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The Effects of Decontaminant Residue on the Viability of *Bacillus* Spores during Wipe Sample Storage

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Abstract

Clearance sampling following a biological terror incident potentially necessitates samples being collected from surfaces recently treated with decontaminant. The impact of residual decontaminant co-collected with surviving biologicals is currently unknown. The purpose of this study was to assess whether residues remaining on non-porous surfaces following decontamination impact estimates of surface contamination. Two experimental approaches were used to determine if agent viability within wetted wipe samples (post-collection) is affected by the presence of pH-adjusted bleach residues, and therefore impacts the quantitative determination of *Bacillus* spore recovery. Results indicated that following sample storage (22°C, overnight) that determined number of colony forming units (CFU) were not statistically different for positive controls and samples containing dry decontaminant residue. These data are necessary for interpretation of post-bioterror or other contamination incident sampling results, and support current use of wetted wipes in non-porous surface sampling protocols for clearance following liquid decontamination activities.

Keywords: Decontamination; Sporicide; Anthrax; *Bacillus anthracis*; Biological agent; Clearance sampling

Introduction

Since the Anthrax attacks of 2001, there has been an increased awareness of bioterrorism and of the capabilities necessary to recover rapidly from such incidents. Following an attack, environmental sampling methods are used to characterize the spatial extent and magnitude of contamination, and verify that decontamination procedures were successful [1,2]. To reduce the risk of post-incident exposures, it is important that decisions regarding building clearance be based upon robust sampling and analysis procedures that are validated and well-characterized [3,4].

To date, numerous studies have reported on the collection efficiencies of various surface sampling methods for spores of *Bacillus anthracis* or its surrogates [1, 5-11]. Others have investigated the effects of storage conditions on bioterror sample integrity [12]. However, none have addressed the potential negative bias associated with co-collection of decontaminant residue that may be present on surfaces during post-decontamination (i.e., clearance) sampling. Evaluation of sample storage and transport methods, under conditions realistic of real-world scenarios, is necessary to determine method performance, overall recovery efficiencies, and to demonstrate sample integrity [13,14].

The purpose of this study was to experimentally assess whether the number of *Bacillus* spores determined by culture analysis from wetted wipe sampling of non-porous surfaces may be affected by the presence of decontaminant residue. Such residues are likely to occur following surface treatment with liquid decontaminants such as pH-adjusted bleach, a sporicidal liquid previously used for *B. anthracis* decontamination [15-17]. Determining the effects of decontaminant residues on sampling and analysis results (i.e., agent viability within wetted wipe surface samples after collection) and demonstrating sample integrity following collection is highly important, as estimates of decontamination efficacy and decisions on clearance may be impacted. The current study is the first the authors are aware of that provide empirical data suggesting no biasing effects on sample viability during storage, from co-collection of dry decontaminant residues.

Materials and Methods

Bacterial spore preparation

Spores of *Bacillus atrophaeus* (ATCC 9372; formerly *B. subtilis* var. *niger* and *B. globigii*) [18] were used as surrogates for the biological agent *B. anthracis*. Spore preparations were obtained from the US Army Dugway Proving Ground (Utah), and have been described previously [5]. These spores were prepared specifically for use as a *B. anthracis* surrogate during surface sampling studies [5]. *B. atrophaeus* is commonly used as a surrogate for *B. anthracis* during decontamination studies [19]. Powdered spores were loaded into metered dose inhalers (MDIs) by the US Army Edgewood Chemical Biological Center (ECBC) according to a proprietary protocol. The MDIs provide a consistent dose of ~1×10^8 aerosolized spores per actuation. For liquid inoculations, the same preparation of *Bacillus atrophaeus* was suspended in a volume of phosphate buffered saline with 0.05% Tween 20 (PBST) (Sigma Aldrich, St. Louis, MO) to produce a 1 x 10^7 Colony Forming Units (CFU) ml^{-1} suspension.

Preparation of Material Coupons

Stainless steel (16-gauge, 304 stainless; Dillon Supply, Raleigh, NC) was used as a representative non-porous surface material, and was cut into 35.6 cm by 35.6 cm coupons from larger pieces of stock material. Coupons were sterilized by subjecting them to a one hour gravity autoclave cycle at 121°C and 103 kPa. Prior to testing, coupon sterility

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was confirmed by swab sampling one coupon from each sterilization batch, streaking the swab onto tryptic soy agar plates (TSA; Difco, Franklin Lakes, NJ) and incubating plates at 35 ± 2°C for 18-24 hours.

Decontaminant residue

Typically, surface decontamination procedures involve spraying sporicidal liquids onto surfaces, maintaining surface wetness over a predetermined contact time, and allowing the surfaces to dry before sampling (occasionally a rinse step precedes the drying step). For the purposes of this study, spraying decontaminant onto surfaces would not have resulted in consistent amounts of residue applied across replicate test samples. For these reasons, the maximum volume of liquid repeatably added (by pipette) to test coupons without spilling from the sides was determined and utilized for decontaminant application. This represents a worst-case scenario with regards to the amount of decontaminant residue present on non-porous surfaces. For all tests, 7.5 ml of freshly-prepared pH-adjusted bleach was added to the surface of each horizontally-oriented coupon and distributed evenly across the surface using a sterile cell spreader. An equal number of control coupons were wetted with the same volume of sterile distilled water (SDW). Coupons were either allowed to dry completely overnight at room temperature (~21°C) or utilized immediately after wetting to demonstrate sporicidal potential before drying.

The pH-adjusted bleach was prepared as described previously [20] i.e., one part Clorox Bleach (Clorox Corp., Oakland, CA) was diluted with eight parts deionized water and one part 5% (v/v) acetic acid (Fisher Scientific, Pittsburgh, PA; Part# 13025). The pH was adjusted to 6.5–7.0 with 5% acetic acid, and the free available chlorine content was adjusted to 6000–6800 ppm with deionized water after preparation. The pH-adjusted bleach was used within three hours of preparation.

Coupon inoculation

The effects of bleach residue on spore viability within wipe samples were determined using two different approaches. Both approaches involved adding viable spores to surfaces or surface samples after the addition or collection of decontaminant residues. While this order of addition is opposite to that of actual decontaminations (spores added to decontaminants), it was necessary as achieving a repeatable and precise number of viable spores after treatment with decontaminant is challenging. The first approach (Approach #1) utilized aerosol-based inoculation of coupons on the day after pH-adjusted bleach (or SDW) was applied to the coupon surface. For this approach, coupon surfaces (with residue) were inoculated with approximately 1×10^8 spores by an aerosol method described previously [21,22]. The targeted minimum recovery from positive control samples was 1.0×10^6 spores. Following the 18-24 hours necessary for aerosolized spore deposition, coupons were wipe-sampled according to the procedures outlined below. This approach offered the most realistic simulation of a real-world situation, as the decontaminant residue and the spores were in direct contact on the material surface, and were co-collected.

The second approach (Approach #2) involved inoculation of wipes with a liquid inoculum, following their use to sample non-inoculated coupons containing pH-adjusted bleach or SDW residue. This approach offered a more repeatable starting titer within the wipes. The inoculum for liquid-based wipe inoculations was 5×10^6 spores dispensed in 0.5 ml from a 1×10^7 CFU ml^{-1} suspension, directly onto the wipe. The target recovery from positive controls was 1×10^6 CFU.

Surface sampling

In all tests, sampling of coupon surfaces was accomplished using gauze wipes (Kendall Versalon 8042, Mansfield, MA) according to the methods described by the US Centers for Disease Control [23]. Three of the most commonly used wipe pre-collection wetting agents [23], Neutralizing Buffer (NB; Hardy Diagnostics K105, Santa Maria, CA), PBST, and SDW, were each evaluated for their effect on recovery of spores from wipes with and without decontaminant residue. Each gauze wipe was wetted with 2.5 ml of wetting agent prior to their use in tests (note: CDC instructions indicate to add 5 ml of wetting agent to one package of sterile gauze, each package contains 2 gauze wipes).

Initially, consistent with previous post-decontamination sampling strategies [17], sampling of coupons was conducted the day following the application of the decontaminant such that all surfaces were completely dry. Subsequently, tests were repeated using (Approach #2) to determine the effects on viability within wipes if surface sampling were to be conducted immediately following decontamination activities. In these tests, wipes were used to sample surfaces containing liquid SDW or pH-adjusted bleach remaining on the coupon surface. Consistent with (Approach #2), wipes were spiked with viable spores after being used to surface sample residue-seeded coupons. During all tests, wipes were stored in 50 ml conical tubes at room temperature (~22°C) for 18-24 hours after sample collection (Approach #1) or wipe inoculation (Approach #2).

Recovery of spores from wipe samples

Spores were extracted from the wipes by adding 20 ml PBST to each tube, then agitating the tubes using a vortex mixer (set to maximum rotation) for 2 minutes in 10 second intervals. Undiluted extracts and 10-fold serially-diluted extracts (in PBST) were spread-plated onto TSA. Plates were incubated at 35 ± 2°C for 18-24 hours and CFU were enumerated. When fewer than 30 CFU were detected on plates, the remainder (1 ml and ~19 ml aliquot) of the extract was analyzed by filtration through 0.2 µm pore-size filters (Nalgene, Rochester, NY), and placing filters onto TSA plates followed by incubation at 35 ± 2°C for 18-24 hours. The CFU counts from these plates were used to calculate recovery in these circumstances.

Recovery (total CFU) was determined for each control (SDW residue) and experimental sample (pH-adjusted bleach residue). Comparisons of control and experimental recoveries were used to assess impacts of residue on sample viability post-collection. For each experimental condition, the average recovery value was calculated using five replicate samples. Recovery data were compared between pH-adjusted bleach residue and SDW residue samples for each test using the Student's t-test. In addition, the effects of wetting agent on recovery were evaluated by ANOVA.

Results

Recoveries from all wipe samples, with dry SDW or dry decontaminant residue, were greater than the targeted minimum 1.0×10^6 CFU. No CFU were recovered from blank, sterility check, or negative control samples.

Recoveries during (Approach #1) (aerosol inoculation of coupons) were between 1.09×10^6 and 1.22×10^7 CFU. The presence of pH-adjusted bleach residue had no effect on recoveries, regardless of wipe wetting agent (t-test, all $p \geq 0.07$) (Figure 1). Interestingly, recoveries from wipes wetted with neutralizing buffer were significantly lower than those wetted with PBST or SDW (ANOVA, $p \leq 0.001$), for both positive control and test samples.

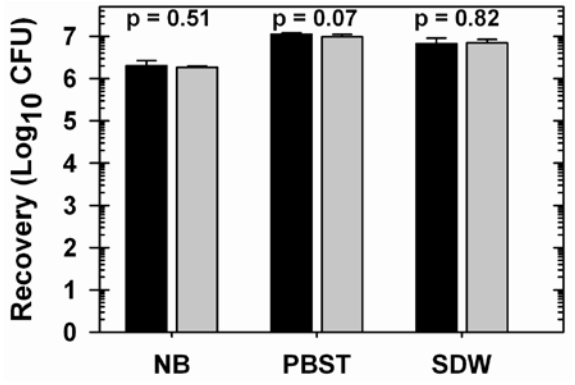

Figure 1: Bacillus spore recoveries from wipe samples during Approach #1 with Dry Residues

Bar heights indicate recoveries from wipes used to collect aerosol-deposited spores (Approach #1), on coupon surfaces with dry bleach (black bars) or sterile distilled water (gray bars) residue. NB indicates wipes pre-wetted with neutralizing buffer, PBST indicates wipes pre-wetted with phosphate buffered saline with 0.05% Tween 20, and SDW indicates wipes pre-wetted with sterile distilled water. Data are presented as the mean recovery (CFU) ± one standard deviation.

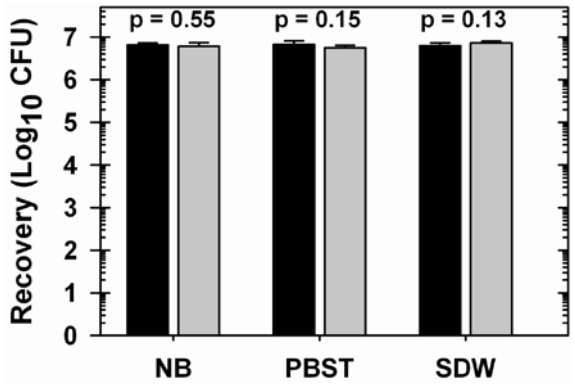

Figure 2: Bacillus spore recoveries from wipe samples during Approach #2 with Dry Residues.

Bar heights indicate recoveries from wipes inoculated with a liquid suspension of spores (Approach #2) following sampling of coupon surfaces with dry bleach (black bars) or sterile distilled water (gray bars) residue. NB indicates wipes pre-wetted with neutralizing buffer, PBST indicates wipes pre-wetted with phosphate buffered saline with 0.05% Tween 20, and SDW indicates wipes pre-wetted with sterile distilled water. Data are presented as the mean recovery (CFU) ± one standard deviation.

Similarly, recoveries during (Approach #2) (liquid inoculation of wipes) were between 4.29×10^6 and 8.13×10^6 CFU for samples collected from coupons with dried decontaminant or SDW residue (Figure 2). For each wetting agent, recoveries were similar for samples with pH-adjusted bleach residue and SDW residue (t-test, all $p \geq 0.13$). Further, recoveries for all samples during (Approach #2) (dry residue tests) were not significantly different (ANOVA, $p \geq 0.45$).

For those samples collected during (Approach #2) from surfaces where the decontaminant was not allowed to dry (i.e., wet residue), all positive control sample (SDW residue) recoveries were above the 1×10^6 CFU target. In contrast, fewer than 22 CFU were recovered from all wet pH-adjusted bleach residue samples, regardless of the wipe wetting agent (Figure 3). Mean recoveries from these samples were 4.4 ± 9.8 CFU, 1.2 ± 2.0 CFU, and 0.24 ± 0.54 CFU for SDW-, NB-, and PBST-wetted wipes, respectively.

Discussion

Many factors can influence recoveries when sampling surfaces for biological agents. Among them are sampling media type used [1,24,25], surface types sampled [26], and technique of the sample collector [27]. An often overlooked factor that may influence sample viability and recovery from post-decontamination samples is the presence of decontaminant residues on the surfaces sampled.

Previously, it was unknown whether dried pH-adjusted bleach (or other liquid decontaminant) residues may impact sample viability when co-collected with biological agent and rehydrated with pre-wetted sampling devices (i.e., wipes or sponge sticks). For instance, does co-collection of biological agent and decontaminant residues from surfaces confound sample analysis and/or result in agent inactivation within sample media following collection, thereby resulting in underestimation of remaining contamination on surfaces? The current study conducted a set of experiments to test the null hypothesis that recoveries from wipes containing decontaminant (pH-adjusted bleach) residue are no different than that of wipes with control (SDW) residues.

It is not possible to reproducibly administer a surface decontamination process in which a suitably repeatable and precise number of viable spores survive treatment and are collected by surface sampling media. Accordingly, a test method was developed that repeatably created surfaces with decontaminant (test samples) or SDW (positive control) residues. Two approaches were then used to spike surfaces (Approach #1) or wipe samples (Approach #2) with a precise amount of viable spores. A worst-case scenario of decontaminant residue was created by adding, to horizontally-oriented stainless steel coupons, a volume of pH-adjusted bleach (or SDW for positive controls) that pooled over the entire coupon surface. For initial tests, all coupons were allowed to dry overnight, consistent with procedures historically used for sampling following a *B. anthracis* contamination incident [17]. The two approaches described above were then used to determine if the pH-

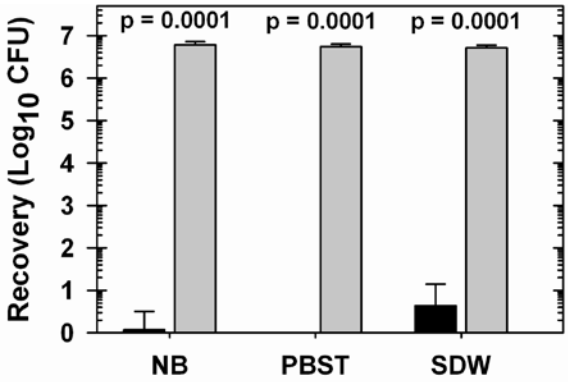

Figure 3: *Bacillus* spore recoveries from wipe samples during Approach #2 with Wet Residues

Bar heights indicate recoveries from wipes inoculated with a liquid suspension of spores (Approach #2) following sampling of coupon surfaces with wet bleach (black bars) or sterile distilled water (gray bars) residue. NB indicates wipes pre-wetted with neutralizing buffer, PBST indicates wipes pre-wetted with phosphate buffered saline with 0.05% Tween 20, and SDW indicates wipes pre-wetted with sterile distilled water. Data are presented as the mean recovery (CFU) ± one standard deviation.

Inoculation Method	Inoc.	Residue	Dry or Wet Residue	Wipe Wetting Buffer[a]	Test Sample Replicates	Negative Control Replicates
Aerosol[b]	1 x 10^8	dH_2O	Dry	NB	5	1
				PBST	5	1
				SDW	5	1
		Bleach	Dry	NB	5	1
				PBST	5	1
				SDW	5	1
Liquid[c]	5 x 10^6	dH_2O	Dry	NB	5	1
				PBST	5	1
				SDW	5	1
			Wet	NB	5	1
				PBST	5	1
				SDW	5	1
		Bleach	Dry	NB	5	1
				PBST	5	1
				SDW	5	1
			Wet	NB	5	1
				PBST	5	1
				SDW	5	1

[a] - NB, neutralizing buffer; PBST, phosphate buffered saline with 0.05% Tween20; SDW, sterile distilled water
[b]- coupons inoculated by aerosol deposition after the addition of pH-adjusted bleach or SDW residue (Approach #1)
[c]- wipes liquid-inoculated following sampling of coupon surfaces containing pH-adjusted bleach or SDW residue (Approach #2)

Table 1: Summary of Test Parameters.

adjusted bleach residue had a negative effect on viable *Bacillus* spores when co-collected. Subsequent tests were conducted to determine the effects if surface samples were collected without a drying period between decontamination and sample collection (Table 1).

Our data indicate that even a worst-case amount of dry pH-adjusted bleach residue on non-porous surfaces has no effect on agent viability within (i.e., recovery from) wetted wipe samples. Both approaches yielded similar results, with no significant difference in recoveries from test samples (pH-adjusted bleach residue) and control samples (SDW residue). It is important to note that the purpose of this study was to assess the impact of decontaminant residues from a non-porous surface on agent viability within wetted wipe samples. It is unknown how these results translate to porous surfaces or other sampling media.

During the current study, samples were held at room temperature (~21°C) for 18-24 hours between collection (Approach #1) or inoculation (Approach #2) and sample analysis, simulating potential storage conditions of samples collected following a biological incident. Such real-world samples would be shipped for next day delivery, thus likely analyzed the day after collection. The effects of longer storage times are not known, however we speculate that the effects will be minor as this study demonstrated no effects for unbuffered (SDW wipe pre-wetting agent) storage conditions following exposure to a "worst-case" amount of decontaminant residue. Further, there was no detectable FAC within dry residue wipe sample extracts.

Considering the liquid-inoculated positive control samples (Approach #2) only, there was no difference (ANOVA, $p \geq 0.34$) in recoveries across the three wipe wetting agents. No difference was expected, as these wipe samples were directly spiked with spores following their use to sample sterile coupons with SDW residue. Since recoveries from controls were not significantly different, we can assert that the test samples were all inoculated with an equivalent amount of agent, and we can therefore compare their means. Similarly, there was no detectable difference in recovery from these test samples across the three wetting agents (ANOVA, $p \geq 0.81$). These results indicate that dried pH-adjusted bleach residue has no detectable sporicidal activity or confounding impact on viable spore recovery or culture analysis, as wipes wetted with SDW would have no quenching or buffering capability against any such activities. If residues possessed any sporicidal activity or confounding impact on sampling or analysis, recovery from SDW-wetted wipes would have been expectantly lower than that of the wipe samples wetted with buffer (PBST and NB). This was not the case.

After obtaining the results discussed above, indicating that dried decontaminant residue has no effect on agent viability post-collection, we conducted another set of tests whereby coupons were sampled immediately after the addition of the pH-adjusted bleach or SDW. Approach #2 (liquid inoculation after residue collection) was used to determine whether wet residual decontaminant has an effect on agent recovery. These data show that recoveries from wipes containing wet pH-adjusted bleach residues were significantly lower than those with wet SDW residue (t-test, all $p \leq 0.0001$) (Figure 3). Interestingly, there was no difference in recoveries between the wetting agents for these test samples (ANOVA, $p \geq 0.51$). This was surprising since NB was specifically designed to neutralize quaternary ammonia and chlorine-containing disinfectants post-sample collection. However, it was apparent that the amount of NB used to pre-wet the wipes was insufficient to neutralize the sporicidal activity of the pH-adjusted bleach collected during sampling. Recovery from the NB-wetted wipe samples was not significantly higher than that of the other two wetting agents. While NB-wetted wipes may quench biocidal activities when a small amount of residual decontaminant is collected, these data suggest that the quenching ability of NB can be overwhelmed when sampling pH-adjusted bleach-wetted surfaces. A larger volume (10 ml) of NB is used to pre-wet cellulose sponge wipe samples and may afford more quenching capacity, however our testing was specific for gauze wipe-based sampling.

During (Approach #1) testing, recoveries from SDW residue samples for NB-wetted wipes were significantly lower than that of SDW- or PBST-wetted wipes. While our data suggest that recovery efficiency for wipes wetted with NB may be lower than that of the other two wetting agents, inoculations for each wetting agent test occurred

on different days. Although the techniques utilized to inoculate the samples were identical between test days, it is impossible to rule out an unknown environmental factor that could have caused the disparity in recoveries. We therefore refrain from making conclusions on the reason behind the observed lower recoveries with NB-wetted wipes. In contrast, for each wetting agent tested, inoculations for samples of both residue types (SDW and pH-adjusted bleach) were conducted simultaneously, therefore comparisons of recoveries from samples with decontaminant and control residues are valid. It is important to note that the only decontaminant tested in this study was pH-adjusted bleach. Recoveries from porous surfaces, using other sampling methods, or other decontaminant residues may have been different from those reported here.

In summary, these data support the use of the current CDC-recommended wetted wipe procedure for sampling of dry non-porous surfaces following liquid-based surface decontaminations, as no effects on sample viability were observed when co-collected with dry pH-adjusted bleach residue. It is important to note that the only decontaminant tested in this study was pH-adjusted bleach. In addition, recoveries from porous surfaces, using other sampling methods, or other decontaminant residues may have been different from those reported here. Nonetheless, this study fills an important knowledge gap surrounding the potential bias of post-decontamination sampling. The data presented herein can be used to increase confidence in clearance sampling results.

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