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SURFACE AND AIR SAMPLING VALIDATION FOR MILLING OF BSL-2 *BACILLUS ANTHRACIS* SIMULANT SPORES

Disclaimer

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RESEARCH AND TECHNOLOGY DIRECTORATE

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14. ABSTRACT:
Biosafety level (BSL) 1 spore-forming bacteria, such as *Bacillus atrophaeus* var. *globigii*, have been traditionally used as surrogate organisms for *B. anthracis* (Ba). There is an increased interest in using the attenuated strain(s) of Ba. The large-scale production of these attenuated strains raises a number of safety concerns about the possible aerosolization and particulate generation of micron-range-sized spores during the drying and milling processes. In this effort, a milling system was placed within a Plexiglas enclosure, and a milling operation was conducted using BSL-1 barcoded *B. thuringiensis* var. *kurstaki* (Btk) to determine whether the engineering controls of the system and the operating procedures would be sufficient to prevent any spillage from BSL-2 materials. To evaluate the integrity of the mill and the enclosure, air and surface samples were tested to learn whether background and BSL-1 Btk barcoded spores could be detected before and during milling operations performed within a dedicated clean-room enclosure. After comparing the similar physical properties of BSL-1 barcoded Btk dried spores and BSL-2 spores, we determined that the milling of Ba Sterne/ Δ Sterne spores under identical engineering controls would not require additional containment measures.

15. SUBJECT TERMS

Bacillus anthracis (Ba) Sterne	Δ Sterne	Simulant
Biosafety level 1 (BSL-1)	Surface sampling	Air sampling
Bacillus thuringiensis var. *kurstaki* (Btk)	Milling process	Drying process

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PREFACE

The work described in this report was authorized under project number 1632G HSHQPM-16-X-00219 DHS. The work was started in November 2017 and completed in February 2018.

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- Members of the ECBC Biosafety Committee for their consideration and approval to include *Bacillus anthracis* (Ba) surrogates (Ba Sterne and Δ Sterne spores) in our revised milling process.

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EXECUTIVE SUMMARY

Biosafety level (BSL) 1 spore-forming bacteria such as *Bacillus atrophaeus* var. *globigii* (BG) have been traditionally used as surrogate organisms for *B. anthracis* (Ba). There has been an increased interest in the use of the attenuated strain(s) of Ba. However, the large-scale production of these attenuated strains gives rise to a number of safety concerns about the possible aerosolization and particulate generation of micron-range-sized spores during the drying and milling processes. In this effort, a milling system was placed within a Plexiglas enclosure that was constructed in conjunction with an existing chemical fume hood. A milling operation was then conducted using dried BSL-1 barcoded *B. thuringiensis* var *kurstaki* (Btk) spores to determine whether the system and operating procedures would have sufficient engineering controls to prevent any spillage from future BSL-2 materials. To evaluate the integrity of the mill and the enclosure, air and surface samples were taken to establish whether background and BSL-1 Btk barcoded spores could be detected before and during milling operations performed within a dedicated clean-room enclosure. After considering the similar physical properties between dried BSL-1 barcoded Btk and BSL-2 spores, our results showed that the milling of Ba Sterne and Δ Sterne spores would not require additional containment measures under identical engineering controls.

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1. INTRODUCTION

Bacillus anthracis (Ba) is a well-known biothreat agent (*1–4*) because of its high virulence and ability to form sturdy and persistent spores that can survive for decades in certain environments (*5*). Traditionally, biosafety level (BSL) 1 nonpathogenic spore-forming bacteria such as *B. atrophaeus* var. *globigii* (BG) have been used as surrogate organisms to simulate Ba (*6, 7*). Genetically tagged BSL-1 barcoded *B. thuringiensis* var. *kurstaki* (Btk) has been chromosomally altered (*8*) to enable better detection of a simulant of interest among background Bacillus species already present in the environment (*8*).

Parallel to the effort to develop a BSL-1 simulant for Ba, there is much interest in using attenuated Ba strains (e.g., Ba Sterne and Δ Sterne) to study their effects on the environment (*9*) and their susceptibility to γ irradiation for inactivation (*10*). The Sterne strain (named after a South African researcher) is a benign form of Ba, as it has naturally lost the pXO2 plasmid that encodes the capsule (i.e., a layer of polysaccharides outside the cell wall that protects the bacteria against phagocytosis). Therefore, the Sterne strain does not have the ability to produce a capsule, and consequently, it is widely used for research. The Ba Sterne strain produces sublethal amounts of the toxin that induces the formation of a protective antibody. Thus, this strain is used in preparations of vaccines against virulent Ba. The Δ Sterne strain was derived from the Sterne strain by further curing the pXO1 plasmid that produces sublethal amounts of the toxin.

The attenuated strains could serve as better simulants for Ba. This study was performed in anticipation of future work including a 1000 L scale-up production of Ba Sterne and Δ Sterne spores and downstream processing that includes spray-drying and milling as a part of the U.S. Department of Homeland Security (Washington, DC)-funded Biowatch program at the U.S. Army Edgewood Chemical Biological Center (ECBC; Aberdeen Proving Ground, MD). However, handling and large-scale production of the attenuated strains were met with healthy doses of safety concerns, particularly at the drying and milling stages because of possible aerosolization and particulates of micron-range-sized spores. Hence, in this report, we discuss the air and surface sampling results of our testing with dried BSL-1 barcoded Btk spores before and during a milling operation in a dedicated clean-room enclosure. In addition, we tested the ability of the mill and the enclosure to contain the product. The data gathered in this study was presented to the ECBC Biosafety Committee to promote the milling of Ba Sterne and Δ Sterne spores in future projects.

2. MATERIALS AND METHODS

2.1 Materials

The materials that were used during testing are listed in Table 1.

Table 1. Materials Used during Testing

Material	Manufacturer	Model No.	Dimensions (in.)
Plexiglas enclosure	Simplex Isolation Systems; Fontana, CA	N/A	186 × 94 × 60
Fume hood	Lab Fabricators Company; Cleveland, OH	N/A	60 × 72 × 48
BSL-1 organism	N/A	N/A	N/A
Barcoded Btk	ECBC	1.16.4	N/A
HEPA filters	Dayton Electric Manufacturing Company; Niles, IL	6B614	24 × 24 × 11.5
3M sponge stick	3M; Maplewood, MN	SSL10NB	N/A
Neutralizing buffer	N/A	N/A	N/A
Phosphate buffer	3M	FTPHB9066	N/A
TSA plates	Fisher Scientific; Hampton, NH	FB0875712	N/A
L shape spreader	Thomas Scientific; Swedesboro, NJ	1221X64	N/A
SMA portable air sampler	Veltek Associates, Inc.; Malvern, PA	SMA-P190-C	6 × 10
Variable rate auger feeder	AccuRate; Kansas City, MO	580-351300	17 × 15 × 13
Exhaust filter bag	Sturtevant; Hanover, MA	BAG10X54 SPGS	12 × 60
Incubator	VWR International; Radnor, PA	3025	16 × 38 × 24
Autoclave	TOMY; Tokyo Japan	SS-325	40 × 19 × 22

N/A: not available.
HEPA: high-efficiency particulate air.
TSA: tryptic soy agar.
SMA: sterilizable microbial atrium.

2.2 Methods

2.2.1 Description of Enclosure

A Plexiglas enclosure was constructed over two existing chemical fume hoods for powder processing and to isolate airflow into the chemical fume hood from the general laboratory area. Room air was passed through four HEPA filters before it was drawn into the chemical fume hood, which caused the air pressure to drop as air flowed from the hallway through the room to the enclosure, and eventually, into the chemical fume hood.

2.2.2 Surface Sampling and Locations

Surface samples were taken before and during the milling operations. Each surface sampling was performed over 100 in.^{2} of area with a 3M sponge stick that was premoistened with 10 mL of neutralizing buffer (in accordance with the manufacturer's recommendations). After swabbing was completed, each sampling head sponge was aseptically broken off into a dilution bottle containing 90 mL of sterile phosphate buffer. A field blank (FB; no swabbing) was also sampled as a negative control. A volume of 100 mL, including the FB and 10 mL of each original sample from the 100 in.^{2} sampling areas, was further serially diluted to 10^{-2} and 10^{-3}. Then, 0.1 mL of each dilution was plated onto a TSA plate using an L shape spreader. Each plate was placed in the incubator at 37 °C for 24 h. Figure 1 shows four sampling locations within and out of the enclosure. Sampling location designations are presented in Table 2.

Table 2. Sampling Locations

Sample	Location
S1	Outer glass of chemical fume hood sash
S2	Floor immediately in front of the chemical fume hood
S3	Floor immediately inside the door of the enclosure
S4	Floor outside the enclosure

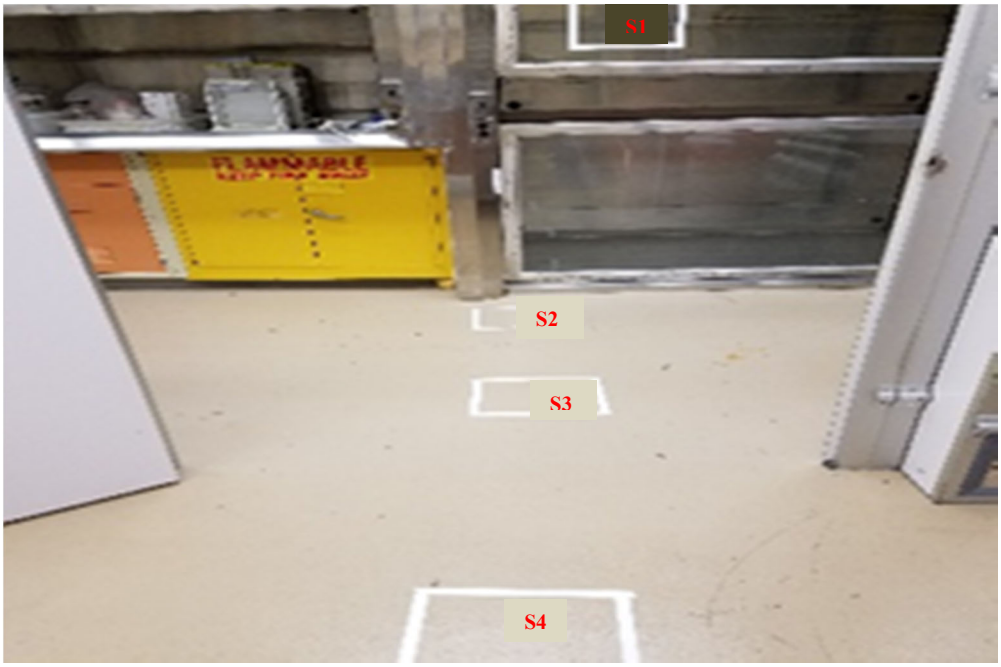

Figure 1. Surface sampling locations. (Perimeters were highlighted with white tape, S4 is in the foreground, and S1 is on the outside glass of hood sash.)

2.2.3 Air Sampling

Air sampling was accomplished using an SMA microportable air sampler. The perforated stainless steel sampling head and cover of the air sampler were autoclaved at 121 °C for 20 min before each use. A TSA plate was inserted into the sampling head, which was covered when it was placed at the sampling location inside the biosafety hood. The cover was removed when the unit was activated. Air was collected for 1 h at 1 cfm (cubic feet per minute). As soon as approximately 1800 L of air sample was exposed to the TSA plate, the sampling head was recovered and replaced in the biosafety hood. The TSA plate was removed and incubated at 37 °C for 24 h, which allowed for any colony development.

Figure 2 shows the location of the air sampler on the floor before the mill was installed in the enclosure, and Figure 3 shows the location of the air sampler during the milling operation. In Figure 3, the air sampler was placed close to the milling apparatus so that potential leaks from critical components of the mill could be easily detected.

Figure 2. Location of air sampler before mill installation in enclosure.

Figure 3. Location of air sampler during milling operations.

2.2.4 Milling Operations

Simulation of milling a BSL-2 organism was achieved by milling a BSL-1 organism of previously milled barcoded Btk inside the built-in enclosure (*8*) (Figure 4).

Figure 4. Milling operation setup with approximately 500 g of barcoded Btk spores.

The variable rate auger feeder was filled with approximately 500 g of dried barcoded Btk spores in the chemical fume hood. The feeder was sealed around the cover and auger feed opening with duct tape before it was connected to the mill for processing. The feeder was set at a constant 20% of the maximum feed rate for 1 h at 100 psi of both inlet and grinding pressure. Micronized particles were directed through 1.5 in. long stainless steel piping into the exhaust filter bag located within the chemical fume hood for collection.

3. RESULTS AND DISCUSSION

Before the milling process was started, initial surface samples (1st swabbing in Figure 5) along with a FB were taken to establish a baseline level of organisms inside and outside the built-in enclosure.

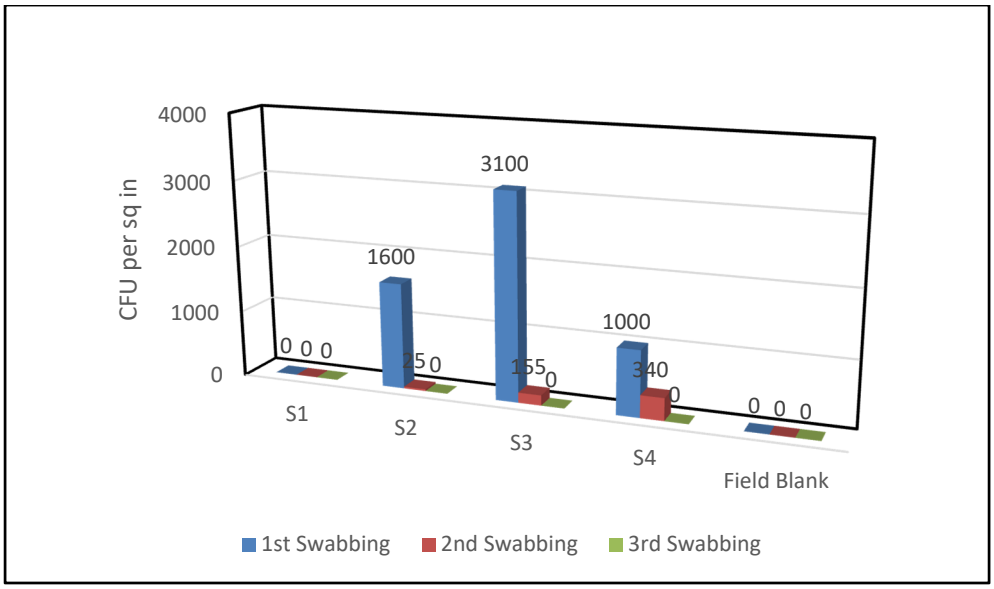

Figure 5. Surface sampling before milling.

As expected, a relatively high level of contaminant organisms that resembled the *Bacillus* species from TSA colony morphology (data not shown) were detected in the samples taken from sample locations S2 through S4. However, these were not present in location S1 or the FB. Detection of organisms in locations S2 and S3 may be attributed to the previous processing of BSL-1 organisms through spray-drying and milling without a thorough cleaning. On the other hand, the detection of organisms in location S4 (outside the enclosure) was unexpected because the air pressure in the enclosure was negative, as compared with the air pressure in the room. It is possible that foot traffic in and out of the enclosure may have contributed to the presence of organisms in location S4 if the surface floor inside the enclosure (e.g., S2 and S3) was already contaminated prior to testing.

The location areas were not cleaned, and a second set of samples was taken to further determine swab sampling efficiency (second swabbing in Figure 5). Although the results

of the second swabbing were much lower, residual contaminant levels in locations S2, S3, and S4 of 25, 155, and 340 cfu/in.^{2}, respectively, indicated that swabbing may not have resulted in 100% recovery. Air sampling was needed to determine if there had been any further settling of air-borne contaminants between each swabbing. In the second swabbing, the FB and location S1 samples were again negative, which confirmed that, if present, contaminants are more likely to be found on horizontal rather than vertical surfaces. In addition, Stoke's terminal settling velocity of barcoded Btk spores was estimated at 1 cm/min. This estimate was based on the previously determined average size of the barcoded Btk spores of 3–5 µm and density of 0.3 g/cm^3 (data not shown). The assumed sphere of particles further indicated that settling during the six days between the first and second swabbing was unlikely to be the contributing factor for the residual contaminant level in the second swabbing. Rather, the results from the swabbing technique may have indicated <100% recovery.

Then, the inside and outside areas of the enclosure were cleaned and disinfected with a 10% bleach solution for a contact time of 30 min to properly test the effect of milling for any contaminant level in the enclosure. After the cleaning, a third swabbing was performed. The results from the third swabbing (Figure 5) indicated the effectiveness of cleaning with 10% bleach; negative results were achieved in all the samples. In addition, after the cleaning with 10% bleach, the first air sampling (Figure 6) showed no airborne contaminants. Therefore, having established a clean environment inside and outside the enclosure, we set up a mill inside the enclosure and operated it as described in Section 2.2.

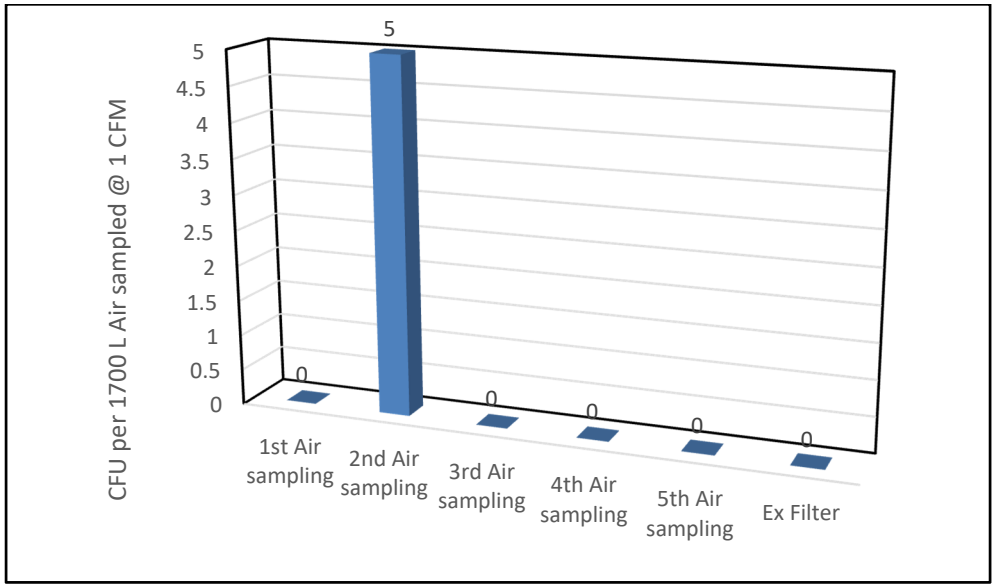

Figure 6. Air sampling before and during milling operations.

Surface samples for the fourth swabbing during milling (Figure 7) and air samples for the second air sampling at the end of milling (Figure 6) were collected. The samples from locations S2 and S3 showed positive results of 5.5 and 14.5 cfu/in.^{2}, respectively. The air sampling result at the end of the milling process was positive and showed 5 cfu/1800 L of air.

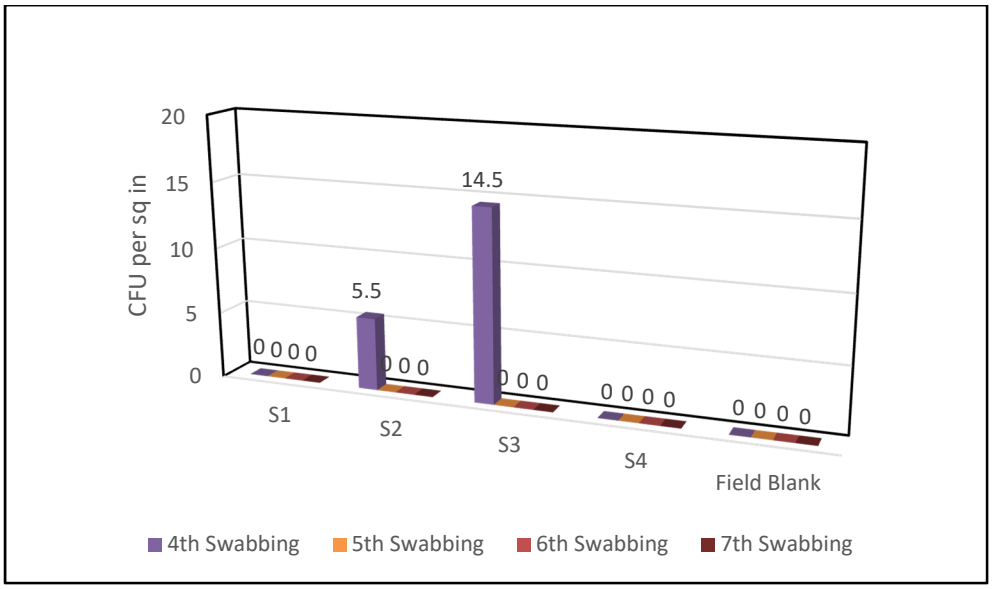

Figure 7. Surface sampling during milling operations.

Upon review of the procedures, it became apparent that the brief removal of the feeder lid and aid of the auger mechanism with a disposable spatula may have contributed to the spore spillage inside the enclosure. Thus, to ascertain and remedy the contaminant inside the enclosure, the inside and outside of the enclosure were again treated with 10% bleach solution to re-establish a clean environment. The samples from the fifth swabbing (Figure 7) and third air sampling (Figure 6) indicated negative results. Subsequently, the identical milling setup and operating conditions were repeated under a closed system throughout the testing period. During milling, all openings were sealed to prevent any leakage of spores being processed through the mill. With these measures in place, the sampling results from the surface during milling (sixth swabbing in Figure 7) and air at the end of milling (fourth air sampling in Figure 6) were negative. Hence, it became apparent that milling must be performed in a closed system. Negative results for the seventh swabbing (Figure 7) and fifth air sampling (Figure 6) were obtained after the area was again treated with 10% bleach after the milling operation. Furthermore, the effectiveness of the exhaust filter collection bag inside the chemical fume hood was tested by exposing a TSA plate for approximately 30 s directly next to the filter bag. The TSA plate showed negative results (Figure 6), which indicated that milled spores were effectively being captured inside the collection bag.

4. CONCLUSIONS

A milling system was set up inside a built-in Plexiglas enclosure in conjunction with an existing chemical fume hood. BSL-1 barcoded Btk spores were tested to determine whether the setup and procedures would be sufficient to provide engineering controls to prevent any spillage of potential candidates of dried BSL-2 materials. Surface and air sampling were performed during and at the end of the milling period to demonstrate the feasibility of milling barcoded Btk without any spillage as long as the milling setup remained “closed” to the enclosure and exhaust was released into a chemical fume hood. Because of the similar physical

properties attributed to dried BSL-1 barcoded Btk spores and BSL-2 Ba Sterne or Δ Sterne spores, milling of BSL-2 Ba Sterne or Δ Sterne under identical engineering controls will not require additional containment measures.

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ABBREVIATIONS AND ACRONYMS

Ba	*Bacillus anthracis*
BG	*Bacillus atrophaeus* var. *globigii*
BSL	biosafety level
Btk	Bacillus *thuringiensis* var. *kurstaki*
DHS	U.S. Department of Homeland Security
ECBC	U.S. Army Edgewood Chemical Biological Center
FB	field blank
HEPA	high-efficiency particulate air
SMA	sterilizable microbial atrium
TSA	triptic soy agar

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