ASSESSMENT OF ENVIRONMENTALLY BENIGN DECONTAMINANT TOWARDS ANTHRAX SPORES

Vipin K. Rastogi,
GEO-Centers, Inc., Gunpowder Branch, PO Box 68, APG, MD 21010, USA

Tu-chen Cheng and Abe Turetsky*
US Army, AMSSB-RTL, ECBC, APG, MD 21010, USA

Phillip Bartram and George Wagner
US Army, AMSSB-RRT-CE, ECBC, APG, MD 21010, USA

In recent years, production and storage of CBW agents in large quantities by a number of rogue nations and extremist groups have raised the probability of their use in armed conflicts and against civilian populations around the world. While the CW agents include nerve agents, G-type and V-type, and blistering agents such as mustard gas; the BW agents include pathogenic viruses, bacterial cells, dormant spores, and protein-based toxins. The current decontaminant used by first responders and the US armed forces is not environmentally friendly. Consequently, development of alternative decontaminants is urgently needed. Three environmentally benign decontaminant formulations, two based on hydrogen peroxide and a third based on hypochlorite, have recently been developed for CW agents. We were interested in evaluating these formulations against BW agents in a quest to develop environmentally benign CBW decontaminants. In this study, these formulations were evaluated against *Bacillus anthracis* (NNRΔ1 strain) spores. While, both peroxide-based formulations resulted in a 7-log reduction in spore viability, the hypochlorite-based formulation was much less effective. These results provide support for continued efforts in a quest for the development of an environmentally benign universal CBW decontaminant.

Currently, decontaminants such as the U.S. Army's DS2, the German C8 emulsion, the British and Canadian CASCAD, the commercial Italian BX-24, the German GD-5, Sandia National Laboratory's SNLF and other NATO decontamination and experimental solutions are designed for use primarily against chemical agents. These decontaminants have also been

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
00 JAN 2002	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Assessment Of Environmentally Benign Decontaminant Towards Anthrax Spores	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **GEO-Centers, Inc., Gunpowder Branch, PO Box 68, APG, MD 21010, USA; US Army, AMSSB-RTL, ECBC, APG, MD 21010, USA**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

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

13. SUPPLEMENTARY NOTES
This article is from ADA409494 Proceedings of the 2001 ECBC Scientific Conference on Chemical and Biological Defense Research, 6-8 March , Marriott's Hunt Valley Inn, Hunt Valley, MD., The original document contains color images.

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**UU**	**7**	

assessed for biological decontaminant efficacy[1] and although CASCAD and SNLF have demonstrated a significant reduction of *B. anthracis* spores and several vegetative bacterial forms, none were sufficiently efficacious to warrant further laboratory study. Although these decontaminants presented activity toward the chemical agents, none were active against all chemical agents assessed. The goal of this study is to initiate research which would contribute to development of a single effective non-corrosive environmentally benign dual use chemical and biological agent decontamination formulation, initially against all common chemical warfare agents and realistic simulants for *B. anthracis*, *Yersinia pestis*, and ricin bio-toxin . Strong oxidants such as halogen and oxygen donors are known to degrade both chemical and biological agents. With this property in mind, three typical formulations were prepared. The investigation was initiated by examining the decontamination properties of one calcium hypochlorite and two hydrogen peroxide based decontaminants. Hydrogen peroxide is inherently environmentally benign since its reaction product is water. Initial results are promising. In particular the hydrogen peroxide formulations demonstrated activity toward all chemical agents assessed as well as significant activity toward the three biological agents included in the study to date.

MATERIALS AND METHODS

1. PREPARATION OF SPORES:

Bacillus anthracis cells of a non-virulent strain (NNRΔ1; plasmid-free) were grown in sporulation media (Turnbull, 1999; personal communication) containing peptone, yeast extract, sodium chloride, and dextrose. The cells were seeded on the plates containing the media solidified with 1.5% Difco Bacto agar, and grown for 2-3 weeks at 28ºC, until the surface was 99-100% phase bright. The spores were suspended in sterile water using a sterile spreader, and collected in sterile tubes. The spore suspension was heated at 62.5ºC for 15 min, and then washed five times with sterile distilled water. Aliquots of spores (10^8-10^9/ml) were kept at 4ºC.

2. "DECON GREEN" FORMULATIONS:

Three "DECON GREEN" formulations, DG1, DG2, and DG3, were prepared after Bartram (personal communication). The DG1 was prepared by first mixing sulfolane and methyl sulfolane (45% and 55%, respectively). The final formulation contained 8 parts saturated HTH, 42 parts water and 50 parts sulfolane mixture. The second formulation, DG2 contained (volume basis) 73 parts propylene carbonate plus 2 parts Triton X-100 plus 25 parts hydrogen peroxide (the stock hydrogen peroxide used was at 50% concentration) and just before mixing it was made 0.2M with respect to both potassium carbonate and potassium bicarbonate. The third formulation was prepared by first preparing the solvent mixture, 33.33 parts 2-ethyl hexanol plus 6.66 parts Triton X-100 plus 6.66 parts dodecyl pyrrolidinone plus 53.33 parts methyl pyrrolidinone. 75 parts of this solvent mixture was then mixed with 25 parts hydrogen peroxide. The hydrogen peroxide used was at 50% concentration and just before mixing, it was made 0.2M with respect to both potassium carbonate and potassium bicarbonate.

3. SPORE VIABILITY ASSAYS:

In initial experiments, freshly-prepared "DECON GREEN" formulations were spread on to nutrient agar plates, and let dry. 100 µl of spore suspension ($5x10^8$/ml) was spread evenly over

the control and test plates. Appropriately diluted spore suspension was spread to observe accurate cfu (colony-forming units). The plates were incubated at 30C for 18-36 hrs, and cfu were accurately counted.

4. TOXIN SIMULANT ASSAY:

Two protein simulants, the ricin A chain purified from native Ricin D (Inland Lab. Inc., Austin, TX) and BSA (bovine serum albumin), were used as toxin simulants. In initial experiments, the effect of hydrogen peroxide on BSA was analyzed using a spectrophotometric assay (absorbance @ 260 nm). Due to overlapping extinction bands of BSA and peroxide at 260 nm, this procedure was rejected for further use. An SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) assay was used to analyze the effect of the "DECON GREEN" active ingredient on protein toxin. The gel was stained with Coomassie Brilliant Blue R-250 (0.025% R 250 prepared in 40% methanol,7% acetic acid), and destained using destaining solution (40% methanol and 7% acetic acid).

RESULTS

1. ANTHRAX SPORES:

Figure 1 shows anthrax spores as prepared by the method described in the Materials and Methods section. Over 99% of the structures in the viewing area are spores. These spores were used in the present study.

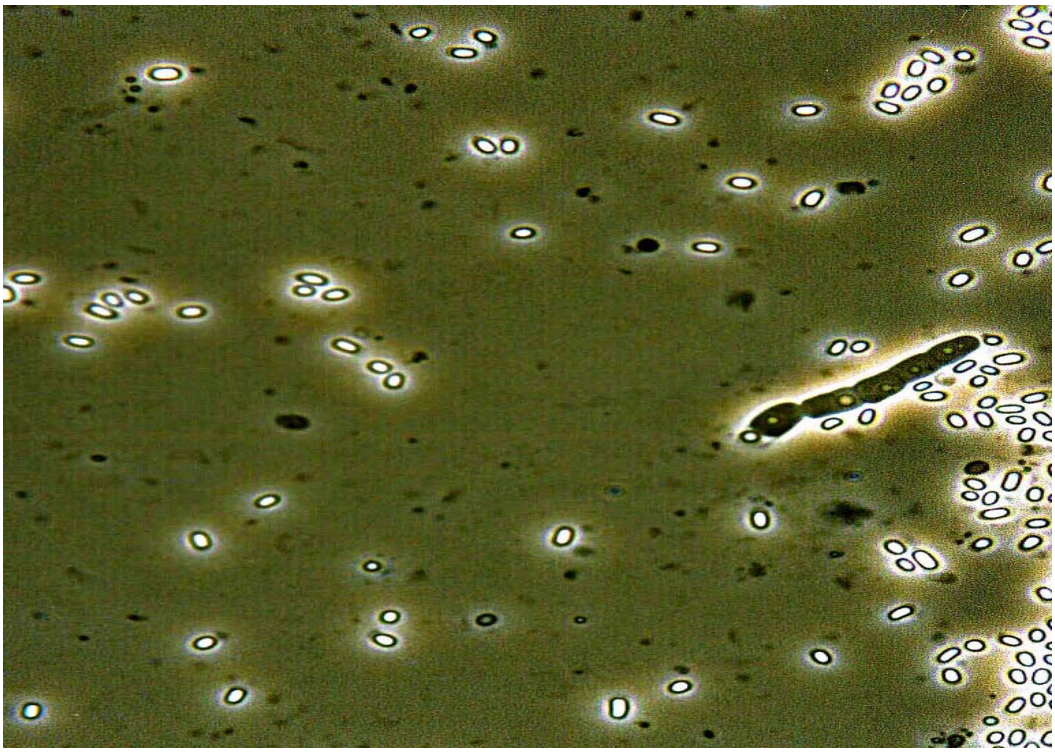


Figure 1. Anthrax Spores seen as Refractile Structures.

2. EFFICACY OF THE THREE "DECON GREEN" FORMULATIONS:

The three formulations, DG1, DG2, and DG3, were prepared as described in the Materials and Methods section. The number of cfu was determined after counting the colonies on the plates 24 hours after plating and incubation. As seen in Table 1, the DG1 formulation was ineffective as a decontaminant against anthrax spores. However, the other two formulations, DG2 and DG3, were highly effective as spore decontaminants. There were no survivors out of $2x10^7$ spores plated. These findings demonstrate that both these formulations result in a 7-log reduction of spores.

TABLE 1. Number of Anthrax Spore Survivors in the Presence of Three "DECON GREEN" Formulations.

Dilution	**Control Cells/mL**	**DG1 Cells/mL**	**DG2 Cells/mL**	**DG3 Cells/mL**
10^{-1}			0	0
10^{-3}			0	0
10^{-5}			0	0
10^{-5}	$6x10^8$	$1.1x10^8$		
10^{-6}	$2x10^8$	$1.2x10^8$		
10^{-7}	$2x10^8$			

The two formulations, DG2 and DG3 were highly effective as spore decontaminants, however, since the formulations were spread on the plates, spores were in constant contact with these chemicals. The effect of the decontaminant on viability or survival could be due to its activity on the vegetative cell resulting from germination of the spore. To resolve this question, the spores were first mixed with each the two formulations, and after overnight treatment, the spores were washed 3x with sterile saline solution. An aliquot was plated on the agar plate and the number of colonies was counted the next day. The results are summarized in Table 2. As seen in Table 2, both formulations were effective in decontaminating anthrax spores within 15 min of treatment. Since, no decontaminants were spread on the plate, the observed lack of any survivor must have resulted from the effect of these chemicals on the spore itself.

TABLE 2. Number of Anthrax Spore Survivors in the Presence of Three "DECON GREEN" Formulations.

	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Control	**	**	**	390, 440	43, 40	4, 3
DG2	0^*	0	0	0	0	
DG3	0^*	1,0	0	0	0	

* 10 million spores treated and approximately 400,000 spores plated on each plate
** TNTC, too numerous to count

3. EFFECT OF HYDROGEN PEROXIDE ON SPORE SURVIVAL:

The active component of the DG1 formulation is HTH (chlorine donor), and hydrogen peroxide is the active component in the other two formulations, DG2 and DG3. It was of interest to quantitatively determine if the active component by itself was an effective decontaminant. The active component was spread on a nutrient agar plate before plating the spores. The colonies were counted after overnight incubation. The results are summarized in Table 3. In the presence of 0.8% HTH, an approximately 10% reduction in number of cfu was observed. In contrast, presence of 12.5% peroxide resulted in complete loss of spore/vegetative cell viability.

TABLE 3. Number of Anthrax Spore Survivors in the Presence of Active Components.

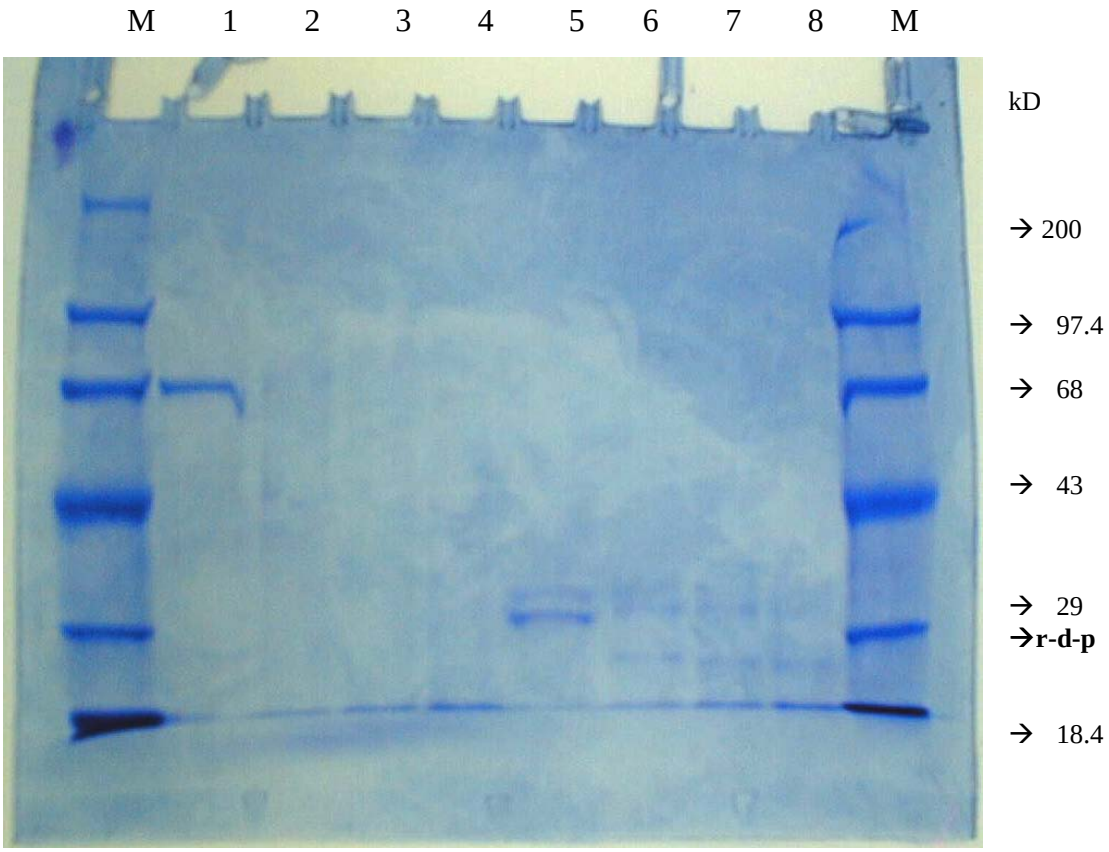
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Background	*	*	*	*	$5.4x10^{7}$	$4x10^{7}$
HTH Test	*	*	*	$3.2x10^{6}$	$3x10^{6}$	
H_2O_2 Test	0	0	0			

* = too numerous to count accurately

An experiment was designed to observe the effect of a brief -time exposure to hydrogen peroxide on spore viability. A spore suspension ($2x10^{8}$/0.5ml) was mixed with hydrogen peroxide (final concentration = 6.25%), and after 25 min incubation, the spores were washed 3x with sterile water before plating. The same number ($2x10^{8}$/0.5 ml) of cfu was recovered in the control sample, however in the test sample, $1\text{-}2x10^{4}$ (a 4-log reduction) was observed in spore viability. These results are consistent with the conclusion that a brief exposure of anthrax spores to 6.25% hydrogen peroxide results in a significant reduction in spore survival.

4. EFFECT OF HYDROGEN PEROXIDE ON TOXIN SIMULANTS:

A large number of toxins, such as botulinum and ricin, are binary (require two chains for activity) and proteinaceous in nature. Ovalbumin and BSA are among the common simulants used in lieu of active toxins. In this study, we used the A chain of ricin derived from ricin D and BSA as toxin simulants. Results are displayed as an electropherogram of the proteins, comparing hydrogen peroxide treatment to that of controls.



Lanea 1-4 = BSA and 5-8 = Ricin. Lanes 1,5 = control; 2,6 = 7.5% peroxide; 3,7 = 15% peroxide; 4,8 = 22.5% peroxide. Six µl sample was mixed with an equal volume of loading buffer containing SDS and heated for 10 min at 95C. The arrow (r-d-p) in the electropherogram shows the position of degradation products of the A chain of ricin D.

Figure 2. Effect of Varying Concentration of Hydrogen Peroxide on Protein Toxin Surrogate BSA and the A chain of ricin D.

CONCLUSIONS

In conclusion, the present study demonstrates:

1. Of the three DECON GREEN formulations, the two formulations based on hydrogen peroxide are very effective decontaminants;
2. The active component, hydrogen peroxide, itself brought about a 4-log reduction in spore number;
3. Hydrogen peroxide, was effective against the protein toxin simulants BSA and the ricin A chain.

REFERENCES

1. Lee, Fred K. and Turetsky, Abe L. Internal report, The Evaluation of Eight Candidate Decontamination Formulations for the Joint Services Fixed Site Decontamination System on Simulants of Potential Biological Warfare Agents. December, 2000.