Validation of Inactivation Procedures for Burkholderia and Brucella Species

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Introduction

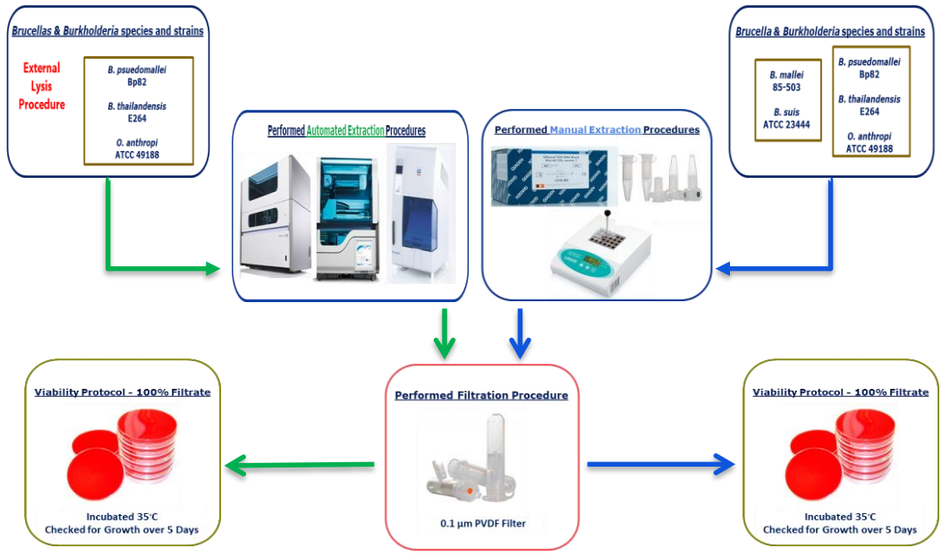
To fulfill the requirements described in FSAP Guidance on the Inactivation or Removal of Select Agents and Toxins for Future Use (7 CFR Part 331.17, 9 CFR 121.3, 42 CFR 73.4), the Biological Rapid Response and Advanced Technology (BRRAT) Laboratory conducted experiments to confirm that nucleic acid extractions from the genera *Burkholderia*, *Brucella*, and a near neighbor met the exclusion criteria found in sections 3(d)(4), 3(d)(5), 4(d)(4), and 4(d)(5) of the SAR. Previously published work showed the ability of commercial manual and automated extraction kits to effectively remove *Bacillus anthracis*, *Yersinia pestis,* and *Francisella tularensis* from nucleic acid preparations[1-4]. Marques *et al*., showed that six commercially available DNA extraction kits (QIAamp Mini Kit, Easy-DNA Kit, ChargeSwitch gDNA, Bilatest Genomic DNA Kit, UltraClean Microbiol DNA Kit and High Pure PCR Template Preparation Kit), and thermal lysis and proteinase K DNA extraction were effective to inactivate viable *Burkholderia pseudomallei* strain No589 (ATCC 23343). *Brucella* spp., including *B. abortus*, *B. suis*, and *B. melitensis*, were shown to be inactivated after standard heating (e.g., 95°C for 15 minutes) or chemical treatment, including formalin and methanol inactivation[6]. Spiked serum containing *B. abortus*, *B. melitensis*, and *B. suis* were also shown to be nonviable after passing through a 0.22 µm filter[6]. Additionally, other laboratory methods, such as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, have also incorporated a filtration step with highly virulent bacteria, including *B. anthracis*, *B. mallei* , *B. pseudomallei*, *Brucella* spp., *Francisella tularensis*, and *Yersinia pestis,* to ensure all viable organisms were removed from sample extracts[7-8].

Objective

The objective of this study was to determine if three automated and three manual extraction procedures followed by 0.1 µm filtration would generate nucleic acid extracts that were free of viable, vegetative cells, which were thus safe to transfer to lower level containment laboratories. Inactivation procedures tested included three automated nucleic acid extraction platforms (MagNA Pure 24, MagNA Pure 96, and Qiagen EZ1 Advance XL) using three surrogate organisms (*B. thailandensis*, avirulent *B. pseudomallei* strain Bp82, and *Ochrobacrum anthropi)* and three manual extraction procedures (QIAamp Qiagen DSP Blood Mini Kit, rapid preparation of cell lysates ["boil preps"], and Filter Capture DNA Isolation [FCDI][5]) using *B. thailandensis*, *B. pseudomallei* (Bp82), and *O. anthropi*, and the two select agents *B. mallei* and *B. melitensis*.

Methods

Figure 1: Automated and Manual Extraction and Filtration Workflow



Culture and Quantification of Bacterial Suspensions

- A pure, overnight culture on chocolate agar (CHOC) or Tryptic Soy Agar with 5% sheep blood agar (SBA) were used to prepare a bacterial suspension in phosphate buffered saline (PBS), inoculum saline, urine, or nuclease-free (NF) water. Tenfold serial dilutions were prepared and plated on CHOC or SBA in triplicate and incubated overnight at 35°C ± 2°C . Colonies were counted and used to calculate the bacterial concentration of the original bacterial suspension.

Extraction and Filtration (Figure 1)

- The following procedures followed by 0.1 µm filtration were tested: (1) MagNA Pure 24 (Roche) automated method using the MagNA Pure 24 Total Nucleic Acid Isolation Kit in combination with 3 external lysis procedures; (2) MagNA Pure 96 (Roche) automated method using the MagNA Pure 96 Small Volume Total Nucleic Acid Isolation Kit in combination with 3 external lysis procedures; (3) EZ1 Advanced XL (Qiagen) automated method using the EZ1 XL blood and serum kit in combination with a heat lysis and proteinase K procedure; (4) QIAamp DNA DSP Blood Mini Kit (Qiagen); (5) Rapid preparation of cell lysates ("boil prep") procedure, which uses heat inactivation (95°C, 20 min) followed by centrifugation at 4°C; and (6) Filter Capture DNA Isolation[5].
- External lysis procedures: 200 µL of sample were added to 250 µL of the following lysis buffers and incubated for 15 minutes before proceeding to automated extraction on the MP24 and MP96: (1) MagNA Pure Bacteria Lysis Buffer (Roche); (2) MagNA Pure LC DNA Isolation Kit I - Lysis/Binding Buffer (Roche); (3) MagNA Pure 96 External Lysis Buffer (Roche).

Viability check

- 100% volume of filtered sample nucleic acid extracts were spread plated on CHOC or SBA and checked daily for bacterial growth over a five-day incubation period at 35°C ± 2°C . Nucleic acid extracts were confirmed to be inactivated if no growth of any colonies was observed.

Controls

- One negative control (PBS, saline, urine, or NF water) was processed with each extraction as an additional sample. Growth on the negative control plate indicated potential reagent contamination, rendering filtered sample nucleic extracts processed with the negative control invalid.
- Positive controls consisted of each organism spiked into PBS, saline, or NF water and plated directly onto CHOC or SBA. Bacterial growth confirmed organism viability; colony morphology consistent with that of the organism plated confirmed culture purity.

Inhibition

- A matrix control using extraction elution buffer was included per each automated and manual extraction procedure. Each organism was spiked into elution buffer and concentration (in CFU/mL) was confirmed in parallel via colony count. No growth on the matrix control plate indicated potential inhibition by the buffers from the extraction methods, rendering filtered sample nucleic extracts processed with the matrix control invalid.

Results

Organism	Strain	Concentration 10^8 CFU/mL or greater	Qiagen†	Rapid Preps†	FCDI†	MP96†	MP24†	EZ1†	200 uL Heat Inactivation Followed by Filtration	Inihibition from Elution Buffers?	Sterility/Viability Test Status for All Procedures?
Burkholderia thailandensis	E264	Yes	0/5	0/5 broth 0/1 agar	0/5	0/12 (BLB) 0/12(96LB) 0/12 (LCLB)	0/5 (BLB) 0/5 (96LB) 0/6 (LCLB)	0/5	0/5	Manual - No EZ1 - No MP24 - No MP96-No	Pass
Burkholderia psuedomallei	Bp82	YesΔ	0/5	0/5 broth 0/5 agar	0/5 saline 0/5 urineΔ	0/5 (BLB) 0/5 (96LB) 0/5 (LCLB)	0/5 (BLB) 0/5 (96LB) 0/5 (LCLB)	0/5	0/5	Manual - No EZ1 - No MP24 - Not performed MP96 - Not performed	Passr
Burkholderia mallei	85-503	Yes	0/5	0/5 broth 0/5 agar	0/5	not performed	not performed	not performed	not performed	No-manual only	Passr
Brucella suis	ATCC 23444	Yes	0/5	0/5 broth 0/5 agar	0/5	not performed	not performed	not performed	not performed	No-manual only	Passr
Ochrobacrum anthropi	ATCC 49188	Yes	0/5	0/5 broth 0/1 agar	0/5	0/5 (BLB) 0/5 (96LB) 0/5 (LCLB)	0/5 (BLB) 0/5 (96LB) 0/5 (LCLB)	0/5	0/5	Manual - No EZ1 - No MP24 - No MP96-No	Pass

†: Number of Replicates with Growth/Total Number of Replicates
Δ: FCDI in urine matrix only 10^7 CFU/mL achieved
€: 200 µL @ 95°C for 20 minutes + 56°C proteinase K for 15 minutes followed by EZ1 extraction
r: excludes 200 uL heat + filtration procedure

Conclusions

- **Viability testing using 100% volume of the filtered nucleic acids from each organism demonstrated that all four procedures in combination with filtration completely inactivated or removed any live bacteria.**
- **High bacterial load (10^8 CFU/mL or greater) did not impact the procedures' ability to inactivate the bacterial species tested.**
- **Viability testing was not inhibited by the extraction elution buffer in each method.**

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