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REVIEW ARTICLE

Recent literature review of soil processing methods for recovery of *Bacillus anthracis* spores

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Abstract Identifying virulent *Bacillus anthracis* within soil is a difficult task due to the number and diversity of other organisms and impeding chemical constituents within soil. Regardless of the detection assay, the initial sample must be processed efficiently to ensure that debris, chemical components, and biological impurities do not obstruct downstream analysis. Soil sample processing protocols can be divided into two general types: indirect and direct. There are two requirements for successful indirect isolation of *B. anthracis* from soil samples: dissociate the spores from the soil particles and physically separate the free spores from the soil particles. Adding an aqueous carrier medium to a soil sample creates a sample slurry for easier manipulation. Centrifugation, high specific gravity separation, immunomagnetic separation, filtration, and settling have been used to physically separate spores from soil. Direct processing utilizes a soil sample without first separating the spores from the bulk sample and falls under two principal types: culturing on *B. anthracis* selective agar and bulk DNA extraction. Direct and indirect processing steps each have associated advantages and disadvantages. The objective of this review was to consolidate information acquired from previous research, focusing primarily on data gleaned in the last decade, on the processing of soils contaminated with *B. anthracis*. As shown in this review, an optimized soil-processing protocol with a known recovery rate and associated confidence intervals is needed. A reliable processing protocol would allow for multiple investigators and laboratories to produce high-quality, uniform results in the event of a *B. anthracis* release.

Keywords *Bacillus anthracis* spores · Soil · Indirect processing · Direct processing

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Introduction

Bacillus anthracis, the etiological agent of anthrax, is a naturally occurring Gram-positive spore-forming bacteria found in many soil environments (Van Ert et al. 2007). Exposure of humans to *Bacillus anthracis* spores has been historically associated with agricultural contact with infected animals or animal products. Outbreaks of anthrax in livestock in the United States have been reported since the early 1800s (Mikesell et al. 1983) and were historically reported along cattle trails (Blackburn et al. 2007). In many instances, recent anthrax cases were associated with old graves of anthrax-stricken animals and adequate soil conditions (Pepper and Gentry 2002; Griffin et al. 2009; Hugh-Jones and Blackburn 2009).

There are a number of alternative theories regarding the lifecycle of *B. anthracis* in soil (Minett 1950; Lindeque and Turnbull 1994; Dragon and Rennie 1995; Atlas 2002; Coker 2002; Dragon et al. 2005; Saile and Koehler 2006; Johnson 2007; Lee et al. 2007; Hugh-Jones and Blackburn 2009; Schuch and Fischetti 2009; Schuch et al. 2010; Dey et al.

2012). Regardless of how *B. anthracis* spores came to a soil, it is generally accepted that some soils are better at harboring spores than others, and weather conditions influence the occurrence of environmental anthrax cases. *B. anthracis* is most often found in dry conditions with soils that are high in organic matter and calcium and are relatively alkaline (above pH 6) (Van Ness 1971; Johnson 2007; Hugh-Jones and Blackburn 2009). Spores are metabolically dormant and extremely resistant to environmental stresses (Ghosh and Setlow 2009, 2010). Spores can persist in soil for years (Graham-Smith 1930; Wilson and Russell 1964; Lewis 1969; Manchee et al. 1981; Lindeque and Turnbull 1994; Purcell et al. 2007; Sinclair et al. 2008) and are thought to migrate within the soil following the flow of water (Kim et al. 2009).

Soil is a complex matrix with multiple components and a plethora of microbial activities, and the properties of soil change with the seasons and over extended periods of time (USDA 1999). One gram of soil reportedly contains up to 10 billion microorganisms, and thousands of different species (Delmont et al. 2011a), and close relatives of *B. anthracis* can be collocated in the soil environments (Kuske et al. 2006). Chemical constituents of soil such as organics, humic acids, etc., can interfere with the chemistry involved in downstream microbiological detection assays (Zhou et al. 1996; Sjostedt et al. 1997; Beyer et al. 1999; Cheun et al. 2003; Robe et al. 2003; Balestrazzi et al. 2009; Dineen et al. 2010; Gulledge et al. 2010). Due to the number and diversity of organisms and impeding chemical constituents within soil, identifying *B. anthracis* within a soil sample is a difficult task.

While there are many *B. anthracis* detection assays, only a few of them can be utilized directly with environmental soil samples. The initial sample must be efficiently processed to ensure that debris, chemical components, and biological impurities do not obstruct microbiological detection. Without appropriate sample processing, the most sensitive detection assay will be ineffective. There is a need for a universal sample processing protocol to separate, concentrate, and purify target agents from any sample type (Lim et al. 2005). There have been multiple reviews detailing the various detection assays for *B. anthracis* (Edwards et al. 2006; Rao et al. 2010; Irenge and Gala 2012), but previous reviews have not included an in-depth discussion of various soil sample-processing protocols. Multiple processing protocols have been developed either to separate spores from soil samples before microbiological assessment or to directly extract bulk DNA to identify the initial organism(s) present within the soil. However, these studies have never been integrated to determine the overall breadth of knowledge regarding the processing efficiency. Therefore, the objective of this project was to consolidate information acquired from previous research and provide a summary regarding the direct and indirect processing of soils contaminated with *B. anthracis* that have been utilized in the recent literature.

Methods

Open-literature searches of PubMed®, Google Scholar, and the Battelle Library using the search criteria "*Bacillus anthracis*," "soil," and "soil microbiology" were used to collect nearly 100 pertinent documents. The search focused primarily on data gleaned in the last decade. A brief summary of the literature review findings are summarized here. Literature for processing protocols of similar pathogens or similar matrices were also included in the summary if found during the search and deemed to be applicable.

Results

Two types of processing protocols, indirect and direct, were identified in the literature by Delmont et al. (2011b). For indirect processing, spores are extracted from soil particles and concentrated prior to downstream detection with an analysis assay such as culture or polymerase chain reaction (PCR). Conversely, direct processes refers to utilization of a soil matrix with a detection assay without first extracting and concentrating the spores from the bulk sample. Direct and indirect processing are discussed in more detail in the subsequent sections.

Indirect processing: Separating *B. anthracis* from soil

Because spores have the potential to adhere to large soil aggregates (Nicholson and Law 1999), there are two requirements for successful isolation of *B. anthracis* from soil samples: dissociate the spores from the soil particles and separate the free spores physically from the soil particles. Protocols for spore purification from soil particles prior to use of detection assays (for example culture or PCR) involve steps to accomplish both of these objectives. The most common types of processing protocols can be broken down into three steps with the first two working together to disrupt spore–soil interactions: (1) introduce an aliquot of soil to an aqueous carrier medium; (2) mix the soil with the liquid to aid in chemical and/or physical dissociation of spores from soil aggregates; and (3) separate and concentrate spores away from soil particulates. In some cases, additional steps are taken to concentrate and further purify the final spore sample.

Aqueous carrier media

The hydrophobic exosporidium of *B. anthracis* interacts with solid soil particles and requires treatment prior to efficient spore recovery (Saikaly et al. 2007; Naclerio et al. 2009). Adding an aqueous carrier medium to a soil sample creates a sample slurry that can be manipulated. While deionized water has been utilized (Dragon and Rennie 2001), chemical

additives (buffers, chelating agents, surfactants, salts, emulsifiers) are often included to aid spore–soil dissociation. Chelating agents [e.g., ethylenediaminetetraacetic acid (EDTA), Chelex® 100 and surfactants (e.g., Triton™ X-100, TWEEN® 20, TWEEN® 80, sodium dodecyl sulfate (SDS)] promote detachment of spores from soil particles, whereas salt solutions (sodium chloride, aluminum sulfate) form a complex and precipitate extracellular DNA and humic acids present within the soil (Lombard et al. 2011). The carrier medium (or spore extraction solution) has been said to be the most important factor influencing the efficiency of extracting spores from wipes (Da Silva et al. 2011).

Within the reviewed studies, there were many different aqueous media used to separate spores from soil samples. The most common type of carrier medium was a buffered solution or a buffer solution with a surfactant. Recovery efficiency data are lacking in many studies. Three *B. anthracis* studies contained spore recovery data for various soil types. The aqueous carrier media used in these studies included: Triton™ X-100 in water (Dragon and Rennie 2001), Nonidet™ P-40 in water (Dragon and Rennie 2001), TWEEN 20 in phosphate buffered saline (PBS) (Marston et al. 2008; Bradley et al. 2011), sucrose (Dragon and Rennie 2001), Triton™ X-100 in sucrose (Dragon and Rennie 2001; Bradley et al. 2011), and TWEEN 20 in sucrose (Dragon and Rennie 2001). Spore recovery efficiency varied depending on the soil type and aqueous carrier medium. A number of other parameters such as sample age, sample amount, and dissociation protocol may also have influenced the overall extraction efficiency. Carrier media that were typically used for comparisons of recovery of pathogens other than *B. anthracis* in soil in the literature included sterile deionized water (Dabiré et al. 2001; Ehlers et al. 2008), PBS (Fitzpatrick et al. 2010; Isabel et al. 2012), NaCl solution (Ehlers et al. 2008; Santana et al. 2008), and NaOH (Dabiré et al. 2001). Studies in which recovery efficiency data were lacking (Rastogi et al. 2009) or which looked at aqueous carrier media for matrices other than soil (Tims and Lim 2004; Hong-Geller et al. 2010; Leishman et al. 2010; Da Silva et al. 2011) were also found but are not discussed in this review. While no study provided statistical evidence for an optimized aqueous carrier medium, the individual studies each concluded that the addition of a surfactant aided spore recovery when compared to PBS or sucrose solutions alone (Dragon and Rennie 2001; Da Silva et al. 2011). Determination of an optimum aqueous carrier medium from the available information is therefore difficult.

Spore–soil dissociation

Microbial cells are tightly bound to soil colloids with clay and organic matter posing particular challenges in spore–soil separation (Zhou et al. 1996). One experiment found that 99 % of the natural spores present in a sandy test soil were associated with the soil aggregates and not within the aqueous carrier medium, indicating that additional steps are needed to dissociate the spores from the soil (Nicholson and Law 1999). Chemical additives added to the aqueous carrier medium are used to help dissociate spores from soil; however, physical means are also utilized. Physical agitation has taken the form of manual shaking, gentle agitation, use of a Stomacher®, use of blenders, vortexing, sonication, and/or bead beating.

More energetic dispersion protocols may yield greater spore recovery efficiencies (Dabiré et al. 2001). Dissociation of large soil aggregates was suggested as the primary cause for the increased spore recoveries. Other studies have confirmed that more energetic dispersion protocols aid in overall recovery rates. Vortexing was found to be statistically superior to sonication for separating *B. anthracis* spores from wipe samples (Da Silva et al. 2011) and enhanced homogenization has been achieved using a Waring® blender over sonication or chemical treatment alone (Courtois et al. 2001). Ultrasonication treatment and shaking have been found to be inferior dispersion protocols when compared to using a Waring® blender (Lindahl and Bakken 1995). Even with significant physical disruption, spore-soil interactions are powerful and may be only slightly interrupted by physical agitation (Nicholson and Law 1999). An estimated 35–55 % of the spores remained with large stable aggregates following total soil disruption with agate marbles (Dabiré et al. 2001).

Physical separation of spores from soil

After spore–soil dissociation, spores can be separated physically from soil particles using methods such as density separation [e.g., high and low speed centrifugation, high specific gravity separation (HSGS)], affinity capture [e.g., immunomagnetic separation (IMS)] or filtration (Fig. 1). While some protocols do not require debris-free sample material for downstream detection assays (culture, direct DNA extraction followed by molecular detection), many assays have higher sensitivities with purified samples.

Density separation methods include low-speed centrifugation, high-speed centrifugation, and HSGS. Low-speed centrifugation precipitates only dense soil particles leaving the more buoyant free dissociated spores within the supernatant. Spores remaining bound to soil particles after dissociation steps are removed with the soil particles. Spores within the supernatant can be detected directly or concentrated through additional steps. Two studies used low-speed centrifugation speeds of 123 *g* (Fitzpatrick et al. 2010) and 2,900 *g* (Roh et al. 2006) to separate soil particles from the microbial cell fraction before DNA extraction. However, neither study specifically targeted *B. anthracis* within the soil samples. The study using the 2,900 *g* centrifugation speed concluded that separation of

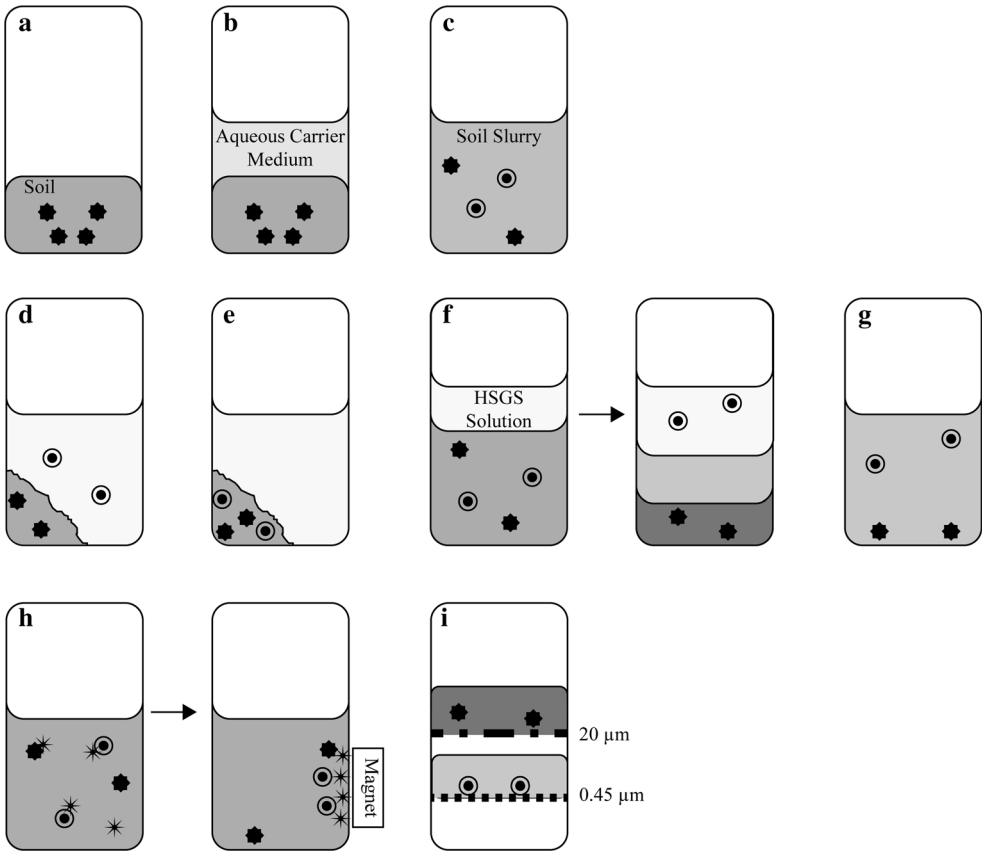

Fig. 1 Indirect soil processing steps. **a** Initial soil sample with soil-bound spores (✹). **b** Soil sample with added aqueous carrier medium. **c** Soil slurry with soil-bound spores and dissociated spores (◉). **d**–**i** Separation and concentration methods; density separation via: **d** low-speed centrifugation; **e** high-speed centrifugation; **f** high specific gravity separation; **g** settling. **h** affinity capture (imperfectly specific) using antibody-labeled magnetic beads (✶); **i** filtration with 20-and 0.45-µm pore size filters

cells prior to DNA extraction (indirect DNA extraction) yielded a lower quantity of higher quality DNA extracts when compared to directly extracted soil samples (Roh et al. 2006). Low-speed centrifugation (657 *g* and 2,000 rpm, respectively) was also used as part of the isolation steps of the GABRI (ground anthrax *Bacillus* refined identification) protocol and was successful in recovering *B. anthracis* from soil samples; however, the study did not specifically target determination of the recovery efficiency of the low-speed centrifugation step (Fasanella et al. 2013a, b).

In contrast to low-speed centrifugation, high-speed centrifugation precipitates free spores along with other microorganisms or soil particles present in the initial suspension. Therefore, high-speed centrifugation is typically used to wash away humic acids and extracellular DNA within a soil sample before further analysis (Gulledge et al. 2010). Seven studies utilized a high-speed centrifugation step to aid in pre-washing the soil samples (Maarit Niemi et al. 2001; Cheun et al. 2003; Bielawska-Drózd et al. 2008; Dauphin et al. 2009; Gulledge et al. 2010; Hong-Geller et al. 2010; Jain et al. 2011). A maximum 1 g aliquot of soil was utilized in these studies. In all but one study (Jain et al. 2011), soil particles were not separated from the spores before lysis and DNA extraction. In one study, additional soil pre-washing before DNA extraction was found to diminish PCR inhibition (Jain et al. 2011), but in a second study it was determined that pre-washed soil samples were not significantly different from soil samples placed directly into the extraction kit process (Gulledge et al. 2010). A settling period following vigorous shaking has also been used in combination with other separation procedures such as high-speed centrifugation (EPA 2013).

The studies discussed in this review used four types of HSGS solutions used as a density separation method: sucrose solutions (Pillai et al. 1991; Dragon and Rennie 2001; Ryu et al. 2003; Bradley et al. 2011; Stratilo and Bader 2012), Nycodenz® density gradient medium (Lindahl 1996; Courtois et al. 2001; Ehlers et al. 2008; Pote et al. 2010; Delmont et al. 2011b), sodium bromide solution (Nicholson and Law 1999), and two-phase liquid systems (Sacks and Alderton 1961; Agarwal et al. 2002; Parachin et al. 2010). Irrespective of gradient medium, HSGS utilizes differences in specific gravity to separate *B. anthracis* spores from other organisms and soil components. Depending upon the sub-species, *B. anthracis* spores range in density from 1.162 to 1.184 g mL^{-1} (Carrera et al. 2008) and are concentrated in the upper layers of most density gradient solutions post-centrifugation. Sucrose and Nycodenz® solutions are utilized at densities of 1.22 and 1.3 g mL^{-1}, respectively, allowing spores to concentrate within the uppermost layer following centrifugation. Two comparative studies concluded that HSGS with 1.22 g mL^{-1} sucrose was the most effective protocol for spore separation, though yields were not high

(Dragon and Rennie 2001; Ryu et al. 2003), while results from a third study were variable (Bradley et al. 2011). The utility of Nycodenz® HSGS for recovering *B. anthracis* spores is unknown. Conflicting efficiency results were found in the literature for use of Nycodenz® density gradient medium to prepare soil samples for total indigenous DNA extraction (Lindahl and Bakken 1995; Courtois et al. 2001). Two-phase liquid systems and sodium bromide include a wider range of liquid densities within a single centrifugation tube (1.0–1.3 g mL^{-1}) (Nicholson and Law 1999; Parachin et al. 2010). The spore-rich layer in these solutions is midway within the tube; the uppermost layers with lower density cell debris must be removed prior to spore collection. The added step of removing the uppermost layer significantly reduced the spore yield within the final sample. The addition of sodium bromide HSGS has been shown to decrease indigenous spore yields from 2–4 % to less than 0.1 % (Nicholson and Law 1999). Using a two-phase liquid HSGS protocol, recovery of *B. anthracis* Sterne spores from garden soil and sand samples was approximately 9–20 % and >50 %, respectively (Agarwal et al. 2002).

IMS is an affinity capture method that utilizes anti-*B. anthracis* spore antibodies associated with magnetic beads to capture and concentrate *B. anthracis* spores. Following the addition of the aqueous carrier medium and spore–soil dissociation steps, magnetic beads conjugated with *B. anthracis* antibodies are added to the soil sample suspension. Spores present in the sample bind to the antibodies. A magnet is used to concentrate the bead–antibody–spore complex away from the soil slurry and allows the transfer of the bead–antibody–complex to a buffer solution for further washing and purification. The recovered spores can be assayed using either culture, chemical, or molecular protocols. One study compared automated IMS recovery efficiencies for four different soil types (Arizona test dust, Minnesota loam, potting soil, and sand) and found an overall minimum limit of detection (LOD) of 10^2 spores g^{-1} of soil (Bradley et al. 2011). Recoveries ranged from 17 to 51 % among the four soils with the Minnesota loam and potting soil being the most recalcitrant (Bradley et al. 2011). However, microorganisms other than *B. anthracis* were detected after culture with sand and potting soil, and may have been transferred to the final sample as an aggregate with magnetic soil particles (Bradley et al. 2011). Antibody specificity was tested using time-resolved fluorescence, and results indicated that the *B. anthracis* antibody can differentiate between closely related and nonrelated bacterial strains (only *B. anthracis* spores were tested, not vegetative cells) (Bradley et al. 2011). In an effort to improve the selectivity of IMS-treated soil samples, a separate study directly extracted small acid-soluble spore protein-B from the spores for highly sensitive liquid chromatography–tandem mass spectrometry detection (Chenau et al. 2011). While selectivity was improved, the added processing/detection steps decreased overall sensitivity to a LOD of 7×10^4 spores g^{-1} soil. Fig. 1h illustrates how both bound spores and dissociated spores can be captured.

The adsorption of *B. anthracis* to immunoglobulin G labeled magnetic beads has been shown to be increased significantly with the addition of didecyldimethylammonium bromide in pure laboratory standards; however, adsorption efficiencies may decrease by 20–40 % for environmental samples (Yitzhaki et al. 2006). While IMS adsorption efficiencies for environmental samples may be of concern, IMS does have the advantage of being rapid. Researchers (Fisher et al. 2009) developed a rapid IMS-lateral flow protocol for identification of *B. anthracis* spores in milk samples within approximately 40 min. IMS may be an attractive option for detecting *B. anthracis* spores in soil due to its simplicity, speed, and utility for large numbers of samples (Bruno and Yu 1996). "Liquid-phase" immunoassays have been used for spore capture of *B. anthracis* spores from dust by adding anti-*B. anthracis* antibodies to spore suspensions, incubating, and further processing the sample (Hang et al. 2008).

Filtration is another method that has been used to dissociate spores such as *Pasteuria penetrans* and *B. atrophaeus* spores from soil samples (Dabiré et al. 2001; Isabel et al. 2012). *Pasteuria penetrans* spores have been concentrated into the 0- to 20-μm sample fraction, but a significant number of spores were also associated with larger clay aggregates (Dabiré et al. 2001). One study used dual syringe filters to establish rapid filtration separation-based sample processing (Isabel et al. 2012). Their protocol utilized a 5-μm pore-sized filter to separate spores from a variety of matrices including soil, dust, silica, and bentonite. An additional 0.45-μm pore-sized filter was used to concentrate the spores recovered from the 5-μm pore-sized filter. On average for all matrices tested, 68 and 51 % of the *B. atrophaeus* spores were recovered using the capture filtration step only (0.45-μm pore-sized filter) and the dual filter protocol, respectively.

Direct processing

Direct processing protocols include direct culturing of soil and bulk DNA extraction. It has been said that clinical identification of *B. anthracis* is not a problem; it is the presence of organic and inorganic compounds and extraneous bacterial flora (particularly other spore-forming *Bacillus* species) in environmental samples that interferes with *B. anthracis* detection and identification (Bielawska-Drózd et al. 2008). While selective media have been used to isolate other *Bacillus* species from soil (Travers et al. 1987), and DNA extraction has been evaluated for isolation of *B. anthracis* from other matrices such as food, powders, and clinical samples (Panning et al. 2007; Wielinga et al. 2011) or for other bacterial organisms in soil (Jacobsen and Rasmussen 1992), direct processing of *B. anthracis* in soil requires more research. Extensive testing must be done to develop a selective culture medium that

allows differentiation between *B. anthracis* and other *Bacillus* spp. In addition, DNA obtained directly from soil samples must be purified carefully and DNA signature specificity must be carefully selected to ensure species selectivity.

Selective culture media

Although culturing is time consuming and laborious for large sample sets, there are times when it is critical to determine the quantity of viable *B. anthracis* within a sample or to assess the antimicrobial susceptibility of an environmental strain (Tomaso et al. 2006; Luna et al. 2009). Researchers have sought a *B. anthracis*-specific agar-based medium that deters background cultures and other *Bacillus* species, yet allows *B. anthracis* to flourish. Sheep or horse blood is often included within a *B. anthracis*-selective medium to evaluate hemolysis. *B. anthracis* is non-hemolytic, and the agar will remain red surrounding the cultures. Conversely, the near-neighbor bacterium *Bacillus cereus* is hemolytic and produces an enzyme that lyses red blood cells and changes the appearance of the agar surrounding *B. cereus* growth. This review found several culture media selective for *B. anthracis* within the open literature as discussed below.

Mannitol-egg yolk-polymyxin B agar (MEP) has been used as a selective medium (Luna et al. 2005). *B. anthracis* colonies on MEP are colorless with a weak lecithinase production giving an opaque zone just beneath the colony, whereas other organisms turn yellow with mannitol fermentation and are translucent without lecithinase production. While MEP can distinguish *B. anthracis* from a number of *Bacillus* species, MEP is not sufficiently reliable (Luna et al. 2005).

R & F® *anthracis* chromogenic agar (ChrA) has also been used to distinguish *B. anthracis* from other *Bacillus* species (Juergensmeyer et al. 2006; Marston et al. 2008). ChrA includes the substrate 5-bromo-4-chloro-3-indoxylcholine phosphate, which converts to a water-insoluble blue dye in the presence of phosphatidylcholine-specific phospholipase C (PC-PLC). Among *Bacillus* species, only *B. anthracis*, *B. cereus*, and *B. thuringiensis* produce PC-PLC. For *B. cereus* and *B. thuringiensis*, the color change occurs within 24 h, whereas for *B. anthracis*, the color change is seen only after 48 h due to a nonsense mutation that reduces PC-PLC activity and eliminates its hemolytic activity (Juergensmeyer et al. 2006). Selective ingredients within ChrA spiked into soil or other materials have been found to reduce the number of background soil flora capable of growing to approximately 10^3 colony-forming units (CFU) g^{-1} (Juergensmeyer et al. 2006). The color changing properties of *B. anthracis* colonies on the ChrA allowed them to be distinguished easily among the remaining background flora. However, *B. anthracis* colonies are harder to identify when *B. cereus* and *B. thuringiensis* growth is overwhelming (Juergensmeyer et al. 2006).

The utility of Cereus Ident Agar™ (CEI) and Anthrax Blood Agar™ (ABA) has been examined (Tomaso et al. 2006). CEI contains a chromatogenic substrate similar to ChrA. Only the turquoise coloration of non-*anthracis* spp. can be used to discriminate *B. anthracis* from its near-neighbors (Tomaso et al. 2006). ABA is a nutrient medium containing sheep blood and supplements to inhibit many fast-growing organisms. The hemolysin gene of *B. cereus* has been found within *B. anthracis* strains on a few occasions, so hemolytic morphology is not a definitive assessment (Tomaso et al. 2006). *B. anthracis* could be identified appropriately 71 and 72 % of the time on CEI and ABA, respectively, when tested against 92 environmental *B. anthracis* isolates and 132 other *Bacillus* spp. (Tomaso et al. 2006).

Polymyxin B, lysozyme, ethylenediaminetetraacetic acid, thallous acetate (PLET) is another selective medium described in the literature. Bradley et al. (2011) compared PLET agar to Trypticase® soy agar amended with 5 % sheep red blood cells (TSA II) and determined the two media to be comparable (PLET CFU were within 72–77 % of the TSA II CFU counts). In a comparison of PLET with ChrA, PLET was found to be more sensitive and more selective against other *Bacillus* and non-*Bacillus* species than ChrA (Marston et al. 2008). However, PLET and ChrA had similar *B. anthracis* recovery rates for the bacteria when spiked into Texas soil and Arizona test dust. Selective PLET agar has been used to differentiate *B. anthracis* colonies from other organisms, but it was found that PLET was not specific for *B. anthracis* (Moazeni Jula et al. 2007; Vahedi et al. 2009). After confirmatory biochemical testing of multiple *B. anthracis*-like colonies, *B. cereus*, *B. circulans*, *B. megaterium*, *B. subtilis*, and *B. sphaericus* were all found on the original formulation of PLET agar. However, in one study, only approximately 33 % of the *B. anthracis*-like colonies tested were in fact *B. anthracis* colonies (Moazeni Jula et al. 2007). Columbia blood agar with trimethoprim, sulfamethoxazole, methanol, and polymyxin has also been used for recovery of spores from soil samples, but no recovery efficiencies were recorded (Fasanella et al. 2013a).

Researchers have sought to improve the original 1966 formulation of PLET medium for better selectivity (Dragon and Rennie 2001; Luna et al. 2009). In 2001, Dragon and Rennie compared non-selective sheep blood agar (SBA) to PLET and PLET amended with 5 % defibrinated horse blood. Results demonstrated that, although the original PLET was more selective than PLET amended with horse blood, SBA recovered significantly more *B. anthracis* than PLET. These findings led Dragon and Rennie (2001) to conclude that, although PLET is selective for *B. anthracis*, PLET is not an ideal recovery medium and may underestimate the number of spores within a sample. Luna et al. (2009) sought to improve the utility of the original PLET medium further with the addition of the antibiotics sulfamethoxazole (38 μg mL^{-1})

and trimethoprim (2 μg mL^{-1}). Plates were incubated at both 30 and 35 °C. The modified PLET medium was tested against 283 environmental isolates, including 23 isolates of *B. anthracis*, and could be used in a liquid broth or solid agar state. Results indicated that the additional antibiotics in the PLET medium delayed the appearance of resistant *B. cereus* and inhibited the growth of other *Bacillus* species. Additionally, the concentrations of polymyxin B and lysozyme, both components of the original formulation of PLET, were found to be optimal at 15,000 and 150,000 units L^{-1}, respectively. Work-safety regulations in some countries prevent the use of PLET due to the high concentrations of toxic thallium acetate (1.9 mg ml^{-1}) within its composition (Tomaso et al. 2006; Luna et al. 2009). Based upon the breadth of data known regarding the specificity of modified PLET medium, modified PLET medium is the most promising selective culture medium for *B. anthracis* documented within the literature.

A compounding difficulty for spore culturing is the existence of superdormant spores of *Bacillus* species (Ghosh and Setlow 2009, 2010) which require elevated concentrations of germination compounds and/or extended incubation periods before they germinate (Ghosh et al. 2009). Even after a suitable processing or culturing protocol for most spores is employed, any superdormant spores present within a sample might not germinate. Previous work has indicated that *B. anthracis* superdormant spores might react in a manner similar to *B. cereus* and *B. megaterium* superdormant spores. However, no studies were found that specifically outline how to process soil-borne superdormant spores (Ghosh and Setlow 2010).

Direct DNA extraction from bulk soils

Prior to performing PCR analysis, DNA must be extracted from the sample. For direct DNA extraction, a small amount of soil (0.1–10 g) is added to a DNA extraction buffer. Cells from all organisms present in a sample are lysed through both chemical and physical means. DNA-identifying reactions are used to seek, amplify, and detect the DNA segments of interest within the total mass of extracted DNA. The DNA extraction protocol influences the quantity and quality of template DNA available.

DNA can be extracted directly from bulk soils or from spores already removed from the soil. Two studies found that direct DNA extraction produced over 33 times more DNA per gram of soil than indirect HSGS separation and over 100 times more DNA per gram of soil than low-speed centrifugation separation (Roh et al. 2006; Delmont et al. 2011b). While indirect DNA extraction had a reduced concentration of DNA, the overall quality of DNA was increased compared to direct extraction protocols. It has been estimated that as much as 40 % of the total microbial DNA contained within a soil sample is lost during direct DNA extraction, and an additional 30 % can be lost during downstream purification procedures (Lombard et al. 2011). The initial soil conditions also have an effect on the quality and quantity of the DNA extracts. Increased carbon content within the bulk soil sample has been shown to correspond to increased DNA yield (Zhou et al. 1996), while the organic content in the soil is directly proportional to humic acids, known PCR inhibitors (Sjostedt et al. 1997). Therefore, appropriate measures must be taken to reduce PCR inhibitors in soil DNA extracts.

Numerous kits are available from vendors that are specific for DNA extraction from soil samples. There are two critical steps to cellular DNA extraction: cell lysis and DNA separation. The components of most kits are proprietary, but there are a few general types of lysis and DNA separation protocols. Many extraction kits utilize a combination of chemical disruption (detergents) and physical agitation (bead beating) for effective lysis of cellular membranes and release of spore DNA. In one study, 40 freeze–thaw cycles with liquid nitrogen were not sufficient to lyse *B. atrophaeus* spores, but a combination of chemical and physical agitation showed promising lysing efficiency (Kuske et al. 1998). Once released, DNA is often bound to silica filters or magnetic beads for purification. Humic acids, polysaccharides, and urea show solubility properties equivalent to DNA and are often co-extracted, especially at higher pHs (Frostegard et al. 1999; Balestrazzi et al. 2009). Washing steps are utilized to reduce the presence of co-extracted compounds post-lysis before purified DNA is concentrated in an elution buffer. In particular, polyvinylpyrrolidone is used to adsorb inhibiting phenols, including humic acids (Frostegard et al. 1999). The final elution buffer often contains Tris and EDTA to protect the extracted DNA from nuclease activity over time (Frostegard et al. 1999).

While there are a multitude of commercial extraction kits available for soil samples, determining the overall best kit is difficult. This literature search found only three studies that directly compared two or more extraction kits for analyzing *B. anthracis* in environmental soil samples (Dineen et al. 2010; Gulledge et al. 2010; Bradley et al. 2011). Gulledge et al. 2010 concluded that no one kit from the five tested was superior [UltraClean™ Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA); SoilMaster™ DNA Extraction Kit (Epicentre Biotechnologies, Madison, WI, USA); Fast DNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA); MagNa Pure®LC (Roche Diagnostics, Indianapolis, IN, USA); and the Qiagen® BioRobot M48 Workstation (Qiagen, Valencia, CA, USA)]. Bradley et al. (2011) determined that the QIAamp® DNA Blood Mini Kit (Qiagen) was more efficient for Arizona test dust, while the UltraClean® Soil DNA Isolation Kit (MO BIO Laboratories) was more efficient for potting soil. The most comprehensive comparison looked at six commercial DNA extraction kits and found that the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon,

OH, USA) yielded significantly higher amounts of spore DNA from each of the three tested soil types (sand, clay, and loam) compared to the other kits tested (Dineen et al. 2010).

The most commonly used commercial extraction kits for soil samples found in the literature search were the UltraClean® Soil DNA Isolation Kit and the Powersoil® DNA Isolation Kit, both produced by MO BIO Laboratories. Both kits require approximately 90 min for bead-beating lysis followed by a silica spin filter to concentrate the extracted DNA. While the UltraClean® Soil DNA Isolation Kit can process a larger quantity of soil (1.0 vs. 0.25 g), the primary difference between the two kits is the presence of an Inhibitor Removal Technology® within the Powersoil® DNA Isolation Kit. In addition, each kit has a large volume companion that uses the same technology to process 10 g samples. Researchers (Whitehouse and Hottel 2007) compared the two kit technologies and found that the UltraClean® Soil DNA Isolation Kit outperformed the PowerMax® Soil DNA Isolation Kit; however, the differences were minimal (Whitehouse and Hottel 2007). The soil conditions apparently have a pronounced effect on the quality and quantity of extracted DNA. Additional information on DNA extraction kits which have been used for similar biological agents and soils or other sample types can also be found in the literature (Maarit Niemi et al. 2001; Cheun et al. 2003; Luna et al. 2003; Roh et al. 2006; Panning et al. 2007; Saikaly et al. 2007; Whitehouse and Hottel 2007; Balestrazzi et al. 2009; Dauphin et al. 2009; Griffin et al. 2009; Fitzpatrick et al. 2010; Hong-Geller et al. 2010; Irenge et al. 2010; Parachin et al. 2010; Pote et al. 2010; Delmont et al. 2011b; Rose et al. 2011; Wielinga et al. 2011; Isabel et al. 2012; EPA 2013).

Care should be taken when using different lots of DNA extraction kits. If different lots of extraction kits are to be used, the lots should be checked for consistency, quality control measures should be used, and new standard curves should be run with each new lot (Bushon et al. 2010).

Enrichment steps

Enrichment steps have been added to processing protocols to help improve recovery of spores from samples that contain a low density of spores (Sjostedt et al. 1997; Cheun et al. 2003; Gulledge et al. 2010; Patel et al. 2013). Addition of an enrichment medium to the sample allows both germination of spores and growth of vegetative cells. As nutrients are depleted, spore-forming bacteria begin sporulation, while the proportion of vegetative cells and other non-spore-forming bacteria decreases or are killed (Patel et al. 2013). Incubation and heat treatment have been used to kill remaining vegetative cells (Patel et al. 2013). The use of selective enrichment agar significantly lowered the detection limits in three studies (Sjostedt et al. 1997; Cheun et al. 2003; Gulledge et al. 2010). The relatively new process of rapid-viability (RV) PCR also incorporates an enrichment step between two PCR reactions to determine the presence of germinated *B. anthracis* spores rapidly within a collected sample, although, to date, no soil samples have been analyzed using this technique (Kane et al. 2009; EPA 2011).

Purification protocols

Because endospores of *B. anthracis* are highly resistant to unfavorable environmental conditions in comparison to vegetative cells (Dragon and Rennie 2001; Koehler 2009), purification protocols such as heat treatment and treatment with ethanol are used to help improve recovery of spores from soil and may be used during either direct or indirect processing of the sample. Heat treatment is a method of purification that has been used as part of the soil processing protocol to kill off vegetative cells in soil samples while leaving viable spores (Moazeni Jula et al. 2007; Santana et al. 2008; Vahedi et al. 2009; Gulledge et al. 2010; Jain et al. 2011; Patel et al. 2013). *Bacillus* spores have been shown to be resistant to ethanol, therefore ethanol has alternatively been used for removing vegetative cells from the sample and is comparable to heat treatment (Dragon and Rennie 2001).

Conclusions

As evident through this review, a significant amount of work has been done to ascertain the most efficient protocol for processing soil samples for *B. anthracis* detection. Direct and indirect protocols for sample processing were reviewed in detail and both have their associated advantages and disadvantages.

Indirect processing uses multiple steps to separate spores from other organisms and particles prior to analysis and increase the proportion of target spores within the final detected sample; however, spore loss prior to analysis also increases. The presence of a detergent in the aqueous carrier medium was consistently found to improve the separation of spores from soil particles, but no consensus on an optimum aqueous carrier medium could be determined among the reviewed works. Future research focusing on the aqueous carrier medium for processing multiple soil types under uniform dissociation and separation conditions is needed. Spore/soil separation is a critical step in determining the overall recovery efficiency of indirect processing protocols. IMS is an attractive option for separating *B. anthracis* in soil due to its simplicity, speed, and utility for large numbers of samples, but continued work on IMS and its ability to bind *B. anthracis* selectively at low concentrations is needed. The overall utility of HSGS as a separation protocol needs to be determined before HSGS is applied within large-scale projects. Although

novel dual syringe filtration has shown promise for being able to separate spores rapidly from diverse matrices, future work that combines an optimized aqueous carrier medium with the dual filter steps may be needed to increase recovery rates further.

Direct processing utilizes bulk sample aliquots without first separating spores from soil particles and falls under two principal types: culturing *B. anthracis* on selective agar and bulk DNA extraction. When samples are directly processed, there is a potential for background organisms to overwhelm the detection assay and prevent target spores from being observed. Researchers have sought a *B. anthracis*-specific medium that deters background cultures and other *Bacillus* species and yet allows *B. anthracis* propagation and identification. Based upon the amount of specificity testing, modified PLET medium was identified as the most promising selective culture medium for *B. anthracis* documented in the reviewed literature. To date no studies have utilized modified PLET agar as an enrichment step prior to *B. anthracis* detection. Future recovery efficiencies could be dramatically increased with such an effort. There are multiple commercial DNA extraction kits available for bulk soil samples. While there are numerous advantages to using a commercial kit for sample processing, due to the difference in study designs, an overall optimum DNA extraction kit was not determined. A study which compares multiple soil DNA extraction kits uniformly across multiple soil types to determine their overall DNA recovery is needed.

The type of sample processing employed, direct or indirect, depends upon the desired downstream applications (Lindahl and Bakken 1995). For DNA detection assays, direct bulk DNA extraction with suitable DNA purification steps may be more appropriate. However, indirect processing might be more appropriate if viability testing is required. Regardless of whether direct or indirect processing protocols are employed, the overall recovery rates and confidence intervals are critical pieces of information for downstream human health and consequence decisions. As shown through this review, an optimized soil processing protocol with a known recovery rate and associated confidence intervals is needed. A reliable processing protocol would allow for multiple investigators and laboratories to produce high quality uniform results in the event of a *B. anthracis* release. A laboratory evaluation of the processing protocols described in this review is necessary in order to provide a recommendation for a standardized processing protocol.

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