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Spore Disruption Analysis and Detection Limit Determination at Low Volume Amplifications (2-10 uL) of Bacillus globigii Using eTags

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Spore Disruption Analysis and Detection Limit Determination at Low Volume Amplifications (2-10 μL) of *Bacillus globigii* Using eTags

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ABSTRACT

Spore Disruption Analysis and Detection Limit Determination at Low Volume Amplifications (2-10 μL) Using eTags. LAUREN TRACY (University of California Berkeley, Berkeley, CA 94720) SHANAVAZ NASARABADI (Lawrence Livermore National Laboratory, Livermore, CA 94550).

In the post 9/11 world the threat of bioterrorism attacks in public venues has ignited a demand to develop a cost effective autonomous pathogen detection system capable of detecting the multitude of biological agents that can pose a threat to public safety. The major cost of such a pathogen detection system is the large volume of reagents it must expend. With the goal of reducing the reagent consumption, and therefore cost, of a pathogen detection system, we used the spore-forming bacteria *Bacillus globigii* (Bg) as a surrogate for the pathogen *Bacillus anthracis* (anthrax) to determine the lowest amplifiable volume and lowest concentration of amplified sonicated and unsonicated Bg spores that would still be detectable using capillary electrophoresis. We created a serial dilution of unsonicated Bg spores ranging in concentration from 10^8 to 10^1 cfu/mL. From each of these unsonicated spore dilutions we formed three aliquots that were sonicated to disrupt the spores. These sonicated aliquots were analyzed alongside the unsonicated spore samples for each dilution at reaction volumes of 25, 10, and 2 μL. All samples were amplified through a polymerase chain reaction (PCR) in the presence of small fluorescent molecules known as electrophoretic tags (eTags), which were analyzed with capillary electrophoresis to detect the presence of certain nucleic acid signatures.

Using this process, Bg samples with concentrations as low as 10^1 cfu/mL and total reaction volumes of amplification as small as 2 μL were readily detectable. Interestingly, detection was more consistent for Bg samples with initial spore concentrations between 10^6 and 10^3 cfu/mL, with the higher and lower concentrations yielding less compelling results. The volume of the sample also affected the efficacy of detection, with detection for 2 μL samples compromised in relation to 25 and 10 μL samples. Detection of sonicated Bg spores appeared to be just as efficient and reliable as detection of unsonicated Bg spores. This work is a small portion of a much larger project being researched at LLNL and Sandia National Laboratory to develop a low cost briefcase size autonomous detector for small-scale 24/7 detection of a variety of common biothreat agents.

INTRODUCTION

In recent years the United States has been rife with fear of bioterrorist attacks, which could be difficult to detect in a timely and cost-effective manner and could infect large populations relatively rapidly. The constant threat of biological attack has spurned the need for the development of an economically feasible autonomous pathogen monitoring system. According to McBride, et al [4], "an ideal [pathogen] monitoring system …should run unattended for long periods of time, require infrequent maintenance, and be inexpensive to operate. The platform must exhibit detection limits such that life-threatening doses of airborne pathogens can be detected."

In order to meet these requirements, an ideal pathogen detection system must be capable of working with low volumes of reagents in order to minimize cost and reduce the need for frequent re-filling maintenance. By simply reducing the sample volume from 25 μL to 2 μL, the cost of running a pathogen detector would decrease from $10,041 to $701 (see Figure 1). The system must also be capable of detecting low concentrations of pathogens in order to alert the public of a life-threatening dispersal, given that aerosolized spores can diffuse rapidly throughout an enclosed space, further diluting the pathogen concentration [2]. Additionally, in order to further reduce cost and decrease the system's size, all unnecessary steps in the detection process should be eliminated. We are interested in particular if sonication of bacterial spores improves detection ability, or if intact bacterial spores are just as readily analyzed, making sonication a causeless step.

Our work focused on determining the smallest volume and lowest concentration of amplified bacterial spores (both intact and sonicated) that would still be detectable using capillary electrophoresis. We used the spore-forming bacteria *Bacillus globigii* (Bg) as a surrogate for the biothreat agent anthrax. All of the concentrations and volumes discussed refer to those measurements before the Bg underwent amplification during a polymerase chain reaction (PCR). In order to detect the Bg through capillary electrophoresis we added electrophoretic tags known as eTags. These small fluorescent molecules act like a biomarker, binding to a certain pattern of DNA and releasing a distinguishably charged electrophoretic molecule after enzymatic cleavage [3]. Although the results of these capillary electrophoresis analyses are not quantitative, they can be compared roughly against each other because we used the same ratio of reagents in each sample.

In this paper we compare the detection results for concentrations of Bg ranging from 10^8 to 10^1 cfu/mL in volumes of 25,10, and 2 μL for each level of dilution. We also compare the detection efficiency of intact Bg spores and sonically disrupted Bg spores.

MATERIALS AND METHODS

We utilized all reagents and materials as received. The Bg spores used in this study were cultured from a Bg powder produced by Dugway Proving Grounds. The original Bg spores were at an undiluted concentration. We ran a serial dilution of the Bg spores by adding nuclease-free water (Ambion; cat. #9932; 2130 Woodward, Austin, TX 78744-1832) to create a concentration range from 10^8 to 10^1 cfu/mL with each

dilution having an initial volume of 1000 µL. For each concentration level of these intact spores, three 100 µL aliquots were removed for sonication. The spores of each of these 24 samples (3 samples for each level of dilution) were sonicated over unwashed glass beads with particulate sizes at least as small as 106 µm (Sigma; #: G8893-100G, 3050 Spruce St, St. Louis, MO, 63103) in a Bransonic 1210 Ultrasonic Cleaner (Branson Ultrasonics Corp.) for approximately one minute each.

To amplify the Bg DNA through PCR we used a mixture of Accuprime Supermix I (Invitrogen; cat. #: 12342-028; Carlsbad, CA, 92008), nuclease-free water (Ambion; cat. #9932; 2130 Woodward, Austin, TX 78744-1832), eTags (Aclara; 1288 Pear Ave., Mountain View, CA, 94043), and Bg specific 40/104 oligonucleotides (BioSearch Technologies; 81 Digital Drive, Novato CA 94949-5750). We attempted to maintain a similar ration of the above-mentioned PCR reagents for each sample volume. Every 25 µL sample contained: 12.5 µL Accuprime Supermix, .5 µL oligonucleotides, 11 µL nuclease-free water, .5 µL eTags, and 1 µL of the relevant Bg dilution. Every 10 µL sample contained: 4.8 µL Accuprime Supermix, .2 µL oligonucleotides, 4.3 µL nuclease-free water, .2 µL eTags and .5 µL of the relevant Bg dilution. Each 2 µL sample contained 1 µL Accuprime Supermix, .04 µL oligonucleotides, .88 µL nuclease-free water, .04 µL eTags and .08 µL of the relevant Bg sample. All the samples were amplified on a Gene Amp PCR System 9600 by Perkin Elmer, which heated the samples to 94°C for 21 minutes, then repeated 40 times a cycle of heating to 94°C for 30 seconds, cooling to 55°C for 1 minute, then reheating to 72°C for 1 minute. The samples were held at 72°C for 5 minutes before being stored at 4°C.

In order to analyze the 25 µL samples, a10 µL portion of the total amplified volume was drawn off into a 96-well plate and mixed with 10 µL of eTag Separation Reagent 2 Marker (henceforth marker) (Aclara; 1288 Pear Ave., Mountain View, CA, 94043). To analyze the 10 µL samples, a 5 µL portion of the total amplified volume was drawn off into a 96-well plate and mixed with
6 µL of marker. To analyze the 2 µL samples, a 1 µL portion of the total amplified volume was drawn off into a 96-well plate and mixed with 10 µL of marker. The samples were then subjected to capillary electrophoresis on ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

The above described set-up was repeated twice each for the 25 and 2 µL sample volumes, and three times for the 10µL sample volume.

RESULTS

The data received from the capillary electrophoresis analyses represents an un-quantified endpoint average of the fluorescent markers given off by eTags in the form of a peak representing the presence of Bg. Although the peaks do not represent quantified data, they can be roughly compared to one another given that all the samples had roughly the same ratio of reagents and approximately the same final volume after the addition of eTag Separation Reagent.

Table 1 illustrates the average peak height for every sample concentration for each sample volume. The peak heights represent the endpoint average fluorescence emitted from the eTags during analysis. The fact that an average peak height is available for every sample concentration at every volume indicates that we were able to detect the

presence of Bg for every experimental condition. Although it was possible to detect Bg at all experimental dilutions and volumes, reaction volumes of 10 and 25 µL and concentrations between 10^6 and 10^3 cfu/mL yielded the best results. Figure 2 displays the average peak height for all the samples with 25, 10 and 2 µL volumes respectively. All the samples were run in triplicates at their individual concentrations for every sample volume. Figure 3 shows the average peak height for each sample concentration from 10^8 to 10^1 cfu/mL. As Figure 1 demonstrates the average peak height for all the 25µL samples was 125,035 fluorescence units (FU). For the 10 µL samples the average peak height was 131,780 FU; and for the 2 µL samples the average peak height was 27,162 FU. Likewise, the average peak height was 60,809 FU for the 10^8 cfu/mL samples; 28,806FU for the 10^7 cfu/mL samples; 115,752 FU for the 10^6 cfu/mL samples, 134,979 FU for the 10^5 cfu/mL samples, 106,942 FU for the 10^4 cfu/mL samples, 122,498 FU for the 10^3 cfu/mL samples, 98,776 FU for the 10^2 cfu/mL samples, and 88,712 FU for the 10^1 cfu/mL samples. A higher average peak height indicates the efficiency and reliability of that sample volume to be detected. A comparison of the average peak height of sonicated Bg spores versus intact Bg spores for each sample volume is presented in Figure 4. For volumes of 25 µL, unsonicated spores gave off 162,446 FU on average, while sonicated spores on average gave off 125,035 FU. For volumes of 10 µL, unsonicated spores gave off 99,080 FU on average and sonicated spores on average gave off 131,780 FU. For volumes of 2 µL, unsonicated spores gave off 58,424 FU on average, while sonicated spores gave off an average of 27,162 FU.

DISCUSSION AND CONCLUSION

As part of the development of an economically manageable autonomous pathogen detection system the limit of detection for bacterial spores must be known in order to minimize the amounts of reagents used, while still ensuring accurate detection of low concentrations of pathogenic material. Our analyses found that while sample volumes as small as 2 μL with concentrations as low as 10^1 cfu/mL can be readily detected (as shown in Table 1), certain conditions provide more accurate and dependable results.

There is little difference in detection dependability between samples with volumes of 25 and 10 μL, with each volume demonstrating an average peak height of around 130,000 FU (as shown in Figure 2). Samples volumes of 2 μL while still detectable, display a degradation of detection ability, with an average peak height much lower than those of the larger sample volumes (see Figure 2). The increased unreliability of detection for the 2 μL sample is due to the very small quantities used to produce each sample. With only .08 μL of Bg spores for each 2 μL sample, the slight expected mechanical error of the pipette or a small human error would result in a compromised analysis. In addition, during refrigeration storage after amplification, portions of the 2μL samples were lost, resulting in even less accurate detection.

The role that Bg spore concentration had on sample detection was unexpected. Overall, samples with concentrations between 10^6 and 10^3 cfu/mL were identified with greater ease than samples of higher or lower concentrations (see Figure 3). Oddly, the samples with the highest concentrations of Bg spores (10^8 and 10^7 cfu/mL) gave off the least fluorescence (60,809 and 28,806 FU respectively), meaning that these concentrations exhibited lowered detection results (see Figure 3). This suggests that the

higher concentrations (10^8 and 10^7 cfu/mL) of Bg could inhibit the polymerase enzyme during amplification, resulting in less overall nucleic acid material. This result could also be due to an inadequate balance of oligos to Bg DNA in the more highly concentrated samples, again resulting in less overall Bg nucleic acids after amplification.

As Figure 4 illustrates there appears to be no correlation between ease of detection and sonification of Bg spores. In both the 25 and 2 μL volumes, the unsonicated spores had a greater average peak height than the sonicated spores. In the 10 μL samples however, the sonicated spores had a greater average peak height. Although the spores had been cleaned of their external DNA from the mother cell, the process is inefficient and there is a sufficient amount of DNA stuck to the cortex of the spores. Thus this data indicates that sonication of spores may not be necessary in order to have a good limit of detection.

The results of this experiment were based on a limited number of electrophoretic analyses. The data could be focused and improved greatly by introducing more Bg spore samples for analysis. Additionally many of the Bg spore samples utilized in this experiment were stored for periods of up to 14 days in 4°C after amplification, which most likely resulted in a loss of amplified DNA through condensation.

With this research we have shown that sample volumes for pathogen detection can be safely reduced to at least 10μL without risking reduced detection ability. We have also shown that a variety of Bg spore concentrations can be detected, with a moderate concentration of Bg being the most efficacious. With this work we have come one tiny step closer to developing a cost-manageable autonomous pathogen detection system.

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TABLES AND FIGURES

Table 1:

Concentration	Sample volume (μL)	Ave. peak height (FU)
10^-1	25	50979.7
10^-2	25	6691
10^-3	25	217670.3
10^-4	25	167019.3
10^-5	25	107485.5
10^-6	25	152647.3
10^-7	25	148494.3
10^-8	25	149295.2
10^-1	10	103926.1
10^-2	10	72626.7
10^-3	10	88558.9
10^-4	10	154469.7
10^-5	10	192794
10^-6	10	181710.3
10^-7	10	145315
10^-8	10	114840.1
10^-1	2	27521.3
10^-2	2	7098.8
10^-3	2	41028.7
10^-4	2	83447.8
10^-5	2	20546
10^-6	2	33138.7
10^-7	2	2519
10^-8	2	2001

Compiled average peak height data in Fluorescence Units for each concentration and sample volume.

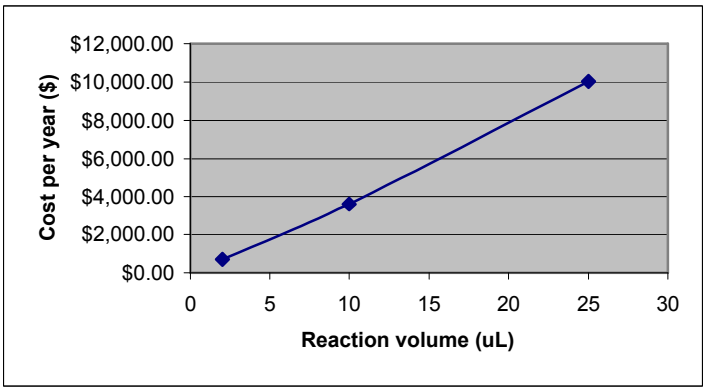
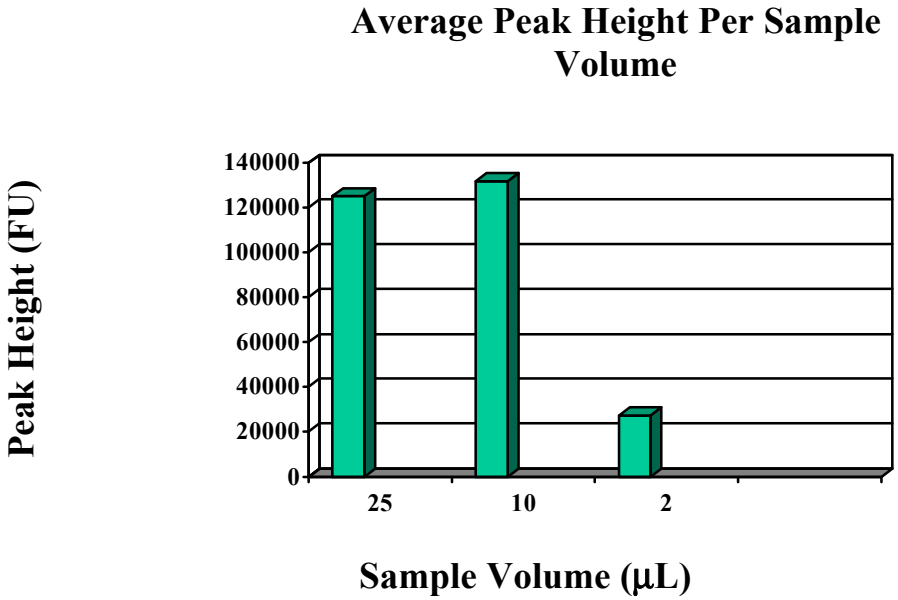


Figure 1:

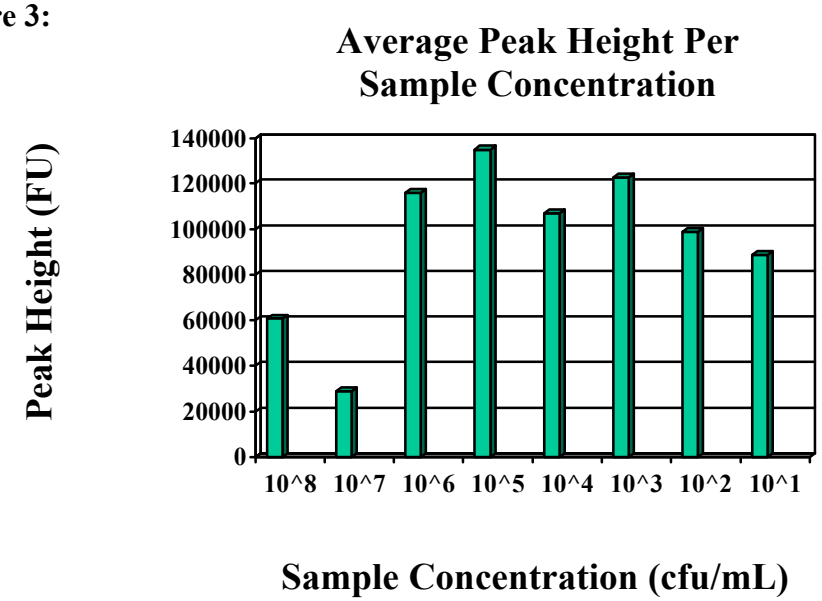
Data taken from S. Nasarabadi, et al. This figure illustrates that as reaction volume decreases, price decreases.

Figure 2:



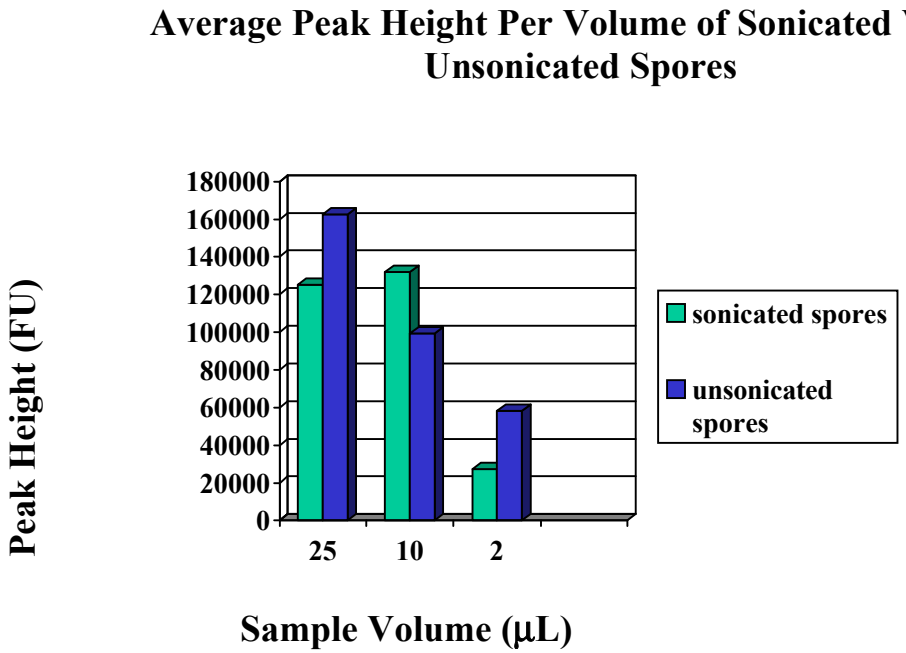
Average peak height in Fluorescence Units (FU) for each sample volume.

Figure 3:



Average peak height in Fluorescence Units (FU) for each sample concentration.

Figure 4:



Average peak height of sonicated spores of each sample volume versus unsonicated spores of each sample volume in Fluorescence Units (FU).