Characterisation of clinical isolates of *Staphylococcus aureus* collected from the UK and Malta

By

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Abstract

Staphylococcus aureus (*S. aureus*) and especially methicillin-resistant *S. aureus* (MRSA) have become a major problem in hospital acquired infections worldwide. These organisms may be resistant to multiple numbers and classes of antibiotics making their treatment complicated and challenging. As well as being resistant to antibiotics, *S. aureus* produces numerous pathogenicity factors including toxins and extra-cellular enzymes. Previous investigations into the relationship between antibiotic resistance and toxin production in both MRSA and methicillin sensitive *S. aureus* (MSSA) strains have found small or no differences in toxin production. However, no investigation has examined the production of pathogenic enzymes and their relationship to antibiotic resistance.

A study panel of 680 isolates of *S. aureus* was collected from the UK and Malta. This investigation addressed several critical phenotypic characteristics of this clinically important pathogen. The isolates were tested against several significant antibiotics, analysed for selected pathogenic factors, analysed by Pulse Field Gel Electrophoresis (PFGE) to determine molecular epidemiological distribution and subjected to novel differentiation methods.

The isolates were tested against a range of fourteen antibiotics. Additionally, the production of three enzymes, DNase, lipase and proteinase, and one toxin, haemolysin was determined for each isolate. The level of antibiotic resistance between the isolate groups for both countries was found to be similar. However, some significant differences were seen in resistance levels to certain antibiotics. No differences in the production of pathogenic factors could be detected between the isolates of MRSA and MSSA from both countries. The isolates were also divided into 47 strain types via *SmaI* digestion, with DNA fragments separated by PFGE. Three UK-recognised epidemic MRSA strain profile types were identified and, interestingly, these types were also observed in the Maltese strain panel.

Novel chromogenic substrates for detecting lipase activity were tested against the *S. aureus* strains. Results suggest these substrates would have limited applicability as a diagnostic tool for differentiation between MRSA and MSSA. However, these compounds may have an application as a research tool for the study of *S. aureus*. A further study examined the effect of antimicrobials on the growth of phenotypically resistant organisms. This pilot study of fourteen isolates, showed significant differences in the final cell population and significant decreases in the production of some pathogenic enzymes. The results of this experiment warrant further investigation.

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List of suppliers of materials and reagents

The following reagents were supplied by

BDH Ltd, UK: Acetone, alcohol, boric acid, ethylene-diaminotetraacetic acid (EDTA), glycerol, methanol, sodium chloride and Tris base.

bioMérieux, UK: McFarland standards.

Biorad Ltd, UK: CHEF DNA size standard Lambda ladder and Plug moulds

Cambridge Diagnostic service Ltd, UK: E-test (containing vancomycin)

Fisher Ltd, UK: Carbol fusion, crystal violet, iodine, porcine fibrinogen, Tween 80.

Mast diagnostic Ltd, UK: Amikacin, chloramphenicol, ciprofloxacin, clarithromycin, clindamycin, erythromycin, gentamicin, linezolid, methicillin, penicillin G, rifampicin, teicoplanin and tetracycline

Nunc Ltd, UK: 96-well polystyrene plates

Tesco Ltd, UK: Skimmed milk powder.

The following materials were supplied by

Oxoid Ltd, UK: Brain heart infusion broth, DNase agar, Iso-Sensitest agar, Iso-Sensitest broth, nutrient agar, nutrient broth, phosphate buffer solution, Ringer solution, Staphalase kit and whole sheep blood.

PPR Diagnostics Ltd, London, UK: SRA-propanoate (C3), SRA-bButyrate (C4), SRA-octanoate(C8), SRA-decanoate(C10), SRA-laurate

(C12), SRA-myristate(C14), SB_ZTM-acetate (C2) and SB_ZTM-bButyrate(C4).

Simga Ltd, UK: Bovine serum albumin, Brij 58, ciprofloxacin, clarithromycin, clindamycin, deoxycholate, erythromycin, ethidium bromide, high strength gel agarose, low melting pointing, lysostaphin, penicillin G, Sarkosyl, *SmaI* endo-restriction nuclease, sodium aceate and vancomycin.

List of abbreviations

agr – accessory gene regulator

Aur – aureolysin

BHI - brain heart infusion

BSA - bovine serum albumen

BSAC – British Society for Antimicrobial Chemotherapy

CBP – collagen binding protein

ClfA – clumping factor A

C-MRSA – community acquired-methicillin-resistant *S. aureus*

CSE - chromogeneic *Salmonella* esterase

DFP – diisopropylphosphofluridate

DNase – deoxyribonuclease

EARSS – European Antimicrobial Resistance Surveillance System

EDTA – ethylene-diaminotetraacetic acid

EMRSA – epidemic methicillin-resistant *S. aureus*

ETA – exfoliative toxins A.

ETB – exfoliative toxins B

FAME – fatty acid-modifying enzyme

FBP – fibronectin-binding protein

FCF – fibrinogen-biding protein clumping factor

G+C - guanine cytosine

HPA –Health Protection Agency

LTC- long term care centres

MIC- minimum inhibitory concentration

MRSA – methicillin-resistant *S. aureus*

MSCRAMM – microbial surface components recognising adhesive matrix molecules

MSSA – methicillin sensitive *S. aureus* .

NaCl – sodium chloride

OD - optical density

ORSAB - oxacillin resistance screening agar base

PBS - phosphate buffer solution

PCR - polymerase chain reaction

PFGE - Pulse Field Gel Electrophoresis

PPA – plasmid profile analysis

PT – pyrogenic exotoxin

PV-leukocidin - Panton-Valentine leukocidin

RLFP – restriction fragment length polymorphism

sar –staphylococcal accessory gene regulator

SCC*mec* – staphylococcal cassette chromosome *mec*

ScpA – staphopain

SE – staphylococcal enterotoxines

SFP – *S. aureus* food poisoning

SNP – single nucleotide polymorphisms

SspA – staphylococcal serine protease

SspB – staphylococcal cysteine protease

SSSS – staphylococcal scalded skin syndrome

Stp – staphylococcal Thiol protease

TSS- toxic shock syndrome

TSST1- toxic shock syndrome toxin 1

VRE – vancomycin resistant entercocci

VRSA – Vancomycin resistant *S. aureus*

1.0 Introduction

Staphylococci are Gram-positive cocci about 0.5 to 1.5 μm in diameter, which can occur singly, in pairs or in small irregular grape-like clusters (Murray *et al.*, 1999). These bacteria were first observed and cultured by Pasteur and Koch (Cookson *et al.*, 2003) and initial detailed studies were carried out by Ogston in 1881 and Rosenbach in 1884. Ogston (1881, cited in Cookson *et al.*, 2003) gave the bacteria their genus name *Staphylococcus*, due to his microscope observations of grape-like clusters of the bacteria from samples of pus from human abscesses (Cookson *et al.*, 2003). The first *Staphylococcus* named was *Staphylococcus aureus* (*S. aureus*) because Rosenbach (1884, cited in Cookson *et al.*, 2003) grew isolates of pure culture of the bacteria and noted a yellow to orange pigment appearance within the colonies.

These bacteria are widespread in nature, but can mainly be found on the skin, the skin glands and on the mucous membranes of mammals, birds and humans. Generally these organisms have a benign or symbiotic relationship with their host (Murray *et al.*, 1999).

1.1 Classification

Staphylococci are members of the *Micrococcaceae* family, which also contains micrococci and early work highlighted three species: *Staphylococcus aureus, Staphylococcus saphrophyticus* and *Staphylococcus epidermidis* (Mandell *et al.*, 1979). Early classification of staphylococci was based on morphological characteristics and physiological properties. However, it has since be shown that classification via physiological characteristics such as the formation of acid from carbohydrates, extracellular enzymes and toxin production is limited due to genomic rearrangement and loss of accessory genetic elements (Witte *et al.*, 2000).

Later work with DNA-DNA hybridisation studies highlighted the fact that staphylococci form a defined genus that can be divided into 27 species (Witte *et al.*, 2005). Developments in analysis have allowed the demonstration of a number of differences between staphylococci and micrococci, namely guanine cytosine (G+C) content of DNA (30-50 molarity % in staphylococci, compared with 70-75 molarity % in micrococci) and staphylococcal sensitivity to lysostaphin (Witte *et al.*, 2000).

1.2 Characteristics of staphylococci

Staphylococci are catalase-positive, non motile, non-flagellated, non-sporing organisms (Wilson *et al.*, 1955). They are Gram-positive cocci, with an average diameter of 1.0μm; however, size varies both between species and the age of the culture and the medium they were grown on (Wilson *et al.*, 1955).

1.3 Culture characteristics

Staphylococci are not fastidious and grow easily on nutrient agar or in nutrient broth within 1 to 2 days at 37°C. Addition of blood and glucose to the medium can slightly increase the growth of staphylococci, however, the addition of serum has no benefits (Wilson *et al.*, 1955). These organisms are facultative anaerobes which grow best in the presence of oxygen. On agar plates in the presence of oxygen they form thick, raised, pigmented, convex colonies (Wilson *et al.*, 1955). However, anaerobic growth results in flat, non-pigmented colonies that tend to spread out over the surface of the media (Wilson *et al.*, 1955). Staphylococci can grow over a wide range of temperatures, varying from 12°C to 45°C. However, the optimum temperature for growth is 37°C (Wilson *et al.*, 1955). As well as growing on nutrient agar, they can be grown on selective agar, since staphylococci are able to tolerate salt (sodium chloride) at concentrations of 5-10% and bile salts (Sleigh *et al.*; 1998). Another selective media that is used in the detection of staphylococci, especially *S. aureus* in faeces, milk, food and other sources, is Baird-Parker agar containing 5% egg yolk tellurite. Colonies on these plates usually appear shiny, black and convex, about 1-5mm in diameter with a halo around the biomass.

Staphylococci are pigment producing bacteria. *S. aureus* forms golden colonies, *Staphylococcus citreus* produces lemon-yellow colonies and *Staphylococcus epidermidis* develops porcelain-white coloured colonies. Pigment development is dependent on a number of factors, such as optimum temperature which is 22°C, age of culture and presence of oxygen (Wilson *et al.*, 1955). Pigment in cultures incubated at 37°C can sometimes deepen in tint when left at room temperature. The age of the colony also affects pigment production. For example, fresh cultures are rich in colour however pigment can be lost overtime (Wilson *et al.*, 1955). Oxygen is required for the pigment development in staphylococci. For example, in anaerobic conditions they appear colourless. Movement of these cultures into aerobic conditions however, will result in pigment development (Wilson *et al.*, 1955).

1.4 Biochemical reactions

Staphylococci are able to ferment a variety of sugars including galactose, glucose, lactose, maltose, mannitol, sucrose and xylose (Brückner *et al.*, 2000) although this varies according to the species (Wilson *et al.*, 1955). This variety in sugar fermentation means that it is not possible to classify all staphylococci using this method.

1.5 *S. aureus* identification

S. aureus can be differentiated from other staphylococci by a number of biochemical tests, including extra-cellular and cell-wall bound protein A, cell-bound clumping factors, extra-cellular coagulase and extra-cellular DNase (Witte, 2000). Most strains of *S. aureus* produce protein A which can agglutinate normal human serum. Clumping factors can provide a rapid means of identifying *S. aureus*, however 12% of strains (including MRSA) are negative for its production and, in addition, these factors can be hidden by large capsules. DNase can be detected using commercially available DNase agar (Oxoid Ltd) in which the test isolates are spotted on to the agar and grown for 24 hours at 37°C, then the plates are flooded with hydrochloric acid, a zone of clearing in the agar denotes DNase activity.

Currently the "Gold standard" method for identification of coagulase is the tube coagulase test with rabbit serum which provides a result in 4 hours. More rapid tests are commercially available kits, such as the Staphylase kit (Oxoid, DR595A) that can give results in approximately 30 seconds. This kit detects the presence of coagulase through its action with fibrinogen-sensitised sheep red blood cells (Anon 1).

1.6 *S. aureus* genome

To date seven complete *S .aureus* genomes have bee sequenced, the first to be sequenced was NCTC 08325 which is a reference laboratory strain. The genomic map of NCTC 08325 (phage group III) can be seen in Figure 1.1, this map was constructed using fragmentation data from four restriction endonucleases and from this map a number of resistance and virulence genes can be identified (see Table 1.1). The remaining six strains of *S. aureus* that have been sequenced are all clinical isolates; COL was an early strain of MRSA at appeared in the 1960s (Gill *et al.*, 2005), N315 and Mu50 are hospital-acquired MRSA strains, with Mu50 having decreased susceptibility to vancomycin (Kuroda *et al.*,

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2001). Furthermore MRSA 252, a hospital acquired EMRSA-16 strain, which is responsible for approximately 50% of the UK MRSA infection (Holden *et al.*, 2001). The complete genome for two community-acquired *S. aureus* have also been determined, MW2, which is an MRSA (Baba *et al.* 2002) and MSSA 476 (Holden *et al.*, 2001). The sequenced genomes range in size from 2.8 to 2.9 Mb and overall have a conserved structure with the major of gene exhibiting a high level of DNA identity (>97%) (Lindsay *et al.*, 2006).

Figure 1.1. Physical and genetic map if the genome of *S. aureus* NCTC 08325 (phage group III) (Iandolo 2000).

Table 1.1: Shows the precision of a number of antibiotic resistance and virulence factor genes within the genome of *S. aureus* NCTC 08325 (phage group III) (Iandolo 2000).

Gene	Precision
Resistant	
Beta lactamases (*bla-pen*)	F
Fluoroquinilone (*flqA*)	A
Quinolone (*flqB*)	D
Fusidic acid (*fus* A_7)	D
Novobiocin (*nov*)	G
Rifamapin (*rif*)	D
Methicillin (*mecA*)	G
Tetracycline (*tet*)	E
Virulence factors	
Coagulase (*coa*)	E
Clumping factor (*clfA*)	B
Collagen adhesion (*can*)	G
Glycerol ester hydrolas (geh^e)	E
Staphylokinase (sak^F)	F
Pathogenicity island 1 containing TSST (*SaPI 1*)	A
Pathogenicity island 2 containing TSST (*SaPI 2*)	A
Fibrinogen-binding protein (*fib*)	B
Fibronectin-binding protein A&B (*fmbA/B*)	C

The *S. aureus* genome has a core region comprising of approximately 75% of the whole genome and consists of genes present in all strains. Genetic diversity in the core genome that can be detected between strains of *S. aureus* occurs in a number of ways and is most often due to single nucleotide polymorphisms (SNP). The effects of these SNP depends on is position; however many SNP's result in phenotypically silent change ie. not changing a amino acid, although when a SNP does change an amino acid this creates a mutant allele (Lindat *et al.* 2006). An example of a SNP that results in a phenotypic change is fusidic acid resistance (Lindat *et al.* 2006).

1.6.1 Mobile genetic elements

The variable components of the *S. aureus* genome that contain genes that are of non-essential functions, include genes for extra virulence factors, plus resistance to antibiotics and metals. Many of these genes are contained on mobile genetic elements which can be transferred between strains via bacteriophage, *S. aureus* pathogenicity islands (SaPI), staphylococcal cassette chromosomes (SCC), plasmids and transposons. Bacteriophage can carry for example, virulence factors including enterotoxin A (*sea*), exfoliatiove toxin (*eta*) (Yamaguchi *et al.*, 2000) and PV-leukocidin (*lukSF*-PV) (Kaneko *et al.*, 1998) to name but a few. *S. aureus* pathogenicity islands are large mobile elements that can carry a number of virulence genes together, including genes for superantigens, TSST-1 and enterotxins B and C (Lindsay *et al.*, 2006), currently two of these islands have been identified. Staphylococcal cassette chromosomes normal carry genes for resistance to antibiotics, for example SCC*mec* carries the gene for methicillin resistance (*mecA*). Currently four different SCC*mec* have been discovered SCC*mec* type I-III are found in hospital-acquired MRSA strains and SCC*mec* type IV is found in community-acquired MRSA (Ito *et* al., 2001; Hiramatsu *et al.*, 2002). There are three classes of plasmids found in *S. aureus*, these range in size with class I being the smallest and class III being the largest and these plasmids also often carry genes for antibiotic resistance (Lindsay *et al.*, 2006). Transposons often encode genes for resistance, for example Tn*554* denotes erythromycin resistance which is often contain within SCC*mec* type II (Lindsay *et al.*, 2006).

1.6.2 SOS response

The transfer of some of these mobile genetic elements can be promoted by the SOS response. The SOS response is activated due to damage to the DNA of a cell and/or interference with cell viability, this can happen due to environmental agents such as radiation (*i.e.* UV light) and certain chemicals, other factors such as antibiotic have been show to induce this response (Phillips *et al.*, 1987; Lewin 2004; Ubeda *et al.*, 2005; Maiques *et al.*, 2006). The SOS response promotes replication and expression of many genes whose function include repair, but other genes such as virulence factors can be up regulated. Induction of virulence factors due to antibiotic exposure was first noted in *Escherichia coli* (Phillips *et al.*, 1987). SOS responses have also been noted in *S. aureus*, this can lead to the up regulation of virulence genes, thereby potentially increasing the severity of the infection. Additionally it has been noted that horizontal transfer of virulence

factor, such as SaPI and resistance genes can occur during a SOS response (Ubeda *et al.*, 2005; Maiques *et al.*, 2006).

1.7 Pathogenic factors produced by *S. aureus*

S. aureus produces a large number of virulence factors which can be divided into two areas: surface-associated factors and secreted factors that can further be divided into toxins and enzymes. These factors help in the adhesion of the organism to the host cell, in immune system evasion, invasion into deeper tissue and tissue destruction (Arvidson, 1983). Many of these factors have been known for sometime, with a lot of work being carried out in the 1960s -1970s (Arvidson, 1983). More recently new factors have been discovered, such as six new proteases (Reed *et al.*, 2001). The understanding of known pathogenic factors has also improved, such as with proteinase, DNase, and lipase (McGavin *et al.*, 1997, Rao *et al.*, 2002, Rosenstein *et al.*, 2000). The roles of the various pathogenic factors are detailed in the following sections.

1.7.1 Surface-associated factors

For an organism to infect a host, it must first adhere to the surface of the host. Adherence to the host cell is a multi-step process and initial adhesion depends upon a number of diverse components such as gravitation, chemotaxis, Van der Waals force, electrostatic force and surface tension (Jones *et al.*, 1983). Following this initial adherence specific processes that involve bacterial cell surface structures called "Adhesins" and complementary receptors on the surface of the susceptible cells, anchor the bacterium to host cells (Koneman, 1997). The adherence of *S. aureus* cells is mediated by protein adhesins belonging to the Microbial Surface Components Recognising Adhesive Matrix Molecules (MSCRAMM) family (Foster *et al.*, 1998). These proteins are in most cases covalently anchored to the cell wall. Currently, there are 3 types of MSCRAMM recognised in *S. aureus*: fibronectin-binding protein A (FBP), collagen binding protein (CBP) and fibrinogen-binding protein clumping factor (FCF) (Foster *et al.*, 1998).

Fibronectin-binding protein (FBP) was first identified and characterised by Espersen and Clemmensen in 1982 and is a very common property of *S. aureus* binding (Foster *et al.*, 1998). Most strains express two related forms: FBPA and FBPB, which are encoded by two closely linked genes. The primary ligand-binding domain (D) of FBPs binds to the amino terminus of fibronectin (Foster *et al.*, 1998). The D domain is located

close to the cell-wall spanning domain and consists of 3-5 repeats of less than 40 amino acids. The amino-acid terminus of fibronectin also comprises of repeated motifs. Once bound, the D-domain of FBPs undergoes a structural change. These epitopes are called ligand-induced binding sites. Interestingly the antibodies of the immune system recognise FBPs only once they are bound, therefore this important pathogenic factor can not be blocked by the immune system (Foster *et al.*, 1998).

A *S. aureus*-specific collagen binding protein (CBP) was first identified by Patti in 1993. CBP adheres to collagen substrates in collagenous tissues and is required for *S. aureus* binding to human cartilage (Patti *et al.*, 1993). Unlike FBPs that appear in most *S. aureus* strains, CBP is expressed in 38% to 56% of strains (Foster *et al.*, 1998). Structural CBP consists of two domains, A and B. The A-domain contains the collagen-binding site. It is connected to the cell wall-spanning region by the B-domain that consists of repeats of 187-amino acids. The number of B-domain repeats, vary from between 1-4 in different strains. The importance of the B-domain is as yet unclear (Foster *et. al.*, 1998).

Fibrinogen-binding protein clumping factor (FCF) adheres to fibrinogen and fibrinogen-containing substrates. It was first described by Much in 1908 and called clumping factor A (ClfA). Recently Edhin *et al.*, (1998) described a second form the ClfB. This new form of FCF only appears when bacteria cells are grown aerobically until the early exponential phase, whereas ClfA is present in all stages of bacteria growth. Currently it remains unclear why *S. aureus* produces two forms of FCF. The two forms have been shown to recognise different parts of the host ligand and might act synergistically to allow the bacteria to attach more firmly to thrombi in the blood stream. Structurally FCF consists of two domains, A and R. The A-domain contains the fibrinogen-binding site. Which is connected to the cell wall-spanning region by the R-domain that consists of largely Asp-Ser dipeptide repeats (Foster *et al.*, 1998).

Apart from specific binding proteins, teichoic acid and its derivative, lipoteichoic acid, are also important adhesions. Teichoic acid mediates the binding of *S. aureus* to fibronectin especially in mucosal surfaces such as nasal epithelial cells. The lipoteichoic acid mediates the binding of *S. aureus* to host cell such as red blood cells and buccal epithelial cells. Structural teichoic acid is an octamer of ribitol phosphate, where the sugar moieties are coupled through D-alanine and *N*-acetyl-glucosamine (Foster *et al.*, 1998).

1.7.2 Secreted factor 1 -Toxins

The main role toxins play in the pathogenicity of *S. aureus* infection is to inactivate the host immune system. This is achieved through direct cytotoxic effect on the cells or by promoting cytokine release via superantigen-mediated action. Damage caused to the host cell through a direct cytotoxic effect also causes the release of macromolecules that are subsequently degraded by enzymes such as DNase, Lipase and Proteinase (Arvidson, 1983). *S. aureus* toxins can be divided into three categories:

- Cytotoxins
- Enterotoxins
- Epidermolytic toxins

1.7.2.1 *S. aureus* cytotoxins

S. aureus cells may produce five different cytotoxic molecules: four different haemolysins (alpha, beta, delta and gamma) and Panton-Valentine leukocidin (PV-leukocidin) (Arvidson, 2000). The main role of these toxins is to disrupt or lyse the cell membrane of host tissue. These various toxins were discovered during the early to mid 20th century. Haemolysins predominately affect erythrocytes and PV-leukocidin has also been shown to affect these cells. However PV-leukocidin has also been shown to affect the different cells of the immune system and can be both dermonecrotic and neurotoxic (Dinges *et al.*, 2000).

Alpha haemolysin affects a wide range of mammalian cells, particularly rabbit erythrocytes. Its effect on human erythrocytes is, in contrast, very low. The effect on rabbit erythrocytes is 1000 times more potent than the effect seen on human erythrocytes (Bhakdi *et al.*, 1991). The toxin is encoded on the *hla* gene, but unlike other factors its regulation is not controlled by the accessory gene regulator (*agr*) gene (Wadström, 1983). Ohlsen *et al.*, (1997) showed that environmental factors such as temperature, osmolarity and carbon dioxide concentration affect the production of α-haemolysin. The toxin works by integrating into the host cell membrane and forming a pore of 1 to 2 nm in diameter. This pore subsequently leads to an influx of sodium and calcium ions into the cell, causing osmotic swelling and rupturing of the cell (Fluit *et al.*, 2003). The α-haemolysin also has effects on other cells including lymphocytes, fibroblasts and endothelial cells. The pores formed in these cells result in arachidonic acid metabolism, leading to the production of thromboxane and prostacyclin, resulting in vasoconstriction (Fluit *et al.*, 2003).

Beta haemolysin was first described in 1935 by Glenny and Stevens. Like α-haemolysin, it is highly haemolytic but does not affect rabbit erythrocytes (Wadström, 1983). It was also noted that the toxin activity was enhanced when first incubated at 37°C and then below 10°C, leading to being called "Hot-Cold" haemolysin (Dinges *et al.*, 2000). Although β-toxin is produced in a large number of *S. aureus* strains, its production is particularly seen in about 88% of animal isolates, compared with ~11-45% of human strains. Its role in disease however, remains unclear (Dinges *et al.*, 2000).

Delta haemolysin is unique, as it is small, heat stable, has surfactant active properties and can affect erythrocytes from a broad spectrum of species including humans (Wadström, 1983). The toxin is produced by about 97% of *S. aureus* strains and is encoded by the *hld* gene. The toxin not only affects the cell membrane but also the sub-cellular structures such as membrane-bound organelles and bacterial protoplasts. Delta haemolysin has also dermonecrotic activity (Dinges *et al.*, 2000).

Gamma-haemolysin and PV-leukocidin are two types of bicomponent toxins. Each toxin is made up of two non associated secreted proteins referred to as slow- and fast-luting proteins (S and F). PV-leukocidin has two encoded components, one S (LukS-PV) and one F (LukF-PV). Gamma-haemolysin has two encoded S components (HlgA and HlgC) and one F component (HlgB) (Dinges *et al.*, 2000). Therefore there are six possible forms of γ-haemolysin or PV-leukocidin that can be formed. γ-haemolysin is produced by 100% of *S. aureus* strains whereas PV-leukocidin is produced by 2 to 3% of strains. The toxins affect neutrophils and macrophages and γ-haemolysin also lyses erythrocytes (Dinges *et al.*, 2000).

1.7.2.2 *S. aureus* enterotoxins (SEs)

Staphylococcal enterotoxins (SEs) belong to a large group of pyrogenic exotoxins (PT) that are found in both staphylococcal and streptococcal isolates. PT share a common phylo-genetic relationship, structure, function and sequence homology. SEs have been implicated as the causative agent of staphylococcal induced food poisoning, toxic shock syndrome (TSS) and several allergic and autoimmune diseases (Balaban *et al.*, 2000). Currently 9 different SEs have been identified. SEA-SEE and SEG-SEJ and toxic shock syndrome toxin-1 (TSST-1, previously named SEF) and are secreted during the post-exponential growth phase (Bergdoll, 1983). Each toxin is a single protein chain with a

molecular weight between 26000 to 30000 Da which is synthesised as a precursor protein. It contains a terminal signal sequence that is cleaved during export from the cell. In order to belong to the large group of PT, SEs must exhibit at least three biological properties (Balaban *et al.*, 2000):

- Pyrogenicity
- Superantigenicity
- Capacity to enhance the lethality of endotoxin in rabbits up to 100,000-fold

In addition to having similar biological properties, they also share genetic and structural similarities. Toxin genes are either carried on plasmids, bacteriophages or on a pathogencity island (Balaban *et al.*, 2000). Their regulation is controlled by at least three global regulatory systems: *agr*, staphylococcal accessory gene regulator (*sar*) and a catabolite repression system (Balaban *et al.*, 2000). Both *agr and* sar are regulators for genes of many *S. aureus* pathogenic factors, however the catabolite repression system seam only to control enterotoxins. In the presence of sugar such as glucose and pyruvate, the production of enterotoxin such as B are repressed, this system has also been noted in other ~Gram positive and negative bacteria like *Bacillus, Streptococcus* and *E.coli* (Morse *et al.*, 1973; *Saier et al.*, 1996). Structurally the toxins are heat stable and resistant to proteolytic enzymes such as trypsin, chymotrysin, rennin and papain. They are also stable over a wide range of pH (pH 3-10). There are two main diseases that result from the effects of SEs: Food poisoning and toxic shock syndrome (Balaban *et al.*, 2000).

The symptoms of *S. aureus* food poisoning (SFP) include nausea, vomiting, abdominal pain and diarrhoea and in rare cases signs of systemic toxicity are seen, giving rise to fever and hypotension. SE-induced SFP can be defined by a set of histological abnormalities, inflammation of several areas of the gastrointestinal tract, with the most severe lesion observed in the stomach and upper part of the small intestine. Symptoms normally occur rapidly after ingestion of food, within 1 to 6 hours and last from between 24 to 48 hours (Balaban *et al.*, 2000).

1.7.2.2.1 *S. aureus* toxic shock syndrome

Toxic shock syndrome (TSS) was first recognised in 1978 by Todd *et al.* (Cited in Bergdoll, 1983). It is involved in an acute and potentially life threatening illness. The illness is normally associated with women during the use of feminine hygiene products but it has also been associated with abscesses and wound infections. Signs and symptoms of

TSS include a high fever (>38.9°C), hypotension, dizziness and diffuse palmar erythroderma, followed by desquamation of the skin on the hands and feet, vomiting, diarrhoea, impaired hepatic and renal function and cardioplumonary dysfunction (Dinges *et al.*, 2000)

1.7.2.2.2 Epidermolytic toxin

Epidermolytic toxin or exofoliative toxin is the causative agent of staphylococcal scalded skin syndrome (SSSS). SSSS affects neonates and appears as widespread erythema, sometimes intense, followed by loosening skin which peels in places leaving extensive raw areas. There are at least two serotypes of the toxin, exofoliative toxins A and B (ETA and ETB) (Bohach *et al.*, 2000). Both of these toxins act by separating the cells, causing the formation of fluid filled gaps between the cells. These subsequently cleave along the horizontal plane leading to the formation of intra-epidermal cleft, with the disappearance of small vesicles normally present in this space (Bohach *et al.*, 2000).

1.7.3 Secreted factors 2 - Enzymes

S. aureus produces a large number of enzymes. Their role in pathogenicity includes host cell evasion, metastasis of the organism from the site of infection into deep tissue and throughout the body and nutrient acquisition, through the degradation of macro-molecular to micro-molecular (i.e. DNA to nucleic acids).

1.7.3.1 Proteinase

Early work, carried out in the 1960s, suggested that most strains of *S. aureus* were proteolytic (Baird-Parker, 1965, cited in Arvidson, 1983). Although work carried in the 1970s showed that at least 3 different types of proteinases are produced by *S. aureus:* staphylococcal serine-protease (Darpeau *et al.*, 1972, cited in Arvidson., 1983), staphylococcal metallo-protease (Arvidson *et al.*, 1972 cited in Arvidson, 1983) and staphylococcal thiol-protease (Arvidson *et al.*, 1973 cited in Arvidson, 1983).

Staphylococcal serine-protease (SspA) or V8 protease was first described in 1972 by Darpeau *et al.*, (1972), who demonstrated that the enzyme was inhibited by diisopropylphosphofluridate (DFP), suggesting that the enzyme contained a serine residue in its active site and hence called a "serine-protease". SspA was shown at the time to be

produced by 67% of *S. aureus* strains, during the post-exponential growth phase (Arvidson, 1983). Its production is stimulated by a number of substrates: Casein, peptides, glycin, serine and theronine, all of which SspA can hydrolyse. SspA is produced as an inactive precursor which is activated by a second proteinase, namely staphylococcal metallo-protease. In mutant strains where staphylococcal metallo-protease is not produced, the inactive SspA precursor accumulates in culture fluid (Arvidson, 1983). SspA cleaves specifically the peptide bonds on the carboxyl terminal side of either glutamic acid or aspartic acid residues at pH7.8 whereas only glutamyol bonds at pH4.0 (Arvidson, 1983).

McGavin *et al.*, (1997) showed that SspA played an important role in the spread of the infection from the initial site into deeper tissue. McGavin's group suggested that SspA was able to digest the binding domain of FBP, which has a glutamic acid residue that is essential for fibronectin binding. SspA also plays an important role in the maturing of another protease namely Staphylococcal cysteine protease (SspB). In addition, SspA plays a role in evasion of host defence where it has been shown to cleave the heavy chain of all human immunoglobulin classes (Prokesova *et al.*, 1992)

A study by Reed *et al.*, (2001) identified a unique operon (*spl*) that encodes six serine protease-like proteins. Although the precise action of these enzymes is unknown, the study showed that in mutants where the operon was missing, no difference in the pathology was seen. However this does not necessarily mean that these enzymes do not play a role in the pathologic process, their mode of action may not have been detected in this study.

Staphylococcal-metallo-protease or Aureolysin (Aur) was first described in 1972 by Arvidson, who demonstrated that the enzyme was inhibited by ethylene-diaminotetraacetic acid (EDTA) (Arvidson, 1983). Aureolysin cleaves the precursors of SspA and it is therefore assumed that it is produced in a similar number of *S. aureus* strains as SspA (67%). Aur cleaves peptide bonds by involving the amino terminal side of hydrophobic residues at a neutral pH. Aur also plays a role in evasion of host defences. It has been shown to modulate immunologic reaction, affecting the stimulation of both T and B lymphocytes and also inhibiting immunoglobulin production (Prokesova *et al.*, 1992). In human plasma Aur can also activate prothrombin which has led to the speculation that it may have a role in disseminated intravascular coagulation that is seen in some cases of systemic infection (Wegrzynowicz *et al.*, 1980). Aur may also aid in the colonisation of healthy carriers of *S. aureus*. Lindsay *et al.*, (1999) suggested that Aur was able to degrade

toxins such as α-haemolysin, thereby down regulating the virulence of the bacteria and aiding in its colonisation of the skin and nares.

Staphylococcal thiol (cysteine)-protease (Stp) was first described in 1973 by Arvidson and it was classified as a thiol-enyzme because it was only active in the presence of reducing agents and was inhibited by Hg^{2+}, Ag^{2+} and Zn^{2+} (Arvidson, 1983). Currently three forms of Stp have been described, but only two have been named. The first named staphylococcal cysteine protease (SspB) is released as a pre-enzyme and is matured by SspA. It is also encoded in an operon contiguous to the gene for SspA (Shaw *et al.*, 2004). Although the role of SspB is unknown it is suggested that it may play a role in the break down of FBP (Rice *et al.*, 2001). The second named Stp is the Staphopain (ScpA) also released as an inactive pre-enzyme, although it is unclear whether it is auto activated or whether it is matured by a second enzyme. ScpA has elastinolytic properties, leading to the speculation that the enzyme could participate in tissue invasion and tissue destruction observed in staphylococcal ulceration (Potempa *et al.*, 1988).

In the last few years there has been an increase in the understanding of the role that proteases play in the pathology of staphylococcal infection and the number of proteases produced by *S. aureus*. However there are still unresolved questions and these issues require further study.

1.7.3.2 Nuclease\DNase

First described in 1956 by Cunningham, the production of extra cellular deoxyribonuclease (DNase) has been shown in almost 100% of *S. aureus* strains and 20% of coagulase-negative staphylococci. The enzyme consists of a single peptide chain of 149 amino acid residues that contain no sulphydryl group or disulphide bonds. Early work suggested that DNase production appears to be parallel to growth but later work showed an increase at the end of the exponential growth phase. Its production can be inhibited by the sugars glucose, maltose and glycerol. DNase hydrolyses DNA to yield predominantly 3'-mono- and di-nucleotides (Arvidson 1983).

The exact role played by DNase in *S. aureus* pathogenesis is unknown at present. A recent study by Rao *et al.*, (2002) highlighted the outbreak of EMRSA-15 in which the strains did not produce DNase. In this study it was suggested that these strains were

clinically significant and may have the same epidemic potential as their DNase positive counter parts (Rao *et al.*, 2002).

1.7.3.3 Lipase & Fatty Acid-Modifying Enzymes (FAME)

Lipase is active against a number of natural and synthetic lipid substrates and water-soluble triglycerides and Tweens. These substrates are broken down to their constituent alcohol and fatty acids by ester linkage hydrolysis (Arvidson, 1983). To date, five different staphylococcal lipase genes from different species have been cloned and sequenced, two from *S. aureus*, two from *Staphylococcus epidermidis* and one from *Staphylococcus hyicus* (Rosenstein *et al.*, 2000).

Of the two lipases present in *S. aureus*, only one is described as a true lipase that is able to hydrolyse water-insoluble long-chain triglycerols as well as water-soluble triglycerols. The second enzyme can only hydrolyse water-soluble short chain triglycerols and should be referred to as a short-chain glycerol ester hydrolyase (Arvidson, 2000). Short-chain glycerol ester hydrolyases are produced by most strains of *S. aureus* whereas true lipases are thought to be less common. Both of the enzymes are released as pro-enzymes, as with protease, in order to prevent damaging the producing cell (Arvidson, 1983). The pro-enzymes are then matured extracellularly by an extracellular protease. The pathogenic role played by lipase during a *S. aureus* infection remains unclear; however, it has been shown that lipase is produced during infection and appears to interfere with phagocytosis (Arvidson, 2000). Free fatty acids produced by the hydrolysis of lipids are also known to impair the immune system (Arvidson, 2000). However, long-chain free fatty acids are bacteriocidal. Most strains of *S. aureus* produce Fatty Acid-Modifying Enzymes (FAME) which inactivate the bactericidal activity of long-chain free fatty acids by catalyzing the esterification to alcohols or cholesterol. In murine models it has been shown that FAME producing *S. aureus* strains are more pathogenic (Mortensen *et al.*, 1992).

1.7.3.4 Coagulase

Coagulase is a vitally important enzyme in the differentiation of *S. aureus* strains from other staphylococci. Coagulase can bind with human plasma on a 1:1 basis, forming a substance called staphylothrombin which in turn can convert fibrinogen to fibrin. Staphylothrombin activates prothrobin in a non-proteolytic manner, unlike the normal

physiological processes where it is proteolytically cleaved by prothrobin to form thrombin. It is still not understood how this process is achieved by *S. aureus*.

The role of coagulase in pathogenesis is still unknown. One suggestion is that in the early stages of the infection it forms a fibrin clot around the bacteria thereby protecting it from host defence mechanisms. However in experiments using mutants where coagulase was missing, there was no impairment of virulence, leading to the suggestion that coagulase may only be important in some types of infection.

As with DNase, a recent report by Olver *et al.*, (2004) highlighted a case of EMRSA-15, that was tube-coagulase negative. The tube coagulase test is the "gold standard test" for coagulase activation. Although rare, as with DNase negative isolates, this is a development of concern, especially as coagulase is the principal test for the definition for *S. aureus* strains.

1.7.3.5 Hyaluronidase

Extracellular hyaluronidase, also known as spreading factor, aids the spread of the organism through connective tissue by depolymerising hyaluronic acid, the ground substance responsible for cell-to-cell adhesion (Arvidson, 2000). The action of the enzyme involves the elimination of one molecule of water from the uronic acid portion of the repeating unit. It has been shown that nearly all coagulase-positive staphylococci (between 95-100%) produce this enzyme (Arvidson, 2000).

In summary *S. aureus* produces a large range of different virulence factors that aid the organism in establishing an infection, through the action of adherence factors. Then once established, factors including the extra-cellular toxin and enzymes, aid the organism in evasion and destruction of the host defence system, destruction of local tissue, breakdown of macro-molecule to micro-molecule and invasion into deeper tissue.

1.8 Diseases caused by *S. aureus*

S. aureus infections are commonly characterised by intense suppurative inflammation of tissue which often leads to abscess formation in the infected area. The most common staphylococcal skin infection is the furuncle or boil. In some cases, the furuncle can spread into deeper subcutaneous tissue resulting in one or more abscesses

called carbuncles. Although less common these days, they are still seen in people suffering from diabetes mellitus. Other skin infection include styes, an infection of the eyelid, were the sebaceous gland becomes swollen, red and tender. Pyogenic paronychia is another infection that forms alongside the nail fold, which becomes red, swollen and tender and produces a small amount of pus below the nail. This is a self limiting infection lasting about 5 to 8 days. (Mandell *et al.,* 1979) Another common illness is self-limited food poisoning, with an approximate duration of 24 hours (Cookson *et al.*, 2003).

Some *S. aureus* strains can cause bullous impetigo. This is a highly contagious superficial skin infection on the face and limbs that is characterised by large blisters which form and burst. It is often seen in infants and children, where direct contact can occur. Extensive impetigo can also be associated with HIV sufferers (Cookson *et al.*, 2003).

S. aureus can also cause more serious infections such as nosocomial wound infection, normally post-surgery. This infection can lead to a variety of deep tissue infections including: bacteraemia, septicemia, osteomyelitis, bacterial arthritis, endocarditis, toxic shock syndrome and cerebral, pulmonary, renal and breast abscesses. *S. aureus* can cause pneumonia, although this is almost always a secondary infection, the primary infection is commonly influenza or another viral infection (Cookson *et al.*, 2003).

Some aspects of *S. aureus* infection (e.g. food poisoning) can affect healthy individuals however the most at risk groups of contracting a more serious infection are immuno-compromised patients. Other risk factors include patients with impaired cellular immunity (e.g. diabetes mellitus or renal failure), post-operative patients and those with open wounds. Moreover, due to the ability of *S. aureus* to form biofilms on artificial devices, patients with catheters or other invasive devices are also at risk (Murray *et al.*, 1999).

1.9 Carriage of *S. aureus*

S. aureus can colonise various sites on the skin and the mucosa membrane of humans as well as several animal species. The most frequent site in the human body where *S. aureus* colonises is the nares. Other sites include the skin, perineum and pharynx and less common sites include the gastrointestinal tract and the vagina (Wertheim *et al.*, 2005).

The reported carriage rate of *S. aureus* varies in different studies, but in the healthy adult population approximately 20% are persistent carriers, 60% are intermittent carriers and 20% are non-carriers (Klytmans *et al.*, 1997). Carriage rates also vary with age. In the first eight weeks of life, approximately 45% of infants carry the organism; however, by six months this figure has decreased to 21% (Klytmans *et al.*, 1997).

Nasal carriage of *S. aureus* is one of the major risk factors associated with contracting a staphylococcal disease (Peacock *et al.*, 2001). Approximately 80% of nosocomial *S. aureus* infections are caused by the patient's own *S. aureus* cells that were present on their skin before admission to hospital (Wertheim *et al.*, 2005). Recent studies have highlighted a threefold increase in the risk of acquiring nosocomial *S. aureus* bacteraemia in patients who are nasal carriers of the organism compared with non-carriers (Wertheim *et al.*, 2004). These patients can act as a reservoir which can then lead to transmission of the infection to other patients via the hands of health care workers (McBryde *et al.*, 2004). This route of transmission was noted in the early part of the 1980's by Thompsom *et al* (1982) who reported the primary route of transmission of MRSA in hospitals appeared to be from the carriage of organism from one patient, to another via the hands of hospital workers. The most effective way to reduce the transmission of *S. aureus* via the hands of health care workers is through the adherence to proper hand hygiene *ie.* hand washing and/or use of alcohol-based products (Boyce *et al.* 2002).

However, other possible routes of infection include hospital fabrics. Neely *et al.*, (2000) demonstrated that *S. aureus* could survive on fabrics including cotton, cotton-polyester blend, polyester and polyethylene for a period of 1 to 90 days. This could possibly lead to these fabrics acting as reservoir for the spread of *S. aureus* to the patients. Bebbington *et al.*, (2003) showed that *S. aureus* could be isolated from patients' medical-notes. From a sample group of two hundred and twenty-eight sets of medical notes, *S. aureus* was positively identified on nine sets. All of these surfaces can act as possible route by which *S. aureus* cells may be transmitted to patients via health care professionals.

1.10 History of *S. aureus* infection and development of methicillin-resistant *S. aureus*

Before the use of the first antibiotic in the 1940s, *S. aureus* caused numerous nosocomial wound infections and led to high morbidity and mortality levels. The discovery of penicillin dramatically changed this situation, since for the first time *S. aureus* infection

could easily be treated. This resulted in a large reduction in the mortality rates due to *S. aureus* infections. However it was not long before *S. aureus* strains resistant to penicillin started to emerge, and by 1948 about 60% of *S. aureus* present in the UK were resistant to penicillin (Cookson *et al.*, 2003). To combat this problem, new semi-synthetic penicillin-like antibiotics as well as other classes of antimicrobials were developed including methicillin, cephalosporins, monobactams and carbapenems.

Methicillin was introduced in 1959, however in 1965, only six years later the first case of Methicillin Resistant *S. aureus* (MRSA) was reported at the Royal Prince Alfred Hospital in Sydney Australia (Rountree *et al.*, 1968, cited in Givney *et al.*, 1998). By the 1980s MRSA started to emerge as a major clinical problem in hospitals worldwide (Murray *et al.*, 1999). Methicillin resistance differed from other types of resistance mechanisms in two ways. Methicillin resistance gave the isolates of *S. aureus* resistance to all beta-lactams. This differed from other types of resistance where the uptake of a resistant gene only donated resistance to one or two antibiotics and not a whole class of antibiotics. For example if an organism was resistant to aminoglycosides such as amikacin, it would still be sensitive to other aminoglycosides like gentamicin. The second difference was that MRSA isolates were generally more resistant to other classes of antibiotics than their methicillin sensitive counterparts, therefore making them a bigger problem.

1.11 The current picture of the problem posed by *S. aureus* and MRSA

Today, nosocomial *S. aureus* and MRSA infections are once again an increasing problem in hospitals worldwide. In the UK during the years from 1991 to the year 2000 the number of cases of *S. aureus* rose from approximately 5000 cases to 11,000 respectively (see Figure 1.2). The percentage of these cases that were due to MRSA rose from 2 to 42% respectively, an increase of 40% over 10 years (Anon 2, 2002).

Figure 1.2. Shows the rise in of the number of cases of *S. aureus* and the percentage of those cases that were due to MRSA of a ten year period form 1991 to 2000 (Anon 2, 2002).

In 2004 the European Antimicrobial Resistance Surveillance System (EARSS) annual report stated that Malta, Great Britain, Ireland, Italy, Greece, Israel, France, Portugal and Hungary all had levels of MRSA over 30%, with Malta being the highest at 56% and the UK fourth highest at 44%, whereas countries including Sweden, Denmark, Netherlands, Finland and Iceland had MRSA levels of less than 1% (Anon 3, 2004).

1.12 Problems posed by *S. aureus* infection

The issues posed by this organism are many. The number of cases of both *S. aureus* and the percentage of those cases that are due to MRSA are increasing, even though in recent years in the UK the percentage of cases due to MRSA have leveled out to about 42% (Anon 2, 2002). The second issue is the continuing development of resistance to new antibiotics. As new antibiotics have been developed and cleared for medical use, *S. aureus* has often very quickly become resistant to them. This resistance has normally developed within about half a decade of the antibiotic being licensed. Although there is still not, at the moment, a single strain of *S. aureus* that is resistant to all antibiotics, there are a number of different strains that if combined could be totally resistant to all antibiotics. In addition, nosocomial infections often result in long hospital stays and increased treatment costs, since new and often more expensive drugs are needed to treat these resistant organisms.

1.13 Multiple antibiotic resistant *S. aureus*

Isolates of *S. aureus* are not just resistant to β-lactam antibiotics, but are potentially resistant to multiple classes of antibiotics. As with β-lactam antibiotics resistance, resistance to other antibiotic classes developed shortly after the introduction of the drug. For example, erythromycin was first clinically used in 1952 (McGuire *et al.*, 1952, cited in Morrissey *et al.* 2003), however in 1956, just four years after its introduction, the first case of resistance was reported (Chabbert, 1956, cited in Morrissey *et al.* 2003).

Resistance to newly developed antibiotics is a reoccurring trend in *S. aureus* isolates. A current example includes resistance to glycopeptides, such as vancomycin, teicoplanin and linezolid. In 1996 the first clinical case of an MRSA with reduced susceptibly to vancomycin emerged in Japan (Hiramatsu *et al.*, 1997). Other cases have subsequently been reported in Asia, Europe and USA (Anon 4, 2002). The first case reported in Britain was in May, 2002 (Anon 4, 2002). In all of these cases the minimum inhibitory concentration (MIC) of vancomycin was between 4-32μg/ml and none of the isolates were found to contain the *vanA* gene that encodes for vancomycin resistance in entercococci. In 2002 however, a vancomycin resistant–*S. aureus* (VRSA) was isolated in USA that had a MIC above 32μg/ml and contained the *vanA* gene (Anon 5, 2002). Although at the moment cases are very rare world-wide, VRSA potentially poses a greater problem clinically in hospitals than MRSA. Currently, vancomycin is the main drug of choice for the treatment of MRSA, as it still remains an effective treatment for this organism. Resistance to vancomycin removes this possible treatment, therefore reducing the number of available antibiotics that can be used against an organism that is often multi-drug resistant. In 2001 the first clinical case involving resistance to linezolid was reported in the USA (Tsiodras *et al.*, 2001). Although cases of linezolid resistance are rare, it is still of concern.

Some strains of MRSA as well as being resistant to multiple antibiotics also appear to be well adapted to the hospital environment (Enright *et al.*, 2002). These organisms can often be isolated in numerous hospitals within a country. Additionally, some strains appear to have spread internationally; these strains are referred to as epidemic MRSA (EMRSA) (Enright *et al.*, 2002). In the UK EMRSA first appeared during the 1980s. During the subsequent 6-month period, thirteen additional EMRSA strains were identified (EMRSA-2 to -14) (Aucken *et al.*, 2002). In the early 1990s two additionally strains were identified, EMRSA -15 and -16, with both now being widespread in the UK (Aucken *et al.*, 2002).

Between 1993 and the first quarter of 1997, there was a significant rise in the number of hospitals with caseS of EMRSA -15 and -16. In 1993 approximately 20 hospitals had reported cases of these two EMRSA strains, whereas by the first quarter of 1997 approximately 145 hospitals had cases of EMRSA-15 and approximately 125 hospitals had cases of EMRSA-16 in the UK (Anon 9, 1997). Since this time both EMRSA-15 and -16 became widespread through hospitals in the UK (Aucken *et al.*, 2002).

1.14 Modern evolution of *S. aureus*

During a similar time period to the emergence of VRSA, an apparently novel strain of MRSA emerged, that appeared to have heightened pathogenicity. This new strain, known as Community acquired-MRSA (C-MRSA) has been reported outside the hospital environment (Naimi *et al.*, 2001). This is converse to the established situation where MRSA has really only been observed in hospitals and long term care centres (LTC). Four different cases of children have been highlighted dying from C-MRSA without associated risk factor (hospitalisation, hospital visitation or LTC visitation) (Anon 6, 1999). Although in these cases C-MRSA resulted in death, C-MRSA shows resistance to fewer antimicrobials than the hospital strains of MRSA. To date resistance has only been reported to the benzyl-penicillin class of agents (Baba *et al.*, 2002). The emergence of C-MRSA is thought to be due to two possible mechanisms: either the movement of nosocomial MRSA into the community or the transfer of genetic material from methicillin resistant Gram positive organisms to *S. aureus* (Bukharie *et al.*, 2001). Genetic studies of C-MRSA have shown that the staphylococcal cassette chromosome *mec* (*SCCmec*) that containing the *mec A* gene that encodes methicillin resistance in C-MRSA is smaller, more than half the size of the smallest *SCCmec* found in nosocomial MRSA (Hiramatsu *et al.*, 2002).

C-MRSA poses a potentially greater problem than nosocomial MRSA and VRSA due to a number of factors. Although VRSA is potentially more life-threatening, due to limited possible treatment, the genetic element of vancomycin resistance is unstable and can be lost, whereas the methicillin resistance in C-MRSA appears stable. In addition C-MRSA has infected people that have been considered not at risk *i.e.*, patients who are not hospitalised or in LTC. This therefore presents a problem in controlling the spread of infection. With nosocomial MRSA, the spread of the organism can be prevented because it is in a semi-enclosed environment. As C-MRSA affects people in the community, the traditional methods of controlling the spread of the infection are lost, since it is occurring

in a more dynamic environment. Finally, although at the moment C-MRSA is only resistant to the benzyl-penicillin agents there is the possibility that C-MRSA will develop resistance to other agents therefore decreasing possible treatments.

1.15 Aims of the present study

Research into staphylococci and more importantly *S. aureus* has been carried out over a number of decades. However with regard to an evolving pathogen, findings from work carried out a decade ago may not mirror the same organism in modern times. The majority of work focusing on the pathogenic factors produced by *S. aureus* was carried out in the 1960-1970s, at which time resistance to antibiotics was less common. Current research focusing on *S. aureus* covers a number of different fields including development of new antibiotics, investigation of emerging antibiotic resistance (for example VRSA), rise and spread of MRSA, evolution of *S. aureus* (*e.g.* C-MRSA) and pathogenicity of *S. aureus*.

The area of research for the present study is the pathogenicity of *S. aureus* and MRSA. Current work in this area has focused on the relationship between the production of the pathogenic toxins and antibiotic resistance (Coia *et al.*, 1992; Schmizt *et al.*, 1997). To date, this work has shown no major relationship between the production of toxins and the level of antibiotic resistance. The aim of the present study was to focus on the production of pathogenic enzymes and antibiotic resistance in *S. aureus*, as well as to grow isolates under antibiotic pressure. Thus, the following aims were:

1. The determination of an antibiotic sensitivity profile for each isolate of *S. aureus* in a collection of *S. aureus* strains from a variety of sources, using accredited methods.

2. The determination of the production of three pathogenic enzymes (DNase, lipase and proteinase) and the production of one toxin (haemolysin) by the isolates within the collection. Additionally employing novel chromogenic substrates to examine of the activity of staphylococcal lipase.

3. The sub-species typing of *S. aureus* strains within the collection by Pulse Field Gel Electrophoresis (PFGE).

4. Investigate the potential relationship between antibiogram profiles and pathogenic factor production in clinical isolates of *S. aureus* and MRSA.

2.0 Materials and Methods

2.1 Laboratory test

2.1.1 Bacterial strains

Isolates of *S. aureus* (n=705) were collected from three UK based hospital sources: the Royal Brompton hospital, London; Kingston Hospital, Kingston-upon-Thames and the Royal Marsden hospital, London and one hospital in Malta, St. Luke's Hospital. The Royal Brompton hospital is a specialist cardiac and respiratory hospital. Kingston hospital is a general hospital and the Royal Marsden hospital is a specialist cancer hospital. St. Luke's hospital in Malta is a general hospital. All samples were stored at the hospitals on nutrient agar slopes. Due to lack of information from the hospitals it was not possible to determine if more then one sample was from the same patient.

Isolates were transported to Kingston University either on nutrient agar slopes or as swabs in transport medium (see Table 2.1. for when sample arrived at Kingston University).

Table 2.1: Shows the data and sample number range of when isolates arrived at Kingston university from St. Luke's hospital, Malta.

Sample No. range	Hospital	Date of arrival at Kingston university
1-41	Royal Brompton	July/2001
42-71	Royal Brompton	November/2001
72-101	Kingston	April/2002
102-200	Kingston	November/2002
201-382	Kingston	December/2002

Sample No. range	Hospital	Date of arrival at Kingston university
M1-35	St. Luke's	Feberary/2002
M36-85	St. Luke's	August/2002
M86-160	St. Luke's	August /2001
M161-255	St. Luke's	September/2002
M254-323	St. Luke's	October/2002

Upon arrival, all isolates were transferred to nutrient agar and then stored on nutrient agar slopes, at room temperature, until used. All isolates were also transferred to 1ml aliquot of Brain-heart infusion broth, containing 15% glycerol and frozen at -80°C, for long term storage. The following *S. aureus* strains were used as controls during the experiment: MRSA NCTC 12493 (Control 1) a methicillin resistant strain of *S. aureus* and Oxford *S.*

aureus NCTC 06571 (Control 2) a strain that is sensitive to all antibiotics (Health Protection Agency, UK).

2.1.2. Resuscitation of isolates prior to use (frozen and slopes cultures)

Cultures were remove from the -80°C (maximum of ten at a time) and using aseptic techniques a swab was take on the inside of the tube and swabbed onto one half of a nutrient agar plate. Plates were incubated overnight at 37°C.

Isolates were cultured from slopes by aseptically transferring a small amount of bacteria onto one half of a nutrient agar plate. Plates were incubated overnight at 37°C.

2.1.3 Initial Isolate Identification

A total of 705 isolates were collected (382 UK and 323 Maltese). All samples were Gram stained using a standard method. Briefly a single colony was suspended in a drop of water on a glass slide and air dried. After drying, the slide was fixed by passing through a flame. The slide was then stained, first with crystal violet and then iodine, de-stained with alcohol and counter-stained with carbol fusion and viewed under a 1000X oil-immersion magnification.

The samples were also tested for coagulase activity using a Staphalase kit . Briefly, a single colony was placed on each circle of the test card (six circles in total). Then a single drop of either test reagent or control reagent was added to the opposite circle. The reagents and colony were then mixed and the card was rotated for 30 seconds and coagulation was noted. All tests were carried out in triplicate.

2.1.4 Antibiogram testing

A panel of 14 different antibiotics was tested, 13 using standard operating procedures for disc diffusion assay defined by the British Society for Antimicrobial Chemotherapy (Anon 8, 2002) and 1 (for vancomycin) using a methodology proposed by Hubert *et al.*, (1999). The 13 antibiotics tested using BSAC methodology were, 30μg amikacin, 10μg chloramphenicol, 1μg ciprofloxacin, 2μg clarithromycin, 2μg clindamycin, 5μg erythromycin, 10μg gentamicin, 10μg linezolid, 5μg methicillin, 1 unit penicillin G, 2μg rifampicin, 30μg teicoplanin and 10μg tetracycline (antibiotic concentration/disc). Hubert. *et al.*, (1999) proposed the use of vancomycin at a concentration of 5μg/ml.

Overnight cultures were suspended in Ringers solution to a turbidity of 0.5 McFarland ($1.5X10^8$ cfu/ml) and swabbed evenly in 3 directions on two plates of Iso-Sensitest agar and on one quarter of Mueller-Hinton agar supplemented with 2% sodium chloride (NaCl). The antibiotic disks were dispensed onto the Iso-Sensitest agar using a 6-chambered disk dispenser (Plate 1: amikacin, chloramphenicol, ciprofloxacin, clarithromycin, clindamycin, erythromycin; Plate 2: gentamicin, linezolid, penicillin G, rifampicin, teicoplanin, tetracycline). The methicillin disks were dispensed aseptically using forceps onto the Mueller-Hinton plates. The Iso-Sensitest plates were then incubated at 35°C for 20 hours and the Mueller-Hinton plates were incubated at 30°C for 20 hours. After incubation the diameter of the zone of clearing around the disks was measured. All tests were carried out in duplicate.

2.1.5 Vancomycin susceptibility testing

Vancomycin susceptibility was tested using the method previously described by Hubert *et al.*, (1999). Briefly, overnight cultures were suspended into Ringers solution to a turbidity of 2 McFarland ($6.0X10^8$ cfu/ml) and swabbed evenly in 3 directions on Mueller-Hinton agar containing 2% NaCl and 5μg/ml of vancomycin. Plates were incubated at 35°C for 48 hours and at the end of the time period they were checked for growth. All tests were carried out in duplicate.

If growth was present on the vancomycin plates, the minimum inhibitory concentration (MIC) of the isolate was determined using the E-test method. Overnight cultures were suspended in Ringers solution to a turbidity of 2 McFarland ($6.0X10^8$ cfu/ml) and swabbed evenly in 3 directions on Mueller-Hinton agar with 2% NaCl and allowed to dry for 15 minutes. One E-test strip containing vancomycin was applied to each plate and then the plates were incubated at 35°C and examined after 18 and 48 hours. All tests were carried out in duplicate.

2.1.6 Breakpoint testing

Micro-dilution plates were prepared with the stated antibiotics in a range of concentrations comprising a quarter and a half of the MIC value, the stated MIC value and double and four times that stated minimum inhibitory concentration (MIC) value for any given antibiotic. The antibiotics were prepared in sterile distilled water using guideline

MIC values as advised by BSAC, which take into count correction for activity. The stated MIC advised by BSAC were as follows: ciprofloxacin (2mg/L), clarithromycin (1mg/L), erythromycin (1mg/L), gentamicin (2mg/L) and tetracycline (2mg/L) (Anon 8, 2002). A template of the micro-dilution plates can be seen in Figure 2.1. The plates were prepared as follows, 50µl of double strength Iso-Sensitest broth was added to wells B1 through to F12, G1-G6 and H1-H6. Antibiotic was added to double strength Iso-Sensitest broth at four times the MIC, i.e., for 2mg/L tetracycline MIC a concentration of 8mg/L would be used. 100µl aliquots of this broth were added to wells A1-A12. Aliquots (50µl) of broth from row A was transferred to row B and mixed, this process was continued to row E. Finally, 50µl of broth was removed from well E and discarded. The same procedure was carried out in well G1-G5 and H1-H5. Once the plates had been prepared, they were frozen at -20°C until used (Rosenblatt *et al.*, 1979).

Plates were left at room temperature to thaw before use. Then the overnight cultures were suspended in Ringers solution to a turbidly of 0.5 McFarland ($1.5X10^8$ cfu/ml). 50µl of suspension were added to well A-through to F. All samples were carried out in duplicate. The control isolates, MSRA NCTC12493 (control 1) and Oxford *S. aureus* NCTC06571 (control 2) were added to either row G or H as showed in Figure 2.1. All plates were incubated at 37°C for 24 hours and were read using a spectrophotometer at 540nm (Dynatech MR5000).

	1	2	3	4	5	6	7	8	9	10	11	12
A	4	4	4	4	4	4	4	4	4	4	4	4
B	2	2	2	2	2	2	2	2	2	2	2	2
C	1	1	1	1	1	1	1	1	1	1	1	1
D	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
E	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
F	0	0	0	0	0	0	0	0	0	0	0	0
G	4	2	1	0.5	0.25	0	Control 1		X	X	X	X
H	4	2	1	0.5	0.25	0	Control 2		X	X	B	B

X: Empty wells, B:Blanks

Figure 2.1: Plate template for the breakpoint of clarithromycin with a MIC of 1 mg/L.

Row C of the template shows the position of the MIC concentration of the antibiotic. Rows A and B contain four times and twice times the MIC of the antibiotic and Rows D and E

contain half and a quarter the concentration of the antibiotic. Row F contains no antibiotic and is used to confirm growth of test isolates. In Rows G and H the concentration pattern runs from column 1 to 6, with column 3 containing the MIC. MRSA (control 1) is placed in Row G and Oxford *S.* aureus (control 2) in Row H. Wells H11 and 12 are used as sample with no antibiotic as controls. Values within the figure represent the antibiotic concentration in mg/L.

2.1.7 DNase detection

Overnight cultures were suspended in Ringers solution (Oxoid Ltd, UK) to a turbidity of 0.5 McFarland ($1.5X10^8$ cfu/ml) and 10µl aliquots were dispensed onto one quarter of a DNase agar plate. Plates were incubated at 37°C for 18 hours. After incubation the plates were flooded with 1M HCl and after one minute the presence or absence of a zone of opalescent around colonies was recorded. Radius zone of opalescent was measured from the edge of the colony to the edge of the zone using a standard ruler.

2.1.8 Haemolysis detection

The assay was carried out on nutrient agar plates overlaid with 5ml nutrient agar supplemented with 5% whole sheep blood. Overnight cultures were suspended in Ringers solution to a turbidity of 0.5 McFarland ($1.5X10^8$ cfu/ml) and 10µl aliquots were dispensed into one quarter of the plate. Plates were incubated at 37°C for 24 hours. After incubation the presence or absence of haemolysis under or around the colony was recorded. Radius zone of clearing was measured from the edge of the colony to the edge of the zone using a standard ruler.

2.1.9 Lipase detection

The assay was carried out on nutrient agar plates supplemented with 1% Tween 80. Overnight cultures were suspended in Ringers solution to a turbidity of 0.5 McFarland ($1.5X10^8$ cfu/ml) and 10µl aliquots were dispensed into one quarter of the plate. Plates were incubated at 37°C for 48 hours. After incubation the presence or absence of opalescent zones was recorded. Radius zone of opalescent was measured from the edge of the colony to the edge of the zone using a standard ruler.

2.1.10 Proteinase detection

The assay was carried out on nutrient agar plates overlaid with 5ml of nutrient agar supplemented with 5% skimmed milk. Overnight cultures were suspended into Ringers solution to a turbidity of 0.5 McFarland ($1.5X10^8$ cfu/ml) and 10µl aliquots were dispensed into one quarter of the plate. Plates were incubated at 37°C for 18 hours. After incubation the presence or absence of a zone of clearing was recorded. Radius zone of clearing was measured from the edge of the colony to the edge of the zone using a standard ruler.

2.1.11 Adherence to polystyrene 96-well plates

Three colonies of an overnight culture grown on nutrient agar were transferred to 5ml nutrient broth and grown for 15 hours at 37°C. This suspension was standardised to a given optical density (OD) reading absorbance of 0.6 OD units at 620nm as described by Genevaux (1996). Aliquots of each culture (20µl) and 200µl of Nutrient broth were placed in the wells of a polystyrene microtiter plate in triplicate. Control isolates (in triplicate) MRSA (NCTC 12493) and Oxford *S. aureus* (NCTC 06571) were dispensed on each plate. One set of wells (three wells) was left blank as a control with 220µl Nutrient broth. Isolates were grown and allowed to adhere for 2 hours at 37°C. After incubation, absorption (at 620nm) of the culture was measured (FLUOstar OPTIMA, BMG Lab technologies). Unbound cells were removed by inversion of plates, followed by vigorous tapping on absorbent paper. Adhered cells were then fixed for 30 mins at 80°C in an oven.

Adhered cells were stained by addition of 220µl of crystal violet (0.1%) for one minute then removed and exhaustively washed with distilled water. Plates were then allowed to dry and 220µl of decolouring solution (ethanol:acetone, 80:20%v/v) was added to each well for 15 minutes. The absorption of the eluted stain was then measured at 620nm (Genevaux, 1996).

2.1.12 Adherence to fibrinogen coated plates

The polystyrene microtiter plates were first coated with 220µl of porcine fibrinogen (10µg ml^{-1}) dissolved in phosphate buffer solution (PBS) (pH 7.0) and incubated for 1 hour at 37°C. Plates were then inverted and washed extensively with PBS (pH 7.0) and 220µl of bovine serum albumin (BSA) (5mg ml^{-1}; pH 7.0) was then added to the wells and incubated for 1 hour at 37°C. Plates were then inverted and washed extensively with PBS

(pH 7.0) (Francois *et al.*, 1996). Then, isolates were added, incubated, fixed and stained as above (section 2.1.10).

2.1.13 Pulse-Field Gel Electrophoresis (PFGE) protocol

This bacterial typing protocol was carried out as described by Bannermen *et al.*, (1995) with modifications taken from the HARMONY project (Murcham *et. al* 2003). Cultures were grown in 5 ml of Brain Heart Infusion (BHI) broth overnight. Then 0.7ml of broth was transferred to a sterile microcentrifuge tube and centrifuged at 3330 g for 2 minutes. The cells were first washed with 1ml of Ringers solution, re-centrifuged, washed with 1ml of sterile TEN buffer (0.1M Tris base, 0.15M NaCl, 0.1M EDTA, at pH7.6) and re-centrifuged. The cell pellets were re-suspended in 0.3ml sterile EC buffer (6mM Tris base, 1M NaCl, 0.1M EDTA, 0.5% Brij 58, 0.2% Deoxycholate, 0.5% Sarkosyl, at pH 7.6). To this, 2µl of 1mg/ml Lysostaphin dissolved in 20 mM sodium acetate was added and the mixture was briefly vortexed. Then 0.3ml of 2% low melting point agarose in EC buffer was added to the mix and briefly vortexed and quickly pipetted into plug moulds. The plugs were allowed to solidify at room temperature for 10 minutes. These were transferred into tubes containing 3ml EC buffer and left to stand for 1 hour at 37°C. After which the buffer was removed and replaced with 3 ml of sterile TE buffer (10mM Tris base, 5mM EDTA at pH 7.6) and incubated for 1 hour at 55°C. The plugs were transferred to fresh TE buffer for storage at 4°C until further analysis.

Small slices (2mm) were cut from the plug and placed in 125µl of restriction enzyme mixture (restriction buffer plus sterile distilled water) containing 20U of *SmaI* and incubated for 4 hours at 25°C while shaking at 80rpm. The trimmed plugs were loaded into 1% high strength gel agarose prepared in 0.5% TBE buffer (5xTBE: Tris base 54g, Boric acid 27.5g , EDTA 2.92g at pH8.0) consisting of 20 wells. The plugs were sealed with 0.8% low melting pointing (LMP) agarose and allowed to solidify. The plugs were loaded into the gel in a set order: The side wells (1 and 20) were not loaded with sample and were just sealed with LMP agarose. Well 2 was loaded with a CHEF DNA size standard Lambda Ladder. Wells 5, 10 15 and 19 were loaded with a control plug (NCTC 08325) and test samples were loaded into the remaining 13 wells.

Gels were run in a CHEF-DR II system using the following parameters derived from the HARMONY protocol (Murchan *et. al* 2003):

Block 1: Initial pulse 5s, Final pulse 15s, Voltage 200V or 6V/cm, Time 10 hours, at 14°C

Block 2: Initial pulse 15s, Final pulse 60s, Voltage 200V or 6V/cm, Time 13 hours, at 14°C

Total Time: 23 hours.

2.1.14 Staining, visualising and photographing the gel

The gels were stained with ethidium bromide at a final concentration of 1μg/ml mixed with 400ml distilled water for 45 minutes, de-stained with distilled water (400ml) for 45 minutes and visualised using a 320nm UV lamp and photographed using a Gel Cam (Polaroid, USA) fitted with a 40.5mm 15 deep yellow lens (Tiffan, USA) and a electrophoresis hood (Polaroid, USA).

2.1.15 Analysis of banding patterns

Band patterns were loaded into Bionumerics programme and were analysed to form a dendrogram. This was carried out in collaboration with Dr. Laura McAuliffe at the Veterinary Laboratory Agency (VLA) Weybridge, UK.

2.1.16 Interpretation of banding patterns

Banding patterns were interpreted using the criteria (Table 5.1) devised by Tenover *et al.* (1995).

Table 2.2: Criteria for interpreting PFGE Banding Patterns (*reproduced from*: Tenover, *et al.*, 1995).

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

2.1.17 Chromogenic substrates

All chromogenic substrates were kindly provided by PPR Diagnostics Ltd, London, UK. The chromogenic substrates used were SRA-propanoate (C3), SRA-butyrate (C4), SRA-octanoate(C8), SRA-decanoate(C10), SRA-laurate (C12), SRA-myristate(C14), SB_ZTM-acetate (C2) and SB_ZTM-butyrate(C4). An example of the chemical structure of the chromogenic substrate can be seen below.

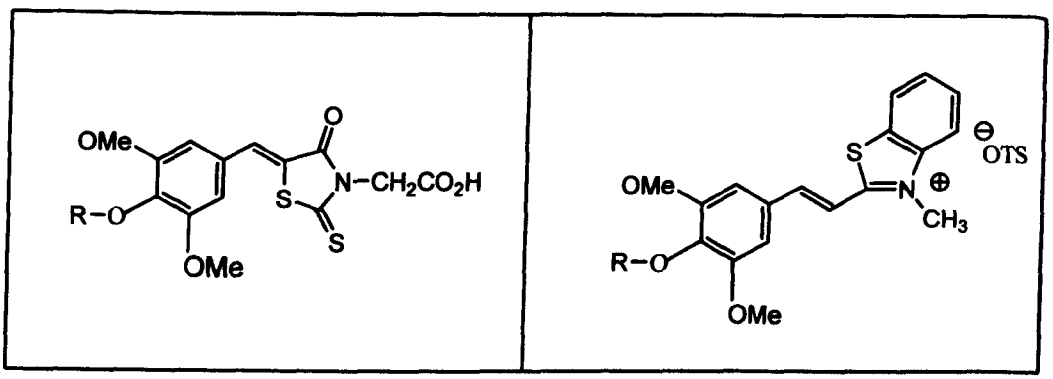


Figure 2.2. Structure of the chromogenic substrates.
Left hand panel SRA (5-(4-hydroxy-3,5-dimethoxyphenylmethylene)-2-thioxothia-zolidin-4-one-3-ethanoic acid), R= propionate (C3), butyrate (C4), octanoate (C8), decanoate (C10), laurate (C12), or myristate (C14). Right hand panel SBzTM-tosylate (2-[2-(4-hydroxy-3,5-dimethoxyphenyl)-vinyl]-3-methy-benzothiazolium salt), R is either acetate (C2) or butyrate (C4). (Dr Dick Richardson of PRR diagnostic provided structures)

2.1.18 Chromogenic medium

The chromogenic substrates were added to nutrient agar to a final concentration of 0.3g/l. The substrates were dissolved in 4ml methanol-water mix (v/v). The substrates SRA-propanoate and SB_ZTM-acetate were dissolved in a 2% (v/v), SRA-laurate and SRA-myristate were dissolved in a 4%(v/v), SRA-butyrate and SRA-decanoate were dissolved in a 8% (v/v), SB_ZTM-butyrate was dissolved in a 10% (v/v) and finally SRA-octanoate was dissolved in a 66% (v/v). The substrates were then added to cooled (55°C) pre-sterilised nutrient agar and poured into 90-mm petri dishes.

2.1.19 Inoculation of test media

Overnight cultures were streaked onto the chromogenic agar plates as a single line, in a radiating pattern from the centre using six isolates per plates. All plates were incubated at 37°C for 48 hours and all tests were carried out in triplicate. Any colour changes were noted after 24 and 48 hours. Uninoculated control plates were incubated under similar conditions.

2.1.20 Growth under antibiotic pressure

Isolates were transferred to nutrient agar containing either 0.125mg/L penicillin G or 0.5mg/L clindamycin and incubated overnight at 37°C. Three colonies were then transferred to 15ml of nutrient broth and incubated for 15 hours at 37°C. Nine suspensions of 2ml each were then centrifuged at 3300g for 2 minutes. The cells were washed in Ringers solution, re-centrifuged and then suspended in 1ml nutrient broth (A_{630} of ≈0.6OD units). Aliquots of the suspension (1 ml) were then added to one of either three flasks. The flasks were prepared as follows: the first was the blank flask that contained nutrient broth with no antibiotic. The second flask contained nutrient broth plus either penicillin G or clindamycin at the reported MIC level (0.25mg/L and 1mg/L respectively) (Anon 8, 2002). Finally, the third flask contained nutrient broth plus penicillin G or clindamycin at double the reported MIC level (0.5mg/L and 2mg/L respectively) (Anon 8, 2002). All flasks were prepared in triplicate and incubated in a shaking incubator at 37°C, at 80rpm for 10 hours. Aliquots of 40µl were taken at 1, 2, 3, 4, 6, 8, 10, hours and decimal dilution was prepared in Ringers solution (10^{-1}-10^{-6}). Then aliquots (10µl) were placed on nutrient agar and incubated at 37°C for 24hours.

2.1 Statistical analysis

2.2.1 For the statistical analysis the Z-Test was used (i.e. testing the difference between a sample mean and the population mean to determine if this difference is large enough to be statistically significant), with the following equation:

$$z = \frac{\hat{P}_1 - \hat{P}_2}{\sqrt{\hat{P}(1-\hat{P})(\frac{1}{n_1} + \frac{1}{n_2})}}$$

where $\hat{P} = \frac{x_1 + x_2}{n_1 + n_2}$

and

X_1- Number of positive UK isolates

X_2- Number of positive Maltese isolates

N_1-Total number of UK isolates

N_2-Total number of Maltese isolates

P_1- Population UK (X_1/N_1)

P_2- Population Malta (X_2/N_2)

The z-score obtained determines the distance from the population mean in units of the population standard deviation. In order to determine if the sample mean in the present study is different from the population mean the level of confidence needs to be determined (i.e. $p<0.05$ or $p<0.005$).

2.2.2 Single factor analysis of Variance (ANOVA) was determined using Microsoft Excel containing the "Analyze it" add in software (Microsoft).

3.0 Determination of antibiotic resistant profiles in clinical isolates of *S. aureus*

3.1 Introduction

Antibiotic resistance has been and is an increasing problem worldwide, in both clinical and veterinary settings (Anon 7, 2002). The world health organisation (WHO) has also recognized that antibiotic resistance is one of the major threats facing the world in the future (Brundtland, 2005). Diseases of concern include AIDS, tuberculosis, malaria, Leishmaniasis, gonorrhea and hospital-acquired infections including vancomycin–resistant enterococci (VRE) and MRSA (Brundtland, 2005). From 1991 to 2000 bacteraemia levels caused by MRSA in the UK rose from 2% to 40% (Anon 7, 2002). In April 2001 the National Health Service introduced a mandatory bacteraemia surveillance scheme for *S. aureus*, with levels reported every 6 months. During the first four years of this project from 2001 to 2005, the MRSA levels in the UK were approximately 39% (Anon 2, 2005). However, these reports only state the level of methicillin resistance and do not include the level of resistance to other antibiotics. It should be remembered that methicillin-sensitive *S. aureus* (MSSA) and MRSA are resistant to multiple antibiotics, therefore when treating these bacteria, full antibiotic profiles are required to aid successful therapy.

The aim of this study was to carry out an epidemiological assessment of antibiotic resistance profile in clinical isolates of *S. aureus,* collected from the UK and Malta. The European Antimicrobial Resistance Surveillance System (EARSS) annual report in 2004, showed that Malta had the highest level of MRSA bacteraemia in Europe (56%) and the UK had the fourth highest (44%) (Anon 3, 2004). Both these countries have had levels of MRSA bacteraemia >40% since 2001 and therefore represent counties where MRSA is an important problem. Isolates of *S. aureus* were collected from three London based hospitals and the main general hospital in Malta and antibiotic profiles for each isolate were determined against a panel of fourteen different antibiotics using the test procedures as described by British Society for Antimicrobial Chemotherapy (BSAC, Anon 8, 2002). This antibiotic resistance data may be used to assesses whether antibiotic prescription policies, from the two countries sampled, need to be adjusted to increase the effectiveness of treatment.

3.2. Materials and Methods

3.2.1 Bacterial strains and initial isolate conformation

The bacterial strains were cultured and maintained as described in section 2.1.1. Isolate identity was confirmed as described in 2.1.2

3.2.2 Antibiogram testing.

The methods for antimicrobial testing were carried out as described in section 2.1.3

3.2.3 Vancomycin susceptibility testing.

The method for vancomycin susceptibly testing was carried out as described in section 2.1.4

3.2.4 Breakpoint testing.

The method for breakpoint testing was carried out as described in section 2.1.5

3.2.5 Statistical analysis

Statistical analysis was carried out as described in Section 2.2.1

3.3 Results

From a total of 705 isolates, 3.5% (25/705) of isolates (11 UK and 14 Maltese) were removed as they tested negative for the production of coagulase.

Figure 3.1 and Figure 3.2 (MSSA and MRSA respectively) show the percentage of isolates resistant to the fourteen antibiotics tested. In both figures it can be seen that all isolates from both countries were sensitive to linezolid, teicoplanin and vancomycin. Additionally all MSSA isolates were sensitive to methicillin.

Figure 3.1 shows the results for the MSSA isolates from both countries. Statistical analysis using the Z-test showed that there was a statistically significant difference ($p<0.05$) between sensitivity to five of the antibiotics from the two countries for the MSSA isolates. In detail, the UK isolates showed a higher percentage resistance to the antibiotics clarithromycin and gentamicin, whereas the Maltese isolates showed increased levels of ciprofloxacin, erythromycin and tetracycline resistance.

Figure 3.2 shows the results for the MRSA isolates from both countries. In this figure the level of resistance for each antibiotic is greater than those seen with the MSSA isolates. It can also be seen that 50% of the isolates from both countries had resistance to six of the antibiotics tested and 80% of the isolates were resistant to five of these antibiotics. Also, 100% resistance to penicillin G and methicillin was observed in all isolates of MRSA from both countries. The Z-test showed statistically significant differences in the MRSA data ($p<0.05$), showing increase in resistance. The UK isolates showed increases in clarithromycin and gentamicin resistance and the Maltese isolates showed increase in tetracycline resistance.

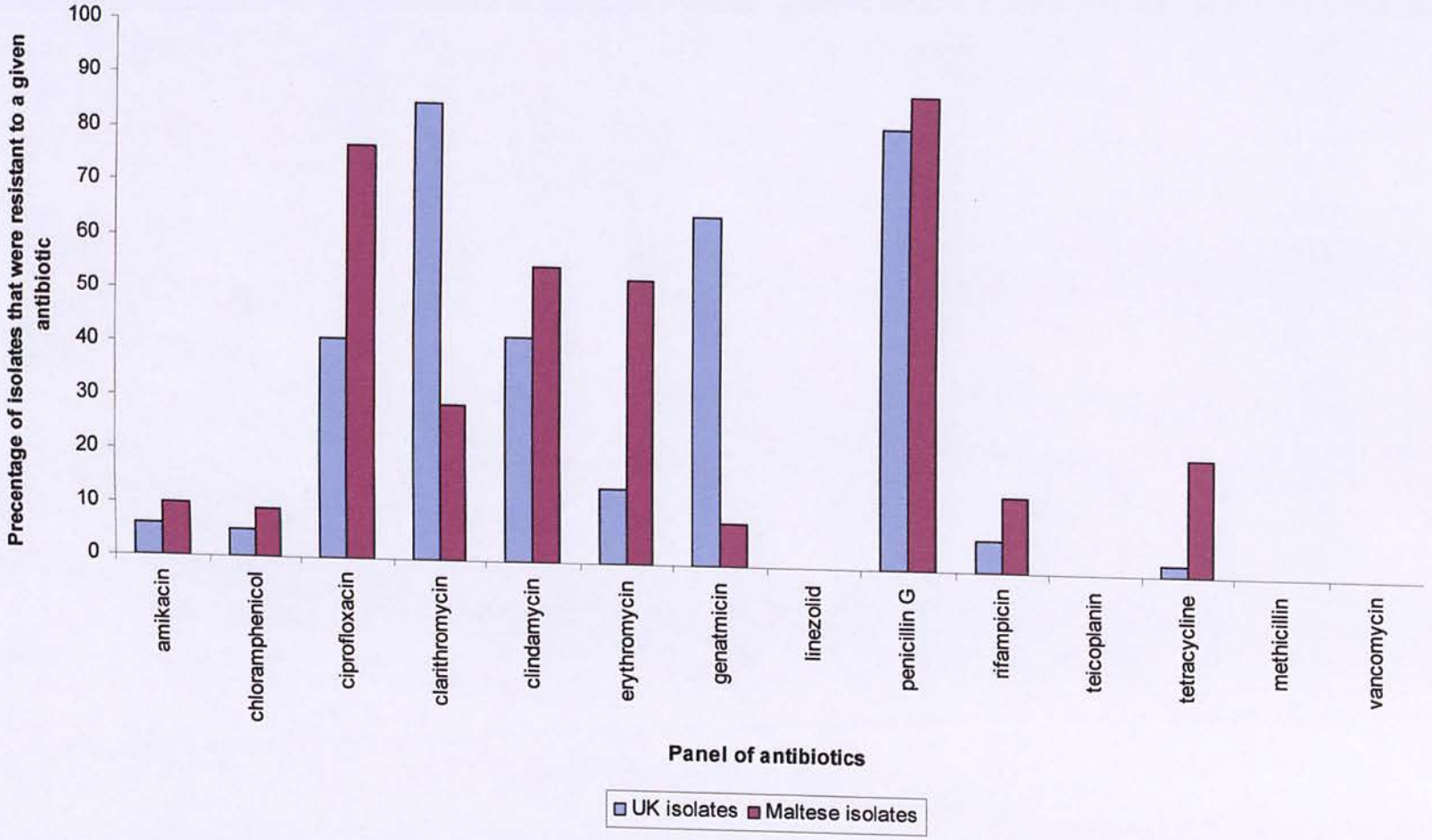


Figure 3.1: Percentage of methicillin-sensitive *S. aureus* isolates showing resistance to a panel of fourteen antibiotics. MSSA Isolates were collected from UK (n=114) and Malta (n=93).

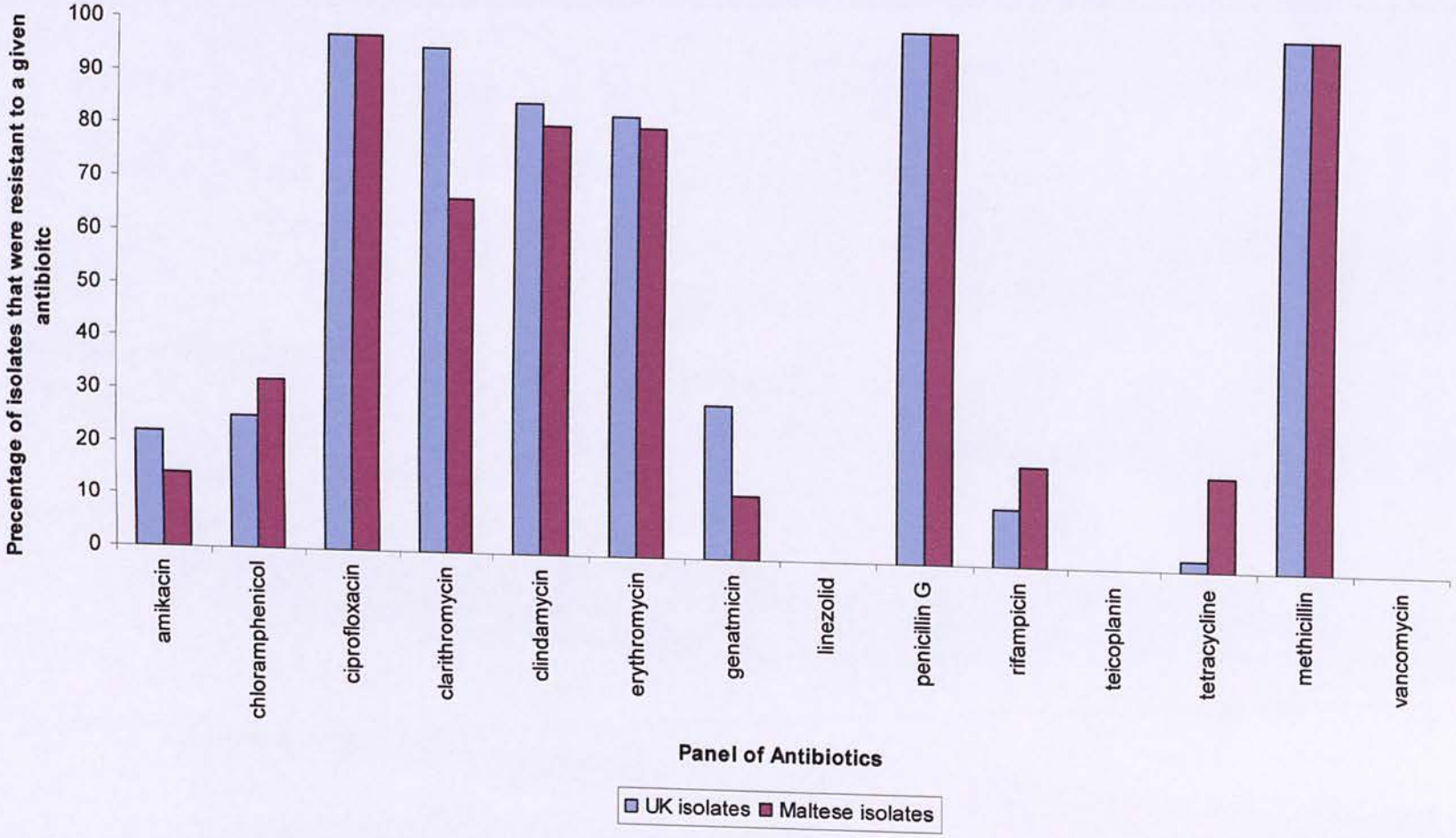


Figure 3.2: Percentage of methicillin-resistant *S. aureus* isolates showing resistance to a panel of fourteen antibiotics. MRSA isolates were collected from UK (n=257) and Malta (n=216).

Figures 3.3 and 3.4 show the number of antibiotics the isolates were resistant to in both countries, for both MSSA (Fig. 3.3) and MRSA (Fig. 3.4). The MSSA isolates (Fig. 3.3) showed resistance to various antimicrobial agents in the range of zero up to eight different antibiotics, with a mean resistance to 4 and 3 antibiotics (UK and Malta respectively) and a median resistance of 3 antibiotic agents. The data from both countries appear to be similar on the graph, although the UK isolates show a slightly higher level of resistance to the median number of antibiotics, 28 % (33/114), compared to the Maltese isolates 26% (24/93).

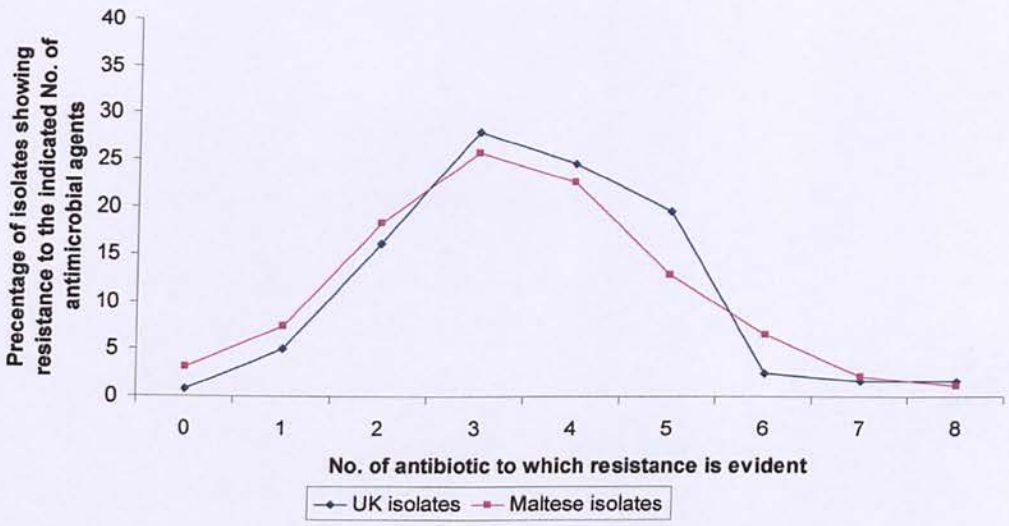


Figure 3.3: Percentage of methicillin-sensitive *S. aureus* isolates showing resistance to between zero to eight different antibiotics.
MSSA isolates were collected from UK (n=114) and Malta (n=93).

Figure 3.4 for MRSA shows a range from two to ten antibiotics to which the isolates were resistant, with a mean resistance of six drugs, and a median resistance of 7 and 6 (UK and Malta respectively) antibiotics. The mean level of resistance seen in the MRSA isolates is double the result seen with the MSSA isolates. As with the MSSA isolates, the MRSA data for both countries appear similar, with no great different trend in the graphs. However, the UK MRSA isolates showed a higher percentage of resistance to the mean number of antibiotics, 37% (95/257), compared with the Maltese isolates, 29% (64/216) (see figure 3.4).

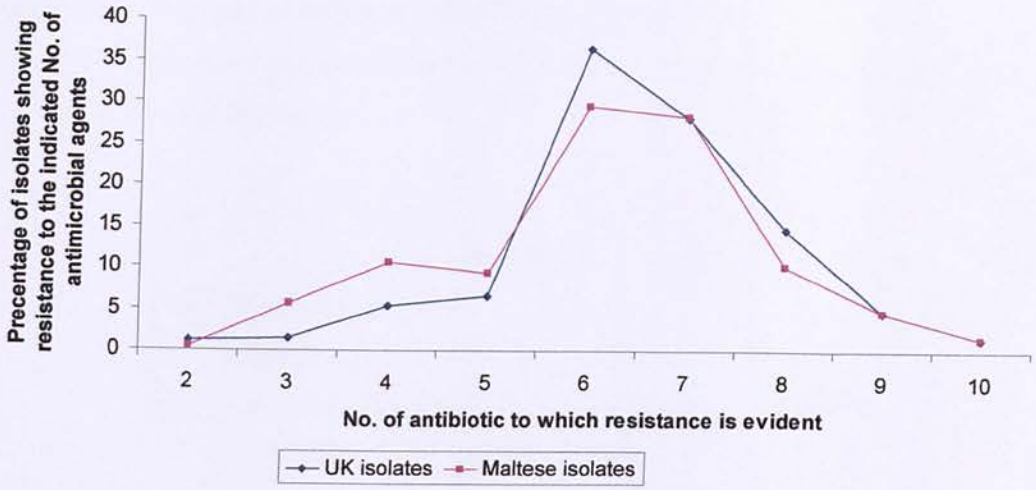


Figure 3.4: Percentage of methicillin-resistant *S. aureus* isolates showing resistance to between zero and ten different antibiotics.
MRSA isolates (were collected from UK (n=257) and Malta (n=216).

Tables 3.1 and 3.2 show the antibiotic breakpoint levels for isolates that were found to have statistically significant different levels of resistance in Figures 3.1-3.2. Table 3.1 shows the result for the MSSA isolates where breakpoint levels were determined for a total of five different antibiotics, namely ciprofloxacin, clarithromycin, erythromycin, gentamicin and tetracycline. In the majority of the results, some isolates gave a breakpoint reading below the resistant level, such as ciprofloxacin (breakpoint level of 2mg/L 65% and 57% (UK and Malta respectively), gave results below this level. This can be seen with other antibiotics tested. Only the UK isolates tested for tetracycline did not give any results below the break point level.

Table 3.2 shows the results for the MRSA isolates. Breakpoint levels were determined for a total of three different antibiotics, clarithromycin, gentamicin and tetracycline. Once again some isolates gave breakpoint levels below published levels of resistance as defined by BSAC. For example, isolate No.13 showed breakpoint of less than 0.05mg/L of gentamicin whereas the published data indicates the breakpoint should be greater than 2mg/L.

Table 3.1: Survival of MSSA isolates from the UK and Malta at the breakpoint value of given antimicrobials, as well as a range of concentration around the stated breakpoint.

ciprofloxacin					
Drug concentration (mg/L)	<0.5	1	[2]	4	8>
UK isolates (n=58)	60	5	10	5	19
Malta isolates (n=58)	43	14	10	9	24
clarithromycin					
Drug concentration (mg/L)	<0.25	0.5	[1]	2	4>
UK isolates (n=94)	50	11	7	13	19
Malta isolates (n=22)	36	9	5	5	45
erythromycin					
Drug concentration (mg/L)	<0.25	0.5	[1]	2	4>
UK isolates (n=14)	14	7	7	0	71
Malta isolates (n=39)	54	10	3	10	23
gentamicin					
Drug concentration (mg/L)	<0.5	1	[2]	4	8>
UK isolates (n=69)	3	0	13	42	42
Malta isolates (n=6)	1	0	1	1	4
tetracycline					
Drug concentration (mg/L)	<0.5	1	[2]	4	8>
UK isolates (n=3)	0	0	0	33	67
Malta isolates (n=17)	35	0	0	0	65

The values in this table represent the percentage of isolates that were detected at a given antibiotic concentration. The number contained within the square parenthesis in each section of the table represents the minimum inhibitory concentration as determined by BSAC for that antibiotic.

Table 3.2: Survival of MRSA isolates from the UK and Malta at the breakpoint value of given antimicrobials, as well as a range of concentration around the stated breakpoint.

clarithromycin					
Drug concentration (mg/L)	<0.25	0.5	[1]	2	4>
UK isolates (n=237)	9	4	2	2	83
Malta isolates (n=120)	5	1	0	1	94
gentamicin					
Drug concentration (mg/L)	<0.5	1	[2]	4	8>
UK isolates (n=73)	14	1	10	26	32
Malta isolates (n=18)	11	0	2	3	12
Tetracycline					
Drug concentration (mg/L)	<0.5	1	[2]	4	8>
UK (n=9)	11	0	0	0	89
Malta (n=36)	25	0	3	0	72

The values in this table represent the percentage of isolates that were detected at a given antibiotic concentration. The number contained within the square parenthesis in each section of the table represents the minimum inhibitory concentration as determined by BSAC for that antibiotic.

3.4 Discussion

Previously published data for MRSA antibiotic resistance only states the level of resistance to methicillin. *S. aureus* isolates however are mostly multi-drug resistant (Anon 7, 2002). The aim of this study was to determine the antibiotic resistance profiles for each isolate in the collection against a panel of fourteen antibiotics. Of the fourteen antibiotics tested, the isolates were completely sensitive to only three antibiotics: linezolid, teicoplanin and vancomycin. This was unsurprising since these antibiotics are relatively new and resistance to these antibiotics is currently rare. However, resistant strains of MRSA to these antibiotics have been identified in other studies. Vancomycin and teicoplanin resistance has been reported in Japan, USA, Europe, Asia and the UK in a few cases (Trakulsomboon *et al.*, 2001; Anon 5, 2002). Linezolid resistance is relatively uncommon, with only a few cases reported in USA (Tsiodras *et al.*, 2001) and the UK (Wilson *et al.*, 2002). These studies have shown that it is possible for MRSA to develop resistance to linezolid, teicoplanin and vancomycin. It is likely therefore that resistance to these antibiotics will increase with time, as has been observed with resistance to other antimicrobials in the past.

Of the remaining eleven antibiotics tested a correlation between the MSSA and MRSA can been seen. Where the resistance is low in the MSSA it is also low in the MRSA and *vice versa*, although there is a greater percentage of overall resistance seen in the MRSA isolates. Also the level of resistance between the two countries was similar although some statistically significant differences in resistance levels between the two countries were noted.

In the MSSA isolates there was a statistically significant greater level of resistance to clarithromycin and gentamicin ($p < 0.05$) in the UK isolates compared to the Maltese isolates. In contrast a greater level of ciprofloxacin, erythromycin and tetracycline resistance was observed in the Maltese isolates ($p < 0.05$) compared to the UK isolates. Once again, in the MRSA isolates greater levels of resistance were recorded with clarithromycin and gentamicin in the UK isolates and from the Maltese isolates a greater level of tetracycline resistance was recorded.

With the isolates that showed statistically significant differences in antibiotic resistance, breakpoint levels were determined. Interestingly in the majority of these tests, isolates gave resistance levels below the breakpoint, except for one group, namely the UK

tetracycline resistant MRSA. Also the largest percentage in isolates that gave lower results than the breakpoint level, were MSSA isolates.

There are two possible explanations for why some isolates gave resistant levels below the breakpoint level. Firstly, the antibiogram profiles were determined by disc diffusion assay. This method of determining resistance is widely used in hospital laboratories; however, it is not as sensitive as breakpoint determination. Interpretation of resistance is determined by a zone of inhibition of growth, around a paper disc containing an antibiotic. The difference between a resistant identification and a sensitive identification is a measurement of 1mm, for example penicillin G: resistant (<24mm), sensitive (>25mm). Therefore it is possible that in the case of these isolates they appear to be resistant by disc diffusion assay, however were determined to be sensitive to the antimicrobial by the more sensitive breakpoint test.

A second possible explanation is the time frame the isolates were collected in. All isolates were collected from external collaborators and therefore the time frame between initial culturing and reception of the isolates into this current studied is unknown. Previous studies have shown that if antibiotic pressure is removed from isolates of *S. aureus*, phenotypic resistance profile can be lost. A study by Boyle-Vavra *et al.*, (2000) has shown that if vancomycin resistant *S. aureus* are serially grown on media containing no antibiotic, the resistance is lost. Similar findings have also been reported by others (Sugino *et al.*, 2000) and has also been shown with other antibiotics such as linezolid (Meka *et al.*, 2004). However, this loss or reversion of phenotypic resistance has been so far reported only in emerging resistances. Loss or reversion of resistance has also been shown to occur in stable resistant genes. van Griethuysen *et al.*, (2005), showed loss of the *mecA* gene in isolates of MRSA after long term storage (2 years) at -80°C. In the current study 14.4% (36/250) of isolates showed loss of the gene. Whether long term storage on slopes, as well as frozen sample could lead to loss or reversion of resistance profile to other antibiotics, needs to be tested. It is possible to suggest that in some of the isolates collected in this study, reversion or loss of resistance may have occurred. Although the isolates were determined to be resistant by disc diffusion, during the time between initial culturing, disc diffusion assay and breakpoint determination possible loss or reversion of resistance may have occurred, therefore resulting in the conflicting results seen in these two tests.

However, it is interesting to note that the highest percentage of isolates to give lower than breakpoint levels, occurred in the MSSA isolates. This may suggest that MRSA isolates activity retain the resistance profile better then their MSSA counterparts.

When comparing the results between MSSA and MRSA isolates, it could be seen that MRSA isolates (see Figure 3.2) had two levels of resistance, either low (<30%) or high (>80%). Concentrating on the MRSA isolates from the UK, it can be seen that >80% of the isolates were resistant to six of the fourteen antibiotics, namely ciprofloxacin, clarithromycin, clindamycin, erythromycin and two beta-lactams (methicillin and penicillin G). Using the number of cases of bacteraemia recorded from 2004 to 2005 (7212 cases) (Anon 2, 2002), the level of resistance recorded in this study would mean that four-fifths of these isolates (5770 cases) may not respond to these antibiotics. It is difficult to ascertain whether the resistance levels to these antibiotics, such as, ciprofloxacin, clarithromycin, clindamycin, erythromycin, will increase to the 100% resistance, as seen with the beta-lactams antibiotics in MRSA. Likewise it is difficult to speculate whether resistance levels will increase to antibiotics that showed isolate resistance levels of <30%, although only future monitoring will detect any such increases in resistance levels.

The other difference between the two populations for both countries was the mean number of antibiotics to which the isolates were resistant. The MSSA population was resistant to a mean of three antibiotics, whereas the MRSA isolates were resistant to double that level (*i.e.* six antibiotics). This fact highlighted one of the main reasons why MRSA infections are difficult to treat due to the higher level of multi-antibiotic resistance, as displayed by these isolates.

The data presented here can be used to improve current antibiotic prescription policies. In the UK current policies on conditions such as endocarditis, advise the use of benzyl penicillin and gentamicin for penicillin sensitive isolates and flucloxacillin and gentamicin for isolates that are penicillin resistant. For isolates that are methicillin resistant, it is advised to use vancomycin (Leach *et al.*, 2002). However, using the results from this study, the use of gentamicin in the MSSA isolates would be limited, since high levels of resistance were recorded (60%). The use of only vancomycin in cases of MRSA is required, but is of concern. The use of two antibiotics would be preferable as this will help to prevent resistance by challenging the bacteria in two different areas of its metabolism and/or cell structure. Therefore the use of two antibiotics suggest that it will be more difficult for the bacteria to develop resistance, whereas the use of one antibiotic will

make this easier, as seen by resistance to this antibiotic recorded in other isolates of MRSA.

In Malta, endocarditis is treated in a similar way to cases as in the UK. MSSA isolates are treated with flucloxacillin and gentamicin, whilst cases of MRSA are treated with vancomycin (Leach *et al.*, 2002; Borg *et al.*, 2004). However, in other illnesses such as superficial skin infection and post traumatic wounds different antibiotic combinations are used, where mainly clarithromycin and clindamycin are advised for *S. aureus* treatment (Borg *et al.*, 2004). However, the results from this study in the case of MRSA isolates suggest that these two antibiotics would have little effect, with resistance levels to clindamycin being 80% and resistance to clarithromycin being over 60%. In the cases of MSSA the levels are less, but resistance to clindamycin is over 50% and resistance to clarithromycin is 30%. Although resistance levels in MSSA isolates are not as high as the levels seen in the MRSA isolates, correct resistant profiles are needed for effective treatment of MSSA, due to this resistance.

In conclusion, this study shows that two countries that are geographically separated by large distance have similar antibiotic resistance profiles in their hospital *S. aureus* isolates, even though they use different antibiotics in their hospital policies. The importance of monitoring the level of not only methicillin resistance but also the resistance levels of other antibiotics is also demonstrated. Currently in the UK, the government has introduced a mandatory reporting scheme for methicillin resistance in *S. aureus*. Due to the high level of resistance recorded to some of the antibiotics used in this study, it could be suggested that this type of scheme needs to be extended to include other antibiotics where there is the potential for resistance level to reach 100%. This type of information is needed in order for suitable changes to current antibiotic policies to be made so as to make them more effective in treating infections caused by *S. aureus* and the principle may be potentially expanded to other organisms in the future.

4.0 Determination of virulence factor production in clinical isolates of *S. aureus*

4.1 Introduction

S. aureus is known to produce a number of different virulence factors. The pathological damage seen in a *S. aureus* infection is not directly caused by the bacterium, but by the virulence factors produced by the cell. The majority of the research on these factors was carried out during the 1960s and 1970s, at which time the resistance to antibiotics was less common. Since the beginning of the new century, antibiotic resistance has increased and is now a major problem (for detailed account, refer to section 3.1-3.4). Some studies have focused on the difference in production of *S. aureus* toxins in isolates of MRSA and MSSA (Coia *et al.*, 1992; Schmizt *et al.*, 1997). However there is limited research focussing on the production of *S. aureus* enzymes in isolates of MRSA and MSSA.

One of the main objectives of this study was to investigate whether there is a difference in the production of virulence factors in MRSA isolates compared to MSSA isolates. This was determined by the observation of the production of three extracellular enzymes: DNase, lipase, proteinase and one toxin: haemolysin. Whilst the toxins produced by *S. aureus* have a local and systemic effect, the extracellular enzymes mainly have a local effect. Increased research into the action of these enzymes has highlighted their importance in *S. aureus* infection (See section 1.62.3.1-1.6.2.3.3), although for some factors, their role in pathogenicity is still unknown, for example DNase. For two of the enzymes tested in this study some of their roles are known, for example lipase has been shown to interfere with phagocytosis (Arvidson, 2000). Whilst proteinase has been shown to affect the stimulation of both T and B lymphocytes and cleave the heavy chain of all human immunoglobulin classes (Prokesova *et al.*, 1992). Additionally, the adhesion ability of the isolates was determined by the binding to plastic multi-well plates and fibrinogen coated plates.

In the present study, there are three hypotheses: firstly that there was going to be a decrease or lack of production of the virulence factor due to resistance mechanism acqustion, secondly that there will be no difference in the production when compared to MSSA isolates and thirdly that there will be an increase in production due to increase need in micro-nutrition because of the demand of the resistance mechanism.

4.2 Materials and Methods

4.2.1 Bacterial strains

A total of 680 isolates were used in this part of the study, taken from the culture collection. The isolates consisted of: 257 and 216 MRSA and 114 and 93 MSSA isolates from UK and Malta respectively.

4.2.2 Pathogenicity factor tests

The methods for pathogenicity factor testing were carried out as described in sections 2.1.6, 2.1.7, 2.1.8 and 2.1.9 (DNase, haemolysis, lipase, and proteinase respectively)

4.2.3 Adhesion to polystyrene 96-well plates and fibrinogen coated polystyrene 96-well plates

The methods for adhesion to polystyrene 96-well plates and fibrinogen coated plates polystyrene 96-well plates were carried out as described in sections 2.1.10 and 2.1.11.

4.2.4 Statistical analysis

Statistical analysis of the pathogenic enzymes was preformed using a Z-Test (see section 2.2.1) and statistical analysis of the adherence data was determined using single factor analysis of variance (ANOVA) (section 2.2.2).

4.3 Results

All isolates were tested for the detection of three extracellular enzymes, namely DNase, lipase and proteinase and one extracellular toxin, haemolysin. Figure 4.1 shows the percentage of isolates that tested positive for the extracellular enzymes and toxin.

4.3.1 DNase

The majority of isolates tested positive for the production of DNase, although some isolates did not show DNase activity (Fig 4.1). Only the MSSA isolates from the UK tested 100% positive for the production of this enzyme. Of the UK MRSA isolates 0.8% (2/260), tested negative for the production of DNase. In the Maltese group 0.5% (1/216) of the MRSA isolates and 3.2% (3/93) of the MSSA isolates produced negative result for the DNase test. In isolates that showed no DNase activity, conformational tests showed the isolates being tested were *S aureus*. There was no statistically significant difference in the production of DNase when MRSA were compared to MSSA. This was observed for both country groups.

Figure 4.2 shows the size of radius of the zones of clearing (mm) the isolates produced on the DNase agar for the population of MRSA and MSSA isolates from UK and Malta. It can be seen that the UK isolates had a range from 2-9mm (MSSA) and 0-9mm (MRSA), the MSSA and MRSA isolates had a mean reading of 6mm and a median and mode value of 5mm. The Maltese isolates had a range from 0-6mm (MSSA) and 0-7mm (MRSA), both the MSSA and MRSA isolates had a mean reading of 3mm, median of 3mm and a mode of 2mm. The UK isolates gave higher values compared to the Malta isolates in the range, mean, mode and median.

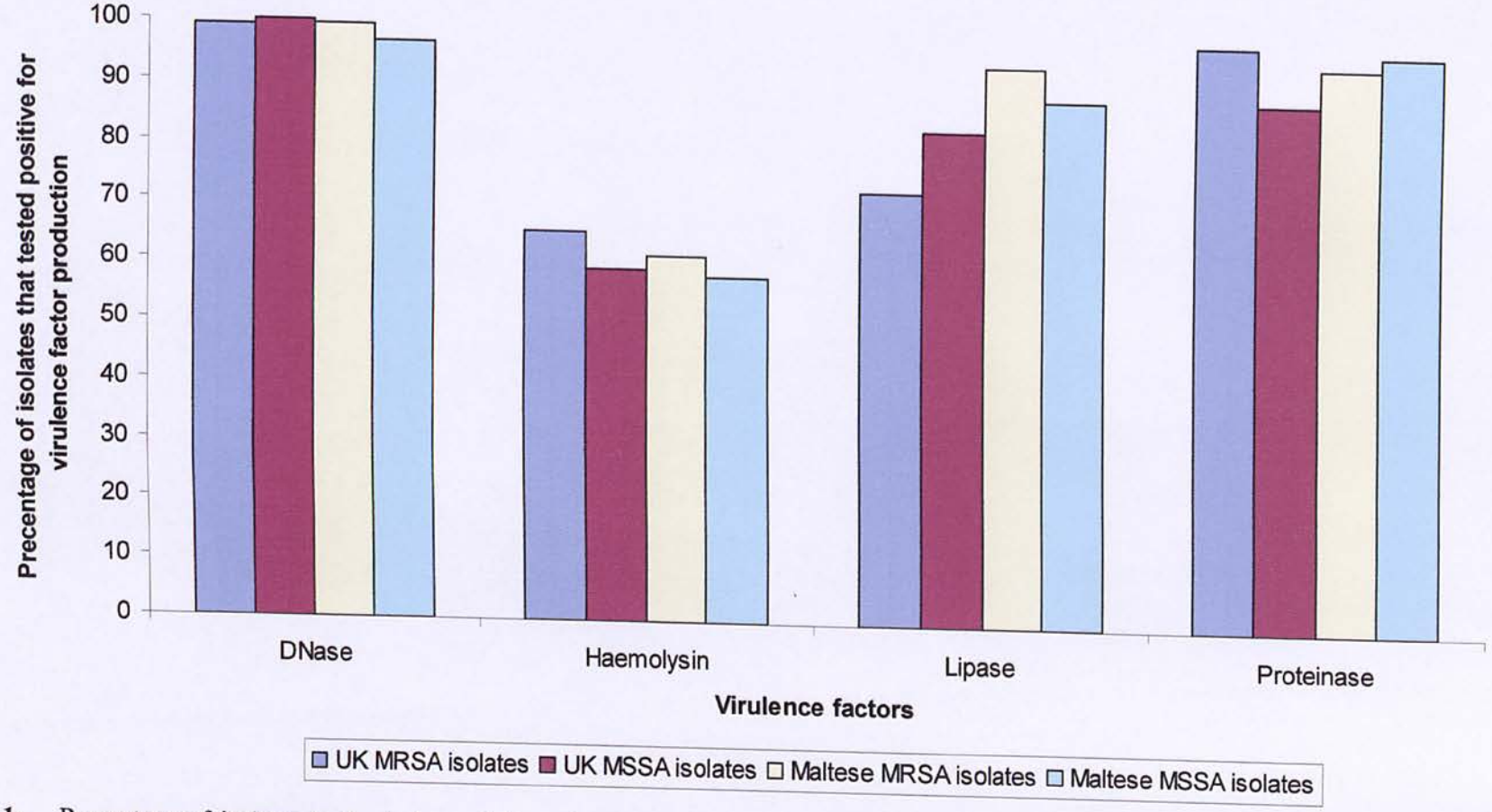


Figure 4.1: Percentage of isolates in the test population of MRSA and MSSA isolates from UK and Malta that tested positive of the production of different virulence factors. The tests include detection of three extracellular enzymes – DNase, lipase and proteinase and one extracellular toxin – haemolysin.

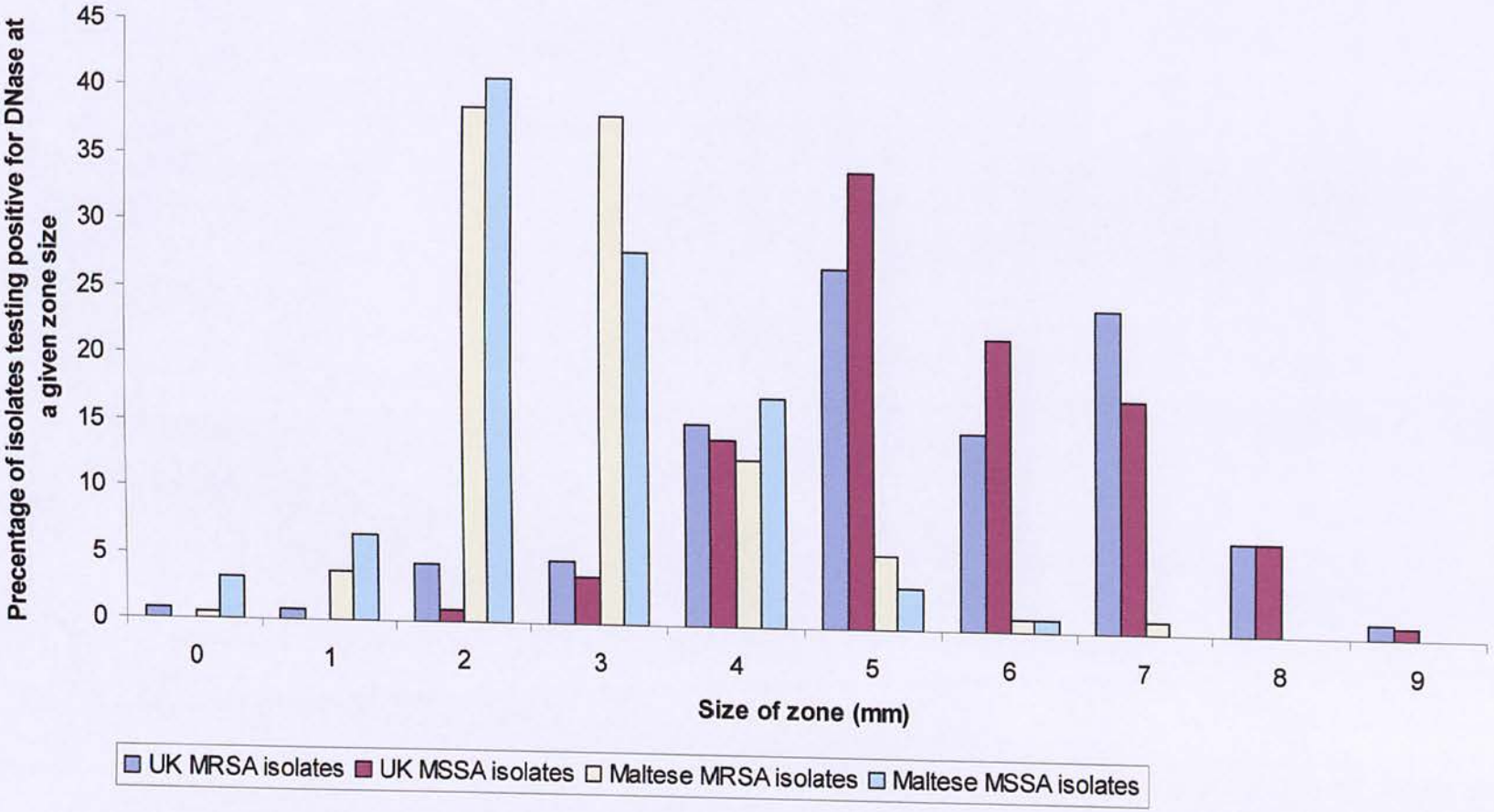


Figure 4.2: The radius of clearing (mm) which the isolates produced on the DNase agar for the population of MRSA and MSSA isolates from UK and Malta.

4.3.2 Haemolysin

The production of haemolysin was observed in approximately 60% of the isolates, the lowest of all the factors tested (Figure 4.1). As seen in the DNase test there was no statistically significant difference in the production of haemolysin, although some variability was observed amongst the strains. With regards to the UK isolates, the MRSA strains produced more haemolysin than the MSSA isolates, with 65% (170/260) and 59% (70/114) respectively showing haemolysin production. This pattern was mirrored with the Maltese isolates where production levels were 62% (133/216) in MRSA isolates and 58% (54/93) in the MSSA isolates.

Figure 4.3 shows the radius of the zones of clearing (mm) the isolates produced on the agar for the population of MRSA and MSSA isolates from the UK and Malta (radius was measured from the edge of the colony to the edge of the zone of clearing). As shown in figure (Fig 4.3), between 35 to 45% of the isolate did not produce the enzyme. The UK isolates had a range from 0-3mm (MSSA) and 0-4mm (MRSA), the MSSA and MRSA isolates had a mean reading of 1mm and a median of 1mm and a mode value of 0mm (MSSA) and 2mm (MRSA). Whereas the Maltese isolates had a range from 0-4mm for both MSSA and MRSA and both groups of isolates had a mean reading of 1mm, median of 1mm and a mode of 0mm.

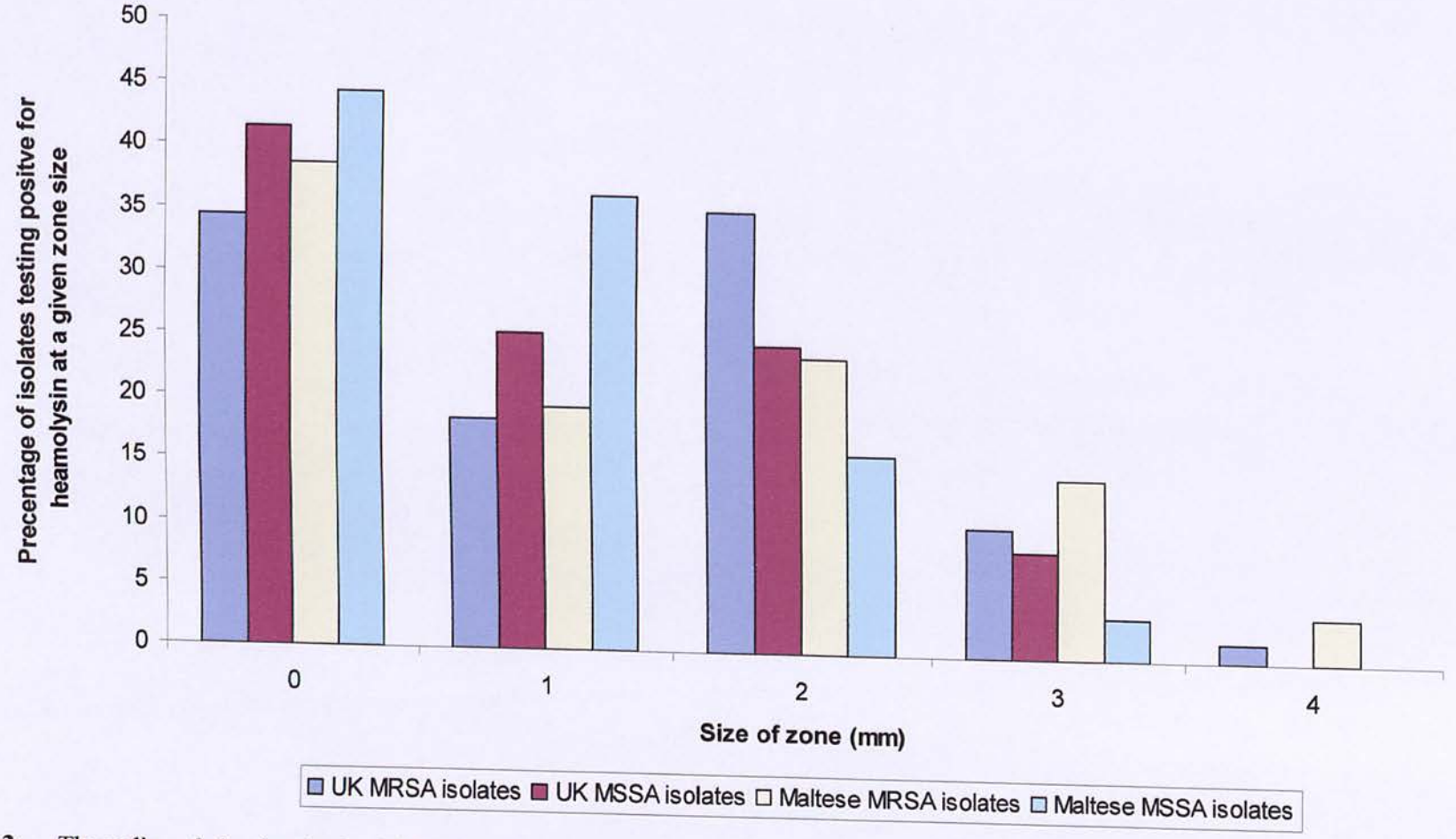


Figure 4.3: The radius of clearing (mm) which the isolates produced on the Sheep blood agar for the population of MRSA and MSSA isolates from UK and Malta

4.3.3 Lipase

These results showed the greatest level of variation (Fig. 4.1) However, despite this observation the Z-test showed that there was no statistically significant difference between the groups of isolates. The lowest readings were recorded in the MRSA isolates from the UK at 72% (190/261) being positive for lipase activity and the highest was recorded in the MSSA isolates from Malta of 94% (82/93). For both countries, the MSSA isolates produced more lipase than the MRSA isolates. The percentage of UK MSSA isolates that produced lipase was 83% (98/114) whereas 72% of the MRSA isolates demonstrated this trait. In the Maltese group the reverse was seen. In the MRSA isolates, 94% (198/216) produced lipase contracting with 88% (82/93) of the MSSA isolates producing lipase.

Figure 4.4 shows the radius of the zones of opalescence (mm) the isolates produced on the nutrient agar supplemented with 1% Tween 80 for the population of MRSA and MSSA isolates from UK and Malta. It can be seen that the UK isolates had a range of activity from 0-8mm (MSSA) and 0-9mm (MRSA), the MSSA and MRSA isolates had a mean reading of 4mm and a median of 4mm and mode value of 5mm (MSSA) and 0mm (MRSA). The Maltese isolates had a range from 0-9 (MSSA) and 0-10 (MRSA), and both groups of isolates had a mean reading of 6mm, median and a mode of 7mm.

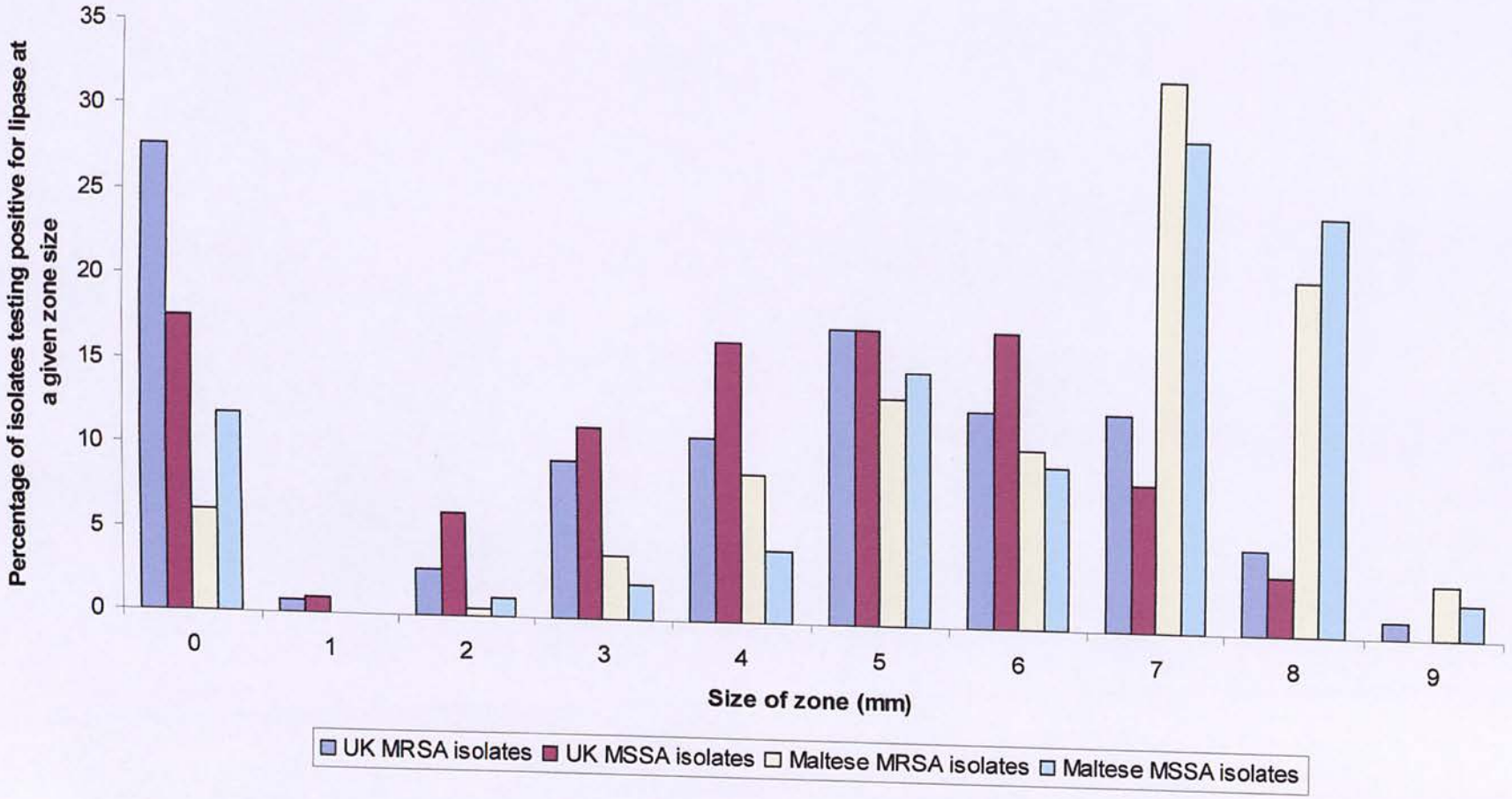


Figure 4.4: The radius of precipitation (mm) which the isolates produced on nutrient agar supplemented with 1% Tween 80 for the population of MRSA and MSSA isolates from UK and Malta.

4.3.4 Proteinase

The majority of the isolates produced proteinase with high levels seen in each category (Figure 4.1). In the UK group the MRSA isolates produced slightly more proteinase than the MSSA isolates, with 98% (254/260) and 88% (104/116) respectively. In the Maltese isolates the MSSA strains produced more proteinase than the MRSA isolates with levels of 97 (82/93) and 94% (204/216) respectively. These tests showed that there was no statistically significant difference between the groups of isolates.

Figure 4.5 shows the radius of the zones of clearing (mm) the isolates produced on the nutrient agar supplemented with 5% skimmed milk for the population of MRSA and MSSA isolates from UK and Malta. In Figure 4.5 it can be seen that the UK isolates had a range from 0-5mm (MSSA) and 0-6mm (MRSA). The MSSA and MRSA isolates had a mean reading of 3mm and a median of 3mm and mode value of 3mm (MSSA) and 2mm (MRSA). Whereas the Maltese isolates had a range from 0-9mm (MSSA) and 0-7mm (MRSA), the MSSA had a mean reading of 4mm, whereas the MRSA was 3mm. Both had a median value of 3mm and a mode of 3 and 2mm in the MSSA and MRSA respectively.

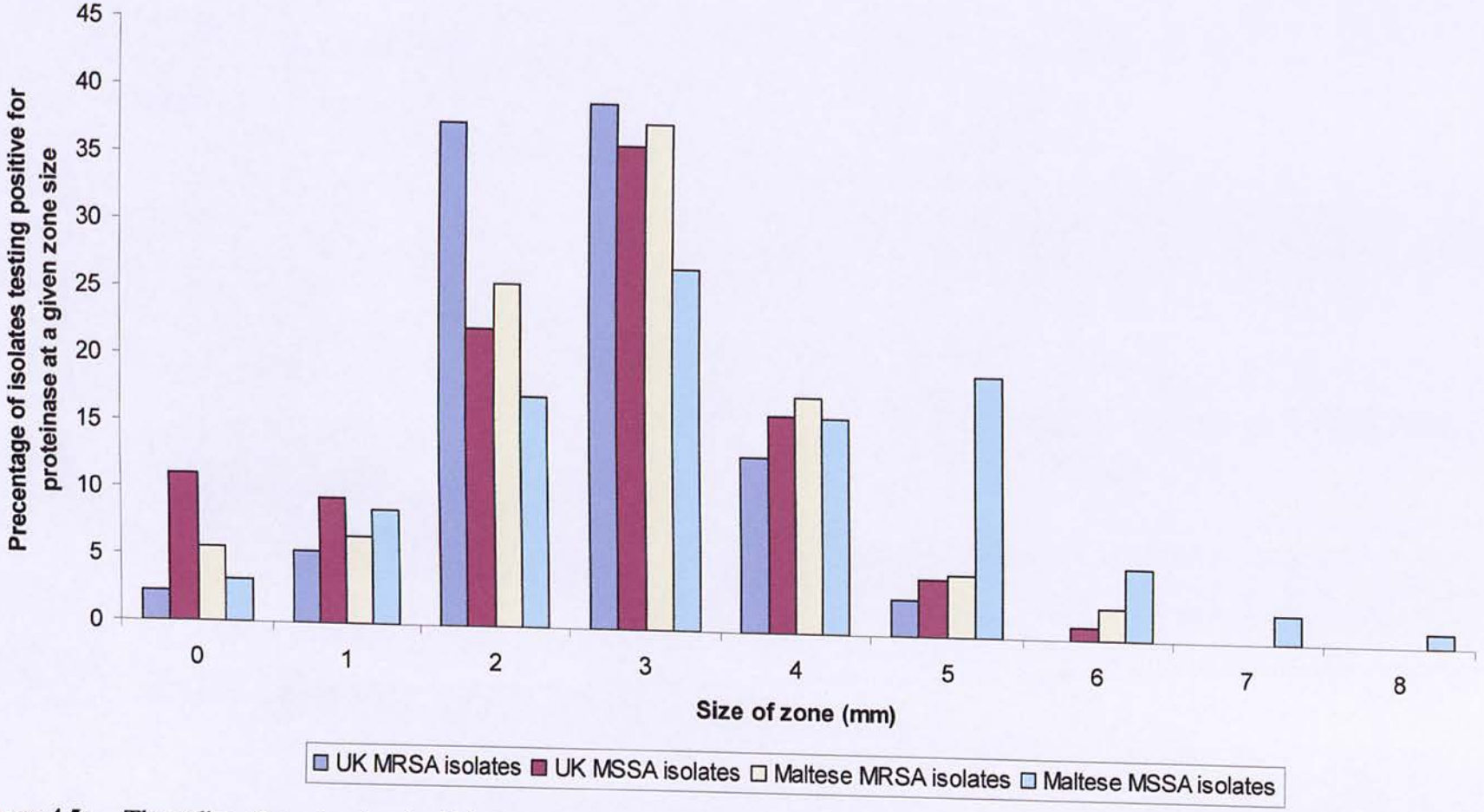


Figure 4.5: The radius of clearing (mm) which the isolates produced on nutrient agar supplemented with 5% skimmed milk for the population of MRSA and MSSA isolates from UK and Malta

4.3.5 Adhesion to polystyrene 96-well plates

Isolates were incubated to allow adherence to polystyrene plates in nutrient broth for two hours. Table 4.1 shows the absorption of the isolates after this two hour period. A reading was recorded to compare any difference in the absorption rate of the sample. Table 4.1 shows the absorption of the MRSA isolates from UK and Malta. The mean reading for the control MRSA strain (NCTC 12493) was 0.17 OD units at 620nm, with a standard error of ±0.01. The absorption readings ranged from 0.0 to 0.35 OD units at 620nm, with the largest percentage of isolates (46% of UK and 44% of Maltese isolate) giving absorption readings ranging from 0.16-0.2 OD units at 620nm. The second largest cluster of isolates had a reading ranging from 0.21-0.25 OD at units 620nm (37% of UK and 34% of Maltese isolates). The percentage of isolates with absorption readings ranging from 0.00-0.1 OD units at 620nm was 1% of UK isolates whereas 4% of Maltese isolates gave readings in this range. Interestingly, the reverse was seen with isolates with absorption readings ranging from 0.26-0.30 OD units, whereas 9% of UK isolates gave readings in this range compared to 3% of Maltese isolates. Table 4.1 also shows the absorption of the MSSA isolates from the UK and Malta. The mean reading for the control strain (NCTC 06571) was 0.18 OD units at 620nm, with a standard error of ±0.01. The absorption readings of the UK and Maltese MSSA isolates produced a range of 0.00 to 0.35 OD units at 620nm, with the largest percentage of isolates (39% of both UK and Maltese isolates) giving absorption readings ranging from 0.16-0.2 OD units at 620nm. The second largest cluster of isolates had readings ranging from 0.21-0.25 OD units (36% of UK and 33% of Maltese isolates). The percentage of isolates with absorption readings ranging from 0.11-0.15 OD units was 10% of UK isolates whereas 20% of Maltese isolates gave readings in this range. Interestingly, the reverse was observed in isolates with absorption readings ranging from 0.26-0.35 OD units where 15% of UK isolates gave readings in this range compare to 7% of Maltese isolates.

At this stage of the test there were no statistically significant differences in the absorption of the UK and Maltese MRSA isolates. Additionally no statistically significant differences could be observed between the MRSA and MSSA isolates for both countries. However there was a difference in the MSSA isolates between the two countries (UK greater than Malta, $p=0.005$).

Table 4.2 shows the absorption of the eluted stain from the isolates, after staining with 1% crystal violet and de-staining with ethanol/acetone (80:20 vol/vol) mixture. Table

4.2 also shows the absorption of the MRSA isolates from the UK and Malta. The mean reading for the control strain was 0.2 OD units at 620nm, with a standard error of ±0.01. The absorption reading ranged from 0.0-0.7 OD units, with the largest percentage of isolates (23% of UK and 33% of Maltese isolates) giving absorption reading ranging from 0.11-0.15 OD units at 620nm. The second largest cluster of isolates showed readings ranging from 0.16-0.20 OD units (20% of UK and 28% of Maltese isolates). The third largest cluster of isolates had readings ranging from 0.21-0.25 OD units (14% of UK and 12% of Maltese isolates). The percentages of isolates with absorption reading ranging from 0-0.1 OD units at 620nm, was 19% of UK isolates whereas 23% of Maltese isolates gave readings in this range. Interestingly, the reverse was seen with absorption readings ranging from 0.26-0.7 OD units at 620nm where 24% of UK isolates gave readings in this range compared to 5% of Maltese isolates. In the previous table (Table 4.1) it was noted that a higher percentage of UK MRSA isolates gave reading of absorbance between 0.26-0.35 OD units at 620nm compared to the isolates from Malta (15% and 7% respectively). This may partly be due to the higher percentage of UK MRSA isolates giving readings between 0.26-0.7 OD units when compared to the Maltese isolates.

Table 4.2 shows the absorption of the MSSA isolates from the UK and Malta. The mean reading for the control strain was 0.13 OD units at 620nm with a standard error of ±0.01. The absorption readings ranged from 0.0-0.8 OD units at 620nm, a slightly larger range than seen in the MRSA isolates. With the UK isolates 8%, readings ranging from 0-0.1 OD units at 620nm were observed compared to 35% of Maltese isolates. Similar percentages were seen at absorbance readings ranging from 0.11-0.15 OD units at 620nm, 20% and 23% (UK and Maltese isolates respectively) at absorbance readings ranging from 0.16-0.2 OD units at 620nm, 28% and 17% (UK and Maltese isolates respectively) were observed. Similar levels were observed with absorbance readings ranging from 0.21-0.25 OD units at 620nm, 14% of UK and 15% of Maltese isolates showing absorbance at this level. At absorbance readings ranging from 0.26-0.8 OD units at 620nm, 30% of UK isolates and 10% of Maltese isolates gave readings within this range.

Although statistically significant differences were observed between the UK and Maltese MSSA isolates in the unstained results, no difference could be detected within the stained group results. However, differences were detected in the MRSA isolates when comparing the two countries (UK>Malta, $p<0.005$). Differences were also detected when comparing the stained results between MRSA and MSSA isolates within the UK cohort. The MSSA isolates showed a higher binding capacity than the MRSA isolates

(MSSA>MRSA $p<0.005$). In the Maltese isolates cohort no significant difference was detected between the MRSA and MSSA isolates.

Table 4.1: Percentage of isolates at a range of absorption values of bacterial suspension after two hours growth in nutrient broth incubated in 96-well polystyrene plates

	Percentage of isolates (%)						
Absorbance ranges at620nm	0.0-0.05	0.06-0.1	0.11-0.15	0.16-0.2	0.21-0.25	0.26-0.3	0.31-0.35
UK MRSA	0	1	6	46	37	9	1
UK MSSA	0	0	10	39	36	13	2
Maltese MRSA	0	4	14	44	34	3	1
Maltese MSSA	0	1	20	39	33	7	0

Table 4.2: Percentage of eluted stain from adhered isolates at a range of absorption values to the 96-well polystyrene plates

	Percentage of isolates (%)															
Absorbance Ranges at 620nm	0.0-0.05	0.06-0.1	0.11-0.15	0.16-0.2	0.21-0.25	0.26-0.3	0.31-0.35	0.36-0.4	0.41-0.45	0.46-0.5	0.51-0.55	0.56-0.6	0.61-0.65	0.66-0.7	0.71-0.75	0.76-0.8	0.81-0.85
UK MRSA	9	10	23	20	14	7	8	2	2	3	1	1	0	0	0	0	0
UK MSSA	2	6	20	28	14	9	5	4	6	2	1	2	0	0	0	1	0
Maltese MRSA	1	22	33	28	12	2	1	2	0	0	0	0	0	0	0	0	0
Maltese MSSA	5	30	23	17	15	9	1	0	0	0	0	0	0	0	0	0	0

4.3.6 Adhesion to Fibrinogen coated 96-well plates

Table 4.3 shows the absorption of the isolates after this two-hour period where a reading was taken to compare any difference in the absorption rate of the samples. Table 4.3 shows the absorption of the MRSA isolates from UK and Malta and the mean reading for the control MRSA strain of 0.16 OD units at 620nm, with a standard error of ±0.004. The absorption reading ranged from 0.0-0.35 OD units, with the largest percentage of isolates (47% of UK and 47% of Maltese isolates) giving absorption reading ranging from 0.16-0.20 OD units at 620nm. The second largest cluster of isolates had readings ranging from 0.21-0.25 OD units at 620nm (24% of UK and 24% of Maltese isolates). The third largest cluster of isolates had readings ranging from 0.11-0.15 at 620nm (17% for UK and 24% for the Maltese isolates). The percentage of isolates with absorption readings ranging from 0-0.01 OD units was 2% for UK isolates whereas 2% of Maltese isolates gave reading in this range. The percentage of isolates with absorption readings ranging from 0.26-0.35 OD units were 10% of UK isolates compared to 3% of Maltese isolates.

Table 4.3 also shows the absorption of the MSSA isolates from the UK and Malta respectively to fibrinogen coated plates. The mean reading for the control strain was 0.168 OD units at 620nm, with a standard error of ±0.004. The absorption readings ranged from 0.0-0.35 OD units at 620nm, with the largest percentage of isolates (52% of UK and 45% of Maltese isolates) giving absorption readings ranging from 0.16-0.20 OD units at 620nm. The second largest cluster of isolates had readings ranging from 0.11-0.15 OD units at 620nm (17% of UK and 31% of Maltese isolates). The third largest cluster of isolates had readings ranging from 0.21-0.25 OD units at 620nm (17% of UK and 22% of Maltese isolates). The percentage of isolate with absorption reading ranging from 0-0.01 OD units was 1% from both countries. The percentage of isolates with absorption readings ranging from 0.26-0.35 OD units were 13% of UK isolates compared to 1% of the Maltese isolates.

At this stage of the test there was no statistically significant difference in the absorption between the MRSA and MSSA isolates of both countries. However, a difference in both the MRSA and MSSA isolates between the two countries was found (MRSA UK greater than Malta, $p<0.05$; MSSA UK greater than Malta, $p<0.05$).

Table 4.4 shows the absorption of the eluted stain from the isolates after staining with 1% crystal violet and de-staining with ethanol/acetone (80:20 vol/vol) mixture. Table 4.4 shows the absorption of the MRSA isolates from the UK and Malta. The mean reading

for the control strain was 0.28 OD units at 620nm, with a standard error of ±0.02. This was a higher reading than the one seen in the previously stain results (Table3.2). The absorption readings ranged from 0.0-0.7 OD units at 620nm, although differences were seen with the previous stained result. The isolates from both countries differ in the percentage of isolates in each range of absorbance. In the low absorbance reading 24% of UK isolates compared to just 3% of Maltese isolates that gave readings in the 0-0.15 OD units range at 620nm. 21% of the UK isolates gave readings range of in the 0.16-0.25 OD units at 620nm, compared to 19% of the Maltese isolates, whereas in the reading 0.26-0.35 OD units at 620nm, 41% of the Maltese isolates gave results in this range compared to 18% of UK isolates. Similar results were seen in the readings ranging from 0.36-0.45 OD units at 620nm with 22% of UK strain compared to 28% of Maltese strains in this range. In the final reading ranging from 0.46-0.7 OD units at 620nm 15% of the UK isolates compared to 9% of the Maltese isolates gave reading in this range.

Table 4.4 also contains the absorption for the MSSA isolates from the UK and Malta. The mean reading for the control strain was 0.257 OD units at 620nm with a standard error of ±0.01. The absorption readings ranged from 0.0-0.85 OD units at 620nm, which were different compared to the ranges seen in the MRSA isolates. At low absorbency ranges from 0.0-0.15 OD units at 620nm, little difference could be seen with 8% of UK and 6% of Maltese isolates in this range. In the range between 0.16-0.3 OD units at 620nm, 32% of UK and 50% of Maltese isolates were observed. A similar percentage was seen in absorbance reading ranging 0.31-0.45 OD units at 620nm with 38% of UK and 37% of Maltese isolates in this range. In the high absorbance range from 0.46-0.85 OD units at 620nm 23% of the UK isolates fall in this range compared to 7% of the Maltese isolates.

As in the previous adhesion experiment, statistically significant differences were detected between the MSSA and the MRSA group in the UK isolates (MSSA>MRSA, $p<0.005$). Similar results were seen in the fibrinogen coated plates (MSSA>MRSA, $p<0.005$). The reverse of these results was observed in the Maltese isolates (MRSA>MSSA, $p<0.005$). Differences were detected in the MRSA and MSSA between the two countries. In the unstained results for the MRSA isolates there was a statistically significant difference between the UK compared to the Maltese isolates (MRSA UK>Malta, $p<0.05$). The reverse was seen in the stained results (MRSA Malta>UK, $p<0.05$). In the unstained results for the MSSA isolates there was a statistically significant

difference between the UK compared to the Maltese isolates (MSSA UK>Malta). A similar finding was observed in the stained results (MSSA UK>Malta, $p<0.005$).

Table 4.3: Percentage of isolates at a range of absorption values of bacterial suspension after two hours growth in nutrient broth incubated in fibrinogen coated 96-well polystyrene plates

	Percentage of isolate (%)						
Absorbance ranges at 620nm	0.0-0.05	0.06-.01	0.11-0.15	0.16-0.2	0.21-0.25	0.26-0.3	0.31-0.35
UK MRSA	0	2	17	47	24	8	2
UK MSSA	0	1	17	52	17	11	2
Maltese MRSA	0	2	24	47	24	3	0
Maltese MSSA	0	1	31	45	22	1	0

Table 4.4: Percentage of eluted stain from adhered isolates at a range of absorption values to fibrinogen coated 96-well polystyrene plates

	Percentage of isolates (%)															
Absorbance ranges at 620nm	0.0-0.05	0.06-.01	0.11-0.15	0.16-0.2	0.21-0.25	0.26-0.3	0.31-0.35	0.36-0.4	0.41-0.45	0.46-0.5	0.51-0.55	0.56-0.6	0.61-0.65	0.66-0.7	0.71-0.75	0.76-0.8	0.81-0.85
UK MRSA	2	6	16	10	11	8	10	11	11	4	6	4	1	0	0	0	0
UK MSSA	0	4	4	5	13	14	18	13	7	2	5	6	3	2	4	0	1
Maltese MRSA	0	1	2	5	14	21	20	16	12	5	3	1	0	0	0	0	0
Maltese MSSA	0	1	5	16	14	20	17	14	6	6	1	0	0	0	0	0	0

4.4 Discussion

The aim of this study was to investigate how the production of four virulence factors, namely DNase, haemolysin, lipase and proteinase, differed in clinical isolates of MRSA compared to MSSA. During the course of this investigation 680 isolates of *S. aureus* (476 MRSA and 204 MSSA) were tested for all four virulence factors. Although some differences in the production of these factors were seen in isolates from the two different countries, no statistically significant differences were observed between isolates of MRSA and MSSA.

The majority of isolates tested positive for the production of DNase, although six isolates tested negative (two: UK MRSA, one: Maltese MRSA and three: Maltese MSSA). Findings of DNase negative isolates have been reported before (Rao *et al.*, 2002). As DNase production is used to differentiate *S. aureus* isolates from other *Staphylococcus* spp., which do not produce this enzyme, the discovery of DNase negative isolates is of interest. Widespread loss of this enzyme in *S. aureus* isolates could lead to difficulty in identification for both clinical and other microbiology laboratories. The exact role of DNase in *S. aureus* pathogenesis is unknown, although DNase negative isolates have been reported to be of clinical significance (Rao *et al.*, 2002). Of the MRSA isolates in this study that tested as DNase negative, all had increased levels of antibiotic resistance. The UK isolates were resistant to nine and ten antibiotics and the Maltese isolates were resistant to eight antibiotics (See Table 4.5 for isolates antibiogram profile). Although an insignificant number of isolates tested DNase negative compared to the number of strains tested, it may be significant that the loss of this enzyme has occurred in isolates of high resistance. Of the three MSSA isolates that tested negative for the production of DNase, one was resistant to two antibiotics and two were resistant to four antibiotics (See Table 4.6 for isolate antibiogram profile). Although once again this is an insignificant number of isolates, plus these isolates were not as highly resistant as the MRSA isolates, these finding are potentially of concern. An investigation into the type of antibiotic to which the MRSA were resistant to does not show a major similarity between the three isolates except that there were all resistant to β-lactam antibiotics. Similar findings were observed when the resistance patterns of the MSSA were examined, although all these isolates were found to be resistant to ciprofloxacin.

Table 4.5 Antibiogram profiles of the MRSA isolates (two UK isolates and one Maltese isolate) that tested negative for DNase production.
KEY:R-Resistant, S-Sensitive. AK-amikacin, C-chlorampenicol, Cip-ciprofloxacin, 2-clarithromycin, CD-clindamycin, E-erythromycin, G-gentamicin, LZD-linezolid, M-methicillin, PG-penicillin G, Rip-rifampicin, T-teteracycline, Tec-tecioplanin and V-vancomycin.

Isolates	Panel of antibiotics														No. of antibiotic to which
No.	AK	C	Cip	2	CD	E	G	LZD	M	PG	Rip	T	Tec	V	the isolates are resistant
7	R	R	R	R	R	R	S	S	R	R	R	R	S	S	10
38	R	R	R	R	R	R	R	S	R	R	S	S	S	S	9
M278	S	S	S	R	R	R	R	S	R	R	R	R	S	S	8

Table 4.6 Antibiogram profiles of the MSSA isolates (three Maltese isolates) that tested negative for DNase production.
KEY:R-Resistant, S-Sensitive. AK-amikacin, C-chlorampenicol, Cip-ciprofloxacin, 2-clarithromycin, CD-clindamycin, E-erythromycin, G-gentamicin, LZD-linezolid, M-methicillin, PG-penicillin G, Rip-rifampicin, T-teteracycline, Tec-tecioplanin and V-vancomycin

Isolates	Panel of antibiotics														No. of antibiotic to which
No.	AK	C	Cip	2	CD	E	G	LZD	M	PG	Rip	T	Tec	V	the isolates are resistant
M98	S	S	R	S	S	R	S	S	S	R	S	R	S	S	4
M280	S	S	R	S	S	S	S	S	S	R	S	S	S	S	2
M281	S	S	R	R	R	R	S	S	S	S	S	S	S	S	4

The majority of isolates tested positive for the production of the DNase and no statistically significant differences between MRSA and MSSA were found. The amount of enzyme produced by each isolate was also measured. Once again, no statistically significant difference between MRSA and MSSA was found. However, there was a major difference in the radius of the zone of test between the UK and Maltese isolates. It was found that the Maltese isolates peaked at a radius of 2-3 mm and the largest zone record was 7mm, whereas the UK isolates peaked at 6mm and produced zone radius up to 9 mm. It can be speculated that the difference in zone radius was due to the different strains present in the sample group.

Haemolysin, unlike the other factors tested, is not an extracellular enzyme but a toxin. Of the four factors tested, haemolysin, gave the highest level of negative results (of approximately 40% in each group) *i.e.* did not produce the factor. The fact that 40% of the isolates tested did not produce this factor is interesting as iron has been recognized to be a crucial factor in infection (Trivier *et al.*, 1996). However in the 60% of isolates found to produce this toxin and are therefore potentially more pathogenic then the haemolysin negative counterparts. Although these factors are not produced until post-exponential phase, it is believed that other mechanisms not tested in this study might aid the intake of iron before this stage and in the absence of haemolysin production. Of the isolates that produced the toxin some differences were seen. One difference was the radius of the zone produced on the agar plate. The result observed was between 1 to 4 mm, although only the MRSA isolate produced zone radius of 4mm. Slight differences were seen between MRSA and MSSA isolates in the country specific sample base. Almost double the number of Maltese MSSA isolates produced zone radius of 1mm compared to the Maltese MRSA. In the zone radius from 2 to 4mm a higher percentage of MRSA isolates produced zone radius in this range. Similar results were observed in the UK sample base, although the difference was not as pronounced. This may indicate that MRSA requires more iron than MSSA in the post-exponential phase when these factors are produced. The number of antibiotics to which the MRSA isolates were resistant to, varied from three to eight antibiotics, therefore suggesting that there was no link between the level of antibiotic resistance and iron dependency in MRSA isolates.

In the lipase test a higher percentage of Maltese isolates produced this enzyme when compared to the UK isolates. The majority of UK isolates, both MRSA and MSSA, produced zone of radius between 4 to 6 mm. The Maltese isolates also matched this pattern. However there was a large increase observed in the percentage of Maltese isolates

that produced zone radii of 7 to 8mm that occurred in both MRSA and MSSA. This increase could be proposed to occur due to strain variance between the two countries.

The final test carried out was the production of total proteinase in which >88% of the isolates produced this factor although once again no statistically significant difference could be detected between MRSA and MSSA. The majority of isolates from both countries produced a zone radius of 3mm, although a similar percentage of UK MRSA isolate produced zone radius of 2mm. Interestingly at a radius of 5mm there was a sudden increase in the percentage of Maltese MSSA isolates compared to the other isolates that had decreased to below 5%. This observation was probably due to strain variance.

Of the four factors tested no statistically significant difference between MRSA and MSSA could be detected, although a difference in the production of the factors was observed between the two countries.

During the course of this investigation the adherence ability of the isolates was also tested. The isolates were allowed to adhere to both polystyrene and fibrinogen coated plates. The isolates were compared between the two countries (UK MSSA vs. Maltese MSSA) and comparison was made between the different isolates within countries (UK MRSA vs. UK MSSA). Comparison of the MRSA isolate between the UK and Malta, showed that there was a statically significant difference between the stained isolates in the polystyrene plate (UK was great than Malta, $p<0.005$). Interestingly the reverse was seen in the fibrinogen coated plate (Malta was great than UK, $p<0.05$). This higher binding affinity to fibrinogen could suggest that in an infection, the Maltese isolates may have a pathological advantage over the UK isolate. As the bacteria may be able to infect a wound easier than the UK isolate (through binding to fibrinogen); however, this was not determined in this study. In the unstained results for the fibrinogen plates, the significant difference observed was reversed (UK was great than Malta, $p<0.05$); however, this does not effect the result in the stained data (*i.e.* if the Maltese results were higher in the unstained, this would suggest a higher cell population and therefore a greater number of cell available to bind. This would therefore explain the higher reading seen in the stained data).

In the MSSA isolates, no statistically significant difference could be detected between the two countries in the isolates binding to the polystyrene plates. In the

fibrinogen coated plates the results suggested a statistically significant difference between the two countries (UK was great than Malta, $p<0.005$). However, in the unstained results this statistically significant difference between the two countries (UK was great than Malta, $p<0.005$) was also present. In the unstained results the absorption of the bacterial suspension was measured in order to asses the cell density. For example the higher the absorbance value, the higher the number of bacterial cells and *vice versa*. In the fibrinogen coated plates there was a significant difference in this reading between the two countries. Therefore in the stained group it can not be determined whether the significant difference recorded was due to a higher cell population in the UK isolates or to an increased binding capacity of the cells to the plate. Hence difference in the stained results is negated by the result in the unstained result.

In the UK isolates no statistically significant differences could be detected in the unstained results between the MRSA and MSSA isolates. However, in the binding to the polystyrene plates and the fibrinogen coated plate there was a statistical difference between the MSSA to the MRSA isolates ($p<0.005$). In the Maltese isolates the only statistically significant difference was observed between the isolate on the fibrinogen coated plates. In these isolates the reverse was seen for the UK isolates (MRSA was great than MSSA, $p<0.05$).

This is the first study to look at the binding affinity between MRSA and MSSA isolates as well as the comparison between MRSA and MSSA isolates between these two countries. The differences in the binding affinity demonstrated in this study are most likely due to strain differences between the sample groups.

During the course of the investigation, four extra-extracellular factors and the adherence ability of isolates of *S. aureus* were recorded to detect whether there was a difference in the production of this various factors between MRSA and MSSA. Although slight differences were detected in the sample groups from each country, no statistically significant differences could be detected between MRSA and MSSA isolates. Similar findings have been reported by other groups that have concentrated on other virulence factors produced by *S. aureus*. For instance Schmizt *et al.*, (1997) studied the production of enterotoxin A-D and TSST-1 in 181 MRSA and 100 MSSA isolates and showed no difference in the production of these factors between the isolates. Similar findings were reported by another group that looked at the production of haemolysin (Coia *et al.*, 1992) in which no differences were reported in the haemolysin production between MRSA and

MSSA. Interestingly, differences were recorded between isolates of MRSA that were either sensitive or resistant to amminoglycosides. However, this was related to strain variation within these isolates (Coia *et al.*, 1992).

Moreover, when examining MRSA and MSSA isolates between the years 1980-1984 and 1999, differences were reported in the production of the toxin–gene (TSST-1 and Enterotoxin-C) (Endo *et al.*, 2004). This group showed the prevalence of strain production of these gene-increases between the two time scales. In the MRSA isolates, production increased from 67 to 91% and in the MSSA isolates from 30 to 55%. Whether these sorts of changes have occurred before, it is difficult to say and would require further investigation.

In summary no difference could be detected in the production of pathogenicity factors in the MRSA and MSSA isolates in this study, regardless of origin. This is the first study to compare the production of extra-cellular enzymes produced by MRSA and MSSA since the majority of work to date has been focusing on the production of toxins (Coia *et al.*, 1992; Schmizt *et al.*, 1997; Endo *et al.*, 2004). This present study and work of others on toxin production suggest that MRSA are no more or less pathogenic then MSSA. However due to increasing antibiotic resistance MRSA are hard to treat and may persist in the body for longer and hence the severity of the infection may be greater due to prolonged exposure to pathogenic factors. Further study is required to monitor the production of all *S. aureus* pathogenicity factors. This could be achieved by looking for the loss or gain of factors to help provide a better understanding of the pathogenesis of this continually evolving bacterium.

5.0 Strain determination of clinical isolates of MSSA and MRSA by *SmaI* digest, with DNA fragment analysed by Pulse-Field Gel Electrophoresis

5.1 Introduction

Typing *S. aureus* beyond the species level has become an important tool in helping to identify the number of strains of *S. aureus* in a disease outbreak and monitoring the spread of different strains of *S. aureus,* both nationally and internationally (Tenover *et al.*, 1995). Phagetyping was the main way by which *S. aureus* was typed for decades (Witte, 2000). This method was based on isolates carrying bacteriophages that lysed some epidemiologically unrelated strains. This lead to the development of a panel of specific bacteriophages that could recognise different *S. aureus* strains (van Leeuwen, 2003). However, phagetyping has now been replaced by molecular typing methods due to a number of factors including high technical skill, effort in maintaining high quality phage set and poor type ability amount MRSA strains (van Leeuwen, 2003).

There are a number of different genotyping methods utilised with *S. aureus* strains that include Plasmid Profile Analysis (PPA), Southern hybridization analysis of digested chromosomal DNA, Polymerase chain reaction (PCR) (including arbitrarily primed PCR, restriction digest PCR, rRNA gene spacer PCR, randomly amplified polymorphic DNA), and Restriction Fragment Length Polymorphism (RFLP) (Witte, 2000). All of these methods have advantages and disadvantages. PPA has a high degree of reproducibility but a low discriminatory power. In contrast, PCR methods have good to high discriminatory power, however they lack reproducibility (Witte, 2000).

Currently, RFLP using the *SmaI* endonuclease, with fragments separated by Pulse-Field Gel Electrophoresis (PFGE) is currently accepted as the "gold standard" method for typing *S. aureus* and MRSA due to the high degree of reproducibility and excellent discriminatory power (Witte, 2000).

How long PFGE will remain the gold standard method for typing *S. aureus* remains to be seen; however, it is likely to be superseded by new techniques such as Multi Locus Sequence Typing (MLST) and sequencing of the polymorphic X region of the protein A gene (spa typing) (Hallin *et. al.*, 2006). Both of these methods have similar discriminatory powers as PFGE, Hallin *et. al* (2006) show that PFGE and spa typing have discriminatory power of 96% and 98% respectively. Additional both MLST and spa typing are faster, easier to use and interpret, then conventional PFGE typing (Shopsin *et al.*, 2001)

Internationally accepted guidelines for the interpretation of PFGE band patterns were proposed by Tenover *et al.*, in 1995. These guidelines were designed for a small set of isolates (typically ≤30) possibly involved in an outbreak of illness. However, they could be used for a larger collection. A key factor in these the guidelines was the criteria for interpreting the banding patterns and the criteria placing the banding pattern into one of four categories, which are summarised in Table 2.1.

However, although interpretation of the banding pattern could then be standardised across laboratories, different PFGE running parameters used by such laboratories meant that multi-centre comparison of banding patterns still could not be carried out.

Recently, twelve international laboratories across ten different countries in the European Union harmonised the protocols for PFGE by forming one method that each laboratory would use, thereby allowing the comparison of results from one country to another (Murchan *et al.*, 2003). The key to this harmonisation method was the running parameter at which the PFGE units were run. A number of different studies suggest numerous methods. However, the parameter set out by the HARMONY project was a two block run (for more information refer to PFGE protocol: section 2.1.12), as this was found to give the best results, in regard to discrimination and reproducibility.

PFGE is used to type strains of MRSA during an outbreak, to determine how many strains are involved. However, it has been used to monitor the spread of specific MRSA strains, such as the Brazilian-clone (Aires DE Sousa *et al.*, 1998) and the Iberian clone (Heym *et al.*, 2002).

Malta and the UK are included in the top five European countries showing high levels of MRSA (Anon 3, 2004). In section 3.1-3.4 the similarity in antibiotic resistance levels between the two countries was investigated while in this chapter the strain similarity between the two countries has been determined. This is the first time strain profiles from these two countries were compared. The isolates represented a large cohort of Maltese isolates from the main general hospital and a local London based cohort of samples from the UK. The aim of this chapter was to type the *S. aureus* isolates collected in this study and thereby divide them into different strain based group. Furthermore the strain profile from the two countries was compared. The isolates were typed using PFGE, the HARMONY protocols and the band patterns were interpreted by the criteria devised by Tenover *et al.* (1995).

5.2 Materials and Methods

5.2.1 Bacterial strains

A total of 680 isolates were used, in this part of the study, taken from the culture collection held at Kingston University. The isolates consisted of: 257 and 216 MRSA and 114 and 93 MSSA isolates from UK and Malta respectively. A control reference standard NCTC 8325 was used due to variable mobility of lambda concatemers.

5.2.2 Pulse-Field Gel Electrophoresis (PFGE), staining, visualising and photographing the gel

The methods for PFGE analysis, staining visualising and photographing the gel were carried out as described in sections 2.1.13 and 2.1.14

5.2.3 Analysis and interpretation of banding pattern

The methods for banding pattern analysis and interpretation were carried out as described in sections 2.1.15 and 2.1.16.

5.3 Results

Of the 680 isolates tested, PFGE profiles were determined for a total of 84.3% of isolates (573/680). Profiles were determined for 84.1% of UK isolates (312/371) and 83.2% of Maltese isolates (263/309). Profiles for 15% of isolates (105/680) could not be determined. These included 15.9% of UK isolates (59/371) and 14.8% of Maltese isolates (46/309).

Appendix 4 shows the complete dendrogram for the 573 isolates tested (the 84.1% of the isolates), analysis of which was determined using three criteria. These criteria were the guidelines determined by Tenover *et al.*, (1995), the similarity in banding patterns using published data (Anon 7, 2000) and the isolates similarity of 60% or greater. Due to the size of the dendrogram it was divided into 10 main groups to make analysis easier. However similarity within these groups was less than 60%. Only isolates with a similarity of greater than 60% could be seen as related.

Table 5.1 show an overview of division, with Appendix 3 and 5 showing the division of the isolates into the main groups, the strain types and sub-strain types. A total of 47 different strains of *S. aureus* were determined in the dendrogram (See appendix 4). Of the 47 strains, 3 were identified as possible UK epidemic MRSA (EMRSA) strains and 1 strain type was identified as a possible local Maltese strain.

Main group one contained 4 strain types (see Table 5.1, Appendix 3 and 5a). The group had a similarity of 33% and the majority of these strains only contained one or two isolates. Main group two also contained 4 strains types with strain type 8 having five sub-types (see Table 5.1, Appendix 3 and 5a). This group had a similarity of 40%. Main group three contained 4 strain types, with strain type 9 having four sub-types and strain type 12 having two sub-types (see Table 5.1, Appendix 3 and 5a). This group had a similarity of 48%. The predominant isolates in these first three groups were MRSA for the UK, then equal numbers of MRSA and MSSA from Malta and the smallest number of isolates were MSSA from the UK.

Main group four contained 17 strains and the predominant number of isolates tested (see Table 5.1, Appendix 3 and 5b). The group had a similarity of 43%. Strain 13 had a main group and 18 sub-types (Appendix 3; 13a-13r). This strain also had similar banding pattern to UK EMRSA-16 (Figure 5.1a). The main group was identified to be the most

similar to this epidemic strain and the sub-type had either insertions or deletions compared to the main banding pattern. The predominant isolates in this group were UK MRSA followed by Maltese MRSA and then the MSSA from both countries.

Strain 14 and 15 consisted of 3 sub-types each with no main banding type and mainly containing UK MRSA isolates. The largest strain group was strain 16. This strain had 8 main groups and 38 (Appendix 3; 16a-16al) sub-types. Moreover, this strain main group had similar banding pattern to UK EMRSA-15 (Figure 5.1b). This strain also contained the largest amount of isolates from both countries the predominant isolates however were MRSA. The sub-types varied in the number of isolates they contained with some containing two to three isolates and the largest containing thirty eight. As with strain 13, the sub-type varied from the main group by either insertion or deletion compared to the main banding pattern.

The remaining strains in this main group could not be linked to existing banding profiles and all varied in the number of isolates within the strain type and the number of sub-type if any. Strains 18, 21, 24, 26, 27, 28, 29 contained no sub-type and only contained a few isolates. Strain 17 had three sub-types and contained mainly Maltese MRSA whereas strain 19 and 20 both had six sub-types and contained mainly UK MRSA. Strain 22 had two sub-types, and strain 23 had four whereas the final strain, 25, had seven sub-types.

Main group five contained 8 strains and had a group similarity of 46% (see Table 5.1, Appendix 3 and 5e). Strain 30 contained three sub-types (Appendix 3; 30a-30c) and all these isolates were UK MSSA. Strain 31 contained one isolate and this was also a UK MSSA. Strain 32 contained one main type and 18 sub-types (Appendix 3; 18a-q) and had a similar banding pattern to UK EMRSA1 (Figure 5.1c). However the two patterns were not an exact match. In addition, the predominant isolates in this strain were UK MSSA therefore it was not UK EMRSA 1. However it could be the MSSA strain from where the MRSA strain came from.

Strains 33 and 36 both contained three sub-types. Strain 34 had no sub-types whereas strain 35 and 36 had 5 and 4 sub-types respectively.

Main group six contained 3 strains and the majority of isolates in this group were Maltese isolates although there were some UK isolates (see Table 5.1, Appendix 3 and 5f). This group had a similarity of 52%. Strain 38 had 4 sub-types and except for one isolate all

were Maltese isolates. Strain 39 had no sub-types. Strain 40 contained 18 sub-types and only two isolates in this strain were UK isolates, whereas the majority were Maltese isolates. Additionally the predominant isolates were MRSA therefore suggesting that it could be a local Maltese epidemic strain.

Main group seven contained one strain type (No. 41), that had 4 sub-types and contained only Maltese isolates (see Table 5.1, Appendix 3 and 5g).

As with group seven, main group eight contained one strain, No. 42 that had 3 sub-types and contained both UK and Maltese isolates (see Table 5.1, Appendix 3 and 5h).

Main group nine contained two strains with a group similarity of 50%. Strain 43 had no sub-types and contained one isolate of MSSA from both countries (see Table 5.1, Appendix 3 and 5j). Strain 44 had 4 sub-types (Appendix 3; 44a-d) and the majority of isolates were UK MSSA isolates.

The final main group, group ten, had three strains with a group similarity of 33%. Strain 45 and 47 both contained one isolate (see Table 5.1, Appendix 3 and 5k). Strain 46 contained 3 sub-types and the predominant isolates within this strain were Maltese MRSA.

To summarise banding patterns were determine for 573 isolates which were grouped into a total of 47 strain types. Strains 13, 16 and 32 were found to have similar banding pattern to three UK EMRSA strain (UK EMRSA-16, -15 and -1 respectively), additionally the majority of the isolates were grouped into strain type 16. Strain type 40 and 41 mainly contain only Maltese isolates (except for two UK isolates) and therefore could represent of local individual strain types.

Table 5.1 Represents a brief overview of the PFGE profile for the 573 isolates tested. The isolates were divided into ten main group's, addition the table also shows the isolates divided into forty-seven strain types and sub-types range as well as the percentage of group similarity.

Main group	Type	Sub-type	Group similarity %
1	1		33
	2		
	3		
	4		
2	5		40
	6		
	7		
	8	a-d	
3	9	a-d	48
	10		
	11		
	12	a-b	
4	13	a-r plus main	43
	14	a-c	
	15	a-c	
	16	a-al plus main 1-8	
	17	a-c	
	18		
	19	a-f	
	20	a-f	
	21		
	22	a-b	
	23	a-d	
	24		
	25	a-g	
	26		
	27		
	28		
	29		
5	30	a-c	46
	31		
	32	a-r plus main	
	33	a-c	
	34		
	35	a-e	
	36	a-c	
	37	a-b	

Main group	Type	Sub-type	Group similarity %
6	38	a-d	52
	39		
	40	a-r	
7	41	a-d	54
8	42	a-c	51
9	43		50
	44	a-d	
	45		
10	46	a-c	33
	47		

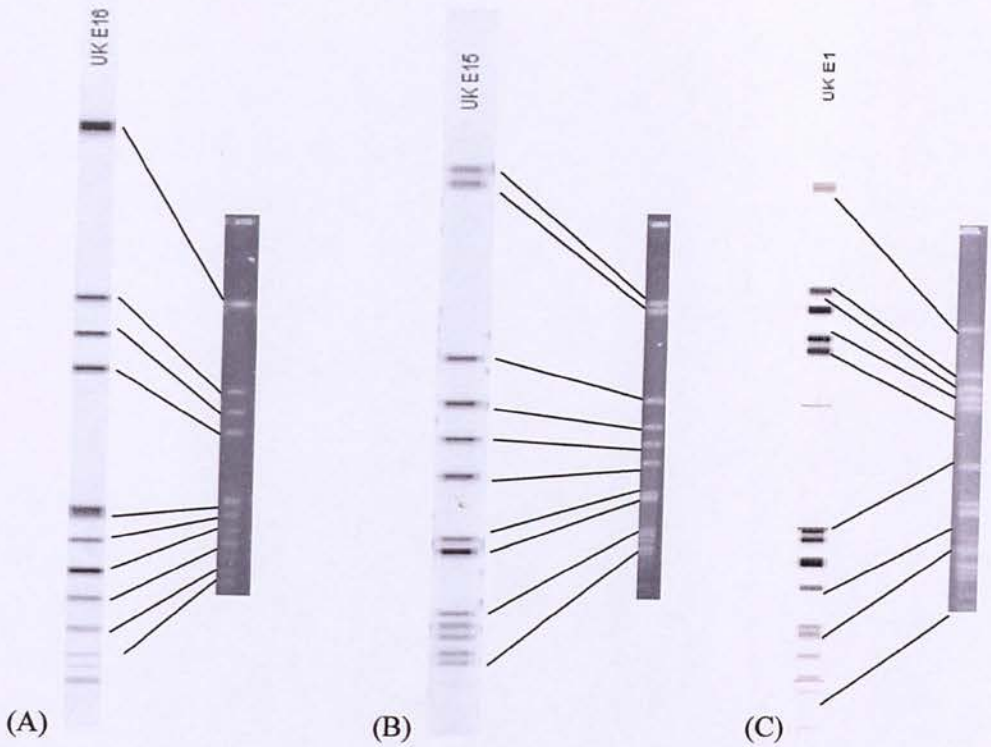


Figure 5.1: A comparison between the published banding pattern (of EMRSA strains 1, 15 and 16) from the HARMONY project (left hand band with a white background) (Murchan *et al.,* 2003) and patterns derived from current this study (right hand band with a black background).

A: Banding pattern for EMRSA UK16 (UK E16)

B: Banding pattern for EMRSA UK15 (UK E15)

C: Banding pattern for EMRSA UK1 (UK E1) and banding pattern of a EMRSA-like MSSA from strain 32

5.4 Discussion

The aim of this chapter was to type the isolates used in this study by PFGE using the endonuclease *SmaI*. Of the 680 isolates tested, PFGE banding patterns were determined for 84.3% (573/680) of isolates. Profiles were determined for 84.1% of UK isolates (312/371) and 83.2% of Maltese isolates (263/309). A dendrogram was constructed using these banding patterns and a total of 47 different strains were identified, with varying number of sub-types within the strains. The number of isolates in each strain group varied from 1-2 and up to 255 in the largest strain group (strain 16).

Of the 47 strains, two were identified as being similar to published banding patterns for EMRSA and these were strains 13 and 16. Strain 13 consisted of a main type and 18 sub-types with the main type being the most similar to the published banding pattern (Murchan *et al.*, 2003). This strain (13) was similar to the UK EMRSA 16 strain of MRSA which is one of the most common types of EMRSA in UK hospitals. The majority of isolates in this strain group were UK MRSA however there were also some UK MSSA isolates present. Interestingly, Maltese isolates were also present in this strain group. In the main type, nine MRSA Maltese isolates were present, these isolates having very similar profile to published data of UK EMRSA 16. Maltese isolates of both MRSA and MSSA were also present in the sub-type group. The presence of MSSA isolates in the sub-type could suggest the clonal MSSA ancestor of the EMRSA 16.

Strain 16 consisted of 8 main types and 38 sub-types with the main type being the most similar to the published banding pattern. These strains were similar to the UK EMRSA 15 strain, once again one of the most common types of EMRSA in UK hospitals. This strain group contained the highest number of isolates (255 isolates). As with strain 13, the majority of isolates were MRSA; however, this time an approximately equal number from each country were present (125 UK and 115 Maltese isolates). Strain 16 contains 8 main types and were dispersed between sub-types 16e and 16r (see Table 5.1 and Appendix 4d) having a 64% similarity between the groups. However, all of the isolates in these main groups were very similar in banding pattern to UK EMRSA 15 and contained no gene insertion or deletion. There are two possible reasons why these patterns did not group all together. Firstly, although the dendrogram was generated using a computer programme, the position of the band on photo of the gel had to be marked by the programme user. Therefore small amount of human error could account for slight differences in banding patterns. Secondly, potentially small differences in the temperature

of the run buffer used in the PFGE unit may have affected the distance the bands moved down the gel. However, this affect is dealt with by the bio numerical programme, therefore minimizing this affect.

The possible identification of strain 13 and 16 as EMRSA 16 and 15 respectively was interesting, in that, these strain types were seen in the Maltese isolates. This is the first time that these strains have been identified in Maltese isolates of *S. aureus*. However a similar apparent spread of isolates from one country to another has been reported in other studies (Heym *et al.*, 2002; da Sliva Combra *et al.*, 2003). One such strain that has been reported the "Iberian clone" (Aires de Sousa *et al.*, 1998; Heym *et al.*, 2002; da Sliva Combra *et al.*, 2003). This EMRSA has been isolated in Spain, Portugal, Belgium, Scotland, Italy, Germany and New York (USA) (da Sliva Combra *et al.*, 2003). Another strain of EMRSA that has been isolated in numerous countries is the "Brazilian Clone" which has been isolated in Brazil, Argentina, Uruguay, Portugal and the Czech Republic (Aires de Sousa *et al.*, 1998; da Sliva Combra *et al.*, 2003). Therefore the possible spread of EMRSA 15 and 16 to Malta from the UK is feasible.

Strain 32 was identified as being similar to a published banding pattern for UK EMRSA 1; however, the majority of the isolates in this strain were MSSA (from the UK) and not MRSA, due to them being methicillin sensitive. Therefore this could mean that they are either isolates of UK EMRSA1 that have lost the *mecA* gene through deletion or a possible MSSA related clone or ancestor.

Strain 40 could not be identified as being similar to any published banding patterns; however, interestingly the majority of isolates were Maltese, except to two UK isolates, 159 (MRSA) and 185 (MSSA). In addition, the majority of the isolates were MRSA, however there were some MSSA. It could be proposed that this is a local Maltese strain of *S. aureus*. Furthermore due the large amount of MRSA isolates it could be suggested that this is a local EMRSA. However this was not confirmed in this study.

Of the remaining isolates both MRSA and MSSA from both countries showed no similarity with published information. Moreover, no further clusters of isolates were found.

Of the remaining 15% (105/680) of undetermined profile isolates, 15.9% were from the UK (59/37) and 14.8% were Maltese isolates (46/316). Interestingly, for both countries the percentage of isolates for which their profile could not be determined was very similar,

with only 1.1% difference. Where profiles were not achieved, isolates were re-tested and Gram stain and coagulase tests were also carried out in order to check the identification of the culture.

There are a number of possible reasons why profiles could not be determined for these isolates. Firstly, these isolates may have been resistant to lysostaphin since this resistance has also been documented in other studies (Boyle-Vavra *et al.*, 2001). However this resistance has been seen in association with the development of vancomycin resistance. This was not displayed by any of the isolates that were tested in this study. In some isolate profiles, a DNA smear could be seen, therefore suggesting that the cell wall had been lysed. The DNA however, could have become damaged and how this damage occurred could not be determined but may have occurred during plug preparation or during storage of DNA plugs.

In conclusion, from the isolate collection a total of 573 DNA banding profiles were determined with a total of 47 strain types identified. Of these strains, two, namely 13 and 16 were possible UK EMRSA and one strain group was identified as a local Maltese strain. The remaining strains could not be matched to any published banding pattern and varied in the number of MRSA and MSSA isolates from both countries.

6.0 Novel Chromogenic ester Agar Medium for *Staphylococcus aureus*

6.1 Introduction.

Lipase is an enzyme, active against a number of natural and synthetic lipid substrates as well as water-soluble triglycerides and Tweens (Arvidson, 1983). These substrates are broken down into their constituent alcohols and fatty acids by ester linkage hydrolysis (Arvidson, 1983). To date five different staphylococcal lipase genes from different species have been cloned and sequenced, two from *S. aureus*, two from *S. epidermidis* and one from S. *hyicus* (Nostro *et al.*, 2001). Of the two lipases produced by *S. aureus*, only one is described as a true lipase, an enzyme that is able to hydrolyse water-insoluble long-chain triacylglycerols as well as water-soluble triacylglycerols (Arvidson, 2000). The second enzyme can only hydrolyse water-soluble short chain triacylglycerols, and should be referred to as a short-chain glycerol ester hydrolase (Arvidson, 2000).

Currently, there are two established ways of detecting lipase activity involving the use of nutrient agar supplied with 1% Tween 80 or nutrient agar supplemented with egg yolk (Cooke *et al.*, 1999). Tween 80 degradation is specific for the lipase enzymes, whereas egg yolk plates demonstrate both lipase and phospholipase activity (Cooke *et al.*, 1999). Lipolysis of Tween 80 is characterised by the appearance of a zone of opacity, due to precipitation of liberated fatty acids that combine with calcium to form salts (Nostro *et al.*, 2001). The Tween 80 assay is normally used for qualitative detection of *S. aureus* lipase activity as the results are difficult to quantify (Nostro *et al.*, 2001).

Chromogenic media are increasingly useful tools in diagnostic laboratories for the rapid detection of bacterial species traits and strain characterisation. Examples of these media include CHROMagar for the detection of *Candida sp.* (Cooke *et al.*, 2002), oxacillin resistance screening agar base (ORSAB) for the detection of MRSA (Perry *et al.*, 2004) and Chromogeneic *Salmonella* esterase agar (CSE) for the detection of Salmonellae (Cooke *et al.*, 1999).

The aim of the current study was to evaluate the variability of clinical strains in terms of lipase activity and to establish whether any variance could be exploited in a diagnostic or analytical context. The clarity and rapidity of resolution was also compared to traditional Tween 80 based tests.

6.2 Materials and Methods

6.2.1 Bacterial strains

In this part of the study a total of 680 isolates were used taken from the culture collection held at Kingston University. The isolates consisted of: 257 and 216 MRSA and 114 and 93 MSSA isolates from UK and Malta respectively.

6.2.2 Lipase detection

The methods for Tween 80 assays were carried out as described in section 2.1.8.

6.2.3 Chromogenic substrate and medium preparation

The methods for chromogenic substrate and media preparation were carried as described in sections 2.1.16 and 2.1.17.

6.2.4 Inoculation of test chromogenic media

The methods for inoculation of test chromogenic media were carried out as described in section 2.1.18.

6.3 Results

6.3.1 Lipolytic activity against Tween 80

Analysis of the UK isolates demonstrated that 72% (190/260) of the MRSA and 83% (98/118) of the MSSA were Tween 80 positive. A higher number of the Maltese isolates 94% (203/216) of the MRSA and 88% (82/93) of the MSSA proved to be Tween 80 positive.

6.3.2 Lipolytic activity against chromogenic substrate

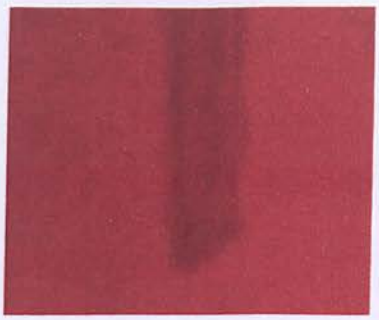
Before incubation the different substrates produced a range of different coloured agars. SRA-propanoate and SRA-butyrate, were orange, SRA-octanoate deep yellow in colour, with a yellow oily film on the surface of the agar. SRA-decanoate, SRA-laurate and SRA-myristate all appeared pale yellow in colour, whilst SB_ZTM-butyrate appeared a light purple and SB_ZTM-acetate appeared maroon.

Following incubation for 48 hours colour changes were seen with all but one of the substrates, namely SB_ZTM –butyrate. This data is summarised in Table 6.1. The colour changes varied with the different substrates and the size of the zone of colour produced around the isolates also varied in size. The coloured zone ranged from 3-4mm from the isolates when SRA-myristate was used, to total colour change of the plate with SRA-propanoate. All isolates produced colour changes with SRA-propanoate and SRA-butyrate (see Figure 6.1), both of which resulted in a coloured agar, with the colour radiating across the plate. This colour change was not seen in the control plates. The colouration also appeared strongest immediately adjacent to the colony. Isolates on both SRA-propanoate and SRA-butyrate showed activity that diffused away from the colony causing the entire plate to turn red within 24 hours.

All isolates produced colour changes on SRA-octanoate, although the changes were only seen within the colony, turning a deep purple. These colour changes were observed after 24 hours of incubation. No defined colour change was observed in the agar; however, most isolates exhibited a loss of the base media colour intensity in the agar immediately around the colony (Figure 6.2).

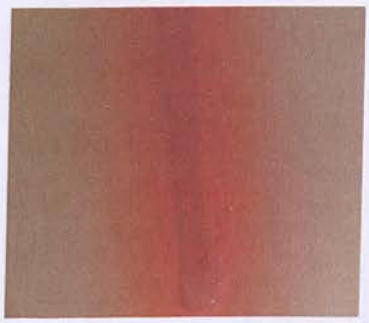
Table 6.1: Summary of test result showing the colour of both the isolate and the agar plate, the time in which the changes were seen and the percentage of isolates from each country that were positive.

Substrate	No. of Hydrocarbons in side chain "R"	Colour change in		Time for detection of colour development (hrs)	UK Isolates showing positive test (%)	Maltese isolates showing positive test (%)
		Colony	Agar			
SRA-propanate	3	Red	Red	24	100	100
SRA-butyrate	4	Red	Red	24	100	100
SRA-octanoate	8	Purple	Yellow	48	100	100
SRA-decanoate	10	Pink	Pink Zone	48	97	98
SRA-laurate	12	Pink	Pink Zone	48	92	90
SRA-myristate	14	Pink	Pink Zone	48	0.5	2
SB_ZTM-acetate	2	Purple	Purple	48	100	100
SB_ZTM- butyrate	4	No change	No change	48	100	100



6.1.

6.2



6.3

Figures 6.1-3: The range of colours produced on three different types of chromogenic substrate media.

Figure 6.1: SRA-propanoate, where the colony and surrounding agar has turned red.

Figure 6.2: SRA-octanoate where only the colony was turned purple.

Figure 6.3: SRA-decanoate where the colony and some of the surrounding agar has turned pink producing a zone of colour around the colony.

Both SRA-decanoate and SRA-laurate plates produced a dark pink zone around the isolates (see Figure 6.3). These zones of colour were larger on the SRA-decanoate plates then on the SRA-laurate plates. The rest of the plate remained the same pale yellow colour. The majority of colonies on both SRA-decanoate and SRA-laurate plates did not change in colour; however, some isolates showed a slight change in colour to pale pink. Most of the isolates changed colour on these two different plates, 97% (362/372) of the UK isolates and 98% (301/308) of the Maltese isolates changed colour on the SRA-decanoate plates. The UK isolates showed similar levels of substrate metabolism in both the MRSA and MSSA group 97% (253/257) and 98% (109/115) respectively. The Maltese isolates showed similar results with both MRSA and MSSA showing 98% (215/216, 90/92 respectively) of isolate metabolism of the substrate. On SRA-laurate plates 92% (345/372) of the UK isolates and 91% (279/308) of the Maltese isolates metabolised the substrate. The UK isolates showed similar level of metabolism when comparing the MRSA and MSSA group, showing results of 91% (235/257) and 94% (108/115) respectively. The Maltese isolates also showed similar results when comparing MRSA and MSSA, 90% (194/216) and 92% (85/92) respectively.

SRA-myristate was metabolised by the lowest number of isolates. A small zone of pale pink could be seen around the isolates, but the biomass did not change in colour. Only 0.5% (2/372) of UK isolates and 1.6% (5/308) of the Maltese isolates produced any colour change, with a 0.4% (1/372) of MRSA and 0.9% (1/115) of MSSA of the UK isolates and 0.9% (2/216) of MRSA and 3.2% (3/92) of MSSA groups of the Maltese isolates showing any apparent metabolism of this substrate.

As mentioned previously none of the *S. aureus* isolates produced any notable colour change on SB_ZTM-butyrate plates. Some slight colour changes were observed following incubation; however, when compared with controls no colour difference was evident, suggesting this slight change was a result of substrate degradation upon incubation. In addition no pigment uptake was observed in any of the isolates.

All isolates produced colour changes on SB_ZTM-acetate with the plates showing deep purple colouration which spread to cover the entire plate. The isolates also appeared to have

taken up the colour into the colony. The intensity of the colour of the agar may have made the colony to appear to have changed colour in some case.

6.3.3 Comparison of chromogenic substrates and Tween 80

Both Tween 80 and the chromogenic agar detected lipase activity of the isolates panel to varying degrees. The most notable difference between the two methods was the ease with which the result on the chromogenic plate could be read, due to the formation of colour on the chromogenic plate. Results from the Tween 80 assay can be difficult to determine, possibly sensitivity of the test due to low lipase activity. In these cases there is very little precipitation observed in the medium, which may lead to a false negative result being reported. When comparing the results obtained using the two different assay types, it could be seen that four of the chromogenic media, SRA-propanoate, SRA-butyrate, SRA-octanoate and SB_ZTM-acetate, were hydrolysed by all of the clinical isolates in the study. This outcome contrasts with the results of the Tween 80 assay which ranged from 73% (UK MRSA) to 94% (Malta MRSA) lipid hydrolysed by the same isolates.

Tween 80 medium required an incubation time of 48 hours before results could be read. However, both SRA-propanoate and SRA-butyrate results could be read clearly within 24 hours. This represented a dramatic reduction in the time required to read the completed test.

6.4 Discussion

In the current study two different agar based assays were examined for their efficiency in detecting lipase activity in clinical isolates of MRSA and MSSA from UK and Malta. The media compared nutrient agar supplemented with Tween 80 (1%) or one of eight novel chromogenic substrates. Of these two types of media, the action of lipase was most apparent when using the chromogenic agar plates due to the production of colour when compared to the precipitate seen with the Tween 80 based assay. A higher percentage of isolates demonstrated lipase activity on both media. All isolates demonstrated metabolism of the chromogenic substrates with fatty chain length up to and including octanoate (C=8); however, some of these isolates were recorded as having no activity according to the Tween 80 assay. This demonstrated that these short-chain fatty acid chromogenic substrates have a higher sensitivity than the traditional Tween 80 agar plates to the activity of the lipase enzyme. In the cases of MRSA isolates from the UK 27% (71/260) were recorded as being lipase negative on Tween 80 plates, but all isolates were positive on the short-chain fatty acid chromogenic substrates.

Of the eight different chromogenic substrates only two gave no or potentially discriminating results. SB_ZTM-butyrate gave no colour changes, whereas $\leq$2% of isolates showed colour change with SRA-myristate. One isolate each of MRSA, MSSA in the UK cohort and two MRSA and three MSSA isolates in the Maltese cohort produced colour on SRA-myristate plates.

The amount of colour that developed on the remaining plates appeared to be linked to the size of the fatty acid side-chain. On the plates that contained either the substrate SRA-propanoate, SRA-butyrate or SB_ZTM-acetate the colour change radiated across the plate. However on the plates containing SRA-octanoate the colour change was only seen within the colony and did not radiate out across the plate. Whereas the SRA-decanoate and SRA-laurate substrates, both produced colour changes that radiated away from the colonies, although the size of this zone of colour was greater with the SRA-decanoate plate than on the SRA-laurate substrate plates. The differences observed between SRA-decanoate and SRA-laurate may be explained by the fact SRA-decanoate is smaller than SRA-laurate by two hydrocarbons. This may suggest a variation in activity due to side chain length, in that the greater the side chain length the lower the activity of the enzymes. It has been suggested that the free fatty acids may

play a role in inhibiting phagocytosis (Arvidson, 1983) and it has also been shown that the long chain fatty acids are bactericidal (Arvidson, 1983). However, 80% of *S .aureus* strains produced an enzyme called fatty acid-modifying enzyme (FAME), that catalyses the esterification of the lipid to alcohols or cholesterol (Arvidson, 2000). However, it should be noted that this enzyme is most effective on saturated fatty acids with 15 to 19 carbons, therefore the reduction in hydrolysis of the substrate seen between SRA-decanoate and SRA-laurate, may be a mechanism to prevent the production of free long-chain fatty acids.

The differences in the amount of colour production may possibly be related to the different action of the two types of lipases. Avidson (2000) reported that *S. aureus* has two lipase genes *geh* (isolated in PS54 strain) and *lip,geh* (isolated in NCTC 8530 strain), encoding two different lipase activities, *geh* encoding for the true lipase and *lip,geh* encoding the short-chain glycerol ester hydrolase. Therefore it is possible that the zone of the colour seen may be due to the action of these two enzymes.

Colour changes seen with the SRA-propanoate, SRA-butyrate, SRA-octanoate and SB_ZTM-acetate maybe due to the action of short-chain glycerol ester hydrolase. Rosenstein *et al.*, (2000) reported a similar finding, referring to the enzymes as SAL-1. Rosenstein reported that SAL-1 has a strong preference of action against short-chain fatty acids, with maximal activity toward butyric acid esterified to glycerol, *p*-nitrophenol or umbellifrone (Rosenstein *et al.*, 2000). This activity decreases with acyl chain length of one methyl group above or below and hardly hydrolysed the larger chain trioctanyloglyercol (Kloos *et al.*, 1991). Zones of colour were seen following metabolism of the small fatty acid chain molecules, SRA-propanoate and SRA–butyrate and SB_ZTM-butyrate, as the hydrocarbon side chain length fell within the active range of short-chain glycerol ester hydrolase. True lipase can also hydrolyse short chain length water soluble triacylglycerols therefore the hydrolysis of these substrates may also be due to the true lipases as well as the short-chain glycerol ester hydrolases.

Colour changes with in SRA-decanoate, SRA-laurate and SRA-myristate may possibly be due to the action of the true lipase, as these can hydrolyse long-chain triacylglycerols. Interestingly, short-chain glycerol ester hydrolases are believed to be produced by most *S. aureus* isolates, whereas true lipases are less common (Arvidson. 2003). If the hydrolysis of SRA-decanoate and SRA-laurate are due to true lipase activity, these results suggest that in the

clinical isolates used in this study, there is a higher level of true lipase activity at 90% and above. This may contrast with other reports (Arvidson, 2003) and could represent a salient feature of hospital strains of MRSA and MSSA.

The colour changes seen with SRA-octanoate pose a problem, as the change in colour was only seen within the colony and did not radiate out from the colony as seen with the other substrate plates. It is tempting to speculate that the hydrolysis of the substrate was due to the action short-chain glycerol ester hydrolase, which shows little activity against the larger chain trioctanyloglyercol. This may explain the poor hydrolysis and lack of radiating colour around the colony. The resulting colour was only seen within the biomass where the concentration of the enzymes was greatest. However, true lipases have a wide range of activity and it has already been suggested that the hydrolysis of the short-chain triacylglycerols may be due to either true lipase or short-chain glycerol ester hydrolase, therefore we can speculate that SRA-octanoate should be hydrolysed by a true lipase. The SRA substrates (propanoate, butyrate, octanoate, decanoate, laurate and myristate) differ only in the size of the side chain "R". However SRA-octanoate required 66% (v/v) methanol/water to dissolve, whereas the substrate with longer side chains only required 4-8% (v/v). It was also noted that the SRA-octanoate plates, once set had an oily film on the surface of the plates. Therefore the lack of colour production on the SRA-octanoate may possibly be due not to lack or poor hydrolysis, but in fact lower concentration of available substrate due to difficulties experienced in dissolving the substrate and subsequent partial precipitation into the media.

The lack of colour change seen in SB_ZTM-butyrate was probably due to substrate failure rather then lack of hydrolysis by either short chain glycerol ester or true lipase. The size of the chain was within the range at which the enzyme works, in that it was not over eight hydrocarbons long. The colour that was seen also appeared on the control plates, therefore suggesting that the colour change was not due to metabolism.

In conclusion we have shown that seven of the eight chromogenic substrates tested demonstrated lipase activity in *S .aureus* more clearly than in the Tween 80 assay. Due to the lack of strain variability it is unlikely that the chromogenic substrates would be a useful tool diagnostically in relation to *S. aureus* although other pathogens might well benefit from their uses. However a role in research is indicated, there is limited knowledge in the area of *S.*

aureus lipase, the main focus of the work so far being directed toward *S. hyicus* (Rosenstein *et al.*, 2000). Therefore it is possible that these substrates may play a role in future *S. aureus* lipase research. One possible area where these substrates may prove useful is in the investigation over the genetic control of lipase production. Lipase activity is regulated by two genes; *agr* which positively regulates lipase production and the *sar* gene which negatively regulates lipase production. As yet, it is unknown which lipase is regulated by these genes as the substrate used did not discriminate between the lipases (Arvidson. 2000). Therefore these new chromogenic substrates could be used for this type of investigation as we have suggested that SRA-decanoate and SRA-laurate can only be hydrolysed by the true lipase. Therefore further work in the area of lipase and fatty acid metabolism in *S. aureus* should be encouraged.

7.0 Effects of antimicrobials on the growth, adherence, pathogenic factors production and pathogenic factors efficacy in isolates of MRSA

7.1 Introduction

There are an increasing number of reports showing evidence that sub-inhibitory levels of antibiotics can affect different virulence factors in *S. aureus*, including isolates of MRSA (Ohlsen *et al.*, 1998; Herbert *et al.*, 2001; Gemmell *et al.*, 2002). These reports have shown a difference in the production of toxins, extracellular enzymes and the adherence ability of the isolates when grown with an antibiotic. So far a large number of different antibiotics have been tested (Ohlsen *et al.*, 1998; Herbert *et al.*, 2001; Gemmell *et al.*, 2002). In 1998 Ohlsen *et al.*, (1998) tested a total of 31 different antibiotics (17 β-lactams, 2 glycopeptides, 5 amino glycosides, 2 fluoroquinolones, 1 macrolide and 4 of other classes) against a total of 19 clinical isolates of MRSA and MSSA (n=14 and n=5 respectively). Ohlsen and colleagues investigated the effect of these antibiotics on the toxins produced by *S. aureus* with special focus on alpha (α-) haemolysin (Ohlsen *et al.*, 1998). Whilst not all the antibiotics affected α-haemolysin, all penicillins and cephalosporins increased expression of this protein. In the same study clindamycin was shown to inhibit expression of α-haemolysin (Ohlsen *et al.*, 1998). In a later report clindamycin was shown to decrease production of other factors including serine protease and protein A even though it was found that production of coagulase and fibronectin binding protein increased (Herbert *et al.*, 2001). Decreases in the production of, α- and delta haemolysin as well as coagulase have also been shown with linezolid (Gemmell *et al.*, 2002). In addition, decreased adherence with increased killing has been observed with gemifloxacin (Sasso *et al.*, 2003). However, to date no studies have been carried out to investigate the effect of inhibitory levels of antibiotic on the rate of growth or production of virulence factors in isolates of MRSA.

The aim of this pilot study was to investigate the effect on MRSA virulence factors using two of the previously tested antibiotics, clindamycin and penicillin G and also to test the effect of these antibiotics at inhibitory and double inhibitory level on isolates of MRSA. The MRSA isolates tested were collected from UK and Malta and all were resistant to both antibiotics tested. The isolates varied in strain type and the number of antibiotics to which they were resistant. The virulence factors tested were DNase, haemolysin, total lipase and proteinase. Adherence to 96-well polystyrene plates and fibrinogen coated plates was also tested.

7.2 Materials and Methods

7.2.1 Bacterial strains

The following *S. aureus* strains were used as controls during the experiment: MRSA NCTC 12493 (Control 1) a methicillin resistant strain of *S. aureus* and Oxford *S. aureus* NCTC06571 (Control 2) a strain that is sensitive to all antibiotics. Antibiogram profiles for isolates used in this study were previously determined from studies detailed in chapter 3 (sections 3.2.3-3.2.4). A total of fourteen test isolates of *S. aureus* (all MRSA) were selected. These isolates are detailed in table 7.1

Table 7.1: Antibiogram profile for isolates used in the experiment.
Antibiogram key: Ak, amikacin; G, gentamicin; PG, penicillin G; M, methicillin; C, chloramphenicol; CD, clindamycin; 2, clarithromycin; E, erythromycin; Rip, rifampicin; Cip, ciprofloxacin; T, tetracycline

Lab Ref. No.	Country of origin	Antibiogram	Number of Antibiotic to which strains are resistant
7	UK	Ak, PG, M,C, CD, E, 2, Rip, Cip, T	10
27	UK	Ak, PG, M,C, CD, E, 2, Rip, Cip	9
195	UK	Ak, G, PG, M, CD, E, 2, Cip	8
60	UK	Ak, PG, M, CD, E, 2, Cip	7
63	UK	PG, M, CD, E, 2, Cip	6
193	UK	PG, M, CD, 2, Cip,	5
134	UK	PG, M, CD, E, 2, Cip	6
M309	Malta	Ak,PG, M,C, CD, E, 2, Rip, Cip, T	10
M21	Malta	Ak, G, PG, M, CD, E, Rip, Cip, T	9
M60	Malta	G, PG, M, CD, 2, Rip, Cip, T	8
M150	Malta	Ak, PG, M, CD, E, 2, Cip	7
M90	Malta	PG, M, CD, E, Cip, Rip,	6
M279	Malta	PG, M, CD, 2, Cip	5
M176	Malta	PG, M, CD, Cip	4

7.2.2 Growth under antibiotic pressure

The methods for growth of test isolate under antibiotic pressure were carried out as described in section 2.1.19

7.2.3 Virulence assays

Pathogenicity factor tests were carried out as described in sections 2.1.6-2.1.9, with the following changes. The 1ml aliquots that were removed from the flask after the 10-hour incubation, prior to 10µl (in triplicate) aliquots being spotted onto DNase, lipase, proteinase and haemolysin plates. A second (1ml) aliquot was filtered sterilized through a Millipore filter (0.2µm) (Galeman, UK) and the supernatant was collected. 10µl of the supernatant was then spotted in triplicate onto the DNase, lipase, proteinase and haemolysin plates.

7.2.4 Adherence assay

At the end of the 10-hour incubation, two aliquots of 1ml each were removed from each flask and kept for the virulence factor testing. The remaining sample was then used to determine adherence of cells to both fibrinogen coated and non-coated polystyrene 96-well plates as described in sections 2.1.10 and 2.1.11.

7.3 Results

7.3.1 Growth of isolates with penicillin G

Figures 7.1-7.16 show the growth curve of the isolates when grown in nutrient broth containing either the MIC (0.25mg/L) or double the MIC of penicillin G (0.5mg/L) and a sample with no antibiotic (SNA) added. Figure 7.2 shows the result of the Oxford *S. aureus* (control 2).Since this isolate was sensitive to the antibiotics cell decline occurred during the 10-hour growth period with total cell death in the broth containing double the MIC. Further testing of the virulence factor levels in this control could not be carried out tests; however, were carried out on the sample containing no antibiotic. Cell decline was also seen in isolates number 63 and M21 (Figures 7.7 and 7.11) with limited recovery of growth after six hours.

Of the remaining twelve isolates two different growth patterns appeared. Isolates 27, 60, 193 and M309 grew similarly to the MRSA control (Control 1) with a slight retardation in growth compared to the control curve (Figures: 7.4, 7.6, 7.8, 7.10 and 7.1 respectively). Additionally, in all of these curves the final population counts of cells were very similar. Within this group of isolates statistically significant difference ($p<0.05$, SNA was greater than MICx2) within the final population size was only observed for isolates 60 and 193.

The growth curve pattern for isolates 7, 195, M60 and M279 were similar (Figures: 7.3, 7.5, 7.12 and 7 .15 respectively) in which the first four hours a pronounced retardation in growth was observed. After 4 hours there was an apparent increase in cell growth resulting in similar number of cells as in the sample containing no antibiotic by the end of the 10-hour growth period. Isolates 7, 195 and M279 showed statistically significant difference ($p<0.05$, SNA was greater than MICx1 and SNA was greater than MICx2) in the final population size for both samples containing the antibiotic.

The growth curve patterns for isolates 134, M150, M90 and M176 were also similar as can be seen in Figures 7.9, 7.13, 7.14 and 7.16. All of these isolates grew similarly to the previous isolates (7, 195, M60 and M279), in that there was an initial retardation or cell death seen in the growth curve. After this initial retardation an increase in cell growth followed. However, there was a larger difference in the final cell number when compared to the sample with no antibiotic. Isolate M176 at both penicillin concentrations and isolate 7 at the MIC had

a statistically significant difference of $p<0.05$ (SNA was greater than MICx1). Whereas at both antibiotic concentrations (0.5mg/L and 0.5mg/L) isolates M150 and M90 and isolate 134 at double the MIC had a statistically significant difference of $p<0.005$ (SNA was greater than MICx1, SNA was greater than MICx2).

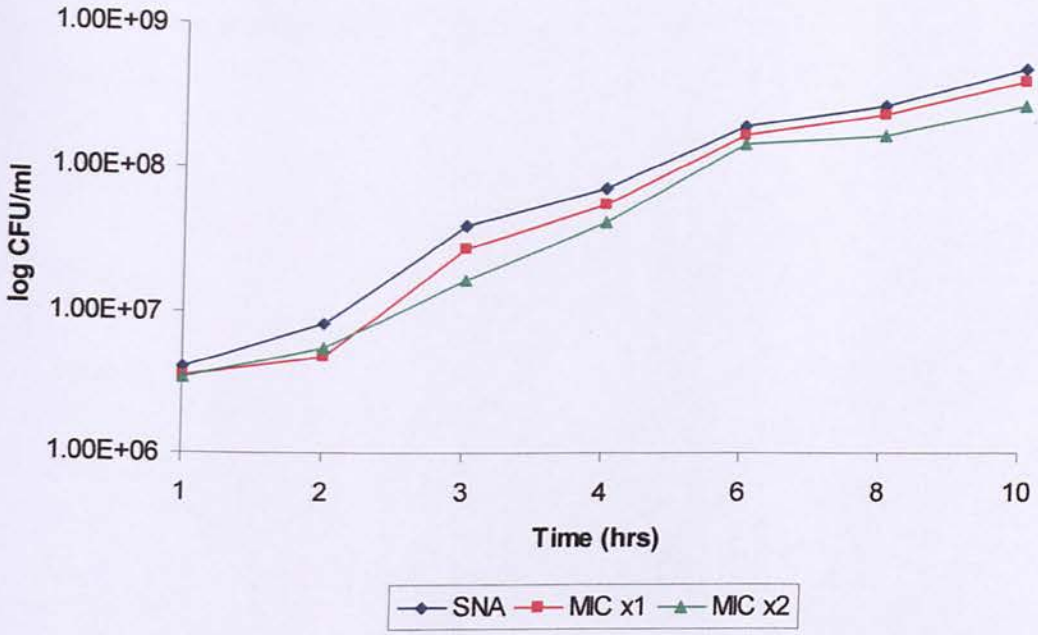


Figure 7.1: Growth of MRSA NCTC 12493 (control 1) recorded over a 10-hour period (± log S.E.M.).
The isolate was grown in nutrient broth containing no antibiotic or either the MIC (0.25mg/L) or double the MIC of penicillin G (0.5mg/L).

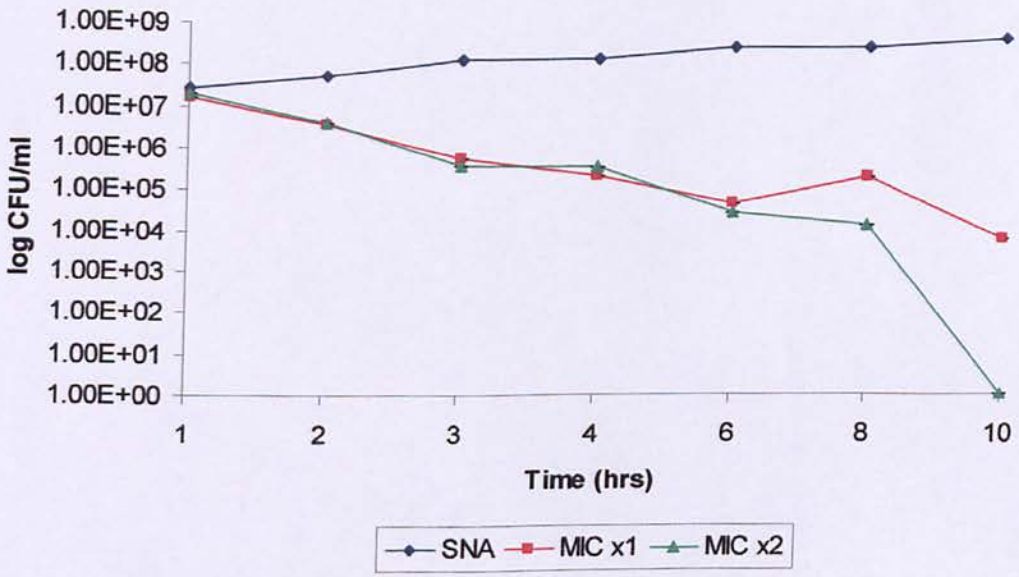


Figure 7.2: Growth of Oxford *S. aureus* NCTC 08325 (control 2) recorded over a 10-hour period (± log S.E.M.).

The isolate was grown in nutrient broth containing no antibiotic or either the MIC (0.25mg/L) or double the MIC of penicillin G (0.5mg/L).

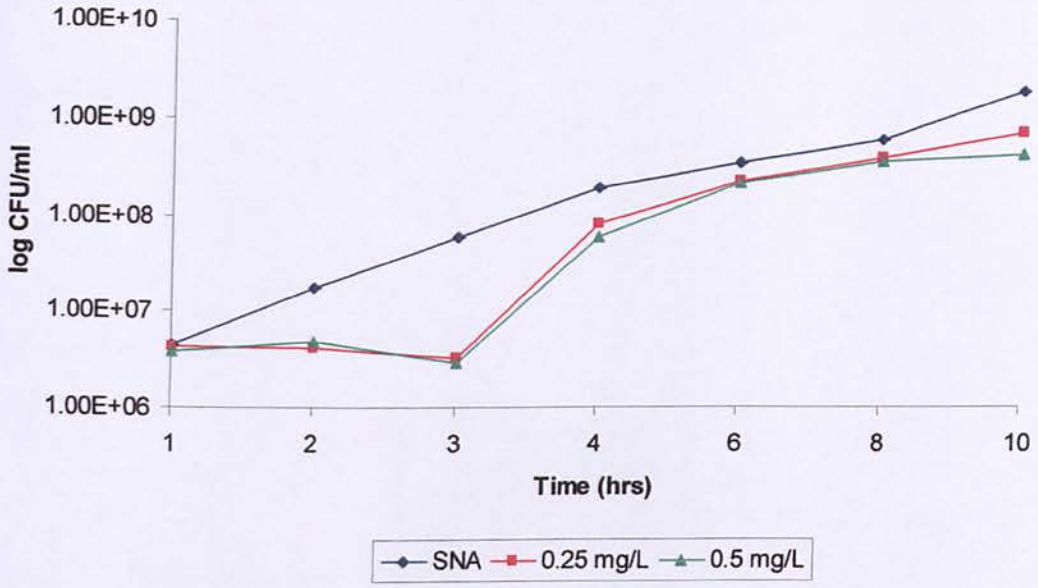


Figure 7.3: Growth of isolate No.7 in nutrient broth containing no antibiotic or either the MIC (0.25mg/L) or double the MIC of penicillin G (0.5mg/L) over a 10-hour period (± log S.E.M.).

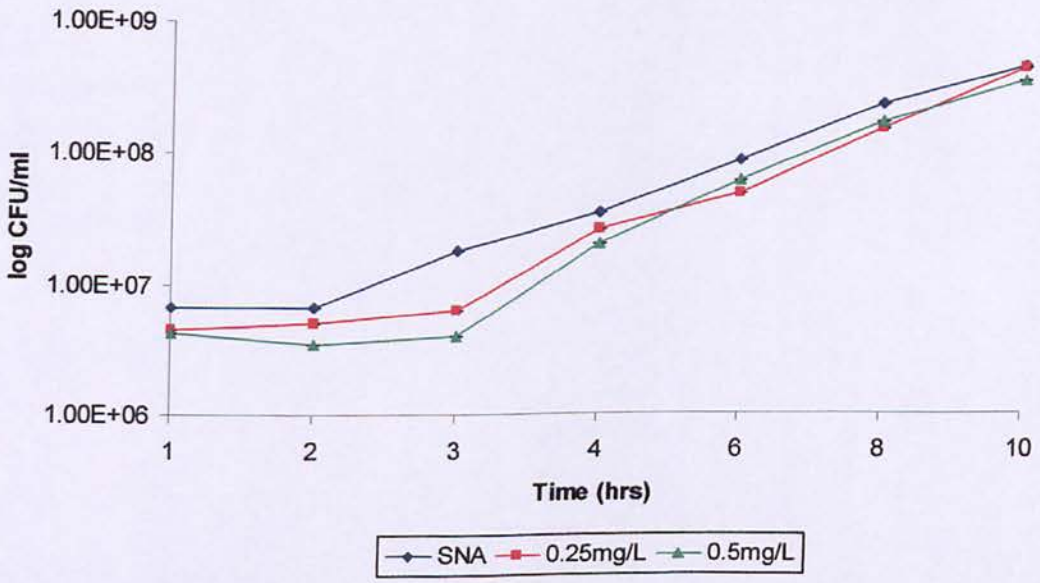


Figure 7.4: Growth of isolate No.27 in nutrient broth containing no antibiotic or either the MIC (0.25mg/L) or double the MIC of penicillin G (0.5mg/L) over a 10-hour period (± log S.E.M.).

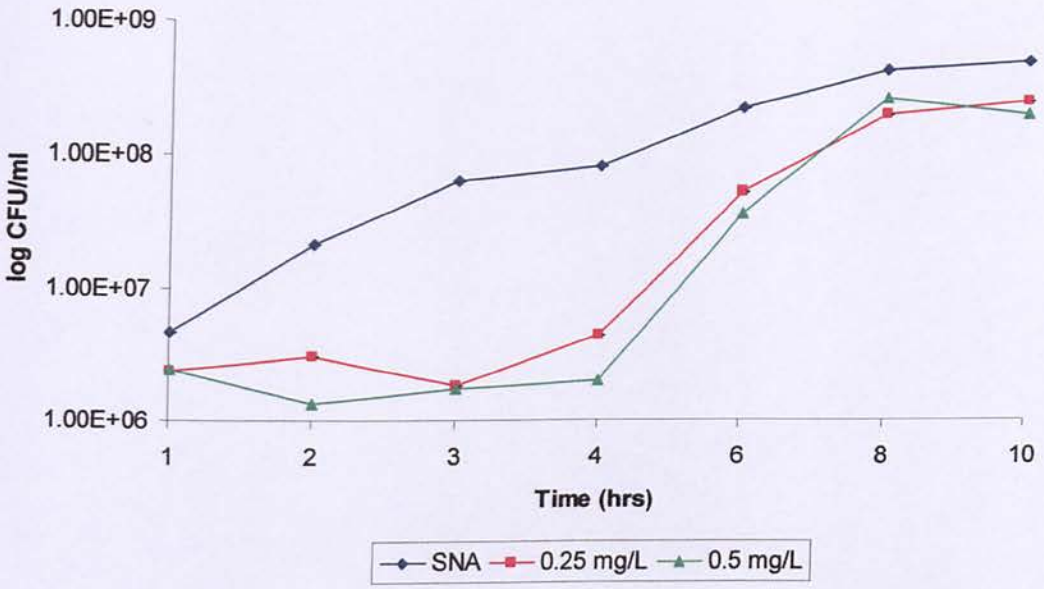


Figure 7.5: Growth of isolate No.195 in nutrient broth containing no antibiotic or either the MIC (0.25mg/L) or double the MIC of penicillin G (0.5mg/L) over a 10-hour period (± log S.E.M.).

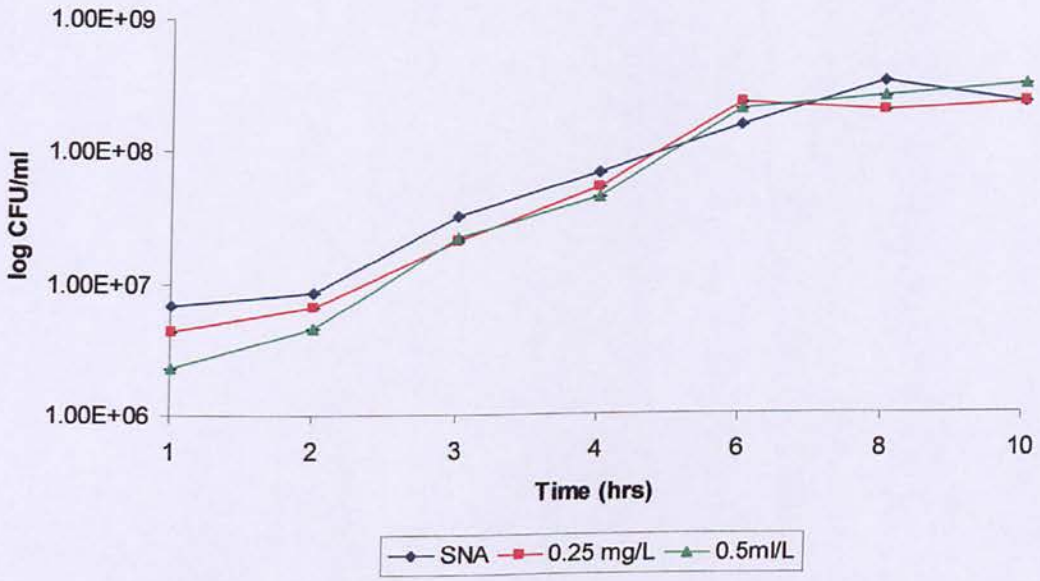


Figure 7.6: Growth of isolate No.60 in nutrient broth containing no antibiotic or either the MIC (0.25mg/L) or double the MIC of penicillin G (0.5mg/L) over a 10-hour period (± log S.E.M.).

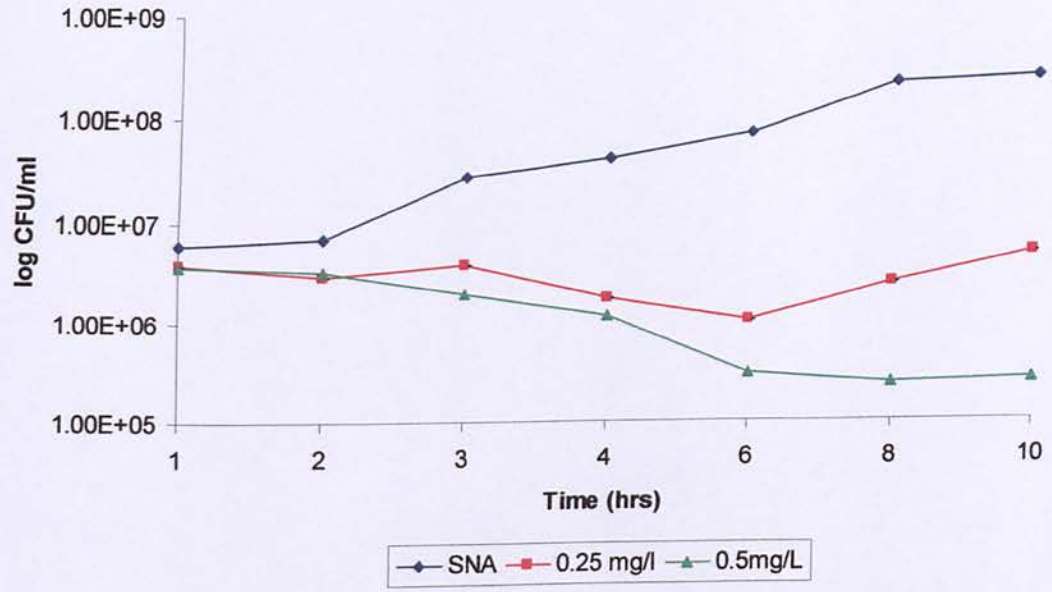


Figure 7.7: Growth of isolate No.63 in nutrient broth containing no antibiotic or either the MIC (0.25mg/L) or double the MIC of penicillin G (0.5mg/L) over a 10-hour period (± log S.E.M.).

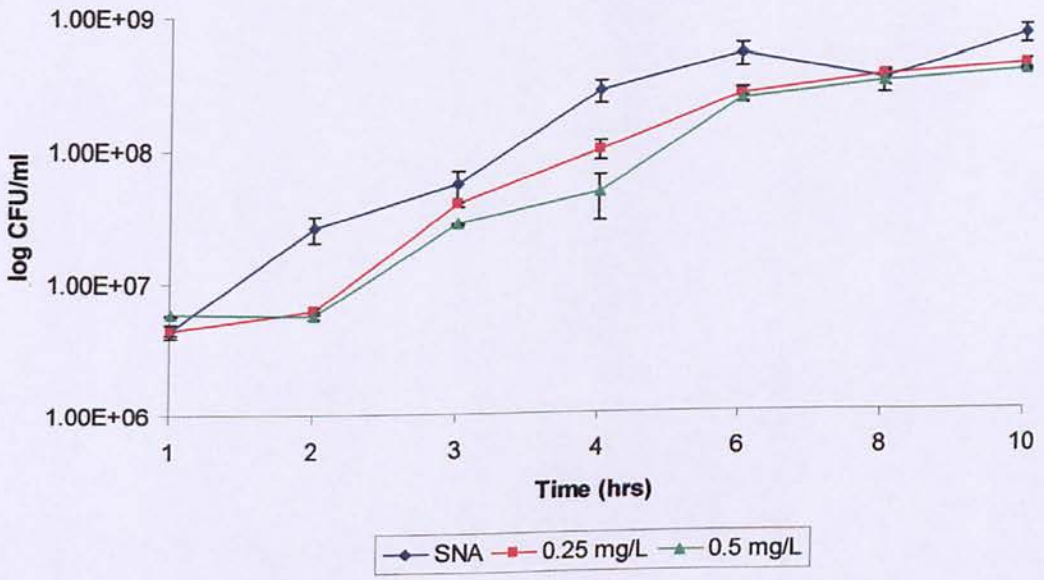


Figure 7.8: Growth of isolate No.193 in nutrient broth containing no antibiotic or either the MIC (0.25mg/L) or double the MIC of penicillin G (0.5mg/L) over a 10-hour period (± log S.E.M.).

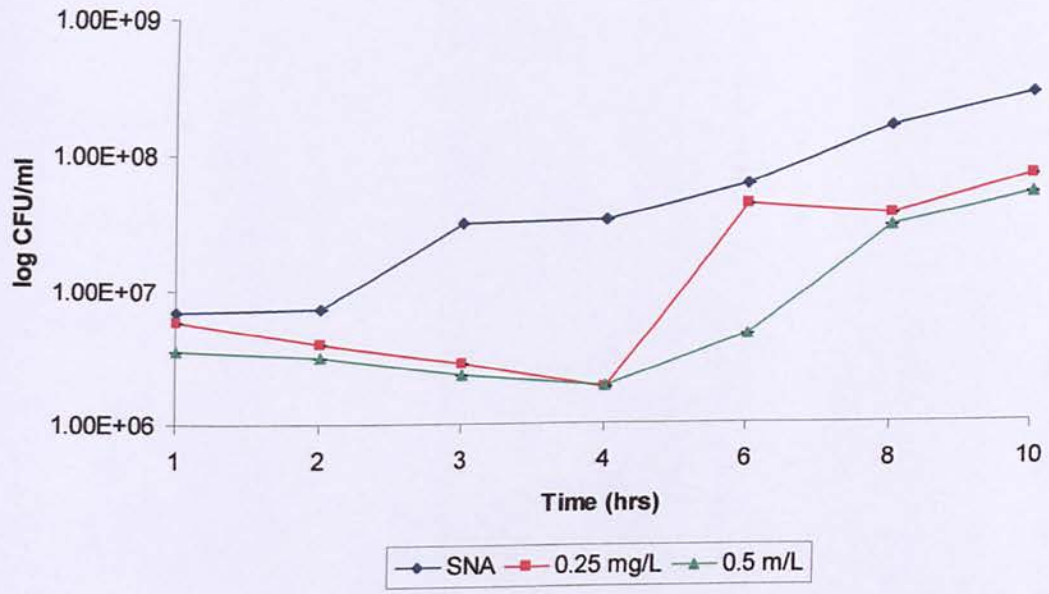


Figure 7.9: Growth of isolate No.134 in nutrient broth containing no antibiotic or either the MIC (0.25mg/L) or double the MIC of penicillin G (0.5mg/L) over a 10-hour period (± log S.E.M.).

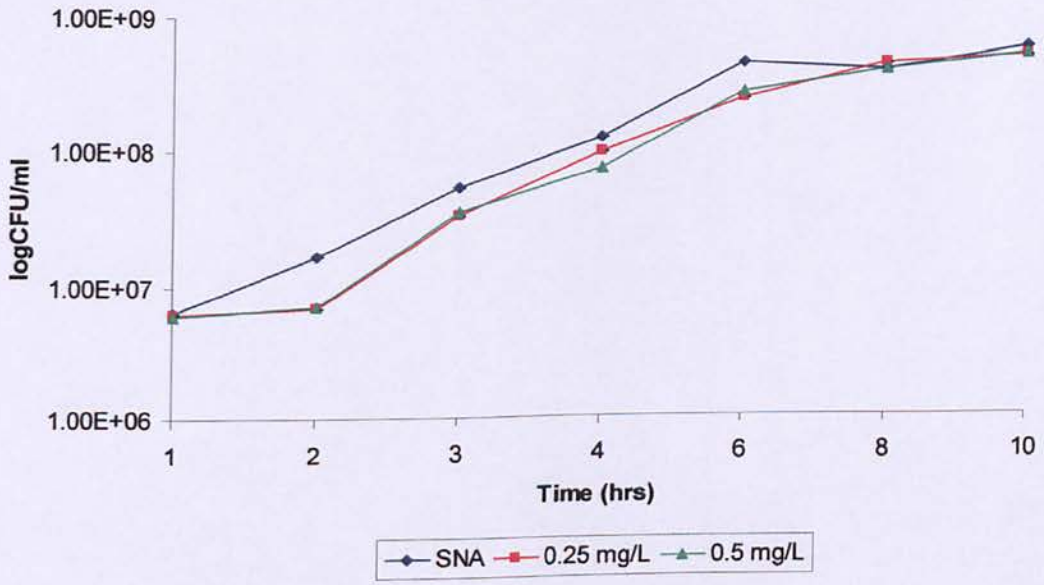


Figure 7.10: Growth of isolate No.M309 in nutrient broth containing no antibiotic or either the MIC (0.25mg/L) or double the MIC of penicillin G (0.5mg/L) over a 10-hour period (± log S.E.M.).

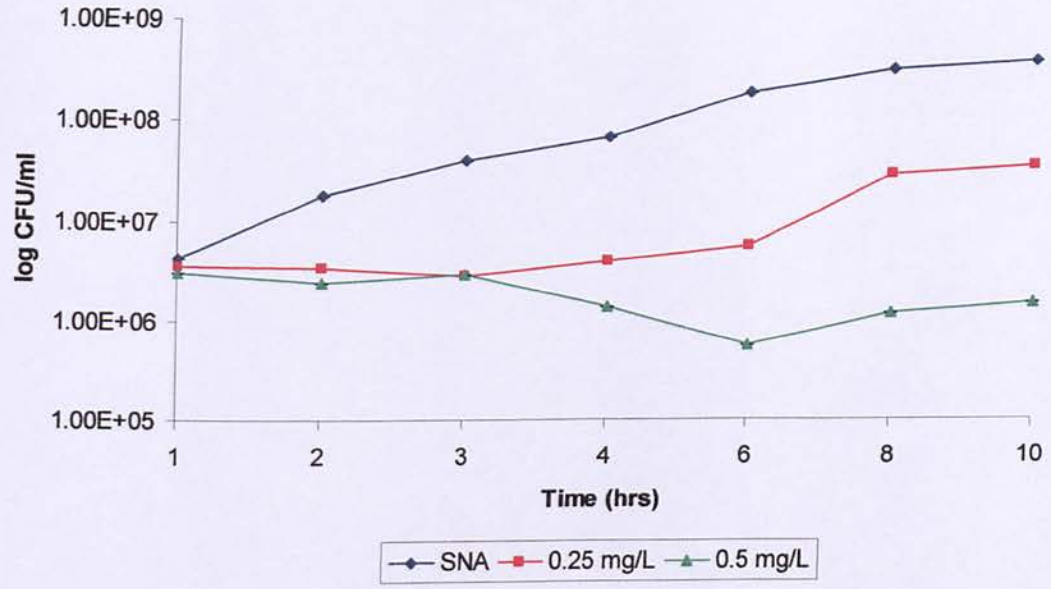


Figure 7.11: Growth of isolate No.M21 in nutrient broth containing no antibiotic or either the MIC (0.25mg/L) or double the MIC of penicillin G (0.5mg/L) over a 10-hour period (± log S.E.M.).

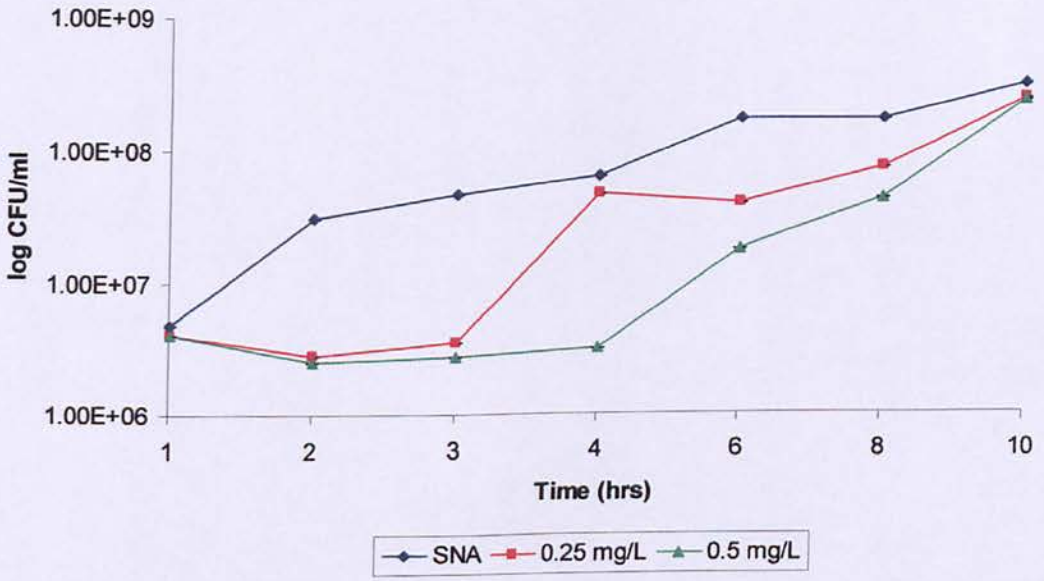


Figure 7.12: Growth of isolate No.M60 in nutrient broth containing no antibiotic or either the MIC (0.25mg/L) or double the MIC of penicillin G (0.5mg/L) over a 10-hour period (± log S.E.M.).

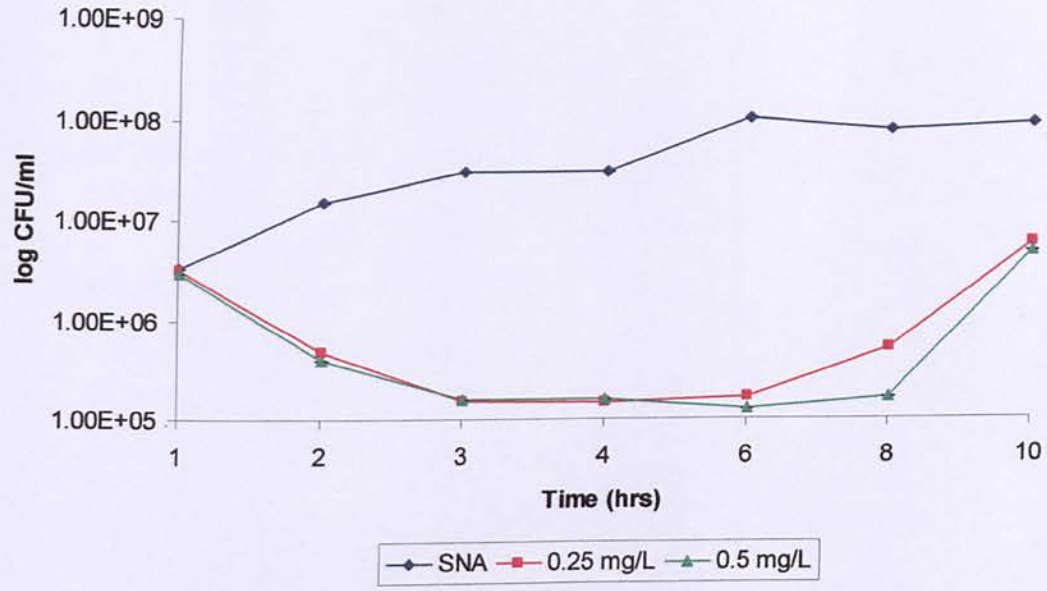


Figure 7.13: Growth of isolate No.M150 in nutrient broth containing no antibiotic or either the MIC (0.25mg/L) or double the MIC of penicillin G (0.5mg/L) over a 10-hour period (± log S.E.M.).

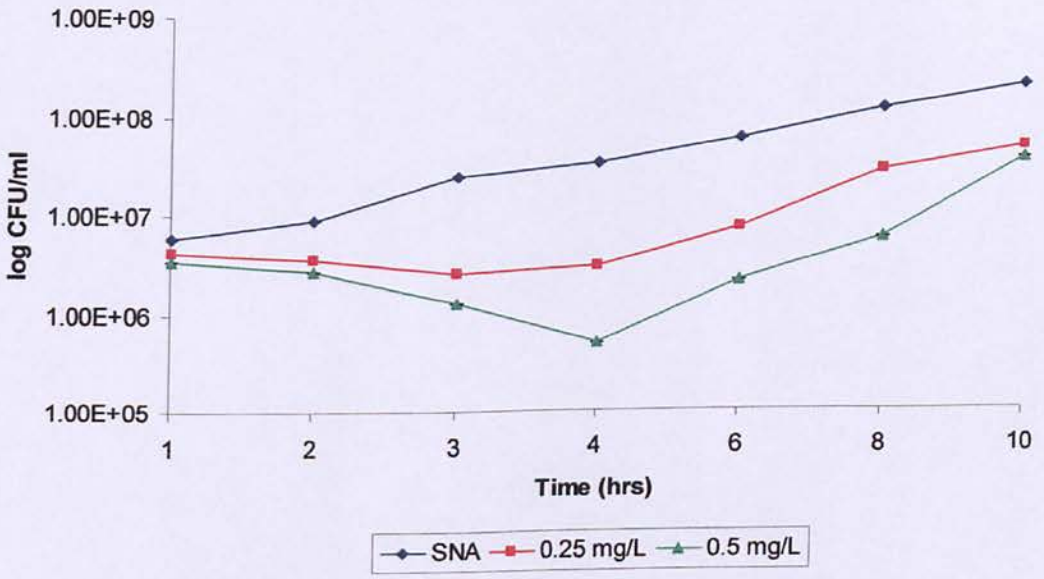


Figure 7.14: Growth of isolate No.M90 in nutrient broth containing no antibiotic or either the MIC (0.25mg/L) or double the MIC of penicillin G (0.5mg/L) over a 10-hour period (± log S.E.M.).

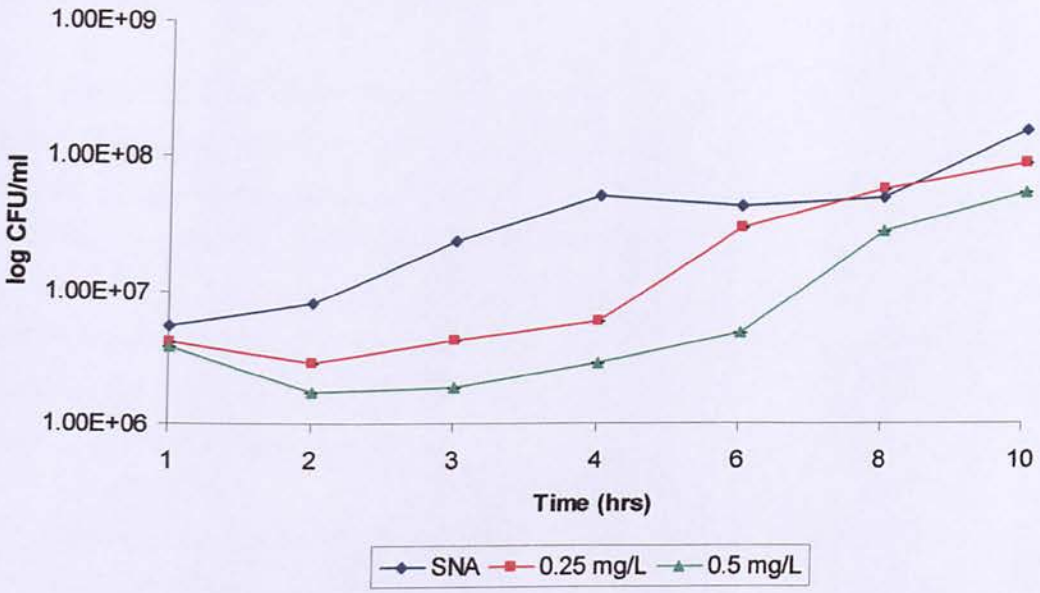


Figure 7.15: Growth of isolate No.M279 in nutrient broth containing no antibiotic or either the MIC (0.25mg/L) or double the MIC of penicillin G (0.5mg/L) over a 10-hour period (± log S.E.M.).

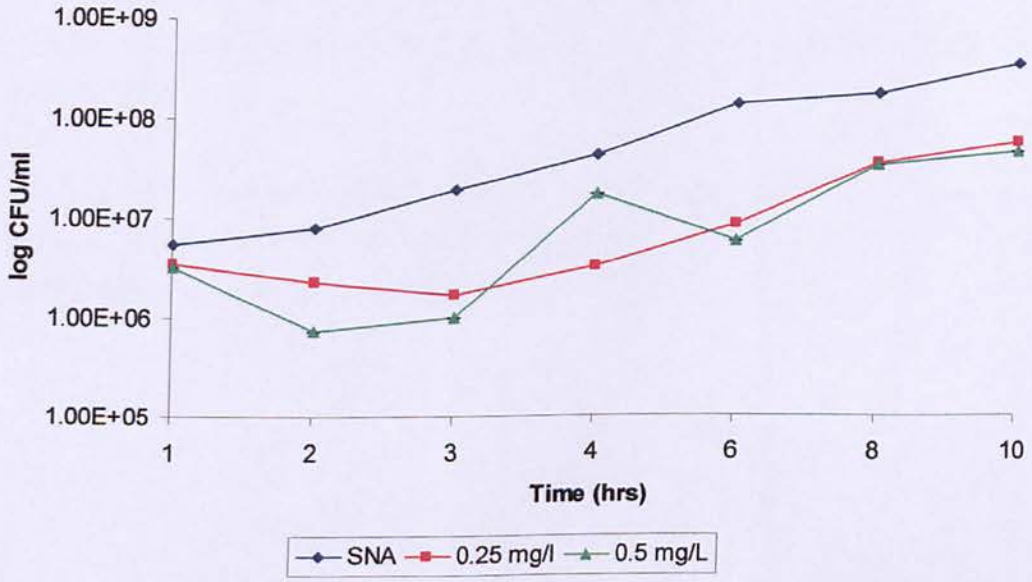


Figure 7.16: Growth of isolate No.M176 in nutrient broth containing no antibiotic or either the MIC (0.25mg/L) or double the MIC of penicillin G (0.5mg/L) over a 10-hour period (± log S.E.M.).

7.3.2 Growth of isolates with clindamycin

Figures 7.17-7.32 show the growth of the isolates in nutrient broth containing either the MIC (1mg/L) or double the MIC of clindamycin (2mg/L) compared to media with no antibiotic. In this experiment three different curves were produced. The first group included MRSA NCTC 12493 (Control 1) and isolates number 60 and M150 (Figures: 7.17, 7.22 and 7.29 respectively). All showed similarity with only the MICx1 and MICx2 lines showing any difference with the control. Statistically significant differences in the size of the final cell population were seen with isolate 60 ($p<0.05$, SNA was greater than MICx1 and $p<0.005$, SNA was greater than MICx2).

The second group contained the Oxford *S. aureus* (control 2) and isolates 7, 27, 195, 137 and M3098 (Figures: 7.18-7.21, 7.25 and 7.26 respectively). All strains showed initial retardation of growth up to 4hours. After this time the growth appeared to recover. Isolates 7 and 134 showed a statistically significant difference ($p<0.05$) when compared to the normal MIC (SNA was greater than MICx1) and a statistically significant difference ($p<0.005$) when compared with double the MIC (SNA was greater than MICx2). Isolate 27 was compared with double the MIC and a statistically significant difference was observed (SNA was greater than MICx2) ($p<0.05$).

The final group containing isolates 63, 193, M21, M60, M90, M279 and M176 (Figures: 7.23-7.24, 7.27-7.28 and 7.30-7.32 respectively) showed a gradual decrease in the cell population with no recovery in the growth.

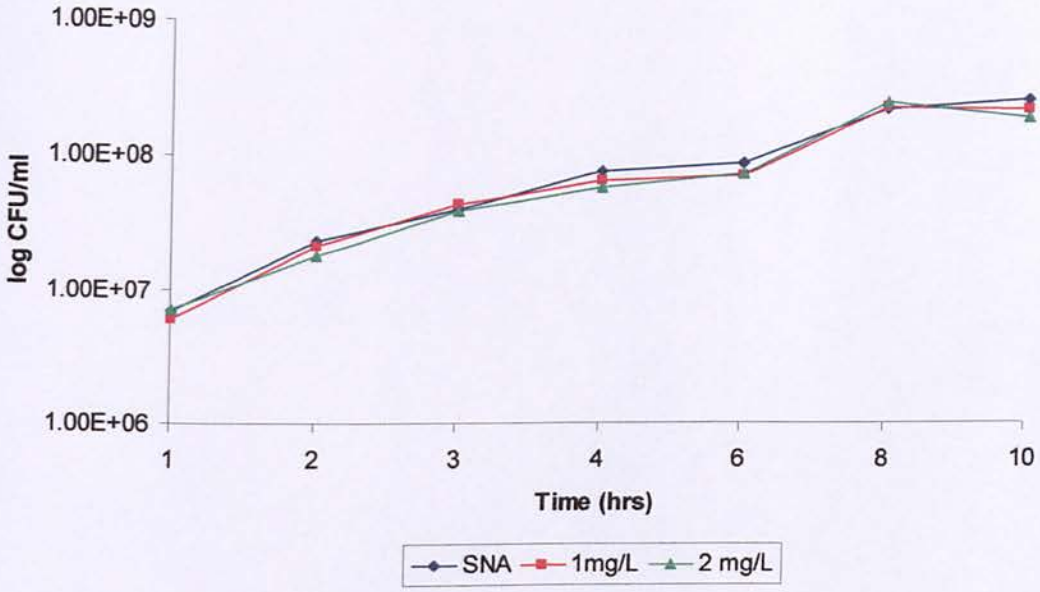


Figure 7.17: Growth of MRSA NCTC 12493 (control 1) in nutrient broth containing either the MIC (1mg/L) or double the MIC of clindamycin (2mg/L) or no antibiotic over a 10-hour period (± log S.E.M.).

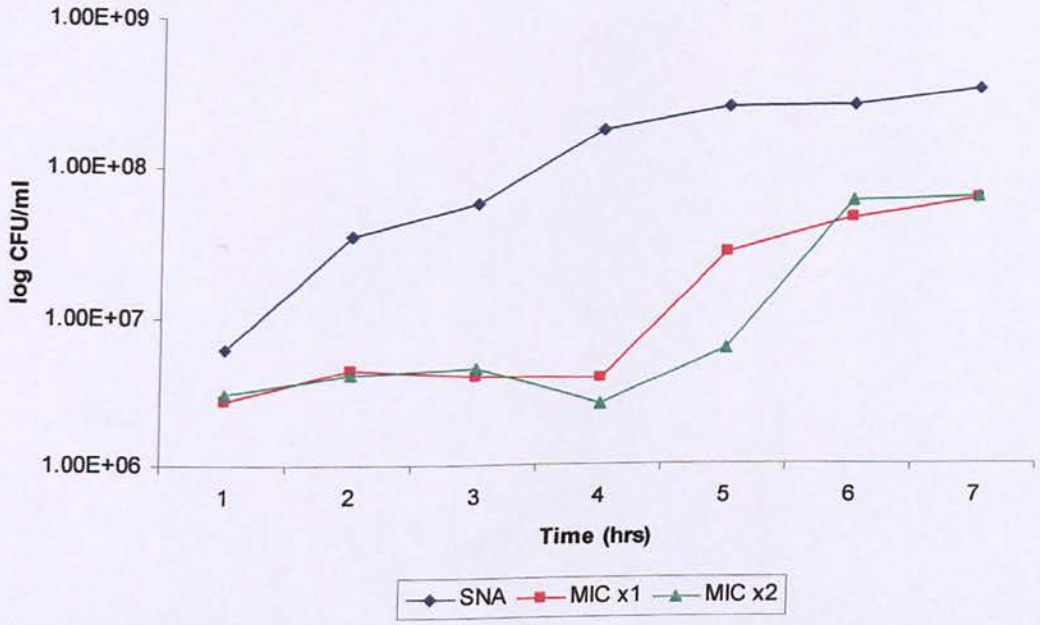


Figure 7.18: Growth of Oxford *S. aureus* NCTC 08325 (control 2) in nutrient broth containing either the MIC (1mg/L) or double the MIC of clindamycin (2mg/L) or no antibiotic over a 10-hour period (± log S.E.M.).

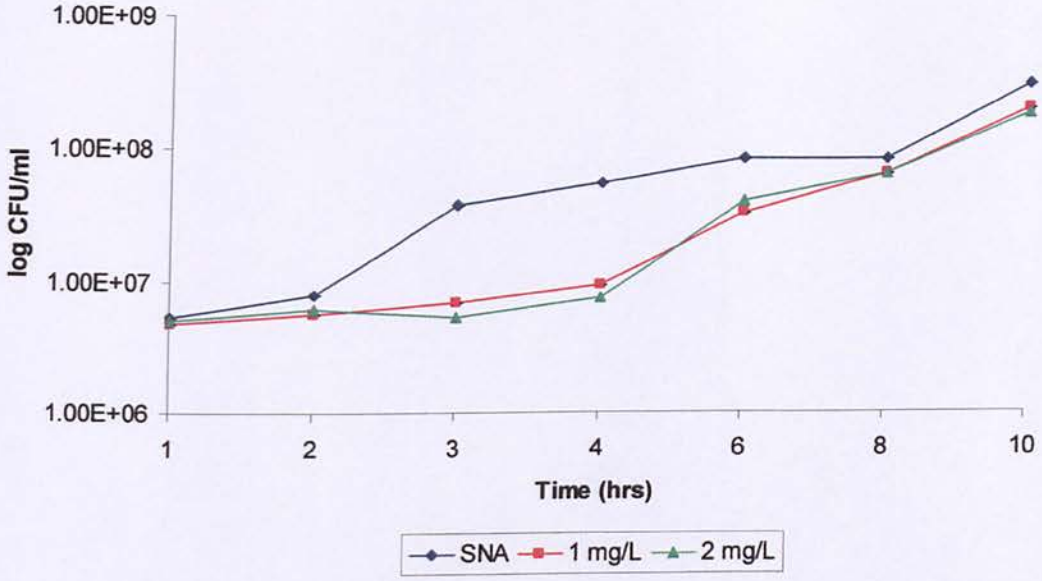


Figure 7.19: Growth of isolate No.7 in nutrient broth containing either the MIC (1mg/L) or double the MIC of clindamycin (2mg/L) or no antibiotic over a 10-hour period (± log S.E.M.).

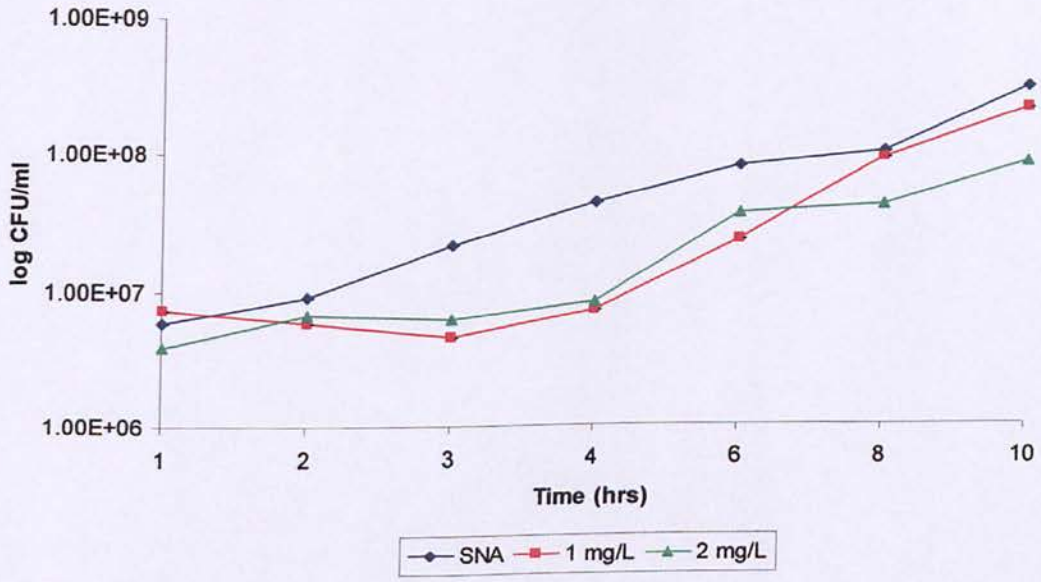


Figure 7.20: Growth of isolate No.27 in nutrient broth containing either the MIC (1mg/L) or double the MIC of clindamycin (2mg/L) or no antibiotic over a 10-hour period (± log S.E.M.).

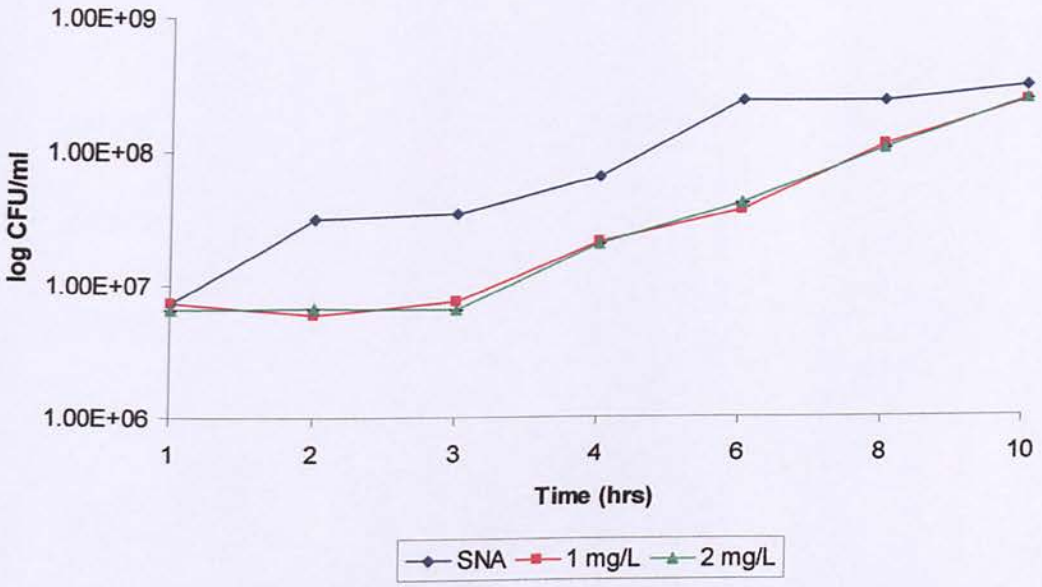


Figure 7.21: Growth of isolate No.195 in nutrient broth containing either the MIC (1mg/L) or double the MIC of clindamycin (2mg/L) or no antibiotic over a 10-hour period (± log S.E.M.).

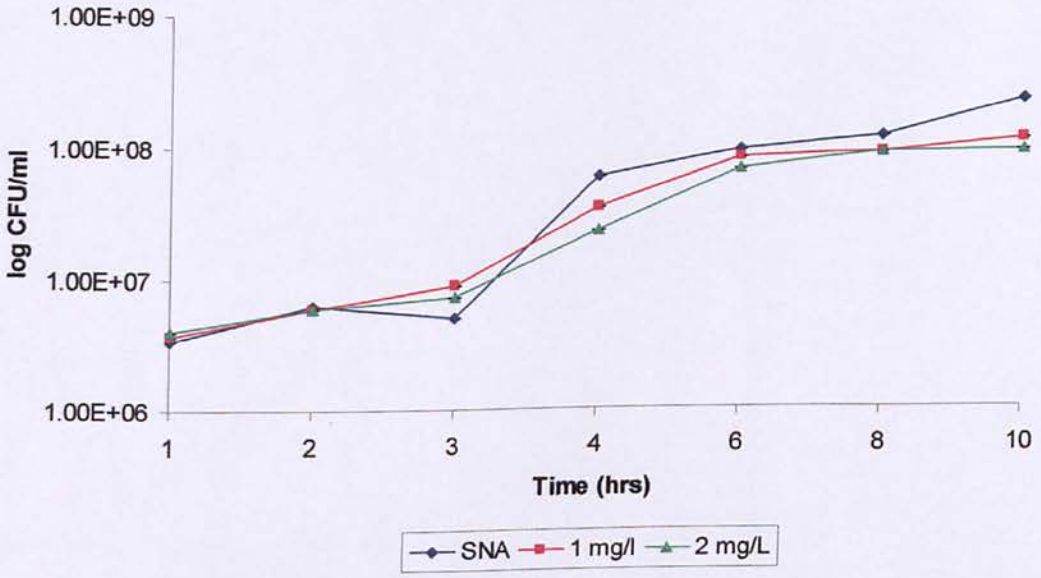


Figure 7.22: Growth of isolate No.60 in nutrient broth containing either the MIC (1mg/L) or double the MIC of clindamycin (2mg/L) or no antibiotic over a 10-hour period (± log S.E.M.).

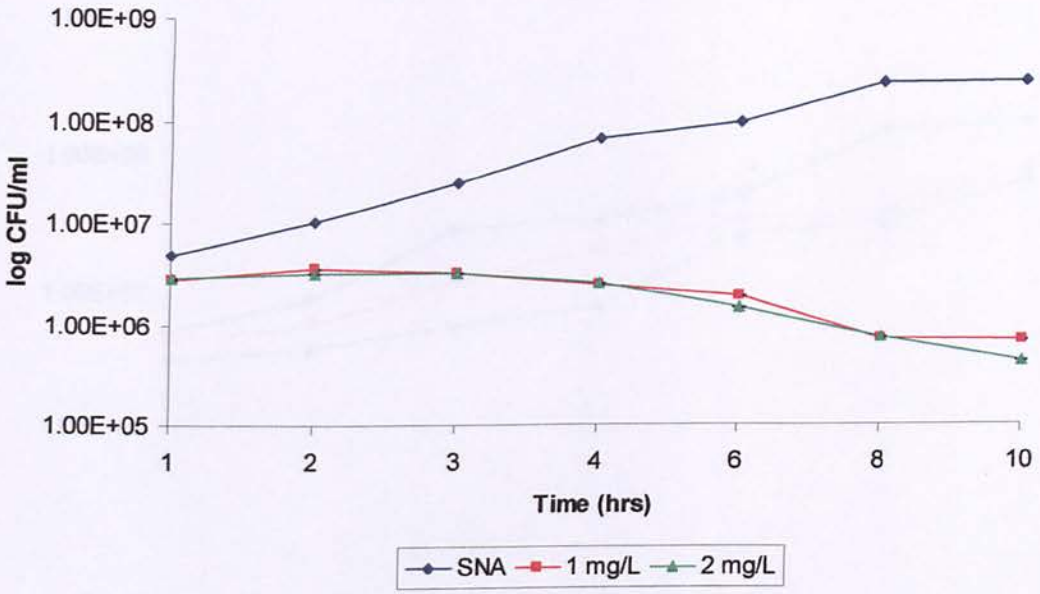


Figure 7.23: Growth of isolate No.63 in nutrient broth containing either the MIC (1mg/L) or double the MIC of clindamycin (2mg/L) or no antibiotic over a 10-hour period (± log S.E.M.).

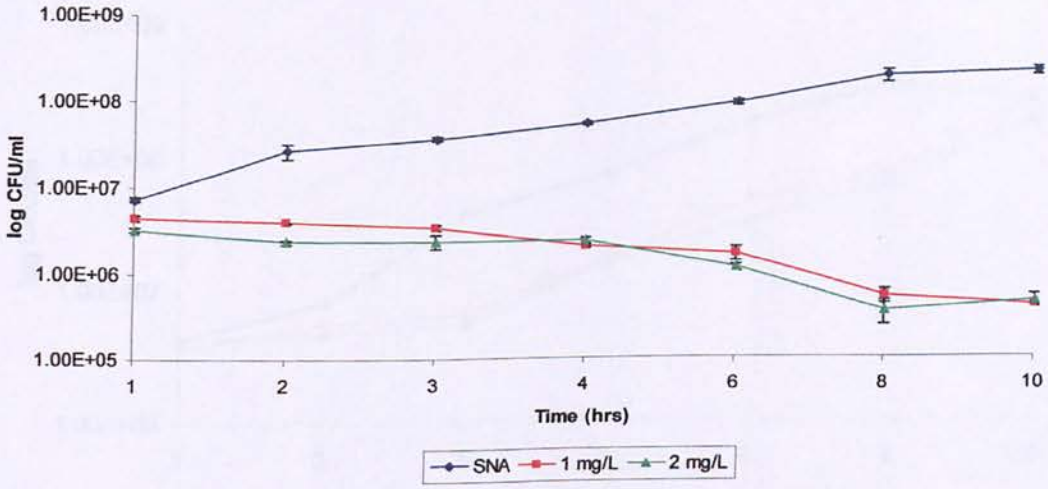


Figure 7.24: Growth of isolate No.193 in nutrient broth containing either the MIC (1mg/L) or double the MIC of clindamycin (2mg/L) or no antibiotic over a 10-hour period (± log S.E.M.).

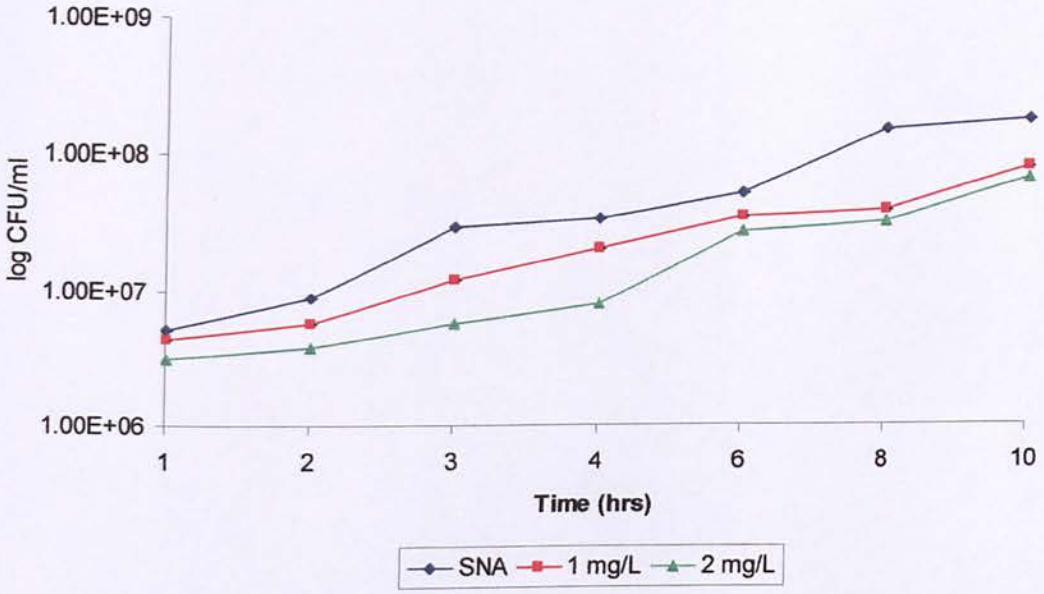


Figure 7.25: Growth of isolate No.134 in nutrient broth containing either the MIC (1mg/L) or double the MIC of clindamycin (2mg/L) or no antibiotic over a 10-hour period (± log S.E.M.).

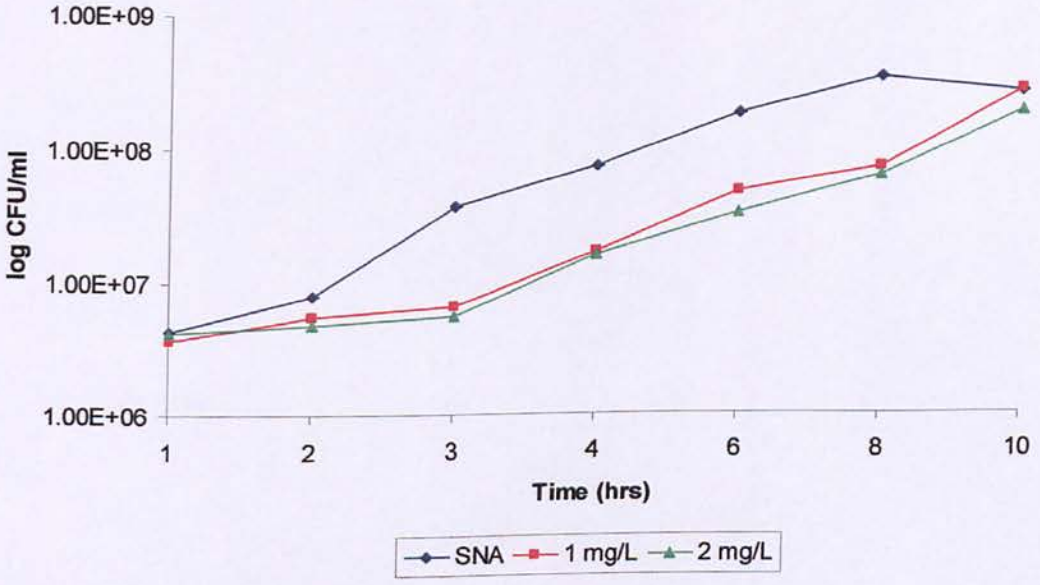


Figure 7.26: Growth of isolate No.M309 in nutrient broth containing either the MIC (1mg/L) or double the MIC of clindamycin (2mg/L) or no antibiotic over a 10-hour period (± log S.E.M.).

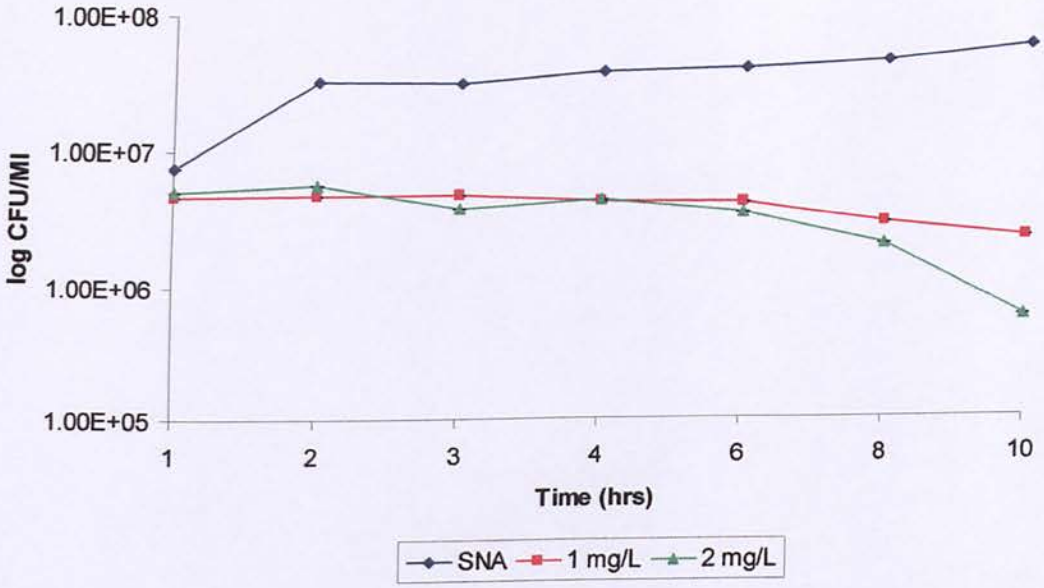


Figure 7.27: Growth of isolate No.M21 in nutrient broth containing either the MIC (1mg/L) or double the MIC of clindamycin (2mg/L) or no antibiotic over a 10-hour period (± log S.E.M.).

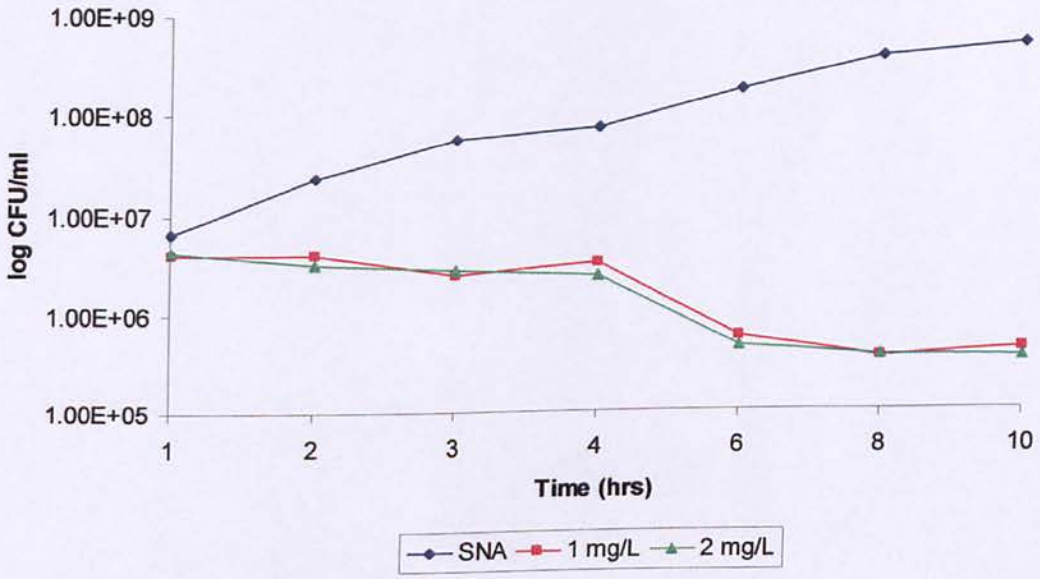


Figure 7.28: Growth of isolate No.M60 in nutrient broth containing either the MIC (1mg/L) or double the MIC of clindamycin (2mg/L) or no antibiotic over a 10-hour period (± log S.E.M.).

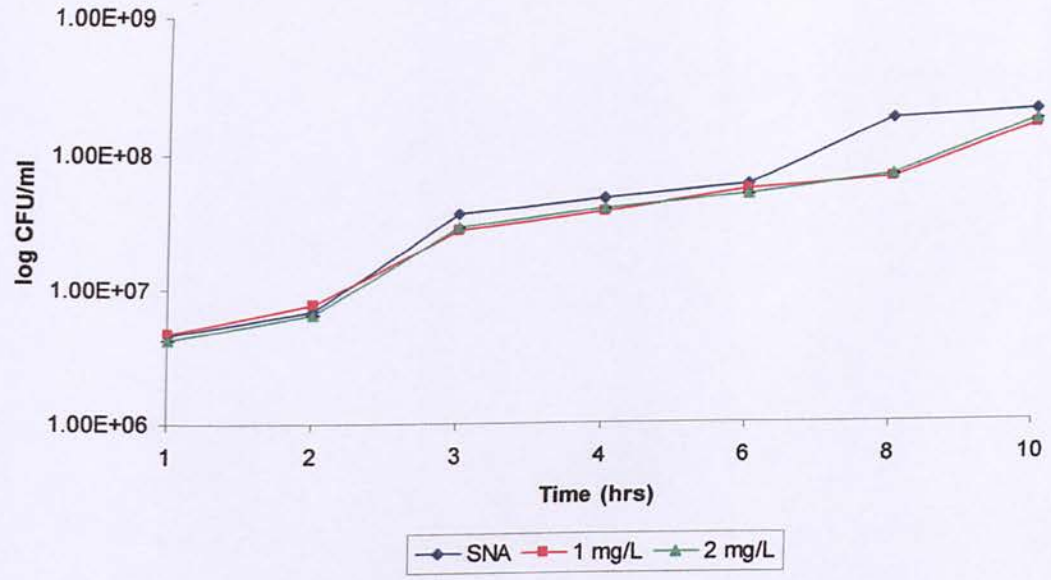


Figure 7.29: Growth of isolate No.M150 in nutrient broth containing either the MIC (1mg/L) or double the MIC of clindamycin (2mg/L) or no antibiotic over a 10-hour period (± log S.E.M.).

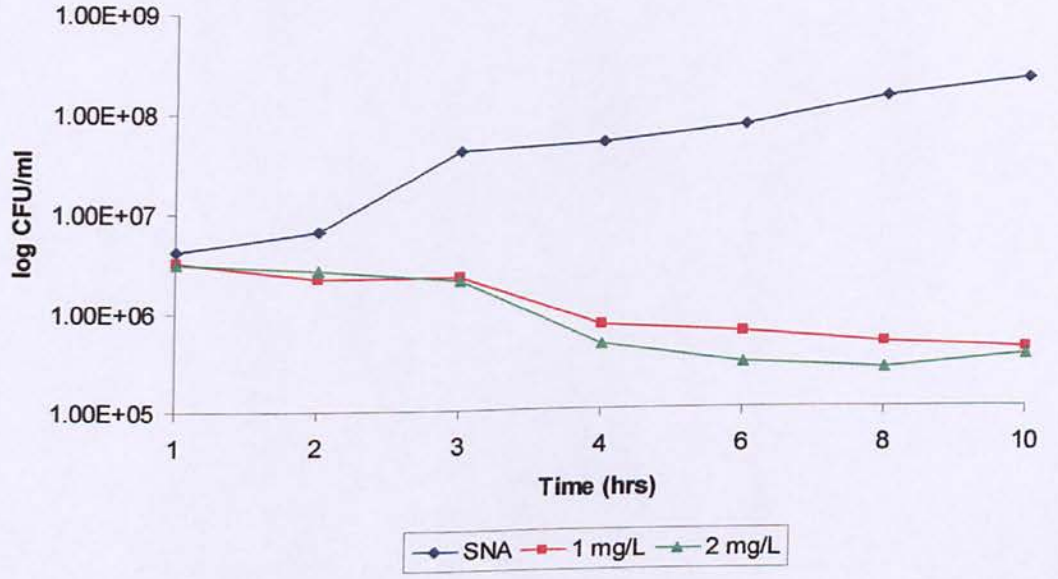


Figure 7.30: Growth of isolate No.M90 in nutrient broth containing either the MIC (1mg/L) or double the MIC of clindamycin (2mg/L) or no antibiotic over a 10-hour period (± log S.E.M.).

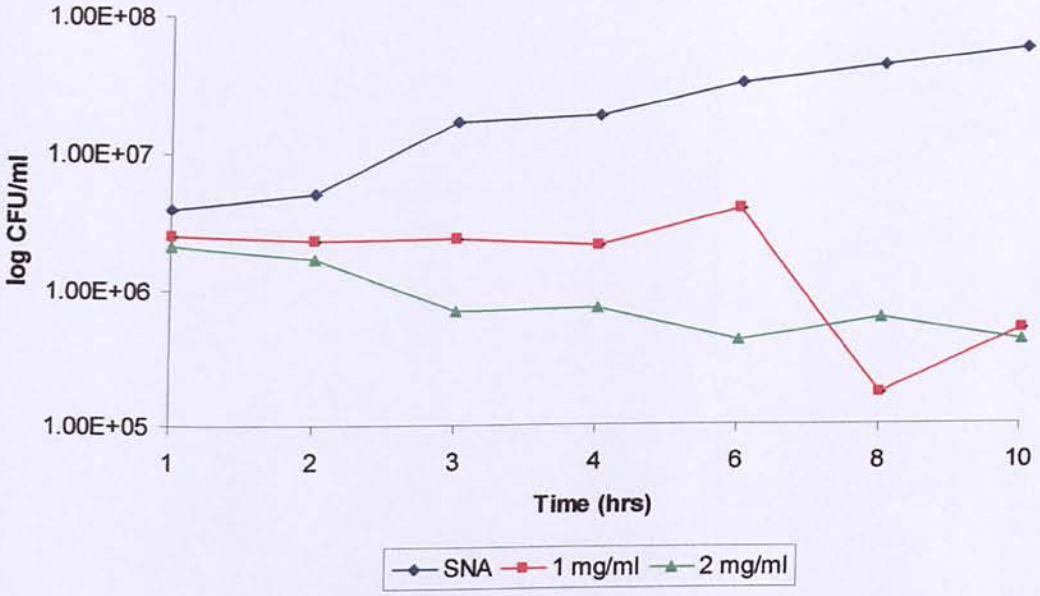


Figure 7.31: Growth of isolate No.M279 in nutrient broth containing either the MIC (1mg/L) or double the MIC of clindamycin (2mg/L) or no antibiotic over a 10-hour period (± log S.E.M.).

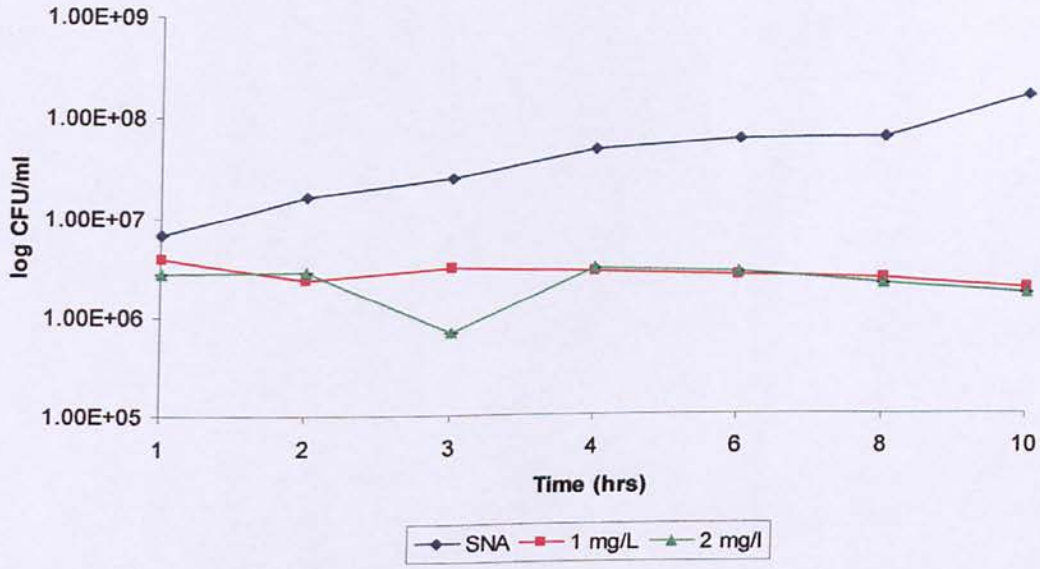


Figure 7.32: Growth of isolate No.M176 in nutrient broth containing either the MIC (1mg/L) or double the MIC of clindamycin (2mg/L) or no antibiotic over a 10-hour period (± log S.E.M.).

7.3.3 Virulence factor activity in isolates grown with penicillin G

The virulence tests were carried out on aliquots of the broth and filtered supernatant. For the supernatant samples no enzyme or toxin activity could be detected. From Figures 7.33-7.36 it can be seen that some isolates did not produce one or more of the virulence factors tested, six isolates did not produce any haemolysin and two did not produce any proteinase. Moreover, in these figures no result was observed for any of the factors tested with Oxford *S. aureus* (control 2) samples at either concentration of the antibiotic.

Figure 7.33 shows the production of DNase at both antibiotic concentrations by all the isolates except the Oxford *S. aureus* control. Isolates 60 and M279 as well as the MRSA control (Control 1) did not show any difference in the production of the enzyme. One isolate showed an increase in the production of DNase and of the remaining isolates ten showed some decrease in DNase production. Isolates 7, 195 and M90 showed a trend-wise decrease in production of DNase with a statistically significant difference of $p<0.05$ (SNA was greater than MICx1) and $p<0.005$(SNA was greater than MICx2). Isolates 193 ($p<0.05$, SNA was greater than MICx2), 134 ($p<0.05$, SNA was greater than MICx1, SNA was greater than MICx2), M150 and M21 showed a decrease in DNase production at both antibiotic concentrations, with the largest decrease seen in isolate M150 and M21 ($p<0.005$, SNA was greater than MICx1, SNA was greater than MICx2). Isolates M309 and M176 ($p<0.005$, SNA was greater than MICx2) showed decreases in production of DNase from samples collected at double the MIC of antibiotic concentration. Interestingly isolate number 27 showed a decrease in production of DNase in the sample collected at the standard MIC concentrations, whereas there was no difference in production between the sample with no antibiotic added and the sample at double the MIC concentration.

Figure 7.34 shows the production of lipase in which only the MRSA control (control 1) and isolate M21 did not produce any of this enzyme. Four of the isolates, 7, 134, M60 and M150, did not show any difference in the production of this enzyme (2, 5, 3 and 3mm respectively). Five isolates (27, 195, 60, 193 and M90) showed an increase in production and isolates 27, 195, 193 and M90 all showed an increase in production at both antibiotic concentrations. Isolate number 60 showed no production in the sample with no antibiotic added and the standard MIC sample but only in the double the MIC sample ($p<0.05$, MICx2

greater than SNA). Four samples (63, M309, M279 and M176) showed a decrease in the production of lipase. Isolate number M309 showed production of the enzyme in the sample with no antibiotic added and then no production in both samples that were grown in the presence of antibiotic ($p<0.005$, SNA was greater than MICx1 and MICx2). Sample M279 showed a slight decrease in the enzyme production in double the MIC of antibiotic whereas isolates 63 and M176 showed a decrease in production at both concentrations of the antibiotic ($p<0.05$, SNA was greater than MICx1 and MICx2).

Figure 7.35 shows the production of proteinase in which two isolates, M150 and M21, did not produce any of this enzyme. Of the remaining eleven isolates, seven showed no difference in the enzyme production and only four showed difference. Isolates M309 and M60 showed a decrease in production from cells collected at a penicillin G concentration of double the MIC. Isolate number 27 showed a decrease in production at both concentrations of penicillin when compared to the sample with no antibiotic. Isolates 27 and M60 showed a statistically significant difference of $p<0.05$ (SNA was greater than MICx2) whereas no other statistically significant difference was observed amongst the sample groups. Only one isolate was observed to have an increase in production, namely isolate 60. For this isolate a trend-wise increase was observed in the production of proteinase from 4cm radius produced by the sample with no antibiotic to 6cm radius produced by the sample grown in double the MIC of the antibiotic concentration.

Finally, Figure 7.36 shows the production of haemolysin in which only three isolates (134, M90 and M279) showed any difference in the results recorded. Isolate number 134 showed an increase in production when grown with double the MIC of penicillin G, whereas isolate number M279 showed a decrease in production of haemolysin. Interestingly, isolate number M90 only showed production of haemolysin after being grown with double the MIC of penicillin G. No production was recorded in the sample with no antibiotic or the standard MIC sample. Only one isolate, number M90 showed a statistically significant difference compared with the sample with no antibiotic ($p<0.05$ MICx2 greater than SNA sample) whereas no difference was recorded for the remaining isolates that produced haemolysin.

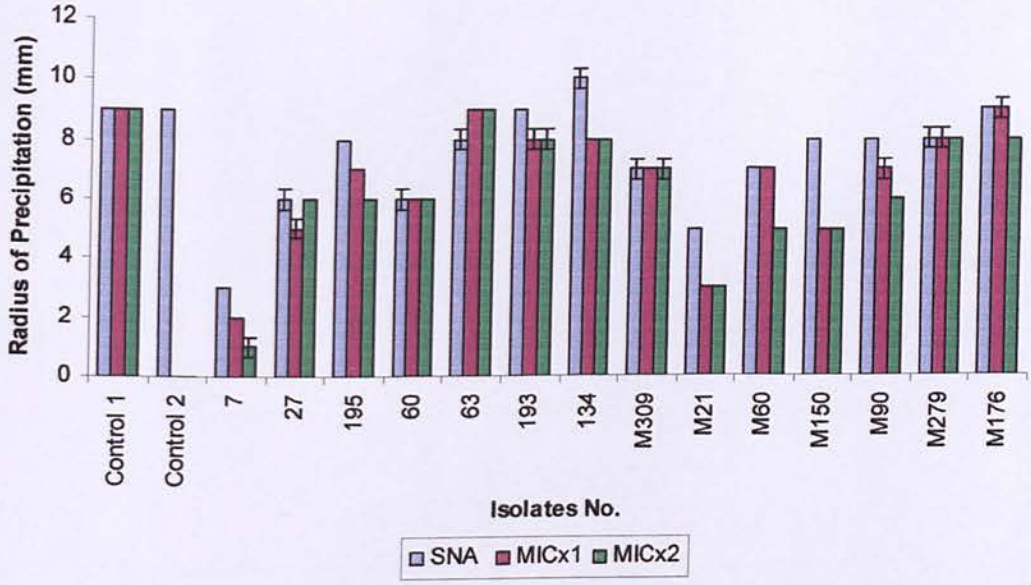


Figure 7.33: Production of DNase (± S.E.M.) by isolates harvested after 10 hours growth in nutrient broth containing either the MIC (0.25mg/L) (Red) or double the MIC of penicillin G (0.5mg/L) (Green) or no antibiotic (Blue).

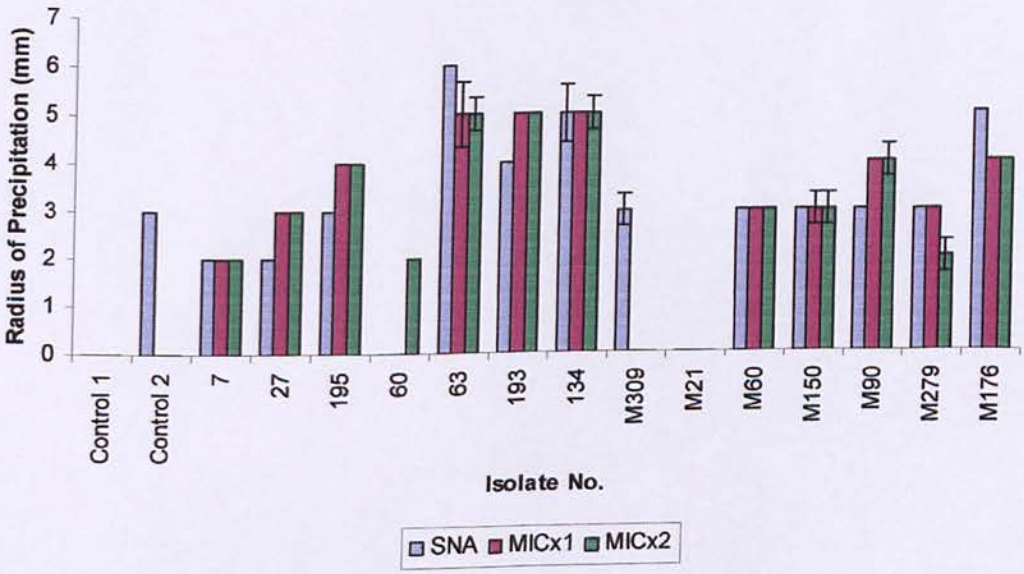


Figure 7.34: Production of lipase (± S.E.M.) by isolates harvested after 10 hours growth in nutrient broth containing either the MIC (0.25mg/L) (Red) or double the MIC of penicillin G (0.5mg/L) (Green) or no antibiotic (Blue).

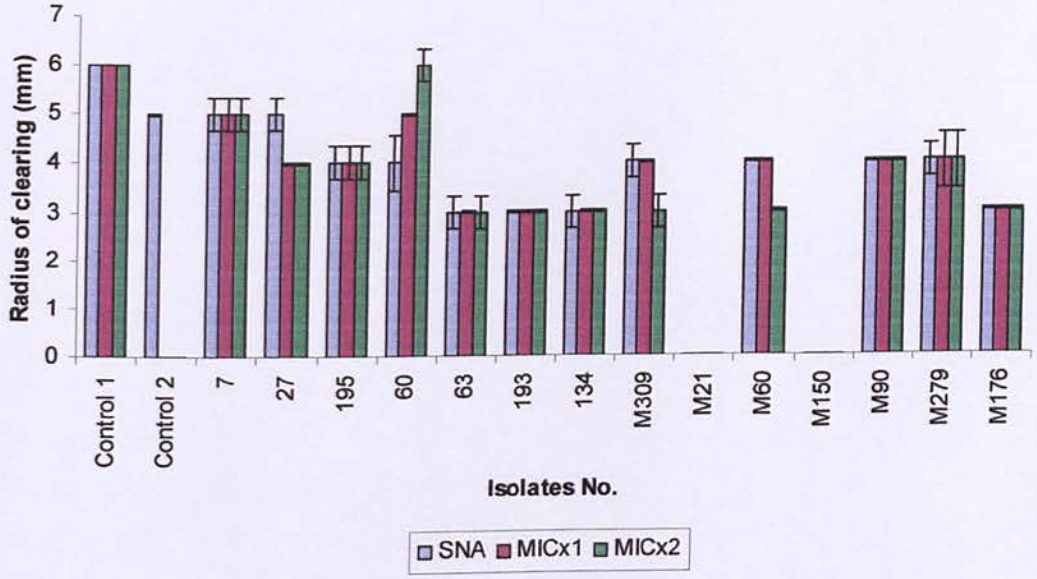


Figure 7.35: Production of proteinase (± S.E.M.) by isolates harvested after 10 hours growth in nutrient broth containing either the MIC (0.25mg/L) (Red) or double the MIC of penicillin G (0.5mg/L) (Green) or no antibiotic (Blue).

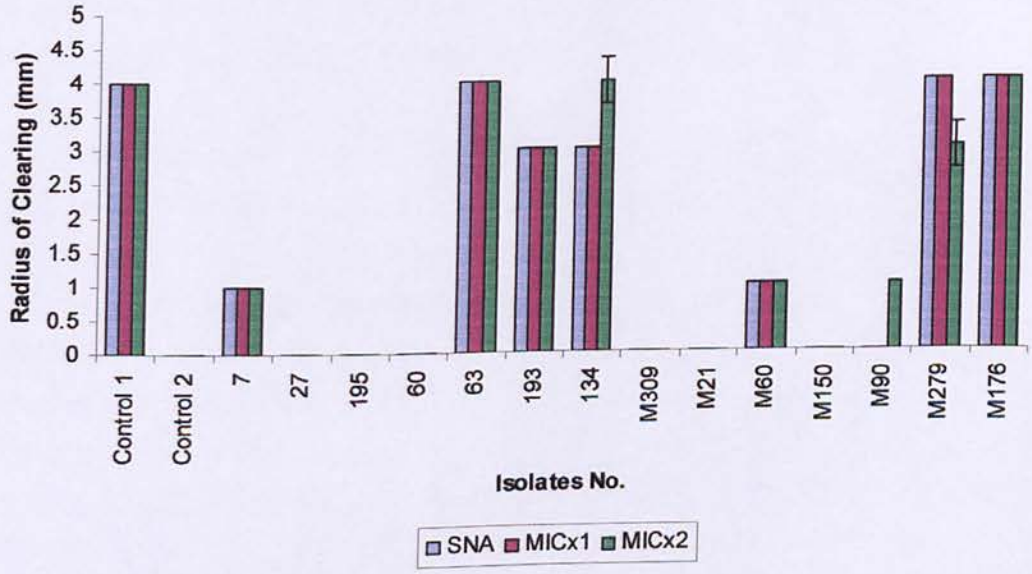


Figure 7.36: Production of haemolysin (± S.E.M.) by isolates harvested after 10 hours growth in nutrient broth containing either the MIC (0.25mg/L) (Red) or double the MIC of penicillin G (0.5mg/L) (Green) or no antibiotic (Blue).

7.3.4 Virulence factor activity in isolates grown with Clindamycin

The virulence tests were carried out on aliquots of the broth and filtered supernatants. For the supernatant samples no enzyme or toxin activity could be detected. From Figures 7.37-7.40 it can be seen that some isolates did not produce one or more of the virulence factors tested. A total of four isolates did not produce haemolysin whereas four did not produce any lipase.

Figure 7.37 shows the production of DNase that was observed for all isolates. Four isolates (195, 134, M176 and M279), showed no difference in the level of production of the enzyme. Decreases in production were seen in five isolates (control 1, 7, 60, 193 and M150). There was a slight decrease in production at both levels of the antibiotic added observed in isolates 60, 193, M150 and the MRSA control (control 1) sample (60, M150 and MRSA, $p<0.05$, SNA was greater than MICx1, SNA was greater than MICx2). Isolate number 7 was the only isolate to show a trend-wise decrease in the production of the enzyme ($p<0.005$, SNA was greater than MICx1, SNA was greater than MICx2). Increases in the production of DNase were seen in isolates 27, 63, M309, M21, M60 and the Oxford *S. aureus* control (control 2). The increase in DNase production in isolate number 27 and M21 was seen in both antibiotic concentrations ($p<0.05$ MICx2 greater than no antibiotic for both isolates).

Figure 7.38 shows the production of lipase. In this Figure it can be seen that isolates 7, 27, 195 and 60 did not produce any lipase, however isolates 7, 27 and 195 did produce detectable level of lipase in the sample grown in penicillin (fig. 7.35). No difference in production of enzyme was detected in isolates number M21 and M150. Decreases in production was seen in isolates number 63, 193, 134, M60, M279 and M176 Oxford *S. aureus* control (Control 2). Isolates number 193, 134 and M176 showed a slight decrease in production of the enzyme at both antibiotic concentrations compared to the sample with no antibiotic added ($p<0.05$, SNA was greater than MICx1, SNA was greater than MICx2). M60, M176 and Oxford *S. aureus* (control 2) had no detectable enzymes in either sample at both antibiotic concentrations ($p<0.005$, SNA was greater than MICx2 for both isolates). Only two isolates, number M309 and M90 showed an increase in production. Isolate M90 showed an increase in the enzyme production in the sample with the addition of double the MIC of antibiotic ($p<0.05$, MICx2 greater than no antibiotic). For isolate M309 however no

production was detected in the sample with no antibiotic added or the lower MIC sample ($p<0.05$, MICx2 greater than SNA).

Figure 7.39 shows the production of proteinase. All isolates produced this enzyme, however, eight isolates (MRSA control (control 1) and isolates 134, M309, M21, M60, M150 and M90) showed no change in production. Isolates 7, 195, 60 and 193 showed a decrease in production with the addition of double the MIC of antibiotic sample ($p<0.05$ SNA was greater than MICx2). This decrease however varied among the isolates. Isolate number M176 was the only one that demonstrated a trend wise decrease in production of the enzymes ($p<0.05$ SNA was greater than MICx1, SNA was greater than MICx2). Two isolates, number 27 and the Oxford *S. aureus* (control 2), showed an increase in the enzyme production. Isolates 27 showed an increase with the addition of double the MIC of antibiotic sample, whereas Oxford *S. aureus* (control 2) showed an increase at both antibiotic concentrations ($p<0.05$). Isolate 63 showed a slight decrease in enzyme production in the sample containing the MIC concentration of antibiotic however, no change in production of the enzyme was detected in the sample containing double the MIC concentration of drug.

Figure 7.40 shows the production of haemolysin in which five of the isolates Oxford *S. aureus* (control 2) and 195, 60, M309 and M90 did not produce any haemolysin. No difference in production of the enzyme was seen in samples 7, 63 and 134. Decreases in production of haemolysin were seen in six isolates (control 1, 193, M150, M60, M279 and M176). The MRSA control (Control 1) and isolate M60 showed a decrease in the sample when grown in double the MIC of antibiotic. Isolate number M150 had no detectable level of the enzyme at both antibiotic concentrations ($p<0.05$, SNA was greater than MICx1 and SNA was greater than MICx2). Isolates M279 showed slight decrease in production of enzyme at both antibiotic concentrations. Both isolates 193 and M176 showed a slight decrease in production of enzyme between the sample with no antibiotic added and the sample containing the antibiotic at the MIC concentration. However no enzyme production was detected in both samples containing double the MIC concentration of drug ($p<0.05$ SNA was greater than MICx2). Only isolate number 27 showed an increase in production of the enzymes with detectable levels in both antibiotic concentrations. However, no enzyme production was observed in the sample with SNA added ($p<0.05$, MICx1 greater than SNA and MICx2 greater than SNA).

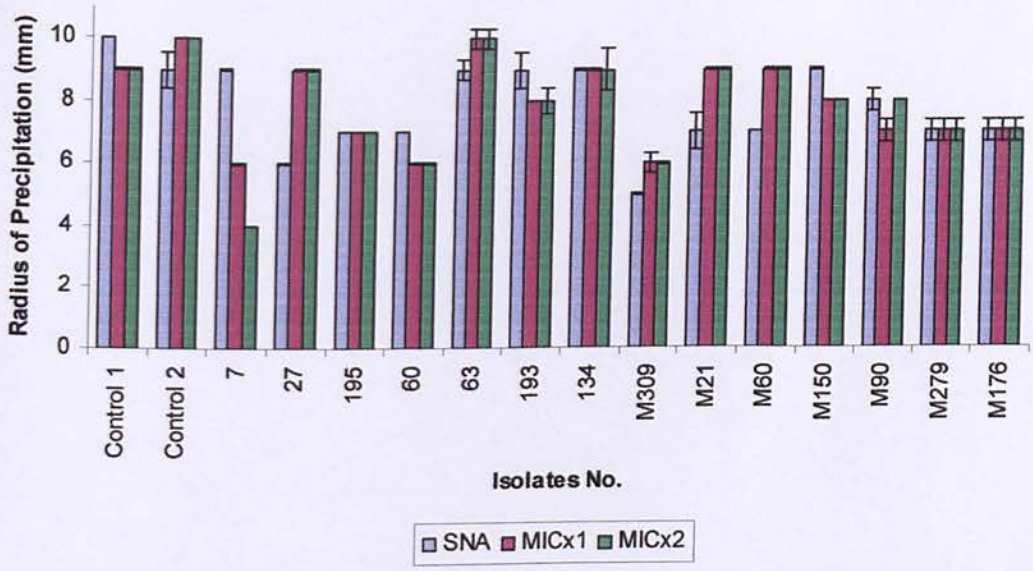


Figure 7.37: Production of DNase (± S.E.M.) by isolates harvested after 10 hours growth in nutrient broth containing either the MIC (1mg/L) (Red) or double the MIC of clindamycin (2mg/L) (Green) or no antibiotic (Blue).

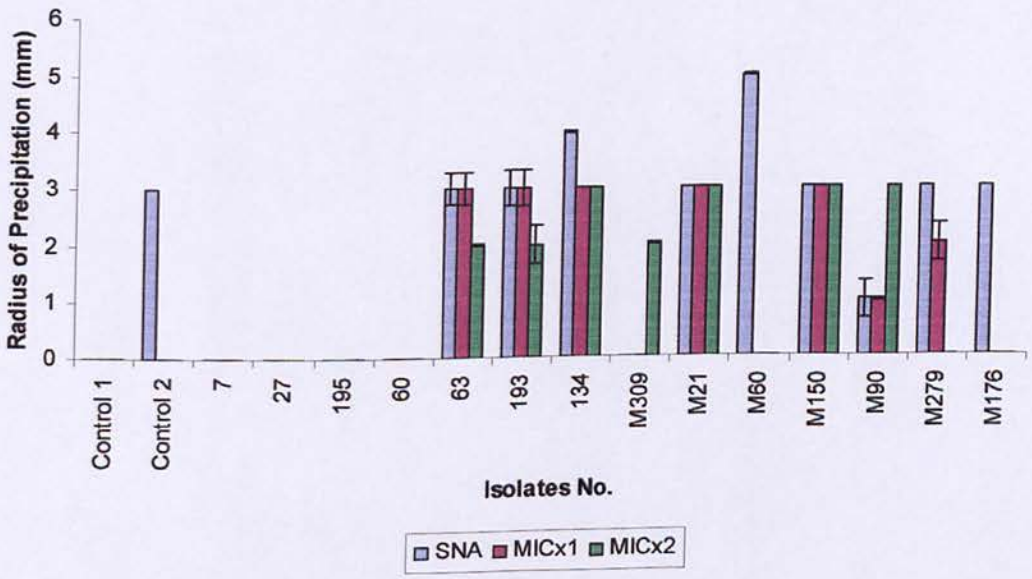


Figure 7.38: Production of lipase (± S.E.M.) by isolates harvested after 10 hours growth in nutrient broth containing either the MIC (1mg/L) (Red) or double the MIC of clindamycin (2mg/L) (Green) or no antibiotic (Blue).

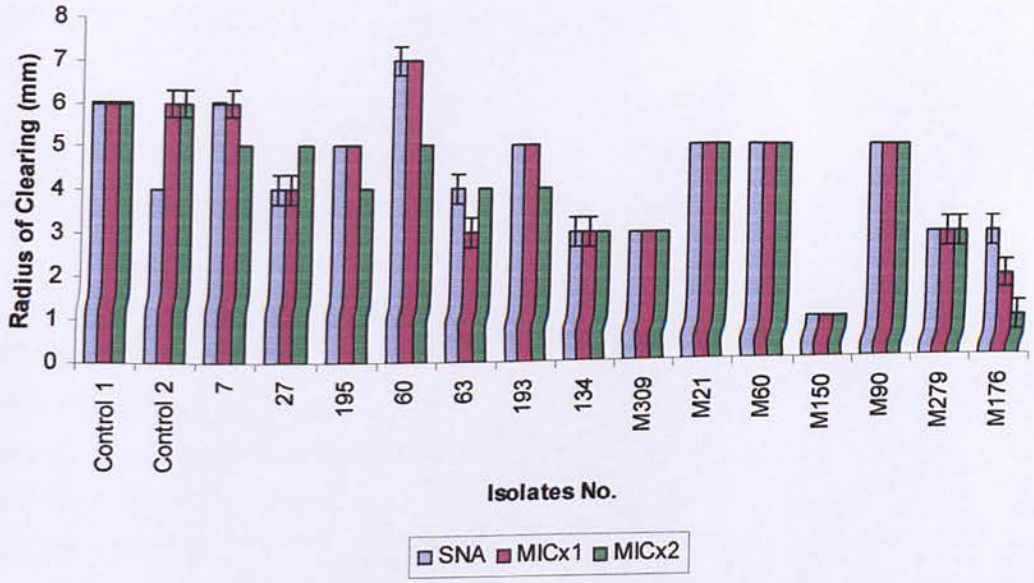


Figure 7.39: Production of proteinase (± S.E.M.) by isolates harvested after 10 hours growth in nutrient broth containing either the MIC (1mg/L) (Red) or double the MIC of clindamycin (2mg/L) (Green) or no antibiotic (Blue).

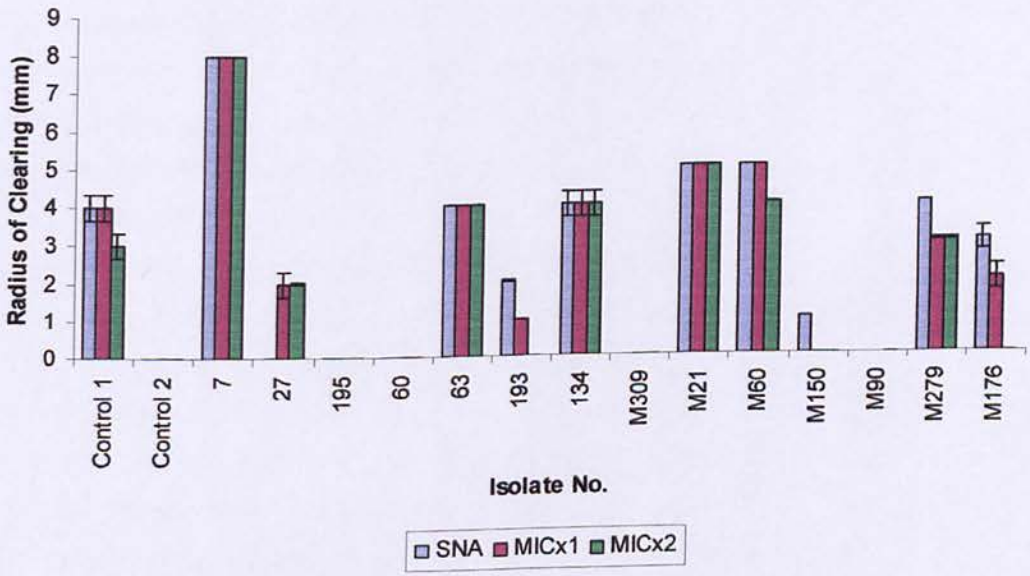


Figure 7.40: Production of haemolysin (± S.E.M.) by isolates harvested after 10 hours growth in nutrient broth containing either the MIC (1mg/L) (Red) or double the MIC of clindamycin (2mg/L) (Green) or no antibiotic (Blue).

7.3.5 Adherence levels of isolates grown in the presence of penicillin G

Figure 7.41 shows the absorption of the isolates after the 2-hour period in which a reading was taken to compare any differences in the absorption rate of the sample taken from sample with no antibiotic added and the two levels of antibiotic for each isolate.

From Figure 7.41 it can be seen that the biggest difference in the results was given by the Oxford *S. aureus* (control 2). Isolate number 60 showed a higher absorption in the sample with no antibiotic added compared to the MICx1 and MICx2, with only the MICx2 value showing statistical significance ($p<0.05$ SNA was greater than MICx2). Isolates number 63 and M60 had a higher absorption in the MICx1 sample compared to the sample with no antibiotic added ($p<0.05$ MICx1 greater than SNA). Isolate M21 showed a decrease in absorbance, with the largest decrease seen between the sample with no antibiotic added and the double the MIC of antibiotic sample ($p<0.05$, SNA was greater than MICx2).

Figure 7.42 shows the absorption of the eluted stained for the adhered cell (N1). As with the enzyme tests the results could be divided into three groups. The control 1 and isolates 7, 195 and M279 showed no real difference in adherence, whilst there was a decrease in absorbance seen with isolates 27, 63, 193, 134, M21 and M176. The greater decrease observed with isolate number M21 and M176 when comparing the sample with no antibiotic added to the two samples that were grown with antibiotic ($p<0.05$, SNA was greater than MICx1, SNA was greater than MICx2). Isolate number 27 and 63 showed a statistically significant difference between the sample with no antibiotic added and the sample containing double the MIC of antibiotic ($p<0.05$ SNA was greater than MICx2). There was a trend wise increase seen with isolates M309 ($p<0.05$ MICx1 greater than SNA), M60 ($p<0.05$ MICx1 greater than SNA, MICx2 greater than SNA) and M90, although the increase in isolate number M90 was very small. Isolate 60 showed an overall increase in adherence especially when the sample with no antibiotic added and the standard MIC were compared ($p<0.05$ MICx1greater than SNA).

Figure 7.43 shows the absorption of the isolates in the plates coated with fibrinogen after 2 hour incubation. As previously seen in figure 7.41, no results were determined for the Oxford *S. aureus* (control 2) due to lack of sample. However, there were significant

differences observed in isolates 63, M60 ($p<0.05$ MICx1 greater than SNA, MICx2 greater than SNA) and M90 ($p<0.05$ MICx1 greater than SNA) and these differences were taken into account when looking at the eluted stain results. Isolate 60 showed a statistically significant difference when the sample with no antibiotic added was compared to the MICx1 sample ($p<0.05$, SNA was greater than MICx1).

Figure 7.44 shows the absorption of the stained results, as before (N1), with plates coated with fibrinogen. In this figure minimum difference was observed for isolates 7, 27, 134 and M21 between SNA and the MIC samples. Trend-wise decrease was observed in isolates 193, M90 and M176 with statistically significant differences seen only in isolates 193 and M176 ($p<0.005$, NSA greater than MICx1; $p<0.05$, NSA greater than MICx2). Trend-wise increases were observed for isolates 63, M60 and M279 with statistically significant difference seen only for 63 and M60 ($p<0.05$, SNA less than MICx2).

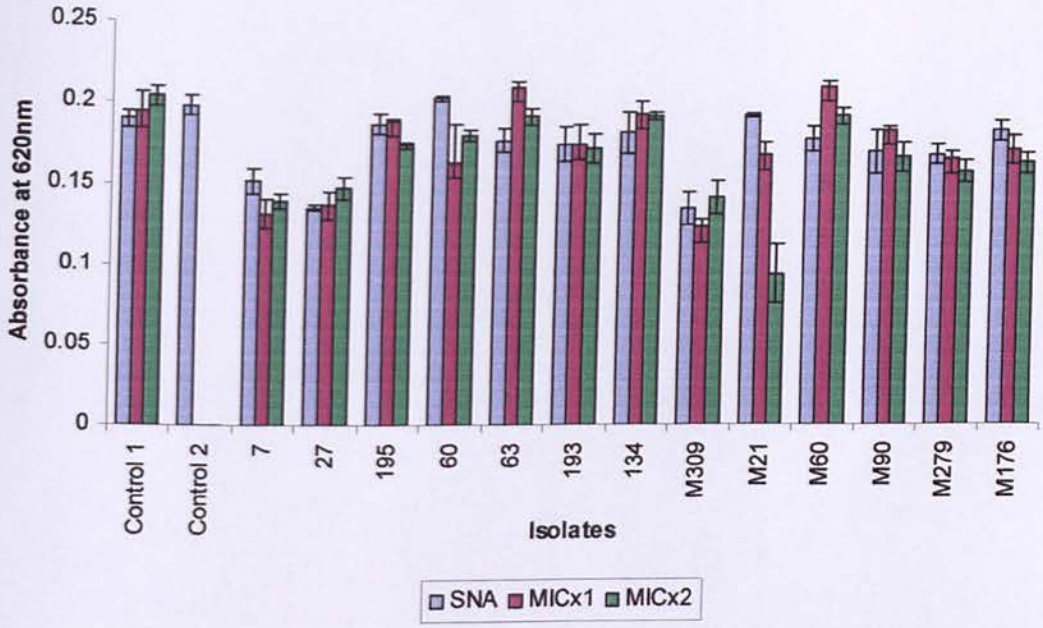


Figure 7.41: Absorbance of bacterial suspension in the polystyrene plates containing nutrient broth after 2 hours incubation (± S.E.M). Isolates were harvested after a 10-hour growth in nutrient broth containing either the MIC (0.25mg/L) (Red) or double the MIC of penicillin G (0.5mg/L) (Green) or no antibiotic (Blue).

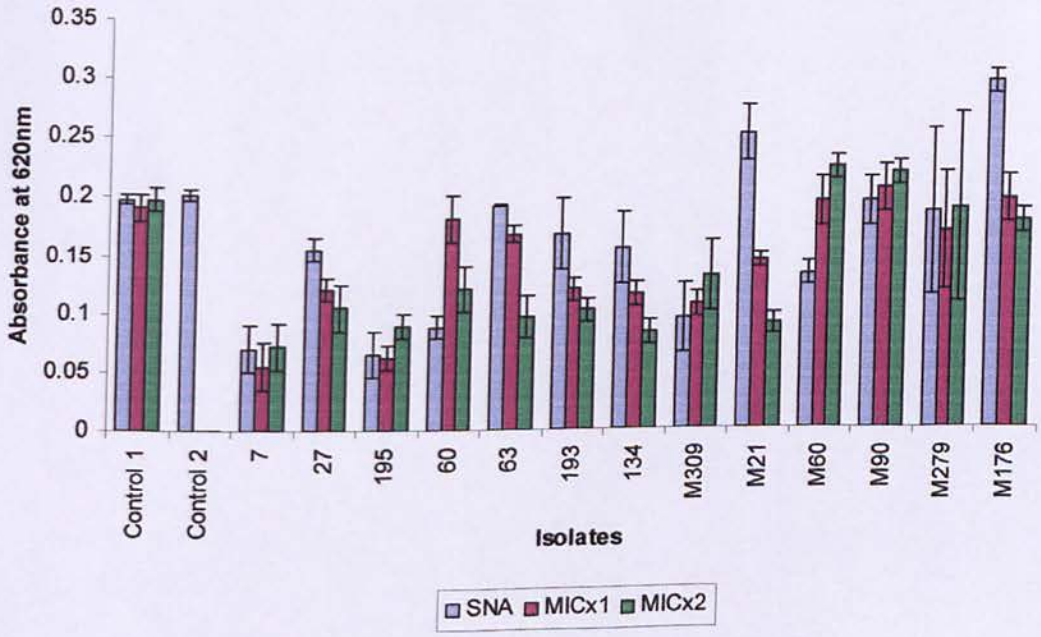


Figure 7.42: Absorbance of eluted stain from bacterial cell bound to the polystyrene plates (± S.E.M.). Cells were stained with 0.1% crystal violet and the stain was eluted with 80:20 (v/v) ethanol: acetone mixture.

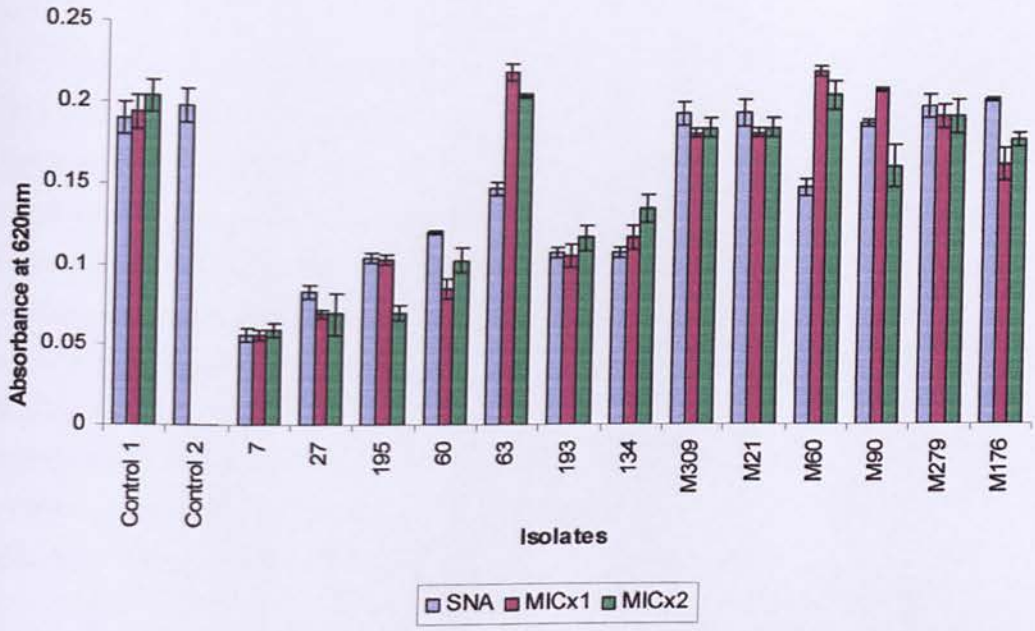


Figure 7.43: Absorbance of bacterial suspension in the polystyrene plates coated with fibrinogen containing nutrient broth after 2 hours incubation (± S.E.M.). Isolates were harvested after a 10-hour growth in nutrient broth containing either the MIC (0.25mg/L) (Red) or double the MIC of penicillin G (0.5mg/L) (Green) or no antibiotic (Blue).

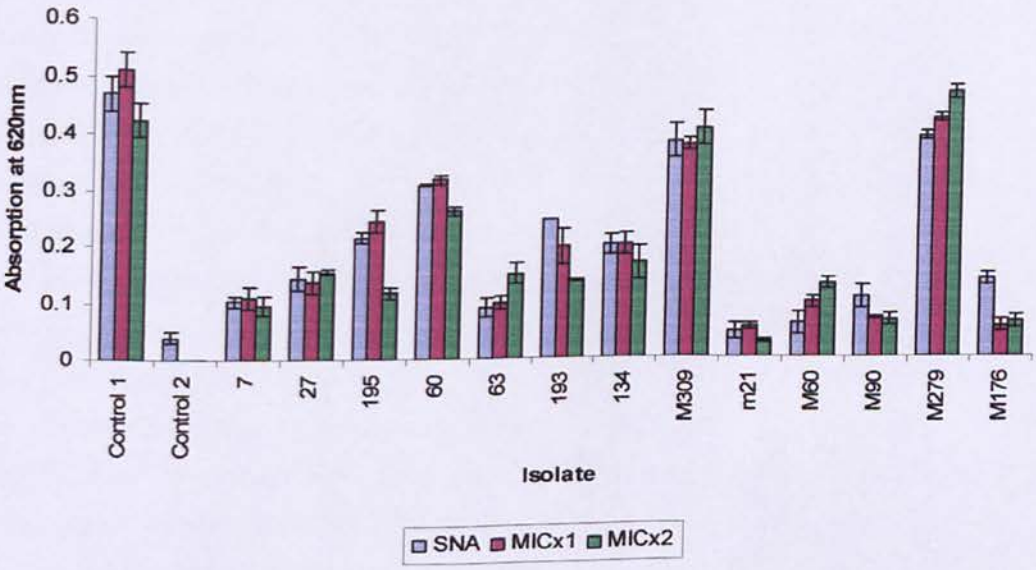


Figure 7.44: Absorbance of eluted stain from bacterial cell bound to the polystyrene plates coated with fibrinogen (± S.E.M.). Cells were stained with 0.1% crystal violet and the stain was eluted with 80:20 (v/v) ethanol: acetone mixture.

7.3.6 Adherence levels in isolates grown in the presence of clindamycin

The majority of isolates (Figure 7.45) showed slight differences in adherence. Isolates 134 and M21 showed a trend-wise increase in adherence. This had to be taken into consideration when looking at the stain data (134, $p<0.05$, MICx2 greater than SNA). Isolates 63 showed an increase in absorbance at both antibiotic concentrations, although the sample containing the MIC concentration of antibiotic gave a slightly higher reading than the sample containing double the MIC of clindamycin ($p<0.05$ MICx1 greater than SNA, MICx2 greater than SNA). Isolate M279 showed a decrease in absorbance ($p<0.05$, SNA was greater than MICx2), whereas isolates M150, M90 and M176 showed a decrease at both antibiotic concentrations. However the decrease in the sample containing the MIC concentration of antibiotic was greater than the decrease seen in the sample containing double the amount of antibiotic (M90 and M176 ($p<0.005$ SNA was greater than MICx1).

Figure 7.46 shows the eluted stained data for the cells that had adhered to the polystyrene plate. The Oxford *S. aureus* (control 2) and isolates 7, 27, 195 ($p<0.05$, SNA was greater than MICx1, $p<0.005$ SNA was greater than MICx2), 63, 190, M309 ($p<0.005$, SNA was greater than MICx1, SNA was greater than MICx2) and M176 showed decreases in adherence. Of these isolates, numbers 7, 195, 63, M309, M279 and M176 all showed a decrease in absorbance. Isolates 60 and 134 both showed an increase in the adherence of the cells to the plate however this was only a slight increase. The MRSA control (control 1), M21 and isolate M150 did not fit into these two groups since the MRSA control showed a sharp increase to just over double that of the sample with no antibiotic added (Mean absorbance of MRSA control 1 = 0.11 OD units with ±S.E.M. of 0.011 for the sample with no antibiotic added and 0.24 OD units with S.E.M. of 0.88 for the MICx1 sample), but then the adherence seen in the double the MIC was similar to that observed in the sample with no antibiotic added. Isolate M21 and M150 showed an increase in just the double the MIC sample, with a slight decrease in adherence seen in the standard MIC when compared to the sample with no antibiotic added. Isolate M90 showed the largest decrease in absorbance at both antibiotic concentrations, however there was a larger decrease seen in the sample containing the MIC concentration of antibiotic compared to the sample containing double the amount of drug ($p<0.005$, SNA was greater than MICx1, SNA was greater than MICx2).

Figure 7.47 shows the absorption of the isolates on the plate coated with fibrinogen after 2 hours incubation. In this figure, the most notable difference in absorption can be seen for Oxford *S. aureus* (control 2) and isolates 63, 193, M309, M60, M150, M90, M279 and M176. There was an increase in binding seen with Oxford *S. aureus* (control 2) ($p<0.05$ MICx1 greater than SNA, $p<0.005$, MICx2 greater than SNA). An increase was also observed in isolate M60 ($p<0.05$ MICx1 greater than SNA), whereas isolates M309, M279 and M150 ($p<0.05$, SNA was greater than MICx2) showed a decrease in binding. Isolates 63, 193, M90 and M176 all showed an initial decreases in absorbance and then, subsequently a slight increase. In samples 63 and 93 the increase in the absorbance in the sample contain double the MIC value of antibiotic, was slight more then the absorbance of the sample with no antibiotic added.

Figure 7.48 shows the stained data for cells that had adhered to the fibrinogen coated plates. The MRSA control (control 1), 193, M21 and isolate M150 all showed a slight trend decrease in the adherence. In sample M176 decreases were also seen at both concentrations of antibiotic ($p<0.05$ SNA was greater than MICx1 and SNA was greater than MICx2). The second control and isolates 7, 27, 60, 134, M60 all showed a slight increase in absorbance. Isolates 195, 63 and 134, all showed an initial slight increase in adherence followed by a decrease observed with the double the MIC sample. This trend was also seen in isolates M90, however the decrease in the sample containing double the concentration of antibiotic is more pronounced ($p<0.005$ SNA was greater than MICx1, $p<0.05$, SNA was greater than MICx2).

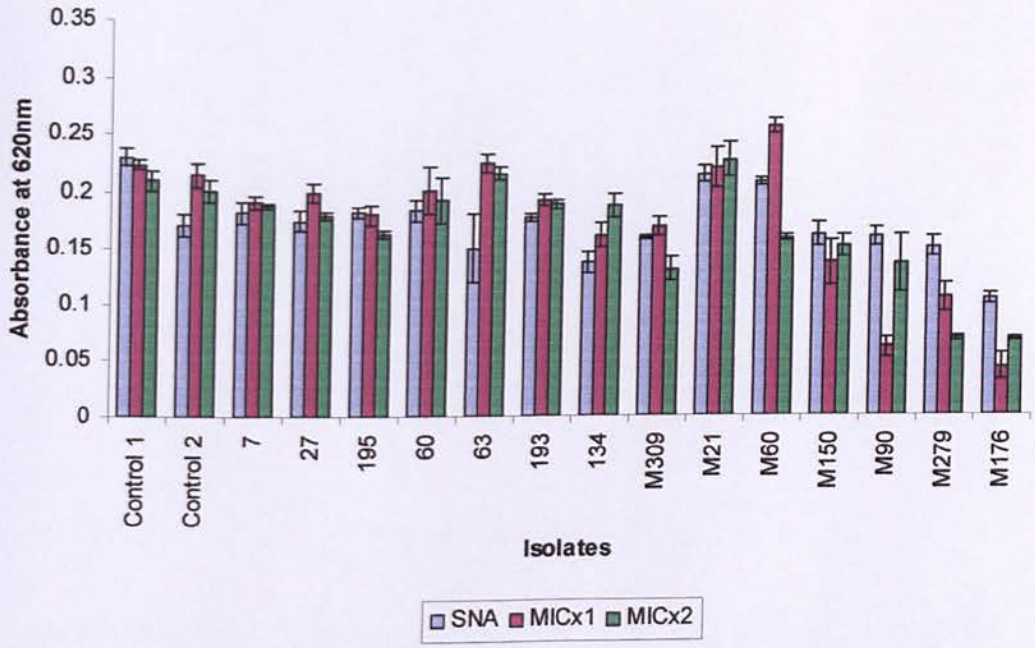


Figure 7.45: Absorbance of bacterial suspension in the polystyrene plates after 2 hour incubation containing nutrient broth (± S.E.M.). Cells were harvested after a 10-hour growth in nutrient broth containing either the MIC (1mg/L) (Red) or double the MIC of clindamycin (2mg/L) (Green) or no antibiotic (Blue).

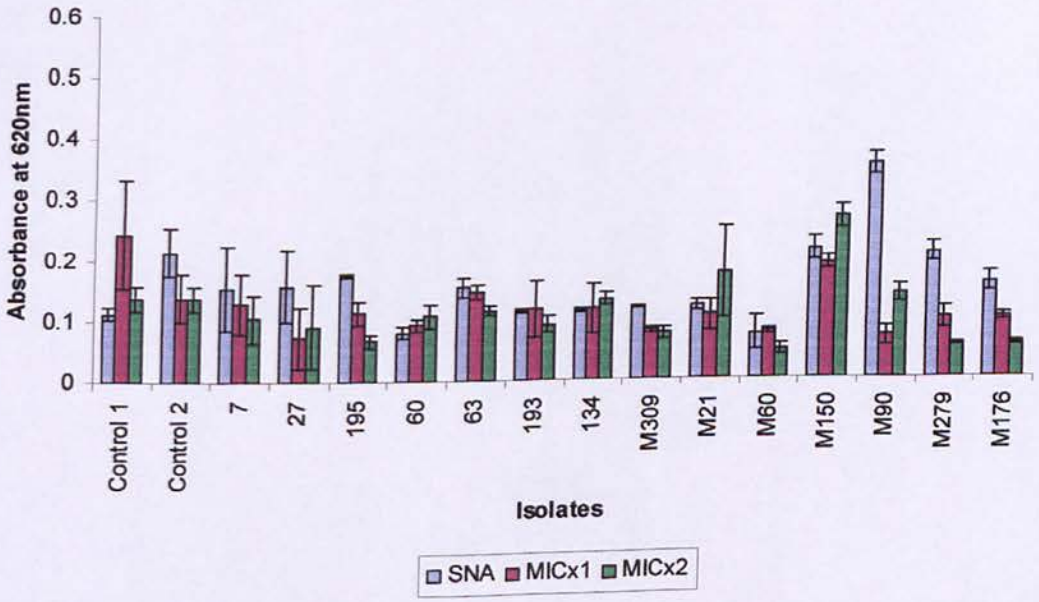


Figure 7.46: Absorbance of eluted stain from bacterial cell bound to the polystyrene plates (± S.E.M.). Cells were stained with 0.1% crystal violet and the stain was eluted with 80:20 (v/v) ethanol: acetone mixture.

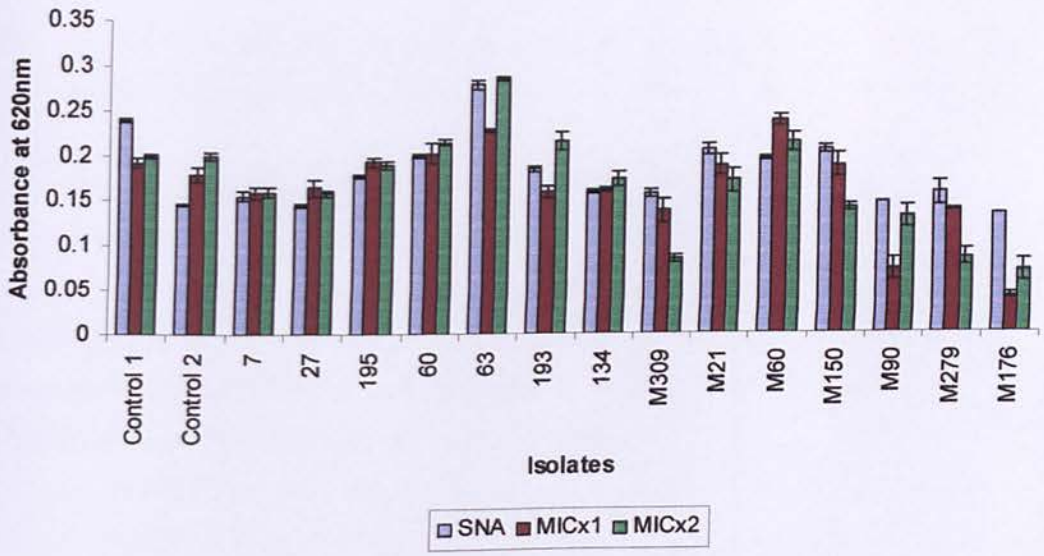


Figure 7.47: Absorbance of bacterial suspension in the polystyrene plates coated with fibrinogen containing nutrient broth after 2 hours incubation (± S.E.M.). Cells were harvested after a 10-hour growth in nutrient broth containing either the MIC (1mg/L) (Red) or double the MIC of clindamycin (2mg/L) (Green) or no antibiotic (Blue).

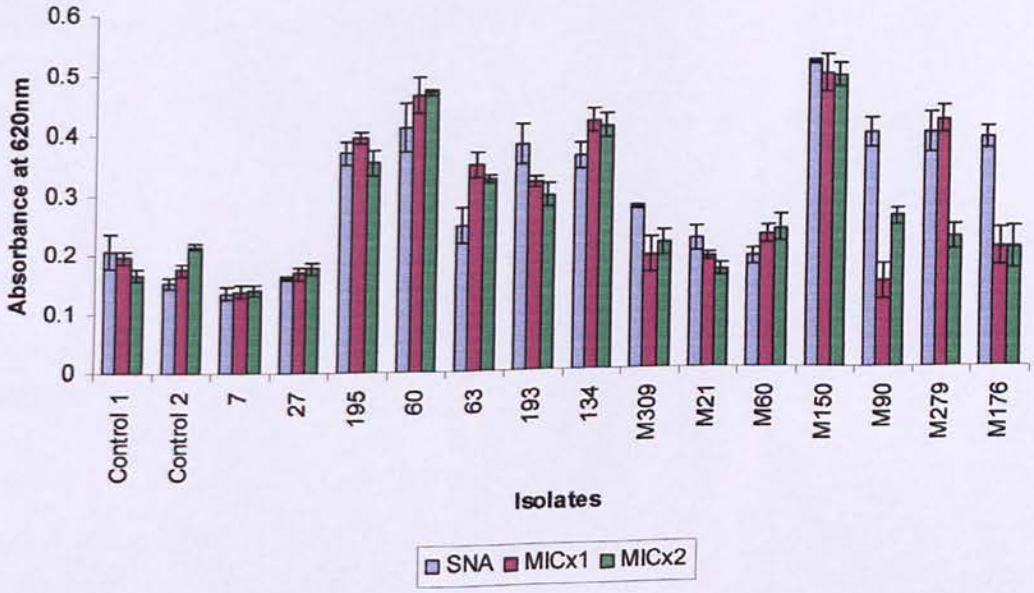


Figure 7.48: Absorbance of eluted stain from bacterial cell bound to the polystyrene plates coated with fibrinogen (± S.E.M.). Cells were stained with 0.1% crystal violet and the stain was eluted with 80:20 (v/v) ethanol: acetone mixture.

7.3.7 Analysis of individual isolates in request of changes in activity of virulence factors: an overview of statistical significance of growth in penicillin G.

Table 7.2 shows an overview of the statistically significant differences in the isolates grown in the presence of penicillin G compared to the sample with no antibiotic added. It can be seen that three of the isolates, 27, M309 and M150 and the control 1 (MRSA) had no statistically significant differences in their final cell population. All of the isolates did show a statistically significant difference to at least one of the virulence factors tests. Of the virulence factors tested the most differences were observed in the DNase test and only isolate 60 showed significant increase in production of one factor, lipase. No other isolates had significant differences to each of the factors tested.

Table 7.3 shows an overview of the statistically significant differences of the isolates adherence ability. No differences were detected in any of the adherence tests for isolates 7, 134, M90, M279 and control 1. Isolates 27, 63, 193, M21 and M176, all showed significant decrease in the stained results, whereas 60, 63 and M309 showed increases. Interestingly isolate M60 showed a significant increase in absorbance in adherence to the polystyrene plate and decreases in adherence the fibrinogen coated plate.

Table7.2: Statistically significant differences in a range of tests with isolates grown in penicillin G, including final cell populations from the growth curve and production of DNase, lipase, proteinase and haemolysin. Isolates were grown in broth containing antibiotic compared to isolates grown without antibiotics).

	Growth curves		DNase		Lipase		Proteinase		Haemolysin	
Isolate No.	SNA*vsMICx1	SNA*vsMICx2	SNA*vsMICx1	SNA*vsMICx2	SNA*vsMICx1	SNA*vsMICx2	SNA*vsMICx1	SNA*vsMICx2	SNA*vsMICx1	SNA*vsMICx2
7	p<0.05	p<0.05	p<0.05	p<0.005	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS
27	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS	p<0.05	NO SS	NO SS
195	p<0.05	p<0.05	p<0.05	p<0.005	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS
60	NO SS	p<0.05	NO SS	NO SS	NO SS	p<0.05(MIC>SNA)	NO SS	NO SS	NO SS	NO SS
63	p<0.005	p<0.005	NOSS	NOSS	NO SS	P<0.05	NO SS	NO SS	NO SS	NO SS
193	NO SS	p<0.05	NO SS	p<0.05	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS
134	p<0.05	p<0.005	p<0.05	p<0.05	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS
M309	NO SS	NO SS	NO SS	NO SS	p<0.005	p<0.005	NO SS	NO SS	NO SS	NO SS
M21	p<0.005	p<0.005	p<0.005	p<0.005	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS
M60	NO SS	NO SS	NO SS	p<0.005	NO SS	NO SS	NO SS	p<0.05	NO SS	NO SS
M150	p<0.005	p<0.005	p<0.005	p<0.005	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS
M90	p<0.005	p<0.005	p<0.05	p<0.05	NO SS	NO SS	NO SS	NO SS	NO SS	p<0.05
M279	p<0.05	p<0.05	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS
M176	p<0.05	p<0.05	NO SS	p<0.005	p<0.05	p<0.05	NO SS	NO SS	NO SS	NO SS
Control 1	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS
Control 2	NO SS	NO SS	----	----	----	----	----	----	----	----

SNA*: Sample with no antibiotic added; NO SS: Not Statistically significant; ---- No comparable test made

Table 7.3: Statistically significant differences in adherence to polystyrene 96-well plates and fibrinogen coat polystyrene 96-well plates of isolates grown in penicillin G. These tests include unstained and stained adherence to a 96-well polystyrene plate either coated with fibrinogen or uncoated.

	Unstained plate		Stained plate		Unstained fibrinogen-plate		Stained fibrinogen-plate	
Isolate No.	SNA* vs. MIC1	SNA* vs. MIC2	SNA* vs. MIC1	SNA* vs. MIC2	SNA* vs. MIC1	SNA* vs. MIC2	SNA*vs. MIC1	SNA*vs. MIC2
7	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS
27	NO SS	NO SS	$p<0.05$	NO SS	NO SS	NO SS	NO SS	NO SS
195	NO SS	NO SS	NO SS	NO SS	NO SS	$p<0.005$	NO SS	$p<0.005$
60	NO SS	$p<0.05$	$p<0.05$ (MIC>SNA)	NO SS	$p<0.05$	NO SS	NO SS	NO SS
63	$p<0.05$ (MIC>SNA)	NO SS	$p<0.05$	$p<0.05$	$p<0.05$ (MIC>SNA)	$p<0.05$ (MIC>SNA)	NO SS	$p<0.05$
193	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS	$p<0.005$
134	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS
M309	NO SS	NO SS	NO SS	$p<0.05$ (MIC>SNA)	NO SS	NO SS	NO SS	NO SS
M21	$p<0.05$	$p<0.005$	$p<0.005$	$p<0.005$	NO SS	NO SS	NOSS	NOSS
M60	$p<0.05$ (MIC>SNA)	NO SS	$p<0.05$ (MIC>SNA)	$p<0.05$ (MIC>SNA)	$p<0.05$ (MIC>SNA)	$p<0.005$ (MIC>SNA)	NO SS	$p<0.05$
M90	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS
M279	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS
M176	NO SS	NO SS	$p<0.05$	$p<0.05$	$p<0.05$	NO SS	$p<0.005$	$p<0.05$
Control 1	----	----	----	----	----	----	----	----

SNA*: Sample with no antibiotic added; NO SS: Not statistically significant; ---- No comparable test made

7.3.8 Analysis of individual isolates in request of changes in activity of virulence factors: an overview of statistical significance of growth in clindamycin.

Table 7.4 shows an overview of the statistically significant differences in the isolates grown in the presence of clindamycin compared to the sample with no antibiotic added. It can be seen that five of the isolates, 195, M309, M60 and M150 and control 1 (MRSA) showed no statistically significant differences in their final cell population. As shown in Table 7.2 the majority of the statistically significant differences were observed in the DNase and lipase tests. A statistically significant increase in the production of virulence factors can be seen in isolate number 27 for DNase and haemolysin tests and in isolates M21 and M60 for DNase production. In all other tests however, there was a statistically significant decrease in the production of virulence factors.

Table 7.5 represents an overview of the statistically significant differences of the isolate adherence ability. The control 2 (Oxford *S. aureus*) showed an increase in adherence ability whereas for isolates 7, 27, 60, 134, M21, M60, M150 and control 1 (MRSA) there was no statistically significant difference observed.

Table 7.4: Statistically significant differences in a range of test with isolates grown in clindamycin, including final cell population from the growth curve and production of DNase, lipase, proteinase and haemolysin. Isolates grown in broth containing antibiotic were compared to isolates grown in broth without antibiotics.

	Growth curves		DNase		Lipase		Proteinase		Haemolysin	
Isolate No.	SNA*vsMICx1	SNA*vsMICx2	SNA*vsMICx1	SNA*vsMICx2	SNA*vsMICx1	SNA*vsMICx2	SNA*vsMICx1	SNA*vsMICx2	SNA*vsMICx1	SNA*vsMICx2
7	p<0.05	p<0.005	p<0.005	p<0.005	NO SS	NO SS	NO SS	p<0.05	NO SS	NO SS
27	NO SS	p<0.05	p<0.05 (MIC>SNA)	p<0.05 (MIC>SNA)	NO SS	NO SS	NO SS	NO SS	p<0.05 (MIC>SNA)	p<0.05 (MIC>SNA)
195	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS	p<0.05	NO SS	NO SS
60	p<0.05	p<0.005	p<0.05	p<0.05	NO SS	NO SS	NO SS	p<0.05	NO SS	NO SS
63	p<0.05	p<0.005	NOSS	NOSS	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS
193	p<0.005	p<0.005	NOSS	NOSS	NO SS	p<0.05	NO SS	p<0.05	p<0.05	p<0.05
134	p<0.05	p<0.05	NO SS	NO SS	p<0.05	p<0.05	NO SS	NO SS	NO SS	NO SS
M309	NO SS	NO SS	NO SS	NO SS	NO SS	p<0.05	NO SS	NO SS	NO SS	NO SS
M21	p<0.005	p<0.005	p<0.05 (MIC>SNA)	p<0.05 (MIC>SNA)	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS
M60	p<0.005	p<0.005	p<0.05 (MIC>SNA)	p<0.05	p<0.005	p<0.005	NO SS	NO SS	NO SS	p<0.05
M150	NO SS	NO SS	p<0.05	p<0.05	NO SS	NO SS	NO SS	NO SS	p<0.05	p<0.05
M90	p<0.005	p<0.005	p<0.05	p<0.05	NO SS	p<0.05	NO SS	NO SS	NO SS	NO SS
M279	p<0.05	p<0.05	NO SS	NO SS	NO SS	p<0.005	NO SS	NO SS	p<0.05	p<0.05
M176	p<0.05	p<0.05	NO SS	NO SS	p<0.005	p<0.005	p<0.05	p<0.005	NO SS	p<0.05
Control 1	NO SS	NO SS	p<0.05	p<0.05	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS
Control 2	p>0.005	p<0.005	NO SS	NO SS	p<0.005	p<0.005	NO SS	NO SS	NO SS	NO SS

SNA*: Sample with no antibiotic added; NO SS: Not statistically significant

Table 7.5: Statistically significant differences to polystyrene 96-well plates and fibrinogen coat polystyrene 96-well plates of isolates grown in clindamycin. These tests include unstained and stained adherence to a 96-well polystyrene plate either coated with fibrinogen or uncoated.

	Unstained plate		Stained plate		Unstained fibrinogen-plate		Stained fibrinogen-plate	
Isolate No.	SNA* vs. MIC1	SNA* vs. MIC2	SNA* vs. MIC1	SNA* vs. MIC2	SNA* vs. MIC1	SNA*vs. MIC2	SNA*vs. MIC1	SNA*vs. MIC2
7	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS
27	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS
195	NO SS	$p<0.05$	$p<0.05$	$p<0.005$	NO SS	NO SS	NO SS	NO SS
60	NO SS	NO SS	NO SS	NO SS	NO SS	$p<0.05$	NO SS	NO SS
63	$p<0.05$	NO SS	NO SS	NO SS	$p<0.05$	NO SS	$p<0.05$ (MIC>SNA)	NO SS
193	$p<0.05$ (MIC>SNA)	NO SS	NO SS	NO SS	$p<0.05$	$p<0.05$	NO SS	$p<0.05$
134	$p<0.05$	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS
M309	NO SS	NO SS	$p<0.005$	$p<0.005$	NO SS	NO SS	$p<0.05$	$p<0.05$
M21	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS	NO SS
M60	$p<0.05$ (MIC>SNA)	$p<0.05$	NO SS	NO SS	$p<0.05$ (MIC>SNA)	NO SS	NO SS	NO SS
M150	NO SS	NO SS	NO SS	NO SS	NO SS	$p<0.05$	NO SS	NO SS
M90	$p<0.005$	NO SS	$p<0.005$	$p<0.005$	$p<0.005$	NO SS	$p<0.005$	$p<0.05$
M279	$p<0.05$	$p<0.005$	$p<0.005$	$p<0.005$	NO SS	$p<0.05$	NO SS	$p<0.05$
M176	$p<0.005$	$p<0.05$	$p<0.005$	$p<0005$	$p<0.005$	$p<0.05$	$p<0.05$	$p<0.05$
Control 1	NO SS	NO SS	$p<0.05$ (MIC>SNA)	NO SS	NO SS	NO SS	NO SS	NO SS
Control 2	NO SS	NO SS	NO SS	NO SS	$p<0.05$ (MIC>SNA)	NO SS	$p<0.005$(MIC>SNA)	$p<0.005$(MIC>SNA)

SNA*: Sample with no antibiotic added; NO SS: Not statistically significant

7.4 Discussion

Previous reports have highlighted that sub-inhibitory levels of antibiotics affect the virulence factor production of *S. aureus* cultures that are sensitive to said antibiotics (Ohlsen *et al.*, 1998; Herbert *et al.*, 2001; Gemmell *et al.*, 2002). In this pilot study the effects on growth and production of various virulence factors in isolates of MRSA was observed when grown in the presence of antibiotic at either the MIC or double MIC concentration. Isolates were grown in antibiotics to which they were phenotypically resistant. The antibiotics used were penicillin G and clindamycin. The aim of the study was to investigate if these levels of antibiotics would have similar effects on the virulence factor production in these resistant isolates. During the course of this investigation fourteen MRSA isolates collected from two different countries (UK and Malta) were tested. All isolates were resistant to both penicillin G and clindamycin as well as a total of 4 to 10 different antibiotics.

Of the isolates grown in the presence of penicillin G two isolates (63 and M21) showed only a downward trend in the rate of growth for the first six hours followed by a slight recovery in growth. There are two possible explanations for the results observed. Firstly it could be due to possible loss or reversion of resistance in the isolates tested. The isolates used in this test were taken from the culture collection; therefore it is possible that during storage, loss or reversion of resistance may have occurred. van Griethuysen *et al.* (2005), showed loss of the *mecA* gene in isolates of MRSA after long term storage (2years) at -80°C. As the isolates used in this study were stored in similar conditions and time periods, it is possible that loss or reversion of resistance may also have occurred.

A second possibility for the results observed could be due to the way the resistance profiles were carried out. Resistance profiles were determined by disk diffusion assays using an accredited method produced by the BSAC. There is a small margin between resistant and sensitive identification, therefore in any future studies MIC determination of antibiotic resistance levels would be more useful. Therefore, MIC determination of antibiotic resistance levels could illustrate possible losses or reversion of resistance in isolates that have been in long term storage more clearly.

Of the fourteen isolates tested twelve had significant differences in their final cell populations compared to their respective samples with no antibiotic added (see Table 7.2). Ten out of twelve isolates showed an initial growth retardation phase of about 4 hours after

which there was an increase in the rate of growth, so that the final population count was in a similar range as the sample with no antibiotic added. Although Isolates 63 and M21 had a longer retardation phase of six hours ($p<0.005$, SNA was greater than MICx1, SNA was greater than MICx2) and isolate M150 had a much longer retardation phase of up to 8 hours, growth recovered after this period ($p<0.005$, SNA was greater than MICx1, SNA was greater than MICx2). This data shows the range of effects in terms of growth dynamics that is obtained following challenges of an organism with an antibiotic to which phenotypic resistance is evident.

Of the isolates grown in the presence of clindamycin, seven isolates (63, 193, M21, M60, M90, M279 and M179) showed only a downward trend in the rate of growth with no recovery in growth. All of these isolates had statistically significant differences of $p<0.005$ in the sample with no antibiotic added compared to both concentrations of antibiotic. Possible reasons for these results have been discussed above *i.e.* reversion of resistance.

Of the remaining seven isolates tested, four showed statistically significant differences in their final cell population compared to their respective sample with no antibiotic added. In addition statistically significant differences were also seen in control 2. Five of the seven isolates (7, 27, 195, M309 and control 2) showed an initial retardation phase of about 4 hours, after which there was an increase in the rate of growth so that the final population count was in the same range as the sample with no antibiotic added.

Growth in the presence of penicillin G did have an affect on the production of virulence factors in the isolates. Decreases in production were noted, with the highest number of reductions seen with the enzyme DNase. Nine of the fourteen isolates (No.7, 195, 193, 134, M21, M60, M150, M90 and M176) showed a statistically significant decrease ($p<0.05$). It is tempting to speculate that this large decrease in the production of DNase may be due to the pre-cursor units being retained in the cell due to disruption of the cell wall and therefore disruption to possible transport mechanism due to the action of penicillin G. Statistically significant decreases ($p<0.05$) were seen in haemolysin with one isolate (number M90), lipase in two isolates (number M309 and M176) and proteinase in two isolates (number 27 and M60).

Of the fourteen isolates, one showed an increase in DNase activity, four in lipase activity, two in proteinase activity and one in haemolytic activity. However, significant increases in virulence factor activity were only observed in one test, where isolate 60

showed a significant increase in lipase activity in the sample at double the MIC concentration of the antibiotic compared to the sample with no antibiotic added ($p<0.05$, MICx2 greater than SNA).

Growth in the presence of clindamycin was also seen to have an affect on the production of virulence factors in the isolates. The highest number of reduction in virulence factors was seen in the isolates treated with clindamycin. Statistically significant reductions ($p<0.05$) were seen in DNase in four isolates (number 7, 60, M150 and control), lipase in nine isolates (number 63, 193, 134, M309, M60, M90, M279, M176 and control 2), proteinase in four isolates (number 7, 195, 60 and M176) and haemolysin in three isolates (number 193, M150 and M279). Increases were also noted in this test however the only significant increases ($p<0.05$) were seen in DNase (isolates, 27, M21 and M60) and haemolysin (isolate 27).

Reduction of virulence factor by clindamycin has been previously reported (Herbert *et al.*, 2001) as a reduction in transcription of the gene encoding protein A, alpha-haemolysin and serine protease. Interestingly, the reported effects were abolished in a resistant strain of *S. aureus*, whereas in this study it was shown that standard MIC level of clindamycin could have an effect in decreasing the virulence factor tested.

One of limitations in this pilot study was the way the virulence factor activity was detected. The aim of this study was to carry out an initial investigation into whether inhibitory levels of antibiotic, to which the isolates were resistant too, could effect the growth and activity of different virulence factors. The methods used to test the virulence factors, were used in a previous chapter (Chapter 4) to test for the presence or absence of various factors. Additionally the zone of enzyme activity was also recorded in chapter 4. Although differences were detected in the activity of the virulence factors in this study, a time period of 15 hours was needed to obtain a result. During this time the isolates were under no antibiotic pressure and therefore any effects the antibiotic might have had on the production of virulence factors may have been reduced or negated. However it must be remembered that differences were recorded. During the experiment measurements of the virulence factor present in the supernatant were taken. However, all results recorded were negative and this may have possible been due to the sensitivity problems of the tests used. The possible advantages of measuring supernatant levels for the virulence factor activity are that this would give a better indication of the effects of the antibiotic on enzyme activity. This would give a "snap-shot picture" of the levels virulence factor activity at the

end of the growth period, therefore giving a better indication of any affect on enzymes activity. Therefore in any future study, methods such as western blotting, that may be able to detect the enzymes in the supernatant could be used. However as stated above the purpose of this initial study was to detect any changes in virulence factor activity which was achieved with the test used.

The effects of the different antibiotics on the adherence of the isolates varied, with neither of the two antibiotics showing any greater ability for increase or decrease adherence. Herbert *et al.*, (2001) reported an increase in levels of fibronectin binding protein B using clindamycin. In this study the opposite was shown, *i.e.* a slight decrease in the adherence to both plate tests (polystyrene plastic plates and fibrinogen coated plates). However, two isolates on both plates did show an increase (number 134 and control 2). With the penicillin G treated isolates the result for increase and decrease was almost even on both plates, with three isolates showing either an increase or decrease. However in the uncoated plate the three increases in adherence were statistically significant ($p<0.05$) compared to only one statistically significant decrease. However, all of the decreases observed in the fibrinogen coated plate were statistically significant ($p<0.05$).

The reason for these increases or decreases is unclear. Penicillin G is known to disrupt the cell wall so this may be a possible answer for the decrease in adherence, whereas clindamycin is known to disrupt protein synthesis and therefore may disrupt the production of the adhesion. In both cases however, increases in adherence was observed.

It is well documented that over the past decade not only the levels of MRSA in western hospitals have increased, but also the number of antibiotics to which these MRSA are resistant (Anon 3, 2002). Vancomycin, teicoplanin and linezolid are all antibiotics which are of increasing importance in the fight against MRSA, as nearly all MRSA are sensitive to these antibiotics. Resistance to vancomycin and teicoplanin has been reported in the UK (Anon 5, 2002) as well as in Europe and the United State (Anon 5, 2002). In addition, one case of linezolid resistance has been reported in the United States (Tsiodras *et al.*, 2001) and one in the UK (Wilson *et. al.*, 2002).

It is now important to find ways in which to treat and combat MRSA in hospitals. One possible solution may be the use of sub-inhibitory or inhibitory level of antibiotic to reduce the production of virulence factor produced by these isolates. This idea has also been suggested by others. Gemmell *et al.*, (2002) wrote "as the number of therapeutic

options is reduced as resistance to traditional antibiotics rises, modulation of virulence factor expression by antibiotic treatment may be of increasing importance." It should be remembered that during an *S. aureus* infection it is not the bacteria that directly cause the pathology seen, but the factors produced by the bacteria. The effects of these factors have been discussed earlier (Section 1.6). However just looking at one group of enzymes, the proteinase family, this group of enzymes affect adherence and play a role in host immune system evasion, nutrient and maturing of other factors (Prokesova *et al.*, 1992; McGavin *et al.*, 1997; Lindsay *et al.*, 1999). Hypothetically by reducing the level of virulence factors it may be possible to reduce the pathogenic effect of *S. aureus* infection and therefore reduce the effect on the patient. It must be taken into account however, that this type of treatment is unlikely to kill the bacteria, but merely reduce the pathogenic effects of the bacteria. This could have a number of advantages. Firstly, it may reduce the degree of pathological effect in the patient and therefore possible reduce the severity of the infection. Secondly, this may make the bacteria more susceptible to the host immune system and/or the possible effects of other antibiotic treatments. However, more work is required.

It has also been shown that the antibiotics do not decrease the population of bacteria and have no effect of the growth of the bacteria compared to antibiotic free control (Ohlsen *et al.*, 1998; Herbert *et al.*, 2001). In this study a statistically significant decrease ($p<0.05$) in the population of bacteria was shown, however the level of bacteria were still sufficient to cause infection.

Therefore this sort of hypothetical treatment would have to be given along side an antibiotic that would also kill the bacteria. Stevens *et al.*, (1998) showed that the addition of clindamycin to standard penicillin therapy caused a more rapid resolution of invasive streptococcal infection, therefore suggesting that this type of treatment can work.

This is the first time this data have been seen where MIC level of antibiotics have been used. Other studies to date have used sub-inhibitory level of antibiotics (Ohlsen *et al.*, 1998; Herbert *et al.*, 2001; Gemmell *et al.*, 2002), but this use creates a dilemma. It is well known that if bacteria are grown in sub-inhibitory level antibiotic over time they will develop resistance to this antibiotic (Pfeltz *et al.*, 2000). The effect of some sub-inhibitory level of antibiotic did not show any effect on virulence factor in resistant strain (Herbert *et al.*, 2001). The use of MIC overcomes both of these problems. In isolates that were sensitive to the antibiotic, the use of clinical MIC concentration would result in the death of these isolates. This would also mean that the changes of the isolate developing

resistance would be greatly reduced. With the antibiotic used in this study, resistance levels were already high (Section 3.3-3.4). The rate of clindamycin resistance in isolates of MRSA from the UK and Malta was high (85% and 81% respectively), therefore negating any effect sub-inhibitory clindamycin would have had on the majority of MRSA.

In conclusion this pilot study indicates that MIC level of two different classes of antibiotics can have an effect on cell population size and the production of virulence factors produced by MRSA isolates. Further research is required with more sensitive measures to study the effects of MIC level of antibiotics on isolates of MRSA in order to determine their possible useful role to treatment.

8.0 General discussion and future work

8.1 General discussion

The main aim of this study was to investigate the relationship between antibiotic resistance and the production of pathogenic factors in clinical isolates of *S. aureus*. Isolates were collected from hospitals in the UK and Malta and tested against a panel of fourteen antibiotics. The isolates were then tested for the production of four different pathogenic factors, namely DNase, haemolysin, lipase and proteinase, additionally the adhesion properties of the isolates were also determined. Isolates were also strain typed by PFGE using the DNA cut *SmaI*, this is the first time isolates of *S. aureus* from these two countries have been compared. As stated in section 1.10, Malta and the UK are both in the top five countries with the highest level of MRSA in Europe (Anon 3, 2004).

In chapter three the antibiogram profile of each isolate was tested against a panel of fourteen different antibiotics using accredited methods (Hubert. *et al.*, 1999; Anon 8, 2000). Isolates were then divided into either MRSA or MSSA groups. No resistance to linezolid, teicoplanin and vancomycin was detected in any of the isolates from both countries. The numbers of antibiotics the isolates were resistant to varied between two to eight antibiotics in the isolates of MSSA and two to ten antibiotics in the MRSA isolates.

The main findings of these tests was that the two countries had similar antibiotic resistance profiles in their hospital isolates of *S. aureus*. This is interesting as the countries are geographically separated and currently use different antibiotics in their hospital policies. However, resistance to antibiotics and the percentage of isolates resistant to those antibiotics develops over time. For resistance to develop to an antibiotic, the antibiotic must be in use, therefore to achieve the resistance profiles seen in this study, the antibiotic would need to have been previously used to treat *S. aureus* infection. As no data of prescription policies before 2000 were available it is difficult to say if the two countries had similar policies historically, that might explain the similarity in resistance levels. Although it is tempting to speculate that at some time in the past, the two countries used the same, or closely related antibiotics and thereby developed the similar resistance profiles demonstrated in this study.

Using the DNA strain profiles from chapter 5 and concentrating on the high level resistance (nine to ten antibiotics) in the MRSA group, it can be seen that these isolates are spread over a number of different strain types. In the UK isolates, that were resistant to ten antibiotics, the isolates were shown to belonged to strain type 16 (possible EMRSA UK-

15), 32 (possible EMRSA UK-1) and strain type 42. In the isolates resistant to nine antibiotics, there was some grouping, with five isolates belonging to strain 13, which is possibly UK EMRSA 16. However, isolates resistant to nine antibiotics, were also seen in DNA strain types 9, 16, 19, 25 and 32. In both groups with high level resistance, the isolates possibly belong to the EMRSA group, such as, strain 13 (EMRSA 16), strain 16 (EMRSA 15) and strain 32 (EMRSA 1). In the UK MSSA group isolates that were resistant to eight antibiotics, were grouped in strain 8, 9 and 32. As with the MRSA, strain 32 has been highlighted as a possible EMRSA; however, in this instance, the isolates are MSSA. This therefore demonstrates that high levels of resistance to antibiotics can be found in the most common EMRSA in the UK (EMRSA 15 and EMRSA 16). This is important, if EMRSA is found to be the cause of outbreak of MRSA in hospitals, these isolates could potentially be resistant to a high number of antibiotics and this could present a major problem in a hospital.

In the Maltese isolates that were resistant to ten antibiotics, a PFGE profile could only be determined for one isolate (strain 40). In the isolates that were resistant to nine antibiotics two were strain 40 and four were strain 13, with the remaining isolate profiles undetermined. In the Maltese isolates it can be seen that the isolates with increase number of antibiotic resistances clustered more closing in terms of relatedness then their UK counterparts. In the Maltese isolates strain 40 is highlighted as being a possible EMRSA strain and therefore these isolates would potentially cause simple problems as mentioned for the UK isolates.

This shows that high level resistance can develop in a number of different strains of MRSA and MSSA, including epidemic MRSA strains. However, only through monitoring of resistance levels and the strain type where high resistance levels are seen, can adequate measures be taken to recognise and possibly prevent outbreaks due to these isolates.

Additionally only through monitoring of resistant level can effective antibiotic prescription policies be derived. The isolates in this study were shown to be highly resistant to a number of antibiotics (≥80%). If these antibiotics were regularly used in the treatment of *S. aureus* infection in patients that had the resistant form of *S. aureus*, no beneficial effect of the antibiotic would be seen. This could possibly lead to longer stays in hospital or other LTCs and potentially lead to a more serious infection. Therefore monitoring resistance levels and updating prescribing policies is not only good for the

patients, but also more cost effective for the hospitals by preventing long hospital stays and preventing the use of antibiotics to which the bacterium may be resistant.

Most published data on MRSA from the HPA in the UK and EARSS, focus on the level of methicillin resistance in *S. aureus* isolates. Other published data often only highlights new cases of resistance to antibiotic such as vancomycin (Hiramatsu *et al.*, 1997, Anon 4, 2002) and linezolid (Tsiodras *et al.*, 2001), as resistance to these antimicrobials is currently rare. In this current study the importance of understanding the resistance profile of isolates within a hospital and how this information is needed to improve antibiotic prescription policies, has been highlighted. Of the fourteen antibiotics tested, >80% of the MRSA isolates were resistant to six of the antibiotic and >50% of the MSSA isolates were also resistant to six antibiotics (refer to Section 3.4). When this data was compared to antibiotic prescription policies, it was noted that in the UK the use of gentamicin for the treatment of MSSA may be impaired due to high resistance level (60%). In the Maltese isolates high level of clindamycin resistance (80%) and clarithromycin resistance (60%) in the MRSA group may also impair treatment. Currently in the UK national levels of resistance to methicillin are reported annually by HPA. Although national resistance levels to other antibiotics would be interesting, its use may be limited to national antibiotic resistance epidemiological studies. However, local resistance levels to other antibiotics could be used to improve antibiotic prescription policies within a individual hospital, thereby improving treatment.

In chapter four the production of different pathogenic factors was measured in both the MRSA and MSSA. The main findings of this part of the investigation were that there was no difference in the production of pathogenic factors in MRSA compared to MSSA. Similar findings have been found in other studies. Schmizt *et al.*, (1997) studied the production of enterotoxin A-D and TSST-1 and found no difference in the production between MRSA and MSSA. No relationship between the production and the level of resistance could be detected. Similarly in the isolates that did not produce individual factors, once again no detected relationship between the production and the level of resistance was found. In both cases there was a range of different resistance and production of factors.

Interestingly, it was noted in this chapter that a small number of the isolates from both countries did not produce DNase, however all isolates of MSSA from the UK did test positive for this trait. Using the strain profile from chapter 5 it can be seen that these

isolates were found in different strains. In the UK MRSA isolates, strain 25 and 32 were found to contain one isolate each, which was DNase negative. In the Maltese MRSA isolates, no DNA profile could be determined for these DNase negative isolates. However in the MSSA isolates, the isolates were found to belong to strains 32 and 37. As with the high level antibiotic resistant isolates, these DNase negative isolates appear not to belong to a single strain but are seen in multiple strains.

The study also highlighted that in an evolving pathogen, the production of pathogenic factors (in this study mainly extracellular enzymes) are similar in isolates of both MRSA and MSSA from two different countries. These findings mirror previous studies which have investigated the production of extracellular toxin and found no difference in production between MRSA and MSSA (Coia *et al.*, 1992, Schmizt *et al.*, 1997). The work carried out here and in other studies suggest that virulence factors production in *S .aureus* is similar regardless of antibiotic resistance. However, has been noted not all isolates produced every factor tested. Although much is known about individual virulence factors, the way they work in combination in an infection is still unclear, as is one factor is more important then another. The fact that pathogenic factor production seems stable in isolates of *S. aureus* is an advantage, as these factors could be targeted by drug companies for new treatment. However, whether this *status quo* will remain, only further monitoring can determine.

The fact that there appeared to be no relationship between antibiotic resistance and pathogenic factor production, is not surprising. If an isolate was highly antibiotic resistant, with a loss of a key pathogenic factor, there is a chance that this isolate may not survive and therefore spread. If an isolate showed antibiotic resistance to a large number of antibiotics and also was highly pathogenic it may suggest that this type of isolate would be very successful pathogen. However this type of isolate was not detected in this study. Only through future monitoring of pathogenic factor production and a better understanding of the role that these factors play in disease generally, can infection of *S. aureus* be controlled and potentially be reduced. This along with better hygiene and improved asepsis in hospitals.

A large epidemiological study of the strains type of both MRSA and MSSA present in the UK and Malta was carried out, this is the first time isolates from these to country were compared. A total of forty-seven different strains type were identified, of these two strains had banding patterns similar to published data. The banding pattern of strain 13 in

this study was similar to that of EMRSA-16 and the banding pattern of strain 16 was similar to that of EMRSA-15. Both of these EMRSA strains are commonly found in UK hospitals; however, the banding pattern of some Maltese isolates were found to be similar to these EMRSA strains. This is the first time that the possibility of EMRSA-15 and -16 being reported in Malta, suggesting possible spread of the isolate within Europe. As discussed in Section 5.4 international spread of other strains of *S. aureus* has been reported, for example the "Iberian clone", this EMRSA has been isolated in Spain, Portugal, Belgium, Scotland, Italy, Germany and New York (da Sliva Combra *et al.*, 2003). This current study also highlights the possibility of unique local Maltese strain of *S. aureus*. Interestingly, the majority of the isolates in this strain group were MRSA and therefore it may suggest that this is a local EMRSA strain, however this was not confirmed in this study.

Further aims of this project were to investigate the possible use of a range of novel lipase substrates, to differentiate between isolates of MRSA and MSSA. Also to carry out a pilot study to investigate the effect on the growth rate and pathogenic factors production in MRSA isolates, grown under antimicrobial stress.

In the first of these studies eight novel chromogenic substrates for lipase activity were tested to determine their possible use as a diagnostic testing to differentiate between isolates of MRSA and MSSA. The isolates from both countries were tested on nutrient agar containing these substrates and no difference in the lipase activity between the MRSA and MSSA isolates could be determined. Lipase activity to some of the substrates was seen in all isolates tested, namely SRA-propanoate, SRA-butyrate and SRA-octanoate; however, when the isolates were tested for lipase activity on Tween 80 plate, activity was recorded in 73-94% of isolates. High levels of lipase activity (>90%) were also record in SRA-deconoate and SRA-laurate.

Although the results show little potential for these substrates to be used as a diagnostic tool with isolates of *S. aureus*, the results did suggest that these substrates may be used to investigate the genetic control of lipases in *S. aureus*. There are currently two form of lipases (see section 1.6.2.3.3), the genetic control of these two forms is currently unknown. However, the two forms can de divided by substrate activity and it is possible that these substrates may be use determine activation between the two types and therefore be used to studies genetic control of lipase.

The final component of this study was to carry out a pilot study, growing isolates of MRSA in the presence of antibiotic to which they resistant (Chapter 7), to determine the effect on growth rates and pathogenic factors production. In this pilot study a statistically significant decrease in the final cell population size was seen in the sample grown in the presence of antibiotic compared to a blank sample. This decrease was seen in eleven of the fourteen isolates grown in the presence of penicillin G and eleven of the fourteen isolates grown in the presence of clindamycin. Statistically significant decreases were also seen in the production of various pathogenic factors; however, some statistically significant increases in these factors were also noted. Although some problems did arise during this pilot study (Refer to section 7.4), the initial results warrant further investigation.

It may be speculated that the use of either sub-inhibitory or inhibitory levels of antibiotic, may be used as a possible support-treatment to retard the growth rate of the isolate and possibly reduce the production or activity of virulence factors within *S. aureus* infection. Thereby possibly making these cells more susceptible to the host immune system or to the action of other antibiotics to which they are sensitive. Although a great deal of further work needs to be carried out in this area.

Although our current understanding of *S. aureus* is extensive, the role of some pathogenic factors is still unclear, such as DNase and the newly discovered proteinase (Reed *et al.*, 2001) and how the various pathogenic factors interact with each other is still unknown. Therefore increased research and surveillance of this evolving pathogen is required.

8.2 Furture work

There are a number of possible avenues that could arise from this work. In chapter 2 the antibiotic resistance profile of each isolate was determined indicating that two possible areas of further work have arisen. Firstly when the antibiotic MIC levels were determined for a number of antibiotics, it was noted in many cases that the MIC levels were below the resistance breakpoint level. In the discussion of this chapter the finding of a recent paper was highlighted in which it was suggested that MRSA isolates in cold storage (-80°C) were found to lose the *mecA* and therefore Methicillin resistance (van Griethuysen *et. al.*, 2005). The isolates in this collection were approximately 3 years old, therefore re-testing the isolate antibiotic resistance profile after a set period of time could highlight whether loss of resistance has occurred in the present study collection. If loss of

phenotypical resistance had occurred, these isolates could be tested for the genetically loss of the resistance gene using PCR analysis. This work could be carried out on both the frozen isolates and isolates stored on agar slopes. As the isolates in this study are going to be used for further research, any loss of change of resistance profile has to be determined otherwise incorrect conclusions may be drawn.

A second area of possible research would be a retrospective and prospective study to evaluate antibiotic resistance in hospitals using hospital data. The levels of MRSA in the UK are reported every year; however, resistance to other antibiotics is not. A retrospective study could be used to look at resistance to other antibiotics in order to evaluate whether this resistance has risen rapidly as seen with methicillin, or more slowly. If there has been a rapid rise in resistance to antibiotic, it is possible to suggest that it will rise to 100%. However, only a prospective study will confirm this by once again using hospital resistance information. The findings of this work could be used to adjust prescription policies to improve antibiotic treatment, as discussed earlier.

In chapter 3 the production of a number of pathogenic factors was assessed. Although no difference in the production of these factors was found between MRSA and MSSA, it can not be concluded that change in production will not occur in the future. Only through monitoring of these factors could changes in production be determined. Increases in factors could potentially lead to more virulent strains of *S. aureus* and only monitoring could detect this. Virulence factor production in hospital isolates of MRSA could also be compared to the produced of factors in C-MRSA. To investigate if there is a difference in these two type of MRSA that affect people in very different setting.

Equally, the loss of pathogenic factors is important, as this could possibly open new avenues of antibiotic treatment. Additionally, the loss of key factors such as coagulase and DNase are important because these two enzymes are key factors since they are used to identify *S. aureus* from other staphylococci. Large scale loss of these factors would make differentiating *S. aureus* from other staphylococci difficult. Investigation into the loss of coagulase would need to focus on hospital isolates of coagulase-negative staphylococci, as any coagulase-negative *S. aureus* would have already been mis-identified. This type of investigation is important to evaluate the current number, if any, on coagulase-negative *S. aureus*. Potentially this type of test could be carried out using the detection of DNase as a marker since loss of this pathogen enzyme at the moment appears rare.

In this study a small number of isolates were found to be negative for the production of DNase. However it was not determined whether this loss was just phenotypic or also genenotypic, therefore future investigation on these isolates is required. The detection of whether this loss is phenotypic or genenotypic is important. If the loss is just phenotypic, this is a question that needs to be answered in itself as well as if the loss is both phenotypical and geneotypic. However the ramification to the pathogencity of *S. aureus* due to the loss of either coagulase or DNase is currently unknown therefore this may affect the detection of *S. aureus* infection in hospital laboratories.

In chapter 5 the growth of isolates in broth containing antibiotics to which the isolates were resistant to was investigated. A number of interesting findings arose from this study. It has to be remembered however, that this was just an initial pilot study. The whole investigation needs to be repeated, with method changes highlighted in the discussion of that chapter (chapter 5) such as checking the MIC breakpoint level of resistance of the isolates tested. Additionally the use of fresh hospital cultures would be better, rather than lab stored cultures, due to possible changes in resistance pattern. The number of pathogenic factors tested could also be increased to include toxins such as TSST-1 and exotoxins. The introduction of antibiotics to the broth could also be carried out using similar procedures used in patient antibiotic therapy to investigate whether additional doses of the antibiotic has a more profound effect on any decrease or increase in growth or virulence factor production. Finally after the growth period (10 hours in this study), the addition of an antibiotic to which the isolates are sensitive to could be introduced in order to investigate whether this changes the time needed to kill isolates in culture, such as to increase or decrease time.

In chapter 6 lipase activity in the isolates was tested against 8 novel chromogenic substrates for the possible use of these substrates as a diagnostic tool to discriminate between MSSA and MRSA. No difference in lipase activity could be determined between MRSA and MSSA. However a role in research was indicated since there is limited knowledge in the area of *S. aureus* lipase as the main focus of the work so far being directed toward *S. hyicus*. One possible area where these substrates may prove useful is in the investigation of genetic control of lipase production. Lipase activity is regulated by two genes, *agr* which positively regulates lipase production and the *sar* gene which negatively regulates lipase production. As yet it is unknown which lipase is regulated by these genes as the substrate used did not discriminate between the lipases. Therefore these new chromogenic substrates could be used for this type of investigation. It has been suggested

that SRA-deconoate and SRA-laurate can only be hydrolysed by the true lipase therefore these substrates could be used for determining genetic control of lipases.

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Appendix list

Appendix 1: UK isolates antibiogram profiles

Appendix 2: Maltese isolates Antibiogram profiles

Appendix 3: Breakdown of PFGE results showing strain type and sub-type, group, sub-group and isolates similarity.

Appendix 4: Main dendrogram

Appendix 5a-j: Ten main group dendrogram

See attached CD: all files save as Microsoft Powerpoint