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Intensive care unit environmental surfaces are contaminated by multidrug-resistant bacteria in biofilms: combined results of conventional culture, pyrosequencing, scanning electron microscopy, and confocal laser microscopy

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SUMMARY

Background: Hospital-associated infections cause considerable morbidity and mortality, and are expensive to treat. Organisms causing these infections can be sourced from the inanimate environment around a patient. Could the difficulty in eradicating these organisms from the environment be because they reside in dry surface biofilms?
Aim: The intensive care unit (ICU) of a tertiary referral hospital was decommissioned and the opportunity to destructively sample clinical surfaces was taken in order to investigate whether multidrug-resistant organisms (MDROs) had survived the decommissioning process and whether they were present in biofilms.
Methods: The ICU had two 'terminal cleans' with 500 ppm free chlorine solution; items from bedding, surrounds, and furnishings were then sampled with cutting implements. Sections were sonicated in tryptone soya broth and inoculated on to chromogenic plates to demonstrate MDROs, which were confirmed with the Vitek2 system. Genomic DNA was extracted directly from ICU samples, and subjected to polymerase chain reaction (PCR) for *femA* to detect *Staphylococcus aureus* and the microbiome by bacterial tag-encoded FLX amplicon pyrosequencing. Confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM) were performed on environmental samples.

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Findings: Multidrug-resistant bacteria were cultured from 52% (23/44) of samples cultured. *S. aureus* PCR was positive in 50%. Biofilm was demonstrated in 93% (41/44) of samples by CLSM and/or SEM. Pyrosequencing demonstrated that the biofilms were polymicrobial and contained species that had multidrug-resistant strains.
Conclusion: Dry surface biofilms containing MDROs are found on ICU surfaces despite terminal cleaning with chlorine solution. How these arise and how they might be removed requires further study.

Introduction

Hospital-acquired infections (HAIs) are a major problem. A recent study estimated that 648,000 patients have 721,800 HAIs annually in acute care hospitals in the USA.[1] This has been estimated to cost US hospitals US$28–34 billion annually.[2] 'ESKAPE' organisms (*Enterococcus* spp., *Staphylococcus aureus*, *Klebsiella* spp., *Acinetobacter* spp., *Pseudomonas aeruginosa*, and Enterobacteriaceae) continue to dominate, and *Clostridium difficile* is now the micro-organism most frequently causing HAIs.[1]

The cost-effectiveness of infection prevention and control programmes has been demonstrated, with hand hygiene being the most critical activity for controlling infection transmission.[3,4] However, sustained improvements in compliance rates are difficult to maintain, and infection control programmes targeting only hand hygiene are not necessarily associated with declining HAI rates.[5,6] By contrast, multiple strategies or bundles including active surveillance, patient isolation/cohort and improved hand hygiene have been shown to be successful in reducing meticillin-resistant *S. aureus* (MRSA) rates, even in hyperendemic regions.[7,8]

The Healthcare Infection Control Practices Advisory Committee (HICPAC) recommends a strategy to control multidrug-resistant organisms (MDROs) that consists of seven elements: administrative support, education, judicious use of antibiotics, MDRO surveillance, infection control precautions, environmental measures, and, where possible, decolonization.[9] An integrated approach to infection prevention should address environmental contamination. HAIs increase length of hospital stay, during which time patients contaminate their surrounding inanimate environment.[10,11] The risk of a patient developing an HAI increased by 73% if the patient previously occupying the room had a vancomycin-resistant enterococcus (VRE), MRSA, *C. difficile* or *Acinetobacter baumannii* infection.[12] Investigations focusing on the recovery of planktonic organisms from patient records and computer keyboards has helped to emphasize the importance of 'hand touch surfaces'.[11,13–15] Enhanced cleaning decreases, but does not eliminate, MRSA and other MDRO environmental isolation rates.[11] However, decreased environmental contamination rates have been associated with decreased MRSA acquisition rates.[16]

We recently showed the presence of dry surface biofilms containing viable MDROs on five out of six furnishings from an ICU, including a sterile supply box, privacy curtain, venetian blind cord, see-through ward entrance door, and rubber from around a sink.[17] As bacteria within biofilm are many more times resistant to desiccation, removal by detergents, and inactivation by disinfectants, we suggested that the presence of biofilms may contribute to the maintenance of environmental contamination in the face of cleaning.[17–20]

In this study we investigated the prevalence of biofilms in the environment immediately surrounding the patient and the frequency with which *S. aureus* was incorporated into these biofilms. In addition 15 samples were subjected to next-generation sequencing to determine the mix and ratio of microbial species present in biofilms contaminating dry surfaces.

Methods

Sample collection

Samples were obtained from an intensive care unit in a fully air-conditioned hospital and stored in a fully air-conditioned laboratory (temperature range 22–25°C, humidity 57–72%). Following a two-step terminal cleaning protocol using neutral detergent followed by disinfection with 500 ppm chlorine (sodium dichloroisocyanurate dehydrate, Diversol5000, Johnson Diversey, Smithfield, NSW, Australia), items from the patient bedding ($N = 11$), patient surrounds ($N = 19$), and fixed furnishings ($N = 14$) were aseptically sampled by cutting out a segment of the furnishing using sterile gloves, forceps, pliers, scissors, or scalpel blades, depending on the material being sampled. Samples were stored in sterile containers and gloves and instruments were changed between each sample.

Aerobic culture

Sample sections, up to 2 cm^2, were sonicated in 4 mL of tryptone soya broth for 5 min, prior to 100 μL being spread over horse blood agar plates (HBA) as a general non-selective medium, Brilliance MRSA agar plates for the detection of MRSA, Brilliance VRE Agar Plates for the detection of VRE, and Brilliance ESBL agar plates for the detection of extended-spectrum beta-lactamase (ESBL)-producing Gram-negative bacteria (Oxoid Adelaide, Australia).[17] MRSA plates were incubated for 18–24 h, and VRE, ESBL and HBA plates up to 48 h, aerobically at 37°C. Positive MDROs were confirmed using a combination of Vitek2 GPS-IX or Vitek2 AST-N149 cards (for Gram-positive or -negative isolates respectively) (bioMérieux-Vitek, Hazelwood, MO, USA) and partial sequencing of the 16S rRNA universal eubacterial gene according to the method described by Kidd *et al.*[21]

Staphylococcus aureus-specific PCR

Samples were sonicated in 300 μL digestion buffer (50 nM Tris/HCl pH 7.5, 150 nM NaCl, 2 mM ethylenediamine tetra-

acetic acid, 1% sodium dodecyl sulphate) in an ultrasonic bath (Soniclean, JMR, Sydney, NSW, Australia) for 15 min with a sweeping frequency of 42–47 kHz at 20°C. Lysozyme (Sigma, Sydney, NSW, Australia) was added to a final concentration 0.5 mg/mL and incubated at 50°C for 2 h. Proteinase K (Sigma) at a final concentration of 1 mg/mL was added, followed by a 2 h incubation at 56°C. Genomic DNA was extracted using phenol/chloroform and then ethanol-precipitated.

Sample DNA was subjected to *S. aureus*-specific real-time PCR targeting the *femA* gene using the following cycling conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 56°C for 30 s, and 72°C for 60 s.[22]

Microbiome of biofilm contaminating dry hospital surfaces

Fifteen samples were subjected to bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) of the V1–V3 regions of 16s rRNA gene to determine the bacterial community of dry surface hospital biofilm.

Pyrosequencing was performed using the Titanium platform (Roche, Basel, Switzerland) in a commercial facility (Molecular Research DNA Lab, Shallowater, TX, USA), as previously described.[23] Pyrosequencing data were analysed by QIIME software (Werner Lab, Cortland, NY, USA).[24] Operational taxonomic units were assigned against the RDP database (Ribosomal Database Project II).[25]

Scanning electron microscopy (SEM)

Following fixing in 3% glutaraldehyde, samples (up to 1 cm^2) were dehydrated in ethanol, prior to immersion in hexamethyldisilazane (HMDS, Polysciences, Inc., Warrington, PA, USA) for 3 min and sputter-coating with 20 nm gold film as previously described.[17] Samples shown to have bacteria attached to a surface and surrounded by extracellular polymeric substances (EPSs) were classified as biofilm positive.

Confocal laser scanning microscopy

Eighteen of the samples, which had been stored for 12 months in sterile containers at room temperature, were stained with a Live/Dead® BacLight™ Bacterial Viability Kit (Life Technologies), using the manufacturer's instructions. SYTO® 9 labels live bacteria with green fluorescence while the propidium iodide component labels membrane-compromised bacteria with red fluorescence. Stained samples were examined using an Olympus Fluoview 300 inverted confocal laser scanning microscopy system.

Statistical analysis

Student's *t*-test was used to compare the number of bacterial species in dry biofilms on patient bedding or patient surrounds with number of species in biofilms on the floor using SigmaPlot11 statistical program. The statistical analysis of the bTEFAP data was performed by QIIME scripts, Calypso software (http://bioinfo.qimr.edu.au/), and FigTree software (http://tree.bio.ed.ac.uk/software/figtree/).[24] Alpha diversity was calculated using the Shannon index and OUT Richness in QIIME. The default number of Monte Carlo permutations was used to calculate the *P*-values and the significance threshold was $P < 0.05$. Phylogenetic analysis was calculated by FigTree with the default setting.

Ethics and safety approvals

Ethics approvals were obtained from South Western Sydney Local Health District Research and Ethics Office (Reference: LNR/14/LPOOL/14) and the University of Western Sydney Human Research Ethics Committee (Reference: H10659). Safety approval was obtained from the University of Western Sydney Biosafety and Radiation Safety Committee (Reference B10072).

Results

Aerobic culture

Twenty-three of the 44 (52%) samples were cultured on HBA. MRSA-, VRE-, and ESBL-positive organisms were detected in eight, three, and five samples, respectively (Table I). At least one MDRO grew in 12 of the 23 (52%) culture-positive samples. Most of these MDROs were in the immediate patient vicinity, with 33% of mattresses and privacy curtains being positive for MRSA. One-third of mattresses were also positive for VRE and one mattress was positive for MRSA, VRE, and ESBL. The ESBL plates in general grew *Sphingomonas paucimobilis*. MDROs were less prevalent on fixed furnishings ($N = 14$) with isolation of MRSA from one floor sample and *S. paucimobilis* present in the wall biofilm.

Detection of S. aureus

Staphylococcus aureus was detected by *S. aureus*-specific PCR in 50% of the samples, including the eight out of 11 (72%) samples from patient bedding, eight of 19 (42%) samples from the patient's immediate environment, and six of 14 (42%) samples from fixed furnishings.

Visual confirmation of biofilm contamination

Forty-one out of 44 samples (93%) were visually confirmed to have biofilm infecting their surfaces either by SEM and/or by CLSM (Table I and Figure 1). SEM of biofilm sourced from areas not routinely cleaned and disinfected such as curtain cords and entrance doors showed bacteria of various morphologies embedded in thick amorous EPS (Figure 1A and B). Bacilliary, filamentous, and coccoid forms were evident on the mattress (Figure 1C) whereas cocci were more prevalent on hand touch items (Figure 1A, B, and D).

All 18 samples stained with bacterial viability stain showed live bacteria despite 12 months of storage at room temperature, demonstrating the stability of biofilm on dry surfaces (see Table I and Figure 2).

Microbiome of biofilm contaminating dry hospital surfaces

Dry surface biofilms were all polymicrobial by pyrosequencing and culture. The average number of species representing at least 1% of the biofilm was 29 (range: 11–42) for patient bedding and 23 (range: 10–32) for patient surrounds. Significantly fewer species were present in biofilms on the floor

Table I
Prevalence of biofilm on intensive care unit dry surfaces

Item	*N*	Biofilm	Live at 12 months (*N* = 18)	PCR positive: *S. aureus*	Culture positive:			
					Non-selective media	MRSA	VRE	ESBL
Patient bedding								
Mattress	6	6	5	4	5	2	2	1
Pillow	5	5	3	4	3	1	0	1
Patient surrounds								
Curtain	9	8	4	5	5	3	0	1
Patient notes wire clip	2	2		0	0	—	—	—
Supply box	4	4	2	1	3	0	0	0
Glove box Velcro	1	1	1	1	1	0	1	1
Notice	3	3		1	2	1	0	0
Fixed furnishings								
Floor	3	3		1	3	1	0	0
Basin rubber	4	3		1	0	—	—	—
Bench top	2	1	1	2	0	—	—	—
Wall	1	1		0	1	0	0	1
Ward entry door	4	4	2	2	0	—	—	—
Total	44	41	18/18	22	23	8	3	5

N, the number of items collected; 'Biofilm', the number of samples with visual confirmation of biofilm presence by microscopy; 'Live at 12 months', the confirmation of live bacteria following 12 months of storage; PCR, polymerase chain reaction; MRSA, meticillin-resistant *Staphylococcus aureus*; VRE, vancomycin-resistant enterococci; ESBL, extended-spectrum beta-lactamase Gram-negative bacilli.

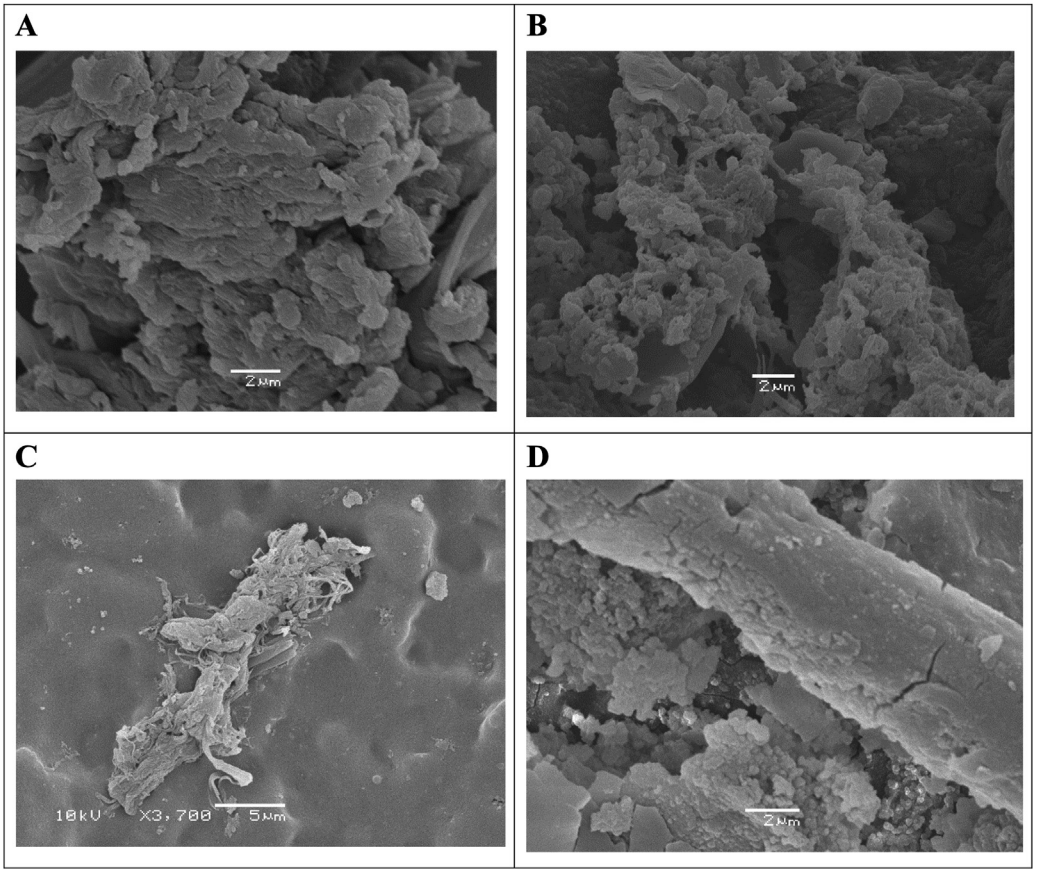

Figure 1. Scanning electron micrograph of biofilms contaminating surfaces in an intensive care unit. (A) Sample from a privacy curtain. (B) Sample from the ward entry door showing coccoid bacteria embedded in a thick amorphous extracellular polymeric substance (EPS). (C) Sample from a mattress showing biofilm containing bacteria of various morphologies including rod-shaped organisms, contaminating especially the natural depressions in the mattress. (D) Sample from a wire clip for holding patients' notes showing dense EPS with embedded coccoid bacteria.

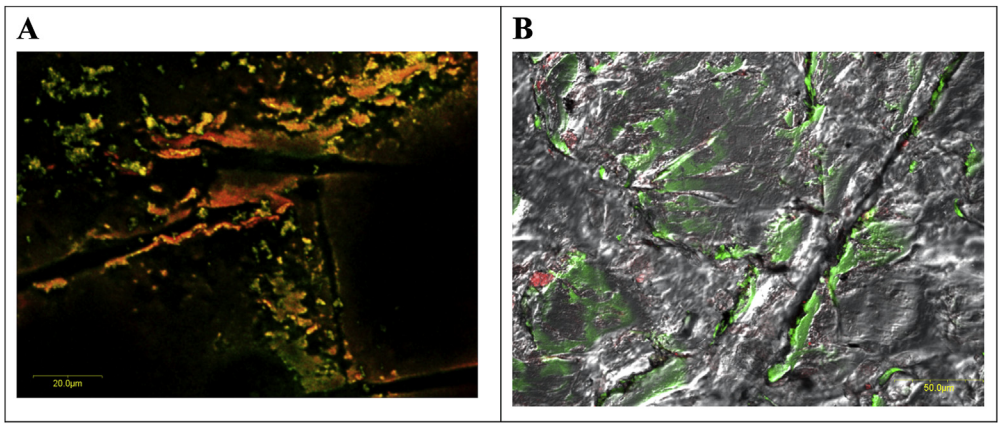

Figure 2. Confocal laser scanning micrograph of biofilm stained with BacLight Live/Dead stain showing the presence of live bacteria (green) and dead bacteria (red) on dry hospital surfaces. Staining conducted after 12 months of storage at room temperature. (A) Sample from a storage box used to hold sterile supplies of single-use patient equipment; this surface cultured *Staphylococcus aureus*. (B) Ward entry door confocal image of live/dead bacteria superimposed on a visual of ward entry door, showing relationship of bacteria to door topography (culture negative but positive for *S. aureus* by polymerase chain reaction).

(average: 8; range: 3–14) ($P = 0.02$). In all biofilms some bacterial species were more prevalent, representing 10% or more of the biofilm mass. The organisms detected with the highest frequencies are detailed in Supplementary Table I (online).

However, the most common bacterial species, on a percentage of biofilm sequence reads, across all the biofilms were *Faecalibacterium prausnitzii*, *Massilia timonae*, *S. aureus*, coagulase-negative staphylococci, *Pseudomonas* species and *Propionibacterium acnes* (Figure 3A). *Pseudomonas* species were found in 14 biofilms but only two contained *P. aeruginosa*: one floor sample and the sterile supply box. The most frequently occurring *Pseudomonas* species were *P. mendocina* and *P. stutzeri* both of which have caused rare opportunistic infections. *P. acnes* was also found in 14 biofilms, coagulase-negative staphylococci in 13, *F. prausnitzii* in 10, *S. aureus* in 11, and *M. timonae* in seven of the 15 biofilms. Other bacterial species were frequently found in the biofilms but only formed a small percentage of the biofilm. For example, *Acinetobacter* species including *A. lwoffii*, *A. calcoaceticus*, *A. haemolyticus*, *A. guillouiae*, *A. estunensis*, and *A. junii*, were found in nine biofilms, but in only three samples did they form more than 1% of the biofilm population. *Acinetobacter calcoaceticus* formed 4% of the population on the poster and *A. guillouiae*, *A. junii*, and *A. lwoffii* made up 8% of the biofilm population on the glove box Velcro®.

Most of the biofilms contained a mixture of organisms with various degrees of oxygen tolerance ranging from aerobic organisms to obligate anaerobes (Figure 3B). Only one floor sample had no anaerobic organisms, with the strictly aerobic organism *P. stutzeri* forming 72% of the biofilm. By contrast, a second floor sample incorporated very few aerobic organisms, with two out of the three bacteria being the anaerobic organism *P. acnes*. All the biofilms contained environmental organisms (Figure 3C). Patient bedding and patient surrounds items not surprisingly incorporated organisms normally encountered on the skin. Significantly more of the bowel-dwelling *Coprococcus* species and significantly fewer *Streptococcus* species were found on pillows compared to mattresses ($P < 0.05$).

Phylogenetic analysis showed that the microbiomes contaminating similar items were more closely related than the microbiomes contaminating different items for mattresses, curtains, pillows, and pillowcases (Figure 4). It is not surprising that three floor microbiomes were more divergent as contaminated by shoes carrying bacteria moving from place to place.

Discussion

More than 90% of the ICU surfaces contained demonstrable bacteria residing in biofilms, and these organisms included those that are important in healthcare-associated infections such as *S. aureus*. The presence of the pathogenic multidrug-resistant species was demonstrated by conventional bacterial cultures using chromogenic agar plates. These species were found together with other, non-pathogenic species, and in the hospital environment. Two microscopy techniques showed that these bacteria were present in biofilms. Furthermore, the confocal laser scanning microscopy showed living bacterial cells within the biofilms. These surfaces had been 'terminally cleaned' twice using cloths and hypochlorite solution and stored for more than 12 months. Nevertheless we demonstrated viable MDROs within biofilms using multiple techniques, confirming our hypothesis that MDROs reside in biofilms and are resistant to being removed. The mere detection of frank, viable pathogens within the built environment near the patient is of concern and increases the risk to the patient for acquiring an HAI as has been shown by several studies.[11,12,26]

This study adds to the weight of evidence that MDRO contamination of the hospital environment is significant, and that these organisms may remain viable for prolonged periods within dry surface biofilms. Australian hospitals are required to manage for quality and risk as part of their service provision under National Guidelines aimed at improving healthcare services.[27] The role of bacterial biofilms that support MDRO growth adds a level of difficulty to this risk management of environmental contamination that has not previously been recognized.

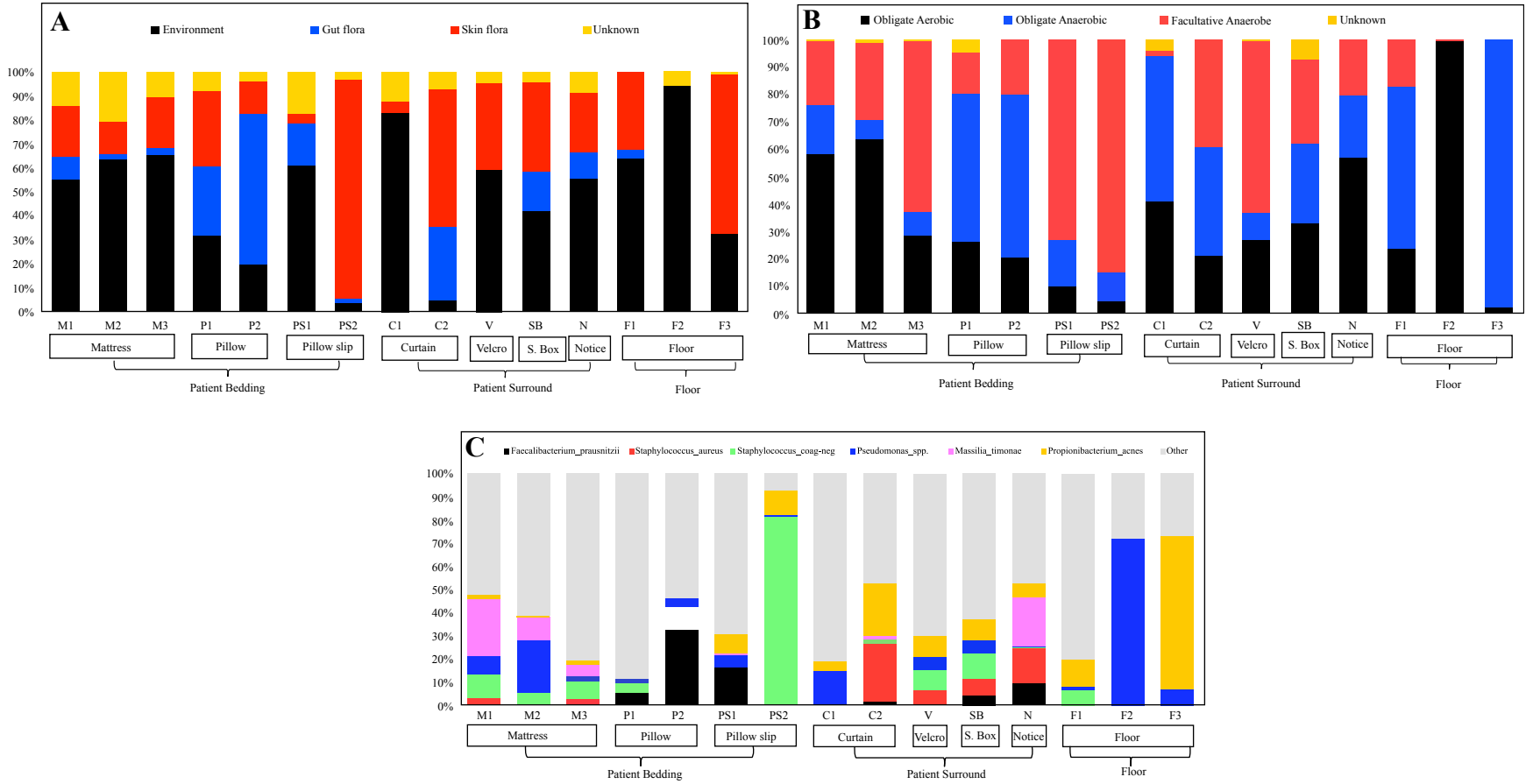

Figure 3. Composition of dry surface biofilms based on pyrosequencing. Pyrosequencing results are divided into types of surfaces by proximity to the patient, i.e. the bedding, (mattress, pillow, and pillow slips), the patient surrounds (curtains, Velcro on storage boxes, storage boxes, and display notices), and the floor of the intensive care unit. (A) The most prevalent species demonstrated on the various biofilm-containing surfaces. (B) Bacterial species grouped by aerotolerance (i.e. obligate aerobic species, facultative anaerobic and obligate anaerobic species; only a small number of identified species were of unknown aerotolerance). (C) Species grouped by their usual niche, i.e. skin flora, gut flora, or environmental species. S. Box, storage box.

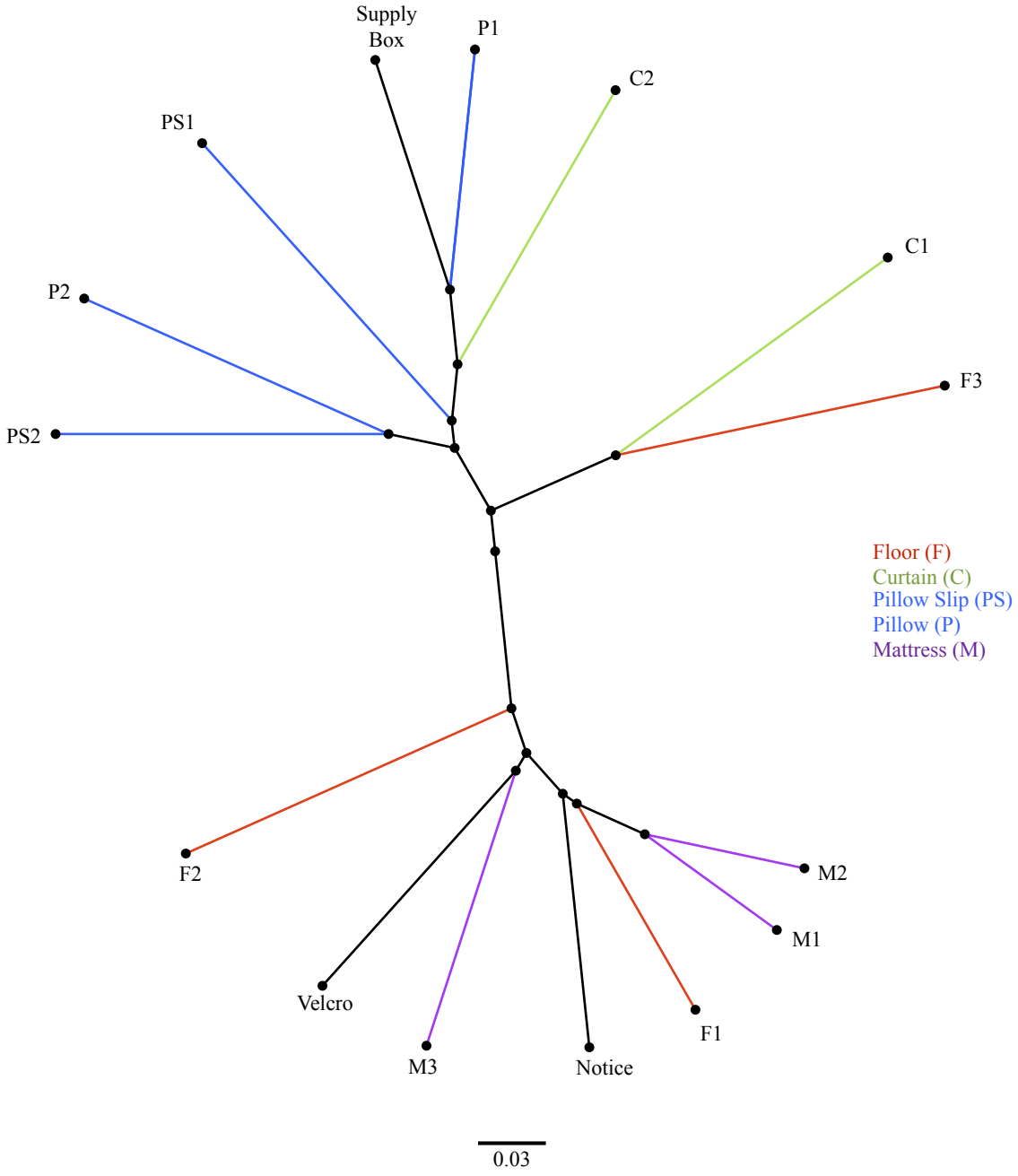

Figure 4. Radial phylogenetic tree showing the relatedness of biofilms on different dry surfaces based on the composition of bacteria within the biofilm. Phylogenetic analysis showed that the microbiomes contaminating similar items were more closely related than the microbiomes contaminating different items for mattresses (purple), curtains (red) and pillows and pillowcases (blue). However, the three floor microbiomes (green) were more divergent.

With the two microscopy techniques we can show structures embedded in biofilms that have the size and morphology consistent with the various species of pathogenic bacteria; however, non-pathogenic species also have the same appearance. Demonstrating the biofilms requires destructive sampling, and thus it is not possible to process the same material for microscopy, culture, and pyrosequencing.

Environmental contamination plays a major role in transmission of infection, particularly of MRSA.[11] In addition, the hands of healthcare workers are twice as likely to be contaminated with MRSA from environmental sources than by direct contact with infected patients.[28] We therefore conducted *S. aureus*-specific PCR to determine the frequency of non-culturable contamination. Half of the samples were contaminated with *S. aureus*; not surprisingly, more of the patient bedding was contaminated than samples obtained more distantly from the patient or the floor. Similarly, surfaces closest to the patient have been found to be more heavily contaminated with regard to total contaminating bacteria, MRSA, and VRE, than surfaces further away.[29,30]

We have shown that 93% of dry hospital furnishings are contaminated with biofilm. This is worrying in view of the difficulty in killing bacteria incorporated into biofilm. Many biofilm bacteria typically survive more than 50 times the amount of disinfectant needed to kill the same bacteria growing planktonically (free swimming) in liquid culture (reviewed in Bridier *et al.*[31]). The increased resistance of biofilms to biocide is thought to be due to changes in bacterial gene regulation (resulting in phenotypic adaptation) and to the EPS surrounding the bacteria. The EPS slows penetration of biocides into the biofilm, inactivates some disinfectants by binding to them, and inactivates some disinfectants by excretion of enzymes, for example catalase destruction of hydrogen peroxide. Additionally there is phenotypic adaption of cells to sub-lethal disinfectant concentration and increased lateral gene transfer and mutation rates.[31] Biocide resistance is due to the biofilm lifestyle, as, when the biofilm structure is disrupted, the bacteria once again become susceptible to biocides.

Biofilms are usually found in aqueous environments, yet we found them on dry surfaces. We surmise that there must be a source of water and nutrients, and a seeding by bacteria to initiate the process. Further studies are underway to find out how this might occur.

Rehydration may occur by contact with patient secretions, such as perspiration, blood, urine or vomitus, and inefficient cleaning may deposit additional solids, thus supporting biofilm growth on environmental surfaces. Additionally, exposure of biofilms to disinfectants can increase EPS production fivefold.[32] Many of the biofilms contaminating dry surfaces in the ICU appear to have very thick biofilm (Figure 1) which would contribute to their desiccation and disinfectant resistance. Indeed viability staining of biofilms, maintained in a fully air-conditioned laboratory 12 months after collection, showed that all 18 tested were principally composed of live bacteria. This included seven samples that were culture negative at collection. Just under one-half of the samples were culture negative at collection and these may reflect the basic aerobic culture conditions. However, it is well known that biofilm bacteria are difficult to culture, which is thought to be due to their low metabolic rate.[33] Of the samples that were culture positive, approximately half grew an MDRO. The presence of MDROs, protected from disinfectant action in biofilms, has implications for infection control, as biofilms intermittently release planktonic bacteria back into the environment which can then infect new niches, or infect patients.[34]

Species interactions within polymicrobial biofilms can have adverse effects on cleaning and disinfection. *Acinetobacter calcoaceticus* and *A. lwolffii* have both been shown to enhance production of other species' biofilm mass when co-cultured.[35,36] In this study *Acinetobacter* species were incorporated into nine of 15 dry surface biofilms. Additionally, polymicrobial biofilms are more resistant to disinfectants than mono-species biofilms.[36] The mechanism of this increased resistance is unknown but could result from increased disinfectant inactivation due to a more complex EPS or shielding of sensitive organisms by externally situated disinfectant tolerant organisms.

The number of species forming the biofilm was highest closest to the patient, followed by items in close proximity to the patient. Despite the biofilm on the floor being multilayered, it was composed of significantly fewer species ($P = 0.02$). The moist microclimate closer to the patient may increase survival of planktonic bacteria, allowing the incorporation of more species into the biofilm; whereas more aggressive/frequent cleaning and chemical disinfection of the floor could kill more planktonic organisms, thus decreasing the number of species incorporated into the biofilm. Or it may be that the higher number of species incorporated into the biofilm is directly related to the distance from a sick patient transmitting large numbers of bacteria.

Although the samples obtained in this study were from a fully air-conditioned hospital, the hospital is situated in a temperate region of Australia, so the type and number of bacteria contaminating fomites might not be generally applicable to hospitals situated in climates that experience extreme cold, such as in northern Europe, or hot and dry conditions as experienced in desert regions. However, there is an important implication for the widespread reliance on chlorinated disinfectants which are widely recommended by the Australian Federal Government and others.[37,38] Previous work has shown that well-applied cleaning protocols have superior performance over poorly used chlorinate-based disinfecting products.[39] The value of wiping removal of common nosocomial pathogenic species is also important, having more impact than a strongly formulated surface disinfectant.[40] The apparent survival and flourishing of the broad array of HAI-related bacteria within these biofilms strongly suggests that these bacteria should be viewed as resident rather than transient.

This study demonstrated that dry surface biofilms containing MDROs may be present on inanimate surfaces in a hospital environment, and were detected despite cleaning with hypochlorite. It suggests yet another reservoir of organisms that may be transmitted to patients to cause HAIs. More research is needed to determine the extent of this problem, and the cleaning agents and techniques required to remove dry surface biofilms from hospital environments.

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Conflict of interest statement
None declared.

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.jhin.2015.05.016.

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