Is a reduction in viability enough to determine biofilm susceptibility to a biocide?

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Abstract (249/250)

Objective

The abundance and prevalence of dry surface biofilms (DSB) in hospitals constitute an emerging problem, yet studies rarely report the cleaning and disinfection efficacy against DSB. Here, the combined impact of treatments on viability, transferability and recovery of bacteria from DSB was investigated for the first time.

Methods

S. aureus DSB was produced in alternating 48h wet/dry cycles for 12 days on AISI 430 stainless steel discs. The efficacy of 11 commercially available disinfectants, 4 detergents and 2 contactless interventions were tested using a modified standardized product test. Reduction in viability, direct transferability, cross-transmission (*via* glove intermediate) and DSB recovery post treatments were measured.

Results

Nine out of 11 disinfectants were effective in killing/ removing bacteria from *S. aureus* DSB with more than 4 $\log_{10}$ reduction. Only two disinfectants, sodium dichloroisocyanurate 1,000ppm and peracetic acid 3,500ppm, were able to lower both direct and cross-transmission of bacteria (< 2 adpression contacts positive for bacterial growth). Eight out of 11 disinfectants could not prevent DSB recovery for longer than 2 days. Treatments not involving mechanical action (vaporised hydrogen peroxide and cold atmospheric plasma) were ineffective, producing < 1 $\log_{10}$ reduction in viability, DSB regrowth within 1 day and 100% transferability of DSB post treatment.

Conclusions

Reduction in bacterial viability alone does not determine product performance against biofilm and might give a false sense of security to consumers, manufacturers and regulators. The ability to prevent bacterial transfer and biofilm recovery post-treatment provides a better understanding of the effectiveness of biocidal products.

Introduction

Environmental cleaning and disinfection of clinical surfaces is essential for infection prevention programs to be effective (1,2). There is now a strong evidence of the role played by contaminated healthcare surfaces in the transmission of pathogens that cause healthcare associated infections such as *Clostridium difficile*, methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococci* (VRE), norovirus, and multidrug-resistant (MDR) gram-negative bacteria including *Acinetobacter baumannii*. These pathogens have been shown to survive on surfaces for prolonged periods (days, months and even years) and remain virulent (3). Their transmission can occur directly or through secondary vehicles such as hands (3).

Recent studies have highlighted the fact that current cleaning and disinfection practices might not be effective (4), despite good adherence to infection control guidelines (5, 6). One possible explanation for this failure encountered in practice is thought to be the presence of bacteria in complex dry surface biofilms (DSB) (7, 8, 9). Multidrug-resistant bacteria have been shown to be present in DSB on bedding, surrounds and furnishing items taken from hospitals' intensive care units, despite cleaning with chlorine solution (10).

There is still a lack of internationally accepted standardised protocols to test disinfectants/detergent product effectiveness against "hydrated" biofilms (11), let alone DSB. In addition, most standard efficacy tests investigate a reduction in microbial viability, but rarely consider microbial transfer post-treatment or the ability of biofilm to reform rapidly after treatment (12, 13). DSB have been shown to be transferred via hands to multiple fomites (14), and the implications of this transfer in pathogens transmission has been identified (15). The role of gloves as DSB transfer

vehicles has been also recognised (14). In addition, DSB can recover even following very strong, 20,000 ppm chlorine treatment (8).

In this study, we investigated the combined reduction in viability, transferability and recovery of bacteria from DSB following treatments with 11 commercially available disinfectants, 4 detergents and 2 contactless interventions. We believe that those three factors combined can give a full picture of product effectiveness against DSB, and hopefully help in development of improved infection control guidelines for routine and terminal cleaning of hospital surfaces.

Methods

Bacterial growth and maintenance

Staphylococcus aureus NCTC10788 was propagated in tryptone soya broth (TSB; Oxoid Limited, Hampshire, UK) at 37°C overnight and washed in tryptone sodium chloride [1 g/L of tryptone (Oxoid, Thermo Scientific™, Loughborough, UK) and 8.5 g/L of sodium chloride (Sigma- Aldrich®, Dorset, UK)] following centrifugation at 1,400g. The bacterial suspension was adjusted to $1x10^6$ CFU/mL.

Products tested

Fifteen commercially available products were tested and summarised in Table 1.

Neutraliser preparations

The universal neutraliser was prepared according to modified protocol (16). Briefly, lecithin 3 g/L (ACROS Organics™, Fisher Scientific, Loughborough, UK), Tween80 30g/L (ACROS Organics™, Fisher Scientific, Loughborough, UK), sodium

thiosulphate 5 g/L (Fisher Bioreagents™, Fisher Scientific, Loughborough, UK), L-histidine 1 g/L (Sigma- Aldrich®, Dorset, UK), saponin 30 g/L (ACROS Organics™, Fisher Scientific, Loughborough, UK) and sodium dodecyl sulphate 5 g/L (Sigma-Aldrich®, Dorset, UK) in sterile tryptone sodium chloride solution were mixed together and made up to 1L with distilled water. The solution was then autoclaved at 121°C for 15 minutes.

Dry surface biofilm formation

Dry surface biofilm was formed and grown based on Ledwoch et al. (17). Briefly, *S. aureus* NCTC 10788 overnight culture was centrifuged at 1,400g in Biofuge Primo R centrifuge (Heraeus, Thermo Fisher Scientific, Newport, UK) and washed with fresh tryptone soya broth (TSB; Oxoid, Thermo Fisher Scientific, Newport, UK). *S. aureus* culture was then diluted to 10^6 CFU/mL in the presence of 3 g/L bovine serum albumin (BSA, Sigma® Life Science, Dorset, UK). Culture was inoculated on stainless steel AISI 430 discs (0.7 +/- 0.07 mm thickness; 10 +/- 0.5 mm diameter, Goodfellow Cambridge Limited, Huntington, UK) by transferring 1 mL of the inoculum and single disc in each well of the Corning™ Costar™ flat-bottom cell culture plates (Fisher Scientific™, Loughborough, UK). The well plate containing discs and inoculum was then placed on Orbit P4 plate rocker (Labnet International, Edison, NJ, USA) at 180 RPM and 21°C for 2 days- this was so-called “wet phase”. Following wet phase, the liquid was drained out from the wells and the well plate was moved into Universal Oven 100-800 incubator (Memmert, Schwabach, Germany) at 37°C for 2 days- this was so-called “dry phase”. After the dry phase, 1 mL of TSB with 3 g/L BSA was added to each well and another wet phase begun. The biofilm was grown in alternating wet-dry phases for a total duration of 12 days.

ASTM E2967-15 Wiperator test

The effectiveness of commercial products commonly used in hospitals was investigated according to a modified ASTM E2967 test (18). All products were tested in a wipe form, but for VHP and CAP. If the product was supplied in a liquid form, it was combined with sterile Rubbermaid® HYGEN™ disposable microfiber cloth (Rubbermaid Products, Surrey, UK) allowing 2.5 mL of disinfectant per 1 g of wipe. The surface of the disc with DSB was wiped with the Wiperator (Filtaflex Ltd, Ontario, Canada) from both sides using separate wipes. Dry surface biofilms were wiped for 10 s under 500 g pressure and left at room temperature for 2 min (contact time). Transfer of viable bacteria from used wipes to clean sterile disc was not performed.

Vaporised hydrogen peroxide test

Vaporised hydrogen peroxide (VHP) test was performed at GAMA Healthcare Ltd, 3 Regal Way, Watford, UK with a RHEA VHP device (Airinspace®, Élancourt, France). Biocide used in the system consisted of 7% of hydrogen peroxide, 5% acetic acid and 0.4% peracetic acid. Briefly, *S. aureus* DSB samples were placed on a stand at 1.5m height and arranged vertically 3 meters away from VHP unit. The treatment ran for 90 minutes with 6 ml of biocide used per cubic meter of the room. In total, 290 ml of biocide was consumed per cycle. Following treatment, samples were neutralised by placing each disc in separate well containing 1 ml of Dey-Engley (DE) neutralizing broth (Neogen® Corporation, Ayr, UK) for 24 h.

Cold atmospheric plasma treatment

Cold atmospheric plasma (CAP) treatment was performed using a surface barrier discharge device developed at the Centre for Plasma Microbiology, Department of Electrical Engineering and Electronics, University of Liverpool (19). The distance between *S. aureus* DSB disc and the plasma generating electrode was 2 mm, providing a spatial separation between the plasma layer and sample surface. Coupons were exposed to an RNS dominated gas-phase chemistry created using a high power discharge plasma ($P_{discharge}$ = 34.5 W). Treatments were performed for 240 s. Discs were treated from both sides. Following treatment, the samples were neutralised by placing each disc in separate well containing 1 ml of DE neutralizing broth for 24 h.

Reduction in bacterial viability

Reduction in bacterial viability (Log_{10} reduction in CFU per ml) gave the number of bacteria that were removed and/or killed following wiping. The test was based on Ledwoch et al. (17). Briefly, following 2 min contact time (or VHP/CAP treatment), the discs were neutralized by placing each disc into universal neutraliser for 5 min.

Following neutralisation, discs were moved to McCartney bottles containing 2 mL of TSB with 1 g of glass beads and incubated for 2 hours at 37°C. After incubation, the bottles with discs were vortexed for 2 minutes to remove DSB from disc surface and the solution was then drop plated on tryptone soya agar (TSA; Oxoid, Thermo Fisher Scientific, Newport, UK). The log_{10} reduction was evaluated against controls consisting of untreated samples. Reduction in viability was measured for disinfectants only.

Direct transfer test

The surface transferability test was designed to investigate how much bacteria can be transferred directly from treated surface. Direct transfer test was performed according to Ledwoch et al. (17). Following wiping and 2 min contact time, or VHP and CAP treatment, each disc was picked by Shaw Magnets Alnico Rod Magnet (Rapid Electronics, Essex, UK) and pressed 36 consecutive times with 100 g pressure on the surface of Dey-Engley (DE) neutralising agar (Sigma Aldrich, Dorset, UK) (Fig. S1, left). Following the transfer test, DE agar was incubated overnight at 37°C. Positive growth/total number of adpressions was recorded. Direct transferability was measured for disinfectants only.

Cross transmission via glove (glove transferability)

The test imitates a single touch of a gloved finger onto treated surface, and further subsequent transfers from the contaminated glove to the environment. Following wiping and 2 min contact time, disc was pressed once with 100 g pressure by gloved finger (Fig. S1, right). Then, gloved finger was pressed 25 consecutive times with 100 g pressure on the surface of Dey-Engley (DE) neutralising agar (Sigma Aldrich, Dorset, UK). Following the transfer test, DE agar was incubated overnight at 37°C. Positive growth was recorded and transferability expressed as the number of positive contact/total number of adpressions (Fig. S3). Cross transmission was measured for both disinfectant and detergent products. Two different glove types were tested: ecoSHIELD™ powder free Nitrile gloves (Appleton Woods, Birmingham, UK) and SemperGuard® 100% latex powder free inner coated gloves (Appleton Woods, Birmingham, UK).

Regrowth test

Regrowth measures the time needed for the DSB to recover following treatment. Regrowth test was based on Ledwoch et al. (17). Following wiping and 2 min contact time, samples were placed in 30 mL capacity flat bottom glass bottle with 2 ml of Dey-Engley (DE) neutralizing broth (Neogen® Corporation, Ayr, UK). The number of days for the DE neutralising broth colour to change from purple to yellow indicative of bacterial growth was recorded. Regrowth was measured for disinfectants only.

Pass/fail criteria

The ASTM E2967 protocol does not have any pass or fail criteria. Here, we arbitrarily set it to > 4 $\log_{10}$, to reflect the minimal reduction requirement indicated in European standards (20).

We also suggested that an effective biocidal treatment would reduce bacterial transferability to a maximum 2 positive contacts.

The NHS specifies 0 - 48h timeframe for cleaning low-risk functional areas (21). Due to the potential low cleaning compliance in hospitals and that some surfaces are being overlooked in routine cleaning (22), we suggested that successful treatment must prevent biofilm regrowth for at least 3 days.

Statistical analysis

Statistical significance of data sets was evaluated with GraphPad PRISM® (ver. 7.04) using single factor ANOVA. All measurements were performed in triplicates. The sample standard deviation was evaluated with Bassel's correction.

Results

Reduction in viability

Overall, most disinfectants tested passed the *S. aureus* DSB viability reduction test (Table 2). Six disinfectants performed particularly well achieving ≥ 6 $\log_{10}$ reduction including 4 NaDCC and 2 PAA formulations (Table 2). ClO_2 (1,000 ppm, pH 4.31) and NaOCl-2 (1,000 ppm, pH 13.13) were the least effective treatments. There was no statistically significant difference between the performance of ClO_2 and NaOCl-2 treatments and just wiping with water (ANOVA: single factor, p=0.06 for ClO_2 and p=0.14 for NaOCl-2). This was a surprising result given that both ClO_2 and NaOCl-2 products have the same level of available chlorine (i.e. 1,000ppm) to the other chlorine-based products that achieved a >6 $\log_{10}$ reduction. However, the relation between pH of tested disinfectant and the $\log_{10}$ reduction in dry surface biofilm viability showed that products underperforming had extreme pH (ClO_2, pH= 4.31 and NaOCl-2 pH= 13.13) comparing to the rest of the products tested. Biocides formulated at pH ~8 were found to be the most effective among tested products (Fig. 1).

Both VHP and CAP interventions that did not involve mechanical wiping performed poorly (< 1 $\log_{10}$ reduction) against DSB (Table 2).

Direct transfer (surface transferability)

Only 5 biocides (BZK, NaDCC-1, NaDCC-3, NaDCC-5 and PAA-1) were successful at preventing direct bacterial transfer from DSB (Table 2). There was no difference in bacterial transfer (i.e. 100% transfer; Fig. S2, right panel) between wiping the surface with just water and applying ClO_2, NaDCC-2, NaOCl-Ref, PAA-2, VHP or CAP treatments (ANOVA: single factor, p>0.05).

Cross transmission (glove transferability)

Both peracetic acid treatments successfully controlled the transmission of bacteria via nitrile and latex gloves. All chlorine – based formulated disinfectants were effective at preventing the transferability of bacteria from DSB via latex glove. The majority of chlorine- based products were also successful when nitrile glove was used. However, some treatments failed to control bacterial transferability, despite the high reduction in bacterial viability reduction, as tested in previous experiments. The inverse phenomenon was observed with NaOCl-2, when bacteria were not transferring from DSB even though the treatment was not effective at significantly lowering biofilm viability.

Bacterial regrowth

Only one treatment, PAA-1, could prevent recovery of bacteria for longer than 3 days (Table 2). The recovery of biofilm was observed within 2 days for the vast majority of the treatments, even when their biofilm viability reduction or transferability prevention was satisfactory.

Discussion

It is now well established that environmental surfaces in healthcare settings act as reservoirs for the transmission of pathogens (23).

Here, we argued that current practices investigating the effectiveness of environmental surfaces disinfection are insufficient. Efficacy surface tests are based on planktonic bacteria dried onto surfaces (e.g. ASTM E2967), and do not reflect nor mimic biofilms

(24) that are actually present on hospital surfaces (7, 8, 25). *S. aureus* was used in our model as it is a well know pathogen that can survive desiccation, and which presence in DSB is widespread (7, 25). Moreover, wiping mechanical action is thought to contribute to biofilm removal with disruption of EPS matrix, weakening its effectiveness as a protective barrier to disinfection. We combined our *S. aureus* dry surface biofilm (DSB) model with the ASTM E2967 product test, with a contact time of two minutes that was considered more realistic on practical field (26). This test acknowledges the importance of mechanical wiping in evaluating the efficacy of products (27). Here, non-mechanical treatments tested (CAP and VHP) showed little activity (<1 $\log_{10}$ reduction) against *S. aureus* DSB (Table 2), with no decrease in bacterial transfer, or delay in biofilm regrowth (Table 2). In contrast, wiping with water only produced 2.3 ± 0.4 $\log_{10}$ reduction in viability (Table 2). The nature of DSB might also have impacted on the lack of efficacy of CAP. Indeed, a 'wet' *S. aureus* ATCC 9144 biofilm was shown to be completely eradicated following CAP treatment for 1 min (19). Where wiping was used, the majority (9/11) of the disinfectant tested decreased *S. aureus* viability by > 4 $\log_{10}$, which was used as a criterion for efficacy (28). We highlighted that formulations' pH might have been a contributing factor (Fig. 1) since actives formulated outside their optimum pH range may be less stable (29).

Our study argued that measuring the biocidal efficacy of a formulation might not be solely indicative of product performance particularly when biofilm eradication is studied. Indeed, when combining efficacy data with impact of treatment on bacterial transfer direct or via gloves, a different picture emerges (Table 2). Only formulated PAA (PAA-1, 3,500 ppm) achieved all set criteria combining efficacy, decreasing direct and indirect transfer and preventing biofilm regrowth.

The meaning of “rendering a surface safe” can be debated. It is however appropriate to reflect to the role of bacterial transmission with gloved hands. The role of gloves to transfer pathogens has been reported (24, 30) with latex and nitrile gloves when bacteria were suspended in tryptone soya broth + 5% horse serum (30). Treatment with detergent was reported to increase the transferability of bacteria from DSB (14). Here, however, we showed that most interventions reduced bacterial transfer from surfaces to gloves (Table 2).

Bacteria in biofilm surviving a biocidal treatment can initiate the formation of a new biofilm (13). Indeed, studying biofilm regrowth post treatment provides important information of product efficacy, but also indicates the frequency of product application, to ensure surfaces remain safe (25). Biofilms have been shown to recover quickly even after decontamination (12). DSB regrew within 2 days at 37°C following 1,000 ppm and 5,000 ppm chlorine treatment, as reported by Almatroudi et al. (8). Our study confirmed that observation with DSB recovering quickly (<2 days) following the use of 8/11 treatments.

In this study, we show that measuring a reduction in viability does not provide enough information to ensure a surface will be safe post-treatment. A significant number of treatments tested (8/13) failed to prevent bacteria transfer from treated surfaces and almost all treatments tested (12/13) did not delay biofilm recovery, despite most treatments (9/13) being effective at reducing viability of biofilm. By determining the impact of treatment on bacterial transfer and recovery from DSB we provided additional practical information to manufacturers and end users. Performing these tests in combination would increase the stringency to demonstrate efficacy, providing reassurance that a surface would actually be safe to touch post treatment.

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Conflict of interest

K Ledwoch is partially employed by GAMA Healthcare Ltd.

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