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Major Article

Klebsiella pneumoniae survives on surfaces as a dry biofilm

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A B S T R A C T

Background: Dry surface biofilms (DSB) are widespread in healthcare settings presenting a challenge to cleaning and disinfection. *Klebsiella pneumoniae* has been a focus of attention due to antibiotic resistance and the emergence of hypervirulent strains. Few studies have demonstrated *K pneumoniae* survival on surfaces following desiccation.

Methods: DSB were formed over 12 days. Bacterial culturability and transfer were investigated following DSB incubation up to 4 weeks. Bacterial viability in DSB was investigated with live/dead staining using flow cytometry.

Results: *K pneumoniae* formed mature DSB. After 2 and 4 weeks of incubation, transfer from DSB was low (<55%) and reduced further (<21%) following wiping. Culturability at 2 and 4 weeks varied although viability remained high indicating viable but non culturable state (VBNC).

Discussion: *K pneumoniae* was removed from surfaces by mechanical wiping as shown with DSB of other species. Although culturability was reduced over time, bacteria remained viable up to 4 weeks incubation, proving the need for robust cleaning regimens.

Conclusions: This is the first study confirming *K pneumoniae* survival on dry surfaces as a DSB. The presence of VBNC bacteria indicated that *K pneumoniae* can for extended periods, raising questions about its persistence on surfaces.

Healthcare associated infections are responsible for a significant economic burden globally, costing NHS England, approximately £2.1 billion for the year 2016/17.[1] The rapid spread of multidrug resistant organisms (MDRO) continues to put pressure on healthcare environments. MDRO are associated with increased mortality and prolonged hospital stays leading to increased cost of care per patient.[2] *Klebsiella pneumoniae* is a common opportunistic bacterium found frequently in the environment, in soils, and surface waters,[3] and in humans, where it colonises the intestines and faeces. *K pneumoniae* is a pathogenic, nonmotile bacterium, associated with pneumonia, septicaemia, and surgical site infections and is the second most common gram-negative bacterium causing invasive infections.[4] It is often classed as an MDRO, due to its widespread carbapenem resistance.[5] The rise of carbapenemase producing *Klebsiella* species has resulted in more deaths and fewer treatment options, due to this increasing threat, hospitals worldwide are implementing stricter infection control measures to prevent further spread of the organism, but currently the effectiveness of each measure still remains unknown.[6]

It has been reported that *K pneumoniae* readily form biofilms on catheters and other medical devices.[7] Folliero et al[8] reported that 72.7% of *K pneumoniae* isolates detected on medical devices were biofilm producers although they remained susceptible to different classes of antibiotics. Biofilms are self-sufficient, complex communities of microorganisms embedded in a matrix of exopolymeric substances (EPS), adherent to both abiotic and biotic surfaces.[9] Recently, biofilms in a dry state have been found colonising dry surfaces such as keyboards, bed rails, curtains and ceilings[10,11] and the importance of DSB in healthcare has been reflected upon since 2012.[12] DSB differ from the more traditional "wet" biofilms, as they have been exposed to lower nutrient resources, reduced water potential and periodic disinfection within hospital environments.[13,14] The presence of DSB with a thicker EPS was first confirmed by Hu et al,[14] who associated this thickness with the ability of DSB to tolerate standard cleaning and

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disinfection treatments. Vickery et al[10] confirmed reservoirs of pathogenic MDRO in hospitals residing in a dry biofilm state. Since then, DSB containing MDRO have now been reported in healthcare settings across the world.[11,15] One of the main concerns is that DSB cannot be detected through routine wet swabbing of dry environmental surfaces and subsequent efforts to culture swabs.

It is known that the longer microbes can survive in the environment, the greater the risk of infection to the patient.[16] Microorganisms in a dry state can survive on surfaces for extended periods compared to those residing in a planktonic form, increasing the chance of patient infection.[17] To date, the literature available supporting the survival of *K pneumoniae* in a dry state is limited and contradictory. Hirai [18]reported the absence of detectable *K pneumoniae* following desiccation on a range of surface materials. However, Kramer et al[19] reviewed *Klebsiella* spp. survival from 2 hours to 30 months on inanimate surfaces. Our study is the first to describe the ability of *K pneumoniae* to survive desiccation as DSB, to persist on, and be transferred from surfaces after weeks of incubation in conditions found in healthcare settings.

MATERIAL AND METHODS

Bacterial growth

K pneumoniae ATCC 13883 was suspended in tryptone soya broth (TSB) and grown overnight at 37 ^{0}C at 120 rpm. The bacterial suspension was centrifuged at 5,000 G and washed with sterile TSB. The bacterial suspension was adjusted to 1×10^6 CFU/mL.

Dry surface biofilm preparation

The DSB formation model follows that described by Ledwoch et al[20] which utilizes alternate hydration and desiccation phases. DSB were grown on sterile stainless-steel discs AISI 430 (0.7 ± 0.07 mm thickness; 10 ± 0.5 mm diameter) placed in a Corning Costar flat bottom 24 well cell culture plate. At the initial hydration phase, 1 mL inoculum (10^6 CFU/mL) with 3 g/L of bovine serum albumin (BSA) was dispensed into wells. Well plates were incubated at room temperature (20-23 ^{0}C) for 48 hour, with continuous shaking at 200 rpm. Media was then drained out of each well and incubated for 48 hour at 37 ^{0}C. This alternating hydration and desiccation sequences were repeated until a mature DSB was formed after 12 days. Each hydration phase included the addition of 1 mL of TSB and BSA at 3 g/L.

Culturability of bacteria embedded in DSB

Bacterial culturability was investigated with 12-day DSB (DSB_0). Discs were removed from well plates and incubated in TSB for 2 hours at 37 ^{0}C to allow bacteria to become metabolically active without bacterial population growth, and then vortexed for 2 min with 1 g of glass beads, serially diluted and spread plated on tryptone soya agar to be enumerated. Other DSB_0 were incubated for a further 2 weeks (DSB_2) and 4 weeks (DSB_4) in 55% ± 5% relative humidity at room temperature (21 ^{0}C). Following that incubation period, discs were processed as described above.

Viability of bacteria embedded in the DSB

Bacterial viability was investigated by live/dead staining using a BD LSR Fortessa flow cytometer. DSB were vortexed for 2 minutes in phosphate buffer saline (PBS) with 1 g of glass beads to ensure that all the biofilm was removed from the disc surface. The resulting suspension was diluted to approx. 10^7 cells and stained using the LIVE/DEAD BacLight bacterial viability kit (Invitrogen, Thermo Fisher Scientific) with syto 9 and propidium iodide in a 1:1 ratio. Live (washed bacterial suspension) and dead (washed bacterial suspension exposed to 85 ^{0}C for 10 min) planktonic cultures were used as a control. The flow cytometer was initially adjusted using a population of unstained cells. The FITC-A and Pe-Texas Red-A channels were used to detect live and dead bacteria. For each sample, 10,000 events were recorded to yield mean values for green and red fluorescence. Data obtained by flow cytometry were analyzed using FlowJo flow cytometry analysis software version 10·8·1.

Transferability of dry surface biofilm

Dry Transfer: Discs containing DSB_0, DSB_2, or DSB_4 were pressed 36 times on Dey-Engley Neutralising agar at a pressure of 100 g. Following transfer, each plate was incubated at 37 ^{0}C overnight. Positive growth was recorded, and transferability (% transfer) was calculated as the number of (positive contacts/ total adpressions) x 100.

Wet Transfer: Discs containing DSB_0, DSB_2, or DSB_4 were wiped with a detergent wipe (TRICLEAN) or a sterile water containing wipe (Rubbermaid microfibre cloth). Each disc was wiped for 10 seconds using the Wiperator (Filtaflex Ltd) with a weight of 350 g, left to air dry for 30 seconds before being pressed 36 times onto Dey-Engley Neutralising agar at 100 g pressure. Plates were then incubated and enumerated as described above.

Scanning Electron Microscopy (SEM) imaging

DSB samples were prepared by overnight incubation of each disc in 2·5 % glutaraldehyde solution, followed by a series of ethanol washes with increasing concentration; 5, 25, 50, 75, 90, and 100 % for 10 minutes each.[21] Prior to imaging, discs with DSB were coated with a thin layer of gold-palladium (20 nm) using a SC500 Bio-rad sputter coater. Images were acquired with a beam energy of 5 kV using an in-lens detector on a Philips XL30 field emission gun-scanning electron microscope at ×5,000, ×1,0000 and ×20,000 magnification and a 5-7 mm working distance.

Virulence of K. pneumoniae

A virulence assay using *Galleria mellonella* larvae (TruLarv, Biosystems) assessed pathogenicity of *K pneumoniae* in DSB compared to planktonic state.[21] Only DSB_0 was investigated, as culturability at DSB_2 and DSB_4 was variable. Planktonic cultures of *K pneumoniae* were prepared overnight in TSB and centrifuged at 5000 g for 10 min at 21 ^{0}C. Cultures were resuspended in tryptone sodium chloride (TSC) and adjusted to 10^8 CFU/mL at OD_{625nm}. For injection into *G mellonella* larvae, planktonic suspensions were diluted to 10^6 CFU/mL corresponding to CFU/mL recovered from DSB_0. DSB_0 suspension was prepared by vortexing disc containing DSB in TSC with 1 g of glass beads for 4 min. All suspensions were serially diluted and plated to ensure the or same concentration was being used.

G mellonella weighing between 0.18 − 0.35 g were kept and stored in the fridge at 4 °C for a maximum of 2 weeks from delivery. Prior to injection, groups of 10 larvae, were placed in a 9 cm petri dish and left to acclimatise at room temperature (21 °C). Three repeats were performed for each test condition (ie, planktonic control) to give a total of 30 larvae sample size. Larvae were injected into the hemocoel via the last left proleg using a 50 μL Hamilton syringe with 22-guage needle. Needles were replaced after 5 injections and were decontaminated with 70 % (v/v) ethanol before each injection. Controls of 100 % DMSO (dead) and TSC (alive) were used to ensure injection accuracy. An untreated control was also used to ensure death was not due to larval health. After injection with 10 μL of either planktonic or DSB_0 suspension containing 10^6 CFU/mL, which corresponded to an injection of 10^4 CFU/mL. Larvae were then placed in an incubator at 37 °C and survival was monitored every 24 hours for a

total of 5 days. A total of 10 larvae were used for each condition, which was performed in triplicate. Larvae were determined as "dead" if they did not respond, through movement, to the light touch of a sterile pipette tip.

Statistical analysis

Where appropriate, statistical testing was conducted using GraphPad PRISM 9 (version 9.3.1) using one−way ANOVA. All measurements comprised a minimum of 3 biological replicates.

RESULTS

Culturability of K pneumoniae *from DSB*

Bacterial culturability varied between the different incubation periods (Fig 1). An average 5·15 ± 0.60 $\log_{10}$ CFU/mL were recovered from DSB_0. After 4 weeks of incubation at 21 °C and 55% ± 5% RH, 4.01 ± 1.64 $\log_{10}$ CFU/mL were recovered, but this number had dropped to 1.58 ± 0.66 $\log_{10}$ CFU/mL after just 2 weeks of incubation. There was a statistically significant difference between Log_{10} CFU/mL recovered from all 3 ages of DSB (One-way ANOVA, P <.05) (Fig 1). Results from DSB_2 and DSB_4 varied between batches of biofilms and not just between biological repeats. Results included some repeats where there were no viable bacteria recovered on agar media, contributing to variability of data. No statistically significant difference was identified between DSB_2 and DSB_4 following post-hoc Tukey test (P >.05).

Viability of bacteria in DSB

Viability of *K pneumoniae* in DSB was measured with live/dead staining using flow cytometry. Manual gating was performed to quantify the presence of live/dead cells (Fig 2) and % fluorescence was calculated from mean fluorescence values (Fig 3). Both viable and dead cells were identified in DSB_0, DSB_2, and DSB_4. The overall percentage of viable cells was much higher than percentage of dead cells in all DSB samples (Fig 4). DSB_0 had the overall lowest % of viable cells (74.5%), compared to DSB_2 (85.6%) and DSB_4 (88.1%). The presence of viable cells in both 2- and 4-week-old DSB is indicative of viable but non culturable (VBNC) state as culturability was low with these DSB (Fig 1).

Transferability of DSB

The highest percentage transfer was recorded following dry transfer compared to wet transfer by wiping with detergent or water (Fig 5). DSB_0 resulted in the greatest percentage transfer and DSB_4 the lowest, with no transfer recorded when DSB_4 wiped with water or detergent (Fig 5). There was a statistically significant difference between all dry, water and detergent transfer for both DSB_0 and DSB_4. respectively (one-way ANOVA, P < .05). Although transfer was recorded from DSB_4, there was a high level of variability between biological replicates (Fig 5), similar to observations made with culturability experiments (Fig 1). Overall, no significant difference was found between dry or wet transfer from DSB_2 (One-way ANOVA, P >.05).

Scanning electron microscope analysis

DSB of *K pneumoniae* were homogenous and uniform within the same biofilm age (based on multiple pictures taken of 3 replicates of each biofilm, data not shown). However, there were marked differences in the overall structure of the biofilm matrix between DSB_0, DSB_2, and DSB_4 (Fig 6). An uneven covering of bacteria over the disc surface

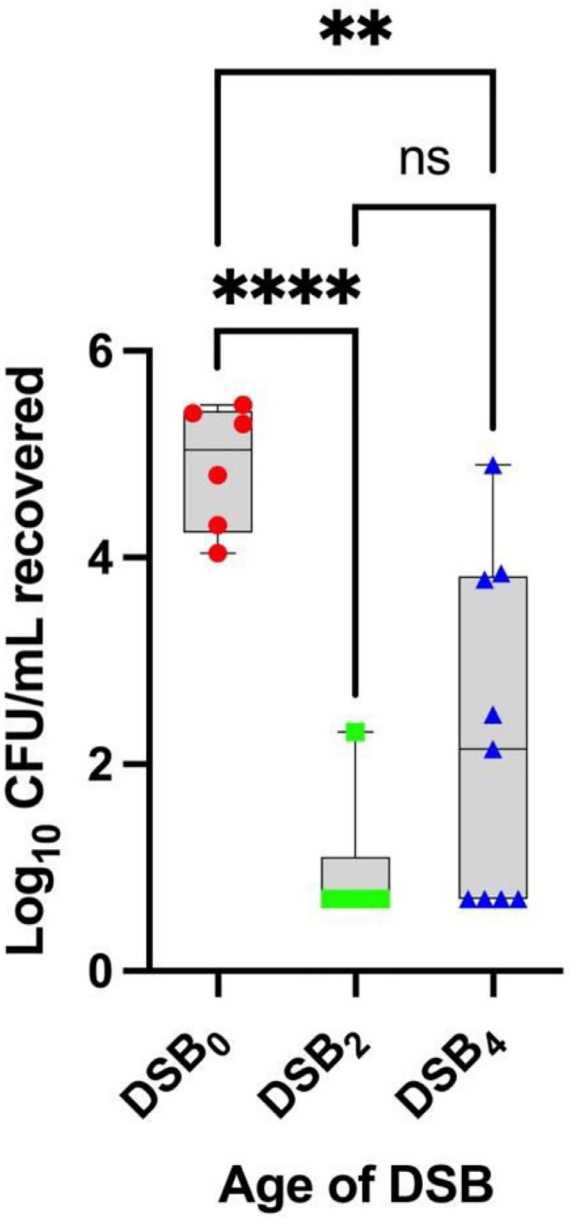

Fig 1. Culturability of *K pneumoniae* in DSB. Culturability was measured after DSB formation (DSB_0), and with 2- (DSB_2) and 4- (DSB_4) week-old DSB. Data are based on ≥6 biological replicates. Culturability of DSB_0 was more consistent than with DSB_2 and DSB_4. There was a statistically significant difference between all DSB ages (one-way ANOVA, P < .05). There was no statistical significance was between 2- and 4-week DSB (ANOVA, Tukey, P > .05). **colour to be used for this graph**

was present at DSB_0, where there was evidently a higher concentration of cells that at DSB_2 (Fig 6 A and B). Bacteria embedded in an organic load matrix (EPS) were observed (Fig 6C, D). DSB_4 showed spatial separation of bacterial cells within the biofilm, with little EPS identifiable (Fig 6E and F). The number of observable bacteria is also lower than DSB_0 and DSB_2 (Fig 6A-D).

Effect of K pneumoniae *DSB on virulence*

Survival rate remained at 100% after 5 days when *G mellonella* larvae were inject with TSC or left untreated (Fig 7A). All larvae had died within 48 hours of injection with 100% DMSO, the positive death control, with only 10% survival rate after 24 hours (Fig 7A). DSB_0 counts corresponded to 10^6 CFU/mL, and so this concentration was

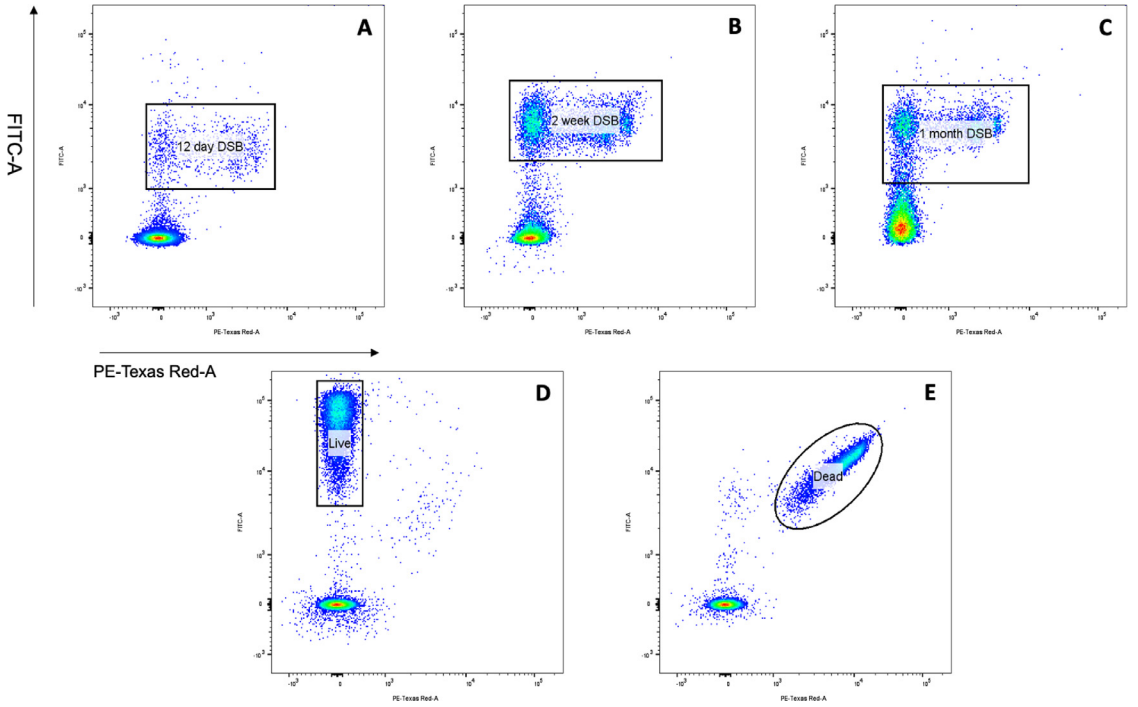

Fig 2. Dotplots created after flow cytometry of DSB_0, 2-week (DSB_2) and 4- (DSB_4) week-old *K pneumoniae* DSB to show the amount of live/dead cells within a sample. Areas gated by black rectangles/squares show the population that was analyzed for live/dead cells in the sample. This gated area eliminates any background noise within the sample. FITC-A is presented on the y axis, and PE-Texas red-A is presented on the x axis. (A) DSB_0, (B) DSB_2, (C) DSB_4, (D) planktonic live control, (E) planktonic dead control. (n = 3) **colour to be used for this graph**

also used for planktonic suspension injections. After 3 days, larvae survival remained at 100% when injected with either DSB_0 or planktonic suspension (Fig 7B). There was no significant difference (two-way ANOVA, $P > .05$) between overall larval survival throughout 5 days of DSB_0 compared to the control planktonic suspension (Fig 7B). Survival rates were reduced to 97% after 5 days for both DSB_0 and planktonic suspension, which indicated that this particular species of *K pneumoniae* is not virulent. Over the 5-day period, larval survival did not differ between DSB_0 and planktonic suspension (Fig 7B).

DISCUSSION

A large proportion of hospital-acquired pneumonia and ventilator-acquired pneumonia is associated with *K pneumoniae*, typically affecting vulnerable patients in intensive care units.[22] Acquisition of this bacteria has been shown from several sources of transmission within hospitals, including direct person-to-person contact and from contaminated surfaces and instrumentation.[23] DSB are prevalent within healthcare environments and reside on most surfaces.[10-13]

Here, we report the ability of *K pneumoniae* to form a DSB and survive in a desiccated state over a 4-week period. Examining pathogen transmission from surfaces, the question was whether bacteria in DSB were transferable or not. To evaluate transmission, we investigated both “dry” and “wet” transfer following wiping with water or a detergent commercial product. Dry transfer test resulted in the highest percentage of transferred *K pneumoniae* from DSB, since the mechanical action of wiping with wet transfer tests likely resulted in removing some bacteria from the disc surface[24] in agreement with other studies where wiping contributed to releasing more microorganisms.[25,26]

K pneumoniae has been identified as one of, approximately, 85 known species, mainly Gram-negatives, to enter a VBNC state supporting long term survival under stressful conditions.[27] One of the main limitations around analysis of cells in a VBNC state, is the lack of methods to quantify the live/dead presence within a sample. Here, staining was employed as it was considered most appropriate for biofilm analysis. The dormant state of bacterial cells has been proven to last several months in Gram-negative species, and some species have been proven to remain infectious following resuscitation from dormancy.[28] Our results show that *K pneumoniae* remains in a VBNC state following 2 weeks in defined dry conditions, up to 4 weeks. At this point, data showed low culturability and bacteria in a VBNC state on surfaces will not be detected by swabbing which can be an issue in healthcare settings.[29] Although both remained low, we observed a greater culturability of *K pneumoniae* after 4 weeks (DSB_4) compared to 2 weeks (DSB_2), an interesting observation which requires future work on the VBNC state of *K pneumoniae* in DSB.

K pneumoniae has received attention globally from its resistance to numerous antibiotics, and a requirement for models to test virulence has become increasingly popular.[30] Here we presented novel work on the lack of effect of DSB virulence of *K. pneumoniae*.

Single species DSB models, with *K pneumoniae* (this study), *Staphylococcus aureus*[14,21] or *Candida auris*[31] provide robust, reproducible platforms for testing culturability and viability over time and following cleaning or/and disinfection. However, these laboratory models do not represent DSB *in situ* where more complex multispecies DSB have been identified.[12,13,15] In addition, environmental DSB isolated from healthcare settings seemed to be associated with more exopolymeric substance[10] than our single species DSB, even after 12 days formation (DSB_0) (Fig 6). We reported some variability in culturability results, not just between repeats, but within batches that used the same original inoculum. A similar observation was made by Ledwoch et al,[20] whereby the *S aureus* DSB studied had a high standard deviation following treatment with sodium hypochlorite. Data variability when studying biofilms is not uncommon and has largely been attributed to the overall biofilm architecture and development.[32]

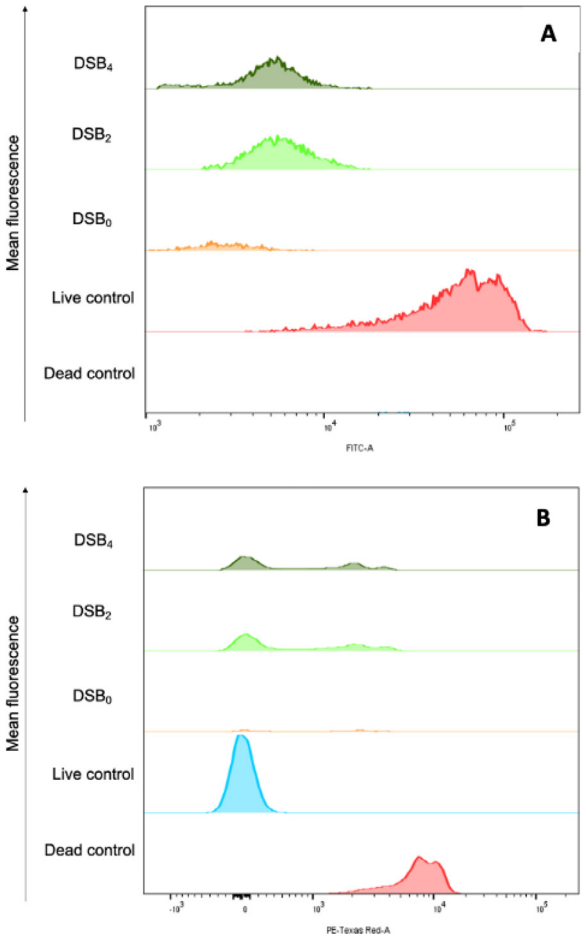

Fig 3. Histograms of mean fluorescence peaks of FITC-A and PE Texas Red-A channels following live/dead staining. Histograms of FITC-A channel (A) show the live staining of cells within all samples, histograms of PE Texas red-A (B) show dead staining of cells within all samples. There is a reduced mean fluorescence of all DSB samples when observing the PE-Texas red A channel. The shift of histograms to the right indicates a decreased viability as shown by the dead control sample. **colour to be used for this graph**

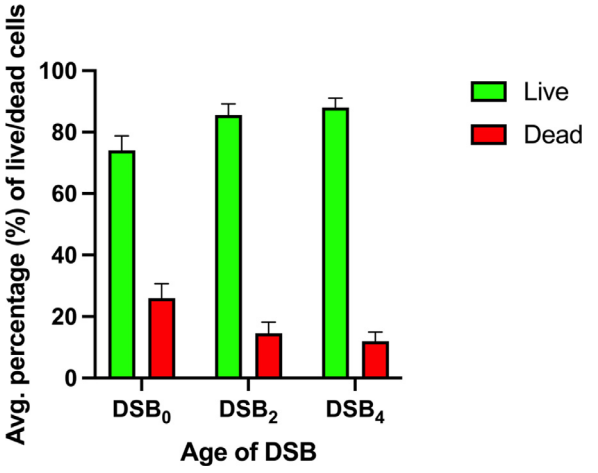

Fig 4. Percentage of live and dead cells within DSB_0, DSB_2, and DSB_4 of *K. pneumoniae*. **colour to be used for this graph**

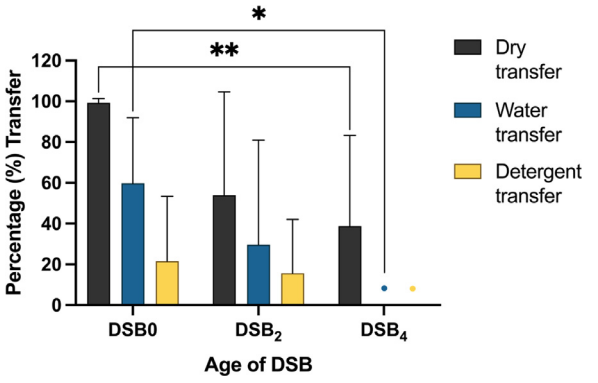

Fig 5. Percentage transfer of bacteria from DSB_0, 2- (DSB_2) and 4- (DSB_4) week-old DSB following dry transfer and wet transfer with detergent or water wipes. Dots (•) DSB_4 indicate no transfer of bacteria following wiping with either water or detergent wipes. There was a statistically significant difference between dry and water wipe transfer of DSB_0 and DSB_2 (ANOVA, Tukey, $P < .05$). ($n > 3$) **colour to be used for this graph**

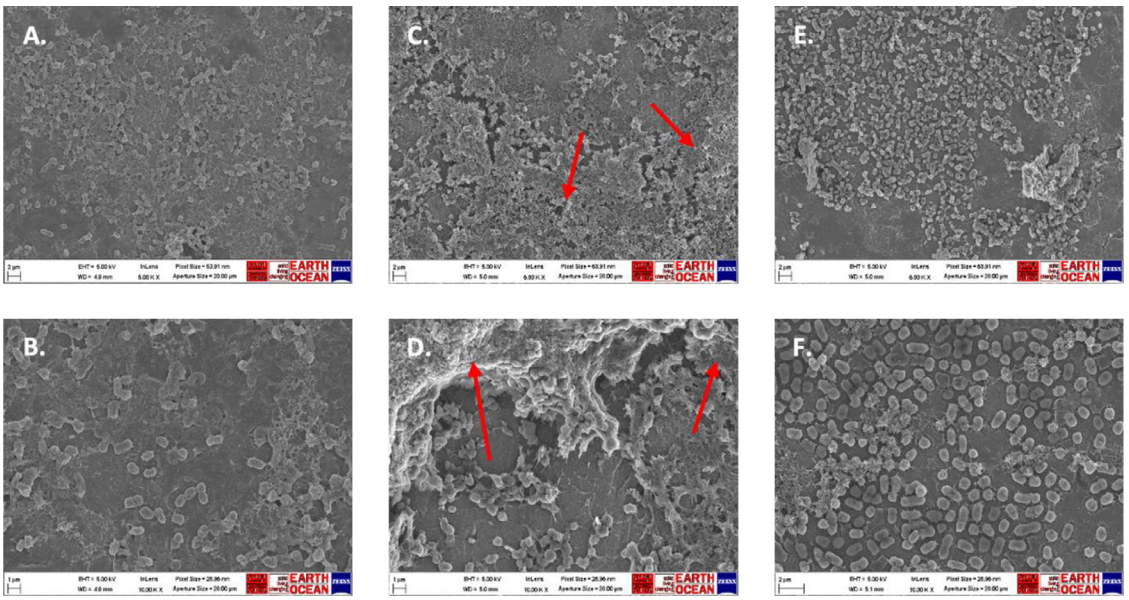

Fig 6. Scanning electron microscope images of *K pneumoniae* dry surface biofilm, ×5,000 and ×10,000 magnifications. Images presented are representative of the whole disc surface. DSB_0 ×5,000 (A) and ×10,000 (B), DSB_2 ×5,000 (C), and ×10,000 (D), DSB_4 ×5,000 (E) and ×10,000 (F). Arrows indicate the presence of cells well embedded into matrix of DSB. **colour to be used for this graph**

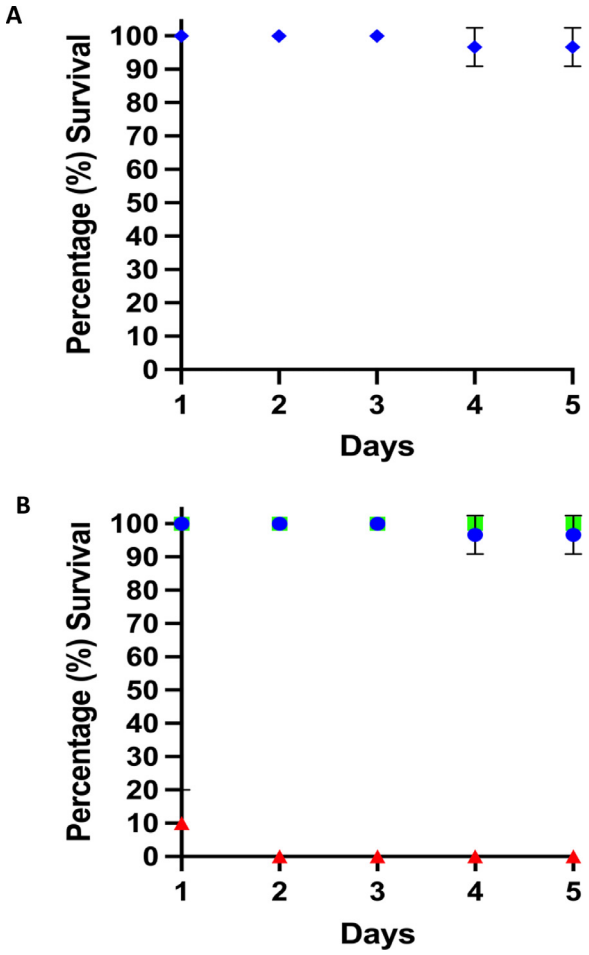

Fig 7. Total percentage survival of *G mellonella* larvae when injected with *K pneumoniae* DSB_0 and relevant controls (planktonic suspension, TSC and DMSO). (A) % larval survival after injection with DSB_0 (avg. 10^6 CFU/mL). (B) % larval survival when injected with control groups—TSC (tryptone sodium chloride) (green), 100% DMSO (red) and planktonic suspension (10^6 CFU/mL) (blue). There was no statistically significant difference between larval survival after injection with DSB_0 or planktonic suspension (two-way ANOVA, $P > .05$). Each condition was tested by injecting 30 larvae. **colour to be used for this graph**

Here we show that *K pneumoniae* remains viable in a desiccated state (55% RH, 21 °C) as DSB on surfaces for at least 4 weeks. We have shown that *K pneumoniae* in DSB can be transferred to another surface directly or following wiping after a month. In addition, we identified *K pneumoniae* persistence in a VBNC state. Altogether these data indicate the ability of *K pneumoniae* in DSB to persist in, and be transferred from, the environment and emphasize the need for robust and increase compliance in infection prevention and control regimens.

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