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Title

Efficiency and novelty of using environmental swabs for dry surface biofilm recovery.

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Abstract

Studies on the epidemiology of dry surface biofilms within healthcare has shown an almost universal distribution across frequently touched items. Despite a growing body of evidence for dry surface biofilms in hospitals little attention has been paid to the recovery capacity of techniques used to detect these microbial communities. Biofilms are inherently difficult to remove from surfaces due to adhesive substances within their matrix and may act as sources of infection but to what extent is largely unknown. In this study we evaluate the recovery efficiencies of commonly used environmental swabs against dry surface biofilms containing 7.24-Log_{10} *Acinetobacter baumannii*/cm^2, using a drip flow reactor and desiccation cycle. Biofilm presence was visually confirmed using episcopic differential interference contrast microscopy combined with epifluorescence and quantified using sonicated viable plate counts. The swab materials used comprised of foam, viscose and cotton, all of which were pre-moistened using a buffer solution. The surfaces were vigorously swabbed by each material type and the resultant microbe populations for both swabs and remaining dry surface biofilms were quantified. Our results found foam tipped swabs to be superior, detecting on average 30% of the original dry surface biofilm contamination; followed by viscose (6%) and cotton (3%). However, no distinct difference was revealed in the concentration of microbes remaining on the surface after swabbing for each swab type suggesting there is variation in the capacity for each swab to release biofilm associated microorganisms. We conclude whilst environmental swabs do possess the ability to detect biofilms on dry surfaces; the reduced efficiencies are likely to cause an underestimation of the microbes present and should be considered during clinical application.

Data summary

The authors confirm all supporting data and protocols have been provided within the article.

Introduction

Every year healthcare-associated infections affect millions of hospitalised patients. Those admitted to intensive care units (ICUs) attribute to 25% of these infections[1,2]. The vast majority of HAIs originate from the ESKAPE pathogens; a list of six top global antimicrobial resistant pathogens (*Enterococcus facium*, *Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* species) [3,4]. Healthcare environments, such as the ICU, are recognised as high risk areas for the proliferation of multidrug-resistant organisms (MDROs) due to the extensive use of antibiotics which applies an inherently selective pressure on the microbiome[5,6].

Transmission of MDROs, and thus HAIs, will readily occur in hospital environments through contact between the patients, healthcare workers and clinical equipment[7,8,9]. Studies have shown environmental surfaces pose a significant risk to patients developing HAIs upon admission if the prior occupant was known to be colonised by MDROs in spite of efforts in infection prevention and routine cleaning of surfaces[10,11]. Nosocomial pathogens have been shown to survive on desiccating hospital surfaces for several months and are suspected of acting as a reservoir for HAIs. Recently, dry biofilms have been found on these types of surfaces and are being recognised as potential cause for the persistence of MDROs on healthcare surfaces[12,13,14,15,16]. Biofilms are communities of microorganisms attached to a substrate and surrounded by a protective structure of extracellular polymeric substance (EPS). Microbes residing within a biofilm are phenotypically more tolerant to antimicrobials such as cleaning agents and can be up to 1000 time less susceptible then their planktonic counterpart[17]. Referred to as dry surface biofilms (DSB), these communities have been detected in abundance across surfaces within an ICU and remain viable despite extensive cleaning using bleach-based disinfectants[15]. During dehydration biofilms increase the production of EPS which can to lead increased tolerance to disinfectants, as a result many authors postulate DSB could be used to explain why basic hospital cleaning with previously approved efficacy to planktonic organisms are failing to achieve desired results[16,17].

Environmental monitoring of clinical surfaces is a fundamental requirement to assess the effectiveness of infection control measures[18]. As no standardised test currently exists, studies monitoring for DSB often result in physical or destructive removal of surfaces from the hospital room, with subsequent microscopy and culture analysis required to confirm biofilm presence[19,20]. This approach is not common practice nor readily feasible in most settings. Instead, clinicians will use less-accurate alternatives such as culture swabs, bioluminescence or contact agar[8,21,22].

Johani *et al.,* used next generation sequencing of *in situ* DSB samples to demonstrate that environmental swabs alone failed to sample the entire microbiome in comparison to destructive sampling[2]. Swabbing only detects planktonic or loosely-bound cultures on the surface and detection efficiencies for environmental swabs of differing tip and substrate materials is well documented. For instance, the foam swabs used by Johani *et al.,* are known to exhibit superior recovery of microorganisms across a vast range of materials, including brushed stainless steel and polypropylene, than cotton or nylon alternatives[23]. In contrast,

few studies report on these efficiencies against biofilms and as a result, swabs results can be deemed unreliable for DSB detection. In this study we demonstrate the performance of three swab material types (foam, viscose and cotton) against *in vitro* DSB with similar characteristics to those found on clinical surfaces.

Methodology

Bacterial strains. The bacterial strain used in this study possessed genes capable of expressing drug resistance mechanisms to the extended-spectrum beta-lactamase antibiotic group. The strain used was: *A. baumannii* (NCTC: 13301). This species was chosen for its ability to form biofilms and known persistence on healthcare surfaces.

Inoculum preparation. The strain was sub-cultured into 10 mL of tryptic soya broth (TSB) (Sigma Aldrich) overnight at 37°C. The number of CFU per mL of bacterial suspension was quantified using serial dilutions and incubation on tryptic soya agar (TSA) (Sigma Aldrich) for 24 hours at 37°C.

Biofilm model. The biofilms were generated in a drip flow reactor (DFR) (BioSurface Technologies, Bozeman, MT) assembled as per ASTM E2647-13 using 316 stainless steel coupons as the substrate[13]. The coupons were inoculated with 1 mL of culture inoculum at a population of 7 to 8-$\log_{10}$ CFU and incubated for 6 hours at room temperature (≈20°C), referred to here as the batch phase. The reactor was then tilted to a 10° angle to allow sufficient drainage of waste and shear force across the coupon surface. A sterile supply of 5% TSB solution was initiated by means of a 6 channel peristaltic pump (Cole Palmer™, UK) at a flow rate of ≈0.9 mL min^{-1} per channel for 36-48 hours at room temperature, referred to here as the media phase. At the end of the media phase the coupons were washed three times with sterile water to remove loosely bound or planktonic cells.

Desiccation model. Swab performance was measured against biofilm in a dry state. A dry state biofilm uses a previously described method for biofilm dehydration by means of an aquatic air pump (Hailea, UK) passing room air, via a 0.2 µm in-line filter (Fisher Scientific™, Loughborough, UK) across the media surface at 3 L min^{-1} in a sealed 0.01 m^3 container for 48 to 66 hours; referred to here as the drying phase[23]. All biofilm coupons were exposed to a single dehydration cycle. The control coupons were removed at this stage and their population and biofilm formation quantified alongside the test coupons once processed.

Swabs and surfaces tested. Three swab types were used: sterile foam swabs (Technical Service Consultants Ltd, Lancashire, UK), sterile viscose swabs (Technical Service Consultants Ltd, Lancashire, UK) and sterile cotton swabs (Fisher Scientific™, Loughborough, UK). Prior to each experiment, all swabs were sufficiently moistened with Phosphate Buffer Solution (Oxoid™, Basingstoke, UK), excess liquid was removed by pressing the tip against the tube. Sampling was performed as previously described by sweeping the swab from side to side across the surface whilst rotating the swab; before repeating the process perpendicular to the first sweeping direction (Fig. 1).

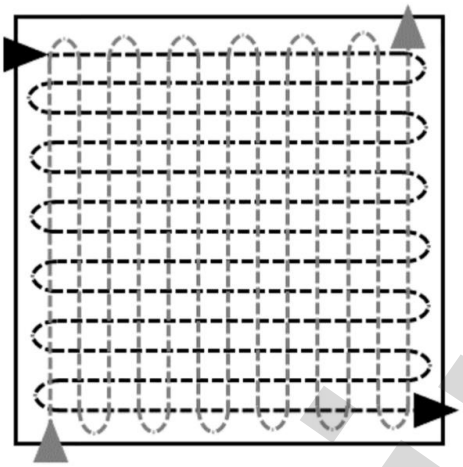

Figure 1. Diagram of the sampling procedure as shown by Jansson et al., [24]. The black arrow heads depict the first sweeping motion whilst the grey arrow heads the second time.

Quantification of swab and coupon population. The Log_{10} CFU per cm^2 of each swab and coupon was quantified in accordance to Johani *et al.,* and ASTM E2647-13[2,25]. In brief, for swabs the tip was aseptically removed into 2 mL of PBS and sterile glass beads; and allowed to soak for up to 15 minutes at room temperature, after which each sample was vortexed twice for 5 seconds intervals. For the coupons, the surface was scraped and rinsed into 20 mL of PBS and sterile glass beads; and the solution homogenized using a vortex twice for 15 seconds. The number of CFU per cm^2 for each of the vortexed samples were quantified using serial dilutions and incubation on TSA for 24 hours at 37°C. The number of colonies on each plate were recorded and reported as colony forming units (CFU) per cm2 using the following calculation:

$$CFU/cm^2 = \left[\left(\frac{(mean\ cfu/plate)}{volume\ of\ sample\ plated}\right) \times \left(\frac{volume\ scraped\ into}{surface\ area\ scraped}\right) \times (dilution)\right]$$

Volume scraped into = 2 mL

Surface area scraped = 18.75 cm^2

Quantification of biofilm colonies. Samples were stained with LIVE/DEAD BacLight™ bacterial viability kits (Invitrogen, UK); this included both SYTO-9 (green) and propidium iodide (red). EF microscopy was used to visualise 'live' and 'dead' bacterial cells, as previously described[23]. ImageJ version 1.52a (National Institutes of Health), with the area measurement tool was used to determine the percentage area of biofilm in each ROI for swabbed and control coupons[26]. In accordance to Korber *et al.,* a minimum area of 100,000µm^2 was analysed at six different areas across the coupon[27]. The datasets generated and analysed during this study are available from the corresponding author upon reasonable request.

Experimental design. The study consisted of three experimental runs; and within each experimental run, a minimum of 2 control coupons were used to ensure sufficient DSB present. The same technique and technician was used to conduct all experiments.

Statistical analysis. The surface loading (CFU) recorded for each coupon were transformed to Log_{10} CFU/cm^2 and all statistical calculations were performed using these values. A Mann-Whitney test was used to compare the quantity of DSB recovered from the processed coupons and swabs with the control; and one-way ANOVA was used to test the statistical significance in the percentage coverage for biofilms quantified during microscopy.

Results

Our drip flow reactor model, using a strain of multidrug-resistant *A. baumannii*, successfully generated substantial DSBs with distinct structural features indicative of clinical biofilms as previously described[23]. Episcopic differential interference contrast (EDIC) and epifluorescence (EF) microscopy highlighted microcolony formation across the topography of the stainless steel (316) substrate surface (Figure 2). The colonies were seen to aggregate along the cracks and crevices of the steel representative of those found *in* situ[28]. The density and distribution of biofilm across the surface was shown to vary in relation to the anticipated nutrient gradients, which form along the longitude and latitude axes. This is a unique feature of drip flow reactors due to the directional flow of media over the coupon[25,29].

Surface bioburden loadings for our control model DSBs averaged 7.24 ±0.57 Log_{10} colony forming units (CFU) per cm^2 which were comparable to worst case scenarios reported on ICU surfaces (7.20 Log_{10} CFU/cm^2) [30]. For this study, the estimated standard deviation for this methodology was 0.23; all DSB control coupons remained within two standard deviations of the mean. There was minimal variation between each experimental run of swab material with a Coefficient of Variation of 5.80%, on par with other published data (10.1%)[23,30].

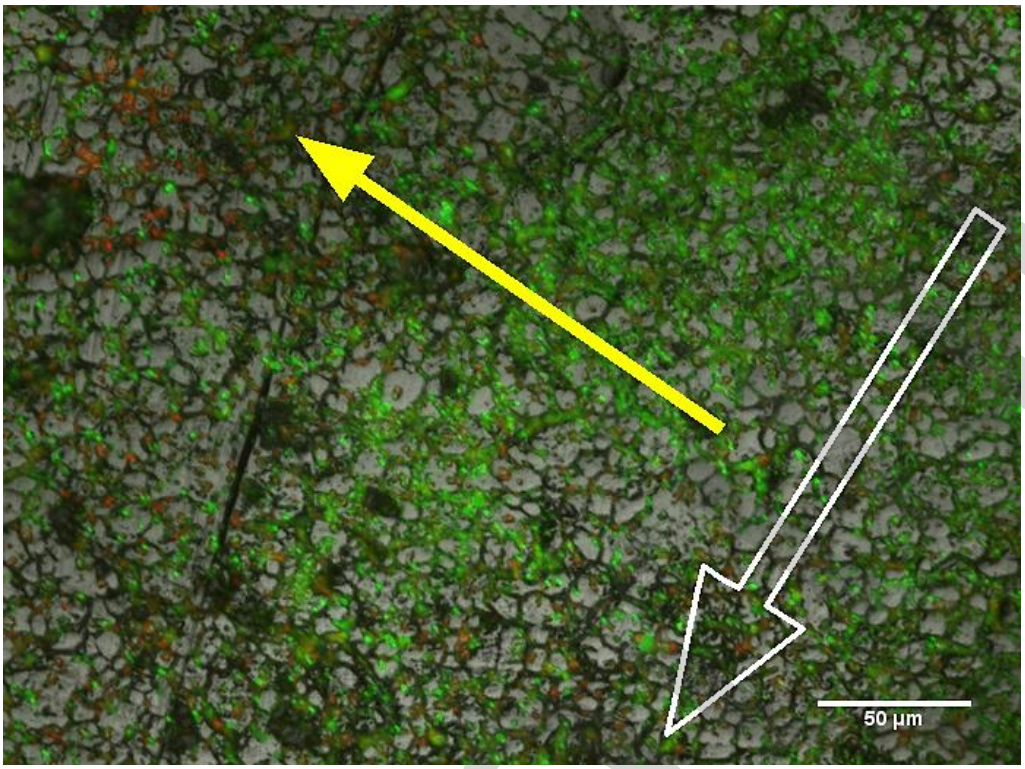

Figure 2. Micrograph of *A. baumannii* DSB taken from the edge of the coupon. The outlined white arrow indicates the direction of flow; whilst the solid yellow arrow indicates a change in microcolony density association with nutrient gradients. There appears to be a great proportion of PI-labelled cells towards outside of the nutrient gradient versus the centre which is more abundant in SYTO-9-labelled cells.

Table 1 shows the mean number of *A. baumannii* (CFU) recovered from the surfaces by foam, viscose and cotton swabs; premoistened using a buffer solution. There was a statistically significant difference in the number of recovered bacteria for foam swabs in comparison to both viscose and cotton swabs (P = 0.0094 and P = 0.0045, respectively). Foam swabs were able to recover up to 18 times more bacteria than the other swab types per surface area sampled. As a result, the microbial load removed by foam swabs demonstrated a closer likeness, in terms of CFU, to those of our control coupons (P >0.9999). Whereas, viscose and cotton swabs were significantly lower (P <0.0001). As shown in previous studies, foam swabs exhibited superior recovery rates, yet none of the swab types were able to recover more than a third of the total biofilm present[31].

Table 1. Data for *A. baumannii* recovered per cm^2 from a stainless steel coupon using three swab material variants (n = 9 per swab type). Each experimental run included a minimum of 2 control coupons which were exposed to identical desiccating conditions and quantified alongside the test coupons. Recovery rates were given as a percentage of the average CFU on the control coupons for each run.

Swabs	Recovered (Log_{10} CFU/cm^2)	Standard deviation	Recovery Rate (%)	Standard deviation
Foam	6.90	0.50	29.79	18.35
Viscose	5.61	0.34	5.75	6.56
Cotton	5.56	0.49	2.87	3.06

Following enumeration of swabbed coupons, the removal rate of surface-bound bacteria averaged 72.28 ±33.80% when compared to the total biofilm originally present (Table 2). No statistical difference was observed between the material types for number of bacteria removed (Log_{10} CFU/cm^2) in spite of those recovered from the swab tips as shown above (*P* >0.9999). A comparison between our swabbed and untouched control coupon results indicated that between 38 - 75% of the microbes removed from the DSBs were unaccounted for. This would imply they had failed to be released from the swab tip during vortexing.

Table 2. Data for average number of *A. baumannii* (CFU) recovered from the surfaces after swabbing (n = 9 per swab type). All swabbed coupons demonstrated a significant drop in bioburden in comparison to our control coupons ($P < 0.04$). Each experimental run included a minimum of 2 control coupons. Removal rate of bacteria through swabbing was given as a percentage of the average CFU on the control coupons for each run.

Swabs	Swabbed coupons (Log_{10} CFU/cm^2)	Standard deviation	Recovery Rate (%)	Standard deviation
Foam	6.57	0.77	67.39	45.43
Viscose	6.42	0.52	80.49	23.77
Cotton	6.71	0.34	70.60	19.72

The pronounced differences observed above between swab types were not clearly identified during EDIC and EF microscopy. Using bacterial viability stains SYTO-9 and

propidium iodide (PI), we could reveal the directional movements of the swab tip across the substrate surface (Fig. 3). Fluorescent staining of microcolonies indicate streak marks visible through the biofilm matrix and the removal of large areas of microcolonies. Within the swabbed areas we can distinguish non-viable bacteria, stained by PI, left behind within the crevices of the substrate. Areas adjacent to these showed no indication of disruption suggesting some areas experienced no physical contact with the swab tip.

Micrographs of swabbed and un-swabbed coupons, for each swab type, revealed statistically significant reductions in DSB coverage across the coupon, in terms of overall fluorescence per 1000 μm^2 (P <0.0001). However, no such statistical difference could be distinguished amongst the surfaces swabbed with either foam, viscose or cotton swabs (P ≥0.0550). This aligns with our CFU values as stated in Table 2 for the same surfaces (Fig. 4).

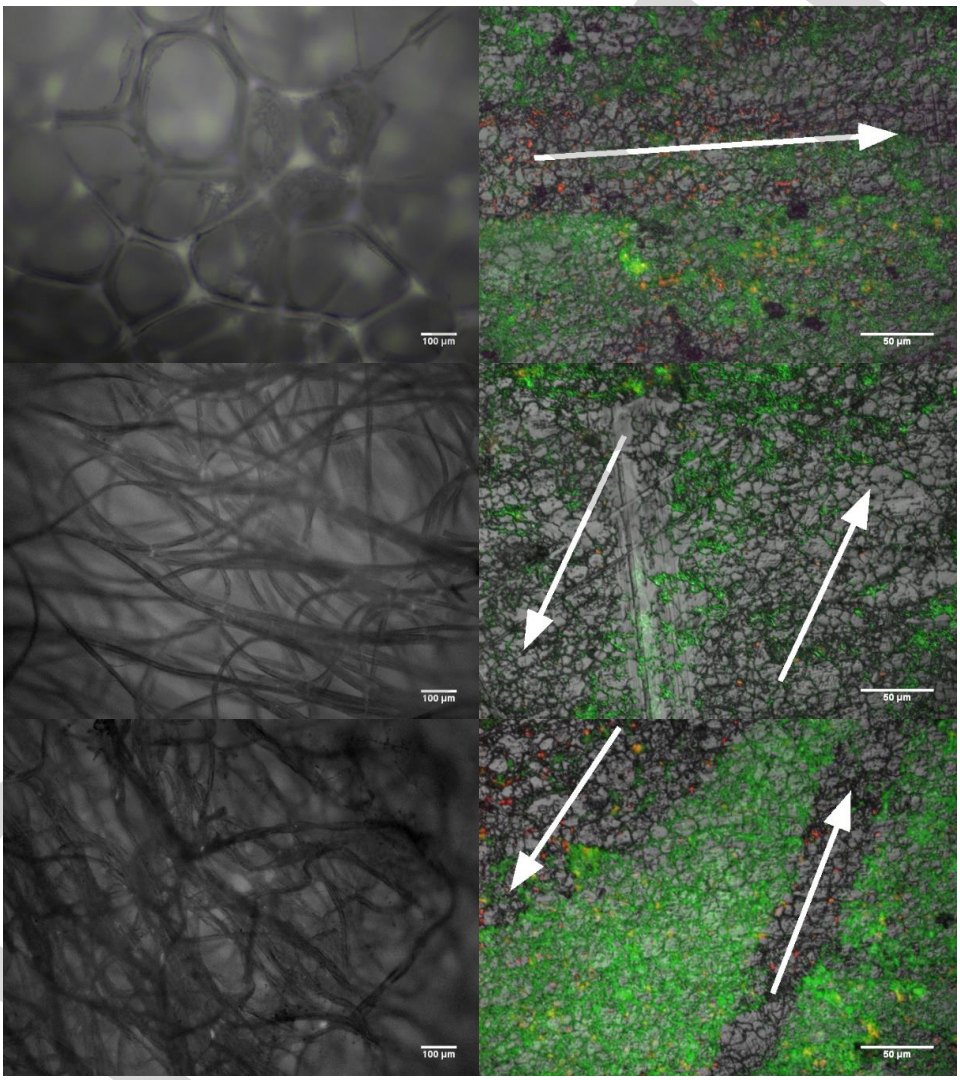

Figure 3. EDIC and EF micrographs of the structure for each swab type (left) and the biofilm surface (right) after being samples using foam (top), viscose (middle) and cotton (bottom)

swabs. The directional arrows (white) indicate the path taken by the swab tip. The direction of flow for the media across the surface was left to right. Traces of dead cells, stained red by PI, can be seen left behind within the path of the swab.

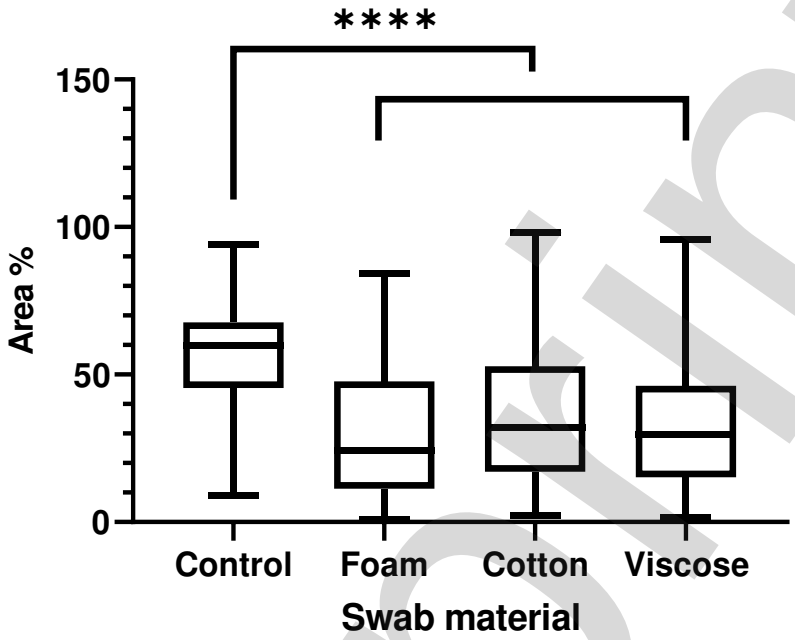

Figure 4. The area of DSB coverage, given as a percentage, remaining on the coupons after swabbing by foam, viscose and cotton tipped swabs. All three swab types demonstrate a reduced level of DSB coverage in comparison with an un-swabbed control (P <0.0001).

Discussion

Patients colonised by epidemiologically important pathogens such as *A. baumannii* will readily shed into their surrounding environment. Studies show once shed, pathogens can form biofilms and survive on dry surfaces for months and act as reservoirs for future infection[30]. This poses a known risk to newly admitted patients. The same studies highlight the presence of MDROs even when the prior occupant was not a known harbourer, demonstrating the importance of effective surveillance and cleaning[32]. Accurately defining the composition of any environmental microbiome in hospitals is crucial for developing infection prevention and control such as targeted disinfection[33]. This study has shown surfaces heavily burdened by DSB can be effectively detected using basic environmental swabbing techniques.

The biofilm model used in the study was selected for its unique air-liquid-solid interface deemed indicative of the conditions found on clinical surfaces. The temperature (≈20˚C) at which the model was run as well as the use of low nutrients media also reflects the limiting conditions of these surfaces[23]. The heterogenous formation of microcolonies in this model,

shown during microscopy, closely resemble *in situ* examples of DSB[14,15,16]. We observed clusters of viable and non-viable colonies suggesting micro-niche formation in areas of differing nutrients or oxygen concentration. Additionally, the flow of media across the coupon surface introduces a mechanical shear force which affects the attachment and construction of the biofilm's matrix such as EPS[34]. We postulate the biofilm's dense non-uniform formation is more representative of those naturally found in hospitals[20].

Post swabbing of the surfaces, viable colonies remained embedded within the crevices of the substrate material. We perceive these to be basal cells associated with the base of the biofilm matrix and expressing reduced or no metabolic activity. As a result, this population of cells are often more tolerant of specific antimicrobials[35]. Biofilm adhesion to the substrate is dependent on many factors including relative humidity and substrate topography. The rate of detachment is generally considered to reduce in the lower layers of biofilm[36,37,38]. These residual traces of biofilm seen here after swabbing demonstrates a potentially irreversibly bound conditioning film of organic matter allowing rapid recontamination of surfaces - not detected by routine swabs[34,39].

The impact of swab material on microbe detection is well documented for a vast range of microbes, and materials[31]. Absorbance and subsequent release capacity of a swab is dependent on the properties and architecture of the material, such as flexibility and liquid retention as well as the osmotic, electrostatic, and hydrophobic properties, and cell size of the target microorganism[40,41]. For example, foam swabs are designed to have a more flexible and open structure which enables the swab to sample hard to reach spots and enhanced microbe release during sonication or vortexing. The inverse is true for the tightly woven fibres of cotton swabs[42,43,44]. We postulate similar performance characteristics can be shown here, whereby foam swabs more accurately identified the total biofilm population present. However, no such difference could be shown for the release capacity across each swab type where a notable proportion of the DSB removed was not released on any of the swabs tested. Biofilms discovered on similar stainless steel surfaces in food processing facilities have been found to adhere more readily compared to transient bacteria[45,46]. Almatroudi *et al.,* revealed *in situ* DSB samples taken from an ICU ranged between 420 to 1.60×10^7 bacteria per cm^2 [16]. The average values of DSB recovered in our study by the three swab materials fall within this range (Foam = 7.94×10^6 CFU/cm^2, Viscose = 4.07×10^5 CFU/cm^2, Cotton = 3.63×10^5 CFU/cm^2) and either equal or exceed the group's published average value of 5.50×10^5 bacteria/cm^2. It is clear from our EF micrographs, in combination with the enumeration values, that the reported bioburden levels for swabbed surfaces were a significant underestimation of the overall contamination present. This reduced efficiency is a known trait when sampling dry surfaces and biofilm formation[47,48,49]. The inherent 'sticky' nature of polysaccharides, liposaccharides, and proteins found in abundance in EPS result in poor release capacities in swabs[34,50,51,52].

Published literature shows environmental biofilms are highly abundant on dry surfaces within healthcare facilities – most notably ICUs where the majority of studies occur. In all the studies, abundance and overall complexity of the biofilms were confirmed by

281 microscopy or culture analysis of surfaces physically removed from the environment. For
282 example, Ledwoch *et al.,* (2018) was able to confirm sessile microbes in 95% of
283 decommissioned equipment samples in spite of negative culture results post swabbing with
284 sterile cotton swab[20]. Due to the strong surface interactions of DSBs, these studies
285 conclude that microbe surveillance using basic environmental sampling techniques is likely
286 to only collect planktonic organisms[53]. In most circumstances these are microbes
287 associated with the skin flora readily transmitted by patients and healthcare workers[2,54]. In
288 the study presented here we ensured all planktonic or loosely bound microbes were
289 removed prior to processing. Therefore, culture results only reflect the detachment and
290 detection of DSB from the surface, and thus advocate the use of swabs for detecting
291 environmental biofilms as well as surface-bound microorganisms in hospitals.

292 In this study, we were able to demonstrate routine environmental swabs are capable of
293 detecting DSB presence, using a clinically relevant strain and model, at levels similar to
294 those found in healthcare settings. *A. baumannii* was chosen for its ability to form biofilms
295 and importance in HAI. However, it is known DSB are comprised of multiple species and
296 future models should incorporate mixed species biofilms found most frequently, such as
297 *Bacillus* spp. and *S. aureus*[2,15,20]. We would anticipate this to have further influence on
298 absorbance and release capacities during swabbing as microbiome composition differs.

299 The drip flow reactor model described here uses a unique air-liquid-solid interface deemed
300 representative of clinical surfaces as opposed to other common models where the biofilms
301 are generated on immersed coupons[26,55,56]. The low laminar flow of media across the
302 biofilm surface results in comparatively weaker adhesive biofilm on hard surfaces. Based on
303 previous studies the model used here has a low Reynold's number (approx. 12 – 20) [57]. This
304 will have influenced the detachment of DSB fragments from the surface. We acknowledge
305 additional mechanical stresses, such as wiping during cleaning or physical contact from
306 patients, will impact upon the structure of biofilm and its resistance to removal during
307 swabbing. Adaptations of this model should be considered to further support our results.

308 Environmental sampling, though not mandatory, is used routinely in hospitals to assess
309 surface cleanliness[18,58]. In part due to the abundance of nutrients used in our study, the
310 average bioburden levels greatly exceed those found *in situ* and the concentration of
311 inoculum per unit of area is an important variable when evaluating sampling methods.
312 Similar swab studies have shown detection efficiencies are directly linked to inoculation
313 levels, with poor recovery rates for lower concentrations i.e. $< 10^4$ CFU[41,57,59]. Our study
314 highlights good recovery efficiencies for high concentrations ($>10^6$) but failed to assess
315 performance through progressively lower levels of bioburden. This could be used to explain
316 why previous DSB studies report poor culture results using swabs.

317 Biofilm contamination on dry surfaces is rapidly becoming a recognised reservoir for
318 nosocomial pathogens in conjunction with the well-established background in resistance to
319 physical removal and antimicrobials during *in vitro* studies[60]. Developing methods for
320 efficiently detecting surface bioburden such as biofilms is key in combatting outbreaks of
321 HAI associated pathogens; for example, piezoelectric sensors which utilise a quartz crystal to

monitor changes in frequency as mass accumulates on the surface[61]. However we acknowledge without visualisation of surfaces biofilm presence cannot be definitively proven; and basic environmental sampling, as shown here, remains capable of capturing an overall estimation albeit a notable underestimation.

Author Contributions

Fergus Watson conceived and coordinated the study, analysed the data, and prepared the paper. Professor Bill Keevil, Dr Sandra Wilks and John Chewins provided technical assistance. All authors reviewed the results and approved the final version of the manuscript.

Conflict of interest statement

Professor Bill Keevil and Dr Sandra Wilks do not have any conflicts to disclose. Fergus Watson and John Chewins are disclosed as employees of BIOQUELL UK Ltd a provider of hydrogen peroxide decontamination systems.

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Dear Editorial Board Member, *Access Microbiology*.

We would like to take this opportunity to thank you, the editor, and the reviewers for the very kind and positive feedback on this article and the timely manner of your responses. We can appreciate how disruptive things can be at times given the current climate, so thank you.

We are very appreciative of feedback and helpful comments, and we hope we have been able to address these fully in our responses below. If there are any further comments or a need for further discussion, please do not hesitate to get in touch.

Reviewer 1 Comments:

In response to the reviewer's suggestions for conduct three repeats; this was indeed completed across three experimental runs. We have updated the methodology to reflect this. We suspect the confusion came from the controls for each experimental run consisting of two coupons. This is an inherent limitation of the drip flow model with limited under of coupons being available.

Reviewer 2 Comments:

In response to the reviewer's suggestions for confocal microscopy; this would indeed provide a three-dimension representation of the biofilm's density and distribution of the 'live' and 'dead' cells on the surface. Unfortunately, due to resource and time constraints this could not be completed using such microscopy techniques. We believe the content on the publication supports the subjective nature of the EDIC/EF microscopy completed and is being used to make suggestions at the biofilm's characteristics.

With regards to the supplementary data requests, we believe this in an appropriate method of reporting on the study and that there is no added benefit to including further supplementary data unless requested by the reader.

In addition to these changes, supplementary figures have been included as per the requirement and included within the submission to support the microscopy analysis conducted; subsequently the methodology has been revised in conjunction.

Kind regards,

Fergus Watson