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U.S. DEPARTMENT OF ENERGY

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Richland, Washington 99352

Summary

Surface sampling for *Bacillus anthracis* spores has traditionally relied on detection via bacterial cultivation methods. Although effective, this approach does not provide the level of organism specificity that can be gained through molecular techniques. False negative rates (FNR) and limits of detection (LOD) were determined for two *B. anthracis* surrogates with modified rapid viability-polymerase chain reaction (mRV–PCR) following macrofoam-swab sampling. This study was conducted in parallel with a previously reported study that analyzed spores using a plate-culture method. *B. anthracis* Sterne (BAS) or *B. atrophaeus* Nakamura (BG) spores were deposited onto four surface materials (glass, stainless steel, vinyl tile, and plastic) at nine target concentrations (2 to 500 spores/coupon; 0.078 to 19.375 colony-forming units [CFU] per cm^2). Mean FNR values for mRV–PCR analysis ranged from 0 to 0.917 for BAS and 0 to 0.875 for BG and increased as spore concentration decreased (over the concentrations investigated) for each surface material. FNRs based on mRV–PCR data were not statistically different for BAS and BG, but were significantly lower for glass than for vinyl tile. FNRs also tended to be lower for the mRV–PCR method compared to the culture method. The mRV–PCR LOD_{95} was lowest for glass (0.429 CFU/cm^2 with BAS and 0.341 CFU/cm^2 with BG) and highest for vinyl tile (0.919 CFU/cm^2 with BAS and 0.917 CFU/cm^2 with BG). These mRV–PCR LOD_{95} values were lower than the culture values (BAS: 0.678 to 1.023 CFU/cm^2 and BG: 0.820 to 1.489 CFU/cm^2). The FNR and LOD_{95} values reported in this work provide guidance for environmental sampling of *Bacillus* spores at low concentrations.

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This report was initially written as a manuscript for submission to a journal, and hence uses the organizational structure required by the journal. Standard PNNL report formatting was applied for this version of the manuscript. This report contains appendices not contained in the journal manuscript.

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Acronyms and Abbreviations

%RSD	percent relative standard deviation
BAS	*Bacillus anthracis* Sterne
BG	*Bacillus atrophaeus* Nakamura
BSC	biosafety cabinet
BSL	Biosafety Level
CDC	Centers for Disease Control and Prevention
CFU	colony-forming units
DHS	U.S. Department of Homeland Security
FNR	false negative rate [the probability of a positive (contaminated) sample incorrectly being identified as a negative (not contaminated) sample]
GAO	Government Accountability Office
LOD	limit of detection
LRN	Laboratory Response Network
mRV–PCR	modified rapid viability-polymerase chain reaction
PBS-T	phosphate buffered saline containing 0.02% Tween® 80
RE	recovery efficiency
SD	standard deviation
TGA	thermogravimetric analysis
TSA	tryptic soy agar
WP	whole plot

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1.0 Introduction

Sampling techniques used to detect indoor *Bacillus anthracis* contamination have been scrutinized following the intentional release of this pathogen via U.S. mail in 2001 (Henderson et al. 2002). During this incident, the Laboratory Response Network (LRN) processed over 125,000 samples, yet the results were inconsistent across sampling locations (U.S. General Accounting Office 2003). In subsequent years, various sampling methods have been analyzed and rigorously tested. Of these methods, the macrofoam-swab and cellulose sponge-wipe sampling methods have been recommended by the Centers for Disease Control and Prevention (CDC) and the LRN (CDC 2012, Hodges et al. 2010, Rose et al. 2011). A number of studies have been conducted to determine the efficiency of the macrofoam-swab collection method (Hodges et al. 2010, Hodges et al. 2006, Brown et al. 2007, Buttner et al. 2001, Buttner et al. 2004, Estill et al. 2009, Frawley et al. 2008, Probst et al. 2010, Rose et al. 2004, and Valentine et al. 2008). The majority of these studies relied on traditional culture-based detection ("culture" subsequently) followed by confirmation via serological methods or real-time polymerase chain reaction (PCR).

Rapid viability PCR (RV–PCR) has been proposed as a sensitive and accurate method to detect *B. anthracis* spores from swab and wipe samples (Kane et al. 2009, Létant et al. 2010, Létant et al. 2011, EPA 2012, and Kane et al. 2013). PCR analysis is first conducted on the initial sample and then again after a broth culture incubation step. The change in PCR signal between the two time points is used to determine the presence of viable spores (Kane et al. 2013). The utility of RV–PCR has been demonstrated with clean samples, dirty samples, and environmental samples (Kane et al. 2009, Létant et al. 2010, Létant et al. 2011, and Kane et al. 2013). Several improvements, including the use of robotics and reducing incubation time, have reduced the sample turnaround time to less than 15 hours (Létant et al. 2010, Létant et al. 2011) compared to the 24 to 48 hours required when using a culture analysis. In terms of detecting a known number of spores, results from the RV–PCR and culture methods were reported to be in good agreement and differences in results from the two approaches were not statistically significant (Kane et al. 2009, Létant et al. 2010, and Létant et al. 2011). Additional studies reported by Kane et al. (2009) implemented RV–PCR for spores recovered with wipes following chlorine dioxide fumigation and determined that there was no significant difference in the total number of samples that were positive with RV–PCR compared to culture. Létant et al. (2010) used a most probable number RV–PCR approach that involves diluting the sample to determine if the dilutions are positive or negative for the presence of spores. Estimates of the total spore numbers using that approach were within an order of magnitude of the values obtained using culture. Also, RV–PCR detection was successful from swab samples used to sample material contaminated with ~40 colony-forming units (CFU) in the presence of Arizona Fine Test Dust. Létant et al. (2011) further refined the RV–PCR method (specifically the incubation time was reduced from 14 hours to 9 hours and magnetic bead-based DNA extraction was implemented).

Minimizing false negatives, wherein a biological agent is present but sampling and detection/analysis generate a negative result, is critical. However, few studies have investigated the influence of surrogate type, spore concentration, surface material, and/or downstream detection method for low numbers of microorganisms.

In the study discussed in this report, we used a modified rapid viability-polymerase chain reaction (mRV–PCR) analysis method to detect *B. anthracis* surrogates at low concentrations. The modifications of the RV–PCR method were mainly: 1) robotics were not used, 2) heat lysis was employed as a DNA extraction method, and 3) samples were enriched for 16 hours. The goal of this study was to understand how surrogate identity and concentration on various surface materials impact the false negative rate (FNR) and LOD values. In addition, the results of this mRV–PCR study were compared with results from a parallel culture-analysis study to assess the performance of the two methods with macrofoam-swab samples.

This study evaluated two *B. anthracis* surrogates [*B. anthracis* Sterne (BAS) and *B. atrophaeus* Nakamura (BG) spores] that were recovered from four surface materials (glass, stainless steel, vinyl tile, and plastic light panels). Spores were deposited at low levels, recovered using a macrofoam swab, extracted, and analyzed using culture and mRV–PCR methods. The data and the statistical analyses conducted provide metrics for interpreting negative results. The results from the culture study are discussed by Piepel et al. (2015). In this report, we discuss the mRV–PCR results and compare them to the culture results.

2.0 Materials and Methods

2.1 Study Overview

For a detailed overview of the design and implementation of the culture and mRV–PCR studies, see Piepel and Hutchison (2014). Briefly, a split-split-split-plot experiment (Jones and Nachtsheim 2009, Kowalski et al. 2010) was designed to test the performance of the macrofoam-swab method for two *B. anthracis* surrogates (BAS and BG) at low concentrations using two analytical detection methods (plate culturing and mRV–PCR). Spores in liquid suspension were deposited onto coupons made of four surface materials (glass, stainless steel, vinyl tile, and plastic [acrylic ceiling light panel]). Spores were collected using the CDC protocol (CDC 2012) for surface sampling of *B. anthracis* spores from smooth, nonporous surfaces with a macrofoam swab. The spores then were dislodged from the swabs as described in the LRN method.[1] Samples were analyzed using either culture or mRV–PCR analysis (see details in Section 2.8). The surrogate and spore concentration were varied in 34 test runs. Even though BG is rated as Biosafety Level 1, Biosafety Level 2 procedures were used for both BG and BAS. All testing was conducted in two biosafety cabinets (which had statistically similar results in preliminary testing). Each test run used the two biosafety cabinets, where the samples from one cabinet were analyzed by culture analysis and samples from the second cabinet were analyzed using mRV–PCR.

Table A.1 in Appendix A lists the macrofoam-swab study tests with both culture and mRV–PCR analyses. Test Runs 1–8 and Test Runs 17–25 used the BAS surrogate, while Test Runs 9–16 and Test Runs 26–34 used the BG surrogate. The target spore concentrations for Test Runs 1-16 were 2 CFU/coupon (0.078 CFU/cm^2), 5 CFU/coupon (0.194 CFU/cm^2), 10 CFU/coupon (0.388 CFU/cm^2), 15 CFU/coupon (0.581 CFU/cm^2), 20 CFU/coupon (0.775 CFU/cm^2), 25 CFU/coupon (0.969 CFU/cm^2), 100 CFU/coupon (3.875 CFU/cm^2), and 500 CFU/coupon (19.375 CFU/cm^2). For Test Runs 17–34, the same target concentrations were used as in Test Runs 1–16, with one exception. The highest target concentration (500 CFU/coupon, 19.375 CFU/cm^2) was replaced with a new, second-lowest target concentration (4 CFU/coupon, 0.155 CFU/cm^2). This change was made to ensure testing was conducted at levels that would result in false negatives. The target spore concentrations were randomly assigned to the test runs prior to the experiment.

Figure 2.1 shows the layout of coupons for the 24 samples for a given test within a biosafety cabinet. There were six coupons of each surface material for a given test. Within a biosafety cabinet, 12 quantification positive controls were generated by directly plating spores onto Tryptic Soy Agar plates (TSA). In addition, within a biosafety cabinet with samples to be analyzed by mRV–PCR, four PCR positive controls were generated by inoculating the spore suspension into 5 mL of extraction buffer. Negative controls consisted of a process negative and coupon negative for each coupon type (further described below). Two personnel conducted the sampling and analysis process (surrogate deposition, sample collection, extraction, and sample analysis), and these three tasks were randomized in a balanced way according to the experimental design (Piepel and Hutchison 2014).

[1] Personal communication between Janine Hutchison (PNNL) and Laura Rose (CDC).

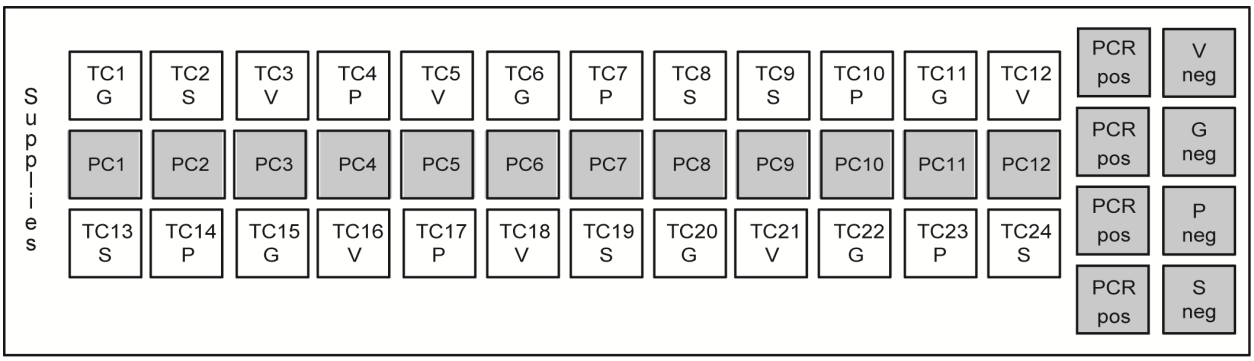

Figure 2.1. Testing Configuration in a Biosafety Cabinet Showing the Locations of the 24 Test Coupons (TC1–TC24), the 12 Quantification Positive Controls (PC1–PC12; gray-fill), the Four PCR Positive Controls (gray-fill), and the Four Negative Coupon Controls (gray-fill) for Each Test Run. The surface materials are denoted by G = glass, S = stainless steel, V = vinyl tile, and P = plastic light cover panel. In addition, the needed supplies (including pipettor, tips, and stir plate) and trash were located to the left of the test coupons. The working surfaces of the biosafety cabinets and coupon locations are not drawn to scale and are a pictorial representation only.

2.2 Bacterial Strains and Culture Methods

The BAS (pX01+ pX02-) was kindly provided by Dr. David Wunschel (Pacific Northwest National Laboratory), and BG ATCC 9372 was purchased from American Type Culture Collection (Manassas, Virginia). Spores were prepared as described previously by Buhr et al. (2008). Briefly, overnight cultures for each strain were grown in tryptic soy broth (#286220; BD Bioscience, Franklin Lakes, New Jersey) at the appropriate temperatures (35°C for BAS and 30°C for BG). These cultures were diluted 1:100 in 1.6% nutrient broth with CCY salts (Buhr et al. 2008). Spore cultures were sporulated at the appropriate temperature with shaking at 200 rpm for 72 hours (BAS) and 7 days (BG). Spores were harvested by centrifugation for 10 min at 10000 × *g* at 4°C. Pellets were resuspended in sterile water and stored at 4°C for 7 days (to enhance vegetative cell lysis), then washed three times in ultra-pure (18 Ω) sterile water prior to use. Spores were passed through a 41-µm filter (EMD Millipore; Billerica, Massachusetts) to remove remaining cellular debris. Spore culture purity was assessed by phase contrast microscopy, with final preparations being >95% phase bright. To reduce variability, three independent spore cultures were pooled for each surrogate. Spore stock suspensions were enumerated by dilution series in phosphate buffered saline (#10010049; Invitrogen, Waltham, Massachusetts) containing 0.02% Tween-80 (#P4780; Sigma-Aldrich, St. Louis, Missouri), which is denoted PBS-T.

2.3 Sample Surface Materials

Materials for coupons were purchased from vendors in the CDC-recommended size for macrofoam-wipe sampling (2 in. × 2 in. [25.8064 cm^2]) per Hodges et al. (2006) or were cut to that size as part of this work. Stainless steel sheets (316L) with 2B Finish and 18G (0.0480 in.) were purchased and cut by Stainless Supply Architectural Metal Solutions, Monroe, North Carolina. Daltile Circa Glass Spring Green tiles (#CG0222HD1P, Store SKU #354111) were purchased from Home Depot. Armstrong Excelon Vinyl Composition Tile (#51830; Armstrong World Industries Inc., Lancaster, Pennsylvania)

was purchased from Home Depot and cut to size. Acrylic, clear cracked-ice, ceiling light panels were purchased from Professional Plastics (Fullerton, California) and plastic coupons were cut from the light panels.

Coupons were washed in a 1% solution of Liqui-nox® (Alconox Inc., New York, New York), rinsed three times in deionized water, and then air dried. After washing, all coupons were sterilized by autoclaving on a dry cycle for 60 minutes in Chex-All® Sterilization Pouches (Propper Manufacturing Company Inc., Long Island City, New York). Sterile coupons were placed in plastic petri dishes within the biosafety cabinet in a consistent and specified order for each test run (Figure 2.1).

Materials surface roughness and porosity were characterized previously (Piepel et al. 2015). The surface roughness of the stainless steel, vinyl tile, and glass coupon materials was measured using a NT1000 Optical Profiler (Veeco Instruments Inc., Plainview, New York) with a 5× magnification. For the plastic ceiling tile, a DekTak® Contact Profilometer (Veeco Instruments Inc., Plainview, New York) was used because this material was incompatible with the Veeco NT1000 instrument. Coupon porosity was measured using classical adsorption/desorption nitrogen isotherms with a Quadrasorb SI analyzer (Quantachrome Instruments, Boynton Beach, Florida). The specific surface area and pore size of each sample were determined by the Brunauer–Emmett–Teller method and the Barrett–Joyner–Halenda method, respectively (Piepel et al. 2015).

2.4 Spore Deposition

Prior to each test, spores were diluted in PBS-T to the target spore concentration: 20 CFU/mL, 50 CFU/mL, 100 CFU/mL, 150 CFU/mL, 200 CFU/mL, 250 CFU/mL, 1000 CFU/mL, or 5000 CFU/mL. Spores were continually stirred to maintain a homogenous suspension using a stir plate. Ten 10-µL drops of the target spore suspension were deposited on the surfaces working from top to bottom and right to left within the biosafety cabinet using a 200 µL electronic repeat pipettor (Rainin E4 XLS; Rainin, Oakland, California). After all coupons, quantification positive-control (TSA) plates, and PCR positive controls (5 mL PBS-T) were inoculated, they were air dried or stored in the biosafety cabinet for approximately two hours with the sash closed.

2.5 Experimental Controls

For each test there were 4 coupon negative controls, 4 process negative controls, 12 quantification positive controls, and 4 PCR positive controls. For each coupon surface material, there was one coupon negative control that contained no deposited spores and was processed in the same manner as the test coupons. For each test, there were four process negative controls, each of which consisted of a swab that was simply removed from the packaging and transferred into the collection tube. Twelve TSA plates were used for the quantification positive controls. These plates were located in the middle row of the samples in a biosafety cabinet (Figure 2.1) and were inoculated with the same spore suspension as the test coupons. The 5000 CFU/mL spore suspension was diluted 1:10 and then cultured to have a countable number of colonies for the quantification positive controls. For each of the four PCR positive controls per test (in cabinets for which mRV–PCR analyses would be performed), 100 µL of the spore suspension (values listed at the start of Section 2.4) was deposited directly into 5 mL of PBS-T.

2.6 Swab Sampling Method

The CDC protocol (CDC 2012) for surface sampling *B. anthracis* spores from smooth, nonporous surfaces with a macrofoam swab was used for the test coupons and to sample the coupon negative controls. Macrofoam swabs (#25-1607 1PF SC; Puritan, Guilford, Maine) were pre-moistened in PBS-T buffer and excess liquid was removed by pressing the swab to the side of a sterile tube. Using sterile technique, each test coupon was swabbed using an overlapping 'S' pattern with horizontal strokes, rotating the swab and swabbing the same area again with vertical 'S' strokes, and rotating the swab once more and swabbing the area with diagonal 'S' strokes to expose all surfaces of the swab to the coupon.

2.7 Spore Extraction

Spores were extracted using the LRN method for processing macrofoam swabs of environmental surfaces.[1] After collection, the end of the macrofoam-swab stick was removed and discarded, and the remaining swab/stick was placed into the pre-aliquotted 5 mL of PBS-T within a screw-cap conical tube. The tubes were vortexed for 2 minutes at the highest setting, using 10-second pulses. The swab was removed using sterile forceps and excess liquid was removed by pressing the swab to the side of the tube.

2.8 mRV–PCR

We used a modified RV–PCR that omitted the DNA extraction in favor of rapid heat lysis, and manually transferred liquids rather than using robotics. PCR analysis was conducted pre- and post-enrichment and was performed according to the protocol described by Létant et al. (2010) with slight modifications. A total of 12 coupons were sampled for each combination of surrogate, concentration, and coupon surface material. The spore suspension was enriched in duplicate and three PCR technical replicates were conducted on each for a total of 72 PCR measurements for a given surrogate, spore concentration, and coupon material. The only exception was the test run with a target of 500 CFU/coupon (19.375 CFU/cm^2), where only six coupons were sampled and there were 36 PCR measurements. In some cases, the filter plate used to concentrate and enrich the spore samples leaked, and results were obtained for 11 rather than 12 test coupons for a given surface material. For each PCR positive control, a single aliquot was processed using the mRV–PCR protocol, and three technical PCR replicates were conducted for a total of 24 PCR measurements.

A Multiscreen™ Deep Well Solvinert plate (#MDRLN0410; Millipore, Billerica, Massachusetts) was used to collect the spores on a 0.45 µm hydrophilic polytetrafluoroetheylene (i.e., Teflon) filter. The filtration manifold was assembled as follows (top to bottom): 1) 96-well Solvinert plate, 2) collar adapter for deep well plate, 3) deep well 96-well plate to collect flow-through, and 4) Multiscreen™ Vacuum Manifold. Each well of the Solvinert plate was first washed with 100 µL PBS-T. One milliliter of suspension from a test coupon or control was added to the Solvinert plate following a predetermined layout (see Appendix B). After the sample was filtered through the plate, each well was washed with an additional 100 µL of PBS-T. The vacuum pump was turned off, the Solvinert plate was removed from the manifold, and the bottom was dried completely using a paper towel and a Kimwipe. The bottom of the

[1] Personal communication between Janine Hutchison (PNNL) and Laura Rose (CDC).

plate was sealed with an adhesive plate seal (#1044-39-4; Thermo Scientific, Waltham, Massachusetts) and 1 mL Brain Heart Infusion (BHI) broth (BD Bioscience, Franklin Lakes, New Jersey) was added to each well. A 5-µL aliquot (T_0 = 0 min) was transferred from each sample to a 96-well PCR plate (#4346906; Applied Biosystems, Grand Island, New Year). The plate was sealed with a static cover seal, and stored at 4°C overnight so that it could be analyzed at the same time as the experimental samples. The Solvinert plate containing the samples was covered with a static cover, placed into a plastic sealable bag, and then incubated at the appropriate temperature for 16 hours with shaking at 220 rpm. After enrichment (T_F = 16 hours) samples were mixed by pipetting vigorously 10 times using a 1.2-mL multichannel pipette to homogenize the sample. Sample aliquots of 5 µL were transferred to each of three PCR plates.

The DNA standards were generated from genomic DNA from BAS or BG. DNA was isolated from log phase cultures using MasterPure™ Complete DNA and RNA Purification Kit (Epicentre Biotechnologies, Inc., Madison, Wisconsin). DNA concentrations were measured by PicoGreen assay (#P7589; Invitrogen, Grand Island, NewYork). The final DNA concentrations were 1 ng/well, 100 pg/well, 10 pg/well, 1 pg/well, and 100 fg/well in TE buffer (Tris EDTA #AM9849; Invitrogen, Grand Island, New York). For each PCR plate, two replicates of DNA standard were added in a volume of 5 µL. Duplicate wells of the no-template controls (BHI or TE buffer) were also analyzed on each PCR plate.

PCR primer/probes (see Table 2.1) were purchased from IDT (Coralville, Iowa), and aliquots were stored at −20°C. Each PCR reaction was performed in a final volume of 20 µL (10 µL of 2× TaqMan® Fast Universal Master Mix, 1 µL of 20x Primer/Probe, 4 µL of nuclease-free water, and 5 µL of sample from the Solvinert plate). The master mix was generated as follows: 3.5 mL 2× TaqMan® Fast Universal Master Mix (#4367846; Life Technologies, Grand Island, New York), 350 µL 20× Primer/Probe Mix (PrimeTime® qPCR Assays; IDT, Coralville, Iowa), and 1.4 mL nuclease-free water (#AM9938; Ambion, Grand Island, New York). Fifteen microliters of the master mix was added to each sample within the PCR plates, which were then sealed (#4311971; Applied Biosystems, Grand Island, New York). PCR was conducted using two Applied Biosystems 7500 Fast Real-Time PCR systems. Thermal cycling parameters were as follows: 95°C for 10 minutes (heat lysis), followed by 40 cycles of denaturation at 95°C for 3 seconds, and annealing/extension at 60°C for 30 seconds. The C_T (Threshold Cycle) to make positive calls was standardized to 0.1, and auto-baseline was used. A C_T of 40 was assigned to samples for which spores were not detected.

Table 2.1. Primers and Probes used for MRV–PCR Analyses in this Macrofoam-Swab Study

Surrogate	Primer Name	Sequence (5′ – 3′)
B. anthracis Sterne[a]	CAAX_F	TCC GTT TAC CAA TTC ACT ATG AAT CAA T
	CAAX_R	ATG CGT TGT TAA GTA TTG GTA TAA TCA TC
	CAAX_Probe	FAM/CC CAC TTG G/Zen/A TTA TAT CCT GAG TAT CGT GA/3IABkFQ/
B. atrophaeus Nakamura[b]	Bg42F	ATG TCA AGA AAC CGC CGT C
	Bg104R	CGC GCC CGA GGA CTT AA
	Bg60FT	FAM/TCT CGT AAA GGG CAG CCC GCA AG/36-TAMSp

[a] Source of *B. anthracis* Sterne chromosomal primer sequence (Wielinga et al. 2011).
[b] Source of *B. atrophaeus* Nakamura primer sequence (Kane et al. 2009).

2.9 Quantification of Positive-Control Concentrations and Uncertainties

The counts from culture analyses of the 12 quantification positive-control plates for each test were averaged, where $\overline{C}_{hij}$ denotes the mean CFUs for the j^{th} target concentration of the i^{th} surrogate in the h^{th} block. There were two blocks of tests, which consisted of Test Runs 1–16 and 17–34, respectively. The $\overline{C}_{hij}$ values were converted to mean concentrations (CFU/cm^2) via

$$\overline{c}_{hij} = \overline{C}_{hij} / 25.8064 \tag{1}$$

and used to assess how FNRs (for test coupons) relate to actual concentrations of the surrogates.

The mean and standard deviation (SD) of the actual concentrations for the j^{th} target concentration of the i^{th} surrogate were calculated using different formulas depending on whether the target concentration was investigated only in Test Runs 1–16 (500 CFU/coupon), only in Test Runs 17–34 (4 CFU/coupon), or in Test Runs 1–16 and 17–34 (the remaining concentrations). The formulas for the mean are

$$\overline{c}_{ij} = \begin{cases} \overline{c}_{hij} & \text{4 and 500 CFU/coupon} \\ (\sum_{h=1}^{2} \overline{c}_{hij}) / 2 & \text{Remaining target concentrations} \end{cases} \tag{2}$$

while the formulas for the SD are

$$SD(c_{ij}) = \begin{cases} SD(c_{ij}^{Plate}) & \text{4 and 500 CFU/coupon} \\ \{[SD(c_{ij}^{WP})]^2 + [SD(c_{ij}^{Plate})]^2\}^{0.5} & \text{Remaining target concentrations} \end{cases} \tag{3}$$

In the second formula of Eq. (3), the squared quantities are the two variances affecting the positive-control concentration data for a given *ij* combination associated with whole plots (WP) and plates. The percent relative standard deviation (%RSD) of c_{ij} was calculated as

$$\%RSD(c_{ij}) = 100\ SD(c_{ij}) / \bar{c}_{ij} \tag{4}$$

using the appropriate formulas in Eqs. (2) and (3) for each target concentration.

2.10 C_T Values

The C_T values for PCR positive controls and coupon tests are summarized subsequently as medians. Medians were used as measures of central tendency because values of C_T that were above 40 (or reported as ">Y" where Y >40) were set equal to 40.

2.11 False Negative Rate and Uncertainties

The notation FNR_{hijkm} represents the FNR of the macrofoam-swab method with mRV–PCR analysis for the m^{th} coupon of the k^{th} material with the j^{th} concentration of the i^{th} surrogate in the h^{th} block. As discussed previously, there were three PCR measurements each of duplicate enriched samples from a given test coupon. Each of the six PCR results was identified as a "detect" or "non-detect" of the surrogate for that test coupon. A FNR estimate of 0, $^1/_6$, $^1/_3$, $^1/_2$, $^2/_3$, $^5/_6$, or 1 for each test coupon was assigned based on the proportion of non-detects obtained for the six PCR analyses.

The mean and SD of FNRs over replicate coupons of the k^{th} material with the j^{th} concentration of the i^{th} surrogate are denoted as $\overline{FNR}_{ijk}$ and $SD(FNR_{ijk})$. These quantities were calculated using different formulas depending on the target concentration, as discussed previously. The formulas for the mean are

$$\overline{FNR}_{ijk} = \begin{cases} \sum_{m=1}^{6} FNR_{hijkm} / 6 & 500\ \text{CFU/coupon}\ (h=1) \\ \sum_{m=1}^{12} FNR_{hijkm} / r & 4\ \text{CFU/coupon}\ (h=2) \\ \sum_{h=1}^{2} (\sum_{m=1}^{6} FNR_{hijkm} / r) / 2 & \text{Remaining target concentrations}\ (h=1,2) \end{cases} \tag{5}$$

where r = 5 or 6 depending on how many test coupons have results for a given *hijk* combination. The formulas for the SD are

$$SD(FNR_{ijk}) = \begin{cases} SD(FNR_{ijk}^{Coupon}) & 4 \text{ and } 500\ \text{CFU/coupon} \\ \{[SD(FNR_{ijk}^{WP})]^2 + [SD(FNR_{ijk}^{Coupon})]^2\}^{0.5} & \text{Remaining target concentrations} \end{cases} \tag{6}$$

In the second formula of Eq. (6), the squared quantities are the two variances affecting the FNR data for a given '*ijk*' combination. The standard error of $\overline{FNR}_{ijk}$ was calculated as

$$SE(\overline{FNR}_{ijk}) = \begin{cases} SD(FNR_{ijk}) / 6 & 500\ \text{CFU/coupon}\ (h = 1) \\ SD(FNR_{ijk}) / s & 4\ \text{CFU/coupon}\ (h = 2) \\ \left(\dfrac{[SD(FNR_{ijk}^{WP})]^2}{2} + \dfrac{[SD(FNR_{ijk}^{Coupon})]^2}{s} \right)^{0.5} & \text{Remaining target concentrations} \end{cases} \quad (7)$$

where the WP variance is reduced by a divsor of 2 because of the two WPs while the coupon variance is reduced by a divisor of s = 11 or 12 depending on the number of test coupons with test results for a given *ijk* combination.

The variances in Eqs. (3), (6), and (7) were estimated from the experimental data using the restricted maximum likelihood method for variance component estimation (Harville 1977).

2.12 False Negative Rates as Functions of Surrogate Concentrations

For a given surrogate and surface material, the FNR for the macrofoam-swab method with mRV–PCR analysis will increase as the surrogate concentration decreases below the level at which false negatives begin to occur. The three-coefficient, cumulative-distribution form of the Johnson SB model (Hahn and Shapiro 2009, Mathwave 2011)

$$FNR_{hijkm} = 1 - \Phi\left(\gamma_{ik} + \delta_{ik} \ln\left(\frac{\overline{c}_{hij}}{\lambda_{ik} - \overline{c}_{hij}} \right) \right) \quad (8)$$

was used to relate FNR to the surrogate concentration for each combination of the two surrogates (i = 1, 2) and the four surface materials (k = 1, 2, 3, 4). The notations FNR_{hijkm} and $\overline{c}_{hij}$ were defined previously, where $0 \le \overline{c}_{hij} \le \lambda_{ik}$; Φ is the standard normal (Gaussian) cumulative-distribution function. The coefficients γ_{ik}, δ_{ik} (> 0), and λ_{ik} (> 0) depend on the surrogate and surface material. The three coefficients were estimated from the experimental data for each combination of surrogate and surface material using nonlinear weighted-least-squares regression (Seber and Wild 2003). The high correlations among some coefficient estimates made it necessary to manually select the value of the λ_{ik} coefficients (for each combination of surrogate and surface material) to be slightly higher than the lowest actual concentration (CFU/cm^2) for which all FNR_{hijkm} values were zero. The weights for the FNR_{hijkm} values were reciprocals of the estimated variances of the FNR values for the six coupons at a given *hijk* combination.

Models of the form in Eq. (8) allow the FNR to be predicted at any concentration within the range where false negatives occur. Also, statistical methods enable calculation of the uncertainty in the predicted FNR at a given concentration (Seber and Wild 2003).

2.13 Limits of Detection and Uncertainties

In this report, the LOD of the macrofoam-swab method for each combination of a surrogate and surface material is defined as the surrogate concentration for which there is a 95% probability of correct detection, denoted LOD_{95}. An estimate of the LOD_{95} is calculated for a given combination of surrogate and surface material using the corresponding FNR-concentration equation of the form given in Eq. (8). Specifically, the LOD_{95} is the concentration at which the equation predicts FNR = 0.05 (i.e., the probability of correct detection = 0.95). A method for nonlinear models was used to calculate the approximate SD of the LOD_{95} of each combination of surrogate and surface material (Seber and Wild 2003).

2.14 Statistical Data Analyses

Statistical analyses of the FNR data (FNR_{hijkm}) accounting for 1) the split-split-split-plot structure of the combined culture and mRV–PCR data and 2) split-split-plot structure of the mRV–PCR data were conducted using PROC MIXED in the SAS software (SAS Institute, Inc. 2014). The restricted-maximum-likelihood and Kenward-Rogers methods were used. Because FNR_{hijkm} values are between 0 and 1, a logit transformation ($FNR_{hijkm}/(1 - FNR_{hijkm})$) was employed to satisfy the assumptions of the statistical analyses performed. The effects of the test factors (surrogate, concentration, analytical method, and surface material) and two-factor interactions were declared statistically significant if the confidence level was 95% or greater.

3.0 Results

3.1 Actual Spore Concentrations

For each test, the number of spores deposited on test coupons was estimated by culturing spore suspensions on TSA. The means and %RSD of actual concentrations ($\bar{c}_{ij}$ and $\%RSD(c_{ij})$), calculated from quantification positive-control data using Eqs. (2) to (4), are listed in Table 3.1 for BAS and Table 3.2 for BG. The mean actual concentrations ranged from 0.090 to 21.119 CFU/cm^2 for BAS and from 0.081 to 20.860 CFU/cm^2 for BG.

3.2 Surface Material Characterization

The four surface materials selected for our macrofoam-swab study are hard, nonporous surfaces (except for vinyl tile, which had pore sizes of 4 to 6 nm and displayed some liquid-absorption capability). The materials also varied in surface roughness, with glass at 0.019 µm, stainless steel at 0.118 µm, vinyl tile at 2.55 µm, and plastic light panel (the roughest) at 139.7 µm. See Piepel et al. (2015) for further discussion of these topics.

3.3 Detection of BAS and BG Spores from Swab Samples by mRV–PCR

In our study, all T_0 samples failed to amplify and were determined to be negative for the presence of the target DNA. Process negative controls and coupon negative controls also were negative at T_{16}.

Previous papers in the literature have reported RV-PCR results as ΔC_T [$\Delta C_T = C_T(T_0) - C_T(T_{16})$]. Recall that the C_T results greater than or equal to 40 were represented in our data as values of 40. Hence, $C_T(T_0) = 40$ for all samples in our study. This would result in $\Delta C_T = 40 - C_T(T_{16})$ for all samples, which provides no added value over just reporting $C_T(T_{16})$ values. Previous studies evaluating the utility and application of RV-PCR concluded that C_T values < 36 have at least a 2 log increase in DNA from the initial samples and would be considered positive for the presence of the target organism. For our study, C_T values < 40 were considered positives because the signal from these samples crossed the instrument cycle threshold (0.1). A more stringent C_T value could have been assigned, such as < 36. However, this would have resulted in data from samples with low target concentration being omitted, and such data were valuable in calculating FNRs.

Table 3.1 and Table 3.2 summarize the medians of $C_T = C_T(T_{16})$ values across the sample and technical replicates for the PCR positive-control samples and test coupons. The ranges of median C_T values for the T_{16} PCR positive-control samples (over the concentrations and coupon materials) were 24.76 to 40 for BAS and 24.76 to 37.05 for BG. The ranges of median C_T values for the test coupons were 24.15 to 40 for BAS and 24.97 to 40 for BG. These are similar to the ranges of median values for the PCR positive-control samples. Hence, inefficiencies resulting from swab sampling and extraction for the coupon samples did not substantively affect the C_T results.

Table 3.1. Performance Measures of the Macrofoam-Swab Method with mRV–PCR Analysis and Liquid-Deposited Bacillus anthracis Sterne Spores on Coupons of Four Surface Materials

Target Deposition, CFU/coupon (CFU/cm^2)[a]	Test #s[b]	Positive-Control Samples: Quantification CFU/sample (CFU/cm^2) Mean[c]	Positive-Control Samples: Quantification CFU/sample (CFU/cm^2) %RSD[c]	Positive-Control Samples: PCR C_T Median	Surface Material[e]	# Test Coupons[f]	Test Coupons: C_T Median	Test Coupons: FNR Mean[g]	Test Coupons: FNR SD[g]
2 (0.078)	5, 39	2.32 (0.090)	70.0	40	S	12	40	0.917	0.289
					G	12	38.33	0.792	0.344
					V	12	40	0.917	0.195
					P	12	38.42	0.694	0.324
4 (0.155)	35, 49	2.84 (0.110)	54.7	40	S	11	34.48	0.500	0.316
					G	11	38.32	0.727	0.344
					V	12	35.21	0.625	0.311
					P	12	37.86	0.708	0.334
5 (0.194)	10, 44	4.46 (0.173)	54.0	35.22	S	12	37.82	0.694	0.324
					G	12	32.96	0.292	0.257
					V	12	35.06	0.500	0.302
					P	12	32.51	0.333	0.408
10 (0.388)	7, 42	10.53 (0.408)	30.7	27.93	S	12	29.43	0.250	0.417
					G	12	28.86	0.125	0.226
					V	12	32.31	0.375	0.382
					P	12	31.86	0.292	0.433
15 (0.581)	13, 48	12.70 (0.492)	26.5	28.20	S	12	26.85	0.111	0.205
					G	12	27.33	0.083	0.204
					V	12	29.54	0.208	0.237
					P	12	26.42	0.014	0.048
20 (0.775)	2, 33	21.57 (0.836)	20.6	27.10	S	12	25.57	0.056	0.192
					G	11	25.75	0	0
					V	12	27.30	0.083	0.204
					P	12	25.76	0.028	0.065
25 (0.969)	4, 37	24.05 (0.932)	19.2	26.10	S	12	25.65	0.028	0.096
					G	12	27.55	0.083	0.204
					V	12	29.55	0.167	0.289
					P	12	26.42	0.042	0.144
100 (3.875)	11, 46	94.50 (3.662)	14.1	24.76	S	12	25.09	0.014	0.048
					G	12	24.15	0.014	0.048
					V	12	25.35	0.014	0.048
					P	12	25.03	0	0
500 (19.375)	15	545.01 (21.119)	15.2	26.36	S	6	24.52	0	0
					G	6	24.71	0.028	0.068
					V	6	24.23	0	0
					P	6	24.58	0	0

[a] Target number and concentration of spores deposited per 2 in. × 2 in. coupon (25.8064 cm^2).
[b] Test numbers are from Piepel and Hutchison (21) and include tests analyzed by culture as well as mRV–PCR, thus not all of the test numbers are included in Tables 3.1 and 3.2.
[c] The means and %RSDs of actual CFU/cm^2 were calculated using Eqs. (2) and (4) for each target concentration. The CFU/sample mean values were obtained via multiplying the CFU/cm^2 values by 25.8064. The %RSD values are the same for both CFU/sample and CFU/cm^2.
[d] Median of C_T values (threshold set at 0.1) for each combination of concentration and surface material. C_T values of 40 were assigned to tests where $C_T \geq 40$. A median of 40 indicates that at least half of the test coupons had C_T values ≥ 40.
[e] S = stainless steel, G = glass, V = vinyl tile, P = plastic light cover panel
[f] The number of test coupons for each combination of surrogate concentration and surface material. The numbers are 11 instead of 12 in a few cases because the filter plate leaked for samples from one coupon.
[g] Mean and SD values of false negative rates for each combination of concentration and surface material, calculated using Eqs. (5) and (6).

Table 3.2. Performance Measures of the Macrofoam-Swab Method with mRV–PCR Analysis and Liquid *B. atrophaeus* Nakamura Spores on Coupons of Four Surface Materials

Target Deposition, CFU/coupon (CFU/cm^2)[a]	Test #s[b]	Positive-Control Samples: Quantification CFU/sample (CFU/cm^2) Mean[c]	Positive-Control Samples: Quantification CFU/sample (CFU/cm^2) %RSD[c]	Positive-Control Samples: PCR C_T Median	Surface Material[e]	# Test Coupons[f]	Test Coupons: C_T Median	Test Coupons: FNR Mean[g]	Test Coupons: FNR SD[g]
2 (0.078)	21, 57	2.09 (0.081)	58.2	37.05	S	12	37.23	0.708	0.334
					G	12	40.00	0.833	0.246
					V	12	40.00	0.875	0.323
					P	12	40.00	0.875	0.226
4 (0.155)	53, 67	5.03 (0.195)	42.0	37.07	S	12	35.73	0.583	0.345
					G	12	33.09	0.375	0.377
					V	12	37.15	0.736	0.329
					P	12	35.52	0.542	0.450
5 (0.194)	25, 62	5.55 (0.215)	44.2	34.58	S	12	34.51	0.458	0.334
					G	12	30.49	0.208	0.257
					V	12	34.98	0.528	0.316
					P	12	36.21	0.583	0.382
10 (0.388)	24, 59	11.85 (0.459)	35.1	27.83	S	12	30.42	0.208	0.257
					G	12	30.25	0.208	0.344
					V	12	31.22	0.292	0.396
					P	12	28.97	0.208	0.334
15 (0.581)	30, 65	17.03 (0.660)	26.4	27.12	S	12	28.55	0.083	0.204
					G	12	28.35	0.083	0.204
					V	12	31.39	0.25	0.261
					P	12	28.23	0.042	0.144
20 (0.775)	18, 52	22.01 (0.853)	25.1	27.48	S	12	27.99	0.083	0.204
					G	12	27.64	0	0
					V	12	30.02	0.194	0.244
					P	12	28.25	0.097	0.194
25 (0.969)	20, 56	27.87 (1.080)	22.3	27.06	S	12	27.48	0.042	0.144
					G	12	27.32	0	0
					V	12	28.07	0.083	0.289
					P	12	27.71	0.042	0.144
100 (3.875)	27, 64	106.04 (4.109)	11.5	26.37	S	12	26.60	0	0
					G	12	26.65	0	0
					V	12	27.10	0	0
					P	12	26.92	0	0
500 (19.375)	32	538.32 (20.860)	14.4	24.76	S	6	24.97	0	0
					G	6	25.12	0	0
					V	6	25.48	0	0
					P	6	25.06	0	0

[a] Target number and concentration of spores deposited per 2 in. × 2 in. coupon (25.8064 cm^2).
[b] Test numbers are from Piepel and Hutchison (2014) and include tests analyzed by culture as well as mRV–PCR, thus not all of the test numbers are included in Tables 3.1 and 3.2.
[c] The means and %RSDs of actual CFU/cm^2 were calculated using Eqs. (2) and (4) for each target concentration. The CFU/sample mean values were obtained via multiplying the CFU/cm^2 values by 25.8064. The %RSD values are the same for both CFU/sample and CFU/cm^2.
[d] Median of C_T values (threshold set at 0.1) for each combination of concentration and surface material. C_T values of 40 were assigned to tests where $C_T \geq 40$. A median of 40 indicates that at least half of the test coupons had C_T values ≥ 40.
[e] S = stainless steel, G = glass, V = vinyl tile, P = plastic light cover panel
[f] The number of test coupons for each combination of surrogate concentration and surface material. The numbers are 11 instead of 12 in a few cases because the filter plate leaked for samples from one coupon.
[g] Mean and SD values of false negative rates for each combination of concentration and surface material, calculated using Eqs. (5) and (6).

3.4 False Negative Results and Uncertainties

False negatives occurred at low target levels that were at or below the detection limit of the assay. In these cases, spores were presumed to be present, but at concentrations below what could be detected by our mRV–PCR method. The false negative results for quantification positive controls, PCR positive controls, and test coupons are discussed in this section.

At the lowest target concentrations, it is possible that no spores were in the spore suspension deposited on a particular test coupon, quantification positive-control plate, or PCR positive-control sample. In such cases, negatives would be "true negatives" instead of "false negatives." However, for conservatism in quantifying FNRs, all negatives were treated as false negatives, regardless of the target concentration of the spore suspension. For the quantification positive controls at target levels of 2 and 4 CFU/sample, there were, respectively, 2 and 1 negatives for BAS, and 2 and 1 negatives for BG (out of 24 possible samples). For the PCR positive controls, there were three mRV–PCR analyses per sample, with eight samples total at a given target concentration (except for 500 CFU/sample, for which there were only four samples). For BAS at target levels of 2, 4, 5, 10, 15, and 20 CFU/sample, there were, respectively, 21, 21, 11, 7, 0, and 1 negatives out of a total of 24 possible sample measurements. For BG at target levels of 2, 4, and 5 CFU/sample, there were, respectively, 15, 15, and 12 negatives out of 24 possible sample measurements. The FNRs for the PCR positive controls were higher than the FNRs for the quantification positive controls (which were analyzed by culturing). This observation is interesting in that the literature reports that PCR and culture are equivalent for LODs, or PCR is more sensitive. The high FNR of the PCR positive controls could be attributed to the numerous manipulation steps used for mRV–PCR. These steps include filtration, buffer exchange, and overnight incubation, which are not conducted for culture analysis. After overnight incubation, the vegetative BAS cells form long filaments that tend to clump into visual aggregates that were not observed with BG samples. Mechanical homogenization by pipetting was used to disrupt the BAS aggregates. However, if clumps remained, then a truly representative sample of BAS may not have been obtained, resulting in an increased FNR.

FNRs were estimated for each test coupon based on the mRV–PCR data, with possible values of 0, $^1/_6$, $^1/_3$, $^1/_2$, $^4/_6$, $^5/_6$, and 1 as discussed previously. The mean ($\overline{FNR_{ijk}}$) and standard deviation [$SD(FNR_{ijk})$] values for each combination of surface material and concentration of BAS and BG are listed in Table 3.1 and Table 3.2, respectively. The $\overline{FNR_{ijk}}$ values range from 0 to 0.917 for BAS and 0 to 0.875 for BG. The values of $SD(FNR_{ijk})$ are relatively high (ranging up to 0.433 for BAS and 0.450 for BG) because they are the uncertainties in FNR values for a single test coupon.

Previous work by Krauter et al. (2012) found that surface roughness significantly affected the FNR results for sponge-wipe samples. Piepel et al. (2015) used the same surface materials as used in this study. They reported that the two smoothest surfaces (glass and stainless steel) had the smallest FNRs, but that vinyl tile had the highest FNR even though the plastic light panel was the roughest material. They concluded that the FNR results for macrofoam-swab sampling were affected by multiple factors including surface roughness, porosity, and surface chemistry. The FNR results from our mRV–PCR study agree with the finding of Piepel et al. (2015) that a combination of surface factors affects FNRs.

Table 3.3 lists the coefficients and R^2 statistics from fitting Eq. (8) to FNR (FNR_{hijkm})) versus concentration ($\bar{c}_{hij}$) data from the mRV–PCR study for each combination of surrogate and surface material. The R^2 statistics range from 0.633 to 0.860 for BAS and 0.592 to 0.776 for BG because of the relatively large uncertainty in FNR values for the individual test coupons used to fit Eq. (8). The FNR for a given surrogate, concentration, and surface material can be predicted by substituting the coefficients from Table 3.3 into Eq. (8).

Table 3.3. Coefficients and R^2 Values for the Johnson SB Equations in Eq. (8) Fitted to **mRV–PCR** Data. These equations relate false negative rate to concentration for each combination of surrogate and surface material.

	Bacillus anthracis Sterne				*Bacillus atrophaeus* Nakamura			
	Coefficient[a]				Coefficient[a]			
Surface Material	γ	δ	λ	$R^{2\,b}$	γ	δ	λ	$R^{2\,b}$
Glass	4.543	1.383	3.914	0.727	1.658	1.277	0.685	0.622
Stainless steel	3.766	1.267	1.621	0.860	1.270	0.790	1.144	0.592
Vinyl tile	2.829	1.002	3.914	0.738	0.554	0.782	1.144	0.776
Plastic	2.630	1.160	3.914	0.633	1.469	1.099	1.144	0.715

[a] The coefficients γ and δ are two shape parameters, while λ is a scale parameter. For this application, λ essentially represents the concentration (CFU/cm^2) at which FNR reaches the zero asymptote. The regression estimates of γ and δ were highly correlated with the estimates of λ for all surface materials. Hence, λ was set equal to a value slightly larger than the lowest mean actual concentration for which the FNR was zero for all test coupons at a given combination of surrogate and surface material.

[b] R^2 values were calculated using a formula that accounts for the model fits being performed using weighted-least-squares regression. The R^2 values are lower than typically considered desirable because of the limited number of FNR values for a given test coupon (and hence there is relatively large uncertainty in the FNR values). However, this is compensated by having 12 test coupons for each combination of surrogate, concentration, and surface material. The data for the target concentration of 500 CFU/coupon were not used in the model fits.

Figure 3.1 shows the mean FNR results for macrofoam-swab sampling of the test coupons for each of the four surface materials ($\overline{FNR}_{ijk}$) plotted against mean concentrations of the surrogates ($\bar{c}_{ij}$) from the quantification positive-control data. The left and right panels display the results for BAS and BG, in respectively. Figure 3.1 also shows the FNR-concentration curves corresponding to the fitted equations Table 3.3 for the mRV–PCR data and in Table 3.4 of Piepel et al. (2015) for the culture data. The top and bottom rows of plots in Figure 3.1 display the results for the culture and mRV–PCR analyses, respectively. The FNR results for the mRV–PCR data are discussed now, and are compared to the culture results (20) in Section 4. The differences in the FNRs for BG and BAS were not statistically significant. There were statistically significant differences in FNRs for some surface materials when concentrations were in the target range of 5 to 20 CFU/coupon (0.194 to 0.775 CFU/cm^2). The mRV–PCR plots in Figure 3.1 show that the FNR curves for glass and vinyl tile differ most in this concentration range, though the difference is larger for BG than BAS. Still, there was no statistically significant interaction between surrogate and surface material for any of the concentrations.

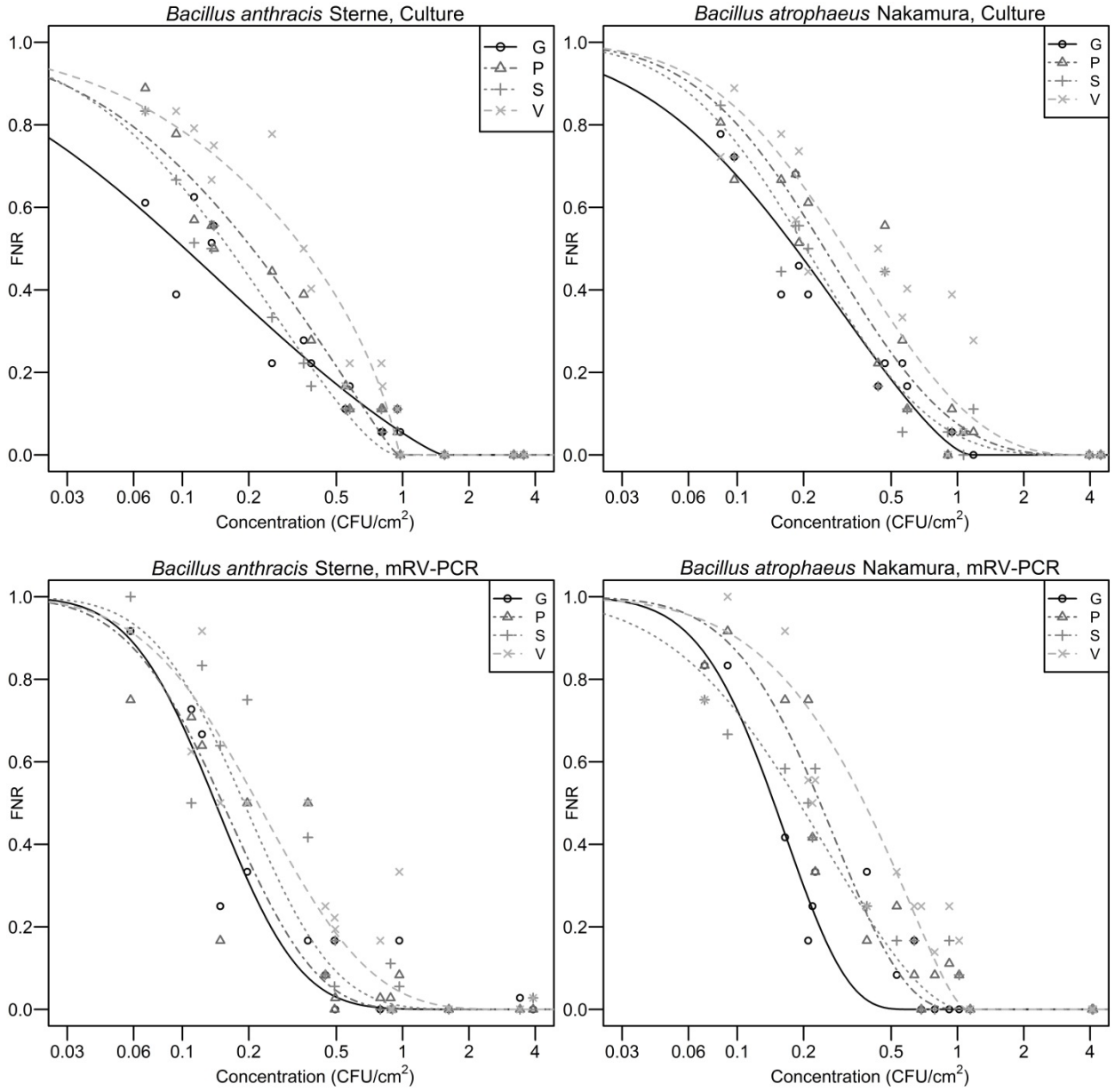

Figure 3.1. Average False Negative Rate Data and Fitted Equations as Functions of *B. anthracis* Sterne and *B. atrophaeus* Nakamura Concentrations (from quantification positive controls) for Each of Four Surface Materials. Results for culture and mRV–PCR analyses are in the top and bottom rows of the figure, respectively.

3.5 Limits of Detection and Uncertainties

The estimates of LOD_{95} for the macrofoam-swab sampling method and mRV–PCR analysis are listed in Table 3.4 for each combination of surrogate and surface material. The LOD_{95} values were calculated using the FNR-concentration equations of the form in Eq. (8) with coefficients listed in Table 3.3. The lowest LOD_{95} values occurred for glass, with 0.429 CFU/cm^2 for BAS and 0.341 CFU/cm^2 for BG. The

highest LOD_{95} values occurred for vinyl, with 0.919 CFU/cm^2 for BAS and 0.917 CFU/cm^2 for BG. Table 3.4 also lists the approximate SDs of the LOD_{95} estimates. The differences in LOD_{95} values for glass and vinyl tile were statistically significant for both BAS and BG. In addition, for BG, glass had a statistically significantly lower LOD_{95} compared to stainless steel. The LOD_{95} values for BAS and BG were not statistically different for any surface material.

Table 3.4. Estimates of the LOD_{95} and Approximate Standard Deviation Values when Sampling Four Surface Materials with the Macrofoam-Swab Method and using **mRV–PCR** Analysis

		Bacillus anthracis Sterne		*Bacillus atrophaeus* Nakamura	
Analysis Method	Surface Material	LOD_{95}[a] (CFU/cm^2)	Approximate SD(LOD_{95}) (CFU/cm^2)	LOD_{95}[a] (CFU/cm^2)	Approximate SD(LOD_{95}) (CFU/cm^2)
mRV–PCR	Glass	0.429	0.071	0.341	0.049
	Stainless steel	0.618	0.088	0.705	0.077
	Vinyl tile	0.919	0.133	0.917	0.066
	Plastic	0.486	0.069	0.617	0.081
Culture	Glass	1.023[b]	0.139	0.820	0.081
	Stainless steel	0.678	0.072	0.981	0.138
	Vinyl tile	0.920	0.025	1.489	0.181
	Plastic	0.800	0.051	1.186	0.156

[a] LOD_{95} is the concentration at which the contamination would be correctly detected 95% of the time, calculated as the concentration corresponding to the 5th percentile of the FNR-versus-concentration equation for each combination of surrogate and surface material.

[b] See Piepel et al. (2015) for a discussion of this value.

4.0 Discussion

4.1 Culture and mRV–PCR Studies In Parallel

Detection of *B. anthracis* surrogates via mRV–PCR was carried out in parallel with culture-based analysis as designed by Piepel and Hutchison (2014). An ideal experimental design would have allowed both culture and mRV–PCR analyses for the same sample, but that would have reduced the total number of analyses performed. Conducting the studies in parallel allowed for more analyses for each analytical method, which was important because of the expected variability in these tests and analyses.

4.2 Comparison of Results from Culture and mRV–PCR Studies

This section compares the FNR results from the culture analysis presented by Piepel et al. (2015) to the mRV–PCR analysis discussed in this report. An inherent challenge of comparing culture-based results to PCR results occurs because the PCR method requires an enrichment step to germinate spores. However, this is not an impediment to comparing, FNR results for the two analysis methods.

FNR ranges when using culture analysis were 0 to 0.833 for BAS and 0 to 0.806 for BG, while the ranges when using mRV–PCR were 0 to 0.917 for BAS and 0 to 0.875 for BG. In both cases, the ranges are for all combinations of the concentrations and surface materials. The FNR-concentration curves for the culture and mRV–PCR data are presented in Figure 3.1. The FNRs for both the culture and mRV–PCR analyses are strongly dependent on the concentration of the surrogate. There are smaller, but still statistically significant, differences in FNRs for glass and vinyl tile with both analytical methods. Also, FNR values for the mRV–PCR method tended to be smaller than for the culture method at several concentrations of both surrogates. These differences were not statistically significant at the 95% confidence level, but were large enough with high enough confidence levels to be noteworthy. Hence, our study indicates that the mRV–PCR method may tend to yield lower FNRs than the culture method for some concentrations. Finally, Figure 3.1 suggests there is somewhat better agreement in FNR results between the culture and mRV–PCR methods for the BG spores than for the BAS spores. The reason for this is not clear. For culture analysis, spores are plated directly onto the agar plates and are not subject to an overnight enrichment step as are the mRV–PCR samples. After mRV–PCR enrichment, the BG samples were homogenous culture suspensions, whereas the BAS samples tended to form large clumps of filamentous vegetative cells. Samples were disrupted mechanically by vigorous pipetting, but some cell clumps could have remained, thus preventing a homogenous representative sample from being collected. The higher FNR at lower spore concentrations could be attributed to not being able to collect and analyze a truly homogenous representative sample. Also, differences in particle deposition (dry vs liquid) have also been shown to affect recovery and other metrics (e.g., FNR, LOD) (Edmonds et al. 2009). An additional hypothesis is that phenotypic differences, including the fact that BG spores lack an exosporium and tend to be smaller than BAS spores, could account for differences in FNR (for a review, see Greenberg et al. 2010).

Table 3.4 lists the LOD_{95} values and their approximate SDs for 1) the culture method (from Table 3.4 in Piepel et al. 2015) and 2) the mRV–PCR method (discussed previously in this report). For tests analyzed with the culture method, the LOD_{95} ranged from 0.678 (stainless steel) to 1.023 (glass) for BAS and 0.820 (glass) to 1.489 (vinyl tile) for BG (20). For tests analyzed with mRV–PCR, the LOD_{95} range was 0.429 (glass) to 0.919 (vinyl tile) for BAS and 0.341 (glass) to 0.917 (vinyl tile) for BG (see Table 3.4). As seen in Table 3.4, the LOD_{95} values are generally lower for mRV–PCR analysis than culture analysis for all combinations of surrogates and surface types except BAS on vinyl tile. The LOD_{95} values with the mRV–PCR method are statistically significantly smaller than the LOD_{95} values with the culture method 1) for BAS and BG on both glass and plastic and 2) BG on vinyl tile.

4.3 Comparison of PCR Results from this Study and Previous Studies

Several studies have evaluated the use of RV–PCR for analysis of *Bacillus* spores collected using wipes or swabs from environmental samples (Kane et al. 2009, Létant et al. 2010, Létant et al. 2011, and Kane et al. 2013). Of these studies, Létant et al. (2010) used RV–PCR to detect BAS spores collected using the macrofoam-swab protocol from stainless steel surfaces. Significant advances made in the RV–PCR protocol have reduced assay time and the LOD (Létant et al. 2011, Kane et al. 2009, and Kane et al. 2013). In our study, we followed the protocol reported by Létant et al. (2010), which was the most relevant since RV–PCR was conducted on macrofoam swab samples rather than other collection media. The LOD of BAS reported by Létant et al. (2010) was 40 CFU/coupon, but lower concentrations were not tested. Using a refined RV–PCR method, Létant et al. (2011) established the LOD for virulent *B. anthracis* as 10 to 99 CFU/sample. Using mRV–PCR in our study, the LOD_{95} for BAS on stainless steel was 0.618 CFU/cm^2 or 15 CFU/coupon. Further, in our study, the LOD_{95} for BG on stainless steel was 0.705 CFU/cm^2 or 18 CFU/coupon. Taken together, these results provide additional evidence that mRV–PCR is an effective method to detect spores at low concentrations.

Because of the number of test variables investigated in our culture and mRV–PCR studies, these studies did not investigate the effects of dust, grime, or other background organisms on swab sampling results. Previous work by Kane et al. (2009) and Létant et al. (2011) tested the effect of Arizona test dust and background organisms on RV–PCR performance. These studies concluded that in general the presence of dust and background organisms did not significantly change selectivity and sensitivity of the assay. These findings suggest that the FNR and LOD values in our studies would be similar for dirty samples compared to clean. However, further testing is needed to evaluate the effect of heavily soiled samples that could contain higher concentrations of PCR inhibitors.

4.4 Liquid versus Dry Aerosol Deposition

In our study, BAS and BG spores were deposited on samples using liquid inoculation (see Section 2.4) because it was not possible to reliably achieve the desired low concentrations using dry-particle inoculation. However, it is of interest to consider how the FNR and LOD results of our mRV–PCR study might differ for dry-particle deposition.

Edmonds et al. (2009) compared recovery efficiency (RE) means of liquid-deposited and dry-aerosol-deposited BG spores (at high concentrations) when using four different types of swabs to sample each of four surface materials. All tests used a culture method to assess RE. For some combinations of surface material and swab type, the RE means were statistically significantly larger for liquid-deposited spores than for dry-aerosol-deposited spores. For other combinations the opposite was the case, while still other combinations showed no statistically significant difference in RE means from the two deposition methods.

The range of results for the Edmonds et al. (2009) study, as well as several differences compared to our study (high versus low spore concentrations, culture versus mRV–PCR, RE versus FNR and LOD), make it difficult to assess how the FNR and LOD results of our mRV–PCR study may have been affected by dry-particle deposition versus liquid deposition.

5.0 Conclusions

This mRV–PCR study and the previous culture study (Piepel et al. 2015) provide information to better understand collection, extraction, and detection of two *B. anthracis* surrogates at low concentrations using a macrofoam-swab method with culture or mRV–PCR analysis. The mRV–PCR method can decrease FNR values and detection limits compared to culture methods. Also, the mRV–PCR method can be completed more quickly than culture methods, which are dependent on a secondary PCR confirmation of bacterial colonies. The ability to rapidly determine the identity, presence, viability state, and extent of bacterial presence enables more efficient sampling and decontamination approaches following an exposure event. The FNR and LOD_{95} values determined in this report and by Piepel et al. (2015) provide valuable data to help understand the extent of contamination of a spore-forming bacterium such as *B. anthracis* on various surfaces. Further, these data can help improve models and sampling plans and thus increase confidence that part or all of a contaminated facility has been decontaminated appropriately before building re-entry.

6.0 References

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Appendix A

Test Matrix for the Macrofoam-Swab Study

Appendix A

Tests Matrix for the Macrofoam-Swab Study

Table A.1 is a revised version of Table 6.1 from Piepel and Hutchison (2014) that lists the tests performed in the macrofoam-swab study. The column containing the number of PCR positive-control samples is new in Table A.1 compared to Table 6.1 from Piepel and Hutchison (2014). The tests analyzed by culturing and by mRV–PCR are included in the test matrix.

Table A.1. Test Matrix for the Split-Split-Plot Experimental Design of the Macrofoam-Swab Study[a]

Test Run	Test[b]	BA Surrogate[c]	Target # Spores[d]	Analytical Method[e]	Biosafety Cabinet[f]	# Test Coupons				# Positive Controls		# Negative Controls		Testing Task[k]		
						Stainless Steel	Glass	Vinyl Tile	Plastic	Quant.[g]	PCR[h]	Coupons[i]	Swabs[j]	A	B	C
1	1	BAS	20	C	BSC1	6	6	6	6	12	–	4	4	2	1	2
	2	BAS	20	mRV–PCR	BSC2	6	6	6	6	12	4	4	4	2	1	2
2	3	BAS	25	C	BSC2	6	6	6	6	12	–	4	4	1	2	1
	4	BAS	25	mRV–PCR	BSC1	6	6	6	6	12	4	4	4	2	1	2
3	5	BAS	2	mRV–PCR	BSC2	6	6	6	6	12	4	4	4	1	2	1
	6	BAS	2	C	BSC1	6	6	6	6	12	–	4	4	1	2	1
4	7	BAS	10	mRV–PCR	BSC1	6	6	6	6	12	4	4	4	1	2	1
	8	BAS	10	C	BSC2	6	6	6	6	12	–	4	4	2	1	2
5	9	BAS	5	C	BSC1	6	6	6	6	12	–	4	4	2	1	2
	10	BAS	5	mRV–PCR	BSC2	6	6	6	6	12	4	4	4	1	2	1
6	11	BAS	100	mRV–PCR	BSC1	6	6	6	6	12	4	4	4	2	1	2
	12	BAS	100	C	BSC2	6	6	6	6	12	–	4	4	2	1	2
7	13	BAS	15	mRV–PCR	BSC1	6	6	6	6	12	4	4	4	2	1	2
	14	BAS	15	C	BSC2	6	6	6	6	12	–	4	4	1	2	1
8	15	BAS	500	mRV–PCR	BSC1	6	6	6	6	12	4	4	4	1	2	1
	16	BAS	500	C	BSC2	6	6	6	6	12	–	4	4	1	2	1
9	17	BG	20	C	BSC2	6	6	6	6	12	–	4	4	1	2	1
	18	BG	20	mRV–PCR	BSC1	6	6	6	6	12	4	4	4	1	2	1
10	19	BG	25	C	BSC1	6	6	6	6	12	–	4	4	2	1	2
	20	BG	25	mRV–PCR	BSC2	6	6	6	6	12	4	4	4	1	2	1
11	21	BG	2	mRV–PCR	BSC1	6	6	6	6	12	4	4	4	2	1	2
	22	BG	2	C	BSC2	6	6	6	6	12	–	4	4	1	2	1
12	23	BG	10	C	BSC2	6	6	6	6	12	–	4	4	2	1	2
	24	BG	10	mRV–PCR	BSC1	6	6	6	6	12	4	4	4	1	2	1
13	25	BG	5	mRV–PCR	BSC2	6	6	6	6	12	4	4	4	1	2	1
	26	BG	5	C	BSC1	6	6	6	6	12	–	4	4	2	1	2
14	27	BG	100	mRV–PCR	BSC1	6	6	6	6	12	4	4	4	1	2	1
	28	BG	100	C	BSC2	6	6	6	6	12	–	4	4	2	1	2
15	29	BG	15	C	BSC1	6	6	6	6	12	–	4	4	1	2	1
	30	BG	15	mRV–PCR	BSC2	6	6	6	6	12	4	4	4	2	1	2

Table A.1. Test Matrix for the Split-Split-Split-Plot Experimental Design of the Macrofoam-Swab Study[a] (contd)

						# Test Coupons										
Test		BA	Target	Analytical	Biosafety	Stainless		Vinyl		# Positive Controls		# Negative Controls		Testing Task[k]		
Run	Test[b]	Surrogate[c]	# Spores[d]	Method[e]	Cabinet[f]	Steel	Glass	Tile	Plastic	Quant.[g]	PCR[h]	Coupons[i]	Swabs[j]	A	B	C
16	31	BG	500	C	BSC2	6	6	6	6	12	–	4	4	2	1	2
	32	BG	500	mRV–PCR	BSC1	6	6	6	6	12	4	4	4	2	1	2
17	33	BAS	20	mRV–PCR	BSC2	6	6	6	6	12	4	4	4	1	2	1
	34	BAS	20	C	BSC1	6	6	6	6	12	–	4	4	2	1	2
18	35	BAS	4	mRV–PCR	BSC2	6	6	6	6	12	4	4	4	2	1	2
	36	BAS	4	C	BSC1	6	6	6	6	12	–	4	4	1	2	1
19	37	BAS	25	mRV–PCR	BSC1	6	6	6	6	12	4	4	4	1	2	1
	38	BAS	25	C	BSC2	6	6	6	6	12	–	4	4	1	2	1
20	39	BAS	2	mRV–PCR	BSC1	6	6	6	6	12	4	4	4	2	1	2
	40	BAS	2	C	BSC2	6	6	6	6	12	–	4	4	2	1	2
21	41	BAS	10	C	BSC1	6	6	6	6	12	–	4	4	1	2	1
	42	BAS	10	mRV–PCR	BSC2	6	6	6	6	12	4	4	4	2	1	2
22	43	BAS	5	C	BSC1	6	6	6	6	12	–	4	4	1	2	1
	44	BAS	5	mRV–PCR	BSC2	6	6	6	6	12	4	4	4	2	1	2
23	45	BAS	100	C	BSC1	6	6	6	6	12	–	4	4	2	1	2
	46	BAS	100	mRV–PCR	BSC2	6	6	6	6	12	4	4	4	2	1	2
24	47	BAS	15	C	BSC2	6	6	6	6	12	–	4	4	1	2	1
	48	BAS	15	mRV–PCR	BSC1	6	6	6	6	12	4	4	4	1	2	1
25	49	BAS	4	mRV–PCR	BSC2	6	6	6	6	12	4	4	4	2	1	2
	50	BAS	4	C	BSC1	6	6	6	6	12	–	4	4	1	2	1
26	51	BG	20	C	BSC2	6	6	6	6	12	–	4	4	2	1	2
	52	BG	20	mRV–PCR	BSC1	6	6	6	6	12	4	4	4	2	1	2
27	53	BG	4	mRV–PCR	BSC2	6	6	6	6	12	4	4	4	1	2	1
	54	BG	4	C	BSC1	6	6	6	6	12	–	4	4	1	2	1
28	55	BG	25	C	BSC1	6	6	6	6	12	–	4	4	1	2	1
	56	BG	25	mRV–PCR	BSC2	6	6	6	6	12	4	4	4	2	1	2
29	57	BG	2	mRV–PCR	BSC2	6	6	6	6	12	4	4	4	2	1	2
	58	BG	2	C	BSC1	6	6	6	6	12	–	4	4	1	2	1
30	59	BG	10	mRV–PCR	BSC1	6	6	6	6	12	4	4	4	2	1	2
	60	BG	10	C	BSC2	6	6	6	6	12	–	4	4	1	2	1
31	61	BG	5	C	BSC1	6	6	6	6	12	–	4	4	2	1	2
	62	BG	5	mRV–PCR	BSC2	6	6	6	6	12	4	4	4	1	2	1

Table A.1. Test Matrix for the Split-Split-Plot Experimental Design of the Macrofoam-Swab Study[a] (contd)

Test Run	Test[b]	BA Surrogate[c]	Target # Spores[d]	Analytical Method[e]	Biosafety Cabinet[f]	# Test Coupons: Stainless Steel	Glass	Vinyl Tile	Plastic	# Positive Controls: Quant.[g]	PCR[h]	# Negative Controls: Coupons[i]	Swabs[j]	Testing Task[k]: A	B	C
32	63	BG	100	C	BSC2	6	6	6	6	12	–	4	4	1	2	1
	64	BG	100	mRV–PCR	BSC1	6	6	6	6	12	4	4	4	2	1	2
33	65	BG	15	mRV–PCR	BSC1	6	6	6	6	12	4	4	4	2	1	2
	66	BG	15	C	BSC2	6	6	6	6	12	–	4	4	1	2	1
34	67	BG	4	mRV–PCR	BSC1	6	6	6	6	12	4	4	4	1	2	1
	68	BG	4	C	BSC2	6	6	6	6	12	–	4	4	1	2	1

[a] Specifics of the test matrix for each test run not shown in this table are given in Figure 2.1.
[b] The Test number also represents the order in which the tests were run.
[c] BAS = *Bacillus anthracis* Sterne, BG = *Bacillus atrophaeus* Nakamura.
[d] The target numbers of spores per coupon or positive-control sample, where the coupons are 2 in. × 2 in. (25.806 cm^2). The target numbers of spores were assigned to test runs in a pseudo-random manner.
[e] C = culture, mRV–PCR = modified Rapid Viability-Polymerase Chain Reaction. The two analytical methods were randomly assigned to BSC1 and BSC2 for each pair of tests in a test run at the same concentration, such that each analytical method is used the same number of times within the sets of Tests 1–16, 17–32, 33–50, and 51–68.
[f] Two biosafety cabinets (BSCs) were located adjacent to each other on one wall of a laboratory. The left one was designated BSC1 and the right one was designated BSC2. The two BSCs ran tests simultaneously, with the one started first in each pair of tests determined randomly.
[g] There was one quantification positive-control sample associated with the two test coupons on either side of it as shown in Figure 2.1.
[h] For each test in a BSC with samples to be analyzed using mRV–PCR, there were four PCR positive controls. These controls consisted of the spore suspension being placed directly into 5 mL PBS-T.

Appendix B

mRV–PCR Solvinert Filter Plate (HTS) and PCR Layouts

Appendix B

mRV–PCR Solvinert Filter Plate (HTS) and PCR Plate Layouts

Figure B.1 shows the test layouts for the mRV–PCR Solvinert plates (HTS) and PCR plates.

HTS Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	TC 1A	TC 2A	TC 3A	TC 4A	TC 5A	TC 6A	TC 7A	TC 8B	TC 9A	TC 10A	TC 11A	TC 12A
B	TC 1B	TC 2B	TC 3B	TC 4B	TC 5B	TC 6B	TC 7B	TC 8B	TC 9B	TC 10B	TC 11B	TC 12B
C	TC 13A	TC 14A	TC 15A	TC 16A	TC 17A	TC 18A	TC 19A	TC 20B	TC 21A	TC 22A	TC 23A	TC 24A
D	TC 13B	TC 14B	TC 15B	TC 16B	TC 17B	TC 18B	TC 19B	TC 20B	TC 21B	TC 22B	TC 23B	TC 24B
E	qP1	qP2	qP3	qP4	PN 1	PN2	PN3	PN 4	Neg 1	Neg 2	Neg 3	Neg 4
F												
G												
H												

PCR Tech Rep 1	1	2	3	4	5	6	7	8	9	10	11	12
A	TC 1A	TC 2A	TC 3A	TC 4A	TC 5A	TC 6A	TC 7A	TC 8B	TC 9A	TC 10A	TC 11A	TC 12A
B	TC 1B	TC 2B	TC 3B	TC 4B	TC 5B	TC 6B	TC 7B	TC 8B	TC 9B	TC 10B	TC 11B	TC 12B
C	TC 13A	TC 14A	TC 15A	TC 16A	TC 17A	TC 18A	TC 19A	TC 20B	TC 21A	TC 22A	TC 23A	TC 24A
D	TC 13B	TC 14B	TC 15B	TC 16B	TC 17B	TC 18B	TC 19B	TC 20B	TC 21B	TC 22B	TC 23B	TC 24B
E	qP1	qP2	qP3	qP4	PN 1	PN2	PN3	PN 4	Neg 1	Neg 2	Neg 3	Neg 4
F												
G											BHI NTC	BHI NTC
H	STD 1	STD 1	STD 2	STD 2	STD 3	STD 3	STD 4	STD 4	STD 5	STD 5	TE NTC	TE NTC

PCR Tech Rep 2	1	2	3	4	5	6	7	8	9	10	11	12
A	TC 1A	TC 2A	TC 3A	TC 4A	TC 5A	TC 6A	TC 7A	TC 8B	TC 9A	TC 10A	TC 11A	TC 12A
B	TC 1B	TC 2B	TC 3B	TC 4B	TC 5B	TC 6B	TC 7B	TC 8B	TC 9B	TC 10B	TC 11B	TC 12B
C	TC 13A	TC 14A	TC 15A	TC 16A	TC 17A	TC 18A	TC 19A	TC 20B	TC 21A	TC 22A	TC 23A	TC 24A
D	TC 13B	TC 14B	TC 15B	TC 16B	TC 17B	TC 18B	TC 19B	TC 20B	TC 21B	TC 22B	TC 23B	TC 24B
E	qP1	qP2	qP3	qP4	PN 1	PN2	PN3	PN 4	Neg 1	Neg 2	Neg 3	Neg 4
F												
G											BHI NTC	BHI NTC
H	STD 1	STD 1	STD 2	STD 2	STD 3	STD 3	STD 4	STD 4	STD 5	STD 5	TE NTC	TE NTC

PCR Tech Rep 3	1	2	3	4	5	6	7	8	9	10	11	12
A	TC 1A	TC 2A	TC 3A	TC 4A	TC 5A	TC 6A	TC 7A	TC 8B	TC 9A	TC 10A	TC 11A	TC 12A
B	TC 1B	TC 2B	TC 3B	TC 4B	TC 5B	TC 6B	TC 7B	TC 8B	TC 9B	TC 10B	TC 11B	TC 12B
C	TC 13A	TC 14A	TC 15A	TC 16A	TC 17A	TC 18A	TC 19A	TC 20B	TC 21A	TC 22A	TC 23A	TC 24A
D	TC 13B	TC 14B	TC 15B	TC 16B	TC 17B	TC 18B	TC 19B	TC 20B	TC 21B	TC 22B	TC 23B	TC 24B
E	qP1	qP2	qP3	qP4	PN 1	PN2	PN3	PN 4	Neg 1	Neg 2	Neg 3	Neg 4
F												
G											BHI NTC	BHI NTC
H	STD 1	STD 1	STD 2	STD 2	STD 3	STD 3	STD 4	STD 4	STD 5	STD 5	TE NTC	TE NTC

PCR Time Zero P	1	2	3	4	5	6	7	8	9	10	11	12
A	TC1 A t0	TC2 A t0	TC3 A t0	TC4 A t0	TC5 A t0	TC6 A t0	TC7 A t0	TC8 A t0	TC9 A t0	TC10 A t0	TC11 A t0	TC12 A t0
B	TC1 B t0	TC2 B t0	TC3 B t0	TC4 B t0	TC5 B t0	TC6 B t0	TC7 B t0	TC8 B t0	TC9 B t0	TC10 B t0	TC11 B t0	TC12 B t0
C	TC13 A t0	TC14 A t0	TC15 A t0	TC16 A t0	TC17 A t0	TC18 A t0	TC19 A t0	TC20 A t0	TC21 A t0	TC22 A t0	TC23 A t0	TC24 A t0
D	TC13 B t0	TC14 B t0	TC15 B t0	TC16 B t0	TC17 B t0	TC18 B t0	TC19 B t0	TC20 B t0	TC21 B t0	TC22 B t0	TC23 B t0	TC24 B t0
E	qP1 t0	qP2 t0	qP3 t0	qP4 t0	PN 1 t0	PN2 t0	PN3 t0	PN 4 t0	Neg 1 t0	Neg 2 t0	Neg 3 t0	Neg 4 t0
F												
G											BHI NTC	BHI NTC
H	STD 1	STD 1	STD 2	STD 2	STD 3	STD 3	STD 4	STD 4	STD 5	STD 5	TE NTC	TE NTC

TC# = test coupon number (1 to 24) after 16 hours of growth in HTS plate
A = 1 mL from 5 mL aliquot 1
B = 1 mL from 5 mL aliquot 2
C = 1 mL from 5 mL aliquot 3
STD = DNA standard with designated mass of DNA in the reaction
NTC = no template control

TC1 A t0 = Test Coupon 1 of 24, Time 0, technical replicate 1 of 3
qP1A = qPCR positive control 1 of 12, technical replicate 1 of 3
PC1 A = process control 1 of 4, technical replicate 1 of 3
Neg1 A = Negative Coupon Control 1 of 4 (1 =G, 2=SS, 3=V, 4=P), technical replicate 1 of 3

Figure B.1. Filter Plate Layout (HTS) and PCR Plate Layout Used for mRV–PCR Analysis. A single coupon was sampled using the macrofoam-swab protocol. From this spore suspension, two, 1-mL samples were enriched for two culture replicates. For the control samples, only one 1-mL sample was enriched for one culture replicate. After overnight growth to germinate the spores, PCR was conducted in triplicate for each culture replicate. To accommodate the number of PCR reactions conducted, samples were analyzed in three PCR plates.

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