The Consequences and Prevention of Bacterial Biofilm Infection of Silicone Breast Implants

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BSc (Hons) Grad Dip MBBS

In Memoriam

I dedicate this thesis and degree to my Father

Colin George Leslie Whicker

(28/1/1942 – 8/2/2013)

You brought me into this world,

you believed in me

and encouraged me to aim for the stars!

"Beyond a wholesome discipline, be gentle with yourself. You are a child of the universe, no less than the trees and the stars; you have a right to be here. And whether or not it is clear to you, no doubt the universe is unfolding as it should.

Therefore be at peace with God, whatever you conceive Him to be, and whatever your labours and aspirations, in the noisy confusion of life keep peace with your soul. With all its sham, drudgery, and broken dreams, it is still a beautiful world. Be cheerful. Strive to be happy."

From Desiderata 1927

May you Rest in Peace

STATEMENT OF ORIGINAL WORK

This thesis contains no material that has been accepted for the award of any other degree or qualification at any other university. To the best of my knowledge this thesis is original and contains no material previously published or written by another person, except where due references or acknowledgements are given in the text and publications indicated below from this material.

Publications

Hu H, Johani K, Gosbell I, **Jacombs, A**, Almatroudi A, Whiteley GS, Deva AK, Jensen S, Vickery K. Intensive care unit environmental surfaces are contaminated by multiresistant bacteria in biofilms: combined results of conventional culture, pyrosequencing, scanning electron microscopy and confocal laser microscopy. *Journal of Hospital Infection.* Accepted – *in Press* DOI: 10.1016/j.jhin.2015.05.016

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Abstract

Bacteria are one of the most successful organisms on the Earth's surface. Mankind has evolved to utilise many of the actions of bacteria to our benefit and advantage, such as synergistic bacterial colonisation of the gut and its function in digestion. Bacteria also cause many human diseases. Indeed one of the most important human advances over the past 200 years has been the rapid development of the Discipline of Microbiology as it has given medicine the knowledge and ability to now treat and cure many of these previously life-threatening infections.

The discovery of bacteria living within communal structures, or biofilms, in the 1970's challenged much of our scientific and medical knowledge of bacteria in health and disease. Bacterial biofilms are now acknowledged to be important in the aetiology of many infections, including infections of surgical implants. Biofilm infection is estimated to occur in between 1-9% of all surgical implants, depending on the device. These infections are characterised by a chronic indolent inflammatory process that can be punctuated by localised or embolic septic events. They are very difficult to diagnose and treat, commonly requiring surgical removal with or without implant replacement and are associated with rapidly increasing health care costs that are not sustainable into the future.

Silicone breast implants are just one type of surgical implant where bacterial biofilm infection has been implicated in a chronic fibrotic inflammation of the implant capsule. There is now increasing evidence that this may lead to capsular contracture, the most common long-term complication of silicone breast implants.

This thesis reviews the evidence for the role of bacterial biofilm infection of breast implants and the formation of capsular contracture. It also uses the porcine model of biofilm infection of silicone implants to:

1) further investigate the role of endogenous breast bacteria in the development of capsular contracture;
2) evaluate the ability of a novel antibiotic prosthetic cover to prevent of bacterial biofilm infection,

3) investigate the cellular immune response to biofilm infection of silicone implants, and
4) postulate whether biofilm-related chronic inflammation and its immune response may be implicated in the neoplastic process of the new entity of breast implant-associated anaplastic large cell lymphoma.

Chapter 1

Where did this Story Begin?

"*The important thing is not to stop questioning.*
Curiosity has its own reason for existing."

- Albert Einstein

1 Introduction

1.1 Early History of Microbiology

The idea that microorganisms may be the cause of disease was first postulated by Marcus Terentius Varro, a Roman polymath, over 2000 yrs ago.[1] In his only surviving intact work *De re rustica*, a treatise on farming practices published in 36 BC, he advised:

"*Care must be taken in swampy locations … since in such places there grow certain minute animals (animalia quaedam minutia) which cannot be followed by the eye and which are carried with the air into the body by way of the mouth and nostrils, there giving rise to serious disease*".[1]

Whilst the concept of minute animals, later called small beasts (*bestiolae*), causing disease was proposed by Varro and repeated by his contemporaries: scholar Scrofa and architect Vitruvius and later writers Columella and Palladius, it did not change the biological and medical thoughts on disease at that time.[1]

Over the next 2000 years the theories of disease suggested they were due to "spontaneous generation" and the popular concepts of "miasmas" or "vapours in the air" or "supernatural retribution for sinful behaviour" predominated.[2, 3] During this time very few scholars suggested that microorganisms may cause disease. Girolamo Fracastoro, a physician, scholar, poet and colleague of Copernicus at the University of Padua, made several studies on epidemic diseases. He named and documented, in rhyme, the disease

Syphyllis in "*Syphillis sive morbus Gallicus"* (Syphilis or the French Disease) in 1530. In 1546 he detailed his concept of epidemic disease in *"De contagion et contagios morbis"* (On Contagion and Contagious Disease). In this study Fracastoro stated that each epidemic disease was caused by a different rapidly multiplying "*seminaria*" (seed of disease)[2, 4] and these *seminaria* were spread in three ways: direct contact, carriers including soiled clothing and linen and by air.[1] His ideas, whilst praised during his life, subsequently waned after his death and fell into disrepute. It took another 350 years, the invention of the microscope, identification of micro-biological life, and development of culture techniques before the true understanding of the role of microbes in the pathogenesis of disease was elucidated.[1]

1.2 The Microscope and Microorganisms

It wasn't until the latter half of the 17th century that microorganisms were first documented. The first crude microscope had been made in 1590, but it was Robert Hooke, an English physicist, who first developed a microscope to document microbial life.[5] In his 1665 publication *Micrographia* he made a detailed study of white and blue mould and attempted to explain how they reproduced without seed.[5] The first descriptions of bacteria were written between 1676 and 1684 by Antonie van Leeuwenhoek, a Dutch draper with a keen interest in grinding lenses to study the microscopic natural environment. In a large series of letters to the Royal Society of London, commencing in 1673, van Leeuwenhook commenced documenting the microbial life he observed from under his microscopes. His first documentation of a microorganism was from *"pepper-water"* where he documented several sorts of *"creatures"* about 100th the size of a *"grain of course sand"*.[5] These were probably protozoa.[5] His first documentation of bacteria resulted from looking at the dental plaque from his teeth in a letter dated 9th October 1676, when he observed *"with great wonder that there were many very little animalcules, very prettily moving*."[5] Numerous microscopists went on to describe and classify bacteria, but it took almost another two hundred years before their importance in disease was understood.

1.3 Germ Theories of Disease

By the 19th century there was increased understanding of the microbiology of bacteria and science was also expanding the understanding of physiology, animal chemistry, biology and medicine.[2, 3] These advances in knowledge and understanding of biology led to increased interest in understanding the basis of disease. Leading thinkers began questioning the ideas of spontaneous generation and miasmas, supernatural forces and retribution, and began to focus on the environment and changes within the body to explain pathology and disease.[2, 3] It wasn't until the mid to late 19th century when experiments by Louis Pasteur, a French chemist, and Robert Koch, a German physician, amongst others, provided the proof that microorganisms caused disease.

The first evidence that a disease could be caused by another living organism came in the 1850's from Pasteur's studies on the process of fermentation of beer and wine.[6, 7] He investigated the processes of lactic, ethanolic, butyric and acetic fermentation. In his initial studies on the process of lactic fermentation, Pasteur isolated and cultured lactic bacillus from a greyish deposit present in souring milk.[6] He demonstrated visually that they were all alike, and then confirmed that when lactic bacillus was mixed with glucose it produced lactic acid.[6] Pasteur then demonstrated that yeast induced ethanolic fermentation and a rod-shaped bacillus microorganism (different to lactic bacillus) was responsible for butyric fermentation.[6] With these early studies on fermentation Pasteur demonstrated that specific microbiological agents were required for specific fermentation processes.

In his experiments on butyric fermentation Pasteur observed under the microscope that the bacillus was more active in the centre rather than at the edge of the coverslip.[6] He also observed that butyric fermentation was stopped when exposed to air. This was the reverse of the behaviour of lactic bacillus, yeast and other microorganisms. From these observations he determined that life without oxygen was possible and developed the terms "aerobic" and "anaerobic" for life in the presence of oxygen and life without oxygen, respectively.[6]

Subsequent to these experiments Pasteur was instrumental in rebutting the accepted wisdom on the aetiology of disease – "spontaneous generation of life". In 1859, the year Darwin's "On The Origin of Species" was published; Pasteur joined the debate on the origin of life. In a series of elegant studies Pasteur refuted experiments by Pouchet, the then Director of the Natural History Museum in Rouen, who supported spontaneous generation. In his famous experiments using swan-necked flasks, he demonstrated that a broth that is sterilised in a flask with a "S"-shaped neck remains sterile indefinitely even when open to air, as the humidity within the long curved tube sterilised the "outside" air. The flask however did retain the elements necessary for life as once the neck was broken microbial life developed quickly within the broth. These experiments demonstrated that provided microbes were excluded "spontaneous generation" did not occur and non-heated air can only trigger microbial growth if it contained living germs.[6, 8]

Pasteur went on to demonstrate that microorganisms were unevenly distributed within the atmosphere. In a series of experiments with sterile flasks opened in many different places, Pasteur discovered that contamination was higher at low altitudes and near inhabited and cultivated areas.[6] These experiments were the genesis for the development of aseptic manipulation, sterilisation and autoclave techniques that are still used today.

Koch graduated from Medicine in 1866 and started investigating anthrax, which was a major cause of death in both humans and farm animals across Europe at that time. Using blood from infected animals he identified *"rod-shaped"* structures that were not present in healthy animals.[9] In a series of elegant studies he inoculated healthy animals, with blood from diseased animals, and they went on to suffer from the disease.[9] At autopsy, Koch was the able to retrieve the rod structures from the inoculated animal's blood, lymph nodes and spleen. He was able to repeat this process.[9] During these experiments he also determined the life cycle of *Bacillus anthracis*, including the formation of spores that enabled the bacteria to survive in the environment between infections.[9] His results were published in 1876 and were the first definitive proof that a specific bacterium causes a specific disease.

Koch's experimental designs led to the development of 4 principles to prove causation between a microbe and a disease. These principles, known as Koch's Postulates, state that:

1) the microorganism must be found in all diseased animals;
2) the microorganism can be isolated and cultured outside the diseased animals;
3) administration of the isolated culture to inoculate another animal will reproduce the same disease;
4) the same microorganism can be isolated from the inoculated animal.[3, 9]

Pasteur's experiments underwrote the movement away from "spontaneous generation" and defined the role of bacteria in the causation of disease. His experiments also formed the basis of the development of vaccines, bacterial virulence and immunity and he is considered by some as the grandfather of immunology.[6] Koch and colleagues developed many of the microbiological principles and laboratory techniques that still underpin modern microbiology.[3, 9] Koch and Richard Petri, developed culture media, "Petri" culture plates, fixatives and stains that remain common tools and techniques in microbiological laboratories.

1.4 Non-culturable Bacteria and Disease

Free floating, or planktonic, bacteria are the foundation of our understanding of infection and disease. The ability to isolate and grow pure cultures from a single bacterium is the basis of microbiology techniques and medical diagnostics that extends back to the 1870's and the work by Pasteur & Koch.[10, 11] The *in vitro* techniques they developed using planktonic bacteria still underpin our understanding of bacterial physiology and pathogenesis of disease and sepsis, yet this understanding does not reflect how we now know bacteria exist in our environment.[10, 11]

The observation that bacteria prefer to grow in communal groups on surfaces was made almost 80 years ago.[12] Marine scientists, in the 1930's and 1940's, were the first to observe that bacteria in water-borne environments preferred to attach to solid surfaces.[13] These bacteria were sessile and lived in small microcolonies attached to

submerged surfaces. The bacterial numbers were much higher in the colonies than in the surrounding water and they were covered with an irregular film of complex organic matter.[14-16] Studies showed that the attached bacteria within these colonies were not removed by numerous solvents including acids (hydrochloric and sulphuric), alcohols, caustic, ammonia, or bicarbonate solutions.[15] Also the bacteria were not seen in pure cultures when grown in nutrient-rich media.[10] Yet these bacteria were metabolically active and capable of growing and multiplying within these enclosed micro-environments.[14, 15, 17]

Various studies in the 1960's on dental plaque and water pollutants demonstrated that the organic film was produced by the bacteria themselves and was rich in polysaccharides.[18-20]. Further experiments showed that the long polysaccharide chains were anchored to lipopolysaccharides on the bacterial cell wall and adhered to other bacterial cells and adjacent surfaces.[19, 21] This substance was initially referred to as slime or "glycocalyx', until Costerton coined the term "biofilm" to define sessile microbial communities that attached to surfaces and surrounded themselves in this extracellular polymeric substance.[21, 22]

Historically, van Leeuwenhoek was the first person to detail biofilm bacteria by observing microbial life from the plaque growing on the surface of his teeth under crude handmade microscopes.[5, 23] With this observation he described microscopic microbial life for the first time, which was the first key step in the development of modern microbiology. However the significance of these observations, with respect to bacterial biofilms and disease, was not realised for almost 300 years.[5]

In 1978, a landmark paper reported the ability to calculate the bacteria in a pristine mountain stream and demonstrated that sessile bacteria counts within biofilm, attached to rocks and other surfaces, predominated over planktonic bacteria counts by several orders of magnitude.[17] These results were consistent with observations from previous studies of aquatic ecological sites, including marine and soils, where sessile bacteria within biofilms vastly outnumbered the planktonic bacteria.[13-16]

1.5 The Biofilm Hypothesis

The biofilm hypothesis states that bacteria in all nutrient-sufficient ecosystems grow predominantly in matrix-enclosed surface-associated communities, or biofilms, which provide protection from external bacterial factors.[21] It is now acknowledged that over 99% of all bacteria grow in biofilms in virtually all nutrient-sufficient aquatic environments including natural,[17] industrial,[24-26] and medical ecosystems.[10, 23, 26-28] Biologically, sessile bacteria living within biofilms display markedly different bacterial physiology compared to their planktonic counterparts, resulting in their failure to be cultured and analysed using standard liquid culture techniques that have been the mainstay of microbiology over the past 100 years.[10, 24, 26, 29] Advances in enhanced culture, microscopy and, more recently, molecular techniques over the past few decades have led to an increasing understanding of the biology of biofilms and their function in ecosystems and industry.[10, 12, 26, 27, 30]

Bacterial biofilms have been well accepted as the cause of numerous complications across a diverse range of industries, causing significant financial costs to industry.[24] Biofilms in marine industries cause biofouling resulting in increased drag on boats[24, 25, 31] and corrosion due to biofilm production of hydrogen-sulphide metabolites.[10, 24, 32] In petroleum and water distribution systems biofilms readily grow on the internal pipe surfaces causing numerous problems. These include metal corrosion, via several different physiochemical interactions,[24, 33] and increased fluid frictional resistance, due to biofilm growth on the luminal surfaces reducing pipeline cross-sectional area and thus fluid flow and also changing the surface viscoelasticity of the fluids.[24, 34-36] In water storage and supply systems biofilms cause nitrification in response to water purification agents and this conversely results in promotion of coliform bacteria.[37-39] This enables pathogenic bacteria and their toxins to persist within water storage and sanitation systems, despite complex purification processes.[39-41] Biofilms in water-cooling, water-heating and air conditioning devices decrease heat exchange capacity[24] and harbour pathogenic bacteria including *Legionella*, *Chlamydiae*, *Coxiella*, *Listeria*, mycobacteria and numerous other human and respiratory pathogens.[42-49]

In the food industry biofilm growth and contamination is caused by numerous species including *Shigella, Staphylococcus, Escherichia coli, Bacillus cereus* and *Listeria* and results in food spoilage and food poisoning.[50-52] In the seafood industry known biofilm contaminants include *Pseudomonas, Listeria, Vibrio, Nisseeriaceae, Enterobacteriaceae* and *Serratia* species and have been found to contaminate stainless steel surfaces, food preparation and packaging equipment and water storage containers.[53] These infections can persist despite strict cleaning and decontamination processes and cause contamination in tin processed seafood.[53-55] These findings have far-reaching implications across the whole spectrum of food provision from production-harvesting, processing and handling, to food preparation, packaging and storage.[51, 55]

Even the electronics industry is affected, as the electrolytic characteristics of biofilm extracellular polymeric substance interact with electron movements in computer chip signalling causing their malfunction.[35] Indeed research and development over the last 40 years has progressed from observation and investigation of the natural behaviour of biofilms to research and development into prevention and treatment as well as exploitation of biofilm actions in numerous industries.[35]

The acceptance that bacterial biofilms cause disease in humans, however, made slow progress within the medical fraternity.[12] By the 1980's, over 120 years since Pasteur and Koch were instrumental in the development of the Germ Theory of Disease, the discipline of Medical Microbiology had developed an ever-increasing knowledge of infectious diseases using standard liquid culture techniques and diagnostic criteria for many important infectious diseases in humans and animals. This knowledge has also led to the development of antibiotics and vaccines for the effective treatment and prevention of many of these serious infectious diseases.[10, 12, 23, 56] Thus, when initial data suggested that bacteria can form biofilms within the human body without causing acute suppurative disease and overwhelming sepsis, the *status quo* of bacterial disease in medicine was challenged.[12]

Initial studies identifying bacteria within biofilms were discovered on various temporary and permanent implantable medical devices from patients with unusual infective

presentations. The bacteria included *Staphylococcus aureus* biofilm on infected cardiac pacemaker leads and Staphylococcal species, *Escherichia coli* and *Pseudomonas aeruginosa* biofilms on Tenckhoff peritoneal dialysis catheters.[23, 57-59] These infections had several similarities:

1) they lacked the classical signs of suppuration, instead having a more indolent course punctured with periods of active and often life-threatening infection;[10, 23]
2) the causative organisms were often human commensal organisms;[23, 57, 58]
3) the infections were not effectively treated and eradicated with standard antibiotic regimes,[10] and
4) they often relapsed when antibiotic therapy was ceased.[23]

Several chronic infective diseases were known to have similar clinical progression. Progression of lung disease in cystic fibrosis is due to infection with *P. aeruginosa* biofilms,[60] vegetative bacterial endocarditis is due to biofilm growth on the endocardium[27, 61] and *Proteus, Providencia, Klebsiella* and *Pseudomonas* species are frequent causative agents in recurrent urinary tract infections secondary to biofilm infected renal calculi.[62, 63] Subsequently, bacterial biofilms have been identified on many implantable medical devices and from tissues with chronic infections (Table 1-1). Bacterial biofilm infections are now increasingly, albeit slowly, being recognised by the medical and surgical fraternity as an important cause of chronic disease and failure of surgical implantable medical devices.[23, 27, 64, 65] Biofilm infections are associated with high rates of morbidity, and to a lesser extent with mortality, but are associated with significant and rapidly increasing health care costs that are unsustainable into the future.[66-70]

1.6 What is a Bacterial Biofilm?

The definition of a bacterial biofilm has evolved over the past 30 years. Initially biofilms were thought to be simple aggregations of bacteria contained within a polysaccharide matrix.[21] It is now known that bacterial biofilms are a complex and dynamic structure that allows survival, growth, reproduction and protection for the bacteria from a hostile external environment and suggests an important selective advantage over their planktonic

1) Table 1-1: Biofilm Infections of Humans Without and With Implantable Medical Devices

Human Infection or Disease	**Common Biofilm Bacterial Species**	
	Primary Organism	**Secondary Organisms**
Cystic fibrosis[60]	*P. aeruginosa*,	*Burkholderia cepacia, S. aureus*, *Haemophilus influenza*
Osteomyelitis[71, 72]	*S. aureus*	*P. aeruginosa, Streptococcus* spp., *Salmonella*, *Bartronella henselae*, *Pasteurella multicida*, *Aspergillus* spp., *Mycobacterium* spp., fungi
Bacterial prostatitis [27, 73]	*E. coli*	*Klebsiella*, eneterobacteria, *Proteus*, *Serratia*, *P. aeruginosa*, *Staphylococcus epidermidis, S. aureus*, *Gardnerella* spp., *Cornyebacterium* spp.
Otitis media[27, 74]	*H. influenza*, *S. pneumonia*	*Moraxella catarrhalis*, β-haemolytic streptococci, enteric bacteria, *S. aureus*, *S. epidermidis*, *P. aeruginosa*
Biliary tract infections[75]	Enteric bacteria	
Native valve endocarditis[27, 76]	*Streptococcus* spp (viridians group, enterococci, pneumococci, *S. bovis) Staphylococcus* spp	*S. aureus*, *S. epidermidis*, GNB, fungi (*Candida* and *Aspergillus* spp)
Medical device Associated Infections		
Endotracheal tubes and ICU pneumonia[70]	Gram-negative bacteria	*P. aeruginosa, Streptococcus* spp., *Staphylococcus* spp.
Urinary catheter and stent infections[77, 78]	*E. coli*, *P. aeruginosa*, *Escherichia faecalis*	*Proteus mirabilis*, *Candida tropicalis, S. aureus*
Central venous catheters[79, 80]	*S. epidermidis*	Other CoNS, *S. aureus*, *Stenotrophomonas*, *Pseudomonas*, Enterbacteriaceae, *Candida*
Mechanical cardiac valves[76, 80]	*S. epidermidis*	*S. aureus*, *Enterococcus* spp., *Streptococcus*,GNB, fungi
Cardiac Pacemakers[70, 76]	*S. epidermidis*	*S. aureus*, *Candida* spp., *P. aeurginosa*
Vascular grafts[80]	*S. epidermidis*	*S. aureus,* GNB
Orthopaedic prostheses[70, 81-83]	*S. aureus*, *S. epidermidis*	*S. pneumonia, Streptococcus* spp., *Propionibacterium acnes,* GNB
Arteriovenous shunts[84]	*S. epidermidis*, *S. aureus*	
Sutures[23, 85]	*S. epidermidis*, *S. aureus*	
Contact lenses[80]	*P. aeruginosa*, *S. epidermidis*	
Peritoneal dialysis catheters[86]	*S. aureus*,	CoNS, Enterobacteriaceae *Streptococcus* spp., *Pseudomonas* spp, diptheroids, *Enterococcus* spp, *Candida* spp
Ventricloperitoneal shunts & neurosurgical devices[87]	*S. aureus*, *S. epidermidis*	*Streptococcus* spp., *Corynebacterium,* GNB
Penile implants[70, 78]	*S. epidermidis*	*S. aureus*, enteric GNB, *P. aeruginosa*, *Serratia* spp., fungi
Breast implants[70, 88, 89]	*S. epidermidis*	*S. aureus, P. acnes, E. coli, Clostridium perfringens*

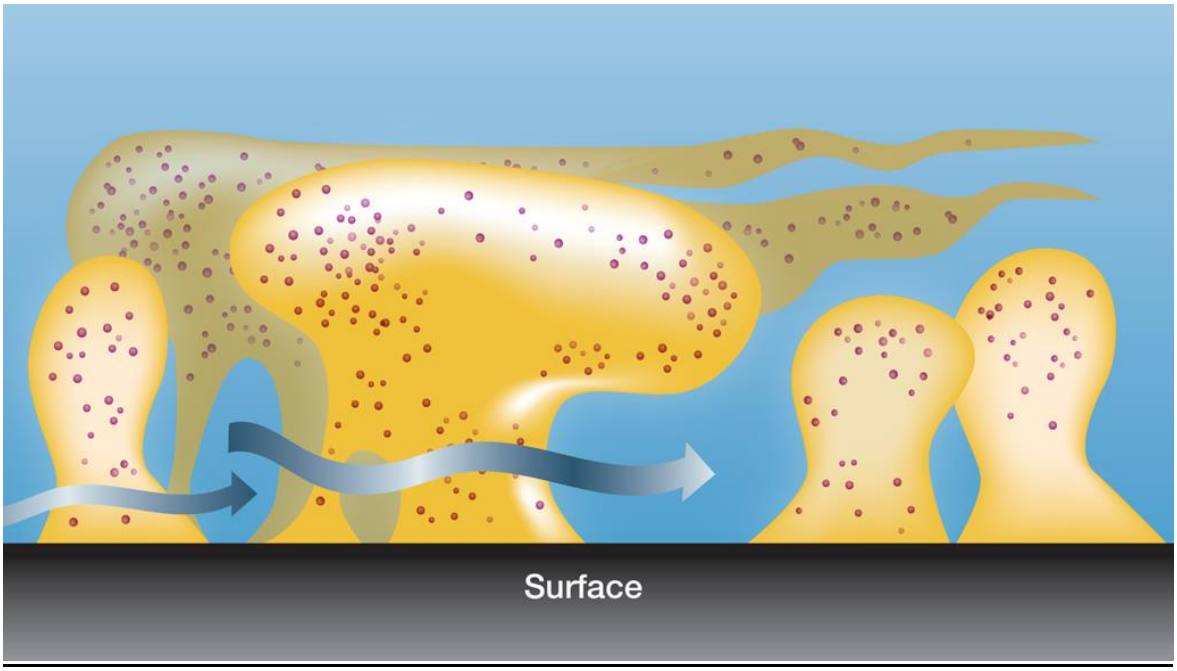
GNB – Gram-negative bacteria, CoNS – coagulase negative *Staphylococcus* species

counterparts.[10, 11, 15, 23, 90] Donlan and Costerton acknowledged this complexity with their definition of a biofilm as:

"*a microbially derived sessile community characterised by cells that are irreversibly attached to a substratum or interface or to each other, embedded in a matrix of extracellular polymeric substances that they have produced and exhibit an altered phenotype with respect to growth rate and gene transcription*".[27]

Biofilms are complex three dimensional structures comprised of bacterially-produced extracellular matrix (75-95% by volume) and contain a small bacterial cellular component (5-25% by volume).[91] The extracellular matrix is fundamental to biofilm formation and is responsible for its attachment to the substratum, the structure and cohesion of the biofilm and the survival of the resident bacteria it encases. [26, 92, 93] The matrix is composed of an assembly of different biopolymers, known as extracellular polymeric substances and these are fundamental for the formation and survival of the multicellular community.[94] When fully hydrated, the extracellular polymeric substance is largely hydrophilic and contains amino acids, inorganic ions and extracellular deoxyribonucleic acid (DNA).[92, 95] These molecules are distributed between the bacterial cells and interact via weak physiochemical interactions that stabilise and form the biofilm structure.[92] The basic biofilm structure is a microcolony which contains bacteria within extracellular polymeric substance punctured by water channels that form a microcirculation for nutrient and oxygen provision and waste excretion (Figure 1-1).[96] These structures can form various shapes from a simple cone shape to a large inverted mushroom, depending on the resident microorganisms, nutrient supply and external factors, such as shear forces.[26, 96] The extracellular polymeric substance matrix also has numerous protective functions including:

1) strong physical adhesion anchoring the biofilm to a substratum;[21, 56]
2) providing a physical barrier with resistance to specific and non-specific host immune defences and as a barrier to some antimicrobials;[93, 97-99] and
3) extracellular DNA that facilitates horizontal gene transfer and transfer of antibiotic resistance genes.[92, 100]



Adapted from *Center for Biofilm Engineering Montana State University – Bozeman* 1996

Figure 1-1: Bacterial Biofilm Structure

Conceptual illustration of bacterial biofilm showing the individual microcolonies, at various stages of development, that encase the resident bacteria. Water channels are present within and between the microcolonies forming a microcirculation for provision of oxygen and nutrients and removal of waste products.

1.7 Formation of a Bacterial Biofilm

The formation of a bacterial biofilm is a multistep process of bacteria/surface adhesion, biofilm formation, maturation and detachment (Figure 1-2). These processes are coordinated by various environmental, surface, bacterial and genetic factors.[90]

1.7.1 Bacterial Adhesion

Bacterial adhesion is a two-step process whereby bacteria is attracted to and loosely attaches to a surface (primary adhesion/docking).[90] Then the bacteria undergo a molecularly-mediated binding process to irreversibly bind to the attached surface and subsequently commence producing extracellular polymeric substance (secondary adhesion/locking).[90, 101]

Primary Adhesion/Docking is a serendipitous event when a surface, either biotic or abiotic, and a planktonic bacterium meet.[90] It is influenced by various physiochemical variables including surface conditioning and bacterial cell-surface binding molecules.[90, 101] The initial step in this process occurs when the planktonic bacterium is brought into close proximity (<1nm) to a surface.[90] Bacteria are propelled by numerous mechanisms; randomly with Brownian motion and convective mass transport, or directly via chemotactic mechanisms such as flagella, pili and fimbriae common in Gram-negative bacteria.[64, 102, 103] Once close to a surface, attractive and repulsive forces, including hydrophobic interactions, van der Waals, steric, electrostatic and hydrodynamic forces, occur between the surface of the cell and substratum.[90, 104, 105] Initial attachment occurs when the net sum of these forces is attractive.[90, 106]

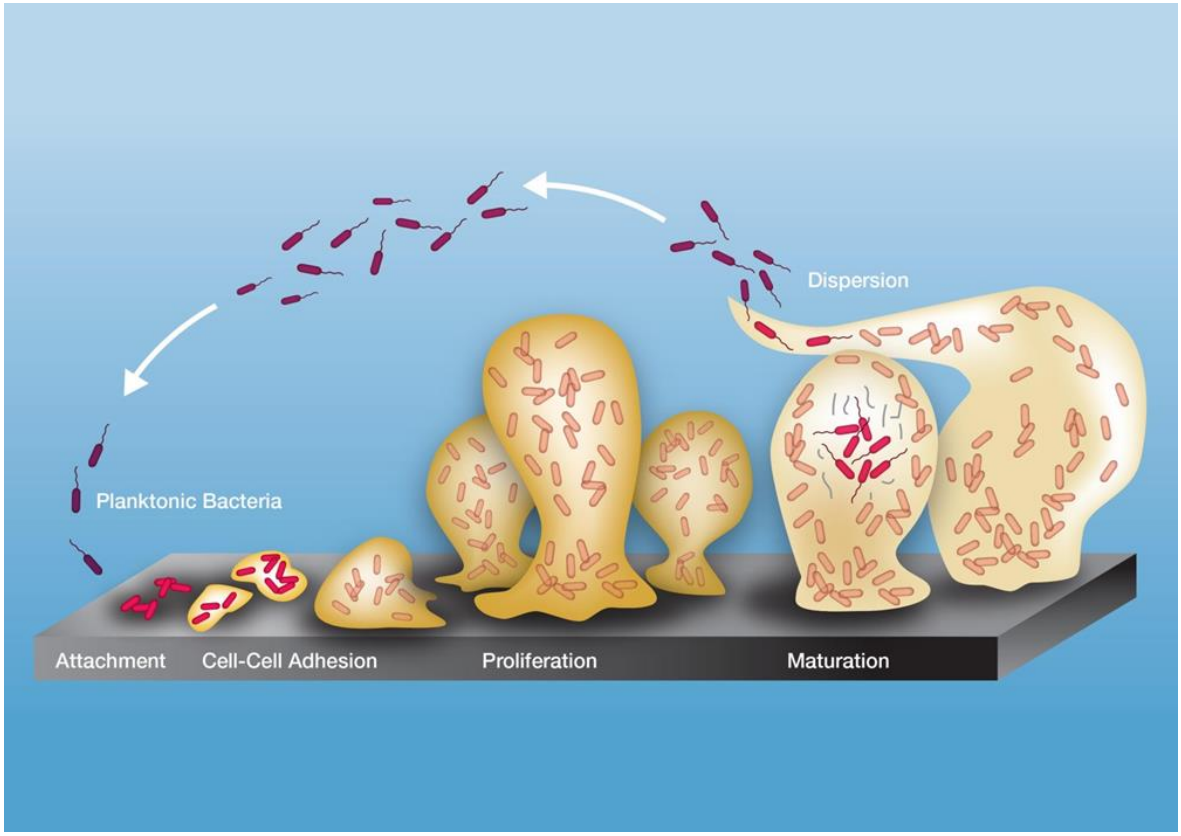
Secondary Adhesion/Locking. Loosely bound bacteria congregate and up-regulate biofilm promoting genes to produce extracellular polymeric substance and promote adhesion/locking reactions between the bacteria cell surface and the target surface.[12, 90, 107, 108] The chemical reactions include oxidation and hydration to consolidate bacteria-surface bonding.[106] In addition, bacteria produce specific bacterial proteins, broadly termed adhesins, which mediate binding to abiotic surfaces.[106, 107] Adhesins include

autolysins in *Staphylococcal* species[109, 110] and Type 1 fimbriae proteins in *E coli*.[111] This process occurs rapidly with numerous studies showing the transition time between reversible and irreversible attachment occurs between 60 seconds to ten minutes depending on the bacteria involved and the surface properties.[101, 112-114]

In many environments bacterial interaction and primary adhesion occurs between bacteria and a conditioned surface.[90, 106] Surface conditioning occurs when there is an accumulation of molecules at the solid/liquid interface and can alter the underlying surface properties with the potential to enhance bacteria-surface attraction and adhesion.[90, 101, 106] Within the human body, after insertion of a surgical implantable medical device, the implant surfaces are commonly conditioned with numerous acute phase proteins. These include extracellular matrix proteins, coagulation products (platelets and thrombi) and host-derived plasma proteins, such as fibrinogen, fibronectin, collagen, thrombospondin, elastin, von Willebrand Factor and vitronectin.[90, 115-118]

Bacteria have numerous adhesins that target these proteins to enhance bacterial/substratum adhesion,[117] including the Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMM) commonly found on Gram-positive bacteria, in particular *Staphylococci* species.[115, 117-119]

MSCRAMM are covalently bonded surface anchored proteins and virulence factors. *S aureus* has over 20 surface-anchored adhesin genes and *S. epidermidis* has over 12 adhesin genes for these specific host matrix and plasma proteins including: laminin binding protein (*eno*), elastin binding protein (*ebps*), fibrinogen binding protein (*fib*) and fibronectin binding protein A/B (*fbnA*/*fbnB*).[118, 120] A few recent *in vitro* studies have demonstrated that several of these genes are up-regulated within the first 12-24 hours from the switch from planktonic to biofilm growth.[121] This activity is further enhanced within 30-60 minutes of exposure to human plasma proteins and supports their role in adhesion and early biofilm cell aggregation.[110, 120, 122, 123]



Adapted from *Center for Biofilm Engineering Montana State University – Bozeman* 2003

Figure 1-2: Biofilm Life Cycle

Under appropriate conditions planktonic bacteria attach to a surface. Once attached the bacteria undergo genotypic and phenytypic changes and begin to produce its own extracellular polymeric substance (EPS) matrix. The bacteria proliferate within the EPS matrix and mature into complex three-dimensional structures. Once mature the biofilm disperses bacteria, either individually or in small biofilm clusters, back into the environment to enable new bacterial biofilm fomation in new locations.

1.7.2 Biofilm Maturation

Once attached the maturation process is a balance between adhesive processes that promote agglomeration of bacteria and disruptive processes that help formation of void spaces.[119, 124] Cells aggregate into microcolonies surrounded by extracellular polymeric substance with bacteria accumulation via replication of attached cells or attraction of additional planktonic bacteria.[125-127] This can result in high bacterial densities up to 10^7 cell/cm^2 within 48 hours in some species.[128]

The resulting three-dimensional structure exhibits a dynamic process of interaction between the bacteria building the biofilm and their surrounding environmental stimuli (Figure 1-1).[129] The size, shape and density of the microcolonies and mature biofilm are influenced by multiple variables including:

1) Bacterial Species: different bacterial species generate biofilm species-specific arrangements of structural components including bacterial cell distribution, integrity of extracellular matrix and void spaces, thus influencing biomass densities and shapes. *P. aeruginosa* biofilms *in vitro* show increased cell density at their attached interface and more diffuse density towards peripheries forming mushroom shapes, whereas a *Vibrio* species under same conditions displays the reverse formation.[30, 91]
2) Bacterial Composition: single-species biofilms develop as a result of their innate biofilm processes in response to other environmental stimuli. Bacteria within multispecies biofilm, however, will interact with one another resulting in the synergistic or antagonistic actions that determine the development and shape of the biofilm community.[26, 91, 130, 131] Dental biofilms are polymicrobial and demonstrate complex synergistic interactions including metabolic co-operation: for example, *Streptococcus oralis* produces lactic acid from fermented sugars that is the energy source for *Veillonella* sp.[132] In contrast, *Streptococcus gordonii* and *Streptococcus sanguinis* biofilms inhibit growth of other oral biofilm-forming bacteria with the production of hydrogen peroxide.[133]
3) Nutrient availability: the availability and type of carbon/energy supply, iron and oxygen have been shown to influence biofilm cellular density and size. Numerous

studies have shown nutrient deprivation results in smaller size with less bacterial density and this effect is rapidly reversed when nutrients are returned.[26, 30, 134-136] Studies using *P. aeruginosa* demonstrated that the biofilm grew into a mushroom shape when grown with a glucose carbon source[137, 138] but developed into a densely packed flat form on citrate.[139, 140]

4) Hydrodynamics: laminar and turbulent fluid flow, shear stress and flow rates affect biofilm growth rates, surface shape and density of biofilm.[11, 26, 103, 141] Biofilms grown in fast water (*in vivo*) or in turbulent flow (*in vitro*) develop with filamentous streamers tapering in the downstream direction compared to the mushroom and mound-shaped clusters in more stationary environments.[41, 129, 142-145]

Ultimately the void spaces enlarge and coalesce to form water channels interspaced within the microcolonies.[26, 91] The channels penetrate deep into the maturing biofilm with smaller branches between microcolonies providing an anastomosing network of water channels.[29, 91] These water channels act as conduits for directional liquid flow around and between the microcolonies, creating a microcirculation to provide oxygen, nutrients and remove waste.[135, 146] This complex dynamic water channel structure has been compared to a "*…primitive circulatory system analogous to that of higher organisms. Because of this remarkable biofilm architecture, bacterial cells within a microcolony have a degree of homeostasis, optional spatial relationships with cooperative organisms, and effective means of exchanging nutrients and metabolites with the bulk fluid phase.*"[26] Biofilm development is highly co-ordinated and controlled by the bacteria using a complex cell-density signalling process called quorum sensing.[147-149]

1.7.3 Biofilm Detachment

Detachment or dispersal is an integral part of the dynamic nature of bacterial biofilms that contributes to biological dispersal, bacterial survival and spreading of disease.[127, 136, 150, 151] Detachment is a heterogeneous process involving numerous environmental signals, signal transduction pathways and other effectors that have yet to be identified in a process that remains poorly understood.[136, 151, 152]

Observational studies have identified 3 distinct detachment strategies: 1) swarming/seeding dispersal – where individual cells are continually released into bulk fluid or surrounding substratum; 2) clumping dispersal – small aggregates or emboli of biofilm cells are shed in to bulk fluid; 3) surface dispersal – whereby biofilm structures move across surfaces.[129, 136, 150, 151]

Swarming dispersal is best characterised in non-mucoid *P. aeuriginosa* biofilms. It occurs when microcolonies differentiate into an outer layer of sessile bacteria (biofilm phenotype) that surrounds an inner layer that liquefies, resulting in motile bacteria which return to planktonic phenotype that "swim-out" of the biofilm.[126, 129, 153] Liquefaction in this process has been associated with a lysogen prophage mediated cell lysis and death.[129, 152] As lysogenic phages, rarely cause lysis within their host, scientists have hypothesise that as the available nutrition decreases within the centre of the microcolony, there is an increase in oxidative stress and reactive oxygen species.[152] Thus initiating a SOS response and adaptive mutations, initiating prophage lysis and ultimately resulting in bacterial dispersal.[152, 154]

Clumping dispersal is commonly observed in *S. aureus* biofilms where whole aggregates are continually shed from the biofilm.[127] The underlying mechanism is unknown, but may be influenced by variable hydrodynamic shear due to changes in flow rates.[136, 141, 150] Dispersal direction is not controlled, and is determined by the direction of the surrounding fluid. These clumps can contain hundreds of bacteria, maintain a biofilm phenotype and readily reattach in distal locations.[136, 151, 155] This process may readily explain the high frequency of infectious metastasis seen with *Staphylococcal* infections.[156]

Surface dispersal has been observed with whole biofilms migrating slowly across surfaces in both laboratory and natural environments.[129] Migratory ripple structures moving at rates of 1 mm/hr have been visualised in biofilms of *P. aeruginosa* and mixed species biofilms.[142, 157] *S. aureus* biofilms have been shown to use rippling migration to roll along a glass tube in an *in vitro* central venous catheter model.[129] Ripple structures have also

been observed in biofilms moving along endotracheal tubes, and thus are hypothesised to be important in the aetiology of ventilator-associated pneumonia.[158, 159]

1.8 Quorum Sensing

Bacteria within biofilms demonstrate sophisticated signalling activity with the production, secretion and activation of small, hormone-like signalling molecules, or autoinducers, in a process called quorum sensing.[149] These extracellular autoinducer molecules activate and regulate gene expression to promote collective behaviour in a concentration-dependant function of cell density.[149, 160-162] Quorum sensing co-ordinates and controls important biofilm functions including virulence, competence, antibiotic production, motility and biofilm formation.[149]

First detailed in the Gram-negative marine bacterium *Vibrio fischeri*, a bacterium specific LuxI/LuxR autoinducer was found to activate luminescence in a cell-density manner (Figure 1-3).[163, 164] LuxI is an enzyme that synthesises the autoinducer *N*-(3-oxohexanoyl)-homoserinelactone (acyl-HSL) which diffuses freely across the cell membrane, until the intracellular and extracellular concentrations are equal.[149, 164] At low cell density, low levels of the LuxI protein are present both inside and outside the cell but no luminescence is triggered.[164, 165]

As the cell density increases the concentration of LuxI protein increases until the threshold level is reached and it binds to LuxR, an intracellular receptor.[160] Activated LuxR exposes a DNA binding domain and binds to the promoter region of *luxCDABE* operon. The transcription of several genes is up-regulated including the luciferase gene for production of bioluminescence resulting in a 1000-fold increase in light production.[160, 163-165] The positive feedback LuxI-light amplification pathway is balanced by negative feedback regulation of *luxR* gene by the activated LuxR protein. Negative feedback results in decreased intracellular LuxR protein levels, decreased LuxI production and subsequently decreased luciferase gene and light production.[160, 166]

The LuxI/LuxR quorum sensing system is highly conserved with over 70 Gram-negative bacteria synthesising acyl-HSL homologues to regulate gene expression in a cell-density

Figure 1-3: The LuxI/LuxR Autoinducing Quorum Sensing system of *Vibrio fischeri*

In *V. fischeri* biofilms the LuxI enzyme produce the quorum sensing molecule acyl-HSL ( ) that freely diffuses across the cell membrane:

1-3(a) In low bacterial numbers there is not enough acyl-HSL to activate the LuxR quorum sensing system.

1-3(b) As bacterial numbers increase, there is increasing production of acyl-HSL. Once the critical threshold is reached the LuxR is activated and initiates binding to the *luxCDABE* operon (Target Genes) and activation of the bioluminescence pathway.

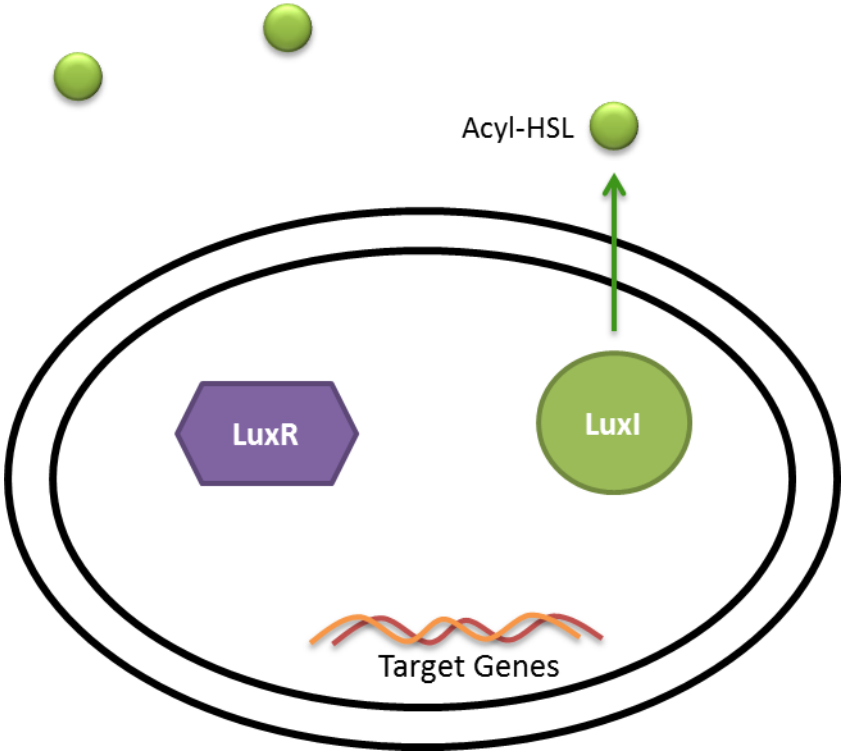


Figure 1-3(a)

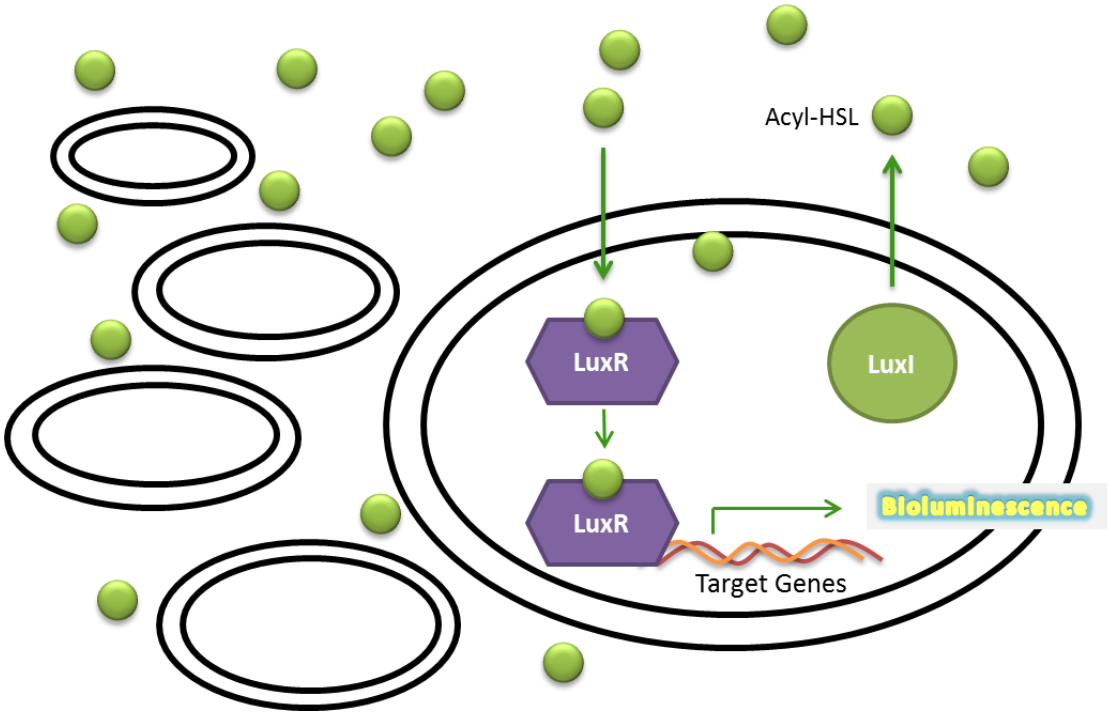


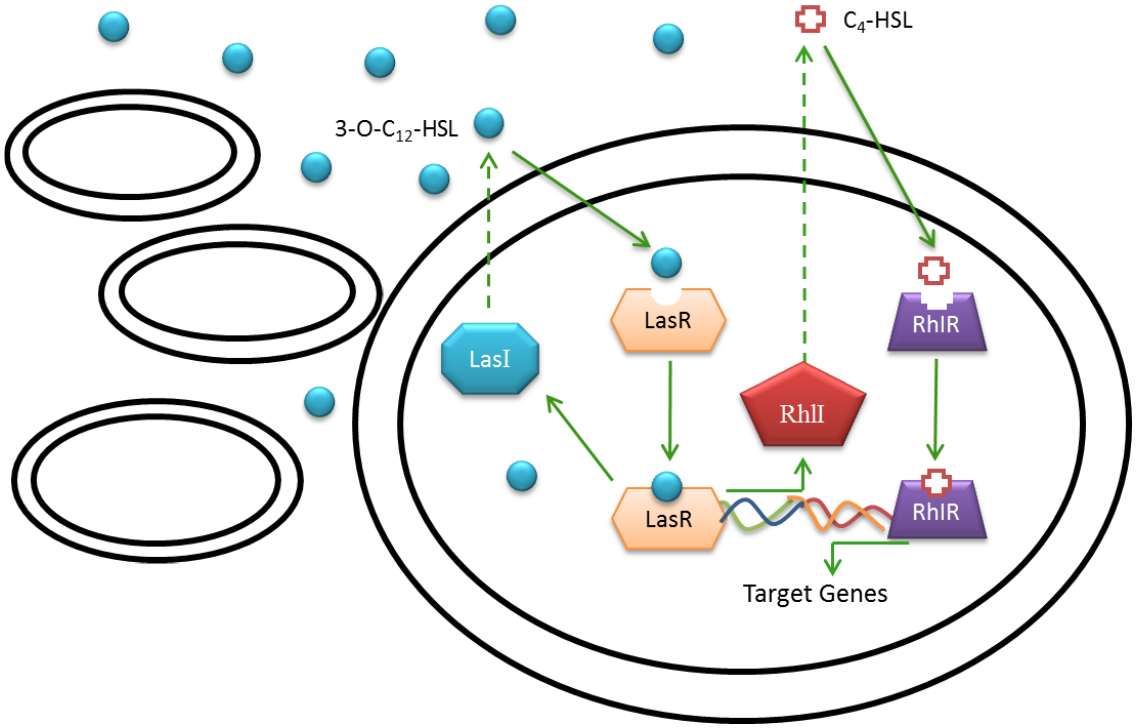
Figure 1-3(b)

Adapted from Jayaraman and Wood[160]

manner.[167, 168] The HSL core is the conserved component of the autoinducer molecule with a variable acyl side chain conferring a high degree of specificity between Gram-negative bacterial species.[169-171] Thus bacteria within multispecies biofilm can synthesize and recognise their specific acyl-HSL autoinducer. This enables species-specific self-regulated gene expression, including production of virulence factors, to confer a species-wide advantage over other bacterial species within the biofilm.[160]

Other Gram-negative quorum sensing systems are homologous to the LuxI/LuxR system.[172] *Pseudomonas aeruginosa*, an important opportunistic human pathogen, has two key quorum sensing systems.[173-175] The LasI/LasR and RhlI/RhlR quorum sensing systems are arranged in series and share gene sequence and structure homology with LuxI/LuxR genes.[160, 172, 176-178] They regulate over 10% of the *P. aeruginosa* genome including production of numerous virulence factors and regulators of biofilm formation.[162, 179] The *lasI* gene encodes an autoinducer synthase (LasI) that synthesizes a *N*-3-oxododecanoyl homoserine lactone (3-O-C_{12}HCL) autoinducer. The 3-O-C_{12} HCL intern binds with the response regulator (LasR) to positively-feedback on the *lasI* gene and also regulates expression of multiple target genes including the RhlI/RhlR quorum sensing system (Figure 1-4).[176, 177] Once activated the *rhlI* gene produces the lactone autoinducer *N*-butanoylhomoserine lactone (C4-HSL) (Figure 1-4).[162] C4-HSL binds to and activates the transcriptional regulator RhlR that regulates the synthesis of rhampholipids[180] as well as regulation of other virulence factors that are fundamental for biofilm maturation, swarming, detachment and neutrophil disruption.[160, 162, 174, 180] Rhamnolipids are amphipathic glycolipids that play a central role in the *P. aeruginosa* biofilm lifecycle including microcolony and mushroom cap formation, maintenance of open channels and biofilm detachment.[181-185]

Other HSL-based quorum sensing systems in Gram-negative bacteria control numerous virulence factors including virulence genes in *Burkholderia* cepacia,[60] antibiotic production in *Erwinia carotovora,*[186-188] cholera toxin and toxin-coregulated pilus production in *Vibrio cholera*,[189] and the "swarming" surface motility capacity of *Serratia liquefaciens,*[190] *P. aeuriginosa,*[191] and *B. cepacia.*[192]



Adapted from Jayaraman and Wood[160]

Figure 1-4: LasI/LasR-RhlI/RhlR Quorum Sensing System of *Pseudomonas aeruginosa*

The LasI/LasR and RhlI/RhlR are a hieratical two step quorum sensing system that regulates production of numerous virulence factors, including rhampholopids, in a cell-density dependent manner. Once activated the LasI synthase produces a 3-O_{12}-HSL ( ) that intern binds with and activates the LasR regulator. The activated LasR activates the RhlI/RhlR quorum sensing system. The RhlI synthase produces a C_4-HSL ( ) that binds to and activates RhlR, The activated RhlR then binds to its target genes, including the rhampholipids genes that are important for biofilm formation and dispersal.

Gram-positive bacteria use a different quorum sensing cell density-dependent process. Their autoinducer molecules are peptide based and do not freely diffuse across the cell membrane.[160] The autoinducers are peptide signals that are cleaved from longer precursor peptides and commonly undergo posttranslational modification into the active-autoinducer peptide.[160, 186] Structurally, active-autoinducer peptides are commonly small polypeptides (seven to nine amino acids long) and contain thiolactone rings with a cysteine residue in the fifth amino acid from the carboxyl terminal.[186]

Active-autoinducer peptides are actively exported from the cell via a dedicated membrane-bound ATP-binding cassette (ABC) exporter (Figure 1-5).[149, 193] The extracellular autoinducer peptide activates the species-specific cell via a two-component signal transduction system. The autoinducer peptide binds to a membrane-bound histadine kinase sensor that stimulates autophosphorylation. The phosphate molecule is subsequently transferred to the response-regulator protein, commonly a regulatory RNA (RNAIII), which regulates a highly diverse range of gene expression.[160, 193] As with Gram-negative biofilms, the Gram-positive peptide quorum sensing systems are also density-dependent, with autoinducer peptide at low cell density unable to trigger the quorum sensing circuit.[160]

The first Gram-positive quorum sensing process identified was in *Streptococcus pneumonia* (Figure 1-6).[194] It produces a competence-stimulating peptide that initiates the development of natural competence, a process of uptake of exogenous DNA for integration into the host bacterial cell genome or horizontal gene transfer.[194, 195] Competence-stimulating peptide is a 17 amino acid peptide, produced by a 41 amino acid precursor called ComC.[149] The levels of competence-stimulating peptide increase slowly until a threshold concentration, related to bacterial cell density, is reached triggering a cascade of gene expression.[160] Competence-stimulating peptide is actively transported out of the cell by a ComAB-ABC transporter. The extracellular competence-stimulating peptide binds to and activates ComD, a cell surface histadine kinase receptor.[194] ComD is autophosphorylated and then the phosphoryl group is transferred to the ComE, a cognate response regulator.[149, 194] Phospho-ComE activates a ComX, an alternate σ factor and

global transcription modulator, which regulates the transcription of numerous structural genes involved in DNA uptake (competence), late competence functions, stress-related functions, protein synthesis and early biofilm formation.[149, 194, 196, 197] ComX also regulates a competence-induced release mechanism, whereby *S. pneumoniae* cells within the biofilm population that are "competent-deficient" are induced to lyse and release DNA to be utilised by "competent-enabled" cells for genetic exchange.[194, 198]

S. aureus and coagulase-negative Staphylococcal species, such as *S. epidermidis* a skin commensal, are medically important bacteria and are the most frequent cause of nosocomial infections including biofilm infections of implantable medical devices.[66, 199-202] Over 50 genes are involved in pathogenesis and encoded by proteins that are displayed on the cell surface or are released into the local environment.[203] These proteins have functions including evading the host immune system, cell adherence, degradation of cell and tissues, nutrition and protection.[203] Expression of many of these exoproteins is controlled by **a g**lobal **r**egulator (*agr)* quorum sensing system*,* in a group of divergent transcription units encoded over a 3 kb locus, driven by two promoters P2 and P3.[203-205] The P2 operon encodes Agr A, the response regulator, AgrC, the membrane histadine kinase sensor and the precursor peptides AgrB and AgrD. P3 transcribes RNAIII which is an intracellular effector of target gene regulation (Figure 1-7).[203, 206] The *agr* system is induced by an extracellular ligand that activates the P2 promoter in a cell-density manner.[203, 207] This leads to the production of AgrB, a membrane bound enzyme, and AgrD to form the pro-autoinducing peptide.[206] The pro-autoinducing peptide is post-translationally processed by the membrane bound AgrB into autoinducer peptide, a nine amino acid peptide with a unique thiolactone ring between the central cysteine and C-terminus, and actively secreted by a membrane-bound ABC transporter.[208] The N-terminal transmembrane domain binds to the membrane bound AgrC kinase triggering the autophosphorylation cascade of the AgrA regulator.[209] Once activated the Phosphyl-AgrA activates the P2 and P3 promoters leading to positive feedback of the P2 cycle and production of RNAIII, respectively.

Figure 1-5: The Gram-positive Quorum Sensing Systems with Autoinducer Peptide

Autoinducer Peptide ( ) is produced as a precursor peptide. The peptide is modified and actively exported outside the cell by a membrane transporter protein.

Figure 1-3(a) In low bacterial numbers the autoinducer peptide level is low and there is no activation of the quorum sensing system.

B) As bacterial numbers increase more autoinducer peptides are exported into the extracellular environment and bind to the membrane bound histidine kinase receptor. This leads to phosphorylation of the response regulator (RR) which intern activated the target genes.

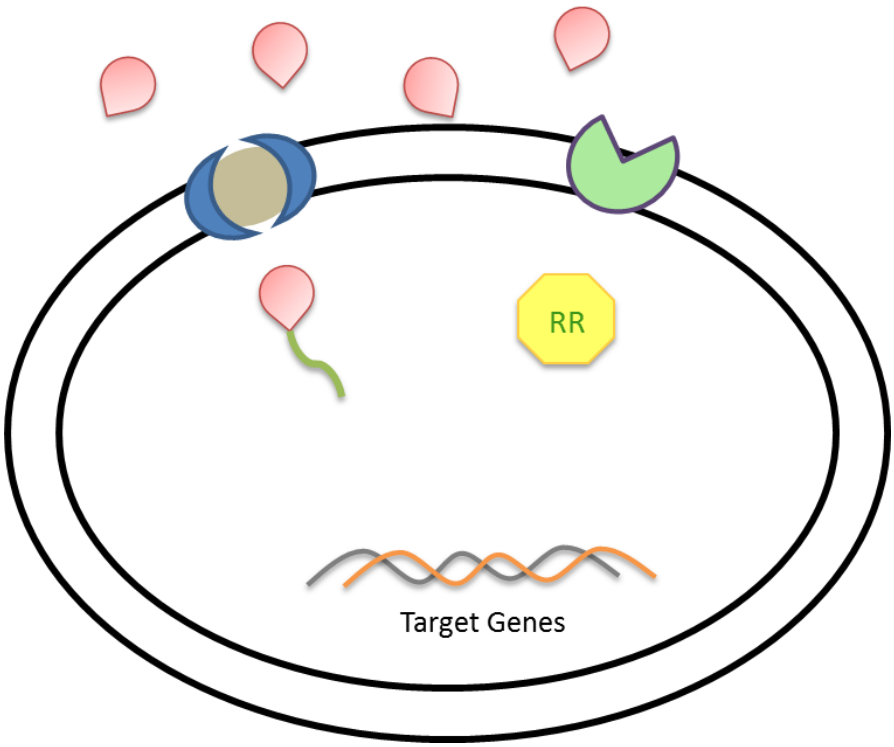


Figure 1-5(a)

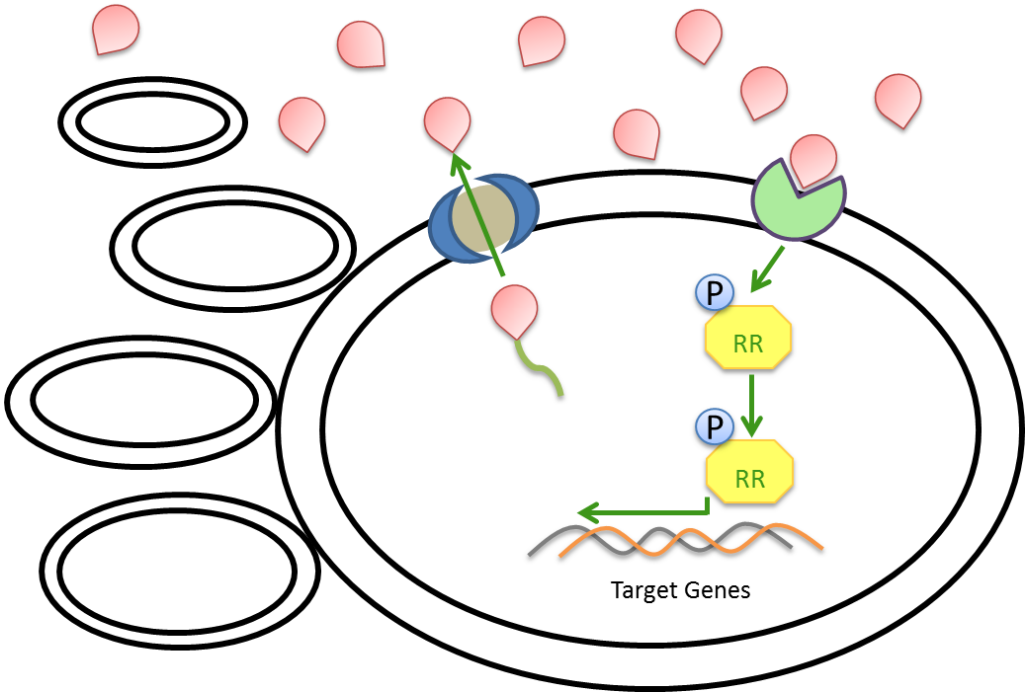
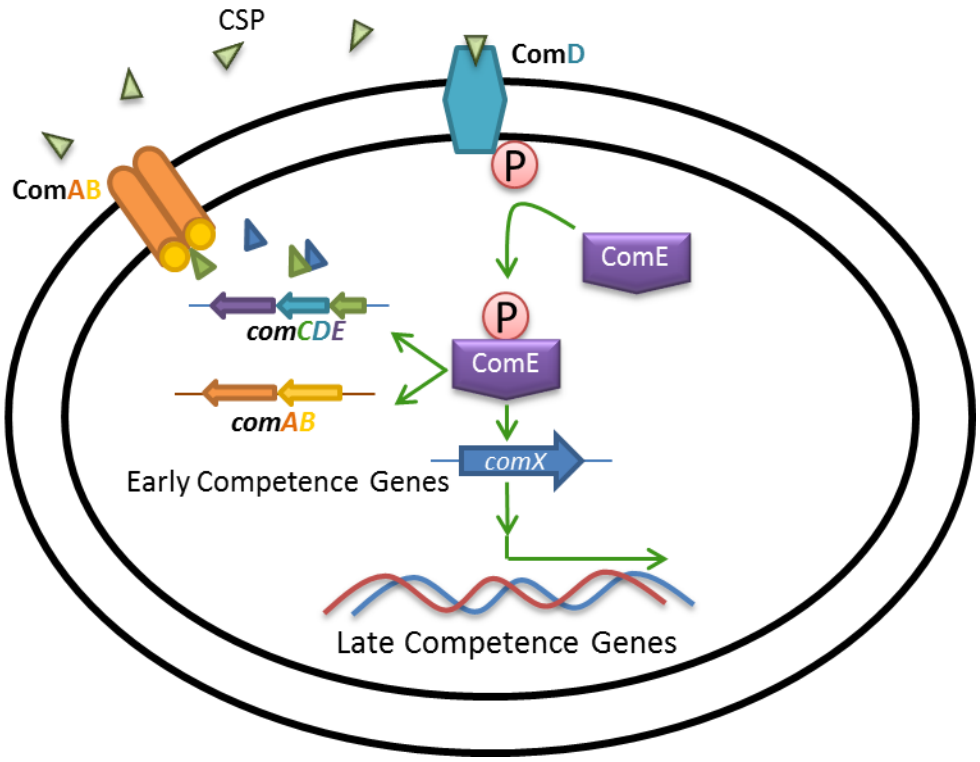


Figure 1-5(b)

Adapted from Jayaraman and Wood[160]



Adapted from Suntharalingam and Cvitkovitch[194]

Figure 1-6 Genetic Competence Quorum sensing pathway of *Streptococcus pneumoniae*

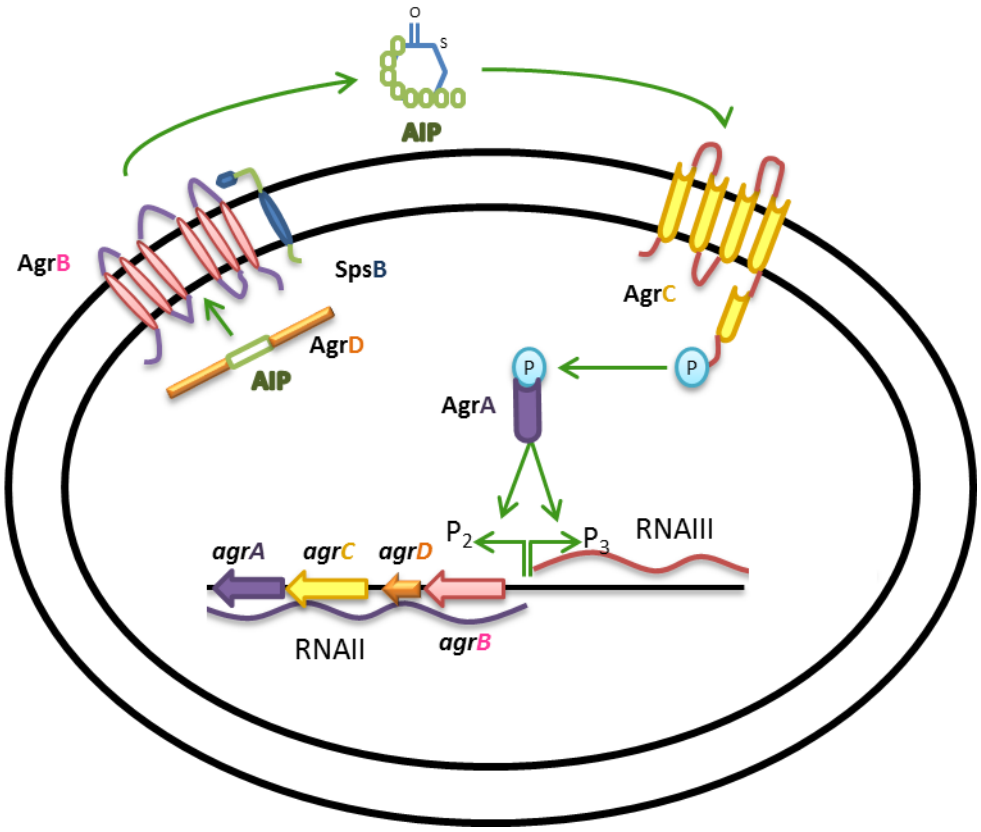
Genetic competence in *S. penumoniae* is regulated by a quorum sensing pathway regulates the production of both *E*arly competence and *L*ate competence genes. The *E*arly competence quorum sensing pathway commences with the induction of the polypeptide ComC (▲) that is actively exported out of the cell by the ATP-binding cassette transporter (ComAB) that also processes the 17 amino-acid competence-stimulating peptide (CSP▲). This activates the histadine kinase CSP receptor (ComD) to phosphorylate ComE that in turn activates ComX an alternate sigma factor. Once activated ComX will bind to the promoter region of numerous Late competence genes required for genetic transformation

RNAIII controls transcription of multiple target genes via numerous intracellular regulatory mediators. Generally it up-regulates transcription of most extracellular protein genes and represses cell surface protein gene expression.[203]

The *agr* system is highly conserved within the Staphylococcal family with *S. aureus* having at least four specific *agr* systems and one or more in twelve other *Staphylococcus* species.[210] Genetic variability occurs within a hypervariable region that spans the *agrB*, *agrD* and *agrC* genes, thus conferring species-specific autoinducing peptide and histadine kinase sensor.[210] The N-terminal third of *agrB* and the C-terminal histadine protein kinase domain of AgrC are highly conserved with the intervening sequences highly divergent. The RNAIII is highly abundant with a complex and highly conserved secondary structure, although there is variability at the sequence level, that enables interspecies cross reactivity.[211]

S epidermidis has a single *agr* quorum sensing system that regulates two proteins, autolysin Alt E and an amphiphillic σ toxin.[212] The *agr* system negatively regulates AltE, which is a cell surface protein that positively mediates primary adhesion.[109, 212] Alternatively, *S. epidermidis* RNAIII encodes a σ toxin that is a member of the phenol-soluble modulins and increases once the *agr* quorum sensing system is activated. Once the σ toxin is present it acts to decrease biofilm attachment, however it does not affect primary adhesion.[212] The level of *agr* is low in the early exponential growth phase of biofilm where attachment predominates and is regulated by autolysin Alt E and other adhesive promoting processes.[119, 213] Activation of the *agr* quorum sensing system occurs in a cell density-dependent manner in the postexponential phase where AltE is down-regulated and PSM increases and has been shown to promote biofilm maturation and detachment.[119, 212] Thus in *S. epidermidis* the *agr* quorum sensing system had been shown not to be involved with primary adhesion but regulates and limits biofilm growth and detachment after primary adhesion.[119, 212, 213]

A *Lux* based quorum sensing system that has been identified in *S. epidermidis* and numerous other bacteria. *LuxS*, and its homologues, have been identified in *S. epidermidis, S. aureus* and over 530 other Gram-negative and Gram-positive bacterial



Adapted from Novick and Geisinger 2008[214]

Figure 1-7: *Staphylococcus* species *agr* Quorum Sensing System

The *Staphylococcal agr* locus had two divergent transcripts, RNA II and RNAIII, driven by two promoters, P2 and P3, respectively. The RNA II operon encodes 4 genes *agrBDCA*. These encode the core machinery for the *agr* system. AgrD is the autoinducer peptide (AIP) precursor that is processed and exported via AgrB-SpsB. The extracellular AIP binds to and activates the AgrC kinase resulting in the phosphorylation of AgrA. Phosphorylated-AgrA activates the P3 promoter and activates the RNAIII. Phosphorylated-AgrA also activates the P2 promoter up-regulating the *agr* system.

species.[215, 216] The *LuxS* system produces an AI-2 molecule that is not species specific and can cross-regulate gene expression in other species in a cell density-dependent manner.[217] The *LuxS* system has shown cross-species regulation of bioluminescence in *V. fischeri*;[218], growth of *E. coli* and *B anthracis*;[219, 220] and virulence factors including flagella motility and biofilm formation in numerous bacteria including important human pathogens such as *E. coli*,[221] *Streptococcus pyrogenes*,[222] *Clostridium perfringens*,[223] *S. pneumoniae*,[224, 225] *S. gordonii*,[226] *Porphyromonas. gingivalis*,[226] *H. pylori*,[227] *P. aeruginosa*,[228] *S. aureus*, and *S epidermidis*.[217] Thus it is now acknowledged that the LuxS quorum sensing system is also used for interspecies communication within mixed-species biofilm,[217] with numerous synergistic and antagonistic actions described.[216, 229]

Quorum sensing systems demonstrate that bacteria are social organisms. They predominately live in biofilm communities and communicate amongst themselves and each other via complex small molecule mechanisms both within and between bacterial species, akin to the hormone control of the human body with autocrine and paracrine hormone regulation. This communication is intricate and sophisticated and has been shown to regulate numerous processes in the production and maturation of bacterial biofilms.

1.9 Antibiotic Resistance

Antibiotics have been the mainstay of treatment for bacterial infections since their discovery by Alexander Fleming over 80 years ago. Failure of antimicrobial treatment and increased antibiotic resistance has long been observed within bacterial biofilms.[98, 230] Sessile bacteria within mature biofilm require antimicrobial drug concentrations much higher than those required to kill planktonic cells; for example the dose of ampicillin levels required to kill *K. pneumonia* in a biofilm is 2,500 fold the dose for their planktonic form.[98, 231] Yet, when the bacteria are removed from the biofilm matrix and returned to their planktonic state they also return to their original susceptibility.

Bacteria within biofilm have numerous physical, molecular and physiological barriers that vary dependant on the bacteria contained within the biofilm and antibiotics.[232-234] These mechanisms include:

1) Reduced penetration of antibiotics;
2) Differential growth states;
3) Persister cells;
4) Extracellular DNA gene regulation;
5) Cellular stress response and antioxidant pathways;
6) Expression of efflux pumps;
7) Horizontal gene transfer.

1.9.1 Reduced Antibiotics Penetration

The biofilm matrix is a mixture of extracellular polymeric substance, extracellular DNA and proteins and presents a physical barrier that molecules need to traverse before they reach the enclosed bacteria.[97, 232, 235] It has long been implicated as a mechanism for antibiotic resistance, however the mechanisms are only now being elucidated.[97, 98, 236-238] The evidence for the physical barrier retardation of antibiotics is variable with some antibiotics readily penetrating certain biofilms and resisted by others depending on the bacteria biofilm and antibiotics combinations.[239] In *P. aeruginosa* biofilms which can produce alginate and have a negatively charged matrix, positively charged antibiotics, such as aminoglycosides and polypeptides are readily bound by the extracellular polymeric substance and have poor penetration.[97, 232, 240, 241] Whereas other antibiotics including β-lactams, imipemen, and some fluoroquinolones, including ciprofloxacin, readily penetrate the *P. aeruginosa* biofilm.[97, 239, 241, 242] Similarly in biofilms of *S. aureus* and *S. epidermidis,* β-lactams and vancomycin are significantly retarded by the extracellular polymeric substance, but amikacin and ciprofloxacin readily pass through.[243] The biofilm matrix also facilitates the accumulation of antibiotic-degradation enzymes including β-lactamases in *Klebsiella pneumoniae*.[231]

Two exopolysaccharide controls and one genetic control of biofilm-specific antibiotic tolerance has been identified in *P. aeruginosa.*[97] Two of the major extracellular matrix

proteins fundamental for *P. aeruginosa* biofilm formation are Pel, a glucose rich exopolysaccharide and Psl, a neutrally-charged repeating (D-mannose-D-Glucose-L-rhamnose) pentasaccharide .[244] Two recent *in vitro* studies have demonstrated that Pel inhibits the aminoglycoside antibiotics tobramycin and gentamicin[245] and Psl inhibits tobramycin, ciprofloxacin and the "last-resort" antibiotic colistin.[236] Interestingly the inhibitory effect of Psl was: 1) concentration-dependent with increased inhibition observed with increased Psl expression, 2) time-dependent with maximal inhibition within the first 24 hours of biofilm growth, and 3) extendible with the inhibitory effect able to encompass *S. aureus* and *E. coli* in mixed biofilms.[236]

The *ndvB* gene in *P. aeruginosa* was one of the first biofilm specific genes linked to antibiotic tolerance. The *ndv* gene produces periplasmic glucans and when expressed in biofilm *in vitro* it increased the tolerance to tobramycin, gentamicin, ciprofloxacin, ofloxacin and chloramphenicol.[246] More recently the *ndvB* gene product glycerol-phosphorylated β-(1,3)-glucans was shown to directly bind to the aminoglycoside, kanamycin,[247] and regulates the expression of over 24 other genes including genes for ethanol oxidation that increase resistance to tobramycin.[248]

These results suggest that the biofilm matrix is not just a simple diffusion barrier but that the bacteria may produce specific matrix molecules as distinct mechanisms to resist antimicrobial actions.

1.9.2 Differential Growth States

The complexity of biofilm communities results in heterogeneous populations of bacterial cells in differential growth states.[98, 233, 238] Within the biofilm microenvironment there is local variation in metabolic substrates, including oxygen, nitrite, nitrate, ammonium, sulphide and methane, resulting in nutrient gradients with the bacteria closer to the surface having access to higher concentrations of nutrients, compared to bacteria within the centre of the biofilm.[233, 235, 238] Thus bacteria have differential states of growth, differentiation and metabolic activity, from active growth and metabolism closer to the surface, where nutrients and oxygen are in better supply, to slow/no growth towards the

centre of the biofilm.[27, 90, 238] This has important implication for chemotherapeutic agents, such as antibiotics that often rely on cellular activity or target specific parts of the cell cycle.[249] Indeed, experimental data has shown that numerous antibiotics, including β-lactams, carbenicillin, ciprofloxacin, and tobramycin were all ineffective in killing bacteria in biofilm and stationary planktonic phase at 6 hours, yet effectively kill bacteria in the logarithmic (active) planktonic phase.[99, 250] Imaging of biofilm exposed to ciprofloxacin and tobramycin demonstrated that the bacteria lysed by these antibiotics were predominantly at the biofilm surface, with none towards the centre.[99] Similarly a study using *P. aeruginosa* biofilms showed differential antibiotic action, with ciprofloxacin and tetracycline being effective in killing the metabolically-active bacteria whereas colistin was only effective in killing bacteria with low metabolic activity.[251] Interestingly this study also demonstrated that the metabolically-active cells developed a tolerance to colistin via mechanisms mediated by the *pmr* operon and the *mexAB-oprM* genes.[251]

1.9.3 Persister Cells

The observation that a viable sub-population of bacteria persists after lethal antibiotic exposure was first made in a series of elegant experiments using penicillin on *in vitro* cultures of *Staphylococcus pyrogenes* reported in 1944.[252] Bigger demonstrated that there was a small proportion of staphylococci present in a dormant, non-dividing phase and described these bacteria as “persisters”.[252] In this phase he demonstrated that the persisters were insensitive to the bactericidal effects of penicillin and were able to survive this treatment. Once the penicillin was removed the persister cells reverted from the dormant phase and commenced normal division, they were able to repopulate the culture and reverted to being sensitive to penicillin treatment.[252]

It has been estimated 0.001-0.1% of the original bacterial population may be in a persister state at any one time.[235, 253] Persister bacteria that spontaneously enter a dormant, non-dividing state occur in both planktonic and sessile bacteria colonies.[254-256] Thus when bacteria within biofilm are subjected to effective bactericidal antibiotics, even for prolonged periods that are common in clinical practice, the persisters survive.[254, 256] The biofilm matrix provides persister bacteria protection from the host immune system. This

protection enables biofilm persisters to survive in greater numbers compared with planktonic persisters, which are readily disabled by normal host immune functions, and readily repopulate the biofilm infection.[256, 257] This has been demonstrated clinically, with high-persister (*hip*) mutants of *P. aeruginosa* and *Candida albicans* surviving after prolonged antibiotic and antifungal treatment, respectively, and re-establishing the patient's infection once therapy is ceased.[258-261] Thus biofilm persister bacteria subpopulations do not have a specific resistance mechanism, but survive due to a transient hyper-resistance phenotype that reverts to a wild–type phenotype and rapidly repopulate once the antibiotic concentration decreases.[255, 256]

The genetic control of the persister phenotype is beginning to be elucidated. Gene mapping of *hip* mutant *E. coli* cells has identified the *hipA* that is part of a toxin/antitoxin (TA) loci.[262, 263] The TA loci commonly encode a two components system: a stable "toxin", which is always a protein that inhibits cell growth, and a labile "antitoxin" that encodes for either a RNA molecule or protein that regulates toxin activity in a feed-back control loop.[264, 265] The antitoxin binds the toxin to form a TA complex and neutralise the toxin's activity, thus enabling normal cell growth metabolism (Figure 1-8). The antitoxin and TA complex represses expression of the TA operon, inhibiting transcription of TA locus and the toxin.[264] The TA locus system can be induced to express the toxin by numerous environmental factors; including starvation, oxidative stress, decreased pH, heat shock and DNA damage; and quorum sensing signals have also been implicated.[266-268] Once the TA locus is activated, there is an excess of free toxin, which in turn inhibits cellular growth and protein synthesis via numerous mechanisms including:

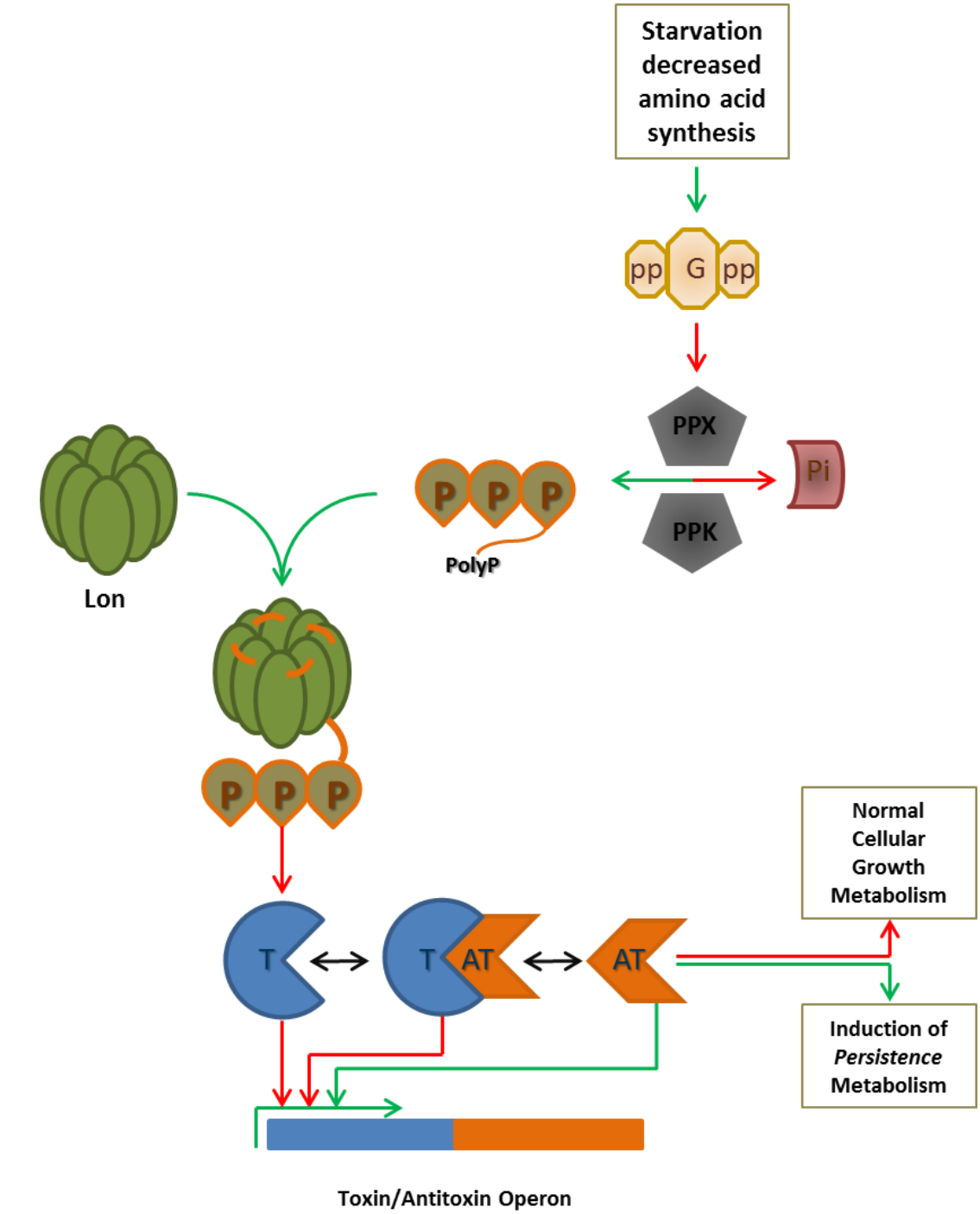
1) inhibition of DNA replication by inhibiting DNA gyrase;[269]
2) degradation of mRNA and inhibition of translation;[270-273] and
3) phosphorylation of the $tTNA^{Glu}$-bound GltX that stimulates activation of the ppGpp pathway, a key inducer of persistence.[257, 265]

The ppGpp molecule is a ubiquitous nutritional "alarmone" that is activated upon amino acid deprivation, causes down-regulation of genes involved in macromolecular biosynthesis and induces entry into the persister state.[265, 274, 275] Once activated ppGpp inhibits the enzyme exopolyphosphatase that degrades the PolyP molecule. PolyP then

Figure 1-8: The Toxin/Antitoxin Mechanism of Bacterial Cell Persistence

Environmental signals, including starvation, oxidative stress, low pH and DNA damage, stimulate the accumulation of the ppGpp regulator molecule that inhibits the enzyme exopolyphosphatase. Inhibition of exopolyphosphatase, which acts to degrade the inorganic polyphosphate (PolyP), results in the accumulation of PolyP which also had positive feedback on polyphoshate kinase (PPK), further promoting PolyP synthesis.

PolyP combines with the Lon protease to degrade the Antitoxin molecules thus leaving the Toxin molecule unbound. The decrease in Antitoxin also decrease the inhibition on expression of the TA locus, combined with the activation by the free Toxin molecule, increase TA locus expression and formation of more free Toxin. Free Toxin acts to inhibit cellular growth and protein synthesis and promote persister cell formation.



Adapted from Maisonneuve and Gerdes 2014[265]

accumulates by the constitutive activation of poly-phosphate kinase. PolyP combines with the cellular proteases Lon and stimulates it to degrade the antitoxin molecules consequently removing the negative feedback on the TA operon resulting in increased TA gene activation (Figure 1-8).[265] Recent evidence suggests that ppGpp is a key pathway for induction of persistence in both *P. aeruginosa* and *E.coli* biofilms.[265, 266, 276]

1.9.4 Extracellular DNA

Extracellular DNA is a key structural component of the biofilm matrix and has numerous roles in the development and stability of biofilm structure.[92] The average size of extracellular DNA within the matrix is 30 kDA and is predominantly formed from programmed bacterial cell lysis under quorum-sensing control.[277-279] Extracellular DNA within biofilm is also secreted by non-cell lysis excretion and from other origins including other bacteria and host cells, such as polymorphonuclear leukocytes that are lysed in the biofilm/host immune response.[280-283]

Two recent studies in *P. aeruginosa* biofilms have demonstrated that extracellular DNA may induce antibiotic resistance mechanisms.[283, 284] DNA is negatively charged and with the biofilm matrix acts as a cation chelator whereby the negatively charged extracellular DNA efficiently binds divalent metal cations, Mg^{2+}, Ca^{2+}, Mn^{2+} and Zn^{2+}.[285] This stimulates a cation-limited environment that activates the *pmr* genes, PhoPQ and PmrAB, inducing expression of antibiotic resistance genes, resistant to cationic peptides and aminoglycosides up to 2560-fold and 640-fold, respectively.[284] In a more recent study exogenous extracellular DNA, from salmon sperm and lysed human polymorphonuclear leukocytes, was shown to readily incorporate into *P aeruginosa* biofilm.[283] Once incorporated the exogenous extracellular DNA conferred increased resistance to the aminoglycosides tobramycin and gentamicin via a yet unknown non-*pmr* mechanism.[283]

1.9.5 Cellular Stress Response and Antioxidation

Lethal doses of numerous bactericidal antibiotics, such as aminoglycosides, β-lactams and fluoroquinolones, have been shown to induce hydroxyl radical formation that promotes the formation of highly deleterious reactive oxygen species. Reactive oxygen species

cause damage to cellular macromolecules and ultimately cause cell death.[286-288] Whilst this mechanism is commonly observed in planktonic bacteria, bacteria within biofilms show little or no oxidative stress response and require much higher concentrations of antibiotics to induce a small reactive oxygen species response.[289, 290]

Bacteria have a global SOS response to DNA damage whereby the cell cycle is arrested to enable DNA repair. This has been well investigated in planktonic bacteria, but poorly understood in biofilms.[239, 291] Within biofilms differential gene expression with up-regulation of numerous SOS response genes is a general feature.[239, 290, 292, 293] Recent studies have identified that nutrient limitation, of both carbon and nitrogen sources, initiates a stringent SOS response in biofilms.[267, 292, 294] Several studies have demonstrated that enhanced antibiotic resistance is correlated to expression of antioxidant-stress and SOS responses, with nutrient starvation being a key initiator.[267, 290, 294]

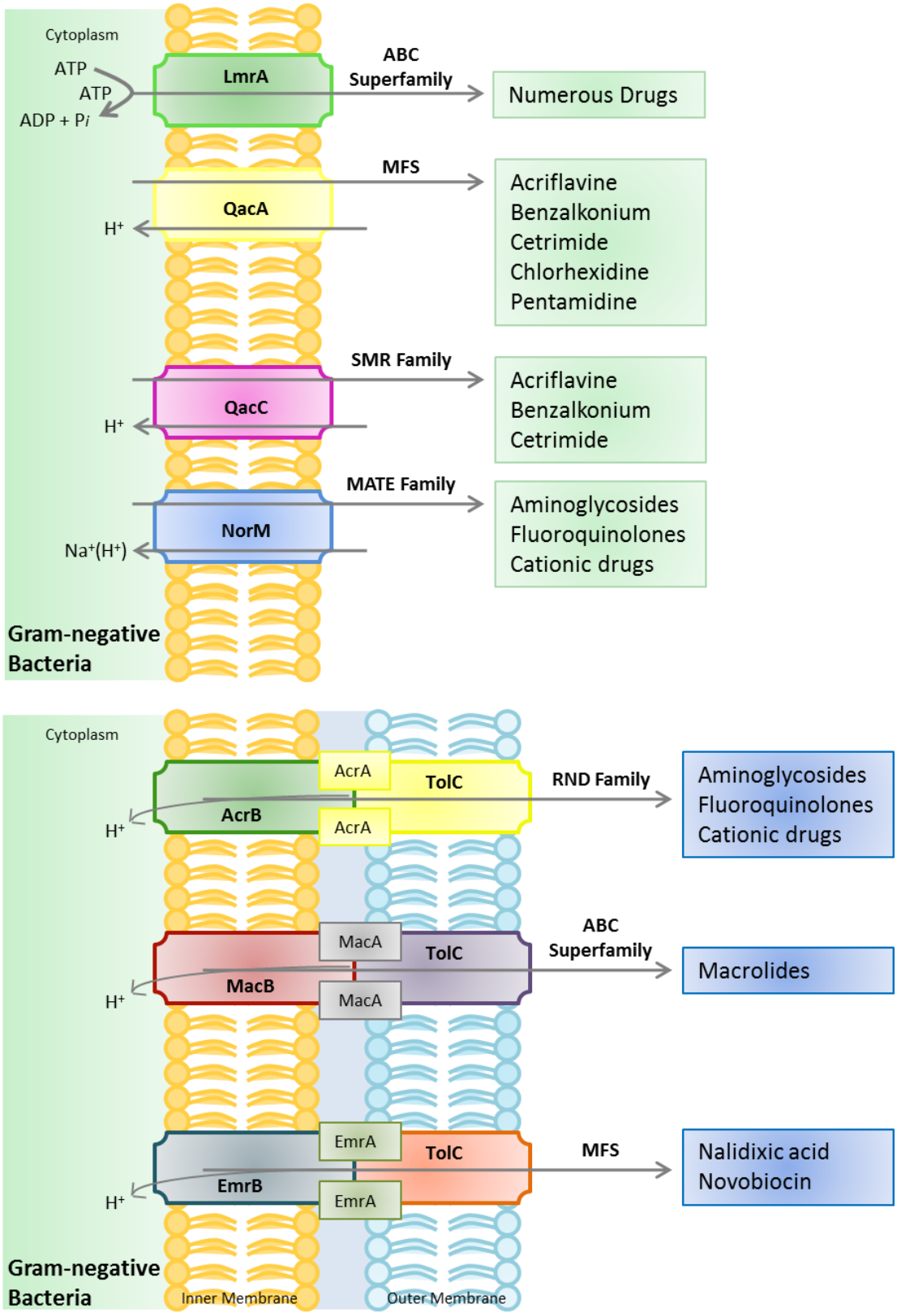
1.9.6 Efflux Pumps

Efflux pumps are energy-dependent transmembrane pumps that actively transport multiple substrates, including numerous antibiotics, out of bacteria.[295-297] Numerous efflux systems have now been described in both Gram-negative and Gram-positive bacteria in both planktonic and biofilm environments (Figure 1-9).[295, 298] Multidrug resistance (MDR) efflux pumps are divided into five families: the major facilitator super (MFS) family, the resistance nodulation division (RND) family, the small multi-resistance (SMR) family, the multidrug and toxic compound extrusion (MATE) family and the ATP-binding cassette (ABC) family.[297, 298] A single organism can express multiple efflux pumps from a single family and/or several pumps from different MDR families.[297]

MDR efflux pumps confer intrinsic resistance with a basal level of efflux.[297] Acquired antibiotic resistance results from a constitutive increase in expression of efflux-pumps. Several mechanisms can result in permanent up-regulation of efflux pump expression including: mutation in local repressor genes; mutations in the transcriptional activator that regulates gene expression, mutation in the promoter region of the efflux-pump

Figure 1-9: Multidrug-resistance Membrane bound Efflux Pumps of Gram-Positive and Gram-Negative Bacteria

Diagrammatic representation of the five families of multidrug-resistance of efflux pumps: 1) ATP-binding cassette (ABC) superfamily, 2) the major facilitator superfamily (MFS), 3) the multidrug and toxic-compound extrusion (MATE) family, 4) the small multidrug resistance (SMR) family, and 5) the resistance nodulation division (RND) family. The structure and membrane location of the efflux pump proteins in both Gram-positive and Gram-negative bacteria are indicated. Common antibiotic substrates for each superfamily are identified.



Adapted from Piddock 2006[297]

genes; or mutations in insertion elements located upstream of the efflux-pump gene.[297] The genes encoding these efflux pumps are commonly plasmid-borne genes and harboured on mobile elements that facilitate transferral via horizontal transmission.[296, 299-301]

Multidrug resistant efflux pumps have been well studied in planktonic bacteria, yet their role in biofilm growth and biofilm-mediated antibiotic resistance is still poorly understood.[297, 302, 303] Early studies suggested that four well-studied RND efflux pumps in *P. aeruginosa* had no impact on biofilm-specific resistance in mature biofilm.[304] In *P. aeruginosa* biofilms two efflux pumps, MexAB-OprM and MexCD-OprJ, have now been shown to be active in biofilm-specific resistance, including resistance to azithromycin.[305] Also a novel MDR efflux pump, PA1874 confers resistance to aminoglycosides and fluoroquinolones in *P. aeruginosa* biofilm, but not in the planktonic state.[302]

In *E. coli*, YhcQ is a putative biofilm-specific MDR pump that confers resistance to penicillin G, but it may also have extra cell wall functions and assist in biofilm formation.[306] Mutations of multiple MDR pumps, of both *Salmonella enterica* and *E. coli* biofilm species, did not affect bacterial attachment, but significantly affected subsequent formation of the three-dimensional biofilm structures.[307, 308] Similarly, efflux pump inhibitors to *E. coli*, *K. pneumonia*, *P. aeruginosa* and *S. aureus* biofilm species show impaired biofilm formation.[303, 307] The resultant biofilm showed decreased resistance to antibiotics with between 33-93% bacteria death between two to six hours post antibiotic exposure.[303] This has prompted much interest in identifying suitable efflux pump inhibitors to potentiate antibiotic therapy as one of many prospective therapeutic strategies for biofilm disease.[309, 310]

1.9.7 Horizontal Gene Transfer

Bacteria were first observed to share or “transform” genetic material in pneumococci by Griffith in 1928, well before the discovery of DNA.[311, 312] Over the next two decades the transforming factor was determined to be DNA in origin and three mechanisms, horizontal genetic transfer/transduction, transformation and conjunction, were

identified.[313] Over thirty years later the "transforming" factors or mobile genetic elements including phages and plasmids were identified.[312] Horizontal gene transfer is now well recognised for its fundamental role in the adaption and evolution of bacteria, including the spread of antibiotic resistance genes.[312, 314]

Bacterial biofilm offers a unique microenvironment that enhances horizontal gene transfer with high cell density, close cell/cell proximity, presence of extracellular DNA and protection from the surrounding environment.[312, 315, 316] Horizontal gene transfer contributes to biofilm development stabilisation and expansion via multiple mechanisms. Expression of prophage proteins has a function in biofilm physiology, metabolism and formation, and phage-induced bacterial lysis contributes to extracellular DNA and biofilm matrix structure.[312, 317, 318] Conjugation, the most common mechanism of horizontal gene transfer in biofilm, encodes adhesive structures, such as fimbriae, and enhances biofilm formation, stability and transfer of antibiotic resistance genes.[100, 277, 315, 316, 319]

Scientific understanding of the mechanisms of antibiotic resistance in bacterial biofilms is still in its infancy.[97] However, the overall mechanisms used by the bacteria residing within biofilms are likely to be multifactorial and influenced by bacterial species involved, biofilm host and/or environmental pressure and antibiotics used. The mechanisms may also vary over time.[320] Biofilms do, however, offer a protected environment that facilitates bacteria to express and rapidly exchange and acquire new antibiotic resistance mechanisms to enable their long term survival.

1.10 Bacterial Biofilms in the Health Care Environment

Nosocomial, or hospital-acquired infections, are defined as any infections that develop in a hospitalised patient, not present at the time the patient presented to hospital.[69] Estimates of hospital-acquired infections for hospital inpatients are similar in both European and American studies; 3-9%[321-323] and 5-10%,[69] respectively. Hospital-acquired infection rates post-discharge are likely to be even higher with one study conservatively estimating a post-discharge infection rate of 19%; two to six times

inpatient rates. This is likely to underestimate hospital-acquired infection rates in post-discharge patients due to the deficiencies in data collection post-discharge.[324]

Not surprisingly inpatient hospital-acquired infections are associated with a significant increase in mortality and health care costs. A review of hospital-acquired infections in Belgium for two years from 2005 to 2007 calculated that there were 125,000 hospital-acquired infections per annum during this period. This was a prevalence of 6.2/100 patient admissions with the majority (86.5%) infected outside the intensive care setting.[321] These infections were associated with an increased mortality of 2.8%, an increased length of stay of 7.3 days and an estimated increase in health-care costs of €290 million per annum.[321]

In 2006 a three month study across 190 English hospitals, as part of a national prevalence survey, assessed the hospital-acquired infection rate to be 8.2%.[325] In the same study surgical site infections, infections in surgical wound or deeper tissues/organ space, accounted for 14.5% of all hospital-acquired infections and occurred in 4.65% of all patients undergoing operations.[325] An earlier review from data collected over a 4 year period (1997-2001), as part of the voluntary English Nosocomial Infections National Surveillance Service (NINSS), showed a similar rate of surgical site infections at 4.2% over 140 participant hospitals.[326] The highest rate of surgical site infections was observed in limb amputation surgery (14.3%) and bowel surgery (10.0%) and lowest rates for abdominal hysterectomy (2.5%) and joint prosthesis surgery (knee 1.9% and hip 3.1%).[326] This was associated with an overall increased mean length of stay of 9 days (range 3-21 days), increased health costs of £959 to £6,103 per patient and significant increased mortality in patients with surgical site infections after vascular, large bowel and hip/knee prosthetic surgery.[326]

Since these studies the National Health System in England has developed a mandatory hospital-acquired infection reporting process via Public Health England (PHE) and their most recent data shows a decrease in hospital-acquired infections (6.4%) with surgical site infections accounting for 15.7% of hospital-acquired infections in 2011.[323] The percentage of surgical patients developing surgical site infections between 2008 and 2013

decreased to 1.4% of patients undergoing operations with one third of surgical site infections developing in the post-discharge period that required hospital readmission.[327] The highest surgical site infection rates were in patients undergoing large bowel (10.6%), small bowel (6.4%) and biliary surgery (6.5%), consistent with the published NINSS rates.[327] Interestingly there was a marked decrease in surgical site infections after limb amputation surgery (3.3%) and persistently low rates of surgical site infections after abdominal hysterectomy (1.5%), joint prosthesis surgery (knee 0.6% and hip 0.7%) and breast surgery (1.0%). Hospital-acquired infections were associated with an overall mean increased length of stay of 10 days (range 1-29 days) and increased healthcare costs of £5,239 per patient (range £1,469/breast surgical site infection to £21,439/gastric surgical site infection), although these data are likely to underestimate the rate and economic burden of surgical site infections that develops post discharge period.[328]

In the United States, the Centers for Disease Control and Prevention, has a voluntary centralised hospital-acquired infection reporting and surveillance programme called National Healthcare Safety Network (NHSN), previously known as National Nosocomial Infections Surveillance System (NNIS) prior to 2005.[329] A study by NNIS estimated a hospital-acquired infection rate of 4.4% in 38.5 million hospitalisations in 2002 with a mortality rate of 5.8% of patients affected by hospital-acquired infections, the fourth leading cause of death per annum.[66, 330] More recent data from the NHSN estimated a hospital-acquired infection rate of 4.0%, with 22% of these infections due to surgical site infections.[331] Surgical site infections have been estimated to increase the mortality rate by 3% with 75% of these deaths directly attributable to the surgical site infection.[332]

In Australia, to date, there is no national approach to reporting or assessing the burden of hospital-acquired infections or surgical site infections.[333] The last national hospital-acquired infection prevalence study, conducted in 1984, found 6.3% of inpatients developed hospital-acquired infections.[334] More recent Government reports estimate there are 200,000 hospital-acquired infections annually across Australia, although the background data for this calculation is unclear.[333, 335, 336] Smaller studies have demonstrated surgical site infection rates between 2.0% for prosthetic hip surgery, 8.9%

for cardiac surgery, 9.8% for prosthetic knee surgery and 12.7% after colorectal surgery.[335, 337, 338] These rates are higher than comparable rates in either England or the United States.

A NHSN study identifying the causative pathogens involved in hospital-acquired infections in the United States demonstrated that approximately one third were caused by *Staphylococcus* species (coagulase negative Staphylococcal species 15% and *S. aureus* 15%), followed by *Enterococcus* species (12%), *Candida* species (11%), *E. coli* (10%), *P. aeruginosa* (8%) and several others.[329] Of these infections 16% were from multidrug-resistant pathogens methicillin-resistant *S. aureus* (MRSA-8% of hospital-acquired infections), vancomycin-resistant *Enterococcus faecium* (VRE-4%), carbapenem-resistant *P. aeruginosa* (2%).[329] These results are consistent with other studies that show *Staphylococcal* species, especially coagulase negative *Staphylococcal* species which are endogenous skin flora, are the most commonly identified pathogens.[339-342]

1.10.1 Biofilms on Hospital Surfaces

The importance of bacterial biofilms in hospital-acquired infections is still poorly understood. There is increasing evidence that bacterial biofilms cause hospital-acquired infections from both patient contact with their surrounding hospital environment and colonisation of body surfaces and implantable medical devices (Table 1-1).[66, 343, 344] In the natural environment bacteria commonly live within biofilms, particularly within moist and wet areas.[10, 12] This is also true within the hospital environment. A reported outbreak of multiresistant *P. aeruginosa* in an ICU and transplant ward was found to be due to biofilm contamination of handwashing sinks.[345] The biofilm was not controlled by multiple decontamination processes and over a sixteen month period the splash contamination of the surrounding environment caused infection of thirty-six patients resulting in seventeen patient deaths.[345]

There is increasing indirect and direct evidence that hospital-acquired infections can be acquired from direct contact with surrounding contaminated surfaces.[346, 347] Transfer of important clinical pathogens has been demonstrated between patients, staff and the

hospital environment.[348, 349] Several studies have demonstrated an average 73% increased risk of acquiring a multi antibiotic-resistant organism infection, including MRSA and VRE, when the bed has previously been occupied by a patient carrying these organisms.[350, 351] Clinically important pathogens, including multiresistant organisms, have been cultured from numerous clinical surfaces and surgical equipment after standard cleaning and sterilisation processes in intensive care units,[344, 352] on endoscopes[353] and surgical instruments.[354] Our research group was the first to demonstrate viable bacterial biofilm of clinically relevant pathogens on numerous environmental surfaces, including bed and mattress surfaces, entry/exit doors, storage containers, etc, in an intensive care unit using a combination of culture, PCR, scanning electron microscopy and confocal laser-scanning microscopy (Chapter 3).[344] Taken together these results suggest that bacterial biofilms on clinical surfaces may represent an important bacterial reservoir that enables clinically relevant pathogens to persist and infect future patients (1.12).[344]

1.10.2 Biofilms on Medical Implants

Sixty to 70% of hospital-acquired infections are associated with temporary or permanent implantable medical devices.[66] Central intravenous catheters and indwelling urinary catheters (urinary catheter) contribute up to half of these infections with estimates suggesting that combined they cause one-third of all hospital-acquired infections in the United States in 2007.[355] Surgical implantable medical devices and engineered tissues are also susceptible to microbial colonisation and infection, with biofilm infections having been identified on most, if not all, such devices (Table 1-1).[23, 70, 230] In the U.S. alone over five million implantable medical devices are inserted per annum and globally this $180 billion industry is rapidly growing. Biofilm infections of implantable medical devices have significant clinical and economic implications.[66, 69] Infection rates vary from 1-3 % for mammary and penile implants, 4-10% for neurosurgical, orthopaedic and some cardiac devices and up to 40% in ventricular assist devices.[356] Conservative estimates suggest mortality rates of patients with infected implantable medical devices also vary with low rates (>5%) for mammary, penile and orthopaedic implants and vascular grafts, up to 10%

for cardiac pacemakers and central venous catheters, 10-30% for urinary catheters and over 25% for cardiac assist devices.[70, 357, 358] Whist the mortality for mammary, penile and orthopaedic implants is low the morbidity related to the personal cost, disfigurement and impacts on mobility and function, whilst difficult to assess and quantify, is not insignificant to the patients affected.

Surgical excision and debridement and prolonged antibiotics +/- replacement in a single or multiple step process remains the mainstay curative treatment for patients with infected surgical implantable medical devices.[356, 359-364] A recent report has suggested that surgical debridement and antibiotic therapy without removal of biofilm infected implants was as efficacious as standard treatment in early infection, although reported numbers to date are small.[365, 366] Novel non-operative strategies have been postulated but none have made it into clinical practice to date.[367, 368] Lifelong suppressive antibiotics are indicated in patients no longer fit for surgical treatment or in whom surgical treatment has failed.[359] Inpatient treatment costs for infected surgical implantable medical devices vary between $US15,000 for fracture fixation devices, up to $US50,000 for neurosurgical implantable medical devices, $US70,000-$US95,000 for prosthetic hip devices and up to $US146,000 for cardiac implantable medical devices.[356, 369, 370] Projected estimates suggest the cost will almost triple and exceed $US1.6 billion by 2020 for the management of infected orthopaedic prosthetic joints in the U.S. alone and is unsustainable in the longer term.[369] Thus understanding of the pathogenesis of biofilm disease of surgical implantable medical devices and development of prevention and treatment strategies are crucial to the long term viability of this burgeoning industry.

1.10.2.1 Indwelling Urinary Catheters

Insertion of an indwelling urinary catheter is one of the most common invasive procedures in hospitalised patients.[371] In acute care patients 12-25% will require an indwelling urinary catheter during their hospitalisation, with higher rates in surgical and critical care patients.[371-373] Long-term (>30-days) indwelling urinary catheter are required for 5-10% of care facility residents[372, 373] and all spinal injury patients require either permanent or intermittent urinary catheter insertion.[374] Urinary tract infections are the

most common nosocomial infections, with an estimated incidence of 40%.[375-377] Catheter-associated urinary tract infections account for 80% of nosocomial urinary tract infections[378, 379] and increasing to 95% in critical care patients.[380]

Once inserted the risk of developing bacteriuria has been estimated at 3%-10% per day.[375] Of the patients with bacteriuria 10-25% develop symptoms of a localised urinary tract infection and 1-4% develop bacteraemia.[77, 373, 376, 377] By 28 days all patients will have bacteriuria.[381] The mortality rate of patients with bacteraemia urinary tract infections, with and without an indwelling urinary catheter, has been estimated as ~13%, accounting for <1% of hospital deaths.[382] In patients with indwelling urinary catheters the development of bacteraemia has been shown to increase mortality by almost 5 fold (from 4% to 19%) compared to patients without bacteraemia.[383] Hospital costs associated with the treatment of symptomatic catheter-associated urinary tract infections were estimated be between US$758 and up to US$2836 for catheter-associated bacteraemia.[377, 384]

Bacterial biofilm are the main cause of the common complications of indwelling urinary catheters including catheter-associated urinary tract infections and recurrent catheter obstruction. Once inserted the urinary catheter quickly becomes coated by various components including Tamm-Horsfall glycoprotein, polysaccharides and other organic molecules.[63, 78, 385] These molecules produce a proteinaceous conditioning film that provides receptor sites for bacterial adhesions that facilitate primary adhesion and docking, the first step in biofilm formation.[77, 78]

Bacterial attachment and adhesion quickly follows. Uropathogens are commonly faecal contaminants or skin microflora that colonise the periurethral area and include *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Morganella morganii, Proteus* spp (esp *Proteus mirabillis* in recurrent infections), *Providencia* spp, *S. aureus* and coagulase negative *Staphylococci*.[378, 385] Bacteria gain access to the extraluminal surface by direct inoculation and ascend into the bladder and account for approximately 65-70% of catheter-associated urinary tract infections.[77, 378] Intraluminal contamination also occurs, accounting for the remainder of catheter-associated urinary tract infections, and

is related to failure of the closed drainage system, contamination from the collecting bag, and cross contamination from health care workers.[77, 378, 379] Once the biofilm is established the biofilm will ascend the tubing into the bladder. Bacteria are continually shed, resulting in bacteriuria within 1-2 weeks post insertion.[371] Bacteriuria is often asymptomatic with short term catheterisation (< 4 days) and will resolve on removal of the catheter.[77, 371] Biofilm in patients with prolonged and long-term urinary catheterisation can cause several complications including symptomatic bacteriuria, catheter-associated urinary tract infection, sepsis, catheter encrustation and blockage, and struvite urolithiasis.[386] Over time the biofilm often progresses from single species to multispecies and acquisition of antibiotic resistance is common due to the dynamic nature of biofilm development, further complicating medical management.[371]

Uropathogens have several characteristics that enhance bacterial attachment. Planktonic bacteria sense their proximity to the surface by releasing protons and signalling molecules. Signalling molecules released from bacteria distant from a surface will diffuse away and no response is made. If, however, the bacteria is adjacent to a surface the signalling molecule concentration increases, thus allowing the bacteria to sense proximity to the surface and initiate adhesion.[78] Several common uropathogens have innate motility that enhances bacterial adhesion including: 1) flagella-mediated motility on *E. coli* and *P. aeruginosa*;[385, 386] and 2) flagella-mediated motility and swarming ability of *P. mirabilis* enables its rapid movement over solid surface after attachment.[385, 387]

Once attached bacteria secrete their exopolysaccharide coat, undergo genotypic and phenotypic adaptation to the low nutrient urinary tract environment and rapidly mature into complex bacterial biofilms.[385] Scanning electron microscopy has demonstrated extensive bacterial biofilm in urinary catheters removed at seven days post insertion, including biofilm extending the length of the catheter.[386, 388, 389] Patents with long-term urinary catheters often develop polymicrobial biofilm, predominantly Gram-negative nosocomial organisms (esp: *P. mirabilis, P aeruginosa, Klebsiella pneumoniae* and *Providencia stuartii*.[386]

Catheter-associated urinary tract infection occurs when bacteria from the catheter gain access to a disrupted uroepitelial mucosa. The presence of a urinary catheter increases uroepithelial mucosal inflammation, increasing binding sites for bacterial adhesins and causes pooling of urine around the catheter bulb.[385] This is also aided by the bacteria which cause nosocomial urinary tract infections having fewer virulence factors required to colonise and cause infection.[390]

Catheter encrustation and blockage is a common reason for removal and replacement of urinary catheters in patients with long-term indwelling urinary catheters and is required on a routine basis every 4-6 weeks. Several of the common uropathogens, including *P mirabilis, Providencia* spp*, P aeruginosa* and *K. pneumoniae*, have the ability to produce the enzyme urease.[385, 386, 391, 392] Urease-producing bacteria hydrolyse urea to ammonia and raise the pH in the bladder.[391, 393] In this alkaline environment magnesium and calcium phosphates precipitate to form struvite crystals (ammonium magnesium phosphate hexahydrate) and hydroxyapatite, a crystalline form of calcium phosphate.[386, 391, 394] These minerals precipitate in the bladder and the developing catheter biofilm until the catheter lumen is blocked, necessitating its removal. Removal and replacement of the urinary catheter often results in repeat bacterial attachment, biofilm formation and recurrent blockage with struvite/hydroxyapatite encrustation.[391]

To date the mainstay of treatment still remains the removal +/- replacement of the indwelling urinary catheter.[373] Over recent years there has been extensive research into and development of biomaterials and coatings that can decrease biofilm formation.[371] These coatings include antibiotics, silver alloy, heparin, hydrophilic polymers and antiseptic agents. To date all clinical investigations have failed to demonstrate any statistical decrease in biofilm formation and its sequelae.[78, 371, 373]

1.10.2.2 Orthopaedic Prosthetic Devices

Orthopaedic implants are the most common surgical implantable medical devices inserted and are highly susceptible to bacterial infection.[327, 395, 396] Periprosthetic joint infection remains a devastating complication post-arthroplasty and is a common

indication for revision surgery.[397, 398] The most recent national 5 year English PHE report had a surgical site infection rate of 0.6% from 183,566 total knee arthroplasty (TKA) operations and 0.7% from 170,158 total hip arthroplasty (THA) operations.[327] US studies report increasing prosthetic joint infection rates for both THA (1.99% to 2.18%) and TKA (2.05% to 2.18%) between 2001 and 2009.[369] Australian data from the National Orthopaedic Registry Data 2013 report infection rates for revision arthroplasty of 1.25% for THA and 1.8% for TKA, being the third and second most common indications for revision surgery respectively.[399] Over 70% of prosthetic joint infections will present within the first 2 years after surgery.[397, 400] Surgical site infections rise noticeably after revision surgery with reported rates between 11.2% to 27% after revision TKA and 4.5% to 18.4% after revision THA.[398, 401, 402]

Internal fixation devices, such as metal plates and screws, for management of fractures are also susceptible to infection.[395] The risk of a surgical site infection is higher than that for primary arthroplasty, although the disease overall burden is lower due to lesser number of operations. The same 5 year PHE data reported a surgical site infection rate of 1.2% from 13,640 operations on long bone fractures and 1.5% from operations on 74,311 neck of femur fractures.[327, 400, 403] Australian Orthopaedic Registry data report an infection rate of 1.1% at 12 years post neck of femur fracture surgery.[399]

Infection of orthopaedic devices has a multimodal pattern of early (< 3 months), delayed (3-12 months) and late (> 12 months) infection.[395, 404] Early and delayed post-operative infection are characterised by acute pain, joint effusion, localised erythema, delayed wound healing and wound discharge.[405] High fever and sepsis symptoms are uncommon and occur in <10% of patients.[405] These infections are thought to be due to contact or aerosol contamination at time of surgery.[395, 404, 406] This is consistent with the bacteriological results showing that 60% of all early infections are due to *S. aureus* (38%) and coagulase negative Staphylococcal species (22%).[406] Anaerobic Gram-negative *bacilli* are present in 24% of early infections and 31% of early infections are polymicrobial.[406]

Late infections can be either exogenously or haematogenously acquired.[404, 406] Typically these are low-grade indolent infections, characterised by pain from inflammation and

implant loosening and recurrent sinuses.[404] Most frequent sources of haematogenous spread are skin, respiratory, dental and urinary tract infections.[83, 405] *Staphylococci* spp remain the most common bacteria identified and account for half of all bacteria identified, followed by polymicrobial (15%) and culture-negative (14%) infections.[406] The risk of a haematogenous acquired prosthetic joint infection is 30-40 % after acute *Staphylococcus* bacteraemia.[407]

Bacterial biofilms have long been implicated in prosthetic joint infections and infection of orthopaedic devices due to the indolent and chronic nature of many orthopaedic infections, their diagnostic difficulty in culturing aspirates and recurrent symptoms despite antibiotic treatment. Bacteria biofilm were first observed attached to orthopaedic devices, osteomyolytic bone debridement specimens and intermedullary methymetacrylate removed from chronically infected wounds 30 years ago.[408-410] More recent studies on infected orthopaedic implantable medical devices using sonication-enhanced culturing techniques, scanning and transmission electron microscopy and confocal microscopy have demonstrated viable bacteria biofilm from multiple devices including Kirschner wires, internal fixation plates & screws, numerous orthopaedic prosthetic devices and surrounding bone and soft tissue.[411-414]

It is now well recognised that bacterial contamination with biofilm formation is a common pathogenesis in exogenous and haematogenous prosthetic joint infections and fixation device infections, and guides both diagnostic procedures and principles of treatment.[395, 406, 415] Unfortunately many of these research techniques to identify biofilm have not been easily transferable to medical diagnostic processes and until recently there has not been standardized diagnostic criteria.[406] Several groups have now published diagnostic criteria with similar requirements based on clinical features and pathological results.[406, 416, 417]

The gold standard surgical treatment for a prosthetic joint infection is a two-stage revision total arthroplasty with the aim of removing the implant and all of the infected tissue and ultimately replacing the prosthesis and returning function without pain.[395] Ideally, this process requires resection of implant and insertion of antibiotic-impregnated cement spacer followed by the delayed reimplantation of joint prosthesis.[404, 406, 418, 419]

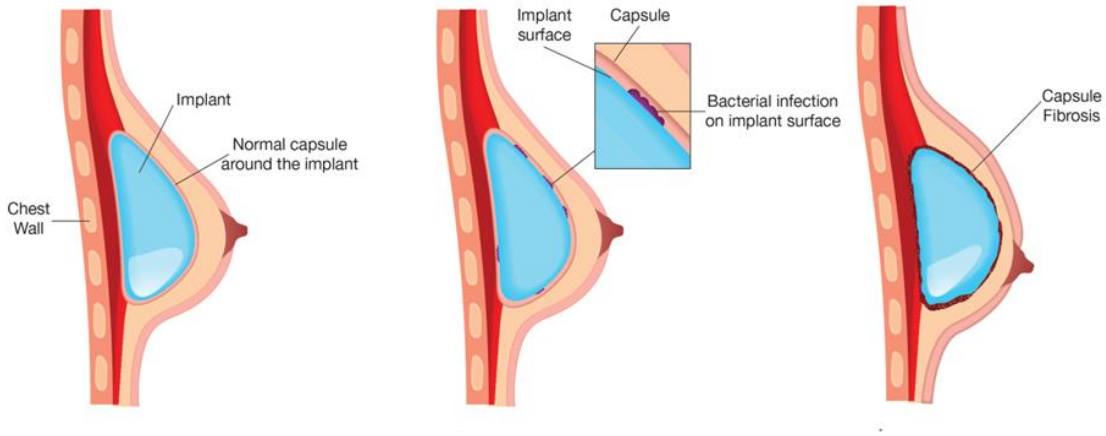
Interestingly the aim of treatment for infected fracture fixation devices is not focused on infection eradication, but maintaining fracture alignment to support fracture union.[395]

Currently no treatment guidelines exist, but standard treatment options include surgical debridement of infected tissues, suppressive antibiotics and maintenance of the fracture fixation device until evidence of bony healing.[395, 420] Not surprisingly failure of union rates of up to 32%, have been reported in the literature suggesting this strategy may need further evaluation.[421-423]

1.10.2.3 Silicone Breast Implants

Breast augmentation and reconstruction, using breast implants, are amongst the most common procedures performed in plastic surgery.[424] In 2011 over 310,000 breast augmentation procedures were performed in the United States alone.[425] Pathological contracture of the periprosthetic capsule and subsequent implant distortion, abnormal firmness and pain remains the most common complication following insertion of breast implants.[424, 426] Reported rates for capsular contracture range from 10-50% for primary aesthetic breast augmentation[426-431] and up to 80% following reconstructive surgery.[426] Pre-market approval data by implant manufacturers Mentor and Allergan report capsular contracture rates of 15% after breast augmentation and 15-30% after breast reconstruction.[426, 431, 432] The Food and Drug Administration reported capsular contracture rates of 10.9-19.1% for primary augmentation and 15.3-24.6% after primary breast reconstruction at 8-10 year follow-up studies.[433]

Until recently the aetiology remained poorly understood. A number of theories have been put forward for the genesis of capsular contracture. These include development of hypertrophic scar, presence of subclinical infection, associated periprosthetic haematoma, silicone bleed and implant surface texture.[426, 434] The role of subclinical infection, however, has gained support from both clinical and animal studies that suggest bacterial biofilm on the surface of implants may be an important pathogenic pathway to development of capsular contracture (Figure 1-10).[89, 426, 435-438] The supporting evidence



1-10(a) **1-10(b)** **1-10(c)**

Figure 1-10: Progress of Biofilm Induced Capsular Contracture

1-10(a) Normal Capsule: When the silicone breast implant is inserted into the breast the body responds to the foreign material and forms fibrous capsule around the implant.

1-10(b) Infected Capsule: If the capsule is infected with bacteria it can adhere to the capsule and proceed to form biofilm.

1-10(c) Capsular Contracture: The biofilm infection results in inflammation and progress fibrosis of the capsule resulting in a thick/stiff capsule and deformity of the implant and breast.

is circumstantial but collectively it supports the emergence of the Subclinical Infection Theory in the Aetiology of Capsular Contracture.

Courtiss and colleagues were the first to observe that infected implants, salvaged with drainage and antibiotics were firmer with increased capsular contracture.[439] Two studies in the early 1980's were able to culture *S. epidermidis* from contracted capsules after capsulotomy.[440, 441] Shah and colleagues were then able to reproduce capsular contracture in rabbits when the implants were inoculated with the clinical *S. epidermidis* strain. [440] Burkhardt and colleagues demonstrated that the antibiotics cephalotin and gentamicin can pass through the silicone shell when used in solution to inflate the silicone-shelled inflatable Heyer-Schulte implant (a standard 1970's implant).

In vitro and then *in vivo* studies with these implants demonstrated that when injected with the antibiotic solution (intraluminal antibiotics), containing either cephalotin or gentamicin in standard therapeutic doses, antibiotic activity was still present for up to 12 months, with no adverse events.[441] When intraluminal antibiotics were used clinically in both primary augmentation and redo-surgery, with open capsulotomy for capsular contracture, they were able to demonstrate a reduction in their capsular contracture rates to 3% at 12 months, compared with their previous contracture rate of 50%.[441] Importantly this study was observational only and not randomised, blinded or case-matched thus making the results difficult to interpret. Regardless a drop from 50% to less than 10% suggests that there was a treatment effect.

In a prospective study Burkhardt and colleagues, using the same inflatable implants, compared intraluminal antibiotics with or without pocket irrigation with either 5% povidone-iodine solution or an antibiotic foam (containing 50,000U bacitracin, 250mg oxacillin and 40 mg triamcinolone) or normal saline (control) to control implants, infiltrated with normal saline pocket irrigation.[442] They found the incidence of clinically significant capsular contracture (Baker Grade III and IV) to be lowest in the groups that had antimicrobial pocket irrigation treatment (povidone-iodine or antibiotic foam, 18% and 14%, respectively) regardless of the instillation fluid within the inflatable implant.

This resulted in 50% reduction in capsular contracture compared to the saline pocket irrigation groups which was statistically significant (P<0.01).[442]

By 1990 the roles of intraluminal antibiotic and antibacterial cleaning of the implant pocket were questioned by several authors, including Dr Burkhart himself.[443] In light of the finding that, in a randomised double-blind trial of prophylactic antibiotics or 0.9% saline (control) one hour prior to surgery, there was no statistical difference in capsular contracture rates between the two groups at 12 months.[444] Interestingly, however, they noted a significant decrease in positive bacteriological cultures from implant pocket specimens taken at time of surgery. Cultures were negative in 93% of the antibiotic groups compared to 21% of control groups, with the most common bacteria grown in control groups being the skin flora *S. epidermidis* (32%) and *P.acnes* (42%).[444]

Virden and colleagues demonstrated a significantly higher rate of bacterial retrieval from the breast implants or peri-prosthetic capsule when removed from patients with capsular contracture (15 of 27 implants; 56%) compared to those without capsular contracture (5 of 28 implants; 18%; p<0.05) using enhanced culture techniques.[88] Interestingly only 3 from 55 implants were culture-positive using standard microbiological culture techniques. *S epidermidis* was the main bacteria identified and they were able to visualise bacteria fixed to the silicone implant surface. This was the first group to suggest that chronic subclinical infection from bacteria contained within biofilm may be responsible for capsular contracture.[88]

Similarly, Ahn and her colleagues were able to culture one third of the capsules and implants from 139 consecutive implants that were removed for various indications including capsular contracture in 92% of cases.[437] *P. acnes* (58%) and *S. epidermidis* (41%) were the common bacteria identified, accounting for 99% of culture positive results.[437] Subsequently similar bacteriological findings have been observed in other studies with positive cultures significantly associated with high grade capsular contracture with *S. epidermidis* and *P. acnes* the two most common bacteria identified, with occasional infection by *S. aureus*, *E. coli, Corynebacterium* and *Streptycoccus pneumoniae*.[445-448]

A case reported in 1999 provided the first positive results associating bacterial biofilm with capsular contracture.[436] In this case a woman suffered persistent recurrent capsular contracture after two previous surgical capsulotomies and removal/replacement of implants. At the time of the third capsulotomy the capsules were thick and calcified with a slimy outer coating noted on the left prosthesis. Bacteriological and fresh tissue samples of the breast and capsules were taken. Standard microbiological testing was negative. Enhanced culture testing grew a pure culture of *S. epidermidis* and transmission electron microscopy demonstrated a thick fibrocalcific capsule adjacent to bacteria embedded in an amorphous protein consistent with a biofilm.[436]

Subsequently Deva and colleagues went on to conduct a prospective blind trial of 27 consecutive implants that were removed for various indications, including 19 for capsular contracture (Baker Grade III or IV) over a 22 month period.[89] Routine microbiological testing was negative for all samples. Enhanced culture techniques were positive for 17 of 19 samples with capsular contracture compared with 1 of 8 samples from non-contracted breast implants (p=0.0006). *S. epidermidis* was the most common bacteria identified (14/17 positive samples) as well as *Bacillus* spp, (4/17) *Propionibacterium* spp (2/17, both specimens demonstrated polymicrobial infection with *Bacillus* spp) and other coagulase negative Staphylococcal species (1 each in 4 specimens).[89] Bacteria contained within biofilm or microcolonies was visualised with scanning electron microscopy in 11 of the 19 contracted breast, implant or capsule samples. From this study the presence of coagulase negative *Staphylococcal* species, especially *S. epidermidis*¸ was significantly associated with capsular contracture (p=0.01), but there was no association between implant surface (saline or textured) and capsular contracture (p=0.885). These results support the findings of Virden, and gave the first credible evidence for the role of bacteria, particularly *S. epidermidis*, in the form of a biofilm in the chronic subclinical infection of silicone breast implants as a causative agent for capsular contracture.

Several groups have developed animal models to support some of these findings. In a rabbit study custom made 20 ml, textured silicone implants were filled with saline using standard septic techniques and impregnated with a minocycline/rifampin solution.[449]

This antibiotic combination had previously been shown to be effective *in vitro* against *S. epidermidis*, *S. aureus* and to a lesser degree *E. coli*.[450] These antibiotic infused implants were subsequently incubated with a clinical strain of *S. aureus* prior to insertion into a subcutaneous pocket on the rabbits' back using standard sterile surgical techniques. The implants were retrieved at either 2 or 4 weeks and demonstrated that the antibiotic impregnated implants were significantly less likely to be colonised (2/24 versus 23/24; $p<0.01$) or develop implant abscess (0/24 versus 21/24; $p<0.01$).[449]

The Deva group have developed a pig model to further investigate the role of bacterial biofilm disease of silicone implants and its role in capsular contracture.[435] In this model, modified silicone implants are surgically inserted into a submammary pocket using standard sterile techniques. Up to 8 implants can be inserted per adult female pig and the implants can be subjected to different treatments both within one animal and between different animals. This enables each project to be designed with adequate numbers of implants and minimising the number of animals needed to achieve statistically significant results. It has been demonstrated that the female adult pigs can tolerate the surgical procedure and placement of mammary implants with minimal distress or morbidity.[435]

The initial project inserted 51 modified textured silicone implants into 6 adult female pigs.[435] Thirty-six implants were inoculated with a clinical strain of *S epidermidis* with variable doses ranging from 5×10^1 to 1×10^6 colony-forming units. The remaining 15 implants were non-inoculated controls. The implants were retrieved using sterile surgical techniques at 13 weeks. The clinical strain of *S. epidermidis* developed biofilm at doses above 3×10^5 colony forming units, this resulted in 26 of the 36 (72%) inoculated implants developing biofilm identified using scanning electron microscopy.[435] Clinically significant capsular contracture (Baker Grade III/IV) was identified in 21 of the 26 (80%) of the biofilm positive inoculated implants and in 28 of the 36 (77%) inoculated implants. Surprisingly, 7 of the 15 (47%) non-inoculated implants developed significant capsular contracture and 5 of these7 implants were biofilm positive with cocci bacteria identified using scanning electron microscopy. These cocci were identified as a native porcine *Staphylococcus* spp.[435] Further analysis showed that pocket inoculation was strongly

associated with biofilm formation (p=0.0095), and the presence of biofilm was associated with an increased risk of developing capsular contracture (OR 4.2, 95%CI 1.2-14.5).[435]

This study demonstrated that:

1) the pigs tolerated the *S. epidermidis* inoculation with no adverse systemic or localised symptoms;
2) this porcine model can replicate biofilm formation with an appropriate bacterial stimulus;
3) there is a causal relationship between the presence of bacteria causing a subclinical infection, the formation of periprosthetic biofilm and subsequent capsular contracture;
4) interestingly the non-*S. epidermidis* inoculated implants were inoculated with endogenous porcine bacteria which also resulted in biofilm formation and subsequent capsular contracture.

There is now increasing evidence to support the emergence of the Subclinical Biofilm Infection Theory in the Aetiology of Capsular Contracture (Figure 1-11). Human clinical studies have identified endogenous skin flora, *S. epidermidis* and *P. acnes*, as the two most common bacteria identified from contracted breast implant capsules. Improvements in surgical techniques, including pocket irrigation, have resulted in decreased capsular contracture rates. Animal studies have demonstrated a causal link between presence of biofilm formation and development of capsular contracture and have suggested a role of antibiotic impregnated implants in the prevention of bacterial contamination at the time of surgery.

1.11 Conclusion

Bacteria are ubiquitous in their presence throughout Earth's surface and waterways and have survived the extremes of climate and temperature to make them one of the most successful organisms on our planet. They are essential to life with most, if not all, higher organisms including humans co-existing and often benefiting from the bacteria that colonise their skin and mucosal surfaces. Bacteria also present some of the most

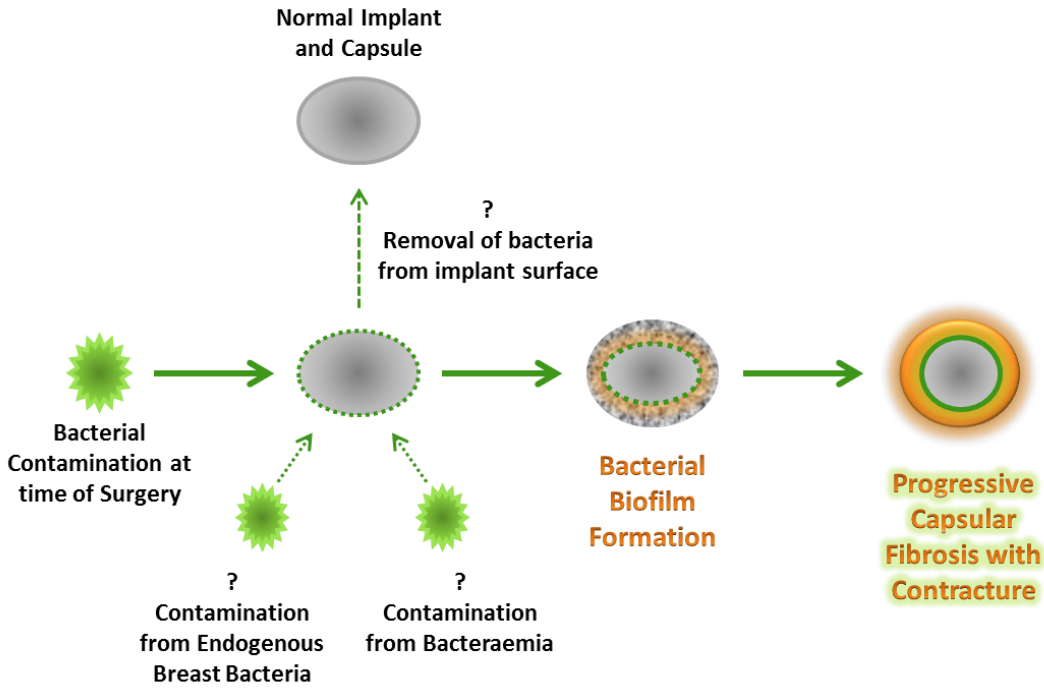


Figure 1-11: Subclinical Biofilm Infection Theory of Breast Implant Infection and Capsular Contracture[89]

Bacteria contaminate and attach to the silicone implant via direct contact at time of insertion due to breakdown in aseptic technique or possibly due to:

1) contamination from endogenous breast flora, or
2) haematogenous spread from an infective source or bacteriaemic episode.

Once attached the bacteria undergo genotypic and phenotypic changes to a sessile physiology and biofilm formation is established. Unless the bacteria can be removed by, the host's immune system or other, as yet, poorly defined interventions.

Biofilm formation results in progressive capsular fibrosis and chronic low-grade subclinical inflammation. This results in progressive stiffening of the capsule surrounding the malleable implant resulting in implant deformity and clinical capsular contracture.

significant threats to human health with bacterial infections and sepsis common causes of morbidity and mortality in the hospital system.

Yet it has only been in recent history, since van Leewenhoek, Pasteur and Koch, amongst others that scientists have identified and begun to understand the natural biology of bacteria in a planktonic form. The modern era of Microbiology and Infectious Diseases has been the mainstay of our knowledge and treatment of these diseases over the past 150 years. More recently, however, science has acknowledged that bacteria preferentially exist in communal structures or biofilms. Bacterial biofilms represent the form/structure in which bacteria preferentially exist and account for over 90% of the world's bacterial biota.

There is now acknowledgement that bacterial biofilms play an important role in human disease including many chronic diseases and diseases of implantable medical devices. Unlike acute septic infections, biofilm infections result in a chronic subclinical inflammatory process that affects surrounding tissue or bone. Most if not all temporary and permanent implantable medical devices are susceptible to bacterial attachment and biofilm formation. Biofilm formation on implantable medical devices is now increasingly identified as a significant disease process and a common reason for device failure. This has important therapeutic implications for both the patients affected, with implant failure, prolonged and complicated treatment requirements and increased morbidity and mortality; and the health system with increasing cost burdens associated with the treatment of biofilm implant disease.

Capsular contracture remains the most common complication of silicone breast implants requiring further surgical intervention. There is now increasing evidence that subclinical biofilm infection may be an important aetiological factor in the formation of capsular contracture. The Subclinical Biofilm Infection Theory hypothesises that implant contamination occurs at the time of implantation from contact between skin and implant either directly or indirectly via surgeon or instruments (Figure 1-11). Bacterial contamination of breast implants may also occur post-implantation from local breast flora or haematogenous spread, although there is limited evidence to support this to date.

There is increasing investigation to develop therapeutic strategies and novel surfaces to prevent and treat biofilm disease on surgical Implantable medical devices. These strategies will need to provide antibacterial protection at the time on surgical insertion and potentially for the life of the implant to protect against endogenous +/- haematogenous spread.

1.11.1 Thesis Outline

In this thesis I have used the porcine model of biofilm infection of silicone implants to further investigate the as yet unproven components of the Subclinical Biofilm Infection Theory of Breast Implant Infection and Capsular Contracture (Figure 1-11):

1) The Role of Endogenous Contamination and/or Haematogenous Spread in the Aetiology of Biofilm Infection (Chapter 5): was investigated using silicone breast implants surgically inserted into three pigs and left *in situ* for up to 36 weeks.
   At time of surgical removal the implants were assessed for clinical evidence of capsular contracture (Baker Grade, 2.2). The implant capsules were assessed using microscopic techniques for presence or absence of biofilm within the capsule (Scanning Electron Microscopy, 2.4.1).
   The implant capsules were further assessed using molecular and polymerase chain reaction techniques to identify the bacteria involved with the biofilm infection and calculate the bacteria load associated with the biofilm infection (Molecular Bacterial Identification, 2.5.3.1 and Molecular Bacterial Count, 2.5.3.2).
2) The Role of Implant Surface in the Prevention of Bacterial Biofilm Formation (Chapter 6): In this study we pooled all the porcine data on the pigs that have been operated on to date and have analysed the clinical and laboratory findings, including Baker Grade (2.2) and Scanning Electron Microscopy (2.4.1), and compared these results between the two types of implant surfaces (smooth and textured).
3) The Prevention of Bacterial Biofilm Formation using a Novel Antibacterial Implant Surface in the (Chapter 7): by using an antibiotic-impregnated implant surface mesh surrounding breast implants surgically inserted into five pigs. The antibiotic mesh was randomly inserted around only half the implants. All the implants inserted were

inoculated with a clinical strain of biofilm forming *S. epidermidis*, as used in the previous pre-clinical pig study.[435] The implants were left *in situ* for 16 weeks.
At time of surgical removal the implants' capsules were assessed for clinical evidence of capsular contracture using both Baker Grade (2.2) and Applantation Tonometry (2.3.1)
The implant capsules were assessed using microscopic techniques for presence or absence of biofilm within the capsule using Scanning Electron Microscopy (2.4.1).

During the research two interesting findings occurred that enabled further investigation of potential complications of bacterial biofilm infection of silicone breast implants.

4) The Finding a Two Double Capsules (Chapter 4). Two fellow researchers and I removed a double capsule from one of the pigs participating in the antibiotic mesh study. At the same time a Plastic Surgeon, attached to our department, removed a double capsule from a woman undergoing elective removal of her breast implants. Double capsule, or a capsule within a capsule, is a rare and often unexpected finding noted at time of removal of a breast implant. The underlying aetiology is unknown. I examined the porcine double capsule with Scanning Electron Microscopy (2.4.1).

5) Examination of the Implant Surface with Microscopy (Chapter 8). Towards the end of my research I began to examine the surfaces of the implants with Scanning Electron Microscopy (2.4.1). Initial assessment of a couple of smooth-surfaced implants showed that they had little if anything attached to their surfaces. Initial assessment of a textured-surfaced implant showed the presence of small amounts of capsule attached to its surface. Unexpectedly, these capsule remnants had large numbers of cells that resembled activated lymphocytes. I used Scanning Electron Microscopy (2.4.1) and developed an Immunofluorescent staining (2.4.3) to identify T-cell and B-cell lymphocytes to further assess the capsular tissue attached to the silicone implants.

Finally, early in my research I, and two research colleagues, had the opportunity of taking samples of clinical surfaces from a functioning intensive care unit prior to its renovation.

Samples were taken of surfaces that came into direct contact with patients or their care-givers (including doctors, nurses, etc) and included surfaces such as entry/exit doors, bedding surfaces and bedside containers and equipment.

6) Investigation of Intensive Care Unit Surfaces for Dry Biofilm (Chapter3). Clinical surfaces were destructively collected, identified and stored in closed containers to prevent cross-contamination.

   The presence or absence of biofilm was detected visually using Scanning Electron Microscopy (2.4.1). The viability of the bacteria within the biofilm was determined using Live-Dead®Backlight™ Fluorescent Confocal Microscopy (2.4.2)

   The identification of clinically relevant-multi drug resistant bacteria was achieved using Enhanced Bacterial Culture (2.3.2.3) and Diagnostic Chromogenic Selective Media plates for several clinically relevant antibiotic-resistant bacteria (2.3.2.4)

1.12 Review Article:

A review of bacterial biofilms and their role in device associated infection

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Contribution

Dr Anita Jacombs: Secondary Author.

I performed all the scanning electron microscopy and confocal laser-scanning microscopy images for the paper, except figure 3.

I assisted with editing the manuscript.

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Review

A review of bacterial biofilms and their role in device-associated infection

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Abstract. ***Background*:** Most of the world's bacteria live in biofilms, three-dimensional clusters attached to surfaces. Many hospital-acquired infections are associated with biofilm infections of implantable medical devices such as orthopaedic prostheses and intravascular catheters. Within biofilms, bacteria are significantly less susceptible to antibiotics and host defences, making biofilm infections difficult to diagnose and treat, and often necessitating removal of the infected implant.

***Method*:** In this review article we describe the process of biofilm formation, quorum sensing, and biofilm infection of the healthcare environment, surgical instruments and implantable medical devices.

***Conclusion*:** The inability to treat biofilm-infected devices means that therapies targeting biofilm-specific processes and targeting prevention of biofilm formation are required.

Additional keywords: biofilms, biomaterial-related infections, environmental contamination, implant-related infections, infection control, staphylococci, surgical infection.

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Introduction

Bacterial biofilms have a major impact on society, not only contributing to hospital-acquired infections (HAIs), but colonising our environment causing among other things, corrosion, fouling of water pipes, and food and pharmaceutical spoilage.[1] Bacteria can attach to and infect all medical devices and up to 60% of HAIs are associated with biofilm infections of implantable medical devices such as orthopaedic prostheses and intravascular catheters.[2]

Over 99% of the world's bacteria are thought to live in biofilms[3] so it must be assumed that they gain an advantage living in this state. Biofilms consist of three-dimensional aggregations of sessile microorganisms surrounded by hydrated, extracellular polymeric substances (EPS). The EPS is secreted by the microorganisms following surface attachment. It consists principally of polysaccharides, nucleic acids, proteins and lipids and comprises between 50 and 90% of the mass of the biofilm.[4] Bacteria within biofilms are significantly less susceptible to antibiotics and host defences than the planktonic (free-swimming) forms of the same organisms[5,6] making them difficult to treat, often necessitating the removal of the infected implant.[7,8] In this review we describe the process of biofilm formation, quorum sensing, and how biofilm infection impacts on healthcare with particular reference to implantable medical devices.

Formation of biofilms

Immediately upon insertion of a medical device into the patient, macromolecules such as fibrinogen and immunoglobulins are deposited on the implant's surface.[2] This is called the conditioning film and generally makes it easier for bacteria to attach to the implant. Several proteins produced by Staphylococcaceae produce proteins that bind specifically to host factors in the conditioning film.[9]

Bacteria can randomly come in contact with implant surfaces by sedimentation and Brownian motion while motile microbial cells may actively seek the implant surface. Initial bacterial adhesion occurs due to van der Waals forces and

Implications

- Bacteria live attached to surfaces in structured communities called biofilms.
- Biofilms colonising surfaces have increased resistance to removal by detergents and disinfectants.
- Biofilms colonising implantable medical devices are resistant to antibiotic treatment and the host immune response.
- It is imperative that methods to prevent biofilm colonisation are developed.

surface structures such as fibrils or polymers create a link between the substrate and individual bacteria. Once attached, the bacteria produce diffusible signalling molecules in a process called quorum sensing which, among other things, induces the bacteria to secrete EPS. It is the EPS that cement the bacteria to each other and the surface and provides mechanical stability for the three-dimensional development of the biofilm. The biofilm structure itself is dynamic with redistribution of reversibly-attached cells, recruitment of cells from the surrounding fluid, replication of attached cells and dispersal of cells back into the surrounding areas.[1,2]

Quorum sensing

Quorum sensing (QS) allows communication between bacteria, synchronising alteration in genetic expression of the whole bacterial population, thus coordinating activities such as biofilm formation and the production of virulence factors.[10,11] Each bacterial species produces its own QS chemical signal.

Gram-negative bacteria use N-acylhomoserine lactones (AHL) as QS molecules. These consist of a conserved homoserine lactone ring attached to an acyl side chain. Species specificity is provided by various chemical modifications including the length of the acyl side chain (4 to 18 carbons). Generally, AHLs are synthetised by homologues of *Vibrio fischeri* Lux1 protein.[10] Short-chain AHLs diffuse passively, in and out of the bacterial cell, along a concentration gradient, whilst long-chain AHLs may be actively transported. Each bacterial cell makes small amounts of AHLs, but with increasing numbers of bacteria the concentration of AHL increases in the local environment. Once a critical concentration is reached, AHL binds to a cytoplasmic transcription factor (homologous to LuxR proteins of *V. fischeri*), which then bind directly to bacterial DNA and regulate genetic transcription.[10]

Gram-positive organisms use small peptides of 5 to 17 amino acids in length as QS molecules. Species specificity is determined by modification of the peptide's side chains, for example QS molecules of Staphylococcaceae spp. contain a thiolactone ring.[12] The peptides are synthetised in the cytoplasm, modified and then excreted. Gram-positive organisms have two component QS detection systems, including sensor and regulator proteins.[13] High concentrations of QS peptides are detected by the membrane-bound sensor protein, which activates the cytoplasmic regulator protein which in turn regulates genetic transcription.

In addition to intra-species communication, bacteria can communicate with other species of bacteria. Auto-inducer 2 has been found in over 50 different species of bacteria may be the QS signal for universal communication.[10]

Antibiotic resistance

Biofilm bacteria can survive up to 1500, typically 100 to 250 times, the amount of an antibiotic needed to kill the same bacteria growing in liquid culture. This increased antibiotic resistance is due to the biofilm lifestyle, as when biofilm bacteria are dispersed they once again become susceptible to antibiotics.[14] The mechanism of increased resistance of biofilm bacteria is multifactoral and may be different for different bacteria;[5,6] also resistance increases as the biofilm ages.[14]

The diffusion of both nutrients and oxygen has been shown to be limited in the deeper layers of the biofilm, which affects bacterial phenotypic expression, resulting in bacteria growing very slowly or not at all. One consequence of this decreased metabolism is antibiotic resistance e.g. an ampicillin-resistant biofilm phenotype in cells genotypically sensitive to ampicillin.[15] These metabolically-inactive cells are also very difficult to culture and thus make diagnosis of implant infection very difficult.[5] Other mechanisms of antibiotic resistance include binding and inactivation by the EPS – as occurs with some antibiotics such as tobramycin[16] – and induction of a distinct biofilm phenotype or 'dormant resistant cell.'[6]

Biofilms in the healthcare setting

Healthcare environment

Environmental biofilms are generally found in wet areas such as water pipes.[17] Indeed in healthcare settings, due to organisms being protected in biofilms, HAI outbreaks have centred around drains[18] and showers. For example, a hand hygiene sink drain infected with a biofilm composed of multidrug-resistant *Pseudomonas aeruginosa*, resulted in bacterial contamination of adjacent medication and the sterile dressing preparation area, due to splashing when the faucet was turned on. Disinfection of the sink failed to remove the biofilm and the outbreak was only terminated when the sinks were renovated to prevent splashing.[18]

Contamination of the inanimate environment around patients in the hospital setting constitutes an important reservoir of bacteria[19] and the risk of obtaining a HAI is increased on average by 74% if the previous patient occupying that room had a multi-antibiotic resistant organism (MRO), as reviewed by Carling.[20] We propose that bacteria exist on these dry surfaces as biofilms and are protected from desiccation and moreover have increased resistance to removal by detergents[21] and increased resistance to disinfectants.[6] A more

detailed discussion on biofilms resistance to various disinfectants can be found in a review by Bridier.[22] The presence of MROs in biofilms may help explain why MROs continue to be isolated from the environment despite enhanced cleaning protocols.[20] We have demonstrated biofilms containing MROs on dry surfaces, such as supply boxes, and furnishings, such as curtains, obtained from an ICU.[23] Although we were unable to culture organisms from all the surfaces, using confocal laser-scanning microscopy (CSLM) we were able to show live bacteria on all surfaces (see Fig. 1). Bacterial biofilm contamination of surfaces in clinical workspaces is likely ubiquitous, and serves as a potential source of infection.

Surgical instruments

Despite pre-cleaning and disinfection protocols, decontamination of heat-sensitive instruments, such as endoscopes, often fails due to the highly-resistant nature of biofilm to removal by cleaning products.[21] Failure to remove patient soil and infecting biofilm compromises disinfection and promotes bacterial survival. In large studies ~1.8% of patient-ready endoscopes remained infected with the previous patient's contaminating bacteria.[24]

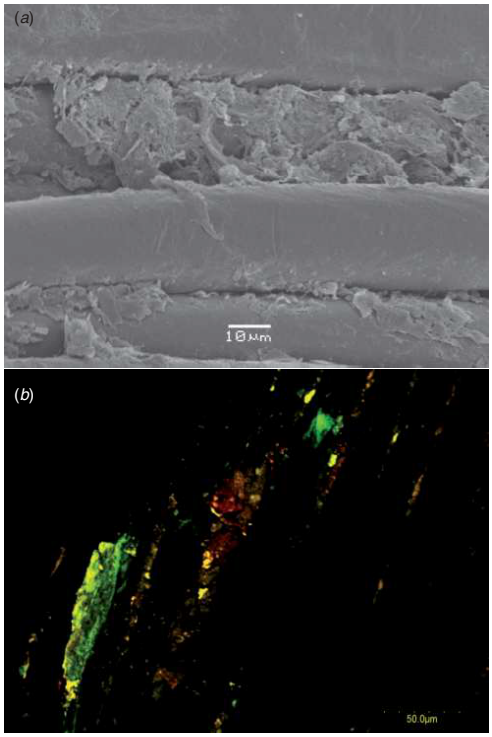

Fig. 1. Biofilm colonisation on dry surfaces in an ICU. (*a*) Scanning electron micrograph of biofilm colonising a curtain showing large amounts of EPS. (*b*) Confocal laser-scanning micrograph (CLSM) of curtain colonised by biofilm showing live bacteria (green) and dead bacteria (red).

Implantable biomedical devices

Worldwide production of biomedical devices and engineered medical tissue is rapidly increasing.[2] In parallel, the cost of treating biofilm-infected devices continues to rise and varies from a few hundred dollars for infected catheters[25] to $50 000 for infected cardiac devices and neurosurgical ventricular shunts.[26] Infection of these devices is also associated with increased mortality. The highest mortality occurs with cardiovascular medical devices ranging from 5% for vascular grafts to 25% in cardiac-assist devices. A recent French study has found that 6% of patients with infected orthopaedic implants require admission to the ICU and infection results in increased mortality with a 4.6% case mortality rate.[27] Similarly, a multi-centre Australian study found that 5.5% of patients with infected prosthetic joints died as a direct result of their infection.[28]

Any implanted medical device may become infected with a bacterial biofilm.[2] The organisms most commonly isolated from biofilm-infected biomedical devices are *Staphylococcus aureus* and coagulase-negative staphylococci[29,30] followed by Streptococcaceae and Gram-negative bacilli[30] but any organism, including fungi such as *Candida*[26] can be involved. Clinical consequences range from surrounding fibrosis, to bone resorption, to persistent life-threatening sepsis. Two examples of impaired clinical outcomes from common implant procedures are given below.

Breast prostheses and capsular contracture

Capsular contracture is the progressive thickening and then shrinkage of the normally thin fibrous capsule surrounding the breast prosthesis which results in distortion of the prosthesis. On average capsular contracture occurs in 2% of patients[31] but the prevalence can be up to 30%.[32] Contamination of the prosthesis, with low numbers of bacteria, occurs during surgery and results in development of a biofilm and ongoing inflammatory response resulting in a very thick fibrous capsule covered in EPS (see Fig. 2). Biofilm confirmed visually by scanning electron microscopy (SEM) was significantly associated with contracture ($P = 0.0006$) in a prospective randomised study.[29] Bacteria were isolated from 17 of the 19 contracted breasts, and 14 of these were coagulase-negative *Staphylococci* sp.

We confirmed that contamination of the prosthesis at surgery can lead to biofilm formation and subsequent contracture by implanting miniature breast prostheses contaminated with a human strain of *S. epidermidis* into pigs. Biofilm developed on 72% of contaminated implants and it was significantly associated with development of contracture ($P < 0.05$).[33]

Conversely, reducing the degree of bacterial contamination on an implant surface decreases the amount of biofilm

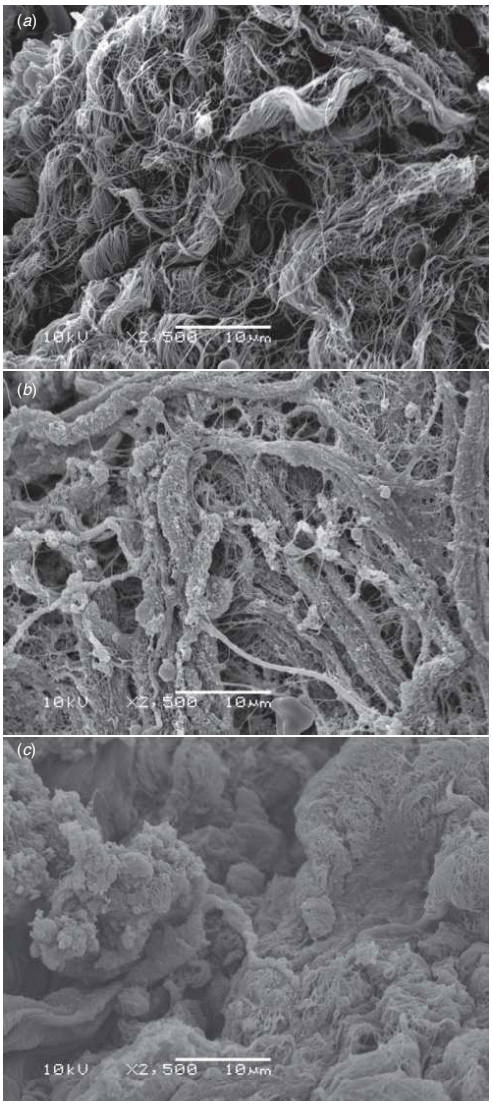

Fig. 2. Scanning electron micrograph of human breast capsule surrounding mammary prostheses. (*a*) Normal, uncontracted breast demonstrating easy-to-see collagen fibres. (*b*) Contracted breasts showing biofilm infection of capsule. Individual collagen fibres are covered in EPS in which coccoid bacteria are embedded. (*c*) A separate contracted breast showing extensive EPS deposition and evidence of coccoid-shaped bacteria.

formation and subsequent contracture. In a prospective study, we contaminated 28 implants with *S. epidermidis*. Half of the prostheses were treated with an antibiotic-impregnated mesh. At 16 weeks post-surgery all the control breasts were contracted whilst no contracture was identified around breasts with treated implants ($P < 0.001$).[34]

Joint replacement prostheses

Deep infection rates for total hip and knee arthroplasties are usually quoted as being ~1–2% over a 2-year post-operative period.[35] The Australian National Joint Replacement Registry reports that primary total hip replacements (THRs) and total knee replacements (TKRs) will require revision, for any cause, in 6.2% and 5.7% of cases, respectively, at 10 years post-implantation. Infection is listed as the third leading cause of such failures for THRs (following loosening and/or lysis and dislocation), being identified as the cause of failure in 16.8% of cases. For TKRs infection is the second leading cause at 22.2%, following loosening and/or lysis.[36]

However, interpretation of infection rates in joint arthroplasty is a complicated diagnostic issue as it has been shown that many revision cases attributed to 'aseptic loosening' are likely in fact infected with low-grade biofilm infections.[37] Bone lysis and subsequent loosening of the implant may be consequences of infection; however, genuinely aseptic loosening (most often due to immune response against polyethylene debris from the artificial joint surface as it wears) presents clinically very similarly to chronic biofilm infection.[38] Whilst an acute post-operative infection is usually obvious, identifying whether an implant has a chronic biofilm infection presents a diagnostic challenge using currently recommended diagnostic approaches.[39]

Diagnosis and treatment implications of biofilm infection

As described above, diagnosing infected implantable biomedical devices can be problematic. Laboratory techniques to establish a diagnosis of infection are culture-based, and may not detect bacteria in biofilm as biofilm bacteria are difficult to culture.[5] However, physical disruption and sonication of the sample can improve isolation rates.[29] Due to the difficulty in culturing biofilm bacteria from clinical samples, it is likely that molecular methods will be increasing used to guide both diagnosis and treatment in the future.[40,41] The concern is that molecular methods are prone to sample contamination.[42–44] SEM (Fig. 3) has been used to visually confirm biofilm infection and combining multiple methods of detection may be the best strategy for diagnosis.[29] Unfortunately, SEM is not readily available commercially.

Similarly, the biofilm mode of growth which confers antibiotic resistance, confounds treatment of biofilm-infected devices and makes these infections difficult to resolve by non-operative means.[5,6] Antibiotic treatment may modulate acute exacerbations of disease but once antibiotics are withdrawn the biofilm is rapidly repopulated from cells that persisted during the treatment,[45] resulting in recurrent infections and chronic low-grade inflammation. In clinical trials of targeted antibiotic treatment of patients with documented biofilm disease, antimicrobial therapy failed in all cases if it was

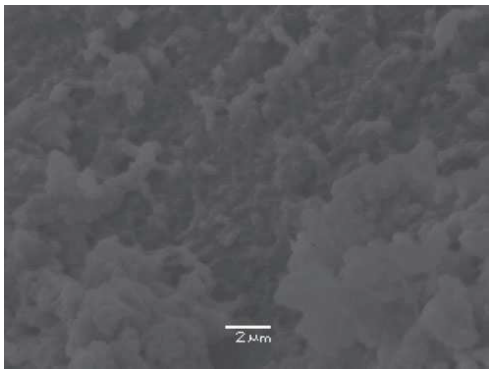

Fig. 3. Scanning electron micrograph of biofilm infecting bone surrounding an infected orthopaedic implant. Coccoid-shaped bacteria are encased in EPS.

instituted before device removal, whilst implant removal coupled with antibiotic therapy was effective in all cases.[46]

Conclusion

Infection of implantable medical devices can have catastrophic complications and the consequences of implant removal are very large in terms of morbidity, mortality, and financial burden.[47,48] Thus preventing contamination of devices is the ultimate goal, however the ubiquitous nature of common infecting organisms throughout the healthcare environment makes this challenging. Nevertheless, enhanced cleaning strategies, breaking the chain of transmission by such methods as the 'Five Moments of Hand Hygiene'[49] and use of disposable equipment should be pursued. At present, careful attention to surgical technique and the use of perioperative antibiotics are the mainstay approaches, but there is considerable active research into the use of enhanced anti-biofilm surface coatings, surgical-site disinfectants, and other specific anti-biofilm therapies.[50]

Conflicts of interest

No conflicts of interest exist for any of the authors.

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Chapter 2

"Nature composes some of her loveliest poems for the microscope and the telescope."

- Theodore Roszak, 1972

2 Materials and Methods

2.1 Animal Surgery

All animals were adult female, non-lactating, domestic Large White pigs (*Sus domesticus*) weighing between 350 and 400kg. They were all housed as a group in designated animal care facilities at the University of Sydney Farms in Cobbitty, New South Wales, Australia. All anaesthetics were performed and monitored by appropriately trained specialist large animal veterinary anaesthetists at the University of Sydney's Veterinary Hospital on the Farm at Cobbitty.

2.1.1 Insertion of Mammary Implants

Anaesthetic Induction

Medications per adult pig (300-400kg)

1. Ketamine 500 mg (100mg/mL Ilium, Troy Laboratories, Smithfield, NSW, Australia)
2. Xylazine 500 mg (100mg/mL Ilium, Troy Laboratories, Smithfield, NSW, Australia)
3. Tiletamine 500mg + Zolezepam 500mg, (combination drug called Zoletil 100mg/ml Virbac Animal Health, Milperra, Australia)

All agents were given as an intramuscular injection in an animal holding bay. Once injected and the pig was sedated the animal was moved by utility vehicle to the animal surgery room and placed on to the operating table. The animal was then intubated with an appropriately sized endotracheal tube and connected to routine anaesthetic monitoring. Anaesthesia was maintained with an isoflurane USP (Baxter Healthcare Pty Ltd, Old Toongabbie, NSW, Australia)/oxygen mixture.

In the surgical area the animal was placed in a supine position and secured (Figure 2-1). Her trunk with her mammary glands were prepared by clipping all hairs and sterilising the skin with 70% ethanol (Chem-Supply Pty Ltd, Gillman, South Australia) and allowed to dry. The skin was then prepared for surgery with standard liquid 10% povidione-iodine solution (Orion, Welshpool, Western Australia, Australia).

The animal was then draped using sterile cloth or adhesive surgical drapes, keeping the prepared teats exposed. The surgical field was then sealed with a sterile Iodan™ antimicrobial incise drape (3M™ Health Care, St Paul, Minnesota). Iodan™ is a surgical anti-microbial film containing iodophor-impregnated adhesive that releases slowly providing continuous antimicrobial activity throughout a surgical procedure.[(451)]

Trained surgeons performed routine surgical scrub protocol and donned surgical gown and sterile gloves. After identifying the glands to be used for augmentation an inframammary skin incision was made. A submammary pocket was fashioned using blunt dissection and haemostasis was performed with diathermy. Sterile gloves were changed for implant insertion and the modified silicone breast implants were inserted with a no-touch technique to minimise contamination between skin and the implant at time of surgery. The pocket was then treated with either 1 mL sterile1 x phosphate buffered saline (control implants, Medicago AB, Uppsala, Sweden) or 1 ml of pure growth of *S. epidermidis #7* inoculum containing between 10^5 and 10^6 colony forming units (inoculated implants).

Each pocket was closed in two layers with a deep interrupted suture layer and a continuous subcuticular layer with absorbable 4-0 undyed Monocryl (Ethicon INC, Somerville New Jersey). Sterile gloves and instruments were changed between each implant to minimise any cross contamination between augmented teats until all implants were inserted. At the conclusion of surgery the wounds were sprayed with OpSite spray dressing (Smith & Nephew, Hull, England).

The animals were removed to their holding pen and allowed to awake under the care of the animal husbandry staff at the University of Sydney Farms in Cobbitty. The animals

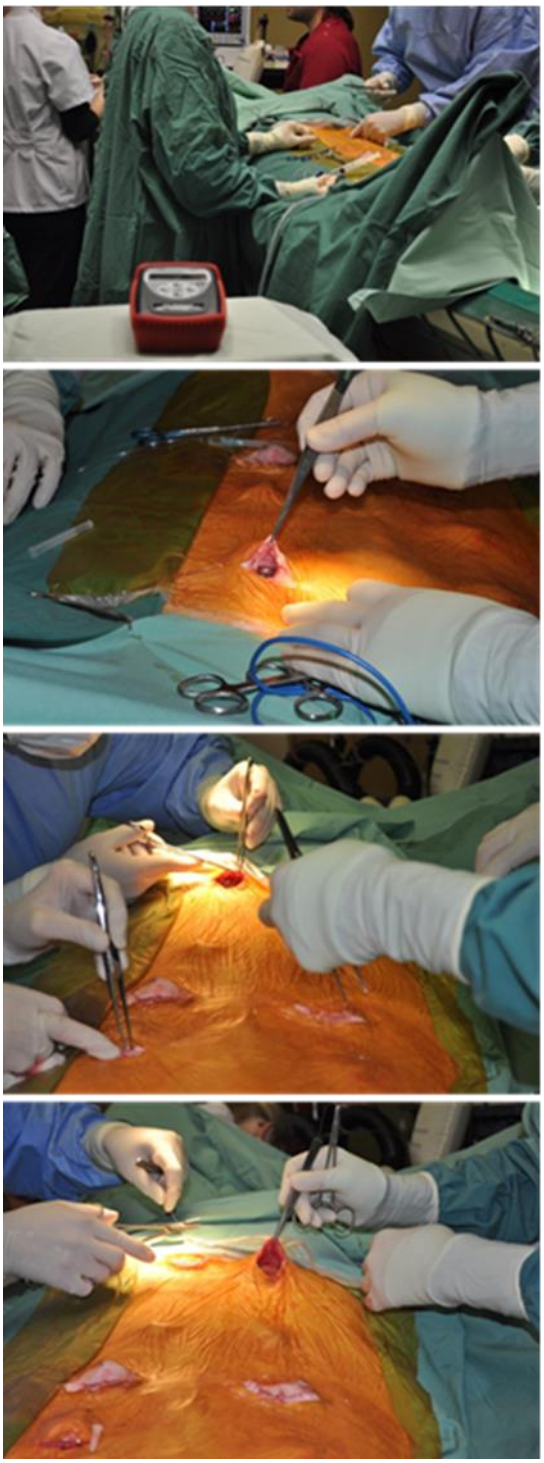


Figure 2-1: Photos of Pig Surgical Technique – Insertion of Implant

Photos of insertion pig augment mammoplasty with modified implants.

1) Standard surgical preparation and setup.

2) Pocket formation with blunt dissection,

3) and 4) Closing in layers.

were returned to their normal group housing and activities when deemed safe. The animals were checked daily by the animal husbandry staff to monitor for any signs of localised or systemic infection.

2.1.2 Retrieval of implants

After the implants had been left *in situ* for the required period of time, the animals were sedated, transferred to the operating room and positioned as detailed above. The animal was euthanized with 60-80 mL pentobarbitone (325mg/mL Virbac Animal Health, Milperra, Australia) and Baker Grading was performed by two surgeons independently. The surgeons were blinded to the original treatment status of the implant.

Following Baker grading the trunk skin was prepared and draped for surgical explantation as detailed above (Figure 2-2). The surgeons performed routine preoperative surgical scrub, sterile gown and gloving as described above. Sterile gloves and instruments were changed between implants to prevent cross-contamination between specimens.

The skin was excised and the implant encased in the capsule was dissected *en bloc* with minimal extraneous tissue and also without entering the capsule or rupturing the implant. Once removed the capsule and implant were individually placed in a sterile transport container containing 1 x phosphate buffered saline (Medicago, Uppsala, Sweden) and stored at 4^{O}C until returned to the laboratory usually within 2 hours but up to 5 hours of explantation.

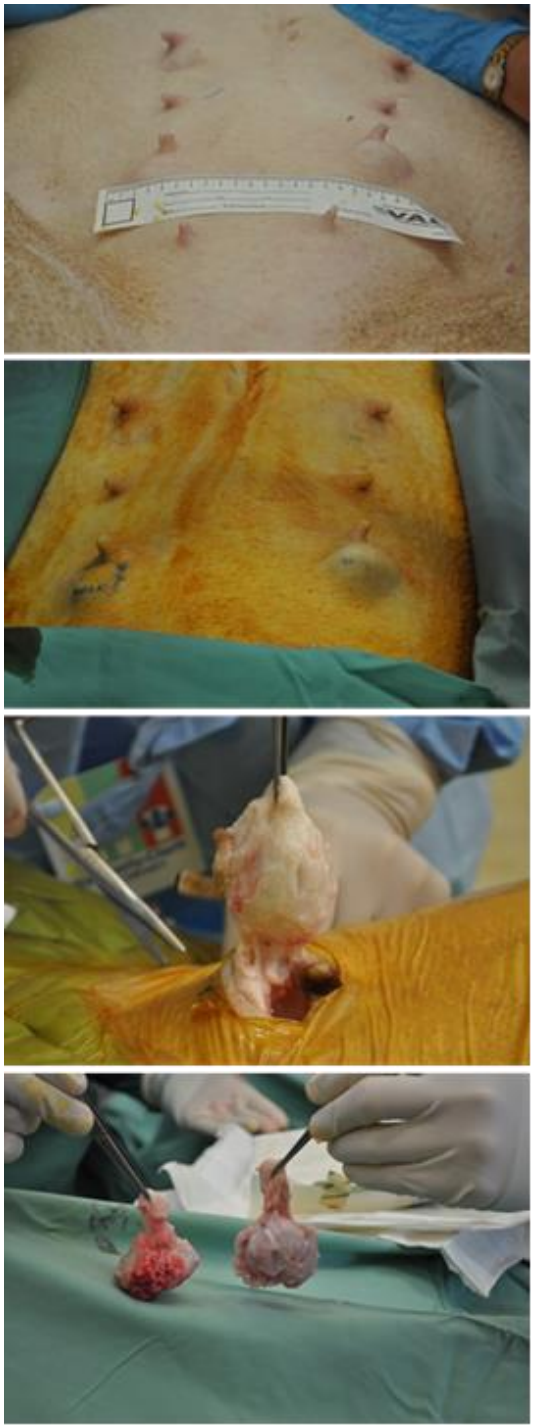


Figure 2-2: Photos of Pig Surgical Technique – Removal of Implant

Photos of removal of implants.

1) Comparison of augmented teats, showing one breast with obvious deformity. This is clinically assessed at time of removal using Baker Grade (2.2),

2) Standard surgical preparation and draping,

3) and 4) Removal of capsule containing an implant.

2.2 Baker Grading

Clinically, capsular contracture was routinely measured by palpation of the augmented breast to feel for the degree of "firmness" of the implant capsule and to determine if the implant could be identified separate to the remaining breast tissue. This process of assessment and a grading system to denote the severity of capsular contracture was first described and graded by Dr James Baker, an American plastic surgeon, in 1978.[452] This was modified in the 1990's but The Baker Grade (Table 2-1) is still the standard assessment tool for capsular contracture in clinical practice today.[452] Baker Grades III and IV are considered significant capsular contracture and, as well as implant firmness, other symptoms include deformity and pain.[426, 453] Baker Grade III and IV contracture commonly requires further surgical intervention.[453]

Table 2-1: Baker Classification of Capsular Contracture after Augmentation Mammaplasty

Descriptive analysis of physical findings of breast examination after augmentation mammoplasty with breast implants to assess the degree of capsule fibrosis and deformation

Class (now commonly referred to as Baker Grade)	Description
I	Breast absolutely natural; no one could tell breast was augmented
II	Mildly firm reconstructed breast with an implant that may be visible and detectable by physical examination
III	Moderately firm reconstructed breast. The implant was readily detectable.
IV	Severe capsular contracture with an unacceptable aesthetic outcome and/or significant patient symptoms requiring surgical intervention

2.3 Laboratory Procedures

In the laboratory, samples were kept on ice until ready for aseptic processing using sterilised equipment and in a sterilised Class II Biosafety cabinet.

2.3.1 Applantation Tonometry

Applantation tonometry is a simple protocol to measure the distensability of the intact implant and capsule as a surrogate marker of the degree of fibrosis or "stiffness" of the capsule. Each intact implant-capsule was placed in a sterile petri-dish and a sterilised aluminium foil placed over the top. A petroleum jelly (Vaseline®, Unilever) covered petri dish containing 200g standard weight was balanced on top of the implant-capsule to distend it. As the implant deforms the contact between the foil covering the implant and the Vaseline covered petri dish increases and the Vaseline transfers to the foil sheet. The greater the deformation the bigger the area that should be covered by the petroleum jelly (Vaseline®, Unilever, Epping Australia). The perimeter of the petroleum jelly (Vaseline®, Unilever, Epping Australia) on the foil was marked. This was repeated three times for each intact implant-capsule. The outline was then transferred to a paper hardcopy and scanned 1:1 as a JPEG file for further analysis.

The surface area of the distended implant-capsule was measured using ImageJ 1.46r software (Wayne Rasband, National Institute of Health, USA Java 1.6.0_20 (64-bit) programme; http://imagej.nih.gov/ij). The mean surface area (cm^2) was calculated for each of the intact implant-capsules from the three individual results for further statistical analysis.

2.3.2 Microbiology

2.3.2.1 Counting of Bacteria

Bacterial count of liquid bacteria cultures was measured in colony forming units per millilitre (CFU/mL). One mL of pure bacterial culture was added to nine mL of sterile phosphate buffered saline (10^{-1} dilution) and mixed for ten seconds. This process was repeated for the required number of dilutions.

A Columbia horse blood agar (HBA) nutrient plate (Oxoid, Threbarton, South Australia) was inoculated with one hundred microliters (µL) of each dilution. The inoculated plates were incubated aerobically overnight at 37^{O}C. The colony forming units of each plate were counted and recorded for colony counts between 30 and 300. The bacterial count was calculated by multiplying the number of colonies by the dilution factor.

2.3.2.2 Standard Curve

Staphylococcus epidermidis #7 (API Staph no. 6706113; API SystemS.A., La Balme-les-Grottes, France) is a clinical isolate previously recovered from a patient with recurrent capsular contracture.[435] A single colony forming unit from a pure *S. epidermidis #7* culture was taken from a haemoglobin blood agar plate and inoculated into 100 mL of sterile 100% tryptone soya broth (Oxoid, Thebarton, South Australia). The culture was grown at 35^{O}C and rotated at 2 g. At regular intervals readings were taken using a Beckman DU 640 spectrophotometer (Beckman, Fullerton, USA) on visible light at 600 nm and reading was recorded using a sterile non-inoculated tryptone soya broth (Oxoid, Thebarton, SA, Australia) blank (Table 2-2). For each time period 1 mL was aliquoted in to 9 mL sterile 1 x phosphate buffered saline (Medicago, Uppsala, Sweden) and shaken. Serial 10-fold dilutions of 1 mL into 9 mL 1 x phosphate buffered saline (Medicago, Uppsala, Sweden) were made to required dilutions. An aliquot of 100µL from each serial dilution was plated on a warmed haemoglobin blood agar (Oxoid Thebarton, SA, Australia) and incubated aerobically at 37^{O}C overnight. The number of colony forming units (CFU) was determined as per 2.3.2.1 (Table 2-2).

Standard curve of *S. epidermidis #7* was constructed by plotting the bacterial colony count against its respective optical density (Figure 2-3). For subsequent experiments, such as preparing inoculation dose for in vivo pig surgery, bacterial concentration of *S. epidermidis #7* culture grown in nutrient broth was determined by measuring absorbance and referring back to this standard curve. Colony forming unit numbers were confirmed by direct bacterial counts

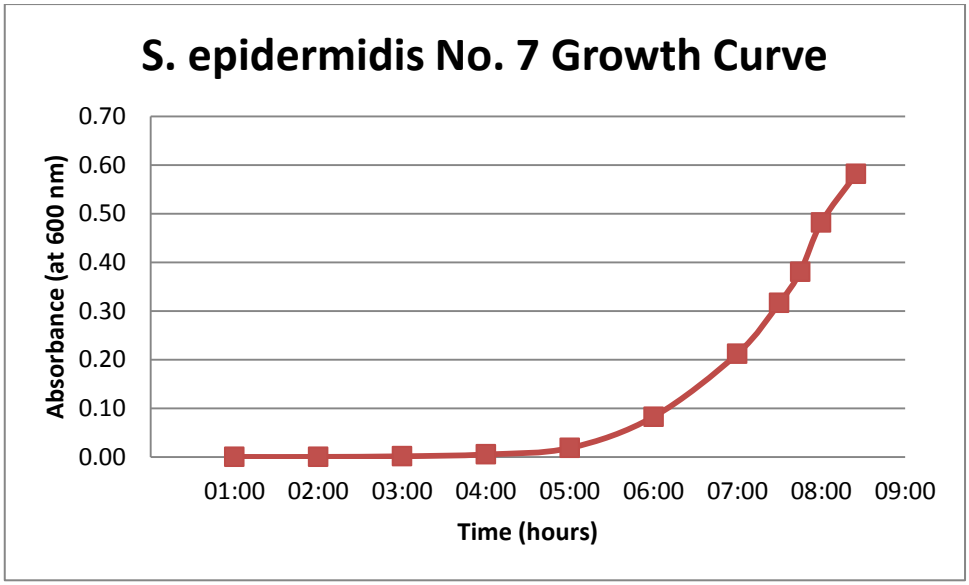


Figure 2-3(a) *S. epidermidis* No. 7 Growth Curve

Bacterial culture optical density measured at regular time intervals after inoculation.

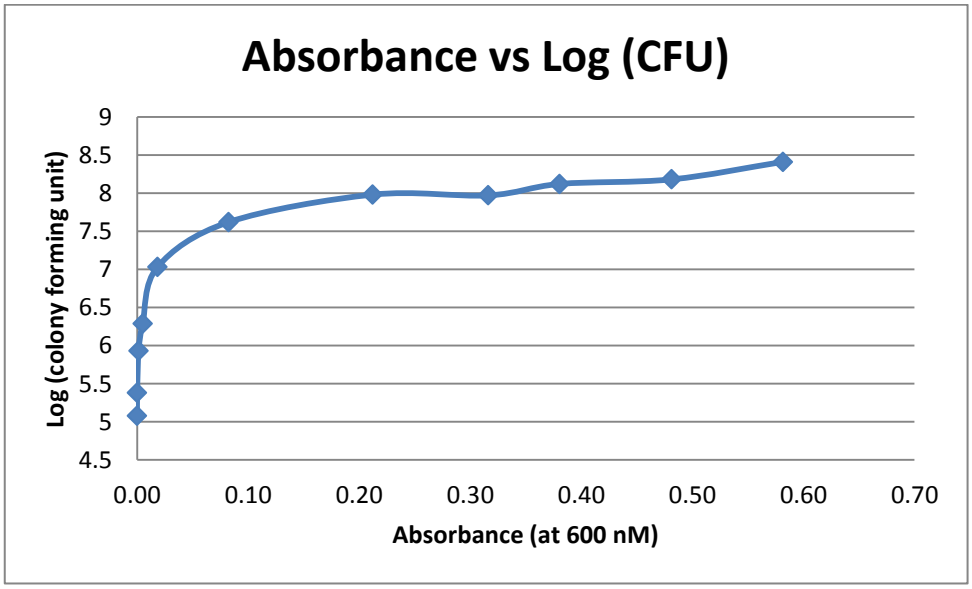


Figure 2-3(b) *S. epidermidis* No. 7 Absorbance verses Log (CFU) Curve

Standard *S. epidermidis* growth curve in nutrient broth plotted in log units versus absorbance reading.

Table 2-2: Hourly absorbance measurements and colony count (plating technique) for *S. epidermidis #7* growth curve

Time (hours)	Average Absorbance (600nm)	Bacterial Count (CFU/mL)	Log CFU
1:00	0.00	1.30×10^5	5.08
2:00	0.00	2.38×10^5	5.38
3:00	0.00	8.50×10^5	5.93
4:00	0.01	1.97×10^6	6.29
5:00	0.02	1.06×10^7	7.03
6:00	0.08	4.20×10^7	7.62
7:00	0.21	9.60×10^7	7.98
7:30	0.32	$9.4\ 0x\ 10^7$	7.97
7:45	0.38	1.32×10^8	8.12
8:00	0.48	1.51×10^8	8.18
8:30	0.58	2.57×10^8	8.41

2.3.2.3 Enhanced Bacterial Culture

Capsules: A fresh 2 x 2 cm piece of capsule was removed, macerated and placed in a sterile 25 ml tube containing 10 mL of sterile 10% tryptone soya broth (Oxoid, Thebarton, SA, Australia).

Implants: A fresh 2 x 2 cm piece of implant was removed, macerated and placed in a sterile 25 ml tube containing 10 mL of sterile 10% tryptone soya broth (Oxoid, Thebarton, SA, Australia).

The samples were sonicated for 15 minutes in an ultrasonic bath (Soniclean: JMR, Sydney, Australia) at 50-60Hz with water temperature maintained below 37^OC. The samples were then removed and shaken vigorously for 2 minutes. Serial 10-fold dilutions of 1 mL into 9

mL 1 x phosphate buffered saline (Medicago, Uppsala, Sweden) were made to required dilutions. An aliquot of 100µL from each serial dilution was plated on a warmed haemoglobin blood agar (Oxoid Thebarton, SA, Australia) and incubated aerobically at 37^{O}C overnight. The number of colony forming units was determined by counting the number of colonies on plates with between 30 and 300 colony forming units.

2.3.2.4 Diagnostic Chromogenic Selective Media for Multiresistant Organisms

Chromongenic molecules produce a coloured product following the hydrolysis of a specific enzyme.[454] A wide range of chromogens have been developed including those that target specific enzymatic functions of pathogenic bacteria.[454] These chromogenic microbial enzymes can be incorporated into a solid-agar-based matrix and enable rapid culture and identification of pathogens directly from clinical samples with high specificity.[454, 455] Oxoid (Thermo Fisher Scientific) have developed several chormogeneic based solid-agar-based diagnostic plates (*Brilliance*™) for clinically important multiresistant microorganisms including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE, including *Enterococcus faecium* and *Enterococcus faecalis*) and extended spectrum β-lactamase-producing organisms (ESBL, including *E.coli*, *Klebsiella*, *Enterobacter*, *Serratia* and *Citrobacter*.)

Each surface sample was placed into 5 ml sterile 100% tryptone soya broth (Oxoid, Thebarton, SA, Australia) and sonicated as per 2.3.2.3. A sterile loopful of specimen was used to inoculate each of the *Brilliance™* MRSA, *Brilliance™* VRE, *Brilliance™* ESBL plates and incubated at 37^{O}C for 24 hours. The plates were assessed for colony growth and colour and bacteria identified as per chromogen chart and manufacturer's instructions.

2.4 Microscopy

2.4.1 Scanning Electron Microscopy

Scanning electron microscopy has become an important tool in the identification and evaluation of bacterial biofilms. The most important obstacle to overcome, however, has

been the specimen preparation techniques as standard specimen processes with solvent dehydration process and heavy metals were shown to degrade and distort these delicate hydrophilic structures.[456-459] Several novel techniques have been developed to overcome the dehydration of the hydrophilic exopolysaccharide matrix and the degradation artefact.[457, 459, 460] Some techniques also have the advantage decreasing the processing time and increasing the sample throughput by bypassing critical point drying, a common rate limiting step due to the limited number of baskets that can be processed at any one time.[460] I compared two techniques for the preparation of biofilm for scanning electron microscopy; a low vacuum-freeze drying technique[461, 462] and a chemical dehydration method with hexamethyldisilazane.[457, 460, 463]

Ultrarapid freeze drying rapidly drops the specimen temperature by 10^4-10^5 ^{O}C/s. This results in the vitrification of cellular water thus avoiding formation of ice crystals.[461] Several freeze drying methods have been developed including supercooling in liquid nitrogen followed by freeze-drying in a vacuum and conductive coating.[459, 464] This technique has been shown to preserve the biofilm ultrastructure and maintain the specimen in a more native state compared to chemical fixation.[459, 464, 465]

Hexamethyldisilazane is a solvent that acts as dehydration agent with low surface tension and evaporates from a specimen in room air.[457] In soft tissues and fragile specimens it has been shown to effectively dehydrate and dry the specimen without introducing artefact or distortion.[457, 460, 463] It also has several advantages as it is relatively quicker than, cheaper than, and does not have the chemical disposal implications of, traditional heavy metal techniques.[463, 466]

2.4.1.1 Low-vacuum Freeze Drying Method[459, 461, 462]

Biofilm specimens were fixed in a 3% gluteraldehyde solution and stored at 4^{O}C overnight. The samples were washed in 0.1M phosphate buffer pH 7.4 for 10 minutes. This was repeated for a total of 3 washes. The sample was then mounted on an aluminium scanning electron microscopy stub (ProSciTech, Thuringowa, QLD, Australia) and secured with a mounting carbon tab (ProSciTech, Thuringowa, QLD, Australia) and

conductive carbon/graphite paint (ProSciTech, Thuringowa, QLD, Australia). The sample was maintained in a hydrated state throughout the preparation under a small drop of MiliQ water. The mounted hydrated specimen was rapidly freeze dried in liquid nitrogen until the covering water was frozen. The specimen was carefully loaded into a JEOL 6480VL scanning electron microscope previously prepared for a low vacuum mode. The sample was brought to a pressure of 40 Pa for 20 minutes before the microscope was vented. The sample was removed and sputter coated with 20 nm gold film using an Emitech K550 gold sputter coater using an argon gas-gold protocol.

2.4.1.2 Hexamethyldisilazane Dehydration Method[457, 460, 463]

A fresh 0.5 x 0.5 cm piece of capsule or implant was fixed in a 3% gluteraldehyde solution and stored at 4^{O}C for overnight. Each sample was washed 3 times with 0.1M phosphate buffer pH 7.4 for 10 minutes each wash. The samples were then processed with serial ethanol dehydration with analytical grade ethanol (Sigma-Aldrich Pty Ltd, Castle Hill, NSW, Australia) starting with 1 ml of 30% ethanol for 10 minutes. The ethanol was aspirated and then followed by 1 ml of 50%, 70%, 80%, 90%, 100% and 100% ethanol (Sigma-Aldrich Pty Ltd, Castle Hill, NSW, Australia) each, for 10 minutes. This was followed by a 1:1, 100% ethanol:hexamethyldisilazane (HMDS, Sigma-Aldrich, Castle Hill, NSW, Australia) for 10 minutes and then 3 x 100% HMDS for 10 minutes each. After the last HMDS step the liquid was aspirated and the samples were allowed to dry at room temperature in a fumehood overnight.

The HMDS prepared and dried samples were mounted on an aluminium scanning electron microscopy stubs (ProSciTech, Thuringowa, QLD, Australia) and secured with a mounting carbon tab (ProSciTech, Thuringowa, QLD, Australia) and conductive carbon/graphite paint (ProSciTech, Thuringowa, QLD, Australia).

The HMDS prepared and mounted specimens were sputter coated with 20nm gold using an Emitech K550 gold sputter coater (West Sussex, England), using an argon gas-gold protocol.

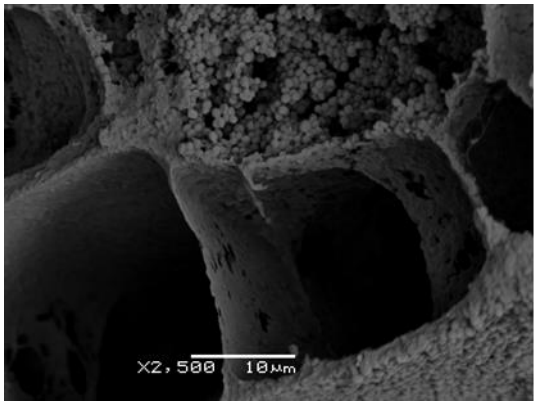
2.4.1.3 Scanning Electron Microscope

All scanning electron microscopy was conducted on a JEOL 6480LV scanning electron microscope (Tokyo, Japan). The standard settings on the microscope were 10 KV with a working distance of 20 mm.

Both techniques provided adequate preservation of bacteria and biofilm structures and were acceptable for comparison and analysis (Figure 2-4). There were several advantages of the HMDS technique over the LVFD technique, detailed below, and the HMDS protocol was used for the remainder of the study.

The advantages included:

1) Reliability and reproducibility – The HMDS protocol did not require judgement was required as to the amount of water to cover the specimen and duration in liquid nitrogen. The HMDS process gave more consistent imaging results and specimen stability, particularly in the early stages of learning the techniques;
2) Ease of process - the HMDS protocol could be batched, throughput was more consistent as the LVFD method was limited by the number of samples at the liquid nitrogen stage, volumes were standardised and did not require any variation;
3) Safety - whilst HMDS is a corrosive agent and has to be handled with care in a fumehood, it is relatively less dangerous than handling liquid nitrogen, often on or beside the electron microscope.



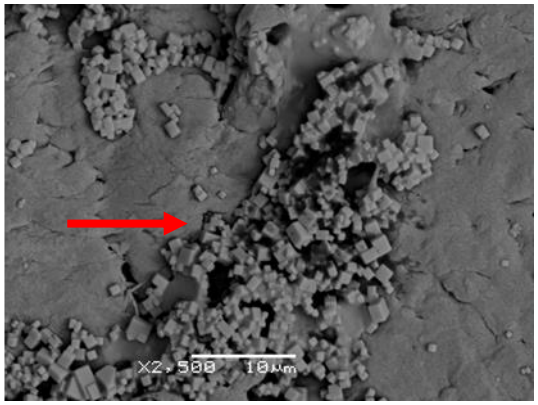
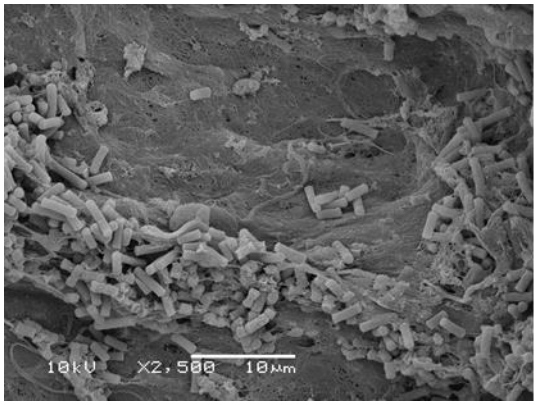


Figure 2-4 (a) and (b)



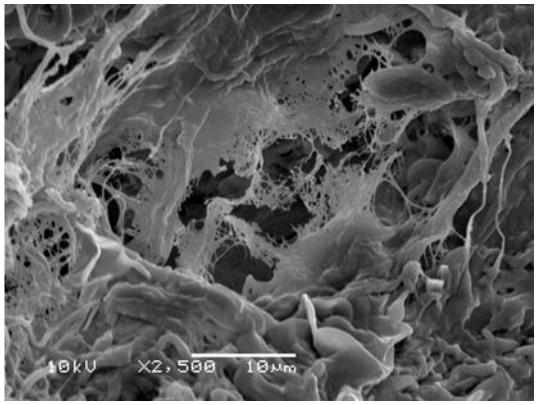


Figure 2-4 (c) and (d)

Figure 2-4: Comparison of Scanning Electron Microscopy Images

2-4 (a) and (b) are from LVFD technique showing maintenance of biofilm architecture although there was often technique artefact. 2.4 (b) shows cuboidal cocci artefact, marked with the red arrow, thought to be salt crystals from the phosphate buffered saline solution.

2-4 (c) and (d) are from HMDS protocol showing similar level of detail and maintenance of biofilm architecture.

2.4.2 Live-Dead®*Bac*light™ Fluorescence Confocal Microscopy

Live-Dead®*Bac*light™ (Molecular Probes, Eugene, Oregon) is a two-colour fluorescence assay kit that stains bacteria nucleic acid with SYTO®9 green and propidium iodide (red-fluorescent). The SYTO®9 will label all bacteria with and without intact cell membranes. Propidium iodide labels on bacteria with damaged membranes. Thus the dead bacteria stain red and the live bacteria stain green.[467]

Environmental specimens and implant surface specimens were cut into 0.5 x 0.5cm pieces. Specimens were fixed in 3% gluteraldehyde overnight at 4^{O}C. The specimens were washed three times with 1 x phosphate buffered saline for 10 minutes each. Each specimen was incubated with an equal volume mix of STYO®9 ™ (Molecular Probes, Eugene, Oregon) and propidium iodide™ (Molecular Probes, Eugene, Oregon), enough to cover the specimen, for 15 minutes at room temperature in the dark. The specimens were washed with 1 x phosphate buffered saline and maintained in a hydrated state in 1 x phosphate buffered saline in the dark until imaged. Specimens were imaged with Olympus Fluoview 300 inverted Confocal Laser Scanning Microscope.

2.4.3 Immunofluorescent Identification of T-cells and B-cells in Porcine and Human Implants and Capsules

Immunofluorescence is a biological assay that can detect specific targets in cells and tissues by combining the use of antibodies and fluorescent molecules.[468] It is a very sensitive and versatile technique used widely in biological, immunological and medical research and can be used on fresh and fixed tissues.[468, 469] The two main methods of immunofluorescent labelling are direct and indirect.[469] Indirect immunofluorescence requires a primary and secondary antibody. The primary antibody is an antibody specific to the molecule of interest and is unlabeled.[469] The secondary antibody is an anti-immunoglobulin antibody directed towards the constant portion of the primary antibody. The secondary antibody is also tagged with a fluorescent dye that when excited has a known optimal wavelength.[469] The main advantage of the indirect method is greater signal sensitivity, as more than one secondary antibody will bind to the primary antibody

resulting in amplification of the immunofluorescence signal.[469] The main disadvantage of the indirect method is the potential for cross-reactivity or non-specific binding between the specimen and the secondary antibody causing false-positive fluorescence.[469]

When the biofluorescent label is exposed to light, of an appropriate wavelength, it will absorb the energy from the photon of light. This in turn excites the electrons around the atom's nucleus to jump to a higher, less stable, energy level. An electron stays in this state for a short time, half-life is less than ten seconds, and as it returns to its resting state the energy is released as a small amount of heat energy and the rest as a photon (light).[469] The emitted light is of a lower energy level than the absorbed light, thus has a longer wavelength and is detected as a distinct colour by the fluorescent microscope. (469) Each fluorochrome has a specific excitation wavelength range, with a peak at the optimal wavelength, as well as a defined emission wavelength range with an emission peak. Thus commercially-available secondary antibodies can be chosen to provide differing flurochromes enabling the labeling of two or more target molecules.

2.4.3.1 Specimen Preparation and Fixation

Fresh specimens were collected from the patient (either human or porcine) after removal of capsule and implant under surgical aseptic technique. The samples were put into sterile 1 x phosphate buffered saline and transferred to the laboratory on ice and stored at 4^0C until dissection. A 1 cm x 1 cm piece of capsule or implant was dissected from the fresh specimen and fixed with 2% paraformaldehyde for 2 hours at room at 4^0C. The sample was washed in 1 x phosphate buffered saline for 10 minutes. The wash was repeated a total of three times and the specimens ware stored in 1 x phosphate buffered saline at 4^0C until required.

2.4.3.2 Primary Antibodies

The primary antibodies were:

1. Monoclonal Mouse Anti-Human CD79αcy, Clone HM57, Code M7051 DakoCytomation, Glostrup Denmark and labels mature B-cell lymphocytes in multiple species including humans and pigs.[470]

2. Polyclonal Rabbit Anti-Human CD3, Code A0452, DakoCytomation, Glostrup Denmark and labels T-cell lymphocytes of multiple species including humans and pigs.[471]

2.4.3.3 Secondary Antibodies

The secondary antibody-fluorochromes used were mouse-antirabbit-Alexa Fluor®448 (Invitrogen, Life Technologies Australia Pty Ltd, Mulgrave, Vic Australia) and goat-antimouse Alexa Fluor® 543 (Invitrogen, Life Technologies Australia Pty Ltd, Mulgrave, Vic Australia). The Alexa Fluor®448 fluorochrome has a maximum absorption of 495 nm and maximum emission of 519 nm with a green emission colour. The Alexa Fluor® 543 fluorochrome has a maximum absorption of 541 nm and a maximum emission of 560 nm with an orange-red emission colour.

2.4.3.4 Staining Protocol

Fresh, fixed specimen was washed in a 1 x phosphate buffered saline (Medicago, Uppsala, Sweden)/0.5% Triton™X-100 (Sigma-Aldrich, Castle Hill, Australia) solution for 30 minutes. The specimen was blocked in a 1 x phosphate buffered saline/0.5% Triton™X-100/5% bovine serum albumin (Promega Corporation Alexandria, Australia) solution for 30 minutes. The primary antibody was diluted with 1 x phosphate buffered saline/0.5% Triton™X-100/5% bovine serum albumin to 1:25 dilution for CD 79 and 1:50 dilution for CD3. The specimen was incubated with the primary antibody for 60 minutes, at room temperature in the dark. The specimen was rinsed with 1 x phosphate buffered saline/0.5% Triton™X-100 for 10 minutes in the dark and repeated for a total of three times. The secondary antibodies were diluted to 1:400 in 1 x phosphate buffered saline and incubated for 60 minutes at room temperature in the dark. The specimen was washed with 1 x phosphate buffered saline for 10 minutes in the dark and repeated a total of three times. The specimen was stored in 1 x phosphate buffered saline at room temperature in the dark until imaged with Olympus Fluoview 300 inverted Confocal Laser Scanning Microscope.

2.5 Molecular Techniques

Specimen preparation: a 0.25 x 0.25 cm piece of capsule or implant was dissected from a fresh specimen. The specimen was then placed in a sterile 1.5mL capped tube and stored at -20^{O}C until use.

2.5.1 Tissue Digestion

Fifty to one hundred grams of each sample was added to 275µl of digestion mix (50 mM Tris/HCl pH7.5, 150mMNaCl, 2mM EDTA, 1% sodium dodecyl sulphate) containing 400µg proteinase kinase 60µl/ml (Sigma-Aldrich, Castle Hill, Australia). The tissue was incubated at 50^{O}C in a water bath overnight. The proteinase kinase was inactivated in boiling water for 5 minutes. Lysozyme (Sigma-Aldrich, Castle Hill, Australia) was added to a final concentration of 0.5 mg/ml and incubated at 56^{O}C for 2 hours. Proteinase kinase 200 µg was added and incubated at 50^{O}C for 2 hours.[(472)]

2.5.2 Phenol-Chloroform DNA Extraction

The procedure was conducted in a fumehood with all reagents kept on ice. All tips and tubes were sterilised prior to use.

The digestion mix samples were removed from the water bath and 300µl of ice cold phenol (pH 7.5-8.0 Sigma-Aldrich, Castle Hill, NSW, Australia) was added. The mix was shaken well for 20 seconds and spun for 3 minutes at 20 000g in a bench centrifuge. The supernatant was aspirated and placed in a sterile 1.5mL tube. This was repeated twice if there was excessive protein precipitation.

Then 300µl of ice cold phenol/chloroform/isoamyl alcohol mix (Sigma-Aldrich, Castle Hill, NSW, Australia) was added. The mix was shaken well for 20 seconds and spun for 3 minutes at X 20, 0000 g in a bench centrifuge. The supernatant was aspirated into a sterile 1.5mL tube and 300µl of ice cold chloroform:isoamyl alcohol (24:1)(Sigma-Aldrich, Castle Hill, NSW, Australia) was added. The mix was shaken well for 20 seconds and spun for 3 minutes at X 20, 0000 g in a bench centrifuge.

The supernatant was removed and measured into a sterile 1.5 mL tube. To precipitate the DNA, sodium acetate 3M pH 5.2 at $^{1}/_{10}$ the volume of the measured supernatant was added and a *total volume* calculated. Then ice cold absolute alcohol was added at 2 x *total volume*. This was placed in a -20^{O}C freezer overnight to precipitate the DNA.

The DNA was spun down in a pre-prepared centrifuge at 4^{O}C for 15 minutes at X 20,0000g in a bench centrifuge. The ethanol mix was aspirated and the tube was blotted to remove excess fluid covered and left to dry in an environment to protect it from contamination.

Once dry the DNA pellet was resuspended in 100 mL Tris-EDTA mix (10mM Tris/0.1mM EDTA) and stored at -20^{O}C until required.

2.5.3 Molecular Microbiology using Partial 16S rRNA Gene Sequencing

Molecular techniques are increasing being used to identify and characterise bacteria within many different environments.[473] In clinical infections molecular identification is particularly useful in bacteria that are slow-growing and difficult or impossible to cuture.[474] Numerous bacterial genes are now used as a genetic targets, however the *16S rRNA* gene remains the primary gene for molecular identification.[473] In bacteria there are 3 rRNA genes that are transcribed from a 30S rRNA precursor molecular that is cleaved by RNase III into 5S, 16S and 23S rRNA molecules.[475] The 16S rRNA gene has several key features making it a key genetic target for molecular identification including:

1) the ubiquitous occurrence of the gene within the bacteria genera;[473, 474]
2) the highly conserved region, used for general bacteria detection;[474, 476]
3) the variable and hyper-variable species-specific region that enable individual species identification;[474, 476] and
4) a sequence size around 1500 bp which is relatively easy to sequence.[473, 477]

   The operon size, nucleotide sequence and secondary structure of 16S rRNA genes is highly conserved.[478] The gene sequences for over 2400 different bacteria species are now known and stored on readily accessible databanks to enable comparison and identification.[478, 479]

In our laboratory Dr Honghua Hu developed and/or optimised several molecular bacterial techniques using partial 16S rRNA and 18s rRNA gene sequence alignments using Clustal Omega service in EMBL-EBI[480] and SeqMatch service in Ribosomal Database Project II.[478]

2.5.3.1 Molecular Bacterial Identification

PCR amplification for 16s rRNA gene was performed using universal eubacterial primers 16s rRNA_341F 5'-CCTACGGGAGGCAGCAG-3' and 16s rRNA_951R 5'-ATGCTCCRCCGCTTGTG-3' to amplify a 610bp amplicon. This amplicon was designed from the variable part of the 16S rRNA genome to enable individual species identification.

PCR was carried out in 50 µl reaction volume containing 250 nM of each primer, 1x ImmoBuffer (Bioline Aust Pty Ltd), 2.5mM MgCl2 (Bioline, BIO-21046), 0.2 mM of each dNTPs, 2.5 U of Immolase DNA Polymerase (Bioline, BIO-21046) and 2 µl of DNA template. PCR fragments were amplified in FlexiCycler (Analytik Jena, Germany) with the following thermal cycle conditions: 95^{O}C for 10 minutes to activate DNA Polymerase; followed by 32 cycles of denaturation at 95^{O}C for 15 seconds, annealing at 56^{O}C for 30 seconds, extension at 72^{O}C for 90 seconds; then followed by a final extension at 72^{O}C for 10 minutes.

PCR amplicons were purified by QIAquick PCR Purification Kit (Qiagen, 28104 Chadstone Centre, Vic, Australia) according to manufacturer's instructions. Sequencing of PCR amplicons (both forward and reverse) was performed using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions. Capillary separation was achieved using an AB3130xl genetic analyser (Applied Biosystems) in Macquarie University's sequencing facility centre.

The bacterial sequences were identified by comparison with known bacterial gene sequences available in the GenBank, EMBL and DDBJ databases by Dr Honghua Hu. The overall quality of each sequence was verified by Sequence Scanner v1.0 (Applied Biosystems) software and sequence editing was performed using BioEdit Sequence Alignment Editor version 7.0.5.3. The identity of each edited sequence was analyzed with

BLASTN. Isolates were allocated to a genus and/or species if the sequence yielded a similarity score of ≥98%.

2.5.3.2 Molecular Bacterial Count

Quantitative PCR was used to quantify the bacterial load on each capsule using molecular primers for a conserved region of the 16s rRNA gene. This ubiquitous gene and Quantitative PCR enables a sensitive quantification test for presence and relative amount of bacteria.[481]

The total number of bacteria in a piece of tissue of know weight was determined by real-time quantitative PCR using universal eubacterial primer pair 16s rRNA_341F 5'-CCTACGGGAGGCAGCAG-3' and 16s rRNA_534R 5'-ATTACCGCGGCTGCTGG-3' to amplify a 194bp amplicon of 16s rRNA gene of all bacteria.[482] This amplicon was designed from the conserved regions of 16S rRNA to ensure its ability to count all bacteria present.

The number of bacteria in each tissue sample was expressed as per mg of capsule based on the average number of copies of the 18S gene in a mg of pig tissue measured by real-time quantitative PCR. This was achieved using the primer pair 18s rRNA_756F 5'-GGTGGTGCCCTTCCGTCA-3' and 18s rRNA_877R 5'-CGATGCGGCGGCGTTATT-3' to amplify a 122 bp amplicon based on the pig (*Sus scrofa*) 18S rRNA reference gene (GenBank: AY265350.1).

Between 50mg and 100mg of capsular tissue and between 40mg and 100mg of implants were digested using a combination of proteinase K and lysozyme digestion (2.5.1) and the genomic DNA extracted using phenol/chloroform extraction followed by ethanol precipitation (2.5.2).

The extracted DNA underwent real-time quantification PCR using a 25 µL reaction mix containing 1 x Brilliant II SYBR Green qPCR Master Mix (Agilent Technologies, Santa Clara, California), 400 nM forward and reverse primers and 20 ng DNA template. The PCR fragments were amplified in Corbett Rotor-Gene 6000 with the following thermal cycle conditions: 95^{O}C for 10 minutes to activate DNA Polymerase; followed by 40 cycles of

denaturation at 95^{O}C for 15 seconds, annealing at 56^{O}C for 30 seconds, extension at 72^{O}C for 90 seconds; then followed by a final extension at 72^{O}C for 10 minutes.

Each quantitative PCR was run with standard samples of known concentrations (copies/µl) – 10^8, 10^7, 10^6, 10^5, 10^4, 10^3 and 10^2 copies of the genome – to generate a standard curve. The quantitative PCR standards were prepared from 16s rRNA gene or 18s rRNA gene PCR fragments purified by QIAquick Gel Extraction Kit (Qiagen, Chadstone, VIC Australia). The calibration standard curve was created by plotting the threshold cycle corresponding to each standard versus the value of their corresponding number of concentration expressed as copies/µL. The threshold cycle is the number of cycles required to reach the defined threshold level. The copy number of the sample was calculated by Rotor-Gene 6 by plotting the sample threshold cycle value against a standard curve generated in the same run.

Concentration of the purified nucleic acid was calculated by measuring the absorbance at 260 nm and its corresponding concentration was converted into copies/µl of PCR amplicon by using the Avogadro constant (6.023X 10^{23}) and its molecular weight, number of bases of the PCR product multiplied by the average molecular weight of a pair of nucleic acids (660 Da).[(483)] The nucleic acid concentration (copies/mL) of the resultant solution was determined by spectrophotometry using the equation:

$$\text{Copies/mL} = \frac{6.023\text{x}10^{23} \text{ x C x } OD_{260}}{\text{MWt}}$$

Where:

- 6.023 x 10^{23} = Constant, Avogadro's number;
- C = 5 x 10^{-5} g/mL for DNA;
- OD_{260} = Optical density at 260nm;
- MWt = molecular weight of PCR product (base pair x 660 Da).

2.5.4 Statistical Analysis

All statistical analyses were carried out using using the statistical package Sigma Plot version 11-13 (Systat Software, Inc., San Jose, California).

Chapter 3

"If you think research is expensive, try disease."

- Prof Suzanne Cory, 2014

3 Presence of Biofilm Containing Viable Multiresistant Organisms Despite Terminal Cleaning on Clinical Surfaces in an Intensive Care Unit

3.1 Introduction

Nosocomial infections, or hospital-associated infections, are an important cause of morbidity and mortality in the healthcare environment.[321, 484] Recent studies have shown an average HAI rate of 4.0% in US Hospitals,[331] 6.0 % across European Hospitals (range 2.3% - 10.8%)[485] and 6.4% in English Hospitals.[486] Hospital-associated infections are much higher in intensive care units with reports rates varying between 19.% - 28% in large population studies in the US, England and Europe.[322, 330, 485, 486] Hospital-associated infections in Belgium result in an increase in length of stay of 7.3 days, an increase in mortality of 2.8% and cost the health-care budget €290 million to treat.[321] In the US hospital-associated infections are estimated to cost between US$26-$33 billion and result in 99,000 deaths annually.[355, 487]

Not surprisingly the bacteria most frequently involved with these infections include *Staphylococcal* species (*S. aureus* and coagulase negative staphylococci), *Enterococcus* species, *Candida* species, *E. coli*, *P. aeruginosa* and *Klebsiella* species, with each involved in 7-15% of hospital-associated infections in several large population studies.[322, 329, 485] In these same studies *Enterobacter* species, *Proteus* species and *Acinetobacter* species commonly cause 3-5% of hospital-acquired infections.[322, 329, 485] In a population study across US hospitals multi-drug resistant bacteria were identified in 1 in every 6 (16.7%) HAI[329] with rates significantly increasing over the past two decades.[487, 488]Various population studies have reported methicillin-resistance is identified in 34% to 57% of

S. aureus infections, vancomycin-resistance in *E. faecalis* infections between 2% to 10.7%[322, 329, 485, 488] and carbpenem-resistance in *P. aeruginosa* infections between 23 to 32% and in up to 82% of *Acinetobacter baumannii* infections.[322, 485]

Within the intensive care environment the two most common sources of health-associated infections are device-associated infection from the patient's endogenous bacteria (40% to 60%) and cross infection from medical staff (20% to 40%).[484, 489] Up to 20% of hospital-associated infection is related to patient contamination from other sources.[484] More recent data has shown that, over a five year period (2008-2012) in a single tertiary hospital, improvements in prevention strategies resulted in a decrease in both device-associated infections (n=517 decreased to n=288) and total hospital-associated infections (n= 704 decreased to n=498).[490] Thus non-device related infections now account for over 40% of hospital-associated infections.[490]

Patient contamination from the hospital environment is increasingly recognised as an important bacterial reservoir.[345, 347, 484, 491-494] There is now increasing evidence that many important pathogens, including *S. aureus* (including MRSA), *Enterococcus* species (including VRE), *A. baumannii*, *P. aeruginosa*, *Clostridium difficile* and norovirus, can be shed from colonised and infected patients into their local environment, including hospital surfaces.[345, 493, 495, 496] Numerous bacteria have been shown to persist for a variable duration: with short lived bacteria ,including *Haemophilus influenza, Helicobacter pylori*, *Campylobacter jejunii, Vibrio cholera* and *Neisseria gonorrhoeae*, being shown to last only a few hours to less than 2 weeks; whereas *S. aureus*, *C. difficile*, *P. aeruginosa*, *E.* coli, *Enterococcus*, *Acinetobacter*, and *Klebsiella* species can survive from four and up to 46 months or more in a dry environment.[492, 497, 498] Indeed the survival of these bacteria within the hospital environment is associated with an increased risk of contracting a hospital-associated infection when the room was previously occupied by an infected/contaminated patient despite cleaning.[484, 490, 492, 493] This risk varies from 1.5 times for methicillin-resistant *S. aureus*, 1.75 times for *P. aeruginosa*, 2 times for vancomycin-resistant *Enterococcus* and up to 3.5 times for *A. bumannii*.[492, 497-501] Cross contamination has been demonstrated to occur via several routes including:[493, 502] direct

colonisation of healthcare workers' hands between patients,[495, 503] air contamination,[345, 504, 505] and contaminated room surfaces and equipment.[495, 503]

There is increasing evidence that dry environmental contamination is an important reservoir for clinically relevant bacteria including many multi-resistant organisms.[347, 484, 490, 492] Despite current cleaning practices, viable bacteria can persist for prolonged periods.[484] Bacteria contained within biofilm may provide a mechanism for these pathogens to persist in the hospital environment.

In 2010 our research team was given access to an intensive care unit in a tertiary referral hospital the day after it was decommissioned. Two colleagues and I destructively sampled and individually stored samples from clinical surfaces from two wards in an intensive care unit. The first ward contained non-infectious patients, the second ward contained patients infected with or colonised with multi-resistant organisms. We used microscopic, microbiological and molecular techniques to investigate these clinical surfaces for the presence of dry-surface biofilms and the bacteria that may be housed within them.

3.2 Original Paper:

Presence of Biofilm Containing Viable Multiresistant Organisms Despite Terminal Cleaning on Clinical Surfaces in an Intensive Care Unit

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Contribution

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I co-ordinated the sample procurement, destructive sampling, storage and management of all samples.

I performed all the scanning electron microscopy

I performed microbiology and identification of the specimens.

I assisted with the manuscript preparation.

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Presence of biofilm containing viable multiresistant organisms despite terminal cleaning on clinical surfaces in an intensive care unit

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SUMMARY

Background: Despite recent attention to surface cleaning and hand hygiene programmes, multiresistant organisms (MROs) continue to be isolated from the hospital environment. Biofilms, consisting of bacteria embedded in exopolymeric substances (EPS) are difficult to remove due to their increased resistance to detergents and disinfectants, and periodically release free-swimming planktonic bacteria back into the environment which may may act as an infection source.
Aim: To establish whether reservoirs of MROs exist in the environment as biofilms.
Methods: Following terminal cleaning, equipment and furnishings were removed aseptically from an intensive care unit (ICU) and subjected to culture and scanning electron microscopy (SEM). Samples were placed in 5 mL of tryptone soya broth, sonicated for 5 min before plate culture on horse blood agar, Brillance MRSA and Brilliance VRE agar plates. Samples for SEM were fixed in 3% glutaraldehyde and hexamethyldisilizane (HMDS) prior to sputter-coating with gold and examination in an electron microscope.
Findings: Biofilm was demonstrated visually on the sterile supply bucket, the opaque plastic door, the venetian blind cord, and the sink rubber, whereas EPS alone was seen on the curtain. Viable bacteria were grown from three samples, including MRSA from the venetian blind cord and the curtain.
Conclusion: Biofilm containing MROs persist on clinical surfaces from an ICU despite terminal cleaning, suggesting that current cleaning practices are inadequate to control biofilm development. The presence of MROs being protected within these biofilms may be the mechanism by which MROs persist within the hospital environment.

Introduction

Healthcare-associated infections (HAIs) are a widespread problem, affecting 5–10% of all patients.[1] In the intensive care unit (ICU), the presence of very sick, elderly and immunocompromised patients results in a disproportionate percentage

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(20%) of patients developing HAI.[2] This problem is compounded by the spread of multiresistant organisms (MROs), making treatment difficult or ineffective.[3] HAIs add considerable morbidity, increase hospital stay times, increase mortality, and add costs to patient care.[1,2,4]

Contamination of the inanimate environment around patients constitutes an important reservoir of MRO with the risk of HAI increased by an average of 73% if the patient previously occupying the room had MRSA, vancomycin-resistant enterococcus (VRE), acinetobacter, *Clostridium difficile* or other pathogens.[3,5,6] Numerous studies have shown persistence of these organisms in the environment even in the face of enhanced terminal cleaning.[7–9]

Biofilms are generally found in moist environments, causing infection on implantable medical devices such as catheters and breast implants or on instruments routinely immersed in fluid.[10–12] We hypothesize that, despite the decreased moisture availability on dry surfaces, bacteria within the ICU environment also reside in biofilms, and that within these biofilms, MROs are protected from physical removal and chemical disinfection.

A biofilm is a structured community of organisms encased and attached to a surface by exopolymeric substances (EPS). The EPS makes up to 90% of the biofilm providing protection from environmental desiccation and this EPS is extremely difficult to remove using detergents.[13–15] Additionally, bacteria within biofilms are up to 1500 times (typically 100–250 times) more resistant to biocides than the same 'planktonic' bacteria growing in liquid culture.[13] These properties of biofilms result in decreased efficacy of cleaning and disinfection, thereby promoting the persistence of bacteria, including MROs, in the environment.

In this study we investigated whether biofilms can be found on furnishings in the ICU.

Methods

Following terminal cleaning in a 16-bed ICU, i.e initial cleaning with neutral detergent, followed by disinfection with 500 ppm chlorine (Diversol5000, Johnson Diversey, Smithfield, Australia), equipment and furnishings were aseptically removed from patient and common-use areas.

Sample collection

Items were destructively sampled using sterile gloves, forceps, pliers, scissors, or scalpel blades, depending on the material being sampled. Gloves and instruments were changed between each sample. Samples were then placed into sterile containers for transport to the laboratory. Small items, such as a sterile supply reagent box, were transported intact to the laboratory; larger items, such as the mattress and door, had sections removed (up to 8×10 cm in size) into sterile containers. Following transport to the laboratory, these large pieces were further sectioned into smaller pieces, using a sterile technique.

Scanning electron microscopy (SEM)

Samples up to 1 cm^2 were fixed in 3% glutaraldehyde, dehydrated through ethanol, immersed in hexamethyldisilizane (HMDS; Polysciences Inc., Warrington, PA, USA) for 3 min before sputter-coating with 20 nm gold film and examined in an SEM microscope as previously described.[12] An item was classified as being biofilm positive if bacteria attached to a surface and surrounded by EPS could be visualized.

Microbiology

Sections of equipment or furnishings up to 2 cm^2 were placed in 4 mL of tryptone soya broth, sonicated for 5 min and 100 μL spread over horse blood agar plates (HBA), Brilliance MRSA agar plates for the detection of multiresistant *Staphylococcus aureus* (MRSA) and Brillance VRE agar plates for the detection of vancomycin-resistant enterococcus (Oxoid, Adelaide, Australia). MRSA plates were incubated for 18–24 h and VRE and HBA plates up to 48 h.

Results

Six samples were examined by SEM (Table I). We failed to demonstrate biofilm on only one sample. Four samples had principally coccoid-shaped bacteria encased in large amounts of EPS and the sample from the curtain had 'strings' of dehydrated EPS evident. (Figure 1).

Bacteria grew on HBA from four of the six samples, demonstrating the presence of culturable organisms. The venetian blind cord and curtain, positive for biofilm by SEM, also grew MRSA. The mattress grew MRSA and *E. faecium* but we were unable to demonstrate biofilm visually on this sample (Table I). Two samples positive for biofilm were culture negative, using the procedure described above.

Discussion

Many studies have shown that contamination of the environment makes an important contribution to HAI and that enhanced cleaning protocols reduce environmental contamination, which translates into decreased incidence of HAI.[5,6] In Dancer *et al.*'s study, the addition of one extra member of cleaning staff, five days a week, resulted in a 32.5% reduction in microbial contamination of hand-touch sites and a 26.6% reduction in new MRSA infections, saving the hospital an

Table I
Scanning electron microscopy (SEM) and culture results for environmental surfaces

Sample	SEM	Culture plates		
		HBA	MRSA	VRE
Curtain	Positive EPS	Growth	Positive	Negative
Venetian blind cord	Positive biofilm	Growth	Positive	Negative
Mattress bay	Negative	Growth	Positive	*E. faecium*
See-through plastic door	Positive biofilm	Negative	Negative	Negative
Wash basin rubber	Positive biofilm	Negative	Negative	Negative
Sterile supply reagent bucket	Positive biofilm	Growth	Negative	Negative

HBA, horse blood agar; MRSA, multiresistant *Staphylococcus aureus*; VRE, vancomycin-resistant enterococcus.

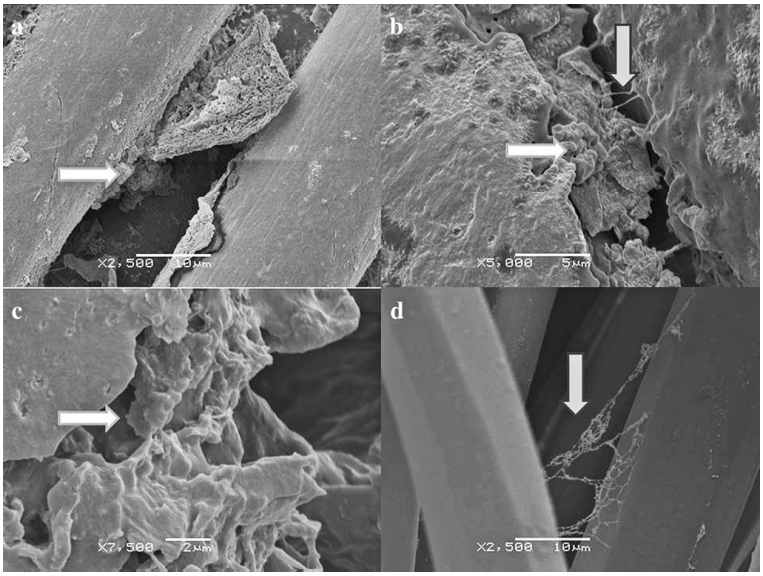

Figure 1. Scanning electron micrographs of: (a) blind cord (original magnification ×2500); (b) see-through ward door (original magnification ×5000); (c) red reagent box (original magnification ×7500); (d) curtain (original magnification ×2500). Horizontal arrows indicate coccoid bacteria embedded in exopolymeric substance (EPS). Vertical arrows indicate residual strings of EPS dehydrated during processing.

estimated £30,000 to £70,000.[7] Termination of the extra cleaner resulted in new clusters of MRSA infection within two to four weeks. However, even with enhanced cleaning, MROs can still be isolated from the environment.[7—9]

We hypothesize that surface condensation occurs, producing a thin film of water, or that the relative humidity in the ICU is high enough to allow biofilms to develop on ICU surfaces. Once formed, the EPS would protect the bacteria from desiccation and make them harder to remove.

We further hypothesize that MROs persist in the environment, in the face of enhanced cleaning, as biofilms. Although detergents are good at removing patient soil and planktonic bacteria, they are less effective at removing biofilm, rendering current cleaning protocols less efficient.[14,15] In industry, extreme measures including physical scraping and use of concentrated biocides are often required to remove biofilm, such as when removing legionella from water-cooling towers.

Of the six furnishings sampled bacteria were demonstrated to be embedded in EPS on four samples and residual EPS on one, whereas only the mattress sample was negative for biofilm by SEM. SEM of the non-porous covering of the hospital mattress shows that the surface is not completely level but has many microscopic dips. This is similar to the dips and imperfections that have been observed on new Teflon endoscope tubing.[12] With use, many of these dips or imperfections in endoscope tubing became contaminated with biofilm.[12] A similar situation may exist with the hospital mattresses and, if a larger area were to be inspected, biofilm may be found.

Using destructive sampling followed by sonication and broth culture, bacteria were grown from three of these biofilm-positive samples. Both the venetian blind curtain cord and the curtain grew MRSA. Even the mattress, the sole sample for which we failed to visually demonstrate biofilm, grew MRSA and VRE. It is worrying that we demonstrated biofilm on the reagent bucket that was used to contain sterile supplies, such as catheters and bandages. Although we did not detect MRSA or VRE, we were able to show that viable bacteria were present in the biofilm. Additionally the rate of acquisition of new resistant determinants is increased in bacteria residing in biofilm.[16] A significant correlation has been shown to exist between class 1 integron resistance genes, biocide resistance and biofilm formation in clinical strains of *Acinetobacter baumannii*.[17] Whether this occurs when water is limited is unknown.

Despite visual confirmation of biofilm, neither the wash basin nor the plastic door grew bacteria when aerobic culture and HBA were used. These bacteria could have been dead, or not culturable using the conditions used, or unculturable due to their state of growth in the biofilm. Bacteria growing as biofilm are notoriously difficult to culture, although sonication of the sample in broth increases the rate of recovery.[13]

Dancer *et al.* found that antibiotic-resistant environmental bacteria were more prevalent in wards with a high level of antibiotic prescribing.[18] The combination of high antibiotic use and environmental biofilms in the ICU may be the mechanism whereby increased genetic exchange occurs between bacteria residing in biofilms, leading to persistence of antibiotic-resistant environmental bacteria, despite enhanced cleaning.

Using destructive sampling, followed by SEM and culture, we have demonstrated the presence of biofilm and biofilm containing MROs on clinical surfaces from an ICU despite terminal

cleaning, suggesting that current cleaning practices are inadequate to control biofilm development. The presence of MROs being protected within these biofilms may be the mechanism by which MROs persist within the hospital environment.

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None declared.

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3.3 Original Paper:

Intensive care unit environmental surfaces are contaminated by multidrug-resistant bacteria in biofilms: combined results of conventional culture, pyrosequencing, scanning electron microscopy, and confocal laser microscopy.

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I co-ordinated the sample procurement, destructive sampling, storage and management of all samples.

I performed the majority of the scanning electron microscopy.

I performed the majority of Live-Dead®Baclight™ fluorescence confocal microscopy.

I assisted with the manuscript preparation.

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Intensive care unit environmental surfaces are contaminated by multidrug-resistant bacteria in biofilms: combined results of conventional culture, pyrosequencing, scanning electron microscopy, and confocal laser microscopy

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SUMMARY

Background: Hospital-associated infections cause considerable morbidity and mortality, and are expensive to treat. Organisms causing these infections can be sourced from the inanimate environment around a patient. Could the difficulty in eradicating these organisms from the environment be because they reside in dry surface biofilms?
Aim: The intensive care unit (ICU) of a tertiary referral hospital was decommissioned and the opportunity to destructively sample clinical surfaces was taken in order to investigate whether multidrug-resistant organisms (MDROs) had survived the decommissioning process and whether they were present in biofilms.
Methods: The ICU had two 'terminal cleans' with 500 ppm free chlorine solution; items from bedding, surrounds, and furnishings were then sampled with cutting implements. Sections were sonicated in tryptone soya broth and inoculated on to chromogenic plates to demonstrate MDROs, which were confirmed with the Vitek2 system. Genomic DNA was extracted directly from ICU samples, and subjected to polymerase chain reaction (PCR) for *femA* to detect *Staphylococcus aureus* and the microbiome by bacterial tag-encoded FLX amplicon pyrosequencing. Confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM) were performed on environmental samples.

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Findings: Multidrug-resistant bacteria were cultured from 52% (23/44) of samples cultured. *S. aureus* PCR was positive in 50%. Biofilm was demonstrated in 93% (41/44) of samples by CLSM and/or SEM. Pyrosequencing demonstrated that the biofilms were polymicrobial and contained species that had multidrug-resistant strains.
Conclusion: Dry surface biofilms containing MDROs are found on ICU surfaces despite terminal cleaning with chlorine solution. How these arise and how they might be removed requires further study.

Introduction

Hospital-acquired infections (HAIs) are a major problem. A recent study estimated that 648,000 patients have 721,800 HAIs annually in acute care hospitals in the USA.[1] This has been estimated to cost US hospitals US$28–34 billion annually.[2] 'ESKAPE' organisms (*Enterococcus* spp., *Staphylococcus aureus*, *Klebsiella* spp., *Acinetobacter* spp., *Pseudomonas aeruginosa*, and Enterobacteriaceae) continue to dominate, and *Clostridium difficile* is now the micro-organism most frequently causing HAIs.[1]

The cost-effectiveness of infection prevention and control programmes has been demonstrated, with hand hygiene being the most critical activity for controlling infection transmission.[3,4] However, sustained improvements in compliance rates are difficult to maintain, and infection control programmes targeting only hand hygiene are not necessarily associated with declining HAI rates.[5,6] By contrast, multiple strategies or bundles including active surveillance, patient isolation/cohort and improved hand hygiene have been shown to be successful in reducing meticillin-resistant *S. aureus* (MRSA) rates, even in hyperendemic regions.[7,8]

The Healthcare Infection Control Practices Advisory Committee (HICPAC) recommends a strategy to control multidrug-resistant organisms (MDROs) that consists of seven elements: administrative support, education, judicious use of antibiotics, MDRO surveillance, infection control precautions, environmental measures, and, where possible, decolonization.[9] An integrated approach to infection prevention should address environmental contamination. HAIs increase length of hospital stay, during which time patients contaminate their surrounding inanimate environment.[10,11] The risk of a patient developing an HAI increased by 73% if the patient previously occupying the room had a vancomycin-resistant enterococcus (VRE), MRSA, *C. difficile* or *Acinetobacter baumannii* infection.[12] Investigations focusing on the recovery of planktonic organisms from patient records and computer keyboards has helped to emphasize the importance of 'hand touch surfaces'.[11,13–15] Enhanced cleaning decreases, but does not eliminate, MRSA and other MDRO environmental isolation rates.[11] However, decreased environmental contamination rates have been associated with decreased MRSA acquisition rates.[16]

We recently showed the presence of dry surface biofilms containing viable MDROs on five out of six furnishings from an ICU, including a sterile supply box, privacy curtain, venetian blind cord, see-through ward entrance door, and rubber from around a sink.[17] As bacteria within biofilm are many more times resistant to desiccation, removal by detergents, and inactivation by disinfectants, we suggested that the presence of biofilms may contribute to the maintenance of environmental contamination in the face of cleaning.[17–20]

In this study we investigated the prevalence of biofilms in the environment immediately surrounding the patient and the frequency with which *S. aureus* was incorporated into these biofilms. In addition 15 samples were subjected to next-generation sequencing to determine the mix and ratio of microbial species present in biofilms contaminating dry surfaces.

Methods

Sample collection

Samples were obtained from an intensive care unit in a fully air-conditioned hospital and stored in a fully air-conditioned laboratory (temperature range 22–25°C, humidity 57–72%). Following a two-step terminal cleaning protocol using neutral detergent followed by disinfection with 500 ppm chlorine (sodium dichloroisocyanurate dehydrate, Diversol5000, Johnson Diversey, Smithfield, NSW, Australia), items from the patient bedding ($N = 11$), patient surrounds ($N = 19$), and fixed furnishings ($N = 14$) were aseptically sampled by cutting out a segment of the furnishing using sterile gloves, forceps, pliers, scissors, or scalpel blades, depending on the material being sampled. Samples were stored in sterile containers and gloves and instruments were changed between each sample.

Aerobic culture

Sample sections, up to 2 cm^2, were sonicated in 4 mL of tryptone soya broth for 5 min, prior to 100 μL being spread over horse blood agar plates (HBA) as a general non-selective medium, Brilliance MRSA agar plates for the detection of MRSA, Brilliance VRE Agar Plates for the detection of VRE, and Brilliance ESBL agar plates for the detection of extended-spectrum beta-lactamase (ESBL)-producing Gram-negative bacteria (Oxoid Adelaide, Australia).[17] MRSA plates were incubated for 18–24 h, and VRE, ESBL and HBA plates up to 48 h, aerobically at 37°C. Positive MDROs were confirmed using a combination of Vitek2 GPS-IX or Vitek2 AST-N149 cards (for Gram-positive or -negative isolates respectively) (bioMérieux-Vitek, Hazelwood, MO, USA) and partial sequencing of the 16S rRNA universal eubacterial gene according to the method described by Kidd *et al.*[21]

Staphylococcus aureus-specific PCR

Samples were sonicated in 300 μL digestion buffer (50 nM Tris/HCl pH 7.5, 150 nM NaCl, 2 mM ethylenediamine tetra-

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acetic acid, 1% sodium dodecyl sulphate) in an ultrasonic bath (Soniclean, JMR, Sydney, NSW, Australia) for 15 min with a sweeping frequency of 42–47 kHz at 20°C. Lysozyme (Sigma, Sydney, NSW, Australia) was added to a final concentration 0.5 mg/mL and incubated at 50°C for 2 h. Proteinase K (Sigma) at a final concentration of 1 mg/mL was added, followed by a 2 h incubation at 56°C. Genomic DNA was extracted using phenol/chloroform and then ethanol-precipitated.

Sample DNA was subjected to *S. aureus*-specific real-time PCR targeting the *femA* gene using the following cycling conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 56°C for 30 s, and 72°C for 60 s.[22]

Microbiome of biofilm contaminating dry hospital surfaces

Fifteen samples were subjected to bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) of the V1–V3 regions of 16s rRNA gene to determine the bacterial community of dry surface hospital biofilm.

Pyrosequencing was performed using the Titanium platform (Roche, Basel, Switzerland) in a commercial facility (Molecular Research DNA Lab, Shallowater, TX, USA), as previously described.[23] Pyrosequencing data were analysed by QIIME software (Werner Lab, Cortland, NY, USA).[24] Operational taxonomic units were assigned against the RDP database (Ribosomal Database Project II).[25]

Scanning electron microscopy (SEM)

Following fixing in 3% glutaraldehyde, samples (up to 1 cm^2) were dehydrated in ethanol, prior to immersion in hexamethyldisilazane (HMDS, Polysciences, Inc., Warrington, PA, USA) for 3 min and sputter-coating with 20 nm gold film as previously described.[17] Samples shown to have bacteria attached to a surface and surrounded by extracellular polymeric substances (EPSs) were classified as biofilm positive.

Confocal laser scanning microscopy

Eighteen of the samples, which had been stored for 12 months in sterile containers at room temperature, were stained with a Live/Dead® BacLight™ Bacterial Viability Kit (Life Technologies), using the manufacturer's instructions. SYTO® 9 labels live bacteria with green fluorescence while the propidium iodide component labels membrane-compromised bacteria with red fluorescence. Stained samples were examined using an Olympus Fluoview 300 inverted confocal laser scanning microscopy system.

Statistical analysis

Student's *t*-test was used to compare the number of bacterial species in dry biofilms on patient bedding or patient surrounds with number of species in biofilms on the floor using SigmaPlot11 statistical program. The statistical analysis of the bTEFAP data was performed by QIIME scripts, Calypso software (http://bioinfo.qimr.edu.au/), and FigTree software (http://tree.bio.ed.ac.uk/software/figtree/).[24] Alpha diversity was calculated using the Shannon index and OUT Richness in QIIME. The default number of Monte Carlo permutations was used to calculate the *P*-values and the significance threshold was $P < 0.05$. Phylogenetic analysis was calculated by FigTree with the default setting.

Ethics and safety approvals

Ethics approvals were obtained from South Western Sydney Local Health District Research and Ethics Office (Reference: LNR/14/LPOOL/14) and the University of Western Sydney Human Research Ethics Committee (Reference: H10659). Safety approval was obtained from the University of Western Sydney Biosafety and Radiation Safety Committee (Reference B10072).

Results

Aerobic culture

Twenty-three of the 44 (52%) samples were cultured on HBA. MRSA-, VRE-, and ESBL-positive organisms were detected in eight, three, and five samples, respectively (Table I). At least one MDRO grew in 12 of the 23 (52%) culture-positive samples. Most of these MDROs were in the immediate patient vicinity, with 33% of mattresses and privacy curtains being positive for MRSA. One-third of mattresses were also positive for VRE and one mattress was positive for MRSA, VRE, and ESBL. The ESBL plates in general grew *Sphingomonas paucimobilis*. MDROs were less prevalent on fixed furnishings ($N = 14$) with isolation of MRSA from one floor sample and *S. paucimobilis* present in the wall biofilm.

Detection of S. aureus

Staphylococcus aureus was detected by *S. aureus*-specific PCR in 50% of the samples, including the eight out of 11 (72%) samples from patient bedding, eight of 19 (42%) samples from the patient's immediate environment, and six of 14 (42%) samples from fixed furnishings.

Visual confirmation of biofilm contamination

Forty-one out of 44 samples (93%) were visually confirmed to have biofilm infecting their surfaces either by SEM and/or by CLSM (Table I and Figure 1). SEM of biofilm sourced from areas not routinely cleaned and disinfected such as curtain cords and entrance doors showed bacteria of various morphologies embedded in thick amorous EPS (Figure 1A and B). Bacilliary, filamentous, and coccoid forms were evident on the mattress (Figure 1C) whereas cocci were more prevalent on hand touch items (Figure 1A, B, and D).

All 18 samples stained with bacterial viability stain showed live bacteria despite 12 months of storage at room temperature, demonstrating the stability of biofilm on dry surfaces (see Table I and Figure 2).

Microbiome of biofilm contaminating dry hospital surfaces

Dry surface biofilms were all polymicrobial by pyrosequencing and culture. The average number of species representing at least 1% of the biofilm was 29 (range: 11–42) for patient bedding and 23 (range: 10–32) for patient surrounds. Significantly fewer species were present in biofilms on the floor

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Table I
Prevalence of biofilm on intensive care unit dry surfaces

Item	*N*	Biofilm	Live at 12 months (*N* = 18)	PCR positive: *S. aureus*	Culture positive:			
					Non-selective media	MRSA	VRE	ESBL
Patient bedding								
Mattress	6	6	5	4	5	2	2	1
Pillow	5	5	3	4	3	1	0	1
Patient surrounds								
Curtain	9	8	4	5	5	3	0	1
Patient notes wire clip	2	2		0	0	–	–	–
Supply box	4	4	2	1	3	0	0	0
Glove box Velcro	1	1	1	1	1	0	1	1
Notice	3	3		1	2	1	0	0
Fixed furnishings								
Floor	3	3		1	3	1	0	0
Basin rubber	4	3		1	0	–	–	–
Bench top	2	1	1	2	0	–	–	–
Wall	1	1		0	1	0	0	1
Ward entry door	4	4	2	2	0	–	–	–
Total	44	41	18/18	22	23	8	3	5

N, the number of items collected; 'Biofilm', the number of samples with visual confirmation of biofilm presence by microscopy; 'Live at 12 months', the confirmation of live bacteria following 12 months of storage; PCR, polymerase chain reaction; MRSA, meticillin-resistant *Staphylococcus aureus*; VRE, vancomycin-resistant enterococci; ESBL, extended-spectrum beta-lactamase Gram-negative bacilli.

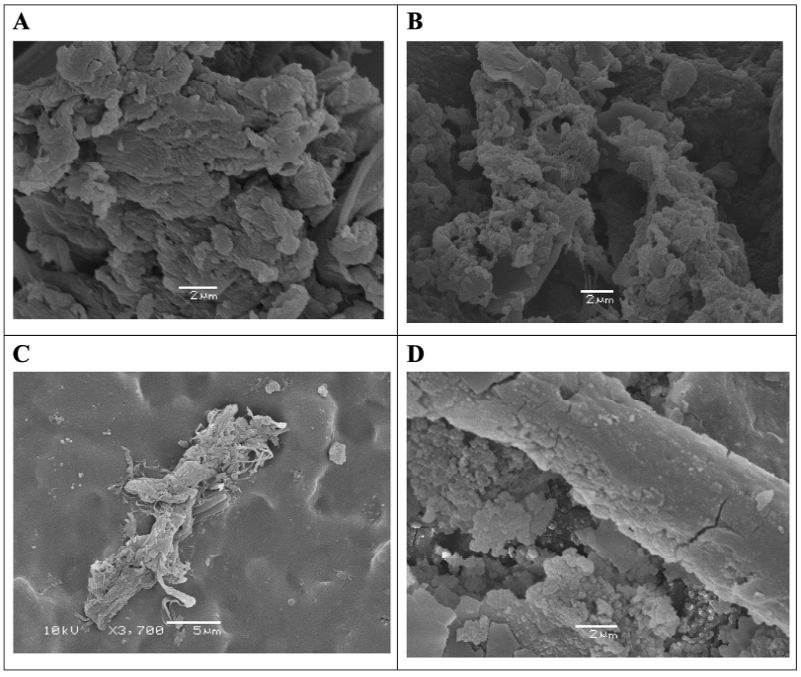

Figure 1. Scanning electron micrograph of biofilms contaminating surfaces in an intensive care unit. (A) Sample from a privacy curtain. (B) Sample from the ward entry door showing coccoid bacteria embedded in a thick amorphous extracellular polymeric substance (EPS). (C) Sample from a mattress showing biofilm containing bacteria of various morphologies including rod-shaped organisms, contaminating especially the natural depressions in the mattress. (D) Sample from a wire clip for holding patients' notes showing dense EPS with embedded coccoid bacteria.

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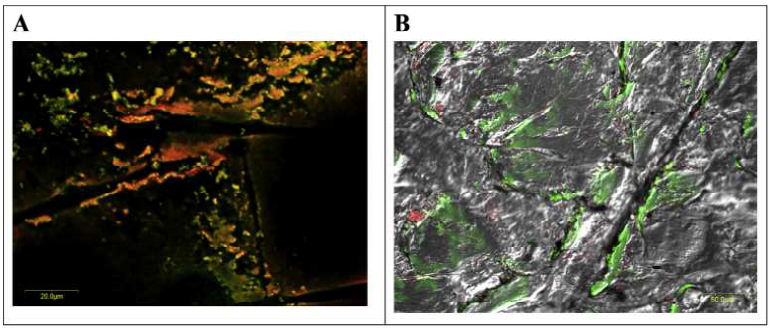

Figure 2. Confocal laser scanning micrograph of biofilm stained with BacLight Live/Dead stain showing the presence of live bacteria (green) and dead bacteria (red) on dry hospital surfaces. Staining conducted after 12 months of storage at room temperature. (A) Sample from a storage box used to hold sterile supplies of single-use patient equipment; this surface cultured *Staphylococcus aureus*. (B) Ward entry door confocal image of live/dead bacteria superimposed on a visual of ward entry door, showing relationship of bacteria to door topography (culture negative but positive for *S. aureus* by polymerase chain reaction).

(average: 8; range: 3–14) ($P = 0.02$). In all biofilms some bacterial species were more prevalent, representing 10% or more of the biofilm mass. The organisms detected with the highest frequencies are detailed in Supplementary Table I (online).

However, the most common bacterial species, on a percentage of biofilm sequence reads, across all the biofilms were *Faecalibacterium prausnitzii*, *Massilia timonae*, *S. aureus*, coagulase-negative staphylococci, *Pseudomonas* species and *Propionibacterium acnes* (Figure 3A). *Pseudomonas* species were found in 14 biofilms but only two contained *P. aeruginosa*: one floor sample and the sterile supply box. The most frequently occurring *Pseudomonas* species were *P. mendocina* and *P. stutzeri* both of which have caused rare opportunistic infections. *P. acnes* was also found in 14 biofilms, coagulase-negative staphylococci in 13, *F. prausnitzii* in 10, *S. aureus* in 11, and *M. timonae* in seven of the 15 biofilms. Other bacterial species were frequently found in the biofilms but only formed a small percentage of the biofilm. For example, *Acinetobacter* species including *A. lwoffii*, *A. calcoaceticus*, *A. haemolyticus*, *A. guillouiae*, *A. estunensis*, and *A. junii*, were found in nine biofilms, but in only three samples did they form more than 1% of the biofilm population. *Acinetobacter calcoaceticus* formed 4% of the population on the poster and *A. guillouiae*, *A. junii*, and *A. lwoffii* made up 8% of the biofilm population on the glove box Velcro®.

Most of the biofilms contained a mixture of organisms with various degrees of oxygen tolerance ranging from aerobic organisms to obligate anaerobes (Figure 3B). Only one floor sample had no anaerobic organisms, with the strictly aerobic organism *P. stutzeri* forming 72% of the biofilm. By contrast, a second floor sample incorporated very few aerobic organisms, with two out of the three bacteria being the anaerobic organism *P. acnes*. All the biofilms contained environmental organisms (Figure 3C). Patient bedding and patient surrounds items not surprisingly incorporated organisms normally encountered on the skin. Significantly more of the bowel-dwelling *Coprococcus* species and significantly fewer *Streptococcus* species were found on pillows compared to mattresses ($P < 0.05$).

Phylogenetic analysis showed that the microbiomes contaminating similar items were more closely related than the microbiomes contaminating different items for mattresses, curtains, pillows, and pillowcases (Figure 4). It is not surprising that three floor microbiomes were more divergent as contaminated by shoes carrying bacteria moving from place to place.

Discussion

More than 90% of the ICU surfaces contained demonstrable bacteria residing in biofilms, and these organisms included those that are important in healthcare-associated infections such as *S. aureus*. The presence of the pathogenic multidrug-resistant species was demonstrated by conventional bacterial cultures using chromogenic agar plates. These species were found together with other, non-pathogenic species, and in the hospital environment. Two microscopy techniques showed that these bacteria were present in biofilms. Furthermore, the confocal laser scanning microscopy showed living bacterial cells within the biofilms. These surfaces had been 'terminally cleaned' twice using cloths and hypochlorite solution and stored for more than 12 months. Nevertheless we demonstrated viable MDROs within biofilms using multiple techniques, confirming our hypothesis that MDROs reside in biofilms and are resistant to being removed. The mere detection of frank, viable pathogens within the built environment near the patient is of concern and increases the risk to the patient for acquiring an HAI as has been shown by several studies.[11,12,26]

This study adds to the weight of evidence that MDRO contamination of the hospital environment is significant, and that these organisms may remain viable for prolonged periods within dry surface biofilms. Australian hospitals are required to manage for quality and risk as part of their service provision under National Guidelines aimed at improving healthcare services.[27] The role of bacterial biofilms that support MDRO growth adds a level of difficulty to this risk management of environmental contamination that has not previously been recognized.

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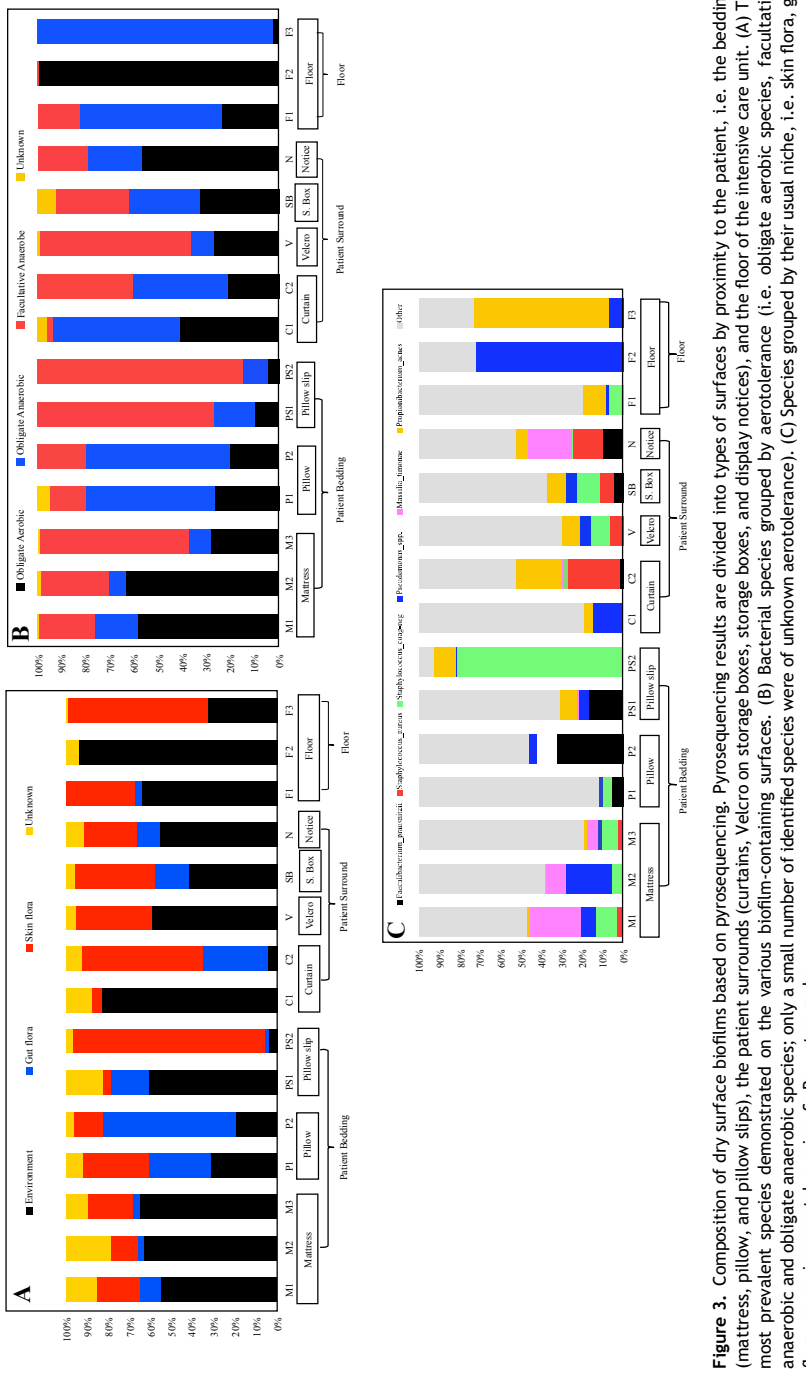

Figure 3. Composition of dry surface biofilms based on pyrosequencing. Pyrosequencing results are divided into types of surfaces by proximity to the patient, i.e. the bedding, (mattress, pillow, and pillow slips), the patient surrounds (curtains, Velcro on storage boxes, storage boxes, and display notices), and the floor of the intensive care unit. (A) The most prevalent species demonstrated on the various biofilm-containing surfaces. (B) Bacterial species grouped by aerotolerance (i.e. obligate aerobic species, facultative anaerobic and obligate anaerobic species; only a small number of identified species were of unknown aerotolerance). (C) Species grouped by their usual niche, i.e. skin flora, gut flora, or environmental species. S. Box, storage box.

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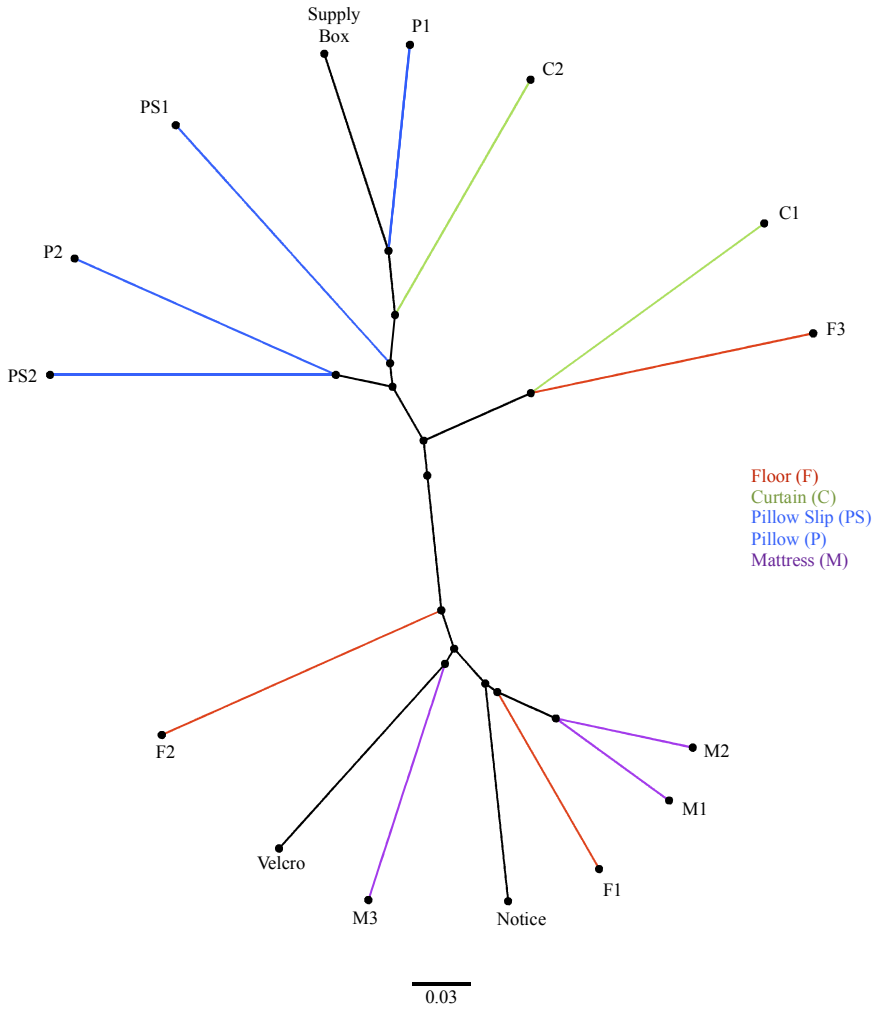

Figure 4. Radial phylogenetic tree showing the relatedness of biofilms on different dry surfaces based on the composition of bacteria within the biofilm. Phylogenetic analysis showed that the microbiomes contaminating similar items were more closely related than the microbiomes contaminating different items for mattresses (purple), curtains (red) and pillows and pillowcases (blue). However, the three floor microbiomes (green) were more divergent.

With the two microscopy techniques we can show structures embedded in biofilms that have the size and morphology consistent with the various species of pathogenic bacteria; however, non-pathogenic species also have the same appearance. Demonstrating the biofilms requires destructive sampling, and thus it is not possible to process the same material for microscopy, culture, and pyrosequencing.

Environmental contamination plays a major role in transmission of infection, particularly of MRSA.[11] In addition, the hands of healthcare workers are twice as likely to be contaminated with MRSA from environmental sources than by direct contact with infected patients.[28] We therefore conducted *S. aureus*-specific PCR to determine the frequency of non-culturable contamination. Half of the samples were contaminated with *S. aureus*; not surprisingly, more of the patient bedding was contaminated than samples obtained more distantly from the patient or the floor. Similarly, surfaces closest to the patient have been found to be more heavily contaminated with regard to total contaminating bacteria, MRSA, and VRE, than surfaces further away.[29,30]

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We have shown that 93% of dry hospital furnishings are contaminated with biofilm. This is worrying in view of the difficulty in killing bacteria incorporated into biofilm. Many biofilm bacteria typically survive more than 50 times the amount of disinfectant needed to kill the same bacteria growing planktonically (free swimming) in liquid culture (reviewed in Bridier *et al.*[31]). The increased resistance of biofilms to biocide is thought to be due to changes in bacterial gene regulation (resulting in phenotypic adaptation) and to the EPS surrounding the bacteria. The EPS slows penetration of biocides into the biofilm, inactivates some disinfectants by binding to them, and inactivates some disinfectants by excretion of enzymes, for example catalase destruction of hydrogen peroxide. Additionally there is phenotypic adaption of cells to sub-lethal disinfectant concentration and increased lateral gene transfer and mutation rates.[31] Biocide resistance is due to the biofilm lifestyle, as, when the biofilm structure is disrupted, the bacteria once again become susceptible to biocides.

Biofilms are usually found in aqueous environments, yet we found them on dry surfaces. We surmise that there must be a source of water and nutrients, and a seeding by bacteria to initiate the process. Further studies are underway to find out how this might occur.

Rehydration may occur by contact with patient secretions, such as perspiration, blood, urine or vomitus, and inefficient cleaning may deposit additional solids, thus supporting biofilm growth on environmental surfaces. Additionally, exposure of biofilms to disinfectants can increase EPS production fivefold.[32] Many of the biofilms contaminating dry surfaces in the ICU appear to have very thick biofilm (Figure 1) which would contribute to their desiccation and disinfectant resistance. Indeed viability staining of biofilms, maintained in a fully air-conditioned laboratory 12 months after collection, showed that all 18 tested were principally composed of live bacteria. This included seven samples that were culture negative at collection. Just under one-half of the samples were culture negative at collection and these may reflect the basic aerobic culture conditions. However, it is well known that biofilm bacteria are difficult to culture, which is thought to be due to their low metabolic rate.[33] Of the samples that were culture positive, approximately half grew an MDRO. The presence of MDROs, protected from disinfectant action in biofilms, has implications for infection control, as biofilms intermittently release planktonic bacteria back into the environment which can then infect new niches, or infect patients.[34]

Species interactions within polymicrobial biofilms can have adverse effects on cleaning and disinfection. *Acinetobacter calcoaceticus* and *A. lwolffii* have both been shown to enhance production of other species' biofilm mass when co-cultured.[35,36] In this study *Acinetobacter* species were incorporated into nine of 15 dry surface biofilms. Additionally, polymicrobial biofilms are more resistant to disinfectants than mono-species biofilms.[36] The mechanism of this increased resistance is unknown but could result from increased disinfectant inactivation due to a more complex EPS or shielding of sensitive organisms by externally situated disinfectant tolerant organisms.

The number of species forming the biofilm was highest closest to the patient, followed by items in close proximity to the patient. Despite the biofilm on the floor being multilayered, it was composed of significantly fewer species ($P = 0.02$). The moist microclimate closer to the patient may increase survival of planktonic bacteria, allowing the incorporation of more species into the biofilm; whereas more aggressive/frequent cleaning and chemical disinfection of the floor could kill more planktonic organisms, thus decreasing the number of species incorporated into the biofilm. Or it may be that the higher number of species incorporated into the biofilm is directly related to the distance from a sick patient transmitting large numbers of bacteria.

Although the samples obtained in this study were from a fully air-conditioned hospital, the hospital is situated in a temperate region of Australia, so the type and number of bacteria contaminating fomites might not be generally applicable to hospitals situated in climates that experience extreme cold, such as in northern Europe, or hot and dry conditions as experienced in desert regions. However, there is an important implication for the widespread reliance on chlorinated disinfectants which are widely recommended by the Australian Federal Government and others.[37,38] Previous work has shown that well-applied cleaning protocols have superior performance over poorly used chlorinate-based disinfecting products.[39] The value of wiping removal of common nosocomial pathogenic species is also important, having more impact than a strongly formulated surface disinfectant.[40] The apparent survival and flourishing of the broad array of HAI-related bacteria within these biofilms strongly suggests that these bacteria should be viewed as resident rather than transient.

This study demonstrated that dry surface biofilms containing MDROs may be present on inanimate surfaces in a hospital environment, and were detected despite cleaning with hypochlorite. It suggests yet another reservoir of organisms that may be transmitted to patients to cause HAIs. More research is needed to determine the extent of this problem, and the cleaning agents and techniques required to remove dry surface biofilms from hospital environments.

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Conflict of interest statement
None declared.

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.jhin.2015.05.016.

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Chapter 4

An Interesting Finding.

"Do not despise the creatures
because they are minute...
doubt not that in these tiny
creatures are mysteries more
than we can ever fathom."

- Charles Kingley, 1855

4 Detection of Bacterial Biofilm in Double Capsule Surrounding Mammary Implants: Findings in Human and Porcine Breast Augmentation

4.1 Introduction

The finding of a double capsule surrounding breast implants is a rare and poorly understood complication of breast augmentation surgery. In a recent review, the largest cohort published to date, double capsules were identified in 14 out of 626 (2.2%) breast implants over a twenty-seven year period in a single surgeon's practice.[506] This likely represents an under-reporting of its true incidence as the diagnosis of a double capsule was unexpected and only detected after surgical intervention for other complications, including asymmetry (6/14), capsular contracture (5/14) and late seroma (3/14).[506] All the cases had Biocell (Allergan Inc, Irvine, Ca) textured surface implants at initial surgery.[506] These findings are consistent with other case reports with diagnosis of a double capsule being associated with late seroma and textured implants,[507-509] implant bleed and textured implants,[510] asymmetry and double capsule[511, 512] and minor trauma and implant rotation.[513-515]

Several theories have been postulated to explain formation of double capsules. Some authors have suggested that mechanical forces, shear forces and textured implant surface

may be important in the underlying aetiology.[506-508, 512, 513, 515] These mechanical forces cause shear between the capsule, the tightly adherent textured implant and breast tissue resulting in capsule separation from the implant, then formation of seroma/hematoma and subsequent organization into a double capsule arrangement.[506, 515]

To date there has been limited scientific analysis of double capsule specimens to investigate this phenomenon. Histopathology analysis showed dense fibrous tissue with many giant cells, a marked active chronic non-specific inflammatory cell infiltrate.[510] For our analysis, we have used microbiological, molecular and scanning microscopic techniques to investigate 4 double capsules, 2 obtained from a human patient and 2 obtained from our previously described porcine model.[435]

4.2 Article – Case Report

Detection of Bacterial Biofilm in Double Capsule Surrounding Mammary Implants: Findings in Human and Porcine Breast Augmentation

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Plastic and Reconstructive Surgery 2012;129(3):579e-580

Acknowledgement/Contribution

Dr Anita Jacombs: Co-Author

I performed scanning electron microscopy for porcine specimens.

I prepared manuscript.

Ethics Approvals: University of Sydney N00/12-2010/2/5420 (Appendix 1)

Macquarie University 5201100027 (Appendix 1)

Table 1. Reported Cases of B-Cell Lymphomas Associated with Breast Implants

Reference	Age (yr)	Sex	Implant	Implant Compromised	Capsular Contracture	Lymphoma Type	Location	Time from Implantation to Lymphoma (yr)	Presentation
Cook et al., 1995[1]	56	F	Silicone	Yes, leakage*	Yes	Extranodal follicular mixed lymphoma	Left breast, medial to implant and bone marrow	6	2-cm palpable nodule
Said et al., 1996[3]	46	F	Silicone	No	NR	Primary effusion lymphoma	Right breast within capsule	5	Swelling in right breast, fluid surrounding implant within capsule
Kraemer et al., 2004[2]	55	F	Silicone	Yes, leakage	NR	Lymphoplasmacytic lymphoma	Bone marrow	25 (implants removed after 17)	Low-grade fevers, enlarged lymph node, high levels of IgM, and monoclonal gammopathy
Present study	58	F	Saline silicone	Yes, rupture	Yes	Nodal marginal zone B-cell and follicular lymphoma	Left axilla	20 since saline, 9 since silicone	3-cm palpable lymph node, fatigue

F, female; NR, not reported; IgM, immunoglobulin M.
*The capsule was no longer intact, and nonpolarized, refractile foreign material was found within the foreign body giant cells. Cook et al. conclude that this is most likely consistent with silicone migration or leakage, but confirmatory tests were not available.

DISCLOSURE

The authors have no financial disclosures or conflicts of interest to report.

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Detection of Bacterial Biofilm in Double Capsule Surrounding Mammary Implants: Findings in Human and Porcine Breast Augmentation

Sir:

The finding of a double capsule surrounding breast implants is a recognized complication of breast augmentation surgery. In a recent review, double capsules were identified in 14 of 626 breast implants.[1] We report the detection of incidental double capsules both in a patient and from our previously described porcine model.[2]

A 30-year-old woman with rapid development of bilateral Baker grade III capsular contractures and seroma was found to have bilateral double capsules at the time of surgery in November of 2010. Also, six custom smooth breast implants (TyRx, Inc., Monmouth Junction, N.J.) were inserted into two adult female nonlactating white pigs using sterile surgical techniques as part of a larger study. Each breast implant was inoculated with 10^6 *Staphylococcus epidermidis*. The implants were removed at 20 weeks, and two were noted to have double capsules. Samples were subjected to enhanced microbiology, scanning electron microscopy, and polymerase chain reaction to detect the *icaA* gene, a potentiator of biofilm formation.

Enhanced cultures from each breast capsule grew *S. epidermidis*. Polymerase chain reaction analysis of the isolated bacteria showed *icaA* positivity. Scanning electron microscopy analysis of both capsules detected the presence of cocci-shaped bacteria colonies encased in exopolysaccharide as biofilm on the surfaces of both capsules (Fig. 1).

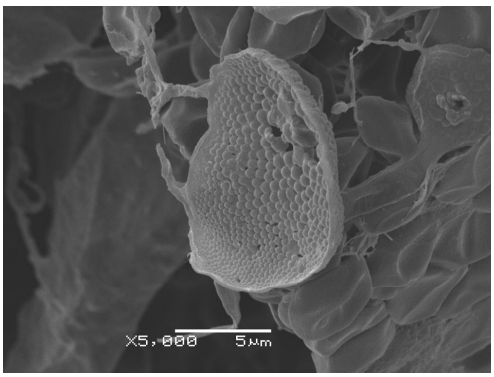

Fig. 1. Biofilm on the surface of the human double capsule showing cocci encased in bacterial proteins.

Blinded analysis of the pig before explantation revealed Baker grade III and IV capsular contracture around these implants. Further laboratory dissection revealed the presence of double capsules in both specimens (Fig. 2). Enhanced cultures revealed the growth of *S. epidermidis* and polymerase chain reaction analysis showed *ica* gene positivity. Scanning electron microscopic analysis of both capsule specimens confirmed the presence of cocci-shaped bacterial colonies encased in exopolysaccharide as biofilm.

The detection of double capsule in association with bacterial biofilm is a novel and potentially important finding. The double capsules in our human samples were associated with both seroma and Baker grade III capsular contracture. The finding of a double capsule in the porcine model represents the first in a nonhuman species and was associated with both contracture and deliberate inoculation with *S. epidermidis*. Microscopic, molecular, and microbiological analyses have identified staphylococcal bacterial biofilm infection of the capsule from all four specimens. This is consistent with our previous findings that subclinical bacterial biofilm infection is an important factor in the pathogenesis of capsular contracture.[2]

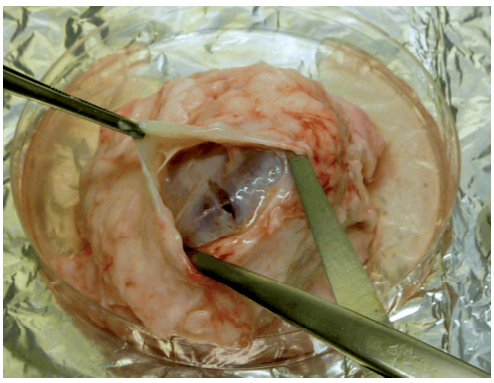

Fig. 2. Dissection of pig breast capsule demonstrating a double capsule surrounding a breast implant left in situ.

Our findings suggest that chronic infection with a bacterial biofilm may also be an important etiologic factor in double capsule formation. The reported association by Hall-Findlay of double capsule with textured implants and late seroma is also interesting,[1] as both these clinical parameters have been reported in patients with anaplastic large cell lymphoma following breast augmentation.[3] There is strong evidence that chronic bacterial infection can cause lymphoma in both humans[4] and animals.[5] It could be that the finding of bacterial biofilm in these double capsule specimens may point to an important and as yet unreported causative association between chronic bacterial biofilm infection and the genesis of this rare malignancy.

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DISCLOSURE

Dr. Deva has been a consultant to Johnson & Johnson Medical. The Surgical Infection Research Group is currently engaged in contract research with Allergan, Inc.

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A Novel Technique for Nipple-Areola Complex Reconstruction: The Acellular Dermal Matrix Onlay Graft

Sir:

In breast reconstruction, the final surgical step is creating the nipple-areola complex. Nipple reconstruction using a skate flap, star flap, or C-V flap uses available skin from the breast and leaves an open wound that is closed primarily or skin grafted to avoid distortion or flattening of the breast.[1] Skin grafting for areola reconstruction gives an aesthetically pleasing texture and color difference to the areola but necessitates a secondary donor-site deficit. We present a series of 12 nipple-areola complex reconstructions using acellular dermal matrix (AlloDerm; LifeCell Corp., Branchburg, N.J.) as an onlay graft. Acellular dermal matrix use in breast reconstruction as an implantable material is well documented for primary expander/implant reconstruction, for secondary contour deficits, and for projection in nipple reconstruction.[3,4] Our technique is a novel use for acellular dermal matrix in breast reconstruction. The principles outlined in the Declaration of Helsinki were strictly observed in this case series study. Informed consent was obtained from all participants. A formal institutional review board process was not available.[2]

Nine patients and 12 nipples of breast cancer or *BRCA*-positive patients following mastectomy were treated. Skate flaps were used for nipple reconstruction. The planned areola area was then marked on the breast dome with sizes ranging from 40 to 45 mm, matching the contralateral areola if present. This area was thinly deepithelialized, leaving a thick dermal base. Acellular dermal matrix was reconstituted by standard practice and cut to size. A central cruciate opening was created to accommodate the nipple flap. The acellular dermal matrix graft was then sewn into place using 5-0 chromic vertical mattress sutures and a petroleum jelly gauze bolster. The bolster remained in place for 5 days, following which Aquaphor was applied until epithelialization was complete.

All 12 areolae revascularized with 100 percent graft take. The average time to complete reepithelialization was 6 weeks (Fig. 1). All patients had satisfactory transition from native skin flap to nipple-areola complex.

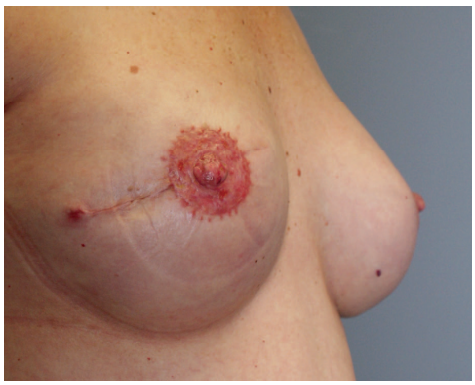

Fig. 1. A 41-year-old woman presented with invasive breast carcinoma of the right breast following right mastectomy and left nipple-sparing prophylactic mastectomy 4 weeks after nipple-areola complex reconstruction with acellular dermal matrix. Epithelialization is taking place over the granulation tissue that has formed in the acellular dermal matrix.

Two patients required subsequent nipple projection. Six areola complexes were tattooed for color, with plans to tattoo others after adequate healing. All patients have been satisfied with the appearance of their reconstructed nipples (Fig. 2).

In our innovation for areola reconstruction, the risk of a donor site for a full-thickness skin graft is weighed against the average cost of a 4 × 7-cm or 4 × 12-cm acellular dermal matrix sheet ($31 per cm^2, or $480 to $1500). The price hurdle can possibly be overcome by banking excess acellular dermal matrix at the time of breast reconstruction for later use.[5] Epithelialization of the graft takes up to 6 weeks and requires patient in-

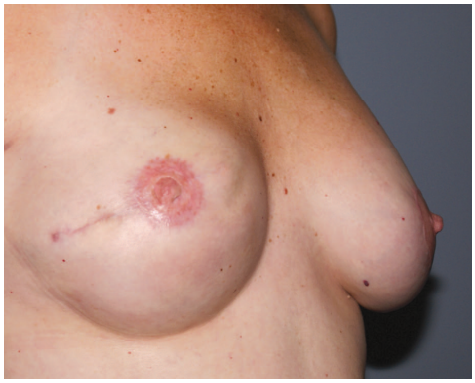

Fig. 2. At 7 weeks postoperatively, the reconstructed areola is fully epithelialized, with good color and texture distinction of the nipple-areola complex.

Chapter 5

Is there Infection after Implant Insertion?

"A fact is a simple statement that everyone believes.

It is innocent, unless found guilty.

A hypothesis is a novel suggestion that no one wants to believe.

It is guilty, until found effective."

- Edward Teller

5 The Subclinical Biofilm Infection Theory of Breast Implant Infection – Is There a Role for Endogenous Breast Flora in Biofilm Infection of Silicone Breast Implants in a Porcine Model?

Unpublished Data

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Acknowledgement/Contribution

Dr Anita Jacombs: Author

I performed pig surgery, specimen retrieval, applantation tonometry.

I performed scanning electron microscopy.

I performed the DNA extraction and polymerase chain reaction for bacterial counts

Ethics Approvals: University of Sydney N00/12-2010/2/5421 (Appendix 1)

5.1 Introduction

It is 50 years since silicone breast prostheses were first used for breast augmentation surgery and capsular contracture still remains the most common postoperative complication, accounting for a significant number of re-operations.[427, 516, 517] The reported rates of capsular contracture vary from less than 10% to over 50% for aesthetic breast augmentation and up to 80% following insertion of implants for breast reconstruction after mastectomy.[426, 428-430]

Formation of a fibrous capsule is a normal healing process after a surgical implant is placed within the human body, which is mediated by the immune system in response to the foreign material.[66, 453, 518, 519] Capsular contracture will occur when the actions of pro-inflammatory potentiators, such as bacteria, tissue trauma and blood, exceed the effects of inflammatory suppressors, such as antibiotics, antimicrobial irrigation, surgical technique or possibly implant surface. If the peri-prosthetic inflammatory process has a stimulus to persist, pathological fibrosis of the capsule can occur.[426, 453, 518] The capsule then becomes stiff and tight, compressing the malleable implant.[518] Clinically this can result in an increase in breast firmness, deformation and even displacement of the implant as well as discomfort and pain.[518, 520]

The subclinical infection hypothesis of breast implant infection states that the prosthetic surface becomes contaminated by bacteria, and that once attached to a surface the bacteria undergo genotypic and phenotypic changes to a sessile physiology and irreversibly bind to the surface ultrastructure (Figure 1-11), (1.10.2.3).[23, 90] The attached sessile bacteria begin secreting a highly adherent mucoid glycoprotein coat of exopolysaccharides to form a biofilm.[12, 129, 521] The biofilm is a key stimulus of peri-prosthetic inflammation that ultimately results in a chronic fibrosis formation of capsular contracture. There is now compelling clinical and pre-clinical evidence that bacterial contamination at the time of implant insertion is the major cause of capsular contracture, as discussed in 1.10.2.3.[522, 523]

The Subclinical Infection Hypothesis also suggests that there are two other potential sources of bacterial contamination, namely local spread from endogenous bacteria within the breast glands and ducts, and systemic haematogenous spread.[89] The human breast is an ectodermally derived modified sweat gland. It has 15-20 glands placed radially within the breast tissue that drain via their lactiferous ducts to openings on the nipple in the centre of the areola.[524] Several studies have demonstrated that the nipple and periareolar region harbours numerous endogenous bacteria commonly *S. spidermidis* and *P. acnes*, as well as *S. aureus* and other coagulase-negative Staphylococcal species with the occasional *Lactobacillus* spp, *Streptoccus* spp, *Enterococcus,* and *Clostridium* species.[439, 525-527] Breast secretions, sampled at the time of breast augmentation, have also been cultured by one group, with *S. epidermidis* the most common bacteria cultured as well as a small number of *Bacillus subtilis* and Diptheroids.[439] Another group has shown that, in one third of patients, these bacteria persist in the nipple and areola throughout the duration of implant augmentation surgery despite routine preoperative skin preparation.[526] Interestingly, in a recent review of 1400 consecutive implant augmentations by a single unit, women falling pregnant post-implant augmentation had double the rate of capsular contracture compared to the remainder of the group.[431] Pregnancy, with or without breastfeeding, is a time for significant growth and activity of breast tissue and this may increase the risk of endogenous contamination of the implant. Together these studies show that the bacteria that commonly colonise the breast, mammary gland and ducts are the same as the bacteria commonly identified in capsular contraction samples and hence they are a possible source of endogenous implant contamination.[88, 89, 437, 440, 445, 446]

Currently there is limited evidence for haematogenous spread of breast implants from the clinical literature on capsular contracture. In fact the main evidence is indirect from research on other implants including orthopaedic prosthesis and implantable cardiac devices. Prosthetic joint infection is a devastating complication that occurs in 0.6-2.4% of primary arthroplasty operations.[327, 369, 399] With more than 550,000 joint arthroplasy operations in the US in 2007 and 85,000 knee and hip arthroplasties done in Australia in 2013, this would result in over 5,500 and 850 prosthetic joint infections per annum in

these two countries alone.[395, 399] Haematogenous spread secondary to a bacteriaemia is one of the most common aetiologies responsible for up to 40% of prosthetic joint infections.[364, 407, 528] The most common sources for the bacteraemia are *S. aureus* sepsis, skin infections and uropathy.[528-530] Interestingly, the evidence for the role of inoculation from dental bacterial, previously speculated in the orthopaedic literature, has not been supported by clinical evidence.[530, 531] With haematogenous spread an important source for prosthetic joint infections, there has been speculation as to the importance of this route with silicone breast prostheses.[89, 426, 523]

The role of endogenous bacterial infection of breast implants was evaluated using the pre-clinical porcine model and evaluated whether bacteria within biofilm infected breast implants changes overtime.

5.2 Material and Methods

5.2.1 Surgical Procedure

Three adult, female, nonlactating, domestic Large White pigs (*Sus domesticus*), weighing approximately 350 kg each, individually received 8 miniature silicone implants (2 cm saline filled Biocell™ or smooth implants Allergan Inc, Irvine, CA).

The implants were placed into 8 submammary pockets that had been dissected using surgical methods as described in 2.1.1 and by Tamboto *et al.*[435] A total of 24 implants were inserted, 12 smooth surface and 12 Biocell™ textured surface implants. Half of all implants were inoculated with 1 ml of 10^5 colony-forming units of a human clinical strain of *S. epidermidis*#7 (Chapter 2.3.2.2)[435] into the pocket after insertion of the implant. The pockets were closed in two layers using standard surgical techniques. The implants were left *in situ* for 28 or 36 weeks. At time of removal Baker Grade assessment was completed on all implants by two surgeons that were blinded to the implant type and treatment. The implants were removed as described in 2.1.2 and by Tamboto *et al.*[435]

5.2.2 Laboratory Procedures

The excised capsules were investigated using Scanning Electron Microscopy (2.4.1) to identify the presence or absence of biofilm and characterisation of the structure of bacteria that may be involved.

The bacteria were identified using molecular identification using 16S rRNA sequencing (2.5.3.1). The bacterial loads within the biofilm were assessed using quantative PCR of the 16sRNA gene and expressed as bacterial numbers/mg based on quantitation of the pig 18s gene (2.5.3.2).

5.3 Results

A total of 24 implants were surgically implanted in 3 adult sows and left *in situ* for 28 or 36 weeks. Four implants were extruded from their pockets and where lost prior to surgical removal (one of the 28 week implants and three of the 36 week implants), resulting in seven implants for 28 weeks and thirteen for the 36 week implants. No pig exhibited systemic signs of infection relating to either bacterial inoculation and/or presence of a surgical implant.

Bacterial biofilm formation on the capsules was confirmed with scanning electron microscopy in sixteen capsules of the twenty capsules. Biofilm positive capsules demonstrated thick biofilm over the imaged surface (Figure 5-1 and 5-2). This was associated with loss of normal fibrous architecture and presence of coccoid bacteria in all samples (Figure 5-1 and 5-2). The remaining four capsules demonstrated normal fibrous architecture and all had scant patches of biofilm, all but one capsule had coccid bacteria visualised (Figure 5-3).

Bacterial number/mg of tissue increased from Baker Grade I to Baker Grade II and III with a plateau between Baker Grade III and IV (Figure 5-4).

Nineteen of the 26 capsules were culture positive and individual isolates were molecularly identified as per 2.5.3.1. Fourteen capsules had two or more bacteria identified, four capsules from the 28 week group and ten capsules from the 36 week

Figure 5-1 Low Magnification (x1500) Images of Biofilm Infected Breast Capsules

A – Thin lace biofilm covers the capsular fibres. Coccoid bacterium identified with a blue arrow.

B – Thick biofilm encases the capsule with loss of normal fibrous architecture. Biofilm lace visible, marked with a red arrow.

C – Patchy thick and thin biofilm with small clusters of coccoid bacterial visible marked with the orange arrows.

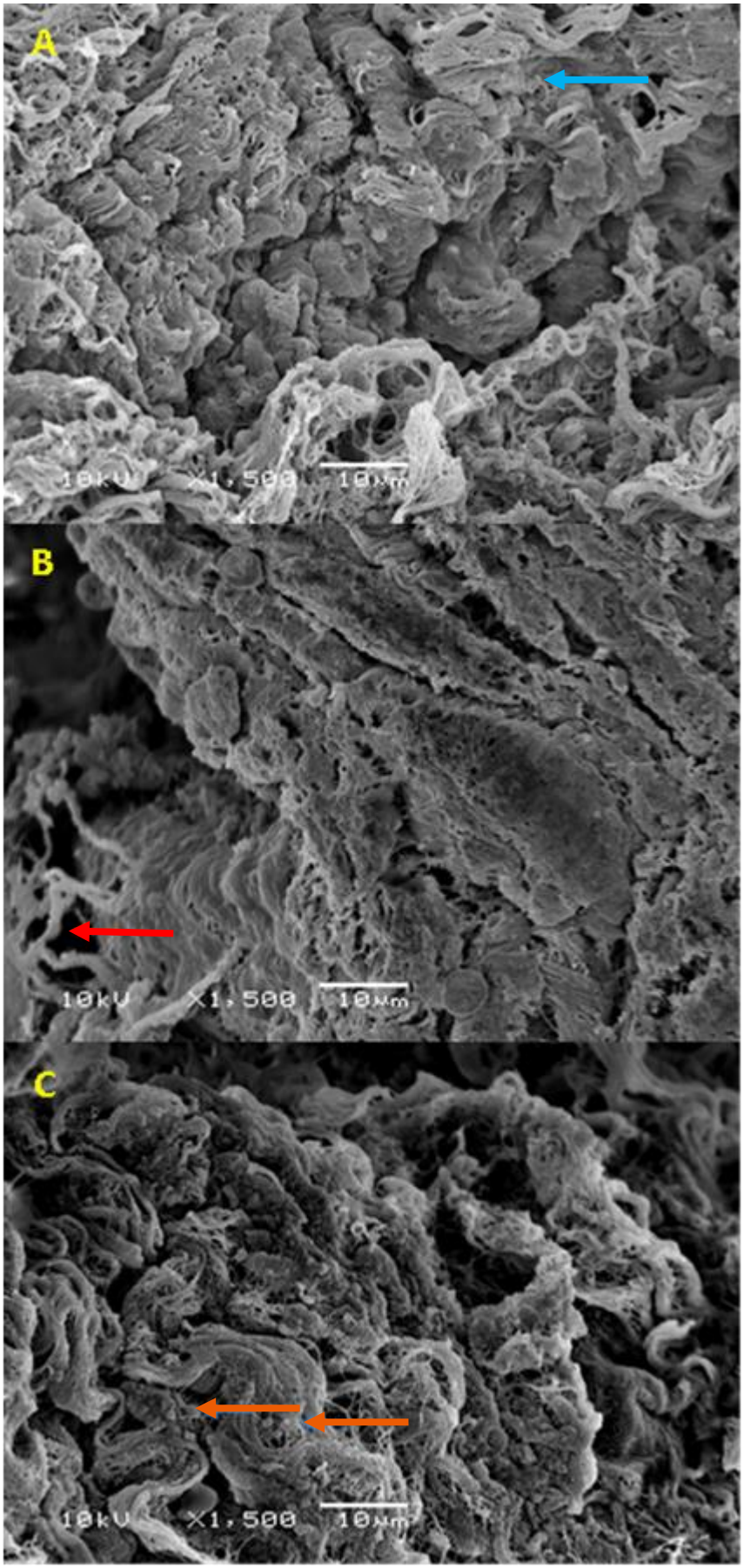

A
10kV X1,500 10µm
B
10kV X1,500 10µm
C
10kV X1,500 10µm

Figure 5-2: High Magnification (x5000-x6500) Images of Biofilm Infected Breast Capsules

A – Thick biofilm covering with scattered coccoid bacteria clusters throughout the frame, some are marked with blue arrows and loss of normal fibrous architecture.

B – Thick biofilm blanketing the capsule and coating individual strands.

C – Extensive biofilm and lace covering the fibrous capsule. Coccoid bacteria marked with orange arrow.

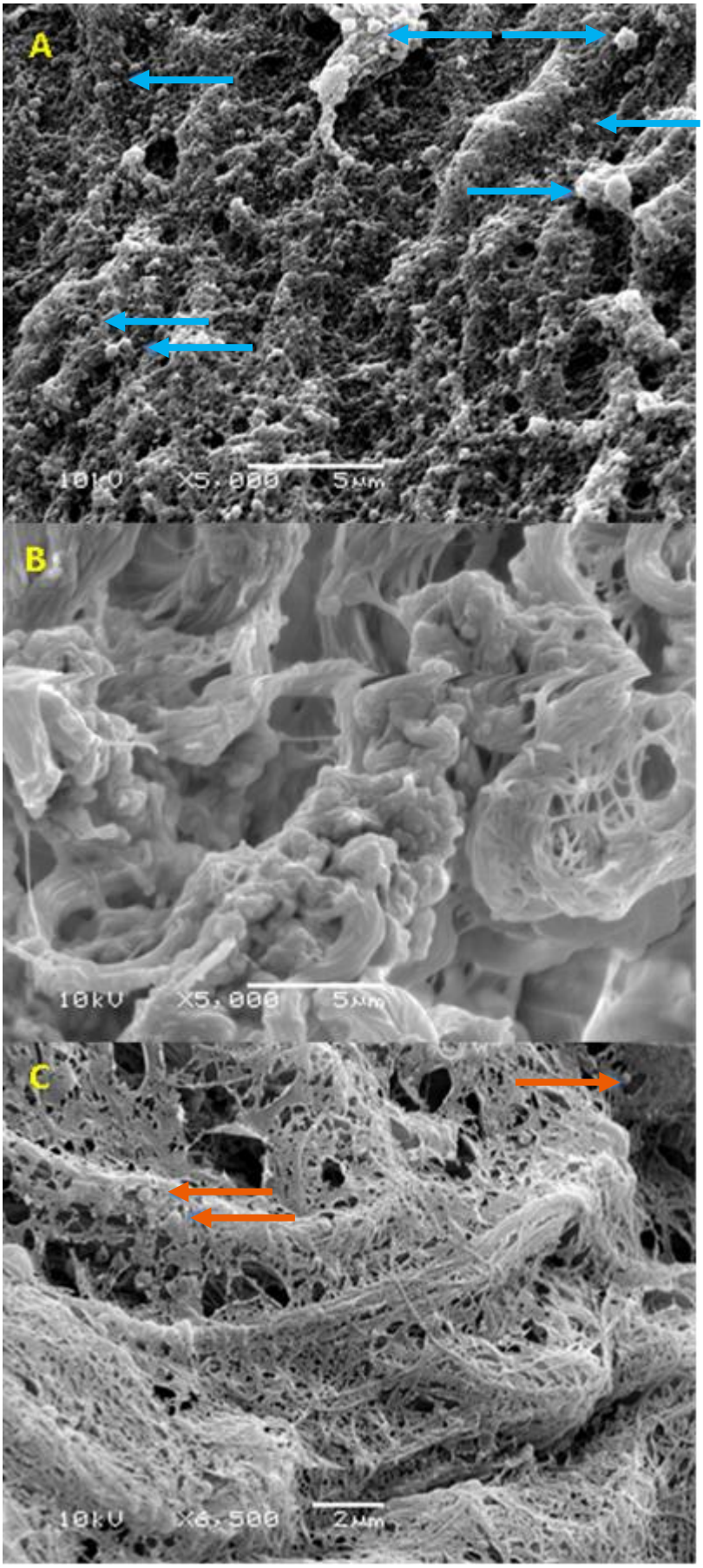
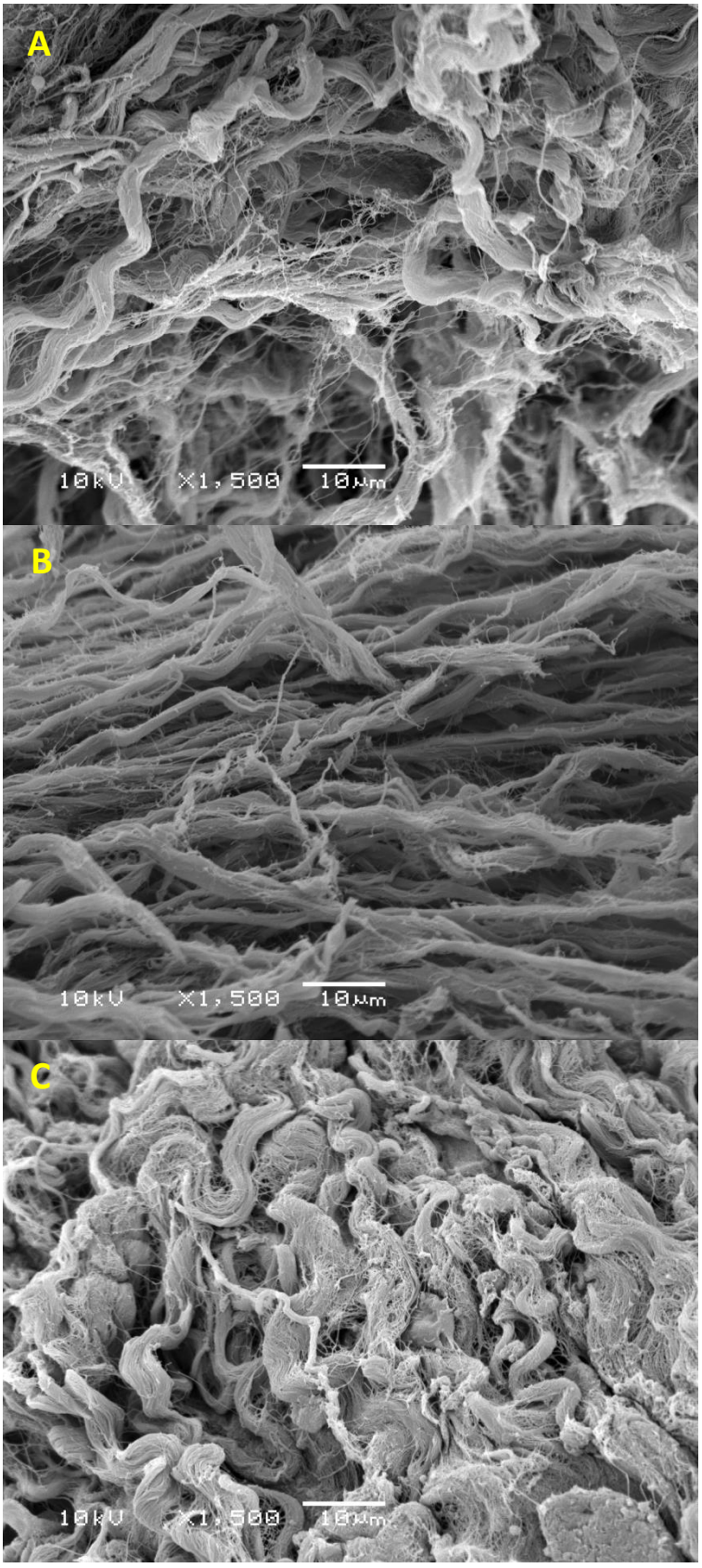

A
10kV X5,000 5μm
B
10kV X5,000 5μm
C
10kV X8,500 2μm

Figure 5-3 Low Magnification (x1500) Images of Normal Breast Capsules

A, B and C – Normal fibrous capsules.

There are minimal to no cellular structures or debris, nil lymphocytes or bacteria.

No biofilm extracellular polymeric substance, lace or biofilm material.


A
10kV X1,500 10µm
B
10kV X1,500 10µm
C
10kV X1,500 10µm

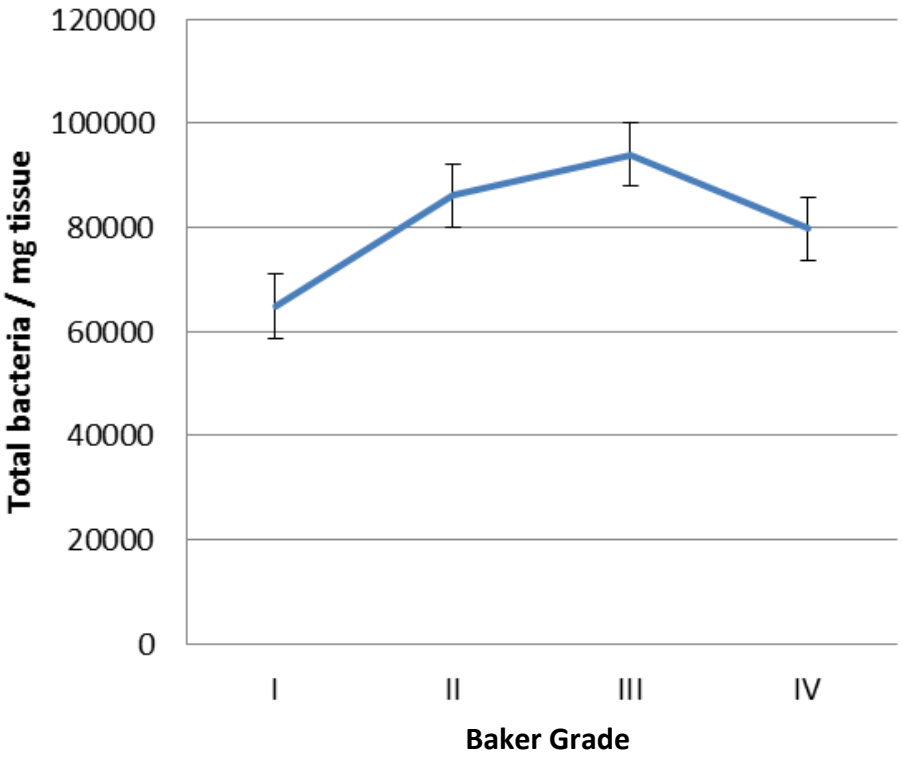


Figure 5-4: Trend in Bacterial Load with Clinical Capsular Contracture

Mean Bacterial Count based on simultaneous quantitative PCR of the universal bacterial gene 16S rRNA and the pig 18s gene with standard error bars.

Bacterial counts showed a significant increase in bacterial counts from Baker Grade I to Backer Grade II, III and IV capsules. There was a plateau between Baker Grade III and IV, although this was not significant.

group. Six capsules had four or more bacteria identified and one capsule had seven bacteria identified. The most common bacteria identified were *Staphylococcal* species including *Staphylococcus simulans* (common aetiological agent for bovine mastitis[532, 533]) and these were found in all but 3 capsules (Table 5-1).

Staphylococcus lugdunensis (important biofilm bacteria causing soft tissue infection in humans [201]) was found in 8 capsules, and at least one coagulase negative Staphylococcus species was found in all but one capsule. Numerous other bacterial skin commensals and

Capsules Explanted at 28 weeks from one pig	**Number of Capsules (6 capsules)**	**Capsules Explanted at 36 weeks from two pigs**	**Number of Capsules (13 capsules)**
Staphylococcus simulans	3	*Staphylococcus simulans*	12
Staphylococcus lugdunenis	3	*Staphylococcus lugdunenis*	6
Corynebacterium confusum	2	*Streptococcus parauberis*	3
Micrococcus luteus	1	*Arthrobacter gandavensis*	2
Staphylococcus condimenti	1	*Escherichia coli*	2
Aerococcus viridans	1	*Staphylococcus chromogenes*	2
Staphylococcus epidermidis	1	*Staphylococcus saprophyticus*	2
Corynebacterium xerosis	1	*Bacilus foraminis*	1
		Corynebacterium xerosis	1
		Leclercia adecarboxylata	1
		Staphylococcus epidermis	1
		Other CoNS*(6)	1 each
		Streptococcus porcinus	1
		Trichococcus pasteurii	1

* CoNS - (Coagulase Negative Staphylococcal) - *S. equorum*, *S. haemolyticus*, *S. intermedius*, *S. microti*, *S. warneri* and *S. xylosus*.

Table 5-1: Molecular Identification of Bacteria from Pig Breast Capsules at 28 and 36 weeks

Bacteria identified from sequence homology with the number of capsules containing each bacterial species. Fourteen capsules (74%) contained two or more bacteria.

1) 14 capsules had 2 or more different bacterial species present (4 from 28 week group and 10 from 36 week group),
2) 6 capsules had 4 or more different bacterial species present (1 from 28 week group and 5 from 36 week group),
3) 1 capsule had 7different bacterial species present. (36 week group)

pathogens of domestic livestock were identified including *Aerococcus viridians* (common cause of mastitis in livestock),[534, 535] *Arthrobacter gandavensis* (teat commensal),[536] *Streptococcus parauberis* (common cause of mastitis in livestock),[537] and *Streptococcus porcinus* (pyogentic skin commensal, common cause of swine abscess/lymphademopathy).[538] Interestingly, two bacteria were of environmental origin: *Bacillus foraminis*[539] and *Trichococcus pasteurii*,[540] found in ground water and waste water, respectively.

5.4 Discussion

In this study the implants were left *in situ* for up to 36 weeks, almost three times as long as our previous study that utilised a time point of thirteen weeks.[435] In this time no animal displayed localised or systemic symptoms of sepsis, consistent with human experience where overt infection is rare.[427, 541-543] Bacterial biofilm was identified by scanning electron microscopy in 75% of implants. This is consistent with previous results using the porcine model that showed that both the clinical *S. epidermidis* inoculant and endogenous porcine bacterial were both important aetiological agents in the formation of bacterial biofilm in this model.[435]

Comparison of the bacterial load and Baker Grade showed that there was increasing bacterial load between Baker Grade's I, II and III, with a plateau between Baker Grades III and IV (Figure 5-4). This suggests that, once bacteria have attached to the implant/capsules, there is a rapid multiplication of bacteria. Once there is a critical mass the biofilm progresses and causes a chronic fibrotic immune response resulting in fibrosis and stiffening of the capsule. The plateau between Baker Grade III & IV is consistent with a mature biofilm in a stable equilibrium.

The bacterial identification results demonstrate several important findings. In the initial study using the porcine model the bacteria identified at 13 weeks were predominantly coagulase negative Staphylococcus species of both inoculant and porcine origin.[435] This is consistent with infection at time of insertion by the inoculant and/or porcine skin

commensal, as postulated with the Subclinical Biofilm Infection Theory (Figure 1-11), (1.10.2.3).

The implants in this study were left *in situ* for 28 or 36 weeks, up to three times longer than our previous study, with no overt wound or systemic infection. This study shows a progressive change in bacterial species identified within the capsules from the inoculant, *S. epidermidis*, to the porcine coagulase negative Staphylococci (*S. simulans*), other staphylococcal spp. and other teat commensals (*Aerococcus viridans*, *Arthrobacter gandavenisi*, *Streptococcus* porcinus). By 36 weeks there were also environmental bacteria *Bacillus foraminis* and *Trichococcus pasteuri* involved within the biofilm. The polymicrobial nature of the biofilm also increased with time with 77% of the implants by 36 weeks possessing two or more bacterial species, and 29% possessing four or more species. Interestingly one capsule had 7 species identified by molecular techniques. Whist many of these bacterial isolates are not relevant to human disease, these results do indicate that:

1) Skin coagulase negative Staphylococci species, including *S. epidermidis*, are important aetiological agents in biofilm formation;
2) Colonised bacteria already on the breast can readily colonise and infect biofilm around breast implants;
3) Implant biofilm is dynamic with numbers and types of bacteria involved within the infection changing with time. The bacteria residing within the biofilm may reflect both the local endogenous bacteria and bacteria that have invaded the biofilm via systemic routes of infections;
4) Implant biofilm can be polymicrobial and the number of bacteria involved increases with the time the implant remains *in situ*.

These results demonstrate that there is now evidence in the pre-clinical porcine model that supports contamination from endogenous breast bacteria being involved in the instigation and/or preservation of biofilm infection of breast implants as hypothesised in the Subclinical Biofilm Infection Theory (Figure 5-5). There is now compelling evidence from both clinical and preclinical studies that bacteria, in the formation of a biofilm, is an

important aetiological factor in the development of capsular contracture. Emphasis now needs to be focused on the development of anti-biofilm implant adjuncts and strategies to prevent and treat implant biofilm infection, in order to decrease capsular contraction rates.

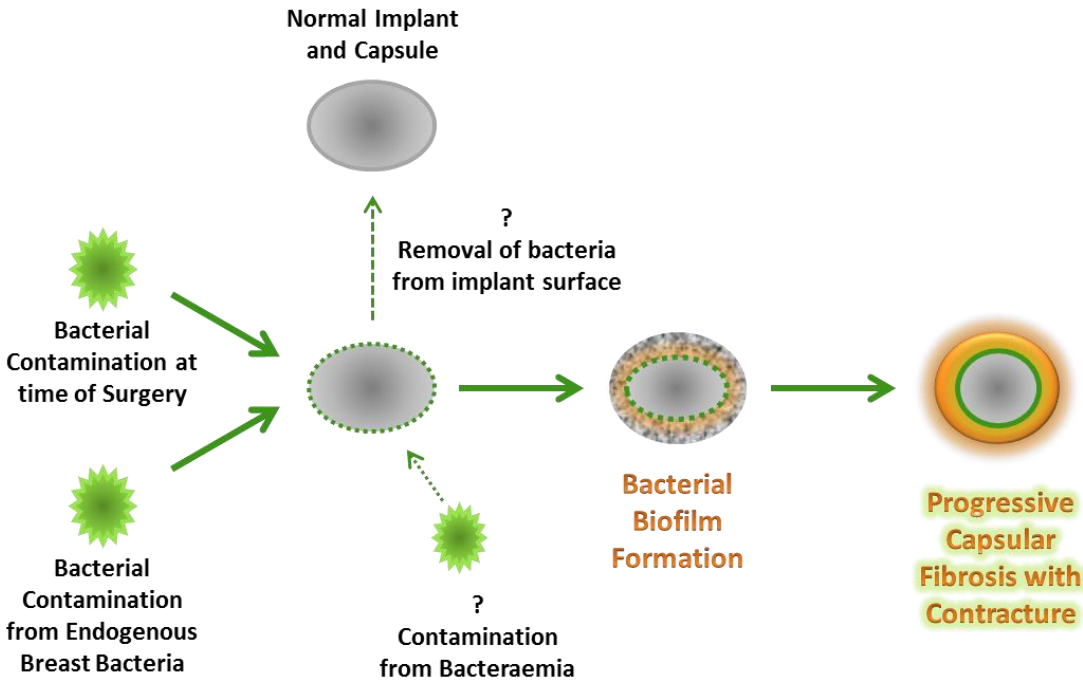


Figure 5-5: Progress of the Subclinical Biofilm Infection Theory of Capsular Contracture

There is now evidence that bacteria contamination of the breast implant can occur via either direct contact at time of insertion due to breakdown in aseptic technique or contamination from endogenous breast flora.

Biofilm is a dynamic structure with bacteria able to move into and out of the extracellular polymeric matrix. Knowledge of the common or potential bacteria contained within the biofilm may be important in the development of non-surgical treatment strategies.

Chapter 6

Does the Implant Surface Play a Role in the Formation of Capsular Contracture in the Porcine Model?

"...by the help of Microscopes, there is nothing so small, as to escape our inquiry; hence there is a new visible World discovered to the understanding."

- Robert Hooke, in Micrographia, 1665

6 *In vitro* and *In vivo* Investigation of the Influence of Implant Surface on the Formation of Bacterial Biofilm in Mammary Implants

6.1 Introduction

Contracture of the periprosthetic capsule with associated implant distortion, abnormal firmness and pain remains the most common complication following insertion of breast implants.[424, 426] Capsular contracture has, for many years, been assumed to be multifactorial in aetiology, as no one theory has yet to be identified to account for all disease. Whilst there is increasing evidence for subclinical biofilm infection being a key pathogenic pathway[89, 426, 435-437, 544] there is still ongoing debate about other possible aetiologies including the role of implant texture in the development of capsular contracture.[426, 545]

Texturisation of the implant surface and its role in preventing contracture was first proposed following the introduction of polyurethane foam-covered implants in the late 1960's.[546, 547] These implants were smooth-surfaced silicone implants covered with a 1.5-2 mm polyurethane coat that resulted in an open-pore texture that promoted tissue ingrowth.[547] Numerous cohort studies of patients with polyurethane-surfaced implants in the early 1980's reported low capsular contracture rates between zero[548, 549] and four percent.[550-552] The highest capsular contracture rate in polyurethane coated implants

was eight percent over the 29 month follow-up period.[541] In the same cohort study the authors reported an identical capsular contracture rate, of eight percent, in textured-surfaced silicone implants, however the rate increased to 19.5% in patients with smooth-surfaced silicone implants. Together these results suggested that surface texture may contribute to capsular contracture and that the rough surface may have a role in decrease capsular contracture.

Whilst preliminary clinical studies supported this claim, more recent studies have shown that the capsular contracture benefit was related to biochemical effects and not texture.[426] Polyurethane stimulates a delayed chronic inflammatory process.[553] This process recruits macrophages and multinucleated giant cells that ingest the polyurethane fragments and forms microcapsules that prevent capsular contracture.[553] Indeed with time the chronic inflammation causes the polyurethane coat to fragment and erode away exposing the smooth surface silicone shell beneath it.[546] This and other safety concerns in the late 1980s, related to potential toxicity of the degradation by-products of the polyurethane coating (including 2,4 toluenediamine (TDA) - a known carcinogen in animals), led to the voluntary withdrawal of polyurethane implants from the market in the USA.[541, 554, 555] However, several studies and a report by the Food and Drug Administration have failed to demonstrate any significant increase in urine TDA or cancer risk.[553, 556-558]

The next generation of texturisation was introduced in the late 1980s, using a number of techniques to modify the external silicone shell.[426, 559] Studies comparing the capsular contracture rate between smooth and textured implants have delivered conflicting results. Several initial studies of the insertion of one smooth and one textured implant per patient yielded differing outcomes, with some reporting reduced contracture rates with textured implants[560] and others reporting no difference.[559, 561] Subsequently, several randomised control trials have shown more consistent results, with textured implants showing lesser rates of capsular contracture.[434, 562-565] Published studies, either randomised controlled or cohort studies, are evenly divided as to whether texturisation showed benefit[560, 563-569] or conferred no difference.[434, 557, 559, 561, 570, 571] Two meta-analyses have reported a small, but significant, association between textures surfaced

implants and decreased capsular contracture rates.[424, 572] Both authors identify significant limitations in the data including: small sample size, short follow up period (only one study had a follow up period over 5 yrs[563]), and variations in texture pore size, surgical technique (including different incisions and pocket positions), use of antibacterial pocket irrigation, antibiotic utilisation and use of postoperative drainage, supporting the need for further investigation.[424, 572] This is consistent with a more recent systematic analysis where the authors argued that "*there is a deficiency of reliable and reproducible sound data*" to support any results or recommendations.[545]

Two large cohort studies were published in 2013 that provided three[568] and five[569] year outcomes data for textured and smooth implants from industry funded and authored clinical trials of their own implants. The study by Namnoun and colleagues reported the 3 year results for over 4400 patients enrolled in the Core (NCT00689871) and 410 (NCT00690339) clinical trials.[568] The capsular contracture rate was 3.6%, with the majority of women (94.1%) receiving textured surfaced implants. Their analysis showed that capsular contracture was significantly higher in the smooth implant group and in patients with periareolar and axillary incisions and subglandular placement.[568] The study by Stevens and colleagues reported the 5 year outcomes for 2560 implant augmentations[569] They reported a capsular contracture rate of 7.6% per device, but in contrast, the majority of women (62%) received smooth implants. Their results, however, are similar to those above with multivariate analysis showing that capsular contracture was significantly associated with the smooth implant group, periareolar incision and subglandular placement as well as haematoma/seroma formation, small implant size and early post-operative use of a bra.[569] These results support the role for implant surface texture as an aetiological factor, however surgical technique and decision making regarding surgical incision, dissection plane, haemostatic technique, implant size as well as post-operative care regimes may also be important.

With this background in mind, the pre-clinical porcine model was used to investigate whether the presence of a smooth or textured outer surface offers any advantages in preventing both biofilm formation and capsular contracture following deliberate bacterial inoculation.

6.2 Article – Original Paper

In vitro and In vivo Investigation of the Influence of Implant Surface on the Formation of Bacterial Biofilm in Mammary Implants

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Acknowledgement/Contribution

Dr Anita Jacombs: Co-Author

I performed pig surgery, specimen retrieval, Baker Grading, applantation tonometry, sample preparation, scanning electron microscopy and DNA extraction for animal study.

I performed the polymerase chain reaction for bacterial counts.

I contributed to the manuscript preparation.

Ethics Approvals: University of Sydney N00/12-2010/2/5420 (Appendix 1)
University of Sydney N00/12-2010/2/5421 (Appendix 1)

In Vitro and In Vivo Investigation of the Influence of Implant Surface on the Formation of Bacterial Biofilm in Mammary Implants

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Background: Capsular contracture remains the most common complication following breast augmentation surgery, and evidence suggests that bacterial biofilm on the implant surface is responsible. The authors investigated whether the interaction of bacterial biofilm with implants independently determines progression to capsule formation. They also studied the rate of bacterial growth and adhesion to implants.
Methods: Sixteen adult female pigs had 121 breast implants inserted. Sixty-six implants—23 smooth and 43 textured—were inoculated with a human strain of *Staphylococcus epidermidis* and received no other treatment. After an average period of 19 weeks, Baker grading was performed and implants were retrieved. For the in vitro study, samples underwent both quantitative bacterial analysis and imaging using confocal laser scanning and scanning electron microscopy.
Results: At explantation, there was no significant difference ($p = 1.0$) in the presence of capsular contracture (Baker grade III and IV) between smooth (83 percent) and textured implants (84 percent). Biofilm was confirmed on 60 of the 66 capsules. Capsules from smooth and textured implants had the same number of infecting bacteria (textured: 3.01×10^8 bacteria/g; smooth: 3.00×10^8 bacteria/g). In vitro, the surface of textured implants showed 11-, 43-, and 72-fold more bacteria at 2, 6, and 24 hours, respectively, compared with smooth implants ($p < 0.001$). These findings were confirmed by imaging analysis.
Conclusions: These results show that textured implants develop a significantly higher load of bacterial biofilm in comparison with smooth implants. Furthermore, in vivo, once a threshold of biofilm forms on either smooth or textured implant surfaces, there seems to be an equal propensity to progress to capsular contracture. (*Plast. Reconstr. Surg.* 133: 471e, 2014.)

Breast augmentation and reconstruction using breast implants are among the most common procedures performed in plastic surgery.[1] In 2011, over 310,000 breast augmentation procedures were performed in the United States alone.[2]

Contracture of the periprosthetic capsule with associated implant distortion, abnormal firmness, and pain remains the most common complication following breast augmentation.[1,3] A number of theories have been put forward for the genesis of capsular contracture. The role of subclinical infection, however, has gained support from both clinical and preclinical studies, and there is now wide acceptance that bacterial biofilm on the surface of implants is the principal pathogenic pathway to development of capsular contracture.[3–8]

Texturization of the implant surface and its role in preventing contracture were first proposed following the introduction of polyurethane foam–covered implants. The next generation of texturization was introduced in the late 1980s,

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Disclosure: *Dr. Deva and Dr. Vickery are consultants to Allergan, Mentor (Johnson & Johnson), and Kinetic Concepts, Inc. They have previously coordinated industry-sponsored research for these companies relating to both biofilms and breast prostheses. The remaining authors have no financial interests to disclose.*

using a number of techniques to modify the external silicone shell.[3,9] Several initial studies inserting one smooth and one textured implant per patient yielded differing outcomes, with some reporting reduced contracture rates with textured implants[10] and others reporting no difference.[9,11] Subsequently, several randomized controlled trials have shown more consistent results, with textured implants showing lesser rates of capsular contracture.[12,13] There are now 10 published randomized controlled or split breast studies that are evenly divided as to whether texturization shows benefit[1,10,13–15] or confers no difference.[6,9,11,12,16] Texturization of the surface of implants has been shown to have biological benefits in enhancing biocompatibility and achieving optimal integration of living host and the alloplast.[17] These effects include enhancing tissue adhesion, growth and proliferation of host blood supply, enhancement of cellular migration, and fibroblast adhesion.[18,19]

With this background in mind, we sought to use our well-established porcine model of capsular contracture to investigate whether the presence of a smooth or textured outer surface offers any advantages in preventing both biofilm formation and capsular contracture following deliberate bacterial inoculation. We also designed an in vitro attachment assay to determine the influence of implant surface on the growth and attachment of bacterial biofilm.

MATERIALS AND METHODS

Approval for the all study protocols was obtained from the University of Sydney Animal Ethics Committee.

Surgical Procedure

A total of 16 adult, female, nonlactating, domestic Large White pigs (*Sus domesticus*) weighing approximately 350 kg each were used. One hundred twenty-one implants (average diameter, 3 cm) were inserted, with each pig receiving between six and eight implants. Of these, 66 implants (23 smooth and 43 textured) were all inoculated with an average of 10^5 colony-forming units of a human strain of *Staphylococcus epidermidis* and received no other treatment. These 66 implants were used in this study.

Implants were inserted into submammary pockets as described by Tamboto et al.[4] The implants were left in situ for an average of 19 weeks, after which clinical assessment by Baker grading was performed.

Baker Grade

Contracture of the 66 implants was assessed blinded using the four-grade Baker scale[20] while the implants were in situ.

Total Number of Bacteria in Capsules and Attached to Implants

The total numbers of bacteria in capsular tissue surrounding 14 smooth and nine textured implants and the numbers of bacteria attached to 11 smooth and nine textured implants were determined by real-time quantitative polymerase chain reaction using universal eubacterial primer 16S rRNA_341F 5′-CCTACGGGAGGCAGCAG-3′ and 16S rRNA_534R 5′-ATTACCGCGGCTGCTGG-3′ to amplify a 194–base pair amplicon of 16S rRNA gene of all bacteria as described previously.[21]

Between 50 and 100 mg of capsular tissue and between 40 and 100 mg of implants were digested using a combination of proteinase K and lysozyme digestion, and the genomic DNA was extracted using phenol/chloroform extraction followed by ethanol precipitation as described previously.[21] The number of bacteria in each tissue sample was normalized to the amount of tissue digested by real-time quantitative polymerase chain reaction of the 18S ribosomal RNA reference gene (GenBank accession no. AY265350.1) using the primer pair 18S rRNA_756F 5′-GGTGGTGCCCTTCCGTCA-3′ and 18S rRNA_877R 5′-CGATGCGGCGGCGTTATT-3′ as described previously.[21]

Scanning Electron Microscopy

The presence of biofilm was confirmed visually on all implants and capsules using scanning electron microscopy. Samples were fixed in 3% glutaraldehyde, dehydrated through alcohol, and then immersed in hexamethyldisilazane (Sigma-Aldrich, St. Louis, Mo.) 50% for 10 minutes and 100% for 10 minutes, three times, before being aspirated dry and evaporated dry overnight. They were mounted on metal stubs with carbon tabs and coated with 20-nm gold film in a sputter coater. The samples were imaged using a JEOL 6480LV scanning electron microscope (JEOL Ltd., Tokyo, Japan) with a voltage of 10 kV and a viewing distance of 20 mm.[21]

In Vitro Assay

Fourteen miniature, textured, 2-cm-diameter and 14 miniature, smooth, 2-cm breast implants were incubated in 20 ml of 10% tryptone soya broth (Oxoid, Cambridge, United Kingdom) containing 5.8×10^6 colony forming units/ml of *S. epidermidis*, originally obtained from a contracted

human breast[4] at 37°C. In addition, eight separate pieces of implant shell (four textured and four smooth) were included in the incubation chamber for the purposes of imaging.

Four implants of each type were removed for quantitative bacterial analysis from the chamber at 2, 6, and 24 hours for colony-forming unit determination. They were washed twice in phosphate-buffered saline and then placed in 20 ml of phosphate-buffered saline to be subjected to sonication for 20 minutes followed by 1 minute of vigorous shaking as described previously.[5]

Quantitative numbers of bacteria attached to whole implants were determined by 10-fold serial dilution and subsequent plate culture. Bacteria attached to the implant sections were visualized by using confocal microscopy and scanning electron microscopy.

For confocal microscopy, one implant of each type was removed from the chamber at the three time points. Sections of the implant were stained with a DNA stain Live/Dead *Bac*Light Bacterial Viability Kit 7012 (Molecular Probes, Life Technologies, Grand Island, N.Y.) according to the manufacturer's instructions. Live cells appeared green and dead cells appeared red because of preferential binding of propidium iodide. Stained samples were examined using an Olympus FluoView 300 inverted confocal laser scanning microscopy system (Olympus Corp., Tokyo, Japan). Implants for scanning electron microscopy were prepared and imaged as described above.

Statistical Analysis

The Fisher's exact test was used to examine differences in contraction rate between smooth and textured implants using the statistical package Sigma Plot 13 (Systat Software, Inc., San Jose, Calif.). The *t* test was used to examine for differences in the number of bacteria associated with contracted implants compared with noncontracted implants and for comparing the number of bacteria associated with textured and smooth implants. The data for the in vivo study were distributed normally and had equal variance. The data for the 6- and 24-hour time point analysis in vitro had to be transformed to ensure normality and equal variance. The Mann-Whitney rank sum test was used to examine for differences in the number of bacteria attached to different implants and in capsular tissue surrounding those implants.

RESULTS

In Vivo Study

No pig exhibited systemic signs of infection relating to either bacterial inoculation and/or the presence of a surgical implant.

Baker Grade

There were no significant differences between smooth and textured implants regarding the proportion of breasts that developed contracture (Baker grade III and IV) following artificial inoculation of *S. epidermidis* ($p = 1.0$). At explantation, 83.7 percent of the capsules around the textured implants and 82.6 percent of the capsules around the smooth implants had capsular contracture (Baker grade III and IV). Seven (16.3 percent) of the 43 textured and four (17.4 percent) of the smooth implants had no clinical capsular contracture (Baker grade I and II) (Table 1).

Total Number of Bacteria in Capsules and Attached to Implants

Although all implant pockets were inoculated with the same number of bacteria, those that went on to develop contracture had 250 percent more bacteria associated with them than those that failed to develop contracture. In artificially inoculated implant pockets, there was no significant difference in total bacterial numbers in capsular tissue surrounding smooth ($n = 14$) and textured implants ($n = 9$). Capsular tissue surrounding textured implants contained an average of 3.00×10^8 bacteria/g of tissue and tissue surrounding smooth implants contained an average of 3.01×10^8 bacteria/g of tissue.

Interestingly, there were 20-fold more bacteria attached to the textured implants (1.18×10^8 bacteria/g of implant) than smooth implants (5.75×10^6 bacteria/g of implant). This difference was significant ($p = 0.006$).

Scanning Electron Microscopy

Bacterial biofilm was confirmed by scanning electron microscopy on all of the Baker grade III/IV implant capsules. We were unable to confirm

Table 1. Baker Grading of Smooth and Textured Implants

Implant Texture	No.	Noncontracted			Contracted		
		Baker I	Baker II	%	Baker III	Baker IV	%
Smooth	23	2	2	17.4	10	9	82.6
Textured	43	1	6	16.3	22	14	83.7

biofilm on four capsules from Baker grade I and II implants. Most of the capsules displayed thick biofilm, with loss of the normal capsule fibrous architecture, over a large area of the imaged specimen (Fig. 1, *above*). Coccoid bacteria embedded in biofilm exopolymeric substances were frequently identified. The remaining capsules showed occasional or patchy biofilm, and normal fibrous capsule architecture was maintained (Fig. 1, *below*). More bacteria were attached to the surface of textured implants compared with smooth implants (Fig. 2).

IN VITRO STUDY

Microbiology

Our analysis showed that at 2 hours, the mean number of bacteria attached to the textured implants was 3.8×10^6 (range, 1.6 to 6.6×10^6) as compared with 3.4×10^5 (range, 1.6 to 6.3×10^5) on smooth implants. This difference was significant ($p = 0.015$). At the 6-hour time point, the number of bacteria attached to the textured implant had increased by a factor of 43-fold on the textured implants compared with the smooth implants [6.8×10^7 (range, 6.0 to 7.5×10^7) for textured versus 1.6×10^6 (range, 0.8 to 2.4×10^6) for smooth]. At the 24-hour time point, the textured implants had 72 times the number of bacteria attached to their surface compared with the smooth implants [6.0×10^9 (range, 2.8 to 6.4×10^9) for textured versus 8.2×10^7 (range, 6 to 12.8×10^7) for smooth]. The differences at the 6- and 24-hour time points were highly significant ($p < 0.001$). These data are summarized in Figure 3.

Imaging

Confocal microscopy confirmed that the number of bacteria attached to textured implants was far greater than the number attached to smooth implants at each time point (Fig. 4). Scanning electron microscopy of the smooth and textured

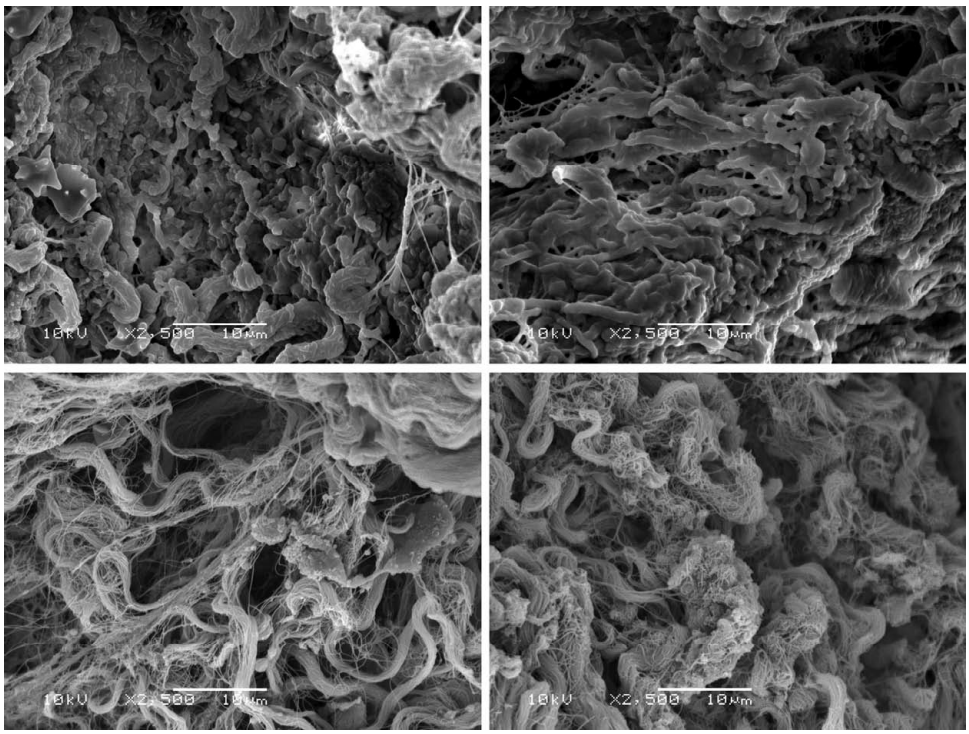

Fig. 1. Comparison of biofilm in periprosthetic capsules for smooth and textured implants. (*Above*, *left*) Smooth implant capsule with excess biofilm and loss of capsular architecture. (*Above*, *right*) Textured implant capsule with excess biofilm and loss of capsular architecture. (*Below*, *left*) Smooth implant capsule with minimal biofilm and normal capsular architecture. (*Below*, *right*) Textured implant capsule with minimal biofilm and normal capsular architecture.

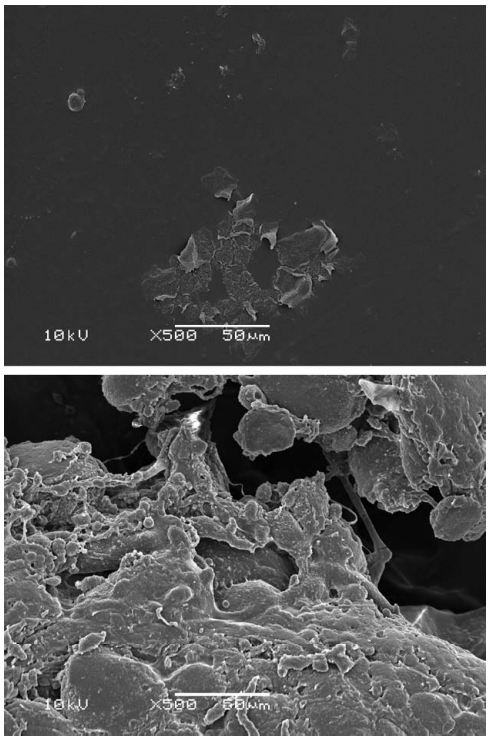

Fig. 2. Comparison of biofilm on implant surface for smooth and textured implants in vivo. (*Above*) Scanning electron micrograph of smooth implant showing minimal patchy residual biofilm. (*Below*) Scanning electron micrograph of a textured implant demonstrating greater numbers of bacteria attached to the textured implant surface. This finding was confirmed on quantitative polymerase chain reaction (see text).

outer shells at 24 hours also confirmed our findings on microbiology, showing dense and mature biofilm on the surface of textured implants and patchy biofilm on the surface of smooth implants (Fig. 5).

DISCUSSION

In the 50 years since the initial silicone breast implant was used surgically for breast augmentation, capsular contracture has remained the most common complication, often requiring further surgical intervention.[3,22,23] The cause of capsular contracture remains poorly understood but is likely to be multifactorial in origin.[3,23] However, a recent large clinical study by Rieger et al.[24] has confirmed that Baker grade of contracture directly correlates with the number of bacteria identified by sonication and culture. These findings further support the subclinical infection theory. In this study, we investigated whether a smooth or textured implant surface offered any advantage in preventing the development of capsular contracture in artificially inoculated pockets of an in vivo pig model.

There was no significant difference in the rate of capsular contracture between smooth surface implants and textured surface implants following deliberate inoculation with human *S. epidermidis*. Following inoculation, 82 percent of breasts implanted with textured prostheses and 83.4 percent of breasts implanted with smooth prostheses developed contracture. These rates are consistent with our previous findings.[4] Contracted breast capsules had 250 percent more bacteria compared with noncontracted capsules. These data once again reinforce the pathway from initial

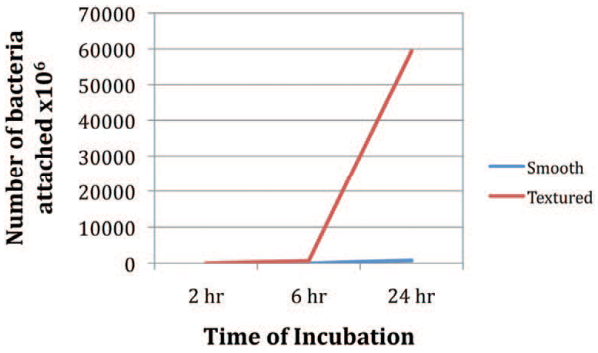

Fig. 3. Mean number of bacteria ($\times 10^6$) attached to miniature implants with smooth and textured surfaces following 2, 6, or 24 hours of incubation in 10% tryptone soya broth containing *S. epidermidis*.

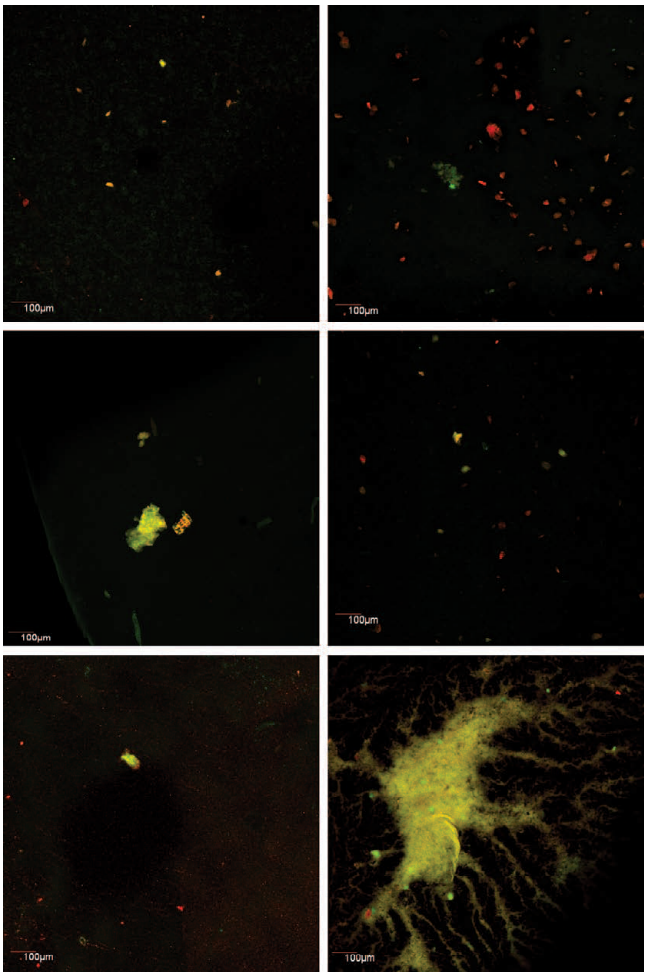

Fig. 4. Confocal laser scanning microscopic images of bacteria attached to miniature implants with smooth and textured surfaces following 2 hours (*above*), 6 hours (*center*), or 24 hours (*below*) of incubation in 10% tryptone soya broth containing *S. epidermidis*. Live bacteria appear *green*; dead bacteria appear *red*.

contamination of breast implants with bacteria progressing to established biofilm and subsequent contracture.

The subclinical infection theory has been further validated by these data. We have shown once again that deliberate inoculation with *S. epidermidis* results in progression to biofilm and contracture in approximately 80 percent of implants. It is likely that there is a threshold of biofilm load, which, once crossed, leads to significant potentiation of capsule formation. This threshold seems to be independent of whether the implant is smooth or textured. The finding of higher numbers of bacteria on textured implants (up to 20-fold more in vivo and 72-fold in vitro) is consistent with the subclinical infection hypothesis, as additional bacteria above this threshold results in the same outcome (i.e., development of contracture) (Fig. 6).

The Baker grading was consistent with our scanning electron microscopic findings, which demonstrated either obvious thick biofilm or patchy biofilm in all but six textured implants.

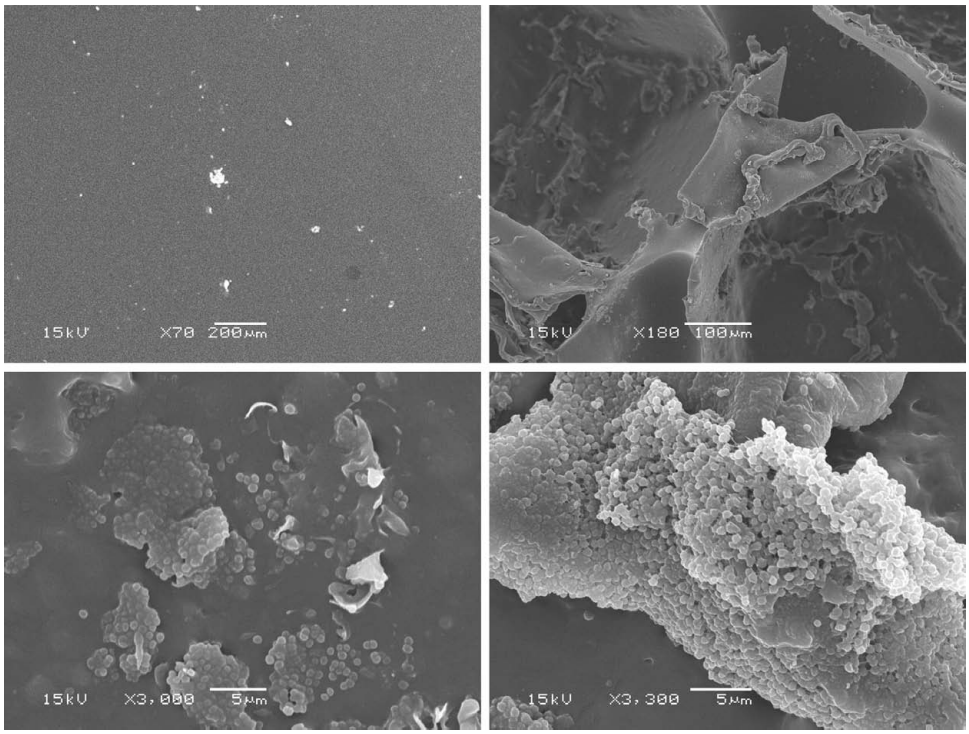

Fig. 5. Scanning electron micrographs of breast implants incubated with *S. epidermidis* for 24 hours. (*Above, left*) Low-magnification image of a smooth implant showing little biofilm coverage (original magnification, × 70). (*Above, right*) Low-magnification image of a textured implant showing heavier biofilm development, especially into the surface crypts (original magnification, × 180). (*Below, left*) High-magnification image of a smooth implant showing coccoid organisms embedded in exopolymeric substances (original magnification, × 3000). (*Below, right*) High magnification image of biofilm from surface crypt of a textured implant showing large numbers of coccoid organisms (original magnification, × 3300).

Four of the six implants in which we failed to visually confirm biofilm were culture-positive, demonstrating the patchy nature of biofilm infection and likely sampling error when using scanning electron microscopy as a sole diagnostic test for the presence of biofilm.

These results show that both implant surface types will readily form biofilm under experimental conditions using deliberate inoculation of *S. epidermidis*. A constant finding was that, as the degree of contracture increased, as measured by Baker grading and tonometry, capsular architecture became less organized and the collagen fibers were covered in biofilm exopolysaccharide (Fig. 1). This effect was seen in capsules from around both smooth and textured implants. A further long-term study to investigate the correlation between the amount of biofilm load and degree of capsular contracture using the porcine model is currently underway.

The in vitro analysis has demonstrated clearly that the presence of a textured outer shell on breast implants encourages a higher rate of biofilm growth. This finding is consistent with a growing body of research from industry and more specifically from investigations on biofouling of metal and plastic surfaces. Biofouling refers to an undesirable development of biofilm on a membrane surface, involving accumulation of deposited microbial cells embedded within a matrix of extracellular polymeric substances.[25] Biofouling has become increasingly recognized as a leading cause of failure in a range of industrial and domestic areas, including water purification systems, hydraulics, home appliances, and food processing.[26–28]

Wenzel[29] was the first to note that rough surfaces were more susceptible to wetting because

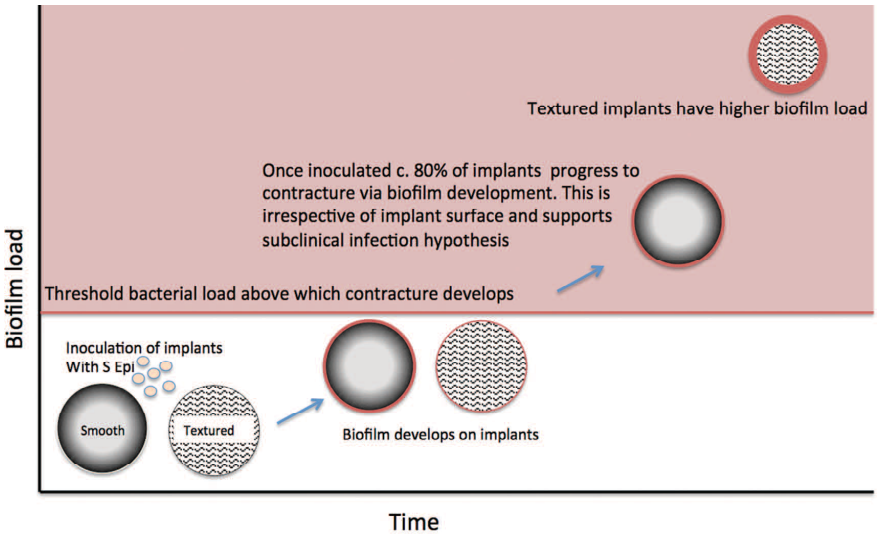

Fig. 6. Support for the subclinical infection hypothesis. Schematic depiction of the progression in smooth and textured implants from inoculation to contracture.

of an increase in both contact angle and surface area. As predicted by Wenzel, as the surface complexity or roughness increases, so too does the propensity for biofilm growth.[30]

Substrates with varying roughness have now been subjected to biofilms in vitro. Arnold and Bailey[31] have demonstrated that electropolished steel has less propensity for early biofilm attachment and formation compared with rough steel substrates. Myint et al.[32] have similarly shown that for polyamide nanofiltration membranes, increased surface roughness potentiates initial biofilm cell attachment, aggregation, and colony formation.

The finding that textured breast implants significantly potentiate biofilm formation compared with smooth implant surfaces has implications for both clinicians and scientists. As the evidence for subclinical infection as a potentiator for contracture increases, surgeons using textured implants need to be especially aware of strategies to prevent the access of bacteria to the implant at the time of surgical implantation. Nipple shields,[33] pocket irrigation,[3,34] no-touch insertion,[35] perioperative antibiotic prophylaxis,[36] and avoiding the transareolar incision[37] have all been recommended as strategies for reducing the risk of bacterial contamination at the time of breast implant insertion. It would be prudent, especially when using textured implants, to recommend mandatory use of these strategies to prevent the initial attachment and subsequent formation of bacterial biofilm on breast implants. The biological advantages of texture, including better tissue ingrowth and potentially less contracture, need to be balanced by the higher risk of bacterial contamination. Surgeons should be aware of this risk and modify their intraoperative strategy to reduce the likelihood of bacterial contamination of breast implants.

For industry and researchers alike, the search for the ideal combination of surface morphology and intrinsic bacterial resistance should be made a priority. The development of this technology would have implications not only for breast prostheses but also for all other implantable medical devices. In orthopedic surgery, for example, failure of hip and knee arthroplasties has been shown to be attributable to biofilm infection, and strategies for intraoperative prevention have shown early promise in reducing the risk of device-associated infection.[38]

The goal to develop an alloplastic surface that resists or limits bacterial adhesion and biofilm formation continues. A number of surface modification techniques have been applied to biomaterials, including antiadhesive, antiseptic, and antibiotic coatings; surface grafting; chemical modification; and biological membranes.[39–41] The techniques aim to create a new interface between the host and the alloplast by modifying topography, surface functional groups, hydrophobicity, and surface charge, and improving the ability of the surface to kill bacteria. The successful design of a truly biofilm-resistant surface, however, remains a significant challenge because of the

variation in required physical properties of the biomaterials, the variety of design and regulatory demands for medical devices, and the changing patterns of resistance of the microbial population.

The finding of an increased number of bacteria attached to the surface of the textured implant is novel. It will be interesting to see whether this higher bacterial load is responsible for chronic immune activation, which in turn may predispose to potential lymphocytic hyperplasia.

The complex interactions between the potentiators and suppressors at play in this hypothesis are not yet fully understood. In this study, we have investigated the interactions between subclinical infections and surface texture. Almost all capsules demonstrated positive findings of bacterial biofilm at the time of removal, with neither surface type demonstrating any reduction in capsular contracture rates. These results suggest that the potentiating stimulus of subclinical infection far exceeds any potential advantage that may be conferred by implant surface type.

CONCLUSIONS

In breast implants, although textured implants may confer better tissue ingrowth, they also have been shown to potentiate the early and rapid formation of *S. epidermidis* biofilm in vivo and in vitro compared with smooth implants. The use of textured implants should especially be combined with stringent intraoperative attention to the prevention of bacterial contamination to reduce the risk of biofilm formation and subsequent capsular contracture.

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Chapter 7

Can Bacterial Biofilm Infection be Prevented?

"Medical science has proven time and again that when the resources are provided, great progress in the treatment, cure, and prevention of disease can occur."

~ Michael J. Fox

7 Prevention of Biofilm-Induced Capsular Contracture with Antibiotic-Impregnated Mesh in a Porcine Model

7.1 Introduction

Biofilm–associated infection of implants, including orthopaedic, cardiac, vascular, urological and silicone implants can result in the infection of bone, soft tissues and systemic sepsis with devastating consequences to the patient and rapidly increasing health care expenses.[110, 573, 574] Whilst improvements in antibiotic prophylaxis and surgical techniques have been shown to decrease infection rates, alone they are insufficient to prevent biofilm infection.[426, 575] Anti-infective biomaterials to modify implant surfaces and coatings are rapidly being developed with the hope that they will become the mainstay in the prevention of biofilm-associated implant infection.[575]

The requirements for these novel anti-infective biomaterials and surfaces are broad and must address numerous variables including patient factors, microbiology, prosthesis type and function variables.[574] These include, but are not limited to, type of device required, duration of device (temporary or permanent), the mode of insertion (percutaneous versus open surgery) and end location for device such as:

1. External: dressings/intrauterine devices; percutaneous: external fracture fixation devices;
2. Mucosal: dental prosthesis/biliary stents/urinary indwelling catheters, ureteric stents;

3. Internal: orthopaedic fracture fixation devices/joint prosthesis/cardiac assist devices/valve replacements/surgical mesh/reconstructive silicone implants.[574]

Not surprisingly numerous anti-biofilm biomaterial strategies have been developed to address these variables.[575] Bioactive antibacterial surfaces and implant coatings are one key area of biomaterial development. The basic aim of this strategy is to deliver the anti-infective agent at the prosthesis-tissue interface to prevent initial bacterial attachment and hence prevent subsequent biofilm formation.[575, 576] Numerous anti-microbial compounds have been integrated into these surface biomaterials including:

1. Antimicrobioal molecules: triclosan, chlorhexidine, benzalkonium;[575, 577, 578]
2. Polymers containing molecules with bactericidal actions: tertiary amines, nitric oxide, reactive oxygen species;[579, 580]
3. Polymers of antibacterial metals: including silver, titanium, platinum and copper compounds;[575, 581, 582]
4. Polymers containing antibiotics.[583]

Antibiotic-impregnated biomaterials have been developed and are now in clinical use for an increasing number of medical devices including: catheters, such as indwelling urinary (Foley's) catheters and central venous catheters; antibacterial loaded cements and grafts for joint prostheses; antibiotic cement spacers for revision prosthesis surgery; and neurosurgical ventriculoperitoneal shunts.[575, 584-586]

Antibiotic-impregnated biomaterials, as well as other antimicrobial biomaterials, have been used in various catheters for over thirty years. The different catheters and different antimicrobial strategies have demonstrated variable clinical efficacy. Several studies including a multicentre randomised trial,[587] systematic reviews[588] and a meta-analysis[589] have investigated of the efficacy of urinary catheters impregnated with either antibiotics (nitrofurazone) or antimicrobial agent (silver). These studies have consistently demonstrated no difference in infection with silver-impregnated catheters compared to non-impregnated catheters.[587-590] Nitrofurozone-impregnated catheters have been found to have a small, but clinically insignificant decrease in infection rates.[587-589] Interestingly the nitrofurozone-impregnated catheters were associated with increased

catheter-related discomfort.[587, 588] These results may in-part be due to the decrease in effectiveness of the antibiotic/antimicrobial agent by 3 weeks post insertion.[591] Local bacterial resistance may also limit impregnated catheters' effectiveness, although there have been no microbiology results to demonstrate this concern. Several authors have also identified that there are limited quality randomised trials on which to conduct systematic analyses.[588, 589]

Investigations into the outcomes of catheter-related bloodstream infections after insertion of central venous catheters made with antibiotic/antimicrobial-impregnated biomaterials show better outcomes. Numerous antimicrobial agents have been bonded to central venous catheters including heparin, silver-platinum, chlorhexidine-silver sulfadiazine and numerous antibiotics including cefazolin, minocycline-rifampicin and miconazole-rifampicin over the last thirty years.[592, 593] Several systematic reviews[593, 594] and a meta-analysis[592] have demonstrated that both chlorhexidine-silver and minocycline-rifampicin impregnated central venous catheters significantly reduce the rates of catheter-related bloodstream infections in intensive cate units.[592-594] Minocycline-rifampicin impregnated catheters have been shown to be more efficacious, with significantly more reduced rates of colonization and catheter-related bloodstream infections, when compared to the chlorhexidine-silver impregnated catheters.[592, 593, 595] Heparin, chlorhexidine-silver sulfadiazine and silver/platinum impregnated catheters showed no significant reduction in catheter-related bloodstream infections when compared with non-impregnated catheters.[593, 595] An Australian cost effectiveness study suggested that the use of minocycline-rifampicin impregnated catheters saved 15 infections per 1000 catheters used with a cost saving of AU$948/catheter.[596]

Antimicrobial biomaterials have been increasingly used in orthopaedic surgery over the last four decades.[585] Initially used in drug eluting bone cement, there are now numerous antibiotic-impregnated/antibiotic eluting spacers and bone graft materials used in orthopaedic operations. Antimicrobial/antibiotic loaded bone cement was first used by German Orthopaedic Surgeons, Buchholz and Engelbrecht, in the mid-1970's when they added gentamicin to standard bone cement.[597] Their initial results showed a decreased infection rate from 6% to 1.6%for elective total hip arthroplasty.[597] Subsequently, several

cohort and randomised trials were performed over the next two decades using different combinations of antibiotics. Two recent reviews of these studies have shown that, whilst the quality of many of these studies is not as robust as current trial guidelines would recommend, antibiotic-loaded cement significantly decreased deep-space infection in total hip replacements, but resulted in no difference in superficial wound infections.[598] Further analysis suggested gentamicin was more effective than cefuroxime, the cephalosporin most commonly used in bone cement.[598, 599]

More recent studies of antibiotic-loaded cement in primary total knee replacement have shown conflicting results. A limited number of studies have shown a decrease in revision rates in patients with antibiotic-loaded cement,[600] with most showing no difference at twelve months[601-603] and one study finding increased infection in patients with antibiotic-loaded cement.[604] Unfortunately, none of these studies are prospective randomised studies and there is no consistency with antibiotic choices and doses making systematic analysis difficult. A single retrospective cohort study of antibiotic-loaded cement in shoulder hemiarthroplasty showed no difference in deep-space infections at three years post-surgery.[605]

Orthopaedic spacers, used in staged surgery for the management of infected prostheses and bone grafts are now available with antibiotic impregnation. Three recent reviews of these devices have demonstrated that the limited initial studies on these products are also inconsistent and a well-designed prospective randomized data is required for effective assessment of the efficacy of these products.[606-608]

Thus, to date the clinical efficacy of antibiotic-loaded bone cement, as well as antibiotic spacers and antibiotic bone grafts, has not been appropriately assessed using prospective randomised studies. However, initial studies of antibiotic-loaded bone cement in primary total hip arthroplasty were promising. Two clinical prospective studies are now underway to assess the efficacy of antibiotic-loaded cement in hemiarthroplasty for neck of femur fractures[609] and a neurosurgical antibiotic-impregnated ventriculoperitoneal shunt.[586] These studies will hopefully begin to provide robust clinical data on the use of antibiotic surfaces in surgical implants. Currently there are no antibiotic or anti-infective surfaces in clinical practice for silicone implants.

This study used the pre-clinical porcine model to investigate the efficacy of an antibiotic-impregnated polypropylene mesh (TYRX inc). The mesh is a tyrosine polyarylate-coated multifilament absorbable mesh envelope that contains minocycline and rifampicin.[610] Minocycline is a bacteriostatic antibiotic that inhibits protein synthesis and is effective against Gram-positive bacteria including *S. aureus* and Gram-negative bacteria including *E. coli*, *Haemophilus influenza, Enterobacter aerogenes* and *Acinetobacter baumannii*.[610] Rifampicin is a bactericidal antibiotic that interferes with DNA-dependent RNA polymerase activity and is effective against Gram-positive bacteria *S. aureus*, including MRSA, *S. epidermidis* and Gram-negative bacteria including *H. influenza.*[610] The reabsorbable antibiotic mesh has a minimum seven days antibiotic activity and is fully reabsorbed within nine weeks.[610]

7.2 Article – Original Paper

Prevention of Biofilm-Induced Capsular Contracture with Antibiotic-Impregnated Mesh in a Porcine Model

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Acknowledgement/Contribution

Dr Anita Jacombs: Co-Author

I performed pig surgery, specimen retrieval, Baker Grading, applantation tonometry, sample preparation, scanning electron microscopy and DNA extraction.

I performed the polymerase chain reaction for bacterial counts.

I contributed to the manuscript preparation.

Ethics Approval: University of Sydney N00/5-2010/2/5283 (Appendix 1)

Research

Prevention of Biofilm-Induced Capsular Contracture With Antibiotic-Impregnated Mesh in a Porcine Model

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Abstract

Background: A growing body of evidence implicates subclinical (biofilm) infection around breast implants as an important cause of capsular contracture (CC).

Objectives: The authors use an in vivo porcine model to investigate the potential of antibiotic-impregnated mesh as a prophylactic measure against biofilm formation and CC.

Methods: A total of 28 implants (14 untreated controls, 14 treated with antibiotic mesh) were inserted into 5 adult female pigs. All implants and pockets were inoculated with a human clinical strain of *Staphylococcus epidermidis*. The implants were left in situ for 16 weeks and then analyzed for contracture using both Baker grading and applanation tonometry. The presence of biofilm infection was assessed by subsequent microbiological analysis of implants and capsules.

Results: One untreated implant had extruded and was excluded from analysis. The tissue surrounding the 13 untreated control implants had Baker Grade III/IV CC, whereas no CC was identified around the 14 antibiotic mesh-treated implants. This difference was highly significant ($P < .001$). Tonometry findings were consistent with the Baker assessments. Although bacterial biofilm was detected on all implants and capsules, the biofilms on the antibiotic-treated implants and surrounding capsules were generally single-layered or isolated in contrast to the multilayer biofilms found on untreated implants and capsules.

Conclusions: Based on the findings from this study of a porcine model, the use of antibiotic-impregnated mesh reduces bacterial access to breast implants at the time of surgical insertion and may subsequently protect against subclinical infection and CC.

Keywords

breast implant, biofilm, capsular contracture, breast surgery, research

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Since the 1970s, capsular contracture (CC) has remained the most significant clinical complication of breast augmentation, with reported rates of 1.3% to 30%.[1] Evidence from clinical studies[2-8] and from in vivo porcine models of CC[9] supports the hypothesis that the majority of CC is due to subclinical infection around breast implants caused by bacterial biofilm. This hypothesis is further strengthened by early reports that antibacterial strategies can reduce the risk of implant infection.[10-13] We have previously reported data from a prospective, blinded study that showed isolation of *Staphylococcus epidermidis* in 73% of samples obtained from capsules and implants of women with symptomatic contracture following augmentation mammaplasty.[4] This is consistent with a growing body of literature that has established the role of *S epidermidis* in contamination of urinary catheters, cardiac valves, orthopedic prostheses, vascular grafts, and contact lenses.[14]

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Using an established porcine model,[9] we tested the efficacy of placing antibiotic-impregnated mesh at the time of device implantation to prevent biofilm formation and subsequent contracture.

METHODS

Study approval was obtained from the University of Sydney Animal Ethics Committee. All pigs were housed as a group in designated animal care facilities at the University of Sydney Farms in Cobbitty, New South Wales, Australia.

Five adult, female, nonlactating, domestic Large White pigs (*Sus domesticus*) weighing approximately 350 kg each individually received up to 6 miniature, smooth, silicone gel–filled implants of 4 cm in diameter (TYRX, Inc, Monmouth Junction, New Jersey).

The implants were placed into submammary pockets that had been dissected under the cranial, middle or caudal sets of teats. Surgical methods were as described by Tamboto et al.[9] All implants and surrounding pockets were then inoculated with 10^5 colony-forming units (CFU) of a human clinical strain of *S epidermidis*, originally isolated from a contracted breast in a human patient. A total of 28 implants were implanted into the 5 pigs: 14 control implants (untreated) and 14 implants inserted with antibiotic-impregnated mesh (treated).

Implants were randomly selected by the investigators before implantation to be inserted either alone (untreated) or with a circular disk of antibiotic-impregnated polypropylene mesh (AIGISRx antibacterial flat sheet; TYRX, Inc) (treated), which was placed directly beneath the implant during surgery. Coated with a resorbable polymer carrying minocycline and rifampicin (which are released over 7 to 10 days), the mesh has previously been shown to be effective against *S epidermidis* for a period of at least 7 days (data on file, TYRX, Inc). The implants were left in situ for 16 weeks. They were then assessed for contracture and harvested with the capsule for additional contracture assessment and biofilm analysis.

Contracture of the implants was first assessed using the 4-grade Baker scale[15] while the implants were in situ, with Grade I indicating a relatively normal breast and Grade IV indicating severe CC. Contracture was also assessed by applanation tonometry, as adapted from Minami et al.[16] For assessment by applanation tonometry, the implant and surrounding capsule were aseptically dissected and transported on ice to the laboratory, where they were overlaid with sterile foil. A 300-g weight coated in petroleum jelly was applied to the specimen, and the area of touch was outlined. The surface area of touch was then calculated using Adobe Acrobat X Pro software (version 10.0; Adobe Systems Incorporated, San Jose, California). All assessments of contracture were performed by an investigator (AKD) who was blinded to the original treatment status of the implant.

Biofilm presence on the implant and capsule was assessed with bacterial viability counts, total bacterial counts by real-time quantitative polymerase chain reaction (qPCR), and scanning electron microscopy (SEM). Quantitative aerobic cultures, viability counts, and bacterial identification were obtained following sample maceration and ultrasonication as described previously.[9] All laboratory assessments were performed by personnel blinded to the original treatment status of the implant.

The total number of bacteria was estimated using quantitative real-time PCR. For DNA extraction prior to qPCR, 50 to 100 mg of each pig capsule was added to 275 µL digestion buffer (50 nM Tris-HCl [pH 7.5], 150 nM NaCl, 2 mM EDTA, 1% sodium dodecyl sulfate) containing 400 µg proteinase kinase (Sigma-Aldrich, St Louis, Missouri) and incubated at 50°C overnight. The proteinase kinase was inactivated by boiling for 5 minutes, and lysozyme (Sigma-Aldrich) was added to give a final concentration of 0.5 mg/mL. Following 2 hours of incubation at 56°C, an additional 200 µg proteinase kinase was added, followed by another 2 hours of incubation at 50°C. DNA was extracted using the phenol-chloroform extraction technique followed by ethanol precipitation. Extracted DNA was resuspended in 200 µL of a Tris EDTA buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA), and the DNA concentration was quantified by a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, Massachusetts).

Extracted DNA was subjected to qPCR using universal eubacterial primer 16S rRNA_341F 5′-CCTACGGGAGGCAGCAG-3′ and 16S rRNA_534R 5′-ATTACCGCGGCTGCTGG-3′ to amplify all bacteria.[17] In addition, extracted DNA was subjected to amplification of the pig 18S rRNA gene, which was used as a reference gene to normalize the amount of pig tissue used in DNA extraction. The primer pair used in 18S rRNA gene real-time PCR was 18S rRNA_756F 5′-GGTGGTGCCCTTCCGTCA-3′ and 18S rRNA_877R 5′-CGATGCGGCGGCGTTATT-3′ to amplify a 122-bp amplicon based on the pig (*Sus scrofa*) 18S ribosomal RNA gene sequence (GenBank: AY265350.1).

The 25-µL reaction mix contained 1 × Brilliant II SYBR Green qPCR Master Mix (Agilent Technologies, Santa Clara, California), 400 nM forward and reverse primer, and 20 ng DNA template. The mix was incubated at 95°C for 10 minutes, followed by thermal cycling (95°C for 15 seconds, 56°C for 30 seconds, and 72°C for 20 seconds, repeated 40 times).

The presence of biofilm in capsules was confirmed visually by SEM. Sections of implant and capsule were fixed in glutaraldehyde for 1.5 hours, dehydrated with ethanol, and immersed in hexamethyldisilazane (HMDS; Polysciences, Inc, Warrington, Pennsylvania) for 3 minutes before being aspirated dry and stored desiccated. The dried sample was coated with 20 nm gold film in a sputter coater and then examined in the SEM.

Statistical Analysis

The χ^2 test was used to compare differences in contracture rates between treatment and control groups, and an unpaired *t* test was used to examine differences between treated and untreated contracted implants. Analysis of

Table 1. Baker Grading of Capsular Contracture According to Anatomical Insert Positions of Control and Treated Implants

	Control Implants			Antibiotic Mesh-Treated Implants		
	Position			Position		
	Cranial	Middle	Caudal	Cranial	Middle	Caudal
Pig 1	IV	III	IV, IV	—	—	—
Pig 2	III	—	IV	II	II, II	I
Pig 3	IV	IV	III	II	I	I
Pig 4	—	—	III, lost	I, I	I, II	—
Pig 5	III	III	IV	II	I	I

Blank field indicates that implant(s) were not inserted at this position. Two numbers in one cell represent implantation in both the right and left breast.

Table 2. Summary of Baker Grade Assessment: Grades and Percentage of Contracted Implants by Treatment

Status	Baker Grade	Contracture
Untreated control implants (n = 14)	Grade III = 6 Grade IV = 7 Extruded = 1	Yes = 13/13 (100%)
		No = 0/13 (0%)
Antibiotic mesh-treated implants (n = 14)	Grade I = 8 Grade II = 6	Yes = 0/14 (0%)
		No = 14/14 (100%)

variance (ANOVA) with the Holm-Sidak method of multiple comparisons was performed to identify any variation in bacterial numbers or implant tonometry between multiple tests. All statistical analyses were conducted using the SigmaPlot version 11 statistical program (Systat Software, Inc, San Jose, California).

RESULTS

Of the 28 total implants (14 control; 14 antibiotic-impregnated), 1 untreated control implant was found to have been extruded and was excluded from analysis. No pigs exhibited any obvious systemic effects from bacterial inoculation and/or treatment with the mesh.

Assessment of contracture by Baker grading revealed that all 13 evaluable, untreated, control implants were contracted (graded III/IV). In contrast, the 14 implants treated with antibiotic-impregnated mesh remained noncontracted (graded I/II). This difference was highly significant (χ^2 = 23.14, df = 1, P < .001). Results for individual pigs are found in Table 1 and summarized in Table 2.

Tonometry confirmed the Baker assessment findings. Implants with a higher Baker grade (ie, untreated, control implants) had a reduced surface area, indicating greater capsule thickness compared with antibiotic mesh-treated implants (Table 3). Despite the observed trend of decreasing surface area with increasing Baker grade, these differences were not statistically significant.

Table 3. Surface Area Measured by Applanation Tonometry for Control (Baker Grades III/IV) and Antibiotic Mesh-Treated (Baker Grades I/II) Implants

Baker Grade	Mean Surface Area, mm^2	Group	Surface Area by Group, mm^2, Mean ± SD	Range
I	940	Treated	913 ± 217	613-1379
II	870			
III	836	Control	772 ± 196	470-1250
IV	716			

On enrichment culture, most implants grew coagulase-negative staphylococcus of pig origin, generally *Staphylococcus simulans*. Quantitative counts were generally low, with only 7 implants having more than 10 colonies. There was no relationship between quantitative counts and implant treatment.

The number of bacteria per milligram of pig tissue as measured by PCR was lowest from sites assessed as Baker Grade I (2.39×10^5 CFU/mg), gradually increasing nonsignificantly until Baker Grade III (2.68×10^5 CFU/mg) before decreasing slightly at Baker Grade IV (2.61×10^5 CFU/mg). However, the volume and mass of the capsules from noncontracted breasts were significantly less than the volume and mass of the capsules from contracted breasts, making the total number of bacteria in contracted breasts at least 10-fold higher.

As all implants were inoculated with 10^5 CFU at implantation, it was expected that some degree of biofilm would be present on all implants. Scanning electron microscopy confirmed the presence of bacterial biofilm on implant surfaces and capsules obtained from both untreated and treated breasts (Figure 1). However, although multilayered biofilm was easily detected and present in 100% of SEM views on untreated implants (Figure 1A) and capsules (Figures 1B,C), the biofilm was generally single-layered or composed of isolated bacterial cells and difficult to find on treated implants (not shown) and capsules (Figure 1D,E).

DISCUSSION

We have previously hypothesized that bacteria such as *S epidermidis* gain access to the breast implant at the time of placement and, once in contact with the prosthetic surface, form a biofilm causing inflammation and the establishment of CC.

Our current findings reinforce the validity of the infection hypothesis, which is also supported by clinical trial data[2-8] as well as data from our previous investigation in a porcine model.[9] In that prior study, we placed miniature, textured, silicone gel–filled breast implants (McGhan,

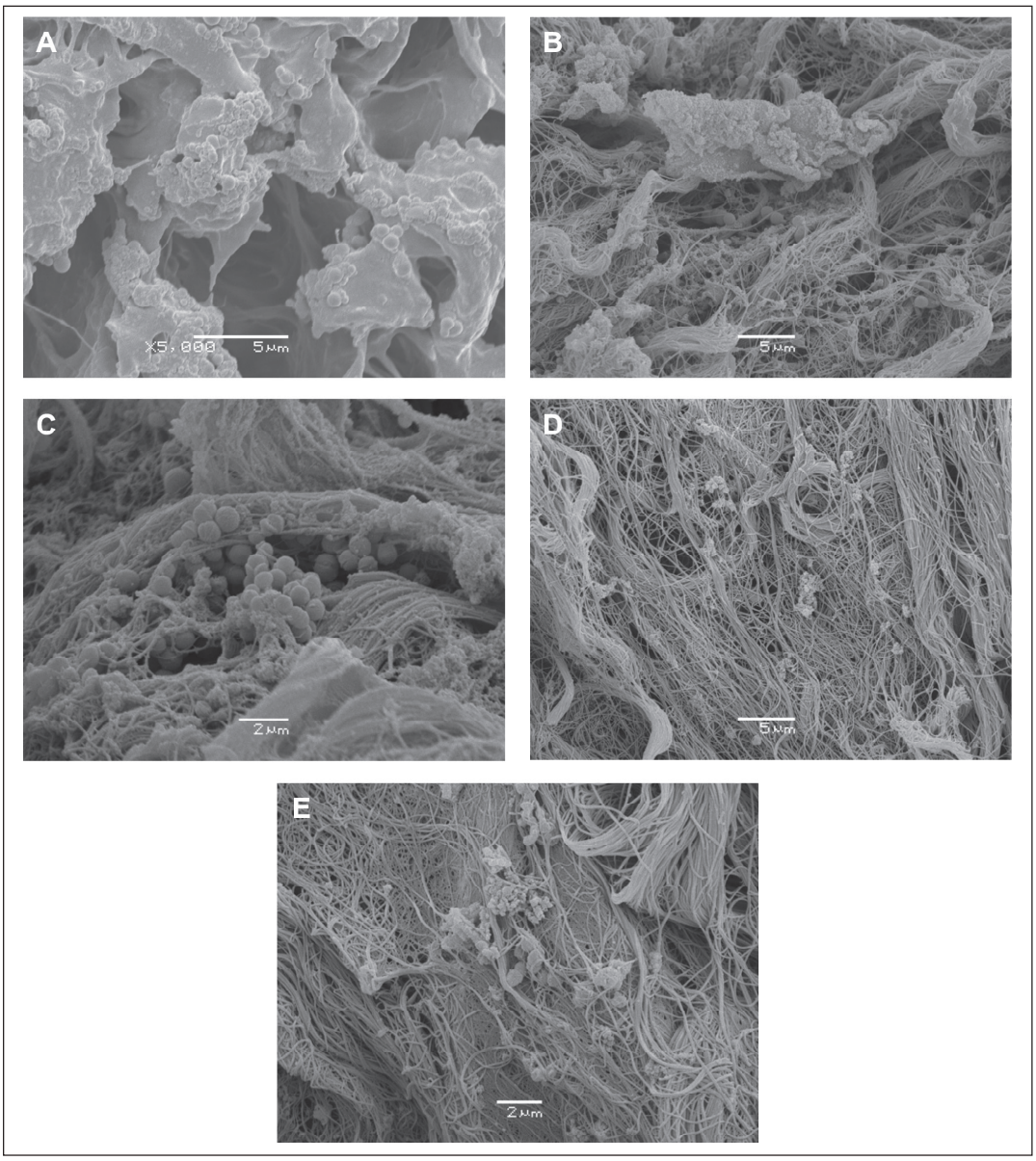

Figure 1. Scanning electron micrographs (SEM) showing presence of bacterial biofilm on untreated (control) and treated breast implant surfaces and capsules. (A) Untreated porcine implant, magnified × 5000. The biofilm consists of coccoid bacteria and large amounts of excreted polymeric substances (EPS). (B) Porcine capsule surrounding an untreated implant, magnified × 4000. Clusters of coccoid-shaped bacteria encased in EPS can be identified. Individual cocci are also frequently found within the capsule fibers. (C) Same capsule as in (B), magnified × 6500 and showing a large clump of cocci overlaced with strings of EPS. (D) Capsule surrounding an antibiotic mesh-treated implant, magnified × 4000 and showing scant cocci. (E) × 6500 magnification of capsule shown in (D), showing a few individual cocci and little EPS.

Dublin, Ireland) in the submammary pockets of pigs using the same standard sterile operative techniques as employed in human surgery. Implant pockets were inoculated with a serially diluted clinical sample of *S epidermidis* cultured from a contracted human breast. A total of 51 mammary augmentations were performed on 6 pigs. Pocket inoculation was strongly associated with biofilm formation ($P = .0095$); moreover, biofilms were significantly associated with CC ($P = .0213$), with 80.6% of biofilm-positive implants developing contracted capsules.

The current findings show that strategies to prevent bacterial attachment at the time of implantation can lead to a decrease in biofilm formation and subsequent contracture. The presence of antibiotic-impregnated mesh had a clear protective effect against the development of contracture when assessed using the Baker grading scale. In addition, the tonometry data indicated that antibiotic mesh-treated implants tended to have greater surface area compared with untreated implants, which was consistent with our findings upon palpation. These data are also supported by a recent study reporting a reduction in biofilm formation on breast implants by applying antibiotic coatings in vitro.[18] The use of antibiotic solution[19] and betadine[10] in the implant pocket has further been shown to significantly reduce the risk of subsequent implant infection and contracture.

The identification of endogenous porcine staphylococcus was consistent with our previous study,[9] in which contracture resulted from contamination with either human or endogenous bacteria. The causative organism in this study was porcine staphylococcus rather than the human staphylococcus inoculum. In our earlier study,[9] we showed that endogenous pig staphylococcus can form biofilms around implants, which subsequently leads to contracture. Interestingly, the original human inoculum was not recovered at implant harvest in this study. This may be due to the later sampling (at 16 weeks) than was undertaken previously (13 weeks). An alternative explanation is that the human inoculum initiates the development of a biofilm by overcoming local immunity. Endogenous porcine staphylococcus species, which are present in higher numbers, may then outcompete the original inoculated human strain.

The analysis of total bacterial counts showed that the number of bacteria within biofilms was consistently low. This finding is consistent with the understanding of biofilm structure. Although not statistically significant, the relationship between bacterial counts and Baker grade is an interesting finding. It is possible that in mature, thickened capsules, the bacterial numbers are reduced relative to connective tissue. Further evaluation of this finding is warranted. The SEM findings in this study reinforce the findings from microbiological analysis and show the presence of mature biofilm colonies attached to both capsule and surface of the implant.

There are some limitations to these findings. Although the 7- to 10-day drug diffusion activity of the antimicrobial mesh seems to be effective at reducing bacterial contact around the time of implantation, exposure of the implants to bacteria following this period, such as through bacteremia from other subsequent procedures, will not be prevented. In addition, the harvest of breast implants at 16 weeks in this study precluded longer follow-up to determine whether any of the treated implants would progress to contracture. In addition, we did not include analysis of the mesh alone (without antibiotic impregnation). We are therefore unable to exclude any local protective effects of a mesh barrier on the prevention of biofilm formation.

CONCLUSIONS

This study demonstrates that strategies to reduce bacterial access to breast implants at the time of surgical insertion can protect against subclinical infection and subsequent CC. Anti-biofilm technologies should be further investigated as a means of preventing infections associated with implantable medical devices.

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Disclosures

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Chapter 8

Biofilm at the Implant Interface

"Dans les champs de l'observation le hasard ne favorise que les esprits préparés. "

("In the fields of observation chance favours only the prepared minds.")

- Louis Pasteur, 1854

8 Chronic Biofilm Infection in Breast Implants is Associated with an Increased T cell Lymphocytic Infiltrate – Implications for Breast Implant-Associated Anaplastic Large Cell Lymphoma

8.1 Introduction

Nonepithelial tumours of the breast are rare and account for less than 1% of all breast malignancies.[611, 612] Non-Hodgkin lymphoma (NHL) is one of the most common of these tumours and may be primary (<1% of all NHL) or extranodal (1.5-2% of all NHL) from primary tumours outside of the breast.[611, 613, 614] Most breast non-Hodgkin lymphomas are B-cell in origin, with diffuse large B-cell lymphoma the most common type, with less than ten percent being T-cell lymphomas.[613] Anaplastic large cell lymphoma (ALCL) is a rare T-cell lymphoma that accounts for up to three percent of all adult and six percent of breast non-Hodgkin lymphomas and 12% of T-cell lymphoma.[613, 615, 616] Histologically ALCL is characterised by large malignant cells with abundant cytoplasm and indented/kidney-shaped nuclei and prominent nucleoli with uniform expression of CD30.[617-619] Anaplastic large cell lymphoma has two phenotypes, systemic and cutaneous. Systemic lymphoma can be further subdivided into ALK-positive and ALK-negative ALCL, based on expression of a unique anaplastic lymphoma kinase (ALK) protein, due to a (2;5)(p23;q35) translocation.[618, 620, 621] The t(2;5)(p23;q35) is the result of a fusion between the anaplastic lymphoma kinase (*ALK*) gene on chromosome 2 and the nucleophosmin (*NPM*) gene on chromosome 5.[622, 623] This neoplastic translocation

encodes an 80-kDa NPM-ALK chimeric protein with constitutive tyrosine kinase activity and results in overexpression of the ALK protein.[624]

Systemic anaplastic large cell lymphoma is a heterogeneous disease with numerous morphological variants including small cell, lymphohistiocytic, Hodgkin-like, sarcomatoid, neutrophil-rich and common forms.[621, 625] It can present with nodal or extranodal disease including in skin, liver, bone or soft tissue.[621] AKL-positive ALCL is predominantly a lymphoma of paediatric and young adult patients, has a male predominance (1.7:1 male:female ratio) and more likely to have bone, bone marrow, subcutaneous and splenic extranodal disease.[626] AKL-negative ALCL is more common in older patients (median age 55-60 years), also has a male predominance (1.5:1 male:female ratio) and is more likely to have cutaneous, hepatic or gastrointestinal extranodal disease.[616] ALK-positive ALCL has a good prognosis with a 60-80% 5-year overall survival, where ALK-negative ALCL has a much poorer prognosis with a 15-40% 5-year overall survival.[616, 618, 620, 621, 626]

Cutaneous ALCL is more common in the older male patient (mean age 55 years; 2-3:1 male:female ratio).[618, 627] The majority of patients (80%) present with solitary or localised cutaneous tumours that are commonly ulcerated.[627] Disseminated disease is uncommon (10%) and mainly involves regional lymph nodes.[627] ALK phenotype is rarely present. Prognosis is good, regardless of presence of multifocal or regional disease, with a five-year survival rate of over 90%.[618, 627]

In 1995 an article detailed three cases of women with breast implants who had developed a cutaneous T-cell lymphoma in the skin overlying breast skin.[628] One patient had evidence of regional lymphadenopathy at time of diagnosis. Implants were removed in two patients, one of whom had resolution of clinical symptoms with nil further treatment.[628] The other had chemotherapy but failed to go into remission, her disease progressed and she died three years after diagnosis. The third woman had localised radiotherapy without removal of her implants and showed resolution of her symptoms and lymphadenopathy.[628] Two years later a second case report was published detailing a single case of a localised swelling of a breast capsule, 5 years after the initial implant was inserted.[629] The capsule was biopsied and was diagnosed as CD30+ anaplastic large cell lymphoma. The patient had resolution of her symptoms after treatment with

chemoradiotherapy without removal of the implant.[629] Whilst the cutaneous T-cell lymphomas in the first three patients were not identified as ALCL at the time, these cases are now recognised as the first documented cases of a possible new entity of breast implant-associated anaplastic large cell lymphoma.[630-632]

The next cohort of women with this disease was not reported until 2008.[633] Five women with breast implants were all diagnosed with CD30+, ALK- anaplastic large cell lymphoma between one and twenty three years after insertion of their implants.[633] Patient treatments and outcomes were not detailed. There are now almost forty articles that have detailed 79 women with breast implant-associated anaplastic large cell lymphoma, with another article published ahead of print detailing 94 previously unreported cases.[630] Large cohort studies have shown that these women had implants inserted for both aesthetic and breast cancer reconstruction in almost equal numbers (75 versus 62, respectively) where indication has been documented.[630, 634]. Similarly implant fill is distributed almost equally between silicone and saline-filled implants (61 versus 48) where documented.[630] However, one implant manufacturer, Allergan Inc (Irvine, Ca), has produced over 80% of the implants associated with breast implant-associated anaplastic large cell lymphoma reported to date.[630, 631]

Clinically, the majority of patients presented with disease localised to the capsule/breast with over 90% of patients presenting with stage I or stage II disease.[630, 634] In the largest cohort review fewer than ten patients presented with disseminated disease with systemic or “B” symptoms of lymphoma, including fever, night sweats, anorexia, generalised fatigue and weight loss.[630] The most common clinical feature is the late presentation of breast swelling secondary to subcapsular effusion/seroma. This finding is consistently present in 60-90% of patients in several large case series, published to date.[630, 631, 634, 635] The estimated volumes involved range from small volumes (<50 ml) to over five hundred mls and the fluid has been described from clear serous-like fluid to a turbid white or yellow liquid.[635, 636] Interestingly a 10-year retrospective review of all subcapsular/peri-implant effusion specimens received by a single pathology service demonstrated that, whilst samples from breast implant effusions are rare (one per year, n=10), four (40%) were diagnosed with breast implant-associated ALCL.[636] Together these findings have

prompted the US Food and Drug Administration and an American Multidisciplinary Expert Review Panel both to recommend the aspiration of the subcapsular fluid, with or without biopsy of implant capsule, for every patient presenting with late breast swelling secondary to subcapsular seroma, for diagnostic investigation including cytology, CD30 and ALK analysis.[637, 638]

Therapeutic options for breast implant-associated anaplastic large cell lymphoma are still evolving, as currently there have been no trials on which to base treatment guidelines.[631] Capsulectomy and removal of implant(s) is the recommended treatment for localised breast disease, as spontaneous remission has been reported in many of these patients.[634, 638, 639] Several different adjuvant chemotherapy and/or radiotherapy options have been used, presumably in-line with local guidelines from the treating hospitals, and a small number of women have undergone a stem-cell transplant either as treatment or salvage therapy.[634, 638, 639] Clinical progression is also variable, with most localised disease commonly having indolent disease course that responds to surgery and/or adjuvant therapy.[630, 634, 640] In contrast, patients presenting with nodal disease, extracapsular involvement or B symptoms have a poorer prognosis.[613, 630, 639, 641, 642] Importantly nine deaths have been reported, all patients presented with metastatic disease, their disease was resistant to systemic treatment and they died from disseminated disease.[630, 640, 641, 643] One patient with systemic disease has been documented to respond to her treatment and remained tumour-free at two years post-treatment.[615]

As the number of patients affected with breast-implant-associated anaplastic large cell lymphoma increases the aetiology is now being questioned.[613, 621, 630, 641, 644, 645] Breast implant-associated anaplastic large cell lymphoma is considered by several investigators to be a disease of the fibrous capsule, and not the breast parenchyma, *per se*.[613] As with oncogenesis, neoplastic transformation results in the progressive accumulation of genetic lesions resulting in the clonal expansion and establishment of a solid or leukemic tumour.[646] During this process DNA damage, with the formation of aberrant chromosomal translocations, is common and identified in up to 90% of non-Hodgkin lymphomas.[646, 647] It is increasingly recognised that environmental agents can play a crucial role in the initiation of the neoplastic transformation of T-cell or B-cells.[646, 648]

These include infectious agents, both viruses and bacteria, and autoimmune and inflammatory diseases.[646, 649] Several authors have suggested that neoplastic transformation in breast implant-associated anaplastic large cell lymphoma is likely to be secondary to chronic inflammation; however the underlying stimulus of the chronic inflammation is still unknown.[613, 621, 630, 641, 644] Similarly suggested potential aetiological agents include silicone surface, implant texture, and implant surface manufacture technique, however to date no causal relationship has be identified.[621, 630, 641, 645]

This article proposes a fourth potential mechanism of chronic inflammation, via the role of chronic inflammation due to bacteria biofilm of the implant capsule. An initial observation of the increased cellularity of the tissue retained on a textured implant and prompted further investigation. Scanning electron microscopy and immunohistochemistry techniques, identifying T-cells and B-cells, have been used to image the implants, both smooth and textured, from a previous study and provided unexpected results. These preliminary results were investigated in both porcine and human patients with imaging and molecular techniques to further used to assess the immune activity at the implant-capsule interface of breast implants that had Baker Grade IV capsular contracture and were positive for biofilm.

8.2 Preliminary Materials and Methods

Silicone implants from the previous porcine study investigating the role of implant texture and capsular contracture (Chapter 5) were examined. Two 1 x 1 cm sections were removed using aseptic techniques and fixed for both scanning electron Microscopy (2.4.1) and Immunofluorescence (2.4.3).

8.3 Preliminary Results

8.3.1 Scanning Electron Microscopy

Cellular and capsular tissue remained attached to surface of the textured-surfaced implants (Figure 8-1). There was limited to no cellular or tissue attached to the smooth implants (Figure 8-2). The attached tissue was either in occasional small thin layered

clumps (Figure 8-2, image B) or single layered scattered clusters (Figure 8-2, images C and D). Within the tissue attached to the textured implants there was a high level of cellular activity on and adjacent to the implant surface (Figure 8-3). These cells, numbering in the tens to hundreds, were situated at the implant-capsule interface. They measured 5-21µm and had a morphology more consistent with activated lymphocytes.

Figure 8-1: Low Magnification-Scanning Electron Microscopy of Textured-Surfaced Implants

Low magnification images of textured-surfaced implants showed thick capsular retained on the implant crypts and surfaces (Images A and B).

Medium magnification images of textured-surfaced implants showing tissue remnants and cells adherent to the implant's irregular surfaces (Images C and D).

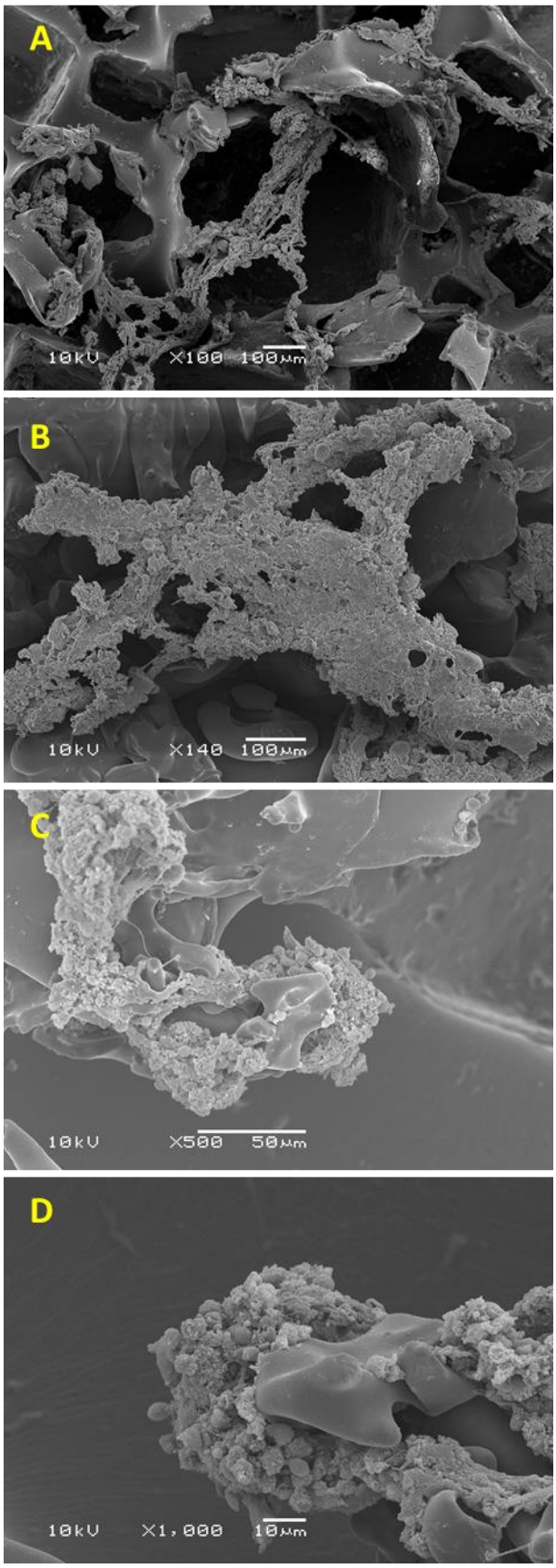

A
10kV X100 100µm
B
10kV X140 100µm
C
10kV X500 50µm
D
10kV X1,000 10µm

Figure 8-2: Low Magnification-Scanning Electron Microscopy of Smooth-Surfaced Implants

Medium magnification images of smooth-surface implants showed minimal attachment. Most implants had large areas of void surfaces (Image A) with occasional small areas of clumps of thin layered capsular tissue (Image B) or scattered single layered clusters (Images C and D). One implant (Image D) showed widespread single layered clusters.

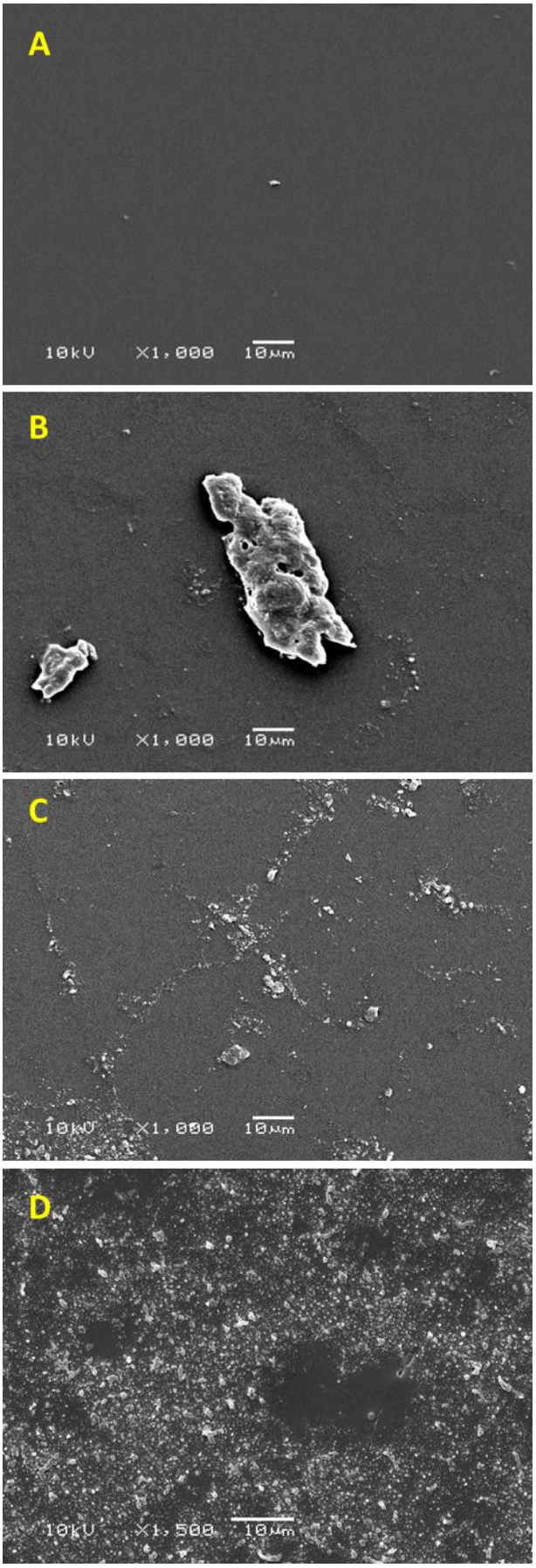
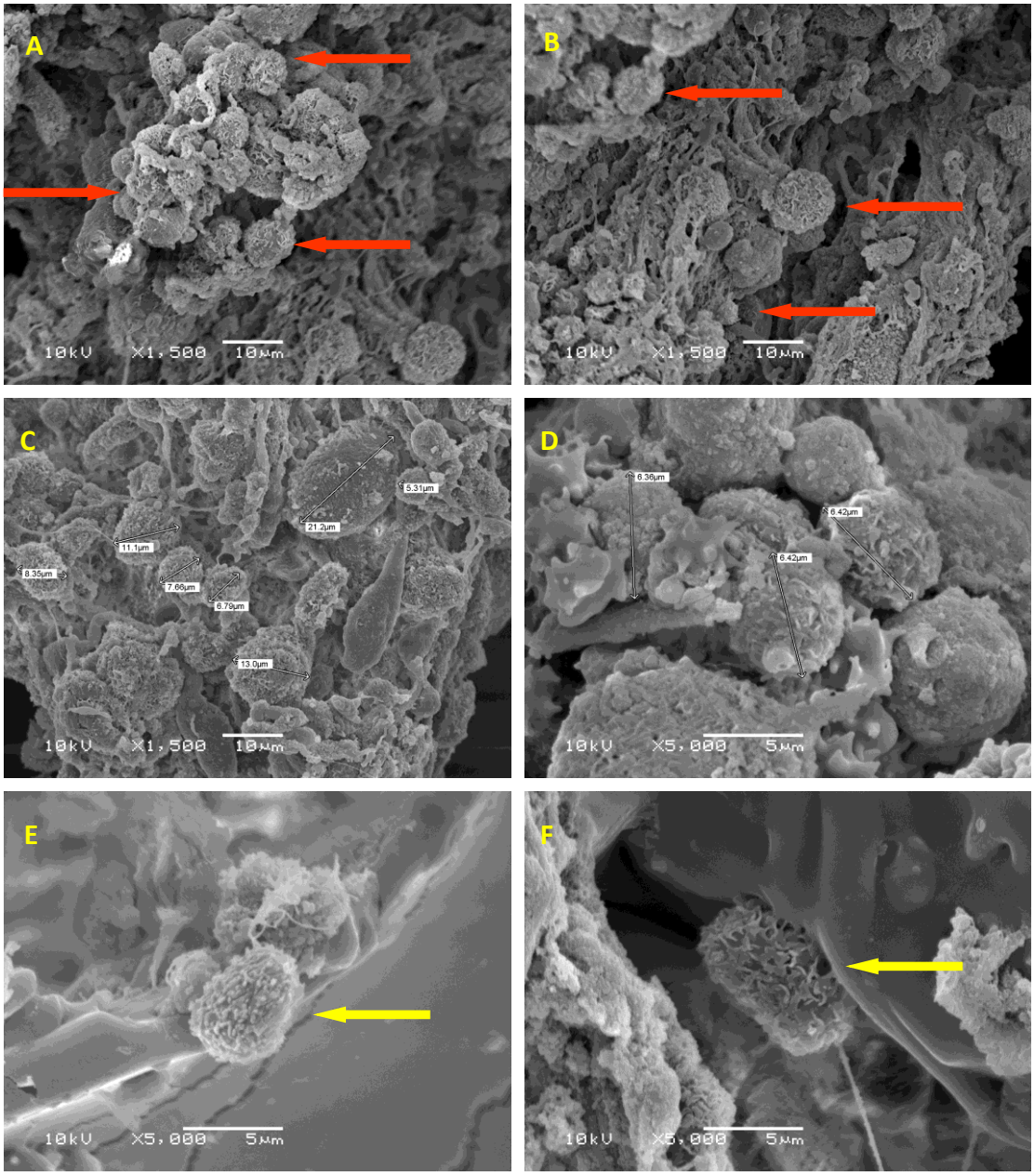

A
10kV X1,000 10μm
B
10kV X1,000 10μm
C
10kV X1,000 10μm
D
10kV X1,500 10μm

Figure 8-3: High Magnification-Scanning Electron Microscopy of Textured-Surfaced Implants

High magnification images of tissue attached to textured-surface implants showed a high level of cellular activity, (orange arrows, Images A and B).

The cells ranged in size from 5 µm, yellow symbol, up to 21 µm, blue symbol. The majority of cells were between 6 µm and 15 µm. These cells were consistent with activated lymphocytes (Images C and D).

Two implants demonstrated the presence of lymphocytes (yellow arrows) directly adherent to the implant surface (Images E and F).


A
10kV X1,500 10µm
B
10kV X1,500 10µm
C
5.31µm
21.2µm
11.1µm
8.35µm
7.66µm
6.79µm
13.0µm
10kV X1,500 10µm
D
6.36µm
6.42µm
6.42µm
10kV X5,000 5µm
E
10kV X5,000 5µm
F
10kV X5,000 5µm

8.3.2 Immunofluorescence

Immunofluorescence with T-cell marker (CD3 with secondary antibody Alexa Fluor®448 that emits a green light) and a B-cell marker (CD79 with secondary antibody Alexa Fluor®448 that emits an orange light) were used to stain the tissue on the textured-surface implants. There was no non-specific fluorescence by the unstained implant. There was mild non-specific binding of the CD79 to the implants, but this did not preclude further analysis.

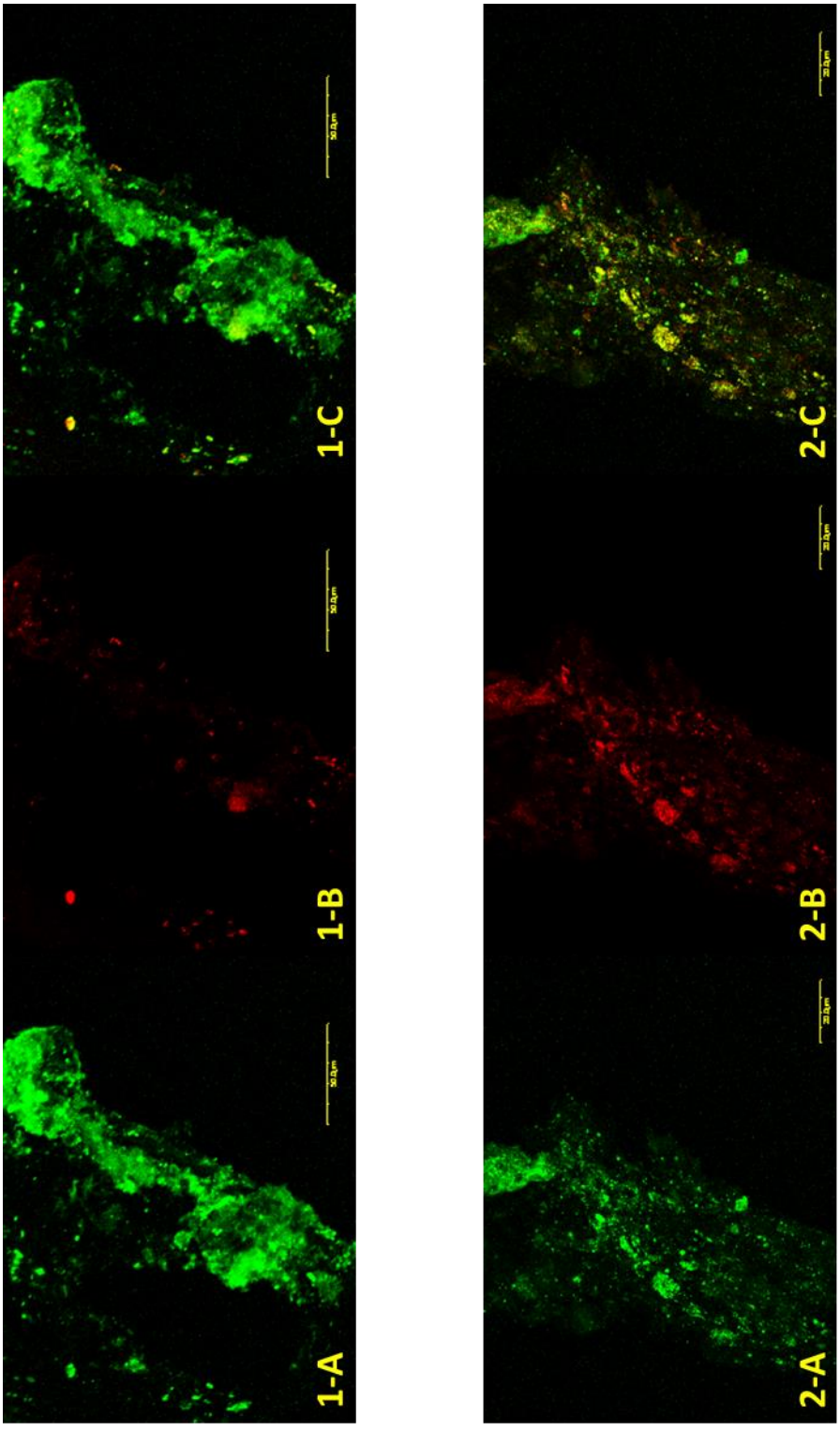
All retained tissue on the implants stained strongly green with the CD3 antibody. There was also red light emission with the CD79 antibody. However, it was a weaker emission and was not always related to the area of tissue attachment, consistent with weaker B-cell response and non-specific binding (Figure 8.4 and 8.5)

Figures 8.4(1) and 8.4(2): T-Cell and B-Cell Immunofluorescence of Tissue attached to Two Textured-Surface Implants

A – T-cell staining with strong binding of the CD3 antibody to the tissue attached to the surface of both implants.

B – B-cell staining with weak binding of the CD79 antibody to the tissue attached to the surface of both implants.

C – Combined T-cell and B-cell staining shows predominant T-cell staining of the tissue attached to the surface of both the textured-surface implant.


1-A
1-B
1-C
2-A
2-B
2-C

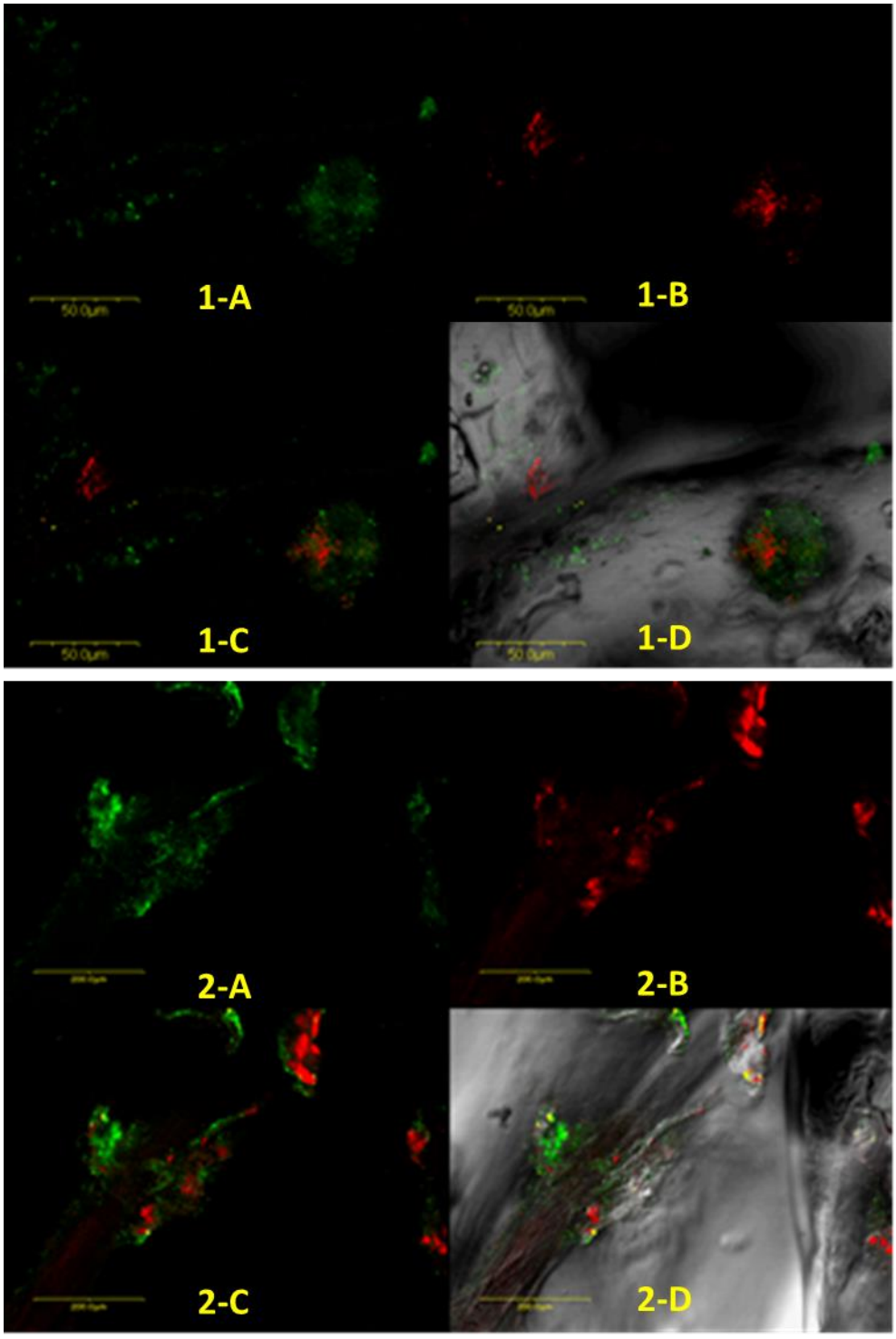
Figures 8.5(1) and 8.5(2): T-Cell and B-Cell Immunofluorescence of Tissue attached to Two Textured-Surface Implants

A – T-cell staining with strong binding of the CD3 antibody to the tissue attached to the surface of both implants.

B – B-cell staining with weak binding of the CD79 antibody to the tissue attached to the surface of both implants. With an area of stronger binding in the second implant (B – 2)

C – Combined T-cell and B-cell staining shows predominant T-cell staining of the tissue attached to the surface of both the textured-surface implant.

D – The T-cell and B-cell stained tissue superimposed over the implant surface where it was attached. These images show strong areas of T-cell staining over the tissue surfaces, with much weaker B-cell staining


1-A
1-B
1-C
1-D
2-A
2-B
2-C
2-D

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8.4 Preliminary Discussion

Whilst these observations were preliminary, together they suggested that instead of a biologically dormant interface, excluded from the immune-system by capsule formation, there is a biologically active interface with increased lymphocyte activity. The lymphocytes appear to be predominantly of T-cell in origin.

These preliminary results prompted a more in depth investigation of implants removed from both porcine and human patients, with the results recently accepted for publication.

8.5 Article – Original Paper

Chronic biofilm infection in breast implants is associated with an increased T cell lymphocytic infiltrate – implications for breast implant associated lymphoma

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Acknowledgement/Contribution

Dr Anita Jacombs: Co-Author

I performed pig surgery, specimen retrieval, Baker Grading, applantation tonometry, sample preparation, scanning electron microscopy and DNA extraction.

I contributed to the manuscript preparation.

Ethics Approvals: University of Sydney N00/12-2010/2/5421 (Appendix 1)

Chronic Biofilm Infection in Breast Implants Is Associated with an Increased T-Cell Lymphocytic Infiltrate: Implications for Breast Implant–Associated Lymphoma

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Background: Biofilm infection of breast implants significantly potentiates capsular contracture. This study investigated whether chronic biofilm infection could promote T-cell hyperplasia.
Methods: In the pig study, 12 textured and 12 smooth implants were inserted into three adult pigs. Implants were left in situ for a mean period of 8.75 months. In the human study, 57 capsules from patients with Baker grade IV contracture were collected prospectively over a 4-year period. Biofilm and surrounding lymphocytes were analyzed using culture, nucleic acid, and visualization techniques.
Results: In the pig study, all samples were positive for bacterial biofilm. There was a significant correlation between the bacterial numbers and grade of capsular contracture ($p = 0.04$). Quantitative real-time polymerase chain reaction showed that all lymphocytes were significantly more numerous on textured compared with smooth implants ($p < 0.001$). T cells accounted for the majority of the lymphocytic infiltrate. Imaging confirmed the presence of activated lymphocytes. In the human study, all capsules were positive for biofilm. Analysis of lymphocyte numbers showed a T-cell predominance ($p < 0.001$). There was a significant linear correlation between the number of T and B cells and the number of detected bacteria ($p < 0.001$). Subset analysis showed a significantly higher number of bacteria for polyurethane implants ($p < 0.005$).
Conclusions: Chronic biofilm infection around breast prostheses produces an increased T-cell response both in the pig and in humans. A possible link between bacterial biofilm and T-cell hyperplasia is significant in light of breast implant-associated anaplastic large-cell lymphoma. (*Plast. Reconstr. Surg.* 135: 319, 2015.)
CLINICAL QUESTION/LEVEL OF EVIDENCE: Risk, V.

Capsular contracture continues to be the most common complication following the use of breast implants in both aesthetic and reconstructive surgery.[1,2] Infection of mammary implants with bacterial biofilm has been shown to be a significant potentiator of capsular contracture.[3–5] The subclinical infection theory, first proposed by Burkhardt et al.,[6] has now been validated by both clinical and laboratory evidence.[2,3,7–11] Bacteria that live on the skin and within the breast ducts can contaminate the surface of the breast implant at the time of insertion. These bacteria subsequently form a biofilm, defined as a combination of bacterially derived sticky glycoprotein and nearly dormant bacteria, which binds irreversibly to the underlying silicone elastomer. Bacteria within the biofilm are resistant to antibiotics and antiseptics.[12] If the biofilm reaches a threshold that overwhelms the local host defenses, it will continue to proliferate and

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expand, eventually causing local inflammation and subsequent fibrosis, leading to the establishment of capsular contracture (Fig. 1).[5]

We have established a valid experimental model for studying the progression of biofilm contamination to contracture in mammary implants using the pig.[3] In addition, we have shown that the use of local antibiotics at the time of implant insertion can significantly reduce the incidence of capsular contracture.[13] This has been further supported in a recent clinical study showing a reduction of capsular contracture from 6 percent to 0.6 percent using intraoperative pocket irrigation.[14] Our most recent findings have shown that, once contaminated, the textured outer surface of a breast implant supports a significantly higher load of bacteria than an implant with a smooth outer shell.[15] The issue of lymphocyte response to breast implants has been raised recently by the reporting of breast implant–associated anaplastic large-cell lymphoma.[16]

To further analyze the interaction among biofilm load, capsular contracture, and host response in the pig, we left biofilm-infected implants in situ three times longer than in our previous experiments. We also prospectively examined human capsules around textured implants from patients with Baker grade IV capsular contracture undergoing revision surgery to generate comparative findings.

PATIENTS, MATERIALS, AND METHODS

Approval for all of the study protocols was obtained from the University of Sydney Animal Ethics Committee. All pigs were housed as a group in designated animal care facilities at the University of Sydney Farms, New South Wales, Australia. Human ethical approval was obtained from Macquarie University Human Ethics Committee.

Pig Study

A total of 24 implants, 12 textured and 12 smooth implants (Allergan, Inc., Irvine, Calif.), were inserted into submammary pockets into three adult, female, nonlactating, domestic, large white pigs (*Sus domesticus*) weighing approximately 400 kg using methods described by Tamboto et al.[3] This model has been reported previously in detail and has become accepted as a valid model for studying the development of capsular contracture following biofilm infection of breast implants.[3,13,17] In summary, miniature implants were inserted into a submammary pocket and inoculated with a dose of human *Staphylococcus epidermidis* previously titrated to generate consistent periprosthetic biofilm infection.[3] The animals were monitored daily in the postoperative period by a veterinary surgeon until their wounds were healed and during their period of housing by support staff at the veterinary school. Implants were left in situ for a mean of 8.75 months, after which Baker grading[18] was performed (Table 1). Baker grading was performed by qualified plastic surgeons, blinded to the implant type, and has been supported as a valid outcome measure of contracture.[3,13,17] Baker grading has also been shown to be consistent with

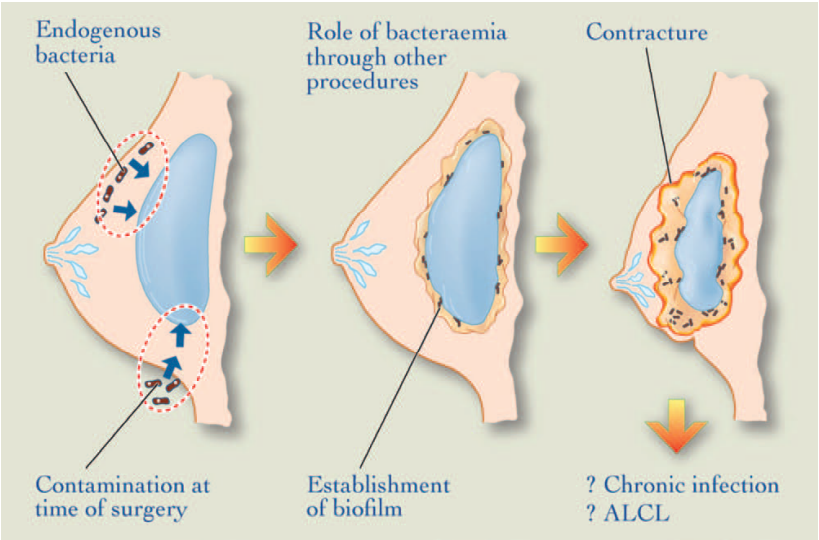

Fig. 1. Subclinical theory of capsular contracture. *ALCL*, anaplastic large-cell lymphoma.

Table 1. Baker Grading for Capsular Contracture*

Baker Grade	Clinical Findings
I	The breast appears natural on examination. There is no evidence of thickening around the implant and minimal palpability of the implant on examination.
II	There is palpable thickening around the implant but no visible change in shape.
III	There is a palpable capsule and hardening of the breasts on examination. There is a visible change in the shape of the breast.
IV	There is palpable thickening of the breast tissue and tissue capsule. Grossly visible changes with severe distortion are apparent on examination.

*From Spear SL, Baker JL Jr. Classification of capsular contracture after prosthetic breast reconstruction. *Plast Reconstr Surg.* 1995;96:1119–1123; discussion 1124.

applanation tonometry when measuring degree of capsular contracture using the porcine model.[13]

Human Study

A prospective collection of capsules from patients undergoing total capsulectomy and removal of implants for Baker grade IV contracture was commenced in January of 2009.

Sample Analysis

Bacterial Culture and Identification

The capsule was separated from the implant using aseptic techniques in a class II laminar flow cabinet. The capsule was macerated and transferred to a 10-ml tryptone soya broth (Oxoid; Hampshire, United Kingdom) and sonication at a mean sweeping frequency of 43 kHz in an ultrasonic bath (Soniclean; JMR, Sydney, Australia) for 20 minutes followed by vigorous shaking for 2 minutes.[3] The numbers of colony-forming units were determined by serial dilution and standard plate culture.

Polymerase Chain Reaction

Fifty or 100 mg of pig or human capsule or breast implant was digested using a combination of proteinase K and lysozyme and the genomic DNA extracted using phenol/chloroform extraction followed by ethanol precipitation as described previously.[15] Total bacterial number in each sample was determined by real-time quantitative polymerase chain reaction of the eubacterial 16 rRNA gene present in all bacteria.[15]

The number of T cells and B cells in each pig or human capsule tissue or attached to breast implants removed from pigs was quantified by real-time quantitative polymerase chain reaction of *CD3* gene (total T cell), *CD4* gene (T helper) and *CD8a* gene (T cytotoxic), and *CD79a* gene (total B cell). Primers specific to these genes in pig and human capsule are listed in Table 2.

The total number of bacteria and lymphocytes in human and pig capsules was expressed per milligram of capsule based on the average number of copies of the *18S* gene in 1 mg of pig or human tissue. Quantitative real-time polymerase chain reaction was carried out in 25 μl of reaction mix containing 1X Brilliant II Sybr Green qPCR Master mix (Stratagene, La Jolla, Calif.), 400 nM forward and reverse primer, and 100-ng DNA template with the following cycling conditions: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 56°C for 30 seconds, and 72°C for 20 seconds as described previously.[13] Each quantitative real-time polymerase chain reaction was run with standard samples of known concentrations ranging from 10^2 to 10^8 copies/μl.

Scanning Electron Microscopy

The presence of biofilm was confirmed visually on all implants and capsules using scanning electron microscopy as described previously.[3]

Confocal Laser Scanning Microscopy

Monoclonal antibodies directed against *CD3* (T cell) and *CD79a* (B cell) (Alexa Fluor Antibody labeling kit; Life Technologies, Carlsbad, Calif.) were used to obtain confocal images of lymphocytic infiltrate on breast implant surface samples using methods described previously by Malisius et al.[19] Stained samples were fixed in 4% paraformaldehyde for 1 hour followed by three rinses in phosphate-buffered saline before examination under an Olympus FluoView 300 (Olympus Corp., Tokyo, Japan) inverted confocal laser scanning microscopy system at Macquarie University Microscopy Unit.

Statistical Analysis

One-way analysis of variance using the statistical package Sigma Plot 11 (Systat Software, Inc., San Jose, Calif.) was used to test the relationship between cultured bacteria and Baker grade. A *t* test was used to examine for differences in the number of bacteria and number of lymphocytes attached to different implants and in capsular tissue surrounding those implants. If the data were not normally distributed, the Mann-Whitney rank sum test was used. Linear regression analysis, on $\log_{10}$-transformed data, was

Table 2. Primers Used for Quantitative Polymerase Chain Reaction of Biofilm and Lymphocytic Response Obtained from Pig Capsules/Implants and Human Capsules

Gene	GenBank Accession Number	Amplicon Size (base pair)	Primer Pair Sequence (5′–3′)
16S rRNA (*Eubacteria*)		194	CCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG
18S rRNA	AY265350.1 NR_003286.2	122	GGTGGTGCCCTTCCGTCA CGATGCGGCGGCGTTAT
Sus scrofa CD3	AY323829.1	125	TCCCTGGGCAAATCTTGGAC AATATCCTTGGGCTGGGTG
Sus scrofa CD4	NM_001001908.1	61	CGCGTGGGACTGGACCTG ACCATGACTGCCCTGTGCTT
Sus scrof CD8a	AY590798.1	114	AACGCAGACCCGAGGAAG GCGGTGGCAGATGATGGTGA
Sus scrofa CD79a	NM_001135962.1	181	TGCTGATCTGTGCCGTGGTG TCCTGGTAGGTGCCCTGGAG
Homo sapiens CD3e	NM_000733.3	66	TGCTGCTGGTTTACTACTGG CCGCTCCTCGTGTCAC
Homo sapiens CD4	BT019811.1	74	TTCATTGGGCTAGGCATC ATCTGAGACATCCGCTCTG
Homo sapiens CD8a	NM_001768.6 NM_171827.3	71	CAGCGGTTCTCGGGCAAGA TCGTTCTCTCGGCGGAAGTC
Homo sapiens CD79a	NM_001783.3 NM_021601.3	54	ACTTCCAATGCCCGCACAAT CGCGCCACCAGGTGACGTT

used to determine the relationship between T cells and number of bacteria in human capsules.

RESULTS

Pig Study

Of the 24 implants, four were lost during the course of the experiment, presumably because of exposure and subsequent extrusion. These lost implants were placed caudally in the pig and were therefore subjected to pressure from the pigs' haunches with resulting wound dehiscence and extrusion. There were 10 capsular specimens surrounding both smooth and textured implants available for analysis, which was sufficient to allow comparative statistical analysis of outcomes.

Bacterial Culture

Twenty capsular samples were subjected to culture analysis. Of the 20 implants, blinded assessment yielded two Baker grade I, six Baker grade II, nine Baker grade III, and three Baker grade IV contracted implants. The overall contracture rate was 60 percent. Figure 2 summarizes the colony-forming unit counts of cultured bacteria per milligram of capsule for each Baker grade. They confirm a significant increase in the number of bacteria for increasing Baker grade ($p = 0.045$).

Total Bacterial Number Detected by Quantitative Real-Time Polymerase Chain Reaction

Twenty capsule samples were also subjected to quantitative polymerase chain reaction to detect the bacterial 16S RNA gene. There was no significant difference in the number of bacteria per milligram in capsules surrounding different types of implants (2.7×10^5 versus 3.5×10^5 bacteria/mg of capsular tissue for smooth and textured implants, respectively). Consistent with our findings on culture, there was an increasing amount of detectable bacterial 16S RNA gene with increasing Baker grade (Fig. 3). This did not reach significance. Significantly more bacteria were attached to textured implants (4.2×10^5 bacteria/mg implant) compared with smooth implants (1.52×10^3 bacteria/mg implant; $p < 0.001$).

Lymphocyte Response

Capsules

Quantitative polymerase chain reaction analysis of lymphocytes in breast implant capsules showed a significant predominance of T cells (CD3; mean, 3.7×10^7) compared with B cells (CD79a; mean, 4.7×10^4; $p < 0.001$). There was no significant difference in the number of CD3, CD4, CD8, or B cells per milligram of capsular tissue surrounding smooth or textured implants ($p > 0.7$).

Implants

Analysis of the lymphocytic infiltrate on implants showed that textured implants had a significantly higher number of both B and T cells on their surface compared with smooth implants (Table 3). There were a mean of 8.23×10^5 lymphocytes/mg of implant for textured as compared with a mean of 1.3×10^4 lymphocytes/mg of implant for smooth ($p < 0.001$), a 63-fold increase for textured implants. The majority of the lymphocytes

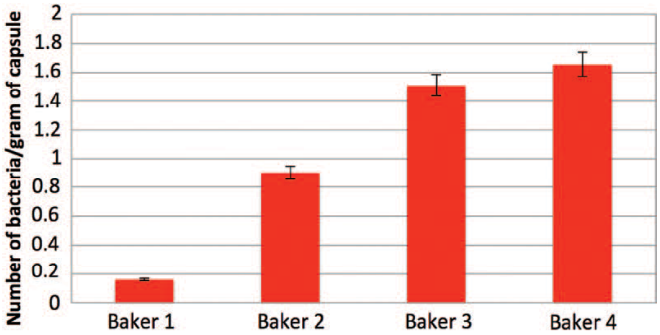

Fig. 2. Colony-forming unit counts of cultured bacteria per milligram of capsule for each Baker grade in the pig cohort. They confirm a significant increase in the number of bacteria for increasing Baker grade ($p = 0.045$) (standard error shown).

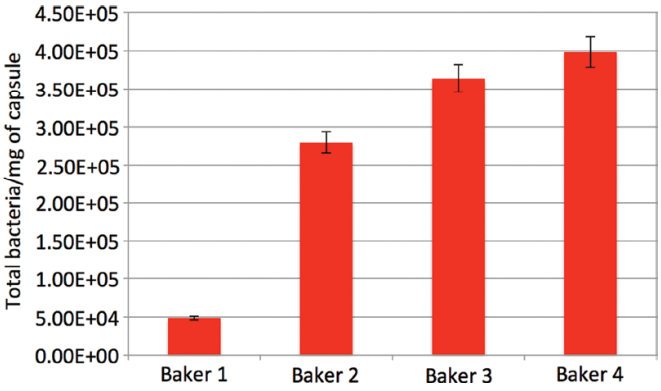

Fig. 3. Total number of culturable and nonculturable bacteria, as determined by quantitative polymerase chain reaction per milligram of capsule for each Baker grade in the pig cohort (standard error shown).

Table 3. Mean Number of Lymphocytes as Detected by Quantitative Polymerase Chain Reaction per Milligram of Implant for Textured versus Smooth Implants in the Pig Cohort*

	CD3 Total T Cells	CD4 Helper T Cells	CD8a Cytotoxic T Cells	CD79a B Cells
Textured	8.23×10^5	6.44×10^4	1.71×10^3	3.94×10^2
Smooth	1.30×10^4	1.10×10^3	8.27	2.37

*$p < 0.001$.

associated with the surface of implants contaminated with bacterial biofilm were T cells.

Immunohistochemistry

Six textured and four smooth implants were subjected to immunohistochemical staining with antibodies against CD3 (T cell) and CD79a (B cell) followed by confocal laser scanning microscopic imaging. All textured implants showed a high number of $CD3^+$ lymphocytes and scant $CD79a^+$ lymphocytes (Fig. 4). Smooth implants had much less biological material to image but also showed a predominant $CD3^+$ (T-cell) infiltrate (Fig. 4).

Confocal laser scanning microscopic images of lymphocytes attached to textured and smooth implants stained with CD3 stain (labeled with Alexa Fluor 488, green) and CD79a stain (labeled with Alexa Fluor 543, red).

Scanning Electron Microscopy

Scanning electron microscopy confirmed the presence of numerous activated lymphocytes on the surface of textured implants (Fig. 5).

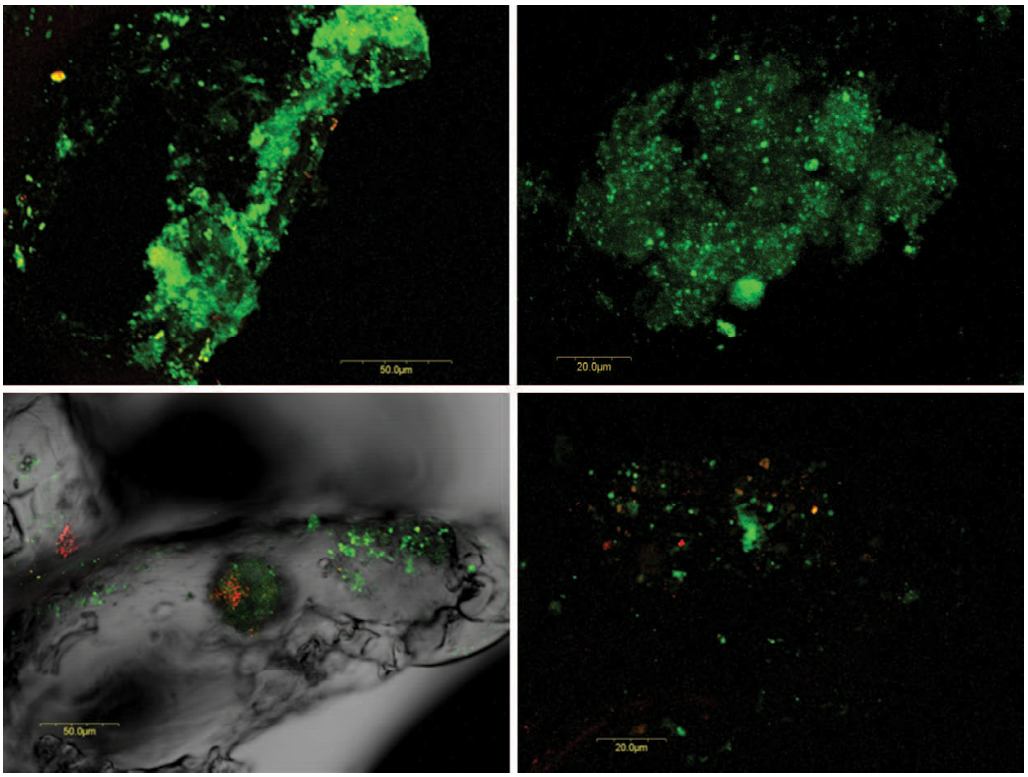

Fig. 4. T- and B-cell staining for textured versus smooth implants in the pig cohort. (*Above, left*) Textured implant at low power (original magnification, × 200) showing predominant CD3 (*green*) staining of material attached to the surface of the implant. (*Above, right*) Textured implant at high power (original magnification, × 400) showing dense $CD3^+$ (*green*) lymphocytes. (*Below, left*) Smooth implant at low power (original magnification, × 200) showing scant CD3 (*green*) and some CD79a (*red*) material attached to surface of implant. (*Below, right*) Smooth implant at high power (original magnification, × 400) showing predominance of $CD3^+$ (*green*) lymphocytes.

Activation was indicated by an increase in lymphocyte size, membrane ruffling, and active replication.[20,21] By comparison, smooth implants had little or no attached infiltrate (Fig. 6).

Human Study

Lymphocyte Response

A prospective collection of 57 periprosthetic capsules from 34 patients undergoing capsulectomy and removal of breast implants for Baker grade IV contracture was undertaken over a 4-year period. All removed implants were textured, reflecting the greater use of textured implants by surgeons who contributed to the study.

Capsules were subjected to quantitative polymerase chain reaction analysis to determine the number of lymphocytes, their CD status, and the total number of bacteria as with the porcine samples. All capsules were positive for biofilm bacteria, with a mean of 2.52×10^7 bacteria/mg of capsule. In this cohort, there were a number of varying texture types. There were 34 implants with Biocell (Allergan) texture, 14 implants with Siltex (Mentor Worldwide, Santa Barbara, Calif.) texture, five implants with Poly Implant Prothèse texture, and four implants with polyurethane texture.

There were significantly more T cells ($CD4^+CD8a$) compared with B cells ($p < 0.001$). The number of lymphocytes correlated with the number of bacteria per milligram of capsular tissue (Fig. 7) ($p < 0.001$; CD3, $r = 0.71$; CD4, $r = 0.83$; CD8a, $r = 0.71$; CD79a, $r = 0.74$). Analysis of bacterial number versus texture type showed that polyurethane implants had significantly more bacteria compared with other textured implants ($p < 0.005$) (Fig. 8).

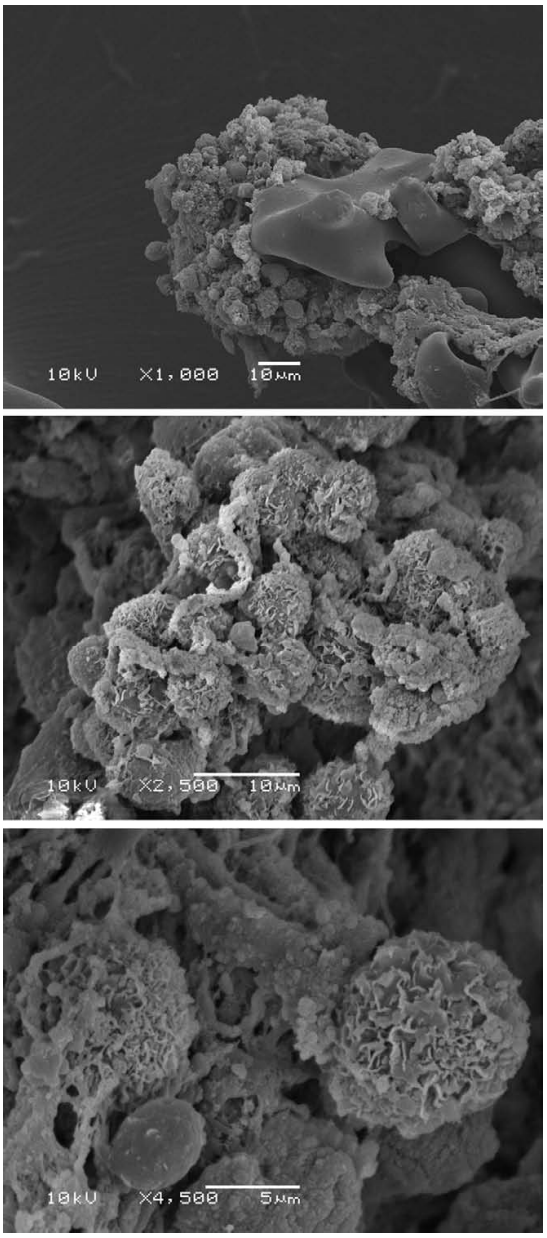

Fig. 5. Scanning electron microscopic images of activated lymphocytes on the surface of textured implants in association with biofilm. (*Above*) Biofilm and lymphocytes attached to a textured implant (original magnification, × 1000). (*Center*) Higher magnification image showing many activated lymphocytes in close association with large quantities of biofilm attached to a textured implant (original magnification, × 2500). (*Below*) Closeup of two activated lymphocytes in close association with biofilm attached to a textured implant (original magnification, × 4500).

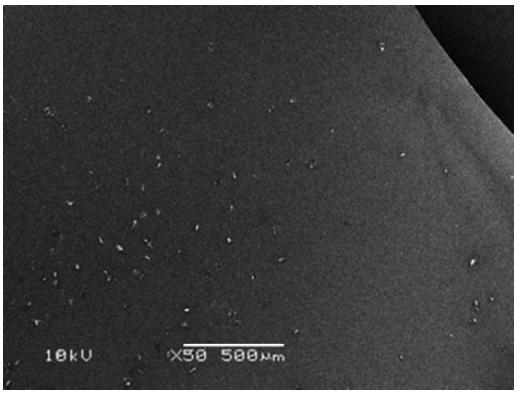

Fig. 6. Surface of smooth implant showing scant biological material.

DISCUSSION

It has been over 30 years since Burkhardt et al. proposed the subclinical infection hypothesis to explain capsular contracture and recommended the use of povidone-iodine irrigation of the pocket.[6] Since that time, with advances in our understanding of bacteria and their propensity to form biofilm on medical devices, the underlying science to support this theory has been elucidated.[5] Biofilm contamination of other medical devices is being recognized as an increasingly important cause of device-associated infection and revision surgery, with associated cost and patient morbidity.[5]

Our study sought to further investigate the important interactions between established biofilm and the surrounding host tissue around infected mammary implants. A number of important findings have arisen from this study.

From our pig model, we have shown that there are increasing numbers of bacteria for increasing Baker grade (Fig. 2). These data suggest that there is a threshold of bacterial biofilm above which host responses are triggered that ultimately lead to contracture. In Baker grade I implants, the host is able to clear or contain the biofilm to a level that does not produce further inflammation. Although it is unlikely that all bacteria are removed, the symbiosis between bacterial load and host immunity is able to restrict the inflammatory response. Once a critical load is reached, however, bacteria overwhelm the host response, continue to proliferate, and trigger an inflammatory response, leading to subsequent fibrosis and contracture. It is likely that this threshold will vary depending on host immunity, bacterial pathogenicity, and the type of implant surface.

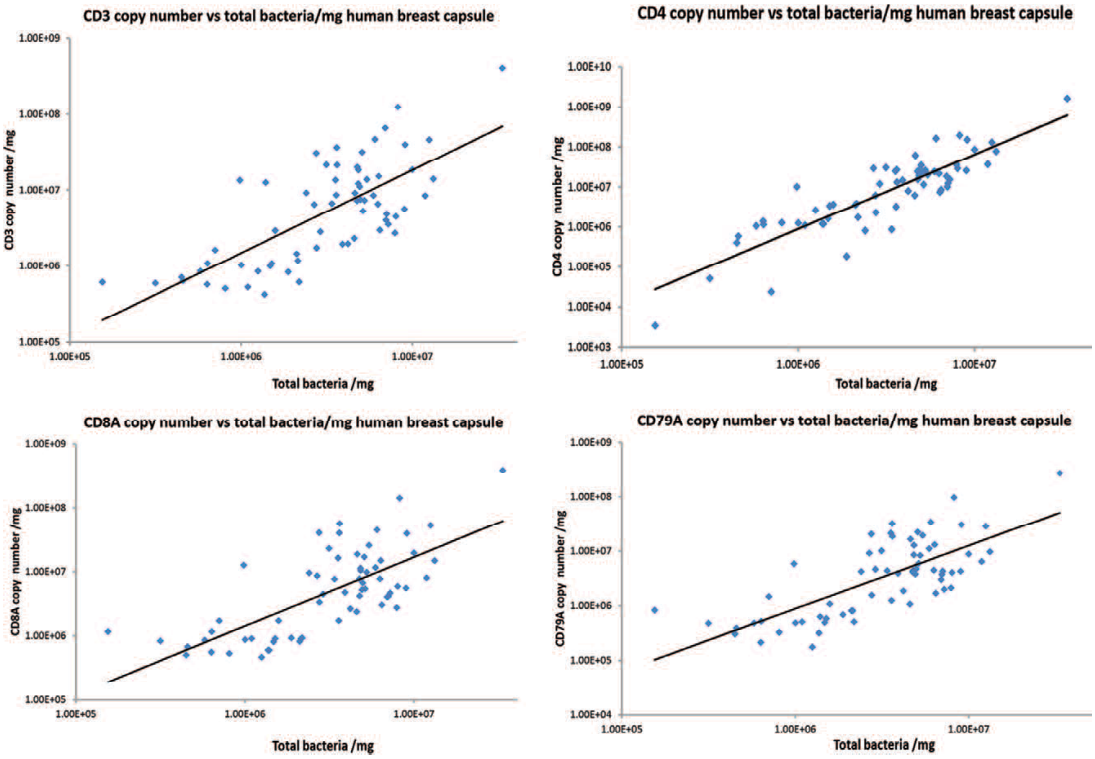

Fig. 7. CD3, CD4, CD8, and CD79a from human capsules versus total bacteria per milligram of capsular sample.

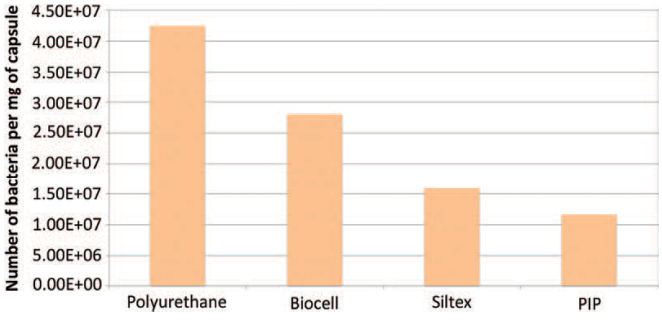

Fig. 8. Bacterial numbers versus type of textured implants from human cohort (standard error shown). *PIP*, Poly Implant Prothèse.

The use of polymerase chain reaction identification of total bacterial 16S RNA gene provides an alternative to traditional culture techniques as a means of detecting biofilm. Polymerase chain reaction is a more sensitive tool for diagnosis and will potentially detect contaminating bacteria as well.[22] It is important to corroborate findings of polymerase chain reaction with imaging of bacterial biofilm and/or with culture techniques. The polymerase chain reaction data did show a similar increasing bacterial load with Baker grade for capsules surrounding both smooth and textured implants. Unlike the culture data, however, this did not reach significance.

The analysis of lymphocytes in both capsules and on the surface of implants contaminated with biofilm has shown that there is an overwhelming T-cell response to the presence of bacteria.

Furthermore, in the pigs, biofilm-infected textured implants elicited a 63-fold increase in the number of T cells compared with smooth implants. In our previous study,[15] we have shown, both in vitro and in vivo, that textured implants, once contaminated, support up to 72 times more biofilm bacteria compared with smooth implants. In this study, we have confirmed that if textured implants become contaminated they support 30 times more biofilm bacteria than contaminated smooth implants. The use of texturization, although conferring biological benefits for tissue incorporation, also increases the surface area, producing a more ideal surface for biofilm to form. The higher bacterial load on textured implants may explain the observed higher lymphocyte numbers and predominantly T-cell hyperplasia.

The analysis of capsules from patients with grade IV contracture and textured implants confirmed the predominance of T cells in the infiltrate and a linear relationship between the numbers of lymphocytes with increasing bacterial load. These findings are consistent with our prior findings in the pig. Analysis of capsules from the varying textured implants showed that the polyurethane-coated implants had significantly higher numbers of bacteria compared with other textured implant types ($p < 0.005$). These findings, however, have to be tempered by the variation in time to explantation in this cohort and small comparative numbers of both Poly Implant Prothèse and polyurethane implants compared with Biocell and Siltex textures. It has been suggested that textured implants cause less capsular contracture, but comparative data to support this are conflicting.[17] Meta-analyses are limited by significant variation in surgical technique, sample size, follow-up period, variations in texture pore size,[23] implant placement, the use of antibacterial pocket irrigation, antibiotic use, and the use of postoperative drainage[23,24] when pooling clinical studies. This variability in clinical practice does bring into question the "dictum" that it is surface texture alone that is responsible for consistently lower capsular contracture. Adams has postulated a cumulative effect of potentiators and suppressors that interplay to determine whether a patient will progress to capsular contracture.[2] Avoidance of contamination, careful atraumatic dissection of surgical pockets, and the use of pocket irrigation, for example, may be more significant suppressors of capsular contracture compared with the suppressive contribution of surface texture alone. A recent study by Giordano et al.,[14] for example, has shown a 10-fold reduction in capsular contracture with the use of povidone-iodine pocket irrigation alone.

Our human data suggest that textured implants present a larger surface area to bacteria and support a higher bacterial load in the setting of established biofilm infection. In the setting of a higher biofilm load, any advantage conferred by surface texture could be potentially negated by more rapid biofilm growth, subsequent contracture, and associated T-cell hyperplasia. The infectious hypothesis does not necessarily predict that textured implants will progress to higher rates of contracture, as this is determined by the threshold levels of infection, above which local inflammation is both initiated and perpetuated. We have shown, for example, that smooth implants with significant biofilm contamination will also equally progress to contracture. It is interesting to note the finding of a decreasing trend of bacterial counts with decreasing aggressiveness of implant texture[25] (Fig. 8). This supports our previous study showing that textured implants present an increased surface area for bacterial biofilm to form.[15] We plan to subject varying implant textures to our previously reported in vitro model[15] to further study this.

A possible link between bacterial biofilm and T-cell hyperplasia is significant in the context of recent reports of breast implant–associated anaplastic large-cell lymphoma (ALCL),[16,26–28] a rare T-cell lymphoma. Interestingly, the majority of cases have been associated with textured implants and, more particularly, the more aggressive (Biocell) texture.[28] The use of Biocell textured implants has also been implicated in late seroma and double capsule, which is a common presentation of breast implant–associated ALCL.[29] Allan et al. have also previously reported the finding of biofilm in double capsules in both human and pigs, pointing to a possible role for biofilm in the pathogenesis of both double capsule and ALCL.[30] Interestingly, the $CD4^+$ (T helper) cells showed the most significant correlation ($r = 0.83$) with increasing numbers of bacteria. It is these cells that undergo malignant transformation in ALCL.

The finding of higher T-cell numbers in periprosthetic capsules taken from both humans and pigs with chronic biofilm infection raises the question of a biological link between the presence of biofilm, inflammation, T-cell stimulation, and the development of lymphoma. Chronic bacterial infection has been shown to be a causal agent in the development lymphoma in humans (e.g., *Helicobacter pylori* and gastric lymphoma).[31] In *H. pylori* infection, chronic inflammation of the

gastric mucosa has been cited as the foundational mechanism underlying the occurrence and development of gastric lymphoma.[32] Deregulation of T-cell stimulation has been identified as a potential pathway to the development of malignancy.[33] More recently, virulence factors from *H. pylori* such as the cytotoxin-associated gene A protein have been shown to deregulate intracellular signaling pathways and promote lymphomagenesis.[34] It is biologically plausible that chronically infected breast implants may mediate similar inflammatory and neoplastic processes that lead to development of a T-cell lymphoma. This is in contrast to mucosal-associated and other implant-associated lymphomas, which are primarily of B-cell origin, and could reflect the unique microenvironment in which breast implants reside. The pathway to malignancy, however, is likely to be a multistep process, with possible variation in bacterial phenotypes, patient genotypes, and other immune-mediated factors contributing to the eventual development of breast implant–associated ALCL. This would explain why some patients with biofilm infection around breast implants proceed to contracture and why other patients (less commonly) proceed to lymphocytic hyperplasia and breast implant–associated ALCL. It would also account for the variation in breast implant–associated ALCL aggressiveness seen in patients who have other phenotypic or immune-mediated risk factors for the development of malignancy. Further study of the relationship among bacterial biofilm, lymphocytes, and the local breast environment is needed to more clearly elucidate the pathway from biofilm infection to the development of malignancy.

A recent study published by Kellogg et al.[35] describes lymphoma (both T and B cell) arising in association with a variety of other prosthetics, including stainless steel, pacemakers, venous access devices, cardiac prostheses, and orthopedic hip prostheses. It is possible that device-associated infection with biofilm and subsequent inflammation combined with a maladaptive immune response is the common causal link to the development of these rare malignancies. These findings further reinforce the importance of applying known and effective intraoperative strategies[5] to reduce the risk of biofilm contamination at the time of insertion, especially when choosing textured implants.

CONCLUSIONS

Our study has confirmed that there is a progressive increase in the number of bacteria as capsular contracture becomes more apparent. This points to a likely threshold for the establishment of a proliferating biofilm and development of capsular contracture on mammary implants. Chronic biofilm infection of mammary prostheses in both pigs and humans is associated with a predominantly T-cell lymphocytic infiltrate, which is directly linked to the bacterial load attached to the implant. In the pig, the T-cell numbers were highest for infected textured implants, consistent with our previous finding of higher bacterial numbers on these implants. The finding of activated lymphocytes on electron microscopy and proliferation of T cells on the surface of textured implants is significant in light of recent reports of breast implant–associated ALCL. Further analysis of the immune response to biofilm around mammary implants and other medical devices should be undertaken as a matter of priority to investigate the link between biofilm-mediated inflammation and malignancy.

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9 Conclusion

"The seeds of great discoveries are constantly floating around us,
but they only take root in minds well-prepared to receive them."

- Joseph Henry, 1877

Bacteria are some of the most successful microorganisms to survive on planet Earth. A crucial part of their success is their ability to form and live in communal structures called biofilms. These structures house over 99% of the bacterial biomass and provide a unique, dynamic microenvironment that ensures their survival, reproduction and protection from the external environment. Without a doubt, it is the incredible success of these complex and protected structures that have made bacterial biofilms elusive to scientific investigation until the latter part of the twentieth century.

John William (Bill) Costerton, the father of the science of bacterial biofilms, only began investigating biofilms when he became interested in bacteria that were attached to the gut or cellulose fibres via a complex matrix. [650] His first paper on what was to be later called biofilms was published in 1977.[651] Since this time our scientific knowledge about bacteria within biofilms has rapidly expanded. However the acceptance by the medical fraternity that bacteria within biofilms could cause disease took almost another 20 years.[10, 23] It is now acknowledged that bacteria within biofilms are important in the aetiology of an increasing number infectious diseases.[65, 70] Biofilm infection of surgical implants is a significant proportion of biofilm-related disease and is associated with a rapidly escalating cost burden to health budgets, which is not sustainable into the future.[66, 69]

This thesis has explored the Subclinical Biofilm Infection Theory of silicone breast implants using a pre-clinical porcine model. There is now extensive clinical and pre-clinical evidence that bacterial biofilm infection of breast implants is important in the aetiology of capsular contracture. Contamination of the implant at the time of insertion, from patient skin, or surgical gloves and equipment, remains the most significant portal of entry. However, the porcine model with silicone breast implants retained *in vivo* for over six

months has demonstrated that the bacteria within the biofilm is dynamic and has origins from skin and teat commensals as well as water borne/environmental bacteria. These results support the role of endogenous spread from translocation of skin/breast commensal bacteria, and possibly haematogenous bacterial spread, in implant infection.

Now that the Subclinical Biofilm Infection Theory of breast implants and its role in the formation of capsular contracture is becoming accepted, the focus must now move towards prevention of bacterial biofilm implant infection. To date, improvements in surgical techniques have helped to reduce capsular contracture rates to less than 15% in aesthetic patients and 15-30% in reconstructive patients. With over 220,000 implant-based breast augmentation operations performed in 2011 in the US alone, putting a minimum of 33,000 women at risk of developing capsular contracture, there is an imperative to decrease this risk further. Rapid advances in biomaterials and implant surface adjuncts are hoped to provide newer and safer implants that have an inherent ability to prevent bacterial attachment.

One of the first modifications of silicone breast implants was the development of textured implants after early results with polyurethane covered implants suggesting that the texture surface decreased capsular contracture. Numerous trials and several meta-analyses have shown a negligible to small decrease in capsular contracture rates with textured-surface implants. On the contrary, results using the porcine model suggest that there is an increased bacterial load attached to textured-surfaced implants, although the rates of capsular contracture between smooth and textured-surfaced implants were not significantly different. These results suggest that modification to surface texture alone is unlikely to significantly decrease rates of bacterial biofilm infection and subsequent capsular contracture.

The emphasis is now moving towards developing novel surface technologies to afford more significant improvements. Antibiotic impregnated surfaces and implant sleeves are one strategy being developed for numerous implants. Early orthopaedic data suggests that this may be a useful strategy, however further research, including appropriately designed and conducted randomised trials, is required. In a trial using a porcine model, a novel antibiotic-impregnated implant sleeve surrounding silicone breast implants was

inoculated with *S. epidermidis*. These implants failed to develop clinically significant capsular contracture and the bacterial biofilm, whilst occasionally present, was single layered and scant. This result was statistically significant compared to the implants that were inoculated without the antibiotic cover, which developed significant capsular contracture in all breast implants and demonstrated extensive thick multi-layered biofilm. This pre-clinical trial demonstrated proof of concept that an antibiotic impregnated implant sleeve can significantly reduce bacterial load and subsequent biofilm formation and capsular contracture. These results suggest that novel antibiotic coatings, or any other novel surface, that can prevent biofilm formation may be an important strategy in the prevention of biofilm-implant disease and subsequent capsular contracture surrounding breast implants.

Towards the end of my research I began to investigate what, if anything was happening on the implant surface. This led to the interesting observation of a highly active cellular interface between the implant and capsule surfaces. The cells were not only bacterial in origin, but were outnumbered by much larger cells consistent with activated immune cell morphology. Preliminary immunofluorescence data identified these cells as predominantly T-cell in origin. These preliminary results coincided with increasing reports of a rare T-cell anaplastic large cell lymphoma being identified in increasing numbers in women with breast implants. The knowledge of this probable new entity, of breast implant-associated anaplastic large cell lymphoma, is still very embryonic but early data in the porcine model shows a correlation between bacterial biofilm load and increasing T-cell activity, suggesting that the chronic infective state of bacterial biofilm may have a role in the tumorigenesis of this lymphoma (Figure 9-1). With over 170 reported cases and 10 deaths attributed to this new disease, further investigation into the aetiology of this rare, but important, lymphoma is required to understand what role, if any, the implants and/or bacterial biofilm play in its aetiology.

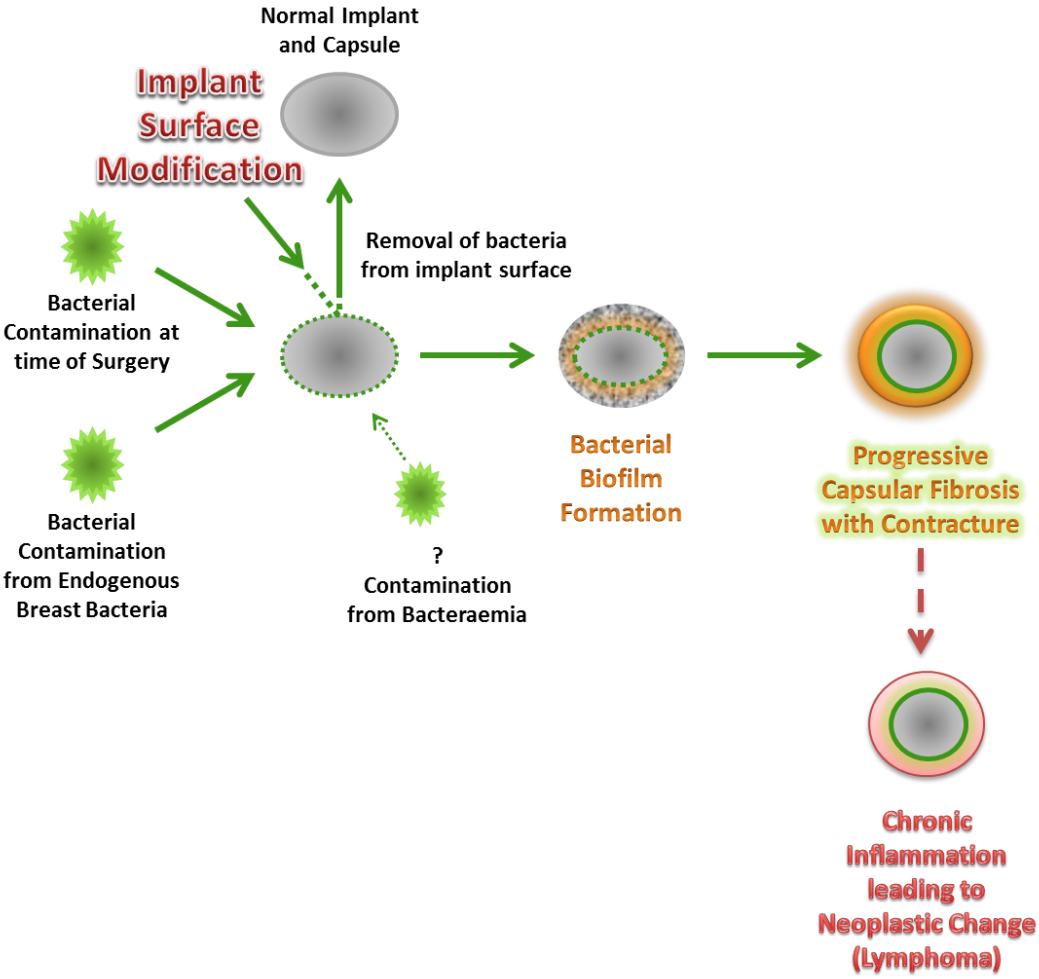
Figure 9-1: Subclinical Biofilm Infection Theory of Capsular Contracture and Lymphoma

Bacterial infection on breast implants results in formation of bacterial biofilm. Chronic inflammation from the biofilm causes progressive fibrosis and development of capsular contracture.

There is now increasing evidence that infection occurs not only at the time of implant insertion (predominant route of infection), but also via contamination of endogenous breast bacteria and this may have a role in initial infection and/or propagation of the biofilm infection.

In this thesis I have demonstrated the proof of concept of the use of implant surface modification as a mechanism of preventing biofilm formation on the implant surface.

Preliminary investigation of the capsule-implant surface interaction has demonstrated that there is a highly active interface involving a predominant T-cell immune response. The significance of this finding, along with the current dilemma of an increase in diagnosis of breast implant associated-anaplastic large cell lymphoma, is unclear but suggests that chronic fibrotic inflammation secondary to biofilm infection may have be a stimulant in the neoplastic progress of this disease.


Normal Implant
and Capsule
Implant
Surface
Modification
Removal of bacteria
from implant surface
Bacterial
Contamination at
time of Surgery
Bacterial
Contamination
from Endogenous
Breast Bacteria
?
Contamination
from Bacteraemia
Bacterial
Biofilm
Formation
Progressive
Capsular Fibrosis
with Contracture
Chronic
Inflammation
leading to
Neoplastic Change
(Lymphoma)

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11 Appendix 1: Ethics approvals

THE UNIVERSITY OF SYDNEY

RESEARCH INTEGRITY
Animal Ethics Committee
Web: http://sydney.edu.au/research_support

Ref: AA/rt

13 September 2011

Dr Jean Zou
Infectious Diseases and Immunology
Blackburn Building – D06
The University of Sydney
Email: jean.zou@sydney.edu.au

Dr Karen Vickery
Australian School of Advanced Medicine
Macquarie University
Email: karen.vickery@mq.edu.au

Dear Dr Zou / Dr Vickery

Title: **Type of implant surface and development of biofilm**

Protocol Number: **N00/12-2010/2/5420**

Your request to modify the above application was considered by the Animal Ethics Executive Committee (AEEC) at its meeting on **Thursday, 1 September 2011** and the following has been approved:

1. To increase the number of implants from 6 to 8 per animal. As detailed in the approved protocol the animals will receive pain relief for 24 hours post implantation.

The additional information will be filed with the original application.

Conditions of Approval Applicable to All Projects

1. The Animal Ethics Committee (AEC) reviews and approves protocols for their compliance with the NSW Animal Research Act (and it's associated Regulations) and the 2004 NHMRC 'Australian code of practice for the care and use of animals for scientific purposes'. All personnel named on the protocol should be conversant with these documents.

2. This approval is in accordance with your original submission together with any additional information provided as part of the approval process.

3. Any changes to the protocol must be approved by the AEC before continuation of the experiment (refer to website http://sydney.edu.au/research_support/ethics/ for a Modification Form). This includes notifying the AEC of any changes to: named personnel, source of animals, animal numbers, location of animals and experimental procedures.

4. All cages/pens/tanks/paddocks used for holding animals must be clearly labelled with the Chief Investigator's name, approval number and cage/pen/tank number.

5. A copy of this approval letter, together with all relevant monitoring records, must be kept in the facility where your animals are housed. These records must be updated regularly as breeding and husbandry events occur and current copies must be maintained in the animal house. Where electronic breeding records are kept instead of records on cage cards, printed copies of the records should be placed in a folder in the relevant animal house, where they can be inspected by the AEC.

6. Investigators should promptly notify the AEC of any unexpected adverse events that may impact on the wellbeing of an animal in their care (refer to Clauses 2.2.28 and 3.1.12 in the 'Australian code of practice'). Please refer to the website http://sydney.edu.au/research_support/ethics/, and complete "Report of adverse or unexpected events during the conduct of an approved project" form.

Address for all correspondence:
Level 6 Jane Foss Russell Building G02
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AUSTRALIA
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ABN 15 211 513 464
CRICOS 00026A

THE UNIVERSITY OF SYDNEY

7. In the event an animal dies unexpectedly, or requires euthanasia for welfare reasons, an autopsy should be performed by a person with appropriate qualifications and/or experience and the AEC should be notified promptly.

8. All animals must be provided with environment enrichment appropriate for their species, unless approved by the AEC.

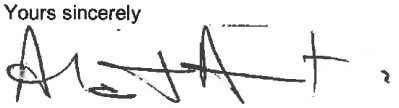
9. Animals should not be housed singly unless approved by the AEC.

10. Animals must not be euthanased within sight or sound of other animals (refer to Clause 3.3.20, of the Australian code of practice).

11. The AEC will make regular announced inspections of all animal facilities and/or specific research protocols. The Animal Welfare Manager will be conducting unannounced inspections of all animal facilities and/or specific research protocols.

Please do not hesitate to contact Research Integrity (Animal Ethics) should you require further information or clarification.

Yours sincerely



Associate Professor Alaina Ammit
Chair
Animal Ethics Committee

THE UNIVERSITY OF SYDNEY

Please complete and return to the Research Integrity (Animal Ethics), Level 6, Jane Foss Russell Building – G02, The University of Sydney

ACKNOWLEDGEMENT OF APPROVAL FOR MODIFICATION
Type of implant surface and development of biofilm

N00/12-2010/2/5420

I KAREN VICKERY agree/acknowledge the following:
PRINT NAME

Please tick:

☑ All modifications that are set out in the approval letter dated 13 September 2011

☑ All Conditions of Approval that are set out in the approval letter dated 13 September 2011

Karen Vickery
SIGNATURE

13.9.11
DATE

THE UNIVERSITY OF SYDNEY

RESEARCH INTEGRITY
Animal Ethics Committee
Web: http://sydney.edu.au/research_support

7 December 2010

Dr R Dixon
Faculty of Veterinary Science
JL Shute Building – C01
Camden Campus
The University of Sydney
Email: r.dixon@sydney.edu.au

Dear Dr Dixon

The correspondence from Associate Professor Karen Vickery dated 10 November 2010 addressing comments made by the Animal Ethics Committee was considered by the Animal Ethics Executive Committee (AEEC) at its meeting on **Thursday, 25 November 2010**. After considering the additional information relating to this protocol, it was the Executive Committee's recommendations that there were no ethical objections to the project, and you have approval to proceed.

The approval of this project is conditional upon you adhering to the conditions outlined in this letter and your continuing compliance with the Animal Research Act (1985 – Animal Research Regulation 2005) and the 'Australian code of practice for the care and use of animals for scientific purposes' (7th Edition 2004). Please sign and return the Acknowledgement of Approval.

The project is approved for an initial period of 12 months with approval for up to three (3) years following receipt of the appropriate report (refer to clauses 2.2.37 and 2.2.38 "Reporting of projects", of the Australian code of practice). Your report will be due on **31 December 2011**.

Details of the approval are as follows:

Title:	**Breast implants, biofilm development and anaplastic large cell lymphoma**
Protocol Number:	**N00/12-2010/2/5421**
Approval Period:	**December 2010 to December 2011**
Authorised Personnel:	**Dr Robert Dixon** **Dr Karen Vickery** **Dr Anand Deva** **Dr Anita Jacombs** **Dr Pedro Miguel Valente**

Species/Strain	TOTAL animals approved for duration of project
Pigs	6

Animal House/Location where Animals will be held:	**Werombi Road Piggery or Mayfarm Piggery - Camden**
Documents Approved:	**Monitoring Form**

Address for all correspondence:
Level 6 Jane Foss Russell Building G02
The University of Sydney NSW 2006
AUSTRALIA
E: animal.ethics@sydney.edu.au

Animal Welfare Manager
Dr Lucie Nedved
T: +61 2 8627 8175
E: lucie.nedved@sydney.edu.au

AEC Secretariat:
Roslyn Todd
T: +61 2 8627 8174
E: roslyn.todd@sydney.edu.

ABN 15 211 513 464
CRICOS 00026A

Special Conditions of Approval Applicable to this Project

1. Please correct your statement regarding Section 1.8, as this protocol does involve the commercial partner, Allergan Pty Ltd link. For the University of Sydney's Department of Finance purposes please provide the following details:
   - Contact name
   - Company name – Allergan Pty Ltd
   - Company Postal Address
   - Telephone number
   - Facsimile number
   - Email address of contact person

2. Please provide a Version 2 of the AEC application form incorporating all changes.

Conditions of Approval Applicable to All Projects

1. The Animal Ethics Committee (AEC) reviews and approves protocols for their compliance with the NSW Animal Research Act (and its associated Regulations) and the 2004 NHMRC 'Australian code of practice for the care and use of animals for scientific purposes'. All personnel named on the protocol should be conversant with these documents.

2. This approval is in accordance with your original submission together with any additional information provided as part of the approval process.

3. Any changes to the protocol must be approved by the AEC before continuation of the experiment (refer to website http://sydney.edu.au/research_support/ethics for a Modification Form). This includes notifying the AEC of any changes to: named personnel, source of animals, animal numbers, location of animals and experimental procedures.

4. All cages/pens/tanks/paddocks used for holding animals must be clearly labelled with the Chief Investigator's name, approval number, title of project and cage/pen/tank number.

5. A copy of this approval letter, together with all relevant monitoring records, must be kept in the facility where your animals are housed. These records must be updated regularly as breeding and husbandry events occur and current copies must be maintained in the animal house. Where electronic breeding records are kept instead of records on cage cards, printed copies of the records should be placed in a folder in the relevant animal house, where they can be inspected by the AEC.

6. Investigators should promptly notify the AEC of any unexpected adverse events that may impact on the wellbeing of an animal in their care (refer to Clauses 2.2.28 and 3.1.12 in the 'Australian code of practice'). Please refer to the website http://sydney.edu.au/research_support/ethics, and complete "Report of adverse or unexpected events during the conduct of an approved project" form.

7. In the event an animal dies unexpectedly, or requires euthanasia for welfare reasons, an autopsy should be performed by a person with appropriate qualifications and/or experience and the AEC should be notified promptly.

8. All animals must be provided with environment enrichment appropriate for their species, unless approved by the AEC.

9. Animals should not be housed singly unless approved by the AEC.

10. Animals must not be euthanased within sight or sound of other animals (refer to Clause 3.3.20, of the Australian code of practice).

11. The AEC will make regular announced inspections of all animal facilities and/or specific research protocols. The Animal Welfare Manager will be conducting unannounced inspections of all animal facilities and/or specific research protocols.

Please do not hesitate to contact Research Integrity (Animal Ethics) should you require further information or clarification.

Yours sincerely

Dr Ian Johnston
Acting Chair, Animal Ethics Committee

cc: Associate Professor Karen Vickery, email: karen.vickery@mq.edu.au

Please complete and return to the Research Integrity (Animal Ethics), Level 6, Jane Foss Russell Building – G02, The University of Sydney

ACKNOWLEDGEMENT OF APPROVAL
Breast implants, biofilm development and anaplastic large cell lymphoma.
N00/12-2010/2/5421

I _____ agree/acknowledge the following:
PRINT NAME

Please tick:

☐ Special Conditions of Approval that are set out in the approval letter dated 7 December 2010.

- ☐ Please correct your statement regarding Section 1.8, as this protocol does involve the commercial partner, Allergan Pty Ltd link. For the University of Sydney's Finance please provide the following details:
  - Contact name
  - Company name – Allergan Pty Ltd
  - Company Postal Address
  - Telephone number
  - Facsimile number
  - Email address of contact person
- ☐ Please provide a Version 2 of the AEC application form incorporating all changes.

☐ All Conditions of Approval that are set out in the approval letter dated 7 December 2010.

☐ The monitoring forms approved by the AEC for protocols involving animals should be used by researchers in all circumstances where experimental work on the animals has commenced.

☐ Personnel associated with the protocol. If training is required for any students (PhD, Masters, Honours) the Committee must be notified of their competency to do the tasks.

☐ The total number of animals that have been stated in the approval letter dated 7 December 2010.

_____ _____
SIGNATURE DATE

Dear A/Prof Vickery

Re: "Identification of bacterial species associated with capsular contracture" (Ethics Ref: 5201100027)

Thank you for your recent correspondence. Your response has addressed the issues raised by the Human Research Ethics Committee and you may now commence your research.

The following personnel are authorised to conduct this research:

A/Prof Karen Vickery- Chief Investigator/Supervisor

Dr Anita Jacombs- Co-Investigator

NB. STUDENTS: IT IS YOUR RESPONSIBILITY TO KEEP A COPY OF THIS APPROVAL EMAIL TO SUBMIT WITH YOUR THESIS.

Please note the following standard requirements of approval:

1. The approval of this project is conditional upon your continuing compliance with the National Statement on Ethical Conduct in Human Research (2007).

2. Approval will be for a period of five (5) years subject to the provision of annual reports. Your first progress report is due on 08 April 2012.

If you complete the work earlier than you had planned you must submit a Final Report as soon as the work is completed. If the project has been discontinued or not commenced for any reason, you are also required to submit a Final Report for the project.

Progress reports and Final Reports are available at the following website:

http://www.research.mq.edu.au/for/researchers/how_to_obtain_ethics_approval/human_research_ethics/forms

3. If the project has run for more than five (5) years you cannot renew approval for the project. You will need to complete and submit a Final Report and submit a new application for the project. (The five year limit on renewal of approvals allows the Committee to fully re-review research in an environment where legislation, guidelines and requirements are continually changing, for example, new child protection and privacy laws).

4. All amendments to the project must be reviewed and approved by the Committee before implementation. Please complete and submit a Request for Amendment Form available at the following website:

http://www.research.mq.edu.au/for/researchers/how_to_obtain_ethics_approval/human_research_ethics/forms

5. Please notify the Committee immediately in the event of any adverse effects on participants or of any unforeseen events that affect the continued ethical acceptability of the project.

6. At all times you are responsible for the ethical conduct of your research in accordance with the guidelines established by the University.

This information is available at the following websites:

http://www.mq.edu.au/policy/

http://www.research.mq.edu.au/for/researchers/how_to_obtain_ethics_approval/human_research_ethics/policy

If you will be applying for or have applied for internal or external funding for the above project it is your responsibility to provide the Macquarie University's Research Grants Management Assistant with a copy of this email as soon as possible. Internal and External funding agencies will not be

informed that you have final approval for your project and funds will not be released until the Research Grants Management Assistant has received a copy of this email.

If you need to provide a hard copy letter of Final Approval to an external organisation as evidence that you have Final Approval, please do not hesitate to contact the Ethics Secretariat at the address below.

Please retain a copy of this email as this is your official notification of final ethics approval.

Yours sincerely

Dr Karolyn White

Director of Research Ethics

Chair, Human Research Ethics Committee

THE UNIVERSITY OF SYDNEY

RESEARCH INTEGRITY
Animal Ethics Committee
Web: http://sydney.edu.au/research_support

Ref: AA/rt

20 June 2011

Dr Jean Zou
Infectious Diseases and Immunology
Blackburn Building – D06
The University of Sydney
Email: jean.zou@sydney.edu.au

Dr Karen Vickery
Australian School of Advanced Medicine
Macquarie University
Email: karen.vickery@mq.edu.au

Dear Dr Zou / Dr Vickery

Title: **Breast implants, biofilm development and anaplastic large cell lymphoma**

Protocol Number: **N00/12-2010/2/5421**

Your request to modify the above application was considered by the Animal Ethics Executive Committee (AEEC) at its meeting on **Thursday, 9 June 2011** and the following has been approved:

1. The breast implants will remain *in situ* for up to 9 months instead of the applied 20 weeks.
2. The animals will be maintained in their normal housing for the extra period of time instead of being euthanased.

The additional information will be filed with the original application.

Conditions of Approval Applicable to All Projects

1. The Animal Ethics Committee (AEC) reviews and approves protocols for their compliance with the NSW Animal Research Act (and its associated Regulations) and the 2004 NHMRC 'Australian code of practice for the care and use of animals for scientific purposes'. All personnel named on the protocol should be conversant with these documents.

2. This approval is in accordance with your original submission together with any additional information provided as part of the approval process.

3. Any changes to the protocol must be approved by the AEC before continuation of the experiment (refer to website http://sydney.edu.au/research_support/ethics/ for a Modification Form). This includes notifying the AEC of any changes to: named personnel, source of animals, animal numbers, location of animals and experimental procedures.

4. All cages/pens/tanks/paddocks used for holding animals must be clearly labelled with the Chief Investigator's name, approval number and cage/pen/tank number.

5. A copy of this approval letter, together with all relevant monitoring records, must be kept in the facility where your animals are housed. These records must be updated regularly as breeding and husbandry events occur and current copies must be maintained in the animal house. Where electronic breeding records are kept instead of records on cage cards, printed copies of the records should be placed in a folder in the relevant animal house, where they can be inspected by the AEC.

6. Investigators should promptly notify the AEC of any unexpected adverse events that may impact on the wellbeing of an animal in their care (refer to Clauses 2.2.28 and 3.1.12 in the 'Australian code of practice'). Please refer to the website http://sydney.edu.au/research_support/ethics/, and complete "Report of adverse or unexpected events during the conduct of an approved project" form.

7. In the event an animal dies unexpectedly, or requires euthanasia for welfare reasons, an autopsy should be performed by a person with appropriate qualifications and/or experience and the AEC should be notified promptly.

8. All animals must be provided with environment enrichment appropriate for their species, unless approved by the AEC.

9. Animals should not be housed singly unless approved by the AEC.

10. Animals must not be euthanased within sight or sound of other animals (refer to Clause 3.3.20, of the Australian code of practice).

11. The AEC will make regular announced inspections of all animal facilities and/or specific research protocols. The Animal Welfare Manager will be conducting unannounced inspections of all animal facilities and/or specific research protocols.

Please do not hesitate to contact Research Integrity (Animal Ethics) should you require further information or clarification.

Yours sincerely

Associate Professor Alaina Ammit
Chair
Animal Ethics Committee

Please complete and return to the Research Integrity (Animal Ethics), Level 6, Jane Foss Russell Building – G02, The University of Sydney

ACKNOWLEDGEMENT OF APPROVAL FOR MODIFICATION
Breast implants, biofilm development and anaplastic large cell lymphoma

N00/12-2010/2/5421

I _____ agree/acknowledge the following:
PRINT NAME

Please tick:

☐ All modifications that are set out in the approval letter dated 20 June 2011

☐ All Conditions of Approval that are set out in the approval letter dated 20 June 2011

_____ _____
SIGNATURE DATE

THE UNIVERSITY OF SYDNEY

RESEARCH INTEGRITY
Animal Ethics Committee
Web: http://sydney.edu.au/ethics

23 April 2010

Dr R Dixon
Faculty of Veterinary Science
JL Shute Building – C01
Camden Campus
The University of Sydney
Email: r.dixon@usyd.edu.au

Dear Dr Dixon

Thank you for your correspondence dated 7 April 2010 addressing comments made by the Animal Ethics Committee. The Executive Committee has considered, and has no ethical objections to, the additional information provided regarding the project.

The approval of this project is conditional upon you adhering to the conditions outlined in this letter and your continuing compliance with the Animal Research Act (1985 – Animal Research Regulation 2005) and the 'Australian code of practice for the care and use of animals for scientific purposes' (7th Edition 2004). Please sign and return the Acknowledgement of Approval.

The project is approved for an initial period of 12 months with approval for up to three (3) years following receipt of the appropriate report (refer to clauses 2.2.37 and 2.2.38 "Reporting of projects", of the Australian code of practice). Your report will be due on **30 April 2011**.

Details of the approval are as follows:

Title: **Investigation of implant surface modification on prevention of biofilm development**

Protocol Number: **N00/5-2010/2/5283**

Approval Period: **1st May 2010 to 30th April 2011**

Authorised Personnel:
Dr R Dixon
Mr G MacNamara
Mr D Palmer
Mr C Kristo
Dr K Vickery
Dr A Deva
Dr A Jacombs
Dr J Allan
Dr P M Valente

Species/Strain	TOTAL animals approved for duration of project
Pigs – Large White [female, 1-3 years old]	5 + 1 contingency = 6

Animal House/Location where Animals will be held: **May Farm Piggery**

Approved Documents:
Monitoring Form
Surgical Monitoring Form

Address for all correspondence:
Level 6 Jane Foss Russell Building G02
The University of Sydney NSW 2006
AUSTRALIA

Animal Welfare Manager
Dr Lucie Nedved
T: +61 2 8627 8175
E: lnedved@usyd.edu.au

AEC Secretariat:
Roslyn Todd
T: +61 2 8627 8174
E: rtodd@usyd.edu.au

ABN 15 211 513 464
CRICOS 00026A

THE UNIVERSITY OF SYDNEY

Conditions of Approval Applicable to All Projects

1. The Animal Ethics Committee (AEC) reviews and approves protocols for their compliance with the NSW Animal Research Act (and its associated Regulations) and the 2004 NHMRC 'Australian code of practice for the care and use of animals for scientific purposes'. All personnel named on the protocol should be conversant with these documents.

2. This approval is in accordance with your original submission together with any additional information provided as part of the approval process.

3. Any changes to the protocol must be approved by the AEC before continuation of the experiment (refer to website http://sydney.edu.au/ethics/ for a Modification Form). This includes notifying the AEC of any changes to: named personnel, source of animals, animal numbers, location of animals and experimental procedures.

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Please do not hesitate to contact Research Integrity (Animal Ethics) should you require further information or clarification.

Yours sincerely

Associate Professor Alaina Ammit
Chair, Animal Ethics Committee

12 Appendix 2: Reagents and Chemical Solutions

12.1 Microscopy

3% Gluteraldehyde (Sigma-Aldich, Castle Hill, NSW Australia)

25% Gluteraldehyde stock solution was diluted to a 3% gluteraldehyde with deionised water.

Dilute ethanol

100% analytical grade ethanol (Sigma-Aldrich Pty Ltd, Castle Hill, NSW, Australia) was diluted with deionised water to the prescribed percentage.

In 100 ml solution:

- 30% ethanol was 30 mL ethanol and 70 mL water,
- 50% ethanol was 50 mL ethanol and 50 mL water,
- 70% ethanol was 70 mL ethanol and 30 mL water,
- 80% ethanol was 80 mL ethanol and 20 mL water, and
- 90% ethanol was 90 mL ethanol and 10 ml water.

2% (w/v) Paraformaldehyde

2 g Paraformaldehyde (Sigma-Aldrich) mixed with 100 mL1 x phosphate buffered saline. The mixture was stirred and heated (using a heating, magnetic stirring platform) until completely dissolved. Once dissolved, allowed to cool and stored at 4^{o}C. Paraformaldehyde is toxic; all steps were performed in a fume hood.

1 x Phosphate Buffered Saline (tablets Medicago AB, Uppsala, Sweden)

1 tablet makes 500ml and contains 0.14M NaCl, 0.0027M KCl and 0.010M Phosphate buffer pH 7.4. The tablet was dissolved in 500 ml deionised water. Autoclaved for 20 minutes at 121^{o}C and stored at room temperature.

0.1 M Potassium Phosphate Buffer

87.09 g K_2HPO_4 (Univar) dissolved in 500 mL deionised water. 68.045 g KH_2PO_4 (Univar) dissolved in 500 mL deionised water. Mixed 80.2mL of K_2HPO_4 solution and 19.8 mL of KH_2PO_4 solution and made up to 1000 mL with deionised water. Autoclaved for 20 minutes at 121^{o}C and stored at room temperature.

1 x phosphate buffered saline/0.5% Triton™X-100

For every 10 mL 1 x phosphate buffered saline (Medicago, Uppsala, Sweden), 0.5 mL of 0.5% Triton™X-100 (Sigma-Aldrich, Castle Hill, Australia) was added and stored at room temperature.

1 x phosphate buffered saline/0.5% Triton™X-100/5% bovine serum albumin

For every 10 mL of 1 x phosphate buffered saline/0.5% Triton™X-100 solution 0.5 mL of bovine serum albumin (Promega Corporation Alexandria, Australia) was added and stored at room temperature.

12.2 Molecular Biology

500 mM Ethylene diamine tetracetic acid (EDTA)

18.6g of EDTA (AnalaR, Muarrie, Australia) was dissolved in 100 mL of dH_2O and adjusted to pH of 8.0 with NaOH and stored at room temperature.

5M NaCl

292.2 g NaCl (Merck, Frenches Forest, Australia) dissolved in 1L dH_2O. Autoclaved for 20 minutes at 121^{o}C and stored at room temperature.

1M Tris (hydroxymethyl) aminomethane (Tris)-HCl

60.57 g Tris-HCl (Merck, Frenches Forest, Australia) was dissolved in 500 mL of dH_2O and adjusted to a pH of 7.5 with HCl and stored at room temperature.

10% sodium dodecyl sulphate (SDS)

1 g SDS (Sigma-Aldrich, Castle Hill, Australia) dissolved in 100 mL sterile dH_2O and stored at room temperature.

Phenol

Proprietary mixture of phenol equilibrated in 10 mM Tris HCl, pH 8.0, 1 mM EDTA, (pH 7.5-8.0 Sigma-Aldrich, Castle Hill, NSW, Australia). Stored at 4^{O}C.

Phenol/chloroform/isoamyl alcohol mix

Proprietary mixture of phenol; chloroform; isoamyl alcohol in the ratio of 25:24:1 (v/v/v); saturated with 100 mM TRIS pH 8.0; contains ~0.1% 8-hydroxyquinoline (Sigma-Aldrich, Castle Hill, NSW, Australia). Stored at 4^{O}C.

Chloroform:isoamyl alcohol

Proprietary mixture of mixture of chloroform and isoamyl alcohol in the ratio of 24:1 (v/v) (Sigma-Aldrich, Castle Hill, NSW, Australia). Stored at 4^{O}C.

Tris-EDTA Mix (10mM Tris/0.1mM EDTA)

1 mL of 1 M Tris and 200 µL of 500 mM EDTA were added to 100 mL of dH_2O. and autoclaved for 20 minutes at 121^{O}C and stored at room temperature.

3M Sodium acetate

40.83 g Sodium acetate·$3H_2O$ (AnalaR, Muarrie, Australia) dissolved in 80 mL dH_2O. pH adjusted to pH 5.2 with Glacial Acetic Acid (Ajax Chemical Ltd.). Adjusted the volume to 100 mL with dH_2O. Dispensed into aliquots and autoclaved for 20 minutes at 121^{O}C. Stored at room temperature.

Digestion Mix - 50 mM Tris/HCl pH7.5, 150mM NaCl, 2mM Ethylene diamine tetracetic acid (EDTA), 1% sodium dodecyl sulphate (SDS)

14 µL 1 mM Tris/HCl, 8 µL 5M NaCl, 1 µL 500mM (EDTA), 28 µL 10% SDS, 224 µL sterile dH_2O per sample.