

Biofilms: Biomaterials and Chronic Wounds

By

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Abstract

Healthcare associated infections (HCAIs) are a large and growing problem. Bacterial infections of patients and on the medical devices used to treat them represent a significant source of morbidity and mortality. There is also a significant economical impact to the healthcare system attributed to HCAIs. While bacterial infections *per se* are not a novel problem, the discovery of an adherent polymicrobial phenotype called a biofilm is. A biofilm is defined by its structure and the community of bacteria therein. This study investigated bacteria biofilms in a number of pertinent clinical scenarios. To achieve this, samples were taken from five different but related clinical areas where biofilms are known to infect or are suspected to, namely endotracheal tubes, tracheostomy tubes, burn wounds, chronic wounds and chronic wound dressings. Samples were analysed using microbiological and molecular analysis techniques, the latter included polymerase chain reactions, species-specific PCR and denaturing gradient gel electrophoresis to assess microbial diversity. Fluorescent in-situ hybridization was used subsequently to analyse species orientation and biofilm structure within the biofilm. This study showed a diverse bacterial population in all the samples, with the presence of oral biota in the ETT specimens, changing to commensal bacteria over time. Large three-dimensional biofilm structures were present in the specimens confirming the presence of biofilms, and within one of the chronic wound dressings where a complex biofilm was visible within the matrix of the dressing itself. These findings have considerable significance clinically, not only in demonstrating the need for biofilm targeted diagnostic techniques, but also in highlighting the need for specific biofilm treatment modalities in critical care, burn services and chronic wound management.

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List of Abbreviations

AHL	N-acylhomoserine lactone
ALU	Arterial Leg Ulcer
AMP	Antimicrobial Peptides
ARDS	Acute Respiratory Distress Syndrome
BA	Blood Agar
BHI	Brain Heart Infusion Broth
BHL	<i>N</i> -Butanoyl-L-homoserine lactone
CDC	Center for Disease Control
CFU	Colony Forming Units
CLSM	Confocal Laser Scanning Microscope
CPIS	Clinical Pulmonary Infection Score
DA-HCAI	Device associated Healthcare Acquired Infections
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
DNU	Diabetic Neuropathic Ulcer
DU	Duodenal Ulcer
DVLO	Derjaguin-Landau-Verwey-Overbeek
ECP	<i>E. coli</i> Common Pilus
ELF	<i>E. coli</i> laminin-binding fimbriae
EPS	Extracellular Polymeric Substances
ETT	Endotracheal tube

FAA	Fastidious anaerobic Agar
FimH	Fimbrial component H
FisH	Fluorescent <i>in-situ</i> Hybridization
FITC	Fluorescein isothiocyanate
HCAI	Healthcare associated Infections
HCP	Haemorrhagic Coli Pilus
HHL	<i>N</i> -Hexanoyl-L-homoserine lactone
HSLs	acyl-Homoserine Lactones
ICU	Intensive Care Unit
MAC	MacConkey Agar
MIC	Mean Inhibitory Concentration
MRSA	Meticillin Resistant Staphylococcus Aureus
NHS	National Health Service
OHHL	<i>N</i> -(3-oxohexanoyl)-L-homoserine lactone
PIA	Polysaccharide Intercellular Adhesion (molecule)
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PNA	Peptide Nucleic Acid
PU	Pressure Ulcer
QS	Quorum Sensing
QQ	Quorum Quenching
REC	Research Ethics Committee
RHE	Recombinant Human Epithelium

RNA	Ribonucleic Acid
SAH	Subarachnoid Haemorrhage
SDA	Sabouraud's Dextrose Agar
SDS	Sodium Dodecyl Sulfate
SPRE	Surface Protein-Releasing Enzyme
TAE	Tris-Acetate EDTA
TBSA	Total Burn Surface Area
TEMED	Trimethylethylenediamine
TT	Tracheostomy Tube
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
USA	United States of America
UV	Ultraviolet
VAP	Ventilator Associated Pneumonia
VLU	Venous Leg Ulcer

Chapter 1: Introduction

Biomedical sciences are constantly evolving often with the aim of improving patient outcomes and promoting efficiency at reduced fiscal outlay. One area into which research and health promotion initiatives are focussed is that of healthcare-acquired or associated infections (HCAIs).

There is a wealth of media coverage both abroad and in the United Kingdom regarding commonly acquired or difficult to manage organisms such as Meticillin Resistant *Staphylococcus aureus* (MRSA) or *Escherichia coli* (Boyce *et al.*, 2009). The topic of HCAI is an especially emotive one, and one that is often quickly highlighted in the media. In the modern era many elective operations are seen as routine, yet the introduction of HCAI into this environment turns a routine procedure into a potentially life-threatening one; a recent meta-analysis by Hu *et al* (2011) shows that infection of total knee joint replacement prostheses is still the most common complication in this type of surgery. The advent of the antibiotic era was meant to herald an end to bacterial infection as a significant source of mortality, yet the main advances in promoting life-expectancy have come from epidemiological interventions, from hand-washing in labour wards to improvements in sanitation and vaccination. Even as mankind once felt it was on the verge of eradicating malaria, so resistant forms of the *Plasmodium falciparum* parasite begin to predominate. Similarly wherever antibiotics are employed, bacterial resistance emerges. The debate on where the fault lies with the creation of antibiotic resistance continues. Some

theories place the blame with the food industry, for regular prophylactic administration of antibiotics to poultry to prevent the large scale financial disaster resulting in an untamed respiratory infection spreading within the broiler industry (Hughes *et al.*, 2008).

Other theories feel that over-dispensation of antibiotics in Primary Care medicine is at fault, that the relatively quick and easy solution to manage a self-limiting viral illness with a prescription of entirely unnecessary antimicrobials is providing an evolutionary selection pressure which favours antimicrobial resistance (Gonzales *et al.*, 1997). A recent study performed at Intensive Care Units in Havana, Cuba by Medell *et al.* (2012) showed moderate to very high resistance by *Acinetobacter spp.* *Pseudomonas spp.* and *Klebsiellas spp.* to almost all modern antibiotics including meropenem, aztreonam, and piperillin/tazobactam (Medell *et al.*, 2012) and they concluded that the liberal use of these agents as preventative therapy was actively inducing resistance in these species. However, while it easy to suggest preventing General Practitioners or farmers from using antibiotics in an injudicious manner, in an Intensive Care setting where patient survival may depend on rapid application of an antimicrobial based on empirical or best-guess reasoning it is not so clear or easy to propose a dogmatic restriction on antibiotic prescription.

Rapid emergence of bacterial resistance to antibiotics is not a modern phenomenon however; while Alexander Fleming (1881-1955) is said to have discovered Penicillin in 1928, by the Normandy landings in June 1944 there had been only 2.3 million doses of Penicillin manufactured, yet the first reports of antibiotic resistance were being published within two years

of that date (Dowling *et al.*, 1946). While it is often portrayed as an arms race between mankind and bacteria, it is felt by many that eventually we will be unable to come up with new antibiotics, or we will remain unwilling to save them until the situation is dire enough. This leads us on to the question of alternative strategies that may be employed.

Where Semmelweiss introduced hand-washing by medical students in the labour ward, and Mussolini advocated the drainage of swamp land to reduce the incidence of malaria (Hite and Hinton 1998) so current studies of microbial species are also being undertaken looking at the nature and pathology of bacterial species. Current clinical initiatives such as the National Hand Hygiene NHS Campaign (Health Protection Scotland, 2007) aiming to decrease transmission of fomites, or the MRSA pre-admission screening policy operated by many NHS trusts to decrease the spread of MRSA (Department of Health, 2006) illustrate that new solutions to microbial infections are being sought. Clinical and laboratory based studies form another important aspect of the current research agenda in this area; a key aspect of this agenda is to develop a deeper understanding of the life-cycle of bacteria.

Recently it has become clear that 19th century theories on the nature of bacteria only consider one phenotype, that of the planktonic or free floating cells. With the advances made in the latter half of the 20th century such as scanning electron microscopy and molecular analysis techniques including polymerase chain reaction (PCR) technology, newer theories regarding bacterial phenotypes emerged. Transmission electron microscopy was introduced by Ernst Ruska in 1931, and by 1939 Piekarski and Ruska had

published pictures of bacterial flagellae (Linke, 2011). The subsequent use of scanning electron microscopy in the 1950s allowed closer examination of bacterial morphology, and theories regarding bacterial evolution and their familial relationships could be expounded (Bartlett 1967).

It has become clear that many microorganisms have evolved to protect themselves, usually as a response to adverse conditions, by being able to encase themselves in a complex matrix called a biofilm. Biofilms were first recognised in some marine species of microorganisms, which could adhere to and grow on surfaces in communities (Zobell 1943; O'Toole *et al.*, 2000). The relevance of this became apparent when it was recognised that organisms were growing in micro-colonies within the biofilms, of their own making (Costerton *et al.*, 1978), as stable and adherent phenotypes (Lawrence *et al.*, 1991); this approach is very different to the concept of free-floating planktonic forms noted by van Leeuwenhoek, Pasteur and Lister as the cause of spoilt wine and surgical infection. One current definition of a biofilm is that of 'a surface-associated microbial community, that is composed of various phenotypes and commonly various genotypes, which encases itself in a three dimensional matrix of extracellular polymeric substances (polysaccharides, nucleic acids, proteins) and demonstrates increased resistance to cellular and chemical attack' (Thomas 2008).

Current research is centred on the study of biofilms and bacteria in this stable phenotype form (Percival and Cutting 2009), principally because in this state, bacteria are resistant to both host defences and antimicrobials (antibiotics and antiseptics). This may be, in part, why bacterial biofilms are so topical and relevant in the arena of chronic wounds and wound-healing;

being uniquely placed at the interface between host defences, trying to heal the wound intrinsically and antimicrobial wound therapies, trying to heal the wound extrinsically.

The term biofilm remains poorly understood and, although they do exist in open chronic wounds healing by secondary intention, the commonly seen 'slimy' appearance on the surface may be mistaken for a biofilm. It is usually only when infection is overt and progressive that organisms can be cultured from simple swab specimens; it is difficult to mimic the conditions that some microorganisms exist in within biofilms in the laboratory setting (Munson *et al.*, 2002). It is felt that biofilms may have quiescent periods and acute exacerbations, and infection in a surgical wound or related to an implanted prosthesis, such as a hip replacement or vascular graft, may be delayed or concealed by a biofilm. The acute exacerbations may be successfully treated by clinicians using antibiotics, but the biofilm nidus is not resolved (Costerton, 2005). This often causes the initial diagnosis to be missed or incorrectly assessed leading to delay in patient therapy and a subsequent increase in morbidity (Nayeemudin *et al.* 2008, Nadeem and Hadden 1999). The treatment of infected or exposed (and therefore presumably colonised) prostheses of all types remains debatable.

When breast implants have been used for augmentation procedures and have become infected, multiple different strategies have been attempted to salvage the prosthesis including antibiotic therapy, debridement, curettage, pulse lavage, capsulectomy, device exchange, primary closure, and/or flap coverage with varying degrees of success (Spear *et al.*, 2004). In the case of infected knee arthroplasty prostheses, which have the potential to be

significantly more debilitating for the patients, early infections may be treated in such a way as to allow retention of the primary prosthesis, using antibiotics and vigorous surgical debridement. However prosthetic infections that occur late (*i.e.* after 30 days of initial implantation), and may therefore be indicative of a biofilm infection, necessitate the removal of the prosthesis and a two-stage reconstruction at significant risk and morbidity to the patient (Hanssen, 2002).

The incidence of prosthetic joint infection has been estimated at 1 to 3%, with a small but significant number of these cases remaining negative on standard microbiological culture due in part to the presence of the abiotic matrix which forms part of the biofilm (Peel *et al.*, 2011). The matrix of a biofilm is not only protective but allows communication between different bacterial species, by a process known as quorum sensing, and even transfer of plasmid-DNA. Aerobic Gram-negative bacilli in particular are able to attach to surfaces by pili or flagella and are resistant to adverse conditions (Canals *et al.*, 2006; Toutain *et al.*, 2007; Jagnow and Clegg 2003; O'Toole *et al.*, 1998). In addition, biofilms have a three-dimensional structure and an increased surface area, which allows absorption of nutrients through built in water channels.

Aside from medical interest, biofilms have been found extensively in water-treatment facilities and, for example, ice cream factories face a constant battle to keep the luminal surfaces of their pipes free of biofilms (Gunduz and Tuncel, 2006). Biofilms have been recognised for a long time in dental plaque, often containing fungal elements as well as common oral pathogens such as *Streptococcus mutans* and *Porphyromonas gingivalis* (Le Magrex *et*

al., 1993; Bowden and Hamilton, 1998). Biofilms have also been associated with medical devices as diverse as contact lenses, breast implants, urinary catheters and hip prostheses (Costerton *et al.*, 2005; Weisbarth *et al.*, 2007; Kuhn and Ghannoum, 2004).

Biofilms therefore have the potential to have a significant impact on patient care, and both clinical and microbiological research is now directed at elucidating the makeup and pathogenicity of bacterial biofilms and subsequently how to diagnose and treat them.

This study will look at the scope of the problem of HCAs within the critical care environment as well as in the arena of chronic wound healing and provide an up to date review of biofilm literature, their composition, life-cycle and pathogenic traits. The research itself will focus on diagnosing the presence of a bacterial biofilm, elucidating the different bacterial populations within a biofilm and their orientation to each other within medical devices used in critical care, on burn and chronic wounds, and within dressings used in the management of chronic wounds. This will be done using different techniques including standard microbial cultural analysis, DNA analysis, both species-specific PCR and denaturing gradient gel electrophoresis, and finally using confocal laser scanning microscopy to delineate the structure of a bacterial biofilm.

The overall aim of this study was to examine bacterial biofilms in a clinical context, especially as pertaining to healthcare-acquired infections and those on medical devices. A specific objective of the study was to evaluate different methodologies for the identification and and quantification of

bacterial biofilms, and also to compare the performance of these different methodologies across a range of different clinical samples.

The first clinical setting selected was that of endotracheal tubes (ETTs). These devices are in contact with a known biofilm forming area (the oral cavity) and have also been shown to have a role in healthcare-acquired infections such as ventilator associated pneumonia (VAP). It was then decided that evaluation of tracheostomy tubes could provide deeper insights into the evolution and ecology of biofilms within the cannulated airway. Tracheostomy tubes are sited within the airway and give rise to VAP but are not in direct contact with the oral cavity. Any oral bacteria found in these samples would have to either come from previous inoculation of the airway by the endotracheal tubes, or migrate from the lungs or oral cavity. Since the tracheostomies would be coming from burn injured patients, the third set of samples chosen to be examined were those of burn wounds themselves. Although not from the same patients, burn injured tissue is known to be a harbinger of infection, and the detrimental role of bacteria in burn wound healing is well known. This deleterious effect of bacterial colonisation on burn wound healing is mirrored in the delayed healing of chronic wounds, especially those heavily contaminated by bacteria, presumably in the biofilm form.

These different sample types have a common theme of healthcare-associated infection running through them, and by using cultural, molecular and structural analysis techniques the role of bacterial biofilms in these HCAs may be assessed. Finally, to elucidate whether information about biofilms may be obtained using non-invasive or destructive techniques, it

was decided to assess and examine the dressings of chronic wound patients to investigate whether dressing analysis might be used as a surrogate in the diagnosis of biofilm infections.

Current methods of attempting to diagnose biofilm infections involve the use of painful debridement and biopsy techniques to physically disrupt the biofilm (methods that are unpleasant and unpopular with patients), or expensive ultrasonication of the biofilm to disrupt the abiotic matrix, causing damage to the architecture, while at the same time being too expensive or sophisticated for routine everyday use. The concept of a surrogate dressing to mirror the biofilm on a wound may provide a simple and painless method of biofilm diagnosis.

Chapter 2: Literature Review

Introduction

Healthcare-Associated Infections

Infection has been a problem for mankind since its inception. Evidence of *Brucellosis* destruction of spinal vertebrae has been identified in the vertebrae of *Australopithecus africanus* (a prehistoric ancestor of man) and is considered to be between 1.5 to 2.8 million years old (D’Anastasio *et al.*, 2009). This infection was thought to have been contracted from the consumption of infected meat products, as indicated by nearby animal bones which had been scored by human-like canines and incisors. By the 19th century, a clearer understanding of infection, and post-operative sepsis was beginning to form. Semmelweiss (1858) noted the high levels of post-puerperal sepsis in patients treated by medical students contaminated by ‘cadaverous particles’ and instituted hand washing techniques, lowering mortality from 18.3% to 1.2% in just two months, although it should be noted pregnant women themselves had long been aware that it was more hazardous to deliver on a medical student led ward, than a midwife-led ward. Subsequently, Joseph Lister (1867) became aware of the need to try and control infection in injuries, and famously instituted the use of carbolic acid as pre-operative skin prep and described the use of antiseptic dressings in attempts to control infection, usually in the post-operative period.

Healthcare-associated infections (HAIs) are infections acquired by contact with healthcare services, devices or staff. Every year at least 300,000 patients develop an HAI and it is estimated that around 1 in 10 patients

acquire an infection during their stay in a UK hospital (HCAI Research Network, 2009). This has a significant impact on the care of patients, their outcomes and their subsequent morbidity. HCAIs are a leading cause of death in United States health care settings, with an overall estimated annual incidence of 1.7 million (Welsh *et al.*, 2011). A recent Scottish study showed an increase in HCAIs with a linear relationship to age; in the most elderly groups (75-84 and 84+ years) the commonest type of infection was urinary and gastro-intestinal, but in under 75s year-olds, surgical site infection represented the largest healthcare burden (Cairns *et al.*, 2011). HCAIs have received widespread attention in recent times in both the media and in healthcare settings, with those caused by ‘superbugs’ such as Methicillin Resistant *Staphylococcus aureus* (MRSA) and *Clostridium difficile*, a particular focus of attention. The national mandatory reporting system for surveilling MRSA rates has been in place since 2006. Between 2006 to 2009 there were 4404 cases of MRSA bacteraemia reported in England, approximately one third of these cases suggested a probable source of the infection, of these 20% were related to the use of intravascular devices and a further 28% to skin and soft-tissue infections (Wilson *et al.*, 2011). However, other bacterial species are actually the primary causes of the majority of HCAIs ranging from infections acquired in the highly interventionist areas of healthcare, such as Intensive Care Units (ICUs) and Burn ICUs through to chronic wounds such as venous leg ulcers (VLUs), which are often managed in the community.

Infection in patients being treated in ICUs can have a significant effect on the patient, including increased mortality (Malacarne *et al.*, 2010). Not only

is there the potential for the patient to develop an infection as a direct consequence of their existing condition, but the possibility also exists for the medical devices used to treat them to introduce infection (Eisenberg, 2009). This, in turn, causes increased mortality, morbidity and increased incidence of device failure. In addition to the detrimental effect on the health of the patient, the failure of medical devices is a source of significant additional expenditure to the healthcare provider. One study from New Zealand estimated the cost of a healthcare associated bloodstream infection as being between \$11,000 and \$20,000 per patient per episode (Burns *et al.*, 2010).

The insertion of peripheral venous cannulae is associated with local risks such as haematoma formation or extravasation injury, but these risks are magnified when the device is being replaced (Lepor and Maydeen, 2009). The same applies for a central line or airway management device, such as an endotracheal tube (ETT) or tracheostomy tube. Even replacement of something as seemingly benign as a urinary catheter has been implicated in prostatic damage, and of course, the introduction of infection and sepsis (Syed *et al.*, 2009). It is thought the use of urinary catheters is a likely point of entry into the blood stream for nosocomial fungal infection by *Candida albicans* (Tiraboschi *et al.*, 2000), with the mortality from candidaemia being greater than 40% (MacPhail *et al.*, 2002). Fungal infection in an Italian ITU showed a healthcare-associated infection rate from mycoses as high as 10.08 per 1000 admissions with *C. albicans* infection rates shown to be as high as 60% of those infections; 77% of these occurred in surgical patients (Tortorano *et al.*, 2011).

A recent Polish study, showed that device-associated healthcare-associated infections (DA-HCAIs) in ITU patients caused a significantly longer stay in ITU; compared with an average stay of 6.9 days, those with central-line associated bloodstream infection stayed for 10.0 days, those with VAP 15.5 days and those with catheter-associated urinary tract infection stayed on average for 15.0 days (Kubler *et al.*, 2011). There is a wide variation of reported incidence rates of DA-HCAI but one study showed rates in a paediatric and neonatal ICU of up to 15.5% (Duenas *et al.*, 2011). Indeed, it has been shown that the presence of a centrally placed indwelling venous catheter is an independent risk factor for the conversion of nosocomial MRSA colonisation into a MRSA HCAI (Harinstein *et al.*, 2011). Central line associated infections have a reported mortality of between 12 and 25% (Centers for Disease Control and Prevention, 2011).

The same device-related problems exist for burn injured patients, but these are coupled with the (usually) extensive burn wound, which also frequently becomes infected. Burns Units along with Intensive Care Units have the highest rates of HCAI, cited as up to 34% in some cases (Lahsaeizadeh *et al.*, 2008). Burn sepsis is still the leading cause of mortality in burn-injured patients (Wang *et al.*, 2009) and control of bacterial colonisation of these wounds is of paramount importance; however, it has been shown that the presence of healthcare-associated blood stream infections has a significant effect on the duration of hospitalisation of burn injured patients (Brusselaers *et al.*, 2010).

Chronic wounds, such as venous leg ulcers(VLUs) or Pressure Ulcers (PUs),are defined as wounds that have failed to proceed through an orderly

and timely reparative process to produce anatomic and functional integrity over a period of three months (Mustoe *et al.*, 2006); these wounds often provide an area where bacteria are able to grow and even thrive. As a consequence, these wounds are predisposed to recurrent incidents of infections, and delayed wound healing.

Chronic wounds are a major cause of morbidity, affecting more than 1% of the population and with treatment costs of at least £2-3 billion per year in the UK and \$25 billion in the USA (Sen *et al.* 2009; Thomas and Harding, 2002). The impact of infected wounds on a patient should not be underestimated. Pain and subsequent lack of sleep, and overpowering odour along with delayed wound healing are a few of the complications of infection that patients cope with on a daily basis (Price and Harding, 1996; Ebbeskog and Ekman, 2001). The combat of infection in such patients is often a prime objective of wound management strategies (Price 2005; Edwards 2009). A significant delay in wound healing may occur with a contaminated or infected wound (Robson, 1997), and the number of bacteria present is directly related to the success of wound healing strategies (Krizek and Davis, 1967; Robson and Heggers, 1969). There is no clear theory as to why this happens but it is felt that the relative hypoxia within a chronic wound may be compounded by the presence of bacteria, which causes an impaired migratory response from the keratinocytes present leading to a delay in wound healing (Xia *et al.*, 2001). Another theory is that the healing wound is damaged through the host's attempt to combat the bacteria, since the lysozymal enzymes released into the wound environment equally damage host tissue as well as microbial contaminants (Glaros and Larsen,

2009). It is felt that the bacteria/host interaction within the wound induces a chronic inflammation, and this causes a subsequent delay in wound healing (Rhoads *et al.*, 2008). Despite this, the majority of wounds will heal if the underlying cause is treated, *i.e.* the VLU will heal if treated with compression therapy or the diabetic ulcer will heal if off-loaded (Kirketerp-Moller *et al.*, 2011). It should, also be mentioned that wound healing can still occur even in the presence of bacterial contamination (Krizek and Robson, 1975).

It is, however, becoming increasingly clear that the current concepts of infection are flawed. While Louis Pasteur (1822-1895) identified bacteria growing within a broth, and Leeuwenhoek's (1632-1723) free-floating 'animalcules' led microbiological theories down a 'free-floating' or planktonic train of thought, it now appears that a more persistent and adherent part of the bacterial life cycle exists. Specific bacterial phenotypes, which instead of behaving like free-floating plankton, exist in a semi-permanent adherent colony; these have become known as biofilms.

It is now accepted that many bacteria exist within a biofilm for the majority of their existence. Certain adherent species, such as *Streptococcus viridians*, the bacterium often responsible for bacterial endocarditis, are recognised by clinicians (Cunha *et al.*, 2010). This may be in part due to the relative ease of diagnosing bacterial 'vegetations' on diseased mitral valves, using echocardiography, and due to the significant complications that bacterial embolisation from the valve leaflets can cause. However, *S. viridians* is a notable exception, and many other bacterial biofilm-related infections are poorly understood or recognised in the clinical context. A

recent study by the Center for Disease Control (CDC) in the USA estimated that approximately 80% of HCAs are due to a biofilm related infection and it is here that modern research is now focused (Gristina and Costerton, 1984; Percival and Cutting, 2009).

Biofilms

In 1943 it was reported that the preference of some marine bacterial species was to adhere to, and grow on, surfaces (Zobell, 1943). The implication of such adherent bacteria often encased within a matrix material did not become apparent until 1991, when living organisms were identified growing in micro-colonies within these biofilms (Lawrence, 1991). O'Toole *et al.* (2000) subsequently defined biofilms as communities of surface attached organisms. Therefore, a currently accepted definition of a biofilm is one of 'a polymicrobial community of adherent organisms within an extracellular polysaccharide matrix, of their own making' (Costerton *et al.*, 1999). This definition was subsequently further expanded to:

A surface-associated microbial community that is composed of various phenotypes and commonly various genotypes, which encases itself in a 3-dimensional matrix of extracellular polymeric substances (EPS; *e.g.* polysaccharides, nucleic acids and proteins) and demonstrates increased resistance to cellular and chemical attack (Thomas, 2008).

This definition reflects the complexity of the composition and functions of a biofilm and incorporates its potential to resist various treatment modalities.

In order to understand why biofilms are so problematic in the healthcare setting, as well as within the water treatment and food preparation industries, we need to recognise why biofilms are resistant to removal by host defences and administered therapeutics, and also to identify potential therapeutic targets. To do this we first need to understand the dynamics of a biofilm population from its inception, to its eventual break-up and dispersion.

Biofilm formation

Microbial adherence

The adherence of microorganisms to a surface, be it in the lumen of a medical device or on a eukaryotic surface, is the critical first step required in the formation of a biofilm. Adherence may be divided into primary or secondary adhesion or may be classified according to the host surface: for example, the biomaterial of a medical device or host tissue in a wound bed.

The first stage of adherence involves the microorganisms coming into contact with a surface to which they can adhere. This may occur randomly (as is the case for bacteria being buffeted along in an airflow stream within an ETT), it may occur as a result of contamination (*e.g.*, a hip prosthesis making contact with the patient's skin during insertion), or as a result of bacterial motility. Certain bacteria such as *Aeromonas*, *Pseudomonas* and *Klebsiella* spp. are all noted for their pili and flagella (Jagnow and Clegg, 2003; Canals *et al.*, 2006; Toutain *et al.*, 2007) and enhanced motile

capacity. It is not surprising therefore that *Pseudomonas aeruginosa* is reported as being able to attach and form biofilms under almost any conditions that allow its growth (O'Toole, 1998).

Primary adhesion depends upon the net effect of repulsive and attractive forces being attractive. The hydrophobicity of bacteria, especially oral bacteria, is known to be a key element in their binding to coated surfaces (George and Kishen, 2007). Oral bacteria bind to salivary glycoproteins, collectively known as the salivary pellicle (Gibbons and Etherden, 1983). It is thought that it is these types of conditioning films, which allow the bacteria to overcome the physico-chemical forces acting at the interface between microbial and host surfaces. These forces include electrostatic and hydrophobic interactions, steric hindrance, van der Waals forces and hydrodynamic forces (An, 2000). Electrostatic charges tend to militate against attachment due to the overall net negative charge of both the bacteria and biological surfaces, meaning hydrophobic attraction is a more crucial element (Carpentier, 1993). This phenomenon is outlined by the Derjaguin, Landau, Verwey and Overbeek (DLVO) theory, which describes particle adherence within a colloid (Geoghegan *et al.*, 2008). It is best thought of as two particles moving towards each other. There is a double layer repulsive force acting on the two particles that attempts to keep them separate, yet if they can come together with sufficient energy to overcome the repulsive force, and then they will bind strongly and irreversibly together (*Figure 1*).

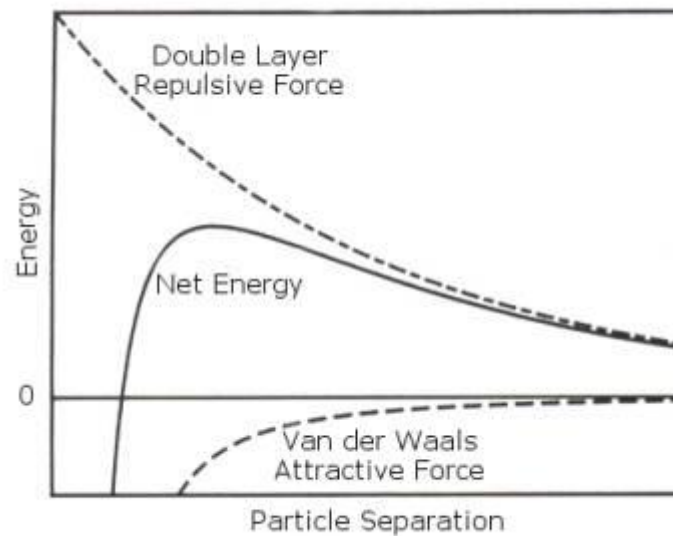


Figure 1: Derjaguin, Landau, Verwey and Overbeek theory, showing that a certain amount of net energy is required to overcome the repulsive force, but which then allows binding within a colloid. Reproduced from www.malvern.com

The attachment of bacteria to a surface is also dependent on surface factors. These include the conditioning of the surface, whereby an initial protein or glycoprotein layer is laid down. The most commonly used example of this is the covering of dental enamel by saliva, thus forming the salivary pellicle, where agglutinin or other proteins within saliva, allow the initial attachment phase to occur. Bacterial factors are also heavily implicated in the initial adherence step, where the expression of bacterial adhesion molecules, called adhesins, can play a vital role. For example, *Streptococcus mutans* has a specific surface protein adhesin (P1), which has a specific binding domain on it for the purpose of interacting and binding to immobilized and fluid phase salivary agglutinin (Crowley *et al.*, 1993). The initial ‘net

repulsion' between cells and surfaces can be overcome by covalent molecular binding mechanisms and it is these 'adhesins' which are responsible for both secondary adhesion and primary adhesion to eukaryotic surfaces (Bos, 1999). However, the progress from the single planktonic phenotype which has adhered to a surface to a mature polymicrobial biofilm is not clear; Hodl *et al.* (2011) have proposed that there is an initial clustering of planktonic bacteria which occurs in a non-random pattern, and they feel this clustering and progression to early biofilm formation is mediated by various biotic interactions and the role of adhesins.

Adhesins have been identified for different bacterial species (Table 1) and examples include the fimbrial component H (FimH) for *E. coli* and the cell surface protein antigen I/II (Ag I/II) for *S. mutans* (Hajishengallis, 1992; Schembri and Klemm, 2001). A key adhesin associated with *S. aureus* is a polymer of β -1,6-linked N-acetylglucosamine, called polysaccharide intercellular adhesion (PIA; Gotz, 2002). It is also clear that the exact mechanism is not clear, and that certain pathological strains may exhibit one or more adhesin type molecules; a recent study in America, showed enteropathic strains of *E. coli* expressed only certain adhesions, namely the *E. coli* common pilus (ECP), but not other known adhesins such as the haemorrhagic coli pilus (HCP) and *E. coli* laminin-binding fimbriae (ELF) as was expected (Hernandes *et al.*, 2011). The authors felt that these findings indicate that these strains bear several pili operons that could potentially be expressed in different niches favouring colonization and survival in and outside the host. Spurbeck and colleagues showed that one subtype of *E. coli* responsible for pyelonephritis carried operons for 12

different fimbriae and that the genes responsible for these were significantly more frequently expressed in those strains responsible for infection compared to human commensal strains (Spurbeck *et al.*, 2011). Conversely, however, a recent Israeli study suggest that the initial adherence step for *Mycoplasma pneumoniae* is solely mediated via the interaction of the tip of its organelle with sialic residues of serum glycoproteins (Kornspan *et al.*, 2011) possibly mediated by only one or two operons, although this was solely an *in vitro* study and may add credence to the theory that many of these factors are environmental or exogenous. Indeed the American Center

Species Name	Adhesin Name	Shorthand Nomenclature	Reference
<i>Escherichia coli</i>	Fimbrial component	Fim H	Hajishengallis 1992
<i>Streptococcus mutans</i>	Antigen I/II	Ag I/II	Schembril and Klemm 2001
<i>Staphylococcus aureus</i>	B,1-6-linked N-acetyl glucosamine	PIA	Gotz 2002
<i>Streptococcus pyogenes</i>	Protein F	PrtF	Okada 1998
<i>Neisseria gonorrhoeae</i>	N-methylphenyl alanine pili	T4 pili	Swanson 1973
<i>Pseudomonas aeruginosa</i>	Pseudomonas aeruginosa lectin	PA-IL	Boteva 2005

Table 1: A table showing a selection of adhesin molecules associated with bacterial interaction with host surfaces which may be associated with initial adherence steps.

for Meat Safety and Quality showed that *E. coli* attachment to beef-contact surfaces was influenced by the type of soiling and temperature. Interestingly, attachment occurred not only at a temperature representative of beef fabrication areas during non-production hours (15°C), but also during cold storage (4°C) temperature, suggesting current procedures may not be adequate (Dourou *et al.*, 2011).

Orgad *et al.* (2011) showed that as well as genetic variation between strains being responsible for alginate production and biofilm thickness in *P. aeruginosa* strains, local concentrations of calcium and elevated ionic strength of the biofilm affected adherence and steric hindrance. Adhesins, therefore, have an additional role beyond that of the initial adherence phase. Many adhesins or fimbrial pili are coded for on genes that appear to have multiple functions which influence general virulence; for example, the MP65 gene expressed in *C. albicans* as well as having a role in adherence, exhibits increased cell wall stability and biofilm formation compared to an MP65 mutant strain (Sandini *et al.*, 2011). This co-coding of virulence factors on certain genes is seen in *S. epidermidis* with the CcpA gene (Sadykov *et al.*, 2011) and the *Yad* and *Ygi* genes in *E. coli* coding for motility, adherence, biofilm formation and *in vivo* fitness respectively (Spurbeck *et al.*, 2011). Biofilms also use adhesins in a role referred to as steric cohesion, and this involves the addition of polymers to the colloid to overcome net repulsive forces, to keep the biofilm stable and prevent flocculation or ‘flaking off’ of parts of the biofilm. However, flocculation in biofilms is an essential part of their life-cycle, allowing the bacteria to disperse and colonise elsewhere.

Biofilm dispersal

Dispersal or detachment is an important part of the biofilm 'life-cycle' and represents a return to the free-floating or planktonic state for some of the biofilm bacteria (Kierek-Pearson, 2005). Different definitions are applied to different forms of detachment, *sloughing* is where large portions of the biofilm itself detaches (Stoodley, 2001), compared with the release of free swimming cells, which is called *erosion* (Kierek-Pearson, 2005). Erosion may be responsible for the dispersal of bacteria within a flow-system, such as in endotracheal or tracheostomy tube sited in an airway. It has been shown the free swimming bacteria revert to a planktonic mode of growth, and therefore the 'life cycle' can turn full circle (Sauer *et al.*, 2002). However, these types of dispersal mechanisms were generally thought of as passive processes, occurring as a result of shear forces and other environmental factors acting on the biofilm surface (Purevdorj *et al.*, 2005). Only more recently has it become clear that dispersal is the result of an active process.

Biofilm dispersal is multi-faceted and a necessary modulation of the biofilm architecture and population for many different reasons. Living within a surface-associated multicellular biofilm has disadvantages, as well as advantages. Growth is restricted and the rate of bacterial DNA transcription resembles that of stationary phase cells (Waite *et al.*, 2005). Another of the disadvantages of bacteria cohabiting within a biofilm matrix assembly is an inability to move easily, either to more advantageous locations or to escape a hostile environment. This is seen in the Barraud and Hasset study in

2006, where nitric oxide was shown to initiate biofilm dispersal and inhibit biofilm aggregation, even at levels insufficient to be bactericidal (Barraud *et al.*, 2006) indicating a possible therapeutic role for nitric oxide in the management of biofilm infections. Nitric oxide was also found to sensitise the bacteria, so that they were vulnerable to concentrations of antimicrobials, which would not previously have been bactericidal. This potent effect of nitric oxide may be due, in part, to the fact that it is an endogenous product of anaerobic metabolism (Ren *et al.*, 2008), and therefore may represent an indicator of an impending hostile environment. It is, also, one of the methods that host defences fight infection. Nitric oxide has been shown to be used by phagocytes to kill bacteria, which have been ingested intracellularly (Forslund and Sundqvist, 1997), but also, that phagocytic activity increases in the presence of increased concentrations of nitric oxide (Zagryazhskaya *et al.*, 2010). Interestingly, some species have developed mechanisms to try to counteract intracellular antimicrobial activity to survive even after being phagocytosed (Fernández-Arenas *et al.*, 2009).

The regulation of adhesin synthesis could govern the cohesion and attachment of bacteria in biofilms (Thormann *et al.*, 2006), but this would depend on environmental erosion factors to actively decrease the size of the biofilm in the face of adverse circumstances. Alternatively the oral pathogen *Aggregatibacter actinomycetemcomitans* secretes a hydrolytic enzyme that cleaves poly- β -1,6-N-acetyl-D-glucosamine, which is a polysaccharide adhesin common to many species (Itoh *et al.*, 2005). It may

therefore be that stasis of biofilm mass is dependent on two balanced competing forces, one for cohesion and one for disaggregation.

Detachment has been shown to be crucial in the shaping of the morphological features and structure of mature biofilms (Piciooreanu *et al.*, 2001), which adds weight to the concept that detachment is integral to the function and behaviour of biofilms in general. This may be further fine-tuned for biofilm streamlining, as exhibited by *P. aeruginosa* biofilms existing within a flow system. Here the biofilm produces surfactants to remove some of the surface attached cells to further model the three-dimensional architecture, in effect, to make the biofilm itself more aerodynamic (Davey *et al.*, 2003), which would be advantageous if the biofilm was, for example, existing within a patient's airway.

Some biofilms can exhibit quite dramatic and devastating incidences of disaggregation; most notably the *P. aeruginosa* 'seeding' dispersal. This tends to occur in ageing *P. aeruginosa* biofilm biofilms, where internal microcolonies undergo disintegration leaving behind voids within the ageing biofilm (Purevdorj-Gage *et al.*, 2005). Seeding dispersal is caused by mediators released by the bacteria in anaerobic environments. This form of dispersal is often associated with a period of cell death as the biofilm attempts to lose some of its dependent bacteria, however, among the bacteria released, viable cells are also present that can generate new colonies elsewhere (Sauer *et al.*, 2004). A similar migratory behaviour is exhibited by *S. mutans* populations where the agglutinin specific adhesin P1 can be lysed by surface protein-releasing enzyme (SPRE) allowing the bacteria to detach from the pellicle to move to a less inhospitable site (Vats

and Lee 2000, Lee *et al.*, 1996). The marine bacteria *Bacillus licheniformis* has been shown to produce a powerful supernatant containing an extracellular DNase which rapidly breaks down the biofilms of both Gram-positive and Gram-negative bacteria; the authors hypothesise that this product not only disperses established biofilms but also acts to prevent the formation of new competing biofilms (Nijland *et al.*, 2010). Similarly the BdcA gene which had previously been shown to mediate dispersal of *E. coli* biofilms has been found to increase dispersal in other bacterial biofilms, including *P. fluorescens* and *Rhizobium meliloti* (Ma *et al.*, 2011).

The ability to trigger biofilm dispersal either through targeting cell-to-cell signalling apparatus or by using exogenous glycoproteins that hold the biofilm together, such as using SPRE, may prove to be important therapeutic tools to use against microorganisms that exist within a three-dimensional biofilm. When nutritional limitation or other unfavourable environmental factors limit further biofilm development in a given site (O'Toole *et al.*, 2000), it is felt that Quorum Sensing (QS) plays a role in detachment and dispersal.

Quorum sensing

Quorum sensing (QS) is the mechanism by which microorganisms within a biofilm recognise environmental changes and react to them, by the expression of certain genes only when a sufficiently high cell concentration has been reached (Fuqua *et al.*, 1996). This phenomenon is common to both

Gram-positive and Gram-negative bacteria using cell signalling to coordinate biofilm activity (Davies and Geesey, 1995). As the biofilm develops, sufficient numbers of bacteria produce the signalling molecules to generate a required response, by expressing specific sets of genes (De Lancey Pulcini, 2001).

QS systems generally consist of a four component circuit; *e.g.*, a LuxI-type signal synthase, a N-acylhomoserine lactone (AHL) signal molecule, a LuxR-type signal receptor, and the target gene (Atkinson *et al.*, 1999). The signal synthase enzyme synthesizes the AHL signal molecule at a constant low basal rate. As the bacterial population increases, so does the concentration of AHL in the environment as it diffuses from the growing cells. At a threshold local AHL concentration the signal receptor is triggered, to modulate the expression of the QS regulated genes (Hentzer *et al.*, 2003a) (Figure 2). Some QS pathways are subject to positive feedback, or auto-induction loops to allow a rapid and ever increasing production of signal molecules, in response to environmental stimuli, or a ‘critical mass’ of bacteria numbers. It has been postulated that this may be used to overwhelm host immunity (Donabedian, 2003). The biofilm essentially is stable until it has sufficiently high numbers of bacteria that lead to modulation of virulence genes, greatly increasing the biofilm’s chances of inducing an infection, allowing increased bacterial reproduction and enhanced spread (Hentzer *et al.*, 2003b). Similarly, the light producing bacteria *Vibrio harveyi* and *Vibrio fischeri* do not emit light unless they detect a high enough concentration of their own AHL (Gray and Garey, 2001).

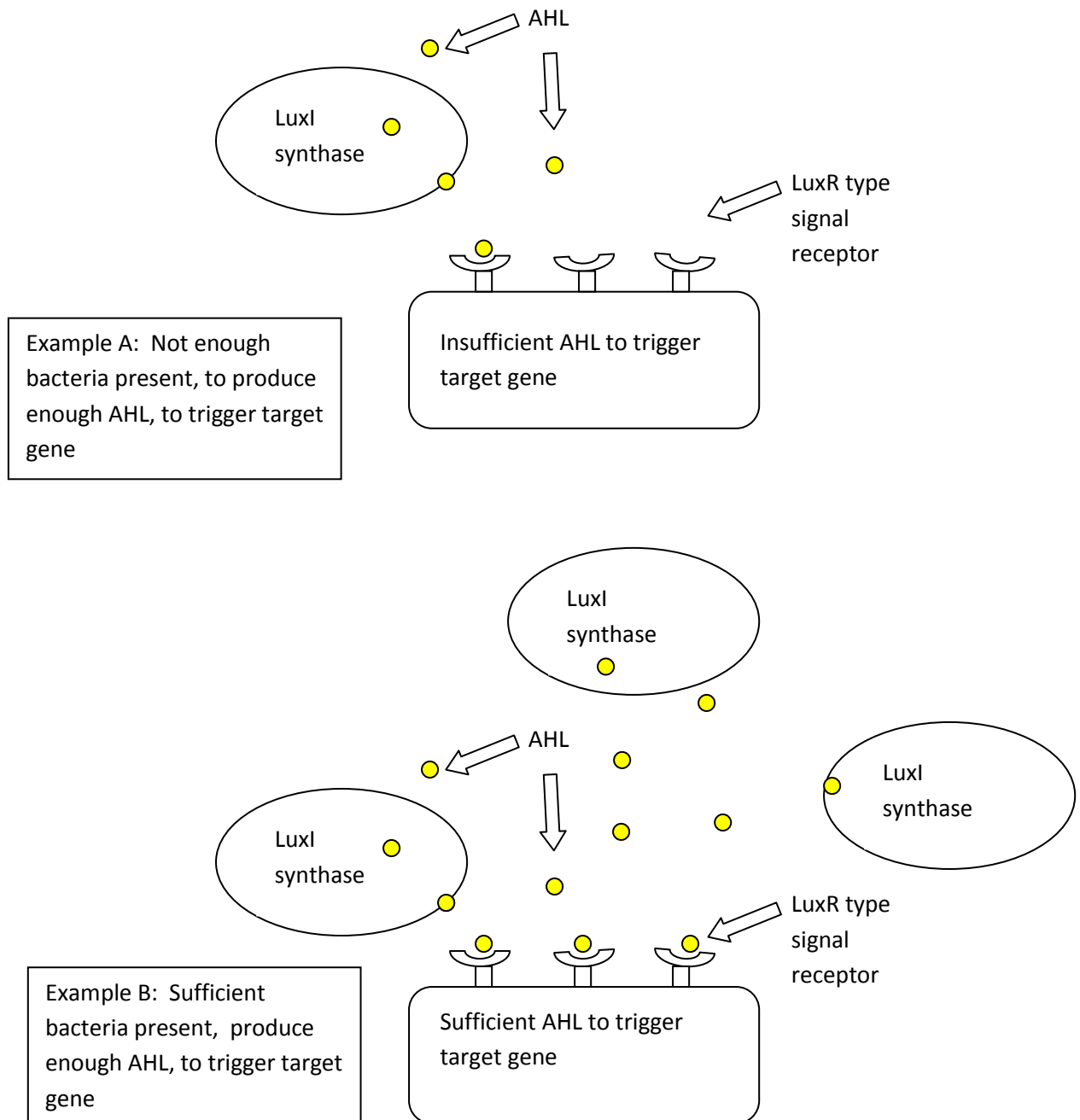


Figure 2. A pictorial schematic of Quorum sensing, showing that as bacterial concentration increases, so does concentration of AHL eventually reaching sufficiently high concentrations to trigger the target gene.

This suggests each bacterium's behaviour is affected by the presence of its own kind, and in sufficient numbers, in this case to make generating light worthwhile (Donabedian, 2003).

As well as mediating light production, or virulence factors, QS has been implicated as having a role in biofilm formation, structure and motility. Rapid motility or swarming of bacteria has been identified as being QS dependent in *Serratia liquefaciens* (Morohoshi *et al.*, 2007). Here the QS system is based around an *N*-butanoyl-L-homoserine lactone (BHL), which is also seen in *Aeromonas hydrophilia* biofilms in the gut of the common leech (Swift *et al.*, 1997).

As well as bioluminescence, virulence and motility, QS has also been shown to play a role in controlling metabolic rate in *Rhizobium leguminosarum*. In the case of the latter, expression of growth factor inhibitors occurs under conditions of carbon and nitrogen starvation, which serve to protect the biofilm in times of nutritional limitation (Thorne and Williams, 1999).

QS also has a role in biofilm defence mechanisms. For example, the plant pathogen *Erwinia carotovora* produces the antibiotic carbapenem under the influence of *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL) (Bainton *et al.*, 1992) and *Pseudomonas aureofaciens* produces phenazine antifungals via an *N*-hexanoyl-L-homoserine lactone (HHL) dependent QS circuit (Wood *et al.*, 1997). These give the biofilm enhanced defence capabilities against competing bacterial or fungal species. Atkinson *et al.* (1999) showed that a more complicated hierarchical QS system was involved in the

motility and clumping of *Yersinia pseudotuberculosis*, where an interactive regulatory cascade was able to respond to at least three different AHLs to affect secretion, motility, flagellin expression and morphology. A study by Davies *et al.* (1998) shows that the QS molecules produced by some Gram-negative bacteria, called acyl-homoserine lactones (HSLs), are responsible for modulating biofilm architecture. When the genes responsible for this molecule are ‘knocked-out’ of mutant strains of *P. aeruginosa*, they produce biofilms that lack the characteristic towers and water channels (Davies, 1998). Furthermore, the addition of HSL to a *Pseudomonas fluorescens* biofilm culture resulted in a significant increase in the biofilm mass and density of the extracellular polysaccharide matrix (Allison *et al.*, 1998). A table summarising QS molecules which are mediated through gene regulation is included below (Table 2).

Targeting of these QS systems for clinical reasons is called Quorum Quenching (QQ). This involves the specific targeting of a QS system, such as that linked to dispersal, to treat a biofilm infection, or to be used in conjunction with antimicrobials to make them more efficacious. By April 2012, 45 patents had been filed for QQ agents and this number is increasing (Romero *et al.*, 2012).

Specific QQ agents would be able to prevent dispersal or aggregation within a biofilm, and this would clearly have a clinical benefit, in preventing the spread of bacterial infections throughout a patient, for example.

Bacterium	Primary Signal Molecules	Regulatory proteins	AHL regulated properties
<i>Aeromonas hydrophilia</i>	BHL, HHL	AhyI/AhyR	Unknown
<i>Agrobacterium tumefaciens</i>	OOHL	TraI/TraR	Ti plasmid conjugal transfer
<i>Burkholderia cepacia</i>	OHL	CepI/CepR	Protease, lipase, and ornibactin synthesis
<i>Chromobacterium violaceum</i>	HHL	Unknown	Production of violacein pigment, exoproducts
<i>Erwinia cartovora</i>	OHHL	ExpI/ExpR-CarR	Carbapenem antibiotic synthesis
<i>Pantoea stewartii</i>	OHHL	EsaI/EsaR	Extracellular polysaccharide capsule, virulence factors
<i>Photobacterium fischerii</i>	OHHL, OHL	LuxI/LuxR	Bioluminescence
<i>Pseudomonas aeruginosa</i>	OdHL, BHL	LasI/LasR, RhlI/RhlR	Extracellular virulence factors, biofilm formation, rhamnolipid synthesis, twitching motility
<i>Pseudomonas aureofaciens</i>	HHL	PhzI/PhzR	Phenazine antibiotics
<i>Ralsontia solanacearum</i>	HHL, OHL	SolI/SolR	Unknown
<i>Rhizobium etli</i>	AHL	RaiI/RaiR	Root nodulation
<i>Rhodobacter sphaeroides</i>	7,8-cis-tDHL	CerI/CerR	Clumping factor
<i>Serratia liquefaciens</i>	BHL, HHL	SwrI/SwrR	Swarming motility, serrawettin W2 synthesis
<i>Vibrio anguillarum</i>	ODHL	VanI/VanR	Unknown
<i>Vibrio harveyi</i>	HBHL	LuxM/LuxN-LuxU-LuxO	Bioluminescence, polyhydroxybutyrate synthesis
<i>Yersinia enterocolitica</i>	OHHL, HHL	YenI/YenR	unknown
<i>Yersinia pseudotuberculosis</i>	?	YspI/YspR	Motility, morphology
<i>Xenorhabdus nematophilus</i>	HBHL	Unknown	virulence

Table 2: A selection of known Quorum Sensing (QS) molecules present in different bacterial populations and the genes and virulence factors they trigger; adapted and abridged from: Eberl, L. *et al.* 1999

Extracellular Polysaccharide Matrix

Extracellular Polysaccharide Matrix (EPS) constitutes 85% of the biofilm, with only 15% of the volume due to microorganisms (De Beer, 1994). Polysaccharide Intercellular Adhesin molecule (PIA) produced by *S. aureus* an important molecule in *S. aureus* biofilm formation, not only because it forms the basis of the matrix within which the staphylococci are embedded, but also for its role in promoting adherence (Costerton *et al.*, 2005). Synthesis of PIA is fundamental in terms of biofilm accumulation, because it ensures cell to cell adhesion of proliferating cells (Cramton, 1999).

Exopolysaccharides are divided into two main groups, homopolysaccharides and heteropolysaccharides (Sutherland, 2001); the difference being the makeup of each type, of exopolysaccharide. Homopolysaccharides consist of one monosaccharide molecule that is constantly repeated, whereas heteropolysaccharides are made up of large and different polysaccharide molecules (Pereira *et al.*, 2009).

Exopolysaccharide structure can vary significantly between biofilms produced by different bacterial species. Such differences occur not only in non-sugar aspects such as lactate, glycerol, acetate and phosphate (De Vuyst *et al.*, 2001), but also the carbohydrate compounds can contain varying amounts of acetylated amino sugars (Girard and Schaffer-Lequart, 2007).

While the composition of bacterial exopolysaccharides varies between species, it also differs with surrounding environmental factors, such as nutrient availability and bacterial growth phase (De Vuyst and De Geest,

1999; Panhota *et al.*, 2007; Fischer *et al.*, 2003). An understanding of the structure of various exopolysaccharides, is considered necessary to predict their physicochemical properties (De Philippis and Vincenzini, 1998). For example, certain biopolymers have been shown to have rheological properties, affecting viscosity, and may therefore be integral to the colloidal structure of biofilms (Sutherland, 1996). It is felt that the resistance of many bacterial biofilm species is due in part, to the poor antibiotic penetration through the EPS (Sutherland, 2001), and a mature biofilm exhibits significantly increased resistance to antimicrobial agents when compared with its planktonic counterparts (Jefferson, 2004).

Mechanically, the biofilm matrix allows bacteria to thrive in hostile environments, such as those encountered in device related infections such as in heart pacemakers and haemodialysis catheters (Costerton, 1999). Indeed, electron micrographs of these devices show layers of bacteria embedded within the matrix (Marrie, 1982; Marrie and Costerton, 1983). In addition to the mechanical resistance shown by these biofilms, the matrix protects the bacteria from dehydration, phagocytosis, antibody recognition and viral lysis (Pereira *et al.*, 2009).

The actual composition of the EPS matrix also allows bacteria to survive in hostile conditions, with species of cyanobacteria living within sand dunes and secreting UV-screen pigments (Bohm *et al.*, 1995). In addition, a number of cyanobacteria have been shown to be able to survive virtually without water, secreting both internal and external polysaccharides, which help to stabilize not only the external cell wall, but also internal macromolecular structures (Grilli Caiola *et al.*, 1993).

Microscopic imaging of biofilms using confocal scanning laser microscopy (CLSM) can reveal the structural complexity of a biofilm, with mushroom-like towers and water channels throughout (Stoodley, 1994; Figure 3). As the biofilm matures it develops into a heterogeneous structure interspersed with channels which allows nutrients and gaseous access to the deepest parts of the biofilm, as well as waste removal (De Lancey Pulcini, 2001). The heterogeneity of the biofilm allows different bacteria to exist within it, or even the same species to exist in different physiological states, for example in a *Pseudomonas* biofilm, some bacteria may be living in aerobic conditions while at lower oxygen concentrations, some will be living in anaerobic conditions (Xu, 1998). Until recently it had been assumed that diffusion of nutrients throughout a biofilm was constant, and various mathematical models had been proposed to describe it, however a recent paper by Van Wey *et al.* (2012) showed that diffusion within a biofilm is often depth dependent and not isotropic as previously thought, with enhanced diffusion within horizontal layers of the biofilm to offset the decreased diffusion through the horizontal layers of the biofilm (Van Wey *et al.*, 2012). These environmental differences within the biofilm allow for many separate ‘microenvironments’ to co-exist within the same matrix, and as a result different bacterial phenotypes can co-exist to the extent that antimicrobial uptake will vary in different areas of the biofilm based on bacterial metabolic rate (Stewart, 1994a; Stewart 1994b; Anderl, 2000).

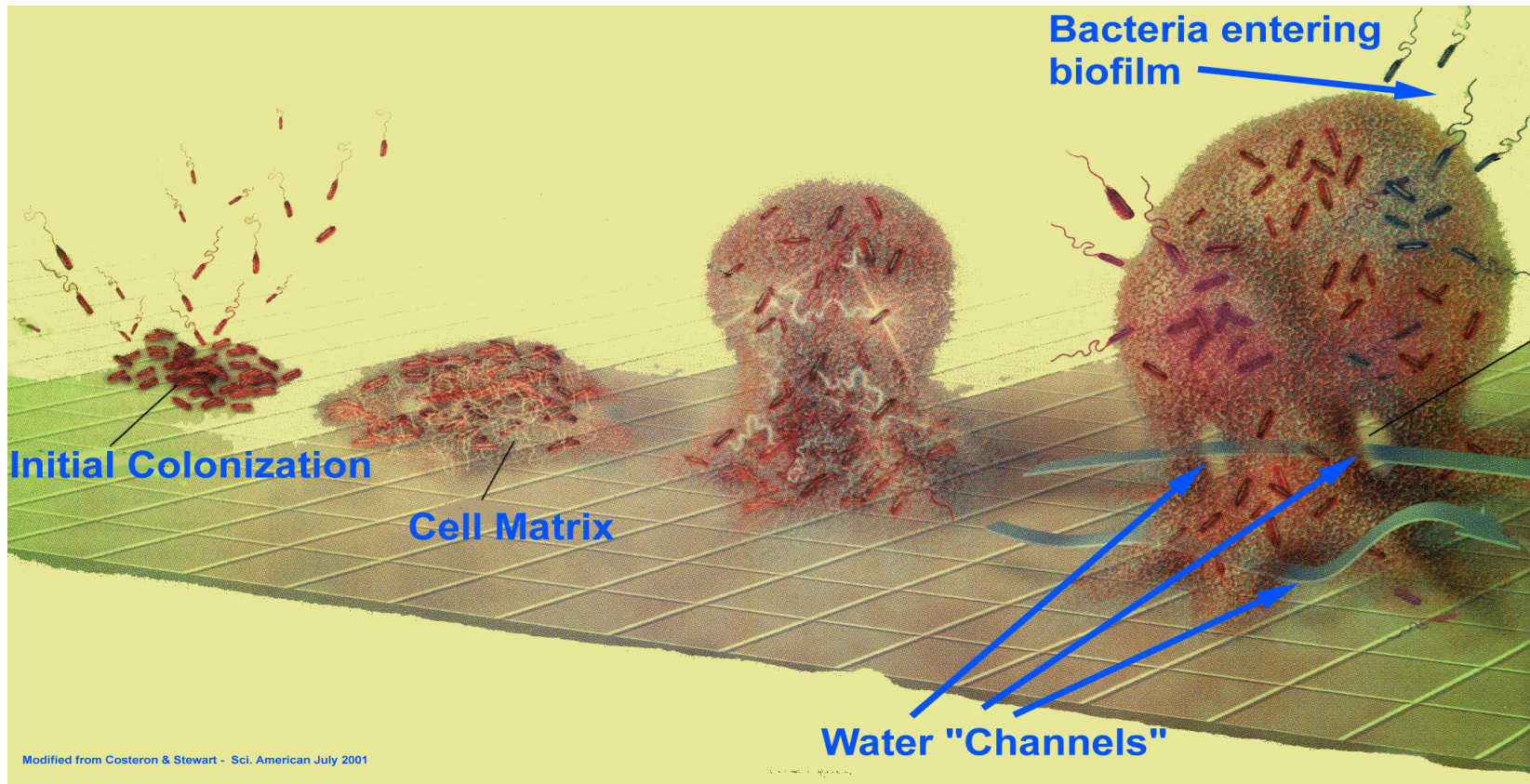


Figure 3: A stylised picture of biofilm development (Costerton and Stewart 2001), showing initial adherence and biofilm colonisation, subsequent biofilm growth, and maturation. There is also a diagrammatic representation of how the surface flora of a biofilm may be added to or altered by the addition of further bacterial numbers.

Biofilm resistance to antimicrobials and host defences

With the variation in bacterial metabolic rate evident within the biofilm, there is logically a variation in the effect of growth dependent antibiotics within the biofilm (Anderl, 2000) which, coupled with the impedance of the biofilm to antibiotic penetration, make the biofilm innately resistant to many antibiotics. For example, quinolones have poor biofilm penetration while glycopeptide or lipopeptide antibiotics (such as vancomycin or daptomycin) exhibit good penetration of staphylococcal biofilm matrices (Dunne, 1993; Stewart, 2009). This may explain the high prevalence of ciprofloxacin resistance exhibited by pseudomonads in chronic wounds (Howell-Jones, 2005). However, some studies have shown that most antibiotics diffuse freely across the biofilm (Shigeta, 1997), and that the biofilm matrix should not impede the diffusion of the small antibiotic molecules (Stewart, 1998) or that once the biofilm is saturated free diffusion could follow (Walters, 2003). Yet even where bactericidal concentrations are reached, bacterial biofilms remain resistant to killing (Darouiche, 1994), suggesting other mechanisms are responsible for the increased resistance seen in biofilm phenotypes (Hoyle, 1991). The use of daptomycin is a common treatment for MRSA, however, in biofilm models, such as implant-associated infection, a concentration of 500 µg/ml failed to kill adherent bacteria, when previously a concentration of 0.625µg/ml killed planktonic bacteria (John *et al.*, 2011). Notably, however, when calcium ions were increased to a physiological concentration, bactericidal activity was enhanced and prevention levels rose to almost 60% of normal.

Periplasmic sequestration of antibiotics by bacteria within a biofilm has been shown to reduce antibiotic efficacy by preventing the antibiotic reached its target site (Mah and O'Toole 2001). Analysis of the genetics of antibiotic resistant forms of *P. aeruginosa*, which can still form biofilm architecture, reveals the presence of the *ndvB* locus. This encodes for a periplasmic glucan which interacts physically with the antibiotic and these glucose polymers prevent the antimicrobials reaching their target, and sequester the antibiotics periplasmically (Mah, 2003). Antifungal resistant strains of *Candida albicans* have been shown to exist in biofilm form, but also interestingly in the absence of the specific antifungal (fluconazole), the antifungal sensitive strain of *C. albicans* was a much more virulent biofilm former and responsible for greater mortality in a mouse model, suggesting adaption to survival in different environments (Schulz *et al.*, 2011), but also that the genetic resistance prevalent in biofilms is not solely a survival benefit.

A further hypothesis extended from the decreased metabolism theories is that of the existence of persister cells within the biofilm (Moker *et al.*, 2010). This is based on the observations that even when 99% of a bacterial population is killed by minimum doses of bacteriocides, the remaining 1% of cells survive even in the face of greatly increased concentrations (Keren *et al.*, 2004), this may be due to a highly resistant phenotype, analogous to microbial spores (Stewart 1998; Lewis 2001). It is felt that the ability of bacteria to recolonise seemingly clean wounds, and the presence of multidrug tolerant biofilms can be explained by the theory of persister cells, and their ability to enter a dormant or non-dividing state (Lewis 2007).

Persister cells are also postulated to produce specific endogenous proteins which can turn off vulnerable areas of their metabolism. For example, glycopeptide antibiotics target translation within a metabolically active bacterium, and it has been shown that *E. coli* encodes for toxins such as ReIE, which will inhibit its own protein translation (Lewis 2005). It may be said, therefore, the persister cells are cells which altruistically forego propagation in order to ensure biofilm survival.

Additionally, the close proximity of bacteria within a biofilm microenvironment provides an excellent milieu for horizontal transfer of resistance genes. This, coupled with the accumulated density of mobile genetic elements from the variety of phenotypes present, means the biofilm state is an excellent environment in which to spread antibiotic resistance (Costerton *et al.*, 2005).

Horizontal transfer of mobile genetic elements, or conjugation between different species of *Pseudomonas* has been shown to be significantly increased in biofilms compared with planktonic cells (Ehlers, 1999). Conjugation is an important mechanism by which resistance is spread through bacterial populations (Gehring *et al.*, 2009). A number of environmental factors, including antibiotic exposure, and water and nutrient availability, can affect the frequency of conjugation (Johnsen and Kroer, 2007). These factors are all influenced by biofilm formation, and their relative resistance to penetration by antimicrobial agents, and the formation of water channels within the EPS. There is experimental evidence to show that the use of antibiotics against a bacterial population will actually promote the horizontal transfer of mobile genetic elements responsible for

resistance between individuals within a bacterial population (Showsh and Andrews, 1992; Sorensen *et al.*, 2005). The polymicrobial biofilms favoured by oral and intestinal bacteria favour horizontal gene transfer, using transposons or plasmids, to enhance virulence of normally apathogenic strains both within and between species (Hausner, 1999; Roberts, 1999), and allows bacteria such as enterococci, which are already inherently resistant to several antibiotics (Fabretti, 2005), to gain resistance to newer antibiotics such as vancomycin and teicoplanin (Leclercq, 1988; de Allori *et al.*, 2006).

Biofilm cells are also resistant against host defences such as neutrophils which eliminate bacteria by ingestion and the production of antimicrobial peptides (AMPs; Otto, 2006). However, there is increasing evidence that bacteria within biofilms are able to evade neutrophils (Leid, 2002) and protect themselves from phagocytosis (Jesaitis, 2003). Additionally, the EPS present in staphylococcal biofilms protects the bacteria from the action of AMPs possibly due to the cationic nature of the EPS repelling the positively charged AMPs (Otto 2006). The PIA may play a similar protective role in impeding the activity of positively charged aminoglycoside antibiotics (Kierek-Pearson, 2005). The N-acyl homoserine lactone QS produced by *P. aeruginosa* signals for the production of virulence factors such as rhamnolipids, which have been shown *in vitro* to eliminate neutrophils (van Gennip *et al.*, 2009). Indeed, examination of two different *Streptococcus pneumoniae* strains showed an increased ability of the biofilm forming strain to create EPS and also to translocate to the lungs

and brains of mice, suggesting extracellular matrix formation enhances the ability of pneumococci to cause disease (Trappetti *et al.*, 2011).

Biofilms involved in ‘healthcare associated infections’

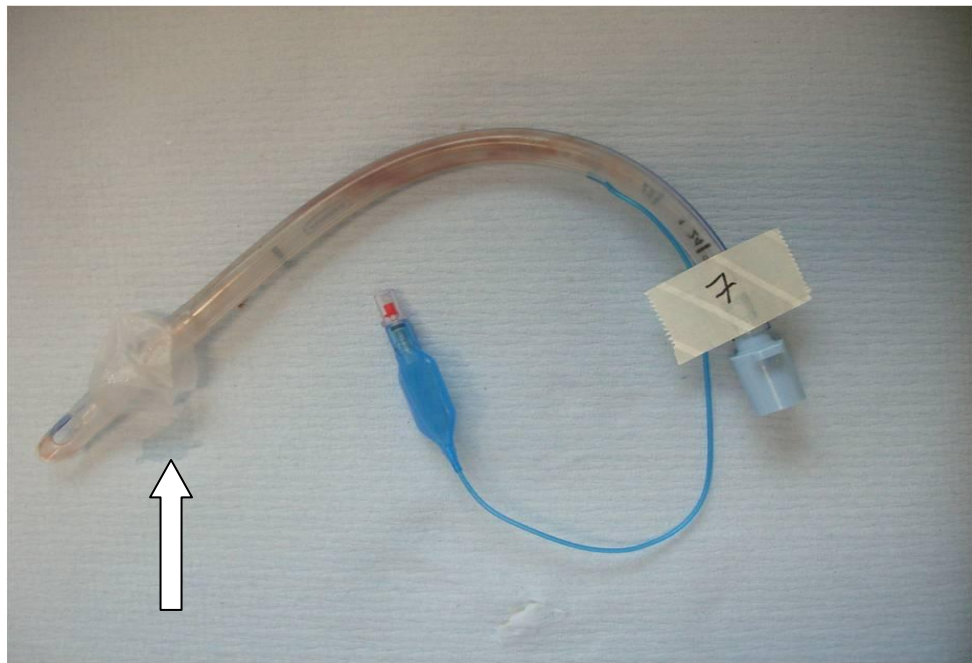
Biofilms on medical devices

Biofilms have been shown to exist on the surfaces on a diverse array of medical devices including contact lenses, breast implants and urinary catheters (Table 3) (Nickel, *et al.*, 1989; Garcia-Saenz *et al.*, 2002; Reiger *et al.* 2009; Costerton *et al.*, 2005). The presence of biofilms on medical devices can have significant implications for patient care. Not only do such biofilms cause increased rates of device failure necessitating the removal and possible replacement of the device with an increased risk to the patients, but their presence also increases the financial burden on the health care system (Bayston *et al.*, 2007). Furthermore, the presence of biofilms within medical devices also predisposes the patient to infections at other distal body sites such as septicaemias, urinary sepsis and pneumonia (Hussain, *et al.*, 1993; Hustinx and Verbrugh, 1994; Bauer, *et al.*, 2002; Di Filippo and De Gaudio, 2003).

Biofilms on endotracheal tubes (ETTs)

Endotracheal tubes are often used in the management of patients receiving intensive care. The ETT is inserted into the patient’s trachea usually through the oral cavity and is then secured in place with an inflatable balloon (Figure 4). The tube essentially closes off the alimentary tract from

Figure 4: Endotracheal tube (clinical specimen number 7) with arrow denoting inflatable balloon cuff at most distal end, there is a port at the most proximal end of the tube for inflating and deflating this ballon, and an adapter to allow the tube to be connected to the air circuit. Of additional note is the heavy soiling in the centre of the tube, at the point of maximum flexion, which most likely is an amalgamation of mucus, saliva and biofilm.



Device	Organism	Reference
Urethral catheter	Uropathogenic <i>E. coli</i>	Nickel <i>et al.</i> 1989
Contact lenses	<i>S. epidermidis</i>	Garcia-Saenz <i>et al.</i> 2002
Breast implants	<i>S. aureus</i> , <i>Propionbacterium acnes</i>	Reiger <i>et al.</i> 2009
Hip implant	<i>S. maltophilia</i>	Trampuz 2007

Table 3: Table showing commonly colonised medical devices, the bacteria found therein and the reference of the paper which first described it.

the patient's airway and this serves two functions; 1) it protects the patient's airway from aspiration of their gastric contents, and 2) it allows mechanical ventilation of the patient through a relatively robust and secure airway.

ETTs, therefore, tend to be used in patients who are unable to maintain and protect their own airway, for example if they are unconscious, or if they require increased respiratory support, for example in pneumonia or other inflammatory lung conditions.

Complications associated with ETT insertion may be due to the initial procedure, cannulation of the oesophagus, or impaction of the tube on the tracheal wall. Certain complications relate to the length of intubation, such as VAP or tracheal stenosis or tracheomalacia (Divatia, 2005; Table 4).

The incidence of VAP varies between 30.67 and 15.87 per 1000 ventilator days in a tertiary care facility (Joseph *et al.*, 2009). VAP is a significant cause of mortality in intubated patients (Chastre and Fagon, 2002) and biofilms within the ETT are heavily implicated in VAP (Sottile *et al.*, 1986; Adair *et al.*, 1999; Ramirez *et al.*, 2007).

Many bacterial species along with fungi of the genus *Candida* have been identified within the lumen of ETTs with species such as *Staphylococcus epidermidis*, *Klebsiella* spp., *P. aeruginosa* and *S. aureus* (Adair *et al.*, 1999; Feldman *et al.*, 1999; Bauer *et al.*, 2002; Boddicker *et al.*, 2006; Ader *et al.*, 2008) frequently encountered. Enteric bacteria have also been isolated (Adair *et al.*, 1999), as have other Gram-negative bacterial species

Complications of Endotracheal Tube Insertion

<u>At the time of Intubation</u>	<u>While ETT is in-situ</u>
Failed Intubation	Tension pneumothorax
Spinal cord injury	Pulmonary aspiration
Occlusion of central artery/blindness	Airway obstruction
Corneal abrasion	Disconnection
Orofacial trauma	Dislodgement
Noxious autonomic reflexes	Fire
Hypertension, tachycardia, bradycardia and arrhythmia	Unsatisfactory seal
Raised intracranial and intraocular pressure	Leaky Circuits
Laryngospasm	Swallowed ETT
Bronchospasm	Tracheomalacia
Laryngeal Trauma	Stenosis
Cord avulsions, fractures and dislocation of arytenoids	Ventilator Associated Pneumonia
Airway perforation	
Airway, oesophageal trauma	
Oesophageal intubation	
Bronchial intubation	

Table 4. Table showing the many complications associated with Endotracheal Intubation, both from the initial insertion of the tube, and the longer term complications that come from being intubated. Adapted from Divatia and Bhomwick 2005.

(Chen, *et al.*, 2007), showing that the ETT can play host to a wide range of microbial species. Dispersal from the luminal biofilm of viable cells, allows mobilisation of bacterial and fungal elements into the lower respiratory tract, enabling the development of potentially fatal respiratory infections (Pneumatikos *et al.*, 2009).

Many strategies have been employed to suppress the influence of the bacterial biofilms in VAP. The effectiveness of such interventions, including the use of nebulised antibiotics (Adair *et al.*, 2002), silver coated tubes (Kollef *et al.*, 2008), silver sulfadiazine and antiseptic coated tubes (Adair *et al.*, 1993; Jones *et al.*, 2002; Orhan-Sungur and Akca 2006; Berra *et al.*, 2008), along with tube sterilisation and sterile insertion techniques (Adair *et al.*, 2002; Triandafillu *et al.*, 2003; Balazs *et al.*, 2004; Cheung *et al.*, 2007), remain difficult to assess. This is due, in part, to the difficulty of diagnosing VAP, as opposed to respiratory infections already present prior to intubation, but not yet clinically apparent. Additionally the mortality of intensive care patients is often multi-factorial, and as such VAP may be an attributable factor in the patient's death, yet not be the main causative factor (Kollef *et al.* 2008). This makes it difficult to ascribe any decrease in mortality to one particular intervention

Ultimately, any intervention at the site of the ETT or tracheostomy tube, may reduce bacterial numbers at this site, yet have no measurable impact on overall patient mortality. The study performed by Rello *et al.* (2006) looked at 156 patients, in Spain and North America. The patients were intubated with either a silver-coated ETT (n=78) or a control device (n=77). This study looked at the rates of ETT and tracheal colonisation, and amount of

bacterial burden in ETT and tracheal aspirates. The researchers reported the differences between the two groups when comparing rates of colonisation were significant but allowed the fact that study limitations, including non-blinding, and non-matching of the population samples in terms of their Clinical Pulmonary Infection Score (CPIS) and variation of infection control practices in different centres may have impacted the results. Part of the problem is in examining sufficient numbers of patients, although the North American Silver-Coated Endotracheal Tube (NASCENT) trial examined 1932 patients who required endotracheal intubation in 54 North American centres. Kollef *et al.* (2008) found there was a reduced frequency of VAP from 7.5% for the control group to 4.8% to the treatment group. However, there was no difference in mortality rates, duration of intubation, or duration of ITU or hospital stay. Kollef *et al.* (2008) acknowledge that their study had limitations, notably, non-blinding of the participants, and the fact the study population did not reflect the greater population as whole, due to the inability to consent emergency intubations for the study. Also, three additional episodes of VAP in the control group would have rendered the results insignificant (Chastre, 2008).

The use of hexetidine (a lipophilic nonantibiotic antimicrobial agent) as a method for decreasing initial bacterial adherence to ETTs has been described with good effect in *in vitro* conditions (Jones *et al.*, 2002), but has limited effect after 21 days. Similarly Balazs *et al.* (2004) showed that sodium hydroxide and silver nitrate impregnated ETTs *in vitro* decreased bacterial adhesion, particularly that of *P. aeruginosa*, but this was only measured up to 72 h post-inoculation. Selective gastric decontamination

has been examined with a view to potentially decreasing the incidence of VAP. A study in 1993 (Adair *et.al.*, 1993) examined 11 ETTs from patients undergoing selective decontamination of the digestive tract using the antibiotics amphotericin B, tobramycin and polymyxin. These tubes were sectioned at their distal tip to look for any adherent bacteria present, as well as tracheal aspirates. The study showed a predominance of Gram-positive bacteria when assessed using standard cultural identification techniques. Interestingly, there were wide variations in the concentrations of antibiotics present, and in particular the Mean Inhibitory Concentrations (MICs) were deficient, and in the case of tobramycin the MIC was uniformly less than the median MIC. Despite limited success in *in vitro* and animal models, these techniques have yet to be translated to clinical practice. This may be due in part to the difficulty in recognising biofilm infections, and inherent difficulty in culturing the full range of microorganisms present using standard hospital culture techniques (Davey, 2000; Spratt, 2004), coupled with the relatively low bacterial numbers that detach and disperse from the sessile colonies (Costerton, 1999).

Biofilms in tracheostomy tubes

Under certain circumstances it may be necessary to replace the ETT with a longer term tracheostomy tube (Figure 5).

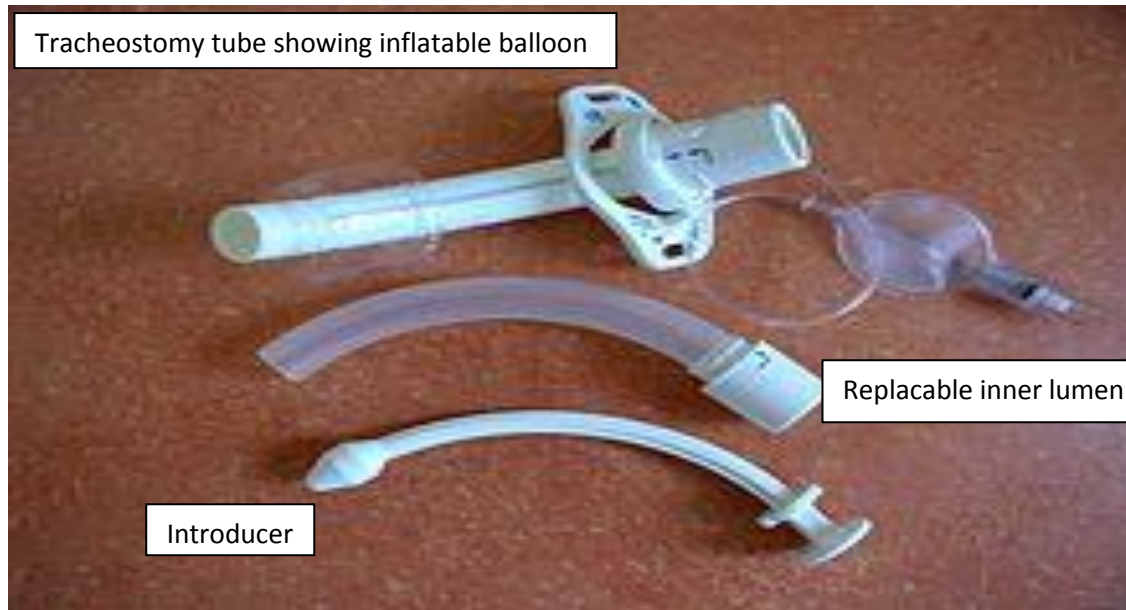


Figure 5: Photograph of a tracheostomy tube. The uppermost tube is the tracheostomy tube itself, which like the endotracheal tube has a balloon to inflate to secure it in the airway, beneath this is an inner liner for the tracheostomy tube, which in the model depicted may be removed so a clean inner may be inserted. The lowermost device on the picture is the introducer, which is inserted into the tracheostomy tube prior to being placed in the airway, so the rounded tip allows for less traumatic placement.

Tracheostomy tubes are usually surgically sited directly through the skin in the neck, below the level of the larynx, directly into the trachea, under direct vision. Tracheostomy tubes tend to be used where a patient is liable to need ventilation over a longer period of time, *i.e.* where the patient has significant underlying medical conditions, such as a large burn injury, or where existing lung morbidities suggest a quick extubation would be unsuccessful, and that a slow wean off the ventilator might be preferable (Jaeger *et al.*, 2002).

The main benefits of tracheostomy tubes over ETTs include a reduction in pulmonary workload (by eliminating dead space), reduced need for sedation, and assistance in weaning a patient off ventilation (Table 5).

In contrast to the large body of work regarding biofilms in ETTs, relatively little research has been done on tracheostomies, yet these too are associated with VAP (Keith, 1996). One study suggests a potential method for decreasing the incidence of ETT related VAP is for patients to undergo early tracheostomy, yet the authors admit this is still controversial (Ramirez *et al.*, 2007). Logically, however, tracheostomies would be susceptible to colonisation in a fashion analogous to ETTs, and indeed have been shown to harbour similar bacterial species (Jarrett *et al.*, 2002) interestingly, however, it has been shown that the flora within the tracheostomies of children can be subject to significant changes in bacteria or antibiotic sensitivity in two consecutive cultures (Cline *et al.*, 2012). It is possible that tracheostomy tubes, with their potential to be *in-situ* for longer, may have a greater risk of colonisation or device failure.

A recent study found that patients with significant burn injuries (Total Burn Surface Area (TBSA) >60%) were more likely to undergo tracheostomy, and were associated with a higher prevalence of chest infection (Aggarwal *et al.*, 2009), although this may have been due to a greater incidence of inhalational injury, and prolonged mechanical ventilation in this group.

Comparison of tracheostomy and endotracheal intubation	
Tracheostomy	Endotracheal intubation
Reduced need for sedation	Easier and quicker to perform compared with tracheostomy
Reduced damage to glottis	Tolerated well for short periods
Reduced work of breathing	Weaning more difficult after long period of placement
Reduced patient discomfort	Need to be sedated
More invasive and complicated insertion	Prevents aspiration of secretions
Scar formation	Can be used to give certain medications e.g. adrenaline
Tracheostomy site can bleed or become infected	Gases need to be warmed and humidified
Very skill intensive	Improper placement can occur
May be associated with long-term complications e.g. swallowing difficulties	

Table 5: Comparison of the possible advantages and disadvantages between using tracheostomy tube or endotracheal tube intubation.

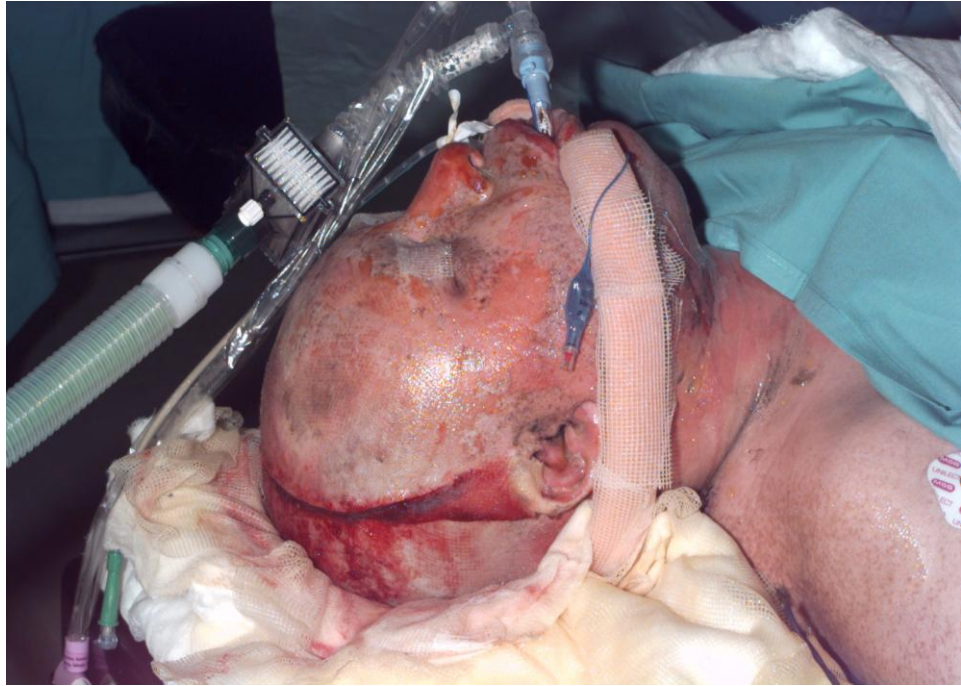


Figure 6: Facially burn injured patient, with an endotracheal tube in place, which can be seen entering the airway via the oral cavity.



Figure 7: Tracheostomy tube in place on a burn injured patient, with evidence of a constricting burn around the neck which has been released superiorly, the tracheostomy can be seen entering the airway through the anterior neck skin.

Biofilms in burn wounds

Burn wound sepsis is a frequent complication in the management of burns, and it is felt that wound infection is a common cause of wound progression or deepening (Singh *et al.*, 2007) and septicaemias. Early debridement or surgical removal of areas of full-thickness skin loss has significantly improved morbidity and mortality of burn-injured patients (Gray *et al.*, 1982). However, along with low platelet counts and ventilator dependence, sepsis remains a key source of mortality in these patients (Wang, Tang *et al.*, 2009). Despite the prevalence of infective complications, there is little evidence regarding biofilm formation within burn wounds. A recent study by Kennedy *et al.* (2010) shows that biofilms can form on burn wounds, and may therefore play a role in the delayed healing of some burn wounds. *Pseudomonas aeruginosa* is a common opportunistic pathogen in burn wounds, and has been shown to be an excellent biofilm former (Figure 8). Genome analysis of *P. aeruginosa* taken from a burn wound shows that genes responsible for infection, iron acquisition, toxin production and QS were expressed (Bielecki *et al.*, 2008).

Infection is the most frequent and most severe complication of burn injuries (Wassermann, 2001). The innate resistance shown by bacterial biofilms is often potentiated by the presence of highly resistant microbes which commonly infect burn-injured patients (Pruitt, McManus *et al.*, 1998) *e.g.* *Acinetobacter baumannii* is a frequent pathogen in burns and other ICUs.

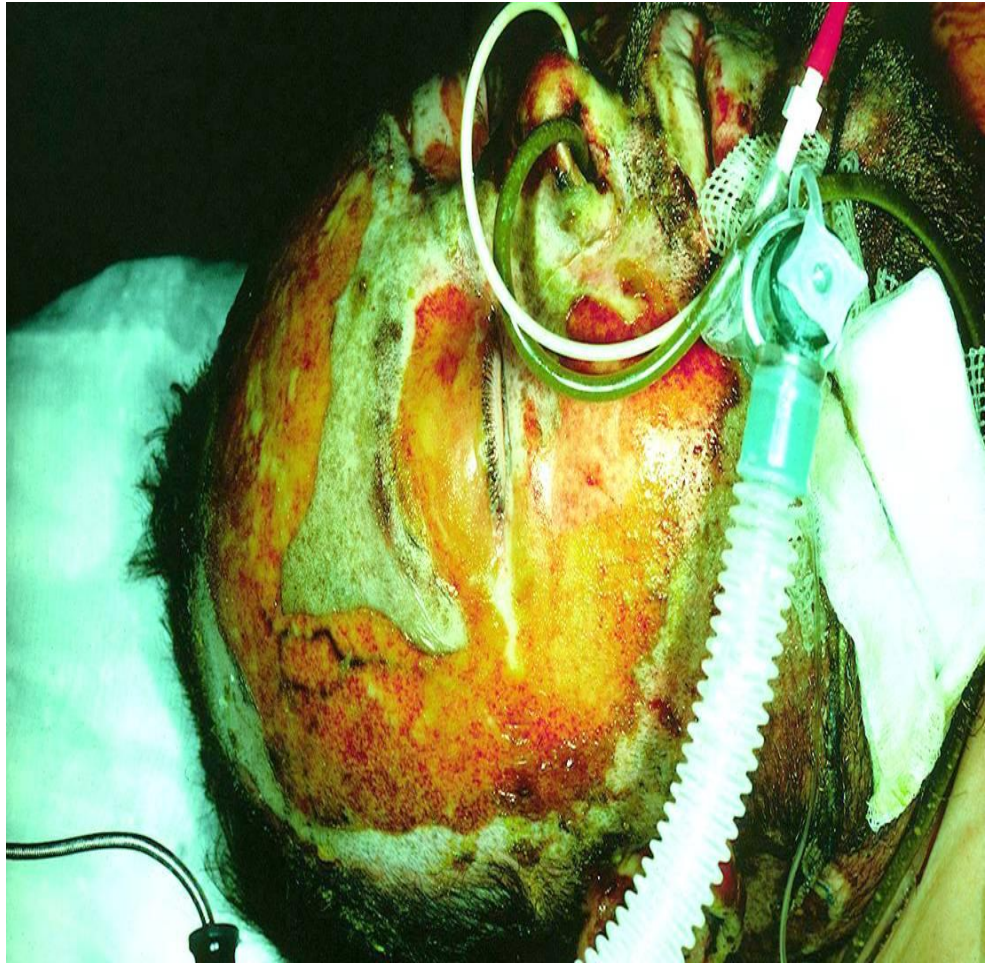


Figure 8: Severe facial burn, with an endotracheal tube in-situ, the large amount of facial swelling resulted in this being replaced with a tracheostomy tube. *P. aeruginosa* was grown from this burn.

A study looking at resistant rates in *A. baumannii* found in ICUs showed between 16 and 55% of isolates were resistant to ampicillin, 72-90% resistant to gentamycin, and up to 4% resistant to meropenem (Babiket *et al.*, 2008). Burns which are not treated with topical antimicrobials have been shown to contain up to 10^7 microorganisms per cm^2 , with normal skin microflora becoming opportunistic pathogens (Jones *et al.*, 1990). A Tunisian study looking at the incidence of multiple-drug resistant bacteria in intensive care settings found that 24.1% of isolates were resistant to two or

more antibiotics. The vast majority of these (82.6%) came from the burns unit. Enterobacteria resistant to third generation cephalosporins were the most common (47%), along with MRSA (29.2%), *A. baumannii* and *P. aeruginosa* were present in 14.8% and 9% of isolates respectively, with over half (51.7%) of the *A. baumannii* resistant to imipenem and ceftazidime, along with 20.5% of the *P. aeruginosa* (Thabet *et al.*, 2008).

The burn injury itself is known to cause a burn-related immunosuppression (Wurtz, Karajovic *et al.*, 1995). The burn causes a lowering in the levels of endogenous AMPs, which would ordinarily inhibit microbial colonisation, and therefore a predisposition to the formation of a biofilm within the wound occurs (Bhat and Milner, 2007). The presence of biofilm-forming bacteria within burn wounds has implications for their management. Burns of a partial-thickness depth, which would in the normal clinical course be expected to heal without surgery or complications, may then go on to become infected and deepen requiring surgical intervention. This scenario is becoming increasingly likely as silver dressings, which are commonly used in burn management, are having less effect. This is due to bacteria, and especially those within a biofilm becoming increasingly resistant to silver (Loh *et al.*, 2009; Woods *et al.*, 2009).

Management of Burn wounds

The decision of whether to treat a burn wound surgically or conservatively is largely based around a clinical estimation of the depth of the burn wound. The rationale being that a burn which has penetrated all of the layers of the dermis would take much longer to heal, and therefore, predispose to scar formation and the contracture problems inherent therein (Jaskille *et al.*, 2009). Many techniques have been employed to improve the estimation of burn depth with varying success (Jaskille *et al.*, 2010; Still *et al.*, 2001). Yet the goal of them all is the same: is there a reason that this burn wound would take longer than 21 days to heal? It is known that bacterial biofilms delay wound healing (Robson, 1997) and while there is a paucity of information regarding the presence of biofilms within burn wounds, it follows that a biofilm infection of a burn wound could induce a delay in healing in this scenario too. A method, therefore, to diagnose the phenotype of bacteria colonising a burn wound surface may allow the clinician to make a more accurate assessment of whether or not there is likely to be a delay in wound healing, and thus influence treatment modalities down a surgical or non-surgical route.

Biofilms in chronic wounds

Chronic wounds are a major cause of morbidity, affecting more than 1% of the UK population and are associated with treatment costs of at least £1 billion per year (Thomas and Harding, 2002). Chronic wounds are defined as any interruption in the continuity of the body's tissue that requires a

prolonged time to heal, that do not heal, or that recur (Wysocki, 1996). Examples of chronic wounds are VLU, arterial ulcers, diabetic neuropathic ulcers and pressure ulcers (Figures 9-12). Among the hallmarks of chronic wounds are the loss of tissue, underlying conditions such as diabetes, cancer, or arterial disease and the presence of bacterial colonisation (Wysocki 2002). The concept of a sterile wound is now obsolete, and microbial contamination of open wounds is now accepted (Schultz *et al.*, 2003).



Figure 9: Venous leg ulcer, on the medial side of the lower leg. Note the haemosiderin staining of the skin around the ulcer, which is a common outcome of venous stasis in the lower limb.



Figure 10: Arterial leg ulcer, note the absence of haemosiderin deposition, the necrotic centre of the ulcer, and generalised muscle wasting.



Figure 11: Diabetic neuropathic ulcer. This patient has an ulcer over the weightbearing point of the head of the fifth metatarsal which is a common site for these ulcers, additionally the dry cracked foot skin is typical of the sudomotor skin changes often seen in Diabetes.



Figure 12: Pressure ulcer. The sacrum is a bony prominence which often has problems with pressure damage. Here a healthy bed of granulation tissue can be seen in the base of the wound, which suggests it has been offloaded and treatment has already begun.

It is now considered that most wounds exist within a spectrum of infection, from surface colonisation through to frank spreading infection (Edwards and Harding, 2004). Microbial contamination of wounds, whilst being relatively ubiquitous, is still a problem, and can lead to significant morbidity and mortality, such as septicaemia. A mortality rate of 8%, which is attributed to septicaemia, is associated with pressure ulcers after spinal cord surgery (Reuler and Cooney 1981).

The presence of microbes within chronic wounds has been shown to significantly delay wound healing (Robson, 1997), although wound healing can still occur (Krizek and Robson, 1975). The number of microcolonies present has been related to the success of wound healing, within areas such as burns, pressure ulcers and skin graft take (Krizek and Davis, 1967; Robson and Heggers, 1969). However, the actual phenotype or species of bacteria present within wounds might be a more important correlator, with the biofilm phenotype being strongly implicated in delayed wound healing. Interestingly it has been shown that the presence of four or more bacterial species co-existing in a wound, perhaps in a biofilm phenotype, strongly correlates with delayed wound healing (Tregrove *et al.*, 1996; Hill *et al.*, 2003).

Biofilms have been shown to exist within chronic wounds (Davis, *et al.*, 2008) and the presence of biofilms is thought to have a deleterious effect on many of the treatment modalities usually used to control bacterial numbers, specifically silver-based dressings and antibiotics, both topical and systemic (Saye, 2007; Davis *et al.*, 2008; Morgan *et al.*, 2009). It has therefore been speculated, that different treatment options targeting the physiology of

biofilms should be adopted, such as QS antagonists (Smith and Bu, 2003), enzymatic debridement agents (Singh *et al.*, 2002) or antibiotics used to inhibit biofilm formation (Mitsuya, *et al.*, 2000).

It is clear therefore that a link exists between biofilms and HCAs, and that this link is often poorly highlighted in the clinical setting. Part of this is due to the difficulty in recognising biofilm infections and their colonisation of medical devices. Many hospital trusts in the UK have policies for the management of medical devices, whether it is the removal and replacement of cannulae at 48 hours or the role of a specific team in managing semi-permanent venous access lines, such as those used in long-term total parenteral nutrition patients. For UK-based practising clinicians, the microbiological load of a wound or device is still by and large, assessed using standard microbiological culture techniques.

Management of Chronic Wounds

There exists a massive multimillion pound industry to diagnose and manage chronic wounds. The crucial first step in managing a chronic wound is in diagnosis of aetiology. There are countless case reports of neoplastic lesions masquerading as chronic wounds (Schottler *et al.*, 2009; Nishimura *et al.*, 2010) as well as the eponymous Marjolin's ulcer, where a cancer can arise *de novo* within a chronic burn wound. While the clinical signs of a venous leg ulcer, or the symptoms associated with arterial disease are often clear, in a significant cohort of patients further investigation is warranted. Once, however, the diagnosis is made, an orderly progression to healing is expected. Unfortunately, this is not always the case, and it is now clear that

the presence of bacteria within a wound can significantly delay healing (Robson, 1997). Using an *in vivo* polymicrobial biofilm to infect mice wounds with a biofilm infection, it was noted by Dalton *et al.* (2011) that the polymicrobial wounds displayed an increased delay in wound healing and resistance to antimicrobials compared to mice infected with a single species biofilm, suggesting that a polymicrobial biofilm may contribute synergistically to a delay in wound healing (Dalton *et al.*, 2011). Currently detection of bacteria within wounds relies solely on the use of standard microbiological analysis using various culture techniques. This method does not tell the clinician about the phenotype present and whether or not a biofilm is present. Additionally, molecular techniques are being employed to assess the number of genera present in a wound (Han *et al.*, 2011). If a method could be found that allows rapid diagnosis of a polymicrobial biofilm infection in a chronic wound, this would have a significant impact on the potential treatment modalities the clinician could employ

Treatment of Biofilms

With a greater acceptance of the concept of biofilm infections, clinicians are attempting to combat them through a variety of means. Simply washing the wounds or cleaning medical devices such as tracheostomy tubes was not sufficient to remove biofilms (Silva *et al.*, 2012). Initially radical surgical debridement has been proposed as a method to physically remove the biofilm, although this has the downsides of often being skill and resource intensive, as well as often being only a temporary measure (Wolcott *et al.*, 2010). If a fungal element is suspected, often as a result of wound location (e.g. *Tinea pedis* the organism responsible for athlete's foot, if a wound is

on a foot), then antifungals may be employed to target the hyphal element of the biofilm. There have been reported uses of low-frequency ultrasound to break-up the biofilm and thus make it more susceptible to antimicrobials or to gentle debridement (Seth *et al.*, 2012; Apatzidou, 2012). Finding new agents to directly target the biofilms is currently following two paths. On one hand we have the scientifically sought Quorum Quenching (QQ) molecules to directly target known QS pathways (Romero *et al.*, 2012), and on the other, a group from Arkansas is examining whether traditional medicine applications that have been used to treat wounds historically have any effect on the formation of biofilms (Hobby *et al.*, 2011). Yet, successful treatment of any condition is first reliant on accurate diagnosis, and it is in this arena that this study concentrates.

Current methods of identifying and characterising bacterial biofilms

There exist multiple approaches for studying bacterial biofilms, including examining aspects relating to their formation, morphology and relationship with disease. In this present study, standard clinical microbiological culture methods were used with molecular and structural analytical methods. Standard culture techniques provide quantifiable data regarding the viable numbers of bacteria present, on specific media to assist characterisation. The role of standard culture media to analyse the bacteria present is invaluable, however it should be noted that many bacteria are simply not culturable using current methodologies due to a lack of understanding of their natural habitat and the inherent difficulty in recreating these

environments in the laboratory (Sprat 2004; Munson *et al.*, 2002). This is further complicated by difficulties in sampling the bacteria within a biofilm, and any co-dependencies on each other. Isolating these bacteria may deprive them of the complex intercellular cytokine signalling pathways they require to grow (Kell and Young, 2000). It is also felt there are a number of bacteria which exist in a viable, but essentially non-culturable state, maintaining a very low metabolic state, but not actually replicating (Lleo *et al.*, 2005). Molecular methods were also utilised in this present study to identify bacteria based on DNA content. Molecular analysis of bacterial DNA allows a more comprehensive investigation of the bacteria present. The presence of the 16s rRNA gene within all bacterial species makes it a highly useful tool for examining bacteria using ‘universal’ primers (Clarridge, 2004), thus providing accurate identification of both viable bacteria and non-viable bacteria which may be within the biofilm.

In the present study, identification techniques were supplemented with the use of molecular probes, capable of binding to specific bacteria in the biofilm, and thus allow them to be imaged using Confocal Laser Scanning Microscopy (CLSM). Species-specific peptide nucleic acid (PNA) probes can be used to identify multiple bacterial species within a biofilm (Malic *et al.*, 2009). This not only provides information about which bacteria are present, due to the specificity of the probes, but also on their spatial orientation within the biofilm. It could be argued that since the Extracellular Polymeric Substances itself have not been imaged, that the “true” biofilm is not being investigated. However, it is the bacteria which make the EPS, which form the biofilm, and which have the greatest clinical impact.

Aims

The key aims of this study were therefore as follows:

1. Collection of appropriate clinical specimens for analysis including, ETTs, tracheostomy tubes, excised burnt tissue, chronic wound biopsies and wound dressings.
2. Cultural analysis of biofilms from the clinical specimens using standard microbiological methods to determine the presence of microbial species.
3. Molecular analysis of clinical specimens to further elucidate the microbial diversity and composition of biofilms.
4. Utilise specific molecular probes to establish the biofilm structure and distribution of targeted species within biofilms.

Chapter 3: MATERIALS & METHODS

DESIGN

The design of this study was to examine bacterial biofilms in a clinical context, especially as pertaining to HCAs and those on medical devices. The initial aim of the study was to evaluate different methodologies for the identification and quantification of bacterial biofilms, and also to see how these different methodologies fared with different clinical samples. The first clinical setting chosen was that of endotracheal tubes, these devices are in contact with a known biofilm forming area (the oral cavity) and have also been shown to have a role in healthcare acquired infections such as ventilator-associated pneumonia (VAP). In addition, analysis of tracheostomy tube biofilm was incorporated into the study for comparison with the endotracheal tubes. Tracheostomy tubes are sited within the airway and may be associated with the occurrence of VAP but are not in direct contact with the oral cavity (Keith, 1996). Any oral bacteria found in these samples would have to either come from previous inoculation of the airway by the endotracheal tubes, or migrate from the lungs or oral cavity. Since the tracheostomies would be coming from burn-injured patients the third set of samples chosen to be examined were those of burn wounds themselves. Although not from the same patients, burn injured tissue is known to be a harbinger of infection, and the detrimental role of bacteria in burn wound healing is well known (Murless, 1940; Fadeyibi *et al.*, 2012). This deleterious effect of bacterial colonisation on burn wound healing is mirrored in the delayed healing of chronic wounds, especially those heavily contaminated by bacteria, presumably in the biofilm form. These different

sample types have a common theme of healthcare associated infection (HCAI) running through them, and by using cultural, molecular and structural analysis techniques the role of bacterial biofilms in these HCAIs may be assessed. Finally to elucidate whether information about biofilms may be obtained using non-invasive or destructive techniques, it was decided to assess and examine the dressings of the chronic wound patients to investigate whether dressing analysis might be used as a surrogate in the diagnosis of biofilm infections.

SPECIMEN ACQUISITION

3.1 Ethical considerations

Ethical approval was sought for this study for a number of reasons. The endotracheal and tracheostomy tubes, although ordinarily discarded after use, were being taken from patients who were critically-ill and therefore needed careful explanation of what was being undertaken. Additionally, wound dressings and wound biopsies, all potentially contain samples of human DNA, and therefore are covered under the Human Tissue Act (2004). The ethics committee required details of sample storage and subsequent disposal, and to ensure the researchers were blinded to patient identifiers. Subsequently, ethical and trust approval was obtained for the collection of all clinical specimens used in this study. (REC reference number: 08/MRE09/48 SSA reference number: 08/WMW02/61). For the endotracheal component of this study, ethical approval was sought, and following discussion with the ethics panel, it was deemed approval would not be required for the use of these discarded devices.

The consent for ETT specimen collection was obtained within the Intensive Care setting by a Senior Consultant anaesthetist. In all cases, for the tracheostomy tubes, wound dressings, chronic wound samples and burned tissue, written consent was obtained by the principal investigator prior to specimen collection. Written patient information was supplied to the patients 24 h prior to expected decannulation for the endotracheal and tracheostomy tube patients. The patients with chronic wounds and dressings were initially contacted in an out-patient setting, and supplied with written information. Arrangements were made at this time for the patients to return to The Department of Wound Healing, Cardiff, UK the next day to sign a consent form, and for sample collection.

Patients in the chronic wound and dressings cohort had the dressing removed from their wound and stored in a sealed bag, along with piece of moistened gauze to prevent dehydration of the specimen. In order to collect the biopsy, an area on the wound which appeared to be representative was identified. 5ml of 1% Lignocaine anaesthetic was injected subcutaneously around the identified area using a sterile technique. When sufficient time had elapsed to allow complete anaesthesia of the region a punch biopsy was performed (Figure 13) using a standard biopsy punch (Brymill Cryogenic Systems, UK). The specimen was then transferred immediately to the laboratory for specimen preparation. Patients admitted for surgery on their burn wounds were contacted by the principal investigator the day before their surgery, and supplied with written information. The next day, patients were approached to sign a consent form prior to entering the theatre suite. At all times, and with all the patient cohorts, great care was taken to

separate the principal investigator from the medical team caring for the patient, to ensure patients understood that refusal to participate in the study would not jeopardise their standard of care.

Once the patient had been anaesthetised and prepared on the operating table, the burn surgeon used an instrument called a Modified Braithwaite knife (Downs Surgical, Sheffield, UK) to shave off the top layer of burned skin (Figure 14), burned tissue is removed incrementally in a procedure called tangential excision, until a healthy base, onto which a skin graft could be placed, is reached. After the first pass of the knife, which usually removes surface tissue including biofilm, down to mid-dermis level, the excised tissue was passed out to the principal investigator who stored it in a sealed bag, along with a piece of moistened gauze, and then transferred to the laboratory for immediate specimen preparation.

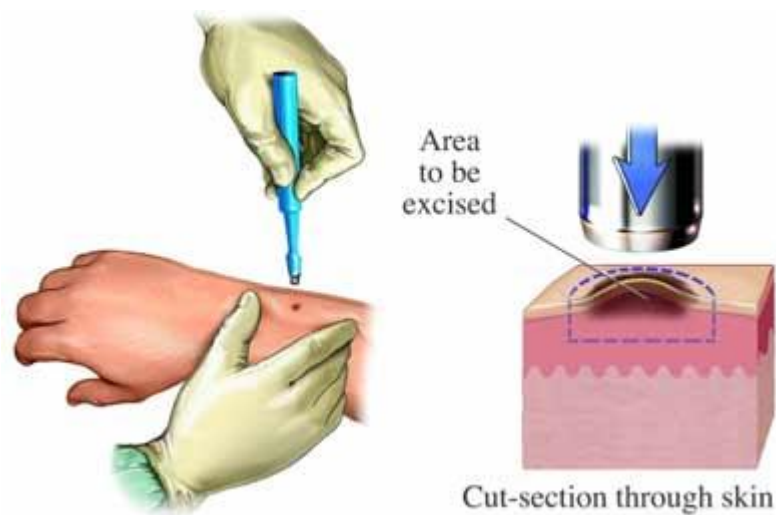


Figure 13: A drawing showing the technique used to perform a Punch biopsy, the cut-section of the left demonstrates how a specimen including the full-thickness of the epithelium may be obtained.



Figure 14: In this picture a Watson knife is being used to shave off the burnt upper layers of epidermis and dermis from the patient's right upper arm. This shaving is repeated until a healthy, non-burnt, bed is reached.

3.2 SPECIMEN PREPARATION

3.2.1 In vitro control biofilm preparation on endotracheal and tracheostomy tubes

A series of controls were employed in this study, predominantly to show that biofilms would form on the material of the tubes, and were subsequently recoverable.

Sections (1 cm) of sterile unused endotracheal tubes (ETTs) and tracheostomy tubes (TTs) were placed in glass universals with either Brain Heart Infusion (BHI) or Fastidious Anaerobic (FA) broth, and inoculated with known microbial species. The reference strains used were; *Staphylococcus aureus* NCTC 1671, *Candida albicans* ATCC 90028,

Pseudomonas aeruginosa ATCC 15692, *Streptococcus mutans* DSM 20523^T, and *Porphyromonas gingivalis* NCTC 11834^T. All media were inoculated to give a final bacterial concentration of 10⁴ cells/ml based on turbidity comparisons with McFarland indices.

Different incubation conditions were initially assessed for *in vitro* biofilm development. Incubation at 37°C for 48 h in an aerobic atmosphere for aerobic organisms was undertaken, whilst for the anaerobic species *P. gingivalis*, the standard incubation was 48 h at 37°C in 10% v/v CO₂, 20% v/v H₂, 70% v/v N₂ 36-37°C. In addition, tubes were also incubated in an anaerobic environment for 48 h before being transferred to an aerobic environment, whilst other replicate preparations were retained in an anaerobic environment for 96 h. Throughout the incubation period the glass universals were constantly rolled on a mixer to promote standardised biofilm formation. The tubes were then subjected to the same analytical preparation as described for the clinical specimens, outlined below (Section 3.2.2).

3.2.2 Collection and preparation of clinical endotracheal and tracheostomy tubes

A total of 25 ETTs were obtained from intensive care unit (ICU) patients (n=20), mean age 58.7 years (range 20 – 79 years) at the University Hospital of Wales, Cardiff, UK (Table 6). In addition, 10 tracheostomy tubes were obtained from the Burns Intensive Care Unit at the Welsh

Regional Centre for Burns and Plastic Surgery, Morriston Hospital, Swansea, UK (Table 7).

All tubes were immediately transported to the microbiology laboratory for urgent processing (Dental School, Cardiff) in sterile sealed plastic bags, containing dampened tissue to prevent sample drying. No tubes required storage and the longest period between decannulation of the patient and commencement of specimen preparation was 2 h.

Table 6: Demographics of endotracheal tube patients

ETT Number	Patient	Age	Gender	Admission diagnosis	Hours intubated
1	1	48	Male	Cerebral infarct post cranioplasty	33
2	2	78	M	Fourniers gangrene	108
3	3	20	M	Subarachnoid Haemorrhage (SAH)	60
4	4	77	M	Cardiac arrest	95
5	5	59	F	SAH/Crohns	43
6	6	41	M	Cerebellar infarct	25
7	2	78	M	Fourniers gangrene	94
8	7	62	M	Peritonitis	289
9	8	76	M	Abdominal Aortic Aneurysm	173
10	6	41	M	Bilateral cerebellar infarcts	41
11	9	74	F	Peritonitis	198
12	10	71	F	Peritonitis: perforated colon	56

ETT Number	Patient	Age	Gender	Admission diagnosis	Hours intubated
13	11	79	M	Gall bladder empyema	137
14	12	76	M	Perforated Duodenal Ulcer (DU)	12
15	12	76	M	Perforated DU	20
16	12	76	M	Perforated DU	96
17	13	49	M	Pancreatitis	224
18	14	77	M	Cardiac arrest-comm	49
19	15	52	F	SAH + pneumonia	29
20	16	38	M	Gastric varices/laparotomy	67
21	17	76	M	Trauma perf colon	20
22	18	77	F	Acute Subdural	76
23	19	44	F	SAH	32
24	17	76	M	Trauma perf colon	143
25	20	66	F	ARDS C.diff	125

Table 7: Demographics of Tracheostomy tube patients

Tracheostomy tube number	Patient number	Age	Gender	% of total body surface area burned	Hours intubated
1	1	34	M	55	1128
2	2	54	M	52	840
3	3	26	F	31	1209
4	4	42	M	33	912
5	5	32	M	26.5	504
6	6	38	M	37	240
7	7	41	M	23	135
8	8	34	M	0	696
9	9	41	M	85	422
10	10	18	F	46	552

The lumen of the tubes were then rinsed with 5 ml of Phosphate Buffered Saline (PBS; 7 mM Na₂HPO₄, 130 mM NaCl) to remove any loosely adherent bacteria,. Sectioning of the tubes was performed using sterile scalpel blades and scissors and three 1 cm long sections were generated. The portion of the tube chosen to be sectioned was the area with greatest curvature, and therefore where the airflow would likely create the highest level of turbulence. This also corresponded to the area with the highest amount of visible soiling. The most distal tube section was immersed in 4% (v/v) formalin prior to structural analysis, whilst the remaining more

proximal or cranial sections were divided longitudinally to give to semicircular lengths of tube.

Sampling involved the physical removal of the biofilm using a sterile number 11 scalpel blade to scrape off the adherent biofilm on the luminal surface into 1 ml of PBS in a Petri dish. The resulting solution was transferred to a microcentrifuge tube for subsequent cultural or molecular analysis as described in sections 2.3 and 2.4, respectively.

3.2.3 Preparation of recombinant human epithelium for construction of biofilms *in vitro*

Specimens of recombinant human epithelium (RHE) were obtained from SkinEthic Laboratories (Nice, France). These 0.5 cm² tissues consisted of human keratinocytes (human foreskin derived) with a well differentiated epidermis consisting of 4 separate layers of epidermis, the stratum basale or germinativum, stratum spinosum, stratum granulosum and finally the stratum corneum.

The bacterial species *S. aureus* NCTC 1671 and *P. aeruginosa* ATCC 15692 were cultured on blood agar at 37°C overnight in an aerobic environment. The resulting growth was used to inoculate 10 ml of BHI which was incubated for a further 24 h in aerobic conditions at 37°C. Bacterial cells were harvested by centrifugation and washed three times with PBS. The pelleted bacterial cells were resuspended in RHE tissue MCDB 153 maintenance medium (SkinEthic Laboratories). This chemically defined medium contained 5 µg/ml of insulin and 1.5 mM calcium chloride with no antibiotics. A 200-µl volume of the resulting

bacterial suspension added to the RHE and this was incubated for 24–28 h at 37 °C in a humidified atmosphere, enriched with 5% CO₂.

After a minimum period of 48 h, the RHE was transferred to and stored in 4% (v/v) formalin (Fisher Scientific Laboratories, Loughborough, UK). These were used as a baseline for the three dimensional structural analysis using confocal laser scanning microscopy (CLSM); see Section 2.5.3, allowing evaluation of the species specific probes.

3.2.4 Collection and preparation of burned tissue, chronic wound biopsies and chronic wound dressings

Five patients, who underwent excision of their burn wounds, consented for the discarded burn tissue to be used in this study (Table 8). The most superficial layer of burn tissue was collected and stored within moist gauze inside a sterile container for transport. From the larger portions of tissue provided, three 1-cm² sections were cut using either scissors or a scalpel blade. One section was then placed in 4% (v/v) formalin for a minimum of 24 h and the other two sections were placed into a Petri dish containing 1 ml of PBS. The surface of the wound or dressing was then scraped into the 1 ml of PBS to remove any biofilm, and then the resultant solution was transferred to a 1.5-ml microcentrifuge tube.

Ten patients receiving treatment for chronic wounds at the Department of Wound Healing, Cardiff, UK were approached and asked to donate their

used discarded dressings to the study. The dressings were placed in a clear plastic bag, with some moist gauze, for transport to the laboratory. In the laboratory, the dressings were cut into three 1-cm² sections, and prepared in an identical fashion to the burn tissue outlined above.

Of the ten ‘dressings patients’, five were additionally asked to provide a tissue sample, in the form a punch biopsy taken under local anaesthetic. For these patients, the total surface area of sample received was 6 mm², and this was further divided into 3 pieces and prepared in the same way as the burn tissue and dressings (Table 9).

Table 8: Burn patient demographics, days post-burn is included as this will have affected how long the burn has been exposed to antiseptics in the form of silver-based burn dressings.

Burn Specimen Number	Patient number	Age	Gender	% burned of body surface area	Days post-burn
1	1	28	M	2	19
2	2	64	M	1	5
3	3	26	M	4	16
4	4	54	M	3	18
5	5	32	M	3	24

Table 9: Demographics for Chronic Wound and Dressings patients, The wounds consisted of varying aetiologies, the dressings used were either hydrocolloid dressings (Aquacel), with or without silver (in the form of Aquacel Ag or Flamazine), or using a Iodine based non-adherent dressing (Inadine).

Wound and dressing number	Patient number	Age	Gender	Aetiology of Ulcer	Dressing regime
1	1	64	M	Venous	Aquacel Ag
2	2	44	F	Vasculitic	Aquacel Ag
3	3	67	M	Venous & arterial	Flamazine and Aquacel
4	4	76	M	Venous	Aquacel Ag
5	5	75	F	Diabetic	Inadine

3.3 MICROBIOLOGICAL CULTURAL ANALYSIS

Biofilm specimens were vortex mixed in 1.5 ml microcentrifuge tubes, and serial decimally diluted in PBS to 10^1 , 10^3 , 10^5 and 10^7 -fold. Fifty μ l was then plated on to BA, FAA and Sabouraud's Dextrose Agar (SDA) using a spiral plating system (Don Whitley Scientific, WASP); BA and FAA were supplemented with 5% (v/v) defibrinated sheep blood (TCS Biosciences Ltd., Buckingham, UK). The ETTs and tracheostomy samples were also plated on to Mitis-salivarius Sucrose Bacitracin (MSB) agar (supplemented with 0.2 U/ml bacitracin and 20% [w/v] sucrose 20% w/v; agar from Difco, supplements from Sigma). This agar is both selective and differential for *Streptococcus mutans* (Schaeken and Van Der Hoeven 1986). Wound

samples were additionally plated on MacConkey Agar (Lab M Limited). Agars were incubated at 37°C for 48 h in a standard aerobic atmosphere, or for anaerobes in 10% v/v CO₂, 20% v/v H₂, 70% v/v N₂ 36-37°C for 72 h.

Visual analysis was then undertaken for macroscopic/phenotypic variations of resulting colonies, and counting of the colony forming units present. Any yeast colonies subsequently cultured on SDA were sub-cultured on to Chromagar® *Candida* agar for presumptive species identification (in accordance with the manufacturer's recommendation). Bacterial colonies on the remaining plates were purified to monoculture prior to identification.

3.3.1 Identification of cultured isolates by PCR sequencing

DNA extraction was performed on isolated and purified colonies obtained by culture and directly on ETTs samples. The extraction was performed using a Puregene DNA isolation kit (Qiagen, Crawley, UK) using the manufacturer's Gram-positive bacteria and yeast extraction protocol (Appendix 9). Five colonies were suspended in 300 µl of Puregene cell suspension solution, and 1.5 µl of Puregene lytic enzyme was added. The solution was inverted several times to mix the reagents before being incubated for 30 min at 37°C. This was then centrifuged at 16,000g for 1 min, and the subsequent supernatant was carefully discarded. The cells were resuspended in 300 µl of Puregene Cell Lysis solution, along with 1.5 µl of Puregene RNase A solution, inverted to mix, and then incubated for 60 min at 37°C. This was rapidly cooled on ice, before the addition of 100 µl of

Protein Precipitation solution, and subsequent vigorous vortexing for 20 s. A further 3 min of centrifugation at 16,000g followed, the resultant supernatant was decanted into a fresh 1.5 ml microcentrifuge tube containing 300 µl isopropanol (Sigma-Aldrich, Dorset, UK) and inverted 50 times. A further 1 min of 16,000g centrifugation followed, prior to drainage of the supernatant and subsequent washing of the pellet in 70% (v/v) ethanol (Sigma-Aldrich). After air-drying the pellet, the DNA was dissolved in 100 µl of Puregene “DNA Hydration solution” prior to storage.

DNA was extracted from pure monocultures grown from the ETTs, and PCR was performed using previously described universal bacterial primers, d88 and e94 (Table 10; Paster *et al.*, 2001).

PCR thermal cycling was performed using the G-storm GS1 (AlphaMetrix Biotech, Rodermark, Germany) with 25 cycles of denaturation at 96°C for 10 s and annealing and extension at 60°C for 4 min. The PCR products were purified by adding a 40-µl volume of 5 M sodium chloride and 40% polyethylene glycol (mol. wt. 8000; Sigma) and centrifuging at 16,000g for 15 min. The supernatant was carefully aspirated and replaced with 200 µl of 70% ethanol, which was then centrifuged for a further 15 min. This previously described wash step was performed twice. The supernatant was then decanted, and the remaining specimen left to aseptically air-dry overnight.

The purified amplified DNA fragments were sequenced using 27F and 1492R primers (Table 10, Lane 1991) and an automatic DNA sequencer

(ABI Prism 3100 genetic analyser; Applied Biosystems). Sequences were identified by comparison to those held in the GenBank DNA sequence database (Benson *et al.*, 2005). Comparisons were done using the FASTA sequence homology search program found at www.ebi.ac.uk/Tools/fasta33/nucleotide.html (Pearson *et al.*, 1990). A >99% homology to the 16S rRNA gene sequence of the type strain, or other suitable reference strain, was the criterion used to identify an isolate to the species level. If there were no significant matches to known reference strains the identity of the isolate was based upon the results of the indiscriminate GenBank search. See Table 10 for a list of all primers used in this study.

3.4 Molecular Analysis

3.4.1 Extraction of DNA from clinical specimens

For the clinical specimens, both endotracheal and tracheostomy tubes, along with burn tissue, chronic wound tissue and dressings, 1 cm² sections were prepared as outlined in the sections above (section 3.2.2 and 3.2.3). These were placed into sterile plastic containers, and the biofilm on the surface was scraped into 1 ml of PBS. DNA was then isolated as described previously using the Puregene kit (Qiagen) and following the “DNA Isolation From Gram-positive Bacteria Culture Medium protocol” (see Section 3.3.1.).

3.4.2 Detection of bacterial species by species specific PCR

Species-specific PCRs was used to detect two oral bacterial species, namely *Streptococcus mutans* and *Porphyromonas gingivalis*, and the opportunistic pathogens, *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

For *S. mutans* PCR, the primers 479F and 479R were used (Table 10, Chen *et al.*, 2007). The PCR cycling parameters involved initial template DNA denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 30 s, 65°C for 30 s and 72°C for 1 min, with a final product extension cycle of 5 min at 72°C. In the case of *P. gingivalis*, the primers used were pGing 16S-1 and pGing 16S-2 (Table 10; Kulekci *et al.*, 2007). Cycling parameters used included an initial 3 min cycle of denaturing at 94°C, prior to 36 cycles of 94°C for 45 s, 55°C for 30 s and 72°C for 45 s, before a final elongation step at 72°C for 10 min. The primers for *S. aureus* were Vick1 and Vick2 (Table 10; Liu *et al.*, 2007), these samples were denatured for 5 min at 94°C, before undergoing 35 cycles of 94°C for 40 s, 50°C for 40 s and 72°C for 1 min, prior to a final single 10 min cycle at 72°C. Finally, for *P. aeruginosa*, the primers ECF1 and ECF2 were used (Lavenir *et al.*, 2007) See Table 10. PCR cycling consisted of a single denaturation cycle of 95°C for 5 min, before undergoing 35 cycles of 94°C for 45 s, 58°C for 45 s and 72°C for 1 min, before a final 5 minutes 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 40mM Tris, 20mM acetic acid and 1mM EDTA buffer at 40 V/cm² for 45 min. The products were run alongside standard 100 base pair marker DNA ladders (Promega). Completed agars

were visualised under ultraviolet light using a GelDoc system (Bio-Rad, Hemel Hempstead, UK).

3.4.3 Denaturing Gradient Gel Electrophoresis (DGGE) to determine microbial diversity within samples

Denaturing Gradient Gel Electrophoresis (DGGE) profiling was used alongside the microbiological culture approach to assess the diversity of the biofilm.

3.4.4 Preparation of parallel denaturing gradient gels

Polyacrylamide gels were cast using a model 385 gradient former (Bio-Rad). All gels consisted of 1×Tris-acetate-EDTA (TAE) buffer comprising 40mM Tris, 20mM acetic acid and 1mM EDTA, with 10% (w/v) acrylamide, 0.1% (v/v) TEMED, and 0.1% (w/v) ammonium persulphate (all materials from Sigma). Gels also contained a parallel 30-60% gradient of denaturants (where 100% denaturant concentration consisted of 7 M urea and 40% [v/v] deionised formamide; Sigma, Dorset, UK).

3.4.5 Resolution of PCR products by denaturing gradient gel electrophoresis

Parallel DGGE was performed using the Bio-Rad D-Code system. Following DNA extraction using the Puregene protocol outlined above

(Section 2.3.1.). PCR was performed using the 341F and 534R primers (Rolleke *et al.*, 1996). Denaturing of PCR product was carried out at 94°C for 1 min, primer annealing was performed at 55°C 1 min, and primer extension was performed at 72°C for 3 min. Products from these ETT PCRs were run alongside a marker comprising of an equal mixture of PCR products from the three known bacterial stains. The use of a marker allowed later normalisation of the gels and an early indication of possible target microorganisms in the clinical samples.

DGGE was run in 1×TAE buffer at 56°C with a current of 170 V/cm² for 20 min, followed by 40 V/cm² for 16 h. In order to stain PCR products in the gel, the gel was placed in a solution of 300 ml of 1x Tris-Acetate EDTA and 30 µl of SYBR green I (Sigma), and agitated for 30 min. Stained gels were then visualised under ultraviolet light using a GelDoc system (Bio-Rad). DGGE banding profiles were then analysed using GelCompar II software (Version 5.1, Applied Maths Software) for calculation and construction of comparative dendrograms. A dendrogram of genetic similarity between samples was calculated. The presence of individual bands in each profile was assessed using the same software, set to a 1% tolerance of band matching. This study used Dice's coefficient, in preference to Jaccard or Sorensen similarity matrices, due to Dice's being relatively more common, with fuzzy logic and the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering method to assess similarity in construction of a dendrogram.

Species targeted	Primer name	Primer (5'-3')	Number of base pairs	Reference
Most	d88	GAGAGTTTGATYMTGGCTCAG	Unknown	Paster <i>et al.</i> 2001
	e94	GAAGGAGGTGWTCCARCCGCA		
Universal	27F	AGAGTTTGATCMTGGCTCAG	Unknown	Weisburg <i>et al.</i> 1991
	1492R	ACCTTGTTACGACTT		
<i>S. mutans</i>	479F	TCGCGAAAAAGATAAACAACA	479	Chen <i>et al.</i> 2007
	479R	GCCCC TTCACAGTTGGTTAG		
<i>P. aeruginosa</i>	ECF1	ATGGATGAGCGCTTCCGTG	528	Lavenir <i>et al.</i> 2007
	ECF2	TCATCCTTCGCCTCCCTG		
<i>S. aureus</i>	Vick 1	CTAATACTGAAAGTGAGAAACGTA	289	Liu <i>et al.</i> 2007
	Vick 2	TCCTGCACAATCGTACTAAA		
<i>P. gingivalis</i>	pGing 16S-1	AGGCAGCTTGCCATACTGCG	404	Kulecki <i>et al.</i> 2007
	pGing 16S-2	ACTGTTAGCAACTACCGATGT		

Table 10: Primers used in PCR in this study, their target genes and the references which validated their use.

3.5 BIOFILM STRUCTURAL ANALYSIS

Biochemical structural analysis was determined using a combination of peptide nucleic acid (PNA) probe hybridization and CLSM. Initially, the specificity of PNA probes for target species was established through evaluation using planktonic cultures.

3.5.1 PNA probe evaluation on planktonic cultures

Initial CLSM studies were undertaken on known species of microorganisms, using a universal and species specific PNA probes to optimise the technique and confirm probe specificity. The probes were manufactured by Boston Probes for Applied Biosystems, USA. The universal bacterial probe (Bac-Uni-CY3) was labelled with the fluorophore cyanine 3 (Cy-3), and excited at a wavelength of 543 nm. The *P. aeruginosa* probe was labelled with fluorescein isothiocyanate (FITC) and was excited at 488 nm, whilst the *S. aureus* probe was coupled with cyanine 5 (Cy-5) and excited at 633 nm. Additionally, a fourth custom probe was manufactured specific to *S. mutans* with a label that excited at 405 nm (Alexa 405). (Table 11).

Target	Fluorescent label	Nucleotide sequence (5'-3')	Conc. (nM)	Wavelength (nm)	References
PNA <i>P.aeruginosa</i>	FITC	AACTTGCTGAACCAC	300	488	Coull & Hyldig-Nielsen (2003)
PNA <i>S. aureus</i>	Cy-3	GCTTCTCGTCCGTTTC	500	633	Perry-O'Keefe <i>et al.</i> (2001a)
PNA <i>S. mutans</i>	Alexa 405	ACTCCAGACTTTCCTGAC	150	405	Thurnheer <i>et al.</i> (2001)
PNA Universal bacteria	Cy-5	CTGCCTCCCGTAGGA	150	561	Perry-O'Keefe <i>et al.</i> (2001b)

Table 11: Summary of fluorochromes used in this study. Table listing targeted organism and the sequence of the PNA probes, with their associated fluorophore label and the frequency at which it fluoresces.

The test species were *S. aureus* NCTC 1671, *P. aeruginosa* ATCC 15692, *S. mutans* DSM 20523^T, *P. gingivalis* NCTC 11834^T, and *C. albicans* ATCC 90028, no specific probe was used for *C. albicans* as this was visible without a fluorescent label. The organisms were cultured on the appropriate culture medium (BA, FAA or Sabouraud's dextrose agar) for 24 h, and colonies sub-cultured into corresponding liquid medium (BHI, FAA or Sabaroud broths) for incubation under appropriate gaseous conditions overnight at 37°C. The resulting microbial growth was pelleted by centrifugation (13,000g for 5 min) and resuspended in PBS. These suspensions were again centrifuged and resuspended in PBS with 4% (w/v) paraformaldehyde (Sigma) and fixed for 1 h. The fixed cells were rinsed in PBS, resuspended in 50% (v/v) ethanol and placed at -20°C for at least 30 min.

A 100-µl volume of fixed cells was then pelleted by centrifugation and rinsed with PBS and resuspended in 100 µl of hybridization buffer (25mM Tris-HCl, pH 9.0; 100mM NaCl; 0.5% (w/v) SDS) containing 150 nM of the fluorescently labelled probe. The preparation was incubated for 30 min at 55°C and then centrifuged (13,000g, 5 min) and cells resuspended in 500 µl of wash solution (10mM Tris, pH 9.0, 1mM EDTA). The cells were then incubated at 55°C for a further 10 min prior to pelleting by centrifugation. This procedure was repeated on two occasions.

Following the last wash, the cells were suspended in 100 µl of wash solution, 2 µl of this preparation was then spread onto clean microscope slides and allowed to air dry, before being treated with Vectashield mounting medium (Vector Laboratories Ltd, Peterborough, UK) and then

overlaid with a coverslip. These were then visualized using a Confocal Laser Scanning Microscope (Section 3.5.4.).

3.5.2 Processing of RHE samples and chronic wound and burn tissue samples for CLSM

The 0.5 cm² of RHE specimens, the 1 cm² sections of burn tissue, and the 6 mm² of chronic wound biopsy tissue which had previously been fixed in 4% (v/v) formalin (see sections 3.2.3 and 3.2.4 for specimen preparation) for a minimum of 24 h, were then processed through a graded alcohol series before embedding in paraffin wax. A section of material was then cut (20 µm), placed on microscope slides and rehydrated to water through a decreasing alcohol concentration gradient.

One hundred µl of hybridization buffer (25 mM Tris-HCl, pH 9.0; 100 mM NaCl; 0.5% (w/v) SDS; Sigma) containing 150 nM of the fluorescently labelled probe was then applied to the surface of the slide. The slides were incubated for 60 min at 55°C. The probes were then removed from the section by gentle pipetting. The slides were washed using 100 µl of wash solution (10mM Tris, pH 9.0, 1mM EDTA; Sigma) and then incubated at 55°C for a further 10 min. This was repeated on two further occasions.

Following the last wash, the slides were mounted with Vectashield (Vector Laboratories Ltd, Peterborough, UK) mounting medium and overlaid with a coverslip. These prepared slides were then submitted for examination by CLSM (Section 3.5.4.)

3.5.3 Processing of clinical and control endotracheal tubes and tracheostomy tubes for CLSM

Initially, the tubes were processed on to microscope slides, at section thicknesses of 5 μm , 10 μm and 20 μm . In addition, 2 mm sections were also similarly prepared and placed into the wells of standard 12 well microtitre plates. These microtitre plate sections came directly from 4% paraformaldehyde immersion and required no rehydration or wax removal steps.

For the tube sections four previously validated PNA probes were utilised, the *S. aureus* specific PNA probe, *P. aeruginosa* probe, *S. mutans* probe and the universal probe. These PNA probes were pipetted into the lumen of each tube, and then incubated in a dark 55°C incubator for 1 h. These probes were then carefully pipetted off and replaced with pre-warmed wash solution; this was repeated on two more occasions. Prepared samples were examined using CLSM. Segments of tissue and dressing samples were examined using both the slide and microtitre plate preparation methods prior to CLSM.

3.5.4 Confocal Laser Scanning Microscopy

The Leica TCS SP2 AOBS spectral confocal microscope (Leica Microsystems, Wetzlar, Germany) was used to analyse specimens in both a standard upright and inverted mode. The upright stand was used for applications involving slide-mounted specimens; whereas the inverted

stand, which had a 37°C incubation chamber and CO₂ enrichment accessories, was used for live cell applications. The TCS SP2 AOBS confocal system had four lasers (one diode, one argon and two helium neon lasers) allowing excitation of a broad range of fluorochromes within the UV, visible and far red ranges of the electromagnetic spectrum. The upright microscope also had a transmission light detector making it possible to overlay a transmitted light image upon a fluorescence recording. The microscope was controlled by Leica confocal software, which allowed 3D reconstruction of the images and measurements.

Chapter 4: RESULTS

4.1 Bacterial and fungal growth on culture media from control and clinical specimens

4.1.1 Cultural analysis of control endotracheal and tracheostomy tubes

The plates from the control tubes which had previously been inoculated with strains of *P. gingivalis*, *P. aeruginosa*, *S. aureus* and *S. mutans* were examined at 24, 48 and 72 h. No quantitative measurements of bacterial numbers were made from control tubes. The isolates which were recovered by cultural methods were subsequently identified using molecular analysis to confirm that DNA from the inoculated species could be recovered. In all cases, *S. aureus* and *P. aeruginosa* were readily detected. *Streptococcus mutans* was detected from one of the six tubes, and no *P. gingivalis* was detected.

4.1.2 Cultural analysis of clinical endotracheal tubes

Agars were examined at 48 h for the number of visibly different colony types present, and these were enumerated to give a number of colony forming units (CFUs) per ml since each ml was taken from a 1cm² section of endotracheal tubes, the number of CFUs detected on the plates represented the number of CFUs per cm² of endotracheal tube lumen. The number of CFUs varied between 2.1 x 10⁸ per cm² on Blood Agar for tube number 20, to no growth on Blood Agar on tubes 2, 3, 8, 21 and 24. Of these, only tubes 21 and 24 had no bacterial growth, although specimen

number 24 was among those positive for *Candida albicans*. In total, seven ETT specimens were positive for growth of *C. albicans*. These specimen numbers were 5, 10, 15, 16, 19 and 23.

Strictly anaerobic or facultatively aerobic bacteria were grown on Fastidious Anaerobic Agar were recovered from 16 of the 25 specimens, with a range of 2.1×10^8 per cm^2 from specimen number 13, down to no growth on FAA on tubes 1, 2, 7, 9, 10, 19, 21, 24. The only specimen to not have grown any bacterial or fungal colonies was tube 21 (Table 12)

ETT Num.	Pt.	Media:	BAP	MAC	FAA	SAB
		Hours intubated	CFUs per cm ²	CFUs per cm ²	CFUs per cm ²	CFUs per cm ²
1	1		4 x 10 ⁴	8 x 10 ⁴	0	No growth
2	2	108	0	2 x 10 ⁴	0	No growth
3	3	60	0	4 x 10 ⁴	2.2 x 10 ⁵	No growth
4	4	95	4 x 10 ⁴	0	1.2 x 10 ⁵	No growth
5	5	43	1.8 x 10 ⁵	0	1 x 10 ⁶	Positive
6	6	25	0	0	0	No growth
7	2	94	2 x 10 ⁴	0	0	No growth
8	7	289	0	2 x 10 ⁴	8 x 10 ⁴	No growth
9	8	173	1.96 x 10 ⁶	1.52 x 10 ⁶	0	No growth
10	6	41	1.78 x 10 ⁶	1.96 x 10 ⁶	0	Positive
11	9	198	1.3 x 10 ⁷	6.9 x 10 ⁶	0	No growth
12	10	56	1.4 x 10 ⁷	60,000	2 x 10 ⁹	No growth
13	11	137	2.8 x 10 ⁷	2.7 x 10 ⁷	2.1 x 10 ⁹	No growth
14	12	12	6.1 x 10 ⁶	2 x 10 ⁴	8 x 10 ⁴	No growth
15	12	20	6.3 x 10 ⁶	0	6 x 10 ⁴	Positive
16	12	96	8.2 x 10 ⁶	6 x 10 ⁴	7 x 10 ⁵	Positive
17	13	224	7.7 x 10 ⁶	3.2 x 10 ⁵	5.2 x 10 ⁵	No growth
18	14	49	7.7 x 10 ⁶	9.2 x 10 ⁵	6.4 x 10 ⁵	No growth
19	15	29	4 x 10 ⁴	0	0	Positive
20	16	67	2.1 x 10 ⁹	1.8 x 10 ⁹	1.3 x 10 ⁹	No growth
21	17	20	0	0	0	No growth
22	18	76	3 x 10 ⁷	2.3 x 10 ⁷	3.7 x 10 ⁷	No growth
23	19	32	1.6 x 10 ⁵	0	6.6 x 10 ⁵	Positive
24	17	143	0	0	0	Positive
25	20	125	2.3 x 10 ⁷	2.3 x 10 ⁷	3.7 x 10 ⁷	No growth

Table 12: Cultural analysis of clinical endotracheal tubes, on different media, Blood agar (BA) to detect fastidious organisms and evidence of haemolysis; MacConkey agar (MAC) to select for Gram negative bacteria; Fastidious Anaerobic Agar (FAA) to select for anaerobic bacteria, and Sabouraud's Dextrose Agar (SDA) with a low pH and containing gentamycin to select for fungal growth.

4.1.2.1. Cultural analysis of clinical tracheostomy tubes

From the ten tracheostomy tubes, the CFUs per cm² ranged from 3.4 x 10⁹ per cm² to 400 CFUs per cm² on Blood Agar, all of the specimens grew bacteria on the BA plates. The range of CFUs on FAA ranged from 1.4 x 10⁹ on specimen number 9, to no growth from specimens numbered 2 and 7. Four out of the ten tracheostomy tubes (numbers 3, 4, 6 and 8) were also found to be positive for *C. albicans* (See Table 13).

4.1.3.1 Cultural analysis of Chronic Wound Dressings and Chronic Wound Biopsies

Each 1 cm² of dressing was analysed for number of CFUs present. Number ranged from 120 × 10⁶ CFUs per cm² from specimen number 5, to 2800 per cm² from specimen number 4. The only specimen to have fungal growth, subsequently confirmed as *C. albicans*, was number 5. (Table 14)

Specimen 1 to 5 of the chronic wound biopsies correspond to Chronic Wound Dressing specimen numbers 1 to 5. The biopsy specimen with the highest numbers of CFUs per cm² was specimen 5, with 4.3 × 10⁸ CFUs per cm² correlating with specimen number 5 of the Chronic Wound Dressing cohort being most heavily infected. However, dressing specimen number 4 was the least populous from the dressings cohort, whereas biopsy specimen number 2 had the least number of CFUs per cm² with only 4.2 x10⁵ CFUs per cm². None of the Chronic Wound Biopsy specimens grew any fungal elements, corresponding with the findings from the Chronic Wound

Dressing cohort, *i.e.* only of the specimens number five in the Chronic Wound Dressings group had positive fungal growth (Table 14).

			BA	FAA	SAB
Tracheostomy	Pt.	Hours intubated	CFUs per cm ²	CFUs per cm ²	CFUs per cm ²
1	1	1128	2.4 x 10 ⁶	1.4 x 10 ⁷	No growth
2	2	840	7.5 x 10 ⁵	0	No growth
3	3	1209	3 x 10 ⁷	2.8 x 10 ⁸	positive
4	4	912	1.1 x 10 ⁶	2,000	positive
5	5	504	3 x 10 ⁸	2.8 x 10 ⁸	No growth
6	6	240	3.4 x 10 ⁹	7 x 10 ⁷	positive
7	7	135	400	0	No growth
8	8	696	7.5 x 10 ⁵	5.6 x 10 ⁵	positive
9	9	422	2.8 x 10 ⁹	1.4 x 10 ⁹	No growth
10	10	552	7.6 x 10 ⁶	6.2 x 10 ⁵	No growth

Table 13: Cultural analysis of clinical tracheostomy tubes using selective culture media for aerobic bacteria, anaerobic bacteria and for fungus.

			BA	FAA	SAB
Dressings	Patient	Dressing type	CFUs per cm ²	CFUs per cm ²	CFUs per cm ²
1	1	Aquacel Ag	7 x 10 ⁵	0	No growth
2	2	Aquacel Ag	3.8 x 10 ⁶	0	positive
3	3	Flamazine and Aquacel	6.2 x 10 ⁵	0	No growth
4	4	Aquacel Ag	2.8 x 10 ³	0	No growth
5	5	Inadine	1.2 x 10 ⁹	0	No growth

Table 14: Cultural analysis of clinical dressing samples using selective culture media for aerobic bacteria, anaerobic bacteria and for fungus.

			BA	FAA	SAB
Wounds	Patient	Aetiology	CFUs per cm²	CFUs per cm²	CFUs per cm²
1	1	VLU	8.4 x 10 ⁵	200	No growth
2	2	Vasculitic	4.2 x 10 ⁵	0	No growth
3	3	Mixed	3.7 x 10 ⁶	0	No growth
4	4	VLU	4.2 x 10 ⁶	0	No growth
5	5	DFU	4.3 x 10 ⁹	0	No growth

Table 14 and 15: Cultural analysis of chronic wound samples using selective culture media for aerobic bacteria, anaerobic bacteria and for fungus.

			BA	FAA	SAB
Burns	Patient	Days post-burn	CFUs per cm²	CFUs per cm²	CFUs per cm²
1	1	19	2 x 10 ³	0	No growth
2	2	5	2.8 x 10 ⁴	3.3 x 10 ⁴	No growth
3	3	16	0	0	positive
4	4	18	7 x 10 ⁴	0	No growth
5	5	24	0	0	No growth

Table 16: Cultural analysis of burn wound samples using selective culture media for aerobic bacteria, anaerobic bacteria and for fungus.

4.1.3.2. Cultural analysis of burn wounds

Two of the samples (Specimens 3 and 5) had no bacterial growth, although specimen 3 did grow *C. albicans*. Specimen 4 was the most heavily infected specimen recording 70,000 CFUs per cm² whereas Specimen 1 had only 2,000 CFUs present and specimen 2 had 28,000 present. Other than Specimen 3, none of the other specimens contained *C. albicans* (Table 16).

4.2.1 Identification of cultured isolates

In an attempt to further elucidate the microbiological composition of the specimens, the DNA extracted from distinct cultured colonies was subjected to 16S rDNA PCR sequence analysis and then compared with the EMBL prokaryote database to identify microbial species. These ranged from the expected *S. aureus* to the rarer *Neisseria Perflava* and *Stenotrophomonas maltophilia*. These data are summarised in Tables 17-21.

Specimen Number ETT no.	Species recovered
1	Bacillus pumilus
2	Staphylococcus hominis
3	Kocuria hominis
4	Streptococcus pneumoniae
5	Pseudomonas aeruginosa
6	No growth
7	No DNA recovered
8	Pseudomonas aeruginosa
9	Staphylococcus aureus
10	Neisseria perflava
11	Staphylococcus aureus, Stenotrophomonas maltophilia
12	Enterobacter hormaechei
13	Pseudomonas aeruginosa
14	Staphylococcus hominis
15	Pseudomonas aeruginosa
16	Staphylococcus haemolyticus
17	Enterococcus faecium
18	Staphylococcus aureus
19	Staphylococcus aureus, Bacillus pumilus, Neisseria perflava
20	Staphylococcus aureus, Pseudomonas aeruginosa
21	Staphylococcus aureus
22	Staphylococcus aureus
23	Staphylococcus aureus
24	No DNA recovered
25	Pseudomonas aeruginosa

Table 17: Culture dependent identification of bacterial species cultured from clinical endotracheal tubes using 16s rDNA PCR and gene sequencing.

ETT Num.	Pt.	Media: Hours intubated	BAP CFUs per cm2	MAC CFUs per cm2	FAA CFUs per cm2	SAB CFUs per cm2	
1	1		4 x 10 ⁴	8 x 10 ⁴	0	No growth	
2	2	108	0	2 x 10 ⁴	0	No growth	
3	3	60	0	4 x 10 ⁴	2.2 x 10 ⁵	No growth	
4	4	95	4 x 10 ⁴	0	1.2 x 10 ⁵	No growth	
5	5	43	1.8 x 10 ⁵	0	1 x 10 ⁶	Positive	
6	6	25	0	0	0	No growth	
7	2	94	2 x 10 ⁴	0	0	No growth	
8	7	289	0	2 x 10 ⁴	8 x 10 ⁴	No growth	
9	8	173	1.96 x 10 ⁶	1.52 x 10 ⁶	0	No growth	
10	6	41	1.78 x 10 ⁶	1.96 x 10 ⁶	0	Positive	
11	9	198	1.3 x 10 ⁷	6.9 x 10 ⁶	0	No growth	
12	10	56	1.4 x 10 ⁷	60,000	2 x 10 ⁹	No growth	
13	11	137	2.8 x 10 ⁷	2.7 x 10 ⁷	2.1 x 10 ⁹	No growth	
14	12	12	6.1 x 10 ⁶	2 x 10 ⁴	8 x 10 ⁴	No growth	
15	12	20	6.3 x 10 ⁶	0	6 x 10 ⁴	Positive	
16	12	96	8.2 x 10 ⁶	6 x 10 ⁴	7 x 10 ⁵	Positive	
17	13	224	7.7 x 10 ⁶	3.2 x 10 ⁵	5.2 x 10 ⁵	No growth	
18	14	49	7.7 x 10 ⁶	9.2 x 10 ⁵	6.4 x 10 ⁵	No growth	
19	15	29	4 x 10 ⁴	0	0	Positive	
20	16	67	2.1 x 10 ⁹	1.8 x 10 ⁹	1.3 x 10 ⁹	No growth	
21	17	20	0	0	0	No growth	
22	18	76	3 x 10 ⁷	2.3 x 10 ⁷	3.7 x 10 ⁷	No growth	
23	19	32	1.6 x 10 ⁵	0	6.6 x 10 ⁵	Positive	
24	17	143	0	0	0	Positive	
25	20	125	2.3 x 10 ⁷	2.3 x 10 ⁷	3.7 x 10 ⁷	No growth	

Specimen Number	Species recovered
1	Bacillus pumilus
2	Staphylococcus hominis
3	Kocuria hominis
4	Streptococcus pneumoniae
5	Pseudomonas aeruginosa
6	No growth
7	No DNA recovered
8	Pseudomonas aeruginosa
9	Staphylococcus aureus
10	Neisseria perflava
11	Staphylococcus aureus, Stenotrophomonas maltophilia
12	Enterobacter hormaechei
13	Pseudomonas aeruginosa
14	Staphylococcus hominis
15	Pseudomonas aeruginosa
16	Staphylococcus haemolyticus
17	Enterococcus faecium
18	Staphylococcus aureus
19	Staphylococcus aureus, Bacillus pumilus, Neisseria perflava
20	Staphylococcus aureus, Pseudomonas aeruginosa
21	Staphylococcus aureus
22	Staphylococcus aureus
23	Staphylococcus aureus
24	No DNA recovered
25	Pseudomonas aeruginosa

Tables 12 and 17: Provided for comparison between cultural results, and outcomes of culture dependent PCR identification

Tracheostomy tube no.	Species recovered
1	<i>Pseudomonas aeruginosa</i> , <i>Streptococcus pneumoniae</i> , <i>Staphylococcus aureus</i> .
2	<i>Staphylococcus epidermidis</i> , <i>Pseudomonas aeruginosa</i> , <i>Staphylococcus epidermidis</i> , <i>Microbacterium trichotecenolyticum</i>
3	<i>Kocuria marina</i> , <i>Pseudomonas aeruginosa</i>
4	<i>Lactobacillus rhamnosus</i> , <i>Staphylococcus epidermidis</i> , <i>Pseudomonas aeruginosa</i>
5	<i>Delftia</i> spp
6	<i>Pseudomonas fulva</i>
7	<i>Pseudomonas aeruginosa</i>
8	<i>Pseudomonas aeruginosa</i>
9	<i>Pseudomonas aeruginosa</i> , <i>Delftia tsuruhatensis</i>
10	<i>Pseudomonas aeruginosa</i>

Table 18: Culture dependent identification of bacterial species cultured from clinical tracheostomy tubes using 16s rDNA PCR and gene sequencing.

Burn Specimen No.	Species recovered
1	<i>Pseudomonas fulva</i> , <i>Oceanobacillus caeni</i> , <i>Staphylococcus aureus</i>
2	<i>Staphylococcus aureus</i>
3	<i>Staphylococcus</i> sp.
4	<i>Staphylococcus aureus</i>
5	<i>Staphylococcus aureus</i>

Table 19: Culture dependent identification of bacterial species cultured from clinical burn specimens using 16s rDNA PCR and gene sequencing.

Tracheostomy	Pt.	Hours intubated	BA	FAA	SAB
			CFUs per cm ²	CFUs per cm ²	CFUs per cm ²
1	1	1128	2.4 x 10 ⁶	1.4 x 10 ⁷	No growth
2	2	840	7.5 x 10 ⁵	0	No growth
3	3	1209	3 x 10 ⁷	2.8 x 10 ⁸	positive
4	4	912	1.1 x 10 ⁶	2,000	positive
5	5	504	3 x 10 ⁸	2.8 x 10 ⁸	No growth
6	6	240	3.4 x 10 ⁹	7 x 10 ⁷	positive
7	7	135	400	0	No growth
8	8	696	7.5 x 10 ⁵	5.6 x 10 ⁵	positive
9	9	422	2.8 x 10 ⁹	1.4 x 10 ⁹	No growth
10	10	552	7.6 x 10 ⁶	6.2 x 10 ⁵	No growth

Tracheostomy tube no.	Species recovered
1	<i>Pseudomonas aeruginosa</i> , <i>Streptococcus pneumoniae</i> , <i>Staphylococcus aureus</i> .
2	<i>Staphylococcus epidermidis</i> , <i>Pseudomonas aeruginosa</i> , <i>Staphylococcus epidermidis</i> , <i>Microbacterium trichotecenolyticum</i>
3	<i>Kocuria marina</i> , <i>Pseudomonas aeruginosa</i>
4	<i>Lactobacillus rhamnosus</i> , <i>Staphylococcus epidermidis</i> , <i>Pseudomonas aeruginosa</i>
5	<i>Delftia</i> spp
6	<i>Pseudomonas fulva</i>
7	<i>Pseudomonas aeruginosa</i>
8	<i>Pseudomonas aeruginosa</i>
9	<i>Pseudomonas aeruginosa</i> , <i>Delftia tsuruhatensis</i>
10	<i>Pseudomonas aeruginosa</i>

Tables 13 and 18: Provided for comparison between cultural results, and outcomes of culture dependent PCR identification in tracheostomy tubes

Chronic Wound No.	Species recovered
1	Streptococcus parasanguinis, Lactobacillus jensenii
2	Enterobacter hormaechei
3	No DNA recovered
4	Staphylococcus aureus
5	Streptococcus parasanguis

Table 20: Culture dependent identification of bacterial species cultured from clinical chronic wounds 16s rDNA PCR and gene sequencing.

Dressing No.	Species recovered
1	Staphylococcus aureus
2	Staphylococcus epidermidis, Micrococcus luteus
3	Staphylococcus aureus
4	Staphylococcus aureus
5	No DNA recovered

Table 21: Culture dependent identification of bacterial species cultured from clinical dressings samples using 16s rDNA PCR and gene sequencing.

4.3 Molecular analysis of bacterial composition and diversity

4.3.1 Detection of bacterial species by PCR

The tracheostomy and endotracheal tube control specimens were analysed using PCR for specific bacterial species, to which they had already been exposed. *Pseudomonas aeruginosa* and *S. aureus* were detected from all the control tubes. *S. mutans* and *P. gingivalis* were recovered from three tubes each (Figures 15-18).

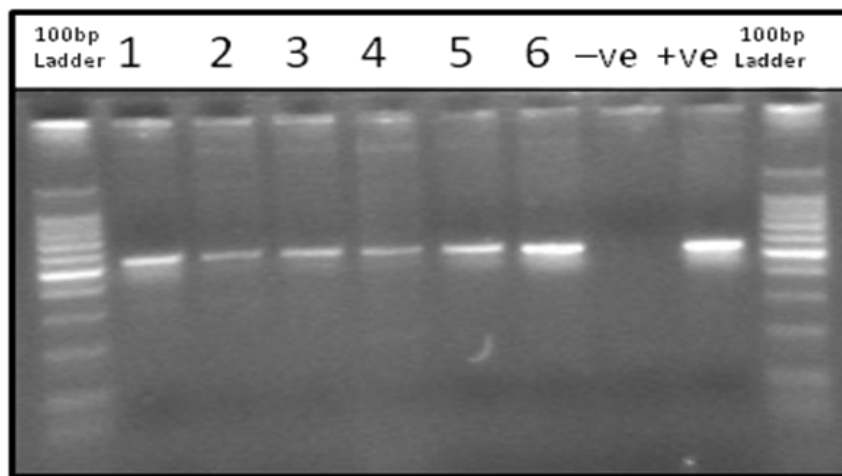


Figure 15: Gel showing PCR result of *P. aeruginosa* DNA species specific PCR from control ETT, with expected band of 528 base pairs (Lavenir *et al.* 2007), alongside 100bp DNA marker ladders (Promega).

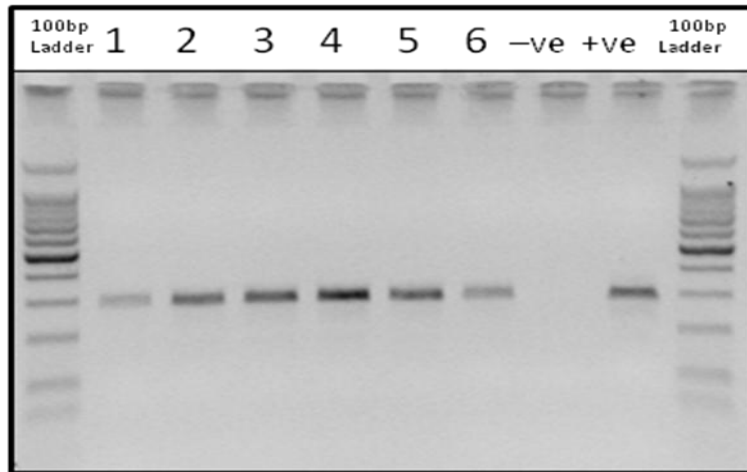


Figure 16: Gel showing PCR result of *S. aureus* DNA species specific PCR from control ETT, with expected band of 289 base pairs (Liu *et al.* 2007), alongside 100bp DNA marker ladders (Promega).

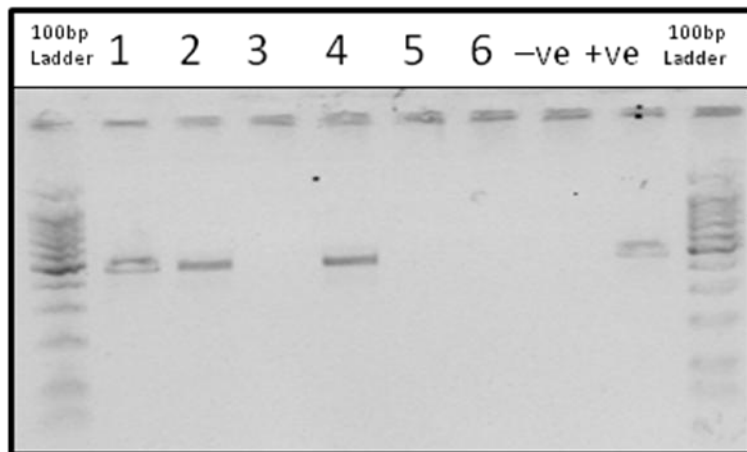


Figure 17: Gel showing PCR result of *S. mutans* DNA species specific PCR from control ETT, with expected band of 479 base pairs (Chen *et al.* 2007), alongside 100bp DNA marker ladders (Promega).

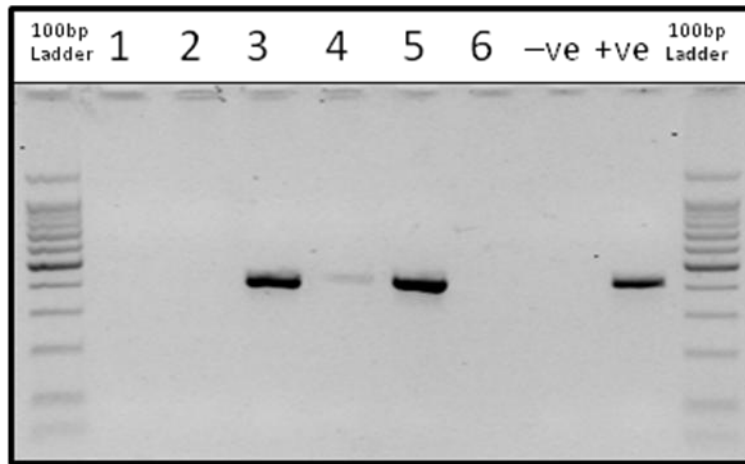


Figure 18: Gel showing PCR result of *P. gingivalis* DNA species specific PCR from control ETT, with expected band of 404 base pairs (Kulecki *et al.* 2008), alongside 100bp DNA marker ladders (Promega).

Clinical ETT and tracheostomy tube specimens were then processed using the specific primers for oral pathogens, *S. mutans* and *P. gingivalis*, and two opportunistic bacterial species, *P. aeruginosa* and *S. aureus*. (Figures 19-22). *Streptococcus mutans* was present in six of the twenty five ETT specimens, *P. gingivalis* in five, *P. aeruginosa* and *S. aureus* were also present in five of the twenty five specimens. In the tracheostomy tubes PCR products specific to the species *S. aureus* was found in five of the ten specimens, and *P. aeruginosa* in seven of the ten. None of the tubes yielded PCR products from either *S. mutans* or *P. gingivalis* (Table 22).

However, when comparing duration of intubation versus change in microbial composition, it can be seen that *P. gingivalis* is present in those who had been intubated for less than five days, compared with a preponderance of *P. aeruginosa* in those who had been intubated for longer than 5 days (Table 23).

ETT number	<i>P.aeruginosa</i>	<i>S.aureus</i>	<i>P.gingivalis</i>	<i>S.mutans</i>
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	-	-	-	-
5	-	+	-	-
6	-	-	-	-
7	-	-	-	-
8	+	-	-	-
9	-	+	-	+
10	-	+	+	-
11	+	+	-	-
12	-	-	+	+
13	+	-	-	-
14	-	-	-	-
15	-	-	-	-
16	+	-	-	+
17	-	-	-	-
18	-	+	+	-
19	-	-	-	-
20	+	+	-	+
21	-	-	-	-
22	-	+	+	-
23	-	-	+	+
24	-	-	-	-
25	-	-	-	-

Table 22: Table showing the results of species specific PCR for each of the target species *P. aeruginosa*, *S. aureus*, *P. gingivalis* and *S. mutans* on clinical endotracheal tube specimens.

Period of Intubation	Less than 5 days	Greater than 5 days
<i>P. gingivalis</i> +ve	5/12 (41.67%)	0/9 (0%)
<i>P. aeruginosa</i> +ve	1/12 (8.33%)	5/9 (55.55%)

Table 23: Table showing change in microbiota over time, with a cut-off of 96 hours there is demonstrable difference with a decrease of *P. gingivalis* numbers and a corresponding increase in *P. aeruginosa* numbers.

Chronic wound, dressing and burn wound specimens were examined by species specific PCR analysis for the presence of the opportunistic pathogens *S. aureus* and *P. aeruginosa*. The chronic wound dressings all revealed the presence of *P. aeruginosa*, but only one was *S. aureus* PCR positive. Interestingly four dressings were positive for *S. aureus* by PCR whilst only one was positive for *P. aeruginosa*.

In the five burn wounds, *P. aeruginosa* was recovered from two of the specimens, but no DNA was recovered from the remaining three dressings.

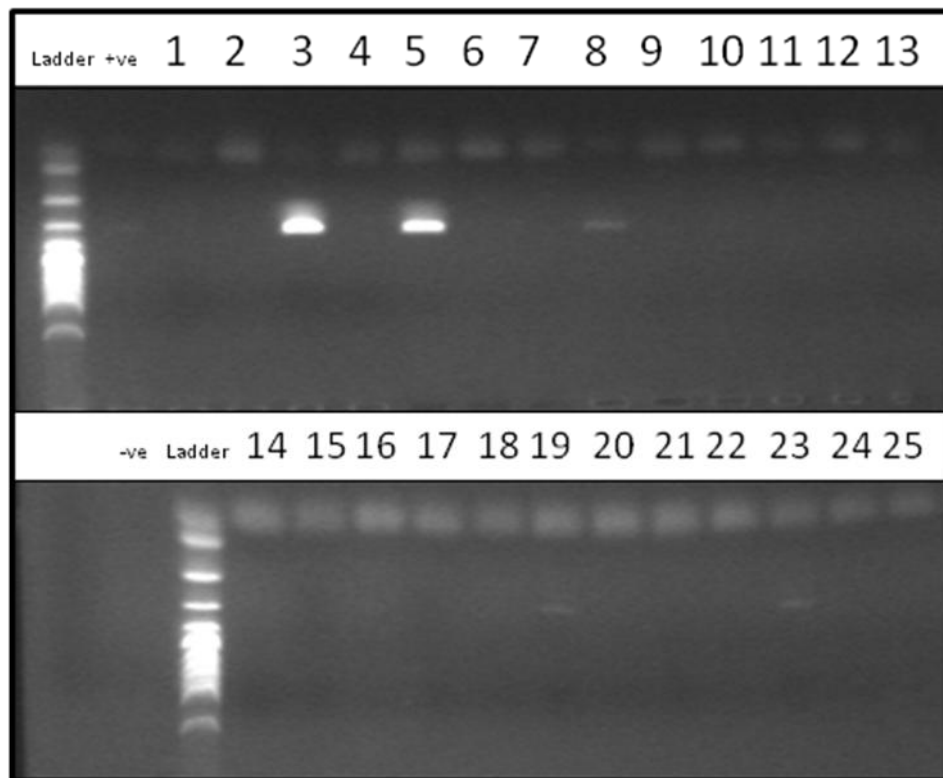


Figure 19: PCR gel showing presence of *P. aeruginosa* in ETT, with expected band of 528 base pairs (Lavenir *et al.* 2007), alongside 100bp DNA marker ladders (Promega).

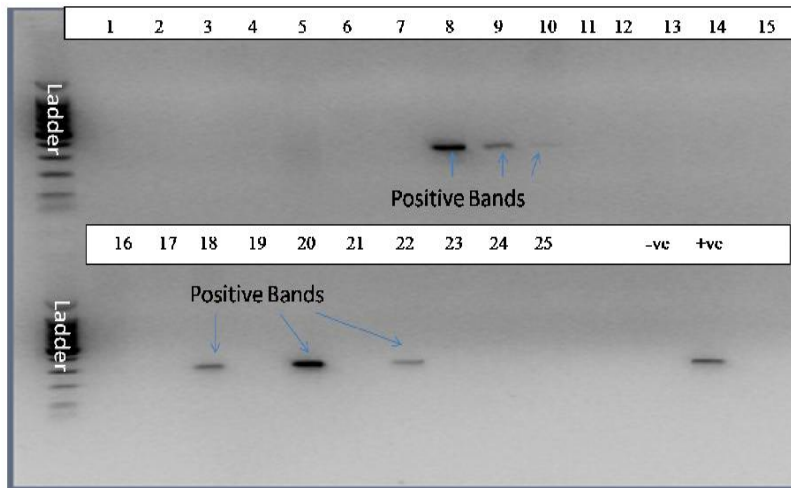


Figure 20: PCR gel showing presence of *S.aureus* in ETT, with expected band of 289 base pairs (Liu *et al.* 2007), alongside 100bp DNA marker ladders (Promega)

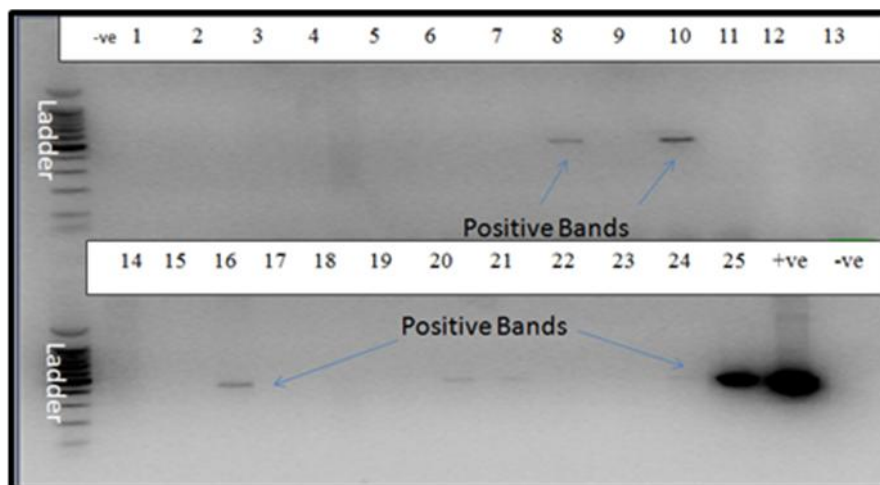


Figure 21: PCR gel showing presence of *P. gingivalis* in ETT, with expected band of 404 base pairs (Kulecki *et al.* 2008), alongside 100bp DNA marker ladders (Promega).

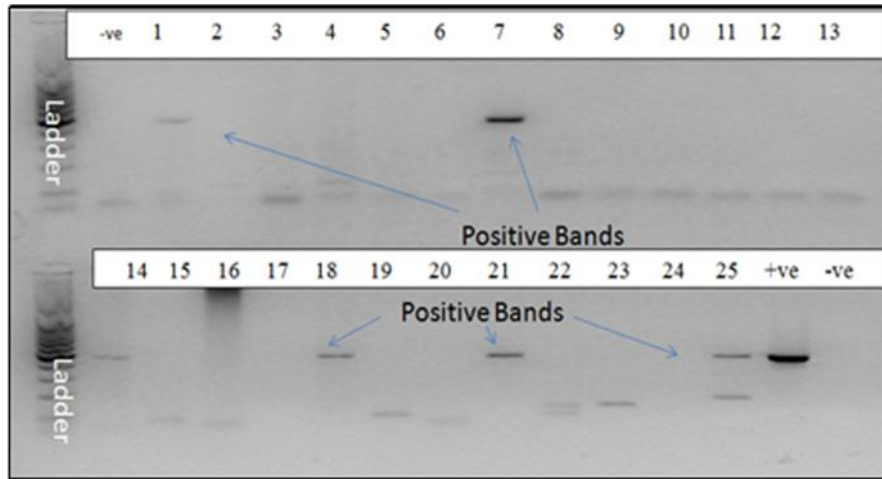


Figure 22: PCR gel showing presence of *S.mutans* in ETT, , with expected band of 479 base pairs (Chen *et al.* 2007), alongside 100bp DNA marker ladders (Promega).

Table 24: A table showing the results of species specific PCR on Chronic Wounds, Dressings and Burns, assessing the presence or absence of the target species *P. aeruginosa* and *S. aureus* in the clinical specimens

Specimen			Specimen			Specimen		
Chronic Wound	<i>P.aeruginosa</i>	<i>S.aureus</i>	Dressings	<i>P.aeruginosa</i>	<i>S.aureus</i>	Burns	<i>P.aeruginosa</i>	<i>S.aureus</i>
1	+	-	1	+	+	1	+	-
2	+	-	2	-	+	2	+	-
3	+	-	3	-	-	3	-	-
4	+	+	4	-	+	4	-	-
5	+	-	5	-	+	5	-	-

4.3.2 DGGE to determine bacterial diversity of biofilm communities on ETTs and tracheostomy tubes

Denaturing Gradient Gel Electrophoresis (DGGE) allows for assessment of multiple specimens at the same time, and gives an indication of the bacterial diversity present. The initial PCR performed with the universal primers gives multiple 194 base pair length products. These products are denatured by the gel at different rates depending on the proportion of A-T or C-G bonds between the nucleotides. This means that the different products will be spread out through the gel. Some bands may overlap within the gel, and some specimens may produce more than one band. On average, however, each separate band within the gel corresponds to a separate bacterial species. This, therefore, gives us a DNA “fingerprint” for each specimen allowing rapid comparison of the populations present in each clinical sample. DGGE profiling of the ETT specimens showed multiple bands present within many of the specimens, and showed objective similarities between the specimens and the positive controls (Figure 23). An average of 6 bands (range 3 to 22) per endotracheal tube sample was detected and the similarity in banding profiles ranged between 20 and 90%. There was no apparent relationship between the duration of intubation and the number of DGGE bands detected, nor was there a high level of similarity for the DGGE profiles obtained for multiple endotracheal tubes from the same patients (*i.e.* patient 2, tubes 2 and 7; patient 17, tubes 21 and 24) The mixed marker containing DNA from *P.aeruginosa*, *S.aureus* and *P.gingivalis* showed bands consistent with those in Tubes 9, 18 and 20,

which all had the presence of *S.aureus* as determined by the species-specific PCR.

Tracheostomy tube specimens also showed multiple bands, with an average of 5 bands (range 3 to 10) per sample (Figure 24). These showed a similarity in banding profiles between 30 and 67% (Figure 25). They were also compared to ETT specimens and to the duration of placement within patient.

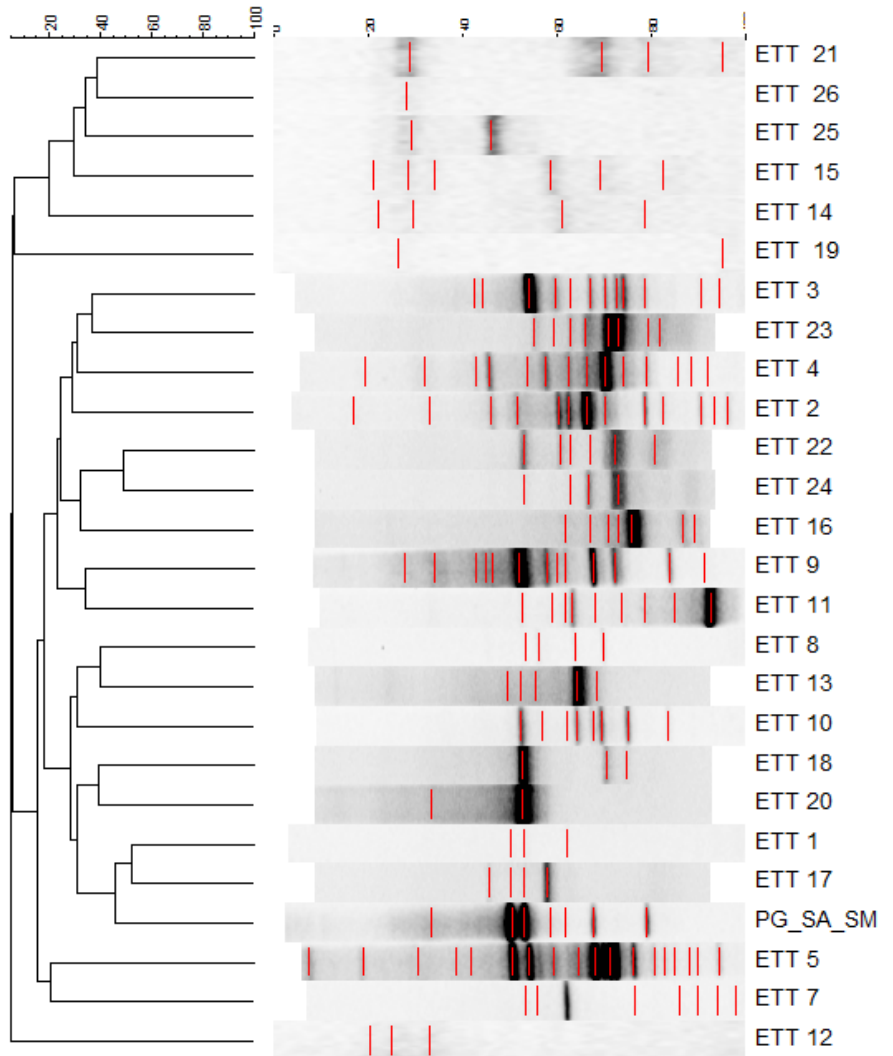


Figure 23. Cluster analysis with Ward's algorithm based on the Dice coefficient demonstrating the diversity in DGGE profiles generated from endotracheal tube (ETT) biofilms. This figure shows the similarity or otherwise between different clinical endotracheal samples, with the most similar tubes being tubes 1 and 17, possibly due to a relative paucity of bands in these samples

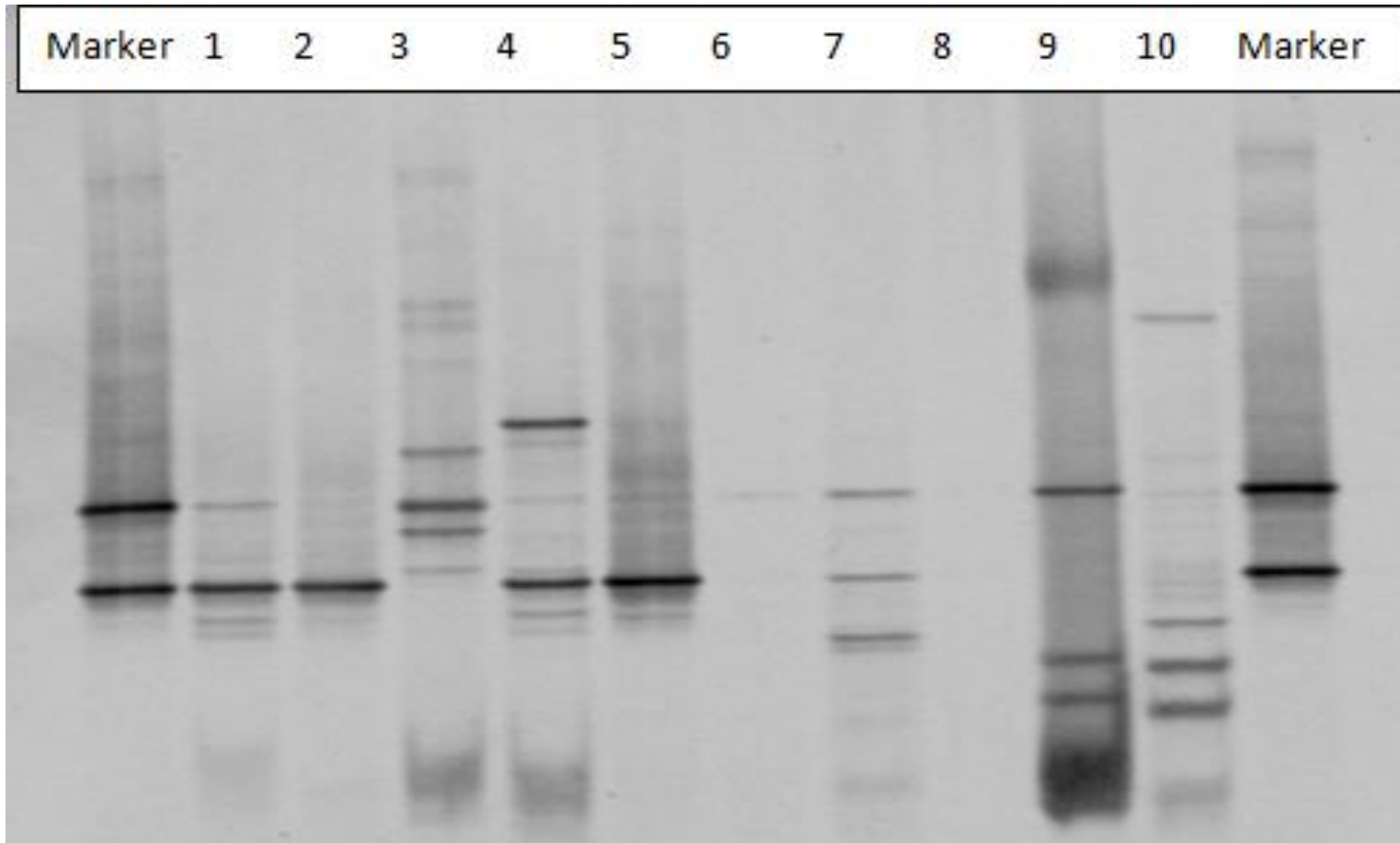


Figure 24: DGGE of Tracheostomy tubes, bracketed by marker species. This gel shows the number of different bands present in each sample, while some bacteria may produce two or more bands, and some bands may occupy the same location, this gel shows a quick fingerprint of the diversity of bacteria present within each specimen.

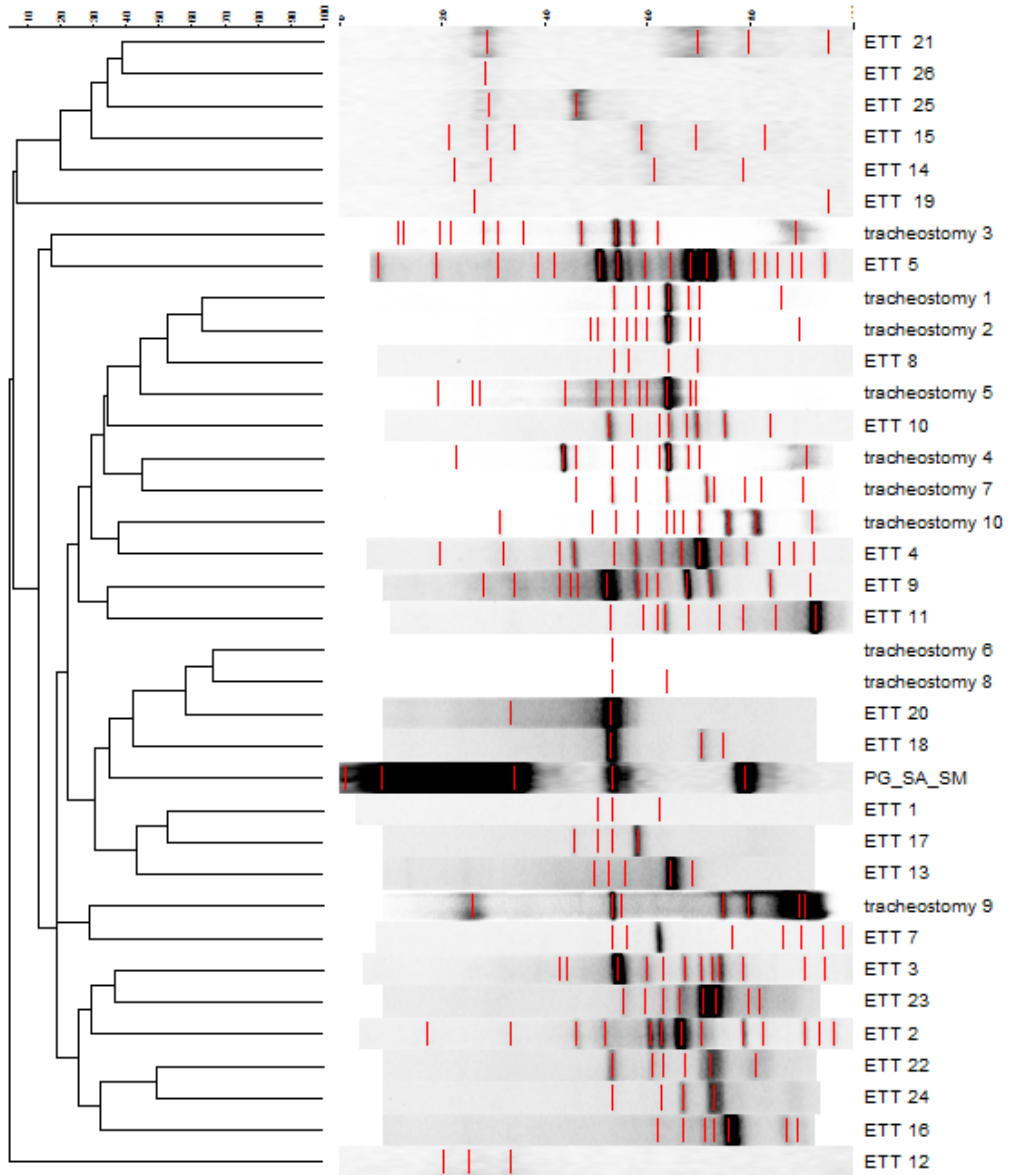


Figure 25. Cluster demonstrating the diversity in DGGE profiles generated from endotracheal and tracheostomy tubes. This dendrogram shows a comparison between the populations of both the ETT and TT, there is little grouping between the different tubes.

Subsequently DGGE was used as a method to compare PCR products from chronic wounds and the PCR products from their associated dressings (Figure 26).

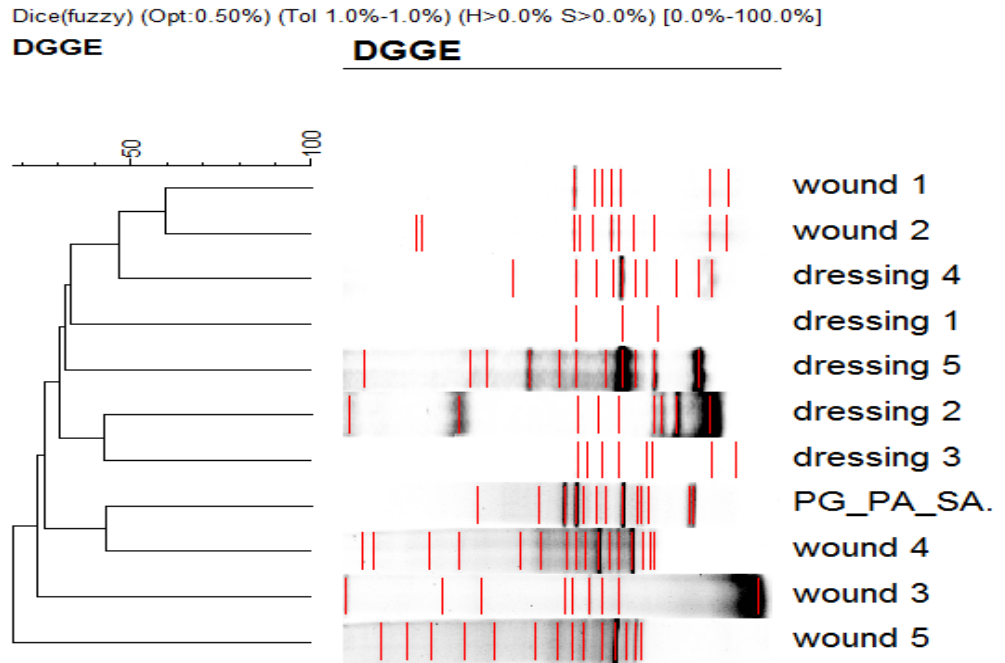


Figure 26. Dendrogram analysis comparing similarities of chronic wound and dressing DGGE band distribution. In this image comparing the bands produced by each sample, if the dressings populations were a good allegory for the wound populations, I would expect to see dressing 1 grouped with chronic wound 1 and so forth.

4.3 Structural analysis of bacterial distribution and biofilm structure using confocal laser scanning microscopic (CLSM) analysis

4.3.1 Validation of PNA probe specificity using planktonic bacterial cultures

In order to assess the functionality of the PNA fluorescent probes, the probes were validated using fixed planktonic cells of known species (*S. aureus*, *P. gingivalis*, and *P. aeruginosa*) by hybridization and CLSM (Figures 26-28). In this scenario, all probes worked as anticipated, as they were used on monocultures there was no evidence of cross reactivity.

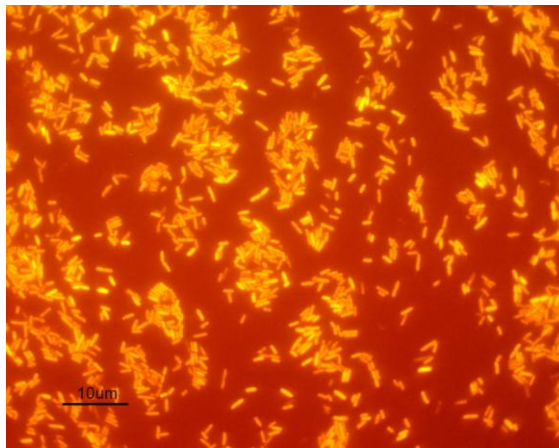


Figure 27. Planktonic *P. aeruginosa* control labelled with FITC fluorescent label using the specific *P.aeruginosa* PNA probe.

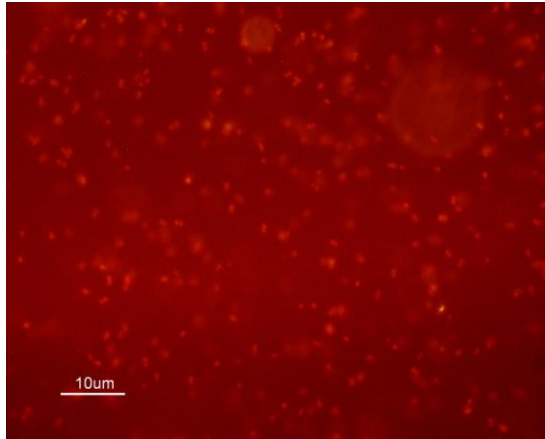


Figure 28. *P. gingivalis* in a planktonic phenotype, stained with the universal probe and labelled with the Cy-5 fluorochrome.

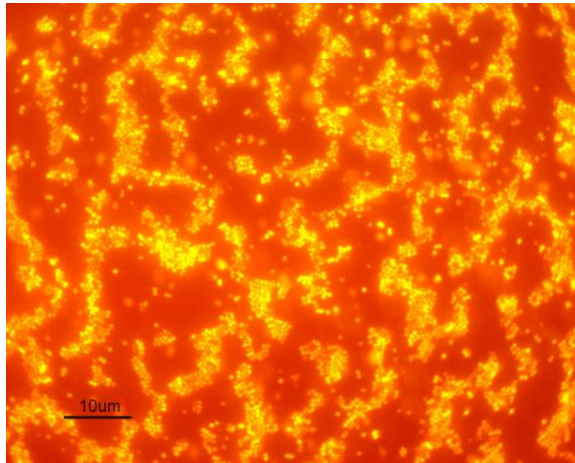


Figure 29. Planktonic *S. aureus* labelled with the specific *S. aureus* PNA probe and Cy-3 fluorescent label. The labels are all displayed as orange in the above images as this is the default setting for the confocal microscope.

4.3.2 CLSM analysis of bacterial biofilms on recombinant human epithelium

Sections of pre-infected fixed specimens of recombinant human epithelium were examined using PNA fluorescent probes. Staining of both *S. aureus* and *P. aeruginosa* could readily be seen (Figures 30 and 31). The bacteria were visible in aggregates along the length of the RHE section, on both sides, with expanses of the tissue showing no bacteria at all. In no areas on the RHE did the target species occupy the same tissue location, but instead showed up as separate and distinct clusters.

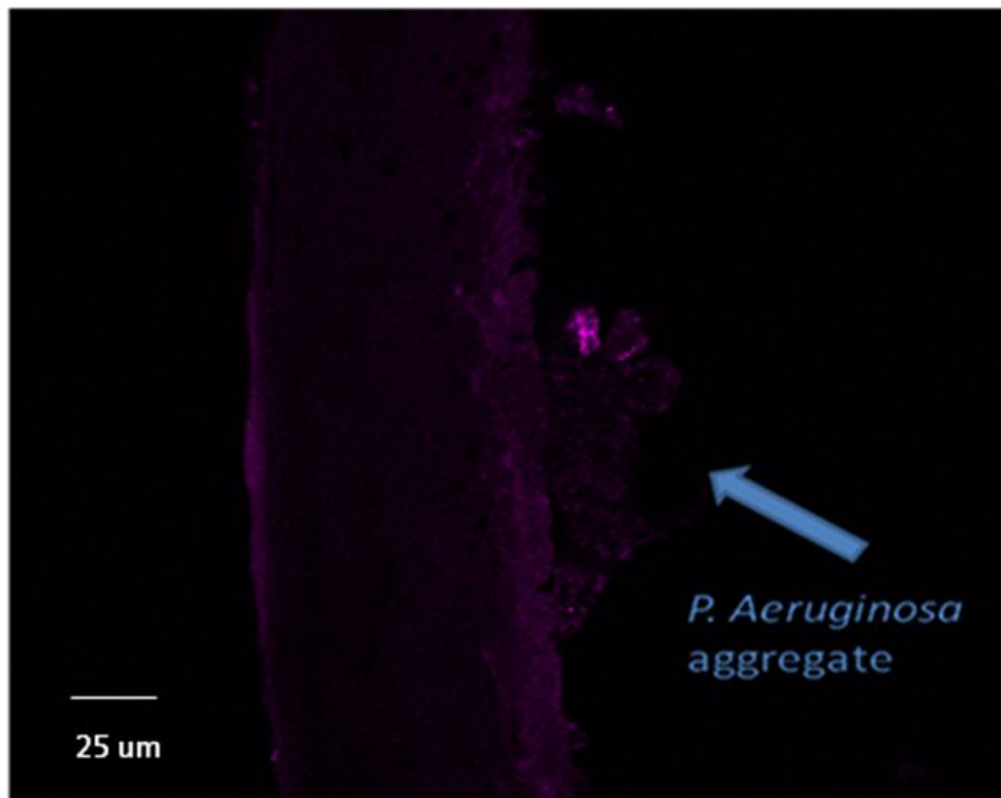


Figure 30. Species specific PNA probe staining an RHE section showing aggregates of *P. aeruginosa*

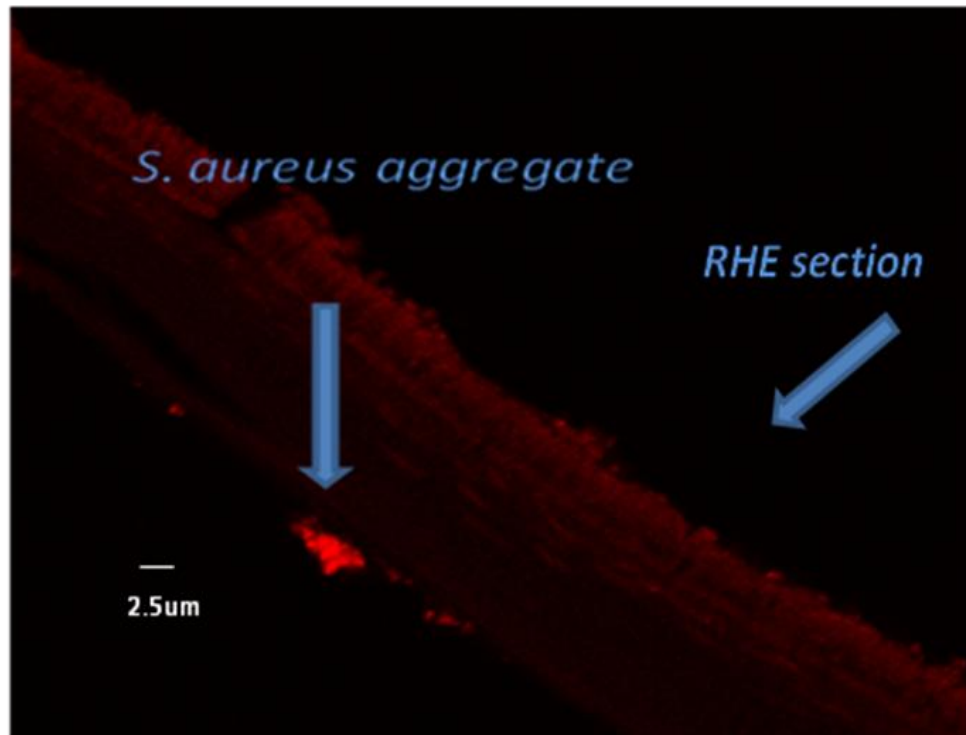


Figure 31. Species specific PNA probe staining an RHE section showing aggregates of *S. Aureus*.

4.3.3 CLSM analysis of bacterial biofilms on *in vitro* ETT

Sections of control tubes were stained with the Universal, *S. aureus* and *P. aeruginosa* PNA probes. These images showed a predominance of *S. aureus* and *P. aeruginosa* bacteria (Figures 32 and 33). Due to the large numbers of brightly staining *S. aureus* (depicted blue) and *P. aeruginosa* (depicted green) it was impossible to determine whether smaller numbers of other bacterial species were being detected by the universal stain. In these images it was, however, possible to see the bacteria forming three-dimensional structures, and not merely assuming a laminar colony. The bacteria have formed on both the inside and outside of the control tubes

equally, which were cultured *in vitro* and therefore lacking in external incentives to colonise one wall or another of the control tube.

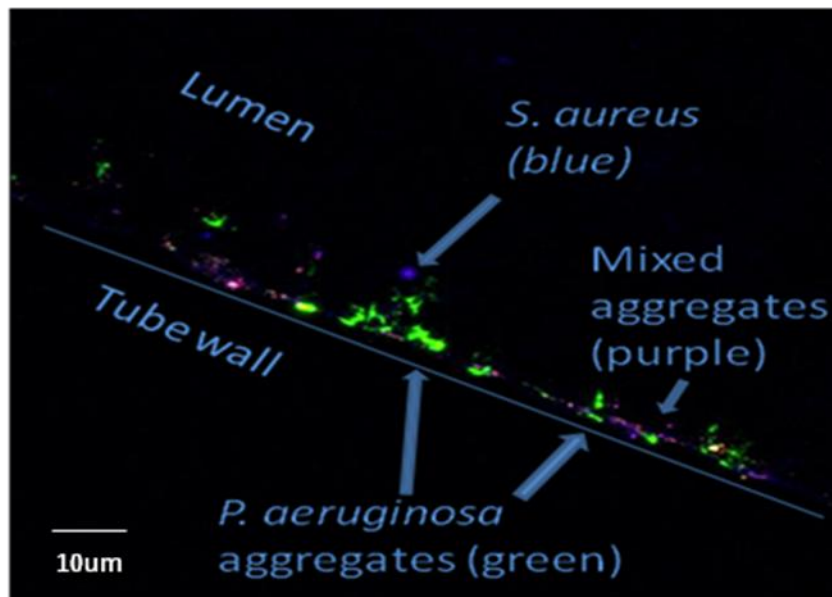


Figure 32. Mixed bacterial aggregates on control tube labelled with Universal probe, and species specific probes for *S. aureus*, *P. aeruginosa*, and *S. mutans*

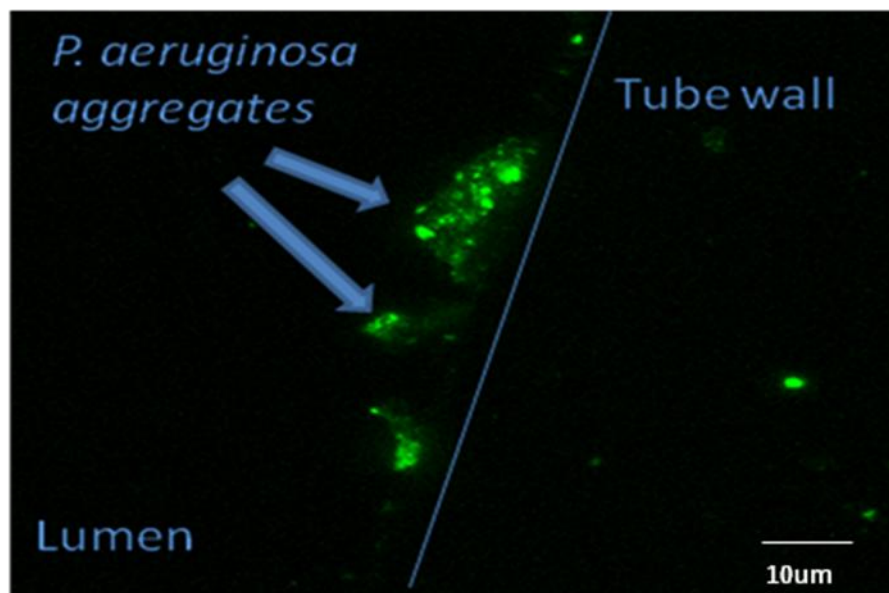


Figure 33. Species specific probes for *P. aeruginosa* on a control tube

4.3.4 Confocal microscopy on clinical samples of endotracheal and tracheostomy tubes

Imaging of clinical tracheostomy tubes showed both thick layering of *S. aureus* (Figure 34) and of other potent biofilm formers (Figure 35) and mixed biofilms of different species (Figure 36). Some of the three-dimensional bacterial layering projected significantly into the lumen, whereas other colonies remain flattened against the tube wall. Interestingly those aggregates which projected the most appeared to be comprised of a more uniform bacterial population, and those with a mixed population appear flatter. Figure 36 shows an aggregate present both within and without the tube and across the sectioned end of the tube, this is unlikely to have occurred *in vivo* and may be as a result of the staining process dislodging the biofilm.

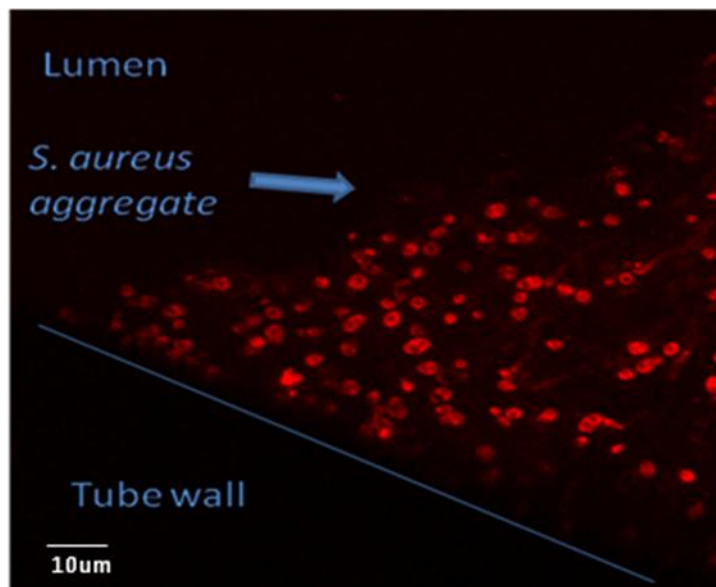


Figure 34. Tracheostomy tube stained with Universal and species-specific probes for *S. aureus*, *P. aeruginosa* and *S. mutans* probes, showing only *S. aureus* aggregates.

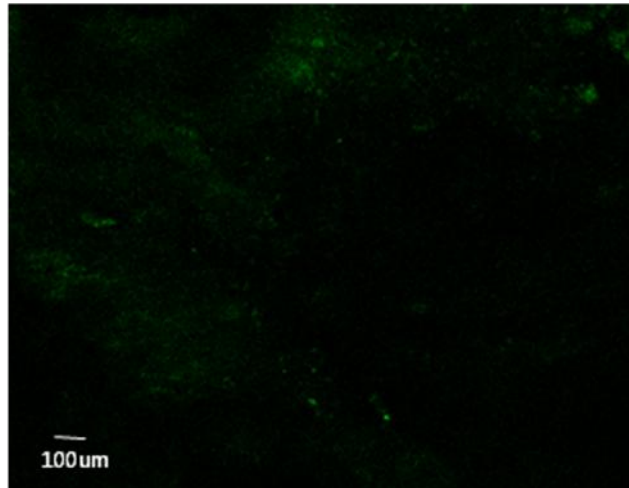


Figure 35. Bacterial aggregates labelled with *P. aeruginosa* species specific label within endotracheal tube lumen

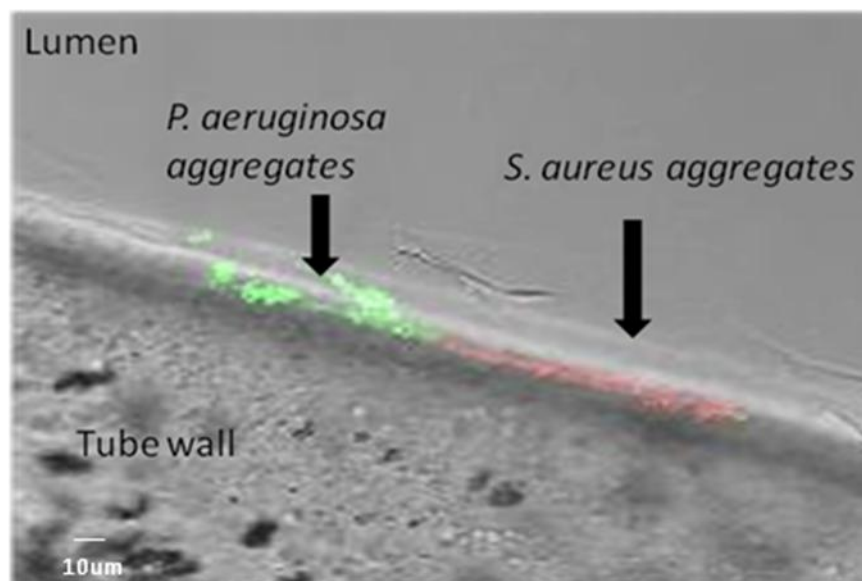


Figure 36. Clinical tracheostomy tube specimen labelled using *P.aeruginosa* specific label (green), *S. aureus* label (red), Universal (yellow), and *S. mutans* specific label (blue). This shows two separate colonies, one of *P. aeruginosa* and the other of *S. aureus*.

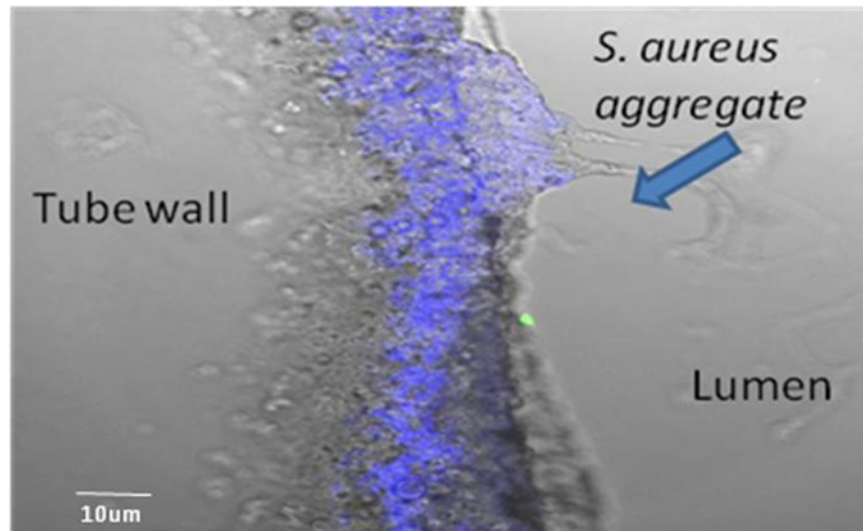


Figure 37. Clinical tube specimen stained with all four probes, *S. aureus* here depicted as blue visible inside and outside of tube lumen

4.3.5 Confocal microscopy on Chronic Wounds, Dressings and Burn Wounds

Chronic Wounds and Burn Wounds revealed limited amounts of bacteria, but those that were present tended to be aggregated (Figures 38 and 39), this possibly reflects a harsher environment in treated wounds when compared with the relative sanctuary of the lumen of an endotracheal tube. Conversely within the interstices of dressings a highly organized mixed species biofilm was clearly visible (Figures 40 and 41) adherent to the edges of the dressing matrix and forming a large three-dimensional biofilm (Figure 41). This appeared to consist of only one bacterial type (those successfully stained with the *S. aureus* PNA probe, cyanine 5 exciting at 633 nm), possibly those which were most resilient to the antiseptic with which the dressing was impregnated.

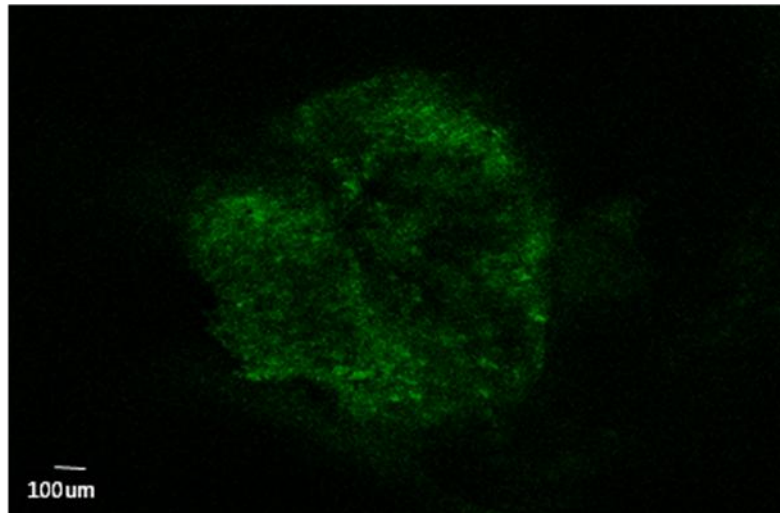


Figure 38. Chronic wound specimen labelled with *P. aeruginosa* (green), *S. aureus* (blue) and Universal (yellow) specific probes, showing diffuse *Pseudomonas* aggregate

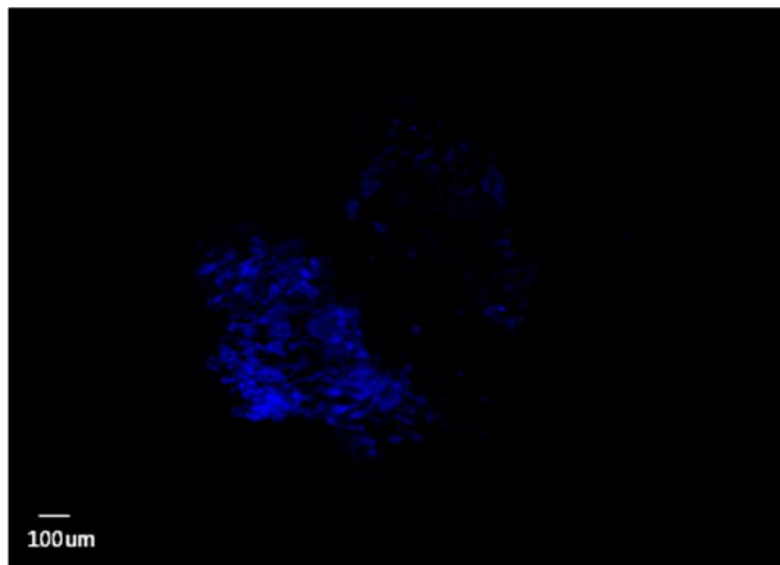


Figure 39. Burn wound specimen labelled with *P. aeruginosa* (green), *S. aureus* (blue) and Universal (yellow) specific probes, showing diffuse *S. aureus* aggregate

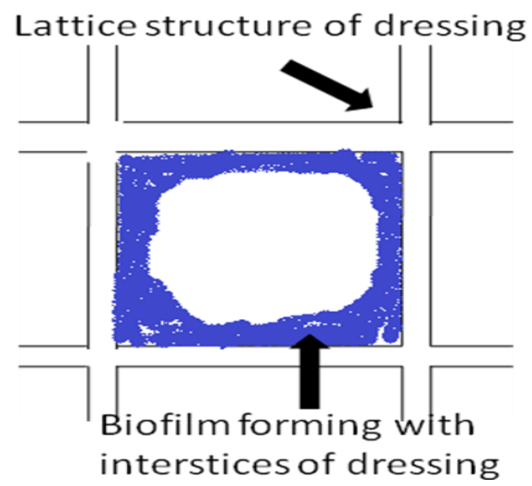
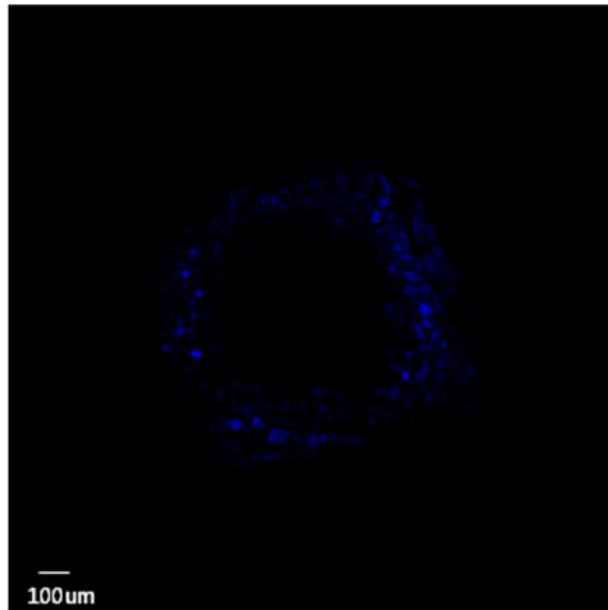


Figure 40. Bacteria within an Inadine dressing, fluorescing after having been labelled with the species specific probes for *P.aeruginosa* (green) and *S. aureus* (blue).

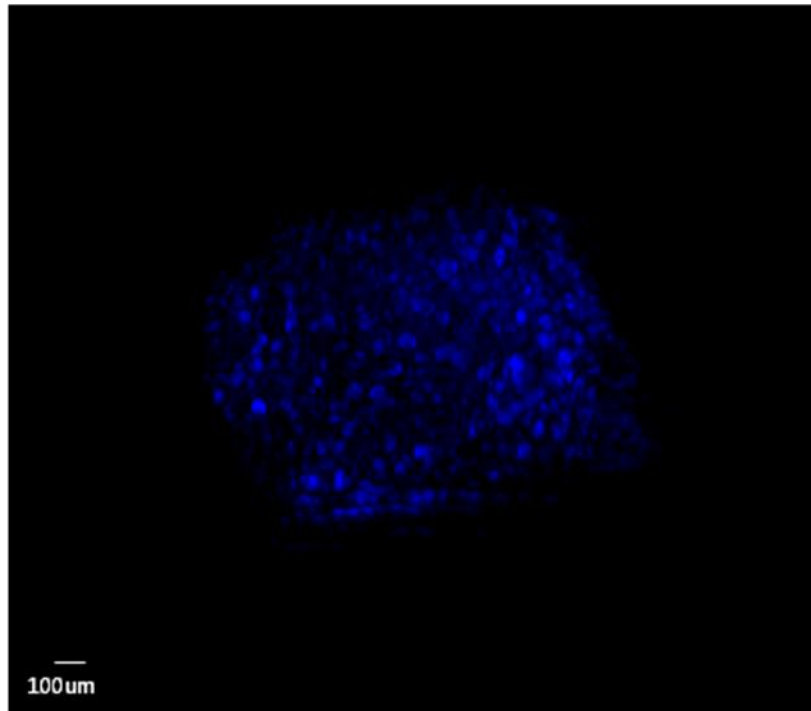


Figure 41. Bacterial aggregate within a dressing, showing the distinct square shape of the inner walls of the dressing (Inadine).

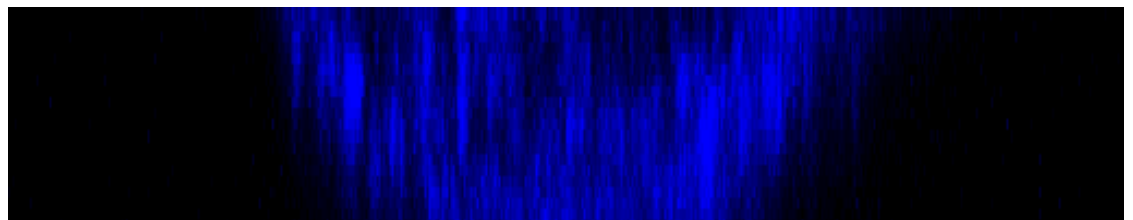


Figure 42. Side of view of bacterial biofilm within a dressing interstices, denoting its thickness and three-dimensionality

Figures 40 – 42: Clinical dressing specimen labelled with Universal probe, and species specific probes for *P. aeruginosa* (green) and *S. aureus* (blue).

Chapter 5: Discussion

This study is centered around biofilms, and their interaction with medical devices and patients. Different techniques have been employed to examine the biofilms, and the chapters have been laid out in a fashion to explore each of the different techniques. This study has focussed on the bacterial and, to a lesser extent, the fungal components of the biofilms. The Extracellular Polymeric Substances (EPS), while making up the majority of the biofilm in terms of volume, are not the focus of this MD. The majority of the pathogenicity of a biofilm comes from its bacterial progenitors and inhabitants, and it is for this reason that techniques such as Scanning Electron Microscopy (SEM) and similar were not employed. In the following sections, the results of each technique have been drawn together under their specimen sub-headings, to try to provide an over-arching understanding of how the biofilms interact in these arena, and to formulate further theories on how future studies may target them.

Endotracheal tubes

Biofilms seem to readily colonise artificial surfaces such as the lumen of an endotracheal tube (ETT) or venous cannulae. Many treatments exist to target bacteria and even to reduce biofilm formation in these areas (Chastre 2008). Oral microflora within biofilms (*i.e.* dental plaque) has been shown to have a role in Ventilator-Associated Pneumonia (VAP) (Adair *et al.*, 1999; Ramirez *et al.*, 2007). The ETT provides a point where a biofilm may form and subsequently disperse into the lung fields (Pneumatikos *et al.*, 2009). The immunological devoid haven provided by an ETT is a moist

environment, with turbulent airflow that favours adherence and subsequent biofilm formation. The use of oral hygiene methods to prevent ‘seeding’ of oral biofilm forming bacteria into the lungs, potentially causing pneumonia, has been proven effective (Di Filippo and De Gaudio 2003) and there is mounting evidence to support the concept that poor oral health is related to the aetiology of bacterial VAP and a number of studies have suggested improved oral hygiene may be effective at reducing its incidence (Koeman *et al.*, 2006; O’Keefe-Mcarthy 2006; Tantipong *et al.*, 2008).

Cultural analysis of the ETTs in this study showed large numbers of readily recoverable aerobic bacteria often with a preponderance of *P. aeruginosa* and *S. aureus*, these bacteria showed large numbers of CFUs in both the *in vitro* and *in vivo* studies, possibly outcompeting other bacteria (Table 9, Chapter 3.1.2). Standard methods of cultural analysis have been shown to be imperfect tools for analysing biofilm communities (Donlan and Costerton, 2002). This was previously elucidated in 2007 by Bahrani-Mougeot *et al.* who showed the presence of oral bacteria in VAP patients using clonal analysis techniques (Bahrani-Mougeot *et al.*, 2007). This present study was also able to demonstrate the recovery of DNA from potent oral biofilm formers from the lumen of the ETT confirming the theories of Sottile *et al.* (1996) that oral microflora does form biofilms with ETT. This shows the potential benefits of the work started by Adair *et al.* in attempting to decrease patient VAP morbidity by modulating oral microflora. Whether nebulised antibiotics (Adair *et al.*, 1999) will prove to be sufficiently efficacious remains to be seen, or whether the use of biofilm

behaviour modifiers such as dispersal initiating quorum-sensing molecules can be applied clinically applied or not.

Species-specific PCR for the oral marker species *Streptococcus mutans* and *Porphyromonas gingivalis* showed that these bacteria were present in 9 of the 20 ETT specimens (45%). These marker bacteria were chosen because *S. mutans* is a recognised former of oral biofilms and has a proven role in dental caries (Burne 1998; Schaeken *et al.*, 1986) and *P. gingivalis* as an indicator of strictly anaerobic oral flora and is commonly the responsible pathogen for subgingival abscess formation (Saini *et al.*, 2003; Kulecki *et al.*, 2008). However, the analysis of tongue swabs in the 2007 paper by Bahraniir-Mougeot *et al.* showed that in the patients with VAP there was no evidence of *S. mutans* or *P. gingivalis*, although the Perkins paper in 2010, found that of the 1263 gene sequences recovered from ETT in VAP patients, 348 were streptococcal, 179 were from *Prevotella*, and 143 from *Neisseria* species (Perkins *et al.*, 2010).

Candida albicans is yeast, which may be found as a common coloniser of the oral cavity. The presence of *Candida* as an oral microbe is well founded, and its occurrence in cases of VAP has previously been documented (Azoulay *et al.*, 2006; Wood *et al.*, 2006). The role of *C. albicans* in the formation of biofilms within the ETT does however remain unclear. The incidence of *Candida albicans* recovered in this study is approximately twenty five percent, which is analogous to the endogenous incidence within the general population. *Candida albicans* was identified using cultural techniques in 5 of the specimens taken from the ETTs, and it is therefore tempting to postulate that it is involved in the formation of a

pathological biofilm with the ETT. The interaction between different microflora to form a stable biofilm is complex, and the relationship between *Candida* and oral bacteria is based on co-aggregation and the facilitation of luminal binding, (Branting *et al.*, 1989; Shinada *et al.*, 1995; Shirtliff *et al.*, 2009) it is postulated the filamentous hyphae of *Candida* can add structural support to the biofilm (Lopez-Ribot, 2005), which may be particularly advantageous in the high shear environment of the ETT lumen. Some loss of microbial viability between the time of removal of the ETT from the patient and its subsequent processing for culture is likely, despite the time between these procedures being kept as short as possible. The fact that microbial DNA was obtained from these ETTs would suggest microbial cells (viable or non-viable) had been present and highlights the benefit of supplementing the culture analysis with molecular investigation.

Confocal Laser Scanning Microscopy (CLSM) of clinical specimens showed both thick biofilm layering by *S. aureus* and of other potent biofilm formers, such as *S. mutans* and *P. aeruginosa*. Mixed biofilms of other bacteria, which were not specifically targeted, were also present. Some of the three dimensional bacterial layering projected significantly into the lumen, whereas other colonies remained flattened against the tube wall, it is believed the inner surface of the ETT provides a unique environment of high shear forces, bi-directional flow, and a restricted immunological environment, which favours a sessile rather than planktonic bacterial life form and thus biofilm formation. Interestingly those colonies which project the greatest height appear to have a more uniform bacterial population, and those with a mixed population appear more sessile. Comparing duration of

intubation with types of flora present may explain this phenomenon. Specimens which were examined after a relatively short period of intubation (<96 h) showed a preponderance of oral microbiota, whereas those specimens from patients who had been intubated for a longer period (>96 h) showed an increase in numbers of the traditional hospital acquired bacteria, namely *P. aeruginosa* and *S. aureus* (Table 15 and Figure 43).

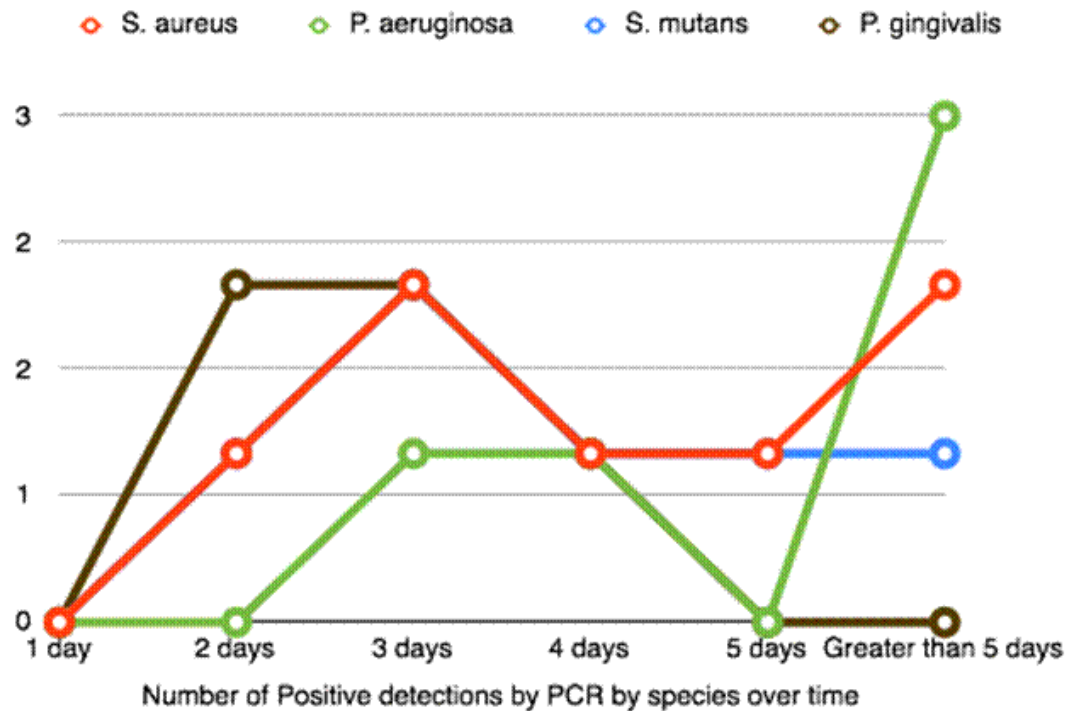


Figure 43: A graph showing how bacterial population with an ETT changes over time, based on positive detection rate on species specific PCR.

This corresponds to the 2010 study by Perkins *et al.* which showed approximately 70% of the sequences retrieved from eight ETTs had typical oral microflora, whereas the tube which had been *in-situ* for 23 days had a 95% prevalence of *P. aeruginosa* (Perkins *et al.*, 2010). The presence of oral microflora within the biofilm and as possible instigators of the biofilm has significant implications in the management of these patients. If the population of oral microflora can be decreased or modulated this may decrease the numbers involved in the initial formation of a biofilm, and the oral cavity provides an easily accessible portal to influence the ecology

within an endotracheal tube, compared with attempting to access the lumen itself.

If instead of a cohesive symbiotic relationship between bacteria, what is actually being observed is hospital acquired bacteria ‘out-competing’ existing host bacteria, it could potentially explain why a polymicrobial biofilm is flatter and not in a vertical growth phase. In a symbiotic scenario there would be less competition for available nutrients and other building blocks of the abiotic matrix, whereas in a competitive scenario energy and nutrients would be at a premium and allow for less expansive growth. A recent study by Mitria *et al.* (2011) showed that when nutrient competition is strong, the addition of new species can inhibit co-operation, whereas when nutrients are abundant co-operation is enhanced, and there was even evidence of species which are advantageous to the biofilm being “protected” within the biofilm to protect them from competition from non-advantageous species (Mitria *et al.*, 2011). Alternatively, in a monocultural biofilm, if all bacteria of the same type had a role in producing EPS it could in theory produce a thicker and more prominent biofilm structure compared to a multicultural biofilm where there would be differing abilities and genetic aptitudes to biofilm formation.

Analysis of the biofilms using DGGE showed multiple bands present in all specimens indicating the presence of many different bacterial species, although the DNA detected need not necessarily be from viable bacteria, and may represent earlier inhabitants of the biofilm.

Accurate counts of colony forming units per 1 cm section of ETT were obtained for both aerobic and anaerobic bacteria using a number of different culture media; however subsequent DNA analysis to confirm species identity was only undertaken on aerobic bacteria. Similarly, samples stored in formalin for CLSM perished and therefore three-dimensional analysis of the biofilm within the ETT lumen was not possible.

Already, the use of improved oral hygiene has been shown to decrease rates of VAP, this study shows oral microbiota are found in endotracheal tubes upto the first five days of intubation and may therefore play an important role in the initiation of a biofilm within the lumen. Treatments targeted at this initial stage, such as the coating the inner lumen of the ETTs or targeted antibiotics at *S. mutans* and other oral biofilm formers prior to intubation may help. Additionally, as it is the passage of bacteria around the outside of the ETT balloon into the lung airways that allows inoculation of the bacteria within the ETT, then redesign of these tubes may prevent this crucial step. Whether it is more pliable balloon material, or more sizes available so that the tubes fitted more snugly remains to be seen. With the tracheostomy tubes there are tubes which have replacable inner linings, something similar could be designed to allow frequent changes of the inner lumen of the ETT.

Tracheostomy tubes

The presence of biofilms within medical devices is well documented (Kuhn and Ghannoum 2004; Costerton *et al.*, 2005; Weisbarth *et al.*, 2007). A recent observational study using scanning electron microscopy identified biofilms present on 4 out of 7 tracheostomy tubes in long term ear, nose and throat patients. This study identified paired Gram-positive cocci in the biofilms, which they felt were consistent with *Staphylococcus epidermidis* biofilm formation (Meslemani *et al.*, 2010). This reaffirms the findings in the study by Jarrett *et al.* demonstrating the presence of both *S. epidermidis* and *P. aeruginosa* (Jarrett *et al.*, 2002).

Standard cultural analysis showed that there were viable bacteria present in tracheostomy tubes and subsequent DNA analysis, showed the presence of *S. epidermidis* in three of the specimens, and *P. aeruginosa* in five of the specimens confirming the findings of the above studies. This was further underlined using species-specific analysis where *S. aureus* was found in five of the ten specimens, and *P. aeruginosa* in seven of the ten. None of the tubes yielded DNA from either *S. mutans* or *P. gingivalis*. Additionally, other bacterial species were isolated such as *S. pneumoniae*, *Lactobacillus rhamnosus* and *S. hominis* suggesting a polymicrobial aspect to the biofilm colonies within tracheostomy tubes not previously elucidated. The oral bacterial species *Streptococcus mitis* was isolated from one of the tubes, and *C. albicans* was recovered using standard microbiological culture techniques in four other cases, suggesting that there is an element of oral

seeding of the tracheostomy tube such as that seen in ETTs. The subsequent structural analysis echoed the work of Jarrett *et al.* (2002) showing staphylococcal and *Pseudomonas* biofilms, but again it additionally showed polymicrobial colonies present in biofilm phenotype with the lumen.

Comparing the data from ETT and tracheostomies there are a few notable differences, while the oral species thought to play a vital role in ETT biofilm formation, *S. mutans* and *P. gingivalis* were both present in the ETT, none was recovered from the Tracheostomy tubes. In almost all cases of surgical tracheostomy insertion the patient has a preceding ETT in their airway. It is possible in this way that the bacteria are inoculated into the patients' airways before subsequently re-infecting the tracheostomy tube. The pathway of a bacterium would be thus, oral cavity and teeth, into ETT lumen, blown into the airway by the process of ventilation, and then onto the tracheostomy tube as it is inserted surgically. The absence of *S. mutans* and *P. gingivalis* in the tracheostomy tubes may be due to these bacteria never having been present in the patients, or perhaps is due to the demise of these bacteria. Alternatively, these bacteria may have been present in the initial ETT inserted prior to tracheostomy insertion, and remained there adherent to the luminal wall and not been transferred to the patients' airways. These ETT are often only present for a fairly short time, typically less than 48 hours before being exchanged for a formal tracheostomy tube. If the *C. albicans* found in approximately 25% of patients in this study were indeed endogenous then it could have infected the tracheostomy tubes without needing the additional vector of the ETT.

Another reason for the variation between the two populations is that unlike an ETT which passes through the oral cavity, the tracheostomy tubes pass through the skin directly onto the anterior surface of the neck. This provides bacteria with another potential entry point into the airway, and may explain the presence of the skin commensural *S. epidermidis* within the Tracheostomy tube specimens.

Burn wounds

Burn wound sepsis is a frequent complication in the management of burns, and it is felt that wound infection is a common cause of wound progression or deepening (Singh., 2007). Infection is the most frequent and most severe complication of burn injuries (Wassermann 2001). A recent study by Kennedy *et al.* (2010) showed that biofilms could form on burn wounds, and may therefore play a role in the delayed healing of some burn wounds. *P. aeruginosa* is a common opportunistic pathogen in burn wounds, and has been shown to be an excellent biofilm former (Bielecki *et al.*, 2008). Standard cultural analysis of the 5 burn tissue specimens revealed sparse growth, with 3 of the 5 specimens having no recoverable, viable bacterial growth; the other two specimens had growth of 2000, and 28,000 CFUs/cm² respectively.

Using species-specific DNA analysis, *P. aeruginosa* was detected in 40% of the burn wound tissue, which corresponds with a recent paper by Rezaei *et al.* showing the presence of *P. aeruginosa*, *Acinetobacter* and *Klebsiella* spp. in burn wounds (Rezaei *et al.*, 2011). This was further confirmed by the use of CLSM which showed aggregated colonies of bacteria which

fluoresce after having been treated with the *Pseudomonas* specific probes (836), while the tissue specimens analysed showed some degree of polymicrobial contamination, they were much less diverse than the ETT colonies and this may represent a less favourable environment for biofilm formation. The relatively sparse diversity identified within the burn biofilms may therefore instead represent the apogee of competition within the biofilm. Further weight is added to this hypothesis when looking at diversity within the tracheostomy tubes, with an average intubation time of 664 h, where the degree of diversity is somewhere between that of ETTs and burn wounds. The theory of social evolution in biofilms suggests there is competition, as well as a degree of collaboration in biofilms between bacterial species (Poltak and Cooper 2011; Mitri *et al.*, 2011) and whether the paring down of diversity represent finessing of collaboration, or the outcome of competition remains to be seen.

Chronic Wounds and Dressings

Chronic wounds may have many and varied aetiologies, ranging from vascular insufficiency, such as venous leg ulcers or arterial ulcers, to those caused by direct damage to the epithelium of the skin, such as trauma or pressure ulcers. Chronic wounds are defined as wounds, which have failed to proceed through an orderly and timely reparative process to produce anatomic and functional integrity over a period of three months (Mustoe *et al.*, 2006), these wounds provide an area where bacteria are able to grow and even thrive. Often treating the underlying cause of an ulcer will allow

it to heal, however a significant delay in wound healing may occur with a contaminated or infected wound (Robson, 1997). The combat of infection in such patients is often a prime objective of wound management strategies (Price 2005; Edwards 2009). It should, however be mentioned that wound healing can still occur even in the presence of bacterial contamination (Krizek and Robson, 1975). The impact of infected wounds to a patient should not be underestimated. Pain and subsequent lack of sleep, and overpowering odour along with delayed wound healing are a few of the complications of infection that patients cope with on a daily basis (Price and Harding 1996, Ebbeskog and Ekman 2001).

In all chronic wound specimens (tissue biopsies and dressing samples), viable bacteria were recovered. All of the tissue biopsies had *P. aeruginosa* and one biopsy had *S. aureus*. Conversely all of the dressings had viable staphylococcal bacteria on them, with only one yielding *P. aeruginosa*. With the dressings and the wounds in close proximity, one might expect there to be a similarity between the bacterial populations sampled. For example, dressing 1 was taken from the same wound which was biopsied for wound 1. However, using denaturing gradient gel electrophoresis (DGGE) to obtain a genetic fingerprint of each specimen, and subsequently comparing them using GelCompar software, showed almost no similarity between the populations. This may be due to the properties of the dressing used, causing a selection bias of bacteria able to colonise the dressing compared to the wound surface. Some dressings are designed to absorb the bacteria within the body of the dressing such as Aquacel Ag. Additionally, the presence of Silver in both the Aquacel Ag dressings and the Flamazine

dressings will have a marked effect of the bacterial population therein. Indeed, structural analysis of the bacterial colonies present in both types (dressing and biopsy) of specimen show marked differences. CLSM of the wound surface shows quite flat aggregates of bacteria, with very little in the way of biofilm architecture, with the appearance more in keeping with a clumping effect; compared to the bacteria of the dressings, which shows a definite three dimensional structure in keeping with biofilm formation. The biofilm within the dressing has formed within the interstices of the dressing mesh, which may have provided enough of an original scaffold for the biofilm to form on, almost analogous to the use of candidal hyphae in oral biofilms. Additionally, the bacteria on the surface of the wound may have formed a biofilm between wound surface and dressing which has been disrupted and destroyed in the process of removing the dressing and sampling the wound. This would explain why the biofilm within the dressing interstices have survived relatively unscathed, while the biofilm remnants left on the wound surface have collapsed into shapeless aggregates. This has significant impact in respect of being able to use dressings as surrogates in the diagnosis of biofilms. While the presence or absence of abiotic biofilm matrix might be visible on the removed dressing, the structure of the biofilm both on the dressing and on the wound will have been irreversibly damaged by the removal of the dressing. Currently this would impact our ability to tell the age of a biofilm, by the presence of water channels and so forth, but as our understanding of biofilm architecture and ecology grows, so greater forensic information may be lost in this manner. Similarly, only those bacteria closest to the uppermost surface of

the biofilm or in contact with the dressing will be identified in this manner, and a potentially significant portion may be left behind within the collapsed biofilm architecture.

Study limitations

As in the case of the chronic wound dressings and chronic wound biopsies, specimen sampling may negatively impact on the biofilm which is under investigation. While the haven of the mesh dressing allowed direct visualisation of the biofilm there, any biofilm architecture stretching from wound surface to dressing was inevitably disrupted. Similarly the methods for processing samples may disrupt the biofilm architecture, biofilms which are free standing on a luminal, or wound surface are exposed to shear forces during the preparation and staining of the specimens. The use of different species-specific probes to enable different species to be detected gives evidence and ideas about the polymicrobial nature of the colonies present, but little idea of any biofilm architecture not directly related to the presence of bacteria, the abiotic portion of the biofilm may not be visualised in this way. Some of the images obtained in control experiments, such as using the CLSM to look at infected recombinant human epithelium surfaces clearly show bacterial aggregates on both sides of the epithelium, when only one side was infected (Figure 30, Chapter 3.3.2). Many of the biofilms fragments have been flipped over or transposed during the preparation process. While the firmer bases of the ETT, tracheostomy tube, and dressing

material allow for preservation of biofilm architecture, the finer specimens are much more easily disrupted.

The use of fluorescent *in-situ* hybridization (FISH) enables visualisation of bacterial species present and their orientation within the biofilm and to some extent allows postulation of the abiotic architecture, it has its limitations such as sampling and preparation problems, along with being unable to assess viability or accurately identify all species and bacterial subtypes present. Many of the methods used throughout this study are able to accurately analyse one aspect of bacterial behaviour, but are deficient in other areas. Cultural analysis is constrained by our current understanding of conditions in which bacteria may grow *in-vitro*, whatever environment we provide in an anaerobic incubator on specific growth media, it is unlikely to be a perfect match for those conditions found in the deepest parts of a biofilm. Cultural analysis provides an estimate of viable microbial numbers present and as such represents a guide to the relative population of each specimen. It does not, however, reveal anything about the natural history of the biofilms, which species may have taken the initial adherence steps before being swept away and out-competed by other bacteria. In wounds which have been treated for many weeks, such as burn wounds, or even many months in the case of the chronic wounds, with antiseptics, and antibiotics, the bacteria which are available for culture may not be the same as those which originally initiated the biofilms, or may be existing within them as persister cells.

Some of these limitations are offset with the use of DNA analysis techniques. Species-specific PCR will tell us if certain choice bacteria are

present, such as oral pathogens in the ETT and tracheostomy samples, but will not reveal their viability. The presence of the DNA shows only that the bacterial DNA was present, and not whether the bacteria it came from was viable or had indeed even been so.

Subsequent DNA analysis using PCR techniques of the bacteria recovered using standard culture techniques provides another strand of information about the nature of the polymicrobial community. This was performed on the bacteria which were recovered and grown in the laboratory from the original specimens. It is therefore subject to the same problems as standard cultural analysis, that of sampling problems, and accurate culture conditions. Assuming no environmental contamination of the specimens, this technique does, at least, tell us that some viable bacteria were present, and provides an accurate identification thereof.

Another method used to gain an estimate of degree of bacterial diversity within a population is with DGGE. DGGE can provides a fingerprint profile of a microbial population present based on different PCR product sequences, frequently these products are obtained from the 16s rRNA genes. The subsequent profile of bands for each specimen gives an indication of the richness of a population (number of bands) and the similarity of species (band positions). However, bands at the same positions are not necessarily of the same size or indeed sequence and may therefore not originate from the same species. DGGE technique is not culture dependent, but instead takes DNA samples directly from each specimen. As such the method does not differentiate between living and dead bacteria present, but rather looks at all bacterial DNA present at a single point in time. This method does not

tell us about the phenotype or potential for biofilm formation, its strength lies in its ability to compare many diverse samples, and with the help of the GelCompar software, give a measurement of similarity between specimens. In this way we can see that there is significant compositional complexity and inter-patient diversity between samples.

Future Work

This study has provided confirmatory data to support some of the existing work in the area of oral biofilms, such as that performed by Sottile and colleagues (Sottile *et al.*, 1996) and the role of maintaining good oral hygiene in ventilated patients is already happening in the clinical environment, although a recent study by Needleman *et al.* (2011), showed no benefit on VAP rates when using a powered toothbrush on patients compared to using a tooth spongette.

The presence of oral microbiota in the biofilm phenotype within ETT and tracheostomy tubes is a novel finding for this study. However, further work needs to be directed into this area to continue to drive down morbidity and mortality in the critical care environment. For example, some critical care units use one disposable airway suction catheter for the duration of the patients' stay in critical care, and this may be an important source of bacterial inoculation into the airway, especially considering the discovery of biofilms within endotracheal and tracheostomy tubes which may be acting as a reservoir of pathogens. The presence of a biofilm within the lumen of an ETT means that the lumen will be narrowed, and as such a

proportionally higher ventilatory pressure will be required to ventilate the patient. This incurs the problems of potentially blowing pathogens into the lung parenchyma, and has increased risks of iatrogenic barotrauma. Nebulised antibiotics have been prescribed with the aim of reducing the incidence of VAP (Adair *et al.*, 2003); it may be that targeted quorum-sensing or biofilm dispersal signalling agents delivered in small nebulised doses either at preintubation or during the course of the patients' stay in critical care may improve survival figures.

The insertion of tracheostomy tubes is a difficult and skill intensive procedure with a high number of possible complications (Table 4, Chapter 2), and as such it is often delayed until such time as it is apparent that there is no other alternative for safe patient ventilation. However, as this study shows, there is an increase in the number of hospital-based pathogens within the biofilm population within a patient's airway over time. It may, therefore, prove to be of benefit if the patient has their ETT replaced with a tracheostomy earlier in the course of their illness. A study comparing rates of VAP in selected populations with their timing of surgical tracheostomy insertion may show a significant decrease in morbidity associated with VAP; however, such a study would need to also assess whether there was a concomitant increase in complications associated with the tracheostomy tube itself. Furthermore using DGGE, patients' oral microflora could be assessed pre-tracheostomy insertion and those patients with a favourable bacterial population could have tracheostomies inserted earlier as they could (in theory) be at a higher or lower risk dependent on the makeup of their oral flora.

This study looked solely at tracheostomy tubes garnered from burn injured patients, and therefore, studying a wider more varied population would also be recommended, as this may also allow for tracheostomies to be examined at an earlier stage in the patients' illness. All of the tracheostomies in this study were obtained at the point of the patients' final extubation. There exist, however, tracheostomies that have a separate inner tube or lumen; the use of this type of tube would allow for earlier examination of the nascent biofilms, as these inner tubes are changed much more often in critical care, indeed several tubes from one patient could be studied in this way in order to build up a picture over time.

The impact biofilms have on burn wound healing has not been fully elucidated; that biofilms exist on burns has been proven both in this study and elsewhere (Kennedy *et al.*, 2010). Whether or not, or to what extent, biofilms affect the speed of burn wound healing has not been fully assessed. Currently, it is understood that a burn which has damaged the dermal blood supply as well as the skin adnexal structures (such as sweat glands or hair follicles) will take longer to heal than a burn which is more superficial. This is also coupled with the many confounding factors affecting burn healing (temperature and duration of insult, adequacy of first aid, and patient comorbidities). Some burn centres use a device called a laser Doppler to assess dermal blood flow, and use this as an indicator of likely time that a burn will take to heal; it should therefore be possible to quantify the dermal blood flow data, and compare it against the bacterial biofilm measures that have been used in this study. For example, does an area with little or no blood flow predispose to the formation of complex biofilm structures as

may be seen using CLSM, or does a 'wetter' burn such as a partial thickness burn wound from a scald have a higher number of bacterial bands on DGGE than a 'drier' wound produced by a flame burn. This in turn would have an impact on both the use of particular dressings to manage the burn wound, but also may direct surgical choices regarding timing of burn wound debridement and skin grafting. Already standard microbiological techniques are used prior to skin grafting, mainly to rule out the presence of streptokinase producing bacteria which would cause the skin graft to fail due to fibrinolytic non-adherence. Whether further in-depth assessment techniques allow for better quantifying of bacterial numbers, the presence of bacterial biofilms and therefore the subsequent risk of graft failure remains to be seen.

Similarly, the healing of chronic wounds is multifactorial; studies have already shown the presence of bacterial biofilms and large diverse bacterial populations in wounds (Hill *et al.*, 2003), but the healing of these wounds is often difficult to predict based as it is on a number of factors (arterial or venous blood supply, patient co-morbidities, and various treatments employed). A further study looking at the nature of the bacterial biofilm within wounds of differing aetiologies, and the variability of these populations would be interesting and could lead to the development of predictive markers of healing. Levels of tissue blood oxygenation may be measured relatively simply using near-infrared spectroscopy, therefore a study comparing bacterial band numbers versus tissue oxygen saturation may prove interesting answers about the nature of wound bed biology and its subsequent impact on biofilm formation. A commonly held hypothesis

in the management of chronic wounds is that reduction of wound depth will more easily allow for epithelial migration over the wound bed subsequently leading to epithelial closure and hence full healing. No studies have been undertaken comparing depth of wound with diversity of bacteria or even depth and maturity of the biofilm.

In this study, samples were only obtained following debridement of the burn wound in the operating theatre. Partial thickness burns are notoriously painful and tissue sampling prior to making the decision of whether or not to operate is liable to be a difficult clinical issue to resolve. A similar problem is faced in the management of chronic wounds: not only are they often very painful, they are often in tissue that does not respond well to local anaesthetic infiltration. Additionally, removal of the full depth of the wound bed risks making the chronic wound, or burn, more complicated and more difficult to heal. While some success has been achieved using *in vitro* techniques such as a constant depth film fermenter to artificially create a biofilm, these by their nature cannot adequately duplicate the complex myriad of biochemical processes occurring in a biofilm wound bed interface. This study used full thickness punch biopsies of wounds, which sampled the full depth of the biofilm, but were limited in the surface area sampled. Nevertheless, it was hoped that the information garnered from this technique would correlate with the data from the wound dressings. The technique of using a dressing or a spray-on foam to sample biofilms has been tried in various centres, but unfortunately the validation of the data has proved difficult. In essence the sampling of the biofilm, by scraping or by ripping it in half using an interface dressing disrupts the very thing which is

being investigated. The techniques used in this study can simultaneously measure different aspects of a biofilm and by inference try to build a complete composite picture of the biofilm being studied. A finessing of these techniques would be welcomed, for example the development of more PNA probes which are specific for different bacterial species yet with a limited fluorescent spectrum would allow more probes to be used simultaneously.

Chapter 6: Conclusion

Comparing the outcomes of this study with the stated aims,

Aims:

1. Collection of appropriate clinical specimens for analysis including, ETTs, tracheostomy tubes, excised burnt tissue, chronic wound biopsies and wound dressings.
2. Cultural analysis of biofilms from the clinical specimens using standard microbiological methods to determine the presence of microbial species.
3. Molecular analysis of clinical specimens to further elucidate the microbial diversity and composition of biofilms.
4. Utilise specific molecular probes to establish the biofilm structure and distribution of targeted species within biofilms.

We can see that all of the initial aims have been achieved. Samples were successfully collected for analysis, and subsequently underwent cultural analysis using standard microbiological techniques to prove the presence of viable microbial species. After this, specimens were subjected to molecular analysis, to provide a deeper understanding of the diversity of microbial genera present, ranging from oral flora to skin commensurals, and finally specific molecular PNA probes were used to provide visual information on the structure of biofilms, and the bacterial species therein.

Whilst the study has certain limitations, it has shown the benefit of multiple techniques for investigating biofilm populations in a wide and varied clinical environment. Some of the work herein has confirmed that already postulated by others, yet it has also posed new and challenging questions of its own, and highlighted many new and potentially fruitful and ultimately clinically beneficial avenues of further study. There are growing concerns both within the clinical environment and the public at large regarding microbial infections, antibiotic resistance and the associated public health concerns. The inherited antibiotic resistance which is now prevalent in many healthcare areas means that a deeper insight and understanding into the life cycle and physiology of bacteria is crucial. Biofilms do not seem to be a modern evolution of bacteria and fungi, but it is only latterly that mankind has become aware of the biofilm phenotype particularly in relation to the preponderance of medical devices now available. The data from this study form part of an important first step by increasing our understanding of the characterisation of biofilms and the ways in which they can be detected across a range of devices; such data are vital if we are to develop products and treatments targeted at preventing device related infections.

The work contained in this study, deepens our understanding of this phenotype, and provides insights into avenues where bacterial biofilms may be combated. In the future absolute reliance of antibiotics to combat bacterial infections will not be enough, instead we will need to rely on early identification of a potential biofilms, and then to prevent initial adherence, disrupt quorum sensing, artificially induce biofilm dispersal, and physically

expunge residual bacteria from the site. It is in these arenas that this study hopes to provide the first steps in a clinician's antibacterial armamentarium.

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Appendices

Appendix 1: Consent form for Chronic Wound patients

Department of Wound Healing
Adran Gwella Clwyfau



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Ysgol Feddygaeth
Llawr Gwaelod Uchaf
Mynydd Bychan
Caerdydd CF14 4XN

Patient Consent Form (Chronic Wounds)

version 1.0, date 18/06/2008

Patient Identification Number for this trial: _____

Title of Project: Biomaterials: Biofilms, Infection and Wound Healing

Name of Researcher: Mr Scott Cairns

Please initial box

I confirm that I have read and understand the information sheet version 1.0 dated 17th June 2008 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.

I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected and that if I decide to withdraw, or are withdrawn, for any reason any data gathered to the point of withdrawal will be used in the data analysis for this study.

I understand that relevant sections of any of the data collected during the study, may be looked at by responsible individuals from; Cardiff University regulatory authorities Cardiff & Vale NHS Trust or Swansea NHS Trust, where it is relevant to my taking part in this research. All personal details will be treated as strictly confidential.

I agree to my used dressings being taken. These dressings will be stored in a manner in which I will not be identifiable.

I agree to these dressings being used for scientific, and regulatory purposes in such a way whereby I cannot be identified personally

I understand that I am free to withdraw from this study: at any time

- without having to give a reason
- without it affecting my future medical care
- details of my participation up to the time of withdrawal may be stored anonymously and may be used in the final analysis of data

Biomaterials: Biofilms, Infection and Wound Healing. Version 1.2. 17/06/2008.

I agree to my GP being informed of my participation in the study

I agree to take part in the above study.

I agree to having a 6mm tissue

biopsy of my wound being taken.

YES / NO

Name of Patient Date Signature

Name of Person taking consent Date Signature
(if different from researcher)

Researcher Date Signature

Appendix 2: Information sheet for Chronic Wounds

Department of Wound Healing
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Mynydd Bychan
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Patient Information Sheet (Chronic Wounds)

Department of Wound Healing

Version 1.2, date 18th June 2008

Part 1.

1. **Study title:** Biomaterials: Biofilms, Infection and Wound Healing

2. Invitation paragraph

You are being invited to take part in a research study. Before you decide if is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish.

- Part 1 tells you the purpose of this study and what will happen to you if you take part.
- Part 2 gives you more detailed information about the conduct of the study.

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

3. What is the purpose of the study?

The purpose of this study is examine long standing wounds in patients, to assess for the presence of bacteria which may not be in a readily detectable state, such as in an adherent colony, called a biofilm
To assess whether certain wounds contain higher numbers of bacterial biofilms resulting in a delayed healing time.

4. Why have I been chosen?

You have been approached to participate in this study because you have been identified as having a chronic wound which, has not healed. It is our theory that your wound may contain bacterial biofilm.

5. Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are free to withdraw at any time and without giving a reason. Data collected up to the point of your withdrawal may be used in the final data analysis for this study.

6a. What will happen to me if I take part?

If you take part in the study, your used dressings, which are normally discarded, will be collected and analysed for the presence of biofilm. This material will be allocated an anonymous specimen number, and will not be traceable back to you.

You may also be asked to provide a small portion of tissue from your wound (6mm punch biopsy), although you can still donate your dressings without having to give a tissue biopsy.

6b. What will happen if I agree to have a biopsy taken?

Local anaesthetic, similar to that given at the dentists, will be injected around your wound. The doctor will use a 6mm punch biopsy to remove a small piece of your wound. Although the local anaesthetic numbs most of the pain, there may still be a small degree of discomfort.

Your wound will then be dressed and you will be offered pain killers if necessary.

You can specify your preference on the consent form.

7. Expenses and payments:

Due to the fact that your tissue would ordinarily be discarded, there are no expenses or payments available.

8. What will happen to my tissue samples?

The specimens will be taken into the laboratory and sectioned into five parts. Each portion will be examined using different techniques to look for the presence of bacteria. The results will be used to assess how the bacteria are behaving in different areas of your wound. At the end of the research period your tissue samples will be destroyed.

9. Will this affect how my wound heals?

No, we will only use dressings which would be discarded. Your ongoing care will therefore remain unchanged.

10. What are the alternatives for diagnosis or treatment?

Your treatment protocol will remain unchanged from that which you would usually receive.

11. What are the side effects of any treatment received when taking part?

In this study you will receive no additional treatments, outside of that which you would normally receive.

12. What are the other possible disadvantages and risks of taking part?

There are no specific risks or complications from taking part in the study, any complications that arise due to your normal medical care, will be dealt with by the doctors who usually look after you.

13. What are the possible benefits of taking part?

There are no potential benefits from taking part. However we hope the study will identify whether biofilms are present and, if so help us improve treatment in the future.

14. What if there is a problem?

If you have any worry about the way you have been dealt with during the study please contact, Mr Scott Cairns, Tel: 029 2074 2316

If you are however unhappy with the treatment you have received from a member of research staff you are entitled to make a complaint, have it considered, and receive a response from Cardiff University. Detailed information is given in Part 2

15. Will my taking part in the study be kept confidential?

Yes. All the information about your participation in this study will be anonymised and stored in a locked cupboard. You will be identified only by study number on all data collected.

16. Contact Details:

For further details you may contact Mr Scott Cairns- Tel: 02920 742316

This completes Part 1 of the Information Sheet.

If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decision.

Part 2

17. What if relevant new information becomes available?

Sometimes during the course of a research project, new information becomes available about the condition that is being studied. If this happens, your research doctor will tell you about it and discuss with you and your hospital team, the best way to continue your treatment. If you decide not to carry on the data collected to the time of your withdrawal may be included in the final analysis. If you decide to continue in the study you will be asked to sign an updated consent form.

18. What will happen if I don't want to carry on with the study?

We would like to emphasise that it is completely voluntary to participate in this study. If you decide to participate you are still free to withdraw from the study at any time without giving a reason; however we will use the data collected up to your withdrawal. If you do not wish to participate, or if you later wish to withdraw from the study, it will not affect the standard of care you receive or your legal rights as a patient.

19. Will my taking part in this study be kept confidential?

If you join the study the data collected for the study will be looked at by representatives of regulatory authorities and authorised people from Cardiff University and your NHS Trust to check that the study is being carried out correctly. All will have a duty of confidentiality to

you as a research participant and nothing that could reveal your identity will be disclosed outside the research site.

20. What is there is a problem?

Harm

In case of any injury caused by participation in this study the Products Liability Insurance of the sponsor, Cardiff University, will cover you.

If you participate you still have the right to complain about your treatment.

Complaints

If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions. Contact details are provided at the end of Part 2.

If you remain unhappy and wish to complain formally, you can do this through Cardiff University or the appropriate NHS Trust Complaints Procedure. Contact details are provided at the end of Part 2.

21. Involvement of the General Practitioner/Family doctor (GP)

Your GP will be notified of their participation in the trial, with your consent.

22. What will happen to the results of the research study?

The results of this study will be reported to Cardiff University, the study sponsor, and may be published or presented at medical/scientific conferences or journals. You will be identified only by study number in any report, publication or presentation of this data.

23. Who is organising and funding the research?

The Department of Wound Healing is supporting Mr Cairns to undertake this study.

24. Who has reviewed the study?

This study was given a favourable ethical opinion for conduct in Cardiff University by the South East Wales Research Ethics Committee.

Further Information and Contact Details

General information about research

Involve, Promoting public involvement in NHS, public health and social care research have a very informative website: <http://invo.org.uk> which you may wish to browse

Specific information about this research project

If you have any questions, comments or problems in connection with this study please feel free to contact:

Mr Scott Cairns, Tel: 029 20 744505, or

Professor Keith Harding, Tel: 029 20 744505.

Cardiff University, Department of Wound Healing, School of Medicine, Upper Ground Floor, Heath Park, Cardiff, CF14 4XN

Advice as to whether you should participate

If you wish to, please discuss with family members and or any person you feel comfortable would give you impartial advice.

Who to approach if you are unhappy about this study

If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions.

Mr Scott Cairns Tel: 029 2074 2316, or Professor Keith Harding Tel: 029 2074 4505

If you remain unhappy and wish to complain formally, you can do this through the Cardiff University Research Governance Office or the appropriate NHS Trust Complaints Procedure.

Research Governance, Research and Commercial Division, Cardiff University, 30-36 Newport Road, Cardiff, CF24 0DE

Tel: 029 208 79277

Cardiff & Vale NHS Trust, University Hospital of Wales, Heath Park, Cardiff, CF14 4XN

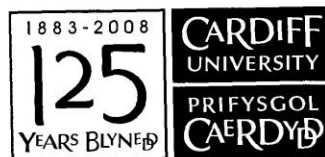
Tel; 029 2074 7747 and ask to speak to the Patient Complaints Manager

You will be given a copy of this Information Sheet and your signed consent form to keep.

***Thank you for considering taking part
and taking time to read this sheet.***

Appendix 3: Consent form for Burns Patients

Department of Wound Healing
Adran Gwella Clwyfau



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Mynydd Bychan
Caerdydd CF14 4XN

Patient Consent Form (Burns)

version 1.0, date 17/06/2008

Patient Identification Number for this trial: _____

Title of Project: Biomaterials: Biofilms, Infection and Wound Healing

Name of Researcher: Mr Scott Cairns

Please initial box

I confirm that I have read and understand the information sheet version 1.0 dated 17th June 2008 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.

I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected and that,

If I decide to withdraw, or are withdrawn, for any reason any data gathered to the point of withdrawal will be used in the data analysis for this study.

I understand that relevant sections of any of the data collected during the study, may be looked at by responsible individuals from; Cardiff University regulatory authorities Cardiff & Vale NHS Trust or Swansea NHS Trust, where it is relevant to my taking part in this research. All personal details will be treated as strictly confidential.

I agree to my discarded wound tissue being taken. I have been reassured that my privacy and dignity will be protected during the taking of these specimens and that they will be stored in a manner in which I will not be identifiable.

I agree to these specimens being used for scientific, and regulatory purposes in such a way whereby I cannot be identified personally

I understand that I am free to withdraw from this study: at any time

- without having to give a reason
- without it affecting my future medical care
- details of my participation up to the time of withdrawal may be stored anonymously and may be used in the final analysis of data

Biomaterials: Biofilms, Infection and Wound Healing. Version 1.2. 17/06/2008.

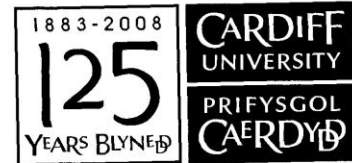
I agree to my GP being informed of my participation in the study

I agree to take part in the above study.

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent (if different from researcher)	Date	Signature
_____	_____	_____
Researcher	Date	Signature

Appendix 4: Patient Information Sheet for Burns Patients

Department of Wound Healing
Adran Gwella Clwyfau



Patient Information Sheet (Burns)

Welsh Regional Centre for Burns and Plastic Surgery

version 1.2, date 17th June 2008

Part 1.

1. **Study title** : Biomaterials: Biofilms, Infection and Wound Healing

2. Invitation paragraph

You are being invited to take part in a research study. Before you decide if is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish.

- Part 1 tells you the purpose of this study and what will happen to you if you take part.
- Part 2 gives you more detailed information about the conduct of the study.

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

3. What is the purpose of the study?

The purpose of this study is examine long standing wounds in patients, to assess for the presence of bacteria which may not be in a readily detectable state, such as in an adherent colony, called a biofilm
To assess whether certain burn wounds contain higher numbers of bacterial biofilms resulting in a delayed healing time.

4. Why have I been chosen?

You have been approached to participate in this study because you have been identified as having a burn wound which, despite initial clinical impression, has not healed and therefore requires surgery. It is our theory that your wound may contain bacterial biofilm.

5. Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are free to withdraw at any time and without giving a reason. Data collected up to the point of your withdrawal may be used in the final data analysis for this study.

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6. What will happen to me if I take part?

If you take part in the study, the burnt tissue which is removed in theatre and which is normally discarded, will be collected and analysed for the presence of biofilm. This tissue will be allocated an anonymous specimen number, and will not be traceable back to you.

7. Expenses and payments:

Due to the fact that your tissue would ordinarily be discarded, there are no expenses or payments available.

8. What will happen to my tissue samples?

The specimens will be taken into the laboratory and sectioned into five parts. Each portion will be examined using different techniques to look for the presence of bacteria. The results will be used to assess how the bacteria are behaving in different areas of your wound. At the end of the research period your tissue samples will be destroyed.

9. Will this affect how my wound heals?

No, this will not change your operation in anyway, and will therefore have no effect on your post-op care.

10. What are the alternatives for diagnosis or treatment?

Your treatment protocol will remain unchanged from that which you would usually receive.

11. What are the side effects of any treatment received when taking part?

In this study you will receive no additional treatments, outside of that which you would normally receive.

12. What are the other possible disadvantages and risks of taking part?

There are no specific risks or complications from taking part in the study, any complications that arise due to your normal medical care, will be dealt with by the doctors who usually look after you.

13. What are the possible benefits of taking part?

There are no potential benefits from taking part. However we hope the study will identify whether biofilms are present and, if so help us improve treatment in the future.

14. What if there is a problem?

If you have any worry about the way you have been dealt with during the study please contact, Mr Scott Cairns, Tel: 029 2074 2316

If you are however unhappy with the treatment you have received from a member of research staff you are entitled to make a complaint, have it considered, and receive a response from Cardiff University. Detailed information is given in Part 2

15. Will my taking part in the study be kept confidential?

Yes. All the information about your participation in this study will be anonymised and stored in a locked cupboard. You will be identified only by study number on all data collected.

16. Contact Details:

For further details you may contact Mr Scott Cairns- Tel: 02920 742316

This completes Part 1 of the Information Sheet.

If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decision.

Part 2

17. What if relevant new information becomes available?

Sometimes during the course of a research project, new information becomes available about the condition that is being studied. If this happens, your research doctor will tell you about it and discuss with you and your hospital team, the best way to continue your treatment. If you decide not to carry on the data collected to the time of your withdrawal may be included in the final analysis. If you decide to continue in the study you will be asked to sign an updated consent form.

18. What will happen if I don't want to carry on with the study?

We would like to emphasise that it is completely voluntary to participate in this study. If you decide to participate you are still free to withdraw from the study at any time without giving a reason; however we will use the data collected up to your withdrawal. If you do not wish to participate, or if you later wish to withdraw from the study, it will not affect the standard of care you receive or your legal rights as a patient.

19. Will my taking part in this study be kept confidential?

If you join the study the data collected for the study will be looked at by representatives of regulatory authorities and authorised people from Cardiff University and your NHS Trust to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and nothing that could reveal your identity will be disclosed outside the research site.

20. What is there is a problem?

Harm

In case of any injury caused by participation in this study the Products Liability Insurance of the sponsor, Cardiff University, will cover you.

If you participate you still have the right to complain about your treatment.

Complaints

If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions. Contact details are provided at the end of Part 2.

If you remain unhappy and wish to complain formally, you can do this through Cardiff University or the appropriate NHS Trust Complaints Procedure. Contact details are provided at the end of Part 2.

21. Involvement of the General Practitioner/Family doctor (GP)

Your GP will be notified of their participation in the trial, with your consent.

22. What will happen to the results of the research study?

The results of this study will be reported to Cardiff University, the study sponsor, and may be published or presented at medical/scientific conferences or journals. You will be identified only by study number in any report, publication or presentation of this data.

23. Who is organising and funding the research?

The Department of Wound Healing is supporting Mr Cairns to undertake this study.

24. Who has reviewed the study?

This study was given a favourable ethical opinion for conduct in Cardiff University by the South East Wales Research Ethics Committee.

Further Information and Contact Details

General information about research

Involve, Promoting public involvement in NHS, public health and social care research have a very informative website: <http://invo.org.uk> which you may wish to browse

Specific information about this research project

If you have any questions, comments or problems in connection with this study please feel free to contact:

Mr Scott Cairns, Tel: 029 20 744505, or

Professor Keith Harding, Tel: 029 20 744505.

Cardiff University, Department of Wound Healing, School of Medicine, Upper Ground Floor, Heath Park, Cardiff, CF14 4XN

Advice as to whether you should participate

If you wish to, please discuss with family members and or any person you feel comfortable would give you impartial advice.

Who to approach if you are unhappy about this study

If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions.

Mr Scott Cairns Tel: 029 2074 2316, or Professor Keith Harding Tel: 029 2074 4505

If you remain unhappy and wish to complain formally, you can do this through the Cardiff University Research Governance Office or the appropriate NHS Trust Complaints Procedure.

Research Governance, Research and Commercial Division, Cardiff University, 30-36
Newport Road, Cardiff, CF24 0DE

Tel: 029 208 79277

Cardiff & Vale NHS Trust, University Hospital of Wales, Heath Park, Cardiff, CF14 4XN

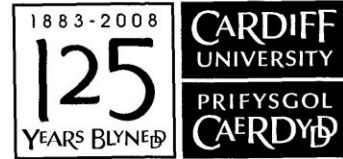
Tel; 029 2074 7747 and ask to speak to the Patient Complaints Manager

You will be given a copy of this Information Sheet and your signed consent form to keep.

***Thank you for considering taking part
and taking time to read this sheet.***

Appendix 5: Consent form for Tracheostomy patients

Department of Wound Healing
Adran Gwella Clwyfau



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Llawr Gwaelod Uchaf
Mynydd Bychan
Caerdydd CF14 4XN

Patient Consent Form (Tracheostomy)

version 1.0, date 17/06/2008

Patient Identification Number for this trial: _____

Title of Project: Biomaterials: Biofilms, Infection and Wound Healing

Name of Researcher: Mr Scott Cairns

Please initial box

I confirm that I have read and understand the information sheet version 1.0 dated 17th June 2008 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.

I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected and that,

If I decide to withdraw, or are withdrawn, for any reason any data gathered to the point of withdrawal will be used in the data analysis for this study.

I understand that relevant sections of any of the data collected during the study, may be looked at by responsible individuals from; Cardiff University, regulatory authorities Cardiff & Vale NHS Trust or Swansea NHS Trust, where it is relevant to my taking part in this research. All personal details will be treated as strictly confidential.

I agree to my discarded tracheostomy being taken. I have been reassured that my privacy and dignity will be protected during the taking of these specimens and that they will be stored in a manner in which I will not be identifiable.

I agree to these specimens being used for scientific, and regulatory purposes in such a way whereby I cannot be identified personally

I understand that I am free to withdraw from this study: at any time

- without having to give a reason
- without it affecting my future medical care
- details of my participation up to the time of withdrawal may be stored anonymously and may be used in the final analysis of data

I agree to my GP being informed of my participation in the study

I agree to take part in the above study.

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent (if different from researcher)	Date	Signature
_____	_____	_____
Researcher	Date	Signature

Appendix 6: Letter to GP

Department of Wound Healing
Adran Gwella Clwyfau

GP NOTIFICATION LETTER

Direct Line +44 (0)29 20744505

Email: cairns001@googlemail.com

our ref: SC/C625/06

GP Ltr version 1, 10/06/2008

Date

(GP address)

Dear Dr

Re: Patient Details

Study Title: Biomaterials: Biofilms, Infection and Wound Healing

Your patient has recently agreed to participate in the above study, which is taking place at the Department of Wound Healing, Cardiff University.

The study is to determine the nature of biofilms within medical devices and in non-healing wounds. Your patient has been identified as having a burn injury and is either in the category of having a tracheostomy in-situ or a non-healing burn wound.

In this study the used tracheostomy or debrided burn injured tissue will be collected and analysed to look for, and to characterise any adherent bacterial colonies, or biofilms, which may be present.

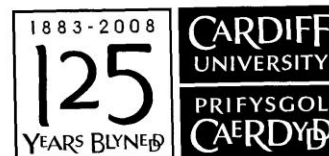
In either case, since we are using devices or tissue which are to be discarded, we do not anticipate our results to affect the ongoing care of your patient, however, should we discover information which has a bearing in your patient's care, we will inform both yourself and the hospital physician responsible for that patient's care.

If you require any further information please do not hesitate to contact me.

Yours sincerely

Mr Scott Cairns BM MRCS

Research Fellow, Department of Wound Healing



Cardiff University
Department of Wound Healing
School of Medicine
Upper Ground Floor
Heath Park
Cardiff CF14 4XN

Tel Ffôn +44(0)29 2074 451
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Website: <http://www.whru.co.uk>

*Prifysgol Caerdydd
Adran Gwella Clwyfau
Ysgol Feddygaeth
Llawr Gwaelod Uchaf
Mynydd Bychan
Caerdydd CF14 4XN*

Appendix 7: Confirmation letter from REC



**Canolfan Gwasanaethau Busnes
Business Services Centre
South West Wales REC**

Swansea
36 Orchard Street
SWANSEA
SA1 5AQ

Telephone: 01792 607416
Facsimile: 01792 607533

30 June 2008

Mr Scott Cairns
Clinical Research Fellow
Cardiff University
Department of Wound Healing
School of Medicine
Cardiff
CF14 4XN

Dear Mr Cairns

Full title of study: Biomaterials: Biofilms, Infection and Wound Healing
REC reference number: 08/MRE09/48
SSA reference number: 08/MMW02/61

Thank you for your application to conduct the above research as local Principal Investigator for Welsh Centre for Burns and Plastic Surgery. We can confirm that the application was received on 29 June 2008.

Professor S Bain will make an assessment of the suitability of the local Principal Investigator, site and facilities, and will notify this Committee whether or not there is any objection to the research being conducted locally. We will then notify the main Research Ethics Committee REC for Wales within 25 days of receiving your application.

It is the responsibility of the main REC to confirm the favourable opinion for each research site, taking account of the advice from site-specific assessors. The main REC will notify the decision to the Chief Investigator for the study and provide a list of approved sites (on form SF1). It is the responsibility of the Chief Investigator to notify the local Principal Investigator at each site.

It is your responsibility to ensure you have final approval from the R&D office for the relevant NHS care organisation before commencing any research procedures.

Yours sincerely

**Ms Penny Beresford
Committee Co-ordinator**

Copy to:

[R&D Office for NHS care organisation at lead site]
Canolfan Gwasanaethau Busnes GIG Cymru
Canolfan Henffordd, 36 Stryd y Benllan,
Abertawe, SA1 5AQ
Ffôn: 01792 458066
WHTN: 1780
Ffacs: 01792 607533
TNT QW5/QW3 32

NHS Wales Business Services Centre,
The Oldway Centre, 36 Orchard Street,
Swansea, SA1 5AQ
Telephone: 01792 458066
WHTN: 1780
Fax: 01792 607533
TNT QW5/QW3 32



NHS
WALES
CYMRU

CORRESPONDENCE 2008. CORRESPONDENCE scott cairns(ssa val let)29-6-08.rtf

rhan o Addysgu Bwrdd Iechyd Lleol Powys / part of Powys Teaching Local Health Board

Appendix 8: Standard Operating Procedure for the use and of Human Tissue

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

1. Purpose
2. Scope
3. Responsible Personnel

4. Definitions
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 waste¹.

Material taken from the living should normally be disposed of by incineration in accordance with current guidelines². Fetal tissue must be dealt with as per HTA advice³.

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

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.

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:

- 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 5⁴

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)

⁴ Code of Practice – the removal, storage and disposal of human organs and tissue, Human Tissue Authority, 2006.

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 in sealable containers, which are then labelled correctly.

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 9: Puregene protocol for the extraction of bacterial DNA

- DNA extracted from suspensions of pure bacterial isolate (1 – 5 colonies in 0.5 ml suspension solution) using a Puregene[®] DNA isolation kit (Qiagen) and following the “DNA Isolation From 1ml Gram-positive Bacteria Culture Medium” protocol (http://www.gentra.com/technical_assistance/protocols.asp).
- 16S rRNA gene PCR reaction mix:
 - 0.5 μ M of forward & reverse primer
 - e.g. either d88 / e94 (Paster *et al*, 2001) or 27F/1492R (Dymock *et al*, 1996)
 - EITHER working concentration of Promega PCR master mix (25 μ L) OR working concentration of Promega GoTaq green buffer, 3mM MgCl₂, 200 μ M dNTPs + 1.25U GoTaq polymerase
 - between 1 – 5 μ l gDNA extract (approx 0.2 – 0.5 μ g)
 - Total reaction volume of 50 μ L
- Use appropriate protocol on PCR machine
 - Protocol for d88 / e94 primer pair:
 - 95 °C for 5 min
 - 30 cycles of 95°C for 45 s denaturation, annealing at 60 °C for 60 s, and extension at 72 °C for 105 s. The extension step is extended by 5 s per cycle.
 - Final extension step, 72°C for 5 min
 - Reactions held at 4°C
 - Protocol for 27F / 1492R primer pair:
 - 94 °C for 5 min
 - 12 cycles of denaturation at 94 °C for 1 min, annealing at 65 °C for 1 min, and extension at 72 °C for 2 min, with the annealing temperature being reduced by 2 °C per cycle
 - 25 cycles of denaturation at 94 °C for 1 min, annealing at 41 °C for 1 min, and extension at 72 °C for 2 min
 - Final extension step, 72 °C for 10 min
 - Reactions held at 4°C
- For sequencing the PCR products should be purified by precipitation and washing with ethanol:
 - Firstly, add 15 μ l of 5M NaCl and 15 μ l of 40% polyethylene glycol (Mol. Wt. 8000; Sigma) to each PCR reaction volume.
 - Centrifuge (16,000g, 15 min)
 - Aspirate supernatant and replace with 200 μ l 70% ethanol (v/v)
 - Repeat centrifugation, aspiration and ethanol washing steps
 - Centrifuge again after 2nd ethanol wash
 - Dry PCR products, either in a vacuum (approx. 30 min) or under a fume hood (overnight) – better results with slow drying in fume hood
 - Re-suspend with 30 μ l nuclease-free water. Products can then be stored in freezer as necessary prior to sequencing.

Appendix 10: Molecular analysis of microbial communities in endotracheal tube biofilms – Paper generated from this MD

Published:

Cairns S, Thomas JG, Hooper SJ, Wise MP, Frost PJ, et al. (2011) Molecular Analysis of Microbial Communities in Endotracheal Tube Biofilms. PLoS ONE6(3): e14759. doi:10.1371/journal.pone.001475

Abstract

Background

Ventilator-associated pneumonia is the most prevalent acquired infection of patients on intensive care units and is associated with considerable morbidity and mortality. Evidence suggests that an improved understanding of the composition of the biofilm communities that form on endotracheal tubes may result in the development of improved preventative strategies for ventilator-associated pneumonia.

Methodology/Principal Findings

The aim of this study was to characterise microbial biofilms on the inner luminal surface of extubated endotracheal tubes from ICU patients using PCR and molecular profiling. Twenty-four endotracheal tubes were obtained from twenty mechanically ventilated patients. Denaturing gradient gel electrophoresis (DGGE) profiling of 16S rRNA gene amplicons was used to assess the diversity of the bacterial population, together with species specific PCR of key marker oral microorganisms and a quantitative assessment of culturable aerobic bacteria. Analysis of culturable aerobic bacteria revealed a range of colonisation from no growth to 2.1×10^8 colony

forming units (cfu)/cm² of endotracheal tube (mean 1.4×10^7 cfu/cm²). PCR targeting of specific bacterial species detected the oral bacteria *Streptococcus mutans* (n=5) and *Porphyromonas gingivalis* (n=5). DGGE profiling of the endotracheal biofilms revealed complex banding patterns containing between 3 and 22 (mean 6) bands per tube, thus demonstrating the marked complexity of the constituent biofilms. Significant inter-patient diversity was evident. The number of DGGE bands detected was not related to total viable microbial counts or the duration of intubation.

Conclusions/Significance

Molecular profiling using DGGE demonstrated considerable biofilm compositional complexity and inter-patient diversity and provides a rapid method for the further study of biofilm composition in longitudinal and interventional studies. The presence of oral microorganisms in endotracheal tube biofilms suggests that these may be important in biofilm development and may provide a therapeutic target for the prevention of ventilator-associated pneumonia.

Introduction

Ventilator-associated pneumonia (VAP) is the most frequent nosocomial infection in the intensive care unit (ICU) occurring in 8-28% of mechanically ventilated patients [1], [2]. Mortality rates are high (15-70%) and length of stay is increased, adding a cost of approximately \$40,000 per patient [3], [4]. The presence of an endotracheal tube is an independent risk factor for developing VAP and whilst tracheal intubation is necessary to facilitate mechanical ventilation, it also circumvents elements of patients' innate immunity. The endotracheal tube disrupts the cough reflex, promotes accumulation of tracheobronchial secretions and mucus, and provides a direct conduit for pathogenic microorganisms to reach the lower respiratory tract, increasing the risk of infection [5]. Significantly, the endotracheal tube may also act as a reservoir for pathogens by providing a surface to which they can adhere and form biofilms [5], [6], [7]. Polymicrobial biofilms develop rapidly following intubation along the inner lumen of the endotracheal tube, with well-organised antibiotic-resistant structures detectable within twenty-four hours [6], [7]. Furthermore, 70% of patients with VAP have been reported as having identical pathogens present within the endotracheal tube biofilm as encountered in the lung [8], suggesting that the biofilm represents a significant and persistent source of pathogenic bacteria.

There is mounting evidence to support the concept that poor oral health is related to the aetiology of bacterial VAP and a number of studies have suggested improved oral hygiene may be effective at reducing its incidence [9]-[12]. Moreover, potential respiratory pathogens such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*, that are not generally regarded as normal inhabitants of the oral microflora, are isolated more frequently in the dental plaque of ICU patients than that of the general population [13], [14]. Given that these potentially pathogenic bacteria are capable of adhering to and forming biofilms with the microflora of the

oral cavity (*i.e.* dental plaque), it seems plausible that oral plaque bacteria may also be present in biofilms of endotracheal tubes. The presence of oral microorganisms in a biofilm within endotracheal tubes may have significance beyond facilitating the adherence of potential respiratory pathogens. Co-aggregation of different microbial species can enhance the virulence characteristics of certain bacteria, as well increasing their tolerance to antimicrobials [15].

The composition of biofilms in general [16], and those originating in the oral cavity, in particular, is often misrepresented by traditional cultural analysis [17]. This distortion is apparent even for microorganisms amenable to culture, since recovery from a biofilm environment is subject to many biases including the ability of species to 'out-compete' neighbouring species in a given environment and variable replication times. The oral microflora is the most diverse bacterial community in the human body with over 700 species present, and cultural analysis is complex. In addition, given that over 50% of the oral microflora is considered to be unculturable or, more correctly, 'not-yet-cultured' the community composition is likely to be considerably distorted by cultural analysis [17].

Numerous culture independent approaches are available for the analysis of mixed microbial communities. One of the most useful has proven to be the community profiling technique known as denaturing gradient gel electrophoresis (DGGE), a method used extensively by microbial ecologists for the study of environmental communities [18], [19]. This approach has been successfully applied to the analysis of wounds, corneal ulcers, gastrointestinal tract and oral cavity [20]-[24]. DGGE is based on the PCR amplification of phylogenetically useful molecules (typically 16S ribosomal DNA) from mixed bacterial communities and the subsequent separation of the amplicons on a denaturing electrophoretic gel. The conditions allow the separation of bands of the same length that have different nucleotide composition. The

resulting banding profiles can be analysed to reveal differences in the predominant bacterial composition of samples in cross-sectional and longitudinal studies.

The aim of this study was to characterise microbial biofilms on the inner luminal surface of extubated endotracheal tubes from ICU patients. More specifically the presence of oral marker bacteria and VAP associated pathogens was determined by PCR and the usefulness of DGGE community profiling for the analysis of the endotracheal tube biofilm was assessed.

Materials & Methods

Collection and processing of endotracheal tubes

A total of 24 extubated endotracheal tubes were obtained from 20 patients (12 male and 8 female; age range 20-79 years; mean 61 years) who were intubated and mechanically ventilated on the ICU of a university teaching hospital. Two endotracheal tubes were obtained from each of two of these patients and one patient provided three endotracheal tubes. The duration of intubation prior to endotracheal tube collection was 12-289 h (mean 92 h). Prior to the study the South East Wales IRB confirmed that the project raised no ethical issues and therefore informed consent was not required.

Collected endotracheal tubes were placed in a sealed sterile bag and transferred immediately to the microbiology laboratory for processing (undertaken within 1 h of endotracheal tube collection). From the central region of each endotracheal tube two 1 cm sections were cut and processed. One was used for quantitative microbial culture and the other for molecular analysis. The biofilm was aseptically removed from the endotracheal tube lumen using a sterile scalpel and this was resuspended in 2 ml of phosphate buffered saline (PBS). The preparation was vortex mixed for 30 s with sterilised glass beads to disrupt any biofilm aggregates.

Quantitation of microbial colonisation of the endotracheal tube

The PBS solution containing recovered biofilm was serially diluted 10⁶-fold. Using a spiral-plater system (Don Whitley Scientific, Shipley, UK), 50 µl volumes of each dilution were deposited on to agar media. Quantitation of aerobic microorganisms was achieved by culture on blood agar (BA; LabM, Bury, UK) whilst CHROMagar[®] *Candida* (CHROMagar, Paris, France) was used to detect *Candida*. Inoculated agars were incubated for 37°C for 48 h. A total aerobic microbial count from the sections was obtained and expressed as total colony forming units (cfu) per cm² of endotracheal tube section. In addition, *Candida* species were identified based on colony colour and appearance on CHROMagar[®] *Candida* [25] and by biochemical profiling using the Auxacolor 2 system (Bio-Rad, Hemel Hempstead, UK) [26].

16S ribosomal RNA gene-defined bacterial colonisation of endotracheal tubes

Sections from each region of the endotracheal tubes were analysed using molecular procedures. The method involved the extraction of total DNA from 500 µl of the PBS specimens using a Puregene[®] bacterial DNA isolation kit (Qiagen). A universal bacterial primer pair, 341F and 534R [27] was then used to amplify the 16S rRNA gene targets within the extracted samples using standard PCR and reaction conditions. Negative controls included a reagent control (sterile water served as PCR template) and a sample preparation control (sterile water used in place of the original sample and exposed to the entire extraction protocol). Positive controls of template DNA from three known species (*Porphyromonas gingivalis* NCTC 11834^T, *Staphylococcus aureus* NCTC 6571 and *Streptococcus mutans* NCTC 20523^T) were also included. PCR products were then resolved by denaturing gradient gel electrophoresis (DGGE) as outlined

below with the products from the control microorganisms serving as markers to facilitate gel normalisation.

Denaturing Gradient Gel Electrophoresis

Polyacrylamide gels were cast using a model 385 gradient former (Bio-Rad). All gels comprised of 1×Tris-acetate-EDTA (TAE) buffer with 10% (w/v) acrylamide, 0.1% (v/v) TEMED, and 0.1% (w/v) ammonium persulphate (all materials from Sigma, Poole, UK). Gels also contained a parallel 30-60% gradient of denaturants (where 100% denaturant concentration was equal to 7 M urea and 40% [v/v] deionised formamide; Sigma).

Parallel DGGE was performed using the Bio-Rad D-Code system. Products from endotracheal tube extract PCRs were run alongside a marker comprising of an equal mixture of PCR products from the three known bacterial stains. DGGE was run in 1×TAE buffer at 56°C and 160 V/cm² for 30 min, followed by 40 V/cm² for 16 h.

DGGE gels were stained with SYBR® Green I nucleic acid gel stain (Sigma) at approximately 4°C for 30 min. Banding patterns were visualised under UV light using a GelDoc system (Bio-Rad). Gels were then aligned and standardised using the reference markers and GelCompar II software (Applied Maths, Sint-Martens-Latem, Belgium). The presence of individual bands in each profile was assessed using the same software, set to a 1% tolerance of band matching. Using Dice's coefficient and the UPGMA clustering method, a dendrogram of genetic similarity between samples was calculated.

Detection of bacteria by species-specific PCR

Species-specific detection of the oral marker organisms *S. mutans* and *P. gingivalis*, together with the accepted VAP causing organisms *P. aeruginosa* and *S. aureus* was also undertaken according to previously published protocols [28]-[31].

Results

Culture of microorganisms from biofilms recovered from endotracheal tubes

The results of microbial culture are summarised in Table 1. In the case of five endotracheal tube samples, no microbial growth was observed. The viable bacterial aerobic counts ranged between 2×10^4 and 2.1×10^8 cfu/cm² of endotracheal tube section. In 5 endotracheal tubes no viable aerobic bacterial growth was detected, although in one of these tubes *Candida* was cultured. There was no apparent relationship between the determined bacterial load on the sample and the duration of intubation. In six endotracheal tubes from five patients, *C. albicans* was isolated following subculture on CHROMagar[®] *Candida* and biochemical identification.

DGGE estimation of species richness

The number of bands obtained by DGGE analysis is shown in Table 1 and typical profiles are presented in Figure 1. An average of 6 bands (range 3 to 22) per endotracheal tube sample was detected and the similarity in banding profiles ranged between 20 and 90% (Fig. 2). There was no apparent relationship between the duration of intubation and the number of DGGE bands detected, nor was there a high level of similarity for the DGGE profiles obtained for multiple endotracheal tubes from the same patients (*i.e.* patient 2, tubes 2 and 7; patient 17, tubes 21 and 24). However, similarity at 75% was evident for the profiles of two endotracheal tubes (14 and 15) which were obtained from patient 12 (Fig. 2). DGGE was shown to be reproducible with consistent profiles being generated upon both repeat and duplicate testing. The community profiles obtained demonstrated considerable inter-patient and intra-patient diversity.

Detection of marker bacteria by species specific PCR

At least one of the oral marker bacterial species (*S. mutans* or *P. gingivalis*) was detected by PCR for 9 different patients. Of these 9 patients, PCR was positive only for *S. mutans* in 4 patients, as was the case for *P. gingivalis*, the endotracheal tube from one patient (Patient 19, endotracheal tube 23) was positive for both oral bacterial species (Table 1). Figure 3 shows a typical result of the *P. gingivalis* specific PCR using template DNA obtained from the biofilms recovered from the endotracheal tubes. The detection of the VAP associated species *P. aeruginosa* and *S. aureus* occurred in 4 and 6 patients, respectively. The patient distribution of these microorganisms is presented in Table 1.

Discussion

Sottile and colleagues first suggested a link between the endotracheal biofilm and pulmonary infection [32], and this was supported by identical pathogenic bacteria being present in the lung of patients with VAP and the endotracheal biofilm [8]. This concept has been substantiated by a number of experimental and clinical studies, which have used antimicrobial (silver coated) endotracheal tubes to inhibit biofilm formation [33]-[36], reduce colonisation of the airway [33]-[36] and decrease the incidence of VAP [37]. The reduction in the number and delay of onset of VAP in a large clinical trial is encouraging, however, bacteria can develop silver resistance [38] and other strategies, which target biofilm formation, may prove to be valuable. In order to prevent endotracheal tube biofilm formation, knowledge of the source of the microorganisms involved is essential.

Improved oral hygiene has proved to be an effective strategy for reducing VAP [9]-[12] and since potential respiratory pathogens are isolated from dental plaque (a biofilm) of mechanically ventilated patients [13], [14], we hypothesised that microbes which constitute the normal oral flora might be present in the endotracheal biofilm. In an *in vitro* Ventilator-Endotracheal-Lung model incorporation of representative species of the oral microflora prior to the addition of pathogens traditionally associated with respiratory infection, augments the subsequent biofilm (JG Thomas, unpublished data), raising the possibility that normal oral microflora may represent pioneering colonising species and promote subsequent endotracheal tube biofilm development. The inner surface of the endotracheal tube provides a unique environment of high shear forces, bi-directional flow, and a restricted immunological environment, which favours a sessile rather than planktonic bacterial life form and thus biofilm formation. The endotracheal biofilm is a complex three-dimensional structure with cyclical

bacterial communities many of which are difficult to isolate by conventional planktonic culture techniques [39]. Bacterial species regarded as appropriate markers of the oral microflora (*P. gingivalis* and *S. mutans*) were detected in 9 of the 20 patients using species specific PCR. *Streptococcus mutans* was selected as a target species due to its recognised ability to promote biofilm formation, whilst *P. gingivalis* was included as a representative species of strictly anaerobic bacteria.

Interestingly, *Candida albicans*, which is also a common coloniser of the oral cavity, was detected by culture in the endotracheal tubes from 5 patients. This yeast produces filamentous growth forms, which could readily enhance formation and structural stability of the biofilm [40]. Interactions between individual elements of the oral microflora and their capacity to form biofilms are complex. *Streptococcus mutans* enables *C. albicans* to bind to synthetic surfaces, coaggregation being dependent on the production of water insoluble glucans and direct cell-cell contact [41], [42], [43]. *Candida albicans* also demonstrates synergistic and antagonistic interactions with *S. aureus* and *P. aeruginosa*, which account for 20% and 24% of VAP respectively [44]. *Pseudomonas aeruginosa* can neither bind nor kill yeast-forms of *C. albicans*, however this bacterium can form a dense biofilm on *C. albicans* filaments [45]. Significantly, *P. aeruginosa* virulence factors associated with human disease (e.g. secreted phospholipase C) have also been linked with biofilm formation and killing of the filamentous forms of *C. albicans* which might then provide nutrients for bacterial biofilm development [45]. Colonisation of the respiratory tract with *C. albicans* is associated with an increased risk of *Pseudomonas* VAP [46], and conversely antifungal treatment of patients demonstrating airway colonisation with *Candida* spp. have less *P. aeruginosa* VAP [47]. An anti-candidal approach has also been successful in reducing the biofilm on laryngeal prosthetic devices [48]. The variation in aerobic bacterial counts between patients ranged between 0 and 2.1×10^8 cfu/cm². The reason for such

variation (including the absence of bacterial growth in 5 endotracheal tubes) could in part relate to the effectiveness of the seal between endotracheal tube cuff and mucosal wall of the trachea. A totally effective seal would prevent leakage of pooled secretions from above the cuff leading to subsequent contamination of the endotracheal tube lumen and lungs. The effectiveness of the seal will vary with pressure variation between the balloon of the cuff and by the extent of 'folding' in the cuff material. Micro-channels generated by such folds can serve as conduits for microbial passage. Oral hygiene parameters of the patients were not assessed in this study and it may be the case that patients with poorer oral hygiene exhibited higher endotracheal tube contamination and *vice versa*. Some loss of microbial viability between the time of removal of the endotracheal tube from the patient and its subsequent processing for culture is likely, despite the time between these procedures being kept as short as possible. The fact that microbial DNA was obtained from these endotracheal tubes would suggest microbial cells (viable or non-viable) had been present and highlights the benefit of supplementing the culture analysis with molecular investigation.

The present study has shown that DGGE can be used to generate community profiles of the microflora associated with endotracheal tubes. This non-cultural approach has revealed significant microbial diversity with between 3 and 22 (mean 6) bands present per sample. These results are similar to those of other studies of microbial communities assessed by non-cultural methods. In the majority of systems studied, molecular methods detect the presence of a greater number of species than culture alone. For example, in the analysis of failed root canal lesions, between one and 26 bands (mean six) were detected by molecular methods compared to 3-4 isolates detected by culture [49].

There is no direct correlation between the number of bands detected by DGGE and the number of species present in a sample since numerous factors affect band number including sampling, PCR and differential DNA extraction biases, heteroduplex formation and divergence within multicopy number rDNA families. However, following excision and sequence analysis of individual bands present on DGGE gels, it has been reported that on average each band represents not just one, but an average of 2.7 phlotypes or bacterial species [50]. Therefore, the species richness within the endotracheal tube may be even greater than that implied in the current study.

Previous studies of complex human microbial communities using DGGE have revealed similar levels of diversity to that encountered in the present study. In a study of the microflora associated with dental abscesses from two different geographical locations a total of 99 distinct bands were detected [51]. In both this and an earlier study of root canal lesions many of the observed bands were shared between patient profiles [49]. Although a small number of shared bands were observed in our current study, the inter-patient profile diversity was generally greater when compared to previous studies. Moreover, there was significant intra-patient diversity for the three patients from whom more than one endotracheal tube community profile was obtained. The marked diversity revealed is perhaps not surprising given that the oropharyngeal microflora is considered to be the most complex in the human body with more than 700 different species present of which over 50 % have not yet been cultivated [17].

Of the numerous culture-independent approaches that are available, DGGE has proven useful in the analysis of many different microbial communities, both environmental and those associated with human infection. The principle advantage is the ability to analyse those bacteria which are either difficult to culture or which have not yet been cultured. The presence in

bronchoalveolar lavages (BALs) of both novel microorganisms and those that are already 'known' on the basis of sequence characterisation but have not yet been cultivated, has been demonstrated [52]. This research employed a ribosomal RNA cloning and sequencing approach which provided valuable information on the microbial composition of BALs obtained from traumatic ICU patients. The approach demonstrated considerable diversity of bacterial types present in BAL samples with over 54 different bacterial types present, of which 38 had not previously been detected at this site.

Both DGGE and the cloning/sequencing approach have a role to play in the analysis of complex microflora including VAP associated bacteria. DGGE is less technically demanding, less labour intensive and more affordable. Consequently, it may be used for the simultaneous analysis of multiple samples, facilitating the direct comparison of bacterial communities from numerous sources and the effects of interventions in longitudinal studies. DGGE has been used successfully to demonstrate differences in profiles between two distinct patients groups, both in geographic and clinical contexts. The method has been used to demonstrate that both the diversity and complexity of the plaque microflora was less in children with caries compared to caries free children [50]. Differences in DGGE profiles of dental abscesses of Brazilian and USA origin have also been shown [51]. In contrast to many other profiling techniques, DGGE has the advantage that if marker signatures or bands appear to be significant, the bands can be excised and sequenced for further characterisation. In this way, a better characterisation of the microflora associated with dental caries has been demonstrated [51]. Furthermore, as in the current study, the technique can incorporate marker profiles of species of interest [53].

Care must however be taken if DGGE is used as a technique for species identification as it does have severe limitations in this regard. Since sequences from different species can co-

migrate to the same position in the gel [54] identification based on excised band sequencing requires every fragment to be singularised before sequencing. This can involve either another gradient gel electrophoresis step or a time-consuming cloning process [55]. Furthermore if comparison is made with specific PCR analysis then consideration has to be made to the lower sensitivity of DGGE when compared to PCR directed against a specific microorganism [53]. It has been reported that the sensitivity of DGGE in a mixed community is about 10^3 cells [56]. However, in terms of community profiling this may not necessarily be a disadvantage since DGGE will reflect the predominant microorganisms present and in the field of medical microbiology, numerical predominance is a fundamental principle.

In summary, a combination of PCR, molecular profiling and culture was used to ascertain the bacterial diversity and presence of specific marker microorganisms in endotracheal tube biofilms. In this context, molecular profiling using DGGE demonstrated considerable biofilm compositional complexity and inter-patient diversity and, as a method that facilitates the study of shifts in the ecological balance of biofilms, will prove invaluable in future longitudinal or interventional studies. We hypothesised that oral microorganisms form part of the endotracheal tube biofilm and, indeed, demonstrated that these bacteria were present. This observation offers the possibility of alternative therapeutic strategies, not necessarily based on anti-infective agents, for preventing VAP which may include mechanically reducing oral plaque, the targeting of quorum sensing signal molecules [57], [58] or modulation of the oral microflora [59].

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Figure 1. DGGE profiles of PCR amplified 16S rRNA from marker bacteria and endotracheal tube (ETT) biofilm samples

Figure 1a: 1, *P. gingivalis*; 2, *S. aureus*; 3, *S. mutans*; 4, mixed marker of *P. gingivalis*, *S. aureus* and *S. mutans*; 5, ETT1; 6, ETT2; 7, ETT3; 8, ETT4; 9, ETT5; 10, ETT7; 11, ETT8; 12, ETT9; 13, ETT10; 14, ETT11; 15, mixed marker of *P. gingivalis*, *S. aureus* and *S. mutans*.

Figure 1b: 1, mixed marker of *P. gingivalis*, *S. aureus* and *S. mutans*; 2, ETT12; 3, ETT14; 4, ETT15; 5, ETT19; 6 ETT21; 7, ETT25; 8, ETT26; 9, mixed marker of *P. gingivalis*, *S. aureus* and *S. mutans*.

Figure 1c: 1, Plaque sample control; 2, mixed marker of *P. gingivalis*, *S. aureus* and *S. mutans*; 3, ETT13; 4, ETT16; 5, ETT17; 6, ETT18; 7, ETT20; 8, ETT22; 9, ETT23; 10, ETT24; 11, mixed marker of *P. gingivalis*, *S. aureus* and *S. mutans*.

Figure 2. Cluster analysis with Ward's algorithm based on the Dice coefficient demonstrating the diversity in DGGE profiles generated from endotracheal tube (ETT) biofilms.

Table 1. Band numbers obtained by DGGE analysis, PCR and culture results for selected organisms

Patient number	Endotracheal tube	DGGE		PCR detection					Culture
		Hours	Number of bands	<i>S. mutans</i>	<i>P. gingivalis</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>C. albicans</i>	Total aerobic bacterial count
		intubated							cfu/cm ² endotracheal tube
1	1	33	5						2.3×10 ⁷
2	2	108	16	+				+	6.3×10 ⁶
2	7	94	4						2.8×10 ⁷
3	3	60	17					+	1.8×10 ⁶
4	4	95	14			+			6.1×10 ⁶
5	5	43	22					+	2.0×10 ⁴
6	10	41	9		+		+		0
7	8	289	8						3.0×10 ⁷
8	9	173	15	+		+	+	+	4.0×10 ⁴
9	11	198	10				+		2.1×10 ⁸
10	12	56	6		+				2.0×10 ⁶
11	13	137	5						7.7×10 ⁶
12	14	12	3						4.0×10 ⁴
12	15	20	5					+	0
12	16	96	6	+				+	1.6×10 ⁵
13	17	224	4						0
14	18	49	5		+		+		0
15	19	29	3						4.0×10 ⁴
16	20	67	7	+			+		1.3×10 ⁷
17	21	20	5			+			0
17	24	143	5			+			7.7×10 ⁶
18	22	76	4		+		+		1.4×10 ⁷
19	23	32	9	+	+				1.8×10 ⁵
20	25	125	3						8.2×10 ⁶

**Appendix 11: DVD - Bacterial sequences from 16S rDNA
PCR**

Appendix 12: DVD – Further images of PNA FiSH CLSM