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

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ABSTRACT

The environment has been shown to be a source of pathogens causing infections in hospitalised patients. Incorporation of pathogens into biofilms, contaminating dry hospital surfaces, prolongs their survival and renders them tolerant to normal hospital cleaning and disinfection procedures. Currently there is no standard method for testing efficacy of detergents and disinfectants against biofilm formed on dry surfaces. Aim: The aim of this study was to develop a reproducible method of producing *Staphylococcus aureus* biofilm with properties similar to those of biofilm obtained from dry hospital clinical surfaces, for use in efficacy testing of decontamination products. The properties (composition, architecture) of model biofilm and biofilm obtained from clinical dry surfaces within an intensive care unit were compared.

Methods: The CDC Biofilm Reactor was adapted to create a dry surface biofilm model. *S. aureus* ATCC 25923 was grown on polycarbonate coupons. Alternating cycles of dehydration and hydration in tryptone soy broth (TSB) were performed over 12 days. Number of biofilm bacteria attached to individual coupons was determined by plate culture and the coefficient of variation (CV%) calculated. The DNA, glycoconjugates 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 and confocal microscopy (CM) and scanning electron microscopy (SEM). Model biofilm was compared to naturally formed biofilm containing *S. aureus* on dry clinical surfaces.

Results: The CDC Biofilm reactor reproducibly formed a multi-layered, biofilm containing about 10^7 CFU/coupon embedded in thick extracellular polymeric substances. Within run CV was 9.5% and the between run CV was 10.1%. Protein was the principal component of both the in vitro model biofilm and the biofilms found on clinical surfaces. Continued dehydration and ageing of the model biofilm for 30 days increased the % of protein, marginally decreased gylcoconjugate % but reduced extracellular DNA by 2/3. The surface of both model and clinical biofilms was rough reflecting the heterogeneous nature of biofilm formation. The average maximum thickness was 30.74 ± 2.1 μm for the in vitro biofilm model and between 24 and 47 μm for the clinical biofilms examined.

Conclusion: The laboratory developed biofilm was similar to clinical biofilms in architecture and composition. We propose that this method is suitable for evaluating the efficacy of surface cleaners and disinfectants in removing biofilm formed on dry clinical surfaces as both within run and between run variation was low, and the required equipment is easy to use, cheap and readily available.

1. Introduction

As the public and health care systems become less accepting of healthcare associated infections (HAI), preventing transmission of infection within the healthcare environment is assuming prime importance. Breaking the chain of transmission includes many strategies such as hand hygiene and barrier protection, however, an important but often overlooked aspect is environmental decontamination. Current

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decontamination agents are effective against planktonic organisms but are less effective against biofilms resident on hospital clinical surfaces (Vickery et al., 2012). We have confirmed the presence of biofilms surrounded by thick extracellular polymeric substances (EPS) on the majority of dry surfaces within the hospital environment. Over a quarter of these contained live multi-antibiotic resistant bacteria (Hu et al., in press). From an infection control perspective, biofilms importance lies in their significant tolerance to destruction by cleaning agents (Vickery et al., 2004) or disinfectant action (Smith and Hunter, 2008).

A common pathogen causing HAI is *Staphylococcus aureus* and we demonstrated its incorporation into 50% of the dry surfaces biofilms (Hu et al., in press). We, therefore, aimed to develop a semi-dehydrated surface biofilm model using *S. aureus* as the target organism in order to test environmental decontamination strategies.

Various methods of biofilm growth have been used to test decontamination efficiency against laboratory grown biofilms. Many of these have been developed for testing specific applications such as Teflon tubing simulating clinical endoscopes (Vickery et al., 2004), or in venous catheters for investigating prevention of biofilm using catheter lock solutions (Wiederhold et al., 2005). Numerous workers have used the micro-titre plate format (Stepanović et al., 2000) which grows biofilm in batch phases, particularly when testing antibiotic sensitivities. The 96 well plate format has the advantage of ease of handling, is readily available and provides a platform for multiple testing (Pitts et al., 2003) but as the biofilm is usually grown for less than 24 h without shear it is less well attached and presents an easier target for biocides. Modifications of the 96 well format to provide shear, such as the minimum biofilm eradication concentration (MBEC) device, formally called the Calgary Device, have good reproducibility and produce a better attached and hence harder to kill biofilm (Parker et al., 2014). Tubular devices, such as the Modified Robins Device that produces biofilm on removable specimen stubs, are also available but are cumbersome to use. However, none of the published models are suitable for producing and evaluating the semi-dehydrated biofilm seen contaminating dry hospital surfaces.

The CDC biofilm reactor has been used to produce hydrated biofilm using either batch or flow through systems. Biofilm is produced under shear producing a well attached and statistically reproducible biofilm on 24 removable coupons (Goeres et al., 2005; Hadi et al., 2010). This combined with versatility in substrate selection (coupons made of different materials) has made the CDC bioreactor ideal for efficacy testing of biocides and antimicrobials and the method has been standardized for *Pseudomonas aeruginosa* in ASTM International, protocol ASTM E2562-12 (ASTM, 2012). By modifying the growth conditions we were able to produce *S. aureus* semi-dehydrated biofilm in the CDC biofilm reactor. We then compared our laboratory grown biofilm with biofilm obtained from clinical surfaces with respect to appearance, thickness and percentage of protein, carbohydrate and DNA.

2. Materials and methods

2.1. Development of a model to simulate S. aureus biofilm forming on a dry surface

S. aureus ATCC 25923 biofilm was grown on 24 removable, sterile polycarbonate coupons in an intensively cleaned, brushed and steam sterilised (121 °C for 20 min) CDC biofilm reactor (BioSurface Technologies Corp, Bozeman, USA). The CDC biofilm reactor is described in Goeres et al. paper (Goeres et al., 2005).

Semi-dehydrated biofilm was grown over 12 days with cycles of batch growth during which time 5% tryptone soya broth (TSB) was supplied alternating with prolonged dehydration phases at room temperature (22–25 °C) as described in Table 1. The biofilm generator was located in an air-conditioned laboratory and filter-sterilised room air (average relative humidity 66%) was pumped across the media surface at an airflow rate of 3 l/min using an aquarium air pump.

Table 1
Culture conditions for formation of semi-dehydrated biofilm. Initial inoculum of about 10^8 CFU of *S. aureus* was added at the beginning of Stage 1 batch phase.

Stage	Culture conditions	Cumulative operating time (hours)
1	48 h batch phase in 5% TSB followed by 48 h dehydration	96 h
2	6 h batch phase in 5% TSB followed by 66 h dehydration	168 h
3	6 h batch phase in 5% TSB followed by 42 h dehydration	216 h
4	6 h batch phase in 5% TSB followed by 66 h dehydration	288 h

Biofilm development was initiated by inoculation of about 10^8 colony forming units (CFU) of *S. aureus* at the beginning of the first batch phase. During batch phases, all biofilms were grown in 5% TSB at 35 °C and subjected to shear by baffle rotation at 130 rpm/min producing turbulent flow.

To remove loosely attached bacteria, rods containing biofilm covered coupons were removed from the generator and placed in 1 l of phosphate buffered saline (PBS) for 5 min. Then, the 3 coupons from each rod were washed an additional two times by placing them into 50 ml PBS before being placed in individual sterile Bijou containers. The number of CFU per coupon was determined by sonication in an ultrasonic bath (Soniclean, JMR, Australia) for 5 min and vigorous shaking for 2 min in 4 ml of media followed by sequential 10-fold dilution and plate count as previously described (Ngo et al., 2012).

The CDC biofilm generator has eight rods which hold three removable coupons in a vertical orientation. For two separate experiments CFU was determined for each coupon position and the within-run % Coefficient of Variation (%CV) calculated. The between run variation was calculated from the CFU using these two runs plus five to six coupons/run from an additional nine separate experiments (total 98 coupons).

A qualitative assessment of the appearance of the semi-dehydrated model biofilm was made by scanning electron microscopy (SEM) and visually compared to clinical dry surface biofilms. Samples were fixed in 3% glutaraldehyde, followed by dehydration in ethanol, prior to immersion in hexamethyldisilazane (HMDS, Polysciences Inc) for 3 min and sputter coating with 20 nm gold film and examination in a scanning electron microscope as previously described (Vickery et al., 2012).

The effect of lack of nutrition and hydration on model biofilm was determined by an additional 18 days of storage at room temperature.

2.2. Characterisation of semi-dehydrated model and clinical dry surface biofilms

2.2.1. Clinical samples

Clinical items were obtained from the Intensive Care Unit of a large teaching hospital after cleaning twice using neutral detergent followed by chlorine disinfection (500 ppm available chlorine). Samples were aseptically cut (Vickery et al., 2012) from two hospital mattresses, one pillow, a box for sterile supplies, curtain cord and Velcro that held glove boxes to the wall. Samples were maintained aseptically until processed at room temperature. All six clinical specimens were shown to contain *S. aureus* by a combination of culture and *S. aureus* specific PCR (Hu et al., in press). Ethical approvals were obtained from South Western Sydney Local Health District Research and Ethics Office (Reference: LNR/14/LPOOL/14) and the University of Western Sydney Human Research Ethics Committee (Reference: H10659).

2.2.2. Spatial dimensions of biofilm

Clinical samples (1 cm^2) and 3 model biofilm coupons were stained with LIVE/DEAD *BacLight* 7012 viability kit (Molecular Probes, Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. The samples were fixed in 4% paraformaldehyde for one hour, washed 3 times in PBS and examined using an Olympus Fluoview 1000 inverted Confocal Scanning Laser Microscope (CSLM) for quantitative analysis.

Biofilm is spatially heterogeneous, therefore, random analysis of a small part of the biofilm may not necessarily reflect the true characteristic of the overall biofilm. Korber et al. (Korber et al., 1993) determined that a minimum statistically relevant area of 100,000 μm^2 was required when analysing *P. aeruginosa* biofilm. Each model biofilm sample was analysed at five different areas giving an average total analysed surface area of 197,000 μm^2. The entire area of the clinical samples was analysed. Images were built with 0.2 μm optical sections using the Imaris 7.7.2 software (Bitplane, Zurich, Switzerland). Biofilm architectural characteristics examined were the minimum and maximum thickness of the biofilm as measured in μm from the top of the biofilm to the substratum and the biofilm mass or the volume of the biofilm that was composed of bacterial cells. The surface area:volume ratio was calculated by dividing the total surface area by the total biomass. This ratio reflects the proportion of biofilm being directly exposed to the external environment.

2.2.3. Biofilm composition

The glycoconjugate component of biofilms was stained using a 1:10 dilution of Alexa-488-labelled *Aleuria aurantia* lectin (Vector Laboratories, Burlingame, CA) for 20 min (Staudt et al., 2004). Proteins were stained using a 1:1000 dilution of SyproOrange (Molecular Probes, Invitrogen, USA) in water for 15 min (Neu et al., 2002). Biofilm nucleic acid was stained in a 1:1000 dilution of SYTO 60 (Molecular Probes, Invitrogen, USA) in water for 5 min (Staudt et al., 2004). Samples were fixed and CLSM examined as described above. The EPS composition of each sample was determined using ImageJ software (1.46r, National Institute of Health, USA).

2.3. Statistical analysis and Image analysis

To examine for variation in biofilm formation between the top, middle and bottom position of the coupon in the holding rod an Analysis of Variance (ANOVA) with the Holm-Sidak method of multiple comparisons was performed.

The %CV for the number of bacteria incorporated into the semi-dehydrated biofilm was calculated as a dimensionless measure of variance for the within-run and between-run variation. It was derived by dividing the Standard Deviation by the Mean (100 × SD/Mean).

3. Results

3.1. Development of a model to simulate S. aureus biofilm formation on a dry surface

The semi-dehydrated growth conditions resulted in an average of $\log_{10}$ 7.13 ± 0.04 CFU of *S. aureus* biofilm per coupon. There was little variation in the amount of biofilm covering individual coupons with CFU of 95/98 coupons being within 2STD of the mean and the within run variation being 9.5%. There was no significant difference in the amount of biofilm growing on coupons in the different rod positions, the mean CFU being $\log_{10}$7.15, $\log_{10}$7.15 and $\log_{10}$7.16 for coupons in the upper, middle and bottom rod position, respectively. Similarly there was little run-to-run variation in the number of CFU in the semi-dehydrated biofilm with between-run variation being 10.1%.

SEM of semi-dehydrated model biofilm showed a multi-layered biofilm with very thick extracellular polymeric substances (Fig. 1a) which was of similar appearance to many of the clinical dry surface biofilms, particularly those on hard surfaces, such as the sterile supply box (Fig. 1b).

3.2. Characterisation of semi-dehydrated model and clinical dry surface biofilms

3.2.1. Spatial dimensions of biofilm

The surface of both model and clinical biofilms was rough reflecting the heterogeneous nature of biofilm formation (Figs. 1 and 2).

The heterogeneous nature of the biofilm was also reflected in the minimum and maximum thickness of the biofilm with some areas of the biofilm having few cells (Fig. 2). The average maximum thickness of the in vitro biofilm model was 30.74 ± 2.1 μm. The maximum thickness of the clinical biofilm varied from 24 μm for the biofilm found on the supply box for sterile supplies to 47 μm for both the curtain cord and pillow biofilms (Fig. 3). The amount of biofilm directly exposed to the external environment, and hence cleaning products, as expressed by the surface area to volume ratio varied 0.34 up to 0.60 for the clinical biofilm whilst the model biofilm had a ratio of 0.52 (Fig. 3).

3.2.2. Biofilm composition

Protein was the principal component of both the in vitro model biofilm and the biofilms found on clinical surfaces, composing between 42 and 95% of the biofilm (Figs. 4 and 5). Glycoconjugates were then next most prevalent component for all the biofilms except those formed on the pillow where DNA was more prevalent. For the model biofilm a further 18 days of ageing and dehydration increased the proportion of protein from 56 to 73%, marginally decreased the proportion of gycoconjugates from 24 to 21%, but reduced the amount of DNA present in the biofilm from 20 to 6%.

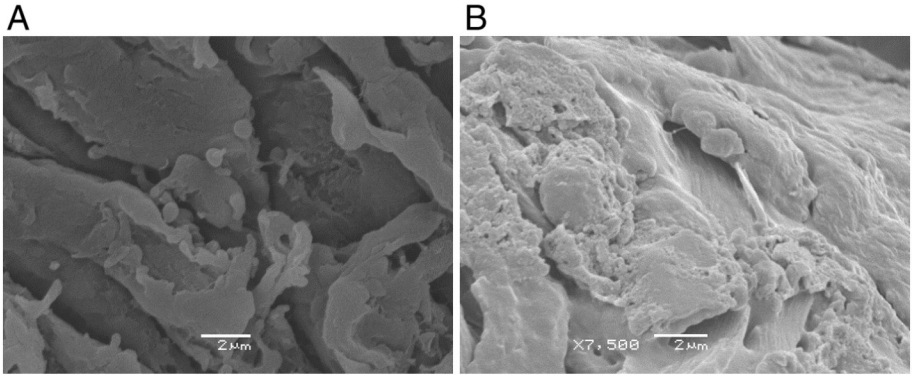

Fig. 1. Scanning electron micrographs of a) in vitro model *S. aureus* semi-dehydrated biofilm and b) clinical biofilm on sterile supply box. Showing multi-layered biofilm with large amounts of amorphous extracellular polymeric substances. Magnified 7500×.

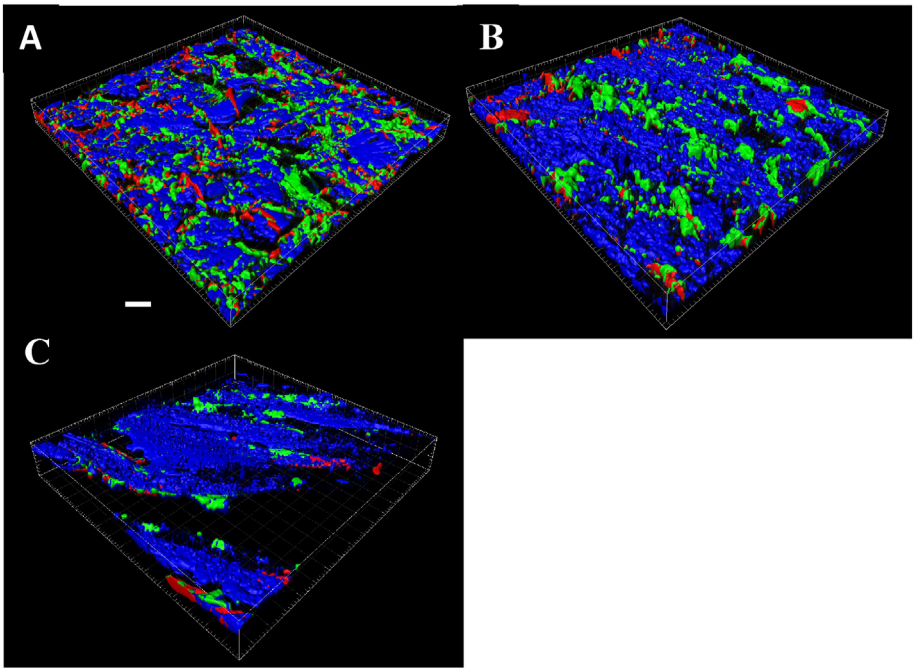

Fig. 2. 3D views of biofilms showing heterogeneous nature of biofilms with areas of multi-layered biofilm interspersed with areas with few cells. A) Model biofilm following 12 days of growth, B) Model biofilm following an additional 18 days of ageing without any nutrition and C) Clinical glove box Velcro biofilm. Extracellular polymeric substances are stained for extracellular DNA (red), protein (blue) and glycoconjugate (green). Bar = 30 μm.

4. Discussion

Biofilms on clinical surfaces are subjected to both lowered water potential and periodic disinfection. SEM of clinical samples show a multi-layered biofilm with the bacteria covered with very thick EPS (Fig. 1b). This is consistent with the finding that biofilms subjected to severe environment stresses (Chang et al., 2007) and/or disinfectants (Machado et al., 2012) produce more EPS, which serves to increase protection of incorporated bacteria.

We have produced a model biofilm, with similar physical attributes to clinical biofilms, by adapting biofilm culture conditions to include periodic dehydration. We utilised the CDC biofilm reactor, thus maintaining the advantages of growing a reproducible, better attached biofilm under shear, without the necessity of using media additives such as sodium chloride or PEG200–PEG8000 to reduce water availability as has been done to model soil biofilms (Chang and Halverson, 2003; Halverson and Firestone, 2000).

The ESKAPE pathogens — *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella* sp., *Acinetobacter baumanii*, *Pseudmonas aeruginosa* and *Enterobacter* sp. — have been classified as the top 6 multi-antibiotic resistant pathogens by the Infectious Diseases Society of America (Llaca-Díaz et al., 2013). *S. aureus* frequently contaminates hospital surfaces causing nosocomial infections (Dancer et al., 2008) and we have previously shown that staphylococci were incorporated into about 86% of clinical dry surface biofilms, 50% specifically contained *S. aureus*. *S. aureus* was used for our model biofilm and compared it to biofilms containing *S. aureus* previously found on hospital surfaces (Hu et al., in press).

The maximum thickness of the clinical biofilm varied depending on the site. Biofilms formed on fabrics such as the curtain cord, pillow and Velcro where an average of 45.6 μm thick which was greater than the average of 26.5 μm for biofilms formed on hard surfaces, such as the box for sterile supplies and vinyl covered mattress. As the thickness of the biofilm reflects the distance that cleaning agents and biocides have to diffuse/penetrate, the model biofilm thickness of 31 μm was a good compromise between the thickness of biofilms on hard and soft clinical surfaces.

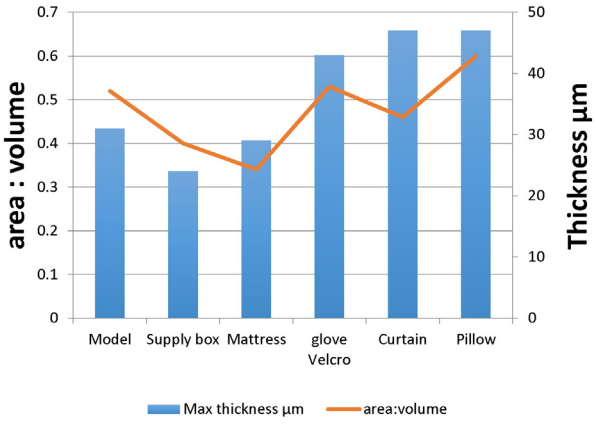

Fig. 3. Maximum thickness (bars) and area:volume ratio (line) of in vitro model biofilm compared to biofilm found on clinical surfaces.

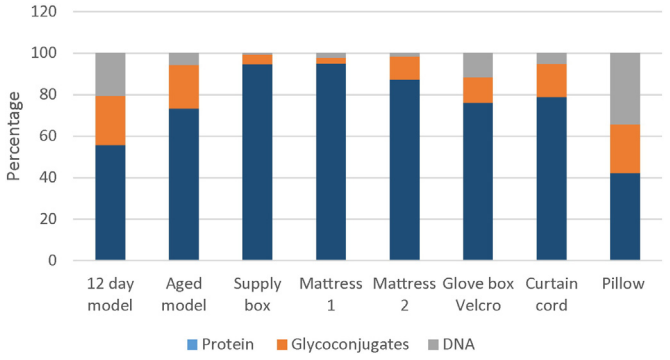

Fig. 4. Composition of in vitro semi-dehydrated model biofilm and biofilm found on clinical surfaces.

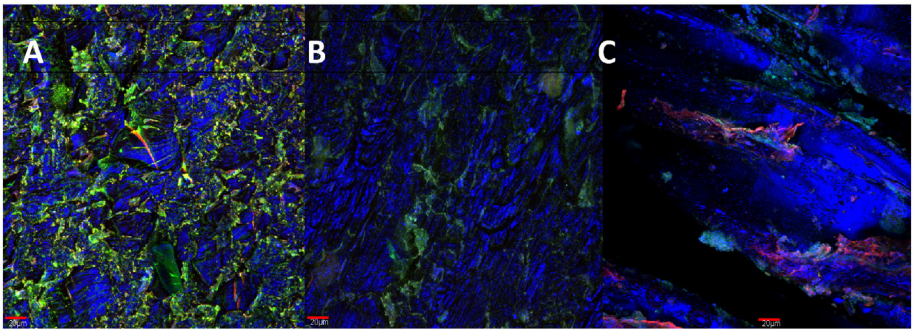

Fig. 5. Composition of biofilm extracellular polymeric substances. Extracellular polymeric substances are stained for extracellular DNA (red), protein (blue) and glycoconjugate (green). A) Model biofilm following 12 days of growth. B) Model biofilm following an additional 18 days of ageing without any nutrition showing a decrease in extracellular DNA and glycoconjugates and an increase in protein components. C) Clinical glove box Velcro biofilm. Bar 20 μm.

The surface area:volume ratio was calculated by dividing the total surface area examined by the total biomass. This ratio reflects the proportion of biofilm being directly exposed to the external environment. As the biomass increases, the proportion of biofilm being directly exposed to the external environment and hence cleaning products/disinfectants decreases. A biofilm with a small ratio might therefore, be expected to be harder to remove. Biofilms on the box for sterile supplies and a mattress had the lowest surface area:volume ratio whilst the model ratio was higher and was more reflective of biofilm found on clinical soft surfaces (Fig. 3).

The excreted EPS immobilizes the biofilm to the substrate and forms the polymer scaffold of the biofilm thus spatially fixing individual bacteria in place and ensuring that cell to cell communication can proceed. Not only does EPS contribute to biofilm tolerance to biocides but it also serves as a store for extracellular enzymes, nutrients and DNA required for horizontal gene transfer (Arciola et al., 2012). During desiccation the EPS becomes concentrated increasing the number of non-specific binding sites within the biofilm (Flemming and Wingender, 2010). The increased number of bonds would undoubtedly adversely affect detergent and disinfectant action increasing the chance of ineffective cleaning and residual soil. Residual surface soil, of any type, promotes additional bacterial contamination and compromises future disinfection (NHMRC, 2010).

Knowledge of the major constituents of the biofilm EPS may aid development of targeted removal strategies. However, separation of EPS from biofilm cells is challenging as some EPS remains attached to the bacteria, extraction methods favour water soluble EPS components, and extraction can damage the cells leading to cellular leakage of contents (Flemming and Wingender, 2010). A combination of lectin, protein and nucleic acid staining and confocal imaging to differentiate EPS components in situ was used to minimise bias of results.

EPS composition varies depending on bacterial species composition and the environmental conditions in which the biofilm grows (Arciola et al., 2012). Considering the desiccating conditions of dry clinical surfaces, biofilms show a remarkable variability containing an average of 23 species of bacteria (Hu et al., in press). Given the species variability it is somewhat surprising that protein is the predominant component making up these diverse clinical biofilms. A possible explanation is that during starvation bacteria utilise the polysaccharide component of the EPS at a faster rate than the protein component, thus increasing the protein concentration (Zhang and Bishop, 2003). Prolonging the period of desiccation and starvation of the model biofilm by 18 days also increased the protein component and slightly decreased the carbohydrate component of the biofilm. The protein component of activated sludge EPS has been shown to increase whilst the polysaccharides remain the same as biofilm ages (Zhang et al., 2007).

In conclusion, thorough cleaning of hospital surfaces is necessary to ensure adequate decontamination and prevention of infection transmission but as yet there is no standardized method for testing the removal of dry surface biofilm. We propose that this study's method is suitable for efficacy testing of decontamination products against biofilms grown on different substrates due to its low within- and between-run variation, and the ready availability/low cost of required equipment and consumables.

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