Dry surface biofilm a persistent source of pathogens in hospital: decontamination and infection control.

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Dedicated to

My Father

Habibur Rahman Chowdhury
(1951 – 1999)

and

My Mother

Rashida Khanam
(1956--)

You brought me into this world,
you believed in me
and encouraged me to aim for the stars!
There is no word to describe what you mean to me.

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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List of Publications

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Table of Contents

Contents **Page number**

List of Publications…… i
Conference proceedings…… ii
Acknowledgement…… iv
Lists of Figure……xi
Lists of Tables…….. xv
Lists of Abbreviation…… xviii
Abstract……..xx
Introduction.....1
Chapter One: Review of Literature.....3
Part One: Bacterial Biofilm and Its Formation 3
1.1 Biofilm history and hypothesis 3
1.2 What is bacterial biofilm? 4
1.3 Formation of Biofilm 5
1.3.1 Stages of biofilm formation 5
1.3.2 Regulatory Mechanism of Biofilm formation.....13
1.4 Mixed species biofilm coexistence and cooperation.....18
1.4.1 Struggle and growth inhibition.....19
1.4.2 Mixed biofilm-spatial distribution 19
1.4.3 Gene expression in mixed species biofilm.....20
1. 4.4 Why it is important to know about multispecies biofilm? 21
1.4.5 Conclusion 22
Part Two: Bacterial Biofilms in the Health Care Environment---Relevance to Hospital Acquired Infections.....23
1.5 Hospital Acquired Infections 23
1.5.1 Hospital acquired infections: relevance of biofilms.....24
1.5.2 Organisms associated with HAIs 30
1.5.3 Conclusion 32
Part Three: Transmission 33
1.6 Definition:.....33
1.7 Transmission of microorganisms 33
1.7.1 Transmission Routes of Infectious Disease 33
1.7.2 Fomites and the spread of Infectious Diseases.....35

1.7.3 History of environmental transmission 35
1.7.4 Epidemiology of infection caused by environmental transmission.....37
1.7.5 Important factors to contribute to transmission of organisms via contaminated hand/glove of health care personnel: 39
1.8 Dry Surface Biofilm (DSB) in Health-care Environment. 48
1.8.1 Biofilm in water distribution system of health care facilities 48
1.8.2 Dry surface biofilm in hospital environment 49
1.9 Prevention of transmission of DSB in hospital environment 52
1.10 Conclusion 54
Part Four: Disinfection 55
1.11 What is disinfection.....55
1.12 Types of disinfectants (Rutala and Weber, 1999) 55
1.12.1 High level disinfectants.....55
1.12.2 Low-level disinfectants 56
1.12.2 Intermediate-level disinfectants 56
1.13 Commonly used disinfectants in hospital (Rutala and Weber, 2013b) 57
1.13.1 Alcohol.....57
1.13.2 Chlorine.....57
1.13.3 Hydrogen Peroxide (H_2O_2).....59
1.13.4 Peracetic acid 59
1.13.5 Peracetic Acid and Hydrogen Peroxide 60
1.13.6 Phenolics 61
1.13.7 Quaternary Ammonium Compounds (QAC) 61
1.13.8 The aldehyde-based disinfectants.....61
1.13.9 Iodophors 62
1.14 Factors affecting disinfectants efficacy.....64
1.15 Difficulties in biofilm eradication.....64
1.15.1 Poor penetration 64
1.15.2 The transfer and co-transfer of genetic determinants.....65
1.15.3 The arbitrary presence of cells showing a resistant phenotype (known as “persisters”).66

1.15.4 The presence of cells that triggered stress responses under unfavourable chemical conditions within the biofilm matrix. 68

1.15.5 Biofilm composition....68

1.16 The eradication of an established biofilm 69

1.17 Cleaning 72

1.18 Terminal (Deep) Cleaning....73

1.19 Conclusion 73

Part Five: Challenges of creating and analysing *in vitro* dry surface biofilms 75

Part Six: Proteomics and genomics of biofilm formation 77

1.21 Important protein responsible for Staphylococci biofilm development 77

1.22 Expression of genes in *S. aureus* biofilm....78

1.23 Conclusion 81

Part Seven: Detection of hydrated biofilm. 82

1.34 Methods of biofilm detection 82

1.34.1Colony Forming Unit (CFU)....82

1.34.2 Protein detections assays....82

1.34.3 Imaging methods....83

1.34.4 Polymerase chain reaction (PCR) 84

1.35: Conclusion 85

Part eight: Hypothesis, Aims and Objectives....86

1.36 Study hypothesis 86

1.37 Aims and Objectives 87

Chapter Two: Materials and methods 89

Part One: Growth of single species dry surface biofilm 89

2.1 Bacterial Strain used 89

2.2 *S. aureus* culture preparation....89

2..2.1 Short term maintenance and growth....89

2.2.2 Long and short term bacterial storage 89

2..2.3 Colony forming unit counting....89

2.3 Growth protocol for biofilm....90

2.3.1 12-day Dry surface biofilm (DSB)....90

Batch phase 91
2.3.2 12-day Wet biofilm (WB) 92
Part two: Standard assays for biofilm studies 93
2.4. Standard protein assay.....93
2.4.1 Crystal violet (CV) assay 93
2.4.2 Bicinchoninic (BCA) Protein assay 94
2.4.3 Coomassie (Bradford) Protein Assay 96
2.5 Microscopic analysis.....97
2.5.1 Confocal laser scanning microscopy.....97
2.5.2 Scanning electron microscope.....103
2.6 Biofilm image analysis.....104
2.7 Colony forming unit (CFU) determination 105
2.8 Real time PCR for ICU samples 106
2.8.1 Bacterial DNA extraction.....106
2.8.2 Quantitative real-time PCR: 106
2.9 Detergent optimisation 107
Part one: Detergent efficacy testing; Optimisation and comparison of methods 107
2.9.1 Efficacy testing.....108
Part Two: Confirmation of disinfectant neutralizer 130
2.9.2 Planktonic control assay with disinfectants along with neutralizer validation in the presence and absence of soil. 131
2.9.3 Toxicity determination of Speedy clean detergent on planktonic bacteria in the presence of neutralizer and soil. 133
2.9.4 Neutralisation of H_2O_2 based disinfectants testing in the presence and absence of soil .135
2.10 Bacterial proteomics and transcriptomic profiling.....137
2.10.1 Microorganism and Culture Conditions.....137
2.10.2 Protein Extraction and Fractionation.....138
2.10.3 nanoLC-ESI-MS/MS.....139
2.11 RNA extraction from biofilm and reverse transcription 139
2.11.1 RNA extraction 139
2.11.2 Reverse Transcription 140

2.11.3 Primers 140

2.11.4 qPCR 140

2.11.5 Gene Quantification 141

Chapter Three: Biofilm: transmission and inactivation by heat 142

Part One: Transmissibility of dry surface biofilm bacteria 142

3.1 Introduction 142

3.2 Author's contribution 143

3.3 Original paper 144

3.4 Original paper 151

3.5 Original paper 166

Part two: Inactivation of biofilm -heat treatment efficacy 181

3.1 Introduction 181

3.2 Original paper 182

Chapter four: Developing novel chemistries for removing environmental surface biofilms to reduce hospital acquired infections 190

4.1 Introduction 190

4.2 Original paper 192

Chapter Five: Development of multispecies dry surface biofilm model 211

5.1 Introduction 211

5.2 Bacterial strains used 212

5.3 Development of *in vitro* multispecies dry surface biofilm (DSB) model 213

5.3.1 First Trial 214

5.3.2. Second trial 214

5.3.3 Third trial 215

5.4 *Pseudomonas putida* in mixed species biofilm 221

5.5. Development of mixed species biofilm model with three bacterial species. 222

5.5.1 Mixed species biofilm development 222

5.5.2 Inoculation of *S. aureus* 24 hours before the addition of other species with 5% TSB in all phases. 224

5.5.3 Inoculation of *S. aureus* 24 hours before the addition of other species with 10% TSB followed by 5% TSB in next four phases. 225

5.5.4 Inoculation of *S. aureus* 24 hours before the addition of other species with 10% TSB in first two phases followed by 5% TSB in next three phases. 227

5.6 Original Paper 235

Chapter Six: Comparative proteomic profile; new insight of *Staphylococcus aureus* dry surface biofilm 251

6.1 Introduction 251

6.2 Original Paper 252

Chapter Seven: General discussion and conclusion 279

7.1 Discussion 279

7.2 Conclusion 285

References 286

Chapter Nine: Appendix 316

9.1 Table: Number of residual viable bacteria/coupon recovered after treatment with detergents. 316

9.2 Standard preparation of biological material for SEM analysis 317

9.2.1 0.1 M phosphate buffer: 317

9.2.2 3% glutaraldehyde: 317

9.3: Copyright permission 317

List of figures

Figure 1- 1: Phases of biofilm formation. Biofilm development includes irreversible attachment to the surfaces or host matrix protein, maturation and final detachment. 8
Figure 1- 2: Bacterial quorum sensing. After entering the bacterial cell Quorum sensing molecules (QSMs) bind to specific regulatory proteins and form protein complex. Protein molecule complex activates transcription of virulence factors and biofilm formed with subsequent production of more QSMs. 15
Figure 1- 3: Spatial distribution within mixed-species biofilm (Elias, 2012). (a) separate mono-species microcolonies (Nielsen et al. 2000); (b) co-aggregation (Rickard et al. 2006)]; (c) arranged in layers (Hansen et al. (2007). Microscopy image was used with permission from original authors (appendix 9.3). 20
Figure 1-4 : Model of biofilm resistance based on persister survival. An initial treatment with antibiotic kills planktonic cells and the majority of biofilm cells. The immune system kills planktonic persisters, but the biofilm persister cells are protected from host defenses by the exopolysaccharide matrix. After the antibiotic concentration drops, persisters resurrect the biofilm and the infection relapses (Lewis, 2008). 67

Figure 2- 1: Centre for disease control bioreactor (CBR) 90
Figure 2- 2: Crystal violet standard curve for calculating the amount of CV (µg/ml) eluted from samples on the basis of the optical density at 590nm wavelength. 94
Figure 2- 3: BSA standard curve for calculating the amount of protein (µg/ml) from samples on the basis of the optical density at 590nm wavelength. 95
Figure 2- 4: BSA standard curve for Bradford assay to calculate the amount of protein (µg/ml) from samples on the basis of the optical density at 595nm wavelength. 97
Figure 2- 5: CLSM image of stained dry surface biofilm with SYTO 60 demonstrating biofilm DNA (A), Lectin showing glycoconjugates (B) and Sypro orange showing protein (C) stain. 99
Figure 2- 6: CLSM image of stained dry surface biofilm with Live/dead stain (backlight 7012) 100
Figure 2- 7: BSA standard curve for OPA assay to calculate the amount of protein (µg/ml) from samples 101
Figure 2- 8: Biofilm coated coupon observed under fluorescence microscope 103
Figure 2- 9: Scanning electron microscope images showing polycarbonate coupon covered with biofilm. Picture showing the aggregation of biofilm bacteria with thick EPS.....104
Figure 2- 10: *S. aureus* biofilm bacteria colonies grew on HBA plate at 10^{-3} dilution after 10-fold serial dilution of sonicated fluid with sterile PBS. 105
Figure 2- 11: Crystal violet absorbance value after treatment with detergents (mean value) 112
Figure 2- 12: Absorbance value in BCA assay after treatment of biofilm coupons with detergents for 10 minutes (mean).....115
Figure 2- 13: Absorbance level in Bradford assay after treatment with detergents for 10 minutes.....116
Figure 2- 14: Calibration curve for TOC analysis.....122
Figure 2- 15: Detected TOC (µg) level after treatment of dry biofilm with detergents.....124
Figure 2- 16: Number of residual viable bacteria (CFU) recovered after treatment with Surfex, Na-HOCl and Chlorclean tablet in presence of soil. 126
Figure 2- 17: Standard curve for Micro BCA Assay to determine residual protein after treatment with detergents 128
Figure 2- 18: Percentage reduction of biofilm protein detected by micro BCA assay after treatment with Surfex, Chlorclean Tablet (1000 ppm) and liquid chlorine (hypochlorite solution) 1000 ppm.....129

Figure 5- 1: Schematic diagram of CFU counting after development mixed species biofilm 213
Figure 5- 2: Number of colony forming units of four species of bacteria incorporated into DSB obtained after increasing hydration time in 3rd phase. 216
Figure 5- 3: Percentage of four bacterial species incorporated into DSB, obtained after increasing hydration time in 3rd phase.....216
Figure 5- 4: Percentage of four bacterial species incorporated into DSB obtained after decreasing dehydration time in all phases.....217

Figure 5- 5: Percentage of four bacterial species incorporated into DSB obtained after increasing hydration time in 3rd & 4th phase and dehydration time (66 hrs.) at 5th phase. 218
Figure 5- 6: Number of colony forming units of four species of bacteria incorporated into DSB obtained after a decrease dehydration time and increase hydration time in 3rd and 5th phase. 219
Figure 5- 7: Number of colony forming units of four species of bacteria incorporated into DSB obtained showing the absence of P. putida after each phase of dehydration. 221
Figure 5- 8: Percentage of four species of bacteria incorporated into DSB obtained showing very low percentage of P. putida after increasing hydration time in all phases. 221
Figure 5- 9: Number of CFU of three species incorporated into DSB. Addition of three species at commencement of the first phase with 5% TSB in all phases. 223
Figure 5- 10: Percentage of three bacterial species incorporated into DSB when all three species were added at the commencement of the first phase. 223
Figure 5- 11: Number of CFU of three bacterial species incorporated into DSB following addition of S. aureus 24 hours before addition of other species, with 5% TSB in all phases. 224
Figure 5- 12: Percentage of three bacterial species incorporated into DSB after addition of *S. aureus* 24 hours before addition of other species, with 5% TSB in all phases. 225
Figure 5- 13: Number of CFU of three bacterial species incorporated into DSB following after addition of S. aureus 24 hours before addition of other species, with 10% TSB followed by 5% TSB in next four phases. 226
Figure 5- 14: Percentage of three bacterial species incorporated into DSB after addition of S. aureus 24 hours before addition of other species, with 10% TSB followed by 5% TSB in next four phases. 226
Figure 5- 15: Number of CFU of three bacterial species incorporated into DSB following after addition of S. aureus 24 hours before addition of other species with 10% TSB followed by 5% TSB in next three phases. 228
Figure 5- 16: Percentage coefficient of variation of three bacterial species obtained from DSB after addition of S. aureus 24 hours before addition of other species, with 10% TSB followed by 10% TSB in next three phases. 228
Figure 5- 17: Total number of CFU obtained from each coupon (total of three bacterial species incorporated into DSB) after addition of S. aureus 24 hours before addition of other species, with 10% TSB in 1st and 2nd phase followed by 5% TSB in next three phases. 229
Figure 5- 18: Percentage coefficient of variation (CV) obtained between each coupon after addition of S. aureus 24 hours before addition of other species, with 10% TSB in 1st and 2nd phase followed by 5% TSB in next three phases. 230
Figure 5- 19: Number of colony forming units of the three bacterial species incorporated into DSB after addition of S. aureus 24 hours before addition of other species, with 10% TSB in 1st and 2nd phase followed by 5% TSB in next three phases. 230
Figure 5- 20: Percentage of three bacterial species incorporated into DSB after addition of S. aureus 24 hours before addition of other species, with 10% TSB in 1st and 2nd phase followed by 5% TSB in next three phases. 231
Figure 5- 21: Coefficient of variation (CV) of three species, obtained from develop mixed species biofilm, after addition of S. aureus 24 hours before addition of other species, with 10% TSB in 1st and 2nd phase followed by 5% TSB in next three phases. 231
Figure 5- 22: Interexperimental variation in number of CFU of the three bacterial species incorporated into DSB after addition of S. aureus 24 hours before addition of other species, with 10% TSB in 1st and 2nd phase followed by 5% TSB in next three phases. 232
Figure 5- 23: Between run coefficient of variation (CV) for the three bacterial species incorporated into DSB after addition of S. aureus 24 hours before addition of other species, with 10% w/v TSB in 1st and 2nd phase followed by 5% TSB in next three phases. 232
Figure 5- 24: Scanning electron microscope images showing polycarbonate coupon covered with mixed species biofilm bacterial aggregation within thick EPS A) magnified 6500X; B) magnified 16000X. 233
Figure 5- 25: Composition of 13-day dry mixed species biofilm extracellular polymeric substances (EPS). EPS are stained for extracellular DNA (red), stained with Syto 60; glycoconjugate (blue), stained with Alexa-488-labelled *Aleuria aurantia* lectin and protein (green), stained with Sypro Orange. 233

List of Tables

Table 1- 1: Biofilm maturation and detachment determinants in Staphylococci spp.....12
Table 1- 2: Some examples of LuxR/LuxI/AHL-dependent quorum sensing systems in Gram negative bacteria (Williams et al., 2007).....17
Table 1- 3: Sources and transmission of hospital-acquired organisms in hospital environment (Kolmos, 2012).....34
Table 1- 4: Survival times of multidrug-resistant organisms (MDROs) on dry inanimate objects. Range depends on experimental design and methods of assessing contamination and material used as test surface. Ratio of increased risk associated with the room being previously occupied by patients infected with common MDROs. (Kramer et al., 2006; Dancer, 2008; Otter et al., 2013; Havill et al., 2014)....40
Table 1- 5: Typical bacterial loads for healthcare related surfaces.....43
Table 1- 6: Effect of colonization or infection status of the prior room occupant on the acquisition of pathogens by subsequent occupants of the same room.....44
Table 1- 7: Studies of spread of pathogens associated with bed components.....52
Table 1- 8: Characterization of disinfectants according to their class (Rutala and Weber, 2001; Gilbert and McBain, 2003; Rutala and Weber, 2004).....56
Table 1- 9: Summary of advantages and disadvantages of disinfectants used as low-level disinfectant (Rutala and Weber, 2014).....63

Table 2- 1: Culture condition for formation of 12-day dry surface biofilm (DSB). Initial inoculum of about 108 of S. aureus was added at the beginning of stage -1 phase.....92
Table 2- 2: Culture conditions for formation of 12-day wet biofilm (12DWB) with an initial inoculum of about 10^8 CFU of S. aureus, at the beginning of Stage-1 batch phases.....92
Table 2- 3: Fluorescence values of OPA assay with 72-hour wet biofilm.....102
Table 2- 4: Number of residual viable bacteria recovered after treatment with detergents 111
Table 2- 5: Absorbance value of crystal violet with different detergents based on Blank corrected (590) 113
Table 2- 6: Absorbance level of detergents in BCA (A) and Bradford assay (B).....117
Table 2- 7: The amount of ATP measured in triplicate as relative light units (RLU) detected following detergent treatment of coupons contaminated with dry biofilm. ATP was measured by a) swabbing the coupon directly, b) sampling the liquid that treated coupons were sonicated in and c) swabbing the coupon directly after sonication.....120
Table 2- 8: Results of negative control after TOC analysis 123
Table 2- 9: TOC analysis of background control coupon (detergent treated sterile clean coupon without biofilm).....123
Table 2- 10: TOC analysis of biofilm coated coupon after treatment with detergents 124
Table 2- 11: Disinfectant testing procedure against control planktonic bacteria with Surfex, SDIC and Chlorclean tablet.....131
Table 2- 12: CFU obtained- after neutralizer toxicity test with three disinfectants; Surfex, SDIC and Chlorclean tablet 131
Table 2- 13: Control CFU obtained- after testing with Chlorclean, SDIC solution and Surfex with or without soil.....132
Table 2- 14: Log_{10} obtained from Positive control and neutralizer toxicity test control.....132
Table 2- 15: Log_{10} obtained after treatment with Surfex, Chlorclean tablet and SDIC solution after five minutes contact 132
Table 2- 16: Disinfectant testing procedure against planktonic S. aureus control bacteria with speedy clean detergent followed by Surfex, SDIC and Chlorclean tablet 133
Table 2- 17: Control CFU obtained- from positive control and neutralizer toxicity test.....134
Table 2- 18: Log_{10} obtained from positive control and neutralizer toxicity test after speedy clean treatment followed by disinfectants 134
Table 2- 19: Disinfectant testing procedure against planktonic S. aureus control bacteria with 0.6% active H_2O_2 (A), 0.6% diluted H_2O_2 (B) and Proxitane (C). 136
Table 2- 20: Control CFU obtained after treatment with three disinfectants in the presence and absence of soil 136

Table 2- 21: CFU obtained from positive control and neutralizer toxicity test 137
Table 2- 22: Amount of protein obtained from 12-day dry surface biofilm (from each run of bioreactor;3) and planktonic bacteria from 24-hours liquid culture (three episodes), maintaining similar growth condition in each episode of growth of DSB and planktonic culture.....139

Table 5- 1: Culture condition for formation of mixed species dry biofilm. Initial inoculum of about 10^8 of each (four) bacterial species was added at the beginning of stage -1 phase. 214
Table 5- 2: Culture condition for formation of mixed species dry biofilm. Initial inoculum of about 10^8 of P. aeruginosa was added at the beginning of stage -1 phase and 10^8 of each of the four bacterial species was added at the beginning of stage -2 phase.....215
Table 5- 3:Culture condition for formation of mixed species dry biofilm. Initial inoculum of about 10^8 of S. aureus was added at the beginning of stage -1 phase and 10^8 of four bacterial species was added at the beginning of stage -2 phase with increase hydration time in 3rd phase.....215
Table 5- 4: Culture condition for formation of mixed species dry biofilm. Initial inoculum of about $10^8$of P. aeruginosa was added at the beginning of stage -1 phase and 10^8 of four bacterial species was added at the beginning of stage -2 phase and duration of dehydration phases were decreased.....217
Table 5- 5: Culture condition for formation of mixed species dry biofilm. Initial inoculum of about 10^8 of P. aeruginosa at stage 1 and then 10^8 of the four bacterial species was added at the beginning of stage 2 phase was combined with decreasing dehydration time in 2nd and 3rd phase and an increase dehydration in 5th phase. 218
Table 5- 6: Culture condition for formation of mixed species dry biofilm. Initial inoculum of about $10^8$of *P. aeruginosa* and 10^8 of four bacterial species was added at the beginning of stage -1 and 2 phase respectively with decreasing dehydration and increase hydration time in 3rd and 5th phase.....219
Table 5- 7: The within and between experimental coefficient of variation for individual bacteria grown in a mixed biofilm.....220
Table 5- 8: Culture condition for formation of mixed species dry biofilm. Initial inoculum of about 10^8 of all three-bacterial species were added at the beginning of stage -1 phase.....222
Table 5- 9: Culture conditions for formation of mixed species dry biofilm. Initial inoculum of about $10^8$of S. aureus was added at the beginning of stage -1 and 10^8 of three bacterial species were added at the beginning of stage -2 phase.....224
Table 5- 10: Culture conditions for formation of mixed species dry biofilm. Initial inoculum of about 10^8 of S. aureus was added with 10% TSB at the beginning of stage -1 and $10^8$of the three bacterial species were added at the beginning of stage -2 phase. 225
Table 5- 11: Culture conditions for formation of mixed species dry biofilm. Initial inoculum of about $10^8$of S. aureus was added at the beginning of stage -1 and 10^8 of three bacterial species was added at the beginning of stage -2 phase.....227

List of abbreviation

agr	Staphylococcal accessory gene regulator genes
AI-2	Autoinducer 2
AIPs	Autoinducing peptides
ATCC	American Type Culture Collection
AHL N	Acylhomoserine lactone
ALCL	Anaplastic large-cell lymphoma
Aap	Accumulation associated protein
BCA	Bicinchonic acid
BSA	Bovine serum albumin
BCS	Bovine calf serum
βME	Beta marcaptoethaen
°C	Degrees Celsius
CDI	*C. deficilli* infection
CDC	Centers for Disease Control and Prevention
CFU	Colony forming unit(s)
CLSM	Confocal laser scanning microscopy
cm	Centimetre(s)
CoNS	Coagulase-negative Staphylococci
CH	Chloral hydrate
CV	Crystal violet
%CV	Percentage Coefficient of variation
DSF	Diffusible signal factor
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DCA	Drichloroacetic acid
eDNA	Extracellular deoxyribonuclease
Embp	Extracellular matrix binding protein
EDTA	Ethylenediamine-N, N’-tetraacetic acid
EPS	Extracellular polymeric substance(s)
EPA	Environmental Protection Agency
ESBL	Extended spectrum beta lactamase
HCW	Health care worker
HCD	High cell density.
HP	Hydrogen peroxide
HCP	Healthcare personnel
HAI	Hospital acquired infections
HCl	Hydrochloric acid
HMDS	Hexithyldisilazane
ica	Intercellular adhesion genes

ICU	Intensive care unit
MS-DSB	Mixed species dry biofilm
MDRO	Multi drug resistant organisms
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
MSCRAMM	Microbial Surface Components Recognizing Adhesive Matrix Molecules
n	Number
OD	Optical density
PNAG	Poly-N-acetylglucosamine
PIA	Polysaccharide intercellular adhesin
PSMs	Phenol-soluble modulins
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodide
PCB	Polychlorinated biphenyl
QS	Quorum sensing
rpm	Revolutions per minute
SasG	*S. aureus* surface protein G
SCVs	Small colony variants
SDIC	Sodium dichloroisocyanurate
SP-DSB	Single species dry surface biofilm
SEM	Scanning electron microscopy
TSB	Tryptone soya broth
TCA	Trichloroacetic acid
UTI	Urinary tract infection
µL	Microlitre
VRE-	Vancomycin-resistant Enterococci
WHO	Word health organisation

Abstract

Environmental surface cleaning and disinfection is crucial for preventing HAI and maintaining patient safety. However, biofilms incorporating multiple bacterial species form on dry hospital surfaces. These biofilms are tolerant to biocides making their eradication difficult. Persistence of pathogens on surfaces increases the risk of transmission and development of HAI.

Aims:

1) To determine if bacteria can be transmitted from dry surface biofilm (DSB) to healthcare worker's hands and through cotton sheets.
2) To develop and test new removal chemistries against dry surface biofilm utilising laboratory models.
3) To develop a mixed species biofilm model analogous to the hospital surface DSB to determine its composition, the effect of multiple species on biofilm's susceptibility to detergents and disinfectants and analyse proteomics and transcriptomics technique.
4) To investigate protein regulatory changes in DSB compared with traditional hydrated biofilm and confirmation with RT PCR.

This study revealed that:

1) DSB is highly transmissible. The total bacterial number transferred are sufficient to cause disease.
2) Soil inactivates the commonly used disinfectants. Peracetic acid reduces 64% of the biofilm mass and >6 Log_{10} viability in single species DSB, 48% of the biofilm mass in mixed DSB in the presence of soil.
3) This study developed a mixed species DSB model with reproducible cell number within and between repeated experiments. This mixed biofilm model was employed to assess the effectiveness of disinfectants.
4) These findings clearly imply that *S. aureus* biofilms represent a unique growth condition in terms of overall numbers of differentially expressed distinct proteins and genes.

This study establishes the transmissibility of bacteria from DSB affirming it to be a potential source of HAI and determined that currently surface disinfectants and methods are insufficient to completely eradicate DSB. This study also established different changes in protein and genetic expression in biofilm, this might act as target group to invent new effective disinfectant for complete eradication of DSB.

Introduction

Infections acquired in health care settings are a major contributor to increased mortality and are associated with a significant increase in morbidity and economic losses. Hospital acquired infections (HAI) occur worldwide and affect both developed and resource poor countries. Pathogens responsible for HAI vary between different patient populations, different health care settings and different countries. Over the past decade, the role that contaminated hospital environmental surfaces are play in the transmission of several significant healthcare associated pathogens including methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin resistant *Enterococcus spp.* (VRE), *Clostridium difficile*, *Acinetobacter spp.* etc. (Weber et al., 2010; Dancer, 2014; Mitchell B.G. et al., 2015). The environmental contamination was thought to be transient planktonic bacteria but Vickery et al. (2012) reported that environmental surfaces such as sterile supply bucket, a ward door, a venetian blind cord and rubber from around a sink were colonised by biofilms incorporating pathogens. This indicates that, the medical environment is favourable for biofilm development, and the fight against their formation is a great challenge to ensure the safety of health care services and to reduce the associated microbiological risk.

Microbes have a tendency to attach to available surfaces and form biofilms. Traditionally biofilms are associated with wet surfaces such as indwelling medical devices and tubing on medical equipment ‘or environmentally in sink or drains. However, microbes can survive for extended periods in a desiccated state on dry hospital surfaces (Hu et al., 2015a). After attachment and formation of biofilm on to the surface, bacteria are less susceptible to biocides, antibiotics and physical stress. Therefore, surface attachment and biofilm formation may explain how vegetative bacteria can survive on surfaces for weeks to months (or more). After formation of biofilm, bacteria embrace a mixed bacterial population for the horizontal transfer of resistance genes (Otter et al., 2015).

Biofilm rarely exist as a single species, but generally incorporate a significant range of microorganisms to form multispecies biofilm (Søren Møller et al., 1998; Elias and Banin, 2012). This is particularly true of environmental biofilm. (Elias and Banin, 2012).

One of the most alarming consequences of synergistic interactions of multispecies biofilm is resistance to antimicrobial agents (Al-Bakri et al., 2005; Burmølle et al., 2006; Bennett, 2008). Antibiotic resistant mechanism of mixed biofilm does not necessarily require direct acquisition

or transfer of resistant genes, but rather depend on the communication ability of the microbes to cooperate in such a way that it can be able to make them antimicrobial agent tolerant (Elias and Banin, 2012).

Once biofilm forms in the hospital environment, it becomes very difficult and expensive to control and often impossible to eradicate due to the high intrinsic tolerance to clinically used disinfectants (Cortés et al., 2011). Currently available disinfection products and processes are not sufficient to control and prevent the spread of pathogens responsible for nosocomial infection (Simões et al., 2007). Hence, new accurate products and technologies with 'permanent' antimicrobial and biocidal activity without the risk of generating resistant microorganisms is paramount for effective patient care and prevention of HAI.

Chapter One: Review of Literature

Part One: Bacterial Biofilm and Its Formation

1.1 Biofilm history and hypothesis

Planktonic bacteria are the foundation of our understanding of infection and disease and were thought to be the primary bacterial form existing in the environment. Van Leeuwenhoek was the first person who saw bacteria coexisting together after observing microbial life in the plaque growing on his own teeth in 17th century (Gest, 2004). With this observation he described microscopic microbial life for the first time, which was the first key step in the development of modern microbiology. However the significance of these observations, with respect to bacterial biofilms and disease, was not realised for almost 300 years (Gest, 2004).

In 1978, a study reported that sessile bacterial counts within biofilm, collected from rocks and other surfaces in a pristine mountain stream, predominated over planktonic counts by several orders of magnitude (Geesey et al., 1978). These results were consistent with observations from previous studies of aquatic ecological sites, including marine and soils, where sessile bacteria within biofilms vastly outnumbered the planktonic bacteria (Lloyd, 1930; Henrici, 1933; Zobell and Anderson, 1936; Zobell, 1943).

Costerton et al. (1978) hypothesized that, in all nutrient-sufficient ecosystems, biofilm bacteria grow in matrix-enclosed surface-associated communities, which provide protection from external adverse factors (Costerton et al., 1978). It is generally thought that over 99% of all bacteria form biofilms in almost all nutrient-sufficient aquatic environments including natural (Geesey et al., 1978), industrial (Dempsey, 1981; Costerton et al., 1995), and medical ecosystems (Costerton et al., 1995; Costerton et al., 1999; Donlan and Costerton, 2002). Bacteria within biofilms exhibit different physiological characteristics including lower metabolic rate, than their planktonic counterparts, making biofilm bacterial culture and analysis, using standard liquid culture techniques difficult. (Costerton et al., 1987; Costerton et al., 1994; Costerton et al., 1995). However, over the past few decades, advancement in culture, microscopy and molecular techniques have led to an increased understanding of the biology of biofilms and their function in ecosystems, industry and clinical fields (Costerton et al., 1987; Costerton et al., 1995; Donlan and Costerton, 2002).

Bacterial biofilms cause significant financial costs to industry, for example- causing corrosion- in marine industries (Dempsey, 1981), in petroleum and water storage and distribution systems (Regan et al., 2003; Beech and Sunner, 2004; Berry et al., 2006).

Since Pasteur and Koch developed the Germ Theory of Disease, the discipline, medical microbiology had developed an ever-increasing knowledge of infectious diseases using standard investigative criteria. This knowledge has also led to the development of antibiotics and vaccines for the effective treatment and prevention of many of these infectious diseases (Costerton et al., 1987; Costerton, 1999; Costerton et al., 1999). Thus, in the 1980s, when initial data suggested that bacteria can form biofilms within the human body without causing acute suppurative disease and overwhelming sepsis, the *status quo* of bacterial disease in medicine was challenged (Costerton, 1999). Bacterial biofilm infections are now frequently recognised by the medical and surgical fraternity as an important cause of chronic disease and surgical implant failure (Donlan, 2001a; Donlan and Costerton, 2002; Lindsay and Von Holy, 2006).

1.2 What is bacterial biofilm?

Biofilm can be defined as:

"*a microbially derived sessile community characterised by cells that are irreversibly attached to a substratum or interface or to each other, embedded in a matrix of extracellular polymeric substances that they have produced and exhibit an altered phenotype with respect to growth rate and gene transcription*" (Donlan and Costerton, 2002).

This diverse community of organisms are physiologically distinct from swarming cells of the same organism. The biofilm structure supports incorporated bacteria by increasing their survival, growth, reproduction whilst simultaneously protecting them from a hostile external environment and suggests that biofilm have an important selective advantage over planktonic bacteria (Davey and O'toole, 2000; Costerton and Stewart, 2001).

Study showed that biofilm develops as complex three dimensional microcolony composed of 75-95% (by volume) extracellular matrix and 5-25% (by volume) cellular component (Lawrence et al., 1991). The extracellular matrix (EPS) encloses the bacteria and is an essential ensuring attachment to the substratum, biofilm development and for the survival of the resident bacteria it encloses (Flemming and Wingender, 2010; Bjarnsholt, 2013). Within the biofilm exists a microcirculation for nutrient and oxygen provision and waste excretion (Costerton and Stewart, 2001).

1.3 Formation of Biofilm

During biofilm formation planktonic bacteria undergo changes in gene expression which results in a change in bacterial physiology (Kostakioti et al., 2013). During the transition, numerous newly activated regulatory networks influence the change of concerted gene expression thereby mediating the spatial and temporal reorganization of the bacterial cell (Pratt and Kolter, 1998; Prigent-Combaret et al., 2001; Monds and O'Toole, 2009).

1.3.1 Stages of biofilm formation

Bacterial biofilm is a multistep process with four common stages

Bacterial adhesion or surface attachment

Micro colony formation

Biofilm maturation

Dispersion or detachment.

1.3.1.a Bacterial adhesion

Many different non-specific interactions and interfacial forces like hydrophobic interactions and hydration forces also influence cell attachment. To form biofilm, regulation of adsorption, adhesion, diffusion, physiology of bacteria and their growth depends on the properties of substrates, eg. stiffness, mechanical stability and elasticity. Consequently in response to surface properties, cells secrete DNA, proteins, lipids and lipopolysaccharide that accumulate and form the EPS, influence the stiffness and elasticity of biofilm (Flemming and Wingender, 2010). High surface free energy or wettability of the surface is an important issue for bacterial attachment (Kokare et al., 2009). Surface with high surface free energy like glass and stainless steel are more hydrophilic and show greater bacterial attachments than hydrophobic surfaces such as Teflon, Buna-n rubber and fluorinated hydrocarbon (Kokare et al., 2009). Adhesion of bacteria to hydrophilic surfaces is enhanced if the surface tension of the bacterial cell wall is higher than the surface tension of the surrounding liquid (Absolom et al., 1983; Davidson and Lowe, 2004).

Bacterial attachment increases as the surface roughness increases (Donlan, 2002) as rough surfaces have a greater surface area for cell attachment. Ra (µm/µin) is representation of surface roughness which is the average of a set of individual measurements of a surface's peaks and valleys.

Ra can be calculated by using following formulas (Ståhl et al., 2011):

$$Ra = (1/L)\int_0^L |Z(x)|dx$$

L = evaluation length

Z(x) = the profile height function

A study evaluated that *Staphylococcus epidermidis* (ATCC35984) has the ability to adhere to the solid biomaterial's surface at different levels of roughness. Five kinds of biomaterials: oxidized zirconium-niobium alloy (Oxinium), cobalt-chromium-molybdenum alloy (Co-Cr-Mo), titanium alloy (Ti-6Al-4 V), commercially pure titanium (Cp-Ti) and stainless steel (SUS316L), samples of which were categorized into a fine group had comparatively smooth surfaces and recorded low average roughness (Ra: 1.8-8.5 nm, <10 nm); and the specimens in the coarse group exhibited comparatively rougher surfaces (Ra: 7.2-30.0 nm). Study revealed that larger amounts of *S. epidermidis* adhered to each specimen in the coarse group than in the fine group, in particular, Oxinium, Ti-6Al-4 V and SUS316L were statistically significant (Yoda et al., 2014). A study on bacterial attachment on titanium surfaces demonstrated that roughness on the nanometre scale increases the attachment of bacteria than micrometre scale (Truong et al., 2009). Several studies have demonstrated that, bacteria more than surface roughness at the nanometre scale enhances colonization efficiency of biofilm bacteria on titanium surfaces (Whitehead et al., 2005) and polymer surfaces (Bakker et al., 2004). A different study on relation between bacterial adhesion and surface roughness (nanostructured titania thin films) showed that, roughness increased up to 20 nm enhances bacterial adhesion and biofilm formation, however, the further increase of roughness improves protein adsorption, that leads to a significant decrease of bacterial adhesion and inhibits biofilm formation (Singh et al., 2011). However, it is not absolute that there will always be "one size fits all" relationship between roughness and attachment, as bacterial strains- even with in the same species can vary significantly (Young, 2006).

Bacterial adhesion is a two-step process. In the first step, bacteria loosely attach to a surface, in a process called primary adhesion or docking (Dunne, 2002). In the second step, bacteria undergo a molecule-mediated binding process where bacteria bind irreversibly to the attached surface followed by production of extracellular polymeric substance, in a process called secondary adhesion or locking (Dunne, 2002).

Primary adhesion or docking

Planktonic bacteria can attach to either biotic or abiotic surfaces at the initial step of biofilm formation. Presence of various physiochemical variables; such as bacterial cell-surface binding molecules, polymers and surface conditioning substances might influence the primary attachment of biofilm bacteria (Dunne, 2002). Planktonic bacteria tend to attach when they come within <1nm of a surface (Dunne, 2002). Movement to a designated surface can be mediated by Brownian motion and convective mass transport, or directly with flagella, pili and fimbriae (especially in Gram negative bacteria) in response to chemotactic mechanisms (Rosenberg et al., 1982). Once bacteria get close to a surface, numerous attractive and repulsive mechanisms; such as- van der Waals, hydrophobic interactions, electrostatic and hydrodynamic forces occur and if the net sum of these forces are attractive, then primary attachment might occur (Dunne, 2002).

Electrostatic forces are among the first interactions that influence bacteria to attach to the surface. Bacteria that are negatively charged attach rapidly and tightly to positively charged surfaces and vice versa. Extra cellular organelles such as fimbriae, flagella, curli and pili promote adhesion and help to overcome destabilizing interactions between cells and anionic surfaces at the initial stage of attachment (Bullitt and Makowski, 1995). High ionic strength liquids eliminate the charge discrimination of surfaces. The part of bacterial cell wall that exposed to the extracellular environment, related with various groups including carboxylate, hydroxyl, phosphate and amine moieties are more complex and promotes the adsorption of bacteria and their growth into biofilms (Renner and Weibel, 2011).

Hydrophobicity also plays an important role in determining the ability of bacteria to form biofilms. After overcoming electrostatic repulsions, the preferential alignment of hydrophobic functional groups on surfaces and hydrophobic moieties on the bacteria cell wall, and extracellular organelles, stabilizes interfacial interactions (Donlan and Costerton, 2002).

During this stage of reversible adhesion, before secretion of EPS and irreversible adhesion the bacteria can exhibit several species-specific behaviours, which include rolling, creeping and aggregate formation (Korber et al., 1995). In addition, some of the adherent cells will detach and resume the planktonic lifestyle.

Secondary Adhesion or Locking

The transition from reversible to irreversible attachment occurs between 60 seconds to ten minutes depending on the bacteria involved and the surface properties (Palmer et al., 2007). It is frequently mediated by the up-regulation of biofilm promoting genes which promote EPS production and adhesion reactions between the bacteria and the target surface (Dunne, 2002) Bacteria have numerous adhesins including the Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMM). MSCRAMM are commonly found on Gram positive bacteria, particularly Staphylococci species and recognise different host matrix and plasma proteins thus enhancing bacterial/substratum adhesion (Wang et al., 1993; Mack et al., 2008). *S. aureus* has over 20 surface-anchored adhesin genes and *S. epidermidis* has over 12 adhesin genes for these specific host matrix and plasma proteins.

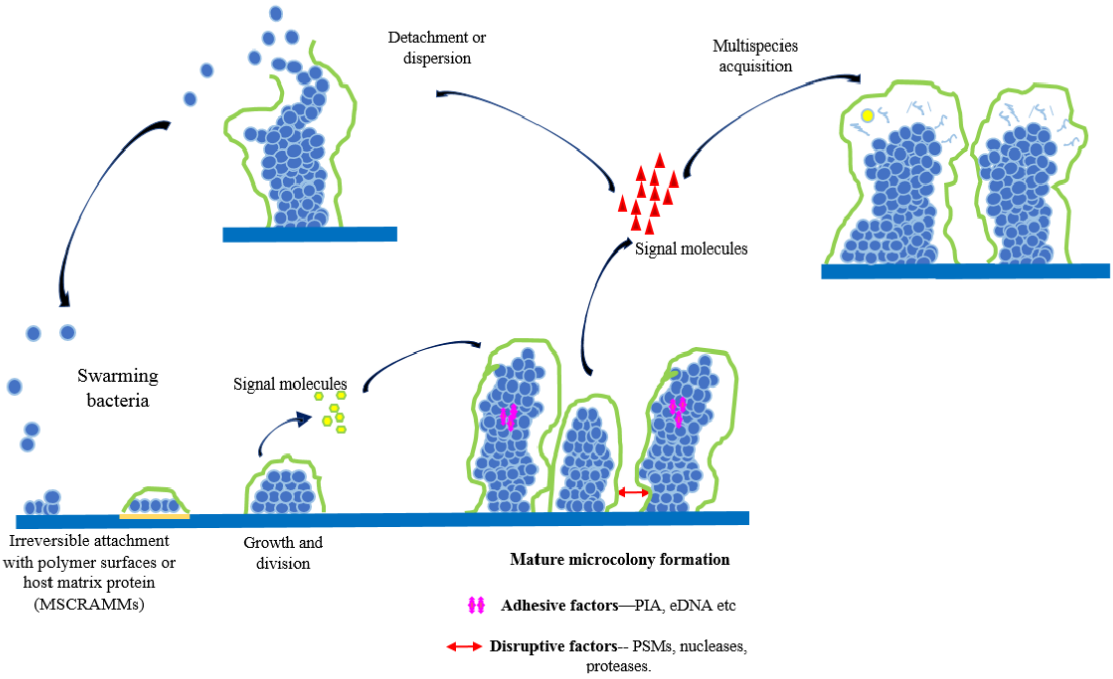

Figure 1- 1:

Phases of biofilm formation. Biofilm development includes irreversible attachment to the surfaces or host matrix protein, maturation and final detachment.

(Atshan et al., 2013). An *in vitro* study demonstrated that, some of the genes coding for MSCRAMM proteins are up-regulated within the first 12-24 hours of switching from planktonic to biofilm growth and are further enhanced within 30-60 minutes of exposure to human plasma proteins supporting their role in adhesion and early biofilm cell aggregation (Arciola et al., 2012; Atshan et al., 2013).

1.3.1.b Micro colony formation

Once attached, the adhesive processes promote accumulation of bacteria and cells aggregate into microcolonies within the EPS by accumulation of growth of attached bacteria, promoted by various complex substances in bacterial cell wall including carboxylate, hydroxyl, phosphate and amine moieties (Renner and Weibel, 2011) or by attracting additional planktonic bacteria (Hall-Stoodley and Stoodley, 2002). This can result in high bacterial densities up to 10^7 cell/cm^2 within 48 hours in some species (Hall-Stoodley and Lappin-Scott, 1998).

1.3.1.c Biofilm Maturation

Micro colony formation helps to establish a three-dimensional structure which exhibits a dynamic interacting/adhesive process between the biofilm building bacteria and their surrounding environmental stimuli (Hall-Stoodley et al., 2004). Adhesive processes link bacteria together during proliferation and disruptive processes that form channels in the biofilm structure (O'Toole et al., 2000). Different bacterial species use different adhesin molecule within the EPS to link to each other. In Staphylococci, one of the most important adhesive biofilm molecule is an exopolysaccharide named polysaccharide intercellular adhesin (PIA), also termed poly-N-acetylglucosamine (PNAG) encoded by the *ica* gene (Mack et al., 1996). PIA was considered indispensable for both in vitro and in vivo biofilm formation (Rupp et al., 1999; Rupp et al., 1999). However, more recent studies identified a biofilm forming staphylococcal strains without ica genes and thus do not produce PIA (Kogan et al., 2006; Rohde et al., 2007). The other important identified biofilm adhesive proteins are accumulation associated protein (Aap) (Kogan et al., 2006; Conrady et al., 2008), extracellular matrix binding protein (Embp) (Christner et al., 2010), protein A (Merino et al., 2009), *S. aureus* surface protein G (SasG) (Geoghegan et al., 2010) and others. However, the mechanisms of actions of these proteins in cell–cell adhesion has not demonstrated yet and are under intense current investigation.

Phenol-soluble modulin (PSM) surfactant peptides is the key biofilm structuring factor for biofilm-forming pathogen *S. aureus* that participate in structuring and detachment processes (Periasamy et al., 2012). A study by Periasamy et al. (2012) showed that PSMs mutation (deletion) in *S. aureus* strongly hampers the formation of biofilm channels and abolish the characteristic waves of biofilm detachment and regrowth followed by loss of control of biofilm expansion. Furthermore, study also demonstrated that PSMs facilitated dissemination of biofilm-associated infection from an infected catheter in a mouse model. Study also showed that formation of the biofilm structure was associated with strongly variable, quorum sensing-controlled PSM expression in biofilm microenvironments (Periasamy et al., 2012).

Knowledge about the structural components of biofilm is crucial for development of removal agents. The appearance (size, shape and density) of the mature biofilm are influenced by numerous variables including:

Bacterial Species

Bacteria generate species-specific arrangements of biofilm (Rupp et al., 1999) structure due to differing cellular distribution, extracellular matrix integration and void spaces. These structural components influence biomass densities and shapes of the biofilm (Costerton, 1995). For example, *Pseudomonas aeruginosa* biofilms *in vitro* show increased cell density at their attached interface and more diffuse density towards peripheries forming mushroom shapes, whereas a *Vibrio* species under same conditions display the reverse formation (Costerton, 1995). In multispecies biofilm, cells interact with one another resulting in the synergistic or antagonistic actions that help to organize development and shape of the biofilm community (Lawrence et al., 1991). For instance, in polymicrobial dental biofilms either complex synergistic interactions such as *Veillonella* spp. using lactic acid produced by *Streptococcus oralis* (Periasamy and Kolenbrander, 2010) or antagonistic actions such as *Streptococcus gordonii* and *Streptococcus sanguinis* biofilms producing hydrogen peroxide inhibiting the growth of other oral bacteria occurs (Kreth et al., 2005).

Nutrient availability

Several studies demonstrated that nutrients are necessary to reach cells in deeper biofilm layers. The availability and type of nutrients, such as the carbon-energy supply, iron and oxygen availability influence biofilm cellular density and size. Nutrient deprivation results in smaller sized biofilm with less bacterial density and this effect is rapidly reversed if the nutrient supply is increased (Costerton et al., 1995) . The type of carbon-energy source can also influence biofilm structure. *P. aeruginosa* grown with glucose as the carbon source, resulted in a mushroom shaped biofilm (Stewart et al., 1993; Davies et al., 1998) but if grown on citrate a densely packed flat form of biofilm developed (Heydorn et al., 2000). Glucose has a positive effect on biofilm formation and it stimulates biofilm formation (Shera et al., 2006) . However, increased glucose concentration leads to a reduction in the expression of the M-protein (McIver et al., 1995) and other virulence factors (Nakamura et al., 2004) in Group A *Streptococcus*. Eventually the void spaces, formed at the microcolony stage, enlarge and coalesce to form water channels which penetrate deeply into the maturing biofilm and provide an anastomosing network of water channels (Lawrence et al., 1991). These water channels flow liquid in different

directions around and between the microcolonies, delivering oxygen and nutrients to the biofilm and removing waste (De Beer et al., 1994).

Hydrodynamics

Study showed that, biofilms grown under laminar flow are found to be patchy and consist of rough cell aggregates separated by interstitial voids whereas under turbulent flow cells are also patchy but are elongated "streamers" that oscillate in the bulk fluid (Davey and O'Toole, 2000). Bacteria form filamentous biofilms, or mats, in fast-flowing environments such as hot springs (Reysenbach and Cady, 2001). A study demonstrated that smooth and dense biofilm grow at higher shear than those grow at low shear (Liu and Tay, 2001). Thus, it is clear that physical effects, besides biological phenomenon have important effect on biofilm structure.

1.3.1.d Dispersion or detachment

After maturation, biofilm bacteria able to achieve ways to perceive environmental changes and can determine whether it is still beneficial to reside within the biofilm or time to detach (Kostakioti et al., 2013). Detachment is a generalized term used to describe the release of cells (either individually or in groups) from a biofilm or substratum (Stoodley et al., 2002). Dispersal contributes to bacterial survival and spreading of disease (Hall-Stoodley and Stoodley, 2005) . This process involved numerous environmental signals, signal transduction pathways (Wilson et al., 2004) and can be caused by various issues, such as nutritional unavailability , oxygen fluctuations and toxic products, or other stress-inducing conditions (Karatan and Watnick, 2009; Hong et al., 2010; Rowe et al., 2010).

Three distinct strategies have been identified for biofilm detachment (Wilson et al., 2004):

1) swarming/seeding dispersal –continuous shedding of individual cells.
2) clumping dispersal – biofilm shed as small aggregates or emboli.
3) surface dispersal – whereby biofilm structures move across surfaces.

Scientists have hypothesised that due to poor nutrient supply within the centre of microcolonies, bacterial cells are subjected to oxidative stress that initiates a SOS response and adaptive mutations, initiating prophage lysis and ultimately resulting in bacterial dispersal (Karatan and Watnick, 2009). Starvation may also lead to detachment by an unknown mechanism and allows bacteria to search for nutrient-rich habitats (O'Toole et al., 2000). One such example is the Gram positive organism *Streptococcus mutans*, which produces a surface protein releasing enzyme (SPRE) that mediates the release of cells from biofilms.

Table 1- 1:

Biofilm maturation and detachment determinants in *Staphylococci spp*.

Determinants	Species	Proposed function	References
PSMs			
PSMα peptides	*S. aureus*	Disruption of noncovalent molecular interactions	(Periasamy et al., 2012)
PSMβ peptides	*S. aureus*	Disruption of noncovalent molecular interactions	(Periasamy et al., 2012)
δ-toxin	*S. aureus*	Disruption of noncovalent molecular interactions	(Periasamy et al., 2012)
PSMβ peptides	*S. epidermidis*	Disruption of noncovalent molecular interactions	(Wang et al., 2011)
Protease			
Aureolysin	*S. aureus*	Digestion of protein-based biofilm matrix by proteolytic activity	(Boles and Horswill, 2008)
Nucleases			
Nuc1	*S. aureus*	Digestion of DNA-based biofilm matrix by nucleolytic activity	(Kiedrowski et al., 2011; Beenken et al., 2012)
Nuc2	*S. aureus*	Digestion of DNA-based biofilm matrix by nucleolytic activity	(Kiedrowski et al., 2011; Beenken et al., 2012)
Regulators			
CidA	*S. aureus*	Control of autolytic activity, which leads to eDNA release	(Mann et al., 2009)
LrgAB	*S. aureus*	Inhibitor of CidA-mediated lysis	(Mann et al., 2009)
Agr	*S. aureus* *S. epidermidis*	Control of PSMs and proteases	(Periasamy et al., 2012)
SarA	*S. aureus*	Control of proteases	(Beenken et al., 2010)

A study utilising two-dimensional-gel protein patterns demonstrated that dispersing cells of *Pseudomonas aeruginosa* are more similar to planktonic than to mature biofilm cells (Sauer et al., 2002). This finding indicates that dispersing biofilm cells revert to the planktonic mode of growth; thus, the biofilm developmental life cycle comes full circle. *P. fluorescens* biofilms experienced detachment, coincident with a reduction in EPS (Allison et al., 1998). Thus it

could be inferred that an increase in the concentration of an inducer molecule is responsible for the release of matrix polymer-degrading enzymes, which results in detachment of cells from the biofilm (Stoodley et al., 2002b).

Dispersal of whole biofilms initiates a slow migration across surfaces (Hall-Stoodley et al., 2004). Migratory ripple structures moving at rates of 1 mm/hr have been visualised in biofilms of *P. aeruginosa* and mixed species biofilms (Purevdorj et al., 2002). *S. aureus* biofilms have been shown to use rippling migration to roll along a glass tube in an *in vitro* central venous catheter model (Hall-Stoodley et al., 2004). Ripple structures have also been observed in biofilms moving along endotracheal tubes, and thus are hypothesised to be important in the aetiology of ventilator-associated pneumonia (Inglis et al., 1995).

The importance of biofilm detachment for infection resides in the dissemination of bacteria from biofilm surfaces, for example, biofilm growing on indwelling medical device to other sites via the lymph and bloodstream respectively. Detached biofilm bacteria may establish secondary biofilm infections elsewhere, possibly with increased severity, such as endocarditis. Additionally, detached bacteria may cause acute, non-biofilm infections, such as sepsis (Otto, 2013).

1.3.2 Regulatory Mechanism of Biofilm formation

Attaching to surfaces and formation of biofilms (Hall-Stoodley et al., 2004) results in cellular reprogramming altering the expression of surface molecules, nutrient utilization and virulence factors and equips bacteria with an arsenal of properties that enable their survival in unfavourable conditions (Whiteley et al., 2001; Schembri et al., 2003; Stanley et al., 2003).

1.3.2.a Physical interaction

Many different non-specific interactions and interfacial forces like hydrophobic interactions and hydration forces also influence cell attachment. To form biofilm, regulation of adsorption, adhesion, diffusion, physiology of bacteria and their growth depends on the properties of substrates, e.g. stiffness, mechanical stability and elasticity. Consequently in response to surface properties, cells secret DNA, proteins, lipids and lipopolysaccharide that accumulate and form the EPS, influence the stiffness and elasticity of biofilm (Flemming and Wingender, 2010).

1.3.2.b. Signalling --quorum sensing

Unless an adequate number of neighbouring cells are present, the costs of biofilm production, in unfavourable conditions, to an individual bacterium outweigh the benefits as quorum sensing (QS), a signalling process, benefits the bacteria by allowing them to sense the presence of neighbouring bacteria and thus respond to varying conditions (Rutherford and Bassler, 2012). Most of the published studies on quorum sensing demonstrated the primary function of QS is to enable bacteria to make collective decisions with respect to the expression of a specific set of genes (Miller and Bassler, 2001; Bjarnsholt and Givskov, 2007; Jayaraman and Wood, 2008). When biofilm cells adhered on surfaces, to regulate their physiology and behaviour including growth, motility, biofilm formation, and pathogenicity, they secrete EPS, QS molecules, and secondary metabolites (Bassler and Losick, 2006).

Quorum sensing uses signalling molecules, sometimes also known as auto inducers (AI), that are often continuously produced by bacteria and are transported passively by diffusion or by active transport mechanisms into the surrounding extracellular milieu (Reading and Sperandio, 2005; Boyen et al., 2009). The concentration of QS signalling molecules in the region is positively correlated with bacterial numbers, increased cell number results in increased auto inducer (Kaplan and Greenberg, 1985). QS signalling molecules can interact with specific repressor or activator sequences in DNA thereby controlling the production of mRNA, and therefore proteins produced, encoded by dozens of genes, including the genes for biofilm production (figure 1-2) (Bjarnsholt and Givskov, 2007). In a study, Bjarnsholt, and Givskov (2007) also showed that, laboratory strains of *P. aeruginosa* lacking the gene encoding the synthesis of a specific acylated homoserine lactone QS signal will not develop into normal biofilms but pile up into a disorganized heap.

Gram positive and Gram negative bacteria use different types of QS systems (Rutherford and Bassler, 2012). Gram positive bacteria use peptide based autoinducer molecules, called autoinducing peptides (AIPs), as signalling molecules (Jayaraman and Wood, 2008) and three different families of AIPs are known to date: (i) the oligopeptide l antibiotics, typified by the lactococcal nisin, which are characterized by the presence of lanthionine-mediated thioether macrocyclic features and dehydroamino acid residues (Quadri, 2002) . Once produced in the cell, AIPs are processed and secreted. The extracellular autoinducer peptide activates the species-specific cell via a two-component signal transduction. AIP binds to a membrane-bound histidine kinase receptor and activates the receptor’s kinase activity followed by autophosphorylation, and these phosphate molecules subsequently transferred to the cognate

cytoplasmic response regulator protein, commonly RNAlll (Kleerebezem et al., 1997; Jayaraman and Wood, 2008).

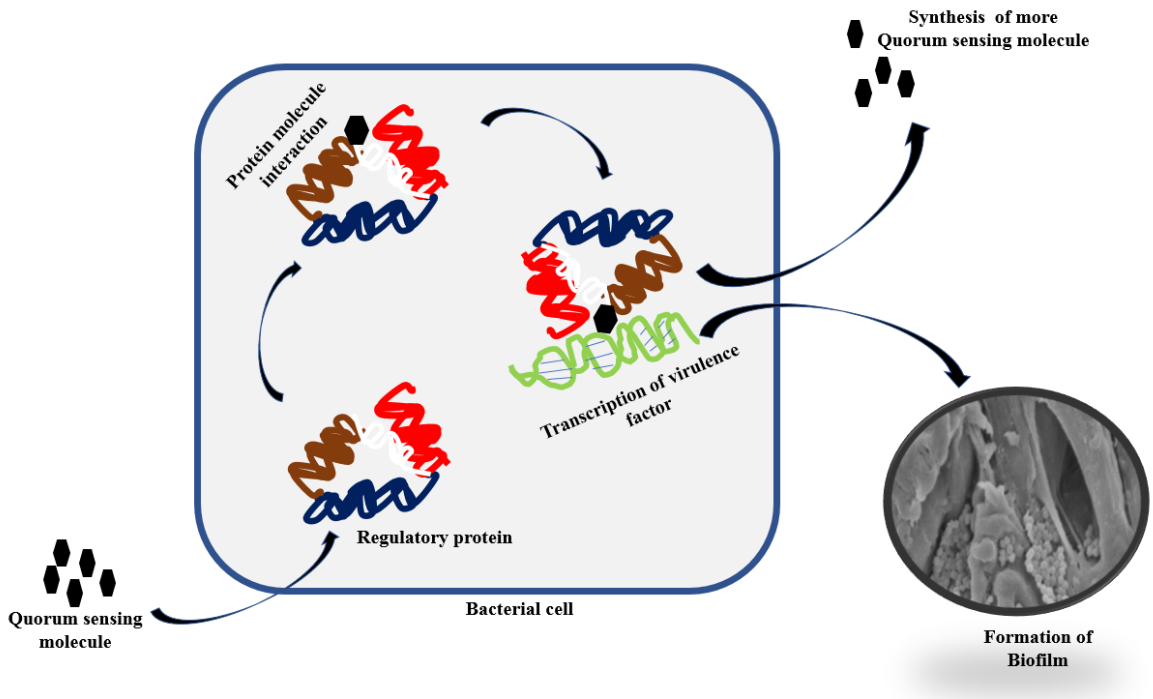

Figure 1- 2:

Bacterial quorum sensing. After entering the bacterial cell Quorum sensing molecules (QSMs) bind to specific regulatory proteins and form protein complex. Protein molecule complex activates transcription of virulence factors and biofilm formed with subsequent production of more QSMs.

The phosphorylated response regulator activates transcription of the genes in the QS regulon (Figure-1-2). In some cases of Gram positive bacterial QS, AIPs are transported back into the cell cytoplasm where they interact with transcription factors to modulate the transcription factor's activity and, in turn, modulate gene expression changes. *S. aureus* and coagulase-negative staphylococcal species have over 50 genes, encoding for proteins that are displayed on the cell surface or are released into the local environment and are involved in pathogenesis (Novick, 2003).

QS peptides in the staphylococcal family are encoded by *agrBDCA* operon (Peng et al., 1988; Novick et al., 1995). *S. aureus* having at least four specific agr systems and one or more in twelve other *Staphylococcus* species (Dufour et al., 2002). Genetic variability occurs within a

hypervariable region that spans the *agrB, agrD* and *agrC* genes, thus conferring species-specific autoinducing peptide and histadine kinase sensor. Although the sequence level has variability, the RNAIII in *S. aureus* is highly abundant with a complex and well-maintained secondary structure, that permits interspecies cross reactivity (Benito et al., 2000). The *agrBDCA* operon is also partially responsible for the level of RNAIII in *S. aureus* (Peng et al., 1988; Novick et al., 1995). RNAIII functions as an effector to activate the expression of an array of secreted virulence factors (Miller and Bassler, 2001).

Gram negative bacteria communicate with each other using small molecules acylhomoserine lactones (AHLs) or other molecules whose production depends on S-adenosylmethionine (SAM) as AIs (Wei et al., 2011). AIs are produced in the cell and freely diffuse across the inner and outer membranes. LuxI/LuxR autoinducer mediated quorum sensing system is highly conserved with over 70 Gram negative bacteria synthesising acyl-HSL homologues to regulate gene expression in a cell-density manner (Kaufmann et al., 2006; Lazar, 2011). AIs are produced in the cell and freely diffuse across the inner and outer membranes. In some cases of Gram negative bacterial QS, AIs are detected by two-component histidine kinase receptors that function analogously to those above for Gram positive QS bacteria. Quorum sensing systems of other Gram negative bacteria are homologous to the LuxI/LuxR system (Brint and Ohman, 1995); for example—the LasI/LasR and RhII/RhIR quorum sensing systems in *P. aeruginosa* (Bjarnsholt and Givskov, 2007; Jensen et al., 2010). Specifically, the autoinducer-2 (AI-2) system is considered universal that mediates interspecies communication and facilitates regulation of bacterial behaviours such as biofilm formation and virulence. (Waters & Bassler, 2005; Federle, 2009). Several bacterial species have the ability to sequester and process the AI-2 present in their environment, thereby interfering with the cell-cell communication of other bacteria (McDougald et al., 2003).

In several Gram negative bacteria, the expression of genes, involved in secondary metabolite production, plasmid transfer, bioluminescence, motility, biofilm maturation, and virulence are controlled by AHL-dependent quorum sensing circuitries (table 1-2). AHL (N-acylhomoserine lactone) system is not considered universal, however, many studies have demonstrated their involvement in the development of multi-species biofilms. Studies have found AHL-mediated quorum sensing in various Gram negative proteobacteria belonging to α, β and γ subdivisions (table1-1), but no Gram positive bacteria have so far been identified that produce AHL (Withers et al., 2001; Cámara et al., 2002; Chhabra et al., 2005).

Table 1- 2:

Some examples of LuxR/LuxI/AHL-dependent quorum sensing systems in Gram negative bacteria (Williams et al., 2007).

Organism	Major AHLs	LuxR	Lux1	Phenotypes
Aeromonas hydrophila	C4-HSL	AhyR	Ahy1	biofilm exoproteases
Burkholderia cenocepacia	C6-HSL, C8-HSL	CepR, CciR	CepI, CciI	biofilm formation swarming motility, virulence
Burkholderia mallei	C8-HSL, C10-HSL	BmaR1-R5	BmaI 1&3	virulence
Chromobacterium violaceum	C6-HSL	CviR	CviI	exoenzyme, pigment, cyanide
Pseudomonas aeruginosa	C4-HSL; 3-oxo-C12-HSL	LasR, Rh1R, QscR, VqsR	LasI, Rh1I	exoenzyme, secretion, HCN, biofilms
Pseudomonas putida	3-oxo-C10-HSL, 3-oxo-C12-HSL	PpuR	PpuI	biofilm formation
Rhodobacter sphaeroides	7-cis-C14-HSL	CerR	CerI	aggregation
Serratia spp.	C4-HSL	SmaR SwrR SpnR	SmaI	swarming motility, exoprotease, biofilm development, antibiotic, pigment, exoenzymes
Vibrio fischeri	3-oxo-C6-HSL	LuxR	LuxI	Bioluminescence
Yersinia enterocolitica	C6-HSL, 3-oxo-C6-HSL, 3-oxo-C10-HSL, 3-oxo-C12-HSL, 3-oxo-C14-HSL	YpsR, YtbR YenR, YenR2	YpsI, YtbI YenI	motility, aggregation swimming and swarming motility

Diffusible signal factor (DSF) (Barber et al., 1997) is a fatty acid based another quorum-sensing system that mediates interspecies interactions, has been identified in several bacterial species including *Burkholderia cepacia* (Deng et al., 2010) and *P. aeruginosa* (Davies and Marques, 2009). DSF plays a role in bacterial virulence, biofilm formation, and antibiotic resistance (Ryan et al., 2009). Gene analysis of *Xanthomonas campestris pv. campestris* (Xcc), strain

ATCC33913, showed that, many DSF-regulated genes perform 12 functional categories: extracellular enzymes, lipopolysaccharide and EPS synthesis and secretion, multidrug resistance and detoxification, flagellum biosynthesis, motility, and chemotaxis, hypersensitive response and pathogenicity (Hrp) system, iron uptake, protein metabolism, tricarboxylic acid (TCA) cycle, aerobic and anaerobic respiration, transcription regulators, membrane components and transporters, and fatty acid metabolism and others (da Silva et al., 2002; He et al., 2006),. DSF signal identified in Xcc, characterizes a novel class of the QS signal, that conserved in a wide range of bacterial species. The Xcc DSF-type QS-signalling system that possessed a unique autoinduction mechanism to modulates DSF biosynthesis along with a second messenger cyclic-di-GMP, enhances the coupling of QS signal sensing to the bacterial intracellular regulatory networks.

QS has been suggested as a novel target for antimicrobial therapy (Amer et al., 2008). Multiple drug-resistant strains of bacteria are emerging continuously. Therefore, it is necessary to find a novel strategy for treating bacterial infections and new anti-virulence therapeutics to control virulence factor production a viable option.

1.4 Mixed species biofilm coexistence and cooperation

Biofilm rarely exist as a single species of bacteria but rather incorporate a substantial range of microorganisms as a complex multispecies biofilm coexist and interact with each other (Søren Møller et al., 1998; Røder et al., 2016). Multispecies biofilms have now been demonstrated in various bacterial habitats including medical, industrial, and ecological habitats.

Mixed biofilms are ubiquitous and found in both ecological and clinical environments. Bacteria within mixed species biofilms can show both synergistic and antagonistic behaviours. They can compete for nutritional resources or alternatively can coordinate to better utilize nutrients or withstand harsh conditions (Elias and Banin, 2012). Synergistic interactions, particularly synergies that facilitate a robust coexistence between species are predominant over antagonistic ones (Periasamy and Kolenbrander, 2009). Many bacterial species do not have the ability to form mono-species biofilms, but they can participate in mixed-species biofilms. In some cases, the capability of species specific co-aggregation is extremely depends on the cell surface components to adhere with each other (Elias and Banin, 2012). One study has found that, formation of mixed biofilm by *Enterococcus spp.* relies on the presence of *E. coli*, that adheres more easily to surfaces. In that study it was observed that, *E. coli* cells attached initially and facilitated the attachment and subsequent formation of a mixed biofilm with *Enterococcus spp.* (Leung et al., 1998). In mixed species biofilm, bacteria also exert metabolic cooperation that

can be observed both in clinical and environmental biofilm. In this cooperating system one species metabolize nutrients while the other species utilize the biproduct of the metabolic procedure. This metabolic commensalism phenomenon was evidenced in biofilm model composed of *Pseudomonas putida* and *Acinetobacter* (Christensen et al., 2002) and *Veillonella spp*. and *Streptococcus oralis* (Periasamy and Kolenbrander, 2010).

1.4.1 Struggle and growth inhibition

Interspecies interactions within the mixed species biofilm bacteria are not always positive but can be competitive. To define the structure and activity of a multi-species biofilm, competition between species plays a significant role. When bacteria aggregated under limited resource conditions, they become more competitive. Sometimes this competitive attitude inhibits the growth of others, by producing inhibitory compounds or consuming essential nutrients (Elias and Banin, 2012). For example, *Enterococcus faecium* produces an inhibitory protein that restricts biofilm formation by three oral streptococci, *Streptococcus sobrinus, S. sanguinis* and, the leading cause of dental caries, *S. mutans* (Kumada et al., 2009). Competition among species within a mixed biofilm is not only influenced by nutritional availability but also environmental conditions such as the availability of oxygen (Kreth et al., 2008) and presence of H_2O_2 (Kreth et al., 2005), antibacterial compound, surface motility etc (An et al., 2006). Any change in one or more of these factors can dramatically impact the structure and dynamics of the biofilm community (Elias and Banin, 2012).

1.4.2 Mixed biofilm-spatial distribution

Bacteria within the mixed-species biofilm show three different general form of organization (Figure 1-6).

Formation of single-species microcolonies, where each species forms a separate microcolony side by side and their interaction depends on available nutritional resources (Nielsen et al., 2000).

Co-aggregation, where cells of both species are mixed and can be found together throughout the biofilm (Rickard et al., 2006).

Layered structure, where one species can be found in the upper layers and another species can be found in the lower layers of the biofilm (Hansen et al., 2007). This type of biofilm structure can be either synergistic (Christensen et al., 2002), or competitive (An et al., 2006).

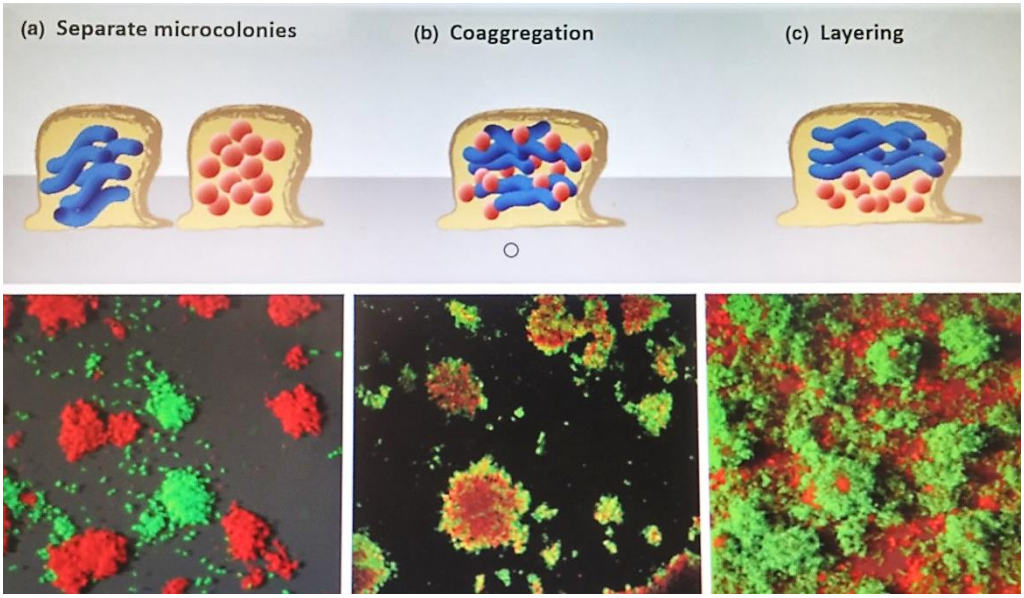

Figure 1- 3:

Spatial distribution within mixed-species biofilm (Elias, 2012). (a) separate mono-species microcolonies (Nielsen et al. 2000); (b) co-aggregation (Rickard et al. 2006)]; (c) arranged in layers (Hansen et al. (2007). Microscopy image was used with permission from original authors (appendix 9.3).

It could be assumed that the interaction within biofilms composed of separate microcolonies can be different compared to the interaction occurring within co-aggregated biofilms (e.g. calling distance of quorum-sensing molecules, both intra and interspecies). However, what processes govern the formation of these three different architectures and whether they provide the community with different phenotypic properties or not, it is still undistinguishable. Thus, the species spatial distribution adds yet more complexity when examining possible interactions within the biofilm community.

1.4.3 Gene expression in mixed species biofilm

A profound change in the gene expression takes place during the formation of mixed biofilm. This transformed gene expression patterns cause both synergistic and antagonistic interactions that help to organize the architecture and function of the multi-species biofilm. Several studies have reported the changes in gene expression when a single species biofilm transformed into mixed-species biofilm. In *V. parvula* and *S. mutans* mixed biofilm, *S. mutans* displayed many changes including altered expression of genes for amino acid metabolism, protein synthesis etc. Similarly, a mixed biofilm of *S. aureus* and *P. aeruginosa,* produced significantly higher amounts of exotoxin A (Goldsworthy, 2008). Numerous *S. mutans* genes, involved in purine, amino acid and intracellular polysaccharide metabolism and protein synthesis, showed altered

expression during co-culture (Luppens et al., 2008). Bacterial interactions within mixed biofilms can lead to evolution of species variants in some cases. For example, in a study researcher observed that *P. putida* and *Acinetobacter spp*. grown in a flow-cell together, an evolutionary variant of *P. putida* had emerged with rough colony variants after several days of intimate contact. This phenotype was heritable and a specific mutated gene was found (Hansen et al., 2007). Jakubovics et al. (2008) have found 9 *Streptococcus gordonii* genes involved in arginine biosynthesis and transport are highly induced in co-aggregates, but not in co-cultures with *Actinomyces naeslundii*. Another study by Wen et al. (2010) reported the significantly reduced expression of *S. mutans* virulence genes in multi-species biofilms with *Streptococcus oralis* or *Lactobacillus casei*.

Bacterial interactions in multi species biofilms can induce resistance to antimicrobial agents. In a study, Harriott et al. (2010) reported induction of *S. aureus* vancomycin resistance gene during the formation of multi-species biofilm with *C. albicans*. In another study, Adam et al. (2002) showed that in multi-species biofilms of *C. albicans* and *S. epidermidis*, EPS produced by *S. epidermidis* can inhibit penetration of antifungal drug fluconazole while *C. albicans* can protect the slime-negative *S. epidermidis* against vancomycin. Thus, it is evident that, formation of the mixed biofilm triggered alterations in gene expression that resulted in the expression of a virulence gene.

Evolution of species variants may be led by bacterial interaction within mixed biofilms. An evolution of variant species was observed by Hoffman et al. (2006) in *S. aureus* and *P. aeruginosa* mixed biofilm. Presence of *P. aeruginosa* induces the emergence of small colony variants (SCVs) of *S. aureus* by producing inhibitory substance 2-heptyl- 4-hydroxyquinoline N-oxide (HQNO). Therefore, it is proclaimed that interactions between species within biofilms should be considered when designing and choosing therapies.

1. 4.4 Why it is important to know about multispecies biofilm?

It is evident that in the environment and even in human disease process, biofilm communities have tendency to be polymicrobial (del Pozo and Patel, 2007). Inclusion of multiple bacterial and/or fungal species in biofilms, it obtains numerous advantages, such as passive resistance (Weimer et al., 2011), an enlarged gene pool with more efficient DNA sharing (Madsen et al., 2012), and many other synergies, which give them a competitive advantage. Mixed species biofilm exhibits an altered phenotype with respect to growth rate and gene transcription. Therefore, it can be seen as a single entity possessing multiple genetic resources that allow it to adapt and thrive regardless of the stresses that it encounters. In general, the greater the diversity,

i.e. the larger the gene pool, the more robust the biofilm is in terms of its survivability (Tuttle et al., 2011).

Bacteria pursuing a biofilm strategy for infection have molecular mechanisms for recruiting other bacteria (Kuramitsu et al., 2007; Wolcott et al., 2013)). It seems that biofilms actively attempt to become polymicrobial, apparently to improve their survivability. A multispecies biofilm thus has an increased chance of persistence and may act as a source of hospital acquired infections (HAI). Formation of biofilms by clinically relevant microorganisms is at the root of many chronic and recurrent infections that has been estimated to account for 80% of all microbial infections in the human body (Wolcott et al., 2013). A recent study by Hu et al. (2015) on ICU environmental surfaces have showed that 93% samples have biofilm and all dry surface biofilms were polymicrobial, 52% of which incorporated MDRO. Among the isolated MDRO bacteria *Staphylococcus aureus* was predominant, of which 18% of were MRSA, 7% were VRE of the isolated *Enterococcus spp.* and 12% of Gram negative bacteria was ESBL producer.

Mixed-species biofilms are unquestionably the dominant form in nature and in the human host. In the oral cavity and the diabetic foot ulcers (McFarland and Stamm, 1986; Johani et al., 2017) are prominent examples in human body infection by multispecies biofilm. Thus, more research directed at explaining interactions within multi-species biofilms and the effects of such interactions on the development, nature and survival of the biofilm community is a demanding need for current days.

1.4.5 Conclusion

Microorganisms within a mixed biofilm interact via quorum sensing and/or metabolically. Interactions can be synergistic or antagonistic and result in phenotypic changes, such as increased resistance to antimicrobial agents or to host defence systems, spatial distribution or emergence of variants (SCVs). Nutritional interaction can be either competitive or cooperative. It should be emphasized that in most cases, mixed-species interactions are driven by physiological processes. However, genetic changes have been reported in SCVs and many cases of antibiotic resistance and metabolic interactions. Therefore, we are in need of a new optimized, reproducible method that can provide information on the spatial interactions and localization in multispecies biofilms, as the number of possible community combinations can be overwhelming as the number of co-inhabitants increases. The challenge of exploring and controlling multispecies biofilm will be the focus for many years to come; however, with the new advances in technology, and thorough experimental designs, we will be able to take up this challenge.

Part Two: Bacterial Biofilms in the Health Care Environment---Relevance to Hospital Acquired Infections.

1.5 Hospital Acquired Infections

Hospital acquired infections (HAI) are infections that develop in a hospitalised patient, not present at the time the patient presented to hospital (Wenzel, 2007) and accounts for a major risk factor for serious health issues leading to death (Brusaferro et al., 2015). The estimated rate of hospital-acquired infections in European and American studies are 3-9% (Vrijens et al., 2012; Zarb et al., 2012) and 5-10% (Wenzel, 2007) respectively which are almost similar. Approximately 15% of HAIs in the USA occur in intensive care units (ICUs) (Magill et al., 2014). Australia does not have a national HAI surveillance system. The Carling report estimated 6% of inpatients from the state of NSW (Garling, 2008) developed HAI which would equate to approximately 200,000 HAI per year occurring in Australian ICU facilities (Cruickshank et al., 2009). A more recent systematic review (Mitchell et al., 2017) has reported the incidence burden of some HAIs in Australian hospitals from 2010 to 2016. Data suggested that Australia has 83,096 HAIs per year, comprising 71,186 urinary tract infections, 4902 *Clostridium difficile* infections, 3946 surgical site infections, 1962 respiratory infections in acute stroke patients and 1100 hospital-onset *S. aureus* bacteraemia. However, this review lacks data on common infections such as pneumonia, gastroenterological and bloodstream infections beyond *S. aureus*. In that case, the incidence of HAIs in Australia may be closer to 165,000 per year (Mitchell et al., 2017) and basically unchanged since 2008. HAIs are continuing and leading to morbidity, mortality and increased healthcare expenditure (Yokoe et al., 2014). In 2011, of the 700,000 United States patients who developed a HAI, 75,000 died (Magill et al., 2014). HAIs are estimated to be the fourth leading cause of death in France (Kaoutar et al., 2004) and sixth leading cause of death in the United States (Kung et al., 2008). According to CDC, approximately 65% of HAIs are caused by biofilms (Coenye and Nelis, 2010), leads to increase length of hospital stay twice than average (Madani et al., 2009). This causes a significant increase in the overall treatment costs. It has been estimated that HAIs associated costs per annum is around $9.8 billion in the United States (Zimlichman et al., 2013).

According to centre for disease control (CDC), HAI constitutes a condition caused by pathogen related toxin(s) contacted after hospitalization. More specifically, a HAI is considered, have developed, if a patient manifests signs of infections on or following the third day of admission to hospital or clinical institution (Horan et al., 2008). There are two sources of infection agents causing HAI

Endogenous source--- Patient's own colonised bacteria cause infections in immune suppressed condition and/or during invasive procedure; example *S. epidermidis* infection in surgical wound.

Exogenous source--- Patient is infected by organisms originating external to themselves. This can be from the hospital environment via HCWs hands, contaminated medical equipment, visitors, or from other colonised patients etc. (Horan et al., 2008).

Although infection control programs and practices are improving with time, the prevalence of HAI still remains as a serious public health problem.

1.5.1 Hospital acquired infections: relevance of biofilms

Hospital acquired infections are divided into the following categories (Kowalski, 2007).

Urinary tract infection (UTI)
Surgical site infections (SSI)
Bloodstream infection
Pneumonia
Others

Most common and well-studied and costly HAIs are catheter-related bloodstream infection (CRBSI), ventilator-associated pneumonia (VAP), surgical site infection (SSI), and catheter-associated urinary tract infection (CAUTI) and aerosolised (Lobdell et al., 2012). Out of all nosocomial infections, more than a third of possibly involving airborne transmission at some point (Kowalski, 2007). Biofilms play a pivotal role in healthcare-associated infections (HAIs), with (Srinivasan et al., 2003; Tena et al., 2005). 60 to 70% being associated with biofilm contamination of temporary or permanent implantable medical devices (Bryers, 2008). Several studies have demonstrated bacterial biofilm on medical devices and instruments, including urinary catheter, endotracheal tube, orthopaedic tourniquets and stethoscope are responsible for many HAIs (Ren-Pei et al., 2014; Sahu et al., 2015; Souza et al., 2015). Central intravenous catheters and indwelling urinary catheters contribute almost half of these infections with estimates suggesting that combined they cause one-third of all hospital-acquired infections in the United States in 2007 (Scott, 2009). Estimated mortality rates of patients with infected implant varies from low rates (>3%) for mammary, penile, orthopaedic implants and vascular grafts, up to 10% for cardiac pacemakers and central venous catheters, 10-30% for urinary catheters to high rate (> 25%) for cardiac assist devices such as; Implantable cardioverter defibrillator (ICD), artificial pump etc. (Weinstein and Darouiche, 2001; Lynch and Robertson,

2008). Although the mortality for mammary, penile and orthopaedic implants is low, the morbidity related cost, disfigurement, immobility and loss of functions are difficult to assess. It has been estimated that, treatment costs for infected surgical implanted devices range from $US15,000 to $US146,000 depending on types of implants and suggested that the cost will be almost triple and exceed $US1.6 billion by 2020 to treat infected orthopaedic prosthetic joints in the U.S. alone and is unsustainable in the longer term (Darouiche, 2004; Greenspon et al., 2011; Kurtz et al., 2012). Thus, understanding of the pathogenesis of biofilm disease of surgical implantable medical devices and development of prevention and treatment strategies are crucial to the long-term viability of this expanding industry.

1.5.1.a Catheter-related bloodstream infection (CRBSI)

CRBSI is a clinical condition when a patient becomes infected by/through a venous or arterial catheter. CRBSI is commonly suspected when a patient develops local and/or systemic signs/symptoms consistent with infection after 48 hours of having an intravascular catheter; for example, a central venous catheter (CVC) and other identifiable sources of infection don't exist (Horan et al., 2008). CVC is prevalently used in the intensive care unit (ICU) setting. In the United States of America, CRBSIs have been estimated at 250,000 cases per year, with peripheral venous catheters incurring fewer clinically apparent infections (0.6 per 1000 catheter-days) than non-tunnelled CVCs (2.3 per 1000 catheter-days) (Maki et al., 2006).

Implantable devices provide an artificial pathway through the body's natural defences. Study on catheter associated infection evidenced approximately 18% of catheters become colonised where the incidence of catheter-related sepsis was up to 10% and skin inflammation at catheter insertion sites up to 17% (Vallés et al., 2008). Venous catheter usually colonised by the patient's own bacteria residing in the area of the device. For example, it might be colonised by skin organisms, and/or may originate from exogenous microflora from health-care personnel, or contaminated infusates. (Donlan, 2001; Valles et al., 2008). Nosocomial sepsis due to implantation of central venous catheters (CVCs) and parenteral nutrition (TPN) remains an important cause of morbidity in neonatal intensive care units. A study on central venous catheters in a neonatal intensive care unit found 16.3% of neonate got nosocomial sepsis due to intra venous catheter (van den Hoogen et al., 2006). Sepsis has a mortality rate up to 25% (Fletcher, 2005).

Bacterial biofilm formation might compromise the use of indwelling venous catheters. After colonising in to the indwelling catheters (CVCs), microorganisms, such as; *S. epidermidis* and *S. aureus, Candida albicans, P. aeruginosa, K. pneumoniae, and Enterococcus*

faecalis form biofilms in catheter surfaces (Elliott et al., 1997; Donlan, 2001), that lead to the blood stream infections (BSIs) (Curtin and Donlan, 2006). Donlan (2001) found that, biofilm on central venous catheters is common worldwide, and the degree and site of its formation depends on the duration of catheterization; short term (<10 days) catheter had more biofilm on the external surface whilst long term (30 days) had on the inner lumen. The number of organisms present on the catheter tip is associated with the incidence of BSIs in the patient (Raad et al., 1992; Gahlot et al., 2014) supporting the concept of a critical level of biofilm development above which substantial cell detachment and embolism occur.

Catheter-associated infections due to biofilms is a major challenge in health care setting. Available preventive measures, such as antimicrobial agent-impregnated catheters and cuffs, in-line filters, and antimicrobial lock therapy (O'Grady et al., 2001), appear to reduce the risk of CRBSI (Mermel, 2000), however, not completely able to prevent catheter-associated biofilm formation, particularly those caused by antimicrobial-resistant strains.

1.5.1.b Ventilator-associated pneumonia (VAP)

VAP is a pneumonia associated with mechanical ventilation. VAP affects 8% to 28% of mechanically ventilated patients (Shorr and Kollef, 2005) or approximately 52,000 patients per year affected with VAP in the United States (Scott, 2011). A newly published data demonstrated that approximately 10% of patients with mechanical ventilation developed VAP and that this rate has not declined over the past decade (Wang et al., 2014). Despite of having advanced knowledge about the causes and prevention, VAP continue to be frequent complications of hospital care. VAP is considered as one of the most common HAIs, accounting for 22% of all HAIs in a multistate point-prevalence survey (Magill et al., 2014) . Average increase of cost for VAP range from $12,000 to more than $40,000 per case (Scott, 2011; Kollef et al., 2012) and is expected to be higher now. VAP is the HAI with the highest risk of mortality ranging from 24% to 76% (Chastre and Fagon, 2002).

Biofilm can form on endotracheal tube (ET) like any other indwelling tube. One study from 1999 isolated various pathogenic bacteria; *S. aureus*, enterococci, *Enterobacteriaceae*, pseudomonads and *Candida spp*. from the biofilm, formed on ET surface (Adair et al., 1999). Study also reported 70% of patients with VAP had identical pathogens isolated from both ET biofilm and tracheal secretions. The oral cavity act as an important source of bacteria and dental plaque, a tooth-borne biofilm, strongly influence the initiation and progression of pneumonia due to relocalization of the biofilm bacteria into the respiratory tract from the dental plaque

(Paju and Scannapieco, 2007). Study has suggested that oral bacterial load and formation of dental plaque increases during intubation predict the risk of pneumonia (Munro et al., 2006) and has been proposed to be the major source of bacteria implicated in the etiology of VAP (Garcia, 2005). Colonisation of anaerobic bacteria at the lower respiratory tract in mechanically-ventilated patients is also a common incident (Robert et al., 2003).

1.5.1.c Surgical site infection (SSI)

Approximately 15% of HAIs are due to SSI. SSIs contribute significantly to morbidity and mortality, and the economic impact of SSI is tremendous. In the United States, approximately 45,000,000 patients undergo surgical procedures and developed 290,485 to 400,000 SSIs per year (Owings and Kozak, 1998; Platt et al., 2001). The costs was attributed to SSI are $11,087 to $29,443 per case and as much as $3,450,000,000 to 10,000,000,000 per year in the United States (Scott, 2011). A study on Healthcare Cost and Utilization Project net (HCUPnet) observed low baseline rate of complex *S. aureus* SSI but total increased rate of 39% (Schweizer et al., 2015). A single SSI could add from $13 000 to $100 000 to the cost of health care (Schweizer et al., 2014). Schweizer et al. (2014) in his study also explained mean unadjusted costs $31 580 and $52 620 for patients without and with an SSI, respectively. In the risk-adjusted analysis, the relative costs increased by 1.43 times for patients with moderate SSI and 1.93 times for deep SSI than a patient without SSI.

Formation of biofilm by colonised bacteria disrupts the healing process and increases the development of chronicity of wound infection (James et al., 2008). Existence of (71.4%) polymicrobial biofilm bacteria such as; *A. baumannii, K. pneumoniae and Escherichia coli*, were demonstrated as a cause of recurrent skin and soft tissue infections which resulted in a greater bioburden, aggravated infections, enhanced resistance to antibiotics, and an augmented inflammatory reaction in affected tissues (Akers et al., 2014).

Although any permanent implantable device or tissue can be contaminated with biofilm the two most studied devices are orthopaedic and breast implants. Use of orthopaedic implants is increasing as the world's population ages, with over 700,000 knee and 300,000 hip replacements conducted in the USA annually (CDC, 2015). However, after surgery, as time goes, incidence of loosened/failing hip and knee arthroplasties increases, and at 15–20 years of operation, the survival rate of patients are very low (<50%). Currently, in the US, 40,000 hip arthroplasties need to be revised each year because of painful implant loosening and the predicted rates of revision will increase by 137% for total hip and

601% for total knee revisions over the next 25 years (Kurtz et al., 2007). Studies have reported aseptic loosening (no infection) as the main cause for revision surgery over the mid- and long-term and is responsible for >70% of hip and >44% of knee replacement (Herberts and Malchau, 2000; Robertsson et al., 2001). However, higher rate (25.2%) of failure of knee arthroplasty due to infection than the aseptic loosening (16.1%) was reported by another study (Bozic et al., 2010). Bacterial biofilms have long been implicated in prosthetic joint infections and infection of orthopaedic devices due to the indolent and chronic nature of many orthopaedic infections, their diagnostic difficulty in culturing aspirates and recurrent symptoms despite antibiotic treatment. The implant is thought to be contaminated at time of surgery by contact or aerosol (Legout and Senneville, 2013; Tande and Patel, 2014) but haematogenous spread also occurs (Tande and Patel, 2014; Zimmerli, 2014). Implant infection are caused by a wide variety of bacteria as determined by culture, most prominently *S. aureus* and *S. epidermidis*, account for close to 70% of orthopaedic implant infections, while *P. aeruginosa* accounts for another 8% of infections (Campoccia et al., 2006).

Breast augmentation and reconstruction, using breast implants is a common procedure performed in plastic surgery. Pathological contracture with subsequent implant distortion, firmness and pain are the most common complication following breast implantation and occurs in 20% of cosmetic patients and up to 30% of reconstructive patients by 10 years post implantation (Hidalgo and Spector, 2014; Ajdic et al., 2016; Wan and Rohrich, 2016) . Though the aetiology remained poorly understood, infection as a predisposing factor for capsular contracture has been supported by numerous microbiological studies that have cultured organisms from the capsule (Pajkos et al., 2003) (Burkhardt et al., 1981; Shah et al., 1981), visual demonstration of biofilm on implant and in the capsule (Pajkos et al 2003) and fulfillment of Koch's postulates showing artificial contamination of implant pockets lead to contracture (Tamboto et al., 2010; Rieger et al., 2013). Utilising traditional culture the organisms most commonly associated with high grade capsular contracture are *S. epidermidis* and *Propionibacterium acnes*, with occasional infection by *S. aureus, E. coli, Corynebacterium, Streptococcus pneumoniae* and *Ralstonia* (Schreml et al., 2007; Del Pozo et al., 2009; Rieger et al., 2013; Hu et al., 2016).

1.5.1.d Catheter-associated urinary tract infection (CAUTI)

Insertion of an indwelling urinary catheter is one of the most common invasive procedures in hospitalised patients with 12-25% of critically ill patients receiving catheters (Lo et al., 2008; Hooton et al., 2010; Nicolle, 2012). It is thus not surprising that catheter associated urinary tract infection (CAUTI) causes 70-80% of HAI urinary tract infections (Nicolle, 2014). Generally,

100,000 colony-forming units (CFU)/ml of urine is thought to be diagnostic of a urinary tract infection (UTI). However, smaller numbers of organisms could become problematic if remaining untreated or if the patient is immune-suppressed with an indwelling urinary catheter. Therefore, CAUTI is commonly defined as > 1000 to 10,000 CFU/mL of urine. CAUTI is the second most common cause of bacteraemia in hospitalized patients (Maki, 1981) as well as the most common HAI (Vincent et al., 1995; Jahani-Sherafat et al., 2015). Around 449,334 cases of CAUTI occur in the United States per year. The presence of a urinary catheter is the most important risk factor for bacteriuria. The risk of bacteriuria increases by 3-10% each day a patient has an indwelling urinary catheter (Yu et al., 2017). Between 10% and 30% of patients develop asymptomatic bacteriuria who undergo short-term catheterization (i.e., 2-4 days) and 90% and 100% of patients develop symptomatic bacteriuria who undergo long-term (>4 days) catheterization (Nicolle et al., 2005).

Bacterial biofilm is the main cause of catheter-associated urinary tract infections and recurrent catheter obstruction. As soon as the urinary catheter is inserted, it becomes coated with various organic molecules, protein and polysaccharides components (Trautner and Darouiche, 2004; Jacobsen et al., 2008; Tenke et al., 2012) producing a proteinaceous conditioning film that facilitates primary adhesion and docking of biofilm bacteria, the first step in biofilm formation (Saint and Chenoweth, 2003; Tenke et al., 2012). Recent U.S. data indicated *E. coli* as the most common etiologic Gram negative organism, followed by descending order of frequency by *P. aeruginosa*, *Klebsiella spp.*, *Enterobacter spp.* and *A. baumannii* (Hidron et al., 2008) and *S. aureus,* coagulase negative staphylococci spp. and *Enterococcus spp.* as Gram positive bacteria as the cause of CAUTI (Lark et al., 2000).

CAUTI occurs when bacteria from the catheter gain access to a disrupted uroepithelial mucosa. The presence of a urinary catheter increases uroepithelial mucosal inflammation consequently increases the binding sites for bacterial adhesins (Jacobsen et al., 2008). This is also aided by the bacteria which cause nosocomial urinary tract infections having fewer virulence factors required to colonise and cause infection (Johnson, 2003). Removal and replacement of the urinary catheter often results in repeat bacterial attachment, biofilm formation and recurrent blockage with struvite/hydroxyapatite encrustation (Stickler and Feneley, 2010). However, the mainstay of treatment of CAUTI is still remains the removal +/- replacement of the indwelling urinary catheter (Hooton et al., 2010) and to date all clinical investigations for biofilm such as CFU, detection have failed to demonstrate any statistical decrease in biofilm formation and its sequelae (Tenke et al., 2012).

1.5.2 Organisms associated with HAIs

The non-viral causes of HAI with *Staphylococcus, Enterococcus* and *Pseudomonas spp.* causing over half of HAI. However, due to difficulty in treating *E. faecalis, S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa, and Enterobacter spp.* are the most important organisms (Revdiwala et al., 2012). These pathogens are known as "ESKAPE" and are so-named to emphasize their ability to "escape" from the antimicrobial and biocidal treatment due to acquisition of resistance genes as well as formation of biofilms (Bales et al., 2013). *S. epidermidis* is also an important cause of HAI and designated as one of the major cause of CRBSIs (Cherifi et al., 2013). It has been reported that, CRBSI are mostly caused by Gram positive organisms (64%) (Parameswaran et al., 2010) and in some cases 50% of infections were caused by coagulase-negative *Staphylococci* (Seifert et al., 2003). In another study, 14% CRBSI due to coagulase-negative *Staphylococci* was reported out of 50 episodes (Almuneef et al., 2006).

1.5.2.a Staphylococcus aureus

S. aureus is a Gram positive organism, found in different parts of human body in approximately 20-33% of humans (Kluytmans et al., 1997; Tong et al., 2015; CDC, March 25, 2016). Both MRSA and methicillin sensitive *S. aureus* (MSSA) frequently cause HAIs and have been responsible for outbreaks. More than 20% of HAI are due to *S. aureus* (Magill et al., 2014). Nosocomial blood stream infection caused by MRSA are associated with increased mortality and longer hospital stays, has 35% mortality rate (Garling, 2008). In many studies, MRSA have been isolated from hospital surfaces especially close to patients' areas, such as bed linen, curtains and over-bed tables (Telang et al., 2010; Chen et al., 2014; Mirzaii et al., 2015). These sites act as an important reservoir of *S. aureus* in the hospital environment (Dancer, 2011) which can be transmitted to new patents as it can survive in the environment for up to 9 months (Wagenvoort et al., 2000).

1.5.2.b Pseudomonas aeruginosa

U.S. National Healthcare Safety Network has reported that Gram negative bacteria are responsible for more than 30% of hospital-acquired infections (Peleg and Hooper, 2010). Unfortunately, multidrug-resistant organisms are increasingly being reported worldwide where *P. aeruginosa* has potent contribution. *P. aeruginosa* can grow in nutritionally constrained environment, and has been responsible for causing outbreaks in urology, haematology, burns units and ICUs (Schneider et al., 2012; Gómez-Zorrilla et al., 2014; Davis et al., 2015). Approximately 51,000 HAIs along with 400 mortality cases by hospital acquired *P. aeruginosa* infection has been reported in the United States per year (CDC, 2015). These organism is highly efficient at up-regulating or acquiring genes that code for mechanisms of antibiotic drug

resistance, especially in the presence of antibiotic selection pressure (Peleg and Hooper, 2010) and the most alarming is, infections caused by this organism is now resistant to all currently available antibiotics (Paterson and Lipman, 2007).

1.5.2.c Vancomycin resistant Enterococcus spp.

Enterococcus spp. are Gram positive organism of which *E. faecalis* and *E. faecium* live in the human body as bowel commensal organisms, but very frequently cause UTI, bacteraemia and endocarditis. When *Enterococci* treats with different antibiotics such as; ampicillin, penicillin and vancomycin, it becomes resistant by altering its susceptibility to these drugs. (Higuita and Huycke, 2014). *Enterococcus spp.* are highly resistant to aminoglycoside and β-lactam antibiotics thus vancomycin is the treatment of choice for most of the cases (Kristich et al., 2014). However, emergence of vancomycin-resistant enterococci (VRE) has made treatment of these organisms difficult. VRE is usually acquired nosocomially and has been reported responsible for several incidents of outbreaks in hospitals (Iosifidis et al., 2013; Cilo et al., 2014). Even increasing infection control practices has not reduced the prevalence of VRE; for example, the morbidity rate of VRE has increased three times from 2007 to 2012 in Germany ICUs (Gastmeier and Vonberg, 2014). According to the National Health-care Safety Network (NHSN), from 2009 to 2010, 35.5% of VRE was isolated and ranking as the second most common cause of nosocomial infections in the US. In contrast, according to CANWARD, from 2007-11, prevalence of VRE was only 6% in Canada (Zhanel et al., 2013)and according to the European Antimicrobial Resistance Surveillance System (EARSS), in 2013, Europe has much lower prevalence (4%) of VRE (The European Antimicrobial Resistance Surveillance System EARS-Net Results, 2015).

Nosocomial acquisition of VRE has been attributed to environmental contamination with the pathogen able to survive for up to four years on hospital surfaces (Wagenvoort et al., 2011). Touching the environment contaminated with relatively low pathogen concentrations in a room occupied by a VRE colonized patient is associated with approximately the same risk of VRE acquisition on hands as touching an affected patient directly (Bonten et al., 1996). Environmental contamination becomes widespread, with 36-58% of chairs and couches in outpatient areas used by VRE-positive patients being contaminated (Grabsch et al., 2006).

1.5.2.d Acinetobacter spp.

The significance of Gram negative *Acinetobacter* species as pathogens resulting in HAI has increased in significance due to it being a principal cause of pneumonia in ICUs in the United States (Weinstein et al., 2005; Royer et al., 2015; Tsiatsiou et al., 2015). *Acinetobacter spp.*

also readily acquires antibiotic resistant genes which has posed important therapeutic challenges. Hidron et al. (2008) reported about 36.8% of ventilator-associated pneumonia cases, caused by *A. baumannii*, were resistant to carbapenems. It has been estimated that 12,000 HAIs were attributed to *Acinetobacter* annually in the US (US Department of health human services, 2014). Infections caused by *Acinetobacter* 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).

It is highly possible that this organism is spread via environmental contamination as it has been frequently isolated from surfaces commonly touched by patients and HCW and survives for prolonged periods, even in dry conditions, on varied materials (Hensley et al., 2010; Thom et al., 2011; Møretrø et al., 2015). Moreover, *Acinetobacter spp.* have strong ability to generate biofilms, which potentiates its virulence and survival in different environments; nutrient limiting conditions, on dry environmental surfaces, increasing the risk of acquisition by patients (Gaddy and Actis, 2009; Kanafani and Kanj, 2014).

1.5.3 Conclusion

There is now acknowledgement that bacterial biofilms play an important role in human disease including many chronic diseases and diseases of implantable medical devices. Unlike acute septic infections, biofilm infections result in a chronic subclinical inflammatory process that affects surrounding tissue or bone. Most if not all temporary and permanent implantable medical devices are susceptible to bacterial attachment and biofilm formation. There is increasing investigation to develop therapeutic strategies and novel surfaces to prevent and treat biofilm diseases on implantable medical devices.

Part Three: Transmission

1.6 Definition:

Transmission is the passing of a communicable disease from an infected host individual or group to a particular individual or group, regardless of whether the other individual was previously infected (Bush, 2001).

Many microorganisms are harmless to human. Pathogenic organisms need to gain access to a susceptible host to cause an infection. The spread of infection requires three elements and to prevent the spread of infection it is necessary to eliminate at least one of these elements:

a source of infecting microorganisms
a means of transmission for the microorganism
a susceptible host

1.7 Transmission of microorganisms

Informed and successful disease control choices are made on the basis of understanding infectious agent transmission routes (Hurst, 1996). Perhaps the most well-known example is also the first: Dr. John Snow's removal of a water pump handle in London in 1854 halted a cholera epidemic. Similar contemporary interventions tailored to impede transmission include hygiene education (Aiello et al., 2008), improved water quality at the source and in the home, improved sanitation (Fewtrell et al., 2005), social distancing (Glass et al., 2006), and respiratory masks (Jefferson et al., 2009). The success of the interventions relies, in part, on the prior justification that the transmission route is a major contributor to the overall disease burden.

1.7.1 Transmission Routes of Infectious Disease

The major transmission routes are not mutually exclusive. Rather, an etiological agent may utilize multiple routes to transfer between infected and susceptible hosts. Similarly, the major routes are not necessarily distinct categories. As an example, indirect contact transmission during preparation may result in a foodborne (i.e., common vehicle) outbreak. Fomite-mediated transmission is a subset of contact transmission, one of the major routes of infectious disease transmission. There are, arguably, four major routes: vector borne, airborne, common vehicle, and contact (Thomas and Weber, 2001; Mangili and Gendreau, 2005).

Table 1- 3:

Sources and transmission of hospital-acquired organisms in hospital environment (Kolmos, 2012).

Sources of infection	Modes of transmission	Examples of risk factors	Examples of infections
Self-infection	Break of natural barriers (skin and mucous membranes)	Surgery. Insertion of peripheral or central line catheters. *Staphylococcus aureus* carriage	Surgical site infections & catheter related infections due to *Staphylococcus aureus.* Urinary tract infections due to *Escherichia coli*
Cross-infection from other patients	Via hands of staff	Failing hand hygiene before and after patient contact	Surgical site infections & catheter related infections due to *Staphylococcus aureus* Respiratory tract infections due to RSV & other respiratory pathogens
	Via instruments & equipment not properly sterilized	Heat-sensitive equipment, e.g. fibre-optic endoscopes	Tuberculosis transmitted by fibre-optic bronchoscope
	Via the environment	Insufficient domestic cleaning leading to accumulation of pathogens on contact surfaces	Diarrhoea due to *Clostridium difficile*
	Via donor blood & drugs	Medication of several patients From the same multi-dose vial	Hepatitis B or C transmitted with drugs from multi-dose vials accidentally contaminated with blood or body fluids
Hospital staff	Hand-borne	MRSA carriage. Insufficient handhygiene in connection with treatment and care of patients	Surgical wound infections. Catheter related infections
	Air-borne during surgery	Carrrier of *Streptococcus pyogenes* in the operating theatre	Puerperal fever & surgical wound infections with *Streptococcus pyogenes*
Hospital environment	Contact with contaminated tap water	Aspiration of oral secretions following ingestion of tap water contaminated with *Legionella.* Immunosuppression	*Legionella* pneumonia
	Inhalation of dust from buildings	Rebuilding of hospitals. Immunosuppression	Lung infection due to *Aspergillus fumigatus*

Activities that involve direct contact with patients, body fluid secretions and/or touching contaminated environmental surfaces may result in the acquisition of pathogens on hands (Boyce and Pittet, 2002). Medical professionals frequently acquired methicillin-resistant *Staphylococcus aureus* (MRSA) on gloves after touching surfaces near colonized patients (Boyce et al., 1997; Harris et al., 2013). Patients colonized with nosocomial pathogens are often unrecognized or not decolonized or isolated and cleaning of the hospital environment may be inadequate. Therefore, it is reasonable that environmental surfaces might be an important source for colonization of healthcare workers' hands.

1.7.2 Fomites and the spread of Infectious Diseases

Fomites are inanimate objects such as doorknobs, elevator buttons, hand rails, phones, writing implements, keyboards, clothing, bedding etc that can become contaminated with infectious agents and serve as a vehicle for transfer between hosts. Fomites can be contaminated with infectious organisms following contact with bodily fluids, body parts, or other contaminated fomites or by the settling of airborne particles produced during talking, sneezing, coughing, vomiting or as a result of mechanical action such as making beds (Weinstein and Hota, 2004; Boone and Gerba, 2007). A study reported that even a stethoscope can serve as a fomite if it isn't cleansed and also reported MRSA contamination on 15% of stethoscopes tested (Merlin et al., 2009). Even clothing from healthcare personnel can be contaminated (Boonie and Gerba, 2007). Contamination of a fomite may provide no obvious or visible evidence of infectious disease presence. As the routes by which an infectious agent contaminates a fomite are equally able to infect a susceptible individual without the intermediate fomite. It is often difficult to determine whether a transmission event occurred directly between an infected host and a susceptible host, or the event occurred indirectly via a fomite.

1.7.3 History of environmental transmission

Indirect contact via inanimate object was first identified by Italian physicist and scholar Girolamo Fracastoro, in 1546 (Ravenel, 1931; Clendening, 1960). He also posited that contaminated fomites may remain so for “two to three years”, and described porous objects (“linen, cloth, and wood”) as more likely to act as fomites than nonporous ones (“iron, stone”) (Clendening, 1960). In 1970s and 1980s, studies suggested that contamination of environmental surface played a lesser important role in the endemic transmission of health care associated infections (Otter et al., 2011). In the following decades (1980s-1990s), research on surface contamination increased four-fold, with studies examining their role in the transmission of respiratory (Dick et al., 1987; Brady et al., 1990), gastrointestinal (Butz et al., 1993) and even

bloodborne pathogens (Ferenczy et al., 1990). Up until 1987 the Centre for Disease Control and the American Hospital Association focused on patient diagnosis due to the belief that nosocomial infections were not related to microbial contamination of surfaces (Cozad and Jones, 2003). The general acceptance of the role of fomites or surfaces in infectious disease transmission was highlighted, perhaps, during this period with the First European Meeting of Environmental Hygiene in Dusseldorf in 1987. Over the years, studies have changed the standpoint of disease transmission to comprise a more complex multifactorial model of disease spread (Goldmann, 2000).

Patients are the leading source of contamination. Many recent studies have established that several major pathogens such as; VRE, *C. difficile*, *Acinetobacter spp*., MRSA and norovirus are shed by patients and contaminate hospital surfaces at concentrations sufficient for transmission (Dancer, 2008; Hayden et al., 2008; Huslage et al., 2010). Surfaces in the vicinity of patients that are touched frequently by health care worker and patients are termed “high touch surfaces”, and have higher occurrence of contamination than other sites (Hayden et al., 2008). These organisms survive for extended periods, can persist despite attempts to disinfect or remove them, and can be transferred to the hands of health care workers (HCWs). A study on hospital environmental screening confirmed the repeated contamination of items, equipment, and general sites in bed spaces (bed rail) and rooms of colonized or infected patients (Lemmen et al., 2004) and as health care workers' are responsible to touch these contaminated surfaces during patient care, that increases the risk of onward transmission to others and often throughout multiple clinical areas in a health care institution (Bhalla et al., 2004; Lemmen et al., 2004).

The hospital bed is comprised of different components, which pose a potential risk of infection for the patient if not adequately decontaminated. In the literature there are many descriptions of outbreaks or experimental investigations involving methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant enterococci, *Acinetobacter spp*., and other pathogens. A study has demonstrated the higher contamination rate of bed covers, bed sheets and bed rails with both Gram positive and Gram negative bacteria (Lemmen et al., 2004). Bed components, including bed frame, sheets and mattress may become contaminated through direct contact with skin and body fluids (Ayliffe et al., 2000; AWMF and practice., 2005). The proportion of contamination may vary depending on the status of the patients. Bedrail and other sites may not be in direct contact with the patient, however, mattress or pillow would be in direct contact. Consequently, a clear focus of investigation was often not apparent, and important reservoirs may have been missed and outbreaks prolonged as a result (Creamer and Humphreys, 2008).

Several studies have reported the contamination of clean linen and linen racks with *S. aureus* and MRSA (Shiomori et al., 2002; Kniehl et al., 2005). Mattress could be contaminated with various organisms (Sherertz and Sullivan, 1985; Andrade et al., 2000). Damaged and wet mattresses have been found to be the source of contamination during outbreaks (Sherertz and Sullivan, 1985). Cleaning and disinfection can remove organisms from the mattress cover, however, the wet foam while inside may still could be able to harbour bacteria that can be released when patient press the mattress physically (Fujita et al., 1981). Contamination with MRSA, VRE, and organisms that cannot be readily eradicated with cleaning or disinfectants make the situation worst (Clarke and Humphreys, 2001). Another study reported the presence of microorganisms in almost half of the tested mattresses (48.1%), 50% of which were positive after disinfection with a high prevalence of *S. aureus* both before and after mattress disinfection (Andrade et al., 2000). Mattress could be disinfected by heat (Medical device agency, 1999, hydrogen peroxide fumigation (up to 5 h or more) or disposable mattress covers can be used (French et al., 2004). Bedframes and mattresses may be cleaned with hot water and detergent followed by thorough drying (Patel, 2005). Detergent impregnated surface wipes are available, are recommended in the HICPAC environmental hygiene guidelines (CDC 2003; Patel, 2005).

1.7.4 Epidemiology of infection caused by environmental transmission

It is now evident that the healthcare environment may play a key role in facilitating the transmission of important pathogens associated with hospital acquired infections (Dancer, 2014). The key pathogens are:

Staphylococcus aureus,
Methicillin-resistant *Staphylococcus aureus* (MRSA),
Vancomycin-resistant *Enterococcus spp.* (VRE),
Acinetobacter spp.,
Norovirus, and
Clostridium difficile.

The occurrence of environmental contamination depends on patient variables such as the body site where the patient is colonized or infected. In one study, 36% of surfaces cultured were contaminated in the rooms of patients having urine or wound infected with MRSA, whereas it was only 6% of surfaces in the rooms of patients with MRSA at other body sites (Boyce et al., 1997). A study by Otter *et al.* (2006) reported that, ten standardized high-touch surfaces were cultured in the rooms of eight patients with heavy gastrointestinal colonization by MRSA and

concomitant diarrhoea (cases) and in the rooms of six patients with MRSA at other body sites, but not in their stool (controls). The investigators found that 59% of surfaces were contaminated with MRSA in the rooms of case patients who had heavy gastrointestinal colonization with MRSA and concomitant diarrhoea. MRSA was recovered most frequently from bedside rails (100% of those cultured), followed by blood pressure cuffs (88%), television remote control devices (75%), bedside tables and toilet seats (63% each), toilet rails and dressers (50% each), door handles (38%) and intravenous pumps (25%). In contrast, significantly fewer (23%) surfaces were contaminated in the rooms of control patients who had MRSA at other body sites, but not in their stool. In the rooms of control patients, bedside rails were also the most frequently contaminated site (67%), followed by toilets and call buttons (33% each). The other seven standardized sites cultured in the rooms of control patients were contaminated less than 20% of the time. In another study, community-acquired strains of MRSA (CAMRSA), were found to contaminate 19% of surfaces in an outpatient clinic (Johnston et al., 2006). Two healthcare workers (HCWs) who worked in the clinic developed infections caused by CA-MRSA strains. One of the infected HCWs who did not have direct contact with patients, became infected with the same strain that was found on environmental surfaces. Although extensive cleaning effectively removed CA-MRSA from surfaces in the outpatient clinic described by Johnston *et al.* (2006) routine cleaning of contaminated environmental surfaces does not always eliminate MRSA from high-touch surfaces in hospitals (Bhalla et al., 2004).

Widespread environmental contamination of *Acinetobacter spp*. has been demonstrated in frequent outbreaks and the rate of environmental contamination in outbreak settings has been stated by investigators to range from 3% to 50% (Weber et al., 2010). The common sites for colonization are bed rails, bedside tables, surfaces of ventilators, sinks, suction equipment, mattresses, resuscitation equipment, curtains, slings for patient lifting, mops, buckets, door handles, stethoscopes, incubators, and computer keyboards (Weber et al., 2010).

It is apparent that hospital environmental surfaces are highly contaminated with pathogenic organisms and have a high potential of transmitting HAIs. Even with apparently adequate cleaning and disinfection, some environmental aspects such as mattresses and pillows are of higher risk due to their direct contact with patients and thus become a source of infection. Therefore, appropriate guidelines should be formulated for environmental decontamination and systems established. Pillows and mattresses should be made easily washable, dried and decontaminated, and must have the lowest potential to harbour organisms and priority should be given to mattresses and pillows, due to their greater degree of contact with the patient.

1.7.5 Important factors to contribute to transmission of organisms via contaminated hand/glove of health care personnel:

The potential for contaminated environmental surfaces to contribute to transmission of health care associated pathogens is be governed by a number of factors (Boyce, 2007).

The ability of a pathogen to remain viable on a variety of dry environmental surfaces.
The frequency with which they contaminate surfaces commonly touched by patient and health care worker
Whether or not levels of contamination are sufficiently high to result in transmission to patients.
Level of cleaning and/or disinfection of the environment (Schultsz et al., 2003)

1.7.5.a The ability of a pathogen to remain viable on a variety of dry environmental surfaces

Pathogens such as MRSA, VRE and *C. difficile* have the capability to remain viable on dry surfaces for days, weeks or even months (Boyce, 2007). In the studies investigating the survival of nosocomial pathogens on surfaces, Kramer et al (2006) showed that under certain conditions, *C. difficile* spores, VRE, MRSA, and *Acinetobacter spp.* can survive for 4-5 months or more, *S. aureus* including MRSA for 7 months on dry surfaces, and norovirus can survive for a week or more. Large variations in survival times in different reports is partly due to species and strain variation but is also due to differences in experimental conditions, including inoculum size, humidity, the suspending medium, and the surface material (Kramer et al., 2006).

Table 1- 4:

Survival times of multidrug-resistant organisms (MDROs) on dry inanimate objects. Range depends on experimental design and methods of assessing contamination and material used as test surface. Ratio of increased risk associated with the room being previously occupied by patients infected with common MDROs. (Kramer et al., 2006; Dancer, 2008; Otter et al., 2013; Havill et al., 2014)

Organism	**Survival time In hospital surfaces**	**Prior hospital room occupancy risk increase**
MRSA (Otter et al. 2013)	7 days to > 12 months	1.5
Acinetobacter spp. (Otter et al.2013)	3 days to >11 months	3.5
P. aeruginosa (Kramer et al., 2006)	6 hours to 16 months	1.75
C. difficile (Otter et al.2013)	>5 months	2.5
VRE (Otter et al.2013)	5 days to 46 months	2.25
E. coli (Kramer et al., 2006)	2 hours to 16 months	
Klebsiella spp. (Havill et al., 2014)	2 hours to > 30 months	
Norovirus (Havill et al., 2014)	8 hours to 7 days	Limited data
Rotavirus (Otter et al.2013)	6 to 60 days	Limited data

1.7.5.b The frequency of contaminating surfaces by patient and HCWs hand

Patients and contaminated surfaces appear to transfer resistant organism to the hands of HCWs at similar frequencies (Duckro et al., 2005). In a recent study, however, compliance with hand hygiene was 80% of 142 opportunities after patient contact compared with only 50% of 196 opportunities after contact with a patient's environment, meaning that contamination acquired from a patient's environment is less likely to be dealt with by hand hygiene (Randle et al., 2010). Acquisition of *S. aureus* on hands is common after contact with contaminated surfaces (Bhalla et al., 2004). In a study it was observed that, 31% of volunteers hands were contaminated with *S. aureus* who touched bed rails and over bed tables in patient rooms and 7 % contaminated their hands in empty rooms that had been terminally cleaned (Bhalla et al., 2004). That means, environmental cleaning is not 100% effective and contamination is contributing to transmission

of healthcare pathogens when healthcare worker's hands or gloves are contaminated by touching contaminated surfaces, or when patients come into direct contact with contaminated surfaces.

Transmission of MRSA from environmental surfaces to gloves or un-gloved hands of HCWs has been documented (Pineles et al., 2017). Hand contamination with MRSA is occurring with equal frequency whether healthcare personnel (HCP) have direct contact with a colonized/infected patient or through touching only contaminated surfaces (Stiefel et al., 2011). The most important risk factor for hand/glove contamination is the contamination with multidrug-resistant pathogens (Morgan et al., 2012). Transmission of MRSA from contaminated environmental sources to patients has occurred in a variety of settings. In a study it was concluded that three patients acquired MRSA from the environment, but did not exclude HCWs as another potential source (Hardy et al., 2006).

Transmission of VRE from environmental surfaces to HCWs hands or gloves has been documented by several investigators. The hands or gloves of a HCW could be contaminated by VRE by touching a contaminated chair (Vernon et al., 2006), items in patient rooms (Tenorio et al., 2001) bedrails and bedside tables (Ray et al., 2002; Bhalla A et al., 2004) without touching patients. Touching of contaminated environmental surface leads to transfer of VRE to another uncontaminated surfaces almost as frequently as touching a colonized patient (Duckro et al., 2005).

Rooms occupied by symptomatic patients with *C. difficile* and asymptomatic patients could be contaminated significantly (McFarland et al., 1989). Several studies have established the widespread and frequent contamination of hospital surfaces and equipment in the rooms of patients with *C. difficile* infections (Jullian-Desayes et al., 2017). *C. difficile* has frequently been detected from the skin and hands of infected patients and the occurrence of skin contamination of patients with *C. difficile* was similar to the incidence of stool detection (Sethi et al., 2010). Hands of healthcare personnel who providing care to patients with *C. difficile* infection, are a source of organisms (Mutters et al., 2009). The frequency of positive hand cultures for healthcare personnel has been shown to be strongly correlated with the intensity of environmental contamination (Mutters et al., 2009). According to one study, when environmental contamination was 0–50%, hand contamination was 0%, and 36% when environmental contamination was greater than 50% (Samore et al., 1996). *C. difficile* spores on gloved hands was as likely after contact with commonly touched environmental surfaces (e.g.

bed rails, bedside table) as after contact with commonly examined skin sites i.e. chest, arm, hand etc (Guerrero et al., 2012).

Environmental contamination is believed to play an important role in hospital outbreaks because clinical isolates of *Acinetobacter spp.* are capable of surviving for extended periods in the environment. A survey from January-April, 2006 on cross-transmission of MDR *A. baumannii* in an orthopaedic hospital ICU in Greece demonstrated the colonization of the hands of health care workers with *Acinetobacter* and recovered *Acinetobacter spp.* from 12 of 42 (28.6%) hand cultures (Markogiannakis et al., 2008). Many outbreaks have been related with extensive environmental contamination, and contamination of the hand of health care workers has been established.

1..7.5.c Whether or not levels of contamination are sufficiently high to result in transmission to patients.

Hospital room surfaces are frequently contaminated and the level of contamination is generally less than 10 organisms per cm^2 (Weber et al., 2013). In a study it was stated that the number of colony forming units (CFU) was less than 100 bacteria per RODAC (25 cm^2) plate (Huslage et al., 2013). It is very important that, there was no statistical difference in the level of contamination despite whether the surface was a high, medium or low touch surface (Weber et al., 2013). Same kind of findings have been reported by other authors (Boyce et al., 1997). However, other studies have showed that less than 15 cells of *S. aureus* were enough to cause infection in experimental lesions (Foster WD and MS., 1960), less than 1 CFU/cm^2 was sufficient to cause *C. difficile* disease in mice (Lawley et al., 2010). Many studies have assessed the level of microbial contamination of *C. difficile* on surfaces in the rooms of patients with CDI. Most of these studies reported that surfaces were contaminated with less than 1–2-log_{10} *C. difficile*, but one study that sampled a larger area of a preoccupied CDI patient room surface, using a sponge technique, reported 1300 colonies. However, for most environmentally associated nosocomial pathogens the infectious dose appears to be low. Importantly, regardless of the relatively low concentration of contamination on surfaces related with that on the skin of patients, touching a VRE-contaminated surface carries almost the similar risk for acquisition of VRE on hands as touching an affected patient (Hayden et al., 2008). Thus, the presence of a pathogen at any concentration on the surface may be a risk for transmission, which is explained in proposed guidelines for microbiological hygiene standards.

Table 1- 5:

Typical bacterial loads for healthcare related surfaces.

Field of study	Site	Bacterial load	References and year
Healthcare environment	Hospital ward surface	<3 cfu/cm^2	(Rutala et al., 1983)
	Ward floor	<5 cfu/cm^2	
	Stethoscope membrane	In >54% of cases >5 cfu/cm^2; in 18% of cases >29 cfu/cm^2	(Bernard et al., 1999)
	Hospital ward surfaces	2.5 to 40 cfu/cm^2; ward cleaning reduced this to <2.5 cfu/cm^2	(Griffith et al., 2000)
	Hospital ward surfaces	2 to 294 cfu/cm^2	(Aycicek et al., 2006)
	Nurse workstation	<9 cfu/cm^2	(Hardy et al., 2007)
	Under ward bed	<25 cfu/cm^2	
	Hospital ward surfaces	55 to 80% of sampled sites had >5 cfu/cm^2	(White et al., 2007)

A room previously occupied with infected or colonised patient with a specific pathogen, increased the risk of acquisition of same infection into a newly admitted patient (Table 1-5). Several studies have identified a strong relationship between the risk of acquisition and prior room occupancy regardless of the organism; VRE, MRSA, ESBL-producing Gram negative bacilli, *A. baumannii* or *P. aeruginosa* (Huang et al., 2006; Datta et al., 2011; Mitchell et al., 2014; Ajao et al., 2015). A review from five different studies, explored the relationship between acquisition of organisms (VRE, MRSA, ESBL etc.) and prior room occupancy in USA,

Table 1- 6:

Effect of colonization or infection status of the prior room occupant on the acquisition of pathogens by subsequent occupants of the same room.

References	Study area and time period	Findings	Percentage acquired
(Martínez et al., 2003)	CU, USA (9-month retrospective case-control study)	Placement within a room from which VRE had been cultured was associated with VRE acquisition in the subsequent room occupant	13%
(Drees et al., 2008)	ICU, USA (14-month prospective cohort study)	Positive VRE room culture results or previous VRE-positive room occupants were associated with VRE acquisition	60%
(Nseir et al., 2011)	ICU, France (12-month prospective cohort study)	Admission to a room previously occupied by an *A. baumannii*- or P. aeruginosa-positive patient was associated with acquisition of these pathogens	*A. baumanni*-28% *P. aeruginosa*-26%
(Huang et al., 2006)	ICU, USA (20-month retrospective cohort study)	Admission to a room previously occupied by a MRSA- or VRE-positive patient was associated with acquisition of these pathogens	VRE-4.5% MRSA-3.9%
(Shaughnessy et al., 2011)	ICU, USA (18-month retrospective cohort study)	Admission to a room previously occupied by a *C. difficile*-positive patient was associated with *C. difficile* acquisition	11%

Australia and France found that, 6.2% patient acquired the same organisms who were admitted into a room where the prior room occupant had any of the studied organisms, whereas, only 3.2% patient acquire the studied organisms who were not admitted in to a room where the prior room occupant had one of these organisms (Mitchell et al., 2015). This might occur due to the higher survival rate of organisms on the hospital environment as described in section 1.7.5.a. and may explain the higher risk for acquisition from prior room occupants with different organisms. In water reservoirs many Gram negative bacteria, such as; *E. coli, Klebsiella spp.* and *Pseudomonas spp.* can survive for more than a year (Kramer et al., 2006). These organisms

can establish biofilms in sink traps have the potential to colonize and infect patients and display greater capacity for antimicrobial resistance and tolerance to chlorine and other disinfectants (Tacconelli et al., 2014). Therefore, contamination of environmental surfaces of room with the prior infected occupants represent a high transmission risk.

1.7.5.d Level of cleaning and/or disinfection of the environment

The public's general understanding is that a hospital is safe for patients if it looks clean. However, pathogenic organisms are invisible to the naked eye rendering naked eye assessment of cleanliness and prediction of infection risk for patients inadequate (Dancer, 2004). A study compared visual assessment against both biochemical and microbiological screening of the hospital environment showed that, less than a quarter of surfaces that looked clean were in fact free from organic soil as measured using ATP testing and less than half were microbiologically clean (Griffith et al., 2000). Despite this it is assumed that the frequency of healthcare-associated colonization or infection will be decreased by cleaning and/or disinfection of the hospital environment. Cleaning is the removal of all foreign material (e.g., soil, organic material) from surfaces and objects, and is normally accomplished using water with detergents or enzymatic products (Rutala and Weber, 1999). However, this process does not kill bacteria, which, under favourable conditions, can redeposit elsewhere and form biofilms and study has revealed that surfaces in hospital rooms are cleaned poorly (less than 50%) during terminal cleaning , (Carling et al., 2010), although methods of evaluating the appropriateness of cleaning varied (i.e., visibly clean, ATPase, fluorescent dye, aerobic plate counts). Similar insufficiencies have been reported for cleaning and disinfection of portable medical equipment between patients (Havill et al., 2011). In another study it was described that many surfaces still remain contaminated with *C. difficile* spores in spite of terminal cleaning of hospital rooms, (Boyce et al., 2008). It happens since many rooms are ineffectively cleaned by the workers and *C. difficile* is not susceptible to most commonly used surface disinfectants (quaternary ammonium compounds).

According to the studies, as there are deficiencies in proper terminal cleaning, presence of many important nosocomial pathogens in the hospital surfaces is not unexpected. The cleaning responsibilities for near-patient hand-touch sites does not always rest with the ward cleaners, however, other areas like beds, drip stands, lockers and over bed tables are generally cleaned by nurses (Bhalla et al., 2004). Nurses are also liable for delicate clinical equipment decontamination. So confusion has been created by this overlapping cleaning responsibilities;

it is very usual that cleaning opportunities of some items are overlooked or abandoned (Goodman et al., 2008). More recently, a ward crossover study by Dancer et al., (2009) conducted to explore the consequence of an extra cleaner, focusing on hand-touch sites. The improved cleaning was associated with a significant reduction in the total aerobic counts on surfaces and in the number of failures to reach a hygienic standard considered to be acceptable (more than 2.5 CFUs/cm^2) and resulted in a significant reduction in MRSA acquisition by patients. However, there was no significant reduction in surface contamination with methicillin-susceptible *S. aureus*. Similar results were shown by a study based on bleach disinfection against VRE. After complete disinfection, study reported 24.8% reduction in the number of new patients colonized and a 66.4% reduction in environmental contamination with VRE, despite a similar proportion of patients already colonized on admission. Study also showed that, whilst VRE bacteraemia decreased by >80%, the rate of vancomycin-susceptible enterococcal bacteraemia did not change during the study period. This suggests that better cleaning may decrease MRO acquisition and HAI.

Cleaning is an important component to control the outbreaks of norovirus, VRE, *C. difficile*, MRSA, and multidrug-resistant (MDR) Gram negative bacilli, including *Acinetobacter spp.* (Dancer, 2011; Donskey, 2013). However, in 1997, Boyce et al. reported the contamination of health care staffs' gloves by touching the MRSA contaminated sites in close proximity to patients and a study after 16 years, showing that thorough cleaning failed to reduce health care worker gown and glove contamination after caring for patients with MRSA and multidrug-resistant *Acinetobacter* spp.(Hess et al., 2013). In contrast, many studies have shown the elimination of MRSA after basic cleaning from the ward environment, for example, number of MRSA identified by extensive environmental (communal shower and a blood pressure cuff) culture was reduced significantly after enhanced cleaning of shared common areas and changing the blood pressure cuff between patients (Layton et al., 1993). Despite of implementing patient isolation and hand hygiene programs, MDRO outbreak in urological ward, persisted for more than a year, significantly reduced the number of new acquisitions when cleaning hours was doubled from 60 h to 120 h per week (Rampling et al., 2001). A study on enhanced disinfection of *C. difficile* by automated UV equipment reported that, from screened sites, the number of *C. difficile* contamination were decreased by 14-89%. Study also recovered positive cultures from two-thirds of CDI rooms before the study began, whereas during periods of study, after disinfection, the percentages of CDI rooms with positive cultures fell by 57% (Sitzlar et al., 2013). A case control study that included multivariate analysis, patients who acquired VRE were significantly more likely than controls to have occupied a room with

persisting VRE environmental contamination, suggesting that inadequately cleaned rooms served as a source of transmission to patients (Martinez et al., 2003). Therefore, surface disinfectants need to have contact with all contaminated surfaces and they should be applied in the appropriate concentrations for appropriate time.

In conclusion, evidence supporting the role of the contaminated surface environment in the transmission of several key healthcare-associated pathogens is summarized as follows (Weber et al., 2013)

1. The surface environment in rooms of colonized or infected patients is frequently contaminated with the pathogen.
2. The pathogen is capable of surviving on hospital room surfaces and medical equipment for a prolonged period of time.
3. Contact with hospital room surfaces or medical equipment by healthcare personnel frequently leads to contamination of hands and/or gloves
4. The frequency with which room surfaces are contaminated correlates with the frequency of hand and/or glove contamination of healthcare personnel
5. Clonal outbreaks of pathogens contaminating the room surfaces of colonized or infected patient are demonstrated to be due to person-to-person transmission or shared medical equipment
6. The patient admitted to a room previously occupied by a patient colonized or infected with a pathogen (e.g., MRSA, VRE, *C. difficile*, *Acinetobacter*) has an increased likelihood of developing colonization or infection with that pathogen
7. Improved terminal cleaning of rooms leads to a decreased rate of infections.
8. Improved terminal disinfection (e.g., vaporized hydrogen peroxide) leads to a decreased rate of infection in patients subsequently admitted to the room where the prior occupant was colonized or infected.

Although transfer of pathogen to a susceptible patient most commonly occurs via the hands (gloved or un-gloved) of healthcare personnel, contaminated hospital surfaces and medical equipment (and less commonly water and air) can be directly or indirectly involved in the transmission pathways. Therefore, it is important to imply new strategies to reduce such transmission of pathogens and breakdown the cycle of transmission from contaminated hospital environment-to- patient-to-patient.

1.8 Dry Surface Biofilm (DSB) in Health-care Environment.

Bacteria can form biofilm on every suitable surfaces both in nutrient sufficient or deficient condition (Prakash et al., 2003). Biofilm can develop on relatively dry surfaces in natural, industrial or clinical environment (Kaplan et al., 2011). Extraordinary physiology and architecture of biofilm help bacteria to escape from assaults to exist prevalently in the environment. This persisting ability strongly supports biofilm to become more abundant in medical environment (Kaplan et al., 2011). Biofilm may develop in heating or cooling system and can act as a source of continuous bacterial dispersion such as; *Legionella, Listeria, Coxiella* etc. into the environmental air. Many study has revealed the frequent presence of biofilm in body tissue (wound) (Lebeaux and Ghigo, 2012), prosthetic devices and any indwelling catheters (Mack et al., 2006). The importance of bacterial biofilms from surrounding hospital environment in hospital-acquired infections is still poorly understood. However, study on dry surface biofilm was first introduced by Vickery et al. (2012) after identification of biofilm bacteria from different hospital surfaces.

1.8.1 Biofilm in water distribution system of health care facilities

It has been recognized that biofilm formed by water borne bacteria can exist in cooling tower (Osawa et al., 2014), sinks (Hota et al., 2009) and shower heads (Kossow et al., 2014) can be a source of nosocomial infection. Despite of disinfecting the source of drinking water (DW) used in hospitals, biofilms formed and got sheltered on water pipelines escaping different disinfection strategies (Soto-Giron et al., 2016) . This increase the chance of patients to be exposed to pathogens via contaminated HCW hands, by bathing and showering (Ozerol et al., 2006; Halachev et al., 2014). For example, the aspiration of *Legionella spp*. contaminated water aerosols during showering is proved to be a major transmission method (Anaissie Ej, 2002) and could be able to persist at low concentration after treatment for 71°C for 30 minutes and flushing (Wong et al., 2006). Study also showed that, despite treatment with chlorine dioxide, *Legionella spp.* could persist in hospital water distribution systems for >5 years and at the end of the study complete eradication could not be achieved (Casini et al., 2008).

The frequency of nosocomial infection due to bacteria located in hospital water supplies is usually thought to be low (Williams et al., 2013). However, in recent years, increase of hospital-acquired infections and the presence of opportunistic pathogens in biofilms located in hospital premise plumbing and medical devices has regained attention (Shin et al., 2007; Szymańska, 2007). A study on biofilm of shower hoses in Ohio hospital found that most

metagenomic sequences were associated with members of the genera Mycobacterium (66%); a close relative of *M. rhodesiae* and *M. tusciae.* , *Erythrobacter, Sphingomonas* , and *Novosphingobium* (Soto-Giron et al., 2016). These two mycobacterial species are considered potential opportunistic pathogens since they have been identified as they cause pulmonary and disseminated infections in immuno-compromised individuals (Rahman et al., 2014)

It has been estimated that, most of the nosocomial infections are associated with biofilms and majority of these biofilms are due to *P. aeruginosa* found mainly in moist environments such as taps and coolers (Anaissie et al., 2002). A study was conducted on *P. aeruginosa* isolation and its ability to form biofilm in hospital water systems (taps and coolers) showed significant biofilm production by all the isolates (Suman et al., 2008). Studies demonstrated that, in water system, different components such as the flow straighteners and solenoid seals (rubberized ethylene-propylene-diene-monomer; EPDM) can become a site of extensive *P. aeruginosa* biofilm proliferation under particular circumstances (Moritz et al., 2010; Walker and Moore, 2015). A study on *P. aeruginosa* outbreak in surgical ICU from 2012 -2014, reported that 66.7% of the cases had been located in one of the rooms with a sink colonized with the outbreak strain that significantly reduced after replacement of colonized sinks. This indicates that, sinks in patient rooms in ICUs can be potential drivers of MDR *P. aeruginosa* outbreaks.

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; Vergara-López et al., 2013). Above discussion could lead to more robust management practices to minimize the risks associated with opportunistic pathogens.

1.8.2 Dry surface biofilm in hospital environment

Environment plays a key role in harbouring and transmitting MDRO and many studies have proved the link between environmental contamination with increased risk of hospital-associated infections (Fisher et al., 2012; Carling and Huang, 2013; Otter et al., 2013). Most of the studies usually focus on MRSA, VRE, and *C. difficile*; however, *Acinetobacter* and norovirus are also frequently sampled. Contamination levels have been reported exceeding the number of microbes necessary for the transmission and infection (Otter et al., 2013). Though it is not confirmed whether an individual surface contamination level is sufficient to be transmitted or cause disease, however, the summation of contamination in a room can pose a significant risk to the next patient which becomes specifically pertinent when considered in conjunction with

extended survival times of MDROs on hard surfaces (Chemaly et al., 2014). It is important to note that this situation not only increase the risk for a patients admitted to rooms in which the prior occupants tested positive for a pathogen but also other patients in the premises (Lee et al., 2011). In order to break this microbe transmission chain, identification of source of pathogen; endogenous or exogenous coming from HCWs hand surrounding environmental surfaces, is crucial point to reduce HAI.

Bacteria tend to attach to both biotic and abiotic surfaces which is problematic in health care settings. Biofilms are traditionally associated with wet or damp surfaces. However, biofilms have been discovered on hard dry surfaces with huge implication (Otter et al., 2015). A study by Vickery et al. (2012) demonstrated the presence of biofilm on hospital dry surfaces after aseptically obtained hard surface sections of additional furnishings and equipment from an intensive care unit after terminal cleaning (two cycles of hypochlorite cleaning). They found evidence of biofilm and bacteria embedded in thick extracellular polymeric substances on 41 of 44 items (93%), which was visually confirmed with scanning electron microscopy. Half of the biofilms were culture-positive, and multi-antibiotic-resistant organisms were found on 52% of the samples.

Another study on dry surface biofilm in intensive care unit (ICU) environmental surface was performed by Hu et al. (2015), surgical infection research group (SIRG), faculty of Medicine and health science, Macquarie University. To investigate the presence of dry surface biofilm in ICU environment, samples were obtained from ICU in a fully air-conditioned hospital and stored in a fully air-conditioned laboratory (temperature range 22–25°C, humidity 57–72%). Following a two-step terminal cleaning protocol using neutral detergent followed by disinfection with 500 ppm chlorine, items from the patient bedding (N = 11), patient surrounds (N = 19), and fixed furnishings (N = 14) were aseptically collected by cutting out a segment of the furnishing using sterile gloves, forceps, pliers, scissors, or scalpel blades, depending on the material being sampled. Samples were stored in sterile containers and gloves and instruments were changed between each sample. To determine biofilm on the surfaces, samples were subjected to aerobic culture, microscopy (SEM &CLSM), PCR. To determine the bacterial community of dry surface hospital biofilm fifteen (15) samples were subjected to bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) of the V1–V3 regions of 16s rRNA gene and Pyrosequencing was performed. Data showed the evidence of dry surface biofilm in the ICU environment with viable and culturable organisms (23/44 samples), and 12 (52%) of these samples were growing MDROs and most of these MDROs were in the immediate patient

vicinity. Study reported 33% of tested mattresses were positive for MRSA, another one-third were positive for VRE and one mattress was positive for all MRSA, VRE, and ESBL. *S. aureus*-specific PCR detected *S. aureus* from 50% of the samples, including the eight out of 11 (72%) samples from patient bedding, eight of 19 (42%) samples from the patient's immediate environment, and six of 14 (42%) samples from fixed furnishings. Presence of biofilm, infecting their surfaces, was visually confirmed in forty-one out of 44 samples (93%), either by SEM and/or by CLSM. This study found number of species forming the biofilm was highest closest to the patient and the items adjacent to the patient and ominously all were viable within the dry surface biofilms. Study explained that, survivability of planktonic bacteria may increase due to moist microclimate closer to the patient which allows incorporation of more species into the biofilm; whereas, the number of species, incorporated into the biofilm, could be reduced by more aggressive/frequent cleaning and disinfection of the floor. The author also elucidated that, the higher number of species incorporated into the biofilm, may be directly related to the distance from a sick patient transmitting large numbers of bacteria. This study on ICU environment by Hu et al. (2015) is a strong evidence of the existence of MDRO-containing dry surface biofilms in the intensive care environment. It was demonstrated that DSB containing MDROs may be present on inanimate surfaces in a hospital environment and were detected despite cleaning with hypochlorite. It also suggested another reservoir of organisms that may be transmitted to patients to cause HAIs and recommended that for further research to determine the extent of this problem, and the cleaning agents and techniques required to remove dry surface biofilms from hospital environments.

Many other studies have also showed the hospital bed as a potential risk factor for infection transmission and demonstrated the higher contamination rate of bed covers, bed sheets, mattress and bed rails with both Gram positive and Gram negative bacteria (Table 1-7).

Microbes including bacterial spores, vegetative bacteria, fungi and viruses can survive on dry surfaces for prolonged periods (Otter and French, 2009). This could explain why vegetative bacteria can survive on dry hospital surfaces for so long, form biofilm and could be part of the reason why they are so difficult to eradicate by disinfectants and how contaminated environmental surfaces become reservoir in the transmission of certain healthcare-associated pathogens (Otter et al., 2011; Salgado et al., 2013).

Table 1- 7:

Studies of spread of pathogens associated with bed components.

References	Location	Pathogens	Intervention	Outcome
(Sexton et al., 2006)	MRSA isolation room with MRSA patients	MRSA	Weekly sampling after admission to isolation room	MRSA isolated in environment, beds and mattresses.
(Shiomori et al., 2002)	MRSA isolation rooms	MRSA	Air and surface sampling before, during and after bed making.	Organism isolated in air, environment, bed linen.
(Sherburn and Jenkins, 2005)	Used cotton and mattress	*S. aureus* and *E. coli*	Simulation head movement on mattresses.	Simulated movement increased the level of organisms in air released from mattresses
(Bonten et al., 1996)	MICU	VRE	Daily sampling from patient and environment.	VRE isolated from bedrail and draw sheet
(Eckstein et al., 2007)	*C. difficile* and VRE isolation rooms	VRE and *C. difficile*	Pre and post-cleaning disinfection samples.	Organisms on bedrails reduced when cleaned by cleaning staff

It is now evidenced that surface-attached microbes are likely to be common on dry hospital surfaces, and harbour established biofilms. The presence of biofilms on hospital surfaces interfere with attempts to recover bacteria from surfaces, that lead to an incomplete idea of both the prevalence of contamination with pathogens and the number of bacteria on the surfaces. This has important implications, particularly for hospital outbreak investigation. Therefore, hospital cleaning and disinfection to reduce microbial attachment and improve its detachment from hospital environmental surface should consider as the prime issue.

1.9 Prevention of transmission of DSB in hospital environment

Several nosocomial pathogens have been demonstrated to persist in inanimate environmental surfaces for months to years and contaminate the hospital surfaces and medical equipment recurrently by colonization of the hands of HCP. This has direct contribution to person-to-person transmission, that can cause epidemics in which environmental transmission was deemed to play a role (Weinstein, 1991; Carling and Huang, 2013). For example, Transmission of MRSA (31-42%) (Boyce et al. 1997; Bhalla et al. 2004) and VRE (10-46%) (Bhalla et al.

2004; Duckro et al., 2005) from environmental surfaces to gloves or hands of HCWs has been documented by several investigators. Furthermore, hospitalization in a room in which the previous patient had been colonized or infected with *S. aureus*, MRSA, VRE, *C. difficile*, multidrug resistant *Acinetobacter spp*., or multidrug-resistant *Pseudomonas* has been shown to be a risk factor for colonization or infection with the same pathogen for the next patient admitted to the room (Huang et al., 2006; Carling and Bartley, 2010). The implementation of room cleaning with immediate feedback to environmental service personnel has been found to reduction in healthcare-associated infections (Carling and Bartley, 2010).

Controlling environmental contamination in patient's zone is important in preventing hospital acquired infections (Dancer, 2009). A novel approach to reducing environmental contamination is "source control" by daily washing of the skin of patients with chlorhexidine (Vernon et al., 2006). This process has been shown to reduce contamination of patient skin, environmental surfaces followed by hands of HCWs. Source control in conjunction with corresponding strategies aimed to improve cleaning and disinfection might help to reduce transmission in advance. However, chlorhexidine is not effective against spores and resistance may emerge, so novel source control strategies are justified.

Advances in hospital design and surface science can help to reduce the potential for contamination (Lankford et al., 2006). Various materials like copper, silver, and other antimicrobial-impregnated materials reduces bacterial survival in vitro (Noyce et al., 2006), and numerous antibacterial surface materials or treatments are now available. Chemically modified surface has an important strategy on regulating bacterial attachment and their development into biofilms. A study showed an example of the influence of surface chemistry on bacterial attachment, where the temperature-responsive switching of poly N-isopropylacrylamide (PNIPAAm) surface, changes the surface energy of the polymer and consequently modulates the adsorption of cells and the attachment of biofilms (Ista et al., 2010). On the other hand, grafting polymer like dextran, poly ethylene oxide (PEO) coatings on surfaces can reduce attachment and affect biofilm organization (Cheng et al., 2008). Hydrophilic, PEO coatings inhibit protein adsorption and repel bacterial adhesion. Some covalently attach bactericidal molecules such as quaternary ammonium groups, N′-disubstituted PEI polymer can inhibit biofilm formation (Wong et al., 2010). This idea of making surfaces with bactericidal and virucidal, might help to eliminate live bacteria on surfaces of medical implants, thus preventing biofilm formation would be a major advancement in the biomedical field. However, such treatments do not remove the need for cleaning, and there is very limited evidence that they have any significant effect on hospital infection rates.

Implementation of improved sampling methods to evaluate the frequency and concentration of surface contamination would be useful. Technological developments to assist with cleaning and disinfection include the introduction of microfiber cleaning materials, which may be more effective than standard cloths for removing pathogens from surfaces.

1.10 Conclusion

Hospital environmental surface contamination contribute negligibly to nosocomial transmission this historical viewpoint has been revaluated. There is now compelling evidence that contaminated surfaces make an important contribution to the epidemic and endemic transmission of *S. aureus*, *C. difficile,* VRE, MRSA, *A. baumannii,* and *P. aeruginosa*. Therefore, measures of infection control should include identifying patients at risk of nosocomial infections, observing hand hygiene, following standard precautions to reduce transmission and strategies to reduce VAP, CR-BSI, CAUTI. Environmental factors and architectural lay out also need to be emphasized upon. Infection prevention by identifying sources of organism, identification of organisms, isolation if required, antibiotic prophylaxis must be executed. Room ventilation, cleaning and decontamination, protective clothing, monitoring and surveillance should be prioritized depending upon the needs.

Part Four: Disinfection

Maintenance of a good hospital environment requires the implementation of adequate cleaning strategies. As described before, hospital surfaces potentially contribute to cross-transmission by acquisition of transient hand carriage by health care personnel due to contact with a contaminated surface or by patient contact with contaminated surfaces or medical equipment. In addition, long-time survivability and tolerance to unfavourable situation of biofilm bacteria on hospital surfaces, increases the opportunity to convert a avirulent bacteria to virulent strain (Lewis, 2001) as a result, new and more virulent microbial phenotypes may be expressed when growing with in biofilm, which are highly resistant to many forms of physical and environmental insults, like host immune response, chemical and disinfectants stress etc. (Cortés et al., 2011). Therefore, to prevent transmission of healthcare-associated pathogens and to reduce the levels of microbial contamination and decrease healthcare-associated infections, more integrated approach to effective environmental surface cleaning and disinfection is required.

1.11 What is disinfection

Disinfection describes a process that eliminates many or all pathogenic micro-organisms on inanimate objects except for the bacterial endospore is achieved by altering or destroying essential structures or functions within the microbe (Rutala and Weber, 1999).

A few disinfectants that kill spores after prolonged exposure (6-10 h) are called chemical sterilant (Rutala and Weber, 1999).

1.12 Types of disinfectants (Rutala and Weber, 1999)

According to its efficiency and ability to kill bacterial spores, an antimicrobial product can belong to one of four distinct groups: sterilant or high-, intermediate- and low-level disinfectants.

1.12.1 High level disinfectants

Disinfectants that kill all micro-organisms with shorter exposure period (≤45 minute) except high numbers of bacterial spores and are called high-level disinfectants.

1.12.2 Low-level disinfectants

Disinfectants that can kill most vegetative bacteria, some fungi, and some viruses within 5 to 10 minutes period of time are called low level disinfectants.

1.12.2 Intermediate-level disinfectants

Disinfectants those are cidal for tubercle bacilli, vegetative bacteria, most viruses and most fungi but do not necessarily kill bacterial spores are called intermediate level disinfectants.

Table 1- 8:

Characterization of disinfectants according to their class (Rutala and Weber, 2001; Gilbert and McBain, 2003; Rutala and Weber, 2004).

Disinfectants	Spectrum of action	Required for	Examples
Sterilant	all microorganisms, including bacterial spores	critical instruments that penetrate tissue or present a high risk if non-sterile (e.g. implants, needles and other surgical instruments)	heat, steam, higher concentrations of hydrogen peroxide and peracetic acid, glutaraldehyde (in 6–10 h)
High-level disinfectants	almost all microorganisms, but not spores	semi-critical items that do not penetrate tissues or contact mucous membranes (except dental) (such as endoscopes, respiratory therapy equipment and diaphragms)	hydrogen peroxide, glutaraldehyde, formaldehyde, ortho-phthalaldehyde, peracetic acid
Intermediate-level disinfectants	almost all vegetative bacteria, fungi, tubercle bacilli and enveloped and lipid viruses	non-critical items that touch intact skin (e.g. thermometers and hydrotherapy tanks	hypochlorite, iodine and iodophor alcohols, disinfectants
Low-level disinfectants	not efficient for most bacteria, tubercle bacilli, spores, fungi and viruses	non-critical items: items such as stethoscopes bedpans, blood pressure cuffs and bedside tables	phenolics, quaternary ammonium compounds

1.13 Commonly used disinfectants in hospital (Rutala and Weber, 2013b)

Disinfection is generally performed using an US Environmental Protection Agency (EPA)-registered hospital disinfectant such as:

Alcohol compound
chlorine-containing compound
Peracetic acid
improved hydrogen peroxide (HP)
quaternary ammonium compounds
phenolics

1.13.1 Alcohol

In the healthcare setting, "alcohol" refers to two water-soluble chemical compounds—ethyl alcohol and isopropyl alcohol. Alcohol disinfectants work by denaturing protein and are act against vegetative bacteria, fungi and viruses, but have no effect on spores (Fraise, 1999; Gilbert and McBain, 2003). Alcohol is bactericidal, tuberculocidal, fungicidal, virucidal. It is fast acting and easy to use. Usually used to disinfect small surfaces such as rubber stoppers on medication vials, oral and rectal thermometer (Tyler et al., 1990; Nelson, 2003), hospital pagers, scissors , stethoscopes, (de Cabo et al., 1978; Babb et al., 1981), has no toxic residue. Alcohol is affected by organic matter and evaporates rapidly. Their cidal activity drops sharply when diluted below 50% (volume/volume) concentration, and the optimum bactericidal concentration is 60%–90% solutions in water (volume/volume) (Ali et al., 2001). *P. aeruginosa* could be killed in 10 seconds by all concentrations of ethanol from 30% to 100% (v/v), and *Serratia marcescens, E, coli* and *Salmonella typhosa* were killed in 10 seconds by all concentrations of ethanol from 40% to 100% (Ali et al., 2001). *S. aureus* and *Streptococcus pyogenes* are slightly more resistant and need 60-95% of ethyl alcohol to be killed in in 10 seconds. Isopropyl alcohol (isopropanol) is slightly more bactericidal than ethyl alcohol for *E. coli* and *S. aureus* (Coulthard and Sykes, 1936; Ali et al., 2001). Alcohol has no detergent or cleaning properties. It can damage some instruments (eg, harden rubber, deteriorate glue) it is flammable (large amounts require special storage) and not registered by EPA (Rutala and Weber, 2008).

1.13.2 Chlorine

Hypochlorites are the most widely used of the chlorine disinfectants, are available as liquid (e.g., sodium hypochlorite) or solid (e.g., calcium hypochlorite). Alternative compounds that release chlorine and are used in the health-care setting include chlorine dioxide, SDIC and

chloramine-T. Chlorine and chlorine based disinfectants are very effective against bacteria -act by oxidizing bacterial membrane proteins leading to inhibit growth within short period of exposure (Rutala and Weber, 2008) with, 100 ppm of free chlorine killing 10^6–10^7 *S. aureus, Salmonella choleraesuis,* and *P. aeruginosa* in <10 minutes (Rutala et al., 1998).. However, these chemical agents can be inactivated by the presence of organic matter (Gilbert and McBain, 2003; Rutala and Weber, 2008). To minimize the effect of soil on the efficacy of chlorine and chlorine-based disinfectants, an increased concentration is required. SDIC at 2,500 ppm is effective against planktonic bacteria in the presence of up to 20% plasma, compared with 10% plasma for sodium hypochlorite at 2,500 ppm (Bloomfield and Miller, 1989). Household bleach contains 5.25%–6.15% sodium hypochlorite, or 52,500–61,500 ppm. For small spills of blood (i.e., drops of blood) on noncritical surfaces, a 1:100 dilution is recommended but for large spills of blood a 1:10 (final concentration) dilution of household bleach (5.25%-6.15% sodium hypochlorite) is recommended which is a very high concentration (Chitnis et al., 2004).

In the last few years, several studies have reported that, different formulation of chlorine disinfectants such as; dichloroacetic acid (DCA), trichloroacetic acid (TCA), and chloral hydrate (CH), polychlorinated biphenyl (PCB), play a role in the development of cancers (Guha et al., 2012; Bermúdez-Aguirre and Barbosa-Cánovas, 2013; Loomis et al., 2013). A potential hazard of chlorine is production of the carcinogen (chloromethyl) bis ether when hypochlorite solutions contact formaldehyde (Gamble, 1977) and produce trihalomethane when hot water is hyperchlorinated (Helms et al., 1987). In addition to this, studies evidenced many bacteria, for example, VRE, *E. coli*, develop resistance to chlorine and chlorine disinfection was not able to destroy antibiotic-resistance genes, though it can decrease bacterial counts of antibiotic-resistant bacteria (ARB) (Cherchi and Gu, 2011; Furukawa et al., 2017). One study has evaluated the efficacy of several disinfectants such as SDIC, chlorine dioxide, and disinfectants from aldehyde group against different strains of mycobacteria and reported that disinfectants based on SDIC were more effective and also concluded that clinical strains were more resistant to biocides than laboratory type strains (Griffiths et al., 1999). A review study on the use of inorganic hypochlorite (bleach) in healthcare facilities to disinfect medical devices and environmental surfaces concluded chlorine as an advantageous disinfectant (e.g. fast microbiocidal activity, cost-effectiveness and good track record) that supports it’s continuous use in healthcare settings (Rutala and Weber, 1997).

1.13.3 Hydrogen Peroxide (H_2O_2)

Hydrogen peroxide works by producing hydroxyl free radicals that destroy membrane lipids, DNA, and other essential cell components. Aerobes and facultative anaerobes produce catalase to protect themselves from metabolically produced H_2O_2 by degrading H_2O_2 to water and oxygen. This defence mechanism has been countered by increasing concentration when used for disinfection (Turner, 1983; Block SS., 2001). H_2O_2 is active against a wide range of microorganisms, including bacteria, yeasts, fungi, viruses, and spores (Block SS., 2001). Hydrogen peroxide in higher concentrations, 6% to 25%, works as chemical sterilant. A study demonstrated 0.5% accelerated H_2O_2 (AHP, US. patent No. 6,346,279) as bactericidal (vegetative bacteria; *S. aureus, E. coli, A. baumannii, P. aeruginosa*, VRE, MRSA, and *S. choleraesuis*) and virucidal in 1 minute and mycobactericidal and fungicidal in 5 minutes of exposure at 20°C, (Omidbakhsh and Sattar, 2006). Organisms with high cellular catalase activity (e.g., *S. aureus, S. marcescens,* and *Proteus mirabilis*) required 30–60 minutes exposure to 0.6% hydrogen peroxide for a 10^8 reduction in cell counts, whereas organisms with lower catalase activity (e.g., *E. coli, Streptococcus species*, and *Pseudomonas species*) required only 15 minutes' exposure (Schaeffer et al., 1980). Commercially available 3% hydrogen peroxide is a stable and effective disinfectant when used on inanimate surfaces. The improved HP-based {very low levels of anionic and/or nonionic surfactants in an acidic product, which act in synergy with hydrogen peroxide, prepared and marketed by several companies in various concentrations (eg, 0.5%–7%) and different products may use different terminology for these products such as "accelerated" or "activated."} environmental surface disinfectants have proved to be more effective (>6-$\log_{10}$ reduction) and fast-acting (30-60 seconds) microbicides in the presence of a soil load (to simulate the presence of body fluids) than commercially available HP (Rutala et al., 2012).

1.13.4 Peracetic acid

Peracetic, or peroxyacetic, shows rapid action against all microorganisms. Lack of harmful decomposition products (i.e., acetic acid, water, oxygen, hydrogen peroxide), enhancement of removal of organic material and no residual are the distinct advantages of peracetic acid. It remains effective in the presence of organic matter and is sporicidal even at low temperatures (Tucker et al., 1996). However, peracetic acid is characteristically unstable, particularly when diluted; for example, a 1% solution loses half its strength through hydrolysis in 6 days, whereas 40% peracetic acid loses 1%–2% of its active ingredients per month (Block SS., 2001). The mechanism of action of peracetic acid is not determined, but it is believed to function similarly to other oxidizing agents—that is, it denatures proteins, disrupts the cell wall permeability, and

oxidizes sulfhydryl and sulphur bonds in proteins, enzymes, and other metabolites (Block SS., 2001). Any Gram positive and Gram negative bacteria, fungi, and yeasts Peracetic acid could be inactivate in ≤5 minutes of exposure at <100 ppm. But to achieve this in presence of organic matter, 200–500 ppm is required (Rutala and Weber, 2008).

Peracetic acid is a corrosive chemical and some harmful effects were observed in humans such as, irritation to skin, mucous membranes and respiratory tract, lacrimation after exposure to concentrations as low as 15.6 mg peracetic acid/m3 (5 ppm) for only 3 minutes and extreme discomfort was noted at concentrations ≥2.5 ppm (7.79 mg/m^3), and 2.0 ppm (6.23 mg/m^3) was considered unbearable (Fraser and Thorbinson 1986). Concentration of peracetic acid ranged from 0.5 to 0.6 ppm (1.56-1.87 mg/m3) are not considered to be immediately irritating, considered as "unpleasant for an extended period" of time (McDonagh 1997). In 2010, the technical documentation supporting an Acute Exposure Guideline Level (AEGL) for peracetic acid was published in the eighth volume of *Acute Exposure Guideline Levels for Selected Airborne Chemicals* published by the National Academies Press which described that exposure to lethal concentrations (>1.3 ppm) of peracetic acid up to 60 minutes causes death/permanent incapacity such as, hemorrhage, edema, and consolidation of the lungs, whereas nonlethal concentrations cause transient weight loss or reduced weight gain in addition to slight to moderate signs of respiratory tract irritation (National Research Council (US) Committee, 2010). In 2014, ACGIH adopted a Threshold Limit Value–Short-Term Exposure Limit (TLV-STEL) for peracetic acid of 0.4 ppm (1.24 mg/m3) as a 15-minute time-weighted average (TWA) exposure that should not be exceeded at any time during a workday

1.13.5 Peracetic Acid and Hydrogen Peroxide

The combination of peracetic acid and hydrogen peroxide is bactericidal inactivating all planktonic microorganisms except bacterial spores within 20 minutes of exposure (Alasri et al., 1992). The 0.08% peracetic acid plus 1.0% hydrogen peroxide product effectively inactivated glutaraldehyde-resistant mycobacteria (Stanley, 1998). This combination has been usually used for disinfecting hemodialyzers (Fleming et al., 1991).

This combination formulations products have oxidizing and corrosive properties. Therefore, safety precautions have to be applied accordingly. The use of personal protective equipment should only be considered a supplementary means or as the last resort to minimize workers' exposure to the hazards. such as, safety goggles, protective gloves and suitable protective clothing must be worn. If necessary, a gas mask should be used with an appropriate filter (Chemical Safety in the Workplace, 2007).

1.13.6 Phenolics

Two phenol derivatives are commonly found as constituents of hospital disinfectants are ortho-phenyl phenol and ortho-benzyl-para-chlorophenol. Phenolics are absorbed by porous materials and can irritate tissue. Phenol acts as a gross protoplasmic poison in high concentrations and easily penetrates and disrupts the cell wall leading to precipitate the cell proteins. Phenol in low concentrations and higher molecular-weight act as bactericidal by inactivation of essential enzyme systems and leakage of essential metabolites from the cell wall (Block, 1983). Phenolics are bactericidal, fungicidal, virucidal, and tuberculocidal (Goddard and McCue, 2001).

1.13.7 Quaternary Ammonium Compounds (QAC)

Quaternary ammonium compounds are widely used as disinfectants. They kill microorganisms by solubilizing the cell membrane, thus inactivating the cell wall energy-producing enzymes and causing essential cell proteins to denature (Rutala and Weber, 2008). The quaternaries are good cleaning agents, but materials such as cotton and gauze pads can decrease their activity because of production of insoluble precipitates or absorption of active ingredients. These compounds are sold as hospital disinfectants are generally fungicidal, bactericidal, and virucidal against lipophilic (enveloped) viruses; but not sporicidal and generally not tuberculocidal or virucidal against hydrophilic (nonenveloped) viruses (Best et al., 1990; Mbithi et al., 1990; Silverman et al., 1999). The quaternaries commonly are used in ordinary environmental sanitation of noncritical surfaces, such as floors, furniture, and walls. EPA-registered quaternary ammonium compounds are appropriate to use for disinfecting medical equipment that contacts intact skin (e.g., blood pressure cuffs) (Rutala and Weber, 2008).

1.13.8 The aldehyde-based disinfectants

The aldehyde based disinfectants disrupt proteins and nucleic acids by alkylation and have antimicrobial activity against spores, bacteria, viruses and fungi (Fraise, 1999). Commonly used aldehyde-based disinfectants are: formaldehyde and glutaraldehyde:

1.13.8.a Formaldehyde

Formaldehyde is used as a disinfectant and sterilant in both its liquid and gaseous states. Formaldehyde is sold and used principally as a water-based solution called formalin, which is 37% formaldehyde by weight. The aqueous solution is a bactericide, tuberculocide, fungicide, virucide and sporicide (Sagripanti et al., 1997). The use of formaldehyde is limited due to its carcinogenic effect. Formaldehyde is used in the health-care setting to prepare viral vaccines (e.g., poliovirus and influenza); as an embalming agent; and to preserve anatomic specimens.

Aqueous formaldehyde solutions (1%–2%) also have been used to disinfect the internal fluid pathways of dialysis machines (Bland and Favero, 1990). To minimize a potential health hazard to dialysis patients, the dialysis equipment must be thoroughly rinsed and tested for residual formaldehyde before use. Bacterial endospores, for example, *Bacillus* and *Geobacillus stearothermophilus* spores are resistant to a wide-variety of treatments, found formaldehyde gaseous decontaminants active against them (Whitney et al., 2003).

A study demonstrated the decontamination efficacy of formaldehyde gas for spores of *B. anthracis* and the two surrogates *B. subtilis* and *G. stearothermophilus* dried on indoor surface materials and observed >6·0 log reduction on all test materials evaluated (Rogers et al., 2007). Due to carcinogenic effect of formaldehyde it has limited use as a decontaminant; however, this hazard could be neutralized by application of ammonium carbonate with formaldehyde to reduce the toxic potential of formaldehyde. This neutralization process leads to the production of the white powder, hexamethylene tetramine, which becomes deposited on the surfaces within the decontaminated area. However, if the formaldehyde is not completely neutralized at the time of administering the ammonium carbonate then there is the potential for formaldehyde gas to leach out of porous materials, thereby posing an additional hazard (Rogers et al., 2007).

1.13.8.b Glutaraldehyde

Glutaraldehyde is a saturated dialdehyde used as a high-level disinfectant and chemical sterilant, most commonly for medical equipment such as endoscopes (Cheung et al., 1999). Also used for spirometry tubing, dialyzers (Husni et al., 1989), transducers, anaesthesia and respiratory therapy equipment, haemodialysis proportioning and dialysate delivery systems and reuse of laparoscopic disposable plastic trocars (Gundogdu et al., 1998).

1.13.9 Iodophors

Iodine solutions or tincture iodine have been used by health professionals primarily as antiseptics on skin or tissue. On the other hand, it also has been used both as antiseptics and disinfectants. Iodine can penetrate the cell wall of microorganisms quickly, and the lethal effects result from disruption of protein and nucleic acid structure and synthesis (Rutala and Weber, 2008). They are bactericidal, mycobactericidal, and virucidal but require prolonged contact times to kill certain fungi and bacterial spores (Terleckyj and Axler, 1987; Rutala et al., 1991). Besides their use as an antiseptic, iodophors can be used for disinfecting blood culture bottles and medical equipment, such as hydrotherapy tanks, thermometers. Antiseptic iodophors are not suitable for use as hard-surface disinfectants because of concentration differences. Iodophors formulated as antiseptics contain less free iodine than do those formulated as disinfectants (Favero and Bond, 1991).

Table 1- 9:

Summary of advantages and disadvantages of disinfectants used as low-level disinfectant (Rutala and Weber, 2014).

Low-level disinfectant	Advantages	Disadvantages
Alcohol	Bactericidal, tuberculocidal, fungicidal, virucidal. Fast acting, easy to use. Used to disinfect small surfaces No toxic residue.	Not sporicidal. Affected by organic matter. No detergent or cleaning properties. Damage some instruments. Flammable. Do not use alcohol for large surfaces.
Chlorine	Broad-spectrum bactericidal, tuberculocidal, fungicidal, virucidal, sporicidal. No toxic residue Inexpensive and fast acting. Unaffected by water hardness. Low incidence of serious toxicity. Reduces biofilms of surfaces. Relatively stable. EPA registered.	At 5% can cause eye irritation, oropharyngeal, oesophageal, and gastric burns. Corrosiveness to metals in high concentrations (>500 ppm). Inactivated by organic matter. Discolouring of fabrics. Release of toxic chlorine gas when mixed with acid or ammonia. Potential hazard is production of trihalomethane.
Improve HP	Broad-spectrum bactericidal tuberculocidal and fungicidal, virucidal and sporicidal. Safe for workers (lowest EPA toxicity category, IV). Benign for the environment. Unaffected by organic matter. Surface compatible. Noncorrosive.	More expensive than some low-level disinfectants
Iodophors	Bactericidal, mycobactericidal and virucidal. Used for disinfecting blood culture bottles	Require prolonged contact to kill fungi. Not sporicidal. Damage silicone catheters. More commonly used as a antiseptic than disinfectant.
Phenolics	Bactericidal, tuberculocidal, fungicidal, virucidal. Inexpensive. EPA registered	Absorbed by porous materials and irritate tissue. Depigmentation of skin caused by certain phenolics. Not sporicidal.
Quaternary ammonium compounds	Bactericidal, fungicidal, virucidal against enveloped viruses (eg, HIV). Good cleaning agents. EPA registered. Surface compatible. Persistent antimicrobial activity when undisturbed.	Not sporicidal, generally not tuberculocidal, and virucidal against nonenveloped viruses. High water hardness and cotton/gauze can make less microbicidal. A few reports documented asthma as result of exposure to benzalkonium chloride. Affected by organic matter

1.14 Factors affecting disinfectants efficacy

The degree of disinfection is affected by a number of factors, that may reduce or nullify the efficacy of the disinfectants. Factors that have been shown to affect efficacy are (Russell, 2008):

The prior cleaning of the object.

Numbers and location of the microorganisms.

The organic and inorganic load.

The type and level of microbial contamination.

The concentration of and exposure time to the germicide.

The nature of the object (e.g., crevices, hinges, lumens) and

The temperature and pH of the disinfection process.

The type nature and condition of a microorganism and its previous history and post treatment handling can all influence the response to the biocidal agent. These aspects are important for not only in designing official tests for evaluating biocidal activity but also in actual in-use situation.

1.15 Difficulties in biofilm eradication

When developed, biofilm provides physical protection to the cells incorporated within it, increasing the difficulty in eradicating biofilm. This protection from killing by innate host defences and antimicrobial agents is unique to and distinct from conventional antimicrobial resistance (Anderl et al., 2000). Biofilm bacteria exhibit resistant to biocide not only due to "classic" genetic mechanisms (i.e., gene mutation, genetic exchange), but also by some peculiarity of biofilm growth (Pozo and Patel, 2007).

A variety of potential mechanisms are implicated in biofilm resistance to antimicrobial agents and biocides (Pozo and Patel, 2007):

1.15.1 Poor penetration

It has been widely reported that biocidal molecules have restricted (partially) penetration into biofilms because of its innate barrier property of the glycocalyx (Gilbert and McBain, 2001). However, this explanation is proved incorrect as the diffusivity of the glycocalyx and water are broadly similar (Suci et al., 1994; Stewart, 1996). Rather, direct inactivation of the agent within and by the glycocalyx might be the reason of poor penetration of drugs or biocides into biofilms. An ionic interaction between the anionic glycocalyx and cationic agents, termed as reaction diffusion limitation, with the extracellular matrix acting as an ion-exchange resin that restricts

the access of biocide to the deeper lying cells and reduces cellular growth rate through nutrient scarcity, to ensure more recalcitrant state of those cells. These two factors together ensure such cells and their ecologic partners to be exposed to sublethal concentrations of biocides during antimicrobial treatments(Gilbert and McBain, 2001). Alternatively penetration failure may result from a covalent reaction with chemically reactive biocides such as iodine, iodine-polyvinylpyrolidone complexes (Favero et al., 1983), chlorine, and peroxygens (Foley and Gilbert, 1996). Deposition of various antimicrobial enzymes, for example, β-lactamases (Giwercman et al., 1991), formaldehyde lyase, and formaldehyde dehydrogenase (Sondossi et al., 1985) may enhance the ability of the glycocalyx to inactivate antimicrobial agents and neutralize antimicrobial agents, such as β-lactams and aldehydes, as they diffuse across it. Studies have also considered microbial maturity as an important factor ranges from cells attached to surfaces to samples extracted (Lechevallier et al., 1988; Lee et al., 2009). Furthermore, study also found cell density as a cause of poor penetration(Nett et al., 2008). Although several studies have not recommended cell density alone as a cause of reduced susceptibility of biofilms to biocides, in many studies it is found difficult to be certain of the impact of the biofilm phenotype independent of cell density (Cochran et al., 2000; Watamoto et al., 2010). Therefore, it might be concluded that, the close juxtaposition of cells within the anionic biofilm matrix (EPS), and incorporating entrapped extracellular enzymes, establishes a reaction-diffusion barrier and leads to poor penetration of chemically reactive biocides, cationic antibiotics, and nutrients.

1.15.2 The transfer and co-transfer of genetic determinants

Transfer and co-transfer of genetic determinants encoding for resistance to antimicrobial drugs following biocide exposure is also of concern, but so far has been poorly evaluated. The existence of drug-resistant phenotypes of biofilm for antibiotics (Ashby et al., 1994) and biocides (Das et al., 1998) has been demonstrated. These researches also demonstrated up to 4-fold decrease in susceptibility toward a broad range of agents after the initial attachment to a surface. Evolution and genetic diversity of natural microbial communities by horizontal gene transfer is not unlikely. Study suggested that, attached bacterial community deploy acyl homoserine lactone communication signals, thus promotes the expression of a biofilm-phenotype involving up to 50 separate gene products responsible for resistance to biocide (Davies et al., 1998). To adapt in an unfamiliar environment, bacteria often gain new genetic traits via horizontal gene transfer rather than gene mutation (Koonin et al., 2001). Horizontal gene transfer between bacteria can be mediated by conjugative plasmids, transposon or bacteriophage (Kokare et al., 2009). This genetic alteration or diversity causes expression of different phenotypic characters in the biofilm forming bacteria than that of planktonic one.

The organism received plasmid can form biofilm but this same organism without plasmid could produces only micro colonies without any further development (Kokare et al., 2009). Surface specific gene expression may happen during biofilm formation procedure; for example—the structural gene specific for attachment to chitin differ from those required for attachment to abiotic, non-nutritive surfaces like plastic and glass (Keyhani and Roseman, 1996). Plasmid may encode for resistance to multiple antimicrobial agents. Therefore, plasmid mediated biofilm association could provide a mechanism for selecting and promoting the spread of antimicrobial resistant bacteria (Kokare et al., 2009).

Antimicrobial agents may be chelated by inactivating enzymes, for example, ampicillin is rapidly destroyed by *β*-lactamases inside *K. pneumoniae* biofilms within the biofilm matrix (Anderl et al., 2000). Biofilm, by promoting horizontal gene transfer, facilitate the spread of conventional antimicrobial resistance and make the situation worst (Pozo and Patel, 2007) . In the harshest environments, high bacterial density within biofilms facilitates metabolic activity (though at reduced rate) are appropriate for horizontal gene dissemination (Otter et al., 2015). Horizontal plasmid transmission also occur in biofilm through conjugation, for example, the transfer of ESBL (CTX-M-15) and carbapenemase (NDM-1) plasmids between Enterobacteriaceae on dry surfaces (Molin and Tolker-Nielsen, 2003; Warnes et al., 2012). Furthermore, many study also reported the increased rate of mutation (the rate at which DNA replication mistakes occur during cell division) in biofilm (Driffield et al., 2008; Høiby et al., 2010). Thus, both horizontal transfer of resistance genes and plasmids and increased mutation rates could be the cause in the acquisition or denovo development of reduced susceptibility to antimicrobial agents.

1.15.3 The arbitrary presence of cells showing a resistant phenotype (known as "persisters")

Biofilm populations are enriched in "persister" cells that exhibit multidrug tolerance. This cells in the biofilm are slow growing, and many are probably in stationary state. These may be either biofilm-specific phenotype or formed because of their protection within the biofilm from immune responses or hostile agents (Lewis, 2000). These cells have criteria to survive in treatment phases and are capable to proliferate in the post-treatment phase, thereby engender the situation obtaining considerable resistance of biofilm community (Gilbert and McBain, 2001).

A study revealed a small subpopulation of cells, persisters, that remain alive irrespective of the concentration of the antibiotic where both biofilm and planktonic cells of *P. aeruginosa* were

eliminated leaving persisters intact (Lewis, 2008). The author also explained that, according to the thickness, dimension and total number of cells obtained from *P. aeruginosa* biofilm, the cell density to be 10^{11}/cm2 which is significantly higher than the planktonic cultures. Study also reported a persister plateau even after increase in the antibiotic concentration which indicates the inadequate killing of persisters even with higher concentration. A study on *E. coli* persisters gene expression profile showed a downregulated biosynthetic pathway, consistent with their dormant nature, and indicated overexpression of toxin/antitoxin (TA) modules that inhibit essential functions (translation) may contribute to persister formation. glpD and plsB were identified as potential persister genes by overexpression cloning of a genomic library and selection for antibiotic tolerance (Lewis, 2008).

It was assumed that the immune system will mop up the persisters cells as it does to bacteriostatic antibiotic treated planktonic persisters. However, the biofilm matrix protects against immune cells (Leid et al., 2002; Jesaitis et al., 2003; Vuong et al., 2004) and its persisters survive (Figure 1-14).

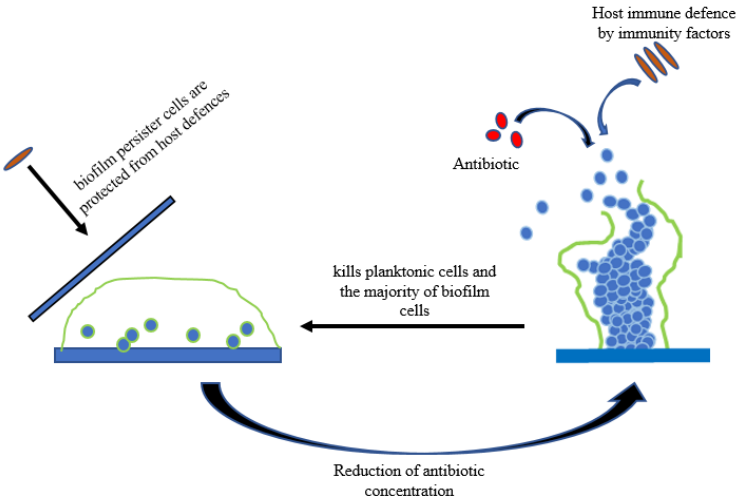

Figure 1-4:

Model of biofilm resistance based on persister survival. An initial treatment with antibiotic kills planktonic cells and the majority of biofilm cells. The immune system kills planktonic persisters, but the biofilm persister cells are protected from host defenses by the exopolysaccharide matrix. After the antibiotic concentration drops, persisters resurrect the biofilm and the infection relapses (Lewis, 2008).

Lewis (2000) demonstrated that after application of bactericidal agent, biofilm bacterial cell lysis does not occur from direct action of the agent rather cell lysis occurs due to a programmed suicide mechanism. Many studies also evidenced similar cells rather than being resistant to the agent are actually defective in programmed cell death (Koch, 1996; Brooun et al., 2000). After

removal of the treatment stress, these cells also have the ability to grow rapidly in the presence of nutrients released from their lysed community partners, and the community would become restored (Gilbert and McBain, 2001).

1.15.4 The presence of cells that triggered stress responses under unfavourable chemical conditions within the biofilm matrix.

Prolong exposure to environmental stress stimulates biofilm bacteria to upregulate stress-response genes, and switch to more resistant phenotypes, for example, prolong exposure of biofilm to β-lactam drugs increase their ability to express chromosomal β-lactamases enzyme (Bagge et al., 2004). Multidrug resistance drugs pumps play an important role in the resistance of planktonic *P. aeruginosa* to antimicrobial agents, that make it logical adopting the biofilm mode of growth might signal cells to increase the expression of efflux pumps (Brooun et al., 2000). A gene, *ndvB*, is only expressed in biofilm cells has been described in *P. aeruginosa* (Mah et al., 2003) which is responsible for the synthesis of periplasmic glucans, the interaction of which with tobramycin prevents it from reaching its site of action (Pozo and Patel, 2007).

1.15.5 Biofilm composition

Susceptibility to biocide also influenced by biofilm composition. Biofilm with higher nutrient concentration and higher cell density are less susceptible to biocides than low-nutrient, low-density biofilms (Stewart et al., 1998; Peng et al., 2002; Condell et al., 2012). Dry hospital surface biofilm usually have low nutrient and cellular density level, however, patient's body fluid contamination might provide an enriched environment in which high-nutrient, high-density biofilms could form on hospital surfaces (Otter et al., 2015). Studies also suggested that microbial ecology of the biofilm is another factor influencing susceptibility. Biofilms composed of multiple species are less susceptible than single-species biofilms, although this is not always the case with the corresponding planktonic cultures (Behnke and Camper, 2012; Bridier et al., 2012).

It has been established that development of bacterial biofilms on the surfaces is the key to the pathogenesis of the associated infections. Therefore, it is essential to extend our knowledge about the mechanisms involved in biofilm resistance to antimicrobial and biocidal agents to develop new and effective strategies to control biofilm-associated infections. New treatment strategies should be focused toward designing substances able to compromise biofilm formation, destabilize established biofilms, and/or target persister cells. Genes and gene

products contributing to this type of resistance may also be a target for the development of new antimicrobial and biocidal agents.

1.16 The eradication of an established biofilm

A mature established biofilm eradication from hospital environment is a great challenge due to its protective capacity from physical removal and reduced susceptibility to biocide (Peng et al., 2002). To control HAI proper cleaning is the only option to be considered as the evidences confirmed the links between infection risk and contaminated hospitals (Nightingale, 1863. Accessed September 2014 ; Dancer, 1999). Several studies reported cleaning as an important control component for outbreaks of norovirus, VRE, *C. difficile*, MRSA, and multidrug-resistant (MDR) Gram negative bacilli, including *Acinetobacter spp.* (Dancer, 2009; Dancer, 2011; Donskey, 2013).

To confront the challenge of biofilm and surface attached cell removal, different approaches are available. Many studies suggested to use physical methods to remove detached bacteria and for effective removal of established biofilms and preventing the development of biofilms, studies also suggested to add detergent treatment with physical approach (Eginton et al., 1998; Mah and O'toole, 2001; Vickery et al., 2004). However, several studies evidenced that, detergent cleaning alone may not be sufficient to remove biofilms. (French et al., 2004; Vickery et al., 2004; Vickery et al., 2012).

Countless studies have been performed regarding the antimicrobial action and efficacy of different disinfectants. A review study by Rutala and Weber (1997) described the use of inorganic hypochlorite (bleach) in healthcare facilities for disinfection of medical devices and environmental surfaces and concluded chlorine as a disinfectant with fast microbiocidal activity with low cost rate and supported its continuous use in healthcare settings. In contrast, a recent study demonstrated that, in vitro 12-day *S. aureus* dry surface biofilm exposed to hypochlorite for 10 minutes reduced CFU by a factor of 7 $\log_{10}$, and biofilm biomass by a factor of 100; however, presence of live bacteria was evidenced from residual biofilm by observing under Confocal Laser Scanning Microscope (Almatroudi et al., 2016). This study also found regrowth and formation of biofilm by *S. aureus* on prolonged incubation suggested that at least up to 20,000 ppm, will not eradicate *S. aureus* if present in a dry-surface biofilm. A study on different strains of mycobacteria disinfection using sodium dichloroisocyanurate (SDIC), chlorine dioxide, 70% industrial methylated spirits, 2% alkaline glutaraldehyde, 10% succinedialdehyde and formaldehyde mixture, 0.35% peracetic acid and a peroxygen compound at 1% and 3%

showed that, SDIC based disinfectants were more effective with a conclusion of clinical strains were far more resistant to biocides than laboratory type strains (Griffiths et al., 1999).

The activities of different broad-spectrum biocides; Ethanol (vol/vol), hydrogen peroxide (HP), hypochlorite, peracetic acid) against on *S. aureus* and *P. aeruginosa* biofilms showed a significant decrease in *S. aureus* biofilm viability with sodium hypochlorite and peracetic acid within 1minute contact and some inhibitory effect on the matrix by HP and sodium hypochlorite was also observed. For *P. aeruginosa* biofilms, Peracetic acid showed highest efficacy on biofilm mass reduction within 1 min of contact and Isopropanol reduced > 99.99% *P. aeruginosa* viability after 30 min of contact (Tote et al., 2010). This study also clearly demonstrated that most disinfectants tested; Hypochlorite, Ethanol, Isopropanol, Benzalkonium chloride, Cetrimide, did not eliminate bacteria in the biofilm even after 60-min contact. Only hydrogen peroxide and chlorine had an impact on the biofilm matrix. A study on Benzalkonium chloride, triclosan and chlorhexidine efficacy against MRSA and *P. aeruginosa* 1-day biofilm found 100 to 1000 times greater MBCs for both MRSA and *P. aeruginosa* biofilms than their planktonic cells (Smith and Hunter, 2008). A disinfectant, Oxsil 320N, tested on 1-day biofilm of *E. coli, Enterococcus hirae, P. aeruginosa* and *S. aureus* showed approximately 10 times more concentrated Oxsil 320N is required to achieve a 5-log reduction for biofilm than planktonic culture (Surdeau et al., 2006).

Other studies have mentioned that, to kill clinically isolated planktonic yeast and its biofilm, 0.5% chlorhexidine is the best choice among several other antiseptics and surface disinfectants including betadine, hydrogen peroxide, sodium hypochlorite, alcohol and ultraviolet radiation (Theraud et al., 2004). A study on disinfection efficacy of several disinfectants used in hospitals against *Enterobacter sakazakii* in suspension, in dried form on stainless steel and in biofilm, stated that, this microorganism in dry and biofilm state cannot be killed by all biocides used in hospitals (Kim et al., 2007). Studies on sodium hypochlorite efficacy against *B. cereus* 8-day biofilm showed 5-log difference between planktonic cells and biofilm (Peng et al., 2002), *S. epidermidis and P. aeruginosa* 16-hours biofilm showed up to >1000-fold less susceptibility to biofilm than planktonic cells (Eginton et al., 1998)and no significant difference in *K. pneumoniae* 1-day biofilm and planktonic cell (Feipeng et al., 1993).

Different studies have established less susceptibility of biocides to mixed species biofilm. Study have shown 0.5% chlorhexidine is more active on both the planktonic and biofilm (single and multiple species) form of fungi than other antiseptics and surface disinfectants including betadine, hydrogen peroxide, sodium hypochlorite, alcohol and ultraviolet radiation (Theraud et al., 2004). Calcium hydroxide was tested on planktonic form of *E. faecalis, Streptococcus*

sobrinus, C. albicans, Actinomyces, Naeslundii, Fusobacterium nucleatum and 5-day mixed species biofilm developed with the same organisms found mixed species biofilm least susceptible after 10 and 100 minutes contact time than single-species biofilm and planktonic cells (Brändle et al., 2008). Similar test results were observed in a study where on the efficacy of isothiazolone compound was tested on planktonic, single species and mixed species biofilm of *Alcaligenes denitrificans, Pseudomonas alcaligenes, Stenotrophomonas maltophilia, Flavobacterium indologenes, Fusarium oxysporum, Fusobacterium solani, Rhodotorula glutinis* (Elvers et al., 2002).

It has been stated that a disinfectant will never be 100% effective against microbes due to the resistance of some bacteria to specific compounds and due to inefficient cleaning protocols (Talon, 1999). Surviving bacterial population can potentially regrow after removal of disinfectant. Moreover, viable spores such as, *C. difficile* (Guerrero et al., 2012) still attached to various materials can remain undetected by current sporicidal tests, resulting in overestimation of the sporicidal activity of sterilizing agents. The efficacy of various disinfectants has been evaluated by several authors against the most clinically relevant bacteria, pathogenic fungi and yeasts. From above review it is now clearly proved that, the concentration required to inhibit biofilm was considerably greater than the concentration required to inhibit growth of planktonic. However, as in most studies, biocides are only tested on wet biofilm, evaluating the impact of a biocide on dry surface biofilm is still require further study. Besides, more research is required to evaluate the prevalence and composition of biofilms in situ on hard and soft hospital surfaces and to optimize methods to tackle biofilms on hospital surfaces, which may include new cleaning and disinfection agents and adjuvants, new technologies (such as microfibre or automated room disinfection technology), and surface modification (Vickery et al., 2012).

Otter et al. (2015) suggested that, disinfectant alone or approaches to interfere quorum sensing can be effective against biofilm, but these procedures may not be able to eradicate biofilm completely because biofilm matrix hinders the biocides to reach microbes protected deep in the biofilm. The author also suggested that, methods, such as; using enzymatic digestion, targeting the biofilm matrix alone may help to reach the organisms within the biofilm matrix to interrupt persistence of the biofilm cells. But this procedure may not have the direct microbicidal activity. Therefore Otter et al. (2015) recommended to target both the microbes in the biofilm and the biofilm matrix simultaneously by applying oxidizing disinfectants or combination approaches to destroy microbes protected deep in the matrix and interrupt the persistence of the biofilm.

Therefore, biocides, those have the ability to reduce biofilm formation can be aided by using surface materials that do not readily support biofilm formation.

1.17 Cleaning

Cleaning is the removal of all foreign material (e.g., soil, organic material) from surfaces and objects, and is normally accomplished using water with detergents or enzymatic products (Rutala and Weber, 1999) . However, this process does not kill bacteria, which, under favourable conditions, can redeposit elsewhere and form biofilms. If it is stated that hospital is clean that means it looks clean. But study have showed that most surfaces looked clean where less than quarter of them were free from organic soil and less than half were free from microorganisms (Griffith et al., 2000). Consequently, cleaning must always precede disinfection and sterilization in order to eliminate infectious microorganisms (Rutala and Weber, 2004). Moreover, it has been already described that organic and inorganic soil can reduce or nullify the efficacy of disinfectants. Thus, before disinfection, surface cleaning has great significance as it has been evidenced that implementation of effective measure for environmental hygiene help to reduce the rate of healthcare-associated infections (Weber et al., 2013). This was exemplified in a study by Sitzlar et al. (2013), who described a reduced frequency of positive surface cultures of *C. difficile* after progressive terminal cleaning and disinfection interventions; monitoring of cleaning by fluorescent markers with feedback, use of a UV-C room disinfection device, and enhanced standard disinfection of *C. difficile* rooms including a dedicated daily disinfection team). Another study by Kundrapu et al. (2013) demonstrated that regular disinfection reduced acquisition of the pathogens on hands of HCWs from high-touch surfaces in rooms of patients with MRSA colonization or CDI. In a review Weber (2013) also demonstrated that after the implementation of methods to measure the effectiveness of room cleaning (e.g., use of fluorescent dye) with immediate feedback to environmental service personnel, cleaning efficacy was improved which consequently reduce the healthcare-associated infections.

In every hospitals, environmental surfaces are routinely cleaned, or cleaned and disinfected, according to programmed hospital cleaning policies (e.g., hourly, daily, twice weekly, and always after patient discharge) or when surfaces appear visibly dirty, such as; spillages, blood.(National Institute of Health and Clinical, 2011). When and how frequently cleaning needs to be done depends upon clinical risk, patient turnover, intensity of people traffic, and surface characteristics (Dancer, 2014). In hospital, disposable or reusable materials are used to lift soil by using neutral detergent. Study determined that >80% of the bacterial load from the hospital floors can be removed by detergent-based cleaning only (White et al., 2007). During

the mopping process, repeated use of same mop and rinsing with water increase the chances of surface contamination and situation become worst when surfaces are heavily soiled and/or have not been cleaned within the previous 24 h (Dancer, 2014) . The water then serves as a medium for spreading microbes around the environment. Thus, it has been suggested that mop should be routinely discarded in favour of fresh detergent solutions between bed spaces or every 15 min, whichever is sooner (NHS et al., 2009). NHS also guided to use ≥ one wipe or cloth for noncritical surfaces in one room or bed space except bathroom areas. Cleaning materials should always be kept separate for different ward surfaces.

1.18 Terminal (Deep) Cleaning

Terminal or deep cleaning is performed after the discharge of a patient colonized or infected with a specific pathogen (NHS et al., 2009). Disinfectants concentration used in this procedure depends upon the pathogen. Dancer SJ (2014) stated that disinfection methods may vary, but a terminal clean procedure typically includes initial removal of all detachable objects from the room, including bedding, screens, and/or curtains. After wiping over of lighting and ventilation components on the ceiling, curtain rails and the upper surfaces of highly placed fixtures and fittings should be wiped. After that all other surface areas should be cleaned downward to floor level. All the removed items and equipment that need to be replaced must wiped over with detergent cloths, alcohol wipes, or disinfectant before being replaced. A terminal clean also implies removal of curtains, drapes, and screens for laundering or cleaning; fixed blinds may be wiped over *in situ*. Unfortunately there is a sustained confusion between nurses and cleaning staffs who cleans what (Anderson et al., 2011). Therefore, a flexible approach in terms of responsibility between nurses and housekeeping services must be adopted to ensure that patient care is not compromised and that the environment and equipment are correctly cleaned without undue delay. In addition, Incorporation of less subjective methods of cleanliness assessment into routine monitoring of the health care environment is a crucial (Dancer, 2004).

1.19 Conclusion

It is now clear that the control of hospital infection must involve cleaning, disinfection and sterilization processes. Although planktonic organisms are readily killed, and significant number of biofilm cells are destroyed by the disinfection process, some biofilm cells remain. When choosing a disinfectant, numerous issues must be considered, such as its effectiveness, compliance with regulations, user acceptability, the types of surfaces and medical equipment, and the pathogenicity, infection rates and persistence of the microorganisms. A disinfectant must be safe, easy to use and effective against a wide range of pathogenic microorganisms and

should not leave toxic residues. Since the environment serves as an important reservoir for infectious organisms, the control of hospital infections is a matter of great concern and a major challenge. Therefore, a new strategy is required to control biofilm formation and development to provide a hospital with rich environmental hygiene which is an absolute requirement now a day. Three general methods are suggested to disinfect hospital environment for lessening the transmission of healthcare-associated pathogens:

- First is the improved cleaning and disinfection of room surfaces (Carling and Bartley, 2010; Dancer, 2011).
- Second is ‘no touch’ methods for terminal room disinfection; ultraviolet light (UV) or hydrogen peroxide (Otter et al., 2013; Rutala and Weber, 2013).
- Finally, use of currently proposed‘self-disinfecting’ surfaces; impregnating or coating surfaces with heavy metals (e.g., silver or copper), germicides (e.g., triclosan), or miscellaneous methods (e.g., light-activated antimicrobials) to reduce the bioburden on environmental surfaces (Weber and Rutala, 2013). Currently proposed self-disinfecting method provides continuous disinfection of environmental surfaces which does not depend on adequacy of cleaning/disinfection by environmental service workers. They have broad-spectrum antimicrobial activity and very low or no toxicity to humans.

Employing nanotechnology, has dramatically improved the development of self-disinfecting surface, has tremendous possibilities. Most importantly, implementation of self-disinfecting surface techniques might minimize the impact of poor cleaning and disinfecting practices during both routine and terminal room cleaning and disinfection.

Part Five: Challenges of creating and analysing *in vitro* dry surface biofilms

Development of in-vitro biofilm model has always been challenging. Many systems for "*in vitro* wet biofilm" development have been introduced. The only 12-dry biofilm (DSB) model has been established by Almatroudi (2015) in the CDC bioreactor with similar physical attributes to hospital surface dry biofilms. Numerous challenges had to face to establish *in vitro* 12-DSB to mimic the biofilm obtained from clinical settings with respect to appearance, thickness and biofilm composition.

Challenge to develop 12-DSB

1. Selection of biofilm strain. It is vital to choose appropriate strain as the species composition can vary depending on the environment of interest which influence bacterial adherence, bacteria-substrate interaction, and biofilm ultrastructure that need to be standardized to develop useful biofilm models for in vitro experiments (Baumgartner et al., 2008).

2. Problem encountered in DSB model is the reproducibility because the complexity of any experimental design mostly deals with the difficulty of reproducibility. Moreover, guideline for evaluating laboratory method usually include a list of desirable features such as repeatability and reproducibility (Feldsine et al., 2002) which must meet to achieve expected valid results.

3. Determination of accurate concentration of nutrition. Appropriate concentration of nutrition is undoubtedly the most important factor influencing the ability of bacteria to produce biofilm under in vitro conditions (Kennedy and O'gara, 2004)

4. Determination accurate time for interphase dehydration. Optimisation of accurate time of dehydration is crucial as extensive period of dehydration may kill the bacteria or decline the biofilm composition.

5. Picking a suitable surface as bacterial attachment varies with surface criteria (Donlan, 2002).

Challenge to analyse 12-DSB

1. The biggest challenge of analysing dry biofilm is to get it off from the attached surface. 12-day DSB is dried and hard enough to detach it even by curettage from the surface which leads to following difficulties

- Extraction of biofilm protein
- Extraction of DNA and RNA

2. Causes of reduced susceptibility to test reagents in DSB are mostly due to reduced penetration, particularly due to the production of thick EPS. This extreme penetration problem that faced by Almatroudi (2016) and this study is remarkable. To analyse detergent and disinfectant efficacy several test methods have been applied which took a lot to optimise the concentration of test reagents and duration of time period of different reagents. For example,

Live/dead stain: To determine the viability of biofilm bacteria, DSB was stained with Live/dead stain after detergent treatment for 60 minutes despite of having instruction of 15 minutes incubation period from manufacturer. This optimisation had to do to ensure the perfect penetration of the dye into the dry biofilm.

Crystal violet (CV) assay: Penetration of CV into dry biofilm is a great challenge. Hadi, Vickery et al. (2010) optimised the incubation period for 90 minutes for 48-hour wet biofilm which was increased up to 2 hours for 12-day DSB in this study, however, the results obtained was insignificant.

Part Six: Proteomics and genomics of biofilm formation

Little is known about the specific factors contributing to biofilm formation in clinical settings, but the bacterial proteome, particularly membrane proteins used in adhesion during biofilm formation and growth are of potential interest in the search for drug targets or biomarkers (Carlsohn et al., 2006).

Proteomics is the comparative identification of all expressed proteins under various circumstances. Proteomics analysis allows the detection of proteins, the functional entities of a cell, and of post-translational protein modifications, which cannot be predicted by mRNA expression analysis (Sauer, 2003). Information about biofilm proteomics is limited, which makes a thorough comparison between transcriptomes and proteomes difficult.

Bacteria express different protein at different stage of biofilm formation. A study on biofilm communities of *P. aeruginosa* by two-dimensional gel electrophoresis combined with reporter-gene analysis and microscopy showed that, *P. aeruginosa* displayed at least five distinct physiologies, where a large number of proteins were found to be differentially produced at different phases of biofilm development. Some of them were differentially expressed after one day of biofilm growth with maximum changes compared with the expression pattern in planktonic cells (Sauer et al., 2002). At each stage, most of the differently produced proteins found to be overexpressed, were involved in oxidative damage, production of exopolymeric substances, aerobic and anaerobic metabolism, and membrane transport. However, when biofilm bacteria revert back to the planktonic mode of growth, most of the differentially produced proteins are repressed. Similar distinct and reproducible protein patterns were observed in *Bacillus cereus* biofilms in its distinct phases of development (Oosthuizen et al., 2002).

1.21 Important protein responsible for Staphylococci biofilm development

Staphylococcus species, especially *S. aureus* and *S. epidermidis* are isolated most frequently from medical devices and other hospital settings due to their capability to grow as a biofilm (Mack et al., 2006). For Staphylococci biofilm, emphasis has been placed on the attachment and accumulation phase, mediated by different types of adhesins, more specifically, a group of surface-exposed proteins collectively referred to as MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) (Patti et al., 1994). MSCRAMMs are considered as the primary determinants responsible for the initial attachment to both native tissues and biomaterials, while poly N-acetylglucosamine (PNAG) also referred to as the

polysaccharide intercellular adhesin (PIA) mediates bacterial adhesion, leading to the accumulation of bacteria on solid surfaces (Götz, 2002; Lin et al., 2015). PIA/PNAG is a β-1,6-linked N-acetylglucosamine homopolymer and synthesized by enzymes encoded by the *ica* operon which consists of four genes (*icaA, icaB, icaC,* and *icaD*) (Lin et al., 2015). Other study also demonstrated the primary attachment of *S. aureus* and *S. epidermidis* is mediated by specific adhesins interacting with extracellular matrix components deposited on the surface (Rohde et al., 2007). In accumulative phase of biofilm formation most Staphylococci do not maintain any direct contact with the surface rather express intercellular adhesive mechanisms within the biofilm. One of the first factors described mediating biofilm accumulation in *S. epidermidis and S. aureus* is the PIA (Mack et al., 1996; Cramton et al., 1999; Lin et al., 2015).

On the contrary, several studies have demonstrated the PIA-independent biofilm formation in both *S. epidermidis* and *S. aureus* (Rohde et al., 2005; Shanks et al., 2005; Sun et al., 2005) and accumulation associated protein (Aap) was identified as a polysaccharide independent intercellular adhesin that mediates *S. epidermidis* biofilm formation (Rohde et al., 2005; Sun et al., 2005). The other important identified Staphylococci biofilm adhesive proteins are extracellular matrix binding protein (EmbP) *S. aureus* surface protein G (SasG). Phenol-soluble modulins (PMSs) *S. aureus* isolates have highly aggressive virulence potential (Peschel and Otto, 2013). PSMs has surfactant-like properties with the tendency to aggregate in oligomers, and have capacity to facilitate spreading on surfaces (Tsompanidou et al., 2013) or to structure biofilms (Wang et al., 2011; Periasamy et al., 2012) are under exceptionally strict and direct regulation by the Agr quorum-sensing system, which promotes strong enhancement of PSM production at high cell density (Cheung et al., 2011; Kretschmer et al., 2012). Recent research indicates that, in a probably receptor-independent manner and in the micromolar range, PSMs cause biofilm structuring and detachment, spreading on surfaces, and cytolysis (Peschel and Otto, 2013). In *S. aureus*, all PSMs have biofilm-structuring activities, indicating that they influence biofilm formation by sharing physico-chemical properties and also biofilm dispersal which is a key mechanism leading to the systemic dissemination of infections involving biofilms (Wang et al., 2007; Kretschmer et al., 2012).

1.22 Expression of genes in *S. aureus* biofilm

After initial characterization of a biofilm by Antoni van Leeuwenhoek the first specific genes that are up- or down-regulated in biofilm were made using transcriptional lacZ reporter-gene fusions (Dagostino et al., 1991; Davies et al., 1993) and this led to acceptance that gene expression changes after initial attachment that culminates in a biofilm phenotype (Costerton

et al., 1995). Biofilm bacteria come across the higher osmolarity conditions, greater oxygen limitation, and higher cell density within biofilm than in the liquid phase and all of these factors are known to influence gene expression in *S. aureus* to adapt this exceptional environmental condition (O'Toole et al., 2000; Novick, 2003). The availability of complete bacterial genome sequences and microarrays with which the expression of the entire genome of an organism can be assayed, has launched the postgenomic era of biofilm research and generated a wealth of additional information. But several DNA microarray studies revealed that no common expression pattern for biofilms has yet emerged. Instead, in different studies different genes are found up and down-regulated in varying numbers ranging from 1% to 38% of the total genome. For example, *P. aeruginosa* biofilm for PA4867, PA0971, PA2128, and PA1080, where about 34% of the 73 biofilm-regulated genes code for hypothetical proteins of unknown function (Whiteley et al., 2001) which is slightly lower then the overall percentage of such genes (44%) derived from the study by Stover et al. (2000) genome-sequencing project of *P. aeruginosa*. In *S. mutans* UA159, the brpA gene (for biofilm regulatory protein) was found to encode a novel protein of 406 amino acid residues (S chembri et al., 2003). A study on *E. coli* MG1655 biofilm global gene expression showed genes encoding proteins involved in adhesion (type 1 fimbriae) and in particular, autoaggregation (*Antigen 43*) were highly expressed (Wen and Burne, 2003). This apparent discrepancy may be due to a sensitive but transient snapshot of gene expression of that DNA microarrays produce and may not necessarily directly correlate with phenotype.

Most *S. aureus* strains comprise the entire *icaADBC* operon responsible for the production of PNAG (Fowler Jr et al., 2001; Rohde et al., 2001). Transcriptomic studies have shown that the *ica* operon in *S. epidermidis* has different phase variation (Ziebuhr et al., 1999), and many studies have specified the *ica* expression in both *S. epidermidis* and *S. aureus* subject to environmental regulation, perhaps most importantly, PNAG production in *S. aureus* is enhanced during in vivo growth. Subsequently one study has demonstrated the *ica* expression by the stress response transcription factor σB and anaerobic environment of biofilm interior supports to induce *ica* operon expression and PIA production in both *S. epidermidis* and *S. aureus* (Cramton et al., 2001). *icaR* encodes a transcriptional repressor (of the TetR family of transcriptional regulators), is located immediately upstream of the *ica* operon is vital for the environmental regulation of *ica* expression in *S. epidermidis*. However, in *S. aureus*, regulation of *ica* expression and biofilm forming ability also involve regulatory elements such as accessory gene regulator *(agr)* and the staphylococcal accessory regulator *(sarA)* other than sigma factor (the subunits of all bacterial RNA polymerases, required for gene transcription to occur, act as initiation factor that helps position the core polymerase to the promoter) σ^B and *IcaR* (Valle et al., 2003).

The *agr* locus encodes a two-component quorum-sensing system that modulates production of a regulatory RNA molecule (RNAIII) results in reduced production of surface proteins (e.g., MSCRAMMs) and increased production of exotoxins (Arvidson and Tegmark, 2001; Novick, 2003) is negatively correlated with biofilm formation (Vuong et al., 2003). This indicates that high level of *agr* expressing strains have a reduced capacity to form a biofilm (Vuong et al., 2003).

The *sarA* locus encodes a binding protein (SarA), used to respond to changing microenvironments, that is required under some growth conditions for maximum expression from the *agr* and RNAIII promoters (Heinrichs et al., 1996). This would suggest that *sarA* mutation would limit RNAIII production and consequently enhance the ability to form a biofilm. However, studies have found that mutation of *sarA* reduced the capacity to form biofilm as it also regulates other genes expression in an *agr*-independent manner (Blevins et al., 1999; Wolz et al., 2000; Dunman et al., 2001). One study found that, *sarA* mutation causes reduced transcription of the *ica* operon led to reduced capacity to produce PNAG and also suggested that SarA may promote biofilm formation in an indirect manner by suppressing transcription of a repressor of PNAG synthesis or a protein involved in the turnover of PNAG. *sarA* is a global regulator of gene expression in *S. aureus*, this suggests that other elements of the *sarA* regulon are also important in biofilm formation both in vitro and in vivo (Beenken et al., 2004).

Bacteria behave differently in different growth condition and undergo massive transcriptional changes. A study on global gene expression of *S. aureus* both in planktonic and wet biofilm condition by Beenken et al. (2004) reported that, in planktonic *S. aureus* expression of genes encoding protein A (*spa*), clumping factor B (*clfB*), collagen adhesion (*can*), coagulase (*coa*), and fibronectin-binding protein (*fnb*) were upregulated in the exponential versus stationary phase. In contrast, expression of the gene (*clfA*) encoding a second fibrinogen-binding protein (ClfA) was upregulated in the post-exponential phase. Expression of the secreted proteins was also elevated in stationary phase such as the genes encoding several cysteine proteases (*sspA*), the Clp proteinase (*sspB*), alpha-toxin (*sspC),* and the genes within the accessory gene regulator (*agr*) operon (*clpC, hla, RNAII, and RNAIII,*). These outcomes indicate that growth conditions reflect the transition between the different growth phases and is not dramatically altered by supplementation of the medium in a manner that promotes biofilm formation. The author also found that, in *S. aureus* biofilm genes were expressed in an altered fashion. The greatest distinction, in terms of overall numbers of differentially expressed genes, was between the biofilm and the exponential growth phase of planktonic culture while a significant number of genes were also differentially expressed in comparison to stationary-phase cultures. In biofilm

48 genes were identified whose expression was enhanced at least twofold in comparison to planktonic cultures such as arginine deiminase cluster (*arc*), a potassium-specific transport system (*kdp*), the pyrimidine biosynthesis operon (*pyr*;), and the urease operon (*ure*;). Interestingly, only one gene in the *ica* locus (*icaD*) was found to be significantly upregulated in a biofilm, and this was limited to the comparison between biofilms and the stationary phase of planktonic growth indicates that *ica* expression is associated with the initial colonization of bacteria but not with its persistence. Genes that were down regulated compared to the planktonic bacteria included oligopeptide transport system (*opp*) and the genes responsible for purine biosynthesis (*pur*). One well-defined gene that was drastically downregulated in biofilms was *spa*, the gene that encodes protein A. In addition to this author has reported differentially expressed genes in biofilms that were part of the *sarA* regulon. Genes positively regulated by *sarA* are *sdhB*, *carA*, an unidentified ORF and negatively regulated by *sarA* are *arc*, *phoP*, *pbp3*, *nuc*, *ndhG*, *spa*, and two hypothetical proteins.

Biofilms are not homogenous populations of cells. Genes identified in *S. aureus* biofilm in different studies may not be the absolute finding and there is a possibility that differentially expressed detect genes that are within the *sarA* regulon may not be detected. At the same time, there is every possibility that, the genes that were identified are either differentially expressed throughout the biofilm or only within more limited regions.

1.23 Conclusion

It is apparent that biofilms have gene-expression patterns that differ from those of planktonic bacteria. It is also clear that we still must decipher the genetic basis of biofilm formation. Much more work is also still needed if we are to completely describe the physiological changes that occur during biofilm formation. The detection of stage-specific physiologies and the display of multiple phenotypes during biofilm development may hold clues to the differences among the various DNA microarray analyses described so far. Until today no study was performed on dry biofilm genomics and proteomics. Information about dry surface biofilm is very limited and this makes it difficult to compare between transcriptomes and proteomes. However, given the different environmental conditions that dry surface biofilm is subjected to it could be expected that *S. aureus* dry surface biofilm might have a different genetic expression profile. Protein and transcriptional profiling of *S. aureus* during different modes of growth will increase our knowledge of biofilm physiology and may open a new era in biofilm related therapeutic and preventive strategies.

Part Seven: Detection of hydrated biofilm.

Methods of detection of the hydrated biofilm removal are quite varied and can be categorised on the basis of biofilm bacterial viability or biomass. Viability tests are usually sensitive and can be a measure of the potential for infection. Biomass measurement is a method where biofilm can be measured directly as they do not rely upon the physiological status of the cells in the sample.

1.34 Methods of biofilm detection

1.34.1Colony Forming Unit (CFU)

Colony Forming Unit (CFU) count is done to estimate viable bacterial load by culturing bacteria through a series of dilution steps. Several studies demonstrated different experiments on dry surface biofilm (DSB) where after *in vitro* development of 12-day DSB, efficacy of various detergents, disinfectants and heat against DSB was determined by CFU along with other microbiological and molecular process (Almatroudi, 2015; Almatroudi, 2016; Almatroudi, 2018). Hydrated biofilm was also experimented such as, treatment with ethanol (Chambers, 2006) followed by detection of CFU, zones of inhibition (Esterla, 2003) or minimum inhibitory concentration (Augustin, 2004). This however, tests an agent's ability to kill bacterial cell rather than to remove it. Depending on the treatment agent there may or may not be a correlation between reduction of colony counts and biofilm removal (Chen, 2000). *C. albicans* biofilm model in saliva was exposed to different antifungal agents at different growth stage. CFU was determined and found that the more mature the biofilm the more bacteria grew and showed more resistance to the drugs (Lamfon, 2004).

1.34.2 Protein detections assays

1.34.2.a Crystal violet assay

Crystal violet is the most common dye used and relies on staining the peptidoglycan component of cell walls. It also stains EPS. Crystal violet assay was performed in different studies on hydrated biofilm, where *E. coli* hydrated biofilm was grown on glass tubes and treated with (Loukili et al., 2004b; Loukili NH et al., 2004) different detergent-disinfectant solutions. The tubes were then rinsed with water and the residual biofilm was stained with crystal violet solution. After removal and rinsing of dye the adherent dye was removed with dimethylsulphoxide and absorbance of the extract measured by spectrophotometry and % reduction of biofilm compared to control was calculated. The advantage of spectrophotometry

in this method is a number is produced enabling quantitative comparisons of biofilm removal. This is an advantage over visual methods of estimating biofilm removal.

1.34.2.b Micro BCA assay

Protein detection by micro BCA assay is another method of estimating biomass. It is based on the assumption that biofilm grown under similar conditions will contain similar amounts of protein as a percentage of total biofilm. It also is based on the assumption that the more efficacious the cleaner, the more protein it will remove. The advantage of this method is that even if the structure of the hydrated biofilm is damaged by the collection process, it will still be included in the estimate. Both extra-cellular and intra-cellular protein are detected. A study by Hadi, Vickery et al. (2010) has detected the percentage reduction of biofilm with micro BCA assay after treatment with NaOH, sodium hypochlorite (0.01%--1%) etc.

Micro BCA Protein Assay is a detergent-compatible bicinchoninic acid formulation for the colorimetric detection and quantitation of total protein. The Micro BCA has been optimized for use with dilute protein samples (0.5-20µg/mL). Bicinchoninic acid (BCA) is the detection reagent for Cu+1, which is formed when Cu+2 is reduced by protein in an alkaline environment. A purple-coloured reaction product is formed by the chelation of two molecules of BCA with one cuprous ion (Cu+1). This water-soluble complex exhibits a strong absorbance at 562nm that is linear with increasing protein concentrations. The macromolecular structure of protein, the number of peptide bonds and the presence of four amino acids (cysteine, cystine, tryptophan and tyrosine) are reported to be responsible for colour formation with BCA (Wiechelman, 1988). The Micro BCA Protein Assay Kit uses concentrated reagents and a protocol that utilizes an extended incubation time at an elevated temperature (60°C, Test Tube Procedure only). The result is an extremely sensitive colorimetric protein assay in a test tube or microplate assay.

1.34.3 Imaging methods

Details about different microscopic procedure and image analysis was described in chapter 2; 2.5.

Observing biofilms under microscope provides a high degree of confidence that biofilm is actually formed on the samples. Scanning Confocal Laser Microscopy enables three-dimensional scanning of samples. It combines laser technology with fluorescent detection. When samples are prepared with live/dead stain, it has the advantage of differentiating viable

bacteria from non-viable, the disadvantage being that samples cannot be stored for later analysis and only a small section of the sample can be assessed at any one time.

In a study Davison et al. (2010) demonstrated that biofilm removal and biocidal activity of products are quite different (Davison, 2010). *S. epidermidis* hydrated biofilm was imaged under confocal laser scanning microscopy (CLSM) to observe the efficacy of chlorine, glutaraldehyde, a quaternary ammonium and nisin. The three-dimensional image of biofilm achieved by CLSM, provides accurate depth analysis of biofilm. Confocal images can also be scanned with colour detection software enabling quantitative analysis to be made (Merod, 2007). The images obtained by CLSM and epifluorescence microscopy can be converted into a grey scale and analysed to give percentages of biofilm coverage (Bredholt, 1999).

FISH or fluorescent in situ hybridisation of rRNA targeted probes may also be used to examine the biofilm structure (Donlan, 2005) with a camera that captures fluorescent images and are particularly useful when studying genetic factors at play within the biofilm. Atomic force microscopy enables imaging at nanometre resolution with no stains or coatings required. It enables proteins, not only the cells involved in biofilm development, to also be studied (Lindsay, 2006).

Scanning electron microscope (SEM) also an effective procedure to analyse biofilm microscopically. Number of studies have been applied SEM to observe different *in vitro* biofilm models. A study on ICU environmental samples were observed under SEM where 41 out of 44 samples (93%) were visually confirmed to have biofilm (Hu et al., 2015). A study on wound model developed with *P. aeruginosa* was assessed under SEM, CLSM and fluorescent microscope to determine the combined effect of pressure and betadin on it, found 100-fold increased biocidal activity (Ngo, 2007). A vascular *S. aureus* biofilm model was tested against various antibiotics and efficacy was detected by CFU and SEM (Edmiston Jr, 2006).

1.34.4 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) has been successfully used in the past to identify and quantify biofilm bacteria. However, there has been some difficulty in obtaining sufficiently high-quality DNA and RNA from biofilm samples owing to their matrix structure. However, boiling, phenolic chaotropic reagents, silica gel, and chemical extraction have been used successfully (Cury, 2008). A study on cost comparison between qPCR and culture techniques described them roughly equivalent, but qPCR is more rapid, quantitative, and specific technique which

detected some pathogens presented in a wound biofilm that were not detected by culture (Wolcott, 2008). In a retrospective study on evaluation of chronic wounds through culture and PCR showed that cultures identified 17 different bacterial genera, whereas molecular method identified 338 bacterial taxa (Rhoads, 2012). A quantitative PCR (qPCR) method has been shown effective for the detection of *Mycobacterium. avium* subspecies, *paratuberculosis* biofilm in drinking water collected from the United States (Beumer, 2010). A study by Hu et al. (2015) has also shown the detection of dry surface biofilm from the ICU samples by using qPCR method.

1.35: Conclusion

Implementation of different methods for the detection of hydrated biofilm might be useful for diagnosis and treatment. However, using the same methods for the detection of DSB is not always fruitful. Therefore, the future will require standard methods to identify and to determine the level of efficacy of disinfectants and detergents against DSB.

Part eight: Hypothesis, Aims and Objectives

Biofilms had never been identified on dry hospital surfaces until discovered by Vickery et al. (2012). Hence, detergents and disinfectant formulation had been aimed at removing planktonic bacteria and only hospital grade disinfectant efficacy testing to be conducted against planktonic organisms. However, studies have demonstrated that, human pathogens can survive incorporated into single and mixed species biofilms on dry surfaces that necessitates a reassessment of this standard. However, a major obstacle to developing better detergents is that the composition and spatial relationship of different bacteria in hospital environment biofilm remains unknown as the EPS composition and its link to the underlying substrate varies between bacterial species.

The objective of this present study is to break the "linkage bond" between the bacteria and the substrate and peel the biofilm off the surfaces and to reduce the risk of HAI by improving environmental cleaning and disinfection by optimising and comparing different cleaning methods.

1.36 Study hypothesis

We hypothesise that:

1. Pathogens in DSB play a significant role in the endemic transmission of healthcare-associated infections. This study aims to determine if DSB can be transferred from hospital surface to healthcare worker's hands.
2. Failure of current detergents and disinfectants to remove dry surface biofilm from the hospital surfaces are the principal reason for continued MRO isolation following hospital cleaning.
3. Growth within multispecies biofilm aids more persistence of bacteria on dry environmental surfaces as they are highly protected from desiccation, detergents and disinfectants actions.
4. Proteomics analysis for exploring the biofilm complex protein composition to identify a target protein that might help to develop an effective detergents and disinfectants in future.

1.37 Aims and Objectives

1. Transmission testing of dry surface biofilm from hospital environment to healthcare worker's gloves and bare hands to determine if bacteria can be transmitted from dry surface biofilm (DSB) to healthcare worker's hands
2. Development of new removal chemistries and efficacy testing of removal chemistries using the original dry surface model; optimisation and comparison of methods.
3. Testing the effect of organic and inorganic soil on the disinfectant's efficacy.
4. Develop a mixed species biofilm model analogous to the properties of mixed species dry biofilms obtained from hospital surfaces to
    a. Determine composition (protein, carbohydrate) dry multispecies biofilms.
    b. Analysis of multispecies biofilm by transcriptomic molecular technique to explore and characterize the complex communities' genetic expression.
    c. The effect of multiple species on biofilm's susceptibility to detergents and disinfectants
4. Investigate protein regulatory changes in DSB compared with traditional hydrated biofilm.

Chapter Two: Materials and methods

This chapter includes methods and in vitro model development of DSB.

Part One: Growth of single species dry surface biofilm

2.1 Bacterial Strain used

Staphylococcus aureus ATCC 25923.

Enterococcus faecium, ATCC 35667

Acinetobacter baumannii, ATCC 19606

Pseudomonas aeruginosa ATCC 25619

2.2 *S. aureus* culture preparation

2..2.1 Short term maintenance and growth

One loopful of *S. aureus* was taken from a vial stored at -80°C (Mazur, 1970) freezer after sufficient amount has been thawed and streaked onto a horse blood agar plate (HBA) (Micro media laboratories, Australia) and incubated overnight at 37^{0}C to generate working bacterial culture. The vial was frozen as quickly as possible. Working bacterial agar plates were sealed with parafilm and are stored at 4^{0}C for a maximum of 2 weeks. To prepare an experimental liquid culture, a colony of bacteria *S. aureus* was aseptically removed from the working agar culture plate and inoculated into 250ml glass bottle containing 100ml of sterile 100% tryptone soya broth (TSB) (Oxoid, Threbarton, Australia) and incubated at 37^{0}C, with constant rotational speed of 120 rpm in an Innova 42^{TM} shaker incubator (John Morris Scientific, Australia) overnight.

2.2.2 Long and short term bacterial storage

For storage, a single pure colony was taken from a fresh overnight culture plate and inoculate into 25 ml of TSB and incubated at 37^{0}C, at a constant rotational speed of 120 rpm in an Innova 42^{TM} shaker incubator. An 850 µl aliquot of bacterial culture was then transferred to a sterile microcentrifuge tube and mixed with sterile 20% glycerol. The tube was then preserved at -20^{0}C for short term and at -80^{0}C for long term storage.

2..2.3 Colony forming unit counting

The culture was diluted with sterile 100% TSB to an optical density of 0.3 at 600 nm wavelength (V- 1200 Spectrophotometer, VWR ^{TM}, Belgium) which was equivalent to approximately 10^{8}

CFU/ml for use in experiments. The exact number of bacteria was determined by serial 10-fold dilution in sterile PBS until dilution 10^{-6}. A 100 µl aliquot of each 10^{-4}, 10^{-5} and 10^{-6} dilution were inoculated onto HBA plates using disposable spreader and incubated at 37^{0}C overnight. The number of CFU was counted in plates with between 30 to 300 colonies (Jackie, 2005) and the original bacterial concentration was calculated from the count obtained from the dilution factor (Mahon et al., 2014; Murray et al., 2015).

2.3 Growth protocol for biofilm

2.3.1 12-day Dry surface biofilm (DSB)

DSB was developed in the CDC bioreactor on polycarbonate and glass coupons (BioSurface Technologies Corporation, US) (Figure 2-1) over 12 days. Each polycarbonate coupon was 1.27 cm diameter, 0.3 cm thick and glass coupon was 1.27 cm diameter, 0.28 cm thick. The CDC bioreactor consisted of a one-litre glass vessel, with a side arm positioned to provide approximately 700 ml operational fluid capacity. A polyethylene top supported eight independent and removable rods. Each rod held three removable coupons (biofilm growth surfaces) for a total of 24 sampling opportunities. Prior to use the CDC bioreactor with coupons was autoclaved to ensure sterility. Biofilm was developed with periodic nutrition interspersed with long periods of dehydration as would be expected for biofilm formed on hospital surfaces.

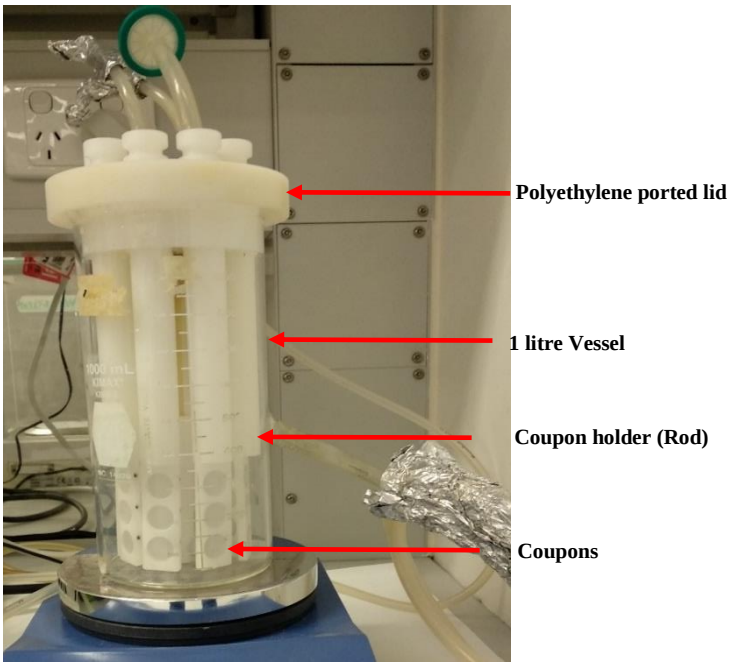

Figure 2- 1:

Centre for disease control bioreactor (CBR)

1 ml of liquid culture of *S. aureus* (10^8/ml) was added to the CDC bioreactor containing sterile 5% (w/v) TSB. During development of the biofilm, filter-sterilised air-conditioned air was pumped into the biofilm generator continuously in order to dehydrate the biofilm. The generator was placed on a digitally controlled stir plate to provide constant rotation of the stir bar at 130 rpm which provided constant mixing and consistent shear to the coupon surfaces. All batch phases were incubated at 35ºC and subjected to shear (baffle rotation at 130rpm). The biofilm went through hydration and dehydration cycles as following (Almatroudi et al., 2015);

Batch phase

First phase

After the above settings, CDC bioreactor (CRB) was left for growing for 48 hrs with 130 rpm stirring at 35ºC. Then, the media was drained out and the CDC biofilm reactor was left on the bench at room temperature for 48 hrs.

Second phase

After 48 hours, 500 ml of 5% (w/v) of fresh sterile TSB was added to CRB for 6 hrs. After that, the media was drained out and the biofilm reactor was left on the bench at room temperature for 66 hrs.

Third phase

After 66 hours of dehydration, 5% (w/v) of fresh sterile TSB was added to CRB for 6 hrs. Subsequently, the media was drained out and the biofilm reactor was left on the bench at room temperature for 42 hrs.

Fourth phase

After this dehydration phase, again 5% (w/v) of fresh sterile TSB was added to CRB for 6 hrs. Next, the media was drained out and the CRB was left on the bench at room temperature for 66 hrs.

Table 2- 1:

Culture condition for formation of 12-day dry surface biofilm (DSB). Initial inoculum of about 10^8 of *S. aureus* was added at the beginning of stage -1 phase.

Phases	Culture conditions	Cumulative operating time
1	48 hr batch phase in 5% TSB followed by 48 hrs dehydration	96 hours
2	6 hr batch phase in 5% TSB followed by 66 hrs dehydration	168 hours
3	6 hr batch phase in 5% TSB followed by 42 hrs dehydration	216 hours
4	6 hr batch phase in 5% TSB followed by 66 hrs dehydration	288 hours

2.3.2 12-day Wet biofilm (WB)

WB also developed on removable coupons polycarbonate coupons (Bio Surface Technologies Corporation) in a CDC biofilm reactor. *S. aureus* was grown in 50% TSB under shear at 37°C in batch phase for 48 hours followed by 20% TSB media exchange every 48 hours for 12 days as shown in table 2-3.

Table 2- 2:

Culture conditions for formation of 12-day wet biofilm (12DWB) with an initial inoculum of about 10^8 CFU of *S. aureus*, at the beginning of Stage-1 batch phases.

Phases	Culture conditions	Cumulative operating time
1	48 hr batch phase in 50% TSB	48 hours
2	48 hr batch phase in 20% TSB	96 hours
3	48 hr batch phase in 20% TSB	144 hours
4	48 hr batch phase in 20% TSB	192 hours
5	48 hr batch phase in 20% TSB	240 hours
6	48 hr batch phase in 20% TSB	288 hours

Part two: Standard assays for biofilm studies

2.4. Standard protein assay

Standard curve for all assay (experiment) was performed individually in each episode of experiment. The equal number of replicates were used for each standard curve, as the number of replicates were used in each experiment.

2.4.1 Crystal violet (CV) assay

Crystal violet stains organic matter such as proteins, carbohydrate (CHO) and DNA and has been used to measure residual biofilm and is a commonly used assay for measuring biofilm mass (Broschat et al., 2005). The majority of biomass is protein. Thus, CV was used to measure protein by following procedure:

Preparation of 0.3% CV solution

200 ml of 19% ethanol and 30 ml of 2.3% crystal violet solution (Sigma-Aldrich Inc, USA) was mixed well to prepare 0.3% CV solution.

Assay procedure:

The amount of residual protein on sample was assessed by placing the samples in 0.3% crystal violet solution for 90 minutes at room temperature (Hadi et al., 2010). Excess crystal violet was removed by rinsing sequentially in two lots of sterile miliQ water, one minute each rinse. Coupons were left to air dry on the bench for approximately 10 minutes and then placed individually into 1ml of 95% Ethanol (EtOH, v/v), to elute the crystal violet solution. The absorbance of the eluted samples was measured by spectrophotometer at 590 mm wavelength and the amount of protein was calculated from the standard curve (see figure 2-2).

Preparation of Crystal violet standard curve.

A standard curve for each crystal violet experiment was also constructed by serially diluting the crystal violet solution in 95% ETOH. The first sample was diluted 1:50 with subsequent cuvettes diluted 1:2 a further 9 times. The absorbency of the 10 dilutions was measured at 540nm. Standard curves were prepared using five replicates for each concentration.

Crystal violet concentration (µg/ml)	0.0234	0.0117	0.0059	0.0029	0.0015	0.000
Absorbance (OD)	4.655	2.381	1.177	0.583	0.266	0.000

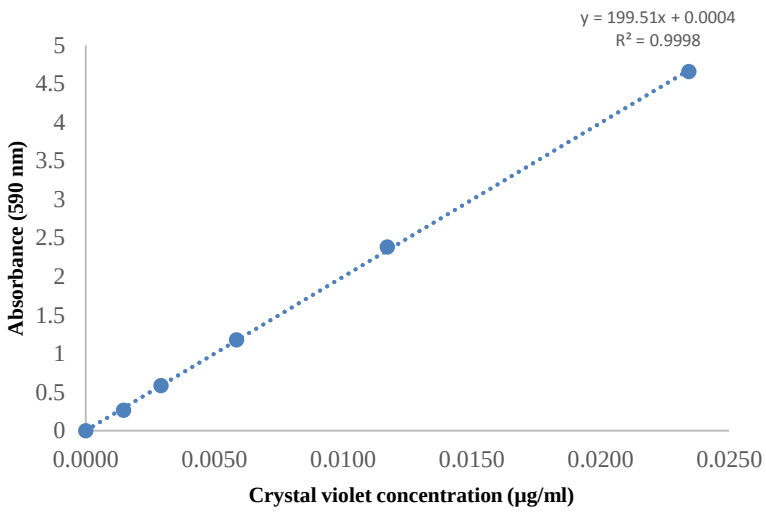

Figure 2- 2:

Crystal violet standard curve for calculating the amount of CV (µg/ml) eluted from samples on the basis of the optical density at 590nm wavelength.

2.4.2 Bicinchoninic (BCA) Protein assay

BCA assay is compatible with many detergent formulations and is hence suitable for measuring the amount of protein left on samples after detergent and/or disinfectant treatment. This assay is used for colorimetric detection and quantitation of total protein. It combines the reduction of Cu+2 to Cu+1 by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of cuprous cation (Cu+1) using a reagent called BSA (Smith et al., 1985).

Assay procedure:

The BCA assay (Pierce^{TM} BCA protein kit assay, Thermofisher, Waltham, USA) reagent solutions was mixed at 1:50 ratio and added to the samples in sufficient amount to cover it. Samples were then incubated at 60°c for 30 minutes. After incubation samples were then kept at room temperature for 10 minutes to cool down. The absorbance at 562 nm wavelength was analysed and the concentration of protein was detected from the standard curve (see Figure 2-3). Standard curves were prepared using five replicates for each concentration.

Preparation for BCA standard Curve

Serial dilution of albumin (BSA) for standard curve			
Vial	**Volume of Diluent or MiliQ water (µl)**	**Volume and Source of BSA (µl)**	**BSA Concentration (µg/ml)**
A	0	200 µl of Stock (1mg/ml)	1000
B	40 µl	160 µl of Stock (1mg/ml)	800
C	80 µl	120 µl of Stock (1mg/ml)	600
D	120 µl	80 µl of Stock (1mg/ml)	400
E	160 µl	40 µl of Stock (1mg/ml)	200
F	100 µl	100 µ**L** of vial E dilution	100
G	100 µl	100 µ**L** of vial F dilution	50
H	200 µl	0	0=Blank

Protein concentration (µg/ml)	1200	800	400	200	100	50
Absorbance	0.57	0.389	0.205	0.124	0.062	0.039

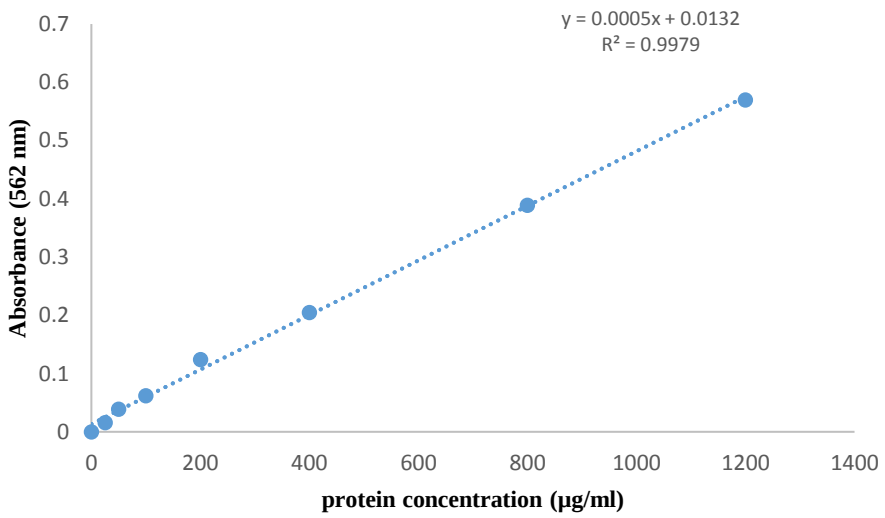

Figure 2- 3:

BSA standard curve for calculating the amount of protein (µg/ml) from samples on the basis of the optical density at 590nm wavelength.

2.4.3 Coomassie (Bradford) Protein Assay

Coomassie (Bradford) Protein Assay (Thermo Scientific™, USA) coomassie-binding, colorimetric method for total protein quantitation. In this procedure, when coomassie dye binds protein in an acidic medium, an immediate shift in absorption maximum occurs from 465nm to 595nm with a concomitant colour change from brown to blue. This assay can be performed in either test tube or microplate.

Microplate Procedure

250µL of the Coomassie Reagent and 5µL of sample was added into each microtiter well plate (Thermo Scientific™ Pierce™ 96-Well Plates) and mixed with plate shaker for 30 seconds. Plates was then incubated for 10 minutes at room temperature. Protein concentrations were estimated by reference to absorbances obtained at 595nm for a series of standard protein dilutions, which are assayed alongside the test samples (see figure 2-4). The colour response with coomassie is non-linear with increasing protein concentration, thus, standard curve has been completed with each assay using five replicates for each concentration.

Preparation of standard Curve for Bradford assay

Preparation of Diluted Albumin (BSA) Standards

Vial	Volume of Diluent or MiliQ water (µl)	Volume and Source of BSA (µl)	BSA Concentration (µg/ml)
A	0	300 µl of Stock (1mg/ml)	2000
B	125 µl	375 µl of Stock (1mg/ml)	1500
C	325 µl	325 µl of Stock (1mg/ml)	1000
D	175 µl	175µL of vial B dilution	750
E	325 µl	325µL of vial C dilution	500
F	325 µl	325µL of vial E dilution	250
G	325 µl	325µL of vial F dilution	125
H	400 µl	100µL of vial G dilution	25
I	400 µl	0	0=Blank

Protein concentration (µg/ml)	1000	800	600	400	200	100	50
Absorbance	0.6395	0.492	0.401	0.306	0.173	0.088	0.077

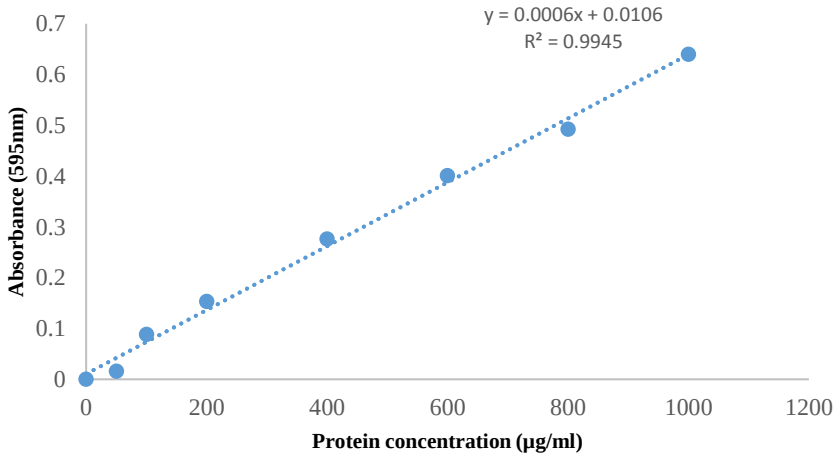

Figure 2- 4:

BSA standard curve for Bradford assay to calculate the amount of protein (µg/ml) from samples on the basis of the optical density at 595nm wavelength.

2.5 Microscopic analysis

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

2.5.1 Confocal laser scanning microscopy

Biofilm is a three-dimensional structure. To examine intact biofilm and the cellular interaction with in biofilm, imaging of their spatial organization is required. This can be achieved by CLSM (Lawrence et al., 1991; de Beer and Stoodley, 1995; Lopez et al., 2005) as it offers non-invasive three dimensional imaging of thick sections and provides horizontal and vertical optical sectioning (Lawrence et al., 1991). CLSM can give horizontal sections up to ~ 0.2 µm thin slide. Various stains have been used to study biofilm properties under CLSM (FV 1000 MCPSU, Olympus, Japan). Many of them are discussed below.

2.5.1.a EPS staining

EPS analysis has considerable influence in developing targeted removal strategy. However, extraction of EPS by chemical method; NaOH and physical method; heat and sonication might lyse bacterial cells and hence contaminate the sample with bacterial cytoplasmic contents. Therefore, a variety of *in situ* methods based on the application of chemical probes has been used in this study. EPS components are diverse. For imaging and analysis, it requires each set of biochemical compounds to be targeted independently.

Lectin: Lectin are non-enzymatic carbohydrate binding protein. In situ procedure using Lectin, facilitates the analysis of three dimensional glycoconjugate in biofilm as different lectins bind to different CHO moieties; preferentially to fucose linked (α -1,6) to N-acetylglucosamine or to fucose linked (α -1,3) to N-acetyllactosamine related structures. AAL also reversibly binds fucose attached to nucleic acids. In this study CHO staining was undertaken with Alexa-488-labelled *Aleuria aurantia* lectin (Vector laboratories, Burlingame, CA), a fructose binding lectin (excitation/emission:495/515).

Protein stain: Several studies have undertaken EPS protein staining with Sypro orange stain as it strongly binds with EPS protein in the biofilm matrix. In this study Sypro orange (Molecular probe, Invitrogen, USA) was applied (excitation/emission:485/590).

Nucleic acid stain: DNA plays a significant role in biofilm composition. Biofilm DNA can be stained with range of Biofilm DNA stain such as SYTO 63, SYTO 84 and SYTO 60. In this research SYTO 60 (Molecular probe, Invitrogen, USA) was chosen as it best suited the available CLSM filters (excitation/emission:652/678).

EPS staining protocol (Neu et al., 2002):

All staining procedure were performed in the dark at room temperature as follows.

1. Samples were fixed with 4% paraformaldehyde for 60 minutes at 4°C.
2. Samples were washed with 1M PBS for 10 minutes each wash
3. Glycoconjugates of biofilm was stained using 1:10 dilution of Alexa-488-labelled *Aleuria aurantia* lectin for 20 minutes and washed three times. The excitation maximum is at 495 nm and the emission maximum is at 515 nm.
4. Protein were stained using 1:1000 dilution of Sypro Orange in water for 15 minutes. Samples were washed for three times. The excitation maximum is at 485 nm and the emission maximum is at 590 nm.
5. Nucleic acid was stained with 1:1000 dilution of SYTO-60 (Molecular probe, Invitrogen, USA) in water for 5 minutes. Samples were washed for three times. Excitation/Emission (nm): 652/678

After staining all samples were observed under microscope (CLSM) within 24 hours (see Figure 2.5).

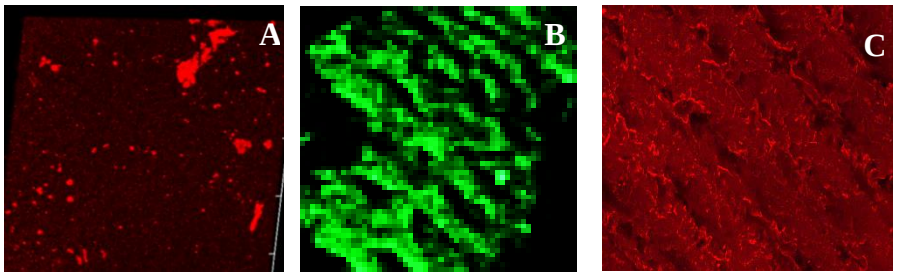

Figure 2- 5:

CLSM image of stained dry surface biofilm with SYTO 60 demonstrating biofilm DNA (A), Lectin showing glycoconjugates (B) and Sypro orange showing protein (C) stain.

2.5.1.b Bacterial viability staining-Live and dead stain (backlight 7012)

Bacterial viability tests performed with Live and dead stain, a mixture of SYTO^{TM} 9 green fluorescent nucleic acid stain and propidium iodide (PI) the red fluorescent nucleic acid stain, based on the detection of membrane integrity. SYTO^{TM} 9 enters live and dead bacterial cells and stains bacteria with intact cell membrane give fluoresce bright green, whereas bacteria with damaged membranes exhibit significantly less green fluorescence. The fluorescent signal of SYTO9 is strongly enhanced when bound to nucleic acid and shows low intrinsic fluorescence signal when unbound. PI penetrate only cells with disrupted membranes and intercalates to DNA (red) and is generally excluded from viable cells. When mixed in recommended proportions, the background remains virtually non-fluorescent, consequently, the ratio of green to red fluorescence intensities provides a quantitative index of bacterial viability.

In this study, staining procedure was performed in the dark room utilising the manufacturer's proportions but increasing the incubation steps to ensure that the dyes had sufficient time to permeate the biofilm.

1. Dilute Live/dead stain component A (1.5 µl: 500µl ratio) and component B (1.5 µl: 500µl ratio) with sterile water. Component A and B were then mixed together and prepared for staining.
2. Apply sufficient amount of stain to cover the samples and incubated at room temperature for 60 minutes. According to manufacturer instruction the incubation period is 15 minutes. However, as dry biofilm is hard to penetrate due to thick EPS, the incubation period for 60 minutes were optimised in this study.
3. After staining samples were fixed with 4% paraformaldehyde for one hour.

4. Rinse with PBS for three times for ten minutes each and left in final PBS wash at 4ºC covered by aluminium foil until imaging.
5. Samples were then observed under CLSM within 48 hours of staining at excitation/ emission 480/500 for SYTO 9 and 490/635 for propidium iodide (see Figure 2.6).

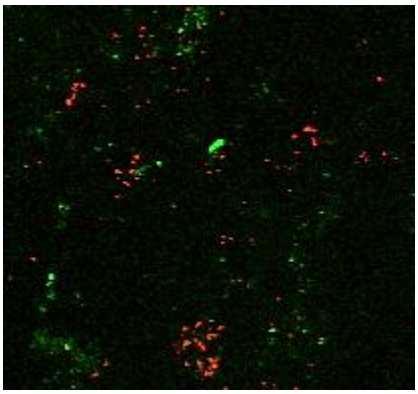

Figure 2- 6:

CLSM image of stained dry surface biofilm with Live/dead stain (backlight 7012)

2.5.1.c Quantitation of Total Protein using o-phthaldialdehyde(OPA)

Total protein content is a measurement common to many applications in basic science and clinical research. Several different fluorescent techniques are available that eliminate many of the problems associated with the traditional absorbance-based colorimetric methods to measure total protein content. Protein quantification from dry surface biofilm is a challenge as it has strong protective structure and that act as a barrier for protein analysis. In this study we have used the compound o-phthaldialdehyde (OPA) to quantitate total protein using the BioTek FL600 fluorescence microplate reader.

Aim:

To verify whether the technique of OPA staining is suitable for detection of residual protein after detergent treatment of dry surface biofilm.

Fluorescence determination Procedure (microtiter plate):

OPA reagent solution (BioTek, Australia) was made by the addition of 0.2 ml of ßmercaptoethanol to 100 ml of OPA reagent solution (incomplete) immediately prior to use. A series of dilutions ranging from 0.0 to 1000 µg/ml of Bovine Serum Albumin (BSA) were made using phosphate buffered saline (PBS) pH 7.4 as the diluent for standard (see figure 2-7). Samples were obtained from detergent (Surfex) treated biofilm coupon. After sonication and

vortexing of treated coupon protein was extracted by hydrolysis method. Samples (five replicates) and standards (five replicates for each concentration) were placed in microplate wells (150 µL per well) and 150 µL of OPA reagent solution was added to each well. Samples were allowed to incubate for 2 minutes with moderate shaking at room temperature and the fluorescence was determined with a 360 nm, 40 bandwidth excitation filters and a 460 nm, 40 nm bandwidth emission filter. The sensitivity setting was at 38 and the data collected from the bottom with a 5-mm probe using static sampling with a 0.35 second delay, 50 reads per well. Individual results are shown in Table 2-2.

Preparation of Diluted Albumin (BSA) Standards

Vial	Volume of Diluent or MiliQ water (ml)	Volume and Source of BSA (ml)	BSA Concentration (µg/ml)
A	4.5	0.5 ml of Stock	200
B	8.0	2.0 ml of vial A dilution	40
C	4.0	4.0 ml of vial B dilution	20
D	4.0	4.0 ml of vial C dilution	10
E	4.0	4.0 ml of vial D dilution	5
F	4.0	4.0 ml of vial E dilution	2.5
G	4.8	3.2 ml of vial F dilution	1.0
H	4.0	4.0 ml of vial G dilution	0.5
I	8.0	0	0=Blank

Protein concentration (µg/ml)					200	40	20	10	5	2
Fluorescence	129306	47805	32125	25729	23092	21217	21130	20638	20344	

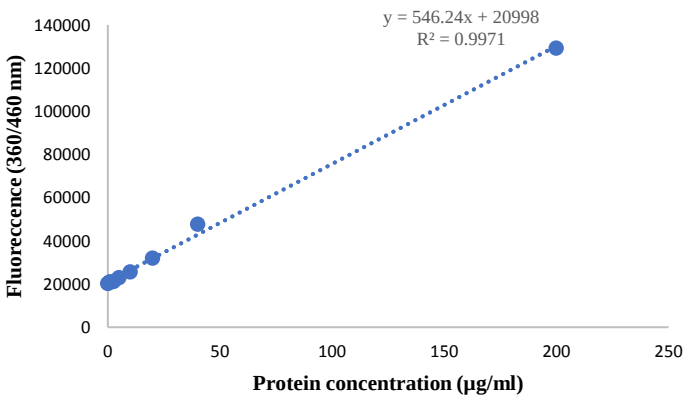

Figure 2- 7:

BSA standard curve for OPA assay to calculate the amount of protein (µg/ml) from samples

Table 2- 3:

Fluorescence values of OPA assay with 72-hour wet biofilm.

Samples	Fluorescence	
	Reading	**Corrected fluorescence**
Treated with Surfex for 10 minutes	94913	-21172
Treated with Surfex for 10 minutes	93667	-22418
Treated with Surfex for 10 minutes	100295	-15790
Treated with Surfex for 10 minutes	94159	-21926
Treated with Surfex for 10 minutes	93164	-22921
Treated with Surfex for 10 minutes	94052	-22033
Treated with Surfex for 10 minutes	99314	-16771
Positive control	108784	-7301
Positive control	99314	-16771
Blank (only (OPA)	116085	0

Conclusion:

Use of OPA reagent with biofilm gave negative fluorescence values when corrected for background fluorescence from the OPA reagent itself, rendering this method unsuitable for future work.

Fluorescence determination Procedure by staining of coupons with OPA reagent

Surfex treated biofilm coated coupons were then stained with one ml OPA reagent for 60 minutes. Washed with mili Q water three times for two minutes for each wash and observed utilising the fluorescence microscope (see figure 2.8).

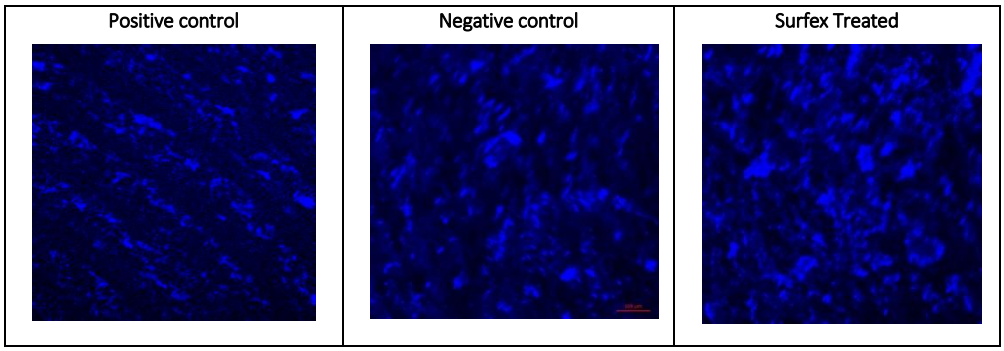

Figure 2- 8:

Biofilm coated coupon observed under fluorescence microscope

Conclusion:

Staining of coupons with OPA reagent and then visualising under fluorescence microscope showed fluorescence was present in treated, positive and negative control coupons rendering this method unsuitable for future work.

2.5.2 Scanning electron microscope

Scanning electron microscopes have high resolution that helps to visualize sample at µm scale (Priester et al., 2007). Sample preparation for scanning electron microscopy utilising a JSM6480LA (JEOL, Tokyo, Japan) requires extreme dehydration. This level of dehydration is highly likely to affect the structure of biofilm EPS as EPS is mainly composed of water. Our samples were sputter coated with a conductive coating which increases resolution and helps to conserve the cellular feature and structure (Priester et al., 2007).

2.5.2.a Biofilm Fixation/HMDS protocol for Scanning Electron Microscopy (SEM)

Samples were fixed overnight in 3% glutaraldehyde followed by 3 washes of 0.1M phosphate buffer prior to serial ethanol dehydration and hexamethyldisilazane (HMDS) treatment followed by gold plating. The following protocol (Vickery et al., 2012) was used in this study to prepare sample for SEM.

- Harvested samples were fixed by completely covering with 3% glutaraldehyde and stored in fridge or at room temperature for 24-48hs.
- Samples were washed, 10min for each wash, with 0.1M of Phosphate buffer solution (see it in appendix) thrice.
- Samples were dehydrated with increasing concentrations of ethanol. The samples were placed in each ethanol concentration for 10 minutes. The dehydration process

commenced with a starting ethanol concentration (v/v) of 30%, and proceeded through the following concentrations 50%, 70%, 80%, 90% and concluded with two treatments with 100% v/v.

Alternatively, samples were critically point dried following ethanol dehydration

Samples were then washed with 1:1 100% ethanol / HMDS (Sigma-Aldrich Co, USA) for 10 min followed by three more washes with HDMS for 10 minutes each.

After removal of all HMDS, samples were air dried in fume hood over 24-48 hrs until dry.

2.5.2.b Observation under SEM

After dehydration specimens were mounted on carbon tabs (ProsciTech, Thuringowa, QLD, Australia). Specimens were coated with 20nm gold using Emitech K550 gold coater (West Sussex, England) and observed utilising a JSM6480LA, scanning electron microscope (JEOL, Tokyo, Japan) at an accelerating voltage of 10KV and spot size 30. Samples with bacteria attached to the coupon surface and encapsulated with EPS were considered as biofilm positive (see figure 2.9).

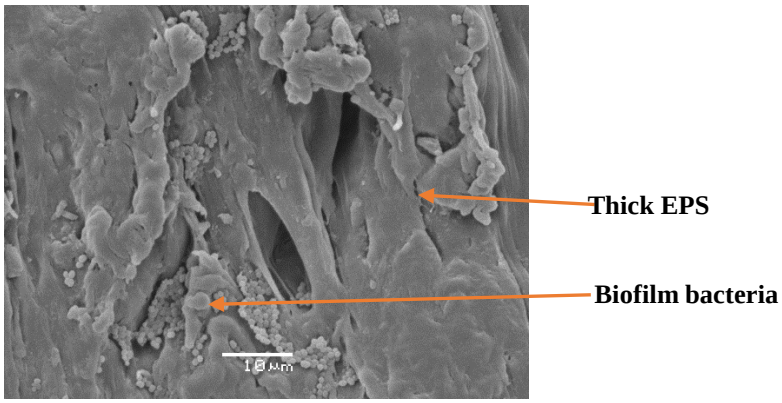

Figure 2- 9:

Scanning electron microscope images showing polycarbonate coupon covered with biofilm. Picture showing the aggregation of biofilm bacteria with thick EPS.

2.6 Biofilm image analysis

To analyse biofilm images obtained during CLSM, Imaris (Bitplane, Zurich, Switzerland) and Image J software (1.46r, National institute of health, USA) were employed for this study. The former was applied to determine biofilm structure; thickness and biomass and the later one was used to determine bacterial viability and EPS composition.

2.7 Colony forming unit (CFU) determination

To ensure that a similar amount of biofilm had grown for each experiment and biofilm generator, the number of viable bacteria; CFU, was determined on a minimum of three coupons by serial dilution and standard plate culture. After development of DSB in the CDC bioreactor, biofilm coated coupons were unscrewed from the rods and washed gently by immersing into sterile 1M PBS to remove loosely attached planktonic bacteria. Coupons were then placed into 2 ml of PBS and subjected to sonication for 20 minutes and vortexing for two minutes. Serial 10-fold dilution were made by adding 1 ml of sonicated fluid into 9 ml of PBS in a test tube and vortexed to mix well. The exact number of bacteria was determined by spreading 100 µl aliquot of the following dilutions 10^{-3},10^{-4}, 10^{-5} and 10^{-6} onto individual HBA plates, using a disposable spreader, and incubating at 37^{0}C overnight. The number of CFU was counted on plates containing between 30 to 300 colonies (Figure 2-10) and the bacterial concentration was calculated using the following formula:

$$\text{CFU/ml} = \frac{\text{Number of colonies X Dilution factor}}{\text{Volume of culture plate}}$$

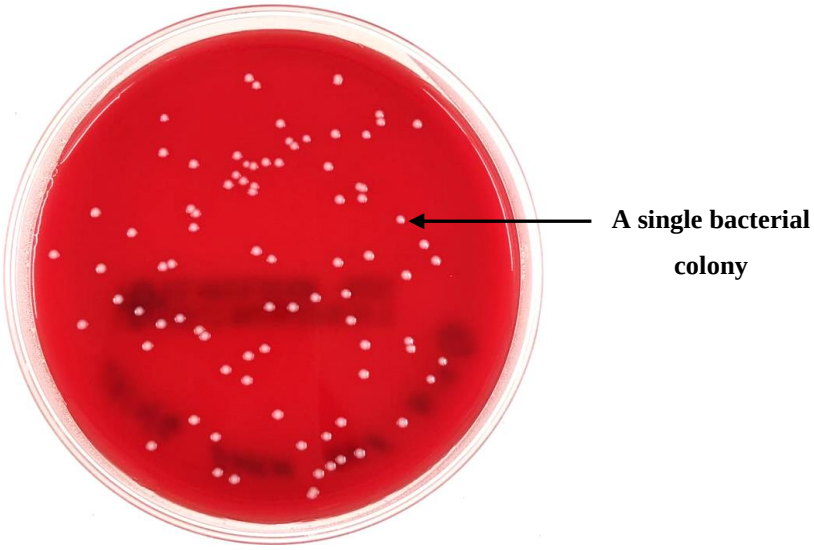

Figure 2- 10:

S. aureus biofilm bacteria colonies grew on HBA plate at 10^{-3} dilution after 10-fold serial dilution of sonicated fluid with sterile PBS.

The purpose of plate counting is to estimate the number of cells present based on their ability to give rise to colonies under specific conditions of nutrient medium, temperature and time.

Theoretically, one viable cell can give rise to a colony through replication. However, solitary cells are the exception in nature, and most likely the progenitor of the colony was a mass of cells deposited together. In addition, many bacteria grow in chains (e.g. Streptococcus) or clumps (e.g. Staphylococcus). Estimation of microbial numbers by CFU will, in most cases, undercount the number of living cells present in a sample for these reasons. This is because the counting of CFU assumes that every colony is separate and founded by a single viable microbial cell (Goldman and Green, 2015).

The accepted range for countable colonies on a standard agar plate is between 30 and 300 for most bacteria (ASTM D5465-93,1998; Breed, 1916). Therefore, to ensure that a sample will yield CFU in this range requires dilution of the sample and plating of several dilutions. Typically, ten-fold dilutions are used, and the dilution series is plated in replicates of 2 or 3 over the chosen range of dilutions. The CFU/plate is read from a plate in the linear range, and then the CFU/g (or CFU/mL) of the original is deduced mathematically, factoring in the amount plated and its dilution factor.

An advantage to this method is that different microbial species may give rise to colonies that are clearly different from each other, both microscopically and macroscopically. The colony morphology can be of great use in the identification of the microorganism present.

2.8 Real time PCR for ICU samples

2.8.1 Bacterial DNA extraction

Biofilms positive samples from a disinfected, decommissioned impervious mattress covers were covered with 200 µl of digestion buffer (50nMTris/HCl pH7.5, 150nM NaCl, 2mM EDTA, 1% SDS) in microcentrifuge tube and sonicated for 15min with a sweeping frequency of 42-47 kHz at 20ºC. 20 µl of 5mg/ µl lysozyme (Sigma, Sydney, NSW, Australia)) was added to final concentration at 0.5mg/ml and incubated at 56ºC for 2 hours. Then 10µl of 25mg/µl (equal to 250µg) proteinase kinase was added and incubated at 56ºC for another 2 hours.
DNA was extracted and purified using QIAamp DNA Mini kit according to manufacturer instruction.

2.8.2 Quantitative real-time PCR:

The quantification of total bacterial DNA in each sample was achieved by real-time quantitative PCR (qPCR) using universal eubacterial primer 16s rRNA_341F 5'-

CCTACGGGAGGCAGCAG-3' and 16s rRNA_534R 5'-ATTACCGCGGCTGCTGG-3' to amplify a 194bp amplicon of 16s rRNA gene of all bacteria.

Real-time PCR was carried out in 25 µl reaction mix containing 1X Brilliant II Sybr Green qPCR Master mix (Stratagene), 400nM forward and reverse primer and 100ng DNA template with the following cycling conditions: activation of Taq polymerase at 95ºC for 10 min, followed by 40 cycles of denaturation at 95ºC for 15 sec, annealing at 56ºC for 30 sec and extension at 72ºC for 30 sec.

Each qPCR was run with standard samples of known concentrations (copies/µl). Tenfold serial dilutions of the quantified 16s rRNA gene were used as external standards of known concentration (copies/µl) in real-time PCR reaction. The standard samples (three replicates for each concentration) were ranged 10–10^6 copies/µl which used to construct a standard curve for each qPCR run. The calibration curve was created by plotting the threshold cycle (Ct) corresponding to each standard vs the value of their corresponding gene concentration (copies/µl).

2.9 Detergent optimisation

Part one: Detergent efficacy testing; Optimisation and comparison of methods

In this study, the detergent efficacy testing was performed and a range of detergents, enzymatic and different oxidizing agents, were used

Aim:

1. To optimise the methods for detecting residual biofilms in the absence of bacterial viability.
2. To determine which method was most accurate yet wasn't affected (chemical interference) with the detergent used.
3. To compare the reliability of residual viability determination and crystal violet assays as a measure of determining cleaning efficacy of test products.
4. To determine if either the BCA assay or Bradford Assay were suitable to estimate the concentration of bacterial protein left after treatment with detergents.
5. To evaluate if measuring ATP was a more accurate method to measure removal of dry biofilm from test coupons.

6. To determine if alkaline hydrolysis followed by protein measurement utilising the Micro BCA test is a more accurate method to measure removal of dry biofilm from test coupons.

This study describes the processes for the development and evaluation of novel cleaners targeting dry biofilm. Performance of cleaning agent in removal of *S. aureus* 12-day DSB was done. Afterwards standard methods were used and optimised to evaluate the efficacy of biofilm removal from coupon surfaces.

Twelve-days dry biofilm was manufactured in the CBR on both polycarbonate plastic coupons and borosilicate glass coupons as described in chapter 2; 2.3.

2.9.1 Efficacy testing

Tested cleaning products are –

Commercial Detergents A, B, C, D, E, F, G, H were sourced from Whiteley Corporation (North Sydney, NSW).

Sample Whiteley corporation, Australia	Product	Dilution used for experiments
A	Fabrisan	Neat
B	Matrix	1:25
C	Zip Strip	1:6
D	Obliter8	1:6
E	Sansol	1:10
F	Phensol	1:50
G	Pasac	10mL/L
H	Fabrisolv	Neat

The company website describes the cleaning products as:

1. Fabrisan® is a specialised carpet sanitiser and deodoriser specifically designed for removing difficult odours from carpets and fabrics. Fabrisan is a unique blend of natural biocidally active oils and is completely biodegradable. It leaves behind a dry crystalline non-resoiling residue on all types of carpets and fabrics. The patented blend of natural oils in Fabrisan are slowly released in the carpet for up to a week after Fabrisan is applied to provide long lasting protection against mould and other fungal growth. In this study, this disinfectant was chosen for the reason that, a significant part of hospital

surface area is covered by carpet which might act as a strong source of DSB in hospital environment.

2. Matrix® has been demonstrated to remove conventional biological soils and bacterial biofilms. Matrix® is specifically designed for use in Endoscopy, Central Sterilizing Departments and Dental Practices. Matrix contained <5% QAC; alkyl dimethyl benzyl ammonium chloride. pH of concentrated solution is 5-7.

3. Zip Strip is for removing floor finishes and wax sealers with a minimum of scrubbing. Zip Strip is a low odour ammonia free stripping system. Zip Strip is caustic free and residue free. Contains <10% ethanolamine and 10-30% 2-Butanoxyethanol.

4. Obliter8 contains a synergistic blend of solvents specifically designed to penetrate and remove floor polish films. Make sure to keep the surface wet at all times when stripping. Contains 10-30% 2-Butanoxyethanol, <10% monoethanolamine, < 10% benzyl alcohol and <10% potassium hydroxide. Obliter8 can be used on vinyl, vinyl asbestos, rubber, no-wax linoleum, lino tile, marble, terrazzo, ceramic quarry tiles and most types of flooring.

5. Sansol is an acidic based, high foaming Hospital Grade Disinfectant containing glycolic acid and zinc chloride with excellent biocidal properties. Sansol effectively removes body fats and oils, hard water carbonate deposits, rust and urine/faeces stains, soap scum, algae and mildew.

6. PHENSOL is a soluble Phenolic Disinfectant/Detergent designed as a wide-spectrum bactericide specifically for use in Hospitals and in other environments where efficient, rapid killing of pathogenic micro-organisms is essential in the general context of infection control. PHENSOL is active in the presence of serum, blood, faeces, urine and other organic matter. active constituent: 18% (w/v) substituted phenols in an alkaline detergent base.

7. PASAC is a liquid neutralising agent based on organic acids (sulphamic acid 10-30%), guaranteeing rinsing of all alkali residues. PASAC is designed specifically for use in miele pasteurizing machines. PASAC is free from surfactants, phosphates and

complexing agents of the EDTA type and is recommended for use in washer disinfectors for the penetration and removal of lime scale and other difficult deposits.

8. Fabrisolv is a synergised combination of D-Limonene solvent and a surfactant blend which combine together to penetrate, remove and suspend all types of spots and stains in carpets. Contains 30-60% dipentene, 10-30% diacetone alcohol, <10% 1-methyl-2-pyrrolidone and <10% propanol.

In addition, the following detergents and chemicals were tested alongside the cleaning products and acted as controls

9. Tween 20 (polyoxyethylene sorbitan manolaurate) (Chem Supply, Australia) as a representative of neutral detergent
10. 1M NaOH as a representative of an alkaline agent suitable for removing biofilm.
11. 12.5% of Sodium hypochlorite (Fronine, Thermofisher Scientific, Australia) – diluted with sterile water to give 1000ppm, 5,000ppm, 10,000 pmm and 20,000ppm. The amount of free chlorine concentration was measured by iodometric titration or by dilution and testing using a commercial pool test kit. The hypochlorite solutions were buffered to a pH between 6.5 and 7.0.

2.9.1.a Experiment--- 1

Comparison of viability and crystal violet assays for determination of efficacy of cleaning products on dry surface biofilm.

Residual viability assay

Procedure

Test biofilm covered coupons (3) placed into 25 ml of detergent or into mili Q water for control coupons and reacted for 10 minutes. Each test parameter was tested 4 times. Each set of 4 coupons were then washed in 30 ml mili Q water for 2 minutes for 3 times prior to being placed in 5 mL of 100% TSB for 10 minutes. This higher duration was applied as Almatroudi (2016) demonstrated that, dry biofilm is extremely hard to clean by 1000, 5000 and 10000 ppm hypochlorite solution even after 10 minutes exposure. Thus, it was expected that 10 minutes exposure to new formulated detergents may help removal of biofilm. After 10 minutes

incubation sonication (Soniclean, JMR, Australia) was done at 80 kHz (Almatroudi, 2015; ASTM G136, 2016) for 20 minutes, which was optimized to ensure the detachment of all residual biofilm bacteria after detergent treatment. After sonication coupons were shaken vigorously for 2 minutes, serially diluted and 100µl of each dilution (neat, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) spread onto horse blood agar (HBA) and incubated at 37°C for 24 hrs. The number of colony forming units (CFU) were counted for the dilution corresponding to between 30 and 300 CFU and results are shown in table 2-3. Results were analysed based on positive control (untreated biofilm coated coupon) count (Log_{10} 6.25±0.12). Individual results for each coupon for each detergent has been given in appendix (9.1).

Table 2- 4:

Number of residual viable bacteria recovered after treatment with detergents

Detergents tested	Average CFU obtained
A	0.00E+00
B	0.00E+00
E	0.00E+00
F	0.00E+00
H	0.00E+00
1 M NaOH	0.00E+00
C	4.64E+04
D	2.10E+05
G	3.90E+05
T20 (.01%)	1.15E+06
Control	3.01E+06

Individual coupon results are found in appendix 9.1

Crystal Violet Assay

Detergent Tested included

A, B, E, F, H (Whiteley Corporation, Australia), T20, NaOH and Hypochlorite diluted to 1000, 5000, 10,000 and 20,000ppm. In hospital disinfection 1000 ppm hypochlorite is recommended. This study tested different concentration of hypochlorite to demonstrate the actual

concentration to eradicate DSB, since the eradication of DSB with 1000ppm chlorine is not possible (Almatroudi et al., 2016).

Three replicates for each detergent has been tested.

Procedure

After formation of biofilm, rods containing polycarbonate (plastic) coupons were placed into 25ml of test products or water for control and incubated for 10 minutes. The rods were then rinsed in 25ml of water for 2 min for three times to remove test products and then placed into 25 ml of 0.3% crystal violet solution and incubated for 90 min at room temperature (Hadi et al., 2010b). Excess crystal violet was removed by rinsing sequentially in two lots of 30 ml water in petri dishes (3 coupons/petri-dish). Coupons were then placed individually into 1ml of 95% ethanol (EtOH, v/v), sonicated for 5 minutes and shaken for 2 minutes and sonicated for 5 minutes again. The absorbance of the eluted samples were measured by spectrophotometer at 590 mm wavelength and the amount of protein was calculated from the standard curve as described in section 2.4.1.

Crystal Violet absorbance value of detergent treated coupons with biofilm was calculated using Equation:

$$\text{Absorbance} = \frac{(Con_{avg} - B_{avg}) - (T\ coupon_{avg} - B_{avg})}{(Con_{avg} - B_{avg})}$$

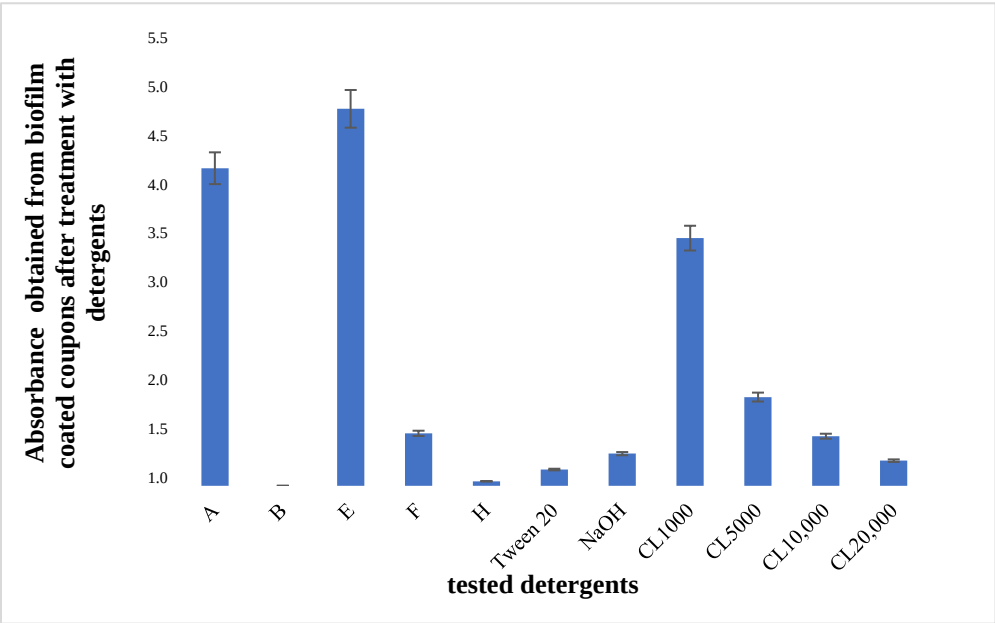

Figure 2- 11:

Crystal violet absorbance value after treatment with detergents (mean value)

Conclusion---

After spectrophotometry we observed higher absorbance value which was not expected. These unexpected results happened may be due to crystal violet was found to stain clean glass or poly carbonate coupon and these background values were deducted from the control and test values. Only coupons treated with test products B, H and Sodium hypochlorite 20000 ppm showed decreased absorbance when compared with the control coupons.

The higher values obtained from other cleaning agents could be due to:

- Test products not removing the biofilm but attaching to the coupon producing an interaction between the test products and crystal violet
- Insufficient rinsing following test product incubation

Crystal violet and detergents interaction assay

Detergent used

A, B, E, F, H (Whiteley corporation, Australia)

Procedure--

The crystal violet assay was repeated using only clean sterile coupons instead of biofilm covered coupons following the same above procedure

Three replicates for each detergent has been tested.

Results---

Table 2- 5:

Absorbance value of crystal violet with different detergents based on Blank corrected at 590 weave length.

Detergents tested	Average of absorbance
A	0.547
B	0.594
E	0.629
F	0.663
H	0.648

Conclusion—

Above table shows the high absorbance value that indicates that there is interaction between detergents and crystal violet suggesting that the crystal violet assay is not suitable for measuring residual soil in this setting.

2.9.1.b Experiment--- 2: Comparison of commercially available protein assays

The suitability of the commercially available BCA (bicinchoninic acid) (Pierce™ BCA protein kit assay, Thermofisher, Waltham, USA) assay and Bradford assay (Thermo Scientific™, USA) were tested.

Detergents used

A, B, E, F, H (Whiteley corporation, Australia),
Tween 20 (Chem Supply, Australia) and
1M NaOH

Three replicates for each detergent has been tested.

BCA protein assay- product testing on dry biofilm protein.

The BCA Protein Assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. It provides a qualitative, rather than quantitative, test of biofilm removal efficacy. Protein assay does not measure biofilm directly, but rather it provides an index of biofilm mass (Hadi ,Vickery et al., 2010). The protein assay kit relies on a protein reduction reaction where Cuþ2/ Cuþ1, which combines with bicinchoninic acid to form a purple-coloured product to provide a quantitative measure of biofilm (Hadi Vickery et al., 2010).

Procedure

The rods containing biofilm covered test coupons were placed into 25ml of test products or water for control and incubated for 10 minutes. Reagent control coupons were clean coupons treated the same way as the test coupons. The rods were then rinsed in 25ml of water for 2 minutes three times prior to individual coupon removal and rinsing in clean water in a new petri dish, to remove test products. Each coupon was added to 2 mL of the bicinchoninic acid protein kit working reagent mix and incubated at 60°C for 30 minutes. Samples were tested in triplicate by adding 200µl of each coupon product supernatant into 3 wells, of a micro titre plate. The

absorbance was reading at 562nm wave length. The absorbency obtained for the reagent control coupons was set as background.

The absorbance of biofilm coupons treated with detergents was calculated using following equation: -

Absorbance of test biofilm coupons = Test coupon $_{avg}$ --- Control reagent coupon $_{avg}$

Results

The absorbance of biofilm coupons treated with detergents was calculated using following equation: -

Absorbance of test biofilm coupons = Test coupon $_{avg}$ --- Control reagent coupon $_{avg}$

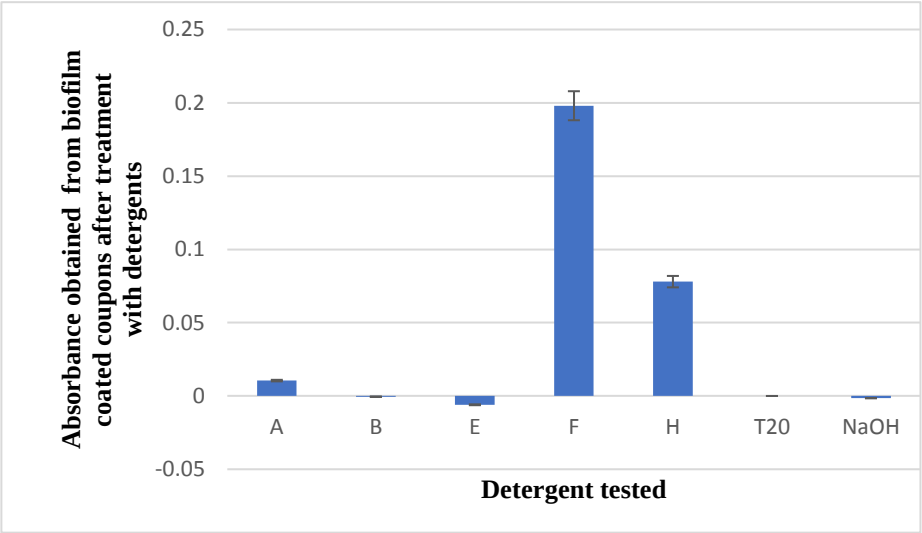

Figure 2- 12:

Absorbance value in BCA assay after treatment of biofilm coupons with detergents for 10 minutes (mean).

Bradford Assay (Thermo Scientific, Austarlia)

Procedure

Biofilm coated coupons and reagent control coupons (coupon without biofilm treated with detergent) were treated with test products and rinsed following the same above procedure (triplicate). Individual coupons were added to 1 ml of mili q water and sonicated for 5 minutes followed by shaking for two minutes. For each coupon, three 5 µl aliquots of supernatant were added to 250 µl of Bradford reagent in three wells of a microtitre plate, mixed and incubated for 10 minutes at room temperature.

Spectrophotometry

A 200 µl aliquot of the mixture was transferred to a new microtitre plate and absorbance at 595nm wavelength was measured in triplicate and protein concentrations estimated from the standard curve (section 2.4.3) The colour response with Coomassie is non-linear with increasing protein concentration, thus, standard curve was completed with each assay as described in section 2.4.3.

The absorbance of biofilm coupons treated with detergents was calculated using following equation: -

Absorbance of test biofilm coupons = Test coupon $_{avg}$ --- Control reagent coupon $_{avg}$

Results

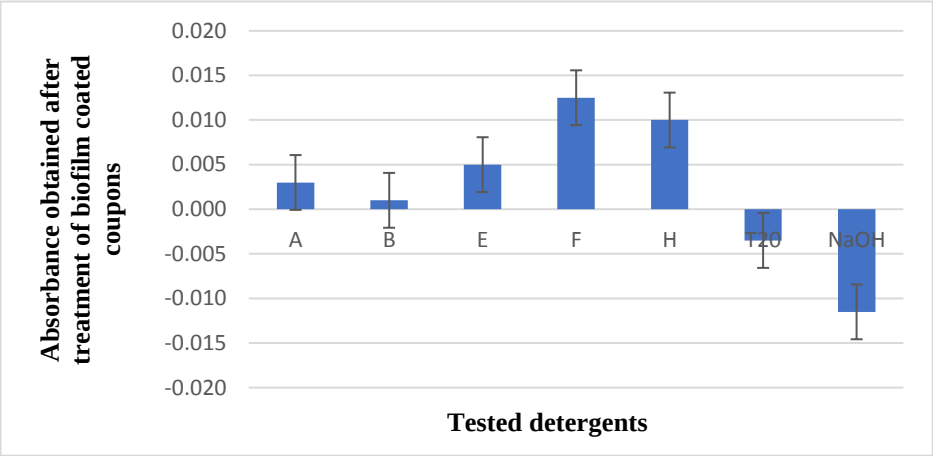

Figure 2- 13:

Absorbance level in Bradford assay after treatment with detergents for 10 minutes.

Conclusion: BCA and Bradford assays

For the BCA assay the high absorbance obtained by detergents F and H and the low results obtained for T20 which is known to be a poor biofilm remover an interaction between the detergents and assay was suspected.

For the Bradford assay the high absorbance obtained by detergents F and H and the negative results obtained for T20 and NaOH it was suspected that there was an interaction between the detergents used and the assay.

The interactions between the assays was confirmed by conducting a detergent interaction test (see below).

BCA and Bradford assay reagent and detergent interaction test

Assays were conducted in triplicate.

BCA assay procedure: A 25 µl aliquot of each detergent was added to 2 mL BCA protein reagent, incubated at 60ºC for 35 minutes and their absorbance measured at a wavelength of 562nm in triplicate.

Bradford assay procedure: A 3. 5 µl aliquot of each detergent was added to 250 µl of Bradford reagent dye incubated at room temperature for 10 minutes and absorbance measured at 595nm wavelength as described above.

Results

Table 2- 6:

Absorbance level of detergents in BCA (A) and Bradford assay (B).

A

Detergents tested	Average of absorbance
A	0.204
B	0.134
E	0.088
F	3.222
H	2.136
T 20	0.097
NAOH	0.081

B

Detergents tested	Average of absorbance
A	0.205
B	-0.123
E	0.019
F	0.924
H	0.874
T 20	0.062
NaOH	0.007

Conclusion

From above results it was shown that there was some interaction with all the tested detergents especially detergents F and H. This combined with the amount of residual biofilm approaching the limit of these assays, we considered them incapable of differentiating between moderate and good biofilm removers and therefore inappropriate for detergent testing of DSB under our testing conditions.

2.9.1.c Experiment-3. Evaluation of ATP bioluminescence assay for detection of residual biofilm protein from detergent treated coupons.

Adenosine triphosphate (ATP) is a chemical substance that acts as an energy source for all living organisms on the planet. The presence of ATP can be considered proof of the presence of a living organism, or of a substance produced by a living organism. Adenosine triphosphate (ATP) luminometers are a widely used cleaning validation tool particularly in the food preparation industry. The use of ATP meters has recently been introduced as a cleaning validation tool into some healthcare facilities.

ATP is detected using "firefly reaction" where any cells in the sample are lysed releasing the ATP which then reacts with luciferase releasing light, that is quantified using a luminometer and expressed as relative light units (RLUs). The amount of light liberated is roughly proportional to the amount of ATP present (Lewis et al., 2008). Despite being a quick and objective method, the use of ATP bioluminescence appears to be still poorly standardized at both the national and international level (Amodio and Dino, 2014). For standard cleaning monitoring an upper ATP limit or cut off value is absent. There are some attempts to set and validate ATP benchmark, determined that an upper limit or cut off value is 200 RLU (3M brand consumable), when assessing the cleaning level of endoscope. However, currently available other ATP luminometers show different reading for the same quantity of ATP, invalidating any direct comparison. Furthermore, ATP bioluminescence has been shown to be also affected by the presence of industrial cleaning solution (Shama and Malik, 2013). Thus, interpretation of any ATP results requires significant care.

Detergent used

A, B, E, F, H (Whiteley Corporation, Australia),

Tween 20 (Chem Supply, Australia) and

1M NaOH

Procedure

ATP testing was done using a swab LuciPan™ pen (Kikkoman, Tokyo, Japan) at a temperature range of 20 °C to 35 °C. The amount of ATP in samples was recorded as relative light units (RLU). After formation of biofilm, rod containing 3 coupons were placed into 25 ml of test products A, B, E, F, H, 1M NaOH and Tween 20 or 25 ml of water for the positive and negative control for 10 minutes at room temperature. Coupons were rinsed in 25 ml of water for 2 min for three times to remove test product. Residual ATP remaining on coupons was determined in two ways:

A. Direct swabbing of coupon surface

The distal 3 cm of a room temperature LuciPac Pen-AQUA sampling stick was wet using water supplied by manufacturer.

Coupon was swabbed and sampling stick was returned to the main body (casing) and pushed firmly onto LuciPac Pen-AQUA casing and agitated to fully dissolve any luminescent reagent.

Inserted the LuciPac Pen-AQUA into the Luminometer and the RLU recorded.

B. Following sonication of treated coupons in 2 ml water

Treated and control coupons were sonicated in 2 ml of sterile Milli q water for 10 minutes and shaken for 2 minutes. Following shaking the distal 3 cm of the sampling stick was soaked in the liquid samples and shaken gently to remove excess fluid and the RLU measured as described above.

Results of ATP bioluminescence assay

Table 2- 7:

The amount of ATP measured in triplicate as relative light units (RLU) detected following detergent treatment of coupons contaminated with dry biofilm. ATP was measured by a) swabbing the coupon directly, b) sampling the liquid that treated coupons were sonicated in and c) swabbing the coupon directly after sonication.

Detergents tested	Directly from coupon	Liquid following coupon sonication	Residual ATP left on coupon following sonication
A	41	503	30
B	57	1025	41
E	263	143	68
F	171	265	57
H	94	652	71
T20	121	1041	89
NaOH	30	20	12
Positive control	28	1205	102
Negative control	27	33	30

Interpretation

- For all detergents and the positive control more ATP was found in the sonicate compared to direct swabbing of the coupon surface
- Following sonication of the coupons, low residual ATP (less than 100 RLU) was found on their surface. This level approaches the lower quantification level of the machine.
- Very low levels of ATP were found on the negative control coupon demonstrating the lower detection level of the machine
- 1 M NaOH returned readings in a similar range to the negative control suggesting removal of the majority of dry biofilm using this method

Conclusion

ATP was able to differentiate between the positive and the negative controls. It could also differentiate poor biofilm removers eg Tween 20 from good biofilm removers eg 1M NaOH. However, it is not sensitive enough to differentiate between the better biofilm removers as the RLU readings approach the limit of quantitation e.g. test product E, are below the limit of

quantitation e.g. 1M NaOH. The limit of quantitation for this system is suggested to be around 100RLU. In addition, measurement of ATP using the currently available commercial kits is highly variable(Whiteley et al., 2012; Whiteley et al., 2015). For these reasons ATP measurement was not conducted in future studies.

2.9.1.d Experiment-4: - Total Organic Carbon (TOC) analysis

Detergent tested

A, B, E, F, H (Whiteley Corporation, Australia),
Tween 20 (Chem Supply, Australia) and
1M NaOH
Surfex (used after 10 and 30 minutes of dissolve of one sachet in 500 ml distilled water)
Chlorine (1000 ppm).

Procedure

To perform TOC analysis of detergent treated biofilm coated coupons, biofilm was developed on glass coupons to avoid any carbon contamination from plastic coupon surface. All the steps were done in a safety hood to avoid atmospheric carbon contamination. Samples were processed as follows:

1. Detergent treatment (as above) was conducted for biofilm coated coupons (samples) and sterile clean coupon (background control) and MilliQ water treatment was conducted for biofilm coated coupon as positive and sterile clean coupons as negative control. Five replicates were used for samples and positive control and thirteen for negative control.
2. All treated coupons were then washed with sterile miliQ water following previous procedure to remove test products with an extra wash with sterile Milli Q water.
3. Each coupon was placed into sterile bijou bottle and covered very carefully with sterile foil without touching the coupons to prevent environmental carbon contamination.
4. TOC level was analysed in triplicate from coupons by Shimadzu-5000A TOC analyser (Columbia, USA).
5. A calibration curve was prepared. (three replications for each carbon concentration were used)

Calibration curve

Carbon concentration (µg)	12.50	6.250	3.125	0
Sample (ppm)	12.50	6.250	3.125	0

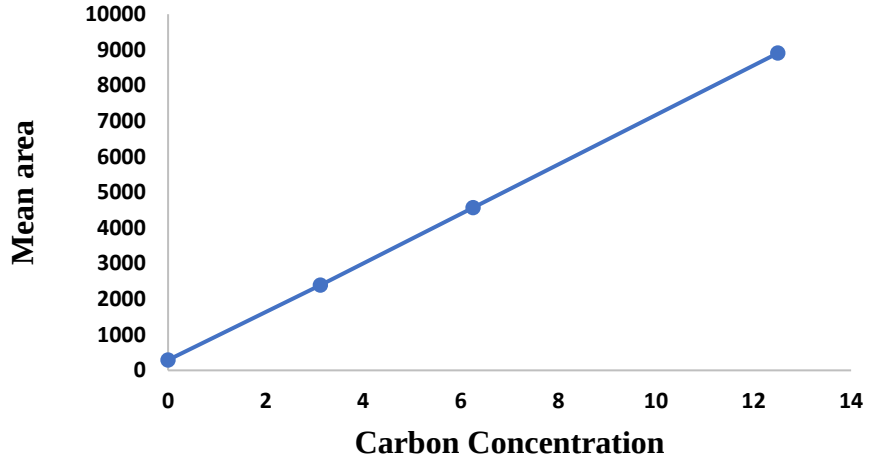

Figure 2- 14:

Calibration curve for TOC analysis

Slope (m)	690.505143	262.8	intercept (b)
m	3.4113028	24.42586	b
R2	0.99995119	31.53366	s(y)
F	40972.5783	2	DF
Regression SS	40741961.3	1988.743	residual ss

Limit of detection 0.12 (3.3 x std deviation of y intercept/slope

Limit of quantification 0.35 (10 x std deviation of y intercept/slope

Results of TOC analysis

Table 2- 8:

Results of negative control after TOC analysis.

Number of controls (Clean Coupon)	Carbon concentration (ug)
1	0.02
2	0.01
3	0.00
4	0.01
5	0.00
6	0.00
7	0.02
8	0.03
9	0.02
10	0.00
11	0.01
12	0.04
13	00
Average	0.01

Table 2- 9:

TOC analysis of background control coupon (detergent treated sterile clean coupon without biofilm).

Tested detergents	TOC concentration (µgm)
A	1.13
B	0.82
E	1.16
F	0.76
H	0.55
1MNaOH	0.17
Surfex (after 10 min of dissolve)	0
Surfex (after 30 min of dissolve)	1.26
Chlorine 100ppm	0.34
Tween 20 (0.01%)	0.20
Negative control	0.06
Blank	00

Total detected TOC concentration was calculated by following equation:

TOC concentration = Average of detergent treated coupons - Background control coupon.

Table 2- 10:

TOC analysis of biofilm coated coupon after treatment with detergents.

Tested detergents	TOC concentration (µgm)
A	4.77
B	6.87
E	5.33
F	3.45
H	3.13
1M NaOH	0.16
Surfex (after 10 min of dissolve)	0.47
Surfex (after 30 min of dissolve)	0.88
Chlorine 100ppm	1.87
Tween 20 (0.01%)	0.72
Positive control	7.43
Blank	00

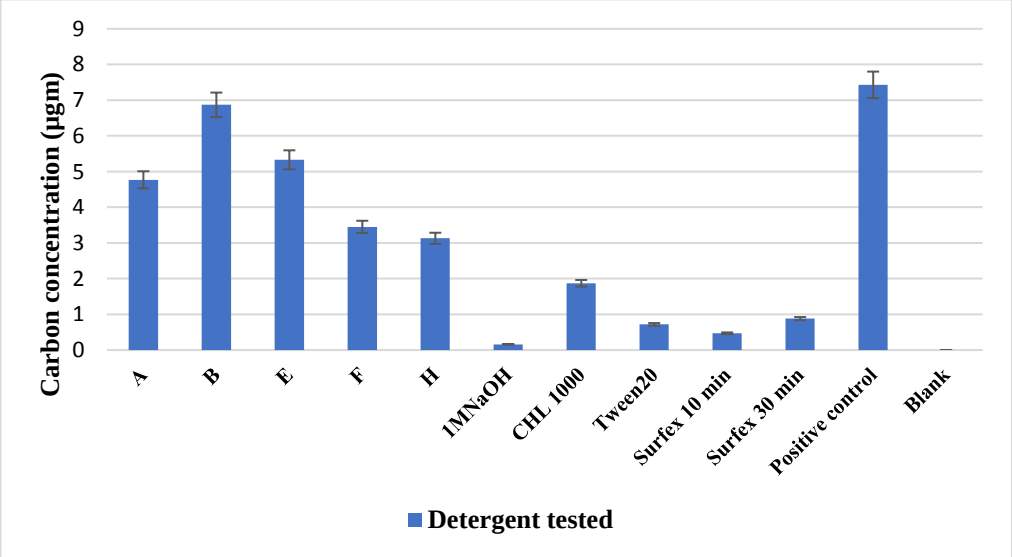

Figure 2- 15:

Detected TOC (µg) level after treatment of dry biofilm with detergents.

Interpretation

After treatment with detergents for 10 minutes, the lowest amount of carbon was found on the 1 M NaOH treated coupon which was below the limit of quantification 0.35 as determined by the calibration curve. Among the five Whitely detergents (A, B, E, F, H), H showed the lowest carbon concentration (3.13) followed by F, A, E and B. Tween 20 and Surfex (both at 10 and 30 minutes) showed low levels of carbon nearing the limit of quantification. Previous testing of Tween 20 showed it to be a poor biofilm remover which had been visually confirmed using live dead staining and CSLM as well as SEM. This suggests that this test is not sensitive enough to discriminate between good, medium and poor biofilm removers. It was therefore, not used in future analyses.

2.9.1.e Experiment-5. MicroBCA test

Cleaning products tested

Surfex (Whiteley medical, North Ryde, Australia)

Na hypochlorite (12.5% hypochlorite solution) 1000ppm, (Fronine, Australia)

Chlorclean tablets 1000 ppm (Whiteley medical, North Ryde, Australia)

Preparation of hard water

Hard water was prepared by dissolving 0.304g anhydrous $CaCl_2$ and 0.065g anhydrous $Mgcl_2$ in distilled water to make one litre (Hadi Vickery et al., 2010).

Preparation of test product

1. Chlorine (Hypochlorite solution)

   Dilute 12.5% hypochlorite solution (Na-HOCl) in to 1000 ppm with hard water. Bovine calf serum was added to 1000 ppm chlorine to make hard water disinfectant with 5% bovine serum.

2. Chlorine Tablet (Chlorclean)

   One tablet was dissolved into 1 litre hard water to get 1000ppm chlorine solution. Bovine calf serum was added to 1000 ppm chlorine to make hard water disinfectant with 5% bovine serum.

3. Surfex (PAA)

   One sachet of Surfex was dissolved in to 500 ml of hard water. Waited till it became blue from red colour. Bovine calf serum was added to sufex solution to make hard water disinfectant with 5% bovine serum.

Residual viability assay (five replicates for each disinfectant, positive and negative control)

1. 2 ml of hard water disinfectant with 5% bovine serum was added into each well for test coupons
2. 2 ml of hard water disinfectant with 5% bovine serum was added into each well for background control (Clean sterile coupon).
3. added 2ml of hard water + 5% BCS for positive control into 6 well-plate.
4. Coupons were placed into well containing test product according to the labelling and reacted for 5 minutes.
5. coupons for CFU were rinsed in 30ml of PBS twice for 5 seconds to remove test product.
6. each coupon was placed for CFU individually into 5 ml tube containing 2ml of PBS with 10% bovine serum albumin (BSA) and sonicated at 80kHz for 20 minutes, shaken vigorously for 2 minutes
7. Plated them out at -1, -2, -3, -4, -5 dilutions

Results

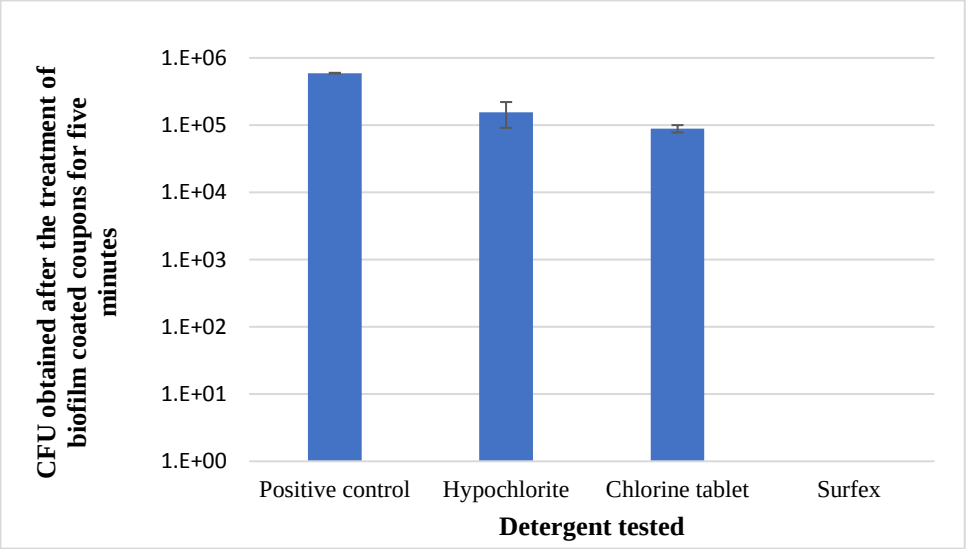

Figure 2- 16:

Number of residual viable bacteria (CFU) recovered after treatment with Na-HOCl, Chlorclean tablet and Surfex, in presence of soil.

Coupons for protein assay--- Micro BCA Assay test tube Procedure (linear working range of 0.5-20µg/mL)

To perform protein analysis of treated DSB, micro BCA assay (Micro BCA^{TM} Assay; Thermo Scientific) was employed as BCA (Pierce^{TM} BCA protein kit assay, Thermofisher, Waltham, USA) assay reagent interacts with detergents.

The amount of residual protein contaminating coupons was determined assay by alkaline hydrolysis as described by Li et al (2006) followed by the Micro BCA assay (Micro BCA^{TM} Assay; Thermo Scientific). The coupons were rinsed in 10 ml of PBS for each, for three times transferred to individual McCartney bottles containing 1mL of ice-cold 20 mM 2M-Morpholino-ethane sulfonic acid 0.9% saline. A 120µL aliquot of 30% NaOH added, the samples sonicated at 60°C for 1 hour vortexed and then incubated at 30°C for 30 minutes followed by incubation in a boiling water bath for 15 minutes. The samples were cooled and 86µL of 32% HCl added prior to centrifuging at 13,000 rpm in a bench top centrifuge for 5 minutes. A 1 ml aliquot of the supernatant was used for protein determination using the Pierce (bicinchoninic acid) Protein micro assay kit according to manufacturer's instructions. Residual protein contaminating samples was determined by measuring sample absorbance at 562nm wavelength, subtracting the absorbance of negative control coupons (n=3) and calculating protein concentration (µg/mL) using a standard curve prepared (five replicates for each concentration) using the supplied (provided in the Pierce Kit) BSA standard.

To determine percentage reduction of protein, following equation have been used:

Absorbance of test biofilm coupons = Test coupon $_{avg}$ --- Control reagent coupon $_{avg}$

Protein concentration= 33.836 X absorbance - 1.5127 (from prepared standard curve)

$$\textbf{Percentage reduction} = \frac{\text{Sample reduced protein X 100}}{\text{Amount of positive control protein}}$$

Results

Serial dilution of albumin (BSA) for standard curve (five replicates for each concentration)			
Vial	**Volume of Diluent or MiliQ water (ml)**	**Volume and Source of BSA (ml)**	**BSA Concentration (µg/ml)**
A	4.5	0.5 of Stock (1mg/ml)	200
B	8	2 ml from A	40
C	4	4 ml from B	20
D	4	4 ml from C	10
E	4	4 ml from D	5
F	4	4 ml from E	2.5
G	4.8	3.2 ml from F	1
H	4	4 ml from G	0.5
I	8	0	0=Blank

Protein concentration (µg/ml)	200	40	20	10	5	2.5	1	0.5	0
Absorbance	5.921	1.298	0.795	0.331	0.149	0.091	0.04	0.023	0

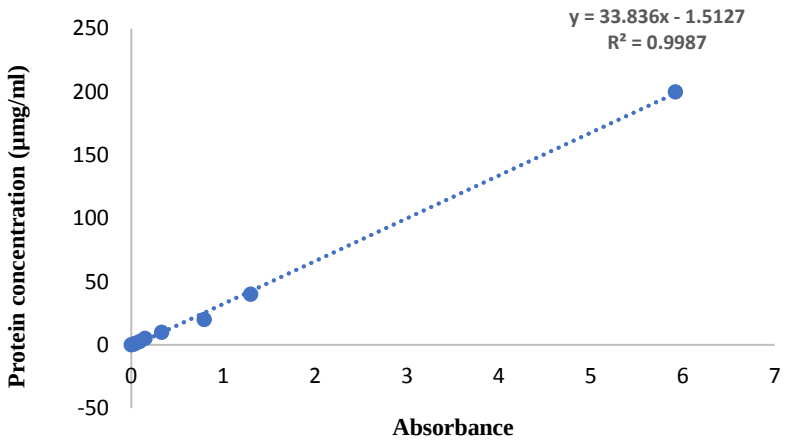

Figure 2- 17:

Standard curve for Micro BCA Assay to determine residual protein after treatment with detergents

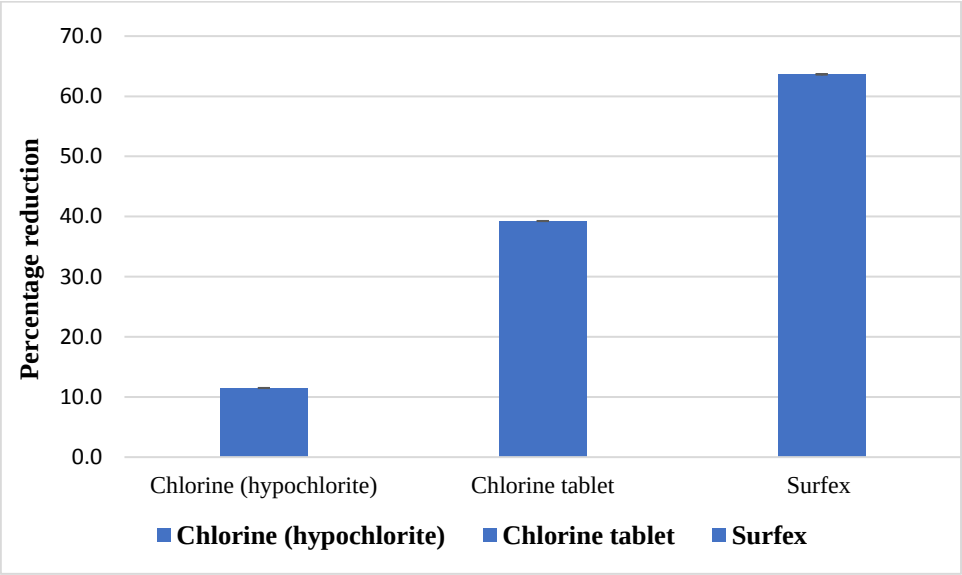

Figure 2- 18:

Percentage reduction of biofilm protein detected by micro BCA assay after treatment with Hypochlorite (1000 ppm), Chlorclean Tablet (1000 ppm) and Surfex 1300 ppm.

Conclusion

1. The efficacy of chlorine-based disinfectants, Chlorclean tablet and Na-HOCL against DSB are affected by soil and showed poor reduction in bacterial numbers while Surfex showed higher level of efficacy.
2. Results obtained by the MicroBCA test mirror the viability results with Surfex removing >60% of the biofilm mass.
3. Alkaline hydrolysis followed by measuring residual protein utilising the MicroBCA test appears to be sensitive enough for measuring biofilm removal from coupons and was used in all further investigations.

Part Two: Confirmation of disinfectant neutralizer

In disinfectant assays it is necessary to inactivate the action of the disinfectant at the end of the specified time period. This is achieved by either adding a chemical, immediate dilution, filtration, and if the test has a solid support, removal from the disinfectant and rinsing. Regulatory authorities and standards require incorporation of a disinfectant neutralise toxicity test as well as a disinfectant neutraliser test to confirm that the disinfectant is no longer active (TGA, 2009). It is evident that the presence of organic and /or inorganic soil strongly affects various disinfectants such as, iodophor and chlorine disinfectants (Pryor and Brown, 1975). They might have the capability to neutralize or to inactivate the disinfectants. This study validated neutralizer along with presence and absence of soil (TGO 54B) to determine whether soil has any negative influence on the efficacy level of these differently formulated disinfectants. The effect of addition of the proprietary ingredients to disinfectant efficacy was also evaluated by comparing the commercial disinfectants with generic equivalents in a bid to determine if biofilm removal is due to the active ingredient alone or if proprietary ingredients act in synergism with the active ingredient.

Preparation of planktonic liquid culture

To prepare liquid culture, a colony of bacteria *S. aureus* was aseptically removed from the working agar culture plate and inoculated into 250ml glass bottle containing sterile 100ml of 100% tryptone soya broth (TSB) (Oxoid, Threbarton, Australia) and incubated at 37^0C, at a constant rotational speed of 120 rpm in an Innova 42^{TM} shaker incubator (John Morris Scientific, Australia) overnight.

Soil and neutralizer used:

Soil---- 5% bovine serum (BCS) plus 10% bovine serum albumin (BSA)

Neutralizer--- 25 ml of 1% Na-thiosulphate + 3 ml of 6 % Tween 20 + 2.5 ml BCS + 5 gm BSA and then add PBS to make it 50 ml.

Test groups:

All the three test products were reconstituted in sterile hard (5.3.1) water to achieve desired concentration;

- Chlorclean tab solution 1000ppm
- SDIC solution 1000ppm and
- Surfex 1300 ppm

2.9.2 Planktonic control assay with disinfectants along with neutralizer validation in the presence and absence of soil.

Procedure:

Table 2- 11:

Disinfectant testing procedure against control planktonic bacteria with Surfex, SDIC and Chlorclean tablet.

Test Condition	Procedure with soil (5%BCS+10%BSA)	Procedure without soil
Test group Surfex (n=5) SDIC (n=5) Chlorclean tab (n=5)	1 ml disinfectant + 1 ml soil + 10 µl 10^9 bacteria --- react for 5 min Add 1ml neutralizer	1 ml disinfectant + 1ml water +10 µl 10^9 bacteria --- react for 5 min Add 1ml neutralizer
Neutralizer control Surfex (n=5) SDIC (n=5) Chlorclean tab (n=5)	1 ml disinfectant +1 ml soil + 1 ml neutralizer+10 µl 10^9 bacteria --- react for 5 min	1 ml disinfectant+ 1 ml water +1 ml neutralizer+10 µl 10^9 bacteria --- react for 5 min
Neutralizer Toxicity test (n=5)	1 ml water+ 1 ml soil +1 ml neutralizer+10 µl 10^9 bacteria --- react for 5 min	2 ml water + 1 ml neutralizer+10 µl 10^9 bacteria --- react for 5 min
Positive Control (n=5)	Dilute 10^9 bacteria-- Serial 10-fold dilutions were made	

After disinfectant treatment, samples were serially 10-fold diluted and 100 ul of neat, 10^{-1} to 10^{-4} dilutions were plated on HBA plates. Plates were incubated at 37°C overnight and CFU counted.

Results of Control Neutralizer

Table 2- 12:

CFU obtained- after neutralizer toxicity test with three disinfectants; Surfex, SDIC and Chlorclean tablet

Test condition	CFU
Neutralizer toxicity with soil	3400000
Neutralizer toxicity without soil	3600000
Positive control	3460000

Table 2- 13:

Control CFU obtained- after testing with Chlorclean, SDIC solution and Surfex with or without soil.

Disinfectants Used	Test CFU in the presence of Soil	Test CFU in the absence of Soil	Neutraliser control with soil	Neutraliser control without soil
Surfex	0.00	0	3320000	3300000
Chlorclean tab	3380000	139	3360000	3460000
SDIC	3340000	0	3260000	3240000

Table 2- 14:

Log $_{10}$ obtained from Positive control and neutralizer toxicity test control

Testing condition	Log 10
Neutralizer toxicity with soil	6.53
Neutralizer toxicity with soil	6.55
Positive control	6.53

Table 2- 15:

Log_{10} obtained after treatment with Surfex, Chlorclean tablet and SDIC solution after five minutes contact

Disinfectants Used	Test CFU in the presence of Soil	Test CFU in the absence of Soil	Neutraliser control with soil	Neutraliser control without soil
Surfex	0.00	0.00	6.52	6.51
Chlorclean tab	6.53	2.14	6.52	6.54
SDIC	6.52	0.00	6.51	6.51

Conclusion

The neutraliser containing Na-thiosulphate, Tween 20, BCS and BSA neutralised the chlorine and peracetic acid containing disinfectants.

2.9.3 Toxicity determination of Speedy clean detergent on planktonic bacteria in the presence of neutralizer and soil.

Aim

To determine if the neutral detergent Speedy clean (Whiteley medical, North Ryde, Australia) is toxic to planktonic *S. aureus* planktonic bacteria.

Procedure

The neutralizer was mixed with one mL of detergent and allowed to react for 5 minutes prior to serial 10-fold dilution and plate culture as detailed in Table 4-13.

Table 2- 16:

Disinfectant testing procedure against planktonic *S. aureus* control bacteria with speedy clean detergent followed by Surfex, SDIC and Chlorclean tablet

Test Condition	Procedure with soil (5%BCS+10%BSA)	Procedure without soul
Neutralizer Toxicity test (n=5)	1. 1 ml detergent +10 µl 10^9 bacteria —react 30 seconds 2. Add 1 ml water + 1 ml soil+1 ml neutralizer--react for 5 min	1. 1 ml detergent+10 µl 10^9 bacteria —react 30 seconds 2. Add 2 ml water + 1 ml neutralizer ----react for 5 mins
Positive Control (n=5)	Dilute 10^9 bacteria-- Serial 10-fold dilutions were made	

Results of Control Neutralizer; treatment with speedy clean

Table 2- 17:

Control CFU obtained- from positive control and neutralizer toxicity test.

Test condition	CFU
Netralizer toxicity with soil	2314000
Netralizer toxicity without soil	2662000
Positive control	2462000

Conclusion

The neutral detergent, Speedy Clean nor the neutralizer were toxic to *S. aureus.*

Table 2- 18:

Log_{10} obtained from positive control and neutralizer toxicity test after speedy clean treatment followed by disinfectants

Test condition	Log 10
Neutralizer toxicity with soil	6.36
Neutralizer toxicity without soil	6.42
Positive control	6.39

2.9.4 Neutralisation of H_2O_2 based disinfectants testing in the presence and absence of soil

Disinfectants tested

- **Sample A** (lot tg-30-105-A)
  It is a third-party disinfectant, which contains 0.6% hydrogen peroxide as its active form with some other (proprietary) ingredients. Used neat concentration of supplied solution.

- **Sample B** (lot tg-30-105-B)
  It is a straight generic 6% hydrogen peroxide solution obtained from the local pharmacy. Diluted with hard water ---10ml sample in 100ml hard water.

- **Sample C** (tg-30-105-C)
  This is Proxitane, a 5% peracetic acid sanitiser typically used in the food industry. In this study we have diluted 4ml disinfectant in 100ml hard water to get 2000ppm PAA, which is the same concentration of PAA in a fresh sample of Surfex.

Aim

To determine if the neutraliser containing Na-thiosulphate, Tween 20, BCS and BSA can neutralise hydrogen peroxide containing disinfectants.

Soil and neutralizer used

Soil---- 5% bovine serum (BCS) plus 10% bovine serum albumin (BSA)

Neutralizer--- 25 ml of 1% Na-thiosulphate + 3 ml of 6 % Tween 20 + 2.5 ml BCS + 5 gm BSA and then add PBS to make it 50 ml.

Procedure

Table 2- 19:

Disinfectant testing procedure against planktonic *S. aureus* control bacteria with 0.6% active H_2O_2 (A), 0.6% diluted H_2O_2 (B) and Proxitane (C).

Test Condition	Procedure with soil (5%BCS+10%BSA)	Procedure without soil
Neutralizer control A (n=5) B (n=5) C (n=5)	1 ml disinfectant +1 ml soil + 1 ml neutralizer+10 µl 10^9 bacteria --- react for 5 min	1 ml disinfectant+ 1 ml water +1 ml neutralizer+10 µl 10^9 bacteria --- react for 5 min
Neutralizer Toxicity test (n=5)	1 ml water+ 1 ml soil +1 ml neutralizer+10 µl 10^9 bacteria --- react for 5 min	2 ml water + 1 ml neutralizer+10 µl 10^9 bacteria --- react for 5 min
Positive Control (n=5)	Dilute 10^9 bacteria-- Serial 10-fold dilutions were made	

After disinfectant treatment, samples were serially 10-fold diluted and 100 ul of neat, 10^{-1} ,10^{-2} ,10^{-3} and 10^{-4} dilutions were plated on HBA plates. Plates were incubated at 37°C overnight and CFU counted.

Results of Control Neutralizer

Table 2- 20:

Control CFU obtained after treatment with three disinfectants in the presence and absence of soil

Disinfectants used	In presence of soil	In absence soil	Neutralizer control with soil	Neutralizer control without soil
A (0.6% H_2O_2)	796000	682000	8060000	7540000
B (diluted 6% w/v H_2O_2)	7460000	7060000	7000000	6960000
C (diluted 5% Proxitane)	658000	139	7240000	7240000

Table 2- 21:

CFU obtained from positive control and neutralizer toxicity test

Disinfectants used	CFU
Netralizer toxicity with soil	8160000
Netralizer toxicity without soil	7160000
Positive control	6340000

Conclusion

The neutraliser containing Na-thiosulphate, Tween 20, BCS and BSA neutralised the hydrogen peroxide containing disinfectants.

2.10 Bacterial proteomics and transcriptomic profiling

2.10.1 Microorganism and Culture Conditions

Proteomics profiling was performed on *S. aureus* (ATCC 25923) planktonic cells and 12-day dry surface biofilm (DSB) and 12-day wet biofilm (WB).

2.10.1.a Sample preparation

Planktonic culture: cells were prepared according to the procedure described in 2.2.1 of part one of this chapter

DSB in vitro model: This model was developed according to protocol described in 2.3.1 in part one of this chapter.

Mixed species biofilm model for qPCR:

13-day Mixed species biofilm was developed with 24 hours prior inoculation of 10^8 *S. aureus* in 10% TSB followed by addition of three bacteria (10^8); *S. aureus*, *E. faecium* and *A. baumanii* in 10% TSB in 2nd phase following DSB protocol for last three phases (Described in 5.5.4).

2.10.2 Protein Extraction and Fractionation

Extraction and fractionation were performed as described (Sadovskaya et al., 2005) with necessary modifications.

2.10.2.a Protein extraction from 24-hour liquid culture

Planktonic bacteria (10^8 CFU at OD 600 nm) were centrifuged at 12,000 x *g* for 10 minutes and the collected supernatant was mixed with lysis buffer containing Triethylammonium bicarbonate (TEAB, pH 8.5) and sodium deoxycholate (1% w/v) at 10:1 ratio (supernatant: lysis buffer) followed by probe sonication (Sonic Ruptor, Omni international, USA) for 2 minutes at 70% pulses. Lysed supernatant samples were loaded on to an Amicon ultra membrane (Sigma Aldrich, Australia) 10KDa molecular weight cut off (MWCO; Sigma Aldrich, Australia) filter tube to concentrate and precipitate proteins from the samples by washing with double volume of PBS for 3 times followed by 3kDa MWCO tube (Sigma Aldrich, Merck Millipore, Ireland) filtration.

2.10.2.b Extraction of biofilm proteins:

Biofilm coated coupons were washed with 1X PBS to remove loosely attached bacteria. Each coupon was placed into a five ml tube containing 2 ml of 1X PBS and 200 µL of lysis buffer and incubated overnight with gentle shaking at 4°C. After incubation, probe sonication was done in an ice-cold environment for 2 minutes at 70% pulses. Collected solutions from each coupon were centrifuged at 12,000 x g for 30 min at 4ºC. Supernatant fractions were transferred, concentrated, and precipitated using 10KDa MWCO followed by 3KDa MWCO, respectively. Finally, BCA protein assay (Thermo Fisher Scientific, Australia) was performed to measure protein concentration at 562 nm wavelength (PHERAstar FS, BMG Labtech, Germany) using supplied bovine serum albumin (BSA) as a protein standard.

2.10.2.c Protein Reduction, Alkylation, and Digestion

Described in chapter 6

2.10.2.d TMT Labelling and High pH Fractionation

Described in chapter 6

2.10.3 nanoLC-ESI-MS/MS

Described in chapter 6

Table 2- 22:

Amount of protein obtained from 12-day dry surface biofilm (from three episodes of 12-day DSB) and planktonic bacteria from 24-hours liquid culture (three episodes), maintaining similar growth condition in each episode of growth of DSB and planktonic culture.

Protein Type (Supernatant)	OD 562 (Blank corrected)		Average	Protein (µg/ml)	Final Volume (µl)	Total Protein (µg)	Cumulative Total Protein (µg)
	1	2					
24 hr Planktonic run-1	1.533	1.571	1.552	2052.85	200	410.57	
24 hr Planktonic run-1	1.521	1.600	1.523	1998.91	200	421.01	**1235.84**
24 hr Planktonic run-1	1.501	1.561	1.574	2087.78	200	404.26	
12DDB (Bioreactor run-1)	1.00	1.07	1.04	1153.67	130	149.98	
12DDB (Bioreactor run-2))	0.68	0.73	0.703	968.08	110	106.49	**397.83**
12DDB (Bioreactor run -3)	1.126	1.13	1.128	1570.69	90	141.36	

2.11 RNA extraction from biofilm and reverse transcription

2.11.1 RNA extraction

Samples were collected into RNA later (Qiagen, Australia) which is an aqueous, non-toxic storage reagent that rapidly permeates cells to stabilize and protect cellular RNA in situ in unfrozen specimens. After harvesting DSB, immediately submerged in RNAlater for storage

without jeopardizing the quality or quantity of RNA and incubated overnight at 4ºC. After washing with RNase-free water coupons were resuspending in a tube containing 200 µl TE buffer [10 mM Tris-HCl, 1 mM EDTA (Sigma-Aldrich, Australia)] containing lysozyme (15mg/ml; Sigma-Aldrich, Australia) and 350 µl RLT buffer containing beta mercaptothion (β ME; 1:50). A 50 µl aliquot of lysostaphin (Sigma-Aldrich, Australia) and 12.5 µl proteinase k (Sigma-Aldrich, Australia) was added and the sample incubated for 20 minutes at room temperature followed by 2 minutes probe sonication (Sonic Ruptor, Omni international, USA) at 70% pulses. The resulting solution was then applied to a Qiagen RNeasy Mini Kit, according to the manufacturer's recommendations for prokaryotic RNA isolation. The RNA was eluted with 35 µL of RNase free water.

2.11.2 Reverse Transcription

Reverse Transcription was conducted with a cDNA synthesis kit using SuperScript™ IV VILO™ Master Mix (Thermo Fisher scientific, Australia) in 20 µL as per the manufacturer's instruction with slight modification of incubation at 37°C for 5 minutes in Eppendorf mastercycler gradient (Eppendorf, Germany). Briefly, 200ng of RNA was added to a 20µL reverse transcription reaction to produce cDNA. A "no" reverse transcription control (-RT control) reaction was also included, which included all reaction components except the reverse transcriptase enzyme, which was replaced with nuclease free water. The cDNA was used immediately in Real Time PCR or stored at -20°C.

2.11.3 Primers

The primers used in this study (describe in chapter 6) were designed using Oligo 6 primer analysis software (www.oligo.net) for selected upregulated genes based on *S. aureus* (ATCC 25923) genome sequence (DDBJ/ENA/GenBank under the accession numbers CP009361). The primers were produced commercially by Sigma Aldrich, Australia. Upon receipt, the lyophilized primers were resuspended with nuclease free water to obtain a 100µM stock solution. From this, 10µM solutions were prepared and frozen in aliquots to prevent freeze-thawing cycles. The primers were stored at -20°C.

2.11.4 qPCR

qPCR was conducted in a Qiagen Rotor Gene Quantitative Real Time PCR (Mx3000P™ qPCR, Stratagene, USA). Samples were analysed using eubacterial universal primers targeting 16s RNA gene (Muyzer et al., 1993). Each qPCR was run with the corresponding standard samples of known concentrations (copies/µl), 10^8,10^7,10^6,10^5,10^4, 10^3 copies of *S. aureus* genome per µl to generate a standard curve for each primer set. A 25µl aliquot of qPCR reaction mix

containing Brilliant® II SYBR® Green QPCR Master Mix (Stratagene, Sandeago, USA), reverse and forward primer and 2 µl of 1:4 diluted cDNA. Controls in each run included a no template control (NTC) for each primer set, which consisted of all PCR components except cDNA template which was replaced by nuclease free water. A no reverse transcription control (RT control) was also included in initial experiments for each targeted gene. RT controls consisted of the cDNA sample with no reverse transcriptase enzyme to determine if there was contaminating genomic DNA in the RNA. Cycling conditions for real time PCR were optimised as follows:

For *fhuC*: an initial activation step of 95°C for 10min, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 47°C for 45 seconds and extension at 72°C for 30 seconds.

For *cysE, mucC, icd* and *murB*: an initial activation step of 95°C for 10min, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 50°C for 40 seconds and extension at 72°C for 30 seconds.

For *prs, femX* and*16s rRNA*: an initial activation step of 95°C for 10min, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 20 seconds.

2.11.5 Gene Quantification

Relative quantitation of gene expression was conducted using the Pfaffl method (Pfaffl, 2001) where changes in gene expression were normalised to a reference gene. Single reference gene, *16s rRNA* was compared for their stable expression in single and mixed species biofilms. In this study, single reference gene was experimented for 12-day WB, 12-day DSB and 13-day mixed species biofilm since all were in stationary phase. Study suggested that, in stationary phase 16s rRNA become more stable (Vandecasteele, 2002). Another study also suggested that, use of *16S rRNA* as internal standard in RTQ-PCR quantification of staphylococcal gene is appropriate as their study results confirmed the use of housekeeping genes as internal standards in relative quantification studies during the exponential growth of bacteria is unsuitable due to the rapid changes in their expression (Eleaume, 2004). These finding provide reference to use the 16S rRNA gene as internal standard to follow the expression of a given gene over time during the stationary phase of *S. aureus* growth (Eleaume, 2004).

Chapter Three: Biofilm: transmission and inactivation by heat

Part One: Transmissibility of dry surface biofilm bacteria

3.1 Introduction

In the hospital environment, patients are the leading source of contamination and can shed several major pathogens into the nearby environment, thus contaminating hospital surfaces at a sufficient concentrations for transmission (Mitchell et al., 2015). Surfaces in the vicinity of patients, that are touched frequently by health care worker and patients are termed as "high touch surfaces", have higher occurrence of contamination than other sites (Hayden MK et al., 2008; Huslage K et al., 2010). These sites are thought to provide the greatest risk for transmission of pathogens in hospital environment just because they are frequently touched. Health care workers' hands can become contaminated by contact with patient and/or touching contaminated environmental surfaces (Rutala and Weber, 2013). As common pathogens such as *S. aureus*, MRSA, VRE, *C. difficile* survive on dry environmental surfaces for prolonged periods and HCW can easily become contaminated. 20%-40% of nosocomial infections are estimated as a consequence of cross contamination of organisms by HCWs hand (Weber et al., 2010; Falagas et al., 2011). As few as 15 *S. aureus* cell (Foster and Hutt, 1960) and 1 CFU/cm^2 (Lawley et al., 2010) can cause infection in experimental lesions, thus, even a few bacteria contaminating surfaces may pose a risk for transmission.

Studies investigating the possibility of DSB contaminating the hands of HCW and then being transmitted to the patient or other surfaces have not been conducted. this study was performed to determine:

- whether DSB bacteria would contaminate hands if touched
- to what extent organisms could be transmitted to other surfaces
- if the hospital bed sheet can protect patients from bacteria present in DSB contaminating mattresses
- if using of gloves can help to prevent transmission of DSB
- If wetting the DSB with detergent to simulate cleaning increases transmission of DSB

In this study, DSB was grown in vitro on polycarbonate and glass coupons in CDC bioreactor for 12 days (Almatroudi et al., 2015a). DSB bacterial transmission was tested using ungloved (bare) and gloved hands to various materials before and after treatment with neutral detergents.

3.2 Author's contribution

1. Transfer of dry surface biofilm in health-care environment- `the role of health care worker's hand as vehicles.

Durdana Chowdhury, Shamaila Tahir, Mark legge, Honghua Hu, Khalid Aljohani, Tania Prvan, Anand Deva, Karen Vickery.

Dr Durdana Chowdhury contribution was:

- Design study protocol with Dr Sharmila Tahir and Associate Professor Karen Vickery
- Carried out in vitro experiments
- Collected data
- Data compilation
- Data analysis.
- Manuscript preparation

Overall contribution: 70%

2. Transmission of dry surface biofilm (DSB) by and through cotton bedsheets: an in vitro study

Durdana Chowdhury, Shamaila Tahir, Mark legge, Honghua Hu, Tania Prvan, Anand Deva, Karen Vickery.
Dr Durdana Chowdhury contribution was:

- Design study protocol with Dr Sharmila Tahir and Associate professor Karen Vickery
- Carried out in vitro experiments
- Collected data
- Data compilation and analysis.
- Manuscript preparation

Overall contribution: 70%

3. Transmission of Staphylococcus aureus from dry surface biofilm (DSB) by different types of gloves.

Shamaila Tahir, **Durdana Chowdhury**, Mark legge, Honghua Hu, Whiteley.G Anand Deva, Karen Vickery.

Dr Durdana Chowdhury contribution was:

- Design study protocol with Dr Sharmila Tahir and Associate professor Karen Vickery
- Carried out in vitro experiments
- Collected data and compilation and analysis.
- Manuscript preparation

Overall contribution: 40%.

3.3 Original paper

Title: Transfer of dry surface biofilm in healthcare environment: the role of healthcare worker's hands as vehicles.

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Transfer of dry surface biofilm in the healthcare environment: the role of healthcare workers' hands as vehicles

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SUMMARY

Background: Dry surface biofilms (DSBs) persist for extended periods in hospital, and may play a significant role in transmission of healthcare-associated infections.
Aim: To determine whether DSBs may be transferred from hospital surfaces to healthcare workers' hands.
Method: Twelve-day *Staphylococcus aureus* DSB was grown on polycarbonate and glass coupons in a CDC Biofilm Reactor®. A total of 1.8×10^6 and 8.8×10^5 bacteria grew on the polycarbonate and glass coupons respectively. Transmission was tested by lifting the coupon with forefinger and thumb of ungloved hands to a height of 30 cm, then touching horse blood agar (HBA) plates 19 sequential times. Transferred bacterial number was determined by colony-forming units. The effect of DSB wetting on biofilm transfer was tested with 5% neutral detergent treatment for 5 s.
Findings: Between 5.5 and 6.6% of the DSB bacteria were transferred to hands with one touch and ~20% were then transferred to HBA with one touch, giving an overall transfer rate of 1.26% and 1.04% for polycarbonate and glass coupons, respectively. Detergent treatment had little effect on bacterial removal from coupons, but, for biofilm grown on polycarbonate, significantly increased transferral to HBA ($P < 0.001$) to 5.2%. Large numbers of bacteria were transferred by bare hands to multiple fomites. One-third of polycarbonate coupons transferred >1000 colonies during the first five sequential touches. Sufficient bacteria to cause infection were transmitted up to 19 times following one touch of the DSB.
Conclusion: DSB bacteria are transferred by hands from one fomite to multiple fomites, suggesting that DSB may serve as a persistent environmental source of pathogens.
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Introduction

Over the decades, the role the environment plays in the acquisition of healthcare-associated infections (HCAIs) has been debated, with some research suggesting that environmental transmission is negligible, but other studies suggesting that the environment plays an important role [1,2]. More recent studies have established that environmental surfaces in rooms with patients colonized with pathogens, such as meticillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE), are frequently contaminated with these organisms at concentrations sufficient for transmission [3–6]. An estimated 20–40% of nosocomial infections have resulted from transmission of micro-organisms present in the hospital environment either directly or via healthcare workers' hands [2].

Although environmental sites frequently touched by the hands of patients and healthcare workers provide the greatest opportunity for transmission of pathogens, there is no significant difference in the microbial bioburden between low-, middle- and high-touch sites [7]. Additionally, 25–79% of non-critical medical devices have been found to be contaminated, so pathogenic organisms may be sourced from any part of the hospital environment [8]. The ease of cleaning hospital surfaces, particularly in proximity to patients, remains a significant challenge.

A single ungloved hand contact with a contaminated surface results in a variable degree of pathogen transfer, with the highest planktonic bacterial transfer rates, of between 38% and 65%, occurring from hard and non-porous fomite surfaces to hands [9]. This is concerning, as important pathogens, such as *S. aureus* (including MRSA), VRE, and *Clostridium difficile* survive on dry surfaces for protracted periods whereas bacteria within dry surface biofilms (DSBs – biofilms growing on dry clinical surfaces) have been shown to survive for more than 12 months as they are protected from desiccation [10–12]. Biofilm bacteria also have increased tolerance to removal by cleaning agents and killing by disinfectants, and we have shown that DSBs are particularly tolerant to chlorine disinfection [13,14].

DSBs may play a role in the endemic transmission of HCAI, but, as biofilm bacteria are attached to a surface and each other, the ease of transfer from fomite to hand is unknown. Clinical DSBs contained up to 10^7 bacteria/cm^2 and 50% of clinical biofilms incorporate the common HCAI pathogen, *S. aureus* [12,14]. We therefore aimed to determine the transfer rate and number of surfaces that could be contaminated following one touch of *S. aureus* DSB, using our DSB model [15].

Methods

Hand preparation

Hands were washed before and after each transfer event by wetting hands, rubbing for 2 min with 2 mL of Microshield neutral liquid hand wash (Johnson & Johnson Pacific, Sydney, Australia) followed by rubbing with 80% v/v ethanol (concentrating on the finger tips). Once dry, the efficacy of the preparation protocol was confirmed by pressing both the thumb and forefinger tips on to HBA and incubation overnight at 37°C.

Transfer protocol

Staphylococcus aureus ATCC 25923 was produced on removable polycarbonate and glass coupons (diameter 1.27 cm, depth 0.3 cm) held in polycarbonate rods (three coupons per rod) in the CDC Biofilm Reactor® (BioSurface Technologies Corporation, Bozeman, MT, USA) over a period of 12 days with four cycles of periodic nutrition interspersed with long periods of dehydration [15]. The rods containing the coupons were rinsed in phosphate-buffered saline (PBS) to remove unattached bacteria. For each individual transfer experiment, the number of colony-forming units (cfu) on a minimum of three untouched coupons (control) was determined to ensure adequate growth of DSB. There was no significant difference in the control coupons' cfu between runs. Control coupons were placed in 2 mL of PBS, sonicated (Soniclean, JMR, Thebarton, SA, Australia) at 80 kHz for 20 min then vortexed for 2 min, followed by serial dilution and standard plate culture at 37°C for 24 h.

Transfer rate of DSB from coupons to agar with one touch

While still in the rod, biofilm-covered coupons ($N = 6$ glass, $N = 6$ polycarbonate) were gripped between the thumb and forefinger and the rod (weight 49.9 g) was lifted to a height of 30 cm as shown in Figure 1. The thumb and the forefinger completely covered the coupon and were then pressed onto the surface of two different HBA plates. Immediately, 100 µL of PBS was applied to the HBA plate and the inoculum spread over the entire surface, incubated and counted. The number of cfu on control coupons ($N = 6$) and cfu remaining on test coupons was determined as described above for control coupons. The experiment was repeated to simulate hospital surface wetting during cleaning. Coupons were exposed to 5% neutral detergent (Lemex; Whiteley Corporation, Sydney, NSW, Australia) for 5 s and excess detergent drained for 5 s prior to hand contact and transfer (Figure 1). This time was based on the estimated time that a surface would remain wet following one wipe with a detergent-impregnated wipe.

The percentage of DSB removed following one touch was calculated using the following formula:

$$\text{Removal rate (\%)} = \frac{100 \times (\text{cfu on control coupons} - \text{cfu left on coupons following one touch})}{\text{cfu on control coupons}}$$

where: cfu left on coupons following one touch was determined by placing test coupons after touching, in 2 mL of PBS, sonicating, serial dilution and standard plate culture as described for control coupons above.

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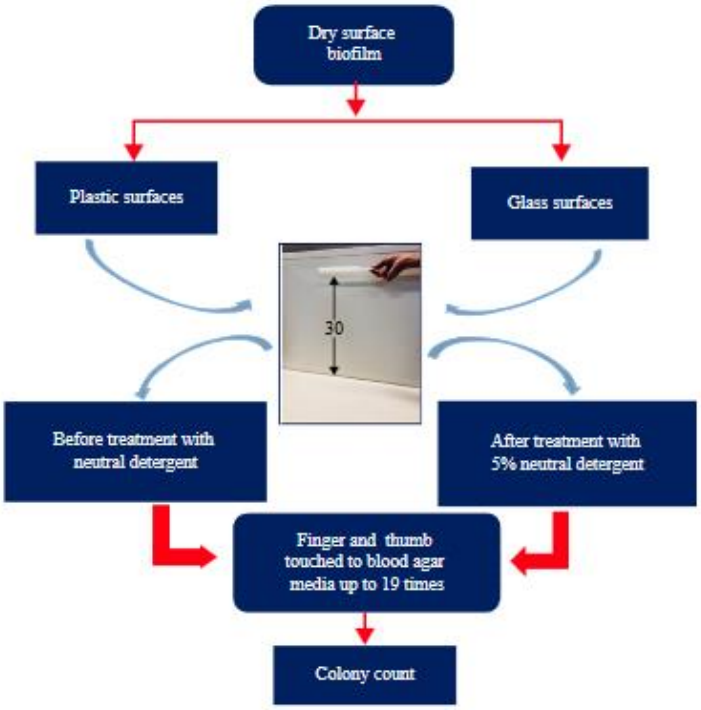

Figure 1. Schematic presentation of transfer testing procedure.

The transfer rate of bacteria from DSB to the hands and thence to HBA plates on the first touch was calculated using the following formula:

$$\text{Transfer rate of DSB (\%)} = \frac{\text{cfu transferred to HBA plate} \times 100}{\text{cfu on control coupons}}$$

Transfer of DSB to multiple surfaces following one touch

The number of surfaces that could be contaminated following touching DSB once was determined by touching the biofilm-covered coupon as described above and then pressing the thumb and the forefinger on to the surface of different HBA plates up to 19 times as detailed in Figure 2 and counting the number bacteria transferred. The protocol was repeated 18 times for both glass and polycarbonate coupons.

Statistical analysis

Data were analysed using SPSS Statistics 23 (IBM, Portsmouth, UK) and Minitab version 17 (Minitab, Inc., Sydney, NSW, Australia). Hierarchical log-linear modelling using backward

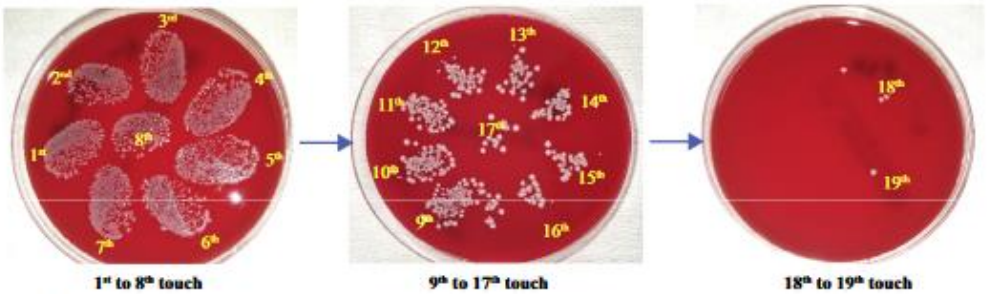

Figure 2. Transfer frequency of biofilm bacteria from 1st to 19th touch on the horse blood agar plate (1st to 19th are touch number).

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elimination was used to test for significant interactions between number of bacteria transferred and number of touches, the type of coupon (polycarbonate or glass), the DSB, and whether detergent was used or not. The χ^2-test was used to test for an association between the number of hand touches to the HBA plates, before and after detergent treatment, and the number of resulting bacterial colonies. For analysis, the number of bacteria transferred per touch was grouped into three categories <100; ≥100 but ≤1000; and ≥1000. The number of touches originating from each coupon ($N = 18$) were grouped as one to five touches; six to 10 touches; and more than 10 touches, yielding a total 342 touches analysed per test group. Student's *t*-test was used to compare, between detergent-treated and control coupons, the percentage of DSB bacteria removed from coupons that were subsequently transferred to HBA.

Results

Transfer rate of DSB from coupons to agar with one touch

Between 1×10^5 and 7×10^5 cfu were removed from DSBs grown on polycarbonate or glass coupons when they were gripped between the thumb and forefinger and lifted to a height of 30 cm. This represented an average of 5.46% and 6.50% of the bacteria incorporated into the DSBs on glass and polycarbonate, respectively (Table I). Of these bacteria, between 3×10^4 to 1.6×10^5 or ~20% were transferred to HBA following one touch. This equated to slightly more than 1% of the bacteria incorporated into DSBs being transferred from the coupon via the hands to HBA with one touch (Table I). A 5 s treatment with neutral detergent had little effect on either the number of DSB bacteria removed from the treated glass coupon or the number of bacteria transferred from the hands to HBA. By contrast, although the detergent treatment had no effect on percentage of DSB removed from polycarbonate coupons, i.e. the number of bacteria contaminating the hands (1.1×10^5 compared to 1.6×10^5), between 67% and 85% of these bacteria were transferred to the HBA plate with one touch ($P < 0.001$). This resulted in significantly greater percentage (5.2% versus 1.31%) of the DSB being transferred from the coupon to the HBA with one touch ($P < 0.001$) (Table I).

Transfer of DSB formed on polycarbonate coupons to multiple surfaces

Each experimental run included a minimum of three control coupons to ensure sufficient DSB numbers on coupons. Polycarbonate control coupons ($N = 15$) were covered with a mean of $\log_{10}$ 6.27 ± 0.4 cfu. Without detergent treatment, large numbers of bacteria were transferred from the polycarbonate coupon to the hands and thence to the HBA plate. Hierarchical log-linear modelling using backward elimination found a significant interaction between the number of bacteria transferred and number of touches, with more bacteria being transferred during the initial touch period and then progressively decreasing ($P < 0.0005$). More than 1000 colonies were transmitted from approximately one-third of coupons for up to 10 touches. Between 100 and 1000 cfu were transferred from >90% of coupons from the 11th to the 19th touches (Figure 3).

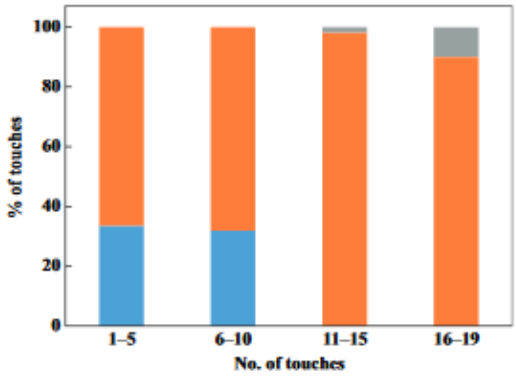

Figure 3. The number of bacteria (cfu) transferred per touch from polycarbonate coupons. Percentage of touches transferring bacteria grouped: >1000 (blue), 100–999 (orange), <100 (grey) and 0 cfu (yellow). The number of touches was grouped 1–5, 6–10, 11–15, and 16–19.

Transfer of DSB formed on glass coupon surfaces to multiple surfaces

Glass control coupons ($N = 9$) were covered with a mean of $\log_{10}$ 5.95 ± 0.37 cfu. Without detergent treatment, as with the polycarbonate coupons, the number of bacteria transferred was significantly associated with the number of touches conducted, with more bacteria transferred during the initial touches ($P < 0.0005$). In contrast to polycarbonate where 100% of coupons and touches transmitted >100 cfu during the first 10 touches, 61% of glass coupon touches transferred <100 cfu during touches 1–5. By the 11th touch, approximately two-thirds of touches failed to transmit any bacteria (Figure 4). From Figure 4 it can be seen that although <1000 colonies were transmitted per touch for up to five touches, some touches transmitted >1000 colonies in later touches, suggesting that the biofilm came away from the fingers in clumps.

Table I
Percentage of dry surface biofilm bacteria removed from coupons and transferred to horse blood agar following one touch

	Mean % removed from coupon		Mean transfer % to HBA plate	
	Untreated dry	Detergent treated	Untreated dry	Detergent treated
Polycarbonate coupons	6.50	6.60	1.31*	5.20*
Glass coupon	5.46	6.18	1.04	1.03

*Significantly different: $P < 0.001$.

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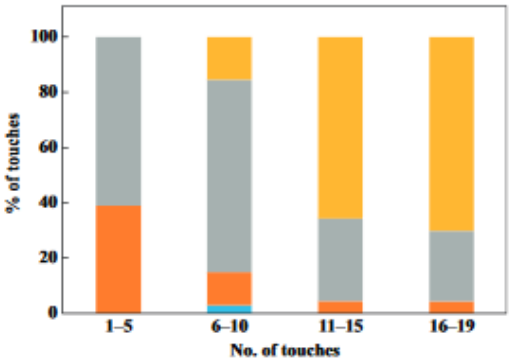

Figure 4. The number of bacteria (cfu) transferred per touch from glass coupons. Percentage of touches transferring bacteria grouped >1000 (blue), 100–999 (orange), <100 (grey) and 0 cfu (yellow). The number of touches was grouped: 1–5, 6–10, 11–15, and 16–19.

Discussion

The transfer of *S. aureus* DSB from polycarbonate and glass surfaces to hands and thence to another surface (HBA plates) was examined to determine whether DSB could act as a persistent source of environmental pathogens, given biofilms' increased tolerance to removal and biocides [13].

The model surfaces used in this study were nominated to signify hard hospital surfaces frequently touched by healthcare staff, that is polycarbonate and glass. When touched with bare hands the majority of DSB remains on the substrate with only 5.46% to 6.5% of the bacteria being removed from the coupons and transferred to the hands. This is in contrast to a transfer rate of between 38.5% and 65.8% from hard surfaces to hands for the non-pathogenic planktonic *Serratia rubidea* and *Micrococcus luteus* [9]. However, the transfer of pathogenic *Acinetobacter baumannii* from fomite to finger pad was only 24% [16]. Bacteria in biofilms are attached to each other, and the layer of bacteria closest to the substrate are also attached to it. The whole colony is incorporated into extracellular polymeric substances, which serves to increase attachment to the substrate and stabilize biofilm architecture. Given biofilms' group cohesiveness it is not surprising that a low percentage of the biofilm is transferred with one touch. However, between 3×10^4 and 1.6×10^5 bacteria were transferred to the hands, and ~20% of these were transferred to HBA, so that ~1% of DSB may be transferred from one fomite to another by one touch. Similarly, Greene and co-workers found that the efficacy of transfer from finger pad to fomite was only ~20% of that from fomite to finger-pad [16]. The average number of bacteria in clinical DSB has been found to be 5.5×10^5 bacteria/cm^2 and many contain pathogens and live bacteria [12,14]. When tested to see how many surfaces could be contaminated by consecutive touching of fomites, sufficient bacteria to potentially cause infection in non-intact skin (>15 cfu) [17] continued to be transferred to the HBA plates for up to 19 touches.

The importance of cleaning is widely recognized in government standards and professional societies' guidelines and the first step of cleaning is very frequently the use of a liquid detergent cleaning product. For these experiments a neutrally charged detergent system was used to replicate the widest possible normal use of cleaning products. To simulate the process of surface wetting during cleaning, the coupons were immersed in neutral detergent for 5 s and then allowed to dry before the transfer experiment was conducted. This treatment had little effect on removal of DSB from glass coupons or transfer to a second fomite (HBA). Nor did detergent treatment affect the percentage removal of DSB from the polycarbonate coupons. However, for biofilm grown on polycarbonate coupons, detergent treatment significantly increased the percentage of DSB bacteria transferred from the hands to the secondary fomite, HBA. This suggests that although a short treatment with detergent fails to remove significant amounts of DSB from its substrate it may act to disperse clumps of biofilm removed by the fingers. The possibility exists that DSB grown on polycarbonate has different physicochemical properties than the same biofilm grown on glass, since the surface free energy of polycarbonate is only 36–46 mN/m (http://www.accudynetest.com/polymer_surface_data/polycarbonate.pdf) whereas that of glass is ~300 mN/m [18].

In the experiment to see how many fomites could be touched consecutively and still transfer sufficient bacteria to cause infection, greater numbers of bacteria were transferred to HBA plates following touching DSB grown on polycarbonate coupons, but this could be explained by the slightly higher number of bacteria incorporated into DSB on polycarbonate coupons ($\log_{10}$ 6.27) versus glass coupons ($\log_{10}$ 5.95). Figure 4 also shows that for biofilm grown on glass coupons, 3% of the six to 10 grouped touches transferred >1000 colonies. This suggests that the biofilm may have been removed from the glass coupon to the fingers in clumps, which, with multiple touches, eventually broke up into smaller clumps or individual bacteria.

The potential role of clinical DSB to act as a reservoir of environmental organisms that may play a role in HCAI has been evaluated in this study. This study suggests that *S. aureus* DSB bacteria are transmitted readily from environmental surfaces. Whether pathogens incorporated into mixed species biofilms, which commonly contaminate hospital surfaces [12], would be transferred in a similar fashion is being investigated. Though the transmission rate of *S. aureus* DSB from one fomite to another is low, when compared with planktonic bacteria, sufficient numbers of bacteria were transferred to cause infection even after 19 consecutive touches as the estimated infective dose in lesions for *S. aureus* is thought to be 15 cells/cm^2 [17]. Thus, even if only one environmental surface is contaminated with DSB, multiple surfaces could be contaminated by healthcare workers touching this item once if hand hygiene is not performed. Once contaminated, the hands transfer up to 41% of planktonic bacteria to the lips, posing a risk to both patients and staff if adequate hand hygiene is not performed [9].

In conclusion, recent scientific evidence has strongly suggested that contamination of surfaces in hospital rooms plays an important role in the transmission of pathogenic organisms. In the environment, microbes exist as either single cell (planktonic) or as biofilm. Transmission of planktonic organisms is accepted but in this study we show that bacteria incorporated into DSB may also be transferred by the hands to multiple fomites, and this highlights the important roles played by environmental cleaning and hand hygiene in infection control.

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Conflict of interest statement

Dr G. Whiteley is the Managing Director and Dr T. Glasbey is the R&D Manager of Whiteley Corporation, a manufacturer of disinfectants and detergents for use in healthcare. Their roles did not lead to any bias in formulating, executing, analysing or writing up the research. The authors declare that no competing interests exist.

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3.4 Original paper

Title: Transmission of dry surface biofilm (DSB) by and through cotton bedsheets: an *in vitro* study.

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Summary

Background The major source of nosocomial pathogens is thought to be the patient's endogenous flora that could be shed by patients in to patient's surroundings especially on to the bed component. It could be contaminated through direct contact of patient and can persist for prolonged periods as dry surface biofilms (DSB). As DSB is highly resistant to cleaning agents and disinfectants it could be a strong source of infection. This study aims to determine if cotton sheet used in hospital bed has transmissibility and skin of hands could be contaminated through cotton sheet.

Method: 12-day DSB of *Staphylococcus aureus* was grown in vitro on polycarbonate coupons in CDC bioreactor with periodic nutrition after a long period of dehydration. Transmission was investigated using polycarbonate coupons by and through cotton sheets before and after treatment with 5% neutral detergents for 5 seconds. Following contamination of cotton sheet with DSB, transmission to 19 subsequent surfaces was performed. ICU mattress cover with a low level of contamination was determined by qPCR and Scanning electron microscopy was performed to confirm the bacterial presence.

Result: For the cotton sheet 150 thread/sq inch, between 100 and 1000 bacteria colonies were transferred before treatment for all levels (100%) of touch whereas after treatment early touches (1st to 5th) range from ≥ 1000 bacteria colonies transferred through to < 100. In the first touch, average number of CFU transferred (755) before treatment, became almost five times higher (3416±453 CFU) after treatment. Both before and after treatment, >100 colonies were transferred from 100% coupon surfaces by 250 thread/sq inch cotton sheet in 1st touch but increased number of bacterial transfer following subsequent touches was observed after treatment than before treatment. After 12 touches was similar to untreated surfaces. For through cotton sheet, in case of 150 thread/sq inch cotton sheet, most of the sequential touches following touching a dry coupon (through the sheet) transferred <100 CFU and in 1st touch the number of CFU transferred was 114±16. However, after treatment, CFU transferred was increased to more than double (259±84) and 100-1000 colonies were transmitted from 100% coupon surfaces up to 5th touch. In case of 250 thread/sq inch cotton sheet, in 1st to 5th touch, before treatment, 10 to 30 colonies were transmitted in 8.3 % cases with <10 colonies transferred from most of the surfaces. However, after treatment >30 colonies were transmitted in the first 5th touches in 8.4% cases. Scanning electron microscopy showed that the impervious mattress covers were not

smooth and that even when contaminated with low numbers of bacteria, bacteria and surrounding EPS were seen to have attached to the surface particularly in the natural pits formed in the material

Conclusion: This study showed that, cotton sheet has higher rate of transmissibility. After treatment with neutral detergent, the transmission rate of DSB bacteria by and through cotton sheet has been increased. DSB could be found in mattress and can be transmitted to the patient from bed mattress, through the cotton sheet. As biofilm bacteria can be transferred through cotton sheets, this study highlights the importance of cleaning bed mattresses to prevent transmission of pathogens.

Introduction

Within the hospital environment patients are the primary source of pathogens and can heavily contaminate their surroundings. Bed covers, bed sheets and bed rails become highly contaminated with bacteria (Lemmen et al., 2006) through direct contact with skin scales and body fluids (Ayliffe et al., 2000, AWMF Working Group, 2005). The degree of contamination may vary depending on the status of the patients with symptomatic patients transferring more bacteria than asymptomatic colonised patients, (Durham et al., 2016). The frequency of acquisition of *Clostridium difficile* by patients is increased with increasing levels of environmental contamination (Wilcox et al., 2003) as is contamination on healthcare workers hands (McFarland et al., 1989). Meta-analysis determining the risk of pathogen acquisition by patients associated with prior room occupancy found that the risk was increased an average of 2.14 (confidence interval 1.65-2.77) if the previous room occupant had been colonized or infected with methicillin resistant *S. aureus* (MRSA), vancomycin resistant *Enterococcus* species (VRE), *C. difficile*, extended spectrum β-lactamase (ESBL) producing coliforms, *Acinetobacter* or *Pseudomonas spp.* (Mitchell et al., 2015). Within the hospital environment, "high touch surfaces," are surfaces that are frequently touched either by health care workers or patients, can carry a higher microbial load when compared to less frequently touched sites (Huslage et al., 2013; Adams et al., 2017).

Controlling environmental contamination in patient's zone is important in preventing hospital acquired infections and reduced environmental contamination can lead to a reduction in HAI (Hayden et al., 2006; Dancer et al., 2009; Hota et al., 2009). An important aspect of the patient zone is the bed linen and mattress. Patients spend most of the day in bed and direct contact with the mattress is only prevented by the bed sheet. Mattresses have been shown to be contaminated with various organisms including multi-antibiotic resistant organisms (Sherertz and Sullivan, 1985; Andrade et al., 2000; Bousquet A. et al., 2017) and as with surgical cloth drapes, the effectiveness of a sheet in preventing bacterial egress from the mattress, is likely to be compromised if it becomes wet with patient sweat or other secretions. Andrade (2000) reported a high prevalence of *S. aureus*, was cultured from 50% of these mattresses after being subjected to disinfection cycle. Thus, an appropriate and effective preventive measure to reduce transmission of organisms through bed materials is paramount for effective patient care and prevention of HAI. A very recent study showed that, *C. difficile* associated HAI was significantly reduced (41%-60%) in six Sentara Healthcare hospitals, USA, after replacing all the regular non-biocidal linens with the copper oxide impregnated biocidal linens (Butler, 2018).

Damage to the impervious mattress lining can go undetected if the external covers are intact (Bradbury et al., 2014) and wet and damaged mattresses have been the source of contamination during outbreaks (Sherertz and Sullivan, 1985; Bradbury et al., 2014). Undetected damage to the inner impervious mattress cover on a therapeutic bed led to blood tinged fluid seeping from a contaminated mattress to the sheet due to the pressure from the patient's body weight (Bradbury et al., 2014). Closer examination of all 656 hospital mattresses found 177 impervious mattress covers were degraded and required replacement (Bradbury et al., 2014).

In addition, to planktonic bacterial contamination bedding can be contaminated with biofilm. A study by Hu et al. (2015), detected dry surface biofilm contaminating 5/5 pillows and 6/6 mattress in one ICU. Despite terminal cleaning with chlorine, the authors were able to culture MRSA from impervious covers of two mattresses and one pillow, VRE from two mattresses and extended spectrum β-lactamase (ESBL) producing organisms from one mattress and one pillow. Bacteria incorporated into biofilms have increased tolerance to disinfectants both due to their lifestyle and the diffusion/inactivation action of the surrounding extracellular polymeric substances (EPS) that the bacteria secrete and which binds them to each other and the substrate reviewed in (Otter et al., 2015). Clinical biofilm that contaminates dry surfaces is visually and chemically similar to laboratory grown biofilm subjected to multiple cyclic periods of nutrition interspersed with long periods of dehydration (Almatroudi et al., 2015). These biofilms have been shown to have extra thick EPS (Vickery et al., 2012; Almatroudi et al., 2015) which may help explain their exceedingly high tolerance to sodium hypochlorite (Almatroudi et al., 2016) and desiccation (Hu et al., 2015).

We hypothesize that mattresses provide a suitable environment for biofilm formation and that bed sheets cannot prevent transmission of organisms from the mattress. The aim of the current study was to test whether bed sheets can transfer biofilm bacteria and whether the sheet prevents movement of biofilm bacteria through them i.e. whether they protect the patient.

Methods

Dry surface biofilm preparation

S. *aureus* (ATCC 25923) biofilms were grown on sterile polycarbonate coupons in a CDC biofilm reactor (BioSurface Technologies Corp, Bozeman USA) over 12 days as previously described (Almatroudi et al., 2015). Growth was initiated with 10^8 cfu *S. aureus* and grown in 5% tryptone soya broth (TSB) with shear (provided by baffle rotation at 130 revolutions per min) for 48-hour batch phase at 35ºC, after which the media was drained and the biofilm

dehydrated for 48 hours at room temperature (22-25 ^{o}C) with filter-sterilised air-conditioned air (average relative humidity 66%) pumped across the media surface at 3L/min. An additional three cycles of batch growth (5% w/v TSB, shear, 35°C for 6 hours) alternated with prolonged dehydration phases of 66, 42 and 66 hours at room temperature. The number of colony forming units on a minimum of 3 coupons/ biofilm generator were determined to ensure that adequate (10^7) and uniform number of biofilm had grown on the coupons. Control coupons were placed in 2 mls of PBS, sonicated (Soniclean, JMR, Australia) at 80 kHz for 20 minutes then vortexed for 2 minutes, followed by serial dilution and standard plate culture at 37°C for 24 hours.

Hand preparation

Hands were washed before and after each transfer event for 2 minutes liquid hand wash followed by rubbing with 80% V/V ethanol until dry (≥3 second). Disinfection was confirmed by pressing both the thumb and fore finger tips on to blood agar media which was incubated aerobically at 37^0 C for overnight.

Transmission testing

Both cotton hospital sheets (250 threads/square inch; 155 gm/M^2 dense) and cotton domestic sheets (150 threads/square inch; 100 gm/M^2 dense) were tested. Sheets were cut into small pieces (3 x 1.5 cm) and autoclaved at 121°C for 15 minutes.

Rod containing coupons were washed with PBSX3 to remove planktonic cells from the coupons. A cotton sheet piece (150 threads/square inch, n=18; 250 threads/square inches, n=12) was held against each surface of the *S. aureus* DSB coupon by the thumb and the index finger as shown in figure 1. The rod (weighing 49.9 g with coupons) was raised up to a height of 30 cm (as shown in figure-1) and then lowered. The cotton sheet piece was pressed on to the media surface of HBA plates up to 19 touches. To determine if bacteria were transferred from the biofilm through the cotton sheet and onto the fingers, the finger and thumb tips were then pressed on to the surface of the HBA media up to eleven times.

The experiment was repeated to simulate hospital surface cleaning. Coupons were exposed to 5% neutral detergent (LEMEX, Whiteley Corporation, Sydney, Australia) for 5 seconds and excess detergent drained prior to sheet contact and transfer (150 threads/square inch, n=18; 250 threads/square inches, n=12). This 5s time was based on the estimated time that a surface would remain wet following one wipe with a detergent impregnated wipe.

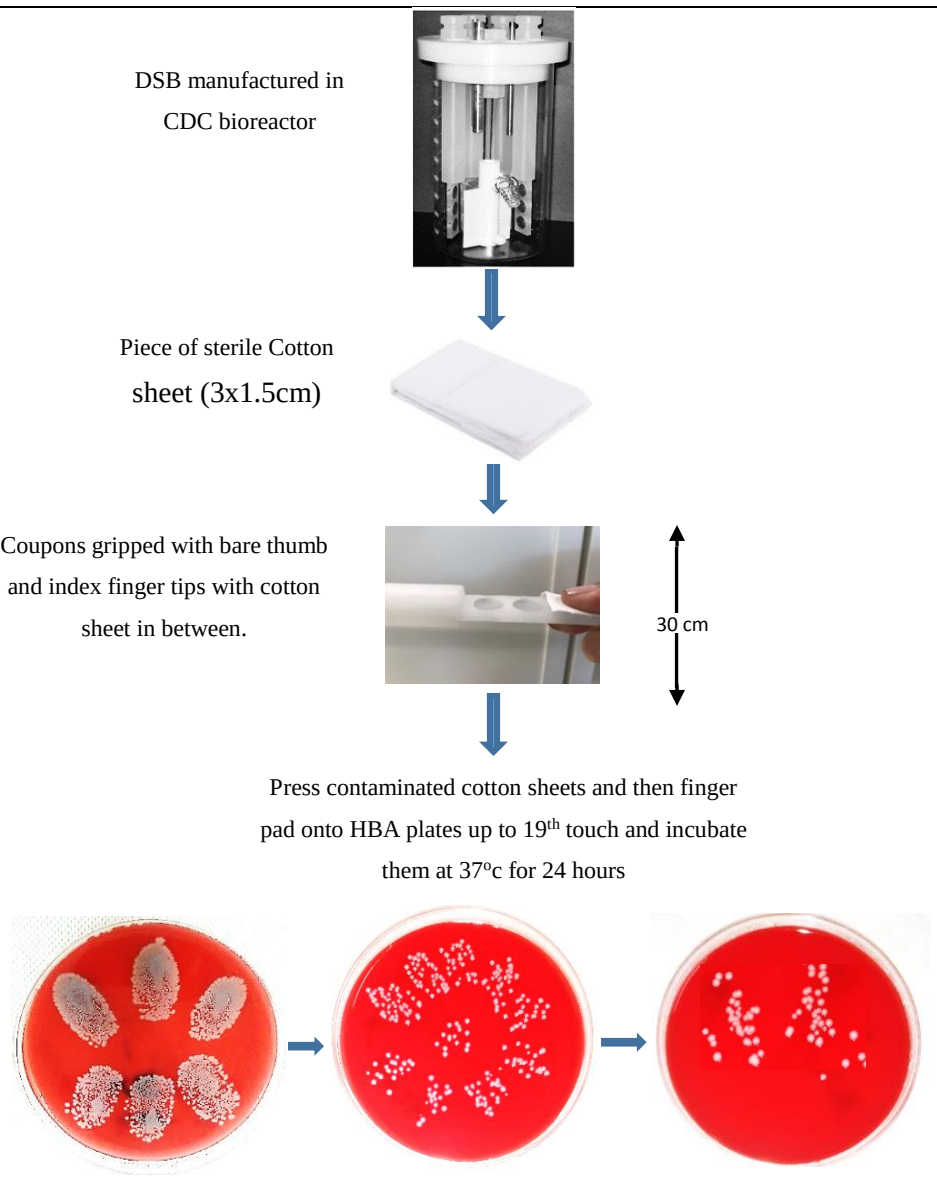

Figure-1: Schematic presentation of procedures for manufacturing DSB and transfer events by and through cotton sheets.

Estimated bacterial number in clinical biofilm contaminating patient bedding

Biofilm positive samples from a disinfected, decommissioned impervious mattress covers (n=6) and the impervious covers from five pillows, aseptically obtained by cutting out segments using sterile gloves, forceps and scissors from intensive care unit, were subjected to real-time quantitative PCR described in chapter 2, 2.8 using universal eubacterial primer16s rRNA_341F 5'-CCTACGGGAGGCAGCAG-3' and 16s rRNA_534R 5'-ATTACCGCGGCTGCTGG-3' as previously described (Jacombs et al., 2012).

Scanning electron microscopy of clinical samples

A mattress cover with a low level of contamination ($1x10^4$ bacteria/cm^2) as determined by qPCR was fixed in 3% glutaraldehyde, followed by dehydration in ethanol, prior to immersion in hexamethyldisilazane (HMDS, Polysciences Inc) for 3 min and sputter coating with 20nm gold film and examination in a scanning electron microscope as previously described (Jacombs et al., 2012).

Statistical analysis:

Data was analysed using SPSS Statistics 23 (IBM, Hampshire, UK) and Minitab version 17 (Minitab, Inc, Sydney, Australia). Hierarchical Loglinear Modelling using Backward Elimination was used to test for significant interactions between number of bacteria transferred and number of touches and whether detergent was used or not. The Chi square test was used to test for an association between the number of sheet and hand touches to the HBA plates, before and after detergent treatment and the number of resulting bacterial colonies. For analysis, the number of bacteria transferred per touch was grouped into 3 categories <100; ≥ 100 but ≤ 1000; and ≥ 1000. The number of touches originating from each coupon were grouped as 1 to 5 touches; 6 to 10 touches; and more than 10 touches giving a total 666 touches analysed for the 150 threads/square inch sheets.

Results

Positive control coupons had a mean of $\log_{10}$ 6.4 ± 0.17 CFU/plastic coupon and $\log_{10}$ 5.94 ± 0.12 CFU/ glass coupon. Transfer of bacterial cells was designated by total CFU counted with each touch directly from HBA plates after 24 hrs incubation keeping into consideration the proposed number of < 2.5 CFU/cm^2 of hospital surfaces as safe value in hospital environment (Boyce et al., 2011; Cloutman-Green et al., 2014; Huang et al., 2015).

Transfer by cotton sheet —150 threads/square inch

Between 100 and 1000 biofilm bacteria were transferred from the DSB covered coupons (n=18) to the dry cotton sheet and thence to HBA for all nineteen sequential touches (total 324 touches) (Figure 2a). In the first touch, average number of CFU transferred (755) before treatment, became almost five times higher (3416±453 CFU) after treatment. In contrast, there was significant interaction between the number of bacteria transferred and number of touches, following a five second treatment of coupons with neutral detergent ($P<0.0005$), with more bacteria being transferred during the initial touch period and then progressively decreasing (Figure 2b). During the first 10 sequential touches significantly, more bacteria were transferred

from coupons that were treated than from coupons that hadn't been treated (P<0.001), with 83% of touches transferring >1000 bacteria/touch from treated coupons. These results indicate that after neutral detergent treatment bacterial transmission has been increased by almost 84%.

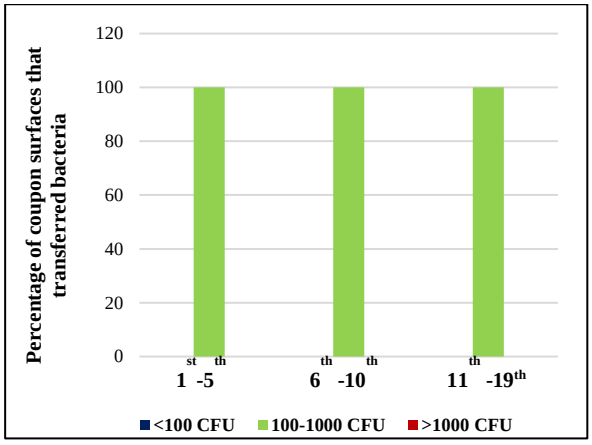

Figure2a

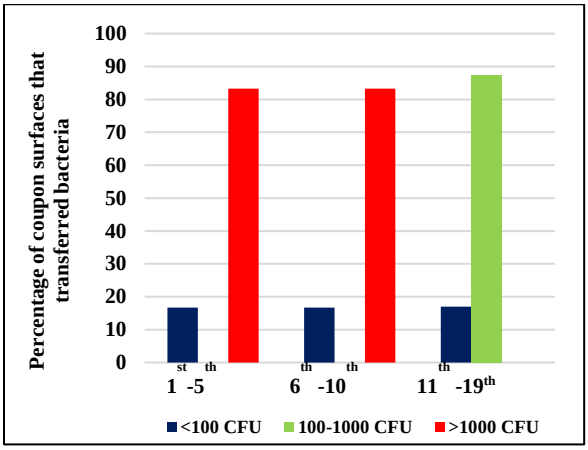

Figure 2b

Figure 2: Percentage of coupons that transferred bacteria (<100, 100-1000 and >1000) to the cotton sheet (150threads/square inch) and thence to horse blood agar with touches before (2a) and after treatment (2b) with 5% neutral detergent (P<0.0005).

Transfer by cotton sheet— 250 threads/square inch

In case of cotton sheet 250 thread/sq inch a Regression model with Box-Cox transformation (after adding 0.5 to all the observed number of bacteria colonies) was fitted to the data using Minitab 17. There is a significant interaction between number of touches and whether the coupon had been treated with neutral detergent or not (p<0.0005) (Figure 3). Before and after treatment, >100 bacterial colonies were transmitted from 100% coupon surfaces in first touch. However, after treatment (Figure-3), more bacterial transmission was observed from the following touches that shows a significant difference in bacterial number at 5th to 7th touch. But after 12 touches it was similar to the untreated condition.

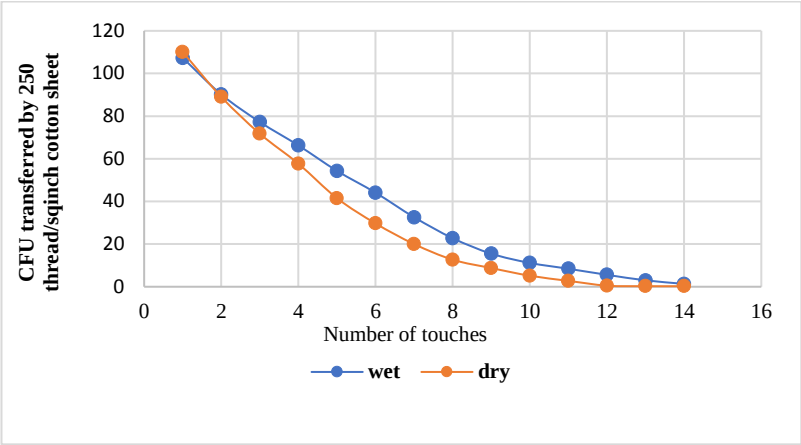

Figure-3: Transfer frequency of organisms by 250 threads /sq inch cotton with touches before and after treatment with 5% neutral detergent

Transfer through cotton sheet —150 threads/square inch

Biofilm bacteria were readily transferred from the biofilm covered coupon through the sheet and onto the hands. In 1st touch the number of CFU transferred to the hand was 114±16 and contaminated fingers then transferred bacteria to another 19 sequential surfaces. The number of bacteria was significantly related to the number of touches, with the number of bacteria transferred from the fingers, to the HBA, decreasing as the number of touches increased ($P<0.0005$) (Figure 4). Wetting the biofilm covered coupon with neutral detergent increased the number of bacteria transferred significantly ($P<0.0005$). CFU transferred in 1st touch was increased to 259±84 which was more than double of the CFU transferred before treatment. Most of the sequential touches following touching a dry coupon (through the sheet) transferred <100 CFU (Figure 4a) but after treatment (Figure-4b), 100-1000 colonies were transmitted from 100% coupon surfaces up to 5th touch.

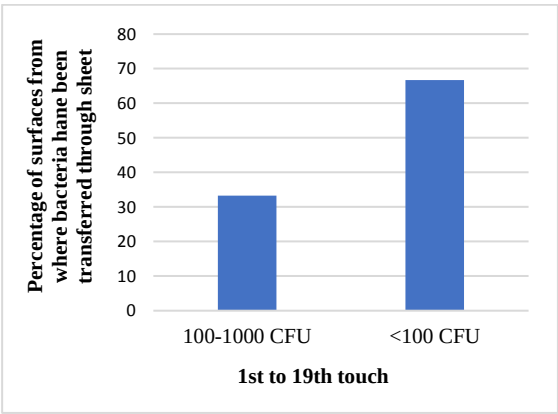

Figure 4a

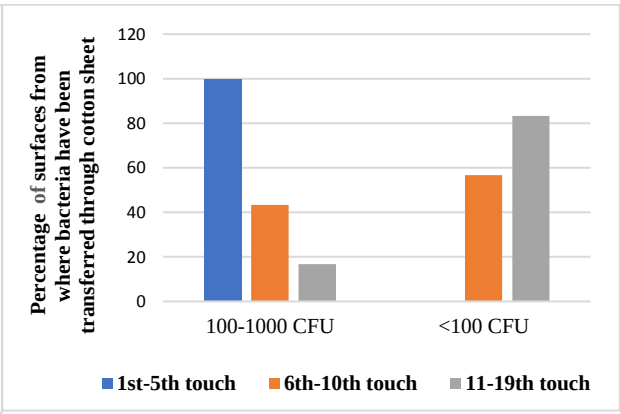

Figure 4b

Figure-4: Percentage of surfaces - biofilm bacteria have been transferred from –through 150 thread/ sq. inch cotton sheet before (4a) and after (4b) treatment with 5% neutral detergent ($P<0.0005$).

Transfer through cotton sheet— 250 threads/square inch

The hospital cotton sheet (250 threads/square inch) transferred significantly less bacteria from the biofilm covered coupons to the hands and thence to the HBA plates than the domestic (150 threads/square inch) sheet (P<0.005). Similar to above, wetting the coupon with neutral detergent increased the number of bacteria that were transmitted through the sheet onto the fingers (P<0.005), (figure 5). In 1st to 5th touch, before treatment, 10 to 30 colonies were transmitted in 8.3 % cases with <10 colonies transferred from most of the surfaces (Figure 5a). However, after treatment, 8.4% cases, the number of transmitted colonies were reached >30 colonies in first 5th touches (Figure-5b).

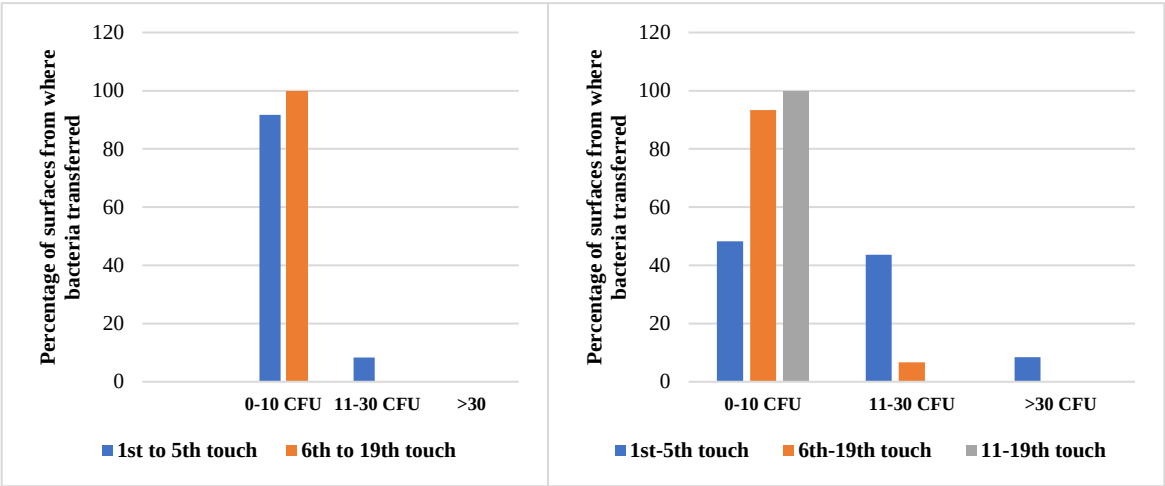

Figure 5a Figure 5b

Figure-5: Percentage of surfaces - biofilm bacteria have been transferred–through 250 thread/ sq. inch cotton sheet before (5a) and after 5b) treatment with 5% neutral detergent (P<0.0005).

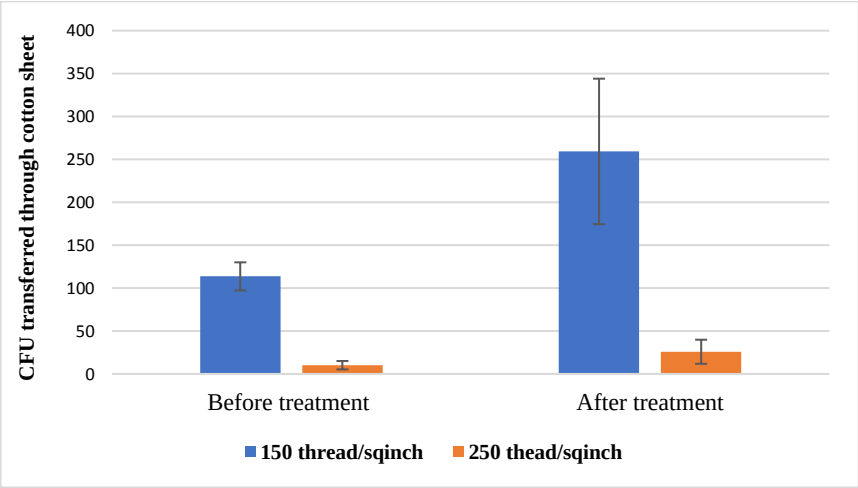

Figure 6: Comparison of number of organisms transferred from coupon surfaces in 1st touch, through 150 and 250 thread/ sq. inch cotton sheet, to the hand before and after wetting with 5% neutral detergent.

Estimated bacterial number in clinical biofilm contaminating patient bedding

Based on qPCR results, the mean number of bacteria contaminating impervious mattress covers was $6.4x10^4$ bacteria/cm^2 (range $9x10^2$ to $2.1x10^5$ bacteria/cm^2) whilst the mean number of bacteria contaminating impervious pillow covers was $4.1x10^4$ bacteria/cm^2 (range $4.8x10^3$ to $1.1x10^5$ bacteria/cm^2). Scanning electron microscopy showed that the impervious mattress covers were not smooth and that even when contaminated with low numbers of bacteria, bacteria and surrounding EPS were seen to have attached to the surface particularly in the natural pits formed in the material (figure- 7).

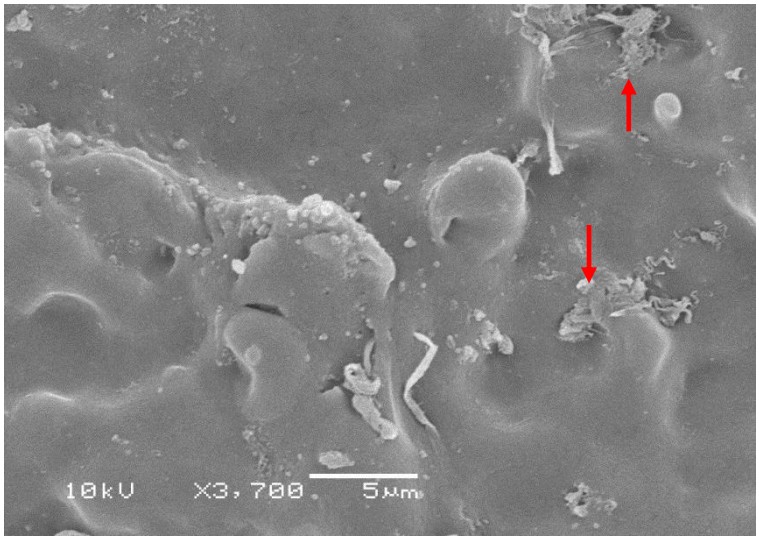

Figure 7: Scanning electron micrograph of an impervious mattress cover showing dips in the material and biofilm contamination (red arrows).

Discussion

In this study, *S. aureus* was selected as the representative biofilm-former as it is frequently isolated from HAIs. *S. aureus* is also a frequent contaminant on hospital surfaces (Huslage et al., 2013; Adams et al., 2017) and we found it in biofilms contaminating 50% of 44 samples taken from an ICU, including 8 of the 11 samples obtained from patient bedding. The average number of bacteria found on patient bedding by qPCR was $5.3x10^4$ bacteria/cm^2 which is less than the bacteria found on the coupon surface of $1.8x10^6$. However, we were trying to mimic the worst-case scenario and therefore used a higher number of bacteria. The transfer of *S. aureus* DSB grown on a plastic coupon to a cotton sheet and thence to HBA was investigated to determine if bed sheets can act as a fomite. We also investigated if DSB could penetrate the sheet and thus contaminate patient skin.

The data from this preliminary study implies that cotton sheets readily transfer bacteria from a source of biofilm onto another surface as cleaning is often hurried and insufficient in the clinical situation and that large numbers of bacteria can be on dry hospital surfaces. In addition, biofilm bacteria were able to penetrate the sheet and contaminate the investigators fingers. Once contaminated the sheet and hands were then able to transfer the bacteria to a further 19 surfaces. Increasing the thickness of the sheet from 150 (100 gm/M^2 dense) to 250 (155 gm/M^2 dense) threads/square inches significantly decreased the number of bacteria that were able to penetrate the sheet. However, wetting the source of the biofilm i.e. the coupon with neutral detergent increased both the transfer of bacteria onto the cotton sheets and through the cotton sheets onto hands.

The number of bacteria that were transferred through the sheets were sufficient to cause disease (Figure-6). This implies that in a clinical setting that transfer of organisms might occur from a biofilm contaminated bed mattress to the patient through cotton sheet. The suggested microbiological standard for hospital linen is 12 cfu/25 cm^2 (Standaert et al.1994). In this study, a much higher number of bacteria was transferred from a single biofilm source and suggests that a clean bed sheet could act as a vector for organism transmission within a very short period of time of being put onto a contaminated bed. The biofilm source in this study was coupons covered with a greater number of bacteria than what we found on clinical bedding. However, we were modelling a worse -case scenario.

This study also highlights the need for cleaning and disinfection of mattresses as detailed in society and government guidelines (NHMRC, 2010; **NHS UK**, 2017) In Australia, common practice is for mattresses to be terminally cleaned using medical surface detergents or detergent wipe in combination with disinfectant. The problem is that the impervious mattress covers are not completely smooth and biofilm forms readily in the dips. As seen with the clinical mattress samples examined the terminal clean doesn't remove all the bacteria, as 5/6 mattress covers grew bacteria and some of the contaminating biofilms incorporated MRSA, VRE and ESBL producing organisms (Hu et al., 2015). The use of removable and washable mattress covers has been advocated and has been shown to reduce the incidence of *C. difficile* by approximately 50% (Hooker et al., 2015). The use of such covers is likely to decrease the formation of biofilm.

In conclusion, biofilm develops on impervious mattress covers and is highly tolerant to disinfectants. Bacteria present in biofilms readily transfer to bed sheets which can then act as a fomite for the spread of HAI. Additionally, biofilm bacteria readily transfer through bed sheets and this transfer is increased if the biofilm is wet.

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3.5 Original paper

Title: Transmission of *Staphylococcus aureus* from dry surface biofilm (DSB) by different types of gloves

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Keywords:

Dry surface biofilms

Transmission

Gloves

Healthcare associated infections

Staphylococcus aureus

Summary

Background: Pathogens can survive for extended periods when incorporated into dry surface biofilm (DSB) formed on dry hospital surfaces exhibiting increased tolerance to cleaning agents and disinfectants. We hypothesise that DSB contaminated gloved hands of healthcare workers (HCW) may transmit pathogens for healthcare-associated infections (HAI's).

Method: *Staphylococcus aureus* DSB [approximately 10^7 colony forming units (CFU)] was grown in vitro on polycarbonate and glass coupons in a bioreactor over a period of 12 days. Transmission was tested with nitrile, latex and surgical gloves, by gripping DSB covered coupons, and then pressing finger tips onto the sterile horse blood agar surface for up to 19 consecutive touches and counting the number of CFU transferred. Coupons were immersed in 5% neutral detergent, to simulate cleaning and the experiment repeated.

Results: Surprisingly *S. aureus* cells were readily transmitted by all three types of gloves, commonly used by HCWs from one DSB touch to cause infection up to 19 individual touches. Six times more bacteria were transferred by nitrile and surgical gloves ($P<0.001$) when compared to latex gloves. Treating the DSB with 5% neutral detergent increased the transmission rate of DSB bacteria ten-fold.

Conclusion: *S. aureus* incorporated into environmental DSB and covered by EPS, readily contaminates gloved hands and can be transferred to another surface, thus confirming the possibility that DSB contributes towards HAI acquisition.

Introduction:

Healthcare associated infections (HAIs) are a worldwide health issue resulting in huge costs to the health system(Al-Tawfiq and Tambyah, 2014; Khan et al., 2017). The World Health Organization (WHO) reports that at one time point, 7% of hospitalized patients in developed countries and 10% of hospitalized patients in developing countries will acquire HAI (2014). In intensive care units, approximately 30% of patients will go through at least one episode of HAI, the longer the stay, more the risk of acquiring infection(Allegranzi et al., 2010). Recently in November 2016, for the first time, WHO released guidelines for prevention of HAI world-wide emphasising on the control strategies of pathogens' transmission(Organization, 2016).

The most important route of transmission of causative HAI pathogens is the contaminated hands of health care workers' (HCW)(Bhalla et al., 2004). Hand hygiene is accepted as the most significant intervention in the preventing HAI (Hygiene, 2017). Bacterial pathogens are transferred to HCWs hands directly by touching patients or after touching environmental surfaces including equipment, devices and furnishings (Bhalla et al., 2004; Dancer, 2004; Kramer et al., 2006; Oie et al.,2012) . Environmental contamination with vancomycin resistant *Escherichia coli* and *Staphylococcus aureus* play an important role in spreading infection via HCWs(Weinstein and Hota, 2004; Boyce, 2007). The 5 moments of hand hygiene dictate that HCWs perform hand hygiene, before and after touching the patient, before and after performing procedures, as well as after touching the patient environment. However, compliance with hand hygiene is often poor, especially when it comes to hand hygiene following touching items such as bed rails and chairs in the patient surroundings (FitzGerald et al., 2013; Kwok et al., 2016).

Infection control guidelines recommend the use of gloves as a practical means of preventing transmission of infective pathogens and decreasing transient contamination of HCWs hands(WHO, 2002; Fraise and Bradley, 2009; NHMRC, 2010). The transmission of pathogens is reduced with gloves as compared to bare hands, but not eliminated and improper use of gloves, such as failure to remove gloves after performing aseptic procedures, is associated with poor hand hygiene compliance(Girou et al., 2004) (Wilson and Loveday, 2014). An observational study found the rate of improper glove use to be as high as 64.4% among HCWs, resulting in possible microbial transmission in almost 1/5 of the time(Girou et al., 2004). Depending upon glove material, the transmission rate of planktonic *Staphylococcus aureus* varies between glove types(Moore et al., 2013). Rate of bacterial transmission was the least with nitrile when compared to vinyl and latex gloves.

Dancer et al emphasized the role of environmental cleaning in controlling HAI, however hospital cleaning alone (Dancer, 2009) fails to prevent the transfer of pathogens (Claro et al., 2014). Otter in his reviews has emphasised the contribution of surface bacteria in the nosocomial transmission of *Acinetobacter baumannii, Clostridium difficile, Pseudomonas aeruginosa* and multidrug resistant organisms like methicillin resistant *S. aureus* (MRSA) and vancomycin-resistant *Enterococci* (VRE)(Otter et al., 2011; Otter et al., 2013). Pathogens shed from infected patients have been shown to survive for up to 7 months (Kramer et al., 2006) on dry hospital surfaces whilst bacteria incorporated into dry surface biofilms (bacteria surrounded by extracellular polymeric substances – EPS) survive over 12 months(Hu et al., 2015). Environmental contamination, is thought to be responsible for new occupants in rooms previously occupied by patients suffering from VRE, MRSA or *C. difficile* being at higher risk of acquiring these pathogens(Boyce, 2007; Nseir et al., 2011; Shaughnessy et al., 2011).

The presence of biofilms on 93% of ICU furnishings suggest also that biofilms escape current cleaning and disinfection protocols and may contribute to nosocomial infection by providing a niche for bacterial persistence and release of free-floating bacteria into the environment(Hu et al., 2015). *S. aureus*, one of the most common pathogens causing HAI, was shown to be incorporated in >50% of ICU dry surface biofilm (DSB) samples(Hu et al., 2015). In this study we used our model of dry surface biofilm (Almatroudi et al., 2015) to determine if biofilm cells can be transferred from one surface to another and whether glove composition affects transmission rate.

Materials and Methods:

Test gloves

Three types of gloves were used to determine transmission rate of biofilm bacteria:

1) Nitrile gloves- latex free, single use, examination gloves (Promed Pty Ltd, Tanawha, Australia)
2) One Hundred percent (100%) latex gloves (Thermo Fisher Scientific Pty Ltd, Scoresby, Australia)
3) Sterile surgical non Latex neoprene (synthetic rubber) gloves (Ansell Gammex® PF DermaPrene® Glove, Richmond, Australia).

The non-sterile nitrile and latex gloves were sanitised by rubbing with 70% w/v ethanol until dry. To confirm sterility, both the gloved thumb and fore finger were pressed onto the surface

of a blood agar plate (BAP), (Edwards Group Pty. Ltd., Narellan, NSW) and incubated at 37° C for overnight.

The hydrophobicity of each glove material was determined three times using contact angle measurements. Briefly, a 10µl drop of water was placed on each glove and photographed using a side mounted camera. The contact angle was then measured using software application, Micro-soft Visio professional 2013.

Preparation of *Staphylococcus aureus* dry surface biofilm:

S. aureus ATCC 25923 DSB was grown on polycarbonate and glass coupons, in Centres for Disease Control biofilm reactor (CBR) (BioSurface Technologies Corporation, Montana, USA), over a period of 12 days as previously developed by Almatroudi et al (Almatroudi et al., 2015). In brief, biofilm was grown for 48 hr in 5% tryptone soya broth (TSB) following which biofilm bacteria were adapted to low water availability by an initial 48hrs of dehydration followed by three cycles of 6 hrs hydration interspersed with prolonged dehydration of between 42 and 66 hrs. The resulting biofilm contained approximately 10^7 colony forming units (CFU) and mimicked hospital dry surface biofilm particularly, regarding the presence of very thick EPS. The biofilm covered coupons were washed 3 times with PBS, whilst still in the rods, to remove planktonic and loosely attached bacteria.

Dry Surface Biofilm transfer test protocol:

The test coupon was then lightly gripped between gloved thumb and index finger tip and the rod lifted to a height of 30cm and returned to the start position (Figure 1). The contaminated finger and thumb tips were then serially pressed, up to 19 times, onto the surface of sterile horse blood agar (HBA) plates and the plates incubated aerobically for 48 hours. DSB transfer was tested for 12 coupons/ glove type.

Effect of wetting DSB with neutral detergent on the transfer of bacteria

The effect of neutral detergent on the transmissibility of DSB was tested by wetting biofilm covered coupons for 5 seconds with neutral detergent containing 10% ethoxylated Nonylphenol (Lemax, Whiteley Medical Pty. Ltd, Tomago, Australia). This was thought to mimick general wetting of surfaces during routine cleaning in the healthcare environment. Excess detergent was allowed to drain before the test procedure detailed above was repeated (n=12/glove type).

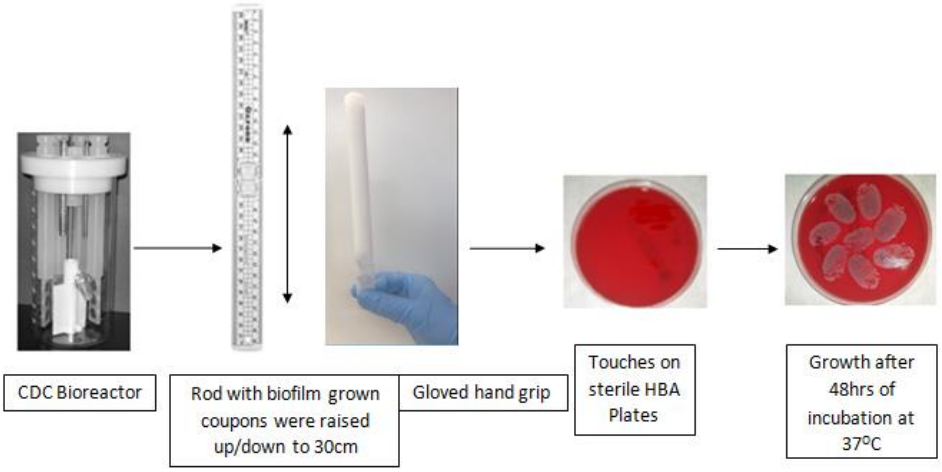

Figure1. Diagramatic representation of the experimental procedure of testing dry surface biofilm transmission

Statistical analysis:

The number of bacterial cells transferred was determined by counting CFU directly from HBA plates. Contact/agar plates have been previously used for environmental sampling in numerous scientific studies as reviewed by Galvin et al(Galvin et al., 2012). This method provides a reasonable estimate of bacterial numbers and is comparable in accuracy, to swab techniques (Galvin et al., 2012).

Initial control CFU/cm^2 of dry surface biofilm and total CFU/cm^2 transferred via each glove type separately was used to calculate the transfer rate(Perez-Rodriguez et al., 2007; Pérez-Rodríguez et al., 2008) according to the following formula:

$$TR = 100 \text{ X CFU blood agar/CFU on the coupon}$$

Data was statistically analysed using Microsoft Excel, 2013 and SPSS program. One way analysis of variance (ANOVA) was done on the data obtained, followed by post hoc Tukey test for comparison within two main groups; detergent treated and untreated. The data was then split into both detergent treated and untreated group subsets to assess the transfer by each glove type.

Results:

Readings obtained for contact angles of the three types of gloves tested for hydrophobicity are tabulated in Table 1. The greater the contact angle the more hydrophobic material is. Hence

latex gloves are most hydrophobic followed by nitrile gloves while surgical gloves are least hydrophobic.

Type of Glove	Contact angle	
	Mean	**STDEV**
Latex	111.01	9.64
Nitrile	88.81	7.02
Surgical	75.43	2.65

Table1. Contact angles for three types of gloves with mean of 3 replicates. STDEV=standard deviation.

Transfer rate of *Staphylococcus aureus* dry surface biofilm bacteria to microbiological media using three types of disposable gloves:

Touching DSB once was sufficient contact to transfer the biofilm bacteria from the coupon to the glove and thence to microbiological media for at least up to 19 times by all three types of gloves tested, (Figure 2). Latex gloves transferred significantly less bacteria than nitrile gloves which transferred significantly less bacteria than surgical gloves ($P<0.01$) (Table 2).
The "number" of touches were found to be significant a factor ($P<0.01$) affecting bacterial transfer. Significantly less bacteria were transferred per each touch as the number of times the fingers touched on the agar plate increased (Figure 3) ($P<0.01$). For nitrile gloves, and to a lesser extent for surgical gloves, a very high number of bacteria were transferred during the first 5 touches (Figure 3 and 4).

Glove type	Untreated		Detergent treated	
	Mean CFU transferred (Log_{10}) ± standard deviation (n=12)	**Transfer rate %**	**Mean CFU transferred (Log_{10}) ± standard deviation (n=12)**	**Transfer rate %**
Nitrile gloves	2.90±0.12	0.0103	3.76±0.66	0.0741
Latex gloves	2.50±0.60	0.0041	2.99±0.08	0.0126
Surgical gloves	3.02±0.30	0.0137	3.74±0.69	0.0705

Table 2. Mean number of *Staphylococcus aureus* dry surface biofilm cells transferred during 19 touches, by three types of commonly used gloves (n=12 glove type).

Effect of wetting with neutral detergent on transmission

Wetting the dry surface biofilm covered coupon with 5% detergent for 5 seconds significantly increased the number of bacteria transferred by latex, nitrile and surgical gloves 3, 5 and 7 times respectively (P<0.01) as shown in Figure 2. Latex gloves transferred significantly less bacteria from detergent treated DSB than either nitrile or surgical gloves (P<0.01) (Table 2, Figure 4). In contrast, to results obtained with untreated DSB, nitrile and surgical gloves transferred similar numbers of bacteria. Significantly more bacteria were initially transferred per touch but as the "number" of times the fingers were touched on the agar plate increased, less bacteria were transferred per touch (P<0.01). After the tenth touch, the average bacterial cell count transferred dropped drastically with all glove types as shown in Figure 4.

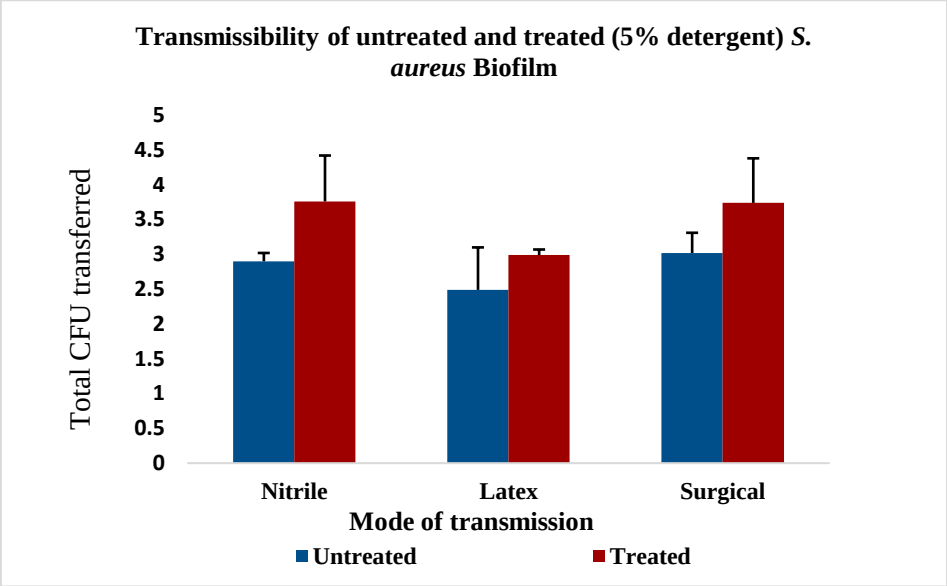

Figure 2. Mean number (colony forming units -CFU) of *S. aureus* DSB cells transmitted via nitrile, latex and surgical gloves before (untreated) and after treatment (treated) with 5% neutral detergent for 5 seconds. Starting number of biofilm bacteria 10^7 S. aureus /coupon.

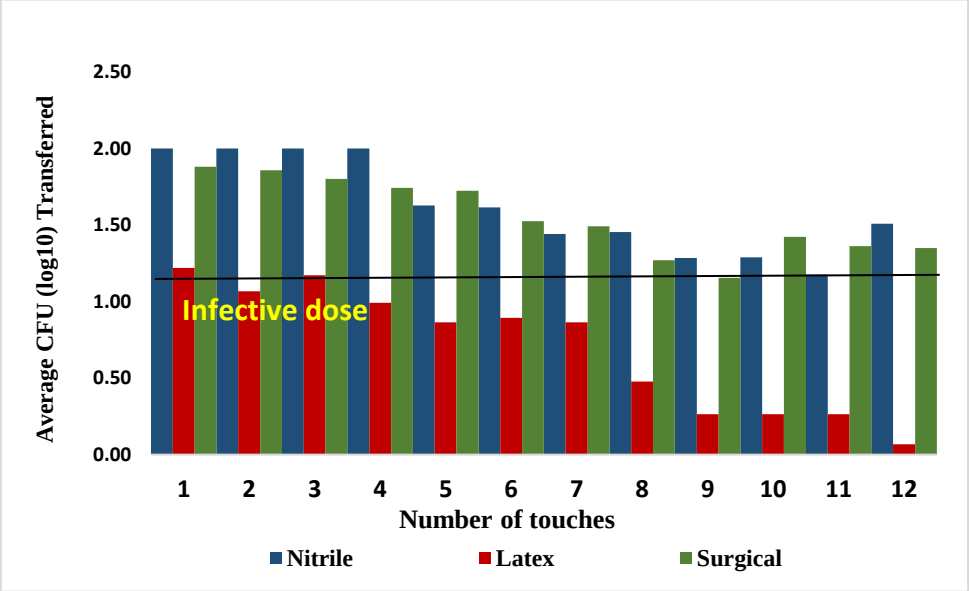

Figure 3. Number of bacterial cells (CFU) transmitted from untreated *S. aureus* DSB/touch. Black horizontal line in the graph indicates the infective dose (15 cells/cm^2 or 1.18 logs/ cm^2).

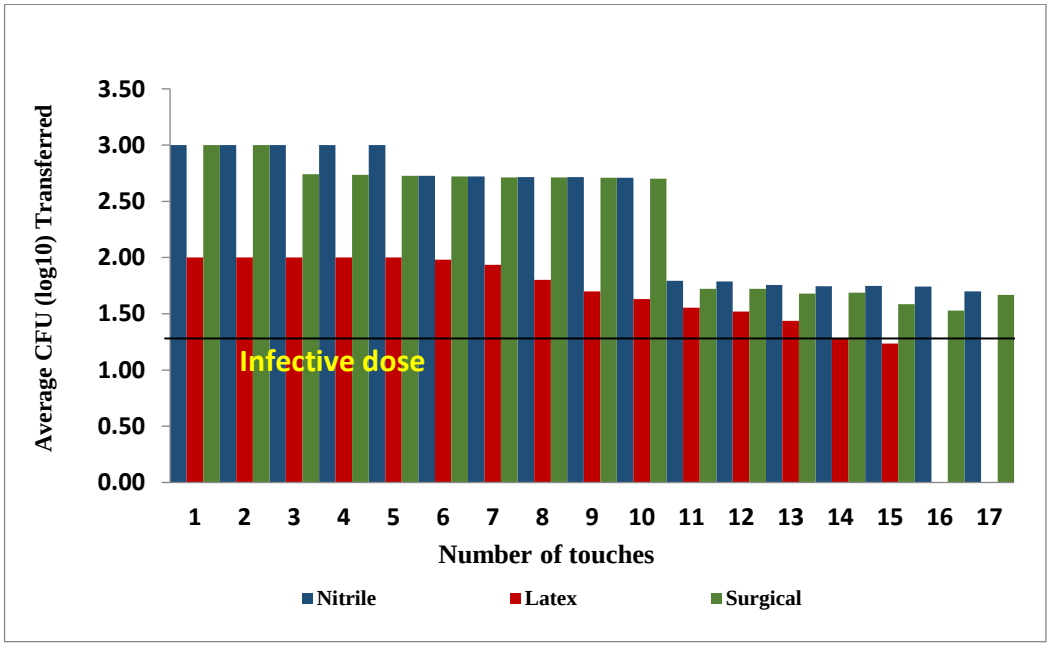

Figure 4. Number of DSB bacterial cells (CFU) transmitted from treated *S. aureus* DSB with the number of touches. The infective dose is 15 cells/cm^2 1.18 logs/ cm^2.

Discussion

Older scientific studies suggested that the hospital environment contributed negligibly to the acquisition of HAI (Mallison and Haley, 1981; Maki et al., 1982). This resulted in the frequency of hospital environmental sampling to decline, and eventually becoming restricted to endemic outbreaks (Sehulster and Chinn, 2003). However, over the last two decades the role of contaminated hospital surfaces in spreading HAIs has been reassessed (Otter et al., 2013).

The existence of DSB containing viable, multidrug resistant and sensitive organisms on high touch surfaces, in the healthcare environment has been confirmed (Hu et al., 2015; Russotto et al., 2015). Biofilms on dry hospital surfaces have been shown to be multispecies and over 50% of these have incorporated *S. aureus*, one of the most common pathogens causing HAI (Hu et al., 2015). Extensive literature exists showing that traditional hydrated biofilm is more tolerant to biocide action, including disinfection and this has recently been reviewed by Otter et al (Otter et al., 2015). A number of scientific studies indicate that there is poor hand compliance by HCWs (Chassin et al., 2015; Luangasanatip et al., 2015; Smiddy et al., 2015). An observational study conducted in respiratory and diabetic wards of Nottingham University hospital measured HCWs, patients' and visitors' compliance with 5 moments of hand hygiene over a period of 24hrs. Compliance by HCWs, patients' and visitors' with hand hygiene was least among doctors (47%) of all the HCWs while overall least compliance with hand hygiene was observed after contact with patient surrounding (50%) (Randle et al., 2010). Pathogens from hospital surfaces surrounding infected patients' such as bed rails, trolleys' and bed surfaces are potential source of HAIs (Huslage et al., 2010; Huslage et al., 2013). Hayden et al observed that 52% of HCWs entering with sterile hands contaminated them after touching the environment of VRE infected patients (Hayden et al., 2008). The actually measured poor hand hygiene compliance demonstrated by HCWs is often in contrast with official reports on hand hygiene compliance (Kwok et al., 2016).

Our current study confirms that viable bacterial cells from DSB touched once are easily transferred up to 19 other surfaces via gloved hands. We demonstrated that *S. aureus* DSB cells can be transferred by three commonly used gloves types worn by healthcare workers. The estimated infective dose for *S. aureus* is 15 cells/cm^2 (1.18 logs/cm^2) and is thought to be sufficient to cause infections in lesions (Foster and Hutt, 1960). The minimum number of bacterial cells transferred by each touch is far more than the minimum infective dose required for initiating HAI for all three types of gloves. Wetting the DSB with neutral detergent increased the transfer rate by 3 to 7 folds.

The adhesive forces between bacterial cells and contact surfaces mainly determine the bacterial transmission where detachment of cells from the donor is followed by these cells sticking to the recipient surface (Vermeltfoort et al., 2004). Surface factors such as hydrophobicity and roughness affect adhesion and hence the attachment/transmission of bacteria. Hydrophilic bacterial cells attach more efficiently to hydrophilic surfaces and vice versa. *S. aureus* being hydrophilic attaches readily to hydrophilic surfaces as compared to hydrophobic surfaces (An and Friedman, 1998; Knobben et al., 2007).

The three gloves types namely nitrile, latex and surgical gloves were non-porous and had smooth surfaces visually. The surgical glove was the most hydrophilic of the 3 gloves and also transferred the greatest number of bacteria from the DSB to the microbiological media. In contrast, latex gloves, being the most hydrophobic transmitted least number of bacteria from DSB irrespective of whether the DSB had been touched dry or treated with detergent. Hence latex gloves are most suitable for use in hospital environment owing to their low transfer rate of pathogens, but development of allergy to latex limits their use. Our results for latex gloves seem consistent with the study by Moore et al who measured transmission of planktonic methicillin resistant *S. aureus* (MRSA) by various types of gloves (Moore et al., 2013). Latex gloves transmitted the least MRSA from and to the fomite.

DSB was treated with 5% neutral detergent for 5 seconds to mimic wetting of surfaces during hospital cleaning. We hypothesise that moisture is readily absorbed into the biofilm structure which changes the semi dried biofilm's physiochemical properties to hydrated type increasing cell detachment and resulting in high transferral rate.

Conclusion

We have shown that although the bacteria incorporated into DSB are attached to a surface and each other, they are readily transferred to new surfaces by gloved hands. The number of bacteria transferred is increased if the DSB is exposed to detergent even for a short time. This finding suggests that removal of DSB as well as planktonic environmental contamination need to be addressed to ensure a safe hospital environment.

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Conflict of interest statement

Dr Greg Whiteley is the Managing Director and Dr Trevor Glasbey is the R&D Manager of Whiteley Corporation, a manufacturer of disinfectants and detergents for use in healthcare. Their roles did not lead to any bias in formulating, executing, analysing or writing up the research.

The authors declare that no competing interests exist.

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Part two: Inactivation of biofilm -heat treatment efficacy

3.1 Introduction

Despite of implementation of standard infection control strategy, SSIs are still prevalent in clinical settings. In fact, among HAIs, SSIs are the second most common type of infection that encountered and cause about 21.8% of infections (Magill et al., 2014). Several studies have reported that inappropriate cleaning of instruments for arthroscopy, urological procedure, orthopedics and ophthalmic surgeries resulted SSI (Kayabas et al., 2008; Dancer et al., 2012; Villar et al., 2015). If used in sterile body tissues these inadequately cleaned and sterilized medical devices pose a substantial risk of transmitting pathogens.

The risk to the patient from contamination of instruments and surfaces is compounded if bacteria form a biofilm. Attachment of microbial cells to the surface and then formation of extracellular polymeric substances (EPS) which enclose the bacteria makes their attachment to the surface secure. Bacteria in biofilm have high level of tolerance to disinfectant action that makes it difficult to eradicate (Zühlsdorf et al., 2002; Hadi et al., 2010; Otter et al., 2015d; da Costa Luciano et al., 2016). In the previous chapter we showed that DSB can be transferred from hard surfaces to the hands and thence to HBA plates. This suggests that DSB present on surgical instruments could be transferred into the patient. Biofilm has been shown to form in numerous surgical instruments over their lifetime(Costa et al., 2017).

To ensure the complete sterility of the surgical instruments, both organic and inorganic material must be removed as the presence of any soil will encourage the development of biofilm. EPS act as a protective barrier for biofilm bacteria against adverse environmental elements, making them more resistant to detergents, disinfectants and other sterilizing procedures and cannot be efficiently removed (Hadi, Vickery et al., 2010; Adukwu et al., 2015). It is unknown if the bacteria surrounded by EPS are more tolerant to heat in a similar fashion to disinfectant tolerance. The production of stress proteins can lead to an increase in planktonic cells tolerance to heat, but this increase is only of a few degrees and is irrelevant to the healthcare environment where heat is applied at much higher temperatures.

In this study we investigated the efficacy of heat to kill planktonic, traditional hydrated biofilm and DSB.

3.2 Original paper

Title: *Staphylococcus aureus* dry-surface biofilms are more resistant to heat treatment than traditional hydrated biofilms.

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Candidate contribution:

- Carried out in vitro experiments
- Determination of dry matter content of dry biofilm
- Determination of water content of dry biofilm
- Data analysis.

Author's contribution was 10%

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Staphylococcus aureus dry-surface biofilms are more resistant to heat treatment than traditional hydrated biofilms

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SUMMARY

Background: The importance of biofilms to clinical practice is being increasingly realized. Biofilm tolerance to antibiotics is well described but limited work has been conducted on the efficacy of heat disinfection and sterilization against biofilms.

Aim: To test the susceptibility of planktonic, hydrated biofilm and dry-surface biofilm forms of *Staphylococcus aureus*, to dry-heat and wet-heat treatments.

Methods: *S. aureus* was grown as both hydrated biofilm and dry-surface biofilm in the CDC biofilm generator. Biofilm was subjected to a range of temperatures in a hot-air oven (dry heat), water bath or autoclave (wet heat).

Findings: Dry-surface biofilms remained culture positive even when treated with the harshest dry-heat condition of 100°C for 60 min. Following autoclaving samples were culture negative but 62–74% of bacteria in dry-surface biofilms remained alive as demonstrated by live/dead staining and confocal microscopy. Dry-surface biofilms subjected to autoclaving at 121°C for up to 30 min recovered and released planktonic cells. Recovery did not occur following autoclaving for longer or at 134°C, at least during the time-period tested. Hydrated biofilm recovered following dry-heat treatment up to 100°C for 10 min but failed to recover following autoclaving despite the presence of 43–60% live cells as demonstrated by live/dead staining.

Conclusion: *S. aureus* dry-surface biofilms are less susceptible to killing by dry heat and steam autoclaving than hydrated biofilms, which are less susceptible to heat treatment than planktonic suspensions.

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Introduction

Heat is used in both the food manufacturing and health industries to sanitize surfaces and equipment. Wet heat, dry heat and pasteurization are all accepted methods, and sterilization in particular is well described in a number of interlocutory standards including ISO 14937:2009, and these methods remain important today [1,2]. Steam sterilization is faster and more reliable than dry heat and is favoured for its sterility assurance level (SAL) of $<1 \times 10^{-6}$ [1,3]. Pasteurization is intended to remove viable planktonic bacteria, and, for applications such as washer-disinfectors, the operating temperatures range from 70 to 90°C with holding times varying from 1 to 100 min [3,4]. Additionally, portable steam vapour systems have been suggested as suitable for decontaminating various healthcare surfaces [5].

Biofilm formation is the principal reason for limiting the duration of manufacturing runs in the food industry; transmission of micro-organisms from biofilms to food products impacts on their quality and safety [6]. In healthcare, dry hospital surfaces, heat-sensitive instruments, and even steam-sterilized instruments have been visually confirmed to be contaminated with biofilms [7–10]. There are more than 234 million medical operations performed globally each year, thus making validation of sterilization an international imperative [11].

We hypothesized that organisms are probably more heat-tolerant when in a biofilm and that dry-surface biofilms are probably more heat-tolerant than hydrated biofilms. *Staphylococcus aureus* is present in 50% of biofilms found on hospital surfaces, it contaminates surgical instruments post use, and it is a frequent causative agent of healthcare-associated and food-borne infections [12–14]. This project investigated the efficacy of heat to kill planktonic organisms compared to bacteria incorporated into traditional hydrated biofilm and biofilm subjected to periodic dehydration to create dry-surface biofilms [15].

Methods

Biofilm formation

Staphylococcus aureus ATCC 25923 biofilm was produced on polycarbonate coupons (1.27 cm diameter) in the CDC biofilm reactor (BioSurface Technologies Corp., Bozeman, MT, USA). During production, filter-sterilized air-conditioned air (average relative humidity 66%) was pumped into the biofilm generator continuously at 3 L/min flow. All batch phases were incubated at 35°C and subjected to shear (baffle rotation at 130 rpm). Traditional hydrated biofilm, representative of biofilm found in fluid environments, was produced in three batch phases, each lasting 48 h. Growth media was initially 50% tryptic soya broth (TSB) which was reduced to 20% TSB for the second and third batch phases.

Dry-surface biofilm was formed under conditions of alternating nutrition and dehydration, as described previously, and as would be expected for biofilm formed on instruments or surfaces subjected to periodic cleaning [15]. Briefly, dry-surface biofilm was grown in 5% TSB for a period of 12 days; the initial batch phase of 48 h was followed by 48 h of dehydration at room temperature (22–25°C). An additional three cycles of batch growth for 6 h alternated with prolonged dehydration phases of 66, 42 and 66 h.

Following biofilm formation, coupons were removed from the holding rods and washed three times in phosphate-buffered saline to remove any loosely attached organisms. The average number of colony-forming units (cfu) of biofilm bacteria per control coupon was 1.67×10^7 (range: 1×10^7 to 2.88×10^7) for hydrated and 1.2×10^7 (range: 1.08×10^7 to 1.5×10^7) for dry-surface biofilms. We chose to test heat efficacy against 10^7 organisms as we have previously confirmed that clinical biofilm samples ($N = 32$) are contaminated with large numbers of bacteria (mean: 5.5×10^5 bacteria/cm^2; range: 4.2×10^2 to 1.6×10^7) [16].

The dry-matter content and hence the water content of the traditional hydrated and dry-surface biofilm grown on eight rods and 24 coupons was determined by drying the biofilm to constant weight at 100°C.

Efficacy of heat disinfection/sterilization

The efficacy of wet and dry heat against stationary-phase planktonic organisms, hydrated biofilm and dry-surface biofilm was determined. Test temperatures ranged from 60 to 134°C and duration of treatment varied from 10 min to 2 h (Table I). Process indicators (Class I Browne chemical and second-generation Browne biological indicators; Albert Browne International Ltd, Leicester, UK) were used in every autoclave load. Lids of Bijou bottles were left loose to ensure direct steam penetration to the sample.

The efficacy of wet heat to inactivate stationary-phase planktonic *S. aureus* was tested against an overnight culture containing 2×10^8 cfu in 2 mL of 100% TSB.

The efficacy of dry heat to inactivate biofilm bacteria was tested by placing biofilm-covered coupons into dry sterilized Bijou bottles with no media, and into a fan-forced hot-air oven at the appropriate temperature.

The efficacy of wet heat to inactivate biofilm bacteria was tested by placing biofilm-covered coupons in individual sterile Bijou bottles containing 4 mL of 100% TSB (which permitted post-sterilization culture without the need to open the Bijou bottle, thereby decreasing possible post-sterilization contamination). Bijou bottles were placed in a heated water bath (temperatures: 60–100°C) or in an autoclave.

The efficacy of a chemical biocidal step, using 1 mol/L NaOH for 1 h, was also assessed [17].

Determination of residual viability: log_{10} reduction in titre

Planktonic cultures and hydrated biofilm were tested in triplicate ($N = 3$) whereas four replicates of dry-surface biofilm were used. Half of the dry-surface biofilm coupons were subjected to 10 min of sonication (42–47 kHz) prior to heat treatment to simulate a cleaning step. All conditions were tested on two separate occasions.

Following heat treatment, control ($N = 5$ per experiment) and test coupons were subjected to 5 min of sonication, 2 min of vortex/shaking, serial ten-fold dilution and plate culture [15]. The $\log_{10}$ reduction in bacterial titre was determined for both planktonic and biofilm cells by dilution and standard plate culture at 37°C. Sigma Plot 13 (Systat Software, Inc., San Jose, CA, USA) was used to test for significant differences in cfu of untreated and treated coupons using Student's *t*-test if

Table I
Log_{10} reduction in titre immediately following heat treatment, in the presence or absence of moisture, of planktonic, traditional hydrated, and dry-surface biofilm

Condition and temperature	Heat duration	Log_{10} reduction in titre		
		Planktonic cfu	Hydrated biofilm cfu	Dry-surface biofilm
Dry				
60°C	1 h	6.22	7.22	0
80°C	1 h	8.30	7.22	0.12
100°C	10 min	8.30	7.22	0.88
121°C	20 min	8.30	7.22	1.88
4 mL TSB				
60°C	1 h	8.30	7.22	7.08
80°C	1 h	8.30	7.22	7.08
100°C	10 min	8.30	7.22	7.08
Autoclave in 4 mL TSB				
121°C	20 min	8.30	7.22	7.08
121°C	30 min	8.30	7.22	7.08
121°C	1 h	8.30	7.22	7.08
121°C	2 h	8.30	7.22	7.08
134°C	4 h	8.30	7.22	7.08
134°C + NaOH	1 h	8.30	7.22	7.08

Starting colony-forming units (cfu): planktonic control 8.30 log_{10}; hydrated biofilm control 7.22 ± 0.16 log_{10} cfu/control coupon; dry-surface biofilm control 7.08 ± 0.04 log_{10} cfu/control coupon.

normally distributed and a Mann–Whitney rank sum test if non-normally distributed.

Determination of residual viability: viability staining and recovery from heat damage

Live/Dead BacLight 7012 viability stain (Molecular Probes, Invitrogen, Mulgrave, VIC, Australia) on control ($N = 3$) and heat-treated coupons ($N = 2$ for dry-surface biofilm and $N = 1$ for hydrated biofilm) was conducted for each temperature tested. The viability stain was incubated with coupons for 60 min to ensure penetration of stain into biofilms. Syto® 9 labels live bacteria with green fluorescence whereas the propidium iodide component labels membrane-compromised bacteria with red fluorescence. The green/red fluorescence was measured, and background fluorescence deducted, for five different areas of each coupon, which gave an average total surface area analysed of 197,000 μm^2. Images of control and non-sonicated heat-treated coupons were built with 0.2 µm optical sections using the Imaris 7.7.2 software (Bitplane, Zurich, Switzerland). The proportion of live cells was analysed using ImageJ software (1.46r, National Institute of Health, USA) and Imaris 7.7.2 software [15].

The ability of planktonic cells ($N = 5$), hydrated biofilm ($N = 5$), and dry-surface biofilm ($N = 4$) to recover from heat damage was determined by prolonged culture in 4 mL TSB. Bacterial recovery (media turbidity) was observed daily, without opening the bottles to avoid contamination, for up to 30 days. If growth was detected the contents were subcultured on horse blood agar plates and inspected for typical staphylococcal colony morphology. For about one-third of the isolates, additional confirmatory tests including DNase reaction (Dnase Test Agar, BD PA-255506) and positive reaction with Prolex™ Staph Xtra Latex Kit (Pro-lab Diagnostics), as well as real-time polymerase chain reaction targeting *S. aureus nuc* genes and *femA* genes was conducted [18,19].

Results

Traditional hydrated biofilm was found to contain 90% water whereas the dry-surface biofilm contained only 61% water.

Determination of residual viability: log_{10} reduction in titre

All biological indicators were negative and chemical indicators were positive, indicating that the intended performance characteristics of the autoclave were within acceptable ranges required for Australian compliance.

Incubating planktonic cultures at 60°C or 80°C for 1 h, 100°C for 10 min, or autoclaving resulted in >8 log_{10} reduction in titre and, despite prolonged culture in TSB, none of these cultures recovered from heat treatment ($P < 0.05$).

Subjecting traditional hydrated biofilm to dry heat at the conditions tested resulted in a significant >7 log_{10} reduction in titre ($P < 0.001$). In contrast, dry-surface biofilm survived all treatments of dry heat tested. The most efficient dry-heat treatment tested was 121°C for 20 min and resulted in <2 log_{10} reduction in titre. Exposing hydrated or dry-surface biofilm to all conditions of wet heat, including autoclaving, or combining NaOH treatment with autoclaving resulted in >7 log_{10} reduction in titre ($P < 0.001$) (Table I).

Determination of residual viability: viability staining and recovery from heat damage

The majority of bacteria in hydrated (94%) and dry-surface control biofilms (97.5%) were alive. Live bacterial cells (viable but non-culturable organisms: VBNC) were also detected by viability staining for all heat treatments tested for both types of biofilms (Figure 1).

Following heat treatment, not all 'live' stained bacteria could reproduce and reconstitute the biofilm despite prolonged

post-treatment culture, suggesting that they were terminally damaged (Figure 2, Table II). None of the traditional hydrated biofilm recovered following autoclaving although there was some recovery at lower temperatures of wet heat and at all temperatures of dry heat tested including 121°C dry heat for 20 min (Table II). Dry-surface biofilm recovered at all temperatures tested except when subjected to prolonged autoclaving at 121°C for 1 or 2 h, or to autoclaving at 134°C (Table II). The time to recovery in days, and release of planktonic organisms into the media, increased with increasing treatment temperature and was longer when treated with moist heat.

At 60°C and 80°C dry heat, the traditional hydrated biofilm took four to eight days to recover whereas at higher temperatures and when treated with moist heat the minimum time to recovery was eight days. Recovery time for dry-surface biofilm was five to eight days at 60°C, 11–12 days at 80°C, 15–16 days at 100°C, but up to 25 days when subjected to autoclaving at 121°C for 20 and 30 min.

Discussion

The hypothesis that *S. aureus* dry-surface biofilms are more heat-tolerant than *S. aureus* hydrated biofilms, which are in turn more heat-tolerant than planktonic suspensions of this species, is supported by our findings.

Subjecting dry-surface biofilm to dry heat (up to 121°C for 20 min) had minimal effect, reducing bacterial numbers by only 2 $\log_{10}$, whereas planktonic cultures and hydrated biofilm counts were reduced by more than 8 $\log_{10}$ and 7 $\log_{10}$, respectively (Table I). In contrast, the application of wet-heat treatments reduced culturable bacterial numbers to zero (Table I) despite the presence of VBNC forming between 46% and 78% of the remaining biofilm (Table II). For some wet-heat treatments (up to 100°C), the VBNC bacteria incorporated into both hydrated and dry-surface biofilm reconstituted the biofilm which eventually released planktonic cells (Table II). Planktonic cells treated with wet heat failed to recover.

Another important finding was that cells within dry-surface biofilms survived up to 30 min of autoclaving at 121°C and reconstituted the biofilm, eventually releasing planktonic cells. This emphasizes the importance of stringent pre-cleaning prior to decontamination or autoclaving. In contrast, autoclaving at the higher temperature of 134°C for 4 min — frequently used for sterilizing surgical instruments — prevented biofilm reconstitution and release of planktonic organisms.

Although it is widely known that bacteria residing in biofilms have increased protection from desiccation and biocide action, conditions necessary for killing biofilm using heat have received little attention. In this study, we compared heat inactivation of stationary-phase planktonic bacteria with bacteria residing within hydrated biofilm and those contained within dry-surface biofilm. Stationary-phase planktonic bacteria were used, as they are more heat-tolerant than exponentially growing cells [20].

At temperatures widely used within Australian healthcare settings, pathogenic vegetative organisms such as *S. aureus* should be killed. Our results confirm that planktonic *S. aureus* is killed at temperatures ≥80°C (Table I).

In contrast, this study found that *S. aureus* survival following heat treatment is increased when incorporated into biofilms (Table II). Some dry-surface biofilm cells were even able to survive autoclaving at 121°C for up to 30 min.

The degree of heat tolerance exhibited by the biofilms was related to the non-temperature-related growth conditions experienced prior to heat application, in that the dry-surface biofilm, subjected to periodic dehydration, survived heat

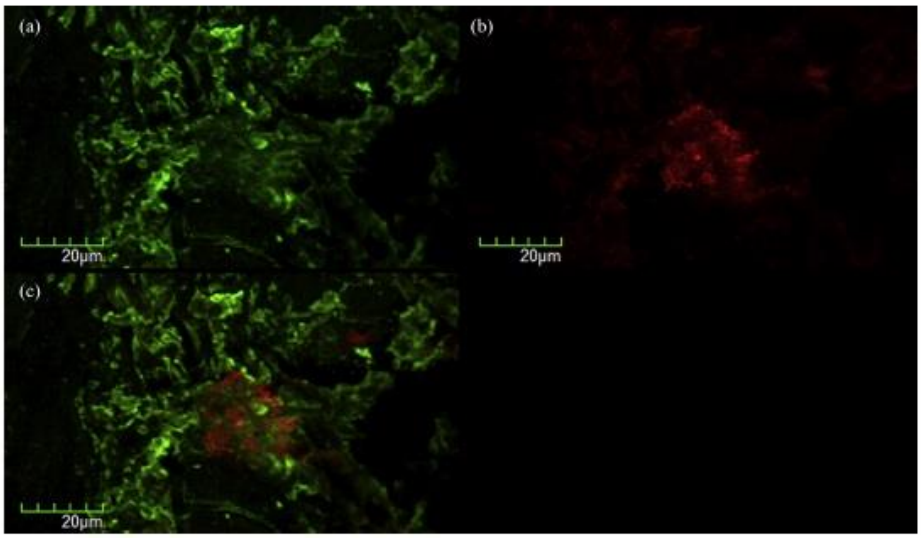

Figure 1. Confocal laser scanning microscope photograph of biofilm after applying sterilization protocol (sonication + autoclaving at 121°C for 20 min). Green cells (a) are live bacteria and red cells (b) are dead bacteria; (c) combined image. Although immediately culture-negative, this biofilm recovered and released planktonic organisms.

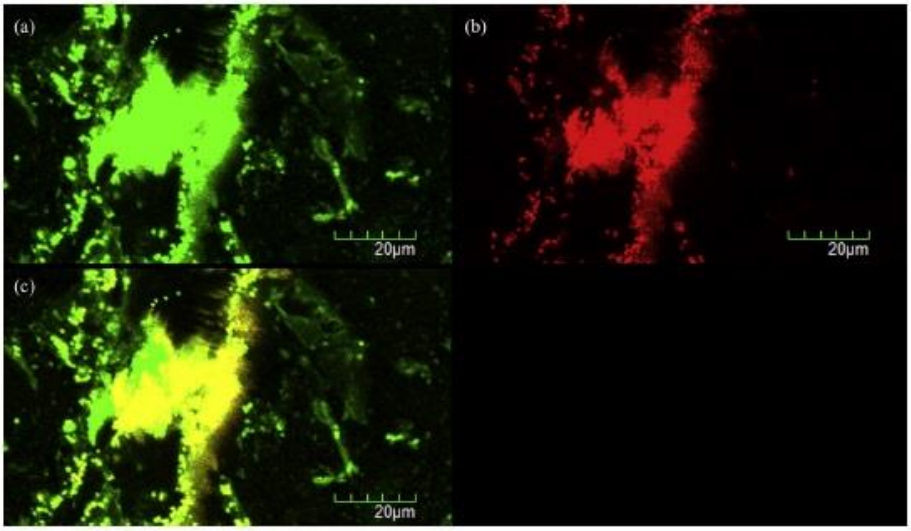

Figure 2. Confocal laser scanning microscope (CLSM) photograph of biofilm after autoclaving at 134°C for 4 min. Green cells (a) are live bacteria and red cells (b) are dead bacteria; (c) composite image combining (a) and (b). Merged panel shows that both live and dead bacteria occupy the same vertical space. Although CLSM detected some live bacteria, these were unable to recover even with prolonged culture.

Table II
Number of coupons where the biofilm recovered and released planktonic bacteria post treatment (hydrated biofilm $N = 5$/treatment, dry-surface biofilm $N = 4$/treatment) and the percentage of viable cells/coupon immediately post heat treatment (positive control $N = 3$; hydrated biofilm $N = 1$ and dry-surface biofilm $N = 2$) as determined by live/dead staining and confocal microscopy

Conditions	Time	No. of coupons recovered		Viable cells (%)	
		Hydrated biofilm ($N = 5$ per treatment)	Dry-surface biofilm ($N = 4$ per treatment)	Hydrated biofilm	Dry-surface biofilm
Positive control		–	–	94%	97%
Dry heat					
60°C	1 h	4	4	51%	57%
80°C	1 h	1	4	71%	46%
100°C	10 min	1	4	55%	78%
100°C	1 h	ND	4	ND	53%
Autoclave					
121°C	20 min	0	4	60%	74%
121°C	30 min	0	4	59%	68%
121°C	1 h	0	0	48%	65%
121°C	2 h	0	0	43%	62%
134°C	4 min	0	0	50%	68%
1 mol/L NaOH then autoclave 134°C	1 h + 1 h	ND	0	ND	66%

ND, not done.

treatment better than the traditional hydrated biofilm and both survived better than stationary planktonic cultures.

In some bacterial species, application of sub-lethal stress has been shown to increase the ability of planktonic cells to withstand subsequent challenge with more extreme stress due to the production of stress proteins [21–23]. For example, subjecting planktonic *Listeria monocytogenes* to osmotic, pH, and sub-lethal heat stresses may increase survival when subsequently exposed to temperatures of 57°C [24]. Similarly, biofilm bacteria can adapt to stresses; heat-shock treatment (70°C for 30 min) of legionella biofilm increased its tolerance to challenge with 70°C heat [25].

It is likely that, in our laboratory-grown dry-surface biofilm, periodic dehydration would lead to osmotic stress during periods of low water availability and this may help explain the difference in susceptibility between traditional and dry-

surface biofilm. In planktonic *L. monocytogenes*, osmotic stress caused higher resistance to thermal stress than prior heat shock [24]. Stressing planktonic *Salmonella* spp., osmotically, by culturing in different solutes including sugars, glycerol or salt, also led to an increase in survival when challenged with heat at 54°C [26].

Steam under pressure easily killed traditional *S. aureus* biofilm. Likewise, traditional *L. monocytogenes* biofilm is easily inactivated by wet heat [27]. Although dry-surface biofilm was immediately culture-negative following autoclaving at 121°C for 30 min, prolonged culture allowed detection of surviving bacteria. A contributing factor towards the increased tolerance of the dry-surface biofilm to steam under pressure may be its low water content, being only 61% compared to the traditional hydrated biofilm which had 90% water content. One of the principal mechanisms responsible for the resistance of spores to heat is their low water content [28].

Comparison of results presented in Tables I and II shows that there is a discrepancy between immediate culture, viability testing, and prolonged culture. The principle behind viability staining is that intact cell walls prevent propidium iodide entering whereas compromised walls allow ingress, staining them red. However, live biofilm cells may be in the VBNC state and hence do not grow on the routine bacterial media whereas prolonged culture in liquid media increases their detection. When bacteria encounter severe environmental surroundings, they shift into a VBNC state, which is thought to be a survival tactic [29]. Although some studies suggest that bacteria in VBNC states are not pathogenic, several reports have found that micro-organisms are virulent [30,31].

The presence of live cells in hydrated and dry-surface biofilm samples subjected to autoclaving where there was a lack of reconstitution despite extended culture suggests that membrane disruption might not be the primary mechanism of heat inactivation in *S. aureus* biofilms. A similar disparity between plate counts and viability staining was found for planktonic *S. aureus* when heated to 80°C [32]. However, this effect may be strain-specific as Kramer and Thielmann found a concordance of results with complete inactivation, membrane rupture, and uptake of propidium iodide in planktonic cells of *S. aureus*, *Listeria innocua*, *Salmonella enterica*, and *Escherichia coli* when heat-treated at 80°C [33].

Our study appears to provide further evidence of the validity of our dry-surface biofilm model in testing various sterilization and disinfection conditions [15]. The study is the first demonstration that *S. aureus* in a dry-surface biofilm is more resistant to heat treatment, including autoclaving, than planktonic suspensions of this species. We did not investigate dry-heat sterilization conditions as our aim was to compare heat tolerance of biofilms with planktonic organisms, and the efficacy of wet and dry heat in conditions that destroy planktonic organisms.

As this study only investigated one strain of *S. aureus*, it remains to be seen if these findings apply to other species of bacteria important in healthcare-associated infections such as *Enterococcus* spp., Enterobacteriaceae, and *Pseudomonas aeruginosa*. Further research should be undertaken to establish if hydrated and dry-surface biofilms of other bacteria and fungi are similarly heat-tolerant. Additionally, further research is needed into real-life dry-surface biofilms on a range of real-life surfaces and instruments which are then subjected to sterilization and pasteurization. The application of steam decontamination of environmental surfaces in the presence of biofilm-mediated bacteria should also be undertaken.

In conclusion, incorporation of *S. aureus* into biofilms increases its tolerance to inactivation by heat. This underlines the critical importance of removal of all organic debris prior to decontamination or sterilization or steam treatment of surfaces. The presence of biofilms is likely to reduce the efficacy of pasteurization, sterilization, and steam treatments.

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Conflict of interest statement

G.W. is the Managing Director of the Whiteley Corporation, a manufacturer of disinfectants and detergents for use in healthcare. His role did not lead to any bias in formulating, executing, analysing, or writing up the research.

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Chapter four: Developing novel chemistries for removing environmental surface biofilms to reduce hospital acquired infections

4.1 Introduction

Cleaning means the removal of contaminants and is necessary to ensure that, disinfection and sterilisation procedures can function to maximum efficacy. In the early 19th century, use of biocides in the healthcare setting commenced (Rotter, 1998). Since then, in health care settings, biocides are used extensively for different purposes such as sterilization of medical devices, disinfection of surfaces, and skin antisepsis (Maillard, 2005). The importance of disinfectants for environmental decontamination is increased as a consequence of increased occurrence of multi-resistant microorganisms associated with high rates of healthcare acquired infection (HAI) worldwide (Donskey, 2013). Many studies have provided evidence of the importance of environmental surfaces in the transfer of microorganisms and HAI (Otter et al., 2011). Thus, the significance of surface disinfection is increasingly accepted and has been included in a number of current national and international infection control policies and recommendations.

The activity of a biocide depends on the biocide itself and the target microorganisms. The degree of disinfection is affected by a number of factors, that may reduce or nullify the disinfectant efficacy. Factors such as the concentration of and exposure time to the germicide, type and level of microbial contamination, the organic and inorganic load, temperature, pH etc. have been shown to affect efficacy (Russell, 2008). Of these the type and level of microbial contamination and organic and inorganic load are considered highly important and as such are their inclusion is detailed in government standards for registration of biocides (TGA, 2009).

The presence of organic soil strongly affects iodophor and chlorine disinfectants (Pryor and Brown, 1975) for example the presence of 2% milk soil reduces the efficacy of hypochlorite solution by factor 8 log10 numbers due to reaction with the disinfectant (Bloomfield and Miles, 1979). While addition of 2% bovine serum albumen binds up to 80% of the active ingredients in ready to use quaternary ammonium compound (QAC) and in the presence of human serum, 10 to 100 times more QAC was required to achieve a definite level of disinfection. (Jono et al., 1986). Hard water also reduces the effectiveness of disinfectants (Davis, 1990; Holah, 1995). Therefore, to achieve optimum cleaning and disinfection, soil removal is the utmost necessity.

Biocides have important role in the prevention of transmission of microorganisms in the healthcare environment and HAI. Thus, many standards regarding the antimicrobial activity of disinfectants have been established by many government and professional organisations (Abreu et al., 2013). Continuous effort to improve manual disinfection of surfaces are needed to reduce the rate of HAIs (Boyce, 2016).

Therefore, the aim of this study was to assess the potential of current and novel chemical disinfectants and detergents to remove and/or kill *Staphylococcus aureus* dry surface biofilm (DSB). The most sensitive method for measuring disinfectant activity is to determine residual bacterial viability by standard culture and plate counting. However, to measure the efficacy of detergents or disinfectants to remove soil optimisation of techniques was necessary due to the small amount of soil components present in biofilm. In our study, we tried the following procedure to establish a useful method to assess the efficacy of detergents.

4.2 Original paper

Title: The effect of disinfectant formulation and organic soil on the efficacy of oxidising disinfectants against biofilms (submitted).

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Summary

Background: Biofilms that develop on dry surfaces in the healthcare environment have increased tolerance to disinfectants. We compared the activity of formulated oxidizing disinfectants versus products containing only active ingredients against *Staphylococcus aureus* dry surface biofilm (DSB).

Methods: DSB was grown in the CDC bioreactor with alternating cycles of hydration and dehydration. Disinfectant efficacy was tested before and after treatment with neutral detergent for 30 seconds and in the presence or absence of soil. Biofilms were treated for 5 minutes with peracetic acid (Surfex and Proxitane), hydrogen peroxide (Oxivir and 6% H2O2 solution) and chlorine (Chlorclean and sodium dichloroisocyanurate [SDIC] tablets). Residual biofilm viability and mass were determined by plate culture and protein assay respectively.

Findings: Biofilm viability was reduced by 2.8 Log10 for the chlorine-based products and by 2 Log10 for Proxitane but these products failed to kill any biofilm in the presence of the soil. In contrast, the formulated Surfex completely inactivated biofilm (6.3log10 reduction in titre) in the presence of soil. H2O2 products had little effect against DSB. Biofilm mass removed in the presence and absence of soil was <30% by chlorine and approximately 65% by Surfex. Detergent treatment prior to disinfection had no effect.

Conclusion: The additives in fully formulated disinfectants can act synergistically with active ingredients and thus increase biofilm killing whilst decreasing the adverse effect of soil. We suggest that purchasing officers seek efficacy testing results and consider whether efficacy testing has been conducted in the presence of biological soil and/or biofilm.

Key Words

Oxidising disinfectants
Disinfectant efficacy
Biofilms
Disinfection
Dry hospital surfaces
Removing biofilms

Introduction

Hospital acquired infections (HAI), particularly with multi drug resistant organisms (MDROs) are the significant contributors to morbidity and is a major risk factor for mortality (Brusaferro et al., 2015). Multiple predisposing factors contribute to the emergence and spread of MDROs such as unjustified or incorrect use of antibiotics, improper hospital cleaning and lack of hand hygiene compliance. An estimated 20-40% of HAI are caused by infectious agent transmission via the hands of health care personnel (Weinstein, 1991). As hands are just as likely to become contaminated from the environment as touching the patient (Bhalla et al., 2004) proper implementation of environmental cleaning and disinfection is of upmost importance (Carling and Bartley, 2010). For some organisms, the healthcare environment plays a key role in facilitating their transmission (Dancer, 2014). The risk of acquiring methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin resistant *Enterococcus* (VRE), extended spectrum β-lactamase producing coliforms (ESBL), *Acinetobacter spp*. and *Clostridium difficile* infections is increased over 2-fold if the previous occupant of that room had the infection (Mitchell et al., 2015).

Under suitable hospital settings organisms can proliferate and survive for prolonged periods of time on environmental surfaces, increasing the probability of transmission to patients. The presence of biofilms on dry hospital environmental surfaces has been confirmed (Hu et al., 2015; Johani et al., 2017). These dry-surface biofilms (DSB) have been shown to be composed of multiple species normally found in both environmental and pathogenic niches and include MDROs such as MRSA, VRE, *Acinetobacter* and ESBL producing Gram negative organisms (Hu et al., 2015). Within DSB, bacteria are highly protected from desiccation with approximately 50% surviving over 12 months without nutrition or hydration (Hu et al., 2015). Bacteria incorporated into traditional hydrated biofilms have increased tolerance to removal by cleaning agents (Hadi Vickery et al., 2010) and disinfectants (Otter et al., 2015; Almatroudi et al., 2016). However, Almatroudi et al. (2016) has shown *S. aureus* DSB to have more tolerance to chlorine disinfection than traditional biofilm, and may, therefore, act as a constant source of pathogenic bacteria.

Typically, disinfectants used in a healthcare environment in Australia are classified as Hospital Grade disinfectants. These disinfectants may be used for the disinfection of environmental surfaces such as walls, floors, benchtops etc. Hospital Grade disinfectants are not however, intended for use on medical devices such as non-critical or semi-critical devices. These medical devices require disinfection using instrument grade disinfectants. These are classified as Low level, Intermediate level and High level instrument grade disinfectants. The choice of

instrument grade disinfectant is typically governed by the Spaulding classification, see Table I (AS/NZS, 2014).

In order to be approved and registered by the Australian Therapeutic Goods Administration, a hospital grade is required to pass the TGA Disinfectant Test, and a bacteriocidal carrier test such as the AOAC Hard Surface Carrier test (see Table I) (TGA, 2009). The TGA test requires challenging diluted disinfectant with a planktonic bacterial inoculum (2x108 – 2x109 organisms) and measuring viability after a given time. Following this a second challenge inoculum is added and the viability determined after a given time. The bacteria tested include Pseudomonas aeruginosa, Proteus vulgaris, Escherichia coli and S. aureus (TGA, 2009). Depending on the product label the test is conducted under either Option A (no organic soil) or Option B (ie addition of organic soil) with Option B being more reflective of clinical conditions than Option A.

Despite the recommendations of the Australian and other jurisdictional regulators, to date there is little or no guidance on disinfectants capable of disrupting biofilm. The ISO standard for automated endoscope reprocessors (ISO 15883-4: 2008) does mandate a cleaning efficacy test against a traditional hydrated model biofilm soil, and several detergent systems with claims against the Annex F biofilm soil in ISO TS 15883-5: 2006 are available on the market (The International Organization for Standardization, Geneva, 2009).

Disinfectants used in hospital such as, alcohol, quaternary ammonium compounds (QAC), oxidizing agents are expected to be effective against organisms in the hospital environment. However, to date, there are no cleaning and/or disinfecting products demonstrated to remove dry surface biofilm from hospital environmental surfaces. Failure to eradicate biofilm and thus pathogens from environmental surfaces, is a great challenge to HAI. Therefore, the aim of this study was to assess the efficacy of three commonly used oxidising agents (actives), peracetic acid, hydrogen peroxide and chlorine against S. aureus DSB and to determine if non-active additives, added to disinfectant formulations, affected efficacy of actives.

Materials and Methods

Bacterial culture preparation

S. aureus (ATCC 25923) dry surface biofilm (DSB) was grown in vitro on polycarbonate coupons (Bio Surface Technologies Corporation) in the CDC bioreactor over a period of 12 days as detailed previously (Almatroudi et al., 2015). Briefly, growth was initiated with 10^8 *S. aureus* and grown in 5% w/v tryptone soya broth (TSB) with shear (provided by baffle rotation

at 130 revolutions per min) for 48-hour batch phase at 35ºC, after which the media was drained, and the biofilm dehydrated for 48 hours at room temperature (22-25 ºC) with filter-sterilised air-conditioned air (average relative humidity 66%) pumped into the bioreactor at 3L/min. An additional three cycles of batch growth (5%TSB, shear, 35ºC for 6 hours) alternated with prolonged dehydration phases of 66, 42 and 66 hours at room temperature and resulted in an average of 2.078×10^6 (Log_{10} 6.30 ± 0.127) CFU of *S. aureus*/ control coupon (n=29).
An overnight culture of *S. aureus* (ATCC 25923) in TSB was used for planktonic challenges.

Test disinfectants

The products used in this study were of two types: fully formulated products and close generic equivalents (Table II). Formulated products were Surfex, (Whiteley Medical, North Sydney, Australia), Chlorclean (Guest Medical, Aylesford, Kent, United Kingdom), and Oxivir Tb (Diversey Australia Pty Ltd, Smithfield, NSW, Australia).

Surfex, a low level instrument grade disinfectant, comprises a powder blend consisting of a hydrogen peroxide source (sodium percarbonate), an acetyl source (tetraacetylethylenediamine, or TAED), chelating agents, and sodium dodecyl sulphate (SDS), which on initial dissolution in water releases a mixture of approximately 1000mg/L hydrogen peroxide and 2100mg/L peracetic acid. The product also has specific claims against a range of organisms and is also indicated the disinfection of environmental surfaces.

Chlorclean is a tabletted hospital grade disinfectant comprising sodium dischoroisocyanurate (**SDCI**) formulated with a foaming anionic surfactant (sodium toluenesulfonate) and binders (adipic acid) which on dissolution in water releases 1000ppm chlorine. The product is a listed hospital grade disinfectant, meaning the product does not have specific claims.

Oxivir Tb is a ready to use hospital grade disinfectant solution comprising 0.5% hydrogen peroxide, formulated with other proprietary ingredients. This product is an example of the "Accelerated® Hydrogen Peroxide" technology licensed from Virox Inc.(Ramirez and Omidbakhsh, 2003). This product is a registered disinfectant, thus has specific claims against a range of organisms.

Generic equivalents of these three disinfectants were Proxitane (Solvay Interox, Botany, NSW, Australia), an equilibrium solution of hydrogen peroxide (27% w/w), acetic (7.5% w/w) and peracetic acid (5.0% w/w), which on dilution in water to give a 4% v/v solution contained

mixture of 10,000mg/L hydrogen peroxide and 2200mg/L peracetic acid on dilution to give a 4% v/v solution in water; an unformulated SDIC tablet (Redox Chemicals, Minto, NSW Australia) containing only sodium diisocyanurate that, on dissolution in water released 1000mg/L; and a 6% solution of hydrogen peroxide (Gold Cross, Biotech Pharmaceuticals Pty Ltd, Laverton North, Victoria, Australia).

Table 1: Disinfectants tested in this study

Product	**Composition**	**Concentration of active ingredients (at use)**
Formulated products		
Surfex powder	Sodium percarbonate 49% Tetraacetylethylenediamine 27% SDS 0.65% Chelating agents 7.9%	1100ppm hydrogen peroxide 2200ppm peracetic acid
Chlorclean tablet	sodium dischoroisocyanurate >30% sodium toluenesulfonate 5-10% adipic acid <12%	1000ppm chlorine
Oxivir Tb Ready to use solution	0.5% (5000ppm) Accelerated® hydrogen peroxide + surfactants	5000ppm hydrogen peroxide
Generic equivalents		
Proxitane solution	Hydrogen peroxide 27% Acetic acid 7.5% Peracetic acid 5%	10,080ppm hydrogen peroxide 2200ppm peracetic acid
20g SDIC tablets	Sodium diisocyanurate	1000ppm chlorine
6% hydrogen peroxide solution	6% hydrogen peroxide	0.6% (6000ppm) hydrogen peroxide

All disinfectants were dissolved or diluted in hard water which was prepared by dissolving 0.304g anhydrous $CaCl_2$ and 0.065g anhydrous $MgCl_2$ in distilled water to make one litre (Hadi Vickery et al., 2010).

Experimental protocol for testing disinfectant efficacy against DSB

Experimental protocol for testing disinfectant efficacy against planktonic and DSB. The efficacy of test disinfectants to kill control planktonic bacteria and biofilm was measured in the presence and absence of organic soil (5% bovine calf serum [BCS] and 10% bovine serum albumin [BSA] in phosphate buffered saline [PBS]). The effect of prior treatment of biofilm with a neutral detergent reconstituted to manufacturer's instruction (Speedy Clean, Whiteley Medical, North Sydney, Australia) on disinfectant efficacy was also tested (Figure 1). Each condition was tested with five replicates for determining residual bacterial number (colony forming units – CFU) and five replicates for determining residual protein contamination.

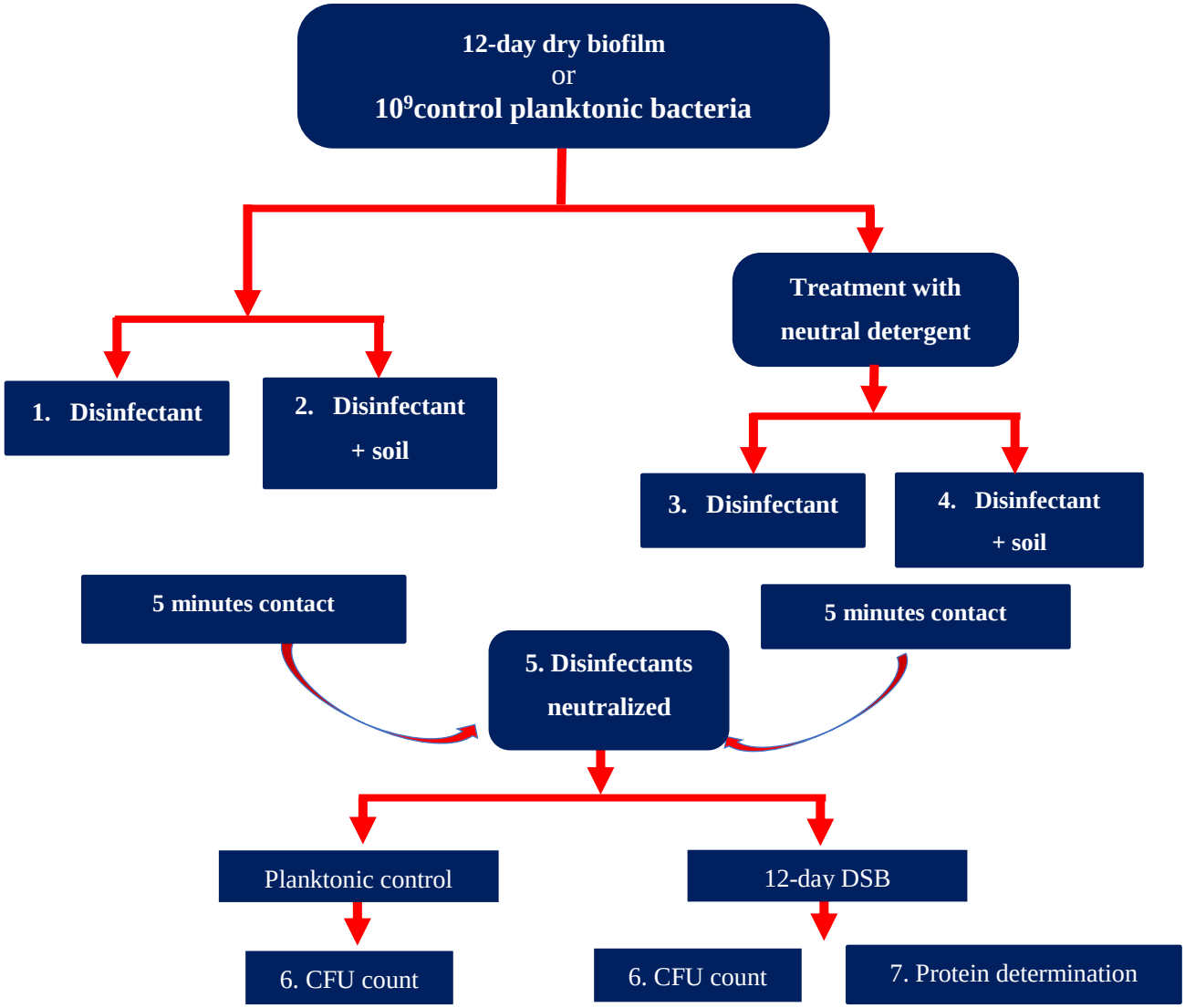

Figure 1: Experimental protocol for disinfection testing

Protocol for efficacy testing against planktonic and biofilm bacteria

The following protocols were followed for efficacy testing of disinfectants against planktonic and DSB. Reaction time was 5 minutes for all tested disinfectants as instructed by manufacturer.

a) Disinfectant efficacy in the absence of organic soil was tested by mixing 1 ml of test disinfectant (all disinfectants) with 1 ml of hard water and immediately adding 10µl of TSB containing approximately 10^9 planktonic bacteria for the planktonic challenge or a biofilm coated coupon for the DSB challenge, for 5 minutes contact time (n=5/disinfectant). (Figure 1, box 1).

b) Disinfectant efficacy in the presence of organic soil was tested by mixing 1 ml of test disinfectant (all disinfectants) with 1 ml organic soil and immediately adding 10µl of TSB containing approximately 10^9 planktonic bacteria for the planktonic challenge or a biofilm coated coupon for the DSB challenge, for 5 minutes contact time (n=5/disinfectant) (Figure 1, box 2).

c) We confirmed that the neutral detergent had no biocide action by mixing 10µl of TSB containing approximately 10^9 bacteria with either 1 ml of Speedy Clean for 30 seconds or hard water (positive control) followed by serial dilution and plate culture (results not shown). The effect of prior biofilm contact with neutral detergent on disinfectant efficacy was tested by soaking a DSB covered coupon in 1 ml of Speedy Clean for 30 seconds, the coupon was removed from the detergent and immediately added to the disinfectant test mixes (Chlorclean, SDIC and Surfex) in the absence of organic soil (n=5/disinfectant) (Figure 1, box 3) or in the presence of organic soil (n=5/disinfectant) (Figure 1, box 4). The DSB coated coupons were left in contact with the disinfectant for five minutes.

d) For parts a-c, at the end of the 5 minutes contact time, disinfectant activity was completely inactivated by the addition of 1 ml of neutraliser containing 1% sodium thiosulphate, 6 % Tween 80, 5% Bovine Calf Serum (BCS) and 10% Bovine Serum Albumin (BSA) in Phosphate Buffered Saline (PBS) (Figure 1, box 5).

e) Residual bacterial viability for planktonic control was determined by serial 10-fold dilution and overnight plate culture at 37_oC and CFU determination (Figure 1, box 6). Biofilm viability for DSB was determined by subjecting control and test coupons to

sonication at 80 kHz for 20 minutes prior to serial 10-fold dilution and overnight plate culture at 37$_o$C and CFU determination (Figure 1, box 6).

f) The experiment was repeated and the amount of residual protein contaminating disinfected coupons was determined using a Bicinchoninic Acid (Micro BCA) Assay (Figure 1, box 7).

Controls:

The positive controls for the planktonic challenge (5 replicates for each disinfectant) were subjected to the same treatments as described above but biocides were replaced with hard water. Positive (DSB covered coupons) and negative (clean sterile coupons; 3 for each disinfectant) controls were subjected to the same treatments as described above but biocides were replaced with hard water.

Neutralisation control: Confirmation that disinfectant activity was completely inactivated by the neutraliser was achieved by the addition of 1 ml of the neutraliser to the disinfectant test mixture prior to adding a DSB covered coupon and reacting for 5 minutes prior to CFU determination (n=10/test disinfectant) (results not shown).

The amount of residual protein contaminating coupons was determined by alkaline hydrolysis of the biofilm as described by Li et al (2006) followed by the Micro BCA assay (Micro BCATM Assay; Thermo Scientific). Briefly, each coupon was rinsed in 10 ml of PBS three times and transferred to individual McCartney bottles containing 1mL of ice-cold 20 mM 2MMorpholino-ethane sulfonic acid 0.9% saline. A 120µL aliquot of 30% NaOH was added, the samples sonicated at 60oC for 1 hour, vortexed and then incubated at 30oC for 30 minutes followed by incubation in a boiling water bath for 15 minutes. The samples were cooled and 86µL of 32%HCl added prior to centrifuging at 13,000 rpms in a bench top centrifuge for 5 minutes. A 1 ml aliquot of the supernatant was used for protein determination using the according to manufacturer's instructions. Residual protein contaminating samples was determined by measuring sample absorbance at 562nm wavelength, subtracting the absorbance of negative control coupons (n=3) and calculating protein concentration (µg/mL) using a standard curve prepared using the kits' standard.

Statistical Analysis

A one way analysis of variance (Anova) combined with the Holm-Sidak all pairwise multiple comparison procedure was used to test for significant differences in Log10 reduction in titre

utilising the SigmaPlot 13 (Systat Software, San Jose, Ca) statistical package. A Mann-Whitney Rank Sum test was used to test for significant differences in the Log10 reduction in microbial titre between coupons subjected to prior detergent treatment and no detergent treatment.

Results

Disinfectant efficacy in the presence and absence of soil.

S. aureus planktonic

In the absence of organic soil and with a five minutes contact time all the disinfectants, used in this study, killed 7 Log_{10} of planktonic organisms. The formulated peracetic acid disinfectant Surfex was unaffected by organic soil, whereas the genetic disinfectant Proxitane's efficacy was greatly reduced. The efficacy of hydrogen peroxide and chlorine based disinfectants were also highly affected by the presence of organic soil (see Figure 2).

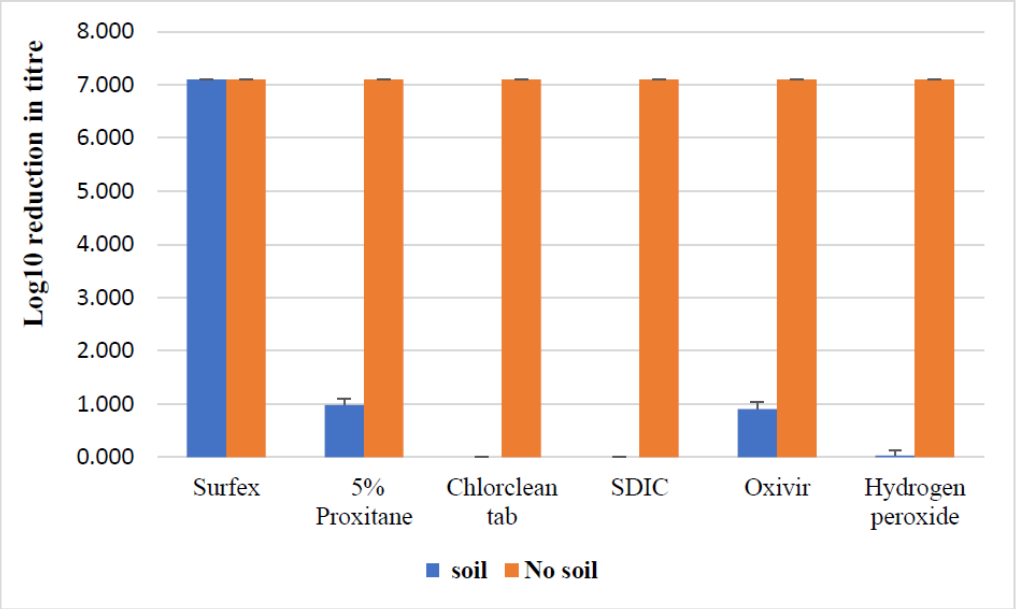

Figure 2. Log10 reduction in planktonic *S. aureus* titre following 5 min contact with disinfectants containing peracetic acid and hydrogen peroxide (Surfex, Proxitane), chlorine (Chlorclean, SDIC) and hydrogen peroxide (Oxivir, hydrogen peroxide) as active ingredients. Disinfectant efficacy was determined in hard water with and without added biological soil.

S. aureus DSB

Positive control DSB coupons had a mean of 2.08×10^6 (Log_{10} 6.32 ± 0.127) CFU of *S. aureus* per coupon (n=29). In the absence of organic soil and with a five minutes contact time the chlorine-based disinfectants, SDIC and Chlorclean reduced biofilm viability by 2.8 Log_{10} (P<0.001). For both SDIC and Chlorclean disinfectant efficacy was significantly decreased in the presence of soil resulting no reduction in titre (P<0.001). In contrast, the addition of the organic soil had no effect on the efficacy of Surfex, completely inactivating DSB resulting in >6 Log10 reduction in titre (P<0.001) (Figure 3). Whilst the generic equivalent to Surfex, Proxitane significantly reduced CFU 4.15 Log10 (P<0.002) in the absence of soil, it failed to kill DSB in the presence of soil. Chemistries based solely on hydrogen peroxide performed poorly against DSB, with only Oxivir Tb reducing biofilm counts by approximately 1 Log_{10} (P=0.01) in the absence of soil and the presence of soil inactivated Oxivir Tb. Generic hydrogen peroxide had no activity. In the absence of soil, Surfex killed 3.5 Log10 (>3000) fold more biofilm bacteria than the next best products and > 6 Log10 more in the presence of soil (P<0.001). In the absence of soil, chlorine-based products (Chlorclean and SDIC) killed significantly more DSB than Proxitane (P<0.001), which killed significantly more bacteria than Oxivir Tb (P<0.001) which in turn had greater efficacy than generic hydrogen peroxide (P<0.001) (Figure 3).

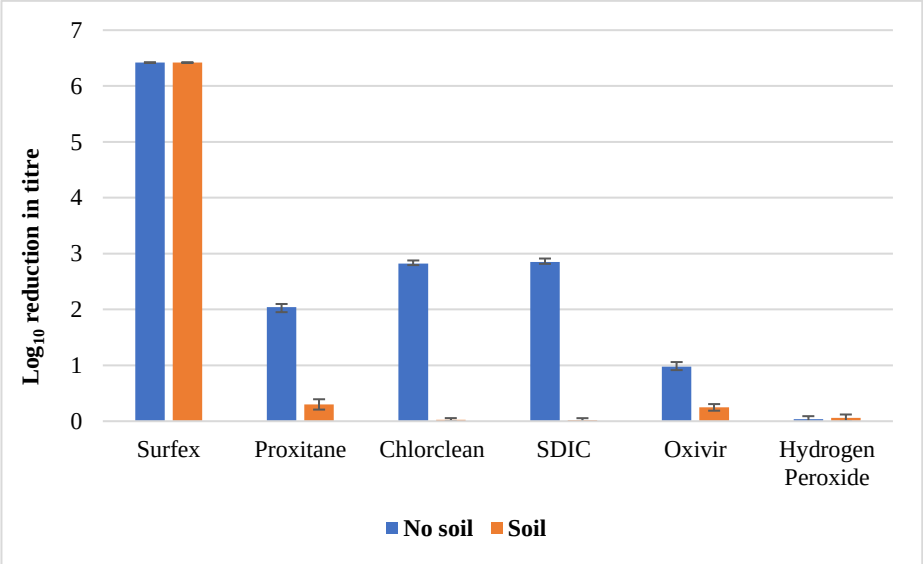

Figure 3. Log10 reduction in biofilm titre following 5 min contact with disinfectants containing peracetic acid and hydrogen peroxide (Surfex, Proxitane), chlorine (Chlorclean, SDIC) and hydrogen peroxide (Oxivir, hydrogen peroxide) as active ingredients. Disinfectant efficacy against Log10 6.32 *S. aureus* DSB was determined in hard water with and without added biological soil.

Disinfectant efficacy following detergent treatment in the presence of absence of soil.

Treatment of biofilm covered coupons with detergent prior to disinfection in the absence of soil, marginally increased the number of biofilm bacteria killed, by chlorine-based products, Chlorclean and SDIC but this was not significant (Figure 4). There was no improvement in kill by prior detergent treatment in the presence of soil. As Surfex resulted in complete kill (>6Log_{10} reduction in titre) under all conditions tested, the effect of prior biofilm contact with detergent was unable to be measured.

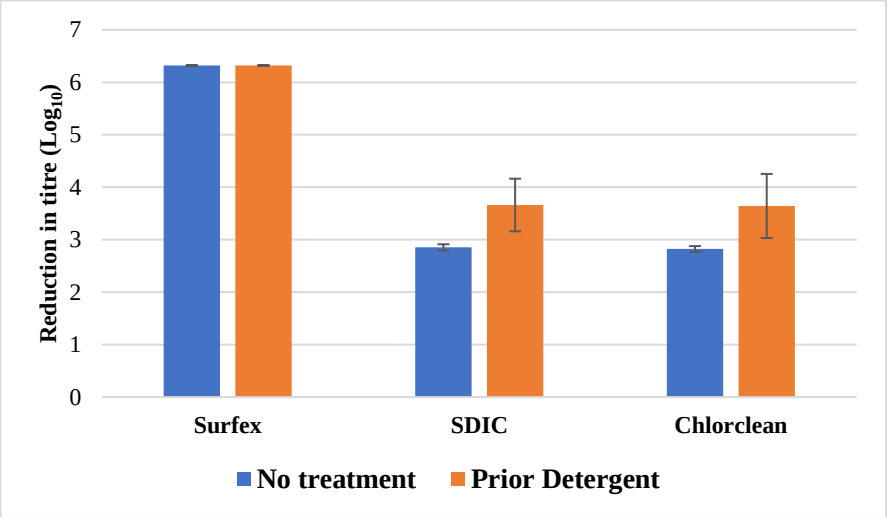

Figure 4: Reduction in DSB titre (Log10), in the absence of biological soil, obtained with (orange boxes) and without (blue boxes) prior biofilm contact with detergent (Speedy clean for 30 seconds) followed by Surfex, SDIC and Chlorclean disinfection for five minutes contact time.

Disinfectant efficacy in removing biofilm mass

The ability of the disinfectants to remove DSB biofilm was evaluated by determining the amount of biofilm protein remaining on the coupons following treatment. Percentage biofilm removal for Surfex in the presence and absence of soil was 64.7% and 65.3% respectively whereas, the reduction in biofilm mass by chlorine-based disinfectants was 17.6% and 22.14% for Chlorclean tablet and 13.12% and 29.71% for SDIC in the presence and absence of soil respectively (Figure 5). As the bacterial viability reduction rate was very low for Proxitane and H2O2 based disinfectants, it was assumed that these disinfectants would have no significant effect on biofilm mass and thus residual protein determination was not conducted for these disinfectants.

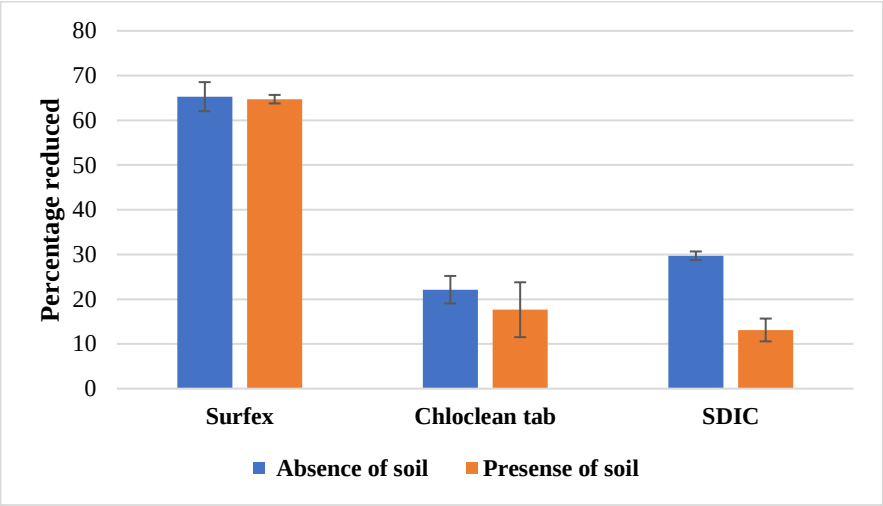

Figure 5: Percentage reduction of biofilm mass (protein) after disinfection with Surfex Chlorclean tablet and SDIC for five minutes in the presence and absence of soil.

Discussion

In this study, *S. aureus* DSB model (Almatroudi et al.,2015) was chosen for testing hospital surface disinfectants as 50% of clinical biofilms incorporate *S. aureus* (Hu et al., 2015) which commonly causes HAI (Tong et al., 2015). The efficacy of three formulated disinfectants, based on three differing active ingredients (chlorine, hydrogen peroxide and peracetic acid) along with generic (unformulated) solutions containing these three actives were evaluated. In this manner, the excipient (non-active) ingredients, as well as the active ingredients themselves could be evaluated. We tested in the presence of organic soil as combined cleaning/disinfecting systems are becoming more popular, in that clinical surfaces are often not precleaned prior to disinfection. Thus, the efficacy testing in the presence of large amounts of organic soil is more reflective of worse-case clinical conditions.

We evaluated three formulated, commercially available disinfectant systems, each of which contained an oxidising biocide, along with other ingredients such as surfactants. The effect of addition of the proprietary ingredients to disinfectant efficacy was evaluated by comparing the formulated disinfectants with generic equivalents in a bid to determine if biofilm removal is due to the active ingredient alone or if proprietary ingredients act in synergism with the active ingredient. The outstanding performer in this study was Surfex which completely inactivated the DSB in the presence or absence of soil. The formulated chlorine-based product Chlorclean, as well as unformulated SIDC tablets were the next best performers, although they killed significantly less biofilm bacteria (3 Log_{10}) than Surfex ($P<0.001$) and only in the absence of

soil. Previous studies have demonstrated that, chemicals such as hypochlorite are consumed by the surface layers of the biofilm causing depletion of the neutralizing capacity before the disinfectant can penetrate into deeper layers (Chen and Stewart, 1996) making traditional hydrated biofilm more tolerant than planktonic cells to these disinfectants (reviewed in Otter). However, a study on the efficacy of hypochlorite against DSB was found DSB as more tolerant to hypochlorite than traditional hydrated biofilm (Almatroudi et al., 2016).

Even in the absence of soil, the hydrogen peroxide-based disinfectants killed significantly less biofilm bacteria than disinfectants based on chlorine or a combination of peracetic acid and hydrogen peroxide ($P<0.001$). Oxivir killed approximated 1 Log_{10} of the biofilm bacteria while hydrogen peroxide solution had no effect, however, Oxivir's manufacturer's recommended contact time for killing bacteria is 10 not five minutes as used in the study and this could explain its lower performance. However, even a contact time of 5 minutes is probably excessive given the way that dry hospital surfaces are cleaned. The majority of disinfectants have no residual effect and remain active only when wet.

The differences in kill rate between Surfex (formulated additives) and Proxitane (no additives) suggests that the activity of Surfex against DSB may be governed not only by the active ingredients (hydrogen peroxide and peracetic acid), but also by other factors such as the added surfactants or excipients, chelating agents or its solution pH. Surfactants may increase diffusion of the active ingredients into the biofilm (due to a lowering of the solution surface tension, and hence improved wetting of the biofilm surface). Increased diffusion is likely to result in increased biofilm kill as all these tested disinfectants, in the absence of organic soil, can kill 7 Log_{10} of planktonic organisms. Chelating agents complex any calcium and magnesium ions present in the hard water, plus any other interfering metals often present in tap water such as iron, manganese and thus increase disinfectant performance in hard water. Additionally, the source of peracetic acid in the two disinfectants is different, which under certain circumstances eg disruption of Proxitane equilibrium may affect levels of active ingredients. Proxitane is an equilibrium mixture formed by the reaction between hydrogen peroxide and acetic acid according to the following formula: $H_2O_2 + CH_3CO_2H \rightleftharpoons CH_3CO_3H + H_2O$ (Ramirez and Omidbakhsh, 2003). However, in Surfex, the PAA is generated by the reaction of hydrogen peroxide with tetraacetylethylenediamine (TAED) (Glasbey, 2015). The source of hydrogen peroxide in Surfex is sodium peroxycarbonate, a 2:3 complex of hydrogen peroxide and sodium carbonate that releases the hydrogen peroxide on dissolution in water.

Except for Surfex, the efficacy of disinfectants was significantly decreased by the addition of soil with little or no reduction of viable bacteria load observed. This result is in agreement with most reports of chlorine disinfectants, where serious loss of efficacy has been demonstrated by the presence of organic matter (Lambert and Johnston, 2001) and hard water (Davis, 1990; Holah, 1995). Both hydrogen peroxide and peracetic acid are effective oxidising biocides. This study showed that the addition of the organic soil had no effect on the efficacy of Surfex whilst the generic equivalent, diluted Proxitane was inactivated. This is most likely due the other ingredients within the formulation, such as chelating agents, or perhaps due to the differences in pH (8.10 for Surfex as compared to 2.6 for a 4% solution of Proxitane). Compared with hydrogen peroxide, peracetic acid does, however, have the disadvantage that it is less stable when diluted: disassociating into acetic acid and hydrogen peroxide over a matter of hours due to the shift in equilibrium conditions brought on by dilution in water.

The very short detergent treatment used in this study was to simulate someone gently wiping over a surface with a damp cloth thus wetting the surface of the DSB with surfactants to increase biocide activity. This detergent treatment had no significant effect on efficacy of the three biocides tested (Chlorclean, SDIC and Surfex). However, even if hospital surfaces are precleaned, the likelihood of DSB being present is high (Hu et al., 2015; Johani et al., 2017; Ledwoch et al., 2018).

Almatroudi (2015) demonstrated that protein was a principal component (56%) of both his in vitro DSB model and biofilms contaminating dry clinical surfaces in hospitals with protein contents varying from 42 to 95%. Therefore, in this study we measured residual protein on the treated coupons to determine the proportion of biofilm mass removed by the oxidising action of the disinfectants. None of the disinfectants were able to completely remove all biofilm protein with 5 min contact time, however a higher percentage reduction of biofilm protein was observed in five minutes with Surfex (65%) than the other tested disinfectants (<30%), both in the presence and absence of soil.

Conclusion

We conclude that disinfectant efficacy against biofilm can vary significantly, despite containing similar levels of biocides due to their formulation/additives. The disinfectant formulation also affected disinfectant action in the presence of soil. Therefore, it is crucial to select clinically efficient disinfectant agents with the potential of effectively eradicating biofilm from hospital environments. We suggest that purchasing officers ask disinfectant manufacturers for efficacy testing results and consider whether efficacy testing has been conducted in the presence of biological soil and/or biofilm.

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Conflict of interest statement

Whiteley Corporation was the industrial partner associated with the Australian Research Council Linkage Project. They are a manufacturer of disinfectants and detergents for use in healthcare and one of their products was tested in this study. Their role did not lead to any bias in formulating, executing, analysing or writing up the research.

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Chapter Five: Development of multispecies dry surface biofilm model.

5.1 Introduction

Multispecies biofilm is complex and exhibits an altered phenotype with respect to growth rate and gene transcription when compared to either mixed species planktonic culture or monospecies biofilm (Elias and Banin, 2012). In multispecies biofilm, there are internal interactions between involved species, which can increase biofilm mass, and due to the increased genetic material available increase the frequency of genetic exchange (Elias and Banin, 2012) and in some cases, decrease individual species susceptibility to antimicrobial agents and disinfectants (Burmølle et al., 2006). Biofilm forming bacteria possess different molecular mechanisms to engage other bacteria and appear to actively attempt to become polymicrobial to improve their survivability (Kuramitsu et al., 2007). Improved survivability increases the opportunity for biofilm to act as a source of pathogens resulting in hospital acquired infections (HAI).

Bacteria show enormous variation when incorporated in to multispecies biofilm. Therefore, development of an in-vitro biofilm model is a great challenge. To develop an in vitro multispecies biofilm several factors are to be considered such as, identifying relevant members to be included in the community; selecting the experimental design for establishment of the biofilm, and determining and executing the appropriate analysis. (Røder et al., 2016)

All the mixed biofilm model developed, and studies undertaken to date involve growth of traditional hydrated biofilm appropriate to studying specific clinical diseases. Although we have strong evidence of existence of mixed dry surface biofilm in hospital surface and their tolerance to disinfection (Hu et al., 2015d; Almatroudi et al., 2016), a dry mixed biofilm model has not yet been developed. Thus, it is now become mandatory to develop the dry surface mixed biofilm model for using it as a tool to evaluate disinfectant and detergent efficacy, as it is already known that dry biofilm eradication is a great challenge. In vitro multispecies biofilm model may optimize a suitable path for bridging the gap between the simple monospecies biofilm and the large-scale metagenomic studies to investigate the complex interactions of mixed biofilm bacteria. Species selection should consider interspecies interactions, coevolution, and other

conditions faced in natural bacterial habitats in the study design. Therefore, Rodor et al. (2016) suggested the following three main guidelines for multispecies biofilm research:

- The highest possible level of diversity should be included in the model system, without compromising the aim of the study or the applicability of the analysis tools applied.
- The natural habitat and prior coevolution of the strains used should be considered and, if possible, strains concurrently isolated from a specific setting should be used.
- Key species and interacting species identified by prior spatial analysis of the habitat or network analysis of the targeted community should be included in the model system.

In this study we chose these strains because development of an in-vitro biofilm model by following these guidelines, might help to explain many critical issues regarding biofilm infection and its resistance phenomenon. Recent advancement in techniques such as multiple fluorophores for designing FISH probes in combination with advanced multi-laser confocal laser scanning microscopy (CLSM) or transcriptomics and proteomics will facilitate exploring and characterizing complex communities.

5.2 Bacterial strains used

In this study, ATCC strain of four bacterial species:

Staphylococcus aureus (25923)
Enterococci faecium (35667)
Acinetobacter baumannii (19606) and
Pseudomonas aeruginosa (25619)

The four bacterial species listed were chosen as

They are highly pathogenic.
Are frequently isolated from HAIs.
They readily form biofilms.
They also possessed a colony morphology that was distinct from other species chosen for the multispecies biofilm reactor, when grown on HBA plate to allow clear identification.

We chose to use type culture strains rather than clinical strains to ensure that other laboratories could replicate our work.

5.3 Development of *in vitro* multispecies dry surface biofilm (DSB) model

Biofilm was grown over a period of 12 days in TSB media varying in concentration from 5% to 10% and bacterial numbers determined. However, the periods of dehydration severely affected *Pseudomonas*. Numerous different protocols were tried to ensure sufficient numbers of all four species (mimicking the percentage, achieved in hospital surfaces) and a few protocols have been described below.

After formation of biofilm CFU was done according to the following protocol:

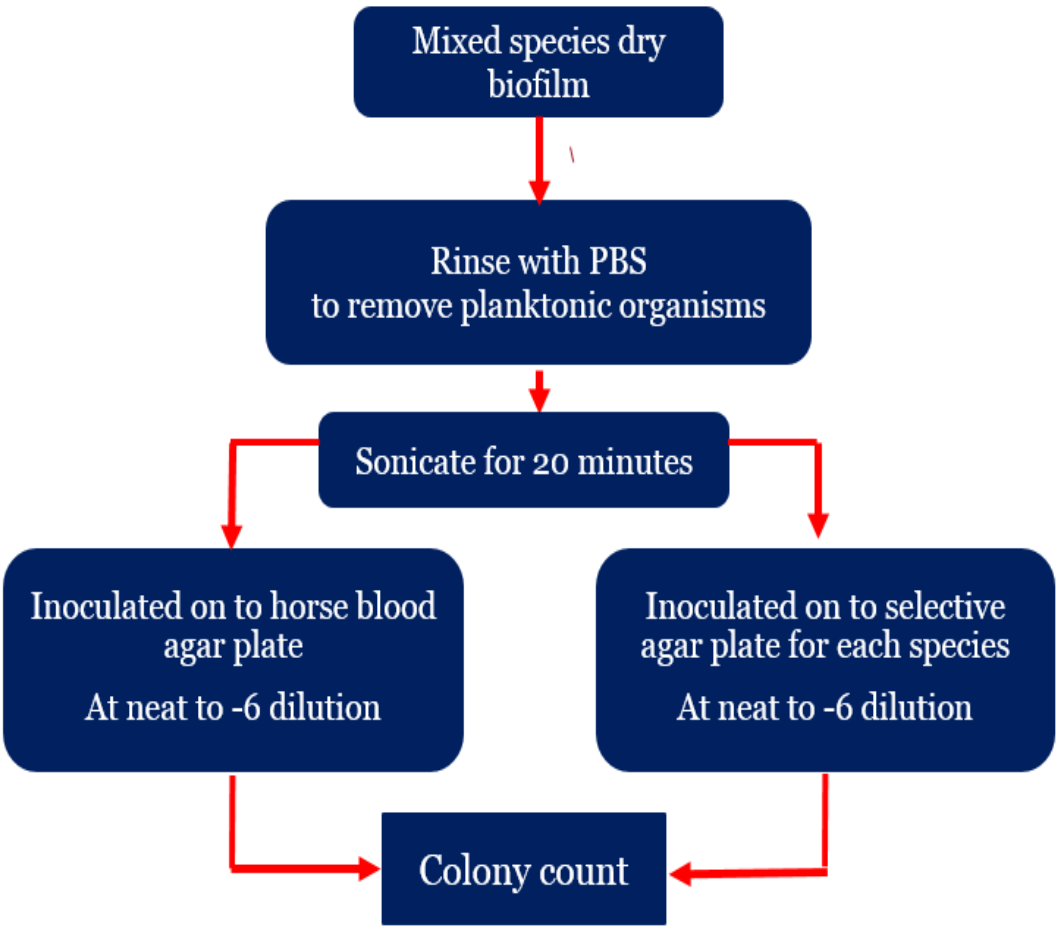


Figure 5- 1:

Schematic diagram of CFU counting after development mixed species biofilm.

The number of individual species bacteria within the biofilm was determined by culture on to the HBA (Micro media laboratories, Australia), UTI (Oxoid, UK) and *Acinetobacter* selective media (Sigma-Aldrich, Australia) plate.

5.3.1 First Trial

To establish a four-species biofilm model, bacteria (liquid culture, approximately 10^8/ml organisms of each species) were added to the CDC biofilm generator at same time point. The initial batch phase of 48 hours (37 ^{o}C), was followed by 48 hours of dehydration at room temperature (22-25 ^{o}C) and three cycles of batch growth for 6 hours, interchanged with prolonged dehydration phases as shown in table 5-1. Batch phases were conducted with shear (baffle rotation at 130rpm). During all phases filtered airconditioned air was pumped into the CDC biofilm generator at 3L/minute.

Table 5- 1:

Culture condition for formation of mixed species dry biofilm. Initial inoculum of about 10^8 of each (four) bacterial species was added at the beginning of stage -1 phase.

Phases	Culture conditions	Cumulative operating time
1	48 hr batch phase in 5% TSB followed by 48 hrs dehydration	96 hours
2	6 hr batch phase in 5% TSB followed by 66 hrs dehydration	168 hours
3	6 hr batch phase in 5% TSB followed by 42 hrs dehydration	216 hours
4	6 hr batch phase in 5% TSB followed by 66 hrs dehydration	288 hours

Results

S. aureus, E. faecium and A. baumannii were all present at considerable percentage of CFU, but *P. aeruginosa*, was completely absent from the coupons.

5.3.2. Second trial

As *P. aeruginosa* failed to survive in the above protocol, we attempted to give it a head start by growing a pure hydrated biofilm of *P. aeruginosa* for 24 hours prior to the addition of the other three species of bacteria. Biofilm was grown in both 5% and 10% TSB according to following protocol:

Table 5- 2:

Culture condition for formation of mixed species dry biofilm. Initial inoculum of about 10^8 of *P. aeruginosa* was added at the beginning of stage -1 phase and 10^8 of each of the four bacterial species was added at the beginning of stage -2 phase.

Phases	Culture conditions	Cumulative operating time
1	24-hour batch phase in 5%/10% TSB only *P. aeruginosa*	24 hours
2	48 hr batch phase (4 bact. sp.) in 5%/10% TSB followed by 48 hrs dehydration	120 hours
3	6 hr batch phase in 5%/10% TSB followed by 66 hrs dehydration	192 hours
4	6 hr batch phase in 5%/10% TSB followed by 42 hrs dehydration	240 hours
5	6 hr batch phase in 5% TSB followed by 66 hrs dehydration	312 hours

Results

S. aureus, E. faecium and Acinetobacter spp. all formed DSB well but *P. aeruginosa*, performed poorly and although it was present on few coupons it was completely absent from most of the coupons irrespective of whether growth occurred in 5 or 10% TSB.

5.3.3 Third trial

The previous protocols failed to isolate *Pseudomonas* from all the coupons. We therefore, increased the duration of hydration time and decreased the duration of the dehydration phases.

5.3.3.a Increase hydration time in 3rd phase;

Table 5- 3:

Culture condition for formation of mixed species dry biofilm. Initial inoculum of about 10^8 of *S. aureus* was added at the beginning of stage -1 phase and 10^8 of four bacterial species was added at the beginning of stage -2 phase with increase hydration time in 3rd phase.

Phases	Cultural conditions	Cumulative operating time
1	24 hr. batch phase in 10% TSB only *P. aeruginosa*	24 hours
2	48 hr batch phase (4 bact. sp.) in 10% TSB followed by 48 hrs dehydration	120 hours
3	16 hr batch phase in 10% TSB followed by 66 hrs dehydration	202 hours
4	6 hr batch phase in 10% TSB followed by 42 hrs dehydration	250 hours
5	6 hr batch phase in 10% TSB followed by 66 hrs dehydration	322 hours

Results

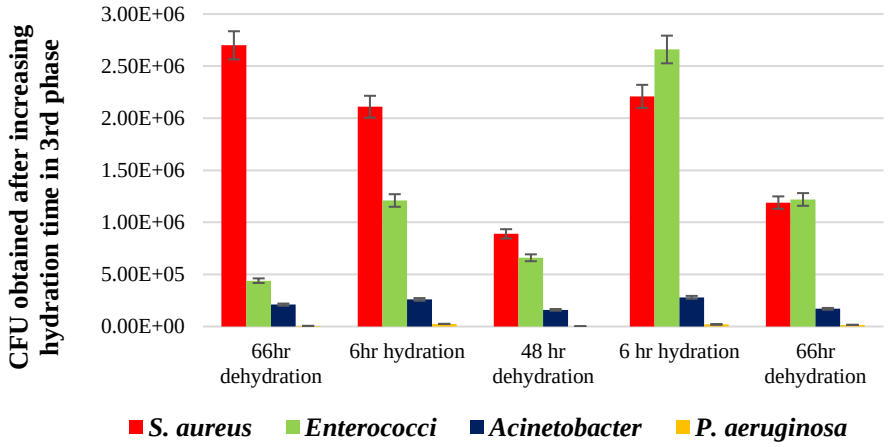


Figure 5- 2:

Number of colony forming units of four species of bacteria incorporated into DSB obtained after increasing hydration time in 3rd phase.

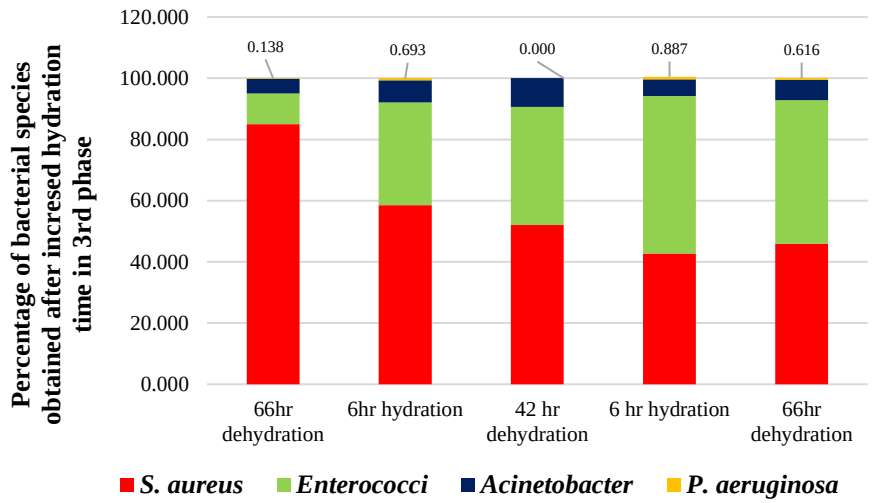


Figure 5- 3:

Percentage of four bacterial species incorporated into DSB, obtained after increasing hydration time in 3rd phase

Conclusion

After increasing hydration time in third phase, we obtained significant number of *S. aureus, Acinetobacter* and *E. faecium.* However, even though *P. aeruginosa* was obtained in each phase it was at very low levels. Following 66 hrs of dehydration in 3rd phase, *P. aeruginosa* formed 0.138 % of the biofilm. However, following the final dehydration phase *P. aeruginosa* formed only 0.62% of the biofilm and individual coupon counts varied considerably.

5.3.3.b Decrease dehydration time in all phases

Table 5- 4:

Culture condition for formation of mixed species dry biofilm. Initial inoculum of about $10^8$of *P. aeruginosa* was added at the beginning of stage -1 phase and 10^8 of four bacterial species was added at the beginning of stage -2 phase and duration of dehydration phases were decreased.

Phases	Culture conditions	Cumulative operating time
1	24 hr. batch phase in 10% TSB only *P. aeruginosa*	24 hours
2	48 hr batch phase (4 bact. sp.) in 10% TSB followed by **42** hrs dehydration	114 hours
3	14 hr batch phase in 10% TSB followed by **42** hrs dehydration	168 hours
4	6 hr batch phase in 10% TSB followed by **42** hrs dehydration	216 hours
5	6 hr batch phase in 10% TSB followed by **42** hrs dehydration	264hours

Results

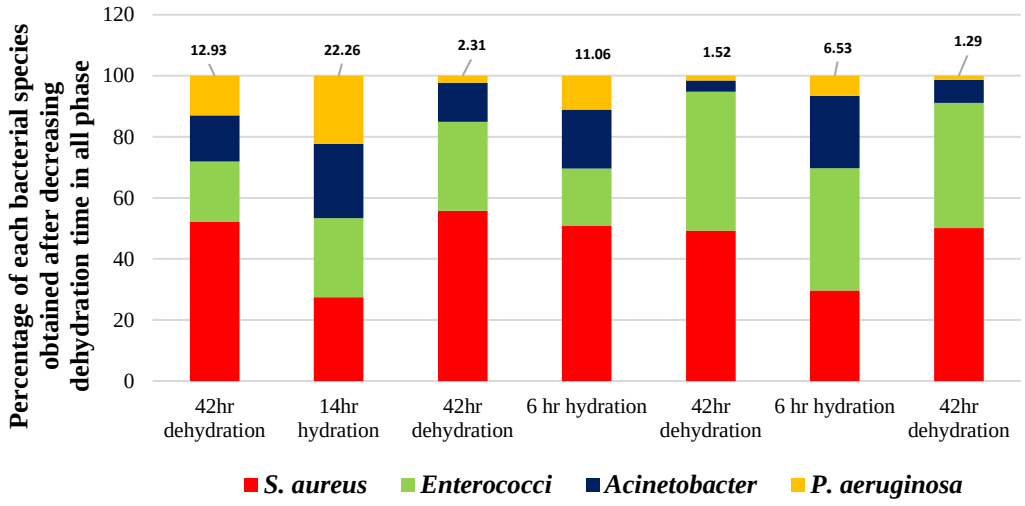


Figure 5- 4:

Percentage of four bacterial species incorporated into DSB obtained after decreasing dehydration time in all phases.

Conclusion

High levels of *P. aeruginosa* grew with prolonged hydration, however its numbers fell sharply during the dehydration phases. For example, in the 3rd phase with prolong hydration time *P. aeruginosa* formed 22.3% of the biofilm but with the following dehydration phase, of 42 hours, it fell sharply to 2.31%. Similar rises and falls were seen with each hydration and dehydration cycle. Following the final dehydration phase on day 12, *P. aeruginosa* formed 1.29% of the

biofilm. Although this represented an increase in the amount of *P. aeruginosa* it was still an insignificant amount and counts on individual coupons were variable (percentage of coefficient of variation >90%).

5.3.3.c Decrease dehydration in 2nd and 3rd phase and increase hydration time in 3rd and 4th phase.

Table 5- 5:

Culture condition for formation of mixed species dry biofilm. Initial inoculum of about 10^8 of *P. aeruginosa* at stage 1 and then 10^8 of the four bacterial species was added at the beginning of stage 2 phase was combined with decreasing dehydration time in 2nd and 3rd phase and an increase dehydration in 5th phase.

Phases	Culture conditions	Cumulative operating time
1	24 hr. batch phase in 10% TSB only *P. aeruginosa*	24 hours
2	48 hr batch phase (4 bact. sp.) in 10% TSB followed by 32 hrs dehydration	104 hours
3	16 hr batch phase in 10% TSB followed by 32 hrs dehydration	152 hours
4	16 hr batch phase in 10% TSB followed by 48 hrs dehydration	216 hours
5	6 hr batch phase in 10% TSB followed by 66 hrs dehydration	288 hours

Results

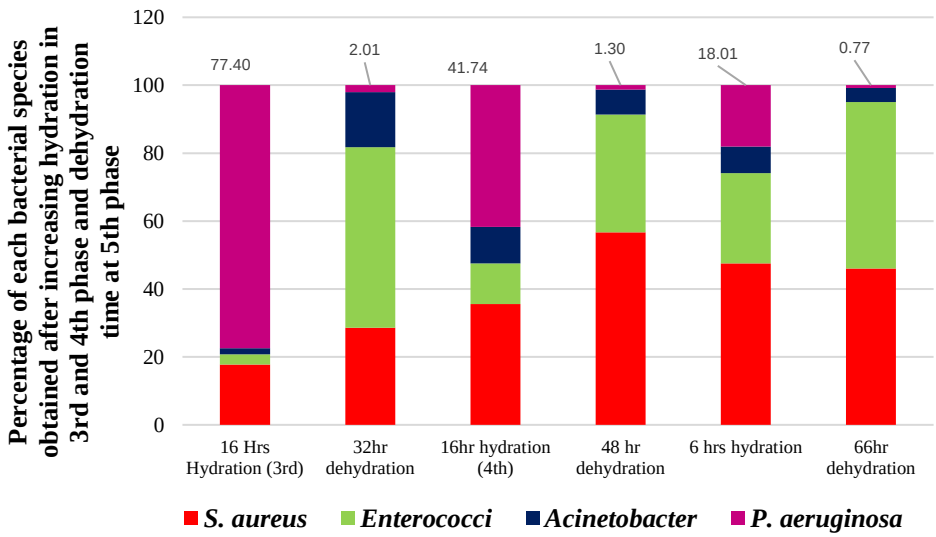


Figure 5- 5:

Percentage of four bacterial species incorporated into DSB obtained after increasing hydration time in 3rd & 4th phase and dehydration time (66 hrs.) at 5th phase.

Conclusion

In this trial, dehydration time was increased to 66 hours at the end phase to make the biofilm more dehydrated thinking that increased hydration time in previous phase (3rd and 4th) would support high *Pseudomonas* growth up to targeted percentage. But after the prolonged 66 hours of dehydration, the percentage of Pseudomonas fell from 18% in 4th phase to 0.8%.

5.3.3.d Decrease dehydration time and increase hydration time in 3rd and 5th phase.

After observing and analysing all above results it was concluded that *Pseudomonas* dies in every time during the dehydration phase. Thus, in this trial we increase hydration time in last phase (Table-5-6).

Table 5- 6:

Culture condition for formation of mixed species dry biofilm. Initial inoculum of about 10^8 of *P. aeruginosa* and 10^8 of four bacterial species was added at the beginning of stage -1 and 2 phases respectively with decreasing dehydration and increase hydration time in 3rd and 5th phase.

Phases	Culture conditions	Cumulative operating time
1	24 hr. batch phase in 10% TSB only *P. aeruginosa*	24 hours
2	48 hr batch phase (4 bact. sp.) in 10% TSB followed by 42 hrs dehydration	114 hours
3	14 hr batch phase in 10% TSB followed by 34 hrs dehydration	162 hours
4	6 hr batch phase in 10% TSB followed by 42 hrs dehydration	210 hours
5	14 hr batch phase in 10% TSB followed by 34 hrs dehydration	258 hours

Results

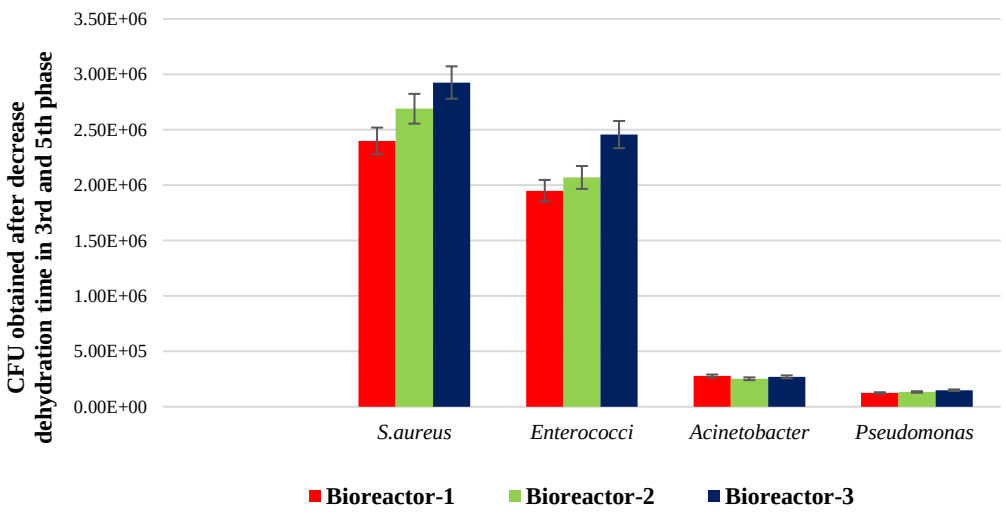


Figure 5- 6:

Number of colony forming units of four species of bacteria incorporated into DSB obtained after a decrease dehydration time and increase hydration time in 3rd and 5th phase.

Conclusion

In this trial, after increasing hydration time in both 3rd and 5th phases the percentage of all four bacteria achieved was close to the percentage; 10-40% *S. aureus*, 7% *Enterococci spp*., 1 to 8% *Acinetobacter spp*. and 0.16 to 2.84%. *P. aeruginosa* found in mixed species biofilm found on hospital surfaces. The biofilm on every coupon included *P. aeruginosa*. Each rods and coupons have *P. aeruginosa* on their surfaces.

Confirmation of results and calculation of coefficient of variation

To confirm these results, this experiment was repeated starting three bioreactor at a time individually following same protocol and the with-in and in-between experiments coefficient of variation calculated (see table 5-7) for each bacterial species using the following formula:

Coefficient of variation =100 x Standard Deviation / mean

Table 5- 7:

The within and between experimental coefficient of variation for individual bacteria grown in a mixed biofilm.

Organisms tested	Within experiment coefficient of variation (CV)			Between experimental CV
	Biofilm Reactor 1	Biofilm Reactor 2	Biofilm Reactor 3	
S. aureus	4	6	13	9.87
E. faecium	15	11	20	12.26
A. baumannii	1	6	5	4.75
P. aeruginosa	105	206	102	137

Within each bioreactor CFU of *S. aureus* was in between 25-30x10^6, *E. faecium* 20-25 x10^6 and *Acinetobacter* was 15-30 x10^5. The CFU of *Pseudomonas* obtained was 10-14 x10^5. However, when considered each coupon of each three bioreactor, still 3 to 4 coupons did not have any *Pseudomonas* on it and the CV was very high. This indicates that the growth of *P. aeruginosa* subjected to long periods of dehydration is inconsistent.

5.4 *Pseudomonas putida* in mixed species biofilm

Then we tried with *Pseudomonas putida*. But after different trial with different hydration and dehydration protocol, *P. putida* showed the equivalent results of *P. aeruginosa* with large variation, occasionally with no growth on many coupons (Figure 5-7 and Figure 5-8). After considering results of both species of *Pseudomonas* it was concluded that in producing laboratory grown DSB in a short period of time the persistence of *Pseudomonas* is challenged and gives inconsistent results. We therefore, abandoned trying to incorporate *Pseudomonas spp*. into the biofilm.

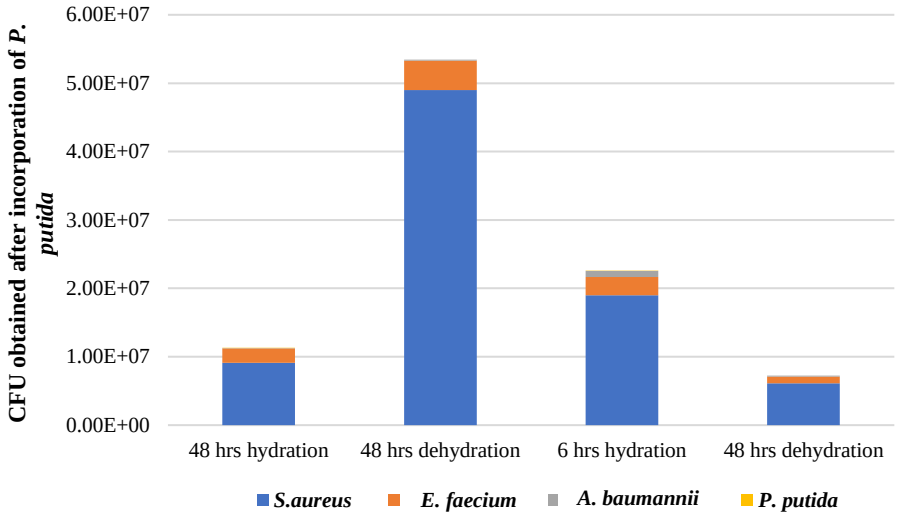


Figure 5- 7:

Number of colony forming units of four species of bacteria incorporated into DSB obtained showing the absence of *P. putida* after each phase of dehydration.

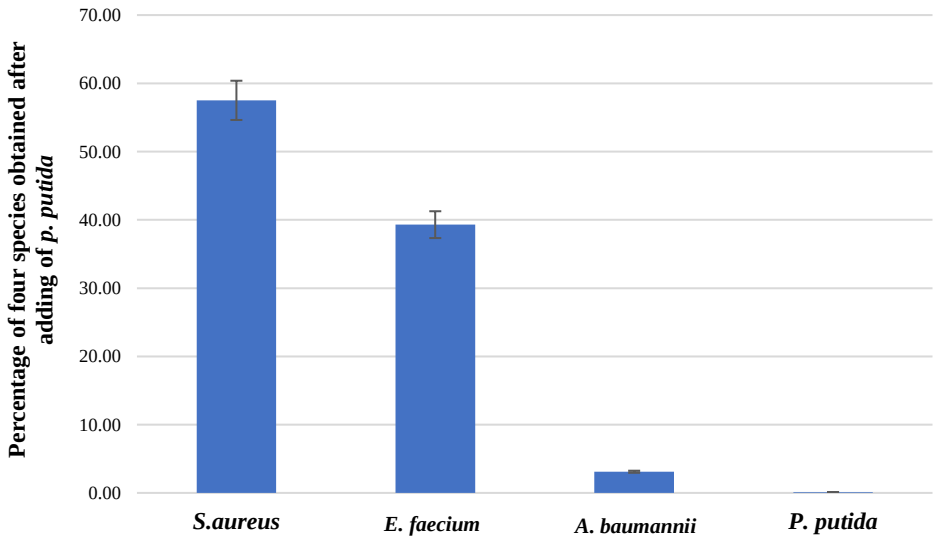


Figure 5- 8:

Percentage of four species of bacteria incorporated into DSB obtained showing very low percentage of *P. putida* after increasing hydration time in all phases.

5.5. Development of mixed species biofilm model with three bacterial species.

The aim of this study was to develop a model dry surface biofilm that incorporated multiple species into the biofilm, thereby ensuring species to species interactions. As shown above *Pseudomonas* species gave inconsistent results following dehydration and thus was not suitable for incorporation into a model to be used for detergent/disinfectant testing which needs consistency and minimal variation between test pieces and between experiments. The following three bacterial species were included:

- *S. aureus* (25923)
- *E. faecium* (35667)
- *A. baumannii* (19606)

The following protocols were tested to determine the optimum protocol for disinfectant testing. Biofilm was grown in the CDC biofilm reactor and individual species cell number determined as described previously.

5.5.1 Mixed species biofilm development

All three bacterial species were added to the CDC bioreactor at same time i.e. at the beginning of stage -1 phase. After inoculation of bacteria (approximately 10^8 bacteria/ species) into the CDC bioreactor, the initial 48-hours batch phase (at 35°C) was followed by 48 hours of dehydration at room temperature (22-25 ^{o}C) and then three cycles of batch growth for 6 hours, interchanged with prolonged dehydration phases as shown in table 5-8. Fresh media was added following each dehydration phase.

Table 5- 8:

Culture condition for formation of mixed species dry biofilm. Initial inoculum of about 10^8 of all three-bacterial species were added at the beginning of stage -1 phase.

Phases	Culture conditions	Cumulative operating time
1	48 hr batch phase (3 bact. sp.) in 5% TSB followed by 48 hrs dehydration	96 hours
2	6 hr batch phase in 5% TSB followed by 66 hrs dehydration	168 hours
3	6 hr batch phase in 5% TSB followed by 42 hrs dehydration	216 hours
4	6 hr batch phase in 5% TSB followed by 66 hrs dehydration	288 hours

Results

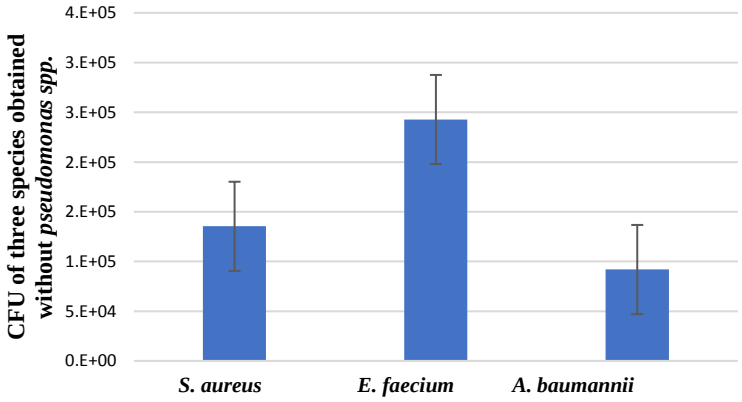


Figure 5- 9*:*

Number of CFU of three species incorporated into DSB. Addition of three species at commencement of the first phase with 5% TSB in all phases.

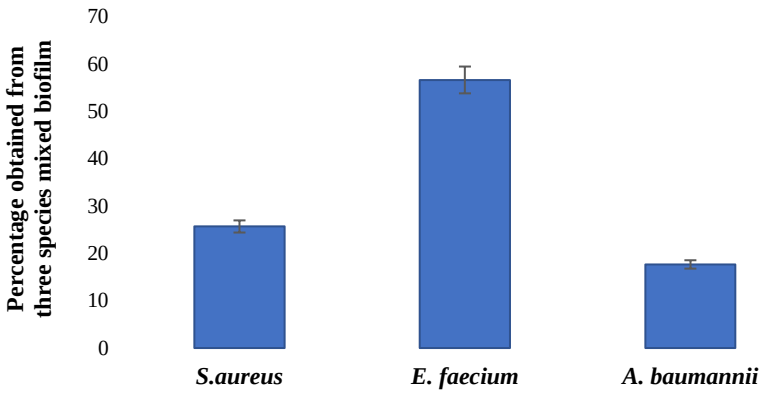


Figure 5- 10:

Percentage of three bacterial species incorporated into DSB when all three species were added at the commencement of the first phase.

Conclusion

This result shows that, among three bacteria, *E. faecium* formed the highest percentage (57%) of the biofilm, this was followed by *S. aureus* (26%) and *A. baumanni* (18%). Several studies have reported the microbiome of mixed species biofilm on hospital surfaces, and *S. aureus* was the predominant organism found and composed between 31-50% of the biomass, while *E. faecium* composed approximately 7 % of the biomass and *A. baumanni* between 1-8% (Hu et al., 2015; Johani et al., 2017). The ideal model would therefore, be composed of principally *S. aureus,* followed by lower percentages of *E. faecium* and *A. baumanni.*

5.5.2 Inoculation of *S. aureus* 24 hours before the addition of other species with 5% TSB in all phases.

S. aureus grows slowly in the low available nutrition in 5% w/v TSB. In this experiment we aimed to give *S. aureus* a head starts by inoculating it 24 hours before addition of the other species. This way it would have formed a immature biofilm in the absence of any other competing bacteria. After 24 hrs growth in 5% TSB, 10^8 *S. aureus* was inoculated again along with the two other bacteria (10^8 bacteria/species) at the start of the second phase (Table 5-9) and biofilm was developed following Almatroudi's (2015) protocol.

Table 5- 9:

Culture conditions for formation of mixed species dry biofilm. Initial inoculum of about $10^8$of *S. aureus* was added at the beginning of stage -1 and 10^8 of three bacterial species were added at the beginning of stage -2 phase.

Phases	Culture conditions	Cumulative operating time
1	24 hr. batch phase in 5% TSB only *S. aureus*	24 hours
2	48 hr batch phase (3 bact. sp.) in 5% TSB followed by 48 hrs dehydration	120 hours
3	6 hr batch phase in 5% TSB followed by 66 hrs dehydration	192 hours
4	6 hr batch phase in 5% TSB followed by 42 hrs dehydration	240 hours
5	6 hr batch phase in 5% TSB followed by 66 hrs dehydration	312 hours

Results

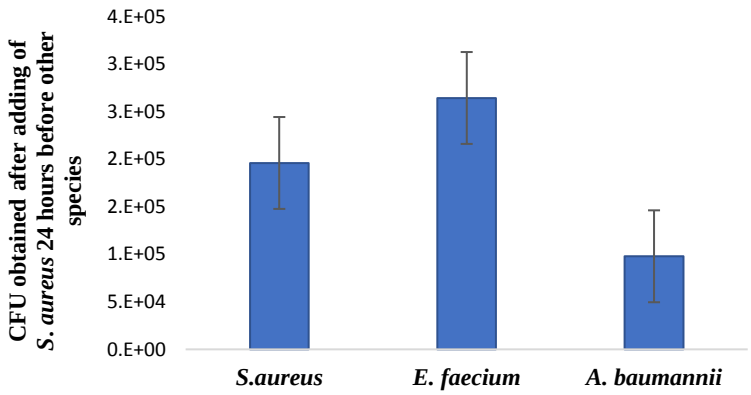


Figure 5- 11:

Number of CFU of three bacterial species incorporated into DSB following addition of *S. aureus* 24 hours before addition of other species, with 5% TSB in all phases.

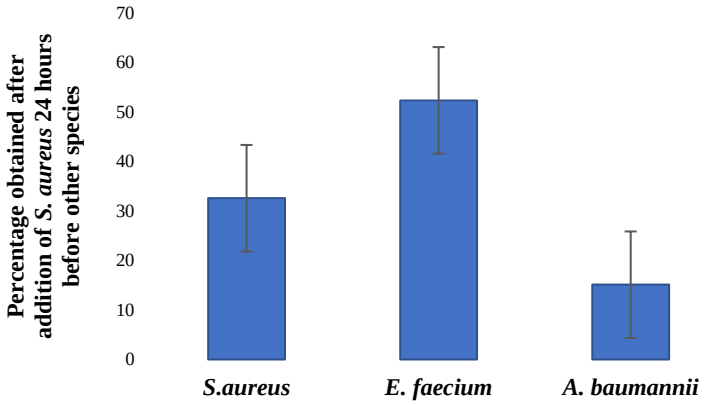


Figure 5- 12:

Percentage of three bacterial species incorporated into DSB after addition of *S. aureus* 24 hours before addition of other species, with 5% TSB in all phases.

Conclusion

Reducing interspecies competition by the addition of *S. aureus*, 24 hours before addition of other species, increased the percentage of *S. aureus* by 7% (from 26 to 33%). However, *Enterococci sp*. still formed more of the DSB at 52% which was not what we were aiming for.

5.5.3 Inoculation of *S. aureus* 24 hours before the addition of other species with 10% TSB followed by 5% TSB in next four phases.

To try and increase the growth rate of *S. aureus* the amount of available nutrients was increased by doubling the % of TSB from 5 to 10%. Competition was eliminated by giving *S. aureus* a 24-hour head start prior to addition of the other species of bacteria as detailed in table 5-10.

Table 5- 10:

Culture conditions for formation of mixed species dry biofilm. Initial inoculum of about 10^8 of *S. aureus* was added with 10% TSB at the beginning of stage -1 and $10^8$of the three bacterial species were added at the beginning of stage -2 phase.

Phases	Culture conditions	Cumulative operating time
1	24 hr. batch phase in 10% TSB only *S. aureus*	24 hours
2	48 hr batch phase (3 bact. sp.) in 5% TSB followed by 48 hrs dehydration	120 hours
3	6 hr batch phase in 5% TSB followed by 66 hrs dehydration	192 hours
4	6 hr batch phase in 5% TSB followed by 42 hrs dehydration	240 hours
5	6 hr batch phase in 5% TSB followed by 66 hrs dehydration	312 hours

Results

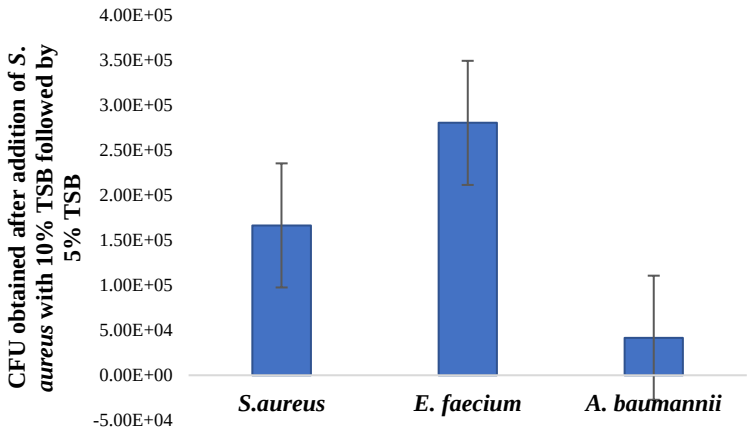


Figure 5- 13:

Number of CFU of three bacterial species incorporated into DSB following after addition of *S. aureus* 24 hours before addition of other species, with 10% TSB followed by 5% TSB in next four phases.

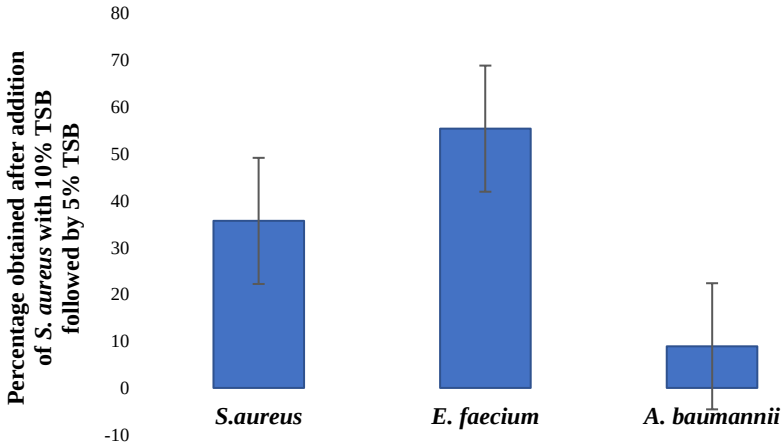


Figure 5- 14:

Percentage of three bacterial species incorporated into DSB after addition of *S. aureus* 24 hours before addition of other species, with 10% TSB followed by 5% TSB in next four phases.

Conclusion

Although the incorporation of *S. aureus* into the DSB was increased marginally, this protocol also failed to reach the desired higher % of *S. aureus* incorporation.

5.5.4 Inoculation of *S. aureus* 24 hours before the addition of other species with 10% TSB in first two phases followed by 5% TSB in next three phases.

Observing the previous results, it may be stated that, *S. aureus* in might be unable to compete *Enterococcus spp*. and its surface attachment might be inhibited by Enterococci in low level of nutrition. Therefore, to augment staphylococcal attachment and to provide additional nutrition, the amount of TSB was also increased in second phase from 5% to 10% TSB (Table 5-11).

Table 5- 11:

Culture conditions for formation of mixed species dry biofilm. Initial inoculum of about $10^8$of *S. aureus* was added at the beginning of stage -1 and 10^8 of three bacterial species was added at the beginning of stage -2 phase.

Phases	Culture conditions	Cumulative operating time
1	24 hr. batch phase in 10% TSB only *S. aureus*	24 hours
2	48 hr batch phase (3 bact. sp.) in 10% TSB followed by 48 hrs dehydration	120 hours
3	6 hr batch phase in 5% TSB followed by 66 hrs dehydration	192 hours
4	6 hr batch phase in 5% TSB followed by 42 hrs dehydration	240 hours
5	6 hr batch phase in 5% TSB followed by 66 hrs dehydration	312 hours

Results

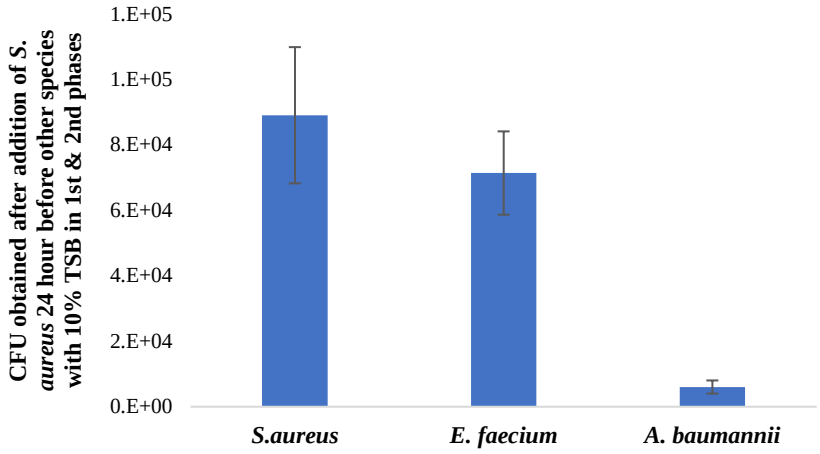


Figure 5- 15:

Number of CFU of three bacterial species incorporated into DSB following after addition of *S. aureus* 24 hours before addition of other species with 10% TSB followed by 5% TSB in next three phases.

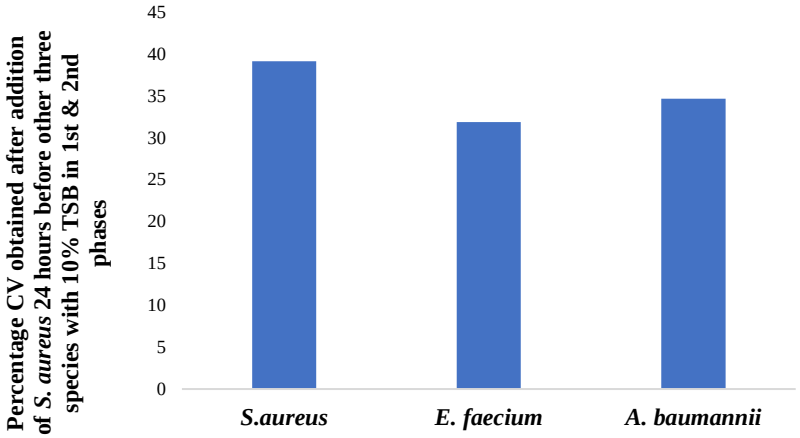


Figure 5- 16:

Percentage coefficient of variation of three bacterial species obtained from DSB after addition of *S. aureus* 24 hours before addition of other species, with 10% TSB followed by 5% TSB in next three phases.

5.5.4.a Replication of inoculation of S. aureus 24 hours before the addition of other species with 10% TSB in first two phases followed by 5% TSB in next three phases.

To determine if the results obtained above in 6.4.4.d were repeatable an additional two mixed species DSB were grown using the same conditions. Following CFU determinations the % coefficient variation was calculated for each individual bacterial species for both within and between run results. Coefficient of variation (CV%) within and between experiment was determined by following equation;

$$\% \text{ Coefficient Variation} = \frac{100 \text{ X Standard Deviation}}{\text{Mean value}}$$

In this experiment following the above protocol, the CDC bioreactor reproducibly formed multi-layered multispecies biofilm containing about—*S. aureus* 10^5/coupon, *E. faecium* 10^5 /coupon and *A. baumannii* 10^4/coupon embedded in thick EPS observed under scan electron microscope (SEM). *S. aureus* was the most prevalent bacteria incorporated into the DSB and contributed 56% of the biofilm mass. This was followed by *E. faecium* which contributed 38% of the biofilm mass and *A. baumannii* which contributed an average of 6% of the biofilm mass. All three-bacterial count with in each bioreactor has minor variation. The within run coefficient variation (CV) is 28% to 39% and the between run CV was 2% to 15% that is considerably low. The total number of CFU achieved from each coupon was 4.3 x 10^5 on an average with interexperimental CV of 11.12.

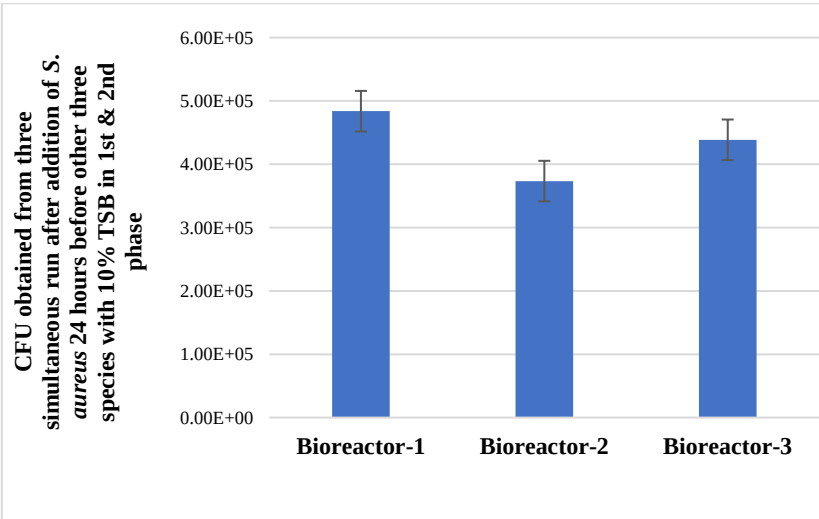


Figure 5- 17:

Total number of CFU obtained from each coupon (total of three bacterial species incorporated into DSB) after addition of *S. aureus* 24 hours before addition of other species, with 10% TSB in 1st and 2nd phase followed by 5% TSB in next three phases.

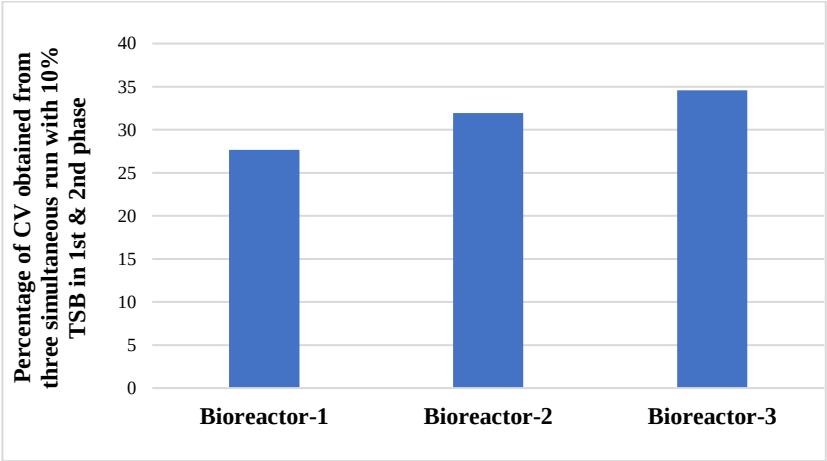


Figure 5- 18:

Percentage coefficient of variation (CV) obtained between each coupon after addition of *S. aureus* 24 hours before addition of other species, with 10% TSB in 1st and 2nd phase followed by 5% TSB in next three phases.

Studies have shown *S. aureus* as the predominant organism found and composed between 31-50% of the biomass, while *E. faecium* composed approximately 7 % and *A. baumanni* between 1-8% of the biomass (Hu et al., 2015; Johani et al., 2017). The percentage of *E. faecium* reported in those studies are lower than the percentage we achieved. This might be due to the involvement of more than three different bacterial species in biofilm on the collected ICU sample. Therefore, it could be stated that, in this study the increased percentage of *E. faecium* might be due to good attachment ability and less competitor. We targeted higher percentage of *S. aureus* because it is the most important cause of HAI and in hospital surface it is evidenced as a cause of HAI in ≥50% cases (Magill et al., 2014; Uneke, 2014).

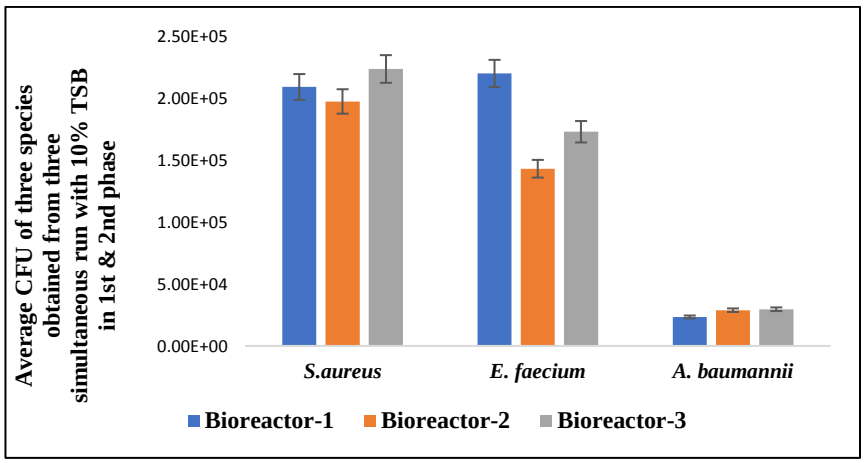


Figure 5- 19:

Number of colony forming units of the three bacterial species incorporated into DSB after addition of *S. aureus* 24 hours before addition of other species, with 10% TSB in 1st and 2nd phase followed by 5% TSB in next three phases.

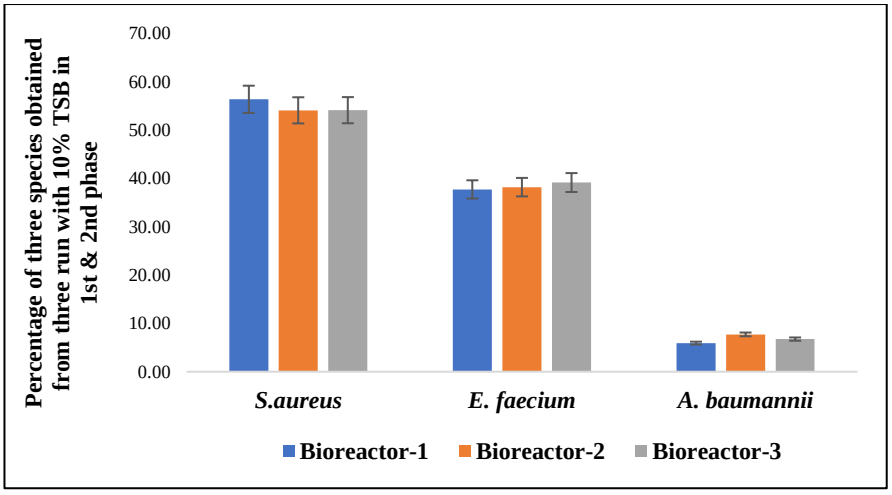


Figure 5- 20:

Percentage of three bacterial species incorporated into DSB after addition of *S. aureus* 24 hours before addition of other species, with 10% TSB in 1st and 2nd phase followed by 5% TSB in next three phases.

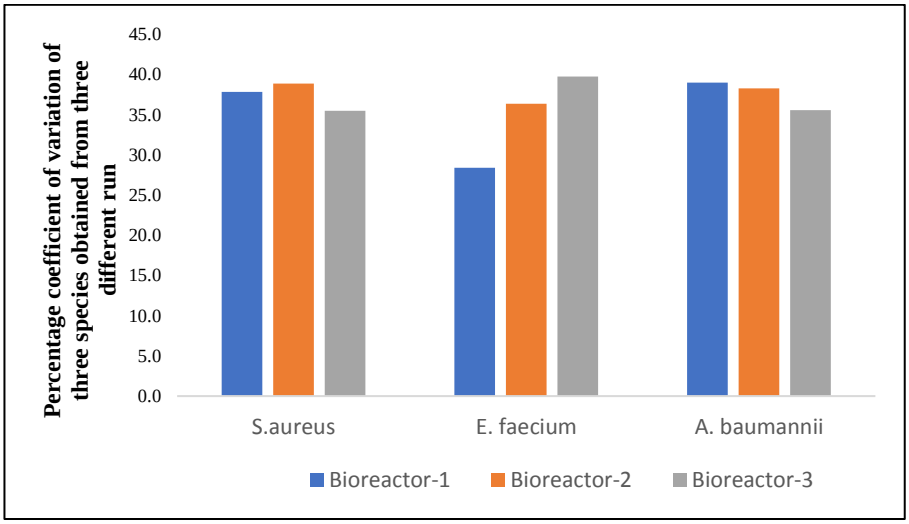


Figure 5- 21:

Coefficient of variation (CV) of three species, obtained from develop mixed species biofilm, after addition of *S. aureus* 24 hours before addition of other species, with 10% TSB in 1st and 2nd phase followed by 5% TSB in next three phases.

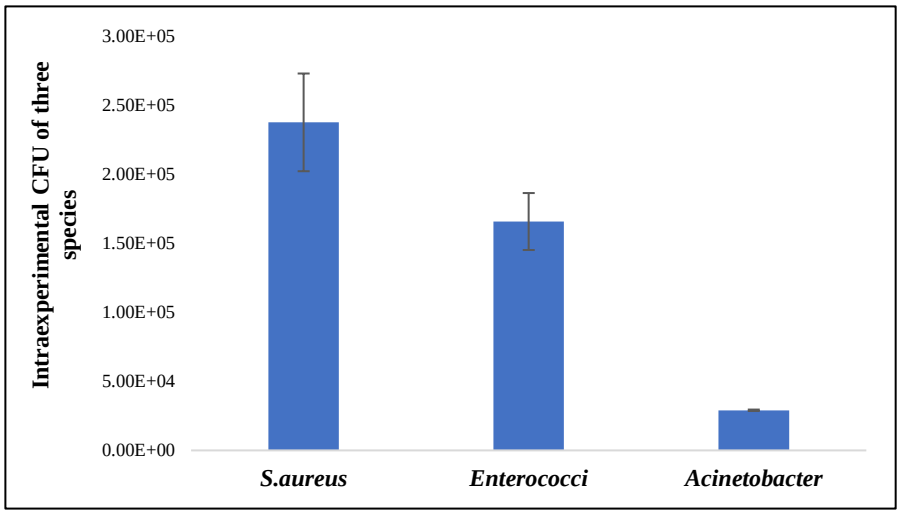


Figure 5- 22:

Interexperimental variation in number of CFU of the three bacterial species incorporated into DSB after addition of *S. aureus* 24 hours before addition of other species, with 10% TSB in 1st and 2nd phase followed by 5% TSB in next three phases.

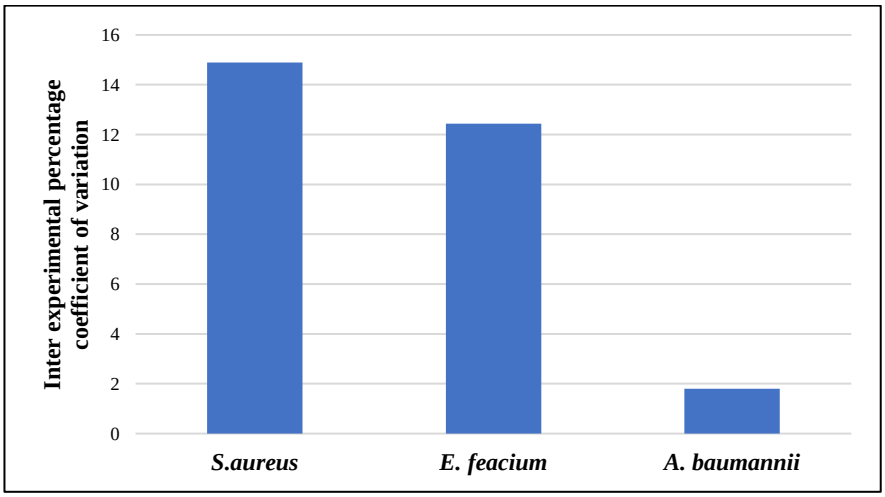
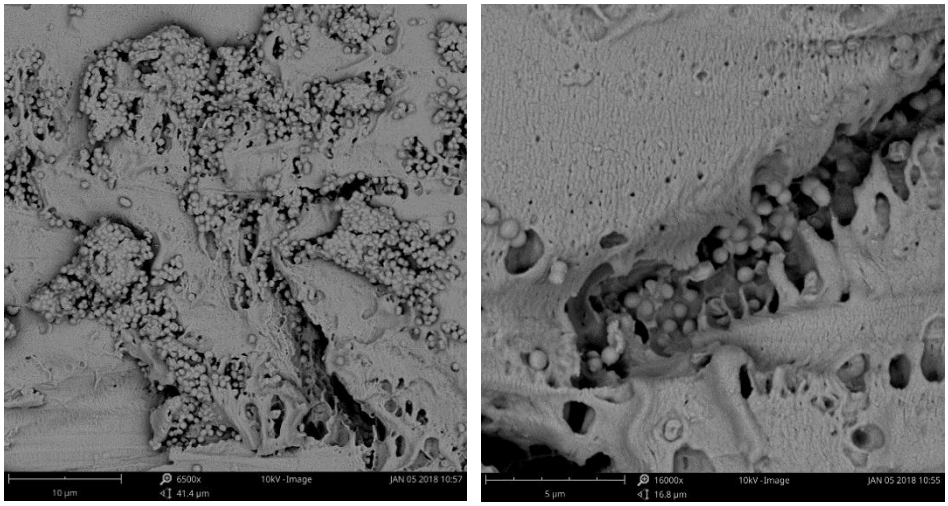


Figure 5- 23:

Between run coefficient of variation (CV) for the three bacterial species incorporated into DSB after addition of *S. aureus* 24 hours before addition of other species, with 10% w/v TSB in 1st and 2nd phase followed by 5% TSB in next three phases.



A B

Figure 5- 24:

Scanning electron microscope images showing polycarbonate coupon covered with mixed species biofilm bacterial aggregation within thick EPS A) magnified 6500X; B) magnified 16000X.

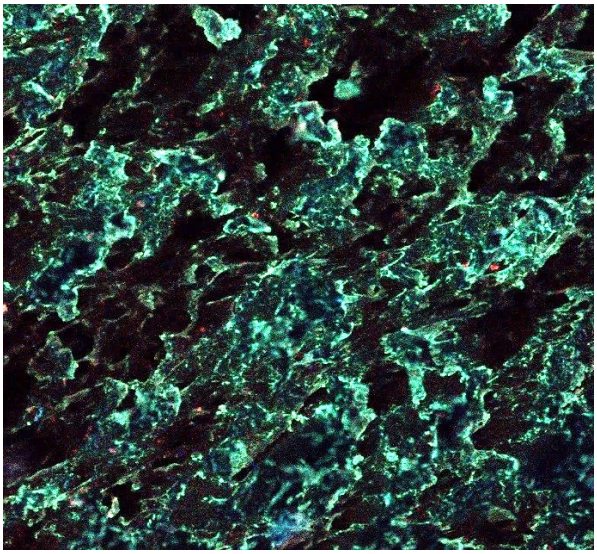


Figure 5- 25:

Composition of 13-day dry mixed species biofilm extracellular polymeric substances (EPS). EPS are stained for extracellular DNA (red), stained with Syto 60; glycoconjugate (blue), stained with Alexa-488-labelled *Aleuria aurantia* lectin and protein (green), stained with Sypro Orange.

Conclusion

In this experiment we achieved incorporation of each bacterial species into the DSB in the percentage that we considered most suitable and representative of clinical biofilms. That is a high percentage of *S. aureus* in conjunction with lower percentages of other important pathogens. considerable amount of three species of bacteria. To observe the reproducibility, we repeated the same protocol in three different runs and determined the coefficient of variation (CV). This model showed low % CV for each three species bacteria. And it is suggested that the lower the CV, the smaller the residuals relative to the predicted value. The between run CV was extremely low and indicates that the model is reproducible suggesting that this model is appropriate for evaluating the efficacy of different detergents and disinfectants commonly used to clean and disinfect hospital surfaces.

5.6 Original Paper

Title: A dry surface biofilm model incorporating three pathogens spread by contact in the hospital environment: A "real life" tool for disinfectants efficacy testing.

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Keywords:
Mixed species biofilms
Disinfectants
Healthcare-associated infections
Surfex
Chlorclean tablet
sodium dichloroisocyanurate (SDIC)

In above publication, Dr Durdana Chowdhury has following contribution

- Design study protocol with Associate professor Karen Vickery
- Carried out all in vitro experiments
- Collected data
- Data compilation
- Data analysis.
- Manuscript preparation

Author contribution 80%

Summary

Background: Biofilm rarely exist as a single species of bacteria but rather incorporate a substantial range of microorganisms as a complex multispecies biofilm. The internal interactions between multiple species has been shown to increase biofilm mass and, in some instances, decrease individual species susceptibility to disinfectants. A multispecies biofilm thus has an increased chance of persistence and may act as a complete source of hospital acquired infections (HAI). At present there is no standard method for testing the level of efficacy of different detergents to eradicate mixed biofilm formed on dry hospital surfaces.

Aim: The aim of this study was to develop a mixed species biofilm model analogous to the properties of mixed species dry biofilms obtained from hospital surfaces to test detergent efficacy and effect of mixed species biofilm on biofilm composition.

Methods: In this study, ATCC strain of three bacterial species, *Staphylococcus aureus, Enterococci faecium* and *Acinetobacter baumannii* that commonly cause HAI, were used. Biofilm was grown in the CDC bioreactor on polycarbonate coupons with periodic nutrition interspersed with long periods of dehydration. The number of three individual species bacteria within the biofilm was determined by plate culture and the within and between experimental coefficient of variation (CV%) calculated. The DNA, glycoconjugate and protein content of the biofilm were determined by analysing biofilm stained with SYTO 60, Alexa-488-labelled Aleuria aurantia lectin and SyproOrange respectively using image J and Imaris software. Biofilm architecture was analysed using live/dead staining, confocal microscopy (CLSM) and scanning electron microscopy (SEM). To perform disinfectant efficacy testing, biofilm coupons were treated with Surfex (1300 ppm), two chlorine-based disinfectants (1000ppm); Chlorclean tablet and sodium dichloroisocyanurate (SDIC) for 5 minutes with neutralizer validation. Residual biofilm viability was detected by standard plate culture, live/dead stain and residual biofilm protein using commercially available protein assay (PA) following hydrolysis of the biofilm. Percentage reduction of biofilm was compared with untreated controls.

Results: Mixed species biofilm was formed with the following % of bacteria incorporated into the biofilm *S. aureus* 54 to 56%, *E. faecium* 37 to 39% and *A. baumannii* 6 to 8%. The individual species within run CV was 28% to 38% and between run was 2% to 15%. After treatment with Surfex, each of the biofilm bacteria; *S. aureus, E. faecium* and *A. baumannii* were inactivated (100%) by factor 6, 5, and 4 $\log_{10}$ in titer respectively. In contrast, after treatment with Chlorclean tablet and SDIC the log number reduction was very low; *S. aureus*-1.9 and 2*, E. faecium*—3.2 and 3 and *A. baumannii* —2.9 and 3.5 respectively. Equally, little of the biofilm mass was removed by chlorine-based disinfectants (<15%) and whilst Surfex removed 48.8% of the biofilm mass.

Conclusion: We have developed a model of dry surface biofilm that incorporates three pathogens that commonly cause HAI. The protocol results in a high percentage of *S. aureus* similar to clinical biofilms which contain large quantities of Staphylococcal bacteria and lower concentrations of *E. faecium* and *A. baumannii* which are also found frequently contaminating hospital environmental surfaces. This combined with the low CV determines that this model is appropriate for evaluating the efficacy of different detergents and disinfectants commonly used to clean and disinfect hospital surfaces. The efficacy level of some commonly used disinfectants against mixed species biofilm are very low.

Introduction

Bacteria exhibit an altered phenotype when incorporated into multispecies biofilm and make them more complex to improve their survivability than the planktonic culture, leading to an active source of pathogens resulting in hospital acquired infections (HAI). (Elias and Banin, 2012; Kuramitsu et al., 2007) Biofilm infections cause many chronic and recurrent infections and are estimated to be associated with 80% of all microbial infections in the human body (Römling and Balsalobre, 2012). A recent study on ICU environmental surfaces by Hu et al., (2015) showed that 93% of samples have biofilm and all dry surface biofilms were polymicrobial. Of the culture positive samples, 52% contained MDRO organisms, including 18% of isolated *S. aureus* strains were MRSA, 7% of *Enterococcus spp*. were VRE and 12% of all Gram negative bacteria were ESBL. The author also found by pyrosequencing, that *S. aureus* contributed highly to biofilm mass, contributing up to 40% of the biofilm. Other pathogens frequently responsible for HAI also contributed to total biofilm mass including *Acinetobacter spp*. that formed 1 to 8% of the biofilm mass and *Pseudomonas aeruginosa* forming between 0.16 to 2.84%.

It is now widely known that biofilms show extreme diversity in different environments. To understand multispecies biofilm many in vitro models utilising multispecies have been established to investigate traditional hydrated biofilm. However, a dry surface mixed species biofilm in vitro model where bacteria are subjected to dehydration during their growth, has not yet been developed. Maintenance of the relevant microenvironment to ensure that each species continues to grow within a multispecies biofilm is a great challenge as many bacterial species find the dehydrating conditions challenging. Despite these challenges, the advantage of developing a dry surface mixed species biofilm model, is worthwhile due to tremendous increase in the number and diversity of interactions present in a multispecies biofilm community. Juggling the growth conditions and selection of additional bacterial species are adding higher demands to the experimental designs and analysis tools used for investigation (Røder et al., 2016).

Establishment of a standard mixed biofilm model might help to elucidate many critical issues such as the development of resistance to antibiotics, disinfectants and detergents. Cleaning and disinfecting are important part of a comprehensive infection prevention strategy. At present there is no standard method for testing the level of efficacy of different detergents to eradicate mixed biofilm formed on dry hospital surfaces. In this study, a multi species dry biofilm model

analogous to the properties of mixed species dry biofilms obtained from hospital surfaces has been developed to test detergent efficacy and effect of mixed species biofilm on biofilm structural and chemical composition (carbohydrate, DNA, protein).

Materials and methods

Development of a model mixed species biofilm on a dry surface:

Multispecies biofilm was developed on removable polycarbonate discs in the CDC bioreactor (BioSurface Technologies Corp, Bozeman, USA) over a period of 13 days. Biofilm growth was initiated by addition of 10^8 colony forming units (CFU) of *S. aureus* ATCC 25923 into 500mls of 10% w/v tryptone soya broth (TSB) and incubated for 24 hours at 35 °C under shear produced by baffle rotation at 130 rpm/min. After 24 hrs the media was replaced with 10% TSB containing 10^8 CFU of each of the three species of bacteria *S. aureus*, *Enterococcus faecium* ATCC 35667 and *Acinetobacter baumannii* ATCC 19606 and incubated for 48 hours. Thereafter, the media was removed and the biofilm subjected to 48 hours of dehydration followed by three cycles of growth in 5% TSB alternating with prolonged dehydration phases at room temperature (22–25 °C) as described in chapter 5, 5.5.4. Filtered airconditioned air was pumped into the CDC biofilm reactor at 3L/minute.

Table 1

Culture conditions for formation of dry surface mixed species biofilm with an initial inoculum of about 10^8 CFU of *S. aureus* in 1st phase and 10^8 CFU of *S. aureus E. faecium* and *A. baumannii,* added at the beginning of Stage 2 batch phases.

Phases	Culture conditions	Cumulative operating time
1	24 hr. batch phase in 10% TSB only *S. aureus*	24 hours
2	48 hr batch phase (3 bact. sp.) in 10% TSB followed by 48 hrs dehydration	120 hours
3	6 hr batch phase in 5% TSB followed by 66 hrs dehydration	192 hours
4	6 hr batch phase in 5% TSB followed by 42 hrs dehydration	240 hours
5	6 hr batch phase in 5% TSB followed by 66 hrs dehydration	312 hours

Determination of colony forming units (CFU) obtained:

After development of biofilm, coupons were rinsed three times in phosphate buffered saline (PBS) to remove loosely attached cells. The number of CFU per coupon was determined by

sonication of the coupon in 5 mL of 100% TSB at 80 kHz for 20 minutes (Soniclean, JMR, Australia), vortexed for 2 min followed by sequential 10-fold serial dilution from neat to 10^{-5} and standard plate count on horse blood agar (HBA) plate and UTI chromagar plate (Fig-1) after overnight incubation at 37ºC. For three separate bioreactor experiments, CFU was determined for each coupon and the within-run % Coefficient of Variation (%CV) calculated. The between run variation was calculated from the CFU using these three runs (n=72).

Qualitative analysis:

A qualitative assessment of the appearance of the dry mixed species model biofilm was made by scanning electron microscopy (SEM) after samples (n=5) were fixed in 3% glutaraldehyde for 24 hours, followed by dehydration in ethanol (30-100%), prior to immersion in hexamethyldisilazane (HMDS, Polysciences Inc) for 10 min. Samples were then air dried for 24 hrs. Specimens were coated with gold with a depth of 20nm using Emitech K550 gold coater (West Sussex, England) and analysed in a JOEL scanning electron microscope (Model-JSM6480LA, JEOL, Tokyo, Japan) at an accelerating voltage of 10KV and spot size 30.

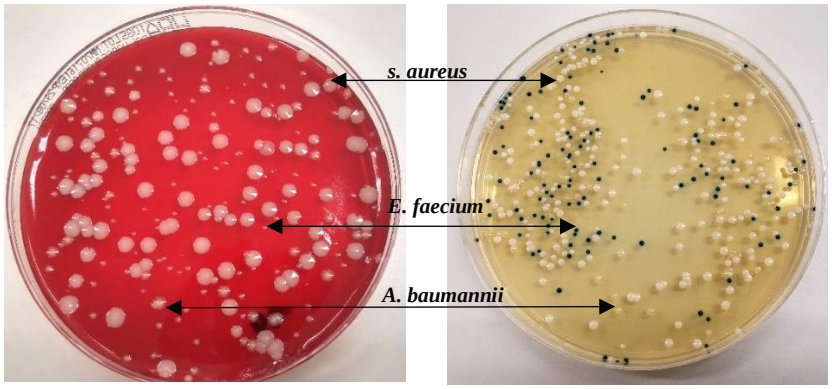


Figure 1: Mixed species biofilm bacteria colonies grew on HBA and UTI plate at 10^{-3} dilution showing three distinct species.

Characterisation of dry mixed species biofilm model

Biofilm coated coupons (n=5) were stained with LIVE/DEAD BacLight 7012 viability kit (Molecular Probes, Invitrogen, Carlsbad, CA, USA) and incubated for 60 minutes. The samples were fixed in 4% paraformaldehyde for one hour, washed 3 times in PBS for 10 minutes and observed under Olympus Fluoview 1000 inverted Confocal Scanning Laser Microscope (CSLM) for quantitative analysis. Each biofilm coated coupon was analysed at five different areas giving an average total analysed surface area of 666400 μm^2. Images were created with 0.2 µm optical sections using the Imaris 8.1.2 software (Bitplane, Zurich, Switzerland). The

minimum and maximum biofilm structural thickness was measured in µm from the top of the biofilm to the surface of the coupon (Almatroudi et al., 2015e).

Determination of Biofilm composition

Biofilm coated coupons (n=5) were stained with a 1:10 dilution of Alexa-488-labelled Aleuria aurantia lectin (Vector Laboratories, Burlingame, CA) for 20 min to label the glycoconjugate, a 1:1000 dilution of Syprorange for 15 minutes (Molecular Probes, Invitrogen, USA) to label proteins and 1:1000 dilution of SYTO 60 for 5 minutes (Molecular Probes, Invitrogen, USA) to stain nucleic acid as described previously.(Almatroudi et al., 2015c) Samples were fixed with 4% paraformaldehyde and examined under CLSM. The EPS composition of each sample was determined using ImageJ software (1.46r, National Institute of Health, USA).

Statistical analysis and Image analysis

To examine for variation in biofilm formation between each coupon, the % CV for the number of bacteria incorporated into the dry biofilm was calculated as a dimensionless measure of variance for the within-run and between-run variation.

Coefficient of variation (CV%) within and between experiment was determined by following equation;

$$\% \text{ Coefficient Variation} = \frac{100 \text{ X Standard Deviation}}{\text{Mean value}}$$

t test was performed to compare control coupon biomass with disinfectants treated biomass using Graph pad prism 7.02 Statistical Program.

Detergent efficacy testing

Test disinfectants used:

In this study, ≥ 1300 ppm a peracetic acid based Surfex (Whiteley Medical, North Sydney, Australia), and two chlorine-based disinfectants at 1000ppm; Chlorclean Chlorine tablets and SDIC tablets (sodium dichloroisocyanurate) (Guest Medical, Aylesford, Kent, United Kingdom), were used as test products (disinfectants). All disinfectants were diluted in hard water (0.304g anhydrous CaCl2 and 0.065g anhydrous MgCl2 in distilled water to make one litre) to obtain desired concentration (Hadi Vickery et al., 2010).

Disinfectant efficacy testing procedure against dry mixed species biofilm:

Each disinfectant treated condition was tested using five replicates for measuring the disinfectant's efficacy against multispecies DSB by determining residual bacterial viability expressed as colony forming units (CFU). An additional five replicates were used to measure the disinfectants ability to remove biofilm from the coupon by determining the amount of residual protein left on the coupon.

Disinfectant efficacy was tested by mixing 1 ml of test disinfectant with 1 ml of hard water and immediately adding the biofilm coated coupon for a contact time of 5 minutes. Then 1 ml neutralizer (1% Na-thiosulphate, 6 % Tween 80, 5% BCS and 10% BSA in PBS) was added. Controls: Positive (biofilm covered coupons) and negative (clean sterile coupons) control coupons were add to 2 mls of hard water for 5 minutes prior to addition of 1 ml of neutraliser. Neutraliser controls: Complete inactivation of disinfectant's efficacy by the addition of neutraliser, was confirmed by adding 1 ml of neutraliser to test mixture (1 ml disinfectant plus 1 ml of hard water) followed by immediately adding a biofilm covered coupon and reacting for 5 minutes. Sonication of the coupon at 80 kHz for 20 minutes (Soniclean, JMR, Australia), vortexed for 2 min followed by sequential 10-fold serial dilution from neat to 10^{-5} was done followed by plating out onto HBA and UTI chromagar plate and incubated overnight at 37ºC.

Residual viability was determined using viability counts and by visual assessment using live/dead staining as described above. Residual protein left was determined by alkaline hydrolysis (Li et al., 2006) followed by the Micro BCA assay (Micro BCA™ Assay; Thermo Scientific, Australia). Residual protein contaminating samples was determined by measuring sample absorbance at 562nm wavelength, subtracting the absorbance of negative control coupons (n=3) and calculating protein concentration (µg/ml) using a standard curve prepared using the supplied (provided in the Pierce Kit) BSA standard.

Results

Developed mixed species biofilm model

The average number of bacteria per coupon was $\log_{10}$±CFU of which $\log_{10}$ 5.3 ± 0.14 CFU were *S. aureus*, $\log_{10}$ 5.2 ± 0.16 CFU were *E. faecium* and $\log_{10}$ 4.4 ± 0.17 CFU were *A. baumannii* within and in between run with negligible variation in the amount of biofilm covering individual coupons (Fig-2a), with an average of 55% *S. aureus*, 38% *E. faecium* and 7% *Acinetobacter* (Fig-2b). The within run variation (CV) being 28- 38% (Fig-2c) for three

bacterial species. Similarly, there was little run-to-run variation in the number of CFU in developed biofilm with the variation being 2-15% (Fig-2d).

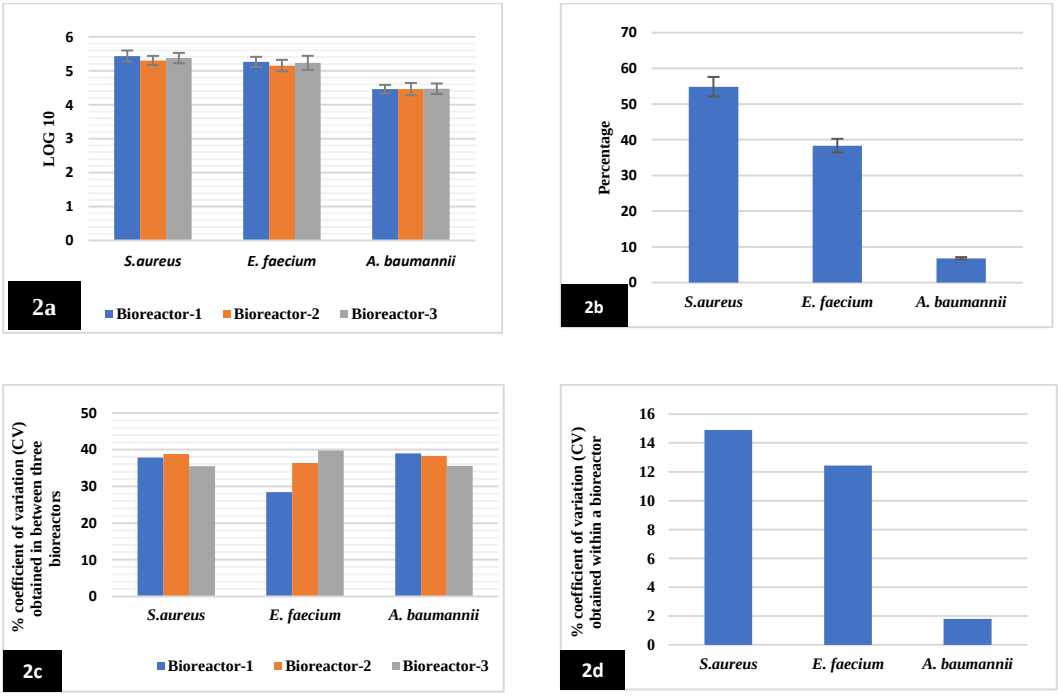
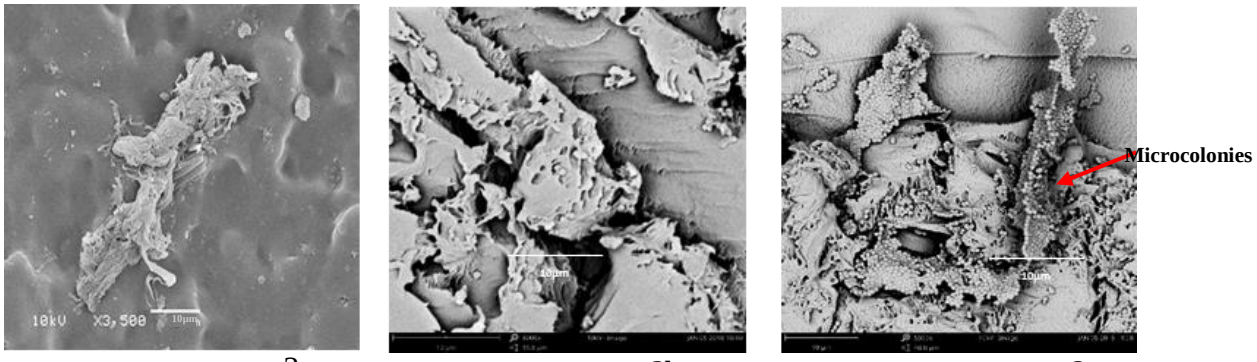


Figure 2: Number of Log $_{10}$ CFU for each three bacteria obtained from three different bioreactors started and experimented simultaneously (Fig-2a). Average percentage of each species contributed to biofilm mass; highest contribution of *S. aureus* followed by *E. faecium* and *A. baumannii* per coupon (Fig-2b).
Percentage coefficient of variation (CV) of three species obtained from within three experiments from three bioreactor (Fig-2c) and interexperiment or within individual bioreactor (Fig-2d).

Characteristics of developed mixed species biofilm model

Under scanning electron microscope (SEM), mixed species model has been observed as a multi-layered biofilm with very thick heterogenous extracellular polymeric substances (Fig. 3 b, c). The spatial organisation of microorganisms was explored, and the model biofilms was found rough, reflecting the heterogeneous nature of biofilm formation (Fig-3b and 4) with dense microbial aggregates (Fig-3c). The heterogenicity also observed under CLSM with maximum thickness of 41 ± 1.3 µm of the biofilm.



3a 3b 3c

Figure-3 Scanning electron micrograph of mixed species biofilm on ICU bed mattress [3a (Hu et al., 2015a)] and in vitro mixed species model biofilm (3b, 2c) showing multilayered heterogenous nature of biofilm with large amount of thick amorphous extracellular polymeric substances (EPS).

Composition of biofilm EPS analysis, performed taking 3D images from different surface area of each stained sample coupon (n=5), exhibited protein as the major component of the model biofilm (73%) and the next most prevalent component was glycoconjugate (23%) and DNA (4%) (Fig 4).

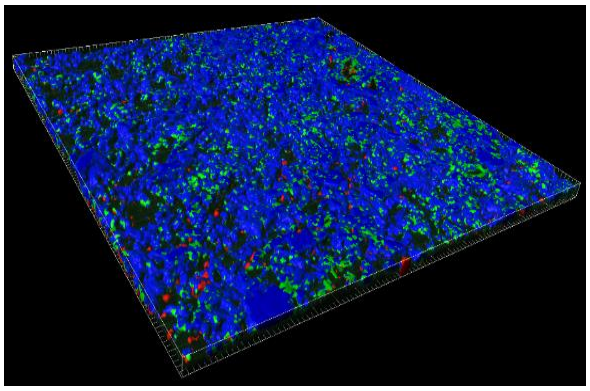


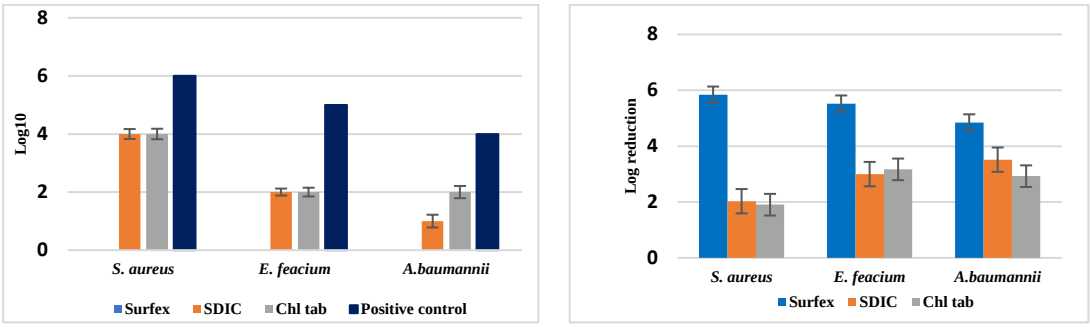
Figure-4: 3D view showing heterogenous nature of mixed species biofilm with multilayered biofilm area and composition of extra cellular polymeric substances with high extracellular Protein (blue) than glycoconjugate (green) and DNA (red).

Disinfectant efficacy testing against mixed species dry durface biofilm

CFU count

In this study, the mean CFU of per positive control (n=5) coupon was 6.9 x10^5 for *S.aureus*, 3.3 x10^5 for *E. faecium* and 7.0 x10^4 for *A. baumannii* (Fig-5a). Treatment of mixed species biofilm with Surfex, no viable bacteria were detected on HBA plate and count was reduced significantly by a factor of 5.8 log $_{10}$ for *S.aureus*, > 5 log $_{10}$ for *E. faecium* and > 4 log 10 for *A. baumannii* (Fig-5b). In contrast, there is a small amount of viable bacterial reduction

occurred by SDIC and Chlorclean tablet ; *S.aureus* reduced by factor 2 ± 0.16 and 1.9 ± 0.17 log 10; *E. faecium* 3 ± 0.11 and 3.2 ±0.14 log 10 ; *A. baumannii* 3.5 ±0.21 and 2.9 ± 0.20 log 10 respectively (Fig-5b).

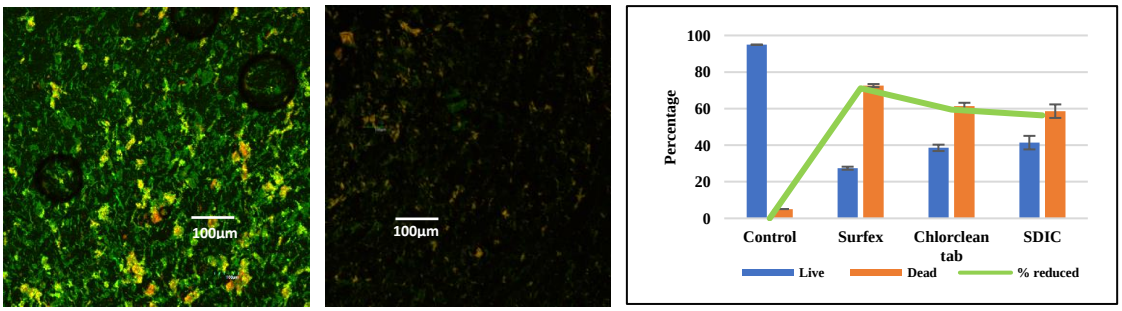


5a 5b

Figure 5: Log_{10} values obtained (5a) and number of log_{10} reduced (5b) of three species after treatment with Surfex, SDIC and Chlorclean chlorine tablet disinfectants for five minutes.

Confocal microscopic analysis

Presence of live bacteria following disinfectant treatment was confirmed by confocal microscopy (CLSM) (Figure 6a, 6b). The total biomass for untreated coupons was 999862µm^3, consisting of 95.1% live bacteria. After analysis of images, it has been determined that, after treatment with Surfex, Chlorclean tab and SDIC, the total biomass reduced by approximately 71.12 %, 59.3% and 56.39% respectively ($P \leq 0.01$) (Fig-6b).



6a 6b 6c

Figure 6. Viability stain of in-vitro developed mixed species biofilm; control (6a) and Surfex treated biofilm (6b) showing the presence of live (green) and dead (red) bacteria. Percentage of live and dead biofilm bacteria and percentage reduction in the total biomass of treated coupons compared to control coupons were determined by live/dead staining. (Fig-6c).

Protein assay

Mixed species biofilm elimination capability of disinfectants was assessed by measuring the amount of protein left on the coupon surfaces after treatment with the disinfectants for five minutes contact time. Percentage reduction of protein with Surfex treatment was 48.8% and the amount of protein reduced by chlorine-based disinfectants were less with Chlorclean tablet only removing13% and SDIC 11% (figure-7)

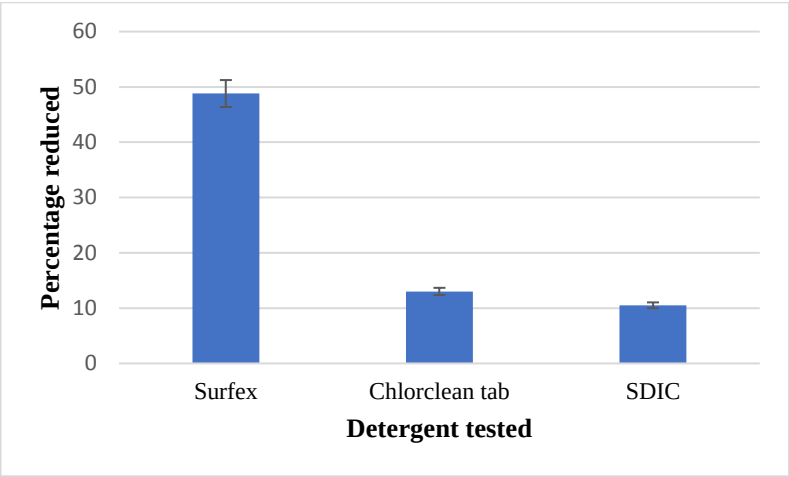


Fig-7: Percentage reduction of protein component after treatment with Surfex, Chlorclean tablet and SDIC for five minutes contact time.

Discussion

In this study, a dry mixed species model biofilm has been developed by adapting biofilm culture conditions including periodic hydration with very low nutrition and dehydration. The CDC biofilm reactor was used to develop this standard protocol with the advantages of growing a reproducible, better attached biofilm under shear rotation. The growth protocol followed the in vitro single species dry surface biofilm model protocol developed by Almatroudi (2015) with some necessary modification. The three strains listed were chosen as the representative biofilm-formers as they are highly pathogenic, are frequently isolated from HAIs. An additional advantage was that they possessed a colony morphology that was distinct from each other and when grown on HBA plate identification of each species was possible. Moreover, *E. faecium*, *S. aureus*, *Klebsiella spp.*, *A. baumannii*, *P. aeruginosa* and *Enterobacter spp*. the ESKAPE pathogens have been classified as the top six MDRO by the Infectious Diseases Society of America (Llaca-Díaz et al., 2012) and it has been also shown that about 86% of clinical dry surface biofilms contains *Staphylococci.* (Hu et al., 2015).

In this experiment, the CDC bioreactor reproducibly formed multi-layered multispecies biofilm containing about—*S. aureus* 10^5/coupon, *E. faecium* 10^5 /coupon and *A. baumannii* 10^4/coupon embedded in thick EPS. *S. aureus* has the highest contribution to biofilm mass that is 56% followed by *Enterococci spp*. 38% and *Acinetobacter spp.* 7% on an average. In this experiment we achieved incorporation of each bacterial species into the DSB in the percentage that we considered most suitable and representative of clinical biofilms. That is a high percentage of *S. aureus* in conjunction with lower percentages of other important pathogens. considerable amount of three species of bacteria. To observe the reproducibility, we repeated the same protocol in three different runs and determined the coefficient of variation (CV). This model showed low % CV for each three species bacteria. And it is suggested that the lower the CV, the smaller the residuals relative to the predicted value. The between run CV was extremely low and indicates that the model is reproducible suggesting that this model is appropriate for evaluating the efficacy of different detergents and disinfectants commonly used to clean and disinfect hospital surfaces.

The percentage of bacteria species in this study have to be different from those found in clinical DSB, as the clinical biofilm contaminating the patient surrounds was found to have an average of 23 different bacteria making up the biofilm mass, albeit most of these were very low percentages of around 1%. In this study an increased percentage of *S. aureus* was targeted because it is the most commonly found pathogen incorporated into the DSB (Hu et al., 2015; Johani et al., 2017) and it is an important cause of HAI being responsible for ≥50% cases (Magill et al., 2014; Uneke, 2014).

The presence of biofilm was visualised and confirmed using either SEM and CLSM. Multiple images were observed under microscope to provide an overall impression. SEM images identified large micro-colonies with a predominance of coccoid cells (fig-3c), which appeared to be coated with very thick EPS (Figure 3b). This result corresponds to appearance of the mixed species biofilm with thick heterogenous EPS, identified from the ICU environment (Fig-3a) (Hu et al., 2015). To study the EPS was stained with a combination of lectin, protein and nucleic acid stain and observed under CLSM to distinguish EPS components. Protein was found as predominant structural component of EPS which was also the case for single species biofilm model composed of *S. aureus* and clinical biofilm where protein forms 42-95% of the biofilm Almatroudi (2015). The biofilm was composed of 73% protein in the mixed species model which was greater than the amount in the monospecies *S. aureus* DSB model which contained

56% protein (Almatroudi et al., 2015). This higher amount of protein may be due to the increased number of bacterial species that stimulate the more rapid utilisation of polysaccharide component of the EPS than the protein component during starvation as described for wet biofilm (Zhang and Bishop, 2003)

According to Almatroudi (2015), the maximum thickness of the clinical biofilm varied depending on the site, for example; an average thickness of biofilms formed on fabrics is 45.6 µm and on hard surface 26.5 µm. In this study the maximum thickness observed in model mixed species biofilm is 41 ± 1.3 µm which is higher than the single species model biofilm (30µm) developed by Almatroudi (2015). This increased thickness may be due to the interspecies interaction, surface characteristics as described in previous studies by Elias (2012) and Almatroudi (2015) respectively. In addition to this, multispecies biofilm is described as a complex structure that exhibits altered phenotype with respect to growth rate and gene transcription, which has been shown as a predisposing cause of increasing biofilm mass and frequency of genetic mutation (Elias and Banin, 2012).

In this study, the developed mixed species model biofilm was investigated to observe the disinfectant efficacy level. We found that a 5 minutes Surfex exposure resulted in a 100% $\log_{10}$ reduction in *S. aureus, E. faecium* and *A. baumannii* and removed over 71% of live bacteria and 48.8% protein component of the biofilm. Despite of complete inactivation of biofilm bacteria by Surfex, the leftover protein concentration is 29%, which could explain that Surfex is not able to remove whole lot of protein contents from the treated surfaces. However, our previous study on single species dry surface biofilm (DSB) model showed 65% reduction of protein component after 5 minutes contact time with Surfex and 17.65% and 13.12% reduction of protein by Chlorclean tablet and SDIC respectively. This reduction level declined to 48.8%, 13% and 11% in case of mixed species model biofilm. These results might be explained by the extra thickness exhibited by the mixed species biofilm as well as extra mass, the extra thickness translates into a greater diffusion distance that cleaning agents and biocides must cross to reach all the biofilm bacteria. The decline of protein reduction in mixed species model biofilm might be due to the increased thickness of the EPS (41µm) that is 36% higher than the single species biofilm model (30 µm) (Almatroudi et al., 2015) and also might be due to the concentrated EPS as it generates a number of non-specific binding sites within the biofilm that certainly affect detergent and disinfectant action (Flemming and Wingender, 2010).

Polymicrobial biofilm has been shown to have improved survivability by decreasing susceptibility to antimicrobial agents and disinfectants (Kuramitsu et al., 2007). This

improvement might help bacteria to persist for longer time in hospital environment and act as a complete source of hospital acquired infections (HAI). Therefore, disinfection and cleaning of hospital surfaces is crucial to prevent transmission of pathogens in hospital environment. However, yet there is no standardized method for testing the removal of mixed species dry surface biofilm. This study recommends that, this mixed species dry biofilm model is suitable for efficacy testing of decontamination products against mixed biofilms grown on dry hospital surfaces. The species percentage of bacteria in the mixed biofilm is similar to that found in dry surface biofilms contaminating hospital surfaces. This combined with the low CV determines that this model is appropriate for evaluating the efficacy of different detergents and disinfectants commonly used to clean and disinfect hospital surfaces.

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Chapter Six: Comparative proteomic profile; new insight of *Staphylococcus aureus* dry surface biofilm

6.1 Introduction

In chapter 4, we showed that the tolerance to biocides and removal of the biofilm mass, are major problems in the decontaminating hospital surfaces of DSB. This study also showed that, bacteria incorporated into a mixed species biofilm environment, had more tolerance to killing and removal than the single species DSB. Limited diffusion of drugs and disinfectants has been detected as a partial cause of higher resistance. A study on fluorescently labelled derivative of vancomycin was been used to treat biofilm and planktonic *S. aureus.* It was demonstrated that the penetration time to contact the bacteria in the deepest layers of a biofilm took 10 times more time than to contact free-floating *S. aureus* (Jefferson et al., 2005). In addition, biocides, particularly highly charged biocides can be inactivated as they diffuse through the EPS. Thus, biofilm bacteria get adequate time to increase their tolerance to the antibiotic/biocide by undergoing stress-induced metabolic or transcriptional changes (Resch et al., 2006). In addition, the slower growth rate and altered metabolism in biofilm state contributes to antibiotic tolerance and may contribute to the higher tolerance towards other therapeutic compounds (Resch et al., 2006).

From above evidence it is now clear that much effort is still needed to gain a better understanding of biofilm formation and growth. Proteomics and transcriptomic analysis could be the ideal approach to explore biofilm bacterial structural and functional characteristics. Several studies have investigated various sub-populations of proteins of *S. aureus* and focused on the comparison of wild-type and mutant strains (Gertz et al., 2000; Ziebandt et al., 2001; Nandakumar et al., 2005). However, so far our knowledge, proteomics study on dry surface biofilm has not performed yet.

In this study, *S. aureus* was chosen as this organism is a major source of HAI and because of its prolong persistence in dry hospital environment (Havill et al., 2014). Therefore, this study aimed to construct a comprehensive reference map of proteins associated with 12-day *S. aureus* wet (WB) and dry biofilm (DSB) and identify proteins that may be differentially expressed in these biofilms. A small number of expressed genes were also targeted to confirm the proteomic profiling and comparing the expression of genes in between DSB and 13-day mixed species biofilm (MDSB) to get an idea how *S. aureus* differs in expression when form a biofilm in a mixed environment.

6.2 Original Paper

Title: Comparative proteomic analysis of laboratory grown *Staphylococcus aureus* traditional and dry surface biofilm and correlation with targeted expressed genes between single and mixed species dry biofilm.

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Keywords:
Dry-surface biofilms
Wet biofilm
Proteomics
Healthcare-associated infections
Staphylococcus aureus

In above publication, Dr Durdana Chowdhury has following contribution

- Design study protocol with Arifur Rahman and Associate professor Karen Vickery
- Carried out in vitro experiments
- Collected data
- Data compilation
- Data analysis.
- Total manuscript preparation

Author contribution 60%

Summary

Introduction and Objectives: *Staphylococcus aureus* infection represents a serious public health burden worldwide and are well known for their biofilm forming ability. Biofilms are problematic, particularly, in healthcare settings where they are thought to be associated with at least 65% of nosocomial infections. *S. aureus* and other pathogens such as *Acinetobacter baumannii* and *Enterococcus spp.* are readily transmitted within the healthcare environment due to their long survival on hospital surfaces. However, recent finding of biofilms on dry hospital surfaces emphasises in the failure of current cleaning practices and disinfectants (Hu et al., 2015b; Johani et al., 2017b). A better understanding of wet biofilm and dry surface biofilm (DSB) specific proteins and genes could help to innovate development of effective detergents and disinfectants by targeting the main proteins found in specific biofilms. and that can be achieved by studying different biofilm specific protein and. This study aimed to construct a comprehensive reference map of proteins associated with *S. aureus* WB and DSB by determining the proteome. Once identified the transcriptome of genes regulating the principally proteins were compared between 12-day single and 13-day mixed DSB followed by changes in metabolic processes.

Methods: *S. aureus* reference strain (ATCC 25923) was used to grow planktonic, WB and DSB grown over a period of 12 days and 12-day mixed bacterial species dry biofilm (MDSB) containing *S. aureus*, *A. baumannii* and *E. faecium*. Bacteria were grown in tryptic soy broth (TSB). Planktonic bacteria were used in the late logarithmic growth stage and the Centres for Disease Control (CDC) biofilm reactor was used to grow WB, DSB and MDSB. Protein extraction, fractionation and concentration were performed using lysis buffer, probe sonication, 10KDa molecular weight cut off (MWCO) followed by 3KDa MWCO, respectively. Samples were subjected to reduction, alkylation and digestion steps prior to Multiplex labelling using Tandem Mass Tag (TMT) 10-plex reagent. The labelled samples were pooled and fractionated by high pH RP-HPLC which followed by loading of the fractions on a nanoflow UPLC system. Mass spectrometry (MS) data was collected on an Orbitrap Elite Mass Spectrometer. Protein identification and relative quantitation of protein levels were performed using Proteome Discoverer and encoded genes were demonstrated by blast analysis. Expression of Targeted genes from proteomic analysis were compared between DSB and MDSB after confirming with RT-qPCR.

Results: The present study showed that considerable proteomic difference exists among *S. aureus* WB and DSB. We identified 1636 total extracted supernatant proteins, of which 113 and 134 proteins were in significantly high abundance in WB and DSB respectively. Of these, significantly up-regulated proteins, 50 were only found in WB and 38 only in DSB. Less than

half of these were in known protein pathways. WB had 52 significantly down-regulated proteins and DSB 55. The genes *cysE, murC, murB, fhuC* regulating high abundance metabolic and structural proteins found in DSB showed relatively low expression in 12-day MDSB compared with DSB by RT-qPCR.

Conclusions: In this study, we constructed a comprehensive reference map of WB and DSB associated proteins and some target genes which might be helpful to diagnose biofilm associated infections as well as designing advanced targeted disinfectants and detergents especially for dry hospital environments.

1. Introduction

Staphylococcal infections impose a threat worldwide. Their biofilm forming ability and huge diversity combined with their long survivability on the dry hospital surfaces increase the difficulties in preventing transmission of infection within the hospital environment. Factors contributing to biofilm formation in clinical settings are still not completely identified. The role the environment plays in transmitting *S. aureus* within the healthcare environment and the continuing development of antibiotic resistance, it is becoming crucial to explore biofilm structural and functional characteristics in the hope of developing novel anti-biofilm biocides. Proteomics analysis allows the detection of proteins, the functional entities of a cell, and of post-translational protein modifications, which cannot be predicted by mRNA expression analysis (Sauer, 2003). Differentially expressed bacterial proteins are of potential interest in the search for drug targets or biomarkers (Carlsohn et al., 2006).

Protein expression changes during the biofilm formation (Sauer et al., 2002). Differentially expressed *S. aureus* biofilm proteins include various types of adhesins or MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) that supports bacterial attachment to the surfaces (Patti et al., 1994) and PSMs that help in biofilm-structuring activities. These proteins are important during biofilm formation and biofilm dispersal (Wang et al., 2007; Kretschmer et al., 2012). However, to date protein expression has only been conducted on WB compared to planktonic growth, but investigating proteins associated with biofilm growth on dry surfaces has not been conducted. The availability of complete biofilm bacterial proteomic analysis might help to generate a wealth of additional information and help target specific proteins for biocide action.

Information about DSB is very limited and this makes it difficult to compare between transcriptomes and proteomes. Biofilm growing on dry surfaces is subjected to different environmental conditions, so it could be expected that, *S. aureus* DSB might have a different genetic expression profile. Protein and transcriptional profiling of *S. aureus* during different modes of growth will increase our knowledge of biofilm physiology and might hold clues for targeting antibiotics, biocides and diagnostic biomarkers.

Therefore, this study aimed to construct a comprehensive reference map of proteins associated with 12-day *S. aureus* wet and dry biofilm and identify unique proteins. A small number of differentially expressed genes were also validated by reverse-transcription quantitative PCR and comparing the expression of genes between DSB and MDSB containing *S. aureus*, *A.*

baumannii and *E. faecium* to get an idea on how *S. aureus* differs in expression when present in a biofilm in a mixed environment.

2. Materials and methods

2.1 Microorganism and Culture Conditions

S. aureus reference strain (ATCC 25923,10^8 CFU at OD 600 nm) was used to grow 12-day wet (WB), dry (DSB) and 10^8 CFU for each species; *S. aureus* (ATCC 25923), *A. baumannii* (ATCC 19606) and *E. faecium* (ATCC 35667) for mixed species biofilm (MDSB). Liquid planktonic culture was prepared in 100% TSB at 37ºC for 24 hour as described in chapter 2, 2.1. Biofilm growth: biofilm was developed on removable coupons in a Centers for Disease Control (CDC) biofilm reactor. For 12-day WB, *S. aureus* was grown in 50% w/v tryptic soya broth (TSB, Oxoid Australia) under shear at 37ºC in batch phase for 48 hours followed by 20% TSB media exchange every 48 hours as described in chapter 2, 2.3.2. DSB was formed with alternating nutrition and dehydration phases, mimicking the dry biofilm on hospital surfaces. Biofilm was grown in 5%TSB over a period of 12 days with an initial batch phase of 48 hours followed by 48 hours of dehydration at room temperature (22-25 ºC). An additional three cycles of batch growth for 6 hours, interspersed with prolonged dehydration phases of 66, 42 and 66 hours (Almatroudi et al., 2015). Mixed species biofilm was developed with 24 hours prior inoculation of *S. aureus* in 10% TSB followed by addition of three bacteria; *S. aureus*, *E. faecium* and *A. baumannii* in 10% TSB in 2nd phase following DSB protocol for last three phases as described in chapter 5, 5.5.4.

Experiments were performed with three replicates for each group. For replication, planktonic liquid culture, 12-day WB and DSB and mixed species dry biofilm were developed maintaining similar growth condition in every run.

2.2 Protein Extraction and Fractionation

Extraction and fractionation were performed as described (Sadovskaya et al., 2005) with necessary modifications. For 24-hour liquid culture, bacteria (10^8 CFU at OD 600 nm) were centrifuged at 12,000 x g for 10 minutes and collected supernatant was mixed with lysis buffer containing Triethylammonium bicarbonate (TEAB, pH 8.5) and sodium deoxycholate (1% w/v) at 10:1 ratio (supernatant: lysis buffer) followed by probe sonication (Sonic Ruptor, Omni international, USA) for 2 minutes at 70% pulses. Lysed supernatant samples were loaded on to an Amicon ultra membrane (Sigma Aldrich, Australia) 10kDa molecular weight cut off (MWCO; Sigma Aldrich, Australia) filter tube to concentrate and precipitate proteins from the samples by washing with double volume of PBS for 3 times followed by 3KDa MWCO tube (Sigma Aldrich, Merck Millipore, Ireland) filtration. Extraction of biofilm proteins: biofilm

coated coupons were washed with PBS to remove loosely attached bacteria. Each coupon was placed into a five ml tube containing 2 mL of PBS and 200 µL of lysis buffer and incubated overnight with a gentle shaking at 4°C. After incubation, Probe sonication was done in an icecold environment for 2 minutes at 70% pulses. Collected solutions from each coupon were centrifuged at 12,000 x g for 30 min at 4ºC. Supernatant fractions were transferred, concentrated, and precipitated using 10KDa MWCO followed by 3KDa MWCO, respectively. Finally, Pierce BCA protein assay (Thermo Fisher Scientific, Australia) was performed to measure protein concentration at 562 nm wavelength (PHERAstar FS, BMG Labtech, Germany) using supplied bovine serum albumin (BSA) as a protein standard.

2.3 Protein Reduction, Alkylation, and Digestion

After protein measurement, a total of 40 µg protein of each sample was processed further for reduction, alkylation and digestion. Each sample was reduced with 5mM dithiothreitol (DTT) for 15 min at room temperature and alkylated with 10mM iodoacetamide (IAA) in the dark for 30 min at RT. Alkylated samples were diluted with 20 µL 100mM TEAB pH 8.5. For digestions, Lys-C was added at a ratio of 1:30 for overnight at room temperature and trypsin (0.2µg/µl; Promega, Madison, USA) was added at a ratio of 1:30 for 5.5 hr at 37°C. Samples were adjusted to 1% (v/v) trifluoroacetic acid (TFA), and the precipitated deoxycholate was removed by centrifugation. Samples were centrifuged at 14,100 x g and desalted using SDB-RPS (3M-Empore) Stage Tips. Briefly, SDB-RPS stage-tips were prepared and samples were added to the stage-tips and centrifuged 1000 x g for 5 min. Stage-tip containing samples were washed twice with 0.2% TFA and centrifuged at 1000 x g for 5 min. Samples were eluted with 5% ammonium hydroxide in 80% acetonitrile (ACN) and centrifuged at 1000 x g for 5 min. Samples were vacuum (miVac, Genevac™, Thermofisher scientific, USA) dried and stored at -20°C until further processing.

2.4 TMT Labelling and High pH Fractionation

Tandem Mass Tag (TMT; Thermo Fisher Scientific) reagents (0.8 mg) were dissolved in anhydrous ACN (85 µL) of which 41 µL was added to the reconstituted (100 µL of 100mM TEAB) samples and incubated for 1hr at RT. 8 µL of 5% Hydroxylamine was added to each TMT labelled samples and incubated for 15 min at room temperature. 2 µL of each labelled sample was pooled and dried and reconstituted in 30 µL 0.1% formic acid (FA) solution, centrifuged for 5 min at 14000 x g and analysed with mass spectrometer.

The data was search using Proteome discoverer 1.3. Based on The Applied Normalization Values from this search result an equal number of peptides were taken from each sample, pooled and vacuum dried (miVac).

The pooled labelled sample was fractionated by high pH RP-HPLC (reversed-phase high-performance liquid chromatography, Agilent technology). The buffer A was 5mM ammonia solution (pH 10.5) and buffer B was 5mM ammonia solution with 90% acetonitrile (pH 10.5). The dried labelled sample was resuspended in loading buffer which was the same as the buffer A. After sample loading and washing with 97% buffer A for 10 min, buffer B (concentration was increased from 3% to 30%) for 55 min; 70% for 10 min; 90% for 5 min at a flow rate of 300 µL/min. The eluent was collected every 2 min at the beginning until 16 min and every 1 min intervals for the remainder of the gradient.

The fractionated sample by high pH RP-HPLC was pooled to 20 fractions, dried in vacuum. Finally, each fraction was resuspended in 55 µL of 0.1% FA for mass spectrometry analysis.

2.5 nanoLC-ESI-MS/MS

The samples were run on two mass spectrometer systems.

2.5.1 nanoLC-ESI-MS/MS using Orbitrap Elite

An Orbitrap Elite (Thermo Fisher Scientific) Mass Spectrometer equipped with PicoView 550 Nanospray Source (New Objectives), an Eksigent Ultra-Performance Liquid Chromatography (UPLC) system (AB SCIEX) consisting of an ekspert™ nanoLC 425 UPLC pump and ekspert™ nanoLC 400 autosampler was used for acquiring data. 20 µL of each fraction was loaded onto a self-packed 100 µm x 3.5 cm trap column with Halo® 2.7µm 160 Å ES-C18 (Advanced Materials Technology) and desalted with loading buffer [0.1% FA] at a flow rate of 4 µL/min for 10 min. Peptides were eluted onto a self-packed analytical column 100 µm x 30 cm with Halo® 2.7µm 160 Å ES-C18 (Advanced Materials Technology) with the linear gradients of mobile phase A [0.1% FA/5% dimethyl sulfoxide (DMSO)] and B [0.1% FA/5% dimethyl sulfoxide (DMSO)] starting with B (1–10%) for 0.1 min, B (10–20%) for 52 min, B (20–32%) for 48 min followed by (32%-43%) for 20 min with a flow rate of 450 nL/min across the gradient. The eluent from the trap was diluted with 100 nL/min of buffer A before reaching the analytical column. The peptides refocused and separated over the analytical column at 60°C. Peptides were ionized by electrospray ionization and data-dependent MS/MS acquisition carried out using a Orbitrap Elite (Thermo Fisher Scientific) consisting of 1 full MS1 (R=120 K) scan acquisition from 380 to 1600 m/z, and 15 HCD type MS2 scans (R=30 K).

2.5.2 nanoLC-ESI-MS/MS using Q Exactive

A Q Exactive (Thermo Fisher Scientific) Mass Spectrometer equipped with Nano spray Source and Easy nLC 1000 (Thermo Fisher Scientific) was used for acquiring data. 10 µL of each fraction was loaded onto a self-packed 100 µm x 3.5 cm reversed phase peptide trap with Halo® 2.7 µm 160 Å ES-C18 (Advanced Materials Technology) desalted with 20 µL of loading buffer [0.1% FA] and the peptide trap was then switched on line with the analytical column a self-packed 75 µm x 3.5 cm Halo® 2.7 µm 160 Å ES-C18column. Peptides were eluted with the linear gradients of mobile phase A [0.1% FA] and buffer B [100%(v/v) ACN, 0.1%(v/v) formic acid] starting with (1–30%) for 110 min, B (30–85%) for 2 min followed by 85% B for 8 min with a flow rate of 300 nL/min. Peptides were ionized by electrospray ionization and data-dependent MS/MS acquisition carried out using a Q-Exactive consisting of 1 full MS1 (R=70 K) scan acquisition from 350 to 1850 m/z, and 10 HCD type MS2 scans (R=70 K).

2.6 Database Searching and Statistical Analysis

The raw data files were submitted to Proteome Discoverer (version 1.3, Thermo Scientific). The data were processed using Sequest and Mascot (Matrix Science, London, UK) against *S. aureus* reference strain (ATCC 25923) genome sequence (DDBJ/ENA/GenBank under the accession numbers CP009361 (Treangen et al., 2014). The parameters for the data processing were as the following:

Enzyme Name: Trypsin
Maximum missed cleavage: 1
Precursor mass tolerance: 10 ppm
Fragment mass tolerance: 0.1 Da
Dynamic modifications: Oxidation (M), Deamidated (N, Q) and Acetyl (N-Terminus)
Static Modification: Carbamidomethyl (C), TMT10plex (K) and TMT10plex (N-term)

BLAST search was performed using highly annotated strains *S. aureus* N315 and *S. aureus* COL. These strains were chosen to investigate differential protein expression in mature *S. aureus* biofilm using Uniprot database of protein sequence and functional information. After the extraction of protein ratios with Proteome Discoverer, additional processing and statistical analysis was done using the TMTPrePro R package (Mirzaei et al., 2017) .

3. RNA isolation and reverse transcription (described in detail in chapter 2, 2.11)

Total RNA was extracted from biofilm coated coupons after fixing with RNA later (Qiagen, Australia) overnight at 4°C. After washing with RNase-free water coupons were resuspending

in a tube containing 200 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA) and 350 µl RLT buffer from Qiagen RNeasy Mini Kit, with 10 µl beta mercaptothion (β ME, Sigma-Aldrich, Australia), with lysozyme (Sigma-Aldrich, Australia, 2.5mg/ml). lysostaphin (Sigma-Aldrich, Australia, 2.5mg/ml) and proteinase K (Sigma-Aldrich, Australia, 2.5mg/ml) and incubated for 20 minutes at room temperature followed by 2 minutes probe sonication (Sonic Ruptor, Omni international, USA) at 70% pulses. Solution was then applied to a Qiagen RNeasy Mini Kit, according to the manufacturer's recommendations for prokaryotic total RNA isolation. Total RNA concentrations were determined by nanodrop 2000.

The contamination of DNA from total RNA extracted was removed by ezDnase (Thermo Fisher scientific, Australia) following manufacturer instruction with slight modification of incubation at 37°C for 5 minutes. Dnase treated RNA was then converted to cDNA using SuperScript™ IV VILO™ Master Mix (Thermo Fisher scientific, Australia) according to manufacturer instruction, incubation at 25°C for 10 minutes, 50°C for 10 minutes and then inactivated at 85°C for 5 minutes.

4. Quantitative real-time PCR (qPCR) and gene quantification

To confirm the results of proteomics data and quantify the relative expression levels of the *murC, murB, cysE, fhuC, femX, prs* and *icd* genes in each growth condition were determined by qPCR (described in detail in chapter 2, 2.11.4 and 2.11.5) with Stratagene Mx3000P QPCR System (Bioscience Corporation, USA), gene-specific primers, and Brilliant® II SYBR® Green QPCR Master Mix (Bioscience Corporation,

Table 1. Sequences of primers used in this study

Gene Name	primer	Position in gene	PCR size (bp)	Found in *S. aureus* ATCC 25923 genome
CysE F	CGGCACGTTCAACATTAGA	53-240	188	Yes
cys R	TATAAATAGACGCTTTCCAA			
murC F	TAATTGGTGATGGCACAGG	395-506	112	Yes
murc R	GTCATAATTGCGTAATCAGG			
murB F	AGGTGGTAATGCCGACTTT	99-253	155	Yes
murB R	TAATTACAATGCCGCGAATA			
icd F	AAGAAGCGGCAGATAAGATT	1124-1207	84	Yes
icd R	CCATTAAACGGGCAAAGT			
fhuC F	TTAATTTCGGACGATTT	287-395	109	Yes
fhuC R	TTACGCTTTCTAATATCTTG			
prs F	TCAGGACCGGCTAAAGAAC	784-918	135	Yes
prs R	AGCTTGTGCGATTAAACCAG			
femX F	ATTAACGAAATGGGCAGAAA	75-144	70	Yes

USA). qPCR was run with the corresponding standard samples of known concentrations (copies/µl), 10^8,10^7,10^6,10^5,10^4, 10^3 copies of *S. aureus* genome per µl to generate a standard curve (three replicates for each concentration) for each primer pair. Cycling conditions for real time PCR were optimised. The sequences of the primers are shown in Table 1.

5. Results and discussion

5.1 Proteomics profiling of *S. aureus* biofilms.

In this study, we focused our efforts on defining the changes in proteomics profile that occur when *S. aureus* is grown within biofilm subjected to differing amounts of water availability. We wanted to investigate whether the expression of proteins in current biofilm model was altered with environmental change. In this study, the threshold for the detection of fold difference in expression was set at > 2-fold, standardized with planktonic bacteria. It is well studied that, a commonly used method, especially for label-based study (e.g. TMT), for identifying differentially abundant proteins is fold change. By this approach, proteins with an FC larger than a defined cut off (e.g. 1.5- or 2-fold) are classified as differentially abundant (Ting, 2009; Kammers, 2015). Therefore, in this study we showed highly significant differentially expressed proteins those are >2-fold difference.

Analysis was performed and compared with three replicates to determine the definite dry biofilm protein. The Pearson correlation coefficients (PCC) were calculated to measure the reproducibility between samples by the TMTPrePro R package. It was found that R^2 ranged between 0.65–0.96 among the 12DWB and 0.68–0.97 among the 12DDB and degree of variation of proteins were counted less than 5%.

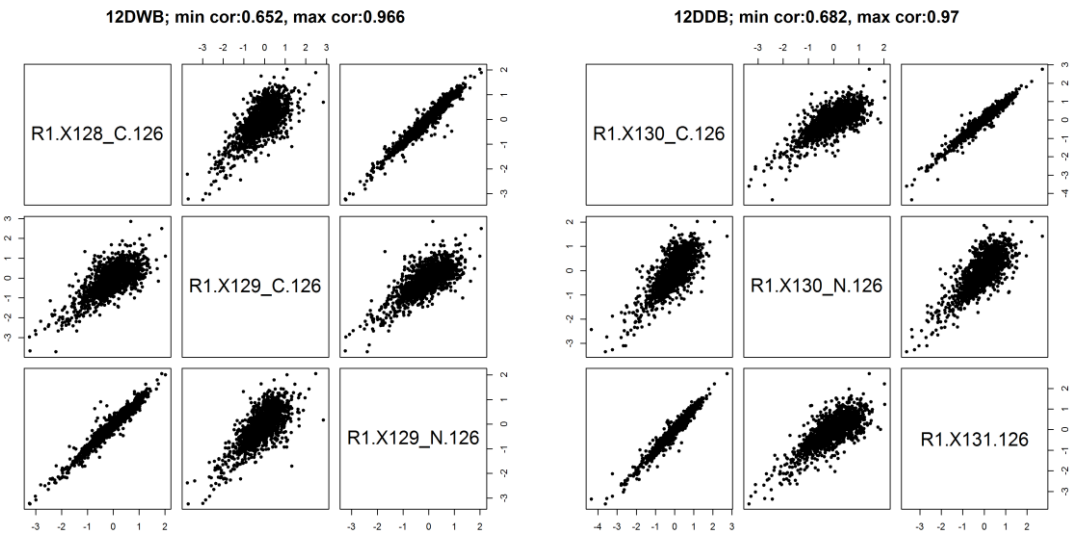

Figure-1: The Pearson correlation coefficients (PCC) of integrated proteins feature intensities are displayed for the comparisons within 12DWB and 12DDB.

This study identified a total 1636 total extracted supernatant proteins, of which 113 and 134 proteins were in significantly high abundance in WB and DSB of these, 50 proteins were significantly up-regulated in WB and 38 proteins in DSB compared to planktonic growth. We identified 264 proteins in WB and 267 proteins in DSB, whose expression was reduced by a factor of at least 2 in comparison with planktonic growth conditions. In this high throughput TMT-based MS study, planktonic bacteria used as a control and control value normalised as a 1 and then compared the effect of wet and DSB over planktonic. Therefore, planktonic data were not shown.

Protein detected in planktonic bacteria are considered as standard) of which 212 proteins were common for both WB and DSB and 52 were only downregulated in WB and 55 in DSB. (Figure-1). Many of the identified proteins conserved hypothetical proteins with no known function.

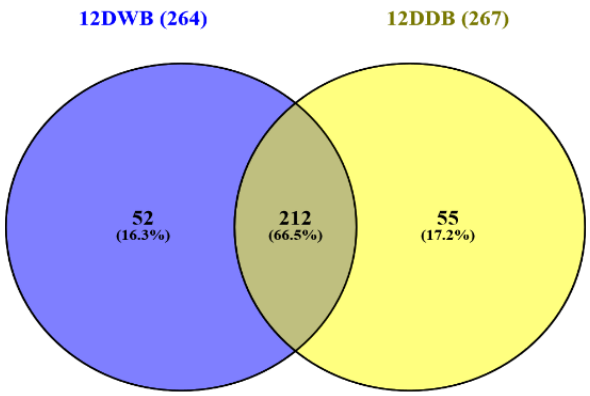

Figure 2: Significantly downregulated proteins in WB and DSB compared to planktonic culture.

In both DSB and WB 19 common proteins were identified (Table-2), whose expression was enhanced at least two to three-fold in comparison to planktonic cultures. Among these proteins were: 2-oxoisovalerate dehydrogenase (*SACOL1561*) responsible for valine, leucine and isoleucine degradation and biosynthesis of secondary metabolites; isocitrate dehydrogenase (NADP; *icd)* and ribose-phosphate pyrophosphokinase (RPPK; *prs)* responsible for microbial and carbon metabolism in diverse environments, biosynthesis of amino acids, TCA cycle, 2-Oxocarboxylic acid metabolism, purine and glutathione metabolism.

These results indicate that biofilm cells are strongly connected with the TCA cycle. The TCA cycle plays and important role in sessile growth in a biofilm and upregulation of amino acids biosynthesis and metabolism of purine and glutathione help to synthesise more complex amino acids in biofilm cells (Resch et al., 2006). Proteins responsible for pyrimidine metabolism; Carbamoyl-phosphate synthase (*carA*), CTP synthase (*pyrG*), Orotate phosphoribosyltransferase (OPRT; *pyrE*) and Uridylate kinase (*pyrH*) were upregulated >two-fold both in DSB and WB from the planktonic culture. In DSB, Holo-[acyl-carrier-protein] synthase (*acpS*) responsible for Pantothenate and CoA biosynthesis and 50S ribosomal protein L13 (*rp1M*) involved in ribosomal pathway, increased one-fold higher than the WB.

Table-2: 19 common protein in 12-day WB and 12-day DSB Detected in KEGG Mapper

Uniprot ID	12DWB MAR	12DDB MAR	Protein name	Encoded gene	Involved protein pathway
A0A0H2 WW67	3.106	3.362	2-oxoisovalerate dehydrogenase, E1 component, beta subunit (EC 1.2.4.1)	*SACOL 1561*	Metabolic pathways, Biosynthesis of secondary metabolites, Propanoate metabolism, Valine, leucine and isoleucine degradation
P99167	3.059	2.173	Isocitrate dehydrogenase [NADP] (IDH) (EC 1.1.1.42) (IDP) (NADP(+)-specific ICDH) (Oxalosuccinate decarboxylase)	*icd*	Metabolic pathways, Biosynthesis of antibiotics, Microbial metabolism in diverse environments, Carbon metabolism, Biosynthesis of secondary metabolites, Biosynthesis of amino acids, Citrate cycle (TCA cycle), 2-Oxocarboxylic acid metabolism, Glutathione metabolism
P65237	2.814	2.785	Ribose-phosphate pyrophosphokinase (RPPK) (EC 2.7.6.1) (5-phospho-D-ribosyl alpha-1-diphosphate) (Phosphoribosyl diphosphate synthase) (Phosphoribosyl pyrophosphate synthase) (P-Rib-PP synthase) (PRPP synthase) (PRPPase)	*prs*	Metabolic pathways, Biosynthesis of antibiotics, Microbial metabolism in diverse environments, Carbon metabolism, Biosynthesis of secondary metabolites, Biosynthesis of amino acids, Pentose phosphate pathway, Purine metabolism
A0A0H3JMR4	2.680	2.380	Octanoyltransferase LipM (EC 2.3.1.181) (Octanoyl-[acyl-carrier-protein]:[GcvH] N-octanoyltransferase)	*lipM*	Metabolic pathways, Lipoic acid metabolism
P99120	2.491	2.347	Diacetyl reductase [(S)-acetoin forming] (EC 1.1.1.304) (Acetoin(diacetyl) reductase) (AR) (Meso-2,3-butanediol dehydrogenase)	*butA*	Butanoate metabolism
Q5HH15	2.454	2.033	Phosphoribosylformyl glycinamidine synthase subunit PurL (FGAM synthase) (EC 6.3.5.3) (Formylglycinamide ribonucleotide amidotransferase subunit II) (FGAR amidotransferase II) (FGAR-AT II) (Glutamine amidotransferase PurL) (Phosphoribosylformyl glycinamidine synthase subunit II)	*purL*	Metabolic pathways, Biosynthesis of antibiotics, Biosynthesis of secondary metabolites, Purine metabolism
P99147	2.410	2.381	Carbamoyl-phosphate synthase small chain (EC 6.3.5.5) (Carbamoyl-phosphate synthetase glutamine chain)	*carA*	Metabolic pathways, Pyrimidine metabolism, Alanine, aspartate and glutamate metabolism
P63469	2.368	3.175	Holo-[acyl-carrier-protein] synthase (Holo-ACP synthase) (EC 2.7.8.7) (4'-phosphopantetheinyl transferase AcpS)	*acpS*	Pantothenate and CoA biosynthesis
Q7A467	2.333	2.195	50S ribosomal protein L18	*rplR*	Ribosome
P99072	2.322	2.241	CTP synthase (EC 6.3.4.2) (Cytidine 5'-triphosphate synthase) (Cytidine triphosphate synthetase) (CTP synthetase) (CTPS) (UTP--ammonia ligase)	*pyrG*	Metabolic pathways, Pyrimidine metabolism
Q5HE15	2.265	2.540	PTS system lactose-specific EIICB component (EIICB-Lac) (EII-Lac) [Includes: PTS system lactose-specific EIIC component (Lactose permease IIC component); PTS system lactose-specific EIIB component (EC 2.7.1.207) (Lactose-specific phosphotransferase enzyme IIB component)]	*lacE*	Metabolic pathways, Phosphotransferase system, Galactose metabolism

Table-2: 19 common protein in 12-day WB and 12-day DSB Detected in KEGG Mapper (continued)

Uniprot ID	12DWB MAR	12DDB MAR	Protein name	Encoded gene	Involved protein pathway
Q7A468	2.151	2.163	Protein translocase subunit SecY	*secY*	Protein export, Bacterial secretion system, Quorum sensing
Q7A459	2.150	2.411	50S ribosomal protein L23	*rplW*	Ribosome
Q7A473	2.136	3.276	50S ribosomal protein L13	*rplM*	Ribosome
P99144	2.129	2.302	Orotate phosphoribosyltransferase (OPRT) (OPRTase) (EC 2.4.2.10)	*pyrE*	Metabolic pathways, Pyrimidine metabolism
P65936	2.066	2.204	Uridylate kinase (UK) (EC 2.7.4.22) (Uridine monophosphate kinase) (UMP kinase) (UMPK)	*pyrH*	Metabolic pathways, Pyrimidine metabolism
Q5HD13	2.065	2.401	PTS system glucoside-specific EIICBA component [Includes: Glucoside permease IIC component (PTS system glucoside-specific EIIC component); Glucoside-specific phosphotransferase enzyme IIB component (EC 2.7.1.-) (PTS system glucoside-specific EIIB component); Glucoside-specific phosphotransferase enzyme IIA component (PTS system glucoside-specific EIIA component)]	*glcB*	Phosphotransferase system, Amino sugar and nucleotide sugar metabolism, Glycolysis / Gluconeogenesis
A0A0H2WVP2	2.037	2.103	Lipoate--protein ligase (EC 6.3.1.20)	*SACOL1034*	Metabolic pathways, Lipoic acid metabolism
P61059	2.030	2.326	50S ribosomal protein L4	*rplD*	Ribosome

In DSB, 40 exclusive high abundance proteins have been detected with >two mean abundance ratio (MAR) when compared with planktonic cells and WB (Table 3). The peptidoglycan pathway is upregulated in DSB cells. Among them, cell wall associated protein, UDP-N-acetylmuramate--L-alanine ligase (*murC*) and UDP-N-acetylenolpyruvoylglucosamine reductase (*murB*) are responsible for peptidoglycan biosynthesis. In a study it has been demonstrated that, ligase enzymes; murC [*Haemophilus influenzae* (Mol et al., 2003)], murD, murF, murB [*E. coli* and *S. aureus* (Benson et al., 2001)] involved in peptidoglycan synthesis are topologically similar to each other, both add peptide groups to the sugar molecules and interrupting these steps would ultimately prove fatal to the bacterial cell (Smith, 2006). These proteins in DSB associated with cell wall biosynthesis may be responsible for modification of bacterial cell wall synthesis in biofilm consequently responsible for increased resistance of biofilm bacteria against antibiotics. Study on genes responsible for methicillin resistance in *S. aureus* reported that, *murE* and some other genes (*-F*, *fmtA* to *-C*, *sigB*, *dlt*, *pbp2*, and *ctaA*, *aux)* are essential for methicillin resistance (Berger-Bächi, 1999). Thus, further investigation of this protein in S. *aureus* dry biofilm would be a guide to identify factors responsible for unusual biofilm bacterial cell wall structure and resistance mechanism.

A protein, iron-hydroxamate transport ATP-binding protein (*fuhC*), similar to the planktonic *S. aureus* ATP-binding cassette (ABC) transporter (*fhuCBG*) associated with ABC transporter function was detected in DSB. This protein increased by 2.4-fold in DSB. This results corelates with the study shown that, expression *FhuC* in *S. aureus* planktonic bacteria help to transport iron complexed to the *S. aureus* siderophore staphylobactin and increase the virulence as either alone or incorporated into heme or iron-sulfur clusters, iron assists the enzymes catalytic centre involved in vital cellular processes such as DNA synthesis and electron transport (Speziali et al., 2006). Multiple iron acquisition strategies of *S. aureus* is thought to be a key to its success as a virulent pathogen as it supports in vivo growth of bacteria and provides cofactor for components of microbial antioxidative stress defence (Garmory and Titball, 2004; Skaar et al., 2004) and mutated iron transport functions damage Gram positive bacterial virulence (Montanez et al., 2005; Speziali et al., 2006; Williams et al., 2006). Therefore, targeting iron-hydroxamate transport ATP-binding protein to hamper iron uptake systems could be an attractive direction for searching for novel biocides to eliminate Gram positive organisms from the hospital environment. In a study, the ABC transporter characterized as a component responsible desiccation tolerance in *Rhizobium leguminosarum*. (Vanderlinde et al., 2010). Another study on *Bradyrhizobium japonicum* revealed that transport genes in this organism are

upregulated in response to desiccation stress (Cytryn et al., 2007). Moreover, a study revealed that, ABC transport proteins includes multidrug efflux pumps causes antimicrobial compounds out of their cells, described as a key method of drug resistance for bacteria (Hassan, 2017). From the above discussion, upregulation of iron-hydroxamate transport ATP-binding protein (*fuhC*) in DSB might be consider as a virulence factor in DSB and for this reason, this could be an attractive target for removal technologies.

Two ABC transporter substrate binding proteins; peptide ABC transporter substrate-binding protein (SACOL0187) and ABC transporter substrate-binding protein (ACOL0996) were increased in 2.8 and 2.6-fold respectively. These proteins are involved in the protein pathway related to quorum sensing (QS). Studies have demonstrated that, Gram positive bacteria use processed oligopeptide autoinducer to facilitate species-specific and interspecies cell-cell communication, responsible for coordinated global behaviour of individual bacterial cells (Miller and Bassler, 2001).

AraC family transcriptional regulator (*SACOL0084*) protein was upregulated in DSB by 2.4-fold and detected as an uncharacterized HTH-type transcriptional regulator SACOL0084. AraC Family transcription regulator protein in *S. aureus* has been shown to be a virulence factor (Gallegos et al., 1997). A review on the role of AraC-type transcriptional regulator (AFTRs) protein in *E. faecalis*, another Gram positive pathogen, explained that, this protein helps to increase the virulence, resistance and pathogenicity, by preventing intracellular phagocytosis (Coburn et al., 2008). Rsp and Rbf from AraC family regulator, present in *S. aureus* MW2 strain, have been shown to be involved in biofilm formation (Gupta et al., 2013; Lim et al., 2004). From above discussed study results the elevated level of upregulation of AraC family transcriptional regulator protein in DSB might contribute to increased virulence.

Five distinct types of hypothetical proteins are upregulated (2-3 fold) in DSB compared to planktonic bacteria and WB include: hypothetical protein KQ76_09700 (*SAS053*), hypothetical protein KQ76-00255 (*SA0092*), hypothetical protein KQ76_07750, hypothetical protein KQ76_07625 (*SACOL0347*) and hypothetical protein KQ76_05275 (*SAS032*). Hypothetical proteins are unknown proteins that exist but have not been characterized or linked to known genes and no known functional or structural domain. One study showed that, hypothetical protein, YP_001317347.1, identified in *S. aureus* planktonic cell has a YbbR domain and is expected to bind ribosomal subunits (Varma et al., 2015). The analysis also reported the importance of YbbR domains which will aid in the development of novel antibacterial agents. A study on 35 hypothetical proteins from the chromosome of planktonic *S. aureus* NCTC 8325

revealed that, some hypothetical proteins related to virulent domains and protein-protein interactions including superoxide dismutase, O-antigen, bacterial ferric iron reductase and siderophore synthesis. Yet other hypothetical proteins detected related to metabolic or transport proteins including ABC transporters, major facilitator superfamily, S-adenosylmethionine decarboxylase, and GTPases (School et al., 2016). The author also recommended that, characterizing of hypothetical proteins might help to identify potential drug targets and thus aid in future drug discovery. Approximately half of all genomic protein sequences are currently annotated as hypothetical (Islam et al., 2015) and thus have great potential, for the discovery of new drug targets. When studied, hypothetical proteins have been shown to be important in different bacterial metabolism, for example, ABC transporter ATPbinding protein, export proteins, and a protein related to the multiple antibiotic resistance family among others (Mohan and Venugopal, 2012). In this study, three-fold upregulation of hypothetical proteins in DSB indicates the importance of more work determining the roles of hypothetical proteins.

Table-3: 40 exclusive high abundance Protein expressed in DSB Detected in KEGG Mapper.

Accession ID	Protein Name	12DDB (MAR)	Gene Name	Protein Pathways	Subcellular Localization
AIO20724.1	DNA-binding protein	3.304979607	SA0975		Cytoplasmic
AIO20264.1	membrane protein	3.048343628	SA0553		Unknown
AIO21178.1	ATPase	2.926134742			Cytoplasmic membrane
AIO21542.1	hypothetical protein KQ76_09700	2.910407158	SAS053		Extracellular
AIO19867.1	peptide ABC transporter substrate-binding protein	2.762768028	SACOL0187	Quorum sensing	Unknown
OOC88015.1	ABC transporter substrate-binding protein	2.584304335	SACOL0996	Quorum sensing	Unknown
AIO20671.1	phosphoribosylglycinamide formyltransferase	2.528475007	purN SACOL1081	Metabolic pathways, Biosynthesis of secondary metabolites, Purine metabolism, Biosynthesis of antibiotics, One carbon pool by folate	Cytoplasmic
AIO20736.1	RNA methyltransferase	2.503124049	SA0984		Unknown
AIO20313.1	iron-enterobactin transporter ATP-binding protein	2.394568782	fhuC	ABC transporters	Cytoplasmic membrane
AIO19772.1	AraC family transcriptional regulator	2.377005084	SACOL0084		Cytoplasmic
AIO22140.1	GntR family transcriptional regulator	2.365616061	gntR		Cytoplasmic
AIO21876.1	30S ribosomal protein S19	2.33251753	rpsS SA2043	Ribosome	Cytoplasmic

Table-3: 40 exclusive high abundance Protein expressed in DSB Detected in KEGG Mapper (continued).

Accession ID	Protein Name	12DDB (MAR)	Gene Name	Protein Pathways	Subcellular Localization
OOC91425.1	serine acetyltransferase	2.322343008	cysE SA0487	Metabolic pathways, Microbial metabolism in diverse environments, Biosynthesis of secondary metabolites, Cysteine and methionine metabolism, Sulfur metabolism, Biosynthesis of amino acids, Carbon metabolism	Cytoplasmic
AIO21414.1	PTS glucose transporter subunit IIBC	2.317849631	ptaA	Amino sugar and nucleotide sugar metabolism, Phosphotransferase system (PTS)	Cytoplasmic membrane
AIO21207.1	membrane protein	2.3139902	SACOL1557		Cytoplasmic membrane
AIO19771.1	hypothetical protein KQ76_00255	2.310688055	SA0092		Unknown
AIO19981.1	Na+/proline symporter	2.271009822	SACOL0311		Cytoplasmic membrane
AIO20708.1	hypothetical protein KQ76_05275	2.252561123	SAS032		
AIO21824.1	6-phospho-beta-galactosidase	2.195559802	lacG SA1991	Metabolic pathways, Galactose metabolism	Cytoplasmic
AIO20667.1	phosphoribosylformylglycinamidine synthase	2.188481716	purQ SACOL1077	Metabolic pathways, Biosynthesis of secondary metabolites, Purine metabolism, Biosynthesis of antibiotics	Cytoplasmic
AIO21431.1	UDP-N-acetylmuramate--alanine ligase	2.173797029	murC SACOL1790	Metabolic pathways, D-Glutamine and D-glutamate metabolism, Peptidoglycan biosynthesis	Cytoplasmic
AIO20726.1	50S ribosomal protein L32	2.16558817	rpmF SA0975.1 SAS033	Ribosome	Cytoplasmic
AIO21014.1	ABC transporter ATP-binding protein	2.146792615	SA1224		Cytoplasmic

Table-3: 40 exclusive high abundance Protein expressed in DSB Detected in KEGG Mapper (continued).

Accession ID	Protein Name	12DDB (MAR)	Gene Name	Protein Pathways	Subcellular Localization
AIO21176.1	hypothetical protein KQ76_07750	2.144296212			Cytoplasmic
AIO20180.1	2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase	2.132762925	folK SACOL0560	Metabolic pathways, Folate biosynthesis	Cytoplasmic
AIO20705.1	inositol monophosphatase	2.127683972	SACOL1116	Metabolic pathways, Streptomycin biosynthesis, Inositol phosphate metabolism	Cytoplasmic
AIO21153.1	hypothetical protein KQ76_07625	2.122257522	SACOL0347		Cytoplasmic
AIO20408.1	UDP-N-acetylenolpyruvoylglucosamine reductase	2.121618822	murB SA0693	Metabolic pathways, Amino sugar and nucleotide sugar metabolism, Peptidoglycan biosynthesis	Cytoplasmic
OOC94205.1	hypothetical protein BWO94_03350	2.110406381	SA2198		Cytoplasmic membrane
AIO21312.1	tetratricopeptide domain-containing protein	2.099682111	SA1448		Unknown
AIO22036.1	nitrite reductase	2.090757113	nasE	Microbial metabolism in diverse environments, Nitrogen metabolism	Cytoplasmic
AIO20589.1	enoyl-ACP reductase	2.086241969	fabI	Metabolic pathways, Biotin metabolism, Fatty acid metabolism, Biosynthesis of antibiotics	Cytoplasmic membrane
AIO21736.1	3-hydroxyacyl-ACP dehydratase	2.073377966	fabZ SA1901	Metabolic pathways, Biotin metabolism, Fatty acid metabolism	Cytoplasmic
AIO21897.1	MarR family transcriptional regulator	2.061220581	sarV SA2062		Cytoplasmic
AIO20821.1	protein phosphatase	2.036022563	SACOL1231		Cytoplasmic

Table-3: 40 exclusive high abundance Protein expressed in DSB Detected in KEGG Mapper (continued).

Accession ID	Protein Name	12DDB (MAR)	Gene Name	Protein Pathways	Subcellular Localization
AIO20891.1	recombinase RecA	2.03567554959138	recA SA1128	Homologous recombination	Cytoplasmic
AIO21702.1	serine/threonine protein phosphatase	2.02712012159594	rsbU		Cytoplasmic
AIO20028.1	chromosome partitioning protein ParB	2.0246801074121	SACOL0432		Cytoplasmic
AIO20673.1	phosphoribosylamine--glycine ligase	2.0108208234719	purD SA0926	Metabolic pathways, Biosynthesis of secondary metabolites, Biosynthesis of antibiotics, Purine metabolism	Cytoplasmic
AIO21758.1	50S ribosomal protein L31 type B	8	rpmE2 rpmE SA1922	Ribosome	Cytoplasmic

Serine acetyltransferase (*cysE*) was upregulated by 2.3-fold in DSB are involved in microbial metabolism including biosynthesis of amino acids and secondary metabolites, cysteine, methionine and carbon metabolism. Protein phosphoribosylglycinamide formyltransferase (*purN SACOL1081*), phosphoribosylamine-glycine ligase (*purD SA0926*) linked with purine metabolism were increased in DSB by 2.5 and 2-fold respectively.

5.2 Confirmation of transcriptional profiling by reverse transcription quantitative PCR

To verify the results of our proteomics experiments, we used reverse transcription quantitative PCR (RT-qPCR) to examine the relative expression levels of selected target genes. These comparisons were done using RNA isolated from two independent cultures representing 12-day single species DSB and 13-day mixed species dry surface biofilm (MDSB) (Table-4).

As observed in our experiments, genes associated with cell wall associated proteins were upregulated. The genes *murB* gene was upregulated six-fold and *murC* 4-fold higher in *S. aureus* DSB. Upregulation of *murB* and *murC* genes that encode cell wall, peptidoglycan, associated protein might confirm the increased cell wall rigidity/or thickness and consequently affect biofilm removal.

Table 4: Comparison of relative expression levels of selected target genes between 12-day single species DSB (12DDB) and 13-day mixed species dry surface biofilm (MDSB).

Tested genes	Fold changed (upregulated) in 12DDB than MDSB
CysE	3
fhuC	5
femX	3
icd	4
murB	6
murC	4
Prs	4

ABC transporter *fuhC* gene found five-fold greater quantities in the single *S. aureus* DSB samples than in mixed species biofilm. Quantification of ABC transporter gene (*fuhC*) undoubtedly pronounced the changes in expression level in different growth environment; single and mixed. Thus, it could be stated that these expressional changes in single and mixed species biofilm might be an important evidence and clue for the need of further investigation of mixed species dry surface biofilm. *Prs* and *icd* genes increased in 4- fold and *femX* and *cysE* increased in three-fold in single DSB compared with mixed species biofilm.

The expression of genes observed in DSB with qPCR were consistent with our protein profiling experiments. Although we did not do mixed species proteomics profiling, and we did not sort out whether these gene expression level in mixed species biofilm is due to the lower number of *S. aureus* resulted from competitive situation with other bacteria, however, it gives us a clear impression about the different genetic expression of mixed species biofilm and a strong need of exploring mixed species biofilm proteome.

6. Conclusion

This study's results provide a new insight for *S. aureus* WB and DSB growth. In this study, DSB biofilm cells appear to higher expression of proteins involved in metabolism of amino acids purine, pyrimidine, as well as proteins involved in sessile growth. Peptidoglycan biosynthesis protein, ABC transport protein and hypothetical proteins are upregulated and we surmise that these protein groups might together strengthen biofilm stability. Further study on these significantly upregulated proteins could be a target for the eradication of dry surface biofilm by developing a novel chemistry. This study confirmed the differential expression of seven targeted genes by RT-qPCR. Differential expression of the target genes in the MDSB indicates that interaction occurs between species within the biofilm as well as the need to conduct proteomic analysis of MDSB. Most of the identified hospital environmental biofilm was mixed species and as shown in Chapter 5, mixed species biofilms are more tolerant of biocides, thus dissecting the effect on bacterial environment on the proteome could be a guide to open a new era to eradicate dry surface biofilm and HAI prevention strategy.

7. Limitations

Although the research has reached its aim, there were some unavoidable limitations. Firstly, because of the time limit, this study could not conduct proteomics analysis of mixed species dry biofilm. Protein specific gene determination to compare between 12-day DSB and mixed species dry biofilm was performed with narrow range.

8. Recommendations

Development of novel detergents and disinfectants, for controlling HAI caused by biofilm in hospital environmental surfaces is crucial now a days. Extensive analysis of dry surface biofilm proteomics might help to find novel protein regulatory pathway and mechanism of action of pathway which might help to perform targeted proteomics to identify specific target protein to develop effective detergents and disinfectants against biofilm.

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Chapter Seven: General discussion and conclusion

7.1 Discussion

Pathogens can transmit in the hospital environment from patient to patient through contact with contaminated medical equipment and the immediate patient environment. HCWs' hands are the main vector through which pathogens are transferred from one contaminated area to other. Healthcare-related pathogens often colonise on the hospital surfaces and can survive on dry inanimate surfaces for days, weeks and even years has been clearly demonstrated (Kramer et al., 2006; Landelle et al., 2014). Furthermore, it was evidenced that, a patient admitted to a room, previously occupied with a patient infected with multi-drug resistant bacteria, such as MRSA, VRE and *Acinetobacter*, has a high risk of becoming infected as well (Dancer, 2014; Rashleigh-Rolls, 2016).

Bacteria arm themselves against detrimental environmental conditions, detergents and disinfectants through the formation of biofilm. In nature, bacterial survival usually takes in the form of biofilms and act as a reservoir of infections. Several studies have shown that low numbers of pathogens can result in infection for most environmentally associated nosocomial pathogens. Thus, the presence of a pathogen at any concentration on the surface may be a risk for transmission. As revealed in **chapter 3**, the transfer efficiency of *S. aureus* biofilm bacteria from plastic and glass surfaces to hands and thence to another surface (HBA plates), found that DSB was readily transferred by bare and gloved hands. Transmission to 19 subsequent surfaces was possible with the first five sequential touches resulting in >1000 colonies from 33% of the plastic coupons. With one touch, approximately $3x10^4$ to $1.6x10^5$ bacteria were transmitted from coupon to the hand and about 20% of these were transferred to the HBA plates. Given that 15 cells of less of *S. aureus* if sufficient to cause infection in wounds then ample bacteria can be transferred from DSB by the bare hands (Foster and Hutt, 1960). Although detergent treatment of the coupons didn't increase removal of bacteria from the DSB, in some cases it significantly increased the number of bacteria transferred to the HBA from the hands between 67 and 85% of these bacteria with one touch ($P<0.001$). This could be due to more bacteria being removed from the hands or the other possibility is that the detergent broke down the EPS joining the bacteria together and that a more accurate count was made as the bacteria were not in clumps. This indicates that a single exposure of cleaning agents although not able to remove dry surface biofilm from hospital surfaces might increase the number of organisms spread. In the experiment to see how many fomites could be touched consecutively and still transfer

sufficient bacteria to cause infection, greater numbers of bacteria were transferred to HBA plates following touching DSB grown on polycarbonate coupons, but this could be explained by the slightly higher number of bacteria incorporated into DSB on polycarbonate coupons (Log10 6.27) versus glass coupons (Log10 5.95). It has been also shown that for biofilm grown on glass coupons, 3% of the 6-10 grouped touches transferred >1000 colonies. This suggests that the biofilm may have been removed from the glass coupon to the fingers, in clumps which with multiple touches eventually broke up into smaller clumps or individual bacteria. This study also demonstrated that, although use of gloves by HCW is mandatory component of contact precautions, they may protect the wearer but still readily transfer DSB from one site to another albeit at a much lower proportion when compared to glove transfer of planktonic organisms. Our study concludes that in case of biofilm transmission, selection of appropriate glove depends upon the hydration of biofilm, glove material and contact surface. This study suggests that the 5 Moments of Hand Hygiene principles should be applied with glove use and gloves changes wherever an equivalent hand hygiene intervention is normally indicated.

Lack of thorough cleaning of hospital surfaces is one of the most important causes of transmission of bacteria that can result in HAI. If blood and other body secretions are allowed to dry and form a film on the surfaces they are extremely difficult to remove (Tosh et al., 2011). In addition, this organic matter encourages bacterial growth and biofilm development increasing the difficulty of cleaning even more as biofilm is not effectively removed by most cleaning products (Edmiston et al., 2013; Hadi, Vickery et al., 2010; Adukwu et al., 2015). Improperly disinfected devices and surfaces can lead to transmission of infectious organisms and has a strong link with HAI. In **chapter 4** we tried to validate the different methods of detection of residual biofilm and soil and then use these methods to test disinfectants and detergents efficacy against DSB. Efficacy testing not only requires a validated method of applying or growing the same amount of soil on each test sample but also requires validated and accurate methods for measuring residual soil. In this study, after treatment of DSB with detergent and disinfectant, several methods were investigated for their specificity and sensitivity in measuring residual soil remaining on treated coupons. The assays included detection of residual protein by the crystal violet assay and commercial BCA and Bradford assays, and ATP determination. These assays proved too insensitive or were too variable eg ATP for our needs. Detection of residual carbon by analysing total carbon showed low levels of residual carbon concentration (0.16) after treatment of biofilm grown on glass coupon with detergents for 10 minutes but as the limit of detection is 0.12 and limit of quantification is 0.35 this method was deemed to be too insensitive. Alkaline hydrolysis followed by protein detection

using the microBCA test proved to be sensitive enough being optimized to measure (A562nm) total protein concentration from 0.5 to 20 micrograms/mL and had the added advantage that multiple replicates of samples could be analysed in one tube thus increasing the sensitivity. This research did not find any detergent interaction with the microBCA reagent assay and was therefore, used for future work.

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 4** showed that in the absence of biological soil, the chlorine-based disinfectants, Na-HOCL, SDIC and Chlorclean reduced 12 day single species *S. aureus* biofilm viability approximately by the factor 3 Log_{10} with a five minutes contact time ($P<0.001$) but their efficacy was significantly decreased by the presence of soil resulting in no decrease in biofilm numbers. These chlorine-based disinfectants were also only able to remove <20% of the protein at the concentrations normally used clinically. The reduction in biofilm viability was even less when the chlorine-based disinfectants were used to treat 12-day mixed species biofilm resulting in $\leq 2Log_{10}$ reduction for *S. aureus.* However, the disinfectants were able to reduce the viability of *E. faecium* and *A. baumannii* approximately $3Log_{10}$. Equally, little of the biofilm mass was removed by chlorine-based disinfectants (<15%). In contrast, after treatment of both single and mixed species biofilm, Surfex efficacy was unaffected in the presence of biological soil and significantly ($P<0.001$) reduced biofilm bacterial counts to zero and removed 65% and 48.8% of the protein contamination associated with single and mixed species biofilm respectively. The reduced amount of protein reduction in mixed species model biofilm might be due to the increased thickness of the EPS (41µm; **chapter 5**) that is 36% higher than the single species biofilm model (30 µm) (Almatroudi et al., 2015) and also might be due to the concentrated EPS as it generates a number of non-specific binding sites within the biofilm that certainly affect detergent and disinfectant action (Flemming and Wingender, 2010). However, CLSM and SEM revealed that biofilms treated with these disinfectants contained live cells with thick EPS. It can thus be inferred that, although after treatment with Surfex, no bacterial growth is achieved on culture plate both in presence and absence of soil and the efficacy of chlorine-based disinfectant is suppressed in presence of soil, complete eradication of DSB is still remain challenging. We did not determine if the biofilm could recover with prolonged culture as Almatroudi did when testing chlorine against single species DSB (Almatroudi et al., 2016). Therefore, it is unknown if the biofilm cells detected by CLSM were alive but too damaged to recover or if they would recover. Moreover, in order to eradicate it completely, a more thorough cleaning strategy,

probably the need of cleaning prior to disinfection is required as residual soil reduces disinfectants efficacy and act as a barrier and protecting organisms from sterilization and disinfection.

Given that biofilm is frequently present on dry hospital surfaces (Hu et al., 2015; Johani et al., 2017) the data produced by this study demonstrates the necessity of testing chemical disinfectants against surface biofilm to ensure that they will be efficient under clinical use conditions. As previously mentioned, both DSB and biofilm composed of multiple species shows significant tolerance to complete removal or eradication when compared to free-floating planktonic bacteria. This calls for urgent assessment of the behaviour of biofilm formed with multi species 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 was necessary to create a mixed species biofilm model that incorporated bacterial species that are commonly transmitted by hand and frequently contaminate the hospital environment.

The development of a biofilm model to resemble a dry hospital surface mixed biofilm was addressed in **Chapter 5**. This process was undertaken with the CDC reactor, which generates a statistically the same hydrated biofilm on 24 removable discs (Goeres et al., 2005; Hadi et al., 2010). Modification of the process by Almatroudi's (2015) produced the first DSB protocol utilising a single bacterial species over a period of 12 days. In this study we modified Almatroudi's protocol to produce a mixed species biofilm over 13 days. 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). In this experiment, the CDC bioreactor reproducibly formed multi-layered multispecies biofilm with the within run CV was 28% to 38% and between run was 2% to 15%. The biofilm contained about 10^5 *S. aureus*, 10^5 *E. faecium* and 10^4 *A. baumannii* per coupon respectively. The bacteria were embedded in thick EPS. *S. aureus* contributed approximately 56% of the bacteria incorporated in the biofilm followed by *E. faecium* and *A. baumannii* contributing 38% and 7% on average. This biofilm composition resembles DSB obtained from clinical surfaces in that it is mostly composed of *S. aureus* which is also the predominant bacterial genus incorporated into clinical DSB. Clinically Staphylococcal bacteria composed approximately 31-50% of biomass, *Enterococcus spp.* 7 % *and, Acinetobacter spp.* 1-8% (Hu et al., 2015; Johani et al., 2017).

The mixed species biofilm was visualised using SEM and CLSM. The biofilm was heterogeneous when observed under CLSM and had a maximum thickness of 41 ± 1.3 µm of the biofilm. SEM images identified the biofilm as having large micro-colonies with a predominance of coccoid cells, which appeared to be coated with very thick EPS. This result corresponds to appearance of the mixed species biofilm with thick heterogenous EPS, identified from the ICU environment (Hu et al., 2015).

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** by staining with a number of fluorescence dyes and analysis using CLSM. The *in vitro* mixed biofilm model was found to have protein as the main element which was also found by Almatroudi's (2015) in his 12-day *S. aureus* DSB model. However, the protein component of the mixed species model was 73% which was higher than the 56% reported by Almatroudi. The next most prevalent component in mixed species DSB was glycoconjugate which contributed 23% of the biofilm mass which was a similar amount to Almatroudi's DSB whilst DNA contributed only 4% of the biofilm mass which was significantly less than the 20% reported by Almatroudi. This higher amount of protein may be due to the increased number of bacterial species that stimulate the more rapid utilisation of polysaccharide component of the EPS than the protein component during starvation as described for wet biofilm (Zhang and Bishop, 2003). Alternatively, it could be in response to growth in a close contact with other species. Results obtained by the present study indicate that it is possible to develop a mixed species dry surface biofilm in vitro model which can then be employed to assess how efficient hospital disinfectants are.

Bacteria experience decrease growth rate and altered metabolism in biofilm state that might be a reason they have a higher tolerance toward therapeutic compounds. Therefore, much effort is still needed to gain a better understanding of biofilm formation and growth. This study constructed **(Chapter 6)** a comprehensive reference map of proteins associated with *S. aureus* traditional hydrated biofilm (WB) and DSB, both grown over 12 days, to identify if unique proteins are associated with the differing biofilm states. The study revealed a considerable proteomic difference exists among *S. aureus* wet and dry biofilms. We identified 1636 total extracted supernatant proteins, of which 113 and 134 proteins were in significantly up regulated in WB and DSB respectively. Of these, significantly up-regulated proteins 50 and 38 proteins

were only found in WB or DSB respectively. Seventeen of the high abundance proteins found in WB and 17 of the high abundance proteins found in DSB were in known protein pathways. In DSB, proteins associated with cell wall biosynthesis, UDP-N-acetylmuramate-L-alanine ligase (*murC*) and UDP-N-acetylenolpyruvoylglucosamine reductase (*murB*) as well as an iron-hydroxamate transport ATP-binding protein (*fuhC*) associated with ABC transporter function were detected in 2.5-fold higher abundance than WB. Several studies also have demonstrated the relatively higher expression of ATP-binding protein in wet biofilm when compared to planktonic bacteria (Benson et al., 2001; Vanderlinde et al., 2010). Increase of these proteins might help explain the higher resistance shown by DSB bacteria. Several other proteins were also identified in high abundance in DSB that are previously described as virulence factors for many bacteria. Additionally, 52 and 55 proteins were significantly down-regulated in WB and DSB.

A small number of expressed genes were also targeted to confirm the proteomic profiling and to compare the expression of genes in between DSB and mixed species biofilm grown over 13 days to evaluate how *S. aureus* gene expression may differ when formed in a biofilm in a mixed environment. In DSB, qPCR evaluation of the level of expressed genes *cysE, murC, murB, fhuC,* encoding for metabolic and structural proteins confirmed the results of the proteomic analysis. However, these genes showed relatively low expression in 12-day mixed species biofilm when compared with DSB.

This study results might suggest understanding general trends in protein expression for *S. aureus* when grown in traditional hydrated (WB) and when subjected to periodic dehydration as happens during growth of DSB. In this study, biofilm cells appear to have up-regulation of proteins involved in metabolism of different amino acid such as purine and pyrimidine synthesis. Peptidoglycan biosynthesis and ABR transport proteins were also upregulated. These two upregulated protein groups might be a target for the eradication of dry surface biofilm by developing a novel chemistry. This study confirmed very small number of expressed target protein gene by qPCR. Although the proteomics profiling of mixed species biofilm has not be conducted, the results of qPCR revealed differences in the regulation of the genes investigated. This suggests that proteomic analysis of clinical DSB need to be conducted to better develop cleaning chemistries. Therefore, further analysis of mixed characteristics such as proteomics profiling could be a guide to open a new era to eradicate dry surface biofilm and prevention of HAI.

7.2 Conclusion

In this thesis we concluded that, DSB is highly transmissible and significant transmission is possible with both bare and gloved hands. Additionally, DSB can readily be transmitted from bed mattress through the bed sheet to the patient. This confirms the possibility that biofilm contributes towards patient colonization with pathogens and development of HAI.

From this study it also could be concluded that many of the assays conducted during biofilm research are too insensitive or not specific enough to use in detergent/disinfectant efficacy testing. The two most sensitive and suitable assays found were CFU for viability determination and the micro BCA assay for determination of residual biofilm protein. Commonly used oxidising disinfectants are inactivated by soil and have limited efficacy against DSB. Detergent treatment, without physical wiping and removal of soil, prior to disinfection has no effect on disinfectant efficacy when treating dry surface biofilm. Peracetic acid preparation with additive (Surfex) showed higher sensitivity to DSB removal and is not affected by soil. Although Peracetic acid is a corrosive chemical, however, in use concentrations do not pose major threats to the environment.

We have developed a reproducible mixed species dry biofilm model that mimics clinical biofilms found on dry hospital surfaces and this model can be used to test new decontamination strategies. Proteomic analysis to obtain structural information of the composition of dry surface biofilm might help guide development of better biocides against DSB. New decontamination strategies, capable of decreasing environmental transmission of pathogens should translate into lower HAI rates making our hospitals safer.

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Chapter Nine: Appendix

9.1 Table: Number of residual viable bacteria/coupon recovered after treatment with detergents.

Detergents	Number of Bacteria/coupon				
	Neat	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**
A_1	00	00	00	00	00
A_2	00	00	00	00	00
A_3	00	00	00	00	00
A_4	00	00	00	00	00
B_1	00	00	00	00	00
B_2	00	00	00	00	00
B_3	00	00	00	00	00
B_4	00	00	00	00	00
C_1	TMTC*	TMTC	157	00	00
C_2	TMTC	TMTC	83	00	00
C_3	TMTC	488	47	00	00
C_4	TMTC	TMTC	84	00	00
D_1	TMTC	TMTC	123	00	00
D_2	TMTC	TMTC	253	45	00
D_3	TMTC	TMTC	157	00	00
D_4	TMTC	TMTC	TMTC	115	08
E_1	00	00	00	00	00
E_2	00	00	00	00	00
E_3	00	00	00	00	00
E_4	00	00	00	00	00
F_1	00	00	00	00	00
F_2	00	00	00	00	00
F_3	00	00	00	00	00
F_4	00	00	00	00	00
G_1	TMTC	TMTC	333	30	4
G_2	TMTC	TMTC	343	37	4
G_3	TMTC	TMTC	305	133	13
G_4	TMTC	TMTC	341	112	11
H_1	00	00	00	00	00
H_2	00	00	00	00	00
H_3	00	00	00	00	00
H_4	00	00	00	00	00
Tween 20	TMTC	TMTC	TMTC	205	42
Tween 20	TMTC	TMTC	TMTC	198	36
Tween 20	TMTC	TMTC	TMTC	298	27
Tween 20	TMTC	TMTC	TMTC	221	12
1 M NaOH	00	00	00	00	00
1 M NaOH	00	00	00	00	00
1 M NaOH	00	00	00	00	00
1 M NaOH	00	00	00	00	00

*TMTC= Too much to count

9.2 Standard preparation of biological material for SEM analysis

9.2.1 0.1 M phosphate buffer:

Solution A:
Na2HPO4 · 12 H2O - 35.82 g/500ml
or Na2HPO4 · 2H2O -17.8 g/500ml

Solution B:
NaH2PO4 · 2 H2O - 15.6 g/500ml
or NaH2PO4 · H2O - 13.6 g/500ml

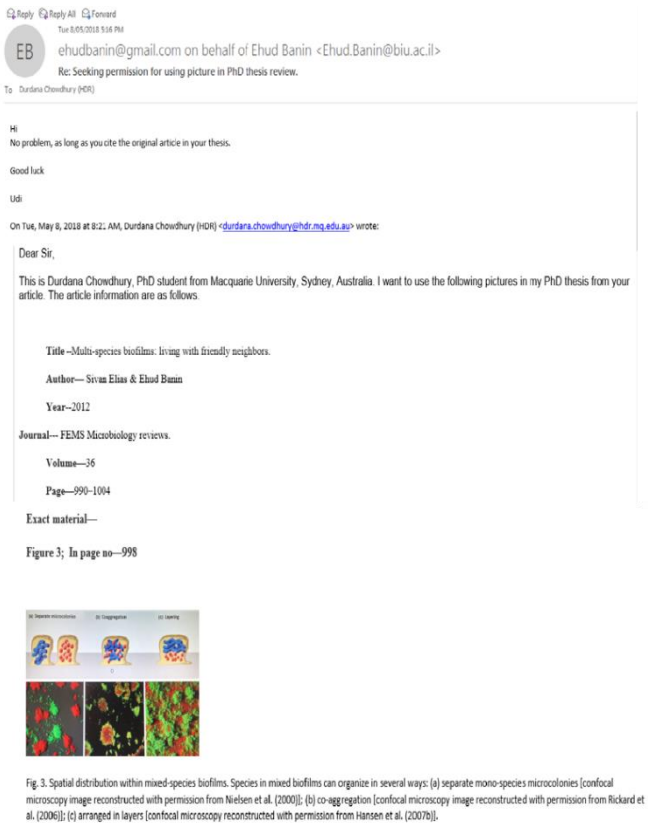
Procedure
add 36 ml solution A to 14 ml solution B (0.2 M phosphate buffer) + 50 ml distilled water 100ml 0.1 M phosphate buffer pH 7.2

9.2.2 3% glutaraldehyde:

50 ml 0.2 M phosphate solution + 12 ml 25% glutaraldehyde + 38ml distilled water

9.3: Copyright permission

Figure 1- 3: Spatial distribution within mixed-species biofilms. (a) separate mono-species microcolonies (Nielsen et al. (2000); (b) co-aggregation (Rickard et al. 2006)]; (c) arranged in layers (Hansen et al. (2007). Original articles were cited.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Hi
No problem, as long as you cite the original article in your thesis.

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Udi

On Tue, May 8, 2018 at 8:21 AM, Durdana Chowdhury (HDR) <durdana.chowdhury@hdr.mq.edu.au> wrote:

Dear Sir,

This is Durdana Chowdhury, PhD student from Macquarie University, Sydney, Australia. I want to use the following pictures in my PhD thesis from your article. The article information are as follows.

Title –Multi-species biofilms: living with friendly neighbors.

Author— Sivan Elias & Ehud Banin

Year—2012

Journal— FEMS Microbiology reviews.

Volume—36

Page—990–1004

Exact material—

Figure 3; In page no—998

Fig. 3. Spatial distribution within mixed-species biofilms. Species in mixed biofilms can organize in several ways: (a) separate mono-species microcolonies [confocal microscopy image reconstructed with permission from Nielsen et al. (2000)]; (b) co-aggregation [confocal microscopy image reconstructed with permission from Rickard et al. (2006)]; (c) arranged in layers [confocal microscopy reconstructed with permission from Hansen et al. (2007b)].