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Alternative fast analysis method for cellulose sponge surface sampling wipes with low concentrations of *Bacillus* Spores

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A B S T R A C T

Environmental sampling is a critical component of the post decontamination verification process following a bioterrorism event. The current work was performed to produce a less labor-intensive method for processing cellulose sponge-wipes used for sampling areas potentially contaminated with low concentrations (i.e., post-decontamination) of *Bacillus anthracis* spores. An alternative fast-analysis processing method was compared to the processing protocol validated by the Centers for Disease Control and Prevention (CDC) for the Laboratory Response Network (LRN). Glazed tile coupons (1102 cm^2) were inoculated with 50, 500, or 5000 spores of *Bacillus thuringiensis* subsp. *kurstaki* (*Btk*), then sampled with cellulose sponges. Sampling was limited to a 25- by 25-cm area and performed in the same manner as the CDC sampling method. Samples were then processed using either the alternative "Fast Analysis" method or the "CDC method". Three different analysts repeated the tests at each concentration utilizing each method. Mean recoveries, labor time, and potentially hazardous waste produced were compared for the two methods. The mean percent recoveries and standard errors for the samples processed using the "CDC method" were 39.9 ± 6.7, 43 ± 7.6, and 36.8 ± 10.1 for the 5000, 500, and 50 spore loading levels, respectively; compared to 54.2 ± 12.9, 64.2 ± 21.7, and 45.2 ± 8.6 for the "Fast Analysis" method. At each titer tested the "Fast Analysis" method resulted in a statistically significant higher percent recovery. Furthermore, analysts processed samples utilizing the "Fast Analysis" method in less than half the time and generated half as much potentially hazardous waste compared to the "CDC method".

1. Introduction

Following the 2001 Anthrax letter attacks, 125,000 samples were analyzed by the Laboratory Response Network (LRN) (Centers for Disease Control and Prevention. 6, 2006). Remediation of the areas affected by these attacks took years to complete with some of the most time-intensive tasks including environmental sampling and sample analysis (Hess et al., 2016). Any future incidents involving the release of *Bacillus anthracis* (*B.a.*) spores will likely require extensive environmental sampling.

Environmental surface sampling for *B.a.* spores following the 2001 attack included a variety of techniques and implements such as swabs, wipes, and vacuum socks (Rose et al., 2011). Following the 2001 incident, numerous research teams have studied the recovery efficiency for several sampling methods using different techniques, as well as, materials and devices for collection including swabs, wipes, and vacuums for *B.a.* spores or surrogates (Brown et al., 2007a; Brown et al., 2007b; Centers for Disease Control and Prevention and National Institute for Occupational Safety and Health, n.d.; Hodges et al., 2006). The goal of this work was to assess a less labor-intensive method for processing sponge-wipe samples. This method, referred to as the "Fast Analysis" method, was designed to quickly and efficiently enumerate low-concentration (i.e., post-decontamination) clearance sponge-wipe samples. In 2011, Rose and colleagues published "National Validation Study of a Cellulose Sponge Wipe-Processing Method for Use after Sampling *Bacillus anthracis* Spores from Surfaces". The Fast Analysis method was compared to the method used by Rose et al., known hereafter as the "CDC method," for the average recovery of spores, labor times, and waste generation. Each method was evaluated against three different spore loading levels (i.e., spore surface concentrations) and processed by three different analysts.

2. Materials and methods

2.1. Bacterial strains and surface inoculation

Bacillus thuringiensis subsp. *kurstaki* (*Btk*) spores, a surrogate for *B.a.* (Tufts et al., 2014), were obtained from Yakibou, Inc. (Apex, NC, USA; *Bacillus thuringiensis* subsp. *kurstaki* 1.0×10^8 CFU/mL mesophilic spore preparation, product MES-185, derived from ATCC 33679). The inoculum suspensions were prepared from a series of ten-fold dilutions of a stock solution. The diluent used was sterile phosphate-buffered saline

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with Tween® 20 (PBST) (Sigma-Aldrich, St. Louis, MO, USA, Product # P3563-10PAK). Target spore titers of 50, 500 and 5,000 colony forming units (CFU)/mL were prepared. Spore inoculum titers were verified by dilution plating on Tryptic Soy Agar (TSA) in triplicate within 3 days of testing. The number of CFU on each plate was manually determined following incubation at 30 °C for 20 h, and the mean of the triplicate plates was used as the reference (i.e., denominator) to calculate percent recovery for each test.

All testing was completed on 33.2- by 33.2-cm glazed ceramic tiles (Lowe's, Morrisville, NC, USA). Each tile was cleaned using deionized water and air-dried. The tiles were sterilized by four-hour exposure to 250 ppm (in air) vaporous hydrogen peroxide (VHP®) generated by a STERIS VHP® ARD hydrogen peroxide (H_2O_2) generator. Swab samples were obtained from sterilized tile coupons and analyzed to confirm sterility of the coupons. The coupons were inoculated inside a Class II Type B2 biosafety cabinet with the use a Rainin® Pipet-Lite LTS Pipette (L-200XLS+, 17014391, Mettler-Toledo Rainin, LLC, Oakland, CA, USA). Each coupon was inoculated with a total of twenty 50-µl droplets deposited in four evenly distributed rows with five droplets per row. A 5-cm. border was maintained around the outer edges of the coupon to limit the inoculation area to a 25.4- by 25.4-cm (10- by 10-in) area. Following inoculation, the coupons were covered with autoclave-sterilized 30.5- by 30.5-cm (12- by 12-in) aluminum covers (P/N 2105–8213, Wilton Brands LLC, Naperville, Illinois, USA) to prevent cross contamination. Coupons were allowed to dry for 20 h under ambient indoor conditions prior to sampling.

2.2. Surface sampling with sponge wipes

Sampling was performed over a 25.4- by 25.4-cm area using cellulose sponges (P/N SSL10NB; 3 M, St. Paul, MN, USA) as previously described (Rose, et al. 2011). The sponge heads were placed in sealable stomacher bags (Stomacher® 400 Circulator Bags, BA6141/CLR, Seward Ltd., Worthing, West Sussex, United Kingdom) and the handles were aseptically removed. To mimic sample shipment, all sponges were held 24–48 h at 4 °C prior to processing. Each test consisted of 9 sponges: 5 test sponges, 2 positive control sponges, 1 procedural blank sponge, and 1 negative sponge sample. For the positive controls, stainless steel coupons measuring 30.5- by 30.5-cm stainless steel (Type 304, #2B mill, unpolished, 0.036 in. thick, McMaster-Carr, Douglasville, GA, USA) were inoculated and sampled in the same manner as the test coupons. The procedural blank coupons were non-inoculated glazed ceramic tiles placed in the biosafety cabinet next to the test coupons. The negative coupons were non-inoculated glazed ceramic tile coupons that remained outside of the biosafety cabinet but contained in un-opened sterilization pouches until sampling. All samples were collected by the same individual to maintain consistency and minimize variation in sampling technique.

2.3. Sponge sample processing

Sponges analyzed using the Fast Analysis method were aseptically placed in stomacher bags (P/N BA6141/CLR Seward Ltd., Worthing, West Sussex, United Kingdom) containing the intact sponge head (i.e. the plastic remnant was no removed) and 100-ml of sterile PBST was added. The sponges were stomached in a Stomacher 400 Circulator (Seward Ltd., Worthing, West Sussex, United Kingdom) for 1 min at 260 rpm. Two Stomacher 400 Circulators were used, each were used to process one sponge sample at a time. The excess liquid was expressed from the sponge while in the bag, and then the sponge head was aseptically retrieved and discarded. The eluent was transferred to a sterile specimen cup (P/N B1202−1O, Starplex Scientific Corp., Cleveland, TN, USA) and sonicated for 10 min in a Branson Ultrasonic Cleaner (Model 8510R-MT, Emerson Electric Co., St Louis, MO, USA) and was then vortexed (Multi-Tube Vortexer, P/N 58816–115, VWR, Radnor, PA) continuously for 2 min prior to plating. Each sample was filter plated using 0.45-µm Microfunnel filter funnels (Metricel Black membrane, P/N 4805, Pall Corporation, Port Washington, NY) with undiluted aliquots of 1, 10 and 80 ml. These volumes were selected to provide a wide range of enumerable plates while retaining 7 to 9 ml of sample for further qualitative or quantitative analysis, if needed. Filter membranes were placed, collection side up, on a TSA plate and incubated at 30 °C for 20 h prior to manual enumeration.

Sponges analyzed using the CDC method were processed as described by Rose et al. (Rose et al., 2011). Briefly, this process includes adding 90 ml sterile PBST and stomaching for 1 min at 260 rpm. The eluent is then evenly divided into two 50-ml conical tubes. Tubes are centrifuged at 3500 ×*g* for 15 min. The supernatant (all but ~3 ml in each tube) is removed and discarded. The pellets are re-suspended, combined into one tube, then sonicated and vortexed. Samples are then serially diluted 100-fold. Spread plates were prepared in triplicate for each of the following dilutions: undiluted, 10-fold, and 100-fold. In addition to the spread plates, two filter plates were prepared, each using 1 ml of undiluted sample. Filter membranes were placed, collection side up, on a TSA plate and incubated at 30 °C for 20 h prior to manual enumeration. Exceptions to the CDC processing method included not removing the plastic remnant inside the sponge head and following extraction, the sponge head was discarded rather than being placed in Tryptic Soy Broth for enrichment analysis.

2.4. Labor time and waste generation

Analysts performed both the Fast Analysis and CDC processing method 3 times, once each at the three (50, 500, 5000 spores) spore loading levels. During tests for each specified processing method, analysts logged individual processing time, as well as, time spent plating and enumerating samples. Total time was divided by the number of sponge wipe samples to determine an average processing time per sample. The total labor time and waste generated for each test were divided by the number of samples in each test to determine the mean labor time and waste generation per sample for each method. All waste generated from each processing method was stored and weighed following completion of enumeration and plate disposal.

2.5. Data analysis

Percent recovery was calculated as previously described (Rose et al., 2011). Samples processed using the Fast Analysis method were enumerated and only plates having CFU between 15 and 150 were used to calculate the recovery. The acceptable CFU range used by Rose, et al. was 30–300 CFU per plate. Sample recovery was calculated as follows: Sample recovery = CFU count/volume filtered × 100 ml (total sample volume). The percent recovery was calculated as follows: percent recovery = sample recovery/inoculum reference check. The mean of the five test coupon percent recoveries was used as a basis to determine the average recovery for each processing method. A single factor analysis of variance (ANOVA) was performed to determine statistical differences between average recoveries for each processing method. Statistical significance was assessed using $\alpha = 0.05$.

3. Results

For each of the three spore loading levels, all test replicates of the Fast Analysis method yielded 1 plate within the countable CFU range that was used to calculate the mean percent recovery. Samples at the 5000-spore inoculum level and processed using the CDC method resulted in triplicate spread plates, with each plate meeting the accepted enumerable range (30 to 300 CFU per plate). At the inoculum level of 500 spores, test samples were enumerated on filter plates in duplicate with each plate having at least 15 CFU. It should be noted that at the 50 CFU inoculum level none of the samples subjected to the CDC method resulted in CFU counts that met the threshold of 15 CFU per

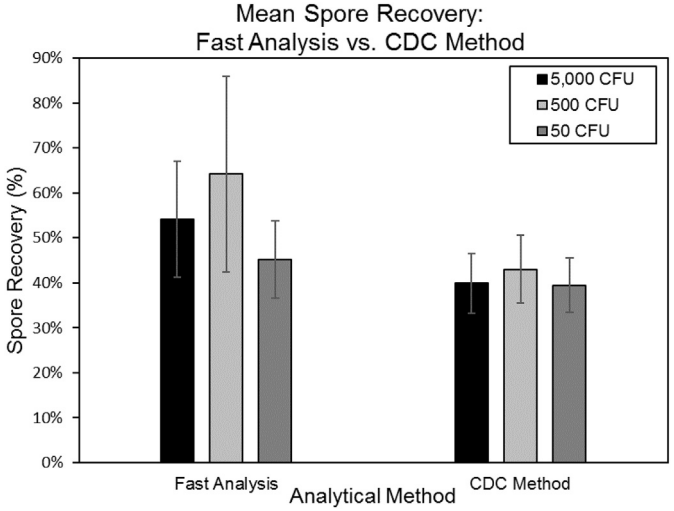

Fig. 1. Mean percent recovery for the fast analysis and CDC methods.

filter plate or 30 CFU per spread plate and all results were based on single digit CFU counts. It is possible that these data would not meet QA requirements set forth by the processing laboratory.

The mean percent recovery during each test is shown in Fig. 1. Each analyst performed two tests at each target inoculation concentration, processing each test with either the CDC method or the Fast Analysis method. The mean percent recoveries and standard errors for the samples processed using the CDC method were 39.9 ± 6.7, 43.0 ± 7.6, and 36.8 ± 10.1 for the 5000, 500, and 50 spore loading levels respectively, compared to 54.2 ± 12.9, 64.2 ± 21.7, and 45.2 ± 8.6 for the Fast Analysis method. The mean percent recovery varied by as much as 19% between inoculum levels for the Fast Analysis, while the variance between inoculum levels for the CDC method was 7%. The standard deviations were also higher in the Fast Analysis method compared to the CDC method.

An ANOVA showed a statistically significant difference (p-value < .007) in spore recoveries obtained with the two methods. Overall, a mean percent recovery of 54.5 ± 17.0 was observed for the Fast Analysis method, as compared to 39.9 ± 8.5 for the CDC method.

The mean sample processing time for each method is shown Fig. 2.

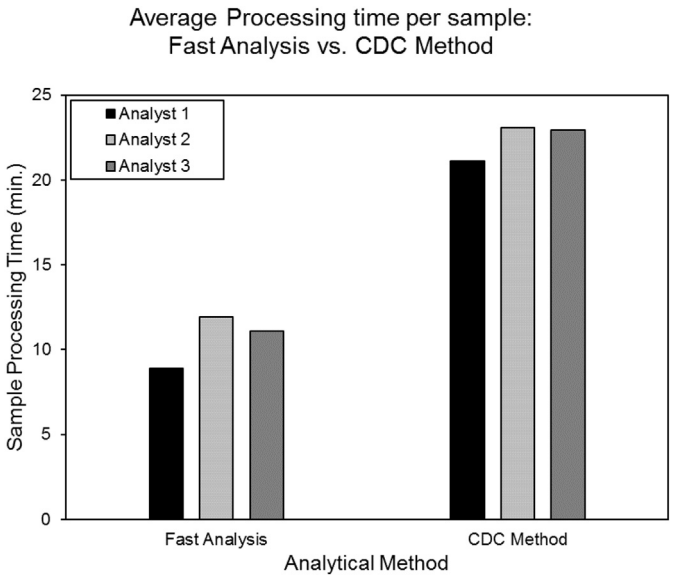

Fig. 2. Mean Processing time per sample for the Fast Analysis and CDC methods.

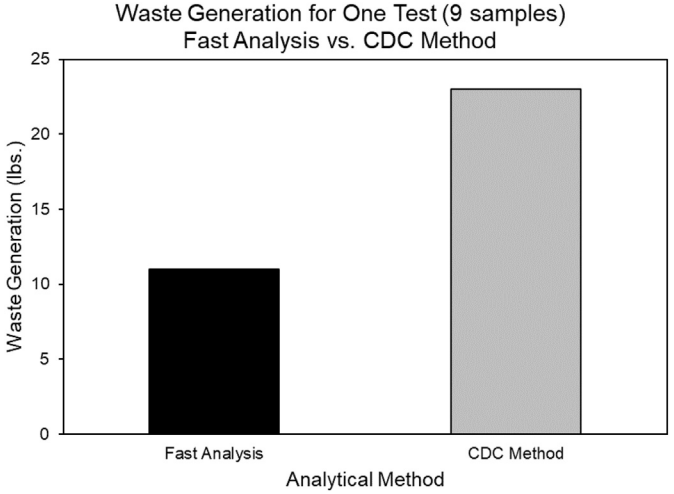

Fig. 3. Comparison of solid laboratory waste generated for the fast analysis and cdc methods. each bar represents waste generated was for one test (nine samples).

The mean processing time using the Fast Analysis method was 10.6 ± 1.7 min per sample, as compared to 22.3 ± 2.3 min per sample for the CDC method (each analyst processed 9 samples per inoculum level for both methods, i.e., each bar in Fig. 2 is the mean of 27 samples). Overall, analysts could process test samples using the Fast Analysis method in less than half the time required to perform the CDC method. The amount of solid laboratory waste generated for one test, involving 9 samples, is shown in Fig. 3. Most of the waste consisted of pipette tips, pall filters, disposable cell spreaders and agar plates. The Fast Analysis method generated less than half the waste compared to the CDC method.

4. Discussion

Sponge wipe methods for recovery of *Bacillus* spores from various materials have been evaluated. Hodges, et al. (Hodges et al., 2006) reported a baseline study in which steel coupons with 10-cm^2 surface areas were inoculated with a protocol that recovers spores from steel surfaces with premoistened macrofoam swabs and vortex processing. The study reported mean recovery percentages of 40.1, 38.0, and 49.1 from 3.8×10^1, 5.9×10^2, and 6.0×10^4 spore loading levels, respectively. Rose, et al. (Rose et al., 2004) evaluated four swab materials to determine recovery efficiency for *B.a.* spores from steel coupons. Cotton, macrofoam, polyester, and rayon swabs were evaluated along with three methods of processing the swabs (vortexing, sonication, or minimal agitation) and two swab preparations (premoistened and dry). Their results indicated that vortexing swabs was more efficacious than sonicating swabs and that premoistened macrofoam and cotton swabs which were vortexed during processing achieved the greatest percent recovery with 43.6 and 41.7, respectively. However, the inoculum levels were much higher at 1×10^6, which is 3 orders of magnitude higher than the highest inoculum concentration used in this study. The Fast Analysis method aims to utilize both stomaching and vortexing in order to optimize the recovery and prevent clumping of spores.

Rose et al. obtained 32.4, 24.4, and 30.1 mean percent recoveries from 1-, 2-, and 4-$\log_{10}$ inocula, respectively (Hess et al., 2016), compared to observed mean percent recoveries for the CDC method applied in this work of 36.8, 43.0, and 39.9 for the 50, 500, and 5000 spore loading levels, respectively. The difference in spore recoveries between the CDC method tested in this study and the mean percent recoveries reported by Rose et al. (Hess et al., 2016) may be attributed to several factors including the type of material sampled, the inoculum suspension liquid, the use of Arizona Test Dust, inoculum suspension drying time,

and the organism used for inoculum preparation. Previous studies have shown differences in the relative recoveries of *B.a.* spores or surrogates from different material types (Centers for Disease Control and Prevention and National Institute for Occupational Safety and Health, n.d.). While non-porous surfaces have shown the highest recoveries, a wide range of spore recoveries from non-porous surfaces have been reported (Brown et al., 2007b; Frawley et al., 2008; Krauter et al., 2012). However, we propose that the difference in mean percent recoveries observed for the Fast Analysis and CDC methods is legitimate because the same surface type was sampled for both analysis methods in this study. The higher mean percent recoveries for the Fast analysis method may be attributed to not concentrating the sample eluent by centrifugation following stomaching the sponge-wipe. Spore loss during supernatant removal, either due to pellet agitation or incomplete sedimentation, is possible and may be a contributing factor to the higher mean percent recoveries observed for the Fast Analysis method.

A Government Accountability Office (GAO) investigation following the 2001 incident concluded that new sampling designs were needed to help substantiate decontamination assertions when all sample results are negative (Government Accountability Office (GAO), 2005). The prevention of false negative results is imperative in the post-decontamination/clearance sampling. The Fast Analysis method attempts to reduce false negative rates by minimizing any loss of spores during processing.

The mean standard deviation for the CDC method was 8.1% compared to a mean of 14.4% for the Fast Analysis method. The difference in standard deviations between the two methods is likely a result of single data points used during the Fast Analysis method compared to triplicate or duplicate data points for the CDC method. Using a single data point to determine contaminant concentration may result in larger variations between subsets. However, the filter plating of various volumes from the same sample can verify the data collected from the single enumerated plate. For example, a sample is processed and plated using the Fast Analysis method and CFU counts of 2, 20 and too numerous to count (TNTC) are observed on the 1-, 10- and 80-ml filter plates, respectively. The CFU counts on the 1- and 80-ml filter plates can be used to verify that the usable count of 20 CFU on the 10-ml filter plate is accurate.

The Fast Analysis method has the potential for all filter plates to be TNTC. However, if the stated volumes of 1, 10, and 80 ml are filter plated initially, additional sample can be subsequently analyzed by dilution plating. This method may not be appropriate for samples with unknown or potentially high concentrations of *B.a.* spores such as characterization samples near the release point, or samples with high numbers of background organisms. Further testing is warranted to evaluate both methods under more challenging conditions that reflect real world samples. Consistent with the current CDC method, the remaining eluate can also be used for enrichment culture, to further lower the limit of detection when no *B.a.* colonies are observed on any of the filters. Similarly, the remaining eluate could also be used for Rapid Viability Polymerase Chain Reaction (RV-PCR) either as a confirmatory test or to help achieve a lower detection limit.

Despite the relatively higher standard deviations found in the Fast Analysis method the higher mean spore recoveries and reductions in labor time and laboratory waste generated indicate this method could be considered for processing of clearance samples or any samples where low concentrations of spores are expected. Utilizing a single data point for each sample and skipping the concentration step in the CDC method were possibly the greatest contributors to these differences. However, the method should be evaluated using *Bacillus anthracis* before being utilized for samples intended to support public health decisions following an anthrax incident. The robustness of this assay was confirmed by the lack of differences between analysts. Each analyst had little to no previous experience utilizing either of these analysis methods prior to beginning these tests, which would give greater credence to the results and impact of the reduction in processing time. There was no statistical difference between analysts in the mean percent recovery from samples, the time spent processing each sample, nor from the amount of waste generated by each analyst (p-values $< .001$). The ability to process samples in half the time could theoretically double a laboratory's throughput rate and the reduction in waste could lead to substantial reduction in costs associated with the disposal of potentially hazardous waste.

5. Disclaimer

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