Bacterial biofilms in healthcare: Transmission and Control

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SYDNEY·AUSTRALIA

March 2017

"Bacterial Biofilms in Healthcare: Transmission and Control"

By

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A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

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Co-supervisor: **Professor MaryLouise McLaws**

March 2017

Dedicated to

The pivotal person in my life

My beloved Father

Engineer Sajid Masood Qureshi

(1938-2016)

Still my inspiration and will always be!

Who taught me to live to the last moment by never giving up

Chase my dreams until I achieve them

Declaration of originality

I hereby declare that the work presented in this thesis has not been submitted for a higher degree to any other university or institution. To the best of my knowledge this submission contains no material previously published or written by another person, except where due reference is stated otherwise. Any contribution made to the research by others is explicitly acknowledged. I certify that all the resources and literature references are indicated in the thesis.

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Date of submission: **March 2017**

List of Publications

Published Papers

I. **Jacombs A, Tahir S, Hu H, Deva AK, Almatroudi A, Wessel L, Bradshaw D, Vickery K. "*In vitro and in vivo investigation of the influence of implant surface on the formation of bacterial biofilm in mammary implants*". *Plastic and Reconstructive Surgery*. April, 2014*, 133*(4), pp.471e-480e.**

II. **Almatroudi A, Gosbell IB, Hu H, Slade J, Espedido BA, Tahir S, Glasbey TO, Legge P, Whiteley G, Deva AK, Vickery K*, "Staphylococcus aureus dry-surface biofilms are not killed by sodium hypochlorite: implications for infection control."* Journal of Hospital Infection. July 2016;93(3):263-270.**

Submitted papers

III. **Tahir S, Slade J, Gosbell IB, Vickery K, "*Staphylococcus aureus - Biofilm specific antimicrobials tolerance and resistance*".**

IV. **Tahir S, Hu H, Deva AK, Vickery K, *"Topical negative pressure therapy can enhance biofilm killing when combined with antiseptic or antibiotic instillation: an in-vitro study."***

V. **Almatroudi A, Tahir S, Hu H, Chowdhary D, Gosbell IB, Slade J, Whiteley G, Deva AK, Vickery K *"Staphylococcus aureus dry surface biofilms survive heat treatment."***

Papers ready for submission

VI. **Tahir S, Emanuel S, Inglis D, Hu H, Vickery K, *"Improved biofilm killing by combination treatment using mild positive pressure with liquid disinfectants."***

VII. **Tahir S, Chowdhary D, Legge M, Hu H, Whiteley G, Deva AK, Vickery K, "*Bacterial transmission via single use gloves from dry surface Biofilm in Health-care Environment*"**

Manuscripts in preparation

VIII. **Chowdhary D, Tahir S, Legge M, Hu H, Aljohani K, Prvan T, Deva AK, Vickery K, "*Transfer frequency of dry surface biofilm in health-care environment- `role of health care worker's hand."***

IX. **Tahir S, Chowdhary D, Hu H, Whiteley G, Deva AK, Vickery K. "**Chlorine ***and bacterial biofilms."***

Acknowledgements

In the Name of Allah, Most Gracious, Most Merciful.

All Praise to Allah

Which of the favours of your Lord will you deny!

Ultimate Thanks to Allah for bringing me up to this stage and my gratitude to all those lovely people who guided me through my research journey.

I can never thank my supervisors enough, A/Prof Karen Vickery and Dr. Honghua (Helen) Hu, who were always there to support and encourage me. I am obliged to them for believing in me, trusting me, accepting my input and providing me with their expertise throughout my research work in the most comfortable environment. I cannot underestimate the contribution of my supervisor A/Prof Anand Kumar Deva whom I met on monthly basis but was always left highly motivated to carry me on with my work until our next meeting. These shining people contributed heaps to my learning and taught me ways in which to tackle with and carry on into my research career. I am so grateful for their continuing support, advice and knowledge with never ending patience.

Special thanks to Dr. Vickery for supporting me through my difficult family situations during PhD thesis writing. I highly appreciate the Macquarie University laboratory and administration staff in the faculty of Medicine and Health Sciences for their continuous help and understanding. I have enjoyed working and learning from my wonderful peers of Surgical Infections Research Group and other research groups in the faculty.

I am also thankful to my co-supervisor Professor MaryLousie McLaws from UNSW for providing me the opportunity to gain knowledge from her evidence informed decision making course in epidemiology and for her time to time cordial assistance and professional input. I cannot forget to appreciate the staff at microscopy unit including Ms Debra Birch (retired) and Dr Nicolle Vella for training me to work on their confocal laser scanning microscope and scanning electron microscope.

Finally, I express my gratitude to the everlasting support from my family for understanding my commitments and bearing with upset routines. My PhD wasn't possible without the constant encouragement from my lovely children Fatima, Abdullah and Khadijah and with a silent backup from my Husband. My late father was the biggest pumping force behind this work that will always stay with me.

May Allah bless all these wonderful people in every aspect of their lives!

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List of Abbreviations

°C	degrees Celsius
agr	staphylococcal accessory gene regulator genes
AI-2	autoinducer-2
AIP	autoinducing peptide
ANOVA	analysis of variance
ASTM	American Society of Testing and Materials
ATCC	American Type Culture Collection
CDC	Centers for Disease Prevention and Control
CFU or cfu	colony forming unit(s)
CLSM	confocal laser scanning microscopy
cm	centimetre(s)
CoNS	coagulase-negative staphylococci
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DSB	dry surface biofilm
eDNA	extracellular DNA
EDTA	ethylenediamine-N, N'-diacetic acid
EPS	extracellular polymeric substance(s)
HCW	health care worker
HMDS	Hexamethyldisilazene
Ica	intercellular adhesion genes
MBC	minimum bactericidal concentration
MBEC	minimum biofilm eradication concentration
MIC	minimum inhibitory concentration
min	minutes
ml	milliliter
MSCRAMM	microbial surface components recognizing adhesive matrix molecules
n	number
NaCl	sodium chloride
NPWT	Negative pressure wound therapy

ICU	intensive care unit
OD	optical density
PBS	phosphate buffered saline
PI	propidium iodide
PIA	polysaccharide intercellular adhesin
QS	Quorum sensing
rpm	revolutions per minute
SEM	scanning electron microscopy
TNP	Topical Negative Pressure
TSB	Tryptone soya broth
V.A.C.®	Vacuum Assisted Closure®
µL	microliter

Abstract: What this study is all about!

It is estimated that more than 99% of bacteria form biofilm where they survive safely in the slime taking refuge from antimicrobials, host immune responses and environmental stress conditions.

Scientific literature demonstrates that more than 85% of chronic infections are currently attributed to biofilm development in human tissues (chronic otitis media, non-healing skin ulcers, persistent perionditis, chronic osteoarthritis) on prosthetic medical devices (contact lenses, implants etc.) and on medical equipment (endoscopes, urinary catheters etc.).

Transmission and control of bacterial biofilm to reduce these infections has been quite a challenge for clinicians and scientists worldwide, especially owing to the tough resistive life style of bacteria inside biofilms. This study investigated if bacteria residing in environmental biofilms are a potential source for healthcare associated infections by determining:

1) The transferral rate of bacteria from dry-surface biofilms via gloved and un-gloved hands
2) Determining the killing efficacy of commonly used disinfectants and sterilisation protocols against dry-surface and traditional hydrated biofilms
3) Investigating the killing efficacy of combining a physical stress (topical negative pressure and positive pressure ≤10 atmospheres) with a chemical stress (disinfectants/antiseptics) on dry and hydrated biofilms.

To study, in-vitro bacterial biofilm was cultured in PC2 laboratory on coupons using Communicable Disease Control and Prevention biofilm reactor and then tested for transmission and various treatments.

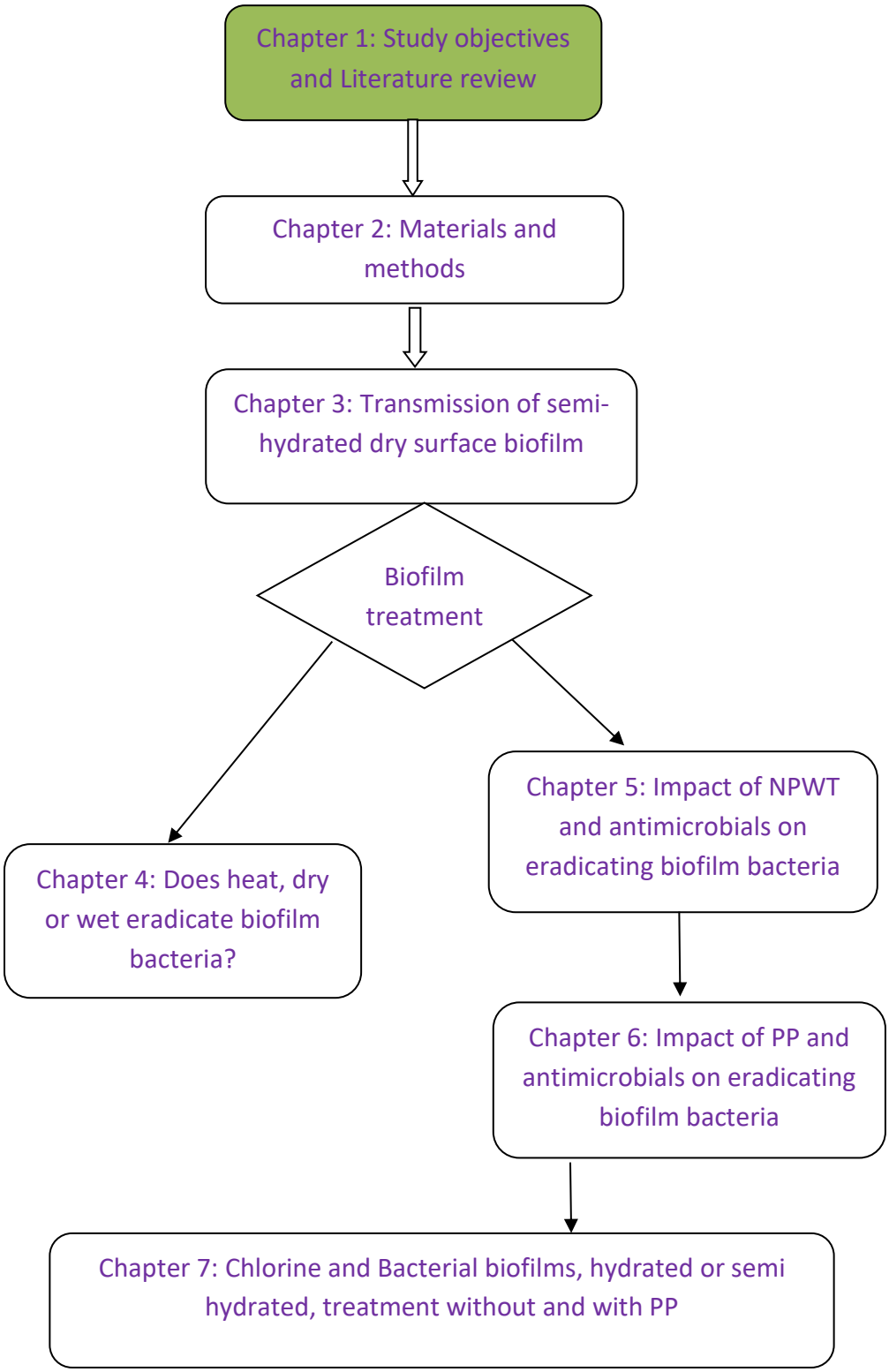
We found that dry-surface biofilm bacteria are highly transmissible. Although gloved hands transferred up to six folds fewer bacteria than bare hands, total bacterial numbers transferred was more than the dose required to cause infection.

Heat treatment as per the prevailing infection control guidelines, successfully eradicated bacteria in hydrated biofilm, but failed to eradicate dry-surface biofilm when autoclaved

at 121^{o}C for up to 30 minutes. Similarly, commonly used disinfectants failed to kill dry-surface biofilm. However, enhanced killing of biofilm bacteria was achieved when compression pressure treatment was combined with biocides.

The current thesis establishes high transmissibility of bacteria from dry surface biofilm affirming it to be a potential source of hospital acquired infections whilst demonstrating that current practised methods of decontamination/sterilization are insufficient to eradicate biofilms forming on dry surfaces. However, promising results were achieved after biofilm treatment with combined pressure and biocides therapy encouraging further evaluation of combination disinfection.

Thesis outline



Introduction: So here the tale begins!

Since ages humans are paying a huge health toll owing to bacterial infections. Despite endless efforts infections remain a major global health issue of which healthcare associated infections (HAI) as well as unresolved chronic infections are a big burden on patient well-being and healthcare system.

Infections in healthcare settings have always been a concern worldwide. Infection control strategies like general use of hygiene reduce the risk of transmission but disinfection/sterilization of equipment and adopting ways of personal protection in healthcare settings play a pivotal role. Although in the developed countries infection control in healthcare settings has improved tremendously the concept of infection transmission free environment remains elusive.

In Australia, The Department of Health has set guidelines for control of infection in healthcare settings but still the cost of patient care due to hospital acquired infections remains a burden on the healthcare budget.

In the past few decades, dispersion of pathogens from biofilms existing in healthcare ecosystem is considered to contribute to these HAIs and other lingering or recurrent bacterial infections[1]. The risk of acquiring multidrug resistance organisms (MDRO) is increased over 70% in the new occupant if the room was previously occupied by patient suffering from MDRO infection. Hu *et al* detected biofilm in 41 out of 44 environmental samples collected from a recently closed ICU[2]. Otter *et al* in his review has called these aggregates on dry hospital surfaces as "environmentome"[3].

Many factors such as type of pathogens, host immune response, infection control measures affect the cycle of infection in healthcare settings. Scientifically it is well established that inside the slime of biofilm, bacteria reside safe from internal as well as external stresses to survive longer and resist adverse circumstances to cause persistent infections. Biofilm formation is now convicted of more than 85% of chronic infections. The thick extracellular matrix or slime of the biofilm acts as a barrier for penetration of antimicrobials to kill infecting bacterial cells.

In this thesis, relevance of bacterial biofilms in healthcare, their tolerance and resistance to antimicrobials and the current strategies of biofilm management have been thoroughly reviewed. Later experimental scientific work focuses on transmission of bacterial biofilms in healthcare and testing various strategies adapted to eradicate them for significant bioburden reduction in healthcare settings.

Chapter 1

Literature review

Part One - Bacterial biofilms

1.1 Historical background

Bacteria were thought to primarily exist in the environment as free floating or in planktonic form. Bacterial existence as biofilm was introduced in 17th century when Antonie Van Leeuwenhoek (1632-1723), the father of microbiology, described plaque on his own teeth as aggregates so different from clusters of micro-colonies. However, it took until 1978, for the acceptance of general theory that bacteria exist predominantly as biofilm, as described in next section[4]. These biofilms were discovered to adhere to all nutrient sufficient-surfaces in aquatic ecosystems. Once inside the biofilms, bacteria undergo phenotypical changes which differ profoundly from their planktonic counterparts[5-7]. Later on wet surface bacterial biofilms were visually identified in abundance by direct microscopic observations and were cultured with the use of quantitative recovery techniques [8]. With water system industries facing serious problems arising from development of biofilms in water pipes, [9,10] these industries became the pioneers in developing methods to assess sessile bacteria in biofilms and to evolve strategies for biofilm control [7]. Biofilm samplers were installed in the water pipes and efficacy of biocides was regularly tested. The significance of bacterial biofilm, in medical and dental areas, has only been established in the past few decades in spite of previous universal acceptance of dental plaque as one form of biofilm[11]. Further development of new investigation methods has shown organisms growing as biofilms on many device-related and other biological (humans and animals) chronic infections. However, detailed understanding of mechanisms of biofilm formation and its dynamics is required to target biofilm eradication for infection control.

1.2 What is bacterial biofilm?

In 1976, Marshall identified very fine polymer fibrils that anchored the bacterial cells to a surface [12]. Costerton *et al* described similar pattern of microbial growth in aquatic systems

where microbial cells were embedded in polysaccharide matrix [4]. Later in their study in 1987, Costerton *et al* stated that biofilms are clusters of microbial cells or single cells embedded in predominantly anionic well hydrated matrix of polymers [5]. Since then the definition of biofilms has kept evolving. Characklis and Marshall explained more by defining biofilm as *".....cells immobilized at a substratum and frequently embedded in an organic polymer matrix of microbial origin ….which is not necessarily uniform in time or space….composed of a significant fraction of inorganic or abiotic substances held together by the biotic matrix"* [13,14].

In 2002 Donlan *et al* came with an even more comprehensive definition as follows:

"A biofilm is a microbialy derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are 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." [15]

Once inside the biofilm, bacteria are safe becoming rather tolerant to the intrinsic and extrinsic devastating factors that normally eradicate planktonic bacteria such as host immune responses, biocides, antimicrobials, physical stresses like heat and lack of nutrition and oxygen[16-18].

1.3 Mechanism of bacterial biofilm formation

Bacterial biofilms follow a complex, rather sophisticated Mechanism of formation. Not all bacterial species form biofilm. However, 99% of the known species have the tendency to form biofilms upon meeting suitable circumstances. The biofilm structure basically consists of the conditioning film which facilitates microbial adherence. Pioneer microbial species make initial contact with the surface and forms the basal layer. As more and more microorganisms attach the biofilm becomes multispecies and ultimately forms a complex, mature biofilm with bacteria embedded in extracellular polymeric substance (EPS) [19-22]. The formation of bacterial biofilms occurs in several subsequent stages as described below and in Figure 1-1

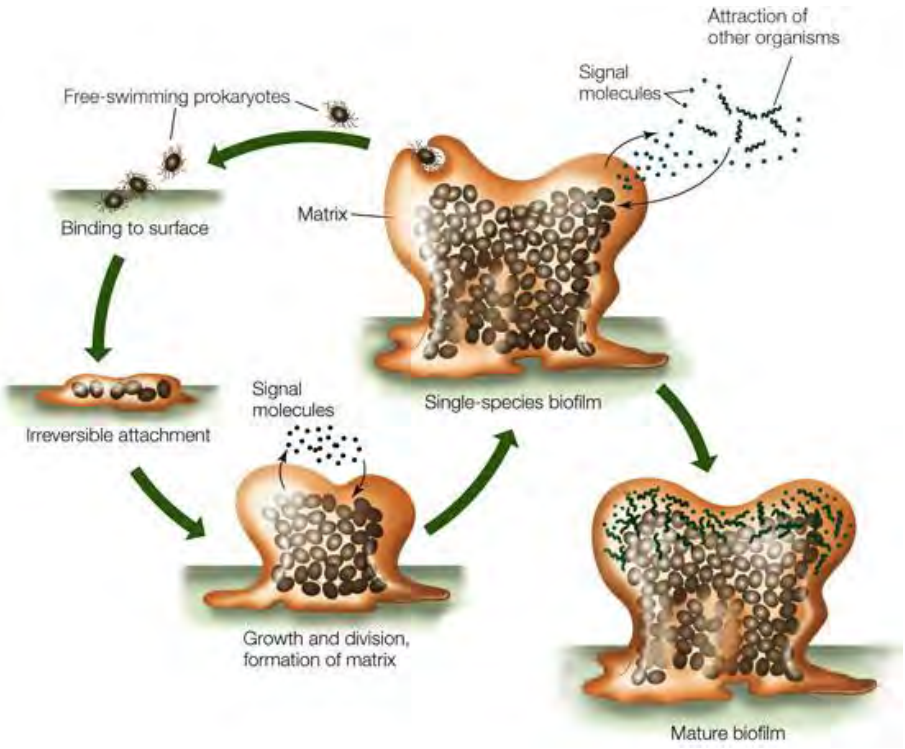


Figure 1-1 The stages of Bacterial Biofilm formation: Anticlockwise from the top (a) binding of bacteria to the surface, reversible followed by irreversible attachment (b) Growth and differentiation of biofilm (c) biofilm dispersion and maturation.

1.2.1 Stage one — Surface conditioning for bacterial biofilms and their surface attachment

To form biofilm, the bacteria must take the mandatory first step, its attachment on to a surface. Almost all surfaces are constantly bombarded by organic/inorganic particles from their surrounding environments. These surfaces may be biotic such as human tissues or abiotic such as human prosthesis or ocean rocks. Initially, reversible attachment of these particles to a suitable surface takes place by the process called surface conditioning. It takes place by the adsorption of macromolecules on to the suitable surface enabling bacterial adherence[22-24].

The bidirectional interaction (surface attachment) is modified by various factors such as the surfaces' chemical composition, roughness, its charge [10,23,25-28], microbial specific factors and the dynamics of colonization [29,30]. Attachment only occurs once compatibility between surface and bacterial factors is achieved utilizing the interactive forces including

hydrodynamics, steric and van der Waal forces activated between attaching surface and the bacterial cell.

Motile organisms such as Gram negative bacteria like *Pseudomonas aeruginosa* and *Escherichia coli* use proteinaceous extensions from the cell surface called pili, fimbriae or flagella as ligands for attachment to a surface. The flagella swim an organism towards the surface aiding its adherence[31,32] whilst, type IV pili serve as adhesives during bacterial cell attachment and further contribute in the process of biofilm maturation [32 33]. Non-motile bacteria like Gram positive *Staphylococcus aureus* and *Staphylococcus epidermidis* are propelled by Brownian motion while surface adhesion takes place by using surface adhesive proteins (detailed in section 1.13).

Once Initial attachment is achieved, contraction of microfilaments and microtubules occur within the bacterial cell which alters the bacterial shape to fit well into the attached surface contour. Irreversible attachment is eventually achieved once the bacterial surface factors as ligands fit well on to the surface receptor sites. Further attachment to a biotic substratum takes place by molecular "docking Mechanism" while in case of abiotic surfaces many non-specific connections, such as bacterial hydrophobicity maintains irreversible adsorption of bacteria to the surface [34,35].

1.2.2 Stage two — Growth and differentiation:

After attaining irreversible attachment, bacteria become member of that chosen micro community. This is followed by continuous EPS secretion from the pioneer microbes which guards the residing bacteria against desiccation and facilitates further accumulation of organic and inorganic substances into the matrix[19,36]. Bacterial cells adjust into appropriate sites within the biofilm housing regarding the availability of enzymes, nutrients and oxygen. Cell density keeps increasing and a specialized cell to cell communication network system of quorum sensing (QS) signaling is activated as soon as the biofilm cell density surpasses the QS threshold in order to build a multispecies biofilm community [37-39]. Gene expression is thence altered throughout the total bacterial population with enhanced expression of QS genes for cellular processes like transcription of antimicrobial peptides, regulation of virulence factors, transfer of genetic material, spore formation and building up of biofilm structure [40]. Bacterial phenotype transforms to suit the biofilm environment and water channels spread throughout the EPS providing a network for nutrient and oxygen

distribution and waste removal[41,42]. With the passage of time, the EPS increases in density and forms a diffusive barrier that prevents both loss of protective compounds like nutrients and seepage of toxic materials into the biofilm[43]. Aggregates of growing and dividing cells develop into small communities with further cell adhesion and biofilm development [22,44]. Outer layers of bacterial cells in biofilm are more metabolically active as they have the advantage of greater availability of nutrients than the inner slow growing cells which rely on nutrients' diffusion[45]. Mature biofilms therefore, exhibit heterogeneity in both phenotype and genotype [46].

The duration of this whole process of biofilm formation depends upon number of main factors like environmental factors, growth rate of the contributing microorganism and the surface type. On solid abiotic surfaces, this initial process takes 4-6 hours, depending upon the growth rate of the species [47]. Further growth and division takes hours or days to build and erect a three-dimensional biofilm structure with continuous production of EPS under complex systems of regulatory processes[48].

1.2.3 Stage three — Dissemination/Dispersal of cells

Once mature, biofilm bacteria start detaching and releasing into the neighbouring environment [41]. The process of dispersion is essential to maintain biofilm architectural integrity owing to the tight housing spaces available inside biofilm. Release of biofilm micro-organisms occurs in planktonic version regulated by numerous internal and external Mechanisms involving specialized signals and complex pathways[42,49,50].

Among the internal factors, a major regulatory Mechanism is stress due to starvation caused by limited nutrients availability resulting in cell lysis. This provides more food to the surrounding bacteria which can result in their active transition into the planktonic forms before dispersing from biofilm. This stress response is often regulated by decreased levels of cyclic di-GMP as seen to occur in Pseudomonas[51-53]. Another stress factor, hypoxia and limitation of environmental carbon serves as driving force for promoting cells' transition to their planktonic form [54]. With the release of materials from lysed cells the genes for EPS synthesis are downregulated with expression of genes regulating chemotaxis and motility are upregulated which aids cell movement out of the biofilm. *Staphylococcus epidermidis* utilizes, *ica* operon "switching off" Mechanism while *Staphylococcus aureus Agr* expression is

decreased that negatively regulates biofilm formation[46,55-58]. Escape of these cells from the biofilm leads to increased provision of food and space for the remaining cells inside the EPS. This prolongs survival of biofilm[59,60].

Bacterial cells disperse from biofilm utilizing distinct external detachment strategies. Biofilm bacteria may actively disperse as clumps or move as a whole biofilm while slowly migrating across surrounding surfaces[61,62]. Passive detachment may also take place owing to the exterior dynamics, such as removal of the sessile cells residing at the outer layers of a centrally liquefied microcolony by the fluid shear or moving currents[63].

Whatever Mechanism of dispersal may be, scientific studies have established dispersal as cause of spread of infection[41,64]. Biofilm clumps propelled by bathing fluid are attached to distil surfaces and cause recurrent infections [65]. Wilson *et al* observed biofilm growth and detachment in their in-vitro catheter model of *S. aureus* biofilm under flow conditions. Four modes of detachment/dispersal were observed by camera imaging and laser microscopy as follows[66].

"(i) continuous growth of cell clusters, (ii) detachment and reattachment of single cells, (iii) detachment and reattachment of clumps and (iv) rolling motility" (online images at www.erc.montana.edu /Res-Lib99-SW/Movies/2003/03-M003_4.htm)

S. aureus micro-colonies resist detachment due to viscoelasticity and have been shown rolling down-stream along the glass flow cell lumen driven by the flow force of fluids [67]. Similar surface migration of biofilms has been observed along endotracheal tubes in patients with ventilator-associated pneumonia[68,69].

1.3 Components of bacterial biofilm EPS and their functions

Mixed species hydrated biofilms are composed of ± 15% of cells by volume, embedded within intercellular matrix consisting of exopolymeric material (± 85% by volume) containing proteins, polysaccharides, nucleic acids, lipids, bio-surfactants, ions and numerous enzymes [15,70]. These EPS components promote maturation of biofilm as evidenced in *P. aeruginosa* biofilms such as participation of eDNA in mushroom cap formation of *P. aeruginosa* biofilm[71-73].

Characklis and Marshall in their book, "Biofilms" described biofilms as 'stiff water' due to water being the largest component of hydrated biofilms [74]. Water being "life" serves as one

of the major savior of biofilm integrity as it buffers the bacterial cells against fluctuations in water potential during variation in environmental hydration. Water also maintains channels within EPS for sequestration of anions, cations and a-polar particles/compounds in various regions of biofilm.

Functions	Relevance for Biofilms	EPS components involved
Adhesion	The long-term attachment of whole biofilms to surfaces	Polysaccharides, proteins, DNA and amphiphilic molecules
Aggregation of bacterial cells	Enables bridging between cells, the temporary immobilization of bacterial populations, the development of high cell densities and cell–cell recognition	Polysaccharides, proteins and DNA
Cohesion of biofilms	Biofilms form a hydrated polymer network (the biofilm matrix), mediating the mechanical stability of biofilms (often in conjunction with multivalent cations) and, through the EPS structure (capsule, slime or sheath), determining biofilm architecture, as well as allowing cell–cell communication	Neutral and charged polysaccharides, proteins (such as amyloids and lectins), and DNA
Retention of water	Maintains a highly-hydrated microenvironment around biofilm organisms, leading to their tolerance of desiccation in water-deficient environments	Hydrophilic polysaccharides and, possibly, proteins
Protective barrier	Confers resistance to nonspecific and specific host defences during infection, and confers tolerance to various antimicrobial agents (for example, disinfectants and antibiotics), as well as protecting cyanobacterial nitrogenase from the harmful effects of oxygen and protecting against some grazing protozoa	Polysaccharides and proteins
Sorption of organic compounds	Allows the accumulation of nutrients from the environment and the sorption of xenobiotics (thus contributing to environmental detoxification)	Charged or hydrophobic polysaccharides and proteins
Sorption of inorganic ions	Promotes polysaccharide gel formation, ion exchange, mineral formation and the accumulation of toxic metal ions (thus contributing to environmental detoxification)	Charged polysaccharides and proteins, including inorganic substituents such as phosphate and sulphate
Enzymatic activity	Enables the digestion of exogenous macromolecules for nutrient acquisition and the degradation of structural EPS, allowing the release of cells from biofilms	Proteins
Nutrient source	Provides a source of carbon-, nitrogen- and phosphorus-containing compounds for utilization by the biofilm community	Potentially all EPS components

Exchange of genetic information	Facilitates horizontal gene transfer between biofilm cells DNA Electron donor or acceptor permits redox activity in the biofilm matrix	Proteins (for example, those forming pili and nanowires) and, possibly, humic substances
Export of cell components	Releases cellular material because of metabolic turnover	Membrane vesicles containing nucleic acids, enzymes, lipopolysaccharides and phospholipids
Sink for excess energy	Stores excess carbon under unbalanced carbon to nitrogen ratios	Polysaccharides
Binding of enzymes	Results in the accumulation, retention and stabilization of enzymes through their interaction with polysaccharides	Polysaccharides and enzymes

Table 1-1. Functions of extracellular polymeric substances[20]

EPS = extracellular polymeric substances.

1.3.1 Polysaccharides

One major component of the dynamic 3-D biofilm matrix is polysaccharide biomolecules. Confocal laser scanning microscopy has been used extensively to study structure/composition of EPS. Staining cell components with fluorescent dyes, antibody labelling, lectin-binding analysis and nucleic acid staining can be used for specific as well as for non-specific binding patterns of polysaccharides in EPS. In 2001, Nue and Lawrence demonstrated that single, double and triple binding lectins are a useful tool to analyze the distribution of glucoconjugates in the complex biofilm EPS [75]. Since then many exopolysaccharides have been isolated to be characterized in bacterial species from diverse environments and their functions are described in table 1-1 and reviewed by Bazaka *et al* [76]. Exopolysaccharides from a range of bacterial species consist of long molecules either branched or arranged linearly. The exopolysaccharides maybe homo-polysaccharides such as glucans and fructans found in oral streptococcal biofilms; or hetero-polysaccharides with a mixture of neutral and charged sugar molecules [77]. Polysaccharides also vary among the strains of the same species such as hetero-polysaccharides in various strains of *Streptococcus thermophilus* that differ in monomer composition and ratio.

Pel and Psl polysaccharides are the major contributors of biofilm maturation in *P. aeruginosa* and are expressed in non-mucoid wild type *P. aeruginosa*. Apart from Pel and Psl, mucoid biofilms of *P. aeruginosa* extensively express alginate, an unbranched high molecular-mass

hetero-polysaccharide that is also proposed to play an important role in biofilm maturation [78 79 80].

An overall charge is imparted on to the biofilm owing to its organic and inorganic substituents[42]. The presence of uronic acid as in alginate in *P. aeruginosa* biofilm, anionic EPS with a net negative charge whereas as in Staphylococcus species, poly-cationic intercellular adhesin exist, composed of poly-N acetyl-Glucosamine and teichoic acid which results in an overall positive charge.

Species that produce less polysaccharides lack the ability to form mature biofilms. However, in mixed biofilms low polysaccharide producing species may be complemented by those which are efficient in production of exopolymers [20].

1.3.2 Extra-cellular proteins

Another key component of biofilm EPS are matrix proteins which may exceed the polysaccharide content. These proteins function as enzymes or form biofilm structural proteins [20].

Structural proteins include cell surface proteins and the proteins that bind to carbohydrates in the EPM. Carbohydrate binding proteins form a network to link the EPS and surface proteins e.g. glucan binding proteins of oral *Streptococcal mutans* biofilm [81] and lectins in *P. aeruginosa* biofilm[82]. CdrA protein that binds to Psl in *P. aeruginosa* strengthens the matrix by anchoring the cell safely to its extracellular polymeric matrix[83]. Similarly, structural integrity in *Bacillus subtilis* biofilm is enhanced by the carbohydrate binding proteins, TasA [84]. In *S. aureus*, surface binding proteins identified as Bap proteins are associated with biofilm formation [85] while functional amyloids are other ubiquitous proteinaceous compounds involved in adhesion of bacterial cells [86].

Enzymatic proteins are either digestive or EPS modifying enzymes. Digestive enzymes break down the EPM components to low molecular mass products to provide energy for growing cells during starvation. The exoenzymes such as dextran, inulin and levan in dental streptococci, themselves are degraded slowly by hydrolases or lyases [87]. The biodegrading property of these enzymes has been utilized for degrading synthetic polymers such as plastics with plasticizers and other additives [88]. Enzymes that degrade matrix promote

dispersal of planktonic cells from biofilms while other enzymes act as virulence factors in medical biofilms e.g. enzyme N-acetyl-β-hexosaminidase produced by pathogen, *Actinobacillus actinomycetemcomitans* degrades the exopolysaccharide, N-acetyl-hexosamine to allow detachment and thence dispersal of planktonic cells from its biofilm[89].

1.3.3 Extracellular DNA

Continuous cell lysis in biofilms causes release of dead cells' DNA into the EPM to become an essential part of the EPS. The released DNA is known as extracellular or eDNA and is found in biofilms of all origins in varying quantities or proportions[90,91]. The formation of eDNA can be both QS dependent or independent (Das, Sehar *et al*. 2013). Basal level of eDNA is released by QS independent pathway during early as well as late exponential growth phases whereas elevated eDNA amounts in EPS is a result of QS-dependent cell lysis occurring during slow stationary growth phase [48,92,93].

eDNA is a major component serving as intracellular connector for the biofilm mass and integrity e.g. in *P. aeruginosa,* eDNA plays a pivotal role in maintaining the cell to cell connection during early stages of biofilm formation while later on it also enhances antimicrobial resistance [94,95]. In *P. aeruginosa,* eDNA determines the flow of cell traffic and large scale self-organization of bacterial cells through building a network of furrows within the expanding biofil [96]. Inside the biofilm, eDNA may be filamentous or form a grid like structure with dense or fine strands of network[92,97,98]. It may locate in the stalk of mushroom like micro colonies in biofilm or accumulate near the mushroom cap of *P. aeruginosa*. Recently, many studies have shown that the use of DNase 1 inhibits formation of bacterial biofilms as seen with *Escherichia coli*, *S. aureus*, *Fusobacterium nucleatum* and *Porphyromonas gingivalis*, hence emphasizing the vital role played by eDNA in keeping the biofilm intact [99,100].

1.3.4 Extracellular lipids and surfactants

EPS also contain lipids as extracellular lipopolysaccharides. In *Thiobacillus ferrooxidants*, lipids are necessary for its adherence to pyrite surfaces [101]. Although the extracellular polysaccharides, proteins and DNA are all hydrated hydrophilic molecules, biofilms may exhibit hydrophobic properties while binding to a surface. This hydrophobicity can be explained by the presence of extracellular hydrophobic lipids and other surface active

substances such as surfactin, emulsion and viscosin as in *Serratia marcerscens* [102,103]. These bio-surfactants facilitate biofilm attachment/detachment such as rhamnolipids in *P. aeruginosa* [104]. Hence their role has been proposed in initial attachment, in all stages of biofilm formation and during detachment.

1.4 Dynamic features of bacterial biofilm

Bacterial biofilms exhibit unique dynamics. EPS, the key component, determines the chemical and biological characteristics of a biofilm, influenced by the constituting species type, its surface dynamics and surrounding environmental factors. Morphologically, the resultant biofilm may be flat or filamentous, smooth or rough with varying porosity. EPS either exists as loosely bound slimy or tightly bound capsular form consisting mostly of soluble and insoluble components[36,105,106]. The dynamic features are described below in detail

1.4.1 Oxygen and nutrition gradient

A unique feature of biofilm is that due to its variability in cell density and thickness, there are regions displaying oxygen and nutrition gradients. Cells on the outer layers are exposed to greater levels of oxygen and nutrients while their supply decreases towards the inner or deeper layers of biofilm[107-109]. As a result, metabolically heterogeneous micro colonies exist within in the same biofilm[64]. Metabolic activity is higher in the bacteria residing in the outer layers while slow growing or stationary phase cells are dominant in the nutrition deficient zones[108,110]. Hence, physiological changes and changes in gene expression take place under altered bacterial mode of growth within different regions of the biofilm [109].

1.4.2 Effect of temperature and hydration on biofilm growth

Formation and constitution of EPS also varies in response to the environmental growth temperatures and to the degree of biofilm hydration. *Listeria monocytogenes* produce dense biofilms at 22-30^{o}C as compared to those cultivated at 37^{o}C while *P. aeruginosa* and *S. aureus* make thicker biofilms at higher temperatures (37^{o}C) than at lower temperatures [111,112].

With regards to hydration, enhanced EPS synthesis on desiccation or drying was observed by Roberson and Firestone in the species identified as *Pseudomonas* extracted from soil unlike

those developed in well hydrated conditions [113]. Similarly, EPS generated under desiccation conserved several times more water by weight due to increased production of alginate with altered fatty acid composition in *Pseudomonas putida* biofilm[114].

Similar protective Mechanism was exhibited by *E. coli* biofilm subjected to dryness when Zhang *et al* observed production of an important non-reducing disaccharide, trehalose among other organic molecules in this EPS as compared to the hydrated counterpart[115]. Previously trehalose was shown to be strongly heat resistant, extreme pH tolerant, a protein stabilizer and a sufficient source of energy for growing cells under stress conditions[116,117].

1.4.3 Quorum sensing

Quorum sensing (QS) is a unique Mechanism by which bacterial cells communicate/talk with one another and form communities[118]. Inside biofilm, bacteria display socially active behaviour via special extracellular signalling molecules that are cell density dependent, hormone like chemicals called auto-inducers and intracellular regulators molecules. These molecules control and coordinate various biofilm activities including expression of virulence factors[15,119-121]. Quorum sensing signals are triggered when an adequate number of bacteria or "quorum" reaches threshold inside a microcolony. Target genes are then activated to alter gene regulation and expression[121].

There are three main groups of QS molecules [37]. Gram positive bacteria use peptides for QS signalling whereas Gram negative bacteria use acyl-homoserine lactones (AHSL). Auto inducer-2 is used by both Gram positive and negative species.

1.4.3.1 Quorum sensing in gram-positive bacteria

Gram positive bacteria use small post transitionally processed peptides for QS signalling to regulate physiological biofilm Mechanisms[122]. For example QS regulates competent bacterial development in *Bacillus subtilis* while it supervises conjugation in *Enterococcus faecalis* [121]. In other bacteria like *S. aureus,* QS takes part in biofilm virulence [123] . The auto-inducing polypeptides (AIPs) for QS are formed inside the cell cytoplasm and are 5 to 17 amino acids long. Precursors of these AIPs are cleaved and modified inside the cell to be transported freely/passively along the concentration gradient out of the bacterial cell [124,125]. At low bacterial cell density, the QS signalling molecules are also at low concentration extracellularly and remain inactive. Once the cell density rises, the concentration of these

signalling molecules in the surrounding cell environment exceeds the QS threshold [126]. Already released AIPs in the outer environment then interact with sensor/receptor component located in the cell membrane of the two-component detection system[37,125]. Once the AIP interact with the sensor component, phosphorus is produced which leads to activation of the response regulator. Response regulator intracellular protein then interacts with the promoter DNA activating an intracellular response pathway as shown in Figure 1-2[127]. The QS regulated genes are thence switched on to alter gene expression of the total bacterial biofilm population for cell to cell synchronisation [128]

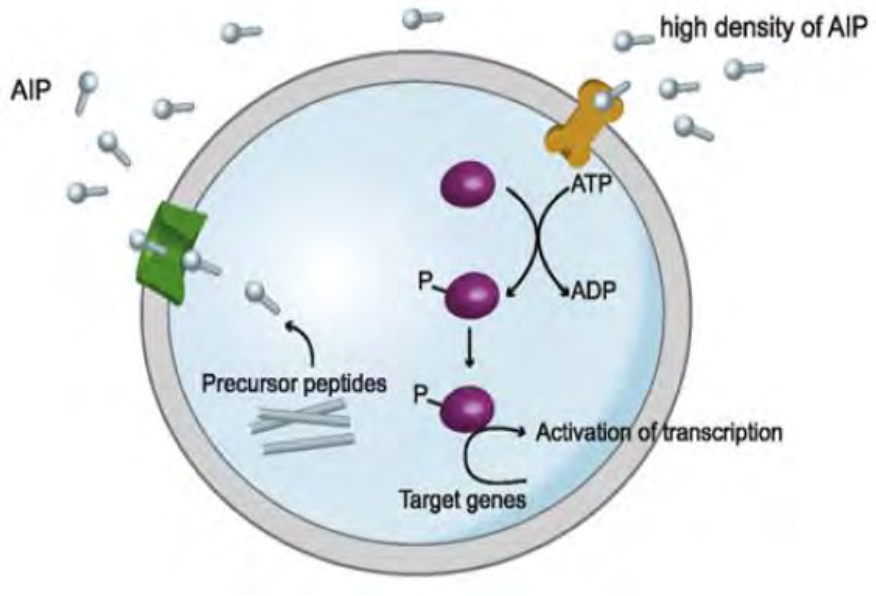


Figure 1-2 Quorum sensing in Gram positive bacteria. AIP = Autoinducing enzymes, ATP = Adenosine triphosphate, ADP = Adenosine diphosphate, P = regulatory protein [125]

1.4.3.2 Quorum sensing in gram-negative bacteria

Gram negative bacteria, at least 70 species, most commonly utilize N-Acylhomoserine lactones (AHL) as auto inducing molecules for QS as shown in Figure 1-3 [129,130]. Enzymatic and regulatory proteins are homologues to LuxI and LuxR-like proteins of *Vibrio fischeri*,[131-133,134,135]. LuxI is an enzyme that synthesises AHL with acyl chains that vary in length from 4 to 18 carbons linked via an amide bond to the homoserine lactone ring[37,38]. The AHL also called autoinducer-1 (AI-1) is synthesized inside the cell cytoplasm and diffuse freely through cell membrane depending upon their size and concentration gradient[136,137]. As the bacterial population increases in size, a critical threshold is reached where AHL re-enters the cell to

bind and activate LuxR like protein receptors of the transcription factor as shown in 1-3 [126,131,138] Direct binding to the DNA then leads to stimulation of altered gene expression [129].

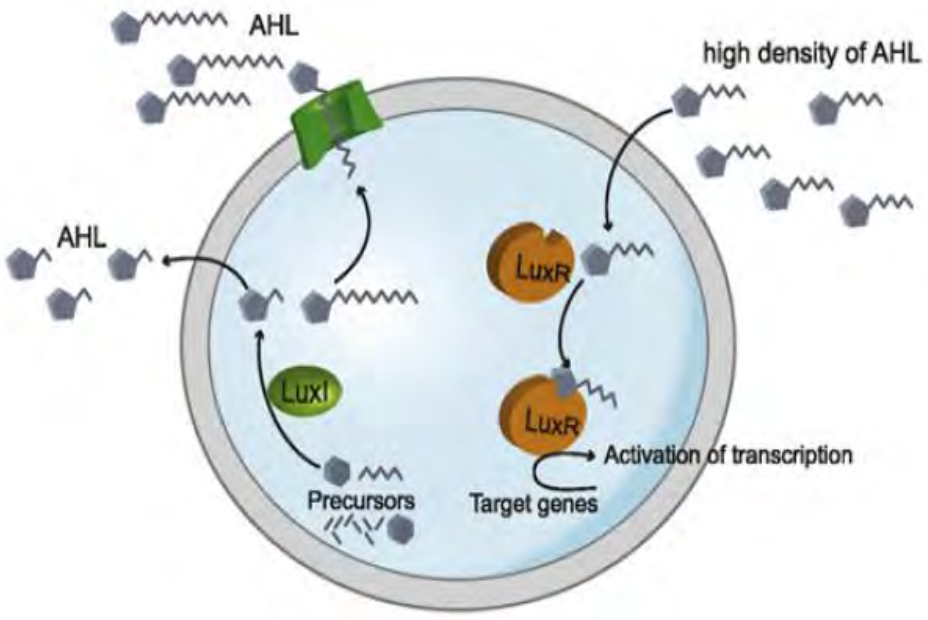


Figure 1-3. Quorum Sensing in Gram negative Bacteria. AHL = N-acylhomoserine lactones [128]

The important nosocomial pathogen *P. aeruginosa* has two key QS components, LasI-LasR and RhlI-RhlR homologous with LuxI-LuxR as shown in Figure 1-4 [138,139]. LasI, homologous to LuxI synthesises the AHL (3-O-C12HCL) auto-inducer which is activated on receiving "quorum" signal to bind with response regulator protein LasR [140]. LasR in turn positively regulates expression of multiple genes including *lasI* for further LasI protein synthesis, *RhlI* gene for RhlI-RhlR QS component activation/regulation [141] while 30 other virulence genes are also upregulated [142]. RhlI-RhlR QS component system uses AHL auto-inducer (C4-HSL) to bind with RhlR response regulator to control synthesis of Rhamnolipids and other virulence factors required to maintain *P. aeruginosa* biofilm throughout its formation, maturation, dispersal and neutrophil disruption[142-145].

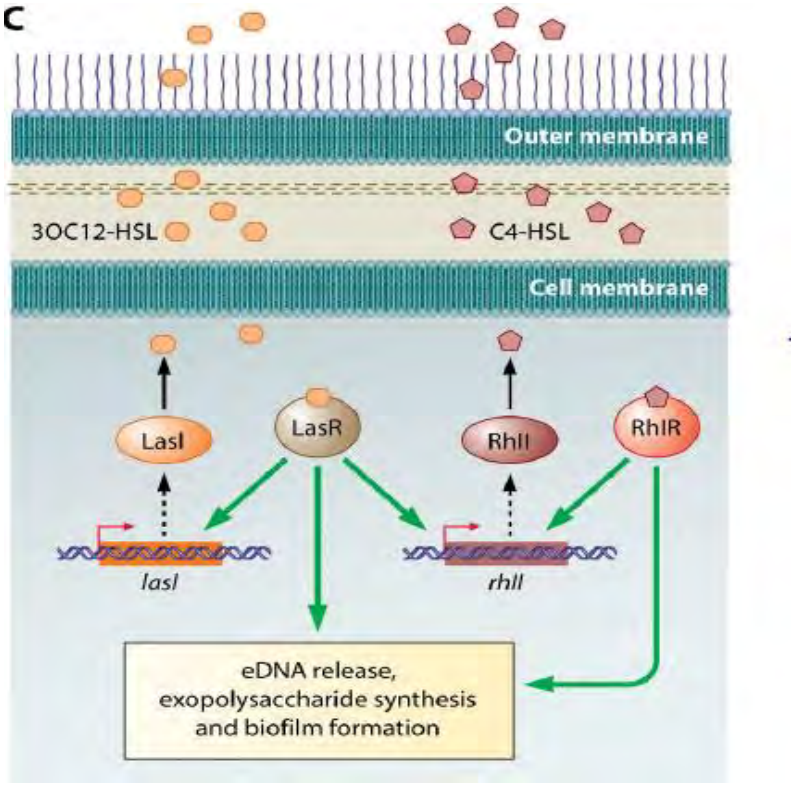


Figure 1-4. Quorum sensing pathway in *P. aeruginosa.* [42]

1.4.3.3 Quorum sensing in both gram-positive and gram-negative bacteria

Gram-positive and Gram- negative bacteria share same mediator autoinducer-2(AI-2) signalling molecules for QS [146-148]. LuxS enzyme produces AI-2, a furanosyl borate diester as a bi product of its involvement in the activated methyl cycle pathway. AI-2 in high concentrations is recognised to bind to the periplasmic LuxP sensor protein of the two-component protein system. This binding activates the response controller domain LuxQ preventing phosphorylation of LuxO to alter gene expression. The QS cycle in low and high cell density biofilm conditions is shown in Figure 1-5. In a recent study on two species of Lactobacillus, Yeo *et al* have suggested the role of autoinducer-2 molecules as one way of adaptation and interaction of bacteria in the intestinal environment [50]

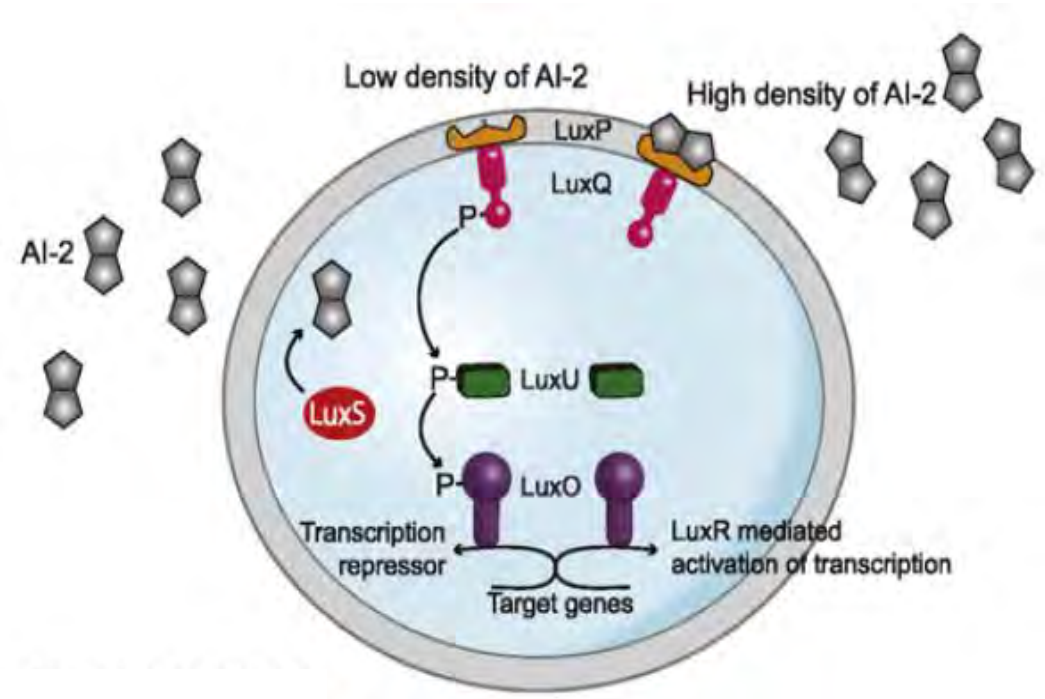


Figure 1-5. Quorum sensing in Gram positive and Gram negative Bacteria. AI-2 = auto-inducer 2 [128]

1.4.4 Antimicrobial tolerance and resistance in bacterial biofilm

Biofilm formation is an efficient way of promoting bacterial survival as they live protected from devastating attacks of antiseptics, antibiotics, disinfectants as well as safe from the host immune responses[149-153]. The main factors responsible for antimicrobial resistance in biofilms are summarized below and discussed further in detail in section 1.13.

1.4.4.1 Delayed penetration of antimicrobials through the exopolymeric substances

Penetration of antimicrobials through the biofilm is delayed by two Mechanisms;

1) The matrix acting as physical barrier depending upon biofilm thickness and its cell density.
2) Interaction or binding of antimicrobials with the components of EPS such as eDNA, polysaccharides, lipids and EPS proteins.

Biofilm matrix increases density of biofilm and hence forms a physical barrier between antimicrobials and bacterial cells giving additional protection from toxins [152]. Physical distance between the antimicrobial at the biofilm surface and bacterial cells is increased while the density of the EPS hinders biocide penetration[154,155]. However, the degree of impairment varies with the type of antimicrobial used, age of the biofilm and constituents of EPS[108,156].

Antimicrobials can bind to components of EPS or cell membranes, such as DNA in EPS matrix of *P. aeruginosa* binds positively charged aminoglycosides or antimicrobial cationic peptides and prevent the antimicrobial agent reaching target cells[157]. In *P. aeruginosa* EPS components Pel and alginate are associated with biofilm tolerance/resistance to Tobramycin and Gentamicin. However, the exact Mechanism of tolerance/resistance is not worked out [158-160].

1.4.4.2 Prolonged growth rate and slow metabolism of bacteria in biofilm

The sessile bacterial masses or micro-colonies within the biofilm differ from one another in their physical and chemical properties [161]. Biofilms exhibit chemical gradients when moving towards their deeper parts. Constituting cells adjust to the stress conditions and survive in anaerobic as well as less aerobic environment[162-164]. Uptake of antimicrobials is reduced by these slowly growing biofilm cells causing tolerance to the biocide as shown by decreased susceptibility of slow growing *Escherichia coli* biofilm cells to antiseptic Cetrimide when compared with planktonic cells growing at faster rate[165,166]. Walter *et al* proved that tolerance of *P. aeruginosa* cells to ciprofloxacin and tobramycin was mainly due to lack of oxygen in deeper, slow growing biofilm cells despite having efficient antimicrobial penetration as visualized via transmission electron microscope[108]. Several other studies on biofilm forming bacteria, using various antimicrobials, have verified this correlation with slow cell metabolism[43,166-168]. Sub-lethal/sub-inhibitory concentration of biocides in deeper layers may enhance biofilm formation as demonstrated by Campanac *et al* in *S. aureus* biofilm after treatment with short C-chain benzalkonium chloride (dilution effect) due to non-specific drastic phenotypical changes in biofilm cells[149,169].

1.4.4.3 Altered gene expression horizontal gene transmission and genetic mutations

Micro communities of bacterial biofilm cells experiencing varying levels of signalling molecules, supply of nutrients, metabolites and waste products, lead to alterations in their gene expression profiles [170,171]. Almost 45 genes differ in expression between *P. aeruginosa* biofilm forming cells and their planktonic counterpart such as genes controlling phosphormannomutase for alginate and polyphosphokinase synthesis are up regulated in *P. aeruginosa* biofilm [15,160,172,173]. These genes encode synthesis of EPS substances like synthesis of alginate (*algC*, *algD*, *algU*, *rpoS*) that provide stability to biofilm architecture for antibiotic tolerance. Out of 25 differentially expressed proteins for adhesion tested by Pulcini, 19 were up-regulated during attachment within 10mins to 3hrs such as upregulation of enzymes glycosyl transferase and glycosyl hydrolase for initial attachment and other proteins presumably increasing antimicrobial resistance or enhancing virulence and toxins delivery to bacteria [158,172-175].

Other than enhanced gene expression, horizontal gene transfer and biofilm specific mutability have been studied. As seen in *P. aeruginosa* biofilm, mutability was increased by 105 folds and 100 folds increase was observed in micro colonies as compared to planktonic cells[176,177]. Horizontal gene transfer takes place easily by transformation, transduction and conjugation in biofilms where cells sit close to one another and there is an abundance of eDNA is also available within the matrix [178]. In *E.coli* biofilm, horizontal gene transfer occurs 7-700 folds more efficiently than in planktonic counterparts[179]. Biofilms hence provide a reservoir of antimicrobial resistant genes and an ideal set of factors that allow convenient inter transfer of these genes in biofilm environment[180,181].

Recent scientific studies have also identified biofilm specific resistance genes for antimicrobial tolerance/resistance. In *P. aeruginosa*, three genes *ndvB, tssCI* and *brlR* are highly expressed specifically in biofilms for resistance to a subset of antibiotics such as tobramycin, gentamicin and ciprofloxacin[174,182-184]. The *ndvB* gene in *P. aeruginosa* encodes a glucosyl-transferase that is required for synthesis of cyclic glucans while it also activates ethanol oxidation genes[182,185]. The glucans in the periplasm then interact with aminoglycosides thus preventing the antibiotic reaching their cellular targets [186]. A similar

Mechanism of extracellular cyclic glucan and antifungals interaction has been proposed for *Candida albicans* biofilms [187].

The gene *tssCI* implicated in T6S, type VI secretion, is expressed in *P. aeruginosa* biofilm. T6S secretion system as seen in *Francisella tularensis*, *V. cholera, P. aeruginosa* and *E. coli*, enhances bacterial virulence, toxin delivery to bacteria and assists its survival during chronic infection[183]. The *brlR*, resistance locus regulator gene is biofilm specific gene in *P. aeruginosa* expressed only in biofilm probably under oxygen limited stress conditions. Deletion of *brlR* gene makes the biofilm sensitive to hydrogen peroxide and antibiotics while its expression translates transitional regulator BrlR which activates multidrug resistance efflux pump for antimicrobial resistance in *P. aeruginosa* biofilm [184,188,189].

1.4.4.4 Efflux pumps

Both Gram positive and negative bacteria possess multidrug resistance efflux pumps which confer basal level of drug resistance in these micro-organisms. Active up-regulation of these multi drug resistant efflux pumps in bacteria occur under biofilm state providing added protection against antimicrobials attacks [149,190-192] The Mechanism takes place under complex and diverse regulatory processes involving numerous transcriptional regulators and modulators. Li *et al* summarised five categories of multidrug resistant efflux pumps as follows:

"The ATP-binding cassette (ABC) superfamily [193], the major facilitator superfamily (MFS) [194], the multidrug and toxic compound extrusion (MATE) family [195], the small multidrug resistance (SMR) family (a subgroup of the drug/metabolite transporter superfamily [196]), and the resistance-nodulation division (RND) superfamily [197]."[190]

In *P. aeruginosa* only, for the first time, a 3-gene locus operon PA1874-1877 was identified to encode an RND multicomponent efflux complex highly expressed in biofilm when compared to planktonic state, conferring high resistance to ciprofloxacin, gentamicin and tobramycin [198]. Another three pumps (MexA-MexB-OprM, MexC-MexD-OprJ, MexE-MexF-OprN) identified, appear to mediate efflux of more antimicrobials such as tetracycline, rifampicin, fusidic acid, erythromycin, J-lactams, chloramphenicol etc. [199]. These pumps are not affected by biofilm formation and growth however specific antibiotic resistance is dependent upon their expression. Ofloxacin resistance and colistin tolerance in *P.*

aeruginosa biofilm depends upon the expression of MexA-MexB-OprM pump and recently, these efflux pumps are shown to express high under oxidative stress conditions as in cystic fibrosis and Ciofu *et al* suggested their play role in biofilm specific resistance [200,201]. Gillis *et al* has also identified MexC-MexD-OprJ pump that is highly expressed in *P. aeruginosa* biofilm and provides azithromycin resistance specific to biofilm environment[202,203]. Concluding, the data suggests more investigation is needed with respect to the role of antibiotic resistance via efflux pumps and their regulation, specific to *P. aeruginosa* biofilm.

1.4.4.5 Persister cells

Persisters or slow growing cells are another challenge for antimicrobials in killing biofilms[204-206]. Basically, conventional bactericidal or bacteriostatic agents target active processes taking place in the cell. Being non-growing, inactive cells, residing deep in the biofilm surrounded by dense EPS, persisters escape these bactericidal impacts of antimicrobials owing to their hyper resistance phenotype rather than any specific resistance Mechanism[207,208],[209]. Once the antimicrobial treatment ceases, persisters rapidly grow to repopulate biofilm with more active cells [210].

The Persisters' hyper resistance phenotype is genetically run by toxin/antitoxin (TA) systems identified in many bacterial genomes to regulate machinery controlling cell survival[211-213]. The TA systems consist of TA locus that encodes 2 genes in an operon to produce a stable toxin protein and a labile easily degradable antitoxin which is either a protein or RNA[214,215]. Toxins proteins act to disrupt normal essential cell functions for cell growth whereas antitoxin neutralizes toxins to enable normal cell metabolism. Antitoxin and TA complexes also regulate the TA activation cycle through feedback mechanism[216,217]. During environmental stress conditions like oxidative stress, high temperatures, decreased pH, starvation etc. with implication of QS signals [218,219], TA systems are stimulated to produce toxin[220,221]. This leaves free toxin in excess to slow down protein synthesis and reduce cell growth for cell persistence and survival via several inhibitory mechanisms and pathways until conditions improve. Biofilm EPS/EPM provides additional shelter for survival of persisters. In *E. coli* persister cells the toxin/antitoxin system is toxic protein HiPA is neutralised by antitoxin protein HipB[208,222,223].

Persister cells are also produced by many other pathways as in *P. aeruginosa*, the *rpoS, spoT*, *relA* and *dksA* genes are involved in formation of persister cells leading to antimicrobial

tolerance [200,224,225]. The *rpoS* gene induces alternative sigma factor RopS, the master stress-response regulator, to regulate many other genes such as genes involved in heat and osmotic induction for persistence during stationary phase. The *rpoS* gene mutant strain was 40% less resistant to imipenem while 70% less resistant to biapenem as compared to parent strain in stationary phase with intact *rpoS* gene in *P. aeruginosa*[225].

1.4.4.6 Reduced host immune response

Finally, biofilm has also been shown to directly impair the host's ability to mount an immune response. Koniaris *et al* [226] have shown that leukocytes have impaired phagocytic activity when entering biofilms. The immune response to *P. aeruginosa* biofilm infection has been thoroughly reviewed by Jenson *et al* [227]. Basic Mechanisms involved in reduced susceptibility of *P. aeruginosa* to the host immune response include: -

- Mucoid strains of *P.aeruginosa* produce large amount of fibrin that trap leukocytes in vivo [228].
- Bacteria enclosed within a polysaccharide matrix do not stimulate oxidative bursts of polymorphonuclear leukocytes to the same extent as non-enclosed counterparts[229,230].
- There is rapid killing of polymorphonuclear leucocytes caused by ramnolipids produced in response to QS system in *P. aeruginosa* biofilm[231].

Thurlow *et al* utilised a mouse model of catheter related *S. aureus* infection to investigate host immune response to biofilms. Several potential mechanisms of biofilms evading innate immunity in *S. aureus* were identified such as limited invasion of biofilm matrix by macrophages, reduced expression of IL-1β, TNF-α, CXCL2, and CCL2 normally needed to kill pathogens by antigen recognition and by inducing inflammatory responses [232]. Even if the *S. aureus* biofilm was invaded by macrophages, cell phagocytosis was limited to eradicate pathogens resulting in persistence of infection[232].

Part Two – Relevance of bacterial biofilm in healthcare

1.5 Introduction

Scientifically it's acknowledged now that over 99% of all bacteria grow as biofilms [5,7,15,233-236] in all nutrient-sufficient hydrated or relatively dry environments that maybe natural[4,237], medical [15,64,238] or industrial [239,240]. Biofilm physiology and architecture shields bacteria from antimicrobials and the host immune response that explains the "abundant" prevalence of biofilm in medical environment[241]. Even the existence of biofilm in water storage systems, food industry [242] and air conditioning equipment indirectly impact human health as these systems form part of the medical ecosystem. During the process of water purification, the growth of coliform bacteria is promoted rendering pathogenic bacteria to persist in the water storage and sanitation systems[243-245],[246]. Similarly, the air conditioning system, whether used for heating or cooling, may harbour biofilm which disperse bacteria into the confined environment via air. Numerous respiratory pathogens including *Legionella*, *Listeria*, *Coxiella*, *Chlamydia* etc. have been transmitted by this route [247,248]. Pathogenic contamination is also frequently encountered in food industry across all stages of product harvesting, processing, handling, preparation, storage, packaging, distribution and consumption[249-251].

In the healthcare environment, bacterial biofilms frequently form on practically every suitable surface, wet or dry, biotic or abiotic e.g. the surface of indwelling catheter lines either for urine excretion or continuous ambulatory peritoneal dialysis or even on contact lenses [252,253]. They also grow on prosthesis like pacemakers, heart valve replacements, artificial joints and other surgical implants. On the other hand bacteria may persist in body tissues after acute infections or colonization as biofilms to cause chronic infections as enumerated in Figure 1-6 [254]. Recently our group, Vickery *et al* has identified dry surface biofilm on furnishings, curtains, bed side rails, writing boards, key boards, tourniquets etc. in in throughout the hospital environment and in intensive care units (ICUs)[255].

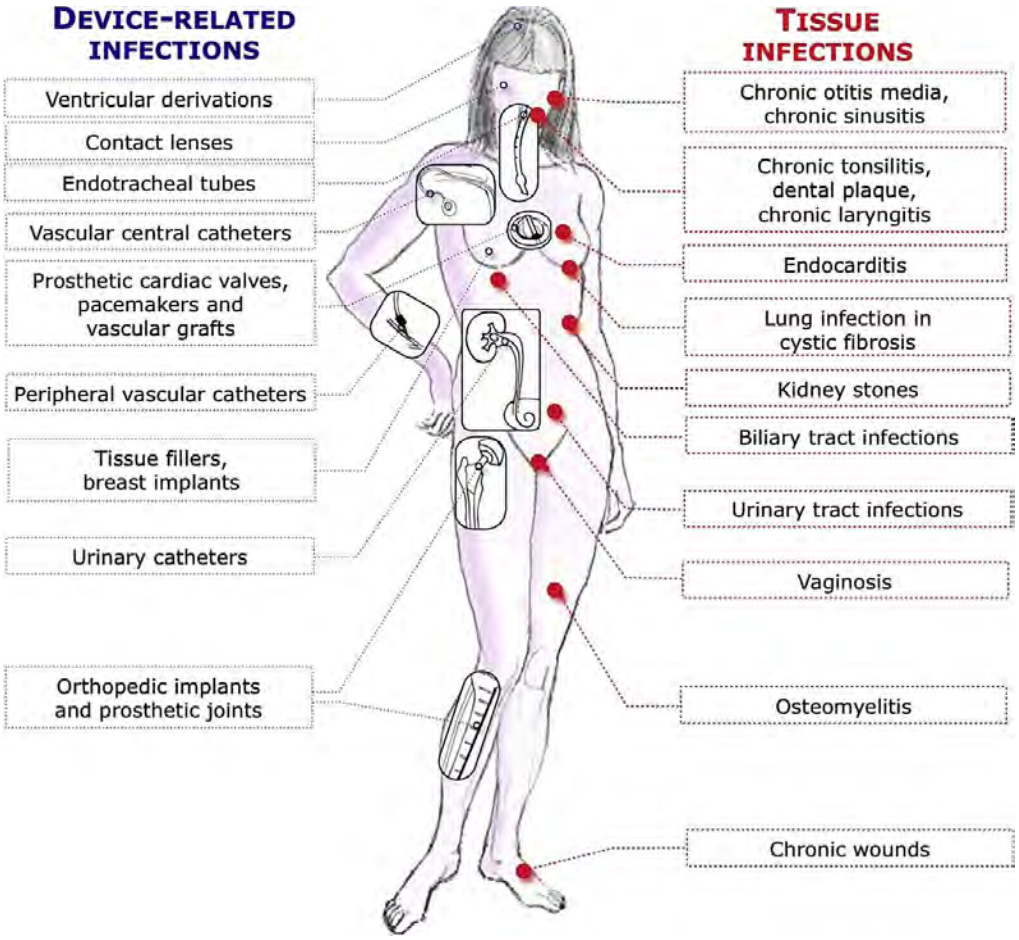


Figure 1-6. Typical biofilm infections[254,256,257]

1.6 Bacterial biofilms and food industry:

Food borne diseases have always threatened human well-being and heavily impacted the healthcare system [258]. Recently, CDC reported 19,507 laboratory-confirmed cases of food borne infections and 4,275 hospitalisations with 75 deaths in 2014 [259]. Outbreaks of *Listeria, Vibrio, Cyclospora, Salmonella, Staphylococcus, Clostridia, Escherichia coli, Bacillus cereus* occurred with food poisoning and many are associated with biofilms[247,260-262].

Scientific research is focussed on deeper investigation of the role of biofilm in food spoilage with the attempt to reduce contamination of food at all stages leading from food production to consumption[260,263-265]. However, despite extensive cleaning and sterilisation strategies eradication of microbial contamination is still a challenge in the food industry.

1.7 Bacterial biofilms on hospital surfaces and hospital acquired infections - a recent discovery

Hospital acquired infections (HAIs) remain a serious public health issue [266]. The CDC (Centres for Disease Control) estimated that over 65% of HAIs or nosocomial infections are biofilm related[267,268]. The overall prevalence rate of HAIs was 7.1% according to the Pilot point survey conducted by European Centre for Disease Control and Prevention in 2010 while in intensive care units the prevalence was 28.1% [269]. A survey conducted in 183 hospitals in 2010-11 across 10 states in USA reported 452 out of 11,282 (4%) patients with at least one HAI [270]while HAI rate was 6% in 9 hospitals in Jacksonville, Florida in 2009[271].

The burden of HAI is even more in developing countries when compared to prevalence rates in USA or Europe. Victor D Rosenthal from the International Nosocomial Infection Control Consortium (INICC) conducted a meta-analysis and systematic review of pooled data from 1995 to 2008. The prevalence rate of HAI was almost double (15.5%) in developing countries than reported by European centre for disease control and prevention in 2008[272-274].
In order to break the transmission of microbes causing these HAI, its mandatory to explore the source of pathogens that can either be endogenous as from the patient's own flora or exogenous coming from healthcare worker's hands (HCWs) or surrounding equipment and environmental surfaces [275]. Regarding pathogen type, *S. aureus* was the most common culprit pathogen found on many occasions including a cohort study in Florida and other studies in developing countries[271,272].

Previously, Claro *et al* reported that commonly touched hospital surfaces, such as toilet door handles to bed side lockers, were heavily contaminated with methicillin resistant *S. aureus* (MRSA) and extended spectrum of beta-lactamase (ESBL) producing *Escherichia coli*[276]. Weber *et al* has recently reviewed the role of environmental surfaces in transferring multidrug resistant organisms including (MRSA, vancomycin-resistant *Enterococcus spp*. (VRE), *Clostridium difficile*, *Acinetobacter spp*., and norovirus in healthcare ecosystem. However, the challenge of eradicating these sources remains. The Weber group suggested that transmission of these environmental organisms can be reduced with improved cleaning and disinfection[277]. Dancer *et al* has emphasised the impact of cleaning with regards to

effective removal of multidrug resistant organisms from hospital surfaces[278-280]. By introducing one more cleaner, there was 32.5 % reduction in microbial load and 26.6% reduction in new MRSA strains on high touch hospital surfaces [281]. In another study during an MRSA outbreak, the MRSA outbreak strain was easily isolated from hospital surfaces, however, with doubling the time spent cleaning from 60hrs/week to 120hrs/week, MRSA could no longer be isolated from hospital surfaces and new acquisitions of MRSA also declined[282].

Recently our research group demonstrated that despite terminal cleaning, multi-resistant organisms can be found in the biofilms formed on 93% hospital surfaces and equipment like key boards, tourniquets, furnishings, clip boards etc. as "dry surface biofilm" (DSB)[255,283] Multidrug resistant organisms like MRSA, ESBL producing organisms and VRE (vancomycin resistant enterococcus) were isolated from 50% of culture positive biofilm samples. . *S. aureus,* the most common causative pathogens for HAI, was identified in 50% of biofilm samples[2]. The study demonstrated that viable and culturable cells can survive as environmental biofilm for weeks or even months. Periodic sloughing off and dispersal of planktonic bacterial cells from these persisting biofilms may contribute to the source of pathogens[284]. Hence adoption of multiple infection control measures is needed to tackle these pathogens in healthcare settings.

Practising proper hand hygiene by HCWs is one of the most cost effective means of breaking the cycle of pathogen transmission in healthcare environment[285-288]. However poor compliance of HCW with hand hygiene is a major barrier in breaking this cycle. An alternative method of personal protection and patient safety is the mandatory use of disposable, single use gloves during invasive procedures as well as routine activities of HCW in patient environment[289,290].

1.7.1 Viable but non-culturable bacteria in biofilm

In 1982, *E. coli* and *V. cholera* were discovered to enter into a viable but non-culturable (VBNC) state[291] that have lost the ability to grow on routine media. Later on many bacterial species were found to enter the VBNC state[292].

These VBNC cells have low metabolic rate and respire with preserved genetic material including plasmids, if any, inside intact cell membranes[293]. Transcription continues inside VBNC cells with uptake of amino acids to incorporate into proteins. Physiologically and

morphologically they differ from their culturable counterparts such as reduction in cell size to minimize energy utilisation. Gene expression alters with enhanced resistance to stress conditions and antimicrobial resistance with prolonged cell survival for months and years[294]. On achieving suitable growth condition the VBNC cells rapidly resuscitate to culturable cells and regain their virulence hence their significance to public health in infections prevention and control[295,296].

Bacterial cells within biofilm acquire VBNC status subjected to nutritional, pH and oxygen conferring antibiotic resistance[297]. Reports have shown that biofilm cultures from water samples do not express their total cell counts attributed to the VBNC cells within biofilm[298]. These VBNC cells have also been retrieved from *S. aureus* and *S. epidermidis* biofilms developed inside intravenous catheters[299].

1.7.2 Development of Dry Surface Model

Discovery of VBNC pathogens residing in dry environmental bacterial biofilms raised the need of developing a reproducible semi-hydrated dry surface biofilm laboratory grown model. Hence an *in-vitro* dry surface biofilm model was finally developed and published by Almatroudi *et al* in 2015 simulating healthcare environmental bacterial biofilm[300]. Both environmental and model dry surface biofilm showed multilayers on SEM with very thick EPS. Their heterogeneous nature was reflected by rough surfaces with varying thickness. The model biofilm had average thickness of 30.74 ± 2.1 µm while thickness of environmental biofilm varied from 24 µm to 47 µm for hard and soft surfaces respectively. Area to volume ratio for environmental biofilm varied 0.34 up to 6.0 whereas the semi hydrated model biofilm had a ratio of 0.52. Protein was the principle component of both types of biofilms. Environmental biofilms contained protein ranging from 42% to 95%. Whereas in the 12 days model biofilm, its protein content was raised (56-73%) while the amount of DNA declined (from 20 to 6%) with further 18 days of dehydration and aging.

Overall the model biofilm simulated environmental biofilm quite suitable for testing efficacy of decontaminating agents such as chlorine in research laboratories at low costs[301].

1.8 Bacterial biofilms - problematic in medical devices and implants

Biofilm infection was identified on indwelling devices like catheters, central venous lines, endoscopes and implantable medical devices like mechanical heart valves, breast implants, prosthetic joints etc. from patients with unusual and prolonged infective presentations as shown in Figure 1-6 [11,238,251,252]. Standard antibiotic therapy was often useless in these cases and the only recourse was to remove the contaminated implant[302]. The proposed reason of these chronic infections was the antibiotic tolerance of biofilm. So, with antibiotic treatment the acute infection was cured but once the antibiotics were discontinued, the biofilm reconstituted eventually leading to another episode of acute infection. In addition, fragments of biofilm emboli on devices such as heart valves and IV catheters, can slough off at intervals and spread infection to distant locations within the body as scientifically demonstrated by Fux *et al* in an in-vitro catheter infection model[66].

Infected devices are also associated with higher morbidity and mortality rates, mortality rates of 25% have been associated with cardiac support devices[303,304]. Similarly as reported in an Australian study across 10 hospitals over a period of 3 years, 5.5% orthopaedic patients died directly due to infected joint prosthesis [305]. The most common causative organism isolated from orthopaedic devices was *S. aureus* followed by *Streptococci* spp., gram negative bacteria and Candida[306,307].

The 2009-2011 CDC surveillance study for HAIs in USA revealed 4.0% HAIs of which 25.6% were only device associated infections[270]. In spite of high rate of biomedical device related infection and associated mortality , manufacturing and application of biomedical devices is rapidly increasing and so are the costs of treating biofilm infected devices such as infected catheters, neurosurgical shunts, prosthetic cardiac valves and pacemakers[307,308] A detailed Australian report on the costs associated with hospital acquired infections was published in 2008[309] indicating huge impacts on human health and state health economy. Hence to address this peaking health issue, considerable active research is needed to improve device disinfection techniques and other patient care strategies by investigating and implementing specific antibiofilm therapies[310-312].

1.9 How bacterial biofilms relate to chronic infections

After extensively revealing the mystery of how bacteria take refuge within the safe shield of biofilms, scientists conclude that non-suppurative infections that do not respond to standard antibiotic therapy and often relapse after completion of therapy are now attributed to biofilm formation [5,11,238,313-315] as shown in Figure 1-6. Nevertheless, there exist no definitive/specific clinical diagnostic criteria or lab screening/confirmatory tests to clinically detect biofilm associated infections.

1.10 Is the infection caused by bacterial biofilm? A question to be solved

Confirming the diagnosis of biofilm infection is still a challenge for clinicians and microbiologists because routine microbiological cultures are frequently negative. The essential clinical indication of biofilm infection is a general chronic inflammatory response [166]. However the rising burden of infections due to biofilms demands a set of guidelines for early diagnosis and treatment [316]. There is no definite/specific indicator of biofilm infection on current clinical routine examinations or laboratory testing such as routine blood tests or tissue swabs[166]. Another indicator of biofilm infection is that organisms sensitive to an antibiotic fail to respond to treatment and/or relapse after completion of antimicrobial therapy[15,154]. Recently, in 2015, European Society of Clinical Microbiology and Infectious Diseases (ESCMID) released useful guidelines for diagnosis of biofilm infections[257].

1.11 Chronic wounds: a continuing healthcare problem

Cutaneous chronic wounds afflict millions of patients worldwide and are mainly classified as venous ulcers, arterial ulcers, diabetic ulcers and pressure ulcers. It is estimated that up to 1-2% of elderly populations living in developed countries suffer from chronic venous ulcers alone[317,318].

1.11.1 Which wounds are chronic?

Chronic wounds fail to heal through an orderly and timely set of reparative stages of repair. These wounds fail to produce anatomic and functional integrity over a period of 3 months and remain lingering in one or more wound healing phases[319].

In Australia, chronic ulcers due to venous insufficiency [320], related to diabetes [321] and pressure ulcers are a major cause of morbidity and mortality [322]. Pressure ulcers are rare, affecting only about 0.5% of the total population of which, 70% cluster in the geriatric population [318]. Approximately 2.5% of patients suffering from diabetes have foot ulcers at any stage of their disease[323]. In Australia there is an estimated 1.7 million diabetics with foot complications such as neuropathy developing in 13% of patients diagnosed with diabetes and 7% in undiagnosed cases [324]. Foot ulcers in these patients were responsible for 65 amputations *per day*, in 2010 [322,325] resulting in millions of dollars per year in medical costs and domestic assistance [324]. To back up Australian data, "The Diabetes UK key stats guidelines, 2014," reported an average prevalence of Diabetes in UK adults within the general population as 6.09% of total. Hence, in 2013, out of 3.2 million diabetic people in UK, 80,000 suffered from chronic ulcers with 100 amputations per week [326].

Chronic wounds/ulcers significantly cost the health care system both financially and in human terms. Hospital costs include nursing care, bed occupancy costs, dressings, disposables and prolonged duration of hospital stay. Graves *et al* recently published an economic study focusing on all four categories of chronic wounds in Australia during the 2010-2011 periods. An estimated total cost of $2.85 billion/year was spent on wound care, however, there was a large uncertainty of the actual costs involved as wound care is not an individual Medicare item and estimates of prevalence varied greatly. Pressure ulcers were the most cost consuming/expensive of the all four categories [327].

Similarly, in America pressure ulcers are expensive. Brem *et al* investigated 20 patients with stage IV pressure ulcers, 11 hospital-acquired and 9 community-acquired, and the direct costs for treatment estimated. The cost for a hospital-acquired pressure ulcer patient averaged $129,248 during 1 hospital stay, whereas the cost for a community-acquired pressure ulcer patient averaged $124,327 for 4 admissions [328]. Costs are greater than previously estimated and are consistently rising. Strategies to improve early management of these wounds are very much needed to eradicate immense pain and patient suffering, reducing morbidity/mortality rate and reduce the health care costs.

1.11.2 Phases of wound healing

Wound healing is broadly divided into following four overlapping phases (Figure 1-7):

1.11.2.1 Homeostasis/coagulation formation

A break in the skins integrity causes in bleeding followed by coagulation and results in haematoma formation.

1.11.2.2 Inflammatory phase

Formation of scab then takes place with pooling of the wound with neutrophils and other cells of initial stage of inflammation within few hours of injury (24-72hrs). Lysosomal granules release enzymes such as collagenase , NADPH oxidase and alkaline phosphatase contributing to the acute inflammatory response [329]. Macrophages (monocytes) while clearing wound debris and contaminating bacteria, release cytokines and some cell growth factors [330]. Simultaneously the basal skin cells undergo mitotic division with epidermal cells migrating towards wound centre.

1.11.2.3 Proliferation

Macrophages continue releasing matrix metalloproteinases (MMPs) for collagen degradation with continuous migration of fibroblasts, epithelial and endothelial cells for wound tissue and subsequent blood vessels repair to replenish blood flow [329].

1.11.2.4 Remodelling

Further development of granulation tissue, protease activity and reorganisation of matrix outside the cells and collagen continues for several weeks. Wound contraction occurs due to transformation of a proportion of fibroblasts into myofibroblasts and balance between MMPs and tissue inhibitors of MMPs (TIMP) during granulation and remodelling phases of wound repair.

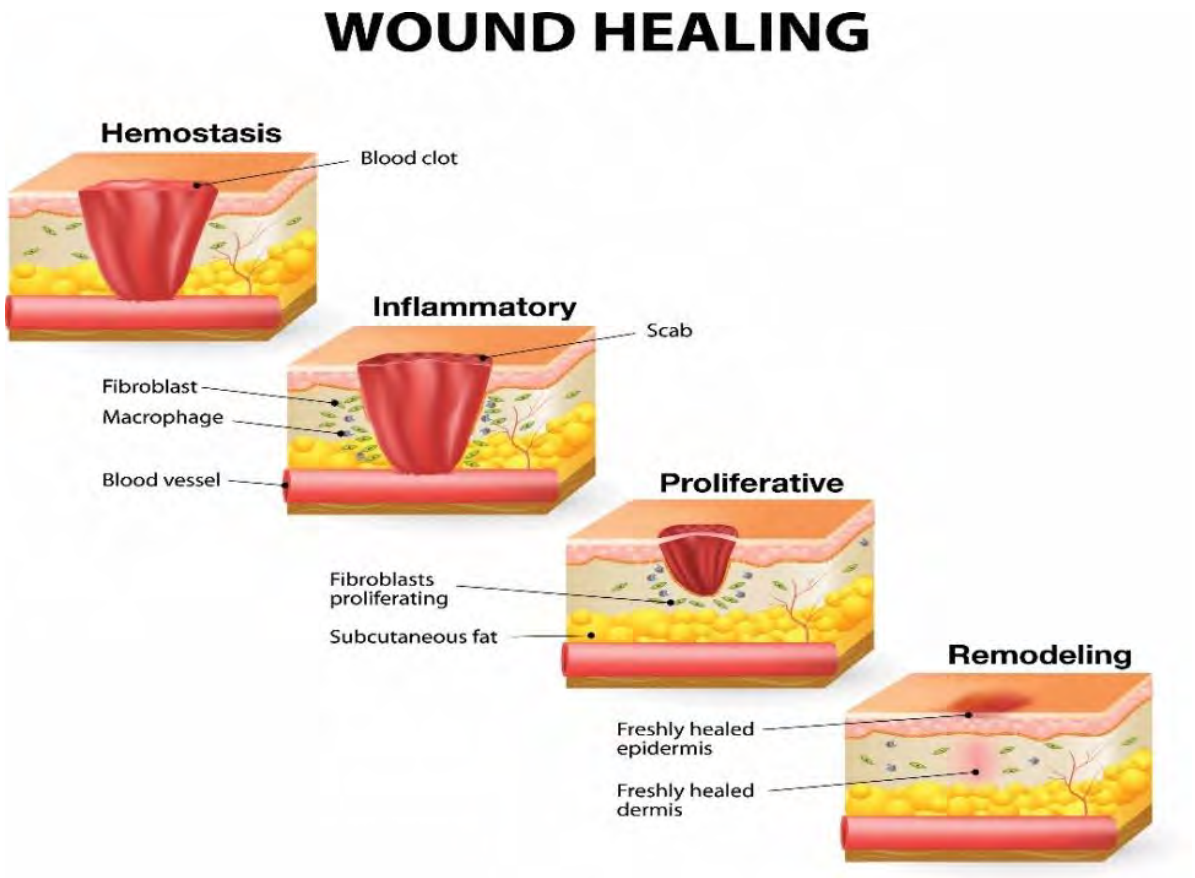


Figure 1-7. The four Phases of wound healing

www.sheildhealthcare.com John Maynard+ on December 18, 2015 [331]

1.11.3 Transition of acute to chronic wounds

Wound healing is a complex and highly regulated process. Disruption of this process results in failure of healing and transition to wound chronicity. Healing, as described above, can be affected both by endogenous and exogenous factors. Endogenous factors being the underlying changed pathophysiological conditions as in arterial imperfusion or venous insufficiency while the main exogenous factor for all wounds is persistent colonization by micro-organisms[332]. Whereas, persistent localized pressure over the skin and/or underlying tissue against a bony prominence, disrupting blood supply is specific major factor in development and persistence of pressure ulcers[333]. Other major potential factors are systemic causes such as poor nutrition, immunosuppression, smoking, aging, infections and metabolic diseases e.g. diabetes[334-337]. Any one of these factors or more may contribute to poor wound healing ultimately leading to local tissue breakdown and subsequent formation of skin ulcer. Identifying and targeting relevant etiological factors is therefore the key to successful wound treatment.

1.11.4 Significance of bacterial biofilm formation in delayed wound healing

Although wound chronicity is multifactorial, microbial biofilms formation is the most common causative factor[334,338]. Recent studies have shown that bacteria in chronic wounds exist as a structured biofilm rather than as individual planktonic cells making it more resistant to conventional antibiotic therapy and hence delaying wound healing [339,340]. Many clinical and case studies support the etiological impact of bacterial biofilm on chronic wounds[313,341-343]. Robson *et al* demonstrated failure of skin graft survival in the wounds contaminated with greater than 100,000/mm^3 of bacteria[344]. Moreover, the authors found that wound healing improves by a reduction in bacterial counts and markers of inflammation as listed in Table 1-2. Hence bacteria in wound persistent as biofilms commonly cause delayed or non-healing of chronic wounds.

The underlying mechanism involves colonizing bacteria that lead to inflammation in chronic wounds with release of bacterial products such as endotoxins, exotoxins, proteases causing damage to host tissues triggering further inflammatory responses[338,345-347]. Tissue response to these exogenous factors increases as the bioburden increases. Research studies on biofilm bacterial typing in chronic wounds have revealed poly microbial or multispecies nature and physiologic heterogeneity of population within harbouring biofilms[340,348].

1.11.5 Investigating bacterial biofilm in chronic wounds:

S. aureus and *P. aeruginosa* hare the principal organisms cultured from chronic wound biopsies [349]. Nevertheless, conventional methods are unable to reveal structured biofilm in wounds as all biofilm bacteria are difficult to recover and culture [350]. The bacterial profile obtained through cultures may underestimate the presence of significant pathogens or difficult to grow organisms like anaerobes while overestimating some other easy to culture species such as *S. aureus* or *P. aeruginosa*. Although Davies *et al* has previously shown the predominance of Staphylococcus and Pseudomonas species by both culture as well as molecular analysis of biopsies from chronic wounds [351], the presence of biofilm was not confirmed. Polymerase chain reaction (PCR) followed by pyrosequencing was later adopted to characterise microorganisms inhabiting delayed or non-healing chronic wounds.

Dowd at el were the first to use bTEFAP-like pyrosequencing for extensive evaluation of chronic wound microbiota [352]. *Staphylococcus, Peptoniphilus, Pseudomonas, Stenotrophomonas, Enterbacter, Serratia,* and *Finegoldia spp* were most numerously identified by using a variety of molecular methods (16S PCR followed by pyrosequencing, shotgun Sanger sequencing, and denaturing gradient gel electrophoresis) on a single pooled sample from 10 diabetic patients [339]. Another study was conducted by the same group on 40 chronic diabetic foot ulcers from different patients with similar diagnostic approach using new bacterial tag encoded FLX amplicon Pyrosequencing (bTEFAP) [352]. Mixed genera of bacteria were revealed in all chronic wound where no single species was the common culprit of delayed infection in all 40 samples. Most prevalent bacteria were Corynebacteria followed by many obligate anaerobes such as *Peptoniphilus, Anaerococcus,* and *Bacteroides.* However, this also led to the possibility of some non-pathogenic bacteria existing symbiotically with the pathogenic genera in the biofilm contributing towards delayed or non-healing of wounds.

Later on Kirketerp *et al* used Peptide nucleic acid fluorescent in situ hybridization (PNA-FISH) to reveal the specific identification and embedding of biofilm in chronic wound irrespective of wound depth and to determine bacterial load[353]. Generally in biological samples, three-dimensional and high resolution images of clean and fouling membranes can be obtained by confocal laser scanning microscopy (CLSM) [354]. Malic *et al* used both these methods to identify, characterize and analyse the spatial distribution of biofilm-forming bacteria in their in-vitro human epidermis wound model and in-vivo non-infected chronic venous leg ulcer biopsy samples [355]. *P. aeruginosa* predominated in all wound biopsies while *P. aeruginosa, S. aureus, Streptococcus sp.* and *Micrococcus sp.* were used in their in-vitro models as these were the most dominant species found in most wound biopsies [355]. Hence PNA-FISH when used in conjunction with CLSM image analysis has shown to be versatile research tool as well as a potentially diagnostic test to detect and identify bacteria as later demonstrated by Fazli *et al* to investigate biopsies from chronic wounds[153]. Since then many scientific studies have explored the biofilm microbiology of chronic wound as listed in Table 1-2. Therefore, for our experimental work, *P. aeruginosa* and *S. aureus* biofilms were chosen owing to their abundant existence in healthcare environment including wounds as well as due to their versatile capability of forming biofilms.

Wound type	No.	Methods	Observations	Reference
Acute wounds	16	Light microscopy, SEM	1 (6%) acute wound contained biofilm	[340]
Chronic wounds	50	Culture, Light microscopy, SEM, PCR, DGGE, Cloning	30 (60%) chronic wounds observed to contain biofilm	[340]
Chronic diabetic ulcers	40	Pyrosequencing (bTEFAP)	Mixed bacterial aggregates most abundantly Corynebacterium, obligate anaerobes, Streptococci, Staphylococci, Serratia	[352]
Chronic wounds Mixed aetiology	22	PNA-FISH, standard culturing, CLSM	13 (60%) chronic wounds contained aggregates suggestive of biofilm majority containing *S. aureus* and *P. aeruginosa*	[353]
Chronic wounds Mixed aetiology	2	FISH, CLSM	Both samples contained micro-colonies, basal structure of biofilm	[356]
Chronic wounds venous leg	10	FISH, CLSM	*P. aeruginosa* aggregates seen deeper in wound bed than *S. aureus*	[357]
Chronic wound venous leg	40	Pyrosequencing (bTEFAP), PCR, Metagenomics	Polymicrobial aetiology predominating with Bacteroidales, staphylococcus, Corynebacterium, anaerobes	[348]
Chronic wounds diabetic	23	Pyrosequencing (bTEFAP),	Polymicrobial aetiology majority with anaerobes, staphylococcus, Corynebacterium,	[358]
Chronic wounds	49	Pyrosequencing (bTEFAP),	Polymicrobial aetiology majority with anaerobes,	[359]

pressure			streptococcus, *P. aeruginosa, S. aureus,* Corynebacterium,	
Full-thickness burns	11	Light microscopy, SEM	Ulcerated areas and escharotomy sites contained biofilm	[360]
Chronic wounds venous leg	10	Conventional aerobic and anaerobic cultures, bTEFAP	Mixed aggregates. Actinomycetales and Bacteroidales more in non-healed wounds	[361]
Chronic wounds venous leg	10	PNA-FISH, CLSM	*P. aeruginosa* aggregates suggestive of biofilm elicited greater inflammation than *S. aureus*	[153]
Mixed aetiologies	15	CLSM	7 (47%) wounds contained biofilm	[362]
Chronic wounds Diabetic	2	CLSM	Both samples contained biofilm	[363]

Table 1-2. Table of wound studies modified from *"Biofilm delays wound healing: A review of the evidence"* [364]

SEM=Scanning Electron Microscopy

CLSM= Confocal laser scanning microscopy

FISH= Fluorescence in-situ Hybridization

DGGE= denaturing gradient gel electrophoresis

PCR= Polymerase chain reaction

PNA-FISH= Peptide nucleic acid based FISH

bTEFAP= bacteria tag encoded FLX amplicon pyrosequencing

1.12 Clinical relevance of *Pseudomonas aeruginosa* in the healthcare environment

Pseudomonas aeruginosa, a gram-negative, opportunistic, bacterium is implicated in numerous chronic infections. *P. aeruginosa* being normal commensal, forming part of normal aerobic intestinal flora, it is not highly invasive[365] but it is readily isolated from

chronic wounds, ear infections, lungs of cystic fibrosis patients and medical devices[366,367]. *P. aeruginosa* widely persists in the environment too due to its robust ability to form biofilm [113,368,369].

Under suitable circumstances *P. aeruginosa* can cause serious infections in susceptible individuals and immune-compromised patients such as in cystic fibrosis, AIDS, malignancy, burns etc. [343,370,371]. It is one of the most common and main opportunistic human pathogen[365,372,373]. For susceptible individuals, the infective dose of *P. aeruginosa* is as low as $10^{\wedge 5}$ [344]. In healthy individuals, it forms part of normal aerobic flora of intestine [374] [366,375] whereas in many chronic infections, *P. aeruginosa* biofilm is one of the major causes of lingering illness [367] such as delaying healing process by invading chronic wounds.

P. aeruginosa biofilm has been recovered from hospital water pipelines and sinks whereas hospital out breaks, due to water contamination with *P. aeruginosa* has also been reported[376-378]. Many other nosocomial outbreaks have been associated with its presence on medical equipment such as outbreaks occurring in intensive care patients over 2 months period due to bronchoscopes and endoscopes contaminated from improperly maintained washer disinfectors[366,375]. Largest ever outbreak of *P. aeruginosa* occurred from contamination of mechanical ventilators during 2001-2002 winters across hospitals in several regions of Norway[379].

Owing to its wide presence in infections and its inherent capacity to form biofilm, *P. aeruginosa* is the preferred model organism for many research based studies conducted for understanding biofilms[32,54,139,380,381]. With long genome of 5570 open reading frames using multitude of survival mechanisms[373,382], this bacteria in biofilm successfully survives host immune responses and detrimental actions of conventional antimicrobial treatments[186,383,384].

1.13 Original review Paper

1.13.1 Author's contribution

Dr Shamaila Tahir reviewed literature and wrote the manuscript for peer review. Her contribution was 95%

"Staphylococcus aureus biofilm: antimicrobial tolerance and resistance"

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Part Three – Strategies of eradicating bacterial biofilms in the healthcare

1.14 Introduction

Current methods of eradicating bacterial biofilms from devices, hospital surfaces and human tissues will be discussed.

1.15 Environmental surfaces:

Healthcare authorities all over the world keep refining the infection prevention and control policies/guidelines aligned with the latest scientific research[385-390]. Progress reports are regularly evaluated and modified strategies implemented. Per these health safety guidelines, protocols of cleaning and disinfecting medical/hospital surfaces whether from instruments, devices or surfaces are set up as described comprehensively in Table 1-3 below.

In spite of all these precautionary measures, the challenge of combating microbes at various fronts still persists[377,391-393]. Pathogens especially the multidrug resistance microbes like MRSA, VRE and *Clostridium difficile* can survive for weeks or even months on medical equipment and other surfaces in healthcare environment as biofilms or micro colonies[394,395].

A classification on how to decontaminate the medical instruments was proposed by Dr Earl Spaulding in 1977 in his book "Disinfection, sterilization, and preservation". According to his classification, medical instruments were categorised to be subjected at three levels of decontamination based on their risk of infection transmission as dealt with in Table 1-3 below[396].

1) Non-critical Instruments used on intact skin fall into low risk category and just need cleaning before reuse.
2) Semi-critical Instruments applied on broken skin and intact mucous membranes had medium risk of infection transmission requiring disinfection after cleaning.

3) Critical lastly the instruments carrying high risk of transmitting infection are those used on broken mucous membranes or on sterile tissues hence need thorough sterilisation.

1.15.1 Cleaning, disinfection and sterilization…… methods of decontamination used in healthcare

As a routine, most hospitals adopt cleaning only protocol for surfaces rather than the two step method of cleaning and disinfection[397-400]. Studies have shown that new patients occupying cleaned rooms previously occupied by patients colonized by multidrug resistance microbes have an increased risk of contracting that same pathogen[280,401,402]. Current infection control scientists hence are emphasising the need of revising/reconsidering Spaulding's classification to suit today's requirements of decontamination[403-405]. Eckstein *et al* reported in 2007 that 16 out of 17 rooms previously occupied by VRE colonised patients, were contaminated with VRE before terminal cleaning. Twelve rooms were still found contaminated after cleaning only, while VRE was not detected in rooms when cleaning was followed by disinfection with 10% bleach[406].

Despite adopting measures of cleaning and disinfection, multidrug resistance (MDR) microorganisms can persist on dry hospital surfaces, in wet areas and on hospital equipment. MDR were found residing in dry biofilms contaminating hospital furnishings, patient bedding and surroundings in an ICU [2]. To ensure biofilm eradication, available scientific studies suggest that cleaning is ineffective unless EPS removal is achieved and bacteria incorporated into the biofilm are killed by disinfection[407].

Table 1-3. Decontamination in Healthcare environment[385-388,408]

Equipment decontaminated		Methods of decontamination		Effectiveness	References
Medical instruments, equipment, liquids or devices	Used on intact skin e.g. ultrasound probe	Cleaning---an essential preliminary procedure		Physically remove organic and inorganic materials, reduces count but does not completely eradicate bacteria or viruses.	[409,410]
	Used on intact mucous membrane and broken skin e.g. dental instruments	Disinfection	Low (Lodophor)	Target vegetative bacteria, enveloped viruses, some fungi.	[411]
			Medium (Phenols and Alcohols)	Target mycobacteria, vegetative bacteria, some viruses, some fungi.	[412,413]
			High (Glutaraldehyde, Formaldehyde, H_2O_2)	Target mycobacteria, vegetative bacteria, spores, fungi, viruses.	[414]
	Used on broken mucous membrane and sterile body tissues.	Sterilization	Wet heat---Boiling and pasteurization (70 ^{O}C for 100mins or 76 ^{O}C for 30mins)	Target pathogenic bacteria, spores, viruses, fungi, 4 common multidrug resistance bacteria.	[415,416]
			Wet heat---steam(autoclave) recommended cycles of 15mins at 121^{O}C or 3mins at 134^{O}C	Inexpensive, non-toxic effective way of sterilization against bacteria, viruses, fungi	[417]
			Dry heat (hot air fan-forced ovens) various cycles, AS/NZS 4187 specifies 180 ^{O}C for 1hr or 160 ^{O}C for 2hrs.	Targets viruses, bacteria and fungi specially used effectively for materials unsuitable for moist heat e.g. powders, airtight containers	[418,419]
Hospital surfaces and inanimate items		One step cleaning only with detergents in most hospitals		Less effective against pathogens especially multidrug resistance bacteria	[420]
		Two step—cleaning and disinfection as using chemicals commonly with 10% sodium hypochlorite (Chlorine) or with combined agent e.g accelerated H_2O_2 (hydrogen peroxide) providing cleaning and disinfection.		More effective against most pathogens including multidrug resistance bacteria	[421-425]
Hospital Environment		Cleaning, disinfection and use of no-touch methods including ultraviolet light exposure, fogging with H_2O_2 (hydrogen peroxide) mist or vapours.		Effective in reducing surface contamination but without absolute decontamination	[426-428]

1.15.1.1 Disinfectants

As shown in Table 1-3, disinfection process is strongly indicated after physical cleaning in order to kill most of the pathogens except spores using low, medium or high level disinfectants at safe to use levels[429]. In general, disinfectants are germicides applied on nonliving objects only, like equipment surfaces; furniture etc., while antiseptics are biocides applied on living tissues or skin[430]. Related to this thesis, 3 disinfectants namely sodium hypochlorite (chlorine), benzalkonium chloride (quaternary ammonium compounds) and ethyl alcohol (ethanol) while 2 antiseptics, Prontosan and Betadine are emphasised. A brief review of these disinfectants and antiseptics is as follows.

1) **Sodium Hypochlorite:** sodium hypochlorite (NaOCl), also known as household bleach, is the most commonly used liquid chlorine disinfectant product. It is colourless, cheap, relatively stable and free of poisonous residuals [408]. Chlorine and other related compounds are active oxidising agents which interfere with DNA synthesis and oxidative phosphorylation while disrupting the cellular activity of microbial proteins [431,432]. Chlorine is available in various concentrations depending upon it targeted use. Although toxic at higher concentrations, in hospitals, it is used at non-toxic levels unless used in conjunction with ammonia or acid detergents when it releases harmful fumes [433].

   Chlorine and related compounds are commonly used in hospitals to disinfect hard surfaces and as such NaOCl used at a concentration of 1000 ppm (parts/million) after cleaning with detergents [434,435]. In an outbreak of VRE, Sample *et al* managed to reduce the rate of VRE cases down to 0.8/1000 from 3.6/1000 patient days in two months after disinfecting haematology-oncology unit with higher concentration of chlorine (5,000ppm).

   However, chlorines efficacy against biofilms is reduced as in wet environments, biofilms are tolerant to commonly used concentrations of NaOCl [436]. While NaOCl effectively removes planktonic organisms dried onto surfaces, recently our group has shown that *S. aureus* dry-surface biofilms are not completely removed, and has the potential to revive after treatment with concentrations of NaOCl used currently in hospitals [301].

2) **Benzalkonium Chloride:** Benzalkonium chloride (BAC) is another disinfectant widely used in healthcare and in the food industry for cleaning, deodorizing and disinfecting hard surfaces[437]. It can also be used as preoperative disinfectant on unbroken skin or as preservative in intranasal solution.[438] BAC is a cationic detergent belonging to the quaternary ammonium compounds (QACs) group of surface active agents or surfactants[439]. BAC being membrane active agent causes bacterial cell lysis via cell wall destruction and disorganization[440]. For hospital surfaces disinfection, the recommended concentration is 10ml/L (2gm/L), while for general cleaning it is 1-2ml/L (0.1-0.2mg/L) of water for most commercially available brands[441]. At lower or sub-lethal concentrations, BAC can stimulate biofilms formation[442,443]. At clinically recommended doses, like other surfactants, BAC effectively destabilises biofilm structures by disrupting hydrophobic interactions within biofilm[444,445] and has been shown to reduce biofilm accumulation [443,444,446]. Some studies have demonstrated enhanced efficacy of NaOCl when NaOCl solution is modified by adding BAC as surfactant such as improved killing of *Enterococcus faecalis* with 5ml of 6% NaOCl and 0.008% BAC from infected extracted teeth[446].

   The bactericidal effect of BAC varies with different bacterial species. Houari *et al* has shown BAC to inhibit *E. coli* GI473 and *P. aeruginosa* POA1 biofilm formation at sub-lethal doses while stimulate growth of *S. epidermidis* biofilm at sub inhibitory doses[443] whilst Bridier *et al* showed that *P. aeruginosa* was more resistance to BAC than *Bacillus subtilis* and *S. aureus*[442]. On the other hand BAC coated surfaces like dentin or polystyrene surfaces has shown to decrease initial cell adhesion for biofilm formation by oral bacteria[447].

   However microbial biofilms can develop resistance/tolerance to BAC either due to slow diffusion and interaction with EPS[448] or due to phenotypic changes of biofilm cells such as high expression of efflux pumps due to oxidative stress owing to decreased oxygen supply[443,449,450] or development of resistance genes[451].

3) **Ethyl Alcohol:** Among "alcohols", ethyl alcohol is the most commonly used compound for disinfection. The other alcoholic product used as disinfectant in health care is isopropyl alcohol, while methyl alcohol is seldom used. At concentrations of 60% to 90% in water, ethanol is excellent for low to medium

level disinfection[408,452]. As an antimicrobial ethanol damages cell membranes and rapidly denatures proteins to interfere with cell metabolism ultimately causing cell lysis[452,453]. Ethanol is most commonly used as topical antiseptic (medical wipes or hand sanitisers), catheter locking agent as well as medicinal solvent[452,454,455]. Sufficient scientific evidence demonstrates that 50% or more concentrated ethanol eradicates planktonic bacteria but limited data is available on elimination of biofilm sessile cells using ethanol. These scientific studies on in-vitro grown biofilms suggest that ethanol can eradicate biofilm bacteria however, efficacy depends upon the adequate combination of its concentration and exposure time[456,457]. Balestrino *et al* determined the bactericidal efficacy of 60% ethanol on planktonic and 4 hr grown *S. aureus, S. epidermidis, P aeruginosa, K. pneumoniae* and *Candida albicans* biofilms. Planktonic bacteria were totally eradicated after 20 min treatment without any revival. Four hour grown *P aeruginosa, K. pneumoniae* and *C. albicans* biofilms were culture negative following 20min and 30min treatments but 20min treated biofilm cells revived on re-incubation in broth for 24 hrs. Gram positive cocci took longer treatment periods for total eradication with revival after 30mins contact time[456].

Efficacy of lower concentration of ethanol (25-30%) in combination with other antiseptics and anticoagulants against biofilm removal or prevention has also been tested with some promising results. Raad *et al* demonstrated complete killing of *S. aureus* and *Candida parapsilosis* in-vitro grown biofilms adhering to central venous catheter with a combination of 25% ethanol with minocycline and EDTA[458]. Takla *et al* used an anticoagulant, 4% trisodium citrate with 30% ethanol that prevented formation of *S. aureus, S. epidermidis, E. coli* and *P. aeruginosa* biofilm during 72 hrs contact treatment in the wells of a Calgary biofilm device[459]. On the other hand scientific studies show that longer exposures of bacterial biofilm to sub-lethal (<50%) ethanol treatment alone promotes biofilm formation by expressing antimicrobial factors such as enhanced expression of efflux pumps [460,461].

1.15.1.2 Sterilization with dry heat

Dry heat is commonly used to sterilize materials sensitive or unsuitable for wet heat [462]. These include powders, airtight containers, petroleum products, paper wrapped things and syringes[463]. Dry heat decontaminates by destroying large biomolecules like proteins and other essential cell constituents by destructive oxidation [418]. Dry heat is applied for sterilisation in fan forced hot air ovens with high temperatures lasting for long durations, higher the temperature, the less the duration of heat application is required [464]. According to AS/NZS 4187 standard specifications, dry heat of 180^{O}C for one hour and 160 ^{O}C for two hours is recommended for sterilisation [465]. However, application of dry heat is a time consuming slow method of sterilisation, unsuitable for items sensitive to high temperatures [463].

1.15.1.3 Sterilization with wet heat

Wet heat is used for sterilisation by boiling, pasteurisation and steam autoclaving [418]. Boiling alone generally isn't an accepted standard method of sterilisation as resistant pathogens as well as spores survive at 100^{O}C [466,467].

Autoclaving utilises various combinations of high temperatures, pressure and time durations with direct exposure of every item to steam in a closed chamber. The method is highly effective, simple, easy to use/monitor, non-toxic and inexpensive [468,469]. According to Australian standard AS/NZS 4187 for instrument sterilisation and the Association for the advancement of medical instruments, medical surgical instruments should be autoclaved at 121^{o}C for 15 mins or at 134^{o}C for 3mins [465,470].

Pasteurisation is used in food and medical industries for sterilising food items and semi-critical items such as respiratory therapy and anaesthetic application devices[415,471]. The French scientist, Louis Pasteur in 19th century invented this process that killed microbes in food without affecting its nutritional value and is popularly applied in food industry to decontaminate canned food, milk juice and other food items [434,472]. During pasteurisation the temperature is maintained around 70^{o}C for half an hour [434] while heating at 70^{o}C is recommended for 100mins by the AS/NZS 4187 for instrumental sterilisation [465]. Higher temperatures have been recommended for half an hour processes such as 75-76^{o}C to kill

S. aureus and *Klebsiella pneumoniae* or multidrug resistance organisms such as MRSA and *Acinetobacter baumannii* [415].

1.15.2 Evolving methods of preventing biofilm contamination

Another challenging approach towards eradicating bacterial biofilm is to inhibit the adhesion and formation of biofilm on devices, implants and environmental surfaces. The methods involved are use of various coating materials that reduce initial attachment of bacteria to surfaces such as silver impregnation or integration of antimicrobial products into surface materials[473-475]. Another method is the development of titanium nanotubular surface structure that is able to capture antimicrobial agents [476]. However none of these methods have proven to be efficient enough due to multiple variables affecting their efficacy and their own adverse effects on the surroundings such as toxicity, effect of soils, cost-effectiveness etc. [477,478]. The cost is too high and the surfaces eventually become depleted of the antimicrobial [479].

1.16 Skin Antiseptics:

1.16.1 Antiseptic agents used in healthcare:

1) **Prontosan:** Prontosan is used as an antiseptic to kill planktonic and biofilm bacteria on live tissue, wounds and skin. Prontosan is a brand product mainly developed by B Braun Wound Care. It has 2 main antimicrobial ingredients; Betaine is an active surfactant containing Iodophor and Polihexanide (PHMB) as antimicrobial agents[480-482]. Small research data is available in scientific literature for its efficacy in eradicating bacterial biofilm from infected tissues such as chronic wounds, however many studies show that Prontosan antiseptic washes are effective in healing wounds[483-485]. Davis *et al* reported a significant reduction of MRSA and biofilms in 48hrs and 72hrs after irrigation with Prontosan solution in a porcine wound model when compared to other treatment groups[486].
2) **Betadine:** Betadine is a brand name of Povidone-iodine (PVP-I) as developed by Purdue Products (Purdue Pharma, Stamford, Connecticut, USA). It is the best

known and widely used Iodophor compound due to germicidal property of iodine with low toxicity and irritancy[487]. Aqueous or alcoholic solution of iodine is used as effective antiseptic for over 180yrs[488,489] specially for preventing and treating wound infections[490]. Iodine in water is unstable hence it is bound to carriers as Iodophor or iodine releasing agents. PVP-I falls under the category of Iodophor [491,492] (Table 1-3). It is lethal for all kinds of bacterial pathogens and fungi while being non-injurious to skin and open wounds[493] but has dark colour and is associated with post use contact dermatitis[494].

The exact mode of its antimicrobial action is not known. It rapidly penetrates inside the target microorganism to attack various major proteins for cell lysis[430,487,495]. Chundamala *et al* reviewed randomised control trials and comparative studies from Medline and EMBASE and found PVP-I to prevent post-surgical site infections significantly when compared to water, saline or no skin irrigation[496]. In a few comparative studies it is less effective surgical site antiseptic when compared to chlorhexidine alone or chlorhexidine made up in alcohol[497,498]. Similarly as compared to other biocides like alcohol or hydrogen peroxide, betadine is less effective in killing bacterial biofilms[499-501] and resistance to iodine can also develop due to its limited diffusion through thick biofilm EPM[502]. Cargill *et al* tested efficacy of PVP-I against attached and unattached *Legionella pneumophila*. Water grown biofilm bacteria were estimated to be 134 times more resistant to PVP-I than planktonic cells [500]. *S. epidermidis* biofilm was less susceptible to PVP-I treatment alone as compared to treatment combined with alcohol or hydrogen peroxide[499].

1.17 Chronic wound biofilm

Non-healing or delayed healing chronic wounds are one of the most common tissues that harbour bacterial biofilm in >60% of cases. Management of these chronic wounds will be discussed in this section as an example of managing biofilm in living tissues. A variety of treatment measures are attempted to heal wounds of arterial, venous, pressure or neuropathic aetiology [503]. Some of these wounds remain refractory even after 6-8 weeks

and fall into the category of chronic wounds as discussed earlier. Available management strategies of these difficult to heal wounds, as established modalities are comprehensively summarized below in Table 1-4.

Bacterial biofilm harbouring chronic wounds is difficult to manage due to their antimicrobial resistance and low grade inflammatory host responses. Current wound care includes debridement, use of antibiofilm agents, oral antibiotics, conventional antimicrobial and negative pressure wound therapy (NPWT) dressings[364,504]. Several innovative devices or physical modalities such as hyperbaric oxygen, bio-surgery, intermittent pneumatic pressure, NPWT, laser therapy etc. have been introduced for wound healing in recent past decades of which NPWT will be discussed below in detail.

Type of Ulcer	Conservative	Indication of surgery	Surgical procedures
Venous	➢ Compression therapy remains the mainstay. Examples include compression stockings, hosiery, Unna's boots, elastic wraps, medicated bandages, orthotic s compression devices and pneumatic compression pumps. ➢ Appropriate moisturising of surrounding skin, control of exudate and eczema and treat infection.	➢ Pure superficial venous incompetence either of the long or short saphenous system	➢ Ligation or stripping of superficial veins or sub-fascial interruption of perforating veins. ➢ Sclerotherapy ➢ Endogenous ligation ➢ Skin grafting for large ulcers after treating the underlying cause
Arterial	➢ Control of any underlying diabetes, hypertension and hypercholesterolemia ➢ Smoking cessation and regular exercise	➢ Non-healing ulcer(s), disabling claudication, rest pain and gangrene	➢ Revascularisation surgery such as bypass grafting or stenting to restore blood supply to the affected limb ➢ Amputation of the affected digit or part of the limb (e.g. below knee) may be indicated in recalcitrant wounds +/- severe rest pain +/-

			ongoing local infection +/- sepsis
Diabetic	➢ Good glycaemic (diabetic) control ➢ Appropriate foot wear ➢ Assess vascular status regularly ➢ Treat infections promptly	➢ Non-healing ulcers and gangrene ➢ Local or systemic infection	➢ Wound debridement ➢ Amputation of the affected digit or part of the limb (e.g. below knee) may be indicated in recalcitrant wounds +/- ongoing local infection +/- sepsis
Pressure	➢ Pressure relief ➢ Regular repositioning of patient ➢ Support surfaces such as air-fluidised mattresses ➢ Regular skin inspection ➢ Optimise nutritional status	➢ Non-healing ulcers and gangrene ➢ Local or systemic infection	➢ Wound debridement ➢ Osteotomy/removal of affected bone ➢ Reconstructive surgery (e.g. flap coverage) to provide tissue over affected bony prominence

Table 1-4. Commonly Used Management options for difficult-to-heal wounds [503]

1.17.1 Negative pressure wound therapy (NPWT)

1.17.1.1 Historical background

From many years, people used suction therapy to heal wounds. Minimal suction improved fluid drainage and kept the wounds clean for fast repair. Historical records show that ancient Greeks used drainage systems as a primitive negative pressure technique to remove discharge from infected wounds. The first reported application of vacuum to facilitate wound healing is dated back to 1932 when Chaffin developed a suction drain for use in post-operative wounds [505]. Later on application of suction techniques continued at a relatively low pace until more formal literature started publishing in 1980s. Five Russians scientists reported the application of 75-80mm Hg negative pressure to wounds in 1986. Chariker *et al* published his results of applying negative pressure therapy in 7 patients with incisional or cutaneous fistulas, later known as Chariker-Jeter technique[506,507]. Subsequently, improvements continued both in NPWT techniques/devices and combining negative pressure with various instillation solutions. In

early 1990s, Flieishman used foam dressing instead of gauze dressing while vacuum assisted closure (VAC) device with integrated catheter was introduced in 1993 [508]. Vacuum therapy was later combined with antiseptic and antibiotics installation from 1996[509].

First commercially based VAC system was introduced in 1993 by Argenta and Morykwas [510]. Since then several companies have introduced their NPWT system. A list 10 manufacturers along with their brand names can be found in the 2011 review paper by Mouës *et al*.[506,511].

1.17.1.2 Current methods for negative pressure wound therapy

There are two main components of current NPWT, namely, the equipment and the techniques[512]. All currently used methods are variations in combination of these two components.

NPWT equipment covers:

1. Vacuum creation system – The systems have been evolved from using wall mounted suction device and surgical vacuum bottle to Vacuum Assisted Closure(VAC) systems with controlled variables, portable systems, and single use systems.
2. Wound filler to fill the wound cavity - Gauze or foam (polyurethane, polyvinyl, polyester layers joined by silicone elastomer, absorbent polymer matrix) are used as wound fillers.
3. Interface material – This material is placed between the wound filler and wound bed to prevent adherence and facilitate dressing changes. Some of the interface materials used are; Vaseline-impregnated gauze, non-adherent silicone and polyester film.
4. Drainage conduit – The conduits can be perforated (used with foam fillers) or non-perforated (usually used with Chariker-Jeter method)

NPWT techniques covers:

1. Magnitude and Periodicity of negative pressure with vacuum – -25 to -200mm Hg, continuous or non-continuous. Non-continuous may be intermittent (negative pressure is applied in intervals) or dynamic (the pressure is varied over time).
2. Instillation or without instillation – for example negative pressure is used with saline or an antimicrobial solution.
3. Duration of treatment – decision is made based on wound time, and wound healing stages.

Almost all manufacturers have built all-in-one NPWT systems with refinements in pressure tracking, instillation port enabling, wound irrigation, portability etc [512]. It is common in the literature to use the term NPWT (Negative Pressure Wound Therapy) which uses topical negative pressure (TNP) systems to heal wounds. Most of the times "NPWT" and "TNP therapy" are used synonymously and the same will apply in this literature review.

The variables of TNP equipment and techniques are chosen depending on wound type, for example one subclass of NPWT is Micro-deformational Wound Therapy (MDWT), in which there is a highly porous interface material placed between the wound and a semi-occlusive dressing and connected to suction. The highly porous interface material helps deform the wound on a micro level increasing cellular proliferation and hence, improve speed of healing. [513]

Among the variations of the traditional TNP therapy, TNP with instillation is the most common and widely used. Evidences show that using instillation solution with TNP helps speed the healing process [514]. The choice of which instillation solution to be used depends upon the wound type. Some of the instillation solutions used are saline, antimicrobial solutions, insulin and protein C etc.[509,515-517]. Another variation includes adding silver to the coating of foam or to add silver dressing as an interface between the foam filler and the wound.

Other possible adjuvants have been proposed in the literature, but not enough evidence is available of their effectiveness [516]. These include adding platelet gel to wound bed in

case of non-healing ileo-cutaneous fistula, administration of vitamin C as anticoagulant and using nutritional supplements.

1.17.1.3 Mechanism of action

NPWT has following 4 primary Mechanisms of action[511,513,518].

1. Contraction of the wound (macro deformation): TNP helps collapse wound pores by exerting force on wound surface through porous wound filler.
2. Stabilization of the wound environment: Fluid removal stabilizes osmotic and oncotic gradient, foam material and dressing maintain thermal insulation to keep warmth and prevent contamination, foam material maintains sub atmospheric pressure at wound bed and reduces evaporative water loss hence keeping the wound moist.
3. Removal of extracellular fluid: Extracellular fluid causes oedema. TNP systems remove the excess fluid.
4. Micro deformation at the foam-wound interface: The mechanical force exerted by TNP devices affects at cellular level causing micro deformation.

1.17.1.4 Outcomes of TNP/NPWT

As a result, following outcomes have been attributed to TNP therapy [506], however not enough scientific evidence is available to prove that all of these outcomes can in fact be attributed to TNP.

1. Creates a moist environment for improved wound healing however, no formal study is available for evidence.
2. By elimination of excess fluid, TNP reduces oedema and interstitial pressure which may occlude wound drainage. Only few studies support this outcome[519,520].
3. Increases local blood flow: Negative pressure of 50-200mm Hg has shown to increase blood flow depending upon the tissue softness. Morykwas *et al* showed that 125mm increased the blood flow in pigs up to 4 times [521,522].
4. Stimulates angiogenesis, increase vascularization and formation of granulation tissue[523]. Scientific studies have proven this Mechanism of TNP therapy in animals while more studies are needed for human wounds [524,525].

5. Stimulates cell proliferation. Due to the vacuum applied to porous wound filler, sub atmospheric force is transmitted to surrounding tissues. This force deforms the extracellular matrix in the cells where it affects various cell structures and signalling pathways [526,527].
6. Reduces size and complexity of the wound. Wound closure and reduction in size and complexity has been observed for many types of wounds threated with TNP therapy [528].
7. Removes soluble healing inhibitors from the wound: Wound exudate may contain an excess of matrix metalloproteinases (MMP) and their inhibitors and may also be associated with poor wound healing and inhibited cell growth. Modulation of MMP concentration is a Mechanism of action induced by TNP therapy. Very little scientific evidence is available [529].
8. Reduces bacterial load: Some studies have claimed that bacterial load reduced [521] while other studies showed increased bacterial load [530,531]. There is no credible scientific evidence that proves that TNP reduces bacterial load. Figure 1-8. taken from [511], though for MDWT, but equally applies to general TNP therapy as a good summary of TNP Mechanisms.

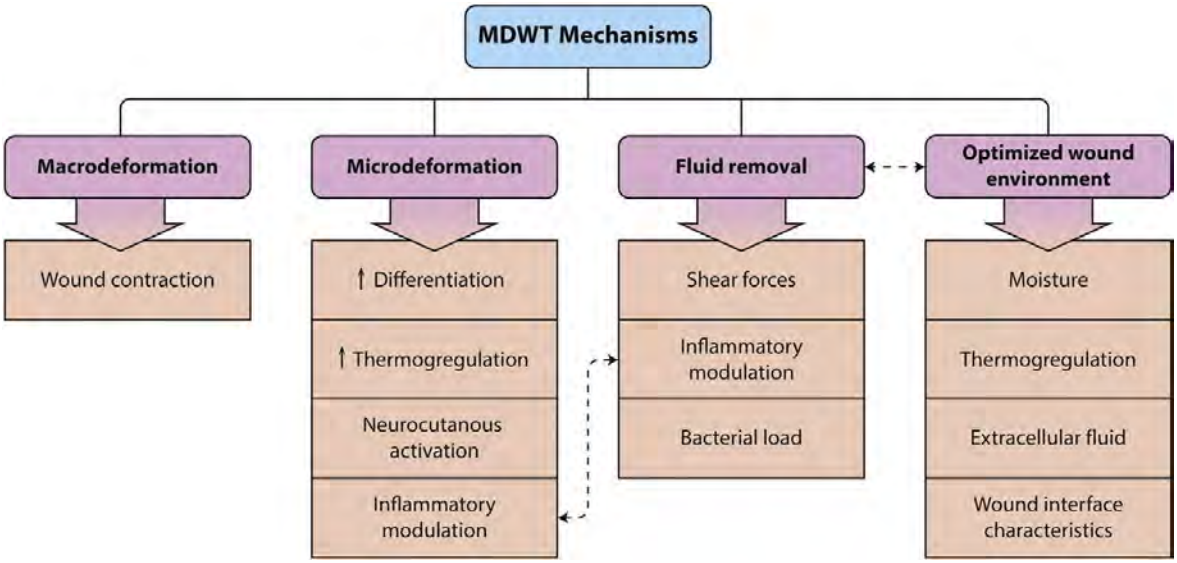


Figure 1-8. Mechanisms of micro-deformational wound therapy (MDWT).

would be impractical to describe all the studies and their outcome in detail; therefore, we will only tabulate in Table 1-5, the wound environment and the results, and then describe the effect due to TNP variables very briefly.

Environment	Treatment	Conclusion	References
In-vitro studies			
Pseudomonas aeruginosa biofilm – in vitro	TNP	Decreased bacterial count, decreased biofilm thickness and diffusion distance.	[532, 533]
Biofilm---in-vitro	silver impregnation of PU foam (V.A.C. GranuFoam Silver® Dressing)	Significant reduction of bacterial count with GranuFoam as compared to PU foam	[532,534]
P.aeruginosa biofilm --- in-vitro study	TNP with periodic instillation of antimicrobial solutions	Bacterial load significantly reduces with instillation. More reduction observed near the VAC port compared to instillation port	[533]
Biofilm in chronic wounds --- in-vitro study	TNP with silver dressing	Reduction in bacterial count in staphylococcal biofilms, *S. aureus*, *S. epidermidis,* MRSA clinical strain and MRSA laboratory strain – reduction is owed more to silver dressing, while role of TNP was less significant	[534]
Animal studies			
Skin graft and dermal scaffold recipient site preparation	TNP without Instillation	TNP assisted in immobilisation and contact between the scaffold and wound surface	[535]
Soft tissue blast injuries	TNP in porcine model	Decreased bacterial load, blocked infection, induced tissue necrosis, earlier	[536]

		initiation of granulation tissue formation	
Porcine excisional wound -	TNP (both continuous and non-continuous) and with instillation	No significant difference in effect due to continuous and non-continuous. A slight improvement in non-continuous TNP. The rate of healing was much faster with instillation	[537]
Human Studies			
Ulcers derived from pressure necrosis, diabetes and venous or arterial pathologies	TNP without Instillation	Reduced wound surface area, volume and depth, improved granulation and reduced frequency of hospitalisation. The improvement rate was disease specific	[528,538-540]
Surgical Wounds	TNP without Instillation	Closure of wound faster, reduced scar height and decrease in infection	[541]
Burns – Clinical	TNP with antimicrobial silver coated foam and/or silver dressing	Number of bacteria is substantially reduced.	[542]
Venous stasis wounds	TNP with intermittent Sodium Hypochlorite instillation	Helped maximise wound bed preparation	[514]
Open wound – clinical study	TNP with continuous	Improved rate of healing	[543]

	Insulin instillation		
Exposed bones and/or joints	TNP	Up to 80% reduction in deep tissue infection, improved granulation of wound bed	[544]
Deep Sternal Wounds	TNP	Reduction of early infection risk and late chronical sternal infection	[545]
Augmented surgical drain in deep neck abscess	TNP	Improved modality for drainage of purulent material	[546]
Deep intra-abdominal wound	TNP	Accelerated abdominal closure, prevention of abdominal compartment syndrome	[547]
Open wound before surgical closure - Clinical	TNP	Reduction in wound surface area. No quantitative reduction on bacterial load	[528]
ischaemic wounds	low-pressure TNP	Successful healing of wound without necrosis at the wound edge.	[548]
multiple significant comorbidities and chronic lower extremity ulcers	TNP	There was a correlation between start time of TNP and healing time. Early TNP showed faster healing	[549]
Pressure Ulcer, Diabetic foot ulcer. Leg ulcer, lower limb, Lower limb ischemia	TNP	TNP effectiveness is shown for grade 3 and 4 pressure ulcers only	[511]
Lymph vessels in wounds	TNP	TNP induced morphological and quantitative alteration on	[523]

		lymph vessels network.	
multiple significant comorbidities and chronic lower extremity ulcers	TNP	Improved healing correlated with early intervention	[549]

Table 1-5. Effect of TNP Therapy in different wound environments

1.17.1.6 Effect due to TNP variable

Effect of varying TNP variables such as negative pressure, type of filler, interface material etc. is still not fully complied in the literature. This is because of large variations in specific wound circumstances and the number of TNP variables involved[512,550]. Birke *et al* has listed evidence based effect of different treatment variables on various treatment goals and has developed 14 recommendations based on choice of wound filler, choice of pressure, choice of wound interface, and some for the use of NPWT in infected wounds[551].

In summary, gauze or Poly Vinyl Alcohol (PVA) foam dressings should be used to reduce pain while Polyurethane (PU) foam dressing is recommended for rapid surface granulation. Foam dressings should be used for deep uniform contractible wound and gauze for shallow non-contracting wounds or complex cavities. An interface layer is recommended with PU foam to bolster a skin graft.

Therapeutic pressure range of -50mmHg to -150mmHg is recommended. Lower negative pressure is helpful to reduce pain, avoid high negative pressure with compromised vascularity, and use higher negative pressure to manage wound exudate or wound fluid.

NPWT alone (without instillation) is recommended to be used only as an adjunctive therapy to combat wound infection, while antimicrobial instillation and silver foam can help infection control significantly. Kim *et al* has also conducted a study on 142 patients to compare the results of TNP with instillation and standard TNP [552]. It is concluded that TNP with instillation is much more beneficial in reducing infection in acutely and chronically infected wounds that require hospital admission [550,553].

1.18 The paradox of negative pressure wound therapy

Since the dramatically expanding successful use of NPWT in advanced wound care, only few studies have been published on the verification of whether vacuum suction increases or decreases pressure at site of application in the wound [554]. Kairinos *et al* conducted an in-vitro study and recorded pressure in the underlying substance at 1cm, 2cm and 3cm from the application site of NWPT dressing in a cavity. An increase in the suction pressure generated hyperbaric pressure in the underlying substance instead of creating decreased pressure. The finding also suggested that tissue hyperbaric pressure was directly proportional to the suction pressure[555]. These finding conflicted with previous studies presuming that NPWT lowers underlying tissue pressure [556]. Hence NPWT produces low positive tissue pressure resulting in mild soft tissue compression.

1.19 Therapy using positive pressure

Compression force can also be obtained by applying direct positive pressure. With regards to microbes, positive pressure up to date is used only in food industry for inactivation of pathogenic microorganisms and enzymes in order to achieve higher health safety levels with preservation of nutrition and taste and obtain longer shelf life [557,558]. Various studies are available on the effect of high pressure to treat several microorganisms especially three major pathogens, namely *Salmonella typhimurium*, *E. coli*, and *Listeria monocytogenes*. However, very limited scientific studies report the effect of positive pressure on bacterial biofilm killing up to date [559-561]. Dommerich *et al* reported improved killing of planktonic and biofilm bacteria in human ossicles colonized by cholesteatoma cells when high hydrostatic pressure was applied with antibiotics, although complete sterilization of ossicles was not achieved [559].

1.19.1 Positive pressure techniques

The most common developed technique is to subject the food under high hydrostatic pressure (HHP). Current food industries using this technique includes all meat products, sea food, fruit juices and jams, fruit beverages, salad dressing, cakes and many ready-to-eat foods [562]. HHP systems generally use pressure in the range from 100 to 800 MPa (1 Atmosphere = 0.1 MPa).

HHP combined with higher temperature can increase stress on microbe that are resistant to pressure alone, for example bacterial spores[471]. Basic components of HHP system are a pressure vessel, fluid (usually water) used to transmit pressure and a pump that generates pressure. Commercially available pressure vessels have the capacities from 35L to 350L. Pressure vessel is filled with fluid and the food package. Food is packaged in a material that does not resist pressure, e.g. polyphone. Use of fluid as pressure transmitter medium ensures pressure is applied uniformly to the food package and does not affect the shape of the food. If the food is fluid itself (e.g. Juice), the whole vessel is filled with fluid food. Pressure is then generated by the pump and the pressurised vessel is left for desired amount of time. The pressure is then released and food taken out.

1.19.2 Mechanism of action

The most prominent action of high pressure is the physical damage of cell membrane, for example bud scars (crater-like rings of chitinous scar tissue located on the parent cell surface) on cell surface of *L. monocytogenes* were observed [563]. It also affects intracellular structure, for example Park *et al* reported empty cavities between cytoplasmic membrane and cell wall of *L. viridescence* [564]. Apart from structure changes, high pressure also inhibits or retards the biochemical reactions which are responsible for an increase in bacterial volume [565]. Enzyme-mediated steps involved in DNA replication and transcription are disturbed by high pressure, e.g. it has been reported that pressure causes a condensation of nuclear material in *L. monocytogenes* and *Salmonella typhimurium* [566].

1.19.3 Effect of high pressure on microbial inactivation

High pressure inactivates many types of micro-organisms however, the sensitivity varies with various microorganisms [565]. Microorganisms are inactivated either by killing or injuring. Generally, vegetative strains such as *E. coli, S. aureus, L. Monocytogenes* and *S. typhimurium* are inactivated at low pressure ranging from 250MPa to 500MPa, but spores strains such as *Clostridium sporogenes, Bacillus amyloloquefaciences* and *Bacillus coaglans* are inactivated at higher pressures of 600-1000MPa [567]. As far as cellular process is concerned, cell motility is inhibited from a low pressure of 10MPa while the DNA structure is affected at 1000MPa [568].

1.20 Hypothesis with aims and objectives of the study

Published scientific studies on the management strategies of bacterial biofilm in healthcare settings indicated difficulties in biofilm eradication for ultimate infection prevention and control. Eradication of bacterial biofilm from delayed or non-healing chronic wounds is still a challenge for infection control scientists and clinicians hence offering gaps in management of such chronic wounds which may lead to amputations.

Recent literature on biofilms has also shown that viable but non-culturable bacteria can survive longer inside semi-hydrated biofilm. However, transmission mode of DSB bacteria has not yet been explored nor enough research work is available to assess antimicrobial resistance of semi-hydrated biofilm when compared to their planktonic counterpart or hydrated biofilm.

This thesis bridges some important research gaps and raises serious questions about the capability of infection control and prevention measures with regards to biofilm transmission and eradication. The ultimate goal is improving patient health and safety with reduced morbidity and mortality and its associated costs.

1.20.1 Study hypothesis

We hypothesise that

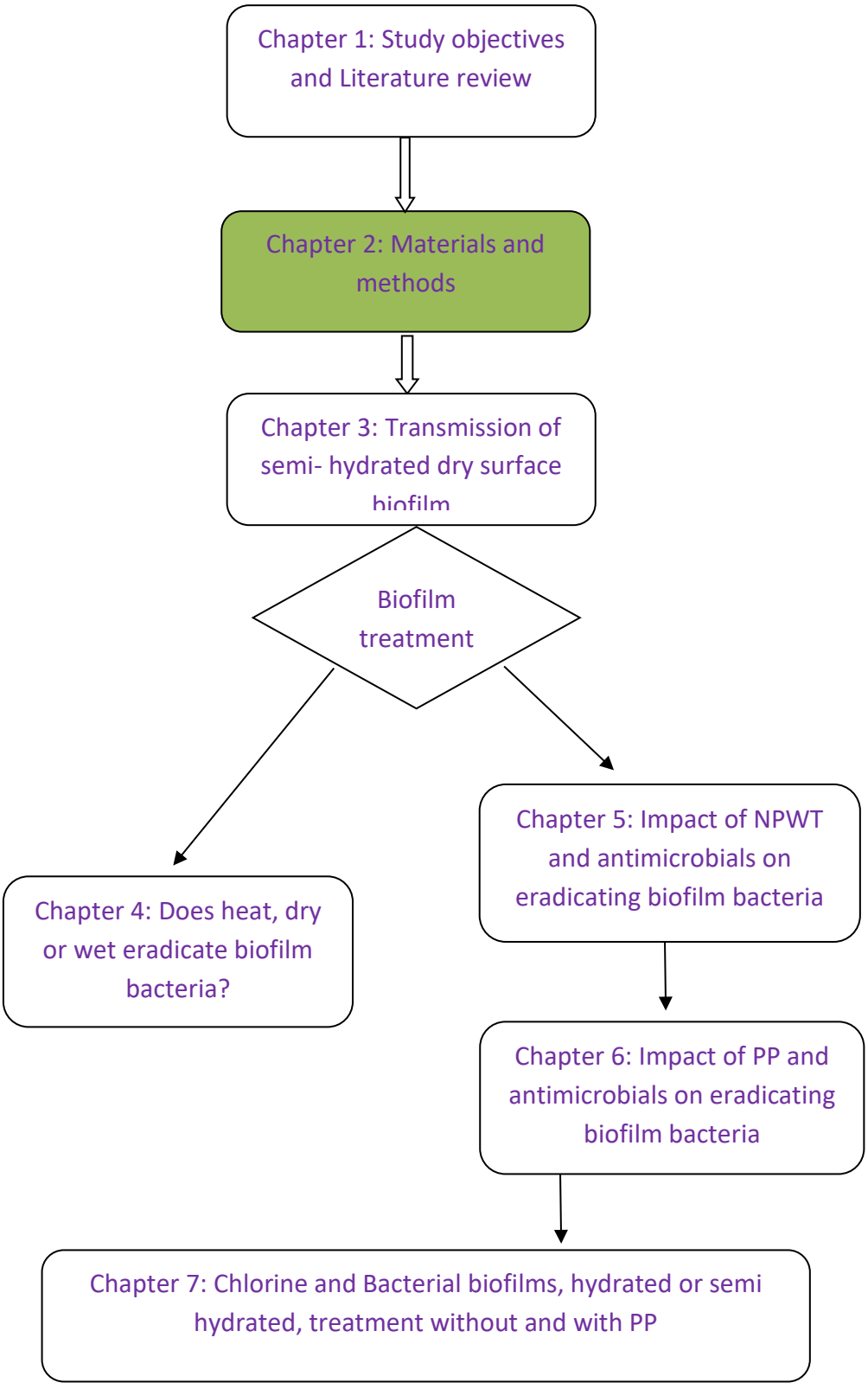
1) Bacteria from dry surface biofilm can transmit via bare and gloved hands as a potential source of pathogen contamination.
2) Semi-hydrated environmental biofilm bacteria display persistence to current decontamination measures in healthcare settings.
3) Antimicrobials killing of biofilm can be improved by physically disrupting thick biofilm matrix using mild compression pressure (negative or positive).

1.20.2 Study aims and objectives

1) We aim for the first time at studying the rate of bacterial transmission from the in-vitro grown dry surface biofilm mimicking “environmentome” previously developed by Almatroudi *et al*[300] via bare and gloved hands.

2) We also aim at determining the killing efficacy of commonly used disinfectants and sterilisation protocols against dry-surface and traditional hydrated biofilms. The study investigates the efficacy of heat and chlorine for comparing killing of planktonic, hydrated and semi-hydrated biofilm bacteria.
3) Finally, we aim at investigating the killing efficacy of combining a physical stress (topical negative pressure and positive pressure ≤10 atmospheres) with a chemical stress (disinfectants/antiseptics) on dry and hydrated biofilm. Hence synergism between compression pressure and commonly used topical antimicrobials or disinfectants for biofilm eradication was studied in a series of well-designed experiments using in-vitro grown *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms.

1.21 Thesis outline



Chapter 2

Part one: Materials and methods

Numerous experimental materials and methods were used during the development of the upcoming studies. Regarding the restricted use chemicals, hazardous chemical risk assessment sheets were completed before use. Protocols were modified or developed for growing in-vitro hydrated biofilms of two selected bacteria, Gram positive *S. aureus* and Gram negative *P. aeruginosa*, in Centre for Disease Control (CDC) biofilm reactor under shear [569], in 96-well microtiters plates [570,571] and on pigs' breast implants for various experimental testing.

Efficacy of treatments was then tested by counting colony forming units (CFU) on horse blood agar (HBA) plates [572] and microscopy. To test the killing of biofilm bacteria by topical negative pressure, biofilm grown coupons were embedded in the modified new chronic wound in-vitro model adapted from the model previously developed by Ngo *et al* [532]. For positive pressure testing to kill biofilm, a pressure chamber was designed and constructed by Dr Inglis in the Engineering Department of Macquarie University.

2.1 Growing hydrated biofilms

S. aureus ATCC 25923 was used for all experiments. *P. aeruginosa* ATCC no 25619 was used to test efficacy of NPWT combined with and without antimicrobials/biocides. All cultures were conducted aerobically. Reagents and chemicals used in the laboratories are explained in the Appendix.

2.1.1 Growing bacteria on nutrient plates

2.1.1.1 Recovery method of bacterial cells

One vial of *S. aureus* stored at -80^{o} C was taken from the laboratory storage freezer [573], thawed by vigorously rubbing between two hands. A single loopful of bacteria was streaked onto horse blood agar (HBA) plate and incubated overnight at 37^{0}C to generate working bacterial cultures in 24hrs. The vial was re-frozen as quickly as possible [574].

Working bacterial culture plates were sealed with paraffin and stored at 4^{o}C for a maximum of 2 weeks.

2.1.1.2 Bacterial culture preparation

A single colony of *S. aureus* ATCC 25923 was aseptically removed from the working agar culture plate and inoculated into 100ml of sterile 100% Tryptone soya broth (TSB) in a sterile 250ml glass bottle and incubated at 37^{0}C, with rotation at 120rpm for 16 hrs. The culture was diluted using sterile 100%TSB to an optical density (OD) of 0.3 which was equivalent to approximately 10^{8}CFU/ml. The exact number of bacteria was determined by serial 10-fold dilution in sterile PBS until dilution 10^{-6}. A 100 µl aliquot of each 10^{-4}, 10^{-5} and 10^{-6} dilution were inoculated onto HBA plates using glass spreader and incubated overnight at 37^{o}C [572]. HBA plates with 30-300 colonies were counted [575] and the bacterial concentration (CFU/ml) calculated by multiplying the number of colonies by the dilution factor[576-578],[579].

2.1.2 Growing *Staphylococcus aureus* hydrated biofilm in the Centres for Disease Control bioreactor

Bacterial biofilm was grown in the Centres for Disease Control (CDC) biofilm reactor (CBR) under shear by batch phase only and by using both batch and flow phases. The method was adapted from ASTM standard method E2562-12 for growing bacterial biofilm in CBR [580]. Desirable bacterial biofilm cells count of around 10^{7}cfu/ml were achieved following 3 days of culture following either of the protocols.

2.1.2.1 Day 1 setup or batch phase biofilm growth

CDC biofilm reactor (CBR) with all its components including magnetic stir rod, 8 rods incorporating 24 polycarbonate coupons were cleaned and installed. Silicon tubing was attached and clamped to waste outlet (Figure 2-1) and the entire assembly was covered with aluminium foil and autoclaved at 121^{0}C, for 20 minutes. Once cooled to room temperature 500ml of sterile 20% Tryptone soya broth (TSB) (3gms TSB/500ml distil water) was added. The magnetic stirrer was adjusted to baffle rotation speed of 130 rpm at 35^{o}C. The CBR was inoculated with 1ml (~10^{8} cfu) of optically adjusted bacterial culture through the inoculation port and re-covered with sterile foil. A 2nm syringe filter

was placed on the inlet port and the outlet tube from an aquarium air filter pump attached which supplied 3L/minute of filtered air into the CBR. To ensure adequate bacterial attachment and uniform biofilm growth the CBR was incubated for 24 hours.

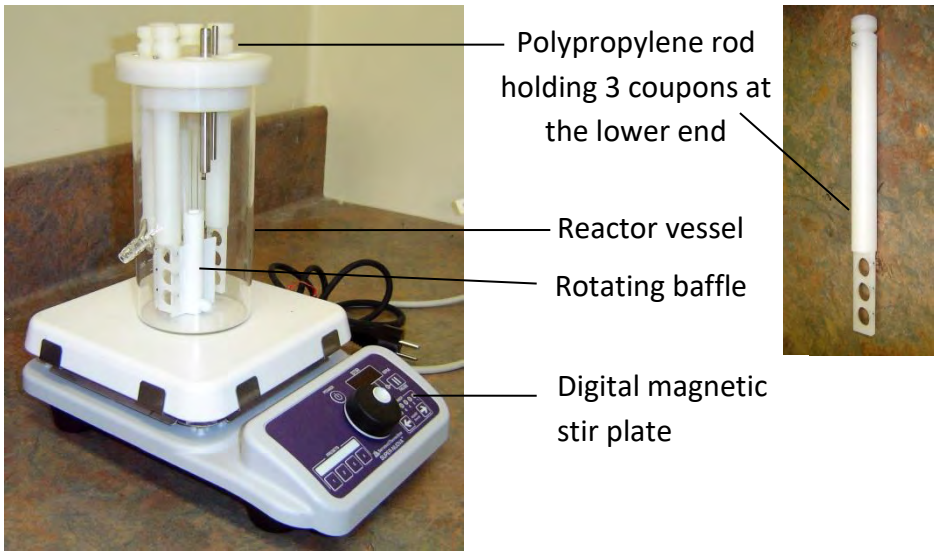


Figure 2-1. CDC biofilm reactor (CBR)

2.1.2.2 Day 2 Flow phase biofilm growth

The peristaltic pump used for nutrient flow was set at 80ml/hrs. Equipment for flow-setup such as glass bottle with 2 L of 20% TSB and silicon tubings previously sterilized were installed between the glass bottle, pump, CDC bioreactor and waste tank as shown in Figure 2-2. The flow phase was run for exactly 24hrs under shear at 35^{o}C.

2.1.2.3 Day 3 Flow phase biofilm growth

Exactly after 24 hrs, the empty nutrient bottle was replaced with another glass bottle containing 2L of fresh sterile 20% TSB under aseptic conditions. Flow phase was run for another 24hrs at the same rate of 80ml/hr.

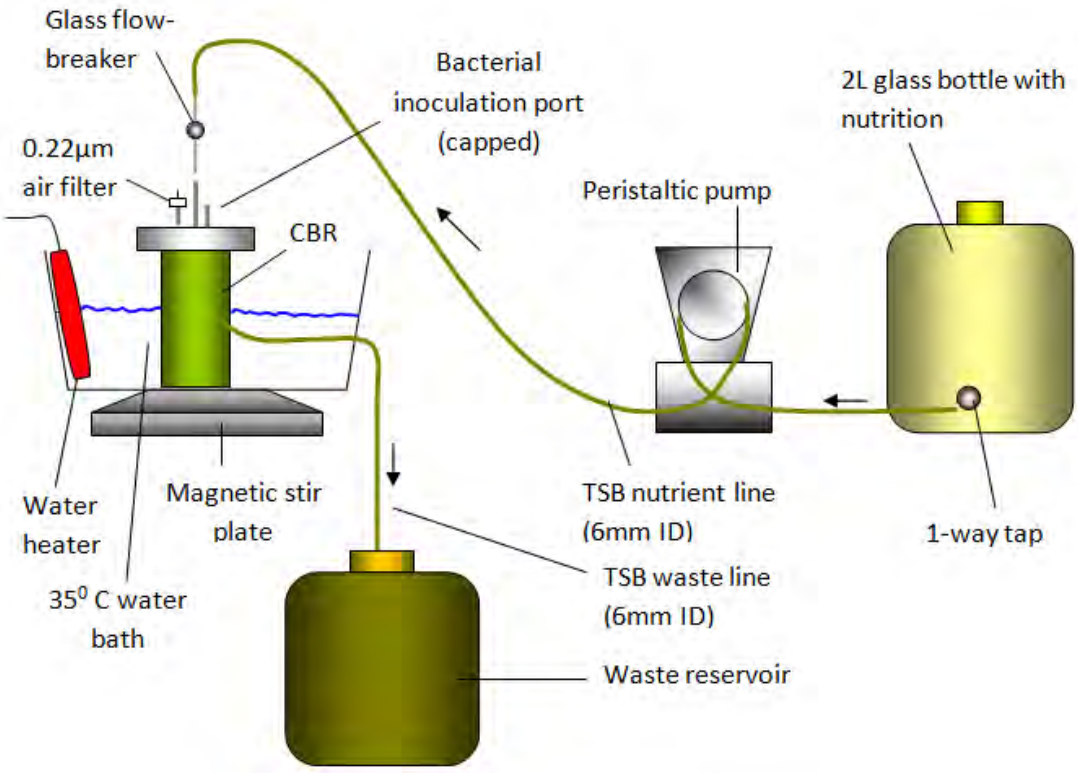


Figure 2-2. Schematic representation of the flow set-up Adapted from Ngo *et al* [581].

2.1.2.4 Growing 6-day hydrated *Staphylococcus aureus* biofilm in CDC bioreactor by batch phase only

Mature *S. aureus* biofilm was grown in CBR by batch phase only, for testing the efficacy of various heat and sodium hypochlorite treatments. The 6-day mature biofilm, was produced using an initial 48hr of batch phase using 50% TSB (15g/L), followed by two 48 hr batch phases each using fresh sterile 20% TSB (6g/L).

2.1.2.5 Harvesting coupons

CDC biofilm reactor was turned off, and the nutrient feed was detached in case of flow set up. All waste was drained into waste container and remaining tubing was detached. Rods were aseptically removed from CBR and placed in a sterile beaker containing 500ml of sterile PBS to remove loosely attached and planktonic bacteria. The 3 coupons from each rod was harvested using aseptic technique by unscrewing the coupons and allowing them to fall into a petri-dish containing 45-50mls of PBS (second rinse). The coupons were rinsed another two times with 45-50mls of PBS before each coupon was placed separately

into 5mls sterile polypropylene containers (TechnoPlas, Pacific laboratory products, Macquarie Park, NSW, Australia) with 4ml of sterile PBS.

The biofilm covered polycarbonate coupons were subjected to test or control treatments and residual biofilm harvested. Coupons were sonicated in an ultrasonic bath (Soniclean, JMR Australia) with an average frequency of 43 kHz for 5 minutes followed by 2 minutes of vigorous shaking MHz followed by 2mins of vigorous shaking. The number of residual culturable bacteria was determined by serial dilutions and standard plate culture as described above in section 2.1.1.2.

Cleaning the CBR

Rods, the CBR and tubings were soaked into detergent (ZEN 1% by Whiteley Corporation, North Sydney, Australia) for at least 30mins before cleaning manually and rinsed with distilled water and set ready for autoclaving for 20min at 123^{o}C. The equipment was placed in dryer

2.1.3 Growing *Pseudomonas aeruginosa* 3-day hydrated biofilm in CDC bioreactor

P. aeruginosa ATCC no 25619 3-day hydrated biofilm (ASTM standard method E2562-12) was grown in-vitro on 24 polycarbonate coupons in CDC bioreactor per the above methods as in sections 2.1.1 and 2.1.2 with following modifications:

a) For the initial 24hrs batch phase, sterile 2% TSB (600 mg of TSB in 500ml of distilled water) was added to the CBR.
b) For the next 48 hr flow phase, sterile 2% TSB was used by adding 2.4 grams of TSB in 2L of distil water.

2.2 Growing bacterial biofilm on 96-well micro-titre plates

Bacteria were grown in 96-well micro-titre plates to evaluate susceptibility of microorganism to antimicrobials by determining the MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration)[582] . According to Jennifer M Andrews in 2001, *"Minimum inhibitory concentrations (MICs) are defined as the lowest concentration of antimicrobial that will inhibit the visible growth of a micro-organism*

after overnight incubation and minimum bactericidal concentrations (MBCs) the lowest concentration of antimicrobial that will prevent the growth of an organism after sub-culture on to antibiotic-free media" [583].

Biofilm were grown on the walls of microtiter plates to determine the MBIC (minimum biofilm inhibitory concentration) and MBEC (minimum biofilm eradication concentration) for test biocides or antimicrobials[571,584-586]. MBIC is defined as the minimum concentration of antimicrobial that will inhibit the visible growth of biofilm microorganism after overnight incubation while MBEC is defined as the minimum concentration of antimicrobial that will prevent the growth of microbial biofilm after sub-culture on to antibiotic-free media.

2.2.1 MIC/MBC of antimicrobials/biocides against planktonic bacterial cultures

An overnight grown bacterial culture as described in section 2.1.1 was adjusted to optical density (OD) 0.300 by photo spectrometer (VWR® international LLC. Spectrophotometers, V-1200) to give approximately 10^8cfu/ml. The culture was then diluted 1 in 10, using sterile 100% TSB (30gm TSB/1L of distil water), to give 10^7 cfu/ml. A 100 µl aliquot of culture was dispensed into each well (except column 2) which gave approximately 10^6 cfu/ well in the manner shown in Figure 2-3.

Column 1 received 100 µl of water in addition to the bacteria as the positive control while sterile media (200µl) was added to column 2 as a negative control. Starting from column 3 of the micro-titre plate, 100 µl of diluted antimicrobial/biocide were added to each well with each column receiving a different concentration of biocide. The 24hr MIC was determined by incubating the plate for 24 hrs at 37^{o}C whereas the MBC was determined by incubating the bacterial cells in test biocide for the specified time and transferring, 20µl into fresh 96-wells plates containing 180µl of sterile 100% TSB incubating for another 24 hours at 37^0 C.

MIC and MBC was the column of lowest antimicrobial/biocide concentration where all the wells remained clear in each plate.

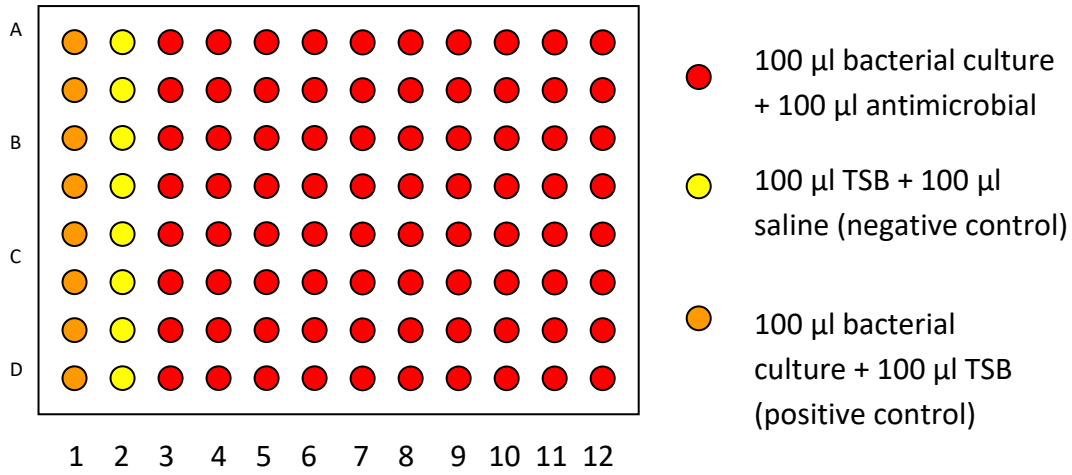


Figure 2-3. 96-well micro titre plate for MIC testing…Adapted from Quan D. Ngo Thesis [581]

2.2.2 MBIC/MBEC of antimicrobials/biocides against biofilm bacterial cultures

The method was developed and documented by Strepanovic *et al* [571,587] previously described by Christensen *et al* in 1985. Christensen *et al* measuring the optical densities of stained of coagulase negative staphylococcus films adherent to the base and side walls of the plastic tissue culture plates [586]. Ceri *et al* described developing biofilms on pegs falling into each well (Calgary device) and counting the residual bacteria after each treatment[587-589].

The bacterial culture 10^7 cfu/ml as prepared above (2.2.1) was diluted 1 in 10, using sterile 100% TSB (30gm TSB/1L of distil water), to give 10^6 CFU/ml. The diluted culture (10^6 CFU/ml) was then used to inoculate 96 well flat bottomed micro-titre plate excluding column 2 as shown in Figure 2-4 while 100 µl of sterile 100% TSB into each well of column 2 for negative control. The micro-titre plate was incubated for 24 hrs at 37^0 C on an orbital shaker at 120 rpm to grow biofilm in inner walls of the wells (covered plate with lid, placed into unsealed plastic bag with a wet paper towel in incubator to minimize evaporation from wells).

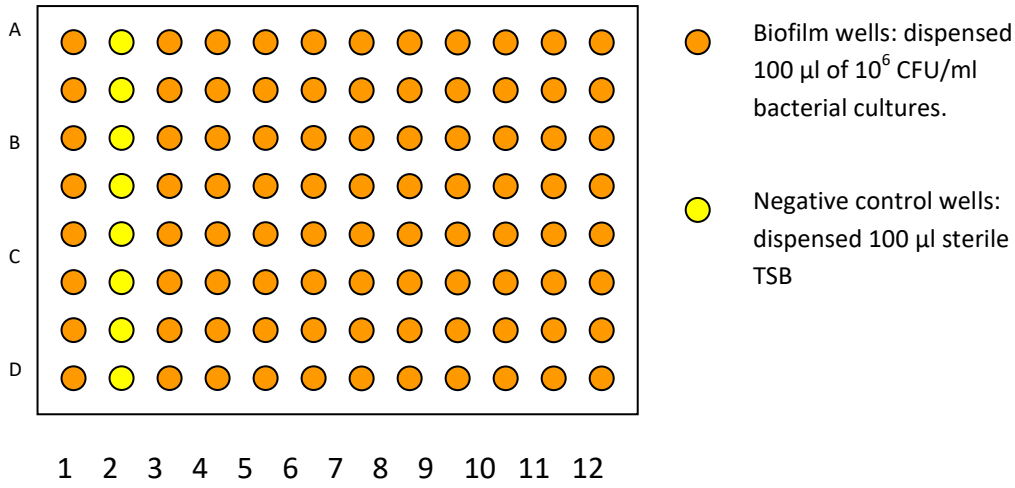


Figure 2-4. 96-wells plate showing biofilm growth for MBEC testing [581].

After 24 hrs incubation, each well should contain approximately 10^7CFU per well of attached biofilm bacteria and the wells were aspirated dry and washed very gently 3 times with 100, 125, 125 µl of PBS to remove loosely attached bacteria. Then 100 µl TSB was added to each well.

In column 1 and 2, 100 µl of water was also added while 100 µl diluted test antimicrobials/biocides were added to wells 3-12 as shown in Figure 2-5 (one antimicrobial/biocide agent per plate). Plates were then incubated at 37^0 C for 24 hours and read. MBIC was the lowest concentration for which all wells in the column remained clear and had no growth.

Following MBIC reading, the wells in the MBIC plates that remained clear were scrapped and mixed using a pipette tip and 20µl aliquots were transferred to fresh plates containing 180µl of TSB. Plates were then incubated at 37^0 C for 24 hours and read for MBEC that was the lowest concentration for which all wells in the column remained clear and had no growth.

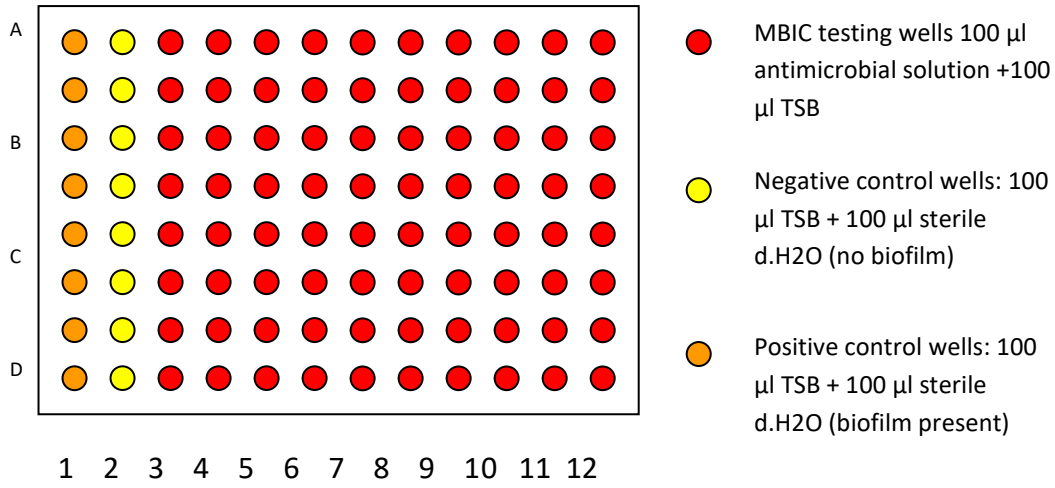


Figure 2-5. 96-wells plate showing volume dilutions for MBEC testing [581].

2.3 Growing bacterial biofilm on smooth and textured breast implants

For the in vivo study, total bacterial numbers in each of the contracted implants and their surrounding capsular tissue was determined by real time quantitative polymerase chain reaction (PCR) irrespective of species identification. The PCR was chosen to compare bacterial counts in contracted smooth and textured implants with surrounding tissue due to their multispecies nature. Multispecies bacterial biofilm with the capsular tissue was visualised using scanning electron microscope.

For the whole implant in-vitro attachment assay, *S. epidermidis* originally obtained from a contracted human breast [590] was grown on mini (Biocell, supplied by Allergan LLC, Irvine, California, USA) smooth and textured breast implants according to the protocol below:

2.3.1 Day 1 preparation of inoculum

S. epidermidis was incubated in 100ml TSB overnight as described in sections 2.1.1.1 and 2.1.1.2.

2.3.2Day 2 and 3 attachment assay

A 0.5 ml from the *S. epidermidis* culture was diluted in 100% TSB to achieve 0.300 OD in the spectrophotometer (gives approximately 10^8cells/ml). Diluted 10ml of 0.300 absorbance culture in 990 ml 10% TSB, (1 in 100 dilution to give 10^6cells/ml). The actual number was determined by serial dilutions and standard plate culture as described in section 2.1.1.2.

Twelve mini textured (Biocell) 2cm diameter and 12 mini smooth 2cm breast implants) were incubated in 20 ml of the above culture containing 10^6cells/ml ensuring the implant was fully covered and incubated at 37^{o}C. The time was recorded in laboratory book.

At each time point of 2, 6 and 24 hrs, four implants were aseptically transferred into a fresh urine pot containing 40mls of PBS. The pot was gently inverted 5 times to remove loosely attached bacteria before draining the PBS. The implant was then transferred to a fresh urine pot containing 40 mls of PBS. The above step was repeated for 2nd wash.

Three implants were transferred into 20mls of PBS in the sterile urine pots, sonicated for 20 minutes and shaken for 1 minute as described previously [591]. Serial 10-fold dilutions were made and 100µl of the neat, 10^{-1} and 10^{-2} (2hrs incubation), neat, 10^{-1}, 10^{-2} and 10^{-3} (6hrs incubation), neat and dilutions 10^{-1} to 10^{-6} (24hrs incubation) were spread on HBA plates. Inoculated HBA plates were incubated at 37^{o}C for 24hrs and the colonies were counted as detailed in section 2.1.1.2. One implant of each type at each time point was retained for CLSM and SEM as detailed in section 2.5 and 2.6.

2.4 Wound model setup

2.4.1 Preparing biofilm covered coupons for insertion into the wound chamber

Three-day traditional hydrated biofilms were grown on 24 polycarbonate coupons using 24hrs of batch and 48hrs of flow through phase as detailed in sections 2.1.2 and 2.1.3.

The rods were removed from the CBR and placed into 2 beakers containing 500ml PBS each.

Sterile paper towel was cut to be placed into 8 sterile petri dishes (one per rod) and then soaked with 12.5% chlorine for 5mins. All the remaining steps were conducted in a Class II Biohazard Cabinet. Approximately 50 ml of sterile PBS was poured into 24 sterile petri dishes (3 for each rod containing 3 coupons). The coupons from each rod were unscrewed using sterile screw driver, and allowed to fall into a petri dish ensuring that the coupon side which was facing inwards in the CBR was facing upwards. Using sterile forceps, the upper surface of each coupon was covered with 100µl of 3% bovine serum albumin (BSA). The BSA acted to protect the biofilm from the action of any chlorine reaching the upper surface. The bottom aspect of the coupon was then gently rubbed across a paper towel soaked in 12.5% chlorine 6 times. The coupon was rinsed twice by immersion in sequential PBS petri dishes to remove residual chlorine and loosely attached bacteria.

Above steps of harvesting were repeated for all 8 rods.

2.4.2 Preparation of wound chambers (Inside the sterile laminar hood)

2.4.2.1 Different wound chambers treatment testing

For each experiment, there were four different wound chambers made as detailed in table below: (Table 2-1)

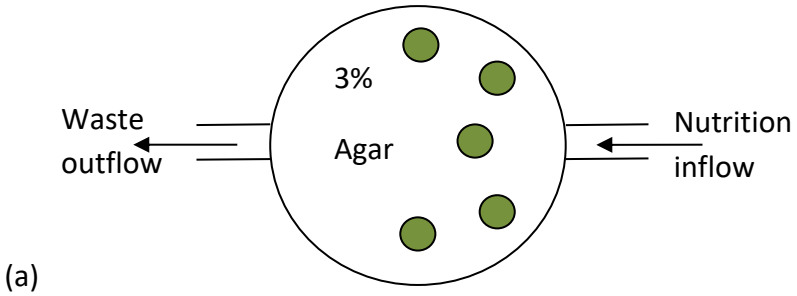
Wound Chambers	Nutrient inflow tube	Waste tube	Suction VAC dressing	Antiseptic reservoir
Control	√	√		
Instil only	√	√		
VAC only	√		√	
VAC Instil	√		√	√

Table 2-1: Set up of four different wound chambers

2.4.2.2 Method of preparation of new wound model (In the sterile laminar hood)

Using aseptic technique approximately 10mls of 3% agar was smoothly poured into each of four petri dishes and the agar allowed setting over 3-5mins. Once the agar was set, 6 biofilm-covered coupons were placed on one side of the petri dish that would be is nearer to the inflow of the nutrients as shown in Figure 2-6 (a). Granulofoam (Acelity and KCI, San Antonio, TX, USA) was cut to the size of petri dish and placed on top of the coupons ensuring that the foam kept the nutrient inflow tube and in control chambers the outflow tube well in place.

Each VeraFlo dressing (Acelity and KCI, San Antonio, TX, USA) was cut in half and used to make an airtight seal around the wound chambers keeping the tubings in place. For models subjected to negative pressure, a cruciate cut was made in the centre of the seal and a VeraFlo dressing applied over the cruciate cut [Figure 2-6 (b)].



(a)

(b)

Figure 2-6. (a) Schematic presentation of in-vitro chronic wound model. (b) New in-vitro chronic wound model. Green circles represent biofilm coated coupons.

2.4.3 Setting up the control, instil only, VAC only and VAC+ instil equipment

Supply of nutrients (all wound models)

Four 2L bottles were filled with 1L of sterile 100% TSB each and connected to the peristaltic pump (2010 Thermo Fisher Scientific Inc.) using sterile silicon tubing (Masterflex, silicon tubing). The nutrient inflow of each wound model was attached to one of the 4 nutrient supply tubing attached to the peristaltic pump. On commencement of the experiment the peristaltic pump was on high to fully soak the dressings and then set to a maintenance flow rate of 40ml/hr.

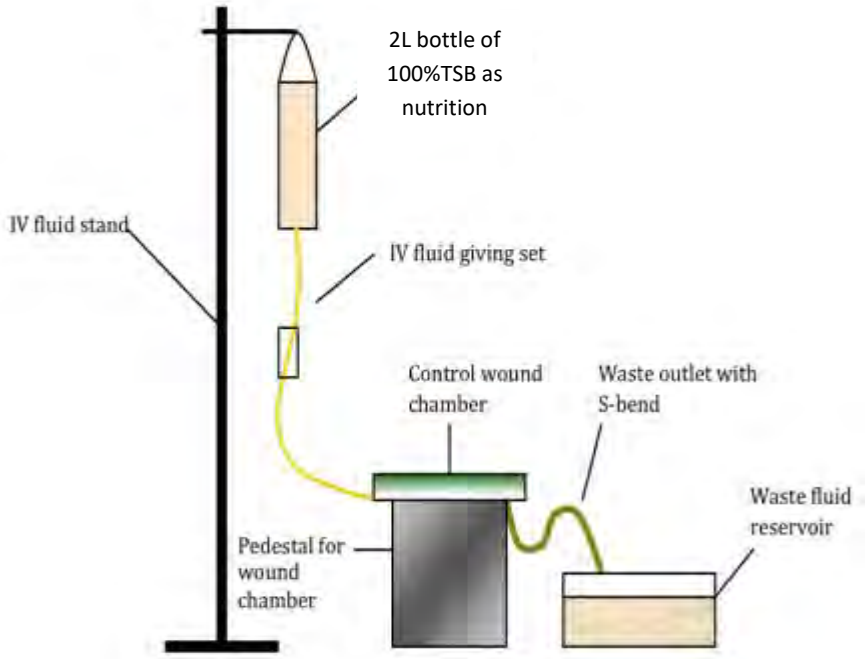
Supply of negative pressure

2 VAC Dressings were connected to the 2 VAC machines (Figures 2-7, 2-8 b and 2-9). One cassette and antiseptic reservoir was attached to one VAC machine with 6 hourly instillation rate(4 instillations in 24hrs) with a calculated dose.

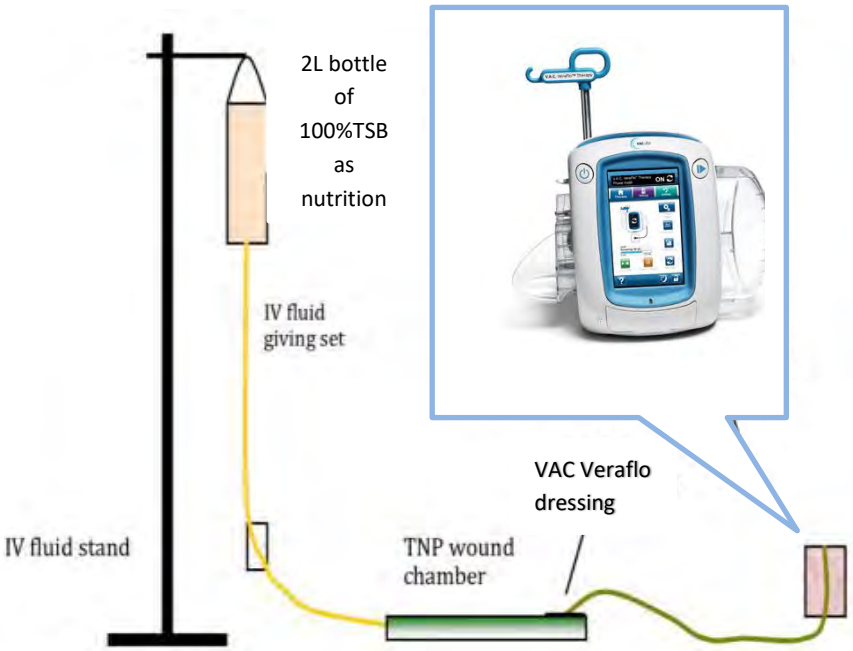
Figure 2-7. VACULTA (VAC) machine with Veraflo dressing

VAC machines as shown in figures (Figures 2-8 b and 2-9) were set to deliver continuous negative pressure of 125mm Hg.The models were observed for 30mins to ensure airtight seals. Antiseptic/antibiotic was instilled in 2 chambers, one through VAC machine instil reservoir and the other by manual instillation after turning the nutrition line off for 20 mins. Negative Pressure was withheld for 20mins and reinstated at the end of this period. Instillation was repeated every 6hrs for 20mins. Each time the models were observed for 30 minutes to ensure that the seals remained airtight. Outflows tubings from the control

and Instil models (Figure 2-8 a) were directed into a 20L container with 2L of 12.5% sodium hypochlorite.



(a)



(b)

Figure 2-8. Experimental methods setup (a) control and antiseptic/antibiotic instils tested without VAC. (b) VAC only and antiseptic/antibiotic instils tested with VAC. [Diagram modified from Ngo *et al* [581]]

Figure 2-9. Set up for chronic wound bacterial biofilm treatment.

2.4.4 Harvesting of control and treated coupons

After 24hrs, the VACULTA machines for VAC application and peristaltic pump supplying the nutrients were turned off, the wound models' inlet and outlet tubings were clamped and the setup disconnected for cleaning and autoclaving. In the Class II Biohazard Cabinet using aseptic technique, each wound chamber was carefully opened by gently removing the dressings and grey foam.

Then each coupon was transferred one by one into separate, appropriately labelled sterile 5ml yellow top flat bottom containers containing 4mls of sterile PBS, shaken for 2mins and sonicated for 5mins. Serial 10-fold dilutions were made and 100µl from each dilution was plated out on HBA plates for counting CFU after 48hrs of incubation at $37^{O}C$ as detailed in section 2.1.1.2.

2.5 Confocal Laser Scanning Microscopy

Confocal Laser Scanning Microscopy (CLSM) is a non-invasive tool utilised to evaluate the 3-dimensional thick biofilm structure by imaging through horizontal and vertical optical sectioning [592,593]. Thin slices' images as thin as 0.2µm were retrieved by adjusting the aperture of objective lens while XYZ 3 dimensional images can be achieved for analysing biofilm architecture, thickness and height by computer controlled adjustments of the

microscope[594-596]. Staining various components of biofilm cells and EPS components can provide useful information [595]. Nucleic acid staining can confirm whether bacteria are still alive and also to determine if viable but non-culturable cells are present[597-601] as shown in Figure 2-10.

2.5.1 Staining protocol for analysing bacterial biofilm cells' viability

Samples were stained according to the manufacturer's instructions, using LIVE/DEAD *BacLight* 7012 kit (Molecular Probes, Invitrogen, Carls- bad, CA, USA) which contains two nucleic acid dyes, green-fluorescent SYTO9 and red-fluorescent propidium iodide [602]. Green-fluorescent SYTO9 stained all bacterial cells whereas red-fluorescent Propidium iodide dye bacterial cells that have damaged membranes (dead cells) [603] .

Staining procedure was performed at room temperature and specimens were kept in dark away from light. Both staining dyes were mixed in sterile Milli-Q water (3µl of equal quantities of STYO9 and propidium iodide for each ml of sterile Milli-Q water) for 15-20 minutes. Samples were then fixed by 3% paraformaldehyde for one hour, washed three times for 10 minutes each with 0.1M PBS. Stained, fixed specimens were then kept covered with PBS in sterile containers (to prevent drying) left at 4^{o}C after final wash.

The stained samples were examined using an Olympus Fluoview 300 inverted confocal laser scanning microscopy system for magnifications 10x, 20x and 40x using water immersion Plan Fluor objectives.

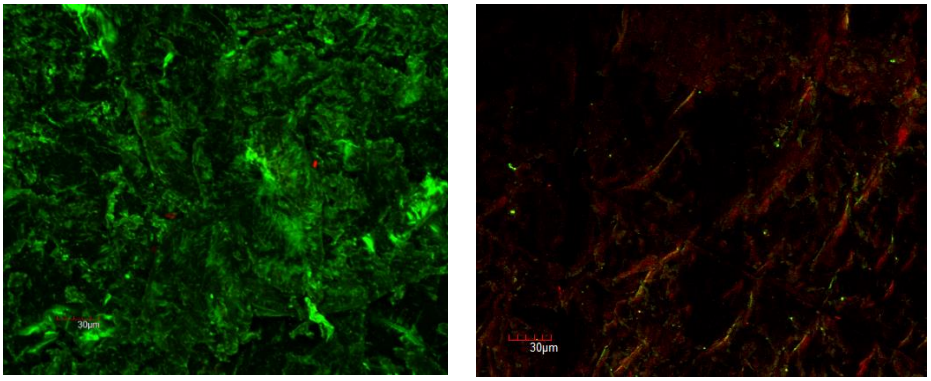


Figure 2-10 . CLSM images of stained *S. aureus* biofilm with LIVE/DEAD® BacLight™ Bacterial Viability Kit. Live bacteria are stained green and dead bacteria are stained red.

2.5.2 Image analysis of stained biofilms

Analysis of biofilm images was carried out using two software programs:

1) ImageJ software developed by Wayne Rasband in 1997 with the National Institute of Health, USA, for image processing and analysis in Java script [604,605]. ImageJ was applied to determine viability percentage of bacterial cells within biofilm.

2) IMARIS 7.7.2 software developed by Bitplane, Zurich, Switzerland was applied to determine thickness and mass of biofilms before and after various treatments. It has been previously used by scientists to study various aspects of bacterial biofilms such as architecture of opportunistic pathogens, effects of Monochloramine disinfection on laboratory grown biofilm or EPS of *P. aeruginosa*[606-608].

2.6 Scanning electron microscopy

Various components of bacterial biofilm have been visualised in many research studies at high resolution and magnification with the use of Scanning electron microscope (SEM) [609-611] as shown in Figure 2-11. It also aided in observation of biofilm contamination of ICU samples [2], wounds [612], soft tissue fillers [613], endoscopes [614], ventilation tubes [615] and other healthcare equipment.

Specimens were prepared for scanning electron microscopy (SEM) according to the protocol used by Vickery *et al*[255]. Bacterial biofilm grown on polycarbonate coupons were fixed overnight in 3% glutaraldehyde solution (Sigma-Aldrich Pty Ltd, Castle Hill, Australia) followed by 3 washes, 10min each, of sterile 0.1 M phosphate buffer.

The specimens were dehydrated by immersion in analytical grade 30%, 50%, 70%, 80%, 90% and 100% ethanol (Sigma-Aldrich Pty Ltd, Castle Hill, Australia) for 10min each. Remaining water was extracted by immersion of the specimen in 100% hexamethyldisilazane (HMDS, Polysciences Inc.Warrington, PA USA) for 10mins 3 times.

After the 3rd immersion the specimen was left to air dry overnight in an open laminar hood to evaporate residual HMDS.

The specimens were mounted on Carbon tabs (ProsciTech, Thuringowa, QLD, Australia) previously glued onto aluminium stubs (ProsciTech, Thuringowa, QLD, Australia). The specimens were finally coated with 20nm gold film in the Emitech K550 gold coater (West Sussex, England) for better resolution and to conserve cellular features and overall structure of bacterial biofilms[616].

Prepared specimens were examined in a JOEL, Ltd., scanning electron microscope (Tokyo, Japan, model 6480LA). Various reagents and chemicals used in the laboratories are explained in the Appendix.

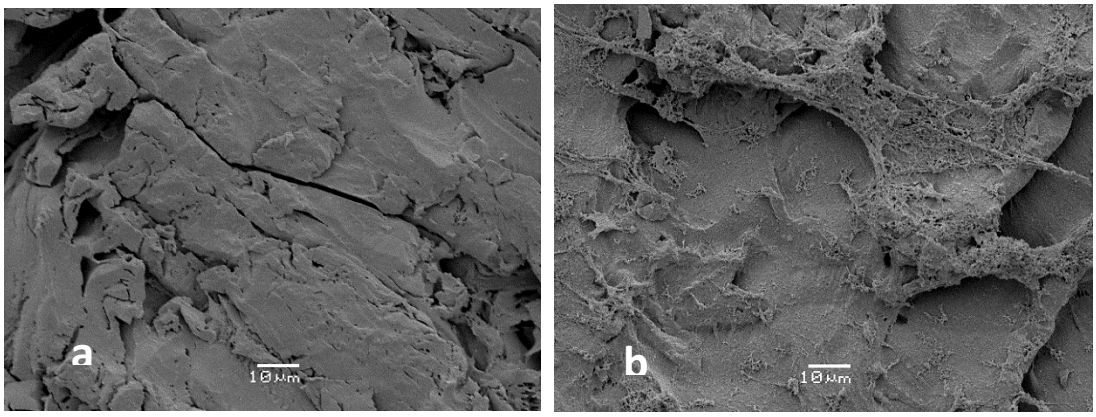


Figure 2-11. Scanning electron microscopy images (magnification 1000x) showing (a) biofilm free, clean polycarbonate coupon with normal corrugations and (b) polycarbonate coupon covered with bacterial biofilm

2.7 Statistical analysis of experimental data

Experimental data obtained after various treatments was analysed statistically using:

1) Microsoft excel 14.4.3 software program.
2) IBM SPSS statistics Grad pack 24. Base statistical program.
3) SigmaPlot 11 Statistical program offered by Systat Software Inc., San Jose, California, USA.

Part Two: Materials and methods

2.8 Original paper

Jacombs A, **Tahir S,** Hu H, Deva AK, Almatroudi A, Wessel L, Bradshaw D, Vickery K**. "*In vitro and in vivo investigation of the influence of implant surface on the formation of bacterial biofilm in mammary implants*". *Plastic and Reconstructive Surgery*. April, 2014*, 133*(4), pp.471e-480e.**

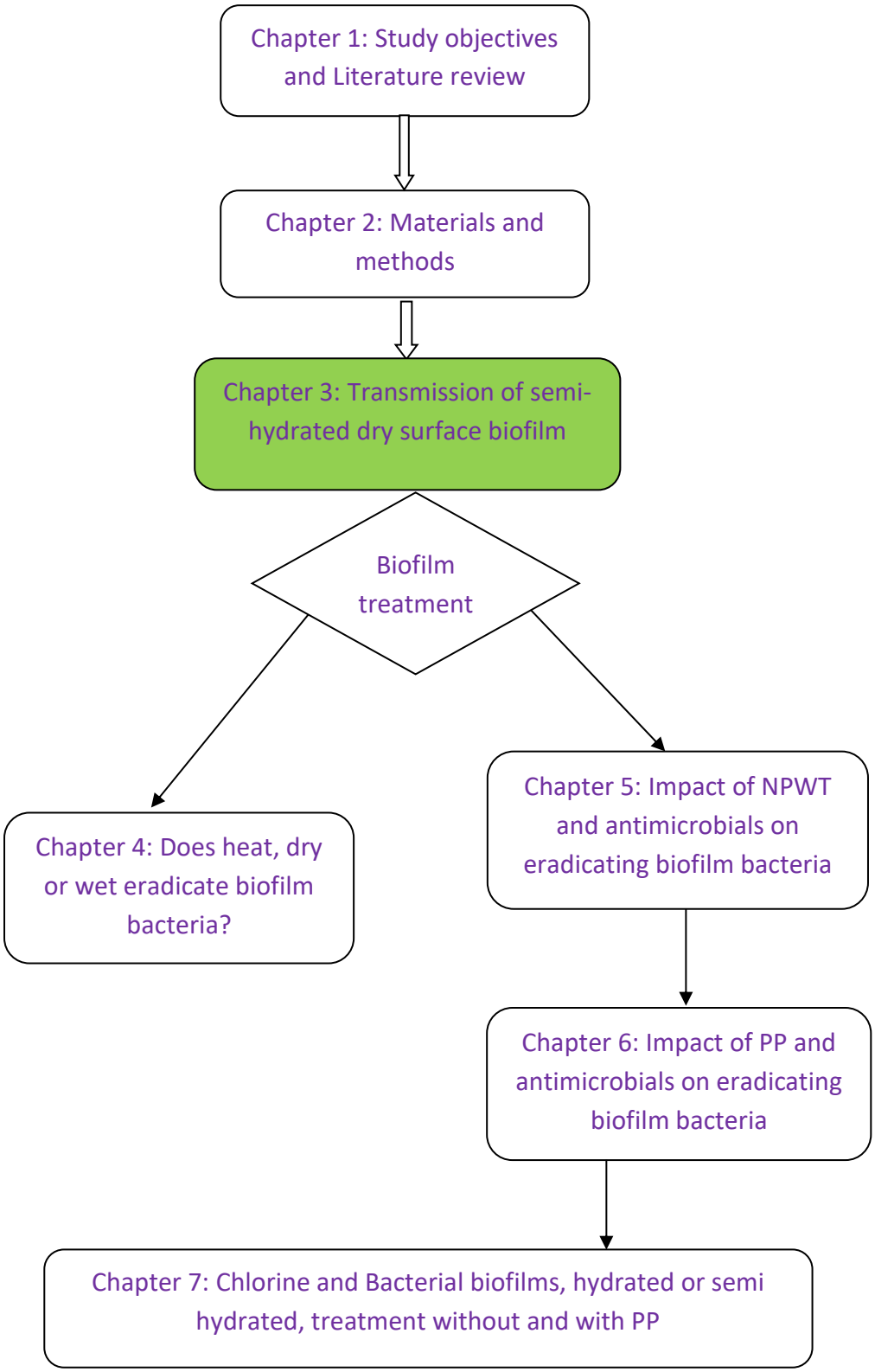
2.9 Author's Contribution

Dr Shamaila Tahir carried out the complete In-vitro experiment and data collection with analysis. Her contribution was 45%.

Pages 115-124 of this thesis have been removed as they contain published material. Please refer to the following citation for details of the article contained in these pages.

APA Jacombs, Anita B.Sc.(Hons.),M.B.B.S.; Tahir, Shamaila M.B.B.S.; Hu, Honghua Ph.D.; Deva, Anand K. F.R.A.C.S.; Almatroudi, Ahmad B.Sc., M.P.H.; Wessels, William Louis Fick M.B.B.S.; Bradshaw, David A. M.B.B.S.; Vickery, Karen B.V.Sc.(Hons.), Ph.D. In Vitro and In Vivo Investigation of the Influence of Implant Surface on the Formation of Bacterial Biofilm in Mammary Implants, Plastic and Reconstructive Surgery: April 2014 - Volume 133 - Issue 4 - p 471e-480e doi: 10.1097/PRS.0000000000000020

Thesis outline



Chapter 3

Transmission of bacteria from biofilms on dry surfaces

3.1 Introduction

Scientifically, biofilms have been reported on numerous dry surfaces in healthcare settings even after terminal cleaning[2,255]. Although semi hydrated, these biofilms retain their persisters, stubborn cells that are viable and culturable once environmental circumstances suit to favour growth. Existence of such biofilms on healthcare dry surfaces is insignificant if viable bacteria inside DSB are non-transmissible in doses that are large enough to spread infections.

On the other hand, practice of proper hand hygiene by HCWs is one of the most cost effective infection control means of breaking the cycle of pathogen transmission in healthcare environment[285-288]. However poor compliance of HCW with hand hygiene is a major barrier in breaking this transmission cycle. An alternative method of personal protection and patient safety is the mandatory use of disposable, single use gloves during invasive procedures as well as routine activities of HCW in patient environment[289,290].

In the light of these scientific facts, numerous queries thence provoked scientific minds:

1) *Whether these viable bacteria are transmitted via hands to surrounding areas or surfaces?*
2) *If transmitted how far the infective dose may be carried to?*
3) *Is the transmission blocked by using single-use gloves?*

In response to the above queries, pioneer experimental protocols were designed to be carried out for studying transmission of viable/culturable bacteria from the laboratory grown *S. aureus* (ATCC 25923) DSB, developed by Almatroudi *et al* [301]. Interesting findings of DSB transmission obtained from these studies are reported for the first time as in up-coming scientific publications.

3.2 Author's contribution

Chowdhary D, Tahir S, Legge M, Hu H, Aljohani K, Prvan T, Deva AK, Vickery K, "Transfer frequency of dry surface biofilm in health-care environment- `the role of health care worker's hand."

In the above co-author publication, Dr. Shamaila Tahir designed the study protocol with Dr. Karen Vickery, performed experiment with Dr. Durdana Chowdhary, collected data and complied results with her. Dr. Shamaila Tahir contributed to manuscript preparation and her overall contribution was 40%.

Tahir S, Chowdhary D, Legge M, Hu H, Whiteley G, Deva AK, Vickery K, "Bacterial transmission via single use gloves from dry surface Biofilm in Health-care Environment:" Ready for submission.

In this co-author publication, Dr. Shamaila Tahir designed the study protocol with Dr. Karen Vickery, performed experiment with Dr. Durdana Chowdhary, collected data and complied results with her. Dr. Shamaila Tahir performed all the data analysis and contributed to manuscript preparation. Her overall contribution was 60%.

3.3 Original papers

“Transfer of dry surface biofilm in health-care environment; Role of health care worker’s hand as a vehicle”

Durdana Chowdhury[a], Shamaila Tahir[a], Mark legge[a], Honghua Hu [a], Khalid Aljohani[a], Tania Prvan[b], Anand Deva [a], Karen Vickery [a]

[a]Surgical Infection Research Group, Faculty of Medicine and Health Sciences, Macquarie University, New South Wales, Australia

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Keywords:

Dry-surface biofilms

Transmission

Disinfectants

Healthcare-associated infections

Staphylococcus aureus

"Transfer of dry surface biofilm in health-care environment; Role of health care worker's hand as a vehicle"

"Transfer of dry surface biofilm in health-care environment; Role of health care worker's hand as a vehicle"

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“Transfer of dry surface biofilm in health-care environment; Role of health care worker’s hand as a vehicle”

“Transfer of dry surface biofilm in health-care environment; Role of health care worker’s hand as a vehicle”

"Bacterial transmission from dry surface Biofilm via Gloves in Health-care Environment:"

Authors:

Tahir, S. MBBS, Grad Dip Inf&Immu[a]

Chowdhury, D. [a]

Legge, M. BAnVetBioSc[a]

Honghua Hu, BSc, Grad DipSc, PhD[a]

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"Bacterial transmission from dry surface Biofilm via Gloves in Health-care Environment:"

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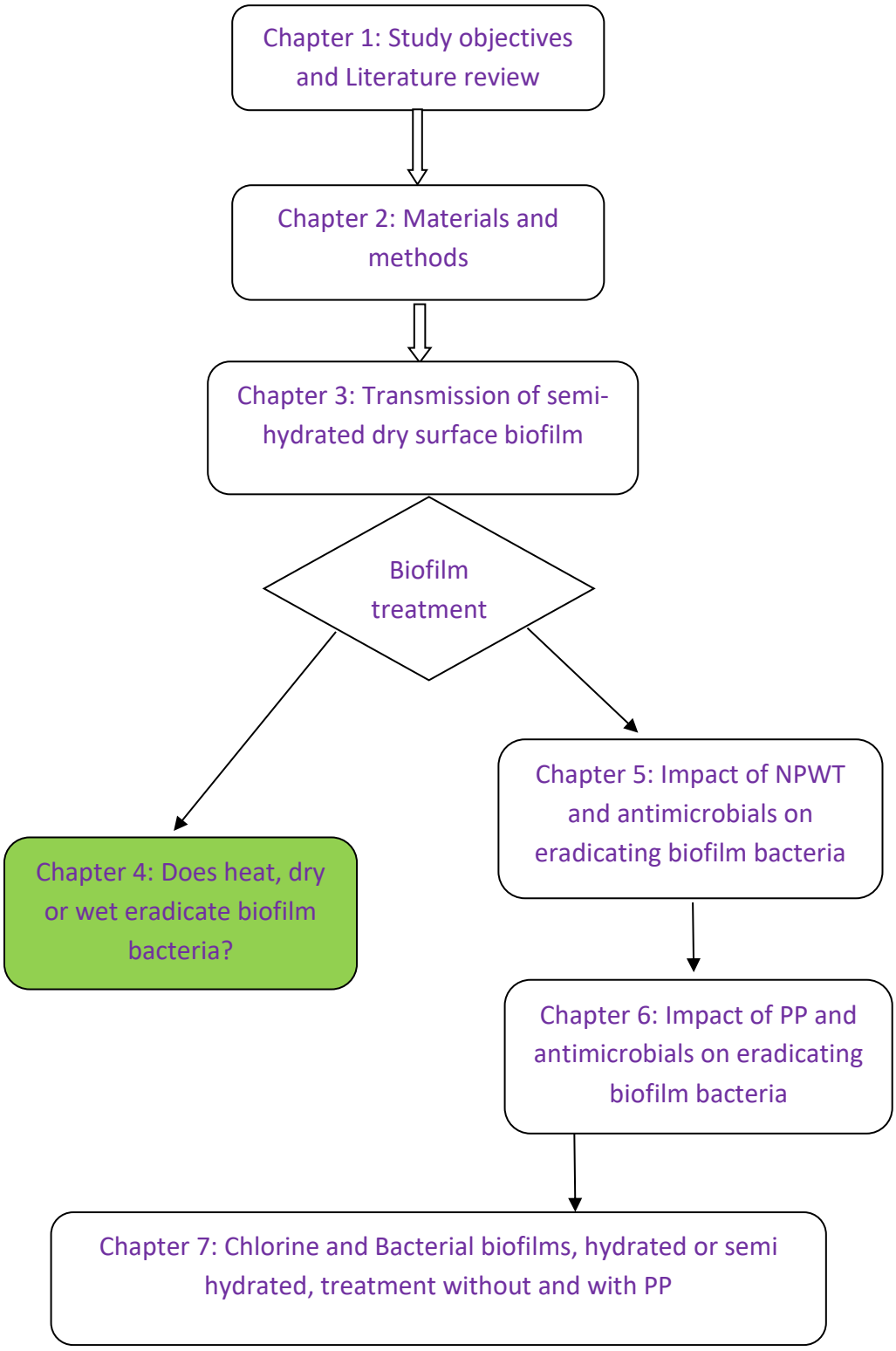
"Bacterial transmission from dry surface Biofilm via Gloves in Health-care Environment:"

3.4 Conclusion:

Both scientific studies conducted to analyse the rate of pathogen transmission from DSB via un-gloved or gloved hands succeeded in demonstrating DSB as the potential source of cross contamination in healthcare environment. This environmental DSB may exist on soft surfaces such as furnishings or hard surfaces such as bed rails. Hence a thorough review of current hospital cleaning and disinfection practicing is highly recommended.

Thesis outline



Chapter 4

Does heat, dry or wet, eradicate bacterial biofilms in healthcare settings?

4.1 Introduction

Once it's scientifically established that pathogens from healthcare biofilm are easily transmitted comes the challenge of its eradication for infection prevention and control. Following scientific studies in the thesis are consequence of various attempts on complete killing of bacterial biofilm in healthcare settings. In this section *S. aureus* hydrated and semi-hydrated biofilm was exposed to high heat (wet or dry) for numerous time periods with and without pressure to analyse killing of biofilm bacteria.

4.2 Author's contribution

Almatroudi A**,** **Tahir S,** Hu H, Chowdhury D, Gosbell IB, Slade J, Whiteley G, Deva AK, Vickery K ***"Staphylococcus aureus dry surface biofilms survive heat treatment."*** **submitted.**

In the above co-author publication, Dr. Shamaila Tahir performed all experimental work on hydrated *S. aureus* biofilm and its microscopic analysis. Her overall contribution was 40%.

4.3 Original Paper

"Staphylococcus aureus dry surface biofilms survive heat treatment"

A. Almatroudi[a,b], S. Tahir[a], H. Hu[a], D. Chowdhury[a], I.B. Gosbell[c,d,e], S.O. Jensen[c,d], G.S. Whiteley[f], A.K. Deva[a], K. Vickery[a,*]

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Running title: Staphylococcus and heat

“*Staphylococcus aureus* dry surface biofilms survive heat treatment”

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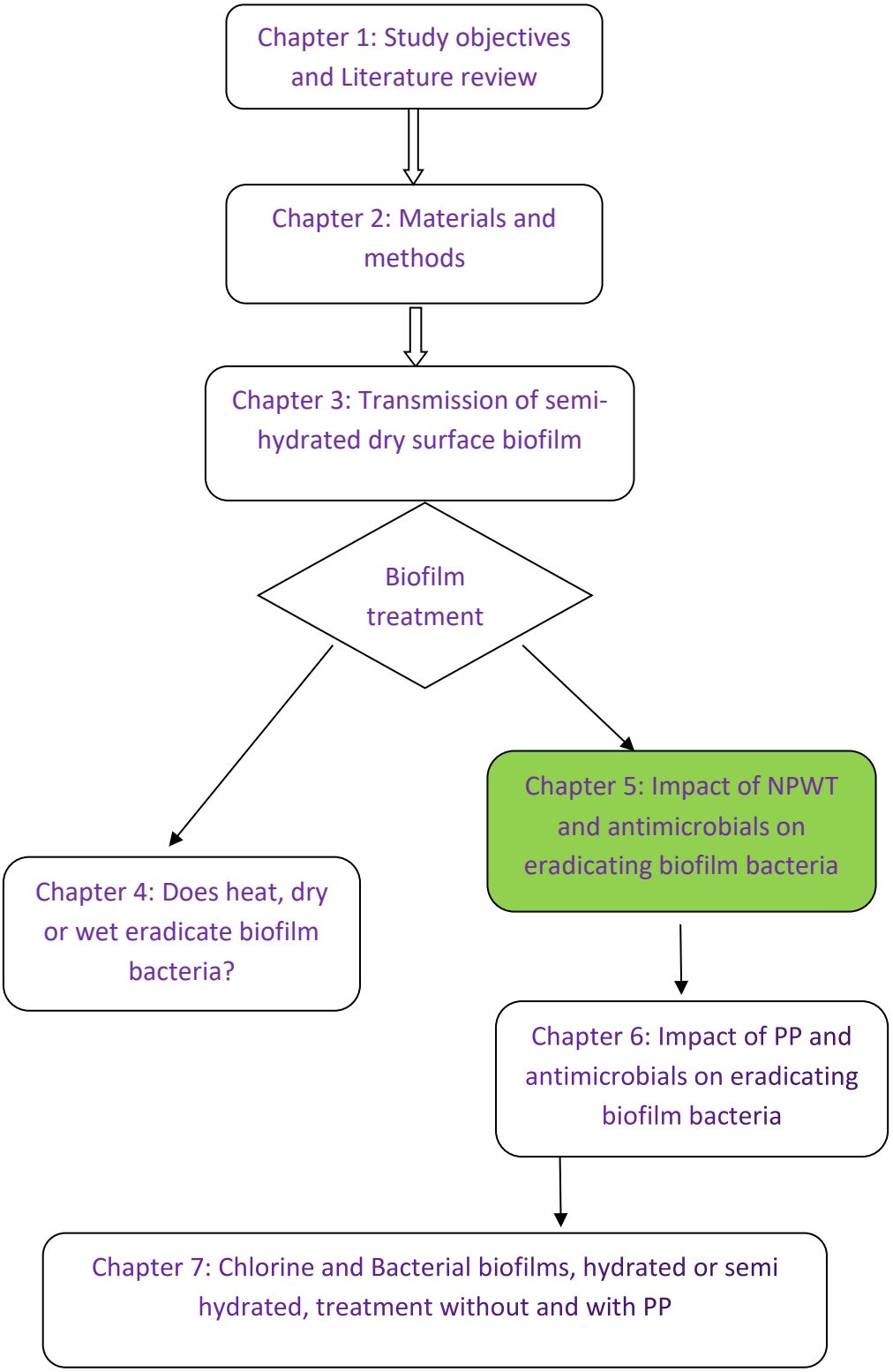
“*Staphylococcus aureus* dry surface biofilms survive heat treatment”

“*Staphylococcus aureus* dry surface biofilms survive heat treatment”

4.4 Conclusion

Above study demonstrates that bacteria in biofilm are comparitively more resistant to high temperatures than their planktonic counterparts. As the water content of biofilm reduces, its inactivation by heat decreases. Hence for healthcare infection prevention and control purposes, cleaning hospital surfaces with steam under pressure should be a consideration.

Thesis outline



Chapter 5

Is combination therapying the way forward to biofilm eradication?

5.1 Introduction

After demonstrating transmission of bacteria from dry or wet biofilms, follows the aim of eradicating bacterial biofilms in healthcare settings. Previously, Ngo *et al* demonstrated reduction in CFU load, biofilm thickness and overall biomass when *P. aeruginosa* biofilm was treated together with silver impregnated foam dressing and NPWT as compared to silver or NPWT treatment alone. Similar synergistic inactivation of *S. aureus* and was shown by Valente *et al* by combing NPWT and silver foam dressing treatment for 3 and 5 days respectively. Hence these studies lead toward testing synergism between combining chemical and topical negative pressure therapy for efficient biofilm killing through following study.

5.2 Author's contribution

Tahir S, Hu H, Deva AK, Vickery K**, *"Topical negative pressure therapy can enhance biofilm killing when combined with antiseptic or antibiotic instillation: an in-vitro study."* Ready for submission.**

In the co-author publication, Dr. Shamaila Tahir contributed to the study design, performed all experimental work, microscopic analysis, data collection and contributed to manuscript production. Her overall contribution was 90%.

5.3 Original Paper

"Topical negative pressure therapy can enhance biofilm killing when combined with antimicrobials instillation: an in-vitro study"

Authors:

Tahir. S, MBBS, GradDip (Inf & Immun)

Hu. H, BSc, Grad DipSc, PhD

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"Topical negative pressure therapy can enhance biofilm killing when combined with antimicrobials instillation: an in-vitro study"

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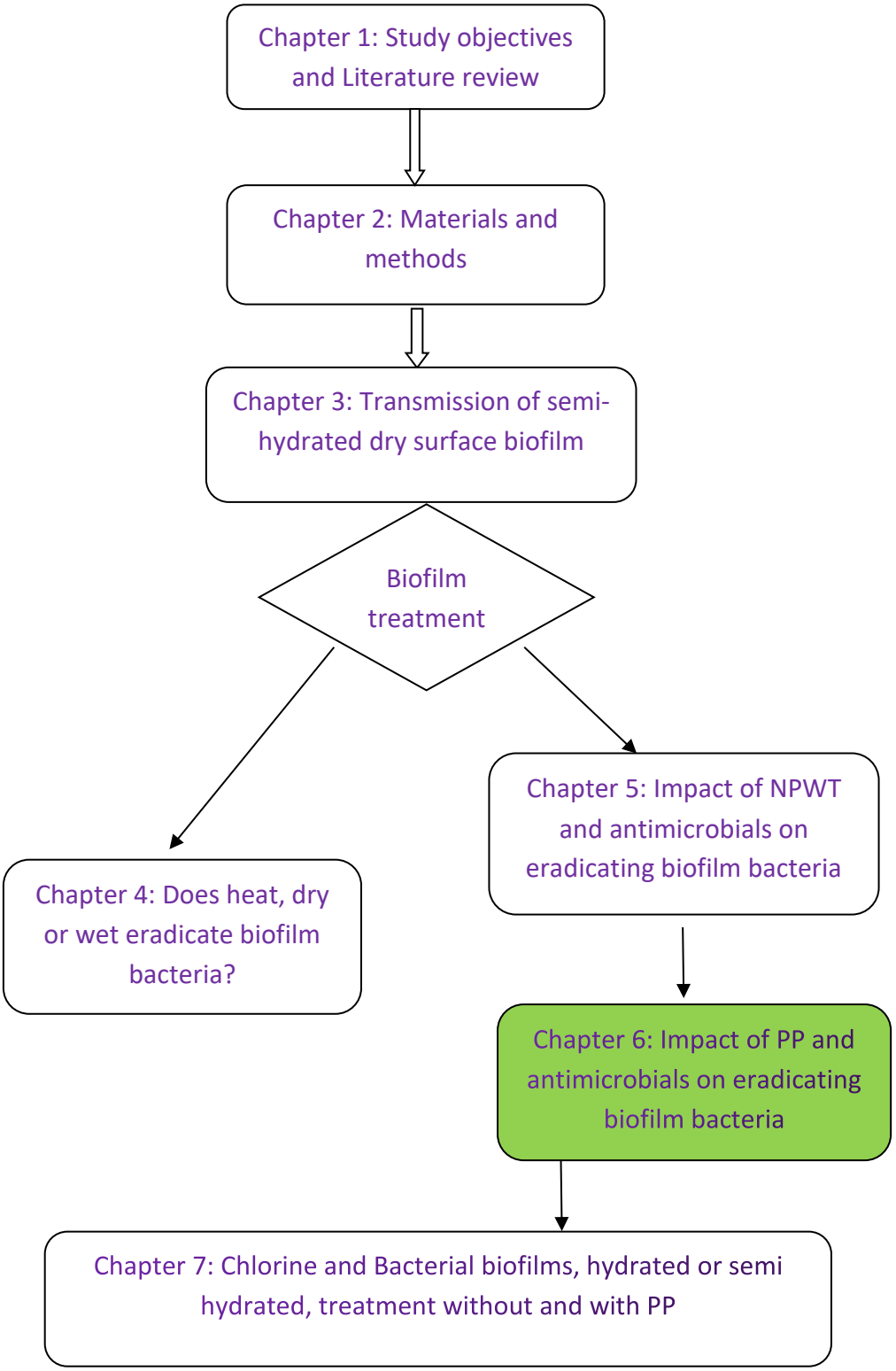
"Topical negative pressure therapy can enhance biofilm killing when combined with antimicrobials instillation: an in-vitro study"

"Topical negative pressure therapy can enhance biofilm killing when combined with antimicrobials instillation: an in-vitro study"

5.4 Conclusion

The above in vitro study clearly supports the hypothesis of synergism between compression pressure and antimicrobial killing of bacterial biofilms. More bacterial cells are approached by the biocide when physical structure of the biofilm is disrupted by pressure. Similar phenomenon can be explored for enhanced killing of multispecies chronic wound biofilm *in vitro* and finally *in vivo* for its future practical implication in the management of delayed or non-healing chronic wounds.

Thesis outline



Chapter 6

Impact of positive pressure use combined with disinfectants on eradicating bacterial biofilms in healthcare settings

6.1 Introduction

After establishing synergism between antimicrobials and NPWT, similar synergism between antimicrobials and mild positive pressure for biofilm killing was put to test.

6.2 Author's contribution

Tahir S, Emanuel S, Inglis D, Hu H, Vickery K, ***"Improved biofilm killing by combination treatment using mild positive pressure with liquid disinfectants"*** **Ready for submission.**

In the co-author publication, Dr. Shamaila Tahir contributed to the study design, performed biofilm growth and disinfectant testing in conjunction with Miss Sara Emanuel. Dr Tahir conducted all microscopic analysis, data collection, data analysis and contributed to manuscript production. Her overall contribution was 85%.

6.3 Original Paper

"Improved biofilm killing by combination treatment using mild positive pressure with liquid disinfectants"

Authors:

Tahir. S, MBBS, Grad Dip Inf&Immu[a]

Emanuel. S, BScMed, MRes[a]

Inglis. D, BScEng, PhD Engineering and Physics[b]

Honghua Hu, BSc, Grad DipSc, PhD[a]

Karen Vickery, BVSc (Hons), MVSc, PhD[a]

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"Improved biofilm killing by combination treatment using mild positive pressure with liquid disinfectants"

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"Improved biofilm killing by combination treatment using mild positive pressure with liquid disinfectants"

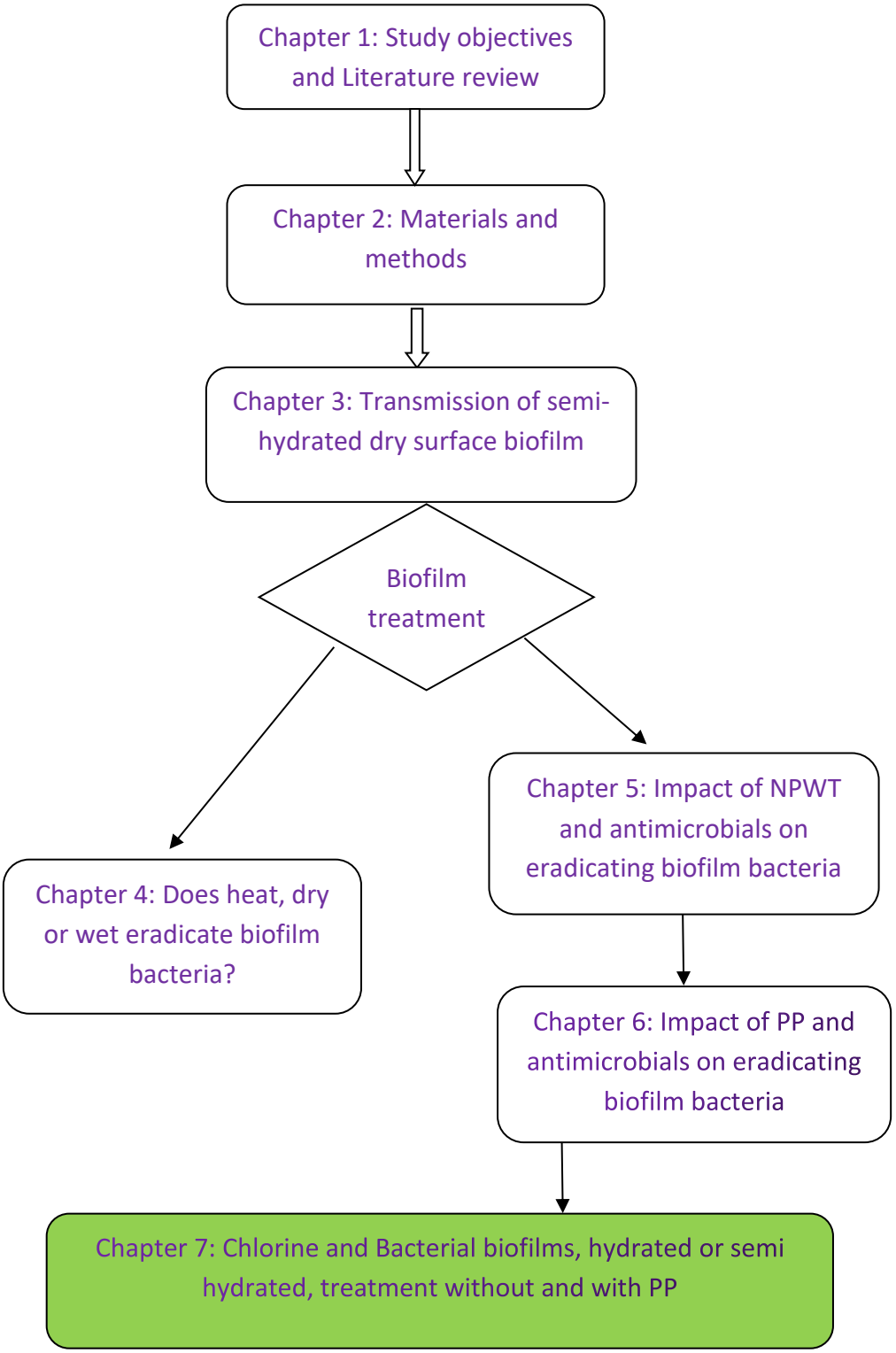
"Improved biofilm killing by combination treatment using mild positive pressure with liquid disinfectants"

"Improved biofilm killing by combination treatment using mild positive pressure with liquid disinfectants"

6.4 Conclusion

Above scientific study once again strongly supports the hypothesis of synergism between antimicrobials and mild postive pressure. This affirmation opened doors for further scientific testing if synergism exists between antimicrobials and compression pressure in killing bacteria in DSB. Also if the phenomenon is as effective as with enhanced cell killing in hydrated biofilms.

Thesis outline



Chapter 7

Use of hospital grade chlorine to eradicate bacterial biofilms from healthcare settings

7.1 Introduction

Chlorine (Sodium Hypochlorite) being a commonly used disinfectant in healthcare was the next chemical to test its efficacy for killing planktonic and biofilm bacteria (as in hydrated and semi-hydrated biofilms) without and with conjunction of mild compression pressure.

7.2 Author's contribution

1) Almatroudi A, Gosbell IB, Hu H, Slade J, Espedido BA, **Tahir S**, Glasbey TO, Legge P, Whiteley G, Deva AK, Vickery K, ***"Staphylococcus aureus dry-surface biofilms are not killed by sodium hypochlorite: implications for infection control."*** **Journal of Hospital Infection. July 2016;93(3):263-270.**

In the above co-author publication, Dr. Shamaila Tahir performed experimental work for MIC, MBC, MBIC and MBEC on planktonic and hydrated *S. aureus* biofilm. Her overall contribution was 15%.

2) **Tahir S,** Chowdhury D, Hu H, Whiteley G, Deva AK, Vickery K***, "Chlorine and bacterial biofilms*:"**

In the co-author publication, Dr. Shamaila Tahir contributed to the study design, performed all experimental work, microscopic analysis, data collection, data analysis and contributed to manuscript preparation. The transmission test was conducted in association with Dr. Durdana Chowdhury. Her overall contribution was 80%.

7.3 Original Papers

Journal of Hospital Infection 93 (2016) 263–270

Available online at www.sciencedirect.com

Journal of Hospital Infection

journal homepage: www.elsevierhealth.com/journals/jhin

Staphylococcus aureus dry-surface biofilms are not killed by sodium hypochlorite: implications for infection control

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ARTICLE INFO

Article history:
Received 13 September 2015
Accepted 18 March 2016
Available online 12 April 2016

Keywords:
Biofilms
Disinfectants
Dry-surface biofilms
Healthcare-associated infections
Hospital cleaning
Infection control
Sodium hypochlorite
Staphylococcus aureus

SUMMARY

Background: Dry hospital environments are contaminated with pathogenic bacteria in biofilms, which suggests that current cleaning practices and disinfectants are failing.
Aim: To test the efficacy of sodium hypochlorite solution against *Staphylococcus aureus* dry-surface biofilms.
Methods: The Centers for Disease Control and Prevention Biofilm Reactor was adapted to create a dry-surface biofilm, containing 1.36×10^7 *S. aureus*/coupon, by alternating cycles of growth and dehydration over 12 days. Biofilm was detected qualitatively using live/dead stain confocal laser scanning microscopy (CLSM), and quantitatively with sonicated viable plate counts and crystal violet assay. Sodium hypochlorite (1000–20,000 parts per million) was applied to the dry-surface biofilm for 10 min, coupons were rinsed three times, and residual biofilm viability was determined by CLSM, plate counts and prolonged culture up to 16 days. Isolates before and after exposure underwent minimum inhibitory concentration (MIC) and minimum eradication concentration (MEC) testing, and one pair underwent whole-genome sequencing.
Findings: Hypochlorite exposure reduced plate counts by a factor of 7 $\log_{10}$, and reduced biofilm biomass by a factor of 100; however, staining of residual biofilm showed that live *S. aureus* cells remained. On prolonged incubation, *S. aureus* regrew and formed biofilms. Post-exposure *S. aureus* isolates had MICs and MECs that were not significantly different from the parent strains. Whole-genome sequencing of one pre- and post-exposure pair found that they were virtually identical.

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http://dx.doi.org/10.1016/j.jhin.2016.03.020
0195-6701/© 2016 The Healthcare Infection Society. Published by Elsevier Ltd. All rights reserved.

Conclusions: Hypochlorite exposure led to a 7-log kill but the organisms regrew. No resistance mutations occurred, implying that hypochlorite resistance is an intrinsic property of *S. aureus* biofilms. The clinical significance of this warrants further study.
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Introduction

Healthcare-associated infections occur because of the complex interplay between patients, antibiotic use, healthcare workers (HCWs) and contaminated hospital environments. Despite action on hand hygiene programmes, antimicrobial stewardship, interventions such as central line bundles and comprehensive infection control systems, healthcare-associated infections are still a problem. Recent attention has been drawn to the importance of the hospital environment as a source of cross-infecting bacteria.[1]

Vickery *et al.* showed that multi-drug-resistant organisms (MDROs) were present in an intensive care environment despite two cycles of hypochlorite cleaning, and that these bacteria resided in biofilms.[2] Further work by Hu *et al.* on 44 samples of furnishings and patient bedding showed that viable and culturable organisms were present in 23/44 samples, and 12 (52%) of these samples were growing MDROs. *Staphylococcus aureus* was detected by polymerase chain reaction in 22/44 (50%) samples, biofilm was demonstrated by confocal and scanning electron microscopy in 41/44 samples (93%), and pyrosequencing showed that these biofilms were polymicrobial and contained species that represent MDRO strains,[3] providing additional evidence of the existence of MDRO-containing dry-surface biofilms in the intensive care environment.

Yezli and Otter postulated that the presence of biofilms would explain the difficulty in controlling MDROs in hospital environments despite enhanced cleaning, and why conventional disinfectants active against pathogenic bacteria in planktonic form are not active against them in biofilm form in the hospital environment.[4] Furthermore, as the bacteria in biofilms are held together tightly, conventional methods of swabbing these surfaces may make them difficult to detect.[4]

The demonstration by Vickery *et al.* of biofilms in a dry environment was enigmatic, as biofilms are generally found in wet environments.[2,3,5] As biofilms were found on the majority of surfaces sampled, conditions for biofilm growth (i.e. warmth, water, nutrients and a surface facilitating bacterial adherence and growth) must exist. Biofilms are found in the intertidal region in marine environments.[6] Could a similar cycle of wetting and drying be responsible for the growth of biofilms on a hospital surface? Using this analogy, a laboratory model was developed to represent biofilms formed on dry hospital surfaces.[7] This study tested the efficacy of chlorine disinfection against the model biofilm.

Materials and methods

Estimated number of bacteria in clinical biofilm contaminating hospital surfaces

Biofilm-positive samples from a disinfected, decommissioned intensive care unit were subjected to real-time quantitative polymerase chain reaction (PCR) using universal eubacterial primer16s rRNA_341F 5′-CCTACGGGAGGCAGCAG-3′ and 16s rRNA_534R 5′-ATTACCGCGGCTGCTGG-3′, as described previously.[8]

Biofilm formation

S. aureus ATCC 25923 dry-surface biofilms were grown as described previously.[7] Briefly, biofilms were grown on sterile polycarbonate coupons in a Centers for Disease Control and Prevention (CDC) Biofilm Reactor (BioSurface Technologies Corp, Bozeman, MT, USA) over 12 days. Growth was initiated with 10^8 colony-forming units (cfu) of *S. aureus* and grown in 5% tryptone soya broth (TSB) with shear (provided by baffle rotation at 130 revolutions per min) for 48-h batch phase at 35°C, after which the media was drained and the biofilm was dehydrated for 48 h at room temperature (22—25 °C) with filter-sterilized air-conditioned air (average relative humidity 66%) pumped across the surface at 3 L/min. An additional three cycles of batch growth (5% TSB, shear, 35°C for 6 h) were alternated with prolonged dehydration phases of 66, 42 and 66 h at room temperature and resulted in 1.36×10^7 cfu/coupon.

Chlorine efficacy testing against dry-surface biofilms

Biofilm-covered coupons were washed three times in phosphate buffered saline to remove loosely attached bacteria before being placed in individual sterile Bijou containers containing 4 mL of diluted hypochlorite solution, at free chlorine concentrations of 1000, 5000, 10,000 and 20,000 parts per million (ppm) (disinfectant test tube) or 4 mL of TSB (control tubes), and allowed to react for 10 min. The free chlorine concentration was measured by iodometric titration. Next, the coupons were washed immediately three times in sterile water before being placed in 4 mL of 100% TSB to inactivate residual chlorine. The hypochlorite solutions were buffered to a pH between 6.5 and 7.0 to reduce any potential variance in solution pH across the concentration range 10—20,000 ppm that would affect the biocidal efficacy of the hypochlorite solution.

Log reduction in bacterial titre was determined by subjecting treated coupons to 5 min of sonication in an ultrasonic bath (Soniclean, JMR, Sydney Australia) with a sweeping frequency of 42—47 kHz and 2 min of vigorous shaking, followed by dilution and standard plate culture at 37°C (control N=5, test coupons/chlorine concentration N=3).

The ability of biofilm cells to repair chlorine damage was assessed by prolonged culture in 4 mL TSB at 37°C (N=2) and at room temperature (22—25°C) (N=2). Biofilm recovery (media turbidity) was observed daily, without opening the bottles to avoid contamination, for up to 16 days. If growth was detected, the contents were subcultured on horse blood agar plates, and the identity of isolated organisms was confirmed to be *S. aureus* by colonial morphology, DNase reaction (Dnase Test Agar, BD PA-255506) and a positive reaction with Prolex Staph

Xtra Latex Kit (Pro-lab Diagnostics Toronto, Canada), and real-time PCR targeting *nuc* genes[9] and *femA* genes.[10]

The above chlorine efficacy testing was conducted on four separate occasions.

An additional three control coupons and two coupons for each chlorine concentration tested were stained with LIVE/DEAD *BacLight* 7012 viability stain (Molecular Probes, Invitrogen, Mulgrave, VIC, Australia) according to the manufacturer's instructions, and subjected to confocal scanning laser microscopy (CSLM) as described by Almatroudi.[7] ImageJ (1.46r, National Institute of Health, USA) was used to determine the proportion of live/dead cells, and Imaris 7.7.2 (Bitplane, Zurich, Switzerland) was used to determine biomass [total amount of bacterial cells and extracellular polymeric substances (EPS)] and height of dry biofilms before and after chlorine exposure, as described by Almatroudi.[7] The height of the biofilm reflects the distance that disinfectants have to diffuse/penetrate to kill all the bacterial cells incorporated into the biofilm, and the biomass reflects the amount of biofilm that needs to be removed or killed.

Whole-genome sequencing

Whole-genome sequencing of one pre- and post-chlorine exposure (20,000 ppm) pair was performed using an Ion Torrent PGM (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Sequence reads were mapped to *S. aureus* JKD6008, an Australasian ST239-MRSA-III isolate (GenBank accession CP002120), and analysed using CLC Genomics Workbench 7.0.3 (CLC bio; Katrinebjerg, Denmark).

Comparison of chlorine resistance of chlorine-survival isolate and parent strain

The minimum concentration of chlorine required to inhibit growth or to kill 10^7 cfu *S. aureus* was determined for both planktonic and hydrated biofilm cultures composed of pre- or post-chlorine-exposure isolates. Ten different concentrations of chlorine were tested (10, 50, 100, 200, 500, 1000, 2000, 5000, 10,000 and 20,000 ppm) with contact times of 10 min and 24 h using a modification of the 96-well-plate format developed by Stepanovic and Ceri.[11,12] The chlorine was inactivated after disinfectant exposure using 100% TSB with 20% bovine serum albumin.

Statistical analysis

Student's *t*-test was performed on $\log_{10}$ transformed data comparing control coupon biomass with hypochlorite-treated biomass (SigmaPlot 11 Statistical Program) Systat Software Inc, San Jose, USA.

Results

Estimated number of bacteria in clinical biofilm contaminating hospital surfaces

Sufficient samples were available from 32 items. The mean number of bacteria was 5.5×10^5 bacteria/cm^2 of sample, and the range was from 4.2×10^2 to 1.6×10^7 bacteria/cm^2. Almost half of the samples were contaminated with over 10,000 biofilm bacteria/cm^2.

Chlorine efficacy testing against dry-surface biofilms

Treatment of dry-surface biofilms with chlorine concentrations from 1000 ppm to 20,000 ppm resulted in a >7 log reduction in the number of *S. aureus*, with all treated coupons being culture negative immediately after treatment. However, even when treated with 20,000 ppm of chlorine, the biofilm recovered with prolonged incubation, releasing planktonic *S. aureus* as evidenced by cloudiness of the media. The time taken for biofilm recovery, production of planktonic organisms and turbidity was correlated with both the chlorine concentration used and the temperature of post-treatment incubation (Figure 1).

The presence of live bacteria immediately after hypochlorite treatment was confirmed by confocal microscopy. Representative images are shown in Figure 2.

The biomass of untreated coupons (N=3) was 379,080 μm^3, consisting of 97.5% live cells and 2.5% dead cells. Hypochlorite treatment (N=2/treatment) reduced the maximum height of biofilm on coupons and significantly reduced the total biomass of the biofilm between 95.5% and 98.6% ($P<0.001$) (Figure 3), resulting in less than 5% of the initial number of bacteria left on coupons treated with 1000 ppm hypochlorite. After treatment with 20,000 ppm hypochlorite, only 1.4% of bacteria remained attached to the coupon, and 60% of these remained alive (i.e. 0.8% of the initial biofilm bacteria remained viable). This appeared to be due to peeling of large sections of the biofilm, as shown in Figure 4.

Whole-genome sequencing

All colonies associated with post-treatment growth were confirmed as *S. aureus* via DNase Test Agar, Prolex Staph Xtra Latex Kit and *S. aureus*-specific quantitative PCR. Furthermore, whole-genome sequencing of a randomly selected colony revealed that it was identical to *S. aureus* ATCC 25923, with the exception of a single nucleotide polymorphism in the *ddl* gene, resulting in amino acid change Ala208Thr. This gene encodes D-alanine-D-alanine ligase, which is involved in peptidoglycan biosynthesis, and the identified mutation is unlikely to be associated (at least directly) with chlorine resistance.

Comparison of chlorine resistance of chlorine-survival isolate and parent strain

The minimum concentration of chlorine to inhibit growth (MIC) or kill (MEC) pre-chlorine-exposed isolates was comparable with that of post-chlorine-exposed isolates when grown in planktonic culture. Similarly, the minimum biofilm inhibitory concentration (MBIC) and the minimum biofilm eradication concentration (MBEC) of chlorine were determined against pre- and post-chlorine-exposed isolates grown as hydrated biofilm (see Table I). Higher concentrations of chlorine were required with both shorter disinfectant contact times and when treating biofilm compared with planktonic cultures.

Discussion

Key findings

Exposure to hypochlorite for 10 min resulted in a 7-log reduction in *S. aureus* plate counts and removed over 95% of

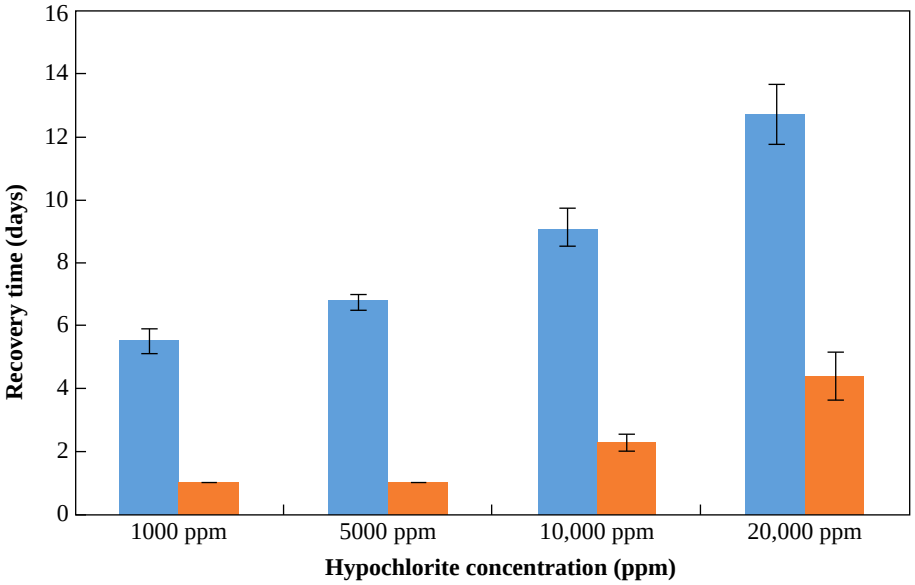

Figure 1. Days required for recovery of biofilm bacteria, release of planktonic bacteria, and development of turbidity in broth following treatment with sodium hypochlorite solution when incubated at 25°C (blue bars) and 37°C (orange bars). Four coupons/treatment. Error bars represent standard deviation. ppm, parts per million.

the biofilm. However, even after exposure to sodium hypochlorite at 20,000 ppm, live *S. aureus* cells were visible by confocal microscopy, and regrew on prolonged incubation, releasing planktonic cells.

The MIC and MEC of the post-exposure *S. aureus* isolate were comparable to the parent strain. Whole-genome sequencing of one *S. aureus* pair before and after chlorine exposure revealed that the sequence was identical apart from a single nucleotide polymorphism in the *dll* gene, a mutation not associated with chlorine resistance. Thus, chlorine resistance of *S. aureus* biofilm is an intrinsic characteristic of the organism's lifestyle.

Strengths and limitations of the study

This study confirmed that the previously described dry-surface biofilm model[7] can be used to test the efficiency of

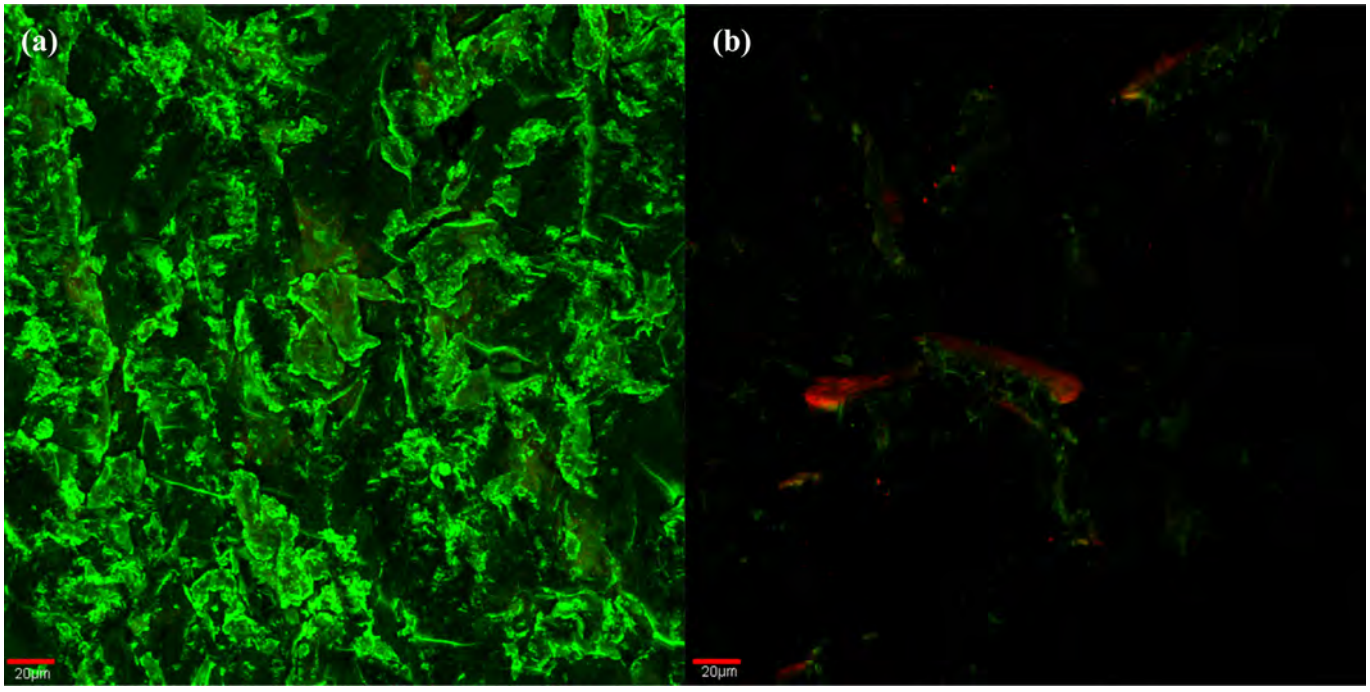

Figure 2. Viability stain of (a) control coupon showing large numbers of live bacteria and (b) hypochlorite-treated (20,000 parts per million) biofilm showing significantly reduced numbers of live (green) and dead (red) bacteria. Bar 20 μm.

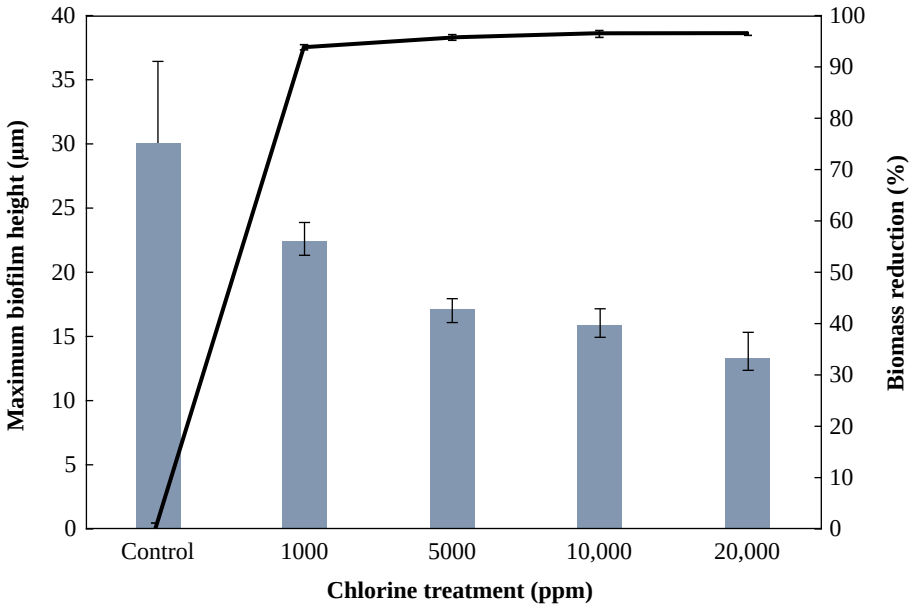

Figure 3. Height of residual biofilm (μm, blue bars) and percentage reduction in the total biomass (black line) of hypochlorite-treated coupons compared with control coupons. Mean of three coupons for control, mean of duplicate samples for hypochlorite-treated groups. Error bars represent standard deviation. ppm, parts per million.

chemicals used in hospital disinfection, and that multiple endpoints can be used in this model, including confirmation of living cells on confocal microscopy, and detection of genetic changes. The thickness of the biofilms and the number of cells in the biofilms in this model were reproducible between repetitions of the experiments, at least for one strain of *S. aureus*. The model biofilm containing 1.36 bacteria/coupon is a reasonable representation of biofilms found on dry hospital surfaces. The mean number of bacteria found in biofilms on dry clinical surfaces was 5.5×10^5 bacteria/cm^2, but the worst-case scenario of 1.6×10^7 bacteria/cm^2 was found in biofilm contaminating the sterile supply box. Thus, 1.36 bacteria/coupon reflects a suitable in-use test concentration, but is lower than planktonic inoculum densities of between 10^8 to 10^9/mL required by governmental organizations[13] for testing hospital-grade disinfectant action. In addition, the authors have shown previously that the model biofilm was similar to clinical biofilm with regards to the amount of biofilm (biomass), EPS composition and biofilm depth, all of which could influence the action of disinfectants.[7]

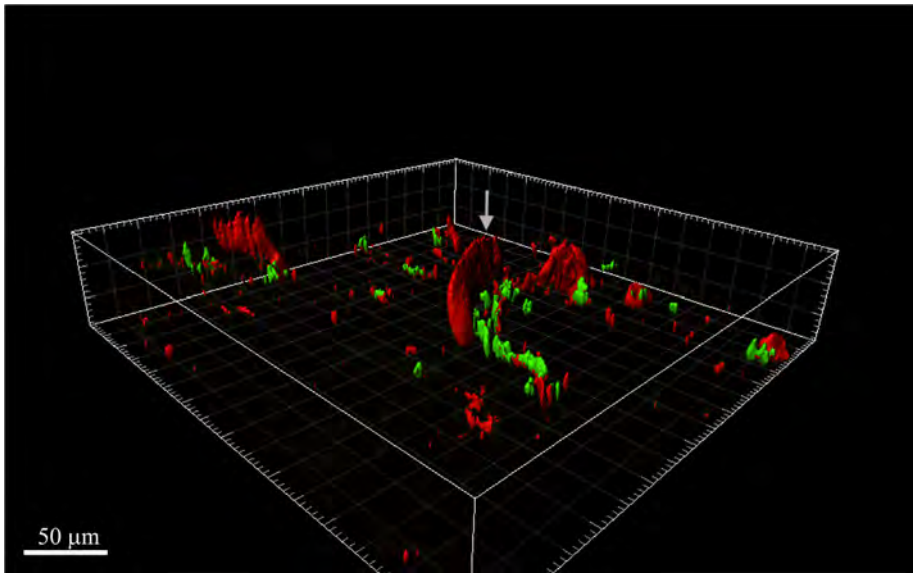

Figure 4. Effect of 20,000 parts per million hypochlorite treatment on dry-surface biofilm. Arrow shows a large section of vertically aligned dead cells suggestive of large sections of biofilm peeling from the coupon.

Table I
Concentration of chlorine (parts per million) required to inhibit growth or kill *Staphylococcus aureus* for planktonic and hydrated biofilm

Sample	Planktonic culture				Hydrated biofilm			
	MIC		MEC		MBIC		MBEC	
	10 min	24 h	10 min	24 h	10 min	24 h	10 min	24 h
Pre-chlorine-exposed strain	1000	500	5000	500	10,000	2000	10,000	2000
Post-chlorine-exposed strain	1000	500	5000	500	10,000	2000	10,000	2000

MIC, minimum inhibitory concentration; MEC, minimum effective concentration; MBIC, minimum biofilm inhibitory concentration; MBEC, minimum biofilm eradication concentration.

This model showed that *S. aureus* cells in biofilms subjected to periodic nutrition and dehydration are not eradicated by high concentrations of sodium hypochlorite. The results need to be confirmed for other strains of *S. aureus*, including clinical strains, meticillin-resistant *S. aureus* (MRSA) strains, and other species of bacteria that are clinically important, such as *Enterococcus faecium*, *E. faecalis*, Enterobacteriaceae and *Pseudomonas aeruginosa*, including multi-drug-resistant strains of these species. Similarly, other compounds used in hospital disinfection need to be tested.

Interpretation and implications in the context of the totality of evidence

Successful infection control programmes require a multi-faceted approach managed using a comprehensive quality system.[14] The Healthcare Infection Control Practices Advisory Committee has indicated that seven elements need attention: managerial buy-in and support; ongoing education; antimicrobial stewardship; MDRO surveillance; imposition of infection control precautions; attention to environmental cleaning; and decolonization of human reservoirs if possible.[15] Antimicrobial stewardship has decreased MDRO acquisition and MRSA bacteraemia rates. Comprehensive hand hygiene programmes have lifted compliance with the World Health Organization's Five Moments of Hand Hygiene, and Pittet *et al.* showed that this decreased MRSA bacteraemia rates.[16] High compliance with hand hygiene is difficult to sustain.[17] Multi-faceted strategies involving active surveillance, patient isolation/cohort and improved hand hygiene have been shown to be successful in reducing MRSA rates.[18,19]

Recent attention has focused on the environment as an important reservoir that needs addressing.[1] Untrained individuals often perform hospital cleaning, and a quality system to evaluate the efficacy of hospital cleaning is usually absent.[20] Contamination of the hospital environment has been implicated in outbreaks of MRSA,[21] *P. aeruginosa*,[22] vancomycin-resistant enterococci (VRE)[23] and other organisms. Dancer has proposed that cleaning efficacy can be measured by dual environmental culturing measuring semi-quantitative bacterial counts and the presence of MDROs.[24]

A study in a newly-built hospital showed that the new rooms and HCWs in the hospital did not return positive cultures of the environment and HCWs' hands until patients positive for MRSA were housed in the new hospital, whereupon 40/142 (28%) HCW handwash samples and 69/319 (22%) environmental cultures were positive for MRSA.[25] The handwash and environmental isolates were indistinguishable from temporally- and spatially-related patient isolates in 63% and 93% of cases, respectively.[25]

What this study adds to the available evidence

Vickery *et al.* and Hu *et al.* did not explain how dry-surface biofilms might develop.[2,3] It was conceptualized that, over a prolonged period of time, the growth of dry-surface biofilms in a clinical environment that is periodically wetted and supplied with nutrients by hospital cleaning with detergent solutions might be analogous to what happens in the intertidal zone in marine environment, where the twice-daily tidal variation subjects this zone to regular wetting and drying, and this results in biofilm formation.[6] The modified CDC Biofilm Reactor model replicated the episodic wetting and drying, and was able to reproducibly produce dry-surface biofilms that had similar physical characteristics to biofilm formed naturally on dry clinical surfaces.[7]

The use of chlorine solutions at 1000 ppm would normally be expected to demonstrate acceptable biocidal performance, and this strength of solution is in conformity with the Australian infection control guidelines.[23] However, despite the failure to kill all bacteria in the model biofilm, hypochlorite treatment caused a reduction in the number of bacteria that could be cultured immediately by 7 log. Following treatment with 1000 ppm hypochlorite, it took a minimum of five days for the biofilm to be reconstituted and release planktonic organisms. This suggests that hypochlorite disinfection of biofilm would reduce the risk of transmission of infection, even without complete bacterial eradication.

Effects on patient care and health policy

This study if other bacterial species might grow these biofilms, test various disinfectants and other compounds for activity against them, and conduct other studies such as how readily dry-surface biofilms might inoculate the hands and clothing of HCWs.

Sodium hypochlorite solutions have had occupational health issues associated with their use and are corrosive to many hospital surfaces. They have high activity in in-vitro testing against many bacterial species important in hospital cross-infection, and thus have been used in the control of outbreaks of bacteria where environmental reservoirs have been important, such as VRE[23] and *Clostridium difficile*.[26] Many MDRO species form biofilms; they have been demonstrated on surfaces in intensive care units treated with two 'terminal cleans' with hypochlorite,[2] and it has also been shown that *S. aureus* dry-surface biofilms are not eradicated by 10 min of static sodium hypochlorite treatment up to 20,000 ppm. In practice, the contact time during cleaning would be expected to be less than the 10 min used in this study, while the effect of

wiping or scrubbing would be expected to impact on biofilm removal. However, disinfectant testing is generally conducted under static conditions,[13] and it is difficult to standardize pressure during wiping.

Possible mechanisms

The concentration of chlorine to inhibit growth or kill *S. aureus* did not change in cells that survived exposure to sodium hypochlorite solution. Also, in the single pre- and post-exposure pair examined, no genetic change was shown that would confer resistance to this compound. This implies that resistance of a subpopulation of *S. aureus* cells is an intrinsic property of biofilm growth, which implies that hypochlorite, at least up to 20,000 ppm, will not eradicate *S. aureus* if present in a dry-surface biofilm. However, at least in the short term, surviving cells are not more resistant to the action of chlorine, suggesting that if the biofilm could be dispersed, surviving bacterial cells would be killed by surface disinfection with hypochlorite.

Future research directions

The efficacy of other commonly used hospital disinfectants needs to be evaluated against dry-surface biofilms composed of pathogens in monoculture and/or mixed species, particularly those species that have multi-resistant strains.

Acknowledgements

The authors wish to thank the Microscopy Department, School of Biological Sciences, Macquarie University for providing expertise in electron microscopy.

Conflict of interest statement
GW is the Managing Director of the Whiteley Corporation; he is currently enrolled in a PhD programme at the University of Western Sydney researching the best ways to clean hospital environments to remove MDRO contamination. TOG is the R&D Manager of Whiteley Corporation, a manufacturer of disinfectants and detergents for use in health care. Their roles did not lead to any bias in formulating, executing, analysing or writing up the research.

Funding sources
KV was in receipt of a Macquarie University Vice Chancellor Innovation Fellowship. This study was funded by an Australian Research Council Linkage Projects Scheme LP130100572 in conjunction with Whiteley Corporation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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"Chlorine and bacterial biofilms"

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7.4 Conclusion

Once again in the above scientific study, synergism between chemistry i-e chlorine and compression pressure was demonstrated. Hence to conclude 3 novel studies it can be established scientifically that physical disruption of biofilm whether hydrated or semi-hydrated occurs by pressure compression. This disruption enhances killing effect of most antimicrobials including antibiotics, antiseptics and disinfectants. Further scientific studies can be confidently performed using more antimicrobials and multi species biofilms.

Chapter 8

General discussion and conclusion

8.1 Discussion

Successful, ubiquitous existence of bacterial biofilm in healthcare ecosystem cannot be undermined. Transmission of pathogens from environmental biofilm during hospitalisation plus the myriad of lingering chronic infections due to biofilm formation require prolonged patient care, diagnostic interventions, and management trials with follow up programs. In addition, these infections are associated with high morbidity and mortality rates. Apart from physical inconvenience, extended patient care including prolonged hospitalisation or repeated hospital/general practice visits place increased financial burden on patients as well as on healthcare systems.

Since the first discovery of medical microbial aggregates by John William Costerton in 1977, the spectrum of biofilm involvement in healthcare continues to expand. This necessitates more scientific investigation of every aspect of biofilm growth, development and removal. It is thought that more than 99% of bacteria form biofilm and >85% of chronic infections are now attributed to biofilm formation[5,11]. Pathogenic micro-organisms not only colonise diseased broken tissues but also reside in biofilm which improves their survival on wet and dry inanimate hospital surfaces, medical devices, and equipment, [3].

Extensive clinical and research evidence is now available to suggest that exogenous or endogenous bacterial contamination of breast implants give rise to biofilm infection that plays important role in the aetiology of capsular contracture[617]. In chapter 2, the in-vitro experimental study demonstrated that *S. epidermidis* biofilm readily grows on both types of breast implants within 2, 6 and 24 hours with 11, 43 and 72 times more bacterial cells growing on textured versus smooth surfaced breast implants respectively. Visual scanning by CLSM and SEM confirmed the CFU findings of this in-vitro study with parallel support from the in-vivo study of porcine model, silicone breast implants retained over 19 weeks

and also by the previously obtained clinical data[590]. These pioneer findings further supported the hypothesis of proposed relationship of bacterial biofilm to the formation of contracted breast implants by emphasising the involvement of implant material texture[302,590].

The environment is known to be a significant source of pathogenic bacteria. The impact of this source is seen with patients admitted into the rooms previously occupied by patients infected with multidrug resistant organisms (MDROs) being at higher risk of acquiring infection with the same MDRO[281,618].

Healthcare worker's hands are the main vehicle of transmitting pathogens from infected patients, contaminated devices or contaminated surfaces. Direct contamination occurring from inadequately sterilised/disinfected reusable medical devices or surfaces may take place in healthcare settings. Pathogenic bacteria survive for weeks, months or even years on inanimate healthcare-related surfaces securing themselves in semi-dried biofilms on dry surfaces[2]. Once formed, bacteria living in semi-dehydrated DSB have the potential to escape killing after following regular cleaning hospital protocols as well as protocols applied for instrument sterilisation. For the first time, experimental study in chapter 3 demonstrated transmissions of high numbers of viable, culturable bacteria from in-vitro grown *S. aureus* DSB via bare and gloved hands. This shows that pathogenic bacteria can be spread from not only planktonic organisms but dry surface biofilm contaminating hospital surfaces. These findings strongly warrant reviewing of cleaning protocols currently practiced in most healthcare facilities.

In Chapters 4 and 7, hydrated *S. aureus* biofilm killing was tested by wet heat, dry heat and chlorine following the same testing protocol developed by Almatroudi *et al* [301,619]. Hydrated biofilm can be successfully eradicated at temperatures more 80^{o}C depending upon the duration of heat application. Biofilm cells were completely eradicated when 80^{o}C heat was applied for 60mins while 10mins treatment was needed at 100^{o}C. DSB proved more resistant to heat and chlorine (1000 ppm) with retention of viable but non-culturable bacteria within biofilm structure.

After establishing the clinical relevance of bacterial biofilm formation in healthcare ecosystem, this thesis then explored optimal strategies/treatments for eradication of biofilm on biotic and abiotic surfaces. Sufficient scientific and clinical evidence is available to support high level of tolerance/resistance of biofilm bacteria towards disinfectants as well as clinically approved antimicrobial treatments[206]. One of the major Mechanisms of antimicrobial tolerance/resistance is the physical barrier afforded by the extracellular matrix which is composed of eDNA, proteins, polymers etc. Despite sufficient antimicrobial concentration, penetration through the biofilm biomass is restricted. In Chapter 5 negative suction pressure was shown to physically disrupt the biofilm architecture to improve penetration of antimicrobials. Synergism between antimicrobials and topical negative pressure was successfully demonstrated to eradicate biofilm cells with certain combinations such as Prontosan and NPWT. Hence Prontosan is recommended to replace saline instillations during NPWT for improved healing of chronic wounds. However, in vivo studies or clinical trials are needed to confirm in vitro results and to determine exact protocols for combined antimicrobial and NPWT in the management of chronic wounds.

Enhanced penetration of antimicrobials through biofilm EPS using suction pressure lead to the idea of testing for a similar synergism using direct positive pressure as studied in Chapter 6. Mild positive or compression pressure was shown to improve penetration of biocide through EPS at sub-lethal doses depending upon the type, time and concentration of the biocide for sterilization. More studies are needed to extend testing for similar synergism between positive pressure and other commonly used biocides for future uses in medicine including the possibility of improving decontamination of heat sensitive equipment

Finally, in Chapter 7, transmission of DSB bacteria was tested after chlorine disinfection followed by hydrated and semi-hydrated *S. aureus* biofilm killing with combined Chlorine and PP treatment. Viable biofilm bacteria were transmitted after chlorine disinfection via strong grip of HCWs' hands. However, bacterial killing of DBS with chlorine was shown to improve with simultaneous application of PP subjected to enhanced penetration through EPS.

8.2 Conclusion

In this thesis, where bacteria from DSB were demonstrated to be highly transmissible, the compression pressure was successfully shown to improve penetration of tested antimicrobials/biocides through biofilm EPS to improve bacterial cell killing. More studies can be conducted to confirm the phenomenon of synergism using other disinfectants. The application of PP with disinfectant in the clinical setting needs to be evaluated.

Almatroudi *et al* showed that current methods of surface decontamination failed to remove DSB. This thesis expanded upon this by determining the efficacy of disinfectants, heat and combining two methods of killing i-e a physical (PP) and a chemical (disinfectant and antiseptic) against DSB and traditional hydrated biofilm. In general, a combination approach was more efficacious. This suggests that additional research needs to be conducted to improve current practices and disinfection protocols to achieve better standards.

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10 Appendix

Chemical reagents and Solutions used

3% Gluteraldehyde (Sigma-Aldich, Castle Hill, NSW Australia)

25% Gluteraldehyde stock solution was diluted with Iodine free water to make 3% gluteraldehyde with solution.

Dilutions of ethanol

Iodine free water was added to 100% analytical grade ethanol (Sigma-Aldrich Pty Ltd, Castle Hill, NSW, Australia) to make following dilutions

In 100 ml solution of V/V:

- 30% ethanol = 30 mL 100% ethanol + 70 mL water,
- 50% ethanol = 50 mL 100% ethanol + 50 mL water,
- 70% ethanol = 70 mL 100% ethanol + 30 mL water,
- 80% ethanol = 80 mL 100% ethanol + 20 mL water, and
- 90% ethanol = 90 mL 100% ethanol + 10 ml water.

2% (w/v) Paraformaldehyde

2 g Paraformaldehyde (Sigma-Aldrich) was stired and mixed with 100 mL of phosphate buffered saline while heated over a heating, magnetic stirring platform until dissolved completely. Solution is then allowed to cool and is stored at 4oC. All these steps were performed in a fume hood as the Paraformaldehyde is toxic.

1 x Phosphate Buffered Saline (tablets Medicago AB, Uppsala, Sweden)

Phosphate tablets come in two sizes. 1 tablet makes 500ml and contains 0.14M NaCl, 0.0027M KCl and 0.010M Phosphate while other double sized tablet makes 1000ml buffer pH 7.4. The tablet depending on the size was dissolved in 500 ml or 1000ml of distilled water then autoclaved for 20 minutes at 121oC and stored at room temperature.

0.1 M Potassium Phosphate Buffer

87.09 g K2HPO4 (Univar) dissolved in 500 mL deionised water. 68.045 g KH2PO4 (Univar) dissolved in 500 mL deionised water. Mixed 80.2mL of K2HPO4 solution and 19.8 mL of KH2PO4 solution and made up to 1000 mL with deionised water. Autoclaved for 20 minutes at 121oC 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.