Decontamination assessment of *Bacillus anthracis*, *Bacillus subtilis*, and *Geobacillus stearothermophilus* spores on indoor surfaces using a hydrogen peroxide gas generator

J.V. Rogers[1], C.L.K. Sabourin[1], Y.W. Choi[1], W.R. Richter[1], D.C. Rudnicki[1], K.B. Riggs[1], M.L. Taylor[1] and J. Chang[2]

[1]Battelle Memorial Institute, Columbus, OH, and [2]US Environmental Protection Agency, TW Alexander Drive, Research Triangle Park, NC, USA

ABSTRACT

J.V. ROGERS, C.L.K. SABOURIN, Y.W. CHOI, W.R. RICHTER, D.C. RUDNICKI, K.B. RIGGS, M.L. TAYLOR AND J . CHANG. 2005.

Aims: To evaluate the decontamination of *Bacillus anthracis*, *Bacillus subtilis*, and *Geobacillus stearothermophilus* spores on indoor surface materials using hydrogen peroxide gas.

Methods and Results: *Bacillus anthracis*, *B. subtilis*, and *G. stearothermophilus* spores were dried on seven types of indoor surfaces and exposed to ≥1000 ppm hydrogen peroxide gas for 20 min. Hydrogen peroxide exposure significantly decreased viable *B. anthracis*, *B. subtilis*, and *G. stearothermophilus* spores on all test materials except *G. stearothermophilus* on industrial carpet. Significant differences were observed when comparing the reduction in viable spores of B. anthracis with both surrogates. The effectiveness of gaseous hydrogen peroxide on the growth of biological indicators and spore strips was evaluated in parallel as a qualitative assessment of decontamination. At 1 and 7 days postexposure, decontaminated biological indicators and spore strips exhibited no growth, while the nondecontaminated samples displayed growth.

Conclusions: Significant differences in decontamination efficacy of hydrogen peroxide gas on porous and nonporous surfaces were observed when comparing the mean log reduction in *B. anthracis* spores with *B. subtilis* and *G. stearothermophilus* spores.

Significance and Impact of the Study: These results provide comparative information for the decontamination of B. anthracis spores with surrogates on indoor surfaces using hydrogen peroxide gas.

Keywords: *Bacillus anthracis*, *Bacillus subtilis*, decontamination, *Geobacillus stearothermophilus*, hydrogen peroxide, spores.

INTRODUCTION

Bacterial endospores can survive in the environment for an extended period of time, and are resistant to a wide-variety of treatments such as heat, desiccation, radiation, pressure and chemicals (Nicholson *et al.* 2000). This spore resistance is the result of various factors such as the thick proteinaceous spore coat, low water content in the spore core, and the α/β-type small, acid-soluble spore proteins (Setlow and Setlow 1993; Nicholson *et al*. 2000; Setlow *et al.* 2000). As potential bacterial spore decontaminants, ultraviolet light, γ-irradiation, wet/dry heat, ozone, aqueous solutions and mixtures, gels, and gases have been evaluated (Sagripanti and Bonifacino 1996; Setlow and Setlow 1996; Khadre and Yousef 2001; Raber and McGuire 2002; Spotts Whitney *et al.* 2003; Young and Setlow 2003; Young and Setlow 2004). Gaseous forms of chlorine dioxide, ethylene oxide, formaldehyde, hydrogen peroxide, methylene bromide, ozone, peracetic acid and propylene oxide have been used

Correspondence to: James V. Rogers, PhD, Battelle Memorial Institute 505 King Avenue, JM-7 Columbus, OH 43201, USA (e-mail: rogersjv@battelle.org).

for the inactivation of *Bacillus* spores (Spotts Whitney *et al.* 2003). These fumigating agents could be advantageous for large-scale decontamination of a room or building, as fumigants are easily dispersed and can potentially penetrate a large volume, thereby accessing all indoor surfaces. However, the toxicity, material compatibility, exposure time, concentration and ventilation requirements vary among gaseous decontaminants and considerations should be made for each of these treatments with respect to the intended decontamination application. Gaseous decontaminants also have the potential to yield better results than conventional surface cleaning with detergent sanitizers, as observed in a hospital environment decontaminated with hydrogen peroxide (French *et al.* 2004).

In aqueous or gaseous forms, hydrogen peroxide exhibits decontamination efficacy against bacterial spores, vegetative bacteria, viruses, amoeba and prions (Klapes and Vesley 1990; Heckert *et al.* 1997; Hiti *et al.* 2002; Fichet *et al.* 2004; French *et al.* 2004; Johnston *et al.* 2005). Hydrogen peroxide is considered less toxic than other fumigants such as chlorine dioxide, ethylene oxide and formaldehyde, and breaks down into water and oxygen. Therefore, gaseous hydrogen peroxide has been used as a decontaminant for treating laboratory and medical equipment, pharmaceutical facilities, hospital rooms and animal holding rooms (Klapes and Vesley 1990; Heckert *et al.* 1997; McDonnell and Russell 1999; Krishna *et al.* 2000; Krause *et al.* 2001; Fichet *et al.* 2004; French *et al.* 2004; Hillman 2004; Wagenaar and Snijders 2004).

In October 2001, the release of Bacillus anthracis spores from envelopes mailed in Trenton, NJ led to subsequent contamination of buildings including the mail processing and distribution centres in Washington, DC and Trenton, NJ, the Hart Senate Office Building, and a number of other mail handling facilities. This contamination led to extensive remediation and clean-up efforts and increased public awareness regarding the possibility of future bioterrorism-related attacks. Since this release of *B. anthracis* spores, there has been a growing interest in methods of detection, sampling and decontamination of *B. anthracis* spores from surfaces, rooms and buildings. A recent review evaluated the information in published literature regarding inactivation of *B. anthracis* spores in which the authors of the review concluded that more research is needed to evaluate the potential application of methods used in decontaminating laboratories and food industry facilities to larger buildings, as well as choosing the most appropriate decontamination technology (Spotts Whitney *et al.* 2003). These authors also note that much of the data available for decontamination of *Bacillus* spores is based on species other than *B. anthracis*; therefore, more testing of *Bacillus* spores should be conducted with or in correlation to B. anthracis (Spotts Whitney *et al.* 2003).

In 2002, the US Environmental Protection Agency responded to the increasing concerns about US homeland security by establishing the National Homeland Security Research Center and expanding its Environmental Technology Verification (ETV) program with a new Building Decontamination Technology Center (http://www.epa.gov/etv). The role of this new Center is to perform testing to verify the performance of commercially available technologies intended to decontaminate indoor surfaces of buildings contaminated with biological and chemical agents. Working through the ETV Building Decontamination Technology Center, the purpose of this study was twofold. First, we wanted to develop and assess a laboratory-scale approach for evaluating decontamination efficacy of *B. anthracis* spores deposited on typical porous and nonporous indoor surface materials using commercially available technologies. Secondly, we wanted to compare the decontamination efficacy of *B. anthracis* with selected surrogates. This paper describes the decontamination efficacies against spores of *B. anthracis* and two surrogates, *B. subtilis* and *G. stearothermophilus*, obtained for the first technology (a hydrogen peroxide gas generator) tested in the ETV Building Decontamination Center.

MATERIALS AND METHODS

Test organisms

Spores of the virulent *B. anthracis* Ames strain were prepared using a BioFlo 3000 fermentor (New Brunswick Scientific Co., Inc., Edison, NJ, USA). A primary culture of *B. anthracis* Ames was grown overnight (16–18 h at 37°C) in nutrient broth (BD Diagnostic Systems, Sparks, MD, USA) on an orbital shaker set at *c.* 150–200 rev $min)^{-1}$. This primary culture was used as an inoculum for a scale-up culture that was grown for 6–8 h in nutrient broth on an orbital shaker set at *c.* 150–200 rev $min)^{-1}$. The scale-up culture was used to inoculate Leighton-Doi Broth (BD Diagnostic Systems) in the BioFlo 3000 fermentor after which cultures were grown in the fermentor for approx. 24 h at 37°C. Cultures exhibiting >80% refractile spores, as determined by phase-contrast microscopy, were centrifuged at approx. 10 000–12 000$\times g$ for 15–20 min at 2–8°C. The resultant pellet was washed twice and resuspended in ice-cold, sterile water. The suspension was heat-shocked by incubating at 60°C for 45–60 min to kill vegetative cells, and centrifuged and washed a minimum of two times in ice-cold, sterile water to remove cellular debris. The spore preparation was purified by centrifuging the sample through a gradient of ice-cold, sterile 58% Hypaque-76 (Nycomed Amersham, Princeton, NJ, USA) at 9000 $\times g$ for 2 h at 2–8°C. The resultant pellet was washed and resuspended in ice-cold, sterile water and evaluated by phase-contrast microscopy. Preparations having >95% refractile spores

with <5% cellular debris were enumerated, diluted to approx. $1{\cdot}0 \times 10^9$ colony-forming units (CFU) $ml)^{-1}$, and stored at 2–8°C.

Commonly used *B. anthracis* surrogates for decontamination testing of spores are *B. subtilis* and *G. stearothermophilus* (Klapes and Vesley 1990; Sagripanti and Bonifacino 1996; Rutala *et al.* 1998; Khadre and Yousef 2001; Melly *et al.* 2002a; Sigwarth and Stark 2003; Young and Setlow, 2003). For hydrogen peroxide decontamination, differences in resistance to hydrogen peroxide gas have been reported between *G. stearothermophilus* and *B. subtilis*, where G. stearothermophilus is the most resistant (Klapes and Vesley 1990). Therefore, stock suspensions of *B. subtilis* (ATCC 19659) and *G. stearothermophilus* (ATCC 12980) spores were purchased from Apex Laboratories, Inc. (Apex, NC, USA) for this study. Samples from these stock cultures were enumerated, diluted to approx. $1{\cdot}0 \times 10^9$ CFU $ml)^{-1}$ in sterile water, and stored at 2–8°C until use.

Test materials

Seven materials representing porous and nonporous indoor surfaces commonly found in buildings were used for testing. These materials included ShawTek EcoTek 6 industrial carpet (Shaw Industries, Inc., Cartersville, GA, USA), bare pine wood (Kingswood Lumber, Columbus, OH, USA), painted (latex, semi-gloss) concrete cinder block ASTM C90 (Wellnitz, Columbus, OH, USA), glass ASTM C1036 (Brooks Brothers Glass & Mirror, Columbus, OH, USA), white formica laminate with matte finish (Solid Surface Design, Columbus, OH, USA), galvanized metal ductwork (Accurate Fabrication, Columbus, OH, USA), and painted (latex, flat) wallboard paper (United States Gypsum Company, Chicago, IL, USA). Samples of each test material were cut from a larger piece of the representative materials to form $1{\cdot}9 \times 7{\cdot}5$ cm coupons. Visual inspection of the physical integrity and appearance of the test material coupons was performed and observations recorded before and after decontamination in order to detect any damage to the test materials.

Decontamination procedure

All portions of this testing were performed under Biosafety Level 3 conditions. Prior to inoculation with either *B. anthracis*, *B. subtilis*, or *G. stearothermophilus* spores, the test materials were cleaned by wiping with 70% isopropanol. Each test coupon was laid flat in a Biological Safety Cabinet (BSC) Class III and contaminated with c. $1{\cdot}0 \times 10^8$ spores. For each type of test material, three coupons were used for decontamination, three coupons were used as controls (inoculated; not decontaminated), and two coupons were used as blanks (not inoculated). Suspensions of the spores were transferred to each coupon using a micropipette by placing the suspension over the surface as small droplets (Fig. 1). Following inoculation, the coupons were allowed to dry overnight, undisturbed. The next day, the inoculated coupons intended for decontamination (and one blank) were transferred to a Plas-Labs Model 830-ABC Compact Glove Box (Plas-Labs, Inc., Lansing, MI, USA; volume of approx. 317 l) and the coupons were placed lying flat, inoculated surface side up on a wire rack lined with Pet-D-Fence Screening (New York Wire Co., Mount Wolf, NY, USA) for support (Fig. 2).

Biological indicators (BI) containing *B. subtilis* (ATCC 19659) and *G. stearothermophilus* (ATCC 12980) and spore strips (SS) containing *B. atrophaeus* (ATCC 9372) were also used to evaluate decontamination. The *B. subtilis* and *G. stearothermophilus* BI consisted of approx. $1{\cdot}8 \times 10^6$ and $2{\cdot}6 \times 10^6$ spores, respectively, on stainless steel discs sealed in Tyvek pouches (Apex Laboratories, Inc.), and the SS consisted of approx. $1{\cdot}8 \times 10^6$ spores on filter paper strips sealed in glassine envelopes (Raven Biological Laboratories, Omaha, NE, USA). For *B. anthracis* decontamination, three

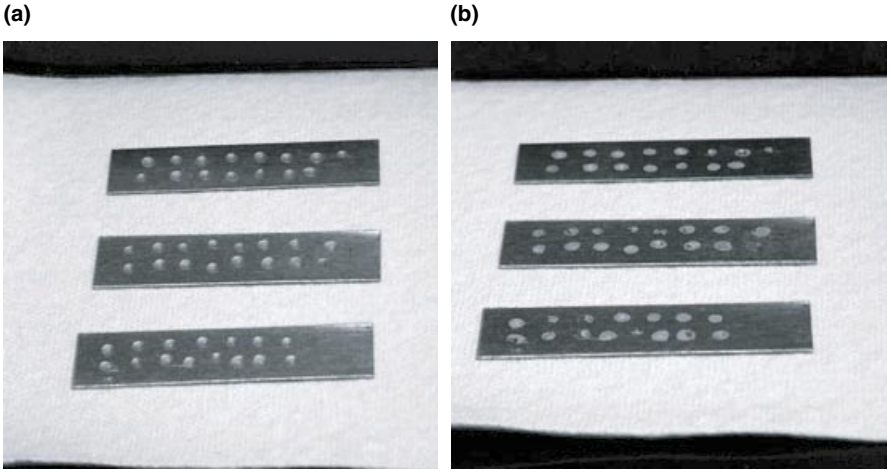

Fig. 1 Representative view of test coupons inoculated with 100 μl droplets containing approx. $1{\cdot}0 \times 10^8$ spores. (a) Image showing droplets immediately after application of spores. (b) Image of spores on test materials following overnight drying

Fig. 2 Representative view of test coupons, biological indicators (BI), and spore strip (SS) inside of the Plas-Labs Compact Glove Box

of each BI and SS were placed inside of the glove box during each decontamination test day. For *B. subtilis* and *G. stearothermophilus* decontamination, three of each respective BI and SS were placed inside of the glove box during each decontamination test day. Three of each BI and SS not subjected to hydrogen peroxide were used as positive controls.

The Clarus™ C hydrogen peroxide gas generator (Bioquell, Inc., Horsham, PA, USA) was used for the decontamination testing. The Clarus™ C unit generated hydrogen peroxide gas by flash evaporation of a 30% hydrogen peroxide stock solution (Sigma, St Louis, MO, USA). Modifications were made to the glove box to accommodate injection/exhaust ports and sensors for the Clarus™ C. The glove box was tested for leaks prior to the initiation of each experimental decontamination run. For each test, a negative pressure equivalent to two inches of a water column was generated by a vacuum pump in the glove box and maintained for a minimum of 2 min. Following this leak test, the decontamination cycle of the Clarus™ C unit was initiated and included conditioning (pressure and relative humidity adjustment), gassing (hydrogen peroxide injection), dwell (contact time) and aeration (hydrogen peroxide gas breakdown) phases. For the purposes of testing under the ETV program, operational parameters were provided by Bioquell, Inc. which included cycle pressure (20 Pa), conditioning (10 min), gassing (20 min), dwell (20 min), hydrogen peroxide injection rate (2·0 g min^{-1}), hydrogen peroxide dwell rate (0·5 g min^{-1}), and aeration time (9999 min). The aeration time was set for 9999 min; however, the actual aeration time was overnight (16–18 h) and the Clarus™ C was turned-off the next day when the hydrogen peroxide concentration was at or below the recommended 8-h exposure limit of 1 ppm (http://www.cdc.gov/niosh). All decontamination testing was performed at room temperature. Time, pressure, relative humidity and hydrogen peroxide concentration were monitored by specific sensors or detectors within the Clarus™ C, and recorded either electronically in real-time using a personal computer or on a paper printout. The Clarus™ C hydrogen peroxide sensor possessed an upper detection limit of 1000 ppm.

Sample processing and data collection

Decontamination efficacy of *B. anthracis*, *B. subtilis* and *G. stearothermophilus* spores was quantified by measuring the viable spores on both exposed and unexposed coupons. Following exposure to hydrogen peroxide gas, each coupon was placed in a 50 ml tube containing 10 ml of sterile phosphate-buffered saline (PBS) to which 0·1% Triton X-100 (Sigma) and approx. 200 μg catalase (Roche, Indianapolis, IN, USA) had been added. The purpose of the Triton X-100 was to minimize clumping of spores, and the catalase was used to neutralize any residual hydrogen peroxide. The inoculated control (not decontaminated) and blank coupons were also placed in a 50 ml tube containing 10 ml of sterile PBS with 0·1% Triton X-100 and catalase. For spore extraction, the tubes were agitated at 200 rev min^{-1} on an orbital shaker for 15 min at room temperature. Each tube was then heat-shocked at 65°C for 1 h to kill vegetative bacteria. Following heat-shock, 1·0 ml of each extract was removed, and a series of dilutions from 10^{-1} to 10^{-7} were prepared in sterile water.

Spore viability was determined by dilution plating in which 100 μl of the undiluted extract and each serial dilution were plated onto tryptic soya agar plates (Remel, Lexena, KS, USA) in triplicate, allowed to dry, and incubated overnight at 37°C for *B. anthracis*, 35°C for *B. subtilis* and 55–60°C for *G. stearothermophilus*. Following 18–24 h incubation, plates were enumerated and CFU ml^{-1} were determined by multiplying the average number of colonies per plate by the reciprocal of the dilution. Data were expressed as the mean ± standard deviation (SD) of observed CFU.

Efficacy calculations and statistical analysis

To calculate the efficacy of the decontamination treatment, the number of viable spores extracted from the decontaminated test coupons was compared with the number of viable spores extracted from the control coupons. Efficacy for biological agents was expressed in terms of a log reduction using the following equation:

$$\text{Log Reduction} = \log(N / N')$$

where N is the mean number of viable organisms recovered from the control coupons (i.e. those not subjected to decontamination), and N' is the number of viable organisms recovered from each test coupon after decontamination. For

decontaminated coupons where viable organisms were not detected, the efficacy was calculated as the log of the mean number of viable organisms recovered from the control coupons. Using the calculated log reduction for each test coupon, the mean (±SD) log reduction was calculated. Mean (±SD) percent recovery was calculated for each type of test material inoculated with each biological agent or surrogate by dividing the number of biological organisms in the treated coupon by the number of biological organisms in the nondecontaminated controls.

For statistical comparisons, the two-way ANOVA and *t*-tests (SAS version 8·2; SAS Institute, Inc., Cary, NC, USA) were used for data analysis. For each material and species combination, log reduction was calculated as described above. The two-way ANOVA was used to assess main effects for each organism and test material and interactions were fitted to the log reduction data. This model was used to compare the mean log reduction for each bacterial species tested, and compare the log reduction in *B. subtilis* and *G. stearothermophilus* spores to *B. anthracis* spores for each test material. The *t*-tests or statistical contrasts were used for the comparisons, with no adjustment for multiple comparisons. The ANOVA model was fitted using the SAS GLM procedure. $P \leq 0{\cdot}05$ was used as the level for significance.

RESULTS

A total of nine decontamination runs were conducted, which included three tests for each test organism (including BI and SS) on all test materials. The conditioning, gassing and dwell phases were initiated and completed within 2 h. In all tests, the hydrogen peroxide concentration inside the glove box was maintained at or above the vendor-recommended 1000 ppm during the dwell phase. Relative humidity reached levels of 60–80% during the dwell phase, in which condensation was prevalent on all surfaces within the exposure chamber. The observed mean cycle pressure was observed to be approx. 20 Pa throughout the entire decontamination cycle. Following all experimental decontamination runs, the test coupons were evaluated qualitatively for visible surface damage and no changes to any of the test materials were observed.

Exposure of test coupons contaminated with *B. anthracis* Ames, *B. subtilis*, or *G. stearothermophilus* spores to hydrogen peroxide gas resulted in a reduction of viable spores that varied according to the type of the test material (Tables 1–3). With respect to the inoculated surface, three of these test materials (industrial carpet, bare pine wood, painted concrete) can be considered porous, while the other four test materials (glass, decorative laminate, galvanized metal ductwork, painted wallboard paper) can be considered nonporous. The mean log reduction of detectable viable *B. anthracis* Ames spores ranged from 3·0 to 7·9 for all seven test materials (Table 1). For all seven test materials, the log reduction of detectable viable *B. subtilis* and *G. stearothermophilus* spores ranged from 1·6 to 7·7 and 0·81–6·0 respectively (Tables 2–3). No viable organisms were detected in any of the blank samples.

Table 1 Decontamination efficacy of *Bacillus anthracis* Ames spores following hydrogen peroxide exposure*

Test material/ treatment	Total spores recovered (CFU)	% Recovery	Log reduction
Industrial carpet			
Control	$6{\cdot}9 \pm 0{\cdot}32 \times 10^7$	$60 \pm 2{\cdot}8$	NA
H_2O_2 treated	$9{\cdot}3 \pm 7{\cdot}2 \times 10^4$	$0{\cdot}081 \pm 0{\cdot}063$	$3{\cdot}0 \pm 2{\cdot}1$†
Bare pine wood			
Control	$9{\cdot}6 \pm 1{\cdot}4 \times 10^6$	$9 \pm 1{\cdot}3$	NA
H_2O_2 treated	$3{\cdot}3 \pm 2{\cdot}9 \times 10^3$	<0·01	$3{\cdot}7 \pm 0{\cdot}67$†
Painted concrete			
Control	$3{\cdot}8 \pm 1{\cdot}7 \times 10^7$	33 ± 15	NA
H_2O_2 treated	$1{\cdot}5 \pm 2{\cdot}6 \times 10^3$	<0·01	$6{\cdot}4 \pm 2{\cdot}1$†
Glass			
Control	$8{\cdot}4 \pm 2{\cdot}2 \times 10^7$	75 ± 20	NA
H_2O_2 treated	0	0	≥7·9 ± 0†
Decorative laminate			
Control	$7{\cdot}0 \pm 1{\cdot}0 \times 10^7$	$61 \pm 8{\cdot}7$	NA
H_2O_2 treated	0	0	≥7·9 ± 0†
Galvanized metal ductwork			
Control	$3{\cdot}5 \pm 0{\cdot}13 \times 10^7$	$31 \pm 1{\cdot}1$	NA
H_2O_2 treated	0	0	≥7·5 ± 0†
Painted wallboard paper			
Control	$8{\cdot}3 \pm 0{\cdot}63 \times 10^6$	$7{\cdot}7 \pm 0{\cdot}59$	NA
H_2O_2 treated	0	0	≥6·9 ± 0†

Values are expressed as mean ± SD from triplicate samples of each test material.

NA, not applicable.

**Bacillus anthracis* Ames spores were subjected to hydrogen peroxide gas exposure and assessed for viability as described in the Materials and Methods. Each test material was inoculated with approx. $1{\cdot}0 \times 10^8$ CFU and dried overnight. Spores were extracted from the test materials and enumerated. Percent recovery and log reduction calculation were based on the number of detectable viable spores in the control and hydrogen peroxide-treated samples.

†Mean log reduction is significantly different than zero ($P \leq 0{\cdot}05$).

Statistical analysis of the data revealed that all mean log reductions were significantly different from zero (Tables 1–3) with the exception of *G. stearothermophilus* on industrial carpet, indicating that exposure to hydrogen peroxide gas significantly reduced ($P \leq 0{\cdot}05$) the mean number of *B. anthracis*, *B. subtilis* and *G. stearothermophilus* spores on all but one of the indoor surface materials. Comparisons within each material indicated that the two selected surrogates had either similar or lower mean log reductions than *B. anthracis*. The mean log reduction in *B. subtilis* spores was significantly lower ($P \leq 0{\cdot}05$) than *B. anthracis* spores for industrial carpet and bare pine wood,

Table 2 Decontamination efficacy of *Bacillus subtilis* (ATCC 19659) spores following hydrogen peroxide exposure*

Test material/ treatment	Total spores recovered (CFU)	% Recovery	Log reduction
Industrial carpet			
None	$4{\cdot}7 \pm 0{\cdot}19 \times 10^7$	51 ± 2·0	NA
H_2O_2	$1{\cdot}2 \pm 0{\cdot}42 \times 10^6$	1·2 ± 0·45	1·6 ± 0·15†
Bare pine wood			
None	$8{\cdot}8 \pm 2{\cdot}2 \times 10^5$	1·0 ± 0·24	NA
H_2O_2	$8{\cdot}1 \pm 6{\cdot}1 \times 10^3$	<0·01	2·2 ± 0·50†
Painted concrete			
None	$1{\cdot}3 \pm 0{\cdot}16 \times 10^7$	14 ± 1·7	NA
H_2O_2	2·2 ± 1·9 × 10	<0·0001	6·1 ± 0·88†
Glass			
None	$3{\cdot}7 \pm 2{\cdot}0 \times 10^7$	41 ± 23	NA
H_2O_2	0	0	≥7·6 ± 0†
Decorative laminate			
None	$4{\cdot}6 \pm 0{\cdot}85 \times 10^7$	51 ± 9·5	NA
H_2O_2	0	0	≥7·7 ± 0†
Galvanized metal ductwork			
None	$3{\cdot}6 \pm 0{\cdot}76 \times 10^7$	44 ± 9·3	NA
H_2O_2	3·3 ± 3·3 × 10	<0·0001	6·4 ± 0·98†
Painted wallboard paper			
None	$3{\cdot}3 \pm 2{\cdot}5 \times 10^7$	41 ± 31	NA
H_2O_2	0	0	≥7·5 ± 0†

Values are expressed as mean ± SD from triplicate samples of each test material.
NA, not applicable.
**Bacillus subtilis* spores were subjected to hydrogen peroxide gas exposure and assessed for viability as described in the Materials and Methods. Each test material was inoculated with approx. $1{\cdot}0 \times 10^8$ CFU and dried overnight. Spores were extracted from the test materials and enumerated. Percent recovery and log reduction calculation were based on the number of detectable viable spores in the control and hydrogen peroxide-treated samples.
†Mean log reduction is significantly different than zero ($P \leq 0{\cdot}05$).

Table 3 Decontamination efficacy of *Geobacillus stearothermophilus* (ATCC 12980) spores following hydrogen peroxide exposure*

Test material/ treatment	Total spores recovered (CFU)	% Recovery	Log reduction
Industrial carpet			
None	$2{\cdot}7 \pm 0{\cdot}051 \times 10^7$	21 ± 0·40	NA
H_2O_2	$4{\cdot}3 \pm 1{\cdot}1 \times 10^6$	3·3 ± 0·84	0·81 ± 0·10
Bare pine wood			
None	$2{\cdot}8 \pm 0{\cdot}081 \times 10^6$	2·2 ± 0·063	NA
H_2O_2	$3{\cdot}0 \pm 2{\cdot}0 \times 10^2$	<0·001	4·1 ± 0·46†
Painted concrete			
None	$9{\cdot}4 \pm 1{\cdot}1 \times 10^6$	7·8 ± 0·87	NA
H_2O_2	$2{\cdot}9 \pm 4{\cdot}1 \times 10^3$	<0·01	4·1 ± 1·0†
Glass			
None	$8{\cdot}7 \pm 0{\cdot}58 \times 10^6$	6·8 ± 0·45	NA
H_2O_2	$2{\cdot}5 \pm 2{\cdot}0 \times 10^2$	<0·001	4·7 ± 0·42†
Decorative laminate			
None	$5{\cdot}9 \pm 1{\cdot}1 \times 10^6$	6·2 ± 1·2	NA
H_2O_2	$1{\cdot}3 \pm 2{\cdot}1 \times 10^4$	0·013 ± 0·023	3·8 ± 1·4†
Galvanized metal ductwork			
None	$1{\cdot}5 \pm 0{\cdot}37 \times 10^7$	12 ± 2·9	NA
H_2O_2	$1{\cdot}6 \pm 0{\cdot}27 \times 10^5$	0·13 ± 0·021	2·0 ± 0·07†
Painted wallboard paper			
None	$9{\cdot}7 \pm 0{\cdot}81 \times 10^6$	8·1 ± 0·68	NA
H_2O_2	2·2 ± 1·9 × 10	<0·0001	6·0 ± 0·88†

Values are expressed as mean ± SD from triplicate samples of each test material.
NA, not applicable.
**Geobacillus stearothermophilus* spores were subjected to hydrogen peroxide gas exposure and assessed for viability as described in the Materials and Methods. Each test material was inoculated with approx. $1{\cdot}0 \times 10^8$ CFU and dried overnight. Spores were extracted from the test materials and enumerated. Percent recovery and log reduction calculation were based on the number of detectable viable spores in the control and hydrogen peroxide-treated samples.
†Mean log reduction is significantly different than zero ($P \leq 0{\cdot}05$).

while the mean log reduction in *G. stearothermophilus* spores was significantly lower ($P \leq 0{\cdot}05$) than *B. anthracis* for industrial carpet, painted concrete, glass, decorative laminate and galvanized metal ductwork (Fig. 3).

For all BI and SS exposed to hydrogen peroxide, no growth was observed as determined by the lack of visibly cloudy liquid cultures at 1 and 7 days postexposure. When not exposed to hydrogen peroxide, all of the BI and SS exhibited growth as determined by the presence of visibly cloudy liquid cultures on both 1 and 7 days.

DISCUSSION

The results of this study show that a 20-min treatment with ≥1000 ppm hydrogen peroxide gas resulted in varying decontamination efficacy against *B. anthracis*, *B. subtilis* and *G. stearothermophilus* spores dried on common indoor porous and nonporous materials. In the present study, the spore deposition from drying of an aqueous suspension on a material surface is a different delivery mechanism than what occurred during the intentional B. anthracis release in Washington, DC and Trenton, NJ. The *Bacillus anthracis* spores that were mailed in envelopes ranged from individual particles to microscopic aggregates and had the consistency of a fine powder. Therefore, it is possible that the spore preparation, mode of deposition, and spore adherence of a fine powder compared with an aqueous suspension could affect the decontamination efficacy of hydrogen peroxide gas.

The observed log reduction values for *B. anthracis*, *B. subtilis* and *G. stearothermophilus* spores inoculated on porous materials were consistently lower than on nonporous materials, suggesting that porosity affects decontamination efficacy. Hydrogen peroxide decontamination of *B. anthracis*

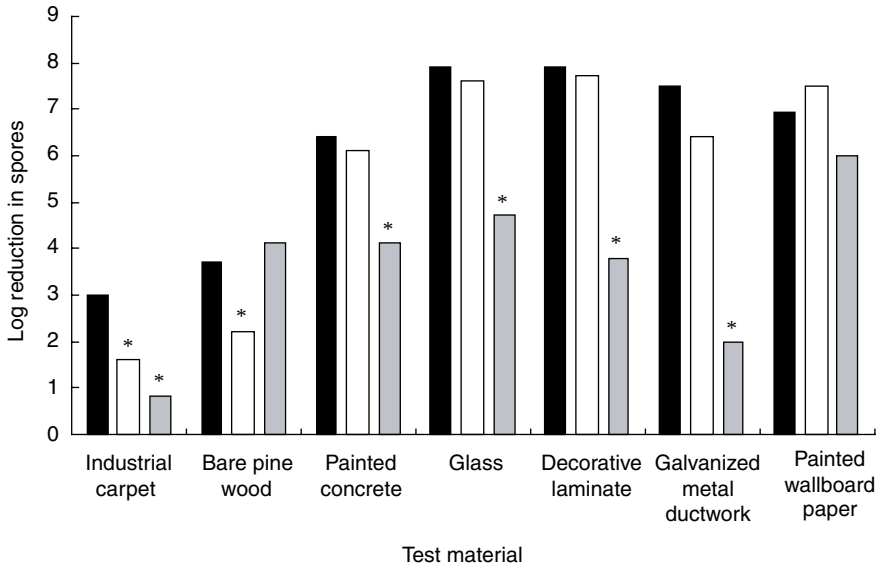

Fig. 3 Mean log reduction values for *B. anthracis* (■), *B. subtilis* (□) and *G. stearothermophilus* (▩) spores on all test materials exposed to hydrogen peroxide gas. Statistical comparisons were made between *B. anthracis* and each of the two surrogates on all test materials as described in the Materials and Methods. Asterisks indicate that the mean value is significantly different ($P \leq 0{\cdot}05$) than the mean value for *B. anthracis*

spores inoculated on nonporous surfaces resulted in >6·0 log reduction. The log reduction in viable *B. anthracis* spores was less (3·0 and 3·7) on the industrial carpet and bare pine wood compared with the other five test materials. For nonporous materials with relatively smooth and hard surfaces, the inoculated spores should remain predominantly at the material surface, enabling interaction of the gaseous hydrogen peroxide and spores with limited obstruction. However, when materials possessing surfaces such as the porous weave of carpet or open-grain of bare pine wood are inoculated with *B. anthracis*, the spores may penetrate into the material and embed in pits and cavities. Such penetration and embedding of spores into the test materials could preclude the interaction of hydrogen peroxide gas with the spores, thereby decreasing the potential for spore inactivation. This may, in part, be the result of the fact that hydrogen peroxide gas must penetrate through carpet fibre or into the wood grain before it can interact with *B. anthracis* spores. Similar to our observations, disinfection of *Paenibacillus larvae* subsp. *larvae* spores in wood using chemical sterilants was not effective because of a limited penetration capacity of the chemicals into the wood (Dobbelaere *et al.* 2001). It is also possible that some of the hydrogen peroxide could decompose through chemical interactions within the carpet fibre or bare pine wood matrices before it reaches the embedded spores. The penetration of bacterial spores into the porous materials is plausible; however, further work evaluating spore deposition, chemical interactions of hydrogen peroxide with test matrices, or possible chemical or physical interactions between the test materials and components of the spore coat will be required.

Most of the data available in the scientific literature on *Bacillus* species spore decontamination utilizes avirulent strains of *B. anthracis* or surrogates. An aim of this study was to compare the decontamination efficacy of the virulent *B. anthracis* Ames strain spores with that of *B. subtilis* and *G. stearothermophilus* spores. With respect to virulent *B. anthracis* spores, a surrogate should simulate the behaviour or responses and result in comparable performance data to hydrogen peroxide gas under controlled conditions. In the present study, *B. subtilis* exhibited nonsignificant differences in log reductions to that of *B. anthracis* for five of the seven materials tested. For the two materials (industrial carpet and bare pine wood) that exhibited statistical differences between *B. subtilis* and *B. anthracis*, the mean log reduction values for *B. subtilis* were lower than the mean log reduction values for *B. anthracis*. When comparing the mean log reductions between B. anthracis and *G. stearothermophilus* spores, nonsignificant differences were observed for only two of the seven materials tested. However, for the industrial carpet, painted concrete, glass, decorative laminate, and galvanized metal ductwork materials, the mean log reduction in *G. stearothermophilus* spores was significantly lower than *B. anthracis*. Therefore, statistical comparisons of the log reductions for all three organisms show that as a surrogate, *B. subtilis* spores appear to reflect *B. anthracis* spore resistance to hydrogen peroxide gas more closely than *G. stearothermophilus* spores. It is possible that the *G. stearothermophilus* spores are a surrogate that could result in relatively conservative results as these spores appear significantly more resistant to hydrogen peroxide gas decontamination than *B. anthracis* spores on most surfaces tested in this study. Our results are consistent with previous work where *G. stearothermophilus* spores were reported to be more resistant to being killed by hydrogen peroxide gas compared with *B. subtilis* spores (Klapes and Vesley 1990).

For the inactivation of *Bacillus* species spores, considerations must be made for the length of exposure time, temperature, decontaminant concentration, pH and relative humidity (Spotts Whitney *et al.* 2003). In the current study, only one combination of the operational parameters for the Clarus^{TM} C unit was utilized. It should be noted that the Clarus^{TM} C hydrogen peroxide sensor measured the circulating hydrogen peroxide concentration inside the glove box and not at the surface of the test materials. Therefore, it is possible that the hydrogen peroxide concentration at the material surface could be different than that measured in the glove box air, which could potentially affect spore killing. The 20-min dwell (contact) time promoted decreases in viable spores for all three organisms on all test materials except *G. stearothermophilus* on industrial carpet. The observed log reductions in viable *B. anthracis* and *B. subtilis* spores were ≥6·0 on painted concrete, glass, decorative laminate, galvanized metal ductwork and painted wallboard paper. A longer dwell time, such as 60 or 120 min, should result in higher decontamination efficacy of *B. anthracis* and surrogate spores on porous and nonporous surfaces. Such time-dependent results for spore killing have been observed using hydrogen peroxide and peroxide-based decontaminants (Klapes and Vesley 1990; Melly *et al.* 2002a; Young and Setlow 2004). The relative humidity reached 60–80% inside of the glove box during the dwell phase of testing and was concomitant with the formation of condensation (fogging) along the walls inside of the glove box. Similar microcondensation has been observed during decontamination of an enclosed BSC in which hydrogen peroxide concentrations exceeded 700 ppm (Hillman 2004). Condensation may enhance the decontaminating effects of hydrogen peroxide (Watling *et al.* 2002), which could promote the penetration of hydrogen peroxide into porous materials to reach embedded spores, or provide a microenvironment for oxidative spore injury. Further investigation is needed to determine whether the formation of this condensation layer is an important aspect for the decontamination of *B. anthracis* spores on porous materials.

The growth assessments of various BI are often used as a qualitative evaluation of decontamination performance (Heckert *et al.* 1997; Sigwarth and Moirandat 2000; Sigwarth and Stark 2003; French et al. 2004; Hillman 2004; Johnston *et al.* 2005). In the present study, we employed this type of growth assessment and evaluation in which the nondecontaminated control BI and SS displayed growth in the liquid cultures at both 1 and 7 days. However, when the BI and SS were exposed to hydrogen peroxide, no growth was observed in the liquid cultures at 1 and 7 days. These results suggest that exposure to ≥1000 ppm hydrogen peroxide for 20 min inactivated both the BI and SS, all of which contain spore loads of approx. $1{\cdot}8–2{\cdot}6 \cdot 10^6$ spores. Using these results, a ≥6·2 log reduction can be calculated based on spore density for the BI and SS. However, <6·0 log reduction was obtained for *B. subtilis* on industrial carpet and bare pine wood, while none of the test materials inoculated with *G. stearothermophilus* exhibited a greater than 6·2 log reduction. When compared with the test coupons used in this study, the observed complete inactivation of purchased BI and SS may reflect differences in inoculation techniques, spore load/distribution and variations in carrier materials of the BI and SS. The spore load on the purchased BI and SS was much less than on the inoculated material coupons prepared in this study, suggesting that the spores may be more evenly distributed with less clumping on BI and SS than on test materials and this even distribution may be a factor in facilitating effective decontamination. Differences in spore carrier materials can affect the performance of BI (Shintani and Akers 2000; Johnston *et al.* 2005), which may also play a role in hydrogen peroxide gas-induced decontamination. It is possible that when using BI and SS as qualitative indicators of decontamination performance, these indicators may overpredict the log reduction of *B. anthracis* spores achieved on porous surfaces.

Spore production conditions and heat treatment of the spores at 65°C could potentially contribute to the spore killing observed in this study. Differences in spore production methods, including growth medium and temperature, have been shown to influence spore resistance (Palop *et al.* 1999; Cazemier et al. 2001; Melly *et al.* 2002b). In the present study, the *B. subtilis* and *G. stearothermophilus* spores were purchased from a commercial source where the growth conditions may have been different from those used in producing the *B. anthracis* Ames spores. Such differences could affect the relative resistance of *B. subtilis* and *G. stearothermophilus* spores to hydrogen peroxide treatment when compared with *B. anthracis* spores. *Bacillus* subtilis spores pretreated with any of eight different oxidizing agents (including hydrogen peroxide) were more sensitive to killing by subsequent incubation at 84°C (Cortezzo *et al.* 2004). In this study, it is possible that viable spores extracted from the material coupons exposed to hydrogen peroxide gas could be killed during the 65°C heat shock. However, further experimentation is needed to determine the effect of the 65°C incubation step decreases the viability of spores damaged by the hydrogen peroxide treatment.

This study demonstrates the decontamination efficacy of hydrogen peroxide gas on spores from *B. anthracis* Ames and the two simulants *B. subtilis* and *G. stearothermophilus*. The capacity for decontamination appears to be influenced in part by the porosity of the contaminated surfaces and differences in decontamination efficacy are prevalent between the biological warfare agent *B. anthracis* and surrogates. The hydrogen peroxide gas exposure demonstrated significant efficacy in spore decontamination on the

seven types of surface materials evaluated; however, future studies will be needed to determine an acceptable reduction in spores (e.g. 6 logs) that would enable safe re-entry back into a building that has been decontaminated. The results of this study support the need for more building decontamination technology testing against *Bacillus* spores conducted with or in correlation to *B. anthracis* spores.

ACKNOWLEDGEMENTS

This work was funded by the National Homeland Security Research Center of the US Environmental Protection Agency as part of the Environmental Technology Verification (ETV) program. The Clarus™ C unit was kindly provided by Bioquell, Inc. for this testing. The authors thank Rick Tuttle, Nancy Niemuth, Claire Matthews, Ed Heller and Nicole Caudill for their excellent assistance.

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