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Analysis of the sporicidal activity of chlorine dioxide disinfectant against *Bacillus anthracis* (Sterne strain)

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Summary

Routine surface decontamination is an essential hospital and laboratory procedure, but the list of effective, noncorrosive disinfectants that kill spores is limited. We investigated the sporicidal potential of an aqueous chlorine dioxide solution and encountered some unanticipated problems. Quantitative bacteriological culture methods were used to determine the log₁₀ reduction of *Bacillus anthracis* (Sterne strain) spores following 3 min exposure to various concentrations of aqueous chlorine dioxide solutions at room temperature in sealed tubes, as well as spraying onto plastic and stainless steel surfaces in a biological safety cabinet. Serial 10-fold dilutions of the treated spores were then plated on 5% sheep blood agar plates, and the survivor colonies were enumerated. Disinfection of spore suspensions with aqueous chlorine dioxide solution in sealed microfuge tubes was highly effective, reducing the viable spore counts by 8 log₁₀ in only 3 min. By contrast, the process of spraying or spreading the disinfectant onto surfaces resulted in only a 1 log₁₀ kill because the chlorine dioxide gas was rapidly vaporised from the solutions. Full potency of the sprayed aqueous chlorine dioxide solution was restored by preparing the chlorine dioxide solution in 5% bleach (0.3% sodium hypochlorite). The volatility of chlorine dioxide can cause treatment failures that constitute a serious hazard for unsuspecting users. Supplementation of the chlorine dioxide solution with 5% bleach (0.3% sodium hypochlorite) restored full potency and increased stability for one week.

Keywords

Bacillus anthracis; Chlorine dioxide; Disinfectant; Sodium hypochlorite; Spores

Introduction

Many disinfectants are available for use in hospital and laboratory settings; however, their potency against many infectious agents is more often presumed than proven. Likewise, the effective concentrations are often based on mixing dilutions with selected bacteria with little focus on contact time, stability, or corrosive effects on metal surfaces (e.g. biosafety cabinets,

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Conflict of interest statement
None declared.

autoclaves, and other expensive equipment). Most studies do not include viruses due the inherent technical degree of difficulty in separating the virions from the disinfectant solution before assay in mammalian host cells, which are even more susceptible to the toxic effects of the disinfectant than the viruses. Consequently, assumptions are often based on minimal data with bacteria.

This report describes our search for a relatively non-corrosive disinfectant that could be used to decontaminate stainless steel biosafety cabinet surfaces and have maximum killing capacity against the spores of *Bacillus anthracis*. An avirulent *B. anthracis* (Sterne) strain was selected as an assay system to evaluate the efficacy of a commercially available disinfectant, Vimoba™ (Quip Laboratories, Wilmington, DE, USA) containing chlorine dioxide as the principal active ingredient. Chlorine dioxide gas has been used to kill *B. anthracis* spores, as reviewed by Spotts Whitney *et al.* following the 2001 bioterrorism attack in the USA.¹

Many laboratories working with *B. anthracis* spores use various concentrations (5-50%) of household bleach (sodium hypochlorite); however, this is corrosive and causes pitting of stainless steel. An alternative to bleach is to use solutions of chlorine dioxide, a gas dissolved in water. Chlorine dioxide is approximately ten times more soluble than chlorine, extremely volatile, and can be easily removed from dilute aqueous solutions with minimal aeration.³ It is also a potent oxidiser, accepting a maximum of five electrons during its reduction to form the Cl⁻ ion.⁴ In this study, we sought to determine whether Vimoba would have biocidal activity against *B. anthracis* spores and reduce the need for high concentrations of bleach in decontaminating laboratory surfaces.

Methods

Bacteria

Bacillus anthracis Sterne was acquired from T.M. Koehler in the Department of Microbiology and Molecular Genetics, University of Texas - Houston Health Science Center Medical School, Houston, Texas.

Preparation of *B. anthracis* spores

Spores were prepared from *B. anthracis* Sterne by growing the bacteria at 37°C on blood agar plates and scraping the growth from the plates into 2× Schaeffer's sporulation medium (pH 7.0) [16 g/L Difco Nutrient Broth, 0.5 g/L MgSO₄ · 7H₂O, 2.0 g/L KCl, and 16.7 g/L 4-morpholinepropanesulphonic acid, 0.1% glucose, 1 mM Ca(NO₃)₂, 0.1 mM MnSO₄, and 1 μM FeSO₄]. Cultures were grown at 37°C with gentle shaking (80-90 rpm) for 24 h, after which the suspension was diluted five-fold with sterile distilled water. After 10-11 days of continuous shaking, sporulation was confirmed at >99% via phase contrast microscopy, and the spores were centrifuged at 587 g in a sealed-carrier centrifuge (Beckman Coulter, Inc., Fullerton, CA, USA) at 4°C for 15 min. Spore pellets were then washed four times in sterile phosphate-buffered saline (PBS) and purified by centrifugation through 58% Ficoll Paque (GE Healthcare, Piscataway, NJ, USA).

Preparation of disinfectant

Vimoba tablets (1.5 g) were purchased from Quip Laboratories, Inc. (Wilmington, DE, USA) and pulverised inside their sealed envelopes with a mortar and pestle immediately before use. Chlorine dioxide was generated by adding indicated milligram amounts of powder from the effervescent Vimoba tablets to water. Disinfectant solutions were prepared fresh for every experiment, unless stated otherwise in the text. For some experiments, the Vimoba powder was added to 2-5% household bleach diluted in water. The latter disinfectant was referred to as Vimoba-bleach cocktail.

Disinfectant assay

All experiments were performed inside a Class II biosafety cabinet. Initial experiments to test the potency of Vimoba in killing *B. anthracis* Sterne were performed by mixing 50 μ L of spores (1×10^8 cfu) with an equal volume of the disinfectant solution diluted as indicated in capped microfuge tubes for 3 min. The spores were quickly separated from the disinfectant by diluting and washing with 1 mL of water and centrifugation (14 000 rpm). Subsequently, the viability of the spores was assessed by serial dilution and plating on to 5% sheep blood agar plates. In later experiments, the spore suspension (1×10^8 cfu) was spread on to 13 mm diameter circular areas on the sterile surface of either stainless steel or polystyrene sheets before spraying with or pipetting 500 μ L of disinfectant on to the spots. After 3 min incubation at room temperature, 1 mL of water was added and the entire suspension was aspirated from the surface and spread on to the surface of four or five blood agar plates. The total number of surviving spores was estimated by plate counts. In some experiments, the disinfectant alone was first sprayed on to surfaces to evaluate the effect of chlorine dioxide vaporisation on the potency of the disinfectant. Samples of the spore suspension (50 μ L; 1×10^8 cfu) were added to the spot for 3 min, the mixture was recovered from the surface and the survivors were determined by serial dilution and plating on 5% sheep blood agar plates.

Results

Initial tube dilution experiments were performed to assess the potency of freshly prepared Vimoba in killing *B. anthracis* Sterne spores. Table I represents a typical experiment in which 50 μ L aliquots of the disinfectant, prepared from 0, 2.5, 5.0, and 10.0 mg/mL Vimoba tablets, were distributed into microfuge tubes. After adding an equal volume of *B. anthracis* Sterne spores (1×10^8 cfu) and incubating at room temperature for 3 min, the microfuge tubes were diluted, centrifuged, and washed twice with 1 mL PBS. Subsequently, the suspensions were diluted and plated on 5% sheep blood agar.

Table I shows the disinfectant potency when mixed in a closed tube with *B. anthracis* Sterne spores for 3 min. Vimoba was highly effective in killing *B. anthracis* Sterne spores in a very short period (3 min), and complete inactivation of $8 \log_{10}$ of spores occurred with 10 mg/mL. The potency was proportionately less with lower concentrations. This dose-response experiment was very reproducible and was also observed with *B. anthracis* Ames spores (data not shown). Consequently, Vimoba was considered as a potential sporicidal disinfectant for routine contact disinfection of biosafety cabinets, carts, animal cages, and other surfaces contaminated with *B. anthracis* Ames spores.

As a further test, we assessed its capacity to kill *B. anthracis* Sterne spores on contaminated surfaces. We spotted 1×10^8 cfu *B. anthracis* spores on to 13 mm diameter circular areas on the sterilised stainless steel work surface within a biosafety cabinet. Without allowing the areas to dry, we sprayed or pipetted various concentrations (10-100 mg/mL) of Vimoba on to the spots, waited 3 min, and then diluted and cultured the areas by transferring the suspension to sectors of blood agar plates with sterile plastic 'L' rods. Qualitative culture of the spots revealed many survivors even at the higher concentrations of the disinfectant with little difference whether the Vimoba was sprayed or pipetted on to the surface (data not shown).

Since the disinfectant would usually be applied by spraying onto surfaces of equipment to decontaminate them, we developed a quantitative experimental approach for testing the effect of spraying or pipetting the disinfectant onto a work surface. Briefly, we sprayed or pipetted ~500 μ L Vimoba onto 13 mm circular areas on each surface (sterilised 304 stainless steel work surface and sterile polystyrene Petri dish lids) and allowed them to remain as a thin film for 3 min. Fifty microlitres of 1×10^8 *B. anthracis* Sterne spores were added to the spots and allowed

to remain for another 3 min. After dilution, quantitative plate counts were performed using blood agar plates incubated at 37°C.

The effect of spraying or pipetting Vimoba onto stainless steel or plastic surfaces for 3 min prior to mixing with 1×10^8 cfu *B. anthracis* spores is summarised in Table II. The negative control is shown in the top row, which shows the number of spores added (1×10^8 cfu). The second row shows the results of a positive control (8 log₁₀ kill) performed by mixing 20 mg/mL Vimoba with a 50 µL spore suspension (1×10^8 cfu). The third and fourth rows show that the Vimoba in contact with a stainless steel surface reduced its killing efficiency to <1 log₁₀ of *B. anthracis* spores. By comparison, spraying or pipetting Vimoba on to a polystyrene plastic surface resulted in a 1 log₁₀ reduction in spore viability.

In order to compensate for the loss of potency of Vimoba when it was sprayed or pipetted onto a surface, an experiment was performed in which various concentrations (2-5%) of household bleach were used to prepare the Vimoba solution, instead of water. Using the disinfectant assay spray method developed for the previous experiment (Table II), four 1 L spray bottles were filled completely with Vimoba solution prepared in 0%, 2%, 4%, or 5% bleach. Each solution was sprayed on to a sterile stainless steel surface and after 3 min, 50 µL aliquots were aspirated and pipetted into microfuge tubes containing 50 µL of 1×10^8 cfu *B. anthracis* Sterne spores.

Table III shows that freshly prepared full bottles of Vimoba alone (5 mg/mL) reduced spore viability by 3.1 log₁₀, but 24 h later it retained little if any potency against *B. anthracis* Sterne spores. When the Vimoba was supplemented with as little as 2% bleach, full potency was restored enabling it to kill 8 log₁₀ of *B. anthracis* Sterne spores with stability for a period of 24 h. Clearly, the optimum concentration of bleach was 5%, because it allowed the disinfectant to be used for at least one week. However, in situations where corrosion-sensitive equipment is being decontaminated, it might be advisable to use a low concentration of bleach (e.g. 1-2%) and prepare it fresh daily. It should also be noted in these experiments that the Vimoba concentration was reduced from 10 to 5 mg/mL, striving to take advantage of the enhanced effect of Vimoba and bleach.

Considering the volatility of chlorine dioxide in solution, a final experiment was designed to determine the effect of residual volume of Vimoba solution remaining in 1 L plastic spray bottles on stability. Reasoning that the surface:air ratio likely is important in the rate with which chlorine dioxide vaporises from the solution. Therefore, using the same assay spray method used in earlier experiments, several 1 L plastic spray bottles containing various volumes (50-1000 mL) of Vimoba (5 mg/mL) were prepared with 5% bleach. We noted that on the day of preparation, there was no difference in potency among the various bottles, with each reducing the viability of *B. anthracis* Sterne spores by 8 log₁₀ (Table IV). It became clear that bottles containing lower volumes of disinfectant were stable for shorter periods of time. For example, a 1 L bottle nearly empty (50 mL) could kill only 4.3 log₁₀ of the 1×10^8 cfu of the *B. anthracis* Sterne spores by 24 h, while by the second day had lost all disinfectant capacity. When the 1 L bottles were filled with 250-500 mL, the disinfectant retained full potency for four days and proportionately lesser kill capacity by the end of seven days. As long as the 1 L bottles were three-quarters full or greater, the disinfectant retained full potency for seven days, that is, the capacity to kill 8 log₁₀ of *B. anthracis* Sterne spores.

Discussion

Chlorine dioxide gas has been used previously to decontaminate indoor materials and sanitise water supplies and equipment; however, we report for the first time that chlorine dioxide in solution rapidly kills *B. anthracis* spores.^{1,4} The disinfectant assay parameters that we established employed chemically resistant *B. anthracis* spores as a target and 3 min as the

maximum period of exposure. We demonstrated by tube dilution that Vimoba had a potent biocidal effect on *B. anthracis* Sterne spores in a closed tube assay system, reducing spore viability by 8 log₁₀ to an undetectable number in 3 min contact time. This was achieved by preparing the chlorine dioxide solution by dissolving various amounts of the crushed effervescent tablet (2.5-10.0 mg/mL) in water. All experiments, except where indicated, were performed with freshly prepared disinfectant solutions. A 10 mg/mL solution produced sufficient chlorine dioxide to completely kill 1 × 10⁸ cfu of *B. anthracis* Sterne spores in a 3 min period. A 50% decrease in chlorine dioxide concentration to 5 mg/mL resulted in a 4.34 log₁₀ reduction in spore viability. Further, by reducing the amount of chlorine-dioxide-generating powder from 10 to 2.5 mg/mL, the disinfectant potency was reduced proportionately to 1.57 log₁₀.

It was noted that the disinfectant exerted a potent sporicidal effect in closed tubes. Typically such observations should be sufficient to justify using the disinfectant in a laboratory or hospital setting; however, additional experiments were performed to mimic the 'real world' scenario of how the disinfectant would be used. Thus, we contaminated a sterile stainless steel work surface with 13 mm spots of a suspension of *B. anthracis* Sterne spores (1 × 10⁸ cfu), and then sprayed or pipetted Vimoba onto them for 3 min. Spraying or pipetting Vimoba onto the stainless steel work surface and spreading it out into a thin film resulted in a significant reduction in disinfectant potential, limiting the kill capacity to approximately 1 log₁₀ in 3 min. Having already demonstrated that chlorine dioxide had a potent sporicidal effect in closed microfuge tubes, we determined why the disinfectant lost so much capacity to kill the spores when it was sprayed onto contaminated surfaces. It was thought that some loss of disinfectant potential may have been due to oxidation of iron from the stainless steel surface, since chlorine dioxide scavenged electrons and was known to be reduced to chlorite, chlorate, and chloride ions. In Table II, we observed that the stainless steel surface played a minimal role, compared with plastic, in reducing the potency of the disinfectant. Additionally, it made no difference whether the disinfectant was sprayed or pipetted onto the work surface; both resulted in the formation of a thin film with poor sporicidal results.

The majority of the loss in potency of Vimoba during application was postulated to result from the rapid vaporisation of chlorine dioxide gas from the disinfectant solution at the work surface. The flow of air within the biosafety cabinet could have promoted evaporation of the chlorine dioxide; however, spreading the disinfectant out into a thin film seemed to be important in diminishing potency. It is only logical that the application process would increase vaporisation of the gaseous chlorine dioxide from the solution. Rather than discarding a potentially excellent disinfectant from further use, we sought to improve its stability and killing capacity by supplementing Vimoba with various concentrations of household bleach to improve its disinfectant action and increase its stability. It was observed upon assay of the Vimoba-bleach cocktail that addition of bleach to Vimoba restored it to full potency and extended its storage life even when sprayed on to surfaces. In doing so, we were able to reduce the Vimoba concentration by 50% (5 mg/mL instead of 10 mg/mL) and prepare it in 2-5% bleach. While 2% bleach supplement worked well when used immediately or within one day, 5% bleach was considered much more reliable in killing *B. anthracis* spores for a period of seven days. The combination of Vimoba and bleach was synergistic in killing *B. anthracis* spores (Table III), resulting in greater combined potency than the anticipated additive effect of the two components.

A disinfectant capable of reducing *B. anthracis* spore viability by 8 log₁₀ in 3 min contact time must be considered an excellent and reliable reagent. Few investigators would argue with the presumption that such a disinfectant would likely exert an equal or greater effect on viruses or vegetative cells of bacteria. The latter are considerably more susceptible to other disinfectants than are spores, which tend to be very resistant to chemicals. As an example, *B. anthracis*

spores are often stored in 1% phenol without loss of viability.⁵ Further, the criteria posed are actually similar to those used as criteria for sterilisers based on steam, vaporised hydrogen peroxide, or ethylene oxide. It is routine practice to expect a 6 log₁₀ reduction in viability of spores from *B. atropheus* or *B. stearothersophilus* as an indicator of sterility. Only one other property that might be expected from an excellent disinfectant is for it to be totally non-corrosive. Vimoba contains corrosion inhibitors, although chlorine dioxide gas is only weakly corrosive.⁶ Corrosion testing is in progress to determine whether the Vimoba-bleach cocktail will be corrosive for metals such as stainless steel.

The Vimoba-bleach cocktail (5 mg/mL; 5%) was shown to be stable for at least seven days when stored virtually full in sealed plastic spray bottles. As summarised in Table IV, we examined the disinfectant potency when bottles were only partially filled. It became apparent that 1 L plastic spray bottles that were at least three-quarters full maintained maximum killing potential for *B. anthracis* Sterne spores for seven days; however, bottles that were one-quarter to one-half full maintained maximum potency in killing *B. anthracis* Sterne spores for four days. An essentially empty bottle (50 mL) was fully potent only when made up fresh.

It was concluded that Vimoba was a potent disinfectant in closed containers; however, substantial reduction in potency occurred when it was sprayed or pipetted on to contaminated surfaces as a thin film. In order to compensate for the loss of chlorine dioxide, Vimoba was prepared in 5% bleach (0.3% sodium hypochlorite) and found to be a potent formulation, remaining stable for at least seven days. Thus, when applied as a spray to decontaminate surfaces, Vimoba should be supplemented with dilute bleach in order to have maximum potency.

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Table IExposure of *B. anthracis* Sterne spores to Vimoba™ in microfuge tubes

Vimoba tablet concentration (mg/mL)	Exposure time (min)	No. of survivors (cfu)	Log ₁₀ reduction	% kill	% survival
0	3	1.0×10^8	0	0	100
2.5	3	2.7×10^6	1.57	97.3	2.7
5.0	3	4.6×10^3	4.34	99.99	0.01
10.0	3	0.0	8	100	0

Table II
Exposure of *B. anthracis* Sterne spores to Vimoba™ after contact with stainless steel or plastic

Vimoba concentration (mg/mL)	Disinfectant treatment	Exposure time (min)	No. of survivors (cfu)	Log ₁₀ reduction	% kill	% survival
0	None	3	1×10^8	0	0	100
10	Plastic tube control	3	0	8	100	0
10	Sprayed on to SS	3	2×10^7	0.7	80	20
10	Pipetted on to SS	3	2.3×10^7	0.64	77	23
10	Sprayed on to plastic	3	9×10^6	1.05	91	9.0
10	Pipetted on to plastic	3	8×10^6	1.1	92	8.0

SS, stainless steel.

Table III

Stability of Vimoba™-bleach cocktail stored in full sealed plastic spray bottles^a

Age of disinfectant (days)	Control (no disinfectant) (cfu)	<i>Bacillus anthracis</i> Sterne spore survival (cfu) (log ₁₀ reduction in viability)			
		Vimoba (5 mg/mL)	Bleach (5%)	Vimoba (5 mg/mL + 5% bleach)	Vimoba (5 mg/mL + 4% bleach)
0 (fresh)	1 × 10 ⁸	6 × 10 ⁴ (3.2)	1 × 10 ³ (5)	0 (8)	0 (8)
1	1 × 10 ⁸	1 × 10 ⁷ (1.0)	1.2 × 10 ³ (4.9)	0 (8)	0 (8)
2	1 × 10 ⁸	1.5 × 10 ⁷ (0.8)	1.4 × 10 ³ (4.9)	0 (8)	4 × 10 ¹ (6.4)
3	1 × 10 ⁸	7.3 × 10 ⁷ (0.14)	2.0 × 10 ³ (4.7)	0 (8)	2 × 10 ³ (4.7)
4	1 × 10 ⁸	6.3 × 10 ⁷ (0.2)	2.1 × 10 ³ (4.7)	0 (8)	1 × 10 ⁵ (3.0)
5	1 × 10 ⁸	7.2 × 10 ⁷ (0.14)	1.0 × 10 ⁴ (4)	0 (8)	6 × 10 ⁵ (2.2)
6	1 × 10 ⁸	1.0 × 10 ⁸ (0)	3.1 × 10 ⁴ (3.5)	0 (8)	1 × 10 ⁶ (2.0)
7	1 × 10 ⁸	-	-	0 (8)	4 × 10 ³ (4.4)

^aDisinfectant sprayed on to stainless steel surface before exposure of *B. anthracis* Sterne spores.

Table IV
Stability of Vimoba™ bleach cocktail stored in various volumes in sealed 1 L plastic spray bottles^a

Storage time (days)	Exposure time (min)	Log ₁₀ reduction in viability				
		50 mL ^b	250 mL ^b	500 mL ^b	750 mL ^b	1000 mL ^b
0 (fresh)	3	8	8	8	8	8
1	3	4.3	8	8	8	8
2	3	0.05	8	8	8	8
3	3	-	8	8	8	8
4	3	-	8	8	8	8
5	3	-	5.5	5.8	8	8
6	3	-	5.0	5.8	8	8
7	3	-	3.7	5.1	8	8

^aDisinfectant sprayed on to stainless steel surface before exposure of *B. anthracis* Sterne spores.

^bDisinfectant storage volume.