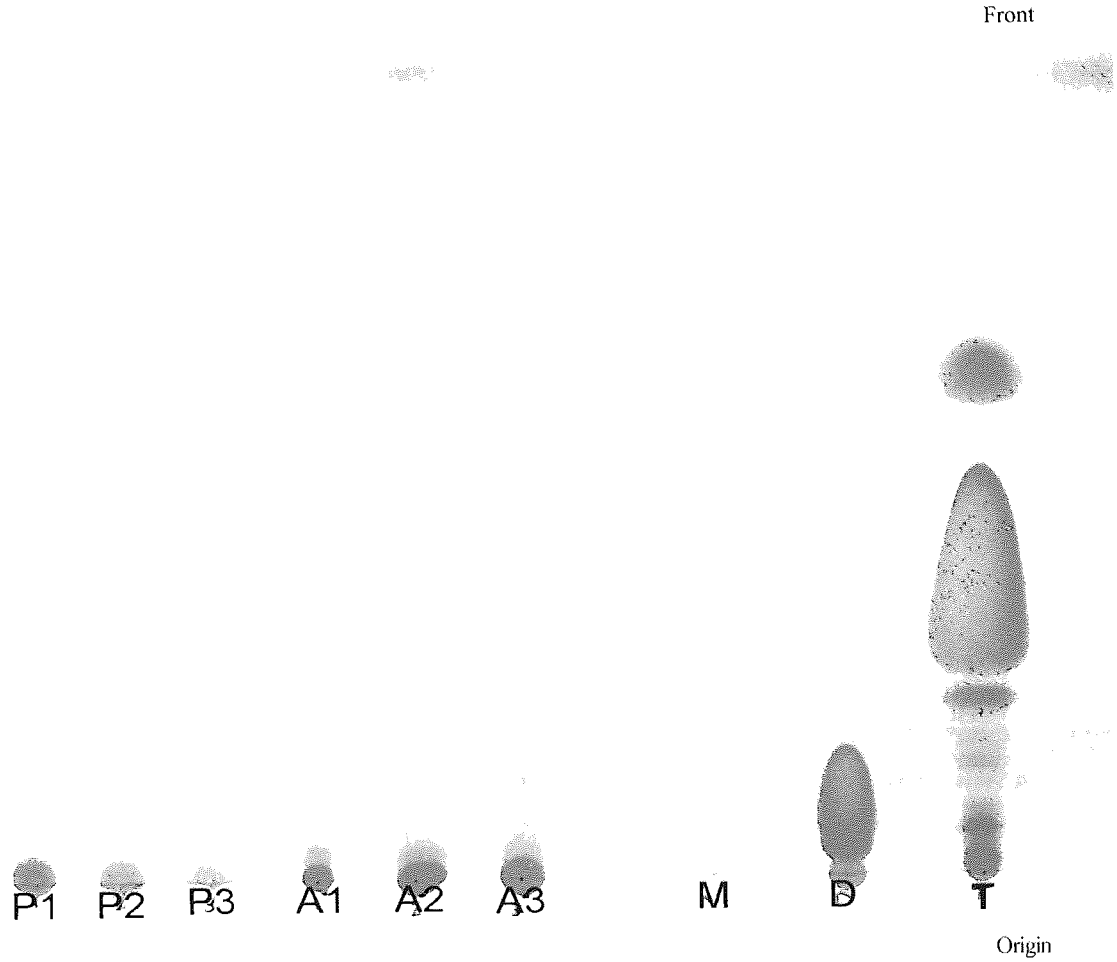


Figure 6.9 Whole cell neutral lipids and fatty acids of (parent and adapted) cells of *S. epidermidis* strains NU11, NU159 and CAPD12 visualised with iodine vapour. P1, P2 and P3 refer to NU11, NU159 and CAPD12 parent strains respectively. A1, A2 and A3 refer to NU11, NU159 and CAP12 respectively, which are the adapted strains after serial passage. M refers for monoolein, D for diolein and T for triolein.

Overall there appeared to be a trend in the proportion of fatty acids and neutral lipids in three adapted strains compared with their parent strains. Adapted strains showed extra bands of fatty acids, diglyceride, triglyceride and monoglyceride. The parental strains only showed one band of monoglyceride on the base line.

6.3.4 Hydrophobicity

The cell surface hydrophobicity of NU11, NU159 and CAPD12 was determined by the MATH assay measuring % cell portioning from an aqueous to a hydrophobic (hexadecane) phase. Results are shown in figures 6:11, 6:12 and 6.13.

The results of the study were analysed by calculating the percent removal of cells from the aqueous phase.

Figure 6.10 Comparison of cell surface hydrophobicity of NU11 adapted and parent cells. P=0.0054 (0.3591)

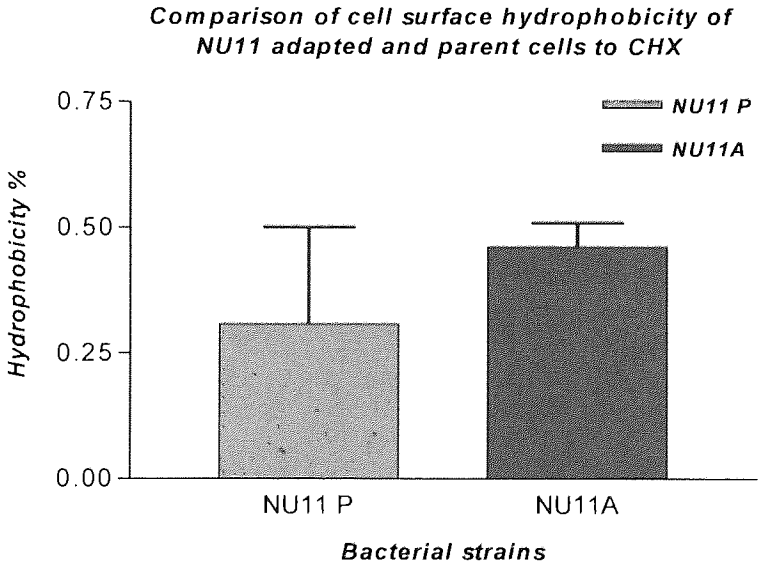


Figure 6.11 Comparison of cell surface hydrophobicity of NU159 adapted and parent cells. P<0.0001(0.0034).

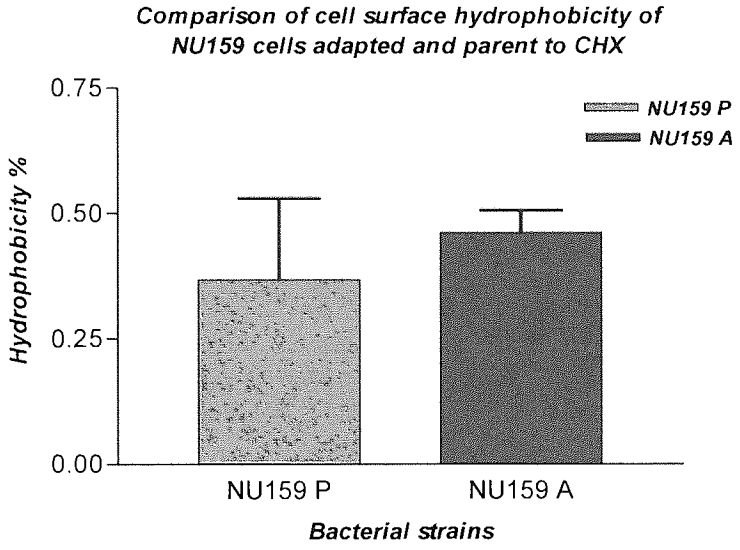
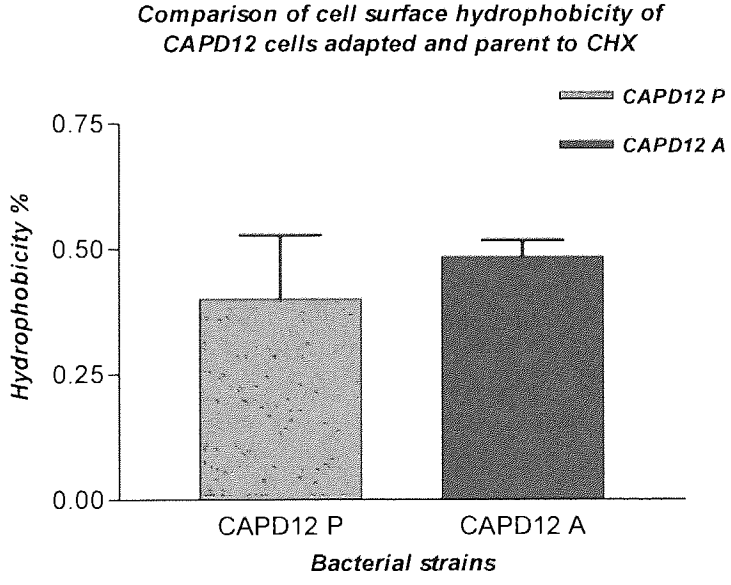


Figure 6.12 Comparison of cell surface hydrophobicity of CAPD12 adapted and parent cells. P=0.0045 (0.0104).



There appears to be a connection between cell surface hydrophobicity of adapted strains and their resistance to CHX. Strains adapted to CHX were more hydrophobic than their parent cells, indicating increased lipid content in their cell wall. P value (t test) calculation indicated that these differences in cell surface hydrophobicity between parent and adapted cells are significant (Prism 3.0 Graph Pad).

6.4 Susceptibility to CHX and other biocides on adapted and parent strains

The susceptibility to other biocides of the CHX-adapted strains was determined. The data are displayed graphically in figures 6.13 –6.24.

Figure 6:13 Effect of CHX on NU11 parent and adapted strains after 1,3 and 5 minutes exposure.

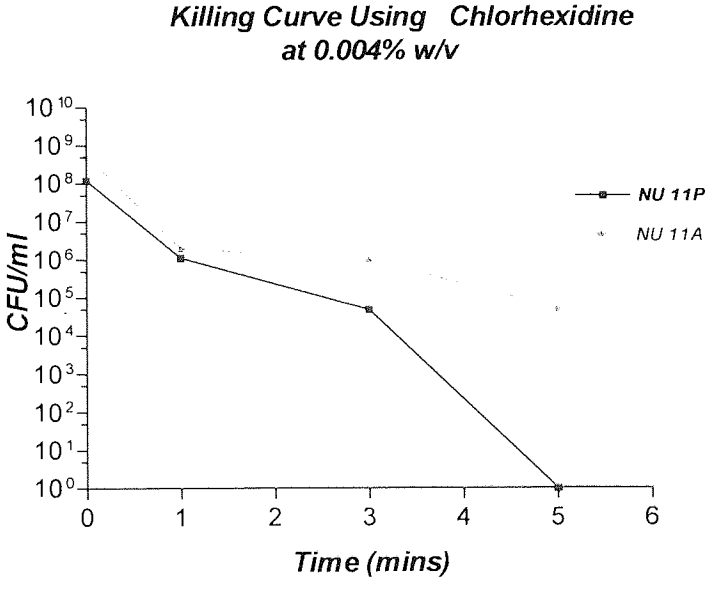


Figure 6:14 Effect of CHX on NU159 parent and adapted strains after 1,3 and 5 minutes exposure.

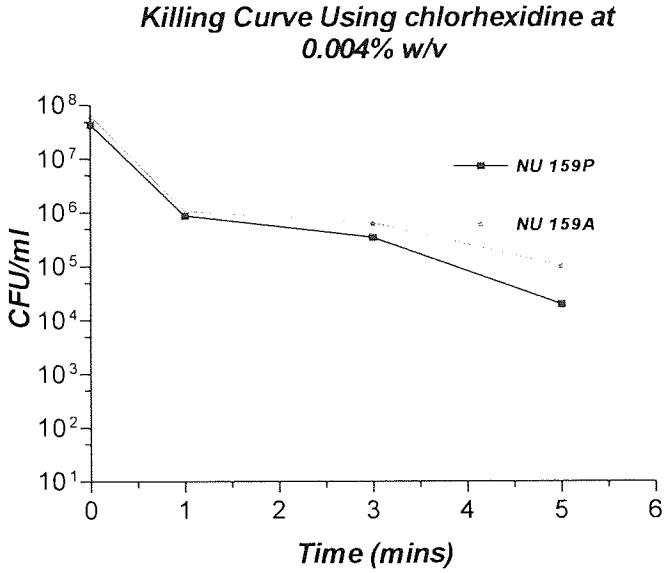
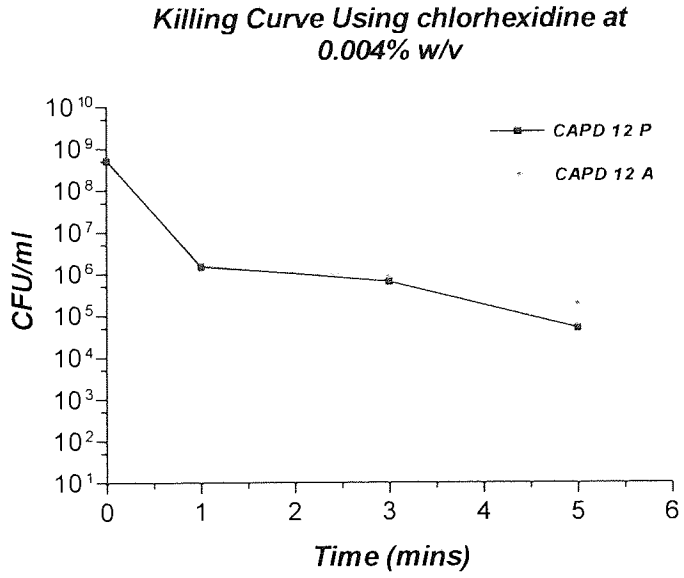


Figure 6:15 Effect of CHX on CAPD12 parent and adapted strains after 1,3 and 5 minutes exposure.



Comparison of the killing curves using CHX at 0.004% between parent and their the adapted strains showed a reduction in susceptibility to CHX in NU11, NU159 and CAPD12 after 5 minutes exposure to CHX.

Figure 6: 16 Effect of triclosan on NU11 parent and adapted strains after 1,3 and 5 minutes exposure.

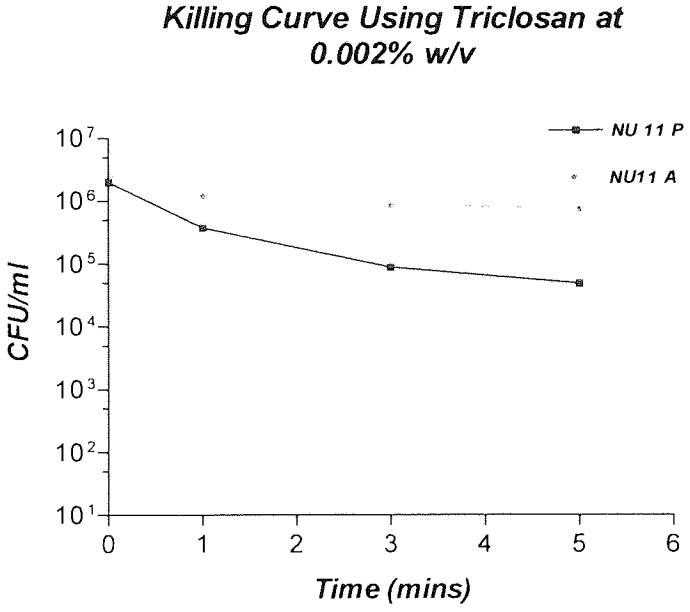


Figure 6:17 Effect of triclosan on NU159 parent and adapted strains after 1,3 and 5 minutes exposure.

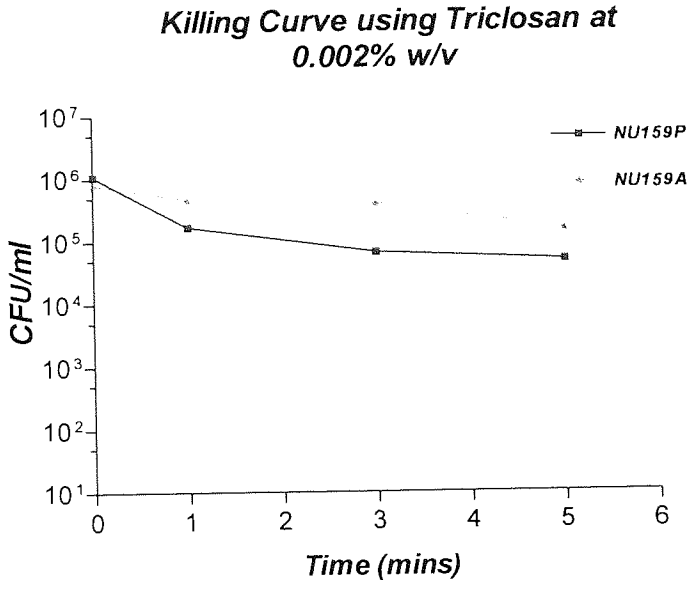
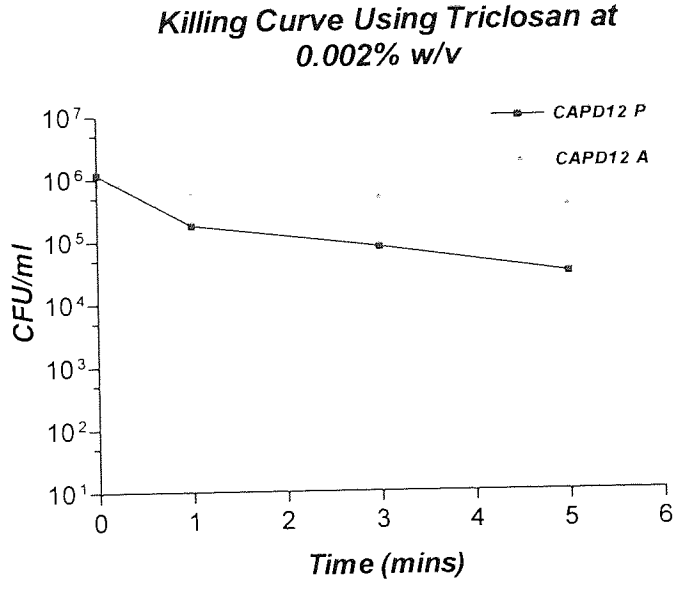


Figure 6:18 Effect of triclosan on CAPD12 parent and adapted strains after 1,3 and 5 minutes exposure.



Overall there was a trend that susceptibility to triclosan was decreased in the adapted strains (NU11, NU159 and CAPD12) after 1 and 5 minutes exposure in comparison with their parent strains.

Figure 6:19 Effect of povidone-iodine on NU11 parent and adapted strains after 1,3 and 5 minutes exposure.

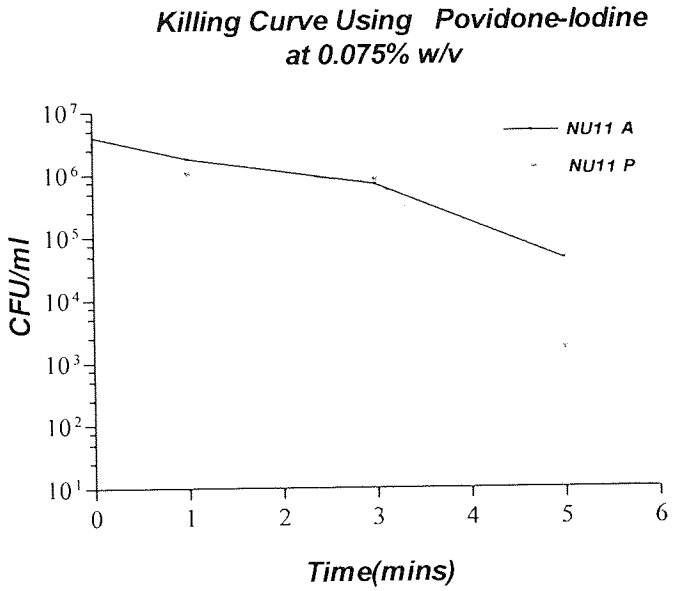


Figure 6:20 Effect of povidone-iodine on NU159 parent and adapted strains after 1, 3 and 5 minutes exposure.

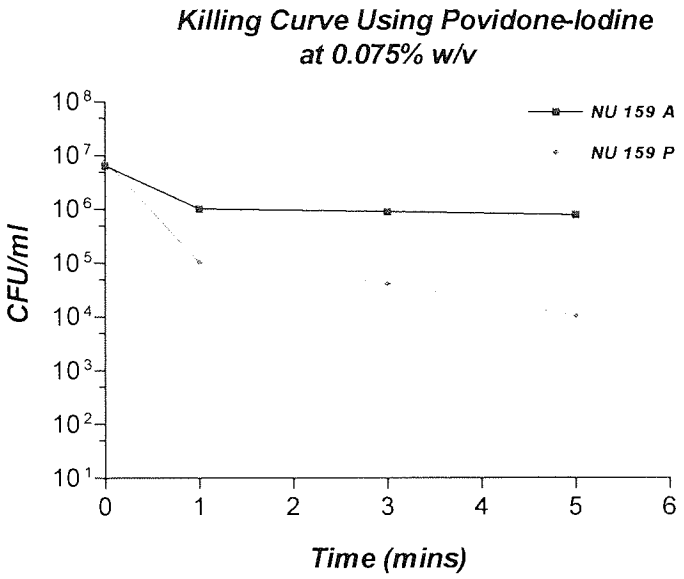
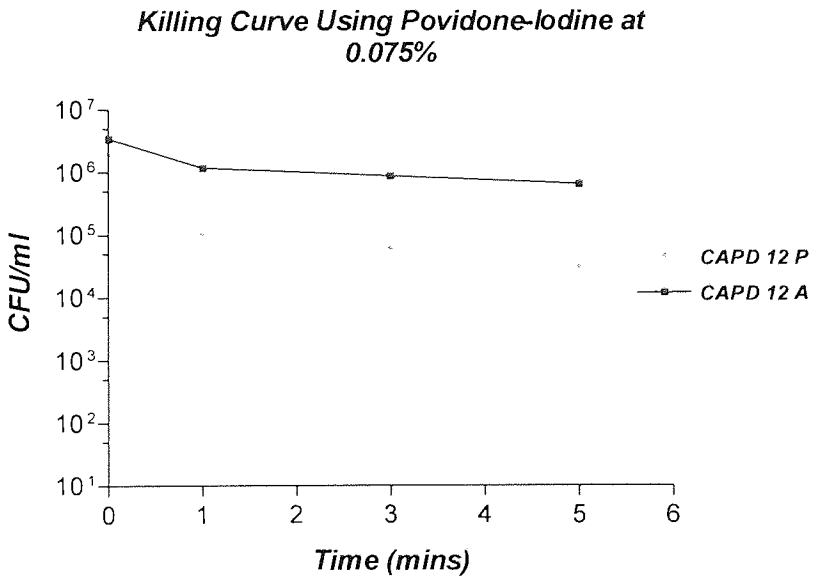


Figure 6:21 Effect of povidone-iodine on CAPD12 parent and adapted strains after 1,3 and 5 minutes exposure



Overall there was a trend that susceptibility to povidone-iodine was decreased in adapted strains (NU11, NU159 and CAPD12) after 1and 5 minutes exposure in comparison with their parent cells.

Figure 6:22 Effect of BKC on NU11 parent and adapted strains after 1,3 and 5 minutes exposure.

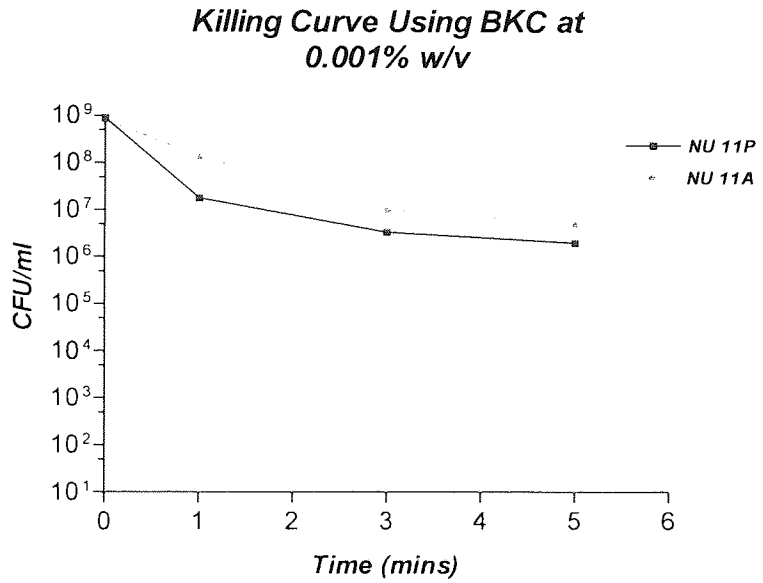


Figure 6:23 Effect of BKC on NU159 parent and adapted strains after 1,3 and 5 minutes exposure.

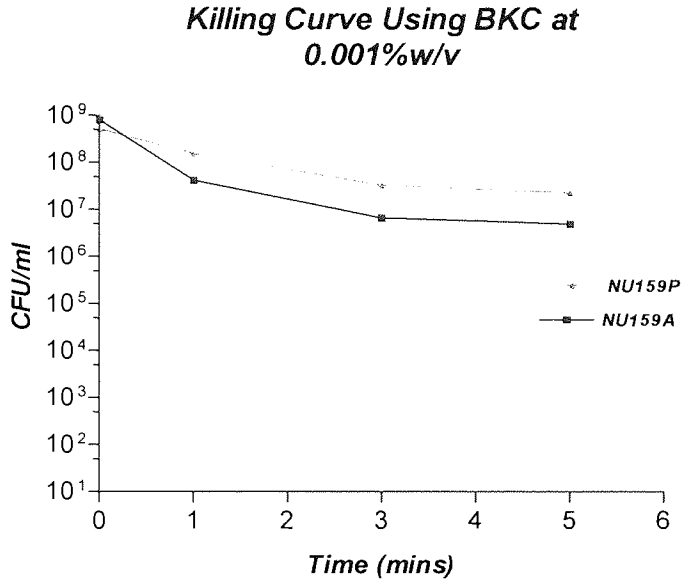
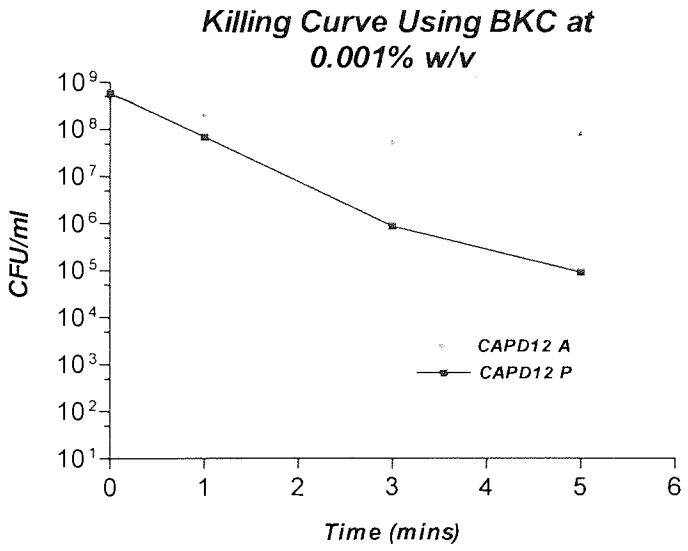


Figure 6:24 Effect of BKC on CAPD12 parent and adapted strains after 1, 3 and 5 minutes exposure.



Overall there was a trend that susceptibility to BKC was decreased in adapted strains (NU11, NU159 and CAPD12) after 1,3 and 5 minutes exposure in comparison with their parent cells.

6.5 Discussion

Biocide and chlorhexidine resistance among Gram-positive cocci is being increasingly recognised. Possible mechanisms for such resistance include efflux, altered target sites and cell wall changes. In order to determine such a mechanism, it must be present in a resistant organism and absent in both the wild type "parent" organism and in the reverting organism having lost the resistant phenotype. While many physiological changes accompanied the development of a CHX resistant phenotype in the three strains of *S. epidermidis* examined, it is impossible to state that they are responsible for resistance, especially in the absence of any revertants to the wild type resistant phenotype. In conclusion, the changes described in the results section and discussed here, can only be said to be associated with development of resistance not responsible for the alteration in resistance phenotype observed.

6.5.1 Efflux

The addition of reserpine to cells adapted to CHX appeared to have no effect upon their resistance to the disinfectant in a manner that would have reflected the presence of an efflux system. The importance of efflux in biocide resistance of Gram-positive cocci is, therefore, not great (Brown and Skurray, 2001). Reserpine has been used in the past to inhibit those efflux pumps associated with Gram-positive organisms such as NorA in pneumococci (Gill *et al.*, 1999). NorA is a multidrug resistant efflux pump in *S. aureus* (Kaatz, Moudgal and Seo, 2002). In the case of NU11, NU159 and CAPD12 cells there was no evidence of cross-resistance to antibiotics normally associated with efflux in *S. epidermidis* cells, and so it is unlikely that such a system was affected by passage in CHX.

6.5.2 Phospholipid and Fatty acid content of cell membranes

For researchers examining the mechanism of resistance to membrane active agents such as CHX and quaternary ammonium compounds (QAC), the composition and properties of bacterial membranes are of particular interest. In this study, cells of *S. epidermidis* strains NU11 and CAPD12 showed an increase in resistance to both CHX and BKC when grown in increasing concentrations of the CHX. These increases in resistance were accompanied by an increase in the proportion of neutral lipids and phospholipids. In all three strains there appeared to be an increased in the proportion of phospholipids. This increase was most marked for diphosphatidylglycerol, each of the adapted strains showed an increased amount of this phospholipid.

There appeared to be no trend in the proportion or nature of fatty acids in whole cells of *S. epidermidis* strains NU11A and NU159A and CAPD12 A as they became adapted to CHX.

In this work the alteration in phospholipid content may alter the properties of the cytoplasmic membrane. This could act as a resistance mechanism to CHX whose site of action is the cytoplasmic membrane and would explain the reduction in cold resistance in adapted cells to CHX in this study. Proportions of phospholipids in *S. epidermidis* adapted cells have not been widely examined with reference to resistance to CHX or other disinfectants.

6.5.3 Hydrophobicity

The hydrophobic nature of a cell surface can be due to a number of chemical species. A number of antibiotics and biocides affecting cell wall synthesis, protein synthesis and nucleic acid synthesis cause a decrease in cell surface hydrophobicity of staphylococci

(Schmidt *et al.*, 1987). Interestingly, studies by Proctor *et al* (1983) showed that chloramphenicol and clindamycin decrease expression of the fibronectin binding protein. Rifampin at sub inhibitory concentrations causes a rapid decrease of surface hydrophobicity and binding of *S. aureus* Cowan I to human nasal cells (Switalski *et al.*, 1983). In addition other work has shown a decrease in cell surface hydrophobicity of *Acinetobacter baumanni* associated with sub inhibitory concentrations of aminoglycosides and β-lactam antibiotics although this is likely to be as a result of the antibiotics acting upon the outer membrane rather that adaptation of the organism to their presence (Hostacka, 2000). The increased binding of *S. aureus* to both human keratinized nasal epithelial cells and human vulvar epithelial cells at low pH suggests that hydrophobic interactions are involved in the binding (Switalski *et al.*, 1983).

In this work the surface of *S. epidermidis* as the strains underwent adaptation to CHX became more hydrophobic. This suggests an increase surface lipid content.

6.6 Conclusions

The properties associated with resistance to CHX seemed to vary between the strains examined. They include alterations in neutral lipids and phospholipids in whole cell membranes, uptake of CHX and cell surface hydrophobicity. This suggests that resistance is due to alterations in the cell wall and whole cell membranes. These alterations seemed to be specific for each strain adapted in this way. Such alterations bring with them biocide cross-resistance properties unique to each strain, making it impossible to generalize about a general method of resistance to the disinfectant CHX. Although the changes in phospholipid alteration were similar in all three adapted strains it was still difficult to confirm that these changes are the only factors responsible for resistance to CHX in adapted cells.

Chapter: 7 Investigation of membrane damage induced by CHX in S. epidermidis

7.1.Aims

In this chapter the membrane properties of the S. *epidermidis* parental and adapted strains were studied. The aim was to determine the potassium (K^+) leakage in three parental strains (NU11P, NU159P and CAPD12P) and their adapted strains (NU11A, NU159A and CAPD12A) following exposure to different concentrations of chlorhexidine.

7.2 Introduction

Membrane-active antibacterial agents like CHX generally cause an initial rapid loss of low molecular weight compounds from within the cell as a consequence of cytoplasmic membrane injury and breakdown of the permeability barrier (Kroll and Anagnostopoulos, 1981). Gilbert *et al.,* (1977) found that leakage of low molecular weight cytoplasmic constituents could be an indication of a reversible disorganization of the cytoplasmic membrane when the concentrations of antimicrobials were bacteriostatic. At more marked bactericidal concentrations, irreversible membrane damage occurs (Salton, 1951; Hugo and Bloomfield, 1971, Broxton *et al.,* 1983, Johnston *et al.*, 2003).

7.2.1Interaction of CHX with cytoplasmic membranes

The interaction of CHX with cytoplasmic membranes of microorganisms is a well-documented phenomenon (Hugo& Longworth, 1964; Harold *et al.*, 1969; Elferink and Booij, 1973). Measurable effects resulting from this interaction include leakage of material absorbing at 260 nm (Hugo and Longworth, 1964), inhibition of membrane bound ATPase (Harold *et al.*, 1969) and potassium ion ($K^{+)}$ leakage (Elferink and Booij, 1973, Lannigan and Bryan, 1985, Hugo and Russell, 1998). Leakage of potassium is an indication of membrane damage (Lambert and Hammond, 1973). Potassium-sensitive electrode and flame photometry have been used to detect potassium leakage from cells after exposure to antimicrobials (Kroll and Anagnostopoulos, 1981; Heipieper *et al.*, 1991, Johnston *et al.*, 2003).

7.3 Material and methods

7.3.1 Bacterial strains

S. epidermidis strains were three clinical isolates, NU11P, NU159P and CAPD12P and the three strains NU11A, NU159A and CAPD12A adapted to chlorhexidine after serial passage.

7.3.2 Reagent

Chlorhexidine gluconate, 20% w/v stock solution was purchased from (Sigma-Aldrich) (Dorset, UK) and used in appropriate dilutions throughout the experiments when required. The concentration range of chlorhexidine was 2, 4, 8, 16, 32, 64 and 128 μg/ml. This range of chlorhexidine concentrations was selected to show K^+ leakage from the organisms at concentrations of chlorhexidine below, around and above the MIC.

7.3.3 Determination of chlorhexidine MICs

MIC's of CHX six *S. epidermidis* parents and their adapted strains after serial passage were determined. These experiments were carried out in the same manner as described in section 5.2.2.

7.3.4 Preparation of whole cells for testing K^+ leakage

Cultures were grown on the surface of a Mueller Hinton Agar plates (MHA) (Oxoid CM 129, Basinstoke, UK) plate at 37°C for 24 hours. Cells were then suspended in 5 ml of de-ionized water (DIW), washed three times by centrifugation and resuspended in 10ml DIW, to give a bacterial concentration of absorbance 2.0 at 470 nm $\cong 2 \times 10^9$ cfu/ml).

7.3.5 Measurement of K^+ leakage

The leakage of potassium and concentration in the supernatant was then measured using a glass potassium specific ion electrode with internal reference electrode (Cosning ISE /BNC 16900/1). Calibration of the electrode was done according to the instruction manual. The solution was stirred continuously with

a magnetic stirrer. The electrode was placed in the solution of CHX at the chosen concentration in DIW and allowed to equilibrate to give a steady reading (Cosning model 240 PH/MV Meter). This reading was the background level of potassium ions in the CHX solution. Once a steady state had been reached, a 1 ml sample of the organism to be tested was added. Readings were taken at 10-second intervals for 1 min, and then at 2 and 4 min over 30 minutes. Control experiments were made using the 1 ml of organisms added to DIW alone. Results were obtained by subtracting the background K^+ concentration from the readings obtained in the presence of chlorhexidine. Table7:1 shows the calibration readings of the glass electrode.

Table 7.1 Calibration and response of the potassium-selective glass electrode.

Sample	**mV 0 sec.**	**mV 30 sec.**	**mV 60 sec**
DIW	-013	-013	-013
CHX (0.04%)	0.56	0.56	0.56
KCl 100 Mmol/L	190	195	195
KCl 10m Mmol/L	142	142	142
KCl 1m Mmol/L	086	087	087
KCl 0.1m Mmol/L	031	031	131
KCl 0.01 Mmol/L	-014	-014	-014
KCl 0.001 Mmol/L	-062	-062	-063
KCl 0.0001 Mmol/L	-113	-114	-113

7.4 Results

7.4.1 Potassium leakage and bactericidal effects

Potassium leakage resulting from exposure of *S. epidermidis* parents NU11P, NU159P, CAPD12P and their adapted strains NU11A, NU159A and CAPD12A to various concentrations of CHX (8, 16 and 32μg/ml) is shown in Figures 7:1-7:9. The figures show the concentration of K^+ measured in the cell suspension (i.e. the extra cellular K^+ concentration) over time after exposure to chlorhexidine (time 0).

Figure 7:1 Chlorhexidine- induced potassium leakage from NU11P & NU11A.

Cultures were exposed to chlorhexidine at 32μg/ml.

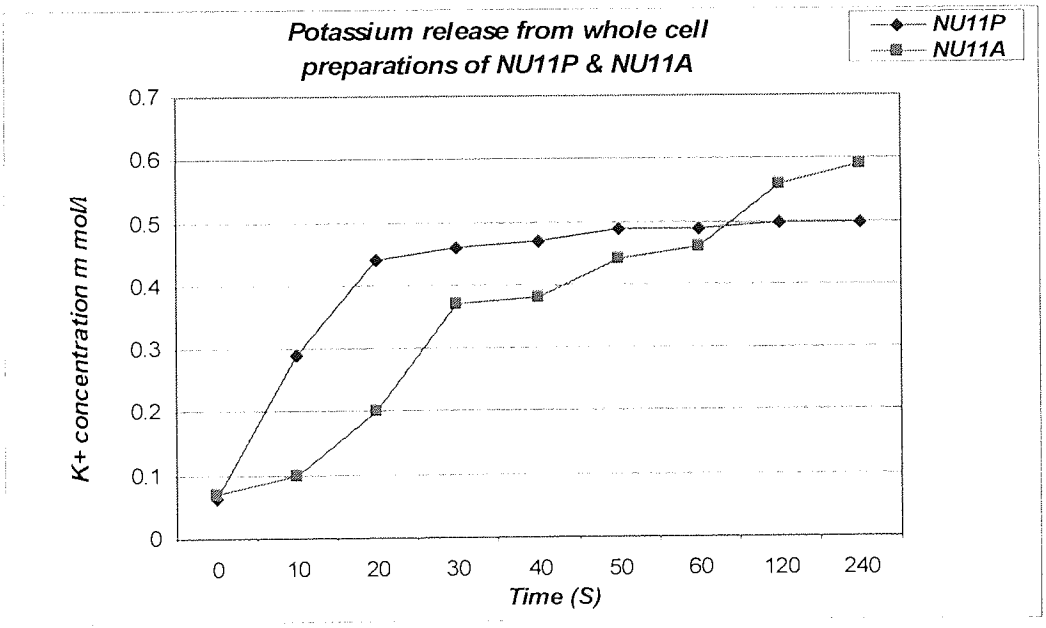


Figure 7:2 Chlorhexidine-induced potassium leakage from NU159P & NU159A. Cultures were exposed to chlorhexidine at 32μg/ml.

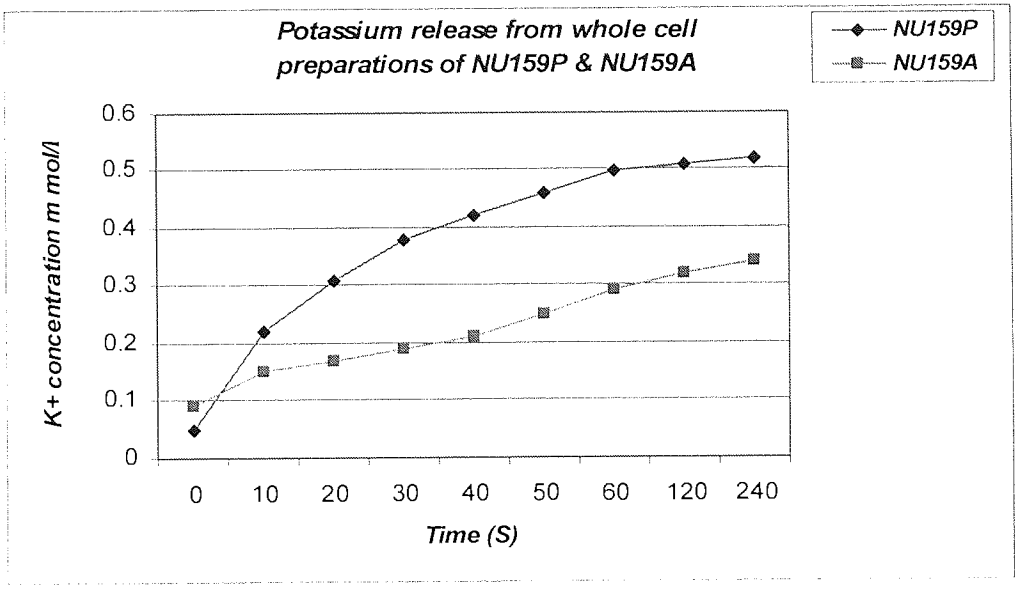


Figure 7:3 Chlorhexidine-induced potassium leakage from CAPD12P & CAPD12A. Cultures were exposed to chlorhexidine at 32μg/ml.

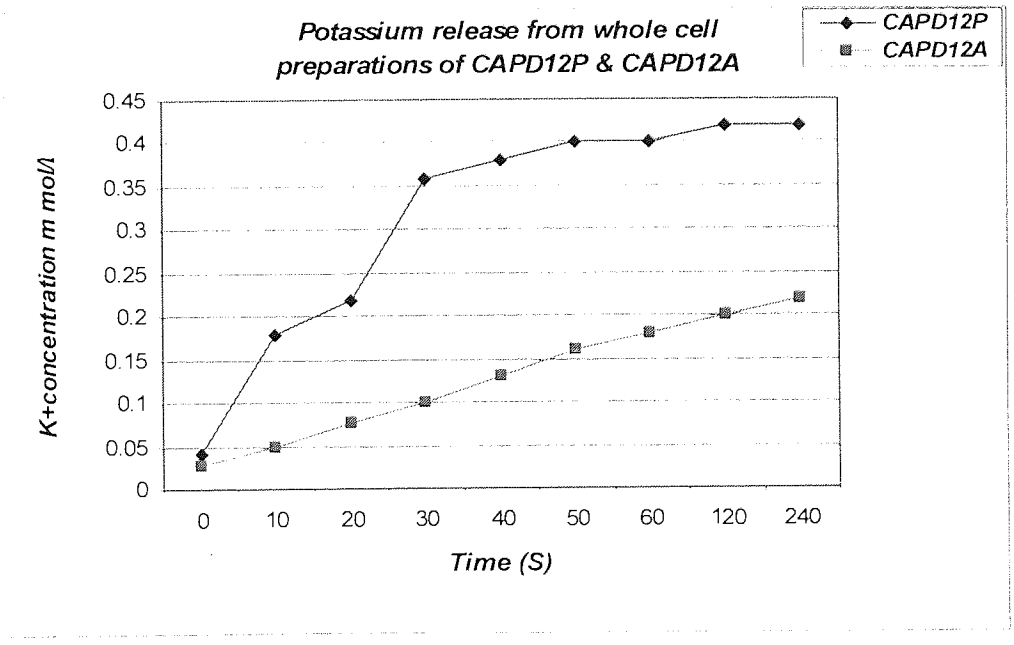


Figure 7:4 Chlorhexidine-induced potassium leakage from NU11P & NU11A. Cultures were exposed to chlorhexidine at 16μg/ml.

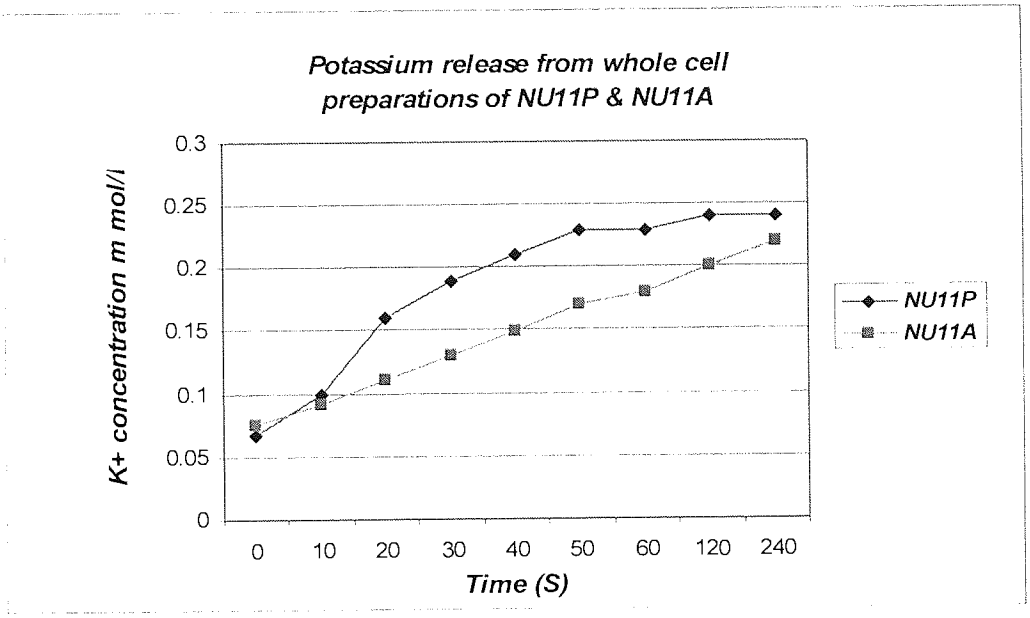


Figure 7:5 Chlorhexidine- induced potassium leakage from NU159P &NU159A. Cultures were exposed to chlorhexidine at 16μg/ml.

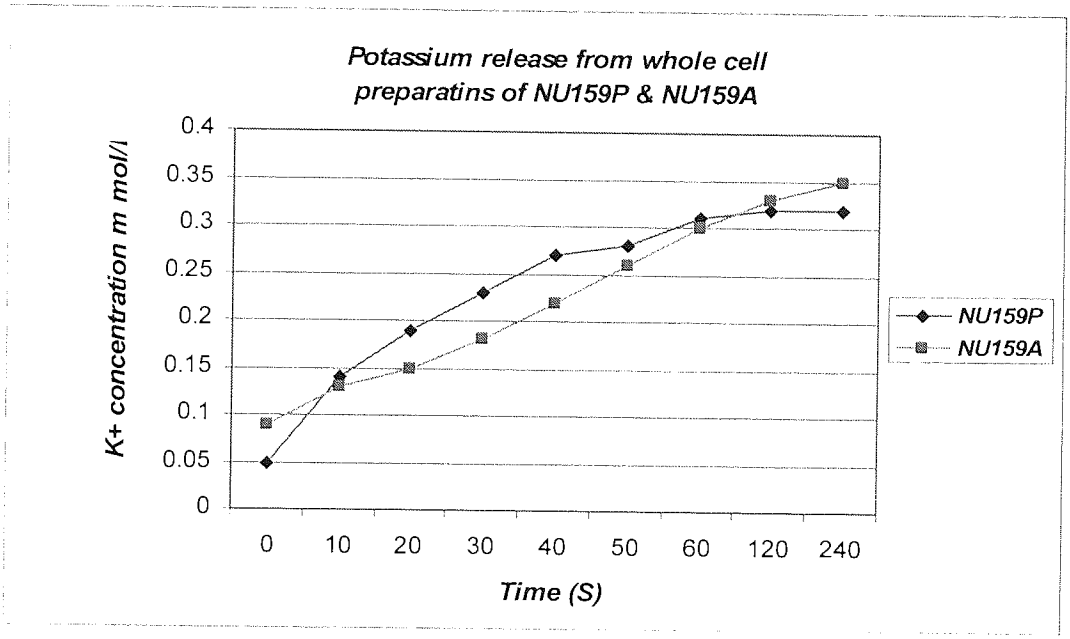


Figure 7:6 Chlorhexidine-induced potassium leakage from CAPD12P & CAPD12A. Cultures were exposed to chlorhexidine at 16μg/ml.

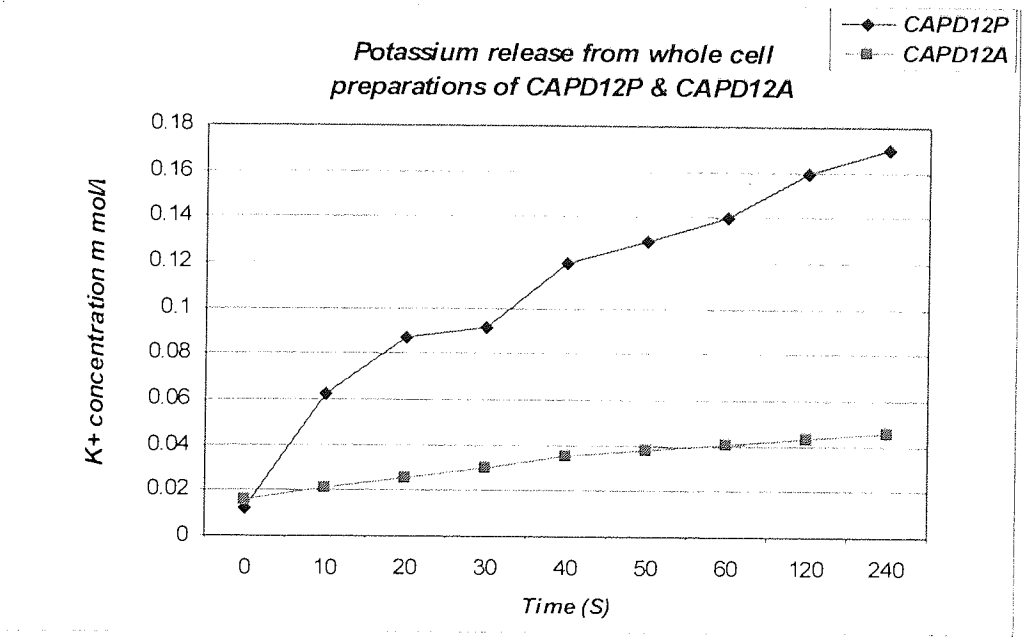


Figure 7:7 Chlorhexidine- induced potassium leakage from NU11P & NU11A.

Cultures were exposed to chlorhexidine at 8μg/ml.

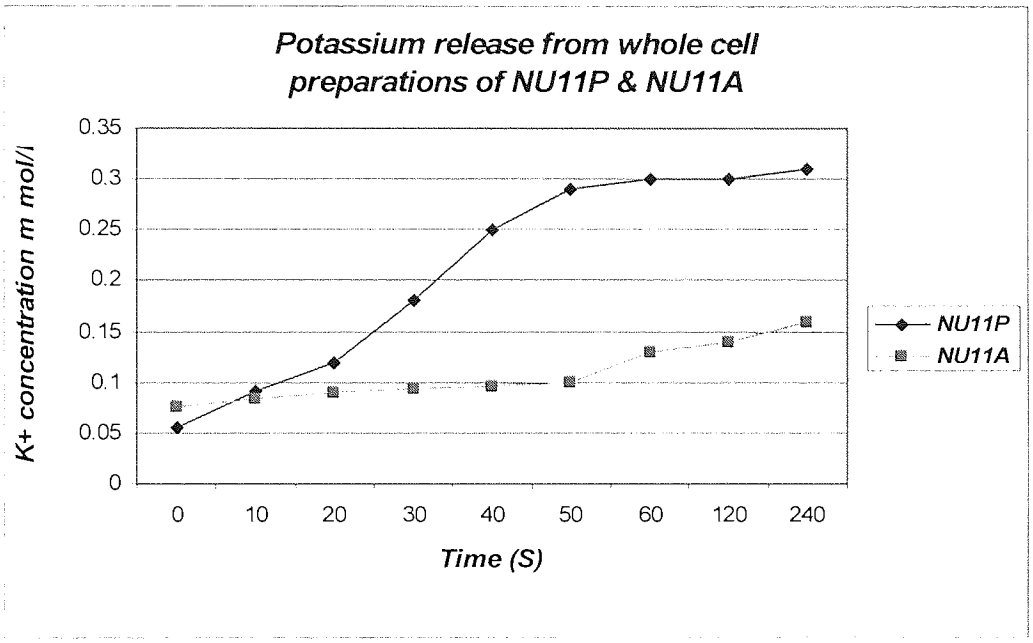


Figure 7:8 Chlorhexidine- induced potassium leakage from NU159P & NU159A.

Cultures were exposed to chlorhexidine at 8μg/ml.

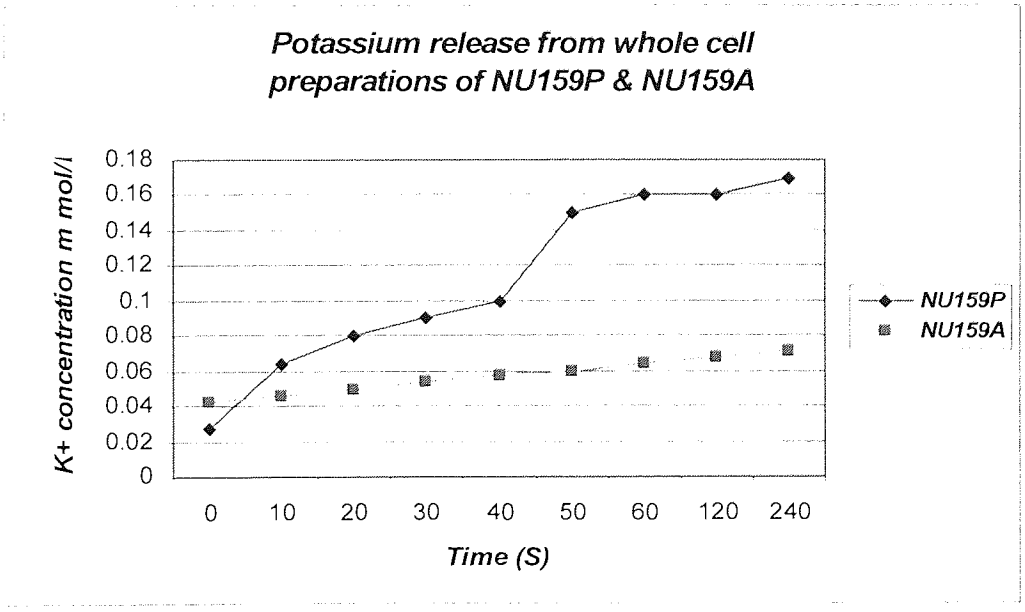
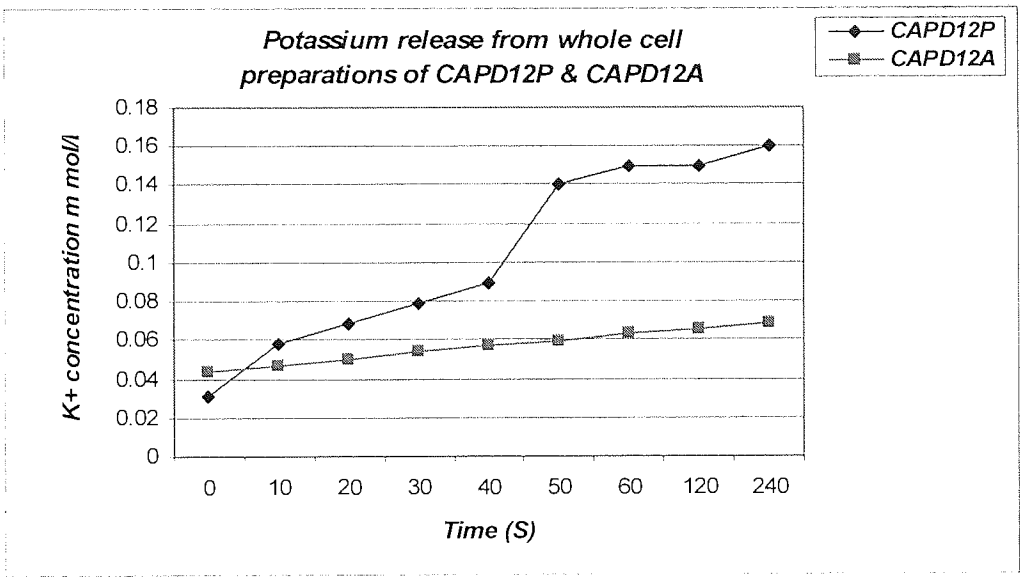


Figure 7:9 Chlorhexidine-induced potassium leakage from CAPD12P & CAPD12A.Cultures were exposed to chlorhexidine at 8μg/ml.



Figures 7:1-7:9 indicate that in treated cells NU11P & NU11A, NU159P & NU159A, CAPD12A & CAPD12A leakage of potassium over time was initially linear, and then the rate decreased and tailed off at a maximum. The leakage rate depended on the concentration of CHX used in the experiment. Leakage of potassium following exposure to chlorhexidine in all three adapted strains NU11A, NU159A & CAPD12A was much slower than in each of the respective parental strains. No leakage was detected in any strain at concentrations of 2 and 4μg/ml. At high concentrations (64 and 128μg/ml) the rate was so rapid that a time curve could not be recorded.

7.5 Discussion and overall comments

The pattern of potassium leakage reflected resistance to CHX in each pair of strains. One explanation could be that the cell wall of each adapted strain did not allow chlorhexidine to penetrate to the underlying cell membrane. Another explanation is that the membrane itself may have become resistant to damage by CHX in each adapted strain.

It is interesting to note the rapidity of the potassium leak, the majority of which occurs within the first sixty seconds of exposure to chlorhexidine. This phenomenon was also noted in yeast cells in which K^+ leakage was found to be almost instantaneous (Elferink and Booili, 1973, Suci and Tyler, 2002).

Harold *et al,* (1969) showed that chlorhexidine inhibited the uptake of K^+ by exchange of Na^+ and H^+, and promoted a stimulus of glycolysis caused by this K^+ uptake. There was no inhibition of ATP formation caused by glycolysis (oxidative phosphorylation). At the concentration of CHX being used (10.8 μg/ml), there was no inhibition of membrane-bound NADH dehydrogenase, neither was the autologous exchange of $^{42}K^+$ for K^+ inhibited. It is significant that chlorhexidine inhibited the solubilized as well as the membrane-bound ATPase.

Addition of chlorhexidine to suspensions of *S. aureus* and *S. epidermidis* cells produces an immediate adsorption of CHX molecules on the bacterial surface. The amount of drug adsorbed depends on the concentration of CHX, the cell density and species of bacterium present and the composition and pH of the suspending medium (Mengistu *et al.,* 1999). Increase in pH of the medium causes an increase in the amount of CHX bound, probably due to an increase in the degree of ionisation of groups on the bacterial surface rather than a change in the ionisation of CHX molecules (Hugo and Longworth, 1964).

The events following adsorption on the surface of the cells depend on the extent of adsorption. At relatively low concentrations of CHX there is a rapid and irreversible loss of cytoplasmic constituents (Rye and Wiseman, 1964). Electron

microscopy reveals the formation of cellular ghosts devoid of cytoplasmic constituents (Hugo and Russell, 1998, Tattawasart *et al.*, 2000).

Increase in the concentration of CHX produces a progressive increase in the amount of drug adsorbed but a decrease in the rate of loss of cytoplasmic constituents. The appearance of cells after treatment with 0.05% CHX solutions is markedly different than those treated with low concentrations. No loss of cytoplasmic constituents being detected (Hugo, 1971; Russell, 1998). The cells remain intact although surface protuberances are detectable and the cytoplasm has a coagulated appearance. The increase in concentration causing increased adsorption and decreased cytoplasmic loss is accompanied by an increase in bactericidal activity. CHX does not interfere with the synthesis of the rigid mucopeptide components of cell walls nor does it cause lysis of isolated bacterial cell walls (Hugo and Longworth, 1966, Hugo, 1971, Russell, 2002).

The report of Tattawasart *et al* (2000) showed that exposure of the chlorhexidine sensitive isolate of *Pseudomonas stutzeri* produced progressive cellular damage, the extent depending on the period of treatment. After exposure of *Pseudomonas stutzeri* cells to CHX for 5 min, numerous small blebs of the outer membrane were seen, with an occasional larger ballooning showing evidence of inner membrane breakage, which resulted in cytoplasmic K^+ leakage.

Whether the potassium leakage is a killing mechanism or simply one measurable effect is in some doubt and depends on the concentration of CHX used in the experiment. The data from Lannigan *et al* (1985) who studied *Serratia marcescens* 303 showed a significant efflux of potassium ions in this strains at a concentration of 6μg/ml of CHX whereas the agar dilution technique showed that the level of susceptibility was between 10 and 20 μg/ml chlorhexidine. This would tend to support the contention that K^+ leakage was not directly related to the killing of the organism but conditions used to measure susceptibility and K^+ leakage were different and may account for this observation. Other authors have noticed a similar dissociation between potassium leakage and death of the organism (Chen, Chou and Feingold, 1978). It seems probable that an early event in the action of CHX in *Serratia marcescens* and possibility other Gram-negative organisms in

contact with the inner membrane which then undergoes permeability changes allowing rapid leakage of intra-cellular potassium. This is however, unlikely to be the complete mode of action (Lannigan and Bryan, 1985).

Authors have suggested that CHX is an inhibitor of membrane ATP-ase in *Streptococcus faecalis* (Harold *et al.*, 1969) and others (Hugo & Longworth, 1964, 1965, Russell and Day, 1993, Tattawasart *et al.*, 2000) on morphological evidence have suggested some action on the cytoplasm causing gelling of intacellular protein.

Chlorhexidine is considered to be a surface-active agent, acting on lipid moieties in the cytoplasmic membrane (Hugo and Longworth, 1964, Russell and Day, 1993, Hugo and Russell, 1998). However, correlation of antibiotic and biocide resistance with lipid content of *S. epidermidis* could be a case of concern. The work of Chang, Molar and Tsang, (1972) has shown that the correlation of antibiotic resistance with lipid content of *Serratia* is poor. A report demonstrating synergism between CHX and polymyxins against *Pseudomonas aeruginosa* (Al- Najjar and Quesnel, 1979, Russell and Day, 1993, Hugo and Russell, 1998, Poole, 2002) suggested that the target molecule for CHX might be a membrane protein rather than a membrane lipid as previously supposed.

In this study no attempt was made to elucidate and clarify the mode of action of CHX further than the potassium leakage effect. The leakage of potassium ion from parental strains of *S. epidermidis* cells NU11P, NU159P and CAPD12P was more rapid than from the adapted cells, NU11A, NU159A and CAPD12A, the majority of which occur within the first sixty seconds of exposure to chlorhexidine.

Results obtained from this chapter provide some insight into a site of action of CHX on the cell membranes of *S. epidermidis* and suggest that the decreased susceptibility of adapted strains to chlorhexidine resulted from changes in the cell membrane. Whether this change is related to lipid (more likely in this study) or protein content or characteristics of the cell membrane or cell wall permeability changes that prevent CHX reaching its target site all may be an avenue for future study.

Chapter 8. Conclusions

Chlorhexidine gluconate is widely used for the management of wound infections, periodontal infection and skin disinfections before surgery. Bacterial resistance to CHX is an increasing problem in staphylococci (Southwood *et al.,* 1996) which have multiple drug resistance to commonly used antimicrobial agents (Cookson, Bolton and Platt, 1991).

Development of resistance by a bacterial cell to biocides such as CHX, which have a multiplicity of cellular target sites, would appear to require an evolutionary solution (Russell and Day, 1993 and Irizarry *et al.,* 1998). By analogy, it is logical to expect to see only small increases in sensitivity, which are not necessarily stable, but not to envisage major changes. (These small increases in resistance might be associated with alterations in the cellular surface. Findings of Fitzgerald *et al* (1992) and Cookson *et al* (1991) confirm this matter. It is also noteworthy that, despite the extensive dental use of CHX, strains of *Strep. mutans* remain sensitive to this important antiseptic (Jarvinen *et al.,* 1993). Furthermore, it has also been demonstrated by Hein and Hambraeus (1992) that the long-term use of a daily scrub with CHX reduced skin colonization with antibiotic-resistant *S. epidermidis.*

Resistance ensues by virtue of intrinsic impermeability mechanisms, biofilm production and possibly even CHX degradation (Ogase, 1992). Resistance does not usually appear to be transferable, and there remains some doubt as to whether organisms can be trained to become highly resistant to the antiseptic (Russell and Day, 1993).

As discussed in chapter five, three out of eleven *S. epidermidis* clinical strains NU11, NU159 and CAPD12 became resistant to CHX after serial passage with 16-fold increase in MIC, while the susceptibility of the other strains treated with CHX did not change. In addition, the CHX resistant phenotype was found to be stable in all three NU11A, NU159A and CAPD12A adapted strains in disinfectant-free media over a period equal to that required to generate the phenotype.

Co-resistance was observed to other disinfectants such as benzalkonium chloride (BKC), triclosan (TLN) and povidone iodine (PI). Such changes differed from strain to strain. In the case of NU11A, NU159A and CAPD12A cells there was no evidence of cross-resistance to antibiotics normally associated with efflux in *S. epidermidis* cells, and so it is unlikely that such a system was affected by passage in CHX.

CHX is a bactericidal antiseptic that disrupts cell membrane functions. The mechanism of resistance of bacteria to CHX has not been well established. Some studies have shown the positive links between resistance to antibiotics and disinfectants (Maris, 1991). In *S. aureus* strains, a single plasmid has been found to be responsible for both disinfectants and antibiotics resistance (Yamamoto, Tamura and Yokota, 1988), while in *S. epidermidis* the equivalent data is poor. Another proposed mechanism of resistance is cell wall permeability changes that prevent CHX reaching its target sites (Tattawasart *et al.*, 2000, Poole, 2002).

In many instances, CHX is incorporated into products (e.g. domestic toiletry products) that have other ingredients, including surfactants and chelators that promote cell damage. These ingredients may also affect resistance by placing additional stress on the bacteria (McDonnell and Russell, 1999).

In this research resistant to CHX was observed. The results, presented in chapter four, indicate that the weight of total lipid and phospholipid content of clinical resistant strains was greater than in the sensitive controls. This might explain the fact that these strains show resistance to CHX. An extra layer of lipid in the cell wall might act as a barrier and prevent CHX penetrating through the bacterial cell wall. On the other hand, *S. epidermidis* has a less complex cell wall than Gram-negative bacteria and it is expected to be more generally sensitive to CHX.

Microorganisms vary in their sensitivity to the action of chemical agents. Some organisms, either because of their resistance to disinfectant or because of their significance in cross-infection or nosocomial infections, merit attention. In this

research experiments showed that it is possible to generate stable adaptive resistance to the disinfectant CHX in clinical *S. epidermidis* strains by serial passage. By contrast the laboratory strains of *S. epidermidis* (ATCC 35984 & NCTC 11047) treated by the same method did not show any changes in MIC after serial passage in CHX.

The properties associated with resistance to CHX seemed to vary between the examined strains. These properties include alterations in fatty acid proportions of whole cell membranes, phospholipid content of cells, uptake of CHX and cell surface hydrophobicity. Alteration in fatty acid and phospholipid content may alter the properties of the cytoplasmic membrane. This could act as a resistance mechanism to CHX which its site of action is the cytoplasmic membrane and explains the reduction in cold resistance in adapted cells to the disinfectant. Proportions of phospholipids in *S. epidermidis* adapted cells have not been widely examined with reference to resistance to CHX or other disinfectants.

In this research the results of surface hydrophobicity of the *S. epidermidis* adapted strains NU11A, NU159A and CAPD12A indicated that as the cells underwent adaptation to CHX they became more hydrophobic.

Determination of potassium leakage ion from parental strains of *S. epidermidis* showed that the leakage was more rapid in parental strains than in respective adapted cells and the majority of leakage occurred within the first sixty seconds of exposure to CHX. These results are also another confirmation of a strong link between cell wall alterations and resistance properties.

The findings throughout the study suggest that resistance is due to alterations in the cell wall and whole cell membranes and increased surface lipid content. These alterations seem to be specific for each strain adapted in this way. Such alterations bring with them co-resistance properties unique to each strain, making it impossible to generalize about a general method of resistance to the disinfectant CHX. Although the changes in phospholipid alteration were similar in all three adapted strains it was still difficult to confirm that these changes were the only factors responsible for resistance to CHX in adapted cells. In other words the changes, which were discussed, could only be said to be

associated with the development of resistance not responsible for the alteration in resistance phenotype observed.

In conclusion, CHX is still an effective antiseptic to suppress or prevent staphylococcal infections in the hospitals. However, there may be a need to monitor the efficacy of the commonly used disinfectants to other bacteria in order to reduce the risk of infection by resistant microorganisms. The increasing rates of drug resistance among bacteria could be a serious threat to the successful antimicrobial therapy. A continuous surveillance of antimicrobial susceptibility patterns and biocide policies is needed to minimize the emergence and spread of resistant pathogenic bacteria.

In summery the effectiveness of CHX as a biocide should be maintained until the mechanisms of resistance and the relative importance of low-level resistance in the environment are better understood. Surveys of the occurrence of resistance to disinfectants in natural settings are needed to determine whether there is a cause for public health concern.

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