Patients' safety and hospital-acquired infections: the size of the problem of biofilms on the health care surfaces

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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.

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Manuscripts in preparation

1. **Almatroudi A**, Hu H, Deva A, Gosbell I, Whiteley G, Jensen SO and Vickery K. Increased tolerance of *Staphylococcus aureus* biofilms to heat when formed on dry surfaces.

In memory of my father

Abdulaziz Ali Almatroudi

28 November 1905 – 30 March 1997

Rest in Peace.

I dedicate this work to my mother and siblings who have always helped and encouraged me. I also dedicate this work to my father, Mr Abdulaziz, who was an exceptional parent and always treated my education and my wellbeing with great importance.

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List of abbreviation

°C	degrees Celsius
agr	staphylococcal accessory gene regulator genes
AI-2	autoinducer-2
AIP	autoinducing peptide
ANOVA	analysis of variance
ATCC	American Type Culture Collection
CDC	Centers for Disease Control and Prevention
CFU	colony forming unit(s)
CLSM	confocal laser scanning microscopy
cm	centimetre(s)
CoNS	coagulase-negative staphylococci
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
eDNA	extracellular DNA
EDTA	ethylenediamine-N, N'-diacetic acid
EPS	extracellular polymeric substance(s)
HCW	health care worker
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
NICU	neonatal intensive care unit
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
TSB	tryptone soya broth
µL	microlitre

Abstract

There is incontrovertible evidence that nosocomial pathogens contaminate inanimate items in the hospital environment, such as surfaces and medical equipment. Transmission of infectious agents from contaminated fomites to patients is a known infectious route, although the contribution to overall hospital acquired infections (HAI) is unknown. However, the risk of developing HAI, has been shown to be increased 73% if the patient previously occupying the room had a multi-antibiotic resistant organism (MRO). *Acinetobacter baumannii*, vancomycin-resistant *enterococci* and methicillin-resistant *Staphylococcus aureus* are the main non-sporing infectious agents that have been clearly demonstrated to survive in environmental reservoirs.

Bacteria readily adhere to surfaces. Once adhered they secrete a slimy matrix principally composed of protein, carbohydrate and DNA which surrounds them protecting them from the external environment. Bacterial reproduction and recruitment, leads to the development of a mature biofilm.

The environmental conditions associated with hospital surfaces, especially in Intensive Care Units (ICU), are conducive to development of biofilm, which could have adverse consequences in these seriously ill patients. Up to 20% of intensive care patients become colonised by MROs increasing their risk of developing a HAI. Dry surfaces biofilms development increases the MDRO persistence rate in the hospital environment, and thereby likely increases the risk of hospital acquired infections and outbreaks. Hence, one cannot emphasise enough the importance of thoroughly cleaning and disinfecting hospital surfaces.

The ultimate aim of this project was to determine if persistence of antibiotic resistant human pathogens in the hospital environment was due to MRO being incorporated into

biofilms contaminating dry hospital surfaces. If incorporated into biofilms it was hypothetised that they would be protected from environmental desiccation, cleaning and disinfectants agents.

The findings of this study revealed that bacteria encased in biofilms, including those causing serious infections, such as *S. aureus*, were present on over 90% of ICU surfaces. We determined that the majority of clinical biofilms incorporated *Staphylococcus* species and therefore, developed an *in vitro* model representative of clinical dry-surface biofilms. The thickness of the biofilms and the number of cells in the biofilms in this model was reproducible between repetitions of the experiments, at least for one strain of *S. aureus*. We then employed this model to assess how effective the commonly used hospital disinfectant 1000 Parts Per Million (PPM) sodium hypochlorite and traditional heat treatment (Autoclaving-121°C for 20 minute, 30 minute, 1 hour and 2 hours) were against dry biofilm. We found that high concentrations of sodium hypochlorite (20,000 ppm) or standard sterilisation (Autoclaving – 121°C for 20 min) did not destroy the *S. aureus* cells in the biofilms. Through such findings, this study makes a valid contribution to existing understanding of hospital surface biofilms. In conclusion, it can be seen from this study that the consequences of biofilm in health care surfaces is underestimated in the existence infection control practices. The efficacy of hospital infection prevention and control policies and decontamination strategies require re-assessment.

Chapter 1: LITERATURE REVIEW

Part One: Patients' safety and hospital-acquired infections

1.1. Introduction

Throughout the world, millions of people receive some kind of healthcare each year. This includes non-invasive care as well as intricate and technically advanced treatments and procedures. One potential consequence of healthcare interventions is that patients develop an infection termed hospital-acquired infection (HAI). According to the Centre for Disease Control and Prevention (CDC), HAI constitutes a condition caused by a pathogen or related toxin(s) contracted after hospitalisation. More specifically, a HAI is deemed to have developed if a patient manifests signs of infection on or following the third calendar day of admission to hospital or clinical institutions, the first calendar day representing the date of admission (Horan et al., 2008, CDC, 2015). There are two sources of infectious agents causing HAIs, namely, endogenous and exogenous sources.

- Endogenous source – the patient's own bacteria cause the infection. Organisms from colonised body locations, such as the nose, mouth, or vagina, can gain access to body areas not normally colonised by them eg. *Staphylococcus aureus* infection of sutures in surgical wounds.
- Exogenous sources are unrelated to the patient's own flora and include healthcare workers (HCW), visitors, equipment, medical instruments, or hospital surfaces (Horan et al., 2008).

Despite the enhancement of infection control practices, the prevalence of HAI remains a serious public health problem *(*Landrigan et al., 2010). HAIs contribute to increased

morbidity and mortality rates in hospitalised patients. In the United States alone, more than 700,000 cases (4% of inpatients) of HAIs were estimated to have occurred in 2011 and this resulted in approximately 75,000 deaths. Approximately 15% of HAIs were among patients in intensive care units (ICUs) (Magill et al., 2014). Furthermore, 6% of Australian inpatients (Garling, 2008) develop a HAI with approximately 200,000/year occurring in Australian ICU facilities (Cruickshank and Ferguson, 2008). In addition, a multicentre epidemiological study evaluating mortality rates due to HAIs, showed that HAIs are the fourth leading cause of death in France (Kaoutar et al., 2004).

The CDC found that approximately 65% of HAIs involve biofilms (Flannery, 1999), leading to lengthy hospital stays of up to two times longer than average (Madani et al., 2009) and a significant increase in the overall treatment costs. It has been estimated that costs associated with HAIs are approximately $9.8 billion in the United States annually (Zimlichman et al., 2013).

1.2. The size of the problem in intensive care units

When a patient is being treated in ICU, he or she has a higher probability for developing a HAI. Fifteen percent of all HAI cases in the US have been reported to be ICU patients (Magill et al., 2014). Similarly, 23.4% of HAIs have occurred in ICU patients in the United Kingdom (Health Protection Agency, 2012). ICU patients are at a greater risk for developing a HAI because they are very sick and have a lowered immune response (Umberger et al., 2015). In addition the high use of antibiotics in ICU encourages selection of antibiotic resistance organisms (Dancer, 2001) as well as promoting contamination of the environment with antibiotic resistant organisms (Cantón et al., 2013, Dancer et al., 2006). The ICU, particularly in the outbreak situation, has a high level of environmental contamination, including the presence of multi-drug resistant organisms. When isolating

ICU environments only, one study showed that 14.6% of screened sites were Methicillin-resistant *Staphylococcus aureus* (MRSA) positive (Wilson et al., 2011). Similarly, one study uncovered that 17.7% of sites, mainly faucets and computer keyboards, in pediatric ICUs were MRO positive (Rastogi et al., 2012). What is more, HCWs responsible for patient care may host antibiotic resistant microorganisms on their hands. In fact, 20.5% of HCWs-patient contacts caused contamination of HCWs' gloves or gowns with MRO. Also, 91% of organisms isolated from HCWs were related to ICU surfaces or patient isolates (Morgan et al., 2012).

1.3. Organisms associated with HAIs

Enterococcus faecium, S. aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and a number of species of the genus *Enterobacter* have been classified as significant threats. This is not due to the severity of the illnesses they cause, but due to the fact that if an infection develops with one of these organisms, they easily develop antibiotic resistance. Collectively known as the 'ESKAPE pathogens', these organisms have determined a reassessment of pathogen development, transmission and resistance, because of their resistance to the effects of antibiotics. In addition to ESKAPE pathogens, *E. coli* and *Stenotrophomonas maltophilia* are among organisms associated with HAIs. In the following section, a discussion and analysis of the impact of ESKAPE organisms on hospital surfaces is provided.

1.3.1. Pseudomonas aeruginosa

P. aeruginosa has the ability to grow in environment that is nutritionally limited, and has been responsible for causing outbreaks in urology and hematology wards, burns units and ICUs (Davis et al., 2015, Gómez-Zorrilla et al., 2014, Rasmussen et al., 2015, Schneider et

al., 2012). It has been estimated that 51,000 HAIs are due to *P. aeruginosa* each year, with approximately 400 mortalities annually in the United States (CDC, 2014).

Many studies have reported outbreaks resulting from *P. aeruginosa*. The sources of these outbreaks were found to be tap water, sinks, water taps and wash basins (Hota et al., 2009, Durojaiye et al., 2011, Knoester et al., 2014, Breathnach et al., 2012). In one instance, a Portuguese hospital suffered a two-year outbreak of *P. aeruginosa*. The pathogen had colonized trays, sinks, beds, countertops, taps, hand gels, soaps and bedside tables. Tap contamination was found to be positively correlated with the contamination levels in hand gels, soaps and sinks ($p < 0.05$), workbench and trays ($p < 0.01$), as well as bed and bedside tables ($p < 0.01$). This information led researchers to conclude that biofilm in the taps was the source of the outbreak (de Abreu et al., 2014).

1.3.2. Staphylococcus aureus

S. aureus is a Gram-positive organism that is resident on approximately 50% of humans principally in their nose, axillae and groin (Frank et al., 2010). MRSA and methicillin sensitive *S. aureus* (MSSA) frequently cause HAIs and have been responsible for outbreaks. More than ten percent of HAI are due to *S. aureus* (Magill et al., 2014).

A prospective cohort study was carried out by Wang et al. (2011) in Taiwan, revealing that 184 cases of both *S. aureus* and MRSA (3% and 97%, respectively) occurred in an adult ICU between 2003 and 2007 (Wang et al., 2011). Another study conducted by Gomes et al. (2012) at a teaching hospital, in Philadelphia, USA, found that 13 out of 179 patients who acquired MRSA died. These 13 patients had relapsed MRSA; there was no mortality with new cases of MRSA. Nosocomial infection with MRSA can result in bloodstream infection which has a mortality rate of 35% and in patients that survive an increased morbidity and

longer hospital stay (Garling, 2008).

In many studies, MRSA have been isolated from hospital surfaces especially those close to patients' areas, such as bed linen, curtains and over-bed tables (Chen et al., 2014, Mirzaii et al., 2015, Telang et al., 2010, Dancer et al., 2009). These sites are the most important reservoirs in the hospital environment as infected patients highly contaminate the surfaces close to them (Dancer, 2011) thus making it easier for these bacteria to be transmitted to new patents. It has been observed that MRSA can persist for up to 9 months in hospital environments (Wagenvoort et al., 2000).

1.3.3. Vancomycin-resistant Enterococci

Gram positive *Enterococcus faecalis* and *E. faecium* are common bowel commensal organisms of man. Clinically they frequently cause urinary tract infections, bacteraemia, and endocarditis which depending on susceptibility is treated with ampicillin, penicillin and vancomycin (Gilmore et al., 2014a). However, this genus has a high level of intrinsic antibiotic resistance especially to aminoglycoside and β-lactam based antibiotics thus vancomycin is often the treatment of choice (Gilmore et al., 2014b). The emergence of vancomycin-resistant *Enterococci* (VRE) has made treatment of these organisms particularly difficult. VRE are typically acquired nosocomially; there have been several incidents of outbreaks in hospitals (Iosifidis et al., 2013, Cilo et al., 2014). Although the infection control practices have been improved, the prevalence of VRE is increasing. For example, morbidity rate of VRE has increased three times from 2007 to 2012 in Germany ICUs (Gastmeier et al., 2014).

The pathogen is able to live for up to four years on hospital surfaces (Wagenvoort et al., 2011). Nosocomial acquisition of VRE has been attributed to environmental

contamination. One study looked at the elements influencing patient contraction of VRE in the private rooms of surgical or medical ICUs. This research relied on Cox proportional hazards when determining the degree of significance each element played in VRE acquisition. The conclusion of the study was that the strongest predictor of VRE acquisition inhabited a room following the occupancy of a VRE-positive patient (Drees et al., 2008). VRE has a high incidence in ICUs; Bonten et al. (1996) found that 12% of 1294 environmental cultures examined contained VRE. The rooms of patients who were positive for VRE at three or four body sites showed even higher levels (60-70%) of environmental contamination (Bonten et al., 1996). This contamination spread to the outpatient facilities as well, with 36-58% of chairs and couches in outpatient areas used by VRE-positive patients being contaminated (Grabsch et al., 2006).

1.3.4. Klebsiella pneumoniae

K. pneumonia is a Gram negative non-motile organism found in the soil but it is also a commonly found as part of the normal flora of the human mouth, skin and intestine (Podschun and Ullmann, 1998). The hospital environment is conducive to the spread of *K. pneumonia* which often causes nosocomial infections and outbreaks, particularly in adult and child ICUs (Vardakas et al., 2015, Sambri, 2014, Mavroidi et al., 2014). During 2009-2010, 8% of 1,749 HAI in American ICUs were caused by *K. pneumonia* (Sievert et al., 2013). Schwaber et al. (2008) concluded that the risk of contracting *K. pneumonia* is significantly heightened by an ICU stay. *Klebsiella* can survive for around three years on dry surfaces (Kramer et al., 2006) and it has been isolated from a wide range of hospital surfaces, such as sink drains, baths, bed bars, bed remote controls and babys' weighing-scales (Guet-Revillet et al., 2012, Tofteland et al., 2013).

1.3.5. Species of Acinetobacter

Acinetobacter species are non-motile, Gram negative environmental bacteria generally found in the soil and water (Dhakephalkar and Chopade, 1994). The species, particularly *A. baumanni*, have become important nosocomial pathogens and outbreaks of clonal *Acinetobacter*, are becoming more frequent especially in immunocompromised patients in ICUs (Tsiatsiou et al., 2015, Royer et al., 2015). It has been estimated that 12,000 HAIs were attributed to *Acinetobacter* annually in the US (US Department of Health Human Services, 2014). *Acinetobacter* infections are associated with a crude mortality rate of the order of 50% and an attributable mortality rate of 8-23% and 10-43% for hospitalised patients and ICU patients, respectively (Weber et al., 2010). *Acinetobacter* can be spread by environmental contamination, as it has been isolated from surfaces frequently touched by patients and HCW and it survives for prolonged periods, even in dry conditions, on objects made from various materials including steel, ceramics, rubber and plastic (Møretrø et al., 2015, Hensley et al., 2010, Thom et al., 2011). Gaddy and Actis (2009) suggested that the ability of *Acinetobacter* to generate biofilms is the reason for its virulence and survival in different environments. Along with it functioning as a nosocomial pathogen, these characteristics make *Acinetobacter* the cause of many HAIs.

1.3.6. Species of Enterobacter

Given that the incidence of *Enterobacter* infections is on the rise, especially in ICUs, *Enterobacter* spp. have recently been acknowledged as significant pathogens. However, not all of the 14 species of the genus *Enterobacter* or biogroups have been identified to be human pathogens (Sanders and Sanders, 1997). The species of *Enterobacter* that cause diseases in humans are *Enterobacter agglomerans*, *Enterobacter sakazakii*, *Enterobacter aerogenes* and *Enterobacter cloacae*; the two former species being the most important

(Gaston, 1988, Chow et al., 1991, Haddy et al., 1991, Hawkins et al., 1991, Andresen et al., 1994). These two species are considered in more detail in the section that follows this one.

In 2010 there were 5,768 HAI in US ICUs caused by *E. cloacae* and *E. aerogenes* (Kallen et al., 2013). Over the past ten years, there has been a considerable rise in faecal carriers and clinically isolation of extended-spectrum beta-lactamase (ESBL)-producing *E. cloacae* in hospitals in France and Germany (Lavigne et al., 2004, Hoffmann et al., 2006). *Enterobacter* is capable of inhabiting a variety of environments and of surviving in dry conditions for sixty days, so it can cause nosocomial infections (Kim et al., 2008).

Many outbreaks have arisen as a result of a contaminated electronic digital thermometer cap (van den Berg et al., 2000), and pre-drawn syringes and the intravenous fluid (Watson et al., 2005). There have also been reports of *Enterobacter* outbreaks in ICU (Boban et al., 2011, Von Baum et al., 2004). An outbreak of *E. cloacae* was documented by Yu et al. (2000) in a neonatal ICU, the authors deriving positive cultures from various items, including sterile gowns, sinks, taps, counter tops, scales and opened milk bottles.

There are ample descriptions of numerous single-clone *Enterobacter* outbreaks likely to have been caused by HCW-facilitated transmission. Talon et al. (2004) detected identical patterns among 18% of *E. cloacae* isolated from a premature unit and paediatric ICU based on pulsed-field gel electrophoresis. Davin-Regli et al. (1996) applied a polymerase chain reaction (PCR) technique to type strains and to examine 185 *E. aerogenes* isolates gathered from two ICUs in a French hospital over twelve months. Their findings were that two-thirds of transmissions resulting in outbreaks in the two ICUs were caused by a single prevalent clone.

The majority of isolates of *E. aerogenes* and *E. cloacae* possess inherent resistance to older antibiotics and can quickly become resistant to new ones. Archibald et al. (1997) conducted an analysis of the level of resistance among *Enterobacter* isolates collected from outpatients and inpatients from eight American hospitals participating in the National Nosocomial Infections Surveillance System, which was initiated by the CDC. The authors observed a trend of rising ceftazidime resistance, which occurred in the proportions of 12% and 26% among outpatients and inpatients, respectively. Furthermore, compared to isolates from other units (26%), those collected from ICUs exhibited higher rates of resistance (36%).

1.4. Routes of transmission

Three major factors form the triangle of HAI risk: patients, HCWs and environmental surfaces. Two decades ago, a study uncovered that the source of pathogens resulting in HAI in ICUs came from the following sources: 40-60% from patients' endogenous flora, 20-40% due to cross infection caused by HCWs' hands, and 20% due to a contaminated environment (Weinstein, 1991). The figure below (Figure 1.1) shows the transmission pathways described above, as well as the means of interrupting these transmissions.

1.4.1 Patients

Based on the data above, it is clear that the patients themselves are the principal source of contamination. Infected patients shed their colonizing bacteria into the healthcare facility that is treating them. Between 18-35% of patients hosting MRSA had contaminated surfaces in their healthcare facilities within 25-33 hours of arrival *(*Chang et al., 2010). Advanced molecular techniques have found that up to 40% of common nosocomial bacterial infections are the result of transmission between patients (Gómez-Zorrilla et al.,

2014, Bloemendaal et al., 2009). However, patient-to-patient transmission is a very broad concept, and may also include transmission from HCWs' hands, or transmission from surface contact.

1.4.2. Health care workers' hands

Hands are widely considered to be one of the primary modes of infection transmission (World Health Organization, 2009). Transmission can be due to both direct contact (hand-to-hand) and indirect contact (hands-to-surfaces). In addition to other factors, the frequency of contact is also a vital determinant when calculating the overall patient exposure to pathogens. McArdle and others (2006) evaluated the contact rates between HCWs and patients and use of appropriate hand hygiene in a 12 bed ICU. For the purposes of their study, the team defined direct contact as HCW contact with skin, wounds, bodily fluids and intravascular equipment. They defined indirect contact as contact with the patient's environment, including handling of case notes, medical equipment and the furniture in the bed space. The study showed that HCWs with more than one patient in their care made 22 direct and 107 indirect contacts on average each day lacking in appropriate hand hygiene; patients were contacted by HCWs 159 times directly and 191 times indirectly. Post-contact hand hygiene rates were found to be 43% for direct contacts and 12% for indirect contacts (McArdle et al., 2006). MRSA transmission from contaminated hands has been observed to occur with equal frequency via direct and indirect contact (Stiefel et al., 2011).

In a prospective cohort study, Morgan and colleagues found that 3.4% of HCWs' hands were contaminated with multi-drug resistant *P. aeruginosa* before room entry. For those

HCW whose hands were free of *P. aeruginosa* 17.4% of their gloves were contaminated following provision of care to patients with multi-drug resistant *P. aeruginosa*. Also, they found a significant association between environmental contamination and transmission to HCW clothing (odds ratio = 4.3) which may help to explain the 3.4% of contamination rate of HCW hands following glove removal (Morgan et al., 2012).

VRE can be transmitted to environmental surfaces from HCWs' hands. In one study that looked at surfaces prior to, and following, routine care administered to VRE-positive patients, 10.6% of sites that had been previously free of VRE were contaminated post-contact (Duckro et al., 2005). Transmission also follows the reverse course – from surface to HCWs' hands. In one study, three HCWs contaminated their gloves simply by touching items in the patient's room, despite never coming into contact with the patient themselves (Tenorio, 2001).

In a health care context, HCWs inadvertently assist in the spread of *K. pneumoniae* by carrying the pathogen on their hands. For instance, Rock et al. (2014) found that the hands of 14% of HCW hands were contaminated with *K. pneumoniae* which served as the source of the infection for the patients in that ICU unit. While Joseph et al found that *K. pneumoniae* was present in 3% and 6% of swabs, respectively, taken from the hands and throats of HCWs (Joseph et al., 2010). Similarly, Markogiannakis et al (2008) isolated *Acinetobacter* from 12 (28.6%) of 42 HCW hands. The spread of *K. pneumoniae* and *Acinetobacter* is facilitated by the fact that the pathogen can survive for a number of hours on the hands of HCWs (Casewell and Phillips, 1977, Musa et al., 1990).

1.4.3. Environment

Although HCWs' hands are often responsible for pathogen transmission, it is also possible

for contaminated surfaces and contaminated medical equipment to contribute to transmission of several pathogens including VRE, *Clostridium difficile*, *Acinetobacter*, MRSA and norovirus (Weber et al., 2010, Creamer et al., 2014, Huang et al., 2014). These pathogens, in particular, are secreted in large numbers from infected patients and hence contaminate nearby surfaces. 'High-touch surfaces' include sinks, side-rails, telephones, bedside tables, call boxes and other surfaces are frequently touched by HCWs and patients and hence are more likely to be a source of transmission (Huslage et al., 2010, Guyot et al., 2013, Rocha et al., 2013, Creamer et al., 2014).

When a patient is admitted to a hospital room following a previous patient who was colonized or infected with MRSA, VRE, *C. difficile*, or multi-drug resistant Gram-negative bacilli, his or her risk of colonization or infection increases (Drees et al., 2008, Datta et al., 2011, Nseir et al., 2011, Shaughnessy et al., 2011). One 20-month retrospective cohort study covering eight ICUs, indicated that patients had an increased risk of 14.5% (P = 0.02) contracting VRE if admitted to a room after it had been occupied by an VRE-infected patient, compared to if patients were admitted to rooms after VRE-negative patients (Huang et al., 2006). The average overall increase in risk has been calculated to be 73% (Carling and Bartley, 2010).

Similarly sharing a room with a MRO infected or colonized patient increases risk of colonization. A retrospective cohort study, over an eight year period in a 472 bed acute-care teaching hospital, found that patients who shared a room with MRSA-positive patients had a substantial increased chance of contracting the pathogen themselves. Of the 198 patients who shared a room with a patient with undiagnosed MRSA, 14.6% acquired MRSA. In all of the cases, the MRSA strain was the same as that of their room-mates (Moore et al., 2008).

A range of multi-drug resistant microorganisms have been found to remain and persist in the environment for extended periods of time, causing increased likelihood of surface and medical equipment contamination, and an ongoing risk of transmission via HCWs' hands (see section 1.3). In such instances, the environment can lead to continuing outbreaks and thus carries a long-term risk of infection (Wendel et al., 2015, Willmann et al., 2015, Lin et al., 2013). Critical care units have been identified to be significant sources of *P. aeruginosa* with 26% of wet and 6% of dry environments being colonised (Quick et al., 2014). This pathogen has been found to persist for up to 2.5 years on a wet surface and for up to 5 weeks on a dry surface (Kampf et al., 1998, Kramer et al., 2006). Snitkin et al. (2012) documented an outbreak of carbapenem-resistant *K. pneumoniae* in 18 patients, which resulted in 11 deaths. Whole-genome sequencing of patient isolates and epidemiological analysis, found that the index patient had been released from hospital three weeks prior to the clinical manifestation of the ensuing outbreak. Genomic comparisons supported the occurrence of environmental contamination (Snitkin et al., 2012).

Remarkably, pathogens have also been reported in uninfected patient rooms. For example, *C. difficile* was discovered in 16% of non-infected patient rooms, commonly on portable equipment or in the physician and nurse working areas (Dumford Iii et al., 2009). A similar study found MRSA contaminating 12.5% of ICU nurses' workstations (Hardy et al., 2007). The occurrence of these bacteria in uninfected patient areas may be caused by HCWs or visitor contact (direct or indirect).

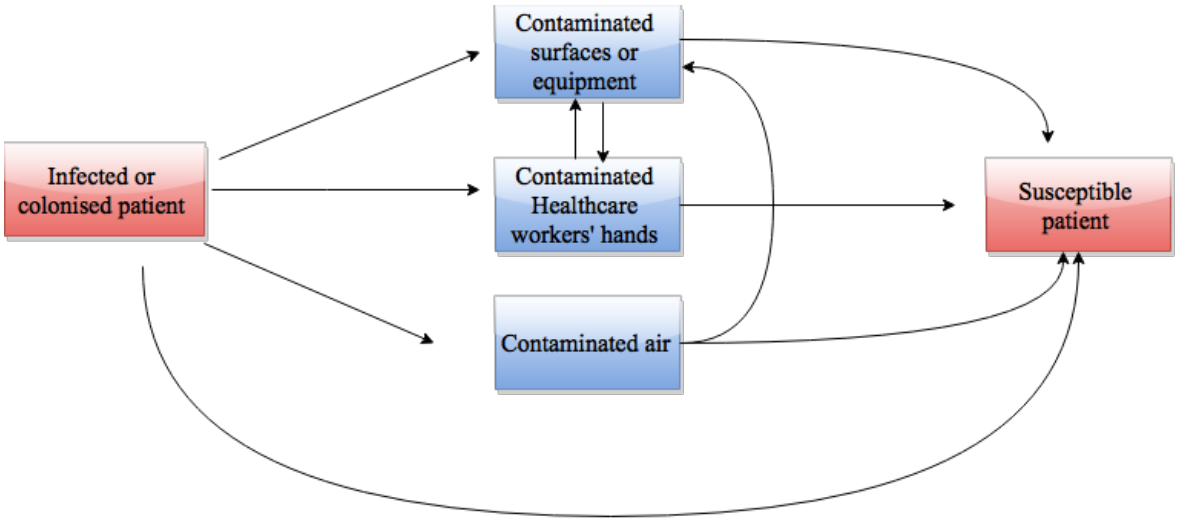

Figure 1.1. Generic transmission routes, adapted from Otter et al. (2011).

1.5. Water distribution systems

It has been recognized that hospital water distribution systems can be a source of nosocomial infection, although this is thought to be controllable. Biofilms, formed by water-borne bacteria, can exist and persist in the piping for a potable water supply, air conditioning cooling towers (Osawa et al., 2014), sinks (Hota et al., 2009) and showerheads (Kossow et al., 2014). Patients are then exposed to water-borne pathogens via contaminated HCW hands, by bathing and showering (Halachev et al., 2014, Ozerol et al., 2006). For some organisms such as *Legionella* spp the aspiration of contaminated water aerosols during showering is believed to be a major transmission method (Anaissie Ej, 2002).

Although water system is treated (by heating to 71°C for 30 minutes and flushing), it may still contain low levels of microorganisms, such as *Legionella* spp. (Wong et al., 2006). The ability of *Legionella* species to persist in hospital water distribution systems was

confirmed by Casini et al. (2008) who found *Legionella* spp., over a period of five years, despite treatment with chlorine dioxide. At the end of the study, the *Legionella* spp count was reduced but complete eradication had not been achieved (Casini et al., 2008).

Unlike with the main water supply, there is usually greater contamination in the plumbing systems of buildings, due to the fact that they contain lower levels of disinfectant. In some areas, such as dead legs and blind ends, there is stagnating water, which promotes bacterial colonisation and proliferation. The complexity of hospital water systems increases the risk of infection, which is particularly high during building or renovation works. The concentration of possible pathogens in the water is fostered by several factors, including the lack of constant use of water and/or an inconsistent flow rate, which can result in stagnation intervals, as well as changing pressure differentials, which can lead to interference by external contaminants and/or descaling *(*Mermel et al*.,* 1995*,* Nygård et al., 2007); while biofilm accumulation and biofilm induced corrosion is influenced by water chemistry, a low water flow, the inadequacy of water treatment, increasing age of water systems and shear stress (Exner et al., 2005).

Bacterial contamination of tap handles, hand-washing basins and waste outlets can occur as a result of contaminated incoming water. It can also be due to transfer from the hands of the HCWs carrying bacteria from patients during hand-washing or during the disposal of water used to bathe patients (Hota et al., 2009, Breathnach et al*.,* 2012*,* Vergara‐López et al., 2013).

1.6. Biofilm infections related to medical devices

1.6.1 Medical equipment (Non-implantable medical devices)

Contaminated medical devices are considered to be major sources of HAIs, and are consequently believed to be significant sources of morbidity and mortality (Tena et al., 2005, Srinivasan et al., 2003). Bacteria have been isolated from several medical instruments, including stethoscope, endotracheal, endoscopes and orthopaedic tourniquets (de Abreu et al., 2014, Souza et al., 2015, Ren-Pei et al., 2014, Sahu et al., 2015). However, it is the endoscope that has been the instrument mostly frequently associated with transmission of infection (Kovaleva et al., 2013).

Medical devices can be contaminated directly due to inadequate cleansing or lack of adherence to infection control practices (Bou et al., 2006) or indirectly via contaminated tap water, HCW hands and contaminated surfaces (Bert et al., 1998).

A study conducted by Kirschke et al. (2003) demonstrated an outbreak of both *P. aeruginosa* and *Serratia marcescens* had been caused by a contaminated bronchoscope. It was found that a biofilm of *P. aeruginosa* and *S. marcescens* accumulated in the biopsy port and inside the cap of the bronchoscope (Kirschke et al., 2003). Similarly, a significant outbreak due to a loose biopsy port cap occurred in Johns Hopkins hospital between June 2001 and January 2002. Of 39 infected patients, three died (Srinivasan et al., 2003). Furthermore, an outbreak reported by Pitten et al. (2001) revealed that a colonoscope, a gastroscope, a suction device, a nasogastric feeding tube and a urinary bag was contaminated with *P. aeruginosa* suggesting frequent non-adherence to infection control guidelines.

There has been a significant focus on endoscopic retrograde cholangio-pancreatography due to the occurrence of several outbreaks. In 2013, there was an outbreak of New Delhi metallo-β-lactamase-producing *E.coli* resulted in 39 infections in Illinois, the United States. New Delhi metallo-β-lactamase – producing *E. coli* isolated from a reprocessed duodenoscope was identical patterns among 92% of all confirmed cases based on pulsed-field gel electrophoresis (Epstein et al., 2014). Another study has reported 7 cases infected with carbapenem-resistant *Klebsiella* from June 2008 and January 2009 in Florida, United States. The author recognized the source which was due to insufficient cleaning of elevator wire (Alrabaa et al., 2013). Several studies have demonstrated a relationship between ERCP endoscopes and transmission of MROs due to the configuration of the ERCP endoscopes, such as elevator piece, is difficult to clean and sterilise and therefore they act as a source of MROs (Aumeran et al., 2010, Wendorf et al., 2015, Ross et al., 2015).

1.6.2 Implantable medical devices

A biofilm can be formed on many devices, including orthopaedic devices, cardiovascular implantable electronic device, spine device and shoulder prosthesis (Sarrazin et al., 2012, Furustrand et al., 2015, Sax et al., 2015, Campoccia et al., 2006). Both Gram-positive and Gram-negative bacteria, including *E. faecalis* (Arciola et al., 2008), *E. coli* (Soto et al., 2007), *K. pneumoniae* (Bellifa et al., 2013), and *Staphylococcus* spp. (Bongiorni et al., 2012) have been isolated from implantable medical devices. *Staphylococcus* spp. is the most common cause of device infection accounting for up to 66% (Campoccia et al., 2006, Gandhi et al., 2012).

As bacteria colonising on implanted medical devices do not necessarily exhibit signs of clinical infection, the occurrence of biofilms on such devices may be underrated. An

investigation of subclinical infection in implanted cardiac medical devices, such as pacemakers and defibrillators, was conducted by Rohacek et al. (2010). Bacteria of many species, primarily *Propionibacterium acnes*, Coagulase-negative staphylococci and Gram-negative rods, were present on 38% of the cardiac medical devices that were extracted for reasons other than infections (there was no clinical signs of infection).

1.6.3 Catheters

In the United States alone, there were more than 93,000 urinary tract infections in 2011, 38% of which were related to the use of urinary catheters (Magill et al., 2014). Approximately 13,000 mortalities are attributed to urinary tract infections yearly in the United States (Gould et al., 2010). It has been estimated that up to 25% of hospitalized people require urinary catheterization as part of their treatment (Gould et al., 2010).

The use of central venous and arterial catheter in ICUs is routine. The incidence rate of central venous and arterial catheter colonization with bacteria after implantation has been found to be approximately 18% (Vallés et al., 2008). The mortality rate associated with the use of venous and arterial catheter are up to 25% (Fletcher, 2005).

Implantable devices that provide an artificial pathway through the body's natural defences such as venous and urinary catheters readily become colonised usually by the patients own bacteria residing in the area of the device. For example, venous catheters are usually colonised by skin organisms whilst urinary catheters are colonised by organisms resident in the perineal area. Wang et al. (2010) discovered that the urinary tract catheters of 45 patients were colonised with a mixture of species of Gram-negative and Gram-positive bacteria, including coagulase-negative Staphylococci, *E. coli, P. aeruginosa*, *K. pneumoniae* and *S. aureus* in the form of a biofilm.

1.7. Associated Surgical Site Infections

Invasive procedures can give rise to wound infections known as surgical site infections (SSIs). According to estimates, every year around 234 million medical operations are carried out throughout the world (Weiser et al., 2008). Furthermore, among all HAIs, SSIs were discovered to be the most prevalent healthcare-related infection (31%), generating costs of $3.5-10 billion in the US alone (Thompson et al., 2011, Magill et al., 2012). SSIs are also associated with significant rates of patient morbidity and mortality. Patients who contract SSI have a five times greater likelihood of being hospitalised again within one month and a twice greater likelihood of dying (Dobson et al., 2011). The general rate of SSIs was estimated, based on the US national healthcare safety network data, to be 1.9% (Mu et al., 2011).

Although it is possible to prevent the majority of SSIs, many infections are acquired through endogenous spread due to contamination of incisions made during surgical procedures with bacteria from the patient's body (Hospital Healthcare Europe, 2008). At the same time, SSIs can also occur due to exogenous spread, which is when a surgical incision is contaminated with microorganisms present on surgical instruments or in the theatre environment or through cross-infection after surgery (National Institute for Health and Clinical Excellence and the National Collaborating Centre for Women's and Children's Health, 2008, Alexander et al., 2013). Bacterial colonisation and infection can disrupt the balance between destructive and healing processes, thus hindering wound healing. James et al. (2008) confirmed that biofilms are one of the causes of chronic wound infection. In recently conducted research, Akers et al. (2014) argued that organisms that generate biofilms are of key significance ($P = 0.024$) in recurrent skin and soft tissue wound infections among post-surgery military staff with injuries acquired during service; this was not found among the cohort group employed as controls. The isolation of multi-

drug resistant organisms, such as *A. baumannii*, *K. pneumoniae* and *E. coli*, led the authors to conclude that recurrent skin and soft tissue infections were due in large part to the existence of such a polymicrobial biofilm (71.4%), which resulted in a greater bioburden, aggravated infections, enhanced resistance to antibiotics, and an augmented inflammatory reaction in affected tissues (Akers et al., 2014).

To facilitate the identification of biofilm-related infections, Hall-Stoodley et al. (2012) put forward a series of diagnostic guidelines, which include:

1. Microbiological evidence of a delimited chronic or foreign body-related infection.
2. Microscopic evidence of microorganism colonisation.
3. Previous conditions associated with biofilm formation, such as an implanted medical device, cystic fibrosis, infective endocarditis and chronic otitis media.
4. Chronic or recurring infections, especially if caused by the same organism.
5. Documentation of unsuccessful antibiotic treatments or of infections that persisted in spite of prompt measures taken to control them.
6. Evidence that infectious symptoms disappear during antibiotic treatment but reappear after the completion of treatment.
7. Evidence of an immune system reaction to the detected microorganisms, such as the presence of antibodies targeting specific pathogens (e.g. cystic fibrosis patients developing antibodies to alginate or other *P. aeruginosa* antigens).

Tice et al. (2003) conducted an investigation of 454 civilian patients suffering from osteomyelitis due to soft tissue wounds and surgery. Their findings revealed that infection was recurrent in 30.6% of cases. Of these, relapses (initial pathogen), reinfections (a different pathogen) and undetermined relapse or reinfection accounted for 15.8%, 16.5% and 67.7% of recurrent infections, respectively.

Part Two: Biofilm

1.9. Introduction

For many decades, there has been a significant focus on microbial biofilms and their effects in different settings, such as medical, environmental and industrial. Biofilm is defined as an accumulation of immobile (sessile) microbial cells that attach to each other on a surface and are encased in an extracellular polymeric substance matrix (EPS), which consists of protein, DNA and a polysaccharide material (Hall-Stoodley and Stoodley, 2009, Høiby et al., 2010).

Biofilm was first discovered by Antonie van Leeuwenhoek when he was examining the plaque on his teeth in the 17th century, cited by Donlan and Costerton (Donlan and Costerton, 2002). In 1940, Heukelekian and Heller concluded that the development and evolution of marine microbes significantly increased with the existence of a substratum to which microbes attach (Heukelekian and Heller, 1940). In 1943, Zobell observed marine microorganisms by direct microscopy and noticed that microorganisms adhered to the walls of bottles (Zobell, 1943). In addition, Marshall noticed a connection between 'very fine extracellular polymer fibrils' and the attachment of microorganisms to a surface (Marshall, 1976). However, the theory behind biofilm formation was established by Costerton et al. (1978).

After it was recognized that many microbes live naturally within biofilm, interest in biofilms increased significantly. It has been well documented that the majority of microorganisms are able to aggregate, attach and form a biofilm on a variety of surfaces (i.e. living surfaces, such as heart valves, and non-living substrata, such as medical devices

and hospital surfaces), causing a range of infections and both economic and environmental issues (Djeribi et al., 2012, Feazel et al., 2009, Soto et al., 2007). Microbial biofilms are found in almost all wet environments where nutrients are present and surface adhesion is feasible (Singh et al., 2006). It has been estimated that up to 99.9% of bacteria join in the biofilm mode when a natural ecosystem is available (Costerton, 2009). The manner in which biofilm forms makes bacteria more tolerant not only to the human immune response, but also to heat, chemical disinfectants and detergents and antibiotics (Mukherjee et al., 2012, Singh et al., 2009, Martinez and Casadevall, 2007, Corcoran et al., 2014). Biofilms cause a serious problem for public health due to their antibiotic tolerance, making them a primary complication of device-associated HAIs (Mulla and Revdiwala, 2011). The CDC found that approximately 65% of HAIs involve biofilms (Flannery, 1999). Therefore, a better understanding of biofilm components is fundamental to infectious disease control.

1.10. Biofilm structure

Biofilm consists of a multicellular population. Although it has been hypothesized that "the biofilm structure is largely determined by the substrate concentration gradient at the biofilm-liquid interface and the detachment forces working on the biofilm" (Van Loosdrecht et al., 1997), it has also been suggested that the basic structure of biofilm is the micro-colony, EPS, water channels and void spaces (Donlan and Costerton, 2002, Dunne, 2002, Flemming and Wingender, 2010). Environmental biofilms are composed of multiple species of organisms. The organisms are spread randomly throughout the EPS, and comprise 10-25% of the biofilm (Costerton et al., 1994, Costerton, 1999). In chronic wounds biofilms *S. aureus* and *P. aeruginosa* have been shown to cluster in single species sections of the biofilm (Fazli et al., 2009). This remains unknown for biofilms on dry hospital surfaces.

In fluid environments the EPS is thickest in the center of the micro-colony and comprises 75-90% of the biofilm (Costerton, 1999, Costerton et al., 1994); while in environments with lower water availability, the EPS is thickest at the exposed surface (Roberson and Firestone, 1992, Chang et al., 2007, Lawrence et al., 1991). The thickness of the biofilm differs with culture conditions and between bacterial species and even strains. *Vibrio parahaemolyticus* and *P. aeruginosa*, for example, have been found to generate two fold thicker biofilm than *Pseudomonas fluorescens* (Lawrence et al., 1991).

The shape of the biofilm depends on several parameters, such as shear stress, the accessibility of nutrients and waste removal (Costerton et al., 1995) with same bacteria forming cone, coral like or mushroom structures (Klausen et al., 2003, Dunne, 2002, Wimpenny and Colasanti, 1997, Stapper et al., 2004).

Apart from nutrients, quorum sensing has also been found to be an important factor in biofilm architecture as it regulates EPS secretion, which impacts on the density of biofilm (discussed below) (Davies et al., 1998, Kierek-Pearson and Karatan, 2005). The structure of biofilm is shown in Figure 1.2.

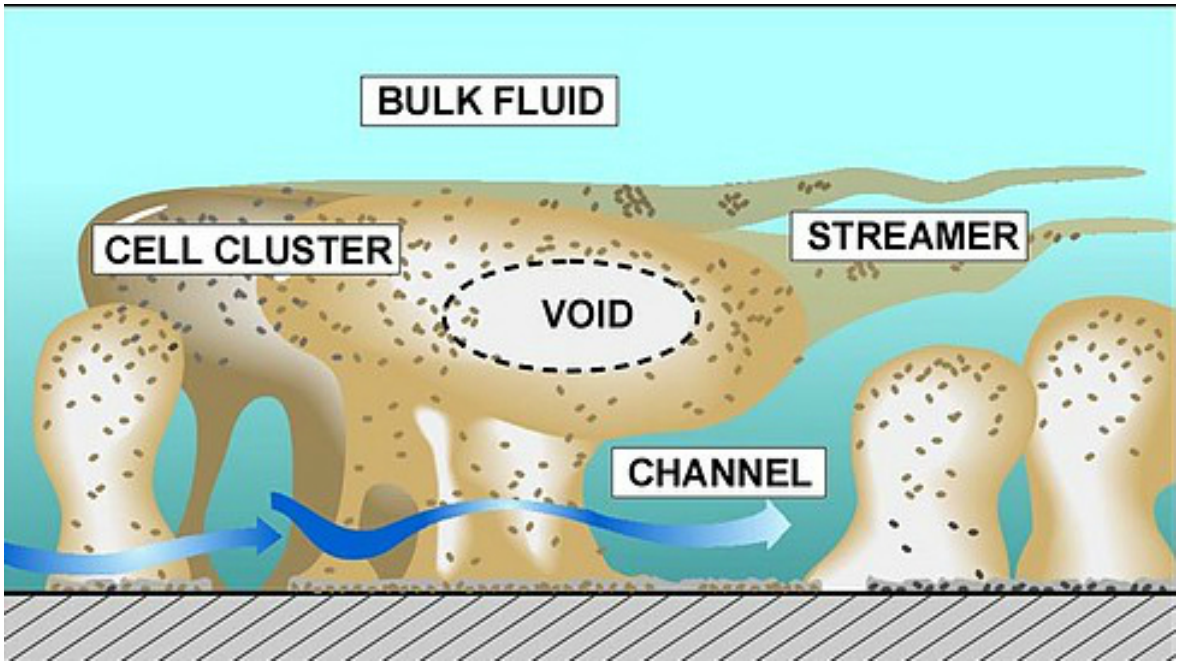

Figure 1.2. Biofilm structure. Theoretical illustration of the heterogeneity of biofilm construction, showing microbial clusters located within an EPS, 'mushrooms', voids and water channels (Dirckx, 1996).

1.11. Biofilm formation

The biofilm life cycle comprises three distinct steps. These steps are discussed below and illustrated in Figure 1.3.

1.11.1. Primary Phase

Biofilm development is initiated when microorganisms sense that the substratum is conditioned (i.e. contains organic molecules), prompting bacteria to switch from the planktonic mode to the biofilm mode (Jain and Bhosle, 2009, Schneider and Marshall, 1994, Schneider et al., 1994). The formation of a biofilm can start with either a solitary species or several (Pratt and Kolter, 1998). Bacteria (planktonic) initially stick to the surface. The initial attachment is facilitated by, 'weak reversible van der Waals interactions', Brownian motion, electrostatic charges and hydrophobic interactions

between the cellular outer surface and the substratum. This attachment is considered to be a reversible (Høiby et al., 2010, Dufour et al., 2010, Hori and Matsumoto, 2010, Gottenbos et al., 2002). Motile and non-motile microorganisms utilize a diverse manner of surface molecules or apendages, such as pili, flagella, and fimbriae (Kierek-Pearson and Karatan, 2005).

Fimbriae, which are proteins attached to the cell surface, are involved in bacteria attachment on the surface in nature. Fimbriae, such as two Aggregative Adherence Fimbriae (AAF/I and AAF/II), are an important in the initial attachment and biofilm formation in *E. coli* (Van Houdt and Michiels, 2005, Wu and Outten, 2009, Boisen et al., 2008). Moreira et al. (2003) found that a significant decrease in biofilm development occurred when type I fimbriae were mutated.

Similarly initial bacterial attachment is facilitated by production of lengthy protein fibres, known as pili (Bradley, 1980, Watnick and Kolter, 1999). *Haemophilus influenza* strains which lack pili, exhibit a remarkable decline in ability to form a biofilm (Murphy and Kirkham, 2002). Furthermore, it has been found that *E. coli* requires not only pili (type I) but also flagella to attach to the surface, whereas only flagella are needed for bacterial motion. Additionally, *P. aeruginosa* is unlikely to be able to form a biofilm in the absence of pili (type IV). This is thought to be due to both insufficient twitching motility (Beatson et al., 2002, Watnick and Kolter, 1999), which is essential for host colonization and also for biofilm development (Comolli et al., 1999, O'Toole and Kolter, 1998) and a considerable decline in the ability of bacteria to attach to a surface (Sauer et al., 2002). Biofilm formation and surface attachment depend significantly on flagella, even though not all bacterial cells in the biofilm are motile (Lemon et al., 2007, O'Toole and Kolter, 1998, Pratt and Kolter, 2002). Flagella, which are defined as filamentous protein structures

adhered to the bacteria surface, are required for bacteria movement . The majority of Gram-negative bacteria need flagella for biofilm development. For instance, flagella were found to enable *Listeria monocytogenes* to adhere to a surface in the early stages of biofilm formation (Lemon et al., 2007). Moreover, it is well established that flagella may perform other functions that allow planktonic to move towards sources of nutrition or respond to signals from other cells on a surface (Pratt and Kolter, 1998).

Non-motile microorganisms use cell surface molecules to colonize a surface. For example, it has been suggested that *S. aureus* and *S. epidermidis* use polysaccharide intercellular adhesion and hemagglutinin, synthesized by *icaADBC* encoded proteins, to adhere to a surface (Rohde et al., 2007). *Staphylococci* species, in particular *S. aureus* and *S. epidermidis,* have also been found to utilize several proteins, including 'microbial surface components that recognize adhesive matrix molecules' collectively named MSCRAMMs, to encourage bacteria to attach to a surface (Vazquez et al., 2011). *S. aureus* can covalently bind to the surface by expressing up to 21 different MSCRAMMs proteins (Roche et al., 2003). Distinctive members of the MSCRAMM family are Clumping factor (Clf) A and B proteins (Walsh et al., 2008), Fibronectin-binding proteins A and B (FnBPA/FnBPB) (Tang et al., 2013, McCourt et al., 2014), fibrinogen binding protein (fib) (Cardile et al., 2014), elastin binding protein (ebps) (Nakakido et al., 2014), laminin binding protein (eno) staphylococcal protein A (SpA) (Cardile et al., 2014).

Additionally, there are some fundamental factors that increase the ability of bacteria to attach to a surface. When the surface is abiotic, non-specific connections, such as those that are hydrophobic, help bacteria to adhere to the substratum. In the case of biotic surfaces, the attachment is achieved by molecular 'docking mechanisms' (Pace et al., 2005).

The probability of bacterial attachment also increases when there is a high degree of surface roughness (Yoda et al., 2014) which is thought to be due to a reduction in shear force and to rough stratums having a higher surface area in comparison to flat surfaces (Donlan, 2002). In addition, it is likely that the stratum's physicochemical characteristics play an essential role in the degree of adherence (Donlan, 2002). In this phase, bacteria are not protected and are thus vulnerable to antimicrobial agents.

1.11.2. Secondary phase (maturation)

The maturation stage starts just minutes after initial attachment, when the early-attached bacteria proliferate and divide, and recruit other species, during which many gene alterations occur. The reversible attachment shifts to become irreversible (Høiby et al., 2010, Aparna and Yadav, 2008). The bacteria start secreting chemical signals, such as acylated homoserine lactones (in Gram-negative bacteria) and peptides (in Gram-positive bacteria), which allow organisms to communicate with each other, and to supervise the population density (Fuqua, 2006, Ahmer, 2004, Cvitkovitch et al., 2003). For example, *S. aureus* increases the expression of accessory gene regulator (Agr) after attachment to the surface. The Agr system utilizes a peptide pheromone (autoinducing peptide) (Periasamy et al., 2012)

When the chemical signals reach a particular threshold level, an EPS is secreted by the sessile cells (Costerton et al., 1999). The biofilm develops more density and complexity due to the multiplication of sessile bacteria and recruitment of other bacteria from the environment. The bacteria become sessile once the coating of accumulated cells thickness exceeds 10 μm (Aparna and Yadav, 2008). In addition, the formation of water channels, designed to spread nutrients and signal molecules, begins (O'Toole et al., 2000). Once mature, biofilms are shear resistant and tolerant to antibiotics and ultraviolet light (Donlan

and Costerton, 2002, O'Toole et al., 2000).

1.11.3. Dispersion phase

The dispersion of bacteria from a biofilm is an essential stage of the biofilm life cycle. There are integral factors (both inside and outside the biofilm) that play important roles in bacterial biofilm detachment. Accessibility to nutrients, the exudation of nutrients to cells in the biofilm and the elimination of waste are important to the maintenance of a biofilm (Dunne, 2002, Kierek‐Pearson and Karatan, 2005).

Bacteria disperse from a biofilm in different ways. Firstly, there is active dispersion, which is induced by bacteria themselves in a specific planned manner during starvation. For example, bacteria produce a degradation enzyme to break the matrix which allows them to disperse and colonise a new conditioned surface. It has been found that *S. aureus* secrets 10 proteases to degrade proteinaceous matrix components and two extracellular nucleases (Mootz et al., 2013, Kumar Shukla and Rao, 2012, Kiedrowski et al., 2014). Also, some physiological changes to the cell induce dispersion (Allison et al., 1998).

The 'switching-off' of the *ica* operon may be used by *S. epidermidis* for dispersal from a biofilm (Ziebuhr et al., 1999, Hennig et al., 2007, Arciola et al., 2015) whilst a decline of Agr expression in *S. aureus* results in biofilm detachment (Periasamy et al., 2012).

Secondly, there is passive detachment, which occurs due to exterior dynamics, fluid shear being an example (Wood et al., 2011).

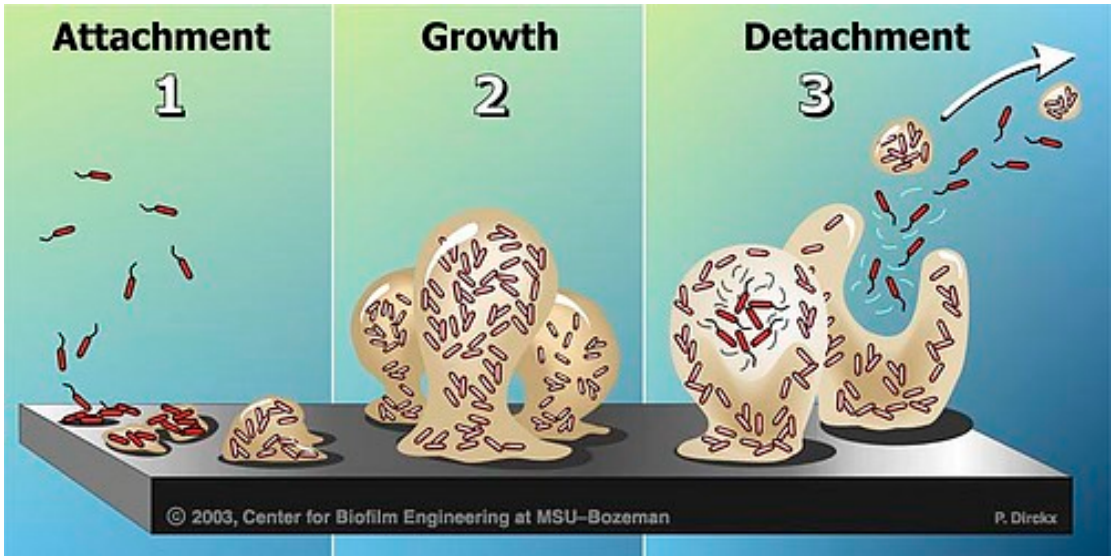

Figure 1.3. Biofilm development. Schematic diagram of the development stages of biofilm. The biofilm life cycle is shown in three stages: 1- the primary phase (attachment), 2- the growth of complex biofilms (maturation), 3- the detachment phase: microorganisms occupy virgin areas and form new biofilms (Stoodley and Dirckx, 2003).

1.12. Extracellular Polymeric Substances

Most bacteria (99%) are surrounded by EPS (Vu et al., 2009) which comprises 75-90% of the biofilm (Costerton, 1999). It has a high molecular weight and is produced by the bacteria, from cell lysis and macromolecule hydrolysis (Liu and Fang, 2003). There are two main forms of bound EPS, that is capsular (tightly bound) or slimy (loosely bound) (Comte et al., 2006, Allison, 2003). EPS can also be soluble. Soluble EPS components probably exist in biofilms formed on dry surfaces, however, the proportion of soluble to non-soluble EPS components is unknown. If soluble components exist then detergents should be able to disrupt the EPS and lead to destabilisation of the biofilm structure.

1.12.1 Composition of EPS

EPS composition, properties and substance percentages vary from one study to another. This is for several reasons including: the particular EPS, the source of the biofilm (i.e. the environmental conditions in which the biofilm grows), the bacterial strain, the growth

phase and the analytical tools used. For instance, aggressive extraction techniques such as NaOH, vapour extraction or prolonged sonication can lead to the contamination of the EPS with intracellular material. In contrast, less aggressive techniques e.g. EDTA, 70°C heating or centrifugation limits the proportion of EPS extracted (Nielsen et al., 1996, Azeredo et al., 1999, Wingender et al., 1999).

EPS may be comprised of polysaccharides, proteins, lipids, uronic acids, teichoic acid and humic substances, which are constituents of the natural organic matter in soil and water environment (Al-Halbouni et al., 2008, Ryder et al., 2007, Tsuneda et al., 2003, Ni et al., 2009). Roughly 50-90% of a biofilm's EPS consists of total organic carbon (Vu et al., 2009) and approximately 89% of EPS is composed of protein and polysaccharides (Tsuneda et al., 2003). Donlan suggests that polysaccharides are the main component and an essential substance within EPS, as they are necessary for the irreversible attachment of the biofilm to surfaces (illustrated below in section 1.12.2.3) (Donlan, 2002).

However, considerable variation in EPS components was found by Sponza (2003) investigating biofilms formed in the wine, petrochemical, textile and pulp-paper industries. They found that the EPS of biofilm formed in the wine industry had the highest protein content 70 mg/g volatile suspended solids (VSS); whilst EPS from biofilm formed in the textile and pulp-paper industries had lower protein content, 38 and 42 mg/g vss respectively. The polysaccharide component varied from 17 mg/g vss in the wine and petrochemical industry and up to 30 mg/g vss in the textile and pulp-paper industries EPS. The lowest level of DNA content was 6 mg/g vss in the wine industry and the highest was 17 mg/g vss in pulp-paper. Consequently, EPS has come to be known as 'the dark matter of biofilms' due to the significant variety in their biopolymers and the difficulty in evaluating them (Flemming, 2011).

1.12.2. Function of EPS

EPS is necessary for the irreversible microbial attachment to the surface and their bioactive products serve to protect microbial cells from the action of antimicrobial agents; guard against surrounding stresses and desiccation by preserving water and nutrients. The EPS also holds microorganisms in close proximity to each other and facilitates the accumulation of microbes (Wingender et al., 1999, Borlee et al., 2010, Looijesteijn et al., 2001). These functions are examined in detail below.

1.12.2.1 Protein

EPS-related proteins play an important role in the functioning of biofilms, as attested by their identification from a wide range of microorganisms (Oda et al., 2006, Eboigbodin and Biggs, 2008). The structural integrity of the matrix, resistance to stress and formation of the biofilm architecture all depend on extracellular proteins (Branda et al., 2006, Islam et al., 2014). For instance in *P. aeruginosa* biofilm the extracellular protein, CdrA in renders the biofilm structurally stable by cross-linking polysaccharides and/or binding cells with polysaccharides (Borlee et al., 2010). Furthermore, the attachment and formation of the biofilm matrix of *S. aureus* requires the involvement of proteins, such as protein A, fibrinogen-binding proteins, *S. aureus* surface protein, biofilm-related protein and clumping factor B (Cucarella et al., 2001, Corrigan et al., 2007, O'Neill et al., 2008, Merino et al., 2009, Geoghegan et al., 2010, Abraham and Jefferson, 2012). The biofilm-associated protein (Bap) is a surface protein that triggers the formation of biofilm in *Staphylococcus* (Cucarella et al., 2001, Tormo et al., 2005). Staphylococcal isolates containing Bap had a high capacity for biofilm formation, despite lacking the *icaADBC* operon (Tormo et al., 2005). Furthermore, in *S. epidermidis*, biofilm formation was suppressed when the *Bap* gene was disrupted; by contrast, a biofilm-negative strain of *S.*

aureus was able to develop biofilm on a surface made of polystyrene as a result of heterologous complementation with the *S. epidermidis* Bap protein (Tormo et al., 2005).

1.12.2.2. Extracellular DNA

Biofilm development by several species of bacteria occurs with the involvement of extracellular DNA (eDNA), including *S. aureus* (Izano et al., 2008), *Streptococcus mutans* (Perry et al., 2009), *Streptococcus pneumoniae* (Moscoso et al., 2006), *Bacillus cereus* (Vilain et al., 2009), *Neisseria meningitidis* (Lappann et al., 2010), *L. monocytogenes* (Harmsen et al., 2010), *P. aeruginosa* (Whitchurch et al., 2002) and *E. faecalis* (Thomas et al., 2008).

P. aeruginosa biofilm eDNA is thought to be derived from membrane vesicles (Whitchurch et al., 2002), whilst in *S. epidermidis* eDNA is thought to be produced by lysis of a subpopulation of bacterial cells (Qin et al., 2007). The eDNA produced by bacterial lysis is essential for the initial attachment of *S. epidermidis* and results in stimulation of the remaining bacterial cells to form biofilm (Qin et al., 2007). Bacteria develop biofilm to protect them from sever environmental insults.

The significance of eDNA as a component of the biofilm matrix and its key function in the formation and dynamics of biofilms and bioaggregates has been highlighted by numerous studies (Watanabe et al., 1998, Dominiak et al., 2011). Mann et al. (2009) found that treating *S. aureus* biofilms with DNase diminished cell attachment to the surface. Watanabe et al. (1998) observed that treatment with nucleic acid-degrading enzymes interrupted flocculation in *Rhodovulum* sp. Likewise, Dominiak et al. (2011) reported that, in comparison to when they used only shear force, activated sludge treated with DNase and shear force, completely disintegrated the flocs. Nemoto et al. (2003) provided evidence that the involvement of extracellular DNA in biofilms takes the form of a cell-to-cell

interconnecting compound.

1.12.2.3. Polysaccharides

Polysaccharides, which are considered to be the most essential of the EPS components, assist microorganisms in adhering to a surface and are needed for biofilm development. *S. epidermidis* and *S. aureus*, for instance, secrete polysaccharide intercellular antigen or poly-N-acetyl glucosamine, which have both been found to assist biofilm formation, virulence and microbial attachment (Cramton et al., 2001). *Staphylococcus* spp. , Gram positive organisms, generally use poly-N-acelylglucosamine and teichoic acid for intracellular adhesion which has an overall positive charge (Mack et al., 1996, Kogan et al., 2006). The Gram negative organism, *P. aeruginosa*, on the other hand, produces uronic acid containing polysaccharides with a net negative charge (Sutherland, 2001). Furthermore, in *P. aeruginosa*, biofilm development was found to involve a minimum of three types of exo-polysaccharides, namely, Psl, Pel and alginate (Ryder et al., 2007, Colvin et al., 2012). Psl is a polysaccharide rich in mannose and galactose, whilst Pel is rich in glucose (Friedman and Kolter, 2004) and they both facilitate attachment of bacterial cells to a substratum and support the biofilm architecture (Friedman and Kolter, 2004, Jackson et al., 2004, Matsukawa and Greenberg, 2004, Ma et al., 2006). Alginate also contributes to the biofilm architecture and, in addition, is involved in antibiotic resistance and offers *P. aeruginosa* protection against adverse conditions (Hentzer et al., 2001, Nivens et al., 2001, Wozniak et al., 2003).

1.12.2.4. Glycoconjugates

The location of glycoconjugates in biofilms is on the surface of the bacterial cell, where they take the form of lipopolysaccharides or lipoteichoic acids, capsules or occasionally adhesive material that can be detected as microbial ‘footprints’ (Neu and Marshall, 1991).

Moreover, glycoconjugates produce microbial mats and bind bacterial cells, micro-colonies, biofilms and bioaggregates (Neu and Lawrence, 2014). Zippel and Neu (2011) also noted that, due to bacterial detachment and dissemination, glycoconjugates in biofilm systems may take the form of cloud-like structures without cells.

1.12.3. Mechanism of EPS secretion

The mechanism of EPS production and the way in which the matrix develops remains poorly understood (Ma et al., 2009). Yet, as pointed out by Flemming, microorganisms produce EPS during initial attachment to the surface. After bacterial cells reversibly attach to a surface, a micro-colony develops and the secretion of EPS begins (Flemming, 2011). In comparison to free-living *P. aeruginosa* cells, attached cells produced 2.5 times more carbohydrates and extracellular polysaccharides (Vandevivere and Kirchman, 1993).

EPS production is thought to occur with the involvement of certain genes. For instance, in studies on *P. aeruginosa* and *Azotobacter vinelandii* respectively, Boucher et al. (1997) and Mejıca-Ruıcz et al. (1997) identified a similar group of genes to be involved in regulating the biosynthesis of extracellular molecules and secretion. This group of genes consists of the genes *algA*, *algD and algE*, which encode gDP-mannose pyrophosphorylase, gDP-mannose dehydrogenase, respectively, and a membrane protein possibly participating in alginate distribution. The reactions of alginate biosynthesis in *P. aeruginosa* were further reported to be inhibited by a defect in the *algK* gene (Aarons et al., 1997, Jain and Ohman, 1998).

The *icaADBC* locus of *S. epidermidis* encodes the PIA synthesis apparatus which is responsible for the synthesis of polysaccharide intercellular adhesion molecules (Heilmann et al., 1996). Inactivation of the *icaADBC* locus results in a biofilm negative phenotype

(Mack et al., 1999). EPS biosynthesis in *B. subtilis* involves the activity of the gene *eps G* (*yveQ*), which encodes a protein with possible involvement in EPS polymerisation, and the gene *epsH* (*yveR*), which encodes a protein participating in glycosyltransferase (Marvasi et al., 2010).

The association of enzymes with certain EPS components has been suggested in many studies. A specific secreted enzyme is responsible for some extracellular polysaccharide syntheses and the synthesis of Levan illustrates this. Levansucrase is the extracellular enzyme possessing saccharose specificity that regulates Levan biosynthesis (Meng and Fütterer, 2003).

1.12.4. Factors affecting EPS production

A wide range of environmental factors can affect the amount of and character of the EPS composition of a biofilm, and thus EPS production.

1.12.4.1. Oxygen levels

Anaerobic conditions lead to a reduction in the EPS formation by aerobic bacteria (Nielsen et al., 1996); whilst anaerobic conditions are essential for EPS production by anaerobic bacteria like *S. mutans* (Ahn et al., 2008). The composition of the EPS can vary with oxygen levels. At high levels of dissolved oxygen, more carbohydrates were produced over time, but the quantity of proteins did not change; however, at low levels of dissolved oxygen, carbohydrates and proteins were of similar concentration in sludge (Shin et al., 2001).

1.12.4.2. Desiccation

EPS production is induced when the biofilm is exposed to dehydration. A species of *Pseudomonas* extracted from soil was used to analyse how desiccation and bacterial

synthesis of EPS are correlated. Roberson and Firestone (1992) observed that, unlike cultures developing in the presence of sufficient water supply, those exposed to desiccation during growth in a sand matrix had more EPS, implying that desiccation prompted the distribution of resources to produce EPS. Furthermore, at low water potential, the *Pseudomonas*-generated purified EPS contained several times its weight in water. The authors suggested that bacterial cultures were protected against some desiccation effects by the EPS matrix, since considerably more water was held by sand amended with EPS in comparison to sand alone, which also dried faster (Roberson and Firestone, 1992).

Similar to *Pseudomonas*, *E. coli* also exhibited greater EPS production when subjected to desiccation. This is consistent with the higher resistance to desiccation manifested by soil *E. coli*. Six important organic solutes (trehalose, proline, glutamine, acetate, valine and glucose) were investigated to determine whether there were any discrepancies in concentration among desiccated and hydrated cells. Of these solutes, the only one that was closely correlated with *E. coli* under desiccation condition was trehalose (Zhang and Yan, 2012). A non-reducing disaccharide, trehalose has great resistance to heat and extreme pH (Saito et al., 1998) and fulfils the functions of energy supply, stress protection, and protein stabiliser (De Virgilio et al., 1990, Jiao et al., 2011).

In the case of *Pseudomonas putida*, the increase in overall production of EPS and alginate was directly proportional to severity of stress. As explained by Chang et al. (2007), the degree of biofilm cell dehydration is decreased by alginate through the use of a biosensor to measure the water potential of separate cells as well as the impact of matric or solute stress shock on desiccation-facilitated alterations in fatty acid composition.

1.12.4.3. Temperature

Attention has also been paid to the extent to which the cultivation temperature affects

exogenous proteins and exopolysaccharide biosynthesis by bacterial cells. For *L. monocytogenes,* the temperature range most conducive to the production of the majority of EPS molecules was determined to be 22-30°C (Perry et al., 2004, Pan et al., 2010). Cultivation of *L. monocytogenes* at 30°C and at 22.5°C led to the formation of higher-density biofilms than at 37°C (Pan et al., 2010). In contrast, *S. aureus* and *P. aeruginosa* biofilm growth was higher at 37 °C than 25°C (Choi et al., 2013).

1.12.4.4. Nutrient content

The presence of carbon and other nutrients is crucial for EPS production. The excess of carbon substrate and lack of nitrogen, potassium and phosphate has been shown to enhance activated sludge EPS (Sheng et al., 2010); whilst a shortage of phosphorus promoted EPS production in *E. coli* (Liu et al., 2006). Moreover, a direct correlation between the carbohydrate content and the ratio of carbon to nitrogen has been proposed by Ye et al. (2011), as a rise in the latter also determined an increase in the former. Conversely, a decrease in the ratio of carbon to nitrogen caused a decrease in the carbohydrate content. In a different study, the incubation of *Pseudomonas* spp. and *Rhodococcus* spp. at high concentrations of ammonium salts accelerated the biosynthesis of exogenous proteins (Sanin et al., 2003).

The polysaccharide matrix content and structure of *S. mutans* biofilms, as well as *S. mutans* gene expression in biofilms are significantly affected by the presence of both starch and sucrose in the medium (Duarte et al., 2008, Klein et al., 2009). A biofilm developed in the presence of a mixture of starch and sucrose has been associated with raised levels of extensively ramified insoluble glucans, which make up the greater part of the biovolume and entrap the bacterial cells. These insoluble glucans were found to make the biofilm more physically stable (Cross et al., 2007) and to provide efficient resistance against

antibiotics and other environmental factors (Kreth et al., 2008).

1.12.4.5. pH Value

The production of EPS molecules is also affected to a considerable degree by the pH of the culture medium. It has been documented that both bacterial growth and extracellular polymer biosynthesis were suppressed by extreme pH values of 2-3, or greater than 10 (Lindsay et al., 2000). For example, for *Antrodia camphorates,* the optimum pH value for production of EPS is 5; whilst biosynthesis was reduced by both high and low pH values (Shu and Lung, 2004). Hoštacká et al. (2010) showed that biofilm production of *V. cholerae, K. pneumoniae*, and *P. aeruginosa* was higher at PH = 8.5 than PH = 5.5.

1.13. Quorum sensing

Gene expression within the biofilm is controlled by a process of cell to cell communication termed quorum sensing (Frederick et al., 2011, Surette et al., 1999, Deep et al., 2011). In a contamination situation involving few bacteria, the environmental concentration of released quorum sensing molecules remains low and therefore not detectable to the bacteria. However, in a contamination situation with adequate numbers of bacteria, significantly more quorum sensing molecules are released and the bacteria are then capable of detecting these which leads to the activation of target genes and alterations in gene regulation (Figure 1.4) (Deep et al., 2011).

Quorum sensing has been recognized to play an important role in the structure and development of biofilm, as it regulates EPS secretions (Hammer and Bassler, 2003, Zhu and Mekalanos, 2003). For example, Davies et al. (1998) found that *P. aeruginosa* lasI quorum signaling mutants produced unstructured biofilms. LasI quorum signaling regulates the creation of the extracellular signal, N-(3-oxododecanoyl)-L-homoserine

lactone (Steindler et al., 2009).

Approximately 4-10% of a microorganism's genome and approximately 20% of its proteins are thought to be affected by quorum sensing, implying that quorum sensing is an important mechanism for virulence regulation in both Gram-positive and -negative microorganisms (Cotter and Stibitz, 2007, Kong et al., 2006, Zhu et al., 2002) and metabolic stress adjustment (Schuster et al., 2003, Wagner et al., 2003, Arevalo-Ferro et al., 2003). Quorum signals have been shown to enhance the metabolism of the bacterial population (Miller et al., 2004, Hentzer et al., 2002).

Three groups of quorum sensing molecules have been recognized:

1- Acyl-homoserine lactones (acyl HSL), which are found in Gram-negative bacteria.
2- Peptides, which are used by Gram-positive bacteria.
3- The autoinducer-2, which is found in both Gram positive and Gram negative organisms.

These groups will be explained in detail below and in the diagram in Figure 1.5.

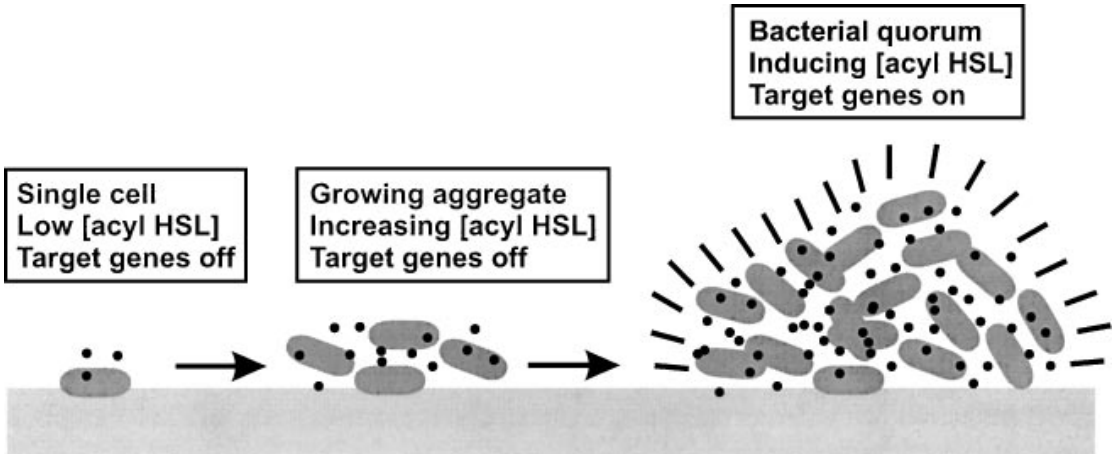

Figure 1.4. Population density-dependent gene regulation in a Gram negative organism. A single population of microorganisms accumulating on a surface is illustrated. Aggregating cell numbers might be a consequence of clonal proliferation or recruitment of other species. Black dots are intercellular quorum signals, in this instance Acyl-homoserine lactones (acyl HSL). At a low cell density, there is a low concentration of quorum molecules and so target genes are switched off. As the cell density increases, the concentration of quorum molecules is increased above a threshold and the target genes are switched on (Fuqua et al., 2001).

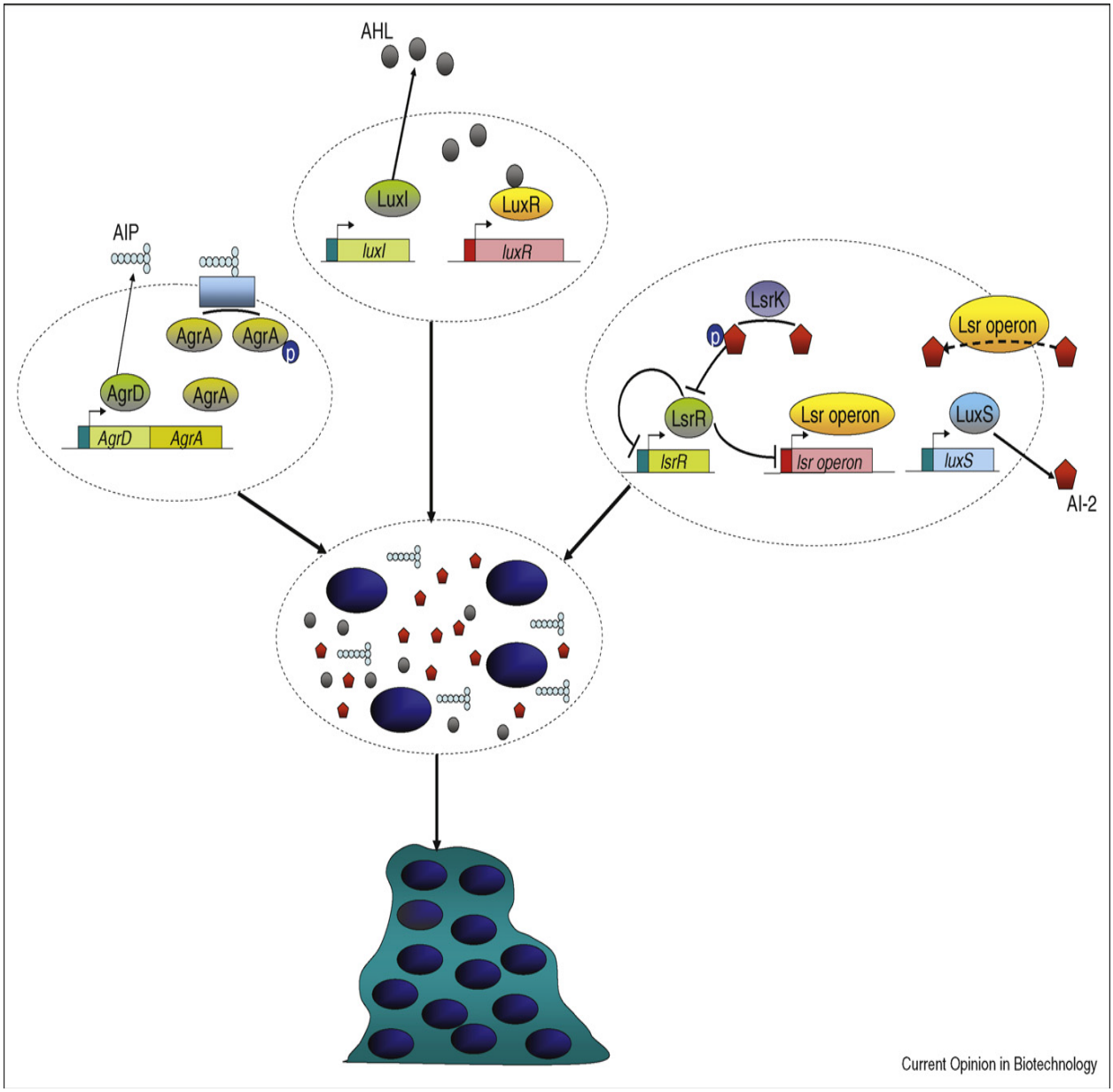

Figure 1.5. Quorum Sensing (QS)-signalling paths. Paradigms of diverse QS-signalling passageways involving signal production, transmission into the extracellular surrounds, and uptake by a micro-colony are indicated as follows: AI-2-signaling passageway (right), Acyl-homoserine lactone (AHL)-mediated regulation (middle) and autoinducing peptide (AIP) regulation (left). QS particles organize population-based performance. One possible reaction to QS is the development of biofilms (bottom) (Hooshangi and Bentley, 2008).

1.14.1 Mechanism 1. Quorum sensing in Gram-negative bacteria (Acyl-homoserine lactones)

The majority of Gram-negative bacteria quorum sensing system research has focused on the homologous system of LuxR-LuxI proteins and N-acyl-homoserine lactones found in *Vibrio fischeri* (Fuqua and Greenberg, 1998, Manefield et al., 1999, Lamont et al., 2002, Riedel et al., 2001).

LuxI produces Acyl-homoserine lactones (AHLs) (Brint and Ohman, 1995). Short chain AHL diffuse in and out of the cell along concentration gradients whilst long chain AHL maybe actively transported out of the cell (Boyen et al., 2009). Once the concentration of released AHLs reaches a threshold of approximately 1 μg/ml, they bind to the LuxR transcription factor, which then binds directly to DNA and leads to the stimulation of target gene expression (Figures 1.5 & 1.6) (Deep et al., 2011, Antunes et al., 2010, Hanzelka and Greenberg, 1995).

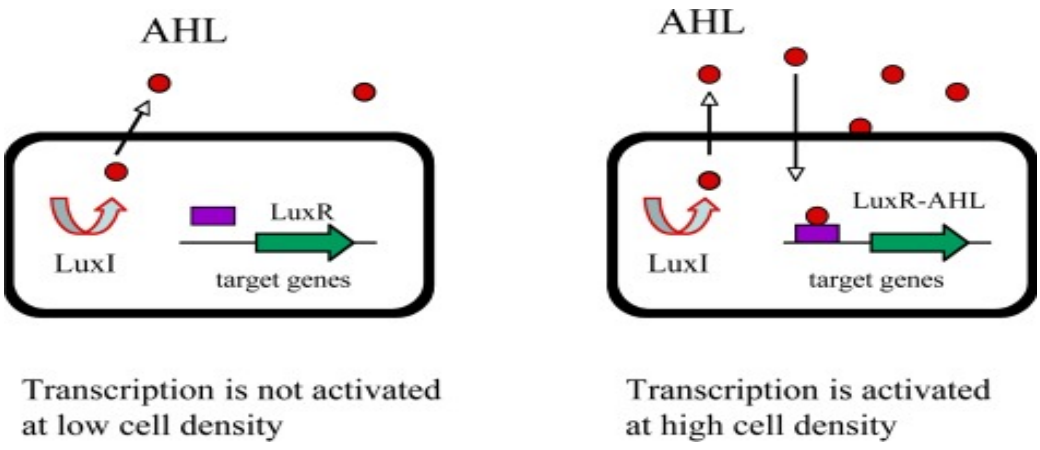

Figure 1.6. The LuxI/LuxR–type quorum signals in Gram-negative bacteria. The LuxI-like protein is an autoinducer that creates AHL (red circle). The AHL easily circulates through the cell membrane at increasing cell density. The LuxR (purple rectangle) is a transcriptional supervisor that joins to the diffusing AHL and then triggers the transcription of its target genes (Li and Tian, 2012).

1.14.2. Mechanism 2. Quorum sensing in Gram-positive bacteria (Peptides)

Quorum sensing in Gram-positive bacteria supervise four physiological mechanisms involving natural competence, sporulation, antibiotic biosynthesis and virulence factor stimulation (Steiner et al., 2012, Pollitt et al., 2014, Vuong et al., 2003). The Gram-positive bacteria quorum sensing system relies on autoinducing peptides of 5 -17 amino acids long encoded by *agrD*, which are produced by an ATP-binding cassette transporter. Species specificity is determined by modification of the peptide's side chains which for *Staphylococcus* species are thiolactone rings (Mayville et al., 1999). Gram positive organisms have a two-component detection system including a histidine kinase sensor and an intracellular response regulator, to mediate the signals (Lyon and Novick, 2004, Cvitkovitch et al., 2003, Novick et al., 1995, Lina et al., 2002, Pestova et al., 1996). The auto-inducing polypeptides are first created in the cytoplasm before undergoing modification and being carried into the environment (Lyon et al., 2002, Lyon and Novick, 2004). When the two-component system recognizes the auto-inducing polypeptides, phosphorus is produced, leading to intracellular response regulator stimulation (Figure 1.7) (Ansaldi et al., 2002, Mayville et al., 1999).

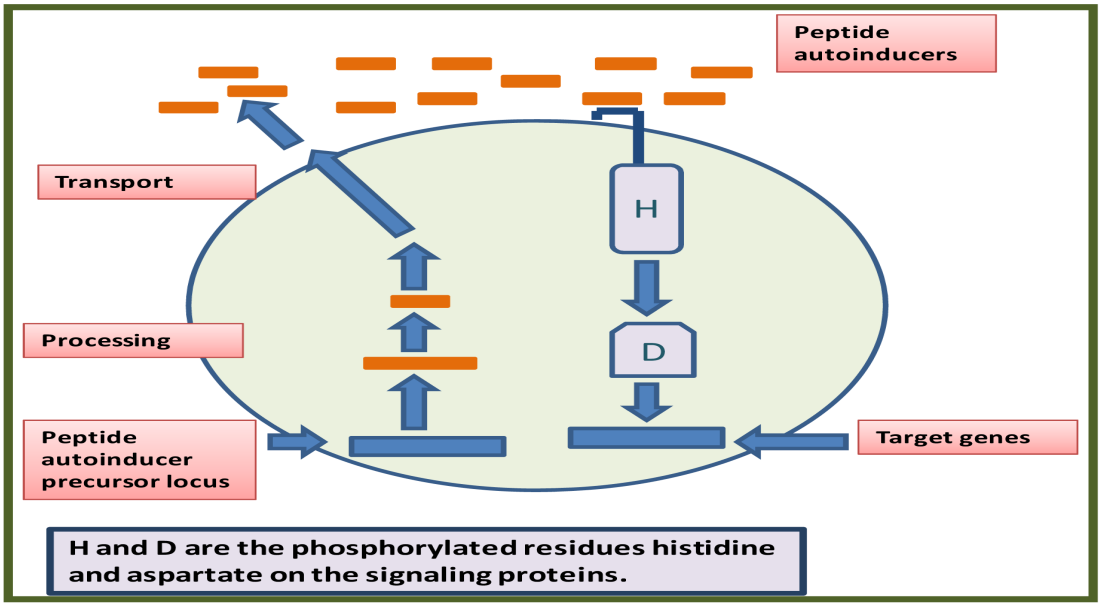

Figure 1.7. A typical model for peptide-mediated quorum sensing in Gram-positive bacteria. A peptide sensing precursor locus is translated into a precursor protein, which is modified before being transported out of the cell via an ATP-binding cassette transporter. Once the extracellular accumulation of the peptide signal reaches a minimal stimulatory level, a histidine sensor kinase protein of a two-component signaling system senses it and phosphorylates the response regulator protein. The phosphorylated response regulator triggers the transcription of target gene(s) (Nazzaro et al., 2013).

1.14.3. Mechanism 3. Quorum sensing in Gram-positive bacteria and Gram-negative bacteria (Autoinducer 2)

Gram-positive and Gram-negative bacteria share the same sensing molecule, Autoinducer 2 (AI-2) (Yeo et al., 2015, Jang et al., 2013). AI-2, a furanosyl borate diester, facilitates cross-species signals and is produced by the LuxS protein (Sun et al., 2015, Sun et al., 2004). AI-2 is detected, when present in high concentrations, by binding with a two-component protein system comprising a sensor kinase domain, the periplasmic LuxP protein, and a response controller domain, LuxQ (Figure 1.5) (Taga et al., 2008, Neiditch et al., 2005). AI-2 binding with LuxP prevents LuxQ from phosphorylating LuxO. This inactivates LuxO alleviating LuxO repression of gene transcription. Additionally, AI-2 has been linked to an increased density of biofilm in *E. coli* (González Barrios et al., 2006).

1.15. Antibiotic and biocide tolerance

Biofilm tolerance to antimicrobial agents is significantly greater than that of planktonic cells, with biofilm found to be 1,000-1,500 times more resistant to antibiotics (Socransky and Haffajee, 2002, Costerton, 1999). Biofilm tolerance to disinfection has major implications for killing of multiantibiotic resistant organisms in the ICU environment. A previous study showed that planktonic cultures of MRSA (8 clinical strains) and multi-resistant *P. aeruginosa* (8 strains) were very sensitive to low concentrations of benzalkonium chloride, chlorhexidine, and triclosan. However, when grown as biofilms, 0-11% of MRSA and 80% of *P. aeruginosa* cells survived, even when exposed to much higher than recommended concentrations of disinfectant (Smith and Hunter, 2008). Similar increases in tolerance to disinfectants have been shown by biofilms produced by bacteria commonly isolated from food preparation equipment, food manufacturing surfaces and spoiled food. These include *Salmonella* spp, *Pseudomonas* spp, *Listeria monocytogenes, Staphylococcus* spp, *Lactobacillus* spp, *Campylobacter* spp and *E. coli* (Van Houdt and Michiels, 2010, Sofos and Geornaras, 2010).

Bacteria develop increased tolerance to biocides at the attachment stage and the degree of tolerance increases as biofilm grows (Patel, 2005). Biocide tolerance is due to the biofilm lifestyle, as when the biofilm structure is disrupted the bacteria, once again, become susceptible to biocides (Anwar et al., 1989). This suggests that biofilm disruption is essential in order to maximise the effectiveness of biocide action. Biofilm biocide tolerance is unlikely to be due to a single factor and there are various theories to explain this phenomenon.

1.15.1. EPS barrier

The extracellular matrix is thought to play an important role in antibiotic tolerance, and acts as shield protecting the enclosed cells from the antibiotic (Davenport et al., 2014, Yamamoto et al., 2015). The biofilm matrix not only interacts directly with some antibiotics, but can also limit antibiotic diffusion through the biofilm thus diminishing their effect. EPS has been shown to interact with disinfectant, such as biguanides and quaternary ammonium compounds, and cationic steroid antibiotics, such as aminoglycosides (da Silva Fernandes et al., 2015, Billings et al., 2013, Chiang et al., 2013).

A study conducted by Souli and Giamarellou (1998), found that EPS accounted for a considerable reduction in bactericidal effectiveness, of Teicoplanin, Vancomycin and Pefloxacin, primarily due to diffusion barriers (Souli and Giamarellou, 1998). The EPS might work as a physical barrier, due to the EPS being highly dense, or interact with antimicrobial agents to significantly increase the size of the bactericide, therefore making them less diffusible (Billings et al., 2013, Davenport et al., 2014). In one study it was pointed out that when the biofilm is ten cells thick, the antibiotic penetration is likely to be 100 times slower than its penetration of a single cell (Stewart, 2003).

However, other studies have shown that an insufficient diffusion of the antimicrobial agent might not be the main contributing factor to biofilm resistance (Darouiche et al., 1994, Rani et al., 2005). For instance, a study carried out by Cochran et al. (2000) showed that a thin biofilm of *P. aeruginosa* had the ability to reduce its susceptibility to both monochloramine and hydrogen peroxide despite easy diffusion of these biocides (Cochran et al., 2000). Supporting this point of view, Dunne et al. (1993) showed that both rifampin and vancomycin diffused into staphylococcal biofilm. However, vancomycin when used alone penetrated 1.5 times less than the penetration achieved when both agents were used

simultaneous. In contrast, the diffusion of Rifampin was reduced by half when it was used in conjunction with vancomycin (Dunne et al., 1993).

In conclusion, the EPS contributes to biofilm tolerance due to quenching chemical reactions associated with agents that are either strongly charged, such as quaternary ammonium compounds and biguanides, or chemically reactive, such as halogens/peroxygens (Stewart et al., 1998, Huang et al., 1995). In addition, the EPS physically hinders diffusion of the biocide, the extent of this is determine by several factors, including the type of agent, the binding ability of the polymeric matrix, the concentration in which the agent is administered, the organisation of the biomass and the local hydrodynamics, as well as the rate of turnover of the micro-colony in relation to the rate of antibacterial dissemination (De Beer et al., 1994, Kumon et al., 1994).

1.15.2. Extracellular Enzymes

Within the EPS are anchor sites for the attachment of extracellular enzymes, which can thus aggregate in the matrix and counteract antibacterial effects. Specific enzymes endows the biofilm with resistance to particular antimicrobial agents (Stewart, 1996, Sutherland, 2001). For instance, both β-lactamases (Giwercman et al., 1991) and the enzymes formaldehyde dehydrogenase and formaldehyde lyase (Sondossi et al., 1986) have been shown to protect biofilm bacteria from penicillin and formaldehyde respectively.

1.15.3. Growth rates and metabolism

The efficacy of antimicrobial agents increases along with the growth rate of bacteria (Evans et al., 1990b, Duguid et al., 1992). The metabolic activity and growth of both biofilm and planktonic bacteria are adversely affected by oxygen and nutrient restrictions (Anwar et al., 1989, Donlan and Costerton, 2002). Costerton et al. (1995) examined both

environmental and *in vitro* biofilms and observed that, under anaerobic conditions, the biofilm surface and centre respectively exhibit high and low concentrations of oxygen. Werner et al. (2004) also noted biofilm discrepancies in development, protein synthesis and metabolic activity, the surface displaying intense activity and growth while the centre had little or no growth.

Antimicrobial agents generally target cellular functions and thus are more effective against bacteria actively metabolising (Corvaisier et al., 2003). Empirical measurements have confirmed that, in comparison to planktonic cultures, the rates of bacterial development within biofilms were significantly less (Anderl et al., 2000, Borriello et al., 2004). Therefore, the antimicrobial agents are less efficacious when used to treat bacteria with low metabolic activity such as planktonic organisms during starvation and bacteria residing in biofilms.

The tolerance of *P. aeruginosa* biofilms to the antibiotics ciprofloxacin and tobramycin was examined by Walters et al. (2003) in relation to antibiotic ingress, oxygen restriction, and metabolic activity limitation. Results revealed that, despite differences in the ingress speed, with ciprofloxacin penetrating the biofilm faster than tobramycin, both antibiotics achieved biofilm penetration, and yet only metabolically active bacteria present in high-oxygen concentration areas were susceptible to their effects. Another study conducted by Evans et al. (1990a) showed that there is a significant relationship between sensitivity to cetrimide and the growth rates of cells- both within planktonic cultures and within biofilms. The influence of cetrimide increases as the growth rate of the biofilm and planktonic bacteria increases.

1.15.4. Persister cells

Based on observations of the reduced susceptibility of a subpopulation of *Streptococcus pyogenes* cells to the effects of penicillin, Bigger (1944) was the first to report the existence of bacterial persister cells. These persisters have been found to endure high concentrations of bactericidal agents (Singh et al., 2009).

Transcriptome analysis has helped to shed light on the molecular processes underlying persisters, especially the processes associated with the reduction in expression of genes involved in biosynthesis and elevated expression of toxin/antitoxin modules. As explained by Schumacher et al. (2009), in ideal conditions for growth, an anti-toxin (e.g. HipB) neutralises the toxin protein (e.g. HipA) and bacterial cell division occurs as usual. However, in conditions that are less than ideal (e.g. exposure to antibiotics), the anti-toxin suffers deterioration leading to toxin release. As a direct consequence, a wide range of cellular processes, including DNA production and translation, are suppressed (Schuster and Bertram, 2013).

The numbers of persisters have been found to increase as the culture density increases, reaching approximately 1% of the population in the stationary stage and in biofilm cultures (Keren et al., 2004). Lewis (2000) suggested that biofilm re-colonisation by persister cells can occur once the antagonistic agent is eliminated, thus causing the recurrence of chronic infection.

1.15.5. Horizontal Gene Transfer

The possibility of the occurrence of horizontal gene transfer was first indicated by transfer of virulent genes among pneumococci in infected mice (Griffith, 1928, Avery et al., 1944). Horizontal gene transfer occurs in bacteria, by one of the following processes − phage

transduction, transformation or conjugation (Hoch et al., 1967, Luchansky et al., 1989).

It has been determined that there are four major types of bacterial cell surface structures that supply DNA or protein effectors to prokaryotic or eukaryotic target cells via direct interaction. More specifically, protein effectors are transferred to eukaryotic host cells by type III secretion systems through a flagellum-like tube (Blocker et al., 2008), whilst DNA and proteins are delivered to target cells by type IV systems through a pilus-based mechanism (Alvarez-Martinez and Christie, 2009). Type V system constitutes a contact-based growth suppression system which interacts with target cell receptors and supplies a growth inhibitory signal via a long β-helical cell surface protein (Desvaux et al., 2004). Type VI systems transfer effector proteins to eukaryotic as well as prokaryotic target cells through a phage-like tube and cell-puncturing mechanism (Leiman et al., 2009, Hayes et al., 2010). Studies have pointed out that type IV pili or type IV pili-like proteins are responsible for the bacterial properties of natural competence and transformability (Hobbs and Mattick, 1993, Kang et al., 2002).

Conjugal plasmid exchange in biofilms has been identified as one mechanism through which horizontal gene transfer takes place (Nguyen et al., 2010, Dahlberg et al., 1997). For example, Roberts et al. (2001) successfully managed to transfer tetracycline resistance encoded by Tn916-like components from four tetracycline-resistant *Streptococcus* spp. to different *Streptococci* in a biofilm. In general, biofilms are conducive to conjugation due to the fact that they offer high population densities as well as minimal distance between cells, which not only enhance genetic competence and aggregated mobile genetic elements in biofilms, but also support effective horizontal gene transfer (Hausner and Wuertz, 1999, Li et al., 2002, Molin and Tolker-Nielsen, 2003). Furthermore, it has been demonstrated that, in comparison to planktonic cells, biofilms of *S. mutans* have a rate of natural

transformation 10 to 600 times higher (Li et al., 2001).

The efficiency of horizontal gene transfer in biofilms is extremely high, and biofilm formation, stabilisation and development occur with the actual involvement of conjugative plasmids (Hausner and Wuertz, 1999, Molin and Tolker-Nielsen, 2003, Reisner et al., 2006).

Due to cell lysis, the extracellular DNA concentrations in biofilms are frequently high (Kadurugamuwa and Beveridge, 1995, Hamilton and Dillard, 2006). It has been estimated that local DNA concentrations undergo an increase of over 100 μg/ml as a result of the lysis of just one cell (Baur et al., 1996). Due to such high DNA concentrations and cell densities, horizontal gene transfer via natural transformation easily occurs in biofilms. As highlighted by Stewart and Carlson (1986), cell lysis offers an ample supply of free DNA and, therefore, a living donor cell is unnecessary for gene transfer through natural transformation (e.g., competence), making this a highly efficient mechanism.

1.15.6. Efflux Pump

Bacterial efflux is an important mechanism via which bacteria withstand the effects of antibiotics and was first identified during research on *E. coli* resistance to tetracycline (Ball et al., 1980, McMurry et al., 1980). The majority of species of both Gram-positive and Gram-negative bacteria possess efflux pumps and the location of the encoding genes is on chromosomes or plasmids (Kaatz et al., 1991, Gill et al., 1999, Poole, 2000b). Efflux pumps are presently divided into five different families of proteins: the multi-drug and toxic extrusion family, the ATP-binding cassette family; the small multi-drug resistant family, the resistance-nodulation-division family, and the major facilitator superfamily (Lewis, 1994, Poole, 2000b, Poole, 2000a).

In general, planktonic bacteria derive their resistance from efflux mechanisms, susceptibility to antibiotics decreasing as these transporters are expressed in cells. More specifically, the antibiotics are eliminated instantly upon penetration, before they have any action on the bacterial cells (Thanassi et al., 1997). It is not clear how active drug efflux is involved in the resistance of biofilms to antibiotics. De Kievit and colleagues (2001) observed that efflux pumps in *P. aeruginosa* biofilms did not offer much protection, prompting them to conclude that these mechanisms do not provide any benefits to the biofilm phenotype.

However, in numerous other studies findings have been reported that contradict this assertion. For example, Zhang and Mah (2008) found that, in *P. aeruginosa*, resistance to aminoglycosides and fluoroquinolones was significantly affected by a new pump but only in biofilms, and not in the planktonic state. Lynch et al. (2007) attributed the enhanced resistance of *E. coli* biofilms to a different efflux pump, YhcQ. In another study, it was recognised that a number of efflux mechanisms are responsible for the elimination of azithromycin from biofilms. In comparison to the constitutive expression associated with planktonic cultures, the expression of the identified efflux mechanisms in biofilm populations was independent of azithromycin exposure (Gillis et al., 2005).

1.16. Surfaces properties influence the formation and existence of biofilms

Microorganisms undergo complicated mechanisms to form biofilms and there are various parameters that affect their development. Bacterial cellular factors, such as flagella, fimbriae and pili, have been found to stimulate the development of biofilm (illustrated in detail above) (Watnick and Kolter, 1999, Moreira et al., 2003, Bradley, 1980). However, the properties of a substrate, such as hydrophilicity, hydrophobicity and surface charge, are critical factors affecting the ability of microorganisms to attach to surfaces (Kwok et al.,

1998, Vieira et al., 1993, Assanta et al., 1998, Rouxhet and Mozes, 1990, Cerca et al., 2005).

Hydrodynamic shear forces are likely to be correlated with biofilm formation and characterization. It has been established that microorganisms prefer to attach and occupy rough surfaces (Quirynen et al., 1993, Yoda et al., 2014). This is thought to be due to the reduction of shear forces and the surface area being greater on rough than on smooth surfaces (Donlan, 2002). Yet, interestingly, once attached the higher the hydrodynamic shear forces, the more stable and dense is the biofilm, illustrating the effect of shear on biofilm structure (Kwok et al., 1998, Chang et al., 1991, Chen et al., 1998). A study conducted by Quirynen and Bollen suggested that high-energy surfaces stimulate bacteria to adhere to them (Quirynen and Bollen, 1995). However, in contrast to this conclusion, Bakker et al. (2004) found that three strains of bacteria (*S. epidermidis*, *A. baumannii* and *P. aeruginosa,* isolated from infected implants) were not inclined to attach to a high-energy surface. Conversely, three other bacterial strains (*Marinobacter hydrocarbonoclasticus*, *Halomonas pacifica* and *Psychrobacter* sp, isolated from a marine environment) exhibited a much greater inclination to attach to a high-energy surfaces (Bakker et al., 2004). It can be concluded from these studies that the surface preferences differ from one bacterial type to another, and therefore, it is difficult or impossible to generalize about the ideal surface for all microorganisms.

Part Three: Decontamination in healthcare.

1.17. Decontamination of equipment

Effective and safe reprocessing of medical equipment is a critical step in preventing transmission of infectious agents to patients. Several Outbreaks have been reported due to insufficient medical equipment reprocessing. Examples of these outbreaks are contaminated bronchoscopes with *P. aeruginosa* and *Mycobacterium tuberculosis* (Machida et al., 2014, Ramsey et al., 2002) and contaminated duodenoscopes with *K. pneumonia* (Gastmeier and Vonberg, 2014). Reprocessing of reusable medical equipment is a multistep process involving cleaning with or without additional disinfection or sterilisation

In 1968, Dr Earl Spaulding (1968) proposed a classification of how medical instruments should be cleansed, depending on the level of infection risk associated with them. Acquiring the name, the Spaulding classification, this system divided medical equipment into three categories, according to their intended use and infection risk. These categories were: critical instruments (for use on sterile tissue), semi-critical instruments (for use on mucous membranes or non-intact skin) and non-critical instruments (for use on skin but not on mucous membranes) (a summary of this classification system is tabulated below) (Spaulding, 1968). Due to its clarity and logic, this classification has been amended and widely adopted by infection management specialists. The Spaulding classification is used to determine the risk of transmission of infection associated with the use of the instrument and therefore, what level of reprocessing that instrument should be subjected to (Table 1.1).

Table 1.1 Spaulding Classification of Risk and Method of Decontamination.

Risk	**Description**	**Measure**
Low	Instruments used on intact skin	*Cleaning*
Medium	Instruments used on non-intact skin or intact mucous membranes	*Disinfection*
High	Instruments used on non-intact mucous membranes or on sterile tissue	*Sterilisation*

The basic aspects of cleaning, disinfection and sterilisation are presented in the next section, special attention being paid to hospital settings and the reprocessing of reusable medical instruments.

1.18. Cleaning

Cleaning is the process by which organic and inorganic materials, such as dirt, dust, or body fluids, are physically removed from the area being cleaned and is a necessary preliminary procedure even when additional processing such as disinfection or sterilisation is conducted. Gross contamination of instruments or equipment can shield pathogens and thus compromise biocide efficacy (Rutala et al., 2008). Thus to ensure that subsequent disinfection and sterilisation are efficient, cleaning must be meticulous and remove all inorganic and organic materials from equipment surfaces (Rutala et al., 2008, Huslage et al., 2010). However, this level of cleaning is often not obtained with between 17 to 60% of

patient-ready autoclaved instruments were found to be contaminated with high levels of protein (Lipscomb et al., 2006, Murdoch et al., 2006). Instruments used for tonsillectomy and adenoid surgery were highly contaminated with protein (Baxter et al., 2006).

Hollow and complex instruments are difficult to clean and highly vulnerable to residual soil and bacterial contamination. Chu et al. (1998) investigated the quantity of microorganisms on rigid lumened medical devices pre- and post-processing. The post-usage bioburden level on medical devices was up to 10^4 CFU per device. After devices were decontaminated, CFU reduced to only 2 log in 83% of devices. *Staphylococcus, Micrococcus, Bacillus, Diphtheroids, Pseudomonas* spp. *and Stenotrophomonas* spp. were the dominant microorganisms. Using the duck hepatitis B virus model, Chaufour et al. (1999), showed that even hepatitis B virus can survive inside inadequately cleaned angioscopes after ethylene oxide sterilization.

Low risk items that are used on intake skin according to the Spauding classification require cleaning only. However, Havill et al. (2011) also reported that portable medical instruments were not cleaned thoroughly by nursing staff. Similarly, cases of *C. difficile* increased almost three times, from 4.08 to 11.75 per month, when construction works interrupted the employed 'Task Team' approach, involving staff members responsible for making sure that ward equipment was thoroughly cleaned (Kiernan et al., 2006). However, once the 'Task Team' resumed their regular activities, the number of *C. difficile* cases declined to 5.74 per month, highlighting the importance of equipment cleaning.

1.19. Disinfectants

After cleaning, the next stage of decontamination is disinfection, which involves the application of different heat or chemical procedures for destruction of bacteria. Disinfectants affect different microorganisms to varying degrees, with the lowest and

highest resistance being displayed by vegetative bacteria and spores, respectively. For many disinfectants bacterial spores may endure (Dubberke et al., 2007). In contrast, some disinfectants and chemical sterilants such as ethylene oxide, can destroy spores through extended exposure (Rutala et al., 2008). Disinfectants can be used on medium-risk items or low-risk items, like hospital textile materials, that are likely to be contaminated with pathogens (Fijan et al., 2005). The wide range of available disinfectants, such as alcohols, chlorine and chlorine compounds, formaldehyde, glutaraldehyde, hydrogen peroxide, peracetic acid, and quaternary ammonium compounds, are employed in the context of healthcare either on their own or in combination (e.g. hydrogen peroxide and peracetic acid) (Shelly et al., 2014, Adukwu et al., 2015, Teker et al., 2015). In the following section, the emphasis is on chlorine and related compounds, given that one of the objectives of the present study was to assess how effective chlorine is in killing or eradicating dry surface biofilms.

In accordance with their effect on pathogens, disinfectants are divided into three categories discussed below (Rutala et al., 2008).

1.19.1. High-level disinfectants

High-level disinfectants are disinfectants that kill vegetative bacteria, viruses (even those that are not enveloped), fungi and mycobacteria. With protracted exposure, these disinfectants may have an effect on bacterial spores as well. They are mainly employed for the disinfection of heat-sensitive and semi-critical instruments (e.g. flexible fibre-optic endoscopes). Furthermore, high-level disinfectants are not intended for use on environmental surfaces, like laboratory benches or floors, but only for medical equipment. The application duration is short, between ten minutes and half an hour. Glutaraldehyde and hydrogen peroxide are examples of high-level disinfectants.

1.19.2. Medium-level disinfectants

Vegetative bacteria, mycobacteria, fungi and the majority of viruses are susceptible to medium-level disinfectants, but spores are not, even following extended exposures. Phenolic and alcohol are examples of medium-level disinfectants.

1.19.3. Low-level disinfectants

Low-level disinfectants kill vegetative bacteria, certain fungi, and enveloped viruses. Iodophor is an example of low-level disinfectants.

1.20. Sterilization

The ultimate measure of decontamination, which destroys all bacteria, even spores, is sterilisation. This procedure is applied to items associated with a high level of risk, such as surgical instruments and other invasive medical equipment. In a healthcare context, the main methods of sterilisation are steam under pressure, dry heat, hydrogen peroxide gas plasma and liquid chemicals (Herwaldt and Rutala, 1996). As this study is primarily concerned with the efficiency of heat sterilisation in killing biofilms, this method is addressed in the next section.

An item is classified as sterile on the basis of the probability of sterility associated with it, which is known as the sterility assurance level (SAL) of the item. Expressed as 10^{-n}, SAL denotes how likely it is for a microorganism to be present on an item following sterilisation. To give an example, if a spore has a survival probability of one in a million, then the SAL will be 10^{-6}. This SAL value is associated with critical items that are body invasive, like implants and scalpels (Rutala et al., 2008, von Woedtke and Kramer, 2008, Bryans et al., 2010).

The risk of pathogen transmission and patient infection is high when critical items are not sterilised properly or when the sterilisation procedure is flawed (Dancer et al., 2012, Tosh et al., 2011).

1.20.1. Steam sterilization

Sterilisation can be achieved most effectively with steam. Steam lacks toxicity when produced from water without volatile chemicals, has an effect on a wide range of bacteria and is capable of good penetration, whilst also being inexpensive, is simple to monitor and leaves no toxic residue (Adler et al., 1998).

Steam sterilisation is undertaken in an autoclave and involves exposure of every item to direct steam, at the established temperature and pressure, for a given amount of time. Thus, steam, pressure, temperature and time are the main variables of steam sterilisation. Dry saturated steam and entrained water are the optimal steam for sterilisation (Association for the Advancement of Medical Instrumentation, 2012). According to the recommendations of the Association for the Advancement of Medical Instrumentation (2013), surgical equipment should be autoclaved for 15 minutes at a temperature of 121°C. Similarly, European (EN 285, 2006, ISO 17665-1, 2006) and Australian standards (AS/NZS 4187, 2014) provide a sliding scale for moist heat sterilisation of 15 minutes at 121°C but reduced to 3 minutes at 134ºC.

1.20.2. Dry-heat sterilization

Dry-heat sterilization is undertaken in hot-air ovens, which must have a fan to distribute the heat uniformly. The sterilisation procedure should only be commenced after preheating the oven. The duration of sterilisation differs according to oven temperature, lasting for one hour, two hours, or two and a half hours at 170°C, 160°C and 150°C, respectively

(Rutala and Weber, 2002); whilst AS/NZS 4187 specifies 180ºC for one hour or 160ºC for 2 hours (Australian/New Zealand Standard, 2003). To destroy bacteria, dry-heat sterilisers oxidise the elements of the bacterial cells. Dry-heat sterilisation is suitable for materials that are either sensitive or impervious to moist heat, such as powders, petroleum products, sharp instruments and airtight containers (Herwaldt and Rutala, 1996, Briggs et al., 2009). Dry-heat has the advantage of being capable of good penetration and does not cause corrosion of metallic equipment. On the downside, it has a slow penetration rate and produces slow bacterial destruction, and numerous items are sensitive to the prolonged high temperatures associated with the procedure (Lewis and McIndoe, 2004).

1.20.3. Pasteurization and boiling

The method of pasteurisation through heating in water can be used to sterilise semi-critical items like devices and instruments for respiratory therapy and anaesthesia (Rutala et al., 2000b). Complete immersion of all components must be ensured throughout the procedure, which usually lasts for half an hour with the temperature maintained at around 70°C (Rutala et al., 2008) while AS/NZS 4187 recommends that holding time should be for 100 minutes at 70°C (Australian/New Zealand Standard, 2003). It has been demonstrated that, apart from spore-producing bacteria, typical pathogens such as *S. aureus* and *K. pneumoniae* are effectively destroyed by 76 °C for 30 minutes (Rutala et al., 2000b). In another study, pasteurisation (75 °C for 30 minutes) was found by Wang et al. (2006) to be efficient in destroying 10^9 CFU/ml of four organisms with multi-drug resistance, which included *A. baumannii, P. aeruginosa,* MRSA, and ESBL-producing *Enterobacteriaceae*.

1.21 Decontamination of hospital surfaces

Pathogenic as well as opportunistic microorganisms can persist for weeks and years on

healthcare surfaces and equipment (Dancer, 2011, Møretrø et al., 2015, Kramer et al., 2006). *P. aeruginosa* can persist for up to 2.5 years on a wet surface and for up to 5 weeks on a dry surface (Kampf et al., 1998, Kramer et al., 2006). *Klebsiella* can survive for around three years on dry surfaces (Kramer et al., 2006).

A patient admitted to a hospital room where the previous patient suffered colonisation or infection with multi-drug resistant bacteria (e.g. MRSA, VRE, *C. difficile*, and multi-drug resistant Gram-negative bacilli) has a high risk of contracting the same pathogen (Dancer et al., 2008, Drees et al., 2008, Nseir et al., 2011, Shaughnessy et al., 2011). Furthermore, the threat posed by multi-drug resistant bacteria is compounded by the fact that they have a high survival rate on hospital inanimate items. For instance, Wagenvoort et al. (2000) showed that MRSA can endure in hospital dust for around twelve months. Thus, effective cleaning is essential to remove these bacteria and thus prevent the spread of such bacteria. Despite this, often environmental service staff and nursing staff fail to clean room surfaces and medical instruments properly. Several studies have reported that the use of chemical germicides was associated with inadequate cleaning and disinfection in more than half of all hospital room surfaces (Carling et al., 2008, Goodman et al., 2008), while Eckstein et al. (2007) reported that current practices of room cleaning by using non-bacterial killing agent, following a patient departure with VRE and *C. difficile* colonisation or infection, were not effective in removing bacteria from hand contact surfaces. However, two-steps (cleaning and then disinfection) were highly effective for surface decontamination. These authors found that, prior to cleaning, one or multiple environmental surfaces were contaminated with VRE in 16 of 17 rooms (94%) where colonised or infected patients had stayed. After only cleaning, contaminated surfaces were still found in 12 of the rooms (71%, $p = 0.125$) whilst after two-step cleaning with detergents followed by disinfection with 10% bleach, all surfaces were VRE free. Similarly, surfaces in all nine of the rooms

housing patients with *C. difficile*-associated diarrhoea, were contaminated prior to cleaning whilst after only cleaning, seven of the nine rooms (78%) were contaminated. Only one room had positive culture after cleaning and disinfection with 10% bleach (Eckstein et al., 2007). Although two-step decontamination involving cleaning followed by disinfection is not commonly applied in health care, as it is considered time-consuming and is labour-intensive, it can be seen from Eckstein et al study it is more effective than one-step cleaning in removing and killing bacteria.

1.21.1. Chlorine and related compounds disinfectants

Chlorine is an effective disinfectant for surfaces and drinking water due to the fact that it has an effect on a wide range of bacteria, kills bacteria quickly, endures for an acceptable length of time in treated drinking water, is simple to use and soluble in water. Also it is more or less stable, does not contain poisonous residuals, is colourless, does not stain and is inexpensive. Chlorine is toxic at high concentration due its fumes and corrosion properties (Rutala and Weber, 1997, Merritt et al., 2000). However, at the concentrations used in hospitals it is not toxic unless it is mixed with ammonia or acid detergents. When chlorine mixed with ammonia or acid detergents, it releases harmful chlorine fume. Also, it leaves low toxic residuals since chlorine degrades quickly and is inactivated by organic and non-organic residue (Rutala and Weber, 1997).

As a highly active oxidising agent, chlorine disrupts the cellular activity of proteins and interferes with DNA synthesis and oxidative phosphorylation (Barrette Jr et al., 1989, Dukan and Touati, 1996, Ogata, 2007).

Chlorine is commonly employed to disinfect hard surfaces. According to the UK Health Department, suspected or known contaminated areas and equipment must be subjected to

decontamination with the use of a neutral detergent followed by 1,000 ppm of chlorine (Department of Health, 2010). Chlorine treatment has also been applied to hospital surfaces to control multi-drug resistant organism outbreaks (Rossini et al., 2012). Sample et al. (2002) attempted the management of a VRE outbreak in a haematology-oncology unit with the use of 5000 ppm sodium hypochlorite. In the two months before treatment application, the VRE incidence rate was 3.6 cases per 1,000 patient-days, while in the two months after disinfection, the incidence rate dropped to 0.8 cases per 1,000 patient-days.

In cases of *C. difficile* outbreaks, healthcare institutions have been advised by the CDC, the Society for Healthcare Epidemiology of America, and the Infectious Disease Society of America to disinfect environmental surfaces with a solution of 5,000 ppm sodium hypochlorite (Dubberke et al., 2014). Additionally, as reported by Barbut (2009), a solution of 5,000 ppm chlorine was found to be effective in diminishing environmental contamination with *C. difficile* spores in rooms vacated by patients with CDI.

1.21.2. No-touch methods of environmental decontamination

The development of "no-touch" methods, such as ultraviolet light (Jinadatha et al., 2014, Rutala and Weber, 2013) or hydrogen peroxide vapour/mist fogging (Passaretti et al., 2013) for disinfecting hospital rooms was prompted by evidence that the terminal room cleaning of room after vacation by patients did not guarantee that room surfaces were completely free of bacteria.

Such methods should not be considered a replacement for standard procedures of cleaning and disinfection but a supplement to these procedures which are indispensable for the physical removal of dirt and debris. Furthermore, the "no-touch" methods require rooms to

be completely empty of people and therefore can be applied only after room occupant discharge (Rutala and Weber, 2013). Automated methods of disinfection require single rooms; they cannot be employed in multi-bedded bays or nightingale wards unless completely emptied, secured and ventilation apertures blocked.

A before-and-after study was undertaken by Haas et al. (2014) to evaluate the efficiency of pulsed xenon UV as a disinfectant in an acute care environment. Prior to the study, hospital-acquired multi-drug resistance organisms and CDI rates were monitored for two and a half years, whilst during UV disinfection they were monitored for almost two years (22 months). Results showed that hospital-acquired MRO and CDI were reduced by 20% ($P < 0.001$) during the application of UV disinfection. Based on such results, the researchers surmised that it was practical to supplement standard room cleaning following discharge with UV disinfection. Nevertheless, the results cannot be said to be incontestable because the study was limited to one hospital (Haas et al., 2014). However, Havill et al. (2012) supported the efficiency of UV disinfection in their study of 15 patient rooms which, prior to decontamination, contained bacterial growth in 68 of the 75 sites (91%) examined, whereas only 33 of 68 (49%, $P = 0.0001$) sites exhibited bacterial growth after UV disinfection.

The efficiency of hydrogen peroxide in outbreak management has been investigated by a number of studies. Best et al. (2014) used aerosolised hydrogen peroxide to disinfect a whole closed stroke rehab unit and reported containment of outbreak, which did not exceed 10.8% prevalence. However, 20 weeks after decontamination, *C. difficile* growth was found in 3.5% of the room sites. By contrast, the use of aerosolised hydrogen peroxide for terminal disinfection was unsuccessful in the study conducted by Landelle et al. (2013), who documented an *Acinetobacter* outbreak in several ICUs 18 months after

decontamination. Vaporised hydrogen peroxide on a whole closed unit followed by aerosolised hydrogen peroxide on two other closed units were equally unsuccessful, the outbreak being managed only by treating patients with intensive measures of infection control. In a different study, Chmielarczyk et al. (2012) attempted to control an *A. baumannii* outbreak among 20 patients by employing multiple measures of infection control concomitantly, including complete isolation, personnel education, hand hygiene, and use of vaporised hydrogen peroxide to disinfect surfaces. None of the surveillance swabs that were gathered showed any trace of an environmental reservoir.

The majority of the studies investigating the efficacy of no-touch methods have shown that these interventions are effective in reducing surfaces contamination. However, complete decontamination of the surfaces has not been accomplished. This might be due to the complete reliance on this intervention without any physical removal of soil and debris which can not only be aesthetically unattractive but may protect bacteria from disinfectant action.

1.22. Removal of biofilms from surfaces.

The main goal of cleaning is the extirpation of unwanted and harmful products on surfaces. Several strategies have been applied using chemicals and detergents, such as the use of a combination of power ultrasound and ozonation on stainless steel surfaces (Baumann et al., 2009), as well as peroctanoic and peracetic acids (Martín-Espada et al., 2014) and hydrogen peroxide (Hota et al., 2009) to eradicate biofilms from different surfaces. The efficacy of these strategies is variable. However, these methods are either not likely to be applicable to all contaminated settings or the biofilm will not be 100% removed, thus rendering the surface susceptible to development of new biofilm and persistence of

pathogenic bacteria. Ozonated water and a chlorinated sanitizer against was effective against *P. fluorescens* and *Alcaligenes faecalis* biofilms, reducing the initial inoculum of $8x10^5$ CFU up to 4.6 log and 5.6 log respectively (Greene et al., 1993).

The efficacy of detergents to remove *E. coli* biofilm from model endoscope tubes was determined. Three types of detergents were used (detergents without enzymes, cleansers with low enzymatic activity and detergents with high enzymatic activity). The findings indicated that both detergents without enzymes (called Matrix produced by Whiteley Medical, Sydney, Australia) and detergents with high enzymatic activity were effective in removing between 60% and 75% of the biofilm (Vickery et al., 2004). However, the remaining biofilm remained could compromise disinfectant action. Other researchers noted that the use of 3.3% citric acid followed by heat disinfection was more efficient than the use of a combination of disinfectants, including peracetic acid, acetic acid and hydrogen peroxide. However, a combination of these chemicals did not show any real efficacy in *P. aeruginosa* biofilm eradication from hydraulic circuit silicone tubing (Holmes et al., 2004). Studies available in the literature suggest that detergents are often inadequate at removing biofilm due to their inefficiency in penetrating the biofilm matrix. The effectiveness of cleaning is completely reliant on the removal of the EPS matrix of the biofilm, to ensure that subsequent disinfectant can target bacterial cells (Simões et al., 2006).

1.23. Control of bacterial adhesion

As microorganisms have the ability to attach to surfaces under a variety of environmental circumstances, the inhibition of biofilm development is challenging (Hori and Matsumoto, 2010). To date no method has been successful in preventing biofilm development, in the long term, without also affecting the surrounding area. Problems with antimicrobial

coating included lack of evidence for shelf life, toxicity (including mutagenicity); effect of organic soil; repeated exposure to bleach; and cost-benefit.

1.23.1. In vitro studies

Various coatings have been used to reduce attachment of bacteria to implant surfaces. *S. aureus* attachment was prevented by a poly (d,l-lactide)/gentamicin coating of titanium implants (Vester et al., 2010); while silver coating of substrates reduced attachment of both *S. aureus* and *S. epidermidis* (Chen et al., 2007), recognized that the use of "low-surface-energy poly (perfluoroacrylate)" was efficient in controlling biofilm formation. However, the financial cost of this method is significant and may not be effective on all settings (Tenke et al., 2004).

The development of titanium nanotubes involves anodising titanium surfaces which results in the formation of nanotubular surface structures. The potential of titanium nanotubes as carriers of different drugs has raised significant interest. They are able to capture antimicrobial agents and effectively transport them to required locations. Popat et al. (2007) observed that, despite not suppressing bacterial adhesion, gentamicin-containing titanium nanotubes significantly reduced surface attachment of *S. epidermidis*.

Also, antibiotic products have been integrated into surface materials. Lysozyme-chitosan was found to inhibit biofilm development and growth rate of *S. faecalis* and *E. coli* (3.8 log and 2.7 log respectively) (Park et al., 2004).

1.23.2. In vivo studies

For implantable medical device various coatings have been applied in an effort to prevent biofilm formation. Nanoparticle silver ion coatings on titanium implants (Secinti et al.,

2011), heparin coated urologic devices (Tenke et al., 2004) and covalently bound furanones (Hume et al., 2004) have been found to prevent biofilm formation.

Other studies have also focused on incorporation of antibiotics in surface materials. In a prospective, randomised study, Chiu et al. (2002) compared the deep infection rate associated with total knee arthroplasties using bone cement loaded with cefuroxime (178 knees) and plain cement, (162 knees). None of the arthroplasties using the antibiotic loaded cement exhibited deep infections, while five of the arthroplasties using plain cement became infected ($p = 0.02$).

A randomised controlled trial, involving over 750 patients, assessed how effective antibacterial catheters were in preventing catheter-related urinary tract infection. The control group, a standard polytetrafluoroethylene catheter, and the silver alloy-coated catheter had the same rate of infection (12.5%) whilst the nitrofural-impregnated catheter had a significantly lower infection rate (10.6%; $p = 0.02$) (Pickard et al., 2012).

A silver zeolite-impregnated central vascular catheter has been shown to be successful in reducing biofilm infections (Khare et al., 2007). However, its effectiveness might only be short term (< 10 days) (Raad, 1998).

Despite their good efficiency, antibiotic-releasing surfaces may foster the development of antibacterial resistance by constantly discharging active compounds for a considerable length of time. These surfaces eventually become depleted an the released antibiotic concentration falls to non-lethal levels (Page et al., 2009, Siedenbiedel and Tiller, 2012).

1.24. Copper surface coating for hospital environmental surfaces

A novel method for reducing bacterial contamination, and hence the rate of HAIs is to coat

environmental surfaces with copper and copper alloys. The inherent and sustained action of metallic copper against a variety of bacteria has been clearly demonstrated (Salgado et al., 2013). Although the underlying mechanism of copper toxicity has not been fully elucidated, it has been thought that it to causes bacterial cell death by rupturing of the cell membrane, production of reactive oxygen species and deterioration of bacterial DNA (Copper Development Association, 2014, Santo et al., 2011). What makes antibacterial copper particularly advantageous is that it can be used in different items, such as bed rails, over-bed tables, door handles, IV poles, lavatory facilities, as well as work surfaces (Copper Development Association, 2014).

A randomised controlled trial conducted by Salgado et al. (2013) confirmed that HAIs in ICUs could be managed by coating surfaces with antibacterial copper. ICU rooms with and without surfaces made of copper alloy were allocated at random to patients and the incident HAI and/or MRSA or VRE colonisation compared. It was observed that, compared to standard ICU rooms (0.123), ICU rooms with copper alloy surfaces had a considerably lower rate (0.071, $P = 0.02$) of HAI and/or colonisation with MRSA or VRE (Salgado et al., 2013). Although Salgado et al study has shown that coating surfaces with antibacterial copper reduced HAI rate, the study has been criticised by Harbarth et al (2013). The criticism was due to issues concerning (a) " the approach taken to reporting study outcomes", (b)" lack of information concerning the determination of study end points" and (c)" a failure to evaluate the biological plausibility of the findings".

Schmidt et al. (2012) assessed how efficient copper was in reducing bacterial surface contamination in ICUs. Bacterial prevalence was substantially diminished after plastic, wood, and stainless steel items within the patient care environment were replaced with copper items. More specifically, by comparison to surfaces made from other materials

(2,674 CFU/100 cm^2; n = 2,831 objects [P < 0.0001]), surfaces made of copper exhibited a decrease in bacterial burden by 83% (465 CFU/100 cm^2; n = 2714 objects).

A similar lower bioburden on copper surfaces was found in a trial lasting 10 weeks (Casey et al., 2010). To reduce bias even more, an interchange of copper and non-copper surfaces was undertaken midway through the trial. A comparison was carried out of toilet seats, brass tap handles and brass door push plates made of copper, plastic, chrome and aluminium in terms of level of microbial contamination. By contrast to control surfaces, copper items were observed to have 90-100% lower counts of bacteria. Importantly MSSA, VRE and *E. coli* were isolated from control surfaces and not found on copper surfaces (Casey et al., 2010).

1.25. Control of biofilm in the water system

The control of biological contamination of the water system is considered to be both crucial and challenging. In many settings, biofilms in rinse water can cause food and equipment contamination. For example, Mitchell et al. (1997) reported that the rinsing of disinfected bronchoscopes with contaminated tap water led to an outbreak of *L. pneumophila* contamination. Therefore, adequate management of water system distribution is needed.

Many approaches, including chemical and physical strategies, have been applied to overcome biofilms and their negative effects. Chemical treatments, such as chlorine, chlorine dioxide and monochloramines, have been widely used (Baron et al., 2014, Baron et al., 2015, Oren et al., 2002). However, these have been found to have only a short-term efficacy and are not suitable for long-term treatment (Oren et al., 2002).

Copper-silver ionization has been commonly used to control *Legionella* (Cachafeiro et al.,

2007), but other workers (Blanc et al., 2005) have noted that ionization had no affected on *Legionella*. Even if effective, Rohr et al. (1999) suggested that copper-silver ionization is only efficient as a short-term treatment and only provides a reduction in the levels of *Legionella*, not complete eradication. The variation in the findings between studies is likely to be due to differences in the concentrations of copper and silver ions used, as copper and silver ion concentrations of up to 400 µg/L and 40 µg/L are needed, respectively. Ozone also was found to be ineffective on *Legionella* (Blanc et al., 2005).

In addition to chemical treatments, physical treatments, such as heat and ultraviolet radiation, have been applied (Lin et al., 2014, Emtiazi et al., 2004). It has been suggested increasing water temperature to 65°C reduced *Legionella* in the water distribution system. After increasing the temperature, there was 51% ($P = 0.0001$) decrease in the positive *Legionella* samples collected (Blanc et al., 2005).

In many studies, a combination of different chemical and physical measures have been used for the removal of biofilm (Blanc et al., 2005, Farhat et al., 2012, Casini et al., 2014). One such study was conducted to stop a *Legionella* outbreak in a hospital water system with a combination of superheating of the hot water tanks to 70°C and outlet flushing, as well as the use of 2,000 PPM hyper chlorination. The authors reported that the outbreak was stopped and *Legionella* was removed. However, a low level of *Legionella* reoccurred after three years (Oren et al., 2002), suggesting *Legionella* may not have been eradicated but persisted in a biofilm below detection levels. Furthermore, chemical and nonchemical approaches have been applied for reducing the microbial density in dental unit waterlines. However, none is effective at eradicating biofilm (Coleman et al., 2009).

Part Four: Aims of the study.

Earlier and recent studies of biofilms have focused on biofilm growing in a fluid environment. Biofilms growing in a non-aqueous environment would be expected to differ in respect to structure, water availability and composition. However, few studies have addressed the properties of dry biofilms, indicating a research gap exists.

The ultimate goal of the present study is to reduce the risk of infections associated with provision of healthcare by improving environmental and surgical instrument cleaning, and thus optimise the decontamination process. As a result, patient safety will be improved, along with the attainment of a reduction in morbidity, mortality and associated health costs.

1.26. Study hypothesis

- High exposure to disinfectants in ICUs induces biofilm growth on environmental surfaces, including multidrug resistant organisms.
- Growth within a biofilm aids persistence of bacteria on dry environmental surfaces as they are protected from desiccation, detergent action and disinfectant action.

1.27. Study Objectives

1. Determine prevalence of biofilms contaminating dry hospital surfaces.
2. Analysis EPS constituents of dry surface biofilms contaminating hospital surfaces
3. Develop a dry surface biofilm model and identify EPS constituents.
4. Test the effectiveness of sodium hypochlorite in eradicating dry surface biofilms.
5. Test the efficacy of heat treatment in eradication of dry surface biofilms.

6. Determine if planktonic bacteria can be carried from the floor to surgical bed sheets by using disposable shoe covers as a fomite of transmission

Chapter 2. Methods of biofilm studies.

2.1. Microscopy

Biofilm recognition and examination are facilitated by microscopy. Many applications have been used to visualize biofilm, including confocal laser scanning microscopy (CLSM), scanning electronic microscopy (SEM), fluorescence microscopy and transmission electron microscopy (Guilbaud et al., 2015, Hu et al., 2015, Ammann et al., 2013, Dohnalkova et al., 2011). In this study, a combination of CLSM and SEM were used to evaluate biofilm presence, structure and viability.

2.1.1. Confocal Laser Scanning Microscopy

Biofilm is a three-dimensional structure, hence in order to examine intact biofilms (i.e. aggregate formation) and the interaction of cells within EPS, imaging of their spatial organization is required. This can be achieved with the use of CLSM (Lopez et al., 2005). CLSM offers noninvasive, three-dimensional imaging of thick sections and provides horizontal and vertical optical sectioning (Lawrence et al., 1991). CLSM can create thin slices (≈ 0.2 μm), depending on the numerical aperture of the objective lens (Pygall et al., 2007). A series of XYZ optical sections can be achieved by using computer-controlled adjustment of the microscope in the Z dimension stage, and by doing so, three-dimensional reconstructions can be achieved (Palmer Jr and Sternberg, 1999). Many studies have used CLSM to examine the spatial structures, voids and channels of biofilms (de Beer and Stoodley, 1995, Lawrence et al., 1991). Various fluorescent stains have been used in conjunction with CLSM to study the properties of biofilms. Many of these fluorescent stains are discussed below in section 2.1.1.1.

2.1.1.1. EPS staining

Understanding the major components of the biofilm EPS might help with development of targeted removal strategies. However, as mentioned in section 1.12.1, EPS extraction by chemical methods, such as NaOH, or physical methods, such as heat and sonication, might result in contamination of the EPS with bacterial contents. Furthermore, it has been suggested that conclusions with respect to biofilm composition and architecture are likely to be speculative, unless investigated by suitable techniques, such as fluorescent lectins (Palmer Jr and Sternberg, 1999). Therefore, a variety of *in situ* methods based on the application of chemical probes will be used. EPS components are diverse requiring each set of biochemical compounds to be targeted independently for imaging and analysis.

2.1.1.1.1. Lectin

The use of lectins is justified given that no general stain for EPS compounds or a fluorescent stain for all polysaccharide types exists. Lectins are non-enzymatic, carbohydrate binding proteins (Staudt et al., 2003, Neu and Lawrence, 2014). In the case of *in situ* procedures, lectins facilitate the analysis of biofilm distribution of glycoconjugates in three-dimensions as different lectins bind to different carbohydrate moieties. A wide range of studies have made use of lectin probes, such as concanavalin A binding to mannose and glucose and hippeastrum hybrid binding to mannose, for staining the glycoconjugates of biofilm EPS (Staudt et al., 2004, Zippel and Neu, 2011, Bennke et al., 2013, Zhang et al., 2015, Bales et al., 2013). At present, glycoconjugates in microbiological samples from the environment can be stained solely *in situ* with fluor-labelled lectins (Neu and Lawrence, 2014). In the present project, carbohydrate staining was undertaken with Alexa-488-labelled *Aleuria aurantia* lectin (Vector Laboratories,

Burlingame, CA) a fructose binding lectin.

2.1.1.1.2. Proteins

A large number of studies have undertaken EPS protein staining with SYPRO orange stain (Neu et al., 2002, Baum et al., 2014, Neu and Lawrence, 2014) as it strongly binds to proteins in the biofilm matrix. In this project, SYPRO orange (Molecular Probes, Invitrogen, USA) is employed.

2.1.1.1.3. Nucleic Acids

The significant role played by DNA as a biofilm constituent has been highlighted by a number of studies (Barken et al., 2008, Dominiak et al., 2011). Biofilm DNA can be stained with a range of biofilm DNA stains, including SYTO 63, SYTO 84 and SYTO 60. In this study, SYTO 60 (Molecular Probes, Invitrogen, USA) is chosen, as it was best suited to the CLSM filter combinations available. SYTO 60 has been employed in numerous earlier studies (Staudt et al., 2004, Yang et al., 2006, Okshevsky and Meyer, 2014).

2.1.1.2. EPS staining protocol

Biofilm covered coupons and clinical samples were stained as described previously (Neu et al., 2002).

1- Samples were fixed in 4% paraformaldehyde for 60 minutes at 4 ºC.
2- Samples were washed three times in 1M phosphate buffered saline (PBS), for 10 min each wash.
3- Glycoconjugates of biofilms were stained using a 1:10 dilution of Alexa-488-labelled *Aleuria aurantia* lectin for 20 minutes and samples were washed three times.

4- Proteins were stained using a 1:1000 dilution of Sypro Orange in water for 15 minutes, subsequently samples were washed three times.
5- Biofilm nucleic acids were stained in a 1:1000 dilution of SYTO 60 (Molecular Probes, Invitrogen, USA) in water for 5 minutes. Samples were then washed three times.

The staining was performed in the dark at room temperature. After staining, samples were taken to CLSM for examination within 24 hours.

2.1.1.3. Bacterial viability staining

To count bacteria and estimate the proportion of live cells, the LIVE/DEAD *BacLight* bacterial viability kit developed by Molecular probes is usually employed (Chadeau et al., 2012, Le et al., 2015). To achieve its objectives, this kit makes use of a combination of SYTO 9 green fluorescent nucleic acid stain and propidium iodide, which is a red fluorescent nucleic acid stain. There are clear distinctions between these two stains in terms of their spectral properties and capacity to permeate healthy bacterial cells. The SYTO 9 stain detects bacteria with both undamaged and disrupted membranes when it is used alone, whilst only bacteria with disrupted membranes are targeted by propidium iodide. When both stains are used together, they compete for nucleic acid attachment sites. If SYTO 9 and propidium iodide are combined in accordance with established guidelines, bacteria with undamaged and disrupted membranes are, respectively, stained green fluorescent and red fluorescent by the two-dye mixture (Leuko et al., 2004) and a quantitative index of bacterial viability can be derived from the green to red fluorescence ratio.

2.1.1.4. Bacterial viability staining protocol

The *BacLight* 7012 viability kit (Molecular Probes, Invitrogen, Carls-bad, CA, USA) was used according to the manufacturer's instructions as follows:

1- Work in the dark and at room temperature.
2- Samples were stained in sterile Milli-Q H_2O containing mixture of SYTO 9 and propidium iodide (3 μl of the dye mixture for each one mL of sterile Milli-Q H_2O) for 15 minutes.
3- Fix samples in 3% paraformaldehyde for 1 hour.
4- Washed three times in 1M PBS for 10 min
5- Samples were taken to CLSM for imaging or left in 1M PBS at 4ºC until imaging. Samples were covered in foil; imaging was carried out withing 48 hours after staining.

2.1.2. Scanning electron microscopy

The advantage of SEM, is that it has a high resolution enabling visualization at the μm scale (Priester et al., 2007). Several research studies have used SEM to study biofilm composition, formation and distribution (Alhede et al., 2012, Takahashi et al., 2015, Kaya et al., 2013). It has been used, for example, to observe biofilm contamination on endoscope tubing (Pajkos et al., 2004), endotracheal tubes (Fernández-Barat et al., 2012), extracellular polymers (Dohnalkova et al., 2011), urinary catheters (Farrag et al., 2015) and ventilation tubes (Tatar et al., 2006).

However, the samples preparation requires dehydration and this adversely affects biofilm structure, as EPS is principally composed of water. Better resolution is usually obtained if

the sample is sputter coated with a conductive coating such as gold which helps to conserve the cellular features and structures (Priester et al., 2007, Kachlany et al., 2001).

2.1.2.1. Sample processing for SEM

The protocol used in this study was according to that of Vickery et al. (2012). Coupon-covered biofilm and 1 cm^2 of clinical samples were fixed in 3% glutaraldehyde solution (Sigma-Aldrich Pty Ltd, Castle Hill, NSW, Australia) and stored overnight at 4°C. Samples were rinsed three times with 0.1M PBS, pH 7.4 for 10 minutes each rinse and dehydrated through analytical grade ethanol (Sigma-Aldrich Pty Ltd, Castle Hill, NSW, Australia) (30%, 50%, 70%,80%, 90% and 100%) for 10 minutes each dilution. Then samples were immersed in 100% hexamethyldisilazane (Sigma-Aldrich, St. Louis, Mo.) for 10 minutes three times and the hexamethyldisilazane aspirated and samples left overnight to enable remaining hexamethyldisilazne to evaporate. Samples were mounted on carbon tab (ProSciTech, Thuringowa, QLD, Australia) onto aluminium SEM stubs (ProSciTech, Thuringowa, QLD, Australia). Finally, samples were sputter coated with 20nm of gold in the Emitech K550 gold coater (West Sussex, England) and visualized using a JEOL 6480LA scanning electron microscope (JEOL Ltd., Tokyo, Japan).

2.2. Analysis of biofilm images

Two software systems were employed for this thesis: Imaris 7.7.2 software (Bitplane, Zurich, Switzerland) and ImageJ software (1.46r, National Institute of Health, USA). The former was applied to determine structural attributes of the biofilm, such as thickness and biomass, while the latter was used to determine bacterial viability and EPS composition.

2.3. Determination of colony forming units

To ensure that biofilm growth was similar from experiment to experiment the number of viable bacteria or colony forming units (CFU) coating a minimum of 3 coupons was determined using serial dilution and standard plate culture. Biofilm covered coupons were removed from the generator and washed 3 times in phosphate buffered saline to remove loosely attached bacteria before being placed in individual sterile Bijou containers containing 4 mls of either PBS or trypon soya broth (TSB). The coupons were subjected to sonication for 5 minutes and vigorous shaking for two minutes. Serial 10 fold dilutions were made by adding 1 ml of the sonicate to 9 ml of PBS in a test tube and vortexing for 10 seconds. A 100 μl aliquot of each dilution was spread over a horse blood agar (HBA) plate (Oxoid, Thermo Fisher Scientific, Australia Pty Ltd) and incubated for 24 hours at 37 °C. The number of colonies on plates showing between 30 and 300 colonies were counted and CFU calculated.

2.4. CLINICAL SAMPLE PREPARATION AND CULTURE

2.4.1. Sample collection

Furnishings and equipment from the ICU were aseptically sampled using sterile gloves, forceps, pliers, and scissors or scalpel blades. Samples were placed in sterile containers for transport to the laboratory at the Faculty of Medicine and Health Science, Macquarie University. Small items were transported whole, whilst large items eg curtain door had sections removed (up to 8x 10cms) for transport as described previously (Vickery et al., 2012).

Clinical samples were cut into 2 cm^2 and placed in 4 ml 100% TSB (Oxoid, Thebarton, SA, Australia). They were then sonicated for 5 min, and 100 μl from each sample was

spread over HBA plates, as a general non-selective media, Brilliance MRSA agar plates, Brilliance VRE agar plates and Brilliance ESBL agar plates (Vickery et al., 2012).

2.4.2. Chromogenic selective media for Multi-resistant Organisms

Various chromogenic media have been created over the past two decades to target pathogenic bacteria with high specificity. These media take advantage of enzyme substrates, when hydrolysed, secrete coloured dyes, leading to the formation of coloured populations of pathogens that are clearly visible among non-targeted bacteria (Orenga et al., 2009, Cellier et al., 2014). Furthermore, the addition of selective agents can lead to complete suppression of commensal thus, making pathogens more conspicuous (Perry and Freydiere, 2007).

Chromogenic selective media are advantageous primarily because they enable multi-resistant bacteria to be screened straight from contaminated environmental specimens quickly and reliably. This study sought to distinguish between bacteria with resistance to multiple drugs and those without by employing a range of *Brilliance* chromogenic agars from Oxoid (Thermo Fisher Scientific), including:

1- Brillance™ MRSA Agar Plates with a sensitivity of 95.4% and specificity of 99.7% were used for the detection of MRSA (Oxoid, 2008). Once inoculated plates were incubated for 19-24 hrs at 37ºC, only colonies that were coloured denim blue were considered MRSA.
2- Brilliance VRE agar was used to detect VRE, displaying a 94.7% sensitivity at 24 hours and 100% sensitivity at 48 hours, as well as 100% specificity (Oxoid, 2009). After inoculation, the plates were subjected to 24-hour incubation at a temperature of 37°C. Another 24-hour incubation was undertaken in the case of negative plates.

The colony exhibiting a light blue colour was identified as *E. faecalis*, while the colony displaying an indigo to purple colour was identified as *E. faecium*.

3- Brilliance ESBL agar was also employed to detect extended-spectrum beta-lactamase (ESBL) producing Gram negative bacteria, exhibiting a 95% sensitivity and 94% selectivity (Oxoid, 2010). After inoculation, the plates were subjected to 24-hour incubation at a temperature of 37°C. Another 24-hour incubation was undertaken in the case of negative plates. ESBL-positive colonies were the coloured ones; thus, the colonies exhibiting a blur or pink colour were identified as *E. coli*, while the colonies displaying a green colour were identified as *Klebsiella*, *Enterobacter*, *Serratia*, and *Citrobacter*.

2.5. CONFIRMATIONARY TESTS

2.5.1. Mannitol salt agar

S. aureus recovered after chlorine and heat treatment were plated out on Mannitol salt agar (Oxoid, Thermo Fisher Scientific, Australia) and incubated for 24 hours. Mannitol fermentation, as indicated by a change in the phenol red indicator, aids in the differentiation of Staphylococcal species. Coagulase positive *Staphylococci* (e.g. *S. aureus*) produce yellow colonies with a surrounding yellow medium while coagulase negative *Staphylococci* produce red colonies and no colour change of the medium.

2.5.2. DNase Activity

DNase test agar (Oxoid, Thermo Fisher Scientific, Australia) was utilized for the detection of DNase activity. One colony was isolated from *S. aureus* recovered after heat and chlorine treatment and streaked onto the surface of the agar medium and incubated at 37ºC for 24-48 hours. Then, 1M hydrochloric acid was poured onto the colonies and after 5

minutes, the hydrochloric acid solution removed. If a clear zone around the streaked colony was present, then this is a positive indicator of DNase activity.

2.5.3. Coagulase Test

Prolex™ Staph Xtra Latex Kit (Pro-lab Diagnostics) was used according to the manufacturer's instructions to detect the presence of coagulase producing bacteria. One colony was isolated from *S. aureus* recovered after heat and chlorine treatment and put on the test reaction area. A colony of *S. aureus* ATCC 25923 was used as a control. One drop of the reagent was added and mixed well with the *S. aureus* colony. If agglutination occurred, then this indicated that the isolate was coagulase positive and if confirmed to be a staphylococcus, it would be coagulase positive *Staphylococci* (e.g. *S. aureus*).

2.6. Minimum inhibitory concentration and Minimum Eradication Concentration

The susceptibility of planktonic bacteria to chlorine was evaluated from the Minimum inhibitory concentration (MIC) and Minimum Eradication Concentration (MEC), as previously described (Stepanović et al., 2000) and adapted from Christensen et al. (1985). MIC is the lowest chlorine concentration that inhibits visible microorganism growth after an overnight incubation. MEC is the minimum concentration of chlorine required to kill all the planktonic bacterial cells.

Chlorine efficacy against *S. aureus* was tested using ten different chlorine concentrations of chlorine (10 ppm, 50 ppm, 100, 200, 500, 1000, 2000, 5000, 10000 and 20000 ppm) and two contact times. The MIC of planktonic cultures was determined by incubating 100 μl of 100% TSB containing 10^8 CFU/ml with 100 μl of diluted chlorine/well in 96 well tissue culture plates for 10 minutes and 24 hours. Following incubation, 20 μl from each well

was transferred onto a fresh plate containing 180 μl TSB and 3% bovine serum albumen/well to inactivate chlorine and the plates were incubated overnight. The MIC (the column of lowest antimicrobial concentration where all the wells remained clear) was determined. The MEC was determined by transferring 20 μl of each well from a MIC plate to a fresh plate, containing 180 μl of 100% TSB and incubated overnight. The MEC was the column of lowest antimicrobial concentration where all the wells remained clear.

2.7. Minimum Biofilm inhibitory concentration and Minimum Biofilm Eradication Concentration

The method established by Ceri et al. (1999) was adapted instead of having biofilm developed on pegs that project downwards from the 96-well plates, the biofilms were grown on the base and side-walls of the 96-well plates, as described previously (Christensen et al., 1985).

Chlorine efficacy against *S. aureus* biofilm was tested using ten different chlorine concentrations of chlorine (10 ppm, 50 ppm, 100, 200, 500, 1000, 2000, 5000, 10000 and 20000 ppm) and two contact times. The minimum biofilm inhibitory concentraion MBIC of chlorine was determined by incubating 100 μl of TSB containing 10^6CFU/ml in wells of a 96 well microtitre plate overnight. The plates were washed 3 times in PBS, removing loosely adhered cells, but leaving 10^7CFU/well of attached cells. Each well had 100 μl of TSB added, followed by 100 μl of diluted chlorine and was incubated for 10 minutes or 24 hours. The chlorine was removed and 200 μl of TSB and 3% bovine serum albumen added to inactivate residual chlorine. The plates were incubated overnight and the MBIC determined.

The MEC of biofilm cultures (MBEC) was determined by transferring 20 μl of each well from MBIC plates into a fresh plate, containing 180 μl of 100% TSB and incubating

overnight. The MBEC was the column of lowest antimicrobial concentration where all the wells remained clear.

2.8. DNA extraction

DNA was extracted from bacterial single colony using High Pure PCR Template Preparation Kit (Roche, Diagnostics, Mannheim, Germany) following manufacturer instruction.

2.9. *S. aureus* -specific PCR

S. aureus was confirmed using *S. aureus* specific real-time polymerase chain reaction (PCR) targeting the *nuc* and *femA* genes. Real-time PCR was carried out in a final volume of 25 μl, consisting of 12.5 μl Brilliant Sybrgreen Master mix, 400nM of each forward and reverse primer, 50ng DNA and H_2O. Cycling conditions were 95ºC for 10 min, followed by 40 cycles of denaturation at 95ºC for 20 sec, annealing at 55ºC for 30 sec and extension at 72ºC for 30 sec. Two primer sets were used to confirm the identity of recovered bacteria: *nuc* gene forward primer 5'-GCGATTGATGGTGATACGGTT-3' and reverse primer 5'-AGCCAAGCCTTGACGAACTAAAGC-3' (Brakstad et al., 1992) and the *femA* gene forward primer 5'- CGATCCATATTTACCATATCA-3' and reverse primer 5'-ATCACGCTCTTCGTTTAGTT-3' (Al-Talib et al., 2009).

2.10. The Use of partial 16S rRNA gene sequencing

Genotypic techniques permit a more precise identification of bacteria than phenotypic criteria. In this respect, the most widely applied genetic method is comparative analysis of the 16S rRNA gene sequence of bacteria. As explained by Kim et al. (2012), this type of analysis enables the detection of novel pathogens and non-cultured bacteria, as it facilitates

the identification of bacterial strains that are seldom isolated, are difficult to culture or have an abnormal phenotype. The 16S rRNA gene is employed by microbiologists as a phylogenetic marker to investigate and categorise the diversity of bacteria, as the majority of bacteria found in natural or artificial environments are difficult to culture (Kim et al., 2011).

With an approximate length of 1,500 bp, the 16S rRNA gene sequence comprises variable as well as conserved regions. Due to its length and ample interspecific polymorphisms, the 16S rRNA gene enables measurements that are differentiated and have statistical validity as well as those that are for informatics purposes (Patel, 2001, Clarridge, 2004). In general, universal primers are selected from conserved regions of the gene whilst the variable region sequence allows for taxonomical identification (Relman, 1999).

Bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) was conducted using the Titanium platform (Roche) in a commercial facility Molecular Research (MR DNA) laboratory in the US as previously explained (Dowd, Callaway et al. 2008). The 16S rRNA gene amplification was undertaken through a single-step PCR comprising broad-range universal primers and a number of 22 amplification cycles. For purposes of identification, the sequencing of the V1-V3 regions of the 16S rRNA gene was carried out.

2.11. Statistical Analysis

All statistical analyses were conducted using Excel 14.4.3 (Microsoft corporation) and the SigmaPlot 11 Statistical Program (Systat Software, Inc., San Jose, California).

The statistical analysis of the bTEFAP data was performed by QIIME scripts (Caporaso et al., 2010), Calypso software (http://bioinfo.qimr.edu.au/), and FigTree software (http://tree.bio.ed.ac.uk/software/figtree/). Alpha diversity was calculated using the

Shannon index and OUT Richness in QIIME. The default number of Monte Carlo permutations were used to calculate the P-values and the significance threshold was $P < 0.05$. Phylogenetic analysis was calculated by FigTree with the default setting.

Chapter 3:

Biofilms harbouring multi-drug resistance organisms in the intensive care unit

3.1 Introduction

The health and safety of inpatients becomes seriously jeopardised if they develop a HAI. As observed by Landrigan et al. (2010), HAI is becoming an increasingly prevalent issue of public health, in spite of improvements in the practice of infection management. HAI increases patient morbidity and mortality. In 2011, the morbidity and mortality rates associated with HAI in the US were 700,000 and 75,000 cases, respectively. Patients in ICUs accounted for about 15% of all HAI cases (Magill et al., 2014). In Europe the bacteria associated most often with HAI were found to include *E. coli* (15.9%), *S. aureus* (12.3%), *Enterococus* spp. (9.6%), *P. aeruginosa* (8.9%), *Klebsiella* spp. (8.7%), and coagulase-negative staphylococci (7.5%) (European Centre for Disease Prevention and Control, 2013). In contrast, the most prevalent bacterial species causing HAI in the US is *C. difficile* which was the reported pathogen in 12.1% of HAI (Magill et al., 2014).

Routine medical care results in contamination of the hospital environment with infectious agents and many studies have suggested that bacteria can survive long term in the healthcare environment (Kramer et al., 2006, Munoz-Price et al., 2013, Landelle et al.,

2013). Non-spore forming pathogenic planktonic bacteria are susceptible to desiccation and are easy to kill with commonly used disinfectants. However, as shown by Vickery et al. (2012) pathogens can be incorporated into biofilms, even on dry hospital surfaces, and this phenomenon could contribute to pathogen survival as once incorporated into a biofilm, bacteria are far less susceptible to desiccation, detergent removal and biocides (Corcoran et al., 2014, Mukherjee et al., 2012, Chang et al., 2007). Mature biofilms can release planktonic organisms back into the environment. The released bacteria can contaminate patients, HCWs' hands and equipment and ultimately colonise patients with the possibility of causing HAI. For patients in the ICU, Weinstein (1991) reported that environmental contamination is the source of contamination for almost 20% of HAIs (Weinstein, 1991). Any environmental surface can become contaminated with bacteria and their extended presence increases the likelihood of contact with either HCW or patient. The risk of transmission is higher for surfaces that are frequently touched (high touch surfaces) by HCWs and patients, such as washbasins, side-rails, telephones, bedside tables, call boxes (Huslage et al., 2010, Guyot et al., 2013, Rocha et al., 2013, Creamer et al., 2014).

Healthcare workers hands can be contaminated from the environment, infected patients or contaminated instruments and an estimated 20-40% of HAI in ICU has been attributed to transmission of pathogens to patients from the HCWs hands (Weinstein, 1991). Surprisingly, HCWs hands' are two times more likely to be contaminated from environmental sources of MRSA than by direct contact with infected patients (Creamer et al., 2010). Hands of healthcare workers have been found to be contaminated with many infectious agents, including multidrug-resistant *A. baumannii* (28.3%), multidrug-resistant *P. aeruginosa* (17.4%) (Morgan et al., 2012), *C. difficile* (24%) (Landelle et al., 2014), *K. pneumonia* (14%) (Rock et al., 2014), and MRSA (5%) (Creamer et al., 2010).

Transmission of pathogens within the healthcare environment is compounded by the increased prevalence of bacteria with resistance to multiple antibiotics, including *S. aureus* and enterococci with resistance to methicillin and vancomycin, respectively. Infection with antibiotic resistance bacteria has a poorer prognosis and results in higher morbidity and mortality (Weber et al., 2010, Creamer et al., 2014, Huang et al., 2014, Zimlichman et al., 2013).

Given these considerations, the purpose of the present study is to determine how prevalent biofilms are in the environment near the patient and how often they include culturable MROs and *S. aureus,* pathogens that commonly causes HAI. The species and ratio of bacteria that exist in biofilms contaminating dry surfaces was determined by undertaking next-generation sequencing. We did not scan new materials such as mattresses as we had no access to new materials and the cost would be prohibitive. However, areas of each of the samples were negative for biofilm suggesting that the sample would have be negative prior to general use in a hospital.

3.2 Original Paper:

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

Authors:

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Candidate Contribution:

- Scanning electron microscopy.
- Scanning confocal laser microscopy.
- Manuscript preparation.

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

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Chapter 4:

Contamination of surgical bed by shoe covers

4.1 Introduction

SSIs are defined as wound infection occurring 30 days following an operative procedure or up to a year following insertion of an implant. SSIs are specifically related to a surgical site, and although the majority are superficial in nature (Cruickshank et al., 2009), they contribute significantly to patient morbidity and mortality (Astagneau et al., 2001, Awad, 2012).

Up to eight percent of hospitalized patients in the United Kingdom develop post surgical site infections (Leaper, 2010). In the US, around 140,000 cases of SSIs were recorded in 2010 alone (Magill et al., 2014).

To reduce SSI, a range of standard measures in the operating theatre have been adopted, such as hand hygiene, personal protective equipment, monitoring of air quality, ventilation and air conditioning, air ducts and vents, as well as terminal cleaning and disinfection practices (Roesler et al., 2010, National Health and Medical Research Council, 2010).

Personal protective equipment, such as gloves, protective eyewear, masks, aprons, gowns, and shoe covers prevents contamination of hands, eyes, clothing, hair and shoes and thus reduces the chance of transmission (Siegel et al., 2007, Ali et al., 2014).

However, Eisen (2011) drew attention to the fact that, shoe covers were not completely effective as they failed to minimise contamination of floors with bacteria. In the previous chapter we showed that a multi-layered biofilm develops on the floor of the ICU. Although

we have not investigated the presence or absence of biofilm on floors in other areas of the hospital, it is likely that they are contaminated with biofilms. Additionally, we were able to culture MRSA from one of 3 floor biofilm samples. Removal of contaminated shoe covers can lead to contamination of surgeon's hands as has been observed by Eisen (2011).

The shoe covers worn by healthcare workers have been the focus of earlier research on good sanitation practices. For example, a comparative analysis of bacterial contamination levels of theatre shoes (at the start and end of the working shift) and outdoor shoes was conducted by Amirfeyz et al. (2007), revealing that 56% of end-of-shift theatre shoes, 68% of start-of-shift theatre shoes and 98% of outdoor shoes were contaminated. The start shift theatre shoes were contaminated with coagulase-negative staphylococci, coliform and *Bacillus* species. One single shoe also showed presence of the *Diptheroid* species. The end-of-shift theatre shoes revealed the presence of Gram-negative *Bacilli* species and coagulase-negative staphylococci. Two of the shoes were shown to be contaminated with *Diptheroid* species. Most of the outdoor shoes were found to have two or more bacterial species present. Of these species, the highest number of shoes were found to have coagulase-negative staphylococci, whilst the second and third-highest number of shoes showed presence of *Bacillus* and coliform species, respectively. Other isolated species included *Micrococci, Neisseria* and *Diptheroid*.

However, as far as the present researcher is aware, the implications of patients wearing shoe covers prior to surgical procedures have never been investigated. Failure to remove shoe covers worn by patients before surgery may lead to contamination of patient hands and contamination of their bed sheets, which will increase the likelihood of the patients contracting an infection. Hence, this study explores whether bacteria can be transmitted from the day surgery floor to surgical bed sheets by disposable shoe covers.

4.2 Original Paper:

Patient shoe covers can transfer pathogens from floor onto bed sheets

Authors:

Justin Galvin, **Ahmad Almatroudi**, Karen Vickery, Anand Deva, Lillian Kelly Oliveira Lopes, Dayane de Melo Costa, Honghua Hu

Candidate Contribution:

- *In vitro* work.
- Scanning confocal laser microscopy.
- Manuscript preparation.

Patient shoe covers: transferring bacteria from the floor onto surgical bed sheets

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Chapter 5:

Development of a dry surface biofilm model

5.1 Introduction

In a clinical context, hard surface materials, including plastic, wood, concrete and rubber, are conducive to the proliferation of various pathogens (Torlak and Sert, 2013, Dawson et al., 2007, Soleimani et al., 2013, Moghaddam and Sargolzaei, 2014). To eradicate populations of bacteria from hard surfaces, chemical biocides are usually employed. However, bacteria may become firmly attached to the surfaces and are protected by a self-generated matrix of extracellular polymeric substances comprising protein, DNA and polysaccharide material, making their removal from surfaces difficult (Hall-Stoodley and Stoodley, 2009, Høiby et al., 2010).

Disinfection practice is greatly shaped by the understanding that the majority of bacteria in nature live not in planktonic suspension but in biofilm communities and that the biocide resistance of cells in biofilms is significantly high. The removal or destruction of bacterial populations, or biofilm, from hard surfaces frequently poses significant difficulties. By comparison to planktonic cells, the resistance of biofilm to biocides was found to be 1,000-1,500 times greater (Vázquez-Sánchez et al., 2014, Socransky and Haffajee, 2002). In spite of this, the majority of biocides are developed and tested not on biofilms but on planktonic cells (Therapeutic Goods Administration, 2015, Environmental Protection Agency, 2012), and as a result, their capacity to remove *in situ* biofilms from clinical surfaces may not be assessed accurately. This calls for urgent testing of chemical antiseptics, disinfectants, detergents and sanitizers to determine how effective they are against biofilms present on

hard surfaces.

Prior to distribution or sale, manufacturers must inform the Environmental Protection Agency (EPA) of any chemical sterilants, disinfectants or sanitizers that they have produced, as these manufacturers are required to employ specific methods to test these products for toxicity, stability and microbiocidal activity. As Rutala et al. (2008) explained, the manufacturer of such products will be granted permission to distribute and sell the registered product on the market as long as the EPA deems the product to be certain not to result in harmful side effects (Rutala et al., 2008). Guidelines exist for microbiological testing of disinfectants and sterilants but as yet, there are no current guidelines for the assessment of detergents.

For testing detergents/disinfectants against hydrated biofilm there is only one standard which is ASTM MBEC (ASTM E2799) method (ASTM E2799-12, 2012). For growing hydrated biofilm, there are two standards which are ASTM E2562-12 (ASTM E2562, 2012) and ISO-15883-4 (International Standardization Organization, 2008); the first of which involves the growth of *Pseudomonas* by CDC biofilm reactor and the latter of which assesses the performance of washer disinfectors through the growth of *Pseudomonas* biofilm on tubing. However, these standards do not apply to *Staphylococcus* spp. and the assessment of detergents/disinfectants on dry surfaces biofilms is supported by no associated standard.

As reported in Chapter 3, in spite of cleaning with hypochlorite, dry surface biofilms containing MROs were still detected on clinical surfaces. This highlights the necessity for assessments of how efficiently antimicrobial agents, like biocides, can destroy biofilm on hard surfaces in a hospital environment. Therefore, the present study aims to test the efficiency of disinfectant products by creating a reproducible dry surface biofilm

resembling that usually found on hospital surfaces. Also, the EPA advises that a mean log density of 6.0 or higher is required for *S. aureus* when assessing disinfectants for hard surface materials. If the mean log density falls below this figure, this renders the test invalid (Environmental Protection Agency, 2012). Therefore, in this study, it is aimed that the concentration of the dry surface biofilm model should be 10^6 cfu/coupon or over.

5.2. Common in vitro models for use in biofilm studies

Various methods of biofilm *in vitro* growth platforms have been invented for the study of different aspects of biofilm development, growth, prevention and modes of killing. Such platforms facilitate the comprehensive investigation of biofilm as many models that can be easily manipulated and replicated.

5.2.1. Microtiter plate-based model systems

Among the most common biofilm models are the microtiter plate-based systems, in which biofilm growth occurs on the microtiter plate bottom and walls, in the case of 96-well plate (Tote et al., 2008) or on the surface of a coupon introduced in the microtiter plate wells, in the case of 6, 12 or 24-well plate (Gião et al., 2015). The main characteristic of these microtiter plate-based systems is that they resemble batch reactors, being closed systems without any inbound or outbound flow during experiments. Hence, the fluid must be changed frequently to avoid adverse environmental transformations, such as nutrient depletion or accumulation of toxic products, in the microtiter plate wells during experiments (Coenye and Nelis, 2010). The major applications of microtiter plates in clinical research include antibacterial compound screening (Erriu et al., 2013, Minardi et al., 2012), as well as biofilm development investigation (Sánchez et al., 2013, Hell et al., 2013) and suppression (de la Fuente-Núñez et al., 2012).

5.2.2. The agar plate:

Numerous biofilm characteristics, especially high cell density and gradients (gas, nutrient and metabolites), are imitated by colonies of bacteria grown on solid media (McBain, 2009). According to Brown and Gilbert (1995), to attain maximum reproducibility in these models, they should be inoculated not as separated colonies, which exhibit size differences due to which variance is enhanced by nutrient availability and gaseous gradient levels, but as confluent bacterial lawns. Elegant studies have investigated the molecular biology of biofilm development based on agar colonies, with biofilm phenotype variance being detected through colonial morphologies of marked complexity (Kearns et al., 2005, Murray et al., 2009, Verhamme et al., 2009, McBain, 2009).

5.2.3. CDC biofilm reactors

The CDC biofilm reactor, (BioSurface Technologies), consists of a modified glass beaker with a polyethylene lid. Eight detachable polypropylene rods, containing 3 coupons hang from the lid so that the coupons are orientated at right angles to the rotating baffle (figure 5.1) (Donlan et al., 2004, Goeres et al., 2005).

The reliability of the CDC biofilm reactor as an experimental instrument for the investigation of biofilm development for various microorganisms has been confirmed by statistical analysis (Goeres et al., 2005, Honraet et al., 2005, Hadi et al., 2010). It produces 24 identical biofilms, while the reactor structure makes it easy to remove discs during experiments (Honraet et al., 2005, Nailis et al., 2009, Hadi et al., 2010, Buckingham-Meyer et al., 2007). Goeres et al. (2005) addressed intra-laboratory reproducibility or repeatability as well as the effects of modifying parameters like flow rate, media concentration and stir rate. The researchers conducted 21 experiments and the evaluation of the findings obtained was undertaken on coupons that were randomly selected from

every experiment. To examine the reproducibility under different conditions, replicate experiments were carried out. Results revealed that the density of the biofilm developed on the surface was not substantially diminished by minor changes in conditions, such as a temperature modification of 2°C or a 10% decrease in media concentration (Goeres et al., 2005).

By altering parameters like shear flow, temperature and nutrient levels, it is possible to produce biofilms that share similarities with different types of environmental biofilms (Paredes et al., 2012, Burgess et al., 2014, Kargar et al., 2014). Furthermore, the fact that it possesses a standard protocol ASTM E2562-12 (ASTM E2562, 2012) makes the CDC biofilm reactor the favourite tool for biofilm development by numerous researchers.

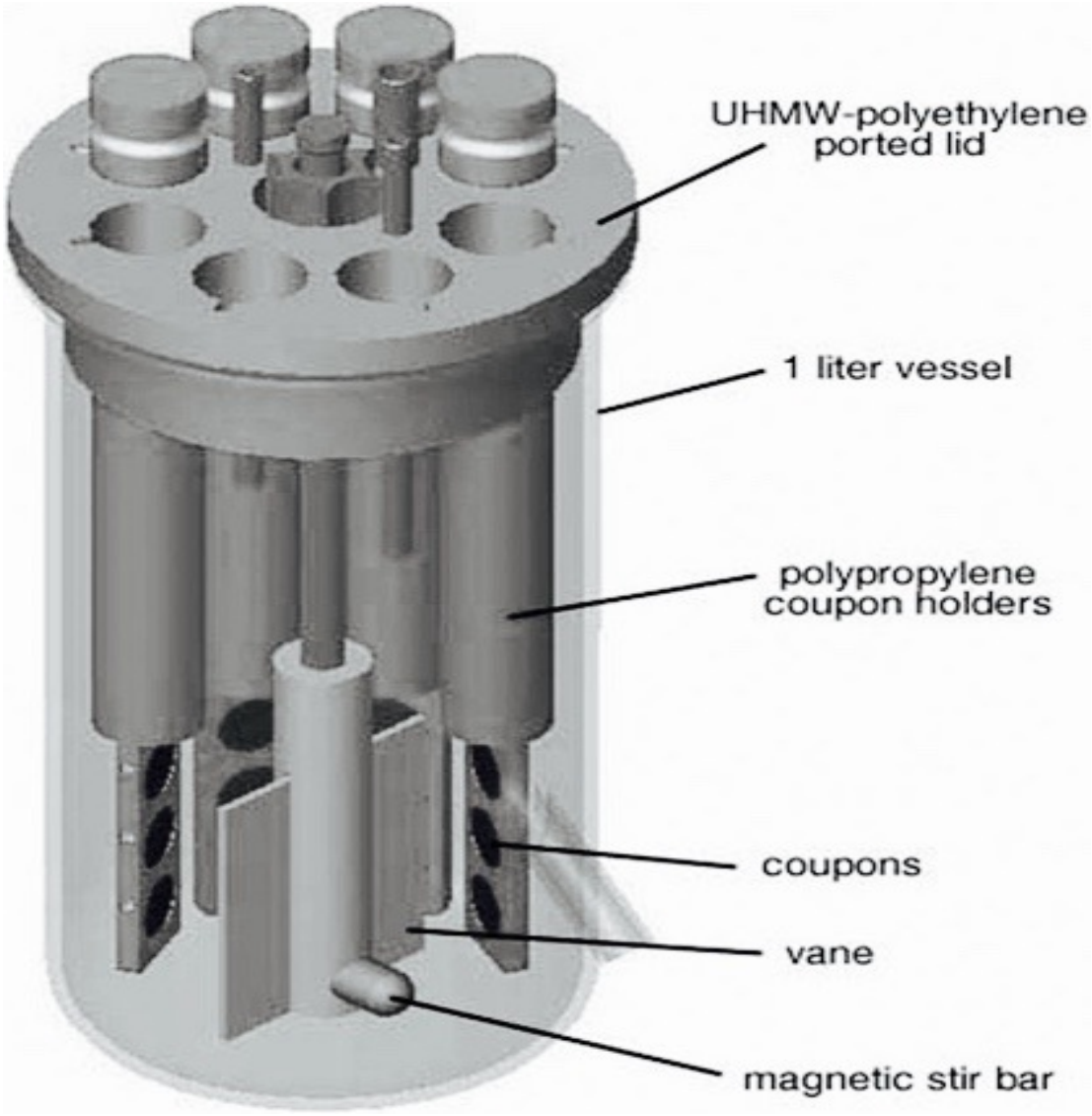

Figure 5.1. Graphical scheme of the CDC biofilm reactor. Biofilms are developed on the faces of coupons in the base of the reactor. Also, biofilms are exposed to shear forces as the paddle (vane) rotates media across the faces of the coupons (Williams and Bloebaum, 2010).

5.2.4. Capillary biofilm reactors

In capillary biofilm reactors, biofilm growth occurs in one or multiple capillary tubes made of glass, under conditions of constant flow. A microscope can be used to observe this growth through the square cross-section of the glass tubes. To prevent them from breaking, the capillary cells are placed in a flow cell holder. The flow cell is linked to a vented feed carboy comprising the medium, a flow break, a filtered air entry, and a peristaltic pump. An inoculation port and waste carboy are additional components of the system (McLandsborough, 2015). Werner et al. (2004) suggested that biofilm formation may be promoted if the fluid is mixed with air in the peristaltic pump. Inoculation requires halting the flow and clamping the downstream tubing. The injection of the culture to fill the glass capillary takes place through the port. Subsequently, the upstream tubing is clamped and the system is maintained without flow for a particular time period. The flow is activated at different flow rates once the culture has attached and begun to grow (McLandsborough, 2015). To enable observation with the confocal scanning laser microscope, the capillary may be injected with a rhodamine B solution to counterstain the biofilms (Rani et al., 2005). Capillary biofilm reactors are excellent for examinations of biofilm development and configuration (Stewart et al., 2009).

5.2.5. Flow cell reactor

In terms of its structure, the flow cell reactor is not significantly different from the capillary biofilm reactor, being made up of a medium-supporting carboy, a flow-regulating pump, and a waste carboy for the collection of the used medium. The flow cell consists of a semi-circular cross-section and seven slides made of stainless steel, which can be removed and are affixed to rectangular Perspex pieces that go into the flow cell openings; the function of these slides is to enable biofilm to be sampled at specific intervals

(McLandsborough, 2015, Huang et al., 1992). Different researchers have employed this reactor for various purposes, including observation of formation of *P. fluorescens* biofilm under conditions of superimposed laminar or turbulent flow (Pereira et al., 2002), *P. aeruginosa* biofilm gene expression (Whiteley et al., 2001), and configuration and properties of biofilm (Hentzer et al., 2001).

5.2.6. Rotating disk reactor

The Teflon disc of the rotating disc reactor supports six coupons and its rotation is made possible by the magnetic stirring bar located at the bottom of the rotating disc, resulting in the occurrence of liquid surface shear over the coupons. The Teflon disc is introduced in a reactor container and, during its rotation, liquid growth medium flows through the container (Coenye and Nelis, 2010). Different studies have used this reactor to investigate biofilm resistance (Cotter et al., 2009, Teitzel and Parsek, 2003), and multispecies biofilm interactions (Komlos et al., 2005).

5.2.7. Drip flow reactors

Biofilm growth in this type of reactor occurs on angled slides which are subjected to constant irrigation with limited quantities of (inoculated) media (Goeres et al., 2009), creating an environment with low shear and dispersive mixing (Stewart et al., 2001, Buckingham-Meyer et al., 2007). Among the applications of drip flow reactors are investigations of biofilm spatial non-uniformity (Hu et al., 2005), physiology of biofilm (Folsom et al., 2010), evaluation of diminished development of *S. epidermidis* biofilm due to the action of bacteriophages (Curtin and Donlan, 2006), and assessment of disinfectant efficiency (Buckingham-Meyer et al., 2007).

5.2.7. Modified Robbins Method

The device employed by the modified Robbins method permits instant biofilm formation in the fluid. The device can be created from stainless steel or plastics consisting of multiple individual sample ports in a linear array via a rectangular cross-section channel (Honraet and Nelis, 2006, Krom et al., 2009). A constant supply of fresh media flows from one end of the device to the other and biofilm develops on the inside of the sample ports providing multiple samples (Krom et al., 2009, Veerachamy et al., 2014). This method is useful in the assessment of biofilm eradication by disinfectant and antibiotic lock therapy (Coenye et al., 2008, Curtin et al., 2003).

5.2.8. Annular biofilm reactor

An inner rotating cylinder for the mounting of multiple slides makes up the annular biofilm reactor. This cylinder is driven by a motor, which supplies liquid/surface shear (Coenye and Nelis, 2010). The main application of this system has been in investigations of drinking water disinfection (Lawrence et al., 2000, Paule et al., 2011).

5.3 Original Paper:

A new dry-surface biofilm model: an essential tool for efficacy testing of hospital surface decontamination procedures

Authors:

Ahmad Almatroudi, Honghua Hu, Anand Deva, Iain B. Gosbell, Anita Jacombs, Slade O. Jensen, Greg Whiteley, Trevor Glasbey, Karen Vickery

Candidate Contribution:

- *In vitro* work.
- Confocal scanning laser microscopy.
- Scanning electron microscopy.
- Data analysis.
- Manuscript preparation.

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Chapter 6:

The efficiency of chlorine for biofilm destruction

6.1 Introduction

In the fight against HAIs, cleaning and disinfection play a major role. Most infection control strategies applied as a result of an outbreak include a procedure of thorough cleaning. Indeed, previous studies have indicated that thorough cleaning and disinfection represents a key instrument for controlling outbreaks of MRSA, *C. difficile*, VRE, and antibiotic-resistant *Acinetobacter* (Wilks et al., 2006, Rampling et al., 2001, Hayden et al., 2006, Denton et al., 2005).

Disinfection of hard surfaces is often undertaken utilising chlorine. The UK Department of Health (2010) has specified that a neutral detergent followed by 1,000 ppm of chlorine should be used to disinfect surfaces that are believed or known to be contaminated. Disinfection of surfaces in the rooms of CDI patients is frequently carried out with chlorine, which is also integrated in strategies for eradicating outbreaks (Rossini et al., 2012, Dubberke et al., 2014).

Surfaces and objects near the patient, such as bed rails, pillows, curtains and bedside tables, tend to be more contaminated than surfaces further away from the patient (Huslage et al., 2010, Weber et al., 2010). These surfaces are often touched by healthcare personnel and patients and frequently have demonstrable biofilm (Vickery et al., 2012, Hu et al., 2015). Given these considerations, it is surprising that, as Carling et al. (2006) observed, cleaning is done most thoroughly not in the case of surfaces and areas that are most likely to accommodate and spread bacteria, but in the case of areas that are considered to be of

aesthetic significance or easy to clean.

The purpose of sanitation practices is to diminish this risk by ensuring that all surfaces with which hospital personnel or patients may have direct or indirect contact are free of infectious agents. As emphasised by Dancer (2009), the likelihood of HAIs is considerably diminished by a high level of hospital cleanliness. On the other hand, if the medical environment is not cleaned or disinfected properly, infectious agents will likely spread among the patients (Eckstein et al., 2007).

The regular usage of disinfectants has raised considerable debate and controversy, even though empirical evidence supports their use within reasonable limits (Dettenkofer and Spencer, 2007, Rutala and Weber, 2004). Every international set of guidelines advocates that adequate surface disinfection is an essential infection preventive strategy (Sehulster et al., 2003, Rutala et al., 2008). In a study conducted by Wilson et al. (2011), high-touch surfaces were disinfected with the standard cleaning procedure involving the use of 1000 ppm of chlorine. The authors observed that within four hours following disinfection, the surfaces became contaminated with bacteria again. This was interpreted as indicative of frequent contact with the surfaces in question and/or reduced efficiency of the disinfectant (Wilson et al., 2011).

Contact time of chlorine to bacteria is an important factor to reach sterility. Many studies have tested the efficacy of chlorine at different contact time. For instance, Jimenez et al. (2010) decontaminated surfaces inoculated with 10^9 of *S. aureus* using a chorline-based spray disinfectant with the active ingredient sodium hypochlorite (0.095%). The researchers found that the log reduction of *S. auerus* was 3.9 following 5 minutes of chlorine exposure. In an earlier study, Rutala et al. (2000a) found that 30 seconds of contact with a 5000 ppm bleach disinfectant led to a 5.0 log reduction in *P aeruginosa*, *S.*

aureus and *E. coli*. The recommended contact time of the majority of disinfectants used in hospitals and registered with the EPA is 10 minutes (Environmental Protection Agency, 2012). For this reason, the current study adopts a 10-minute contact time when testing the performance of chlorine on dry surface biofilm.

Chlorine is the main surface disinfectant recommended by existing guidelines. However, comprehensive testing and assessment are required to establish exactly how efficient it is against dry surface biofilm. Thus, the present study seeks to investigate the capability of chlorine to remove the dry surface biofilms, which has been developed in Chapter 5.

6.2 Original Paper:

Staphylococcus aureus Dry Surface Biofilms are not Killed by Sodium Hypochlorite - Implications for Infection Control

Authors:

Ahmad Almatroudi, Iain B. Gosbell, Honghua Hu, Slade O. Jensen, Björn A. Espedido, Shamaila Tahir, Trevor O. Glasbey, Peter Legge, Greg Whiteley, Anand Deva, Karen Vickery

Candidate Contribution:

- *In vitro* work.
- Confocal scanning laser microscopy.
- Scanning electron microscopy.
- DNA extraction.
- PCR.
- Data analysis.
- Manuscript preparation.

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Chapter 7

Heat treatment efficacy to inactivate biofilm

7.1 Introduction

According to the Centre for Disease Control and Prevention (2010), 51.4 million inpatient surgical procedures were conducted in the US, in 2010. SSIs continue to be prevalent, in spite of the implementation of standards for infection management. In fact, among HAIs, SSIs are the second most commonly encountered type with SSI accounting for 21.8% of infections (Magill et al., 2014).

To ensure that they are completely sterile, surgical instruments must be cleaned to eliminate organic residue. If residual tissue and body fluids are allowed to accumulate, the effective removal of organic material layers will become more difficult (Tosh et al., 2011), while biofilm development will make the situation even more challenging (Edmiston et al., 2013). Bacteria are protected by EPS against the action of adverse environmental elements, making them more resistant to detergents and disinfectants. Indeed, some studies have indicated that biofilm is not efficiently removed by currently used detergents and disinfectants (Hadi et al., 2010, Adukwu et al., 2015).

Many official guidelines emphasise the need of cleaning prior to sterilisation as residual soil or biofilm formation can act as a barrier, protecting organisms from sterilisation (Rutala et al., 2008). Improper cleaning and sterilisation of surgical instruments, has been linked to SSI. For example, Tosh et al. (2011) reported that inappropriate cleaning of arthroscopic instruments in a central decontamination unit was the reason for the outbreak of *P. aeruginosa* in a SSI. Kayabas et al. (2008) also documented that inadequate

decontamination of instruments employed in urological procedures resulted in urinary tract infections with *P. aeruginosa*; while Baruque Villar et al. (2015) found that contaminated laparoscopes were the cause of the outbreak of *Mycobacterium abscessus* infection following laparoscopy. Inappropriate post-sterilisation storage and handling has led to contamination of instruments used in orthopaedic and ophthalmic surgery and resulted in an increase in SSI (Dancer et al., 2012).

On the other hand, in their investigation of patient-ready dental syringes, Vickery et al. (2000) provided evidence that biofilm could also develop due to repetitive use, washing, drying and sterilisation of instruments. The present study intends to determine the efficacy of heat against planktonic and hydrated biofilm of *S. aureus* and compare this to the worst case scenario of semi-dehydrated biofilm formed by periodic wetting and drying.

7.2 Original Paper:

Increased tolerance of Staphylococcus aureus biofilms to heat when formed on dry surfaces

Authors:

Ahmad Almatroudi, Shamaila Tahir, Honghua Hu, Anand Deva, Karen Vickery

Candidate Contribution:

- *In vitro* work.
- Confocal scanning laser microscopy.
- DNA extraction.
- PCR.
- Data analysis.
- Manuscript preparation.

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Chapter 8

General discussion and conclusion

In recent times, there have been ample studies documenting the development of HAIs and their often serious complications. Developing during hospitalisation for medical treatment, these unforeseen infections are associated with high morbidity and mortality rates (Gomes et al., 2012), extended hospitalisation (Madani et al., 2009), and supplementary diagnostic interventions and treatment, increasing patient's healthcare costs (Zimlichman et al., 2013).

HCWs' hands are the main conduit through which pathogens are transferred from infected patients to non-infected ones, but pathogen spread is also aided by contaminated hospital surfaces and medical equipment. Significant healthcare-related pathogens often colonise hospital surfaces and the ability of bacteria to survive on dry inanimate surfaces for days, weeks and even years has been clearly demonstrated (Kramer et al., 2006, Landelle et al., 2013). Furthermore, a patient admitted to a room that was beforehand occupied by a patient infected with multi-drug resistant bacteria, such as MRSA, VRE and *Acinetobacter*, has a high risk of becoming infected as well. There are two main reasons why this happens.

The first reason is that bacteria arm themselves against detrimental environmental conditions, detergents and disinfectants through the formation of biofilm. In nature, bacterial survival usually takes the form of biofilms. As revealed in Chapter 3, bacteria encased in biofilms, including those causing serious infections, such as *S. aureus*, were

present on over 90% of ICU surfaces. Moreover, when analysed under the CLSM, biofilms were found to contain living bacterial cells, despite being derived from surfaces that had been subjected to "terminal cleaning" with cloths and hypochlorite solution and kept in storage for more than a year. In addition, we were able to demonstrate viable MDROs within biofilms using multiple techniques, confirming our hypothesis that MDROs reside in biofilms and are resistant to being removed.

The second reason is related to the lack of thorough cleaning of hospital surfaces. The fact that hospital rooms are not cleaned properly after they are vacated has been highlighted by numerous studies. Carling et al. (2008) and Goodman et al. (2008) showed that the cleaning of over 50% of hospital room surfaces was inadequate when chemical germicides were employed. Hypochlorites are an important component not only for routine disinfection of rooms occupied by patients infected with MROs, but also form part of multiple intervention approaches during tackle outbreaks. Nevertheless, the findings outlined in Chapter 6 indicated that hypochlorite exposure led to a 7-log decrease in CFU when immediately cultured, however, CLSM revealed that biofilms treated with hypochlorite contained live cells. What is more, growth of *S. aureus* cells was observed during protracted incubation following sodium hypochlorite exposure at 20,000 ppm. It can thus be deduced that, although bacterial biofilm is suppressed by hypochlorite, it begins to develop anew when the disinfectant is removed and conditions like temperature and nutrition are favourable. Biofilm can only be temporarily suppressed by disinfecting surfaces with chlorinated products, and in order to eradicate it completely, a more thorough cleaning strategy probably involving physical removal is required.

The data produced by this study show that it is crucial for chemical disinfectants be tested against surface biofilm. As previously mentioned, biofilm poses significant resistance to

complete removal or eradication and is less affected by chemical agents by comparison to free-floating planktonic cells. In spite of this, it is not biofilm but planktonic cells that are usually used in biocide development and testing, and therefore the killing effect of disinfectants against *in situ* biofilms developed on hospital surfaces may be erroneously over estimated. This calls for urgent assessment of the behaviour of biofilm formed on different surface materials when exposed to chemical antiseptics, disinfectants, detergents and sanitizers. Such testing could ensure that treatment failure is prevented. To evaluate these ideas, it is necessary to create a biofilm model that is similar to dry surface biofilm, particularly as found on ICU surfaces.

The development of a biofilm model to resemble a dry hospital surface biofilm was addressed in Chapter 5. This process was undertaken with the CDC reactor, which is capable of generating hydrated biofilm on 24 removable discs (Hadi et al., 2010). What made this model different was that dehydration stress was intermittently introduced through the removal of media from the generator. It was proposed that biofilm forms as a result of the regular hydration and provision of nutrients of the hospital surfaces through cleaning with detergents, which in this sense is similar to the periodic wetting and drying of the intertidal zone in the marine environment (Orvain et al., 2014). The experimental work in this study confirmed that by using cycles of wetting and drying, biofilm formed in the modified CDC reactor model mimicked dry surface biofilms both in composition with regards protein, carbohydrate and DNA content and visually confocal microscopy and scanning electron microscopy.

The results obtained by the present study validate that it is possible to develop dry surface biofilm in an *in vitro* model which can then be employed to assess how efficient hospital disinfectants are. The CDC biofilm reactor generated a reproducible biofilm with multiple

layers comprising 10^7 CFU/coupon entrenched in exopolymeric substances of considerable thickness. Within- and between-run CV was 9.5% and 10.1%, respectively. Similarly, the thickness and amount of biomass was reproducible.

The environmental conditions during biofilm growth might have a potential effect on the extent of detergent penetration, since an increase in the carbohydrate and uronic acid content of the biofilm EPS was reported upon biofilm exposure to desiccating conditions (Chang et al., 2007). Biofilm EPS was characterised in terms of chemical content in Chapter 5. Following the formation of a desiccated biofilm, the EPS was stained with a number of fluorescence dyes and its chemical composition was identified through CLSM. The *in vitro* biofilm model and the biofilms isolated from hospital surfaces were both found to have protein as the main element. As the biofilm model was subjected to additional desiccation and aging for one month, the proportion of protein increased from 56% to 73%, while glycoconjugate and DNA decreased from 24% to 21% and from 20% to 6%, respectively.

A particularly predominant type of HAIs is surgical site infections (SSIs), the prevention of which is prioritised by both HAI control strategies and orthopaedic surgery procedures. Aside from prophylactic antibiotic treatment, other measures for preventing SSIs include sterilisation of the operating theatre environment and compliance with operating room health and safety procedures on the part of medical personnel.

Infection may be caused by endogenous or exogenous bacteria. One major source of endogenous bacteria is the skin, which therefore must be adequately prepared before surgery. The sources of exogenous bacteria include HCWs' hands, uncovered skin and mucous membranes, and/or contaminated inanimate surfaces, medical instruments, or irrigation solutions. Furthermore, as discussed in Chapter 4, bacteria can be spread from

the floor to bed sheets via disposable shoe covers. What is more, there is evidence that bacteria can permeate the non-skid shoe covers made of polypropylene and thus transfer from the floor to the patient's foot.

Another means through which hospital patients can become infected with pathogens is reusable surgical instruments. Hence, to prevent HAIs, these instruments must be thoroughly cleaned and sterilised between uses. As discussed in Chapter 7, biofilm is not inactivated as a result of exposure to different temperatures and heat levels. Additionally, despite the fact that after heat treatment with portable steam vapour systems no cultures were observed in the biofilm-covered coupons, CLSM revealed that the bacteria retained viability even though they were not immediately culturable and even biofilm subjected to autoclaving for 20 minutes and 30 minutes at 121°C revived was reconstituted and commenced releasing planktonic organisms within 23 days. The eradication of biofilm from hospital surfaces and clinical equipment is always necessary, as the biofilm will compromised disinfections and sterilisation and thus might act as a reservoir for pathogens.

Healthcare surfaces sampling can be conducted to reveal the survival of nosocomial pathogens, to determine the environmental contamination source as a part of outbreak investigation (CDC, 2003) or to assess the effectiveness of cleaning and disinfection practices (Galvin et al., 2012). Sampling methods including swabs, dipslides, sponges, and agar settle plates can be used to evaluate inanimate surfaces contamination. When a quantitative analysis is needed, swabs or sponges can be used (Public Health England, 2013). Sponges sampling, which has been assessed as having superior sensitivity than swabs, is usually used for large surfaces (otter et al., 2009) while in less accessible surfaces, swabs are more suitable (Public Health England, 2013). Agar settle plates also can be used, however, this method can only be used on reasonably even surfaces (Galvin et

al., 2012). Another agar settle plates limitation is the probability of clattering of bacterial colonies on the surface, causing underestimating of bacterial counts and differentiation (Pinto et al., 2009). Dipslide method has been found to have a higher sensitivity than swab sampling, especially for dry items. However, this method is limited to sampling even surfaces (Obee et al., 2007).

Before sampling healthcare surfaces, the purpose of the sampling need to be determined and if quantitative or qualitative data are required. The aim of chapter 3 study was to show the existence of biofilm in ICU surfaces despite the terminal cleaning. Destructive environmental sampling allowed the researchers to visually confirm that bacterial contamination was due to biofilm by conducting SEM and CLSM (Vickery et al., 2012). Other methods, such as swabbing, are quantitative and demonstrate the presence of culturable bacteria, however, this method is unlikely to detect bacteria present as biofilms due to biofilms low metabolic state (Fux et al., 2005)

Infection management and prevention revolves around cleaning, disinfection and sterilisation. Alongside these, standard measures of environmental cleaning monitoring, greater compliance with hand hygiene procedures, and proper education and training of both medical and non-medical personnel are also essential. Furthermore, two-step process that includes cleaning and then disinfection should be taken into account as the cleaning step removes patient soil, dust and the physical action might disrupt the biofilm EPS thus making it easier for the disinfectant step to kills the bacteria. The concept of “dry” biofilm is new and therefore, the capability of two-step process in removing dry biofilm is difficult to estimate.

The release of planktonic bacteria from a biofilm is a natural pattern of dispersion stage which is an essential phase of the biofilm life cycle. Planktonic bacteria detachment from

biofilm can be due to internal factors, such as space limitation and nutrient depletion, or external factors friction being an example (Aparna and yadav, 2008). After planktonic detachment from biofilm, free planktonic bacteria move to occupy new niches.

Cleanliness is typically measured via visual assessment (Loveday et al., 2014, Guh and Carling, 2010), even though this does not provide an accurate qualitative or quantitative gauge of bacterial contamination (Malik et al., 2003, Dancer, 2004), since the absence of visual dirt does not necessarily imply the absence of pathogens. Clutter, lack of ward space, excess equipment and fabric deterioration can all give an erroneous perception of cleanliness (Dancer, 2011). Luick et al. (2013) reported that, unlike fluorescent markers and adenosine triphosphate (ATP) assays, subjective visual inspections were less efficient indicators of cleanliness.

Contact time of chlorine to bacteria is an important factor to reach sterility. The recommended contact time of the majority of disinfectants used in hospitals and registered with the EPA is 10 minutes (Environmental Protection Agency, 2012). For this reason, the current study adopted a 10-minute contact time when testing the performance of chlorine on dry surface biofilm. Clinically, surfaces are cleaned with disinfectant soaked wipes. The action of wiping over a surfaces thus deposits a small amount of disinfectant on the surface. Any disinfectant rapidly evaporates which means that prolonged contact times are rarely, if ever accomplished. This can have a large effect on the efficacy of the disinfectant used, in particular for disinfectants that have little residual effect such as chlorine.

Multi-measure strategies should be applied to ensure that pathogens do not spread among patients via contaminated surfaces and should include environment cleaning/disinfection, isolation of patients infected with pathogens resistant to multiple drugs, and strict compliance with hand hygiene standards. The efficiency of such multi-measure strategies

including an emphasis on environmental decontamination, has been confirmed by Sitzlar et al. (2013) and Fisher et al. (2013) reported that fewer surfaces tested positive for *C. difficile* and MRSA as a result of the use of fluorescent markers with feedback to monitor cleaning, the application of a UV-C room disinfection device, and advanced standard disinfection, including daily decontamination.

A limitation of the chlorine study is that the protocol of chlorine study was based on static exposure to the chlorine due to the difficulty in ensuring the same physical pressure was applied to all test surfaces. However, in clinical practice it is expected that chlorine exposure would be combined with the physical action of wiping.

In conclusion, this thesis has shown that biofilms incorporating viable MROs are found on the majority of ICU environmental surfaces. That biofilms which have been subjected to the stress of periodic dehydration have increased tolerance to both chlorine disinfection and heat treatment. We have developed a reproducible model that mimics these surface biofilms that can be used to test new decontamination strategies. New decontamination strategies capable of decreasing environmental transmission of pathogens should translate into lower HAI rates making our hospitals safer.

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10. Appendix A:

Bacterial species present as 10% or more of the microbiome in individual biofilms

Zone	Bacteria name			
Patient bedding				
mattress 10	*Massilia timonae*			
mattress 12	*Pseudomonas parafulva*	*Massilia timonae*		
mattress 29	*Paracoccus aminophilus*			
pillow 20	*Propionibacterium acnes*	*Mycobacterium duvalii*	*Coprococcus clostridium*	
pillow 28	*Faecalibacterium prausnitzii*	*Coprococcus clostridium*		
pillow slip 16	*Staphylococcus hominis*	*Staphylococcus epidermidis*		
pillow slip 31	*Faecalibacterium prausnitzii*	*Bacillus cereus*	*Facklamia miroungae*	*Bacillus thuringiensis*
Patient				

surrounds				
curtain cord 5	*Staphylococcus aureus*	*Propionibacterium acnes*	*Kingella oralis*	*Staphylococcus aureus*
curtain 29	*Rubrobacter radiotolerans*	*Acidithiobacillus ferrooxidans*	*Pseudoxanthomonas mexicana*	*Rubrobacter radiotolerans*
sterile supply box 48	*Propionibacterium acnes*	*Staphylococcus aureus*		*Propionibacterium acnes*
Glove box Velcro® 26	*Propionibacterium acnes*	*Ralstonia pickettii*		*Propionibacterium acnes*
Notices/posters 36	*Massilia timonae*	*Staphylococcus aureus*	*Faecalibacterium prausnitzii*	*Massilia timonae*
Fixed furnishings				
floor 14	*Pseudomonas stutzeri*	*Mycobacterium septicum*		
floor 21	*Propionibacterium acnes*	*Rubrobacter radiotolerans*		
floor 23	*Schlegelella* species	*Gordonia rubripertincta*	*Stenotrophomonas rhizophila*	*Propionibacterium acnes*