

# A Quaternary Ammonium Disinfectant Containing Germinants Reduces *Clostridium difficile* Spores on Surfaces by Inducing Susceptibility to Environmental Stressors

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Exposing *Clostridium difficile* spores to germinants in a quaternary ammonium matrix was an effective method to reduce environmental contamination by sensitizing the spores, leaving them susceptible to ambient conditions and enhancing killing by acid, high-intensity visible light, and radiation.

**Keywords.** *Clostridium difficile* spores; spore contamination; spore germination; surface disinfection; quaternary ammonium compound.

*Clostridium difficile* is a spore-forming anaerobic bacterium that is the most common cause of healthcare-associated diarrhea in developed countries [1]. Because *C difficile* is an obligate anaerobe, its spore form is the agent responsible for infection and recurrence of disease [2]. Spores are highly resistant structures consisting of multiple proteinaceous layers that surround a dehydrated core [3]. Prevention of *C difficile* transmission is challenging in part because spores survive for months on surfaces and are resistant to killing by many commonly used disinfectants such as quaternary ammonium compounds [4, 5]. Sodium hypochlorite (bleach) is a disinfectant with sporicidal activity, but it has several disadvantages, including being corrosive to many materials and irritating to some patients and staff members [6]. Due to these disadvantages, bleach disinfection is often reserved for rooms of patients with known *C difficile* infection (CDI), whereas quaternary ammonium compounds are often used for everyday disinfection of hospital surfaces. This approach may be problematic because recent studies suggest that spore shedding by asymptomatic carriers of *C difficile* have the potential to contribute significantly to disease transmission [7]. Consequently, there is a need for development of sporicidal disinfectants that do not adversely affect surfaces and that are nonirritating to staff and patients.

*Clostridium difficile* spores remain dormant until they are exposed to agents that trigger them to come back to life through the process of germination [8]. Spore germination is defined as the irreversible loss of spore-specific properties and is an essential step required before outgrowth of vegetative cells [9]. *Clostridium difficile* uses a species-specific mechanism to regulate spore germination that is initiated by the presence of bile salts and amino acids [10, 11]. When exposed to bile salts such as taurocholate, a cascade of events occurs that causes the spore's cortex layer to deteriorate and eventually leads to rehydration of the core [11]. Because germinated spores become more susceptible to killing by heat and other stressors, induction of germination has been studied as a possible measure to eliminate *Bacillus* and other *Clostridia* spp spores from food products (ie, addition of germinants to reduce heat resistance of spores) [12]. Initiation of germination has been shown to increase susceptibility of spores of *Bacillus* spp (*Bacillus subtilis*, *Bacillus coagulans*, and *Bacillus cereus*) and *Clostridium botulinum* to killing by radiation and heat [13–17]. We have recently demonstrated that exposing *C difficile* spores to a germination solution containing amino acids, minerals, and taurocholic acid resulted in initiation of germination in room air and significantly enhanced killing by ultraviolet C (UV-C) radiation and heat [18].

In this study, we tested the hypothesis that the addition of germinants to a quaternary ammonium disinfectant solution would result in sensitization of *C difficile* spores, leaving them susceptible to killing by ambient room conditions. In addition, we tested whether the addition of germinants to quaternary ammonium solutions would enhance killing by acid, high-intensity blue light, and UV-C radiation. Our findings suggest that this approach may provide a novel strategy to reduce the burden of *C difficile* spores in the healthcare environment.

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## METHODS

### Spore Strains and Growth Conditions

VA 17 is an epidemic (cdtB+) restriction endonuclease analysis (REA) BI strain, and VA 11 is a nonepidemic (cdtB-) REA J strain; both isolates are toxigenic (tcdA+, tcdB+) strains. American Type Culture Collection (ATCC) 43598 is a toxigenic (tcdA+, tcdB+) strain classified toxinotype VIII/ribotype 017 from serogroup F. *Clostridium difficile* cultures were incubated at 37°C for 48 hours in a Whitley MG1000 anaerobic workstation (Microbiology International, Frederick, MD) on prereduced cycloserine-cefoxitin-brucella agar containing 0.1% taurocholic acid and lysozyme 5 mg/L [19].

### Preparation of Spores

*Clostridium difficile* spores were prepared as previously described by Perez et al [20] with the following modifications. Spores were harvested from the Clospore media after 2 weeks of incubation in a Whitley MG1000 anaerobic workstation. Vegetative material was removed by density gradient centrifugation in Histodenz (Sigma-Aldrich, St. Louis, MO). Before testing, spore preps were confirmed by phase contrast microscopy and malachite green staining to be >99% dormant, bright-phase spores.

### Reduction of *Clostridium difficile* Spores on Carriers by Sensitizing with Germinants in a Quaternary Ammonium Matrix

Initial experiments were performed to determine whether *C difficile* spores would germinate in the presence of a quaternary ammonium disinfectant. Virex II 256 (Sealed Air, Charlotte, NC) containing didecyl dimethyl ammonium chloride (5%–10%) and n-alkyl dimethyl benzyl ammonium chloride (5%–10%) was diluted to the working concentration as directed by manufacturer. *Clostridium difficile* germinants (10 mM taurocholic acid and 100 mM L-glycine) were added, and the pH was adjusted to pH 7.5–8.0 with hydrochloric acid. The final concentration of quaternary ammonium was the same for solutions with or without the addition of germinants. Three strains of *C difficile* spores (described under Spore Strains and Growth Conditions) were inoculated into 1 mL water, quaternary ammonium, germinants, or quaternary ammonium with germinants to a final concentration of 6 log<sub>10</sub> colony-forming units (CFUs) per mL. After incubation under ambient conditions (~22°C) for 30 minutes, spores were viewed under phase contrast microscopy to determine whether they had changed from bright phase (dormant) to dark phase (germinated). In our experience, spores were fully germinated between 2 and 10 minutes of exposure to germinants (our unpublished data).

Once it was determined that spores germinated in the presence of a quaternary ammonium matrix, stainless steel carriers (1 cm<sup>2</sup>) were inoculated with 6 log<sub>10</sub> CFUs of each of the 3 strains of *C difficile* spores, spores were spread to cover the surface of the carrier, and allowed to air dry for 24 hours. Thirty microliters

of water, quaternary ammonium, germinants, or quaternary ammonium with germinants were applied to the inoculated carriers, and the solutions were allowed to air dry. Carriers were processed as described by the American Society for Testing and Materials (ASTM) in “Standard Quantitative Carrier Test Method to Evaluate the Bactericidal, Fungicidal, Mycobactericidal, and Sporocidal Potencies of Liquid Chemicals” after 1 hour, 24 hours, 5 days, 14 days, and 47 days posttreatment [21]. Carriers were processed using Dey Engley neutralizer (Becton, Dickinson and Company, Franklin Lakes, NJ) to assure that any residual quaternary ammonium would not affect viability of cultured spores and subsequent vegetative *C difficile*. Log<sub>10</sub> CFU reduction was determined by comparing recovery of spores from carriers after treatment with solution and recovery of spores from carriers treated with water and processed immediately (T = 0). Each experiment was repeated 3 times.

### Reduction of *Clostridium difficile* Spores on Hospital Surfaces by Sensitizing With Germinants in a Quaternary Ammonium Matrix

A hospital mattress, bedside table, call button, and bedrail were marked with 1-cm<sup>2</sup> circles. The circles were inoculated with 7 log<sub>10</sub> CFUs of ATCC 43598 *C difficile* spores, spores were spread to fill the circle, and air dried for 24 hours. Thirty microliters of water, quaternary ammonium, germinants, or quaternary ammonium with germinants were spread inside each inoculated circle. Sterile premoistened swabs were used to sample each surface immediately after application of each solution (baseline control) or 2 hours, 24 hours, and 7 days postapplication of solution. Each circle was swabbed only once; each time point had a distinct circle for sampling. Swabs were transferred to the anaerobic chamber, applied to *C difficile* Selective Agar plates containing Dey Engley neutralizer, and cultured as described previously. Colony-forming unit recovery was determined using Fotodyne Incorporated TotalLab Quant colony counting software. Log<sub>10</sub> CFU reduction was determined by comparing recovery of spores from swabs sampled immediately after solution treatment (T = 0) and recovery of spores from swabs at each time point. Each experiment was repeated 3 times.

### Reduction of Sensitized *Clostridium difficile* Spores by Low pH

Six log<sub>10</sub> CFUs of ATCC 43598 spores were inoculated into 1 mL of either water, quaternary ammonium, germinants, or quaternary ammonium with germinants. After incubating for 30 minutes under ambient conditions (~22°C), the spore suspensions were split into 2 centrifuge tubes (500 µL in each tube) and washed 3 times by pelleting and resuspended in sterile water. After the final centrifugation, 1 set of treated spores was resuspended in 1 mL neutral pH (~6.5) water, and the other set of spores was resuspended in 1 mL low pH (2.0 with hydrochloric acid) water and incubated at room temperature for 10 minutes. The spores were washed 3 times by centrifugation and resuspended in pH 7.5 phosphate-buffered saline (PBS). The samples

were transferred to the anaerobic chamber, serially diluted, and plated to quantitate viable CFU. Log<sub>10</sub> CFU reduction was determined by comparing recovery of spores treated with neutral pH water and recovery of spores treated with pH 2.0 water. The experiment was repeated 3 times.

#### **Reduction of Sensitized *Clostridium difficile* Spores by High-Intensity Blue Light and Ultraviolet C Radiation**

Eight log<sub>10</sub> CFUs of ATCC 43598 spores were inoculated into 1 mL of either water or quaternary ammonium with germinants. After incubating for 30 minutes under ambient conditions (~22°C), the spore suspensions were washed 3 times by pelleting and resuspended in sterile water. Four sets of stainless steel carriers (1 cm<sup>2</sup>) were inoculated with 10 µL of each spore suspension (~6 log<sub>10</sub> CFUs), spread to cover the surface of the carrier, and allowed to air dry. One set of carriers was processed immediately (baseline control), the second set was irradiated with UV-C (254 nm) light 4 feet from the source (Tru-D Rapid Room Disinfection device; Lumalier, Memphis, TN) for 10 minutes, the third set was placed 2 inches from a high-intensity blue light fixture (Vital Vio) for 24 hours, and the last set was placed under ambient room light for 24 hours. The temperature for each set was monitored and remained between 22°C and 24°C during the duration of treatment. Carriers were processed using the ASTM standard method as described previously [21]. Log<sub>10</sub> CFU reduction was calculated by comparing recovery of spores from carriers processed immediately and recovery of spores from carriers treated with room, blue, or UV-C light. The experiment was repeated 3 times.

#### **Bactericidal Activity of Quaternary Ammonium in the Presence of Germinants**

Virex II 256 was diluted to working concentration and prepared with or without the addition of germinants (10 mM taurocholic acid and 100 mM L-glycine). Stainless steel carriers (1 cm<sup>2</sup>) were inoculated with 10 µL of a PBS solution containing isolates from 24-hour cultures of a pulsed-field gel electrophoresis type USA300 methicillin-resistant *Staphylococcus aureus* (MRSA) and a VanA-type vancomycin-resistant *Enterococcus* (VRE) (~7 log<sub>10</sub> CFUs each). The inoculum was spread to cover the surface of the carrier and allowed to air dry. Thirty microliters of either PBS, Virex II 256, or Virex II 256 containing germinants was applied to carriers and incubated under ambient conditions for 10, 15, 20, or 30 minutes. Carriers were neutralized with 3 mL Dey-Engley medium and processed using the ASTM standard method as described previously [21]. Log<sub>10</sub> CFU reduction was determined by comparing recovery of spores from carriers after treatment with quaternary ammonium containing solutions and recovery of spores from carriers treated with PBS (control). The experiment was repeated 3 times.

#### **Data Analysis**

Data were analyzed with R statistical software (version 3.1.1). Continuous data were analyzed using unpaired *t* tests.

## **RESULTS**

#### **Sensitizing With Germinants in a Quaternary Ammonium Matrix Results in Reduction of *Clostridium difficile* Spores on Carriers**

Exposure of dormant *C. difficile* spores to germinants (taurocholic acid and L-glycine) in a quaternary ammonium matrix resulted in initiation of germination in room air. *Clostridium difficile* spores remained viable on carriers for up to 47 days after exposure to either water or quaternary ammonium and subsequent ambient desiccation. After a single exposure to the quaternary ammonium solution containing germinants, recovery of the 3 spore strains was significantly reduced after 1 hour (1.2 to 2.7 log<sub>10</sub> CFU reduction) with a further reduction by 24 hours (2 to 3.2 log<sub>10</sub> CFUs) ( $P < .01$  for each comparison to water or quaternary ammonium alone) (Figure 1, Supplementary Data 1). For 2 of the 3 strains, the reduction at 1 hour was significantly greater for the quaternary ammonium solution containing germinants than for germinants alone ( $P < .01$ ).

#### **Sensitizing with Germinants in a Quaternary Ammonium Matrix Results in Reduction of *Clostridium difficile* Spores on Hospital Surfaces**

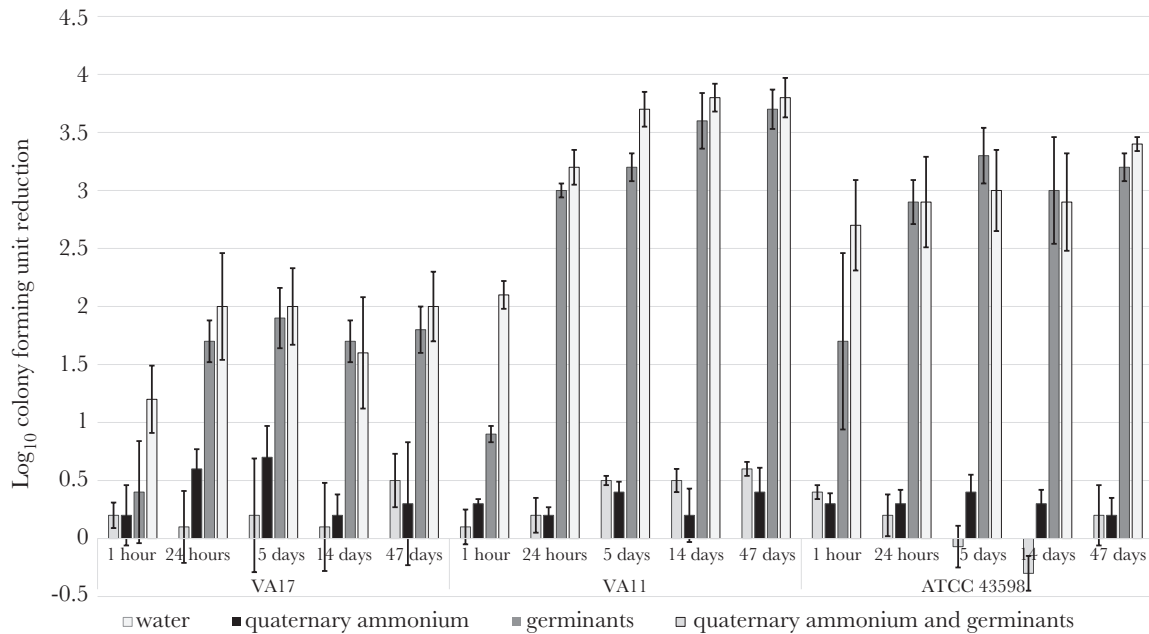
Spores on hospital surfaces treated with water or quaternary ammonium alone were not significantly reduced for up to 1 week after exposure (<1 log<sub>10</sub> CFU reduction in recovery). In contrast, by 2 hours after a single exposure to a quaternary ammonium solution containing germinants with subsequent ambient desiccation, recovery of spores on the test surfaces was significantly reduced by ~1–1.5 log<sub>10</sub> CFUs in comparison to spores exposed to quaternary ammonium or water alone ( $P < .01$  for each comparison) (Figure 2 Supplementary Data 2). The reduction at 2 hours was significantly greater for the quaternary ammonium solution containing germinants than for germinants alone for the spores on the call button ( $P < .01$ ), but not on the other surfaces. For each spore strain, further reductions occurred by 24 hours after exposure to the quaternary ammonium solution containing germinants or germinants alone.

#### **Sensitized *Clostridium difficile* Spores Are Killed by Low pH**

Dormant spores have been previously shown to remain resistant to killing at pH 2.0 [22]. After 30 minutes of incubation in water or quaternary ammonium, *C. difficile* spores remained resistant to killing by subsequent exposure to acidic conditions (pH 2.0) for 10 minutes. In contrast, spores exposed to 30 minutes of incubation in either germinants or quaternary ammonium containing germinants were reduced by >3 log<sub>10</sub> CFU when exposed to acidic conditions for 10 minutes ( $P < .001$  for each comparison) (Figure 3 Supplementary Data 3).

#### **Sensitizing *Clostridium difficile* Spores Enhances Reduction by High-Intensity Blue Light and Ultraviolet C Radiation**

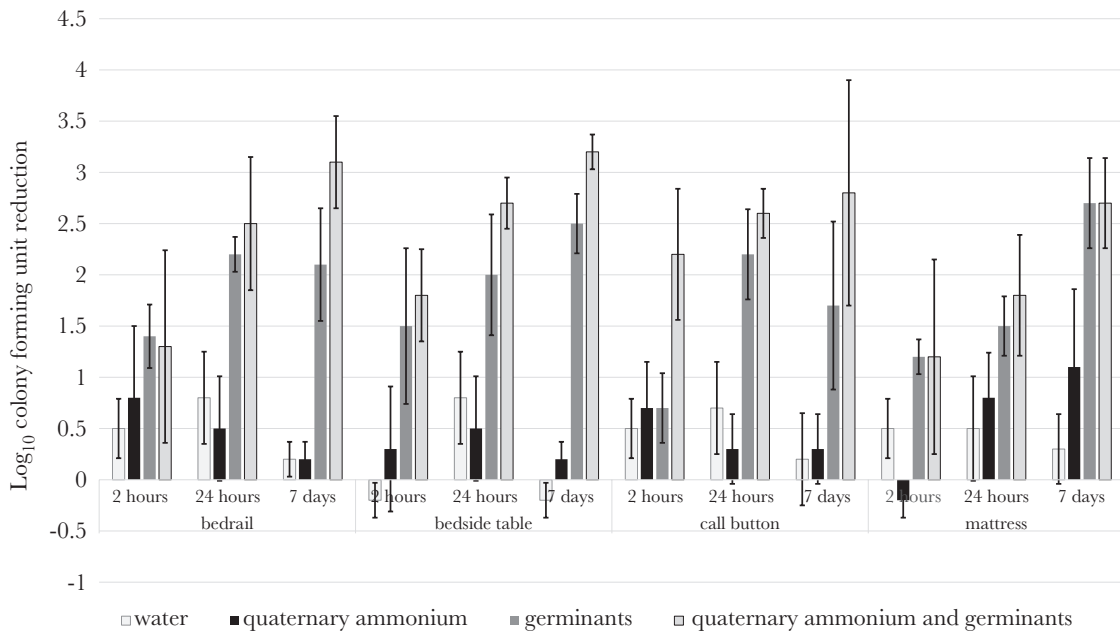
Dormant spores are protected from high-intensity radiation due to the dehydrated state of the vulnerable deoxyribonucleic acid in the spore's core. Dormant *C. difficile* spores



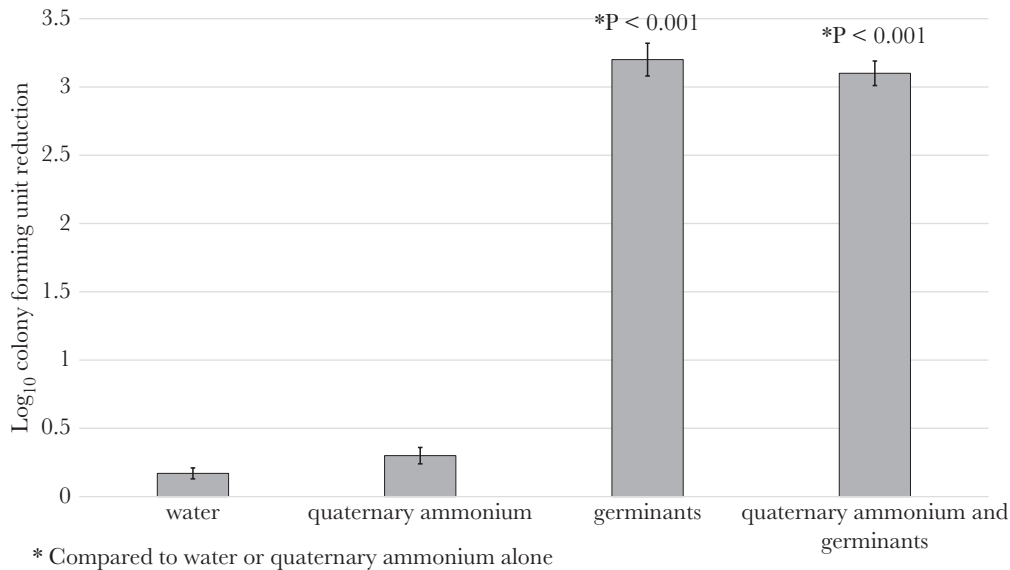
**Figure 1.** *Clostridium difficile* spores on carriers are reduced by sensitizing with germinants in a quaternary ammonium matrix. Six  $\log_{10}$  colony-forming units (CFUs) of *C. difficile* (VA17, VA11, and American Type Culture Collection [ATCC] 43598) on steel disk carriers were inoculated with 30  $\mu$ L water, quaternary ammonium, germinants, or quaternary ammonium containing germinants and allowed to air dry. Carriers were incubated under ambient conditions for up to 47 days.  $\log_{10}$  CFU reduction was determined by comparing recovery of spores from treated carriers and carriers treated with water ( $T = 0$ ). The means of data from triplicate experiments are presented. Error bars indicate standard error.

exposed to water under ambient conditions were not reduced by high-intensity blue light after 24 hours. However, when spores were exposed to a quaternary ammonium solution containing germinants, they were significantly reduced by

$\sim 2.5 \log_{10}$  CFUs under ambient conditions after 24 hours, and this reduction was further enhanced by  $>1 \log_{10}$  CFU in the presence of high-intensity blue light ( $P < .001$  for each comparison) (Figure 4 Supplementary Data 4). Dormant spores



**Figure 2.** *Clostridium difficile* spores on hospital surfaces are reduced by sensitizing with germinants in a quaternary ammonium matrix. Seven  $\log_{10}$  colony-forming units (CFUs) of *C. difficile* (American Type Culture Collection [ATCC] 43598) applied to common hospital surfaces were inoculated with 30  $\mu$ L of either water, quaternary ammonium, germinants, or quaternary ammonium containing germinants and allowed to air dry. Swabs were used to recover spores immediately ( $T = 0$ ), 2 hours, 24 hours, and 7 days after treatment.  $\log_{10}$  CFU reduction was determined by comparing recovery of spores from swabs after treatment and recovery of spores from swabs processed immediately ( $T = 0$ ). The means of data from triplicate experiments are presented. Error bars indicate standard error.

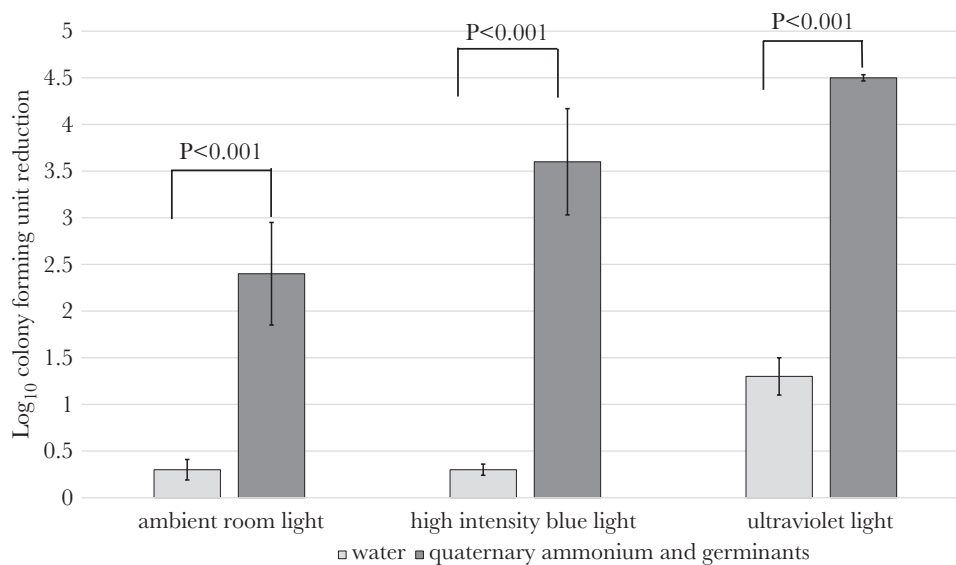


**Figure 3.** Sensitized *Clostridium difficile* spores are killed by low pH. Six log<sub>10</sub> colony-forming units (CFUs) of *C. difficile* (American Type Culture Collection [ATCC] 43598) were inoculated into 1 mL water, quaternary ammonium, germinants, or quaternary ammonium containing germinants. Spore suspensions were incubated under ambient conditions for 30 minutes, split into 2 sets, and then washed 3 times with water. The treated spores were exposed to pH 2.0 water or neutral pH water for 10 minutes. Log<sub>10</sub> CFU reduction was determined by comparing recovery of spores treated with neutral pH water and recovery of spores treated with pH 2.0 water. The means of data from triplicate experiments are presented. Error bars indicate standard error.

exposed to water were nominally reduced by ~1 log<sub>10</sub> CFU after 10 minutes of UV-C radiation. Exposure to quaternary ammonium containing germinants enhanced UV-C spore killing (>3 log<sub>10</sub> CFU reduction) ( $P < .001$ ).

#### Bactericidal Activity of Quaternary Ammonium in the Presence of Germinants

The addition of germinants reduced the killing efficacy of quaternary ammonium against MRSA and VRE by ~1 log<sub>10</sub> CFU



**Figure 4.** Sensitizing *Clostridium difficile* spores enhances reduction by high-intensity blue light and ultraviolet C radiation. Eight log<sub>10</sub> colony-forming units (CFUs) of American Type Culture Collection (ATCC) 43598 spores were inoculated into 1 mL of either water or quaternary ammonium with germinants. After incubating for 30 minutes under ambient conditions, the spore suspensions were washed 3 times by pelleting and resuspended in sterile water. Treated spores were inoculated onto carriers and either processed immediately, 24 hours after exposure to room lighting, 24 hours after exposure to high-intensity blue light, or after 10 minutes of irradiation with ultraviolet C radiation. Log<sub>10</sub> CFU reduction was calculated by comparing recovery of spores from carriers processed immediately and recovery of spores from carriers treated with room, blue, or ultraviolet C light. The means of data from triplicate experiments are presented. Error bars indicate standard error.



after 10 minutes of exposure (Figure 5 Supplementary Data 5). For VRE, quaternary ammonium with or without germinants had equal killing efficacy after 15 minutes of exposure. However, killing of MRSA was reduced by  $\sim 1 \log_{10}$  CFU in the presence of germinants for exposures up to 20 minutes. After 30 minutes of exposure, quaternary ammonium with or without germinants had equal killing efficacy against MRSA.

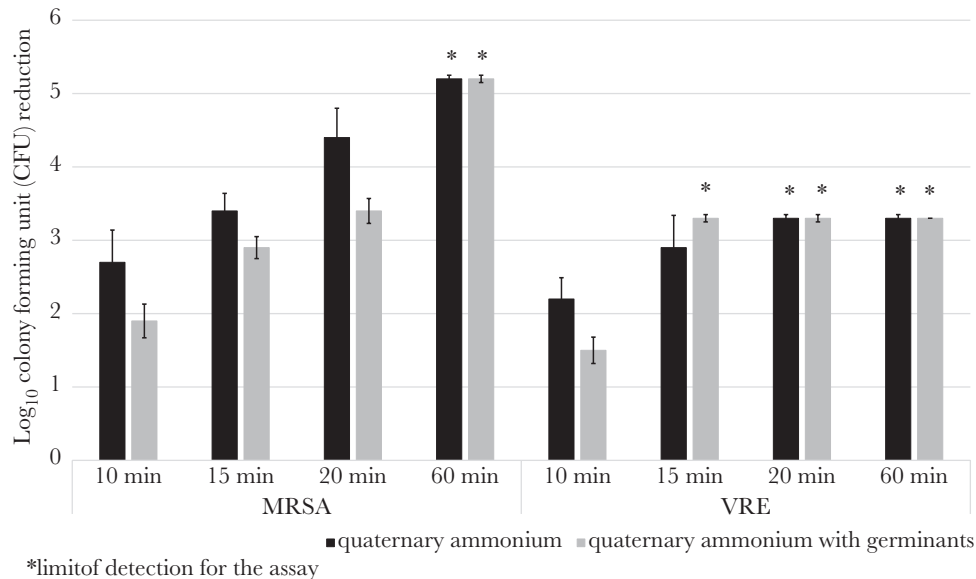
## DISCUSSION

We found that exposure of dormant *C. difficile* spores to germinants in a quaternary ammonium matrix resulted in initiation of germination in room air. After a single exposure, recovery of spores on steel carriers was reduced by 1.2 to 2.7  $\log_{10}$  CFU after 1 hour and by 2 to 3.2  $\log_{10}$  CFU after 24 hours. Similar results were obtained on the porous and textured surfaces commonly found in hospitals; however, there was more variability in reduction due to the type of surface tested (ie, call buttons had a bumpy plastic surface and the mattress had a smooth vinyl surface). Moreover, germinated spores exhibited enhanced killing by acid (pH 2), by blue light at 405 nm after 24 hours of exposure, and by UV-C radiation. The addition of germinants delayed the bactericidal activity of quaternary ammonium within the first 10 minutes of contact time; however, similar activity was achieved after 15 minutes of contact time, suggesting that longer contact time is required for germinant-containing solutions. These findings suggest that the addition of germinants to quaternary ammonium-based disinfectants could represent a novel strategy to reduce the burden of

*C. difficile* spores on hospital surfaces and to enhance killing by technologies such as blue light and radiation.

Triggering germination has been studied as a means to enhance killing of spores of *Bacillus* spp and of other *Clostridia* spp [12–17], due in part to the detailed elucidation of the germination process in these species [9]. For *Bacillus* and other *Clostridia* spp, germination consists of 2 distinct constitutive stages [23]. Stage I is composed of 3 processes: (1) release of  $H^+$ , monovalent cations and  $Zn^{2+}$  from the spore core (increasing core pH from  $\sim 6.5$  to 7.7); (2) release of dipicolonic acid and  $Ca^{2+}$  from the spore core; and (3) increase in the spore core's hydration [23]. Stage II is composed of 2 processes: (1) hydrolysis of the spore's cortex, and (2) swelling of the spore core due to further increase in hydration and expansion of the germ cell wall [23]. In contrast, recent studies have revealed that *C. difficile* has a species-specific germination process that is triggered by bile salts and glycine [10]. Unlike *Bacillus* and other *Clostridia* spp that use inner membrane germinant receptors to sense small molecule nutrients, *C. difficile* uses a subtilisin-like serine protease as a germinant receptor to sense bile salts [24]. As a result of these differences, degradation of the thick protective cortex layer (Stage II germination) precedes release of dipicolonic acid from the core (Stage I) [11].

These differences between *C. difficile* and other spore-forming bacteria have important implications for utilization of germination as means to enhance killing of *C. difficile* spores. Research is needed to identify and optimize chemical germinants that target *C. difficile*'s unique mode of germination. In addition, studies are



**Figure 5.** Bactericidal activity of quaternary ammonium in the presence of germinants. Seven  $\log_{10}$  colony-forming units (CFUs) of pulsed-field gel electrophoresis type USA300 methicillin-resistant *Staphylococcus aureus* (MRSA) and VanA-type vancomycin-resistant *Enterococcus* (VRE) on steel disk carriers were exposed to phosphate-buffered saline ([PBS] control), quaternary ammonium, or quaternary ammonium containing germinants. Carriers were incubated for 10, 15, 20, and 30 minutes under ambient conditions.  $\log_{10}$  CFU reduction was determined by comparing recovery of spores from PBS-treated carriers and carriers treated with solutions containing quaternary ammonium. The means of data from triplicate experiments are presented. Error bars indicate standard error.

needed to identify stressors that enhance killing of germinated *C difficile* spores in room air. It is likely that germinated spores are killed on surfaces in room air due to desiccation and exposure to oxygen, but other factors may contribute. Notably, at 1 hour, spores were reduced more by exposure to quaternary ammonium compounds plus germinants than by exposure to germinants alone, suggesting that the combination enhanced killing. Wheeldon et al [25] recently reported that exposure of *C difficile* spores to taurocholate under aerobic conditions resulted in enhanced killing on copper surfaces [25]. In conjunction with our findings, such reports should stimulate additional research to identify other stressors that enhance killing of germinated spores.

Our findings have important practical applications. First, daily disinfection of non-CDI rooms is typically performed with nonsporicidal agents such as quaternary ammonium compounds. Because spore contamination is not uncommon in non-CDI rooms, the addition of germinants to daily disinfection may reduce hidden reservoirs of contamination [7]. Second, spores that are sensitized by germinants could potentially have a decreased chance of infecting susceptible patients because they may not survive the low pH of normal stomach acid. Further testing in an animal model of *C difficile* colonization is needed to test this hypothesis. Third, our findings suggest that novel lighting fixtures that emit high-intensity visible light could be effective in reducing dormant *C difficile* spores that are exposed to germinants [26]. Spores exposed to germinants change from phase bright (reflecting visible light) to phase dark (visible light can pass through), potentially allowing high-intensity, visible light to reach its target. Finally, our findings suggest that the time required for UV-C devices to achieve a 3 log<sub>10</sub> CFU reduction of spores on surfaces could be reduced from 45 minutes to 10 minutes with the application of quaternary ammonium solution containing germinants [18, 27]. Because precleaning of surfaces is recommended before running UV-C devices, the addition of germinants could increase the feasibility of UV-C use in hospital settings by reducing the time required for device operation.

## CONCLUSIONS

Our study has some limitations. First, only 3 strains of *C difficile* were studied. More studies are necessary to determine whether this strategy is effective against a broader range of strains. Second, the studies on hospital surfaces involved inoculation of spores onto surfaces rather than spore contamination from patients. Additional studies are needed on native *C difficile* contamination. Third, it is possible that factors such as organic matter might reduce the efficacy of the germinants in the quaternary ammonium solutions. Fourth, the presence of germinants reduced the efficacy of quaternary ammonium against vegetative bacteria. Longer exposures were necessary to achieve optimal killing of MRSA and VRE. Additional studies are needed to develop formulations with rapid, high-level bactericidal activity. Finally, as previously described by Gould et al [28], a persistent

“superdormant” fraction of spores remains unaltered by exposure to germinants. More recently, Ghosh and Setlow [29] isolated superdormant spores of *B subtilis* and *Bacillus megaterium* and found that superdormant spores require an increased signal for triggering spore germination compared with most spores in populations. Further studies are therefore needed to optimize germinants for *C difficile* to further enhance killing by triggering germination in the superdormant fraction of spores.

## Supplementary Data

Supplementary material is available at *Open Forum Infectious Diseases* online.

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