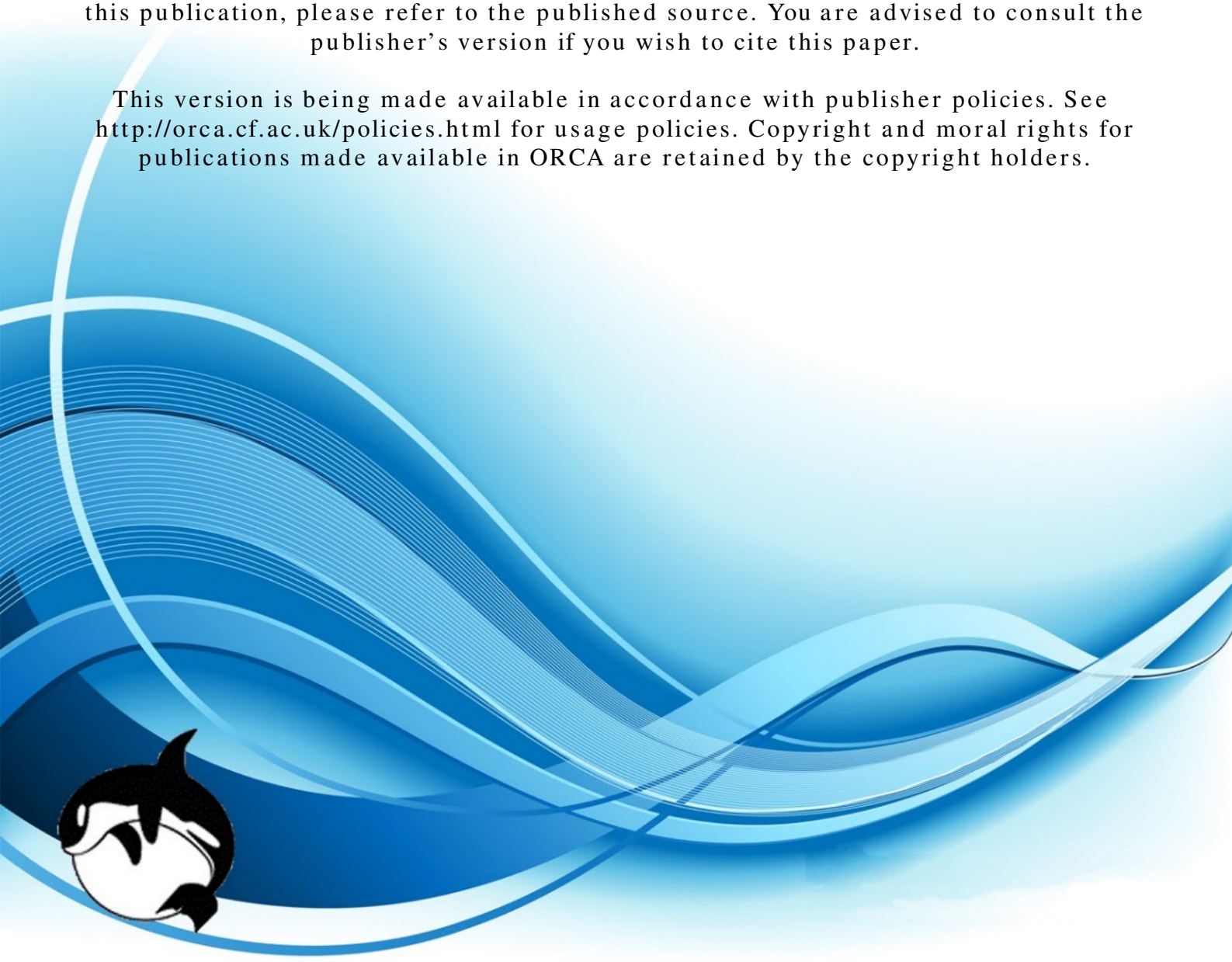

Biocide resistance and transmission of *Clostridium difficile* spores spiked onto clinical surfaces from an American healthcare facility

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Running Head: Transmission and resistance of *C. difficile* spores

Abstract

Clostridium difficile is the primary cause of antibiotic-associated diarrhea globally. In unfavourable environments the organism produces highly resistant spores which can survive microbicidal insult. Our previous research determined the ability of *C. difficile* spores to adhere to clinical surfaces; finding that spores had marked different hydrophobic properties and adherence ability. Investigation into the effect of the microbicide sodium dichloroisocyanurate on *C. difficile* spore transmission revealed that sub-lethal concentrations increased spore adherence without reducing viability. The present study examined the ability of spores to transmit across clinical surfaces and their response to an in-use disinfection concentration of 1,000-ppm of chlorine-releasing agent sodium dichloroisocyanurate. In an effort to understand if these surfaces contribute to nosocomial spore transmission, surgical isolation gowns, hospital-grade stainless steel and floor vinyl were spiked with 1×10^6 spores/ml of two types of *C. difficile* spore preparations: crude spores and purified spores. The hydrophobicity of each spore type versus clinical surface was examined via plate transfer assay and scanning electron microscopy. The experiment was repeated and spiked clinical surfaces were exposed to 1,000-ppm sodium dichloroisocyanurate at the recommended 10-min contact time. Results revealed that the hydrophobicity and structure of clinical surfaces can influence spore transmission and that outer spore surface structures may play a part in spore adhesion. Spores remained viable on clinical surfaces after microbicide exposure at the recommended disinfection concentration demonstrating ineffectual sporicidal action. This study showed that *C. difficile* spores can transmit and survive between varying clinical surfaces despite appropriate use of microbicides.

IMPORTANCE

Clostridium difficile is a healthcare-acquired organism and the causative agent of antibiotic-associated diarrhoea. Its spores are implicated in faecal to oral transmission from contaminated

surfaces in the healthcare environment due to their adherent nature. Contaminated surfaces are cleaned using high-strength chemicals to remove and kill the spores; however, despite appropriate infection control measures, there is still high incidence of *C. difficile* infection in patients in the US. Our research examined the effect of a high-strength biocide on spores of *C. difficile* which had been spiked onto a range of clinically relevant surfaces including isolation gowns, stainless steel and floor vinyl. This study found that *C. difficile* spores were able to survive exposure to appropriate concentrations of biocide; highlighting the need to examine the effectiveness of infection control measures to prevent spore transmission, and consideration of the prevalence of biocide resistance when decontaminating healthcare surfaces.

Introduction

The anaerobic spore-forming Gram-positive bacterium *Clostridium difficile* is the primary cause of antibiotic-associated diarrhea globally (1). *C. difficile* asymptomatically forms part of the microbiota of 1-3% healthy adults (2, 3); however, if the microbiota of the intestine is disrupted, for example as a result of broad- spectrum antibiotic treatment, colonisation of the colon by vegetative cells of *C. difficile* can proceed and escalate into the onset of *C. difficile* infection (CDI) (4). When fulminant infection ensues the patient will suffer from inflammation and diarrhoea. Further complications of CDI include pseudomembranous colitis, sepsis and the fatal toxic megacolon (5).

Hypervirulent PCR ribotypes such as BI/027/NAP1 have spread intercontinentally and caused epidemics in Western countries further adding to CDI incidence (6, 7). Many reports highlight the increasing impact of CDI to public health and the associated economic burden. For example, mortality rates in the United States increased from 25 to 57 per million people for the periods 1999-2000 and 2006-2007, respectively (8). In total approximately 14,000 deaths occurred in 2007 and this statistic increased still further with an estimated 29,300 deaths in

2011 (9). In 2008 alone the estimated cost related to CDI within the United States to health-care facilities was $4.8 billion, ignoring the additional cost to other facilities such as care homes (10). A similar pattern of statistics can be seen in England, with an increase from 1,149 *C. difficile*-related deaths in 2001 rising to 7,916 in 2007 (11).

In response to increasing CDI infection rates, stringent infection control procedures were implemented within hospital environments in England which resulted in a decline in mortality to 1,487 in 2012. This figure surpasses that of MRSA and non-specified *Staphylococcus aureus* infection mortality (262 in 2012) (12) and thus is still a major source of concern globally. Despite implementation of appropriate surveillance and infection control procedures the organism still causing significant levels of morbidity and mortality across nosocomial environments (13).

Incidence of CDI is directly affected by the ability of *C. difficile* to produce resistant spores which can survive on organic and inorganic surfaces for months and remain viable (6). A major source of CDI and transmission in the healthcare environment is through the faecal to oral route; often via the contamination of surfaces. As many as 1×10^7 spores per gram faeces are released into the environment by infected patients through airborne dispersal and soiling further adding to the bioburden (14). Possible causes of transmission include inappropriate biocide use, lack of adherence to infection control guidelines and varying standards of practice across healthcare facilities globally (15, 16, 17).

Chlorine-releasing agents (CRAs) are the predominant form of biocide used in healthcare facilities to disinfect surfaces; namely sodium hypochlorite (NaOCl) and sodium dichloroisocyanurate (NaDCC) (18). These microbicides are fast-acting in aqueous solutions and are relatively inexpensive (19). Low concentrations of 50-ppm available chlorine have shown to kill >99% of vegetative bacterial cells *in vitro*. In addition, when 275-ppm chlorine

was applied to a clinical environment there was a significant reduction in hospital-acquired infections from non-spore forming bacteria (20, 21). However, the inactivation of spores requires much higher concentrations with the current recommendation for application of NaDCC in hospitals in England being 1,000-ppm available chlorine for 10 minutes to deactivate spores of *C. difficile* and *Bacillus* species (22, 23). Although the working concentration of NaDCC has shown to be effective in liquid culture (24), its application to working surfaces is less efficient for inactivation of spores (25) and this reduced activity is exacerbated by the presence of organic substances, such as bodily fluids and faeces, which have a neutralising effect on the biocide (26). The mechanism of action of chlorine-releasing biocides is poorly understood; however, it has been suggested that their action may be due to strong oxidative ability, their effect on cell membranes and inhibition of enzymatic reactions (27).

Our previous study showed that adherence of *C. difficile* spores to inorganic surfaces increased when spores were exposed to sub-lethal concentrations (500-ppm available chlorine) of sodium dichloroisocyanurate (27). This increase was more pronounced for strain DS1748 (002 ribotype) which is not known to produce an exosporium outer layer (28) and suggests that when spores are exposed to sub-lethal levels of biocide they may inadvertently become more adherent to inorganic surfaces. The purpose of the present study was to assess the transfer ability of *C. difficile* spores from clinical surfaces pre- and post-biocide exposure. Surfaces tested include hospital isolation gowns, hospital grade stainless steel and vinyl flooring routinely used within the United States. Spore recovery from spiked clinical surfaces was investigated using a plate transfer assay. Clinical surfaces spiked with spores were exposed to NaDCC to determine sporicidal efficacy and the presence of spores on each clinical surface pre and post NaDCC treatment was examined using scanning electron microscopy.

Results

Transfer of *C. difficile* spores from liquid form to hospital surgical gowns

To examine the ability of *C. difficile* spores (U and P derived from strains DS1748, R20291 and DS1813) to adhere to, and subsequently transfer from hospital surgical gowns, spores were applied directly to the surgical gowns in liquid for 10 s, 30 s, 1 min, 5 min and 10 min before being removed and discarded (Figure 1, Figure 4A and C). This experiment was designed to mimic transfer of infectious bodily fluids in the clinical setting and assess the potential for onward transmission to patients. There was no significant difference between the amount of spores (U and P) recovered from the gowns and the contact time of the spores to the gowns; suggesting that the process of spore transfer between surfaces occurred within the first 10 seconds of contact with the gown (two-way ANOVA; $p = 0.696$). From Figure 1 it appears as though the recovery of DS1748 P Spores increased with contact time; however, this was not statistically significant (one-way ANOVA; $p = 0.144$). Generally, U spore recovery was significantly higher than that of P spores (two-way ANOVA; $p < 0.001$); however, the exception to this trend was the increased recovery of DS1813 P spores when compared to U spores of the same strain (one-way ANOVA; $p < 0.001$). There were no significant differences in spore recovery between DS1748 and R20291 for either U spores or P spores.

Spore recovery from spiked clinical surfaces after direct contact with hospital gowns

To establish whether hospital-grade stainless steel surfaces and vinyl flooring surfaces act as fomites for *C. difficile* spore transmission in the clinical setting, sterile sections of hospital surgical gowns were placed in direct contact with hospital-grade stainless steel and vinyl flooring spiked with 1×10^5 spores and spore recovery from the surgical hospital gowns assessed. The contact times were reduced to 10 s, 30 s and 1 min due to results presented in Figure 1 which confirm that the length of contact time had no significant effect on spore recovery. Similarly, there remained no significant difference in spore recovery from steel and

vinyl between the contact times used and the amount of spores recovered from the strains examined (Figure 2) (two-way ANOVA; $p = 0.892$ and $p = 0.904$ for steel and vinyl, respectively). Spore recovery of U DS1748 was significantly higher from both stainless steel surfaces (one-way ANOVA; $p = 0.034$) and vinyl flooring (one-way ANOVA; $p < 0.001$) when compared to the other strains. DS1748 P spore recovery was higher on stainless steel (one-way ANOVA; $p < 0.001$) and vinyl flooring (one-way ANOVA; $p < 0.001$) than of R20291 and DS1813. DS1748 P spore recovery was approximately 10-fold higher than that of the U Spore equivalent (two-way ANOVA; $p < 0.001$).

Sporicidal efficiency of sodium dichloroisocyanurate (NaDCC)

Two types of spore suspension from three *C. difficile* strains (DS1748, R20291 and DS1813) were exposed to the recommended in-use concentration of NaDCC in solution (1,000-ppm) and spore viability was determined. From Figure 3 it can be seen that there was no recovery of spores which had been treated in liquid form and then spiked onto gowns. Moreover, recovery of NaDCC-treated U spores from the spiked and directly-treated hospital surgical gowns were lower across the three strains tested when compared to non-treated spores, with the lowest relative recovery from strain R20291 (Student t-test; $p < 0.005$). Scanning Electron Microscopy (SEM) images in Figure 4A and 4C support this by showing adhered spores on the fibres of the gowns from strain R20291 before and after treatment with the recommended concentration of NaDCC. Interestingly, Figure 4B shows a single P spore of R20291 after NaDCC treatment with a visible exosporial layer, while Figure 4D shows a U spore of R20291 after treatment that has no visible evidence of an exosporial layer. These differences in spore exosporium show distinct morphological variations within the R20291 strain; but may not necessarily be as a result of NaDCC exposure. It is possible that any damage to the exosporium after NaDCC exposure is not visible via SEM (Figures 4B); thus there is a possibility that NaDCC may have

chemically altered the exosporium structure without changing the spore's overall three-dimensional appearance (28).

Decreased sporicidal activity was observed for strains tested with NaDCC on the varying clinical surfaces (Figure 3). Similar results were observed with DS1813 P spores, but not for P spores of DS1748 and R20291. There was detectable recovery of R20291 U Spores (~73 to ~23 SFU) after NaDCC treatment on stainless steel; although this was not significantly different when compared to the lack of recovery of the other U strains tested (Mann-Whitney Test; $p = 0.40$). Despite the lack of DS1813 spore recovery from stainless steel surfaces after NaDCC exposure (Figure 3C), spores were still present on the steel surfaces indicating lack of viability (Figure 5A).

After NaDCC exposure no DS1748 or R20291 P spores were recovered from the vinyl flooring, whereas U spores from these strains were recovered (Figure 3A and B). SEM results revealed the presence of spores of both types on the vinyl (Figure 5B and D). The recovery of R20291 U spores significantly decreased (Student's t-test; $p = 0.001$) but not for DS1748 (Figure 3A and B). In contrast, the recovery of both U and P spores of DS1813 did not change significantly after NaDCC treatment (Student's t-test; $p > 0.05$ for both U spores and P spores; Figure 3C).

Discussion

Gowns have been used by healthcare professionals to mitigate the risk of transmitting infectious materials between patients, hospital visitors and other healthcare workers (31). Many gowns have shown differences in barrier and textile performance and it is these variations that play a role in the dissemination of microorganisms across healthcare facilities (32). With the advent of modern technology single-use isolation gowns made from fluid-resistant materials, such as polypropylene, are now widely used as a form of barrier protection; however, there is some debate as to their efficiency (31, 33, 34). Our results demonstrated that *C. difficile* spores

were able to transfer and adhere to fibres of the polypropylene spun gowns when spiked in a liquid medium. As there was no significant difference between the contact time of the spores and the recovery of spores from the gown, it appears as though the process of spore transfer occurred rapidly within the first 10 seconds of contact when examining spore recovery from spiked liquid, hospital grade stainless steel and vinyl flooring, respectively. This suggests a clear need to ensure appropriate decontamination of surfaces that a contaminated gown may come into direct contact with in a clinical setting.

The ability of microorganisms to travel through fabrics is related to the physico-chemical properties of the gowns and the characteristics of the microorganism (32). Another interesting observation from this study is the rapid ability of the spores to move from one hydrophobic surface to another hydrophobic surface i.e. fluid-resistant gowns and stainless steel which suggests that the more hydrophobic spores interacted better with the stainless steel surfaces than the gowns (Table 1, Figure 1). Whether this is related to steel surface structure as opposed to gown structure, or the hydrophobic interactions between (i) the individual strains (which possess varying relative hydrophobicity; Table 1) (ii) the liquid and (iii) each test surface warrants further investigation at the molecular level. It is also clear that the single-use gowns act as fomites for *C. difficile* spore transmission. Not only do spores of all strains rapidly attach to the gown fibres from liquid and dry clinical surfaces but the single-use gowns are then ineffective at trapping spores within their fibres and preventing the onward transmission of spores as demonstrated by spore recovery from the gowns (Figures 1& 2). While this ability differs between strains, it does suggest that the adherence ability of the spore to individual gown fibres may be affected by spore hydrophobic properties and exosporium layer which is known to aid spore adherence on hospital surfaces (Table 1; 28). Results also suggest that *C. difficile* spores, after microbicidal exposure to NaDCC at the recommended contact time and concentration, can continue to remain viable, adhere and transmit via hospital gowns (Figure

4A & 4C; 1, 28, 35). This highlights the importance of ensuring that single-use surgical isolation gowns are used appropriately in infection prevention and control; i.e. that gowns are adorned upon entering and disposed of when exiting a single room to prevent onward spore transmission and incidence of CDI (36).

Despite using recommended concentrations of NaDCC to decontaminate gowns, stainless steel surfaces and floor vinyl after spore exposure, spores were still visibly attached to each surface and were viable upon culture (Figures 4 & 5). Decontamination and appropriate cleaning of surfaces is critical in managing the spread of CDI to patients from spores (37). It can be speculated that the hydrophobic properties and weave of the gown fabric may have prevented exposure of spores to NaDCC which explains the increased spore recovery; however this would need to be examined further by exploring the use of fluorescence-based spore viability tests (38). The smooth surfaces of steel and vinyl would theoretically make NaDCC treatment more effective by increasing the test surface area; however, the occurrence of viable spores on both treated steel and vinyl surfaces conflicts with this hypothesis and clearly evidences spore resistance to NaDCC. This resistance was found for all three strains tested and was not limited to hypervirulent R20291 027 PCR ribotype strains (7) (Table 1). Our results confirm that working concentrations of sporicides (with active concentrations of chlorine) applied at the appropriate contact times may not kill *C. difficile* spores. The ability of microbicides, such as CRA's, to kill *C. difficile* spores has been examined previously with similar results (7, 25, 26, 38).

Spores which possess an exosporium-like structure have been demonstrated to have increased adhesion to surfaces *in vivo* and *in vitro*; associated with increased hydrophobicity of the spore (28, 35, 39). The exact function of the exosporium-like structures on certain strains of *C. difficile* spores has yet to be fully elucidated; however, its role in adhesion to intestinal mucosal cells and in *Bacillus* spore adhesion has been more clearly defined (35, 39, 40). Our previous

study established that exosporium-positive spores (DS1813) were more resistant to NaDCC at sub-lethal concentrations than exosporium-negative spores (DS1748) (1, 28), which appears to correlate with the theory that the exosporium layer confers a protective barrier to the spore, preventing it from being damaged (41). It has also been hypothesised that exposure of spores to NaDCC at inappropriate concentrations and contact times can alter and increase spore adhesion ability (1). In the present study, while we observed a lack of exosporium-negative DS1748 and exosporium-positive DS1813 spore recovery from hospital stainless steel, SEM image (Figure 5A) revealed the presence of DS1813 spores adhered onto the stainless steel surface, and the presence of possible damaged spores of DS1748 (Figure 5C). Indeed, the presence of a small number of spores following NaDCC treatment could still produce recovery of zero viable spores. Moreover, the viability of spores from all strains tested was also observed after NaDCC treatment of vinyl flooring (Figure 3). This strongly indicates that recovery of spores from stainless steel and vinyl, two very different materials, has been affected by biocide exposure, either due to biocidal killing or reduced spore adherence; however, the exact mechanism of spore adherence and biocidal activity of NaDCC upon the exosporium layer and spore ultrastructure has yet to be determined.

As seen in Figures 4 and 5, there are exosporium-like projections present on R20291 spores that increase the material surface-spore contact area which correlates to data from other studies (41). It is possible that these projections may increase spore adherence and perhaps biocide resistance by protecting the core from chemical effects. Moreover, as NaDCC was completely effective when spores were exposed in liquid form (Figure 3) when compared to the spore recovery post exposure from spiked surfaces, attests to the potential the exosporium may have for protection of the spore from biocide exposure. Interestingly, hypervirulent DS1813 and R20291 strains have shown an increased adherence ability throughout this study comparative to DS1748; suggesting exosporium- positive spores adhere better and more rapidly with first-

contact to the test surface (Table 1). Additionally, unpurified R20291 spores were recovered from all surfaces tested post-NaDCC exposure which demonstrates the spore's ability to remain viable after biocide exposure (Fig 2 & 3). This concurs with previous studies that have demonstrated CRA resistance in PCR Ribotypes 017, 012 and 027 (R20291) (7). Mechanical removal to remove spores from clinical surfaces has been shown to be effective in studies, however, this may not be appropriate with gowns as they are designed for single-use; therefore effective and immediate disposal of surgical gowns after use needs to be considered when preventing transmission of CDI (6, 25). The impact of the microbicide upon spore structure and resistance warrants further research to fully understand the mechanisms of resistance and to establish up-to-date and effective decontamination protocols. Moreover, our research suggests that the *C. difficile* exosporium may play a key role in biocide resistance of spores and thus could be a potential target for development of novel sporicidal disinfectants.

Materials and Methods

Growth conditions, *Clostridium difficile* strains and spore production

C. difficile cultures were incubated anaerobically at 37 ºC for 48 hours in a BugBox Plus anaerobic workstation (Ruskinn Technology Ltd. United Kingdom) using an 85% nitrogen, 10% carbon dioxide and 5% hydrogen gas mix. Clinical isolates of *C. difficile* (PCR Ribotypes 027 and 002) used in this study are described in Table 1 and were obtained from the Anaerobic Reference Unit, University Hospital Wales, Cardiff, UK. Unless otherwise stated, all organisms were stored as spores at 4 ºC. All experiments described were conducted in triplicate. *C. difficile* spores were produced according to two methods to generate unpurified/crude and purified spore preparations; spores produced *via* Perez et al 2005 (42) methodology were designated as unpurified (U) spores due to being harvested via water-washing and containing vegetative and spore forms of the organism. These were deemed

representative of *C. difficile* commonly encountered within clinical environments. Spores were produced on reduced brain heart infusion (BHI) agar and BHI broth (Oxoid Ltd, Basingstoke, United Kingdom) each supplemented with the germinant 0.1% (w/v) sodium taurocholate (28).

Purified spores (P spores) were produced as described by Sorg and Sonenshein (2010) (43). Briefly, *C. difficile* strains were cultured on reduced BHI agar with 5 g/L yeast extract and 0.1% L-cysteine and were examined after four days anaerobic incubation for characteristic colonies. Spores were harvested using sucrose density-washing. Spore purity was confirmed via phase contrast microscopy. Spore concentration was determined via drop count as described by Miles *et al*. (44) and mean spore-forming units (SFU) per ml calculated (28).

Preparation of clinical surfaces

Single–use hospital surgical gowns were produced by MediChoice, order no. 77752XL (45), made from fluid-resistant spunbond-meltdown-spunbond (SMS) polypropylene laminate at AAMI PB70:2012 (46) standard at level 2. To test the transfer of spores to and from the gowns, gowns were aseptically cut into 7×7 cm sections and testing performed within a drawn circle of 2 cm diameter to confer with the surface area of the hospital grade 2B stainless steel discs and vinyl flooring used in this study.

Spore Transfer to Hospital Surgical Gowns

To test the number of spores transferred to the hospital surgical gown after direct contact, U Spores and P Spores from strains DS1748, DS1813 and R20291 (Table 1) were produced at 1 x10^4 spores/ml. From these, 100 µl were spiked onto the gown surface in triplicate experiments and allowed to remain in static contact for 10 s, 30 s, 1 min, 5 min and 10 min before being removed and discarded. After contact with spores, each section of gown was aseptically mounted onto a plunger pre-affixed with a steel disc so that the disc was aligned with the test

area. A plate transfer test was then performed as described in Joshi *et al*., (28). A force of 100g was used as a simulated "touch" pressure.

Spore Transfer from spiked "high-touch" surfaces to Hospital Surgical gowns

To test the number of spores transferred to the surgical hospital gown from dry "high-touch" surfaces (hospital grade stainless steel and vinyl flooring), U and P spores were produced at concentrations of 1 x10^6 spores/ml. Sterilised hospital grade steel discs and vinyl flooring were inoculated with 100 µl of spores and allowed to dry completely for 120 min in a Category 2 Biosafety laminar flow cabinet. Sections of gown were then placed in contact with the steel and vinyl under 100g pressure for 10 sec, 30 sec and 1 min and the gown was then pressed onto the appropriate agar plate for 10 sec at 100 g pressure (28). All agar plates were then incubated for 48 hrs at 37 ^oC under anaerobic conditions. Following incubation colonies were counted and SFU per ml were calculated.

Exposure of Spores to Sodium Dichloroisocyanurate disinfectant

Spore suspensions (U and P) from strains DS1748, R20291 and DS1813 at a concentration of 1 x 10^6 spores per ml were exposed to 1000-ppm NaDCC for 10 minutes in liquid form (recommended contact time), neutralised with sodium thiosulfate and deposited onto sterile gowns. Spores were recovered as described previously (1, 22). Secondly, spores were also spiked onto the gown surface, as described in the spore transfer section above, and spores were spiked onto the surfaces of hospital stainless steel and hospital vinyl flooring, respectively, for each biological repeat and allowed to dry for 120 min in a Category 2 Biosafety laminar flow cabinet. The three spiked surfaces were then directly exposed to 100µl NaDCC at 1000-ppm for 10 minutes and neutralised with 1% sodium thiosulphate before plate transfer experiments were performed and spore recovery recorded. Three technical repeats of each experiment were

performed. Control experiments where spores were exposed to sodium thiosulfate, sterile deionised water and NaDCC alone were also performed.

Scanning electron microscopy

Gowns, steel and vinyl were analysed using scanning electron microscopy for the presence of characteristic spores before and after treatment with NaDCC. Spores which had not been exposed to NaDCC were used as a comparative control. Test surfaces were sputter coated with metal using a gold palladium sputtering target (60% Au and 40% Pd from Testbourne Ltd) and argon as the sputtering gas. Images were taken on a scanning electron microscope (Zeiss Sigma HD Field Emission Gun Analytical SEM) using an accelerating voltage of 5 kV. Over 100 individual spores were viewed per sample at magnifications of x 4, 890 and x 83,380.

Statistical Analysis

Data are expressed as means ± SEM. Paired T-tests, One way ANOVA, 2-way ANOVA and Mann-Whitney U tests were performed using Minitab 17.

Acknowledgements

Authors wish to acknowledge Cardiff University Earth and Ocean Sciences for assistance with electron microscopy studies. This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors. This work was supported by the Society for Applied Microbiology Summer studentship fund and by Robert Burky. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

References

1. Joshi L T, Welsch A, Hawkins J. & Baillie L. 2017. The effect of hospital biocide sodium dichloroisocyanurate on the viability and properties of Clostridium difficile spores. Applied Microbiology 65:199-205.
2. Fekety R, Shah A B. 1993. Diagnosis and treatment of Clostridium difficile colitis. *JAMA* 269:71-75.
3. Voth D E, Ballard J D. 2005. Clostridium difficile toxins: mechanism of action and role in disease. *Clin Microbiol Rev* 18:247–263.
4. Nelson R L, Suda K J, Evans C T. 2017. Antibiotic treatment for Clostridium difficile-associated diarrhoea in adults. *The Cochrane Database of Systematic Reviews* 3.
5. Lamont, J.T., Kelly, C.P. and Bakken, J.S., 2018. Clostridium difficile infection in adults: Clinical manifestations and diagnosis. *UpToDate*. Waltham, Mass.: *UpToDate*.
6. Hellickson, L.A, and Owens, K.L. (2007). Cross-contamination of *Clostridium difficile* spores on bed linen during laundering. Am. J. Infect. Control 35:32–33
7. Dawson, L.F., Valiente, E., Donahue, E.H., Birchenough, G. and Wren, B.W. (2011) Hypervirulent Clostridium difficile PCR-ribotypes exhibit resistance to widely used disinfectants. *PLoS ONE* 6:25754.
8. Eyre, D.W., Davies, K.A., Davis, G., Fawley, W.N., Dingle, K.E., De Maio, N., Karas, A., Crook, D.W., Peto, T.E., Walker, A.S. and Wilcox, M.H., 2018. Two Distinct Patterns of Clostridium difficile Diversity Across Europe Indicating Contrasting Routes of Spread. *Clinical Infectious Diseases*, p.ciy252.
9. Hall A J, Curns A T, McDonald L C, Parashar U D, Lopman B A. 2012. The roles of Clostridium difficile and Norovirus among gastroenteritis-associated deaths in the United States, 1999-2007. *Clinical Infectious Diseases* 55:216-223.

10. Lessa F C, Mu Y, Bamberg W M, *et al*. 2015. Burden of Clostridium difficile infection in the United States. *The New England Journal of Medicine*, 372:825-834.
11. Dubberke E R, Olsen M A. 2012. Burden of Clostridium difficile on the healthcare system. *Clinical Infectious Diseases* 55 (suppl 2):S88-S92.
12. Office for National Statistics. 2017. Deaths involving Clostridium difficile, England and Wales 1999-2012. Available at: http://www.ons.gov.uk/peoplepopulationandcommunity/birthsdeathsandmarriages/deaths/bulletins/deathsinvolvingclostridiumdifficilereferencetables. Accessed July 2018.
13. Office for National Statistics. 2014. Age-standardised rates for deaths involving Staphylococcus aureus and MRSA and number of deaths by annual registration quarter, England and Wales. Available at: https://www.ons.gov.uk/peoplepopulationandcommunity/birthsdeathsandmarriages/deaths/datasets/deathsinvolvingmrsaagestandardisedratesfordeathsinvolvingstaphylococcusaureusandmrsaandnumberofdeathsbyannualregistrationquarterenglandandwales. Accessed August 2018.
14. Davies K A, Longshaw C M, Davies G L, Bouza E, Barbut F, Barna Z, Delmée M, Fitzpatrick F, Ivanova K, Kuijper E, Macovei I S, Mentula S, Mastrantonio P, von Müller L, Oleastro M, Petinaki E, Pituch H, Norén T, Nováková E, Nyč O, Rupnik M, Schmid D, Wilcox M H. 2014. Underdiagnosis of Clostridium difficile across Europe: the European, multicentre, prospective, biannual, point-prevalence study of Clostridium difficile infection in hospitalised patients with diarrhoea (EUCLID). *Lancet Infect Dis* 14:1208-1219.

15. Best E L, Fawley W N, Parnell P, Wilcox M H. 2010. The potential for airborne dispersal of Clostridium difficile from symptomatic patients. *Clin Infect Dis* 50:1450–1457.
16. National Institute for Health and Care Excellence. 2014. Infection prevention and control. Available at: https://www.nice.org.uk/guidance/qs61. Accessed August 2018.
17. U.S. Department of Health and Human Services Centers for Disease Control and Prevention (CDC). 2003. Guidelines for Environmental Infection Control in Health-care Facilities. Available at: https://www.cdc.gov/infectioncontrol/pdf/guidelines/environmental-guidelines.pdf. Accessed August 2018.
18. World Health Organization. 2004. Practical Guidelines for Infection Control in Health Care Facilities. Available at: http://www.wpro.who.int/publications/docs/practical_guidelines_infection_control.pdf. Accessed August 2018.
19. Coates D. 1996. Sporicidal activity of sodium dichloroisocyanurate, peroxygen and glutaraldehyde disinfectants against Bacillus subtilis. *J Hosp Infect* 32:283–294.
20. Seymour I J, Appleton H. 2001. Foodborne viruses and fresh produce. *Journal of Applied Microbiology* 91:759–773.
21. Bloomfield S F, Arthur M. 1989. Effect of chlorine-releasing agents on *Bacillus subtilis* vegetative cells and spores. *Letters in applied microbiology* 83:101-104.
22. Conlon-Bingham G, Aldeyab M, Kearney M P, Scott M G, Baldwin N, McElnay J C. 2015. Reduction in the incidence of hospital-acquired MRSA following the introduction of a chlorine dioxide 275 ppm based disinfecting agent in a district general hospital. *Eur J Hosp Pharm* 23.

422 23. Department of Health and Public Health Laboratory Service Joint Working Group.
423 1994. Clostridium difficile Infection: Prevention and Management. BAPS Health
424 Publications Unit: Heywood, UK 1-49.
425 24. Guest Medical. 2017. Guest Medical Brochure. Available at: http://guest-
426 medical.co.uk/wp-content/uploads/2017/09/62774-Guest-Medical-Brochure17.pdf.
427 25. Fawley, W N, Underwood S, Freeman J, Baines S D, Saxton K, Stephenson K,
428 Owens R C, Wilcox M H. 2007. Efficacy of hospital cleaning agents and germicides
429 against epidemic clostridium difficile strains. *Infection Control and Hospital*
430 *Epidemiology* 28: 920-925.
431 26. Block C. 2004. The effect of Perasafe® and sodium dichloroisocyanurate (NaDCC)
432 against spores of *Clostridium difficile* and *Bacillus atrophaeus* on stainless steel and
433 polyvinyl chloride surfaces. *Journal of Hospital Infection* 57:144-148.
434 27. Virto R, Mañas P, Álvarez I, Condon S, Raso J. 2005. Membrane damage and
435 microbial inactivation by chlorine in the absence and presence of a chlorine-
436 demanding substrate. *Applied and Environmental Microbiology* 71:5022-5028.
437 28. Joshi L T, Phillips D S, Williams C F, Alyousef A, Baillie L. 2012. Contribution of
438 spores to the ability of *Clostridium difficile* to adhere to surfaces. *Appl Environ*
439 *Microbiol* 78:7671–7679.
440 29. Weber D J, Ruttala W A, Miller M B, Huslage K, Sickbert-Bennett E. 2010. Role of
441 hospital surfaces in the transmission of emerging health care-associated pathogens:
442 Norovirus, *Clostridium difficile*, and *Acinetobacter* species. *Am J Infect Control*
443 38:S25-S33.
444 30. Fawley W N, Parnell P, Verity P, Freeman J, Wilcox M H. 2005. Molecular
445 epidemiology of endemic Clostridium difficile infection and the significance of
446 subtypes of the United Kingdom epidemic strain. *J Clin Microbiol* 43:2685-2696.

31. Rutala, W.A. and Weber, D.J., 2001. A review of single-use and reusable gowns and drapes in health care. Infection Control & Hospital Epidemiology, 22(4), pp.248-257.
32. Balci, F.S.K., 2016. Isolation gowns in health care settings: Laboratory studies, regulations and standards, and potential barriers of gown selection and use. American journal of infection control, 44(1), pp.104-111.
33. Snyder, G.M., Thorn, K.A., Furuno, J.P., Perencevich, E.N., Roghmann, M.C., Strauss, S.M., Netzer, G. and Harris, A.D., 2008. Detection of methicillin-resistant Staphylococcus aureus and vancomycin-resistant enterococci on the gowns and gloves of healthcare workers. Infection Control & Hospital Epidemiology, 29(7), pp.583-589.
34. Wiener-Well, Y., Galuty, M., Rudensky, B., Schlesinger, Y., Attias, D. and Yinnon, A.M., 2011. Nursing and physician attire as possible source of nosocomial infections. American journal of infection control, 39(7), pp.555-559.
35. Husmark, U. and Rönner, U., 1992. The influence of hydrophobic, electrostatic and morphologic properties on the adhesion of Bacillus spores. Biofouling, 5(4), pp.335-344.
36. Barra-Carrasco, J. and Paredes-Sabja, D., 2014. Clostridium difficile spores: a major threat to the hospital environment. Future microbiology, 9(4), pp.475-486.
37. Edwards, A.N., Karim, S.T., Pascual, R.A., Jowhar, L.M., Anderson, S.E. and McBride, S.M., 2016. Chemical and stress resistances of Clostridium difficile spores and vegetative cells. Frontiers in microbiology, 7, p.1698.
38. Laflamme, C., Lavigne, S., Ho, J. and Duchaine, C., 2004. Assessment of bacterial endospore viability with fluorescent dyes. Journal of Applied Microbiology, 96(4), pp.684-692.

471 39. Stewart G C. 2015. The exosporium layer of bacterial spores: a connection to the
472 environment and the infected host. *Microbiology and Microcular Biology Reviews*
473 79:437-457.
474 40. Mora-Uribe, P., Miranda-Cárdenas, C., Castro-Córdova, P., Gil, F., Calderón, I.,
475 Fuentes, J.A., Rodas, P.I., Banawas, S., Sarker, M.R. and Paredes-Sabja, D., 2016.
476 Characterization of the adherence of Clostridium difficile spores: the integrity of the
477 outermost layer affects adherence properties of spores of the epidemic strain R20291
478 to components of the intestinal mucosa. Frontiers in cellular and infection
479 microbiology, 6, p.99).
480 41. Leggett, M.J., McDonnell, G., Denyer, S.P., Setlow, P. and Maillard, J.Y., 2012.
481 Bacterial spore structures and their protective role in biocide resistance. Journal of
482 applied microbiology, 113(3), pp.485-498.
483 42. Perez, J., Springthorpe, V.S. and Sattar, S.A., 2005. Activity of selected oxidizing
484 microbicides against the spores of Clostridium difficile: relevance to environmental
485 control. American journal of infection control, 33(6), pp.320-325.
486 43. Sorg J A, Sonenshein A L. 2010. Inhibiting the Initiation of Clostridium difficile
487 Spore Germination using Analogs of Chenodeoxycholic Acid, a Bile Acid. *Journal*
488 *of Bacteriology* 192:4983-90.
489 44. Miles A A, Misra S S, Irwin J O. 1938. The estimation of the bactericidal power of
490 blood. *J Hyg* 38:732–749.
491 45. MediChoice Product Catalog 2015. MediChoice, Owens & Minor Inc, Richmond,
492 Virginia. Available at: https://www.owens-
493 minor.com/globalassets/docs/medichoice-product-catalog.pdf. Accessed August
494 2018.

46. PB70, A.A.M.I., 2012. Liquid barrier performance and classification of protective apparel and drapes intended for use in health care facilities. Arlington, VA: Association for the Advancement of Medical Instrumentation.

Tables: Table 1 *Clostridium difficile* strains used in the present study.

C. difficile strain	PCR Ribotype	Source	Exosporium Presence	Relative Hydrophobicity
DS1813	027	Hinchingbrooke	Positive	77%
R20291	027	Stoke-Mandeville	Positive	62%
DS1748	002	Leeds	Negative	14%

Figures & Legends

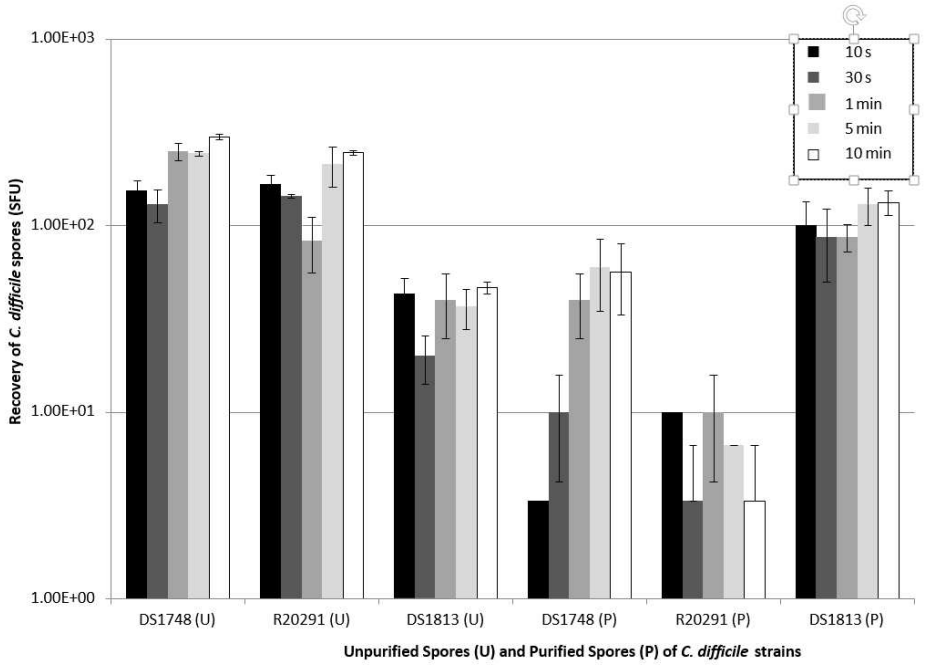
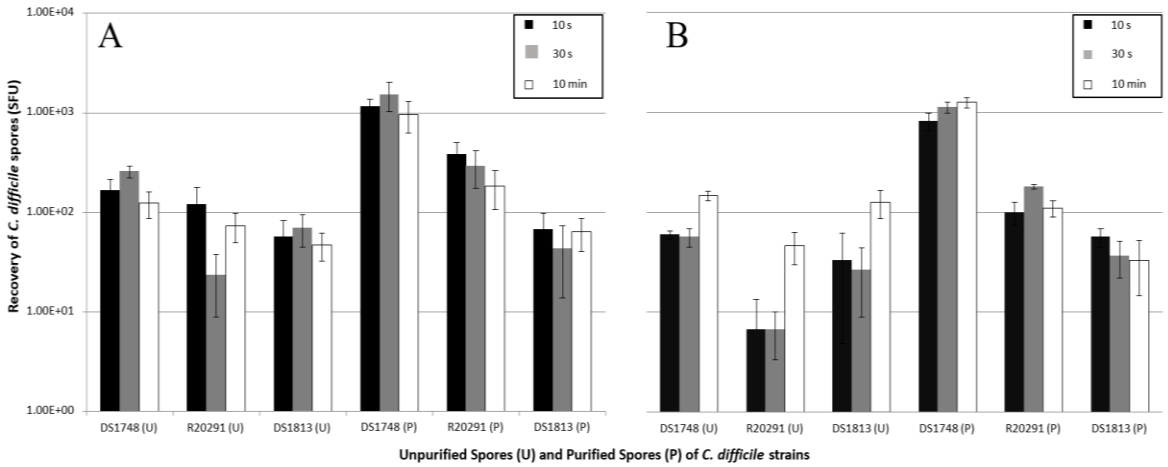


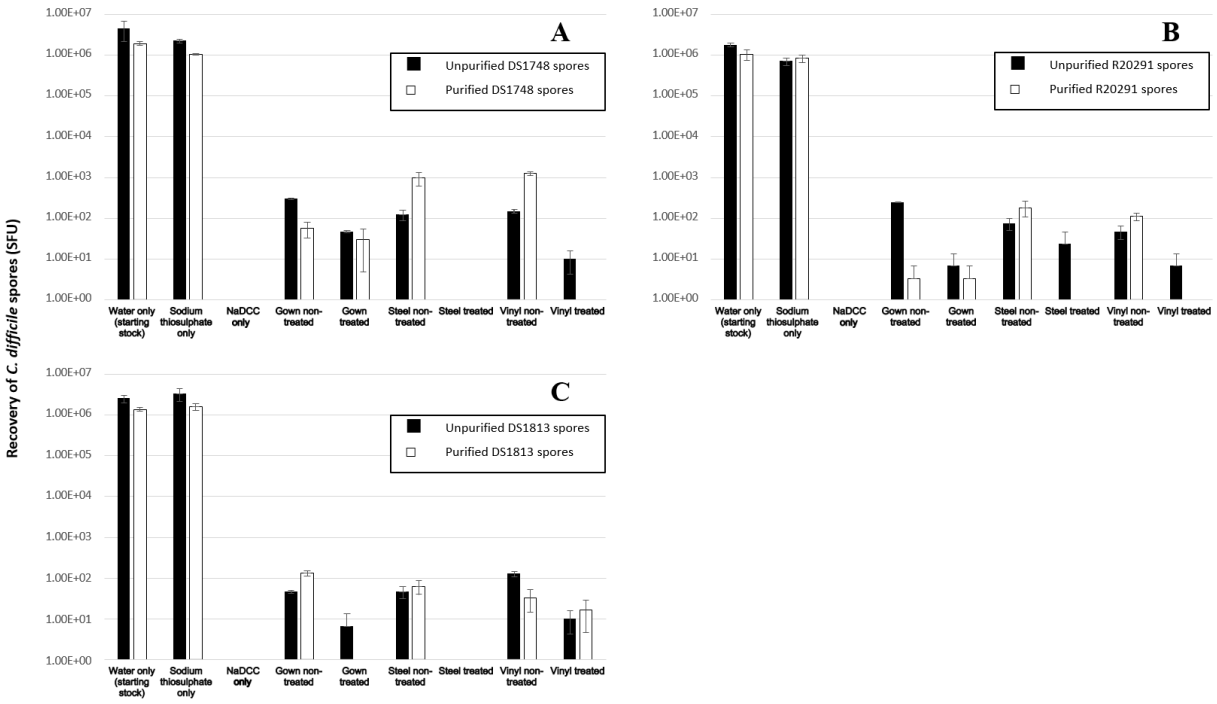
Figure 1: Recovery of two different *C. difficile* spore types (Unpurified [U] and Purified [P]) from spiked hospital surgical gowns. Spores were derived from strains DS1748, R20291 and

503 DS1813 and spores recovered after being exposed to the gowns at contact times ranging from
504 10 s to 10 min. Plots represent mean ± SEM (n = 3).



505

506 Figure 2: Transmission ability of two different *C. difficile* spore types between clinical surfaces.
507 Spores derived from strains DS1748, R20291 and DS1813 were spiked onto hospital stainless
508 steel and vinyl surfaces and their ability to transfer to hospital surgical gowns was tested.
509 Unpurified (U) and purified (P) spores were recovered via transfer test from (A) hospital grade
510 stainless steel and (B) hospital vinyl flooring using hospital surgical gowns applied at a
511 pressure of 100g. Contact times ranged at 10s, 30 s and 1 min. Plots represent mean ± SEM
512 (n = 3).



513

514 Figure 3: Recovery of unpurified and purified *C. difficile* spores from spiked clinical surfaces
515 after treatment with 1000-ppm NaDCC for 10 min. Transfer testing was used to recover U and
516 P spores of *C. difficile* strains (A) DS1748, (B) R20291 and (C) DS1813 from hospital surgical
517 gowns after contact with: spores suspended in NaDCC applied to sterile gown, spiked gown
518 exposed to NaDCC, spiked hospital stainless steel and hospital vinyl flooring exposed to
519 NaDCC. The inoculum was used as the positive control (water only) and was also suspended
520 in sodium thiosulfate to ensure no cross reactivity. Plots represent mean ± SEM (n = 3).

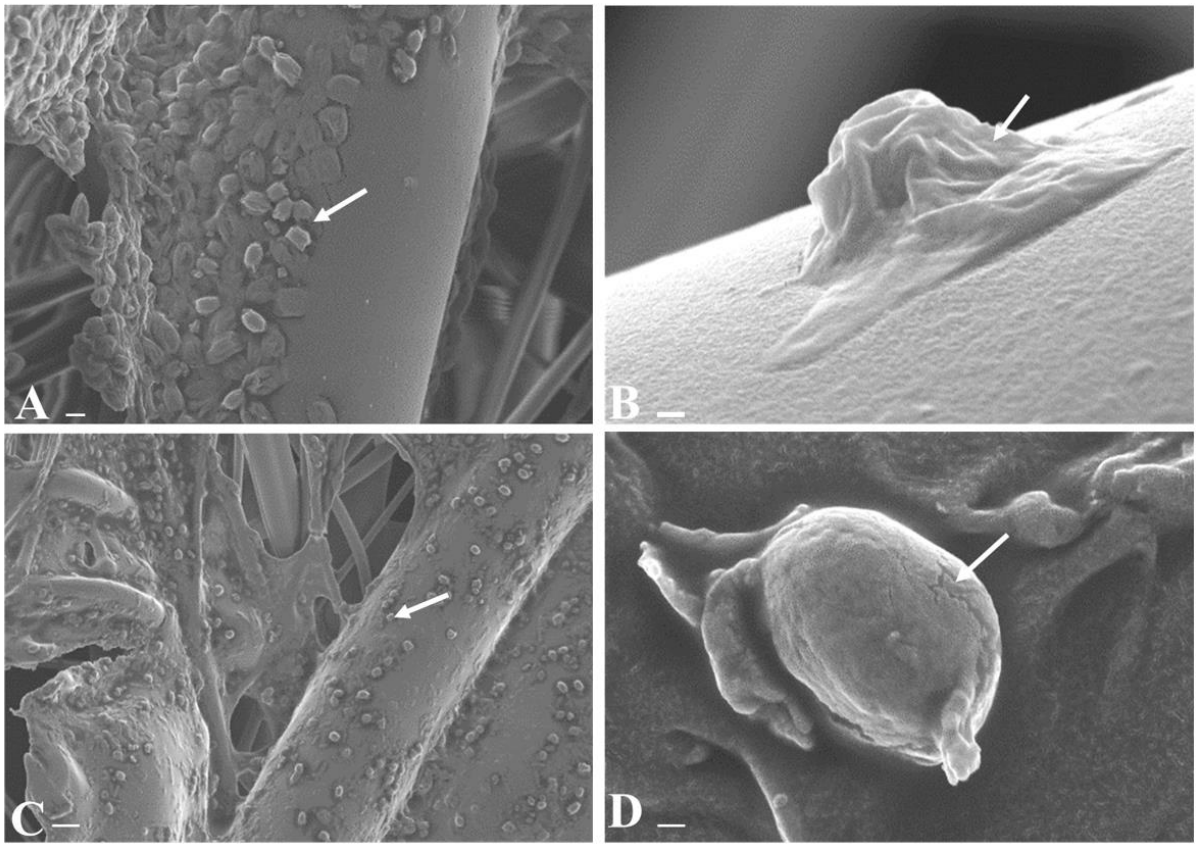


Figure 4: Scanning electron micrographs of *C. difficile* spores present on spiked hospital surgical gowns before and after treatment with NaDCC at 1,000-ppm for 10 min. Images depict untreated (A) R20291 U spores on surgical gown fibres and (B) R20291 P single spore and NaDCC treated (C) R20291 U spores on surgical gown fibres, (D) R20291 U single spore. Arrows highlight spores adhered to gown fibres before (A) and after NaDCC treatment in (C), and morphological changes in exosporium before (B) and after NaDCC treatment (D). Scale bars in B and D are 200 nm, in A 2 µm, and in C 10 µm.

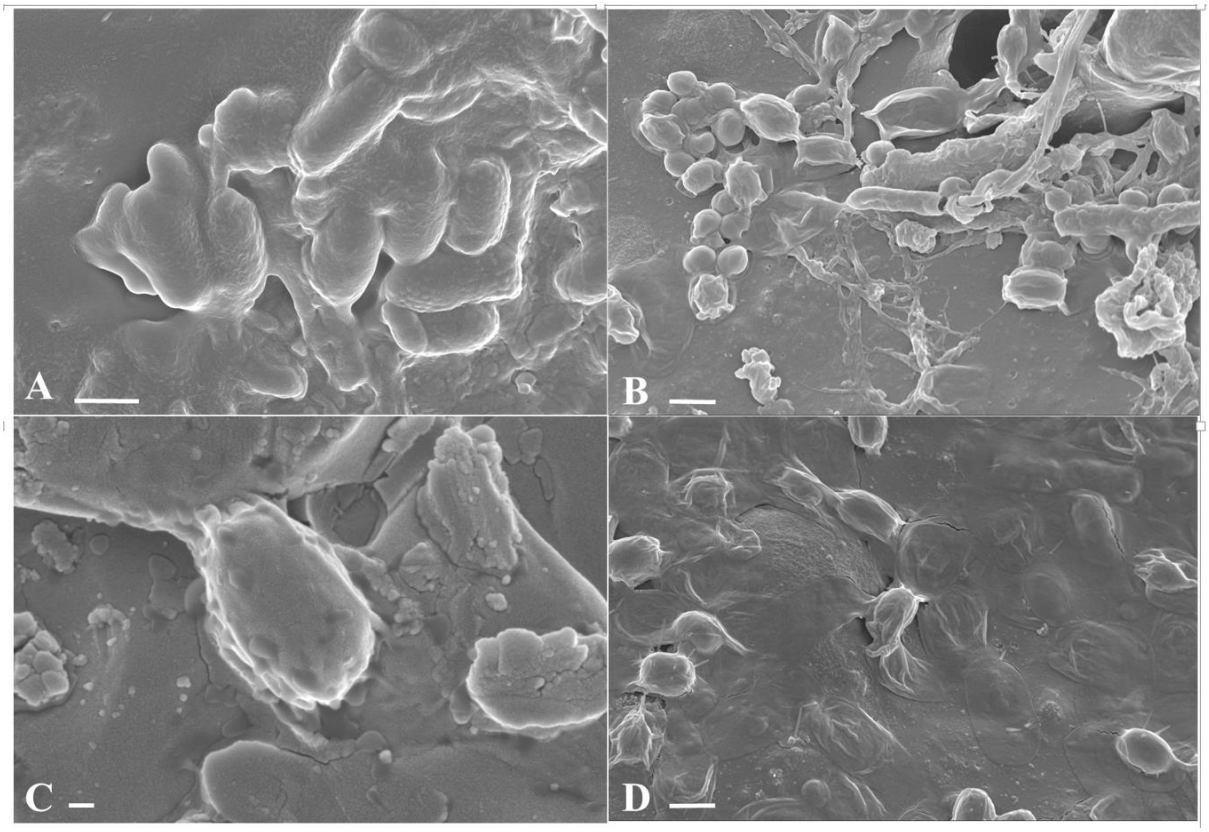


Figure 5: Scanning electron micrographs of *C. difficile* spores present on spiked hospital stainless steel and floor vinyl before and after treatment with NaDCC at 1,000 ppm for 10 min. Images are NaDCC-treated (A) DS1813 P spores on stainless steel; (B) DS1748 U Spores on floor vinyl; (C) DS1748 U spores on stainless steel and (D) R20291 U spores on floor vinyl. Arrows highlight areas in the exosporium layer. Scale bars in A, B and D are 1µm, and in C 200 nm.