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Optimization of a 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, 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/g 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.

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.

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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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 a selective medium 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).

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 effects 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 $<1 \times 10^5$ 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 (BaS) 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 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 BaS CFU per mL. BaS 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 min at 121 °C, 17 psi) with a 10 min drying time. Soil moisture content was measured before and after autoclaving. For sandy soil, moisture content was measured at 7.7 ± 12% prior to autoclaving and 2.3 ± 13% 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 h 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 s. 100 μL of the soil suspension was plated onto three low nutrient Reasoner's 2 Agar (R2A) plates and six high nutrient 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 medium 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 s. 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.