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

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1.4 Keyword

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

2. Abstract

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

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

3. Introduction

Clostridioides difficile is a Gram positive, spore forming enteric pathogen and the causative agent of *C. difficile* associated diarrhoea. *C. difficile* is the primary cause of healthcare associated infections (HCAIs) in the USA, with an incidence of 306, 500 cases in 2011 and 235, 700 cases in 2017, and ~ 15000 deaths per year, with an approximately economic burden $1.5 – 3.2 billion annually [1,2]. This incidence has been attributed to an epidemic strain of *C. difficile*: BI/NAP1/027 that has caused outbreaks in the USA and Europe[3]. This strain is hypervirulent due to fluoroquinolone resistance, production of binary toxin, increased toxin production, and higher sporulation rates [4]. The organism produces spores that are able to resist biocides [5] and persist on a range of clinical surfaces including wheelchairs, flooring, surgical gowns and bedrails [6,7,8]. These surfaces are implicated in spore transmission and may contribute to increasing incidence of *C. difficile* infection (CDI) [9].

Healthcare textiles, such as surgical gowns, are recognised as potential sources of HCAIs; however their exact role has yet to be elucidated due to limited epidemiological studies in the area [10]. These

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

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

4. Methods

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

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

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

***C. difficile* culture:**

To examine whether *C. difficile* was present on the gown ties, which had touched the operating theatre floor, the 15 full samples of gown (20 cm strips) were aseptically placed into 20 ml sterile deionised water (SDW) and vortex mixed to dislodge any possible spores. Each sample was then centrifuged at 5,000 × g and the pellet resuspended in 1 ml of SDW, heated to 80°C for 10 min to inactivate any remaining vegetative cells, and subsequently stored at 4°C. Each sample was then enumerated for spores in reduced brain heart infusion (BHIS) broth (Oxoid Ltd, UK) supplemented with 0.1% (w/v) sodium taurocholate (Sigma Aldrich, UK), and plated on BHIS agar as described previously [14].

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

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

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

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

The C. Diff Quik Chek Complete dual-antigen enzyme immunoassay (EIA) was performed according to manufacturer's instructions (TechLab, UK) and as described previously [18]. Briefly, instead of using stool samples, 25 µl of suspected *C. difficile* mixed with sterilized deionised water (diH2O) was added to the

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

Scanning Electron Microscopy (SEM)

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

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

Multiplex Polymerase Chain Reaction (PCR)

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

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

Molecular Confirmation of *C. difficile*

The final presumptive *C. difficile* isolates were sent for MALDI-TOF[22] analysis and 16S- 23S rRNA PCR Ribotyping [20] confirmation at the ARU, University Hospital Wales, UK.

5. Results

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

The C. DIFF Quik Chek Complete [18] assay only found one out of the four presumptive samples to be *C. difficile* positive, producing visible bands for GDH and toxins A and B [18]. All controls produced positive results, except for strain DS1684 which was toxin negative.

Molecular testing via multiplex PCR[21] confirmed this result with this sample (designated 2B) showing a species specific fragment at approximately 200 base pairs correlating to the presence of the *C. difficile tpi* gene (Figure 2). This fragment was also detected for the positive controls. Non-deleted *tcdA* fragments (A+B+) were detected for *C. difficile* DS1813, R20291 and CD630 at approximately 400 base pairs. The *tcdB* gene was detected for *C. difficile* R20291 and CD630 at approximately 150 base pairs. No fragments for *tpi*, *tcdA* or *tcdB* genes were produced for other samples tested.

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

6. Discussion

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

From SEM analysis (Figure 2) the spores formed by these Gram positive Bacilli appear to have similar features including raised bumps across the spore coat [7, 14, 28, 29]. CDI outbreaks are becoming more 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 HCAIs [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

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

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

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

7. Author statements

7.1 Authors and contributors

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

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

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

RB: Conceptualization, Resources

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

7.2 Conflicts of interest

The authors declare that there are no conflicts of interest.

7.3 Funding information

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

7.4 Ethical Statement

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

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9. Figures and tables

TABLES

Table 1 *Clostridioides difficile* strains used in this study

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

*NCTC -National Collection of Type Cultures

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	+	-	+	+	-	-

*CCFA- Cycloserine and cefoxitin fructose agar

Table 3 Confirmation of Sample Identity

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

FIGURES

Figure 1: Workflow of *C. difficile* Identification

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

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

Agarose gel electrophoresis of a multiplex PCR used for identification and toxigenic type characterisation of presumptive *C. difficile* isolates, previously isolated from used hospital gowns. M represents Quick-Load® 1kb plus ladder. Lane 1 represents sample 2B, lane 2 represents sample 19, lane 3 represents sample 17, lane 4 represents sample 12. Positive controls for the *tpi and tcdA* and *tcdB genes* were in lane 5 -*C. difficile* DS1813, lane 6 - *C. difficile* R20291, and lane 8 - *C. difficile* CD630. Negative control for *tcdA* and *tcdB* genes was lane 7 - *C. difficile* DS1684 and nuclease free-water and an *E. coli* K12 was used in Lane 9 as a non-clostridial strain control. Arrows indicate number of base pairs correlated to the DNA bands detected.

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

Scanning electron microscopy of presumptive *C. difficile* isolated from contaminated single-use hospital gowns. Spores were produced and harvested from each purified sample after selective culture. (A) represents sample 2B- *C. difficile* 027 spore, (B) represents sample 5 a Propionibacterium *spp* (C) represents sample 7a Presumptive *Bacillus cereus* spore and (D) represents sample 8 a *Clostridium tertium* spore. Scale bar is 100 nm.