1 **Biocide resistance and transmission of *Clostridium difficile* spores spiked onto clinical**

2 **surfaces from an American healthcare facility**

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

12   **Abstract**

13   *Clostridium difficile* is the primary cause of antibiotic-associated diarrhea globally. In

14   unfavourable environments the organism produces highly resistant spores which can survive

15   microbicidal insult. Our previous research determined the ability of *C. difficile* spores to adhere

16   to clinical surfaces; finding that spores had marked different hydrophobic properties and

17   adherence ability. Investigation into the effect of the microbicide sodium dichloroisocyanurate

18   on *C. difficile* spore transmission revealed that sub-lethal concentrations increased spore

19   adherence without reducing viability. The present study examined the ability of spores to

20   transmit across clinical surfaces and their response to an in-use disinfection concentration of

21   1,000-ppm of chlorine-releasing agent sodium dichloroisocyanurate. In an effort to understand

22   if these surfaces contribute to nosocomial spore transmission, surgical isolation gowns,

23   hospital-grade stainless steel and floor vinyl were spiked with 1×10^6 spores/ml of two types

24   of *C. difficile* spore preparations: crude spores and purified spores. The hydrophobicity of each

25   spore type versus clinical surface was examined via plate transfer assay and scanning electron

26   microscopy. The experiment was repeated and spiked clinical surfaces were exposed to 1,000-

27   ppm sodium dichloroisocyanurate at the recommended 10-min contact time. Results revealed

28   that the hydrophobicity and structure of clinical surfaces can influence spore transmission and

29   that outer spore surface structures may play a part in spore adhesion. Spores remained viable

30   on clinical surfaces after microbicide exposure at the recommended disinfection concentration

31   demonstrating ineffectual sporicidal action. This study showed that *C. difficile* spores can

32   transmit and survive between varying clinical surfaces despite appropriate use of microbicides.

33   **IMPORTANCE**

34   *Clostridium difficile* is a healthcare-acquired organism and the causative agent of antibiotic-

35   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

60   2011 (9). In 2008 alone the estimated cost related to CDI within the United States to health-

61   care facilities was $4.8 billion, ignoring the additional cost to other facilities such as care homes

62   (10). A similar pattern of statistics can be seen in England, with an increase from 1,149 *C.*

63   *difficile*-related deaths in 2001 rising to 7,916 in 2007 (11).

64   In response to increasing CDI infection rates, stringent infection control procedures were

65   implemented within hospital environments in England which resulted in a decline in mortality

66   to 1,487 in 2012. This figure surpasses that of MRSA and non-specified *Staphylococcus aureus*

67   infection mortality (262 in 2012) (12) and thus is still a major source of concern globally.

68   Despite implementation of appropriate surveillance and infection control procedures the

69   organism still causing significant levels of morbidity and mortality across nosocomial

70   environments (13).

71   Incidence of CDI is directly affected by the ability of *C. difficile* to produce resistant spores

72   which can survive on organic and inorganic surfaces for months and remain viable (6). A major

73   source of CDI and transmission in the healthcare environment is through the faecal to oral

74   route; often via the contamination of surfaces. As many as 1×10^7 spores per gram faeces are

75   released into the environment by infected patients through airborne dispersal and soiling further

76   adding to the bioburden (14). Possible causes of transmission include inappropriate biocide

77   use, lack of adherence to infection control guidelines and varying standards of practice across

78   healthcare facilities globally (15, 16, 17).

79   Chlorine-releasing agents (CRAs) are the predominant form of biocide used in healthcare

80   facilities to disinfect surfaces; namely sodium hypochlorite (NaOCl) and sodium

81   dichloroisocyanurate (NaDCC) (18). These microbicides are fast-acting in aqueous solutions

82   and are relatively inexpensive (19). Low concentrations of 50-ppm available chlorine have

83   shown to kill >99% of vegetative bacterial cells *in vitro*. In addition, when 275-ppm chlorine

84　was applied to a clinical environment there was a significant reduction in hospital-acquired

85　infections from non-spore forming bacteria (20, 21). However, the inactivation of spores

86　requires much higher concentrations with the current recommendation for application of

87　NaDCC in hospitals in England being 1,000-ppm available chlorine for 10 minutes to

88　deactivate spores of *C. difficile* and *Bacillus* species (22, 23). Although the working

89　concentration of NaDCC has shown to be effective in liquid culture (24), its application to

90　working surfaces is less efficient for inactivation of spores (25) and this reduced activity is

91　exacerbated by the presence of organic substances, such as bodily fluids and faeces, which

92　have a neutralising effect on the biocide (26). The mechanism of action of chlorine-releasing

93　biocides is poorly understood; however, it has been suggested that their action may be due to

94　strong oxidative ability, their effect on cell membranes and inhibition of enzymatic reactions

95　(27).

96　Our previous study showed that adherence of *C. difficile* spores to inorganic surfaces increased

97　when spores were exposed to sub-lethal concentrations (500-ppm available chlorine) of sodium

98　dichloroisocyanurate (27). This increase was more pronounced for strain DS1748 (002

99　ribotype) which is not known to produce an exosporium outer layer (28) and suggests that when

100　spores are exposed to sub-lethal levels of biocide they may inadvertently become more

101　adherent to inorganic surfaces. The purpose of the present study was to assess the transfer

102　ability of *C. difficile* spores from clinical surfaces pre- and post-biocide exposure. Surfaces

103　tested include hospital isolation gowns, hospital grade stainless steel and vinyl flooring

104　routinely used within the United States. Spore recovery from spiked clinical surfaces was

105　investigated using a plate transfer assay. Clinical surfaces spiked with spores were exposed to

106　NaDCC to determine sporicidal efficacy and the presence of spores on each clinical surface

107　pre and post NaDCC treatment was examined using scanning electron microscopy.

108　**Results**

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

110   To examine the ability of *C. difficile* spores (U and P derived from strains DS1748, R20291

111   and DS1813) to adhere to, and subsequently transfer from hospital surgical gowns, spores were

112   applied directly to the surgical gowns in liquid for 10 s, 30 s, 1 min, 5 min and 10 min before

113   being removed and discarded (Figure 1, Figure 4A and C). This experiment was designed to

114   mimic transfer of infectious bodily fluids in the clinical setting and assess the potential for

115   onward transmission to patients. There was no significant difference between the amount of

116   spores (U and P) recovered from the gowns and the contact time of the spores to the gowns;

117   suggesting that the process of spore transfer between surfaces occurred within the first 10

118   seconds of contact with the gown (two-way ANOVA; $p = 0.696$). From Figure 1 it appears as

119   though the recovery of DS1748 P Spores increased with contact time; however, this was not

120   statistically significant (one-way ANOVA; $p = 0.144$). Generally, U spore recovery was

121   significantly higher than that of P spores (two-way ANOVA; $p < 0.001$); however, the

122   exception to this trend was the increased recovery of DS1813 P spores when compared to U

123   spores of the same strain (one-way ANOVA; $p < 0.001$). There were no significant differences

124   in spore recovery between DS1748 and R20291 for either U spores or P spores.

125

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

127   To establish whether hospital-grade stainless steel surfaces and vinyl flooring surfaces act as

128   fomites for *C. difficile* spore transmission in the clinical setting, sterile sections of hospital

129   surgical gowns were placed in direct contact with hospital-grade stainless steel and vinyl

130   flooring spiked with 1×10^5 spores and spore recovery from the surgical hospital gowns

131   assessed. The contact times were reduced to 10 s, 30 s and 1 min due to results presented in

132   Figure 1 which confirm that the length of contact time had no significant effect on spore

133   recovery. Similarly, there remained no significant difference in spore recovery from steel and

134   vinyl between the contact times used and the amount of spores recovered from the strains

135   examined (Figure 2) (two-way ANOVA; p = 0.892 and p = 0.904 for steel and vinyl,

136   respectively). Spore recovery of U DS1748 was significantly higher from both stainless steel

137   surfaces (one-way ANOVA; p = 0.034) and vinyl flooring (one-way ANOVA; p < 0.001) when

138   compared to the other strains. DS1748 P spore recovery was higher on stainless steel (one-way

139   ANOVA; p < 0.001) and vinyl flooring (one-way ANOVA; p < 0.001) than of R20291 and

140   DS1813. DS1748 P spore recovery was approximately 10-fold higher than that of the U Spore

141   equivalent (two-way ANOVA; p < 0.001).

142

143   **Sporicidal efficiency of sodium dichloroisocyanurate (NaDCC)**

144   Two types of spore suspension from three *C. difficile* strains (DS1748, R20291 and DS1813)

145   were exposed to the recommended in-use concentration of NaDCC in solution (1,000-ppm)

146   and spore viability was determined. From Figure 3 it can be seen that there was no recovery of

147   spores which had been treated in liquid form and then spiked onto gowns. Moreover, recovery

148   of NaDCC-treated U spores from the spiked and directly-treated hospital surgical gowns were

149   lower across the three strains tested when compared to non-treated spores, with the lowest

150   relative recovery from strain R20291 (Student t-test; p < 0.005). Scanning Electron Microscopy

151   (SEM) images in Figure 4A and 4C support this by showing adhered spores on the fibres of

152   the gowns from strain R20291 before and after treatment with the recommended concentration

153   of NaDCC. Interestingly, Figure 4B shows a single P spore of R20291 after NaDCC treatment

154   with a visible exosporial layer, while Figure 4D shows a U spore of R20291 after treatment

155   that has no visible evidence of an exosporial layer. These differences in spore exosporium show

156   distinct morphological variations within the R20291 strain; but may not necessarily be as a

157   result of NaDCC exposure. It is possible that any damage to the exosporium after NaDCC

158   exposure is not visible via SEM (Figures 4B); thus there is a possibility that NaDCC may have

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

161   Decreased sporicidal activity was observed for strains tested with NaDCC on the varying
162   clinical surfaces (Figure 3). Similar results were observed with DS1813 P spores, but not for P
163   spores of DS1748 and R20291. There was detectable recovery of R20291 U Spores (~73 to
164   ~23 SFU) after NaDCC treatment on stainless steel; although this was not significantly
165   different when compared to the lack of recovery of the other U strains tested (Mann-Whitney
166   Test; p = 0.40). Despite the lack of DS1813 spore recovery from stainless steel surfaces after
167   NaDCC exposure (Figure 3C), spores were still present on the steel surfaces indicating lack of
168   viability (Figure 5A).

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

175   **Discussion**

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

183   were able to transfer and adhere to fibres of the polypropylene spun gowns when spiked in a

184   liquid medium. As there was no significant difference between the contact time of the spores

185   and the recovery of spores from the gown, it appears as though the process of spore transfer

186   occurred rapidly within the first 10 seconds of contact when examining spore recovery from

187   spiked liquid, hospital grade stainless steel and vinyl flooring, respectively. This suggests a

188   clear need to ensure appropriate decontamination of surfaces that a contaminated gown may

189   come into direct contact with in a clinical setting.

190   The ability of microorganisms to travel through fabrics is related to the physico-chemical

191   properties of the gowns and the characteristics of the microorganism (32). Another interesting

192   observation from this study is the rapid ability of the spores to move from one hydrophobic

193   surface to another hydrophobic surface i.e. fluid-resistant gowns and stainless steel which

194   suggests that the more hydrophobic spores interacted better with the stainless steel surfaces

195   than the gowns (Table 1, Figure 1). Whether this is related to steel surface structure as opposed

196   to gown structure, or the hydrophobic interactions between (i) the individual strains (which

197   possess varying relative hydrophobicity; Table 1) (ii) the liquid and (iii) each test surface

198   warrants further investigation at the molecular level. It is also clear that the single-use gowns

199   act as fomites for *C. difficile* spore transmission. Not only do spores of all strains rapidly attach

200   to the gown fibres from liquid and dry clinical surfaces but the single-use gowns are then

201   ineffective at trapping spores within their fibres and preventing the onward transmission of

202   spores as demonstrated by spore recovery from the gowns (Figures 1& 2). While this ability

203   differs between strains, it does suggest that the adherence ability of the spore to individual

204   gown fibres may be affected by spore hydrophobic properties and exosporium layer which is

205   known to aid spore adherence on hospital surfaces (Table 1; 28). Results also suggest that *C.*

206   *difficile* spores, after microbicidal exposure to NaDCC at the recommended contact time and

207   concentration, can continue to remain viable, adhere and transmit via hospital gowns (Figure

208   4A & 4C; 1, 28, 35). This highlights the importance of ensuring that single-use surgical

209   isolation gowns are used appropriately in infection prevention and control; i.e. that gowns are

210   adorned upon entering and disposed of when exiting a single room to prevent onward spore

211   transmission and incidence of CDI (36).

212   Despite using recommended concentrations of NaDCC to decontaminate gowns, stainless steel

213   surfaces and floor vinyl after spore exposure, spores were still visibly attached to each surface

214   and were viable upon culture (Figures 4 & 5). Decontamination and appropriate cleaning of

215   surfaces is critical in managing the spread of CDI to patients from spores (37). It can be

216   speculated that the hydrophobic properties and weave of the gown fabric may have prevented

217   exposure of spores to NaDCC which explains the increased spore recovery; however this would

218   need to be examined further by exploring the use of fluorescence-based spore viability tests

219   (38). The smooth surfaces of steel and vinyl would theoretically make NaDCC treatment more

220   effective by increasing the test surface area; however, the occurrence of viable spores on both

221   treated steel and vinyl surfaces conflicts with this hypothesis and clearly evidences spore

222   resistance to NaDCC. This resistance was found for all three strains tested and was not limited

223   to hypervirulent R20291 027 PCR ribotype strains (7) (Table 1). Our results confirm that

224   working concentrations of sporicides (with active concentrations of chlorine) applied at the

225   appropriate contact times may not kill *C. difficile* spores. The ability of microbicides, such as

226   CRA's, to kill *C. difficile* spores has been examined previously with similar results (7, 25, 26,

227   38).

228   Spores which possess an exosporium-like structure have been demonstrated to have increased

229   adhesion to surfaces *in vivo* and *in vitro*; associated with increased hydrophobicity of the spore

230   (28, 35, 39). The exact function of the exosporium-like structures on certain strains of *C.*

231   *difficile* spores has yet to be fully elucidated; however, its role in adhesion to intestinal mucosal

232   cells and in *Bacillus* spore adhesion has been more clearly defined (35, 39, 40). Our previous

233   study established that exosporium-positive spores (DS1813) were more resistant to NaDCC at

234   sub-lethal concentrations than exosporium-negative spores (DS1748) (1, 28), which appears to

235   correlate with the theory that the exosporium layer confers a protective barrier to the spore,

236   preventing it from being damaged (41). It has also been hypothesised that exposure of spores

237   to NaDCC at inappropriate concentrations and contact times can alter and increase spore

238   adhesion ability (1). In the present study, while we observed a lack of exosporium-negative

239   DS1748 and exosporium-positive DS1813 spore recovery from hospital stainless steel, SEM

240   image (Figure 5A) revealed the presence of DS1813 spores adhered onto the stainless steel

241   surface, and the presence of possible damaged spores of DS1748 (Figure 5C). Indeed, the

242   presence of a small number of spores following NaDCC treatment could still produce recovery

243   of zero viable spores. Moreover, the viability of spores from all strains tested was also observed

244   after NaDCC treatment of vinyl flooring (Figure 3). This strongly indicates that recovery of

245   spores from stainless steel and vinyl, two very different materials, has been affected by biocide

246   exposure, either due to biocidal killing or reduced spore adherence; however, the exact

247   mechanism of spore adherence and biocidal activity of NaDCC upon the exosporium layer and

248   spore ultrastructure has yet to be determined.

249   As seen in Figures 4 and 5, there are exosporium-like projections present on R20291 spores

250   that increase the material surface-spore contact area which correlates to data from other studies

251   (41). It is possible that these projections may increase spore adherence and perhaps biocide

252   resistance by protecting the core from chemical effects. Moreover, as NaDCC was completely

253   effective when spores were exposed in liquid form (Figure 3) when compared to the spore

254   recovery post exposure from spiked surfaces, attests to the potential the exosporium may have

255   for protection of the spore from biocide exposure. Interestingly, hypervirulent DS1813 and

256   R20291 strains have shown an increased adherence ability throughout this study comparative

257   to DS1748; suggesting exosporium- positive spores adhere better and more rapidly with first-

258   contact to the test surface (Table 1). Additionally, unpurified R20291 spores were recovered

259   from all surfaces tested post-NaDCC exposure which demonstrates the spore's ability to remain

260   viable after biocide exposure (Fig 2 & 3). This concurs with previous studies that have

261   demonstrated CRA resistance in PCR Ribotypes 017, 012 and 027 (R20291) (7). Mechanical

262   removal to remove spores from clinical surfaces has been shown to be effective in studies,

263   however, this may not be appropriate with gowns as they are designed for single-use; therefore

264   effective and immediate disposal of surgical gowns after use needs to be considered when

265   preventing transmission of CDI (6, 25). The impact of the microbicide upon spore structure

266   and resistance warrants further research to fully understand the mechanisms of resistance and

267   to establish up-to-date and effective decontamination protocols. Moreover, our research

268   suggests that the *C. difficile* exosporium may play a key role in biocide resistance of spores and

269   thus could be a potential target for development of novel sporicidal disinfectants.

270   **Materials and Methods**

271   **Growth conditions, *Clostridium difficile* strains and spore production**

272   *C. difficile* cultures were incubated anaerobically at 37 °C for 48 hours in a BugBox Plus

273   anaerobic workstation (Ruskinn Technology Ltd. United Kingdom) using an 85% nitrogen,

274   10% carbon dioxide and 5% hydrogen gas mix. Clinical isolates of *C. difficile* (PCR Ribotypes

275   027 and 002) used in this study are described in Table 1 and were obtained from the Anaerobic

276   Reference Unit, University Hospital Wales, Cardiff, UK. Unless otherwise stated, all

277   organisms were stored as spores at 4 °C. All experiments described were conducted in

278   triplicate. *C. difficile* spores were produced according to two methods to generate

279   unpurified/crude and purified spore preparations; spores produced *via* Perez et al 2005 (42)

280   methodology were designated as unpurified (U) spores due to being harvested via water-

281   washing and containing vegetative and spore forms of the organism. These were deemed

282 representative of *C. difficile* commonly encountered within clinical environments. Spores were

283 produced on reduced brain heart infusion (BHI) agar and BHI broth (Oxoid Ltd, Basingstoke,

284 United Kingdom) each supplemented with the germinant 0.1% (w/v) sodium taurocholate (28).

285 Purified spores (P spores) were produced as described by Sorg and Sonenshein (2010) (43).

286 Briefly, *C. difficile* strains were cultured on reduced BHI agar with 5 g/L yeast extract and

287 0.1% L-cysteine and were examined after four days anaerobic incubation for characteristic

288 colonies. Spores were harvested using sucrose density-washing. Spore purity was confirmed

289 via phase contrast microscopy. Spore concentration was determined via drop count as described

290 by Miles *et al*. (44) and mean spore-forming units (SFU) per ml calculated (28).

Preparation of clinical surfaces

292 Single–use hospital surgical gowns were produced by MediChoice, order no. 77752XL (45),

293 made from fluid-resistant spunbond-meltdown-spunbond (SMS) polypropylene laminate at

294 AAMI PB70:2012 (46) standard at level 2. To test the transfer of spores to and from the gowns,

295 gowns were aseptically cut into 7×7 cm sections and testing performed within a drawn circle

296 of 2 cm diameter to confer with the surface area of the hospital grade 2B stainless steel discs

297 and vinyl flooring used in this study.

Spore Transfer to Hospital Surgical Gowns

299 To test the number of spores transferred to the hospital surgical gown after direct contact, U

300 Spores and P Spores from strains DS1748, DS1813 and R20291 (Table 1) were produced at 1

301 $\times 10^4$ spores/ml. From these, 100 µl were spiked onto the gown surface in triplicate experiments

302 and allowed to remain in static contact for 10 s, 30 s, 1 min, 5 min and 10 min before being

303 removed and discarded. After contact with spores, each section of gown was aseptically

304 mounted onto a plunger pre-affixed with a steel disc so that the disc was aligned with the test

305   area. A plate transfer test was then performed as described in Joshi *et al*., (28). A force of 100g

306   was used as a simulated "touch" pressure.

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

308   To test the number of spores transferred to the surgical hospital gown from dry "high-touch"

309   surfaces (hospital grade stainless steel and vinyl flooring), U and P spores were produced at

310   concentrations of 1 x10^6 spores/ml. Sterilised hospital grade steel discs and vinyl flooring were

311   inoculated with 100 µl of spores and allowed to dry completely for 120 min in a Category 2

312   Biosafety laminar flow cabinet. Sections of gown were then placed in contact with the steel

313   and vinyl under 100g pressure for 10 sec, 30 sec and 1 min and the gown was then pressed onto

314   the appropriate agar plate for 10 sec at 100 g pressure (28). All agar plates were then incubated

315   for 48 hrs at 37 °C under anaerobic conditions. Following incubation colonies were counted

316   and SFU per ml were calculated.

Exposure of Spores to Sodium Dichloroisocyanurate disinfectant

318   Spore suspensions (U and P) from strains DS1748, R20291 and DS1813 at a concentration of

319   1 x 10^6 spores per ml were exposed to 1000-ppm NaDCC for 10 minutes in liquid form

320   (recommended contact time), neutralised with sodium thiosulfate and deposited onto sterile

321   gowns. Spores were recovered as described previously (1, 22). Secondly, spores were also

322   spiked onto the gown surface, as described in the spore transfer section above, and spores were

323   spiked onto the surfaces of hospital stainless steel and hospital vinyl flooring, respectively, for

324   each biological repeat and allowed to dry for 120 min in a Category 2 Biosafety laminar flow

325   cabinet. The three spiked surfaces were then directly exposed to 100µl NaDCC at 1000-ppm

326   for 10 minutes and neutralised with 1% sodium thiosulphate before plate transfer experiments

327   were performed and spore recovery recorded. Three technical repeats of each experiment were

328 performed. Control experiments where spores were exposed to sodium thiosulfate, sterile

329 deionised water and NaDCC alone were also performed.

330 **Scanning electron microscopy**

331 Gowns, steel and vinyl were analysed using scanning electron microscopy for the presence of

332 characteristic spores before and after treatment with NaDCC. Spores which had not been

333 exposed to NaDCC were used as a comparative control. Test surfaces were sputter coated with

334 metal using a gold palladium sputtering target (60% Au and 40% Pd from Testbourne Ltd) and

335 argon as the sputtering gas. Images were taken on a scanning electron microscope (Zeiss Sigma

336 HD Field Emission Gun Analytical SEM) using an accelerating voltage of 5 kV. Over 100

337 individual spores were viewed per sample at magnifications of x 4, 890 and x 83,380.

338 **Statistical Analysis**

339 Data are expressed as means ± SEM. Paired T-tests, One way ANOVA, 2-way ANOVA and

340 Mann-Whitney U tests were performed using Minitab 17.

341 **Acknowledgements**

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498   **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%

499   **Figures & Legends**
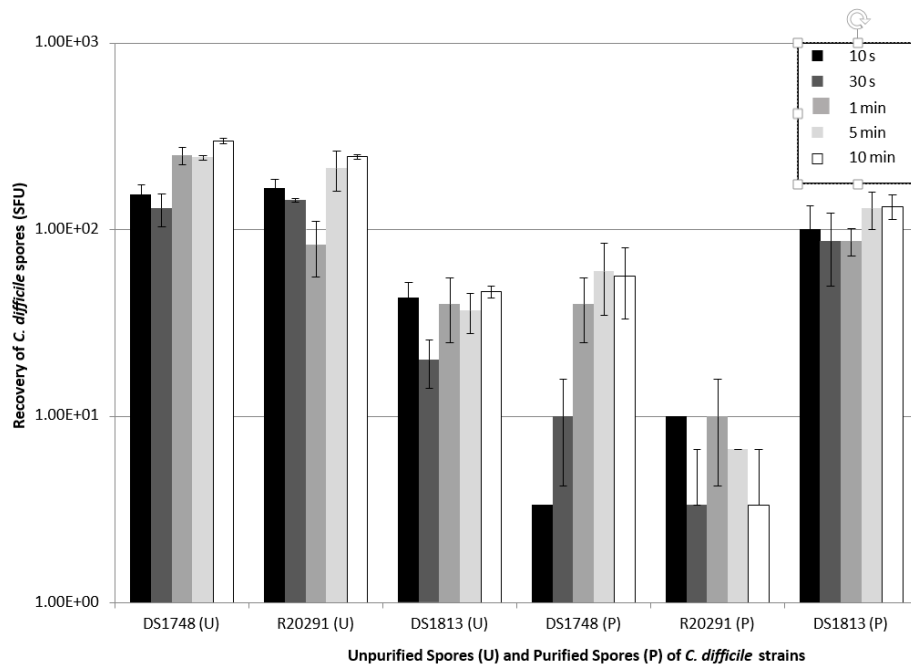
500

501   Figure 1: Recovery of two different *C. difficile* spore types (Unpurified [U] and Purified [P])

502   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).
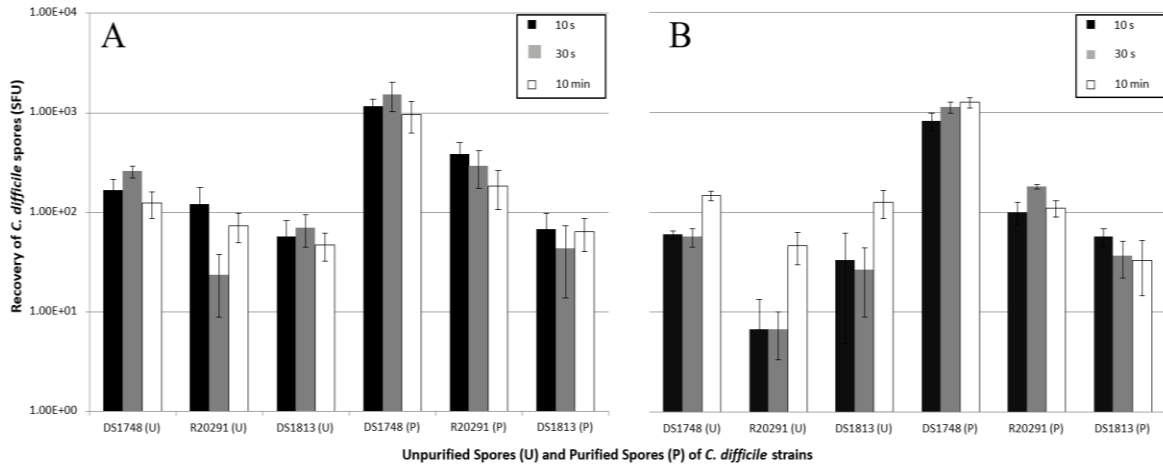
505

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
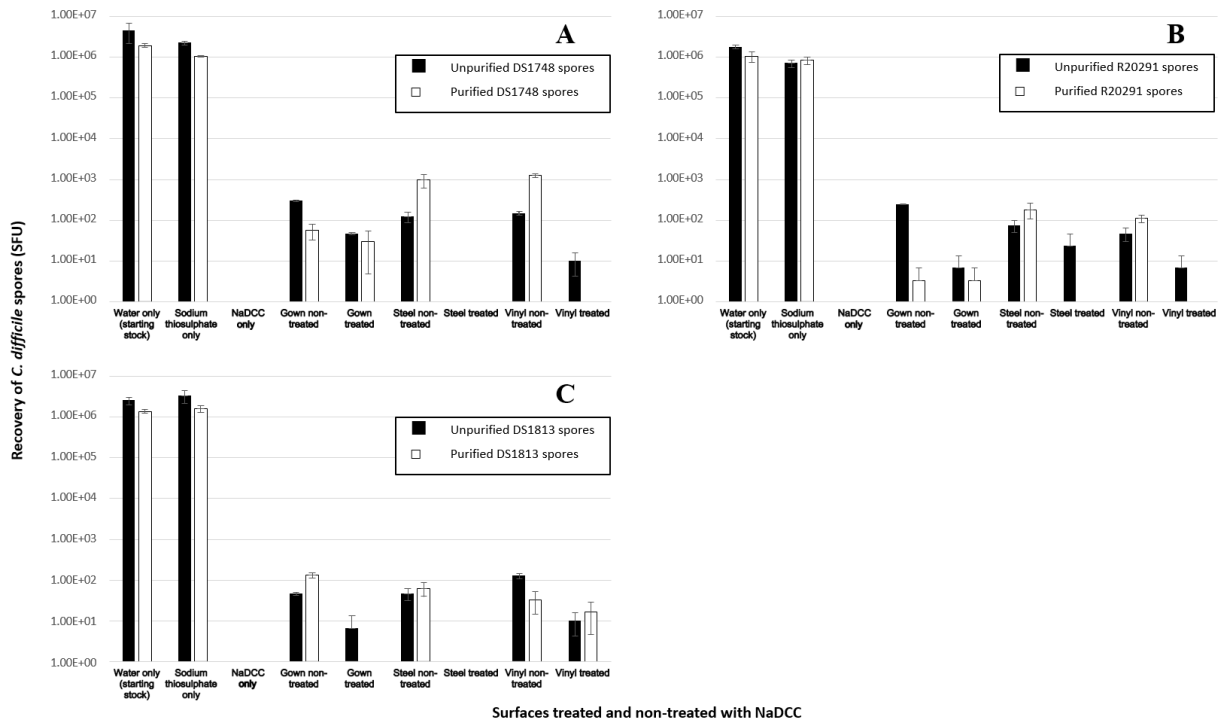
Figure 3: Recovery of unpurified and purified *C. difficile* spores from spiked clinical surfaces

after treatment with 1000-ppm NaDCC for 10 min. Transfer testing was used to recover U and

P spores of *C. difficile* strains (A) DS1748, (B) R20291 and (C) DS1813 from hospital surgical

gowns after contact with: spores suspended in NaDCC applied to sterile gown, spiked gown

exposed to NaDCC, spiked hospital stainless steel and hospital vinyl flooring exposed to

NaDCC. The inoculum was used as the positive control (water only) and was also suspended

in sodium thiosulfate to ensure no cross reactivity. Plots represent mean ± SEM (n = 3).

521
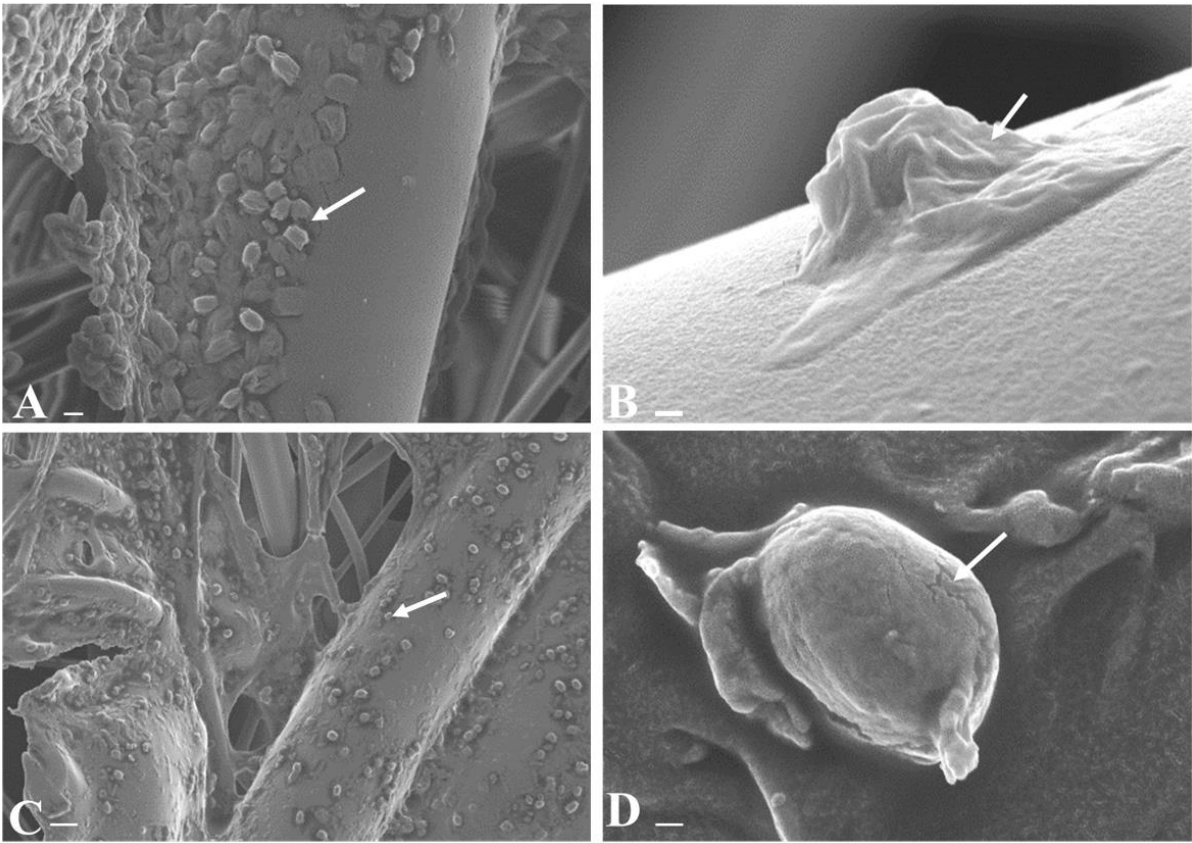
Figure 4: Scanning electron micrographs of *C. difficile* spores present on spiked hospital surgical gowns before and after treatment with NaDCC at 1,000-ppm for 10 min. Images depict untreated (A) R20291 U spores on surgical gown fibres and (B) R20291 P single spore and NaDCC treated (C) R20291 U spores 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.

529
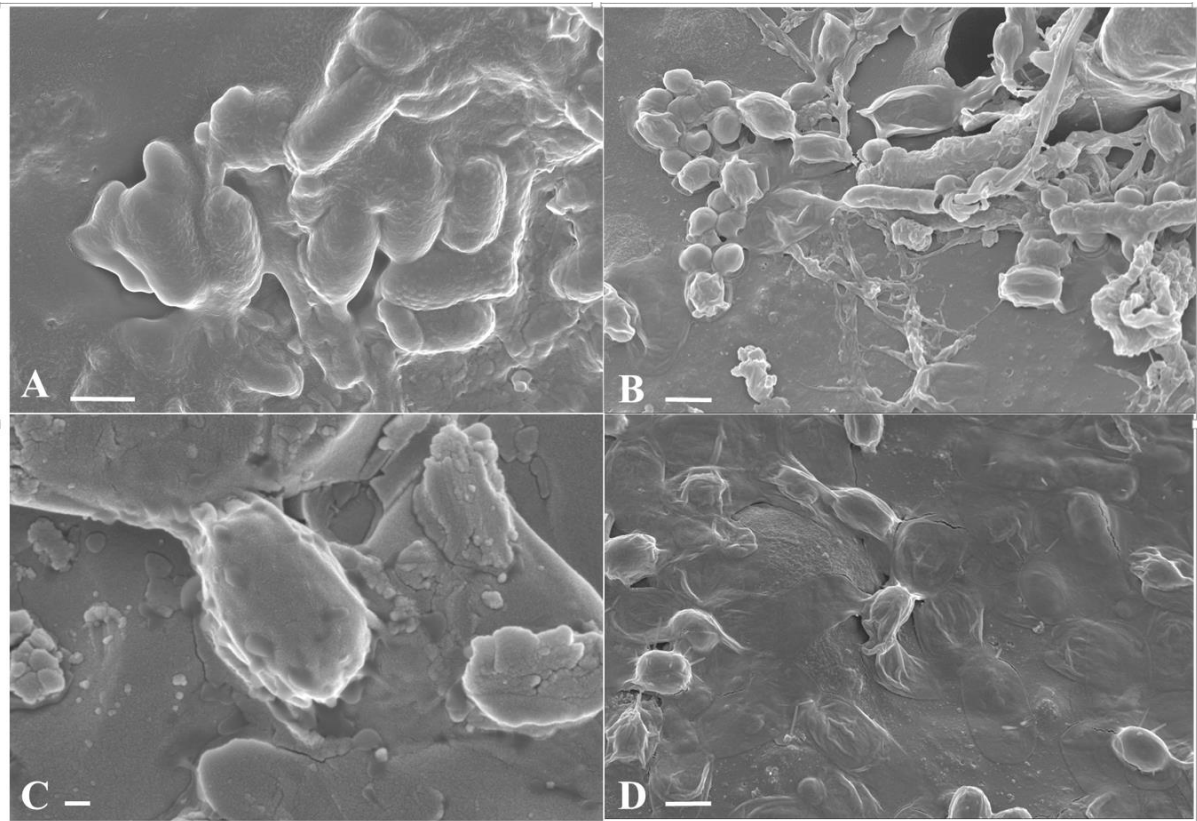
Figure 5: Scanning electron micrographs of *C. difficile* spores present on spiked hospital stainless steel and floor vinyl before and after treatment with NaDCC at 1,000 ppm for 10 min. Images are NaDCC-treated (A) DS1813 P spores on stainless steel; (B) DS1748 U Spores on floor vinyl; (C) DS1748 U 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.

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