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ORIGINAL ARTICLE

The effect of hospital biocide sodium dichloroisocyanurate on the viability and properties of *Clostridium difficile* spores

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Significance and Impact of the Study: This study is the first to report on changes in *Clostridium difficile* spore surface property after exposure to sublethal levels of the commonly used biocide sodium dichloroisocyanurate. The implications of these changes to the spore surface include increased adherence of the spores to inorganic surfaces which can directly contribute to persistence and spread of spores within the hospital environment.

Keywords
biocide, *Clostridium difficile*, spores, sublethal, transmission, viability.

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Abstract

Clostridium difficile is the primary cause of healthcare-associated diarrhoea globally and produces spores which are resistant to commonly used biocides and are able persist on contaminated surfaces for months. This study examined the effect of sublethal concentrations of the biocide sodium dichloroisocyanurate (NaDCC) on the viability of spores produced by 21 clinical isolates of *C. difficile* representing a range of PCR ribotypes. Spores exposed to 500 ppm NaDCC for 10 min exhibited between a 4–6 $\log_{10}$ reduction in viability which was independent of spore PCR ribotype. The effect of sublethal concentrations of biocide on the surface properties of exosporium positive and negative clinical isolates was determined using a spore adhesion to hydrocarbon (SATH) assay. These isolates differed markedly in their responses suggesting that exposure to biocide can have a profound effect on hydrophobicity and thus the ability of spores to adhere to surfaces. This raises the intriguing possibility that sublethal exposure to NaDCC could inadvertently promote the spread of the pathogen in healthcare facilities.

Introduction

Clostridium difficile is a Gram-positive, anaerobic spore-forming bacillus and is a major cause of healthcare-associated infection globally. Epidemics have occurred with the intercontinental spread of hypervirulent PCR ribotypes such as BI/NAP1/027, in Europe, Asia and the United States (Dawson *et al.* 2011). In the United States, the pathogen contributes to 14 000 deaths per year (Frieden 2013), while between 2011–2012 in England and Wales it was responsible for 15·3 deaths per million, representing a tragic loss of life and a significant economic burden (ONS, 2014).

Clostridium difficile infection (CDI) manifests in varying severity from mild diarrhoea to fatal pseudomembranous colitis in antibiotic-treated patients where the gut microbiota has been disrupted (Voth and Ballard 2005). Carriage of *C. difficile* can be asymptomatic and occurs in 1–3% of healthy adults (Kuijpers *et al.* 2008). In the hospital environment the organism is primarily acquired through the faecal–oral route as between 1×10^4 to 1×10^7 spores are excreted per gram of patient faeces (Salyers and Whitt 2002; Bartlett 2006; Best *et al.* 2010). Spores are resistant to biocide treatment and for this reason increased efforts have been made to maintain strict infection control practices within the hospital environment. Approaches used include hand-washing with soap, decreased use of alcohol hand rubs and disinfection with sporicidal agents such as sodium hypochlorite (Department of Health and Public Health Laboratory Service Joint Working Group 1994). While these measures have resulted in a decrease in the incidence of *C. difficile* in the United

Kingdom, the rates of infection still exceed those of MRSA (ONS, 2012; Office for National Statistics 2014).

The most commonly used biocides for *C. difficile* spore decontamination in healthcare facilities in the UK are chlorine-releasing agents such as sodium hypochlorite (NaOCl) and sodium dichloroisocyanurate (NaDCC) (Coates1996). It is recommended that chlorine-releasing agents such as these should be employed at a concentration of 1000 ppm and should remain in contact with *C. difficile* spores for at least 10 min (Department of Health and Public Health Laboratory Service Joint Working Group 1994). Spores are often suspended in complex organic material such as faeces which can interact with the biocide to reduce its antibacterial potential. Hence, this study looks to determine what effect sublethal exposure to NaDCC would have on the viability of a representative panel of *C. difficile* clinical isolates.

The epidemiology of *C. difficile* infection within Europe has changed in recent years with an increase in overall ribotype diversity when compared to previous studies in 2008 (Bauer *et al.* 2011; Davies *et al.* 2016). Interestingly the prevalence of PCR ribotype 027 strains, which are linked to severe CDI outbreaks, has increased across Europe when compared to their uncommon isolation in the 1990s (Davies *et al.* 2016). This increase in ribotype diversity highlights the need to include a wide range of clinical ribotypes as possible in future studies to ensure scientists are able to combat the majority of forms of the pathogen.

In addition to assessing the effect of biocide exposure on spore viability, authors also wanted to determine whether biocide exposure had any effect on the ability of spores to adhere to surfaces. Surface adherence is thought to play an important role in the survival and spread of *C. difficile* spores to susceptible individuals (Kramer *et al.* 2006; Vonberg *et al.* 2008). Spore hydrophobicity has been shown to have a key role in mediating the attachment of spores to surfaces such as stainless steel. In a previous study, authors have shown that the hydrophobicity of spores produced by clinical isolates of *C. difficile* vary widely and that this in turn affects their ability to adhere to stainless steel (Joshi *et al.* 2012). These differences were independent of ribotype but appeared to be linked to the presence of an exosporial-like layer. Thus, in this study, authors also determined the effect of sublethal exposure of NaDCC on the hydrophobicity of exosporium-positive and exosporium-deficient spores.

Results and discussion

Effect of NaDCC on the viability of clinical isolates of *Clostridium difficile*

The viability of 21 clinical isolates of *C. difficile* following exposure to 500 ppm of NaDCC at varying contact times was determined. Spores were exposed to 500 ppm, which is half the recommended concentration, to reflect potential inappropriate daily practice. As the contact times increased the biocide killed more spores (Fig. 1). Spore viability decreased by approximately 4–6 $\log_{10}$ when exposed to biocide at the recommended contact time of 10 min ($P = 0{\cdot}004$) (Guest Medical Ltd, Aylesford, Kent, UK). The strains which showed the greatest reduction in spore numbers were DS1787 (PCR ribotype 106), DS1750 (001), R20291 (027), DS1807 (027) and DS1752 (012). The strains which showed the least susceptibility to biocide exposure under the same test conditions were DS1724 (020) and DS1750 (001). The presence of examples of the same 001 PCR ribotype in both groups suggest that there is no direct correlation between ribotype and susceptibility to NaDCC.

The effect of NaDCC on the viability of spores with and without an exosporium-like outer spore layer

To determine if spore structure contributed to biocide susceptibility, the responses of spores (from two previously characterized *C. difficile* isolates) to different concentrations of NaDCC were compared. The effect of different concentrations of biocide on the viability of exosporium-positive (DS1813) and -deficient (DS1748) *C. difficile* spores was also determined. As can be seen from Fig. 2 exposure to 10 ppm resulted in a reduction in viability of both sets of spores with the DS1748 showing the greatest reduction (Student's *t*-test; $P = 0{\cdot}0001$). While increasing the concentration of the biocide to 100 ppm further reduced the viability of DS1813 spores (two-way ANOVA; $P = 0{\cdot}048$), it had no significant effect on the viability of the DS1748 spores. Indeed, somewhat surprisingly, there appeared to be an increase in DS1748 spore survival which rose in line with the increase in exposure time. At 1 min exposure time using 10 ppm NaDCC there is similar spore recovery to the control sample. This may be due to the low contact time of the biocide, resulting in less spores interacting with the biocide. At 5 min of 100 pm there is increased recovery of the spores when compared to 1 and 10 min (which show similar recovery). This increase in spore viability suggests that the biocide may not have damaged the surface of the spores to a level where germination was prevented, thus allowing spore revival on agar. Therefore, exposure of spores to 100 ppm NaDCC for 5 min is inadequate to kill spores. Exposure to 1000 ppm of biocide across all contact times killed both sets of spores. Taken together, these results suggest that the exosporium-deficient spores are more susceptible to biocide than the spores which possess an exosporium.

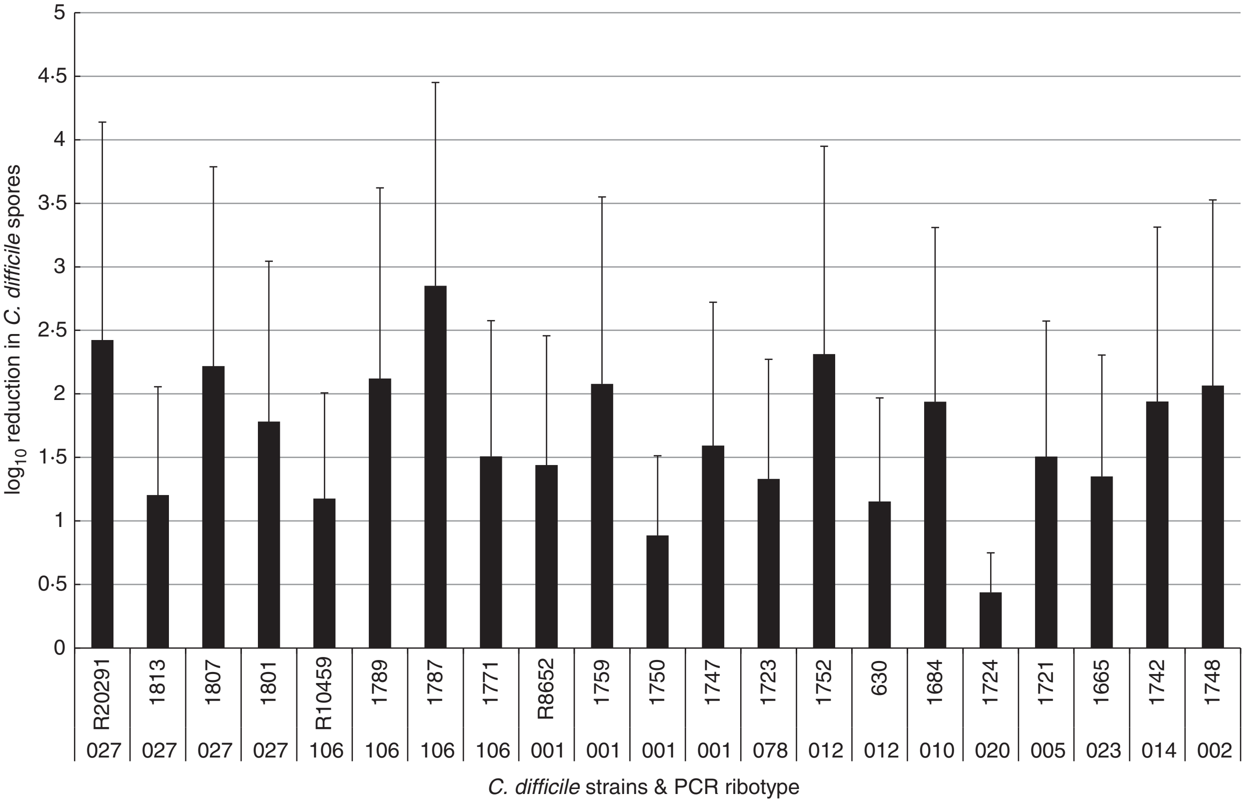

Figure 1 Effect of exposure of spores of clinical isolates of *C. difficile* to 500 ppm NaDCC on viability after 10 min contact time. The susceptibilities of spores from 21 clinical isolate at a low concentration of 500 ppm biocide and compared to the control (T= 0). The log$_{10}$ reduction in spore viability after exposure to 500 ppm NaDCC was determined at the recommended contact time of 10 min and compared to the control (0 ppm) (One way ANOVA; $P = 0{\cdot}004$). Each result is the mean of three repeats.

In a previous study, authors observed that spore structure and properties also differed in a manner unrelated to the ribotype of the strain; hence it is possible that these differences in spore architecture could be linked to biocide sensitivity (Joshi *et al.* 2012). Examination by electron microscopy had revealed the presence of an exosporium-like structure surrounding the spore form of DS1813 (ribotype 027) which may account for its hydrophobic nature (~72% RH) and its ability to adhere to organic and inorganic surfaces (Joshi *et al.* 2012). In contrast, the spores produced by DS1748 (ribotype 002) appeared to lack the exosporial layer, were significantly less hydrophobic (~20% RH) and were not as efficient at adhering to inorganic surfaces as those of DS1813. This study also found that the spores formed by DS1748 were more susceptible to biocide than those produced by DS1813 suggesting that spore structure may contribute to NaDCC susceptibility.

The spore structure of *C. difficile* is similar to that of bacilli; comprising a core, cortex, membrane, coat and, usually, an exosporial layer (Lawley *et al.* 2009). Spores of DS1813 with the exosporial layer showed reduced susceptibility to biocide when compared to DS1748 (which did not possess the layer). The spore coat has been hypothesized to act as a permeability barrier to prevent the entry of nonspecific molecules to the spore and in doing so protects the spore core (Russell 1990). It is possible, however, that the spore coat acts as a protective layer in conjunction with the exosporial layer to protect the contents of the spore core such as its enzymes and DNA from nonspecific molecules and chemicals. This would explain why DS1748 spores were more susceptible to biocide than DS1813.

Effect of NaDCC on the relative hydrophobicity of spores with and without an exosporium-like outer spore layer

In addition to determining whether the structures of the spore contributed to biocide sensitivity, this study also examined the effects of exposure to sublethal levels of NaDCC on spore surface properties. At present there have been no studies to our knowledge which have sought to examine the effects of biocides on spore surface properties

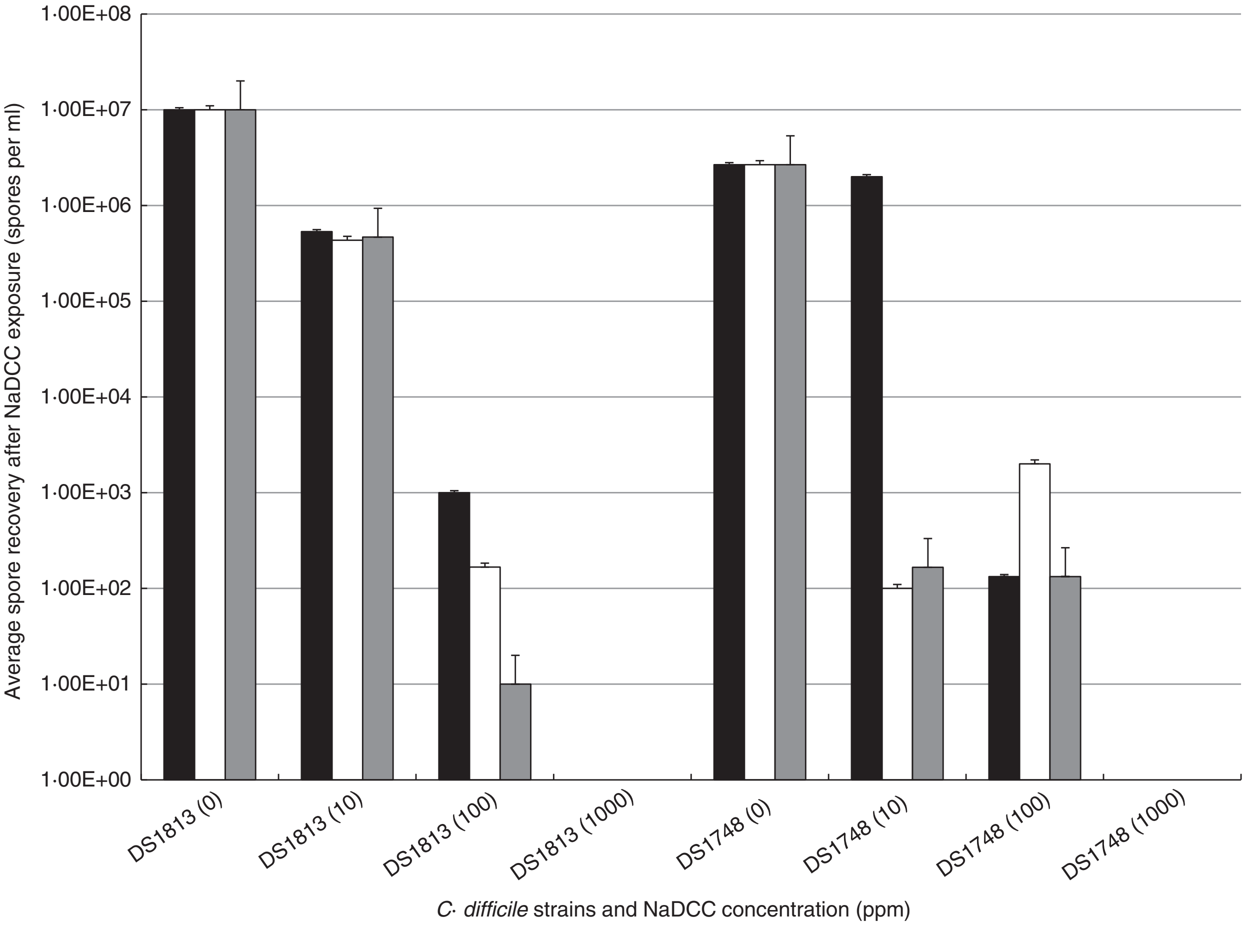

Figure 2 Sensitivity of *C. difficile* spores DS1813 and DS1748 to a range of NaDCC concentrations and contact times. The viability of spores was determined after exposure to NaDCC at a range of concentrations and times. As the concentration of NaDCC increases there appears to be a decrease in spore viability; most significantly with DS1813 when compared to the control at 0 ppm (Two way ANOVA; $P = 0{\cdot}048$). Control spore concentrations were at $2{\cdot}67 \times 10^6$ for DS1748, and 1×10^7 for DS1813 respectively. Each value is the mean of three independent tests. (■) 1 min, (□) 5 min, (▨) 10 min.

and surface proteins. The factors governing spore adherence have yet to be defined, but the spore outer surface is thought to possess a range of factors which facilitate adherence to inorganic and organic surfaces (Panessa-Warren *et al.* 1997, 2007; Paredes-Sabja and Sarker 2012).

The authors hypothesized that biocide exposure could cause changes in spore surface properties which could affect the ability of spores to adhere to inorganic surfaces. To determine if this was the case, exosporium-positive (DS1813) and -deficient (DS1748) spores were exposed to a range of NaDCC concentrations and subsequently the effect on relative hydrophobicity (RH) was measured. As can be seen in Fig. 3, the RH of the DS1813 spores decreased following exposure to 10 ppm of NaDCC at all contact times, and remained within a similar percentage range (20–35%) as the biocide concentration increased. In contrast, spores of DS1748 increased in hydrophobicity following contact with NaDCC from 14% RH to between 40 and 63% RH as the contact times increased (Fig. 3). The increase in hydrophobicity of strain DS1748 appears to be concentration dependent. This marked change in the RH of the exosporium-deficient spores occurred at concentrations of biocide which failed to inactivate all of the spores (Fig. 2), suggesting that chemical changes occurred to the surface of these surviving spores, which in turn may influence their ability to adhere to surfaces. Hence, sublethal exposure to biocide could have profound effects on the spread of spores across healthcare facilities.

In conclusion, we found that spores from *C. difficile* isolates respond differently when exposed to sublethal concentrations of the biocide NaDCC. The outer structures of spores, such as the exosporial layer and coat, may play a crucial role in a spore's ability to withstand or become susceptible to biocide attack. It is these outer structures which also contribute to the ability of the spores to adhere to inorganic and organic surfaces. When spores are exposed

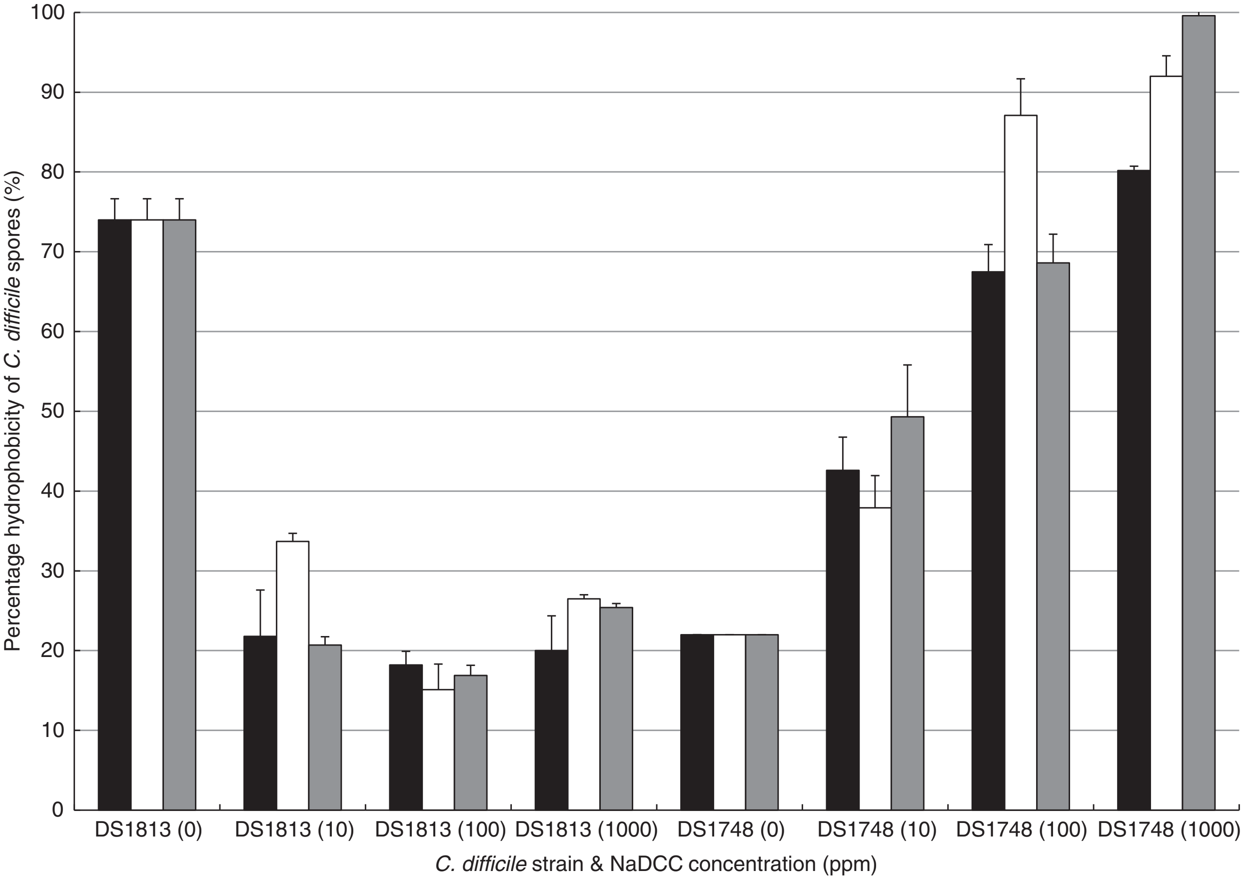

Figure 3 Relative hydrophobicity of exosporium positive (DS1813) and exosporium deficient (DS1748) *C. difficile* spores following exposure to varying NaDCC concentrations. Spores of hydrophobic DS1813 and hydrophilic DS1748 were examined for changes in their hydrophobicity following exposure to NaDCC at a range of concentrations and times using a spore adhesion to hydrocarbon test. The relative hydrophobicity of DS1813 spores remains high upon increasing longevity of exposure to NaDCC. DS1748 appears to significantly increase in percentage hydrophobicity after optimal NaDCC exposure (Two way ANOVA; $P = 0{\cdot}008$). Controls[12] were established by testing the hydrophobicity of DS1813 and DS1748 respectively before biocide exposure: DS1813 hydrophobicity (~75%), DS1748 hydrophobicity (~14%). Each value is the mean of three independent tests. (■) 1 min, (□) 5 min, (▨) 10 min.

to half the recommended concentration of biocide, there are noticeable changes in spore adherence ability; hence it is important to use the recommended concentration of NaDCC when attempting to inactivate spores.

Materials and methods

Strains and growth conditions

The clinical isolates of *C. difficile* used in this study were obtained from the National Anaerobic Reference Unit, Cardiff Wales. Strains used are as described in Joshi *et al.* (2012). Brain Heart Infusion (BHI) agar and Broth supplemented with 0·1% (w/v) sodium taurocholate (Oxoid Ltd, Basingstoke, UK) were used to culture the organisms and produce spores. Cultures were incubated at 37°C in an Bug Box Plus anaerobic workstation (Ruskinn Technology Ltd, Bridgend, UK) using a 85% nitrogen, 10% carbon dioxide and 5% hydrogen gas mix, and were examined at 48 h for the presence of characteristic colonies.

Clostridium difficile spore production and enumeration

As described previously in Joshi *et al.* (2012). The number of spores produced following broth culture was determined using a drop count method based on that of Miles *et al.* (1938).

Susceptibility of spores from two strains postexposure to sodium dichloroisocyanurate

Spores of strains DS1813 (exosporium positive) and DS1748 (exosporium deficient) at a concentration of

~1 × 10^8 CFU per ml were exposed to NaDCC at 10, 100 and 1000 ppm (Guest Medical Ltd) for contact times of 1, 5 and 10 min. These strains were selected based on their differing hydrophobic characteristics. Spores of *C. difficile* were diluted into 9 ml of NaDCC, at the specified concentrations and contact times, to an optical density between 0·500–0·600 nm at OD_{600} and separated into 4-ml aliquots. NaDCC activity in the sample was then neutralized by exposing spores to 1% (w/v) sodium thiosulphate solution (5 g l^{-1}) which has been shown previously to inactivate NaDCC at 0·5% (w/v) (Ungurs *et al.* 2011) (Sigma Aldrich, Dorset, UK) for 1 min at room temperature. After exposure, spores were centrifuged at 5000 ***g*** for 15 min and the supernatant discarded. The pellet was resuspended in 1 ml of fresh sterile deionized water (sdw) and stored at 4°C. The treated spores were then plated onto BHI agar supplemented with the germinant 0·1% (w/v) sodium taurocholate. The experiment was performed three times.

Spore adhesion to hydrocarbon test after biocide exposure

A hexadecane hydrocarbon-based SATH test (Rosenburg *et al.* 1980) was used to determine the relative hydrophobicity of spores of *C. difficile* strains DS1748 and DS1813 after exposure to NaDCC. As described previously in Joshi *et al.* (2012). Changes in hydrophobicity were calculated as a percentage from original OD_{600} to the final OD_{600} posthexadecane exposure.

Spore susceptibility to sodium dichloroisocyanurate at 500 ppm

The chlorine-releasing biocide sodium dichloroisocyanurate (NaDCC) was obtained in tablet form (HazTabs) from Guest Medical (Guest Medical Ltd) and diluted according to manufacturer's instructions to give the required concentrations in parts per million (ppm). The recommended in use concentration of NaDCC is 1000 ppm. Spores generated from 21 strains of *C. difficile*, at concentrations between 1 × 10^6 and 1 × 10^8 spores per ml were exposed to low (when compared to the in use concentration of 1000 ppm) concentrations (500 ppm) of NaDCC for 10 min. After exposure to NaDCC, biocide activity in the spore sample was neutralized with 1% sodium thiosulphate solution which has been shown previously to inactivate NaDCC at 0·5% (w/v) (Ungurs *et al.* 2011) (Sigma Aldrich). Spores were then centrifuged at 5000 ***g*** and the spore pellet resuspended in sdw. The experiment was performed three times. Spore viability was tested by culture after the experiment.

Statistical analysis

Statistical analysis was performed using MINITAB 17. Statistical significant differences were tested for using one way analysis of variance (ANOVA) at the 95% confidence interval with Anderson–Darling normality tests, and a Bartlett's test for equal variances. Two sample *t*-tests were also conducted. A *P* value of <0·05 was considered significant (Bowker and Randerson 2007).

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Conflict of Interest

None declared.

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