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REVISED: Optimization of Sample Processing Protocol for Recovery of *Bacillus anthracis* Spores from Soil

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Abstract

Following a release of *Bacillus anthracis* spores into the environment, there is a potential for lasting environmental contamination in soils. There is a need for detection protocols for *B. anthracis* in environmental matrices. However, identification of *B. anthracis* within a soil is a difficult task. Processing soil samples helps to remove debris, chemical components, and biological impurities that can interfere with microbiological detection. This study aimed to optimize a previously used indirect processing protocol, which included a series of washing and centrifugation steps. Optimization of the protocol included: identifying an ideal extraction diluent and evaluating variation in the number of wash steps, variation in the initial centrifugation speed, sonication and shaking mechanisms. The optimized protocol was demonstrated at two laboratories in order to evaluate the recovery of spores from loamy and sandy soils. The new protocol demonstrated an improved limit of detection for loamy and sandy soils over the non-optimized protocol with an approximate matrix limit of detection at 14 spores/gram of soil. There were no significant differences overall between the two laboratories for either soil type, suggesting that the processing protocol will be robust enough to use at multiple laboratories while achieving comparable recoveries.

Keywords

Bacillus anthracis; spores; sand; loam; soil; indirect processing

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1. Introduction

The etiological agent of anthrax, *Bacillus anthracis,* is a gram-positive spore forming bacteria that is naturally found in many soil environments and that has spores that can persist in soil for many years (Graham-Smith, 1930, Lewis, 1969, Lindeque and Turnbull, 1994, Manchee et al., 1981, Purcell et al., 2007, Sinclair et al., 2008, Van Ert et al., 2007, Wilson and Russell, 1964). *B. anthracis* spores were mailed to members of Congress and the news media in 2001, contaminating many facilities in the Washington D.C. area and Florida (GAO, 2012). Following the release of *B. anthracis* spores, there is a potential for lasting environmental contamination (Turnbull, 2008) and public health risk as spores can be transported into a building following an outdoor release (Van Cuyk et al., 2012), transported within ventilation systems (Sextro et al., 2002), and transported from inside a building to areas outside a building following an indoor release (Silvestri et al., 2015b). Site characterization and remediation activities following an intentional indoor release might need to consider outdoor soil as a potential exposure pathway due to indoor-to-outdoor spore transport. There is a need for a method for detecting *B. anthracis* in soil with lower limits of detection than are currently available. However, identifying *B. anthracis* within a soil sample is a difficult task.

The difficulties with soil detection methodologies are numerous. Soil is a complex matrix containing many microorganisms and an abundance of microbial activities (Delmont et al., 2011, USDA, 1999), which can interfere with detection assays. The chemistry involved in downstream molecular assays can be affected by soil constituents such as organics and humic acids (Balestrazzi et al., 2009, Beyer et al., 1999, Cheun et al., 2003, Dineen et al., 2010, Gulledge et al., 2010, Robe et al., 2003, Sjostedt et al., 1997, Zhou et al., 1996). Also, unlike clinical samples, the density of the target microorganisms in environmental samples is not great. Without an appropriate soil sample processing protocol, the most sensitive detection assay will be ineffective.

Processing soil samples helps to remove debris, chemical components, and biological impurities that can interfere with microbiological detection. While multiple processing protocols have been developed either to separate spores from soil samples or to directly extract bacterial DNA prior to use of a detection assay, a universal sample processing protocol to separate, concentrate, and purify *B. anthracis* from the soil sample is needed (Lim et al., 2005). A recently published review of soil sample processing protocols discussed both direct and indirect processing protocols for soils contaminated with *B. anthracis* spores (Silvestri et al., 2015a). With indirect processing, spores are separated from soil and other organisms prior to analysis in order to purify and concentrate them within the final sample. It is, however, possible that spore loss prior to analysis might increase with such processing. With direct processing, bulk sample aliquots are utilized without first separating spores from soil particles. Direct processing might conclude with bulk DNA extracted for a molecular assay or with the culturing of *B. anthracis* using selective media. Direct processing of soil via culture on selective media is significantly hindered by the presence of other microorganisms in the soil, such as close relatives of *B. anthracis* (Kuske et al., 2006). The selection of either direct or indirect sample processing will be dependent on the downstream analysis and the intended use of the data (Lindahl and Bakken, 1995).

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A recent study, using a direct sample processing protocol, looked at transportation of *B. atrophaeus* subsp. *globigii* spores from a contaminated building to the outside environment (Silvestri et al., 2015b). During the study, petri dishes filled with 45 g sterile sand were placed outside an experimentally contaminated building to simulate the outside environment (Silvestri et al, 2015b). For processing, the sand samples were shaken in a centrifuge bottle after adjusting the volume to 125 mL with phosphate-buffered saline supplemented with TWEEN®-20 (PBST). After washing the sand several times by centrifugation, the entire pellet was used to extract DNA for subsequent quantitative polymerase chain reaction (qPCR) analysis (Silvestri et al., 2015b).

For this study, the authors believed the limit of detection of the protocol used in the above study (Silvestri et al, 2015b) could be improved upon by looking at certain portions of the protocol. For example, one variable considered was the diluent used to wash the samples. Polyphosphate has been used in the hydrometer method of soil particle sizing to disperse soil particle aggregates (Kettler et al., 2001) and 2% polyphosphate has been used to disperse and remove bacterial cells from lake sediment particles (Poté et al., 2010). However, a number of studies also investigated the potential negative affects of various polyphosphate formulations to both vegetative bacterial cells and to bacterial spores (Akhtar et al., 2008, Borch and Lycken, 2007, Lee et al., 1994, Moon et al., 2011, Obritsch et al., 2008, Post et al., 1963). For example, a polyphosphate concentration of 0.05% to 0.1% was found to inhibit *Bacillus cereus* spore outgrowth, while 1% polyphosphate was found to be sporicidal to *B. cereus* and to reduce viable spore plate counts from 2×10^6 to less than 1×10^5 colony forming units (CFU)/ml in a liquid medium (Maier et al., 1999). Sodium hexametaphosphate and detergents such as sodium dodecyl sulfate (SDS) or Triton X-100 were reported to have synergistic antibacterial activity against gram negative bacteria when combined in solution (Vaara and Jaakkola, 1989). This activity could be a benefit if *B. anthracis* spores are unaffected by reducing other background bacteria in the sample, or inhibitory if such a combination of polyphosphate and a detergent has a sporicidal effect. Phosphate buffered citrate is another diluent that has also been used successfully as a diluent to measure viral and bacterial abundance in several soil types (Williamson et al., 2005).

Other optimization steps for the current study included evaluating varying the number of wash steps, the effect of sonication on results, varying the initial centrifugation speed, and shaking techniques. A two laboratory demonstration of the final optimized protocol was also conducted using both sterile loam and sandy soil seeded with *Bacillus anthracis* Sterne (BaS) strain spores in conjunction with a culture assay for a preliminary assessment of reproducibility. Sterile soil was used to eliminate background microorganism to include other target *Bacillus* spp. spores, which would have made quantitation much more difficult. This paper describes the optimized soil processing protocol and results of the evaluation.

2. Methods

2.1 Organisms

Bacillus anthracis Sterne strain was obtained from Laura Rose at the Centers for Disease Control and Prevention (CDC, Atlanta, GA). Spores were prepared in a broth sporulation medium (Coroller et al., 2001) incubated at 35°C with agitation on a rotary shaker for 5 or

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more days. Spores were purified by differential centrifugation using RenoCal-76® (Bracco Diagnostics, Princeton, NJ) and repeating washing and centrifugation cycle three times, as previously described (Nicholson and Setlow, 1990). Purified spore preparations were examined using phase contrast microscopy, which showed <0.1 % vegetative cells. Purified spores were stored in 40% (vol/vol) ethanol/water at 5°C.

2.2 Titer of Stock Spore Suspension

Spore counts in suspensions used to inoculate soils were determined by serial dilution and plating on 5 replicate Trypticase® Soy Agar (TSA) plates. Stock spore suspensions generally contained between 10^8 and 10^9 *B. anthracis* CFU per mL. *B. anthracis* spore suspensions were adjusted further by serial dilution to target concentrations and the titer was confirmed by replicate plating on TSA as above.

2.3 Preparation of Soil

Sterile Agvise Laboratories (Northwood, ND) high organic matter sandy loam soil (DU-L-PF, Pesticide free) and loamy sand soil (RMN-LS, 0-6”) were used for all experiments in this study. Agvise characterized sandy loam (DU-L-PF, Pesticide free) as being 60% sand, 36% silt, and 4% clay (12.4% organic matter) with a pH of 6.5 and loamy sand (RMN-LS, 0-6”) as being 85% sand, 6% silt, and 9% clay (containing 2.2% organic matter), with a pH of 5.9. Soils were sterilized by autoclaving in Pyrex® glass pans using a gravity cycle (45 minutes at 121 °C, 17 psi) with a 10 minutes drying time. Soil moisture content was measured before and after autoclaving. Soil moisture content was measured at 7.7 ±12% prior to autoclaving and 2.3 ±13% for sand after autoclaving. For loamy soil, the soil moisture content was 25 ±2% prior to autoclaving and 15.8 ±14% after autoclaving.

Soil was incubated at room temperature for 24 hours and then autoclaving was repeated. Two methods were used to test soil sterility. The first method included the use of solid agar plate medium. Approximately 1 g of soil was added to 9 mL phosphate buffered saline (PBS) and was vortexed for 30 seconds. One-hundred μL of the soil suspension was plated onto three low nutrient media Reasoner’s 2 Agar (R2A) plates and six high nutrient media TSA plates. The R2A plates and three of the TSA plates were incubated at room temperature (22-27 °C) for 7 to 10 days while the remaining three TSA plates were incubated at 35 °C for 3 days.

The second method used to test soil sterility included incubation in broth media followed by plating onto agar plates. Approximately 1 g of soil was transferred to three 9 mL Reasoner’s 2 Broth (R2B) and to each of six Trypticase Soy Broth (TSB) tubes. All tubes were vortexed for 30 seconds. The R2B tubes and three TSB tubes were incubated at room temperature for 7-10 days. The remaining three TSB tubes were incubated at 35 °C for three days. One-hundred μL from the R2B tube was inoculated onto R2A. One-hundred μL from each TSB tube was plated onto a TSA plate. The R2A plate inoculated with R2B and three TSA plates inoculated with TSB were incubated at room temperature (22-27 °C) for 7 to 10 days. The remaining three TSA plates inoculated with TSB were incubated under the same conditions as the broth tubes being tested for growth.

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2.4. Inoculation of Soil Samples

To inoculate samples, the stock BaS spore suspension was serially diluted to the requisite spore count in a sterile diluent referred to in this paper as “study diluent” and consisting of 0.01 M PBS, 0.01% Tween 80, and 0.001% antifoam A. For initial protocol development studies, inoculation consisted of a suspension of 994 spores in 4.5 mL diluent. *B. anthracis* spore concentrations investigated during the inter-laboratory study included 1, 5, 10, 25, and 50% of the positive control, which was targeted at 350 spores per gram soil.

Forty-five g aliquots of sterile soil were transferred aseptically into sterile 250 mL centrifuge bottles. Then, 4.5 mL of the respective experimental spore suspension was transferred to each 250 mL centrifuge bottle. The 4.5 mL inoculum was dropped onto the soil in 10 different places on the soil surface while rotating on an angle in order to distribute spores evenly throughout the sample. After decontaminating the exterior of the bottles, the samples were agitated by hand in order to further distribute the liquid containing the spores among the soil particles. Inoculated bottles were stored in a biosafety cabinet for 3 days with daily agitation to maximize spore distribution in samples. Negative controls for the inter-laboratory study consisted of 4.5 mL of sterile study diluent added to sterile 45g soil samples in 250 mL centrifuge bottles.

2.5 Preliminary Experiments

The protocol used for soil sample processing optimization consisted of adding 200 mL of diluent to a 45g soil sample inoculated with spores, shaking for 3 minutes, and settling for 15-30 minutes. The supernatant was then transferred to a 250 mL Sorvall® centrifuge bottle (Cat #03937) and centrifuged at 250 x *g* for 5 minutes to remove large particles. The supernatant was transferred to a new bottle and the sample was centrifuged at 5,900 x *g* for 35 minutes to pellet the spores. The pellet was resuspended in 25 mL diluent and transferred to a 50 mL centrifuge tube and centrifuged at 5,900 x *g* for 35 minutes to pellet the spores. The pellet was resuspended in 2.2 mL diluent and heat treated for one hour at 70°C. One-hundred and fifty uL was used for plating. Efforts to optimize the protocol used in the Silvestri et al. (2015b) study included selection of an extraction diluent, varying of the number of wash steps used, evaluation of the benefit of adding sonication to the protocol, varying the initial centrifugation speed, and an evaluation of mechanical versus hand shaking of samples. These efforts are briefly described below.

2.5.1 Extraction Diluent—The study diluent used in the previous study by Silvestri et al. (2015b) was compared to a diluent of 2% Tween 80 and a 4% polyphosphate test solution during two experiments to determine the concentration of polyphosphate that could be sporostatic or sporicidal to *B. anthracis*. BaS spore stock was diluted to 10^{-4} in the test solution and in the control (study diluent). The sample and control were split into two 5 ml volumes. One volume was incubated at room temperature for one hour and one volume for four hours, heat-treated for at least 15 minutes at 70 °C, then further diluted 1:10 in water (the control was diluted in study diluent) and 150 μL of both dilutions plated on quadruplicate TSA plates.

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Additional experiments were done to compare the study diluent to a diluent containing 1% Tween 80 with 2% hexametaphosphate (referred to as Spore Extraction Solution [SES]) to determine if the SES would disperse particles better than the study diluent used in the previous study by Silvestri et al. (2015b). Three samples were processed for each diluent. SES was prepared from stock solutions of 20% Tween 80 (50 mL) and 20% sodium hexametaphosphate (SHMP, 100 mL adjusted to a pH of 7.1-7.4) added to 350 mL Milli-Q® or Super-Q® water. A final concentration of 1% Tween 80 and 2% SHMP was achieved by adjusting the pH to 7-7.2 and the volume of the solution to 1.0 L. The solution was filter sterilized using Coming® 1,000 mL CA membrane bottle top filters (0.22 μm pore size) and stored at 4°C.

In a separate set of experiments, the SES was also compared to a diluent made up of phosphate buffered citrate. A total of four samples were processed for each of these two diluents in this set of experiments. Samples were processed as described above.

2.5.2 Wash Steps—Five washes of the sample were evaluated instead of just a single wash as described above. Experiments were run in triplicate and samples were spiked on the same day. Each wash consisted of three minutes of shaking with a solution of SES. Less solution is used for resuspension when doing multiple washes (~150 mL vs. 175 mL). Approximately 13,600 BaS spores were added per 45 gram sample. After the initial wash, 100 μL sample was used for spread plating, whereas after each subsequent wash, 200 μL of sample was used. The multiple wash step experiment was repeated for the first three washes.

2.5.3 Sonication—A sonication step was added to the protocol, following shaking of the sample for 3 minutes. Each wash consisted of three minutes of shaking with a solution of SES, 15 minutes for bleaching the exterior of the sample container, which was followed by three minutes of bath sonication (Bransonic® 32 Model Branson Ultrasonic Cleaner (Branson Ultrasonics Corp., Danbury, CT) operating at a frequency ~ 55 kHz).

2.5.4 Initial centrifugation speed and sample shaking—The initial centrifugation speed of 250 x *g* was compared to a centrifugation speed of 100 x *g*. Two experiments were performed for this variation of the protocol. Finally, manual shaking of samples was compared to mechanical shaking of samples using the New Brunswick Innova® 2100 Platform Shaker (Eppendorf, Hauppauge, New York). The shaker has two locking bars. The lids of the bottles were modified to allow for locking in the shaker. Six minutes was chosen as the shaking time (preset value on the shaker [l/10^{th} of an hour]). Shaking speeds of 350 and 425 rpm were evaluated.

2.6 Final Optimized Sample Processing Protocol for Laboratory Comparison

The final optimized protocol, which was developed based on the testing described above, is briefly described and is shown in Figure 1. A volume of 175mL SES was added to each 45 g sample (seeded) bottle. The soil was suspended by inverting each bottle repeatedly. Sample bottles were packed in a Nalgene® biotransport carrier (ThermoFisher Scientific, Waltham, MA, model number 7135-0001) which was placed and secured on a shaker (New Brunswick Scientific Platform Shaker [Model Innova 2100, Eppendorf, Hauppauge, New York]). The

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shaker was set for 20 minutes at 450 rpm. Bottles were then centrifuged at 100 x *g* for 5 minutes in a swinging bucket rotor to settle large, dense particles. The supernatant from each bottle was transferred to a new sterile 250 mL centrifuge bottle labeled Wash 1 and the bottle with original soil pellet was set aside. Each of the Wash 1 bottles was centrifuged at 5,900 x *g* for 30-35 minutes to pellet the spores. The resultant Wash 1 supernatant was removed and discarded. One-hundred and fifty mL SES was then added to the original sample bottle and the pellet was resuspended by inversion (Wash 2). The Wash 2 bottles were shaken for 20 minutes at 450 rpm as before. The Wash 2 bottles were then centrifuged for 5 minutes at 100 x *g*, and the Wash 2 supernatant was then added to the Wash 1 pellet. The bottles containing the Wash 1 pellet and Wash 2 supernatant were then centrifuged at 5,900 x *g* for 30 minutes to pellet the spores. The supernatant was removed from each bottle and discarded. Each pellet was transferred to its own sterile 50 mL Corning conical centrifuge tube after being resuspended in 15 mL SES by using a 10 μL Combi Loop® (Fischer Scientific, Pittsburgh, PA). Each 250 mL centrifuge bottle received a final wash with 10 mL of SES, which was then added to the respective 50 mL centrifuge tube. The volume in each 50 ml tube was adjusted to 45 mL with additional SES before being centrifuged at 5,900 x *g* for 20 minutes. The supernatant was removed and discarded. At this point pellets in each tube were stored overnight at 4°C.

Fifteen mL of SES was added to each pellet before it was agitated with a 10 μL Combi Loop. The volume was adjusted to 25 mL by adding additional SES. Each tube was then vortexed at 70% of full speed for 15 seconds. Each sample tube was heat treated for one hour at 68-70 °C in a water bath. For plating, each sample tube was placed on a vortexer (1-2 minutes at a setting near 8) before 200 μL of suspension was transferred to each agar plate (5 TSA plates). Inoculated plates were incubated overnight at 35°C. TSA plates were counted approximately 15-18 hours post plating (Day 1 data). Due to the low spore counts expected for the lower seed levels, an additional 10 TSA plates were inoculated the following day (Day 2 data) using 200 μL of suspension in order to confirm results. Plate counts were recorded on an Excel® spreadsheet.

2.7 Splitting of Samples and Sample Shipment for Inter-Laboratory Demonstration of the Protocol

Four experimental bottles, a negative control bottle, and a positive control bottle were shipped at ambient temperature to the US Geological Survey Laboratory in St. Petersburg, Florida on the third day after spore inoculation for each concentration tested (1, 5, 10, 25, and 50% of the positive control, which was targeted at 350 spores/g). The remaining 6 samples remained at EPA (Cincinnati, Ohio) stored at 4 °C. The Monday following the sample shipment both laboratories began processing their respective samples. Samples were blinded to avoid bias. Time and funding allowed for additional runs of the sand experiment to be completed at 5% and 10% of the positive control as well.

2.8 Statistics for Inter-Laboratory Demonstration

Statistical analysis was done to determine if there were significant differences between the EPA and USGS laboratories when analyzing standardized soil samples that have been seeded with known concentrations of BaS spores. Sample event included data for colony

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count/plate from the first day (Day 1), which included the five TSA agar plates inoculated using spread plate technique, and a second set of ten TSA agar plates, which were plated the next day (Day 2). These data were sorted, organized and coded using Excel, then imported into Minitab® (version 17), which was used to perform all of the statistical analyses.

All plate count data were used in the analyses, including zeros and CFU/mL values less than the calculated detection limit (i.e., 5 CFU/mL or 14 CFU/gram of soil). A decision was made to use this approach instead the other options for assigning values to censored data (e.g., half the detection limit, assigning zero to values less than detection limit). Summary statistics were completed for raw and $\log_{10}$ (x+1) transformed data. Percent recovery data (expressed in percentages) and the back transformed geometric means and associated 95% confidence intervals are also included for comparison.

Tests for outliers were conducted on the transformed data using the Grubbs' Test (alpha = 0.05). Tests for the normal distributions of the transformed data were performed on each data set using the Anderson-Darling test. Several approaches were used to bring those data sets that were not normally distributed into a normal distribution, including using a different transformation.

The comparison of plate count data within and between the EPA and USGS laboratories, with respect to each sample event, was accomplished by using the general linear model (GLM) to perform an analysis of variance (ANOVA). For each sample event, the response variable was the transformed CFU/plate data and the nested model included the variables Laboratories and Sample Tubes with both Laboratories and Sample Tubes being random factors. The variances associated with the different aspects of the process were derived from this analysis. An adjusted Type III sum of squares, with pairwise comparisons using the Tukey method and a confidence interval of 95.0% were constraints on the ANOVA. Additionally, a one-way ANOVA was used to test the differences between laboratories with regard to the CFU/plate data.

Finally, a two-sample t-Test ($\alpha = 0.05$), where the variances between the two data sets were assumed to be unequal, was conducted to see if there was an overall significant difference between the $\log_{10}$-transformed plate count data from the two laboratories. For this comparison, all plate count data from the respective laboratories were combined into a single data sets for the loamy (n=300) and sandy (n=420) soils, respectively.

3.0 Results

3.1 Preliminary Experiment Results

3.1.1 Extraction Diluent—The 2% Tween 80 combined with 4% polyphosphate did not demonstrate any sporicidal effect on countable plates (10^{-6} final dilution) after either one hour or four hours of exposure (data not shown). However, a zone of inhibition was observed on every 10^{-5} final dilution plate at the location where 100 µl of the test solution initially contacted the TSA plate at both time points. Colonies surrounding this zone were small compared to those on control plates, but colonies more than half a centimeter away from this spot were normal in size and morphology. A 1:10 dilution before plating removed inhibition

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and spore outgrowth, with vegetative growth proceeded normally even after four hours of exposure.

The results of the comparison between the study diluent and SES showed improved spore recovery for the SES compared to the study diluent. The average recovery of spores in the study diluent was 10.9% while the average spore recovery using the SES was 36.6%. When the SES was compared to phosphate buffered citrate, spore recoveries averaged 54.2% and 35.9%, respectively.

3.1.2 Wash Steps—When varying the number of washes, initial results showed diminishing return after the second or third washes (See Table 1). Each wash took approximately ¾ hour, making the total processing time approximately 10-12 hours, depending on number of washes done. The multiple wash step experiment was repeated for the first three washes. With each additional wash, a decrease in the final pellet size was seen. Of the four samples repeated with the three washes, recovery averaged 36.3%, 8.3%, and 3.8% for Wash 1, 2, and 3, respectively (data not shown). Following this experiment, the effort was continued using two washes.

3.1.3 Sonication—Samples that were sonicated tended to have a larger pellet, however, spore recovery results for sonicated and unsonicated samples were similar (for processing samples with two washes, total recovery averaged 41.9% and 43.2%, respectively). Introducing an additional step of sonication did not improve spore recovery, but did extend processing time, so the rest of the study proceeded without the sonication step.

3.1.4 Initial centrifugation speed and sample shaking—Centrifugation at 100 x *g* and 250 x *g* gave similar results when trying to remove large particles from the soil suspension. For the two samples for each speed tested during experiment 1, 100 x *g* and 250 x *g* speeds averaged 46.4% and 53.3% spore recovery, respectively. During a repeat of the experiment however, spore recoveries were only 21.7% and 18.8% for the 100 x *g* and 250 x *g* speeds, respectively. This difference in recoveries between the two experiments could be due to the age of the SES being approximately 60 days old (half-life of 90 days) at the time of the second experiment. Therefore, the 100 x *g* centrifugation speed was selected for further study.

Manual hand shaking showed slightly higher results than the mechanical shaking (See Table 2). And the average results for 350 rpm mechanical shaking were slightly higher than the 425 rpm shaking results. The mechanical shaker tended to produce smaller pellets than those observed with hand shaking. A speed of 450 rpm was used for the final optimized protocol as this is the maximum speed resulting in observable effective mixing of the suspended samples in the bottles.

3.3 Intra- and Inter-laboratory Study Statistical Results

No CFU were seen on any of the negative control plates. The positive controls showed colonies on all plates and had similar recoveries across the five experimental concentrations at both laboratories (Tables 3 and 4). Summary statistics are included in Tables 3 and 4 for the loamy and sandy soil experiments, respectively. Each experimental concentration had 4

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sample replicates (each with 5 plates inoculated on Day 1 and 10 TSA plates inoculated on Day 2). The raw data column represents the range of mean CFU counts observed over the four experimental samples per concentration/day. The percent spore recovery column represents the range of mean percent recovery observed for the four experimental samples for each concentration/day. The geometric mean column represents the lowest observed and highest observed geometric mean (of the plate counts) from the four samples from each concentration target along with the corresponding 95% confidence intervals for each geometric mean. The positive control column is the geometric mean data for the positive control (1 per concentration level).

The test for outliers uncovered one outlier each in the transformed data from the 10% and 25% concentrations Day 1 data and two outliers in the 10% day 2 data for loamy soil (data not shown). For sandy soil, there was one outlier in each of the two 10% experiments on Day 1 and one outlier for each of the 5% and 25% experiments for Day 2 (data not shown). The data from these samples were determined to be acceptable, though considered outliers, and included in all data analysis. Tests for normality on the transformed data using the Anderson-Darling test revealed all of the data sets were normally distributed, except for those for the 1% concentration tested for both soil types and both laboratories. Because the seed levels at the 1% target concentration level (3.5 spores/g soil) were not normally distributed, these seed levels will not be discussed further in the paper or displayed in the associated tables.

For the loamy soil, a majority of the samples showed a higher percentage of total variance coming from plating of samples compared to variance caused by the laboratory or the samples themselves. Exceptions included Day 1 and 2 data for the 50% concentration target samples and for Day 1 data for the 5% concentration target samples. For the loamy soil data, the plating process accounted for approximately 30.0-92.0% of the variability in the data for the Day 1 data and approximately 46.0-99.0% for the Day 2 data (data not shown). For the sandy soil experiments, plating accounted for the highest source of variation for all experimental concentrations and days tested with approximately 57.0-100% of the variance in the Day 1 data and approximately 77.0-97% for the Day 2 data (data not shown).

There was no statistically significant difference between the plate count data within the USGS laboratory for all of the Day 1 and Day 2 samples in the loamy soil experiment (Table 5). For these same samples, significant differences were detected within the EPA laboratory in the Day 1 (25%) and Day 2 (10%) data. When comparing the two laboratories, there were significant differences in the Day 1 data for 5%, 25%, and 50% concentration targets and the Day 2 data for 5%, 10%, and 50% concentration targets.

When comparing the processing the sandy samples by two laboratories, there were statistically significant differences within the USGS laboratory data for: both sets of 10% concentration targets on Day 1; the 50% concentration target on Day 1; the first set of 5% concentration target on Day 2; and the first set of 10% concentration target on Day 2 (Table 5). There were no differences within the EPA laboratory data sets for the Day 1 or Day 2 data sets. When comparing the two laboratories, there were significant differences in the plate count data between the two laboratories for the first set of 10%, the 25% and 50%

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concentration target data on Day 1 and the first 5% concentration target and first 10% concentration target on Day 2.

Results of the two-sample t-Test did not show a significant difference between the EPA lab and the USGS lab for either soil type (loamy and sandy).

4.0 Discussion

The goal of this study was to optimize a previously used indirect sample-processing protocol (Silvestri et al., 2015b) to improve recovery of *B. anthracis* spores from soil samples and to subsequently demonstrate that protocol at two laboratories. Optimization steps included identifying optimal extraction diluent, evaluating varying the number of wash steps, evaluating the effect of sonication on results, evaluating varying the initial centrifugation speed, and evaluating hand versus mechanical shaking.

Results of initial diluent experiments suggest that 4% polyphosphate combined with 2% Tween 80 is not sporicidal to BaS, but is sporostatic. However, a 1:10 dilution before plating removes the sporostatic inhibition and restores spore outgrowth, and vegetative growth proceed normally even after four hours of exposure. Based on these results, using concentrations of Tween 80 at 1% and polyphosphate up to at least 2% in a spore extraction medium for soils should not result in the inhibition of BaS spore outgrowth recovered from soil. Results of further diluent tests suggested that the SES might disperse particles better than the study diluent did.

Use of two washes during sample processing appears to be beneficial, however adding a third wash yields diminishing returns due to the additional processing time required. Being cognizant of how long processing takes might be critical given quick results needed in response to a contamination incident. Use of sonication with the protocol did not improve recovery.

When varying centrifuge speeds, there was a large difference in recovery. It was hypothesized that the age of the washing solution (the SES was 60 days old and has a half-life of 90 days), may have affected recovery. It should be noted that the speed at which this step of the protocol can be completed is limited by the number of buckets in the centrifuge.

Results indicated that hand shaking gave slightly higher recovery than mechanical shaking. However, for consistency, use of a mechanical shaker would be preferred when multiple operators are processing samples.

The processing protocol was able to detect spores in sterile soil all the way down to 1% of the positive control spiking level. However, the large number of data that did not follow a normal distribution for the 1% concentration tested is most likely due to a large number of plates in which no colonies were counted on or whose averages were at or below the 14 CFU/gram of soil detection limit of the protocol. In addition, a wider range in spore recovery was also noted with that data. Interpretations made with this data should be considered with caution because numerous low or no CFU plates indicate that the seed concentration was below the detection limit of this recovery protocol. However, the matrix

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limit of detection for the processing protocol used by Silvestri et al. (2015b) was reported at 10^4 spores g/sand, so the processing protocol optimized in the current study has demonstrated improvement over the non-optimized protocol. The current protocol also showed improvement over several other studies also reporting detection limits of 10^4 CFU/g soil (Herzog et al., 2009, Ryu et al., 2003).

According to the results of the ANOVA analysis, a majority of the variability in the data came from plating of inoculum on TSA, rather than from the laboratory or samples themselves. However, it should be noted that ANOVA tests were only defined by the CFU/plate data and did not include the characterization of the other aspects of sample preparation (e.g., weighing out the soil, volume of diluent, adsorption onto soil, dilutions) prior to the plating the suspensions on TSA agar. There was no significant difference overall between the laboratories for either soil type, suggesting the processing protocol will be robust enough to achieve similar recoveries at multiple laboratories.

One limitation to this study was that it was completed using only sterile soil due to budgetary constraints. Ideally the study would be repeated using native soils to test performance of the protocol which would be more representative of a real life situation. Native soils with different composition than the sterile soils used in this study might require additional considerations for processing. For example, the size of the pellet produced during processing could be altered and affect final sample volumes. In addition, background organisms present in native soil might interfere with use of culture following the processing protocol, so the protocol might need to be coupled with a target specific molecular method for detection instead of plating.

5.0 Conclusions

Following an intentional or unintentional release of *B. anthracis* in indoor or outdoor environments, might require sampling and analysis of surrounding soil environments. However, detection of *B. anthracis* spores in soil is a difficult task due to background organisms, inhibitors, and the high limit of detection of most protocols reported in literature. This study optimized a previously used indirect soil sample processing protocol. Optimization steps included; identifying an optimal extraction diluent; identifying the number of wash steps that gave the best recovery while at the same time not adding on unnecessary processing time; varying the initial centrifugation speed, evaluating if addition of sonication would be beneficial to the protocol, and evaluating if there was any difference between mechanical and hand shaking mechanisms. The improved protocol demonstrated an improved limit of detection (14 CFU/g soil) for loamy and sandy soils over the non-optimized protocol (10^4 CFU/g soil). Variability in the data was attributed to the plating procedure. There was no significant difference overall between the two laboratories that completed the laboratory comparison study. Although this protocol was demonstrated using sterile soil, making use of culture for detection possible, use of culture with non-sterile soil samples might be difficult due to the number of background organisms in soil. The results of this study suggest that this processing protocol is robust enough to achieve similar recoveries if used among multiple laboratories. However, additional work is needed to combine the processing protocol with DNA extraction and qPCR protocols and provide a complete

detection method. In addition, additional work is needed to verify the protocol using native soils.

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Abbreviations

ANOVA	Analysis of Variance
BaS	*Bacillus anthracis* Sterne
CDC	Centers for Disease Control and Prevention
CFU	Colony forming units
EPA	U.S. Environmental Protection Agency
PBS	Phosphate buffered saline
qPCR	Quantitative Polymerase Chain Reaction
R2A	Reasoner's 2 Agar
R2B	Reasoner's 2 Broth
SDS	Sodium dodecyl sulfate
SES	Spore extraction solution
SHMP	Sodium hexametaphosphate
TSA	Trypticase® Soy Agar
TSB	Trypticase® Soy Broth
USGS	U.S. Geological Survey

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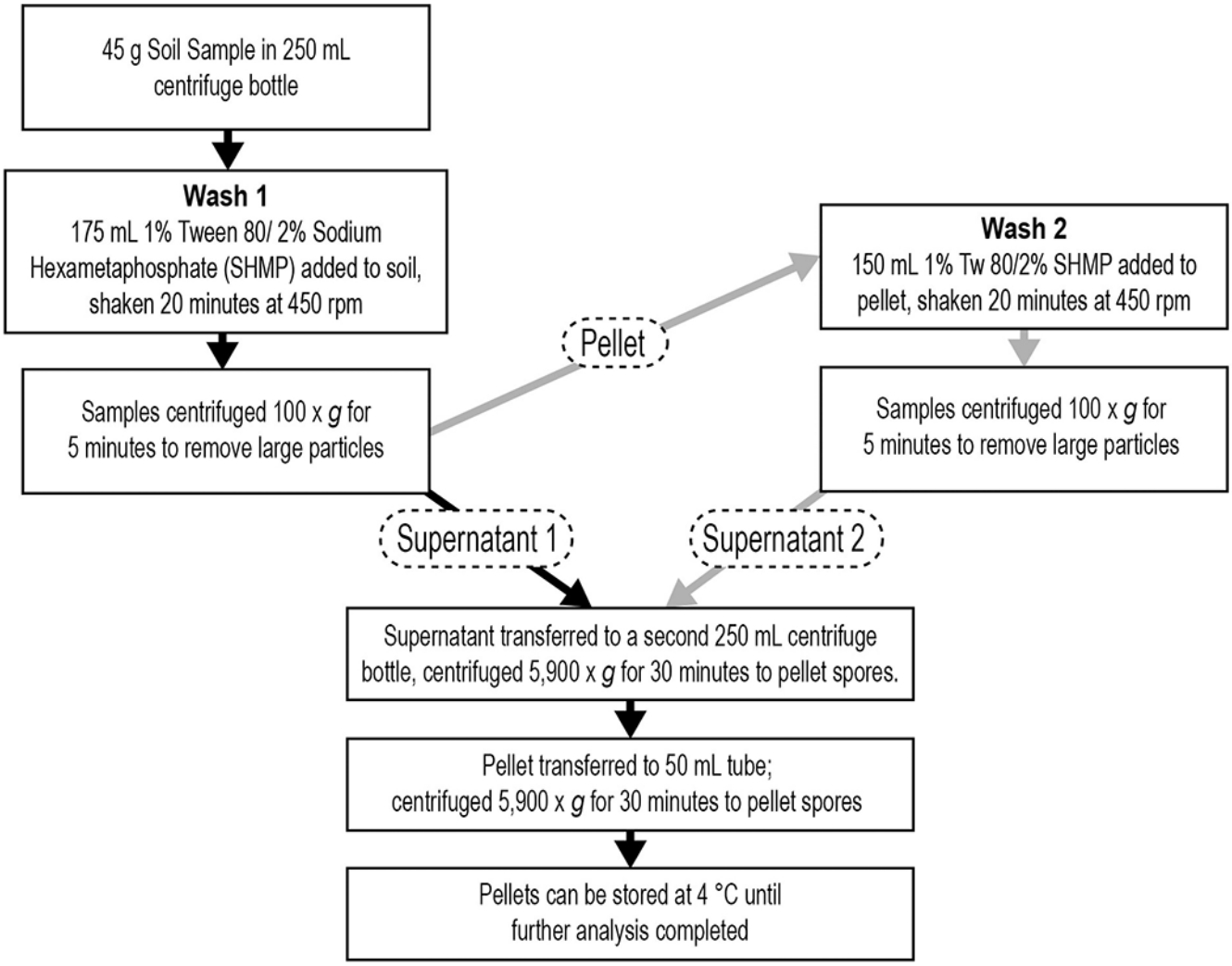

Figure 1.
Optimized Soil Processing Protocol for 45g Soil Samples

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Table 1.

Bacillus anthracis Sterne Spore Recoveries from Sterilized Agvise Loam with Five Wash Procedure

Sample	1	2	3	Average Recovery	Standard Deviation (SD)	% SD	Time Elapsed (Hours)
Washes	Recovery	Recovery	Recovery				
1	23.8%	29.1%	16.6%	23.2%	6.3%	27%	0.75
2	8.9%	3.9%	3.0%	5.3%	3.2%	60%	1.5
3	4.0%	1.2%	1.5%	2.3%	1.6%	69%	2.25
4	1.9%	0.9%	0.9%	1.2%	0.5%	45%	3
5	1.0%	0.4%	0.5%	0.6%	0.3%	56%	3.75
Total Recovery	39.6%	35.5%	22.5%	32.5%	9.0%	28%	

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Table 2.

Average Spore Recovery for Mechanical Shaking versus Manual Hand Shaking

Sample	Mechanical Sample Average (350 RPM)	Manual Control Sample Average
Wash 1 Recovery (%)	52.5	57.4
Wash 2 Recovery (%)	6.2	7.0
Total Recovery (%)	58.5	64.4
Sample	**Mechanical Sample Average (425 RPM)**	**Manual Control Sample Average**
Wash 1 Recovery (%)	42.3	50.6
Wash 2 Recovery (%)	5.7	5.8
Total Recovery (%)	48.0	56.3

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Table 3.

Summary Statistics for Spore Recovery from Loamy Soil in EPA and USGS Labs

Percent of Positive Control (spores/g) Targeted	Spores/g Actually Achieved	Day	EPA Lab					USGS Lab				
			Raw Data Mean CFU/plate Range[a]	Percent Recovery (%R) Range	Geometric Mean			Raw Data Mean CFU/plate Range[a]	%R Range	Geometric Mean		
					Lowest Observed Mean CFU/ plate (95% CI)	Highest Observed Mean CFU/ Plate (95% CI)	Positive Control Mean CFU/ plate (95% CI)			Lowest Observed Mean CFU/ plate (95% CI)	Highest Observed Mean CFU/ plate (95% CI)	Positive Control Mean CFU/ Plate (95% CI)
5% (17.5)	18.0	1	1.6-2.6	24.7-40.1%	1.3 (1.1-4.5)	2.4 (2.1-5.4)	57.0 (47.0-71.6)	3.6-5.0	55.6-77.2%	3.4 (3.2-6.3)	4.9 (4.9-7.2)	95.3 (91.7-101.2)
		2	1.9-2.4	29.3-37%	1.8 (1.8-4.3)	2.3 (2.6-4.1)	64.3 (59.0-72.2)	2.7-3.7	41.7-57.1%	2.5 (2.6-4.6)	3.3 (3.0-6.3)	68.4 (62.3-77.4)
10% (35)	33.1	1	4.4-9.0	37-75.8%	3.9 (2.7-9.0)	8.7 (6.8-13.7)	73.5 (64.4-86.2)	3.4-5.4	28.6-45.5%	3.0 (2.2-7.2)	5.2 (4.7-8.3)	64.5 (57.6-74.5)
		2	3.6-9.1	30.3-76.6%	3.5 (3.6-5.5)	8.9 (8.2-11.9)	67.2 (62.0-75.0)	3.4-5.9	28.6-49.7%	3.0 (2.6-6.2)	5.4 (4.7-8.8)	73.3 (63.8-86.6)
25% (87.5)	65.0	1	12.4-19.6	53-83.8%	12.2 (11.0-16.0)	19.6 (18.9-22.3)	70.2 (57.1-88.7)	10.4-15.8	44.4-67.5%	9.7 (6.8-16.9)	15.7 (15.0-18.7)	56.2 (51.1-64.0)
		2	13.3-17.8	56.8-76.1%	12.7 (10.5-17.8)	16.8 (13.2-23.9)	74.1 (68.7-82.1)	14.9-17.2	63.7-73.5%	14.1 (11.4-20.0)	16.7 (14.5-21.6)	62.5 (59.4-67.9)
50% (175)	162.7	1	15.6-19.6	26.6-33.5%	15.3 (12.6-21.0)	19.3 (16.7-24.9)	57.8 (48.9-70.8)	23.2-28.4	39.6-48.5%	23.0 (20.8-27.8)	28.1 (24.6-34.5)	64.4 (56.9-75.2)
		2	15.8-19.8	27-33.8%	15.4 (13.6-19.8)	19.5 (17.9-23.6)	51.1 (32.9-82.4)	24.0-26.0	41-44.4%	23.7 (21.8-28.0)	25.6 (22.8-31.0)	63.0 (57.3-71.6)

[a]All plates were inoculated with 200 uL of soil suspension.

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Table 4.

Summary Statistics for Spore Recovery from Sandy Soil in EPA and USGS Labs

Percent of Positive Control (spores/g) Targeted	Spores/g Actually Achieved	Day	EPA Lab					USGS Lab				
			Raw Data Mean CFU/plate Range[a]	Percent Recovery (%R) Range	Geometric Mean			Raw Data Mean CFU/ plate Range[a]	%R Range	Geometric Mean		
					Lowest Observed Mean CFU/ plate (95% CI)	Highest Observed Mean CFU/ Plate (95% CI)	Positive Control Mean CFU/plate (95% CI)			Lowest Observed Mean CFU/ plate (95% CI)	Highest Observed Mean CFU/ plate (95% CI)	Positive Control Mean CFU/Plate (95% CI)
5% (17.5)	17.6	1	3.4-7.4	53.7-116.8%	3.3 (3.2-5.8)	7.1 (5.7-11.5)	83.1 (78.0-90.6)	3.6-6.0	56.8-94.7%	3.6 (4.0-5.3)	5.5 (3.9-10.9)	99.5 (94.2-107.1)
		2	4.9-7.8	77.3-123.1%	4.8 (3.9-8.5)	7.4 (6.5-10.9)	106.4 (99.4-116.2)	3.3-6.1	52.1-96.3%	2.6 (2.1-6.2)	5.7 (4.9-9.1)	102.3 (99.0-107.7)
5% (17.5)	19.7	1	4.4-5.6	62.0-79.0%	4.0 (3.0-8.4)	5.2 (3.9-9.9)	88.4 (87.3-91.5)	4.6-6.4	64.9-90.2%	4.1 (2.4-10.9)	6.3 (5.9-9.1)	95.7 (91.0-102.7)
		2	3.9-6.8	55.0-95.9%	3.6 (3.4-6.2)	6.3 (5.4-9.9)	113.8 (105.1-125.3)	5.1-6.7	71.9-94.5%	4.8 (4.4-7.7)	6.2 (5.1-10.1)	118.2 (112.2-126.6)
10% (35)	29.6	1	6.2-9.8	58.2-92.0%	5.4 (3.3-12.4)	9.6 (8.4-13.4)	88.4 (73.7-108.4)	6.2-13.2	58.2-123.9%	5.9 (4.8-9.9)	13.1 (12.2-16.3)	66.3 (63.4-71.5)
		2	8.6-10.7	80.7-100.4%	8.6 (8.8-10.4)	10.4 (9.1-14.1)	96.2 (88.5-106.8)	8.7-13.4	81.6-125.8%	8.6 (8.5-10.9)	12.9 (11.2-17.4)	68.1 (65.7-72.7)
10% (35)	35.6	1	10.8-13.6	84.3-106.1%	10.2 (7.4-17.0)	13.2 (10.3-19.5)	107.6 (97.6-120.9)	7.6-15.4	59.3-120.2%	7.5 (7.3-10.0)	15.1 (12.7-20.4)	112.4 (105.0-122.4)
		2	10.8-13.5	84.3-105.3%	10.3 (8.7-14.6)	13.2 (12.0-16.9)	112.5 (106.7-120.7)	9.1-10.9	71.0-85.0%	8.7 (7.6-12.4)	10.6 (9.5-14.1)	108.0 (99.2-119.7)
25% (87.5)	89.3	1	20.40-26.8	63.5-83.4%	20.3 (18.2-24.8)	26.2 (20.7-35.7)	66.7 (56.1-81.7)	27.4-33.2	85.2-103.3%	27.1 (23.0-34.3)	32.9 (28.6-40.2)	112.3 (98.8-130.0)
		2	27.6-31.7	85.9-98.6%	26.9 (23.2-33.6)	31.2 (27.5-37.7)	93.7 (88.4-101.4)	26.2-31.2	81.5-97.1%	25.9 (23.5-30.7)	30.8 (27.7-36.5)	98.7 (89.7-111.0)
50% (175)	166.7	1	38.4-47.6	61.0-79.3%	38.3 (36.3-42.6)	47.2 (40.4-57.4)	90.0 (78.7-105.3)	37.8-51.4	63.0-85.6%	37.6 (33.4-44.5)	51.3 (48.5-56.4)	105.8 (94.9-120.1)
		2	45.7-48.9	76.2-81.5%	45.5 (42.9-50.4)	48.5 (44.2-55.4)	97.6 (95.4-102.0)	45.2-52.0	75.3-86.6%	44.9 (41.4-50.8	51.6 (47.3-58.5)	96.9 (93.4-102.7)

[a]All plates were inoculated with 200 uL of soil suspension.

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Table 5.

Laboratory Performance Comparisons for Significant Difference

Soil Type	Percent of Positive Control Targeted	Day	Laboratory Comparisons		
			Within		Between
			USGS	EPA	
Loam	5%	1	NSD[a]	NSD	0.003[b]
		2	NSD	NSD	0.026
	10%	1	NSD	NSD	NSD
		2	NSD	<0.001	<0.001
	25%	1	NSD	0.006	0.002
		2	NSD	NSD	NSD
	50%	1	NSD	NSD	<0.001
		2	NSD	NSD	<0.001
Sand	5% (A)	1	NSD	NSD	NSD
		2	0.027	NSD	0.005
	5% (B)	1	NSD	NSD	NSD
		2	NSD	NSD	NSD
	10% (A)	1	0.006	NSD	0.014
		2	<0.001	NSD	0.002
	10% (B)	1	0.005	NSD	NSD
		2	NSD	NSD	NSD
	25%	1	NSD	NSD	0.011
		2	NSD	NSD	NSD
	50%	1	0.004	NSD	0.002
		2	NSD	NSD	NSD

[a] NSD: No statistically significant difference between the respective means.

[b] p-values from the ANOVA table.