EPA 600/R-10/177 | January 2011 | www.epa.gov/ord

United States
Environmental Protection
Agency

Biological Sample Preparation Collaboration Project: Detection of *Bacillus anthracis* Spores in Soil

FINAL STUDY REPORT

Office of Research and Development
National Homeland Security Research Center

Biological Sample Preparation Collaboration Project: Detection of *Bacillus anthracis* Spores in Soil

FINAL STUDY REPORT

Meranda D. Bradley, Ph.D.

Laura Rose, M.S.

Judith Noble-Wang, Ph.D.

Matthew Arduino, M.S., Dr.P.H.

United States Environmental Protection Agency
Office of Research and Development
National Homeland Security Research Center
Cincinnati, Ohio 45268

Centers for Disease Control and Prevention
National Center for Emerging and Zoonotic Infectious Diseases
Atlanta, Georgia 30329

Disclaimer

U.S. Environmental Protection Agency (EPA), National Homeland Security Research Center and the Centers for Disease Control and Prevention (CDC), National Center for Emerging and Zoonotic Infectious Diseases (Proposed), under IA #DW-75-92259701 (CDC IA# Cl10-001), collaborated in the development of the analysis procedure described here.

This report has been peer and administratively reviewed and has been approved for publication as a joint EPA and CDC document. Note that approval does not signify that the contents necessarily reflect the views of the Agency. CDC and EPA do not endorse the purchase or sale of any commercial products or services. The findings and conclusions in this report are those of the author(s) and do not necessarily represent CDC or EPA.

Questions concerning this document or its application should be addressed to:

Erin Silvestri, MPH
Project Officer
U.S. Environmental Protection Agency
Office of Research and Development
National Homeland Security Research Center
26 W. Martin Luther King Drive, MS NG16
Cincinnati, OH 45268
513-569-7619
Silvestri.Erin@epa.gov

Laura Rose, MS
Centers for Disease Control and Prevention
National Center for Emerging and Zoonotic Infectious Diseases (Proposed)
Division of Healthcare Quality Promotion
Clinical and Environmental Microbiology Branch
1600 Clifton Avenue
Atlanta GA, 30329
404-639-2161
Lmr8@cdc.gov

If you have difficulty accessing these PDF documents, please contact Nickel.Kathy@epa.gov or McCall.Amelia@epa.gov for assistance.

Foreword

Following the terrorist events of 2001, the U.S. Environmental Protection Agency's (EPA) mission was expanded to meet critical needs related to homeland security. Presidential Directives identified EPA as the primary federal agency responsible for the country's water supplies and for decontamination following a chemical, biological, and/or radiological attack. To provide scientific and technical support for EPA to meet this expanded role, EPA's National Homeland Security Research Center (NHSRC) was established. The NHSRC research program is focused on conducting research and delivering products that improve the capability of the Agency to carry out its homeland security responsibilities.

As a part of its long-term goals, NHSRC has been charged with delivery of detection techniques that will enable the rapid characterization of threats, identification of specific contaminants to protect workers, and development of plans for recovery operations. Substantial effort and resources have been invested in the development of molecular assays and culture techniques for pathogens; however, initial sample collection and preparation methodologies lag behind in development. To bridge this critical data gap, EPA collaborated with the Centers for Disease Control and Prevention to develop and optimize an immunomagnetic separation (IMS) method for isolation of *Bacillus anthracis* (BA) spores from soil. The developed BA IMS method will support environmental remediation and recovery activities.

This report summarizes the experimental development of the method and the corresponding study results that move EPA one step closer to achieving our homeland security mission, and our overall mission of protecting human health and the environment.

Jonathan Herrmann, Director
National Homeland Security Research

Acknowledgements

The following individuals and organizations served as members of the Project Team and contributed to the development of this document are acknowledged:

U.S. Department of Health and Human Services
Centers for Disease Control and Prevention

Laura J. Rose (Principal Investigator)
Meranda D. Bradley (Co-Principal Investigator)
Matthew Arduino (QA Coordinator)
Judith Noble-Wang (Project Manager)
Michele Howard
Alicia Shams
Heather O'Connell
Stephen Morse
Leslie Dauphin
Jason Goldstein
Betsy Weirich

U.S. Environmental Protection Agency (EPA)
Office of Research and Development
National Homeland Security Research Center

Sanjiv Shah, (Project Technical Lead)
Erin Silvestri (Project Officer)
Sarah Perkins
Frank Schaefer
Eugene Rice

Naval Medical Research Center

Contents

List of Figures

List of Tables

List of Acronyms

%R	Percent Recovery
ABI	Applied Biosystems by Life Technologies
AIMS	Automated Immunomagnetic Separation
ATCC	American Type Culture Collection
ATD	Arizona Test Dust
BA	*Bacillus anthracis*
BSA	Bovine Serum Albumin
CFU	Colony Forming Units
CFU/g	Colony Forming Units per gram of soil
CDC	Centers for Disease Control and Prevention
COA	Certificate of Analysis
DHHS	Department of Health and Human Services
EDTA	Ethylenediaminetetraacetic Acid
EPA	Environmental Protection Agency
h	Hour
HSGS	High Specific Gravity Sucrose
IgG	Immunoglobulin G
IMS	Immunomagnetic Separation
LRN	Laboratory Response Network
min	Minute
ML	Minnesota Loam
NHSRC	National Homeland Security Research Center
NMRC	Naval Medical Research Center
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline with Tween® 20
PCR	Polymerase Chain Reaction
PS	Potting Soil
QA	Quality Assurance
QAPP	Quality Assurance Project Plan
QC	Quality Control
RCF	Relative Centrifugal Force
RO	Reverse Osmosis
RPM	Revolutions per Minute
RV-PCR	Rapid Viability-Polymerase Chain Reaction
PLET	Polymyxin B, Lysozyme, Ethylenediaminetetraacetic Acid (EDTA), Thallous Acetate

s	Seconds
SD	Standard Deviation
SEM	Scanning Electron Microscopy
SNF	Sonicating and filtering
SNFS	Sonicating, filtering, and settling
SNS	Sonicating and settling
TRF	Time Resolved Fluorescence
TSAII	BBL™ Trypticase™ Soy Agar with 5% Sheep Blood

Executive Summary

The objective of this work was to develop and optimize an automated immunomagnetic separation (AIMS) method for isolation of *Bacillus anthracis* (BA) spores from soil. In this study, the AIMS method for separating BA spores from various soil matrices was developed using the Dynabeads® technology (Invitrogen Corporation, Carlsbad, CA). This involved optimizing paramagnetic beads conjugation with antibodies specific to BA spores, designing an automated program (BeadRetriever™, Invitrogen) and developing standard procedures for separating BA spores from four soil types using AIMS.

IMS technology utilizes paramagnetic beads conjugated with BA spore antibodies (polyclonal) to separate BA spores from spiked soil samples. Specifically, conjugated Dynabeads® MyOne™ beads were used for immunomagnetic separation of BA spores (Sterne 34F2; Colorado Serum Company, Denver, CO) from four soil types; Arizona test dust (Powder Technology Inc, Burnsville, MN), Minnesota loam, potting soil, and sand (sources described below). Both sterile and non-sterile soils were evaluated. Spores were recovered from one gram of each of the soil type spiked with 10^1-10^4colony forming units (CFU). Positive controls consisted of buffer (10X phosphate buffered saline with 0.05%Tween® 20, PBST) spiked with the same inocula, negative controls consisted of soils without added spores. The percent of spores recovered (%R) was determined using the ratio of colony forming units per gram (CFU/g) recovered from the soil to the number of CFU of the spore stock inoculated into the gram of soil. The spore stock was cultured onto trypticase soy agar with 5% sheep blood plates (TSAII; BD, Franklin Lakes, NJ) and polymyxin B, lysozyme, ethylenediaminetetraacetic acid (EDTA)-thallous acetate (PLET) agar (non-sterile soils only).

Although the soils were spiked with spore inocula ranging from 10^1-10^4, the lowest limit of reliable quantitation by AIMS varied. The limit of detection for all sterile soil types, as well as non-sterile sand and Arizona test dust was at least 10^2 spores/g. The limit of reliable quantitation (CFU ≥25 on each plate) for sand and Arizona test dust (sterile and non-sterile) was also 10^2 spores/g. The limit of reliable quantitation for Minnesota loam and potting soil, however, was 10^3spores/g (sterile) and 10^4spores/g (non-sterile), respectively. The mean %R (±SD) of BA spores per gram of soil from inoculum levels 10^2 -10^4 varied by soil type, with 61.39% (±9.67) from sand, 38.08% (±4.38) from Arizona test dust, 29.09% (±5.79) from potting soil and 15.39% (±3.44) from Minnesota loam. The %R from the positive control (PBST) was 59.50% (±7.89).

Several published articles have evaluated various techniques for isolating BA spore DNA from soil (Ryu et al. 2003; Herzog et al. 2009; Gulledge et al. 2010). One frequently cited culture based method for recovering spores from soil is the high specific gravity sucrose (HSGS) plus non-ionic detergent method (Dragon and Rennie 2001). When the HSGS method was compared to the IMS method, the %R using HSGS was lower and the limit of reliable quantitation was 1 to 2 orders of magnitude higher, depending upon the soil type. With the HSGS method, the %R ranged from 0.75% - 9.0 % with soils inoculated with 10^4-10^6 spores/g. The AIMS method was demonstrated to improve the efficiency of separating BA spores from soil as compared to the HSGS extraction method.

1.0 Introduction

The U.S. Environmental Protection Agency (EPA) has identified detection of pathogenic microorganisms in environmental samples following a terrorist attack as a critical component of an effective response. Detection of such pathogens would require development and validation of sampling techniques that could be used by multiple laboratories following a homeland security event. To meet this requirement, EPA's National Homeland Security Research Center (NHRSC), along with other EPA divisions and sister agencies, published a compendium of standard analytical methods, the 6th revision in 2010 (EPA 2010). The compendium contains suggested assays for use by laboratories tasked with performing confirmatory analysis of environmental samples following a homeland security event.

Sample preparation, however, remains the limiting step in the detection techniques available today whether for non-culture methods like PCR (polymerase chain reaction), semi-quantitative methods, or quantitative culture-based methods. Sample preparation includes extraction of the analyte from sample matrix for molecular (e.g., nucleic acid extraction) or culture-based techniques. Extracting pathogens from environmental matrices is challenging because the matrices are composed of non-target biological and chemical analytes. These non-target analytes may interfere (compete) with the extraction of the target analyte, and if present in the extracted product, they can inhibit PCR detection of the target

The Centers for Disease Control and Prevention (CDC), part of the U.S. Department of Health and Human Services (DHHS), has extensive knowledge of potential biological hazards. In this project, CDC, in collaboration with EPA, developed and improved methods for extraction of *Bacillus anthracis* (BA) spores from soil. Immunomagnetic separation (IMS) technology utilized tosylactivated beads conjugated with BA spore antibodies (polyclonal) to separate BA spores from spiked soil samples. IMS enables both concentration and purification of spores from soil samples. The IMS method for separating BA spores from various soil matrices in this study was developed using the Dynabeads® technology (Invitrogen Corporation, Carlsbad, CA). Due to the size of BA spores (1-2 µm), Dynabeads® MyOne™ beads (1 µm in diameter) were conjugated with antibodies specific for BA spores (obtained from the Naval Medical Research Center (NMRC, Silver Spring, MD)). Conjugated Dynabeads MyOne beads are added to a slurry of BA spores and soil to allow binding of the spore antigens to the antibodies coating the beads Spore separation is performed in a BeadRetriver™ Automated Immunomagnetic Separation Benchtop System (Invitrogen). The magnetic beads, along with the bound target, are collected using a magnet. The excess solution and most of the soil is left behind, leaving only the beads with spores attached to the magnet. The beads are then washed to remove inhibitors and any remaining soil. The optimum bead conjugation parameters for the IMS protocol were determined in this investigation. The percent recovery (%R) of IMS method was compared to that of the published method for extraction of spores from soil, the high specific gravity sucrose plus non-ionic detergent method (HSGS, Dragon and Rennie 2001).

2.0
Materials and Methods

2.1 Spore preparation

Bacillus anthracis Sterne 34F2 spores (BA) (Colorado Serum) were grown on soil extract peptone beef extract agar (Atlas 1996) at 35°C for seven days, harvested with sterile reverse osmosis (RO) water, and concentrated by centrifugation at 5000 RCF (relative centrifugal force) for 15 min. Centrifugation and washes were performed three times. The pelleted spores were placed in 50% ethanol (25 mL) at room temperature on a shaker table at 100 RPM (revolutions per minute) for 1 h. The suspension was centrifuged and washed with sterile RO water an additional 5 times and the spores were suspended in 5mL sterile RO water for storage at -70 °C. Spore preparations were validated by culture and hemocytometry. The purified spore suspension density was determined by optical density and culture.

2.2 Inoculum preparation

The inoculum was initially prepared by diluting the frozen spore stock in sterile RO water to attain a concentration of 10^8 spores/mL. This standard was diluted in series with RO water, to make four spore concentrations; 10^4, 10^3, 10^2 and 10^1 spores/mL. These spore suspensions were quantified by culturing on TSAII (BBL™ Trypticase™ Soy Agar with 5% Sheep Blood, Becton Dickinson Microbiology Systems, Franklin Lakes, NJ) at 35°C for 18 h. Colony forming units (CFU) were counted and used to determine the number of spores for inoculation of soils. A pilot experiment comparing spore suspensions diluted in sterile RO water and in phosphate buffered saline, pH 7.4 with 0.05% Tween® 20 surfactant (PBST) showed that the suspension in RO water yielded fewer CFU than the spores suspended in PBST. The PBST most likely assisted in disaggregating spores. Thereafter, the spore inoculum consisted of spores suspended in and diluted in series in PBST.

2.3 High specific gravity sucrose extraction (HSGS)

In a 15 mL conical tube, autoclaved soil (2.5 g) was spiked with 10^4-10^6 BA spores. Sucrose extraction solution (12 mL of 1.22 g/mL sucrose/Triton®X-100 surfactant) was added to each soil sample. The mixture was shaken by hand for 1 min, placed on a shaker at 75 RPM for 15 min, and centrifuged at 850 RCF for 45 s. The supernatant (3mL) was transferred to a new 15 mL conical tube containing 6 mL filtered 1% bovine serum albumin in 0.01 mol/L phosphate buffered saline (BSA/PBS) and centrifuged in a swing bucket rotor at 5100 RCF for 10 min. The pellet was resuspended in 1 mL filtered 50% ethanol, gently agitated at room temperature for 60 min, and centrifuged at 5100 RCF for 10 min. The pelleted spores were resuspended in 1 mL 1% BSA/PBS, 0.10 mL of purified samples were spread onto TSAII or PLET (Polymyxin B, Lysozyme, Ethylenediaminetetraacetic Acid thallous Acetate) agar plates, and incubated at 35°C for 24 - 48 h (Dragon and Rennie 2001).

2.4 Soil inoculation

Four soil types were obtained for this project; Arizona test dust (Powder Technologies, Inc.), potting soil (Home Depot, Atlanta, GA), Minnesota loam (Eagan, MN), sterile sand (Fisher Scientific, Sewanee, GA, cat# AC61235-5000), and non-sterile sand (Destin, FL).

The soil physical and chemical characterizations were performed by the Plant, and Water Laboratory at the University of Georgia, Athens, GA (Table 1).

Table 1. Physical and Chemical Properties of Soils

Soil Type	Lime buffer capacity[1]	Soil pH[2]	Equivalent. water pH	Base saturation[3] (Percent)	Cation exchange capacity[4]
Arizona test dust	NA[5]	8.08	8.68	100.00	29.87
Minnesota loam	371	4.51	5.11	61.94	4.27
Sand	NA	7.87	8.47	100.00	0.22
Potting soil	618	7.46	8.06	100.00	26.99

Soil sample	Mineral Composition of Each Soil, ppm						
	Calcium	Cadmium	Chromium	Copper	Iron	Potassium	Magnesium
Arizona test dust	5423	<0.01	<0.02	0.02	0.58	117.90	201.90
Minnesota loam	377	0.07	0.05	0.21	34.30	33.46	75.94
Sand	28	0.02	<0.02	0.04	7.21	4.89	5.56
Potting soil	4020	<0.01	0.06	0.11	3.47	95.44	897.10

Soil sample	Mineral Composition of Each Soil, ppm					
	Molybdenum	Sodium	Nickel	Phosphorus	Lead	Zinc
Arizona test dust	0.05	176.30	0.06	6.69	<0.10	0.79
Minnesota loam	<0.02	9.53	0.42	14.16	0.10	2.20
Sand	<0.02	4.15	0.07	0.71	<0.10	0.62
Potting soil	<0.02	24.99	0.24	30.92	<0.10	3.68

Soil sample	Percent Composition of Each Soil			
	Sand	Silt	Clay	Total carbon
Arizona test dust	0.00	86.00	14.00	0.65
Minnesota loam	46.00	50.00	4.00	1.02
Sand	36.00	62.00	2.00	0.02
Potting soil	ND[6]	ND	ND	ND

[1] ppm $CaCO_3$/pH
[2] Measurement of pH in dilute salt (http://www.caes.uga.edu/publications/caespubs/pubcd/C875.html)
[3] Percent of soil exchange sites occupied by basic cations
[4] Measures the soils ability to retain nutrients; unit of measurement millequivalent/100g of soil
[5] Not applicable
[6] Not done

Soils were inoculated as is, or sterilized (autoclaved at 121ºC, 32.5 psi for 30 min). One mL of spore suspension was added to 1 g of soil, and 2 mL 10X buffer (positive control). Two to 3.5 mL of 10X PBST was added to the soils to form a slurry. An evaluation of two buffer concentrations (1X and 10X PBST) for use in making the slurry was conducted and the 10X was found to provide the optimum spore recovery. The soil slurry and spiked buffer were rotated end over end for at least 10 min, allowed to settle for 10 min, and then filtered through a 30 µm pre-separation filter (Miltenyi Biotec, Auburn, CA), before IMS processing (see Appendix C for final protocols/methods for real-world sample analysis).

2.5 Immunomagnetic Separation

2.5.1 Conjugation of tosylactivated paramagnetic beads

BA anti-spore polyclonal antibodies (goat affinity anti-anthrax antibody (IgG, lot# 031104-01 and lot# 2610006-01) were acquired from NMRC (Bethesda, MD), purified in-house (CDC Core Facility), and initially conjugated by Invitrogen to tosylactivated magnetic beads. Obtaining the conjugated beads was delayed by several months because of the merging of Invitrogen Corporation with Applied Biosystems Inc.™ (ABI) by Life Technologies, and subsequent reorganization of the company. In the reorganization, ABI eliminated the division that performs the conjugation, so arrangements were made for the CDC Core Facility to perform the conjugations. The conjugation method (see attached Appendix: Protocols/methods for real-world sample analysis) was adapted from Invitrogen's "Dynabeads MyOne™ Tosylactivated Product Description and Instructions for Use" (Invitrogen 2006)." Two different temperatures and times of the reactions were evaluated (37°C for 24 h and 20°C for 48 h), as well as two different antibody to bead ratio (50 µg/mg and 40 µg/mg). Optimum conditions for conjugation were found to be 37°C for 24 h with an antibody to bead ratio of 40µg/mg.

2.5.2 Exploratory Bead Retriever methods

Two programs were designed for the immunomagnetic BeadRetriever system (Invitrogen Corporation, Carlsbad, CA) to optimize recovery of spores from sand and Arizona test dust (the soils with the largest and smallest particle size, respectively). The alternative and the final programs were developed using a manual magnetic bead retriever. The parameters evaluated for optimum recovery were (a) the volume of slurry necessary, (b) the time needed for homogenous mixing, and (c) the wash times. The results of these evaluations were used to configure the KingFisher™ software (Thermo Fisher Scientific, Waltham MA) which operates the automated BeadRetriever system. Once the parameters were configured, a pilot run using the automated BeadRetriever to separate BA from spiked sand and Arizona test dust was performed. Approximately 3 mL total of soil slurry was divided into 3 equal portions and placed into wells 1-3 (~1 mL per well) within a tube strip (Figure 1). Each of these 3 tubes contained 20 µL of antibody-conjugated MyOne beads. The automated Bead Retriever™ was started and the beads and soil slurries were mixed vigorously. The instrument then collected the beads, along with the bound target BA spores, from tubes one through three, into a fourth tube in the tube strip where they were washed with PBST. The washed automated immunomagnetic separation (AIMS) product, i.e. the magnetic beads with bound target BA spores, was then transferred to the fifth and final tube within the tube strip and resuspended in 400 µL of PBST. The software program is available at Life Technologies™.

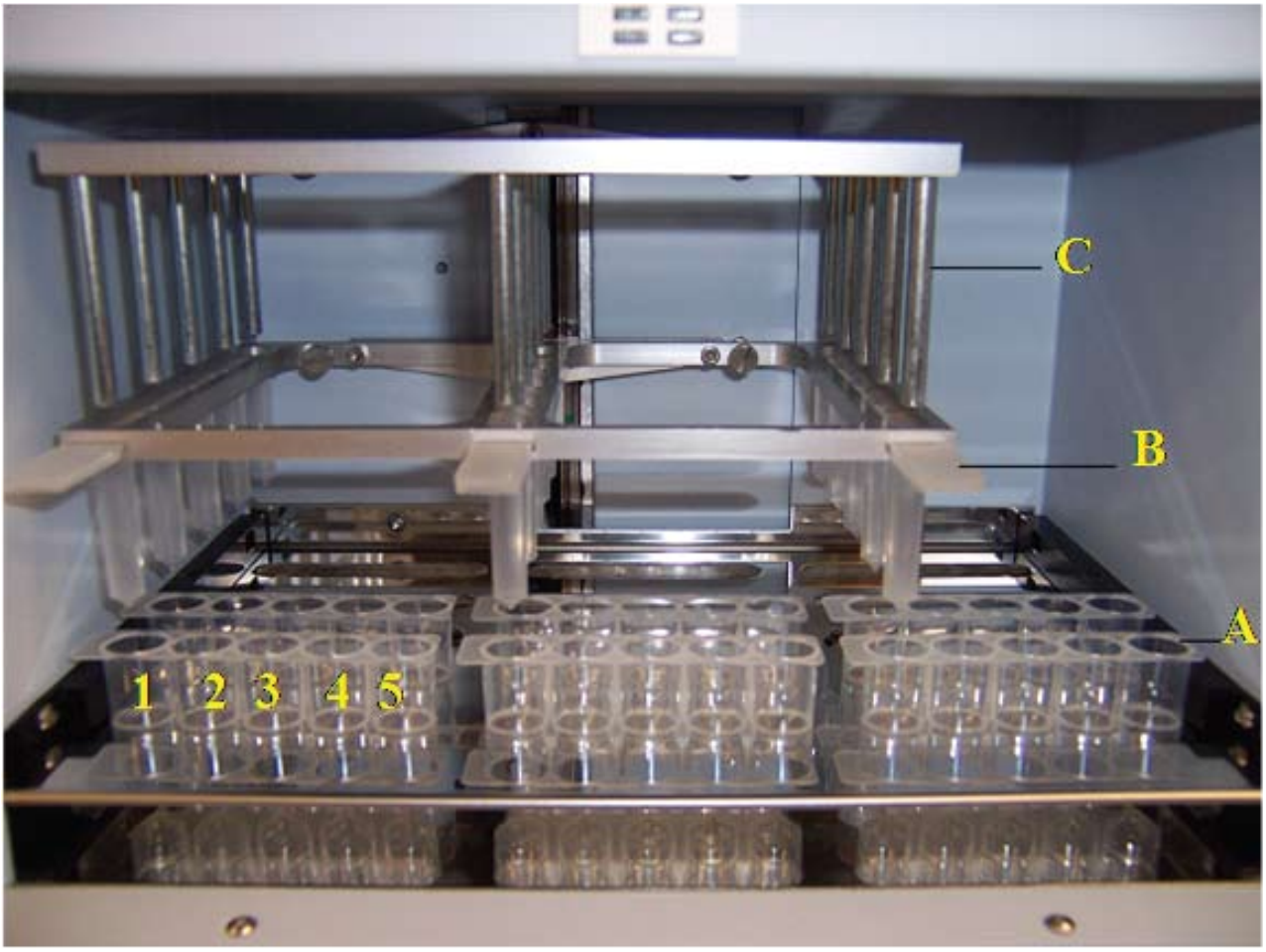


Figure 1. BeadRetreiver™ Automated immunomagnetic seperation bench top system

The BeadRetriever system is capable of processing up to 15 samples at a time. Soil and spore slurry samples are pipetted into tube strips (A) which consists of 5 wells linked together and held on a stationary platform. The samples are processed by a moving platform consisting of plastic tip comb holders (B) and magnetic rods (C) which slide down into the plastic comb holders during AIMS processing. Antibody conjugated paramagnetic beads along with bound spores adhere to the plastic enclosed magnetic rods in the first three wells and are mixed by an up and down motion of the magnetic rods. The platform moves the magnetic rods with captured beads and spores up, over, and releases the collected beads into the fourth well in the tube strip. In the fourth well the beads with bound spores are washed in PBST by a similar mixing action. The final washed bead with bound spores are then collected by the magnetic rod and placed into the fifth well of the tube strip containing PBST (see http://tools.invitrogen.com/content/sfs/manuals/1189_BeadRetriever_Manual.pdf).

2.5.3 Exploratory Method: Miltenyi Biotec Immunomagnetic Separation (IMS)

Miltenyi Biotec (Auburn, CA) super-paramagnetic particles, approximately 50nm in diameter, were also evaluated as an alternative to the MyOne beads. It was thought that the nano-sized beads utilized in this IMS system would increase magnetic retrieval and spore recovery by allowing more beads to bind to each spore. We evaluated the manual IMS system OctoMACS™ (Miltenyi Biotec) along with their anti- mouse IgG bead for its ability to recover 10^4 spores/g of soil. The mouse IgG beads were pre-complexed with monoclonal (mouse) BA spore antibody and used for manual immunomagnetic separation of BA spores from sand. Recovery using this manual system was well below the recoveries observed using the manual Invitrogen® IMS system and the HSGS. A preliminary study comparing MyOne beads conjugated with polyclonal antibodies and MyOne beads conjugated with monoclonal antibodies revealed that the recovery was not sufficient using the

BA monoclonal (mouse) antibody. The BA polyclonal (goat) antibody could give better results, however, the Miltenyi cell separation technology does not have an appropriate microbead strategy available for goat derived antibodies. Nonetheless, we did incorporate the Miltenyi Biotec pre-processing step (filtering the soils with a 30 µm pre-separation filter) into the final AIMS method.

2.5.4 Final AIMS method: Applied Biosystems Inc., IMS

Three mL of the spiked soil slurry was dispensed into three tube strips containing 100µL of 10X PBST and 20 µL of MyOne conjugated beads. Three or more replicates of each soil were performed. The mixture was then gently agitated for approximately 30-35 min. The beads, along with bound spores, were collected into one tube, washed in 1 mL of 1X PBST for approximately 10 min, and concentrated in 300 µL of 1X PBST. This magnetic bead retrieving process was accomplished with the BeadRetriever. This AIMS system utilizes inverse magnetic bead processing technology, which rather than transferring liquids, transfers paramagnetic beads through a series of tubes containing specific reagents with the aid of internal rather than external magnetic rods. The spores and beads captured by AIMS were diluted in series and using the spread plate technique cultured onto TSAII or PLET plates (non-sterile soils only) in triplicate and incubated at 35°C for 24 h. Colonies were counted after 24 h and the %R was determined using the ratio of CFU/g of soil to the CFU of the inoculum (see final protocols/methods for real-world sample analysis). The eluent was stored at -20°C for detection by real-time PCR.

2.6 Spore recovery by culture

The AIMS product was diluted in series and spread plated (100 µL each) onto TSAII and PLET agar, incubated at 35°C overnight and the colonies were then counted. The CFU/g of soil was determined and the %R calculated, relative to the inoculum. In order to confirm that colony counts on both media were comparable, a growth challenge test was conducted. TSAII and PLET agar were inoculated with BA spores with a low level of challenge spore concentration (10-100 CFU). The plates were incubated at 35°C overnight and counted the next day**.** Media acceptance requires that the average number of CFU on the PLET agar must fall within ±70% of the average number of CFU found on the TSAII plates (adapted from USP 32, NF 27, Chapter 1227 *in* U.S. Pharmacopeia 2009).

2.7 Spore detection by real-time PCR

In addition to culture, real-time PCR was evaluated for the ability to detect the spores present in the product of the AIMS. The DNA was extracted from the BeadRetriever product initially using a simple boil prep procedure (100°C for 5 min). The real-time PCR was performed using an ABI 7500 Fast DX platform. The cycling parameters were programmed according to the LRN™ protocol "Detection of *Bacillus anthracis* DNA by fluorogenic 5' nuclease assay using the Applied Biosystems® 7500 Fast DX Real-Time PCR System." The primers and probes were obtained from the LRN™ and are described in Hoffmaster et al. (2002). The only modification made was that the PCR was performed using 1X PCR master mix [Light Cycler FastStart PLUS DNA Master Hybprobe (Roche Molecular Biochemicals, Indianapolis, IN)]. We evaluated two DNA extraction kits, the MO BIO UltraClean® Soil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA) and the QIAamp DNA Blood Mini Kit (Qiagen Inc., Valencia, CA), for DNA extraction from the AIMS product. Both kits are supported by the LRN™. The MO BIO soil kit was used by collaborators at Lawrence Livermore National Labs. Both kits were evaluated for their efficiency of DNA purification from AIMS products (from spiked potting soil and Arizona test dust with 10^3 spores/g of soil).

3.0
Results and Discussions

3.1 High specific gravity sucrose (HSGS) extraction

This study was designed to improve existing methods to extract BA spores from soil samples. The HSGS method exploits the difference in specific gravities between BA spores and spores from other organisms and from background materials in order to isolate the spores. The HSGS method is inexpensive relative to immunomagnetic separation; however, we found the technique to be highly variable. Dragon and Rennie (2001) found that using HSGS, the %R of BA (ATCC 4229) spores (2 x10^5 to 8x10^5) seeded into 2.5 g of sterile field soil was 4.5%. Using this sucrose flotation method, we recovered 0.75 - 9% BA spores on TSA II from 1 g of spiked sterile soils (Figure 2). As seen in Figure 2, the best average %R for each soil were 5% (10^6 spores/g) from Arizona test dust, 3.7% (10^4 spores/g) from Minnesota loam, 9% (10^4 spores/g) from potting soil, and 5.8% (10^5 spores/g) from sand. The standard deviations decreased slightly as the inoculum level increased. We also observed that this method yielded the highest %R from potting soil, regardless of the inoculum level tested. Dragon and Rennie (2001) also found potting soil to yield the highest %R when compared to spiked field soils.

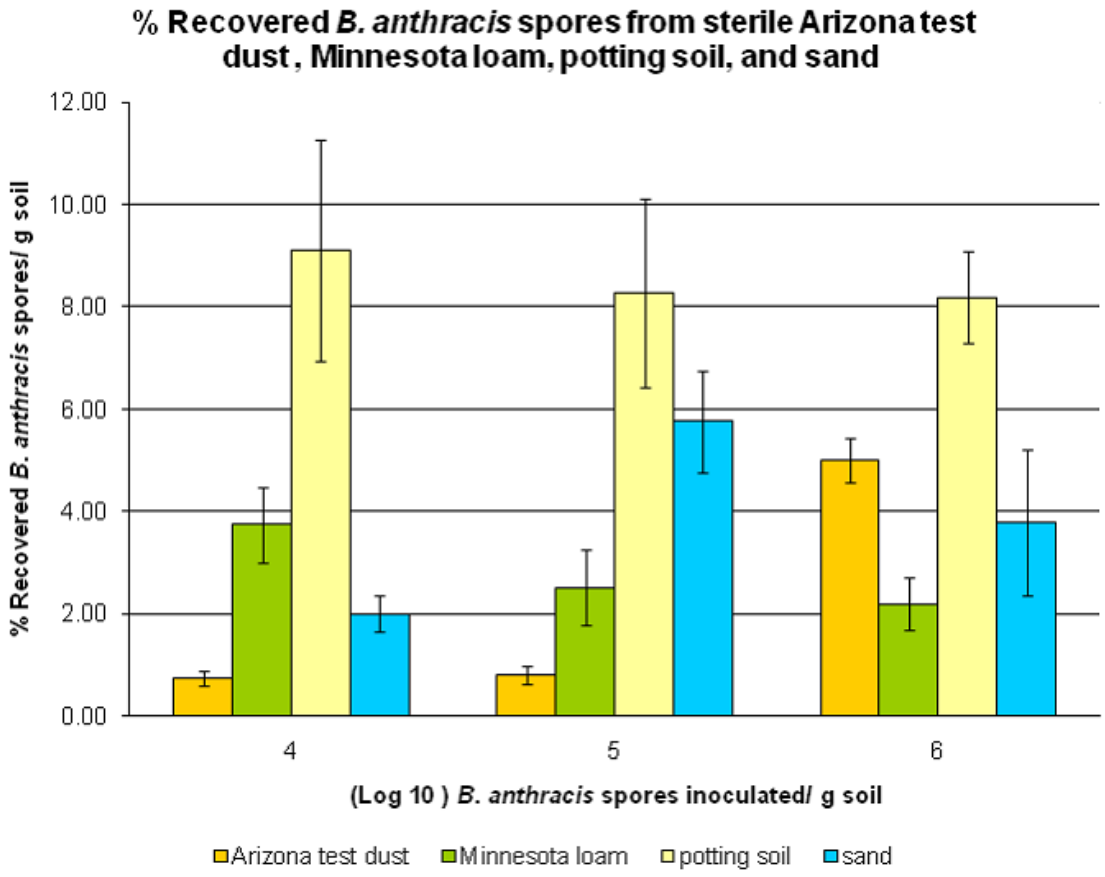


Figure 2. The mean percent of spores recovered using HSGS plus non-ionic detergent extraction (Y-axis) (n≥3) is shown for each spiked BA spore inoculum level (shown on the X-axis) and for each sterile soil type: Arizona test dust, Minnesota loam, potting soil, and sand. Error bars shown in graph represent standard deviations.

3.2 Immunomagnetic Separation

3.2.1 Exploratory methods and challenges

3.2.1.1 Alternate IMS system

Soon after obtaining the BeadRetriever system from Invitrogen, a merger between Invitrogen and Applied Biosystems led to reconstruction of the division responsible for programming the BeadRetriever system and conjugating the MyOne beads with our antibody. This restructuring impeded the programming of the BeadRetriever system and delayed obtaining conjugated beads; therefore, an alternative IMS method was explored. The Miltenyi Biotec IMS system was selected as an alternative because it uses nanoscale paramagnetic beads. Theoretically, smaller beads should allow for multiple beads to bind to each BA spore. This is a different approach than the Invitrogen system's 1µm MyOne beads in which fewer beads bind per spore simply because the spore and the bead are of equivalent size. Unfortunately, all spore recovery attempts with the Miltenyi system failed, most likely due to the use of monoclonal, rather than polyclonal, antibodies.

Fortunately, we were able to get the BeadRetriever King Fisher™ Software programmed by Applied Biosystems technicians, and were able to continue with the BeadRetriever instrument as originally planned.

3.2.1.2 Conjugation of tosylactivated paramagnetic beads

The performance of beads conjugated at 40 µg IgG/mg beads and 50 µg IgG/mg beads were evaluated using the three soil types with lower spore recovery rates. Results showed that the %R of spores from soil was greater when the beads were conjugated with an antibody to bead ratio of 40 µg IgG/mg (Figure 3). The one unexplained exception was potting soil spiked with 10^2 BA spores. The %R of spores from positive controls (buffer) was greater when using beads conjugated with an antibody to bead ratio of 50 µg IgG/mg. We found that conjugation at the different times and temperatures evaluated (20°C for 48 h and 37°C for 24 h) did not affect the %R (data not shown). We therefore chose to conjugate the antibody to the beads at 37°C because of the shorter incubation time.

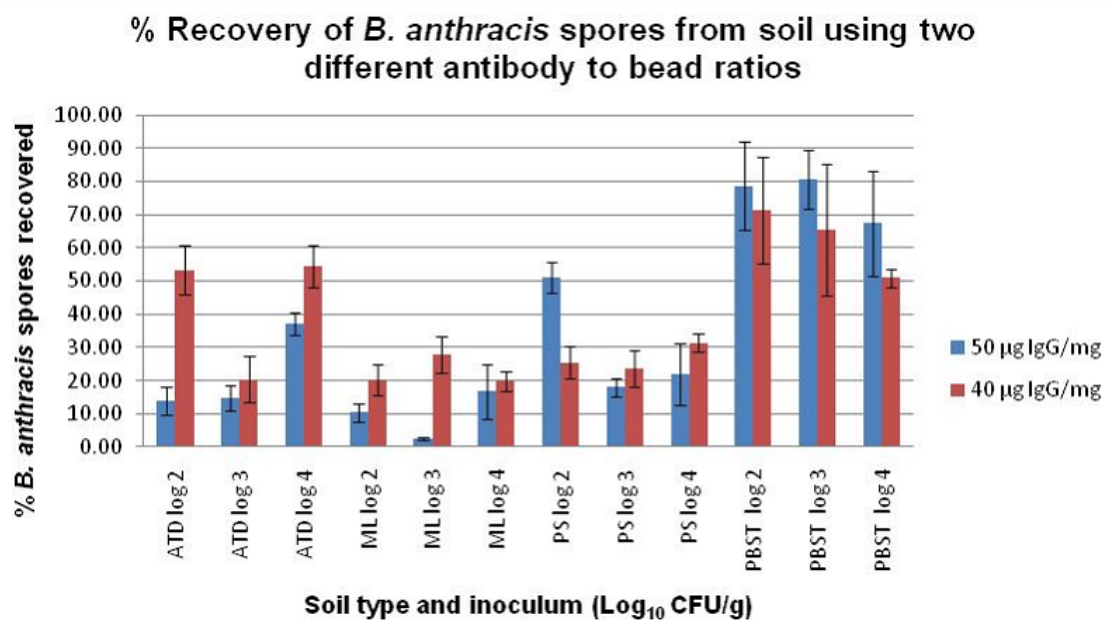


Figure 3. Comparison of the mean percent of BA spores recovered (n≥3) from Arizona test dust (ATD); Minnesota loam (ML); potting soil (PS), and PBST (buffer positive control) spiked with 10^2-10^4 spores/g of soil using conjugated bead concentrations of 50 µg IgG/mg and 40 µg IgG/mg.

During the course of the experiments, we received more antibodies from NMRC with a different lot number than previously used. In order to confirm comparable performance, we evaluated the two antibodies, conjugated to the beads in the same manner (37°C for 24 h), for their ability to recover spores from soil and buffer. The results indicated little difference in the %R when using beads conjugated with either of the two lot numbers of antibody.

3.2.2 Final method and challenges

Our optimization of the AIMS protocol revealed that separation of the BA spores from soil was best accomplished by preprocessing the soil slurry samples, sonicating and vortexing (3 min each) the spore slurry to disrupt clumps, filtering the slurry through a 30 µm pore size filter, allowing the slurry to settle, and removing the liquid from the top of the sediment and placing it in the Bead Retriever tube tray. The preprocessing procedures improved the recovery of BA spores in all soil types and decreased the amount of unrecovered spores in the wash. These improvements were most noticeable with Arizona test dust (Figure 4).

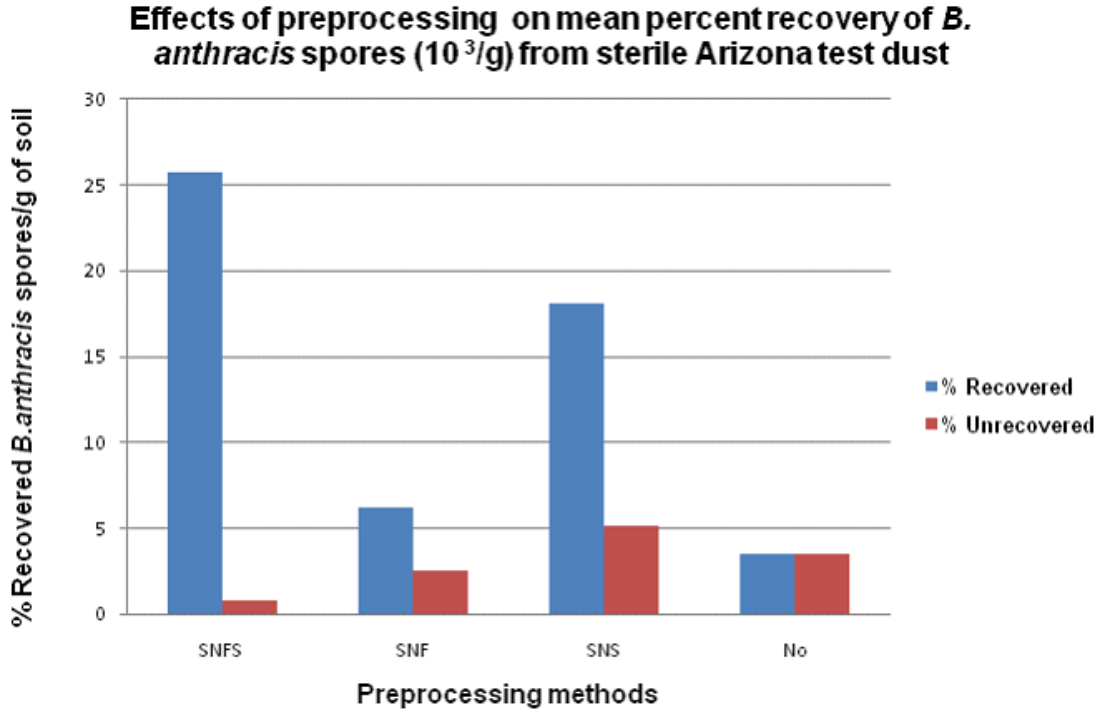
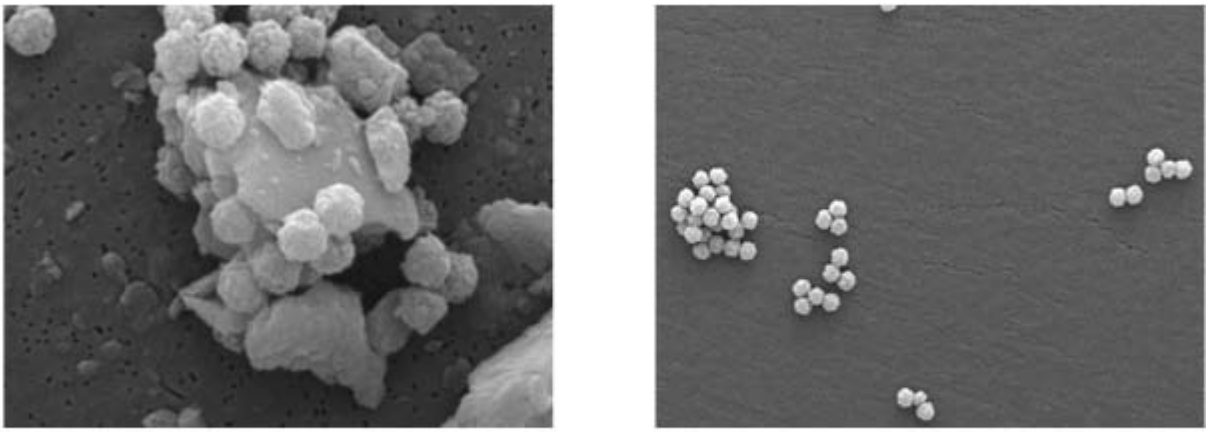


Figure 4. Effects of Preprocessing on the mean recovery of BA spores from sterile Arizona test dust. (Experiment done in duplicate.) Unrecovered spores = percent of spores recovered in IMS wash tube. Preprocessing abbreviations: SNFS – sonicating, filtering, and settling, and; SNF-sonicating and filtering; SNS- sonicating and settling and, No- no preprocessing.

The AIMS system program was designed for the immunomagnetic separation of spores from Arizona test dust and sand. Each program specifies the amount of time the beads are allowed contact with the soil, the amount of vigor used in agitation of the slurry and beads, and the length of time for the wash step. The entire AIMS procedure consists of two steps: soil extraction which includes all of the preprocessing of samples and automated immunomagnetic separation by the BeadRetriever system.

When the conjugated beads are mixed with the soil, the critical step is the binding of the bead to the target BA spore. This binding depends upon the specificity of the conjugated antibody to the spore and the contact time between the beads and the soil slurry. The antibody specificity was tested previously, by CDC's Division of Bioterrorism and Preparedness Response, using time resolve fluorescence (TRF). The results showed that the antibody can differentiate between closely related and nonrelated bacterial strains (see appendix B). In the TRF study, only BA spores, not vegetative cells, were screened.

Our data show that the AIMS method recovered other organisms in addition to BA. It is possible that the soil aggregates contain metallic or magnetic particles that are attracted to the magnetic rod during AIMS processing. These soil aggregates may have other bacterial species adhering to them. In order to determine if this were true, we looked at Arizona test dust, which contains iron oxide, and sand using scanning electron microscopy (SEM). Observation by SEM of non-sterile Arizona test dust processed AIMS without any BA spores revealed possible magnetic particle aggregating with MyOnebeads and other particles (Figure 5).



A B

Figure 5. Scanning electron microscopy image of AIMS (automated immunomagnetic separation) processed non-sterile Arizona test dust (A) and sand (B) (Both without BA spores and at 10,000× and 3,500× respectively). (A) In Arizona test dust, clumps or aggregates containing beads and other particles are seen. (B). In sand, only beads are seen.

Very few other organisms were cultured when recovering spores from sand and Arizona test dust, but the numbers were significant when recovering from potting soil and Minnesota loam. For this reason, the use of the selective agar (PLET) for recovery of BA from unknown soil samples is recommended. A comparison of recovery on both media (PLET and TSAII) was conducted, and the results demonstrated that recovery of BA on PLET agar was comparable to growth on TSAII agar. Specifically the PLET CFUs were within 72-77% of the number of CFUs found on TSAII agar after overnight growth at 35°C for 48 h.

We also noted that the concentration of PBST buffer used in making the soil slurry influenced the %R. Using 10X PBST in culturing methods reduced clumping and increased the %R when compared to 1X PBST. Results from a preliminary study showed the %R from Arizona test dust spiked with 10^3/g BA spores were 9.23% and 0.52% when 10X PBST and 1X PBST, respectively, were used to make the slurries.

3.2.2.1 Soil and Inoculum level evaluations

The average %R for each inoculum level and preprocessed soil type is shown in Table 2. The number of spores recovered from all soils inoculated with 10^1 spores and some soil types inoculated with 10^2 spores were below the limit of reliable quantitation (<25 CFUs recovered by plate count). Therefore, the average %R is only discussed for inoculum levels 10^2-10^4 in this report. The %R was found to be optimal within the 10^2-10^4 range of inoculum.

The number of spores recovered does not necessarily increase with increasing inoculum (Table 2). According to the MyOne Tosylactivated product description and instructions for use (Invitrogen 2006), the amount of ligand per mg of beads is optimal at 40 µg ligand per mg beads. Thus it seems at higher spore (ligand) concentrations, the target sites on the beads become saturated and can no longer bind additional spores.

Table 2. AIMS Mean %R (SD) of BA Spores Recovered From All Four Sterile and Non-Sterile Preprocessed Soil Types and PBST. Percent recovery determined from culture on TSAII and PLET Agar.

Soil Types	Arizona test dust	Minnesota loam	Potting soil	Sand	Phosphate Buffered Saline with Tween® 20
	Avg. % Recovered BA spores/g (Std. Deviation)				
Inoculum level	Sterile TSAII				
10^4	39.44 (4.57)	14.46 (2.18)	31.28 (2.75)	61.95 (10.02)	56.12 (8.98)
10^3	35.32 (1.64)	16.89 (3.33)	23.64 (5.53)	55.67 (3.96)	57.22 (18.42)
10^2	25.05 (5.29)	*14.81 (4.92)	*31.24 (6.45)	68.01 (11.25)	67.82 (11.70)
10^1	*25.25 (5.05)	*24.23 (24.24)	*50.51 (0.00)	‡25.67 (2.14)	*173.86 (117.27)
Overall**	38.08 (4.38)	15.39 (3.44)	29.09 (5.79)	61.39 (9.67)	59.50 (14.88)
	Nonsterile TSAII				
10^4	51.07 (7.39)	10.09 (1.76)	19.52 (4.80)	54.99 (9.02)	61.54 (19.03)
10^3	28.84 (5.03)	*10.86 (1.94)	18.52 (2.20)	56.23 (5.87)	62.96 (3.56)
10^2	48.82 (9.55)	*10.1 (3.64)	*16.84 (3.55)	53.53 (11.25)	70.38 (7.59)
10^1	*131.3 (113.83)	*116.16 (7.14)	*50.51 (0.00)	*202 (0.00)	*148.2 (123.8)
Overall**	41.22 (12.74)	10.35 (2.06)	18.71 (3.64)	55.32 (7.74)	64.48 (16.36)
	Nonsterile PLET				
10^4	37.60 (6.62)	13.31 (3.40)	15.57 (6.42)	53.87 (14.42)	70.99 (19.53)
10^3	24.35 (12.25)	15.49 (5.79)	*20.09 (16.96)	48.60 (18.90)	53.94 (19.40)
10^2	20.2 (9.55)	*29.63 (10.22)	*17.51 (5.74)	51.18 (4.98)	60.00 (8.64)
10^1	*45.45 (28.56)	*121.21 (14.28)	16.84 (0.00)	*117.85 (119.04)	*173.9 (133.01)
Overall**	29.43 (11.75)	17.07 (10.48)	17.78 (11.81)	51.23 (15.32)	62.64 (19.13)

‡ no preprocessing
below limit of reliable quantitation (<25 CFU)
**overall % recovered excluding 10^1 data
Sand 10^1 sterile is from experiment 10-14-2008 no preprocessing was done

3.3 Spore extraction and rapid viability PCR detection

In evaluating the DNA extraction kits, we found that neither kit was able to detect the chromosomal target, most likely because the inoculum was at or below the limit of detection. The extraction efficiencies of the two kits were affected by the soil type. The Qiagen Blood kit extracted DNA from Arizona test dust more efficiently than the MO BIO soil kit. The MO BIO soil kit extracted DNA from potting soil more efficiently than the Qiagen Blood kit.

Both DNA extraction kits were able to extract DNA from the AIMS products, although the results were not consistent between replicates, and the level of detection was still >10^3 spores/g of soil. Additionally, because of the chemistry of the MO BIO soil kit (personal communication with the MO BIO technical support department), a consistent DNA extraction of spores from a suspension of spores in PBS, necessary for a positive control, was not possible.

Considering all of these challenges with the DNA extraction kits, we decided that the best way to reduce the level of detection and to get consistent DNA extraction was by using the rapid viability PCR (RV-PCR) method combined with an enzymatic lysis protocol developed by scientists at Lawrence Livermore Laboratories. The RV-PCR method includes an incubation and replication step to increase the DNA copy number. The enzymatic lysis uses *Bacillus cereus* Zebra Killer lysin, currently in use within another laboratory at CDC, and has been extensively evaluated and found to consistently lyse vegetative BA cells.

Because of the challenges with the DNA extraction from spores, the timeline for the RV-PCR portion of the project was extended to December 2010. The protocol has been finalized and work is ongoing. A separate report will be submitted in 2011 that will include the RV-PCR results and recommendations.

4.0
Conclusion

An optimized AIMS method was successfully developed. The optimized method improved the %R of BA spores from four soil types and lowered the limit of quantitation, as compared to the extraction method, HSGS. Percent recovery of spores from soils inoculated with 10^2 to 10^3 spores/g with the AIMS method ranged from 15% to 68% as compared to ≤9% with the HSGS (range 10^4 – 10^6 spores/g) method. When the number of spores in the sample was greater than 10^3, the amount of target sites on the beads could have become saturated resulting in an inability to bind additional spores. The addition of pre-processing steps improved spore recovery from soil. The pre-processing steps were vortexing, sonication, filtration and allowing for soil to settle before AIMS processing. The %R of spores from soils with more organic matter and background organisms (i.e., Minnesota loam and potting soil) was lower than soil types with less organic matter and fewer background organisms (i.e., Arizona test dust and sand). In cases where soil types contain high levels of background organisms, PLET agar was helpful as a selective agar, but an additional day of incubation was required before countable colonies were visible. The limit of detection of the AIMS may be enhanced with the addition of the RV-PCR method.

5.0 References

Atlas R M. 1996. *Handbook of Microbiological Media.* 2nd edition. New York, NY:CRC Press.

Dragon DC, Rennie RP. 2001. Evaluation of spore extraction and purification methods for selective recovery of viable *Bacillus anthracis* spores. *Lett Appl Microbiol*. 33(2):100-5.

Gulledge J S, V A Luna, Luna, AJ, Zartman R, Cannons AC. 2010. Detection of low numbers of *Bacillus anthracis* spores in three soils using five commercial DNA extraction methods with and without an enrichment step. *J Appl Microbiol.109(5):1509-20.*

Herzog A B, McLennan SD, Pandey, Gerba C P, Haas CN, Rose JB, Hashsham SA. 2009. Implications of limits of detection of various methods for *Bacillus anthracis* in computing risks to human health. *Appl Environ Microbiol.* 75(19): 6331-9.

Hoffmaster AR, Meyer RF, Bowen MD, Marston CK, Weyant RS, Thurman K, Messenger SL, Minor EE, Winchell JM, Rassmussen MV, Newton BR, Parker JT, Morrill WE, McKinney N, Barnett GA, Sejvar JJ, Jernigan JA, Perkins BA, Popovic T. 2002. Evaluation and validation of a real-time polymerase chain reaction assay for rapid identification of *Bacillus anthracis*. *Emerg Infect Dis.* 8:1178-82

Invitrogen. *2006. Dyabeads® MyOne™ Tosylactivated.* [product description and instructions for use] Oslo, Norway: Invitrogen Dynal AS. http://www.siercheng.com/UploadFile/200961193927447.pdf

Ryu C, Lee K, Yoo C, Seong W K, Oh H-B. 2003. Sensitive and rapid quantitative detection of anthrax spores isolated from soil samples by real-time PCR. *Microbiol Immunol* 47(10): 693-9**.**

U.S. Department of Health and Human Services. Public Health Service, Centers for Disease Control, and National Institutes for Health. 2009. *Biosafety in Microbiological and Biomedical Laboratories*. 5th ed. HHS Publication No (CDC) 21-1112. Atlanta, GA: Centers for Disease Control and Prevention. http://www.cdc.gov/biosafety/publications/bmbl5/index.htm

U.S. Environmental Protection Agency. Office of Research and Development. National Homeland Security Research Center. 2010. *Standardized analytical methods for environmental restoration following homeland security events.* Revision 6.0. EPA/600/R-10/122. October, 2010. http://www.epa.gov/nhsrc/pubs/600r10122.pdf

U.S. Pharmacopeia 32. National Formulary 27. 2009. *Validation of Microbial Recovery*. Vol 1 of *The Official Compendia of Standards*. Baltimore, Maryland: United Book Press.

Appendix A
Conjugation of Paramagnetic Beads

http://tools.invitrogen.com/content/sfs/manuals/655_01_02_rev003.pdf

Appendix B
Time Resolved Fluorescence Assay Summary

TRF assay *Bacillus anthracis* (Spore) Antibody Summary Information:

Agent: *B. anthracis* (spore assay)

Strains Tested: *B. anthracis* Pasteur vaccine strain, Sterne vaccine strain, *B. anthracis* strains AO34, AO39, AO62, AO102, AO149, AO158, AO174, AO188, AO193, AO248, AO256, AO264, AO267, AO297, AO328, AO367, AO379, AO419, AO442, AO462, AO463, AO465, AO488, AO489, M36 (vollum), ASC-1, ASC-3, ASC-32, ASC-38, ASC-45, ASC-58, ASC-68, ASC-69, ASC-78, Z-1, Z-6, PB-292, PB-293

Near-neighbor Screen*:* *Bacillus thuringiensis* ssp. *kurstaki, B. thuringiensis* ssp. *israelensis*

Unrelated Screen: *Paenibacillus macerans, Bacillus mycoides, Bacillus cereus* (Laboratory Response Network (LRN) control strain), *Brevibacillus laterosporus, Bacillus licheniformis, B. cereus,* (Fri-42*), Bacillus epiphytus, Bacillus alvei, Bacillus firmus, Bacillus amyloliquefaciens, Bacillus badius, Brevibacillus brevis, Bacillus circulans, Bacillus subtilis, Bacillus subtilis* (*globigii), Paenibacillus polymyxa, Geobacillus stearothermophilus, Bacillus sphaericus, Bacillus lentus,* Thuricide (pesticide 0.8% *B. thuringiensis* ssp. *kurstaki* (A.G Organics, Prosper, TX), Dipel dust.

Negative Control Antigen: *B. cereus* (LRN control strain)

Markers Targeted (if known*):* *B. anthracis* spore coat Ag

Sensitivity: Assay was optimized to detect 100 *B. anthracis* spores total.

Optimization: Antibodies were optimized using a range of 0.1 µg/mL to 10.0 µg/mL for both capture and detection. The assay utilizes 0.75 µg/mL for both goat anti-*B. anthracis* capture and anti-*B. anthracis* mouse monoclonal detector antibodies. Assay requires 90-min antigen incubation.

Limitations: Assay also reacts with *B. anthracis* vegetative cells.

Appendix C
Final Protocols/Methods for Real World Sample Analysis

Procedure Title: Automated Immunomagnetic Separation of *Bacillus anthracis* spores from soil

Purpose: Immunomagnetic Separation (AIMS) of *Bacillus anthracis* spores from soil.

Reagents: Custom coated Dynabeads® (Invitrogen Dynal MyOne™ beads, Carlsbad, CA)

Sample buffer (10X Phosphate buffered Saline pH7.4 + 0.05% Tween 20)

Equipment: Dynal® BeadRetriever system (Invitrogen Dynal cat# 159-50)

End over end rotator (such as VWR cat # 13916-822)

Supplies:

- 50 mL conical tubes
- 1.5 mL Eppendorf tubes
- 30 µm Pre-Separation filter (Miltenyi Biotec cat. No. 130-041-407)
- BeadRetriever tubes and strips (Invitrogen Cat. No. 159-51)
- Polymyxin lysozyme EDTA thallous acetate select agar (PLET)
- Tryptic Soy Agar II with 5% sheep blood (TSA II, BD Diagnostic Systems)
- Cell spreaders
- Pipettors (100 mL, and 1000 mL)
- Pipette tips (100 mL, and 1000 mL)

Specimen: 1 gram of soil sample, potentially contaminated with *B. anthracis* spores

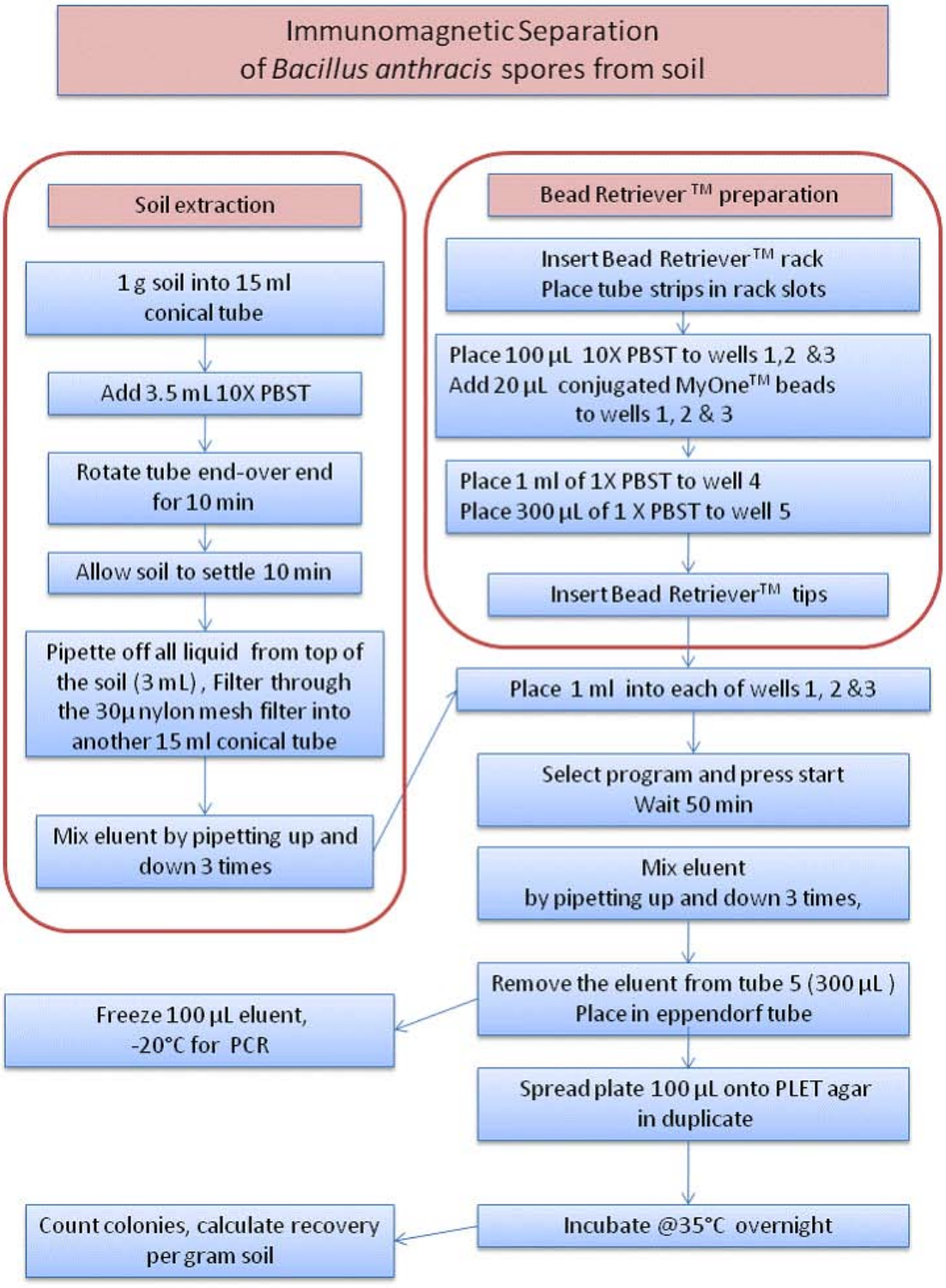
Special safety precautions: Follow all safety precautions as outlined in *Biosafety in Microbiological and Biomedical Laboratories*, 5th edition (U.S. Department of Health and Human Services 2009)

Quality control: Check sterility of PLET agar and TSA II by incubating 10% of media at 35°C for 7 days and check for growth.

Procedure:

1.	Place 1 g of soil sample in a 15 mL conical tube.
2.	Add enough 10X PBST to enable a 3 mL volume to be retrieved for AIMS. This can be estimated by subtracting the volume occupied by the soil from the total volume as observed on the side of the tube.
3.	Place the 15 mL conical tube (s) on the rotator and allow the tube(s) to rotate end over end for 10 min.
4.	Remove tube from rotator, place in a tube rack and allow the soil to settle for 10 min.
5.	Withdraw as much of the liquid from the top of the settled soil and filter it through a 30 µm pore size nylon filter (Miltenyi) that is set on top of a second 15 mL conical tube.
6.	Prepare the BeadRetriever tube strips for AIMS by placing the tube trays which consists of 5 wells into BeadRetriever rack. Add 100µL of 10X PBST to well 1, 2 and 3 of a BeadRetriever tube strip. Add 20µL of beads to well 1, 2 and 3. Add 1 mL of 1X PBST to well 4. Add 300µL of 1X PBST to well 5.
7.	Mix filtered sample eluent by pipetting up and down a few times and place 1 mL of filtered sample to each of tubes 1, 2, and 3.
8.	Insert BeadRetriever tips into slot.
9.	Choose BA sand program and press start. The sample will be ready in ~ 50 min.
10.	After the cycle is finished, remove the cleaned eluent (in the final tube containing 300 µL of buffer) and place in an eppendorf tube.
11.	Mix sample by pipetting up and down a few times. If large numbers of spores are expected, serial dilutions of the AIMS product may be necessary.
12.	Spread 0.1mL from well 5 onto TSAII and/or PLET plates in duplicate. Incubate plates at 35°C for 24 h
13.	Count and record colonies on plates.
14.	Calculate the number of CFU/g soil: $\frac{\text{mean number of colonies per plate} \times \text{total dilution factor}}{\text{g of soil}}$

Flowchart of AIMS method



Limitations of AIMS

(1) False negative results may occur due to high background contamination and may prevent identification of growth of BA on TSAII plates.

(2) The limit of detection as determined by culture is dependent upon the soil type. Based on the four soil types evaluated, the limit of detection for soils with more organic material and background microorganisms was approximately 10^3 spores/g. The limit of detection for soils with less organic material and background organisms was approximately 10^2 spores/g.

(3) Growth on PLET agar requires an additional 24 h of incubation (as compared to growth on TSAII) and the resulting BA colonies are very small with slight differences in colony size and morphology.

(4) During the pre-processing step, filtration of some soil types may reduce recovery of BA spores, due to clogging of the 30µm mesh filter.

(5) Metallic or magnetic particles in soil can bind beads and cause aggregation of particles and other organisms to be recovered.

Interpretation/examination

Examine the serial dilution plates for suspect *B. anthracis* colonies.

To calculate spore recovery per gram of soil from serial dilution plates

containing between 25 and 250 *B. anthracis* colonies:

$$\text{CFU/g} = \frac{\text{mean number of colonies per plate} \times \text{total dilution factor}}{\text{g of soil}}$$

Appendix D
Quality Assurance and Quality Control (QA/QC)

QA/QC Categories	QA/QC implemented and QAPP* deviations
Equipment, media and supplies	As described, the certificate of analysis (COA) for all commercially available media were obtained and kept; sterility was also assessed with no deviations. Quality control of each lot of selective PLET agar media was performed as described.
Bacillus spp. and soil sources	The storage and propagation conditions for the *Bacillus anthracis* Sterne 34F2 veterinary vaccine strain were as outlined in the QAPP with no deviations. Cross reactivity of the obtained antibodies was assessed by CDC's Division of Bioterrorism and Preparedness Response and the findings are included in the report. Physical and chemical properties of each soil type were determined and the results are found in the report.
Immunomagnetic bead separation	AIMS parameters such as buffer types, buffer strength and pH were optimized for the final method as described. For experimental trials positive and negative controls were always included. The negative control consisted of soil slurries made with soil and buffer only. We found this to be a more relevant negative control considering the nature of the beads and soil types. Appropriate interpretation of results was based on the use of these controls.
DNA extraction	Experimental trials of the DNA extraction method included BA spiked sterile soil slurries as a positive control and buffer only as a negative control. Appropriate interpretation of the performance of the DNA isolation kit included considered findings from the controls.
High density spore flotation	Experimental trials assessed spore recovery from spiked sterile soils and positive and negative controls as described. Appropriate interpretation of results was based on the use of these controls.
Real time PCR and RV-PCR	All trials included the use of the 16S Ribosomal Ribonucleic acid (rRNA), and a plasmid containing all select agent primer and probe sets in the detection assays as positive controls and a no template control (water only). No deviations were observed. Findings will be covered in a another report
Training	Standard laboratory procedures were followed and all personnel were trained on using real time PCR and other critical equipment. No deviations were observed.
Data management	All documentation and records of testing and results were kept in a secured laboratory notebook and in project-specific electronic files as described in the QAPP. No deviations were observed.
Data reporting	Monthly and quarterly progress reports were prepared and submitted to EPA's project assistant and project manager.

*QAPP, Quality Assurance Project Plan

EPA
United States
Environmental Protection
Agency

PRESORTED STANDARD
POSTAGE & FEES PAID
EPA
PERMIT NO. G-35

Office of Research and Development (8101R)
Washington, DC 20460

Official Business
Penalty for Private Use
$300