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Transfer of micro-organisms from dry surface biofilms and the influence of long survival under conditions of poor nutrition and moisture on the virulence of *Staphylococcus aureus*

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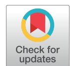

SUMMARY

Background: Biofilms on dry hospital surfaces can enhance the persistence of micro-organisms on dry harsh clinical surfaces and can potentially act as reservoirs of infectious agents on contaminated surfaces.
Aim: This study was conducted to quantify the transfer of viable *Staphylococcus aureus* cells from dry biofilms through touching and to investigate the impact of nutrient and moisture deprivation on virulence levels in *S. aureus*.
Methods: Dry biofilms of *S. aureus* ATCC 25923 and a defective biofilm-forming ability mutant, *S. aureus* 1132, were formed in 24-well plates under optimized conditions mimicking dry biofilm formation on clinical surfaces. Microbial cell transfer was induced through the touching of the dry biofilms, which were quantified on nutrient agar. To investigate the impact of nutrient and moisture deprivation on virulence levels, dry and standard biofilms as well as planktonic cells of *S. aureus* ATCC 25923 were inoculated into *Galleria mellonella* and their kill rates compared.
Findings: Results of this study showed that viable cells from dry biofilms of S. *aureus* ATCC 25923 were significantly more virulent and readily transferrable from dry biofilms through a touch test, therefore representing a greater risk of infection. The biofilm-forming capability of *S. aureus* strains had no significant impact on their transferability with more cells transferring when biofilm surfaces were wet.
Conclusions: These findings indicate that dry biofilms on hospital surfaces may serve as a reservoir for the dissemination of pathogenic micro-organisms in hospitals, thus highlighting the importance of regular cleaning and adequate disinfection of hospital surfaces.
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Introduction

Staphylococcus aureus is estimated to exist as a commensal organism on about 30% of human populations but can become pathogenic in susceptible individuals and it is among the most common healthcare-associated infection (HAI) pathogens globally [1–3]. *S. aureus* is a common cause of a wide range of human infections including, endocarditis, osteomyelitis, meningitis, bacteraemia, pneumonia, skin and soft tissue infections [4]. *S. aureus* infections are complicated by common resistance to meticillin which renders *S. aureus* infections hard to treat [5]. However, their ability to exist within the clinical environment on surfaces, and easily transfer amongst individuals, is an important virulence factor.

Inanimate surfaces in healthcare facilities are prone to contamination with pathogenic micro-organisms, and as such regular decontamination of hospital surfaces is a common infection control procedure [6,7]. However, these decontamination procedures do not always achieve total removal of micro-organisms, which can result in the long-term persistence of pathogenic microbial species on hospital surfaces. These can serve as reservoirs for other disease-causing organisms [8–10]. Pathogenic micro-organisms can be transferred from these existing reservoirs to new surfaces, fomites or susceptible individuals, thus resulting in infection transmission. Dry cell aggregates, supported by extracellular matrix, are one of the key protective factors that have been identified to aid the survival of pathogenic micro-organisms on healthcare surfaces after routine cleaning and disinfection [8,11,12]. Pathogenic micro-organisms have been reportedly isolated from dry biomass on healthcare surfaces even after routine cleaning and disinfection [8,9,12].

A widely recognized means of pathogen transfer in healthcare facilities is through the touching of contaminated surfaces by healthcare workers and patients. Hands have been described as the most critical factor in the transfer of pathogens in healthcare facilities, thus regular hand washing is one of the most recognized strategies for controlling healthcare-associated infection transmission [13,14]. Despite evidence showing significant numbers of HAIs resulting from pathogen transfer through the touching of contaminated surfaces [13], the potential for the transfer of pathogens from dry biofilm on inanimate surfaces through touching has been poorly studied. In this study, we evaluated the potential for the transfer of pathogens from dry biofilms using *Staphylococcus aureus* as the model organism.

In this study, we evaluated the potential of transferring viable cells from the dry biofilms of *S. aureus* through touching and explored the effect of long-term persistence on surfaces as dry biofilm on the virulence of *S. aureus* strains. This was carried out by quantifying cells transferred from dry biofilms of two strains of *S. aureus* of different biofilm-forming phenotypes and by determining the kill rate of *S. aureus* grown in three different states — planktonic cells, standard wet biofilms and dry biofilms — using the *Galleria mellonella* model.

Methods

Bacteria strains used

Two strains of *S. aureus* were used for this study, *S. aureus* ATCC 25923 (a robust biofilm former) and 1132 (a ΔsrtA mutant). *S. aureus* 1132 is a mutant strain of *S. aureus* NCTC 8178 with a defect in the srtA gene, which codes for sortase transpeptidase. SrtA is a Gram-positive membrane enzyme which catalyses the anchoring of many cell surface proteins to the peptidoglycan [15,16]. This mutation hinders the attachment of the organism to surfaces.

The bacteria strains were maintained in glycerol stock kept at -80 °C from where working stocks were made by subculturing on Luria Bertani (LB) agar (Sigma Aldrich, St Louis, MO, USA).

Preparation of dry surface biofilm

Dry biofilms were formed according to Amaeze *et al.* [17]. Briefly, cells were recovered from overnight culture of each test organism grown in LB (Sigma Aldrich, St Louis, MO, USA) at 37 °C and 150 rpm by centrifugation at 10,000 × g for 5 min. The recovered cells were washed twice with phosphate-buffered saline (PBS) and adjusted to a final cfu count of 10^8 cfu/mL in 5% tryptic soy broth (TSB). Two millilitres of each test organism were subsequently transferred into wells of 12-well microtiter plates (Corning, Costar™). The biofilms were initially grown in 5% TSB for 48 h at room temperature before being dehydrated for 48 h, after which 5% TSB was added to the biofilm for 6 h followed by another cycle of dehydration for 66 h at room temperature. The biofilm was subjected to two further 6-h cycles of rehydration with 5% TSB with intermittent 42- and 66-h dehydration at room temperature.

Evaluation of bacteria cells transfer from biofilm through touching

Transfer of bacterial cells from dry biofilms was studied using biofilms described above, following a method adapted from Chowdhury *et al.* [18]. Biofilms were dehydrated for 66 h at ambient temperature and were then touched with a gloved index finger that was subsequently used to touch the surface of sterile nutrient agar plates 24 successive times, each touch on a new sterile surface. The plates were incubated aerobically overnight at 37 °C. Three wells were used for the touch transfer test from each biofilm type with the experiment repeated three times using nitrile latex-free gloves (Fisher Scientific, Leicestershire, UK). The sterility of the gloves used was initially confirmed by touching surfaces of sterile media with the gloves before use. The results from this experiment were accepted only when there was no growth on the media previously touched with the glove before use. The number of cells transferred from the dry biofilms of the two strains of *S. aureus* used in this study were compared using a two-way analysis of variance (ANOVA) and Sidak's multiple comparisons test using GraphPad Prism (version 6.07; San Diego, CA, USA). This procedure was repeated to test transmission of bacteria cells from wet surfaces using dry biofilms, which had been hydrated by washing with 1 mL of PBS twice to remove unbound cells (after 66 h dehydration at ambient temperature).

G. mellonella infection model evaluation of virulence

The virulence of cells recovered from dry biofilms was compared with cells recovered from wet biofilms and planktonic cultures to evaluate the impact of persistence in dry conditions on the pathogenicity of the *S. aureus* ATCC 25923

used for this study. *G. mellonella* model was used as it has the advantages cost and space effectiveness, involves no feeding, and is of similar humoral and cellular components as mammals.

Cells were recovered from 12-day-old biofilms of *S. aureus* ATCC 25923 prepared as explained above by adding 1 mL PBS to the biofilms after being washed twice with PBS to remove unbound cells. The biofilm plates were placed in an ultrasonic bath (Fisher Scientific, Leicestershire, UK) and sonicated for 5 min after which the wells were scraped according to Kobayashi *et al*. [19]. The recovered biomass was transferred into Eppendorf tubes and vortexed at maximum speed for 1 min. Prior to this study, an evaluation of the numbers of cells in the dry and wet biofilms after rinsing with PBS and sonication was carried out. These evaluations were performed in triplicate, and the results consistently showed approximately 1×10^8 cfu/mL in each replicate. The cfu counts of the cells recovered from the 12-day-old biofilm and 48-h standard biofilms were evaluated on nutrient agar by serial dilution in PBS followed by drop plating. After overnight incubation at 37 °C, cfu recovered from 12-day-old biofilm and the 48-h standard biofilms were confirmed to contain 1×10^8 cfu/mL.

Cells were also recovered from wet biofilms of *S. aureus* ATCC 25923 grown by inoculating 1 mL of *S. aureus* ATCC 25923 (10^8 cfu/mL) in TSB into 24-well plates. The plates were incubated at 37 °C for 48 h. The biomass of the wet biofilms was also harvested as described above and the cfu counts were also evaluated on nutrient agar. Cells recovered from overnight planktonic cultures were also used for the *G. mellonella* virulence experiment.

G. mellonella larvae (Livefoods Direct, Sheffield, UK) measuring 20–25 cm in length with no discoloration were allowed to equilibrate to ambient temperature by keeping them overnight in a dark cabinet as described by Olsen *et al.* [20]. Ten larvae were transferred into each Petri dishes and were injected with 10 μL of prepared cells (10^6 cfu) recovered from dry biofilms, wet biofilms and planktonic cultures explained above through their right last proleg using a 50-μL Hamilton syringe with 26 g needle. Prior to injection, the planktonic culture was adjusted to 10^8 cfu/mL to match with the numbers of cells recovered from the dry and wet biofilms and each experimental condition were set up in triplicate. Larvae injected with PBS were used as a control and another group (a sham-inoculated group) used for control for injection trauma [21]. The percentage survival of *G. mellonella* larvae inoculated with cells recovered from the dry biofilms, wet biofilms and planktonic cells as well as PBS was analysed using the Kaplan–Meier survival curves and GraphPad Prism (version 6.07; San Diego, CA, USA).

Results

The potential for the transfer of bacteria cells from dry biofilms was evaluated by quantifying the cfu counts of cells transferred from dry biofilms over 25 touches with a gloved index finger using *S. aureus* as the model organism. The number of cells transferable from dry biofilms of *S. aureus* was compared between two strains of different biofilm-forming abilities. As shown in Figure 1, *S. aureus* ATCC 25925 forms more robust biofilms under dry biofilm-forming conditions used for this study compared with *S. aureus* 1132, which has defective biofilm-forming ability. Using crystal violet assay, significantly

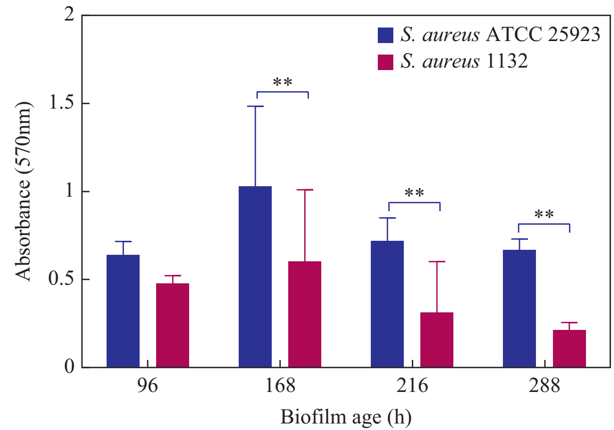

Figure 1. Crystal violet comparison of the total biomass of *Staphylococcus aureus* ATCC 25923 and *S. aureus* 1132 dry biofilms. Data are shown as the mean of three replicates with a standard error of the mean (** = $P \leq 0.01$) .

more biomass was quantified from *S. aureus* ATCC 25925 biofilms compared with the biomass of *S. aureus* 1132 biofilm.

The number of cells transferable from the dry biofilms of *S. aureus* ATCC 25925 and *S. aureus* 1132 are shown in Figure 2. Despite the different biofilm-forming abilities of the two *S. aureus* strains used, there was no significant difference in the number of cells transferable from the dry biofilms of both *S. aureus* test strains over 25 touches. The highest number of cells were transferable from the first touch which decreased with the number of touches. The average number of cells deposited on nutrient agar at the first touch for *S. aureus* ATCC 25925 and *S. aureus* 1132 were 22 and 19, respectively. The transfer rates were higher when the seeded biofilm surfaces were wet than when dry. The numbers of cells deposited on nutrient agar plates were too numerous to count, with >1000 cfu all through the 24 touches tested.

The galleria infection model was used to compare the virulence of cells recovered from dry biofilms to cells recovered from wet biofilms and planktonic cultures. As shown in Figure 3, cells recovered from dry biofilms were more virulent

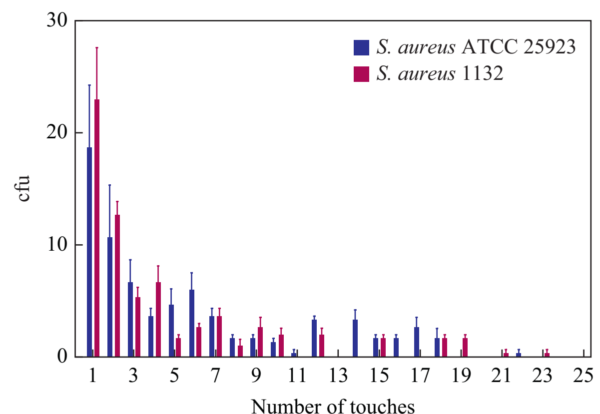

Figure 2. Colony forming units (cfu) of cells transferred from dry biofilms of *Staphylococcus aureus* ATCC 25923 and *S. aureus* 1132 over 25 touches. Data are shown as the mean of three replicates with a standard error of the mean.

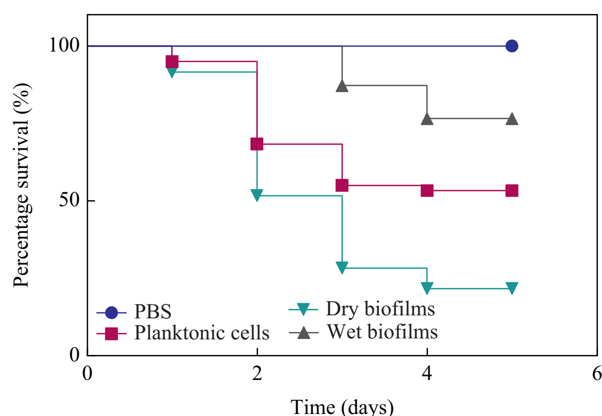

Figure 3. Kaplan–Meier survival curves of the percentage survival of *Galleria mellonella* larvae inoculated with cells recovered from dry biofilms, wet biofilms or planktonic cultures of *Staphylococcus aureus* ATCC 25923 and phosphate-buffered saline (PBS). Dry biofilms caused highest mortality of 60%; planktonic and standard biofilms followed with 53 and 21%, respectively (P=0.0008, <0.0001).

compared with cells recovered from wet biofilms or planktonic cultures of the same organism. After five days, 21, 60 and 53% of *G. mellonella* larvae inoculated with *S. aureus* ATCC 25925 cells recovered from dry biofilms, wet biofilms and planktonic cultures survived, respectively. A statistically significant difference was observed between mortality rate caused by planktonic and dry biofilms in waxworm (P=0.0008) and mortality rate caused by standard and dry biofilms (P<0.0001) with greater mortality exhibited in larvae injected with dry biofilm of *S. aureus* ATCC 25925 in both cases. The response of waxworms to standard and planktonic biofilms was the same.

Discussion

In this study, we evaluated the potential for the transfer of pathogenic microbial cells from dry biofilms on clinical surfaces by gloved fingers. Surfaces in clinical environments are regularly contaminated by potentially pathogenic micro-organisms from patients, staff and even visitors [22]. These micro-organisms can persist on the surfaces from where they can be transferred to susceptible hosts thus resulting in healthcare-associated infection transmission. While the use of gloves by clinicians protects them from infectious agents, gloves touching contaminated surfaces can transfer infectious agents to patients or even to the surfaces of medical devices [23,24]. Pathogens transferred to medical equipment can colonize patients if the equipment is not properly decontaminated before use. Some medical equipment such as endoscopes have complicated surfaces which can also cause the build-up of dry biofilms of potentially pathogenic micro-organisms [25].

In this study, we showed that a single touch of dry biofilms of *S. aureus* with a gloved finger can transmit up to 30 cfu of *S. aureus* cells and transfers further with more bacterial cells when the surface is wet. Hands are crucial vehicles for the transfer of infectious agents in the clinical environment [26]. Contamination of gloves with pathogenic micro-organisms on clinical surfaces has been reported in clinical studies, and this poses a risk of infection transmission to patients [27,28]. Low numbers of cfu of *S. aureus* have been reported to cause infections in humans. Elek [29] showed that 20 cfu of *S. aureus* can cause skin lesions in humans. Thus, this study demonstrated that *S. aureus*-contaminated gloves pose an infection transmission threat in a clinical environment.

The number of *S. aureus* cells transferred from the dry biofilms grown *in vitro* reduced with an increase in the number of touches. A similar observation was reported by Tahir *et al.* [30], however, intermittent wetting of the dry biofilms in their experiment to mimic detergent application on hospital surfaces increased the number of cells transferable from dry biofilms. Thus, intermittent wetting of dry biofilms on surfaces during cleaning can result in an increase in the number of cells transferable from dry biofilms on surfaces through touching with gloves. A notable observation in this study is that the biofilm-forming ability of the *S. aureus* strains had no significant impact on the number of transferable cells from their dry biofilms. This indicates that even poor biofilm strains of pathogenic bacteria can form dry biofilms, which can serve as reservoirs of infectious agents on hospital surfaces. Although the results of this study indicate that dry biofilms can be transferred multiple times, it is challenging to generalize these findings to what may occur in healthcare settings as no studies have yet determined the numbers of viable microbial cells in dry biofilms. In a systematic review conducted by Schapira *et al.* [31], no in-situ studies have been able to quantify the number of cells of recoverable bacteria/cm^2 inside dry biofilms. Currently, the sampling methods used are limited in describing microbial diversity and population size, which may play crucial roles in biofilm persistence, transfer, virulence and decontamination.

In this study, cells recovered from dry biofilms of *S. aureus* ATCC 25923 showed more virulence potential than cells recovered from planktonic cultures of wet biofilms. *S. aureus* has a plethora of virulence factors, which not only protect the bacterium from the host immune system and antibiotics but also produce excessive capsule biosynthesis proteins, hemolysins, leukotoxins and lipase [32]. These enzymes target host cell membranes leading to host cell lysis and death cell. The higher mortality rate observed in *G. mellonella* larvae inoculated with *S. aureus* ATCC25925 cells recovered from dry biofilms compared with cells recovered from wet biofilms and planktonic cultures indicates that the expression of some virulent factors is enhanced under dry conditions. Virulence factors such as staphyloxanthin, have been shown to play a role in bolstering the *S. aureus* resistance to desiccation by facilitating membrane fluidity and enhancing antioxidant characteristics [33]. The increased virulence of cells recovered from the dry biofilms compared with cells recovered from wet biofilms and planktonic cultures may signify a risk in healthcare settings, particularly for immunocompromised patients.

In conclusion, dry biofilms on clinical surfaces can promote the persistence of pathogenic micro-organisms and can also aid their survival of disinfection procedures. Therefore, dry biofilms can act as reservoirs of infectious agents on hospital surfaces. In this study, we showed that infectious cells of *S. aureus* can be transferred from dry biofilms through touching with gloves and subsequently resulting in infection transmission. Thus, dry biofilm poses a significant risk in HAIs, therefore there is need to emphasize the removal of dry biomass on clinical surfaces in decontamination procedures.

Deploying sustainable and environmentally friendly decontamination agents, such as ozone, which leaves no toxic residues on surfaces, may be beneficial in eradicating dry biofilms on surfaces in the healthcare environment [34].

Conflict of interest statement
The authors have no conflicts of interest to declare.

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