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1 Isolation of *Clostridioides difficile* PCR Ribotype 027 from 2 single-use hospital gown ties

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16 17 **1.4 Keyword**

18 *Clostridioides difficile*; transmission; spores; gowns; flooring; infection control.

19 20 **2. Abstract**

21 **Background** *Clostridioides difficile* is a spore-forming pathogen responsible for antibiotic-associated
22 diarrhoea. In the USA high incidence of *C. difficile* infection (CDI) in clinical environments has led to
23 interest in *C. difficile* spore transmission. **Hypothesis** Single use hospital surgical gown ties act as a
24 reservoir for *C. difficile* spores. **Aim** This study sought to examine whether single-use hospital surgical
25 gown ties used in surgery, from an acute healthcare facility, harboured *C. difficile* spores.
26 **Methodology** Used surgical gowns ties worn by clinicians in the healthcare facility were examined for
27 *C. difficile* spore presence via spread plate and anaerobic culture. The colonies isolated from each

28 gown tie were subcultured on *C. difficile* selective agar for phenotypic confirmation. Presumptive *C.*
29 *difficile* colonies were examined using *C. difficile* Quik Check Complete, 16-23S PCR Ribotyping and
30 MALDI-TOF analysis. **Results** In total 17 suspected *C. difficile* colonies were isolated from 15 gown ties
31 via culture. *C. difficile* Quik Check Complete found two isolates as possible *C. difficile*. MALDI-TOF and
32 PCR Ribotyping confirmed one isolate as *C. difficile* PCR ribotype 027 associated with clinical
33 outbreaks. **Discussion** Our study revealed the presence of hypervirulent *C. difficile* ribotype 027 spores
34 on single-use gown ties. This highlights the potential of gown ties as a vector of spore transmission
35 across clinical environments, especially when gowns are not worn appropriately. **Conclusions**
36 Appropriate compliance to infection control procedures by healthcare workers is essential to prevent
37 spore dissemination across clinical facilities and reduce CDI rates.

38

39 3. Introduction

40 *Clostridioides difficile* is a Gram positive, spore forming enteric pathogen and the causative agent of
41 *C. difficile* associated diarrhoea. *C. difficile* is the primary cause of healthcare associated infections
42 (HAIs) in the USA, with an incidence of 306, 500 cases in 2011 and 235, 700 cases in 2017, and ~
43 15000 deaths per year, with an approximately economic burden \$1.5 – 3.2 billion annually ^{1,2}. This
44 incidence has been attributed to an epidemic strain of *C. difficile*: BI/NAP1/027 that has caused
45 outbreaks in the USA and Europe³. This strain is hypervirulent due to fluoroquinolone resistance,
46 production of binary toxin, increased toxin production, and higher sporulation rates ⁴. The organism
47 produces spores that are able to resist biocides ⁵ and persist on a range of clinical surfaces including
48 wheelchairs, flooring, surgical gowns and bedrails ^{6,7,8}. These surfaces are implicated in spore
49 transmission and may contribute to increasing incidence of *C. difficile* infection (CDI) ⁹.
50 Healthcare textiles, such as surgical gowns, are recognised as potential sources of HAIs; however
51 their exact role has yet to be elucidated due to limited epidemiological studies in the area ¹⁰. These

52 porous surfaces act as a reservoir for microorganisms, including spores, which can attach and survive
53 within the fibres ⁷⁻¹². Isolation gowns are the protective garments worn by health care workers (HCWs)
54 as a physical barrier to primarily prevent the transmission of pathogens between HCWs and patients
55 ¹². HCWs may also self-contaminate when removing personal protective equipment (PPE) such as
56 gloves or gowns. Upon contact with biological fluids, gowns must be discarded or laundered
57 appropriately for decontamination so that the gowns themselves do not serve as vectors of
58 transmission ¹³.

59 Two types of gown are available and possess varying properties: (a) single-use made from non-woven,
60 synthetic fibres such as polyethylene or (b) multi-use commonly made from woven fabric such as
61 cotton or polyester ¹². These fibres may also be used in combination with plastic films to offer an
62 enhanced liquid barrier. Contaminated gowns act as fomites, as do gloves or drapes, and can
63 propagate the transmission of microorganisms to further patients, HCPs, or the environment,
64 especially if used inappropriately. In the current study we sought to determine whether used single-
65 use hospital surgical isolation gown ties, worn inappropriately during surgery from a US healthcare
66 facility, act as fomites and harbour *C. difficile* spores.

67

68 **4. Methods**

69 Adventist Health Hospital was noted as being a statistical outlier for hospital acquired *C. difficile* in the
70 region of California. The Californian Department of Health revealed that the Standardised Infection
71 Ratios (SIR) for CDI between 2013- 2016 (SIR 2.13- 1.11) were higher than other hospitals in the USA.
72 In October 2015, Consumer Reports (national US publication) noted that out of 3000 facilities in the
73 USA, Adventist Health Hospital was in the bottom 12 for infection prevention based on incidence rates
74 of CDI and methicillin resistant *Staphylococcus aureus* (MRSA) (Dr R. Burky, Infection Control
75 Consultant, Adventist Health Hospital; *personal communication*). Infection Control staff at Adventist

76 Health Hospital surmised the cause of these hospital-wide CDI cases might be inappropriate use of
77 PPE. This manifested as a problem with staff compliance in securing gown ties and wearing
78 polypropylene isolation gowns inappropriately, which was hospital-wide (Dr R Burky; *personal*
79 *communication*). Moreover, in the affected wards, surgery staff were routinely noted as wearing
80 single-use surgical gowns without the tiebacks being tied, and walking in and out of the operating
81 theatre without disposing the gowns. This resulted in the gown ties dragging around the floor during
82 surgery, potentially picking up infectious material. Thus, to ascertain whether these gown tiebacks
83 were involved in spore transmission and picked up *C. difficile* spores, culture analysis of the tiebacks
84 was conducted between 2016-2018.

85 **Gown Sampling:** Single- use surgical gowns were immediately obtained from (a) the Intensive Care
86 Unit and (b) Medical Surgery units after removal by clinicians in the surgical theatre. Gown ties were
87 selected for sampling after observation of incorrect staff compliance to securing the gowns. The gown
88 ties were then aseptically removed from the gowns (via cutting) and individually packaged at source
89 within Adventist Health and Rideout Hospital, USA. The ties were not handled or used after contact
90 but were instead placed into sterile containers for transport. The gown ties were sent via secure post
91 to the United Kingdom for immediate analysis in late 2016. They were sent to the United Kingdom as
92 researchers had a prior published collaboration and experience of *C. difficile* spore isolation in clinical
93 settings^{7,14}. In total, 15 surgical gown ties were received for analysis. The hospital surgical gowns were
94 produced by MediChoice, as described previously by Dyer et al (2019)⁷ and made from fluid-resistant
95 spunbond-meltdown-spunbond (SMS) polypropylene laminate at American National Standards
96 Institute (AAMI) PB70:2012 level 2. No ethical approvals at Adventist Health were required as only the
97 discarded used gowns were taken for sampling.

98 ***C. difficile* culture:**

99 To examine whether *C. difficile* was present on the gown ties, which had touched the operating theatre
100 floor, the 15 full samples of gown (20 cm strips) were aseptically placed into 20 ml sterile deionised
101 water (SDW) and vortex mixed to dislodge any possible spores. Each sample was then centrifuged at
102 5,000 × g and the pellet resuspended in 1 ml of SDW, heated to 80°C for 10 min to inactivate any
103 remaining vegetative cells, and subsequently stored at 4°C. Each sample was then enumerated for
104 spores in reduced brain heart infusion (BHIS) broth (Oxoid Ltd, UK) supplemented with 0.1% (w/v)
105 sodium taurocholate (Sigma Aldrich, UK), and plated on BHIS agar as described previously¹⁴.

106 D-cycloserine (500µg/mL) and cefoxitin (16µg/mL) fructose agar (CCFA; Oxoid Ltd, UK) was used for
107 selective culture of *C. difficile* from the BHIS agar. All suspected cultures were incubated anaerobically
108 at 37 °C for 48 hours in a Don Whitley Scientific anaerobic workstation using an 85% nitrogen, 10%
109 carbon dioxide and 5% hydrogen gas mix. Clinical toxigenic and a non- toxigenic isolates of *C. difficile*
110 were used as growth and polymerase chain reaction (PCR) controls (Table 1), and were obtained from
111 the Anaerobic Reference Unit (ARU), University Hospital Wales, Cardiff, UK. Unless otherwise stated,
112 all isolated organisms were stored on ProtectBeads™ at 4 °C. All experiments described were
113 conducted in triplicate (n=3).

114 ***C. difficile* phenotypic confirmation:**

115 All spores isolated from the gown ties were streaked to purity on CCFA, and colonies were examined
116 for typical *C. difficile* morphology. Colonies were then checked for anaerobic growth, gamma
117 haemolysis, odour, *chartreuse* fluorescence under Ultra Violet light, and Gram stain (Positive)^{16,17}.

118 ***C. difficile* Quik Chek Immunoassay:**

119 The C. Diff Quik Chek Complete dual-antigen enzyme immunoassay (EIA) was performed according to
120 manufacturer's instructions (TechLab, UK) and as described previously¹⁸. Briefly, instead of using stool
121 samples, 25 µl of suspected *C. difficile* mixed with sterilized deionised water (diH2O) was added to the

122 assay alongside diluent and conjugates. Results were read 10 minutes after the assay was completed.
123 Samples were determined to be *C. difficile* positive if bands were visible for Glutamate dehydrogenase
124 (GDH) and/or toxins. This test was performed twice for each sample (n=2).

125 **Scanning Electron Microscopy (SEM)**

126 SEM was used to examine the morphology of presumptive *C. difficile* spores before and after NaOCl
127 exposure. Samples were fixed with 2.5% glutaraldehyde and were transferred onto Nuclepore
128 membranes (Sigma-Aldrich, UK) which were sputter coated with gold palladium (60% Au and 40% Pd
129 from Testbourne Ltd) and argon was used as the sputtering gas. An accelerating voltage of 15kV was
130 used to view 10 spores per sample at magnifications of x 8,500-20,000 (JEOL JSM-6610 Series SEM).

131 **DNA Extraction from *C. difficile* using Chelex 100** Genomic DNA was extracted from suspected *C.*
132 *difficile* as described previously^{19,20}. DNA was quantified in µg/ml using the Qubit™ dsDNA BR Assay
133 Kit and read using the Qubit 4.0 Fluorometer (Fisher Scientific, UK), following the manufacturers
134 protocol.

135 **Multiplex Polymerase Chain Reaction (PCR)**

136 A multiplex PCR targeting topoisomerase (*tpi*), toxin A (*tcdA*) and toxin B (*tcdB*) genes as described by
137 Lemee et al (2004)²¹ was used for *C. difficile* identification and toxin characterisation (n = 2) using a
138 *Taq* PCR Kit (New England BioLabs, UK). The *tpi* generates 230-bp amplified fragments, *tcdA* generates
139 369-bp amplified fragments (Toxin A+ B+) or 110-bp fragments (Toxin A- B+) and *tcdB* generates 160-
140 bp fragments. PCR was performed on a T100™ thermal cycler (Bio-Rad, UK) using 25µl volumes.
141 Cycling parameters were: 95°C initial denaturation for 3 minutes, 40 cycles of 95°C denaturation for
142 30 seconds, 54°C annealing for 30 seconds, 72°C extension for 1 minute and 72°C final elongation for
143 5 minutes. PCR products were analysed via gel electrophoresis on a 1.2% agarose gel at 80 V for 60

144 minutes (n = 2). Quick-Load® 1kb Plus DNA Ladder (New England BioLabs, UK) was used and GelRed®
145 Loading Buffer (Biotium Inc, USA) was used for gel fluorescent staining.

146 **Molecular Confirmation of *C. difficile***

147 The final presumptive *C. difficile* isolates were sent for MALDI-TOF²² analysis and 16S- 23S rRNA PCR
148 Ribotyping²⁰ confirmation at the ARU, University Hospital Wales, UK.

149 **5. Results**

150 In total 15 samples of contaminated, single-use hospital gown ties were examined for the presence of
151 *C. difficile*. Of the 15 samples, 23 presumptive colonies were grown on CCFA media, and subsequent
152 phenotypic analysis revealed 8 isolates that exhibited phenotypic characteristics (e.g. Gram positive,
153 growth on CCFA, odour, *chartreuse* fluorescence under UV light) similar to those of *C. difficile* colonies
154 ¹⁶ (Table 2). Four samples including 2B were taken for further analysis based on positive phenotypic
155 characteristics. The *C. difficile* identification process used in this study is shown in Figure 1 .

156 The C. DIFF Quik Chek Complete¹⁸ assay only found one out of the four presumptive samples to be *C.*
157 *difficile* positive, producing visible bands for GDH and toxins A and B¹⁸. All controls produced positive
158 results, except for strain DS1684 which was toxin negative.

159 Molecular testing via multiplex PCR²¹ confirmed this result with this sample (designated 2B) showing
160 a species specific fragment at approximately 200 base pairs correlating to the presence of the *C.*
161 *difficile tpi* gene (Figure 2). This fragment was also detected for the positive controls. Non-deleted
162 *tcdA* fragments (A+B+) were detected for *C. difficile* DS1813, R20291 and CD630 at approximately 400
163 base pairs. The *tcdB* gene was detected for *C. difficile* R20291 and CD630 at approximately 150 base
164 pairs. No fragments for *tpi*, *tcdA* or *tcdB* genes were produced for other samples tested.

165 Further identification of these 4 isolates was undertaken through MALDI-TOF ²² analysis at the ARU,
166 Wales, UK (Table 3). The spore forming capacity of isolates 2B and 7 was confirmed via scanning
167 electron microscopy where typical spore morphology can be seen in Figure 3A and 3C. This was not
168 evident in isolates 5 and 8 (Figure 3B and 3C). Sample 7 was confirmed to be presumptive *Bacillus*
169 *cereus* via MALDI TOF, sample 5 to be a *Propionibacterium* species and sample 8 to be *Clostridium*
170 *tertium* ^{23,24}. Importantly, sample 2B was confirmed to be *C. difficile* and subsequent PCR Ribotyping
171 confirmed this isolate to be an epidemic PCR Ribotype 027 ³. This sample was isolated from gown ties
172 sampled within the Medical Surgery department at Adventist Health & Rideout Hospital, USA.

173
174

175 6. Discussion

176 This study examined the ability of single-use surgical gown ties to harbour *C. difficile* spores through
177 inappropriate use of PPE. While an algorithm of phenotypic and molecular methods were used to
178 specifically isolate *C. difficile* spores, such as CCFA, we isolated other Gram positive spore formers
179 which exhibited similar growth characteristics to *C. difficile*, demonstrating the limitations of these
180 isolation methods ²⁵. Isolated species included foodborne pathogen *Bacillus cereus* which can cause
181 severe nosocomial infections ²⁶ and the little-studied *Clostridium tertium* which primarily infects
182 neutropenic patients, causing bacteraemia ²⁷. *C. tertium* is not a common healthcare associated
183 pathogen; however, its isolation from the gown ties can be attributed to its presence on the hospital
184 floor. This is a concern due to *C. tertium's* ability to cause bacteraemia in patients with recent
185 abdominal surgery ²⁷⁷.

186 From SEM analysis (Figure 2) the spores formed by these Gram positive Bacilli appear to have similar
187 features including raised bumps across the spore coat ^{7, 14, 28, 29}. CDI outbreaks are becoming more
188 frequent so “one-step” molecular identification methods for patient diagnosis are becoming

189 increasingly valuable tools ^{18,19}. Thus, we used the C DIFF Quick Chek Complete ¹⁸ to determine toxin
190 production and GDH antigen presence from each isolate. We combined this with a multiplex PCR assay
191 to determine whether our suspected *C. difficile* isolates possessed any specific *C. difficile* genes. The
192 PCR detected *tpi* genes amongst sample 2B only (Figure 2) and the positive controls demonstrating
193 that this sample was likely to be a *C. difficile* strain. Subsequent MALDI TOF analysis and *C. difficile*-
194 specific 16-23S rRNA PCR Ribotyping effectively confirmed the identity of the suspected *C. difficile*
195 species as a 027 hypervirulent ribotype, implicated in severe CDI outbreaks ^{1,3}.

196 Clinical environments comprise of a variety of surfaces that can harbour pathogens able to cause
197 HCAs ³⁰. These contaminated surfaces range from flooring to bedpans, hospital equipment and the
198 hands of HCWs, often leading to increased infection case rates and implementation of infection
199 control practices to prevent pathogen transmission ^{7, 31}. Hospital floors are an important pathogen
200 reservoir lending to the easy spread of disinfection-resilient pathogens such as *C. difficile* ^{32,33}. The role
201 of flooring in aiding the spread of pathogens through vectors such as shoes, socks and even
202 wheelchairs has become a subject of interest in recent years with pathogens including methicillin
203 resistant *Staphylococcus aureus*, *C. difficile* spores and common viruses being studied ^{6,7,32-35}. Indeed a
204 clear limitation of this study is the lack of environmental sampling, meaning that while the presence
205 of *C. difficile* was confirmed on the gown ties, the nosocomial source of *C. difficile* was not identified.

206 Improper and inappropriate use of PPE, including re-using single-use hospital gowns, can also
207 contribute to pathogen spread and increasing patient HCAI case rates ^{8,10-13}. Single-use gowns have
208 yet to be recognised as important sources of pathogen transmission in clinical environments, likely
209 due to the instructions for “single-use” and immediate disposal ^{12,13}. However, with pressures on
210 clinical resources, time and economics, especially during the COVID19 pandemic, HCW compliance to
211 “single-use” may decline ³⁶. This compliance may also be compounded by the fact that society is

212 currently attempting to reduce plastic waste in an effort to combat climate change and protect the
213 environment ³⁷.

214 In the current study we found that single-use gowns harboured spores of healthcare associated
215 pathogen *Clostridioides difficile*. This demonstrates the potential of healthcare textiles as fomites and
216 a medium for pathogen transmission. Single-use gown fabrics are designed to repel fluids and are
217 more commonly employed in the USA (80%) than in Europe ^{11,12}. Differences in the physiochemical
218 properties of gowns have been shown to play a critical role in the dissemination of microorganisms
219 throughout clinical environments ^{7,8,10,14}. In this study, *C. difficile* spores not only adhere to the single-
220 use, repellent fabrics but the fabric fibres did not demonstrate the ability to trap spores effectively as
221 the hospital in question had increasing CDI rates ^{7,11}. Moreover the HCWs were letting the gown ties
222 trail on hospital floors without disposing immediately after use (Dr. R Burky: *personal communication*).
223 A direct link has been found between bacterial colonisation of hospital uniforms and patient
224 morbidity; an example is *C. difficile* spore dissemination in intensive care units and geriatric wards
225 where immunocompromised patients are at a higher risk. This strengthens the need to ensure HCW
226 education and compliance in using PPE appropriately^{38,39}.

227 In conclusions, our findings demonstrate that single-use hospital gowns can act as fomites and in
228 pathogen transmission, especially when not used appropriately. Interventions such as increasing
229 compliance and appropriate use by ensuring gown ties do not drag upon the floor, changing single-
230 use gowns between patients and disposing of them immediately after use may aid in reducing
231 pathogen dissemination. Further research is required to elucidate the chain of transmission between
232 floors, HCWs and gowns and the correlation between patient CDI case rates. This study suggests that
233 current infection control procedures should be audited to ensure compliance and effective
234 disinfection of clinical surfaces.

235 **7. Author statements**

236 **7.1 Authors and contributors**

237 SW: Formal analysis, Data Curation, Investigation, Writing – original draft, Writing – review &
238 editing.

239 HA: Formal analysis, Investigation, Validation, Writing – review & editing.

240 IAJ: Writing – original draft, Writing – review & editing

241 RB: Conceptualization, Resources

242 LTJ: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project
243 administration, Supervision, Resources, Writing – original draft, Writing – review & editing.

244

245 **7.2 Conflicts of interest**

246 The authors declare that there are no conflicts of interest.

247

248 **7.3 Funding information**

249 This work was supported by Robert Burky and the University of Plymouth. The funders had no role in
250 study design, data collection and interpretation, or the decision to submit the work for publication.

251

252 **7.4 Ethical Statement**

253 No Ethical Approvals at Adventist Health & Rideout Hospital were required as only the discarded
254 used gowns were taken for sampling at source. No human work was carried out.

255

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261

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399 **9. Figures and tables**

400 **TABLES**

401 **Table 1 *Clostridioides difficile* strains used in this study**

<i>C. difficile</i> strain	PCR Ribotype	Source
R20291	027	Stoke- Mandeville, UK
DS1813	027	Hinchingbrooke, UK
CD630	012	NCTC
DS1684	010	Brighton, UK

406
407

*NCTC -National Collection of Type Cultures

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409 **Table 2 Phenotypic characterisation of presumptive *C. difficile* colonies.**

Sample Number	CCFA Growth	Odor	Fluorescence under UV Light	Gamma Hemolysis	C. DIFF Quik Chek	
					GDH	Toxin A/B
1, 3, 4,	+	-	-	+	-	-
2B	+	+	+	+	+	+
5, 7,8	+	-	+	+	-	-

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411 *CCFA- Cycloserine and cefoxitin fructose agar

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413 **Table 3 Confirmation of Sample Identity**

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Sample Number	Result
2B	<i>C. difficile</i> Ribotype 027
5	<i>Propionibacterium spp</i>
7	Presumptive <i>Bacillus cereus</i> (MALDI-TOF)
8	<i>Clostridium tertium</i>

422 **FIGURES**

423

424 **Figure 1: Workflow of *C. difficile* Identification**

425 The process of isolation of presumptive *C. difficile* from the gown ties is described. The phenotypic
426 testing on Cycloserine-Cefoxitin Fructose Agar (CCFA), Scanning Electron Microscopy (SEM),
427 Multiplex PCR, Matrix-assisted laser desorption/ionization time of flight mass spectrometry
428 (MALDI-TOF MS) and 16S-23S rRNA PCR ribotyping was used to fully analyse suspected *C. difficile*
429 isolates and identify species.

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431

432 **Figure 2: Multiplex PCR of suspected *C. difficile* isolates**

433 Agarose gel electrophoresis of a multiplex PCR used for identification and toxigenic type
434 characterisation of presumptive *C. difficile* isolates, previously isolated from used hospital gowns.

435 M represents Quick-Load® 1kb plus ladder. Lane 1 represents sample 2B, lane 2 represents sample
436 19, lane 3 represents sample 17, lane 4 represents sample 12. Positive controls for the *tpi* and

437 *tcdA* and *tcdB* genes were in lane 5 -*C. difficile* DS1813, lane 6 - *C. difficile* R20291, and lane 8 - *C.*
438 *difficile* CD630. Negative control for *tcdA* and *tcdB* genes was lane 7 - *C. difficile* DS1684 and

439 nuclease free-water and an *E. coli* K12 was used in Lane 9 as a non-clostridial strain control.
440 Arrows indicate number of base pairs correlated to the DNA bands detected.

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443 **Figure 3: Scanning Electron microscopy of suspected *C. difficile* isolates**

444 Scanning electron microscopy of presumptive *C. difficile* isolated from contaminated single-use
445 hospital gowns. Spores were produced and harvested from each purified sample after selective

446 culture. (A) represents sample 2B- *C. difficile* 027 spore, (B) represents sample 5 a
447 Propionibacterium spp (C) represents sample 7a Presumptive *Bacillus cereus* spore and (D)

448 represents sample 8 a *Clostridium tertium* spore. Scale bar is 100 nm.

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