

Activity of selected oxidizing microbicides against the spores of *Clostridium difficile*: Relevance to environmental control

Justo Perez, PhD, V. Susan Springthorpe, MSc, and Syed A. Sattar, PhD
Ottawa, Ontario, Canada

Background: *Clostridium difficile* is an increasingly common nosocomial pathogen, and its spores are resistant to common environmental surface disinfectants. Many high-level disinfectants (eg, aldehydes) are unsuitable for environmental decontamination because they need several hours of contact to be sporicidal. This study tested the potential of selected oxidative microbicides to inactivate *C difficile* spores on hard surfaces in relatively short contact times at room temperature.

Methods: The spores of a clinical isolate of *C difficile* were tested using disks (1 cm diameter) of brushed stainless steel in a quantitative carrier test. The spores of *C sporogenes* and *Bacillus subtilis*, common surrogates for evaluating sporicides, were included for comparison. The clostridia were grown separately in Columbia broth (CB), and *B subtilis* was grown in a 1:10 dilution of CB. Each disk received 10 μ L test spores with an added soil load, and the inoculum was dried. One disk each was placed in a glass vial and overlaid with 50 μ L test formulation; controls received an equivalent volume of normal saline with 0.1% Tween 80. At the end of the contact time the microbicide was neutralized, the inoculum recovered from the disks by vortexing, the eluates were membrane filtered, and the filters placed on plates of recovery medium. The colony-forming units (CFU) on the plates were recorded after 5 days of incubation. The performance criterion was $\geq 6 \log_{10}$ ($\geq 99.9999\%$) reduction in the viability titer of the spores. The microbicides tested were domestic bleach with free-chlorine (FC) levels of 1000, 3000, and 5000 mg/L; an accelerated hydrogen peroxide (AHP)-based product with 70,000 mg/L H_2O_2 (Virox STF); chlorine dioxide (600 mg/L FC); and acidified domestic bleach (5000 mg/L FC).

Results: Acidified bleach and the highest concentration of regular bleach tested could inactivate all the spores in ≤ 10 minutes; Virox STF could do the same in ≤ 13 minutes. Regular bleach with 3000 mg/L FC required up to 20 minutes to reduce the viability of the all the spores tested to undetectable levels; chlorine dioxide and the lowest concentration of regular bleach tested needed approximately 30 minutes for the same level of activity.

Conclusions: Acidified bleach, Virox STF, and regular bleach (3000-5000 mg/L FC) could inactivate *C difficile* spores on hard environmental surfaces in approximately 10 to 15 minutes under ambient conditions. All of these products are strong oxidizers and should be handled with care for protection of staff, but acidified and regular bleach with high levels of FC also release chlorine gas, which can be hazardous if inhaled by staff or patients. (Am J Infect Control 2005;33:320-5.)

Clostridium difficile-associated diarrhea (CDAD) is a serious and increasingly frequent nosocomial infection,^{1,2} resulting in significant annual health care costs.³ Disruption of the normal gut flora by factors such as antibiotic usage,⁴ advanced age,⁵ cancer therapy,⁶ immunosuppression, and infections with other gastrointestinal pathogens⁷ are among the contributors to an increase in the number of cases of CDAD. Recurrence of the disease or reinfection is

frequently observed.^{8,9} Those infected with *C difficile* excrete large numbers of vegetative bacteria as well as spores in their feces,¹⁰ and recent evidence suggests that *C difficile* is present among human gut bacteria more frequently than previously thought.¹¹ Frequent episodes of loose stools, typical in CDAD, increase the risk of environmental contamination with *C difficile*, which can be linked to the number of CDAD patients^{12,13} and the length of hospital stay.⁵

Spores may remain viable for months on contaminated surfaces¹³ and can often be recovered from environmental samples.^{14,15} Such environmental contamination has been incriminated in the spread of CDAD,^{1,5,14,15} either directly or through the hands of health care personnel. The spores of *C difficile* are also resistant to common types and levels of general-purpose hard surface disinfectants, and their elimination from rooms of CDAD patients may require the use of sporicidal chemicals. Indeed, arbitrary use of poorly effective hard surface disinfectants may lead to dissemination of the *C difficile* spores over a wider area

From the Centre for Research on Environmental Microbiology (CREM), Faculty of Medicine, University of Ottawa, Ottawa, Ontario, Canada.

Reprint requests: Syed A. Sattar, PhD, Director, Centre for Research on Environmental Microbiology (CREM), Faculty of Medicine, University of Ottawa, 451 Smyth Road, Ottawa, Ontario, Canada K1H 8M5. E-mail: ssattar@uottawa.ca.

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during routine environmental decontamination. This study was initiated to identify oxidative, sporicidal, hard surface disinfectants that could inactivate significant numbers of the spores of *C. difficile* under ambient conditions and with contact times relevant to environmental surface disinfection. An internationally accepted quantitative carrier test method¹⁶ was employed to determine the activity of the tested formulations against spores of a clinical isolate of *C. difficile*.

MATERIALS AND METHODS

Test organisms

The *C. difficile* tested was a local clinical isolate from a child and was designated as CHEO. Because the spores of *Bacillus subtilis* (ATCC 19659) and *Clostridium sporogenes* (ATCC 7955) are widely used in assessing the sporicidal potential of disinfectants,¹⁷ they were included in this investigation to compare their susceptibility to that of the spores of *C. difficile*. The bacteria were stored at -80°C in appropriate media (see below), plus 10% glycerol.

Sporulation media and culture conditions

The spores of *C. difficile* were prepared by growing it anaerobically for 7 to 10 days in Erlenmeyer flasks with 25 mL of either Columbia broth (CB; Difco, Detroit, MI) or brain-heart infusion (BHI) broth. The culture was centrifuged at 8000g for 15 minutes at room temperature and was washed 3 times in cold, sterile, distilled water between centrifugations. The final pellets were resuspended in sterile distilled water and heated to 80°C for 10 minutes to kill vegetative cells.

B. subtilis was grown aerobically for 72 hours at $36^{\circ}\text{C} \pm 1^{\circ}\text{C}$ in a 1:10 dilution of CB containing $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$. Full-strength CB inoculated with *C. sporogenes* was incubated anaerobically for 5 days at $36^{\circ}\text{C} \pm 1^{\circ}\text{C}$. Processing of the grown cultures of *B. subtilis* and *C. sporogenes* spores was as described above for *C. difficile*. The spore suspensions, which contained 10^8 to 10^9 colony-forming units (CFU)/mL, were held at 2°C to 4°C until needed but for no longer than 1 month.

Standard hard water (HW)

Water with a hardness of 400 ppm as calcium carbonate (CaCO_3) was prepared according to specifications of AOAC International¹⁷ and was used to dilute disinfectants if required.

Free chlorine (FC) determinations

Levels of FC in the test solutions were measured by the DPD (N, N-diethyl-p-phenylenediamine) method using premeasured reagent pillows (Hach Co., Loveland,

CO). The results were read in a DR/820 Colorimeter (Hach).

Hydrogen peroxide (H_2O_2) determinations

H_2O_2 concentrations in the test solutions were determined by adding titanium sulphate reagent to produce a yellow peroxy-complex as previously described.¹⁸ The sample was then analyzed in a spectrophotometer (Pye Unicam SP6-450) at 410 nm, and the absorption compared with a standard curve.

Disinfectants tested

Chlorine dioxide (ClO_2). To prepare a ClO_2 solution, 124.59 mL distilled water was added to a dark container with a magnetic stir bar, and the following were added in order: 0.263 mL domestic bleach (containing 5.25% sodium hypochlorite; NaOCl), 0.035 mL concentrated hydrochloric acid (HCl), and 0.112 mL 24.67% sodium chlorite (NaClO_2). The solution was mixed for 1 minute and allowed to sit for 10 minutes. This gave a solution (pH 4.0) with FC of 630 ± 60 mg/L.

Acidified bleach. One part domestic bleach, 2 parts 400 mg/L HW,¹⁸ 1 part of commercial white vinegar (5%), and 6 parts of 400 mg/L HW were mixed in that order. The concentration of FC in the acidified bleach was 5000 ± 200 mg/L with a pH 5.3.

Domestic bleach. Dilutions of locally purchased domestic bleach with approximately 5000 mg/L FC were prepared by adding 0.54 mL full-strength product (5.25%) to 5 mL 400 mg/L HW¹⁸; the pH of the diluted bleach was 11.0. Further dilutions of the bleach (3000 mg/L, pH ~ 10.5 , and 1000 mg/L, pH ~ 10.0) were prepared by diluting the 5000 mg/L FC solution with HW as required.

Virox STF. This was a commercially available (Virox Technology, Oakville, ON) high-level disinfectant based on accelerated hydrogen peroxide (AHP) technology. It contained 7% (70,000 mg/L) H_2O_2 and was tested undiluted.

Testing of sporicidal activity

The second tier of a quantitative carrier test (QCT-2), a standard of ASTM International,¹⁶ was used to determine the sporicidal activity of the formulations tested. To simulate the presence of body fluids, each 340 μL spore suspension was mixed with 35 μL 5% tryptone (Difco, Detroit, MI), 25 μL 5% bovine serum albumin (Sigma, St. Louis, MO), and 100 μL 0.4% bovine mucin (Sigma), all in 0.3 mmol/L potassium phosphate buffer, with 0.05% MgSO_4 .¹⁶ Stainless steel disks (1 cm diameter; 0.7 cm thick) acted as a prototypical hard environmental surface, and each one was contaminated with 10 μL test spore suspension.

Table 1. Initial titers of spores on controls as CFU/carrier

Formulation tested	<i>B subtilis</i>	<i>C sporogenes</i>	<i>C difficile</i> CHEO (BHI)	<i>C difficile</i> CHEO (CB)
Chlorine dioxide (600 mg/L free chlorine)	2.50×10^6	9.00×10^6	6.20×10^7	2.70×10^7
Virox STF (70,000 mg/L H ₂ O ₂)	2.85×10^6	1.15×10^7	1.22×10^7	1.53×10^7
Acidified bleach (5000 mg/L free chlorine)	3.12×10^6	1.80×10^7	2.70×10^7	2.83×10^7
Regular bleach (5000 mg/L free chlorine)	3.50×10^6	6.50×10^6	6.50×10^7	5.00×10^7
Regular bleach (3000 mg/L free chlorine)	4.45×10^7	3.50×10^6	1.03×10^7	2.75×10^7
Regular bleach (1000 mg/L free chlorine)	3.00×10^7	3.50×10^6	1.03×10^7	2.75×10^7

The inocula on the disks were first air-dried for 20 minutes in a laminar flow cabinet and then for at least 2 hours under vacuum in a desiccator. One disk each was then placed, with contaminated side up, at the bottom of a sterile 20 mL glass vial, covered with 50 μ L test formulation, and held for the desired contact time at room temperature. Control disks received an equivalent volume of normal saline with 0.1% Tween 80 (Saline-T). At the end of the incubation time, the action of the disinfectant was stopped by the addition of 9.95 mL neutralizer (see below). The contents of the vials were vortexed for 3 to 5 cycles of 30 seconds each to elute the inoculum from the surface of the disks. The eluates and several washes of the vials with Saline-T were passed through a membrane filter (Millipore Corp.; 47 mm diameter; 0.2 μ m pore size). Each filter was then placed on the surface of an appropriate agar recovery medium. The plates were incubated at 36°C \pm 1°C; CFU were counted at 2 days and again at day 5, and log₁₀ reductions in spore titres were calculated.

Neutralizers

For all chlorine-based formulations, the neutralizer was 1% sodium thiosulfate (Na₂S₂O₃) in normal saline (0.85% NaCl), with 0.1% (final concentration) of Tween 80 (Bioshop, Burlington, ON). The AHP-based product was neutralized with Lethen Broth (LB; Difco) containing 0.1% Na₂S₂O₃.

Statistical analysis

All experiments were repeated at least 3 times with duplicate determinations in every one. Analyses used the Statistica 99 program (Stat-Soft Inc, Tulsa, OK); the NCSS-PASS 2000 statistical package (Kaysville, UT); the Excel spread sheet (Microsoft Corp., Redmond, WA), and the CurveExpert version 1.38, by Daniel G. Hyams, Hixson, TN; the latter uses the Levenberg-Marquardt method to address nonlinear regressions.

RESULTS

The numbers of viable spores on the control carriers are summarized in Table 1. Although their titers varied by approximately 1 log₁₀ among experiments, the

minimum was always >6 log₁₀. Each value shown is the average of at least 2 repetitions. The values for the viable spores recovered from the test carriers in a given experiment were compared with the control values normalized to 100%; data were expressed as percentage reductions.

However, for infection control purposes, it is important to know the contact times desirable for elimination of *C difficile* spores in the field. The times required for the microbicides to inactivate ≥ 6 log₁₀ ($\geq 99.9999\%$) of the tested spores were therefore estimated by processing the kinetic data with a nonlinear regression using the Lambert-Johnston model.¹⁹

The findings on the sporicidal activity of the formulations tested are summarized in Fig 1. Acidified bleach and regular bleach both at 5000 mg/L proved to be the most reliable to inactivate all the spore types to a high level within a short contact time; the viability of all 3 types of spores was reduced by $\geq 99.9999\%$ within 10 minutes. However, for the *C difficile* spores grown in either medium, the Virox STF was also effective within 10 minutes. The 2 higher dilutions of regular bleach in hard water required longer contact times for the same level of reduction in the initial titer of *C sporogenes*, *B subtilis*, and *C difficile* spores—the 3000 mg/L concentration needed approximately 15 minutes, whereas the 1000 mg/L concentration needed from 15 to 25 minutes.

The spectrum of susceptibility was different for *C difficile* isolate grown on the 2 different media. For Virox STF, chlorine dioxide or acidified bleach—all acid in reaction—the *C difficile* spores grown in BHI were less susceptible than those grown in CB, whereas, for regular bleach solutions—which are all alkaline—the reverse was true. Although the 600 mg/L chlorine dioxide inactivated the *B subtilis*, the *C sporogenes*, and the *C difficile* spores grown in CB within 15 minutes, approximately twice as long was required to inactivate the *C difficile* spores grown in BHI.

DISCUSSION

Although the role of environmental surfaces in the transmission of nosocomial infections is not always recognized, it seems to be supported for CDAD

spread.^{5,13-15,20-23} Nothing has been published regarding the survival of *C difficile* spores on inert surfaces after treatment with disinfectants; nevertheless, there are anecdotal as well as published reports^{10,24-26} of a decrease in the transmission of CDAD when oxidative disinfectants such as bleach or AHP are used instead of a detergent alone or a quaternary ammonium-based cleaner.

It is known that the nature of the surface to be disinfected, the concentration and volume of germicide, and the time of contact, among other factors, influence the degree of disinfection achieved.²⁷ The emphasis here was on oxidizing formulations likely to be sporicidal in relatively short contact times. Neither detergents nor quaternary ammonium-based formulations were tested in this study because they are both known to be ineffective in killing bacterial spores.²⁴ Even for nosocomial pathogens that are much less hardy than *C difficile*, the quaternary ammonium products frequently applied to certain environmental surfaces have been shown to be inadequate to prevent further dissemination of the pathogen.²⁸ For any benefit to be derived from these cleaners for *C difficile*, one would need to ensure that the cleaning procedure itself would be effective in lifting and removing the spores from the contaminated surfaces; otherwise, cleaning would just move the spores from one place to another.

Therefore, during a CDAD outbreak and/or for known cases of CDAD, to prevent further transmission, it is clearly desirable to understand which types of products can effect an adequate or virtually complete decontamination of personal items and or small and large environmental surfaces. Moreover, the contact time needed for such decontamination is important to understand. This study, therefore, focussed on the use of oxidative products and realistic contact times to rid environmental surfaces of *C difficile* spores. A standardized carrier test protocol with a relatively small volume of disinfectant was employed to assess sporicidal activity.¹⁶ Because the spores of *C sporogenes* and *B subtilis* are frequently used for label claims of high-level disinfectants,¹⁷ standard strains of the 2 organisms were included here for comparison.

All spore preparations studied exhibited a slightly different pattern of susceptibility when exposed to the various disinfectant types. Some of the comparative data may also have been affected by variations in the initial titer for the spores (see for example the data with chlorine dioxide). However, certain general patterns were readily apparent. With the strongest oxidants (Virox STF, acidified bleach, and the higher concentrations of domestic bleach), the inactivation of all tested spores was essentially complete within 15 minutes. Chlorine dioxide (600 mg/L FC) and bleach at 1000 mg/L FC required up to 30 minutes to achieve the same

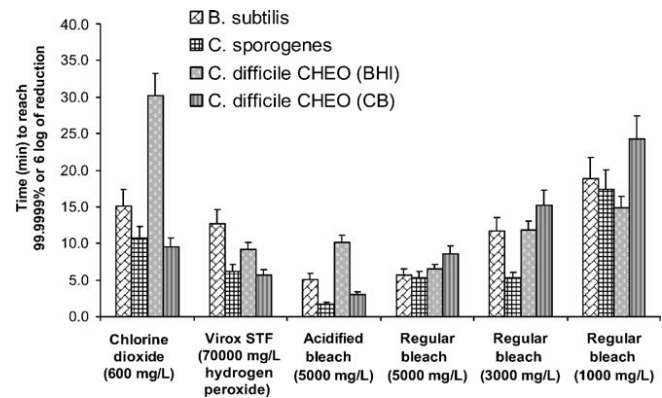


Fig 1. Times required for the microbicides to inactivate $\geq 6 \log_{10}$ (99.9999%) of the spores tested.

level of activity; stronger solutions of chlorine dioxide (eg, 1000 mg/L FC) may have faster sporicidal activity.

The spores of the *C difficile* strain tested proved to be slightly less sensitive than the other spores for all formulations except the AHP. For the chlorine dioxide and the acidified bleach, both of which are used in hospitals only rarely, it was the BHI-grown *C difficile* spores that were the least sensitive. For the regular bleach solutions, it was the *C difficile* spores that were grown in the CB medium that were least sensitive. The reasons for these differences in spore sensitivity are unknown at this point, but may relate to the disinfectant pH, the mechanism of action of the formulation, and/or characteristics of the organisms and any residual soil load from the growth medium. It is recognized that the medium and growth conditions used to generate spores may alter their resistance to microbicides.²⁹ How the microbicide resistance of laboratory-grown spores of *C difficile* relates to those naturally produced in the human gut remains unknown. Therefore, the precise contact times given in these results must be taken with some caution for application to the field, and the main emphasis should perhaps be on the types of products that are likely to be efficacious.

Oxidative formulations also showed efficacy against *C difficile* spores in another study,³⁰ and they are among the most frequently used microbicides in decontamination of environmental surfaces. They are often used at concentrations and contact times unsuitable to inactivate large numbers of bacterial spores. The use of such formulations at higher concentrations on environmental surfaces to reduce contact time to 10 to 30 minutes can be potentially hazardous to the user and materials treated. Thus, workplace safety demands adequate awareness and personal protection for their use.

Solutions of sodium hypochlorite (bleach), widely used in health care and other settings, are relatively inexpensive, fast-acting, and broad-spectrum microbicides. However, the presence of sodium hydroxide for stability makes them highly alkaline in high concentrations and compromising microbicidal activity to some degree.⁵¹ Acidification enhances the power and speed of microbicidal action of bleach with a corresponding reduction in its stability.⁵² Therefore, acidified solutions of bleach must be freshly prepared for use. Because acidification releases chlorine gas more rapidly, such solutions require greater care in handling.

AHP-based technology has been gaining acceptance as a means of formulating safer and environmentally benign microbicides with activity against major classes of nosocomial pathogens.⁵³ The AHP formulation tested in this study contains 7% hydrogen peroxide as the active ingredient and is sold as a high-level disinfectant.

An ancillary objective here was to assess the comparative microbicidal activity of the tested formulations against the spores of *C difficile* and 2 other types of spores (*B subtilis* and *C sporogenes*), commonly used as surrogates in sporicidal tests.¹⁷ Although tests with additional strains of *C difficile* may be needed, available data do not indicate any definitive differences.

Stainless steel is often used as a reference surface in disinfection testing but does not represent all hospital surfaces that may have different demands on the disinfectants applied to them. Also, the numbers of spores used in this study may be higher than would often be encountered on environmental surfaces. However, the volume of disinfectants applied in the test is also higher than may be in contact with the surfaces by wiping. It is suggested that the sporicidal products tested here would be appropriate to eliminate severe or long-standing environmental contamination with *C difficile* but should not be routinely employed because of potential hazards to personnel and patients.

It is generally difficult to produce high-titered pools of spores of *C difficile* in the laboratory. In this investigation, several clinical and standard strains of this organism were initially included, but the one eventually selected was the one that gave the best spore yields. However, the findings should be applicable to *C difficile* in general because of the layers of stringency built into the test protocol and the parallel testing with 2 well-known surrogates for assessing the sporicidal activity of microbicides.

In conclusion, based on quantitative carrier testing, the findings of this study identify oxidative microbicides suitable to rid environmental surfaces of contamination by spores of *C difficile* under ambient conditions and in reasonably short contact times.

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