An investigation, using an in-vitro alginate biofilm model, into locally delivered antibiotic combinations to treat staphylococcal prosthetic infection

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Declaration

I declare that this thesis has been composed by myself, that the work is my own and this thesis has not been submitted in candidature for any other degree, postgraduate diploma or professional qualification.

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Hypothesis

If staphylococcal biofilms are exposed to clinically relevant doses and combinations of antibiotics, a variable amount of biofilm eradication will be observed, as some regimes are more effective than others.

Abstract

Introduction

Joint replacement is a common and effective procedure but unfortunately, a small proportion of patients develop Prosthetic Joint Infection (PJI). The bacteria responsible for these infections exist within a surface-associated community known as a biofilm. When this biofilm phenotype is expressed, it allows the organisms to resist phagocytic host defenses, tolerate the stresses induced by antimicrobials and colonize peri-prosthetic niches. PJI is invariably refractory to standard therapies and clinicians are required to use a combination of systemic and local antimicrobials, repeated debridement and prosthesis exchange to treat the patient resulting in significant morbidity. Standard antibiotic sensitivity tests offer little insight into bacterial susceptibilities in the biofilm state. Furthermore, they neither test at the higher levels, nor in the combinations of antibiotics that are commonly locally delivered during surgery. The aim of this thesis was to develop and validate a biofilm model, and to use it to test staphylococcal biofilms with clinically achievable concentrations of antibiotics in combination.

Methods

Sodium alginate gel was chelated with a calcium chloride solution to form bullet shaped beads with a surface area of 161mm$^{2.}$ These beads were then removed and their surface inoculated with either a methicillin sensitive *Staphylococcus aureus* (ATCC 29213) or a clinical strain of coagulase negative staphylococcus. After being incubated aerobically for 20 hours in a 48-well micro-titre plate, growth controls were sampled and enumerated. The remaining beads were washed to remove non-adherent bacteria and placed into fresh broth containing antibiotics. After 3 hours of antibiotic challenge, they were removed, washed and the biofilm detached by dissolving the bead in a citric acid and Na_2CO_3 solution. Viable organisms were enumerated after micro-dilution and Miles Misra plating onto agar.

Results

Cryo-scanning electron microscopy demonstrated the model allowed a biofilm to develop on the surface of alginate beads. Overall the technique performed with satisfactory resemblance of the control data and acceptable responsiveness after disinfection. The repeatability of disinfection was found to be most variable around the level used to define bacterial eradication. The model was used to compare the minimum inhibitory (MIC) and biofilm eradication concentrations (MBEC) of seven commonly used antibiotics. Poor correlation was found between the susceptibility of the standard planktonic cultures to antibiotics and those that were effective against organisms in biofilm. Gentamicin and daptomycin were found to be the only mono-therapies that were effective against the biofilm at clinically achievable levels. Combining antibiotics that were ineffective as single agents did not confer additional benefit.

Interestingly despite gentamicin being effective when tested alone, combining it with clindamycin, rifampacin or linezolid reduced the bactericidal effect markedly in both strains. This phenomenon was investigated further by varying the concentration of antibiotics within the combinations. Combining the bactericidal antibiotics tested with gentamicin had an additive or synergistic effect. More importantly, a strong antagonistic effect was observed, with between 8 and 32 times more gentamicin being required, when it was combined with antibiotics considered bacteriostatic.

Conclusion

Standard microbiology laboratory testing is inadequate to guide clinical treatment of PJI. Testing of biofilm susceptibility to combinations of antimicrobials at high concentrations should be included in the laboratory testing of PJI. Further research should be directed towards understanding the mechanisms in which bacteriostatic antibiotics induce the organisms to become more tolerant to other antibiotics.

If the antagonistic effect is confirmed in-vivo then it is logical to study a two stage antimicrobial strategy, avoiding potential antagonism by only introducing bacteriostatic agents when the bactericidal drugs dip below their minimum biofilm eradication concentration.

Glossary

Amphiphilic	Chemical compound possessing both hydrophilic and lipophilic properties e.g. detergent.
ANOVA	Analysis of variance is a statistical method for making simultaneous comparisons between two or more means
ALAC spacer	Antibiotic loaded acrylic cement is fashioned into an anatomic shape and used as a temporary prosthesis between surgical procedures.
Anaerobe - facultative	Bacteria that can grow without oxygen but can utilise oxygen if it is present
Anaerobe - aerotolerant	Bacteria which cannot use oxygen for growth, but can tolerate the presence of it
Anaerobe - obligate	Bacteria which is harmed by the presence of oxygen
Arthroplasty	A procedure where a natural joint is reconstructed with an artificial prosthesis.
Arthroplasty - excision	A procedure where the articular ends of the bones are simply excised, so that a gap is created between them, or when a joint replacement is removed and not replaced by another prosthesis.
Arthroplasty - primary	The first time a total joint replacement operation is performed on any individual joint in a patient.
Auxotrophic	Inability of an organism to synthesize a particular organic compound required for its growth
Biofilm	A heterogeneous aggregate of micro-organisms who are adherent to a surface, embedded within a self-produced extracellular matrix and exhibit an altered phenotype with respect to growth rate and gene transcription
Body mass index	A statistical tool used to estimate a healthy body weight based on an individual's height. The BMI is calculated by dividing a person's weight (kg) by the square of their height (m2).
Bone cement	The material used to fix cemented joint replacements to bone – polymethyl-methacrylate (PMMA). Antibiotic can be added to bone cement to try and reduce the risk of and treat established infection.
Chaperone	Protein that assists the folding or unfolding of other macromolecular structures
CNS	Coagulase negative staphylococcus
Debridement	Surgical removal of dead, damaged, or infected tissue to improve the healing potential of the remaining healthy tissue.
ETC	The electron transfer chain is a sequence of electron carrier molecules or membrane proteins that shuttle electrons during the redox reactions that release energy used to power the cell.
HR	Hazards Ratio are an expression of the chance of events occurring in the treatment arm as a ratio of the hazard of the events occurring in the control arm
Homologues	Having the same or allelic genes with genetic loci usually arranged in the same order
Horizontal gene transfer	Acquisition of genetic information by transfers from an organism that is not its parent, typically a member of a different species.
[LR]	Likelihood ratio is the chance that a given test result would be expected in a patient with the disorder compared to a patient without the disorder
LMWH	Low molecular weight heparin.

LD	Log density is a measure of bacterial number. Calculated by $\log_{10}$ converting the number of colony forming units enumerated.
LR	Log reduction is the difference between LD of growth controls and LD of biofilms exposed and antimicrobials.
MSSA	Methicillin sensitive *Staphylococcus aureus*
MBC	Minimum bactericidal concentration is lowest concentration of antibiotic that inhibits subsequent planktonic subculture.
MBEC	The minimum biofilm eradication concentration is concentration of antimicrobial that eradicates 99.9% of the bacterial biofilm.
MIC	Minimum inhibitory concentration is the lowest concentration of antibiotic that inhibits overnight growth in a planktonic culture
NCP	Normal colony phenotype
[OR]	The odds ratio represents the chance that an outcome will occur given a particular exposure, compared to the odds of the outcome occurring in absence of exposure.
Operon	A unit made up of linked genes that is thought to regulate other genes responsible for protein synthesis.
PMF	Proto-motive force
Parenteral	Introduction of substance into the body via a route other than the mouth, especially via intra-venous infusion
Pharmaco-dynamics	Study of relationship between antibiotic concentration and pharmacologic effect or microorganism death.
Pharmaco-kinetics	Study of relationship between antibiotic concentration and time or dosing regimes.
Prosthetic	An artificial body part usually internally implanted but can be removable.
Prosthetic joint infection	Bacterial or fungal infection surrounding an artificial joint replacement. Subcategory of prosthetic related infection that encompasses infection of any implanted material in the host.
Putative	Commonly regarded as such; reputed; supposed
Quorum sensing	Derived from legal term denoting minimum number of members required to make a decision. Bacteria have evolved sophisticated cell–cell communication systems that allow them to orchestrate the expression of different phenotypic states
Revision	Operation performed to remove (and usually replace) one or more components of a total joint prosthesis for whatever reason.
Revision single-stage	A revision carried out in a single operation
Revision two-stage	A revision procedure carried out as two operations, often used in the treatment of deep infection.
Sinus	A channel connecting the skin with an underlying suppurative wound.
SCV	Small colony variant is a bacterial phenotype that displays auxotrophism, forms slow growing poorly pigmented, small colonies on agar plate and expresses an altered pattern of virulence and tolerance to antimicrobials
Standard deviation	Measure of the spread of the data about the average. The smaller the standard deviation, the less spread out the data.
Stochastic	A random probability pattern that may be analysed statistically but may not be predicted precisely.
Tolerant	Reduced efficacy of antibiotics in the absence of genotypic resistance.

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1. Introduction

1.1. Prosthetic related infection

1.1.1. Historical view of prosthetics

The ability to secure resources necessary for the replication of our genome is universal to all life on Earth today. What has distinguished *Homo sapiens* from the other tens of millions of species that exist[1], was our ability to develop technologies which allowed us to augment our hunting ability, migrate to every continent, farm other species and latterly test and rapidly communicate abstract concepts such as the ones contained within this thesis.

Not content just to adapt our environment, humans have demonstrated a widespread desire to modify their own bodies for social, religious or functional reasons. The first recorded example of this is the 5,300 year-old Ötzi the Iceman, who was found in a glacier in Austria sporting some gold earrings[2]. There are also several reports of the Incas performing crainoplasties with gold plates to cover trephine defects 4,200 years ago[3].

These procedures did not advance significantly for the next 4000 years and surgery was limited to amputation or evacuation of pus until modern anaesthesia with chloroform or diethyl ether, was developed in the 1840's by James Simpson and William Morton respectively. Europe's first operation using ether was a leg amputation performed by Robert Liston on December 21st 1846 at the University College Hospital London. A contemporaneous description of the procedure highlighted why the mortality rate from post-operative infection was 50-60%[4]:

'Everyone swore that the first flash of his knife was followed so swiftly by the rasp of saw on bone that sight and sound seemed simultaneous. To free both hands, he would clasp the bloody knife between his teeth.'

It was not until Joseph Lister built on the work of Pasteur and described aseptic surgical techniques in his 1867 thesis[5], that implanting biomaterials became more popular. As the intra-operative and post-operative risks lessened surgeons started to use biomaterials to repair, re-orientate and replace parts of the musculoskeletal system aiming to alleviate pain and restore function to their patients. Hansmann first described reconstruction of fractured limbs with metal plates and screws in 1886. The first endoprosthesis was invented and implanted in 1890 by Themisocles Gluck, in the form of a hinged ivory knee replacement used to replace a tuberculous knee joint[6]. Although it allowed the patient to mobilise pain free after a month, it later had to be removed because of prosthetic infection.

As hip replacement evolved from interpositional and hemi-arthroplasty between 1920 and 1950 it became evident that despite careful antisepsis, infection was prevalent after implantation of prosthetic material and research began to understand the mechanisms. Elek and Conen demonstrated in 26 human volunteers[7], that the minimum inoculum of *Staphylococcus aureus* necessary to cause a superficial abscess in their skin reduced from 10 million to just 200 when they were introduced around a silk suture. A number of researchers have subsequently identified that the granulocytes that accumulate around biomaterials become "frustrated' by their futile attempts to attack the implant, develop a functional defect that impairs superoxide production and phagocytosis [8].

Total hip replacement was popularised after Sir John Charnley made a number of important advances in the 1960's (Figure 1.1). He introduced bone cement as an interface between the prosthesis and bone, he also conceptualised low friction arthroplasty, reducing wear between the bearing surfaces. His initial deep infection rates were 9% but with a series of adaptations, including cleaner air flow reducing intra-operative aerosol contamination, his infection rate fell 10 fold by 1970[9]. Joint replacement is

just one example in medicine today of how our desire to modify our own bodies has presented microorganisms with a new peri-prosthetic niche to colonise and exploit. Although the pathophysiology of prosthetic related infection is similar no matter where it occurs in the body, the focus of this thesis will be when it occurs after joint arthroplasty and causes a Prosthetic Joint Infection (PJI).

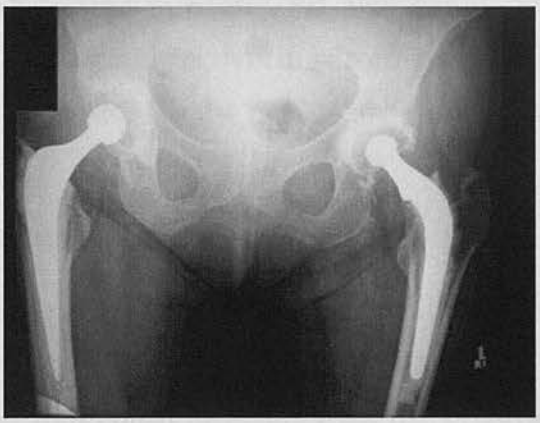

Figure 1.1. A radiograph showing bilateral hip replacements.

The right hand side of the page shows Charnley's design and the left is a stem designed in Exeter that is currently popular. The cement mantle that supports the metal stems and polyethylene cups appears more radio dense than the surrounding bone.

1.1.2. Current use of prosthetic joints in the UK

In 2011, over 160,000 primary total hip and knee replacement procedures were performed in the UK in approximately equal proportion[10,11]. Worldwide sales figures showed 1.4 million hip and 1.1 million knee replacements were sold in 2011[12]. It is one of the most rewarding operations a surgeon can perform, reducing pain and increasing physical function in the vast majority of patients. It also presents good value for money compared to other medical interventions with the mean cost per Quality-Adjusted Life Year (QALY)[13] has been determined to be £5,728 for primary hip and £11,947 for primary knee replacement[14]. A commonly used threshold level for the cost per QALY that is deemed to be good value is £25,000–43,000.

Despite precautions, major complications such as component dislocation, death from medical complications and prosthetic infection are inevitable in a proportion of patients. Specialist arthroplasty units with high throughput generally report a deep infection rate of around 0.5-1% but range up to 4% in some general hospitals[11,15,16]. Although revision surgery costs around £20,500, which is more than three times the cost of the primary procedure[17-19], it is not the greatest associated burden. PJI also entails significant patient morbidity with weeks in hospital recovering from painful surgery and is an emotionally draining experience for the healthcare professionals involved.

1.1.3. Definition of prosthetic joint infection

The recent 'Infectious Diseases Society of America Guidelines'[20] reviewed the current literature and defined definite prosthetic joint infection as: "the presence of a sinus tract that communicates with the prosthesis or purulence surrounding the prosthesis without another known etiology or two or more cultures that yield the same organism". Findings that are also highly suggestive of PJI include the presence of acute inflammation seen on histo-pathologic examination of peri-prosthetic tissue, or the growth of a virulent microorganism (e.g. *S. aureus*) in a single specimen of a tissue biopsy or synovial fluid. The presence of PJI is possible even if the criteria are not met and the clinician should apply clinical judgment.

1.1.4. Diagnosis of prosthetic joint infection

Clinical features of PJI vary depending on the time from surgery and the responsible organism. Around 2/3^{rds} of all PJI present within the first few weeks post-operatively[16]. Persistent wound drainage is typical with systemic upset and local inflammation if caused by a more virulent organism. If infection presents after this period but within the first year, then a less virulent organism is typically responsible and patients frequently report that their original pain was never abolished, or has returned. Clinically the limb can be oedematous with a woody character to the soft tissue envelope,

secondary to the inflammation. If the infection occurs after a year then it is more likely to represent haematogenous spread of a more virulent organism and present with systemic symptoms.

Once PJI is suspected it must be investigated by a thorough history and physical examination. The history should include the onset of and current clinical symptoms, the exact date of implantation, the type of prosthesis and whether Antibiotic Loaded Acrylic Bone Cement was used (ALAC). Enquiries into previous surgical history, wound healing problems following joint implantation, concurrent infections, drug allergies and co-morbid conditions should be made. Relevant microbiology results from aspirations and intra-operative samples, as well as previous antimicrobial therapy and inflammatory indices should be detailed.

1.1.5. Investigation of suspected PJI

Two orthogonal plain radiographs of the joint should be obtained to assess mechanical loosening and remaining bone stock. Routine use of bone scans, leukocyte scans, magnetic resonance imaging, computed tomography, and positron emission tomography scans are not indicated.

Laboratory blood tests including Erythrocyte Sedimentation Rate (ESR) and C-Reactive Protein (CRP) should be performed in all patients and blood cultures should be taken if there is systemic upset with fever. CRP is acute phase protein, produced by the liver and is a maker of systemic inflammation. Its plasma concentration rises dramatically after arthroplasty with peak concentration on day 2 but generally returns to normal (<10 mg/dl) in 3-4 weeks following joint arthroplasty[21]. Berbari *et al.*[22] included 30 studies in their meta-analysis to determine the usefulness of inflammatory markers at predicting the presence of infection in patients suspected of having PJI. They found CRP had a sensitivity of 88% and specificity of 74% whilst ESR had a sensitivity of 75% and specificity of 70%. Combining them showed an increase in their diagnostic Odds Ratios [OR] to 89 reflecting improved accuracy whist remaining independent to prevalence. Berbari's

meta-analysis found interleukin-6 to be the most sensitive and specific marker but as its use is limited to a few specialist centres and it is not currently recommended.

A diagnostic joint aspiration should be performed in all patients with suspected PJI before antibiotics are given. Analysis of the synovial fluid should include a leukocyte count and extended 14-day culture for aerobic and anaerobic organisms. Schafer *et al.*[23] showed in a well-conducted study that 1/4 of organisms (typically *Propionibacterium* spp) were only identified in cultures between 7 to 14 days. Importantly they demonstrated the clinical significance of these organisms with the association between ≥ 2 culture-positive tissue samples and positive histological analysis. The OSIRIS group[24] demonstrated by increasing the cutoff to two or more specimens growing the same organism raises the positive Likelihood Ratio [LR] to 25.9, which is highly predictive of infection (sensitivity, 71%; specificity, 97%; post-test probability of infection, 80.6%).

If antibiotics have been started and the patient is not clinically unwell, then withholding antimicrobial therapy for 2 weeks prior to aspiration increases the positive identification rate from 41% to 77%. However, if patients had received antimicrobial therapy within 2 weeks of revision, then culture after sonication of the prostheses is a more sensitive method than conventional culture of peri-prosthetic tissue (75% and 45%, $p<0.001$)[25]. Interestingly however, it has been demonstrated that a single dose of pre-operative antibiotic at induction of anesthetic does not alter the culture results of positive intra-operative specimens[26].

1.1.6. Risk factors for developing prosthetic joint infection

An understanding of patient, intra-operative and post-operative factors associated with PJI (Table 1.1) is needed if clinicians are to take steps to mitigate them. Some risk factors like the use of surgical drains are easy to avoid once they are known to be detrimental. Others such as previous joint surgery or being male cannot be modified and in these circumstances it is vital for the patient to be counseled and weigh up the advantages and disadvantages of the procedure before granting their consent.

Using bone cement that is loaded with antibiotics is generally accepted to lower the risk of PJI. It is of note though, when Parvizi performed a meta-analysis of 15,000 hip replacements[27] which showed that 6/8 of the studies demonstrated reduced infection with antibiotic cement, only the largest study (with around 75% of all hips studied)[28] actually demonstrated an effect that was statistically significant. This helps explain why their Forest plot, using weighted means, demonstrated overall a statistically significant reduction in infection by around 50% (1.2% vs. 2.3%).

Engesaeter *et al.*[29] analysed the outcome of 56,275 joint replacements collected in the Norwegian arthroplasty register and found that the risk of implant revision was lowest if ALAC was used in combination with 4 doses of systemic antibiotic on the day of surgery. Patients who only received systemic prophylaxis had a 1.3 times higher revision rate for aseptic loosening than the group in whom ALAC was used. This suggests a proportion of revisions carried out for 'aseptic' loosening are probably associated with low-grade infection. Registries do not however, provide information about true infection rates as their primary outcome is a revision or re-operation and can underestimate PJI incidence by a quarter[15].

Factors	Major Risks	Lesser Risks
Patient Related	Malignancy [OR 3][19] Rheumatoid Arthritis BMI >40 [OR 6.4][30,31] Warfarin Bacteraemia Avascular necrosis [OR 3.7][32] Post traumatic arthritis [or 3.2][32]	Male [OR 1.9-2.2][32,33] Diabetes [OR 1.28-2.3][30,32] Psoriasis or dermatitis Immunosuppression Malnourishment BMI >35 [OR 1.5][32] Dental abscess
Intra-operative	Excessive surgical time [OR2.8][32,33] Revision Surgery [OR 2][19] Use of a wound drain [OR 7][34] Preoperative LMWH [OR 6.2][35] NNIS score > 2 [OR 4][19]	Inadequate skin closure Not using prophylactic antibiotics [OR 4][36] Not using ultraclean air filters [OR 2.6][36]
Post-operative	Early wound infection [OR 36][19] Haematoma [OR 12][37] Uncontrolled INR [OR 2.4][34] Wound leakage after 7 days	Skin blisters Concurrent UTI

Table 1.1: Risk factors for developing prosthetic related infection.

[OR]-Odds ratios should the patient be exposed to that particular risk. LMWH-low molecular weight heparin, UTI - urinary tract infection, NNIS - National Nosocomial Infections Surveillance risk index[38]. BMI- Body mass index. UTI – urinary tract infection. INR – International Normalized Ratio of prothrombin time is a measure of coagulation.

Although there have been two recent studies that did not find ALAC lowered the 1-year infection rate, there are important differences between them and previous studies. Namba *et al.*[32] identified 56,216 primary knee replacements using registry data to identify that a higher proportion of patients with deep infection had received ALAC, (18.8% compared to 12.0% of patients without infection p>0.001). Although the multi-variant analysis will have controlled for some of the confounding factors, there were enough other apparently spurious associations to make residual confounding factors very likely. For instance they also found that low volume centers had 0.33 Hazards Ratios [HR] (see glossary) compared to high volume centers and that clean laminar air did not influence the infection rate. It seems likely that they failed to control the factors that differentiated high and low risk patients and this diminished the studies relevance.

Hinarejos *et al.*[33] performed a well constructed prospective study with 2948 cemented total knee replacements that were randomised to use the same bone cement with or without added erythromycin and colistin. They reported no difference (1.4% vs. 1.35%) in the incidence of deep infection in the first year between groups. Their findings do not necessarily conflict with the vast majority of the studies included in Parvizi's meta-analysis that used gentamicin as the antibiotic. The most logical conclusion is that the erythromycin/colistin combination is not as efficacious as gentamicin at preventing infection following primary arthroplasty.

1.1.7. Causative organisms

The resident micro-flora of human skin consists primarily of Gram-positive bacteria, including the genera *Staphylococcus, Propionibacterium* and *Corynebacterium.* Staphylococcal spp are identified as pathogens in around 2/3rds of infected joint replacements[23,39-42] (Table 1.2). Whilst the coagulase negative staphylococci that commonly colonise normal immuno-competent

skin do not normally cause infection, they are frequent opportunistic peri-prothetic pathogens. *S. lugdunensis* is an exception as it is coagulase negative but can cause severe soft tissue infection and should be suspected of being pathogenic if identified[43].

The coagulase positive *Staphylococcus aureus* is another opportunistic pathogen but only colonizes healthy adults transiently, in the groin or axillae, or permanently in the nostrils or in areas of dermatitis[44]. Approximately 45% of *S. aureus* strains isolated in the UK are methicillin resistant (MRSA)[45]. There are two dominant clones of Epidemic MRSA (EMRSA –15 and EMRSA-16) that cause over 90% of all cases in Scotland[46].

Propionibacteria are aero-tolerant anaerobes that thrive in the anoxic environment of sebaceous glands. They have been identified from as many as 62% of hip implants[47] often requiring prolonged anaerobic culture before identification can be made. Schäfer *et al*[23]. published their clinical series of aspirations from PJI and highlighted two important points. The first was that the fastidious organisms (such as *Propionibacterium* and *Peptostreptococcus* spp) accounted for more than 25% of pathogens detected but took longer than a week to become apparent. Obviously, these late cultures are prone to contamination and so they also correlated their microbiological with histological results before considering it an infection. Interestingly, they found similar proportion of true positives in the late compared to early positive cultures (61% vs. 73% p=ns).

A commonly cited series of 112 prosthetic joint infections from Oxford[48] has not been included in Table 1.2, as in that study the definition of infection included a single positive culture without histological corroboration which would have increased their false positive rate. This was counter to the groups previous recommendations that the optimal number of samples is 5 if you accept that isolating three identical organisms indicates infection[24].

	Nickinson n=121[39]	Schafer n=110[23]	Trampuz n= 129[40]	Zimmerli n=600[8]	Ostendorf n=851[42]	Mean %
CNS	49	54	37	23	40	40.6
S. aureus	13	11	34	30	19	21.4
E. coli	7	2	11	6	5	6.2
Enterococcus	6	5	3	-	6	5
Propionibacterium	-	14	8	-	7	9.6
Streptococcus	-	5	7	9	9	7.5
Other bacteria	25	9	-	32	14	20

Table 1.2: Causative organisms in prosthetic joint infection.

Author, study size and relative frequencies (%) of species isolated.

1.1.8. Classification of prosthetic related infection

One obstacle in improving the outcome after prosthetic related infection is the lack of a comprehensive classification system. There are a number of key independent variables that need to be matched to enable comparison between different studies. These include the chronicity of the infection, the virulence and sensitivity of the infecting organism if known, whether the prosthesis is currently stable, the hosts ability to mount a strong immune response, presence and degree of osteomyelits, quantitate bone loss and degree of surrounding soft tissue damage. One can easily envisage, with each factor requiring a number of sub-categories, ending up with a cumbersome classification system. Even attempts such as McPherson's in 2002[49] to produce simplified systems with three categories: infection chronicity, systemic host grade and local extremity grade has not been subsequently used in the literature. Therefore attempts at constructing a comprehensive system for all musculoskeletal infections including PJI, such as the one proposed recently by Romano[50] with 7 categories and 33 subcategories, seems unlikely to be adopted.

Classifications limited to PJI have been more readily used with modifications to the Coventry classification[51] by first Tsukajama[52] and then Zimmerli[53] who defined acute infections as being present for less than 4 weeks. A description of PJI would include information about the source and chronicity of infection. The organisms can either be introduced to the joint at the time of operation, via haematogenous spread or by contiguous inoculation. The chronicity of infection depends upon the source. Therefore, an acute infection occurs within 4 weeks postoperatively and delayed infection presents within a year. Late infection has to be more than a year postoperatively but it treated differently depending if inoculation is peri-operative, haematogenous or contiguous.

1.2. Treatment strategies in prosthetic joint infection

As the bacteria responsible for PJI grow slowly in a sessile biofilm state, in which they are recalcitrant to conventional antibiotic therapy, surgical management is necessary to affect a cure. The most effective way to treat peri-prosthetic infections is to debride all devitalized tissues, remove all the prosthetic material, administer appropriate antibiotics and support the host's immune system in dealing with the remaining bacteria. This is only easily achievable in cases where the prosthesis is easily replaceable (e.g. peripheral cannula) or functionally redundant (e.g. metal work around a healed fracture). The management of prosthetic joint infection is more complicated and necessitates eradicating the infection while preserving mechanical function of the joint. Clinicians tend to use a stepwise approach to selecting the appropriate surgical treatment (Table 1.3).

1.2.1. Debridement, Antibiotics and Implant Retention (DAIR)

Attempts at component revision often can result in reduction of bone stock and peri-operative fracture, making subsequent reconstruction more challenging. Unsurprisingly surgeons have tried to retain stable implants with varying degrees of success. The outcome of DAIR depends heavily on the time between infection onset and treatment with between 69-91% success reported in patients with symptoms of less than one month compared to only 16.6% if present for more than six months[53]

The largest series of 112 patients treated with DAIR was an observational study over a five year period in Oxford[55] . Overall 81% of joints remained *in situ* after 2 years. The majority of patients received both oral ciprofloxacin and rifampicin for a mean of 1.5 years. Multivariate analysis indicated that, in terms of recurrent infection: arthroscopic debridement was less effective than open surgery [HR] 4.2; *S. aureus* was an independent variable associated with failure [HR] 2.9; previously revised

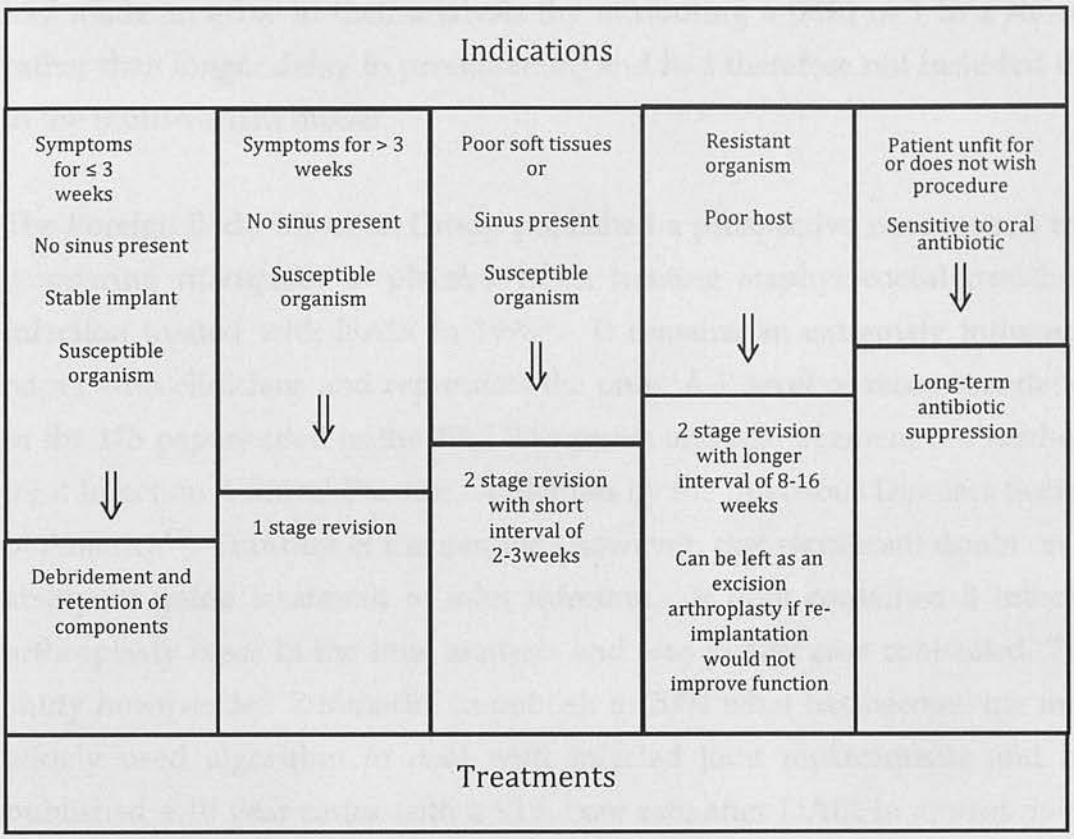

Table 1.3: A stepwise approach to selecting a treatment strategy in prosthetic joint infection.

The left hand boxes represents the minimum surgical procedure required for eradication of PJI. As the operative indications become less favorable, a more extensive surgical tactic is required. Long-term antibiotic suppression is the correct option for a proportion of patients but it will not result in a eradication of infection. See glossary for explanation of terms.

implants did worse than primary ones [HR] 3.1; patients debrided within three days of presentation had after univariate analysis, a [HR] 0.36 compared to patients who were delayed more than two weeks. The authors had made an error in their analysis (by attributing a [HR] of 1 to a shorter rather than longer delay in presentation) and had therefore not included this in the multi-variant model.

The Foreign Body-Infection Group published a prospective randomised trial comparing rifampicin to placebo when treating staphylococcal prosthetic infection treated with DAIR in 1998[56]. It remains an extremely influential paper with clinicians and represents the only 'A-I' level of recommendation in the 175 papers cited in the 2012 'Diagnosis and Management of Prosthetic Joint Infection: Clinical Practice Guidelines by the Infectious Diseases Society of America'[20]. Scrutiny of the methods however, cast significant doubt on its ability to guide treatment of joint infection. It only contained 8 infected arthroplasty cases in the final analysis and was poorly case controlled. This study however led Zimmerli[53] to publish in 2004 what has become the most widely used algorithm to deal with infected joint replacements and has published a 10 year series with a 91% cure rate after DAIR in appropriately selected patients[40].

1.2.2. Single-Stage Revisions

A single-stage revision is a surgical procedure in which the infected tissues and prosthesis are removed, and during the same anaesthetic, a new joint replacement is inserted and the wounds closed. It is gaining popularity especially in Europe and a review in 2008 of more than 1200 infected joint replacements from 12 studies showed that the combined 5 year implant survival rate after single stage revision was 83%[57]. When its use is strictly limited to those patients where a single causative organism is known to be susceptible to appropriate antibiotics, there are no sinuses present, the patient is immuno-competent and the reconstruction after debridement relatively straight forward, the results seem to be even more encouraging.

Oussedik *et al.*[58] selected 11 suitable patients from 50 consecutive infected hip replacements and achieved 100% cure at mean of 5 years after a single stage revision. The same group have reported 94% 2-year success with knee replacements[59]. Joulie *et al.*[60] followed Zimmerli's protocol[53] and cured 94% of their patients after single stage revision of both hip and knee arthroplasty that had been infected with *S. aureus.*

1.2.3. Two-Stage Revisions

A two-stage revision differs from a one-stage as the re-implantation is performed weeks or months after the ex-plant. It remains the current gold standard method of eradicating infection, achieving it in > 90% of patients in most published series[57]. It allows the surgeon two opportunities to debride any devitalized tissue, identify the responsible pathogen and reduce the bacterial load by several orders of magnitude. The majority of surgeons now place an ALAC spacer between stages that helps to preserve a more normal soft tissue envelope and deliver very high local concentrations of antibiotics with few systemic side effects.

The clinician can monitor the patients' recovery and chose the most appropriate time interval to perform the second re-implantation stage. Traditionally, systemic antibiotic have been continued for at least 6 weeks before the second stage. This length of time is reputed to allow necrotic bone to become resorbed. However there seems little published evidence to support the view that an extended course is more efficacious than an appropriately selected shorter one. In two multi-centre studies totaling 749 patients with *S. aureus* bacteraemia, the duration of anti-staphylococcal therapy was not associated with clinical relapse[61,62] and both papers found that the most important factor was prompt initiation of appropriate antibiotics. This is supported by a conclusion from the Oxford DAIR study[55], that an extended course of parenteral antibiotics simply prolonged rather than prevented the infection recurring.

Stockley *et al.*[63] reported 88% cure at 2 years after 114 two-stage hip revisions. This success rate is comparable to the majority of two-stage revisions in the literature however they did not use more than two doses of post operative antibiotics and relied exclusively on locally delivered antibiotics from cement beads to augment the host innate defenses. When the soft tissues envelope was adequate and inflammatory markers were low, the patient was considered for a second stage reconstruction. This interval was on average 6.4 months (2 to 22) and no organism was cultured in 84% of patients at that point. They found no association between the cure rate and the infecting organism, positive second-stage culture or the number of operations prior to their intervention.

1.2.4. Suppressive antibiotics

In general, treatment with suppressive antibiotics is considered in patients who have contraindications, or do not wish further operative intervention. No studies have reported a complete cohort without attempts at debridement. However there are subsets within more heterogeneous groups that can be examined. 7/24 patients within Trebse *et al.*[64] prospective cohort study were treated with combination antibiotic therapy alone and no recurrence was reported during the 3-year follow-up period. Similarly Goulet *et al.*[65] reported that the 8/19 patients selected not to undergo debridement had a greater chance of implant survival at 2 years than the patients that were debrided (80 vs. 30 %). They both postulated that this difference could be explained by pre-selecting the patients with early infections, less aggressive and more sensitive organisms. Although it seems suppression is effective in preventing recurrence in the medium term, it will not be curative and most patients and surgeons would prefer to attempt a curative procedure.

1.2.5. Summary of treatment strategies

By using this step-wise approach (Table 1.3) to treat PJI, clinicians are trying to effect eradication of infection whilst maintaining long-term joint function and reducing patient morbidity as much as possible. Each different surgical option should cure around 85-90% of patients if they are selected carefully, debrided meticulously and appropriate antibiotics administered in a timely fashion.

Consensus about the best class of antibiotic to be given, whether to combine them, their mode of delivery and the length of their administration are still lacking. The published literature is limited in its generalisability because of a number of factors. Approximately 1,500 new patients are treated for PJI each year in the UK but they are divided between 183 NHS Trusts and similar numbers of private providers[10,11], each following slightly different local protocols. Even if a study could recruit large numbers of patients, then the variation within the group of bacterial species, chronicity of infection, host type and implant type, would all have to be controlled for before starting to compare any treatment modalities. Previous prospective studies have demonstrated a 30% drop out rate[56] and long term follow up is required. This kind of multi-centre study is expensive and if not funded centrally, could be prone to bias from the pharmaceutical industry.

There is also likely to be a reporting bias with PJI as registries do not provide information about true infection rates. The England and Wales Joint Register[10] does not record infection as an outcome unless the joint is revised and in Scotland[11] an infection is not recorded if it presents after a year, potentially missing between a third and quarter of all cases[15,16]. The literature is also likely to contain publication bias, with most series originating from units with an expertise in PJI. It is unlikely that smaller units would be able to recruit a cohort large enough to be accepted for publication. It is also conceivable that units with poorer results than expected, would be unlikely submit their results for peer review.

1.3. Staphylococcal biofilm

A biofilm can be defined as an aggregate of micro-organisms which are adherent to a surface, embedded within an extracellular matrix and exhibit an altered phenotype with respect to growth rate and gene transcription[66]. Biofilms offer microorganisms more protection from the extreme physical and chemical stresses they are exposed to as well as acting as an arena, which can facilitate horizontal gene transfer and coordinated gene expression via quorum sensing.

The master lens maker Antonie van Leeuwenhoek was the first to observe a biofilm in 1676 through his handcrafted microscope when he examined his own dental plaque. William Costerton first coined the term 'biofilm' in 1978[67] after he slipped whilst crossing an icy stream in the wilds of British Columbia. After examining the slime, he found it was composed of a very high density of sessile (Latin for seated) bacteria that greatly outnumbered the planktonic (Greek for wandering) bacteria in the same aquatic systems. Since then biofilms have been found to be pervasive both in both natural and man-made ecosystems.

They are the predominant (>95%) form in which microorganisms exist and have been estimated to contain up to 50% of the Earth's biomass[68,69]. Evidence of cyanobacterial biofilms have been found in fossilized stromatolites that existed more than 3 billion years ago[70] and have been isolated from Antarctic ice lakes[71], hydrothermal vents[72], plant roots and leaves[73], industrial and domestic pipes and dental plaque. Bacterial biofilms have been implicated in between 65-80% of human infection[74] and are found to grow on native as well as prosthetic surfaces. The scope of this chapter will however, now focus specifically on staphylococcal biofilms seen on orthopaedic joint replacements.

Staphylococci are Gram-positive, facultative anaerobic prokaryotes. They are spherical with a diameter of 1μm and grow in grape like clusters as they

remain attached to their sister cell whilst they divide sequentially in three perpendicular planes. More than 40 species of staphylococci have been described, and 16S RNA analysis groups them into 11 clusters[75]. The most common groups seen clinically in humans is the coagulase positive *S. aureus* and the Coagulase Negative Staphylococci (CNS) (*S. epidermidis, S. capitis, S. haemolytic, S. hominis, S. lugdunensis, S. saprophytic, S. warneri and S. pasteuri).*

CNS are one of the most populous species of the human skin microbiome[44]. Although they are not generally considered pathogenic, they have all have been recovered from infected implants[76] and *S. lugdunensis* can cause serious soft tissue infection[43]. *S. aureus* is considered an opportunistic human pathogen, most commonly isolated from the anterior nares or groin, where it prefers the higher humidity and temperature. 60% of the population are intermittent carriers, with 20% being persistent carriers[77]. Interestingly there is evidence that a strain of *S. epidermidis* that secretes a serine protease (Esp) acts synergistically with human antimicrobial peptide β active against *S. aureus.* The presence of this protective strain has been epidemiologically correlated to the 20% of humans who do not carry *S. aureus* in their nose[78].

All staphylococci share the same basic ultra-structure that includes a micro-capsule, cell wall, and cytoplasmic space containing the unbound DNA, plasmids and 70S ribosome (with 30S and 50S subunits). The peptidoglycan cell wall is around 50nm thick and is formed from chains of alternating N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) sugars. They are connected by β-glycosidic bonds to short peptides (L-alanine, D-alanine, D-glutamine and L-lysine in *S. aureus*), to form a lattice structure. Traversing through the several layers of these peptidoglycan sheets are negatively charged teichoic acids that have a role in intercellular attachment.

S. aureus biofilm was first visualised in 1982 using electron microscopy to examine an infected heart pacing wire[79]. Since then, staphylococci have been recognised as the major cause of device associated infection, isolated in around 2/3^{rds} of all prosthetic joint infections (see Table 1.2). Their ubiquity as part of the skin flora explains why they are frequently introduced as

contaminants, but it is their ability to form stable biofilms, which are tolerant to the host's and clinicians attempt to eradicate them, that makes them so prevalent in PJI.

1.3.1. Initial attachment

When planktonic organisms come into contact with a substratum, they start to switch to a sessile phenotype and form a biofilm perhaps as a means of surviving the austere environment, ready to colonise new niches when conditions allow. Although it is a temporal process, it is frequently conceptualised in the 4 stages of initial attachment, cell aggregation, biofilm maturation and eventually bacterial detachment (Figure 1.2)

As an implant is inserted, the host's plasma quickly 'conditions' the prosthesis with proteins such as fibrinogen, vitronectin and fibronectin, into which the bacteria are absorbed. The extent to which they are absorbed depends primarily upon a number of non specific factors including: Van der Waals forces, electrostatic interactions and hydrophobic effects with biomaterials with hydrophilic 'wettable' surfaces seen to absorb the fewest bacteria[80]. In addition to this passive process, staphylococci actively secrete a variety of protein adhesins in an effort to bind to the substratum. These include a variety of Microbial Surface Components Recognising Adhesive Matrix Molecules (MSCRAMMs) and Autolysin homologues (Atl and AtL). Adhesins have been demonstrated to mediate attachment to polystyrene, vibronectin and even facilitate internalization into non-professional phagocytes, converting them to intracellular organisms within fibrocytes and ostoeblasts[81].

Figure 1.2: Formation and structure of staphylococcal biofilm.

Diagram adapted from Davies concept 2008[81].

1.3.2. Cell aggregation

Once attached, the bacteria start to aggregate together in layers within Extracellular Polymeric Substance (EPS, commonly known as glycocalyx) that is composed of secreted polysaccharides, extracellular DNA (eDNA), teichoic acids and proteins such as Autolysin associated protein (Aap) (Figure 1.3). Interestingly, Aap activation is catalysed by the host's own innate response to infection. The proteases secreted by granulocytes cleave the Aap precursor stimulting further aggregation and biofilm formation[82].

The most important biofilm associated polysaccharide in staphylococci is Polysaccharide Intercellular Adhesin (PIA), a partially N-acetylated glycan and is regarded as central to their biofilm formation. The de-acetylated portions impart a positive charge to the PIA when it is exposed to acidic environments found in wounds and skin. The resulting electrostatic attraction between PIA and the negatively charged teichoic acids in the cell wall ensure the bacteria are embedded securely within the hydrated matrix. The capacity for PIA synthesis is encoded at the intercellular adhesion (*icaADBC*) locus in all staphylococci except *S. haemolyticus*[83] and *S. saprophytic*[84] but its absence does not prevent biofilm formation, suggesting several ica-independent pathways also play roles[85]. The *ica* operon is up-regulated in conditions of nutrient depletion, anaerobiosis, low cell density, salt stress and ethanol by the global gene regulators SarA and alternative sigma factor $Sigma_B$[86] (Figure 1.4). The subsequent production of PIA facilitates intercellular adhesion and biofilm aggregation, protecting the organisms from physical degradation, desiccation, phagocytosis and oxidative stresses that polymorphonuclear leucocytes attempt to impart[87].

1.3.3. Maturation

The biofilm is seen to mature dynamically over time with spatial re-organisation allowing channels to form between pedunculated structures in the matrix and supply nutrients to the deeper layers[66]. The eDNA found

within the matrix, originates from lysed bacteria and endows structural integrity to this 3 dimensional architecture, as well as possibly enabling horizontal gene transfer[88]. The physical time dependent viscoelastic properties of the biofilm matrix allow it to adapt to high shear stresses, reducing the rate of cell detachment and preventing the rolling migration along a substratum[89,90].

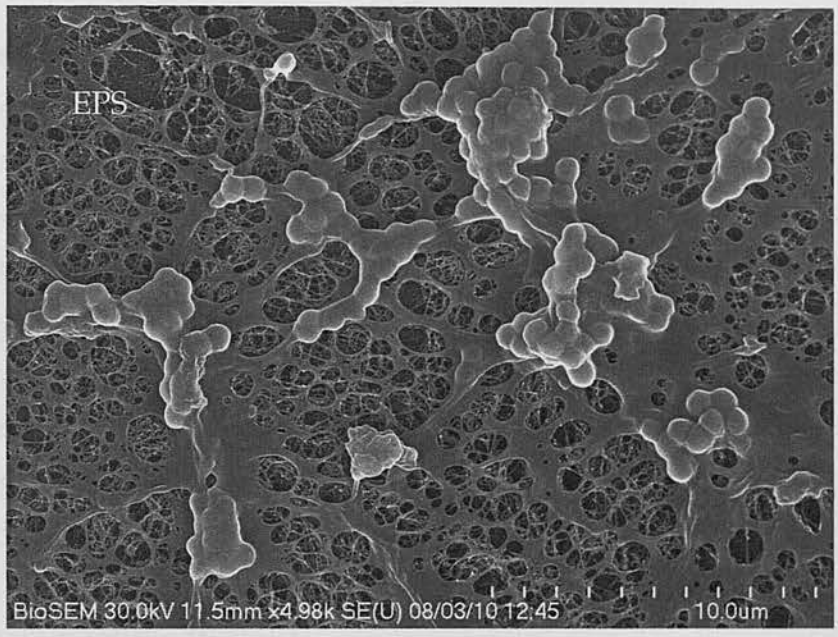

Figure 1.3: Cryo-scanning electron microscopy of biofilm.
Photograph taken taken by the author, perpendicular to a 20-hour-old *S. aureus* biofilm already aggregated into 4 layers within a self-produced Extacellular Polymeric Substance (EPS) (commonly called a glycocalyx).

1.3.4. Detachment

The term quorum sensing (QS) is used to describe the phenomenon, seen in a wide range of bacteria, of cell density-dependent regulation of gene expression. It allows individual staphylococci the opportunity to express virulence factors as part of a timely and co-coordinated multi-cellular response to changing conditions. In doing so, it improves its odds of genomic survival compared to a non co-operative population. Staphylococci are known to have two autocrine quorum sensing systems encoded within

the accessory gene regulator (*agr*) and *LuxS* loci respectively. Agr uses RNA III as an effector to mediate the expression of amphiphillic peptides (also knows as Phenol-Soluble Modulins (PSMs), which act as biofilm detergents[91] (Figure 1.2). Among the many other accessory genes it regulates, Agr also down-regulates the expression of surface proteins such as MSCRAMM's required for surface binding (Figure 1.4).

LuxS inhibits *ica* mediated PIA production[92] whilst metabolic slow down, has the opposite effect, enhancing PIA biosynthesis and facilitating a sessile state. As the biofilm matures and the cell density increases to typically 15% of volume, both Agr and LuxS are up-regulated when the threshold concentration of autoinducers is reached. In contrast to the extensively studied Gram-negative QS systems, staphylococci colonise and form a biofilm more readily when bacterial density and QS are at low levels. The high levels of QS associated with higher cell density have the net effect of causing biofilm detachment. The liberated bacteria may simply migrate a short distance along the substratum to increase the biofilms surface area or switch back to their more virulent planktonic form and migrate to exploit distant sites within the host.

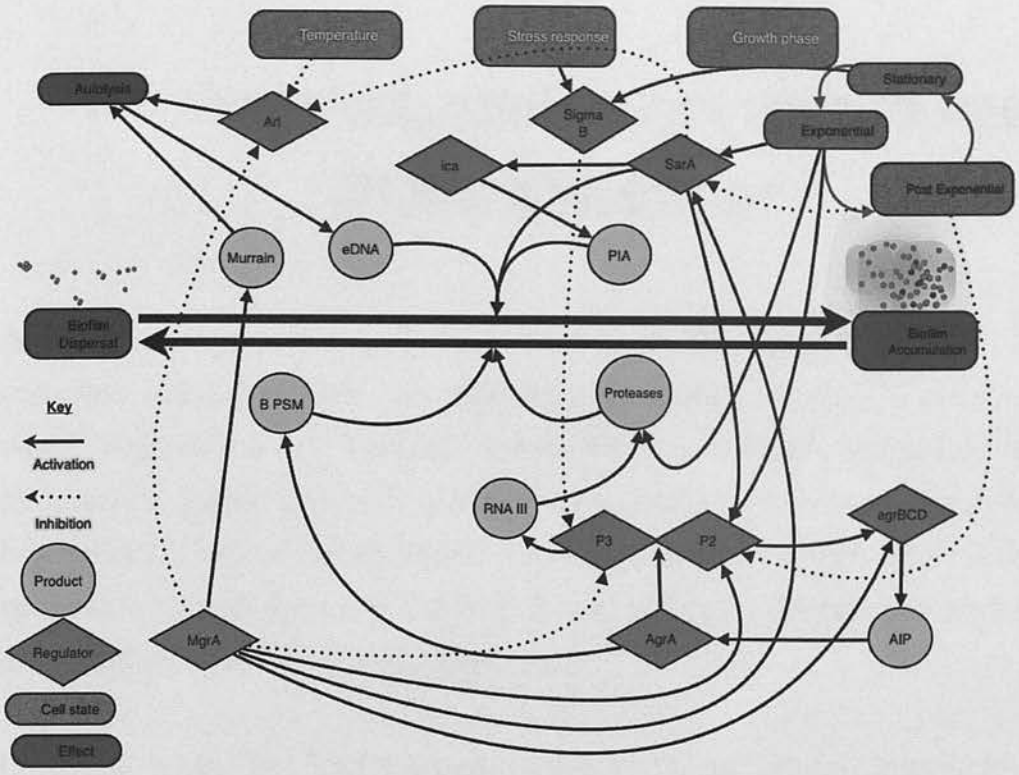

Figure 1.4: Outline of genomic regulation during staphylococcal biofilm formation. Adapted from Arciola et al.[86].

The *agr* locus consists of 2 divergent transcription units driven by promoters P2 and P3. The P2 operon encodes a 2 component-signaling molecule, of which AgrC is the receptor and AgrA is the response regulator.
Agr also encodes 2 proteins AgrB and D, which combine to produce and secrete an Auto Inducing Peptide (AIP) that is a ligand for AgrC. AgrA functions to activate transcription from its own promoter and from the *arg*P3 promoter, which drives the synthesis of RNAIII, the main effector in the Quorum Sensing system [93].

1.4. Persisters, small colony variants and biofilm phenotypes

Although in many clinical bacterial infections, the human host can achieve complete resolution, there are numerous examples of when it cannot, even when augmented by medical treatments. Common examples include *Helicobacter pylori* induced gastritis, reactivation of latent *Mycobacterium tuberculosis*, *Haemophilus influenzae* associated with recurrent otitis media and prosthetic related infection, such as *E. coli* urinary catheter mediated sepsis and staphylococcal joint infections.

Over the years, the bacteria responsible for these chronic infections have been described in a number of discrete physiological states, that can resist eradication. Persisters, Small Colony Variants (SCV) and biofilms are generally thought to be distinct phenotypes but through the course of this section, the argument that these phenotypes all possibly derive from a common intrinsic response to environmental stresses, will be advanced.

1.4.1. Persisters

Persisters are small subpopulations of bacteria that can survive prolonged exposure to high concentrations of antibiotics without any specific, inheritable resistance mechanisms. Persisters are typically very slow growing or dormant. They were first described in 1944 by Joseph Bigger who reported that a small, but consistent proportion of *S. aureus* that survived exposure to penicillin. Subcultures of these persisters could be eradicated however, if exposed to a similar level of penicillin for a second time[94].

Balaban *et al*[95] explored persistence further with experimental and mathematical studies of two strains of *E. coli* that were high persister (hip) mutants that tolerated normally bactericidal concentrations of ampicillin, and found two types of co-existing persisters (Figure 1.5). Type I persisters were generated primarily during sub-maximal growth conditions (where they reach 10^{-2}) and displayed antibiotic tolerance after exposure to significant environmental stresses. Type II persisters are less common (around 10^{-6}) and appear to be generated stochastically throughout the cell cycle without an obvious extrinsic stimulus.

Becoming a persister does not make sense from an individual organism-based perspective, as its growth slows or stops and would be quickly out replicated by its Normal Colony Phenotype (NCP) sisters. It does make sense however if you consider it from the perspective of the whole population acting co-operatively towards the continued survival of that genome. There appears to be different levels of contingency, with a few Type II persisters present at all times in case of a major unforeseen environmental catastrophe. If the population becomes stressed, more organisms appear to take this as a warning of impending death and become Type I persisters but most of the population continues to maximise their metabolic and reproductive potential until the bitter end. This hedge betting[96] ensures the population is maximised if environmental conditions are optimal whilst maintaining a genetic reservoir to repopulate from, should it become austere for a period. Some doubt does remain as to whether Type II persisters are generated without any stimulus or whether that stimulus has not been elucidated as yet[97].

1.4.2. Small colony variants

Small colony variant (SCV) is a descriptive term for an unstable genetic mutant, with low replication rate, that differs from the NCP. *S. aureus* SCV for instance are auxotrophic (lacking the ability to produce metabolites essential for normal growth) resulting in a defective Electron Transfer Chain

(ETC)[96]. At first glance, this appears to hinder *S. aureus*, resulting in non-pigmented colonies 10 times smaller than NCP and decreasing both the α-toxin and coagulase production. However, the ETC suppression also confers several survival advantages, such as the ability to reside intra-cellularly, display increased adhesion expression and tolerate high concentrations of antibiotics through mechanisms that will be discussed in section 1.4.4[98]. SCV are a heterogeneous group within themselves with reports of *S. aureus* displaying at many types of auxotrophy (menadione and haemin, being most completely described)[99,100].

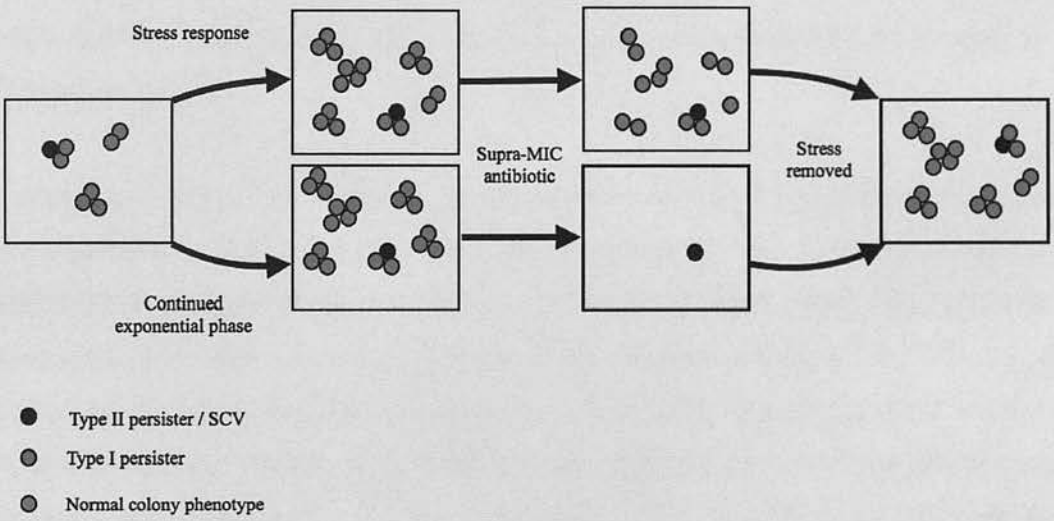

Figure 1.5: Bacterial population response to different environmental conditions.

Type II persisters / SCV are unstable genetic mutants generated stochastically during the exponential phase possibly as a result of imperfect replication. This is a reversible and dynamic phenotypic equilibrium with a prevalence of around one per million NCP. Type I persisters are generated as environmental stresses increase (such as starvation, antibiotics, hypoxia, oxidative stress, heat stress, envelope stress and low pH) with their prevalence depending on the scale and duration of these stresses. A supra-MIC concentration of a bactericidal antibiotic is delivered that will kill the NCP leaving the persisters to repopulate the niche when conditions permit.

In a recent study, 11/31 PJIs were found to be SCV of *S. epidermidis* but despite similar appearances, they varied in their degree of aminoglycoside tolerance, type of auxotrophy and enhanced biofilm formation[101]. SCV have been shown to be present at similar concentrations ($3x10^{-6}$) as type II persisters in the stationary phase with the absence of any selective pressure[102]. They seem to be generated as an inevitable natural consequence during replication in the exponential phase. The population of SCV is dynamic and the bidirectional phenotypic switching seen indicates the genetic mutations are generally unstable[102]. The SCV confers marked aminoglyoside tolerance that is lost when the purified SCV cell is sub-cultured and re-exposed. This description of the *S. aureus* SCV seems one that bears striking resemblance to the description of type II persisters reported by Balaban *et al.* as well as the original description of persisters by Bigger in 1944[94,95].

Continued selection pressure from high levels of gentamicin, commonly encountered around prosthetic joints, would favor the recovery of relatively stable SCV/type II persisters that revert back to NCP when that pressure is removed and this is commonly seen in clinical practice. SCV's are also notorious for their ability to reside intra-cellularly, thereby avoiding extreme environmental stresses and antibiotics. This internalisation phenomenon appears to be mediated by the autolysins AtlA in *S. aureus* and AltE in *S. epidermidis*, both of which are associated with the initial attachment phase of biofilm to a substrate (Figure 1.4).

1.4.3. Biofilm phenotype

As previously described, a biofilm is a aggregate of micro-organisms that are adherent to a surface, embedded within an extracellular matrix and exhibit an altered phenotype[66]. The physicochemical properties of the extracellular matrix confer protection from heat shock, desiccation and phagocytosis. It also has been shown to slow but not preclude antibiotic penetration and so

cannot fully explain the antibiotic tolerance seen in the biofilm phenotype[103]. Numerous studies, in a range of bacteria, have reported mechanisms to explain this antibiotic tolerance and include: limitation of nutrients and oxygen; reduced growth rates; facultative intracellular growth and a high proportion of SCV / persister phenotypes within the biofilm[104].

This invites the questions: are these mechanisms responsible for antibiotic tolerance interconnected?[105] Is an in-vivo biofilm simply a manifestation of the natural relationship between NCP, SCV and persister phenotypes when a suitable substrate for attachment is present?

1.4.4. How do persisters tolerate antibiotics?

Thus far, inhibition of protein synthesis[106] and dissipation of the Proton Motive Force (PMF)[107] appear to be the main mechanisms underlying persister formation. This inhibition of metabolic pathways could be effected by genetic mutation[102], Toxin-Antitoxin (TA) systems[108] or direct inhibition of the ribosome by some antibiotics[106]. All of these result can result in the cell displaying multi-drug tolerance and concentrations of up to 1000x the MIC of planktonic culture are required to eradicate them in mature biofilm[109]. This phenomenon is also seen in planktonic cultures pre-exposed to sub-MIC levels of either gentamicin, ciprofloxacin, oxacillin or vancomycin, where it induces a multi-drug tolerance that results in higher viable cell densities if re-exposed to sub-MIC levels of antibiotics than cultures that were not pre-exposed[110].

Although slow growth or dormancy has been shown as insufficient to explain the persister phenotype[117], less replication does reduce the availability of active binding sites for the penicillins, glycopeptides and quinolones that target the cell wall and DNA. Aminoglyosides uptake into the cell is an energy dependent process requiring a threshold cell membrane potential of −155mV[111] and uptake is proportional to increased PMF. The slower metabolic rate of persisters reduces ATP production and the PMF, thereby limiting the uptake of aminoglyosides and enabling tolerance of

concentrations normally bactericidal to the NCP. Supplementary fructose that enters upper glycolysis via the phosphotransferase system and generates NADH utilised by the ETC to increase PMF, has been shown to facilitate the killing of *S. aureus* persisters by 2 orders of magnitude compared to controls[107]. Lower metabolites that could only be utilized by the pentose phosphate or tri-carboxylic acid pathway did not potentiate aminoglycosides. This fructose-enabled killing was found to be independent of aerobic conditions (as long as conditions supported the PMF) and was seen with aminoglycosides but not penicillins or quinolones. No growth resumption was seen within the 4-hour period tested, however this would not be long enough to exclude that the lag phase of growth was still in progress. Further in-vivo studies are needed to confirm both the safety and efficacy of this promising metabolite enabled eradication.

The stringent response (in response to amino acid starvation) has been shown to up-regulate the anti-oxidant systems[112], presumably to prepare for the onset of post-exponential catabolism of non-preferred carbon sources using the TCA cycle[113]. If the conditions allow post-exponential growth, then hydroxyl radicals are sequentially detoxified, first by Mn superoxide dismutase then by peroxidases and catalase. If environmental conditions deteriorate further and inhibits the TCA cycle, this will reduce the generation of hydroxyl radicals formed during oxidative phosphorylation and perhaps act as a signal transduction pathway that favors the persister phenotype[113]. This mitigation against hydroxyl radicals possibly enables the bacteria to survive the additional hydroxyl radicals promoted by the effects of bactericidal agents[114].

Another possibility that is likely, but remains unproven, is that the quorum sensing system influences the frequency of persister formation in staphylococci. There is evidence that indole, an organic alkaloid secreted by altruistic *E. coli* persisters acts as a cue, influences the cell envelope and oxidative stress response of neighboring NCP cells and so modulates the frequency of persister formation[115]. The closest evidence that this occurs in

staphylococci was reported by Butler *et al.*[116] who interpreted the consistently low MIC of indole, compared to antibiotics, against 39 staphylococcal strains, as a potential therapeutic development. Another interpretation would be that indole is a very effective persister generator and that its use *in vivo* would delay rather than cure any infection.

1.4.5. Do persisters, SCV and biofilms share common molecular mechanisms that allow staphylococci to tolerate stress?

Despite seemingly unique differences, SCV, persister and biofilm phenotypes share important similarities that include being stress inducible, demonstrating dramatically slower growth rates and marked tolerance to antibiotics. All three characteristics invariably present together as antibiotics are known to cause an oxidative stress and slow cellular growth, which leads to reduced antibiotic efficacy, due to target inactivity. A recent study by Orman *et al.*[117] used fluorescent assays to show that a lack of replication or a low metabolic activity was not necessary or sufficient to turn a NCP into a persister but simply increased the likelihood that a cell became a persister after it was stressed with antibiotics. Similarly, another study inactivated the stringent stress response in a *Pseudomonas aeruginosa* biofilm and found that it's inactivation did not change the slow metabolic rate compared to the wild-type but did result in the bacteria being much more susceptible to antibiotics[112].

1.4.6. Toxin-antitoxin systems

As well as nutritional limitation, hypoxia, oxidative stress, heat stress, envelope stress, low pH, and exposure to sub-MIC concentrations of antibiotics have all been shown to elicit a stress response, in a wide range of bacteria including staphylococci, through the Toxin-Antitoxin (TA) system[118,119]. TA systems are composed typically of two genes in an operon that encode both a protein toxin and its unstable RNA (Type I) or protein (type II) anti-toxin that neutralises it. Down-regulation in the expression of

the rapidly degradable antitoxin will allow the more stable toxin to act on its target, which is typically mRNA. TA systems regulate genes other than their own operons, mediate the general stress response, and help direct cells toward the formation of biofilm and Type I persisters[120]. Numerous studies have demonstrated that deleting a single TA system does not reduce persistence though, due to the redundancy of these systems[121]. *S. aureus* has at least one Type I and three Type II TA systems[122,123], all of which code for endoribonucleases. The most studied of these in *S. aureus* is the $mazF_{sa}$ locus for which over-expression was found to result in a growth arrest, although the dormant cells were not examined to determine if they were persisters in this study[124]. The ATP-dependent ClpP protease (and its chaperone ClpC) serves as the functional unit for the degradation of all known antitoxins in *S. aureus*[123].

A comprehensive understanding of why heterogeneous phenotypes are generated in an isogenic population is still lacking[97]. There is an emerging Persistence as Stuff Happens (PaSH) hypothesis however; that it is simply a natural function of replication that confers genomic advantage through the hedge betting strategy and so the genes that control their frequency have been selected as the organism has evolved. These are now being elucidated by several groups[102,110,125]. As the cells divide in the exponential phase, imprecise gene expression can create type II persisters / SCV. During the stationary phase and in biofilm, environmental stresses mediated through the TA systems and possibly the quorum sensing molecule, indole[115], may generate type I persisters. The greater the degree and period of stress a population is exposed to, the greater the proportion of persisters[126] that adapt to tolerate both the original stress and others including antibiotics[112].

1.5. Antibiotics used in PJI

Despite being identified as an important orthopaedic pathogen in 1880 by Scottish surgeon Alexander Ogston[127], an effective treatment for staphylococcal infection remained elusive and it was still associated with an 80% mortality just 75 years ago[128]. The development of penicillin, which remains one of the most significant advances in medicine, was certainly not a sudden breakthrough as one might expect. From its discovery in 1928 through to a clinically useful form in Oxford took 12 years and earned Fleming, Chain and Florey the Nobel Prize for Physiology or Medicine in 1945[129]. Although penicillin was initially effective against a wide range of staphylococci, resistance mechanisms quickly emerged and by the 1960's, strains resistant to both penicillin and the synthetic penicillinase-resistant methicillin had become globally prevalent[130].

Antibiotics remain an important adjunct to surgical debridement of prosthetic infection but need to be used rationally and sparingly to minimise the rate at which resistance to them develops. Currently the central tenets of this approach are to: first identify the relevant organisms causing the infection; determine how sensitive they are to a wide spectrum of antibiotics; selecting an antibiotic that is able to be PharmacoKinetically (PK) delivered, at a concentration that is PharmacoDynamically (PD) effective whilst maintaining an acceptable side-effect profile.

The antibiotics commonly used in PJI are listed in Table 1.4. As can be seen, many of these antibiotics are derived from naturally products of soil bacteria[131]. There is a growing realisation that the molecules we use as antibiotics, are bacterial secondary metabolites. They seem to be used in natural eco-systems, to signal, cue or coerce neighboring bacteria in to altering their genomic expression[132]. It follows then, that bacteria are likely to have developed systems to respond to these antibiotics and this must be kept in mind when trying to eradicate prosthetic infection. It is recognised that exposure to sub-inhibitory levels of one antibiotic leads to high level tolerance to multiple and dissimilar antibiotics[110]. It is feasible that even very high concentrations remain sub-inhibitory to the persister phenotype and encourage them to maintain their tolerance to a variety of stresses[102].

1.5.1. Optimal duration of antibiotic treatment

A recent survey of over 500 clinicians that treat PJI in the USA found that 3/4 of them prefer to give 6-8 weeks of antibiotics aimed at eradicating the infection, with only 16% switching to oral antibiotics when clinical response allowed and 0.4% treating for less than 4 weeks[133]. This dogmatic approach is repeated in the recent Clinical Practice Guidelines by the Infectious Diseases Society of America[20] without any evidence cited to justify it except expert opinion. The arbitrary 6 weeks is likely to be based on a series of animal models in the 1970's and 80's which demonstrated that their bones re-vascularised within 3-4 weeks of surgery[134], and historical reports of shorter duration failures in cases of acute osteomyelitis in children. Important differences between these models and adult PJI exist, which make a meaningful comparison difficult.

Class	Examples	Cellular Target	Effect
Fluoroquinolones	Ciprofloxacin and levofloxacin Synthetic	DNA gyrase	Bactericidal by interfering with DNA replication and cell division
Rifamycins	Rifampin Semisynthetic derivative of naturally occurring Rifamycin SV produced by *Streptomyces mediterranei*	DNA-dependent RNA polymerase	Both bacteriostatic by blocking RNA transcription and bactericidal damaging DNA if oxidised
β-lactams	Penicillin - naturally derived from *Penicillium* fungi Semisynthetic - Ampicillin, Flucoxacillin, Cephalosporins and Carbapenems	Penicillin-binding proteins in cell wall	Bactericidal by preventing peptidoglycan cross linkage in cell wall, cell division and autolysin activation
Glycopeptides	Vancomycin and teicoplanin Semisynthetic derived from *Actinobacteria* spp.	Terminal dipeptide of Peptidoglycan in cell wall	Bactericidal by preventing transglycosylation, transpeptidation of cell wall and autolysin activation. Less rapid than penicillin as larger molecule cant penetrate entire wall.
Lipopeptides	Daptomycin and Polymixin B Derived from *Streptomyces* spp.	Cell membrane	Bactericidal. Rapid depolarisation of the membrane potential.
Aminoglycosides	Gentamicin, Tobramycin, Streptomycin Kanamycin Natural and semi-synthetic amino sugars derived from *Streptomyces and Micromonospora* spp.	30S ribosome	Bactericidal. Protein translation (mistranslation by tRNA mismatching)
Tetracyclines	Tetracycline and Doxycycline. Semi-synthetic -derived from *Streptomyces* spp	30S ribosome	Bacteriostatic by blocking aminoacyl tRNA binding to ribosome and preventing translation of proteins
Macrolides	Erythromycin Clindamycin and Azythromycin. Semi-synthetic derived from *Streptomyces* spp.	50S ribosome	Bacteriostatic by inhibition of elongation and translocation steps during protein translation and free tRNA depletion.
Oxazolidinone	Synthetic - Linezolid	50S ribosome	Bacteriostatic. Inhibits protein translation through inhibition of initiation step.

Table 1.4: Antibiotics commonly used to treat staphylococcal infections. Adapted from Kohanski *et al.*[135]

The systematic reviews[136,137] on this subject, conclude that longer courses of antibiotic expose the patient to more side effects, whilst there is no clinical evidence to suggest they are more effective than a shorter course. Another key point can be extrapolated from a prospective cohort of patients with chronic osteomyelitis from Oxford[138]; All 50 patients were treated with debridement followed by 6 weeks IV and 6 weeks oral antibiotics. 14/35 infections recurred within a year where the margin of normal bone surrounding the resection was less than 5mm, whereas all of the 15 patients with adequate resection remained infection free. This emphasises the importance of adequate surgical debridement when treating PJI.

1.5.2. Pharmodynamics of antibiotics

Antibiotics are broadly classified as either bactericidal or bacteriostatic depending on whether they cause cell death or a reversible antagonism to cell metabolism. In general if the Minimum Bactericidal Concentration (MBC) is equal to or less than 4 times the Minimum Inhibitory Concentration (MIC), the antibiotic is designated bactericidal. This distinction is blurred somewhat depending on drug concentration, bacterial phenotype and what oxidative state is present. For example, rifampicin demonstrates more bactericidal effect when the balance between the cells' superoxide dismutase and free radicals are tipped toward the rifampicin being oxidized, where upon it directly damages the DNA when combined in its RNA polymerase complex[139-141], rather than just blocking protein transcription. Bacteriostatic antibiotics only work if the host immune system can use the bacterial stasis as an opportunity to mount an effective response and eradicate the bacteria. If the response if ineffective and the antibiotics are stopped then bacterial virulence resumes.

Antibiotics can also be classed as being either concentration dependent, or time dependent, depending on the rate and proportion of the population they kill (Figure 1.6). Examples of concentration dependent antibiotics include the aminoglycosides, daptomycin and fluoroquinolones and so the relationship between their peak concentrations and minimum bactericidal

concentrations should be an indicator of outcome. Time dependent antibiotics include vancomycin, beta-lactams, macrolides, clindamycin, tetracyclines and carbapenems. Higher concentrations have no additional effect so it is thought vital to deliver a supra-inhibitory concentration over as much of the day as possible. A post antibiotic effect is one where growth suppression is seen after antibiotic levels have dropped to below MIC and is seen with aminoglycosides, fluoroquinolones and rifampicin.

The 3 most common PK-PD measures are: the duration of time a drug concentration remains above the MIC (T>MIC); the ratio of the maximal drug concentration to the MIC (C_{max} : MIC); the ratio of the Area Under the Curve over 24 hrs to the MIC - ($\underline{AUC}_{0-24}$: MIC).[142]

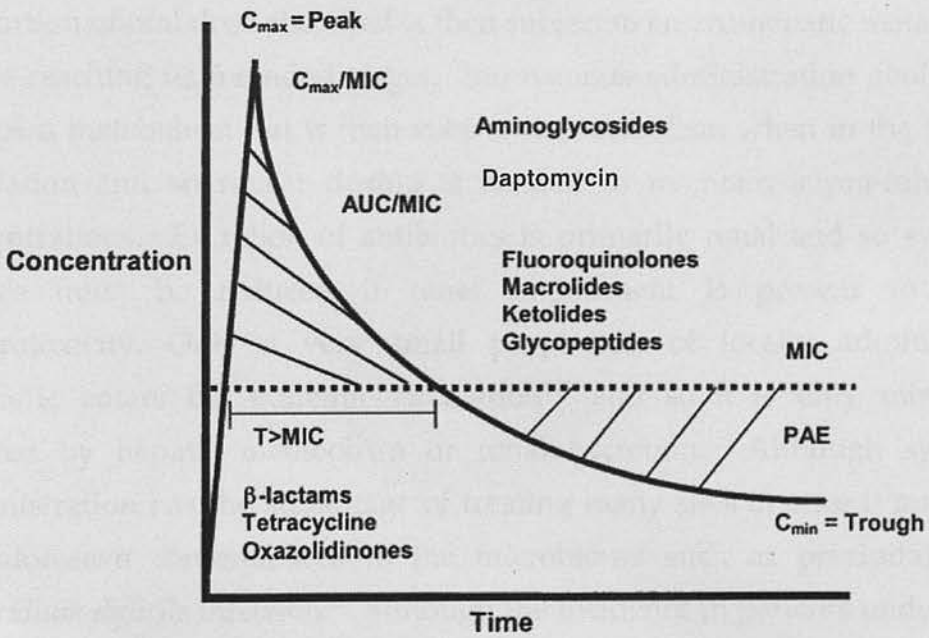

Figure 1.6: Pharmodynamic profile of commonly used antibiotic in PJI.

Reproduced with permission from Rybak MJ Am. J. Med. 2006[143]. C_{max}=Maximum concentration, AUC - area under curve, MIC = minimum inhibitory concentration, PAE = post antibiotic effect, T = time.

1.5.3. Pharmokinetics of antibiotic delivery

The concentration of active antibiotic actually present at the site of infection depends on the pharmokinetic variables of absorption, distribution, metabolism and excretion. Delivering antibiotics to the site of infection can be achieved by local administration at the time of surgery, or systemically via an oral or parenteral intravenous (IV) route. 100% absorption is seen after IV administration but excellent bioavailability can also be achieved using oral fluoroquinolone or rifampicin[144] or when using locally administered antibiotics. Active drug availability is increased by low plasma binding, good vascularity to the infected area, lipophilic antibiotics with small molecular size, favourable pH and ionisation.

The majority of antibiotics are primarily metabolised in the liver and so the proportion of oral dose absorbed is then subject to entero-hepatic metabolism before reaching its intended target. Intravenous administration avoids this first-pass metabolism but is then subject to metabolism when in the arterial circulation and so repeat dosing is needed to maintain supra-inhibitory concentrations. Excretion of antibiotics is primarily renal and so systemic dosage must be reduced if renal impairment is present to avoid nephrotoxicity. Only a very small proportion of locally administered antibiotic enters the systemic circulation[145] and so it is only minimally affected by hepatic metabolism or renal excretion. Although systemic administration has the advantage of treating many sites at once it may lead to unforeseen consequences to the microbiome such as precipitation of *Clostridium difficile* infection[146], although the incidence in patients undergoing primary arthroplasty is extremely low (1.7 cases per 1000)[147].

Even with adequate serum levels, antibiotic concentration in bone can be suboptimal. Landersdorfer *et al.*[148] reviewed the literature and found the mean bone/serum ratios were typically between 0.2-0.5 after systemic administration. They noted substantial heterogeneity in the methods and reporting standards and therefore saw large variance in the data sets. In

general however, the penicillins and cephalosporins penetrated bone least effectively followed in ascending order by glycopeptides, aminoglycosides, rifampicin, clindamycin, linezolid, quinolones, tetracyclines. Macrolides were found to be the most effective bone penetrators with serum to bone ratios of around 1.

Further complicating this situation is that intracellular persisters are known to be prevalent in staphylococcal biofilm infections and appreciation of this cellular level of pharmokinetics is perhaps one of the reasons many in vitro model are not predictive of in-vivo efficacy. Most antibiotics simply diffuse across cell membranes and so smaller lipophilic molecules such as daptomycin and quinolones penetrate more readily and accumulate in the cytoplasm[149]. Polar molecules, such as aminoglyosides and glycopeptides cannot diffuse readily, and rely upon non-specific endocytosis to enter the cell where they accumulate and can act on the persisters that also reside within this lysosomal compartment within human cells[150]. The cytoplasmic activity of these polar molecules is limited however, which may have clinical significance when treating SCV.

1.6. Systemic administration of antibiotics

1.6.1. Parenteral delivery

Invariably, intravenous antibiotics are used during the peri-operative period as entral absorption is initially unpredictable and as the IV route delivers 100% bio-availability it can achieve much higher serum levels. Effective doses of gentamicin, vancomycin and daptomycin can only be achieved using the parenteral route.

Gentamicin is typically given once daily at 7mg/kg. Its half-life of 2 hours is increased with renal impairment. Target serum levels need to be carefully titrated, aiming for peak levels of between 5-10mg/L to ensure trough levels are <1mg/L to avoid renal and vestibular damage. Vancomycin reaches its C_{max} of around 50mg/L within an hour of infusion, has a half life of 8 hours and a target trough level of between 15-20mg/L for PJI[151]. Daptomycin, like gentamicin and ciprofloxacin, displays concentration dependent killing with a C_{max} of 94mg/L after once daily 6mg/kg dosing[152]. Its half-life is 8 hours, it is highly protein bound and renally excreted. Initial side effects of myopathy were overcome by moving to the once daily dosing regime. It has been demonstrated to be safe to use for 6 weeks in patients with PJI[153] . However it has shown mixed efficacy when studied in both animal and human populations[154].

Flucloxacillin is commonly used to treat sensitive staphylococci, as it is rapidly bactericidal and has a favourable side effect profile. It is also an example of an antibiotic with much higher serum concentrations after IV rather than oral administration. Its C_{max} after 2g infusion reaches 244mg/L whilst 500mg oral administration only achieves 14.5mg/L. Although penicillins have a time-dependent bactericidal action, this difference in the C_{max} may still be important, as only a small proportion of the serum level is seen in bone and it does not accumulate intra-cellularly in humans to any

substantial level. Theoretically therefore, higher serum concentrations are needed if it is to be effective in treating PJI.

1.6.2. Entral delivery

The oral route is the most convenient but can be associated with poor outpatient compliance and variations in pharmokinetic profiles between patients. Rifampicin is a good example of this, as its serum level is dependent upon entero-hepatic recirculation as well as renal and hepatic metabolism. This means that if it taken with food, less is resorbed and serum levels drop. If 300mg is taken on an empty stomach, the peak of 4mg/L is reached within 2 hours and the trough at 12 hours would be around 0.25mg/L[155], still above the expected MIC for staphylococci[156]. Rifampicin is reputed to be most useful in eradicating intracellular organisms as it accumulates up to 10 times more in the host lysosomes than their serum and helps overcome the local granulocyte defect that allows the bacteria to reside unchecked[8]. This tendency however, is not exclusive to rifampicin and most other classes of antibiotics, apart from penicillin and linezolid, are seen to do this[150]. Rifampicin should not be used as a single agent as resistance quickly develops especially if there is still a high bacterial load or inadequate dosing established[157].

Clindamycin is strongly plasma bound, hepatically metabolised and has a half-life of 2 hours. A French study of 50 patients with osteomyelitis[158] demonstrated oral clindamycin had 87% bio-availability, good soft tissue penetration, with no significant difference seen in the serum concentration at 8 hours compared to IV administration (2mg/L). Similarly an oral dose of 750mg ciprofloxacin has equivalent C_{max} (3.6mg/L) and AUC (32mg/hr/L) to 400-mg IV dose[159]. Linezolid has 100% bioavailability, and after an hour following a 600mg oral dose, reaches C_{max} of around 20 mg/L. It is only 31% plasma protein bound and so is predictably distributed and its trough level is around 4mg/L at 12 hours[160].

1.7. Local administration of antibiotics

Advantages of treating PJI surgically include debridement of devitalised tissues, reduction of bacterial load and affording the surgeon a chance to administer antibiotics locally. This depot is not dependent on the presence of vascularized tissues, it achieves concentrations that are orders of magnitude higher than systemic administration would be able to be safely deliver and avoids unwanted antimicrobial effects at distant sites. However, there is a possibility that the delivery material may eventually act as an additional nidus for infection should it persist after the depot runs out. Biodegradable systems such as hydroxyapatite crystals[161], bone grafts, collagen sheets[162], polylactide polymers[163] and hyaluronic acid gels[164] have all been developed as carriers. However Antibiotic Loaded Acrylic bone Cement (ALAC) remains the gold standard. Despite systematic reviews focused on the last 40 years of research in this field, no consensus yet exists as to the optimal carrier, its form or the class and dose of antimicrobial it should carry[161,165,166].

1.7.1. Antibiotic loaded acrylic bone cement (ALAC)

PolyMethylMethAcrylate (PMMA) acrylic bone cement is the most widely used antibiotic carrier in PJI since its introduction in 1970[167]. It can be molded into beads to fill dead space temporarily, contoured as an articulating joint spacer, or be used as the interface between the hosts' bone and revision joint replacements. There has been numerous *in vitro* studies quantifying and attempting to optimise the concentration of antibiotics eluted from PMMA. However due to marked differences in study design, drawing conclusions during systematic reviews has been difficult[168-170].

That said, there is a consensus that there is a typical biphasic release seen[171], with the high initial elution rate primarily a function of surface roughness and the prolonged, lower elution rate dependent on the porosity of the cement[172]. The initial rate of elution is approximately 60 $\mu g/cm^2$ in the first hour followed by 5-10 $\mu g/cm^2/hr$ over the next 12 hours[172-175].

The effective concentration of antibiotic will depend on the volume of the compartment (V) that the cement surface area (A) elutes into. Typical estimates of surface area in a primary arthroplasty are in the region of $150cm^2$ and $15mm^3$ of volume yielding an AV ratio of 10 cm^{-1}. Most in-vitro models have a very low A/V ratio of between 0.001 to 0.3[176] and therefore antibiotic concentrations in the first week average around 10 mg/L[172]. Wang *et al.*[177] introduced the concept of interfacial gap between the cement and the prosthesis (Figure 1.7). In a porcine model they demonstrated that there was a 50-500μm gap along 15% of the interfacial circumference, leading to an A/V ratio of around 100. The concentration of gentamicin eluted from ALAC with 1.7 % wt/wt was measured using an immunoassay in an in-vitro model with similar A/V ratio. It was found to be between 2500-4000 mg/L[178] which correlates more closely with previous human in-vivo studies that found the average 24 hour peri-prosthetic concentration to be around 150 mg/L[173].

The difference between these two concentrations might be explained by antibiotic diffusing out of the peri-articular compartment into surrounding tissues. This concept of concentration gradient is supported by previous canine models of infection[179], which found a linear relationship between the antibiotic concentration in bone and the distance from the intra-medullary antibiotic loaded PMMA beads (Figure 1.8). The highest levels were found at the endosteal surface and lowest in the periosteum, both peaking from 1-2 weeks after implantation.

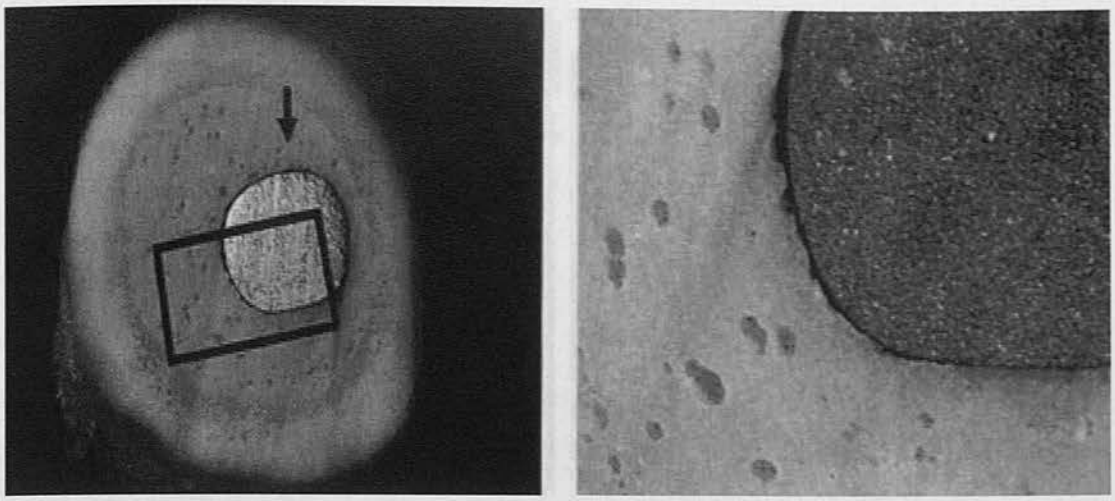

Figure 1.7: Interfacial gap between cement and prosthesis

Cross-section of porcine femur with prosthesis, ALAC, endosteal bone and cortical bone concentrically arranged. The magnified view on the right demonstrates the small gap that exists between the cement and prosthesis. A similar gap exists between the cement and the bone (not shown) and is due to the volume reduction seen as the cement polymerises during curing.
Figure reproduced from Wang *et al.* Interface gap after implantation of a cemented femoral stem in pigs. *Acta Orthop Scand* **70,** 234–239 1999[177].

Figure 1.8: Appearance of ALAC beads.

Typically connected together with steel wire. Figure reproduced from[180]

1.7.2. Optimising ALAC composition

The addition of an antibiotic powder or liquid to PMMA is deleterious to its optimal mechanical properties. If the ALAC is going to be used in a single or 2nd of two-stage revision, then it is expected to also provide long-term structural support to the construct. These mechanical characteristics can either be measured statically in accordance with ISO 5833 standards[181], or using the more physiological fatigue testing method. Up to 13% antibiotic /PMMA powder can be added before the ISO standard is breached[182,183] but significant differences are seen in the fatigue tests still with as little as 2.5% antibiotic per weight[184]. This leaves the surgeon scope to balance the risk between recurrent infection and long-term stability and vary the amount of antibiotic added between 1g and 5.2g of antibiotic per 40g of PMMA. Vacuum mixing the ALAC improves its mechanical characteristics by reducing its porosity [185].

If the ALAC is not required for support, then attempts to maximize elution takes president. The addition of fillers such as glycine, or an additional antibiotic has the effect of increasing porosity and so facilitate a higher release[168]. Therefore optimal elution characteristics from PMMA can be seen strings of ALAC beads (Figure 1.8) , which have the highest surface area, can have high porosity and very high antibiotic/cement ratios. If temporary mechanical support is needed, surgeon can also choose to produce an articulating ALAC spacer, which although also can have favorable porosity and antibiotic ratios, their shape presents a diminished surface area to mass ratio (130 cm^2 vs. 160cm^2 per 40 beads[186]).

Certain antibiotics have historically been thought of as heat unstable during the exothermic polymerisation of PMMA which reaches 56°C for around 3 minutes[187,188]. Studies initially found penicillin isotopes eluted into bone very well[179] . However a bioassay method concluded they were not functional[189], although this has recently been contradicted by a study that demonstrated bactericidal concentration of oxacillin were eluted for the first 2 days

following TKR[190]. Also, whilst tetracyclines were initially felt to be inactive, more recent studies has shown that after heating, around 70% of the compound remained active[191] ; so should not be completely discounted from further study designs. Chloramphenicol does seem to degrade significantly[188,192] and rifampicin delays the polymerisation to such an extent as to render its addition impractical[193,194].

The discussion will now focus on in-vivo models and clinical series of the more commonly used techniques to deliver antibiotics in PJI. Differences in the many delivery devices, antibiotics added, study duration, measurement techniques, and a number of other factors that make comparison infeasible. Therefore the discussion is split into techniques used in one stage revision and two stage revisions such as beads and spacers.

1.7.3. Techniques used in one stage revisions

As discussed previously, between 1 and 5.2g of antibiotic can be added to each 40g mix of PMMA powder before catastrophic mechanical consequences are likely. Wahlig *et al.*[195] recorded the gentamicin concentrations from surgical drains adjacent to 27 primary hip replacements using bioassay. They found a mean gentamicin concentration of 118mg/L (range 56-307mg/L) during the first postoperative day, for the group with 1g antibiotic/ 40g PMMA powder. They also noted peak serum levels to range between 0.96 and 2.9 mg/L, well below toxic levels for gentamicin. 17mg/L of vancomycin has been recorded in the knee joint at 8 hours using HPLC following the addition of 1g/40g PMMA.

A French group[196] investigated vancomycin loaded PMMA (1.3g/40g PMMA) in both primary joint replacements and a sheep model. They found peak drain levels of around 25mg/L at day 1 which tailed off to 1mg/L by day 5. They found the endosteal bone level to peak somewhere between 1-14 days at a level of around 50mg/kg bone. The gradient between endosteal and outer cortex (Figure 1.7) equilibrated by 3 months with a tissue

concentration of 50mg/kg maintained up to 6 months when it tailed off sharply. Surprisingly, only non-peer reviewed literature examining the in-vivo elution of non-aminoglyoside antibiotics from mechanically stable cement mantles was found[197].

There have been also been several studies utilising biodegradable delivery systems suitable for one stage revisions. Cancellous bone graft mixed with vancomycin have been shown to be effective in delivering around 400mg/L for the first day and tailing off to elute nearly all of the added antibiotic by 2 weeks without interfering with graft incorporation, with a dose of 1g vancomycin per 300g of bone[198]. The intra-articular application of gentamicin impregnated collagen fleeces has been examined using a canine knee model[199]. They found peak gentamicin levels of 2397mg/L at 80 minutes with corresponding serum concentrations of 8mg/L.

These very high concentrations fall within the mid-range of other published in-vivo studies on biodegradable collagen fleeces[162], unfortunately gentamicin is the only commercially available option at present. Biodegradable alginate beads have been used to deliver vancomycin at a dose of 154mg/L on day 1, tailing off to 1mg/L at day 28 in a rabbit model[200]. Biodegradable calcium sulphate beads have recently been re-introduced onto the market[201] and although it's very high zero order elution rate and wide variety of possible antimicrobial combinations are attractive, clinical evidence of its efficacy is still lacking.

1.7.4. Techniques used in two stage revisions

Strings of ALAC beads are used to fill dead space and offer an increased surface area to elute from. Beads do not provide mechanical integrity and so surgeons have been tending to use an anatomically molded temporary spacer between revision stages that allows the patient to ambulate better. Anagnostakos *et al.*[186] compared the concentration of antibiotics in drain fluid

in patients who had either beads or spacers inserted at the end of a first stage revision. Both groups had 0.5g of gentamicin and 2g vancomycin per 40g PMMA powder. They found the beads eluted more than 3 times more antibiotic with peak concentrations seen in day 1; 116mg/L gentamicin and 80mg/L of vancomycin was eluted from the beads vs 21 and 37mg/L from the spacer when analysed using fluorescence polarization immunoassay.

A well performed in-vivo model which implanted 5 highly antibiotic loaded PMMA beads into canine tibia, measured the seroma concentration on day 1 using a bioassay[202]. They found peak levels of 7.5mg/L ciprofloxacin, 1516mg/L clindamycin, 11mg/L vancomycin and 154mg/L of tobramycin. Interestingly only vancomycin and clindamycin achieved high levels in bone at 28 days (15 and 30mg/L). A different organism was used to bioassay clindamycin and this, coupled with the fact that they found the *in vitro* elution of clindamycin, similar to most of the antibiotics tested, should call its very high *in vivo* concentration into question.

In a rat model, treating osteomyelitis with 7.5% ALAC beads, Rouse *et al.* demonstrated peak bone levels on day four for daptomycin at 178mg/kg and 49mg/kg for vancomycin[182]. The first case report of daptomycin being incorporated in human ALAC spacer was in 2013[203] and used 2g gentamicin and 2g daptomycin per 40g PMMA powder. The spacer was in situ for 6 months and no adverse effects (e.g. myositis, rhabdomyolysis, peripheral neuropathy, derangement of liver function) were noted. Although linezolid has been tested extensively in-vitro and displays reasonable elution characteristics[193,204], no in-vivo studies have confirmed this yet.

Only a few studies have investigated whether spacers retain their biological activity throughout their lifespan. Fink *et al.*[205] analysed the soft tissue specimens that had been adjacent to antibiotic loaded spacers in 14 patients. They noted that tissue concentrations ranged widely, but found median levels of 10, 60 and 40 mg/kg for gentamicin, clindamycin and vancomycin respectively at 6 weeks.

1.7.5. Potential adverse effects of local antibiotics

There have been a handful of case reports of acute renal failure in patients with articulating spacers *in situ*. No other obvious causes were identified except an aminoglycoside level of 5mg/L[166]. That said Evans *et al.* [63] used 4 g of vancomycin and 4.6 g of tobramycin per 40g of PMMA in their spacers. All 44 patients were without renal, vestibular, or auditory effects at a minimum of 2 years. After single stage revision using a commercial ALAC, a maximum plasma concentration of 0.2mg/L gentamicin at 6 hours and 0.1 mg/L vancomycin at 20 hours was seen, well below toxic levels. A possible explanation for this discrepancy could be due to the cyclic loading that the articulating spacers are subjected to. Propagation of fatigue fractures could expose seams of interior antibiotic that would not normally be eluted so rapidly[206].

1.8. Combining antibiotics

Combining antibiotics both systemically and locally is common clinical practice and has been shown to result in the lowest infection rate after primary arthoplasty [207]. In the context of established infection, it is done for a number of theoretical reasons including: broadening the spectrum of antibiotic activity in empirical therapy; utilising synergistic effects of combinations, preventing resistance mechanisms evolving; enhancing intracellular penetration and to limit bacterial toxin and other virulence factor expression. Although clindamycin is a bacteriostatic agent it displays superior clinical outcomes compared to penicillin in severe streptococcal and staphylococcal infection. It does not kill the organisms but does limit the production of their virulence factors by blocking peptide translocation at the 50S ribosome[208]. In some situations, rapid bacteriolysis has been shown to be detrimental to the host as well as the bacterial population[209].

The influential Swiss infection group led by Zimmerli[210] do not recommend adding rifampicin until the bacterial load has been lowered, perhaps 2 weeks after debridement, in order to reduce the risk of resistance emerging . They cite the high intracellular concentration as important to its clinical efficacy. Apart from their group's randomised control trial[56] which has been critiqued earlier in this introduction, there has been a dearth of well-designed clinical studies and this has led to a significant citation bias in the literature.

A 2008 systematic review into the adjunctive use of rifampicin for the treatment of *S. aureus* infection[211] noted that *in vitro* results of interactions between rifampicin and other antibiotics were heavily method dependent and often did not correlate with in-vivo findings. They concluded that although adjunctive rifampicin in PJI seemed promising they cautioned that more definitive data are lacking.

It is theorised that the β-lactams, daptomycin and vancomycin can yield synergistic effects by opening pores in the cell wall and facilitate access of the fluoroquinolone and aminoglycosides to their target sites within the bacteria[212]. Although this has been demonstrated in a few animal models there is no good clinical evidence to suggest dual therapy improves clinical outcome in established staphylococcal infection[213]. Antagonism was also commonly noted in many of the in-vitro studies on rifampicin combinations which were comprehensive reviewed by Forrest *et al.*[144]. This antagonism has been replicated in a study of 42 patients treated for MRSA endocarditis in which the addition of rifampicin to vancomycin was associated with clinically and significant prolongation of bacteraemia[214].

1.8.1. Future approaches

As our understanding of staphylococcal metabolism and the mechanisms underpinning its pathogenesis has increased, new targets and strategies to exert more control over them have been identified[86,215]. These include manipulating the *agr* locus[216] and quorum sensing systems[217] , which are key to the expression of biofilm phenotype, producing bio-materials coated with antimicrobial substances[218,219] and engineering naturally occurring bacteriophages to become more efficient bactericidal agents[220]. Another promising strategy is metabolite-enabled eradication[107] in which persisters are coaxed into generating a proton-motive force, facilitating aminoglycoside uptake and death, without fully reactivating the bacteria into a NCV.

Intriguingly, persister theory has recently utilised the concept of resonant activation more familiar to physicists. This describes an effect seen in systems with multiple steady states, in which larger fluctuations in the oscillating perturbation, leads to a faster transition rate to a new steady state. In the context of PJI, the inherent biological noise within the bacterial toxin-antitoxin systems could explain and drive the stochastic transit between the normal colony and persister phenotypes. By inducing resonant activation one could potentially prompt a fast and synchronized phenotypic switch back to a less antibiotic tolerant state[221]. This may turn out to be the mechanism for the intermittent sterilisation effect that Bigger described back in 1944[94] with penicillin and staphylococci but a more complete understanding of this process is needed before safe clinical implementation. All of these approaches are still years away from clinical trials and there remains an urgent clinical need to optimise the current treatment of staphylococcal PJI by establishing the most efficacious antimicrobial regime to complement surgical debridement.

1.9. In vitro biofilm models

Although in-vitro biofilm assays frequently do not correlate well with clinical outcomes, they are a vital first step before animal or human models can be justified. The choice of which of the numerous biofilm models would be the best is dependent upon the clinical question requiring to be answered, which is:

"Which antibiotic combination delivered immediately after surgical debridement, is the most effective at eradicating the patches of staphylococcal biofilm that remain?"

To answer this question, several aspects in the design of a relevant in-vitro biofilm model had to be resolved: selection of a suitable device for generating and testing the biofilms; selection of appropriate growth conditions; definition of which end-points should be used to analyse the results; rationalising which antibiotics should be used and for how long.

1.9.1. Current biofilm devices

Several established and reliable biofilm models, summarised very well by McBain[222], such as constant depth film fermentors; rotating reactors; perfused membrane models; drip flow biofilm reactors; and flow cells were considered and discounted as they lacked sufficient throughput to allow the estimated 1200 samples to be processed in the time available. Simple agar plate methods have been used previously but are not suitable as they use the substratum as the source of nutrition, expose the biofilm to air and are influenced by differing rates of antibiotic diffusion throughout the agar medium. This left 3 suitable models: the submerged substratum, the multi-well plate and the modified Robbins device (MRD).

The MRD is a linear array of 6 ports along a rectangular cross-section channel that the media flows through, into which coupons of substrata can be removed during an experiment for susceptibility testing[223]. Although it has been extensively used for bacterial susceptibility testing, it was also deemed unsuitable as it relies on unidirectional shear and so is prone to contamination from an up-stream source. The intermittent sterilisation necessary between batches would also have impaired high throughput.

The multi-well plate technique has become increasingly popular after the Calgary group patented it and made it commercially available as the MBEC™ device[109]. It is essentially a 96-well polystyrene plate with a lid that incorporates 96 removable pegs to use as biofilm substrata. The lid is transferred between inoculated media into antibiotic laden broth and then rinsed to remove non-adherent organisms before sonicating the biofilms at low frequency, into fresh media for quantification. This MBEC™ device has published results for a wide range of bacterial and yeast species and have established protocol in Nature[224]. It is capable of high output and seemed ideally suited to answer the clinical question asked of it.

Unfortunately after many weeks of trying to optimise what is a very exacting and lengthy protocol, despite technical advice from the company, the author was not able to achieve any semblance of reproducibility. These pilot studies seemed to be unduly influenced by either; a heterogeneous ultrasound field used to sonicate the biofilm off the lids or perhaps irregular shear forces during incubation as the author used a rotating rather than a tilting platform. Both these minor deviations from the protocol highlighted that the system was not particularly robust and this prompted our group to develop our own more cost effective method, modifying the established submerged substratum model to suit our clinical question more closely.

1.9.2. Correlating study end points with current clinical practice

The next decision was how to calculate the viability of the organisms after treatments. Groups have used a colorimetric redox marker such as Alamar blue[225] to indicate metabolic activity or simply optical density of the recovered samples, as a proxy for cell death. Attempts were made to stay as close to the validated MBEC™ protocol as possible to facilitate comparison of the results. Ceri *et al.*[109], felt as we did, that the gold standard for quantification of viability was extended culture on agar. Not only could the bacteria liberated from the biofilm be accurately enumerated, one could make comparisons to standard laboratory tests on planktonic cultures.

Currently, microbiologists determine the Minimum Inhibitory Concentration (MIC) of an antibiotic that inhibits growth in a planktonic culture by either the ISO 20776-1 broth micro-dilution or disc diffusion method[156]. Microbiologists then interpret this MIC using a discriminating breakpoint concentration, which reflect the drugs bioavailability and previous clinical outcome data, thereby defining isolates as susceptible, intermediate or resistant. The breakpoints recommended by the British Society for Antimicrobial Chemotherapy (BSAC) Working Party, have recently been brought into line with the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines[226]. In the USA, the Clinical Laboratory Standards Institute (CLSI) publishes such guidance and typically suggests slightly higher breakpoints.

The BSAC breakpoint is calculated as (C_{max}* F/TE) *S.

C_{max} is the maximum concentration of antibiotic at site of the infection;
E is a factor by which the breakpoint should exceed MIC (usually 4)
T is the ½ life (2 if <1hr, 0.5 if >3hrs);
F is a serum binding factor (1 if <70%, 0.2 if >90%);
S is the shift needed to allow for resistant mutants in sensitive strains.

Typically the ***resistant*** breakpoint is set around the C_{max} and the ***sensitive*** breakpoint around 1/4 C_{max}. Staphylococcal spp. are deemed resistant if the

MIC is > 0.5 for clindamycin or rifampicin; > 1 for gentamicin, ciprofloxacin or daptomycin; > 2 for vancomycin; and > 4 for linezolid[227].

Using breakpoints to interpret MIC's fails in a number of ways in the context of PJI. By utilising local delivery of antibiotics at the time of surgery, the C_{max} is potentially increased by orders of magnitude. Therefore, if this susceptibility test is to be used, there is a need to develop higher breakpoints to reflect this. It also fails to consider that the biofilm will already be well established with a high bacterial load before clinical symptoms manifest themselves. Merely inhibiting further growth will only temporarily suppress the infection and fail to eradicate it unless the bacterial load is sufficiently diminished to allow the host's innate immune system to deal with it. Therefore the concentration of antibiotic needed to ***eradicate*** the infection would be a better measure than MIC.

1.9.3. Selection of a study end point

As outlined, the bacteria associated with PJI are in a biofilm rather than a planktonic state. It is well known that, even in the absence of specific antibiotic resistance factors, organisms in biofilm are generally between 100 - 1000 times more tolerant to antibiotics than when they revert back into their actively growing planktonic state[109,228,229].

The Minimal Biofilm Inhibitory Concentration (MBIC) is the lowest concentration of antimicrobial that prevents biofilm re-growth. However it varies between a 50-75% reduction of metabolic activity compared to controls and has not been extensively established as a concept[230,231]. The Minimum Biofilm Eradication Concentration (MBEC) is more widely recognised and is defined as the lowest concentration of antimicrobial that eradicates 99.9% of the bacteria compared to growth controls[109,232]. This reduction has not been shown to assure that the residual infection can be dealt with by the innate immune system but provides the closest approximation to expected in-vivo effects. Thus, MBEC was selected as the primary end point of the study.

1.9.4. Selection of antibiotics

A pragmatic approach prompts the selection of the most commonly used antibiotics in ALAC (gentamicin, vancomycin, clindamycin, ciprofloxacin and rifampicin) as well as reflecting the emerging use daptomycin and linezolid. They should be tested both in isolation and in combination at physiological doses. Studies have shown that this will vary between 4000mg/L and 10mg/L in a time and compartment volume dependent fashion (section 1.7). The length of antibiotic exposure for this in-vitro model was chosen to be 3 hours on the basis of several factors. Firstly, the highest and most predictable concentrations will be in the initial few hours following administration. Previous work has shown that, of all total gentamicin released in the first three days from ALAC, 2/3 of it elutes in the first three hours[174]. Secondly, the initial few hours offer the best opportunity to eradicate the infection as the biofilm has just been physically debrided and in most instances, will not be under preceding antibiotic stress. Thirdly, three hours has previously been demonstrated to be long enough to reveal persister frequency in previous models using rich media[233]. Finally, a shorter challenge time allowed more samples to be processed in a working laboratory week, expanding the choice of which type of biofilm model could be used.

1.9.5. Selection of an alginate biofilm model

One of the parameters we were keen to change from the MBEC™ method was biofilm disruption. Previous groups have scraped of the biofilms[234], but this has shown to be inferior to sonicating the biofilms away from the substratum, resulting in substantial assay variance[235] . Although sonication does not seem to affect the viability of staphylococci (which is protected by its cell wall and spherical shape), it does significantly reduce viability of Gram negative and anaerobic bacteria[236]. In an effort to make this new biofilm assay universally applicable, we sought to design a system that liberated the biofilm from the substratum without sonication. After

literature review a method initially used for cellular immobilisation[237] was adapted to provide a suitable substratum. A French group had shown that sodium alginate could be chelated and then dissolved (using a citric acid and sodium carbonate solution) to liberate the immobilised bacteria without affecting their viability[238].

Sodium alginate is a naturally occurring biodegradable polysaccharide, extracted from brown seaweed. It is a copolymer of 1,4-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) . Its ability to solidify relies on the G blocks being chelated by divalent calcium and forming structures proposed to resemble eggs in an egg box junction[239]. Using an alginate surface to grow biofilm on, before dissolving the bead, and liberating the previously adherent bacteria had not been tried before, but seemed like an elegant solution worth developing further.

For the reasons outlined above, a submerged alginate substratum biofilm model with semi-continuous batch culture was selected. A single species staphylococcal biofilm was cultured in an enriched medium, exposed to *in vivo* levels of antibiotics up to concentration 2048mg/L for 3 hours. The bacteria were directly enumerated after dissolving the alginate substratum and liberating the biofilm. Outcome measures of the model were divided into technical and practical aspects. Practical aspects included cost effectiveness, in terms of materials equipment and technicians time, as well as the ability to guide clinicians' decisions in a real world time frame. A technically satisfactory method would have repeatable control data and reliably demonstrate what proportion of the bacteria was killed by an antimicrobial.

2.Materials and Methods

2.1. Materials

2.1.1. Bacterial Strains

Three staphylococcal strains were used; a reference strain and two clinical strains isolated from patients with PJI at the Royal Infirmary of Edinburgh. All were sub-cultured and stored in 10% glycerol at -70°C. A Methicillin Sensitive *Staphylococcus aureus* **MSSA-'F'** was isolated from infected metal-work used to reconstruct an elbow fracture. A coagulase negative staphylococcus **CNS-'J'**, isolated from an infected hip replacement. **MSSA-'N'** (American Type Culture Collection 29213) was a *S. aureus* reference strain which had been extensively studied in its biofilm state[109,231,240] and was used to facilitate comparison of the final protocol to other techniques.

2.1.2. Media

Luria broth (LB): Difco bacto tryptone (10 g), Difco bacto yeast extract (5 g) and NaCl (10 g) were dissolved up to 1L of dH_2O. The pH was adjusted to 7.2 prior to autoclaving. **Tryptic soy broth** (TSB): Pancreatic digest of casein(17g), peptic digest of soybean (3g), glucose (2.5g), NaCl (5g), dipotassium hydrogen phosphate (2.5g) was dissolved up to 1L with dH_2O and the pH adjusted to 7.3 prior to autoclaving. **Solid LB agar**: Broth was solidified by adding 15 g/L difco agar prior to autoclaving.

2.1.3. Common materials and solutions

Disposables were used as follows: 0.2um sterilising filters, Minisart R Sartorius Biotech GnbH; Pipette tips, Greiner Bio-One Ltd. Gloucestershire GL103SX; Greiner 50ml and 15ml CELLSTAR PP-Falconi tubes; Greiner 96-well round-bottomed micro-titre plates were used for forming the bead in and NUNC 96 well flat bottom microtitre plate 300ul were used to perform the optical density measurements and micro-dilutions, Sigma-Aldrich

Company Ltd. Dorset SP8 4XT; IWAKI flat bottomed 48 well polystyrene plates with 1.76 ml capacity, were used for alginate bioassays; Bibby Scientific Limited, Staffordshire, ST15 OSA, UK.

All chemicals were of reagent grade and obtained from standard suppliers. Solutions were autoclaved prior to use unless stated. A *4% (w/v) sodium alginate solution* was prepared in warm water with agitation. 100ml was enough to make around 200 beads. A 2M solution of *calcium chloride* containing 29.4g made up to 100ml with sterile dH_2O was enough to chelate around 400 beads. A *universal neutralizer* was used to counteract free radicals and minimise antibiotic carry over. It was made up to 20ml in double distilled water and composed of 1.0 g L-histidine, 1.0 g L-cysteine and 2.0 g reduced glutathione. The solution was filter sterilised with a 0.20μm filter and stored at -20°C. A solution containing 0.05M Na_2CO_3 and 0.02M citric acid was used to *dissolve alginate beads*. 10x stock solution made by making up 5.3g of Na_2CO_3 and 3.8g of Citric acid to 1L in dH_2O.

2.1.4. Antibiotics

The antibiotics used in this study (listed below) were purchased via NHS Lothian pharmacy. Liquids and reconstituted powders were used as stock solutions and stored as per manufacturers instructions[156] until being diluted immediately prior to use.

Linezolid - Zyvox ® 2000mg/L Pharmacia, Sandwich, Kent, CT13 9NJ
Gentamicin - Cidomycin ® 4000mg/L Sanofi Aventis, Guildford GU1 4YS
Rifampicin - Rifadin ® 400mg/L Sanofi Aventis, Onslow Street Guildford
Ciprofloxacin - 2000mg/L PLIVA Pharma Ltd, Petersfield, GU32 3QB
Vancomycin – 4000mg/L Wockhardt UK Limited Wrexham LL13 9UF
Clindamycin - Clindamycin -Cleocin ® 4000mg/L Sanofi Aventis, Guildford
Daptomycin – Cubicin ® 4000mg/L Cubist Pharmaceuticals, Lexington,USA.

2.2. Methods

2.2.1. Alginate bead topography

Alginate beads were measured to calculate their surface area using vernier calipers measuring to nearest 0.1mm. The surface area was calculated by dividing the bead into 2 parts.

i. Surface Area of a 1 ended Cylinder = $\pi r^2 + 2\pi * r * h$,

ii. Surface Area of half a Sphere = $2\pi r^2$

Therefore, 1-ended Cylinder is 114.51mm^2 and half sphere is 45.80mm^2, making a total surface area of 160.31 mm^2 (Figure 2.1) and average weight of 213.1μg (Table 2.1).

Sample	Weight (μg)	Height (mm)	Radius (mm)
1	214.6	5.4	2.7
2	215.0	5.5	2.7
3	209.7	5.4	2.7

Table 2.1: Bead topography

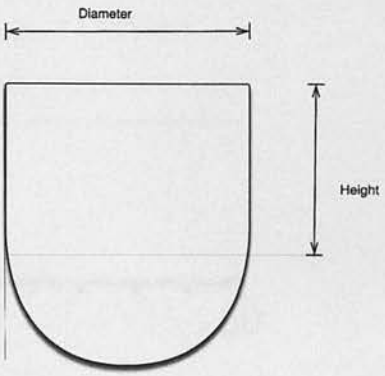

Figure 2.1 Bead shape

2.2.2. Generation time in rich media growth conditions

To ensure the microtitre conditions were resulting in normal exponential planktonic growth, the generation time was calculated for MSSA-F (Figure 2.2). Freezer stocks were cultured overnight in 5ml of tryptic soy broth (TSB) and plated on to agar. A typical colony was then sub-cultured overnight in 5ml TSB. Stationary phase organisms were added to 200μl TSB to make a final concentration of 10^{6}/ml in a 96 well plate and placed in an orbital incubator set at 200 rpm and 37^{O} for 24 hours. Three wells were sampled at 1, 2, 3, 4, 5, 6, 12, 13 and 24 hours, diluted and then plated on to LB agar using

the Miles Misra technique[241] which is detailed below in section 2.2.4. The method of calculation of generation time[242] assumes that as bacteria grow by binary fission, the increase in population is by geometric progression where G (generation time) = (time) / n (number of generations). As n is related to both B and b (number of bacteria at the beginning and end of time interval respectively) by the equation b = B x 2n it can be resolved to n = 3.3 log b/B and then solved as G=t/n.

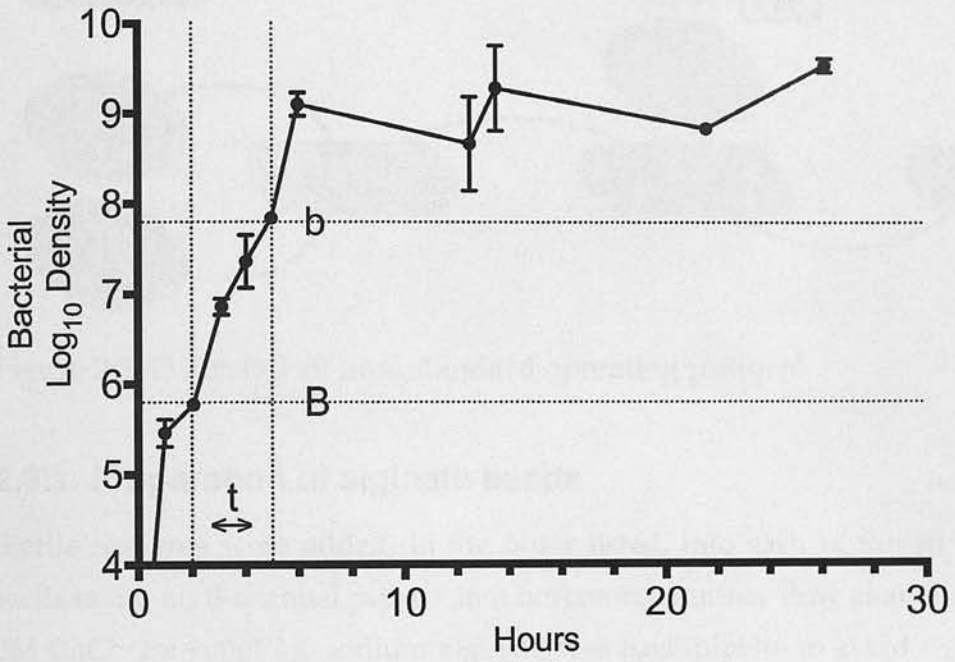

Figure 2.2: Generation time of planktonic MSSA-F in TSB.

Fitted circles are the mean Log 10 CFU recovered with the bars representing the SD.

t is the time interval, B is the number of bacteria at beginning and b is the number of bacteria at the end of the time interval.

Therefore the generation time of the F strain in these conditions, was calculated as:

$G = t/n$

$G = 299 - 187\text{mins} / 3.3 (7.84 - 6.86) = 112 / 3.27$

G = 34.3 minutes per binary division which is in keeping with the normal range for these conditions [242].

2.3. Standard Operating Protocol

After the results of chapter 3 had been taken into account the final version of the Standard Operating Protocol (SOP) (Figure 2.3) was finalised.

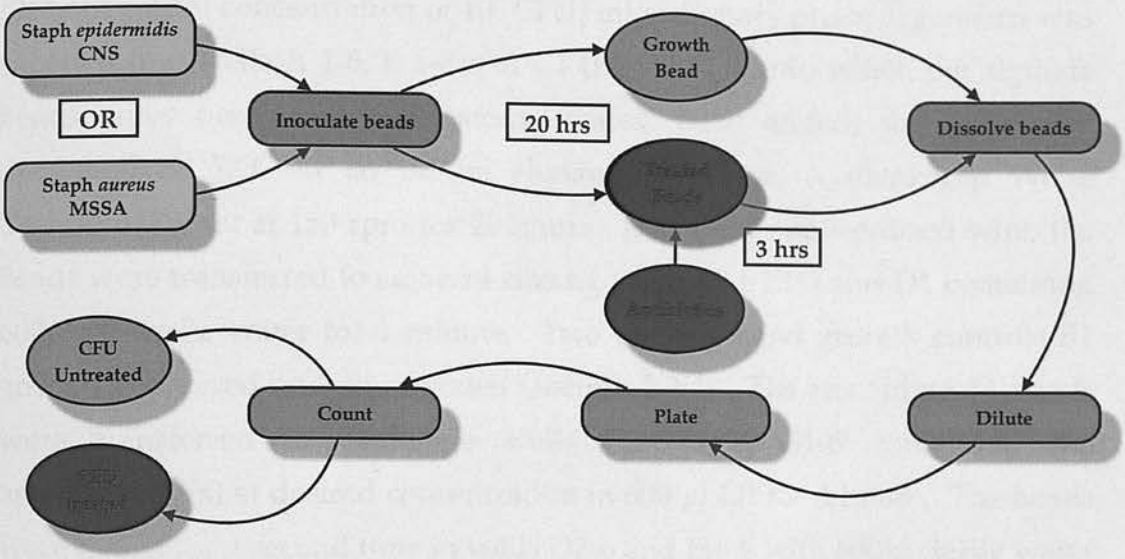

Figure 2.3: Overview of final standard operating protocol

2.3.1. Preparation of alginate beads

Sterile reagents were added, in the order listed, into each of the 96 U shaped wells using an 8-channel pipette in a horizontal laminar flow chamber: 10μl of 2M CaCl; 2 x 100μl 4% sodium alginate was back-pipette to avoid clogging the tips; a fine mist of 2M $CaCl_2$ was sprayed over plate using a sterilized pump; another 20μl of 2M $CaCl_2$ was added once a flat chelated surface had formed; the plate was covered with its lid, sealed with paratape and stored at 4^{O}C.

2.3.2. Preparation of the organisms

An overnight culture of freezer stock in 5ml of LB was aerobically incubated at 37^{O}C, streaked onto agar plates and incubated overnight at 37^{O}C; two morphologically consistent single colonies were sub-cultured overnight in duplicate 5ml LB bottles at 37^{O}C; a starting inoculum of 10^{7} CFU/ml was made up by the measuring the optical density of the overnight stationary phase sub-culture @ 600nm before being confirmed by 10 fold serial dilutions and plating using Miles Misra method, as detailed below in section 2.2.4.

2.3.3. Growing and challenging the biofilms

Reagents were added into each of the 48 flat-bottomed wells in order: 600μl LB with a final concentration of 10^7 CFU/ml stationary phase organisms was pipetted into wells A 1-6, E 1-6 and C1 (Figure 2.4); into which the alginate beads, after being rinsed in sterile water, were added; the plate was incubated at 37°C in an orbital shaking incubator, (Gallenkamp Weiss Technik UK), set at 150 rpm for 20 hours. Using a flame sterilised wire, the beads were transferred to adjacent rinsing wells B1-6 F1-6 and D1 containing 600μl of sterile water for 1 minute. Two unchallenged growth controls B1 and D1 recovered and enumerated (section 2.3.4). The remaining 11 beads were transferred to challenge wells C2-6 and G1-6 containing the antimicrobial(s) at desired concentration in 600 μl LB for 3 hours. The beads were rinsed for a second time in wells D2-6 and H1-6 with 600μl sterile water for 1 minute before being dissolved as detailed below.

2.3.4. Recovering the organisms

The dissolving solution contained 1ml of universal neutraliser made up to 40mls with 0.02M citric acid & 0.05M Na_2CO_3. It was titrated to pH 6.8 and sterilised with 0.2μm filter. Each bead was placed into a separate 15ml Falconi tube containing 1 ml of dissolving solution and crushed using a flame sterilised glass-stirring rod for 10 seconds. Another 1ml of dissolving solution was added and the tube was placed in rotating rack for 15mins. After the bead had dissolved, the tube was vortexed for 10 seconds before being 10 fold diluted six times using PBS in flat bottomed 96 well plate. Each bead was plated using the micro Miles Misra technique onto LB agar and incubated at 37°C for a minimum of 48 hours before resulting no growth.

10μl of each dilution was sampled using a multichannel pipette and dropped onto a LB agar dish from 1cm (Figure 2.5). Five technical replicates were performed. After the drops had been fully absorbed into the agar (20 minutes), the plate was turned upside down and incubated at 37°C. Plates were manually enumerated after 24 hours (48 hours if no growth was seen at 24 hours). It became difficult to read the technical replicates when there were more than 20 CFU per drop and so 3-15 CFU was found to be an optimal number to record as a result. If the CFU numbered less than three on the least dilute row and the colonies were both within the drop and morphologically consistent, with the organism being assayed, they were included in the results.

The Log Density (LD) per biological replicate was calculated by $\log_{10}$ transforming the product of the mean of the five technical replicates and the dilution factor of 200 (2 ml of dissolving solution x 100 to adjust for sample volume). The lowest possible value recordable using this technique was therefore 1 colony in the 5 replicates of the least dilute row, which equates to LD of 2.6. If there was no CFU visible, it was recorded below the minimum detectable limit and assigned at half that level at 2.3 LD, instead of zero. As all the samples were taken from the same well, the mean of all 5 technical replicates was recorded and the median of a minimum of 3 biological replicates for each condition was resulted along with the inter-quartile ranges.

2.3.5. Data handling, graphical illustration and statistical analysis

Data was analysed using GraphPad Prism 6 for Mac OS X (GraphPad Software, San Diego California USA, www.graphpad.com) software for statistical analysis and graphing. The author performed all standard analyses, the only exception being a fully nested ANOVA test that was done using SPSS Version 14 with advice from a senior medical statistician.

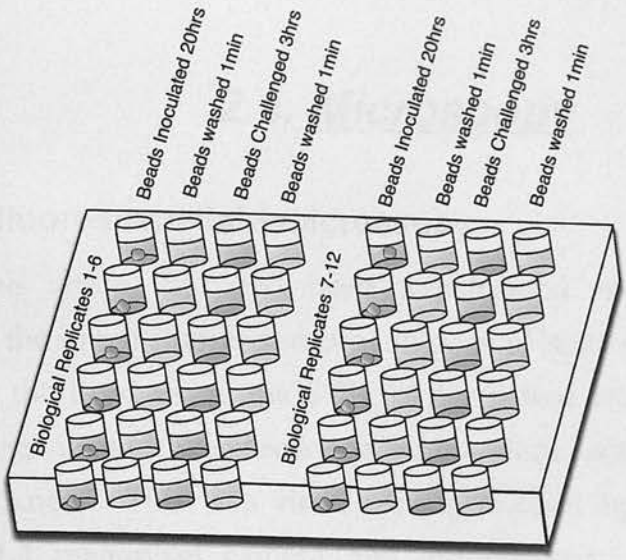

Figure 2.4: Schematic of 48 well plate used in alginate biofilm model.

Two growth controls and 11 biological replicates per plate. Only one growth control shown for simplicity but there was normally a second control in well C1 (wells A-H are left to right).

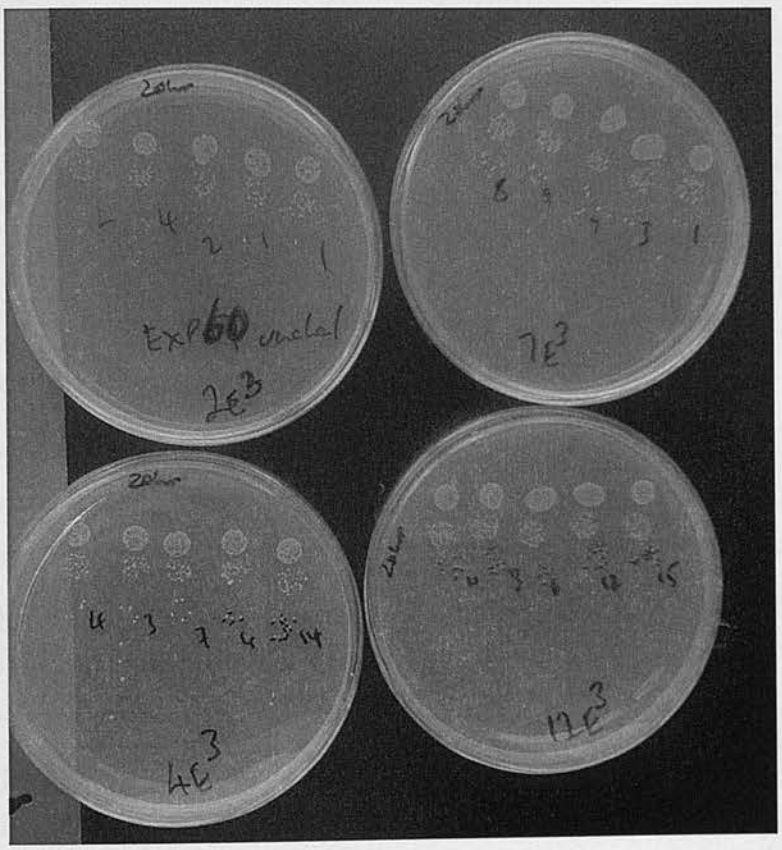

Figure 2.5: Example of Miles Misra enumeration method.

Each agar plate is a different biological replicate. 5 technical replicates are plated from left to right in each plate with 6 dilutions top to bottom from 10 to 10^6 fold. Although median result is written on plate, the mean was used in results. Results from top left clockwise: LD of 5.6, 6.1, 5.9 and 6.4 once dilution and $\log_{10}$ transformation was performed for each biological replicate.

2.4. Microscopy

2.4.1. Epifluorescent light microscopy

A *S. aureus* strain that had been transformed with a plasmid vector containing the green fluorescent protein gene pHC60 was used to generate a biofilm on the bead using the SOP. After it was washed to remove non-adherent organisms it was sectioned with a sterile scalpel to approximately 0.5mm thickness. This was viewed using a Zeiss light microscope with a Axiocam 1.4 megapixel camera and epifluorescent function (Carl Zeiss Microscopy Ltd Cambridge)

2.4.2. Transmission Electron Microscopy

Attempts to section the alginate bead were made using a microtome. Unfortunately both the unprepared bead and one that had been fixed in 2.5% glutaraldehyde solution were too soft to be sectioned to the thickest possible width of 100μm and could not be processed further.

2.4.3. Electron cryo-microscopy

A 20-hour MSSA-N biofilm that had grown on the surface of an alginate bead was washed with water to remove non-adherent organisms. Under the guidance of Dr C Jeffree (University of Edinburgh's electron cryo-microscopy unit), the sample was rapidly frozen using a Gatan ALTO 2500 Cryotransfer module in it's native hydrated state to preserve the biofilm architecture. It was freeze-fractured to expose its internal microstructure and sputter-coated with gold-palladium to allow higher resolution surface imaging before being transferred under vacuum to the microscopy chamber. The FEI F20 electron microscope (200 kV, field emission gun) was equipped with an 8k x 8k CMOS camera. Images were processed using a dual Quad, 8 GB RAM computer that used the IMAGIC processing software (Image Science Software GmbH, Gillweg 3, Berlin, Germany) to convert images into tagged image file Format (tiff) format, typically 5MB in size.

3.Development of an alginate bioassay

3.1. Introduction

Prosthetic Joint Infection (PJI) is a leading cause of morbidity in patients with orthopaedic implants. PJI are typically caused by adherent bacterial biofilms which are refractory to systemic antibiotics alone. It has been appreciated for over 20 years[243], that the Minimum Inhibitory Concentration (MIC) of a planktonic culture is a poor predictor of PJI susceptibility. Despite this, the only method currently clinically available, to evaluate microbial sensitivity, is still based upon the MIC for a single antibiotic using either the ISO 20776-1 broth micro-dilution or a disc diffusion method[156]. The MIC is interpreted as a discriminating breakpoint concentration, thereby defining isolates as susceptible, intermediate or resistant[227] to an individual antibiotic.

The bacteria associated with PJI are in a sessile biofilm rather than the planktonic state they are tested in routinely. It has been established that, even in the absence of specific antibiotic resistance factors, sessile organisms in biofilms are generally between 100 - 1000 times more tolerant to antibiotics than when they revert back into their planktonic state[66,109]. No current clinical method of in-vitro susceptibility testing considers the pathogens' sessile state or accurately reflects the higher antibiotic levels frequently delivered locally, and often in combinations, to treat prosthetic infection.

In the present study, a method described by Mater *et al.*[238] to immobilise cells from a late growth phase *Pseudomonas* spp. within a bead of 2% (w/v) sodium alginate, by chelating the alginate using calcium chloride 0.07M, has been adapted. These authors demonstrated that if the beads were dissolved with a sodium carbonate and citric acid solution then 100% of the organisms remained viable and could be quantified as colony forming units (CFU) by plating. An attractive feature of this approach was that after the bead was dissolved, the biofilm would have no surface to remain attached to and so recovery would be maximized, compared to other methods such as sonication which is commonly used to liberate bacteria from solid surfaces[224,244,245].

3.1.1. Aims

The aim of this chapter was to develop a biofilm model which met the following requirements:

i. Allowed the testing of sessile bacteria in biofilm with multiple antibiotics at high doses achievable with local delivery.

ii. Had the ability to guide clinical decisions in a real world time frame more confidently than breakpoint discriminations of the MIC.

iii. Was cost effective in terms of materials, equipment and technicians' time.

iv. Produced repeatable control data.

v. Demonstrated what proportions of the bacteria are killed by a antimicrobial.

vi. Maintained an acceptable repeatability of disinfection within and between laboratories.

3.2. Results

In order to establish and assess the value of a suitable biofilm model, it was firstly necessary to establish the growth profiles for the isolates under study in both planktonic and sessile conditions. Two clinical isolates of staphylococci were therefore examined: A Methicillin Sensitive *S. aureus* MSSA-F, and a Coagulase Negative Staphylococcus CNS-J, were isolated from patients with prosthetic infection at the Royal Infirmary of Edinburgh.

3.2.1. Optimising planktonic growth

Three different culture media were initially compared to optimise planktonic growth conditions. Tryptic Soy Broth (TSB) is a commonly used medium in staphylococcal biofilm research[230,246]. It was compared to Luria-Bertani broth (LB[247]) which was used routinely by the laboratory in which the investigations were carried out, and also to LB with the further addition of 7.5% (w/v) NaCl (SLB). This additional osmotic stress has previously been shown to enhance biofilm formation in MSSA isolates by up-regulating the *ica* locus that controls PIA secretion[248].

To establish the growth profiles, 96 well plates containing 200μl of fresh medium was inoculated with 10^7 CFU/ml of either MSSA-F or CNS-J, previously grown overnight in 5ml LB at 37°C. A microplate absorbance spectrophotometer with a built-in incubator and plate shaker set to 37°C and at 200rpm (BioRad Laboratories Ltd.) was used for incubation whilst the optical density at 600nm, was measured every 30 minutes. There were 14 biological replicates in each of the six possible organism/medium combinations and sampling continued until stationary phase was well established (Figure 3.1). In order to calculate the bacterial generation time under these conditions (Figure 2.2 in section 2.2.1), sampling of additional wells containing MSSA-F in TSB was undertaken every hour. Samples of these three biological replicates were micro-diluted and then plated on to LB agar using the Miles-Misra technique[241].

Examining figure 3.1, it can be seen that LB with added NaCl (SLB) has the poorest exponential growth rate and lowest peak OD. TSB allowed both organisms to stay in their exponential growth phase for longer. However their generation time in the exponential phase seemed similar. The apparent biphasic growth seen for MSSA-F in SLB is likely to reflect an artifact optical density measurement (discussed in section 3.4.1) as the standard deviation seen was much higher in this group and no other plausible explanation was found. Because CNS-J also grew poorly in SLB the experiment was not repeated.

3.2.2. Optimising sessile growth

The hypothesis that the medium producing the highest bacterial numbers in the planktonic state should also produce the highest sessile numbers on the bead surface was then tested.

The LB, SLB and TSB media were inoculated to a concentration of 10^7 CFU/ml with either CNS-J or MSSA-F. 10mls of each combination was then added to individual 25ml conical flasks, each containing 4 alginate beads, and incubated aerobically in an orbital shaker set at 50 rpm and 37 °C, for 20 hours. Subsequently, the beads were removed and gently rinsed twice with PBS solution to dislodge non-adherent organisms. The beads were then dissolved into 3ml of sodium carbonate / citric acid solution (see section 2.2.4) before micro-dilution and enumeration of the liberated bacteria, using the Miles Misra technique, described in the methods[241], onto LB agar. A minimum of three biological replicates in each group was done and as the variance in each group was found to be unequal, the results were compared using the Kruskal-Wallis test and post tested using Dunn's multiple comparisons test (Figure 3.2).

The results shown in figure 3.2 do seem to show similarity in trend to those obtained for the planktonic culture in figure 3.1, with the TSB having the greatest mean Log$_{10}$ Density (LD) of sessile organisms recovered. However the differences in the means were only statistically significant between the SLB and LB for MSSA-F and between LB and TSB for the CNS-J. The beads in the TSB group had a noticeably softer consistency as they were being transferred between containers. Although advantageous as the alginate dissolved in the solvent more easily, questions remained however as to whether the beads would remain robust enough to allow incubation for a longer time.

3.2.3. Sessile growth over time

To examine how the organisms grew when forming a biofilm over time, five parallel cultures with four alginate beads in each 25ml conical flask containing 10mls of either LB or TSB were prepared. They were inoculated to 10^7 CFU/ml with CNS-J and incubated aerobically in an orbital shaker at 37 °C, 50 rpm. At 3, 20, 45, 77 and 96 hours four beads were washed, dissolved and enumerated as detailed above.

The beads incubated in TSB were found to be too fragile after 45 hours to handle and so were not examined further. Figure 3.3 demonstrates that the adherent organisms divided steadily for the first 45 hours in this closed system, at which point the number of viable organisms recovered declined slowly. Focus moved away from further replicates of this experiment onto modifying the protocol in an effort to improve the robustness of the bead.

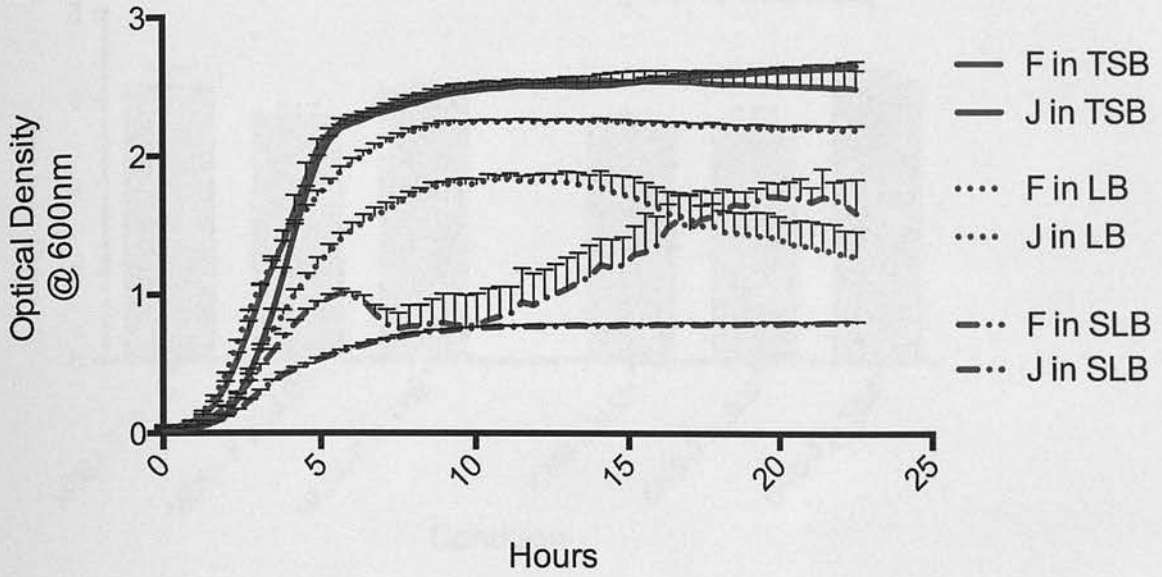

Figure 3.1: Planktonic growth curves comparing 3 different media.

Mean Optical Density at 600nm with SD (bars) is shown for 2 strains and 3 different mediums.

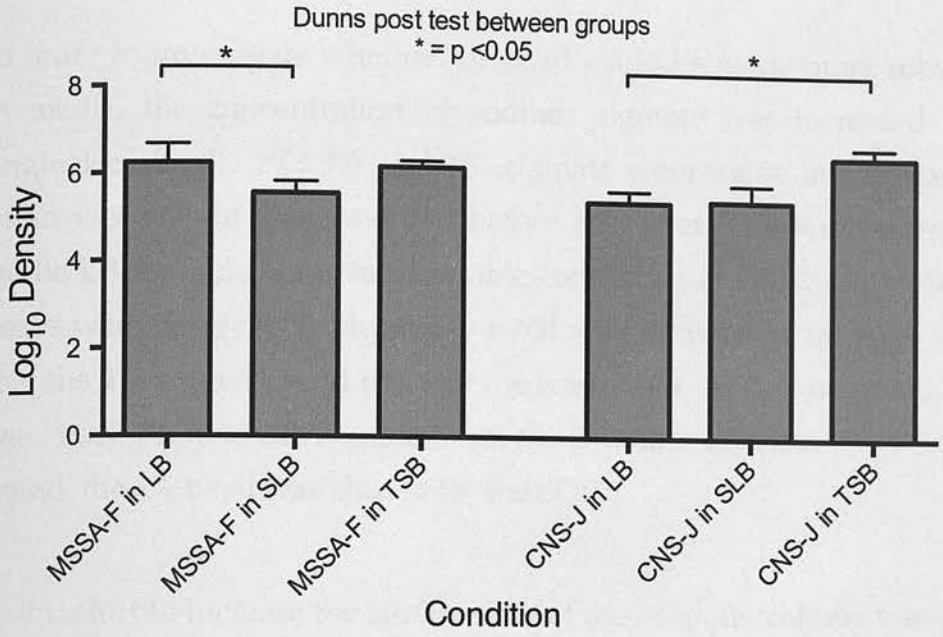

Figure 3.2: Comparison of sessile growth in 3 different media LB, SLB and TSB with both MSSA-F and CNS-J organisms.

LD – Log_{10} density. SD- Standard Deviation denoted by bars.

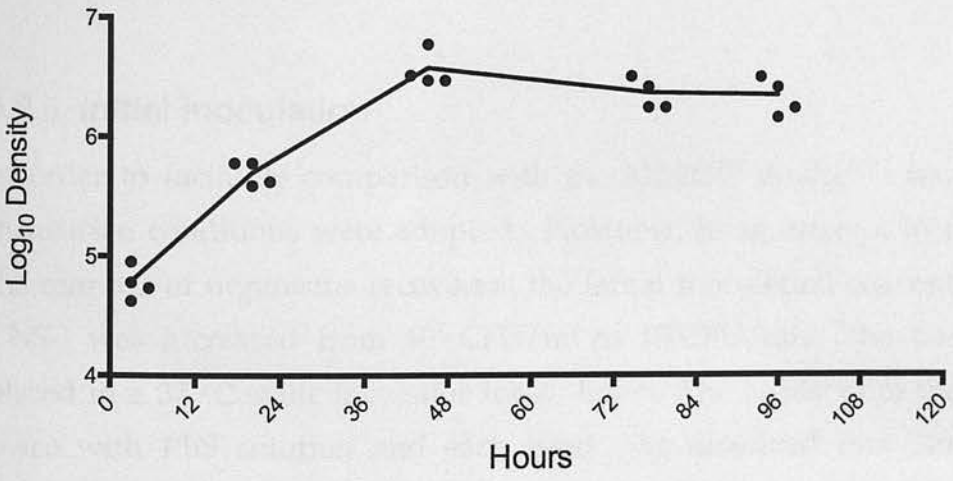

Figure 3.3: Growth curve of MSSA-F biofilm on alginate bead in LB over time

Each closed circle is the mean of the 5 technical replicates and expressed as Log_{10} CFU.

3.2.4. Modifications to the bead

In order to investigate whether the bead could be made more robust whilst in media, the concentration of sodium alginate was increased from the original method. 2%, 3% and 4% alginate were made in triplicate. Their mean wet weight was recorded before and after they were submerged in sterile LB for 6 days in static aerobic conditions at 37°C. Although all the beads were observed to physically swell after immersion, table 3.1 confirmed that the 4% alginate bead had lost the least mass. As it was felt that any loss was most likely to have arisen from the interface between the bead and the liquid, the 4% bead was chosen for the SOP.

In an effort to increase the surface area of the bead, its volume was increased from 100 to 200μl. As a consequence, they were only peripherally chelated and a more concentrated 2M calcium chloride solution was needed to consolidate their centre. The order and volume in which the reagents were added was also crucial to prevent a central lagoon of calcium chloride from forming and to ensure the top surface was flat (section 2.2.1).

3.2.5. Initial inoculation

In order to facilitate comparison with the MBEC™ device[224], many of it's incubation conditions were adopted. However, in an attempt to maximise the number of organisms recovered, the initial inoculation concentration of CNS-J was increased from 10^7 CFU/ml to 10^8CFU/ml. The flasks were placed in a 37 °C static incubator for 20 hours. The beads were then rinsed twice with PBS solution and each bead was dissolved into 3ml, before dilution and plating as described previously. From the data presented in Table 3.2 however, it can be seen that no advantage was gained from using the higher starting inoculums and 10^7 CFU/ml was used in subsequent experiments.

3.2.6. Shear Stress during incubation

Shear stress has previously been shown to impact on the quality of biofilm formation[66]. In order to relate well to the natural situation for biofilm formation, it was important that the shear stresses applied to the beads was sufficient to result in firm biofilms but without disrupting their integrity. To address this point, four beads were added into each 25ml conical flask filled with LB as before. The flasks were inoculated with 10mls of LB containing 10^7 CFU/ml of CNS-J and incubated at 37°C for 20 hours at 0, 50, 150 or 200 rpm in an orbital shaker. The data in Table 3.2 shows that 150 rpm was the optimal setting which maximised biofilm growth without the mechanical failure of the beads that was seen at 200 rpm (figure 3.4).

3.2.7. Rinsing solution

PBS was initially used to rinse away any non-adherent organisms because of its physiological pH and osmolarity. It was noted however to be associated with degradation of bead structure and was substituted with sterile water.

3.2.8. Incubation vessel

The final modification to the SOP was to switch from using 25 ml conical flasks containing five beads in 10mls of media to a single 48 well flat-bottomed polystyrene plate. The beads were transferred between adjacent wells, each containing 600μl of medium (figure 3.5). This was mainly done for logistical reasons as it greatly reduced preparation time, amount of reagents that were needed, and allowed more biological replicates to be done per week. As a further advantage, it minimised the bacterial aerosol biohazard and risk of cross-contamination whilst maintaining the reliability of bacterial recovery from the beads (table 3.2).

% Sodium Alginate	Initial weight of 3 bead (μg)	Weight of 3 beads at 6 days (μg)	% Weight lost
2	634	523	17.5
3	621	562	9.5
4	681	644	5.5

Table 3.1: Sodium alginate concentration influences the wet weight of beads after 6 days in static sterile LB broth at 37 °C

Characteristic	Condition	Handling	Dissolving	LD
Starting inoculation (2 tailed test p = 0.27)	Static test low inocula 10^7 CFU/ml^{-1}	+	+	4.57 ± 0.31 n=4
	Static test high inocula 10^8 CFU/ml^{-1}	+	+	4.37 ±0.05 n=4
Shear stress within orbital incubator	Static	++	-	6.31±0.67 n=4
	50 rpm	++	-	6.19 ±0.57 n=16
	150 rpm	+	++	6.97±0.47 n=41
	200rpm	-	-	NR
Rinsing Agent	PBS	-	++	NR
	Water	+	++	NR
Incubation vessel	25ml conical flask CNS-J in LB (5 beads in 10ml)	+	+	5.42±0.26 n=16
	48 well plate CNS-J in LB (1 bead in 600μl)	++	+	6.12±0.37 n=37

Table 3.2: Summary of bead characteristics dependent on incubation conditions.

LD – Mean Log_{10} Density = Log_{10} CFU, +/- Standard Deviation, ++ Favourable, + Workable, - Unfavourable, NR - Not Recorded, n - number of replicates

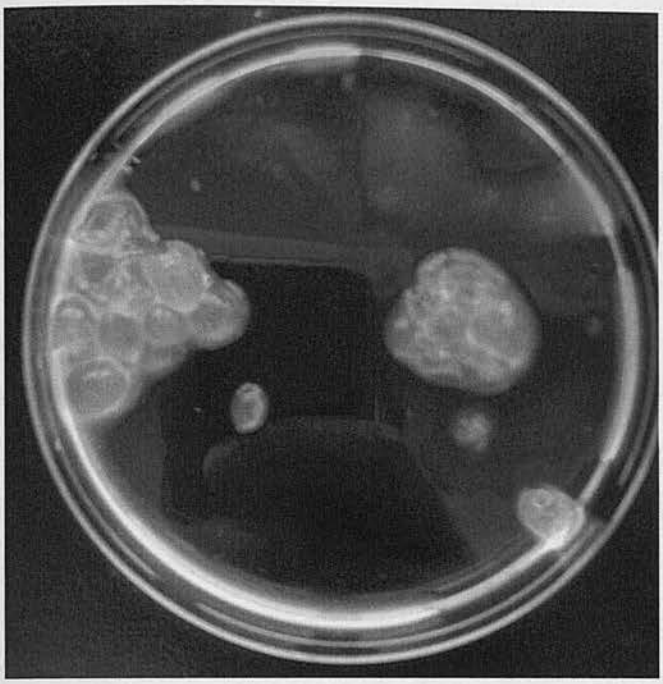

Figure 3.4: Appearance of beads after agitation at 200 rpm for 20 hours

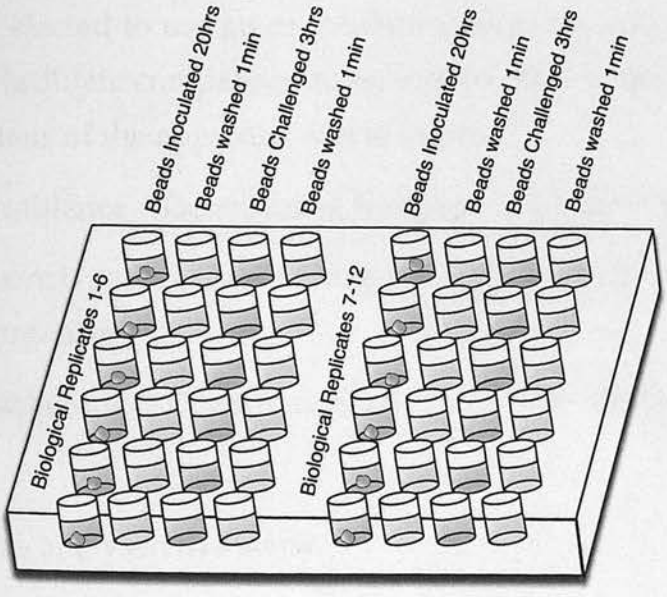

Figure 3.5: Schematic of 48 well plate used in alginate biofilm model.

Two growth controls and 11 biological replicates per plate. Only one growth control shown for simplicity but there was normally a second control in well C1 (wells A-H are from left to right).

3.2.9. Assessment of the alginate biofilm model

The method is fully detailed in section 2.3 but briefly a 4% (w/v) sodium alginate solution was chelated with 2M calcium chloride using a round bottom, 96 well plate to mould bullet shaped beads with a surface area of 161mm^2. Each bead were then removed and incubated in 600μl of LB, inoculated with 10^7 CFU/ml^{-1} of either MSSA-N or CNS-J for 20 hours in a single well of a 48 well micro-titre plate. The beads were washed individually with water to remove non-adherent bacteria, growth controls were sampled before the remaining beads were placed in fresh LB (600μl) containing antibiotics. After 3 hours of antibiotic challenge, the beads were removed, washed with water and dissolved in a solution containing 2mls of 0.02M citric acid and 0.05M Na_2CO_3 , supplemented with a universal neutralizer[249] detailed in section 2.1.4. Viable organisms were enumerated after micro-dilution and Miles Misra plating onto LB agar.

3.2.10. Method of SOP assessment

The author elected to use an established system for analysing biofilm data[250] in order to facilitate comparison to reports by other authors more easily. The 3 central tenets of the approach was to assess:

i. Resemblance - Determining how repeatable the control data was.

ii. Responsiveness - Determining how effectively the antimicrobial killed the organism.

iii. Repeatability - Determining how repeatable the disinfections were.

The data was analysed in 4 steps

i. Log_{10} transform CFU/bead into LD before calculating their descriptive statistics

ii. Perform a fully nested ANOVA of all controls to calculate resemblance. Satisfactory if SD < 0.6 [251]. This also describes the proportion of error that is attributable to either intra- or inter-

experimental factors.

iii. Perform a one-sample Students t-test against a hypothesised mean of 0 to calculate responsiveness. Satisfactory if $p < 0.05$

iv. Use the SD of LR in a one-sample t-test to assess repeatability. Satisfactory if SD < 0.7 [252]

3.2.11. Resemblance of control data

Unchallenged beads that had been inoculated for 20 hours were dissolved and the organisms recovered as detailed above. Data from all 38 experiments that used the CNS-J and 43 experiments that used the MSSA-N reference strain were included. The ANOVA was nested, so that each biological replicate is found in combination with only one value of the higher-level nominal experiment.

Figure 3.6 shows the distribution of 67 CNS-J controls measured over 38 experiments. The mean LD was 6.08 with a SD of nested ANOVA of 0.34. 72% of the variance was attributed to be intra-experimental and 28% of variance inter-experimental.

Figure 3.7 shows all 78 controls from the 41 experiments using MSSA-N. The 2 outliers (circled data points) were more than 3 SD from the mean LD of 6.98. If they were excluded, the SD fell from 0.53 to 0.35. 49% of the variance was attributed to be intra-experimental and 51% of variance inter-experimental. This level of error falls within guidelines that indicate an acceptable resemblance[251] and confirms that the growth control data for both organisms is repeatable in the alginate biofilm model.

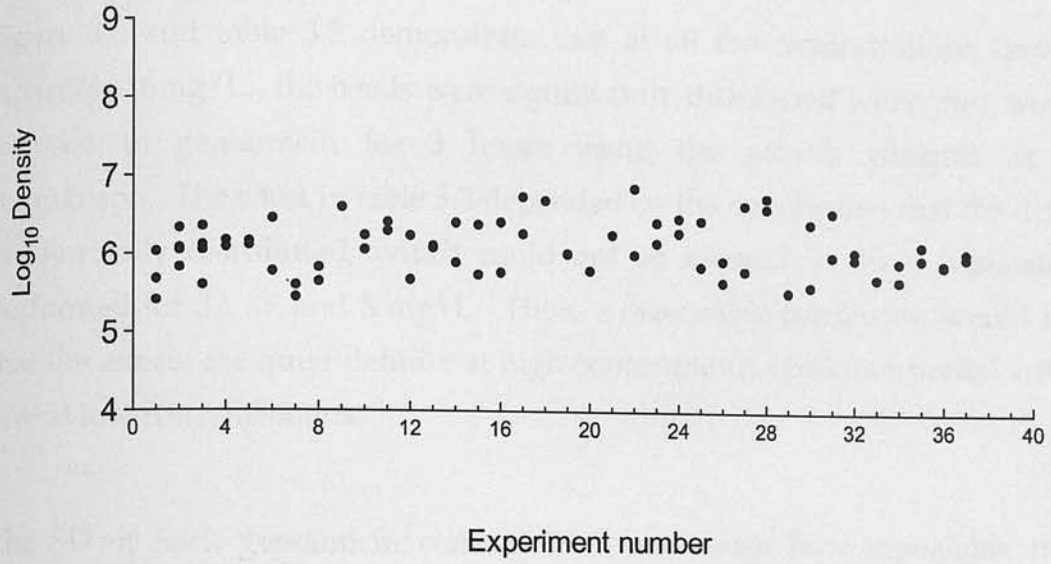

Figure 3.6: Resemblance of CNS-J growth controls

67 growth controls from 38 experiments using the SOP to test CNS-J

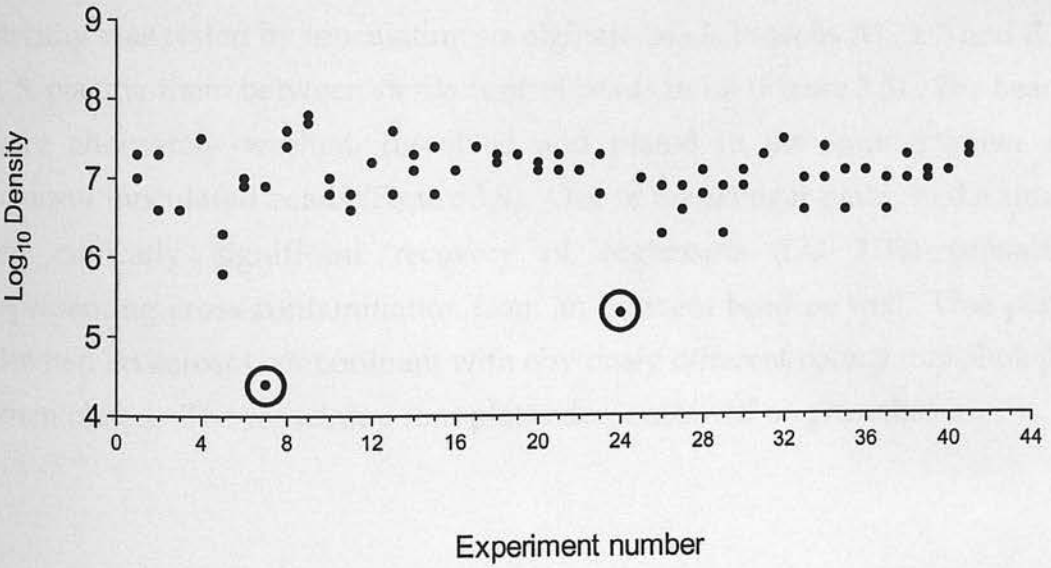

Figure 3.7: Resemblance of MSSA-N growth controls

78 growth controls from 41 experiments using the SOP to test MSSA-N

2 outliers more than 3 SD from mean are circled

3.2.12. Responsiveness and repeatability of antibiosis

Figure 3.8 and table 3.3 demonstrate that at all the concentrations tested (apart for 16mg/L), the beads were significantly disinfected when they were exposed to gentamicin for 3 hours using the growth controls as a comparison. The t-test in table 3.3 depended on the assumption that the data are normally distributed, which could not be assured in the 3 replicates performed for 32, 16 and 8 mg\L. Thus, a reasonable conclusion would be that the effects are quite definite at high concentrations but interpreted with care at low concentrations.

The SD of each gentamicin concentration represents how repeatable the antibiosis was between biological replicates. Guidelines suggested a satisfactory SD would be < 0.7 [252]. All concentrations except 256 and 128mg/l produced repeatable $\log_{10}$ reductions using this definition (table 3.3). These concentrations are important however, as they were around the levels that produced a $\log_{10}$ reduction of 3 that defined a study end-point, MBEC.

3.2.13. Sterility of technique

Sterility was tested by inoculating six alginate beads in wells A1, 3, 5 and E 1, 3, 5, placing them between sterile control beads in LB (Figure 3.5). The beads were alternately washed, dissolved and plated in the same manner as adjacent inoculated beads (Figure 3.9). One of the six agar plates had a small but clinically significant recovery of organisms (LD 3.08) probably representing cross-contamination from an adjacent bead or well. One plate also had an aerosol contaminant with obviously different colony morphology when plated. The remaining four plates demonstrated no growth.

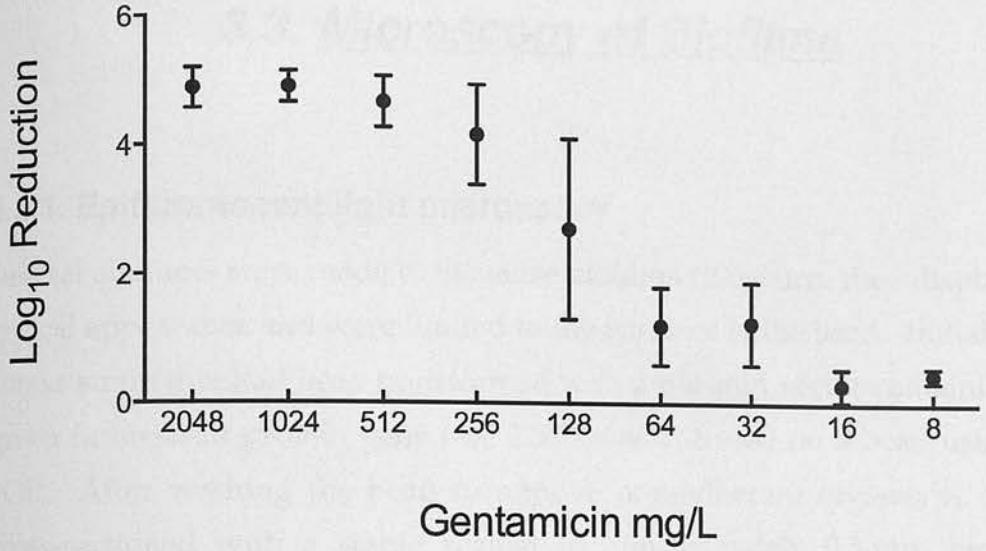

Figure 3.8: Responsiveness and repeatability of disinfection.

MSSA-N exposed to gentamicin for 3 hours.

Gentamicin concentration mg\L	t-test treated vs. control	SD
2048	p<0.001	0.31
1024	p<0.001	0.24
512	p<0.001	0.39
256	p<0.001	0.78
128	p<0.001	1.41
64	p=0.002	0.61
32	p=0.039	0.65
16	p=0.094	0.26
8	p=0.010	0.12

Table 3.3: One sample t test between disinfected and control samples.

MSSA-N exposed to gentamicin for 3 hours.

3.3. Microscopy of Biofilms

3.3.1. Epifluorescent light microscopy

Several attempts were made to visualise biofilms to confirm they displayed a typical appearance and were limited to the surfaces of the bead. Initially a *S. aureus* strain that had been transformed with a plasmid vector containing the green fluorescent protein gene (see 2.3.1) was cultured on a bead using the SOP. After washing the bead to remove non-adherent organisms, it was cross-sectioned with a sterile scalpel to approximately 0.5mm thickness. Slices were viewed using a light microscope with an epifluorescent function (Figure 3.10).

3.3.2. Cryo-scanning electron microscopy images

Attempts at both confocal and transmission electron microscopy were unsuccessful (data not shown) as the alginate could not be adequately fixed to enable thin enough sections to permit light transmission through the samples. This led to attempts to try scanning electron microscopy (SEM); this was avoided initially as it involves vacuum desiccation and can disrupt the micro-architecture of the biofilm[253]. A cryo-SEM technique was chosen which minimised such artefacts and also allowed the beads to be fractured without contaminating the interior surface (Figures 3.11-3.14)

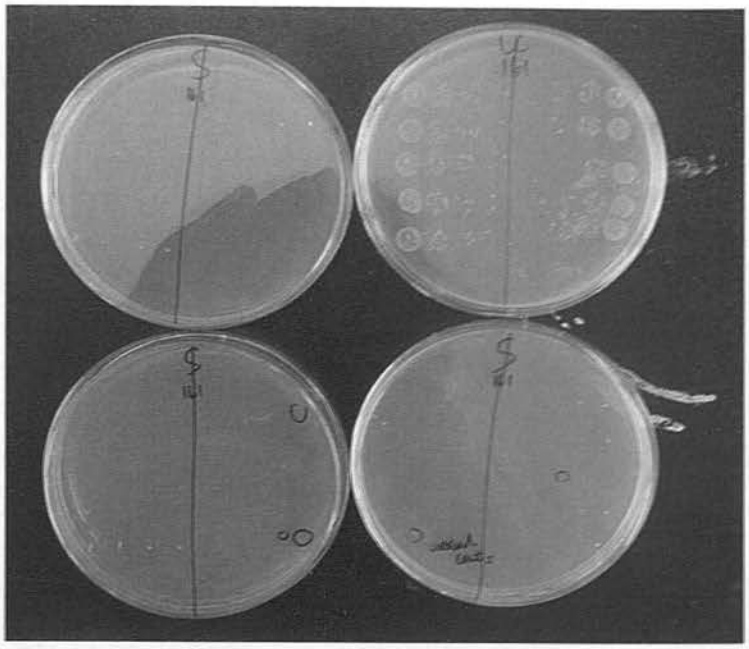

Figure 3.9: Experiment to test sterility of SOP.

The top right plate shows the 2 growth controls for this experiment.

One of the six sterile half plates (right hand side of bottom left plate) had a small but clinically significant recovery of organisms (LD 3.08) probably representing cross-contamination from an adjacent bead or well. One plate also had an aerosol contaminant (left hand of bottom right plate) with obviously different colony morphology when plated. The remaining four plates demonstrated no growth.

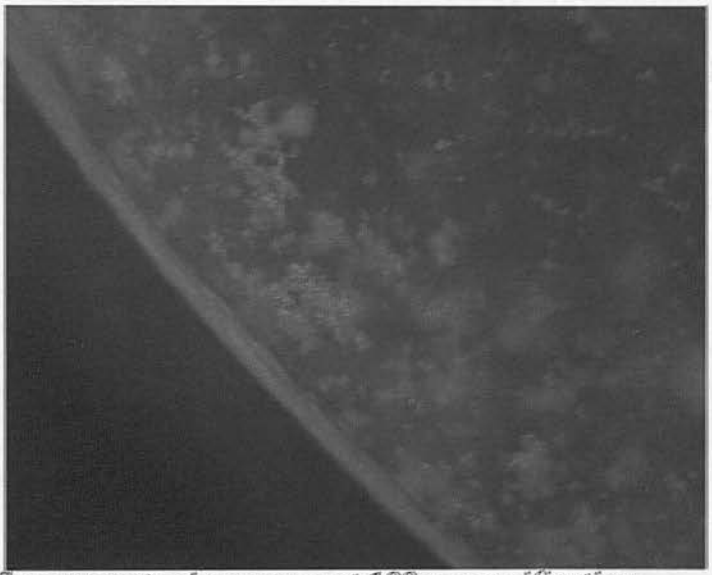

Figure 3.10: Epifluorescent microscopy at 100x magnification

The image hints that the adherent organisms are located on the beads surface. It is not sufficiently detailed however, to demonstrate they are within a biofilm or to exclude subsurface organisms that might be subject to unique nutrient and antimicrobial gradients. The possibility of cross-contamination during sectioning also must be considered.

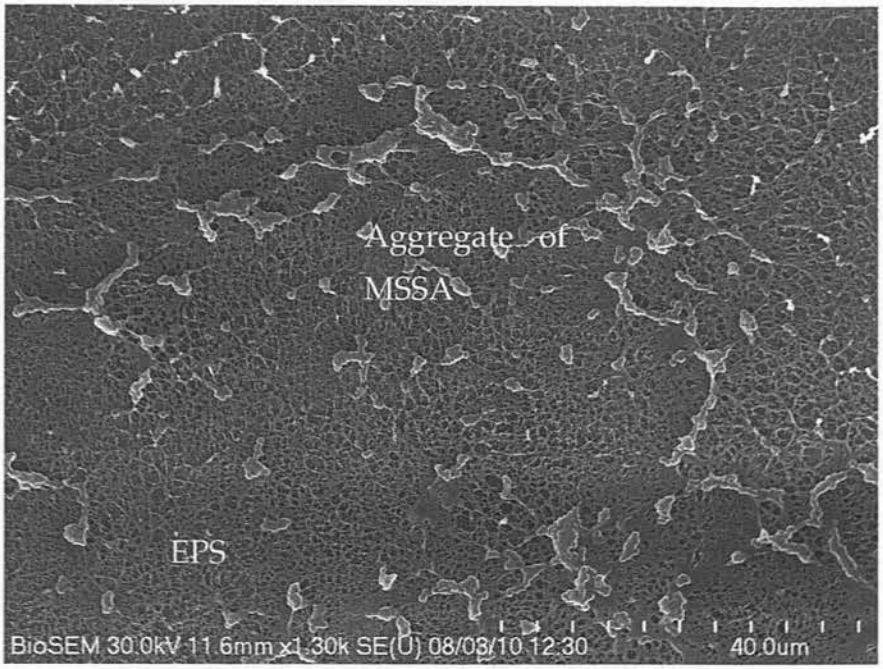

Figure 3.11: A cryo-SEM image taken perpendicular to the bead showing the alginate in the background with the Extracellular Polymeric Substance (EPS), secreted by and encasing the colonies of *S. aureus*.

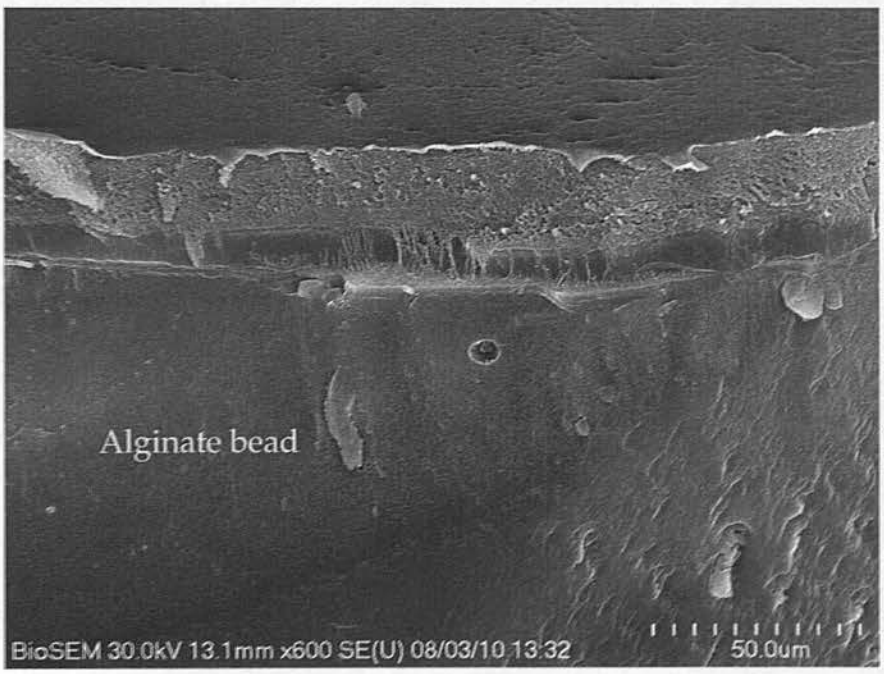

Figure 3.12: A cross section produced by fracturing a frozen bead. A thin layer of an ice artefact can be seen on the top surface, with what perhaps is colony of *S. aureus* standing proud. The EPS appears to be 15-20 um thick and no organisms are seen below this depth within the alginate.

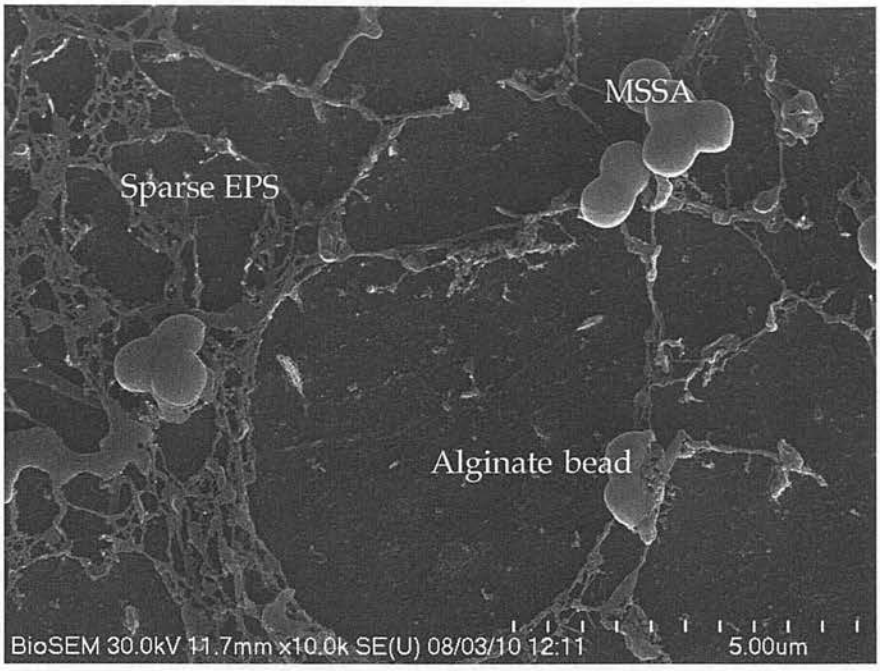

Figure 3.13: A cryo-SEM image taken perpendicular to an area of the bead where the extracellular polymeric substance (EPS), secreted by and encasing the colonies of *S. aureus* are sparse. The alginate bead substratum is clearly seen in the background.

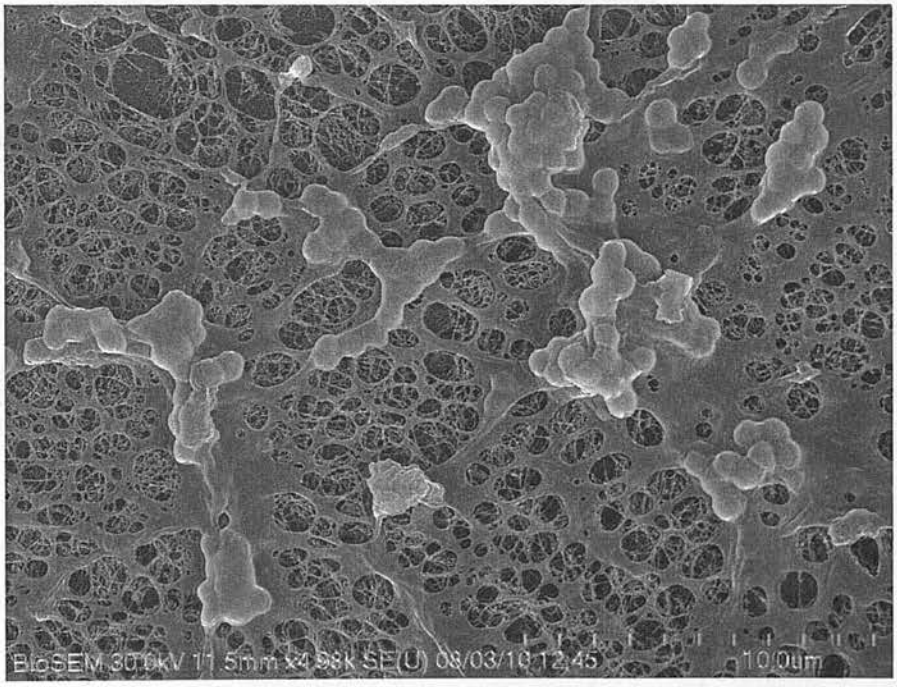

Figure 3.14: An 3 dimensional anaglyph using red and cyan encoding of a cryo-SEM stereogram taken perpendicular to the bead showing the alginate in the background with the extracellular polymeric substance (EPS), secreted by and encasing the colonies of *S. aureus*. **Please use the glasses provided to view.**

3.4. Discussion

3.4.1. Planktonic growth cultures

Typical growth curves were observed for the clinical strains CNS-J and MSSA-F with the latter being shown to have a generation time of 34 minutes (Figure 3.1). As the generation time falls within the normal 30-52 minute range seen in the *Staphylococcus aureus*[254] this suggests that the strain is not a stable small colony variant (SCV), which typically replicate much slower due to a defective electron transfer chain[255].

After a 90 minutes lag phase, both organisms entered a more prolonged exponential phase in TSB than the other media (Fig 3.1). This is explained by its higher glucose concentration, a preferred carbon source for *S. aureus*, allowing glycolysis to continue for longer[256,257] and resulting in a higher final OD. The exponential phase is known to tail off when either the concentration of glucose falls or acetic acid, the by-product of aerobic glycolysis, rises. Therefore, any post exponential growth involves activation of the Tricarboxylic Acid (TCA) cycle[258,259], which facilitates catabolism of non-preferred carbon sources and enables oxidative phosphorolation using the Electron Transfer Chain (ETC).

At the point that one would have expected the rate of cell death to increase, the OD did not decline in most of the groups (Figure 3.1). However there were 2 groups (CNS-J in LB and MSSA-F in SLB) that demonstrated unexpectedly rapid declines in their OD with time. This reduction in turbidity cannot easily be explained biologically and the most likely explanations are bacterial clumping or misalignment of the internal geometry of the spectrophotometer (Fig 3.15) causing false optical density measurements as light is scattered by the sample. The aberrant data were not critical to the aims of these experiments and so were not repeated.

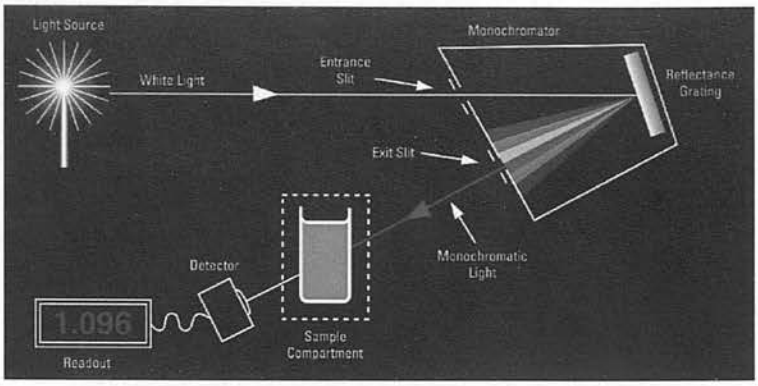

Figure 3.15: Internal geometry of spectrophotmeter.

Misalignment may cause aberrant optical density measurement as light is scattered by the sample. Reproduced from[260].

3.4.2. Sessile growth curves

The addition of more NaCl into the LB (SLB) was investigated, as previous studies[261,262] found both MSSA and CNS isolates were more likely to form biofilms in media supplemented with NaCl. This was reported to be achieved primarily by *icaADBC* operon dependent up-regulation of PIA after the additional osmotic stress (see section 1.3.2). In the current study however, SLB was not found to result in an increase in the number of CFU recovered from either the planktonic or sessile cultures (Figures 3.1 and 3.2) as well as having a detrimental effect upon the robustness of the beads.

Figure 3.3 demonstrated that sessile LD was maximal at 48 hrs. As the experiment used a closed system and planktonic growth was also occurring in the broth, this post-exponential growth is likely to have been TCA dependent. This view is supported by comparing the data in figures 3.1 and 3.2 that show that the differences between media are less pronounced with sessile compared to planktonic growth. The planktonic organisms are expected to use the glycolytic pathway favored by the TSB whilst the sessile organisms are able to catabolise the non-preferred carbon sources that are plentiful in all three media, using their TCA cycle.

3.4.3. Modification to the Standard Operating Protocol

The many alterations in the methods slowly improved both the bead robustness and recovery of more sessile organisms. Utilising the 48 well micro-titre plate as the incubation vessel provided the greatest single improvement. This reduced reagent cost, increased productivity and exposed the investigator to less biohazard when handing the samples.

Both PBS and TSB seemed to be detrimental to the bead's integrity. Sodium alginate is a naturally occurring polysaccharide extracted from seaweed and is a copolymer of α-L-guluronic acid (G) and 1,4-linked β-D-mannuronic acid (M). Divalent calcium ions are proposed to form 'egg box junctions' between the homopolymeric GG blocks, with the alginate chelating the calcium into an alginate gel.

TSB contains dipotassium phosphate and the competing charged function of the phosphate ions, also present in PBS, appeared to strip the bead of its calcium and mechanical integrity. This effect has been seen to increase with temperature and pH > 5.5[263,264]. Further work aiming to extend the bead's endurance in a variety of growth conditions may be achieved by replacing the chelator calcium, with another biocompatible divalent cation which has a higher affinity for alginate, such as strontium or barium[265].

3.4.4. Assessment of the Alginate Biofilm Model

The SOP was found to have satisfactory resemblance of controls (Figure 3.6 and 3.7)[251,266,267] and responsiveness at higher gentamicin levels but repeatability of antibiosis[252] was less satisfactory around the critical levels used to define the MBEC (Figure 3.8 and table 3.3). This trend of has also been seen by other authors who used a submerged substratum method[224,229,246]. This widening of the SD around the MBEC has also been observed in studies that have used a time kill method[230,268,269] rather than the 3 hour time interval employed in this study, which may bias toward antibiotics that kill in a more concentration-dependent manner.

3.4.5. Sterility

The SOP was tested in a rigorous fashion and even though there was evidence of cross-contamination in one of the six replicates (Figure 3.9), it would not have altered the statistical interpretation of the result, as it fell well below the level sufficient to alter the interpretation of the MBEC end-point.

3.4.6. Microscopy of Biofilm

Despite having some surface ice artefact, the cryo-scanning electron microscopy technique, appeared to preserve the EPS and biofilm structure very well. The ability to fracture the frozen samples was useful and demonstrated that the biofilm appeared to be exclusively localised to the beads surface, thus preventing heterogeneous exposures to nutrients and antimicrobials. The findings were consistent with previous models that found 10% of the silastic substratum was colonised with *S. aureus* biofilm 5-6 cells thick within 2 hours of exposure[270].

3.4.7. Conclusion

Returning to the original aims of the chapter, this alginate model allowed testing of staphylococcal biofilms exposed to single or multiple antibiotics at high doses achievable with local delivery. It resulted in repeatable control data and reliably demonstrated what proportions of the organisms were killed, in terms of reduced colony forming ability. Some concern remains about the repeatability of the response to the level of antibiotics that reduce the log density by around 3 (i.e. eradicate 99.9%). However, no other biofilm assay has shown to have more repeatable data in this zone. Tests of robustness (ability to be accurate despite sub-optimal technique) and inter-laboratory repeatability have not been tested yet.

Although the alginate biofilm model is inexpensive in terms of materials, it only lends itself to a moderate throughput (with 33 samples possible per day). Although this could be increased to perhaps 55 if the technician had access to pre-sterilised consumables and media, it still falls well below the level that would make it cost effective overall in a clinical setting. If further modifications to the alginate gel make it more mechanically stable then perhaps it could be used as a dissolvable coating to cover the 96-pin micro-titre lid. Such an approach would benefit from high throughput and enhanced recovery of viable organisms. This is certainly worth pursuing, as the only similar validated technique, the MBEC test[224], is expensive, complicated, and lacks robustness .

This method did go some way to bridging the gap between in-vitro and in-vivo conditions which merits further development.

4. Combining antibiotics to treat prosthetic related infection

4.1. Introduction

In 1970 Buchholz and Engelbrecht[167] first described using bone cement as a means to deliver high levels of antibiotic locally around orthopaedic implants. There is high quality evidence to show that antibiotic loaded bone cement does significantly reduce the clinical infection rate after primary hip and knee replacement [28,52,271]. In cases where Prosthetic Joint Infection (PJI) has developed and local antibiotics have been used as part of the treatment, expert opinion and single centre series have demonstrated between 75-91% clinical success with many diverse protocols [40,63].

Meta-analyses of these trials have concluded that the patient populations and treatment algorithms were too heterogeneous to draw any clinically useful inferences about the antibiotic ideal regime from the available data[136,166]. In lieu good quality clinical evidence, inferences about the optimal antibiotic dose or combination for therapy must be made with in-vitro experiments and with animal models before a properly controlled trial, comparing the most promising ones is commissioned.

4.1.1. Current surgical practice

Surgeons attempt to identify the infecting organisms prior to reoperation and optimise, on the basis the antibiogram, the number and class of antibiotics chosen for treatment [272]. Commercially available Antibiotic-Loaded Acrylic bone Cements (ALAC's) that contain gentamicin, with either clindamycin (Refobacin Revision® or Copal®G+C) or vancomycin (Vancogenx®[273,274]), are commonly used. Surgeons occasionally hand mix a third antibiotic, if it has shown to be sufficiently heat stable and to elute well after the exothermic polymerization of the cement (examples include linezolid[204], daptomycin[275,276] and ciprofloxacin[193]). As well as the local antibiotics and surgical

debridement, intravenous antibiotics are commonly administered for between 1 and 6 weeks post-operatively and converted to oral equivalents on discharge, although there appears to be no evidence to suggest there is a relationship between the length of the post-operative course and the final outcome[277,278]. There is also a paucity of evidence on whether local delivery of a single agent or a combination is superior. Saginur [279] used the Calgary biofilm device to test a number of *Staphylococcal* spp. and found their biofilms were not significantly affected by combinations of up to 3 of the following; linezolid, rifampin, cefazolin, oxacillin, vancomycin, gentamicin, azithromycin, ciprofloxacin or fusidic acid, in concentration ranges up to 128mg/L. A recent review examining the choice and dose of antibiotics in ALACs came to the conclusion that "current recommendations are based on expert opinions and in-vitro studies, rather than on the results of randomized controlled clinical trials" [166]

4.1.2. Rationale for antibiotic choice and concentrations tested

The antibiotics examined in this study reflect the most common and effective in current clinical practice[28,161,202,280]. The concentration at which they are eluted from the cement varies significantly and depends on a number of factors. These include: cement porosity[281]; how hydrophilic the PMMA structure is; the volume of fluid the antibiotics are eluted into[176,178]; the surface area of the cement; the assay used to measure elution; the physical state and chemical structure of the antibiotic[168].

In the current study, insufficient resources were available to perform an exhaustive checkerboard method that varied the antibiotic concentrations within each combination so the concentrations studied had to be rationalized after review of relevant in-vitro and in-vivo studies. In-vitro studies invariably found peak antibiotic concentrations on day one, with more than 2/3 of gentamicin being released in the first hour[174] (Figure 4.1). Concentrations of antibiotics eluted locally were reported as being between 75-426mg/L for gentamicin[176,179,276,282], ciprofloxacin 54-75mg/L[193,202],

clindamycin 407mg/L[202], linezolid 36-64mg/L[193,204],vancomycin 140-200 mg/L[273,283]. Hendriks *et al.*[284]demonstrated however, the possibility of much higher levels of gentamicin; up to 4000 mg/L if was sampled from the 100μm gaps found in the interfaces between the cement and the bone. Such interfacial gaps were seen around 10-15% of the cement mantle circumference with micro-CT [177].

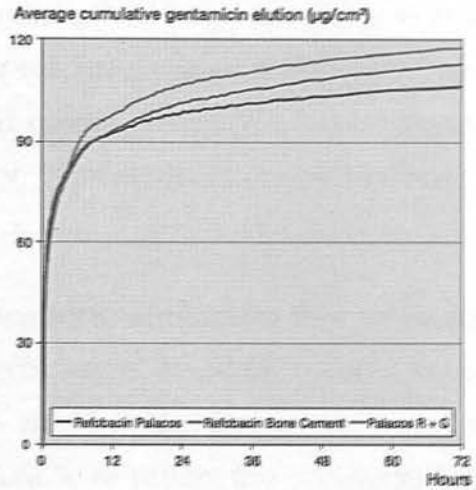

Figure 4.1: Elution profile from 3 commercially available ALAC's over time.

Gentamicin was eluted into PBS and was measured using an fluorescence polarization immunoassay technique over 6 time points[174].

In-vivo studies such as by Hoff *et al.*[179] filled the medullary canals of canine femora with gentamicin loaded cement (2.5% wt/wt) and found the concentration gradient was highest at the endosteal surface with 140 μg/g of bone, compared to 40 μg/g measured at the periosteal surface. Peri-articular seroma has been recorded to contain 20 – 37 mg\L vancomycin (5%wt/wt) and 21-116 mg/L gentamicin (1.25 wt/wt) when recovered via wound drains in first 2 days[196,285]. Fink *et al.*[205]analyzed peri-articular soft tissues around an ALAC spacer containing gentamicin, clindamicin and vancomycin that had been *in situ* for six weeks and found levels of 19, 62 and 54 μg/g respectively.

Rifampacin dramatically alters the mechanical properties of bone cement[193] and is very bio-available orally. Therefore it is typically only used as systemic chemotherapy, rather than being delivered locally, which results in levels of around 4mg/L[286]. Although daptomycin can be successfully eluted from bone cement[276] clinicians have been cautious to incorporate it as even low concentrations can result in myopathy[287] and only a single case has been published to reassure surgeons about its side effect profile[203] after local delivery have been published to date. If used systemically, daptomycin has been shown to have a peak serum level of 33mg/L[288] and a peak bone level of 4.7 mg/L[289] in a rabbit model. Therefore both rifampacin and daptomycin were tested around their typical serum concentrations.

When selecting combinations, antibiotics that were active against the same general bacterial targets were avoided. After rationalisation, a default concentration of 128 mg/L was chosen for the other five antibiotics commonly used in ALAC's to reflect the midrange between the high initial interfacial gap levels and the human in-vivo levels recorded from sites more distant from the cement such as seroma.

4.1.3. Aims

A series of experiments using the alginate model were designed to investigate:

i. Whether high levels of antibiotics, delivered locally and in combination, were effective against the CNS-J and MSSA-N biofilms.

ii. If there is a relationship between the minimum inhibitory, bactericidal, and biofilm eradication concentrations.

4.2. Results

4.2.1. The Minimum Biofilm Eradication Concentrations (MBEC)

To investigate whether a ***single*** antibiotic, delivered at a clinically relevant dose, would be effective at eradicating staphylococcal biofilm, a series of experiments were performed using seven antibiotics; gentamicin, vancomycin, daptomycin, clindamycin, ciprofloxacin, rifampacin and linezolid. Their effects were examined at concentrations between 4 and 2048mg/L with a minimum of 3 biological replicates. Daptomycin was supplemented with Ca^{2+} at a physiological level of 50mg/L as previous studies have shown this to result in an induced conformational change resulting in a more amphiphilic and bactericidal compound[287,290].

The SOP is described fully in the Methods (section 2.3) but in summary, bullet shaped sodium alginate beads were inoculated in 600 μl of LB containing 10^7 CFU/ml of either MSSA-N or CNS-J (section 2.1.1) and individually aerobically incubated for 20 hours in a 48-well micro-titre plate. Growth controls that were not exposed to antibiotic were sampled and enumerated. The remaining beads were gently washed with water to remove non-adherent bacteria and placed into 600μl fresh LB containing antibiotics. After 3 hours of antibiotic challenge, the beads were removed, washed with water and dissolved in a solution containing 2mls of 0.02M citric acid and 0.05M Na_2CO_3, supplemented with a universal neutraliser (section 2.1.3)[249]. Viable organisms were enumerated after micro-dilution and Miles Misra plating onto LB agar. CFU were $\log_{10}$ converted into Log Density (LD) per bead. The minimum concentration needed to reduce the viable cell count by 3 LD's, compared to untreated controls, was defined as the MBEC (Figures 4.2 and 4.3). Since gentamicin is the most commonly utilised local antibiotic in clinical practice[28], the other antibiotics MBECs' were compared against it using the Mann-Whitney U-test (Table 4.1).

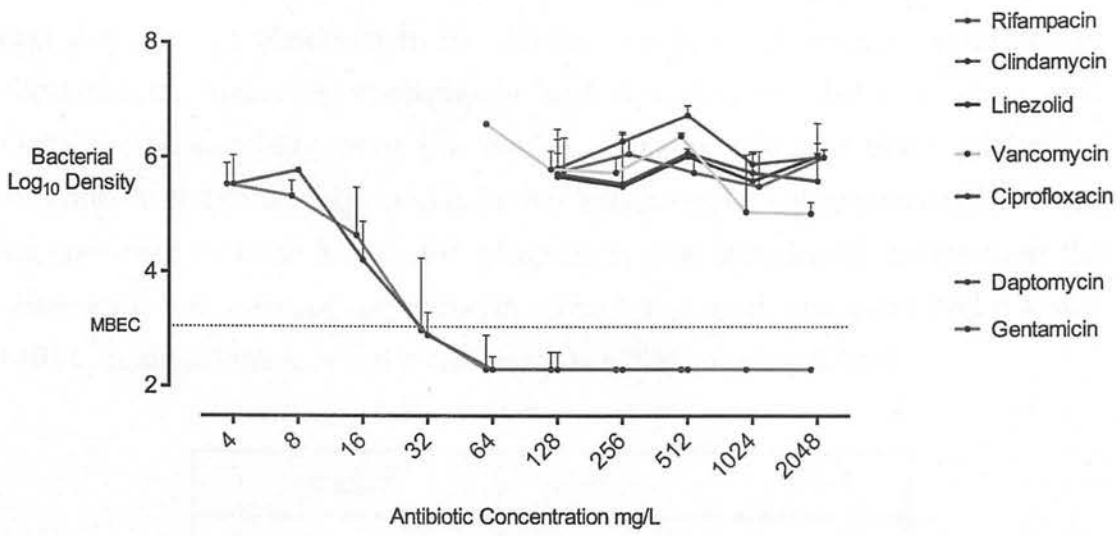

Figure 4.2: Median log density (fitted circles) & inter-quartile ranges (bars) of CNS-J after exposure to single antibiotics. MBEC represents a log reduction of 3 from growth controls.

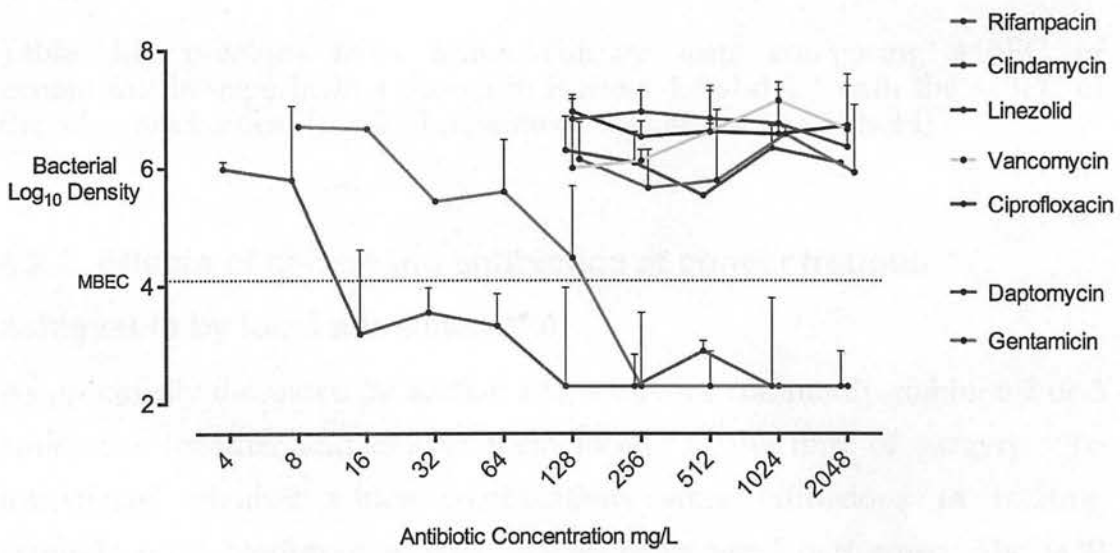

Figure 4.3: Median log density (fitted circles) & inter-quartile ranges (bars) of MSSA-N after exposure to single antibiotics. MBEC represents a log reduction of 3 from growth controls.

Both figures 4.2 and 4.3 demonstrated the same trend with only gentamicin and daptomycin observed to be effective bactericidal agents. Rifampacin, clindamycin, linezolid, vancomycin and ciprofloxacin did not reduce cell viability substantially over the 3-hour exposure despite being orders of magnitude above the expected minimal bacteriocidal concentration [156]. Table 4.1 confirms that the MBEC for gentamicin was statistically lower than the other antibiotics except daptomycin. The latter antibiotic exhibited a lower MBEC against MSSA-N but a comparable MBEC against CNS-J.

Antibiotic	MSSA-N	CNS- J
Vancomycin	**0.004**	**0.006**
Ciprofloxacin	**0.010**	**0.013**
Clindamycin	**0.004**	**0.006**
Rifampacin	**0.001**	**0.013**
Linezolid	**0.010**	**0.013**
Daptomycin	**0.004**	0.34

Table 4.1: p-values from Mann-Whitney tests comparing MBEC of gentamicin in experiments shown in Figures 4.3 and 4.4 with the MBEC of the other antibiotics. ($p < 0.05$ considered significant and in bold).

4.2.2. Effects of combining antibiotics at concentrations achievable by local administration

As previously discussed (in section 1.8), surgeons commonly combine 2 or 3 antibiotics together and deliver them locally at the time of surgery. To investigate whether which combinations were efficacious in treating staphylococcal biofilm, a series of experiments were performed. The SOP was used to test the five antibiotics suitable for local delivery in combinations of two or three antibiotics. The final concentration of each antibiotic was 128mg/L in each well. Three biological replicates were performed for each combination and the recovered CFU/ml were converted $\log_{10}$ and graphed in Figures 4.4 & 4.5.

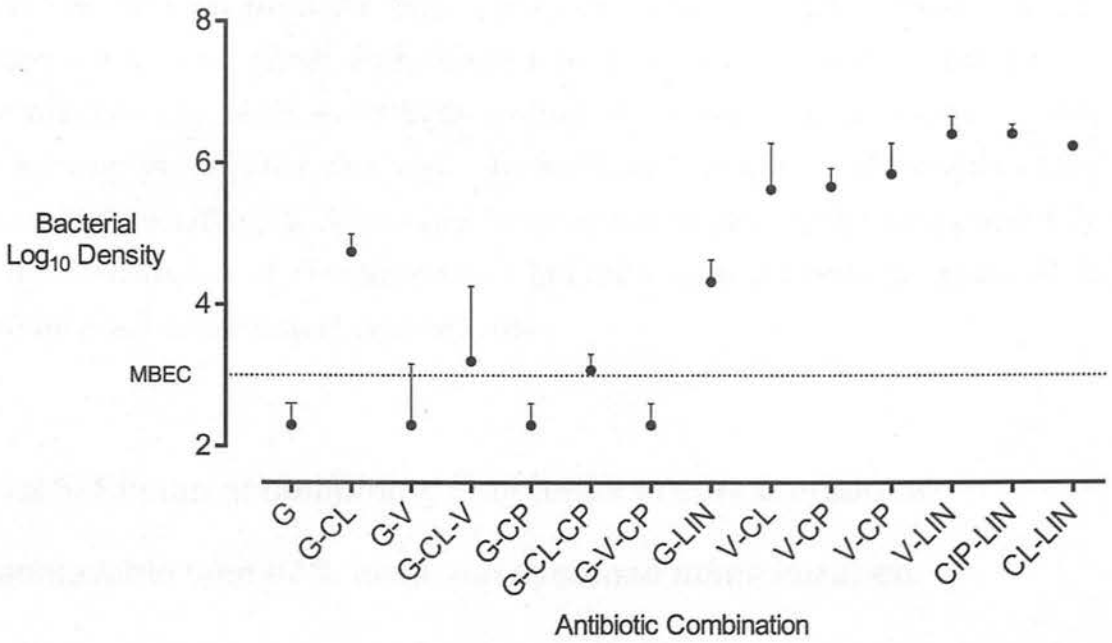

Figure 4.4: Median & inter-quartile ranges (bars) of CNS-J recovery after 3-hour exposure to a combination of 2 or 3 antibiotics at 128mg/L concentrations.
(G–Gentamicin, V-Vancomycin, CL- Clindamycin, CP-Ciprofloxacin, LIN-Linezolid)

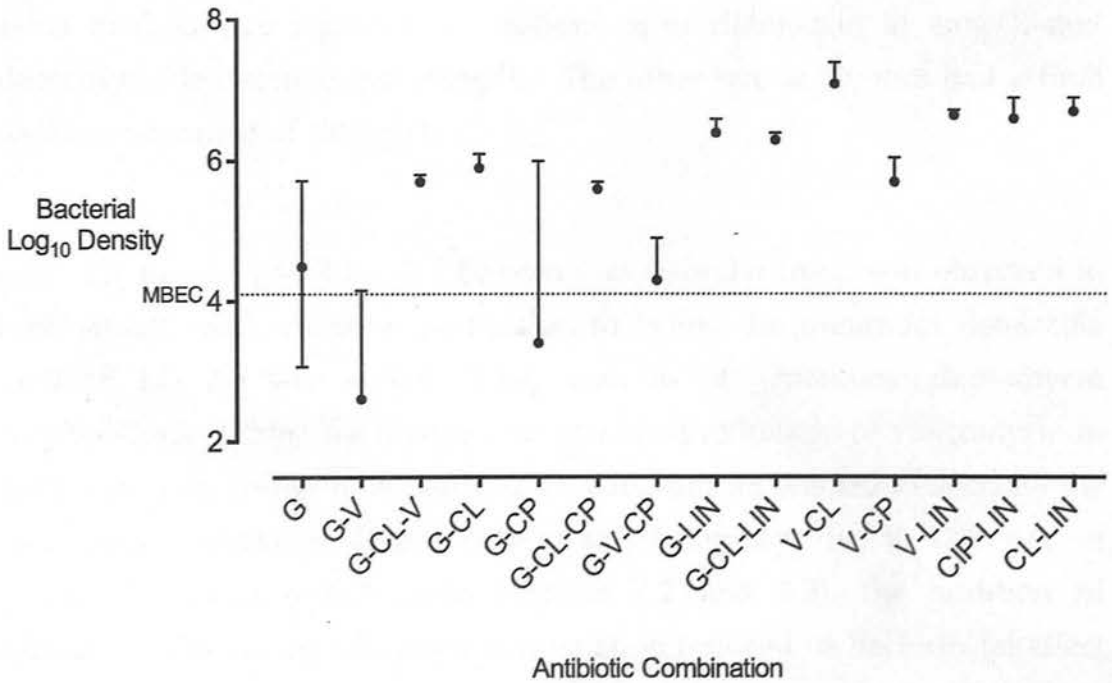

Figure 4.5: Median & inter-quartile ranges (bars) of MSSA-N recovery after 3-hour exposure to a combination of 2 or 3 antibiotics at 128mg/L concentrations.
(G–Gentamicin, V-Vancomycin, CL- Clindamycin, CP-Ciprofloxacin, LIN-Linezolid)

As can be seen from the data shown in Figs 4.4 & 4.5, combinations of gentamicin and either ciprofloxacin or vancomycin, each at 128 mg/L, eradicated the biofilms of both isolates but combining ciprofloxacin and vancomycin together did not. Interestingly, despite both strains being sensitive to 128mg/L of gentamicin when tested alone (Figures 4.2 and 4.3), the combination of clindamycin or linezolid with gentamicin appeared to reduce its bactericidal effect markedly.

4.2.3. Effects of combining antibiotics at concentrations achievable with both local and systemic administration.

As some antibiotics cannot be delivered locally because of practical or safety issues (section 1.6), they are administered systemically in addition to the antibiotics delivered locally. To investigate whether systemic doses of rifampacin or daptomycin had any effect if administered as part of a therapeutic combination, a further two experiments were undertaken. The same method was repeated in triplicate with rifampacin at 4mg/L and daptomycin between 4 and 16mg/L. The other five antibiotics had a final well concentration of 128mg/L.

From Figures 4.6 and 4.7 it can be seen that a similar trend was observed in both strains, with effective eradication to below the minimum detectable limit of LD 2.6 (see section 2.3.4) seen in of gentamicin/daptomycin combinations. Whilst the further addition of ciprofloxacin or vancomycin to gentamicin/daptomycin had little effect, clindamycin seemed to decrease the its efficacy. Similarly, despite both strains being sensitive to 128mg/L of gentamicin when tested alone (Figures 4.2 and 4.3), the addition of clindamycin or rifampacin to the combination reduced its bactericidal effect markedly.

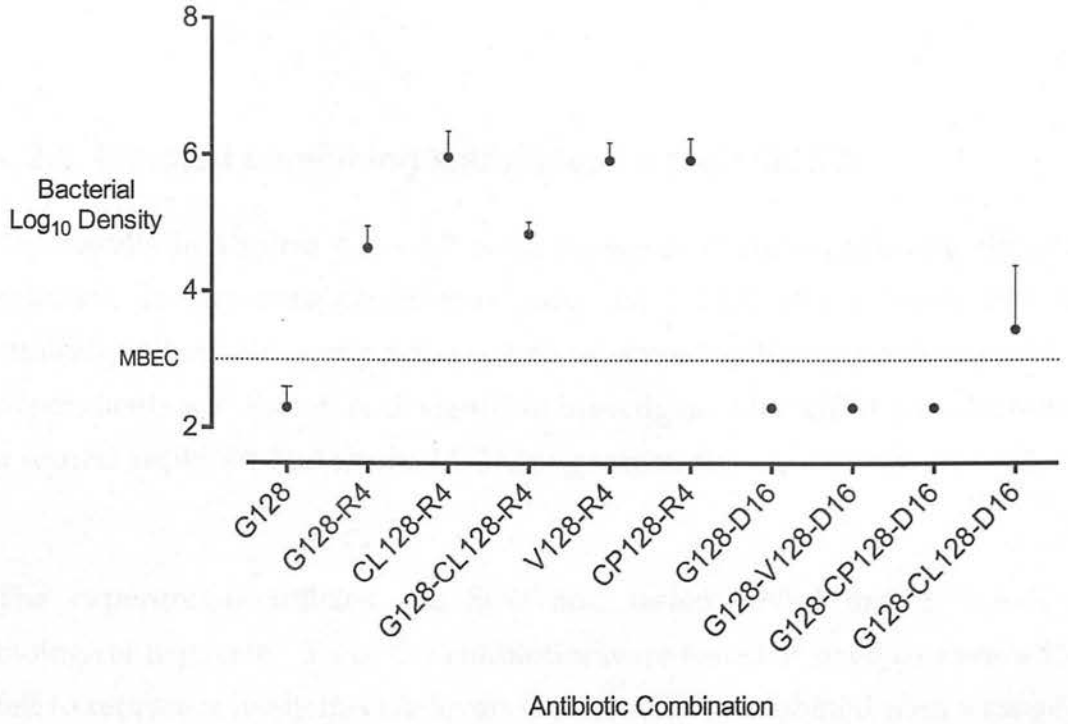

Figure 4.6: Median & inter-quartile range of CNS-J recovery after 3-hour exposure to a combination of 2 or 3 antibiotics at 128, 16 or 4mg/L concentrations. (G128 – 128mg/L Gentamicin, R4 – 4mg/L Rifampacn, D16 – Daptomycin 16mg/L, V-Vancomycin, CL- Clindamycin, CP-Ciprofloxacin, LIN-Linezolid)

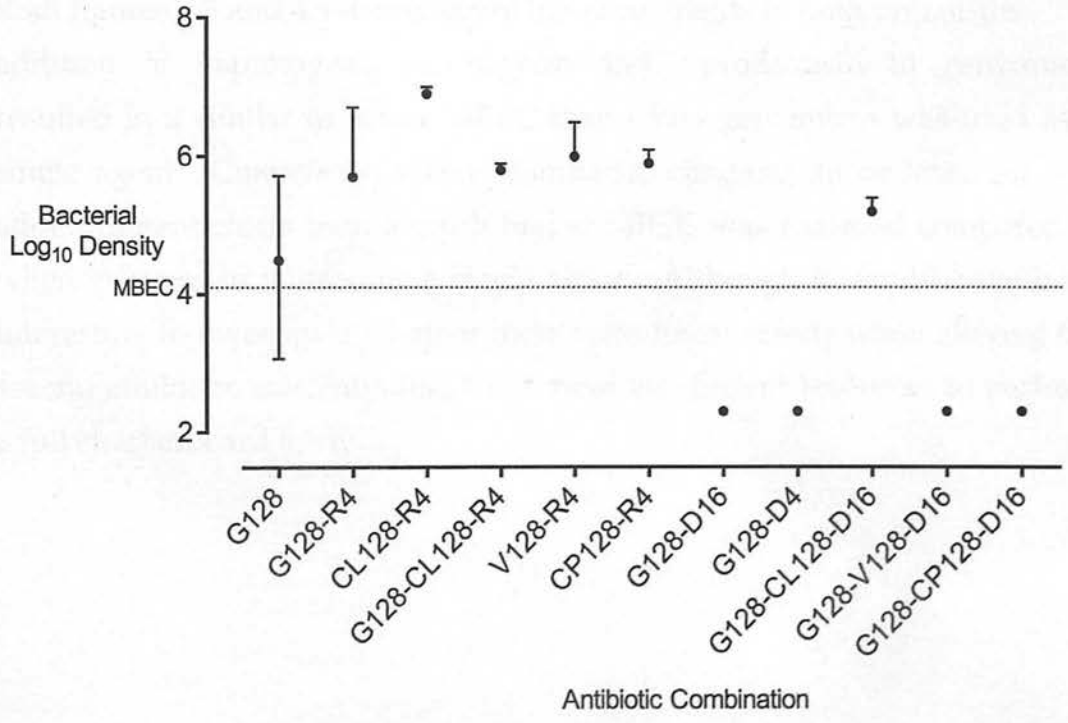

Figure 4.7: Median & inter-quartile range of MSSA-N recovery after 3-hour exposure to a combination of 2 or 3 antibiotics at 128, 16 or 4mg/L concentrations. (G128 – 128mg/L Gentamicin, R4 – 4mg/L Rifampacn, D16 – Daptomycin 16mg/L, V-Vancomycin, CL- Clindamycin, CP-Ciprofloxacin, LIN-Linezolid)

4.2.4. Effect of combining antibiotics on their MBEC

The results in Figures 4.4 – 4.7 were unexpected and potentially clinically relevant, as any antagonism may raise the MBEC above levels that are clinically attainable and predispose to treatment failure. Another series of experiments was therefore designed to investigate what effect the addition of a second antibiotic had on the MBEC of gentamicin.

The experiments utilized the SOP and tested CNS-J and MSSA-N in biological triplicate. Six of the antibiotics were tested at ***fixed*** concentrations felt to represent likely in-vivo levels (section 4.1.2), combined with a range of gentamicin concentrations that ***varied*** from 8-2048 mg/L.

Both figures 4.8 and 4.9 demonstrate the same trends in both organisms. The addition of daptomycin, vancomycin and ciprofloxacin to gentamicin resulted in a similar or lower MBEC than when gentamicin was used as a single agent. Conversely, when rifampacin, clindamycin or linezolid was added to gentamicin then a much higher MBEC was recorded compared to when gentamicin is used as a single agent. Although it would have been interesting to investigate whether there were linear effects when altering the second antibiotic concentration, there were insufficient resources to perform a full checkerboard assay.

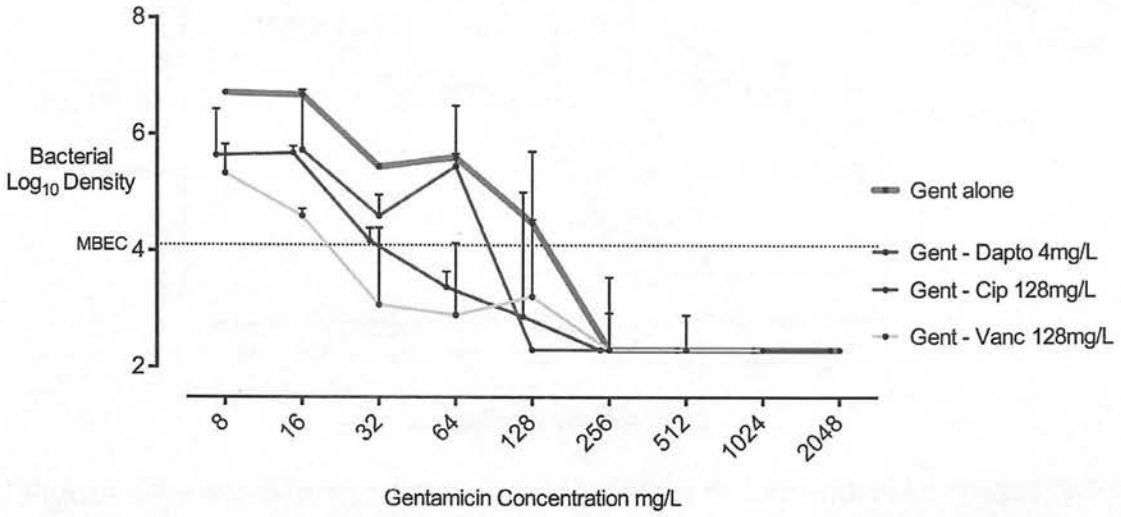

Figure 4.8a: Median log density (fitted circles) & inter-quartile ranges (bars) of MSSA-N recovery after 3-hour exposure to gentamicin alone or in combination with daptomycin, ciprofloxacin or vancomycin.

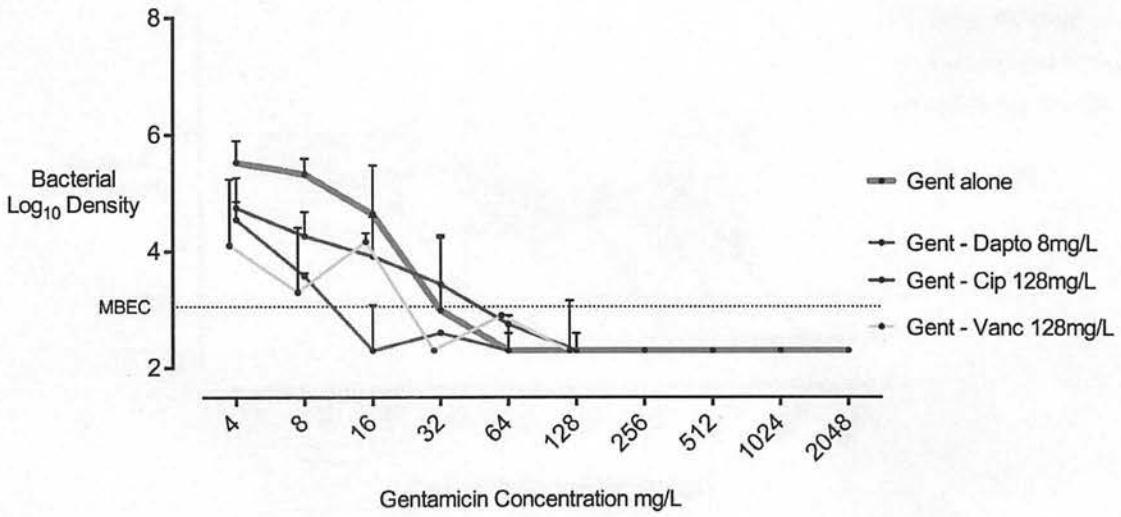

Figure 4.8b: Median log density (fitted circles) & inter-quartile ranges (bars) of CNS-J recovery after 3-hour exposure to gentamicin alone or in combination with daptomycin, ciprofloxacin or vancomycin.

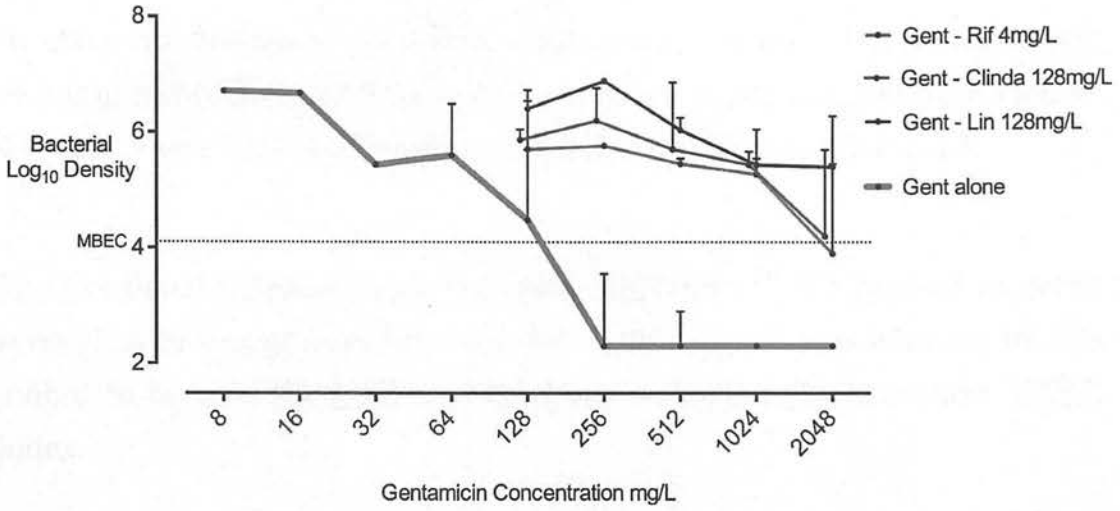

Figure 4.9a: Median log density (fitted circles) & inter-quartile ranges (bars) of MSSA-N recovery after 3-hour exposure to gentamicin alone or in combination with rifampacin, clindamycin or linezolid.

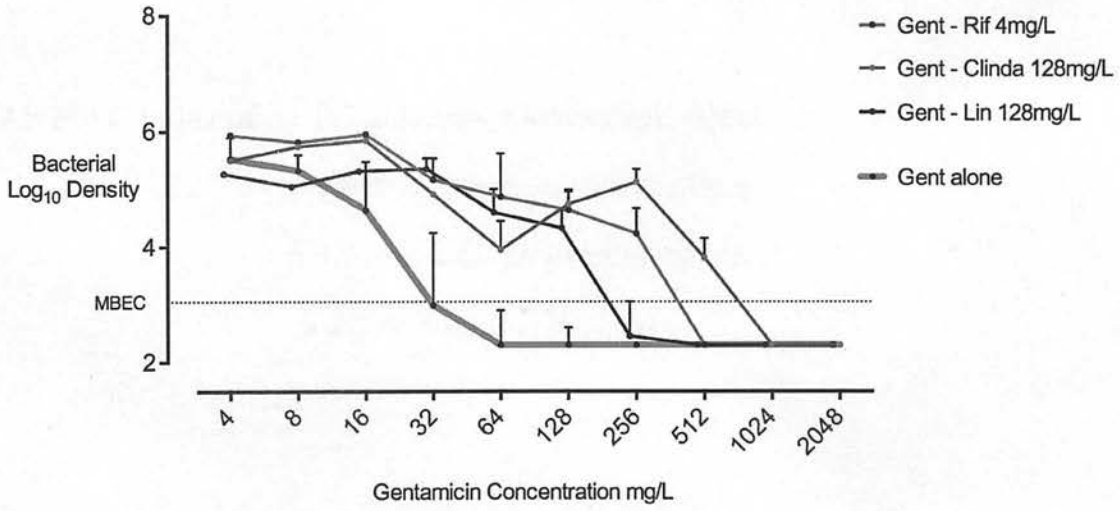

Figure 4.9b: Median log density (fitted circles) & inter-quartile ranges (bars) of CNS-J recovery after 3-hour exposure to gentamicin alone or in combination with rifampacin, clindamycin or linezolid.

4.2.5. The fractional inhibitory concentration index

In order to document the effect size of the apparent interactions seen, enabling comparisons with previous and future work, the data from Figures 4.8 and 4.9 was analysed together with data from Figures 4.2 and 4.3.

The Fractional Inhibitory Concentration (FIC) index[291] is a method to detect synergism or antagonism between two antibiotics. It was adapted by this author to become the Fractional Biofilm Eradication Concentration (FBEC) index.

FBEC = MBEC of antibiotic in combination / MBEC of antibiotic on its own

FBEC index = FBEC (x) + FBEC (g)

Where (x) is an antibiotic tested in combination with gentamicin (g).

An FBEC index of ≤ 0.5 indicates a synergistic effect

> 0.5 and ≤ 1.0 an additive effect

> 1.0 and ≤ 4.0 an indifferent effect

> 4.0 an antagonistic effect.

Antibiotic x	Vanc	Dapt	Clinda	Cipro	Rifampacin	Linezolid
MBEC antibiotic x in combination	128	4	128	128	4	128
MBEC antibiotic x in isolation	2048	16	2048	2048	2048	2048
FBEC x	0.0625	0.25	0.0625	0.0625	0.002	0.0625
Gentamicin MBEC in combination	32	128	2048	64	2048	2048
Gentamicin MBEC in isolation	256	256	256	256	256	256
FBEC g	0.125	0.5	8	0.25	8	8
FBEC Index	Synergistic	Additive	Antagonistic	Synergistic	Antagonistic	Antagonistic

Table 4.2: Fractional biofilm eradication concentration index (FBEC) for **MSSA-N.** Efficacy of combinations in relation to antibiotics tested alone (data from Figures 4.2, 4.8a and 4.9a).

Antibiotic x	Vanc	Dapt	Clinda	Cipro	Rifampacin	Linezolid
MBEC antibiotic x in combination	128	8	128	128	4	128
MBEC antibiotic x in isolation	2048	32	2048	2048	2048	2048
FBEC x	0.0625	0.25	0.0625	0.0625	0.002	0.0625
Gentamicin MBEC in combination	32	16	1024	64	512	256
Gentamicin MBEC in isolation	32	32	32	32	32	32
FBEC g	1	0.5	32	2	16	8
FBEC Index	Indifferent	Additive	Antagonistic	Indifferent	Antagonistic	Antagonistic

Table 4.3: Fractional biofilm eradication concentration index (FBEC) for **CNS-J.** Efficacy of combinations in relation to antibiotics tested alone (data from Figures 4.2, 4.8b and 4.9b).

4.2.6. Establishing minimum inhibitory and minimum bactericidal concentrations

The MIC is the lowest concentration of an antibiotic that will inhibit visible overnight growth of a bacterial culture. The MBC is the lowest antibiotic concentration that inhibits growth when the MIC sample is subsequently sub-cultured onto an antibiotic free substrate. The range is established by doubling up or down from 1mg\L.

The micro-dilution method of the British Society of Antibiotic Chemotherapy (BSAC)[156] was used to determine MIC and MBC for both the MSSA-N (reference ATCC 25923) and CNS-J strains of *Staphylococcus* spp. for 7 antibiotics: gentamicin; vancomycin; daptomycin with 50mg/L Ca^{2+}; clindamycin; ciprofloxacin; rifampacin and linezolid. (The supplementation of 50 mg/L of Ca^{2+} has been shown to increase in-vitro activity of daptomycin when tested in broth and is recommended[292]).

5ml LB overnight broths were cultured from cryo-preservative, plated onto LB agar and incubated overnight. Four morphologically similar colonies from either the MSSA-N or CNS-J strains were sampled from the LB agar plate with a sterile loop and sub-cultured in a 5ml bottle of LB overnight. Growth controls were micro-diluted and plated on LB agar using the Miles-Misra method[241]. Samples were prepared in quadruplicate by adding 75µl of inoculum to 75µl of LB containing antibiotic, in a range either side of the expected MIC [156], into each of the flat-bottomed 96 wells to establish a final suspension of 10^5 CFU/ml. The plates were taped shut, incubated statically in air at 37° for 20 hours and MIC identified as lowest value without visible growth.

To establish the MBC, 20μl samples from each aforementioned well and transferred into a new 96 well micro-titre plate containing 180μl of fresh LB and universal neutraliser (L-histidine, L-cysteine and reduced glutathione)[249]. These samples were also incubated statically in air at 37° for a further 24 hours before the MBC was recorded as the lowest value without visible growth.

LB was chosen to facilitate comparison between the MIC and the MBEC found in the alginate bioassay. The MIC's were the same or within one double dilution of the suggested levels for the BSAC, except for rifampacin, which was, 5 fold different. The most likely reason for this would be the use of LB instead of Muller Hinton broth (MHB) that is more commonly used by the BSAC although other media are permitted for use in the test if they produce values that are within one dilution of the reference level[156]. The other anomaly was that clindamycin, which is normally regarded as bacteriostatic, had a MBC/MIC ratio of 2 (i.e. bactericidal) in both organisms. This was perhaps due to a post antibiotic effect in the sub-culture.

As can be seen from the wide range of MIC/MBEC ratios (that were broader than the range of MBEC's alone) in Table 4.4, there was poor correlation observed between the MIC and MBEC. Rifampacin had the lowest MIC but equal highest MBEC and daptomycin had a relatively high MIC but the lowest MBEC. Therefore the common practice of selecting the antibiotics based solely on the MIC antibiogram seems misguided and may explain a proportion of the failures that are seen in clinical practice.

	Mg / L	Gent	Vanc	Dapto	Clinda	Cipro	Rif	Linez
CNS-J	MIC	0.125	2	0.25	0.125	0.125	0.062	1
	MBC	0.5	2	0.5	0.25	0.25	0.5	8
	MBC/MIC	4	1	2	2	2	8	8
	MBEC	32	>2048	32	>2048	>2048	>2048	>2048
	MBEC/MIC	256	>1024	128	>16,384	>16,384	>33,032	>2048
MSSA-N	MIC	0.25	1	0.25	0.125	0.5	0.125	2
	MBC	0.5	2	0.5	0.25	0.5	1	16
	MBC/MIC	2	2	2	2	1	8	8
	MBEC	256	>2048	16	>2048	>2048	>2048	>2048
	MBEC/MIC	1024	>2048	64	>16,384	>4096	>16,384	>1024

Table 4.4: Ratios of MIC, MBC and MBEC (mg/L) of CNS-J and MSSA-N when exposed to the antibiotics: gentamicin; vancomycin; daptomycin with 50mg/L Ca^{2+}; clindamycin; ciprofloxacin; rifampacin and linezolid.

4.3. Discussion

The principle findings of this chapter were that

i. There appears to be a poor correlation between the MIC of an antibiotic and the MBEC.

ii. When used as single agents at clinically achievable doses, gentamicin and daptomycin were the only effective antibiotics.

iii. Combining antibiotics, that were not effective as single agents, did not confer additional benefit.

iv. Combining bactericidal antibiotics with gentamicin had some additive effects whilst combining it with antibiotics considered bacteriostatic, had a strongly antagonistic effect.

4.3.1. Correlating MIC breakpoints and MBECs

It is generally accepted that bacteria aggregated around a prosthesis in a biofilm are much more tolerant to antibiotics than when they are in a planktonic state[66,109,293,294]. The reasons for this have not been elucidated fully but they are most likely related to the higher proportion of the bacterial population that enter a dormant persister state, thereby protecting themselves from the effects of antibiotics and other stresses[295].

Antunes *et al.*[296] tested 82 staphylococcal *spp.* isolated from patients and found no correlation between the MIC and MBEC for vancomycin with their ratio ranging between 1 and >64. Similar ranges in the MIC/MBEC ratio for multiple antibiotics have previously been published[228,297]. This calls into question whether current clinical susceptibility testing is able to identify the most appropriate chemotherapeutics when treating prosthetic related infection. This view is supported by recent mathematical and continuous biofilm culture model comparing these pharmacokinetic/MIC indices, used

to establish breakpoints, with biological response against a MSSA[298]. They found that the antibiotics, predicted to be effective by the traditional methods, never fully eradicated the biofilms which repopulated from the persisters whenever the antibiotic level receded between dosing periods.

Our finding that gentamicin and daptomycin were the only effective single antibiotics examined in our study, is supported by some previous studies. Ceri *et al.* who tested the same MSSA-N strain ATCC 29213 using the Calgary biofilm device [109] found the MBEC of gentamicin to be within a clinically achievable dose whilst ciprofloxacin, clindamycin and vancomycin were more than a 1000x their MIC. Girard *et al.*[297] tested 21 clinical isolates of MSSA with seven antibiotics and found gentamicin to be the only effective antibiotic, however they also did not test daptomicin and their methods were not detailed enough to draw direct comparison with our results. Raad *et al.*[299] used a modified Robbins device to culture 10 strains of MRSA in a biofilm state and found daptomycin to eradicate them effectively whilst vancomycin, linezolid and rifampacin were ineffective. Daptomicin has also been shown to be active against the stationary growth phase of MRSA which has been used as a proxy for organisms in biofilm[300].

4.3.2. Antibiotic characteristics

The differences seen between the seven different antibiotics cannot easily be explained by their ability to penetrate the polysaccharide intercellular adhesin (PIA). Indeed gentamicin, ciprofloxacin[301], daptomycin[302], rifampacin[303], linezolid[304], and clindamycin[305] have all demonstrated good penetration into staphylococcal biofilm whilst only vancomycin has been shown to be significantly hampered by it [301,304].

This led to an enquiry as to whether gentamicin and daptomycin have a common mode of action that could explain their efficacy against staphylococcal biofilms. Daptomycin is a negatively charged cyclic lipopeptide that rapidly penetrates biofilm [302] and has a distinct mechanism

of action. Ca^{2+} acts as a cross-bridge to enable it to insert into the cytoplasmic membrane, permitting escape of K^{+}, Mg^{2+} and ATP [306,307], thereby depolarizing the membrane potential and leading to cell death . Gentamicin is a positively charged aminoglycoside that, unlike all other classes of ribosome inhibitors, causes the 30S ribosome to produce mistranslated proteins. These abnormal proteins are inserted into the cell membrane that sets up a positive feedback loop of increasing permeability, hyper-stimulation of the electron transfer chain and hydroxyl radical damage to the cell[308]. Although they have different modes of action they both deal a catastrophic blow to the integrity of the cell membrane.

The finding that rifampacin combined with either vancomycin or ciprofloxacin didn't confer additional benefit was a surprise as it is commonly used in clinical situations with apparent success[53]. When Olson *et al.* [309]performed an in-vitro study using CNS and a continuous flow chamber with steady state antibiotics the results were similar to ours, in that the addition of 12mg/L rifampacin to 40mg/L vancomycin did not result in additional killing. However when they performed the experiment using a guinea pig cage tissue model they found it did significantly improve eradication of the 5-day-old CNS biofilm compared to monotherapy. Zimmerli *et al.* has published a similar model with a 24 hour old MSSA biofilm[240] and found the combination of rifampacin (trough 1mg/L to peak 8mg/L) and ciprofloxacin (trough 0.11mg/L to peak 1mg/L) or vancomycin (trough 3mg/L to peak 9mg/L) much more effective than monotherapy.

A possible explanation for this discrepency may be in the dosing regimes, which are ***constant*** in-vitro but ***vary*** in-vivo, between peak bactericidal and trough sub inhibitory concentrations. Lewis[295] previously postulated that cyclic dosing that allowed persisters to start repopulating the biofilm just as they are exposed to another bactericidal wave may eventually eradicate all persisters. The effect of the host's innate immune system might also be another obvious explanation for these discrepancies between laboratory and clinical studies.

4.3.3. Synergism and antagonism

Finding that combining bactericidal antibiotics with gentamicin had an additive or synergistic effect against staphylococcal biofilm has been reported once before by Tsuji *et al.*[310] using an endocardial vegetation model. They also found gentamin and daptomycin to be the most efficacious single antibiotics whilst vancomycin was ineffective. Their study reported for daptomycin, the addition of a single high dose of gentamicin provided synergistic, rapid concentration-dependent killing. The Calgary biofilm device has been used to analyse a large number of two and three drug combinations[279] up to 256mg/L however the results of the study seem inconsistent with the MBC, being commonly less than the observed MIC. It seems possible that they may have had similar problems as this author in optimising the complex protocol [224].

The results that combining gentamicin with the bacteriostatic antibiotics (linezolid, rifampacin, clindamycin) had a strongly antagonistic effect with gentamicin have, to the authors knowledge not been published before. Several commercial bone cements have been designed for treating prosthetic infection, which contain gentamicin and clindamycin. The rationale behind this was to broaden the antibiotic cover and also to improve the bactericidal effect. Neut *et al.*[311] investigated its properties using a modified Robbins device and found that gentamicin / clindamycin cement coupons resisted colonization by MSSA more effectively than gentmicin alone. However the distribution of that data was not presented further than 'a combined SD in all 21 samples of around 50%'. It would also been more enlightening if a biofilm had been allowed to form before adding the antibiotics, as this study might have represented more of a test of biofilm attachment than treatment.

There are three postulates for the observed antagonism found in this present study. It seems the organisms either became more tolerant to the gentamicin, there was a chemical interaction between the two antibiotics rendering them less active, or there was methodological error. Given the effect was observed

with narrow inter-quartile ranges in three antibiotics for whom interactions have not been published before, the former seems most likely.

4.3.4. Mechanisms for antibiotic tolerance

Staphylococci have, as discussed in the section 1.3, a diverse network of regulators that modify gene expression and enable them to tolerate a wide range of environmental stresses which include antibiotics and cationic antimicrobial peptides (CAP's). They are able to alter the proportion of the negatively charged PIA and positively charged teichoic acids in their extracellular polymeric matrix and cell membrane via the GraRS system[312]. This confers them the ability to change their surface charge when stressed, making them more significantly tolerant to the positively charged CAP's, gentamicin and vancomyin. Interestingly this envelope stress response has previously not found to be induced by gentamicin, vancomycin or daptomycin[313] but if they were inducible by bacteriostatic antibiotics this would be a possible explanation for our results and worthy of further study.

An important paper from Kohanski *et al.*[114] postulates the common final pathway for all bactericidal drugs as being an overwhelming oxidative stress. Hydroxyl radical formation, induced by bactericidal antibiotics, are the end product of an oxidative damage cellular death pathway involving a transient depletion of NADH by the electron transfer chain, destabilization of iron-sulfur clusters, and stimulation of the Fenton reaction. They also demonstrated that bacteriostatic drugs do not cause oxidative stresses or initiate the DNA damage response SOS system, even though rifampacin was shown to damage it. This enables one to deduce that the antagonism seen in this study, was not due to the activation of the SOS pathway mitigating the effects of oxidative stress caused by gentamicin. Perhaps the bacteriostatic agent further slows the metabolic rate of the organism and this relative lack of NADH protects it against hydroxyl radical formation induced by bactericidal antibiotics. It is also conceivable that as the bacteria senses the bacteriostatic antibiotic; a hitherto unexplained mechanism induces an envelope response that limits gentamicin penetration (Figure 4.10).

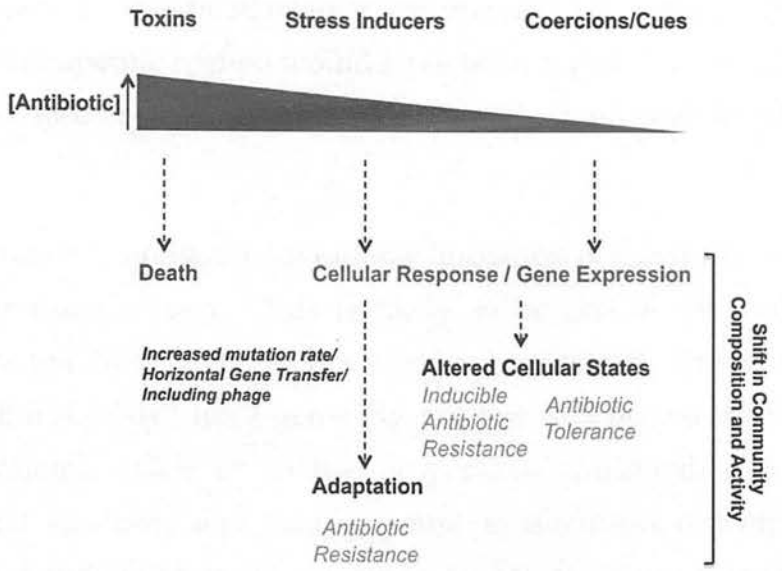

Figure 4.10: Spectrum of bacterial responses that antibiotics can induce. Reproduced from Surette *et al.*[132].

4.3.5. Limitations of our experiments

As Luria broth LB was the most effective media for the biofilm assay, it was also used to facilitate comparison when calculating the MIC and MBC. Compared to the more commonly recommended Mueller-Hinton broth, LB contains less divalent cations (Ca^{2+}, $Mg2^{+}$) and so theoretically can interfere with the binding of cationic antibiotics. It also contains less carbohydrate and so could potentially cause nutritional stress with longer-term growth. That said however, the MIC values for the reference strain MSSA-N were within one dilution (except rifampacin) of the Clinical and Laboratory Standards Institute values and therefore acceptable[156,314].

Another possible bias in our results relates to the relatively short time the bacteria were exposed to the antibiotics so might under estimate the effect of antibiotics that display time-dependent killing[232]. The very high antibiotic concentrations that are seen initially, tail off rapidly[174] (Figure 4.1) and unless

dosing is consistent with *in vivo* pharmokinetics then this short exposure time is reasonable to ensure conservative estimations of *in vivo* effects. Both mathematical and *in vitro* experiment models[298] suggest that a successful chemotherapeutic regime would have to be rapidly bactericidal in the period that the dose dependent antibiotic is present at effective levels.

This leads into another unavoidable limitation of the study, which is a lack of host immune system. This is likely to be one of the main reasons why regimes that have been demonstrated to be most effective in animal models[240] and clinical trials[56] have generally not been replicated in biofilm models[309]. No evidence exists as to the proportions attributable to chemotherapy, bacterial virulence and host response in a success outcome. To minimize financial and ethical costs, it is important to continue to improve the in-vitro models until they more closely parallel biological effects in the clinical situation. This will never fully be realized however, as bacterial eradication is only one end-point to a good clinical outcome. Local and systemic side effects may be worse than the cure, as in the case of severe streptococcal soft tissue infection where the addition of the bacteriostatic agent clindamycin has been shown to reduce mortality compared to the bactericidal agents, by down-regulating the organism's exotoxin production whilst penicillins initially have the opposite effect, due to rapid cell lysis[208].

Acknowledgement is given to the fact that, only two strains of staphylococci were tested and that insufficient resources were available perform a full checkerboard with varying doses of both drugs in combination. The rationale for selecting the most appropriate conditions was listed previously (sections 1.5- 1.7) based on correlation with the literature review and current clinical practice.

4.3.6. Future research

Recent efforts to augment the cellular uptake of gentamicin, either by degrading the integrity of the cell membrane or increasing the proton motive force have been described. Cationic antibacterial peptides (CAP's) are normally produced as part of the host's response but can be isolated from non-pathogenic bacteria and indolicidin cecropin A, melittin A amide (CAMA) and nisin have all shown to be synergistic with gentamicin against MRSA at clinically achievable doses[228]. The CAP's form complexes with the lipids in the cell membrane then aggregate, incorporate additional peptides, and form a pore that allows more gentamicin to enter the cell[315].

Both aminoglycoside uptake[316] and hydroxy radical formation by bactericidal agents[308] are energy dependent via the Proton Motive Force (PMF). Realising this Allison *et al.*[107] did a number of experiments to investigate whether stimulating dormant persisters with metabolites could potentiate the effects of gentamicin. They showed that the if certain metabolites (glucose, mannitol, fructose or pyruvate) are transported to the cytoplasm, some by their specific phospho-transferase system enzymes; they enter glycolysis, where their catabolism generates NADH, and this NADH is then oxidized by enzymes in the electron transfer chain, which contribute to PMF. The elevated PMF facilitates the uptake of aminoglycosides, which bind to the ribosome and cause cell death via mistranslation and oxidative stresses resulting from a positive feedback[135]. These effects were apparently seen without allowing the organisms to revert to a growth state. They also demonstrated this was effective in a mouse catheter biofilm model. However mannitol's diuretic effect was not controlled for, but is worthy of further study.

5.Conclusion

5.1.1. Summary of main findings

The aim of this thesis has been to investigate whether staphylococcal biofilm, found in PJI, is susceptible to specific antibiotic combinations when delivered at clinically relevant doses. In order to do that most effectively, a new method to culture and assay the biofilm had to be developed, and validated to recognised standards.

The research hypothesis has been to shown to be correct as only a minority of the 29 antibiotic combinations tested effectively eradicated the biofilm. An additive/synergistic effect was seen between gentamicin some of the other cidal antibiotics. However, the antagonism seen between gentamicin and the bacteriostatic drugs tested, has important potential implications for current clinical practice.

5.1.2. Different perspectives

Through the course of the thesis, a number of arguments have been advanced. The popularity of joint replacement has provided bacteria with an immuno-incompetent niche to exploit as a biofilm (section 1.3). Rather than representing a distinct phenotye, biofilms seem to share many of the metabolic characteristics of the persister and SCV's (section 1.4.5) and it is feasible all three may be part of the same continuum, driven by schohastic gene responses to the different micro-electrochemical conditions they are exposed to.

Clinical experience dictates that antibiotic therapy alone is insufficient to eradicate PJI and without thought of how to: optimise the host's immune response; debride all necrotic tissues; temporarily remove immuno-incompetent niches; select how and which antibiotics to deliver in order to effectively eradicate both the remaining biofilm and intra-cellular bacteria, then success is more likely to be achieved by good fortune than good clinical care.

5.1.3. The road to rational decision making

Evidence of the very poor correlation between the planktonic susceptibility tests, currently used to guide treatment, and the antibiotics that are actually effective against staphylococcal biofilm, is presented in section 4.2.6. There appears to be a disconnect between the current susceptibility methods using breakpoint determination and the high antibiotic concentrations local delivery can achieve. Also, the results presented in section 4.2.1, that most antibiotics even if delivered in very high concentrations, appear ineffective in this model, prompt further thought. Of the seven antibiotics tested, daptomycin was the only one, if administered systemically, (section 1.6.1) that would have been likely to achieve the levels necessary to be effective against these strains. Gentamicin was the only other effective mono-therapy in this model but at levels only achievable with local administration (section 1.7) presumably due in part to its favorable PK-PD profile (section 1.5.2).

One of the most interesting findings of this research is the observed antagonism between clinically achievable doses of gentamicin and the three bacteriostatic agents (linezolid, clindamicin and rifampacin) that would necessitate between 8 and 32 times more gentamicin being administered before it is effective against the same biofilm (Tables 4.2 & 4.3). Clindamicin is commonly used in conjunction with gentamicin in an ALAC and rifampacin is frequently administered as a systemic adjunct as recommended by the recent American guidelines in PJI[20]. Gentamicin's entry into the cell is diminished when the bacteria becomes less metabolically active, the cell

membrane potential reduces as a consequence thereby conferring gentamicin tolerance. It seems plausible that the bacteriostatic antibiotics might be able to induce the antagonism seen in this study, by changing the membrane potential, either by blocking key metabolic pathways or perhaps more likely given the short time frame in which they seem to be acting, via the toxin-antitoxin system.

Although the alginate model is seen to meet previously defined standards of an acceptable biofilm assay, it is still too labour intensive to be useful in clinical medicine without further modification. Repeatable antibiosis was most variable around the critical MBEC and was perhaps due to threshold response phenomena. Another difficulty in this area of research is defining when an organism is non-viable, as persisters may have a very long lag phase and require specific growth conditions before they are culturable *in vitro.* In terms of the future directions of this thesis, a non-invasive continuous flow biofilm model would be most useful to confirm our findings as well as further investigation of the induction of cell envelope responses and metabolite assisted eradication.

If the same antagonistic trends are found, then an in-vivo model could be justified to test the hypothesis that the addition of bacteriostatic agents initially antagonises the bactericidal effects of gentamicin. Bacteriostatic antibiotics are likely, on the basis of published clinical series, to be beneficial in the long run especially as they readily penetrate and concentrate in human cytoplasm, where gentamicin and daptomycin have minimal penetration. Perhaps a regime where bacterostatic antibiotics are withheld until such time as the antibiotics, found to be rapidly effective against the extracellular biofilm, fall below their MBEC with time.

Whilst would be hubris for man to contemplate exerting ultimate control over staphylococci, our growing understanding of how they are regulated is presenting new therapeutic targets which may allow us to modulate their growth allowing both our species to live together harmoniously.

6.Bibliography

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