Decontamination of *Bacillus anthracis* Spores: Evaluation of Various Disinfectants

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Abstract

The present study compares the efficacy of various disinfectants against Bacillus anthracis *spores. While Bleach Rite® and 10% bleach reduce spore numbers by 90% within 10 minutes, a long contact time is required for complete disinfection. By contrast, although SporGon® did not initially reduce the number of spores as quickly as Bleach Rite or 10% bleach, shorter contact times were required for complete eradication of viable spores.*

Introduction

With renewed interest in the possible use of *Bacillus anthracis* (*B. anthracis*) as a biological weapon, the urgency to understand the biologic characteristics of this organism has increased. *B. anthracis* spores are extremely resistant to harsh environmental conditions (Mock & Fouet, 2001), and as a result, many challenges are encountered when trying to eliminate a spore population. Recent studies addressing this issue have focused predominately on decontamination of large facilities subjected to a bioterrorism event (Buttner et al., 2004; Canter et al., 2005; Kenar et al., 2007). While there are studies examining decontamination of spores on hard, nonporous and porous surfaces (Kolb & Schneiter, 1950; Majcher et al., 2008; Rogers et al., 2007; Rogers et al., 2005; Sagripanti et al., 2007), few studies directly measure the decontamination of spores in liquid suspension as used routinely in many laboratory manipulations. Furthermore, many of these studies examine a variety of disinfectants such as formaldehyde gas (Rogers et al., 2007) and methyl bromide (Kolb & Schneiter, 1950). These disinfectants are considered hazardous if inhaled or exposed to the skin and, therefore, are not applicable to routine laboratory decontamination measures.

Most scientific research with *B. anthracis* is conducted in stainless steel biosafety cabinets, and current recommendations for decontamination by the Environmental Protection Agency (EPA) and the Centers for Disease Control (CDC) suggest a 10% household bleach solution at pH 7 (Decon Laboratories, 2006; Moran, 1999). However, 10% bleach is known to be corrosive for certain grades of stainless steel. Furthermore, bleach solutions are also known to be unstable in diluted form. Rutala et al. (1998) demonstrated that the concentration of chlorine for a 1:5 dilution was only 83%-85% of the initial concentration, and 1:50 or 1:100 dilutions were only 40%-50% of the initial values after 30 days of storage. Moreover, many studies that tested the efficacy of disinfectants used *Bacillus* species other than *B. anthracis* (Buttner et al., 2004; Perez et al., 2005) and do not specifically address decontamination of varied spore concentrations that are routinely used in a laboratory setting. The present study compares the usefulness of various disinfectants in a laboratory where *B. anthracis* spores are routinely used for experimental work.

When choosing a disinfectant it is important to consider the properties of the microbiological agent in use and the equipment and tools that will require decontamination during the course of a laboratory procedure. Several disinfectants are widely used for decontamination in scientific research laboratories; these include SporGon®, 10% bleach, Bleach Rite®, Vesphene®, and Sporicidin®. As described in Table 1, SporGon® is a hydrogen peroxide/peracetic, acid-based disinfectant (Decon Laboratories, 2001) that is considered effective against many different organisms but has an expiration date that extends only 14 days after opening (Decon Laboratories, 2006). Although SporGon® has passed the AOAC sporicidal test against *B. subtilis* spores as well as *Clostridium sporogenes* spores, no published data describe its activity against *B. anthracis* spores (Decon Laboratories, 2006). A 10% solution of household bleach is widely used for decontamination of surfaces and tools exposed to biological agents including *B. anthracis* spores. However, it requires long contact times (Table 1) to inactivate spores, is corrosive for certain grades of stainless steel, and is highly unstable in the diluted form. Bleach Rite®, a pre-mixed stable formula of buffered (pH 12.3) 10% bleach is considered safe for stainless steel (Current Technologies, 2006), but no data are available on its effectiveness for killing bacterial spore populations such as *B. anthracis*. Sporicidin® contains 1% glutaraldehyde and 1.9% phenol as active ingredients (The Sporicidin Company, 2006). While many studies demonstrate the

Table 1
Disinfectant Pros/Cons

	Active Ingredients	Expiration Date	Safe for Stainless Steel	Recommended contact time for bacterial spores
Sporicidin® (The Sporicidin Company, 2006)	Glutaraldehyde	6 months	Yes	N/A
Vesphene® (Steris Corporation, 2001)	Phenol	14 days upon dilution	Yes	N/A
Bleach Rite® (Current Technologies Inc., 2006)	Sodium hypochlorite	1 year	Yes	Unknown
10% Bleach (U.S. Environmental Protection Agency, 2003)	Sodium hypochlorite	1 month	No	60 minutes
SporGon® (Decon Laboratories, 2006)	Hydrogen peroxide/ paracetic acid	14 days after opening	No	180 minutes

sporicidal activity of glutaraldehyde against *Bacillus* species, solutions ranging from 2%-5% are typically used, long contact times are required, and species of *Bacillus* other than *anthracis* were used as test organisms (Dyas & Das, 1985; Kenar et al., 2007; Manchee et al., 1983). Vesphene, the other disinfectant noted in Table 1, is a broad-spectrum disinfectant but does not indicate efficacy against spores (Steris Corporation, 2004). With such a wide range of disinfectant products available, it is important to determine the optimal disinfectant for a variety of biological agents, including *B. anthracis*, under conditions that are consistent with a scientific research environment.

Method

A spore stock was prepared (Heninger et al., 2006) and used to determine the rate of killing *B. anthracis* spores at two different concentrations, 100,000 and 1,000,000 cfu/ml. Spores, at a concentration of 100,000 cfu/ml, were added to a flask containing the recommended concentration of disinfectant (Vesphene® 1:128; bleach 1:9; Sporicidin®, Vesphene®, and Bleach Rite® were used undiluted) and were continuously mixed. One milliliter of the spore/disinfectant mixture was removed immediately after addition and at 10, 20, 30, and 60 minutes post-inoculation. The sample was immediately diluted into 9 ml phosphate buffered saline (PBS, filtered through a 0.2 mm analytical filter (Nalgene; ThermoFisher Scientific, Waltham, MA) and then the filter was washed three times with 50 ml PBS. Because the intent of treatment with disinfectants is to rapidly achieve complete or nearly complete sterilization, we chose not to enumerate the number of spores remaining following the use of a disinfectant that clearly failed to disinfect spore samples. Following the final wash, the filter was removed, placed onto a blood agar plate (Remel, Lenexa, KS), and incubated at 30°C. Twenty-four hours post-inoculation, filters were examined for the presence or absence of *B. anthracis*. Colonies were enumerated and recorded as $\log_{10}$ cfu/ml only if the disinfectant reduced the bacterial load to a discernable number.

Results and Discussion

In the present study, we tested the efficacy of the disinfectants described in Table 1 against *B. anthracis* strain ANR-1 spores (gift from T. Koehler, University of Texas). This strain is a variant of the virulent Ames strain that is devoid of the plasmid responsible for capsule production in the vegetative state ($pXO1^+$/ $pXO2^-$).

As shown in Table 2, when a sample containing 100,000 spores was analyzed, either Bleach Rite® or 10% bleach was able to dramatically reduce (<0.0001% remaining) the number of viable spores at the earliest time point, and no viable spores were detected after 20 minutes of treatment. Complete sterilization was not attained until 20 minutes post-inoculation due to 1 cfu being present at 10 minutes in the 10% bleach-treated groups. We believe this is due to random variation, and that while 10% bleach and Bleach Rite® immediately reduce spore numbers (>0.9999%) at this concentration, an occasional spore can persist. Similarly, no spores were detected after 20 minutes of treatment with Sporgon®, although this agent was not as effective as bleach or Bleach Rite® at the earlier time point. Although SporGon® was unable to greatly reduce the bacterial

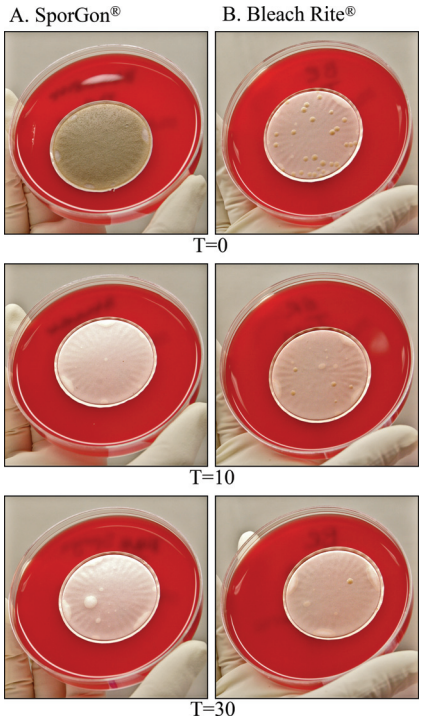
Table 2
Disinfection of 100,000 spores/ml

	T_0	T_{10}	T_{20}	T_{30}	T_{60}
PBS	+ (lawn)	+	+	+	+
Sporicidin®	+ (lawn)	+	+	+	+
Vesphene®	+ (lawn)	+	+	+	+
Bleach Rite®	+ (1cfu)	-	-	-	-
10% Bleach	-	+ (1cfu)	-	-	-
SporGon®	+ (lawn)	+ (2cfu)	-	-	-

Table 3
Disinfection of 1,000,000 spores/ml

	T_0	T_{10}	T_{20}	T_{30}	T_{60}
PBS	+ (lawn)	+	+	+	+
Sporicidin®	+ (lawn)	+	+	+	+
Bleach Rite®	+ (32cfu)	+ (5cfu)	+ (1cfu)	+ (2cfu)	-
10% Bleach	+ (18cfu)	+ (11cfu)	+ (9cfu)	+ (2cfu)	-
SporGon®	+ (lawn)	-	-	-	-

Figure 1



spore numbers at the earliest sample time points, complete sterilization was still achieved in 20 minutes. As expected, Vesphene® and Sporicidin® were completely ineffective in killing spores.

Because scientific manipulations often deal with different concentrations of spores, we evaluated the efficacy of each disinfectant at a higher test dose (Table 3, Figure 1). For this experiment we repeated the procedures above but added 1,000,000 spores per ml of disinfectant. While 10% bleach and Bleach Rite® were each able to reduce viable spore numbers immediately (within 2-3 minutes) at the higher inoculation dose, residual

viable spores remained after 30 minutes of treatment. This demonstrates that long contact times are required to achieve complete sterilization when using 10% bleach and 10% bleach products. By contrast, although treatment with SporGon® does not result in an immediate reduction in viable spore numbers, complete sterilization is achieved within 10-20 minutes.

These experiments demonstrate that 10% bleach, Bleach Rite®, and SporGon® are all effective disinfectants capable of successfully killing *B. anthracis* spores. Either 10% bleach or Bleach Rite® is an appropriate choice for daily decontamination of work surfaces due to the ability of these disinfectants to effectively kill a large portion of *B. anthracis* spores immediately. Bleach Rite® is a pre-mixed stable formula that is not corrosive to stainless steel (Current Technologies, 2006) and therefore ideal for disinfection of stainless steel biosafety cabinets. SporGon® is slightly corrosive for stainless steel; yet, complete sterilization was reached more quickly when compared to 10% bleach or Bleach Rite®. This makes SporGon® an appropriate disinfectant when dealing with biological spills outside the biosafety cabinet or when working with highly concentrated spore stocks. Therefore, we have implemented the use of Bleach Rite® for our daily laboratory decontamination of equipment and SporGon® in the event of a biological spill or when working with high concentrations of bacteria. While we use two separate disinfectants for different situations, it remains important to note that SporGon® and Bleach Rite® cannot be mixed and that each scientific laboratory is unique and should evaluate disinfectants based on its own individual needs.

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