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Effectiveness of calcium hypochlorite, quaternary ammonium compounds, and sodium hypochlorite in eliminating vegetative cells and spores of *Bacillus anthracis* surrogate

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ABSTRACT

Background: The spore-forming bacterium *Bacillus anthracis* causes anthrax, an often-fatal infection in animals. Therefore, a rapid and reliable strategy to decontaminate areas, humans, and livestock from *B. anthracis* is very critical.

Objectives: The aim of this study was performed to evaluate the efficacy of sodium hypochlorite, calcium hypochlorite, and quaternary ammonium compound (QAC) sanitizers, which are commonly used in the food industry, to inhibit spores and vegetative cells of *B. anthracis* surrogate.

Methods: We evaluated the efficacy of sodium hypochlorite, calcium hypochlorite, and a QAC in inhibiting vegetative cells and spores of a *B. anthracis* surrogate. We treated a 0.1-mL vegetative cell culture or spore solution with 10 mL sanitizer. The samples were serially diluted and cultured.




Results: We found that 50 ppm sodium hypochlorite (pH 7), 1 ppm calcium hypochlorite, and 1 ppm QAC completely eliminated the cells in vegetative state. Exposure to 3,000 ppm sodium hypochlorite (pH 7) and 300 ppm calcium hypochlorite significantly eliminated the bacterial spores; however, 50,000 ppm QAC could not eliminate all spores.

Conclusions: Calcium hypochlorite and QAC showed better performance than sodium hypochlorite in completely eliminating vegetative cells of *B. anthracis* surrogate. QAC was ineffective against spores of the *B. anthracis* surrogate. Among the three commercial disinfectants tested, calcium hypochlorite most effectively eliminated both *B. anthracis* vegetative cells and spores.

Keywords: *Bacillus anthracis* surrogate; disinfectants; sodium hypochlorite; quaternary ammonium compounds; spores

INTRODUCTION

Bacillus anthracis is a gram-positive bacterium that causes a zoonotic disease known as anthrax worldwide [1]. In 2001, terrorist attacks in the United States employed *B. anthracis* spores

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Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

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that were transmitted through the postal system [1,2]. Twelve cases of cutaneous anthrax and 11 cases of inhalational anthrax resulted from these attacks, and inhalation anthrax caused five deaths [3]. The U.S. Department of Justice mail facility in Landover, Maryland was contaminated with *B. anthracis* spores, along with numerous other sites either directly or through cross-contamination [4,5]. The U.S. Department of Justice adopted a two-pronged approach to remediate the facility, specifically using aqueous chlorine dioxide to decontaminate hard, nonporous surfaces and paraformaldehyde to fumigate two pieces of mail equipment [6,7]. The facility remained closed for almost 5 months; cleanup activities took approximately 3 months, with source reduction activities as the most time-consuming steps [2-4,8]. Bleach, chlorine dioxide, ethylene oxide, hydrogen peroxide, peroxyacetic acid, methyl bromide, paraformaldehyde, and vaporized hydrogen peroxide were among the disinfectants used during this cleanup process [9]. Therefore, a key lesson learned from the 2001 anthrax attacks is that remediations including fumigations are complex, time-consuming, and costly [10,11]. Hence, a safe decontamination method for *B. anthracis* using commercial disinfectants is urgently needed for simpler and faster remediation.

The ability of alkaline hypochlorite solutions to rapidly oxidize, decarboxylate, and deaminate primary and secondary α -amino acids has been demonstrated, and the antimicrobial efficacy of sodium hypochlorite (NaOCl) is well-recognized [12-14]. This mechanism is concentration-dependent considering available chlorine [13,14]. Calcium hypochlorite (Ca[OCl]₂) is a relatively stable compound with greater chlorine availability than NaOCl [14]. It is available as granules or freshly prepared aqueous solution based on the following reaction: $\text{Ca(OCl)}_2 + 2 \text{H}_2\text{O} \rightarrow 2 \text{HOCl} + \text{Ca(OH)}_2$ [15].

As regulatory hurdles increase, chemical biocides, such as quaternary ammonium compounds (QACs), currently used in homes, are subject to more scrutiny and rigorous investigation than new chemicals [16]. A common feature of QACs is their ability to cause cell leakage and membrane damage, primarily due to their adsorption by the bacterial membrane [17]. Monoalkyl QACs have been reported to bind to microbial membrane surfaces via ionic and hydrophobic interactions, with the cationic head group facing outwards and hydrophobic tails inserted into the lipid bilayer, causing rearrangement of the membrane and subsequent leakage of intracellular contents [17]. The difference in modes of action between QAC agents may be discerned through a scientific approach; for example, this method initially checks for various properties such as bactericidal, bacteriostatic, and uptake isotherm, and then evaluates the membrane sensitivity [18]. To date, decontamination efficacies of various sanitizers with respect to the spores and vegetative cells of a *B. anthracis* Sterne (lacking pXO1 and pXO2), a surrogate strain of virulent *B. anthracis*, have not been reported. Therefore, this study was to evaluate the efficacy of three different sanitizers (calcium hypochlorite, QAC, and sodium hypochlorite), which are commonly used in the food industry, so as to inhibit spores and vegetative cells of *B. anthracis* surrogate.

MATERIALS AND METHODS

Strains

The attenuated vaccine strain *B. anthracis* surrogate was obtained from Drs. Jeff Karns and Michael Perdue (U.S. Department of Agriculture, Agricultural Research Service, USA). Cryopreserved spores (100 μL) stored in 15% (w/v) glycerol at -70°C were thawed, inoculated in 10 mL of tryptic soy broth (TSB, pH 7.0; BBL/Difco, BD Biosciences, USA), and incubated

at 36°C for 24 h. Three consecutive loop transfers of TSB cultures incubated at 36°C for 24 h were prepared immediately before the experiments were conducted.

Media

New Sporulation Medium agar (containing tryptone [3 g/L], yeast extract [3 g/L], bacto-agar [2 g/L], Lab-Lemco agar [23 g/L], and MgSO₄ [0.01 g/L] in distilled water) was used for *B. anthracis* sporulation. Nutrient agar (NA) and tryptic soy agar (TSA) were purchased from BBL/Difco BD Biosciences. Media were autoclaved (121°C), poured into standard 150-mm Petri dishes, and dried at 25°C. Filter-sterilized distilled water was used to suspend and store *B. anthracis* spores. Vegetative cells and spores were enumerated on NA and TSA, respectively.

Sporulation of *B. anthracis*

Aliquots (200 µL) from overnight TSB cultures were spread over the surface of four 150-mm New Sporulation Medium plates and incubated at 37°C. The plates were removed from the incubator, left at 25°C, and then gently scraped with a sterile plastic spreader and added to 3–5 mL of sterile distilled water. This suspension was collected from the four plates and combined in a 50-mL centrifuge tube, which was left at 25°C to promote the lysis of vegetative cells. The tube was then centrifuged at 6,000 × *g* for 10 min, the supernatant was removed, and the pellet was resuspended in 40 mL of sterile distilled water. This procedure was repeated five times. Finally, cells were resuspended in 10 mL of sterile distilled water to form a milky suspension. The spore suspension was observed with a video-microscope (CX21LED, Olympus Corporation, Japan) and was found to contain fewer than 1% vegetative cells. The preparation was enumerated on NA by serial dilution to approximately 5 × 10⁹ colony-forming unit (CFU)/mL and stored at 5°C until use.

Preparation of sanitizers

Sodium hypochlorite solutions were prepared by diluting Clorox bleach (6%, Commercial Clorox; The Clorox Company, USA) with sterile distilled water. Calcium hypochlorite was obtained from Sigma-Aldrich (USA) and used in solutions with sterile distilled water. A QAC solution was prepared at concentrations from 1–50 ppm by diluting BDD™ (Decon Labs, Inc., USA) with sterile distilled water. The concentration of free active chlorine in all solutions was then determined using a residual chlorine meter (Model RC-24P; Analyticon Instrument Corporation, USA) immediately before use. The sodium hypochlorite solution was adjusted to pH 7.0 with 1N HCl; pH was measured using a Thermo Fisher Scientific Orion 2 Star pH meter (USA).

Treatment with sanitizers

B. anthracis vegetative cells and spores were treated with each of the different sanitizers. A vegetative cell culture or spore solution (0.1 mL) was treated with 10 mL of sanitizer in a 50-mL conical tube. After the contact time, Dey-Engley neutralizing broth (Hardy Diagnostics, USA) was used to neutralize the sanitizers [19]. The solution of vegetative cells and spores was serially diluted and enumerated on NA and TSA plates, respectively, and then incubated at 37°C. The colonies were then counted, and the results were recorded as log CFU/mL.

Statistical analyses

All experiments were replicated three times and statistical analysis was performed using GraphPad InStat (version 3.05; GraphPad Software, USA). Mean values were analyzed to determine significant differences ($p \leq 0.05$) in microbial populations detected in samples, after different treatments.

Table 1. Inactivation of vegetative cells of the *Bacillus anthracis* surrogate by various sanitizers

Type of sanitizer	Surviving vegetative cells, according to each sanitizer concentration					
Sodium hypochlorite (pH 7)	Concentration (ppm)	0 (Control)	1	10	50	100
	Log CFU/mL	5.7 ^a	5.7 ^a	5.7 ^a	0.1 ^b	0 ^b
Calcium hypochlorite	Concentration (ppm)	0 (Control)	0.1	1	10	100
	Log CFU/mL	6.32 ^a	6.2 ^a	0 ^b	0 ^b	0 ^b
QAC	Concentration (ppm)	0 (Control)	0.5	1	5	10
	Log CFU/mL	5.7 ^a	5.2 ^b	0 ^c	0 ^c	0 ^c

CFU, colony-forming unit; QAC, quaternary ammonium compound.

^{a-c}Different letters within a row indicate a significant difference ($p < 0.05$).

Table 2. Inactivation of spores of the *Bacillus anthracis* surrogate using various sanitizers

Type of sanitizers	Surviving spores, according to each sanitizer concentration					
Sodium hypochlorite (pH 7)	Concentration (ppm)	0 (Control)	10	100	1,000	3,000
	Log CFU/mL	7.2 ^a	6 ^b	5.23 ^c	3 ^d	0 ^e
Calcium hypochlorite	Concentration (ppm)	0 (Control)	10	50	100	300
	Log CFU/mL	7.7 ^a	7.7 ^a	6.5 ^b	5.84 ^c	0 ^d
QAC	Concentration (ppm)	0 (Control)	7,000	15,000	30,000	50,000
	Log CFU/mL	8.3 ^a	8.2 ^a	8 ^a	8 ^a	7.9 ^b

CFU, colony-forming unit; QAC, quaternary ammonium compound.

^{a-e}Different letters within a row indicate a significant difference ($p < 0.05$).

RESULTS

We examined the effectiveness of sodium hypochlorite, calcium hypochlorite, and QAC in killing vegetative cells and spores of the *B. anthracis* surrogate (**Tables 1 and 2**).

According to the results of neutralizer efficacy, there was no difference between treated and control samples subjected to neutralization immediately after treatment with various concentrations of each sanitizer for 10 min (data not shown).

The minimum concentration required to completely remove vegetative cells of the *B. anthracis* surrogate was 100 ppm of sodium hypochlorite (pH 7), whereas 1 ppm of calcium hypochlorite and QAC were more effective than sodium hypochlorite against vegetative cells (**Table 1**). Interestingly, spores of the *B. anthracis* surrogate were completely removed by exposure to 3,000 ppm sodium hypochlorite (pH 7) and 300 ppm calcium hypochlorite, showing that much higher concentrations are required for spores than for vegetative cells (**Table 2**; $p \leq 0.05$). However, exposure to 5,000 ppm QAC did not completely eliminate these spores showing QAC is ineffective against spores of the *B. anthracis* surrogate (**Table 2**).

DISCUSSION

This study showed that neutralizer treatment was effective for eliminating *B. anthracis* surrogate cells and spores. In general, *B. anthracis* spores are significantly less responsive to biocide than vegetative-type cells [19-21]. After exposure to biocide, the neutralization step is essential [20,21]. This is to ensure there is no residual agent that could target the germinating bacteria [21]. Otherwise, misleading results for the anti-sporicidal activity may be obtained [21].

In 1957, under the authority of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), sodium hypochlorite was registered for use as an antimicrobial pesticide in the United States [9,13]. As a sanitizer or disinfectant to kill bacteria, fungi, and viruses, sodium

hypochlorite was approved for use in households, food processing plants, agricultural settings, animal facilities, hospitals, and human drinking water supplies [11]. pH is an important factor for chlorite disinfectants [3,22-24]. The inactivation of *Bacillus subtilis* spores by glutaraldehyde, formaldehyde, hydrogen peroxide, peracetic acid, cupric ascorbate, and sodium hypochlorite is affected by pH [22-25]. Further, Majcher et al. [8] compared the spores of six strains of *B. anthracis* (four virulent and two avirulent) to those of four other types of spore-forming bacteria to evaluate their resistance to four liquid chemical sporicides (e.g. sodium hypochlorite at 5,000 ppm available chlorine). Similar to the previous study results, our results showed that *B. anthracis* vegetative cells and spores were significantly reduced by treatment with sodium hypochlorite at 100 and 3,000 ppm (pH 7), respectively (**Tables 1 and 2**).

When calcium hypochlorite was used as a water disinfectant, a high free available chlorine (FAC) concentration was required to kill *B. anthracis* spores, and therefore, water treated with calcium hypochlorite to kill anthrax spores is neither potable nor palatable [26,27]. Galanina et al. [28] reported that calcium hypochlorite affected *B. anthracis* spores at a lethal dose (0.2–0.3 mg/mL active chlorine in 1.5 h or 5.6 mg/mL active chlorine in 1 h). In our study, calcium hypochlorite showed the best effectiveness, and vegetative cells and spores were completely removed by treatment with 1 and 300 ppm of calcium hypochlorite, respectively (**Tables 1 and 2**).

Hypochlorite-containing solutions are the most commonly used disinfectants in both household and industrial disinfectant processes [26,27]. For example, the U.S. military has adopted the standard approach of using 0.5–10% high test hypochlorite (HTH) for decontaminating *B. anthracis* spores on skin or surfaces [27]. Treatment with 5% HTH results in an approximate eight log reduction in *B. anthracis* spores [27]. Although HTH is effective for decontaminating these *B. anthracis* strains, it is considered extremely corrosive to metals, skin and mucous membranes, eyes, and respiratory and gastrointestinal tract [27]. Therefore, to avoid material corrosion or toxicity, alternative disinfectants with high efficacy are needed.

QACs are sporostatic, as they inhibit the outgrowth of spores (development of a vegetative cell from a germinating spore) but not the actual germination process comprising development from dormancy to a metabolically active state, albeit via an unknown mechanism [29,30]. Similarly, they are not mycobactericidal but have mycobacteriostatic activity, although the actual effects on mycobacteria have not been extensively studied [31]. Our results showed that 50,000 ppm of QAC did not completely remove *B. anthracis* surrogate spores (**Table 2**). This finding is consistent with the fact that QAC acts by inhibiting the outgrowth of germinating spores; in fact, many antibacterial compounds such as phenols, QAC, mercury compounds, biguanides, alcohols, and parabens are not sporicidal but are sporostatic, inhibiting germination or outgrowth [18,22]. Bacterial spores are considerably more resistant than vegetative cells [3,6,8,12], and similar results were obtained in this study.

In conclusion, our results showed that the vegetative cells of the *B. anthracis* surrogate were easily inactivated compared to spores as previously demonstrated [19,22,24]. Among the three commercial disinfectants compared, calcium hypochlorite and QAC were better than sodium hypochlorite for complete elimination of vegetative cells of the *B. anthracis* surrogate (**Tables 1 and 2**). Calcium hypochlorite was best followed by sodium hypochlorite, while QAC was ineffective against spores of the *B. anthracis* surrogate. This research reveals the usefulness of various sanitizers for inactivating *B. anthracis* vegetative cells and spores.

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