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Research Article

Amperometric Detection of *Bacillus anthracis* Spores: A Portable, Low-Cost Approach to the ELISA

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Antibody-based detection assays are generally robust, a desirable characteristic for in-the-field use. However, to quantify the colorimetric or fluorescent signal, these assays require expensive and fragile instruments which are ill-suited to in-the-field use. Lateral flow devices (LFDs) circumvent these barriers to portability but suffer from poor sensitivity and subjective interpretation. Here, an antibody-based method for detecting *Bacillus anthracis* spores via amperometric signal generation is compared to ELISA and LFDs. This amperometric immunoassay uses antibody conjugated to magnetic beads and glucose oxidase (GOX) along with the electron mediator 2, 6-dichlorophenolindophenol (DCPIP) for production of a measurable current from a 0.4 V bias voltage. With similar sensitivity to ELISA, the assay can be completed in about 75 minutes while being completely powered and operated from a laptop computer. Immunoassay amperometry holds promise for bringing low-cost, quantitative detection of hazardous agents to the field.

1. Introduction

Anthrax spores are a dormant viable offspring derived from the Gram-positive, rod-shaped bacterium *Bacillus anthracis*. They are responsible for 3 well-known disease states: cutaneous, gastrointestinal, and inhalation anthrax, the latter of which is characterized by a high mortality rate [1]. Due to spore stability and high mortality rate, anthrax spores are ranked high among the potential weapons of bioterrorists [2, 3]. Hence, a tremendous interest exists for developing quick, reliable, and field-deployable detection devices. DNA-based assays typically have the best sensitivity and specificity but are not readily suited for in-the-field use due to lack of portability and being highly sensitive to environmental contaminants [4]. Notably, during the investigation of the USA anthrax attack of 2001, a total of 4,639 real-time PCR reactions were performed and all were negative [5]. Positive results were only obtained after culture enrichment when environmental contaminants (dust, debris, and collection buffer surfactants) were eliminated. Alternatively, antibody-based assays are more appealing for in-the-field use due to their environmental robustness and ease of use.

The typical ELISA generates quantifiable results and is fairly sensitive (10^4 cfu/mL) [6] but is too cumbersome and time-consuming to be performed entirely in the field. In addition, expensive and power-demanding instrumentation is needed. Lateral flow devices (LFDs, a.k.a. immunostrips) are a portable and low-cost immunoassay that can be completed in just 15 minutes. However, they are subjectively interpreted and typically characterized by poor sensitivity and frequent false positives. The lower limit for detecting *B. anthracis* spores is typically around 10^5 to as much as 10^7 cfu/mL depending on manufacturer and/or spore strain and preparation method [7, 8]. Ideally, for an immunoassay to meet the demands of hazardous agent detection in the field, it would have the sensitivity and dynamic range of an ELISA and the simplicity and quickness of the LFD.

Among the emerging technologies which are advancing the immunoassay state of the art [9], electrochemical signal generation methods are appealing for high sensitivity, low power, and relatively simple instrumentation, along with other advantages [10]. For the sake of portability and in-the-field use, the advantages of electrochemical detection are quite attractive. To this end, here an amperometric immunoassay is described for the detection of *B. anthracis* spores. A moderate power source (5 V via USB connector) supplied a bias voltage (0.4 V) that enabled the measurement of current generated by the reaction between glucose oxidase (GOX, conjugated to anti-*B. anthracis* spore antibodies) and glucose. 2, 6-dichlorophenolindophenol (DCPIP) mediated the electron transfer from GOX to a gold electrode. Magnetic bead-mediated spore capture eliminates the need for a centrifuge and long binding durations. Once more, magnetic bead-conjugated capture antibodies and GOX-conjugated detection antibodies are added in a single step, and substrate incubation occurs at room temperature, further shortening assay duration. Using this strategy, *B. anthracis* spores can be detected in about 75 minutes.

2. Materials and Methods

All reagents, supplies, and equipment were purchased from VWR (Wayne, PA) unless otherwise indicated.

2.1. Spore Production. All *Bacillus* strains used in this research were purchased from BEI Resources (Manassas, VA) and include *B. anthracis* Sterne strain (cat# NR-1400), *B. thuringiensis* (NR-610), *B. cereus* (NR-608), and *B. mycoides* (NR-612). Each strain was inoculated into 5 mL LB broth (Difco 244620) for 4–8 hours until cloudy before transferring to 50 mL nutrient broth (Fluka 70122). After two days, *B. anthracis* cultures were transferred to Leighton-Doi broth and were monitored daily until greater than 95% spores were observed via microscopy (Western Digital PMD-1 USB2 1.09, software v 2.0.0, Westover Scientific, Mill Creek WA). Cultures were then centrifuged (30 minutes, 10,000 ×g, 4°C). Pellets were rinsed once with cold diH_2O, centrifuged, and then resuspended in 10 mL diH_2O. One mL aliquots were heat treated before spore concentration was determined via plate counting. Immunoreactivity was confirmed by dot blot analysis (data not shown) though spot intensity did not necessarily correlate with amperometry, immunostrip, or ELISA sensitivity.

2.2. Assay Procedure. Capture (cat# T110810-01, Tetracore Inc., Rockville, MD) and detection (cat# C86702M and C86910M, Meridian Life Science Inc. Saco, ME and cat#s T100510-01 and T270605-01, Tetracore Inc.) antibodies were conjugated to magnetic beads (Dynabeads MyOne Tosylactivated cat# 655.01D, Life Technologies Oslo, Norway) or glucose oxidase (Lightning Link cat# 706-0010, Novus Biologicals LLC Littleton, CO), respectively, according to manufacturers' instructions. Briefly, capture antibody was conjugated in 0.1 M sodium borate buffer, pH 9.5 at 37°C. Detection antibody was conjugated in 50 mM sodium phosphate buffer, pH 7.5. Both antibodies were exchanged into respective conjugation buffers using centrifugal filtration devices (Amicon Ultra, cat# UFC505096, Millipore Cork, Ireland). For the assay procedure, 100 μL of master mix (50 ng of each of the 4 detection Ab-GOX conjugates, 10 μg Ab-magnetic bead conjugate) in incubation buffer (1.5x PBS pH 7.2, w/0.15% BSA and 0.075% Tween 20) was added to 100 μL of (5′ vortexed) *Bacillus anthracis* spores (diluted in PBST) in prelubricated microcentrifuge tubes (Costar 3207) and inverted (Labquake, Thermo Scientific Waltham, MA) for 30 minutes at room temperature. The magnetic bead pellets were then washed with 1 mL of incubation buffer and repeated twice with 1 mL wash buffer (140 mM MES buffer, pH 7.0, 0.05% Tween 20). The samples were placed on a magnetic tube holder (cat# 101414-700) for 2 minutes before each wash step. Pellets were stored at 4°C for up to 48 hours before being assayed.

Pellets were assayed for GOX activity by incubating in substrate buffer (50 μL of 40 μM DCPIP, 60 mM glucose in 1.4 M MES, pH 6.2) by inversion for 30 minutes at room temperature and then analyzed on the PHAD instrument. The PHAD software running time was optimized to coincide with the 30-minute incubation. The program instructs the PHAD instrument to perform a series of 22 one second measurement (closed circuit) events, where the largest value from zero is reported (out of 100 measurements per second). Each measurement event is paired with an open circuit interval when buffer exchanges can be performed. The first 10 measurements (A1–A10, see Figure 1) discharge static from the disposable electrode to stabilize the signal; the next 10 cycles (B and C measurements) are used to adjust the baseline signal to maximize the reportable range. The sample is added during the 21st cycle. From this signal the background noise is subtracted.

2.3. Instrumentation. The electrodes were made of 50 nm pure gold sputtered onto 10 mm Toray Lumirror S10 polyester (Materion Corp, Mayfield Heights, OH) backboard. The solder mask (FOC-800 USA, Taiyo America Inc., Carson City, NV) application, laser ablation, and sheet cutting were performed by Conductive Technologies Inc. (York, Pennsylvania). A Plexiglas well (Proto Labs Inc, Maple Plain, MN) is attached via double sided adhesive to form the electrochemical cell.

The PHAD detection box consists of an aluminum housing (2.75″W × 5.5″H × 7.75″D) encompassing a DT9812-2.5V data acquisition board with a 4-way multiplexer, 2 kHz oscillator, and LTC6078 dual op-amp (operation amplifier). A 0.4 V bias voltage is applied to one side of the electrochemical cell, while the other side is connected to a current-to-voltage converter through a 10 k Ohm resistor. Thus, the output ampere signal, amplified 10 V/μA, is reported as volts. The reportable range of the PHAD box is 250 nA, and resolution is 0.25 nA.

The PHAD box is connected to a PC workstation via USB, which supplies the box with the operational power of 5 V while also performing communication operations. The Kit Instrument software (v0.90) was developed using dotNet 2.0 using Microsoft Visual Studio 2005 (Microsoft Corp, Redmond, Washington).

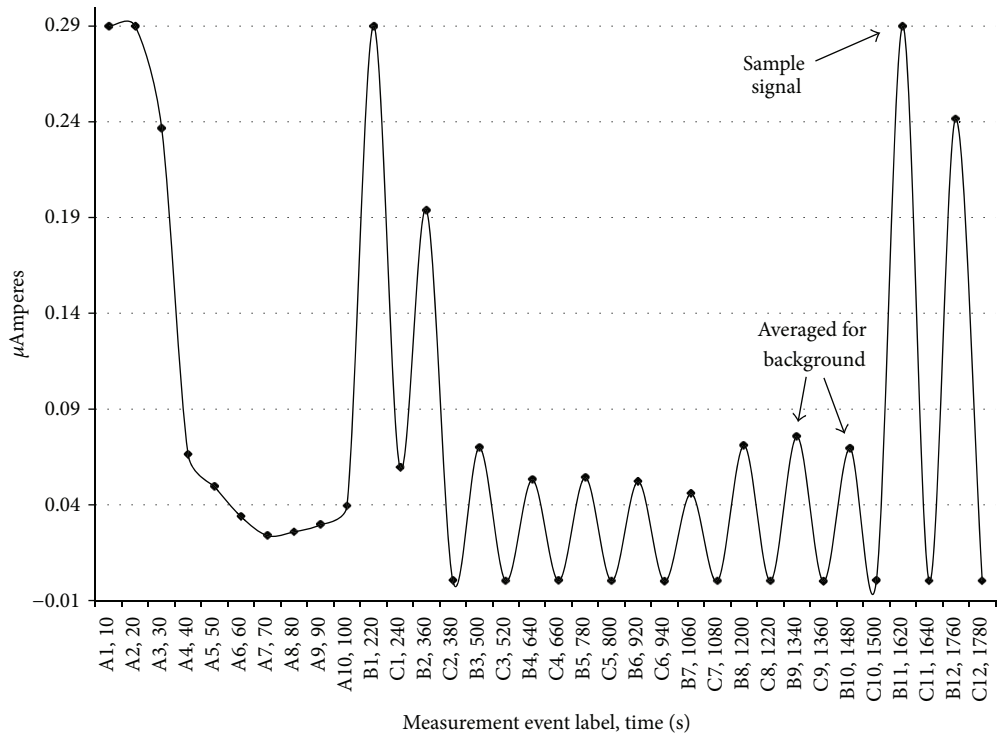

Figure 1: Amperogram of a typical PHAD run. Closed circuit measurements are indicated by a diamond symbol. Otherwise, the PHAD instrument is in "open circuit" mode during which no measurements occur. A1–A10 are used to discharge the electrode. During measurements B1-C10 used blank substrate buffer is replaced with fresh (after C10 blank buffer is replaced with sample substrate buffer) 45–50 seconds prior to subsequent "B" measurement. Between B1 and C8, an offset knob is adjusted after each B, but before subsequent C measurement, to bring background near zero. Averaged background is subtracted from sample signal.

2.4. Immunostrip Procedure. All LFD testings were performed according to manufacturer instructions. Briefly, BADD test strips (Advnt Biotechnologies LLC, Phoenix Arizona) use a swab/capillary that is swirled in a liquid sample and mixed with buffer, six drops of which are added to the LFD. Anthrax spore Smart II (New Horizons Diagnostics Corp, Columbia MD) LFDs instruct that 3 drops of liquid spore sample are added directly to the strip, followed by 2 drops of chase buffer 3 minutes later. The Anthrax Biothreat Alert Test Strip (Tetracore Inc., Rockville MD) dilutes liquid samples 1 : 2 in buffer before 5 drops are added to the test strip. All three manufacturers require that LFDs develop for 15 minutes and are "interpreted" by the presence (positive result) or absence (negative result) of a test line before 30 minutes. All three also include control lines to ensure a properly functioning LFD.

2.5. ELISA Procedure. ELISA kit assays (Tetracore Inc., Rockville, MD) were performed according to manufacturer's instructions. Briefly, plates were coated with capture antibody and incubated overnight at 4°C. Plates were then incubated, in a series of steps, with Blocking buffer, samples, detector antibody, and then conjugate antibody for 1 hour each at 37°C. After each step, the microtiter plates were washed 4x with PBST. The substrate was incubated for 30 minutes at 37°C. Plates were read at 405 nm on a Biotek Synergy HT running Gen 5 software v1.11.4 (Biotek Inc, Winooski VT).

2.6. Calculations. For ELISA, the LOD was calculated following the manufacturer's suggested positive signal cutoff (the average of no antigen controls plus 3x the standard deviation plus 0.15). Dynamic range was determined by the greatest regression coefficient (R^2) value using 3 spore concentrations over the broadest range. Error bars are equal to one SD value. Detection rate was calculated by subtracting the percent false negatives (type 2 error) from 100%. Time-to-results was the amount of time from beginning the assay to signal output. The average of *B. thuringiensis* signals plus 3x SD was used to calculate LoD and 5x SD for LoQ.

3. Results

For each run, the PHAD instrument performs several measurements, labeled A, B and C (closed circuit events, Figure 1), which are separated by open circuit intervals when buffers can be exchanged and the offset adjusted. The 10 A measurements occur during the first 100 seconds and discharge static from the electrode. Then, a series of 12 B-C measurements occur (B1, C1, B2, C2, etc.). The Bs are for background and sample measurements, whereas Cs allow for an additional open circuit interval when the offset knob can be adjusted. The open circuit interval between B and C is 20 seconds. During this time, the offset knob is adjusted to get the next B measurement near zero. The open circuit interval between C and the subsequent B (i.e., C2, B3) is

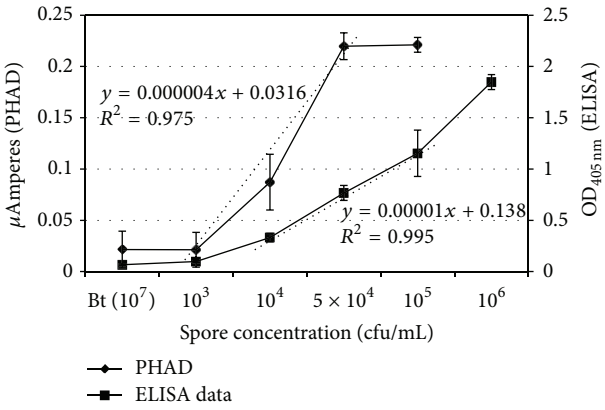

Figure 2: Linear range of PHAD compared to ELISA. PHAD has a dynamic range between 10^3 and 5×10^4 cfu/mL, whereas ELISA has a dynamic range between 10^4 and 10^5 cfu/mL. Spore concentrations that produce statistically saturated signals for PHAD (5×10^4) and ELISA (10^6) are indicated. Average signal for *Bacillus thuringiensis* (*Bt*, tested at 10^7 cfu/mL) is also shown adjacent to 10^3 values for comparison. Error bars = standard deviation, dotted line = linear range.

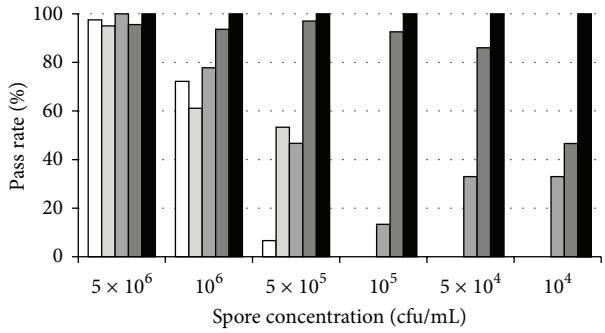

Figure 3: PHAD detection rate compared to LFDs and ELISA. Three LFDs Tetracore, Advnt, and New Horizons; white, light and medium gray bars, resp.), PHAD (dark gray) and ELISA (black bars). were assessed for type 2 error (presented here as "detection rate" or percent true positive signals) at the indicated spore concentrations. $n \geq 15$ (LFDs), 100 (PHAD), and 25 (ELISA).

120 seconds. During this interval, blank substrate buffer is removed and replaced with fresh buffer (or sample buffer prior to B11) 45–50 seconds before the next B measurement. Reproducibility is somewhat dependent upon consistent timing of buffer replacement since less resistance during longer open circuit intervals results in greater current signals. (Hence, C measurements are always lower than prior B measurements due to the shorter open circuit event preceding it). No offset adjustments occur prior to B9 and B10, as these are averaged for the true background signal of the discharged and conditioned electrode. Then, this value is subtracted from the sample signal at B11.

Amperometric detection of *B. anthracis* spores was compared to Tetracore Inc.'s ELISA assay in terms of specificity, limit of detection (LoD), and dynamic range (DR) (Figure 2). Neither PHAD nor ELISA falsely detected 10^7 cfu/mL of

Table 1: Assay parameter comparison.

Assay	Time-to-results (hrs)	Limit of detection (cfu/mL)	Limit of quantification (cfu/mL)	Dynamic range (cfu/mL)
PHAD	1.25	10^4	5×10^4	10^3–5×10^4
LFD	0.25	10^6	NA	NA
ELISA	6.5*	10^4	10^4	10^4–10^5

*Assay time does not include initial overnight ELISA-plate coating step.
NA: not applicable.

B. thuringiensis as *B. anthracis*. Signals generated for this negative control were similar to signals generated by 10^3 cfu/mL *B. anthracis* spores; hence, this concentration is below the level of detection of both assays. The LoD for both PHAD and ELISA was 10^4 cfu/mL. The dynamic range ($R^2 = 0.975$ and 0.995 for PHAD and ELISA, resp.) of both assays was similar (10^3–5×10^4 for PHAD and 10^4–10^5 cfu/mL for ELISA), though PHAD produced saturated signals at 5×10^4 cfu/mL or greater concentrations. ELISA generated saturated signals at spore concentrations of 10^6 cfu/mL and greater (data not shown). In general, PHAD had greater variability in signal output for each spore concentration tested except 10^5 cfu/mL. At this concentration, most signals on the PHAD device are saturated; thus, the true variability for this concentration is not known.

LFDs are rapid, disposable chromatographic strips designed for remote testing and therefore were compared to amperometry and ELISA for sensitivity and reliability (Figure 3). LFDs provide no quantifiable data, as any appearance of a line (color development) within the test area of the strips positively indicates the presence of anthrax spores, and color intensity does not necessarily correlate with antigen concentration. Despite differences of sample application (see Section 2), all 3 LFDs (white, light and medium gray bars) performed similarly. All positively detected *B. anthracis* spores in solutions of 5×10^6 cfu/mL for at least 90% of trials. However, the detection rate dropped greatly for lower concentrations (<80% for 10^6 and <60% for 5×10^5 cfu/mL). Only New Horizon test strips successfully detected spores at 10^5 cfu/mL or lower, albeit at very low rates (<40% of trials). The subjective interpretation of results is often an understated limitation of LFDs. Tetracore LFDs are the easiest to interpret (usually the test line is dark and highly contrasted), whereas Advnt LFDs almost always produce a very faint test line even at very high spore concentrations (data not shown). ELISA (black bars) had 100% positive detection (zero type 2 errors) for all 25 trials of each spore concentration tested. Amperometry detected spores greater than 90% of trials at concentrations of 10^5 cfu/mL or greater. The rate drops to 86% and 47% for 5×10^4 and 10^4 cfu/mL, respectively.

The amount of time to perform an assay is an important parameter in field-detection research. LFDs are unrivaled in time-to-results, with each generating results in as little as 15 minutes of sample application (Table 1). The Tetracore ELISA assay requires over 6 hours for results. The amperometry-based detection described here can obtain results in about 1.5

hours. The LoD of ELISA and PHAD is the same (10^4 cfu/mL) and 100-fold more sensitive than the LFDs. The LoQ, a stricter measure of sensitivity than LoD, is limited to 5×10^4 cfu/mL, 5-fold greater than ELISA.

4. Discussion

Anthrax spores are naturally poorly immunogenic [11]. Thus, many commercially available antibodies claiming to be *B. anthrax* spore specific actually bind to a spore preparation contaminant that originates from vegetative cells [12–14]. A recent mAb development strategy has attempted to circumvent this problem by developing antibodies that recognize *B. anthracis* specific sugar moieties [15]. However, this strategy presumably only worked with inactivated spores. Spore inactivation can increase or decrease sensitivity of mAb and DNA-based detection assays depending on the method [16]. Considering the homogeneity of the *Bacillus* genera, it is also not surprising that cross-reactivity is a major issue [12, 17, 18]. In our testing, we used a single polyclonal antibody for capture (conjugated to magnetic beads) and 4 monoclonal antibodies for detection (conjugated to GOX). This enabled efficient capture of anthrax spores without falsely detecting other *Bacillus* species. None of the monoclonals cross-react with the other *Bacillus* sp. tested (*B. cereus, mycoides*, and *thuringiensis*; data not shown); however, specific strains shown to cross-react with monoclonals were not tested [13, 15]. In addition, fully active spores were used to limit the effects of pretreatment.

ELISAs are a robust, adaptable, and sensitive detection method. Long sample-to-signal times and cost of plate readers have been the ELISA biggest limitation. As the detection assay exits the laboratory and enters the field, the power requirements and delicate optics further limit the traditional and even some more advanced [19] ELISA formats. Amperometry benefits from low cost and power requirements. The PHAD instrument can be made for under $1000 and is powered by laptop battery through a USB cable. For small batch production (less than 10,000 units), the PHAD electrode costs less than $3 and can be reused 2-3 times with minimal drift (data not shown). Here, amperometry was linked with the robustness of antibody-based detection and magnetic-bead capture. Magnetic-bead capture eliminates the need for centrifugation, an activity that is not readily mobilized. The assay was also optimized so that conjugated capture and detection antibodies could all be incubated simultaneously, eliminating several incubation and wash steps typical of ELISA. The reporter enzyme, glucose oxidase, was chosen due to general robustness, a testament to its use in diverse biosensor applications [20, 21]. Immunoassay robustness is partially attributable to the removal of potential signal interferents prior to substrate incubation via rigorous washing steps. Similarly, the PHAD assay tolerates moderate interferent concentrations (data not shown). The greatest limitation of the current method is the narrow reportable range of the PHAD device. The limit of detection was 1×10^4 cfu/mL whereas a maximum signal was achieved with just 5×10^4–1×10^5 cfu/mL, just a 5-fold range. The second generation PHAD will have a maximum reportable range of 32 V with a resolution of 1 part in 32,000, thereby enhancing the reportable range and negating the need for background adjustments.

5. Conclusion

Here, the benefits of generating an amperometric versus colorimetric signal in immunoassay performance were demonstrated. Immunoassay amperometry was comparable to ELISA in terms of limit of detection but was much better in sample-to-signal time. In addition, the low power needs of amperometry allow the assay to be portable, further increasing the appeal of the strategy. The use of magnetic-bead separation was included to eliminate the need of a centrifuge and thus all high-power equipment.

Conflict of Interests

The authors have no direct financial relationship of any kind with any of the commercial identities named in this paper.

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