Evaluation of sporicidal activities of selected environmental surface disinfectants: Carrier tests with the spores of *Clostridium difficile* and its surrogates

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Background: The emergence of *Clostridium difficile* as a major nosocomial pathogen points to the need for safe, effective, and fast-acting environmental sporicides for infection prevention and control. Available and fast-acting sporicides are generally corrosive and unsafe for both humans and the environment.

Methods: We evaluated chlorine bleach (500 and 5000 ppm) and a gel containing 4.5% of accelerated hydrogen peroxide against the spores of *C difficile* and its surrogates *Bacillus subtilis* and *Clostridium sporogenes* with contact times of 1, 5, and 10 minutes at 20°C ± 2°C using a quantitative carrier test (QCT-2), which is a standard (ASTME2197) of American Society for Testing and Materials International.

Results: The gel and the higher level of bleach inactivated ≥6-$\log_{10}$ of viable spores of all 3 types in 10 minutes but were unable to do so after 1 and 5 minutes. The lower level of bleach showed virtually no activity even after 10 minutes. The gel could keep the treated surface wet for the entire 10 minutes, whereas the bleach became visibly dry in approximately 4 minutes and needed reapplication.

Conclusion: The gel, with no off-gassing and designed especially for use on toilet bowls to allow for the required dwell time on vertical surfaces with one application, is a potential alternative to high levels of bleach.

Key Words: *Clostridium difficile*; infection control; disinfectants; sporicides; nosocomial infections; toilet bowl decontamination; microbicides; environmental hygiene; hydrogen peroxide; bleach.

Until the emergence of *Clostridium difficile* as a significant nosocomial pathogen,[1-3] bacterial spore formers were not among the major targets for environmental decontamination in health care settings. The frequent and profuse diarrhea because of *C difficile* can contaminate environmental surfaces with stable and disinfectant-resistant spores[4,5]; indeed, such spores detected in hospital wards[6] may lead to secondary cases.[7] Because toilets are most often contaminated with *C difficile*,[8,9] they require frequent and proper decontamination.

Environmental surface disinfectants such as alcohols, quaternary ammonium compounds (quats), and phenolics are ineffective against bacterial spores even at high concentrations[10]; therefore, to decontaminate such surfaces, a sporicidal agent is required.[11] Although such sporicidal agents may be efficacious under standard in vitro testing conditions, they may fail in the field if the required contact time is not met. It is well-known that the activity of disinfectants in general is time dependent, and, consequently, the rate of evaporation of the active ingredient in a given formulation would influence its ability to produce the desired level of microbicidal effect for the target pathogen. The objective of this study was to investigate the level of sporicidal action that can be obtained before the surface dries out for selected surface sporicides.

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MATERIALS AND METHODS

Disinfectants tested

Two products were tested in this study. (1) accelerated hydrogen peroxide (AHP) sporicidal gel, which is based on 4.5% AHP, a combination of hydrogen

peroxide, surfactants, and stabilizers; and (2) domestic bleach (5.25% sodium hypochlorite). The AHP sporicidal gel was tested undiluted, whereas a locally purchased domestic bleach solution was evaluated with free available chlorine (FAC) concentrations of 500 and 5000 ppm. Water with a standard hardness of 400 ppm as calcium carbonate was made as per the specifications of the Association of Official Analytical Chemists (AOAC) International[12] to make in-use dilutions of the formulations when required.

Test organisms

The spores of *Bacillus subtilis* (ATCC 19659), *Clostridium sporogenes* (ATCC 7955), and *C difficile* (ATCC 43598) were the challenge. The first 2 organisms are commonly used as surrogates to assess the sporicidal activity of disinfectants.

C difficile spores were grown anaerobically in a liquid medium at 36°C ± 1°C and held for a further 15 days at 25°C. To yield the required concentration of the spores, their suspensions were centrifuged for 15 minutes at room temperature at 8000*g*; washed 3 times in cold, sterile, distilled water between centrifugations; and resuspended in smaller volumes of sterile, distilled, deionized water. All spore suspensions were then heated to 80°C for 10 minutes to inactivate any vegetative cells.[11]

B subtilis was prepared by growing aerobically for 72 hours at 36°C ± 1°C in a 1:10 dilution of Columbia Broth containing magnesium sulfate tetra hydrate. Undiluted Columbia Broth inoculated with *C sporogenes* was incubated anaerobically for 5 days at 36°C ± 1°C. The grown cultures of *B subtilis* and *C sporogenes* spores were processed as described above for *C difficile*. The spore suspensions were kept at 2°C to 4°C until used for the maximum period of 1 month.

Testing for sporicidal activity

The second tier of the quantitative carrier test (QCT-2), a standard of American Society for Testing and Materials International,[13] was used to assess sporicidal activity of the test formulations. Each disk (1-cm diameter; 0.7 cm thick) of brushed and magnetized stainless steel, used as a prototypical hard environmental surface, received 10 μL of the test spore suspension in a soil load.[11]

After inoculation of the disks, they were first air-dried in a laminar flow cabinet for 60 minutes and then under vacuum in a desiccator for at least 2 hours. Each disk was then separately placed at the bottom of a 20-mL sterile plastic vial with the contaminated side up, covered with 50 μL of the test formulation, and kept at 20°C ± 2°C for the required contact time.

An equivalent amount of normal saline with 0.1% Tween-80 (Saline-T, Fischer Scientific, Waltham, MA) was added on the control disks. At the end of the contact time, the disinfectant activity was arrested by adding 9.95 mL of a neutralizer (see below). The carriers inside the vials were vortexed for 3 to 5 cycles of approximately 30 seconds each to recover the inoculum. The eluates and subsequent washes of the vials with Saline-T were passed through a 47-mm diameter and 0.2-mm pore size membrane filter (Millipore Corp, Billerica, MA). Filters were then located on the surface of a proper agar recovery medium. For *C difficile* spores, the recovery medium was brain-heart infusion agar (Difco [Bacto Difco/BD, Franklin Lakes, NJ] or Oxoid [Oxoid Corporation, Basingstoke, Hamshire, UK]) supplemented with 0.5% of yeast extract, 0.1% L-cysteine, 0.1% sodium taurocholate, and 1.5% agar.[14] For *B subtilis* and *C sporogenes*, the recovery media were tryptic soy agar (TSA) (TS-Oxoid and Agar QueLab) and fastidious anaerobic agar (FAA) (Oxoid plates), respectively.[13] The plates were placed in an incubator at 36°C ± 1°C; Colony-forming units (CFU) were counted after 5 days, and $\log_{10}$ reductions in spore titers were calculated. Control disks inoculated with *B subtilis*, *C sporogenes*, *and C difficile* were dried and then counted for their CFUs. On average, they showed counts of approximately 10^6 to 10^7 CFU/mL. The count range of the dried inocula may have been a lot higher than the concentration typically expected on hospital surfaces, and, therefore, this test would simulate the worst case scenario that may happen in the field.

Neutralization of sporicidal activity

For sodium hypochlorite solutions, the neutralizer was 1.0% (wt/vol) sodium thiosulfate in normal saline, with 0.1% (final concentration) of Tween-80 (Bioshop, Burlington, ON). The AHP-based product was neutralized with Letheen Broth (Difco) with 1.0% (wt/vol) sodium thiosulfate.

Controls

Suitable controls were incorporated to check for sterility of media, reagents, and carriers; effectiveness of the microbicide neutralization procedure; and carrier population counts. Each experiment included no less than 3 control disks and 5 disks for each contact time or product concentration tested. All experiments were repeated at least 3 times; the average of the log reductions were used in the analysis; and the tests were performed at 3 different contact times: 1, 5, and 10 minutes.

Drying test

Toilet brush was soaked in the test solution and was applied inside the toilet bowl. Melt blown

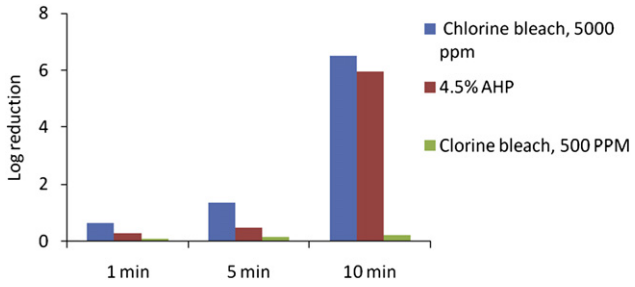

Fig 1. Sporicidal activity of chlorine bleach, 500 ppm and 5000 ppm, and 4.5% AHP gel against *B subtilis* at different contact times using QCT-2.

polypropylene towels (20 cm by 20 cm), saturated with the test solution, were applied to the outside surface of the toilet bowl, ceramic tiles, porcelain toilet sinks, and epoxy resin countertops and allowed to air-dry at room temperature with 60% ± 10% relative humidity). The time was measured for each product to dry approximately on 50% of the wetted surface. The test was repeated 10 times for each product for meaningful statistical analysis. The drying test was performed to find out the actual contact time of the test solution to the surface. This test is important because, in practice, the surfaces are wiped and let air-dry, and, in many cases, they do not meet their required disinfection contact times.

Statistical analyses

The Excel spread sheet (Microsoft Corp, Redmond, WA), was used for *t* test analyses.

RESULTS

Sporicidal tests

Figure 1 shows the activity of 500 ppm of bleach, 5000 ppm of bleach, and 4.5% AHP gel against the spores of *B subtilis* at 1-, 5-, and 10-minute contact times. As shown in Fig 1, neither chlorine bleach (at 500 ppm or 5000 ppm of FAC) nor AHP sporicidal gel was effective in inactivating the spores at 1 and 5 minutes. At all 3 contact times, bleach at 500 ppm FAC had almost the same count as the controls (approximately 5×10^6 CFU). At 1 minute and 5 minutes contact time, bleach at 5000 ppm of FAC had 1.15×10^6 CFU and 3.37×10^5 CFU, respectively, whereas 4.5% AHP had 2.8×10^6 CFU and 1.7×10^6 CFU, respectively. At 10-minute contact time, both of these products had 0 CFU.

Figure 2 shows the activity of 5000 ppm of bleach and 4.5% gel against the spores of *C sporogenes* and *C difficile*. Bleach at 500 ppm of FAC was not included in this experiment because it did not show any activity against *B subtilis* at even 10-minute contact time. The average count for *C sporogenes* on dried control

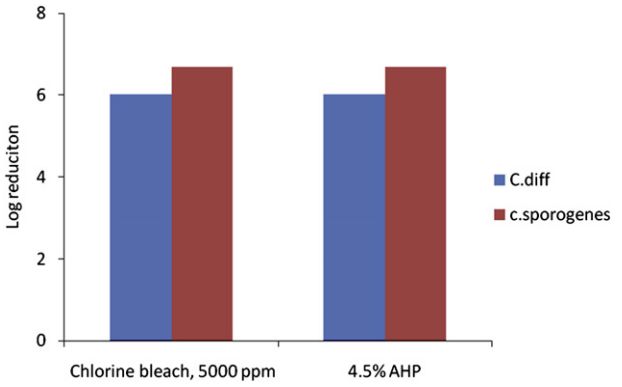

Fig 2. Sporicidal activity of chlorine bleach, 5000 ppm, and 4.5% AHP gel against *C difficile* and *C sporogenes* at 10-minute contact time using QCT-2.

carriers was 5×10^6 CFU, and the average count for both 5000 ppm bleach and 4.5% AHP were 1 CFU. The average count for dried control carriers of *C difficile* was 1.03×10^6 CFU, and the average count for both 5000 ppm bleach and 4.5% AHP were 0 CFU.

Drying tests

As shown in Fig 3, on average, the gel took longer than 10 minutes to become visibly dry on all 5 surfaces. Because of its high viscosity, the gel was uniformly spread and formed a layer on both horizontal and vertical surfaces. Chlorine bleach at 5000 ppm of FAC dried on both horizontal and vertical surfaces in approximately 4 minutes. It was observed that the chlorine solution did not spread uniformly on the toilet bowl surface, or ceramic tiles, and tended to form small droplets unless the surface was over saturated. The formation of small droplets can be attributed to the lack of detergency and may result in inadequate disinfection to the contaminated region. Additionally, it was observed that, in the region at which the surface meets the water, the products tested became diluted. Therefore, regardless of the chemistry used, the decontamination will not be as efficient in this region as well as in the water inside the toilet bowl.

DISCUSSION

Effective prevention and control of the environmental spread of nosocomial infections remains a challenge. In recent years, the task has become even more difficult with the emergence of *C difficile* as a major hospital-acquired pathogen[15] because it represents a major health service burden, and outbreaks are very costly, affecting duration of stay and, finally, disrupting services and patient care.[8,16]

Many commonly used environmental surface disinfectants simply do not have the power to inactivate

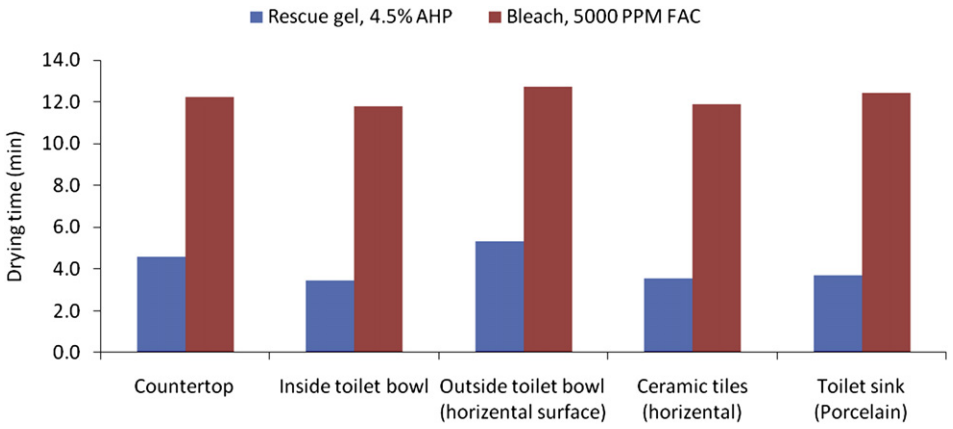

Fig 3. Drying time test for bleach, 5000 ppm, versus 4.5% AHP sporicidal gel on different surfaces.

bacterial spores in general with contact times relevant in field use. Furthermore, sublethal levels of quaternary ammonium compounds, or detergents often used on environmental surfaces, may induce better sporulation in organisms such as *C difficile.*[15]

Bleach at 500 ppm of FAC is not effective at even 10-minute contact time and therefore should not be used as sporicides, whereas, at 5000 ppm of FAC, it is effective against bacterial spores. Different liquid sporicide chemistries are available and might be considered to inactivate *C difficile* spores on such environmental surfaces. Glutaraldehyde is sporicidal at contact times of 3 to 10 hours,[17] which is too long for this application. It is also toxic[18] and not suitable for disinfection of environmental surfaces. Formaldehyde is slow acting,[17] carcinogenic,[19,20] and highly toxic.[21,22] Of chlorine-releasing agents, hypochlorites have strong sporicidal activity, but their activity is reduced in the presence of organic matter.[23] Hypochlorites are more active at low pH[11] but less stable. Typical hypochlorites are sodium or potassium salts and commonly sold as powders or liquids. Their low cost and ready availability make them the most common environmental surface disinfectants against nosocomial pathogens including the spores of *C difficile.* Chlorine dioxide is a very strong oxidizer and a powerful sporicide, but it is very toxic[20] and has poor stability and, therefore, should be generated in situ. Hydrogen peroxide is also a sporicidal agent.[24,25] It is environmentally friendly because it decomposes to water and oxygen. A 6% solution of hydrogen peroxide takes up to 6 hours to inactivate spores,[26] which is too slow. Higher concentrations are faster but are unsafe to store and handle. Peracetic acid is a strong sporicide and remains effective in the presence of organic matter. Although it is faster than hydrogen peroxide, a 0.2% peracetic acid (PAA) solution takes 30 minutes to inactivate spores[25]; furthermore, it is highly corrosive with a pungent odor.

Considering the pros and cons of these chemicals, hypochlorites are currently the most viable option to use as surface sporicides. This study compared the sporicidal activity of domestic bleach (~5% solution of sodium hypochlorite) with an AHP-based surface sporicide. The chlorine bleach solution dried on the surface at the same rate as water (~4 minutes), whereas AHP sporicidal gel took longer than 10 minutes to dry because of its higher viscosity, even keeping it wet on inclined surfaces for at least 10 minutes.

Although bleach is fast acting, inexpensive, and generally readily available, its main drawbacks are off-gassing and high corrosivity. Also, concentrated bleach solutions generally do not contain detergents and do not fully wet hydrophobic surfaces such as many plastics and therefore cannot come into contact with the contaminated surfaces efficiently. Chlorine solutions containing detergents in the formulation have higher pH for better stability, which results in significant loss of microbicidal activity.[27-29] In addition, bleach dries on the surface in approximately 4 to 5 minutes and must be reapplied to meet its 10-minute contact time. Furthermore, bleach does not form a uniform layer on many surfaces, especially hydrophobic surfaces or those that are poorly cleaned, raising the risk of not contacting with the entire contamination zone.

The gel did not meet the performance criterion at 5 minutes of contact but did so at 10 minutes. Its viscous nature allows it to keep even vertical surfaces wet for at least 10 minutes for good sporicidal action, and the presence of surfactants facilitates coverage of hydrophobic surfaces as well.

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