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K. Ledwoch[a], S. J. Dancer[b,c], J. A. Otter[d], K. Kerr[a], D. Roposte[a], J.-Y. Maillard[a*]

[a] School of Pharmacy and Pharmaceutical Sciences, Cardiff University, Cardiff, UK

[b] NHS Lanarkshire, Scotland, UK

[c] School of Applied Sciences, Edinburgh Napier University, Scotland, UK

[d] NIHR Health Protection Research Unit (HPRU) in HCAIs and AMR at Imperial College London, and Imperial College Healthcare NHS Trust, Infection Prevention and Control, London, UK.

* Corresponding author:

Prof Jean-Yves Maillard: School of Pharmacy and Pharmaceutical Sciences, Cardiff University Redwood Building, King Edward VII avenue, Cardiff CF10 3NB, UK

telephone: + 44(0)2920879088

e-mail: maillardj@cardiff.ac.uk

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SUMMARY

Background: Wet biofilms associated with medical devices have been widely studied and their link with healthcare associated infections (HCAIs) is well recognised. Little attention has been paid to the presence of dry biofilms on environmental surfaces in healthcare settings.

Aim: To investigate the occurrence, prevalence, and diversity of dry biofilms on hospital surfaces.

Method: 61 terminally cleaned items were received from three different UK hospitals. The presence of dry biofilm was investigated using culture-based methods and scanning electron microscopy (SEM). Bacterial diversity within biofilms was investigated using RISA-PCR and next generation sequencing.

Findings: Multi-species dry biofilms were recovered from 95% of 61 samples. Abundance and complexity of dry biofilms were confirmed by SEM. All biofilms harboured Gram-positive bacteria including pathogens associated with HCAIs; 58% of samples grew meticillin-resistant *Staphylococcus aureus* (MRSA). Dry biofilms had similar physical composition regardless of the type of items sampled or the ward from which the samples originated. There were differences observed in the dominance of particular species: dry biofilms from two hospitals contained mostly staphylococcal DNA, whereas more *Bacillus* spp. DNA was found on surfaces from the third hospital.

Conclusion: The presence of dry biofilms harbouring bacterial pathogens is virtually universal on commonly used items in healthcare settings. The role of dry biofilms in spreading HCAIs may be underestimated. The risk may be further exacerbated by inefficient cleaning and disinfection practices for hospital surfaces.

Introduction

Healthcare associated infections (HCAIs) affect approximately 20% of National Health Service (NHS) patients in the UK, causing significant mortality and financial losses with yearly cost for the healthcare systems estimated to be at least $1 billion [1,2].

A sizable proportion of HCAIs are preventable through improved practices such as environmental hygiene, hand hygiene, use of personal protective equipment, and screening and isolation; some estimate this preventable portion to be 20-30% [3,4]. Cleaning is used to reduce microbial burden on surfaces, but methods vary widely between hospitals. Most UK hospitals use detergents for routine cleaning. Chlorine-based disinfectants are usually reserved for terminal or specialised cleaning of areas exposed to antibiotic-resistant bacteria and *Clostridium difficile*, or during high-risk incidents or outbreaks. Efficacy of the cleaning process is dependent upon type of equipment, consumables, microbiocidal activity, allotted time and motivation of cleaning and/or nursing staff [5,6]. There is evidence that enhanced cleaning is cost-effective [7].

Although in studies 98% of patients felt that their hospital room or ward was cleaned properly, visual audits used in monitoring the cleanliness of institutions are insufficient [8,9]. The best evidence for the role of the environment comes from studies showing the risk of infection for patients admitted into a cleaned room recently vacated by a patient with the same pathogen [10]. Microbial pathogens can persist on surfaces for days, months and even years unless removed by some cleaning or disinfection process [11]. Bacterial biofilms are commonly identified on some medical devices and are associated with the presence of moisture or/and liquid, for example biofilms on medical devices such as urinary catheters, tracheal tubes, breast implants and endoscopes. Around 65% cases of HCAIs are associated with "wet" biofilms [12]. However, more recently, biofilm has been discovered on dry surfaces, despite effective infection control measures. These biofilms are referred to as "dry" biofilms [13,14].

To date, evidence of the presence of dry biofilms on surfaces in healthcare settings remain limited. This study aims to provide a better appreciation of the scale, extent, and composition of dry biofilms on surfaces that are regularly touched by healthcare workers.

MATERIALS AND METHODS

Samples

Samples were collected from different wards and departments from three different hospitals in Wales (Hospital A), Scotland (Hospital B) and England (Hospital C), including Trauma & Orthopaedics, Adult Intensive Care, Joint Assessment Unit, Acute admission unit, Kidney and transplant, Nephrology, Cardiology, Gastroenterology, Intensive therapy unit and Haematology (Table I). Items collected included hand sanitising bottles, keyboards, patient folders and clipboards. In addition, one hospital provided a commode, a chair and wooden tray (part of a food trolley). Due to usage of electronic notes, hospital C was unable to provide any patient folders. All samples were kept in sterile bags to avoid cross-contamination during transportation. Sample processing was carried out under aseptic conditions.

Selection of sampling area

A key was removed from each keyboard and tested separately. The keys selection was made based on English letter frequency; letters with similar frequencies were tested (E, T, A and O). Folders, clipboards, the chair, commode, sanitising bottles and the trolley were sampled from the "high touch" areas. In order to ensure the same surface area was investigated for the different materials a 1.5 x 1.5 cm squares were cut from each sample.

Sample rinse and "sterility" testing

Samples were placed in 50 mL polypropylene conical Falcon™ tubes (Fisher Scientific, Loughborough, UK) containing 30 mL of sterilised water and mixed with a Fisherbrand® vortex shaker (Fisher Scientific, Loughborough, UK) for 1 minute. After three consecutive

rinsing steps, a sterile cotton swab (ThermoFisher Scientific, Newport, UK) was streaked over the surface of the sample and incubated at 37°C overnight onto PP0280 TSA plate (EO Labs, Bonnybridge, UK). A sample was presumed free from planktonic bacteria if there was no bacterial growth observed after rinsing.

Determination of the presence of sessile bacteria

Following rinsing step, samples were placed into 50 mL polypropylene conical tube containing 30 mL of tryptone soya broth (TSB; Oxoid Ltd., Basingstoke, UK) and incubated in Sanyo orbital incubator (ThermoFisher Scientific, Newport, UK) at 37°C at 180 RPM. The broth was examined for sign of turbidity every 24 hours.

Microbial species determination by genotypic analysis (RISA)

Ribosomal RNA (rRNA) intergenic spacer analysis (RISA) is a method of microbial community analysis to compare samples without culture-dependent bias. Samples were prepared as follows: turbid broths were incubated overnight and centrifuged at 1,400 g for 10 min at 20°C. The supernatant was discarded, and the pellet was re-suspended with 500 µL of TSB. Five hundred µL of 4 M guanidine isothiocyanate (UltraPure™, ThermoFisher Scientific, Newport, UK) was added and the suspension was mixed with Wizard™ infrared vortex mixer (Firsherbrand™, Fisher Scientific, Loughborough, UK) at 2,000 rpm. One mL of culture mix was added into the tubes containing 1 g of 0.1 mm diameter zirconia/silica beads (Thistle Scientific, Glasgow, UK) and suspensions were mixed in Bead Bug homogeniser (Benchmark Scientific, Cole-Parmer®, St Neots, UK) at 2,800 shaking speed for 2 min. DNA was amplified with Maxwell® 16 Instrument (Promega, Southampton, UK) and quantified by Quibit® 3.0 fluorometer (ThermoFisher Scientific, Newport, UK). The internal transcribed spacer (ITS) bacterial region between the 16S rRNA and 23S rRNA subunit genes was amplified with 1406F (TGYACACACCGCCCGT, Eurofins Genomics, Ebersberg, Germany) and 23SR (GGGTTBCCCCATTCRG, Eurofins Genomics, Ebersberg, Germany) primers by running RISA-PCR in thermal cycler (BIO-RAD, Watford, UK) [15].

Microfluidic separation was performed with a 2100 Bioanalyser Instrument (Agilent Technologies Ltd, Craven Arms, UK) using DNA 7500 chip (Agilent Technologies Ltd, Craven Arms, UK) [16].

PCR bands of 55 different hospital samples were cluster-analysed with Bionumerics software (Applied Maths, Gent, Belgium) to determine the similarity of RISA-PCR profiles between hospitals and wards. Similarity was determined using Pearson coefficient [17].

Microbial species determination by DNA analysis (next generation sequencing)

DNA was extracted and quantified as described above. Next generation sequencing (NGS) and quality analysis of FASTQ sequence reads was performed by BaseClear Group, Netherlands. The 16S rRNA gene (V3-V4) was PCR amplified before sequencing. The Illumina MiSeq (PE300) system was used to generate paired-end sequence reads and blc2fastq2 2.18 software was utilised to produce FASTQ sequence files. Reads were filtered and clipped. Raw sequences were analysed with open source software Edge Bioinformatics (v.1.5.1).

Scanning electron microscope (SEM) analysis

Three randomly selected samples from keyboards, folders, hospital commode and chair seating were analysed with SEM to visualise the presence of dry biofilms. Samples were prepared by overnight incubation in 2.5% glutaraldehyde solution (Contain™, Fisher Scientific, Loughborough, UK) followed by immersion in successive concentrations of ethanol for 10 minutes each (10%, 25%, 50%, 70%, 90%, 100%). Prior to SEM scanning, samples were coated with 20 nm AuPd coating with sputter coater (SC500, Biorad, UK). Secondary electron images were acquired with a beam energy of 5kV using an in-lens detector on a Sigma HD Field Emission Gun Scanning Electron Microscope (Carl Zeiss Ltd., Cambridge, UK) at 10,000x magnification and 5-7 mm working distance with the help of Earth and Ocean Sciences Department, Cardiff University, UK.

Statistical analysis

The strength of linear association between PCR-RISA profiles of dry biofilms recovered from hospital samples was calculated using Pearson coefficient with Bionumerics software (Applied Maths, Gent, Belgium). Higher linear association between two data sets is presented by higher absolute value of Pearson coefficient [17]. The Pearson coefficient values were re-calculated into percentage to better reflect the correlation between PCR-RISA data sets.

RESULTS

Bacterial presence on hospital sample surfaces

No planktonic bacteria were recovered from any of the 61 samples investigated after multiple rinsing steps with sterile water. Bacterial growth in a nutrient rich broth was observed in 95% of samples (58/61) (Table I). The assumption was made that bacterial growth resulted from the presence of sessile bacteria embedded within the dry surfaces, which was confirmed by SEM analysis. Most of the samples (76%) produced growth within 24 hours (Table I).

DNA analysis (RISA-PCR)

RISA-PCR bands of bacterial cultures recovered from samples were analysed to determine the consistency of microbial composition between wards from the same hospital and between hospitals (Figure 1). Cluster analysis showed that the majority of bacterial cultures share a part of genome, regardless of the sample type or origin. The PCR profiles of hospital samples are convergent in 49%, meaning that the similarity between samples is high. Similarity of bacterial cultures is consistent within samples originating from hospitals A and B with 53% and 45% similarity, but lower for hospital C with 38% similarity.

DNA analysis – next generation sequencing (NGS)

The DNA analysis of the bacterial species recovered from broth cultures following incubation of hospital samples was performed by next generation sequencing. NGS allowed more precise and detailed analysis of bacterial cultures' composition than RISA-PCR (Figure 2). Bacterial species found from the hospital samples contained mainly staphylococci and *Bacillus* spp. The most common bacteria identified were *S. aureus, S. saprophyticus* and *S. epidermidis*, with *B. licheniformis* and *B. subtilis* being the most common *Bacillus spp.* The only Gram-negative DNA isolated was that of *Pseudomonas* spp. (Figure 2).

An average of 18 different bacterial species were present on each surface analysed from the dry surface samples.

Dominance of particular species was associated with their origin (Figure 3). Samples isolated from hospitals A and B mostly contained *S. aureus* and *S. epidermidis* (34.6% and 24.6% in hospital A, 23.8% and 24.2% in hospital B, respectively). The most common bacteria found from hospital C samples were *S. saprophyticus* and *B. subtilis* (20.6% and 19.1%, respectively).

Staphylococci generally dominated on hospital A and B surfaces (66% and 53% of dry biofilm composition, respectively), whereas in hospital C, *Bacillus* spp. were dominant (68% of dry biofilm composition).

Scanning electron microscope (SEM) analysis

The previous experiments indicated the presence of multiple sessile bacterial species on the surfaces from dry hospital surfaces. SEM was used to visualise the presence and complexity of dry biofilms formed on these surfaces. Samples varied in their composition and presence of matrix. These dry biofilms formed random clusters of bacterial biofilm on surfaces (Figure 4).

DISCUSSION

A biofilm is a complex community of microorganisms embedded in self-produced extracellular polymeric matrix, markedly different from their planktonic state equivalent [18]. Most biofilm studies are associated with wet biofilms sessile microorganisms grown in aqueous habitats. Little attention has been paid to biofilms grown in dry environments, despite their presence on dry hospital surfaces [19]. There is no standardised method for biofilm detection on surfaces [12]. Nevertheless, an approach was developed to identify the presence of dry biofilm on healthcare surfaces. No viable bacteria were grown following rinsing dry surface samples, suggesting that no planktonic bacteria were present. However, by immersing the sample surfaces in nutrient broth and following incubation, we were able to detect multiple bacterial species from the surfaces, and scattered random microbial biofilm were observed by SEM. Indeed, almost all samples (95%) harboured dry biofilms. Similar results were obtained by Hu et al, who confirmed the presence of biofilm on 41/44 (93%) hospital surface samples [14]. It is conceivable that the rinsing step including vortexing may have contributed to dislodging loosely attached bacteria/biofilm [20]. Our results showed, however, that the majority of surfaces bear dry biofilms that were not removed or loosen by the multiple rinsing steps.

Our study shows staphylococci and *Bacillus* spp. to be the main bacterial genera recovered from the dry biofilms. It was surprising to see some variability between hospitals whereby in one study hospital *Bacillus* spp. were the dominant bacteria. These differences may be explained by hospital size, antimicrobial consumption, ventilation and patient infections, since patients shed pathogens onto surfaces around them, cleaning and disinfection regimens, which will impact on soiling, and possibly differences in the item sampled [21]. Here, the three hospitals varied in size: hospitals A and C are 1500 bed teaching hospital in wales and in London, whereas hospital B is a Scottish district general hospital with 550 beds. Detergents are used for routine cleaning in all three hospitals, although in hospital A, there is proportionately greater use of chlorine-based disinfectants. All three hospitals utilised a terminal cleaning regimen with bleach-type agents for areas exposed to known

pathogens such as carbapenemase-producing Enterobacteriaceae (CPE), norovirus, *C. difficile*, MRSA and vancomycin0resistant enterococci (VRE). We cannot comment on the type of patients or their infections as that information was not collected during this study. Despite these differences, it was remarkable that dry biofilms on the sampled surfaces were widespread and that there was a good similarity of the dry biofilm microbial composition from two hospitals. The local environment (urban *vs.* rural) could also play some parts. For example, external building work has been associated with increased number of bacillus spores in healthcare facilities [22,23]. Here, no external building work was reported near or within hospital C at the time of sampling, which would have explained the high level of *Bacillus* spp. observed.

S. aureus, *S. saprophyticus* and *S. epidermidis* were the dominant *Staphylococcus* spp. MRSA was cultured from a high proportion of samples; the clinical risk associated with this finding is yet to be determined. Among *Bacillus* spp., the prevalent species were *B. licheniformis* and *B. subtilis*. These results differ from the study by Hu et al, in which no *Bacillus* spp. were reported, although the authors analysed only samples from the critical care environment [14]. Nevertheless, it is clear that composition of the isolated biofilms is a mixture of environmental, skin and gut microflora, including pathogens. The complexity of composition has been reported for biofilm from different surfaces, although overall composition depends on the object sampled [14]. Our study showed some variability in the detailed composition of the biofilm for the same object between different hospitals. However, variability in microbial composition decreased between different objects from the same hospital. It is likely that each hospital has its own unique environmentome, which is reflected in the composition of biofilm on items and surfaces.

We and others have not addressed the formation of complex dry biofilm on environmental surfaces in healthcare settings [13,14]. Bacterial stress such as desiccation and exposure to chemicals can enhance the production of extracellular polysaccharides [24-26], which can

protect biofilms from adverse chemical and physical effects [27,28]. More of a concern is the report of the ineffectiveness of disinfection, notably chlorine-based agents, against dry biofilms, and how this impacts on infection control [14,29]. The high prevalence of *S. aureus,* and MRSA in particular, from dry biofilm from the surfaces sampled adds to that concern, since MRSA is known to be transmitted through environmental contamination [30]. The presence and transmission of pathogens in dry biofilms from healthcare surfaces warrants further examination [31,32]. The risk associated with bacterial pathogens recovered in dry biofilms with HCAIs is yet to be determined. Some studies link the survival rates of biofilm-forming strains with persistent nosocomial infections and outbreaks [33,34]. The role of *Bacillus* spp. in protecting bacterial pathogens in biofilm from disinfection also needs to be addressed [34-38].

Conclusion

Our study provides more evidence that complex dry biofilms containing bacterial pathogens are virtually universal on hospital surfaces, despite regular cleaning and disinfection. These dry biofilms were shown to occur in clusters on different materials and were predominantly formed by Gram-positive bacteria although occasional *Acinetobacter* spp. were identified. Unlike other studies, we identified a large proportion of *Bacillus* spp. The role of *Bacillus* spp. in protecting mixed biofilm community from environmental conditions and disinfection should be further investigated [38]. Although these dry biofilms are clearly harboring pathogens, their role in transmission needs to be established. This is particularly important in view of the failing of disinfection to control these biofilms and the general absence of testing and biocidal products claiming efficacy against dry biofilms.

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Conflict of interest

K Ledwoch is partly funded by GAMA Healthcare Ltd. J A Otter is a consultant to GAMA

Healthcare Ltd, and has been a consultant to Pfizer in the last three years.

Figure 1 Cluster analysis of PCR-RISA bands of cultures recovered from hospital samples (keyboards and folders). The scale gives the similarity between a pair of two samples (in a range of 0-100%, where 0% indicates no correlation, and 100% indicates complete convergence in RISA-PCR profiles). Red dots indicate pairs of the same samples, which are not taken into account in analysis.

Figure 2 Percentage of samples containing given species following DNA analysis – all samples pooled.

Figure 3 Difference in "dry" biofilm composition between hospitals. *Anoxybacillus flavithermus*: ■, *Bacillus amyloliquefaciens:* ■, *Bacillus anthracis:* ■, *Bacillus cereus:* ■, *Bacillus licheniformis:* ■, *Bacillus megaterium:* ■, *Bacillus pumilus:* ■, *Bacillus sp.:* ■, *Bacillus subtilis:* ■, *Bacillus thuringiensis:* ■, *Staphylococcus aureus:* ■, *Staphylococcus epidermidis:* ■, *Staphylococcus lugdenensis:* ■, *Staphylococcus pasteuri:* ■, *Staphylococcus saprophyticus:* ■, *Staphylococcus warneri:* ■, OTHER:*■*

Figure 4. Examples of "dry" biofilms recovered from surfaces; magnification X10,000. A) and B) Patient folders, C) patient chair, D) keyboard key. Images of biofilms were coloured in purple to help visualisation and contrast using GNU Image manipulation program (GIMP 2.8) software. Images were not otherwise altered.

Table I Samples and time for bacterial regrowth in TSB following rinsing

Hospital A				Hospital B				Hospital C			
Sample number	Department/ Ward	Sample type	Regrowth (days)[1]	Sample number	Department/ ward	Sample type	Regrowth (days)[1]	Sample number	Department/ Ward	Sample type	Regrowth (days)[1]
1	Gastroenterology	folder	1	32	Microbiology	patient folder	1	52	Acute admission unit	keyboard	no regrowth[2]
2		wooden tray (food trolley)	no regrowth[2]	33		patient folder	1	53	Kidney and transplant	keyboard	4
3		hospital commode	1	34		patient folder	1	54	Cancer services and haematological malignancies	keyboard	6
4		leather chair seating	1	35		patient folder	1	55	Kidney and transplant	keyboard	1
5		fabric chair back support	1	36		patient folder	4	56		keyboard	1
6	Trauma & Orthopaedics	patient folder	6	37		patient folder	5	57	Cardiology	keyboard	1
7		patient folder	1	38		patient folder	1	58		keyboard	7
8		patient folder	1	39	Biochemistry	patient folder	1	59	Acute medicine	keyboard	1
9		patient folder	1	40		patient folder	1	60	Haematology	keyboard	1
10		patient folder	1	41	Pharmacy	patient folder	1	61		keyboard	no regrowth[2]
11		patient folder	2	42	General medicine	keyboard	1				
12		patient folder	1	43		keyboard	1				
13		patient folder	1	44		keyboard	1				

14		patient folder	1	45		keyboard	1
15		patient folder	1	46		keyboard	1
16	Adult Intensive Care	keyboard	1	47		keyboard	1
17		keyboard	1	48		keyboard	1
18		keyboard	1	49		keyboard	1
19	Acute Short Stay Medicine	keyboard	2	50		keyboard	1
20		keyboard	1	51		keyboard	1
21		keyboard	2				
22	Gastroenterology	keyboard	4				
23		keyboard	1				
24		keyboard	4				
25	Nephrology	keyboard	6				
26		keyboard	9				
27		keyboard	1				
28		keyboard	7				
29		keyboard	1				
30	Trauma & Orthopaedics	sanitising bottle	1				
31		sanitising bottle	1				

[1] Time for turbidity to developed following incubation in TSB.

[2] No regrowth following > 14 days incubation

Scale

TOTAL

0   20   40   60

Hos	Item	N
A	keyboard	1
A	keyboard	2
A	folder	3
A	folder	4
C	keyboard	5
A	folder	6
A	keyboard	7
B	keyboard	8
A	folder	9
A	folder	10
B	folder	11
B	keyboard	12
A	folder	13
A	keyboard	14
C	keyboard	15
B	folder	16
A	keyboard	17
B	keyboard	18
B	keyboard	19
B	keyboard	20
A	folder	21
A	keyboard	22
A	keyboard	23
C	keyboard	24
C	keyboard	25
B	folder	26
B	keyboard	27
C	keyboard	28
B	folder	29
C	keyboard	30
C	keyboard	31
B	keyboard	32
B	keyboard	33
B	keyboard	34
A	folder	35
B	folder	36
B	folder	37
B	folder	38
C	keyboard	39
B	folder	40
C	keyboard	41
B	folder	42
C	keyboard	43
B	folder	44
A	folder	45
A	folder	46
A	folder	47
A	keyboard	48
A	keyboard	49
A	folder	50
A	folder	51
A	folder	52
B	keyboard	53
A	folder	54
A	keyboard	55

A)

B)

C)

D)