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Reagent-free and portable detection of *Bacillus anthracis* spores using a microfluidic incubator and smartphone microscope

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Abstract

Bacillus anthracis is the causative agent of anthrax and can be contracted by humans and herbivorous mammals by inhalation, ingestion, or cutaneous exposure to bacterial spores. Due to its stability and disease potential, *B. anthracis* is a recognized biothreat agent and robust detection and viability methods are needed to identify spores from unknown samples. Here we report the use of smartphone-based microscopy (SPM) in combination with a simple microfluidic incubation device (MID) to detect 50 to 5000 *B. anthracis* Sterne spores in 3 to 5 hours. This technique relies on optical monitoring of the conversion of the 1 μm spores to the filamentous vegetative cells that range from tens to hundreds of micrometers in length. This distinguishing filament formation is unique to *B. anthracis* as compared to other members of the *Bacillus*

cereus group. A unique feature of this approach is that the sample integrity is maintained, and the vegetative biomass can be removed from the chip for secondary molecular analysis such as PCR. Compared with existing chip-based and rapid viability PCR methods, this new approach reduces assay time by almost half, and is highly sensitive, specific, and cost effective.

Introduction

Anthrax is an acute disease caused by the bacterium *Bacillus anthracis* that can be contracted by humans and other animals through ingestion or inhalation of the spores, or by cutaneous introduction through abrasions.[1] While the natural infection of humans and livestock in the United States is rare, anthrax outbreaks are common in many developing countries where effective means of prevention, diagnosis and treatment are lacking. The hardiness of *B. anthracis* – spores can remain viable for decades – and the ability to produce large quantities of the spores *in vitro* have also led to the use of *B. anthracis* as an agent of biological warfare and bioterrorism. In a 2001 case of bioterrorism in the United States, *B. anthracis*-laced letters sent through the postal system infected 22 individuals, 5 of whom died as the result of the attack.[2]

Since the 2001 incident, substantial research efforts have focused on improving viable *B. anthracis* spore sampling, recovery, detection and confirmation.[3–5] Current detection methods include traditional culture plating, immunoassays, DNA-based detection via PCR, and a variety of biosensors, with each approach presenting advantages and challenges.[6] A particular emphasis has been placed on field-based measurements, where diagnostics can be performed at the point of threat in the case of either a suspected deliberate release or a natural anthrax outbreak. For such field-based measurements, a premium is placed on ruggedness, ease of use and low cost. A

noteworthy advance in fieldable detection was recently reported by Harper *et al.,* in which a self-contained credit card sized device enabled remote detection of *B. anthracis.*[7] The device incorporated on-chip incubation starting with as few as 100 spores for amplification to the $>10^6$ spores needed for detection by an integrated lateral flow immunoassay. The time required for the test was in the range of 8–18 h and the device was self-contained, requiring no external power sources or pumps (novel magnetically actuated valves provided fluidic control). The price per assay was estimated to be ~$5–7, a fivefold reduction compared to current commercial offerings. The reduction in price can dramatically increase the accessibility of the test, particularly in resource-limited settings where natural outbreaks are most common, and additional reductions in assay cost will further promote testing and minimize the impact of outbreaks.

An exciting development in recent years has been the adaptation of ubiquitous smartphone technology for portable chemical and biological detection.[8–16,16–25] Smartphones are now widely available in even the most remote settings, and the combination of high quality cameras and integrated communication capabilities offer tremendous promise for remote and resource-limited analyses. Assays are frequently performed using either paper- or channel-based microfluidic platforms with optical signals being recorded by the smartphone camera. Custom-built applications can then be used for data processing and readout or the acquired images can be transmitted to a centralized facility for further evaluation. Optical attachments can convert smartphone cameras into high powered microscopes for cytological measurements.[26–33]

Here, we have combined a simple microfluidic incubation device (MID) with a high-power, extremely low-cost (< $0.10) smartphone microscope (SPM) for rapid, sensitive and reagent free

detection of viable *B. anthracis* spores. *Bacillus anthracis* spores germinate under appropriate conditions and the metabolically active vegetative cells grow in filamentous rods that can be distinguished visually from other classes of bacteria. Spores are combined with growth media and manually loaded via syringe into a microfluidic incubation chamber. Following incubation, the media is passed through a patterned microfluidic filter where the filamentous rods are retained. The SPM, which has 350× magnification and is based on a Leeuwenhoek design, can readily identify the filamentous rods and confirm the presence of *B. anthracis*.[34] Detection of 50 to 5,000 spores was achieved in 3 to 5 hours, and the platform is readily compatible with secondary confirmation by PCR providing not only genus/species verification but potentially isolate information. Importantly, aside from the smartphone, the entire platform, including the SPM and the MID has a cost of less than $1, providing an order of magnitude reduction in per-assay costs relative to current protocols, which should dramatically increase accessibility of the assay and improve response to potential threats.

Materials and methods

Smartphone microscope

The SPM utilized a glass bead as its lens and was attached to the smartphone using a 3D printed lens holder.[23] The lens holders were created on a Makerbot Replicator 2X using PLA filament at a print speed of 60 mm/s (Makerbot, New York, NY, USA). Two designs were evaluated; the first was a 'clip' style 100× microscope that fits over most smartphones. The second design, which was the primary microscope for the current study, fits snuggly over an iPhone 5 or 5S and provides a magnification of 350×.[35] For the 100× SPM, a 3-mm-diameter glass bead was employed (GL0179B/3000; MO-SCI, Rolla, MO, USA), while a 1-mm-diameter glass bead

served as the 350× microscope lens (11079110; BioSpec, Bartlesville, OK, USA). The glass bead lens was aligned with the 3D printed lens holder and inserted by compression fitting. The magnification was measured using a chrome-on-glass calibration slide from Motic (Motic, Richmond, British Columbia, Canada). The 350× design weighs 2.1 grams, adds 1mm of thickness to the phone, and has a bill of materials cost of $0.07 including the optics. All images were captured using an iPhone 5s (Apple, Cupertino, CA) with the default camera application that is included with the phone. No external light sources were used for any images and ambient light conditions both inside and outdoors are sufficient for imaging at the tested magnifications.

Microfluidic Incubation Device (MID) fabrication

The MID design is depicted in Fig 1. The template used for soft lithography comprised three different feature heights, two of which were photolithographically patterned.[36] A single photomask contained two distinct and aligned layers and was designed and used according to a previously developed procedure.[37] The first layer patterned the 10-µm-tall fine filter (Fig. 2) and was created by spin-coating SU-8 2010 (Microchem, Westborough, MA, USA) on a 100-mm-diameter silicon wafer (University Wafer, Boston, MA, USA) at 3000 rpm for 60 sec and soft baking, exposing, post-exposure baking and developing according to manufacturer instructions. The wafer was then hard baked at 180 °C for 15 min. The remaining patterned features were 25 µm tall and were made from SU 8 25 (Microchem) spin-coated at 2000 rpm for 30 sec and were also processed according to manufacturer instructions followed by a 180 °C hard bake for 15 min. The incubation chamber and waste chamber were made from ~7-mm-tall, 1-cm-diameter cylinders of hot melt adhesive (HMA; Ad tech, Hampton, NH, USA). HMA cylinders were affixed to the template as shown in Fig. 1 by placing the wafer on a hot plate at 85 °C and

pressing the HMA in place. This provided an incubation chamber volume of ~500 µL in the completed devices.

Devices were prepared from the patterned templates as described previously.[38] Briefly, Sylgard 184 (Dow Corning, Midland, MI, USA) was mixed according to manufacturer specifications, poured onto the template to a thickness of ~8 mm, degassed under vacuum and cured at 70 °C for 2 hr. Patterned substrates were then removed from the template and holes were punched at the Inlet and Air Outlet (Fig. 1) using a 20-gauge catheter hole punch (Syneo, West Palm Beach, FL, USA). Substrates were cleaned and irreversibly bonded to 22 mm x 40 mm cover glass (Thermo Fisher, Waltham, MA, USA) by activating the bonding surfaces of the glass and PDMS in an oxygen plasma system. Following activation, the PDMS and glass substrates were brought into contact and then placed in an oven at 70 °C for 1 hr to improve bond strength.

Microorganisms

Bacillus anthracis Sterne spores were prepared reported in Nutrient Broth with CCY salts for 72 hours.[37] Two additional spore preparations, derived from *Bacillus atrophaeus* ATCC 9372 and the filamentous fungus *Neurospora crassa*, were tested and served as negative controls. Spores were diluted in phosphate buffered saline (#10010049 Invitrogen, Waltham, MA, USA) containing 0.02% Tween-80 (#P4780, Sigma-Aldrich, St. Louis, MO, USA) which is denoted PBS-T, to 10 times the target concentration. Spores were then diluted tenfold into germination media [Tryptic Soy Broth (TSB; #286220, BD, Franklin Lakes, NJ, USA) with 10 mM L-alanine (#A7627, Sigma-Aldrich)] to the final concentration (10^6, 10^4, 10^3, or 10^2, CFU/mL) .[39] *Bacillus*

atrophaeus and *N. crassa* spores were tested at a concentration of 10^6 CFU/mL. Viable plating was used to confirm the spore CFU/mL.

Spore germination and optical detection

Five-hundred µL of spore-containing mixture were loaded into a 1 mL plastic syringe (Becton Dickinson, Franklin Lakes, NJ, USA) and supplied to the MID via Tygon tubing (Cole-Parmer, Vernon Hills, IL, USA) by hand. After the entire sample was loaded, the MID was incubated at 37 °C with shaking at 100 rpm for 3 to 5 hours to allow the spores to germinate and form vegetative filaments. Subsequent experiments were conducted at room temperature with no shaking to determine the fieldability of the device. The culture was then injected into the waste chamber to collect the *B. anthracis* vegetative cells on the fine filter. The MID fine filter was aligned with the SPM or a standard light microscope (BX51, Olympus, Waltham, MA, USA) to visualize and record the presence of filamentous bacteria. For SPM operation, the 3D printed lens holder was inserted over the iPhone 5s, auto-aligning with the rear-facing camera on the smartphone. Holding the MID to the lens, with the glass coverslip physically resting on the lens cover, the MID was manually translated relative to the lens until the image on the screen displayed the fine filter region of the device. Once positive pressure was applied to aspirate the sample from the incubation chamber to the waste chamber, an accumulation of filamentous rods was rapidly observed on the fine filter surface.

PCR confirmation

To determine the compatibility of the MID with established *B. anthracis*-specific detection a secondary analysis was performed with real-time PCR. MIDs were seeded with spores in

germination media and were incubated for 3 to 5 hr. For each concentration of *B. anthracis* spores tested, a time zero sample in PBS-T was also analyzed to control for false positives. The sample was removed from the single-use MID by piercing a needle into the incubation chamber and aspirating near the fine filter. Real-time PCR was conducted with an Applied Biosystems 7500 Fast Real-Time PCR system (Grand Island, NY, USA). The FAST cycling conditions were used but were modified to include a thermal lysis step at 95 °C for 10 min prior to cycling. Each PCR reaction was performed in a final volume of 20 µL (10 µL of 2x TaqMan® Fast Universal Master Mix, 1 µL of 20x Primer/Probe, 4 µL of nuclease free water, and 5 µL of template). Auto baseline and auto threshold were used for all analyses. The primers and probes (CAAX) are specific for a *B. anthracis* chromosome marker and were purchased from IDT as a PrimeTime® qPCR Assays (IDT, Coralville, IA, USA).[40] The primer sequences were: Forward primer (5' to 3') TCC GTT TAC CAA TTC ACT ATG AAT CAA T, reverse primer (5' to 3') ATG CGT TGT TAA GTA TTG GTA TAA TCA TC and probe (5' to 3') FAM/CC CAC TTG G/Zen/A TTA TAT CCT GAG TAT CGT GA/3IABkFQ/.

Results and discussion

Smartphone microscope characterization

Over the past several years, several research groups have developed a wide array of microscope attachments for smartphones. These attachments range from highly sophisticated systems with multiple components, to relatively simple, low cost arrangements.[41] For the present application, a system was desired that had a footprint comparable to that of a traditional protective cellphone case and that was also inexpensive enough to be considered disposable if contaminated (Fig. 2). Magnification of at least 100× was critical, with the added resolution provided by 300–400×

magnification being desirable for bacterial morphology inspections. With the small form factor requirement, a Leeuwenhoek design was selected.[34] Leeuwenhoek-style microscopes notoriously suffer from aberrations in image quality around the perimeter of the field of view due to the spherical lens being used to view the sample. The center of the field of view, however, provides a more than adequate resolution for cellular morphology and optical viewing of samples.

The SPM was initially characterized and compared to other microscope arrangements using an *Aspergillus* specimen. As shown in Fig. 3, the specimen was imaged using the iPhone 5s camera with no microscope (a); 100× SPM (b), 350× SPM (c), KingMas 60× Clip-On Microscope Magnifier (d), and an Olympus BX51 fluorescence phase contrast benchtop microscope at 100× (e) and 400× (f). All images were cropped to the same field of view, and no other image manipulation was performed. Insets in Fig. a, b, d and e provide expanded views of the region displayed in Fig. 3c for resolution and detail comparison. The unassisted iPhone 5s camera (Fig. 3a) and the low-cost commercial magnifier (Fig. 3d) are insufficient for resolving the *Aspergillus* conidiophore structures (which range in diameter of 2 to 6 μm) or the ~1 μm diameter *B. anthracis* filaments. Similar resolution images were acquired with the 100× SPM (Fig. 3b) and the upright microscope at 100× (Fig. 3e). While individual conidiophore structures can be imaged, there is low resolution of the vesicle at this magnification. As expected, the image quality and structural resolution was the greatest with the 350× SPM (Fig. 3c) and the upright microscope at 400× (Fig. 3f); as both show resolution of distinct conidiophore and vesicle structure of the *Aspergillus* sample. Impressively, the image quality in the center of the field of view of the <$0.10 SPM is comparable to the ~$10,000 benchtop microscope. Images taken with the SPM can also be easily transmitted via email or text message allowing sharing of data and

input from collaborators on the identification of an unknown sample. Larger magnification SPMs (>350×) can also be developed using smaller diameter lenses, but for the purposes of this study the resolution obtained with the 350× SPM was sufficient to distinguish fungal and bacterial morphology.

The use of a 3D printed lens holder increases the ease of use of a Leeuwenhoek style microscope. Previous work by Smith *et al.* demonstrated the utility of a smartphone microscopes using a spherical 1mm glass lens taped to an iPhone camera.[23] The 3D printed lens holder used here allows for the automatic alignment of the microscope lens and the camera, increasing the ease of use. Costs were minimized for the SPM reported here relative to past approaches by employing glass beads that are sold in bulk at a cost of <$0.01/bead. Similar resolution was achieved with these low cost beads and dedicated optical spheres, and the resulting SPM provides a simple, cost effective microscope for the educational field, environmental sampling, the diagnosis of blood borne diseases, and biothreat detection as described here.

Bacillus anthracis detection by smartphone microscopy

To explore the utility of the SPM for bacterial pathogen detection, *B. anthracis* spores were selected as a model pathogen. *Bacillus anthracis* has a unique dormant spore structure which can be problematic for detection and viability assessment at low concentrations (for a review see Irenge and Gala, 2012).[3] While immunoassays are increasingly popular for rapid detection, they typically require high spore numbers (>10^4 CFU/mL), and require antibodies that are expensive to develop and that can be cross-reactive, reducing the advantage of the rapid assays. Further, immunoassays cannot provide information on the viability, infectivity, or the genetic potential of

the spore. While spores can be imaged directly using the SPM, little information can be gained through optical observation. However, by germinating the spores into the metabolically active vegetative filaments, one can not only determine viability, but sequential molecular assessment can provide valuable information regarding the genetic potential of the organism.

To detect *B. anthracis* spores without the addition of reagents such as an antibodies, a microfluidic incubation device (MID) was developed for the growth and subsequent imaging of germinated spores. Assay operation was kept as simple as possible as a proof of principle for field-based or resource-limited detection. As such, spores were combined with growth media off-chip prior to loading the MID by hand using a syringe and tubing, such that no equipment besides the MID and the SPM was required. In the process of filling the incubation chamber, the spores were passed through a micropatterned coarse filter (Fig. 1), which consisted of 12 rows of 50-µm-diameter pillars with 50-µm spaces in between. The coarse filter was designed to prevent any filamentous, non-spore debris from entering the incubation chamber, as such debris could potentially lead to false positive identifications.

Initial tests were conducted at high spore concentrations to verify that germination occurred and that the filaments could be viewed within the MID using the SPM. For the $5x10^5$ *B. anthracis* spore samples, numerous vegetative filaments can easily be seen on the fine filter both with standard microscopy and with the SPM (Fig. 4a, 4b, respectively). Similar results were observed for spores at $5x10^3$ but were less dense (Fig. 4c, 4d). No filamentous fibers were observed with either the *B. atrophaeus* or the *N. crassa* spores, although the ~5–8 µm diameter *N. crassa* spores did aggregate at the coarse filter and tended to clog the MID (Fig. 4e, 4f). Germination and

vegetative filament growth was observed for samples incubated at both 37 °C with shaking and static incubation at room temperature (data not shown).

Optical detection of $5x10^3$ *B. anthracis* spores in 3 hours offers a vast improvement upon traditional culture methods that rely on turbidity measurements and/or colony and bacterial morphology characterization with selective media. However, detection of lower concentrations of spores is desirable for trace analysis. To evaluate the utility of the MID and SPM for low level detection, 500 or 50 CFU were seeded into the MID. After 3 hours no vegetative filaments could be seen, which is not surprising since at lower concentrations additional time is needed to reach an active doubling time. At 4 hours vegetative filaments were observed with MID seeded with 500 CFU. At the 5 hour time point vegetative filaments were observed with the 50 CFU samples, as shown in Fig. 4g.

In summary, optical detection using the MID and SPM is successful after 3 hours for 5000 CFU, 4 hours for 500 CFU, and 5 hours for 50 CFU. The most appropriate comparison for device performance is to Sandia National Lab's credit card-size anthrax detector, BaDx.[7] The BaDx is a self-contained bio-amplification device that has the capability to detect 500 *B. anthracis* spores within 8 to 18 hours. Overall assay cost is greatly reduced from the $30 diagnostic cost to ~$5–7 for BaDx. The results presented here show successful detection of 50 to 500 *B. anthracis* spores in 3 to 5 hours with a device cost of less than a dollar. Further advancements in MID design will allow for automated loading and integrated optical detection.

Molecular confirmation

Incubation of an unknown sample for 3 to 5 hours in the germination media is unlikely to lead to rapid growth of filamentous species other than *B. anthracis*. Indeed, filamentous rods were not observed in any negative control (Fig. 4e, 4f). However, in some situations, visual observation of the conversion of bacterial spores to filamentous rods may be considered as a presumptive identification of viable *B. anthracis* spores. In these cases it would be advantageous to confirm the identity and genetic potential of the biological sample using an additional detection method. One of the fastest methods for molecular detection is real-time PCR (RT-PCR). RT-PCR adds and additional hour to the assay, but provides a conformation of the optical detection method. Three MID experiments at several concentrations were analyzed in triplicate by RT-PCR for n=9 measurements, and results are summarized in Table 1. At all concentrations, time zero samples failed to amplify nucleic acid and were considered negative for the presence of viable *B. anthracis* cells. Conversely, all samples were positive after incubation in the MID Table 1. These data demonstrate that use of a SPM in combination with a MID allows for rapid detection (3 to 5 hours) of low numbers of spores (50 to 5000).

Recent advances of microfluidic chip PCR propose on-board field-based detection of biological samples [42]. A challenge with these methods is that the target sample needs to be in a form that can be easily detected. As such, spores are not conducive for direct PCR analysis and require extensive sample prep using, e.g., bead beaters and/or chemical lysis. Rapid viability PCR (RV-PCR) has been utilized to overcome these challenges. In this method, spores are germinated for 9 hours in filter cups prior to PCR and PCR is conducted pre- and post- enrichment. The change in CT values from time zero to the final endpoint is used to determine if the sample is positive for *B. anthracis* spores. This method has been extensively used for wipe, swab, and post-decon

samples with a reported detection level of 10 to 99 CFU/sample.[43–46] The advantage of using the MID followed by PCR detection is that the overall enrichment time is decreased to 5 hours for 50 CFU/sample, allowing rapid turnaround of samples for viability assessment of *B. anthracis* spore samples.

Conclusions

In summary, we have developed and implemented a simple microfluidic device with an inexpensive smartphone-based microscope for optical detection of the unique filaments formed by viable *B. anthracis* spores following germination. While *B. anthracis* is closely related to other members of the *Bacillus cereus* group, the vegetative filaments of *B. anthracis* tend to be much longer (>100 μm) and can be used as a distinguishing phenotype from other group members. The approach presented here is simple to use, cost effective, readily implemented in resource-limited settings and is compatible with secondary confirmation assays such as real-time PCR. Only 3 to 5-hour incubation times are required depending on spore concentration, and image collection takes less than 5 minutes. The unique approach also allows data transmission to a subject matter expert for confirmation in minutes. This platform can be modified for the detection of potential biothreat agents in addition to *B. anthracis*.

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Tables

Table 1. RT-PCR values of biomass recovered from MID when seeded with a target spore number and sampled at a specific time. Mean CT values are the average of 3 MID chips analyzed in triplicate for an n=9 PCR measurements.

Target number of *B. anthracis* spores	Growth time	Mean C_T	Standard deviation of the mean
$5x10^5$	3 hours	30.31	0.41
5000	3 hours	35.90	0.40
5000*	3 hours	36.76	0.50
500	4 hours	34.02	0.66
50	5 hours	35.62	1.39

*In addition to *B. anthracis* Sterne spores, *N. crassa* spores were also seeded into the MID

Figure Legends

Fig. 1. Schematic of the MID. The biological sample is injected into the inlet and passes through a coarse filter to remove large debris and particulates before entering the incubation chamber. After incubation for a pre-determined amount of time, the sample is aspirated to the waste chamber. Vegetative bacterial filaments are trapped and concentrated on a fine filter during aspiration for facile optical monitoring.

Fig. 2 Smartphone microscope (SPM) device for iPhone 5 or 5s. Plastic housing is printed using a 3D printer with PLA filament. A 1 mm glass bead that serves as the lens is inserted into the housing after 3D printing.

Fig. 3 Comparison images of an *Aspergillus* specimen taken with a) an iPhone 5s using maximum digital zoom and no optical zoom attachment b) iPhone 5s with 100× SPM c) iPhone 5s with 350× SPM, d) iPhone 5s with KingMas 60x Smarthphone Microscope, e) Olympus BX51 at 100×, f) Olympus BX51 at 400x. Insets in a, b, d and e magnifications of the region displayed in c for resolution and detail comparison.

Fig. 4 *B. anthracis* Sterne spores were germinated for 3 hours in a MID prior to imaging with the 350× SPM. Thin filamentous vegetative cells were observed only in samples that included *B. anthracis* spores, the presence of these filaments was indicative positive viable anthrax. (a) $5x10^5$ spores imaged using standard microscopy; (b) $5x10^5$ spores imaged with SPM; (c) $5x10^3$ spores imaged with standard microscopy; (d) $5x10^3$ spores imaged using SPM; (e) $5x10^5$ *B. atrophaeus*

spores; (f) $5x10^5$ *N. crassa* spores; (g) 50 spores incubated for 5 hours in MID. Arrows highlight *B. anthracis* vegetative filaments.