

Report Submitted to Virox Technologies, Inc., Mississauga, Ontario

**EVALUATION OF THE ACTIVITY OF TWO  
ACCELERATED HYDROGEN PEROXIDE-BASED  
FORMULATIONS AGAINST THE SPORES OF  
*BACILLUS ANTHRACIS* AND *BACILLUS SUBTILIS*  
USING A QUANTITATIVE CARRIER TEST**

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**This study was conducted in collaboration with the National Research  
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## OBJECTIVE OF THE STUDY

The objective of this study was to test the activity of two accelerated hydrogen peroxide-based formulations against the spores of a virulent strain *Bacillus anthracis* and the surrogate organism *Bacillus subtilis* using the second tier of a quantitative carrier test (QCT-2) developed in our lab; the method is now a standard (#E-2197) of ASTM International (ASTM 2002).

## MATERIALS AND METHODS

### Test Facility

The experimental work for this study was performed in a facility certified by Health Canada for handling Biohazard Level 3 infectious agents by a technician fully trained in working with the spores as well as the test protocol.

### The Test Formulations

The test formulations were shipped to us directly by the Sponsor and upon receipt they were stored at room temperature in a secure area with controlled access. The product effectiveness criterion was arbitrarily set at a minimum  $\geq 6 \log_{10}$  reduction in viable spores.

### The Spores

The spores of the test bacterium were grown in a 1:10 dilution of Columbia Broth for 72 hours at 37°C. The bacterial suspensions were heated at 80°C for 10 minutes to ensure the absence of any vegetative cells.

### Soil load

The test spores were first suspended in a tripartite soil load: 25  $\mu\text{L}$  of bovine serum albumin, 100  $\mu\text{L}$  of mucin and 35  $\mu\text{L}$  of tryptone were added to 340  $\mu\text{L}$  of the spore suspension. The soil load mixture contains a level of protein roughly equal to that in 5% serum.

### Test Procedure:

Stainless steel disks (1 cm in diameter) were used as the carriers in QCT-2 developed at CREM (Springthorpe and Sattar, 2003). The test spores were first suspended in the soil load and 10  $\mu\text{L}$  of it placed on each metal disk. Teflon vials were used to hold the disks. The inoculum was allowed to become visibly dry under ambient conditions. The dried inoculum on each disk was then overlaid with 50  $\mu\text{L}$  of the test formulation and control carriers received an equivalent volume of normal saline. The desired minimum number of viable spores in the dried inoculum on each carrier was  $\geq 6 \log_{10}$ . Each test incorporated 3 control and five test carriers.

The contact time between the dried spore inoculum and the test formulation was 10 minutes at 20°C. At the end of the contact time, each vial received 10 mL a diluent/eluent containing Lethen broth+ 1% sodium thiosulfate as the neutralizer. The contents of the vial were vortexed for 30 seconds and the eluate passed through a 47 mm diameter filter membrane (0.22  $\mu\text{m}$  pore diameter). The vial with the disk was rinsed with saline several times and the washes were also

filtered. The total volume of saline for each carrier was about 100 mL. The filters were placed on trypticase soy agar medium as the recovery medium. The plates were examined periodically over a period of 5 days to get the final count of colony forming units (CFU). Log<sub>10</sub> reductions were then calculated.

## Results and Discussion

As can be seen from the data summarized in Table 1, the formulation labeled AHP 5 (lot #12400) showed good activity against the spores of *B. anthracis* and *B. subtilis* used as the challenge organisms here. The contact time was 10 minutes at 20°C. The titre of viable spores on the control carriers was roughly similar for both organisms and was also higher than the target value of 10<sup>6</sup>. No survivors were detected in any of the five test carriers for *B. anthracis* and thus the log<sub>10</sub> reduction obtained was almost 7 log<sub>10</sub>. For *B. subtilis* two of the test carriers showed some survivors. However, the mean log<sub>10</sub> reduction was still higher than the target value of 10<sup>6</sup>.

In the evaluation of the test formulation labeled Accel (lot #13114) the titre of the spores of *B. anthracis* on the control carriers was 3.2 X 10<sup>6</sup> but that on the *B. subtilis* controls was nearly 10-fold lower. Survivors were recovered from two of the test carriers for *B. anthracis* and this gave the mean log<sub>10</sub> reduction for this organism as 5.55. No survivors were found on the test carriers for *B. subtilis* thus giving a log<sub>10</sub> reduction of 5.37.

The test facility was made available to us for a very limited time during which it was necessary for us to develop the basic procedures for working with *B. anthracis* and then conduct the microbicide tests. In view of this, only one test with each formulation could be run at only one contact time. However, these preliminary findings show that the two test formulations have nearly the same level of activity against both the spore-formers tested. This means that the results obtained with *B. subtilis* could be regarded as predictive of the activity of the formulation against *B. anthracis* as well.

As far as we are aware, this is the first study where the spores of both organisms were grown and processed in an identical manner and the microbicide testing was also carried out using the same test protocol. This makes it possible to directly compare the results against the two spore-formers. The test method used in this study meets the requirements of the Canadian General Standard Board's national standard (CGSB 1997).

## LITERATURE CITED

ASTM International (2002). Standard Quantitative Disk Carrier Test Method for Determining the Bactericidal, Virucidal, Fungicidal, Mycobactericidal and Sporocidal Activities of Liquid Chemical Germicides, Document #E 2197-02. ASTM International, West Conshohocken, PA.

Canadian General Standards Board (1997). *Assessment of Efficacy of Antimicrobial Agents for Use on Environmental Surfaces and Medical Devices*. Document number CAN/CGSB-2.161-M97, CGSB, Ottawa, Ontario, Canada.

Springthorpe, V.S. and Sattar, S.A. (September 2003). *Quantitative Carrier Tests to Assess the Germicidal Activities of Chemicals: Rationales and Procedures*. ISBN 0-88927-298-0' Centre for Research on Environmental Microbiology (CREM), University of Ottawa, Ottawa, ON, Canada.

Table 1. Sporicidal Activity of AHP-Based Formulations

Sample	Titre of viable spores in log <sub>10</sub> /carrier		Log <sub>10</sub> Reduction	
	<i>B. anthracis</i>	<i>B. subtilis</i>	<i>B. anthracis</i>	<i>B. subtilis</i>
<b>AHP 5 - Control</b>				
Carrier 1	1.21 X 10 <sup>7</sup>	1.03 X 10 <sup>7</sup>	-	-
Carrier 2	1.19 X 10 <sup>7</sup>	1.11 X 10 <sup>7</sup>	-	-
Carrier 3	4.50 X 10 <sup>6</sup>	1.31 X 10 <sup>7</sup>	-	-
<b>Mean titre on controls</b>	<b>9.50 X 10<sup>6</sup></b>	<b>1.15 X 10<sup>7</sup></b>	-	-
<b>AHP 5 (Lot #12400) - Test</b>				
Carrier 1	0	0	6.98	7.06
Carrier 2	0	0	6.98	7.06
Carrier 3	0	0	6.98	7.06
Carrier 4	0	1.72 X 10 <sup>2</sup>	6.98	5.34
Carrier 5	0	1.43 X 10 <sup>2</sup>	6.98	5.63
<b>Mean log<sub>10</sub> reduction</b>			<b>6.98</b>	<b>6.43</b>
<b>Accel - Control</b>				
Carrier 1	2.5 X 10 <sup>6</sup>	1.6 X 10 <sup>5</sup>	-	-
Carrier 2	3.9 X 10 <sup>6</sup>	1.9 X 10 <sup>5</sup>	-	-
Carrier 3	-	3.5 X 10 <sup>5</sup>	-	-
<b>Mean titre on controls</b>	<b>3.2 X 10<sup>6</sup></b>	<b>2.33 X 10<sup>5</sup></b>	-	-
<b>Accel (Lot #13114) - Test</b>				
Carrier 1	0	0	6.50	5.37
Carrier 2	0	0	6.50	5.37
Carrier 3	0	0	6.50	5.37
Carrier 4	2.0 X 10 <sup>3</sup>	0	3.20	5.37
Carrier 5	1.9 X 10 <sup>1</sup>	0	5.04	5.37
<b>Mean log<sub>10</sub> reduction</b>			<b>5.55</b>	<b>5.37</b>