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Comparison of sampling techniques and sterilization methods for *Bacillus anthracis*-contaminated surfaces

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University of Health Sciences Türkiye, Department of Medical CBRN Defense, Ankara, Türkiye

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Corresponding Author:

Mesut Ortatatlı, M.D., Assoc. Prof.,
University of Health Sciences Türkiye,
Department of Medical CBRN
Defense, Ankara, Türkiye
mortatatl@gmail.com

ORCID:

orcid.org/0000-0002-7899-6157

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ABSTRACT

Aims: Sampling techniques and disinfection methods are essential for surfaces in critical areas for human health, such as food processing areas, hospital operating rooms, and laboratories. Thus, in the case of biological warfare agent use, the detection and decontamination of contamination sites have become a public health concern. From this perspective, our study aimed to evaluate the effectiveness of two distinct sampling techniques and three sterilization methods for surfaces contaminated with *B. anthracis* spores.

Methods: To collect samples from surfaces and for recovery, two methods, including a moistened technique using sterile cotton swabs and a dry cotton swab technique following the suspension of the contaminated surfaces in saline solution, were applied. To sterilize the contaminated surface, 0.5% sodium hypochlorite (NaOCl), 3% H₂O₂, and UVC-254 were applied for various durations.

Results: The suspension method yielded a recovery of 427.50 cu for *B. anthracis*, whereas the moistened method yielded 272.50 cu (p=0.003). Among the decontamination methods tested, complete sterilization was achieved within 15 min using 0.5% NaOCl and within 6 h with 3% H₂O₂, whereas UVC-254 led to a decrease in the spore count by up to 96.73% after 24 h.

Conclusions: In the evaluation of surface contamination and sterilization-disinfection, sampling with a dry cotton swab by forming a suspension on the surface increased recovery. Thus, it was concluded that the use of NaOCl is appropriate for decontaminating *B. anthracis* spores on surfaces resistant to oxidation, whereas H₂O₂ is preferable for more delicate surfaces.

Introduction

The possible use of *B. anthracis* as a biological warfare agent (BWA) has descended from bookshelves into real life and poses a significant threat. The ease of use and low production cost have contributed to the extent of this threat in terms of terrorist purposes (1). *B. anthracis*, a member of the *Bacillus cereus* group, is a gram-positive, aerobic, encapsulated, spore-forming bacterium whose resistance to environmental conditions allows it to remain viable for many years (1). In the event of BWA deployment, the undetectability of biological agents by

the senses, along with incubation periods ranging from days to weeks, complicates the identification of the affected area (2). Like other BWAs, *B. anthracis* in aerosolized form can also cause contamination of various surfaces, posing a long-term risk of contact transmission (3).

A proper and accurate technique for sample collection from suspected surfaces plays a crucial role in ensuring the accuracy of both on-site and laboratory analyses (4). An effective response to a BWA attack includes not only the detection of the agent but also the decontamination and sterilization of



contaminated surfaces rapidly and effectively (4,5). Routine assessments of microbial contamination and sterilization efficacy on surfaces in laboratories, hospitals, and in contact with food are indispensable for ensuring human health (5). Heat and radiation treatment, filtration, and treatment with gases and liquid chemicals are among the primary sterilization methods currently used (6).

Although several studies have been conducted on sampling and sterilization procedures associated with human health risks using surrogates, research using real highly pathogenic live agents, resulting in complete data, is not common (7,8). Thus, it has been emphasized that further research that targets more accurate and proper findings using real live agents is required (8,9).

The present study aimed to determine an appropriate sampling technique for detecting surface contamination following an attack with *B. anthracis* spores and to identify the sterilization method and time required for effective surface decontamination.

Methods

In this prospective study, the preparation of relevant materials, application of sterilization methods, and control evaluation processes were conducted within a class 2 type B2 biosafety cabinet (BLF2000-Bilser, Ankara, Türkiye). The required sample size was 56 for the four groups based on a 95% confidence interval, 85% power value, and an effect size of 0.5 using G*Power V3.1.9 statistical analysis software.

Preparation and surface contamination of *Bacillus anthracis* spores

B. anthracis spores were obtained from stock samples from a previous study (10). Spores were inoculated onto sheep blood agar plates (RDS, Ankara, Türkiye) and incubated at 36 °C in an incubator (Nüve, EN 400, Ankara, Türkiye) for 24 h. For sporulation, the bacteria were maintained at room temperature for 24 h and then refrigerated at +4 °C for 7 days. Daily spore staining (Schaeffer-Fulton Spore Stain Kit, Sigma-Aldrich, Switzerland) confirmed the >90% sporulation rate. The bacterial spore suspensions were adjusted to a McFarland 0.5 standard, equivalent to 1×10^8 /mL) using isotonic saline. From a dilution prepared at a final concentration of 1×10^4 /mL, 10 µL inoculations were made onto sheep blood agar plates to verify the spore counts. A 1 mL suspension of *B. anthracis* spores at a concentration of 1×10^4 /mL was placed in sterile Petri dishes with a diameter of 6 cm and left to rest for 24 h inside a biosafety cabinet with lids closed.

Swab sampling and recovery methods

Two different sampling methods were used: surface sampling and recovery. In the first method (the moistened method), swab samples were collected from previously contaminated

Petri dish surfaces using sterile cotton swabs moistened with saline solution. In the second method (the suspension method), contaminated Petri dish surfaces were suspended in 0.5 mL of saline solution, followed by sampling with dry sterile cotton swabs. All collected swab samples were inoculated onto sheep blood agar plates and incubated at 36 °C for 24 h, after which colony counts were conducted.

Sterilization methods

For sterilization of 1 mL *B. anthracis* spore suspension at a concentration of 10^4 /mL, which was left to dry in Petri dishes for 24 h, the following was used: 3% hydrogen peroxide (H₂O₂) (Kim-Pa Pharmaceutical Lab. Co. Ltd., İstanbul, Türkiye), a 10-fold diluted solution of 5% sodium hypochlorite (NaOCl), commonly known as bleach, and an ultraviolet C (UVC) lamp with a wavelength of 254 nm located inside a type 2 biosafety cabinet (OSAKA T8 30 W TUV; China). The doses and durations of the treatments are presented in Table 1.

Statistical Analysis

Statistical analyses were conducted using Statistical Package for the Social Sciences 21.0 (IBM, Inc., USA). The Shapiro-Wilk test was used to investigate the normality of the variables. The differences in continuous variables among the groups were tested using the Kruskal-Wallis test, and the Bonferroni corrected Mann-Whitney U test was used for post-hoc analyses. The Chi-square test was used to compare nominal

Table 1. The sterilization methods, duration, number of positive Petri dishes, and median and interquartile range values (25-75%) of the colonies

Sterilization method	Application time	Positive Petri dishes (n)	Colony count (cfu) (median and interquartile)
0.5% NaOCl	5 min	6	101.0 (85.25-153.75)
	10 min	4	26.0 (20.25-31.0)
	15 min	0	
3% H ₂ O ₂	1 h	8	118.0 (96.25-140.75)
	3 h	3	29.0 (26.0-33.5)
	6 h	0	
UVC	6 h	10	323.0 (277.75-407.0)
	12 h	8	42.5 (34.5-56.0)
	24 h	3	14.0 (10.0-18.0)
Control		14	427.50 (289.75-470.0)

NaOCl: Sodium hypochlorite, H₂O₂: Hydrogen peroxide, UVC: Ultraviolet C

values. Differences with a p value <0.05 were considered statistically significant.

Results

The median and interquartile range values of the recovery colony count for swab samples taken 24 h later from sterile Petri dishes contaminated with a 1 mL suspension of *B. anthracis* spores at a concentration of 10⁴/mL were 272.50 cu (234.25-339.00) for the moistened method and 427.50 cu (289.75-470.00) for the suspension method (p=0.003) (Figure 1). The recovery rates for the moistened and suspension methods were 2.73% and 4.28%, respectively.

Table 1 demonstrates the treatment time for each sterilization method, the number of positive Petri dishes, and the median and interquartile range values of the colony count for *B. anthracis*. Following the application of 0.5% NaOCl for 5 and 10 min, positive results were observed in 6 and 4 Petri dishes, respectively, and no statistical significance was observed (p=0.430), but a significant difference was found between the numbers of growing colonies (p=0.011). The relationship between the number of positive Petri cells and colony counts in the control group and after NaOCl treatment for 5 and 10 min is presented in Figure 2.

The number of positive Petri dishes after sterilization with 3% H₂O₂ for 1 and 3 h was 8 and 3, respectively, and no significant difference was observed (p=0.053). Figure 3 illustrates the statistical significance found not only between the numbers of positive Petri dishes of the group after 6 h and controls, but also between the colony counts of each group.

Neither of the sterilization studies involving ultraviolet radiation for 6, 12, or 24 h provided complete sterilization, but bacterial growth was observed in 10, 8, and 3 Petri dishes, respectively. No significant difference was detected between the

control and 6-h UV treatment groups concerning the number of colonies. The data related to all other variables are presented in Figure 4.

Discussion

The use of aerosolized spores of *B. anthracis* as a bioweapon or bioterrorism agent remains a concern due to potential environmental contamination. Therefore, it is important to make great efforts toward detection and decontamination for public health measures (4,5). The swab sampling technique is the most common method for identifying pathological microbial contamination on environmental surfaces, including hospital locations and food processing facilities, and for various cleaning procedures (4). Typically, sticks with cotton tips made of plastic

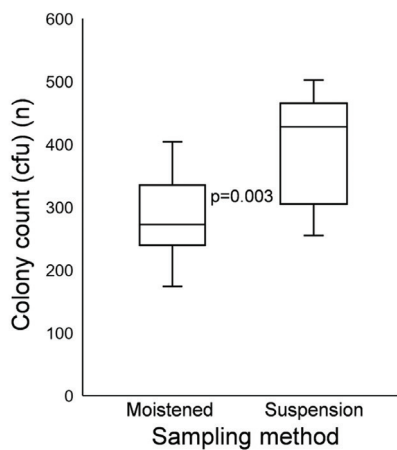


Figure 1. Box plot representing the median values of *B. anthracis* colony counts from surface specimens obtained using moistened and suspension methods and the statistical significance of the differences between these methods

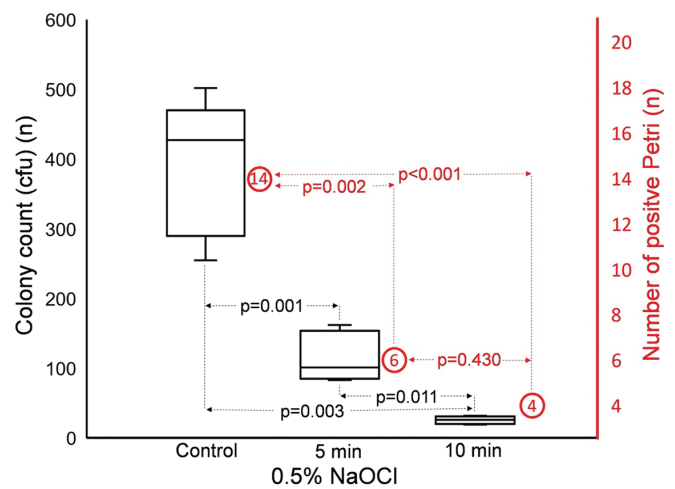


Figure 2. Box plot of median values of *B. anthracis* colony counts after the control group, 5- and 10-min incubations of 0.5% NaOCl, number of positive Petri dishes with growth (data within circles), and p values NaOCl: Sodium hypochlorite

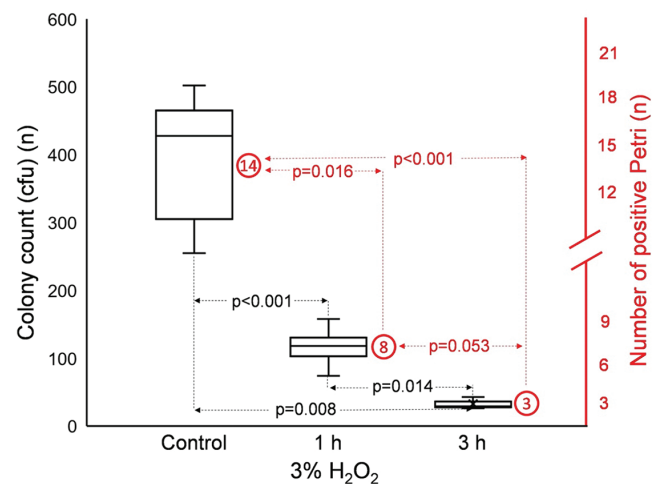


Figure 3. Box plot of median *B. anthracis* colony counts following the control group and 1 and 3 h of 3% H₂O₂ application; number of positive Petri dishes showing growth (data within circles); and p values

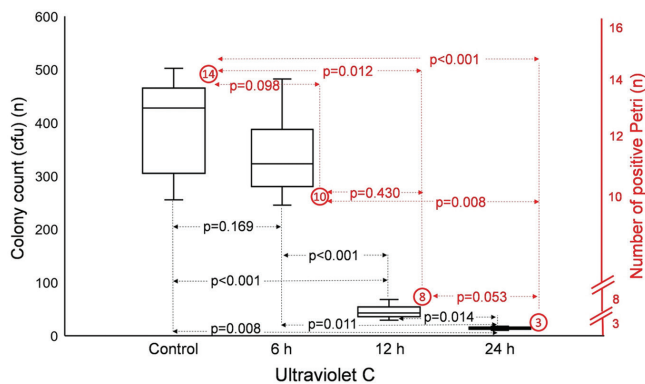


Figure 4. Box plot of median *B. anthracis* colony counts for the control group and after 6, 12, and 24 h of UV light application, number of positive Petri dishes with growth (data within circles), and p values UV: Ultraviolet

or wood are used for this purpose. The principle involves transferring bacteria from the contact surface to the material and then to the culture medium (5). However, it has been reported that the number of bacteria grown (recovery) by the swab sampling technique is significantly lower than that on the surface (4,5). The material used in the swab sample (cotton, foam, polyester, artificial silk, etc.), the extraction method (shaking, sonication, vortexing, etc.), and the type of tip (dry or premoistened) affect the recovery rate (5). In a study of *B. anthracis* spores by Rose et al. (11), the recovery rates for dry and moistened cotton-tipped swabs were 0.5% and 4.7%, respectively, without extraction; and 7.5% and 27.5%, respectively, with extraction. In another study involving live bacteria, the average recovery rate of moistened cotton-tipped swabs was 48.5% (9). In these studies, the cotton on the tips of the swabs was generally premoistened, and the swab samples taken from the dry surface were first transferred to liquid media by various methods and subsequently inoculated in solid culture media (9,11).

In our study, samples were taken from the *B. anthracis*-contaminated surface and left to dry for 24 h using cotton-tipped swabs via two different methods, and inoculations were made directly onto sheep blood agar without pretreatment. Colony numbers recovered using the suspension method were significantly higher than those recovered using the moistened method (Figure 1). It is suggested that wetting the surface with a sterile liquid before taking the swab sample facilitates the removal of existing microorganisms from the surface and enhances their adsorption of liquid on the cotton, leading to an increase in recovery. Keeratipibul et al. (9) also supported this suggestion and found that the recovery decreased by 30-40% in samples after 1 hour of drying compared with that before the suspension was dried. Our results showed that the recovery rate decreased by 36.25% with the moistened method compared with the suspension method (4.28% and 2.73%, respectively) in samples dried on the surface for 24 h. Sterilization of surfaces and materials

often involves both physical methods, including heating, filtration, ionizing radiation, and UV radiation, and chemical methods like ethylene oxide, H_2O_2 , glutaraldehyde, and NaOCl, depending on the characteristics of the surface and material (12). Among the aforementioned methods, NaOCl, which we also applied, is known to affect the inner membrane structure of bacterial spores, whereas UV radiation causes DNA damage (12). Another agent, H_2O_2 , acts on DNA in a vegetative form and causes damage to the core proteins of bacterial spores (13).

DeQueiroz and Day (14) demonstrated in their study that a mixture of H_2O_2 and NaOCl could achieve a more effective sporicidal effect at lower concentrations and for shorter contact times. They observed a 90% reduction in the *B. subtilis* spore count after 5 min of 2.5% NaOCl treatment, and complete sterilization was achieved after 10 min (14).

A 0.5% NaOCl solution was used in our experiment because of its applicability to intact skin. The number of *B. anthracis* spores was reduced by 76% compared with the control group after 5 min of contact time and by 94% after 10 min. Complete sterilization was achieved after 15 minutes. The reduction in colony counts and the number of positive Petri dishes was statistically significant after 5 or 10 min of treatment compared with the control group (Table 1, Figure 2). Although no complete sterilization was observed, the findings suggested that a 10-min contact time with NaOCl significantly reduced the number of *B. anthracis* spores on the surface. When high NaOCl concentrations cannot be used, extending the contact time can increase its effectiveness. This makes it useful for sensitive surfaces, such as intact human skin.

An ideal agent for sterilizing and disinfecting surfaces important for public health and safety, including those in contact with food sources, laboratories, and hospital operating rooms, should be effective and safe without producing toxic waste (14,15). One of the aforementioned agents, H_2O_2 , generates free hydroxyl radicals and can be used in both liquid and gas forms. It is known for its environmentally friendly properties, as it decomposes into water and oxygen (15). Although H_2O_2 is generally recommended for use at high concentrations (6-35%) for its sporicidal effects (13,16), it has been emphasized that even lower concentrations (1-2.5%) can be effective if H_2O_2 is combined with alcohol-and chlorine-containing chemicals (17,18). According to the study of Hayrapetyan et al. (19), as the concentration of H_2O_2 decreases, the exposure time should be extended to increase the sporicidal effect.

We applied 3% H_2O_2 for 1 and 3 h, resulting in a reduction of 72.39% and 93.22% in the number of live spores compared with the control group, respectively, and achieved complete sterilization after 6 h (Table 1). Although there was no significant difference in the number of positive Petri dishes after 1 and 3 h of exposure, a significant reduction was observed in the number

of live spores (Figure 3). Our results indicate that H₂O₂, which is known to leave no toxic waste (15), can be safely used at a 3% concentration as we observed a significant reduction in the number of live *B. anthracis* spores. However, increasing the contact time of H₂O₂ should be considered for the sterilization and disinfection of surfaces in working environments.

“UV sterilization” has been referred to as the “UVC-254 method” since 254 nm is the most commonly used and selected wavelength for sterilization (16). It has been reported that while UVC-254 application rapidly reduces bacterial spore counts in the initial phases, its effectiveness gradually decreases as the exposure continues, which generally leads to incomplete sterilization (16,20). Moreover, despite an extended exposure time and increased dose, UVC might shield live spores and protect them beneath inactivated spores (16,20). In line with previous studies, our study showed a 90% reduction in *B. anthracis* spore counts after 12 h of UVC-254 treatment, with efficacy reaching only 96.73% after 24 h (Table 1, Figure 4). These findings indicate that UVC-254 treatment is a valuable tool for mitigating the risk of *B. anthracis* contamination, although this method cannot achieve complete sterilization.

Conclusion

In conclusion, it is recommended to wet the surface of the *B. anthracis* spore-contaminated environment before obtaining the swab sample because this method has shown significant recovery rates for monitoring surface contamination and sterilization-disinfection. The study also suggests using 0.5% NaOCl for 15 min to decontaminate *B. anthracis* spores and applying 3% H₂O₂ for 6 h on sensitive surfaces. However, UVC-254 did not completely remove all spores from the surface, but significantly reduced the number of viable spores. Additional sampling and decontamination studies on various types of surfaces with live agents should be conducted to determine appropriate procedures following the use of BWA.

Ethics

Ethics Committee Approval-Informed Consent: Not required for this study.

Footnotes

Authorship Contributions

Concept: M.O., Design: M.O., Data Collection or Processing: M.O., B.Ç., Analysis or Interpretation: M.O., L.K., Literature Search: B.Ç., Writing: M.O., L.K.,

Conflict of Interest: No conflict of interest was declared by the authors.

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References

- Ortatatlı M. Biyolojik Silahlar. İçinde: Eryılmaz M, Dizer U, editörler. Afet Tıbbı. Ankara: Ünsal Yayınları; 2005:1207-1255.
- Treadwell TA, Koo D, Kuker K, Khan AS. Epidemiologic clues to bioterrorism. Public Health Rep. 2003;118:92-98.
- Staneck SA, Saunders D, Alves DA, et al. USAMRIID's medical management of biological casualties handbook. 9th ed. Fort Detrick, Maryland; 2020.
- Jones SL, Ricke SC, Keith Roper D, Gibson KE. Swabbing the surface: critical factors in environmental monitoring and a path towards standardization and improvement. Crit Rev Food Sci Nutr. 2020;60:225-243.
- Rawlinson S, Ciric L, Cloutman-Green E. How to carry out microbiological sampling of healthcare environment surfaces? A review of current evidence. J Hosp Infect. 2019;103:363-374.
- Dağlı G, Özyurt M. Hastane Ortamında Sterilizasyon Uygulamaları. In: Haznedaroğlu T, Özgüven V, Pekcan M, editors. Hastane İnfeksiyonları. Ankara: GATA Basımevi; 2001;68-78.
- Spotts Whitney EA, Beatty ME, Taylor TH Jr, et al. Inactivation of Bacillus anthracis spores. Emerg Infect Dis. 2003;9:623-627.
- Setlow P. Resistance of Bacterial Spores to Chemical Agents. Principles and practice of disinfection, preservation and sterilization. Oxford, UK: Wiley-Blackwell;2013;121-130.
- Keeratipibul S, Laovittayanurak T, Pornruangsarp O, Chaturongkasumrit Y, Takahashi H, Techaruvichit P. Effect of swabbing techniques on the efficiency of bacterial recovery from food contact surfaces. Food Control. 2017;77:139-144.
- Ortatatlı M, Karagoz A, Percin D, Kenar L, Kilic S, Durmaz R. Antimicrobial susceptibility and molecular subtyping of 55 Turkish Bacillus anthracis strains using 25-loci multiple-locus VNTR analysis. Comp Immunol Microbiol Infect Dis. 2012;35:355-361.
- Rose L, Jensen B, Peterson A, Banerjee SN, Srdino MJ. Swab materials and Bacillus anthracis spore recovery from nonporous surfaces. Emerg Infect Dis. 2004;10:1023-1029.
- Cho WI, Chung MS. Bacillus spores: a review of their properties and inactivation processing technologies. Food Sci Biotechnol. 2020;29:1447-1461.
- Setlow P, Christie G. What's new and notable in bacterial spore killing! World J Microbiol Biotechnol. 2021;37:144.
- DeQueiroz GA, Day DF. Disinfection of Bacillus subtilis spore-contaminated surface materials with a sodium hypochlorite and a hydrogen peroxide-based sanitizer. Lett Appl Microbiol. 2008;46:176-180.
- Lineback CB, Nkemngong CA, Wu ST, et al. Hydrogen peroxide and sodium hypochlorite disinfectants are more effective against Staphylococcus aureus and Pseudomonas aeruginosa biofilms than quaternary ammonium compounds. Antimicrob Resist Infect Control. 2018;7:154.
- Wood JP, Adrion AC. Review of Decontamination Techniques for the Inactivation of Bacillus anthracis and Other Spore-Forming Bacteria Associated with Building or Outdoor Materials. Environ Sci Technol. 2019;53:4045-4062.

17. Kenar L, Ortatli M, Yaren H, Karayilanoglu T, Aydogan H. Comparative sporicidal effects of disinfectants after release of a biological agent. *Mil Med.* 2007;172:616-621.
18. Mickelsen RL, Wood J, Calfee MW, et al. Low-concentration hydrogen peroxide decontamination for *Bacillus* spore contamination in buildings. *Remediation (N Y).* 2019;30:47-56.
19. Hayrapetyan H, Nederhoff L, Vollebregt M, Mastwijk H, Nierop Groot M. Inactivation kinetics of *Geobacillus* stearothermophilus spores by a peracetic acid or hydrogen peroxide fog in comparison to the liquid form. *Int J Food Microbiol.* 2020;316:108418.
20. Owens MU, Deal DR, Shoemaker MO, Knudson GB, Meszaros JE, Deal JL. High-Dose Ultraviolet C Light Inactivates Spores of *Bacillus Atrophaeus* and *Bacillus Anthracis* Sterne on Nonreflective Surfaces. *Appl Biosaf.* 2005;10:240-247.