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Differential Response of Various Spore Species to Sporicidal Disinfectants

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DIFFERENTIAL RESPONSE OF VARIOUS SPORE SPECIES TO SPORICIDAL DISINFECTANTS

by

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ABSTRACT

DIFFERENTIAL RESPONSE OF VARIOUS SPORE SPECIES TO SPORICIDAL DISINFECTANTS

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Master of Science

In the fall of 2001, letters laced with anthrax spores were delivered to various news organizations in New York and Florida, as well as to two Senators in Washington, D.C. Over 22 anthrax infections and five deaths resulted from exposure to these spores, and decontamination of the affected buildings was both time consuming and costly. Since these attacks, interest in sporicidal disinfectants has increased greatly. Many chemical sporicidal disinfectants are available commercially, but the exposure time required to sterilize can be relatively long. In addition, some spores are simply injured or inhibited by chemical disinfectants, but not necessarily killed. Studies have shown that heat shocking spores after exposure to some disinfectants can aid in the recovery of injured spores, but these studies have not evaluated this effect on spores exposed to peracetic acid-based disinfectants. Recently, our lab has evaluated two novel peracetic acid-based chemical

disinfectants, PeraDox™ and PeraDox Ultra™ for their activity against a variety of bacterial agents. Results indicated that the PeraDox™ solutions had extremely rapid cidal activity on a wide variety of microorganisms, especially those with innate germicide resistance, such as bacterial endospores. However, possible recovery of these spores after heat shock was not evaluated. The purpose of this study was to compare the sporicidal activity of three disinfectants: CIDEX™, PeraDox™, and PeraDox Ultra™ on three species of spores (*Bacillus subtilis*, *Bacillus anthracis*, and *Clostridium sporogenes*) in suspension, with and without heat shocking. Spores in suspension were exposed to disinfectants for specified times and assayed for viable spores. These spore suspensions were then heat shocked (80º C for 20 min) and assayed again. After exposure to peracetic acid-based disinfectants and subsequent heat shock, some *B. subtilis* spores recovered, resulting in up to a one log difference in viable spores. Other species and disinfectants did not show this effect. In addition, the activity of these disinfectants on spores dried onto a surface was evaluated using the standard AOAC sporicidal test. The current AOAC test specifies heat shocking after three weeks of incubation. In this study, we evaluated the AOAC sporicidal test by heat shocking immediately following disinfection and after three weeks of incubation as prescribed. Carrier tests showed a greater number of positive *B. subtilis* carriers when heat shocked immediately following PeraDox™, and PeraDox Ultra™ treatment, than when carriers were heat shocked after three weeks. In summary, results showed that heat shocking increases resuscitation of spores treated with some disinfectants, but not others. Spores in suspension and those dried onto carriers responded similarly to heat shocking. Finally, PeraDox™ formulations had surprisingly rapid sporicidal kinetics.

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Introduction

One week after the terrorist attacks of September 11, 2001, two waves of bioterrorist attacks occurred in the form of letters laced with anthrax spores sent through the U.S. Postal Service. These spores were the virulent Ames strain of *Bacillus anthracis*. Over 22 anthrax infections resulted from these attacks, including 11 cases of inhalational anthrax and five deaths. Decontamination of the buildings involved in the attacks was both time consuming and expensive. The Brentwood postal facility in Washington, D.C. alone took 26 months to decontaminate, at a cost of over $130 million. As a result of these attacks, interest in sporicidal disinfectants has increased greatly. Bacteria of the genera *Bacillus* and *Clostridium* form endospores in response to external stress. Bacterial endospores are extremely resilient and can withstand extremes in temperature and pH. They are also very resistant to ionizing and nonionizing (UV) radiation, chemical germicides, dessication, and the vacuum of outer space (27). Upon returning to a favorable environment, spores can readily convert back to a vegetative state through the process of germination (28). Disinfection of surfaces contaminated with bacterial endospores is not only an issue of bio-security, but also an issue faced repeatedly in clinical settings (32). For example, surgical instruments and endoscopes require sterilization or high-level disinfection between uses (33). Dental instruments must be treated similarly to prevent cross-contamination (1).

Sporulation and Germination

When bacterial cells of the genera *Bacillus* and *Clostridium* are challenged by factors such as starvation or environmental extremes, they will undergo the process of

sporulation (8). The bacterial spore is a complex structure composed of several layers, typically including the core, the plasma membrane, the germ cell wall, the cortex, the inner spore coat, the outer spore coat, and the exosporium. During spore formation, the spore becomes progressively dehydrated, with a final water concentration of about 15%.

Sporogenesis is a complex process which, once initiated, takes approximately six to eight hr to complete (12). Under conditions of starvation, or upon other cell signals, sporulation begins, bringing about a rapid series of morphological changes (13). First, an asymmetrically placed septum forms inside the vegetative cell. The smaller compartment, which is soon engulfed by the mother cell, is known as the forespore and will later become the spore itself (20). This core typically includes the DNA and RNA, carbohydrates, dipicolinic acid, Ca^{2+}, K^{+}, Mn^{2+}, and some proteins (31). While inside the vegetative cell, the forespore is enclosed in two layers of cell wall. The inner layer is known as the germ cell wall and, after germination, will become the cell wall of the vegetative cell. The outer layer, known as the cortex, is composed mainly of peptidoglycan (31) and contributes to the dehydrated state of the spore (8). The spore coat is then formed around the cortex. This layer, which will make up the bulk of the spore, consists mainly of protein, along with some complex carbohydrates and lipids (31). Some species, including *B. anthracis*, posses an exosporium, a loose-fitting layer around the spore (4). Finally, the mother cell lyses and releases the fully formed spore (8).

Germination, or the return of a spore to a vegetative state, is generally triggered by the presence of nutrients, including amino acids, sugars, and nucleosides (9). It has previously been reported that germination can be activated by heat (41). For some spores,

heat activation is not essential but increases the frequency of germination (26). Other factors, such as ions (9), and high pressure (29) also act as germinants. During germination, the spore undergoes the following changes: loss of heat resistance, Zn^{2+}, Ca^{2+}, and dipicolinic acid from the core, hydrolysis of the cortex peptidoglycan, rehydration of the core protoplast, and the resumption of metabolic activity (15, 26).

Disinfectants

Destruction of spores by chemical agents can be difficult and expensive due to the inherent resistance of bacterial spores to chemical attack. Many sporicidal disinfectants, including aldehydes (glutaraldehyde), chlorine-releasing agents (hypochlorite), peroxygens (peracetic acid and hydrogen peroxide), and ethylene oxide are commercially available and effective (31, 36). Some of these are listed in Table 1. However, most require long contact times, are toxic to humans, and are corrosive to various materials.

CIDEX™. Glutaraldehyde has been shown to be an effective sporicide (1, 5, 30, 37) and CIDEX™ has been in use for many years (18). CIDEX™ Activated Dialdehyde Solution is a 2.4% activated gultaraldehyde solution available commercially. CIDEX™ was chosen for use in this study because of its common use and effectiveness as a sporicide. Aqueous solutions of glutaraldehyde are acidic and must be buffered (or activated) by alkalinating agents to a pH of 8.2 to 9.2 in order to have optimal antimicrobial activity (31). Glutaraldehyde inactivates spores by cross-linking the spore's outer protein layers and by blocking normal germination events prior to dipicolinic acid release (37).

PeraDox™ Formulations. Novel disinfectants, PeraDox™ and PeraDox Ultra™, (sBioMed, LLC) have been shown to have very rapid sporicidal activity (6) with very few toxic or corrosive properties (sBioMed LLC, personal communication).

PeraDox™ formulations are peracetic acid (also known as peroxyacetic acid, or PAA)-based disinfectants with additional proprietary active ingredients that work synergistically. Peracetic acid has previously been shown to be an effective disinfectant on a variety of microbes, including vegetative bacterial cells, fungi, viruses, and bacterial spores, including those of *B. anthracis* (3, 14, 16, 19, 21, 34). The primary mode of action of peracetic acid is the oxidation of the double bonds of carbohydrates, nucleic acids, lipids, and the outer cell membrane proteins of vegetative bacterial cells, endospores, yeasts, and mold spores (21). Oxidation by peracetic acid usually occurs through the generation of free radicals. Carbon-centered free radicals such as $CH_3C(=O)O\cdot$ and $CH_3C(=O)\cdot$ have been implicated in the sporicidal action of peracetic acid, as has the hydroxyl radical (·OH) (21). Peracetic acid may also increase cell wall permeability by disrupting sulfhydryl and sulfur bonds (23). Two PeraDox™ formulations were used in this study, one with 0.25% peracetic acid (PeraDox™) and one with 1.3% peracetic acid (PeraDox Ultra™)

Heat Shock

When exposed to chemical disinfectants, spores may be inhibited, injured, or killed. The possibility exists that if spores sub-lethally injured by chemical disinfectants find a favorable resuscitation environment, they may then germinate and cause disease. Several factors have been shown to aid in the resuscitation of spores injured, but not killed, by chemical disinfectants, and it has been proposed that this could be a matter of clinical

importance (23). Some treatments that have been shown to aid in this resuscitation include lysozyme (11), sodium hydroxide (7), and heat (43). Heat shock, or exposing spores to high heat for a specific period of time (usually 80ºC for 20 min), has been shown previously to aid in the resuscitation of spores treated with certain biocides (35, 43). However, no previous studies have evaluated the effect of heat-shock on spores exposed to peracetic acid-based disinfectants, nor were spores of virulent *B. anthracis* evaluated in such studies. While heat shock following exposure to chemical disinfectants is not a typical practice during the clinical use of these agents, it can be a valuable research tool to determine the actual sporicidal efficacy of chemical disinfectants.

Specific Aims of This Study

The purpose of this study was to examine the differential response of various spore species to sporicidal disinfectants, as determined by both suspension and carrier-type tests. There were two specific aims to this study:

Aim 1: Suspension Tests. The first aim was to show the differences in heat shock-induced resuscitation between disinfectant-treated bacterial spores of *B. subtilis*, virulent *B. anthracis*, and *C. sporogenes* in suspension, and to compare sporicidal kill kinetics of the disinfectants.

Aim 2: Carrier Tests. The second aim was to compare the effect of heat shock on the resuscitation of bacterial spores of *B. subtilis* and *C. sporogenes* which have been dried onto porcelain penicylinders and polyester suture loops, as specified in the AOAC Official Method 966.04 – Sporicidal Activity of Disinfectants. The effect of heat

shocking immediately after disinfectant exposure versus following three weeks of incubation (as specified by the AOAC method) was also investigated.

Materials and Methods

Laboratory Conditions. Experiments with *B. anthracis* were performed under Biosafety Level 3 (BSL-3) conditions. All other experiments were performed under BSL-2 conditions.

Bacteria. Bacterial strains used were *Bacillus subtilis* ATCC 19659, *Clostridium sporogenes* ATCC 3584, and virulent *Bacillus anthracis* A0462 (Ames strain). *B. subtilis* and *B. anthracis* were grown on Columbia Agar (CA, Becton, Dickinson, and Company, Sparks, MD). *C. sporogenes* was grown on Reinforced Clostridial Agar (RCA, Becton, Dickinson, and Company) and incubated under anaerobic conditions.

Spore Suspensions. Two-hundred-twenty-five ml of Nutrient Broth (Becton, Dickinson, and Company) were added to each of two 2-L Erlenmeyer flasks and autoclaved. To each, 25 ml of filter-sterilized dextrose-salts solution (1.6% KCl, 0.3% $CaCl_2$, 0.9% dextrose, 0.025% $MgSO_4 \cdot 7H_2O$, 0.002% $MnSO_4 \cdot H_2O$, 0.0003% $FeSO_4 \cdot 7H_2O$) was added to make complete Leighton-Doi Broth (LDB). One hundred µl each of *B. subtilis* and *B. anthracis* freezer stocks (with a titer of approximately $1x10^8$ cfu/ml) were used to inoculate two flasks of LDB. The two Erlenmeyer flasks were placed in a Lab-Line™ 3525 shaking incubator at 32°C and 100 rpm for three days. The suspensions were then placed in conical vials, heated for 30 min at 65°C to kill any remaining vegetative cells, and centrifuged. Spores were then resuspended in 20 ml sterile HPLC water and refrigerated overnight. This washing procedure was repeated three times. Spore content

was monitored with phase-contrast microscopy. The spore suspensions were quantified using serial dilution and triplicate plating by membrane filtration. The *B. anthracis* spore suspension had a titer between $1.80x10^9$ and $1.98x10^9$ spores/ml. The *B. subtilis* spore suspension had an initial titer of between $4.16x10^9$ and $7.02x10^9$ spores/ml. A spore suspension of *C. sporogenes* was purchased from Presque Isle Cultures (Presque Isle, PA), and was quantified using serial dilution and triplicate plating by membrane filtration. This suspension had a titer of between $1.24x10^7$ and $3.12x10^7$ spores/ml.

Disinfectants. All three spore suspensions were tested against three disinfectants: CIDEX™ Activated Dialdehyde Solution, 2.4% gultaraldehyde (Advanced Sterilization Products, Irvine, CA), PeraDox™, 0.25% peracetic acid (sBioMed, LLC, Orem, UT), and PeraDox Ultra™, 1.3% peracetic acid (sBioMed, LLC). All disinfectants were activated immediately prior to use, according to manufacturer's instructions.

Suspension Tests. A kill-time suspension test performed at 20˚C was used to evaluate each disinfectant. Briefly, 0.1 ml of spore suspension was added to 9.9 ml of disinfectant at time zero. The suspension was mixed thoroughly and returned to the water bath. At various times, the spore mixture was sampled. This involved removal of one ml of the spore/disinfectant solution, which was then added to nine ml of an appropriate neutralizer. To neutralize the aldehyde-based disinfectant, a freshly-prepared glycine solution (1%) was used, while a freshly-prepared neutralizer containing tris buffer (500mM, pH 8.0), Tween 80 (12.72%), tamol (6%), lecithin (1.7%), catalase (1.1%), cysteine (1%), and peptone (1%) was used for the peracetic acid-based disinfectants. The neutralized sample was then mixed thoroughly and serially diluted in physiological saline

solution (0.9% NaCl). Neutralization controls were performed for each assay to ensure that adequate neutralization of the disinfectants was achieved.

The number of viable spores in each dilution was quantified by membrane filtration of one ml samples in triplicate. Millipore E-Z Pak 0.45 μm membranes were used. Filter membranes were placed onto CA plates in the case of *B. subtilis* and *B. anthracis*, while pre-reduced RCA plates were used for *C. sporogenes*. Once samples were taken from each time point/dilution, all dilution tubes were then heat shocked in a water bath (80ºC) for 20 min, and the tubes were re-sampled and tested, following the same protocol as above. The plates were incubated at 37˚C, and counted after approximately 24 and 48 hr of incubation. Plates of *C. sporogenes* were counted only after 48 hr to ensure that anaerobic conditions were maintained.

Carriers. Porcelain penicylinders and polyester suture loops inoculated with *B. subtilis* ATCC 19659 and *C. sporogenes* ATCC 3584 were purchased from Presque Isle Cultures. Recent studies showed that polyester suture loops are preferred to silk suture loops, as the latter interact with peracetic acid (22). All carriers were prepared according to the AOAC Official Method 966.04 (15^{th} ed.) and were tested for acid resistance by the manufacturer according to the AOAC Official Method. Spore titers were documented by the manufacturer to exceed $1x10^6$ spores/carrier.

These titers were confirmed in our lab using the following method: carriers were placed in tubes containing modified nutrient broth (2% Tween) and sonicated for ten min in a Sonicor™ SC-200 ultrasonic cleaner to remove the spores from the carrier. After serial dilution, the number of viable spores in each dilution was quantified by membrane filtration of one ml samples. Millipore E-Z Pak 0.45 μm membranes were used. Filter

membranes were placed onto CA plates in the case of *B. subtilis*, while pre-reduced RCA was used for the *C. sporogenes*. Samples from each dilution were plated in triplicate. Counts were averaged for the three replications. The carrier titers are listed in Table 2.

Carrier Tests. Carriers were tested against the three disinfectants used in the suspension tests. Experiments were performed according to the AOAC Official Method 966.04 – Sporicidal Activity of Disinfectants. Briefly, ten ml of each disinfectant was placed into 50 ml conical centrifuge tubes and brought to 20º C. Two suture loops or penicylinders were placed into each tube of disinfectant using a flamed metal hook. After the specified contact time, the carriers were removed using flamed metal hooks and placed individually into tubes containing Sodium Thioglycolate Broth (STB, Becton, Dickinson, and Company). Using sterile metal hooks, each carrier was retransferred into a second tube of STB. Tubes were incubated at 37 ºC for 21 days, and then heat shocked at 80 ºC for 20 min and reincubated at 37 ºC for 72 hr and checked for growth. This test was performed on 30 of each carrier per species.

Exposure time for PeraDox™ was 25 min, the exposure time for PeraDox Ultra™ was 15 min, and the exposure times for CIDEX™ were one hr for *C. sporogenes* and four hr for *B. subtilis*. These contact times were determined experimentally by previous testing (data not shown) to be at the end of the kill curve for each organism/disinfectant combination.

A repeat of the above testing was performed, which differed from the AOAC Official Method in the following respect: instead of incubating for 21 days before heat shock, these tubes were heat shocked at 80 ºC for 20 min immediately following retransfer to the second tube of STB. They were not heat shocked after 21 days. Again,

the test was performed on 30 of each carrier per species. In all, 60 carriers of each type were tested for each species, for each of the three disinfectants.

Statistical Methods. For suspension tests, the susceptibilities of three species of bacterial spores, to three disinfectants were evaluated. On each test day, both *B. subtilis* and *C. sporogenes* were tested with different disinfectants selected at random until each disinfectant was repeated three times. For *B. anthracis*, on each test day, all three disinfectants were tested in random order. Each dilution assayed for viable spores was plated in triplicate and these counts were averaged to obtain the estimate for each dilution. Statistical analysis was performed using the GLIMMIX procedure in SAS. An F-test adjusted for multiple replications was used. Data from all three dilutions plated were used in this analysis.

For carrier tests, the number of positive tests after delayed heat shock was compared to the number of positive tests after immediate heat shock using the FREQ procedure in SAS. For this data, Fisher's exact test was used.

Results

Suspension Tests

The first aim of this study was to show the differences in heat shock-induced resuscitation between disinfectant-treated bacterial spores of *B. subtilis*, virulent *B. anthracis*, and *C. sporogenes* in suspension. To test the hypothesis that spores treated with heat after disinfection had different recoveries than those not heat shocked, the spore suspensions were exposed to a disinfectant, assayed for viable spores, then heat shocked and assayed again.

Upon exposure to PeraDox™, *B. anthracis* showed an average log reduction of 4.99 in 3.5 min among three replications, with an additional average log reduction of 1.68 caused by heat shocking. The kill kinetics of PeraDox™ on *B. anthracis* spores are shown in Figure 1A. PeraDox™ produced an average log reduction of *B. subtilis* spores of 6.12 after one min, but an average log reduction of only 5.30 after heat shock. The kill kinetics of PeraDox™ on *B. subtilis* spores are shown in Figure 1B. *C. sporogenes* showed a log reduction of greater than 5.89 after 15 sec. This data represents complete kill. No increase was seen after heat shock. Figure 4A shows a comparison of the kill kinetics of PeraDox™ on *B. anthracis* and *B. subtilis* spores. Note that *B. anthracis* spores are significantly more resistant to PeraDox™ than *B. subtilis* spores. Statistical analysis confirmed that the heat-shock treatment resulted in a significant difference in both species ($p < 0.0001$), with spores of *B. anthracis* showing potentiated kill and *B. subtilis* spores showing increased resuscitation.

Upon exposure to PeraDox Ultra™, *B. anthracis* showed an average log reduction of 5.18 in 45 sec, with an additional log reduction of 1.56 following heat shocking. The kill kinetics of PeraDox Ultra™ on *B. anthracis* spores are shown in Figure 2A. *B. subtilis* showed an average log reduction of 6.04 in 15 sec, but this value dropped to 4.82 following heat shocking. See Figure 2B for kill kinetics of PeraDox Ultra™ on *B. subtilis* spores. *C. sporogenes* again showed a log reduction of more than 5.67 in 15 sec due to complete kill. A comparison of the kill kinetics of PeraDox Ultra™ on *B. anthracis* and *B. subtilis* spores can be seen in Figure 4B. As was seen with PeraDox™, *B. anthracis* spores are more resistant to PeraDox Ultra™ than *B. subtilis* spores. Again, the difference in log reduction due to heat shock was significant ($p < 0.0001$), with species of *B.*

anthracis showing potentiated kill and *B. subtilis* spores showing increased resuscitation.

After exposure to CIDEX™, *B. anthracis* showed an average log reduction of 4.59 after 4 min, with an additional average log reduction of 0.63 following heat shocking. The kill kinetics of CIDEX™ on *B. anthracis* spores are shown in Figure 3A. *B. subtilis* showed a log reduction of 4.74 after 190 min, with an additional average log reduction of 0.90 after heat shocking. Figure 3B shows the kill kinetics of CIDEX™ on *B. subtilis* spores. *C. sporogenes* showed an average log reduction of 3.23 after 20 min, with an additional average log reduction of 0.69 after heat shock. See Figure 3C for kill kinetics of CIDEX™ on spores of *C. sporogenes*. Figure 4C shows a comparison of the kill kinetics of CIDEX™ on all three species. Note the dramatic resistance of *B. subtilis* spores to CIDEX™. Statistical analysis again showed heat shock to significantly potentiate CIDEX™-mediated killing with higher log reductions following heat-shock treatment ($p < 0.0001$).

Figure 5A shows a comparison of the kill kinetics of all three disinfectants on spores of *B. anthracis*. Note the extremely rapid kill of PeraDox Ultra™ on these spores. Figure 5B shows the same comparison for spores of *B. subtilis*. Again, the extreme speed of kill of the PeraDox™ formulations is evident. All suspension test results are summarized in Table 3. The statistical analysis, using data from all three dilutions plated, produced the data inTable 4. Mean differences in log reductions before and after heat shock for each bacterial species and disinfectant, along with standard errors and p values, are listed.

Carrier Tests

The second aim of this study was to compare the effect of delayed or immediate heat shock on the recovery of disinfectant-treated bacterial spores of *B. subtilis* and *C. sporogenes* dried onto porcelain penicylinders and polyester suture loops. *B. anthracis* was not used in these experiments because it is not mandated by the AOAC Official Method. Again, in one experiment, the carriers were heat shocked immediately after disinfectant exposure and in the other, the carriers were heat shocked following three weeks of incubation, as specified in the AOAC procedure. Each carrier possessed at least $1x10^6$ viable spores. Any viable spores remaining on the carrier after exposure to disinfectant will result in growth, or a positive test. Carriers with no remaining viable spores after exposure to disinfectant will result in a negative test (no growth). The results from the carrier tests are shown in Table 5. In the AOAC Official Method, carrier test results are based on a combined total of carriers (porcelain penicylinders and suture loops) that yielded growth. The results in Table 5 are reported similarly.

For PeraDox™, a total of three *B. subtilis* carriers were positive when the test was performed according to the AOAC guidelines (heat shock after three weeks of incubation). However, the group subjected to immediate heat shock had nine positive carriers, a three-fold increase (p=0.0627). For *C. sporogenes*, only one carrier was positive in each group (p=0.7521).

For PeraDox Ultra™, two *B. subtilis* carriers were positive under AOAC guidelines, as opposed to seven after immediate heat shock, a 3.5-fold increase (p=0.0815). Two *C. sporogenes* carriers were positive under AOAC guidelines, and four were positive when subjected to immediate heat shock, a two-fold difference (p=0.3397).

For CIDEX™, three *B. subtilis* carriers were positive under AOAC guidelines, with five positives when carriers were exposed to immediate heat shock, a 1.67-fold increase (p=0.3585). Three *C. sporogenes* carriers were positive under AOAC guidelines, with four positives produced when carriers were immediately heat shocked, a 1.33-fold increase (p=0.5000).

Discussion

Aim 1: Suspension Tests

Results show that PeraDox™ and PeraDox Ultra™ have extremely rapid sporicidal activity (see Figures 1 and 2). Overall, PeraDox Ultra™ had the most rapid sporicidal activity across all spore species (see Figures 5A and 5B), resulting in complete kill in as little as 15-45 sec (see Table 3). While CIDEX™ had comparable activity to PeraDox™ on spores of *B. anthracis*, it was generally much slower than the two peracetic acid-based disinfectants on the other spore species (see Figure 5). One possible reason for this disparity relates to differences in mechanisms of action of the two types of disinfectants. Glutaraldehyde, the active ingredient of CIDEX™, specifically targets proteins, which are found primarily in the spore coat. Peracetic acid, the active ingredient of PeraDox™ and PeraDox Ultra™, oxidizes a variety of molecules, including nucleic acids, proteins, lipids, carbohydrates, and any other molecule containing a double bond. Thus, the peracetic acid-based disinfectants have conceivably more cellular targets on which to act.

These results also showed important differences between the two *Bacillus* species with respect to sporicide resistance. *B. subtilis* proved to be less resistant than *B. anthracis* to PeraDox™ and PeraDox Ultra™. Greater exposure times (up to 3.5 times

greater) were required for *B. anthracis*, to achieve the level of disinfection seen in *B. subtilis* (see Figures 4A and 4B). However, the opposite is true of CIDEX™. In this case, *B. subtilis* was significantly more resistant than *B. anthracis*, requiring a 186 min longer exposure time to achieve a similar inactivation of spores (see Figure 4C).

Differences in spore coat proteins between *B. subtilis* and *B. anthracis* may be responsible for these differences in resistance. Genome analysis of the two species has shown that spore coat proteins with key roles in spore morphogenesis are present in the genomes of both species (17). This may indicate that the sporulation process is similar for *B. subtilis* and *B. anthracis*. However, this same genomic analysis showed several differences in the proteins that make up the spore coat, which may be responsible for differences in their susceptibilities to disinfectants (17). Under electron microscopy, the spore coats of *B. subtilis* and *B. anthracis* are visibly different. *B. subtilis* has a thick coat with two major layers, while the *B. anthracis* coat is thinner and more compact (2, 10, 42). Another possibility is that resistance or susceptibility to disinfection may be the result of interactions between multiple coat proteins (17). The exosporium, a structure which surrounds the entire spore, including the spore coat, is absent in *B. subtilis* but present in *B. anthracis*. This may also affect resistance to disinfectants (17). Differences between species obviously exist, and the identification of which proteins or combinations of proteins are affected by glutaraldehyde-based disinfectants is a question for future research.

C. sporogenes proved to be extremely susceptible to disinfection with PeraDox™ and PeraDox Ultra™, but less so to CIDEX™. Complete kill was always observed in 15 sec with the two peracetic acid-based disinfectants (a >5-log reduction), while a three-log

reduction took 20 minutes with CIDEX™. It is not surprising, however, that *C. sporogenes*, an anaerobic bacterium, is more susceptible to oxidative damage than aerobic bacteria such as *B. subtilis* and *B. anthracis*. The similarities in kill kinetics of CIDEX™ on *C. sporogenes* to those of *B. anthracis* (see Figure 4C), support this line of thought.

Perhaps most interesting is the resuscitation observed in *B. subtilis* spores when heat shocked immediately following exposure to peracetic acid-based disinfectants. After disinfection with PeraDox™ and PeraDox Ultra™, heat shock of *B. subtilis* spores resulted in statistically significant ($p < 0.0001$) resuscitation of spores (see Table 4). Heat shocking after disinfection with all three disinfectants resulted in further killing of the remaining viable spores in all other species, including *B. subtilis* when treated with CIDEX™ (see Table 3). These results indicate that while peracetic acid causes rapid injury to bacterial spores, heat shock aids in the repair of at least some of this damage. On the other hand, no resuscitation was seen on any of the species exposed to CIDEX™, indicating that heat shock does little to reverse the damage mediated by glutaraldehyde.

Interestingly, the resuscitation observed when *B. subtilis* spores were immediately heat shocked after exposure to PeraDox™ and PeraDox Ultra™ was not observed in *B. anthracis* (Figure 4). *B. subtilis* is often used as a surrogate organism for *B. anthracis* when BSL-3 conditions are not available. Thus, care should be taken when extrapolating data from surrogate organisms.

Taken together, these data indicate that, while PeraDox™ and PeraDox Ultra™ show a much faster kill rate than CIDEX™ for spores in suspension, some of these spores are resuscitated upon heat shock. In a clinical setting, it would be advisable to

increase the PeraDox™ exposure times toward the end-points tested here to ensure complete kill. For disinfection of *B. anthracis*, however, these peracetic acid-based disinfectants are as effective as the glutaraldehyde-based disinfectants, and, in the case of PeraDox Ultra™, much more so.

Aim 2: Carrier Tests

The second aim of this study was to determine if the resuscitation of spores in suspension after heat shock was similar to that of spores dried onto solid surfaces. It was expected that this may be the case, however, the rate of diffusion of the disinfectants into dried films containing spores and other unknown factors may affect this phenomenon.

With PeraDox™, the *B. subtilis* carriers heat shocked immediately following exposure to disinfectant showed a three-fold increase in positives over the carriers tested according to AOAC guidelines. With PeraDox Ultra™, the *B. subtilis* carriers immediately heat shocked after disinfectant exposure showed a 3.5-fold increase in positives over the carriers tested according to the AOAC method. These results are significant at a 90% confidence level, and approached significance at the 95% confidence level ($p=0.0627$ and 0.0815 for PeraDox™ and PeraDox Ultra™, respectively – see Table 5). The *C. sporogenes* carriers showed no heat-shock mediated increase with PeraDox™ and only a two-fold increase with PeraDox Ultra™. These results were not statistically significant ($p=0.7521$ and 0.3397 for PeraDox™ and PeraDox Ultra™, respectively – see Table 5). All spores treated with CIDEX™ showed almost no increase in the number of positive carriers when immediately exposed to heat shock vs. the group tested according to AOAC guidelines ($p=0.3585$ and 0.5000 for *B. subtilis* and *C. sporogenes*, respectively – see Table 5).

Taken together, these results indicate that, when testing PeraDox™, PeraDox Ultra™, and perhaps other peracetic acid-based disinfectants with *B. subtilis*, immediate heat shock after disinfection may be a better indicator of the effectiveness of the sporicidal activity of a disinfectant than the current AOAC guidelines. In 2003, the Environmental Protection Agency initiated research to improve efficacy test methods for sporicides (38). Since then, the AOAC Official Method 966.04 has been reevaluated several times (23-25, 38-40), but none of these evaluations addressed the effect of immediate heat shock. The findings of this study may warrant further evaluation of the AOAC Official Method 966.04 in this respect.

In conclusion, spore species differ widely in their susceptibility to disinfectants and their response to heat shock following disinfectant treatment. These data showed a significant spore species-disinfectant-heat shock interaction. Because these interactions are complex and unpredictable, tests with and without heat shocking should be performed when evaluating the sporicidal properties of a disinfectant. In addition, the reported sporicidal efficacy of peracetic acid-based disinfectants may be overrepresented when heat shock is not performed immediately following disinfection treatment. Therefore, the current AOAC Official Method 966.04 – Sporicidal Activity of Disinfectants may be suboptimal for determining the activity of peracetic acid-based disinfectants on *B. subtilis* spores. Lastly, PeraDox Ultra™ has exceptionally fast sporicidal kinetics, being able to kill *B. subtilis* spores in suspension about 1,000 times faster than CIDEX™. Further confirmation of the reported lack of toxicity and corrosion of the PeraDox™ formulations will likely open the door to countless practical uses of these products in infection control.

Table 1. Various Sporicidal Disinfectants and Their Properties

Active ingredient(s)	Sporicidal time	Disinfection time	Shelf-life
8.3% Hydrogen Peroxide 7.0 % Peracetic Acid	5 hours	5 minutes	5 days
1.0% hydrogen peroxide 0.08% peracetic acid	8 hours	25 minutes	14 days
3.4% glutaraldehyde 26% isopropanol	10 hours	10 minutes	14 days
2.4% glutaraldehyde	10 hours	45 minutes	14 days
Hypochlorite and Hypochlorous acid 650-675 ppm Active free chlorine	24 hours	10 minutes	Single -use
0.55% *ortho*-phthaldehyde	32 hours	12 minutes	14 days

Table 2. Concentrations of Spores Dried Onto Carriers From Recovery Experiments

B. subtilis		
Lot 032107*	**Carrier**	**Average Titer (cfu)**
	Porcelain Penicylinder	1.05×10^6
	Polyester Suture Loop	1.46×10^6
Lot 052507†	**Carrier**	**Average Titer (cfu)**
	Porcelain Penicylinder	1.12×10^6
	Polyester Suture Loop	1.00×10^6
C. sporogenes		
Lot 040207*	**Carrier**	**Average Titer (cfu)**
	Porcelain Penicylinder	1.21×10^6
	Polyester Suture Loop	3.05×10^6
Lot 052607†	**Carrier**	**Average Titer (cfu)**
	Porcelain Penicylinder	1.61×10^6
	Polyester Suture Loop	5.27×10^6

* Lots used for CIDEX™ and PeraDox™ Ultra tests.
† Lots used for PeraDox™ tests.

Table 3. Effect of Heat Shock on Log Reduction Values of Disinfectant-treated Spores in Suspension

		Replication 1			Replication 2			Replication 3			
PeraDox™	Exposure Time	Log Reduction Before HS	Log Reduction After HS	Difference	Log Reduction Before HS	Log Reduction After HS	Difference	Log Reduction Before HS	Log Reduction After HS	Difference	Mean Difference†
B. anthracis	3.5 min	5.02	6.43	-1.41	4.92	6.64	-1.72	5.02	6.93	-1.91	-1.68
B. subtilis	1 min	6.01	5.36	0.65	5.97	5.20	0.77	6.38	5.35	1.03	0.82
C. sporogenes	15 sec	>5.85*	>5.85*	0.00	>5.85*	>5.85*	0.00	>5.97*	>5.97*	0.00	0.00

		Replication 1			Replication 2			Replication 3			
PeraDox Ultra™	Exposure Time	Log Reduction Before HS	Log Reduction After HS	Difference	Log Reduction Before HS	Log Reduction After HS	Difference	Log Reduction Before HS	Log Reduction After HS	Difference	Mean Difference†
B. anthracis	45 sec	5.32	6.89	-1.57	5.19	6.50	-1.31	5.02	6.82	-1.80	-1.56
B. subtilis	15 sec	5.91	4.70	1.21	5.90	4.85	1.05	6.31	4.91	1.40	1.22
C. sporogenes	15 sec	>5.77*	>5.77*	0.00	>5.57*	>5.57*	0.00	>5.68*	>5.68*	0.00	0.00

		Replication 1			Replication 2			Replication 3			
CIDEX™	Exposure Time	Log Reduction Before HS	Log Reduction After HS	Difference	Log Reduction Before HS	Log Reduction After HS	Difference	Log Reduction Before HS	Log Reduction After HS	Difference	Mean Difference†
B. anthracis	4 min	4.54	5.20	-0.66	4.59	5.23	-0.64	4.63	5.23	-0.60	-0.63
B. subtilis	190 min	4.67	5.53	-0.86	4.63	5.88	-1.25	4.93	5.53	-0.60	-0.90
C. sporogenes	20 min	3.15	4.02	-0.86	3.33	3.94	-0.61	3.22	3.83	-0.61	-0.69

Abbreviations: HS = Heat Shock. All calculations were made using the most reliable dilution plated.

*Data represents complete kill. Actual log reduction may be greater.

† Negative values indicate further inactivation of spores by heat shock. Positive values indicate resuscitation.

Table 4. Mean Differences in Log Reduction Values of Disinfectant-Treated Spores in Suspension Following Heat Shock (From Statistical Analysis*)

PeraDox™†	Exposure Time	Mean Difference‡	Standard Error	p§
B. anthracis	3.5 min	-1.3338	0.07157	< 0.0001
B. subtilis	1 min	0.8897	0.1061	< 0.0001

PeraDox Ultra™†	Exposure Time	Mean Difference	Standard Error	p
B. anthracis	45 sec	-1.5452	0.09206	<0.0001
B. subtilis	15 sec	0.8699	0.09445	< 0.0001

CIDEX™	Exposure Time	Mean Difference	Standard Error	p
B. anthracis	4 min	-0.5981	0.09725	< 0.0001
B. subtilis	190 min	-0.3238	0.06632	< 0.0001
C. sporogenes	20 min	-0.4425	0.06569	< 0.0001

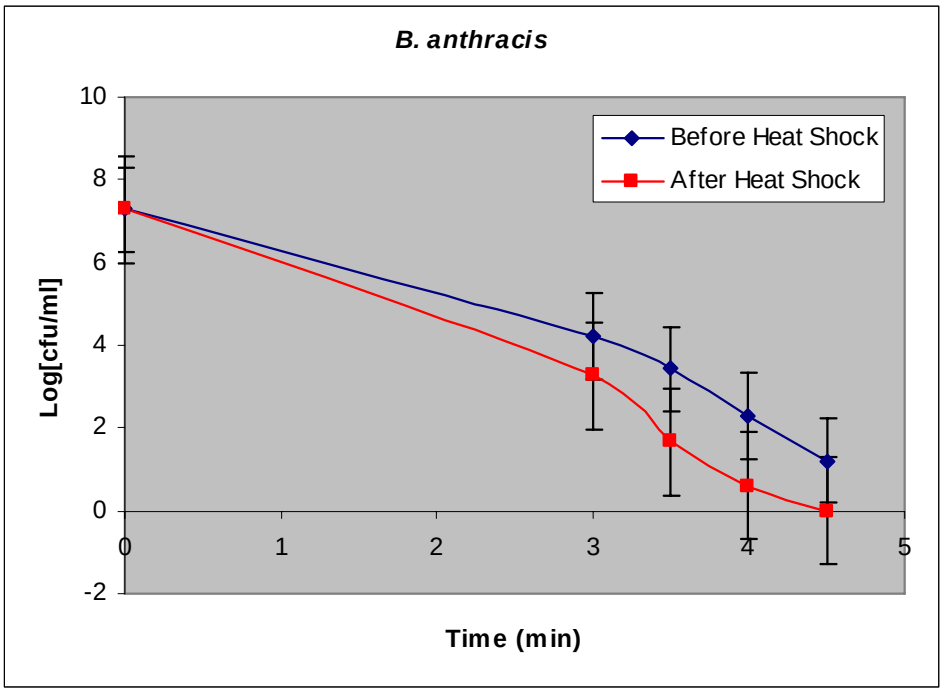
* Data from all three dilutions was used in the analysis.
† *C. sporogenes* was not calculated because complete kill was observed.
‡ Negative values indicate further inactivation of spores. Positive values indicate resuscitation.
§ F-test adjusted for multiple replications used.

Table 5. Effect of Immediate versus Delayed Heat Shock on the Resuscitation of Disinfectant-treated Spores Dried onto Carriers

Species	Disinfectant	Exposure Time	Delayed HS	Immediate HS	p*
B. subtilis	PeraDox	25 min	3/60	9/60	0.0627
C. sporogenes	PeraDox	25 min	1/60	1/60	0.7521
B. subtilis	PeraDox Ultra	15 min	2/60	7/60	0.0815
C. sporogenes	PeraDox Ultra	15 min	2/60	4/60	0.3397
B. subtilis	CIDEX	4 hr	3/60	5/60	0.3585
C. sporogenes	CIDEX	1 hr	3/60	4/60	0.5

Abbreviations: HS = Heat Shock. Results are based on the combined number of positive carriers (porcelain penicylinders and polyester suture loops).
* Fisher's exact test used.

A



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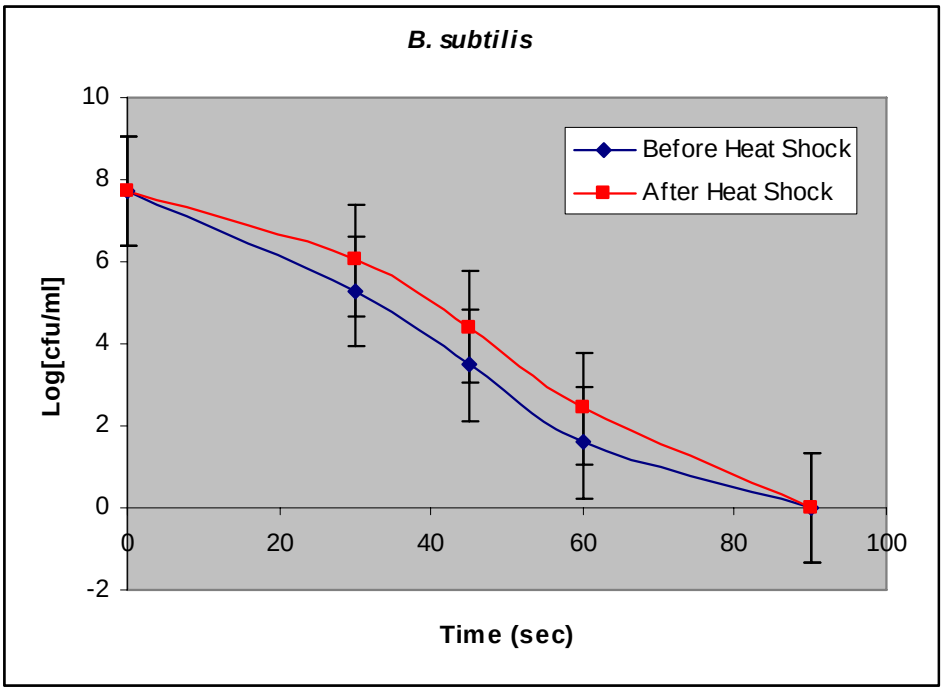
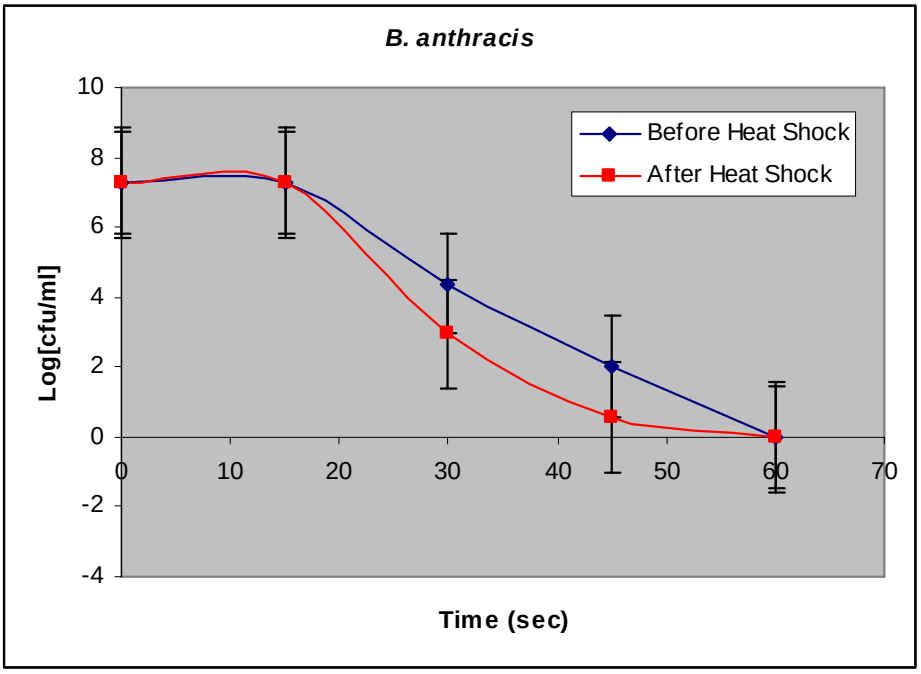


Figure 1. Kill kinetics of PeraDox™ on spores of (A) *B. anthracis* and (B) *B. subtilis* in suspension. Kinetics of *C. sporogenes* kill are not represented because complete kill was observed in 15 sec. Values are means from three replications. Error bars indicate standard errors.

A



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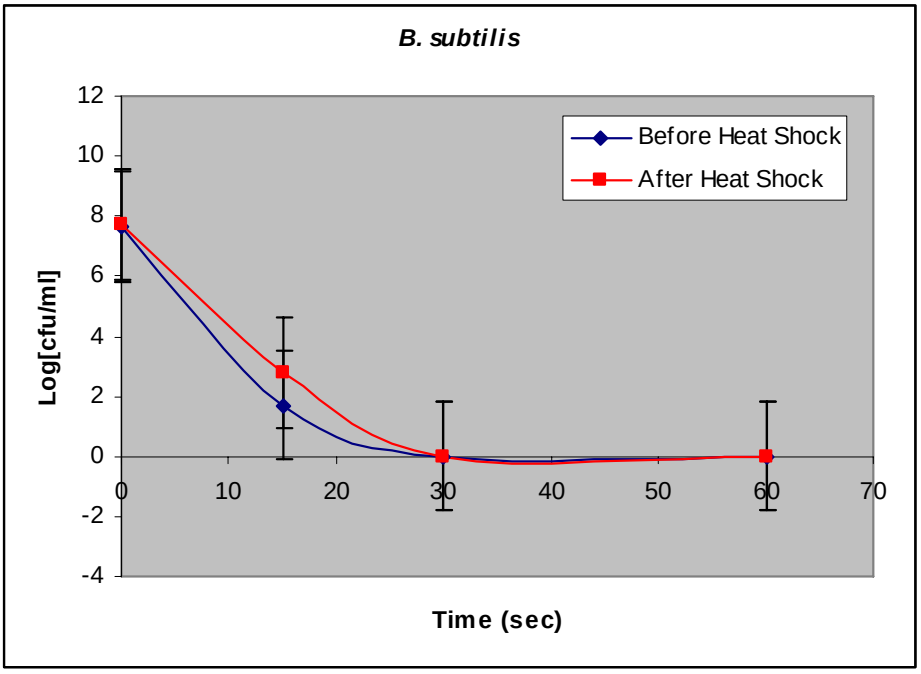
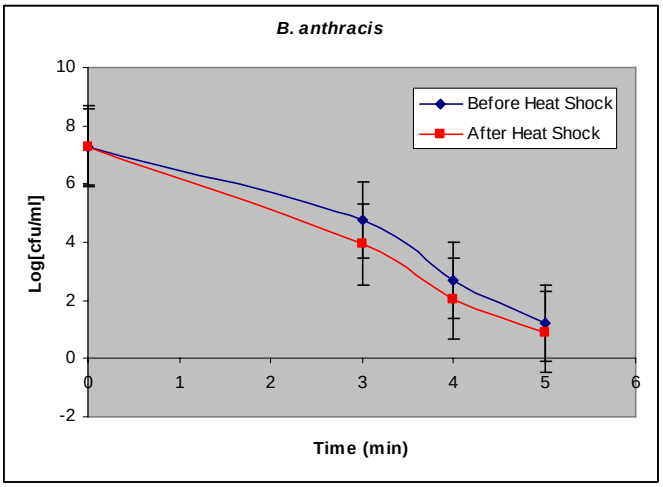


Figure 2. Kill kinetics of PeraDox Ultra™ on spores of (A) *B. anthracis* and (B) *B. subtilis* in suspension. Kinetics of *C. sporogenes* kill are not represented because complete kill was observed in 15 sec. Values are means from three replications. Error bars indicate standard errors.

A



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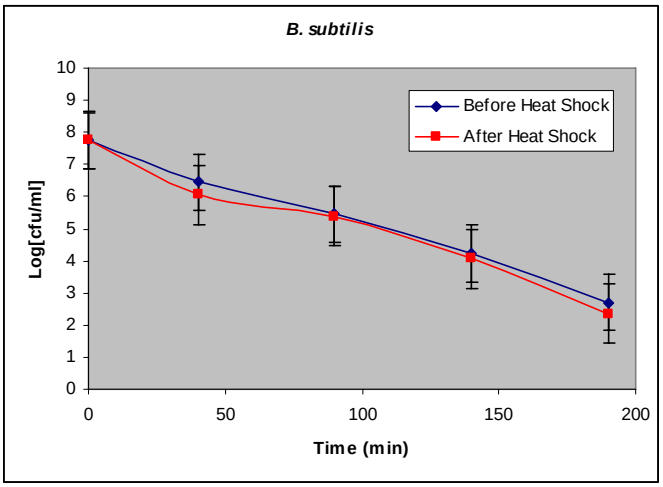

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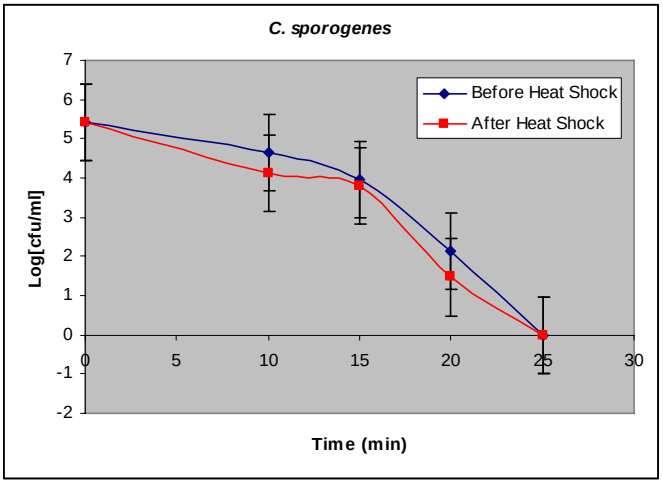
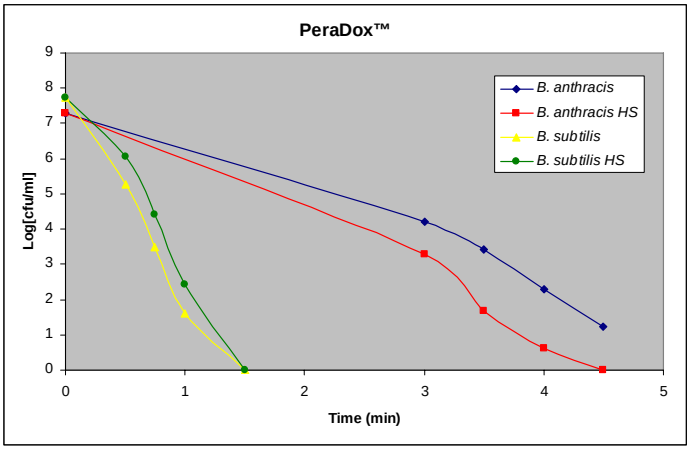


Figure 3. Kill kinetics of CIDEX™ on spores of (A) *B. anthracis*, (B) *B. subtilis*, and (C) *C. sporogenes* in suspension. Values are means from three replications. Error bars indicate standard errors.

A



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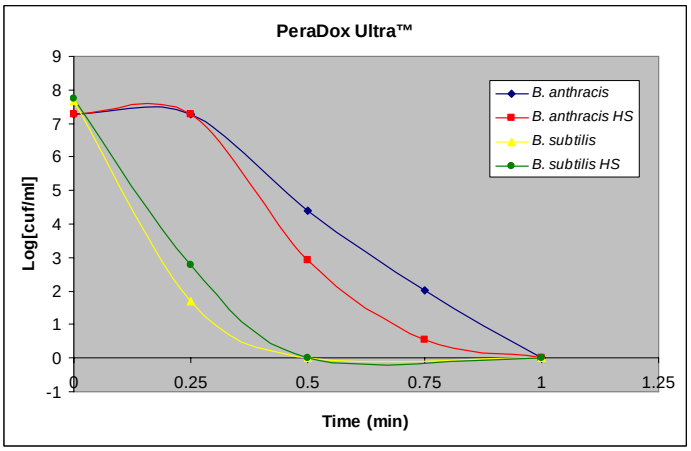

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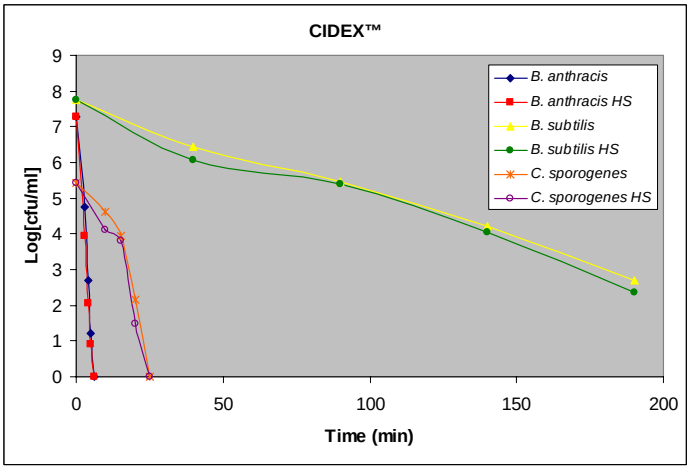
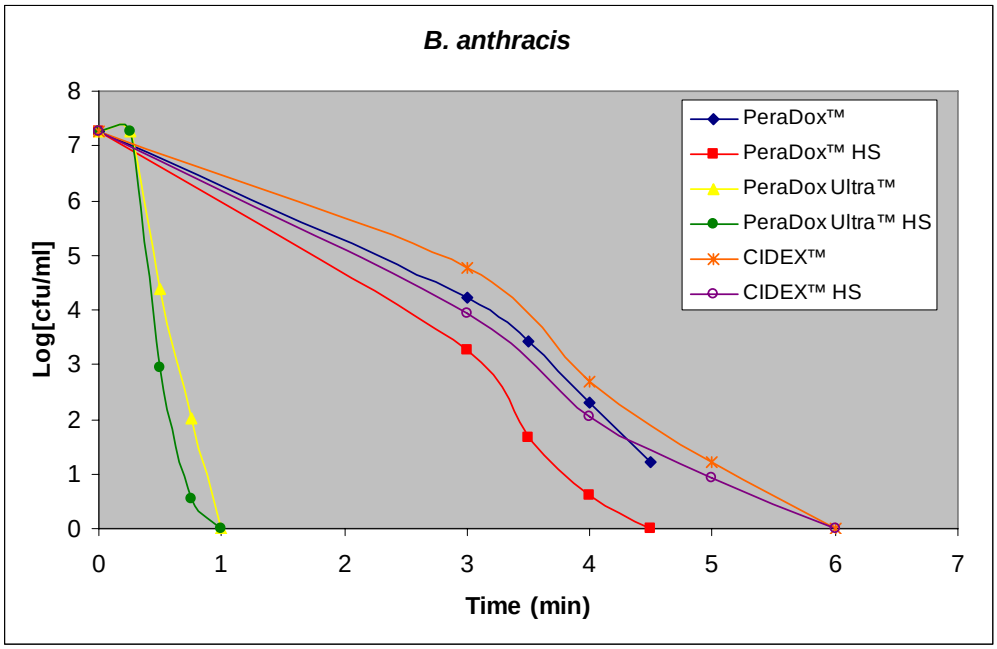


Figure 4. Kill kinetics of (A) PeraDox™, (B) PeraDox Ultra™, and (C) CIDEX™ on *B. anthracis*, *B. subtilis*, and *C. sporogenes* in suspension. Kinetics of *C. sporogenes* is not represented in (A) and (B) because complete kill was observed in 15 sec. Values are means from three replications.

A



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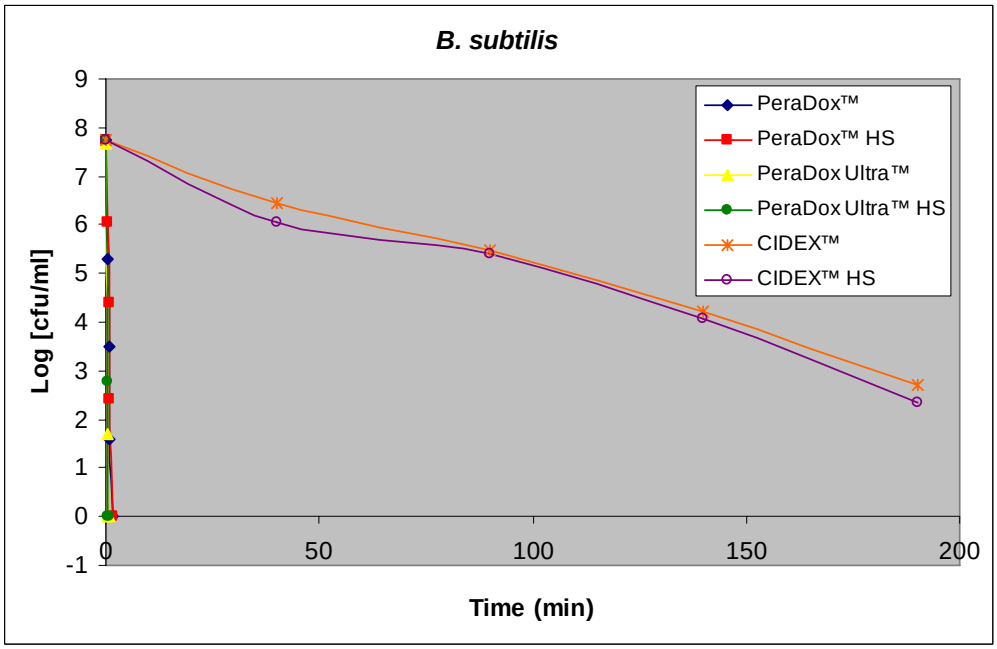

Figure 5. Kill kinetics of PeraDox™, PeraDox Ultra™, and CIDEX™ on spores of (A) *B. anthracis* and (B) *B. subtilis* in suspension. Complete kill was observed in *C. sporogenes* in 15 sec for both PeraDox™ and PeraDox Ultra™. Values are means from three replications.

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