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Discerning Viable from Nonviable Yersinia Pestis pgm- and Bacillus Anthracis Sterne using Propidium Monoazide in the Presence of White Powders

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Abstract

Purpose of the study

To develop and optimize an assay to determine viability status of *Bacillus anthracis* Sterne and *Yersinia pestis* pgm- strains in the presence of white powders by coupling propidium monoazide (PMA) treatment with real-time PCR (qPCR) analysis.

Approach and results

After gaining entry to intracellular space, PMA can be exported by metabolically active cells. The PMA remaining in nonviable cells binds DNA, thereby increasing qPCR assay cycle threshold (C_T) values compared to untreated samples. Dye concentration, cell number and fitness, incubation time, inactivation methods, and assay buffer were optimized for a Gram-positive pathogen, *B. anthracis* Sterne, and a gram negative pathogen, *Y. pestis* pgm-. Differences in C_T values in nonviable cells compared to untreated samples were consistently > 9 for both *B. anthracis* Sterne vegetative cells and *Y. pestis* pgm- in the presence and absence of three different white powders. Our method eliminates the need for a DNA extraction step prior to detection by qPCR.

Significance of the Study

The method developed for simultaneous detection and viability assessment for *B. anthracis* and *Y. pestis* can be employed in forming decisions about the severity of a bio threat event or the safety of food.

Keywords: Bacillus anthracis; *Yersinia pesti*; Propidium monoazide; QPCR; White powders; Rapid viability detection

Introduction

Bacillus anthracis is a spore-forming, Gram-positive bacterium and is the causative agent of the zoonotic disease anthrax. Infection occurs when spores gain access to the host through respiratory, cutaneous, or gastrointestinal routes [1]. *B. anthracis* is listed by the CDC as a category A bio threat agent because of the stability of the spore form, infectivity, and ease of dissemination. In 2001, the intentional dissemination of *B. anthracis* spores through the mail prompted public health officials and the scientific community to improve methods for rapid detection of bio threat agents in general [2]. Of additional concern, *Y. pestis* is also a category a bio threat agent and is the causative agent of plague [3,4]. Given the bio threat potential of both *Y. pestis* and *B. anthracis,* we sought to develop a rapid viability determination method for these two organisms [4-7].

The need for rapid methods to detect DNA signatures from suspected bio threat events has largely been met with real-time PCR (qPCR) methods, which can be used to quickly identify bacterial species using specifically designed probes. However, qPCR assays alone do not assess bacterial viability [8-12], which is critical information to ensure an appropriate and timely response to bio threat events. While many assays have been developed to assess bacterial viability for certain types of bacterial cells, bacterial spores remain challenging due to their dormant nature but infectious potential.

Published work has studied the effectiveness of determining viable from nonviable samples by pretreating with intercalating dyes propidium monoazide (PMA) or ethidium monoazide (EMA) prior to conducting qPCR assays. PMA enters cells and cannot be actively exported from nonviable cells or cells with damaged membranes and forms covalent bonds with DNA after photo activation [13-15]. Previous work by Nocker et al. showed that EMA is capable of penetrating live as well as dead cells; therefore, EMA was not used in our study [14]. The binding action (PMA-DNA covalent bond) renders the bound DNA inaccessible to the polymerase and therefore undetectable in the qPCR reaction, thereby increasing the C_T value compared to equivalent untreated samples [12,16]. Previous studies using intercalating dyes coupled with qPCR (i.e., PMA-qPCR) detection have focused on bacterial species commonly associated with food-borne illnesses, such as *Escherichia coli*, *Listeria monocytogenes*, *Salmonella enterica* serovar *Typhimurium*, and *Campylobacter jejuni,* in which artificially spiked foods were subjected to PMA treatment followed by qPCR to determine the presence and viability of the bacteria. Ultimately, the conclusions of these studies indicate that PMA coupled qPCR is a promising tool to identify viability, presence, and species, but that assay optimization is required for each system [12,16-21]. The purpose of the current study is to assess the suitability of this method for bio threat agents *Y. pestis* and *B. anthracis*, and to define assay conditions and critically test the method

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under laboratory conditions. We found that the assay is well suited to discerning viable from nonviable cells of both of the tested bacteria in a laboratory setting and in the presence of three different white powders. A DNA extraction is not required, thereby streamlining the process and reducing labor requirements compared to other published methods. Therefore, the PMA-qPCR assay described here is a promising method for rapid detection and viability assessment of *B. anthracis* Sterne and *Y. pestis* pgm- vegetative cells in a controlled, laboratory setting and in the presence of the white powders included in this study.

Materials and Methods

Bacterial strains and culture conditions

Yersinia pestis KIM D27 (pgm-) and vegetative *Bacillus anthracis* Sterne were grown by selecting a fresh colony from a Tryptic Soy Agar (TSA; BD Biosciences, Franklin Lakes, New Jersey) streak plate derived from glycerol stocks. The colony was used to inoculate fresh tryptic soy broth without dextrose (TSB; BD) in a glass flask (50 mL culture volume for *Y. pestis* pgm- and 10 mL culture volume for *B. anthracis* Sterne). Overnight cultures were grown at 37°C (*B. anthracis* Sterne) or 30°C (*Y. pestis* pgm-) at 200 rpm for 16 to 18 hours. Log phase *B. anthracis* Sterne cultures were prepared by adding 1 mL of overnight culture to 9 mL of fresh TSB in a 50 mL glass flask and incubating at 37°C and 200 rpm for three to four hours. Log phase *Y. pestis* pgm- cultures were prepared by adding 15 mL of overnight cultures to 15 mL of fresh TSB and incubating at 30°C and 200 rpm for four to five hours.

Bacillus anthracis Sterne spore stocks were generated using nutrient broth supplemented with CCY salts as described by Buhr [22]. Briefly, *B. anthracis* Sterne was inoculated into TSB at 37°C and agitated for 16 to 18 hours at 200 rpm. The sporulation media was then inoculated (1:100) using the overnight culture and incubated at 37°C, 200 rpm for 72 hours. The suspension was then centrifuged for 10 minutes at 10,000 × *g* and the resulting supernatant was discarded. The biomass was suspended in 30 mL of sterile, milli Q water and stored at 4°C for seven days to promote vegetative cell lysis. The sample was then washed three times with 30 mL of sterile, milliQ water by centrifugation for 10 minutes at 10,000 × *g* and resuspended in a final volume of 10 mL. The spores were visualized using phase contrast microscopy and > 95% of observed spores was phase-bright. The spore stocks were maintained at 4°C for the duration of the study. All bacterial stocks were enumerated prior to conducting experiments by plating serial dilutions in PBS (Life Technologies, Carlsbad, CA) containing 0.02% Tween 80 (Sigma Aldrich, St. Louis, MO) on TSA plates at the appropriate temperature.

Generating a template for qPCR reactions by heat lysis or DNA extraction

DNA extractions were performed using the Qiagen DNeasy Blood and Tissue Kit according to the manufacturer's instructions, without any deviations. As described in the qPCR section below, direct heat lysis was conducted by including a single cycle at the beginning of the qPCR method at 95°C for ten minutes.

Sample inactivation

Y. pestis pgm- was inactivated using 50% ethanol. Aliquots of log phase or overnight cultures were centrifuged at 16,000 *x g* for 5 minutes and resuspended in the equivalent volume of 50% ethanol (prepared fresh just before use by diluting 200 proof ethanol at a 1:1 ratio with nuclease free water) and transferred to a new tube. Samples were incubated at room temperature for 30 minutes. Half of the sample volume was plated on TSA plates and incubated for 48 hours at 30°C to verify lack of cell growth and inactivation. The *B. anthracis* Sterne spores and vegetative cells were inactivated using 3% hydrogen peroxide (Sigma Aldrich) (prepared fresh just before use by diluting a 35% stock of hydrogen peroxide with nuclease free water). Aliquots of log phase culture, overnight cultures, and up to 10^9 spores were centrifuged at 16,000 × *g* for 5 minutes and resuspended in the equivalent volume of 3% hydrogen peroxide. Samples were incubated at room temperature for one hour. For all experiments, half of the sample was plated on TSA plates and incubated for 48 hours at 37°C to verify inactivation; all samples were negative for bacterial growth. Following the indicated exposure time to the inactivation agent, the remaining sample volume was centrifuged at 16,000 × *g* for five minutes and the supernatant was then discarded. The resulting sample was resuspended in the equivalent volume of Tris-EDTA pH 8 Buffer (TE, Ambion, Carlsbad, CA) and transferred to a new tube.

Addition of white powders to samples

The white powders were purchased from a local vendor. We selected Peak Dry whole milk powder (referred to in the text as milk powder), Rumford aluminum free baking powder (referred to in the text as baking powder), and Coffee-mate "The Original" coffee creamer (referred to in the text as coffee creamer). Suspensions of each powder were prepared in TE at a stock concentration of 5 mg/mL. To ensure the white powder did not contribute to viable biomass measurements, the powder suspensions were sterilized by autoclaving for 20 minutes on a liquid cycle at 121°C. Sterilization was confirmed by plating 100 µL aliquots on TSA plates and confirming lack of growth after 48 hours at 37°C. Samples containing white powder were prepared as described above; however, the final resuspension of the sample was conducted using fresh TE spiked with the white powder suspension to a final concentration of 0.1 mg/mL white powder in TE.

Preparing and treating samples with PMA

All dilutions were performed using TE buffer. The indicated concentration of PMA was added to 500 µL aliquots of viable or nonviable cells. An assay workflow is depicted in (Figure 1). The 20 mM PMA stock (Biotium, Hayward, CA) was stored at -20°C and diluted to the required concentrations using nuclease free water prior to use. Sample volumes were 500 µL and we prepared concentrations of PMA such that the addition of 1.25 µL to the sample would result in the desired PMA concentration. Samples were incubated in the dark and covered with aluminum foil for five minutes at room temperature. Following the incubation, the samples were photo activated using the PMA-Lite™ LED Photolysis Device (Biotium) as the LED sources to photo activate the PMA for five minutes (Figure 1B).

Quantitative PCR (qPCR) experiments

Primer sets are provided in (Table 1). *Y. pestis* primers and probes targeted the chromosomal gene *yihN* as described by Stewart et al. [3]. *B. anthracis* primer and probe targeted the *CAAX* gene as described by Wielinga et al. [23]. Following PMA treatment, 5 µL of sample was used as the template in the qPCR reaction. Each qPCR reaction was performed in a 20 µL volume with the following reaction components: 10 µL of 2x TaqMan® Fast Universal Master Mix (Life Technologies), 1 µL of 20x Primer/Probe (PrimeTime® qPCR Assays; IDT, Coralville, IA), 4 µL of nuclease free water, and 5 µL of template. TE was used as the template for the no template control reactions. Each sample was run in triplicate, and the experiments were conducted a total of three times independently (n=9). PCR was conducted using the Applied Biosystems 7500 Real-Time PCR system with the default fast platform

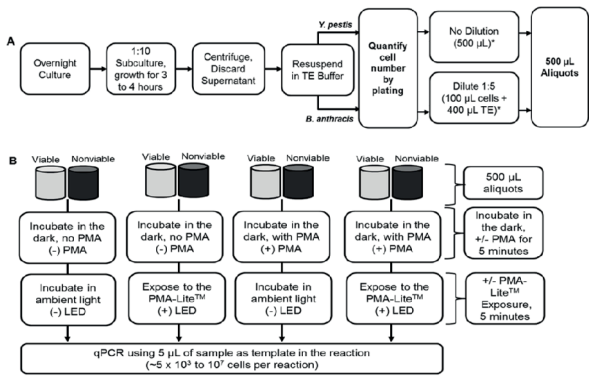

Figure 1: The PMA Assay Workflow. The workflow of (A) preparing cultures of *Y. pestis* pgm- and vegetative *B. anthracis* Sterne cells for the PMA assay. The *symbol indicates the point in the workflow in which a portion of the culture is subjected to inactivation according to the procedures outlined in Materials and Methods. (B) Sample treatments with PMA, the PMA-LITE™ which functions to cross-link the PMA to DNA, followed by qPCR.

Target	Primer Name	Sequence (5' – 3')
B. anthracis chromosome	CAA × _F	TCC GTT TAC CAA TTC ACT ATG AAT CAA T
	CAA × _R	ATG CGT TGT TAA GTA TTG GTA TAA TCA TC
	CAA × _ Probe	FAM/CC CAC TTG G/Zen/A TTA TAT CCT GAG TAT CGT GA/3IABkFQ/
Y. pestis chromosome	Yp_F	CGC TTT ACC TTC ACC AAA CTG AAC
	Yp_R	GGT TGC TGG GAA CCA AAG AAG A
	Yp_ Probe	FAM/TA AGT ACA T/Zen/C AAT CAC ACC GCG ACC CGC TT/3IABkFQ/

Table 1: Primers and probes used in this study.

settings (95°C for ten minutes for heat lysis, 95°C for 10 s, followed by 40 cycles of denaturation at 95°C for 3 s and annealing/extension at 60°C for 30 s). C_T values greater than 35 were considered as undetected by the qPCR assay, abbreviated as ND in the presented data. A workflow of the full assay is depicted in (Figure 1).

The difference in C_T values was used to compare nonviable samples with viable samples to the appropriate non-treatment control using the following formulas:

1) $\Delta C_T = \Delta C_{Tnonviable} - \Delta C_{Tviable}$,

2) $\Delta C_{Tnonviable}$ = C_T nonviable with PMA– C_T nonviable without PMA, and

3) $\Delta C_{Tviable}$ = C_T viable with PMA– C_T viable without PMA.

Quantitation

As a reference method for quantitation of the bacteria, after culture preparation, but prior to PMA or LED treatment, aliquots of bacterial samples were serially diluted and plated on TSA plates to determine total cell numbers in all qPCR reactions. These values could then be correlated back to raw C_T values obtained in the experiments to ensure consistent biomass in all treatment samples and qPCR reactions. The number of cells in each qPCR reaction is provided in the figure legends for each relevant data set. In this way, the C_T value can be used to deduce the amount of bacteria in unknown samples.

Statistical analysis

Statistical analysis was conducted using the student's one-tail t-Test with the Analysis Tool Pak Excel add-in tool. Results were considered significant if the resulting p value was less than or equal to 0.05. The data set for each t-Test consisted of a minimum of three biological replicates for each sample type. That is, three independent experiments were conducted for each sample type. In each independent experiment, a total of three technical replicates were conducted (n=9).

Results

Evaluating PMA treatment with *Y. pestis* pgm-, *B. anthracis* sterne vegetative cells, and *B. anthracis* sterne spores

We first wanted to test if populations of cells could be simultaneously identified and determined as viable or not without performing a DNA extraction. To test this method, we subjected equivalent quantities of viable and nonviable cells or spores to PMA treatment and the PMA-Lite ™ according to the procedures outlined in Materials and Methods and diagrammed in (Figure 1). We empirically determined the optimal incubation time, assay buffer, inactivation method, LED exposure time, and growth phase for *B. anthracis* Sterne and *Y. pestis* pgm- (data not shown). As shown in (Table 2), there was a shift in the C_T values between the viable and nonviable cell populations for both *Y. pestis* pgm- and *B. anthracis* Sterne vegetative cells. For clarity, a workflow of data analysis from raw C_T values to ΔC_T for the vegetative cells from this data set is shown in (Figure 2). For both cell types, qPCR detection was successful without a DNA extraction (untreated and treated). The optimized parameters for the assay are described in (Table 3).

To ensure that the genomic DNA extraction step could be eliminated from the assay workflow, we compared the qPCR signal from viable and nonviable bacteria under our culture and experimental conditions using direct heat lysis and DNA extraction using a Qiagen DNeasy Blood and Tissue kit. The genomic DNA was extracted according to the manufacturer's instructions without deviations. Both *Y. pestis* pgm- and *B. anthracis* Sterne were grown under conditions described in (Figure 1A). The resulting cultures were centrifuged and split into three equivalent aliquots. Two of the aliquots were designated as viable and a

Organism	Viability	Untreated C_T (SD)	LED C_T (SD)	LED + PMA C_T (SD)	ΔC_T viable or ΔC_T nonviable	ΔC_T
B. anthracis Sterne spores	Nonviable	31.87 (0.30)	32.17 (0.22)	ND	N/A	N/A
B. anthracis Sterne spores	Viable	33.67 (0.16)	34.25 (0.31)	ND	N/A	
B. anthracis Sterne Vegetative	Nonviable	21.76 (0.07)	21.80 (0.11)	34.15 (0.40)	-12.4	-7.2
B. anthracis Sterne Vegetative	Viable	21.66 (0.03)	21.87 (0.18)	26.90 (0.24)	-5.24	
Y. pestis pgm-	Non-viable	16.1 (0.06)	16.1 (0.17)	27.5 (1.03)	-11.4	-6.6
Y. pestis pgm-	Viable	16.6 (1.47)	16.6 (1.46)	21.4 (1.19)	-4.8	

Table 2: PMA treatment distinguishes viable from nonviable *B. anthracis* Sterne vegetative cells and Y. *pestis* pgm- cells but not *B. anthracis* spores. Raw C_T values of of viable and nonviable samples. The PMA concentration is 10 µM for *B. anthracis* Sterne spores and vegetative cells and 25 µM for *Y. pestis* pgm- samples. Spore samples contained 15,000 spores. ND, absence of qPCR signal. N/A, not applicable. Measurements are the average value of three independent e × periments. All ΔC_T values are calculated using equations (1), (2), or (3) as described in Materials and Methods.

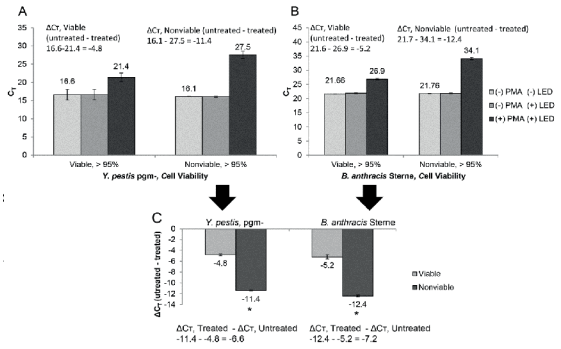

Figure 2: PMA treatment increases the C_T values of nonviable cells. (A) PMA treatment of viable and nonviable *Y. pestis* pgm- (25 µM PMA) and (B) *B. anthracis* Sterne (10 µM PMA) results in a significant shifts in C_T values between treated and untreated samples (ΔC_T). (C)There is a significant difference between the ΔC_T values of viable and nonviable cells, enabling differentiation of viable and nonviable populations. The *symbol indicates a statistically significant difference ($p < 0.05$) when comparing the ΔC_T values of viable and nonviable populations. Among the three independent experiments, the total number of cells per qPCR reaction for *B. anthracis* Sterne ranged from 3.1×10^6 to 3.8×10^6 which corresponded to C_T values of 21.66 to 21.76. For *Y. pestis,* the total number of cells per qPCR reaction ranged from 5.9×10^5 to 6.2×10^5 which corresponded to C_T values of 16.1 to 16.6.

Variable	*Y. pestis* pgm-	*B. anthracis* Sterne
Cell state	Log phase	Log phase, vegetative cells
Cell number in qPCR reaction	5×10^3 to 10^7	5×10^3 to 10^7
Inactivation Method	50% ethanol exposure for 30 minutes	3% H_2O_2 exposure for 60 minutes
PMA Concentration	25 µM	10 µM
PMA Pretreatment Time	5 minutes	5 minutes
Exposure to PMA-Lite™	5 minutes	5 minutes
Assay Buffer	TE	TE

Table 3: Summary of optimized conditions for each model system used in the study, compatible with 0.1 mg/mL concentration of white powders discussed in the te × t.

third was subjected to inactivation methods described in the Materials and Methods. One viable sample was subjected to DNA extraction and resuspended in an equivalent volume as the untreated samples. All samples were then analyzed by qPCR. The C_T value in viable *Y. pestis* pgmd- for the extracted DNA was 15.98 ± 0.09; compared to untreated samples which had C_T values of 16.2 ± 0.02 for the viable sample and 16.41 ± 0.03 for the nonviable sample. Similar results were achieved with *B. anthracis* Sterne. Extracted DNA from viable *B. anthracis* Sterne had C_T value of 18.82 ± 0.02; compared to 19.11 ± 0.01 for the untreated viable sample and 19.19 ± 0.04 for the untreated nonviable sample. Given these results, we determined that the difference between C_T values was not significant in extracted DNA versus cells lysed by direct heat lysis [24] and that a DNA extraction would not be required for either organism used in our study. Lending further support to our decision to eliminate the DNA extraction step can be found in the work done by Nocker et al. which showed that excess PMA (i.e., PMA that did not interact with DNA molecules) is inactivated by reacting with water molecules in solution, prior to DNA extraction, and thus will not affect the DNA from viable cells after cell lysis [14,15].

B. anthracis Sterne spores are expected to be coated in chromosomal DNA because of the interaction with the chromosome of the mother cell which gives rise to the spore [25-27]. For this reason, we postulated that treating spores with PMA would result in the PMA binding to the DNA coating the outside of the spore rather than penetrating to the interior of the spore, and would result in the absence of a qPCR signal. Further, the robust nature of the spore structure would limit the amount of PMA able to enter the spore. Therefore, we expected that treating both viable and nonviable spores with PMA would yield the same result: an absence of a qPCR signal and an inability to differentiate viable from nonviable spores. To test this idea, spores were inactivated using 3% hydrogen peroxide as described in the Materials and Methods to generate nonviable spores. The same number of viable and nonviable spores in each independent experiment were treated with 10 µM PMA with or without five minute exposure to LED light prior to qPCR detection. As shown in (Table 2), we obtained the postulated results for viable and nonviable spores; there is no difference in the qPCR results from viable and nonviable samples. Therefore, we concluded that this assay is not compatible with spores under the tested conditions; spore samples would require germination to a vegetative cell state in order to be compatible with this assay.

Optimizing PMA concentrations for cells

To find the most effective PMA concentration for *Y. pestis*, we tested concentrations of PMA ranging from 5 µM to 25 µM. The number of cells in each sample ranged from 5×10^3 to 1×10^6 CFU/ µL, with most samples containing 10^4 CFU/µL (i.e., approximately 5×10^4 cells per qPCR reaction per 5 µL of sample used in the qPCR reaction). For samples treated with light alone, or PMA alone, there was not a significant difference in C_T values between the viable and nonviable cells (Figure 3A). PMA treatment at 25 µM concentration resulted in the greatest change in magnitude of the PCR signal and was therefore selected as the optimal concentration for *Y. pestis*.

With respect to *B. anthracis* Sterne cells, we observed consistent ΔC_T values in viable and nonviable populations with PMA concentrations between 6.25 µM up to 10 µM (Figure 3B). Neither viable nor nonviable samples could be consistently detected by qPCR with concentrations greater than 10 µM (Figure 3). The difference between the ΔC_T values in viable and nonviable populations were significant ($p < 0.05$) at the 10 µM concentration. Therefore, we chose this highest concentration of PMA, 10 µM, as the treatment concentration for *B. anthracis* Sterne cells in all future experiments.

Discerning viable from nonviable cells in mixed population samples

Mixtures of viable and nonviable cells were used in the PMA-qPCR assay to assess the suitability of PMA treatment in situations with mixed samples. All samples contained the same total number of cells, only the ratio of viable to nonviable cells was adjusted. As shown in (Figure 4), >95%, 10%, 1%, 0.1%, or 0% viable cells were mixed with nonviable cells. For Y. *pestis* pgm- (Figure 4A) and *B. anthracis* Sterne (Figure 4B), the highest ΔC_T values were observed in samples when the majority of the population was comprised of nonviable cells. An analysis workflow depicting the mathematical method for calculating ΔC_T values from raw C_T values is provided in (Figure 2). The assays for both organisms reached a maximal ΔC_T with 1% viable cells, with no significant difference in ΔC_T values from the 0.1% or 0% samples ($p > 0.05$). However, the ΔC_T values were significant ($p < 0.05$) at every tested concentration when compared to the sample with >95% viable cells. The optimized conditions for the PMA assay with the two organisms tested are presented in (Table 3).

Discerning viable from nonviable cells in the presence of white powders.

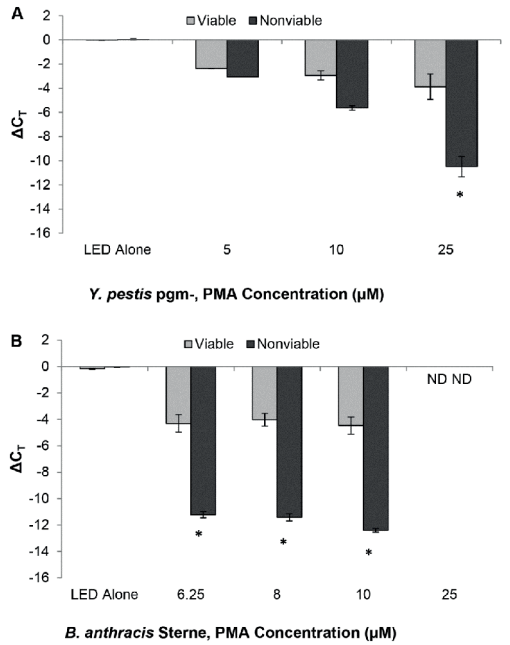

Figure 3: Treatment of *Y. pestis* pgm- with 25 μM PMA and *B. anthracis* Sterne with 10 μM PMA is most effective for distinguishing cell viability. Titration of PMA with (A) *Y. pestis* pgm- cells and (B) *B. anthracis* Sterne vegetative cells followed by a five minute exposure to the LED PMALite™ and detection by qPCR. ND indicates lack of qPCR signal. Cell counts were determined by dilution series plating qPCR reaction. Measurements are the average value of three independent experiments, with three technical replicates in each independent experiment, and error bars are representative of the standard deviation. The * symbol indicates a statistically significant difference ($p < 0.05$) when comparing the ΔC_T values of viable and nonviable populations. For every experiment, the total number of cells was determined by plate counts. Among the three independent experiments, the total number of cells per qPCR reaction for *B. anthracis* Sterne ranged from 5.8×10^3 to 1.33×10^4 which corresponded to C_T values of 26.2 to 27.1. For *Y. pestis,* the total number of cells per qPCR reaction ranged from 6.1×10^5 to 9.8×10^5 which corresponded to C_T values of 15.7 to 16.1.

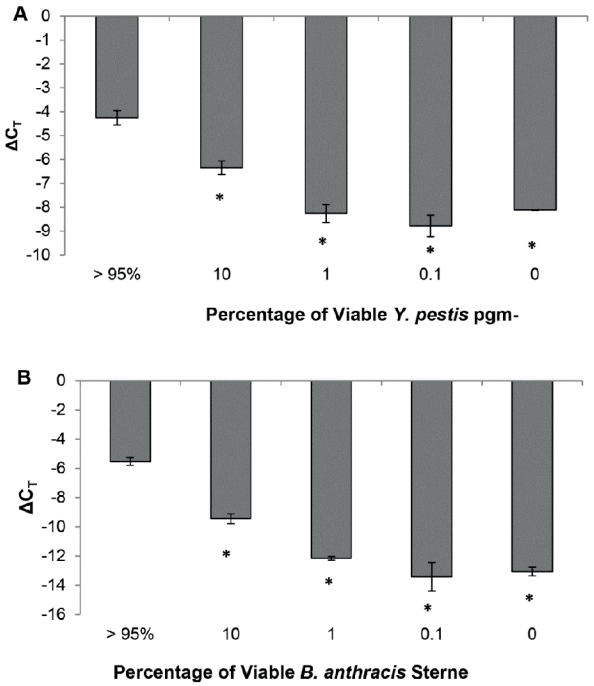

Figure 4: Reduction in C_T values are observed in samples with increasing concentrations of nonviable cells in *Y. pestis* pgm- treated with 25 μM PMA (A) and *B. anthracis* Sterne vegetative cells treated with 10 μM (B). Measurements are the average value of three independent experiments and error bars are representative of the standard deviation. The * symbol indicates a statistically significant difference ($p < 0.05$) when comparing the ΔC_T values to the ΔC_T values of the >95% sample. Among the three independent experiments, the number of cells per qPCR reaction for *B. anthracis* Sterne ranged from was 9.83×10^4 to 1.98×10^5 which corresponded to C_T values of 21.59 to 21.06. For *Y. pestis,* the total number of cells per qPCR ranged from 7.83×10^5 to 1.65×10^6 which corresponded to C_T values ranging from 14.8 to 15.01.

Because intentional release of anthrax spores and stabilized *Y. pestis* could occur in the form of a powder [28], we tested whether or not the PMA assay was compatible with white powders in the sample matrix. The same criterion of ΔC_T value "cut-offs" (refer to equation 1) was used to determine if the white powders interfered with the assay. Using optimized assay conditions (Table 3), we observed ΔC_T values of at least six for both *Y. pestis* pgm- and *B. anthracis* Sterne cells in assay buffer (Figure 3). If similar magnitudes of the ΔC_T value was obtained in samples containing white powders versus samples in buffer alone (i.e., ΔC_T value is ≥ 6), we concluded that there was no interfering effect.

We selected milk powder, baking powder, and coffee creamer in powder form to test the effects of the presence of these powders in the samples at a 0.1 mg/mL concentration. As shown in (Figure 5), the ΔC_T values in both viable and nonviable samples are consistent in the presence and absence of white powders in comparison to samples that did not contain powders. Comparing the ΔC_T values of viable cells to the ΔC_T values for nonviable cells was consistently a magnitude of six or higher for both *Y. pestis* pgm- (Figure 5A) and *B. anthracis* Sterne vegetative cells (Figure 5B), thereby meeting the criteria for simultaneous identification and viability assessment. The data presented in the figures are based on three independent experiments averaged together. For each independent experiment, the ΔC_T (i.e., $\Delta C_{Tnonviable} - \Delta C_{Tviable}$) value was at least 6. However, the ΔC_T values in the three independent experiments ranged from six to 15, thereby resulting in a high standard deviation for this data set. From these data, we determined that this assay is effective even in the presence of the tested white powders.

Discussion

Developing rapid methods for the detection, quantification, and viability determination of *B. anthracis* and *Y. pestis* is relevant to several fields of study, including use in forming decisions about the severity of a bio threat event or the safety of food [7,9,12,18,29]. Two drivers for developing a rapid method that simultaneously identifies bacterial species and determines cell viability are related to situations where there is an environmental exposure or identification of a suspicious white powder. First responders need to know whether the sample contains a biological agent, if the agent is a bio threat, and if so, its viability status. The second driver for a rapid viability detection method is in verifying decontamination of areas such as individual rooms of a building after a bio threat event. After decontamination, one could expect nucleic acid or protein fragments to persist, making it possible for qPCR or immunoassays to yield a positive result; however, the result would most likely be a false positive because the biological material would be nonviable and would not pose a threat to human health. In this case, traditional time consuming methods such as cultivation in a laboratory

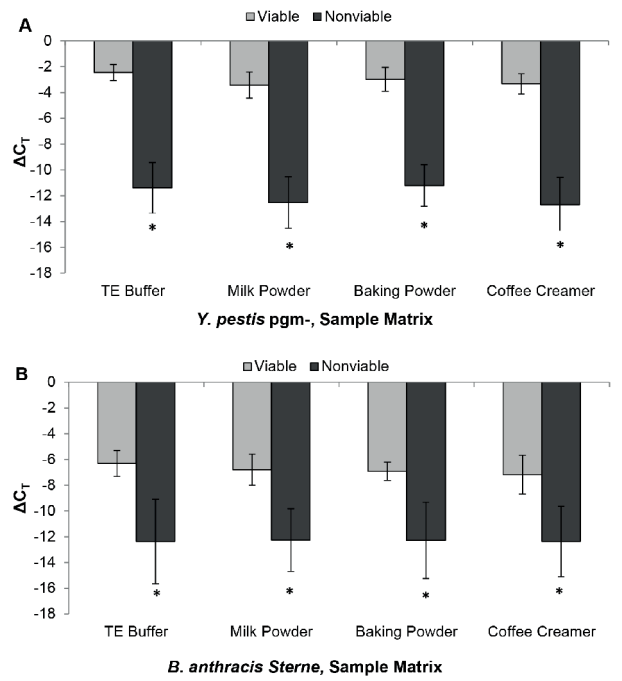

Figure 5: The optimized PMA assay distinguishes cell viability state in the presence of white powders. ΔC_T values remain consistent in viable and nonviable *Y. pestis* pgm- (A) and *B. anthracis* Sterne (B) cells in the presence and absence of white powders. Signal reduction is calculated as the difference in C_T value of a sample that was not exposed to PMA and a sample pretreated with PMA. Measurements are the average value of three independent experiments and error bars are representative of the standard deviation. The * symbol indicates a statistically significant difference ($p < 0.05$) when comparing the ΔC_T values of viable and nonviable populations. Among the three independent experiments, the total number of cells per qPCR reaction for *B. anthracis* Sterne ranged from 8.01×10^4 to 3.97×10^5 which corresponded to C_T values of 21.7 to 22.9 For *Y. pestis,* the total number of cells per qPCR reaction ranged from 1.42×10^6 to 1.99×10^6 which corresponded to C_T values of 14.8 to 14.9.

(which takes 24 hours or more) would be necessary prior to detection.

In this study, we tested the suitability of PMA-qPCR to simultaneously identify and assess the viability of *B. anthracis* Sterne and *Y. pestis* pgm- without DNA extraction and in the presence of white powders. PMA selectively enters nonviable cells, or cells with damaged membranes, and forms covalent bonds with DNA after photoactivation. PMA assays have been developed to assess the viability of food-associated bacterial pathogens including *L. monocytogenes* and *E. coli* O157:H7 [14,30]; however, to the best of our knowledge, this is the first report of a PMA assay for assessing the viability of the bio threat agents *B. anthracis* and *Y. pestis.*

Suitable conditions for PMA treatment and detection of viable and non-viable *B. anthracis* Sterne and *Y. pestis* pgm- cells are summarized in (Table 3). We explored PMA concentration (Figure 3), LED exposure time, and cellular fitness levels for *Y. pestis* pgm- and *B. anthracis* Sterne (data not shown). With respect to cellular fitness, inconsistent results were observed when using overnight cultures that had reached the plateau phase of growth; we identified that consistent results were obtained when cells were in log phase of growth. The consistent change in ΔC_T values between viable and nonviable populations was at least two fold under these conditions. Unfortunately, the method was not suitable for use with *B. anthracis* Sterne spores because viable spores could not be discerned from nonviable spores (see further discussion below). Therefore, the assay is only suitable with cells in a vegetative (i.e., metabolically active) form. Of particular note is that in the more recent of the aforementioned studies [12,31,32], a DNA extraction step was used after PMA treatment to generate the nucleic acid template for the qPCR reaction. In this study, we showed that DNA extraction is not required for obtaining consistent results with the PMA-qPCR assay. The changes in C_T values induced by the PMA treatment were similar to those observed by other groups using DNA extracted from samples as the qPCR template rather than intact cells [12,16,33]. These data further suggest that sufficient lysis is occurring during the qPCR reaction to enable access to the DNA for amplification of viable and nonviable cells, thereby eliminating the requirement for a DNA extraction prior to the detection step. Removing the DNA extraction requirement is a significant time reduction in the methodology, especially with respect to Gram-positive bacteria which typically require up to three hours for DNA extraction. Reduced sample processing for metabolically active (i.e., log phase) cells in this case improves assay reliability and accuracy while reducing labor requirements, cost, and length of time of the assay.

Our findings are consistent with other groups that have used PMA-qPCR in a laboratory setting, with different organisms, demonstrating that nonviable samples had a high C_T shift compared to the equivalent biomass (e.g., CFU) of viable samples after PMA treatment [12,31,32]. As was the case with work done by other groups, the PMA-qPCR assay required evaluation of several variables of the assay for each species used in the study. Specifically, we evaluated the following variables: (1) PMA treatment concentration to ensure a consistent two fold change in C_T values between viable and nonviable samples; (2) biomass (cell number) in each sample; and (3) assay buffer [12,14,16,21,31-33]. From our studies, we also concluded that a PMA-qPCR assay can effectively discern both organism identity and viability in a mixed sample of viable and nonviable cells, with the ΔC_T value increasing as the percentage of nonviable cells increased. We found that for both organisms, the maximal shift in C_T value was obtained in samples in which only 1% of the cells were viable. The assay is sufficiently robust to discern the presence of viable cells in an unknown sample, with the caveat that at least 5×10^3 cells are present in the qPCR reaction.

With respect to evaluation of the PMA treatment concentration, our study is in agreement with the study conducted by Seinige *et al.* with *C. jejuni* which focused on artificially contaminating and then recovering bacteria from chicken leg quarters. They observed that with *Campylobacter* species, the extent of the ΔC_T shift was dependent upon PMA concentration (i.e., increasing PMA concentration increased ΔC_T shifts) even among viable cell populations, indicating that at sufficiently high concentrations, PMA is able to penetrate viable cells [12]. We also observed this type of C_T shift in viable *Y. pestis* pgm- (Table 2 and Figure 3A), but did not observe this with vegetative *B. anthracis* Sterne (Table 1 and Figure 3B). Other bacterial species have similar detection profiles as *Y. pestis* pgm-, such as *L. monocytogenes* and *S. aureus* whereby changes in C_T can occur at higher PMA treatment concentrations in viable samples (e.g., ≥30 μM PMA, data not shown) [14]. In regard to total biomass in the samples used with PMA treatment, we did not observe any significant changes in assay performance between samples containing 10^5 up to 10^8 nonviable cells, however, studies conducted with *Campylobacter* species showed significant changes in assay results in samples containing > 10^4 nonviable cells [12]. The differences between our study and the *Campylobacter* study highlights the need for assay optimization at the level of the species. In this study, we have provided conditions for effective PMA-qPCR viability assay of both *Y. pestis* pgm- and *B. anthracis* Sterne (Table 3).

Common background matrices that have been used in conjunction with the PMA-qPCR assay include human fecal samples with *Bacteroides* [32], raw surface water (untreated river water) and sewage waters with *Cryptosporidium* [31], and recovered solutions from food surfaces with *Campylobacter* [12]. In these previous studies, the application of the PMA-qPCR assay with more complex matrices resulted in difficulties with reproducibility or loss of signal. Matrices generated from recovered food surfaces have been shown to be less problematic in interfering with assay performance compared to wastewaters [34]. Our study indicates that coffee creamer, milk powder, and baking powder do not interfere with the PMA-qPCR viability assay performance (Figure 5). This is a promising result and indicates that the PMA-qPCR assay could be used for screening suspicious white powders. Given that our results show that detection is possible in a complex matrix (i.e., additions of powders to buffers), it is conceivable that further optimization could enable detection of these organisms in biological fluids and recovered rinses from suspected contaminated meat.

The *B. anthracis* lifecycle includes a spore form that is in a dormant state which gives rise to the vegetative cell form. Vegetative cells are metabolically active and, upon gaining access to the host, can produce the toxins causing anthrax infection. The spore form has been proven to be robust and resilient to molecular analysis, highlighting the need for developing a rapid viability assay. Although the presence of whole spores can be detected by qPCR using the tested primer/probe set (Table 1), the qPCR assay coupled with PMA treatment does not provide meaningful information with respect to the viability of the spore (Table 1). Although Rawsthorne et al. demonstrated a C_T shift in *Bacillus subtilis* endospores when subjected to PMA treatment [35], we were not able to replicate these results with *B. anthracis* Sterne spores. We believe there are several reasons for the discrepancy. The first reason is related to the concentration of spores used in our study, which is a relatively small amount of biomass (~15,000 spores). The raw C_T values for DNA extracted from viable spores and spores inactivated with hydrogen peroxide were either not detected or greater than 35 (data not shown). We also attempted autoclaving 10^9 spores and then extracting DNA as was done by Rawsthorne [35], but the extracted DNA from the inactivated samples could not be detected by qPCR either (data not shown). This result is in agreement with the study conducted by Dang et al. which showed that *B. anthracis* Ames spores that were inactivated by autoclaving had significant decreases in signal in qPCR assays [36]. The second issue in using spores with the PMA-qPCR assay is that the assay is dependent upon a significant shift in C_T values between samples treated or untreated with PMA. Because the C_T values of DNA extracted from untreated spores was so high, any shift in C_T would result in an absence of qPCR signal, resulting in a false negative for identification, without which, a viability assessment cannot be conducted. Because spores were incompatible with the PMA assay, a germination step is required to obtain vegetative cells which are compatible with the developed assay.

In conclusion, we optimized a PMA-qPCR assay for rapid identification and viability analysis of *Y. pestis* and *B. anthracis* Sterne vegetative cells, and demonstrated that three different types of white powders do not interfere with the PMA-qPCR assay when conducted in a compatible buffer. However, the assay produces false negative results (i.e., not detected by qPCR) with viable *B. anthracis* spores, and must be conducted with vegetative cells. Therefore, more research is required to overcome the limitations caused by the required germination step to produce vegetative cells in order to use this rapid viability assay for spore identification and viability analysis.

Conclusions

The developed assay enables simultaneous identification and viability assessment for *B. anthracis* Sterne and *Y. pestis* pgm- under laboratory conditions, even in the presence of white powders. Eliminating the DNA extraction step that is typically used reduces total assay time and labor requirements for sample analysis.

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