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LLNL-JRNL-404721

Most Probable Number Rapid Viability PCR Method to Detect Viable Spores of Bacillus anthracis in Swab Samples

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June 17, 2008

Journal of Microbiological Methods

Disclaimer

Abstract

This note presents a comparison of Most-Probable-Number Rapid Viability (MPN-RV) PCR and traditional culture methods for the quantification of B*acillus anthracis* Sterne spores in macrofoam swabs generated by the Centers for Disease Control and Prevention (CDC) for a multi-center validation study aimed at testing environmental swab processing methods for recovery, detection, and quantification of viable *B. anthracis* spores from surfaces. Results show that spore numbers provided by the MPN RV-PCR method were in statistical agreement with the CDC conventional culture method for all three levels of spores tested (10^4, 10^2, and 10 spores) even in the presence of dirt. In addition to detecting low levels of spores in environmental conditions, the MPN RV-PCR method is specific, and compatible with automated high-throughput sample processing and analysis protocols.

Improved methods are needed to rapidly restore facilities contaminated from a biothreat agent release. Current methods involving serial dilution and plating on culture media are labor and time-intensive, such that only 30-40 samples can be processed per lab per day. This note summarizes the results from a comparison of MPN RV-PCR and traditional culture protocols for quantification of *Bacillus anthracis* Sterne spores in environmental samples. Experiments involved analysis of six shipments of blinded samples prepared by the Centers for Disease Control (CDC) for a multi-center validation study aimed at testing environmental swab processing methods for the recovery, detection, and quantification of viable *B. anthracis* spores from surfaces. Although not directly part of the validation study, our laboratory received the same samples as the other Laboratory Response Network laboratories included in the study and analyzed them using both the CDC protocol and the MPN RV-PCR method. We refer the reader to the companion article by Hodges et al. (Hodges et al., 2008) published in the same issue of the Journal of Microbiological Methods for the detail of the CDC culture-based method and the results of the multi-center validation study.

The CDC group prepared 16 blinded samples for each shipment, including 10 dirty macrofoam swabs inoculated with a defined level of *B. anthracis* spores, 2 positive controls (dirty swabs directly-inoculated with a known level of *B. anthracis* spores), 2 blank swabs (no dirt, no *B. anthracis*), and 2 dirty blank swabs (with dirt, no *B. anthracis*). The swabs were held at refrigeration temperature (2-8C) upon reception and processed the next day. The separate shipments focused on three separate spore levels: 10^4, 10^2, and approx. 30 spores per sample; each level being tested with and without reference dirt. Swab preparation was performed at CDC using a slurry of the well-

characterized Arizona Fine Test Dust (Materials Safety Data Sheet, Powder Technologies, 2006), which contains *Bacillus* spores and other bacteria.

Twice the extractant volume specified in the multi-center validation study was added to the swabs, so that sufficient volume could be used for processing with both traditional culture and MPN RV-PCR methods. Traditional culture was performed according to the protocol provided by CDC, which is described in details in the companion article (Hodges et al., 2008). MPN-RV-PCR was performed according to a protocol described in detail elsewhere (Kane et al., 2008).

In order to obtain quantitative information, a modified statistical MPN method was incorporated into the RV-PCR method. The MPN RV-PCR method includes a fully automated dilution series and analysis of 3 to 5 replicates from each dilution via RV-PCR to determine whether the sample dilution is positive or negative. From each of the dilutions, five replicate aliquots are subjected to the RV-PCR protocol, and qPCR data are obtained before and after incubation. The same criteria for starting Ct, endpoint Ct, and ΔCt are used to determine if 1-mL aliquots contained any viable spores and the number of positives is then compared to a standard MPN table to obtain the average spore number for a 95% confidence interval.

Spores were removed from the wipes by vortexing at high speed and a set of two to three 10-fold dilution series was prepared for each sample using a robotic system. Extracted spores were collected for five replicates of each dilution by filtering 1 mL of the vortexed solution using 96-well filter plates. Sterile media was added to the filter plate, and the bottoms were thermally sealed by melting the filter plate bottom. After mixing the media, a 25-µL aliquot (T_0 = 0 min) was removed for each sample and lysed by heat treatment prior to PCR analysis. The sealed filter plate with remaining spore culture was then incubated at 37°C with shaking at 230 rpm until the pre-determined endpoint T_f = 16 h, which constitutes a conservative endpoint allowing

detection of single spores levels even in the presence of background debris. At the endpoint, a second 25-μL aliquot was removed and prepared for real-time PCR analysis, as for the time-zero aliquot. The *B. anthracis* Sterne assay used in this study was adapted from one targeting the *B. anthracis* chromosome (Bode et al., 2004). Taqman PCR reactions were run in 96-well plates with 45 PCR amplification cycles. Samples with a Ct value less than or equal to 32 were identified as positive, and samples with Ct values greater than 32 were identified as negative. The criterion for positive live spore detection was based on an algorithm that uses a combination of the C_{t0}, delta-C_t, and the end point C_f values. The typical threshold value for delta-C_t was set at 6.4.

Figure 1 shows the comparison of the number of *B. anthracis* spores determined by MPN RV-PCR and traditional culture methods on clean swabs containing three levels of spores (10^4, 10^2, and 10^1) and Figure 2 shows the corresponding data sets for dirty swabs. All samples and controls were correctly identified by both methods and the two methods were in statistical agreement for 10^4 spores for both levels and 10^2 spores for clean swabs. The MPN RV-PCR method slightly overestimated (by < 1-log) spore levels for dirty swabs containing either 10^2 or 10^1 spores, while the MPN RV-PCR method was slightly closer to the expected results for clean swabs containing 10^1 spores.

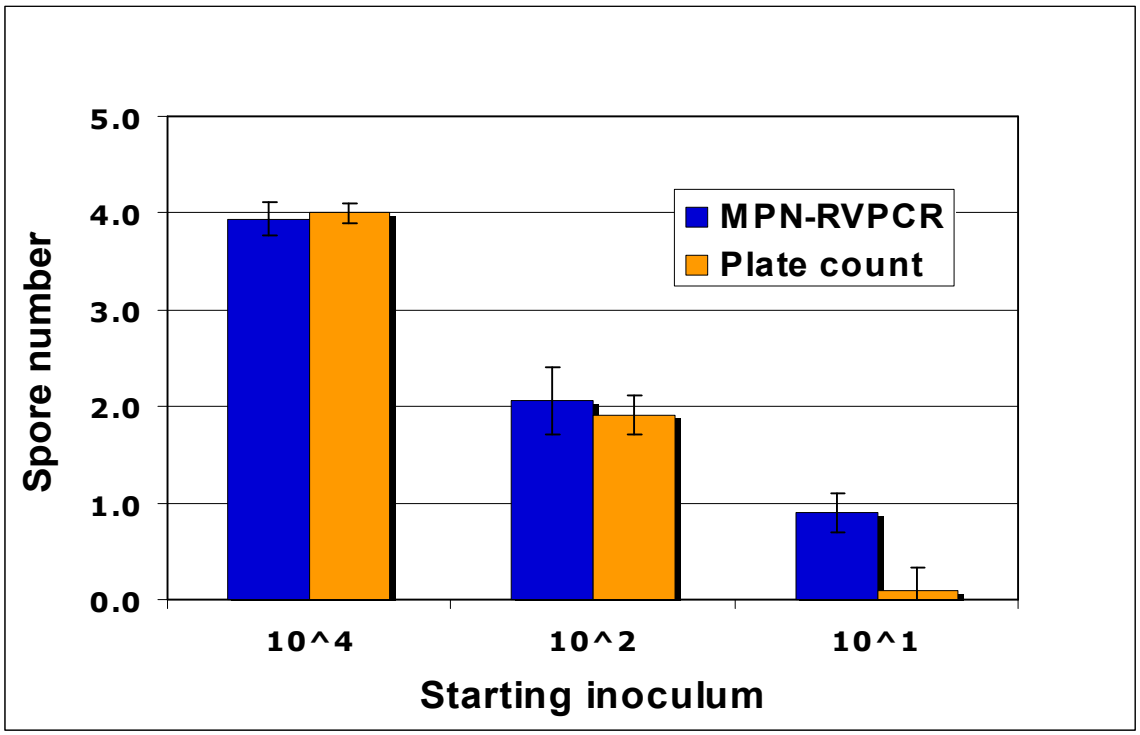

Figure 1. Average spores numbers determined by MPN RV-PCR and traditional culturing for *B. anthracis* Sterne spores recovered from clean swabs with 3 different spore levels (approx. 10^4, 10^2, and 10^1). Experiments included 10 samples for each spore level. Error bars represent 1 SD above and below the average MPN value corrected for dilution.

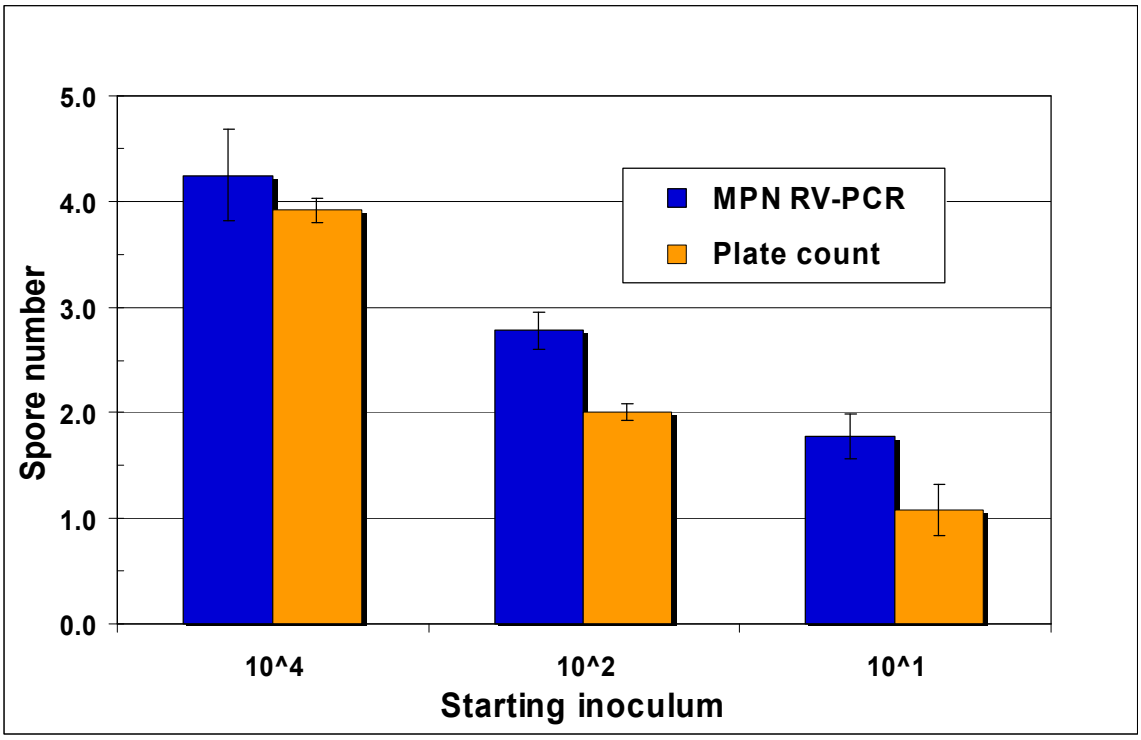

Figure 2. Average spores numbers determined by MPN RV-PCR and traditional microbiology for *B. anthracis* Sterne spores recovered from dirty swabs with 3 different spore levels (approx. 10^4, 10^2, and 10^1). Experiments included 10 samples for each spore level. Error bars represent 1 SD above and below the average MPN value corrected for dilution.

No spore counts were obtained on negative controls, which confirmed the absence of contamination. Results from the MPN RV-PCR method were not statistically significantly different from conventional culture results. Spore count values were slightly greater for the MPN RV-PCR method relative to the plate count method, but still well within 1 log of the values obtained by traditional culture. All samples were correctly identified as positives and/or negatives using both traditional culture and MPN RV-PCR methods.

Based on analysis of colony counts at the endpoint, there was little indication of growth inhibition for swabs with dirt. PCR inhibition was observed at high spore levels but could always be overcome with a 1:10 dilution. The MPN RV-PCR method was shown to work well without modification for spore levels less than 10^4; for levels above 10^4, samples would still be reported as positive, but an additional dilution needs to be performed to obtain accurate quantitative data.

PCR confirmation was performed on colonies counted, following the protocol provided by CDC (Hodges et al., 2008) and all sample identifications were confirmed. This additional step was not required when using the MPN RV-PCR method, which has built-in target confirmation in the Taqman PCR step.

In conclusion, both MPN RV-PCR and culture methods provided correct identification for all samples analyzed in this study and the estimation of the number of spores by MPN RV-PCR was in statistical agreement with the traditional culture method. The addition of dirt to the swabs did not inhibit *B. anthracis* growth and PCR inhibition observed at high spore levels could be overcome with a 1:10 dilution.

These results confirm the effectiveness of the MPN RV-PCR approach to detect low levels of *B. anthracis* spores in the presence of dirt and high numbers of non-target microbial populations. In addition, MPN RV-PCR assays are specific and provide immediate verification of the nature of the pathogen. Automation allows processing of 100-200 samples per day, with quantitative results obtained within 24 hr. It also reduces the risk of exposure to pathogens since robotics perform the liquid handling steps and no plating nor centrifugation is required. Additionally, the significantly decreased labor costs more than offset the additional costs of PCR reagents and consumables for MPN

RV-PCR analysis, which could ultimately enable the implementation of this method for bio-security and decontamination applications.

Acknowledgments

This work was performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344. Funding for this research was provided by the Department of Homeland Security and the Defense Threat Reduction Agency.

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