**Title**

Development of a High-Throughput Method for Processing Sponge-Stick Samples to Detect Viable *Bacillus anthracis* Spores

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**Graphical Abstract**

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**Abstract**

Since the national validation of the sponge-stick based method for detection of *Bacillus anthracis* spores in environmental samples, there have not been focused efforts to address the low throughput nature of the method, which processes only one sample at one time. Sample processing remains a serious bottleneck for rapidly analyzing large numbers of samples expected from a biological warfare attack. Therefore, we developed a high-throughput method to simultaneously process multiple sponge-stick samples to be better prepared for rapid response and recovery after wide area anthrax incidents. In this method, sponges are placed in 50 mL tubes containing 25 mL buffer and shaken to release spores, after which the buffer is recovered for analysis. We determined that an additional rinse step, conducted in the same tubes with 10 mL buffer, further increased spore recovery from sponge-stick by approximately 10%. We determined that orbital shaking and multi-tube vortexing were both more effective than reciprocating shaking for recovering spores. We conducted simultaneous processing of up to 12 sponge-stick samples and demonstrated comparable spore recovery efficiencies to the traditional low-throughput stomacher-based method (approximately 60% recovery at 102-spore level and 75% recovery at 104-spore level for both methods in three replicate experiments, P > 0.05 for two-tailed T-tests for each experiment and spore level). We also demonstrated that our high-throughput method could be integrated with Rapid Viability-Polymerase Chain Reaction (RV-PCR) analysis and could detect levels as low as 40 spores per sponge even when challenged by a PCR particulate contaminant.

**Keywords**

*Bacillus anthracis*, anthrax, high-throughput sample processing, sponge-stick samples, spore viability

**Highlights**

* Traditional pathogen recovery from sponge-stick samples is time/material/equipment intensive.
* The high-throughput method (HTM) can simultaneously process ≥ 12 sponge-sticks, while reducing buffer volumes required.
* *B. anthracis* spore recovery using HTM was comparable to traditional stomacher-based method.
* The HTM is robust to presence of interfering particulate contamination (Arizona Test Dust) and compatible with Rapid Viability-Polymerase Chain Reaction (RV-PCR) analysis.

**1. Introduction**

A wide-area biological warfare attack with *Bacillus anthracis* spores (causative agent of anthrax) remains a significant concern for government agencies focused on bio-preparedness and mitigation planning. As a Tier 1 Select Agent, *B. anthracis* is of particular concern because its spores can be aerosolized to cause widespread infections and contamination [CDC, 2024]. For inhalational anthrax, the median infectious dose was estimated at 8,000 to 50,000 spores [Seigel et al., 2007] and while case-fatality rates have decreased, 45 percent of victims from the 2001 Amerithrax attack perished, even with antibiotics and supportive care [Jernigan et al, 2002]. *B. anthracis* spores are resistant to environmental conditions [Setlow, 2014] enabling their persistence in the environment [Barandongo et al, 2023; Dragon and Rennie, 1995], therefore, widespread contamination and spore resuspension (leading to inhalation) are also of concern.

Following release of a biothreat agent, impacted sites will need to be thoroughly sampled to adequately determine the severity and extend of exposure, resulting in a large and targeted sampling campaign. Based on its features and past contamination incidents [Teshale et al., 2002], an anthrax attack could result in a large number of samples for analysis, making efforts to improve sampling and analysis of this bioterrorism agent of utmost importance to both determine the extent of contamination and to verify decontamination activities were successful. To safely reopen facilities following an anthrax attack, rapid, high-confidence data are needed. Targeted sample testing approaches should include molecular analyses such as real-time Polymerase Chain Reaction (PCR) to first identify the biothreat agent present. Secondly, once the agent is identified, viability-based analysis, such as microbiological plate-culture and Rapid Viability-Polymerase Chain Reaction (RV-PCR) [Létant et al., 2011], should be conducted to accurately determine the extent to which intact, infectious agents are present, since in this form, these agents carry a risk to human health. In RV-PCR, real-time PCR is conducted on samples before and after a short incubation in growth media (9 hours for *B. anthracis*). By comparing real-time PCR results from the before and after incubation samples, this allows detection of growth, and by extension of viable spores, even in a complex matrix with other microorganisms present.

As a front-end to analytical methods to detect viable biothreat agents, sampling devices need to be processed to recover spores of *B. anthracis* for analysis. Sample processing remains a serious bottleneck for rapidly analyzing a large number of samples, and subsequently, providing timely results. The sponge-stick device (Hardy Diagnostics, Cat. No. SH10NB1; prewet with 10-mL Neutralizing Buffer) is a preferred and commonly used surface sampling approach to collect *B. anthracis* spores from contaminated non-porous surfaces. Like macrofoam swabs, these devices have a validated sample processing and analysis method; however, sponge-sticks can be used to sample larger surface areas up to 100 sq. in. [Rose et al., 2011] vs. 4 sq. in. for swabs [Hodges et al., 2010].

A sample processing method to recover *B. anthracis* spores from sponge-sticks was developed and validated by the Centers for Disease Control and Prevention (CDC) in 2011 [Rose et al., 2011]; the method uses a Stomacher with associated plastic bag and a large volume of buffer (90 mL) to process only one sponge-stick sample at a time. Although the method was recently updated to use 45 mL buffer for spore recovery, other steps remained unchanged [Chan-Riley et al., 2024]. Such a low throughput method can create a serious backlog of samples to be analyzed during a wide-area anthrax incident. Therefore, we developed and evaluated a high-throughput sample processing method for analysis of the large number of sponge-stick samples anticipated during a contamination incident involving *B. anthracis*. Following evaluation and optimization of method steps, the high-throughput method was compared with the traditional stomacher method for spore recovery using *B. anthracis* Sterne (lacking the pXO2 virulence plasmid).

**2. Materials and Methods**

***2.1 Bacillus anthracis Spores***

All experiments were conducted with *B. anthracis* Sterne 34F2 spores (confirmed to lack the pXO1 virulence plasmid). Spore suspensions were obtained from Apex Laboratories, Inc. (Apex, North Carolina) as 108 colony forming units (CFU) per ml in 30% ethanol. The spore concentration was verified by serial dilutions with plate counting and microscopic analysis using a hemocytometer, showing similar spore levels of 3.0 ± 0.3 × 108 and 4.8 ± 0.82 × 108 CFU/mL, respectively. Serial dilutions of spore suspension were prepared in phosphate buffered saline with 0.05% Tween 20 (PBST) to produce working spore stocks at different concentrations for experiments. Spore stock concentrations were determined by dilution plating on tryptic soy agar (TSA) plates (triplicates, 100 µL per plate) and incubating overnight (18 to 24 hours) at 37°C. Spore stocks were stored at 4°C when not in use for up to 4 weeks and were periodically checked with dilution plating to ensure that the viable spore concentration did not decrease over time.

***2.2 High-throughput Spore Recovery***

*2.2.1 Preparation of Sponge-Sticks*

Sponge-sticks were inoculated to achieve the desired number of spores (between 2 × 101 and 3 × 104 CFU per sponge depending on the experiment). Sponges were inoculated by pipetting a total of 100 µL of spore suspension, with approximately half of the inoculum deposited across each side of the sponge in a drop-wise manner. Thus, to achieve an inoculum level to 102 CFU, a spore suspension of 103 CFU/mL was prepared in PBST from spore stocks, and 100 µL of that suspension was used to inoculate each sponge, depositing 50 µL of the inoculum in a drop-wise manner across each side.

For some experiments, Arizona Test Dust (ATD; ISO 12103-1, A3 Medium, Powder Technology, Inc.) was spiked onto sponges to simulate interference that could be found in environmental surface samples. An ATD slurry was prepared at a concentration of 10 mg/mL in sterile ultrapure water. The slurry was sterilized by autoclaving (121 °C, 15 psi, 20 min) since presence of indigenous spores [Rose et al., 2011] would interfere with culture-based analysis, while unsterilized ATD was used for experiments with RV-PCR analysis. Sponges were spiked with 200 µL of this suspension (100 µL on each side), for a total addition of 2 mg ATD per sponge (as described in Rose et al., 2011). The ATD slurry was vortexed between each sponge preparation to ensure consistency between sponge applications. Prepared sponges were detached from their sticks and placed in sterile specimen cups held at 4°C until processing (up to 24 hours). Negative control sponges (uninoculated, with or without ATD as appropriate) were prepared for each experiment.

*2.2.2. Sponge Processing*

For each sponge to be processed, a 50 mL conical tube containing 25 mL sterile spore recovery buffer (70% PBST, 30% ethanol) was prepared and used. Using two sets of sterile disposable forceps, each sponge was folded in half and then in half again, and transferred to a prepared 50 mL tube containing recovery buffer. This process was repeated for all sponges. Tubes were then sealed and simultaneously shaken for five minutes using one of three shaking methods: vortexing in a multi-tube vortexer (VWR, Cat. No. 444-7063) at maximum speed, shaking in an orbital shaker (New Brunswick Innova 4080, Mfg. No. M1192-0004; vertical tube orientation) at 400 RPM or shaking in a reciprocating shaker (Eberbach Corp., Cat. No. E6013.00; horizontal tube orientation) at 240 RPM (maximum speed).

After shaking, the buffer containing released spores was recovered and transferred to a new sterile 50-mL conical tube. A 25-mL serological pipette was used to recover the buffer and transfer it to the new tube. To maximize recovery, the tip of the pipette was used to express as much buffer from the sponge as possible. A secondary rinse step was conducted to recover remaining spores in the sponge. After the initial spore recovery, 10 mL buffer (70% PBST, 30% ethanol) was added to each sponge in its 50-mL conical tube. Tubes were sealed and simultaneously shaken using the same shaking method as described for the initial recovery. After shaking, the released spores in buffer were recovered using a 25 mL serological pipette as described above.

For some experiments (comparisons to conventional stomacher processing, ATD addition experiments, and integration with RV-PCR), recovered spores were further concentrated by centrifugation. Tubes containing recovered spores in buffer were centrifuged at 4,000 RPM (3,220 ´ g) for 30 minutes at 4ºC. Using a serological pipette, enough supernatant was carefully removed to leave a final volume of approximately 3 mL. The remaining volume, containing the spore pellet, was vortexed to resuspend spores for analysis.

*2.2.3. Spore Recovery Analysis*

To evaluate spore recovery, spore suspensions were plated on TSA plates by either dilution plating or MicroFunnel filter plating, dependent on starting spore concentrations. For dilution plating, appropriate dilutions were prepared in PBST to achieve spore counts in the range of 25 to 250 CFUs per plate (and up to 100 CFUs per Microfunnel filter) based on the spore level being used. Aliquots (100 µL each) of the spore suspensions, or dilutions thereof, were plated in triplicate on TSA plates. Plates were incubated overnight (18 to 24 hours) at 37°C, after which colonies were quantified. Plating and colony counting of the spore inoculum was also conducted with each experiment to determine the actual number of viable spores spiked onto sponges for each experiment.

For lower spore level experiments (< 103 CFU per sponge), MicroFunnel filter plating was used to quantify spore recovery. MicroFunnel filters (VWR, Cat. No. 55095-060) were pre-wet by filtering 10 mL PBS through the filter under vacuum (10 psi). Spore suspension was split between 2 or 3 MicroFunnel filters (0.45 µm pore size) and vacuum was applied to recover spores onto the filters. Filters were then rinsed with another 10 mL PBS, transferred to TSA plates using sterile forceps, and incubated overnight (18 to 24 hours) at 37°C.

RV-PCR [Létant et al., 2011; Shah, 2017] was also used to determine the sensitivity of detection for the high-throughput sample processing method. For these experiments, the entire volume of the recovered spore suspension (approx. 3 mL) was collected by vacuum filtration (10 psi) onto 0.45 micron autovials (Whatman Cat. No. AV125NPUPSU) using the custom vacuum manifold (Pacon Mfg., Cat. Nos. 1701232-1 and 1701232-2). The subsequent steps followed the Protocol for Detection of *Bacillus anthracis* Spores from Environmental Samples During the Remediation Phase of an Anthrax Incident [Shah, 2017], with differences noted. Briefly, the RV-PCR protocol included washing the filter vial with 70% PBS/30% EtOH (without Tween), followed by High Salt Wash buffer, and then Low Salt Wash buffer. Each rinse step was reduced to 5-mL of cold buffer followed by vacuum. The autovials were then capped on the bottom by transferring the top manifold to the capping tray (Pacon Mfg., Cat. No. 1701233) with Luer lock caps, followed by addition of 5 mL cold Tryptic Soy Broth. The autovials were capped on the top and the manifold/capping tray was double-bagged and vortexed using the multi-tube plate vortexer set to level 7 for 10 min at room temperature. A 1-mL aliquot was then taken from each sponge sample autovial prior to incubation, referred to as the T0 aliquot, and centrifuged at 14,000 rpm at 4°C for 10 min. Then, 800 mL were removed from each tube and the pellets were stored at -20°C until DNA extraction was conducted (up to one week). The autovials were recapped, bagged and vortexed with the multi-tube plate vortexer set to level 7 for 10 min. The autovials were then incubated in a shaker (Innova 4080, New Brunswick Scientific) at 37°C and 230 RPM. After 9 hours of incubation, another 1-mL aliquot was removed (referred to as the T9 aliquot). The T9 aliquots were centrifuged at 14,000 RPM at 4°C for 10 min. Then, 800 mL were removed from each tube and the T9 pellets were stored at -20°C until DNA extraction could be performed (up to one week).

DNA was extracted using the Promega Magnesil kit (Promega, Cat. No. MD1360) with modifications to the manufacturer’s protocol as previously described [Shah, 2017]. The chromosomal assay BC3 and the pXO1 assay EPA-1 (targeting pPCP1) were used on triplicates of each undiluted sample DNA extract [Létant et al., 2011], with T0 and T9 aliquots from the same sample analyzed on the same PCR plate. Each plate included 10-fold serial dilutions of genomic DNA extracted from *B. anthracis* Sterne 34F2 cells using a MasterPure Complete DNA and RNA Purification Kit (Biosearch Technologies, Cat. No. MC89010) and quantified using a Qubit dsDNA HS Assay Kit (Invitrogen, Cat. No. Q32851). Each DNA extract (5 μL) was added to 20 μl of PCR mix containing the following: 12.5 μl of TaqMan 2× universal master mix (Applied Biosystems, Cat. No. 4305719), 1 μL of 2 μM probe, 1 μL of 25 μM each for the forward and reverse primers, and 4.5 μL Nuclease-Free Water (Invitrogen). PCR was performed on T0 and T9 DNA extracts using the ABI 7500 Fast platform (Applied Biosystems) in fast mode with the following parameters: 2 min at 50°C, 10 min at 95°C, followed by 45 amplification cycles (5 s at 95°C for denaturation and 20 s at 60°C for annealing/extension) as previously established (Létant et al., 2011). The assay primers and probe for the chromosomal assay BC3 were (5’ to 3’):

* BC3 Forward Primer: TTTCGATGATTTGCAATGCC
* BC3 Reverse primer: TCCAAGTTACAGTGTCGGCATATT (reverse primer)
* BC3 Probe: FAM-ACATCAAGTCATGGCGTGACTACCCAGACTT-BHQ1.

For the EPA-1 (pXO1) assay, the primer and probe sequences were:

* EPA-1 Forward Primer: GCGGATAGCGGCGGTTA
* EPA-1 Reverse Primer: TCGGTTCGTTAAATCCAAATGC
* EPA-1 Probe: FAM-ACGACTAAACCGGATATGACATTAAAAGAAGCCCTTAA-BHQ1.

To calculate or DCT for PCR samples with no CT values (detected at T0 or T9), these values were set at 45 (the number of amplification cycles performed). The criteria for positive, viable *B. anthracis* spore detection with the RV-PCR method were: T9 PCR CT £ 39.0 and DCT (CT[T0] - CT[T9]) equal to ³ 6.0 (representing at least a ~2-log increase in DNA concentration after 9 h of sample incubation). The average CT values and standard deviations were based on triplicate samples and triplicate PCR analyses per sample.

***2.3. Conventional Stomacher Sponge-Stick Processing***

The Stomacher method for sponge-stick samples [Shah, 2017] was followed using a Stomacher® 400 Circulator (Seward Cat. No. 0400/001/AJ). Briefly, each inoculated sponge was transferred with forceps to a Stomacher bag (Seward, Cat. No. BA6141/CLR) and any remaining liquid in the specimen cup was also transferred to the corresponding bag. Then, 90 mL of sterile PBST was added to the bag using 50-mL serological pipets, the top of the stomacher bag was closed using the whirl-pack type closure, and the bag was placed into the Stomacher. The bag was processed at 260 rpm for 1 min at room temperature and then removed and left to settle for 10 min to allow the foam to dissipate. The sponge was then removed from the bag with a sterile forceps after squeezing out as much liquid as possible. The bag contents were then mixed by 50-ml serological pipet and transferred to two 50-mL conical tubes.

To concentrate the spores, tubes were centrifuged at 4,000 RPM (3,220 ´ g) for 30 minutes at 4 °C. The supernatant was carefully removed and discarded leaving the spore pellet and approximately 3 mL of buffer in each tube. The tubes were vortex-mixed on high setting for 2 min in 10-sec bursts to suspend the pellets. The suspensions from the two tubes were combined into one tube and the volume was measured and recorded. The spore suspensions were plated on TSA plates to determine CFUs as described for the high-throughput method above.

***2.4. Data analysis***

Values for spore counts (CFU) or percent recovery are reported as the mean ± the standard deviation for three or four biological replicates. Data for all individual replicates are indicated in the data tables. Within each experiment, two-tailed Student’s t-tests were used to test for significant differences between treatments in Microsoft Excel. For the statistical analysis of RV-PCR, the overall standard deviation from all sample replicates was calculated using the following equation,



where *n*1, *n*2, and *n*3 = the number of PCR analyses per sample for sample replicates 1, 2, and 3; *s*1, *s*2, and *s*3 = the SD of the CT values for the individual samples; *X*1, *X*2, and *X*3 = the average CT values for the individual samples; = the overall average CT value for the samples. The overall SD equation was modified accordingly for either two or four replicate samples or positive controls.

**3. Results**

***3.1. Effect of Two Rinse Steps on Spore Recovery***

To determine *B*. *anthracis* spore percent recovery and whether a second rinse step was necessary, we initially tested first and second rinse steps separately. Here, shaking was conducted in an orbital shaker (at 400 RPM) and the inoculum level was determined to be 21,000 ± 500 CFU per sponge. The initial rinse step recovered 83 ± 9% of the spores (17,500 ± 1,800 CFU) inoculated onto sponges, and the second rinse step recovered an additional 10 ± 3% of the spiked spores (2,100 ± 600 CFU) (Table 1). This experiment was replicated, and the inoculum level of the replicate experiment was determined to be 25,600 ± 1,000 CFU per sponge. The replicate experiment showed similar recovery to the first experiment (71 ± 17% recovery or 18,100 ± 4,300 CFU for the initial rinse and 11 ± 2% recovery or 2,700 ± 400 CFU for the second rinse). Spore recovery efficiencies for the two experiments were not statistically different for either the first rinse (P=0.30) or the second rinse (P=0.75). Because the second rinse consistently improved recovery—an additional 10% of the initial spore inoculum—subsequent experiments used the two-rinse procedure, and reported recoveries represent the recoveries of both rinses combined.

***3.2. Effect of Shaking Mode on Spore Recovery***

All three shaking methods (orbital shaker, multi-tube vortexer, and reciprocating shaker) were compared to determine the most effective method for spore recovery. Orbital shaking and vortexing were found to produce comparable spore recoveries, while reciprocating shaking produced a lower spore recovery (Table 2). For an initial inoculum of 213 ± 9 CFU per sponge, orbital shaking and vortexing resulted in statistically similar (P=0.21) recoveries of 96 ± 4% and 91 ± 2% (204 ± 10 and 195 ± 4 CFU), respectively. For reciprocating shaking, the spore recovery was 81 ± 6% (172 ± 12 CFU), which was statistically lower than that with the other shaking methods (P < 0.05). This experiment was replicated, but recoveries between different shaking/vortexing methods were not statistically different (P > 0.05 for all pairwise comparisons). The inoculum concentration in the replicate experiment was 251 ± 10 CFU per sponge, and recoveries were 76 ± 7%, 68 ± 4%, and 69 ± 3% (190 ± 18, 171 ± 10, and 172 ± 9 CFU) for orbital shaking, vortexing, and reciprocating shaking, respectively. Based on these results, and considering recovery efficiency and other factors (e.g., the multi-tube vortexer is portable, easily holds a 50-mL tube rack without additional clamps required by the orbital shaker, and is lower in cost), all further experiments were conducted using the multi-tube vortexer for shaking.

***3.3. Comparison of High-throughput and Traditional Stomacher Sample Processing Methods***

The high-throughput method was compared to the traditional stomacher-based method for spore recovery, and the two methods achieved similar percent recoveries at two different inoculum levels (Tables 3 and 4). At the high inoculum level (25,800 ± 600 CFU per sponge), the high-throughput method recovered 74 ± 9% (19,000 ± 2,200 CFU), and the stomacher method recovered 77 ± 2% (19,900 ± 600 CFU). The spore recoveries for the two methods were not statistically different (P = 0.54). At the low inoculum level (227 ± 25 CFU per sponge), the high-throughput method recovered 61 ± 6% (139 ± 14 CFU), and the stomacher method recovered 57 ± 13% (130 ± 30 CFU). These recoveries were also not statistically different (P = 0.63). This experiment was replicated two more times with similar results (Tables 3 and 4), and in each case recoveries achieved with the high-throughput method were not statistically different from the stomacher method (P > 0.05).

***3.4. Impacts of Arizona Test Dust on Spore Recovery***

To evaluate the impacts of external debris on spore recovery, an experiment was conducted to compare spore recoveries from sponges with and without autoclaved ATD additions to assess the impact of interfering particulate matter on recovery of viable spores. Autoclaved ATD was used because non-sterile ATD could introduce indigenous microbes that would interfere with the culture-based detection method for spore recovery used in this experiment. Since RV-PCR analysis is not affected by background microbes, the impact of native ATD was evaluated using RV-PCR as discussed in Section 3.5. A low inoculum level was used for this experiment and was determined to be 250 ± 10 CFU per sponge. During the experiment, we observed recoveries were comparable between the two conditions (Table 5). The percent recovery without ATD was 81 ± 8% (201 ± 20 CFU) and with ATD was 80 ± 2% (200 ± 5 CFU). These spore recoveries were not statistically different (P = 0.96). This experiment was repeated using a low inoculum level of 248 ± 3 CFU per sponge, and recoveries of 81 ± 11% and 76 ± 7% (200 ± 28 CFU and 189 ± 19 CFU) without and with ATD, respectively. These recoveries were not statistically different between the two conditions (P = 0.57).

***3.5 Integration with RV-PCR for Detection from Complex Samples***

The high-throughput sponge-stick processing method was integrated with the high-throughput and rapid analytical method, RV-PCR, to determine sensitivity of detection of *B. anthracis* spores. An experiment was conducted to determine whether the high-throughput sponge-stick processing method could be integrated with RV-PCR to detect *B. anthracis* Sterne spores in the presence of a non-sterile environmental interference matrix (non-autoclaved ATD) (Table 6). The inoculum level for this experiment was determined to be 115 ± 14 CFU per sponge. For the BC3 (chromosomal) assay, the average DCT values (difference between CT of T9 and T0 for given sponge sample) were 26.5 ± 1.5 and 26.1 ± 1.1 for samples with and without ATD, respectively. For the EPA-1 (PXO1) assay, the DCT values were 25.6 ± 1.5 and 25.1 ± 1.1 for samples with and without ATD, respectively. These DCT values indicated positive detection results significantly greater than that required (DCT ³ 6). For both assays, there were no significant differences between DCT values for samples with and without ATD (P > 0.05, two-tailed T-test). All negative controls were non-detectable results. Considering the RV-PCR results analysis algorithm of T9 CT £ 39 and DCT ³ 6, these high DCT values indicated that much lower spore levels could also be detected.

A replicate experiment was conducted with a lower inoculum level of 36 ± 3 CFU per sponge. In the replicate experiment, the average DCT values were 20.3 ± 1.3 with ATD and 21.5 ± 1.0 without ATD for the BC3 assay and 20.7 ± 2.4 with ATD and 22.4 ± 0.8 without ATD for the EPA-1 assay (Table 7). There were no significant differences for samples with and without ATD based on the EPA-1 assay (P = 0.23 for a paired, two-tailed T-test). There was a significant difference for the BC3 assay (P = 0.03), with a higher DCT value for samples without ATD; however, DCT values for samples with and without ATD were more than 3-fold higher than the cut-off value for positive detection (DCT ³ 6). These high DCT values for samples spiked with approximately 40 spores suggested that even lower spore levels would also be detected. Unlike the first experiment, some of the negative controls showed high T9 CT values (43.8 ± 0.2 for 2 of 3 replicate PCR analyses for one negative control for the BC3 assay and 40.6 ± 0.5 and 40.8 ± 0.4 for triplicate PCR analyses from two negative controls with the EPA-1 assay). This was likely due to cross-contamination from adjacent samples with low T9 CT values as PCR amplification curves from these samples were indistinguishable from those of known positive samples. However, the DCT values were less than the required DCT cut-off value of 6 for positive detection (1.2 for BC3 and 4.2-4.4 for EPA-1) indicating a “non-detect” result.

**4. Discussion**

A new high-throughput sample processing method was developed for detection of *B. anthracis* spores from the sponge-stick sampling device. The stomacher-based method [Rose et al., 2011] allows processing of only a single sponge sample at a time, while requiring larger buffer volumes and more processing time for the same number of sponge samples as the newly developed high-throughput method. Within this study, we demonstrated that the high-throughput method performed comparably to the stomacher-based method while processing up to twelve sponge-stick samples simultaneously. This method also reduced the total buffer volume used to 35 mL per sponge-stick sample (25 mL in the initial rinse and 10 mL in the second rinse), compared to 90 mL used in the original stomacher-based method [Rose et al., 2011; Shah, 2017]; the buffer volume thus reducing the waste associated with the method, while shortening the method’s overall procedural time. Additionally, this method does not require specialized equipment (i.e., a Stomacher) and instead relies on equipment commonly available in labs, as we found that both the multi-tube vortexer and the orbital shaker produced similar spore recovery percentages from sponge-sticks. Taken together, this high-throughput method represents a significant improvement over the previous method while maintaining similar levels of performance while reducing costs and shortening overall sample processing times.

In the event of a large-scale *B. anthracis* spore release, large numbers of samples will need to be processed quickly both to assess the extent of the initial contamination and later to confirm decontamination effectiveness. To meet this need, a method must also be able to assess the spore viability in the context of other potentially interfering materials and other microorganisms present in real world samples. Here we show that our high-throughput method is robust to the presence of ATD, a commonly used environmental interferent (Hodges et al., 2010; Létant et al., 2011; Rose et al., 2011). We also show that it is compatible with not only the culture-based analysis method but also the high-throughput RV-PCR-based analysis method for detecting low number of viable spores.

Continued development has the potential to further extend the usefulness of this method. One aspect of that development would be extension of this method for detection of other biothreat agents, including non-spore-forming agents (e.g. *Yersinia pestis,* *Francisella tularensis*, and other Category A Select Agent pathogens). This would require testing and adaptation of the method to ensure that vegetative cells can be effectively recovered while maintaining viability for detection. In addition to applications to other organisms, this method could be further tested to address high levels of interferent materials that may be present in environmental samples. Here we demonstrate that this method was robust for detecting spores in the presence of non-sterile ATD. Future testing could address the sensitivity of this method in the presence of more extensive particulate contamination and microbial complexity. Additional development could also address extension to even higher-throughput sample processing. As presented here, the number of samples processed simultaneously is constrained by the capacity of the multi-tube vortexer—which can accommodate up to 24 sample tubes—as well as the centrifuge capacity per run. Processing larger sample sets simultaneously would require multiple vortexers or use of an alternate, higher capacity shaking method. We demonstrated in this work that an orbital shaker could produce comparable spore percent recoveries to vortexing. Depending on the model, an orbital shaker could accommodate up to 100 or more 50-mL sample tubes simultaneously. Concerning the centrifugation step, the time can be shortened to 15 min if 3,500 ´ g can be achieved [Shah, 2017], and the centrifugation step could be performed in batches, depending on the centrifuge rotor capacity. For example, the centrifuge used in this study only accommodates twelve 50 mL tubes, so larger sample sets would need to be processed in batches.

**5. Conclusions**

In the event of a wide-area anthrax bioattack, large numbers of samples will need to be processed to enable a rapid assessment and effective response. However, the currently used method for recovery of *B. anthracis* spores from sponge-stick samples can process only one sample at a time, and is time, material, and equipment intensive. We developed a new high-throughput method for sponge-stick sample processing to recover *B. anthracis* spores and showed this method has comparable spore recovery percentages to the traditional stomacher-based sample processing method. We also demonstrated this method is robust to presence of non-sterile interfering particulate contamination (ATD) and compatible with RV-PCR-based high-throughput analysis for detection of *B. anthracis* spores from complex samples. This method will enable more rapid processing of large numbers of samples and therefore a more rapid response to a large-scale *B. anthracis* attack.

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**CRediT authorship contributions**

Vanessa L. Brisson’s contributions included Methodology, Investigation, Validation, Data curation, Formal analysis, Visualization, Writing – original draft, and Writing – review and editing.

Staci R. Kane contributions included Methodology, Investigation, Data curation, Formal analysis, Writing – original draft, and Writing – review and editing.

Sanjiv R. Shah’s contributions included Conceptualization, Funding acquisition, Project administration, Data curation, Writing – original draft, and Writing – review and editing.

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**Disclaimer**

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**Tables**

**Table 1.** Recovery of *B. anthracis* Sterne spores from inoculated sponges in two rinse steps.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | Condition |  | First Rinse | | | | |  | Second Rinse | | | | |
|  | Volume Recovered | Colony Counts a | | | Total CFUb |  | Volume Recovered | Colony Counts a | | | Total CFUb |
|  | Plate 1 | Plate 2 | Plate 3 |  | Plate 1 | Plate 2 | Plate 3 |
| Initial Experiment | Positive control  (inoculated buffer without sponge) |  | 24.4 mL | 82 | 97 | 84 | 21391 |  | NA | NA | NA | NA | NA |
|  | 24.4 mL | 84 | 88 | 89 | 21228 |  | NA | NA | NA | NA | NA |
|  | 24.4 mL | 87 | 81 | 83 | 20415 |  | NA | NA | NA | NA | NA |
| Negative Control (uninoculated sponge) |  | 28.6 mL | 0 | 0 | 0 | 0 |  | 10.6 mL | 0 | 0 | 0 | 0 |
|  | 29.6 mL | 0 | 0 | 0 | 0 |  | 9.0 mL | 0 | 0 | 0 | 0 |
|  | 28.5 mL | 0 | 0 | 0 | 0 |  | 9.1 mL | 0 | 0 | 0 | 0 |
| Recovery From Sponge |  | 28.5 mL | 64 | 62 | 55 | 17195 |  | 8.8 mL | 19 | 27 | 15 | 1789 |
|  | 30.3 mL | 59 | 73 | 61 | 19493 |  | 9.6 mL | 33 | 27 | 28 | 2816 |
|  | 30.2 mL | 51 | 50 | 57 | 15905 |  | 9.4 mL | 21 | 16 | 18 | 1723 |
| Replicate Experiment | Positive control  (inoculated buffer without sponge) |  | 24.4 mL | 97 | 101 | 103 | 24481 |  | NA | NA | NA | NA | NA |
|  | 24.0 mL | 107 | 113 | 105 | 26000 |  | NA | NA | NA | NA | NA |
|  | 24.4 mL | 105 | 98 | 121 | 26352 |  | NA | NA | NA | NA | NA |
| Negative Control (uninoculated sponge) |  | 28.9 mL | 0 | 0 | 0 | 0 |  | 9.8 mL | 0 | 0 | 0 | 0 |
|  | 29.6 mL | 0 | 0 | 0 | 0 |  | 9.4 mL | 0 | 0 | 0 | 0 |
|  | 27.8 mL | 0 | 0 | 0 | 0 |  | 10.0 mL | 0 | 0 | 0 | 0 |
| Recovery From Sponge |  | 29.2 mL | 58 | 82 | 83 | 21705 |  | 9.2 mL | 24 | 23 | 28 | 2300 |
|  | 29.2 mL | 54 | 73 | 70 | 19175 |  | 10.0 mL | 26 | 26 | 33 | 2833 |
|  | 26.6 mL | 43 | 55 | 53 | 13389 |  | 9.0 mL | 43 | 32 | 28 | 3090 |

a Plate colony forming units (CFU) are from 100 µL of the final suspension.

b Total CFU recovered was calculated based on the volume recovered and the plate colony counts.

**Table 2.** Recovery of *B. anthracis* Sterne spores from inoculated sponges using different shaking methods.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | Condition | MicroFunnel Filter Colony Counts | | | Total CFU Recovered a |
| Filter 1 | Filter 2 | Filter 3 |
| Initial Experiment | Negative Control (uninoculated sponge) | 0 | 0 | 0 | 0 |
| 0 | 0 | 0 | 0 |
| 0 | 0 | 0 | 0 |
| Orbital Shaker | 73 | 100 | 26 | 199 |
| 100 | 96 | 19 | 215 |
| 92 | 81 | 25 | 198 |
| Multi-tube Vortexer | 64 | 103 | 23 | 190 |
| 83 | 88 | 27 | 198 |
| 83 | 98 | 16 | 197 |
| Reciprocating Shaker | 54 | 74 | 32 | 160 |
| 89 | 67 | 28 | 184 |
| 68 | 80 | 26 | 174 |
| Replicate Experiment | Negative Control (uninoculated sponge) | 0 | 0 | 0 | 0 |
| 0 | 0 | 0 | 0 |
| 0 | 0 | 0 | 0 |
| Orbital Shaker | 91 | 73 | 7 | 171 |
| 105 | 83 | 20 | 208 |
| 85 | 95 | 11 | 191 |
| Multi-tube Vortexer | 73 | 81 | 16 | 170 |
| 63 | 63 | 36 | 162 |
| 79 | 75 | 28 | 182 |
| Reciprocating Shaker | 84 | 63 | 16 | 163 |
| 87 | 79 | 14 | 180 |
| 86 | 66 | 21 | 173 |

a Total CFU recovered was calculated as the sum of the CFU recovered on all MicroFunnel filters for a sample.

**Table 3.** Comparison of recoveries of *B. anthracis* Sterne spores using the high-throughput method described here and the traditional stomacher-based method from sponges inoculated at a 104-spore inoculum level (25,800 ± 600, 25,800 ± 800, and 25,900 ± 1,100 CFU per sponge for the first, second, and third experiments, respectively).

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | Condition | Volume Recovered | CFUa | | | | Total CFUb |
| Plate 1 | Plate 2 | Plate 3 | Plate 4 |
| Experiment #1 | High-throughput | 4.70 mL | 84 | 91 | 102 | NA | 21698 |
| 3.95 mL | 98 | 86 | 93 | NA | 18236 |
| 4.10 mL | 100 | 89 | 98 | NA | 19612 |
| 4.00 mL | 91 | 74 | NA | NA | 16500 |
| Stomacher | 5.20 mL | 79 | 73 | 81 | NA | 20193 |
| 6.10 mL | 63 | 81 | 55 | NA | 20232 |
| 4.45 mL | 84 | 89 | NA | NA | 19246 |
| Experiment #2 | High-throughput | 2.10 mL | 167 | 152 | 172 | NA | 17185 |
| 3.60 mL | 84 | 93 |  | NA | 15930 |
| 3.20 mL | 112 | 106 | 105 | NA | 17227 |
| 3.70 mL | 109 | 102 | 83 | NA | 18130 |
| Stomacher | 5.20 mL | 71 | 65 |  | NA | 17680 |
| -------------------------------Sample Lost----------------------------- | | | | | |
| 4.45 mL | 45 | 69 |  | NA | 12683 |
| Experiment #3 | High-throughput | 3.50 mL | 74 | 90 | 104 | NA | 15633 |
| 3.95 mL | 133 | 125 | 110 | NA | 24227 |
| 2.90 mL | 89 | 87 | 89 | NA | 12808 |
| 2.80 mL | 134 | 141 | 131 | NA | 18947 |
| Stomacher | 4.75 mL | 92 | 80 | 76 | 90 | 20069 |
| 5.55 mL | 64 | 72 | 62 | NA | 18315 |
| 5.35 mL | 92 | 79 | 78 | 92 | 22804 |

a Plate colony forming units (CFU) are from 100 µL of a 1:5 dilution of the final sample.

b Total CFU recovered were calculated based on the volume recovered and the plate colony counts, accounting for the 1:5 dilution ratio.

**Table 4.** Comparison of recoveries of *B. anthracis* Sterne spores using the high-throughput method described here, and the traditional stomacher based method from sponges inoculated at a low inoculum level (227 ± 25, 265 ± 11, and 227 ± 17 CFU per sponge for the first, second, and third replicate experiments, respectively).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | Condition | Volume Recovered | MicroFunnel Filter Colony Counts | | Total Sporesc |
| Filter 1a | Filter 2b |
| Experiment #1 | High-throughput | 3.65 mL | 48 | 80 | 128 |
| 3.05 mL | 59 | 100 | 159 |
| 2.9 mL | 60 | 71 | 131 |
| 4.1 mL | 49 | 88 | 137 |
| Stomacher | 4.5 mL | 22 | 79 | 101 |
| 5.8 mL | 22 | 108 | 130 |
| 5.3 mL | 21 | 139 | 160 |
| Experiment #2 | High-throughput | 3.8 mL | 55 | 91 | 146 |
| 3.05 mL | 82 | 77 | 159 |
| 4.2 mL | 47 | 130 | 177 |
| 1.8 mL | 83 | 35 | 118 |
| Stomacher | 4.5 mL | 31 | 115 | 146 |
| 5.8 mL | 5 | 143 | 148 |
| 5.3 mL | 24 | 128 | 152 |
| Experiment #3 | High-throughput | 3.7 mL | 32 | 127 | 159 |
| 1.7 mL | 78 | 57 | 135 |
| 2.2 mL | 69 | 87 | 156 |
| 4 mL | 49 | 91 | 140 |
| Stomacher | ----------------------------Sample Lost------------------------------- | | | |
| 5.2 mL | 3 | 96 | 99 |
| 4.7 mL | 42 | 135 | 177 |

a Filter 1 was from 1 mL of sample filtered onto a MicroFunnel Filter.

b Filter 2 was from the remainder of the sample, after removal of 1 mL for Filter 1.

c Total CFU recovered was calculated as the sum of the CFU recovered on all MicroFunnel filters for a sample.

**Table 5.** Comparison of recoveries of *B. anthracis* Sterne spores with and without addition of ATD.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | Condition | Volume Recovered | MicroFunnel Filter Colony Counts | | | Total CFUc |
| Filter 1a | Filter 2a | Filter 3b |
| Initial Experiment | Without ATD | 4.2 mL | 65 | 43 | 98 | 206 |
| 3.3 mL | 49 | 58 | 72 | 179 |
| 4.0 mL | 65 | 51 | 102 | 218 |
| With ATD | 3.8 mL | 55 | 52 | 91 | 198 |
| 4.3 mL | 50 | 53 | 103 | 206 |
| 3.3 mL | 66 | 52 | 79 | 197 |
| Replicate Experiment | Without ATD | 3.8 mL | 50 | 64 | 93 | 207 |
| 4.0 mL | 54 | 58 | 88 | 224 |
| 4.1 mL | 44 | 40 | 87 | 170 |
| With ATD | 3.1 mL | 64 | 59 | 58 | 191 |
| 2.4 mL | 77 | 64 | 36 | 169 |
| 3.1 mL | 65 | 68 | 48 | 206 |

a Filters 1 and 2 were from 1 mL aliquots of sample filtered onto MicroFunnel Filters.

b Filter 3 was from the remainder of the sample, after removal of 1 mL each for Filters 1 and 2.

c Total CFU recovered was calculated as the sum of the CFU recovered on all MicroFunnel filters for a sample.

**Table 6.** RV-PCR results for SS samples inoculated with 120 *B. anthracis* Sterne spores with and without ATD. “Avg.” indicates average, and “SD” indicates standard deviation. “ND” indicates not detected.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Condition | Replicate  Number |  | BC3 Chromosomal Assay | | |  | EPA-1 Assay | | |
|  | CT | | DCT |  | CT | | DCT |
|  | T0 | T9 |  | T0 | T9 |
| Without ATD | 1 |  | ND | 18.9 | 26.1 |  | ND | 22.7 | 22.3 |
| 2 |  | ND | 18.6 | 26.4 |  | ND | -- | -- |
| 3 |  | ND | 17.3 | 27.7 |  | ND | 19.5 | 25.5 |
| Avg. |  | ND | 18.3 | 26.7 |  | ND | 21.1 | 23.9 |
| SD |  |  | 0.9 | 0.9 |  |  | 2.2 | 2.2 |
| 1 |  | ND | 18.2 | 26.8 |  | ND | 19.2 | 25.8 |
| 2 |  | ND | 18.0 | 27.0 |  | ND | 19.7 | 25.3 |
| 3 |  | ND | 18.9 | 26.1 |  | ND | 19.7 | 25.3 |
| Avg. |  | ND | 18.3 | 26.7 |  | ND | 19.5 | 25.5 |
| SD |  |  | 0.5 | 0.5 |  |  | 0.3 | 0.3 |
| 1 |  | ND | 21.1 | 23.9 |  | ND | 19.8 | 25.2 |
| 2 |  | ND | 19.6 | 25.4 |  | ND | 20.1 | 24.9 |
| 3 |  | ND | 19.9 | 25.1 |  | ND | 20.0 | 25.0 |
| Avg. |  | ND | 20.2 | 24.8 |  | ND | 19.9 | 25.1 |
| SD |  |  | 0.8 | 0.8 |  |  | 0.1 | 0.1 |
| **Overall Avg** |  | **ND** | **18.9** | **26.1** |  | **ND** | **20.2** | **24.8** |
| **Overall SD** |  |  | **1.1** | **1.1** |  |  | **1.3** | **1.3** |
| With ATD | 1 |  | ND | 18.3 | 26.7 |  | ND | 18.6 | 26.4 |
| 2 |  | ND | 21.0 | 24.0 |  | ND | 19.7 | 25.3 |
| 3 |  | ND | 18.2 | 26.8 |  | ND | 23.0 | 22.0 |
| Avg. |  | ND | 19.2 | 25.8 |  | ND | 20.5 | 24.5 |
| SD |  |  | 1.6 | 1.6 |  |  | 2.3 | 2.3 |
| 1 |  | ND | 17.1 | 27.9 |  | ND | 18.5 | 26.5 |
| 2 |  | ND | -- | -- |  | ND | 18.9 | 26.1 |
| 3 |  | ND | 16.8 | 28.2 |  | ND | 17.1 | 27.9 |
| Avg. |  | ND | 16.9 | 28.1 |  | ND | 18.2 | 26.8 |
| SD |  |  | 0.2 | 0.2 |  |  | 1.0 | 1.0 |
| 1 |  | ND | -- | -- |  | ND | 19.5 | 25.5 |
| 2 |  | ND | 19.1 | 25.9 |  | ND | 19.3 | 25.7 |
| 3 |  | ND | 20.0 | 25.0 |  | ND | 19.6 | 25.4 |
| Avg. |  | ND | 19.6 | 25.4 |  | ND | 19.5 | 25.5 |
| SD |  |  | 0.6 | 0.6 |  |  | 0.1 | 0.1 |
| **Overall Avg.** |  | **ND** | **18.5** | **26.5** |  | **ND** | **19.4** | **25.6** |
| **Overall SD** |  |  | **1.5** | **1.5** |  |  | **1.6** | **1.6** |

**Table 6.** RV-PCR results for SS samples inoculated with 40 *B. anthracis* Sterne spores with and without ATD. “Avg.” indicates average, and “SD” indicates standard deviation. “ND” indicates not detected.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Condition | Replicate  Number |  | BC3 Chromosomal Assay | | |  | EPA-1 Assay | | |
|  | CT | | DCT |  | CT | | DCT |
|  | T0 | T9 |  | T0 | T9 |
| Without ATD | 1 |  | ND | 23.0 | 22.0 |  | ND | 22.0 | 23.0 |
| 2 |  | ND | 23.6 | 21.4 |  | ND | 22.5 | 22.5 |
| 3 |  | ND | 23.7 | 21.3 |  | ND | 22.6 | 22.4 |
| Avg. |  | ND | 23.5 | 21.5 |  | ND | 22.4 | 22.6 |
| SD |  |  | 0.4 | 0.4 |  |  | 0.3 | 0.3 |
| 1 |  | ND | 24.8 | 20.2 |  | ND | 23.6 | 21.4 |
| 2 |  | ND | 24.6 | 20.4 |  | ND | 23.3 | 21.7 |
| 3 |  | ND | 24.4 | 20.6 |  | ND | 23.5 | 21.5 |
| Avg. |  | ND | 24.6 | 20.4 |  | ND | 23.5 | 21.5 |
| SD |  |  | 0.2 | 0.2 |  |  | 0.1 | 0.1 |
| 1 |  | ND | 22.7 | 22.3 |  | ND | 21.6 | 23.4 |
| 2 |  | ND | 22.1 | 22.9 |  | ND | 22.2 | 22.8 |
| 3 |  | ND | 22.2 | 22.8 |  | ND | ND | NA |
| Avg. |  | ND | 22.3 | 22.7 |  | ND | 21.9 | 23.1 |
| SD |  |  | 0.3 | 0.3 |  |  | 0.4 | 0.4 |
| **Overall Avg** |  | **ND** | **23.5** | **21.5** |  | **ND** | **22.6** | **22.4** |
| **Overall SD** |  |  | **1.0** | **1.0** |  |  | **0.8** | **0.8** |
| With ATD | 1 |  | ND | 24.5 | 20.5 |  | ND | 22.9 | 22.1 |
| 2 |  | ND | 24.2 | 20.8 |  | ND | 23.8 | 21.2 |
| 3 |  | ND | 24.3 | 20.7 |  | ND | 24.3 | 20.7 |
| Avg. |  | ND | 24.4 | 20.6 |  | ND | 23.7 | 21.3 |
| SD |  |  | 0.2 | 0.2 |  |  | 0.7 | 0.7 |
| 1 |  | ND | 26.7 | 18.3 |  | ND | 28.5 | 16.5 |
| 2 |  | ND | 25.9 | 19.1 |  | ND | 26.4 | 18.6 |
| 3 |  | ND | 26.2 | 18.8 |  | ND | 26.7 | 18.3 |
| Avg. |  | ND | 26.3 | 18.7 |  | ND | 27.2 | 17.8 |
| SD |  |  | 0.4 | 0.4 |  |  | 1.1 | 1.1 |
| 1 |  | ND | 23.4 | 21.6 |  | ND | 22.1 | 22.9 |
| 2 |  | ND | 24.2 | 20.8 |  | ND | 22.1 | 22.9 |
| 3 |  | ND | 22.9 | 22.1 |  | ND | -- | -- |
| Avg. |  | ND | 23.5 | 21.5 |  | ND | 22.1 | 22.9 |
| SD |  |  | 0.7 | 0.7 |  |  | 0.0 | 0.0 |
| **Overall Avg.** |  | **ND** | **24.7** | **20.3** |  | **ND** | **24.3** | **20.7** |
| **Overall SD** |  |  | **1.3** | **1.3** |  |  | **2.4** | **2.4** |

**Data Statement**

The data used in this manuscript will be available in the online published article.