Journal of Microbiological Methods 73 (2008) 242–246

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Development of a rapid and sensitive immunoassay for detection and subsequent recovery of *Bacillus anthracis* spores in environmental samples

Jun Hang [a,1], Appavu K. Sundaram [a,2], Peixuan Zhu [a], Daniel R. Shelton [b,*], Jeffrey S. Karns [b], Phyllis A.W. Martin [c], Shuhong Li [a], Platte Amstutz [a], Cha-Mei Tang [a]

[a] *Creatv MicroTech, Inc., 11609 Lake Potomac Drive, Potomac, MD 20854, United States*
[b] *U.S. Department of Agriculture, Agricultural Research Service, Animal and Natural Resources Institute, Environmental Microbial Safety Laboratory, Bldg 173, BARC-East, 10300 Baltimore Ave, Beltsville, MD 20705, United States*
[c] *USDA, ARS, Plant Sciences Institute, Invasive Insect Biocontrol and Behavior Laboratory, Beltsville, MD 20705, United States*

ARTICLE INFO

Article history:
Received 4 January 2008
Received in revised form 26 February 2008
Accepted 26 February 2008
Available online 2 March 2008

Keywords:
Bacillus anthracis
Anthrax
Bioterrorism agent
Immunological detection
Spore germination
Spore viability

ABSTRACT

Bacillus anthracis is considered a major threat as an agent of bioterrorism. *B. anthracis* spores are readily dispersed as aerosols, are very persistent, and are resistant to normal disinfection treatments. Immunoassays have been developed to rapidly detect *B. anthracis* spores at high concentrations. However, detection of *B. anthracis* spores at lower concentrations is problematic due to the fact that closely related *Bacillus* species (e.g., *B. thuringiensis*) can cross-react with anti-*B. anthracis* antibodies, resulting in false positive detections. Subsequent polymerase chain reaction (PCR) analysis is required to differentiate virulent strains. We report here on a protocol for the rapid, sensitive detection of *B. anthracis* spore using the Integrating Waveguide Biosensor followed by a method for the rapid release and germination of immunocaptured spores. A detection limit of ca. 10^3 spores was achieved by incubating spores simultaneously with capture and detection antibodies ("liquid-phase" assay) prior to capture on capillary tubes/waveguides. Subsequent incubation with BHI broth directly in capillary tubes allowed for rapid germination, outgrowth, and release of spores, resulting in vegetative cells for PCR analysis.

Published by Elsevier B.V.

1. Introduction

Bacillus anthracis is a spore-forming, Gram-positive bacterium that causes anthrax disease in animals and humans. Due to its virulence and ease with which spores can be aerosolized, *B. anthracis* is considered a major threat as an agent of bioterrorism (Inglesby et al., 1999; Higgins et al., 2003). Its spores are resistant to normal disinfection treatments, including heat, radiation and toxic chemicals such as acids and bases (Cortezzo et al., 2004). The spores may remain dormant for decades but can germinate and multiply once they enter a susceptible host.

Numerous methods have been developed to detect anthrax spores in environmental samples (King et al., 2003). Immunoassays have been successfully used to rapidly detect *B. anthracis* in air, water and powders (Welkos et al., 2004; Tims and Lim, 2004, Farrell et al., 2005), particularly at the higher concentrations consistent with a bioterrorism attack. However, accurate detection at lower concentration can be problematic due to the fact that other closely related *Bacillus* species (e.g., *B. thuringiensis*, *B. cereus*, and *B. mycoides*), that are ubiquitous in the environment, can give cross-reactions with *B. anthracis* immunoassays (DelVecchio et al., 2006), resulting in false positive detections.

With traditional methods, confirmation of *B. anthracis* spores can require several days. After a suspected bioterrorism event or for monitoring cleanup after decontamination, there is a need for rapid and sensitive diagnostic tests to detect the presence of *B. anthracis* spores in environmental samples within a few hours.

Immunoassays can be combined with real-time PCR analysis for confirmation of virulence and verification of viability (McBride et al., 2003). This requires the recovery of captured spore DNA after positive immunoassay detections. However, the strong denaturant and lysis reagents commonly used for dissociating spores from antibodies can interfere with PCR confirmation tests.

Ideally, *B. anthracis* detection would incorporate an immunoassay for spore concentration and detection, an assay to assess spore viability, and a PCR assay to confirm strain identity and virulence. In this paper, we describe an assay that achieves this goal with a protocol that includes (i) a rapid immunoassay procedure using the Integrating Waveguide Biosensor (less than 2 h) followed by (ii) germination and outgrowth of spores in BHI broth to assess viability (less than 1 h) to (iii) provide vegetative cells for subsequent lysis and polymerase chain reaction (PCR) confirmation.

* Corresponding author. USDA/ARS/ANRI/EMSL, Bldg. 173, BARC-East, Powder Mill Rd, Beltsville, MD 20705-2350, United States. Tel.: +1 301 504 6582; fax: +1 301 504 6608.
E-mail address: dan.shelton@ars.usda.gov (D.R. Shelton).
[1] Current address: Edge BioSystems, Inc., 201 Perry Parkway, Suite 5 Gaithersburg, MD 16 20877, United States.
[2] Current address: MassTech, Inc., 6992 Columbia Gateway Drive, Columbia, MD 21046, United States.

0167-7012/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.mimet.2008.02.018

2. Materials and methods

2.1. Bacterial strain and reagents

All the chemicals were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise indicated. *B. anthracis* Sterne strain was provided by U.S. Department of Agriculture, Agricultural Research Service (USDA-ARS) (Beltsville, MD). Samples of affinity purified polyclonal antibody (goat) against *B. anthracis* spores were obtained from the Naval Medical Research Center (Silver Spring, MD).

Biotinylation of antibody was achieved by conjugating 1 mg of antibody using Sulfo-NHS-LC-Biotin (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions, resulting in 4–6 biotin molecules per antibody. Cy5-labeled antibody was prepared by conjugating 1 mg of antibody using a FluoroLink-Ab Cy5 labeling kit (Amersham Biosciences, Piscataway, NJ) using a Cy5 concentration resulting in a Cy5 to antibody ratio of 2:1. NeutrAvidin™ (biotin binding protein) was purchased from Pierce Biotechnology (Rockford, IL). Glass capillary tubes (52 mm long, 1.66 mm O.D., 3 1.23 mm I.D.) were purchased from Drummond Scientific Company (Broomall, PA).

2.2. Spore preparation

The Sterne strain was cultured on agar plates with the New Sporulation Medium (NSM), containing 3 g l^{-1} tryptone, 3 g l^{-1} yeast extract, 2 g l^{-1} Bacto-Agar, 23 g l^{-1} Lab-8 Lemco Agar (Oxoid, Hampshire, England) and 0.01 g l^{-1} $MgSO_4 \cdot 4H_2O$ (Perdue et al., 2003), resulting in sporulation in 5–7 days. Spores were harvested with sterile water, washed five times with 20 mL of sterile water, and finally suspended in 10 mL of sterile water. Spores were stored at 4 °C until use. Spore concentrations (CFU; colony forming units) were determined by plating onto Tryptone Soy Agar (TSA; Oxoid).

Culturing of *B. anthracis* (Sterne strain) and all experiments were conducted in a BSL-2 facility.

2.3. Immunoassays

Glass capillary tubes were prepared as previously described (Ligler et al., 2002). Briefly, capillary tubes cleaned with methanol/HCl followed by sulfuric acid were dried using nitrogen and silanized using 3-mercaptopropyl trimethoxysilane in anhydrous toluene, under nitrogen atmosphere. Capillaries were then incubated with 4-maleimidobutyric acid *N*-hydroxysuccinimide ester (GMBS), followed by NeutrAvidin.

For "sandwich" immunoassays, neutravidin coated capillaries were incubated with biotinylated anti-*B. anthracis* spore antibody (10 μg mL^{-1} in PBSTB (0.05% Tween-20 and 2%BSA in PBS) for 1 h, followed by incubation with PBSTB to block remaining sites. After 1 h, capillary tubes were filled with fresh PBSTB, stored at 4 °C and used within a week. Capillary tubes containing immobilized anti-spore antibody were statically incubated with different concentrations of spores (10^3 to 10^9 spores mL^{-1} in 5 PBSTB) at room temperature for 1 h. After washing with PBSTB, tubes were incubated statically with 10 μg mL^{-1} of Cy5-labeled anti-spore antibody for 1 h to complete the sandwich. Capillaries were then washed with PBST, and analyzed using the Integrating Waveguide Biosensor, previously described Zhu et al. (2005). Each experimental treatment was conducted in triplicate; the mean value ± S.D. are reported.

For "liquid-phase" immunoassays, spore suspensions (in 1 mL of PBSTB) were incubated simultaneously with both biotinylated and Cy5-labeled anti-*B. anthracis* spore antibodies at room temperature for 1 h with gentle mixing. Based on preliminary experiments, the optimal concentration of antibodies was determined to be 5 μg mL^{-1} of each. Free antibodies were then removed from the spores with bound antibodies using an Ultrafree-MC 0.1 μm centrifugal filter unit (Millipore, Bedford, MA). Spores with bound antibodies retained on the filter membrane were washed twice with 1 mL of PBSTB to remove any non-specifically bound antibody. Spores with bound antibodies were then resuspended in 1 mL of PBSTB and used for incubation in capillary tubes that had been blocked with 2% BSA after immobilization of neutravidin. This static incubation was carried out at room temperature for 2 h, followed by washing with PBST to remove any non-specifically bound spores. Capillaries were then analyzed using the triplicate; the mean value ± S.D. are reported.

2.4. Detection of spores in environmental samples

Plastic furniture upholstery was cut to fit into a 150 mm diameter culture dish and used as a surface for environmental testing. Twenty milligrams of dust from an office vacuum cleaner was spread on each test surface. Swabs wetted with PBS were then used for wiping the entire surface by making enough vertical s-strokes. After wiping the surface, the swab was placed in 5 mL of PBSTB in a 50 mL centrifuge tube and vortexed to extract the contents from the swab. The tube was left undisturbed for 15 min to allow particles to settle and 900 μL of the solution transferred to a microcentrifuge tube. Different spore concentrations (in 100 μL of PBSTB) were added to microcentrifuge tubes giving final spore concentrations of 10^3 to 10^7 spores mL^{-1}. These samples were used for spore detection using the "liquid-phase" immunoassay as described in the immunoassay section. Each experimental treatment was conducted in triplicate; the mean value ± S.D. are reported.

2.5. Spore germination and release

Germination experiments were conducted with spore concentrations of 10^4 CFU mL^{-1}. *B. anthracis* spores were incubated at 37 °C in H_2O alone; BHI broth alone; or in 10 mmol l^{-1} L-alanine, 1 mmol l^{-1} inosine, 10 mmol l^{-1} NaH_2PO_4 (pH 8.0) and 100 mmol 19 l^{-1} NaCl; 100 mmol l^{-1} L-alanine and 50 mmol l^{-1} inosine, 1 mmol l^{-1} L-alanine and 1 mmol l^{-1} inosine, or 50 mmol l^{-1} L-alanine and 10 mmol l^{-1} inosine with or without BHI broth. After germination under designated conditions, percent germination was assessed by heating suspensions at 70 °C for 30 min to kill vegetative cells, then plating onto TSA to determine the number of surviving spores (Welkos et al., 2004). Percent germination was calculated as 1 − (CFU after second heating per total CFU).

A number of physical/chemical methods were evaluated for the release of immunocaptured spores from magnetic beads, including: heating at 70 °C for 30 min or 80 °C for 3 min; incubating with 0.1 mol l^{-1} glycine (pH 2.5); incubating with DMSO (20%, 40%, 100%); incubating with ethanol (50%, 70%); or incubating with BHI at 37 °C for 50 min. *B. anthracis* spores were immunocaptured using the "liquid-phase" assay as previously described. Spores (10^5 mL^{-1}) were mixed with 5.0 μg each of biotinylated antibodies and Cy5 conjugated antibodies in 1.1 mL PBSTB (containing 2% BSA and 0.5% Tween-20 in PBS buffer). Four microliters streptavidin-Dynabeads M-280 (Dynal Biotech, Brown Deer, WI, USA) in PBSTB were mixed with the spore–antibody complexes. The mixture was incubated on a rotary shaker for 1 h at ambient temperature. The beads were collected by magnet, washed three times and resuspended in PBSTB. About 70% of spores were bound to immunomagnetic beads. Release experiments were conducted with immunocaptured spore concentrations of 10^4 CFU mL^{-1}. After experimental treatments, beads were collected by magnet, washed twice and resuspended in BHI. Both beads and supernatant (containing released spores) were plated onto TSA to determine percent release.

3. Results and discussion

3.1. Immunoassay development and evaluation

A variety of immunoassays have been developed for the detection of pathogenic agents (e.g., *B. anthracis* spores). For optical detection

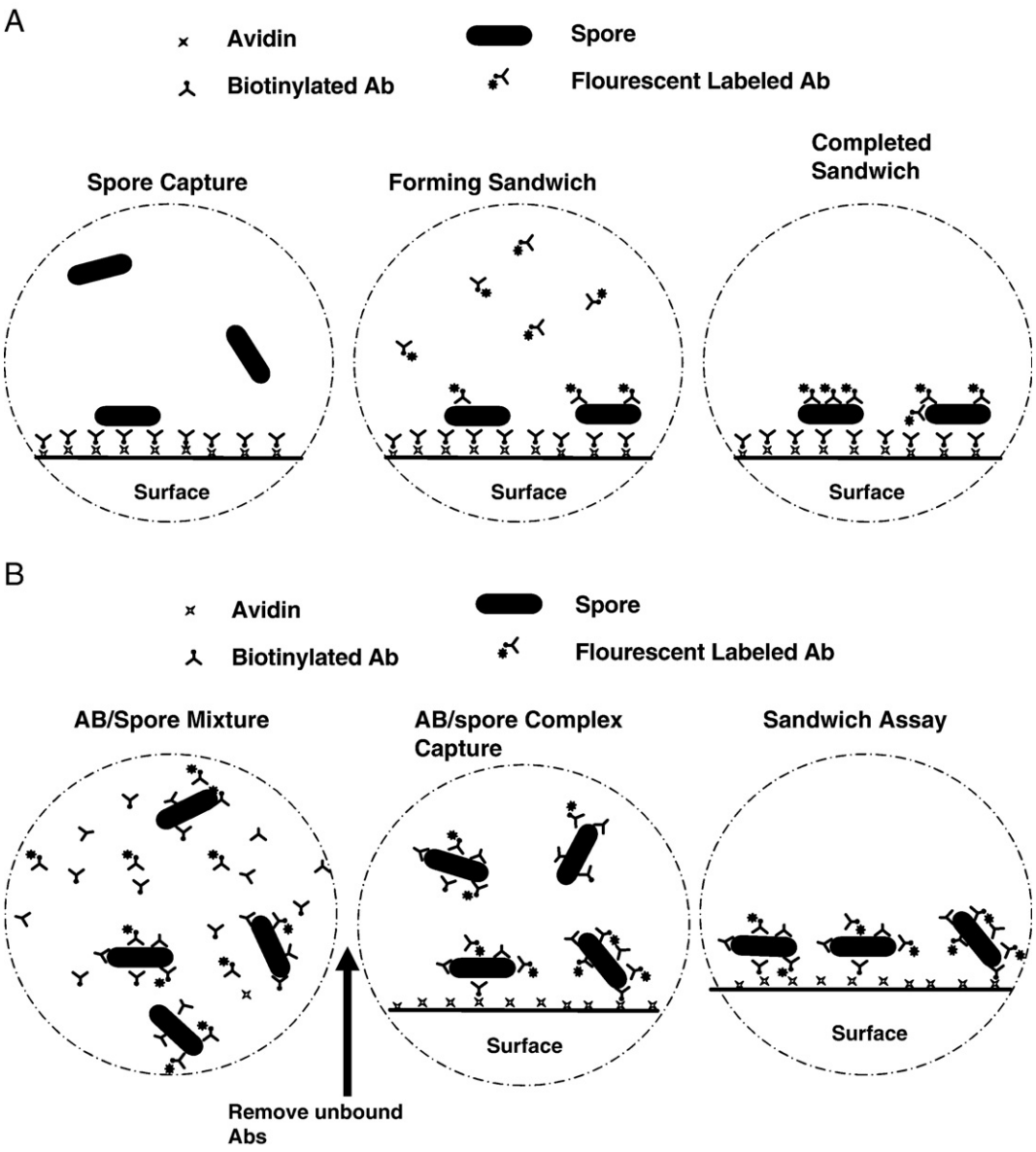

Fig. 1. Schematic of typical "sandwich" (A) and "liquid-phase" (B) immunoassay techniques.

methods, pathogens are typically captured and then labeled with a second detection antibody conjugated to a reporter molecule, resulting in a "sandwich" immunoassay (see Peruski and Peruski, 2003, for a review of immunoassay methods). *B. anthracis* spores, however, present a unique challenge; because of the relatively low concentration of epitopes on the spore surface, capture efficiencies are lower than for other bacterial pathogens. An alternative to the traditional "sandwich" immunoassay, where spores are sequentially captured then labeled, is the "liquid-phase" immunoassay where spores are incubated simultaneously with capture and detection antibodies (Fig. 1). Spores are subsequently captured via the binding of biotinylated antibodies to avidin.

"Sandwich" and "liquid-phase" assays were evaluated using the Integrating Waveguide Biosensor (Zhu et al., 2005). For "sandwich" assays, capillary tubes coated with capture antibodies (inner surface) were incubated statically with different spore concentrations, followed by incubation with Cy5-labeled detection antibodies. The limit of detection (LOD) was about 10^4 spores per capillary (Fig. 2); or 2×10^5 spores mL^{-1} (50 µL capillary volume). Based on a capture efficiency of ca. 10% (unpublished data), the theoretical LOD is about 10^3 bound spores per capillary. For "liquid-phase" assays, different spore concentrations were incubated with a mixture of biotinylated capture antibodies and Cy5-labeled detection antibodies. The spore–antibody suspension was then incubated statically in capillary tubes with neutravidin, allowing for the biotinylated antibody to conjugate with the neutravidin. The limit of detection (LOD) was about 2×10^3 spores per capillary (Fig. 2); or 4×10^4 spores mL^{-1} (50 µL capillary volume). This represents an approx. 5-fold increase in sensitivity over the "sandwich" assay. Although capture efficiency was not determined with capillary tubes, the increased sensitivity is presumably due to enhanced capture efficiency. This would be consistent with studies conducted with immunomagnetic beads in which capture efficiency was 70%. Note that the enhanced sensitivity of the "liquid-phase" assay is dependent on removal of all unreacted biotinylated antibodies prior to incubation in capillary tubes. Otherwise, an excess of unreacted antibodies will bind to avidin sites, preventing the capture of spores.

3.2. Spore detection in environmental samples

Additional "liquid-phase" assays were conducted with dust suspensions amended with different spore concentrations. The presence

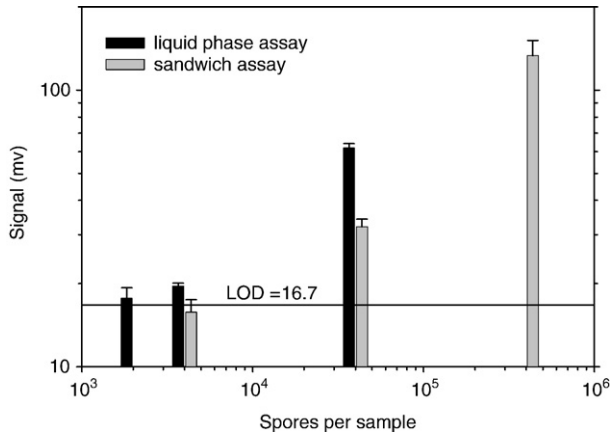

Fig. 2. Results of "sandwich" and "liquid-phase" assays for detection of *B. anthracis* spore suspensions. The average of 3 capillary readings for each concentration is reported. The limit of detection (LOD) shown as a horizontal line is defined as the average reading for the blank control capillaries+3 times the standard deviation. Values for the capillary blanks were 13.7±1.0 mV and 13.1±1.2 mV for "sandwich" and "liquid-phase" assays, respectively.

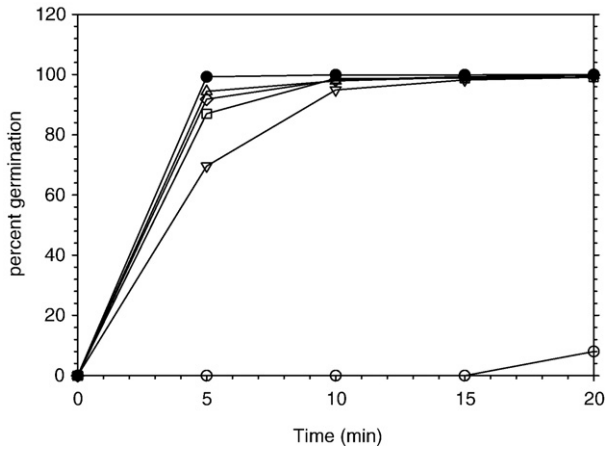

Fig. 4. Germination of *B. anthracis* spores at 37 °C in H_2O alone (○); 1 mmol l^{-1} L-alanine and 1 mmol l^{-1} inosine (▽); 10 mmol l^{-1} L-alanine and 1 mmol l^{-1} inosine (□); 50 mmol l^{-1} L-alanine and 10 mmol l^{-1} inosine (◊); 100 mmol l^{-1} L-alanine and 50 mmol l^{-1} inosine (△); and BHI alone (●). Germination percentage was measured by loss of heat resistance (70 °C for 30 min).

of dust particles did not adversely affect the assay sensitivity (Fig. 3). In fact, the apparent LOD was ca. 10^3 spores per capillary due to the lower background signal. Since spores were added after swabbing of the fabric, no conclusions can be drawn regarding the efficiency of detection directly from the fabric.

3.3. Spore germination and release

Previous research had shown that the polyclonal antibodies used in this study showed low levels of cross-reactivity with other *Bacillus* strains. Consequently, subsequent release of spores and strain identification are necessary to minimize false positive detections. Several methods have been described for the direct extraction of DNA (for PCR) from spores for strain identification (Hoffmaster et al., 2002). However, these methods are extremely rigorous and would require release and recovery of captured spores from capillary tubes. An alternative strategy is spore germination, during which growing vegetative cells are released from the spore coat (Moberly et al., 1966). DNA can be readily extracted from vegetative cells due to the ease of cell lysis. The authors have previously demonstrated that captured *E. coli* cells are readily lysed inside capillary tubes (Zhu et al., 2005).

A variety of supplements, which have previously been reported to accelerate spore germination, were evaluated (Brown et al., 1982; Clements and Moir, 1998; Kiel et al., 2000; Paidhungat and Setlow, 2000; Barlass et al., 2002; Ireland and Hanna, 2002; Setlow, 2003). *B. anthracis* spores were incubated in water or BHI broth containing 8 different concentrations of L-alanine and inosine to assess germination. Rapid germination was observed in all solutions containing both L-alanine and inosine at 10 concentrations >1 mM (Fig. 4). However, when incubated in BHI broth alone, >99% of spores germinated within 5 min, regardless of the presence or absence of supplements (Fig. 4; data with supplements not shown).

Based on the observation that incubation in BHI broth alone resulted in rapid germination, BHI incubation was compared with various physical/chemical methods for the release of spores from antibodies. The majority of physical/chemical methods were unsuccessful. Incubation in 100% DMSO for 10 min at ambient temperature was effective in releasing spores from antibodies, although a 24% decrease in spore viability was observed (data not shown). Incubation in BHI at 37 °C for 50 min was effective in releasing ca. 90% of spores (as vegetative cells) from antibodies. The presence of the virulence plasmid in the vegetative cells was subsequently confirmed using PCR techniques (data not shown). The BHI broth did not interfere with PCR analysis.

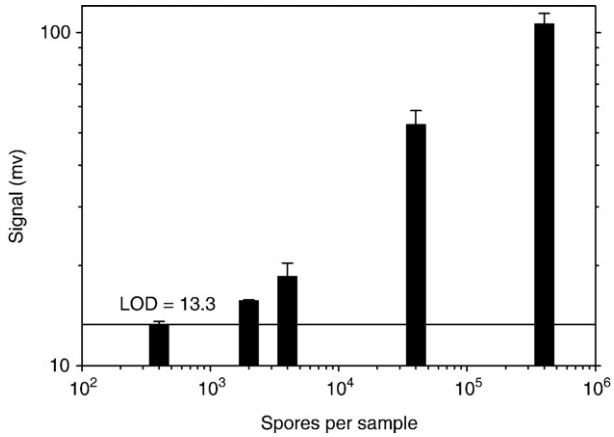

Fig. 3. Results of "liquid-phase" assay for detection of *B. anthracis* spore suspension in environmental dust samples. The average of 3 capillary readings for each concentration is reported. The limit of detection (LOD) shown as a horizontal line is defined as the average reading for the blank control capillaries+3 times the standard deviation. The value for the capillary blank was 11.5±0.6 mV.

4. Conclusions

We report here a simple method for rapid recovery and confirmation of *B. anthracis* spores. Utilizing a "liquid-phase" assay in conjunction with the Integrating Waveguide Biosensor allows for detection of as few as 10^3 captured spores, which is lower than for most commercial biosensors (King et al., 2003). Subsequent germination and release of spores in BHI broth allows for confirmation of viability and strain identity using PCR techniques.

Acknowledgments

This project was supported by SBIR grant R43 EB001731 from National Institutes of Health. We thank Dr. Robert L. Bull, Naval Medical Research Laboratory, for providing affinity purified goat polyclonal antibody against *B. anthracis* spores.

References

Barlass, P.J., Houston, C.W., Clements, M.O., Moir, A., 2002. Germination of *Bacillus cereus* spores in response to L-alanine and to inosine: the roles of gerL and gerQ operons. Microbiol. 148, 2089–2095.

Brown, W.C., Vellom, D., Ho, I., Mitchell, N., McVay, P., 1982. Interaction between a *Bacillus cereus* spore hexosaminidase and specific germinants. J. Bacteriol. 149 (9), 969–976.

Cortezzo, D.E., Koziol-Dube, K., Setlow, P., 2004. Treatments with oxidizing agents damages the inner membrane of spores of *Bacillus subtilis* and sensitizes spores to subsequent stress. J. Appl. Microbiol. 97, 838–852.

DelVecchio, D.V.G., Connolly, J.P., Alefantis, T.G., Walz, A., Quan, M.A., Patra, G., et al., 2006. Proteomic profiling and identification of immunodominant spore antigens of *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*. Appl. Environ. Microbiol. 72, 6355–6363.

Clements, M.O., Moir, A., 1998. Role of the gerI operon of *Bacillus cereus* 569 in the response of spores to germinates. J. Bacteriol. 180, 6729–6735.

Farrell, S., Halsall, H.B., Heineman, W.R., 2005. Immunoassay for *B. globigii* spores as a model for detecting *B. anthracis* spores in finished water. Analyst 130, 489–497.

Higgins, J.A., Cooper, M., Schroeder-Tucker, L., Black, S., Miller, D., Karns, J.S., et al., 2003. A field investigation of *Bacillus anthracis* contamination of U.S. Department of Agriculture and other Washington, D.C., buildings during the anthrax attack of October 2001. Appl. Environ. Microbiol. 69, 593–599.

Hoffmaster, A.R., Meyer, R.F., Bowen, M.P., Marston, C.K., Weyant, R.S., Barnett, G.A., et al., 2002. Evaluation and 12 validation of a real-time polymerase chain reaction assay for rapid identification of *Bacillus anthracis*. Emerg. Infect. Dis. 8, 1178–1182.

Inglesby, T.V., Henderson, D.A., Bartlett, J.G., Ascher, M.S., Eitzen, E., Friedlander, A.M., et al., 1999. Anthrax as a biological weapon: medical and public health management. JAMA 281, 1735–1745.

Ireland, J.A., Hanna, P.C., 2002. Amino acid- and purine ribonucleoside-induced germination of *Bacillus anthracis* ΔSterne endospores: gerS mediates responses to aromatic ring structures. J. Bacteriol. 184, 1296–1303.

Kiel, J.L., Parker, J.E., Alls, J.L., Kalns, J., Holwitt, E.A., Stribling, L.J., et al., 2000. Rapid recovery and identification of anthrax bacteria from the environment. Ann. N. Y. Acad. Sci. 916, 240–252.

King, D., Luna, V., Cannons, A., Cattani, J., Amuso, P., 2003. Performance assessment of three commercial assays for direct detection of *Bacillus anthracis* spores. J. Clin. Microbiol. 41, 3454–3455.

Ligler, F.S., Breimer, M., Golden, J.P., Nivens, D.A., Dodson, J.P., Green, T.M., et al., 2002. Integrating Waveguide Biosensor. Anal. Chem. 74, 713–719.

Moberly, B.J., Shafa, F., Gerhardt, P., 1966. Structural details of anthrax spores during stages of transformation into vegetative cells. J. Bacteriol. 92, 220–228.

McBride, M.T., Masquelier, D., Hindson, B.J., Makarewicz, A.J., Brown, S., Burris, K., et al., 2003. Autonomous detection of aerosolized *Bacillus anthracis* and *Yersinia pestis*. Anal. Chem. 15 (20), 5293–5299.

Paidhungat, M., Setlow, P., 2000. Role of ger proteins in nutrient and nonnutrient triggering of spore germination in *Bacillus subtilis*. J. Bacteriol. 182, 2513–2519.

Perdue, M.L., Karns, J., Higgins, J., Van Kessel, J.A., 2003. Detection and fate of *Bacillus anthracis* (Sterne) vegetative cells and spores added to bulk tank milk. J. Food Prot. 66, 1762–1767.

Peruski, A.H., Peruski Jr., L.F., 2003. Immunological methods for detection and identification of infectious disease and biological warfare agents. Clin. Diagn. Lab. Immunol. 10, 506–513.

Setlow, P., 2003. Spore germination. Curr. Opin. Microbiol. 6, 550–556.

Tims, T.B., Lim, D.V., 2004. Rapid detection of *Bacillus anthracis* spores directly from powders with an evanescent wave fiber-optic biosensor. J. Microbiol. Methods 13 (59), 127–130.

Welkos, S.L., Cote, C.K., Rea, K.M., Gibbs, P.H., 2004. A microtiter fluorometric assay to detect the germination of *Bacillus anthracis* spores and the germination inhibitory effects of antibodies. J. Microbiol. Methods 56, 253–265.

Zhu, P., Shelton, D.R., Karns, J.S., Sundaram, A., Li, S., Amstutz, P., et al., 2005. Detection of water-borne *E. coli* using the integrated waveguide biosensor. Biosens. Bioelectron. 21, 678–683.