U.S. ARMY COMBAT CAPABILITIES DEVELOPMENT COMMAND
CHEMICAL BIOLOGICAL CENTER
ABERDEEN PROVING GROUND, MD 21010-5424

DEVCOM CBC-TR-1785

Decontamination of *Bacillus anthracis* Spores on Military Working Dog Skin

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July 2022

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
XX-07-2022	Final	Jan 2021–Aug 2021

4. TITLE AND SUBTITLE
Decontamination of *Bacillus anthracis* Spores on Military Working Dog Skin

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Rastogi, Vipin K.; Katoski, Sarah (DEVCOM CBC); Cross, Orshuntis (ORISE); and Leija, Brianna M. (Excet)

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Director, DEVCOM CBC, ATTN: FCDD-CBR-BD, APG, MD 21010-5424
Oak Ridge Institute for Science and Education; 4692 Millennium Dr., Suite 101, Belcamp, MD 21017-1543
Excet, Inc.; 6225 Brandon Ave., Suite 360, Springfield VA 22150-2519

8. PERFORMING ORGANIZATION REPORT NUMBER
DEVCOM CBC-TR-1785

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Office of the Surgeon General, U.S. Department of Health and Human Services; 200 Independence Ave. SW, Humphrey Bldg., Suite 701H, Washington, DC 20201-0004
U.S. Army Public Health Center; 8252 Blackhawk Rd., APG, MD 21010-5403

10. SPONSOR/MONITOR'S ACRONYM(S)
OSG DHHS; APHC

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release: distribution unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT: (Limit 200 words)
Military working dogs (MWDs) are deployed in conflict regions for the detection of chemical, biological, radiological, and nuclear threat materials. Protocols such as rinse–wash–rinse and chlorhexidine sponge wipe-downs for MWD skin bio-decontamination rely largely on mechanical removal and not on inactivation of *Bacillus anthracis* (Sterne strain) spores. As a result, viable spores remain in high number on MWD skin. Wipes soaked in 5% Bioxy (Atomes, F.D.; Quebec City, Canada) were tested for effective inactivation of the spores. Shortening MWD fur length was also tested. Relative to just a 1–1.5 log reduction in the number of spores with the rinse–wash–rinse and chlorhexidine sponge wipe-down protocols, Bioxy wipes and fur length shortening resulted in a 3–4 log reduction. Efforts are ongoing to enhance spore inactivation using other commercial wipe types and wipes soaked with 10% Bioxy.

15. SUBJECT TERMS

Bacillus anthracis	Military working dog (MWD)	Spore
Bioxy	Bio-decontamination	Chlorhexidine

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Renu B. Rastogi
U	U	U	UU	20	19b. TELEPHONE NUMBER *(include area code)* (410) 436-7545

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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PREFACE

The work described in this report was authorized under project PHC. The work was started in January 2021 and completed in August 2021.

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This report has been approved for public release.

Acknowledgment

The authors acknowledge the technical help of Savannah Hurst (U.S. Environmental Protection Agency; Fort Meade, MD) in the early phase of the project.

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DECONTAMINATION OF *BACILLUS ANTHRACIS* SPORES ON MILITARY WORKING DOG SKIN

1. INTRODUCTION

Military working dogs (MWDs) actively assist soldiers in the field by performing operations such as search and rescue, scouting, and threat detection. These roles are highly focused on the MWD's ability to detect targets by smell, thus limiting its use of protective equipment.[1] This limitation restricts the use of MWDs in areas contaminated by chemical, biological, radiological, and nuclear (CBRN) threats.[1] However, it is assumed that the MWD plays a critical role in early detection of CBRN agents. With respect to the release of biological warfare (BW) agents, such as spores of *Bacillus anthracis*, MWDs are particularly susceptible to aerosol-based dispersion as they operate low to the ground where aerosols settle and prevail.

MWD fur can initially serve as a barrier to dermal infection. Varied susceptibility reduces symptom severities associated with many common BW agents such as spores of *B. anthracis*, which is the causative pathogen for anthrax. Unfortunately, contaminated MWDs can pose a risk to their handlers by serving as carriers or as sources of transmission for zoonotic infection. Existing decontamination protocols (the rinse–wash–rinse and chlorhexidine wipe-down methods) for MWD skin are focused on full technical decontamination.[2,3] The rinse–wash–rinse method is water-intensive, and the chlorhexidine wipe-down method is generally used when water is unavailable. The rinse–wash–rinse process uses a high volume of low-pressure, soapy water to remove the contaminant.[6] The contaminated runoff is drained to mitigate the risk of ingestion. However, washing can push contaminants deeper through the fur and directly onto the skin surface. In addition, over-washing can compromise the integrity of the skin barrier through drying and removal of natural skin oils.[3] Hypochlorite can be used as an added decontaminant during bathing, but the rinse–wash–rinse process primarily focuses on mechanical removal.

Anthrax, the pathogenic Ames strain of *B. anthracis,* is a notoriously efficient spore-forming, Gram-positive bacterium. Ames spores are easily aerosolized, persistent, and highly infectious via inhalation and the cutaneous and gastrointestinal routes of exposure; thus, the strain is an ideal BW agent. After the 2001 "Amerithrax" attack, the DOD and Centers for Disease Control and Prevention (Atlanta, GA) elevated anthrax to a Tier 1 select agent. Therefore, to date it remains a significant focus of bio-decontamination efforts. In this study, *B. anthracis* Sterne (BaS) was used as a surrogate for Ames to evaluate the efficacy of decontamination protocols used to neutralize the risks posed by BW agents (spores) to MWDs. We compared the efficacy of the rinse–wash–rinse process to the chlorhexidine wipe-down method and assessed their effectiveness in decontaminating different lengths of MWD fur.[4] Drawbacks to the rinse–wash–rinse method include high water usage, focus on mechanical removal of the agent rather than inactivation or neutralization of agent, and lack of rapid and effective interim gross decontamination while away from decontamination tents.[2,3]

As an alternate to using chlorhexidine sponges and the rinse–wash–rinse method to reduce the number of spores on dog skin surface, we used Bioxy, a powder disinfectant

(Atomes, F.D.; Quebec City, Canada) that generates peracetic acid and peroxide when dissolved in water. A 5–10% solution has been documented as a sporicidal on hard surfaces.[5] Wipes soaked in 5% Bioxy were included as test wipes for decontamination of dog skin in this study.

2. METHOD

2.1 BaS Spore Preparation

BaS was streaked onto a tryptic soy agar (TSA) plate from a frozen culture collection (lot 032607). Plates were incubated at 37 °C for 24 h. We used 1 or 2 single colonies from the TSA plate to inoculate a 20 mL tryptic soy broth. The overnight broth culture was spread onto four Lab Lemco plates. Spores from the first sporulation set were assessed to determine the number of vegetative cells. When the microbial growth on the plates showed ~95% sporulation, spores were harvested the next day. The plates were washed three times with sterile water to harvest clean spores.

Harvested spores from the plates were filtered through an autoclaved paper towel and then centrifuged at 5000 rpm for 2 h. The clear supernatant was aspirated, and the pellet was washed with 40 mL of sterile water. The suspension was centrifuged again for 1 h at 5000 rpm. The clear supernatant was aspirated, and the pellet was washed with 40 mL of 0.01% Tween 80. The suspension was centrifuged again and the supernatant was aspirated. The pellet was resuspended in 30 mL of 0.01% Tween 80 and stored at 4 °C prior to being heat-shocked. The spore suspension was heat-shocked at 65 °C for 30 min to eliminate any viable vegetative cells. The stock was sonicated for 5 min and vortexed for 2 min to break up any spore clumps. The stock culture was enumerated and stored at 4 °C for up to 12 weeks.

As analyzed under the microscope, spore numbers were >95% with negligible vegetative cell burden. Based on the number of colony-forming units (cfu) on the enumeration plates, the stock titer averaged ~3.6E9 cfu/mL. A working stock was prepared by adding 10 mL of ~4.0E9 cfu/mL parent stock to 90 mL of 0.01% Tween 80. The working stock was then divided between two tubes so that each had 5 mL of parent stock and 45 mL of Tween 80. The working stock was enumerated separately by two research scientists and was found to have a titer of ~4.0E8 cfu/mL.

2.2 Dog Skin Samples

Skin samples were collected from six dogs (Table) that were euthanized. These dogs were either MWDs or a breed typically used as MWDs. Full-thickness skin, which withers within 3 h of euthanasia, was removed from the left and right sides of the flank, and subcutaneous fat, when present, was excised. The skin from each region was cut into 1.5 in. strips or 1.5 × 1.5 in. coupons before being wrapped in foil and stored long-term at –80 °C.

Table. Dog Skin Sample Collection and Characteristics

Identification	Breed	Sex	Age (year)	Hair Coat	Coat Color
Basco	German shepherd	Male	6	Rough, long	Black
Izmos	German shepherd	Male	7	Rough, medium-long	Sable
Kali	German shepherd	Female	7	Rough, short	Black
Kep	Belgian Malinois	Male	2	Rough	Tan
Viktor	Belgian Malinois	Male	2	Rough	Tan
Usher	Labrador retriever	Male	9	Rough	Black

For testing, samples were thawed overnight in a 4 °C refrigerator. Thawed samples were saturated with approximately 5 mL of 70% isopropanol and then left to dry overnight in a biosafety cabinet II (BSC-2). Larger skin samples were cut down to roughly 1 × 0.5 in. using surgical scissors. Afterwards, fur length was trimmed when necessary.

Clean, disinfected skin samples were inoculated with 50 µL aliquots of BaS spores with an ~1-2E7 cfu/mL titer. The aliquot was left to dry in a BSC-2 for 60–90 min under sterile airflow until visibly dry (Figure 1).

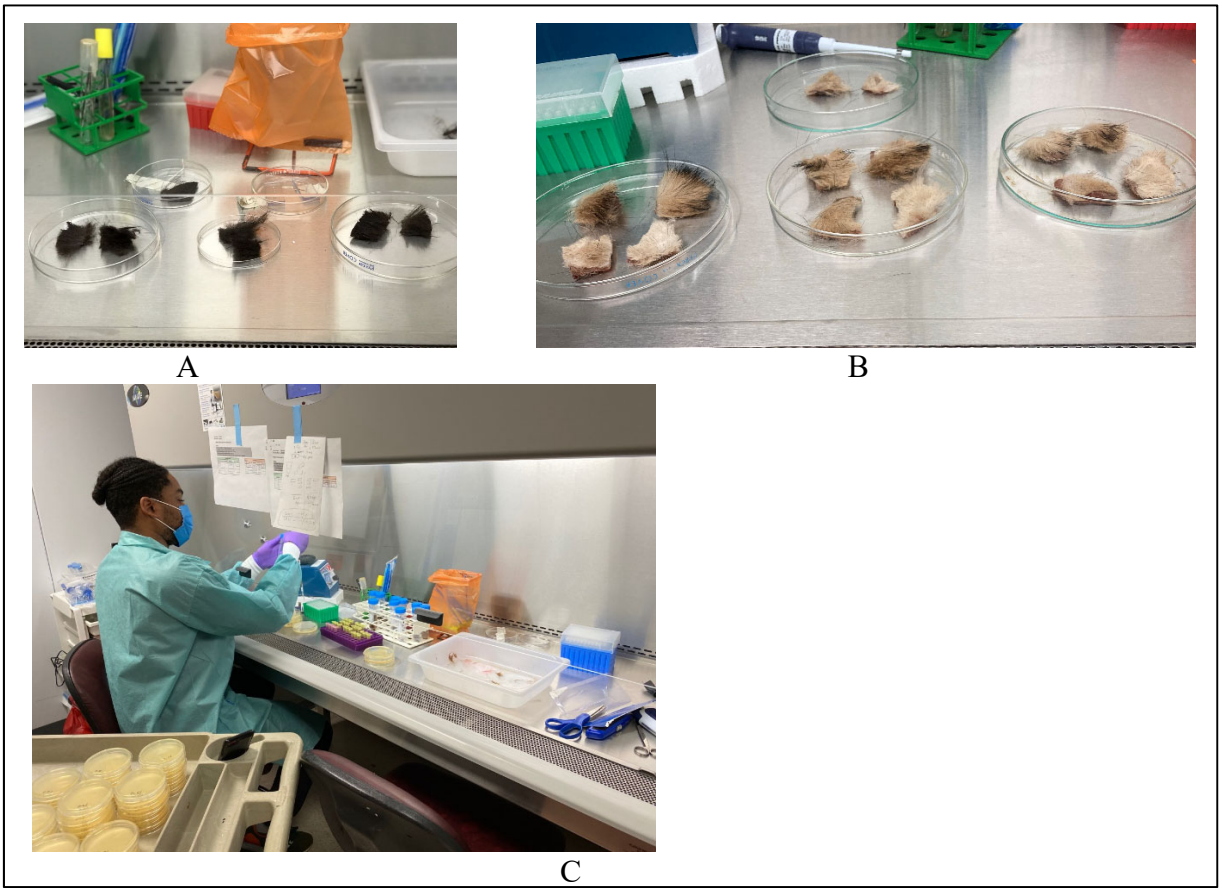

Figure 1. (A and B) Examples of dog skin samples and (C) a typical workstation for decontamination runs.

2.3 Decontamination Approach

Spore suspension of Sterne strain was deposited on dog skin as microdroplets. An aliquot of 50 µL (containing ~7 log) spores was deposited on each skin piece. Typically, three replicate skin samples were used for each set. The spores were allowed to dry overnight. Sterile, prewetted polyester wipes were used to clean the skin surface by repeated mechanical scrubbing. As a control, chlorhexidine gluconate sponges were used to clean the MWD skin surface. Finally, polyester wipes, prewetted with 5% Bioxy solution, were also used as a novel decontamination wipe. Spores removed mechanically were discarded and not enumerated. Skin samples with residual spores were placed in 10 mL of 0.01% Tween 80 solution and vortexed for 2 min. The tubes were sonicated for 10 min to ensure spore recovery from the skin samples. The control and test sets were diluted 10-fold in a final volume of 1 mL of 0.01% Tween-80. A 100 µL aliquot was plated on two TSA plates from dilutions of appropriate control and test sets. The plates were incubated for 24 h at 37 °C. The numbers of colony-forming units were counted from all plates and recorded.

2.4 Data Handling and Reduction

The colony-forming unit numbers were multiplied with a volume factor (10) and a dilution factor to estimate the spore number per sample. Log values were computed from the spore numbers. Average values and standard deviations were computed. Log reduction was estimated by subtracting the log spore of the test sample from the control sample.

3. RESULTS

3.1 Log Recovery of BaS in Test Sets

Across all test sets, the log recovery of BaS spores was consistent in all the runs, as shown in Figures 2 and 3. These figures also show that fur length made no difference in log recovery.

3.2 Log Reduction of BaS Spores in Test Sets

The average log reduction of BaS in all test sets is shown in Figure 4. The mechanical removal of spores using the rinse–wash–rinse method was the least effective with a 2.64 log reduction. The Bioxy wipes were the most effective with a 4.52 log reduction.

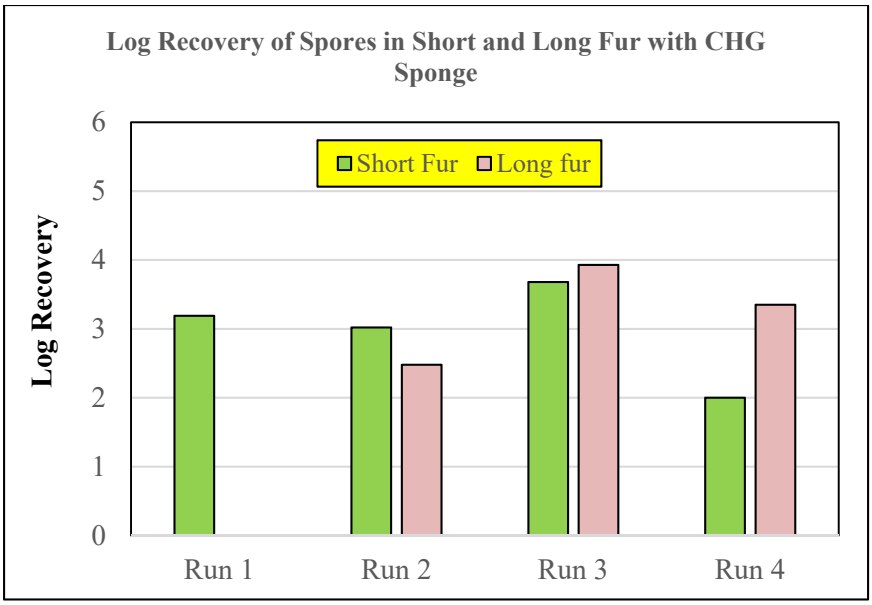

Figure 2. Log recovery of spores from samples treated with chlorhexidine sponge.

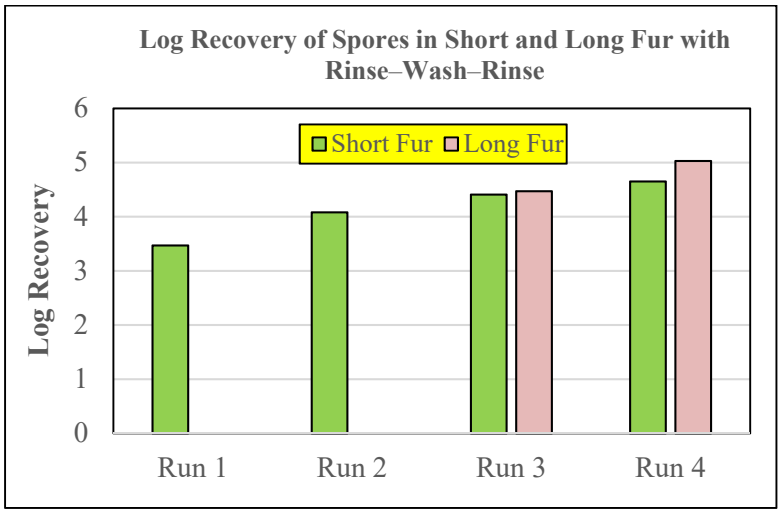

Figure 3. Log recovery of spores from samples treated with standard procedure.

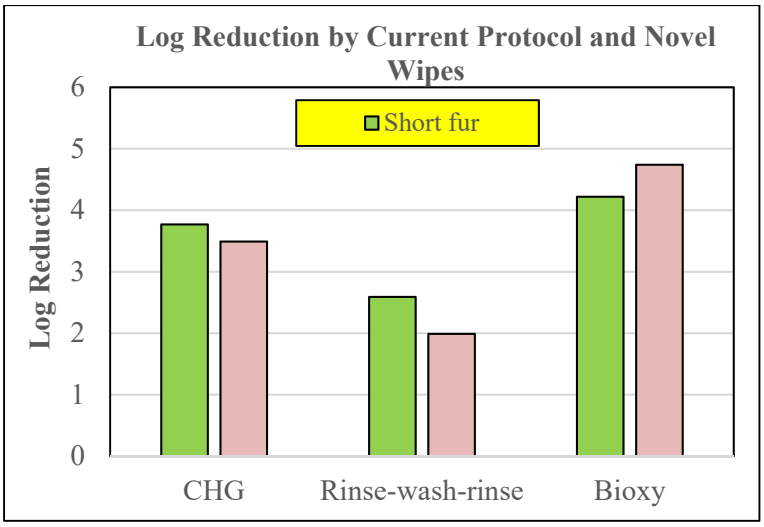

Figure 4. Effectiveness of different approaches in spore inactivation on dog skin.

4. CONCLUSIONS AND DISCUSSION

The primary objective of this study was to evaluate the two BW decontamination methods currently used for MWD skin surfaces. Our results show that the rinse–wash–rinse and chlorohexidine sponge wipe-down methods are not effective on dog skin for spore decontamination. The second objective was to evaluate two novel approaches to MWD skin decontamination. The first approach evaluated the efficacy of wipes soaked in 5% Bioxy for spore reduction on dog skin, and the second approach involved shortening the length of the dog fur as a factor in decontamination effectiveness. It was observed that shortening the dog fur should be restricted to those MWDs suspected of having been exposed to BW agents. Compared with the rinse–wash–rinse and chlorohexidine sponge wipe-down methods, wipes soaked in Bioxy were highly effective in attaining log reduction values of ~5 log. Shortening fur length also resulted in a marginal improvement over rinse–wash–rinse and chlorohexidine sponges in decontamination efficacy. In conclusion, the novel approaches of using Bioxy wipes and fur length shortening appear to be far superior to the rinse–wash–rinse method and practice of using chlorohexidine sponges in decontamination efficacy. Additional tests must be performed to establish the use of Bioxy wipes as an improved technology for BW decontamination of MWDs. These additional tests may include spraying dogs in the field with 5% Bioxy and then wiping them down, which could provide immediate MWD decontamination.

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ACRONYMS AND ABBREVIATIONS

BaS	*Bacillus anthracis* Sterne
BSC-2	biosafety cabinet II
BW	biological warfare
CBRN	chemical, biological, radiological, and nuclear
cfu	colony-forming unit
MWD	military working dog
TSA	tryptic soy agar

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