Validation of Inactivation Procedures for *Bacillus anthracis*

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Abstract

Introduction and objectives: To fulfill Federal Select Agent Program (FSAP) requirements, laboratories must confirm that nucleic acid extracts from *B. anthracis* meet the exclusion criteria found in sections 3(d)(4), 3(d)(5), 4(d)(4), and 4(d)(5) of the Select Agents and Toxins regulations. Extraction inactivates vegetative cells and most viruses, but does not completely inactivate spores. DNA extracts are subsequently filtered through a 0.1 µm filter in accordance with the laboratory's procedures to effectively remove viable cells and spores from nucleic acid extracts. Validation studies were performed to assess the ability of the laboratory's inactivation procedures (extraction followed by filtration) to effectively remove B. anthracis from nucleic acid extracts for use in lower biocontainment facilities.

Methods: These studies were performed using 6 strains: *B. cereus*, commercial suspension of *B. atrophaeus* spores, *B. anthracis* Sterne, *B. anthracis* A0248, *B. anthracis* Pasteur, and *B. anthracis* Ames. All strains were spiked at a high concentration into phosphate buffered saline (PBS), and suspensions were extracted using the QIAamp DSP DNA Blood Mini Kit (QIAGEN), the MagNA Pure Compact (Roche), or by heating for 20 minutes at 95°C. Each extraction technique was followed by 0.1 µm filtration. To evaluate viability, 100% of each filtrate was added to tryptic soy broth (TSB) for 48 hours. Ten percent of this TSB was plated on sheep's blood agar (SBA) and incubated for 2-4 days. To determine the effect of residual lysis buffer and wash solutions on *B. anthracis* growth, PBS was processed using QIAGEN or MagNA Pure Compact extraction procedures followed by 0.1 µm filtration. The extract was spiked with 100-150 cells of *Bacillus cereus* ATCC 11778 and the full volume was immediately plated on SBA plates.

Results: For all 90 samples processed by extraction and filtration, no growth was observed in liquid media, and no colonies were observed on SBA plates. The expected number of colonies were recovered from the spiked elution buffer, indicating that any residual reagents from the extraction process did not interfere with *B. cereus* viability.

Conclusions: These data indicate that the three extraction procedures tested, in combination with 0.1 µm filtration, removed 100% of viable organisms from nucleic acid preparations.

Introduction

To fulfill requirements described in the most recent FSAP Guidance on the "Inactivation or Removal of Select Agents and Toxins for Future Use" (7 CFR Part 331, 9 CFR Part 121.3, 42 CFR Part 73.3 from August 2017), the Biological Rapid Response and Advanced Technology (BRRAT) Laboratory conducted studies to confirm that nucleic acid extractions from *B. anthracis* are safe to transfer to lower containment level laboratories.

There are several methods to extract DNA for use as a template in PCR-based detection assays and, under ideal conditions, the presence of viable organisms in an extract would be a rare event. The regulation states: "If after using the standardized nucleic acid extraction kit, viability is still detected, conduct a separate filtration step using the appropriate pore size filter. Test the filtrate using all of the sample to confirm non-viability during validation. If non-viability is confirmed then this additional step should be added to procedure."

Verification of a selected procedure for nucleic acid extraction meets the requirements outlined in the regulation if the verification study takes into account the following:

- Validation of the procedure is performed in-house
- Validation of the procedure is performed with 100% of the inactivated sample
- Appropriate positive, negative, and process controls are used
- Sufficient experimental replicates are included (*Note: It is each entity's responsibility to determine a sufficient number of replicates.*)
- Bacteria from the same genus can be suitable surrogates
- Start with highest concentration expected as a worst case scenario.

The goal of this study was to validate inactivation procedures and to fulfill the requirements described in FSAP guidelines by demonstrating the effectiveness of laboratory's procedures to inactivate the Tier 1 Select Agent *B. anthracis*.

Methods

Culture: Studies were performed using 6 strains (Table 1). For all strains (except *B. atrophaeus*), glycerol stocks from long-term inventory were recovered on SBA plates. After overnight growth, an isolated colony was suspended in phosphate buffered saline (PBS), and 100 µL of this suspension was streaked onto each of three SBA plates to create bacterial lawns. The following day, cells were harvested into nuclease-free water for the rapid preparation of cell lysate from culture-grown bacteria ("boil prep") procedure or PBS for MagNA Pure Compact and QIAamp DNA DSP Blood Mini Kit (Qiagen) extractions. Cell concentrations in these suspensions were measured using a turbidity meter. Ten-fold dilution series were performed for plating and colony counts, and aliquots were made for extractions.

The *B. atrophaeus* commercial spore prep (VWR) was diluted in PBS and in nuclease free water to 5.0 x10^6 spores/mL, based on the concentration provided in the certificate of analysis. An aliquot of each suspension was diluted and plated to confirm the concentration of viable spores in the commercial spore prep. The final concentrations of bacteria are shown in Table 3.

Table 1. Organisms and Surrogates Tested for Inactivation

Strain	Description
Bacillus cereus ATCC 11778	Gram positive, spore forming bacteria
Bacillus anthracis Sterne	Gram positive, spore forming bacteria
Bacillus atrophaeus ATCC 9372	Purified spore suspension
Bacillus anthracis Pasteur	Gram positive, spore forming bacteria
Bacillus anthracis A0248	Gram positive, spore forming bacteria
Bacillus anthracis Ames	Gram positive, spore forming bacteria

Extractions and filtration (Fig 1.): Three different extraction methods followed by 0.1 µm filtration were used: (1) the MagNA Pure Compact (Roche) automated method using the MagNA Pure Compact Nucleic Acid Isolation Kit in combination with an external lysis procedure, (2) Qiagen, and (3) "boil prep" procedure, which uses heat inactivation (95°C, 20 min) followed by centrifugation at 4°C.

Viability check: Following filtration, 100% of each filtrate was added at a dilution of ≥1:10 to TSB (2 mL) and incubated for 2-4 days at 35°C ± 2°C. Ten percent (220 µL) of the liquid culture was plated onto an SBA plate and incubated for at least 2-4 days.

Controls for validation of inactivation: A negative control (PBS only) was processed with each method and was included with each batch of extractions as an additional sample. Positive controls consisted of each organism spiked into PBS or nuclease-free water and plated directly onto appropriate plates. All controls performed as expected (data not shown).

Matrix control (Table 2): Aliquots of PBS (200 µL each) were processed with extraction and filtration procedures. Filtrates were either plated directly on TSA plates (negative control) to ensure that no contamination occurred or were spiked with 5 µL of *Bacillus cereus* ATCC 11778 (matrix control) and plated to ensure that there are no residual chemicals or additives during the extraction and filtration process that inhibit bacterial growth. To confirm spiking concentration and ensure growth on TSA plates, PBS was spiked with *B. cereus* ATCC 11778 and plated (positive control).

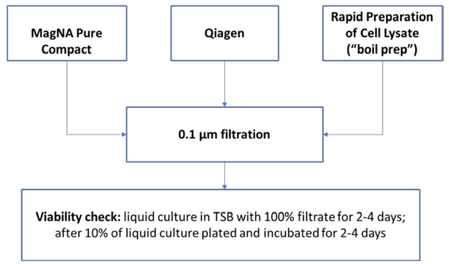

Figure 1. Experiment Workflow

Results

Table 2. Matrix Control per FSAP Guidance

Matrix Controls used to determine the effect of residual buffers on the organism viability (average number of colonies; n= number of replicates)				
Positive control (PBS spiked with *B. cereus* ATCC 11778, no extraction or filtration performed) n=3	**MagNA Pure Compact matrix control** (PBS extracted, filtered and then spiked with *B. cereus* ATCC 11778) n=5	**MagNA Pure Compact negative control** (PBS extracted and filtered) n=1	**Qiagen matrix control** (PBS extracted, filtered and then spiked with *B. cereus* ATCC 11778) n=5	**Qiagen negative control** (PBS extracted and filtered) n=1
124	156	0	160	0

Table 3. Validation of Inactivation Procedures

Strain	Concetration of cells extracted (CFU/mL)	MagNA Pure Compact Plates with growth/ total	Qiagen Plates with growth/ total	Concetration of cells extracted (CFU/mL)	"Boil prep" Plates with growth/ total
Bacillus cereus ATCC 11778	1.3x10^8	0/5	0/5	1.3x10^8	0/5
Bacillus anthracis Sterne	3.2x10^8	0/5	0/5	4.6x10^8	0/5
Bacillus atrophaeus ATCC 9372 spore suspension	3.1x10^6	0/5	0/5	2.1x10^6	0/5
Bacillus anthracis Pasteur	1.9x10^8	0/5	0/5	1.4x10^8	0/5
Bacillus anthracis A0248	1.2x10^9	0/5	0/5	1.1x10^9	0/5
Bacillus anthracis Ames	2.3x10^8	0/5	0/5	2.7x10^8	0/5

Conclusions

- **The BRRAT Laboratory Qiagen extraction procedure followed by 0.1 µm filtration was validated in accordance with FSAP guidance and was found to remove 100% of viable organisms and spores from the nucleic acid extracts tested.**
- **The BRRAT Laboratory MagNA Pure Compact extraction procedure followed by 0.1 µm filtration was validated in accordance with FSAP guidance and was found to remove 100% of viable organisms and spores from the nucleic acid extracts tested.**
- **The BRRAT Laboratory "boil prep" procedure followed by 0.1 µm filtration was validated in accordance with FSAP guidance and was found to remove 100% of viable organisms and spores from the nucleic acid extracts tested.**
- **Residual lysis or wash buffers that may have been present in the Qiagen or MagNA Pure Compact extracts demonstrated no effect on organism viability.**

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