Characterisation of bacterial communities within chronic wounds

By

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DECLARATIONS/STATEMENTS

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Abstract

Chronic wounds are a rapidly growing clinical problem across the world, expensive to healthcare systems and severely injurious to the quality of life of those affected. Such wounds have been shown to contain bacteria within biofilms which can be difficult to identify and treat, and are implicated in the lack of healing. The aim of this study was to improve our understanding of the characterisation of these bacterial communities within chronic wounds. This was achieved through the collection of 18 unique chronic wound samples of various phenotypes and 9 acute wound samples. The two bacterial species, *Staphylococcus .aureus* and *Pseudomonas. aeruginosa,* most commonly isolated from chronic wounds and known to be prolific biofilm formers and formidable human pathogens were the focus of this study. Both PCR and culture demonstrated the presence of one or both species in all chronic wound samples and the presence of S. *aureus* in some acute samples. Novel monoclonal antibodies were developed to TNase (of *S.aureus*) and LPS (of *P. aeruginosa*) and their ability to detect the bacteria *in vitro* in a simulated chronic wound environment was evaluated. The action of these monoclonal antibodies were calibrated and evaluated through the use of fluorescent microscopy, and direct and competition ELISA. Following this biofilms of *S.aureus* and *P. aeruginosa* both single species and mixed were evaluated utilising Peptide Nucleic Acid Flourescent In Situ Hybridisation, and the Confocal Laser Scanning Microscope to demonstrate the architecture of the biofilms produced and the locations of the bacteria within the biofilm. Microbiologically using both culture and PCR, the widespread presence of S. *aureus* and P. *aeruginosa* throughout human chronic wound samples was demonstrated. The ELISA and fluorescent microscopy illustrated the feasibility of MABs as a rapid and accurate detection system for identifying bacteria within chronic wounds. The PNA FISH accurately identified individual species in mixed biofilms through multiplex staining. It also visually demonstrated the tissue invasion of *S. aureus*, adhesive properties of P. *aeruginosa* and the synergy of these virulence factors in mixed biofilms. This study has met its aims in that it has provided further evidence of techniques which could be successfully applied to rapidly identify bacteria within a chronic wound environment, and to characterise the structure and composition of bacterial biofilms formed within these wounds. It provides a basis for the development of future clinically relevant rapid bacterial detection systems, and for the academic study of bacteria within the biofilm phenotype.

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List of abbreviations

AVAP (Avidin alkaline phosphatise)

BHI (Brain heart infusion broth BHI, Oxoid)

BSA (Bovine serum albumin)

CDFF (Constant Depth Film Fermenter)

CFUs (Colony forming units)

CHR (Chronic wound fluid sample i.e.: CHR1, CHR2 etc)

CLSM (Confocal laser scanning microscopy)

CNS (*Coagulase negative staphylococci*)

CVLUs (Chronic venous leg ulcers)

DGGE (Denaturing Gradient Gel Electrophoresis)

DIC (Differential interference contrast microscopy)

DNA (Deoxyribonucleic acid)

DNase (An enzyme which catalyses hydrolytic cleavage of phosphodiester links of DNA)

DVLO (Derjaguin-Landau-Verwey-overbeek theory of colloid stability)

DWT (Debrided Wound Tissue sample i.e.: DWT 1, DWT2 etc)

ELISA (Enzyme Linked Immunosorbent Assay)

EMLA (Eutectic Mixture of Local Anaesthetics)

EPS (Exopolysaccharide)

EPSRC (Engineering and Physical Sciences Research Council)

FISH (Fluorescent in situ Hybridisation)

FITC (Fluorescein isothiocyanate)

IDDM (Insulin dependent Diabetes mellitus)

LPS (Lipopolysaccharide)

MABs (Monoclonal antibodies)

MMPs (Matrix metalloproteinases)

MRSA (Methicillin Resistant *S. aureus*)

MSA (Mannitol Salt Agar)

NETs (Neutrophil extracellular traps)

NHS (National Health Service)

NIDDM (Non Insulin dependent Diabetes mellitus)

PBS (Phosphate Buffered saline)

PCR (Polymerase Chain Reaction)

PDGF (Platelet-derived growth factor)

PMNLs (Polymorphonuclear lymphocytes)

PNA (Peptide Nucleic Acid)

QS (Quorum Sensing)

RHE (Recombinant Human Epithelium)

RNA (Ribonucleic acid)

TGFα (Transforming growth factor alpha)

TNase (Thermostable DNase)

TRIS (Hydroxymethyl aminomethane)

UK (United Kingdom)

USA (United States of America)

WD (Wound dressing sample i.e.: WD1, WD2 etc)

Chapter 1: Introduction to thesis

Chronic wounds are a large and growing problem worldwide, In the UK alone they cost the NHS (National Health Service) 3-5% of the UK total health spend (Posnett and Franks 2008). The annual cost is as high as £3.1 bn in the UK (United Kingdom) (Posnett and Franks, 2008), and up to US$25bn in the United States (Sen *et al.,* 2009). As the population of the world becomes progressively more elderly and the prevalence of diabetes and obesity rises this figure is likely to increase significantly.

Chronic wounds are a burden on society and the individual economically; however for the individual with chronic wounds they are far more significant than that. A chronic wound impacts on quality of life extensively, and data suggests that such wounds have a significant effect on quality of life throughout the developed world (Price and Harding 2004, Ribu *et al.,* 2007). Patients living with chronic wounds experience pain and discomfort with half requiring analgesia (Price 2005) and many on regular opiate analgesia. They can also experience odour and exudate leading to social discomfiture anxiety, social embarrassment and social withdrawal, all severely affecting quality of life (Mudge 2008). Many of these negative experiences associated with chronic wounds can be linked to the complications of bacterial infection.

There are many differing causes for the development of chronicity within wounds however it is well recognised that the bacterial populations found within chronic wounds are significantly different to those found on normal skin (Martin *et al.,* 2009). Infection of

wounds both clinically and sub clinically is a significant factor in the delaying of adequate and timely wound healing. Unlike many other factors in the causation of chronic wounds this factor can be modified through appropriate and timely therapeutic interventions

Bacteria do not exist planktonically throughout the majority of their lifestyle but in the biofilm phenotype (Stoodley *et al*., 2002). This natural state of bacterial existence is of great benefit to the bacteria enabling enhanced protection against immune defence mechanisms and antibiotics. Bacteria infecting chronic wounds exist largely in the biofilm phenotype.
If it were possible to rapidly and accurately assess the bacteria present within a chronic wound, their phenotypic state, which species were present and absolute counts of bacteria of various species, we would then be able to assess which combinations were harmful, which neutral and even which possibly beneficial. With this information, tailor-made rapid and effective treatment targeted only at those who need it would be possible, negating the need for costly overprescribing and needless side effects from antibiotics and conversely reducing the significant population risk of the development of antibiotic resistance.

Currently the most commonly used method of obtaining this information in the clinical setting is the use of wound swabs and classical microbiology to obtain both species and bacterial counts and antibiotic sensitivities. These methods face obvious limitations, in that they provide no information on the bacterial phenotype, no accurate count of bacterial numbers and the possibility of missing microcolonies of culturable bacteria without even mentioning the unculturable bacteria that are not picked up (Davies *et al*., 2002). These swabs frequently lead to overtreatment of wounds that are merely colonised rather than

infected and are slow to heal, leading to empirical and often inappropriate prescribing of antibiotics, with the attendant risks of that.

To date scientifically various methods have been employed in the laboratory setting to try and elucidate the bacterial communities present within chronic wounds, including the use of PCR (Polymerase Chain Reaction), targeted antibodies and the use of FISH (Fluorescent In situ Hybridisation) and ELISA (Enzyme Linked Immunosorbent Assay). These all have various shortcomings, and none have translated to clinical practice in the field of wound healing in contrast to other areas of medicine.

The current state of knowledge of bacterial colonies present in chronic wounds is poor (Dowd *et al*., 2008), both in terms of total species present and arrangement within the biofilm phenotype. If a solution is to be found to this problem a method of rapidly, reliably and accurately detailing bacterial communities within chronic wounds, both in terms of species present and phenotype, is necessary to then correlate against wound healing, and provide a basis for individually tailored treatment.

Therefore there is a need for further work on the characterisation of bacterial communities within chronic wounds to find an accurate method for rapid and accurate detection of bacterial species presence and of their relationship to the biofilm state.

To address this problem the literature was thoroughly reviewed which led to the collection of chronic wound fluid, debrided wound tissue and discarded dressings from patients with infected chronic wounds in order to subject this material to rigorous microbiological testing.

The collected material was utilised in experiments to develop rapid and accurate identification of microbial species within the samples. In order to make the case for these methods it was decided to focus on the identification of two bacterial species commonly present within chronic wounds and known to have both significant virulence factors and be prolific biofilm formers: *Pseudomonas aeruginosa* and *Staphylococcus aureus*

The thesis is structured into six chapters, an introduction, followed by a comprehensive review of the current literature. Three experimental chapters each divided into introduction, materials and methods, results and discussion sections, followed by a final chapter consisting of a general discussion and synthesis of the entire thesis findings.

Chapter Two: Background and Literature Review

Introduction

In order to gain an appreciation of the current literature in this field the following topics were reviewed to help build a picture of the current state of knowledge of bacterial communities within chronic wounds, and in particular biofilm formation and the consequences of this for wound healing.

This review has focused on the normal stages of healing and the reasons for the development of a chronic wound with particular reference to infection. Following this the evidence for the presence of biofilms within chronic wounds is presented, with the associated implications of this for investigation and treatment of biofilms within wounds. Finally this review focuses on the methods currently utilised to characterise bacterial communities within chronic wounds. The causes of chronic wounds have not been investigated in detail nor has the treatment of chronic wounds been exhaustively reviewed as this remains outside the scope of this thesis.

Introduction to chronic wounds

A chronic wound is defined by the Wound Healing Society as a wound, which does not heal in an orderly set of stages and in a predictable amount of time. Wounds that do not heal within three months are considered chronic (Mustoe, 2005). The term 'chronic wound' is often used as an umbrella to encompass a broad spectrum of aetiologies. The three

principal forms of chronic wounds are leg ulcers, diabetic ulcers and pressure ulcers but many other wound types can, and do, become chronic (Mustoe, 2004).

Impact of chronic wounds

The burden of chronic wounds falls disproportionately upon those who are immobilised, diabetic and/or geriatric. Chronic skin wounds affect 3% of individuals over 60 years of age and 3.55 per 1000 in the general population in the UK (Vowden *et al*., 2009). In the aging population this becomes a bigger problem year on year (Posnett and Franks, 2008). More than 90% of chronic wounds fall into the three main categories: diabetic ulcers, venous leg ulcers and pressure ulcers (Price, 2005; Mustoe *et al.*, 2006). Chronic wounds are a major and growing world health problem, the cost of managing chronic wounds in the UK has been estimated at 2.3-3.1bn pounds a year in the UK alone and constitutes 3-5% of the UK total health spend (Posnett and Franks, 2008). These costs are exacerbated by very slow healing rates, with as many as 67% diabetic foot ulcers remaining unhealed at 20 weeks (Kantor and Margolis, 2000) and only 42.6% healed at 24 weeks in a recent UK study (Jeffcoate *et al.,* 2009). Many of these wounds become infected and patients report living with cycles of active infected ulceration (Price *et al.,* 2007).

Chronic human infections including chronic wound infections constitute 60-80% of all human infection diseases (Costerton, 1995). The financial cost of management of chronic wounds is growing exponentially. Aside from the cost of day-to-day management of chronic wounds, such wounds can lead to the amputations of toes, feet and limbs. Over 80000 amputations are performed on patients with diabetes in the US per year the majority of these associated with diabetic ulceration and subsequent non-healing and infection (Centre

for Disease Control, 1997). In the UK it is predicted that the forecasted rise in type 2 diabetes will lead to 25000 new cases of foot ulceration per year (Posnett and Franks, 2008).

Chronic wounds are a cause of significant morbidity, economic costs, and have a significant negative effect on quality of life throughout the developed world (Price, 2004, Ribu *et al.,* 2007) and beyond. The development of a chronic wound has severe consequences for the patient and carers; affected individuals are usually elderly, immunocompromised, or have serious concomitant systemic illnesses. The affect on this already vulnerable population can be dramatic. Physically there is pain and discomfort (with 50% requiring analgesia) (Price, 2005), odour and restricted mobility. Psychologically, there is a fear of social discomfort, worry about current health state and further deterioration, social embarrassment and withdrawal, all of which can severely adversely affect a patients quality of life (Mudge *et al.*, 2008).

Wound healing

Normal wound healing is a dynamic complex biological process that is not completely understood. The process involves a complex cascade of cytokines and growth factors, cell proliferation, differentiation, migration, and neoangiogenesis. Generally the wound healing process is sub-divided into three distinct phases for the purposes of description but it is important to remember that these phases are not purely sequential but have substantial overlap. A summary of these processes is presented in Fig 2.1: each phase will be discussed in turn.

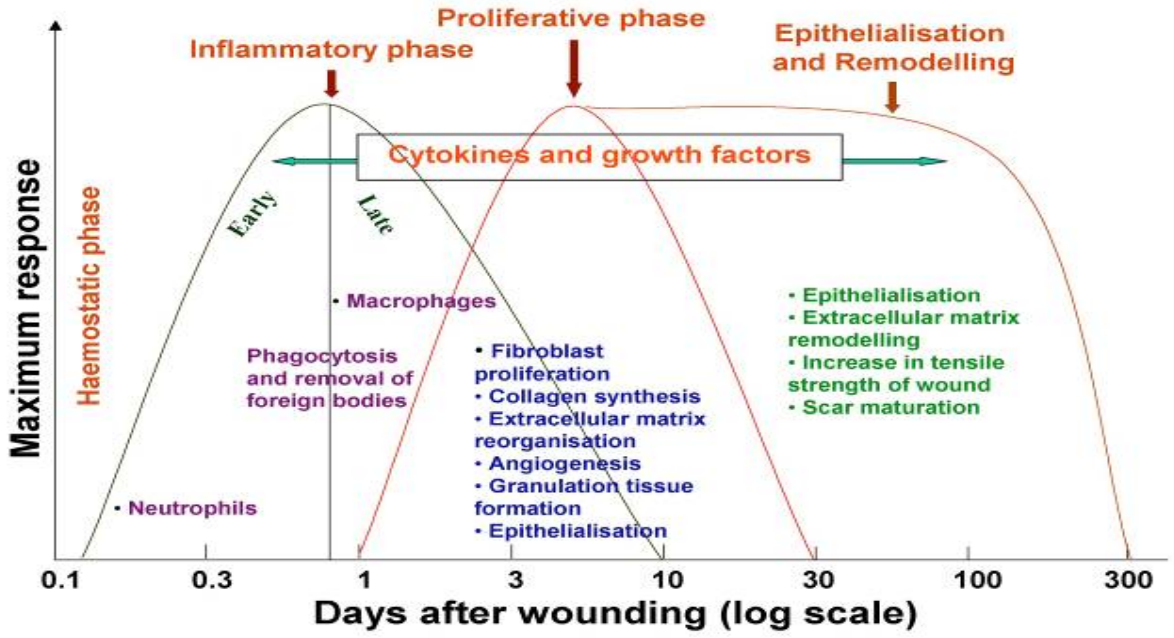


Fig. 2.1 A schematic illustrating the mechanisms involved in the normal wound healing process. [ABC of Wound Healing (Grey and Harding 2006: permission to reprint –Professor K. Harding.)

The Inflammatory phase of wound healing

The inflammatory phase of wound healing begins at the time of injury and can last up to 10 days in normal wound healing (Grey and Harding, 2006); its primary purpose appears to be to cleanse the wound of bacteria and tissue debris. Following tissue damage there is an aggregation of platelets into the damaged area, which forms a 'plug'. Any damage to the endothelial lining of blood vessels triggers the coagulation and plasminogen cascades causing the formation of fibrin, which together with the platelets forms a clot. The purposes of clotting are two-fold; firstly, the clot is haemostatic therefore preventing further haemorrhage and secondly, the clot provides a provisional matrix for the initial stages of tissue repair.

A combination of the degranulation of platelets within the clot and the endothelial damage at the time of injury results in the release of cytokines such as PDGF (platelet-derived growth factor) and TGFα (transforming growth factor alpha)(Barrientos et al., 2008), which will then attract leucocytes into the area of tissue damage. Bacteria will also colonise any open wound usually sourced from the patient's own normal skin flora, and these bacteria will also promote the infiltration of white blood cells into the wound (Edwards and Harding, 2004).

The innate immune system is thus stimulated and (PMNLs) polymorphonuclear lymphocytes flood into the area of tissue damage to phagocytose bacteria and debris. The PMNLs produce hydrolytic enzymes and oxygen derived free radicals to kill phagocytosed bacteria through inducing damage to protein lipids and DNA (deoxyribonucleic acid).

The activity of PMNLs reduces a few days post initial injury and then macrophages infiltrate the site to phagocytose debris. Monocytes and lymphocytes also infiltrate the wound at this point. The macrophages release cytokines and growth factors, which facilitate the recruitment of fibroblasts, keratinocytes and endothelial cells into the wound for tissue repair. To compliment this, enzymes produced by macrophages also degrade the provisional wound matrix (set up by the initial clot) through proteolytic enzymes and down-regulate acute inflammatory signalling, thus orchestrating the next phase of wound healing, the proliferative phase.

The proliferative phase of wound healing

The proliferation phase starts at around 3 days post tissue damage and can last up to 25 days, and is characterised by the replacement of the provisional fibrin/fibronectin matrix with newly formed granulation tissue (Enoch and Price 2004). Fibroblasts are key to this formation, they migrate into the wound (stimulated by cytokines produced by platelets, macrophages and other injured fibroblasts (Slavin, 1996) and proliferate thereby beginning the construction of the new extracellular matrix. Fibroblasts produce fibronectin, a matrix protein which facilitates the migration of cells through the matrix. Subsequently, fibroblasts go on to produce collagen and proteoglycans providing a denser matrix. Angiogenesis and neovascularisation occur in this period and the granular appearance of granulation tissue is secondary to the formation of new capillaries.

Angiogenesis is affected by the migration of endothelial cells from the wound edge and proliferating from the damaged edges of blood vessels. The angiogenic process is mediated by growth factors, matrix proteins and hypoxic conditions (Enoch and Price 2004). Following the formation of healthy granulation tissue re-epithelialisation can occur.

Re-epithelialisation and remodelling phase of wound healing

Although commonly held to be the third stage of normal wound healing, re-epithelialisation begins within hours of injury and is, in fact, concurrent with the previously described phases. Basal cells rapidly divide and migrate from wound edges due to a loss of contact inhibition, until stopped by contact with other epithelial cells (Singer and Clark 1999). Following initial coverage a new basement membrane is formed, further epithelial cell

growth and differentiation then establishes the stratified epithelial architecture of normal skin. As well as contact inhibition, growth factors produced by fibroblasts, macrophages and keratinocytes themselves will all promote keratinocyte proliferation and migration. Some keratinocytes even undergo a marked morphological transformation through which they can phagocytose debris and migrate across the wound bed (Thomas and Harding, 2002). Wound healing does not end with re-epithelialisation as remodelling of the scar can continue for 12 to 18 months (Singer and Clark 1999).

Not all wounds obligingly follow the pattern described above; this is particularly evident in the healing of chronic wounds and many wounds will appear arrested in one phase or progress more slowly than expected. The exact causes of failure of normal wound healing are not known. In routine wound healing, repair is affected by macrophages, keratinocytes, endothelial cells and fibroblasts, which are coordinated via a complex cascade of cytokines and growth factors in cell-to-cell and cell-to-matrix interactions. In chronic wounds this response is impaired; there is prolonged inflammation, a defective wound matrix and failure of re-epithelialisation.

The question that needs to be addressed is therefore why does a wound become chronic? This is a difficult question to answer. It has been suggested that whatever the cause, non-healing hinges on 3 mechanisms, cellular and systemic changes of aging, repeated ischaemia-reperfusion injury, and bacterial colonisation with resulting inflammatory host responses. (Mustoe *et al*., 2006)

Chronic wounds are difficult to study, as they are highly variable in patients and difficult to match for the purposes of randomised controlled trials. Furthermore adequate laboratory models are difficult to construct. Chronic wounds do not occur in animals, and are impossible to accurately reproduce *in vitro*. To date, there are no recognised chronic wound models to facilitate further study of the factors affecting healing.

Factors affecting delayed wound healing

There is currently no single unifying theory which can explain why some wounds fail to progress to full healing and develop chronicity. It is likely that the causes of chronic wounds are multifactorial (see Table 2.1). Both systemic factors, such as metabolic disease states and inflammatory conditions, and local factors, such as bacterial colonisation and inappropriate microcirculation, inflammation and pressure states are all likely to play a role. There is a tendency for research to focus on the wound without considering the patient as a whole; many systemic and local factors have been shown to be associated with delayed wound healing. When treating patients with chronic wounds it is essential to correct as many of these factors as possible. Some, such as poor nutrition and vascular supply, may be treated medically or surgically, many however are impossible to correct. Examples of factors we are unable to correct include inoperable vascular disease, chronic disease states and advanced age and immobility. In these cases care must be taken to optimise these conditions as far as possible.

Local factors	Systemic factors
Inadequate blood supply Increased skin tension Poor surgical apposition Wound dehiscence Poor venous drainage Presence of foreign body and foreign body reactions Continued presence of microorganisms Infection Excess local mobility, e.g., over a joint	Advancing age and general immobility Obesity Smoking Malnutrition Deficiency of vitamins and trace elements Systemic malignancy and terminal illness Shock of any cause Chemotherapy and radiotherapy Immunosuppressant medications, corticosteroids, anticoagulants Inherited neutrophil disorders such as leucocyte adhesion deficiency Impaired macrophage activity (malacoplakia)

Table 2.1 Factors increasing the risk of developing chronic wounds

Adapted from (BMJ 2006) and (Grey and Harding, 2004) (reprinted with permission of Professor Harding)

Interestingly all factors that cause an increased risk of the development of chronic wounds are well recognised to decrease the strength of host defence mechanisms to bacterial invasion. Bacterial presence in any wound is inevitable as the wound provides a warm, moist, nutrient rich environment for bacterial growth and bacteria are ubiquitous in our environment. Thus all wounds provide an environment for interaction between host and

bacteria. It is reasonable therefore to suggest that interplay between host defence factors and bacterial virulence factors will occur in every wound.

A competent host could still develop a chronic wound if the burden and virulence of bacterial presence was high enough and similarly an incompetent host could heal normally in the presence of few bacteria with little or no virulence factors. However wounds in hosts with impaired defences are more likely to develop chronicity than those in the immunocompetent (Landis, 2008). The development of a chronic wound is not an 'all or nothing' event and it is very likely that the interplay between host and bacterial factors described here also explains delayed healing rates.

Prevalence of bacteria in wounds

Staphylococci, *Streptococci*, *Enterococci* and Gram-negative bacilli are the most commonly isolated bacteria from CVLUs (chronic venous leg ulcers) (Davies *et al*., 2007). However, it has become increasingly apparent that hard to culture bacteria may also play an important role in the bacterial microflora of chronic wounds. The most prevalent problem with identification of appropriate species in the literature has been the incomplete isolation of anaerobes, which is both complex and time consuming via culture (Davies *et al*., 2001). However, painstaking anaerobic culture has yielded a high frequency of strictly anaerobic bacteria including *Peptostreptococcus* and *Bacteroides* species, amongst others. Irrespective of our ability to culture microorganisms, the DNA of bacteria which have been in the wound should be present in all samples and molecular techniques exploiting the variations within the bacterial 16s ribosomal DNA sequences are being increasingly utilised.

The 16S rDNA gene sequencing tool provides a means of tracing and identifying bacteria from various sources by assessment of their phylogenetic relationships (Dowd *et al*., 2008). It is a powerful and accurate method that is applicable to small sample sizes and for identifying bacteria that are difficult or impossible to culture. Molecular studies of chronic wounds using PCR and DGGE (Denaturing Gradient Gel Electrophoresis) and exploiting the RNA (Ribonucleic acid) 16S variability of bacteria to identify them has also shown that previously uncultured bacteria are present in wounds. It has been estimated that less than 5% of bacterial species are culturable by traditional methods (Amann *et al*., 2001: Moter and Göbel, 2000). Thus chronic wounds can and do harbour extremely diverse bacterial species, both aerobic and anaerobic and a variety of species not previously recorded.

Significance of bacteria in wounds

A recent study by Davies *et al*. (2000: cited in Davies *et al*., 2007) showed that both bacterial diversity in a wound and bacterial density were independent predictors of increased healing time. Predominant organisms found were *S. aureus* (34.8%) and *P. aeruginosa* (71.2%) for all wounds. This confirmed the findings of a study by Stephens *et al*., (2003) showing that a diverse wound microflora can contribute both directly and indirectly to the 'non-healing' phenotype of chronic wounds.

Commonly isolated chronic wound bacteria

The impact of microorganisms on chronic wounds has been studied and reviewed using many different approaches to detect microorganisms and elicit their effects on wound healing, often highlighting particular species (Schraibman 1990), or groups of organisms (Wall *et al.,* 2002). The microflora of chronic wounds is usually polymicrobial and recent

studies using molecular techniques have emphasized the complex ecology of these wounds (Davies *et al.,* 2003, Davies *et al.,* 2004). Utilisation of conventional techniques has demonstrated a mean number of bacterial species per wound from 1.6-4.4 (Howell-Jones *et al.,* 2005.) Staphylococcus aureus and coagulase-negative staphylococci have been the predominant organisms isolated from both prospective, purpose-collected samples, and retrospective analysis of clinical investigations. In contrast molecular analysis reveals an average of 10 species per chronic wound, approximately four times more than demonstrated with culture (Martin 2009). A 2007 study (Davies *et al.,*) showed that the predominant organisms in chronic wounds from both surface swabs and tissue biopsies were *Staphylococcus spp*. and *P. aeruginosa*, present in 71.2% of patients. In bacterial profiling studies (Gjodsbol *et al.,* 2006) found that chronic venous leg ulcers harboured *S. aureus* in 93.5% and *P. aeruginosa* in 52.2% of cases, making these species ideal for characterisation of bacterial communities in chronic wounds. These are far from the only species present in chronic wounds. Other commonly isolated aerobic bacteria are *Staphylococcus epidermidis, Escherichia coli Enterobacter cloacae, Klebsiella species, Streptococcus species, Enterococcus species,* and *Proteus species*, (Howell-Jones *et al.,* 2005). This is by no means an exhaustive list, but is illustrative of the range of aerobic bacteria that exist in chronic wounds. Harder to culture anaerobic bacteria are also present (Bowler & Davies 1999) found anaerobes in 73% of non infected leg ulcers and 82% of infected leg ulcers. Commonly isolated anaerobic bacteria frequently identified from chronic wounds were *Peptostreptococcus species* and pigmented and non-pigmented *Prevotella/Porphyromonas species, Finegoldia magna (*previously classified as *Peptostreptococcus magnus), Peptoniphilus, asaccharolyticus , Bacteroides Species* and *anaerobic cocci* (Howell-Jones *et al.,* 2005).

Special mention should be made of beta haemolytic streptococci, which although rarely isolated are considered so deleterious to wound healing that the Chronic Wound Care Guidelines of The Wound Healing Society in 2006 (Robson and Barbul 2006) stated that a chronic wound should be treated for infection if there were "greater than 10^6 CFU/g of tissue of bacteria, or any beta heamolytic streptococci" echoing the views of Wiström *et al.,* in 1999. This bacteria is associated with a host of virulence factors and severe tissue destruction, and are particularly concerning in acute setting in which it is implicated in necrotizing fasciitis. Vallalta Morales 2006 *et al.,* state that beta haemolytic streptococci bacteraemia are more common in the "elderly and those with chronic diseases" both of which are common in the chronic wound patient group. The incidence is often quoted at less than 1% of chronic wounds however one study showed a rate of as high as 18.8% of chronic leg ulcers (Schraibman 1990) However it has been stated that "while the β-haemolytic streptococci are associated with tissue destruction, their relevance to chronic (as opposed to acute) wound healing, particularly of non infected wounds, remains unclear". (Davies *et al.,* 2007 Pg 17).

Bacterial colonisation vs. wound infection

Intact skin provides a physical barrier against bacterial infection. When a wound occurs, for whatever reason, microorganisms from the surrounding skin and the wider environment will take advantage of access to a warm moist environment. No wound is sterile and species invading the wound may then establish themselves and colonise the wound. There is some debate as to whether this should be considered an adverse event. Many wounds with

bacteria present will heal normally and many acute wound models deliberately infected have healed at a normal rate or with slight delay (Kanno *et al*., 2010). However, it is generally accepted that microbial colonisation can progress to a stage that inhibits normal wound healing and causes wound deterioration.

Bacteria within chronic wounds will attract leucocytes and therefore a high protease oxidase environment results which is thought to explain that deterioration. A randomised evaluator blind study on venous ulcers showed a modest, but statistically significant difference in healing when wounds are treated with silver sulfadiazine, which could be attributed to an increased rate of healing when the bacterial count decreases (Bishop *et al*., 1992). The best model to explain this is the bacterial load pyramid described by Edwards and Harding (2004) in Figure 2.2

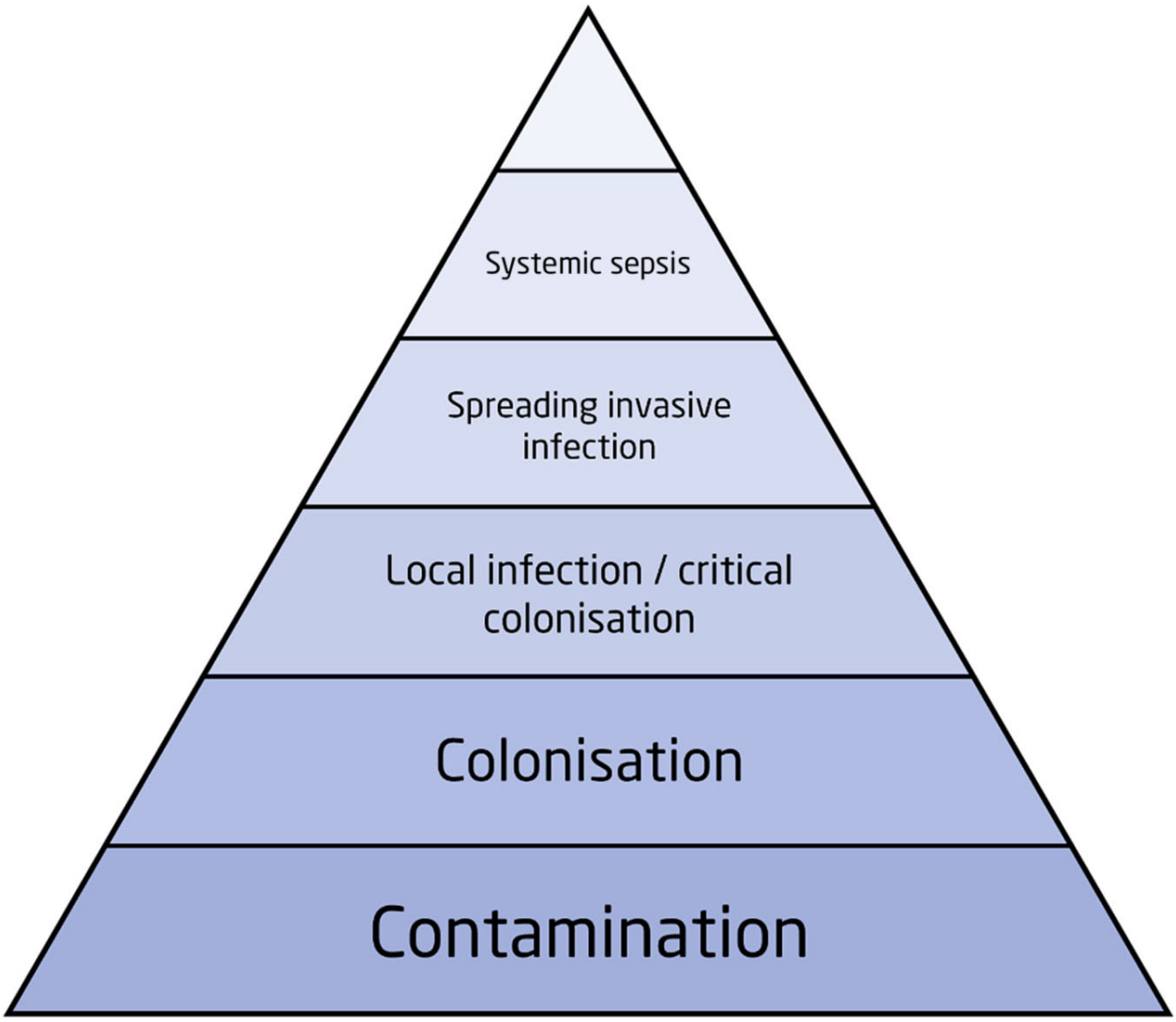


Fig 2.2 Harding/Edwards pyramid model of infection within chronic wounds (Edwards and Harding 2004).

The model neatly illustrates the continuum of bacterial actions within a wound from initial contamination to septicaemia. Where on the pyramid a given wound can be placed depends on a complex interplay of factors, including microbial (e.g., species virulence factors phenotype) and host factors (e.g., tissue, perfusion, immunocompetency, glycaemic

control, necrotic tissue and eschar). The development of infection in chronic wounds is considered to reflect host susceptibility (Edwards and Harding, 2004).

Mechanism of bacteria promoting wound chronicity

It is important to remember that most colonised chronic wounds will heal eventually and they do so in the colonised state (Leaper, 1994). It has been suggested that chronicity in wounds begins with a persistent tissue level of bacteria producing endotoxins resulting in a prolonged elevation of proinflammatory cytokines (Kirketerp-Moller, *et al.,* 2008, Edwards and Harding, 2004). This in turn causes an increase in matrix metalloproteases along with a decreased level of growth factors which shift the balance of the wound from a healing to a more chronic phenotype. Inflammation is a normal and necessary part of normal wound healing, and it has been shown that sub-infective levels of bacteria which cause appropriate levels of inflammation, increase neutrophil and monocyte infiltrates and actually enhance the collagen formation, granulation tissue and wound healing (see table 2.2), (Edwards and Harding, 2004). However some studies have suggested the presence of certain bacteria within a wound seem to prevent the normal healing process from taking place (Madsen *et al.,* 1996).

Phase of Wound Healing Response	Mediators	Effect of Wound Colonization	Effect of Wound Infection
Inflammation	Complement cascade proteins activate platelets causing release of inflammatory mediators, growth factors and fibronectin. Cellular response particularly neutrophils and macrophages	Enhanced White cell accumulation chemotactic and bactericidal activity	Increased consumption of complement proteins, resulting in decreased chemotaxis. Plus depletion of platlets. White cell function impaired in the presence of infection including by short chain fatty acid products by anerobic bacteria. Increased production of cytotoxic enxymes and free oxygen radicals increase tissue damage. Localised thrombosis and release of vasoconstrictive metabolites increase tissue hypoxia and promote bacterial proliferation and tissue destruction
Granulation tissue formation and angiogenesis	Macrophages as stimulus to angiogenesis and granulation tissue formation	Increased granulation tissue formation and angiogenesis	Further increased turnover partly due to production of bacterial enzymes, resulting in oedematous, haemorrhagic friable granulation bed and excessive scar formation. Endotoxins in wounds stimulate production of interleukins and TNF which induce production of MMPs imbalance of MMPs decrease growth factor production
Epithelialisation			Decreased if bacterial load is over 10^5 as bacterial metabolites inhibit migration of epithelium and digest dermal proteins and polysaccharides. Also increased production of neutrophil proteases damage vulnerable epithelium.
Collagen Production		Increased tensile strength of wound	Reduced numbers and proliferation of fibroblasts with disorganised collagen production. Both increased production and Breakdown of collagen with overall effect of decreased wound strength. Endotoxins decrease collagen deposition and cross linking and associated with Surgical debridement.
Wound Contraction			Significantly delayed

Table 2.2 Edwards and Harding 2004- "effects of bacterial colonisation on wound healing" Reproduced with permission.

Diagnosis of wound infection

Wound infection is known to impair wound healing in both acute and chronic wounds. Thus diagnosis of wound infection is extremely important. No wound is sterile and bacterial colonisation will occur in all chronic wounds. Bacterial colonisation can vary in terms of species present and load and is not a constant event. The impact of colonisation on a wound can be evaluated as being from beneficial through to neutral or harmful (Cogen, 2008). Colonisation in itself is widely considered to be a normal phase of wound healing.

However, the progression to 'critical colonisation' (Edwards and Harding, 2004) and the differences between these two states is controversial. Critical colonisation refers to a transitional state between colonisation and invasive wound infection (Edwards and Harding, 2004). It can be defined as the inability of a wound to maintain a balance between increasing bioburden and an effective immune system denoted by a delay in healing but not deterioration of the wound or other overt signs of infection (White *et al*., 2006). Clinically, an unhealthy looking wound bed without signs of invasive infection may be seen. Absolute bacterial CFUs (colony forming units) are difficult to quantify despite several authors postulating ≤ 10^5CFU/g tissue (Heggers, 1998), which is not unreasonable, as bacterial loads above this level have been linked to the rejection of split skin grafts. However, this interpretation does not take into account the difficulty of obtaining accurate CFU numbers of non-randomly distributed bacteria present in a wound from one sample, or the presence of viable but non culturable bacteria, and the virulence and synergistic factors of different species (Percival *et al*., 2010)

Diagnosis of wound infection is best elucidated on the basis of clinical signs by experienced clinicians. Further microbiological investigations are useful, not for the diagnosis of wound infection, but for species identification and antibiotic susceptibilities. A notable wound care committee stated in 1999 (Diabetes Care, August 1999) that current microbiological investigations of wound infection were of limited use for diagnosing most infections. It is important to remember that some wounds may fail to heal in the absence of infective signs and these may be critically colonised as previously described.

Patients with chronic wounds are regularly treated with antibiotics on the basis of wound swab results or empirically following visualisation of the wound. A systematic review of the literature has shown little evidence for the benefit of antibiotics on wound healing (O'Meara *et al*., 2008). However, expert clinicians believe that the judicious use of antibiotics in infected wounds is a vital part of the wound healer's armoury. Undoubtedly many courses of antibiotics are prescribed inappropriately on the basis of a microbiology wound swab report or following an assessment of a wound as infected, when it is only colonised. Excess antibiotic prescribing encourages the development of resistant and multi-resistant strains of bacteria, with serious implications on a population level for human health (Knox and Holmes, 2002).

Management of wound infection

Wound management is currently empirical and based on principles of reducing bacterial load and preventing infection (Fonder *et al*., 2008). Currently wound infection remains a clinical diagnosis and is therefore subjective, risking over diagnosis and inappropriate treatment. Current treatment strategies depend on the extent of wound infection (Landis, 2008). Patients with critical colonisation and localised infection are often treated with dressings containing topical iodine or ionised silver, with topical antiseptics such as Betaine Polyhexanide, or with potassium permanganate soaks all of which are felt to act locally to reduce bacterial load (Landis, 2008, Fleck, 2006, White *et al*., 2006). Systemic or spreading infection should be treated with systemic antibiotics either orally or intravenously.

Biofilms

For much of the twentieth century it was generally accepted that microorganisms existed naturally in a 'free growing' or so-called planktonic form. These planktonic organisms were culturable and easily studied in both medicine and the wider scientific world. Scientific study centred on cultures of single species bacteria grown in a variety of liquid or solid specific media in aerobic or anaerobic conditions.

However, in recent years it has become increasingly clear that most bacteria exist in the 'biofilm phenotype' for the majority of their lifecycle, spending relatively small amounts of time in the free or planktonic state (Stoodley *et al*., 2002). Over the last few decades awareness of the relevance of biofilms to human disease has been recognised, leading to increased research into bacteria in the biofilm phenotype.

Bacteria from biofilms were first described by Antonie van Leeuwenhoek (1632-1723). Van Leeuwenhoek's 1683 report to The Royal Society on the examination of "animalcules" in the plaque scraped from his teeth was the first description of live bacteria recorded.

However, the general theory of biofilm predominance was not postulated until 1978 (Costerton *et al.,* 1978). This theory states that the majority of bacteria grow in matrix enclosed biofilms adherent to surfaces, in a nutrient sufficient aquatic ecosystem and that these sessile bacterial cells differ profoundly from their planktonic counterparts.

The importance of biofilms was slow to be recognised by the medical community. The concept was first developed and recognised by industry, especially in industrial water systems and then in dentistry, a field that had the advantage of dental plaque to study (Costerton *et al*., 1995). Over the last two decades and with advances in microscopy, it has

become clear that rather than just bacterial 'slime' these biofilms are organised and attached in complex ways. Biofilms have been increasingly recognised for their significant role in human disease, predominantly in dentistry but increasingly by the medical community. The National institute of Health in the USA estimates that over 80% of all human infections are biofilm related (NIH 1997). Given the ubiquity of biofilms it seems that extrapolations from planktonic cultures can no longer be considered reliable in determining outcome in clinical infection and its management.

The classic definition of a biofilm is a 'community of microorganisms often adhered to a surface and encased in an EPS (exopolysaccharide) or glycocalyx matrix' (Saye, 2007, O'Toole *et al.*, 2000).

Biofilms in chronic wounds

The incorporation of biofilms into the existing models of chronic wounds allows interpretation of the biochemistry and cellular biology of the chronic wound environment in a different and more logical way. Chronically elevated pro inflammatory cytokines and matrix metalloproteases, and increased elastase could all be explained by the possible effects of biofilms on the host's immune system (Wolcott *et al.*, 2008). They are also thought to influence host fibroblast activity, impair keratinocytes and prevent endothelial cells initiating angiogenesis.

There is evidence that microorganisms within a wound contribute to the non-healing or chronic phenotype and will often form biofilms on the surface (Sutherland, 2001). Biofilms have been identified macroscopically as slime layers in almost every different chronic

wound phenotype from venous leg ulcers, diabetic ulcers and pressure ulcers, and these have subsequently been confirmed to be biofilms at a microscopic level. The ubiquity of biofilms within the chronic wound environment has led to speculation that they may be a causative factor in wounds developing chronicity. Studies of serial wound debridement of chronic wounds have suggested that it may be correlated to improved wound healing rates and more frequent closure (Wollcott *et al*., 2010). This effect is thought to be secondary to the removal of biofilm. It has been suggested that the presence of biofilm phenotype infections can be considered a primary impediment to chronic wound healing (Kanno *et al*., 2010).

Excess bio-burden on a wound bed is thought to be one of the most important barriers to wound healing (Dowd *et al*., 2008). This 'bio burden' comprises bacteria, devitalised tissue, proteinaceous exudate and white blood cells. Given that these surface-associated bacteria will form into biofilms they could be considered the most important element of a wounds bio-burden.

The complexity of biofilms

Initially biofilms were considered to represent a simple 'slab' of matrix material with randomly embedded cells. However the advent of CLSM (confocal laser scanning microscopy) has allowed further study of biofilm structure (Malic *et al*., 2009). Biofilms are not formed by a particular species or set of bacteria but are communities of one or more bacteria living in a structured and ordered environment of microcolonies, water channels and waste channels, with differing microenvironments held together by an extracellular scaffold of polysaccharides protein and DNA associated with the surface. The nature of a

biofilm allows these bacteria to cooperate and thrive in a manner reminiscent of a multicellular organism. Biofilms provide a multitude of microenvironments and indeed some dental biofilms have been shown to harbour over 500 different bacterial taxa (Whittaker *et al.*, 1996). The level of sophistication apparent within biofilms requires a system of intracellular communication and cellular specialization not thought to exist in bacteria prior to recent biofilm studies.

Bacteria and Biofilms

It is important to emphasise that bacterial species have grown in the biofilm phenotype for millions of years, and that this must therefore be a very successful strategy for bacterial survival, propagation and new colonisation. This distinct and ubiquitous phenotype of bacterial growth has only been studied for the last few decades and investigation of its structures, physiology and effects are at an early stage. Within biofilms, development occurs resulting in structures and patterns and metabolic activities, which are not randomised or localised but integrated throughout the biofilm (Stoodley *et al*., 2002). Biofilms are important in understanding how bacteria interact with their environment. They show how bacteria are capable of radically altering their physiology and expressed genes to adapt to, exploit, and thrive in challenging environments. Indeed biofilms are likely to have evolved due to their ability to allow a protected microenvironment for growth within a hostile macroenvironment.

Characteristics of Biofilms

A biofilm can be defined as “a structured community of bacterial cells living in a self-produced polymeric matrix and adherent to an inert or living surface” (Costerton, 1999

p1318). No two biofilms are the same; they are a product of the interaction between bacterial species, a suitable surface, and the external environment. A biofilm's strength is in its heterogeneity, different protein expression, multiple cooperating species, differing metabolic rates and microenvironments affording different levels of protection, nutrient and oxygen availability throughout the biofilm. Those bacteria nearest to the surface are most metabolically active, closest in phenotype to planktonic cells and are most vulnerable to attack by host defences and antibiotics. All biofilms are surrounded by an EPS matrix or glycocalyx, which acts as both a physical barrier to penetration by biological and pharmacological antimicrobials and facilitates the adherence of bacterial cells to each other, surfaces and foreign bodies (Stoodley *et al*., 2002).

Biofilm formation

The development of biofilms is unique in biology and is a remarkable and complex process involving the coming together of independent small prokaryotic cells to form a functional and sophisticated multicellular community.

It is now generally recognised that bacteria will form biofilms preferentially in whatever eco system they inhabit and that planktonic bacteria are the exception rather than the rule. Many aspects of biofilm formation seem counterintuitive. Biofilms will form preferentially in high shear environments, and indeed can form in shear forces of greater than 5000 Reynolds (Peyton *et al*., 1993), a force which greatly exceeds the shear found in heart valves. Indeed biofilms seem to favour turbulent flow. Research from industry has shown that smooth surfaces are colonised as easily as rough surfaces (Costerton, 1995).

Reversible attachment

The first stage of biofilm formation involves conveyance of bacterial species to the appropriate surface. This may be a passive action, *e.g.* convection in liquids or an active one such as flagella induced motility, and there is some speculation as to whether a high flow environment aids this process (Sauer *et al*., 2002).

Biofilm formation begins with bacterial adhesion to a surface. When planktonic bacteria attach to a surface they begin to secrete EPS. Studies have shown adherence within 3 hours (Harrison-Balestra *et al*., 2003). Adhesion of bacteria to smooth surfaces is explained by the classical DVLO (Derjaguin-Landau-Verwey-overbeek) theory of colloid stability, describing the interaction of attractive van der Waal's forces and repulsive interactions resulting from the electrical double layer of the cell (Hermansson, 1999). The first adhesion phase is governed by physical or chemical interactions between surface and cell. However, although DVLO theory explains the experimentally low levels of bacterial attachment to negatively charged surfaces, it does not explain the variety of attachment behaviours seen with other types of surfaces and electrolyte solutions. Despite it not being an all encompassing theory of bacterial attachment it can be argued that it describes one of many components of the adhesion process (Katsikogianni and Missirilis, 2004).

Initial attachment is reversible with the bacteria surrounded by only small amounts of EPS and these often motile bacteria can still leave the surface and resume their planktonic lifestyle. During this period of reversible attachment bacteria can exhibit several species-specific behaviours such as rolling, creeping, aggregate formation and windrow formation (Costerton *et al*., 1995). It is thought that when planktonic bacteria sense appropriate

environmental conditions and a surface to attach to, they may move from the reversible attachment phase to become irreversibly bound through various adhesions (Sauer *et al*., 2002).

Irreversible attachment

Irreversible adhesion is not a random event. Numerous transcription regulators coordinate the phenotypic changes that result in attachment and as many as 800 proteins within a single species alter their expression (Sauer *et al*., 2002). Bacterial phenotypes then alter rapidly. *P. aeruginosa* can alter its metabolism within 10 hours to express a biofilm phenotype (Harrison-Balestra *et al.,* 2003). Once attached, biofilm cell accumulation can occur by 3 mechanisms, first by simple division of the attached bacteria, secondly by recruitment of cells from the bulk fluid and thirdly by redistribution of attached cells via surface motility (particularly relevant for mobile species such as *P. aeruginosa*) (Sauer *et al*., 2002).

Biofilm maturation

Following attachment biofilms undergo a period of maturation transforming themselves from a collection of bacterial cells surrounded by EPS to a complex organised multicellular structure. Channels and pores are formed for nutrient delivery and waste removal and bacteria are redistributed away from the substratum and into microcolonies. Cell to cell communication is thought to occur through various chemicals (quorum sensing agents) (Kong *et al*., 2006) whose release is dependent on cell densities and is thought to organise this structural reconfiguration. Once attached to a suitable surface, like any animal the

biofilm needs nutrients to grow and reproduce. In a suitable environment progression to mature biofilm can be very rapid.

The biofilm structure is largely determined by the 'slime-like' matrix of EPS which provide the structural support for the growing biofilm and consists of polysaccharides, proteins and nucleic acids. The developed EPS matrix consists of very long and thin molecular chains that can associate in a number of different ways (Sutherland, 2001).

The time taken to form a mature biofilm is controversial and estimates range from 10 hours to several months. In reality, the time taken to form the biofilm depends on nutrient availability, inoculation load of bacteria, species of bacteria, motility of bacteria, and of course the definition of a mature biofilm. Therefore time of biofilm development is likely to exhibit extensive variation and can take up to 10 days to reach structural maturity (Stoodley *et al*., 2002).

The exchange of nutrients facilitated by the architecture of biofilms enables the biofilm community to develop considerable thickness and complexity whilst maintaining individual cells in microenvironments that are optimal for them in terms of location within the biofilm and their nutrient requirements. Cells in differing regions of a biofilm have been shown to exhibit differential gene expressions (Stoodley *et al*., 2002), demonstrating biofilm organisation and cell to cell communication.

Biofilm dispersal

Mature biofilms often release planktonic bacteria and groups of bacteria into the local environment. These cells are not merely planktonic bacteria that could not be incorporated

into the biofilm, but deliberately ejected bacterial cells produced by the biofilm (Stoodley *et al*., 2002). The obvious purpose of this is to ensure propagation of the biofilm in new environments. This is a particularly troublesome phenomenon in vascular diseases such as endocarditis, where these groups of bacterial cells form septic emboli, with often severe clinical consequences for the patient.

Physiology of biofilms

The physiology of bacteria within biofilms is profoundly different to that of bacteria in the planktonic state. It has been shown for *Pseudomonas putida* that irreversible attachment to a surface leads to a surface regulated switch from flagella based motility to type IV twitching based motility (Sauer and Camper, 2001). There is also evidence of the production of adhesins binding the cells together and causing the architecture of microcolonies throughout the biofilm. Mature biofilms have a very different protein profile to planktonic colonies; in a recent study of *P. aeruginosa* as much as 50% of the detectable proteome (over 800 proteins) was shown to have a six fold or greater difference in expression, of these more than 300 were undetectable in planktonic bacteria (Sauer and Camper, 2001). These proteins are known to be involved in metabolism, phospholipid and lipopolysaccharide (LPS) synthesis, membrane transport, secretion, adaptation and protective mechanisms.

In mature biofilms, cells will detach and revert to a planktonic form. This is not simple physical forces acting on the biofilm but a physiological process thought to be regulated either by cell density or a lack of nutrients available for the biofilm. (See figure 2.3)

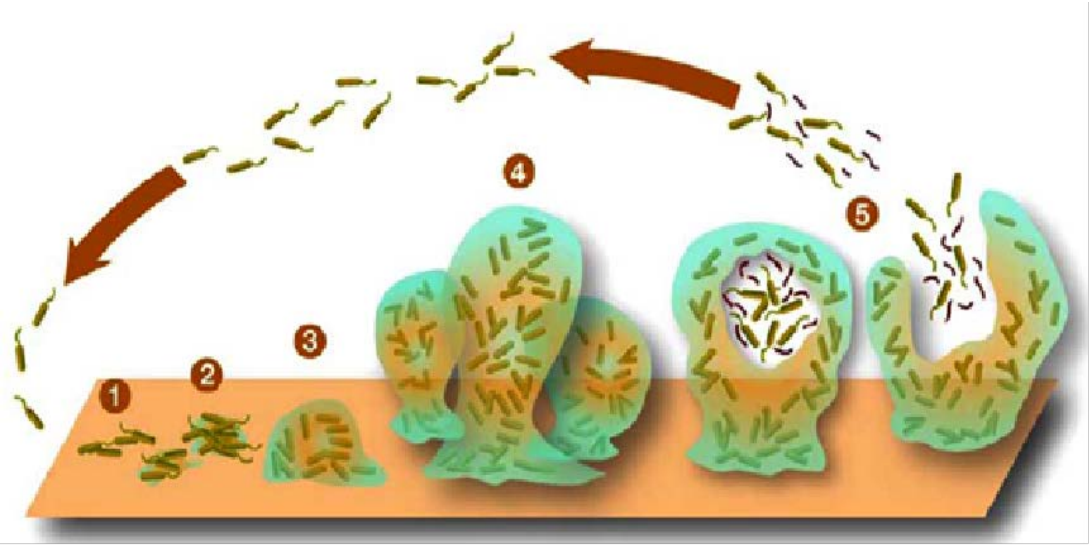


Fig 2.3"Life cycle of a biofilm", (Biofilm hypertextbook, Montana State University Center for Biofilm Engineering) 1: individual cells populate the surface. 2: EPS is produced and attachment becomes irreversible. 3 & 4: biofilm architecture develops and matures. 5: single cells are released from the biofilm.

Structure of biofilms

Biofilms can often be thick enough to be visible without the use of a microscope. Biofilms can consist of millions of bacterial cells, often multispecies and organised to allow a stable structure of physiologically cooperating organisms. Particular species or bacteria are found consistently in certain locations within towers, mushrooms or microcolony biofilm structures (Costerton *et al*., 1997). Some areas of the matrix are devoid of cells, whereas others will be heavily populated often with regular cell spacing. The orderliness of the structure points towards sophisticated cell-to-cell communication systems.

Biofilms can develop structurally into many different forms, from a flat appearance to one of towers and mushroom shaped microcolonies. The biofilm structure is a result of a myriad

of interacting factors including bacterial factors such as twitching, motility growth rate, quorum sensing, EPS production and non bacterial factors such as shear forces, flow direction and available nutrients.

Effect of flow on biofilm structure

Under conditions of low laminar flow most biofilms will form towers and mushroom shaped microcolonies with no evidence of any directionality. Under higher unidirectional flows the biofilms unsurprisingly become elongated and filamentous or mat like in the downstream direction (Stoodley *et al*., 2002).

Effect of shear forces on biofilm structure

Fluid shear also influences the structure of biofilms. Biofilms grown at higher shear have been shown to be smoother and denser than those grown at lower shear (Lui and Tay, 2002). It is as yet unclear whether this effect is due to altered gene expression in response to the environment or simple natural selection of bacteria producing EPS with the strongly adhered remaining attached and the more weakly attached being removed.

Cell to cell signalling within biofilms

Biofilms are not random aggregates of microorganisms but highly structured and organised communities. To develop and maintain this level of differentiation requires an effective system of cell-to-cell communication. Cell-to-cell communication is also essential to orchestrate the expression of virulence factors, the delicate coordination of which are required to successfully invade the host and for survival of the organism within the host

(Kong *et al*., 2006). Amongst bacteria, the regulatory systems involved are collectively referred to as QS (Quorum Sensing) systems.

QS systems have gained the attention of the scientific community due to the possibility of manipulating these communication processes as a novel method of disease control. The signalling mediators of QS are small molecules called autoinducers (QS molecules) (Costerton *et al*., 1999). At a low bacterial cell concentration autoinducers are in low concentrations but at higher densities, a threshold concentration is reached and a transcriptional regulator is activated regulating the expressions of various genes and therefore proteins.

It would be reasonable to postulate that different autoinducers are responsible for cell to cell signalling in different phases of biofilm formation, from reversible attachment through to dispersal. Table 2.3 provides a list of autoinducers of interest in *staphylococcal* biofilm formation.

Function in biofilm phase	**Factor**	**QS system**
Attachment	AtIE[a] PSMs	Agr Agr
Cell-cell adhesion	PIA	luxS
Detachment	PSMs	Agr

Table 2.3 Autoinducers of interest in *Staphylococcal* biofilm formation

(Kong *et al*., April 2006)

The concept of QS as a means of bacterial communication has emerged as being essential to the formation of biofilms with several studies showing impaired biofilm formation when QS genes are knocked-out. It has been shown that discreet QS molecules promote the formation of biofilms and also their dissolution to release planktonic bacteria again (Kong *et al*., 2006).

Studies on P. *aeruginosa* biofilms have shown that the LasR-Lals system controls expression of a battery of extracellular virulence factors and those mutants with a knocked out version of this gene form only thin undifferentiated microcolonies which disperse with weak detergents. When the missing signal is restored P. *aeruginosa* biofilms revert back into healthy biofilms, thereby indicating the requirement of this QS signal in development of the *Pseudomonas* biofilm (Davies *et al*., 1998). Such studies indicate that QS molecules must have an effect on genes expressed in distant cells of the same species and are therefore in effect a bacterial hormone system.

Virulence of biofilms

Biofilms are not just of academic interest as they can have extreme significance in a clinical environment. For all of human history we have been beset with acute epidemic infectious diseases involving the planktonic phenotype bacterial cells of specialised pathogenic bacteria, only recently contained by vaccination schemes and antibiotics. However, more than half of infectious diseases affecting individuals involve commensal or common environmental bacterial species. These can cause devastating and life threatening chronic

infections such as those involving implantable devices, chronic wounds and osteomyelitis which can be secondary to the biofilm phenotype.

Hospital associated infections or nosocomial infections are estimated to kill approximately 99,000 people per year in the USA (United States of America) and around 50,000 per year in Europe (Bryers, 2008). Of these infections, 60-70% are associated with an implantable medical device *i.e.* a biofilm infection (Amman *et al*., 2001). Medical device infections have a directly attributable cost of $5bn per annum in the U.S.A alone (Wenzel, 2007). The UK has no published figures on the cost of medical device infections to the NHS but it is likely to be substantial**.** These figures do not even consider the cost of biofilm disease on body surfaces, especially chronic wounds. Biofilm infections share clinical characteristics, they develop preferentially on inert surfaces such as medical devices, dead tissue or bone but can also grow on living tissues *e.g.* endocarditis (Costerton *et al*., 1999). Due to the nature of biofilms, planktonic cells are released deliberately and periodically, which can give rise to infective emboli or close and distant acute infections.

Since biofilms tend to be slow growing, symptoms generally develop at a comparable rate. Unlike planktonic cells, microorganisms within a biofilm are protected from host defence molecules such as antibodies, which may cause immune complex damage to the surrounding tissues (Jackson and Cochrane, 1988). This damage can allow further nutrient availability and actually increase the opportunity for biofilm growth. Similarly, antibiotic therapy in these cases can arrest symptoms caused by any microorganisms that leave the biofilm and become planktonic, but have a limited or no effect on the biofilm itself. A

patient with biofilm based infection will therefore often have repeating cycles of antibiotic therapy followed by a perceived resolution and then recurrence.

Eradication often requires the surgical removal of the biofilm and the affected surface. Although not always the most dramatic of infectious processes, biofilm infections are notoriously difficult to resolve, for reasons outlined above. Biofilm infection commonly manifests in insidious chronic and recurrent infections. These biofilm infections pose a number of clinical challenges involving rapidly evolving antibiotic resistance, unculturable organisms, chronic inflammation and the ability to shed new biofilm formers in the shape of planktonic bacteria and biofilm microcolonies.

Beneficial biofilms

Are all biofilms 'bad' biofilms? Biofilms are formed by both commensal and pathogenic bacteria and can be considered the predominant phenotype of bacteria in nature. Biofilms form when bacteria and surfaces abound. The gut contains many biofilms, there are biofilms on our teeth and biofilms have also been described in vaginal mucous. These are all biofilms coexisting with humans in a healthy state and could be considered at best neutral and in many respects highly desirable. These biofilms appear to have a passive protective role in preventing harmful bacteria colonising this environment and in the case of gut bacteria an active role in breaking down nutrients for absorption by the body.

Microbes found on the skin are generally categorised as opportunistic pathogens or potential pathogens. However some have advanced the theory that certain members of the ubiquitous skin microflora could be mutually beneficial to the human host without impaired immunity and with intact skin (Cogen *et al*., 2008). In this regard, a symbiotic mutually

dependent relationship with the host similar to bacteria within the gastrointestinal tract has been postulated.

Many bacterial species produce a variety of by-products that can protect the host. *Pseudomonas fluorescence* produces pseudomonic acid (commercially used as the antibiotic mupirocin) potent against staphylococci and streptococci, and also inhibits the transition of fungi from yeast to virulent filamentous forms (Cogen *et al*., 2008). Certain strains of *S. aureus* can produce a proteinaceous bacteriocin (staphylococcin 462), which inhibits the growth of other strains of *S. aureus* (Cogen *et al*., 2008).

Host immunity against biofilm infections

The human immune system is well adapted and able to deal with bacteria in a planktonic state shorn of the protection of a biofilm. However, when encountering a biofilm-based infection the immune system has to deal with a host of different behaviours, phenotypes, and defence mechanisms. Most research into human immunity has been based on planktonic models and does not consider the biofilm with its protective layer of EPS, water and waste channels and deep lying persister cells behaving more like a multicellular organism than a mere aggregation of planktonic cells (Costerton *et al*., 1999).

Antibiotics and biofilms

Researchers working on industrial biofilms found they had higher resistance to bacteriophages, amoeba and chemically diverse biocides (Costerton *et al*., 1993). With respect to medicine, biofilms have been shown to withstand host immune responses and

exhibit greatly reduced susceptibility to antibiotics (Cos *et al.,* 2010). There are many mechanisms through which this may be achieved and these are outlined below.

Barrier to antibiotic penetration

The resistance of biofilms to antibiotics has been shown to be between 10-1000-fold higher compared with the planktonic form (Anderl *et al* 2000). There are many theories postulated for the mechanism of biofilm resistance to antibiotics and one of the most popular is the barrier theory.

It has been suggested that the extracellular polysaccharide matrix surrounding the biofilms acts as a 'defensive barrier', restricting accessibility of antibiotics to the microbial cells. Experiments have shown that although this is the case for some antibiotics, for many there is merely a reduction in diffusion of antibiotics through a biofilm (Singh *et al*., 2010). This alone does not account for the reduced bactericidal effect. Many antibiotics are able to penetrate the biofilm but still do not kill as many bacterial cells as expected (Anderl *et al*., 2000). It is likely, and predicted by mathematical models that if the antibiotic can be deactivated quicker than it diffuses, a formidable barrier is then created to further antibiotic attack of the biofilm (Stewart, 1996). Various enzymes have been shown to be present in biofilms, from catalase to β-lactamases all of which contribute to the resistance of the biofilm (Stewart, 1996). The model of faster deactivation than penetration also holds true for the defences of biofilms versus reactive oxidants like hydrogen peroxide. It is thought poor penetration by oxidative species accounts for the failure of phagocytic cells to destroy biofilm bacteria. In 2003 (Mah *et al*) reported that by knocking out the gene ndvB that synthesised periplasmic glucans they increased sensitivity to tobramycin from 100-fold

reduction to 100000-fold reduction in viability. This indicated that the periplasmic glucans interact with the tobramycin physically sequestering it in the biofilm and preventing its reaching sites of bactericidal action (Mah *et al*., 2003).

Effect of biofilm growth rate on antibiotic susceptibility

A reduced growth rate may also be a factor in the protection of biofilm cells from antimicrobial agents, with transition to a slow growth phase generally recognised as a factor that enhances resistance to antibiotics. It is also important to consider that every microbial cell within the biofilm has its own local microenvironment, with different growth rates and differing gradients of oxygen, nutrients and waste products present.

The high level of heterogeneity of the biofilm may be vital in negating the effects of many antibiotics. It has also been postulated that QS may play a protective role in antibiotic resistance, but this is not yet clear. There appear to be a multitude of interacting conditions within a biofilm combining to form an effective defence against host immunity and antibiotics.

Role of persister cells in resistance of biofilms to antimicrobials

Persister cells are the portion of a bacterial biofilm that remains viable immediately after exposure to antimicrobials. These persister cells do not necessarily have an enhanced or inherent resistance to the administered antimicrobial and are of the normal phenotype of the other biofilm cells. It is hypothesised these cells survive via a mechanism of temporarily disabling their system for programmed cell death which is triggered under certain circumstances such as starvation, which may be replicated in the lower reaches of a biofilm.

Persister cells are capable of reproducing and later repopulating the biofilm. Such persister cells are a unique feature of biofilms and not evident in planktonic populations (Marsh 2003).

Biofilms in other parts of the body

Biofilms are ubiquitous in nature. Humans provide a wide range of accommodating surfaces for microbial attachment and subsequent biofilm development. Dental plaque is perhaps the most studied and described biofilm (Costerton *et al*., 1999). Candidal biofilms on oral and vaginal mucosa and central venous lines have also been reported in the literature (Douglas, 2003).

Introduced foreign bodies, such as endotracheal tubes and urinary catheters (Donlan, 2011) have been shown to provide an environment conducive to biofilm development. Implantable foreign bodies such as pacemakers, prosthetic joints, silicone implants and meshes all provide inviting surfaces for biofilm formation, resulting in significant morbidity and often ultimately necessitate surgical removal.

Biofilms are frequently seen in chronic wounds (see Fig.2.4 assumed biofilm) and are regularly recognised clinically; the warm moist environment exposed to bacteria from the air and surrounding skin is an inviting environment for biofilm formation.

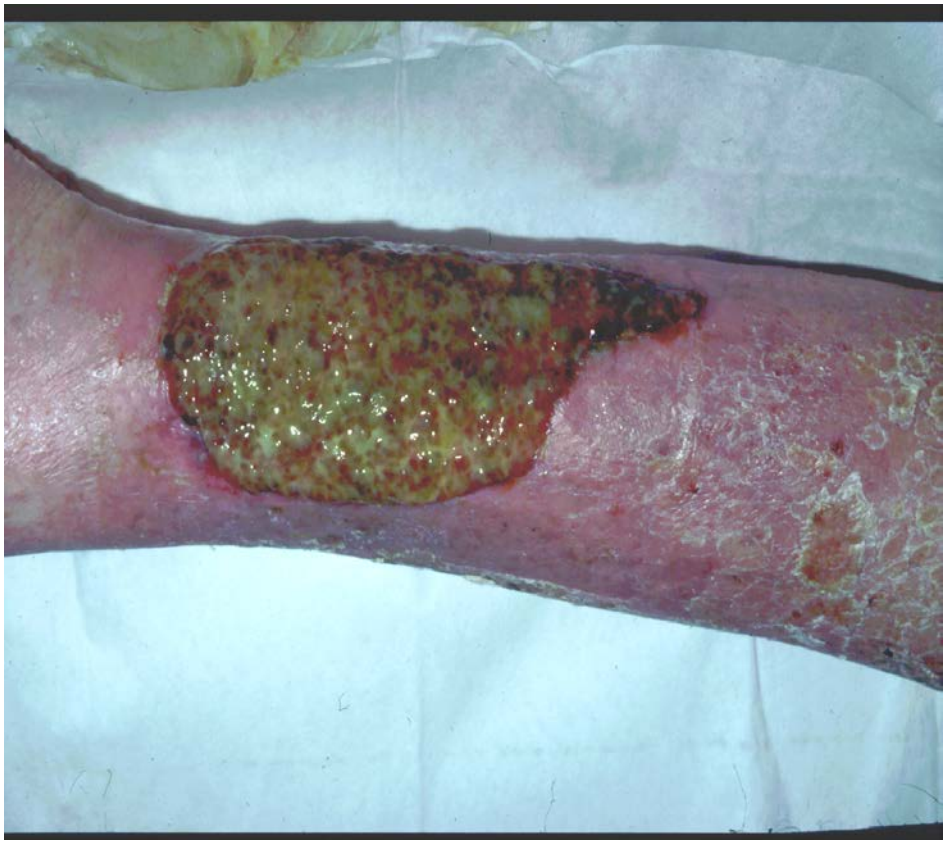


Fig 2.4 Macroscopic image of an assumed biofilm in a chronic wound (biofilm is the shiny green portion of the wound) reproduced courtesy of Prof. D. Leaper (Department of Wound Healing, Cardiff University)

In the case of chronic wounds, it has been suggested that the biofilms source of nutrition is not from locally necrotic tissue but from nutrient rich plasma, neutrophils, inflammatory proteins and iron from lysed red blood cells delivered by the body's circulatory system (Wolcott, *et al*., 2008). It has been suggested that in the case of chronic wounds the biofilm exploits chronic inflammation and capillary transudates to secure a persistent source of nutrition (Wolcott, *et al*., 2008).

It is believed that the biofilm may associate closely with blood vessels to modulate the host inflammatory response (Rumbugh, 2007) as in a diabetic foot ulcer with an inadequate

blood supply and therefore decreased host inflammatory response, increased tissue necrosis is observed. In such instances the biofilm is manipulating its local environment in terms of inflammatory responses by modulating its own behaviour and virulence through QS (Sandal *et al*., 2007).

***P. aeruginosa* biofilms**

Pseudomonas species are common and prolific biofilm formers and can play extensive roles in biofilm infections (Bjarnsholt *et al*., 2009). As such P. *aeruginosa* biofilms are highly studied, both as single and mixed species biofilms.

When treated with antibiotics 6 out of 7 diverse microbial biofilms shifted to *P. aeruginosa* only biofilms. However isolates of these P. *aeruginosa* were susceptible to the antibiotics when cultured in planktonic form, showing the great protective value of the biofilm phenotype for bacteria (Flanagan *et al*., 2007).

Mutant non-biofilm forming strains of P. *aeruginosa* have been identified. One class has flagella and motility mutations resulting in poor initial surface adherence and the other has defective type 4 pili making them unable to form microcolonies. It is thought that the 'twitching' produced by these pili is a form of surface-associated motility and is essential to the formation of micro colonies (O'Toole and Kolter 1998).

The biofilm structure appears to be largely determined by EPS, which consists of polysaccharides, proteins and nucleic acids. These EPS molecules support and shape the biofilm. It has been shown that mucoid strains of *P. aeruginosa* produce more structurally

differentiated biofilms than non mucoid strains (Nivens *et al.,* 2001) and that more specifically that it is a result of increased production and acetylation of alginates (a major component of *P. aeruginosa* biofilms). Mucoid strains and those altered to increase alginate production will form mushroom shaped clusters of cells with corresponding water channels. Following attachment the *algC*, *algD* and *algU* genes are activated immediately after attachment to form the alginate extracellular polysaccharide matrix (Davies and Geesey, 1995).

As previously described, at an appropriate time, microcolonies will aggregate into true differentiated biofilms. Through cell-to-cell communication *P. aeruginosa* can form a biofilm in as little as 8 hours, following inoculation on rat skin wounds (Kanno *et al*., 2010). However despite this, these acute full thickness wounds still progressed to full healing in 7 days, as did the uninoculated controls.

P. aeruginosa is an opportunist pathogen and the cause of many infections, both in wounds and elsewhere. It has a high incidence of antibiotic resistance making its eradication troublesome. The ability to detect *P. aeruginosa* infections early would not only reduce time spent in hospital and pharmaceutical requirements but also improve morbidity and mortality and help curtail the financial burden of wound infections (Sharp *et al*., 2010).

***Staphylococcus aureus* biofilms**

In a study of bacterial genera in diabetic foot ulcers in 2008 staphylococci were found to be the primary organism detected (Dowd *et al*., 2008). Staphylococci are also potent biofilm formers and bring a host of virulence factors and antibiotic resistance genes to a biofilm

Studies on porcine wounds showed that *S. aureus* was capable of forming a biofilm within 48 hours post inoculation. Topical antimicrobials (mupuricin cream and a triple antibiotic) took in excess of 48 hours to achieve a 2 log reduction in number of these still immature staphylococcal biofilms, but was very effective versus planktonic bacteria demonstrating that this clinically prevalent biofilm conveys a significant survival benefit to the bacteria within it (Davis *et al*., 2008). Early and accurate recognition of the presence of *staphylococci* biofilms would also be of huge benefit in reducing morbidity and mortality from chronic wounds.

Management strategies for biofilms

Awareness of biofilms and their structure, virulence, and protective factors inevitably leads the clinician to consider various strategies for the management and control of biofilms in chronic wounds. A number of different approaches to this have been adopted and can be broadly grouped into the following three categories; prevention of bacterial attachment, prevention of biofilm development and treating established biofilms.

Prevention of initial planktonic bacterial attachment

Lactoferrin inhibits biofilm formation by *P. aeruginosa* and *S. aureus*. through inhibition of aggregation (Valenti and Antonini, 2005). In recent studies this was thought to be due to its ability to chelate iron, causing an iron deprived state in the bacteria leading to 'twitching behaviour' in *P. aeruginosa* causing wandering across the surface instead of forming cell clusters which in turn inhibits initial adhesion (Singh *et al*., 2002).

Prevention of biofilm development

Attempts to prevent biofilm development began in industry with efforts to produce a surface that bacteria could not attach to and therefore not develop into biofilms. However all attempts to manufacture such a surface have failed (Costerton *et al*., 1999).

Bacteriophages have been proposed as one method for prevention of biofilm development. Bacteriophages are viruses that propagate in and destroy their bacterial host and some will hydrolyse biofilm extracellular polymers (Donlan, 2009). Bacteriophages appear to penetrate biofilms effectively (Doolittle *et al*., 1996) and due to their replicative nature, a single dose could theoretically treat an entire biofilm. However there are many obstacles to the effective use of bacteriophages as a therapy in humans, including bacterial host range, development of resistance, incorporation of virulent phage genes into bacteria and inactivation by the human immune system (Valenti and Antonini, 2005). As a consequence their potential as therapeutic agents is still being evaluated.

As previously described, QS or cell-to-cell communication is thought to be essential in biofilm formation. Using ribonucleic acid 3-inhibiting peptide (RIP) which inhibits phosphorylation of a molecule called TRAP, which is essential in staphylococcal cell to cell communications; it was possible to reduce biofilm formation in Dacron grafts (synthetic material used to replace normal body tissues) in rats under both flowing and static conditions (Balaban *et al*., 2005).

Treatment of an established biofilm

By the time it is evident that a biofilm will be a problem within a chronic wound the biofilm is usually already established, thus the attempted eradication of established biofilms is the mainstay of treatment. Many differing methods have been described, with varying degrees of success (Fleck 2006, Donlan 2009, Douglas et al., 2009).

Physical Treatment of an established biofilm

Regular sharp debridement has long been held by many to be efficacious in promoting the healing of chronic wounds. A sharp debridement down to bleeding tissue physically removes the bioburden of a wound, the devitalised tissue, bacterial biofilms and inflammatory components. Similarly to the removal of an implantable device, it physically removes the problem. This strategy whilst reducing bacterial counts also allows the removal of fibrinous material and aims to leave a well vascularised wound bed with healthy granulation tissue (Davis *et al*., 2006). However, it is not always possible to completely remove all the bioburden from a wound due to pain or time constraints, so other options also need to be considered.

Ultrasound Treatment of an established biofilm

Some studies have shown an increased rate of wound healing following an ultrasonic debridement. It is felt that ultrasound as well as removing tissue may disrupt QS leading to a decrease in the virulence of biofilms and an increase in their susceptibility to antibiotics (Ennis *et al*., 2005).

Dressings and biofilms

Some wound dressings may have anti-biofilm activity based on their chemical and physical attributes. Iodine and ionic silver impregnated dressings are popular in wound care and used extensively in patients with suspected biofilm infection (Percival *et al.,* 2007). Dressings should be changed regularly as their active anti-biofilm substances may be depleted with time resulting in provision of another surface of attachment for biofilms.

Antimicrobials and biofilms

Antimicrobials and antibiotics are generally felt to be effective against metabolically active cells in the superficial layers of biofilms but not as effective against deeper persister cells (Anderl *et al.,* 2000). These metabolically active cells are thought to be the most detrimental to the host through up regulation of inflammation. Consequently treating biofilms with antimicrobials can improve wounds through a limited bactericidal effect without eradicating the biofilm. Systemic antibiotics are used wherever there is suspicion of systemic infection or severe localised infection. Ischemic wounds have a poor blood supply and thus antibiotic delivery and the effectiveness of systemic antibiotic therapy is reduced. Studies have shown limited effectiveness of antibiotics versus eradication of biofilms for reasons already described (Cos *et al.,* 2010), but their effectiveness against planktonic cells detached by the biofilm and metabolically active cells on the periphery of biofilms means they are still a very useful adjunct in biofilm management.

Antiseptics and biofilms

Topical antiseptics can be used to suppress the growth of biofilm communities. Antiseptics can penetrate biofilms and have a considerable antimicrobicidal effect. However some

antiseptics have been shown to damage human antibodies and cytokines and even kill human cells (Wilson *et al*., 2005). Hence they are used selectively and sparingly on human wounds.

Ionic silver and Biofilms

It has been recognised for some time that silver can exert an antimicrobial effect at low concentrations (Demling and Leslie DeSanti, 2002). The mechanism of action is through damage to the cell membrane, the cell membrane transport system and on the nucleic acid and protein function (Fleck, 2006). Silver is more universal in terms of its activity compared with antibiotics being effective against gram-negative bacteria, fungi, yeast and viruses. Silver has also been found to have greater efficacy than chlorine (Fleck, 2006). As with all antimicrobials, silver can be cytotoxic if the concentration used is too high. Some silver dressings have been shown to prevent biofilm formation *in vitro* (Percival *et al*., 2007) although there is controversy over the concentrations required to achieve this.

Polyhexamethylene biguanide and biofilms

This is a polymeric broad spectrum cationic antimicrobial agent. The mechanism of action is through impairing the integrity of the outer membrane of both Gram positive and negative bacteria, and has been used in chronic wounds with considerable success reported in small trials (Fleck, 2006).

Iodine and biofilms

Iodine has long been used to prevent infection and promote wound healing. However, there is some debate as to its effectiveness as an antimicrobial, together with the possible

discomfort caused to patients and its toxicity towards host tissues. *In vitro* it has been shown to impair the function of cells involved in wound healing (Schreier *et al*., 1997). It does however have a long history of use and established effectiveness *in vivo* against *S. aureus* and MRSA (Methicillin Resistant *S. aureus*) (Mertz *et al*., 1999).

Biomaterials and biofilms

Large portions of biofilm infections involve implantable biomedical devices. Various attempts have been made to produce a biomaterial that is inhibitory to biofilm formation. Liquid/air breaks, antibiotic fills and skin cuffs, tethered antibiotics, silver coatings, slow release antiseptics and antibiotics and mechanical design alternatives have all been trialled, all with little or no effect (Bryers, 2008).

Iron metabolism interferenc

Iron is critical for the growth of bacteria and the function of key metabolic enzymes. The sequestration of iron is an evolutionally early host defence mechanism against bacteria (Bryers, 2008). Gallium can be used as an irreversibly binding non-functional competitor to iron in many bacterial enzyme systems. Increasing concentrations decrease the growth rate of *P. aeruginosa* in a dose dependent manner and encouragingly it appeared to prevent biofilm growth and was bactericidal against mature biofilms when introduced in appropriate concentrations (Kaneko *et al*., 2007).

Rotation of strategies

Many of the above methods are of merit in particular cases and can, for a short time, be effective against biofilms in chronic wounds allowing the underlying wound to improve.

However, none of these methods are perfect and often the best strategy for management of wounds complicated with biofilm infection is to combine or rotate several methods, giving the wound enough respite from the biofilm to progress towards healing.

Wound diagnostics

Several wound diagnostic methods for bacteria in wounds have been available for a long period of time. All those involved in treating wounds will be familiar with the use of wound swabs and classical microbiological tests for bacteria. While useful for identifying bacterial species, classical culture has been shown to identify as little as 5% of bacterial species, missing potentially relevant pathogens, whilst also being time consuming and labour intensive. This can be remedied to some extent by the use of molecular approaches such PCR. However these tests cannot given their reductionist nature or provide us with any idea of the organisation or structure of the bacterial communities in chronic wounds.

Bacterial staining such as Gram or Ziehl-Neelsen stains are fast and inexpensive and of use in giving direct visualisation and crude characterisation, but due to the 'paucity of morphological distinctions among bacteria' (Moter and Göbel, 2000) do not allow reliable identification of species.

The ultimate goal in wound diagnostics would be a rapid acting, economically viable, non invasive test giving accurate information concerning bacterial species present, their numbers, and their organisation into communities or biofilms.

PCR

Molecular diagnostic techniques based upon PCR can now give us rapid and sensitive identification of bacterial species present in a wound independent of their viability in culture. These techniques are however limited in that they give no information on numbers present or spatial distribution of bacteria (Amann *et al*., 2001).

The 16SrDNA gene sequencing tool provides a means of tracing and identifying bacteria from various sources by elucidating their phylogenetic relationships. It is powerful and accurate on miniscule sample sizes and for identifying bacteria that are difficult or impossible to culture. Limitations include a lack of information on antibiotic resistance; the variability between strains of the same species and the fact the database does not include newly discovered microorganisms (Schreier *et al*., 1997).

Confocal Laser Scanning Microscopy (CLSM)

Several different microscopy techniques have been evaluated for the observation of biofilms. Light microscopy, transmission electron microscopy and scanning electron microscopy have all been used to accurately visualise the structure of biofilms. These techniques suffer severe limitations, either through only allowing surface viewing of the biofilms or requiring its dehydration. Dehydration of biofilms has been shown to account for a loss of up to 98% of its *in vivo* mass (Sutton *et al*., 1994).

Confocal Laser Scanning microscopy (CLSM) allows three-dimensional imaging of biofilm sections without the need for dehydration or physical disruption. As a consequence CLSM has quickly become the 'gold standard' for biofilm imaging. To obtain more detailed

analysis of biofilm structure and the species within it, FISH which can provide species specific fluorescent labelling, is often used in association with CLSM.

Fluorescence *in situ* Hybridisation (FISH)

FISH is a method that can provide more detail to the images obtained on a CLSM, through fluorescently labelled probes specific to certain bacteria emitting light of a specific range of wavelengths. FISH is an interesting technique that allows 'simultaneous visualisation, identification, enumeration and localization of individual microbial cells' (Moter and Göbel, 2000). It is hoped that this technique can be culture independent and combine the precision of molecular genetics with the visual information of microscopy allowing bacterial communities to be accurately characterised in terms of structure and composition.

In Situ hybridisation was first developed by two independent research groups (Pardue and Gall 1969, John *et al* 1969) using radioactive labels. The method was initially developed to allow examination of nucleic acid sequences within cells without altering cell morphology. *In situ* hybridisation was first used on bacterial cells by Giovanni *et al.* in 1988. Radioactive labels were replaced with fluorescent ones for microbial detection by Delong in 1989. Fluorescent dyes were clearly superior in terms of safety, speed of process and allowing multiple dyes with different wavelengths to detect several target sequences with a single hybridisation step. FISH has developed in both sensitivity and speed over the last 20 years making it a powerful tool in phylogenetics, ecologics, diagnositics and environmental studies in microbiology (Amann *et al*., 2001). Thurneer *et al.,* 2004, neatly described the use of FISH to investigate a mixed species biofilm with simultaneous 16srRNA targeted oligonucleotide probes.

FISH was originally described with DNA and RNA probes. However over the last decade PNA (Peptide Nucleic Acid) probes have been introduced. PNA probes are DNA analogues with an uncharged polyamide spine instead of sugar phosphates and are stable to degradation. These probes hybridise to complementary sequences with a higher affinity and have different hybridisation characteristics (Prescott and Fricker, 1999). PNA probes are superior in their penetration of hydrophobic cell walls due to their neutral backbone in contrast to oligonucleotide probes.

As with any technique FISH suffers from several limitations. Auto fluorescence of both human and bacterial cells can considerably hamper the gathering of accurate information. It also requires very narrow wavelength probes and multiple sections for negative controls to avoid false positive results. Probe specificity and insignificant penetration of the probe into a biofilm can lead to falsely negative results as can variability between cells of target sequence. Photo bleaching of the probes under the very high laser energy focused upon a small area of the slide requires rapid focusing and data storage and reduces the reproducibility of the technique. FISH also requires specimen processing fixation and dehydration, potentially leading to similar problems encountered in Transmission Electron Microscopy and Scanning Electron microscopy, thus limiting its utility in the observation of dynamic changes in live biofilms.

Enzyme-linked immunosorbent assay (ELISA)

Monoclonal antibodies raised to specific bacterial strains exhibiting immunofluorescence can be highly species specific and fast acting allowing rapid identification of bacterial species. Limitations to this method include non-specific binding, and 'antibody size restricting easy access to their target antigens within tissues or biofilms' (Meyer-Reil *et al*., 1980). This method still requires culture of the bacteria to raise a specific antibody and would involve the use of multiple antibodies on single samples, leading to issues of 'cross-reactivity of monoclonal antibodies' (Moter and Göbel, 2000).

Guggenheim *et al.,* (2001) demonstrated that specifically raised monoclonal antibodies to *Streptococcus mutans* could be detected not only in the planktonic phenotype but also accurately in a 3 species *in vitro* biofilm and in an *in vivo* biofilm. It was postulated that the tight structure of a biofilms EPS could potentially serve as a size exclusion barrier to large molecules entering the biofilm. However immunoglobulin G (IgG) has been shown to be small enough to migrate through biofilm matrix (Zhu *et al*., 2001). More recent studies contradicted this, with Thurnheer *et al*., (2004) demonstrating that IgG penetrates into the biofilm but that movement is impaired by the matrix and possibly blocked in areas with smaller pores.

The use of monoclonal antibodies to identify species of bacteria within biofilms by CLSM is exciting due to its potential to allow study of a dynamic biofilm. It does however have limitations secondary to increased molecular size versus matrix pore size and durability of the associated fluorescent probes.

Why further characterisation?

The microbial communities formed by bacteria present obstacles to the clinician in both diagnosis and management. Biofilms are resistant to many biocides, antibiotics and wound care products. Better characterisation of microbial communities in chronic wounds will allow the development of more targeted strategies to combat this problem.

It is important to find new and effective ways to characterise biofilms. A clinically important biofilm may be present without being amenable to culture; it is thought that only 10% of all bacteria are culturable (Davies et al., 2004). Bacteria within a biofilm, such as persisitor cells with a slow metabolism, may also be unable to divide sufficiently within 48 hours to be cultured. With more information on biofilm composition and structure we will be able to engineer more appropriate treatments.

Knowledge of the exact structural composition of bacterial communities in chronic wounds and the metabolism and manipulation of biofilms should aid us in the development of improved measures against these common diseases (Thurnheer *et al*., 2001).

Investigations *in vivo* are difficult due to the inability to control variability between patient factors, bacteria and the wound environment; no two wounds will be identical, rendering any randomised controlled trials difficult, if not impossible. Further characterisation of the bacterial community in chronic wounds may enable the construction of a more accurate model for research. Animals do not suffer chronic wounds and as such provide no suitable models.

A key goal for management of wound infection is an early warning diagnostic method that would allow the non-invasive detection of the early stages of tissue or implant infection allowing an expedient response (Parvizi *et al*., 2006).

Aim of the thesis

This study aims to demonstrate ways of characterising bacterial communities in chronic wounds by identifying bacteria within biofilms, through the collection of appropriate microbiological samples from wounds, to build up an accurate pool of clinical samples for detection strategies.

The EPSRC project which kindly partly funded this research had developed novel Monoclonal antibodies (MABs) to both *S.aureus* and *P.aeruginosa.* This thesis will concentrate on efforts to characterise bacterial communities of *S. aureus* and *P. aeruginosa.* Both of these species are commonly found within chronic wounds, are selectively pathogenic, easily culturable and prolific biofilm formers making them ideal candidates for demonstrating methods of characterising bacterial communities within chronic wounds.

The following chapters will cover the collection and microbiological typing of chronic wound associated specimens. We will also use novel MABs in the detection of bacteria in these samples, using fluorescent microscopy, ELISA, FISH and the CLSM to identify bacteria within biofilms and demonstrate biofilm architecture. It is hoped that more accurate characterisation of the bacterial communities in chronic wounds will increase our understanding of these communities and lead to novel detection and treatment strategies.

Chapter Three: Collection and Microbiological Analysis of Wound Samples

Introduction

Chronic wounds remain an expensive and growing problem worldwide, particularly in the industrialised world with its ageing and increasingly obese population. Chronic wounds currently cost 2.3-3.1bn pounds per year in the UK alone (Posnett and Frank 2008) and over 10bn dollars per annum in the USA (Bickers *et al.,* 2006). One to two percent of the population in developed countries develops chronic wounds, (Fazli *et al.,* 2009) a condition associated with severe patient suffering, loss of employment and reduced quality of life (Gottrup, 2004).

Whether or not a wound develops chronicity is based upon many factors (Jones *et al.,* 2004). It is however, generally agreed that an unfavourable microbial population can be a significant causative factor for chronic wounds (Mustoe *et al.,* 2004). Bacterial damage in non healing wounds is mediated through the destructive effects of chronic inflammatory reactions inhibiting key processes in wound healing (Jones *et al.,* 2004, Junger and Sorkin 2000) and the creation of a high protease, high oxidase environment resulting from the bacteria and attracted leukocytes. It has been suggested that maintenance of chronicity may be determined by bacterial burden (Bjarnsholt *et al.,* 2008)

In order to better characterise bacterial communities in chronic wounds, it is important to collect information on the likely species present in the chronic wound. It is also pertinent to consider the chronic wound environment, as any potential method of characterising

bacterial communities will have to operate effectively in an environment containing both bacteria and chronic wound fluid.

In order to develop improved methods to characterise bacterial communities in chronic wounds, it would be useful to construct a chronic wound model on which these methods can be tested. There is no animal chronic wound model, as animals do not suffer from chronic wounds due to effective skin contraction and the relative absence of aged animals (Mustoe, 2004). Methods of wounding animals to try and reproduce *in vivo* models has been attempted using various and ingenious ways, from skin banding in guinea pigs (Constantine and Bolton, 1986) to rabbit ear models (Ahn and Mustoe, 1990). Models to reproduce ischaemia have also been trialled ranging from the use of flap surgery (Quirinia *et al.,* 1992) to decrease blood supply, to the injection of chemotherapeutic agents into the dermis to produce localised ischemia (Rudolph *et al.,* 1979). *In vitro* methods include the production of biofilms from wound isolates using the Constant Depth Film Fermenter (CDFF;Hill et al., 2010) and the Lubbock Chronic Wound Biofilm Model (Sun *et al.,* 2008).

Despite the work outlined above, to date there is no recognised standard model to use for the study of chronic wounds. In this thesis, efforts have been made to collect wound fluid eluted from chronic wounds and acute wounds and then characterise the chronic wound microflora. A variety of approaches are used to characterise the bacterial communities, with focus on detecting *S. aureus* and *P. aeruginosa* within chronic and acute wound fluid.

Bacterial species in chronic wounds

As previously described in the literature review (Chapter 2 section: bacterial colonisation vs. wound infection), no chronic wound is sterile; all chronic wounds have a bacterial component ranging from simple colonisation to an aggressively invasive biofilm, and systemic sepsis (Edwards and Harding 2004). The diverse microflora present within many chronic wounds includes both anaerobic and aerobic species (Price *et al.,* 2009). These bacteria originate from skin, oral mucosa, enteric tract, and the wider environment, and are significantly different from populations found on normal skin. The microbial populations are thought to vary between different types of chronic wound (Martin, 2009, Davies *et al.,* 2004). The effect of these bacteria is dependent on many factors including species, composition, concentration, and host response (Frank *et al.,* 2009). The role of these organisms in impairing wound healing is complex and may include both direct and indirect mechanisms (Stephens, *et al.,* 2003, Wall, *et al.,* 2002). See Chapter 2, (section: Mechanism of Bacteria promoting chronicity) for more detail.

Many bacteria have been identified from chronic wounds; and traditionally these have been isolated through culture in an agar medium under aerobic and anaerobic conditions, however, isolation of bacteria in this manner does not identify the full range of bacteria present. Despite the fact that new organisms are being continuously isolated, it is estimated that only a small fraction of all microorganisms have been grown in pure culture and isolated (Botarri *et al.,* 2006). There is a loss of information not just of number of species but also their prevalence, and the architecture and structure of the bacterial communities being identified. Indeed some studies using molecular techniques demonstrate that only 1% of bacteria can be grown in culture (Martin, 2009) and molecular

analysis reveals an average of 10 species per chronic wound, approximately four times more than demonstrated with culture (Martin, 2009). Interestingly normally cultured bacteria were often found only through molecular methods raising the question of competition and overgrowth, with sessile growth states complicating the picture further (Davies *et al.,* 2004).

Chronic wounds support a wide variety of microbial flora due to the different microenvironments present within the wound bed and its accessibility to organisms. A 2007 study (Davies *et al.,*) showed that the predominant organisms in chronic wounds from both surface swabs and tissue biopsies were *Staphylococcus* species and *P. aeruginosa*, present in 71.2% of patients. In bacterial profiling studies (Gjodsbol *et al.,* 2006) found that chronic venous leg ulcers harboured *S. aureus* in 93.5% and *P. aeruginosa* in 52.2% of cases. In view of this predominance, their pathogenicity and biofilm forming ability, it was decided to concentrate on these species for identifying ways of better characterising bacterial communities in chronic wounds.

Staphylococcus aureus **in wounds**

Staphylococcus aureus are characterised by golden yellow circular colonies, and β-haemolysis of blood agar. The coagulase-positive, *S. aureus* is a leading human pathogen with infections ranging from minor self-limiting skin infections to invasive and life threatening disease. *Staphylococcus aureus* is recognised to be a prolific biofilm former, and widely present within chronic wounds (Oliveira *et al.,* 2003).

Staphylococcus aureus is the most commonly isolated bacteria from chronic wounds (Davies *et al.,* 2004, James *et al.,* 2008). There are varying estimates as to its prevalence ranging

from 60% (Kirketerp-Moller *et al.,* 2008) to one study demonstrating *S. aureus* in 93.5% of all investigated chronic venous leg ulcers (Gjodsbol *et al.,* 2006).

***Pseudomonas aeruginosa* in wounds**

P. aeruginosa is a gram negative rod shaped motile aerobic bacteria. It is commonly found on non-sterile areas of healthy individuals and is a normal constituent of human microflora. *P. aeruginosa* are opportunistic pathogenic bacteria widely known to cause chronic biofilm based infections (Fazli *et al.,* 2009); it has been demonstrated to be present in 52.2 % of chronic venous leg ulcers.

*P. aeru*ginosa is a recognised prolific biofilm former, most commonly studied in cystic fibrosis sufferers (Bjarnsholt *et al.,* 2009) and are very resilient to both host defences and antimicrobials (Yang *et al.,* 2008). The ability of *P. aeruginosa* to form EPS encapsulated biofilms resistant to polymorphonuclearneutrophils (PMNs) is thought to be its main survival strategy for hostile human environments (Bjarnsholt *et al.,* 2008)

P. aeruginosa biofilms predominate in those patients who have undergone recent antibiotic therapy shifting the profile of relatively diverse wounds to *P. aeruginosa* dominated colonies (Price *et al.,* 2009). The ability of *P. aeruginosa* to form biofilms, and be resistant to antibiotic eradication, has led to it being hypothesised that *P. aeruginosa* biofilms are the main cause for resistance to host defences and the chronic inflammatory states seen in many chronic wounds, and that their ability to neutralise PMNLs lead to a more attractive environment for other opportunistic bacteria to thrive within the chronic wound (Bjarnsholt *et al.,* 2008).

Wound fluid

Wound fluid or exudate was first described by the Swiss physician Paracelsus in the sixteenth century as “nature's balsam” (Haeger, 1925). Wound fluid is produced as a response to the presence of a wound via the inflammatory process, leading to extravasation of serum. It has a high protein count and specific gravity of 1.020 (White and Cutting, 2006). The production of exudate is part of the normal wound healing sequence in acute wounds. When a wound becomes chronic either through infection or inflammation the exudate has a different composition with proteolytic enzymes and components not seen within acute wounds (White and Cutting, 2006).

Collecting wound fluid was important in this study in order to investigate the bacterial characterisation profiles in more detail; any future bacterial detection system used for research or clinical practice would need to operate effectively in an environment dominated by wound fluid.

Chronic wound fluid

Chronic wounds are different to acute wounds; there is an increased inflammatory response, vascular permeability, and often an infectious state. As previously stated it is felt that bacteria and especially biofilms can up regulate the inflammatory response increasing the volume of exudate considerably. Therefore it can be expected that chronic wound exudate will also be different in both volume and composition to acute wounds.

Several studies have looked at the composition of chronic wound fluid (Trengrove *et al*., 1996, Wysocki *et al*., 1993). Prior to these studies it was assumed that fluid consisted

entirely of tissue or extracellular fluid and was dismissed as modified serum of little interest to wound healing. Recent studies on the composition of chronic wound fluid have shown it is in fact a complex 'soup' consisting of electrolytes, glucose, growth factors, cytokines, proteinases, and adhesion molecules, bacteria, immunoactive cells, and molecules of the extracellular matrix such as MMPs (matrix metalloproteinases) and neutrophil elastase (Beidler *et al.,* 2008, Moor *et al*., 2009).

MMPs influence cellular functions by altering the microenvironment around cells affecting cell migration through matrix degradation (Vu and Werb, 2000). Growth factors are released by selected subsets of cells and usually have a trophic effect upon cells in the wound, however it has been suggested that they may indirectly mediate the inflammatory response, as the many pro inflammatory cytokines do (Baker and Leaper, 2000*,* Moor, *et al.,* 2009). This fluid also contains neutrophils, polymorphonuclear leukocytes, macrophages, and foreign body giant cells. The proteases present in the wound fluid are capable of degrading every known constituent of soft connective tissue, and it has been suggested that chronic wound exudate can be regarded as a wounding agent in its own right (Moor *et al.,* 2009, Palolahti *et al.,* 1993). An imbalance between proteases and their inhibitors may be contributory to the chronic wound state (Grinnell and Zhu, 1996, Rao *et al.,* 1995).

Chronic wound fluid also contains bacteria of many different species, the most common of which are *Staphylococcus* and *Pseudomonas* species. Studies have consistently demonstrated colony counts to be higher in wound fluid than in wound tissue from the same wound (Breuing *et al.,* 2003).

Collection of chronic wound fluid

Chronic wound fluid is not easy to collect. Despite a relatively large population of people presenting with chronic wounds and a significant proportion of these with heavy exudate causing significant excoriation, it has always proved difficult to collect wound fluid in the significant quantities required for reproducible experiments. There is a lack of consensus in the many methods of collection of wound fluid with no singular universally accepted method (Bowler *et al.,* 2001). Many methods of collecting wound fluid have been described from catching wound fluid dripping from the wound in a sterile container to using occlusive dressings, centrifuging them and collecting the supernatant. Several groups have tried aspirating with a sterile needle from under an occlusive dressing (Rayment *et al.,* 2008, He *et al.,* 1999). Typical amounts collected were 50-200µls per wound in recent studies (Moor *et al.,* 2009). In view of these difficulties, it was decided to collect wound fluid from chronic wounds using the catch and release system, and to dilute it in processing with TRIS (hydroxymethyl aminomethane) buffered saline to ensure the collection of sufficient quantities that would allow meaningful and repeatable experimentation, while accepting that this fluid was more dilute than concentrated wound fluid collected using alternative methods. This process would dilute the numbers of bacteria found with within chronic wound samples and the concentration of electrolytes, glucose growth factors cytokines and MMPS. However, this approach would dilute all of these equally and provide enough fluid for meaningful repeatable experimentation.

Acute Wound Fluid

In view of the difficulties previously described in collecting large volumes of chronic wound fluid, it was felt that a chronic wound fluid substitute would be beneficial for analysis. Acute wound fluid was considered to be sufficiently similar to provide an analogous fluid for chronic wound fluid. The most prolific source of acute wound fluid available was deemed to be post mastectomy wound fluid. Mastectomy is a commonly performed operation, and is considered 'clean' surgery and representative of an acute wound (Baker *et al.,* 2008). The surgery leaves a relatively large area which is enclosed and can be drained effectively by a conventional tube drain, or vacuum drainage system. Post mastectomy fluid is available in large quantities post operatively and for several days/weeks following the procedure. It is worth noting that fluid may be of varied with likely sources being post-operative blood, exuded tissue fluid, and lymphatic channel origin (Baker *et al.,* 2008).

The composition of growth factors and MMPs in acute wound fluid has been shown to be highly variable between samples (Baker *et al.,* 2008). Different levels of growth factors have been identified on various post operative days, with growth factors peaking on post operative day (POD) one and then declining, consistent with the acute inflammatory response. MMPs tended to be lower on POD 1 and peak on POD 2 to 3 (Baker *et al.,* 2008). Acute wound fluid can also be described as bloody, serous, as well as ranging between these two descriptors, unsurprisingly bloody samples have been shown to contain more growth factors. Interestingly, the more serous samples appear to contain more MMPs (Baker *et al.,* 2008).

Acute wound fluid is not analogous to chronic wound fluid, it is macroscopically different and is more likely to be bloody or serosanguineous. Acute wound fluid is also different in its composition of growth factors and proteolytic enzymes. In the first 43-72 hours, platelets and fibrin may be present which are not present in chronic wound fluids (White and Cutting, 2008). Comparisons have also shown differences in neutrophil activity. For example, it has been demonstrated that fluid from some venous leg ulcer patients (but not fluid from mastectomy patients) contain high neutrophil elastase activity (Hoffman *et al.,* 1998). Despite not being analogous with chronic wound fluid acute wound fluid is readily available in large amounts, and consists of a soup of extracellular fluid growth factors and MMPs, whilst far from being identical, this similarity makes it a useful alternative to perfect methods prior to chronic wound fluid testing.

Wound imprints

An imprint of a wound was produced by transfering a dressing which had been placed directly on a wound on to a piece of sterile blotting paper. This paper subsequently absorbed the wound fluid and debris. Wound imprints were also collected to allow further information for the development of a wound model. It was considered that the collection of wound imprints would also be of value in the development of any future wound diagnostic method, which theoretically could be embedded within dressings. Any diagnostic embedded within a dressing would have to be effective in detecting bacteria through the fluid and material transferred from the wound to the dressing, therefore wound imprints provide an accurate model of conditions in which any potential dressing based diagnostic would function.

Debrided wound tissue

In order to provide further detailed characterisation of bacterial presence across a range of clinical samples that may be easily collected in an outpatient setting, samples of debrided tissue were also collected. Punch biopsies have previously been utilised for the recovery of bacteria (Gardner *et al.,* 2006), and for the detection of biofilms (Malic *et al.,* 2009). Whilst infected wounds are frequently surgically debrided, to the author's knowledge no previous microbiological studies have been performed on these readily available and potentially useful tissue samples.

Polymerase chain reaction (PCR) analysis of microbial composition in wounds

PCR is a molecular technique that can be exploited for the rapid and sensitive detection and identification of bacteria in a wound. The method relies on the use of oligonucleotide primer pairs that are specific to bacterial DNA sequences. The targets for the primers are selected based on established DNA sequences of target microorganisms or may be generic allowing the detection of a wide range of microorganisms. DNA is extracted from an appropriate sample and this provides the template for PCR. Repeated cycles of primer annealing, primer extention (DNA synthesis mediated by *Taq* polymerase and incorporation of nucleotide bases) and DNA denaturation, permits the exponential amplification of the targeted DNA. As a result, even targets that are in low abundance within a sample can be amplified to a detectable level, thereby offering great sensitivity. The fact that bacterial detection by PCR is not reliant on cell viability means that even those microorganisms that are 'unculturable' using conventional methods can be analysed. This is important as it has been estimated that as few as 1-2% (Dowd *et al*., 2008, Martin, 2009) of bacteria within chronic wounds are culturable by conventional methods. Standard PCR techniques are,

however, limited as they are not really quantitative and therefore give no information on numbers of target microorganisms present nor the spatial distribution of the bacteria within the wound (Amann *et al*., 2001). Other limitations include the lack of information provided on antibiotic resistance, potential sequence variability between strains of the same species and the fact databases do not include sequences of newly discovered microorganisms (Schreier *et al*., 1997). In this chapter, PCR was performed on chronic wound fluid samples specifically targeting *P. aeruginosa* and *S. aureus* DNA within the samples. The use of a universal bacterial PCR targeting 16S rDNA gene sequences as added confirmation of bacterial presence or absence from the collected samples.

Aims

The aims of the chapter were to provide data on the collection, microbiological typing, and storage of clinical samples from chronic and related wounds. These samples were collected to provide a suitable bank of samples to test different methods of characterising bacterial communities in chronic wounds and to provide the components to recreate key parts of the chronic wound environment.

Materials and Methods

Various sample types were collected for this research. All sample sources were from unique patients, and no patient was sampled more than once in the same category, nor were patients sampled in two different categories (such as in chronic wound fluid and debrided wound tissue).

Collection of chronic wound fluid

All samples were taken from a cross section of unique patients with chronic wounds presenting with heavy exudate. Ethical approval was granted and informed consent was obtained (see Appendix 1) to collect samples of chronic wound fluid from recruited patients from the wound healing department of a large teaching hospital within an NHS Trust. Prospective patients were given an information sheet (see Appendix 2) and an appointment was made for them to attend the research clinic at the University Hospital. After information about the study was presented and full written informed consent was obtained (Appendix 1), the wounds were then dressed with RELEASE^{TM} (Systagenix Wound Management Limited, Gatwick, UK) dressings and wrapped in cling film, to prevent moisture loss, for four hours. RELEASE^{TM} (Johnson and Johnson Medical Limited, Ascot, UK) dressings were used as these consist of gauze with a non-adherent layer, and have no reported evidence of bacterial sequestration. Dressings were placed into sterile containers using sterile forceps and these were in turn placed in a secure and appropriately labelled icebox for transportation to the laboratory. The majority of samples were collected in the outpatient clinic, however, three samples were collected from patients at home, as this was more convenient for the patients concerned. All samples collected in and out of the

hospital environment were transported in sealed sterilised containers within iceboxes containing carbon dioxide pellets for no longer than 30 minutes until processing in the laboratory.

On arrival in the laboratory, the samples were removed from the sterile containers and the dressings were cut into 5 cm x 5 cm squares, these were placed in a sterile Petri dish with 12.5 ml of TRIS (hydroxymethyl aminomethane) buffered saline with Tween (Wound fluid elution buffer; 0.1M Tris-HCl buffer, pH 7.4, containing 0.1% Triton X-100) and agitated on a tilt board at a medium speed for at 4°C for 4 hours. The samples were then returned to the laboratory and with sterile forceps the fluid was squeezed out of each dressing; the eluted fluid was then recombined in a sterile bottle. A 100 µl volume of the chronic wound fluid was serially diluted and plated out on blood, MSA (Mannitol Salt Agar), and *Pseudomonas* agar. These plates were incubated for 24 hours at 37°C and colonies counted to give a reading of bacterial load in each sample. Each isolate was then subjected to classical microbiology and PCR to aid speciation. The remaining fluid was then aliquoted into falcon tubes for storage at -80°C in a locked Human Tissue Authority approved freezer. For a full flow chart of chronic wound fluid collection please see fig 3.0 below.

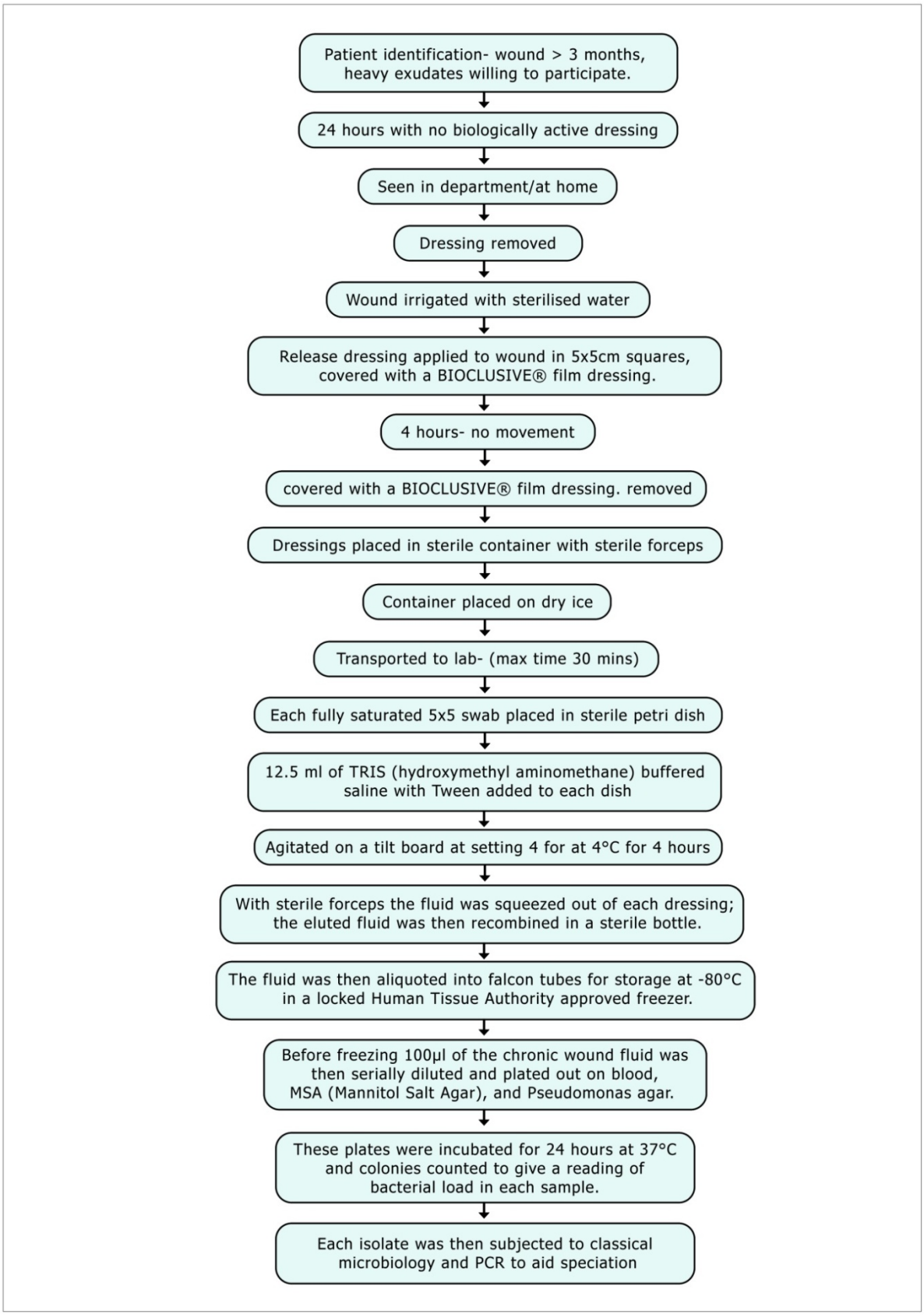


Fig 3.0 Flow chart of collection of chronic wound fluid

Collection of acute wound fluid

Ethical approval was granted and informed consent obtained to collect samples of post mastectomy drain fluid from patients in a large NHS teaching Hospital under the care of a single consultant breast surgeon (Appendix 3). Patients were approached prior to their mastectomies; patient information sheets were given (Appendix 4) and questions answered. On postoperative day one appropriate patients were approached, information reiterated, and written informed consent taken. All patients were sampled on day one and day two post operatively.

Inclusion criteria consisted of a consecutive cohort of patients undergoing mastectomy by a consultant breast surgeon in a large NHS teaching hospital between May and December 2008. No one was excluded except at their own request. Acute wound fluid samples were taken from nine patients on post operative day one and post operative day 2 following mastectomies performed. Most patients had 2 drains and therefore two samples were collected on day one labelled A and B, and 2 samples on day 2 labelled C and D. Patient 4 only had one drain and was therefore only labelled A for day one and B for post operative day 2. One patient refused on moral grounds to enter the study, as antibodies to be used later on the fluid were developed in animals. She has been referred to as patient number 10 in all the tables for comparison to the selected group and her samples safely discarded according to her wishes.

Mastectomy drain fluid bags were removed from the drains, the amount of collected fluid was recorded in the medical and nursing notes, and empty bags fitted to the drains with aseptic technique. The wound fluid bags were then placed in a secured labelled transport

container chilled with dry ice to approx 4°C. On return to the laboratory these samples were decanted into falcon tubes in 5-ml aliquots and stored in a locked -80°C Human Tissue Authority approved freezer.

For the purposes of identification in analysis, samples were labelled 1-9 (patient number 10s samples were not analysed). Most patients had 2 drains so samples from these on post operative day one were labelled *A* and *B*, and on post operative day 2, labelled *C* and *D.* Therefore, samples for each donor were divided into *A, B, C* and *D* except sample 4 in which the donor had one drain only and thus only *A* and *B* samples, a total of 34 samples.

Acute wound fluid (100 µl) from each sample was serially diluted and plated on to blood agar, MSA, and *Pseudomonas* agar. These agars were then incubated for 24 hours at 37°C and resulting colonies enumerated to determine bacterial load in each sample. Each isolate was then subjected to traditional microbiological identification procedures and PCR to allow identification.

Collection of wound imprints and swabs

Inclusion criteria included a wound duration of greater than one year, and all wounds would have been dressed with simple hydrofibre dressing, so no dressing components would be present to affect the imprints.

Wound dressings were collected from patients attending the wound healing outpatient's clinic in a major NHS teaching hospital in accordance with the ethical approval. An information sheet was given and full informed consent taken (Appendix 2). Only patients

with plain Hydrofibre*(Convatec Limited, Uxbridge UK) dressings that were soaked through with exudate were approached to participate in the study. Wound imprints were collected exclusively from patients with chronic wounds which were judged to have dressings which were wet secondary to high exudate, and would give a good imprint onto blotting paper. These wounds were all dressed with plain Hydrofibre dressings as this retains a large amount of wound fluid and is amenable to imprint. Each patient's wound(s) was swabbed and samples placed in a labelled sterile dry-ice filled secure container and transported to the laboratory.

Dressings were imprinted on to clean filter paper in sterile conditions; the dressings were then appropriately discarded. The filter paper was dried overnight in a sealed container and stored in a -80°C freezer in accordance with the Human Tissue Act. Only filter paper, which had become saturated on both sides was removed with the hole punch for further testing from the frozen samples.

Swabs were used to inoculate Blood agar, MSA and *Pseudomonas* agar and incubated at 37°C overnight. Colonies were then Gram-stained and tested for catalase, oxidase and coagulase activity as previously described. Bacterial isolates were then stored as described previously.

Collection of debrided wound tissue and swabs

Debrided tissue was collected from patients attending the wound healing outpatients clinic in a major NHS teaching hospital in accordance with the ethical approval obtained (see Appendix 5). An information sheet was given and fully informed written consent taken.

Only patients with infected wounds at the time of review requiring sharp debridement in the normal course of treatment were considered for participation in this part of the study. No patient underwent sharp debridement solely for the purposes of this study. Debridement occurred following the application of a topical local anaesthetic EMLA (Eutectic Mixture of Local Anaesthetics containing lidocaine and prilocaine, APP Pharmaceuticals) for thirty minutes; debridement was sharp to bleeding tissue. Each patient's wounds were swabbed prior to application of local anaesthetic and debridement. Swabs were placed in an ice filled labelled secure container and transported to the laboratory. Debrided tissue was placed straight into formalin for fixation prior to paraffin embedding by standard histological technique.

Method of Microbiological Identification

AGARs: the following standard methods were used to ensure that the samples were treated in a systematic and consistent way.

MSA-OXOID Mannitol Salt Agar - 111g was suspended in 1 L of distilled water and brought to the boil to dissolve completely. The resultant liquid was sterilised by autoclaving for 15minutes at 121°C and then cooled in a water bath (Laboratory thermal engineering) at 56°C. It was then poured into aseptic Petri dishes and cooled further, stored at 4°C.

PSEUD-LAB M LAB 108 *Pseudomonas* agar base- 48.4g was suspended in 1 L of distilled water soaked for 10 minutes- swirled to mix and added to 10 ml of glycerol sterilised by autoclaving for 15 minutes at 121°C cooled to 47°C and 2 vials of LABM supplement X107 (Cetrimide, Nalidixic acid) diluted in 5 ml of sterile water, and the solution then aseptically dispensed into Petri dishes.

BLOOD-LAB M Blood agar base- 39.5g was dispersed in distilled water soaked for 10 minutes, and swirled to mix, then autoclaved for 15 minutes at 121°C, cooled to 47°C in a water bath, and 5% of defibrinated sheep blood, was mixed in and the solution then aseptically dispensed into Petri dishes.

Gram Stain-protocol

A loop of the liquid culture was transferred to the surface of a clean glass slide, and spread over a small area. For staining of material from a culture growing on solid media, a loop of sterile water was placed on a slide; using a sterile cool loop transfer a small sample of the colony was added to the drop, and emulsified. The film was allowed to air dry. The film was then fixed by passing it briefly through the Bunsen flame two or three times without exposing the dried film directly to the flame. The slide was kept cool so as to not be uncomfortable to the touch.

The slide was flooded with crystal violet solution for 1 min and washed briefly with tap water (no longer than 5 seconds). The slide was then flooded with Gram's Iodine solution, which was allowed to act (as a mordant) for 1 min and then washed off with tap water. Excess water was removed from the slide and the slide blotted, so that alcohol used for decolourisation was not diluted. The slide was then flooded with 95% alcohol for 10 s and washed with tap water. The slide was then flooded with safranin solution and allowed to counter stain for 30 seconds, and washed off with tap water. The slide was then drained and blotted dry with bibulous paper. Slides were were examined by standard light microscopy under oil immersion lens (x1000).

Coagulase test

Coagulase-Oxoid Diagnostic reagents staphylase test (Oxoid Limited Basingstoke, United Kingdom) was used in detecting free and bound coagulase (or clumping factor) which is characteristic of *Staphylococcus aureus*.

Test and control reagents were shaken vigorously to obtain a homogenous suspension. Any reagent cells that were trapped in the dropping pipette were mixed into the suspension. A loop was used to smear 1 to 3 of the suspect colonies on a test circle and a control circle on the Reaction Card. One drop of the Test Reagent was added to the test circle and 1 drop of Control Reagent to the control circle. The contents of the test circle were mixed using a loop. The loop was flamed and then used to mix the contents of the control circle. The circles were observed for agglutination while mixing and the results were recorded. The Reaction Card was safely disposed of in to disinfectant. A positive result was obtained if clumping of the test cell suspensions occurred during mixing and a presumptive identification of *Staphylococcus aureus* was then made. Results were not interpreted if there was any clumping of the control cell suspension.

Oxidase Test

The Oxidase Test (Sigma-Aldrich) consisted of: N,N,N,N,-tetramethyl-p-phenylenediamide dihydrochloride (minimum 95%)- diluted in phosphate buffered saline to 1% concentration on filter paper strips. A sterile inoculating loop was used to transfer a portion of an isolated colony from the agar on to the oxidase disk provided. The reaction was observed within 2 minutes at 25-30°C. If *P. aeruginosa* was present a positive reaction was observed with the

disc changing colour to a deep purple. The result was recorded after 2 minutes of observation.

Catalase Test

A solution of 3 % aqueous hydrogen peroxide, protected from light, on a glass microscope slide was used. Catalase converts hydrogen peroxide into water and oxygen. The catalase test is commonly used to differentiate streptococci (negative) from staphylococci (positive). Using a sterile loop, cells from an isolated colony were transferred to a glass slide. One to 2 drops of 3% hydrogen peroxide was added. Positive results for the presence of catalase were evident by the rapid and sustained appearance of effervesence, 2 minutes after addition of bacterial cells. Positive *S. aureus,* and negative *Streptococcus sp.* controls were used as references for the experiment.

Storage of microbial wound isolates

Microbial Wound isolates were stored using a solution of 25 coloured beads packed in cryo preservative (microbank Pro-lab diagnostics). Fresh monocultures (<36 h) were cultured on *Pseudomonas* agar and MSA, and incubated at 37°C. Using a permanent marker the vials were coded with the origin of the specimen and microorganism to be stored. Under aseptic conditions microbank beads were inoculated with a single colony of pure growth bacteria. Vials were then closed tightly and inverted 4-5 times to emulsify the organisms, at this point the microorganism would have been bound to the porous beads. The vial was reopened and the excess cryopreservative aspirated using a pipette and sterile tip. The inoculated microbank beads were then stored at -80°C. .

Polymerase chain reaction for detection of *S. aureus* and *P. aeruginosa* in chronic wound fluids

Samples of chronic wound fluid collected and stored as previously described were subjected to PCR to identify the presence or absence of *S. aureus* and *P. aeruginosa*. Total DNA extraction was based on the use of the PUREGENE DNA Purification Kit with DNA purification from 1 ml Gram-positive bacteria with an expected yield 6-60 µg DNA.

Cell Lysis

Chronic wound fluid (1-ml) was added to a 1.5 ml tube on ice. This was then centrifuged at 13,000-16,000 x g for 5 s to pellet cells (for some species, centrifugation for up to 60 s was required to obtain a tight cell pellet). As much supernatant as possible was removed using a pipette. Six hundred µl of Cell Suspension Solution was added to the cell pellet and gently resuspended using a pipette. 3.0µl of Lytic Enzyme Solution was added and the tube inverted 25 times to mix. The solution was then incubated at 37°C for 30 min to digest cell walls. The sample was inverted occasionally during the incubation period. The solution was then centrifuged at 13,000-16,000 x g for 1 min to pellet the cells. The supernatant was removed and 600 µl of Cell Lysis Solution was added to the cell pellet and gently resuspended using a pipette to lyse the cells.

RNase Treatment

RNase A Solution (13.0 µl) was added to the cell lysate and the sample mixed by inverting the tube 25 times. The preparation was then incubated at 37°C for 15-60 min.

Protein Precipitation

The sample was cooled to room temperature and 200 µl of Protein Precipitation Solution was added to the cell lysate. The sample was then vortexed at a high speed for 20 s to mix the protein precipitation solution uniformly with the cell lysate (for species with a high polysaccharide content, placing the sample on ice for 15-60 min was required). The sample was then centrifuged at 13,000-16,000 x *g* for 3 min. The precipitated proteins formed a tight white pellet (if the tight protein pellet was not formed, Step 3 was repeated followed by repeated incubation). The sample was then placed on ice for 5 min and then recentrifuged at 13,000-16,000 x *g* for 3 min.

DNA Precipitation

The supernatant containing the DNA was decanted (leaving behind the precipitated protein pellet) into a clean 1.5 ml microfuge tube containing 600 µl 100% isopropanol (2-propanol). The sample was mixed by inverting gently 50 times. The sample was then centrifuged at 13,000-16,000 x *g* for 1 min; at which point the DNA became visible as a small white pellet. The supernatant was removed and the tube drained briefly on clean absorbent paper. Ethanol (600 µl of 70%) was added, and the tube inverted several times to wash the DNA pellet. The solution was then centrifuged at 13,000-16,000 x *g* for 1 min, and the ethanol carefully removed. The tube was inverted and drained on clean absorbent paper and allowed to air dry 10-15 min.

DNA Hydration

DNA Hydration Solution (200 µl) was added (200 µl will give a concentration of 100µg/ml if the yield is 20µg DNA). The DNA was then rehydrated by incubating the sample for 1 h at

65°C and/or overnight at room temperature. The tube was tapped periodically to aid in dispersing the DNA; the DNA was then stored at 4°C.

Detection of bacterial species by species specific PCR

Species-specific PCRs was used to detect two opportunistic pathogens, *Staphylococcus aureus* and *Pseudomonas aeruginosa.* PCR was undertaken in volumes of 25 µl. In the case of *S. aureus*, Each reaction consisted of PCR buffer (x10), template DNA (5 µl), *Taq* polymerase (1.5 units), and 5 µM of each PCR primer. The primers for *S. aureus* were Vick1 and Vick2 Primer designs taken from Liu et al, 2007.

- S.aur_vicK1: 5'-CTA ATA CTG AAA GTG AGA AAC GTA-3'
- S.aur_vicK2: 5'-TCC TGC ACA ATC GTA CTA AA-3'

The reaction mix was denatured for 5 min at 94^{o}C, before undergoing 35 cycles of 94^{o}C for 40 s, 50^{o}C for 40 s and 72^{o}C for 1 min, prior to a final single 10 min cycle at 72^{o}C. For *P. aeruginosa*, the reaction mix was identical but included the primer pair of ECF1 and ECF2 Primer designs taken from Lavenir *et al*, 2007

- Ps.aeru_ECF1: 5'-ATG GAT GAG CGC TTC CGT G -3'
- Ps.aeru_ECF2: 5'-TCA TCC TTC GCC TCC CTG -3'

(Lavenir *et al.,* 2007; Table 10). PCR cycling consisted of a single denaturation cycle of 95^{o}C for 5 min, before undergoing 35 cycles of 94^{o}C for 45 s, 58^{o}C for 45 s and 72^{o}C for 1 min, before a final 5 min at 72°C.

The subsequent PCR products were electrophoresed in a 1.5% (w/v) agarose gel containing 1 µl of ethidium bromide within a 1× Tris-acetate-EDTA (TAE) buffer comprising 40 mM Tris,

20 mM acetic acid and 1 mM EDTA buffer at 40 V/cm^2 for 45 min. The products were run alongside a standard 100 base pair marker DNA ladders (Promega). PCR products were then visualised using ultraviolet light in a GelDoc system (Bio-Rad, Hemel Hempstead, UK). PCR for *S. aureus* and *P. aeruginosa* in chronic wound fluids were repeated 3 times to ensure reproducibility.

Results

Overview of collected samples

Table 3.0 summarises the clinical samples used in this research. All of these samples were subjected to microbiological testing specifically to identify the presence and absence of our target bacteria *S. aureus* and *P. aeruginosa* as is illustrated later in this section.

Sample type	Number collected	Label used to identify	Applied analysis
Chronic wound fluid	10	CHR1- CHR10	Competitive ELISA DNase Agar
Acute wound fluid	9	Acute wound fluid 1-9	Competitive ELISA
Debrided chronic wound tissue	3	DWT 1- DWT3	PNA CLSM FISH
Chronic wound imprints	5	WD1-WD5	Modified competitive ELISA

Table 3.0 Demonstrating samples collected, nomenclature and experiments utilised in throughout the thesis.

Chronic wound fluid collection

In total 10 chronic wound fluid samples were taken. The participants ages ranged from 64 to 90 years (table 3.1), with a mean age of 75.4 years being largely representative of the ageing population most commonly affected by chronic wounds. For purposes of identification of samples they were labelled CHR1-10. Duration of these chronic wounds ranged from 10 months to 27 years (table 3.1), with a mean duration of 99.5 months (8.3 years)

Study number	Age in years	Location of wound	Size of wound	Cause of wound	Duration of Wound in Months
CHR1	90	Right ankle	Semi Circumferential	Unknown	36
CHR2	67	Right ankle	Circumferential	Unknown	13
CHR3	85	Right legs	Circumferential	trauma-burns in accident	216
CHR4	64	Both legs	Circumferential	Unknown	18
CHR5	69	Left ankle	Circumferential	insect bite, venous disease	216
CHR6	80	Both legs	Medial malleolus	venous disease	180
CHR7	81	Right leg	Circumferential	Arterial	10
CHR8	64	Both legs	Semi Circumferential	venous disease	48
CHR9	75	Both legs	Semi Circumferential	venous disease	17
CHR10	79	Right leg	Semi Circumferential	venous disease	138

Table 3.1 Chronic wound Fluid donor patient information

The wounds were all located on the lower legs with 1 being confined to the malleolus, 4 were semi-circumferential and 5 were circumferential wounds (table 3.1). Of these wounds, 4 were bilateral, 5 were right leg only and 1 was left leg only (table 3.1). The aetiology of the ulcers was venous disease in 4, traumatic in 2, arterial in 1, and 3 of unknown aetiology (table 3.1). Despite an N of only 10, the donor's wounds were representative of those most often seen in clinical practice in terms of wound location and aetiology.

Of these wounds, 3 were assessed using an experienced wound healing clinician as being currently locally infected, and the other 7 were not considered locally infected (table 3.2). All three of the patients whose wounds that had been assessed as infected were taking oral antibiotics at the time of wound fluid sampling; the antibiotics taken were as listed in table 3.2. The time of the last course of antibiotics taken was recorded as it is possible that this could have affected the wounds ecology (table 3.2). It must be noted that this information was based on patient recollection and may be unreliable. Prior use of antibiotics was a median of 56 days and mean of 74 days previous to sampling. It should be noted that participant CHR2 was also using potassium permanganate soaks, a topical treatment effective against *Pseudomonas* species, and CHR8 was using Betaine Polyhexanide irrigation solution and gel with antibacterial and antiseptic properties, both of which could be considered likely to affect the wounds ecology.

Study number	Currently Infected	Most recent antibiotic
CHR1	No	10 weeks ago - unsure what antibiotic.
CHR2	Yes	Currently- Augmentin and Potassium Permanganate.
CHR3	No	3 weeks ago – Ciprofloxacin
CHR4	Yes	Currently on Ciprofloxacin
CHR5	No	34 weeks ago - Flucloxacillin
CHR6	No	8 weeks ago – Trimethoprim
CHR7	Yes	Flucloxacillin
CHR8	No	3 weeks ago - intravenous Cefuroxime, Vancomycin, and Teicoplanin
CHR9	No	8 weeks ago - Cefuroxime, and metronidazole
CHR10	No	8 weeks ago - Flucloxacillin, and Benzyl penicillin

Table 3.2 Antibiotic use in patients donating chronic wound fluid

The patients' concomitant medications were recorded as various medications are known to effect wound healing directly or indirectly. It was immediately obvious (table 3.3) that the majority of the patients exhibited polypharmacy, the mean number of medications taken was 8.1, with a range from 3-13 oral medications prescribed (table 3.3). The cohort was also taking large amounts of analgesics ranging from simple paracetamol to strong opiates such as Morphine Sulphate tablets, Oxycodeine tablets and Fentanyl patches (table 3.3). The patient CHR8, was also taking Pregabalin and Amitryptaline for neuropathic pain (table 3.3). Patient CHR4 was taking oral hypoglycaemics; indicating NIDDM (Non insulin dependent

diabetes mellitus) and patient CHR8 was taking both oral hypoglycaemics and insulin indicating IDDM (Insulin dependent Diabetes mellitus) (table 3.3).

Study number	Medications
CHR1	Tramadol, Clonidine, Lisinopril, Asprin
CHR2	Simvastatin, Losaratan, Frusomide, Levothyroxine, Symbicort, Tramadol, Paracetamol, Diazepam, Oramorph
CHR3	Cyclizine, Oxycontin, Paracetamol, Omeprazole, Chloramphenicol eye drops
CHR4	Paracetamol, Tramadol, Metformin, Spironolactone, Frusemide, Salbutamol, Perindopril, Simvastatin, Asprin, Lansoprazole, Novorapid, GTN spray
CHR5	Loperamide, Warfarin, Oxycodone, Ascorbic acid, Paracetamol, Tramadol, Buprenorphine patches, Esomeprazole, Domperidone, Chlorpheniramine
CHR6	Bendroflumethazide, Asprin, Prednisolone
CHR7	Isosorbide Mononitrate, Quinine Sulphate, Atorvastatin, Temazepam, Alphacalciferol, Asprin, Bisoprolol, MST, Perindopril, Bisoprolol, Frusemide
CHR8	Metformin, Calcichew, Ensure, Paracetamol, Ferrous Sulphate, Fentanyl, Insulin, Ibersartan, Pregabalin, Amitryptaline, Bisoprolol, Lidocaine, Epiderm, Warfarin, Betaine Polyhexanide
CHR9	Salbutamol, Fluticasone, Salmeterol, Fentanyl patches, Tramadol, Paracetamol, Naproxen, Moxonidine
CHR10	Ramapril, Simvastatin, Tamsulosin, Quinine Sulphate, Co-codamol

Table 3.3 Concomitant medication of chronic wound fluid patients

Volumes of collected chronic wound fluids

Despite the difficulties experienced in other studies collecting chronic wound fluid, it was possible to collect reasonable amounts using the dilution method described in the preceding

materials and methods section. These were stored in 5 ml aliquots and where necessary in smaller quantities. The mean amount collected was 94.95 ml with a range from 45 ml to 230 ml (table 3.4)

Study Number	Volume and number of aliquots
CHR1	9 x 5 ml
CHR2	13 x 5 ml
CHR3	11 x 5 ml
CHR4	46 x 5 ml
CHR5	13 x 5 ml
CHR6	14 x 5 ml
CHR7	22 x 5 ml, 1 x 3.5ml
CHR8	22 x 5 ml
CHR9	19 x 5 ml, 1 x 1 ml
CHR10	20 x 5 ml

Table 3.4 Volumes of chronic wound fluid collected

Acute wound fluid

Significant quantities of acute wound fluid were collected in comparison to chronic wound fluid samples. It was not unusual to collect over 200 ml (×4) from each patient. A and B samples were empirically serosanguineous whereas C and D samples tended to be more serous as would be expected for fluid collected a further day post operatively.

The patients were exclusively female, consisting of three left mastectomies, four right mastectomies and two bilateral mastectomies. Mean age was 60.7 years old, and median

age of 64 years old. Two of the patients had IDDM. In terms of previous antibiotic exposure, one patient had had a recent course of topical antibiotics (table 3.5), one had a one week long course for a urinary tract infection and three patients had been given a single dose of antibiotics at induction of anaesthesia as a prophylactic measure.

All mastectomies were performed for confirmed breast cancer, except patient 8 who underwent bilateral mastectomy (surgical removal of both breasts) aged 31 as a prophylactic measure following diagnosis of a genetic predisposition, skewing our mean age range slightly. Interestingly no patients developed any post operative wound complications, such as infection, seroma or breakdown.

Patient Number	Age	Mastectomy	Recent Antibiotics	Diabetes	Rheumatoid	complications
1	74	Left	Metronidazole gel- for acne rosacea	IDDM	Nil	Nil
2	63	Right	Nil	Nil	Nil	Nil
3	82	Left	Augmentin at induction	Nil	Nil	Nil
4	50	Right- (Latissimus Dorsi flap+implant)	Augmentin at induction	Nil	Nil	Nil
5	60	Bilateral	Nil	IDDM	Nil	Nil
6	64	Right	Nil	Nil	Nil	Nil
7	72	Right	Nil	Nil	Nil	Nil
8	31	Bilateral	Nil	Nil	Nil	Nil
9	51	Left	Cefalexin 1/52 (UTI)- Augmentin at induction	Nil	Nil	Nil
10	77	Right	Nil	Nil	Nil	Nil

Table 3.5 Acute wound fluid donor information

Wound Imprints

Five patients' dressings were sampled; the majority of the wounds (80%) were located on the lower limbs, but one (20%) abdominal wound was also utilised. The cause of these wounds was 3 venous, 1 arterial, and 1 surgical (table 3.6). Samples WD1, WD2, WD4, and WD5 were all judged to be infected and as such various antimicrobials were used; topically Flamazine, Pronotosan and Trimovate, were all used (table 3.7). WD1 had also undergone a 2-week course of oral Augmentin as treatment for a localised wound infection (table 3.6).

Sample Number	Location of wound	Wound Duration	Wound Cause
WD1	Left leg and thigh	1 year	Arterial
WD2	Right Leg	over 1 year	Venous
WD3	Abdomen	over 1 year	Surgical
WD4	Right Leg	over 1 year	Venous
WD5	Left leg	over 1 year	Venous

Table 3.6 wound dressing donor information

Sample number	Infected	Topical treatments	Antibiotics	Dressing
WD1	Yes	Flamazine	Co-amoxiclav 2 weeks	Hydrofibre*
WD2	Yes	Betaine Polyhexanide	Nil	Hydrofibre*
WD3	No	Trimovate	Nil	Hydrofibre*
WD4	Yes	Nil	Nil	Hydrofibre*
WD5	Yes	Aquafoam	Nil	Hydrofibre*

Table 3.7 Wound dressing donor wound information

Debrided wound tissue

Only 3 patients were recruited due to the small number of patients seen in the clinic requiring sharp surgical debridement; all of these patients were considered to have grossly locally infected wounds with probable biofilm formation. All patients were female and all were suffering from leg wounds secondary to venous disease (Table 3.8). Four separate samples were collected from DWT1, 2 from DWT2, and 3 from DWT3

Patient	Sex	Wound duration	Location	DM/RA	Smoker	Dressings
DWT1	F	Unknown	Right circumfential	No	No	Hydrofibre* Ag
DWT2	F	7 years	Left medial and lateral malleolus	No	Yes	Hydrofibre* Ag
DWT3	F	3years	Left circumfential	No	No	Hydrofibre* Ag

Table 3.8 Debrided wound tissue donor information

Microbiological analysis

Chronic wound Fluid

The results of analysis of the chronic wound fluid samples through traditional microbiological analysis using Blood agar, MSA, and *Pseudomonas* agar are summarised in table 3.9 and Appendix 6.

CHR sample	*S. aureus*	*P. aeruginosa*	Other
1	Yes	Yes	*Corynebacterium*
2	No	Yes	CNS
3	Yes	Yes	CNS
4	Yes	Yes	CNS
5	Yes	Yes	No
6	Yes	Yes	No
7	No	Yes	CNS 2 strains
8	No	Yes	CNS
9	Yes	Yes	*Corynebacterium*
10	No	Yes	CNS

Table 3.9 Culture analysis of chronic wound samples

Acute Wound Fluid

As expected the results of the microbiology for the acute wound fluid were very different from chronic wound fluid; For the purpose of analysis, each patient's samples were split into 4 groups, referred to as A, B, C and D. Groups A and B were from separate drains in the same patient on post operative day one, and groups C and D from day two. None of the samples grew *P. aeruginosa*, so the *Pseudomona*s agar which all demonstrated no growth after 24 h have been excluded from the table of results (Appendix 6). As can be seen from table 3.10, the predominant organisms were CNS (coagulase negative staphylococci) with occasional *S. aureus* cultured. The colony numbers were very low ranging from 0 cfu/ml to 2.7×10^4 cfu/ml, with a median of 50 cfu/ml indicating that the fluid was, as might have been expected, mostly clean with only light skin commensal contamination.

Sample	Oxidase	Catalase	Coagulase	Conclusion
1A	Negative	Positive	Negative	CNS (both clear and pale)
B	Negative	Positive	Negative	CNS
C	Negative	Positive	Negative	CNS
D	Negative	Positive	Negative	CNS
2A	Negative	Positive	Positive	*S. aureus*
B	Negative	Positive	Positive	*S. aureus*
C	Negative	Positive	Positive	*S. aureus*
D	N/A	N/A	N/A	Nil
3A	Negative	Positive	Negative	CNS
B	Negative	Positive	Negative	CNS
C	Negative	Positive	Negative	CNS
D	Negative	Positive	Negative	CNS-haemolytic
4A	Negative	Positive	Negative	CNS
B	Negative	Positive	Negative	CNS- (white and yellow colonies)
5A	Negative	Positive	Negative	CNS
B	Negative	Positive	Negative	CNS
C	Negative	Positive	Positive	*S. aureus*
D	Negative	Positive	Negative	CNS
6a	Negative	Positive	Negative	CNS- (white and yellow colonies)
B	Negative	Positive	Negative	CNS
C	Negative	Positive	Negative	CNS
D	Negative	Positive	Negative	CNS
7A	Negative	Positive	Negative	CNS
B	Negative	Positive	Negative	CNS
C	Negative	Positive	Negative	CNS
D	Negative	Positive	Negative	CNS
8A	NA	NA	NA	NA
B	NA	NA	NA	NA
C	NA	NA	NA	NA
D	NA	NA	NA	NA
9A	Negative	positive	MSA→positive Blood → negative	MSA→*S. aureus* Blood → CNS
B	NA	NA	NA	NA
C	NA	NA	NA	NA
D	NA	NA	NA	NA

Table 3.10 Microbiological analysis and speciation of bacteria present in acute wound fluid samples

Wound imprints

Traditional microbiology results from the wound dressing imprints were similar to those for the chronic wound samples, with CNS present in all samples*, P. aeruginosa* present in three and possibly in the remaining two, but these were obscured by swarming *Proteus* making definitive identification very difficult. *Staphylococcus aureus* was only present in one sample in WD1 (Table 3.11)

Wound Dressing	Agar	Colony appearance	Gram stain	Coagulase	Catalase	Oxidase	Identity
1	Blood	Green colonies	Negative bacilli	-	+	+	*P. aeruginosa*
	MSA	Yellow colonies with mannitol fermentation	Positive cocci-clumps	-	+	-	CNS/*S. aureus*
2	Blood	*Proteus* swarming	Positive cocci, Negative bacilli				*Proteus*
	MSA	White colonies	Positive cocci in chains, Negative bacilli	-	+	-	CNS
3	Blood	White Colonies	Negative bacilli	-	+	+	*P. aeruginosa*
	MSA	White small	Positive cocci	-	+	-	CNS
4	Blood	*Proteus* swarming	Negative bacilli and cocci				*Proteus*
	MSA	white small	Positive cocci	-	+	-	CNS
5	Blood	White/green	Negative rods	-	+	+	*P. aeruginosa*
	MSA	1 colony yellow	Positive cocci	-	+	-	CNS

MSA, mannitol salt agar; CNS, coagulase negative staphylococci

Table 3.11 Wound dressing culture microbiology and speciation

Debrided wound tissue

Classical microbiology demonstrated the presence of P. aeruginosa in all 3 samples, *S. aureus* in two out of three and *Proteus spp* in 2 out of three, with a CNS also cultured from sample one (table 3.12).

Sample	Morphology	Gram stain	Coagulase	Catalase	Oxidase	Conclusion
DWT1	White colonies	Positive cocci	Negative	+	Negative	CNS
	Green colonies	Negative bacilli	Negative	+	+	*P. aeruginosa*
DWT2	Swarming	Negative bacilli	Negative	+	Negative	*Proteus*
	Yellow colonies	Positive cocci	Positive	+	Negative	*S. aureus*
	White/Green	Negative bacilli	Negative	+	+	*P. aeruginosa*
DWT3	Swarming	Negative bacilli	Negative	+	Negative	*Proteus*
	Yellow colonies	Positive cocci	Positive	+	Negative	*S. aureus*
	White/green	Negative rods	Negative	+	+	*P. aeruginosa*

Table 3.12 Debrided wound tissue, culture, microbiology and speciation

Polymerase chain reaction

Chronic wound fluid samples were analysed using species specific PCR and each PCR was repeated on three occasionsTypical results of PCR (Fig 3.1 and 3.2) are shown for wound fluid extracts 1- 10. *Pseudomonas aeruginosa* was detected in chronic wound fluids numbers 3, 6 and 7, whilst *S. aureus* was detected in chronic wound fluids numbers 1, 3, 4, 6 and 8 (table 3.1).

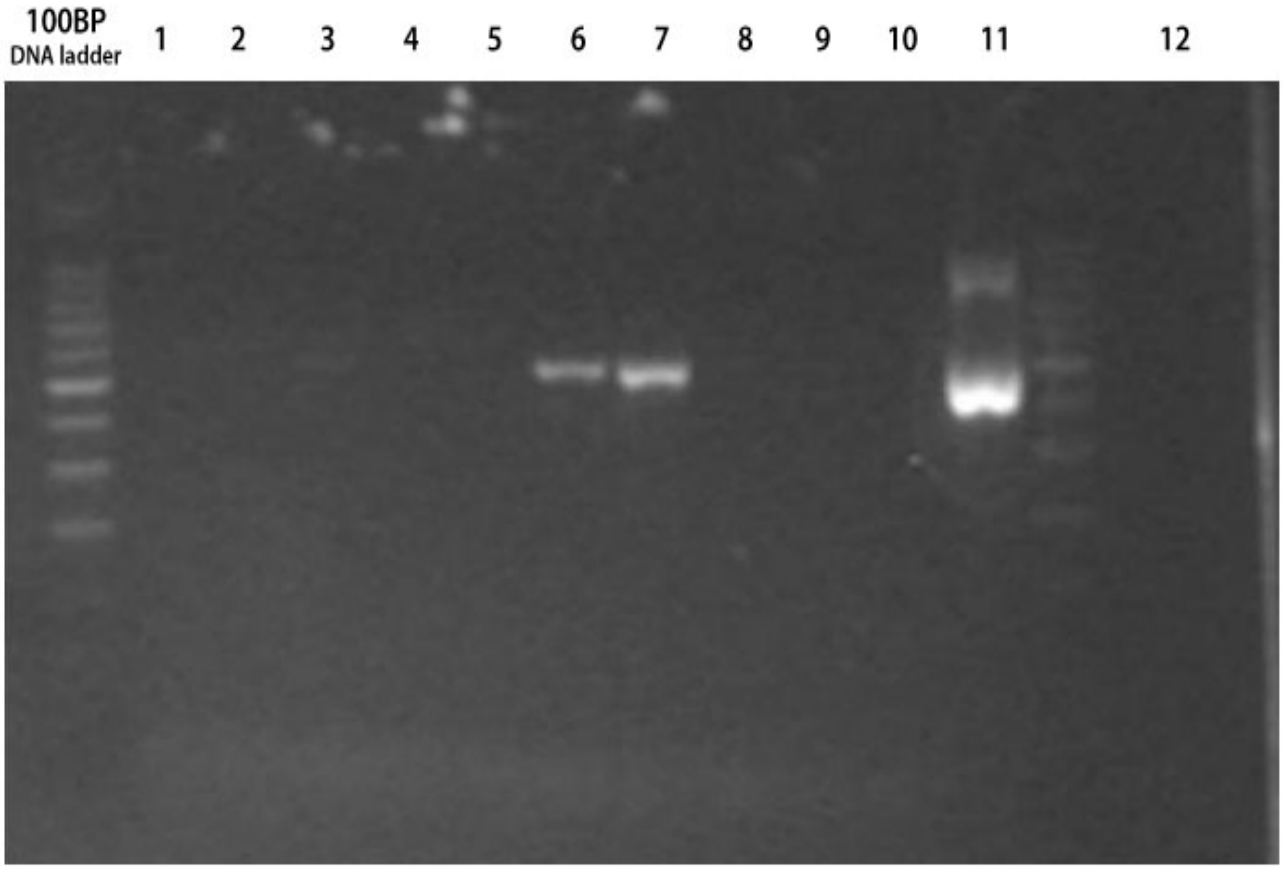


Fig 3.1 PCR of chronic wound isolates for detection of *P. aeruginosa.* Lanes 1-10 represent chronic wound fluid samples CHR1- CHR10. Lane 11 contains *P. aeruginosa DNA* (positive control), Lane 12 is negative control (absence of template) *P. aeruginosa* was detected in chronic wound fluids 3,6 and 7. Lane 3 was positive on all gels but unfortunately too faint to appear in the picture. Left side 100 base pair marker DNA ladders (Promega).

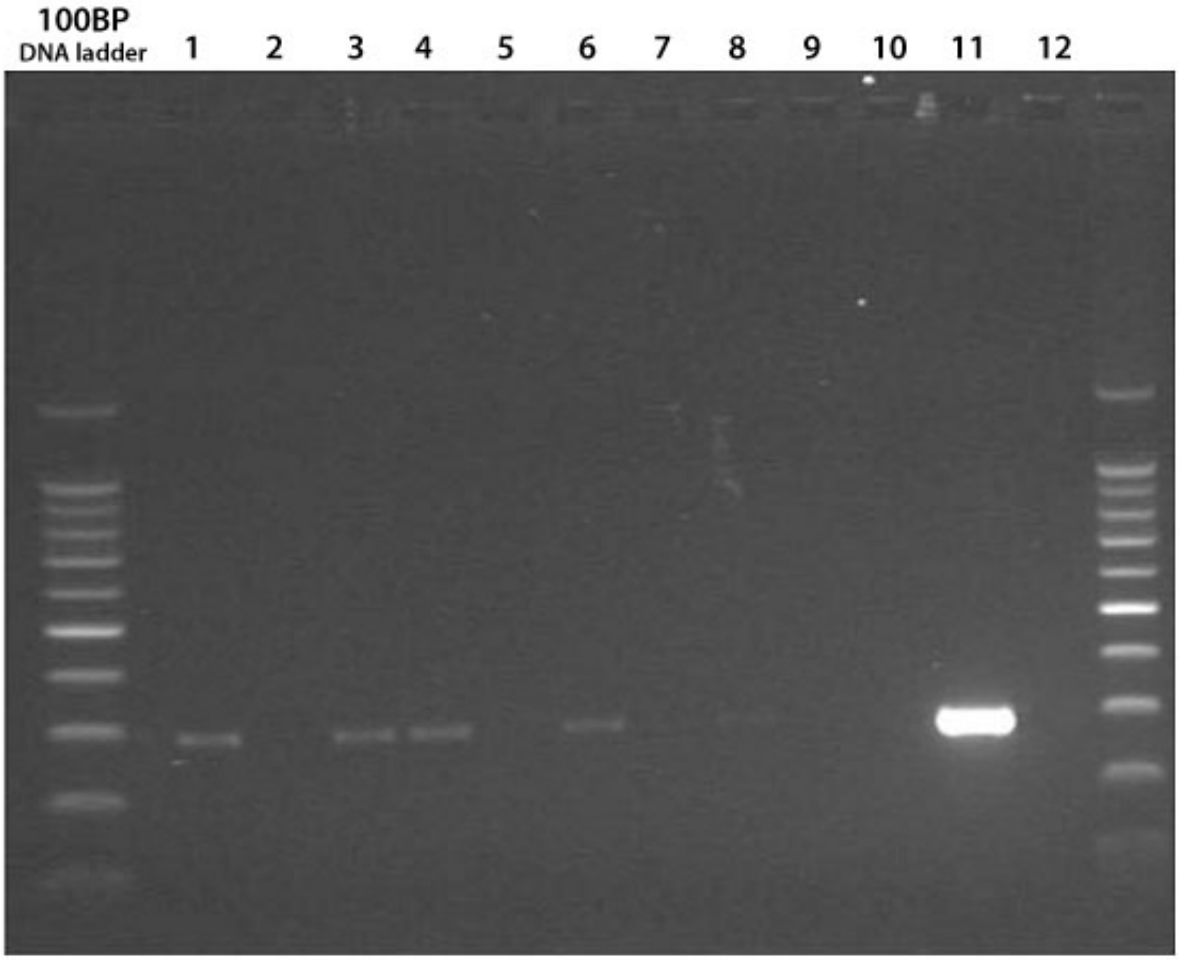


Fig 3.2 PCR of chronic wound isolates for detection of *S. aureus.* Lanes 1-10 represent chronic wound fluid samples CHR1- CHR10. Lane 11 contains *S. aureus DNA* (positive control), Lane 12 is negative control (absence of template), Ladder = 100 base pair marker DNA ladders (Promega) to left and right.

Sample	*P. aeruginosa* cfu/ml	*S. aureus* cfu/ml	Target species detected by PCR
CHR 1	Unknown	1.3×10^5	*S. aureus*
CHR2	6.3×10^6	Not detected	ND
CHR3	6.5×10^4	1.6×10^4	*P. aeruginosa*, *S. aureus*
CHR4	3.4×10^9	2.4×10^7	*S. aureus*
CHR5	4.5×10^6	3.3×10^4	ND
CHR6	4.2×10^6	Not detected	*P. aeruginosa*, *S. aureus*
CHR7	1.02×10^3	Not detected	*P. aeruginosa*,
CHR8	4.8×10^5	Not detected	*S. aureus*
CHR9	1.6×10^6	7.2×10^4	ND
CHR10	7.8×10^6	Not detected	ND

ND, not detected

Table 3.13 Results of traditional microbiology and PCR for enumeration and detection of *P. aeruginosa* and *S. aureus.*

Discussion

Chronic wounds remain a significant and increasing problem worldwide; one factor strongly linked to the development of wound chronicity is bacterial colonisation, infection and biofilm formation. The bacterial flora of chronic wounds has been studied both through culture and molecular methods and consists of bacteria from skin, oral mucosa, enteric tract and the wider environment. Wound microflora is deemed significantly different from that found on normal skin and indeed appears to be different even for different types of chronic wounds (Martin, 2009, Davies *et al.,* 2004).

In order to complete an in-depth study into bacterial communities within chronic wounds it was decided to focus on *S. aureus* and *P. aeruginosa*. It is well recognised that the microflora of chronic wounds is polymicrobial (Fazli *et al.,* 2009). *Pseudomonas aeruginosa* and *S. aureus* are both frequently isolated from chronic wounds and these species are prolific biofilm formers, making them ideal for further characterisation.

Both traditional microbiology and molecular microbiology were used to characterise the bacterial species present within chronic wounds and to provide a bank of acute and chronic wound fluid, debrided wound tissue and wound imprints for the further study and characterisation of bacterial communities within chronic wounds.

Using the dilutional methods previously described, significant volumes of chronic wound fluid were collected, stored and typed in comparison with other studies, which although diluted still contained bacteria of many different species. The chronic wound fluid took a long time to collect (approx 3 months to obtain all 10 samples) even in a large university

wound healing unit. This was due to the limited number of patients with wounds with heavy exudate and the rather onerous 4 h of collection followed by redressing required. It would be very difficult to regularly reproduce this for a diagnostic test. This wound fluid provides a bank of fluid to be utilised in controlled studies for assessing the bacteria within chronic wounds, as any detection system will have to work in the hostile environment that is dominated by wound fluid.

It was possible to collect, store and microbiologically analyse large volumes of acute wound fluid from post mastectomy patients, which although rather sanguineous, provided further fluid for controlled testing of the components of a proposed diagnostic system prior to their use on chronic wound fluid samples. The collection of acute wound fluid was far easier and only limited by the number of patients having mastectomies; it involved no discomfort to the patient and would be easily reproducible in any hospital setting.

Wound imprints successfully obtained from patients with chronic wounds, giving a valuable bank of wound fluid impregnated samples, which can be used to demonstrate the utility of methods of characterising chronic wounds in differing environments. Any dressing based detection system would work on the basis of detecting chronic wound fluid absorbed into the dressing. This was the easiest sample bank to collect as it merely entailed collecting discarded dressings of a commonly used type and imprinting them on blotting paper. As a simple non invasive method of sampling, this could be applied to any patient group and would be the most appropriate to convert to a diagnostic test.

Debrided wound tissue was taken from patients with infected leg wounds and its bacterial communities analysed. Sharp debridement of infected wounds while a well recognised treatment is not common and it was difficult to recruit patients to this group leading to the small number of samples collected. The use of debrided wound tissue for a diagnostic tool would not be convenient, well tolerated, or desirable.

Acute Wound Microbiology

There is currently no data in the literature to compare with the microbiological findings of this study in the mastectomy wound fluid. However, the microbiological results for the acute wound samples were as would be expected following 'clean' surgery with no open wounds or history of infection. Bacteria were isolated in the majority of acute wound fluid samples, the numbers were, however, relatively low. Each of the nine samples was split into 4, except sample 4 which was split into 2 giving 34 samples in total.

Of this bank of samples all showed no growth on *Pseudomonas* agar, but only 8 showed no growth in all agars (samples 2D, 8A, B, C, D, 9B, C, and D). Of the others that yielded bacterial growth, the numbers were low with a median of 50 cfu/ml. The majority of bacteria isolated were CNS, however *S. aureus* was isolated in samples 2 A, B, and C. The bacteria isolated were all normal skin microflora and most likely contaminated the wound in very low numbers during surgery.

It would be expected that samples taken on day two would have a higher bacterial content; however this did not prove to be the case with no significant differences in bacterial counts observed between the days. It is postulated that the large numbers of red blood cells

obviously present in the fluid may have had an antibacterial effect along with the white blood cells and cytokines undoubtedly present. There was no significant difference in bacterial concentrations identified, despite the administration of prophylactic antibiotics at induction, as were given to patients 3, 4 and 9. There were also no significant differences in the concentration of bacteria found or the diabetic status of the patients. On reflection it may have been useful in the creation of a chronic wound model to collect fluid for more days post operatively, as on days one and two there was still a significant red blood cell component to the fluid which becomes more serous, and less sanguineous and therefore would be expected to be more similar to chronic wound fluid over time.

Chronic wound fluid

Chronic wounds are known to support a far more diverse microflora than acute wounds, due to their duration they are exposed to the environment for longer and their microflora changes over time. The patients were fairly typical in age range for chronic wounds with a mean age of 74.1 years, and a range of 62- 88 years at the time of sampling, reflecting that the aging population is most commonly affected. The causes of these chronic wounds were also fairly typical with 40% venous disease, 10% arterial disease, 20% attributed to trauma and 30% of unknown aetiology. The wound locations, exclusively on the lower limbs, were also very typical of the chronic wound population, as was the mean duration of 8.3 years. It was worthy of note, and typical of this population, that patients were on a large number of regular oral medications. Three of these wounds were assessed as currently infected and these patients were on oral antibiotics, and 2 were using topical antimicrobials, again very typical of this group of patients. It was particularly noticeable that 8 out of 10 patients in this group required strong analgesia to help manage the pain of their chronic wounds and 5

of these required controlled drugs, indicating the significant long term reductions in health related quality of life for this cohort.

Chronic wound fluid microbiology

Pseudomonas aeruginosa was successfully cultured and identified in 100% of the chronic wound fluid samples, while *S. aureus* was demonstrated in 70%. CNS were also isolated in 50% of cases and coryneforms in 20%. This is a higher than expected frequency for *P. aeruginosa* than normally cited in the literature, with Fazli *et al.,* (2009) describing them as isolated in only 53.5% of chronic wounds. It is possible that this preponderance of *P. aeruginosa* occurred through selection following the high frequency of antibiotic prescription. As has been described in Table 3.2, all patients had antibiotics of various kinds within a prior timescale ranging from 0- 32 weeks and a mean of 8 weeks. Thus fitting with the evidence of Price *et al.,* (2009) that *P. aeruginosa* biofilms predominate in those patients who have undergone recent antibiotic therapy shifting the profile of relatively diverse wounds to *P. aeruginosa* dominated. This may also be a factor explaining the relative paucity of *S. aureus,* 70% as opposed to the expected 93.5% (Gjodsbol *et al.,* 2006). As seen in Table 3.2, patient CHR4 was on Ciprofloxacin at the time of sampling, for eradication *P. aeruginosa* infection and patient CHR3 had finished the same antibiotics just 2 weeks previously. Patient CHR 7 was undergoing a course of Flucloxacillin, and patient CHR2 was taking Co-amoxiclav at the time of sampling, presumably to counter *S. aureus* infection, which may well account for the lack of *S. aureus* in those samples and bringing the results more into line with expectations.

It must also be considered that 10 samples were of a relatively small sample size and though interesting to speculate, no definitive conclusions can be drawn regarding the microflora without larger studies.

Potassium permanganate topical preparation were being used by patient CHR2, and CHR4 was taking Ciprofloxacin to counter *P. aeruginosa*, while CHR3 was 3 weeks post a course of Ciprofloxacin. It would be expected that levels of *P. aeruginosa* in these samples would be lower than those of the other chronic wound fluid samples containing *P. aeruginosa*. This however was manifestly not the case (Appendix 6). This may be due to the fact that in those undergoing treatment the antibiotics had yet to be fully bactericidal as the course was not completed and in the case of CHR3 due to regrowth post antibiotics. It must also be considered that *P. aeruginosa* is very difficult to eradicate and the antibiotics may be ineffective due to a biofilm phenotype or the development of Ciprofloxacin resistance.

Staphylococcus aureus was isolated in 50% of the 10 sampled patients by culture, with a discrepancy from expected rate of around 90% (Gjodsbol *et al.,* 2006). There appeared to be no relationship between wound duration, causation, or timing of antibiotic courses and the presence or absence of *S. aureus* in the wound fluid samples. However, it should be noted that wound fluid is not a swab or a biopsy and it is possible that some bacteria were not cultured or were adherent to a biofilm and did not survive as well suspended in a wound fluid environment as others.

The results from the PCR (Fig 3.1 and 3.2) of the chronic wound fluid interestingly showed significant discrepancies from those found in the culture of chronic wound fluids (Table

3.13). *Pseudomonas aeruginosa* was only detected in three samples using PCR as opposed to all ten by culture. It is therefore likely that despite selective agars and positive oxidase tests these isolates were not *P. aeruginosa* but related *Pseudomonas* species. *Staphylococcus aureus* was present in 50% of samples using conventional testing and 50% of samples by PCR. However, three of these samples correlated, two samples of wound fluid were found to contain *S. aureus* by culture but not by PCR and *vice versa*. It is important to note that PCR is considered definitive, whereas culture on selected agars and oxidase, catalase and haemolysis tests provide only a presumptive identification. As for those *S. aureus* detected by PCR and not culture, it is possible that these represent non-viable organisms or those in a quiescent non culturable state.

While disappointing that the molecular biological detection methods and classical microbiological methods did not match entirely, this is not uncommon in microbiology and the study of wound ecology, for the reasons mentioned above (Dowd *et al.,* 2008). It has been stated that 'Discrepancies between culture and molecular data were numerous and demonstrate that accurate identification remains challenging' (Bittar *et al.,* 2008, pg 1).

Wound Fluid Quantity

Significant quantities of chronic wound fluid were collected (Table3.4). In those studies that use methods for concentrated wound fluid only small quantities are collected whilst the dilution method results in large volumes suitable for use in multiple confirmatory experiments. The dilution of the chronic wound fluid samples in Tris-HCl was expected to have caused a dilution in the final bacterial counts per ml and may possibly account for some discrepancies in the microbiology with that found in the literature. However, the

large quantities of wound fluid collected allows repeated experiments to be undertaken in order to find effective ways of identifying bacteria within chronic wounds. Bacterial isolates from chronic wounds were stored for use in future experiments.

Wound imprints

Wound imprints were collected from patients recruited on the basis of their highly exudating chronic wounds. The patients selected were typical of the population with chronic wounds in terms of their wound aetiology and location (Table 3.7). There were five patient samples collected and they were labelled WD1-5; four of these wounds were considered to be locally infected and four were on topical antimicrobials with one also on a course of oral antibiotics.

Following analysis by traditional microbiology, the species listed in Table 3.13 were identified. It was interesting to note the presence of *Proteus* in two of these wounds and the presence of CNS in swabs from all patients. *Staphylococcus aureus* was again under represented, present in only one of the samples and *P. aeruginosa* was present in three of the five samples and possibly in the other two, but was impossible to identify secondary to the swarming of *Proteus*. It was interesting to note the presence of *Proteus* in 40% of wounds with high levels of exudate, and the possibility that *Proteus* may be related to this should be considered. *Pseudomonas aeruginosa* species were again commonly found (60% - 100%), which correlates well with our previous findings, if not the literature, which suggests a prevalence of approximately 50%.

Debrided wound tissue

Debrided wound tissue was collected from three patients with infected wounds that were clinically considered to require sharp surgical debridement. All of these patients had chronic leg wounds of venous aetiology; although the population was small the location of the wounds, causation and age range of the population suffering from chronic wounds was typical.

In keeping with the other chronic wound samples, three were positive for *P. aeruginosa*, showing it to be almost ubiquitous in chronic wounds. Two were positive for *S. aureus*, with the remaining containing CNS. Interestingly two samples were positive for *Proteus* species which had not been isolated from any other chronic wound fluid samples analysed. These were patients considered to be so badly locally infected that the treatment of choice was sharp debridement, all were felt to have a macroscopically visible biofilm *in situ*. In view of this, it was interesting that *S. aureus* occured with a greater frequency in these patients than in the other chronic wound samples and that *Proteus* (Appendix 7) was present in 3 of the wounds selected either for excessive exudate of severe local infection, perhaps secondary to the virulence factors present within these species.

The strengths of collecting debrided wound tissue in this manner include the fact that it was readily available and as an accepted treatment modality the patients were put through no extra discomfort in sampling. Weaknesses include the fact that EMLA has some antibacterial properties and that the tissue collected is necessarily disordered giving little information on tissue and biofilm architecture.

Conclusion

All sample sources were unique patients, and no patient was sampled more than once in the same category. No patient was sampled in two different categories. Whilst it could have been useful to compare the microbiology of the same patient sampled in different modalities *i.e.* Chronic wound fluid, wound dressing imprint, and debrided tissue all from the same patient, this idea was discounted. As the wounds would need to be sampled at different times, this would be of uncertain significance, and impractical for the individual patient given the time involved in sample collecting. It was considered that whilst multiple sampling of individuals would increase sample number, it was more useful and more ethical to have unique patients for each sample taken.

Following the collection, microbiological typing and storing of acute wound fluid, chronic wound fluid, wound imprints and debrided wound tissue a large bank of varied microbiologically typed specimens has been built up to allow further experimentation on methods of identifying bacteria in chronic wounds as both individual species and biofilm communities. The bacterial characterisation achieved while not exhaustive could broadly be described as typical of the microflora of the chronic wound population with respect to populations of *S. aureus* and *P. aeruginosa.*

As previously mentioned there are many unculturable bacteria within chronic wounds possibly as many as 98-99% of chronic wound flora are unculturable, therefore to fully characterise bacterial communities in chronic wound molecular methods such as PCR, the use of monoclonal antibodies and ELISA and CLSM FISH are of likely value. The following

chapters involve the application of these methods to further characterisation of bacterial communities in chronic wounds.

Chapter 4: The use of monoclonal antibodies in the detection of specific bacteria in chronic wounds biofilms

Introduction

The microbiology of chronic wounds, as determined both through culture and molecular methods, has continually demonstrated the presence of a diverse microflora (Bowler and Davies, 1999, Davies *et al.,* 2007). This microflora is frequently found to be polymicrobial and comprised of both aerobic and anaerobic bacteria with a significant anaerobic population present in up to 45% of non-infected venous leg ulcers (Bowler and Davies, 1999, Edwards and Harding 2004). Although many studies have examined the microbiology of chronic wounds, a large number of these have classified the various patient groups insufficiently or have not done an adequate or robust microbiological analysis of the microflora itself and subsequently have frequently failed to report any strict anaerobes at all (Davies *et al.,* 2007).

The bacterial species present in chronic wounds have been implicated in impaired wound healing and the development of chronic wound states. A number of mechanisms have been postulated for this bacterial mediated delayed healing, particularly the promotion of a pro-inflammatory state and the direct effects of bacterial exotoxins as discussed previously in the Literature Review of this thesis (Wall *et al.,* 2002; Stephens *et al.,* 2003). Reliable and rapid detection methods for such microorganisms are therefore important in terms of management of these wounds.

Chronic Wounds-current characterisation of microflora

As previously discussed (Literature Review; Bacterial colonisation and wound infection) a wound is considered to be locally infected, or a source of systemic infection, following review by an experienced wound healing medical practitioner, this diagnosis is purely clinical. When making the diagnosis, wound swabs are often sent for microscopy analysis, microbial culture and antimicrobial sensitivity testing of the recovered microorganisms. Microbiological culture of wound samples often requires 1-2 days incubation for aerobic species and between 7-10 days for slow-growing, strictly anaerobic bacteria (Davies *et al.,* 2007). Since clinical treatment of patients cannot be delayed for accurate microbiological typing and antibiotic sensitivity determination prior to treatment, most antibiotic prescribing for these patients is empirical. Broad-spectrum antibiotics are often therefore the preferred course of treatment. As a result of both a lack of accurate diagnosis of infective states and a limited knowledge of the bacterial sensitivities of the species present, over-prescribing of antibiotics for this particular patient group is frequent (Howell-Jones *et al.,* 2005 and 2006).

Indiscriminate antibiotic therapy, whilst possibly being effective for an individual patient, can be problematic as it increases the risk of antibiotic resistance development, thus creating future problems at both a patient and population level (Knox and Holmes, 2002). An accurate and rapid method of determining the presence, species and antibiotic sensitivities of wound pathogens would therefore be beneficial to patient, clinician and the population at large and would allow rapid and appropriate antibiotic use when necessary.

Chronic wound bacteria targeted for detection

As previously discussed (Literature Review; Significance of bacteria in chronic wounds), aerobic bacterial species that are frequently isolated from wounds include both *S. aureus* and *P. aeruginosa* (Davies *et al.,* 2004; 2007) with detection rates as high as 93.5% and 52.2%, respectively (Gjodsbol *et al.,* 2006). *Staphylococcus aureus* is present as a part of the normal skin flora and *P. aeruginosa* can also be found on the skin of healthy individuals (Cogen *et al* 2008), but in chronic wounds both are also opportunistic pathogens, expressing significant virulence factors. In this present study, these two species were targeted for detection due to their high incidence in chronic wounds, association with wound infection and delayed wound healing. The approach used for detection was based on the use of MABs (monoclonal antibodies) that had previously been developed against targets specific for these bacterial species. Following the development of an effective method of detection of these bacteria, this approach would have potential to be extended to the detection of other bacterial species.

Staphylococcus aureus

Staphylococcus species can partly be characterised by their ability to produce deoxyribonuclease (DNase), an enzyme which catalyses hydrolytic cleavage of phosphodiester links of DNA. It is one of several hydrolytic enzymes produced by *S. aureus* and indeed, testing for DNase is often used as a tool in the identification of this species. Importantly, *S. aureus* produces a heat-stable or thermostable DNase referred to as thermonuclease (TNase), which is encoded by the *nuc* gene (Chesneau 1992). Given TNase's previously cited high specificity and sensitivity of 97% and

100%, respectively, for detection in routine blood culture (Lagace-Wiens *et al.,* 2007) and food bacteriology (Brakstad *et al.*, 1995), TNase was selected as a suitable target antigen for monoclonal antibody (MAB) production.

TNase has successfully been used in identifying *S. aureus* from blood cultures as far back as 1983 (Madison and Baselski, 1983). Nevertheless, it must still be recognised that there is no single diagnostic test that can confirm the presence of *S. aureus* despite the seeming high specificity of TNase (Kateete *et al.,* 2010). To highlight this, although initially considered unique to *S. aureus*, it is now thought that some strains of the species *Staphylococcus schleiferi, Staphylococcus carnosus, Staphylococcus intermedius, Staphylococcus hyicus, Staphylococcus caprae* and *Staphylococcus capitis* may also produce a TNase (Kloos and Bannermann, 1994), along with some streptococci and bacilli (Park *et al.,* 1980). It must also be recognised that TNase is generated intracellularly and may therefore remain protected from interaction with extracellular detection systems, or be excreted and detected following the demise of the bacteria.

Pseudomonas aeruginosa

The opportunistic Gram-negative bacterium, *P. aeruginosa*, is frequently isolated from chronic wounds and this species is known to be a prolific biofilm former (Eberl and Collinson 2009). *Pseudomonas aeruginosa* is often considered to be an infecting organism in chronic wounds and populations of this organism recover quickly following antibiotic administration. For such reasons, *P. aeruginosa* often becomes the dominant bacterial species within chronic wound environments (Flanagan,

2007). *Pseudomonas aeruginosa* causes severe infections in hosts with weakened defence mechanisms (Bystrova *et al.,* 2004). The outer component of the *P. aeruginosa* cell wall is predominantly LPS (lipopolysaccharide), which is highly antigenic and plays an important role in the interaction of the bacterium with its host (Pier, 2007). LPS consists of three parts; a lipid component (lipid A), a core of oligosaccharide and an O-polysaccharide chain or O-antigen attached, this antigen defines the immuno-specificity of the bacterium, there have been 11 major O-antigen groups identified (Pier, 2007; Fig. 4.1)

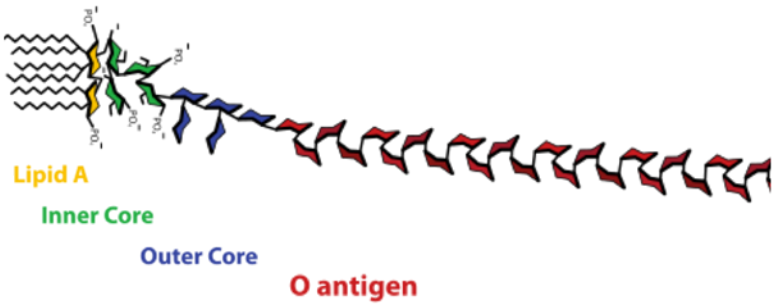


Figure 4.1 Schematic representations of LPS (Picture courtesy of Jones M.D., University of British Colombia Wikipedia 2011)

The structure of *P. aeruginosa* LPS has been fully elucidated (Bystrova *et al.,* 2004); it is located on the outer wall of the bacteria and thus readily exposed to MABs. The O-polysaccharide region is unique to the bacterial species making it an ideal and specific target for the production of MABs to detect *P. aeruginosa.*

Many antigens have previously been used for the detection of *S. aureus* and *P. aeruginosa* previously using differing methodologies for detection. A small selection

of recent commonly used antigens for the development of monoclonal antibodies to *S. aureus* and P. a*eruginosa* is shown in tables 4.1 and 4.2.

Antibody	Study title and year
Staphylococcal enterotoxin B (SEB)	Generation, characterization, and epitope mapping of neutralizing and protective monoclonal antibodies against staphylococcal enterotoxin B-induced lethal shock. 2011
Iron regulated surface determinant B (IsdB) of *Staphylococcus aureus*	A fully human monoclonal antibody to *Staphylococcus aureus* iron regulated surface determinant B (IsdB) with functional activity in vitro and in vivo. 2010
Staphylococcus aureus Fibronectin-binding proteins A and B	Functional analysis of a murine monoclonal antibody against the repetitive region of the fibronectin-binding adhesins fibronectin-binding protein A and fibronectin-binding protein B from Staphylococcus aureus. 2010
Staphylococcus aureus penicillin-binding protein 2'	Development of an immunochromatographic strip for simple detection of penicillin-binding protein 2'. 2011
IsaA of *Staphylococcus aureus*	Functional antibodies targeting IsaA of *Staphylococcus aureus* augment host immune response and open new perspectives for antibacterial therapy. 2011
Staphylococcus aureus MSCRAMM® (Microbial Surface Components Recognizing Adhesive Matrix Molecules)	A humanized monoclonal antibody targeting *Staphylococcus aureus 2004*

Table 4.1 Antigens for *S. aureus* that have been used in previous diagnostic studies (Varshney *et al* 2011), (Ebert *et al* 2010), (Provenza *et al* 2010), (Matsui *et al* 2011), (Lorenz *et al* 2011), (Patti, 2004).

Antibody	Study title and year
O-polysaccharide moiety of *Pseudomonas aeruginosa* serotype O11.	Pharmacokinetics and safety profile of the human anti-Pseudomonas aeruginosa serotype O11 immunoglobulin M monoclonal antibody KBPA-101 in healthy volunteers. 2009
highly phosphorylated and 7-O-carbamoyl-l-glycero-alpha-d-manno-heptopyranose (CmHep) of the inner core of LPS	Monoclonal antibody S60-4-14 reveals diagnostic potential in the identification of *Pseudomonas aeruginosa* in lung tissues of cystic fibrosis patients. 2010
Pseudomonas Aeruginosa serotype O11 LPS	Preclinical *in vitro* and *in vivo* characterization of the fully human monoclonal IgM antibody KBPA101 specific for *Pseudomonas aeruginosa* serotype IATS-O11. 2010
acyl homoserine lactones (AHL) used by *Pseudomonas aeruginosa*	Generation of quorum quenching antibodies, 2011
MAbs against wild-type amidase from *Pseudomonas aeruginosa*	Chromatographic behaviour of monoclonal antibodies against wild-type amidase from *Pseudomonas aeruginosa* on immobilized metal chelates. 2011
Outer membrane protein F (OprF) of *P. aeruginosa*, 3C3B5	Development of a colloidal gold-based immunochromatographic test strip for the rapid, on-site detection of *Pseudomonas aeruginosa* in clinical samples. 2011

Table 4.2 Antigens for *P. aeruginosa* utilised in previous studies (Lazar *et al* 2009), (Schmengler *et al* 2010), (Horn *et* al 2010), (Kaufmann *et al* 2011), (Martins *et al* 2011), (Wang *et al* 2011).

Monoclonal antibodies (MABs)

MABs are identical mono-specific, reproducible antibodies which can be produced in unlimited quantities to almost any antigen. MABs are identical, since they are generated by the same immune cells (B lymphocytes) found within the spleen, which are in turn, clones of a unique parent cell. This cell is then fused with a myeloma cell to make an immortal cell line. As such, MABs already have an extensive range of clinical uses in diagnostic systems and in the treatment of human disease. For the purposes of this project, MABs had previously been raised against *S. aureus* TNase and *P. aeruginosa* LPS as part of an EPSRC (Engineering and Physical Sciences Research Council) project. These antibodies were utilised in the present study for bacterial detection.

Immunofluorescent microscopy

The use of species-specific MABs attached to fluorescent labels for the detection of bacteria by fluorescent microscopy (immunofluorescent microscopy) has been widely reported (Gu *et al.,* 2002). More recently, expansion of this technique to detect bacteria within biofilms using confocal laser scanning microscopy (CLSM) has been described (Gu *et al.,* 2005). This method of study is significant as it allows imaging of biofilms without altering structure and allows analysis over extended periods of time. Despite initial concerns about molecular penetration into biofilms, it is now thought that immunoglobulin G (IgG) is a small enough molecule to migrate through the extracellular matrix of biofilms unimpeded (Zhu *et al.,* 2001). This

process has previously been successfully utilised to allow real-time characterisation of species both within and outside of a biofilm (Gu *et al.,* 2005).

Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-Linked Immunosorbent Assay (ELISA) is a biochemical technique used to detect the presence of an antibody or an antigen within a sample. In its basic form, the principle of ELISA is the use of a captured antigen (immobilised to a solid surface), to which a labelled antibody is then applied, allowing it to bind with the antigen. The reporter label on the antibody is often an enzyme with which an added substrate reacts (usually causing a colour change) yielding a detectable signal. There are several variations of ELISA and these include indirect, sandwich and competitive formats.

ELISA was first described in 1971, and replaced forerunner assays that utilised radioactive labels with the incorporation of 'safer' colour change signals (Engval *et al.,* 1971, Van Weemen and Schuurs, 1971). ELISAs are now widely used in medicine for detection of host antibodies to viruses and in the food industry for detecting food allergens. For the detection of *P. aeruginosa* in cystic fibrosis patients there is a commercially available ELISA to detect IgG antibodies formed as a result of exposure to *P. aeruginosa* (Douglas *et al.,* 2009). ELISAs have also been used in chronic wounds to quantify levels of matrix metalloproteinases (MMPs) (Barone *et al.,* 1998) and cytokines (Secor *et al.,* 2011, Pukstad *et al.,* 2010), but to date have not been employed to detect bacteria within chronic wounds.

Aims of study

The aim of this present study was to use developed MABs against *P. aeruginosa* and *S. aureus* to detect these bacterial species in chronic wounds. Prior to application to clinical specimens it was first necessary to demonstrate specificity and sensitivity of these antibodies in pure culture against planktonic and biofilm growth states, and in testing on previously microbiologically typed wound samples utilising fluorescent microscopy and ELISA, both indirect and competitive.

Materials and Methods

Monoclonal antibodies used in this study

Antibodies had previously been prepared in a separate Engineering and Physical Sciences Research Council funded grant (Grant Number EP/D505437/2) aiming to develop diagnostic systems for wound pathogens. MABs against *S. aureus* TNase had previously been raised by murine BALB/c immunisation with a commercially available TNase preparation (Toxin Technology Inc, Sarasota, Florida, USA) purified from *S. aureus* strain FRI 1151M (Kokan and Bergdoll, 1987). In the case of *P. aeruginosa*, antibodies were raised against the LPS O-antigen portion by murine BALB/c immunisation with phenol chloroform extracted lipopolysaccharide (LPS) from *P. aeruginosa* PAO1 strain (Sigma). Immunisation was done using 10 µg of commercial antigen on alum adjuvant intra-peritoneally. Immunisations were done every 7 days for 4 weeks, with a final immunisation at week 8. Blood serum levels were monitored periodically over time for antigen presence by indirect ELISA and

spleen cells harvested 4 days after the last immunisation. The spleen cells were used to prepare monoclonal antibodies as detailed below. Briefly, murine splenocytes from the immunisations were fused with SP2/0 myeloma cells. Cultured cells were screened for antibodies against commercial antigen (TNase, LPS) using culture supernatants which were tested by indirect ELISA for recognition of TNase antibodies Cultures which appeared positive for antigen production were cloned by limiting dilution. Cultures showing monoclonal growth were tested by indirect ELISA and frozen for long term storage. Large scale antibody production was done by bulk culture in growth medium. Secreted antibodies were enriched and purified by affinity chromatography of culture supernatant on Hitrap protein G HP columns (GE Healthcare Biosciences AB) after centrifugation to remove cells (Randle *et al.,* 2004).

Detection of target bacteria by immunofluorescent microscopy

Preparation of microorganisms

Pseudomonas aeruginosa D10 and *S. aureus* FRI 1151M were cultured from stock isolates on *Pseudomonas* agar (LabM UK) and Blood agar (LabM UK), respectively. Colonies of these bacteria were then resuspended in phosphate buffered saline (PBS) and washed twice by centrifugation (2500*g*) and resuspension of the pellet in fresh PBS.

Immunofluorescent staining of bacteria

A human serum block (Sigma, Poole, UK; 1% of volume of the resuspended culture) was added to the cell suspension and the resulting solution was incubated at room temperature for 1 hour. The primary mouse antibodies used were either anti-*P.*

aeruginosa LPS (A56) or anti-*S. aureus* TNase (B34). These antibodies were previously stored at a stock concentration of 1 mg/ml and added to the cell suspension at a 10% concentration (100 µg/ml) and incubated at room temperature for 30 minutes. The culture was then centrifuged at 600*g* (Heraeus Pico 17 centrifuge) for 1 minute, and washed twice with PBS as described earlier. Secondary antibody (goat anti-mouse IgG FITC (fluorescein isothiocyanate)-conjugated antibody) (Sigma-Aldrich UK) was then added to the culture and allowed to incubate in the dark at room temperature for 30 minutes. This preparation was centrifuged at 2500*g* and washed twice as before. The culture was re-suspended in PBS and smeared on to a microscope slide; the suspension was maintained in a hydrated state under a cover slip and imaged by fluorescent microscopy at the appropriate excitation wavelength for FITC (494 nm) on a Nikon microscope.

Appropriate controls for immunofluorescence included either omission of the primary or secondary antibodies as well as replacement of the primary antibody with an isotype IgG antibody (Sigma-Aldrich UK) not raised against TNase.

Production of Constant Depth Film Fermenter (CDFF) generated biofilms

The CDFF was prepared as previously described (Hill *et al*., 2010) using *S. aureus* FRI 1151M and *P. aeruginosa* PAO1. Overnight cultures of both bacterial strains incubated in BHI on a shaking incubator at 37°C were used, the bacterial growth was harvested by centrifugation (8000g, for 10 minutes) to produce a cell pellet. The culture medium was circulated through the CDFF for 30 min prior to inoculation and rotated with a turntable speed of 20 rev/min. Five µl of each wound isolate was

added separately to BM medium (1000 mL) and was then recirculated through the CDFF for 24 h to 'seed' the system. The inoculum was then disconnected and fresh non inoculated medium was fed into the CDFF. The CDFF waste was collected in a separate bottle. The growth medium was delivered at a rate of 30 mL/h using a peristaltic pump (Watson–Marlow). Biofilms were cultured in a CDFF maintained at 37°C with plug inserts recessed to a depth of 400 µm. CDFF pans were removed after 7 days and biofilms from 4 plugs were pooled and harvested (Hill *et al*., 2010). Harvested plugs with biofilms were then embedded in paraffin wax blocks.

Fig 4.2 Constant depth film fermenter

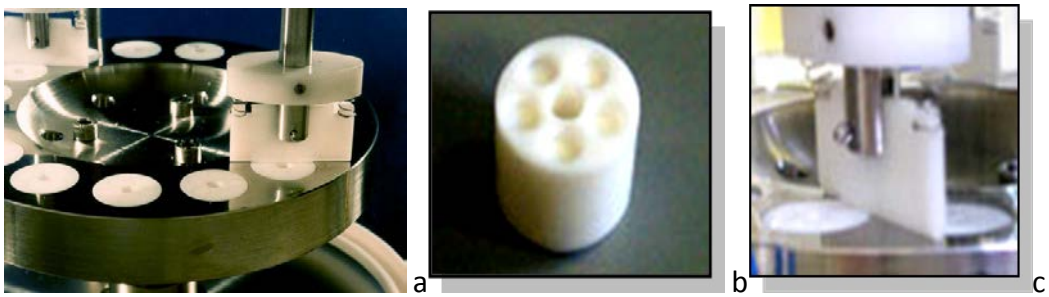


Fig 4.3 (a, b and c) internal components of CDFF

a. Turntable with components *in situ*
b. Plug
c. Sweeper

Preparation of biofilms on Recombinant Human Epithelium (RHE)

Cultivated reconstituted human epidermis (RHE; 0.5 cm^2) was obtained from SkinEthic Laboratories Lyon France. These tissues had previously been cultured for 10 days in a chemically defined culture medium containing 5 µg insulin ml^{-1} and 1.5 mM calcium chloride as well as various growth factors, but with no antibiotics (SkinEthic Laboratories). The RHE consisted of normal human keratinocytes (human foreskin-derived) with a well-differentiated epidermis consisting of basal layers, spinous layers, granular layers and a stratum corneum Fig 4.4.

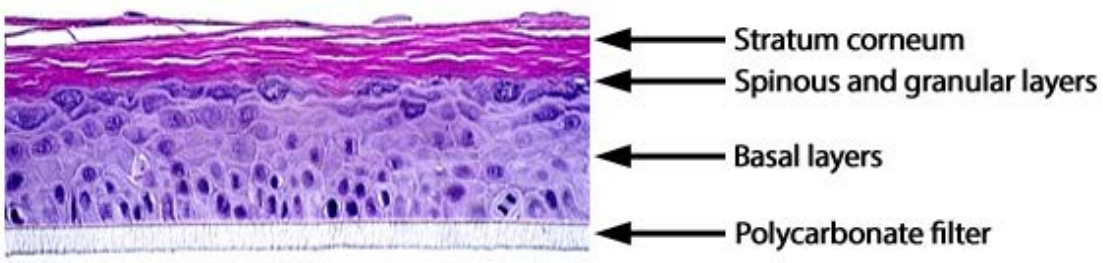


Fig 4.4 The *in vitro* reconstructed human epidermis (RHE). RHE consists of normal human keratinocytes cultured on an inert polycarbonate filter. This model is

histologically similar to that of the *in vivo* human epidermis. (Image and text courtesy of SkinEthic.com/RHE.asp 4-12-2011)

Bacterial strains were inoculated into 5 ml of BHI (brain heart infusion broth BHI, Oxoid) and incubated with shaking at 37°C for 18 hours. Bacteria were harvested by centrifugation at 600*g* (Heraeus Pico 17 centrifuge) for 1 minute and washed three times with PBS. The pelleted bacteria were then resuspended in 1.5 ml of RHE culture medium. The RHE inoculum was 500 µl of the bacterial suspension and this was deposited centrally on to the surface of the RHE tissues. When dual species inocula were used, supernatants from 2 strains of equal volumes (250-µl) of each was applied.

The infected RHE was incubated for 24 hours at 37°C in a humidified atmosphere, enriched with 5% CO_2. The RHE conditioned growth media was then removed and stored at -20°C until required for detection of target *S. aureus* TNase antigen. A non-infected control was included for comparison. The tissue itself was used for immunofluorescent staining and detection of target bacteria (*P. aeruginosa* and *S. aureus*).

Immunofluorescent staining of infected RHE and CDFF generated biofilms

Both RHE and CDFF generated biofilms were fixed in formalin 10% (v/v) overnight and embedded in paraffin wax. The fixed material was then sectioned using a microtome to generate 10 µm sections and these were mounted on poly L-lysine coated microscope slides. Processing of these sections on the slides involved

treating with xylene to remove the paraffin wax, and then rehydration of the specimens through decreasing concentrations of alcohol back to water. The RHE sections were delineated using a wax pen and blocked with 20 μl/sample of 10% (w/v) BSA (bovine serum albumin) in PBS (Phosphate Buffered saline) for 10 minutes, which was then removed by gentle washing. Primary antibody was then added at a concentration of 100 μg/ml was added to the tissue and incubated at room temperature for 1 hour. The section was then gently washed with PBS to remove the primary antibody. Initially, washing of the sections was performed using water bath with agitation, however due to the loss of contact of several sections from the slide the method was refined to three washes by gentle pipetting of PBS. FITC conjugated goat anti-mouse IgG antibody at a concentration of 1mg/ml was then added and incubated on the section in the dark at room temperature for 1 hour. The section was then washed with PBS and resuspended in 20 μl of PBS and maintained under a coverslip and imaged as previously described. Consecutive sections were then imaged by fluorescent microscopy (Olympus Provis AX70). Controls used were the substitution of the monoclonal antibodies (*i.e. P. aeruginosa* MAB challenged with *S. aureus* and *vice versa*), and the substitution of the FITC conjugated anti-mouse IgG antibody with a similarly conjugated anti-goat antibody. Light microscopy was also used to examine Gram-stained sections to demonstrate the presence of bacterial biofilms.

Enzyme Linked Immunosorbent Assay (ELISA)

For the purpose of ELISAs it was decided to focus on the B34 antibody for *S. aureus* only. An enzyme linked immunosorbent assay (ELISA) was initially developed to

determine the optimal concentrations of biotinylated TNase for use in antigen detection using a competitive ELISA**.** To achieve this result the following competitive ELISA method was followed but with no addition of competitor to biotinylated TNase. The sample added to the ELISA contained only dilutions of biotinylated TNase from an initial concentration of 10 μg/ml in doubling dilutions through to 0.005 ng/ml.

Development of a competitive ELISA protocol for detection of *S. aureus* TNase in bacterial supernatants

The wells of a microtitre plate (96-well PVC) were coated with 50 μl of 10 μg/ml of goat anti-mouse IgG (Fc specific) antibody (Sigma) in coating buffer (Carbonate/bicarbonate 0.1M pH 9.6, Sodium carbonate 1.59g/L, Sodium hydrogen carbonate 2.93g/L). This was incubated overnight at 4°C. The antibody coating was then removed and the plate blocked with 100 μl/well of 10% (w/v) bovine serum albumin (BSA) in PBS for 2 hours at room temperature with gentle agitation. The plate was then washed twice with 150 μl/well of 0.05% Tween 20 in PBS (Wash buffer). The anti-*S. aureus* TNase (B34) antibody was prepared at 4 μg/ml in protein buffer (Appendix 1) and added to the plate at 50 μl/well, this was left to incubate at room temperature for 1 hour with gentle agitation. The plate was then washed twice with 150 μl wash buffer.

Competitor antigen samples (unlabelled) were prepared in 35-μl volumes a separate 96 well microtitre plate (Corning, Amsterdam, The Netherlands) in a doubling series of dilutions in protein buffer, a control of protein buffer alone was included. TNase

conjugated to biotin (previously prepared as part of a separate research project) was used at a previously determined 80 ng/ml concentration (determined by the experiment listed above). This was prepared in protein buffer and 35 μl of the biotinylated TNase was added to the competitor antigen preparations in the replica plate wells. The contents of the replica plate were transferred to the washed ELISA plate (50 μl) and allowed to incubate at room temperature for 1 hour with agitation. The antigen mix was then removed and the plate washed twice with wash buffer. A 500-fold dilution of avidin alkaline phosphatase (AVAP, Sigma, Poole, UK) in protein buffer was then added at 50 μl/well (to bind to any captured biotinylated TNase) and the plate incubated at room temperature for 15 minutes. The AVAP was then removed and the plate washed 3 times with 150 μl wash buffer. Any bound alkaline phosphatase enzyme was then detected by adding 50 μl/well of 1 mg/ml para-nitrophenol phosphate in diethanolamine buffer 0.1M pH 9.6 (Appendix 1). The plate was then incubated in the dark for 5-15 minutes and the developed colour from the enzyme-substrate reaction was read on a microplate reader (Fluostar Optima BMG Labtech, Offenburg, Germany) at 405 nm.

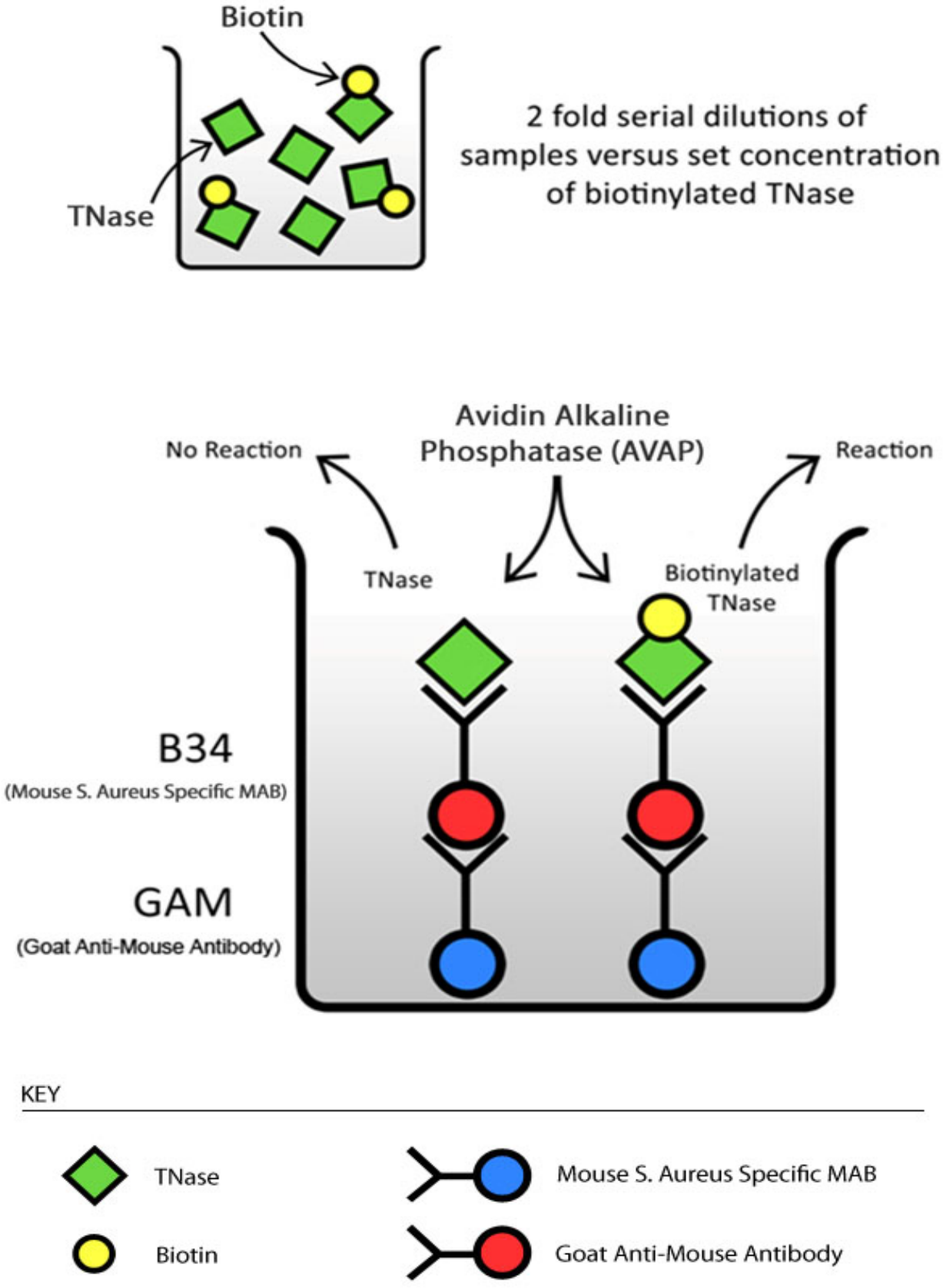


Fig 4.5 Schematic of competitive ELISA

Competitive ELISA for detection of *S. aureus* TNase in artificially contaminated wound fluid

Wound fluid was artificially contaminated with TNase at a concentration of 1 µg/ml to produce samples of chronic wound fluid with a known quantity of TNase. These samples were mixed in a microfuge tube and subjected to the competitive ELISA as described above.

Competitive ELISA for detection of *S. aureus* TNase in wound fluid samples

Wound fluid samples (chronic and acute) were vortexed to maintain consistency of sample constituents. These wound samples were then subjected to the competitive ELISA as described above.

Detection of *Staphylococcus aureus* TNase in chronic wound fluids using DNA agar

An agar test was also used to determine the presence of TNase within chronic wound fluid samples. DNase Test Agar (Oxoid), incorporating DNA into Trypticase Soy Agar, was used as a TNase test for the chronic wound samples collected in the study. Chronic wound samples (1 ml) were heated at 80°C for 30 min to denature any heat-sensitive DNase. A 6 mm punch biopsy was then used to produce a well in the centre of DNA agar plates, to which heat-treated chronic wound fluid samples were added at a volume required to fill the well. The plates were then incubated for 4 h at 37°C before flooding with HCl (1 M). TNase activity was evident through the

presence of a zone of clearing in the agar around the well of any TNase-positive isolates. Positive controls of *S. aureus* FRI 1151M supernatants (previously incubated in BHI) were also tested and uninoculated BHI served as a negative control.

Competitive ELISA for detection of bacterial antigens in culture supernatants

Supernatants from RHE incubated 24 hour biofilms of *P. aeruginosa* and *S. aureus* were extracted using a pipette and subjected to the competitive ELISA as described above.

Sensitivity of the competitive ELISA with respect to *Staphylococcus aureus* FRI 1151M cell number

These experiments aimed to determine the amount of *S. aureus* (colony forming units/ml) required to generate sufficient levels of TNase to be detected by ELISA using the MAB (B34) specific to *S. aureus* TNase.

BHI broth was inoculated with *S. aureus* FRI 1151M using colonies from a blood agar plate and incubated overnight at 37°C with agitation. *S. aureus* FRI 1151M culture (10 ml) was centrifuged, and the resulting cell pellet (estimated at 10^8 CFU/ml) was washed twice in 10 ml of PBS to remove any TNase generated overnight in the culture. A ten-fold dilution series of this cell suspension was then prepared in BHI. The broths were then incubated at 37°C. Using a spiral plater system (Don Whitley Scientific, Shipley, UK), the CFU/ml present in preparation was determined on MSA

at hourly intervals between 0 and 12 h at and also at 16 h and 24 h. All plates were incubated for 24 hours at 37°C and resulting colonies were enumerated. In addition, at each time point a sample was also stored at -70°C for later analysis by competitive ELISA as described earlier. Uninoculated BHI was used for controls.

Concentrating chronic wound fluid

An 11.25-ml volume of each chronic wound fluid sample (Materials and Methods in Chapter 3) was centrifuged at 600*g* for 10 minutes to remove large particulate material and the samples were then filtered using a 0.22 µm syringe filter. Salt and small solutes were removed by dialysis against 10 L of distilled H_2O at 4°C overnight using visking tubing size 1. The contents of the dialysis tubing were transferred to 30 ml universal containers freeze dried for approximately 3 days to remove the water thus concentrating the samples. Resulting samples were resuspended in 150 µl of protein buffer (1% BSA, 1% HEPES in PBS) a concentration of the chronic wound fluid sample of x75, and subjected to the competitive ELISA as previously described.

Filter paper ELISA

A falcon 96-well PVC microtitre plate was coated with 50 µl/well of anti-*S. aureus* TNase B34 antibody at a concentration of 1 µl/ml and the plate was incubated overnight at 4°C. The plate was then washed using wash solution as previously described. To each well a volume of 10% foetal calf serum diluted in PBS was added and incubated for 1 hour at room temperature. The plate was again washed with wash solution.

The chronic wound dressing imprints were produced by pressing saturated dressings against filter paper. Completely saturated filter paper containing this absorbed wound fluid was then stored. Three samples of the filter paper were cut with a hole-punch to 4 mm discs from each wound dressing sample stored. 50µl of Protein buffer was added to the appropriate wells. Three samples of clean filter paper which had not been in contact with wound fluid or dressings were then added as a control to other wells and 50 µl protein buffer added.

A control of serial dilution of stock TNase was then added to generate a standard curve and allow quantitative values of TNase to be determined for the chronic wound fluids. The plate was then incubated for 2 hours on a shaking plate. The layer was removed (including filter paper) and the plate was then washed three times with wash solution. Fifty µl of biotinylated B34 at a concentration of 1.2µl/ml of protein buffer was added to each well and incubated at room temperature for 2 h. The plate was washed as described previously and a 500-fold dilution of avidin alkaline phosphatase (AVAP) in protein buffer was added at 50 µl/well. The plate was incubated at room temperature for 15 minutes. AVAP was then removed and the plate washed 3 times with wash buffer. Alkaline phosphatase activity was detected by adding 50 µl/well of 1mg/ml para-nitrophenol phosphate in diethanolamine buffer 0.1M pH 9.6 (Appendix 1). The plate was then incubated in the dark for 5-15 minutes and optical density read on a microplate reader (Fluostar Optima BMG Labtech, Offenburg, Germany) at 405nm.

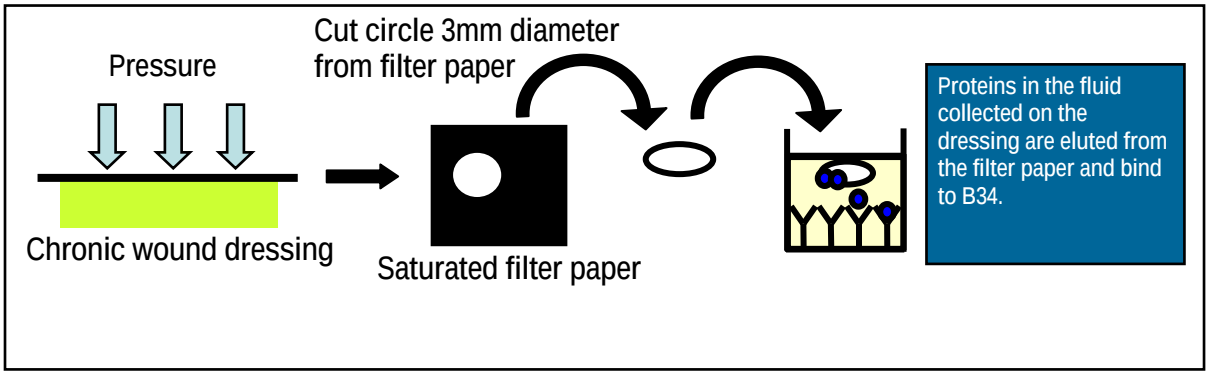


Fig 4.6 The use of a filter paper collection method for chronic wound dressings.

Results

Immunofluorescent microscopy

Immunofluorescent staining of *S. aureus* FRI 1151M with anti-TNase B34 antibody followed by anti-mouse IgG conjugated with FITC was positive (Fig. 4.7). Controls without inclusion of the primary antibody were negative for *S. aureus* detection, although weak background fluorescence was evident on occasion.

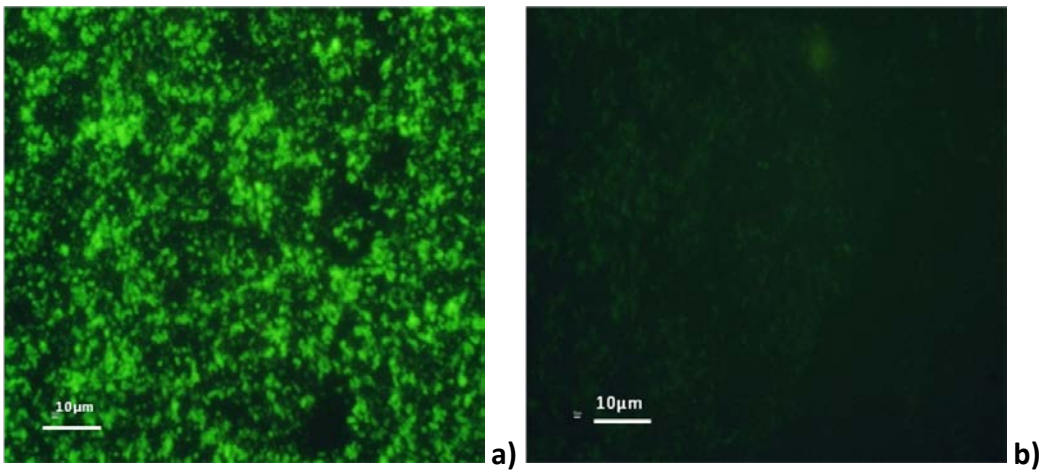
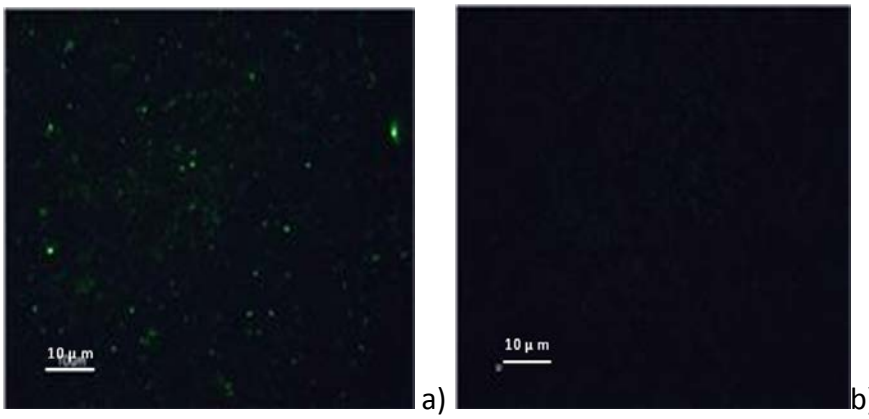


Fig 4.7 Detection of *S. aureus* using a) mouse anti-TNase (B34) antibody and anti-mouse IgG conjugated FITC; (positive) b) control with omission of primary B34 antibody (negative). Original magnification ×1000.

In the case of immunofluorescence staining of *P. aeruginosa* with mouse anti-LPS (A56) antibody and anti-mouse IgG conjugated FITC, cells were visible as small rod shape cells with slightly increased fluorescence evident in the MAB treated specimen compared with controls (Fig. 4.8). Rapid quenching of FITC staining was however evident and coupled with autofluorescence of *P. aeruginosa* made interpretation of reactivity problematic.



a) b)

Fig. 4.8 Detection of *Pseudomonas aeruginosa* using a) mouse anti-LPS (A56) antibody and anti-mouse IgG conjugated FITC; (positive) b) control with omission of A56 antibody (negative). Original magnification ×1000.

Detection of *S. aureus and P. aeruginosa* in CDFF and RHE biofilms by immunofluorescent microscopy

The presence of biofilms on both the PTFE plugs from the CDFF and the RHE was confirmed by light microscopy and Gram-staining with appropriate controls. Immunofluorescent staining of biofilms prepared in these systems all demonstrated significant autofluorescence which precluded bacterial detection (as demonstrated in fig 4.9).

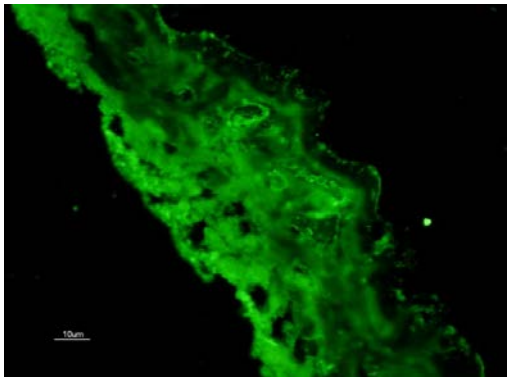


Fig. 4.9 *Pseudomonas aeruginosa* CDFF generated biofilm with omission of A56 antibody demonstrating autofluorescence of the biofilm. Original magnification ×1000.

Development of a competitive Enzyme Linked Immunosorbent Assay (ELISA) for detection of *S. aureus* TNase

Initially, the chosen MAB developed for detection of *S. aureus* TNase was used in an ELISA against two-fold serial dilutions of biotinylated TNase antigen. These experiments allowed the optimal concentration of biotinylated TNase for subsequent use in a competitive ELISA format. As a result of these experiments, standard curves were generated (Fig. 4.10). Optimal biotinylated TNase concentration was deemed to be the level that gave 80% of signal in the competitor ELISA format. As can see from figure 4.10, this concentration was approximately 78 ng/ml of biotinylated TNase.

Concentration in ng/ml	Biotinylated TNase	Biotinylated TNase	Mean of Biotinylated TNase	SD	Blank corrected Mean
10000	3.193	3.712	3.453	0.367	3.214
5000	3.168	3.598	3.383	0.304	3.144
2500	3.281	3.598	3.439	0.224	3.200
1250	3.207	3.536	3.371	0.233	3.132
625	3.193	3.411	3.302	0.154	3.063
312.5	2.996	3.110	3.053	0.081	2.814
156	2.542	2.691	2.616	0.105	2.377
78	1.949	2.055	2.002	0.075	1.763
39	1.360	1.424	1.392	0.045	1.153
19.5	0.896	0.943	0.919	0.034	0.680
9.8	0.610	0.606	0.608	0.002	0.369
4.88	0.419	0.423	0.421	0.003	0.182
2.44	0.334	0.356	0.345	0.015	0.106
1.22	0.315	0.307	0.311	0.006	0.072
0.61	0.304	0.288	0.296	0.011	0.057
0.3	0.258	0.264	0.261	0.004	0.022
0.15	0.258	0.275	0.266	0.016	0.027
0.08	0.250	0.264	0.257	0.010	0.018
0.04	0.262	0.249	0.256	0.009	0.017
0.02	0.246	0.239	0.242	0.005	0.000

Table 4.3 Absorbance values for biotinylated TNase in serial twofold dilutions in competitive ELISA without competitor.

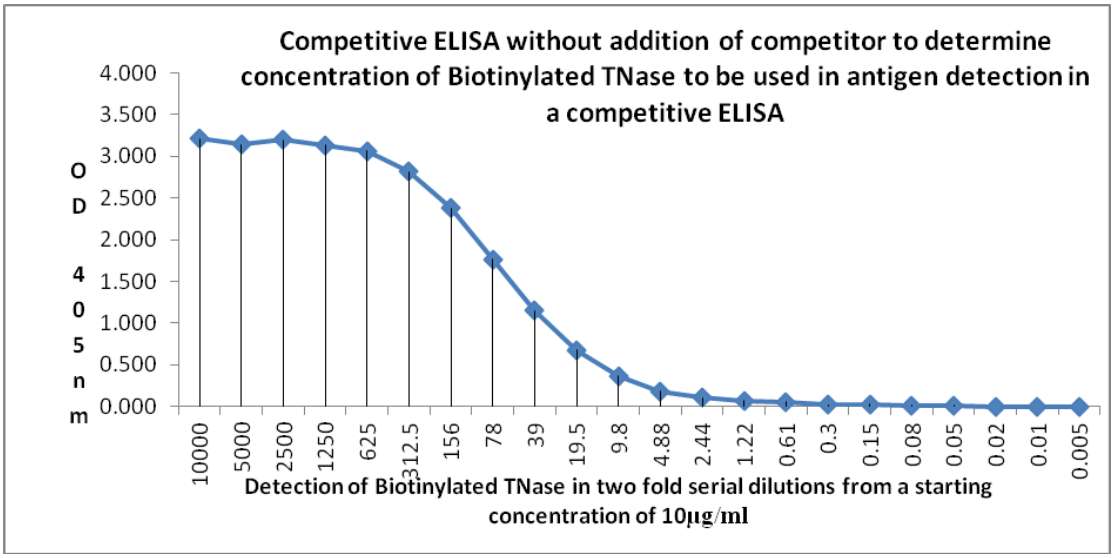


Fig. 4.10 Showing detection of biotinylated TNase using anti-*S. aureus* TNase antibody (B34) by competitive ELISA format, but without competitor. These values are a mean of two runs, further see table, this was completed 3 times (total of six runs) please see Appendix 8 for further data tables.

Detection of TNase in *S. aureus* culture supernatants using the competitive ELISA

It was decided to test the ability of the competitive ELISA developed to detect TNase in the supernatants of different strains of *S. aureus*. It was also felt pertinent to test the ELISA against filtered and unfiltered supernatants of *S. aureus* cultures to ascertain if the debris and cells present in a supernatant significantly affected the ELISA, as demonstrated in Fig 4.11 showing detection of the S. aureus produced TNase from FRI strain supernatant to a sensitivity of 62.5ng/ml.

Dilutions (two fold serial)	No Competition	S.*aureus* B2 supernatant	S. *aureus* FRI115M supernatants	TNase
1	2.218	0.733	1.465	0.230
0.5	2.234	1.920	2.190	0.539
0.25	2.239	2.175	2.228	0.921
0.125	2.261	2.265	2.296	1.496
0.062	2.243	2.274	2.310	1.917
0.032	2.251	2.298	2.305	2.146
0.016	2.247	2.320	2.299	2.214
0.008	2.248	2.313	2.306	2.258
0.004	2.266	2.295	2.316	2.256
0.002	2.215	2.292	2.287	2.268
0.001	2.202	2.289	2.289	2.246

Table 4.4 Absorbance values for S. *aureus* supernatants in competitive ELISA with positive and negative controls.

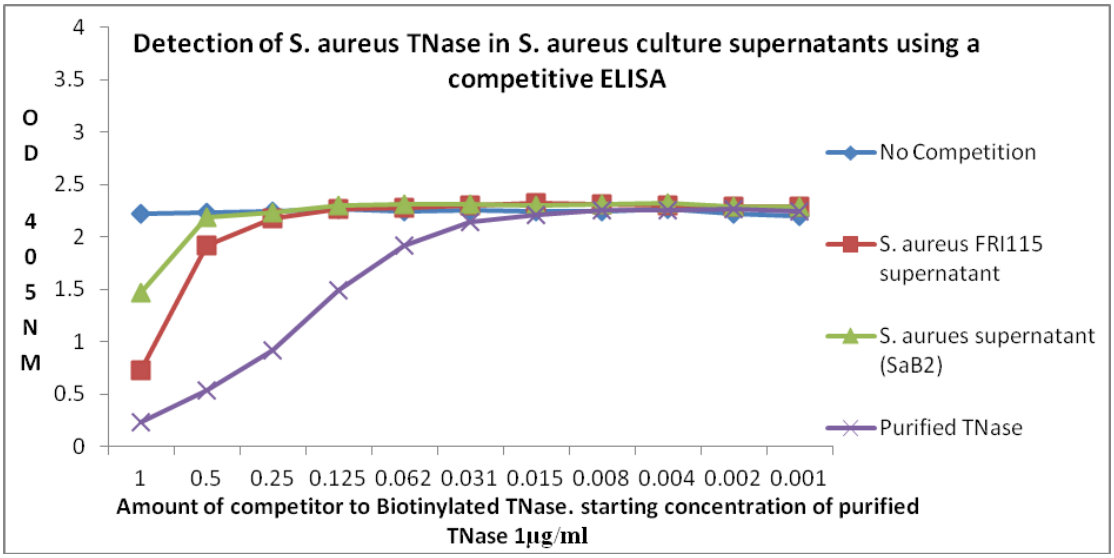


Fig. 4.11 Detection of *S. aureus* TNase in *S. aureus* culture supernatants. A positive control of purified TNase was included, together with an uninoculated BHI broth (negative control). An increased level of competition occurred with *S. aureus* FRI 1151M supernatant compared with supernatants from other strains of *S. aureus*. This data was compiled from the mean of two runs, the full data tables for which can be found in Appendix 8.

Dilution (two fold serial)	S. *aureus* FRI115M supernatant	S. *aureus* FRI115M supernatant and cells	P. *aeruginosa* supernatant	No competition
1	0.2625	0.11615	1.4012	1.5623
0.5	1.1004	0.8413	1.42	1.658
0.25	1.23555	1.14385	1.46035	1.5623
0.125	1.29285	1.2819	1.3943	1.658
0.062	1.3112	1.3382	1.46035	1.5623
0.032	1.3959	1.3864	1.51675	1.658
0.016	1.4442	1.4452	1.54055	1.6451
0.008	1.43745	1.45535	1.50745	1.65195
0.004	1.4513	1.44575	1.51365	1.58695
0.002	1.46	1.4503	1.48535	1.6234
0.001	1.412	1.4201	1.382	1.616

Table 4.5 Absorbance values for S. *aureus* and P. *aeruginosa* supernatants in a competitive ELISA with negative control.

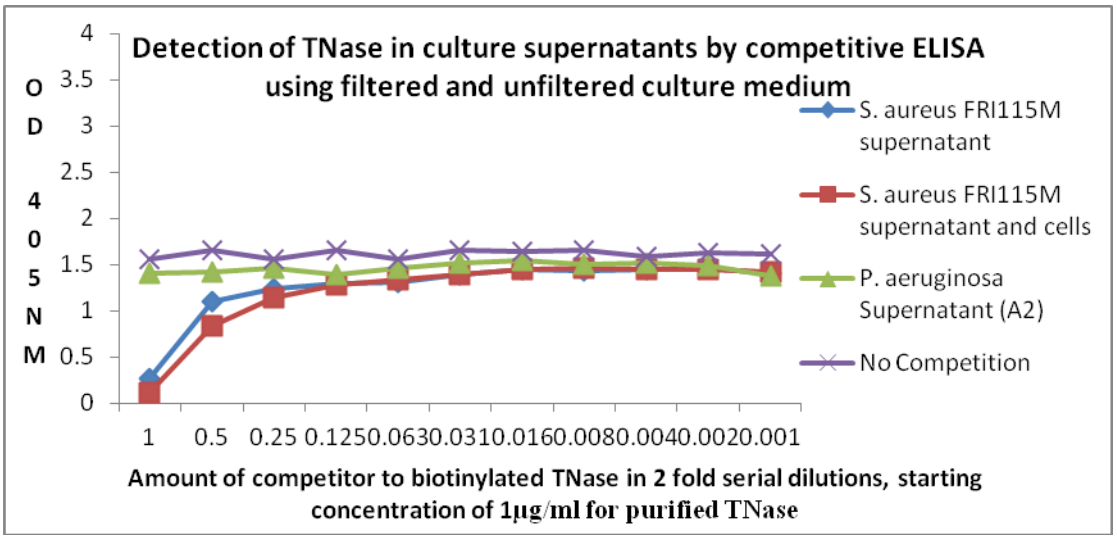


Fig. 4.12. Detection of TNase in *S. aureus* FRI 115M culture supernatants by competitive ELISA using filtered and unfiltered culture medium. Controls included a *P. aeruginosa* culture supernatant and an uninoculated BHI broth showing no competition. This data was compiled from the mean of two runs, the full data tables for which can be found in Appendix 8.

Specificity of competitive ELISA for *S. aureus* TNase

The specificity of the *S. aureus* TNase MAB (B34) for its target (*S. aureus* TNase) in a competitive ELISA was assessed by challenge with different bacterial supernatants from infected RHE utilising purified *S. aureus* TNase as a positive control (Fig. 4.13, 4.14).

Dilution	1.000	0.500	0.250	0.125	0.063	0.031	0.016	0.008	0.004	0.002	0.001
TNase control	0.016	0.026	0.074	0.182	0.343	0.515	0.639	0.659	0.689	0.703	0.673
RHE S. *aureus* supernatants 1.	0.146	0.240	0.434	0.562	0.608	0.619	0.638	0.647	0.630	0.620	0.620
RHE S. *aureus* supernatants 2	0.122	0.233	0.415	0.549	0.620	0.663	0.667	0.684	0.680	0.678	0.686
RHE S. *aureus* supernatants 3	0.150	0.261	0.442	0.555	0.633	0.695	0.688	0.668	0.682	0.676	0.657
RHE S. *aureus* supernatants 4	0.283	0.470	0.613	0.677	0.733	0.769	0.763	0.816	0.806	0.774	0.747
RHE S. *aureus* supernatants 5	0.086	0.173	0.339	0.523	0.644	0.835	0.821	0.860	0.861	0.864	0.869

Table 4.6 Absorbance values for Recombinant human epithelium S. *aureus* biofilm supernatants in a competitive ELISA with positive control of TNase.

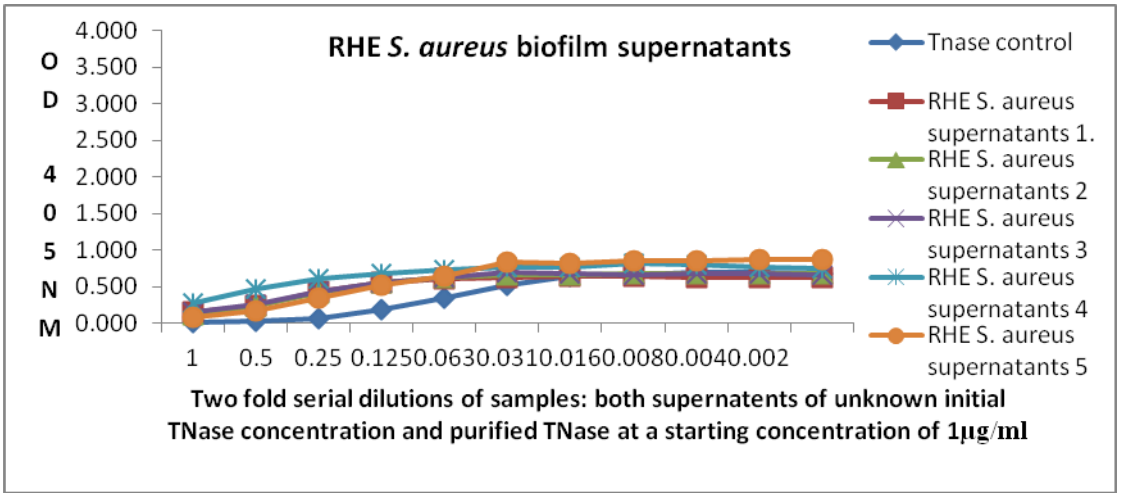


Fig. 4.13. Competitive ELISA demonstrating competition by *S. aureus* RHE biofilm supernatants indicating the presence of TNase in these samples. This data is not a mean and was not repeated.

Dilution	1.000	0.500	0.250	0.125	0.063	0.031	0.016	0.008	0.004	1.000	0.500
TNase control	0.047	0.088	0.189	0.467	0.774	0.934	0.996	1.112	1.151	0.047	0.088
RHE P.*aureginosa* supernatants 1.	0.874	0.829	0.885	0.892	0.877	0.934	0.886	0.866	0.874	0.874	0.829
RHE P.*aureginosa* supernatants 2.	0.909	0.916	0.983	0.969	1.029	1.028	1.070	1.128	1.053	0.909	0.916
RHE P.*aureginosa* supernatants 3.	0.963	1.034	0.975	1.045	1.053	1.040	1.072	1.052	1.096	0.963	1.034
RHE P.*aureginosa* supernatants 4.	0.961	1.130	1.157	1.152	1.170	1.108	1.113	1.098	1.132	0.961	1.130
RHE P.*aureginosa* supernatants 5.	0.865	0.854	0.845	0.836	0.827	0.817	0.832	0.837	0.840	0.865	0.854

Table 4.7 Absorbance values for Recombinant human epithelium P. *aeruginosa* biofilm supernatants in a competitive ELISA with positive control of TNase.

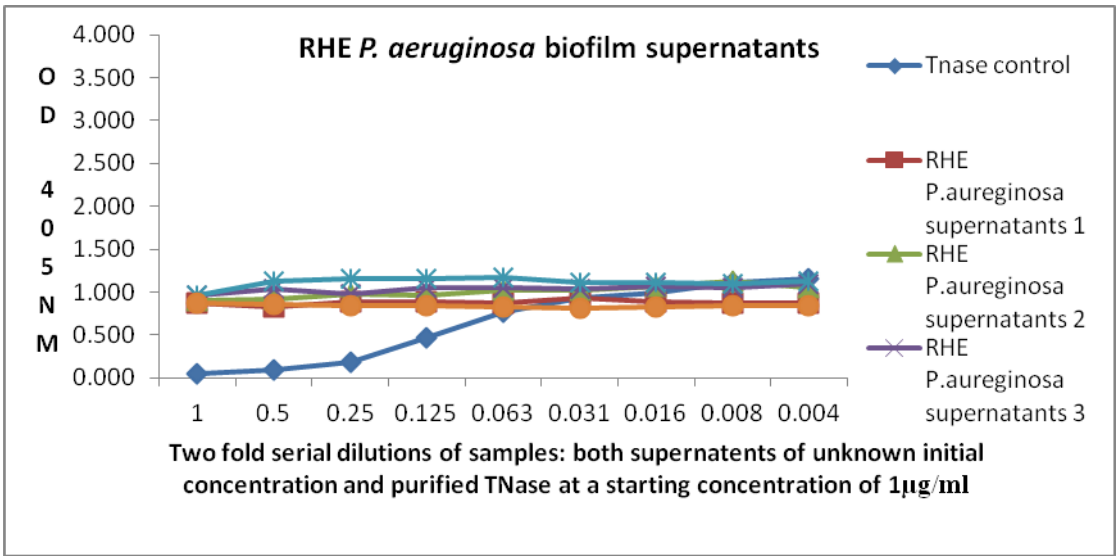
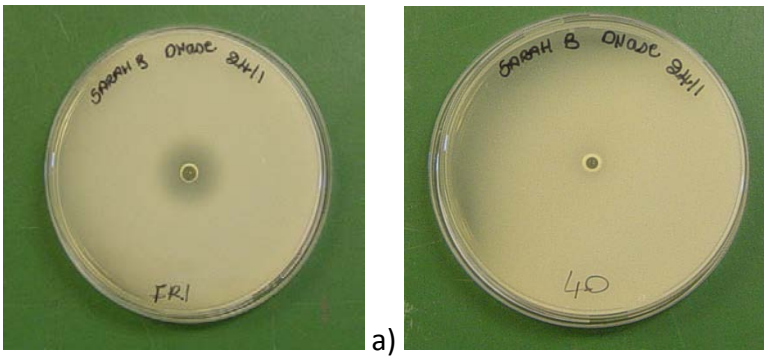


Fig. 4.14 Competitive ELISA for *S. aureus* TNase demonstrating no competition using *P. aeruginosa* RHE biofilm supernatants. This data is not a mean and was not repeated.

Detection of *S. aureus* TNase in wound fluids using DNA agar

Prior to assessing TNase presence by ELISA in chronic wound fluid samples, these were first subjected to agar testing for TNase. Using this detection method all samples were negative (fig4.15 b). When the wound fluids were artificially contaminated with *S. aureus* FRI 1151M positive results were evident (fig4.15 a).



a) b)

Fig. 4.15 Detection of *S. aureus* TNase activity using DNA agar; a) example of positive result, and b) a negative result for detection of TNase.

Sensitivity of competitive ELISA for *S. aureus* TNase with respect to cell numbers of S*. aureus* FRI 1151M

To give an indication of the numbers of colony forming units per ml of *S. aureus* required to produce enough TNase for detection utilising the competitive ELISA developed it was decided to produce a growth curve of *S. aureus* (fig4.16) and take supernatants and a series of time points for competitive ELISA.

Time in hours	0	1	2	3	4	5	6	
CFUs/ml	$4.8x10^2$	$6.4x10^2$	$8.8x10^2$	$2.0x10^3$	$1.7x10^4$	$3.0x10^4$	$6.8x10^4$	

Time in hours	7	8	9	10	11	12	16	24
CFUs/ml	$6.535x10^5$	$2.25x10^6$	$1.4x10^7$	$4.6x10^7$	$1.4x10^8$	$3.35x10^7$	$2.55X10^8$	$9.75X10^8$

Table 4.8 colony forming units per ml recorded over time.

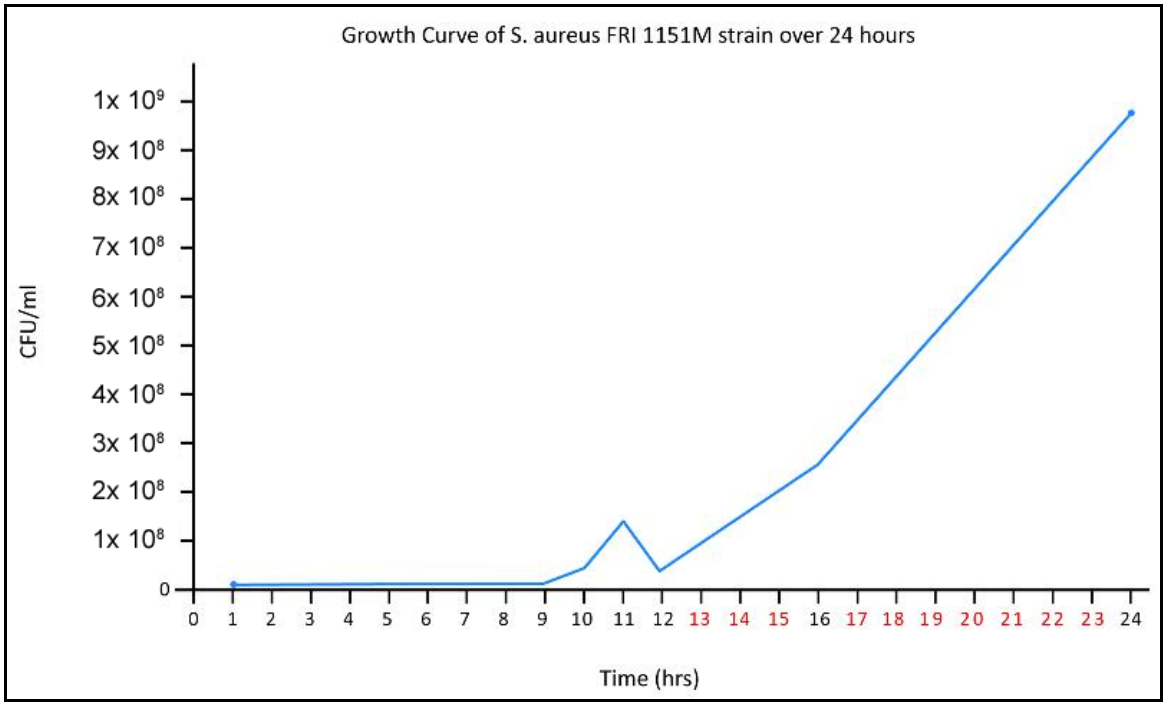


Fig. 4.16 Growth curve (CFU per ml) of *S. aureus* FRI 1151M over 24 hours at 15 different time points. Non measured time points in red.

A satisfactory growth curve for *S. aureus* FRI 1151M was obtained with levels of 480 CFU/ml at time 0 to 9.75 x 10^9 CFU/ml at 24 hours. At each time point (excluding those in red), supernatants were obtained and subjected to the competitive ELISA as previously described.

BHI negative control	**1.887**	**1.886**	**1.944**	**1.948**	**1.924**	**2.034**	**1.986**	**2.039**	**1.939**
TNase Positive control	**1.029**	**1.583**	**1.721**	**1.933**	**1.986**	**2.032**	**2.126**	**2.084**	**2.059**
0hrs	**1.825**	**1.867**	**1.837**	**2.021**	**1.959**	**1.921**	**1.936**	**1.87**	**1.965**
1hrs	**1.856**	**1.959**	**1.863**	**2.011**	**1.912**	**1.909**	**1.864**	**1.873**	**1.889**
2hrs	**1.818**	**1.925**	**1.945**	**1.977**	**1.957**	**1.946**	**1.955**	**1.879**	**1.871**
3hrs	**1.876**	**1.916**	**2.038**	**2.128**	**2.027**	**2.077**	**2.016**	**1.99**	**1.904**
4hrs	**1.843**	**1.927**	**1.999**	**2.063**	**2.058**	**2.079**	**2.004**	**1.998**	**1.933**
5hrs	**1.882**	**2.001**	**1.922**	**1.838**	**1.898**	**1.915**	**1.903**	**1.946**	**1.996**

Table 4.9 Mean absorbance values for FRI115M S. *aureus* supernatants collected at different time points in the growth curve in a competitive ELISA against biotinylated TNase, with positive control of TNase and a negative control of Brain heart infusion (BHI).

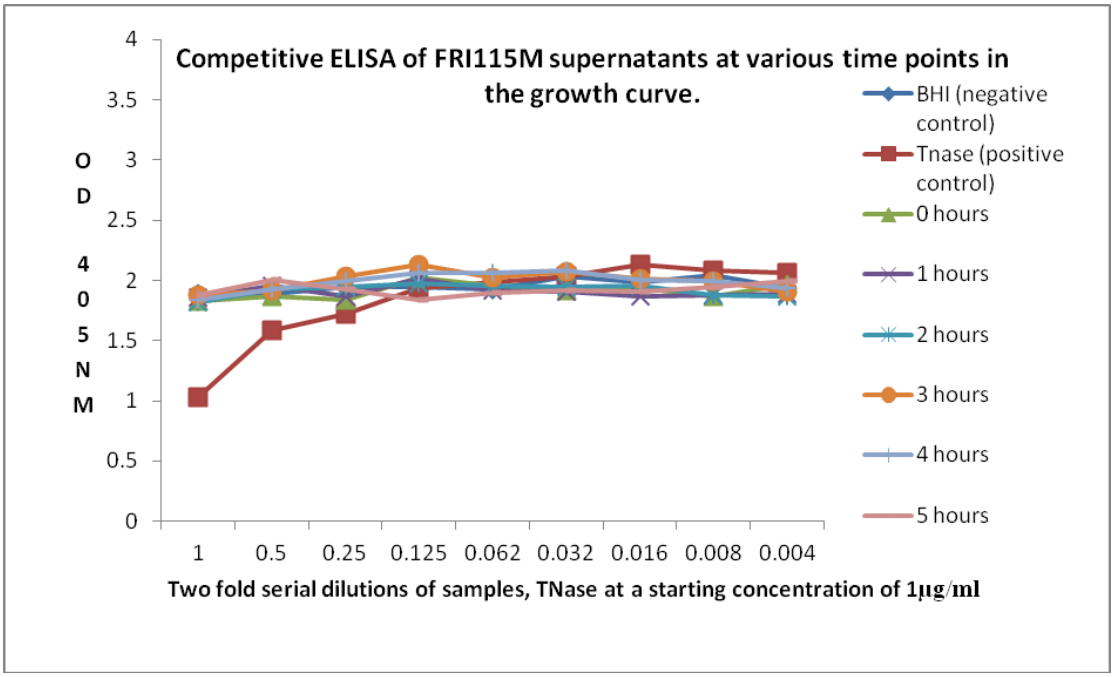


Fig. 4.17 Mean results of competitive ELISAs run on supernatants collected from hours 0 to 5 plus a control of purified TNase- red. This ELISA was repeated a twice further (see Appendix 8 for data tables).

BHI negative control	**1.901**	**1.958**	**1.925**	**1.93**	**1.937**	**1.957**	**1.927**	**1.856**	**1.888**
TNase Positive control	**1.069**	**1.471**	**1.736**	**1.793**	**1.859**	**1.858**	**1.893**	**1.905**	**1.934**
6hrs	**1.825**	**1.867**	**1.837**	**2.021**	**1.959**	**1.921**	**1.936**	**1.87**	**1.965**
7hrs	**1.856**	**1.959**	**1.863**	**2.011**	**1.912**	**1.909**	**1.864**	**1.873**	**1.889**
8hrs	**1.818**	**1.929**	**1.945**	**1.977**	**1.957**	**1.946**	**1.955**	**1.879**	**1.871**
9hrs	**1.876**	**1.916**	**2.038**	**2.128**	**2.027**	**2.077**	**2.016**	**1.99**	**1.904**
10hrs	**1.843**	**1.927**	**1.999**	**2.063**	**2.058**	**2.079**	**2.004**	**1.998**	**1.933**
11hrs	**1.882**	**2.001**	**1.922**	**1.838**	**1.898**	**1.915**	**1.903**	**1.946**	**1.996**

Table 4.10 Mean absorbance values for FRI115M S. *aureus* supernatants collected at different time points in the growth curve in a competitive ELISA against biotinylated TNase, with positive control of TNase and a negative control of Brain heart infusion (BHI).

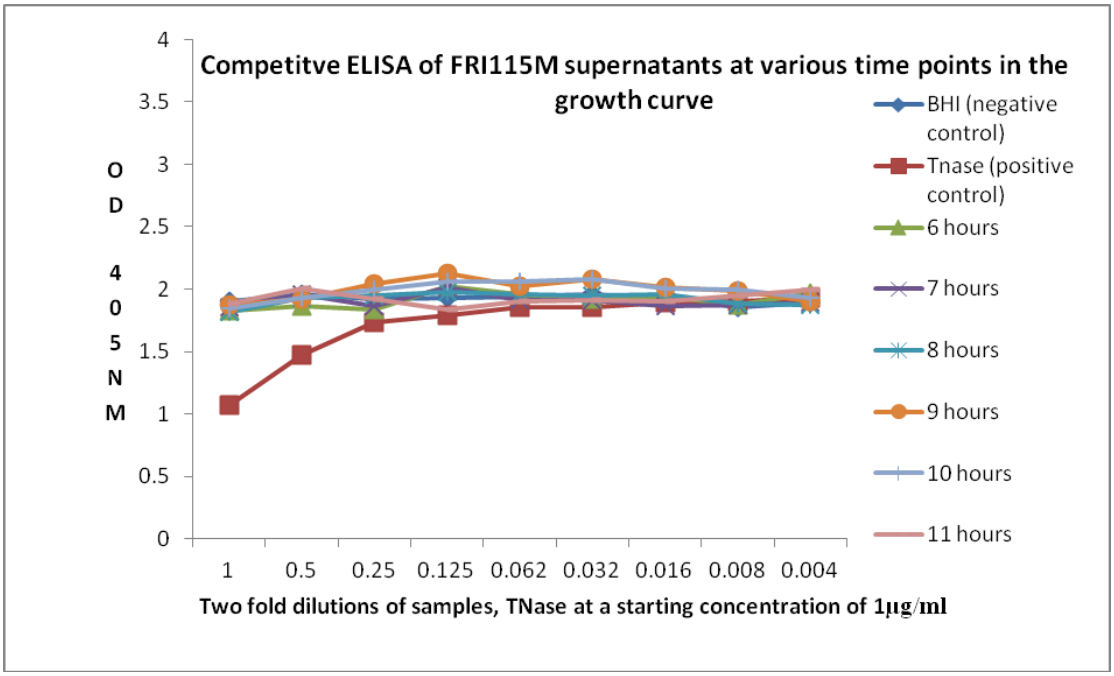


Fig. 4.18 Mean results of competitive ELISAs run on supernatants collected from hours 6 to 11 plus a control of purified TNase- red. This experiment was repeated twice further (see Appendix 8 for data tables).

BHI negative control	**2.074**	**2.136**	**2.148**	**2.265**	**2.206**	**2.272**	**2.287**	**2.202**	**2.129**
TNase Positive control	**1.104**	**1.712**	**2.038**	**2.089**	**2.124**	**2.086**	**2.112**	**1.976**	**1.955**
12hrs	**1.535**	**1.839**	**1.823**	**1.936**	**2.008**	**1.929**	**1.972**	**1.984**	**2.016**
16hrs	**0.768**	**1.292**	**1.487**	**1.803**	**1.756**	**1.803**	**1.898**	**1.915**	**2.052**
24hrs	**0.539**	**1.063**	**1.127**	**1.462**	**1.731**	**1.916**	**2.059**	**2.04**	**2.111**

Table 4.11 Mean absorbance values for FRI115M S. *aureus* supernatants collected at different time points in the growth curve in a competitive ELISA against biotinylated TNase, with positive control of TNase and a negative control of Brain heart infusion (BHI).

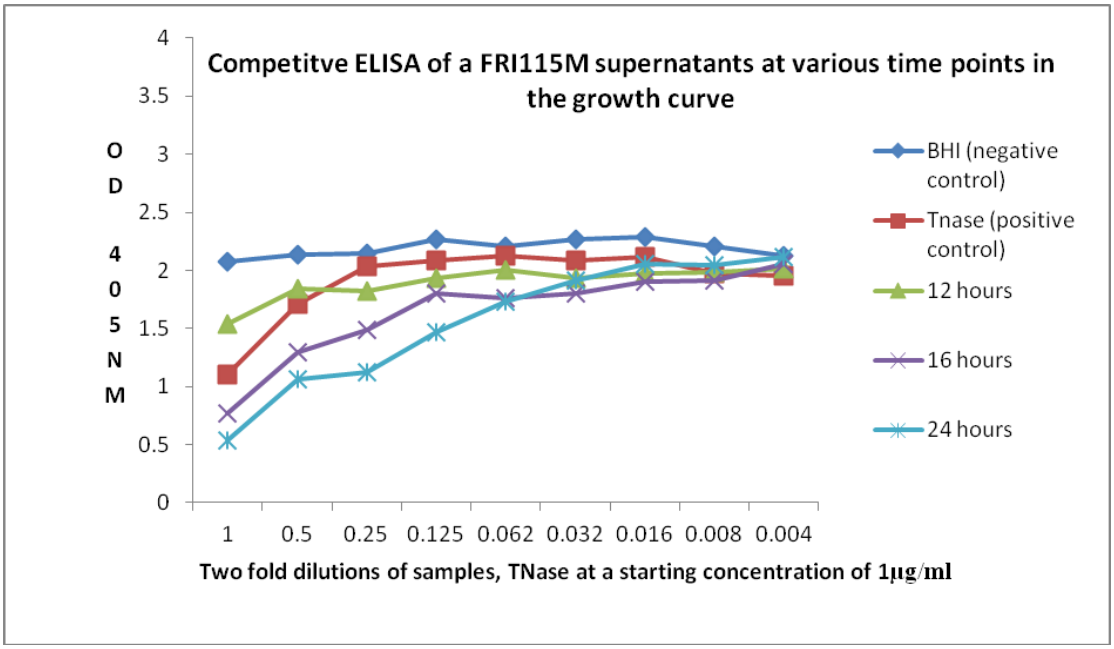


Fig. 4.19 Mean results of three competitive ELISAs run on supernatants collected from 12 h, 16 h, and 24 h plus a positive control of purified TNase and uninoculated BHI. Showing competition at all three time points with increasing quantities of TNase with time. This experiment was repeated twice further (see Appendix 8 for data tables).

There was a trend of increased competition in the ELISA as the number of *S. aureus* CFU/ml increased with time. There was no competition over the first five time points(fig 4.17) (or up to a *S. aureus* concentration of $3.0x10^4$ CFU/ml). Clear competition was evident between 12 and 24 h time points (fig 4.19) or concentrations of S. aureus in excess of $3.35x10^8$ CFU/ml. The 16 and 24 h time points appeared to contain a higher concentration of TNase than the positive control (1µg/ml).

Detection of *S. aureus* TNase in wound fluid artificially contaminated with *S. aureus* TNase to determine the ability of the MAB to function in a wound fluid environment

Following confirmation of the ability of the competitive ELISA to detect *S. aureus* TNase, and therefore the likely presence of *S. aureus* in both bacterial and RHE biofilm supernatants, it was decided to test the antibodies in a wound fluid environment.

Two fold serial dilutions	Wound fluid	Wound fluid spike (1:1) with TNase	Wound fluid spiked (1:2) with TNase	S.aureus supernatant	No competition
1	1.543	0.890	0.737	0.573	1.922
0.5	1.584	1.244	1.161	1.109	1.959
0.25	1.624	1.439	1.244	1.365	1.797
0.125	1.624	1.534	1.385	1.494	1.773
0.062	1.666	1.514	1.506	1.569	1.674
0.032	1.691	1.511	1.471	1.587	1.780
0.016	1.780	1.546	1.577	1.681	1.868
0.008	1.731	1.565	1.505	1.558	1.896
0.004	1.788	1.605	1.537	1.632	1.838
0.002	1.755	1.619	1.565	1.688	1.834
0.001	1.803	1.652	1.598	1.762	1.756

Table 4.12 Absorbance values for wound fluid spiked with TNase in a competitive ELISA with positive and negative controls.

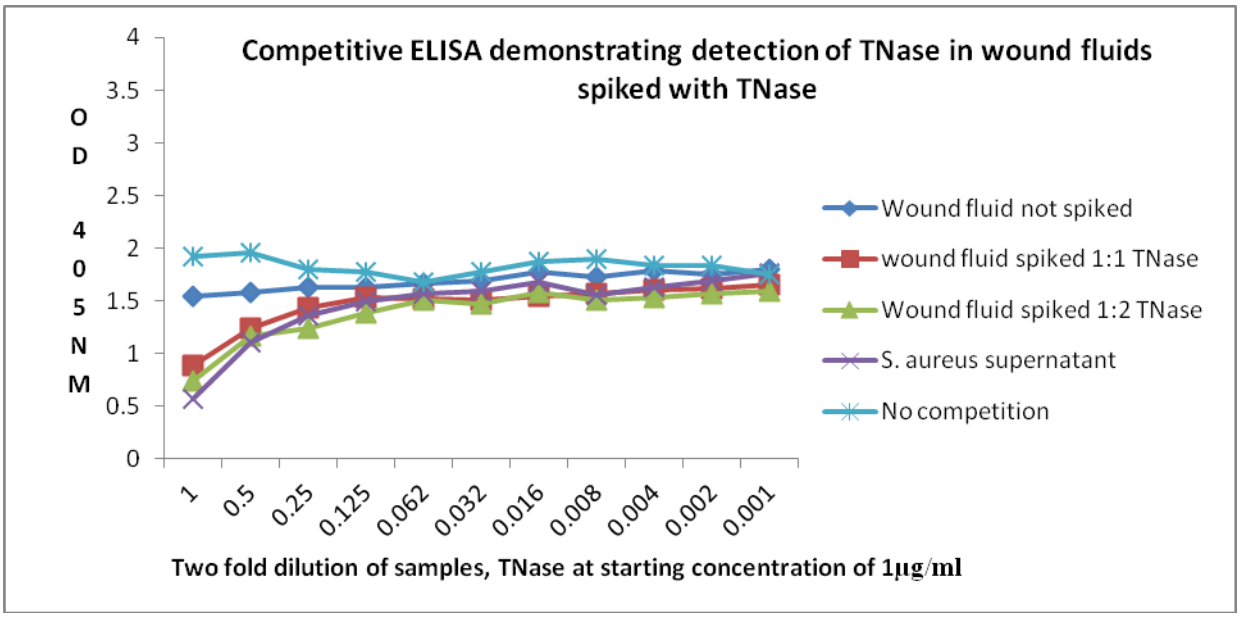


Fig. 4.20. Competitive ELISA for the detection of *S. aureus* TNase within chronic wound fluids artificially and deliberately contaminated with TNase. *S. aureus* culture supernatant was used as a positive control and PBS as a negative control. This data was compiled from the mean of two runs (full data tables for which can be found in Appendix 8).

Detection of *S. aureus* TNase in acute and chronic wound fluids by competitive ELISA

Following the preceding results demonstrating the ability of the MAB (B34) to detect *S. aureus* TNase in a competitive ELISA using artificially contaminated chronic wound fluid, it was decided to test all of the acute and chronic wound fluid samples (Fig. 4.12).

Dilution	Acute 3b	Acute 4a	Acute 2b	TNase
1	0.814	0.947	0.941	0.035
0.5	0.965	1.164	0.903	0.108
0.25	1.124	1.279	1.047	0.073
0.125	1.207	1.303	1.168	0.243
0.062	1.338	1.362	1.295	0.672
0.032	1.340	1.455	1.474	0.918
0.016	1.477	1.480	1.511	1.008
0.008	1.519	1.456	1.486	1.145
0.004	1.531	1.429	1.471	1.203
0.002	1.489	1.430	1.482	1.431
0.001	1.554	1.469	1.512	1.447

Table 4.13 Absorbance values for acute wound fluids in a competitive ELISA with a positive control of TNase.

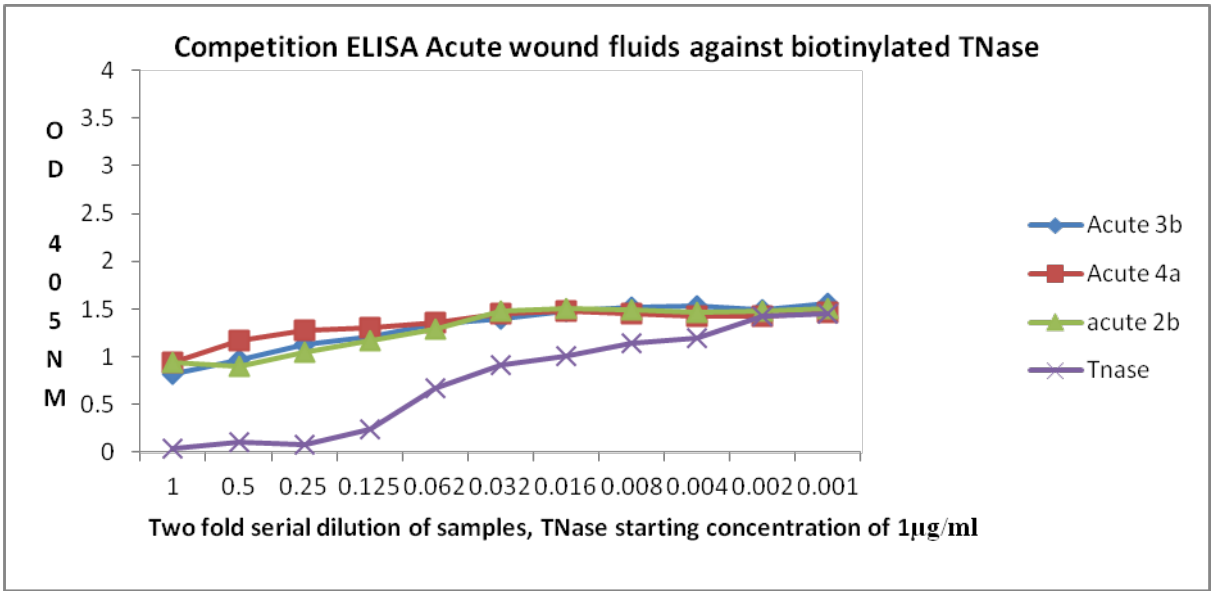


Fig. 4.21 Competitive ELISA for detection of TNase in acute wound fluid samples previously shown to contain *S. aureus* by culture. These samples were negative for TNase. This data was compiled from the mean of two runs, (full data tables can be found in Appendix 8).

Dilution	Chronic 1	Chronic 2	Chronic 3	Chronic 4	Chronic 5	TBS	TNase
1	1.204	1.102	0.919	1.344	1.379	1.272	0.136
0.5	1.312	1.146	1.021	1.388	1.462	1.392	0.305
0.25	1.282	1.091	1.097	1.478	1.329	1.345	0.569
0.125	1.262	1.183	1.135	1.477	1.406	1.359	0.811
0.062	1.276	1.235	1.190	1.420	1.397	1.310	1.022
0.032	1.219	1.179	1.191	1.396	1.353	1.353	1.113
0.016	1.206	1.136	1.147	1.380	1.315	1.300	1.224
0.008	1.190	1.050	1.124	1.390	1.332	1.358	1.274
0.004	1.283	1.130	1.167	1.353	1.382	1.355	1.316
0.002	1.256	1.159	1.149	1.345	1.357	1.346	1.318
0.001	1.248	1.089	1.101	1.354	1.310	1.287	1.392

Table 4.14 Absorbance values for chronic wound fluids in a competitive ELISA with positive and negative controls.

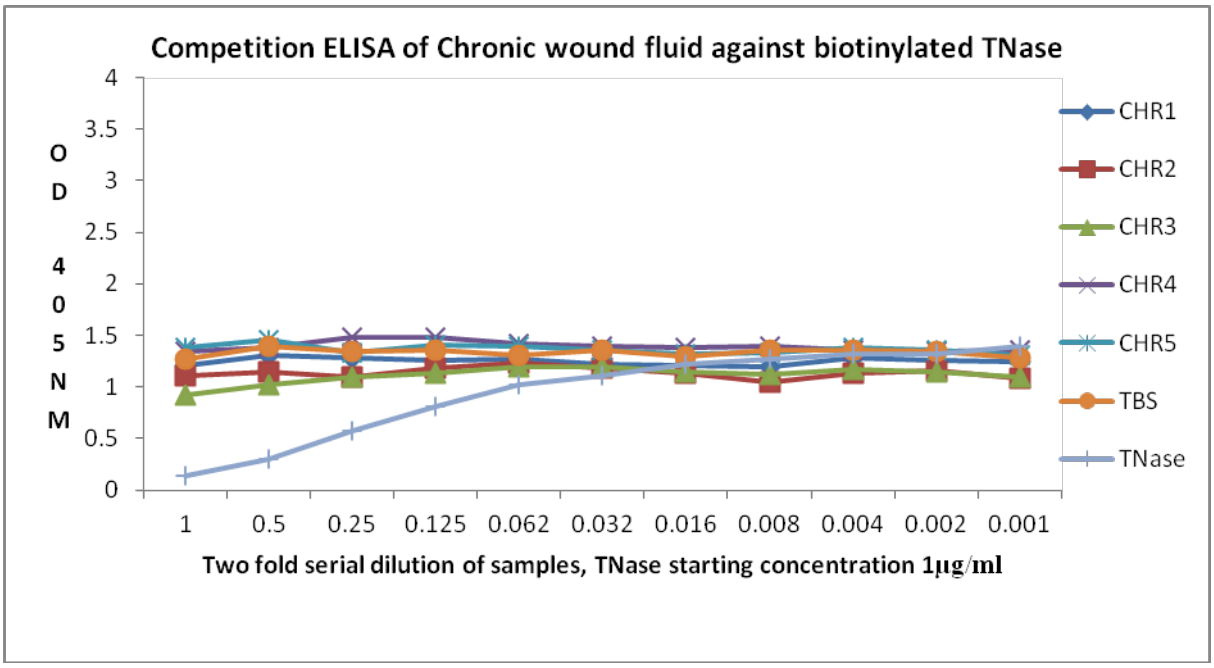


Figure 4.22 Competitive ELISA of chronic wound fluid samples 1-5 demonstrating no TNase detection, with a positive control of purified TNase. A pattern repeated in all chronic wound samples and concurring with the DNA agar results**.** This data was compiled from the mean of two runs, (full data tables can be found in Appendix 8).

Serial two fold dilutions	Chronic 6	Chronic 7	Chronic 8	Chronic 9	Chronic 10	TBS negative control	TNase positive control
1	1.139	1.220	1.058	1.082	1.176	1.272	0.024
0.5	1.087	1.025	0.823	1.125	1.224	1.392	0.053
0.25	1.087	1.025	0.778	0.901	1.004	1.345	0.066
0.125	1.098	1.073	0.852	0.939	0.928	1.359	0.098
0.062	1.295	1.160	1.004	1.006	1.210	1.311	0.196
0.032	1.288	1.149	1.063	1.204	1.258	1.353	0.399
0.016	1.326	1.181	1.113	0.967	1.148	1.300	0.494
0.008	1.265	1.206	1.036	0.960	1.132	1.358	0.856
0.004	1.317	1.209	1.187	1.053	1.220	1.355	0.934
0.002	1.363	1.334	1.301	1.272	1.256	1.346	1.143
0.001	1.372	1.276	1.319	1.188	1.340	1.287	1.221

Table 4.15 Absorbance values for Chronic wound fluids in a competitive ELISA with positive and negative controls.

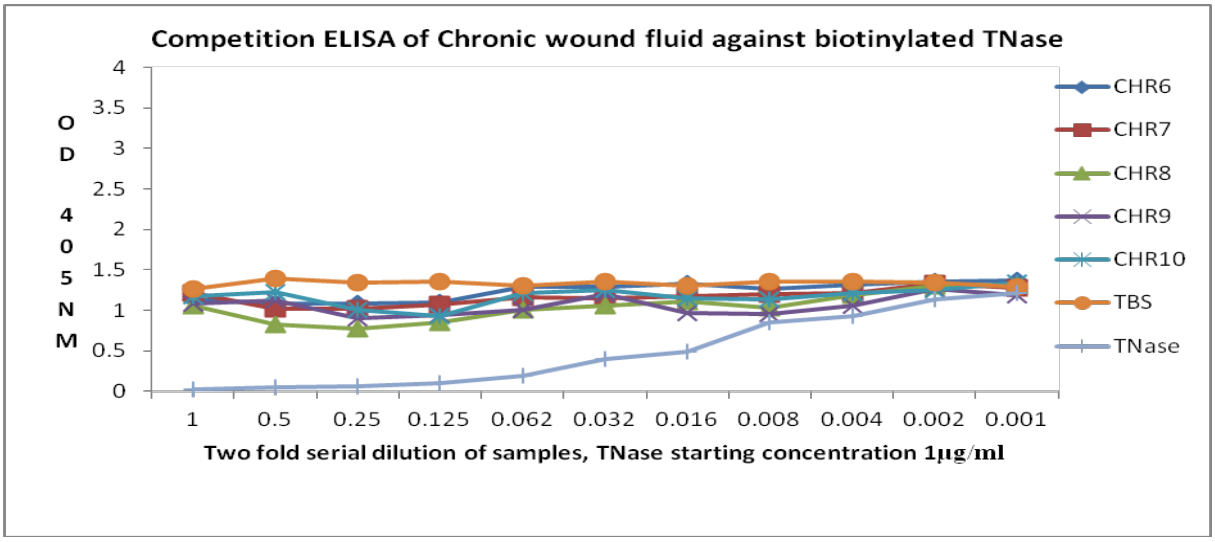


Figure 4.23 Competitive ELISA of chronic wound fluid samples 6-10 demonstrating no TNase detection, with a positive control of purified TNase. A pattern repeated in all chronic wound samples and concurring with the DNA agar results**.** This data was compiled from the mean of two runs (full data tables can be found in Appendix 8).

Concentration of wound fluid samples and application of ELISA

The results appeared to demonstrate definitive TNase detection in chronic wound samples 2-6 and 9 (fig 4.25, fig 4.26, fig 4.27, fig 4.28, fig 4.29, fig 4.32) and possible

detection in samples 10 and 8 (fig 4.31, fig 4.33), with no discernable competition in sample 1 (fig 4.24) figures 4.24-4.34 below illustrate the results of ELISA on these samples.

Two fold serial dilution	1.000	0.500	0.250	0.125	0.063	0.031	0.016	0.008	0.004	0.002	0.001
Negative control	1.061	1.020	0.929	0.963	0.997	0.944	0.961	1.012	1.009	1.078	1.046
TNase positive control	0.021	0.046	0.110	0.213	0.377	0.533	0.695	0.799	0.821	0.906	0.984
1X concentration (mean)	1.021	0.996	0.954	0.962	0.985	0.961	0.960	0.977	0.935	0.985	1.010
75X concentrated (mean)	0.661	0.817	0.846	0.890	0.931	0.909	0.942	0.957	0.916	0.915	0.943
Spiked chronic wound fluid	0.016	0.014	0.091	0.201	0.362	0.572	0.731	0.827	0.871	0.938	0.973

Table 4.16 Absorbance values for wound fluid 1 and concentrated x75 chronic wound fluid 1 in a competitive ELISA with positive and negative controls.

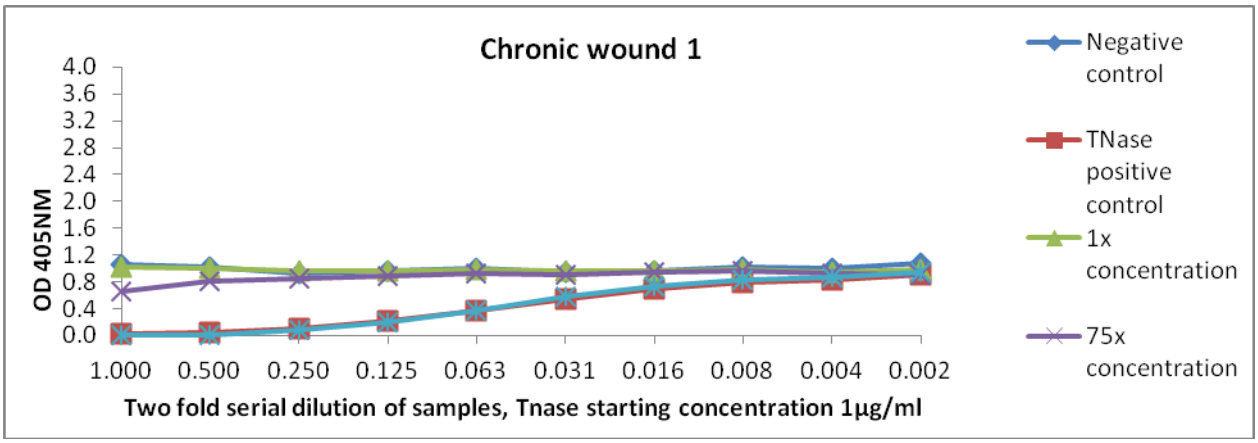


Fig 4.24 Competitive ELISA for detection *of S. aureus* TNase in concentrated wound fluid sample (CHR1). 1x concentration, 75x concentration and artificially contaminated are mean of two runs, controls one run only, (for full data see Appendix 8)

Two fold serial dilution	1.000	0.500	0.250	0.125	0.063	0.031	0.016	0.008	0.004	0.002	0.001
Negative control	1.002	1.102	1.080	1.047	1.018	0.927	0.988	0.984	0.954	0.987	1.027
TNase positive control	0.014	0.057	0.127	0.254	0.365	0.506	0.693	0.775	0.835	0.905	0.920
1X concentration (mean)	0.856	0.841	0.934	0.933	0.942	0.889	0.901	0.893	0.886	0.917	0.924
75X concentrated (mean)	0.080	0.207	0.385	0.630	0.735	0.773	0.818	0.829	0.834	0.895	0.864
Spiked chronic wound fluid	0.019	0.062	0.152	0.276	0.448	0.587	0.742	0.791	0.848	0.870	0.884

Table 4.17 Absorbance values for wound fluid 2 and concentrated x75 chronic wound fluid 2 in a competitive ELISA with positive and negative controls.

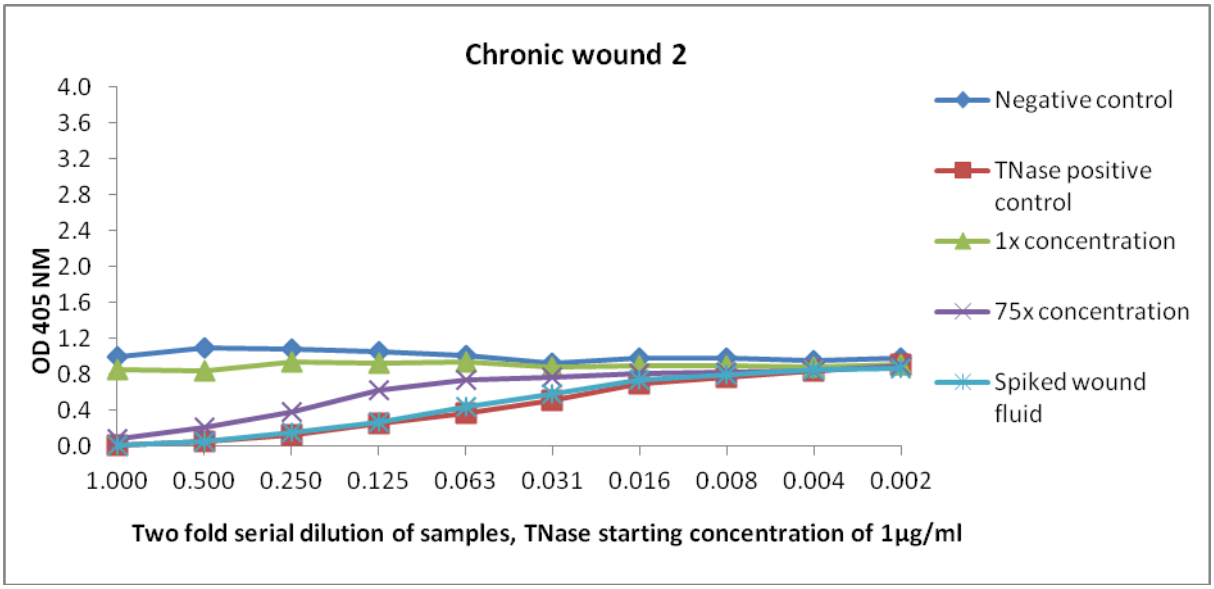


Fig 4.25 Competitive ELISA for detection *of S. aureus* TNase in concentrated wound fluid sample (CHR2). 1x concentration, 75x concentration and spiked are mean of two runs, controls one run only (for full data see Appendix 8).

Two fold serial dilution	1.000	0.500	0.250	0.125	0.063	0.031	0.016	0.008	0.004	0.002	0.001
Negative control	1.428	1.292	1.297	1.312	1.255	1.208	1.250	1.200	1.181	1.270	1.219
TNase positive control	0.045	0.060	0.153	0.307	0.457	0.605	0.772	0.911	0.961	0.898	1.071
1X concentration (mean)	1.045	1.058	1.098	1.135	1.131	1.146	1.174	1.182	1.161	1.197	1.189
75X concentrated (mean)	0.148	0.210	0.284	0.484	0.671	0.847	1.006	1.066	1.063	1.028	1.069
Spiked chronic wound fluid	0.009	0.035	0.136	0.215	0.415	0.611	0.791	0.898	0.954	0.955	1.014

Table 4.18 Absorbance values for wound fluid 3 and concentrated x75 chronic wound fluid 3 in a competitive ELISA with positive and negative controls.

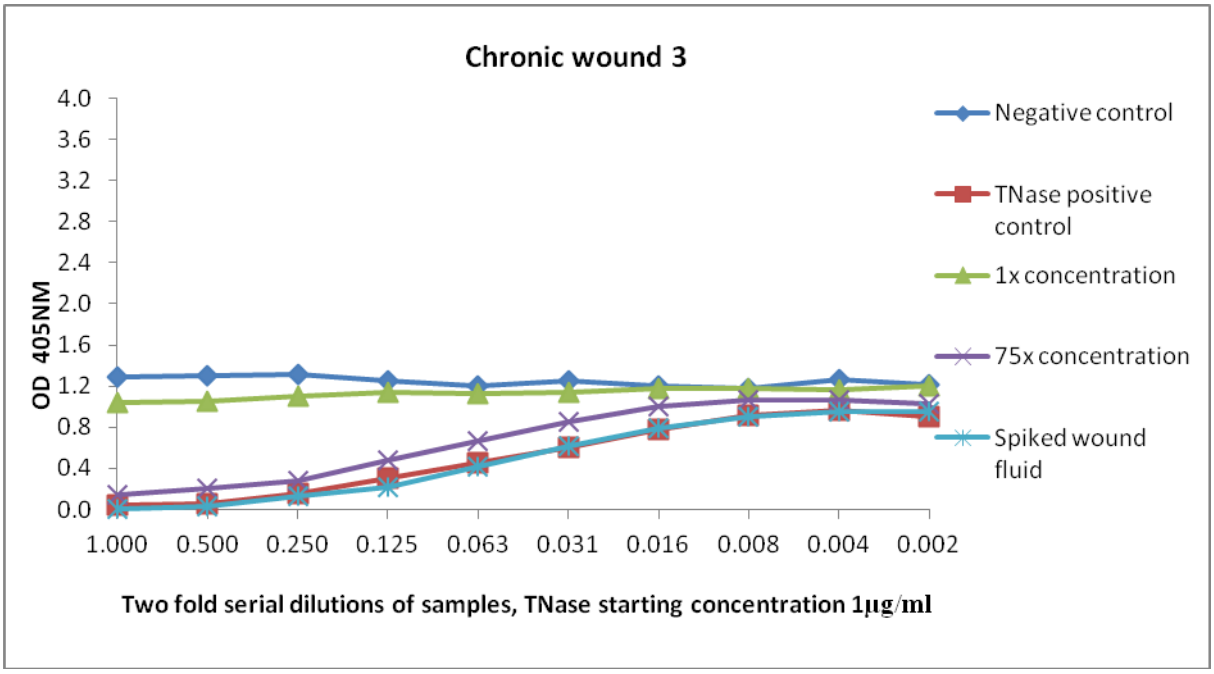


Fig 4.26 Competitive ELISA for detection *of S. aureus* TNase in concentrated wound fluid sample (CHR3). 1x concentration, 75x concentration and artificially contaminated are means of two experiments, controls one experiment only (for full data see Appendix 8).

Two fold serial dilution	1.000	0.500	0.250	0.125	0.063	0.031	0.016	0.008	0.004	0.002	0.001
Negative control	1.353	1.354	1.286	1.330	1.324	1.327	1.302	1.247	1.349	1.398	1.341
TNase positive control	0.014	0.063	0.139	0.255	0.467	0.688	0.829	0.957	1.073	1.122	1.223
1X concentration (mean)	1.109	1.225	1.220	1.265	1.221	1.205	1.220	1.200	1.231	1.160	1.301
75X concentrated (mean)	0.374	0.454	0.564	0.700	0.797	0.954	1.027	1.151	1.184	1.127	1.211
Spiked chronic wound fluid	0.008	0.002	0.089	0.193	0.404	0.689	0.868	0.986	1.077	1.123	1.230

Table 4.19 Absorbance values for wound fluid 4 and concentrated x75 chronic wound fluid 4 in a competitive ELISA with positive and negative controls.

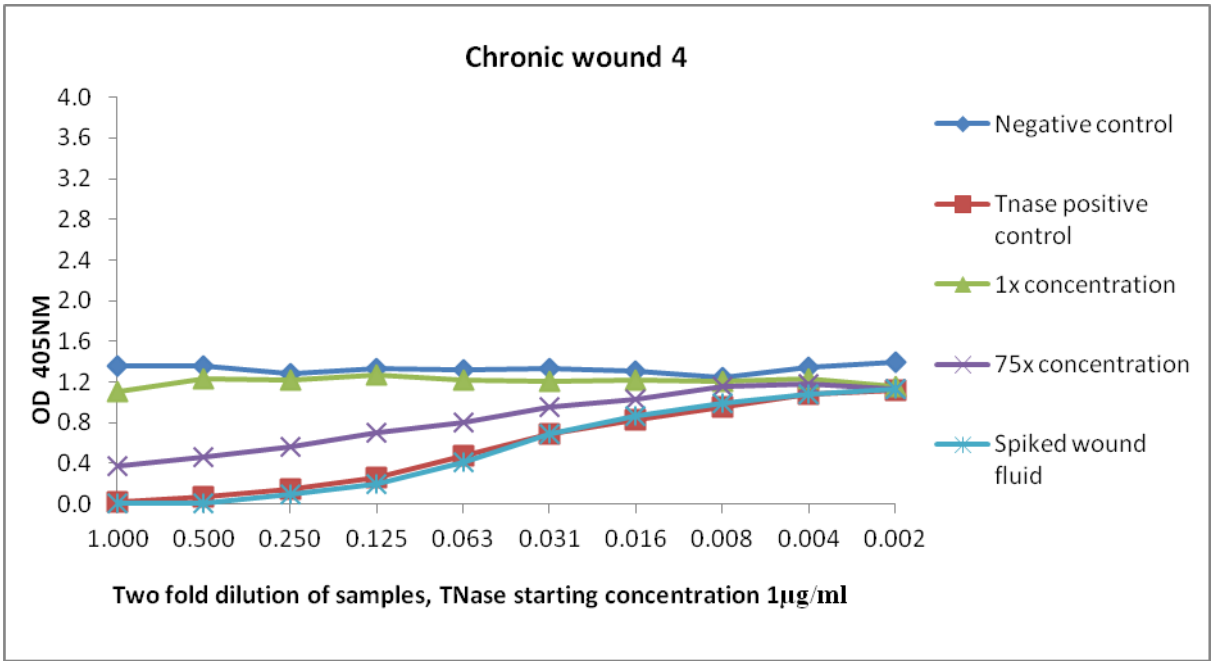


Fig 4.27 Competitive ELISA for detection *of S. aureus* TNase in concentrated wound fluid sample (CHR4). 1x concentration, 75x concentration and artificially contaminated are mean of two runs, controls one run only (for full data see Appendix 8).

Two fold serial dilution	1.000	0.500	0.250	0.125	0.063	0.031	0.016	0.008	0.004	0.002	0.001
Negative control	1.478	1.430	1.602	1.521	1.456	1.473	1.533	1.413	1.434	1.526	1.343
TNase positive control	0.012	0.054	0.155	0.323	0.506	0.807	1.033	1.296	1.296	1.418	1.287
1X concentration (mean)	1.278	1.280	1.245	1.222	1.274	1.269	1.289	1.329	1.292	1.241	1.423
75X concentrated (mean)	0.115	0.282	0.442	0.821	1.069	1.221	1.298	1.361	1.330	1.272	1.236
Spiked chronic wound fluid	0.003	0.044	0.163	0.321	0.559	0.800	1.088	1.286	1.375	1.414	1.229

Table 4.20 Absorbance values for wound fluid 5 and concentrated x75 chronic wound fluid 5 in a competitive ELISA with positive and negative controls.

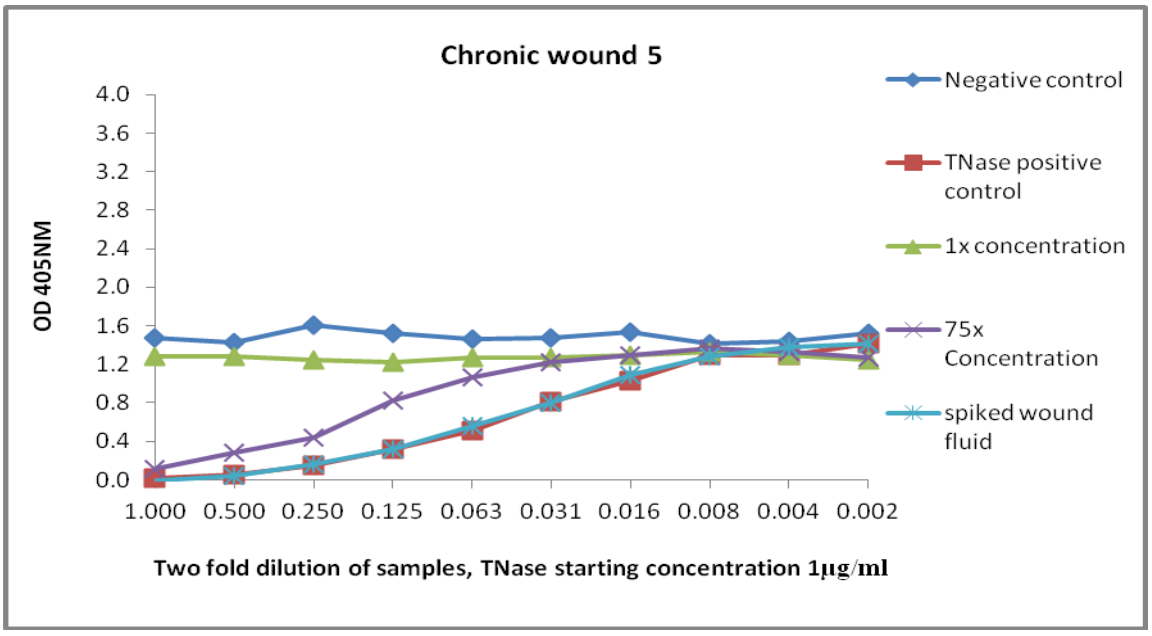


Fig 4.28 Competitive ELISA for detection *of S. aureus* TNase in concentrated wound fluid sample (CHR5). 1x concentration, 75x concentration and artificially contaminated are means of two experiments, controls one experiment only (for full data see Appendix 8).

Two fold serial dilution	1.000	0.500	0.250	0.125	0.063	0.031	0.016	0.008	0.004	0.002	0.001
Negative control	0.667	0.782	0.731	0.715	0.675	0.676	0.710	0.709	0.679	0.619	0.662
TNase positive control	0.024	0.089	0.175	0.270	0.362	0.450	0.486	0.549	0.586	0.645	0.630
1X concentration (mean)	0.482	0.542	0.576	0.419	0.591	0.497	0.609	0.581	0.618	0.614	0.600
75X concentrated (mean)	0.094	0.172	0.188	0.302	0.369	0.416	0.466	0.531	0.581	0.574	0.560
Spiked chronic wound fluid	0.017	0.097	0.221	0.302	0.417	0.494	0.519	0.556	0.591	0.607	0.582

Table 4.21 Absorbance values for wound fluid 6 and concentrated x75 chronic wound fluid 6 in a competitive ELISA with positive and negative controls.

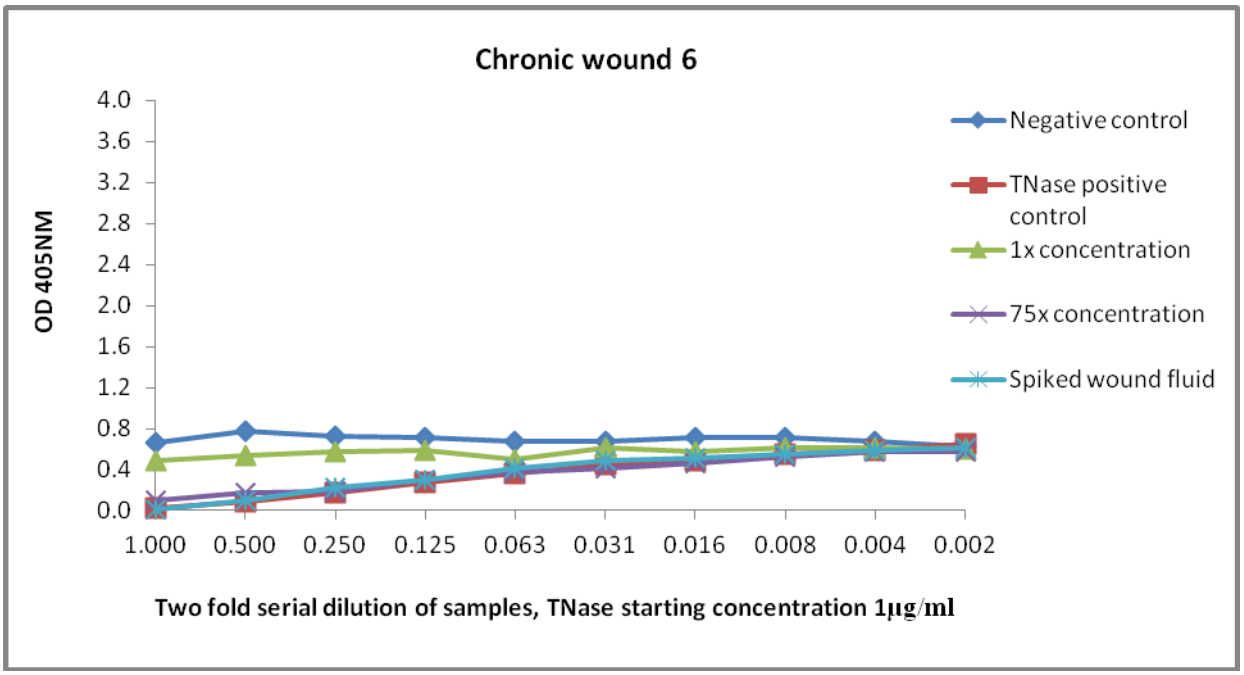


Fig 4.29 Competitive ELISA for detection *of S. aureus* TNase in concentrated wound fluid sample (CHR6). 1x concentration, 75x concentration and artificially contaminated are mean of two experiments, controls one experiment only(for full data see Appendix 8).

Two fold serial dilution	1.000	0.500	0.250	0.125	0.063	0.031	0.016	0.008	0.004	0.002	0.001
Negative control	0.783	0.783	0.728	0.781	0.788	0.761	0.769	0.763	0.764	0.759	0.687
TNase positivel	0.039	0.110	0.236	0.381	0.521	0.636	0.681	0.716	0.727	0.696	0.719
1X concentration (mean)	0.673	0.723	0.789	0.780	0.790	0.809	0.812	0.802	0.797	0.770	0.768
75X concentrated (mean)	0.575	0.624	0.633	0.648	0.648	0.708	0.752	0.791	0.766	0.728	0.742
Spiked chronic wound fluid	0.039	0.100	0.259	0.384	0.534	0.627	0.687	0.703	0.721	0.696	0.694

Table 4.22 Absorbance values for wound fluid 7 and concentrated x75 chronic wound fluid 7 in a competitive ELISA with positive and negative controls.

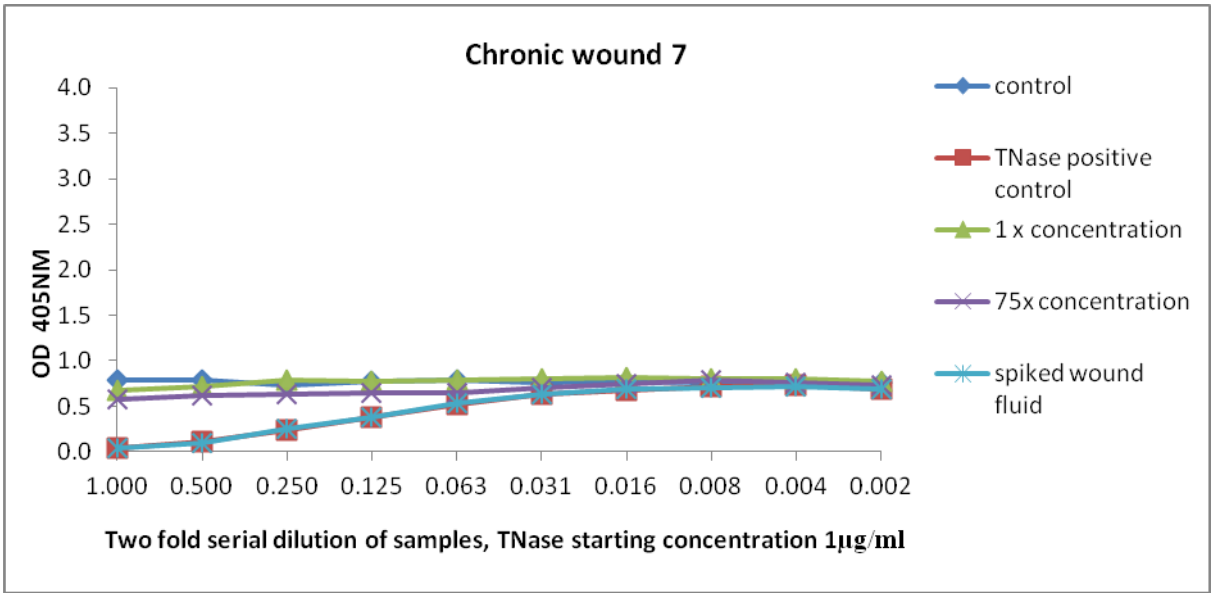


Fig 4.30 Competitive ELISA for detection *of S. aureus* TNase in concentrated wound fluid sample (CHR7). 1x concentration, 75x concentration and artificially contaminated are means of two experiments, controls one experiment only (for full data see Appendix 8).

Two fold serial dilution	1.000	0.500	0.250	0.125	0.063	0.031	0.016	0.008	0.004	0.002	0.001
Negative control	0.859	0.853	0.795	0.797	0.801	0.764	0.804	0.812	0.800	0.769	0.803
TNase positive control	0.045	0.114	0.227	0.403	0.535	0.629	0.685	0.751	0.765	0.754	0.803
1X concentration (mean)	0.588	0.707	0.627	0.793	0.784	0.797	0.763	0.770	0.782	0.773	0.820
75X concentrated (mean)	0.312	0.315	0.370	0.433	0.471	0.543	0.635	0.699	0.727	0.728	0.758
Spiked chronic wound fluid	0.019	0.054	0.196	0.282	0.431	0.605	0.651	0.700	0.696	0.722	0.771

Table 4.23 Absorbance values for wound fluid 8 and concentrated x75 chronic wound fluid 8 in a competitive ELISA with positive and negative controls.

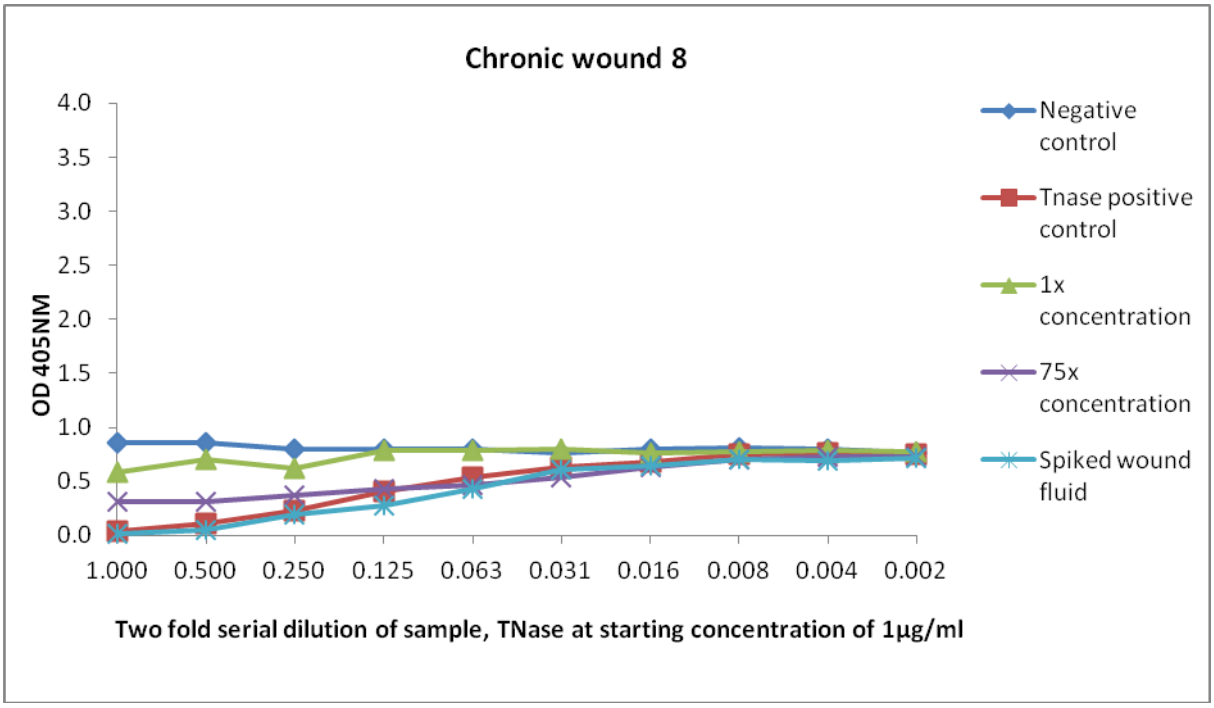


Fig 4.31 Competitive ELISA for detection *of S. aureus* TNase in concentrated wound fluid sample (CHR8). 1x concentration, 75x concentration and artificially contaminated are means of two experiments, controls one experiment only (for full data see Appendix 8).

Two fold serial dilution	1.000	0.500	0.250	0.125	0.063	0.031	0.016	0.008	0.004	0.002	0.001
Negative control	0.925	0.951	0.884	0.899	0.877	0.883	0.896	0.822	0.820	0.830	0.786
TNase positive control	0.052	0.135	0.240	0.383	0.511	0.654	0.658	0.718	0.726	0.740	0.727
1X concentration (mean)	0.725	0.793	0.810	0.836	0.798	0.846	0.827	0.845	0.842	0.823	0.772
75X concentrated (mean)	0.071	0.165	0.260	0.434	0.556	0.621	0.661	0.714	0.758	0.743	0.757
Spiked chronic wound fluid	0.031	0.110	0.224	0.354	0.544	0.660	0.715	0.751	0.731	0.733	0.460

Table 4.24 Absorbance values for wound fluid 9 and concentrated x75 chronic wound fluid 9 in a competitive ELISA with positive and negative controls.

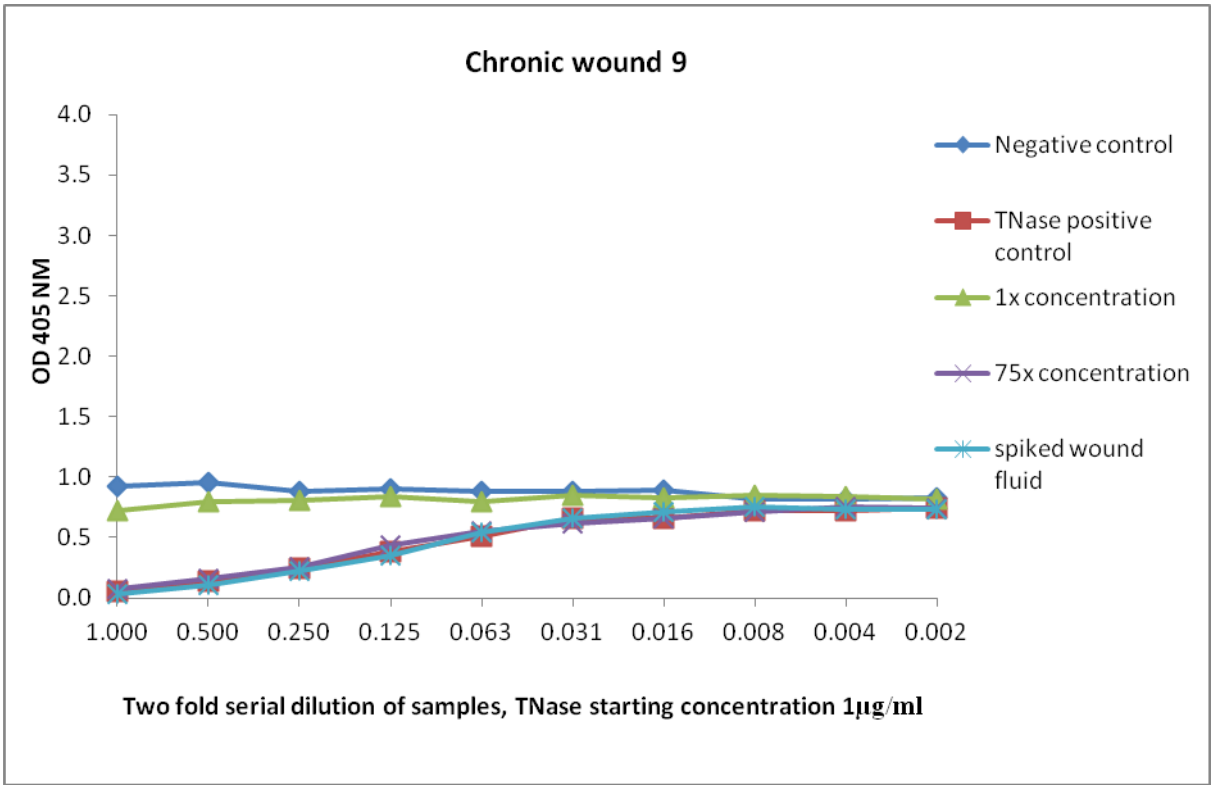


Fig 4.32 Competitive ELISA for detection *of S. aureus* TNase in concentrated wound fluid sample (CHR9). 1x concentration, 75x concentration and artificially

contaminated aremeans of two experiments, controls one experiment only (for full data see Appendix 8).

Two fold serial dilution	1.000	0.500	0.250	0.125	0.063	0.031	0.016	0.008	0.004	0.002	0.001
Negative control	1.062	1.031	1.111	1.114	1.060	1.092	1.104	1.134	1.136	1.082	1.051
TNase positive control	0.052	0.185	0.193	0.457	0.647	0.809	0.884	1.032	0.993	1.046	1.022
1X concentration (mean)	0.910	1.008	1.075	1.089	1.062	1.073	1.025	1.103	1.112	1.025	0.989
75X concentrated (mean)	0.590	0.650	0.615	0.720	0.785	0.796	0.842	0.951	0.947	0.964	0.992
Spiked chronic wound fluid	0.040	0.187	0.195	0.447	0.648	0.761	0.859	0.940	0.970	1.011	1.002

Table 4.25 Absorbance values for wound fluid 10 and concentrated x75 chronic wound fluid 10 in a competitive ELISA with positive and negative controls.

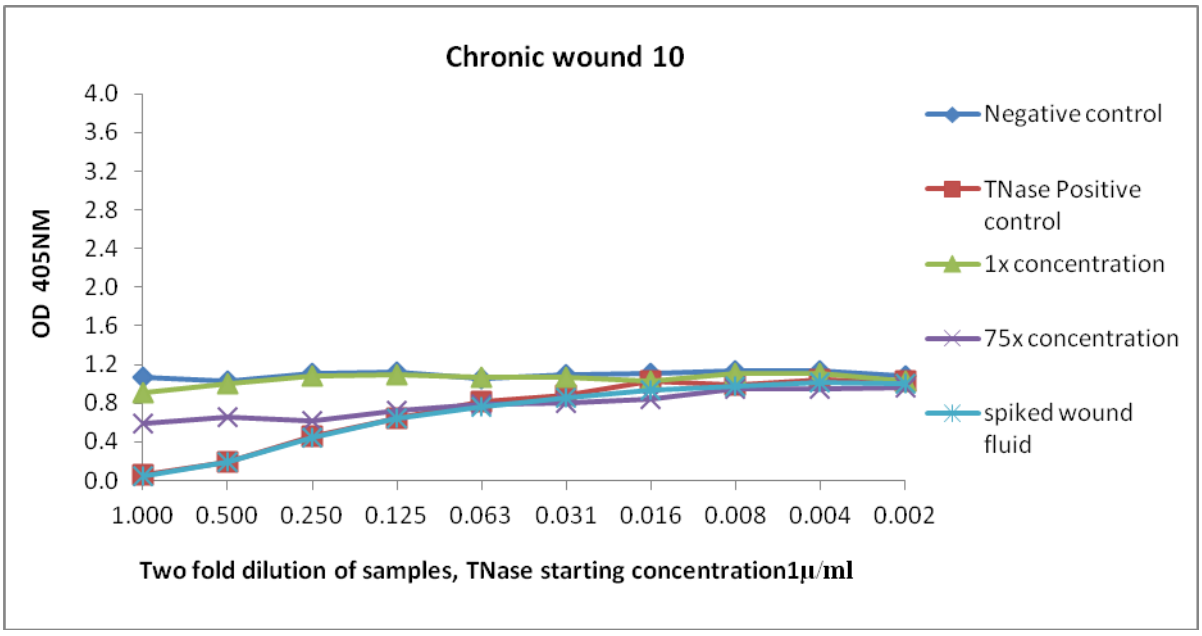


Fig 4.33 Competitive ELISA for detection *of S. aureus* TNase in concentrated wound fluid sample (CHR10). 1x concentration, 75x concentration and artificially contaminated are means of two experiments, controls one experiment only (for full data see Appendix 8).

Detection of *S. aureus* TNase using a modified ELISA format applied to chronic wound dressings

Filter paper from all six patients was assayed using the sandwich ELISA technique. Six samples were taken from each patient and compared to blank filter paper. TNase was not detected in any samples using this method.

Discussion

Monoclonal antibody targets

In this study, LPS present from the cell wall of *P. aeruginosa* was one target for MAB development. LPS consists of three components, the hydrophobic membrane anchoring lipid, a conserved oligosaccharide core region and an O-antigen section distally (Fig. 4.1). The O-antigen is a major component of the outer cell surface of the Gram-negative bacterial cell wall and is a well-established virulence factor of bacteria (Jamasbi and Taylor, 2009) to which patients will commonly develop antibodies. The O-antigen is however a variable molecule with most clinical isolates being classifiable into 10 varieties or serogroups (Hatano and Pier, 1998). A recent study of 16 *P. aeruginosa* strains demonstrated three differing O-antigen components and varying levels of expression within the same serotype (Jamasbi and Taylor, 2009).

LPS has been used as a target for MAB production for bacterial detection in other studies including that of Weisner *et al.,* 2007 who detected LPS in the sputum of patients with cystic fibrosis by various methods including ELISA. Lam *et al.,* 1987 developed two MABs to the O-antigen of *P. aeruginosa* LPS and studied binding by scanning electron microscopy secondary labelled antibodies with gold markers. Despite promising results with one strain, this was not replicated with a further 'wild type' strain of *P. aeruginosa*, possibly highlighting differing O-antigen regions. To counter this variation, previous studies have been attempted to utilise antibodies

raised against 8 of the most common O-antigens rather than just one (Kusama, 1983).

TNase is a thermostable nuclease produced by *S. aureus* and some other species of bacteria and this was also used as an antigen to generate MABs. TNase is a 5'-diphosphodiesterase which yields DNA or RNA hydrolysis products, predominately 3'-mono and dinucleotides (Yazdankhah *et al.,* 1999). TNase is the target of detection in several commercial tests for *S. aureus* with sensitivity rates as high as 96.7% at 2 hours and is the favoured microbiological test for rapid identification of *S. aureus* (Lagace-Wiens *et al.,* 2007).

Recent advances in the understanding of the methods used by neutrophils to combat bacterial infection have led to the discovery of neutrophil extracellular traps (NETs) (Brinkmann *et al., 2004*). These traps consist of a DNA backbone with associated antimicrobial peptides, histones and proteases. These NETs have been shown to form in response to *S. aureus* infection (Berends *et al.,* 2010). It has also been reported that TNase producing *S. aureus* reduce entrapment in NETs, whereas non-TNase producing *S. aureus* does not, indicating possible strain differences for TNase production.

TNase is also not entirely specific for *S. aureus* with some TNase negative *S. aureus* strains having reported (Neville *et al.,* 1991). The number of false negatives is likely however to be low with a recent study, Gonano et al 2008, demonstrating no TNase production in only one out of 48 strains isolated from human wounds. However, a

study by Chesneau *et al.,* (1993) demonstrated TNase production in nine of twenty eight staphylococcal species studied.

Previous studies utilising TNase as a target antigen for MABs have shown sensitivity rates as low as 1 ng/ml of TNase (Yazdankhah *et al.,* 1999) when utilised in ELISA with magnetic beads, which they estimated as corresponding to the amount of TNase produced and secreted by 10^5 CFU/ml of *S. aureus*.

TNase has successfully been used as a marker for the identification of *S. aureus.* In previous studies, detection of this antigen has shown a high positive identification rate, with low numbers of false negatives (<1%) (Gonano *et al.,* 2009). However, it is clear from this, and other studies, that false-positive results can also occur due to TNase activity in some strains of coagulase negative staphylococci (CNS) (Chesneau *et al.,* 1993). Hence, MABs developed against TNase would appear to be good identifiers for *S. aureus* strains, although they show similar limitations, and some false positives may be expected.

Interestingly, previous studies utilising MABs raised to TNase produced by *S. aureus* have demonstrated that some of these antibodies are specific to *S. aureus* TNase whilst some detect all TNase produced by different species of bacteria. This is almost certainly as a function of the binding site of the antibody as there may only be slight differences in TNase configuration between species (Brakstad *et al.,* 1995). It is therefore possible to produce *S. aureus* TNase specific MABs, as although the activity of TNase is not specific to *S. aureus* the nuc encoding gene has *S. aureus*

specific sequences leading to minor differences between *S. aureus* TNase and those produced by other bacteria. MABs specific to *S. aureus* TNase were previously produced by (Brakstad *et al.,* 1995). Unfortunately this information or epitope mapping was not available for the antibody used in the present study.

Monoclonal antibodies for use in the detection of target bacteria

MABs and the ELISA methods allow the rapid and effective detection of bacteria within a few hours (Yazdankhah *et al.,* 1999). The O-antigen of the LPS is expressed on the cell wall of the bacterium and should therefore be easily available for presentation to the antibody and subsequent detection through fluorescent microscopy of ELISA. However the O-antigen of the LPS has variable regions with 11 commonly recognised variants. It was anticipated that the antibody used would be specific to a constant epitope, however it was possible that specificity was to a variable epitope of O-antigen particular to the LPS of the PAO1 strain *P. aeruginosa*. If the latter was the case then use of the antibody would be limited to selected strains of *P. aeruginosa*.

TNase is manufactured intra-cellularly and then secreted by *S. aureus* at which point it is available for presentation of the antigen and subsequent detection through fluorescent microscopy of ELISA. TNase is not specific to *S. aureus* and is expressed by other species such as. *S. schleiferi, S. carnosus, S. intermedius, S. hyicus, S. caprae* and *S. capitis* (Kloos and Bannermann, 1994), along with some streptococci and

bacilli (Park *et al.,* 1980). One other potential problem of using TNase as an antigen was that there is no reliable data as to when it is produced and expressed. If it is constitutively expressed by all strains of *S. aureus* then it would be an advantageous choice of antigen, however if it is produced only at times of stress or when the bacteria are above a certain cell density then variability in the test would be encountered. In the case of the latter this could also be an advantage when testing for *S. aureus* in chronic wounds as undoubtedly a relatively high cell density would be synonymous with the pathogenic state.

Immunofluorescent microscopy

Immunofluorescent microscopy utilising MABs and FITC labelled conjugated anti-mouse antibodies was successful in a limited manner in detection of *P. aeruginosa* and *S. aureus* planktonic bacteria (Fig. 4.7 and 4.8). However, interpretation of these results was difficult due to auto-fluorescence and quenching which was particularly evident with *P. aeruginosa* as well as biofilm specimens (fig 4.9). The use of an alternate non-quenching fluorescent marker (*e.g.* Alexa 594) may have negated this risk of false negatives. If this molecule could have been attached directly to the MAB rather than a secondary antibody a simpler method could have produced more reliable results. There appears to be no reliable method for reducing autofluorescence in bacteria or tissue and therefore no current way of reducing the risks of false positives in this technique.

A very similar method was used by Gu *et al.,* 2002 using MABs to *Streptococcus mutans* which was then treated with FITC-conjugated goat anti-mouse antibodies to

detect the bacteria in salivary samples. This study did not mention FITC quenching or the problems of auto fluorescence and claimed 100% sensitivity and specificity in detecting *S. mutans*.

Immunofluorescence microscopy of CDFF and RHE generated biofilms

The methods for visualising bacteria within biofilms developed in the CDFF and on a RHE model were very similar, both were paraffin embedded and then cut into 10 µm sections and mounted on slides prior to processing. Both had biofilms evident through Gram-staining but the RHE exhibited autofluorescence thus preventing bacterial detection. The biofilms generated in the CDFF autofluoresced to a lower extent and although there were some possible positive results particularly with the *P. aeruginosa* biofilms (fig 4.9), the interpretation of these results was again compromised by rapid quenching. Fluorescent microscopy, depending on observer interpretation of light levels, suffers from autofluorescence particularly when used in conjunction with tissue samples and highly fluorescent bacteria such as *P. aeruginosa.*

Previous studies identifying bacterial species in biofilms using fluorescently labelled MABs (Gu *et al.,* 2005) directly conjugated their MABs to fluorescent labels that did not quench and utilised a CLSM rather than a light microscope to successfully visualise individual bacteria within biofilms. In view of the difficulties with immunofluorescence microscopy it was decided to proceed to an ELISA method for detection of targets in which would negate the problems of autofluorescence and quenching previously encountered.

ELISA for the detection of *S. aureus* TNase

Initial experiments were undertaken to establish the optimal amount of biotinylated *S. aureus* TNase to use in competitive ELISAs. The results indicated that 80% of the maximal ELISA response was generated by ~78 μg/ml of biotinylated TNase (see fig 4.10). This was the concentration used in all subsequent competitive ELISAs and enabled competition with *S. aureus* TNase to be reliably detected.

The competitive ELISA sensitivity and specificity

Using the competitive ELISA, competition was as expected, demonstrated with purified *S. aureus* TNase. The sensitivity of *S. aureus* TNase detection was deemed to be ~62.5 ng/ml. Similarly, competition was evident with *S. aureus* culture supernatants and also supernatants from *S. aureus* biofilms on RHE (Fig. 4.11).

Further ELISAs indicated (Fig. 4.11) that as expected the highest competition occurred using purified *S. aureus* TNase, followed by *S. aureus* FRI 115M culture supernatants (NB it was from this strain that the purified TNase was obtained and to which the MAB was raised) and then *S. aureus* SaB2 supernatant. No competition was evident in the uninoculated BHI control.

It is possible that *S. aureus* FRI 115M strain produces more TNase than SaB2 strain or alternatively that the MAB was more specific to the TNase from *S. aureus* FRI 115M. Additional studies on *S. aureus* FRI 115M supernatant (Fig. 4.12) using filtered and unfiltered supernatants showed little difference in detection, thus indicating that the

cells and other debris within the unfiltered supernatant were not interfering with the ELISA. Using the competitive ELISA no cross reaction between the B34 antibody to *S. aureus* TNase and *P. aeruginosa* RHE biofilm supernatants was evident (Fig. 4.14). These results indicated a high level of sensitivity and specificity and a lack of cross reaction of bacterial products and cells affecting the competitive ELISA developed.

Detection of *S. aureus* TNase using DNA agar

Before using the competitive ELISA chronic wound samples were crudely screened for *S. aureus* TNase using DNA agar. All chronic wound fluids tested were negative in these tests for TNase. This was perhaps surprising given that previous microbiological and PCR analysis had shown the presence of *S. aureus* within some of these samples. The failure of the DNA agar to detect *S. aureus* TNase most likely reflects the low concentration of the enzyme following dilution during wound fluid collection (see methods chapter 1). It was also possible that the *S. aureus* present within these fluids had not secreted TNase and thus the TNase may have been present intracellularly and not available for detection.

Competitive ELISA on wound fluids artificially contaminated with *S. aureus* TNase

To ensure there would be no interference of the ELISA by wound fluid components it was decided to use the competitive ELISA on wound fluids artificially contaminated with *S. aureus* TNase (fig 4.20). Wound fluid, and particularly chronic wound fluid is a complex mix of cells, proteins and growth factors which could inhibit a MABs action through preventing it reaching its target antigen. Chronic wound fluid not previously shown to contain *S. aureus* by culture or PCR was artificially contaminated

with purified *S. aureus* TNase and subjected to competitive ELISA. The TNase in these samples was reliably detected in these assays, indicating that the components of the chronic wound fluid did not inhibit the ELISA.

Wound fluids and the competitive ELISA

All acute wound fluids analysed by competitive ELISA demonstrated no competition, indicating the absence of *S. aureus* TNase. This was expected given that all acute wound samples except sample 2a, b, and c were negative for *S. aureus*, and the amount of CFU/ml cultured within samples 2a, b, and c was low (<833.3 CFU/ml). All chronic wound fluid samples were negative for S. aureus TNase by ELISA (fig 4.22, fig 4.23), including the samples from which *S. aureus* was isolated (counts as high as $2.4x10^7$ CFU/ml). Two possibilities for these results were considered, the first was again due to sample dilution during collection resulting in a *S. aureus* TNase concentration below the sensitivity of the ELISA (<62.5 ng/ml), or that *S. aureus* was present and not secreting TNase, as previously postulated. Further studies, using a more sensitive analysis *e.g.* surface plasmon resonance may be of value in establishing the presence of TNase in these samples.

Correlation between TNase detection and *S. aureus* cell number using ELISA

As the competitive ELISA detects TNase effectively in a wound fluid environment, and the samples had been shown to contain *S. aureus* it was necessary to accurately determine of the levels of *S. aureus* required to produce detectable levels of TNase. It is not known when *S. aureus* produces TNase, and it is possible that this occurs constitutively, however given the nature of the enzyme it is more likely that this

occurs when populations reach a certain density and the supply of available nutrients become deficient.

To determine the number of *S. aureus* required to produce detectable TNase, a *S. aureus* FRI 115M growth curve was produced (fig 4.16) and the culture supernatants at measured time points were subjected to the competitive ELISA (fig 4.17, fig4.18, fig4.19). The results first suggested TNase presence at 12 hours and clear detection occurred at 16 and 24 hours. A cell concentration $3.35x10^8$ CFU/ml was considered necessary to produce TNase at the necessary detectable level. Interestingly this density of *S. aureus* far exceeds that detected within our chronic wound fluid samples, providing a likely explanation that out MAB was not sensitive enough to detect any TNase that may have been produced within our samples. Of course a lower cell number might be able to generate detectable concentrations of TNase provided a sufficient period of time was available and that the TNase remained stable in the environment.

Concentration of chronic wound fluid samples

Diluted chronic wound fluid samples were concentrated in order to improve sensitivity of the assay. After concentration *S. aureus* TNase was detected in samples 2-6 and 9, with possible detection in samples 8 and 10. No TNase was detected in sample 1 see fig 4.24-Fig 4.33 inclusive.

It should be noted that the positive results on these concentrates did not correspond exactly to the cultural and molecular findings for the presence of *S. aureus*. False positives could occur due to the presence of other TNase producing bacterial species such as CNS. However, more likely is the presence of TNase which could persist after the loss of *S. aureus* from the environment (Yazdankhah *et al.,* 1999). When ELISA was negative but culture positive for *S. aureus*, an additional explanation could be the persistence of *S. aureus* within epithelial cells (Yazdankhah *et al.,* 1999) where any secreted TNase may be unavailable for subsequent detection.

ELISA application to chronic wound dressings

An attempt to modify the ELISA format and apply it to filter paper imprints of chronic wound dressings was undertaken. It was hoped that since these imprints were directly from the dressing and not subjected to dilution then greater sensitivity of detection would occur.

The results of the chronic wound dressing ELISAs showed no detectable TNase within the tested samples including WD1 where *S. aureus* had been detected by culture/PCR. The fact that these samples were negative may indicate absence of TNase. Further work is however needed in this area to create a reliable filter paper based ELISA.

Summary

The approach of using MABs and fluorescent microscopy to directly detect bacterial species in clinical samples is of great value as this would greatly reduce the period

required for detection compared with traditional culture. Furthermore, previous studies using permanent non quenching markers directly linked to the antibody and CLSM rather than standard fluorescent microscopy (Gu *et al.,* 2005) hold great promise for the dynamic investigation of bacteria within biofilms.

The method of utilising MABs and ELISA to rapidly detect bacteria is well established and offers a rapid, efficient and reproducible testing format that could be simply translated into a bedside or simple laboratory test. The novel MABs and competitive ELISA used in this present study reliably detected purified *S. aureus* TNase from *S. aureus* culture supernatants, RHE culture medium and artificially contaminated wound fluids. Unfortunately, *S. aureus* TNase was not detected initially from chronic wound fluid samples, which was consistent with the screening analysis using DNA agar. However, concentrating the collected wound fluids did overcome potential problems of low levels of TNase in the samples. Further study in optimising the ELISA and sample collection/processing would be recommended. Possible other *S. aureus* specific targets for future study include the recently described extracellular adherence protein (*EAP)* (Hussain *et al.,* 2008) which showed 100% specificity to *S. aureus* with no false-positives. It would also be of value to consider MABs developed against antibiotic resistance pumps in Methicillin Resistant *S. aureus*. Antigens developed to a constant and specific cellular wall protein or even the mobile extracellular flagella would be useful in further studies of *P. aeruginosa*.

Chapter Five: Characterisation of biofilms on tissues using fluorescent *in situ* hybridisation

Introduction

Previous investigations in this thesis have characterised the bacterial communities in chronic wounds using species-specific monoclonal antibodies raised against *P. aeruginosa*, and *S. aureus* (Chapter 4). This chapter focuses upon the analysis of bacterial biofilm communities colonising surfaces of Reconstituted Human Epithelium (RHE) and human debrided wound tissue using molecular probes. Specifically, studies aimed to determine the type and quantity of bacteria present, together with the structure of the biofilm typical of chronic wounds. An approach based on Fluorescent *in situ* Hybridisation (FISH) utilising bacterial specific Peptide Nucleic Acid (PNA) probes and Confocal Laser Scanning Microscopy (CLSM) was used.

Traditional analysis of microbial communities

A wide range of techniques can be used to identify bacterial species in different fields of microbiology. Simple bacterial culture is routinely employed for the isolation of microorganisms from clinical origin, including chronic wounds. Culture methods are of benefit in the identification of culturable bacteria that commonly cause wound infection. However, the approach clearly requires an ability of the microorganisms to grow on the selected agar medium together with the atmospheric conditions employed. The existence of unculturable microorganisms is

widely recognised, and it has been estimated that for many microbial populations, culturable organisms represent only a small proportion (only 2-3%) of the total bacterial population (Amman *et al.,* 2001, Moter and Göbel, 2000). Cultural analysis can also be time-consuming (Amman *et al.,* 2001) and even in instances where differential agar media are used, supplementary testing including biochemical and serological analysis is required for definitive identification. Traditional microbiological analysis is often limited in terms of the information generated on the bacterial phenotype and structural composition of bacterial communities, when compared with other methods (Thunheer *et al.,* 2004)

Bacterial staining approaches such as Gram-staining coupled with light microscopy can provide detail on bacterial cell walls and a limited amount of information regarding the structure of biofilms. The method broadly delineates two groups of bacteria (Gram-positive and Gram-negative) but does not permit species identification (Moter and Göbel, 2000). Molecular microbiological methods based on the analysis of DNA/RNA extracted from microbial populations can be very effective at species identification, but in mixed communities PCR bias can theoretically occur and the processing steps involved in the nucleic acid extraction methods will generally destroy any structural detail of the bacterial community (Amann *et al.,* 2001).

Analysis of biofilm composition and structure

A more detailed characterisation of biofilms is important since identification of pathogenic bacteria and their quantity and location within biofilms can aid in

assessing the risk status of any infection (Gu *et al.,* 2005). This is perhaps best demonstrated by studies on dental plaque (a well-recognised and studied biofilm) where a correlation between the relative proportion of certain bacteria and the occurrence of dental caries has been established (Marsh, 2003). It is highly probable that the same principle applies to biofilms in other clinical environments. For example in chronic wounds it has been shown that some wounds heal when a biofilm is present (Leaper 1994, Kanno *et al.,* 2010), whilst others have their healing arrested and will deteriorate (Schierle *et al.,* 2009). Marsh (2003) also hypothesised that changes in the oral environment (pH, nutrition, oxygen concentrations, medications) could alter gene expression within oral microflora, increasing the competitiveness of pathogenic bacteria, whilst decreasing the competitiveness of the normal microflora, thus promoting disease. This view has been termed the 'environmental plaque hypothesis' (Marsh, 2003). It would seem logical to extrapolate this hypothesis to chronic wound microflora and if demonstrable may lead to new strategies for altering wound environment to maximise beneficial or non-harmful species within the biofilm allowing a 'healing biofilm' to develop rather than focusing on biofilm eradication. This approach would, however, require an accurate assessment of biofilm structure, distribution and numbers of species involved.

The ideal method for characterisation of bacterial communities within chronic wounds would be one that combines the species specificity of the molecular techniques together with staining and microscopy, allowing the analysis of the biofilm structure. Biofilms communities are dynamic, with continuous changes

occurring in structure and species composition and an appropriate method would facilitate observation of this dynamic biofilm without killing the microbial cells.

Structure of biofilms

As previously discussed (Chapter 1) biofilms occur ubiquitously where microorganisms and a surface come into contact with sufficient nutrition to support microbial growth. Biofilms can be polymicrobial and are structurally organised into multiple microcolonies with interspersed water channels. As such, biofilms consist of bacterial cells, water, and cell aggregates embedded into an exopolysaccharide (EPS) matrix (Costerton, 1999 and 1995).

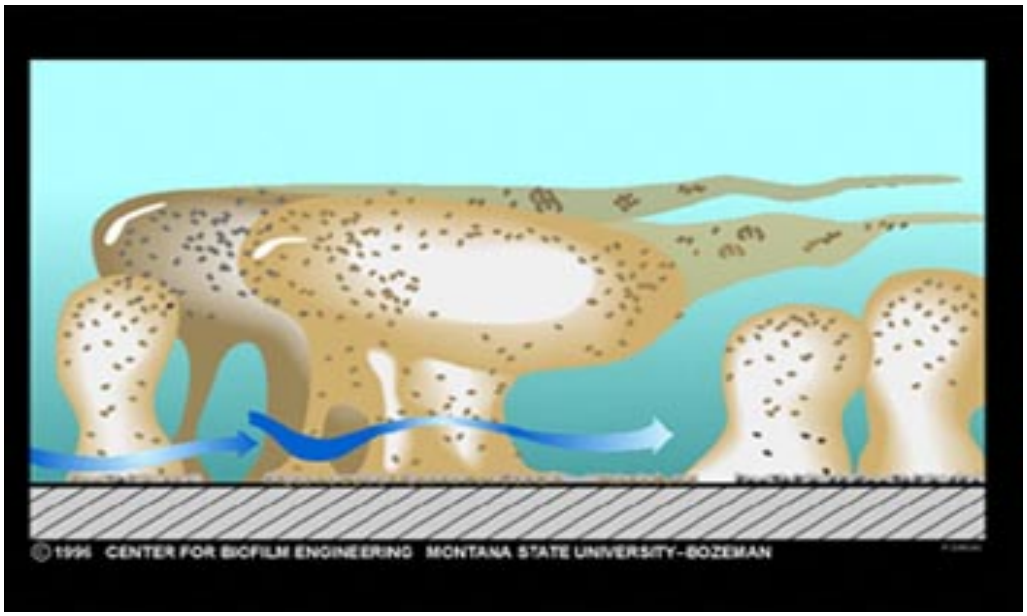


Figure 5.1. Schematic representation of a biofilm (courtesy of Bozeman- Centre for Biofilm Engineering Montana State University)

Fluorescence *in situ* hybridisation (FISH)

In situ hybridisation (ISH) is a technique that was first developed in 1969 (Pardue and Gall, 1969) and allowed nucleic acid sequences to be detected inside cells using radioactive labels without altering the cell morphology or integrity of various components (Moter and Göbel, 2000). The approach was not applied to bacterial cells until 1988 (Giovannoni *et al.,* 1988). Radioactively labelled probes have since been replaced in many applications by fluorescent probes, leading to the development of the fluorescent *in situ* hybridisation (FISH).

FISH detects nucleic acid sequences using a fluorescently labelled oligonucleotide probe that hybridises specifically to its complementary target sequence within the intact cell (Moter and Göbel, 2000). The procedure includes several universal stages (Fig 5.2). Specimens are first fixed prior to exposure to any necessary pre-treatment steps. Hybridisation of the oligonucleotide probe to the target molecule is followed by washing to remove unbound probe. Mounting and imaging of the specimen are the final stages of the process. Fluorochromes with different excitation and emission spectra allow for the simultaneous detection of multiple probes within a single specimen, and therefore the potential to visualise several microbial species. This adaptation is frequently termed as multiplex staining (Thurnheer *et al.,* 2004).

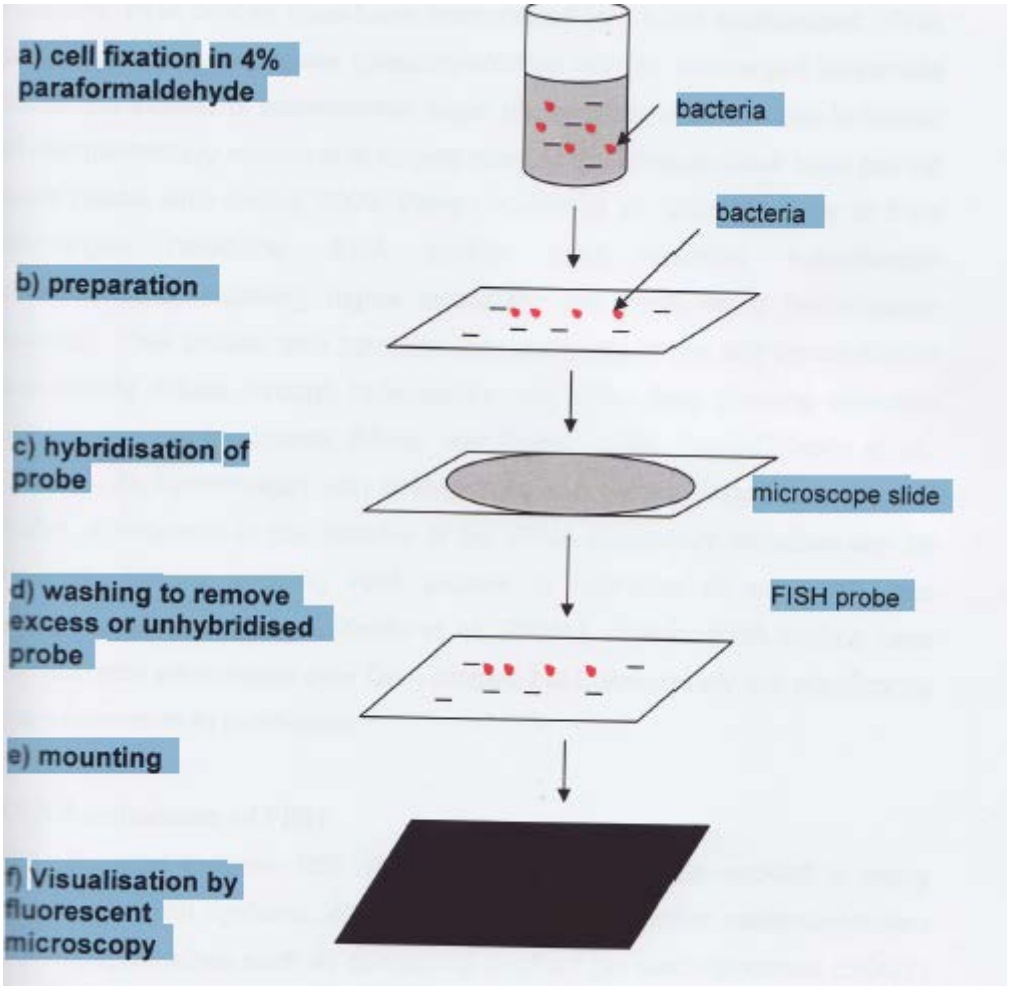


Figure 5.2. Schematic illustrating typical stages involved in FISH (Courtesy of Dr S. Malic, Ph.D. thesis (2008) 'The *In Vitro* Development and Characterisation of Two Clinically Important Biofilms').

FISH has been used for direct imaging of microorganisms since 1999 (Sekiguchi *et al.,* 1999). The method initially targeted 16S ribosomal RNA (rRNA) sequences and was applied to a variety of diverse bacterial systems, such as microbial isolates (Mignard and Flandrois, 2006) and dental plaque (Thuneer *et al.,* 2001). Probes were selected that targeted rRNA sequences because the encoding gene regions are often functionally conserved as well as being abundant (usually more than 10000 per cell)

(Perry-O'Keefe *et al.,* 2001) throughout the genome thus allowing a high degree of sensitivity and specificity.

The 16S rRNA gene sequences for a wide range of bacteria are readily available within genomic databases and this allows the design of probes to target specific species or groups of bacteria. The probes used in FISH are typically 15-30 nucleotides long and are covalently bound at the 5' end to a single fluorescent dye molecule (Helmer-Madhok *et al.,* 2002). Whilst useful, FISH does, like all methods of characterisation have some limitations. DNA probes can bind to intracellular proteins and due to the thick cell wall of Gram-positive bacteria there is sometimes insufficient probe penetration (Malic *et al.,* 2009).

Peptide Nucleic Acid (PNA) probes

Peptide Nucleic Acid (PNA) probes have over the last few years been incorporated into FISH (Malic *et al.,* 2009). PNA probes are DNA analogue molecules consisting of an uncharged polyamine backbone instead of conventional sugar phosphates. These DNA analogues hybridise with DNA sequences through complimentary base pairing (Moter and Göbel, 2000, Pardue and Gall, 1969). As PNA probes have an uncharged backbone, they tend to have superior hybridisation characteristics with higher specificity and exhibit more rapid hybridisation compared with equivalent DNA probes. It has also been proposed that PNA probes can more readily diffuse through hydrophilic cell walls allowing hybridisation to occur without the need for stringent pre-treatment stages (Moter and Göbel, 2000, Pardue and Gall, 1969). As a result, hybridisation with PNA probes can be performed in a low salt buffer and reduced

stability of the rRNA secondary structure can be achieved, allowing PNA probes to hybridise to otherwise less accessible target pairings (Moter and Göbel, 2000, Pardue and Gall, 1969). It could therefore be argued that PNA probes have considerable advantages over DNA probes, although the former are very expensive to synthesise and this does limit their use in routine clinical applications.

The use of PNA probes in bacterial studies is increasingly being reported as the advantages of the technique become more widely recognised. Recent studies have successfully and rapidly differentiated between *Enterococcus* species (Wellinghausen *et al.,* 2007) and allowed the rapid (in less than 2.5 h) identification of *S. aureus* in blood culture bottles with 100% specificity and sensitivity (Oliveira *et al.,* 2003). Recently, a PNA probe was used successfully to detect *Helicobacter pylori* in paraffin embedded gastric biopsy specimens enabling its easy and rapid detection with 100% specificity (Guimarães *et al.,* 2007). Further PNA/FISH work has allowed the definitive detection of *Candida* species cultured from solid and liquid media in 1 h, whilst claiming a specificity and sensitivity of 100% (Trnovsky *et al.,* 2008). Whilst these studies have demonstrated the rapid and specific identification of pure microbial species in culture media, further work by Malic and colleagues (Malic *et al.,* 2009) have showed the effectiveness of the PNA/FISH technique in the analysis of multispecies communities allowing the detection of both *S. aureus* and *P. aeruginosa* strains, both planktonically and within a biofilm.

Aims

The aim of the work described in this Chapter was to use PNA-FISH as an approach to further characterise chronic wound biofilm populations. Detection of *S. aureus* and *P. aeruginosa* was the focus of study, as both of these species are prevalent in chronic wounds and recognised as prolific biofilm formers. As previously mentioned (Literature review) it is important to remember that the presence or absence of *S. aureus* and *P. aeruginosa* biofilms have not been definitively shown to be related to chronicity or healing of wounds.

Materials and Methods

Peptide Nucleic Acid (PNA) Probes

Three PNA probes were used in this present study and consisted of the universal bacterial probe (Bac-UniCY3), *P. aeruginosa* specific probe (PsaerFITC) and a *S. aureus* specific probe (Sta 16S03). Details of these probes are provided in Table 5.1. The sequences of these probes were partially adapted from Perry-O'Keefe *et. al*, (2001). The probes were manufactured by Boston Probes (Foster City, USA) for Applied Biosystems (Warrington, UK) and were labelled either with FITC, Cyanine 5 (CY5) or Cyanine 3 (CY3) and designed to target the 16s rRNA gene of bacteria. All probes were pre-validated using the PNA designer software (Applied Biosystems) before synthesis.

Probes	Target	Nucleotide sequence	Hybridisation conditions	Fluorescent Label	Working concentration (nM)	Excitation wavelengths	Refs.
Psaer	PNA P. aeruginosa	5'-OOAACTTGCTGAACCAC-3'	55°C No Formamide	FITC	300	488	(Coull and hyldig-Nielsen 2003)
Sta 16S03	PNA S. aureus	5'-OOGCTTCTCGTCCGTTC-3'	55°C 30% Formamide	CY5	500	633	(Perry-O'Keefe e al 2001)
Bac Uni1	PNA Universal probe (eubacteria)	5'-OOCTGCCTCCCGTAGGA-3'	55°C No Formamide	CY3	150	561	(Perry-O'Keefe e al 2001)

Table 5.1 summary of the properties of the PNA probes used in this study

To confirm the PNA probes were indeed functional and specific to the target bacterial species and that the probes functioned in a 'fixed' preparation of mixed bacteria, initial studies were undertaken using planktonic suspensions of bacteria. These included pure cultures of *P. aeruginosa* and *S. aureus* species and mixed preparations of these species. These preparations were prepared from pure isolates as previously described in Chapter 2.

Cell fixation and PNA-FISH was performed as described by Perry-O'Keefe *et. al.*, (2001). Bacterial cultures were grown overnight and pelleted by centrifugation at (16000 *g*, 5min). The supernatant was removed and the cell pellets resuspended in PBS (7 nM $NaHPO_4$, 7 mM NaH_2PO_4, 130 mM NaCl). Cell suspensions were centrifuged again and the supernatants removed. The cells were resuspended in PBS containing 4% (w/v) paraformaldehyde (Sigma) and fixed for 1 h. The fixed cells were then rinsed in PBS and resuspended in 50% (v/v) ethanol and kept at -20°C for 30 min prior to use. A 100-µl volume of the fixed cells was then pelleted by centrifugation and the pellet washed in PBS and resuspended in 100 µl of hybridisation buffer (25 mM Tris-HCl, pH 9.0; 100 nM NaCl; 0.5% (w/v) SDS) containing 150-500 nM of fluorescently labelled PNA probe. The cells were then incubated at 55°C for 30 min, centrifuged again, and resuspended in 500µl of wash solution (10 mM Tris pH 9.0, 1 mM EDTA). After a further incubation of 10 min at 55°C, the cells were recentrifuged, and this process was repeated twice for a total of three washes. The cells were finally resuspended in 100 µl of wash solution and 2 µl of this was spread on to a clean microscope slide and air-dried. Vectashield (Vector

laboratories Ltd, Peterborough, UK) mounting medium was applied and the preparation was overlaid by a coverslip, held in place with nail varnish.

Preparation of bacteria for infection of *in vitro* (RHE) Reconstituted Human Epidermis.

SkinEthic Laboratories (Nice, France) produced a 0.5 cm^2 disc of reconstituted human epithelium (RHE) *in vitro* on a polycarbonate filter at the air-liquid interface in a chemically defined medium devoid of antibiotics for 17 days. The RHE consisted of normal human keratinocytes (human foreskin derived) with a well differentiated epidermis consisting of basal, spinous and granular layers with a stratum corneum. At least four viable cell layers were present (fig 5.3).

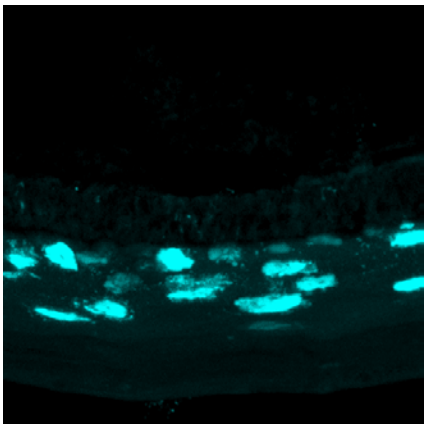


Figure 5.3. Cell layers present in RHE as illustrated using Hoechst 33258 (2 µg/ml; Sigma) staining of the cell nuclei (cyan)

Staphylococcus aureus (FRI 1151M) and *P. aeruginosa* (D40) were cultured on blood agar at 37°C overnight. The resulting growth was used to inoculate 10 ml of brain heart infusion (BHI) medium and this was incubated at 37°C for 24 h. Bacterial cells

were harvested by centrifugation and washed three times with PBS. The pelleted bacterial cells were resuspended in RHE tissue maintenance medium (MCDB 153; SkinEthic Laboratories) containing 5 µg/ml of insulin and 1.5 mM $CaCl_2$ with no antibiotics. Two hundred µl of this bacterial suspension was added to the RHE surface. Once infected, the RHE was incubated at 37°C for time periods of between 24 and 72 h.

Processing of infected RHE

After incubation, the RHE was fixed in 10% (v/v) formalin for 24 h and stored in 10% formalin overnight. The RHE was then embedded in paraffin wax using standard histological techniques. Sections of varying thickness were then cut and placed on poly-L-lyseine coated microscope slides (Polysciences, Inc; Warrington, UK) and fixed overnight at 60°C.

Following attachment of RHE to the poly-L-lyseine coated slides, the sections were de-waxed in xylene and then rehydrated by processing through graded alcohol to water. It was to these rehydrated sections that the technique of PNA/FISH was applied. To aid PNA probe hybridisation, sections were initially treated with 100 µl of lysozyme (10 mg/ml; Sigma) and these were incubated at 37°C for 30 min. The sections were then briefly and carefully washed in pre-warmed wash solution (10 mM Tris, pH 9.0, 1 mM EDTA) prior to probe hybridisation as previously described.
For nuclear context, sections were counterstained with Hoechst 33258 dye (2 µg/ml; Sigma) for 20 min before washing in wash solution and mounting as previously described.

Processing of debrided wound samples

Debrided wound tissue was collected from patients attending the wound healing clinics of the University Hospital of Wales between June and August 2009 and this was in line with the aforementioned ethical approval (Appendix 5) . Full informed written consent was taken and information sheets provided. A charcoal Amies swab was then taken from each wound surface (as detailed in Chapter 3) for microbiological analysis. Analgesia was with EMLA (Eutectic mixture of local anaesthetics). (please see discussion section re antibacterial properties). Following 30 minutes of EMLA application the leg was cleaned with sterile water and a sample of debrided tissue was obtained and fixed in 10% (v/v) formalin for 24 h.

Hybridisation of PNA probes to bacteria in RHE biofilms and debrided wound tissue

One hundred and fifty µl of pre warmed hybridisation buffer (25 mM Tris-HCl, pH 9.0; 100 mM NaCl; 0.5% (w/v) SDS) containing 150-400 nM of fluorescently labelled PNA probe was added to each of the RHE/debrided tissue sections (20 µM). The preparations were placed in a dark humidified chamber and incubated at 55°C for 90 min. The stringency of hybridisation conditions was adjusted by adding 30% (v/v) formamide to the hybridisation buffer for the *S. aureus* specific probe (Sta 16S03). After incubation each slide was washed with pre warmed solution and a magnetic stirrer for 30 min.

Gram-staining of processed sections

Sections of RHE and debrided wound tissue of varying thicknesses from 5 to 20 µm embedded in paraffin were first de-waxed with xylene, rinsed in 100% ethanol and

then in distilled water. After the washing steps sections were stained with crystal violet (0.5% w/v) in 25% (v/v) ethanol for 3 min, rinsed briefly in distilled water and stained with Gram's iodine for 3 min. Sections were then decolourised in acetone for a few seconds and again briefly rinsed in water. Sections were then stained with neutral red for 3 min, dehydrated in alcohol, rinsed briefly in saline and mounted with DPX mounting medium.

Confocal Laser Scanning Microscopy (CLSM)

PNA probe hybridised sections were viewed and analysed by CLSM using a Leica TCS SP2 AOBS spectral confocal microscope (Leica, Wetzlar, Germany). The sections were scanned to full depth using appropriate settings for single, double or triple channel fluorescence recordings of FITC, CY5, CY3 or Hoechst 33258 dye. All wavelengths were assigned a colour as outlined in Table 5.2.

Flurochrome	Laser excitation line (nm)	Emissions Detected (nm)	Colour assigned
Hoescht 33258	405	410-485	Cyan
FITC	488	498-540	Green
CY3	543	550-610	Red
CY5	633	635-700	Blue

Table 5.2 CSLM scan parameters used for simultaneous excitation and detection of the fluorescent labels utilised in the PNA probe study

All multichannel recordings were scanned sequentially to eliminate any spectral overlap between probes (Table 5.2). Selected images were presented either as single confocal optical sections or maximum intensity type reconstructions. The emission detection spectrum was often narrowed further to demonstrate individual probe detection and reduce bleed through into adjoining probe spectrums.

Results

Sensitivity and specificity of PNA probes for target planktonic bacteria

Figures 5.4-5.7 show the results of single and multiplex PNA probe staining of planktonic *S. aureus* and *P. aeruginosa*. In all cases the universal bacterial PNA probe was found to hybridise to all the bacteria tested (Fig. 5.4b, 5.5ba). The respective species-specific probes were found to hybridise to their target species with no cross-reactivity evident with non-target species (Fig 5.6).

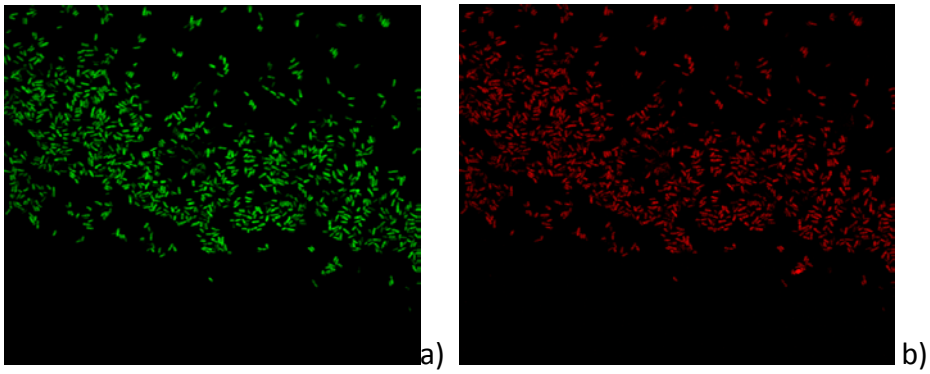


Fig 5.4. Staining of planktonic *P. aeruginosa* using the a) *P. aeruginosa* probe and b) universal probe

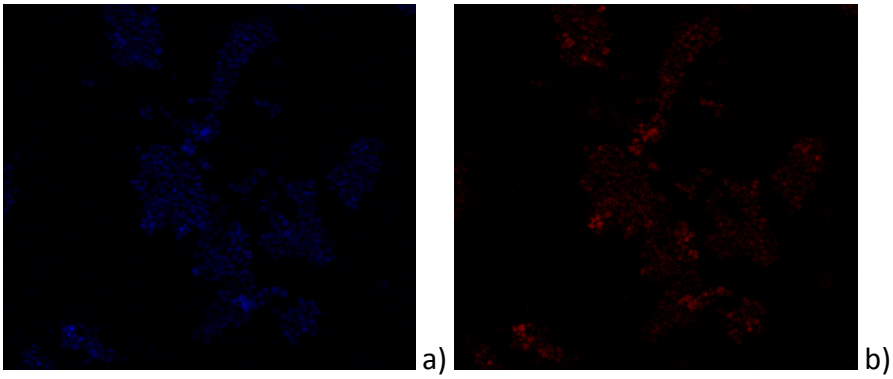


Fig 5.5. Staining of planktonic *S. aureus* using the a) *S. aureus* probe and b) universal bacterial probe

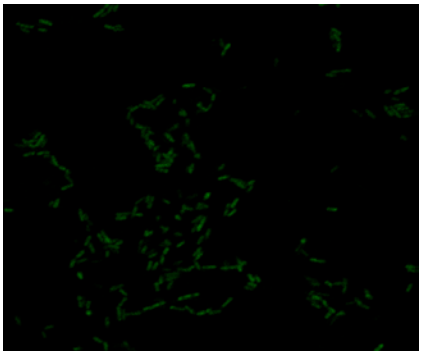
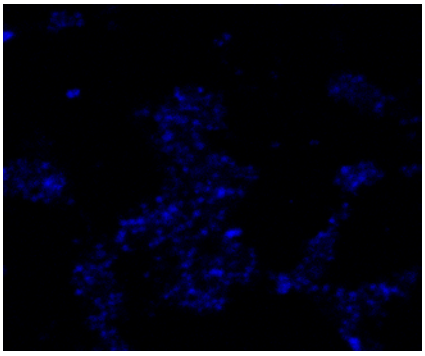
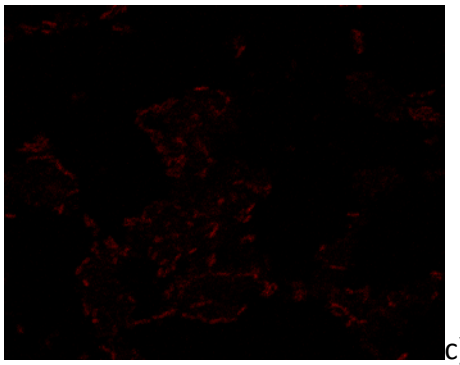

a)

b)

c)

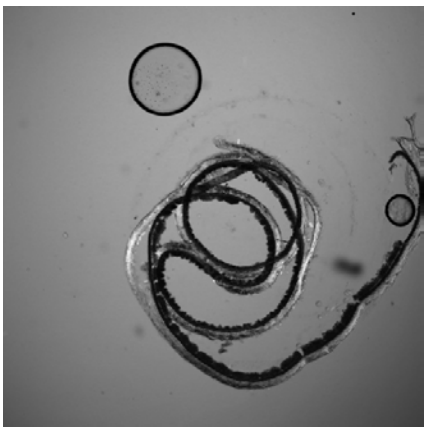
Fig 5.6. Multiplex staining of planktonic *P. aeruginosa* and *S. aureus* using the a) *P. aeruginosa* probe, b) *S. aureus* probe and c) universal bacterial probe

Multiplex staining with all of the PNA probes was also shown to be feasible with differential detection of both *P. aeruginosa* and *S. aureus* occurring within the same specimen (Figures 5.6a-c). Similar performance of PNA probe hybridisation was evident for both fixed and unfixed planktonic preparations.

Detection of target bacterial species using PNA probe staining of biofilms prepared on RHE

Bacteria in biofilms formed on RHE over 24 h, 48 h and 72 h were analysed by PNA probe hybridisation. However, it was found that prolonged incubation of bacteria on the RHE (>24 h) caused the RHE and attached biofilms to separate from the supporting plastic insert making subsequent processing problematic. Furthermore,

any specimens that were successfully embedded often appeared to be folded (Fig. 5.7) and disintegrated into smaller sections making meaningful architectural information difficult to obtain. As a consequence all RHE sections subsequently described and illustrated in these results were generated over a standard period of 24 h.



Fig, 5.7. DIC (Differential interference contrast microscopy) imaging showing partial separation from plastic backing and folding of RHE

In addition to the duration of biofilm formation, the thickness of RHE sections had to be optimised and several section thicknesses (5 µm, 10 µm and 20 µm) were examined. It was evident that the 5 µm and 10 µm sections maintained better contact with the microscope slides during processing compared with the 20 µm sections. The detachment of the 20 µm sections occurred even when longer fixation periods (48-72 h) at 60°C were used. However, a major advantage of using 20 µm sections was that these sections were more readily visible, which made processing easier. As a result 20 µm sections were selected for subsequent analysis by PNA probe staining and CLSM. The most appropriate period for removal of paraffin by

xylene treatment was deemed to be 5 min, with subsequent 5 min washes in graded alcohols suitable for rehydration.

Optimal results were obtained when probe solutions were pre-warmed to 60°C and in the case of multiplex staining when the probes were combined before addition to the specimen. Incubation of the probes for 2 h gave optimal results, with 30 min monitoring and constant hydration of the specimen with the probe solutions was ensured. To maximise probe hybridisation and retention of samples on slides, whilst maintaining sufficient washing to remove non-hybridised probes, various probe washing procedures were examined. Of the methods used, pipetting of the wash solution (×3) directly on to the samples and incubation for 5 minutes was most appropriate.

In the case of *S. aureus*, optimal PNA probe hybridisation required pre-treatment of tissue sections with lysozyme (immediately prior to PNA probe staining). Excessive lysozyme treatment was implicated in detachment of the sections from the microscope slide as well as separation of the biofilms from the RHE, whereas inadequate lysozyme treatment resulted in limited penetration of probes into the Gram-positive *S. aureus*. The most successful lysozyme treatment was therefore 1 h incubation using pre-warmed lysozyme at 60°C, in a moist dark container.

Initially, Gram-staining of RHE biofilms was used to confirm the presence of bacterial biofilms after 24 h on the RHE. It was found that all target bacteria produced

biofilms on the RHE after 24 h of incubation. Gram-stained *P. aeruginosa*, *S. aureus* as well as a dual species biofilms on RHE are presented in fig. 5.8.

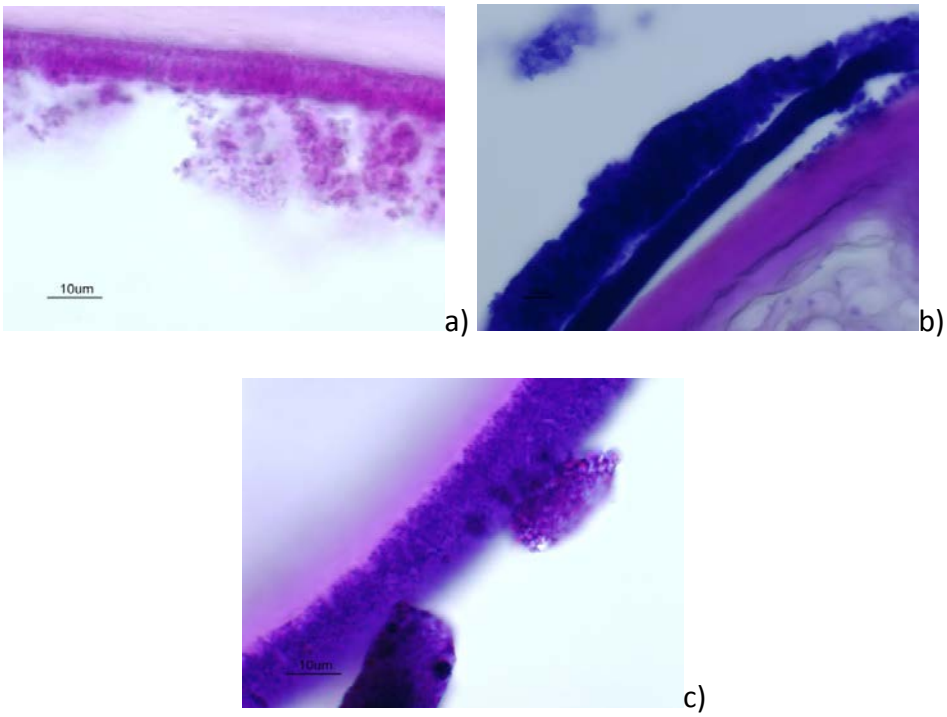


Fig 5.8 Gram-stained images a) *Pseudomonas aeruginosa*, b) *S. aureus* as well as a c) dual species biofilm on RHE (x1000 original magnification)

Figure 5.9a presents an uninfected control (24 h incubated) RHE tissue on its supporting plastic insert and imaged by DIC imaging. No damage to this tissue was evident under these conditions. Figure 5.9b shows the RHE with a mixed species 24 hour biofilm by DIC imaging.

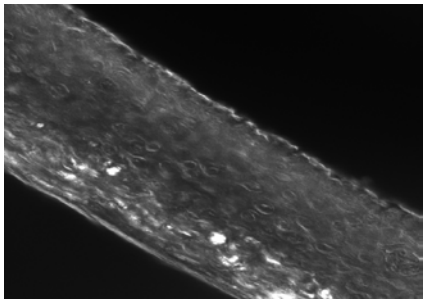
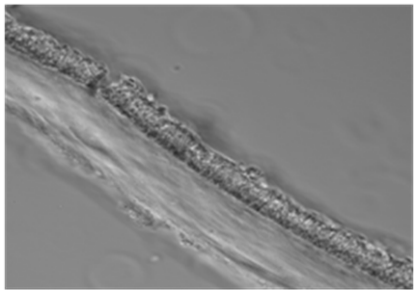
 a)  b)

Fig 5.9 DIC imaging of a) uninfected control RHE and b) infected RHE

The Hoescht stain was found to give nuclear context to the epithelial cells within the RHE layers and this provided greater detail and contrast to location of the biofilm (Fig 5.10).

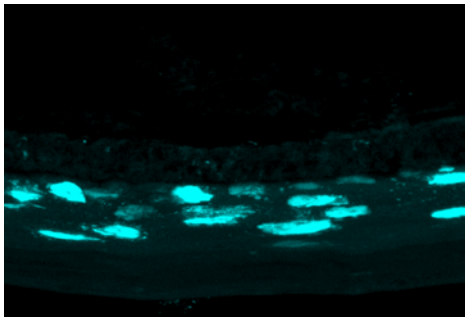


Fig 5.10 Hoescht staining of RHE showing nuclei of the epithelial cells

It was interesting to note that on several occasions, the PNA probes successfully hybridised to bacteria that were in a loose or 'immature' biofilm, and yet failed to hybridise to those bacteria within the 'mature' biofilm lining the tissue (fig 5.11).

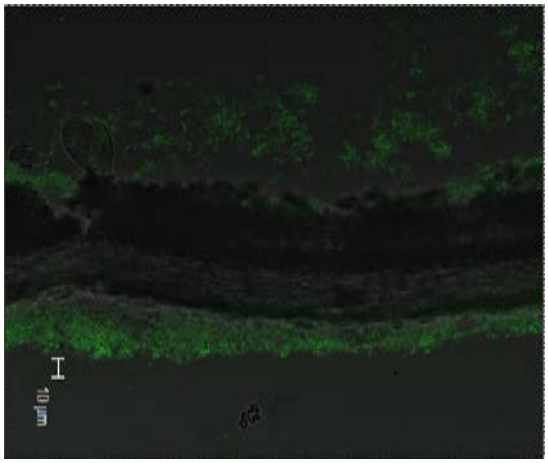


Fig 5.11. *P. aeruginosa* biofilm on RHE stained with the *P. aeruginosa* probe and superimposed by a DIC image demonstrating position of biofilms

Detection of *P. aeruginosa* in biofilms on RHE using PNA probe staining

Utilising the optimised conditions described above, PNA probe hybridisation to *P. aeruginosa* in biofilms coupled with CLSM was able to detect the target bacteria (Fig. 5.12). Typically, an extensive biofilm was present with *P. aeruginosa* occurring in large numbers on the plastic insert support for the RHE. There was no evidence of bacterial invasion or damage to the RHE. On occasion, reduced fluorescence occurred for bacteria in the 'deeper layers' of the biofilm even though bacteria were present as previously demonstrated by gram-staining and DIC imaging. It was also noted that both the universal bacterial and *P. aeruginosa* PNA probes detected *P. aeruginosa* with a very high rate of concordance (fig 5.13) as expected from planktonic studies. Figure 5.13 demonstrates detection of *P. aeruginosa* biofilm on RHE using the *P. aeruginosa* probe, Universal bacterial probe, and Hoechst staining of the RHE epithelial cell nuclei.

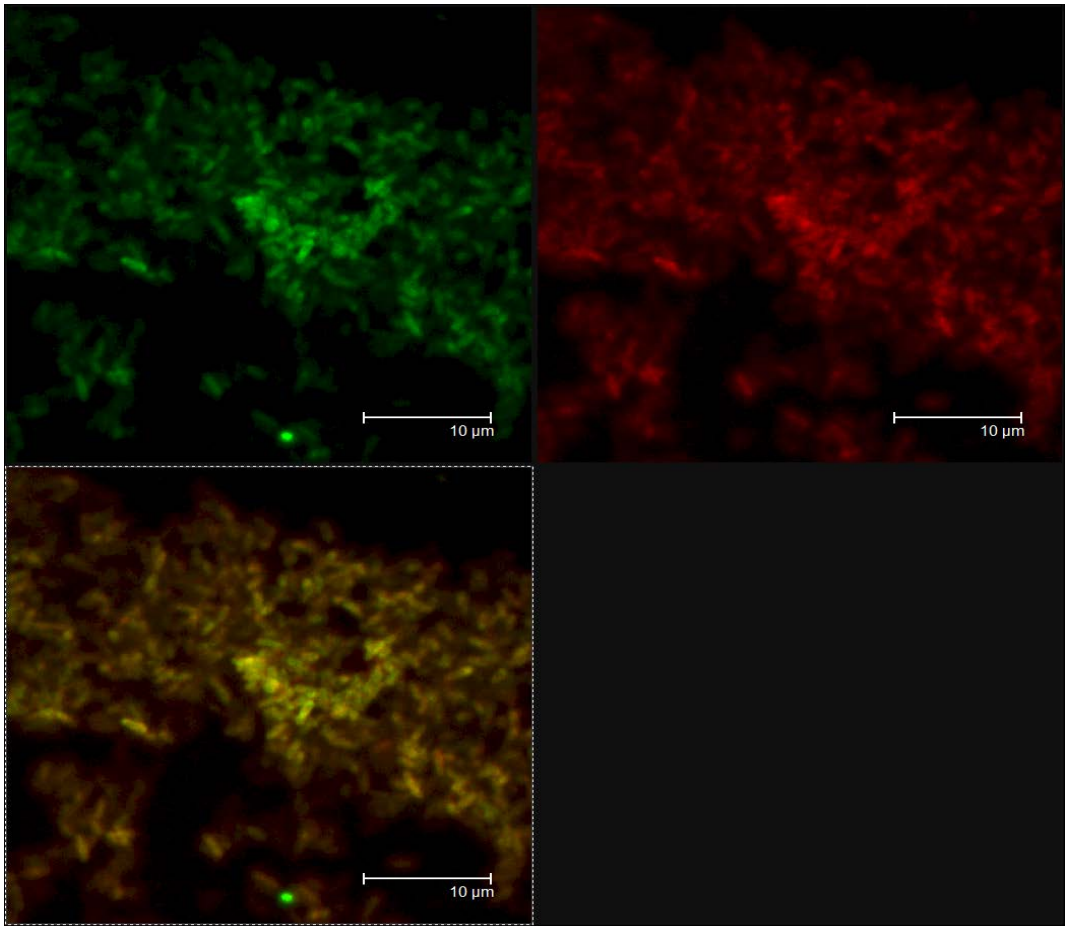


Fig 5. 12. PNA probe detection of *P. aeruginosa* biofilm with top left, *P. aeruginosa* probe, Universal bacterial probe (top right), and combined images (bottom left). Bottom right is blank.

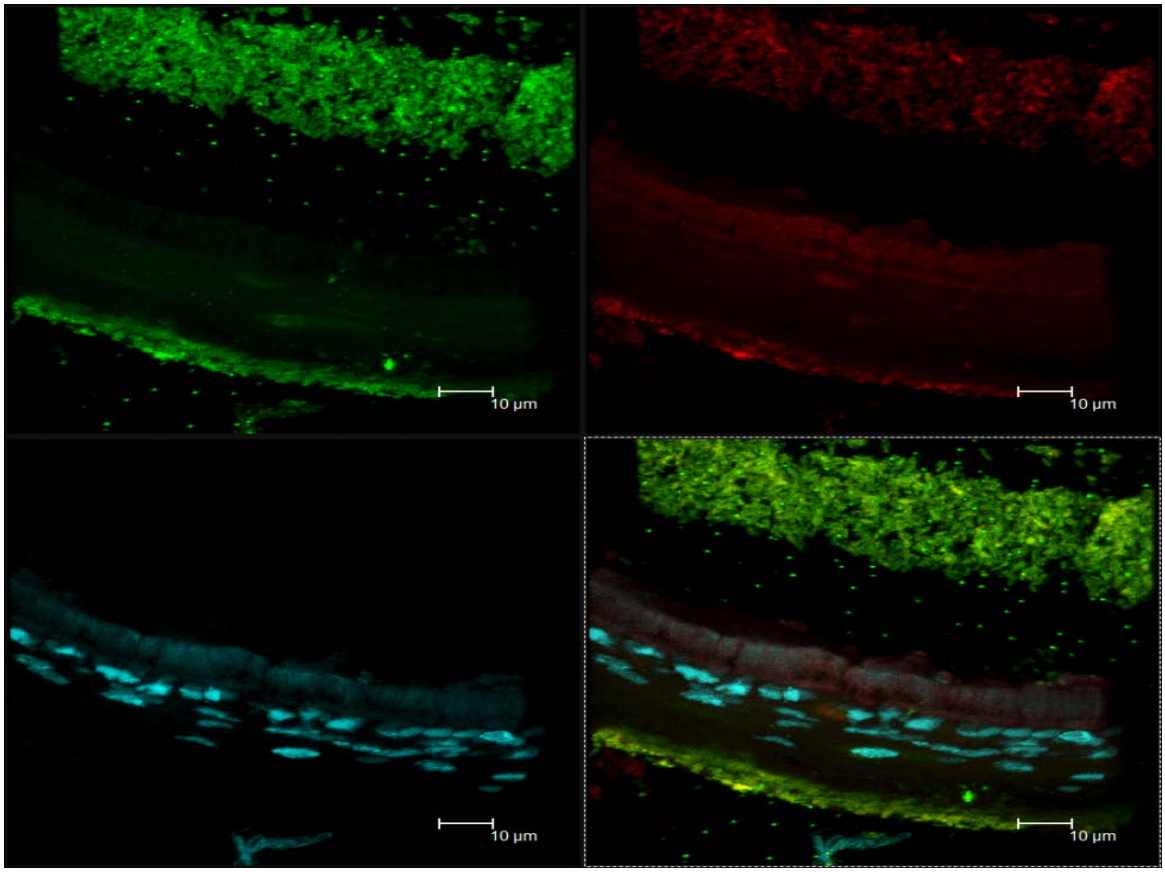


Fig 5.13. PNA probe detection of *P. aeruginosa* biofilm on RHE with *P. aeruginosa* probe top left, Universal bacterial probe top right, Hoescht dye bottom left, and combined images bottom right.

Detection of *S. aureus* in biofilms on RHE by PNA probe staining

The species-specific PNA probe for *S. aureus* was able to detect *S. aureus* within biofilms when coupled with CLSM (Fig. 5.14 and 5.15). Typically, an extensive biofilm was present on the RHE surface after 24 h. Unlike *P. aeruginosa*, *S. aureus* was not detected on the plastic insert of the RHE in single species *S. aureus* biofilms. The staining of the nuclei within the RHE enabled the demonstration of invasion of *S. aureus* from the biofilms into the tissue layers (fig. 5.15), a feature not seen with *P. aeruginosa* biofilms.

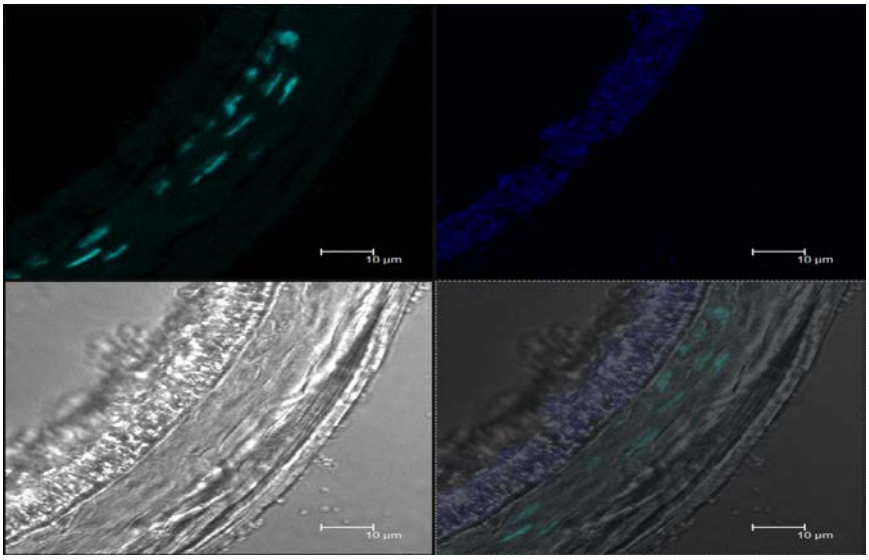


Fig 5.14 PNA probe detection of *S. aureus* biofilm on RHE. Hoescht staining top left, *S. aureus* PNA probe top right, DIC bottom left, and combined images bottom right.

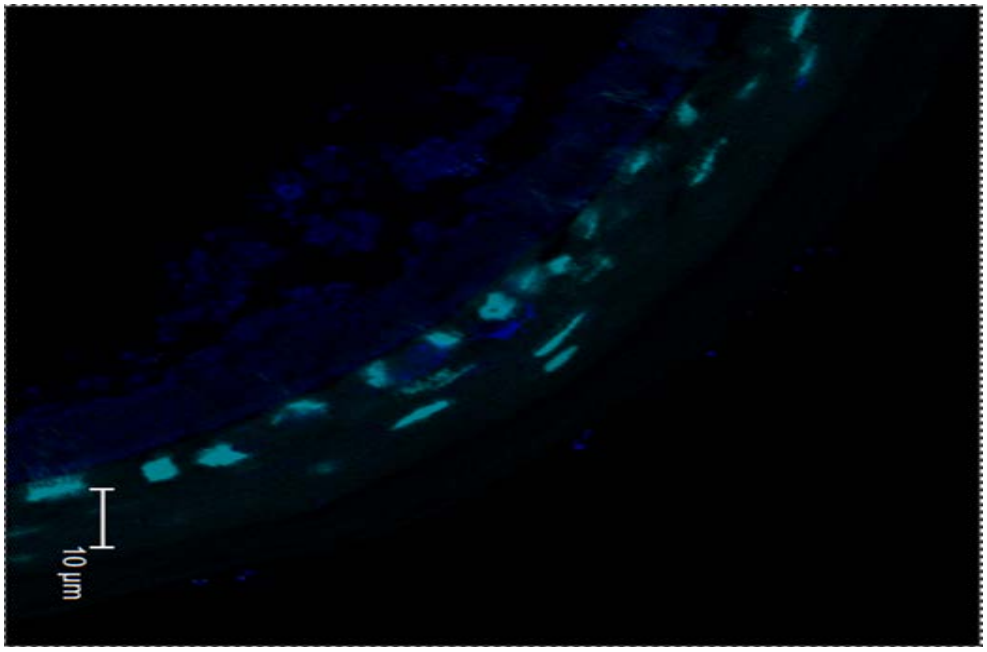


Fig 5.15 Invasion of RHE by *S. aureus* biofilm, stained with *S. aureus* PNA probe and Hoescht dye

Multiplex staining of mixed species biofilms on RHE

RHE was inoculated with both *P. aeruginosa* and *S. aureus* in an attempt to reproduce a mixed species biofilm of the type encountered in chronic wounds. Importantly, multiplex staining of mixed species biofilms revealed the presence and location of each target bacterial species in fixed RHE specimens (fig 5.16, fig 5.17). Similar colonisation patterns and observed levels for each species were evident in these biofilm preparations. The bacterial biofilms were homogenously distributed with no distinct microcolonies present. Biofilm architecture consisted of deep and dense layers of bacteria with superficial and loosely aggregated cells. Features distinctive of biofilm architecture such as water channels were evident (fig 5.18). Appendix I (CD) provides electronic representation of these structures as 3-dimensional images, whilst z stack animations demonstrate the early biofilm architecture throughout the samples.

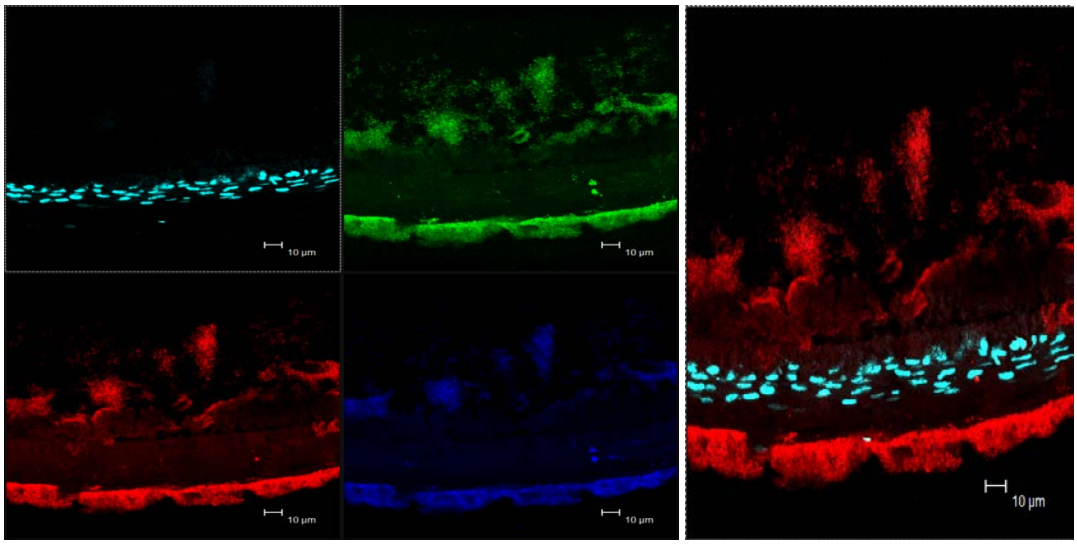
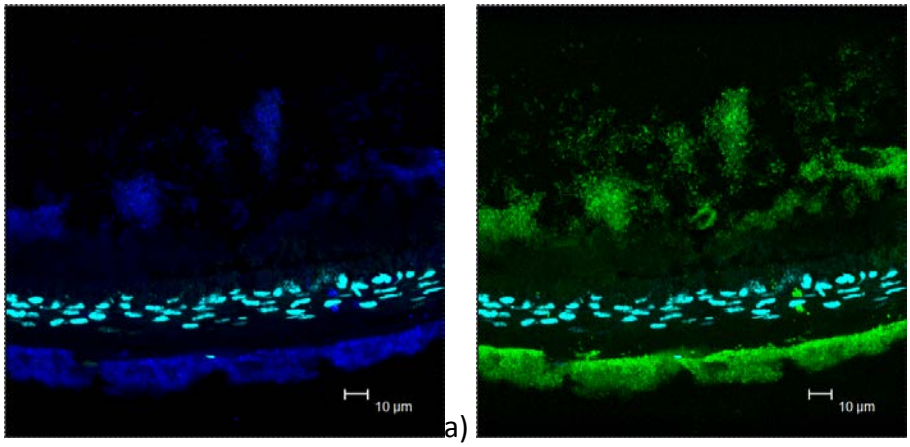


Fig 5.16 Detection of *S. aureus* and *P. aeruginosa* in mixed species biofilms on RHE using Hoechst staining, top left *P. aeruginosa* probe top right, universal bacterial probe bottom left, and *S. aureus* probe bottom right, Combined picture far right

shows mixed *S. aureus* and *P. aeruginosa* biofilm on RHE, with Hoescht staining and universal bacterial probe.



a) b)

Fig 5.17 Mixed *S. aureus* and *P. aeruginosa* biofilm on RHE stained with Hoescht dye and, a) *S. aureus* probe and b) *P. aeruginosa* probe

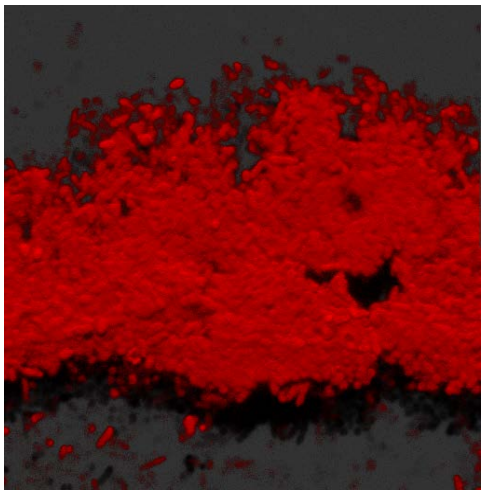


Fig 5.18 Mixed species biofilm stained with the universal probe and demonstrating water channels

Interestingly when PNA probes were used in mixed *S. aureus* and *P. aeruginosa* biofilms, the *P. aeruginosa* specific probe appeared to hybridise not only to the

bacilli (*P. aeruginosa*) but also to cocci shaped cells (*S. aureus*). Whilst the fluorescence of these cocci appeared weaker than that of the bacilli, it was noticeable over background (fig 5.19), but deemed not to be autofluorescence as it only occurred at the probe's specific excitation wavelength. Interestingly, this phenomenon was not present in *S. aureus* only biofilms treated with the *P. aeruginosa* probe or in mixed populations of planktonic bacteria.

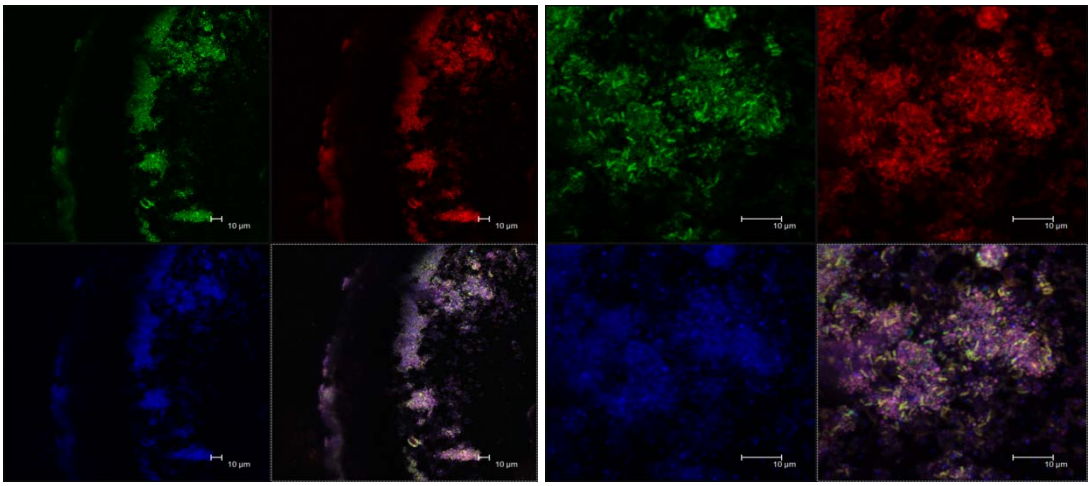


Fig 5.19 Mixed *S. aureus* and *P. aeruginosa* biofilm on RHE, with from clockwise, *P. aeruginosa* probe, universal bacterial probe, *S. aureus* probe and a recombination of the three.

In these mixed species biofilms multiplex PNA probe staining demonstrated *S. aureus* adherent to the plastic underside of the RHE (fig. 5.17) and *P. aeruginosa* invading the tissue in concert with *S. aureus* (fig. 5.17).

Detection of *S. aureus* and *P. aeruginosa* in debrided wound tissue using PNA probe staining

The PNA FISH protocol previously developed on planktonic bacterial preparations as well as infected RHE was used to detect the target bacterial species in debrided wound tissues. Initial Gram staining of the debrided wound tissues did not show the presence of many bacteria and extensive biofilm presence was therefore not evident (fig 5.20). This was despite these wounds having previously been clinically categorised as infected, and the swabs yielding microbial growth upon culture (Chapter 3). Single and multiplex PNA probe hybridisation to these samples did however reveal the presence of occasional isolated aggregations of bacteria (fig 5.19, 5.20), although clear resolution of these aggregates was problematic. Autofluorescence of the tissue was encountered in a number of the tissue samples (fig 5.21), but this could be eliminated from specific probe hybridisation, as the former was present across a range of excitation wavelengths.

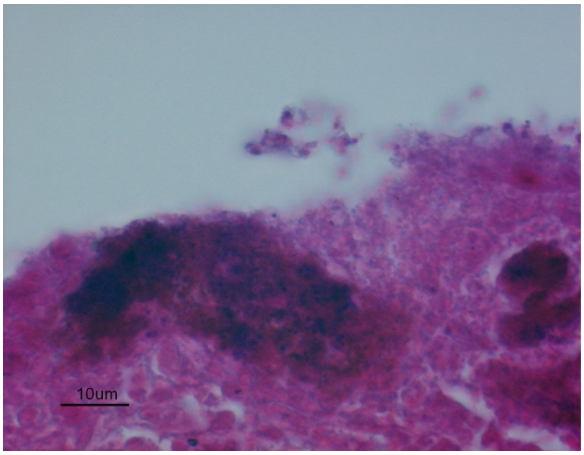
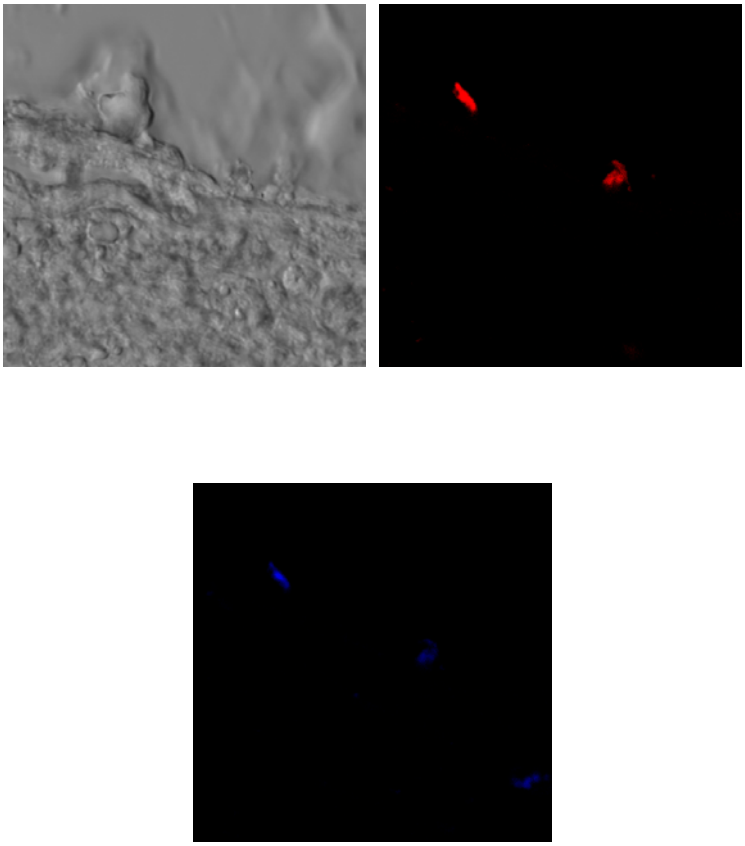


Figure 5.20. Gram stained debrided wound tissue



5.21 FISH images showing overlay of *S. aureus* probe and universal bacterial probe

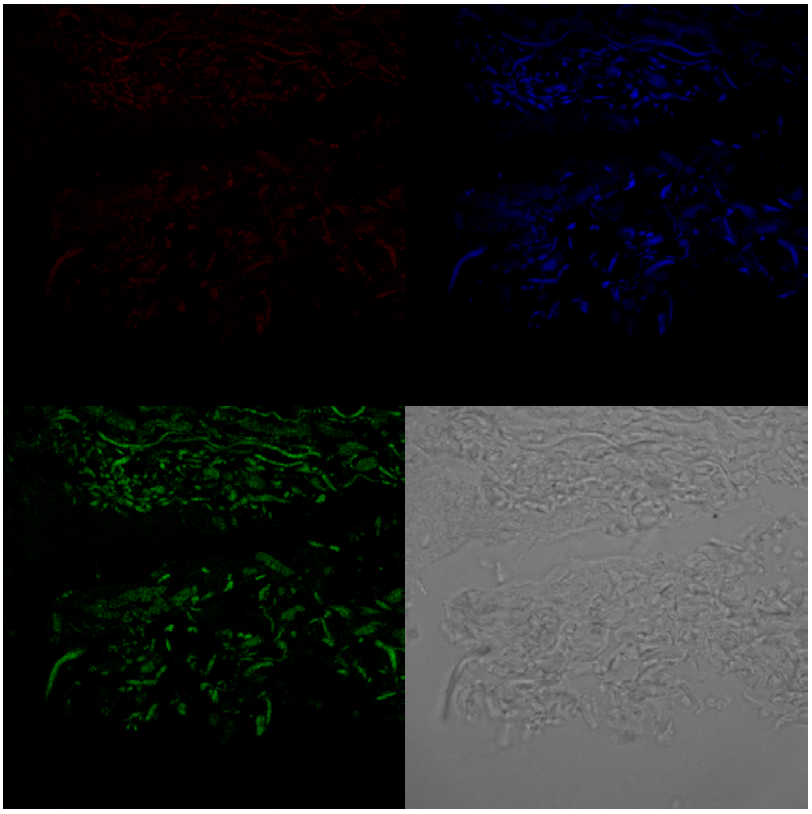


Fig 5.22 Autofluorescence of debrided wound tissue at differing wavelengths

Of the five debrided wound tissue samples examined, single bacteria were evident in samples 1 and 3 using each of the PNA probes. Meaningful colonies and mixed biofilms of *P. aeruginosa* and *S. aureus* were not detected using multiplex staining.

Discussion

The occurrence of biofilms in chronic wounds is an important cause of patient morbidity and a significant financial burden to health care services worldwide. The cost of managing chronic wounds is estimated at £2.3-3.1bn per annum in the UK alone and this constitutes 3-5% of the UK total health spend (Posnett and Franks, 2008). Chronic wounds increase in incidence with patient age and obesity, and as industrialised nations across the world have rapidly increasing aging populations and incidences of obesity and diabetes, this figure would be expected to increase dramatically in the near future (Yach *et al.,* 2006)

It is important to characterise not only the microbial composition of chronic wound biofilms, but also gain insight to their structure and the bacterial distribution within them. By acquiring such information it may be possible to elucidate the processes involved in biofilm development in wounds and develop strategies to combat these. FISH using species-specific PNA probes can be utilised with CLSM for both the identification of target species and their location in the biofilm. The approach enables deciphering of the biofilm architecture using microscopic resolutions that can demonstrate biofilm features such as water channels and areas of differing cell densities between deep and superficial biofilm regions.

In the case of biofilm imaging, CLSM is preferred to standard light microscopy as it allows the three dimensional, non-invasive imaging of cells and the computational reconstruction of mature biofilms without distortion of structure (Thurneer *et al.,*

2004). This is important, as the functional properties of biofilms are intimately related to spatial architecture (Bridier *et al.,* 2010). An Increased awareness of the structure of biofilms serves to improve an understanding of biofilm function in terms of metabolism, tolerance to antibiotics and the immune system. This in turn allows the complex behavioural and survival strategies of biofilms to be elucidated, which may lead new anti-biofilm treatment strategies to be developed based upon interfering with these mechanisms.

The impact of biofilms on wound healing is far from fully understood. However, there is a general agreement that biofilms within chronic wounds have serious detrimental effects on wound healing processes (Bjarnsholt *et al.,* 2008,) and indeed there is a growing consensus that the chronicity of a wound may be related in a large part to its biofilm component

Biofilms have been demonstrated within chronic wounds in several studies (Davis *et al.,* 2008, Kirketerp-Moller *et al.,* 2008) and a biofilm incidence of 60% in chronic wounds has been estimated using scanning electron microscopy (James *et al.,* 2008).

It is well recognised that the microflora of chronic wounds is polymicrobial (Fazli 2009). *Pseudomonas aeruginosa* and *S. aureus* are both frequently isolated from chronic wounds and are prolific biofilm formers. To highlight this, in one study *S. aureus* and *P. aeruginosa* (Gjodsbol *et al.,* 2006) were isolated from 93.5% and 52.2% of chronic venous leg ulcers respectively. In 2008, Kirketerp-Moller *et al.,* (2008) did however show that when compared with FISH analysis *S. aureus* was over-

represented by culture from wound swabs, whilst *P. aeruginosa* was under-represented. These findings were possibly due to the relative positions of these species within biofilms. Both of these bacterial species are widely known to cause biofilm based infections in their hosts (Fazli *et al.,* 2009) and as a result were targeted in the current study.

In this present chapter, FISH using PNA probes and CLSM were used to analyse the biofilm architecture and location of bacteria. Biofilms were generated *in vitro* using an RHE model to simulate the formation of biofilms on a tissue surface. This model had previously been used in the study of both *Candida* biofilms on reconstituted human oral epithelium (Silva *et al.,* 2010) as well as chronic wound biofilms (Malic *et al.,* 2009). Subsequently, tissue debrided from the wounds of patients were analysed using developed methods.

PNA probes have over recent years been increasingly incorporated into FISH applications (Malic *et al.,* 2009). PNA probes are DNA analogues with an uncharged polyamine backbone instead of conventional sugar phosphates and hybridise with complimentary DNA sequences through base pairing (Motor and Gobel 2000). The uncharged backbone of the probes is claimed to allow superior hybridisation characteristics with higher specificity and more rapid hybridisation techniques. It is also proposed that PNA probes can diffuse through hydrophilic cell walls allowing hybridisation to occur without stringent pre-treatment stages (Motor and Gobel, 2000). Hybridisation with PNA probes can be performed in a low salt buffer, therefore reduced stability of the rRNA secondary structure can be achieved,

allowing PNA probes to hybridise with otherwise inaccessible targets (Motor and Gobel, 2000). It could therefore be argued that PNA probes have considerable advantages over oligonucleotide probes and were thus used in this study.

The PNA-FISH methodology used had previously been described by Malic *et. al.* (2009) for imaging of fixed biofilms prepared on RHE, although in this present study, modification and optimisation of the protocol was required. Probe specificity was verified by preliminary investigation using planktonic bacteria preparations. This preliminary work demonstrated the specificity and sensitivity of the PNA probes when applied to *S. aureus* and *P. aeruginosa* in single and mixed species preparations (Figs 5.5 and 5.6). These investigations also provided a protocol for application to biofilms constructed *in vitro* on RHE surfaces as well as clinical specimens of debrided wound tissue.

Analysis of biofilm structure and composition using PNA probes and CLSM

As previously noted by Bridier *et. al.* (2010), the present study was able to obtain detailed structural information of the biofilms using confocal Z stacks. The generation of Z-stacks by sequential imaging of sections through the biofilm allowed subsequent reconstruction into a three dimensional structure (Appendix CD).

It was evident that *P. aeruginosa* biofilms generated over 24 h on RHE exhibited a mushroom shaped structure (Fig 5.8a), whilst biofilms produced by *S. aureus* had a more variable structure in accordance with other studies (Bridier *et al.,* 2010, Pamp and Tolker-Nielsen, 2007, Davey *et al.,* 2003, Harmsen *et al.,* 2010). It was also

possible to demonstrate the formation of hollow voids in the *P. aeruginosa* 'mushroom like' biofilms and these were deemed to be water/waste channels (Fig. 5.18) which are notable components of biofilms. Water/waste channels and a structure of mushroom shaped microcolonies are a typical feature of *P. aeruginosa* biofilms. The water/waste channels allow biofilm cells to obtain nutrients and remove toxic products of metabolism. The presence of water channels have also been shown to facilitate cell-to-cell communication and biofilm organisation expression (Stoodley *et al.,* 2002)

The 24 h infected RHE specimens clearly exhibited a dense biofilm that was adherent to the RHE. The denser regions were seemingly more resistant to penetration by the PNA probes compared with looser aggregates of biofilm-associated bacteria (Fig. 5.13). It is possible that this is secondary to a failure of the probes to penetrate the protective EPS of the biofilm (indicating the early and effective development of a protective EPS covering). EPS is recognised as a barrier to the entry of various chemicals including antibiotics and may explain some of the negative results obtained in other samples with proven biofilm (seen by Gram-staining), but not visible using FISH.

Comparison of *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms

Despite appearing homogenous on viewing prior to probe hybridisation, it was apparent that the single species biofilms had several distinctive characteristics. As noted by Schaudin *et. al.,* (2009) (who utilised both CLSM and SEM to study

endodontic biofilms), the present study was able to demonstrate individual rods and cocci in aggregates within the biofilm matrix (Fig. 5.19).

Pseudomonas aeruginosa biofilms formed not only on the RHE, but also developed on the plastic tissue insert. This was likely to be less a function of bacterial motility (as bacteria were inoculated into the broth surrounding both sides of the RHE) and more as a result of different adhesion characteristics. Single species biofilms of *S. aureus* did not exhibit this trait with biofilm formation restricted to the RHE. It would have been interesting to see if S*. aureus* would have formed biofilms over the plastic surface given more time, however, as previously stated, when incubated for greater than 24 h the RHE tended to detach from the insert making processing difficult. This finding is clinically interesting as the adhesive abilities of biofilm forming bacteria is an essential component of their ability to cause infection and disease, and whilst slightly out of the scope of this thesis biofilms on implantable devices are an increasingly recognised clinical problem (Donlan, 2011).

Both *P. aeruginosa* and *S. aureus* persisted in mixed species biofilms, with the former also appearing to promote attachment of *S. aureus* to the tissue insert. This possibly indicated that the non-attachment previously encountered by *S. aureus* was likely to be related to poorer adhesion factors to this surface. The similar attachment patterns of mixed species biofilms and *P. aeruginosa* may be indicative of increased virulence of a mixed species biofilm by allowing the *S. aureus* to form biofilms in areas it would not be able to attach to independently.

Staphylococcus aureus biofilms showed significant invasion of the RHE, which was depicted in figs 5.14 and 5.15. This demonstration of virulence was not evident in any '*Pseudomonas* only' biofilms, but was apparent with *P. aeruginosa* in a few mixed biofilms. In the mixed biofilms, there is clear evidence of bacteria positive to the *P. aeruginosa* probe invading the tissue (Fig. 5.16), and it is tempting to speculate that these *P. aeruginosa* bacteria are exploiting the tissue invading *S. aureus* capacity to facilitate colonisation of a new environment. This is clinically significant as it provides *in vitro* evidence of increased tissue invasion by *P. aeruginosa* in the presence of *S. aureus*. Extrapolated to a wound, this could result in increased pathogenicity and subsequent difficulties in therapeutic management when both species are involved in infection. Mixed species biofilms as opposed to single species biofilms are therefore likely to be more detrimental to the host in chronic wounds and in other medically significant biofilms. Evidence of synergistic virulence factors although assumed, has not previously been described.

Mixed species biofilms have been described as being more resistant to eradication with disinfectants than single species biofilms of the same bacteria (van der Veen and Abee, 2011, Harriott and Noverr, 2010) and some researchers have shown an inhibitory effect between species within biofilms (Bandara *et al.,* 2010). It is evident that species within biofilms interact in ways that are only now beginning to be understood, sometimes synergistically and sometimes competitively. It has been increasingly suggested that in human disease, the biofilm in its entirety rather than species should be considered the unit of pathogenicity (Siqueira and Rocas, 2009)

Limitations of PNA probe and Confocal Laser Scanning Microscopy methodology

Due to the constraints of wavelength and quenching of fluorescence between adjacent excitation wavelengths for fluorescent labels, only a limited number of different probes can be used in any multiplex analysis of a single specimen. The approach has several limitations, not least the fact that several of the stages involved in the fixation and embedding of the tissue are cidal to biofilm cells, thereby preventing subsequent assessments of viability and associated parameters. Furthermore, it could be argued that dehydration, and subsequent rehydration may adversely affect biofilm tissue and architecture. This was not however evident in this study.

One practical limitation of using PNA probes relates to the cost of the probes themselves, which are currently prohibitively expensive for routine diagnostic use. However, the optimised FISH approach described in this chapter generates efficient and accurate imaging of biofilm structures allowing the spatial location of targeted species to be determined. Whilst the technique provides good imaging of biofilm structure, it does not give specific quantitative information (beyond assessments of volume) or dynamic information. Further, given the multiple steps and corrosive chemicals involved it would not currently be a candidate for conversion to a direct wound diagnostic system due to patient safety concerns. The method could however be utilised for rapid 'off patient' detection of bacteria in chronic wounds, and the rapid turnaround provided by such a test would allow for patient tailored treatment to be initiated far earlier than would be possible with conventional methods.

As reported by Malic *et al.,* (2010), the current study was able to demonstrate specificity of the PNA probes against planktonic bacteria with detection of all bacteria evident when overlaid with DIC images. Unlike Malic *et al.* (2009) the *P. aeruginosa* probe appeared to cross react in mixed species biofilm conditions with the cocci of *S. aureus*. This may partly be due to imaging of 'end on rods' appearing as cocci in various sections. In addition, the production of an adherent EPS by *P. aeruginosa* may have contributed to capture of the probe by EPS coated *S. aureus* cells. Further study is needed to confirm this, although the absence of cross reaction in preparations devoid of *P. aeruginosa* might be suggestive that cross reaction was not through probe hybridisation to DNA.

The study demonstrated the development of a multilayered community of bacteria on the RHE samples. In addition, and in contrast to the study of Malic *et al.,* (2009) invasion of this tissue by *S. aureus* was detected even without previous wounding of the tissue. In agreement with Malic *et al.* (2009) the *S. aureus* probe appeared to have reduced fluorescence when compared with both the *P. aeruginosa* and the universal probe. As with Hartmann *et al.,* (2005) who utilised PNA FISH to detect *S. aureus* in blood the need for pre-treatment of staphylococci with enzymes (lysozyme) to allow entry of PNA probes was demonstrated. The reasons for this apparent lower fluorescence of the *S. aureus* PNA probe could relate to a comparatively reduced probe uptake or the relative number of probe targets within the targeted cells. In this study (as with Malic *et al.,* 2009), the inclusion of 30%

formamide to the hybridisation buffer was necessary, along with lysozyme treatment to facilitate hybridisation of the *S. aureus* probe.

Despite limitations, as with other studies using PNA probes and FISH studying the analysis of biofilms, the images of biofilms obtained through this method were of value (Malic *et al.,* 2009, Kirketerp-Moller, 2008, Fazli, 2009, Bjarnsholt, 2008). The ability to image biofilm structure with minimal disruption of the architecture is invaluable, and the use of fluorescent probes to accurately identify and locate individual species within that biofilm is particularly useful.

Use of the RHE model

Use of RHE as an *in vitro* model of wound infection is the most appropriate system commercially available. However there are limitations of the RHE model in that it is largely devoid of a host immune response and the pro-inflammatory cytokines of chronic wounded tissue, which as previously mentioned may be vital to biofilm formation and regulation. In the present study the RHE was not artificially wounded, but was intact, as it was felt wounding in this fashion would resemble an acute wound than a chronic wound.

In vivo, multiple microbial species form a biofilm and for reasons of simplicity the microbial biofilm composition in this study was restricted to just *S. aureus* and *P. aeruginosa*. As a caveat it should be recognised that the inclusion of other bacterial species could have affected biofilm structure and growth rate (Kroukamp *et al.,* 2010). In this study the RHE samples were only incubated for 24 h and longer

incubation periods would potentially allow more extensive and mature biofilms to develop. A longer incubation period would have enabled the studying of a more mature biofilm in a simulated chronic wound model, however this was not possible to process due to lifting of the RHE and biofilm layer from the plastic insert.

Debrided wound tissue

The CLSM/PNA method was able to demonstrate individual bacteria within debrided wound tissue and this is the first instance of successful application of the method to such specimens. However, the demonstration of biofilms on the debrided wound tissue using gram-staining and CLSM/PNA was not evident.

The results demonstrated both *P. aeruginosa* and *S. aureus* bacteria in debrided wound samples one and three. This correlated with the classical microbiology from swabs of these wounds taken at the time of sampling. It is however worth noting that sample two was also positive for both species on classical microbiological analysis. Recent work by Kirketerp-Moller *et al.,* (2008) has demonstrated a poor concordance between FISH PNA and conventional microbiological culture with the latter approach possibly dependent on relative bacterial positions in biofilms. In this study the absence of positive PNA detection in sample two may well be secondary to the nature of the debrided specimen obtained and processing.

The question remains as to whether there are biofilms in chronic wounds and if so, why could they not be detected on the debrided wound tissue. The current evidence suggests that biofilms abound on almost any surface and are prevalent in

chronic wounds (Davis *et al* 2008, Kirketerp-Moller *et al.,* 2008, James *et al.,* 2008). Indeed biofilms have been visualised through use of FISH on 'punch biopsy' specimens (Malic *et al.,* 2009). The debrided wound tissue obtained from infected chronic wounds was by its nature severely disrupted. In many cases surgical debridement is the treatment of choice in biofilm colonised wounds. Therefore it is not overly surprising that any biofilm that may have been present was severely disrupted, and in all likelihood detached from the specimens through both the actions of physical debridement and then through processing in formalin, leaving only remnants of single bacteria behind as demonstrated by the highly specific CLSM/PNA system.

One additional consideration to be made with regards to the study is the potential antibacterial eactivity ofEMLA (Eutectic mixture of local anaesthetics) contains two anaesthetic drugs: lidocaine 2.5% and prilocaine 2.5%. EMLA is known to have an antibacterial effect (Kerenyi *et al*., 2004), with its effect being described as powerful and rapid with test isolates of typical wound pathogenic bacteria (including *S. aureus* and *P. aeruginosa*) being significantly reduced within 1 hour and killed within 1–3 hours particularly killing *Staphylococcus* species within 1 hour (Berg *et al*., 2006). Despite this it would have been unethical to debride patients without appropriate analgesia, the alternative option of a field block with local anaesthetic injections was considered and discounted. This was due to the fact that the alternative local anaesthetic agents are similarly antibacterial (Thompson et al., 1993) when applied topically, and the multiple injections necessary for a field block were considered inappropriate due to the painful nature increased by using additive free lidocaine. In

an attempt to minimise these antibacterial effects EMLA was applied for 30 minutes and each patient's wounds were swabbed prior to application of local anaesthetic and debridement. Swabs were placed in an ice filled labelled secure container and transported to the laboratory for microbiological analysis. Sample analysis showed expected wound flora (see chapter 3 results) , however it must be considered that as well as debridement disrupting the biofilms the EMLA may have had some bactericidal effect in the 30 minutes of application post swabbing. However the PNA probes should still have been able to detect these bacteria (as molecular metods do not detect only viable organisms) and the antibacterial effects of EMLA should not materially affect the results of the PNA FISH for debrided wound tissue.

Summary

It was encouraging that detection of *P. aeruginosa* and *S. aureus* in single and mixed species biofilms was achieved with PNA probe hybridisation. This allowed characteristics of each biofilm to be observed and described. It was pleasing to be able to image biofilms within the first 24 h revealing definite structure with a densely adherent thick biofilm on the surface with likely EPS protection and more loosely aggregated bacteria superiorly to this. In contrast to the research of Malic *et al.,* (2009) this present study revealed extensive RHE biofilms after 24 h of incubation and that the *S. aureus* containing biofilms could invade epithelial layers in the absence of artificial wounding. As Malic *et al.,* (2010) reported biofilms with discrete clusters of individual bacterial species in microcolonies were evident in this present study, although more commonly the two species were present within microcolonies.

This latter feature was not as evident in the study of Malic *et al*., (2010*)* and may be due to the shorter incubation period for the mixed biofilms in the present research, which could lead to an inadequate time period for biofilms to mature and differentiate into separate species microcolonies.

It was also interesting to visualise the individual rods in an early immature *P. aeruginosa* biofilms and cocci in a *S. aureus* biofilm, and the development of water channels in a mixed biofilm. 3D reconstructions demonstrate that the structure of these early biofilms is beginning to be organised with clusters of cells in microcolonies within the biofilm EPS.

The combination of PNA labelled FISH probes and CLSM has enabled us to demonstrate the specificity of the probes in planktonic bacteria and biofilms. In comparison to other techniques utilised in this thesis it demonstrated biofilm structure more clearly and reliably then the use of fluorescence microscopy, and identified species more quickly and accurately than the use of monoclonal antibodies and ELISA. Uniquely it demonstrated both the structure of biofilms and location of individual species within them. It was also valuable in showing the differing behaviour of different types of single species biofilm and of mixed biofilms.

Chapter 6: General Discussion

Previous discussions have followed all experimental chapters focussing on the points raised within that section, the aim of this chapter is to bring together the salient points relevant to the topic and suggest further and new avenues of research. The complete characterisation of bacterial communities within chronic wounds is an ambitious and ongoing project. In this thesis significant advancements to achieving this goal have been reached.

Collection of varied wound samples and their microbiological typing to aid in the characterisation process is essential in any attempt to further knowledge. Chronic wound fluid was collected in larger quantities than any other study allowing repetition and the use accurate controls in later identification experiments. The microbiological and PCR results for this fluid (and indeed the other chronic wound samples) were consistent with the literature, with both *P. aeruginosa* and *S. aureus* very prevalant, confirming the validity of the decision to focus on these species. The acute wound fluid collected was subjected to classical microbiological analysis on day one and two post-operatively for what appears to be the first time in the literature, and demonstrates the relatively sterile nature of this fluid.

Utilising fluorescent microscopy and specifically developed monoclonal antibodies (MABs) to *P. aeruginosa* and *S. aureus* demonstrated that it was possible to identify planktonic bacteria using this method. Utilising the MABs in a competitive ELISA was

successful in a number of ways. It was demonstrated that MABs can accurately and repeatedly detect both the antigen and the relevant bacteria in planktonic bacterial and biofilm supernatants and more impressively within a chronic wound fluid environment, through the use of a competitive ELISA. It was also possible to demonstrate for the first time the number of CFU/ml necessary to produce enough TNase to be detected by the MAB using a competitive ELISA.

Biofilms of single and multiple species were successfully grown and demonstrated on RHE. The use of FISH/PNA probes and the CLSM was successful at characterising bacterial species, identifying specific species in planktonic and biofilm states. It was particularly useful in demonstrating the spatial arrangements within biofilms, with its non-invasive three dimensional imaging. It allowed identification of individual bacteria within single species biofilms and with the use of multiplex staining, of multiple species biofilms demonstrating the position of the individual bacteria of each species within the biofilm. It was particularly interesting to note the differing virulence factors of *P. aeruginosa* and *S. aureus* single species biofilms. *P. aeruginosa* biofilms were far more adhesive, particularly to the plastic backing of RHE when compared with *S. aureus* only biofilms, and *S. aureus* biofilms actively invading the tissue, in a trait not seen in *P. aeruginosa* only biofilms. It was also demonstrated that combined species biofilms enabled increased adhesion of *S. aureus* and invasion of *P. aeruginosa* indicating a synergy not previously described in multiple species biofilms, these data suggest that these biofilms may increase their virulence factors *in vivo.*

This research has demonstrated the importance of collection of chronic wound fluid samples and their microbiological typing for characterisation of bacteria within chronic wounds.

The MAB work reiterated the potential of MABs for the rapid and accurate identification of bacteria within chronic wounds as previously demonstrated by (Gu *et al.,* 2005). The study has shown the possibility of detecting antigens and bacteria within a chronic wound environment for the first time, demonstrating both the sensitivity and specificity required for a clinical test. The experiment to determine the number of CFUs/ml required for detection of *S. aureus* has not previously been performed, and has demonstrated a useful method of calibrating the sensitivity of MABs.

The use of FISH/PNA probes and CLSM built on the already promising work of Malic *et al.,* (2009) confirming the utility of the method in detecting individual bacteria within in the biofilm state, in both single and multiple species biofilms and, also in delineating biofilm architecture. It also demonstrated for the first time a synergy of virulence factors of *S. aureus* biofilms in terms of invasion and adhesion.

The relevance of this work to the clinical problem of people suffering from chronic wounds is the advancement of the base of knowledge in methods for identification of bacteria and the characterisation of them within chronic wounds. MABs have proved accurate at rapid detection of bacteria within the complex chronic wound environment, as a result, future rapid bacterial detection and identification systems

could be developed leading to more rapid treatment and monitoring of treatment effectiveness in these patients.

The FISH/PNA CLSM work demonstrated the effectiveness of this approach as a tool for examining biofilm architecture and positions of individual bacteria within biofilms. This has already yielded interesting information of a possible synergistic relationship between two pathogens. A wide range of clinically important biofilms can be studied and modelled using this method, with the possibility of developing and testing therapeutic interventions as a result.

Given the opportunity to repeat this research with the benefit of hindsight, there are certain methodological changes that could be considered. Although pleased with the large quantities of wound fluid collected and the advantages previously mentioned, concentrated wound fluid would (despite the problems of collection and repeatability of experiments) have been very useful to fully test the MABs and would have avoided the need for, and attendant difficulties of concentrating the chronic wound fluid. It would have been useful to be in possession of full epitope mapping for the monoclonal antibodies used. Complete information regarding binding sites and detection concentrations would have been very helpful. It may have been possible to choose a different and more specific target for both of the antibodies. The attachment of non-quenching fluorescent markers would have made the interpretation of the MAB fluorescent microscopy work far easier.

Whilst the FISH/PNA CLSM work demonstrated biofilms grown in vitro on RHE it was unsuccessful in demonstrating this in debrided wound tissue. This is likely to be secondary to the disordered nature of the specimens post debridement and punch biopsy from chronic wounds infected with biofilm samples as utilised by Malic et al (2009), may have hopefully demonstrated the efficacy of this method on wound biofilms.

As previously mentioned the majority of bacteria found in chronic wounds are "unculturable" for these bacteria the molecular methods described in this thesis of MABS and ELISA work and PMA FISH CLSM work may prove particularly useful in detection and elucidating the roles of these bacteria within chronic wounds.

To expand on the work in this thesis there are several recommended research avenues. It would be possible to expand with the judicious production of MABs to any other bacteria found within wounds, MAB ELISA has the benefit of being a molecular method and is therefore not reliant on the viability of bacteria. Ideally these MABs would be produced to a prominent unique structure on the cell wall of the organism in question allowing ease of detection. The development of MABs to a unique protein produced by the bacteria in question is also an option to be considered and worked well in these experiments but is limited by the knowledge of the circumstances and quantities in which the protein is expressed. The competitive ELISA model developed in this thesis could then be applied to rapidly detect the presence of many different bacteria within wound fluid. The potential of this ELISA to be developed into a wound diagnostic is exciting. Work on optimising the

Competitive ELISA to function effectively on wound imprints is an important avenue of research for achieving a rapid clinic based diagnostic ELISA. The rapid nature of this test and the non-invasive nature of the sampling method required would make this a very exciting diagnostic.

If non-quenching fluorescent markers could be added to the MABS and the problems of autofluorescence overcome it may be possible to expand on the CDFF and RHE generated biofilm work and produce detailed images of the structure and architecture within biofilms of a comparable nature to FISH.

The obvious first area to expand on the PNA CLSM FISH work described in this thesis is to obtain specimens of biofilms in wounds that are not disrupted as debrided wound tissue was. This may be possible through punch biopsy or even through obtaining complete chronic wounds with biofilms from amputated limbs, and applying the processes described in chapter 5 to demonstrate the structure of biofilms within these chronic wounds, rather than attempting to construct a model.

It is also evident that the RHE biofilm model work could be easily expanded to include different species found in chronic wounds, which could be then analysed using PNA CLSM FISH. maximising our knowledgee of the structure of single and multispecies biofilms. Different combinations of species could be assessed to determine whether tissue invasion/destruction or other virulence factors are maximised with particular combinations of bacteria, as demonstrated with the *P. aeruginosa* and *S. aureus* combinations, or whether with certain species there is

direct competition rather than synergy. It would also be worthwhile to grow biofilms for different time periods prior to observation. This approach could allow the observation of particular bacteria or combinations of bacteria demonstrating differing degrees of tissue destruction/invasion. It would also allow the observation of the effect time had on the relative numbers of bacteria of different species present over time to be elucidated. It would be interesting to see if the trend is towards uniformity or heterogeneity.

It would also be useful to observe the changes to the biofilm in response to external "shocks" such as antibiotic treatment, addition of Ionic silver, iodine, polyhexamethylenen biguanides, or potassium permanganate, and possible recovery from these shocks. It is reasonable to hypothesise that biofilms of differing species are more susceptible to different treatments, or treatment combinations allowing the possibility of developing more tailored treatments to biofilms of known species.

Moving away from direct follow up work from this thesis there are several future avenues of research suggested by this work. A rapid detection system could be developed for bacteria within chronic wounds involving the use of discarded dressings and ELISAs with MABs to rapidly detect the bacteria present whilst the patient is in clinic. This could allow rapid, effective targeted treatments, and indeed test response to treatment

It would also be clinically relevant and highly useful to the health service if a MAB could be developed to the altered penicillin binding regions of methicillin resistant *S.*

aureus, a rapid diagnostic test utilising MABs would not only detect the bacteria but also its antibiotic resistance, leading to faster treatment and isolation of contaminated patients. Currently every patient admitted to a UK hospital is swabbed for MRSA and isolated if this is found, but the process can take over 24hrs at which point other patients could have been colonised or infected. This principle of targeting the cause of antibiotic resistance rather than the bacterial species could be extended to other methods of antibiotic resistance again allowing early targeted treatment.

Future investigations of biofilms could involve the combination of CLSM and non-quenching fluorescent markers which may allow the observation of a biofilm in real time without dehydrating; such an approach would greatly enhance our knowledge of the behaviour of biofilms.

This thesis has implications for clinical practice in the management of chronic wounds. In the short term the need for accurate appropriate and as rapid as possible antibiotic prescription, and together with monitoring in the event of infection within chronic wounds should be emphasised. As a result of the likely synergies in the virulence factors of *S. aureus and P. aeruginosa* multispecies biofilms may benefit from more aggressive treatments and surgical debridement should be considered in this eventuality.

In the short term it should be possible to produce commercially available reliable reproducible and rapid bedside tests to identify bacteria utilising this technology,

In the medium term advances in the characterisation of living biofilms utilising MABs/FISH technology should be possible, leading to increased knowledge of the biofilm phenotype, the development of novel strategies to treat these biofilms, and a means of monitoring treatment efficacy.

In the long term it is to be hoped that these therapies, developed secondary to better knowledge of bacterial interaction within biofilms and life cycles and pathogenicity of bacteria present, will provide an effective method of treating biofilms within chronic wounds and the whole spectrum of biofilm infection and disease. This may be achieved through eradication of certain species, an entire biofilm or the application of a known beneficial biofilm on wounding

References

Amann R, Fuchs BM, Behrens S. The identification of microorganisms by fluorescence in situ hybridisation. Curr Opin Biotechnol. 2001 Jun;12(3):231-6.

Anderl JN, Franklin MJ, Stewart PS. Role of antibiotic penetration limitation in Klebsiella pneumoniae biofilm resistance to ampicillin and ciprofloxacin. Antimicrob Agents Chemother. 2000 Jul;44(7):1818-24.

Ahn ST, Mustoe TA.Ahn. Effects of ischemia on ulcer wound healing: a new model in the rabbit ear. Ann Plast Surg. 1990 Jan;24(1):17-23.

Baker EA, Leaper DJ. Proteinases, their inhibitors, and cytokine profiles in acute wound fluid.Wound Repair Regen. 2000 Sep-Oct;8(5):392-8.

Baker EA, Kumar S, Melling AC, Whetter D, Leaper DJ. Temporal and quantitative profiles of growth factors and metalloproteinases in acute wound fluid after mastectomy.Wound Repair Regen. 2008 Jan-Feb;16(1):95-101.

Balaban N, Stoodley P, Fux CA, Wilson S, Costerton JW, Dell'Acqua G. Prevention of staphylococcal biofilm-associated infections by the quorum sensing inhibitor RIP. Clin Orthop Relat Res. 2005 Aug;(437):48-54.

Bandara HM, Yau JY, Watt RM, Jin LJ, Samaranayake LP. Pseudomonas aeruginosa inhibits in-vitro Candida biofilm development. BMC Microbiol. 2010 Apr 25;10:125.

Barone EJ, Yager DR, Pozez AL, Olutoye OO, Crossland MC, Diegelmann RF, Cohen IK. Interleukin-1alpha and collagenase activity are elevated in chronic wounds. Plast Reconstr Surg. 1998 Sep;102(4):1023-7; discussion 1028-9.

Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. Growth factors and cytokines in wound healing. Wound Repair Regen. 2008 Sep-Oct;16(5):585-601.

Beidler SK, Douillet CD, Berndt DF, Keagy BA, Rich PB, Marston WA. Multiplexed analysis of matrix metalloproteinases in leg ulcer tissue of patients with chronic venous insufficiency before and after compression therapy. Wound Repair Regen. 2008 Sep-Oct;16(5):642-8.

Berends ET, Horswill AR, Haste NM, Monestier M, Nizet V, von Köckritz-Blickwede M. Nuclease expression by Staphylococcus aureus facilitates escape from neutrophil extracellular traps. J Innate Immun. 2010;2(6):576-86.

Berg JO, Mössner BK, Skov MN, Lauridsen J, Gottrup F, Kolmos HJ. Antibacterial properties of EMLA and lidocaine in wound tissue biopsies for culturing. Wound Repair Regen. 2006 Sep-Oct;14(5):581-5.

Bickers DR, Lim HW, Margolis D, Weinstock MA, Goodman C, Faulkner E, Gould C, Gemmen E, Dall T. The burden of skin diseases: 2004 a joint project of the American Academy of Dermatology Association and the Society for Investigative Dermatology. J Am Acad Dermatol. 2006 Sep;55(3):490-500.

Bishop JB, Phillips LG, Mustoe TA, VanderZee AJ, Wiersema L, Roach DE, Heggers JP, Hill DP Jr, Taylor EL, Robson MC. A prospective randomized evaluator-blinded trial of two potential wound healing agents for the treatment of venous stasis ulcers. J Vasc Surg. 1992 Aug;16(2):251-7.

Bittar F, Richet H, Dubus JC, Reynaud-Gaubert M, Stremler N, Sarles J, Raoult D, Rolain JM. Molecular detection of multiple emerging pathogens in sputa from cystic fibrosis patients. PLoS One. 2008 Aug 6;3(8):e2908.

Bjarnsholt T, Kirketerp-Møller K, Jensen PØ, Madsen KG, Phipps R, Krogfelt K, Høiby N, Givskov M. Why chronic wounds will not heal: a novel hypothesis. Wound Repair Regen. 2008 Jan-Feb;16(1):2-10.

Bjarnsholt T, Jensen PØ, Fiandaca MJ, Pedersen J, Hansen CR, Andersen CB, Pressler T, Givskov M, Høiby N. Pseudomonas aeruginosa biofilms in the respiratory tract of cystic fibrosis patients. Pediatr Pulmonol. 2009 Jun;44(6):547-58.

Bottari B, Ercolini D, Gatti M, Neviani E. Application of FISH technology for microbiological analysis: current state and prospects. Appl Microbiol Biotechnol. 2006 Dec;73(3):485-94.

Bowler PG, Duerden BI, Armstrong DG. Wound microbiology and associated approaches to wound management. Clin Microbiol Rev. 2001 Apr;14(2):244-69.

Bowler PG, Davies BJ. The microbiology of infected and noninfected leg ulcers. Int J Dermatol. 1999 Aug;38(8):573-8.

Brakstad OG, Maeland JA. Direct identification of Staphylococcus aureus in blood cultures by detection of the gene encoding the thermostable nuclease or the gene product. APMIS. 1995 Mar;103(3):209-18.

Brakstad OG, Maeland JA, Chesneau O. Comparison of tests designed to identify Staphylococcus aureus thermostable nuclease. APMIS. 1995 Mar;103(3):219-24.

Breuing K, Kaplan S, Liu P, Onderdonk AB, Eriksson E. Wound fluid bacterial levels exceed tissue bacterial counts in controlled porcine partial-thickness burn infections. Plast Reconstr Surg. 2003 Feb;111(2):781-8.

Bridier A, Dubois-Brissonnet F, Boubetra A, Thomas V, Briandet R. The biofilm architecture of sixty opportunistic pathogens deciphered using a high throughput CLSM method. J Microbiol Methods. 2010 Jul;82(1):64-70.

Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y, Zychlinsky A. Neutrophil extracellular traps kill bacteria. Science. 2004 Mar 5;303(5663):1532-5.

Bryers JD. Medical biofilms. Biotechnol Bioeng. 2008 May 1;100(1):1-18.

Bystrova OV, Lindner B, Moll H, Kocharova NA, Knirel YA, Zahringer U, Pier GB. Full structure of the lipopolysaccharide of Pseudomonas aeruginosa immunotype 5. Biochemistry (Mosc). 2004 Feb;69(2):170-5.

Chesneau O, Allignet J, el Solh N. Thermonuclease gene as a target nucleotide sequence for specific recognition of Staphylococcus aureus. Mol Cell Probes. 1993 Aug;7(4):301-10.

Cogen AL, Nizet V, Gallo RL. Skin microbiota: a source of disease or defence? Br J Dermatol. 2008 Mar;158(3):442-55

Constantine BE, Bolton LL. A wound model for ischemic ulcers in the guinea pig. Arch Dermatol Res. 1986;278(5):429-31.

Cos P, Toté K, Horemans T, Maes L. Biofilms: an extra hurdle for effective antimicrobial therapy. Curr Pharm Des. 2010;16(20):2279-95.

Costerton JW, Geesey GG, Cheng KJ. How bacteria stick. Sci Am. 1978 Jan;238(1):86-95.

Costerton JW, Khoury AE, Ward KH, Anwar H. Practical measures to control device-related bacterial infections. Int J Artif Organs. 1993 Nov;16(11):765-70.

Costerton JW. Overview of microbial biofilms. J Ind Microbiol. 1995 Sep;15(3):137-40.

Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM. Microbial biofilms. Annu Rev Microbiol. 1995;49:711-45.

Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. Science. 1999 May 21;284(5418):1318-22.

Costerton JW. Introduction to biofilm. Int J Antimicrob Agents. 1999 May;11(3-4):217-21; discussion 237-9.

Davey ME, Caiazza NC, O'Toole GA. Rhamnolipid surfactant production affects biofilm architecture in Pseudomonas aeruginosa PAO1. J Bacteriol. 2003 Feb;185(3):1027-36.

Davies CE, Wilson MJ, Hill KE, Stephens P, Hill CM, Harding KG, Thomas DW. Use of molecular techniques to study microbial diversity in the skin: chronic wounds reevaluated. Wound Repair Regen. 2002 Sep-Oct;9(5):332-40.

Davies CE, Hill KE, Wilson MJ, Stephens P, Hill CM, Harding KG, Thomas DW. Use of 16S ribosomal DNA PCR and denaturing gradient gel electrophoresis for analysis of the microfloras of healing and nonhealing chronic venous leg ulcers. J Clin Microbiol. 2004 Aug;42(8):3549-57.

Davies CE, Hill KE, Newcombe RG, Stephens P, Wilson MJ, Harding KG, Thomas DW. A prospective study of the microbiology of chronic venous leg ulcers to reevaluate the clinical predictive value of tissue biopsies and swabs. Wound Repair Regen. 2007 Jan-Feb;15(1):17-22.

Davies DG, Geesey GG Regulation of the alginate biosynthesis gene algC in Pseudomonas aeruginosa during biofilm development in continuous culture. Appl Environ Microbiol. 1995 Mar;61(3):860-7.

Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW, Greenberg EP. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science. 1998 Apr 10;280(5361):295-8..

Davis SC, Martinez L, Kirsner R. The diabetic foot: the importance of biofilms and wound bed preparation. Curr Diab Rep. 2006 Dec;6(6):439-45

Davis SC, Ricotti C, Cazzaniga A, Welsh E, Eaglstein WH, Mertz PM. Microscopic and physiologic evidence for biofilm-associated wound colonization in vivo. Wound Repair Regen. 2008 Jan-Feb;16(1):23-9.

Demling RH, Leslie DeSanti MD. The rate of re-epithelialization across meshed skin grafts is increased with exposure to silver. Burns. 2002 May;28(3):264-6.

Donlan RM. Preventing biofilms of clinically relevant organisms using bacteriophage. Trends Microbiol. 2009 Feb;17(2):66-72. Epub 2009 Jan 21.

Donlan RM. Biofilm elimination on intravascular catheters: important considerations for the infectious disease practitioner. Clin Infect Dis. 2011 Apr 15;52(8):1038-45.

Doolittle MM, Cooney JJ, Caldwell DE Tracing the interaction of bacteriophage with bacterial biofilms using fluorescent and chromogenic probes. J Ind Microbiol. 1996 Jun;16(6):331-41.

Douglas LJ. Candida biofilms and their role in infection. Trends Microbiol. 2003 Jan;11(1):30-6.

Douglas TA, Brennan S, Gard S, Berry L, Gangell C, Stick SM, Clements BS, Sly PD. Acquisition and eradication of P. aeruginosa in young children with cystic fibrosis. Eur Respir J. 2009 Feb;33(2):305-11.

Dowd SE, Sun Y, Secor PR, Rhoads DD, Wolcott BM, James GA, Wolcott RD. Survey of bacterial diversity in chronic wounds using pyrosequencing, DGGE, and full ribosome shotgun Microbiol. sequencing. BMC 2008 Mar 6;8:43.

Dowd SE, Wolcott RD, Sun Y, McKeehan T, Smith E, Rhoads D. Polymicrobial nature of chronic diabetic foot ulcer biofilm infections determined using bacterial tag encoded FLX amplicon pyrosequencing (bTEFAP). PLoS One. 2008 Oct 3;3(10):e3326.

Eberl HJ, Collinson S. A modeling and simulation study of siderophore mediated antagonism in dual-species biofilms. Theor Biol Med Model. 2009 Dec 22;6:30.

Ebert T, Smith S, Pancari G, Clark D, Hampton R, Secore S, Towne V, Fan H, Wang XM, Wu X, Ernst R, Harvey BR, Finnefrock AC, Wang F, Tan C, Durr E, Cope L, Anderson A, An Z, McNeely T. A fully human monoclonal antibody to Staphylococcus aureus iron regulated surface determinant B (IsdB) with functional activity in vitro and in vivo. Hum Antibodies. 2010;19(4):113-28.

Edwards R, Harding KG. Bacteria and wound healing. Curr Opin Infect Dis. 2004 Apr;17(2):91-6.

Engvall E, Jonsson K, Perlmann P. Enzyme-linked immunosorbent assay. II. Quantitative assay of protein antigen, immunoglobulin G, by means of enzyme-labelled antigen and antibody-coated tubes. Biochim Biophys Acta. 1971 Dec 28;251(3):427-34.

Ennis WJ, Foremann P, Mozen N, Massey J, Conner-Kerr T, Meneses P. Ultrasound therapy for recalcitrant diabetic foot ulcers: results of a randomized, double-blind, controlled, multicenter study. Ostomy Wound Manage. 2005 Aug;51(8):24-39.

Enoch S, Price P, Cellular, molecular and biochemical differences in the pathophysiology of healing between acute wounds, chronic wounds and wounds in the aged. World Wide Wounds 2004 Aug.

Fazli M, Bjarnsholt T, Kirketerp-Møller K, Jørgensen B, Andersen AS, Krogfelt KA, Givskov M, Tolker-Nielsen T. Nonrandom distribution of Pseudomonas aeruginosa and Staphylococcus aureus in chronic wounds. J Clin Microbiol. 2009 Dec;47(12):4084-9.

Flanagan JL, Brodie EL, Weng L, Lynch SV, Garcia O, Brown R, Hugenholtz P, DeSantis TZ, Andersen GL, Wiener-Kronish JP, Bristow J. Loss of bacterial diversity during antibiotic treatment of intubated patients colonized with Pseudomonas aeruginosa. J Clin Microbiol. 2007 Jun;45(6):1954-62.

Fleck CA. Fighting infection in chronic wounds. Adv Skin Wound Care. 2006 May;19(4):184, 186, 188.

Fonder MA, Lazarus GS, Cowan DA, Aronson-Cook B, Kohli AR, Mamelak AJ. Treating the chronic wound: A practical approach to the care of nonhealing wounds and wound care dressings. J Am Acad Dermatol. 2008 Feb;58(2):185-206.

Frank DN, Wysocki A, Specht-Glick DD, Rooney A, Feldman RA, St Amand AL, Pace NR, Trent JD. Microbial diversity in chronic open wounds. Wound Repair Regen. 2009 Mar-Apr;17(2):163-72.

Gardner SE, Frantz RA, Saltzman CL, Hillis SL, Park H, Scherubel M. Diagnostic validity of three swab techniques for identifying chronic wound infection. Wound Repair Regen. 2006 Sep-Oct;14(5):548-57.

Giovannoni SJ, DeLong EF, Olsen GJ, Pace NR. Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial cells. J Bacteriol. 1988 Feb;170(2):720-6.

Gjødsbøl K, Christensen JJ, Karlsmark T, Jørgensen B, Klein BM, Krogfelt KA. Multiple bacterial species reside in chronic wounds: a longitudinal study. Int Wound J. 2006 Sep;3(3):225-31.

Gonano M, Hein I, Zangerl P, Rammelmayr A, Wagner M. Phenotypic and molecular characterization of Staphylococcus aureus strains of veterinary, dairy and human origin. Epidemiol Infect. 2009 May;137(5):688-99.

Gottrup F. A specialized wound-healing center concept: importance of a multidisciplinary department structure and surgical treatment facilities in the treatment of chronic wounds. Am J Surg. 2004 May;187(5A):38S-43S.

Grey, J. Harding, K. G. ABC of Wound Healing. 2006. *BMJ publishing* .

Grinnell F, Zhu M. Fibronectin degradation in chronic wounds depends on the relative levels of elastase, alpha1-proteinase inhibitor, and alpha2-macroglobulin. J Invest Dermatol. 1996 Feb;106(2):335-41.

Gu F, Lux R, Anderson MH, del Aguila MA, Wolinsky L, Hume WR, Shi W. Analyses of Streptococcus mutans in saliva with species-specific monoclonal antibodies. Hybrid Hybridomics. 2002 Aug;21(4):225-32.

Gu F, Lux R, Du-Thumm L, Stokes I, Kreth J, Anderson MH, Wong DT, Wolinsky L, Sullivan R, Shi W. In situ and non-invasive detection of specific bacterial species in oral biofilms using fluorescently labeled monoclonal antibodies. J Microbiol Methods. 2005 Aug;62(2):145-60.

Guggenheim M, Shapiro S, Gmür R, Guggenheim B. Spatial arrangements and associative behavior of species in an in vitro oral biofilm model. Appl Environ Microbiol. 2001 Mar;67(3):1343-50.

Guimarães N, Azevedo NF, Figueiredo C, Keevil CW, Vieira MJ. Development and application of a novel peptide nucleic acid probe for the specific detection of Helicobacter pylori in gastric biopsy specimens. J Clin Microbiol. 2007 Sep;45(9):3089-94.

Harriott MM, Noverr MC. Ability of Candida albicans mutants to induce Staphylococcus aureus vancomycin resistance during polymicrobial biofilm formation. Antimicrob Agents Chemother. 2010 Sep;54(9):3746-55.

Hatano K, Pier GB. Complex serology and immune response of mice to variant high-molecular-weight O polysaccharides isolated from Pseudomonas aeruginosa serogroup O2 strains. Infect Immun. 1998 Aug;66(8):3719-26.

Hæger, Knut. 1925 *The illustrated history of surgery / Knut Haeger.* 2nd rev. and updated ed. / by Roy Kalne. London ; Chicago, Ill. : Fitzroy Dearborn Publishers, 2000.

Harmsen M, Yang L, Pamp SJ, Tolker-Nielsen T. An update on Pseudomonas aeruginosa biofilm formation, tolerance, and dispersal. FEMS Immunol Med Microbiol. 2010 Aug;59(3):253-68.

Harrison-Balestra C, Cazzaniga AL, Davis SC, Mertz PM. A wound-isolated Pseudomonas aeruginosa grows a biofilm in vitro within 10 hours and is visualized by light microscopy. Dermatol Surg. 2003 Jun;29(6):631-5.

Hartmann H, Stender H, Schäfer A, Autenrieth IB, Kempf VA. Rapid identification of Staphylococcus aureus in blood cultures by a combination of fluorescence in situ hybridization using peptide nucleic acid probes and flow cytometry. J Clin Microbiol. 2005 Sep;43(9):4855-7.

He C, Hughes MA, Cherry GW, Arnold F. Effects of chronic wound fluid on the bioactivity of platelet-derived growth factor in serum-free medium and its direct effect on fibroblast growth. Wound Repair Regen. 1999 Mar-Apr;7(2):97-105.

Heggers JP. Defining infection in chronic wounds: methodology. J Wound Care. 1998 Oct;7(9):452-6.

Helmer-Madhok C, Schmid M, Filipov E, Gaul T, Hippen A, Rosenwinkel KH, Seyfried CF, Wagner M, Kunst S. Deammonification in biofilm systems: population structure and function. Water Sci Technol. 2002;46(1-2):223-31.

Hermansson, M. The DVLO Theory in Microbial Adhesion. *Colloids and surfaces B: Biointerfaces* 1999. 105-119 (15)

Hill KE, Malic S, McKee R, Rennison T, Harding KG, Williams DW, Thomas DW. An in vitro model of chronic wound biofilms to test wound dressings and assess antimicrobial susceptibilities. J Antimicrob Chemother. 2010 Jun;65(6):1195-206.

Hoffman R, Starkey S, Coad J. Wound fluid from venous leg ulcers degrades plasminogen and reduces plasmin generation by keratinocytes. J Invest Dermatol. 1998 Dec;111(6):1140-4.

Howell-Jones RS, Price PE, Howard AJ, Thomas DW. Antibiotic prescribing for chronic skin wounds in primary care. Wound Repair Regen. 2006 Jul-Aug;14(4):387-93.

Howell-Jones RS, Wilson MJ, Hill KE, Howard AJ, Price PE, Thomas DW. A review of the microbiology, antibiotic usage and resistance in chronic skin wounds. J Antimicrob Chemother. 2005 Feb;55(2):143-9.

Hussain M, von Eiff C, Sinha B, Joost I, Herrmann M, Peters G, Becker K. eap Gene as novel target for specific identification of Staphylococcus aureus. J Clin Microbiol. 2008 Feb;46(2):470-6.

Jackson JH, Cochrane CG. Leukocyte-induced tissue injury. Hematol Oncol Clin North Am. 1988 Jun;2(2):317-34.

James GA, Swogger E, Wolcott R, Pulcini E, Secor P, Sestrich J, Costerton JW, Stewart PS. Biofilms in chronic wounds. Wound Repair Regen. 2008 Jan-Feb;16(1):37-44.

Jamasbi, R.J. Taylor, N. 2009. Effect of lipopolysaccharide expression on adhesiveness of clinical isolates of Pseudomonas aeruginosa. *Lab Medicin*e 2010 41:24-30;

Jeffcoate WJ, Price PE, Phillips CJ, Game FL, Mudge E, Davies S, Amery CM, Edmonds ME, Gibby OM, Johnson AB, Jones GR, Masson E, Patmore JE, Price D, Rayman G, Harding KG. Randomised controlled trial of the use of three dressing preparations in the management of chronic ulceration of the foot in diabetes. Health Technol Assess. 2009 Nov;13(54):1-86, iii-iv.

John HA, Birnstiel ML, Jones KW. RNA-DNA hybrids at the cytological level. Nature. 1969 Aug 9;223(5206):582-7.

Jones SG, Edwards R, Thomas DW. Inflammation and wound healing: the role of bacteria in the immuno-regulation of wound healing. Int J Low Extrem Wounds. 2004 Dec;3(4):201-8.

Junger H, Sorkin LS. Isoprostanes induce plasma extravasation in rat skin. Prostaglandins Other Lipid Mediat. 2000 Oct;62(4):335-42.

Kaneko Y, Thoendel M, Olakanmi O, Britigan BE, Singh PK. The transition metal gallium disrupts Pseudomonas aeruginosa iron metabolism and has antimicrobial and antibiofilm activity. J Clin Invest. 2007 Apr;117(4):877-88.

Kanno E, Toriyabe S, Zhang L, Imai Y, Tachi M. Biofilm formation on rat skin wounds by Pseudomonas aeruginosa carrying the green fluorescent protein gene. Exp Dermatol. 2010 Feb;19(2):154-6

Kantor J, Margolis DJ. The accuracy of using a wound care specialty clinic database to study diabetic neuropathic foot ulcers. Wound Repair Regen. 2000 May-Jun;8(3):169-73.

Kateete DP, Kimani CN, Katabazi FA, Okeng A, Okee MS, Nanteza A, Joloba ML, Najjuka FC. Identification of Staphylococcus aureus: DNase and Mannitol salt agar improve the efficiency of the tube coagulase test. Ann Clin Microbiol Antimicrob. 2010 Aug 13;9:23.

Katsikogianni M, Missirlis YF. Concise review of mechanisms of bacterial adhesion to biomaterials and of techniques used in estimating bacteria-material interactions. Eur Cell Mater. 2004 Dec 7;8:37-57.

Kerenyi M, Batai R, Juhasz V, Batai I. Lidocaine/prilocaine cream (EMLA) has an antibacterial effect in vitro. J Hosp Infect. 2004 Jan;56(1):75-6.

Kirketerp-Møller K, Jensen PØ, Fazli M, Madsen KG, Pedersen J, Moser C, Tolker-Nielsen T, Høiby N, Givskov M, Bjarnsholt T. Distribution, organization, and ecology of bacteria in chronic wounds. J Clin Microbiol. 2008 Aug;46(8):2717-22.

Kloos WE, Bannerman TL. Update on clinical significance of coagulase-negative staphylococci. Clin Microbiol Rev. 1994 Jan;7(1):117-40.

Knox KL, Holmes AH. Regulation of antimicrobial prescribing practices--a strategy for controlling nosocomial antimicrobial resistance. Int J Infect Dis. 2002 Mar;6 Suppl 1:S8-13.

Kokan NP, Bergdoll MS. Detection of low-enterotoxin-producing Staphylococcus aureus strains. Appl Environ Microbiol. 1987 Nov;53(11):2675-6.

Kong KF, Vuong C, Otto M. Staphylococcus quorum sensing in biofilm formation and infection. Int J Med Microbiol. 2006 Apr;296(2-3):133-9.

Kusama H. Enzyme-linked immunosorbent assay for detection of Pseudomonas aeruginosa Lipopolysaccharides. J Clin Microbiol. 1983 Feb;17(2):317-22.

Lagacé-Wiens PR, Alfa MJ, Manickam K, Karlowsky JA. Thermostable DNase is superior to tube coagulase for direct detection of Staphylococcus aureus in positive blood cultures. J Clin Microbiol. 2007 Oct;45(10):3478-9.

Lam JS, Lam MY, MacDonald LA, Hancock RE. Visualization of Pseudomonas aeruginosa O antigens by using a protein A-dextran-colloidal gold conjugate with both immunoglobulin G and immunoglobulin M monoclonal antibodies. J Bacteriol. 1987 Aug;169(8):3531-8.

Landis SJ. Chronic wound infection and antimicrobial use. Adv Skin Wound Care. 2008 Nov;21(11):531-40; quiz 541-2.

Lavenir R, Jocktane D, Laurent F, Nazaret S, Cournoyer B. Improved reliability of Pseudomonas aeruginosa PCR detection by the use of the species-specific ecfX gene target. J Microbiol Methods. 2007 Jul;70(1):20-9.

Leaper DJ. Prophylactic and therapeutic role of antibiotics in wound care. Am J Surg. 1994 Jan;167(1A):15S-19S; discussion 19S-20S.

Liu Y, Tay JH. The essential role of hydrodynamic shear force in the formation of biofilm and granular sludge. Water Res. 2002 Apr;36(7):1653-65.

Liu ZM, Shi XM, Pan F. Species-specific diagnostic marker for rapid identification of Staphylococcus aureus. Diagn Microbiol Infect Dis. 2007 Dec;59(4):379-82.

Madison BM, Baselski VS. Rapid identification of Staphylococcus aureus in blood cultures by thermonuclease testing. J Clin Microbiol. 1983 Sep;18(3):722-4.

Madsen SM, Westh H, Danielsen L, Rosdahl VT. Bacterial colonization and healing of venous leg ulcers. APMIS. 1996 Dec;104(12):895-9.

Mah TF, Pitts B, Pellock B, Walker GC, Stewart PS, O'Toole GA. A genetic basis for Pseudomonas aeruginosa biofilm antibiotic resistance. Nature. 2003 Nov 20;426(6964):306-10.

Malic S, Hill KE, Hayes A, Percival SL, Thomas DW, Williams DW. Detection and identification of specific bacteria in wound biofilms using peptide nucleic acid fluorescent in situ hybridization (PNA FISH). Microbiology. 2009 Aug;155(Pt 8):2603-11.

Marsh PD. Plaque as a biofilm: pharmacological principles of drug delivery and action in the sub- and supragingival environment. Oral Dis. 2003;9 Suppl 1:16-22.

Martin C. Recent Developments in Wound Management: intelligent biomaterials to novel antimicrobials. 24 September 2009, London, UK. IDrugs. 2009 Nov;12(11):695-8.

Mertz PM, Oliveira-Gandia MF, Davis SC. The evaluation of a cadexomer iodine wound dressing on methicillin resistant Staphylococcus aureus (MRSA) in acute wounds. Dermatol Surg. 1999 Feb;25(2):89-93.

Meyer-Reil LA, Bölter M, Dawson R, Liebezeit G, Szwerinski H, Wolter K. Interrelationships between microbiological and chemical parameters of sandy beach sediments, a summer aspect. Appl Environ Microbiol. 1980 Apr;39(4):797-802.

Moor AN, Vachon DJ, Gould LJ. Proteolytic activity in wound fluids and tissues derived from chronic venous leg ulcers. Wound Repair Regen. 2009 Nov-Dec;17(6):832-9.

Moter A, Göbel UB. Fluorescence in situ hybridization (FISH) for direct visualization of microorganisms. J Microbiol Methods. 2000 Jul;41(2):85-112

Mudge E, Meume S, Woo K, Sibbald G, Price P. The patients' experience of wound pain: an international perspective. *J EWMA*. 2008;8(2):19–28.

Mustoe T. Understanding chronic wounds: a unifying hypothesis on their pathogenesis and implications for therapy. Am J Surg. 2004 May;187(5A):65S-70S.

Mustoe, T. Dermal ulcer healing: Advances in understanding. Tissue repair and ulcer/wound healing: molecular mechanisms, therapeutic targets and future directions. Paris France, *EUROCONFERENCES*. 2005. .

Mustoe TA, O'Shaughnessy K, Kloeters O. Chronic wound pathogenesis and current treatment strategies: a unifying hypothesis. Plast Reconstr Surg. 2006 Jun;117(7 Suppl):35S-41S.

NIH. 1997. Minutes of the National Advisory Dental and Craniofacial Research Council, 153rd Meeting; Available at: http://www.nidcr.nih.gov/AboutNIDCR/CouncilAndCommittees/NADCRC/Minutes/Minutes153.htm.

Neu TR, Manz B, Volke F, Dynes JJ, Hitchcock AP, Lawrence JR. Advanced imaging techniques for assessment of structure, composition and function in biofilm systems. FEMS Microbiol Ecol. 2010 Apr;72(1):1-21. Epub 2010 Feb 19.

Neville LO, Billington OJ, Kibbler CC, Gillespie SH. Methicillin resistant Staphylococcus aureus without clumping factor, protein A, and DNAse. Lancet. 1991 Aug 24;338(8765):518.

Nivens DE, Ohman DE, Williams J, Franklin MJ. Role of alginate and its O acetylation in formation of Pseudomonas aeruginosa microcolonies and biofilms. J Bacteriol. 2001 Feb;183(3):1047-57.

Oliveira K, Brecher SM, Durbin A, Shapiro DS, Schwartz DR, De Girolami PC, Dakos J, Procop GW, Wilson D, Hanna CS, Haase G, Peltroche-Llacsahuanga H, Chapin KC, Musgnug MC, Levi MH, Shoemaker C, Stender H. Direct identification of

Staphylococcus aureus from positive blood culture bottles. J Clin Microbiol. 2003 Feb;41(2):889-91.

O'Meara S, Al-Kurdi D, Ovington LG. Antibiotics and antiseptics for venous leg ulcers. Cochrane Database Syst Rev. 2008 Jan 23;(1):CD003557.

O'Toole GA, Kolter R. Flagellar and twitching motility are necessary for Pseudomonas aeruginosa biofilm development. Mol Microbiol. 1998 Oct;30(2):295-304.

O'Toole G, Kaplan HB, Kolter R. Biofilm formation as microbial development. Annu Rev Microbiol. 2000;54:49-79.

Palolahti M, Lauharanta J, Stephens RW, Kuusela P, Vaheri A. Proteolytic activity in leg ulcer exudate. Exp Dermatol. 1993 Feb;2(1):29-37.

Pamp SJ, Tolker-Nielsen T. Multiple roles of biosurfactants in structural biofilm development by Pseudomonas aeruginosa. J Bacteriol. 2007 Mar;189(6):2531-9.

Pardue ML, Gall JG. Molecular hybridization of radioactive DNA to the DNA of cytological preparations. Proc Natl Acad Sci U S A. 1969 Oct;64(2):600-4.

Park CE, de Melo Serrano A, Landgraf M, Huang JC, Stankiewicz Z, Rayman MK. A survey of microorganisms for thermonuclease production. Can J Microbiol. 1980 Apr;26(4):532-8.

Parvizi J, Ghanem E, Menashe S, Barrack RL, Bauer TW. Periprosthetic infection: what are the diagnostic challenges? J Bone Joint Surg Am. 2006 Dec;88 Suppl 4:138-47.

Percival SL, Bowler PG, Dolman J. Antimicrobial activity of silver-containing dressings on wound microorganisms using an in vitro biofilm model. Int Wound J. 2007 Jun;4(2):186-91.

Percival SL, Thomas JG, Williams DW. Biofilms and bacterial imbalances in chronic wounds: anti-Koch. Int Wound J. 2010 Jun;7(3):169-75.

Perry-O'Keefe H, Rigby S, Oliveira K, Sørensen D, Stender H, Coull J, Hyldig-Nielsen JJ. Identification of indicator microorganisms using a standardized PNA FISH method. J Microbiol Methods. 2001 Dec;47(3):281-92.

Peyton BM, Characklis WG. A statistical analysis of the effect of substrate utilization and shear stress on the kinetics of biofilm detachment. Biotechnol Bioeng. 1993 Mar 25;41(7):728-35.

Pier GB. Pseudomonas aeruginosa lipopolysaccharide: a major virulence factor, initiator of inflammation and target for effective immunity. Int J Med Microbiol. 2007 Sep;297(5):277-95.

Posnett J, Franks PJ. The burden of chronic wounds in the UK. Nurs Times. 2008 Jan 22-28;104(3):44-5.

Prescott AM, Fricker CR. Use of PNA oligonucleotides for the in situ detection of Escherichia coli in water. Mol Cell Probes. 1999 Aug;13(4):261-8.

Price LB, Liu CM, Melendez JH, Frankel YM, Engelthaler D, Aziz M, Bowers J, Rattray R, Ravel J, Kingsley C, Keim PS, Lazarus GS, Zenilman JM. Community analysis of chronic wound bacteria using 16S rRNA gene-based pyrosequencing: impact of diabetes and antibiotics on chronic wound microbiota. PLoS One. 2009 Jul 31;4(7):e6462.

Price P, Harding, K, . Measuring health related quality of life in patients with chronic leg ulcers. *Wounds.* 1996 8(3):91-94

Price P, Harding K. Cardiff Wound Impact Schedule: the development of a condition-specific questionnaire to assess health-related quality of life in patients with chronic wounds of the lower limb. Int Wound J. 2004 Apr;1(1):10-7.

Price P., An Holistic Approach to Wound Pain in Patients with Chronic Wounds. *Wounds.* 2005 17(3):55-57

Price P, Fogh K, Glynn C, Krasner DL, Osterbrink J, Sibbald RG. Managing painful chronic wounds: the Wound Pain Management Model. Int Wound J. 2007 Apr;4 Suppl 1:4-15.

Provenza G, Provenzano M, Visai L, Burke FM, Geoghegan JA, Stravalaci M, Gobbi M, Mazzini G, Arciola CR, Foster TJ, Speziale P. Functional analysis of a murine monoclonal antibody against the repetitive region of the fibronectin-binding adhesins fibronectin-binding protein A and fibronectin-binding protein B from Staphylococcus aureus. FEBS J. 2010 Nov;277(21):4490-505.

Pukstad BS, Ryan L, Flo TH, Stenvik J, Moseley R, Harding K, Thomas DW, Espevik T. Non-healing is associated with persistent stimulation of the innate immune response in chronic venous leg ulcers. J Dermatol Sci. 2010 Aug;59(2):115-22.

Quirinia A, Jensen FT, Viidik A Ischemia in wound healing. I: Design of a flap model--changes in blood flow Scand J Plast Reconstr Surg Hand Surg. 1992;26(1):21-8.

.

Randle BJ, Scoltock SJ, Scott DK. Integrating molecular detection and response to create self-signalling antibodies. Biochem Biophys Res Commun. 2004 Nov 12;324(2):504-10.

Rao CN, Ladin DA, Liu YY, Chilukuri K, Hou ZZ, Woodley DT. Alpha 1-antitrypsin is degraded and non-functional in chronic wounds but intact and functional in acute wounds: the inhibitor protects fibronectin from degradation by chronic wound fluid enzymes. J Invest Dermatol. 1995 Oct;105(4):572-8.

Rayment EA, Upton Z, Shooter GK. Increased matrix metalloproteinase-9 (MMP-9) activity observed in chronic wound fluid is related to the clinical severity of the ulcer. Br J Dermatol. 2008 May;158(5):951-61.

Ribu L, Hanestad BR, Moum T, Birkeland K, Rustoen T. Health-related quality of life among patients with diabetes and foot ulcers: association with demographic and clinical characteristics. J Diabetes Complications. 2007 Jul-Aug;21(4):227-36.

Robson MC, Barbul A. Guidelines for the best care of chronic wounds. Wound Repair Regen. 2006 Nov-Dec;14(6):647-8. No abstract available.

Rudolph R, Woodward M, Hurn I. Ultrastructure of doxorubicin (adriamycin)-induced skin ulcers in rats. Cancer Res. 1979 Sep;39(9):3689-93.

Rumbaugh KP. Convergence of hormones and autoinducers at the host/pathogen interface. Anal Bioanal Chem. 2007 Jan;387(2):425-35.

Sandal I, Hong W, Swords WE, Inzana TJ. Characterization and comparison of biofilm development by pathogenic and commensal isolates of Histophilus somni. J Bacteriol. 2007 Nov;189(22):8179-85.

Sauer K, Camper AK. Characterization of phenotypic changes in Pseudomonas putida in response to surface-associated growth. J Bacteriol. 2001 Nov;183(22):6579-89.

Sauer K, Camper AK, Ehrlich GD, Costerton JW, Davies DG. Pseudomonas aeruginosa displays multiple phenotypes during development as a biofilm. J Bacteriol. 2002 Feb;184(4):1140-54.

Saye DE. Recurring and antimicrobial-resistant infections:considering the potential role of biofilms in clinical practice. Ostomy Wound Manage. 2007 Apr;53(4):46-8.

Schaudinn C, Carr G, Gorur A, Jaramillo D, Costerton JW, Webster P Imaging of endodontic biofilms by combined microscopy (FISH/cLSM - SEM). J Microsc. 2009 Aug;235(2):124-7.

Schierle CF, De la Garza M, Mustoe TA, Galiano RD. Staphylococcal biofilms impair wound healing by delaying reepithelialization in a murine cutaneous wound model. Wound Repair Regen. 2009 May-Jun;17(3):354-9.

Schraibman IG. The significance of beta-haemolytic streptococci in chronic leg ulcers.

Ann R Coll Surg Engl. 1990 Mar;72(2):123-4.

Schreier H, Erdos G, Reimer K, König B, König W, Fleischer W. Molecular effects of povidone-iodine on relevant microorganisms: an electron-microscopic and biochemical study. Dermatology. 1997;195 Suppl 2:111-6.

Secor PR, James GA, Fleckman P, Olerud JE, McInnerney K, Stewart PS. Staphylococcus aureus Biofilm and Planktonic cultures differentially impact gene expression, mapk phosphorylation, and cytokine production in human keratinocytes. BMC Microbiol. 2011 Jun 21;11:143.

Sekiguchi Y, Kamagata Y, Nakamura K, Ohashi A, Harada H. Fluorescence in situ hybridization using 16S rRNA-targeted oligonucleotides reveals localization of methanogens and selected uncultured bacteria in mesophilic and thermophilic sludge granules. Appl Environ Microbiol. 1999 Mar;65(3):1280-8.

Sen CK, Gordillo GM, Roy S, Kirsner R, Lambert L, Hunt TK, Gottrup F, Gurtner GC, Longaker MT. Human skin wounds: a major and snowballing threat to public health and the economy. Wound Repair Regen. 2009 Nov-Dec;17(6):763-71.

Sharp D, Gladstone P, Smith RB, Forsythe S, Davis J. Approaching intelligent infection diagnostics: Carbon fibre sensor for electrochemical pyocyanin detection. Bioelectrochemistry. 2010 Feb;77(2):114-9.

Silva S, Henriques M, Hayes A, Oliveira R, Azeredo J, Williams DW.Candida glabrata and Candida albicans co-infection of an in vitro oral epithelium. J Oral Pathol Med. 2011 May;40(5):421-7.

Singer AJ, Clark RA. Cutaneous wound healing. N Engl J Med. 1999 Sep 2;341(10):738-46.

Singh PK, Parsek MR, Greenberg EP, Welsh MJ. A component of innate immunity prevents bacterial biofilm development. Nature. 2002 May 30;417(6888):552-5.

Singh R, Ray P, Das A, Sharma M. Penetration of antibiotics through Staphylococcus aureus and Staphylococcus epidermidis biofilms. J Antimicrob Chemother. 2010 Sep;65(9):1955-8.

Siqueira JF Jr, Rôças IN. Community as the unit of pathogenicity: an emerging concept as to the microbial pathogenesis of apical periodontitis. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2009 Jun;107(6):870-8.

Slavin J. The role of cytokines in wound healing. J Pathol. 1996 Jan;178(1):5-10.

Stewart PS. Theoretical aspects of antibiotic diffusion into microbial biofilms. Antimicrob Agents Chemother. 1996 Nov;40(11):2517-22.

Stephens P, Wall IB, Wilson MJ, Hill KE, Davies CE, Hill CM, Harding KG, Thomas DW. Anaerobic cocci populating the deep tissues of chronic wounds impair cellular wound healing responses in vitro. Br J Dermatol. 2003 Mar;148(3):456-66.

Stoodley P, Sauer K, Davies DG, Costerton JW. Biofilms as complex differentiated communities. Annu Rev Microbiol. 2002;56:187-209.

Sun Y, Dowd SE, Smith E, Rhoads DD, Wolcott RD. In vitro multispecies Lubbock chronic wound biofilm model. Wound Repair Regen. 2008 Nov-Dec;16(6):805-13.

Sutherland IW. The biofilm matrix--an immobilized but dynamic microbial environment. Trends Microbiol. 2001 May;9(5):222-7.

Sutton NA, Hughes N, Handley PS A comparison of conventional SEM techniques, low temperature SEM and the electroscan wet scanning electron microscope to study the structure of a biofilm of Streptococcus crista CR3. J Appl Bacteriol. 1994 May;76(5):448-54.

Thomas DW, Harding KG. Wound healing. Br J Surg. 2002 Oct;89(10):1203-5.

Thompson KD, Welykyj S, Massa MC. Antibacterial activity of lidocaine in combination with a bicarbonate buffer. J Dermatol Surg Oncol. 1993 Mar;19(3):216-20.

Thurnheer T, Gmür R, Giertsen E, Guggenheim B.Automated fluorescent in situ hybridization for the specific detection and quantification of oral streptococci in dental plaque. J Microbiol Methods. 2001 Feb 1;44(1):39-47.

Thurnheer T, Gmür R, Guggenheim B. Multiplex FISH analysis of a six-species bacterial biofilm. J Microbiol Methods. 2004 Jan;56(1):37-47.

Trengove NJ, Langton SR, Stacey MC. Biochemical analysis of wound fluid from nonhealing and healing chronic leg ulcers. Wound Repair Regen. 1996 Apr-Jun;4(2):234-9.

Trnovsky J, Merz W, Della-Latta P, Wu F, Arendrup MC, Stender H Rapid and accurate identification of Candida albicans isolates by use of PNA FISHFlow. J Clin Microbiol. 2008 Apr;46(4):1537-40.

van der Veen S, Abee T. Mixed species biofilms of Listeria monocytogenes and Lactobacillus plantarum show enhanced resistance to benzalkonium chloride and peracetic acid. Int J Food Microbiol. 2011 Jan 5;144(3):421-31.

Valenti P, Antonini G. Lactoferrin: an important host defence against microbial and viral attack. Cell Mol Life Sci. 2005 Nov;62(22):2576-87.

Vallalta Morales M, Soriano Navarro CJ, Salavert Lletí M, Montero Alonso M, Pérez Bellés C, López Aldeguer J, Otero MC, Gobernado Serrano M. Group A streptococcal bacteremia: outcome and prognostic factors. Rev Esp Quimioter. 2006 Dec;19(4):367-75.

Varshney AK, Wang X, Cook E, Dutta K, Scharff MD, Goger MJ, Fries BC. Generation, characterization, and epitope mapping of neutralizing and protective monoclonal antibodies against staphylococcal enterotoxin B-induced lethal shock. J Biol Chem. 2011 Mar 18;286(11):9737-47.

Vowden K, Vowden P, Posnett J. The resource costs of wound care in Bradford and Airedale primary care trust in the UK. J Wound Care. 2009 Mar;18(3):93-4, 96-8.

Vu TH, Werb Z. Matrix metalloproteinases: effectors of development and normal physiology. Genes Dev. 2000 Sep 1;14(17):2123-33.

Wall SJ, Bevan D, Thomas DW, Harding KG, Edwards DR, Murphy G. Differential expression of matrix metalloproteinases during impaired wound healing of the diabetes mouse. J Invest Dermatol. 2002 Jul;119(1):91-8.

Van Weemen BK, Schuurs AH. Immunoassay using antigen-enzyme conjugates. FEBS Lett. 1971 Jun 24;15(3):232-236.

Wellinghausen N, Bartel M, Essig A, Poppert S. Rapid identification of clinically relevant Enterococcus species by fluorescence in situ hybridization. J Clin Microbiol. 2007 Oct;45(10):3424-6.

Wenzel RP. Health care-associated infections: major issues in the early years of the 21st century. Clin Infect Dis. 2007 Jul 15;45 Suppl 1:S85-8.

White RJ, Cutting K, Kingsley A. Topical antimicrobials in the control of wound bioburden. Ostomy Wound Manage. 2006 Aug;52(8):26-58.

White RJ, Cutting KF. Critical colonization--the concept under scrutiny. Ostomy Wound Manage. 2006 Nov;52(11):50-6.

White RJ. Cutting KF. Critical colonisation of chronic wounds: microbial mechanisms. *Wounds UK*; 2008 , 4(1): 70-79.

Whittaker CJ, Klier CM, Kolenbrander PE. Mechanisms of adhesion by oral bacteria. Annu Rev Microbiol. 1996;50:513-52.

Wilson JR, Mills JG, Prather ID, Dimitrijevich SD. A toxicity index of skin and wound cleansers used on in vitro fibroblasts and keratinocytes. Adv Skin Wound Care. 2005 Sep;18(7):373-8.

Wiström J, Lindholm C, Melhus A, Lundgren C, Hansson C. Infections and treatment of chronic leg ulcers: the use of antibiotics is too excessive, restrictive prescription is recommended. Lakartidningen. 1999 Jan 6;96(1-2):42-6.

Wolcott RD, Rhoads DD, Dowd SE. Biofilms and chronic wound inflammation. J Wound Care. 2008 Aug;17(8):333-41.

Wolcott RD, Rumbaugh KP, James G, Schultz G, Phillips P, Yang Q, Watters C, Stewart PS, Dowd SE. Biofilm maturity studies indicate sharp debridement opens a time-dependent therapeutic window. J Wound Care. 2010 Aug;19(8):320-8.

Wysocki AB, Staiano-Coico L, Grinnell F. Wound fluid from chronic leg ulcers contains elevated levels of metalloproteinases MMP-2 and MMP-9. J Invest Dermatol. 1993 Jul;101(1):64-8.

Yach D, Stuckler D, Brownell KD. Epidemiologic and economic consequences of the global epidemics of obesity and diabetes. Nat Med. 2006 Jan;12(1):62-6.

Yang L, Haagensen JA, Jelsbak L, Johansen HK, Sternberg C, Høiby N, Molin S. In situ growth rates and biofilm development of Pseudomonas aeruginosa populations in chronic lung infections. J Bacteriol. 2008 Apr;190(8):2767-76.

Yazdankhah SP, Sølverød L, Simonsen S, Olsen E.Development and evaluation of an immunomagnetic separation-ELISA for the detection of Staphylococcus aureus thermostable nuclease in composite milk. Vet Microbiol. 1999 Jun 15;67(2):113-25

Zhu M, Takenaka S, Sato M, Hoshino E. Extracellular polysaccharides do not inhibit the reaction between Streptococcus mutans and its specific immunoglobulin G (IgG)

or penetration of the IgG through S. mutans biofilm. Oral Microbiol Immunol. 2001 Feb;16(1):54-6.

[No authors listed]. Consensus Development Conference on Diabetic Foot Wound Care: 7-8 April 1999, Boston, Massachusetts. American Diabetes Association. *Diabetes Care.* 22(8):1354-60. Review.

Biofilm hypertextbook, Montana State University Center for Biofilm Engineering

Appendicies

Appendix 1

Consent Chronic wound fluid collection

Appendix 2

Patient information sheet chronic wound fluid collection

Appendix 3

Consent acute wound fluid collection

Appendix 4

Patient infomation sheet acute wound fluid collection

Appendix 5

Ethical approvals and HTA Standard Operating procedure

CARDIFF UNIVERSITY

STANDARD OPERATING PROCEDURE FOR THE USE OR STORAGE OF HUMAN TISSUE FOR THE PURPOSES OF RESEARCH OR EDUCATION

DISPOSAL OF HUMAN TISSUE

Author: L Burrow, Corporate Compliance Unit

Procedure Approved by:

Responsible Unit: Corporate Compliance Unit (in conjunction with Research and Commercial Development)

Date issued: September 2007

Review date: December 2007

Version: 1.0

Contents page

5. Procedure
6. References

1 PURPOSE

The purpose of this Standard Operating Procedure (SOP) is to ensure that staff involved in research covered by the Human Tissue Act 2004 understand the procedure and mechanisms for the disposal of human tissue.

The purpose of the Human Tissue Act 2004 is to provide a consistent legislative framework for issues relating to whole body donation and the taking, storage and use of human organs and tissue. It makes consent the fundamental principle underpinning the lawful storage and use of human bodies, body parts, organs and tissue and the removal of material from the bodies of deceased persons. It introduces regulation of other activities like *post mortem* examinations, and the storage of human material for education, training and research. It is intended to achieve a balance between the rights and expectations of individuals and families, and broader considerations, such as the importance of research, education, training, pathology and public health surveillance to the population as a whole.

2 SCOPE

This SOP applies to all Cardiff University staff responsible for the disposal of human tissue for research or teaching purposes.

3 RESPONSIBLE PERSONNEL

The Principal Investigator/Research Supervisor is ultimately responsible for ensuring that this SOP is correctly applied in the conduct of research and each researcher also has individual responsibility for applying this SOP when required to do so.

The individual member of staff will be responsible for ensuring that this SOP is correctly applied when disposing of human tissue.

The Corporate Compliance Unit (in conjunction with the Research and Commercial Development Division) are responsible for ensuring that the SOP remains fit for purpose.

4 DEFINITIONS

Anatomical examination – macroscopic examination of the body of a deceased person, or separated parts of such a body, by dissection for anatomical purposes (teaching or studying, or researching into the gross structure of the human body).

Human Tissue – Any and all constituent parts of the human body formed by cells.

Principal Investigator (PI) – is the appropriately qualified individual at each project site who has responsibility for the conduct of the project at that site.

5 PROCEDURE

5.1 General Principles

Human tissue must be disposed of with respect. As an absolute minimum tissue identified for disposal should be disposed of separately from other clinical waste.

Human tissue samples must be tracked and recorded at all times.

Appropriate methods of destruction, storage and arrangements with people involved must be planned and arranged in advance, and considered when preparing risk assessments.

No human tissue may be transported from one establishment to another unless both establishments have full ethical approval from a recognised ethical approval service (REC) or are subject to an appropriate Human Tissue Authority (HTA) Licence.

Any recipient of transported tissue must be the subject of and compliant with an appropriate ethical approval or HTA licence.

5.2 Organs/tissue removed from the living

The Human Tissue Act 2004 makes it lawful to treat as 'waste' any relevant material which has come from a relevant person who was

- in the course of receiving medical treatment
- undergoing diagnostic testing or

- participating in research

Material no longer used, or stored for use, for any scheduled purpose can be dealt with as human tissue waste[1].

Material taken from the living should normally be disposed of by incineration in accordance with current guidelines[2]. Fetal tissue must be dealt with as per HTA advice[3].

5.3 Patients wishes

Some patients/donors may wish to retain tissue samples or make their own arrangements for disposal. Such requests should be considered on a case-by-case basis assessing the risk to the patient and others. Patients should be given sufficient information to allow them to make an informed decision.

5.4 Organs/tissues removed after death

Tissue and organs should be handled in accordance with any reasonable wishes expressed by relatives or the deceased person, as long as the method of disposal is legal. The time, place and method of disposal must be recorded.

5.5 Surplus material from tissue samples

Such material should be disposed of as human tissue waste. This includes:

[1] Cardiff University Guidance on the disposal of Hazardous Waste http://www.cf.ac.uk/osheu/environment/waste/envwaste.html
[2] Safe disposal of clinical waste, second edition, HSE books, 1999.
[3] Code of Practice – the removal, storage and disposal of human organs and tissue, Human Tissue Authority, 2006.

- tissue fragments trimmed from the tissue sample before it is processed
- tissue in the sections trimmed from a wax embedded block before the usable sections are cut and
- unrecoverable material that is washed out of tissue during its processing into a waxed block.

5.6 Existing holdings of unidentifiable, and identifiable but unclaimed, tissue

Detailed guidance on the disposal of such collections is available in Appendix A of HTA Code 5[4]

5.7 Protective equipment

Personal protective equipment (lab-coat, gloves) should be worn at all times when disposing of human tissue.

5.8 Incineration (witness burns)

Witness burns are carried out by the approved OSHEU vendor.

Material for disposal is identified on the database and marked appropriately. University staff place the material for disposal is sealable containers, which are then labelled correctly.

[4] Code of Practice – the removal, storage and disposal of human organs and tissue, Human Tissue Authority, 2006.

The material is then collected by the company carrying out the witness burn, supervised by the appropriate member of University staff (generally the Person Designated).

5.9 Cremation/Burial

Cremations and burials are carried out by the approved OSHEU vendor and the deceased is moved in a careful and respectful manner in conjunction with local procedures and as per any specifically stated wishes of the relatives of the deceased.

Appendix 6

Tables of microbiological cultural analysis of chronic wound samples

Sample	Blood plate bacteria/ml	MSA plate bacteria/ml	Pseudomonas plate/ml
CHR1	$2x10^5$	$9.7x10^5$	Unknown
CHR2	$2.2x10^7$	$1.0x10^6$	$6.3x10^6$
CHR3	$5.6x10^4$	$1.6x10^4$	$6.5x10^4$
CHR4	$1.7x10^8$	$2.4x10^7$	$3.4x10^9$
CHR5	$3.0x10^7$	$3.3x10^4$	$4.5x10^6$
CHR6	$8.0x10^6$	No growth White colonies from blood reincubated on MSA	$4.2x10^6$
CHR7	$7.1x10^4$	$3.6x10^3$	$1.02x10^3$
CHR8	$4x10^5$	$1.68x10^4$	$4.8x10^5$
CHR9	$1.6X10^6$	$7.2X10^4$	$1.6X10^6$
CH10	$1.11x10^7$	$2.3x10^6$	$7.8x10^6$

Table of bacterial counts for chronic wound samples

Sample	Blood plate appearance	MSA plate appearance	Pseudomonas plate appearance
CHR1	White colonies, yellow with haemolysis, P. aeruginosa like	Yellow, agar turned yellow	White colonies
CHR2	White colonies, yellow with haemolysis, P. aeruginosa like	White and off white- turns agar yellow	White colonies
CHR3	Grey/white	Yellow and White colonies	White colonies agar green
CHR4	Grey white with alpha haemolysis	White/yellow colonies- agar turned yellow	Whitecolonise -agar unchanged
CHR5	White pseudlike colonies	White colonies- agar turned yellow	white/yellow colonies
CHR6	Heavy growth of Pseuds, some small white colonies	no growth reincubated on msa – white colonies from blood agar	flourescent yellow/green colonies
CHR7	Proteus, small white and yellow colonies.	White and yellow colonies-agar-yellow	White colonies- agar fluorescent

CHR8	**White and clear colonies**	**White colonies- agar yellow**	**Flourescent green colonies**
CHR9	**White and clear**	**Clear and yellow**	**Flourescent green**
CH10	**White and clear**	**Yellow**	**Flourescent green**

Table of appeareances of CFUs of chronic wound fluid samples

Sample	**Plate**	**Tests**				
		Gram Stain	**Catalase**	**Oxidase**	**Coagulase**	**Conclusion**
CHR1	**MSA**	**White (W)=+dipplococci Yellow(Y)=+cocci**	**W positive Y positive**	**W negative Y negative**	**W positive Y negative**	**W= coryneforms Y=S. aureus**
	Pseud	**-rods**	**Positive**	**Positive**	**Negative**	**P.**

	omonas					aureginosa
CHR2	MSA	White(W)=+cocci Off White (OW)=+cocci	W positive OW positive	W negative OW negative	W negative OW negative	W=CNS OW=CNS
	Pseudomonas	-rods	Positive	Positive	Negative	P. aureginosa
CHR3	MSA	White(W)=+cocci Yellow(Y)=+cocci	W positive Y positive	W negative Y negative	W negative Y positive	W=CNS Y=S. aureus
	Pseudomonas	-rods	Positive	Positive	Negative	P. aureginosa
CHR4	MSA	White(W)=+cocci Yellow(Y)=+cocci	W positive Y positive	W negative Y negative	W negative Y negative	W=CNS Y=S. aureus
	Pseudomonas	-rods	Positive	Positive	Negative	P. aureginosa

CHR5	**MSA**	**+cocci**	**Positive**	**Negative**	**Postive**	**S. aureus**
	Pseudomonas	**-rods**	**Positive**	**Positive**	**Negative**	**P. aureginosa**
CHR6	**MSA**	**+cocci**	**Positive**	**Negative**	**Positive**	**CNS**
	Pseudomonas	**-rods**	**Positive**	**Positive**	**Negative**	**P. aureginosa**
CHR7	**MSA**	**White(W)=+cocci Pale(P)=+cocci**	**W positive P negative**	**W negative P negative**	**W negative P negative**	**2 strains of CNS**
	Pseudomonas	**-rods**	**Positive**	**Positive**	**Negative**	**P. aureginosa**
CHR8	**MSA**	**+cocci**	**Positive**	**Negative**	**Negative**	**CNS**
	Pseudomonas	**-rods**	**Positive**	**Positive**	**Negative**	**P. aureginosa**
CHR9	**MSA**	**Clear (C)=+rods Yellow(Y)=+cocci**	**C positive Y positive**	**C negative Y negative**	**C negative Y positive**	**C= coryneforms Y=S. aureus**

	Pseudomonas	-rods	Positive	Positive	Negative	P. aureginosa
CHR10	MSA	+cocci	Positive	Negative	Negative	CNS
	Pseudomonas	-rods	Positive	Positive	Negative	P. aureginosa

Table of chronic wound fluid sample derived colonies demonstrating classical microbiological tests and conclusions of likely bacteria present.

Appendix 7

Table of Microbial isolate storage samples

Storage no.	Chronic 1	Yellow	SA
2	Chronic 1	White	Coryneform
3	Chronic 1	Pseud	Pseud
4	Chronic 2	White	CNS
5	Chronic 2	Off White	CNS
6	Chronic 3	Yellow	SA
7	Chronic 5	Pseud	Pseud
8	Chronic 6	clear	CNS
9	Chronic 6	Pseud	Pseud
10	Chronic 7	Pseud	Pseud
11	Chronic 7	Yellow	CNS
12	Chronic 8	Pseud	Pseud
13	Chronic 8	clear	CNS
14	Chronic 9	Yellow	SA
15	Chronic 9	clear	Coryneform
16	Chronic 9	Pseud	Pseud
17	Acute 1	clear	CNS
18	Acute 1	Pale	CNS
19	Acute 2	White	SA
20	Acute 2	Yellow	SA

21	**Acute 3**	**White**	**CNS**
22	**Acute 3**	**Pale**	**CNS**
23	**Acute 5**	**White**	**SA**
24	**Acute 5**	**Yellow**	**CNS**
25	**Acute 6**	**Pale**	**CNS**
26	**Acute 7**	**White**	**CNS**
27	**Acute 7**	**White**	**CNS**
28	**WD4**	**white**	**pseud**
29	**WD4**	**yellow**	**CNS**
30	**WD6**	**White**	**Pseud**
31	**WD6**	**White**	**CNS**
32	**WD7**	**White**	**CNS**
33	**WD8**	**White**	**Pseud**
34	**WD8**	**Yellow**	**CNS**
35	**WD5**	**proteus swarm**	**proteus**
36	**WD7**	**proteus swarm**	**proteus**

Appendix 8
Data for ELISAs Chapter 4

Data for Figure 4.10

ng/ml	OD1	OD2	Ave	SD	Blank corrected
10000	3.566	3.598	3.582	0.023	3.336
5000	3.867	3.712	3.790	0.110	3.544
2500	3.633	3.633	3.633	0.000	3.387
1250	3.598	3.482	3.540	0.082	3.294
625	3.758	3.508	3.633	0.177	3.387
312.5	3.482	3.370	3.426	0.079	3.180
156	3.598	3.434	3.516	0.117	3.270
78	3.411	3.297	3.354	0.081	3.108
39	3.181	3.121	3.151	0.042	2.905
20	2.913	2.820	2.867	0.066	2.621
9.8	2.605	2.557	2.581	0.034	2.335
4.9	2.198	1.889	2.043	0.218	1.797
2.44	1.666	1.515	1.590	0.107	1.344
1.22	1.215	1.069	1.142	0.104	0.896
0.61	0.950	0.791	0.870	0.112	0.624
0.31	0.703	0.559	0.631	0.102	0.385
0.2	0.557	0.429	0.493	0.091	0.247
0.076	0.416	0.382	0.399	0.024	0.153
0.038	0.371	0.348	0.359	0.017	0.113
0.019	0.346	0.334	0.340	0.009	0.094
0.0095	0.318	0.295	0.307	0.016	0.061
0.0047	0.297	0.285	0.291	0.009	0.045

ng/ml	OD1	OD2	Ave	SD	Blank corrected
10000	3.314	3.314	3.314	0.00	3.075
5000	3.350	3.457	3.403	0.075	3.164
2500	3.314	3.314	3.314	0.00	3.075
1250	3.281	3.250	3.265	0.022	3.026
625	3.099	3.168	3.133	0.049	2.894
312.5	2.913	2.988	2.950	0.053	2.711
156	2.396	2.502	2.449	0.075	2.210
78	1.743	1.880	1.811	0.097	1.572
39	1.220	1.350	1.285	0.092	1.046
19.5	0.868	0.892	0.880	0.017	0.641
9.8	0.586	0.613	0.599	0.018	0.360
4.88	0.407	0.400	0.403	0.004	0.164
2.44	0.325	0.328	0.327	0.002	0.088
1.22	0.307	0.273	0.290	0.024	0.051
0.61	0.277	0.280	0.278	0.002	0.039
0.3	0.263	0.257	0.260	0.004	0.021
0.15	0.253	0.238	0.246	0.011	0.007
0.08	0.232	0.242	0.237	0.007	0.000
0.04	0.251	0.242	0.247	0.007	0.000
0.02	0.243	0.239	0.241	0.003	0.000
0.01	0.240	0.243	0.241	0.002	0.000
0.005	0.248	0.229	0.239	0.014	0.000

Data for Fig 4.11
Blank
0.085
0.091
0.085
0.094
0.086
0.087
0.096
0.087 0.088875

FRI115M

1st Run	2nd Run	Ave	SD	Blank corrected
0.843	0.803	0.823	0.028	0.733
1.983	2.037	2.010	0.038	1.920
2.264	2.266	2.265	0.001	2.175
2.353	2.357	2.355	0.003	2.265
2.36	2.367	2.364	0.005	2.274
2.379	2.397	2.388	0.013	2.298
2.41	2.410	2.410	0.000	2.320
2.401	2.404	2.403	0.002	2.313
2.387	2.382	2.385	0.004	2.295
2.39	2.374	2.382	0.011	2.292
2.389	2.368	2.379	0.015	2.289

SaB2

1st Run	2nd Run	Ave	SD	Blank corrected
1.578	1.532	1.555	0.033	1.465
2.275	2.284	2.280	0.006	2.190
2.305	2.330	2.318	0.018	2.228
2.383	2.389	2.386	0.004	2.296
2.401	2.399	2.400	0.001	2.310
2.386	2.404	2.395	0.018	2.305
2.385	2.393	2.389	0.006	2.299
2.384	2.407	2.396	0.016	2.306
2.422	2.390	2.406	0.023	2.316
2.364	2.390	2.377	0.018	2.287
2.387	2.370	2.379	0.012	2.289

TNase

1st Run	2nd Run	Ave	SD	Blank corrected
0.327	0.312	0.320	0.011	0.230
0.632	0.626	0.629	0.004	0.539
0.975	1.046	1.011	0.050	0.921
1.549	1.622	1.586	0.052	1.496
1.939	2.074	2.007	0.095	1.917
2.207	2.265	2.236	0.041	2.146
2.302	2.306	2.304	0.003	2.214
2.358	2.338	2.348	0.014	2.258
2.345	2.347	2.346	0.001	2.256
2.361	2.355	2.358	0.004	2.268
2.335	2.336	2.336	0.001	2.246

No competition

1st Run	2nd Run	Ave	SD	Blank corrected
2.319	2.297	2.308	0.016	2.218
2.317	2.330	2.324	0.009	2.234
2.336	2.322	2.329	0.010	2.239
2.346	2.356	2.351	0.007	2.261
2.34	2.326	2.333	0.010	2.243
2.34	2.341	2.340	0.001	2.251
2.34	2.333	2.337	0.005	2.247
2.329	2.346	2.338	0.012	2.248
2.374	2.337	2.356	0.026	2.266
2.333	2.277	2.305	0.040	2.215
2.294	2.290	2.292	0.003	2.202

Data for figure 4.12
Blank
0.227
0.224
0.230
0.209
0.238
0.244
0.250
0.225 0.23105

FRI115M spt

1st Run	2nd Run	Ave	SD	Blank corrected
0.476	0.509	0.493	0.024	0.263
1.277	1.384	1.330	0.076	1.100
1.411	1.521	1.466	0.078	1.236
1.516	1.529	1.523	0.009	1.293
1.510	1.573	1.541	0.045	1.311
1.631	1.621	1.626	0.007	1.396
1.649	1.700	1.674	0.036	1.444
1.654	1.681	1.667	0.019	1.437
1.681	1.681	1.681	0.000	1.451
1.666	1.714	1.690	0.037	1.460
1.413	1.445	1.429	0.023	1.412

FRI 115M spt and cells

1st Run	2nd Run	Ave	SD	Blank corrected
0.342	0.350	0.346	0.006	0.116
1.019	1.124	1.071	0.074	0.841
1.360	1.388	1.374	0.020	1.144
1.521	1.503	1.512	0.013	1.282
1.604	1.532	1.568	0.051	1.338
1.611	1.622	1.616	0.008	1.386
1.686	1.665	1.675	0.015	1.445
1.680	1.691	1.685	0.008	1.455
1.685	1.666	1.676	0.014	1.446
1.675	1.686	1.680	0.008	1.450
1.368	1.380	1.374	0.009	1.420

Pseudomonas spt

1st Run	2nd Run	Ave	SD	Blank corrected
1.396	1.598	1.497	0.142	1.401
1.602	1.305	1.454	0.210	1.420
1.576	1.614	1.595	0.027	1.460
1.622	1.627	1.624	0.003	1.394
1.656	1.725	1.690	0.049	1.460
1.701	1.793	1.747	0.065	1.517
1.733	1.808	1.771	0.053	1.541
1.743	1.732	1.737	0.007	1.507
1.745	1.743	1.743	0.002	1.514
1.713	1.718	1.715	0.004	1.485
1.452	1.401	1.426	0.036	1.382

No competitor (TNase B only)

1st Run	2nd Run	Ave	SD	Blank corrected
1.666	1.328	1.497	0.239	1.562
1.683	1.663	1.673	0.014	1.658
1.637	1.699	1.668	0.044	1.562
1.727	1.881	1.804	0.109	1.658
1.713	1.872	1.792	0.113	1.562
1.799	1.977	1.888	0.125	1.658
1.724	2.026	1.875	0.214	1.645
1.754	2.010	1.882	0.181	1.652
1.793	1.841	1.817	0.035	1.587
1.777	1.930	1.853	0.108	1.623
1.474	1.652	1.563	0.126	1.616

FRI115M data 1-5 hours
Blank
0.299
0.286
0.313
0.330
0.328
0.023
0.034
0.288 0.23775

0 hours

1st Run	2nd Run	Ave	SD	Blank corrected
2.185	2.164	2.175	0.015	1.938
2.230	3.061	2.645	0.587	2.408
2.262	2.650	2.456	0.274	2.219
2.486	2.562	2.524	0.053	2.287
0.970	2.159	1.565	0.841	1.328

1 hour

1st Run	2nd Run	Ave	SD	Blank corrected
1.909	2.863	2.386	0.675	2.149
2.574	2.811	2.693	0.167	2.456
2.574	2.422	2.498	0.108	2.261
2.325	2.510	2.418	0.131	2.181
2.457	2.505	2.481	0.034	2.244

2 hours

1st Run	2nd Run	Ave	SD	Blank corrected
2.689	3.123	2.906	0.307	2.669
2.741	3.600	3.170	0.608	2.934
2.857	3.413	3.135	0.393	2.898
2.895	3.134	3.015	0.170	2.778
2.370	2.685	2.527	0.223	2.290

3 hours

1st Run	2nd Run	Ave	SD	Blank corrected
3.158	2.863	3.010	0.209	2.773
2.966	2.863	2.914	0.073	2.677
2.440	2.665	2.552	0.159	2.315
2.584	2.459	2.521	0.088	2.284
2.281	2.301	2.291	0.014	2.054

4 hours

1st Run	2nd Run	Ave	SD	Blank corrected
2.701	1.832	2.266	0.615	2.029
3.538	2.982	3.260	0.393	3.023
3.101	2.646	2.874	0.322	2.637
2.943	3.042	2.993	0.070	2.756
2.746	2.839	2.792	0.066	2.555

5 hours

1st Run	2nd Run	Ave	SD	Blank corrected
3.033	3.015	3.024	0.0125	2.787
2.577	2.565	2.571	0.0088	2.334
2.502	2.454	2.478	0.0339	2.241
2.454	2.505	2.479	0.0357	2.242
2.322	2.120	2.221	0.1426	1.984

No competition

1st Run	2nd Run	Ave	SD	Blank corrected
3.484	3.869	3.676	0.273	3.439
3.123	3.568	3.346	0.315	3.109
3.267	3.299	3.283	0.023	3.046
3.081	3.316	3.198	0.167	2.961
2.990	3.252	3.121	0.185	2.884

Repeat of FRI115M Data 0-5 hours

Blank
0.332
0.316
0.372
0.381
0.290
0.333
0.358
0.472 0.3566

0 hours

1st Run	2nd Run	Ave	SD	Blank corrected
2.890	2.691	2.791	0.141	2.434
3.104	3.093	3.098	0.007	2.742
3.254	3.054	3.154	0.141	2.797
3.486	3.073	3.279	0.292	2.923
3.570	3.254	3.412	0.224	3.055

1 hour

1st Run	2nd Run	Ave	SD	Blank corrected
3.637	3.438	3.537	0.141	3.181
3.602	3.570	3.586	0.023	3.230
3.461	3.570	3.516	0.077	3.159
2.583	3.254	2.918	0.475	2.562
1.280	3.093	2.187	1.282	1.830

2 hours

1st Run	2nd Run	Ave	SD	Blank corrected
2.663	2.835	2.749	0.122	2.392
2.968	3.225	3.097	0.182	2.740
2.656	3.239	2.947	0.413	2.591
2.984	3.355	3.169	0.262	2.813
3.225	3.254	3.239	0.021	2.883

3 hours

1st Run	2nd Run	Ave	SD	Blank corrected
3.301	3.570	3.4357	0.190	3.079
3.239	3.336	3.28755	0.066	2.930
3.318	3.374	3.34605	0.039	2.989
3.083	3.269	3.17595	0.132	2.819
3.148	3.336	3.24205	0.133	2.885

4 hours

1st Run	2nd Run	Ave	SD	Blank corrected
2.984	2.802	2.893	0.128	2.537
2.910	3.083	2.996	0.122	2.640
3.374	3.225	3.299	0.105	2.943
3.637	3.540	3.589	0.069	3.232
3.570	3.318	3.444	0.178	3.088

5 hours

1st Run	2nd Run	Ave	SD	Blank corrected
3.512	3.301	3.407	0.149	3.050
3.239	3.000	3.120	0.169	2.763
3.301	3.083	3.192	0.155	2.835
3.301	3.285	3.293	0.012	2.937
3.486	3.301	3.393	0.130	3.037

No competition

1st Run	2nd Run	Ave	SD	Blank corrected
3.415	3.486	3.450	0.049	3.094
2.859	2.945	2.902	0.061	2.545
2.976	3.063	3.019	0.062	2.663
3.318	3.486	3.402	0.118	3.045
3.486	3.637	3.561	0.107	3.205

FRI115M data 6-11 hours

Blank
0.307
0.325
0.356
0.326
0.338
0.336
0.348
0.328 0.333075

6 hours

1st Run	2nd Run	Ave	SD	Blank corrected
2.486	2.419	2.452	0.047	2.119
2.835	3.336	3.086	0.354	2.753
3.026	3.570	3.298	0.385	2.965
3.637	3.675	3.656	0.027	3.323
3.136	3.486	3.311	0.247	2.978

7 hours

1st Run	2nd Run	Ave	SD	Blank corrected
2.435	2.633	2.534	0.140	2.201
2.633	2.853	2.743	0.155	2.410
2.006	2.160	2.083	0.109	1.750
2.044	1.727	1.885	0.224	1.552
2.210	1.957	2.083	0.178	1.750

8 hours

1st Run	2nd Run	Ave	SD	Blank corrected
3.301	3.269	3.285	0.023	2.952
2.787	3.239	3.013	0.320	2.680
3.336	3.336	3.336	0	3.003
3.762	3.637	3.699	0.088	3.366
3.285	3.461	3.373	0.125	3.040

9 hours

1st Run	2nd Run	Ave	SD	Blank corrected
2.748	2.762	2.755	0.010	2.422
3.000	2.637	2.818	0.257	2.485
2.251	2.227	2.239	0.016	1.906
1.749	1.849	1.799	0.070	1.466
1.850	1.933	1.892	0.058	1.559

10 hours

1st Run	2nd Run	Ave	SD	Blank corrected
3.008	3.125	3.067	0.082	2.734
3.114	3.017	3.065	0.069	2.732
3.285	3.336	3.310	0.036	2.977
3.570	3.871	3.720	0.213	3.387
3.225	3.354	3.289	0.092	2.956

11 hours

1st Run	2nd Run	Ave	SD	Blank corrected
2.299	2.411	2.355	0.079	2.022
2.198	Nil	2.198	Nil	1.865
2.005	2.099	2.052	0.066	1.719
1.784	1.740	1.762	0.031	1.429
1.707	1.606	1.657	0.072	1.324

No competition

1st Run	2nd Run	Ave	SD	Blank corrected
3.637	3.461	3.549	0.125	3.216
2.896	3.160	3.028	0.186	2.695
3.318	2.687	3.002	0.446	2.669
3.813	3.762	3.787	0.036	3.454
3.160	3.254	3.207	0.066	2.874

Repeat of FRI115M Data 6-11 hours

Blank
0.304
0.288
0.337
0.366
0.306
0.293
0.322
0.285 0.31275

6 hours

1st Run	2nd Run	Ave	SD	Blank corrected
0.028	2.915	1.4712	2.041275856	1.1592
0.022	3.600	1.81105	2.529957352	1.49905
2.544	3.392	2.9677	0.59962655	2.6557
2.908	3.811	3.35925	0.638588134	3.04725
3.600	3.635	3.61735	0.024536605	3.30535

7 hours

1st Run	2nd Run	Ave	SD	Blank corrected
3.760	3.673	3.7161	0.061659711	3.4041
3.869	3.600	3.7344	0.190070303	3.4224
3.760	3.673	3.7161	0.061659711	3.4041
3.714	3.600	3.65695	0.080539462	3.34495
3.413	3.568	3.49035	0.10953084	3.17835

8 hours

1st Run	2nd Run	Ave	SD	Blank corrected
3.237	3.352	3.2945	0.081600123	2.9825
3.352	3.352	3.3522	0	3.0402
3.237	3.635	3.43575	0.281357788	3.12375
3.635	3.635	3.6347	0	3.3227
3.635	3.568	3.60125	0.047305444	3.28925

9 hours

1st Run	2nd Run	Ave	SD	Blank corrected
3.568	3.568	3.5678	0	3.2558
3.600	3.568	3.5839	0.022768838	3.2719
3.568	3.484	3.52565	0.059609102	3.21365
3.484	3.372	3.4275	0.079195959	3.1155
3.538	3.252	3.39465	0.202444671	3.08265

10 hours

1st Run	2nd Run	Ave	SD	Blank corrected
3.195	3.283	3.239	0.061659711	2.927
3.538	3.568	3.5528	0.021213203	3.2408
3.714	3.673	3.6932	0.029274221	3.3812
3.811	3.635	3.72275	0.124521504	3.41075
3.635	3.538	3.58625	0.068518647	3.27425

11 hours

1st Run	2nd Run	Ave	SD	Blank corrected
3.352	3.372	3.36185	0.013647161	3.04985
3.392	3.372	3.3816	0.014283557	3.0696
3.538	3.568	3.5528	0.021213203	3.2408
3.510	3.568	3.5388	0.041012193	3.2268
3.538	3.435	3.4865	0.072549156	3.1745

No competition

1st Run	2nd Run	Ave	SD	Blank corrected
4.112	3.435	3.77355	0.478499159	3.46155
3.673	3.936	3.80415	0.186181215	3.49215
3.936	3.811	3.8733	0.088388348	3.5613
3.811	3.352	3.5815	0.32427917	3.2695
3.760	3.600	3.67985	0.112924953	0.55985

FRI115M data 12-24 hours

Blank
0.328
0.347
0.349
0.375
0.409
0.367
0.335
0.357 0.3582375

12 hours

1st Run	2nd Run	Ave	SD	Blank corrected
1.515	0.848	1.18175	0.471710934	0.82375
1.695	1.946	1.82035	0.177554513	1.46235
2.393	2.832	2.6126	0.31070272	2.2546
3.195	3.208	3.20125	0.00940452	2.84325
2.200	2.451	2.32525	0.17727167	1.96725

16 hours

1st Run	2nd Run	Ave	SD	Blank corrected
0.568	0.640	0.6043	0.050770267	0.2463
0.781	0.995	0.8877	0.151462273	0.5297
0.739	0.806	0.7727	0.047658997	0.4147
0.864	0.887	0.87565	0.016617009	0.51765
1.187	1.097	1.14175	0.063286057	0.78375

24 hours

1st Run	2nd Run	Ave	SD	Blank corrected
0.027	0.023	0.025	0.002969848	-0.333
0.560	0.649	0.6043	0.063356768	0.2463
1.250	1.119	1.18475	0.09284312	0.82675
1.908	1.709	1.8084	0.140289985	1.4504
1.759	1.532	1.6457	0.160654661	1.2877

No competition

1st Run	2nd Run	Ave	SD	Blank corrected
3.637	3.461	3.54875	0.124521504	3.19075
2.896	3.160	3.02805	0.186181215	2.67005
3.318	2.687	3.0023	0.446467222	2.6443
3.813	3.762	3.7873	0.036203867	3.4293
3.160	3.254	3.20665	0.066397327	2.84865

Repeat of FRI115M data 12-24 hours
Blank
0.312
0.321
0.436
0.334
0.370
0.348
0.301
0.310 0.3415375

12 hours

1st Run	2nd Run	Ave	SD	Blank corrected
1.708	1.815	1.76175	0.075448294	1.42075
2.689	2.806	2.7475	0.082590072	2.4065
3.413	3.413	3.4134	0	3.0724
3.635	3.635	3.6352	0	3.2942
3.635	3.760	3.6977	0.088388348	3.3567

16 hours

1st Run	2nd Run	Ave	SD	Blank corrected
0.796	0.807	0.80115	0.007707464	0.46015
1.374	1.542	1.45805	0.11886465	1.11705
1.759	1.332	1.54535	0.30242957	1.20435
2.085	1.184	1.6345	0.63710321	1.2935
0.921	0.887	0.904	0.024465895	0.563

24 hours

1st Run	2nd Run	Ave	SD	Blank corrected
0.562	0.556	0.5586	0.004242641	0.2176
1.103	1.103	1.10315	0.000353553	0.76215
1.634	1.707	1.67045	0.051265242	1.32945
2.535	2.562	2.54875	0.018879751	2.20775
2.895	2.929	2.912	0.024183052	2.571

No competition

1st Run	2nd Run	Ave	SD	Blank corrected
3.869	4.016	3.9424	0.103379011	3.6014
3.760	4.016	3.88785	0.180524361	3.54685
3.760	3.936	3.84825	0.124521504	3.50725
3.538	3.869	3.7038	0.234052345	3.3628
3.317	3.811	3.5639	0.349876435	3.2229

Data for Fig 4.18
Blank
0.225
0.226
0.233
0.218
0.246
0.252
0.254
0.231 0.2356375

Wound fluid vs TNase

1st Run	2nd Run	Ave	SD	Blank corrected
1.781	1.775	1.77805	0.004596194	1.54305
1.850	1.790	1.8199	0.042992092	1.5849
1.846	1.873	1.8592	0.019233304	1.6242
1.911	1.807	1.85915	0.073892659	1.62415
1.916	1.886	1.90085	0.021708178	1.66585
2.024	1.827	1.9255	0.139017193	1.6905
2.024	2.006	2.0147	0.012869343	1.7797
1.997	1.935	1.96585	0.044335595	1.73085
2.041	2.005	2.0228	0.025597265	1.7878
1.967	2.013	1.99035	0.032456201	1.75535
2.026	2.049	2.03785	0.016192745	1.80285

Wound fluid spike (1:1) vs TNase

1st Run	2nd Run	Ave	SD	Blank corrected
1.908	1.187	1.12455	0.08860048	0.88955
2.249	1.479	1.4788	#DIV/0!	1.2438
2.382	1.612	1.6737	0.087681241	1.4387
2.553	1.699	1.7692	0.099843478	1.5342
2.556	1.682	1.7486	0.093903781	1.5136
2.443	1.670	1.74605	0.107692363	1.51105
2.591	1.726	1.78075	0.077711035	1.54575
2.497	1.771	1.8004	0.041153615	1.5654
2.471	1.811	1.83985	0.040517219	1.60485
2.594	1.812	1.8535	0.059114127	1.6185
2.611	1.906	1.8872	0.026445794	1.6522

Wound fluid spiked (1:2) vs TNase

1st Run	2nd Run	Ave	SD	Blank corrected
1.031	0.914	0.97245	0.082943625	0.73745
1.431	1.362	1.3964	0.048790368	1.1614
1.400	1.559	1.47925	0.112217846	1.24425
1.595	1.645	1.62005	0.035567471	1.38505
1.693	1.789	1.74065	0.067670119	1.50565
1.697	1.714	1.7057	0.011737973	1.4707
1.747	1.877	1.812	0.092206724	1.577
1.733	1.746	1.73965	0.00940452	1.50465
1.767	1.776	1.77185	0.00629325	1.53685
1.781	1.819	1.8001	0.026587215	1.5651
1.754	1.912	1.833	0.111864293	1.598

S.aureus spt vs TNase

1st Run	2nd Run	Ave	SD	Blank corrected
0.812	0.803	0.8076	0.007	0.573
1.376	1.311	1.3436	0.046	1.109
1.561	1.639	1.5998	0.055	1.365
1.736	1.723	1.7297	0.009	1.495
1.770	1.839	1.80445	0.048	1.569
1.862	1.781	1.8216	0.058	1.587
1.918	1.915	1.9162	0.002	1.681
1.806	1.780	1.7928	0.018	1.558
1.886	1.847	1.8665	0.028	1.632
1.930	1.915	1.9225	0.011	1.688
1.964	2.031	1.99725	0.048	1.762

No competition

1st Run	2nd Run	Ave	SD	Blank corrected
2.189	2.125	2.157	0.045	1.922
2.213	2.174	2.194	0.027	1.959
2.018	2.047	2.032	0.025	1.797
2.058	1.957	2.008	0.072	1.773
1.894	1.924	1.910	0.021	1.674
2.065	1.964	2.015	0.072	1.780
2.121	2.085	2.103	0.026	1.868
2.193	2.068	2.131	0.089	1.896
2.189	1.957	2.073	0.164	1.838
2.213	1.924	2.069	0.205	1.834
2.018	1.964	1.991	0.038	1.756

Repeat of Data for fig 4.18

Blank
0.240
0.235
0.247
0.251
0.256
0.240
0.216
0.226 0.2388375

Wound fluid vs TNase

1st Run	2nd Run	Ave	SD	Blank corrected
1.222	1.245	1.234	0.016	0.996
1.220	1.258	1.239	0.027	1.001
1.277	1.282	1.279	0.003	1.041
1.299	1.252	1.275	0.033	1.037
1.221	1.218	1.220	0.003	0.982
1.240	1.268	1.254	0.020	1.016
1.269	1.270	1.270	0.001	1.032
1.241	1.257	1.249	0.012	1.011
1.243	1.269	1.256	0.018	1.018
1.236	1.281	1.259	0.032	1.021
1.257	1.267	1.262	0.007	1.024

Wound fluid spike (1:1) vs TNase

1st Run	2nd Run	Ave	SD	Blank corrected
0.929	0.971	0.060	0.733	0.733
1.149	1.138	1.143	0.008	0.905
1.235	1.215	1.225	0.014	0.987
1.241	1.240	1.240	0.001	1.002
1.281	1.218	1.249	0.044	1.011
1.275	1.216	1.246	0.041	1.008
1.255	1.257	1.256	0.001	1.018
1.271	1.279	1.275	0.006	1.037
1.211	1.209	1.210	0.002	0.971
1.167	1.204	1.186	0.026	0.948
1.181	1.209	1.195	0.020	0.957

Wound fluid spiked (1:2) vs TNase

1st Run	2nd Run	Ave	SD	Blank corrected
0.832	0.861	0.846	0.020	0.608
0.952	0.947	0.949	0.004	0.711
1.082	1.041	1.061	0.029	0.823
1.083	1.085	1.084	0.001	0.846
1.054	1.173	1.113	0.084	0.875
1.197	1.282	1.240	0.060	1.002
1.209	1.285	1.247	0.054	1.009
1.217	1.252	1.234	0.025	0.996
1.220	1.235	1.228	0.011	0.990
1.215	1.220	1.217	0.004	0.979
1.201	1.192	1.196	0.007	0.958

S.aureus spt vs TNase

1st Run	2nd Run	Ave	SD	Blank corrected
0.563	0.558	0.560	0.003	0.323
0.836	0.866	0.850	0.021	0.613
0.990	0.997	0.993	0.005	0.755
1.046	1.105	1.075	0.042	0.837
1.127	1.121	1.124	0.004	0.886
1.160	1.205	1.182	0.032	0.944
1.217	1.269	1.243	0.037	1.005
1.233	1.273	1.253	0.028	1.015
1.224	1.290	1.257	0.046	1.019
1.220	1.296	1.258	0.054	1.020
1.233	1.325	1.279	0.065	1.041

No competition

1st Run	2nd Run	Ave	SD	Blank corrected
1.236	1.257	1.246	0.0150	1.008
1.281	1.267	1.274	0.0102	1.036
1.167	1.181	1.174	0.009	0.936
1.204	1.209	1.206	0.003	0.968
1.215	1.201	1.208	0.009	0.970
1.220	1.192	1.206	0.020	0.968
1.220	1.233	1.226	0.009	0.988
1.296	1.325	1.311	0.020	1.073
1.236	1.257	1.246	0.0150	1.008
1.281	1.267	1.274	0.0102	1.036
1.167	1.181	1.174	0.009	0.936

Data for Fig 4.20

Blank
0.2544
0.2478
0.2722
0.2743
0.2929
0.2761
0.2442
0.2533 0.2644

Acute 3b

1st Run	2nd Run	Ave	SD	Blank corrected
1.067	1.089	1.078	0.016	0.814
1.240	1.218	1.229	0.015	0.965
1.375	1.401	1.388	0.019	1.124
1.463	1.480	1.471	0.012	1.207
1.623	1.582	1.602	0.029	1.338
1.676	1.652	1.664	0.017	1.400
1.736	1.745	1.741	0.007	1.477
1.807	1.760	1.783	0.033	1.519
1.736	1.854	1.795	0.084	1.531
1.769	1.736	1.753	0.023	1.489
1.844	1.792	1.818	0.037	1.554

Acute 4a

1st Run	2nd Run	Ave	SD	Blank corrected
1.185	1.237	1.211	0.037	0.947
1.397	1.458	1.428	0.043	1.164
1.530	1.557	1.543	0.007	1.279
1.551	1.583	1.567	0.023	1.303
1.649	1.604	1.626	0.032	1.362
1.715	1.724	1.719	0.006	1.455
1.758	1.730	1.744	0.020	1.480
1.773	1.666	1.720	0.076	1.456
1.689	1.697	1.693	0.005	1.429
1.702	1.685	1.694	0.012	1.430
1.739	1.727	1.733	0.009	1.469

Acute 2b

1st Run	2nd Run	Ave	SD	Blank corrected
1.231	1.179	1.205	0.037	0.941
1.217	1.116	1.167	0.072	0.903
1.352	1.270	1.311	0.058	1.047
1.440	1.424	1.432	0.011	1.168
1.599	1.519	1.559	0.057	1.295
1.758	1.719	1.738	0.027	1.474
1.763	1.787	1.775	0.017	1.511
1.727	1.773	1.750	0.033	1.486
1.743	1.726	1.735	0.012	1.471
1.735	1.757	1.746	0.016	1.482
1.785	1.760	1.776	0.014	1.512

TNase line

1st Run	2nd Run	Ave	SD	Blank corrected
0.302	0.296	0.299	0.004	0.035
0.367	0.377	0.372	0.007	0.108
0.239	0.436	0.337	0.139	0.073
0.515	0.499	0.507	0.011	0.243
0.920	0.952	0.936	0.023	0.672
1.138	1.225	1.182	0.061	0.918
1.225	1.320	1.272	0.067	1.008
1.368	1.451	1.409	0.058	1.145
1.442	1.492	1.467	0.035	1.203
1.689	1.700	1.695	0.008	1.430
1.721	1.701	1.711	0.014	1.447

Data for Fig 4.21 and 4.22

Blank
0.221
0.249
0.237
0.257
0.248
0.272
0.246
0.243 0.2465125

Chronic 1

1st Run	2nd Run	Ave	SD	Blank corrected
1.502	1.385	1.444	0.0826	1.204
1.611	1.493	1.552	0.0841	1.312
1.579	1.465	1.522	0.0805	1.282
1.504	1.500	1.502	0.0025	1.262
1.520	1.513	1.516	0.0050	1.276
1.502	1.415	1.459	0.0615	1.219
1.502	1.390	1.446	0.0790	1.206
1.439	1.421	1.430	0.0126	1.190
1.516	1.530	1.523	0.0098	1.283
1.508	1.483	1.496	0.0180	1.256
1.542	1.434	1.488	0.0767	1.248

Chronic 2

1st Run	2nd Run	Ave	SD	Blank corrected
1.370	1.315	1.342	0.040	1.102
1.417	1.354	1.386	0.045	1.146
1.377	1.284	1.331	0.007	1.091
1.486	1.360	1.423	0.089	1.183
1.602	1.349	1.475	0.179	1.235
1.442	1.396	1.419	0.033	1.179
1.387	1.365	1.376	0.016	1.136
1.288	1.292	1.290	0.002	1.050
1.374	1.365	1.370	0.006	1.130
1.424	1.374	1.399	0.036	1.159
1.319	1.339	1.329	0.014	1.089

Chronic 3

1st Run	2nd Run	Ave	SD	Blank corrected
1.142	1.176	1.159	0.024	0.919
1.249	1.272	1.261	0.016	1.021
1.301	1.373	1.337	0.052	1.097
1.348	1.402	1.375	0.038	1.135
1.406	1.454	1.430	0.034	1.190
1.395	1.467	1.431	0.051	1.191
1.319	1.454	1.387	0.095	1.147
1.357	1.372	1.364	0.011	1.124
1.377	1.437	1.407	0.043	1.167
1.386	1.392	1.389	0.004	1.149
1.326	1.357	1.341	0.021	1.101

TNase line

1st Run	2nd Run	Ave	SD	Blank corrected
0.372	0.296	0.334	0.054	0.094
0.471	0.380	0.425	0.064	0.185
0.697	0.725	0.711	0.020	0.471
0.952	0.954	0.953	0.002	0.713
1.108	1.236	1.172	0.090	0.932
1.261	1.323	1.292	0.044	1.052
1.177	1.365	1.271	0.133	1.031
1.331	1.348	1.339	0.012	1.099
1.394	1.434	1.414	0.028	1.174
1.403	1.401	1.402	0.001	1.162
1.394	1.407	1.401	0.009	1.160

Blank
0.235
0.255
0.237
0.249
0.238
0.265
0.232
0.231 0.2426875

Chronic 4

1st Run	2nd Run	Ave	SD	Blank corrected
1.546	1.623	1.58425	0.054517933	1.34425
1.619	1.637	1.6282	0.012586501	1.3882
1.759	1.677	1.71805	0.058336309	1.47805
1.697	1.737	1.71675	0.028213561	1.47675
1.649	1.671	1.65955	0.015485639	1.41955
1.606	1.667	1.63635	0.042638539	1.39635
1.658	1.582	1.62025	0.053952247	1.38025
1.599	1.662	1.6305	0.044406306	1.3905
1.554	1.631	1.5925	0.053881537	1.3525
1.528	1.642	1.5848	0.080185909	1.3448
1.625	1.564	1.59415	0.042921382	1.35415

Chronic 5

1st Run	2nd Run	Ave	SD	Blank corrected
1.663	1.575	1.6192	0.061942554	1.3792
1.710	1.694	1.70215	0.011667262	1.46215
1.522	1.617	1.5692	0.006858936	1.3292
1.686	1.606	1.64615	0.056497832	1.40615
1.708	1.566	1.637	0.100833427	1.397
1.620	1.565	1.5927	0.03860803	1.3527
1.565	1.545	1.5551	0.0145664	1.3151
1.540	1.604	1.5721	0.045254834	1.3321
1.632	1.612	1.62235	0.014212846	1.38235
1.598	1.595	1.5966	0.001697056	1.3566
1.552	1.548	1.5499	0.002969848	1.3099

Tris buffered saline

1st Run	2nd Run	Ave	SD	Blank corrected
1.418	1.606	1.51205	0.132582521	1.27205
1.606	1.657	1.63155	0.036415999	1.39155
1.652	1.517	1.5846	0.095742258	1.3446
1.691	1.507	1.59865	0.130036937	1.35865
1.554	1.547	1.5505	0.005091169	1.3105
1.619	1.566	1.5928	0.037476659	1.3528
1.556	1.524	1.54015	0.022698128	1.30015
1.602	1.595	1.59845	0.00516188	1.35845
1.558	1.631	1.5945	0.052043059	1.3545
1.608	1.565	1.5863	0.030547013	1.3463
1.543	1.511	1.5268	0.022203153	1.2868

TNase line

1st Run	2nd Run	Ave	SD	Blank corrected
0.385	0.366	0.37555	0.013788582	0.13555
0.552	0.539	0.5454	0.009050967	0.3054
0.802	0.817	0.8093	0.010606602	0.5693
1.053	1.048	1.0508	0.003676955	0.8108
1.218	1.306	1.2621	0.062083975	1.0221
1.376	1.331	1.3533	0.031536962	1.1133
1.430	1.498	1.4643	0.048224682	1.2243
1.511	1.517	1.51415	0.003889087	1.27415
1.547	1.566	1.5563	0.013717872	1.3163
1.552	1.564	1.5579	0.007919596	1.3179
1.612	1.652	1.63215	0.028496403	1.39215

Blank
0.210
0.214
0.221
0.203
0.232
0.237
0.236
0.213 0.220525

Chronic 6

1st Run	2nd Run	Ave	SD	Blank corrected
1.342	1.361	1.35185	0.01350574	1.13185
1.366	1.249	1.30745	0.082095097	1.08745
1.110	1.192	1.1507	0.057699913	0.9307
1.397	1.240	1.31845	0.111369318	1.09845
1.513	1.517	1.5148	0.002969848	1.2948
1.515	1.501	1.50775	0.010111627	1.28775
1.528	1.564	1.54595	0.025667976	1.32595
1.450	1.520	1.48505	0.049285343	1.26505
1.553	1.522	1.5373	0.021496046	1.3173
1.598	1.568	1.58275	0.021142493	1.36275
1.601	1.584	1.59245	0.011667262	1.37245

Chronic 7

1st Run	2nd Run	Ave	SD	Blank corrected
1.444	1.435	1.4397	0.00622254	1.2197
1.249	1.240	1.24455	0.006858936	1.02455
1.249	1.240	1.24455	0.006858936	1.02455
1.291	1.295	1.29275	0.002899138	1.07275
1.370	1.390	1.38025	0.013930004	1.16025
1.412	1.326	1.36895	0.060740473	1.14895
1.450	1.352	1.40085	0.06880149	1.18085
1.425	1.428	1.42635	0.001767767	1.20635
1.448	1.410	1.42935	0.026799347	1.20935
1.543	1.566	1.55445	0.016192745	1.33445
1.463	1.528	1.4955	0.046103362	1.2755

Chronic 8

1st Run	2nd Run	Ave	SD	Blank corrected
1.340	1.216	1.27765	0.08761053	1.05765
1.050	1.036	1.0432	0.010182338	0.8232
1.009	0.987	0.9979	0.016122035	0.7779
1.163	0.982	1.07245	0.127774195	0.85245
1.308	1.141	1.22445	0.118298964	1.00445
1.340	1.226	1.2828	0.080610173	1.0628
1.430	1.237	1.33345	0.135976634	1.11345
1.303	1.209	1.2559	0.066892302	1.0359
1.469	1.344	1.40665	0.08860048	1.18665
1.585	1.457	1.52105	0.0907218	1.30105
1.572	1.505	1.53875	0.047164022	1.31875

TNase line

1st Run	2nd Run	Ave	SD	Blank corrected
0.244	0.243	0.2438	0.000848528	0.0238
0.282	0.264	0.27325	0.012657211	0.05325
0.309	0.262	0.28555	0.033163308	0.06555
0.343	0.294	0.3183	0.034789654	0.0983
0.481	0.351	0.4156	0.092065303	0.1956
0.726	0.512	0.6191	0.151038008	0.3991
1.115	0.313	0.7137	0.567382481	0.4937
1.142	1.010	1.0761	0.092913831	0.8561
1.251	1.057	1.154	0.136895873	0.934
1.463	1.262	1.3626	0.142269884	1.1426
1.480	1.401	1.4406	0.055437172	1.2206

Blank
0.233
0.253
0.236
0.248
0.238
0.264
0.228
0.230 0.241075

Chronic 9

1st Run	2nd Run	Ave	SD	Blank corrected
1.278	1.368	1.32325	0.0635689	1.08225
1.322	1.411	1.36615	0.062861793	1.12515
1.029	1.255	1.1421	0.159947554	0.9011
1.125	1.235	1.18	0.077781746	0.939
1.290	1.204	1.247	0.061376869	1.006
1.404	1.486	1.4446	0.057841335	1.2036
1.264	1.153	1.20845	0.078842406	0.96745
1.124	1.279	1.2011	0.109742972	0.9601
1.296	1.292	1.29415	0.003181981	1.05315
1.600	1.426	1.5132	0.12303658	1.2722
1.502	1.356	1.4292	0.102954747	1.1882

Chronic 10

1st Run	2nd Run	Ave	SD	Blank corrected
1.446	1.388	1.41695	0.040800061	1.17595
1.485	1.445	1.46505	0.028213561	1.22405
1.232	0.372	1.24455	0.006858936	1.00355
1.345	0.994	1.16945	0.248265191	0.92845
1.421	1.482	1.45115	0.043204224	1.21015
1.532	1.466	1.49885	0.046174073	1.25785
1.383	1.395	1.38925	0.008273149	1.14825
1.467	1.278	1.3726	0.133784603	1.1316
1.467	1.456	1.46135	0.008273149	1.22035
1.490	1.504	1.49705	0.01039447	1.25605
1.558	1.604	1.58125	0.032597623	1.34025

TNase line

1st Run	2nd Run	Ave	SD	Blank corrected
0.302	0.296	0.29855	0.00417193	0.05755
0.367	0.377	0.372	0.007495332	0.131
0.239	0.436	0.3372	0.139441457	0.0962
0.515	0.499	0.50695	0.010818734	0.26595
0.920	0.952	0.9359	0.022344574	0.6949
1.138	1.225	1.18165	0.061164737	0.94065
1.225	1.320	1.2723	0.067316566	1.0313
1.368	1.451	1.4094	0.058265599	1.1684
1.442	1.492	1.4668	0.035213918	1.2258
1.471	1.522	1.49625	0.035850314	1.25525
1.533	1.645	1.5889	0.079195959	1.3479

Concentrating data books

Fig 4.23 blank adjusted data prior to averaging

1 x conc	1.042	1.056	0.979	0.991	0.975	0.957	0.969	1.015	0.945	1.009	1.040
	0.999	0.936	0.928	0.933	0.995	0.965	0.950	0.939	0.925	0.962	0.980
75x conc	0.664	0.795	0.842	0.873	0.917	0.895	0.924	0.946	0.894	0.873	0.894
	0.658	0.839	0.850	0.907	0.945	0.923	0.961	0.967	0.937	0.957	0.991
spiked	0.016	-0.006	0.085	0.204	0.364	0.553	0.726	0.828	0.888	0.953	0.970
	0.016	0.035	0.097	0.197	0.360	0.590	0.735	0.827	0.853	0.923	0.975
TNase control	0.021	0.046	0.110	0.213	0.377	0.533	0.695	0.799	0.821	0.906	0.984
control	1.061	1.020	0.929	0.963	0.997	0.944	0.961	1.012	1.009	1.078	1.046

Fig 4.24 blank adjusted data prior to averaging

1 x conc	0.867	0.833	0.941	0.970	0.948	0.940	0.884	0.883	0.877	0.933	0.926
	0.844	0.849	0.926	0.895	0.935	0.838	0.919	0.904	0.894	0.902	0.922
75x conc	0.065	0.194	0.386	0.604	0.703	0.727	0.796	0.787	0.817	0.880	0.798
	0.094	0.220	0.384	0.656	0.767	0.820	0.840	0.871	0.851	0.911	0.930
spiked	0.012	0.070	0.164	0.293	0.485	0.626	0.771	0.831	0.861	0.868	0.889
	0.026	0.053	0.139	0.259	0.411	0.547	0.713	0.751	0.836	0.872	0.880
TNase control	0.014	0.057	0.127	0.254	0.365	0.506	0.693	0.775	0.835	0.905	0.920
control	1.002	1.102	1.080	1.047	1.018	0.927	0.988	0.984	0.954	0.987	1.027

Fig 4.25 blank adjusted data prior to averaging

1 x conc	0.998	1.053	1.067	1.104	1.093	1.119	1.165	1.174	1.151	1.177	1.239
	1.093	1.064	1.129	1.167	1.168	1.173	1.182	1.190	1.172	1.217	1.138
75x conc	0.133	0.203	0.263	0.490	0.651	0.812	1.008	1.063	1.065	1.028	1.070
	0.162	0.217	0.305	0.477	0.691	0.881	1.004	1.069	1.061	1.028	1.067
spiked	-0.005	0.032	0.115	0.203	0.428	0.609	0.829	0.941	0.961	1.001	1.039
	0.023	0.039	0.156	0.228	0.401	0.613	0.753	0.855	0.946	0.909	0.990
TNase control	0.045	0.060	0.153	0.307	0.457	0.605	0.772	0.911	0.961	0.898	1.071
control	1.428	1.292	1.297	1.312	1.255	1.208	1.250	1.200	1.181	1.270	1.219

Fig 4.26 blank adjusted data prior to averaging

1 x conc	1.084	1.195	1.172	1.232	1.145	1.145	1.175	1.149	1.148	1.122	1.299
	1.135	1.255	1.268	1.297	1.298	1.264	1.265	1.251	1.315	1.199	1.304
75x conc	0.341	0.434	0.543	0.687	0.774	0.918	0.969	1.095	1.145	1.035	1.107
	0.407	0.475	0.584	0.713	0.820	0.991	1.084	1.206	1.223	1.218	1.315
spiked	0.009	-0.009	0.077	0.181	0.400	0.667	0.818	0.982	1.077	1.129	1.216
	0.006	0.014	0.101	0.206	0.409	0.711	0.918	0.990	1.076	1.117	1.244
TNase control	0.014	0.063	0.139	0.255	0.467	0.688	0.829	0.957	1.073	1.122	1.223
control	1.353	1.354	1.286	1.330	1.324	1.327	1.302	1.247	1.349	1.398	1.341

Fig 4.27 blank adjusted data prior to averaging

1 x conc	1.286	1.341	1.297	1.190	1.261	1.236	1.266	1.299	1.318	1.140	1.563
	1.269	1.218	1.192	1.255	1.287	1.302	1.312	1.359	1.266	1.342	1.284
75x conc	0.104	0.258	0.384	0.787	1.048	1.157	1.286	1.295	1.277	1.234	1.206
	0.126	0.305	0.501	0.855	1.090	1.285	1.310	1.427	1.383	1.310	1.265
spiked	-0.015	0.040	0.153	0.317	0.528	0.777	1.068	1.276	1.318	1.398	1.184
	0.008	0.047	0.173	0.325	0.590	0.824	1.108	1.297	1.432	1.430	1.275
TNase control	0.012	0.054	0.155	0.323	0.506	0.807	1.033	1.296	1.296	1.418	1.287
control	1.478	1.430	1.602	1.521	1.456	1.473	1.533	1.413	1.434	1.526	1.343

Fig 4.28 blank adjusted data prior to averaging

1 x conc	0.463	0.520	0.544	0.372	0.569	0.436	0.596	0.532	0.593	0.612	0.628
	0.502	0.564	0.608	0.467	0.613	0.558	0.623	0.631	0.642	0.617	0.571
75x conc	0.076	0.167	0.160	0.299	0.379	0.420	0.471	0.527	0.590	0.569	0.546
	0.113	0.178	0.217	0.306	0.359	0.412	0.462	0.536	0.572	0.579	0.575
spiked	0.012	0.102	0.211	0.283	0.431	0.495	0.532	0.573	0.590	0.614	0.597
	0.023	0.093	0.231	0.321	0.403	0.492	0.505	0.540	0.592	0.599	0.567
TNase control	0.024	0.089	0.175	0.270	0.362	0.450	0.486	0.549	0.586	0.645	0.630
control	0.667	0.782	0.731	0.715	0.675	0.676	0.710	0.709	0.679	0.619	0.662

Fig 4.29 blank adjusted data prior to averaging

1 x conc	0.696	0.752	0.814	0.805	0.771	0.811	0.823	0.805	0.797	0.784	0.798
	0.651	0.694	0.764	0.755	0.808	0.807	0.802	0.799	0.797	0.756	0.739
75x conc	0.630	0.623	0.654	0.675	0.667	0.722	0.768	0.801	0.784	0.742	0.738
	0.519	0.624	0.611	0.620	0.628	0.693	0.737	0.781	0.748	0.714	0.747
spiked	0.034	0.096	0.267	0.408	0.565	0.647	0.700	0.718	0.745	0.702	0.683
	0.044	0.104	0.252	0.359	0.504	0.607	0.674	0.688	0.697	0.691	0.705
TNase control	0.039	0.110	0.236	0.381	0.521	0.636	0.681	0.716	0.727	0.696	0.719
control	0.783	0.783	0.728	0.781	0.788	0.761	0.769	0.763	0.764	0.759	0.687

Fig 4.30 blank adjusted data prior to averaging

1 x conc	0.590	0.712	0.511	0.783	0.784	0.811	0.753	0.756	0.755	0.770	0.853
	0.585	0.702	0.743	0.803	0.785	0.783	0.773	0.785	0.809	0.776	0.788
75x conc	0.245	0.298	0.356	0.432	0.461	0.522	0.622	0.689	0.715	0.721	0.682
	0.380	0.333	0.385	0.434	0.481	0.564	0.648	0.708	0.739	0.736	0.833
spiked	0.012	0.052	0.181	0.293	0.445	0.620	0.677	0.723	0.723	0.711	0.744
	0.027	0.055	0.210	0.270	0.417	0.590	0.625	0.677	0.669	0.732	0.798
TNase control	0.045	0.114	0.227	0.403	0.535	0.629	0.685	0.751	0.765	0.754	0.803
control	0.859	0.853	0.795	0.797	0.801	0.764	0.804	0.812	0.800	0.769	0.803

Fig 4.31 blank adjusted data prior to averaging

1 x conc	0.720	0.809	0.807	0.838	0.786	0.850	0.853	0.873	0.862	0.825	0.821
	0.730	0.777	0.813	0.835	0.810	0.843	0.801	0.818	0.822	0.821	0.723
75x conc	0.049	0.172	0.234	0.443	0.570	0.641	0.660	0.696	0.771	0.761	0.755
	0.093	0.158	0.286	0.424	0.541	0.601	0.662	0.732	0.746	0.725	0.760
spiked	0.020	0.109	0.212	0.355	0.537	0.649	0.721	0.764	0.741	0.751	0.784
	0.042	0.111	0.235	0.354	0.550	0.671	0.710	0.738	0.721	0.714	0.137
TNase control	0.052	0.135	0.240	0.383	0.511	0.654	0.658	0.718	0.726	0.740	0.727
control	0.925	0.951	0.884	0.899	0.877	0.883	0.896	0.822	0.820	0.830	0.786

Fig 4.32 blank adjusted data prior to averaging

1 x conc	0.923	1.025	1.085	1.034	1.108	1.034	0.996	1.053	1.109	1.051	1.057
	0.898	0.990	1.065	1.143	1.016	1.112	1.054	1.152	1.116	0.999	0.921
75x conc	0.508	0.653	0.534	0.699	0.780	0.792	0.824	0.918	0.908	0.879	0.922
	0.671	0.647	0.697	0.741	0.791	0.800	0.861	0.985	0.987	1.049	1.061
spiked	0.029	0.186	0.186	0.450	0.660	0.771	0.891	0.978	0.967	1.034	1.040
	0.051	0.187	0.205	0.444	0.636	0.751	0.827	0.903	0.973	0.988	0.963
TNase control	0.052	0.185	0.193	0.457	0.647	0.809	0.884	1.032	0.993	1.046	1.022
control	1.062	1.031	1.111	1.114	1.060	1.092	1.104	1.134	1.136	1.082	1.051

Appendix 9

CD of Animations Utilising PNA/FISH CLSM