

**REPORT TO VIROX TECHNOLOGIES  
OAKVILLE, ONTARIO**

**MICROBICIDAL ACTIVITY OF AN AHP-BASED HIGH-LEVEL  
DISINFECTANT (PREVENTION) AFTER 14 DAYS OF  
SIMULATED REUSE**

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## EXECUTIVE SUMMARY

**Background:** Heat-sensitive medical devices require chemical disinfection between patients, and certain formulations for this purpose can be reused for several days. Because dilution, evaporation, and breakdown or neutralization of active ingredients can occur during reuse, it is vital to ensure that the solution retains its broad-spectrum germicidal activity even at the end of the recommended reuse period. This study was performed with the product 'Prevention' which is a 2.0% accelerated hydrogen peroxide (AHP)-based formulation. AHP is a synergistic blend of commonly used, safe ingredients that, when combined with low levels of hydrogen peroxide, produce exceptional activity as a microbicide.

**Objective:** The purpose of this study was to combine the U.S. Environmental Protection Agency- and the Food and Drug Administration-recommended simulated reuse method with the first tier of the quantitative carrier test (QCT-1) to assess the broad-spectrum microbicidal activity of 'Prevention' subjected to simulated reuse stress for 14 days.

**Materials and methods:** Twenty liters of each of the three lots (# 3575, 3576 and 3577) of the product were stressed repeated over 14 days by challenging them each day with carriers contaminated with microorganisms dried on them. The microbial concentrations on the carriers were adjusted to get a cumulative bacterial bioburden of at least  $10^4$  CFU/mL of each test solution/day to conform to the requirements of the stress protocol. *Salmonella choleraesuis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Clostridium sporogenes* were used for the contamination of the carriers used for stressing. One complete set of inhalation equipment was added to each batch of test solution three times daily. QCT-1, without an added soil load, was used to determine the sporicidal, bactericidal, mycobactericidal and fungicidal activities of the test formulation at the end of the stress period and the virucidal activity was determined according to the ASTM E-1053/97 Method. The microbicidal activity was tested at 20°C for the spores and at 22±2°C for the other microbial groups.

**Results:** The initial pH of the test solutions was about 2.5 and it remained essentially the same at the end of the stress period. The H<sub>2</sub>O<sub>2</sub> concentration of the three lots at the start of the experiment was 1.73; after the 14 days of stress this value was 1.67. All lots were able to reduce the viability titre of the spores by at least 6 log<sub>10</sub> in a contact time of 6 hours and the same level of reduction was achieved with the vegetative bacteria and the mycobacterium in 5 minutes. The required reduction of ≥5 log<sub>10</sub> in the viability of the fungus and a •4 log<sub>10</sub> in poliovirus titer were also achieved with a contact time of 5 minutes.

The neutralization procedures used effectively arrested the activity of the stressed formulation against all test organisms at the end of the contact time. In tests for virucidal activity, its cytotoxicity was successfully removed and the formulation was also found to be free from any interference with virus plaque formation.

**Conclusion:** The results indicated that all three lots of the test formulation maintained their broad-spectrum microbicidal activity even at the end of 14 days of stress.

## **BACKGROUND AND INTRODUCTION**

Reprocessing of heat-sensitive medical devices between patients requires thorough pre-cleaning followed by disinfection (Bond and Favero, 2001). The working solutions of many high-level disinfectants for this purpose are meant for use only once, but others are formulated for reuse in manual systems for 14 to 30 days. Since dilution, evaporation, breakdown and/or neutralization of the active ingredient(s), as well as the accumulation of soil from the devices being processed, can occur during reuse, it is vital to ensure that the solution can retain its broad-spectrum germicidal activity even at the end of the recommended reuse period (Mbithi *et al.*, 1993). Formulations based on accelerated hydrogen peroxide (AHP) are an alternative to more toxic disinfectants such as those based on glutaraldehyde (Sattar, Springthorpe & Rochon, 1998).

In an earlier study, Virox STF, an AHP-based formulation with 7% stabilized hydrogen peroxide was evaluated after 14 days of stress and was found to retain its broad-spectrum microbicidal activity (Sattar *et al.*, 2002). In the present study, 'Prevention', which is another AHP-based formulation with about 2% of hydrogen peroxide as the active, was submitted to similar evaluation in order to know its sporicidal, bactericidal, fungicidal, mycobactericidal and virucidal activities after it was subjected to simulated reuse for 14 days under ambient conditions.

## **OBJECTIVE OF THE STUDY**

The objective of this study was to determine the microbicidal activity of three lots (# 3575, 3576 and 3577) of 'Prevention' after 14 days of stress.

## **SITE OF STUDY**

The study was carried out at the Centre for Research on Environmental Microbiology (CREM), Faculty of Medicine, University of Ottawa. All members of the laboratory staff at CREM are properly trained in the safe handling of infectious agents and hazardous chemicals.

## **MATERIALS AND METHODS**

### **Test samples**

Three lots of the test solution in 20 L white plastic buckets were received in our laboratory. The solutions were clear, pale yellow in color and had a slight acidic odor. The containers were stored at room temperature in an area with controlled access. The study period lasted from March 16, 2004 to May 28, 2004.

### **Challenge microorganisms**

Standard strains of *Pseudomonas aeruginosa* (ATCC 15442), *Staphylococcus aureus* (ATCC 6538), *Salmonella choleraesuis* (ATCC 10708), *Bacillus subtilis* (ATCC 19659), *Clostridium sporogenes* (ATCC 7955), *Mycobacterium terrae* (ATCC 15755), *Trichophyton mentagrophytes* (ATCC 9533) and poliovirus type 1 (Sabin; ATCC VR-192) were used in this

study. They were cultured as follows:

***P. aeruginosa*, *S. aureus* and *S. choleraesuis*:** Stock suspensions of the vegetative bacteria were prepared by culturing them in trypticase soy broth (TSB; Quelab) for 24 hours at 37°C.

***B. subtilis*** spores were grown aerobically in a 1:10 dilution of Columbia broth (Quelab), with manganese, for 72 hours at 37°C. To yield a concentration of 10<sup>9</sup> CFU/mL, the spore suspension was centrifuged, washed and re-suspended in sterile distilled, deionized water (DDW).

***C. sporogenes*** spores were grown anaerobically in undiluted Columbia broth for 5 days at 30°C. To yield a concentration of 10<sup>9</sup> CFU/mL, the spore suspensions were centrifuged, washed and re-suspended in sterile DDW.

***M. terrae*** was grown in Middlebrook 7H9 broth (Difco) with ADC enrichment and glycerol, in vented plug seal capped tissue culture flasks. The test suspension was prepared from stocks grown for 21 days at 37°C. The suspension was washed 3 times by centrifugation at 1,000 x g for 15 minutes and re-suspended in DDW. The final stock suspension was prepared by re-suspending the bacterial pellets in sterile bijoux bottles containing glass beads to about 10<sup>8</sup> CFU/mL. The stock solution was stored at 4°C.

***T. mentagrophytes***, a stock suspension of the conidia was obtained by inoculating the centre of a Mycobiotic Agar plate (Difco) and incubating it at 28°C for 10 days. Mycelial mats were harvested from the agar surface, homogenized with sterile glass beads in normal saline and filtered through sterile cotton gauze to remove the hyphae.

**Poliovirus:** The virus was grown and its infectivity titrated in monolayers of vero cells. To determine virus plaque forming units (PFU) the cells were fixed in 10% formaldehyde and stained with an aqueous solution of crystal violet.

### Soil load

To increase the level of stress to the disinfectant solution, fetal bovine serum (FBS) at a final concentration of 2%, was added to the each container with the test product. The objective of this was to simulate loading with organic material. FBS is universally accepted as a soil load in testing the germicidal activity of liquid chemical disinfectants (CGSB, 1997). It was non-inhibitory for all the organisms used in this study. The addition of contaminated carriers as bioburden and the soaking of several items of respiratory equipment over the 14-days stress cycle further simulated the challenge the product may face under reuse.

### Carriers

**Carriers for the bioburden:** Two types of carriers were used to challenge the test solution with the bacterial bioburden. Pyrex glass beads of 6 mm in diameter (Corning; New York. Catalogue No: 7268-6) were soaked in separate suspensions of *S. choleraesuis*, *S. aureus* and *P. aeruginosa*. Stainless steel penicylinders (8.0 mm OD, 6 mm ID, 10 mm length; Fisher Scientific, Catalog No; 07-907-5) were soaked separately in suspensions of *B. subtilis* and *C. sporogenes* spores. Holding them for 45 minutes at 37°C before use dried the inocula on all the carriers. The same carriers were used repeatedly in the testing with decontamination, washing and autoclave sterilization in between.

**Carriers for the microbicide test:** Glass vials (Galaxy Co Newfield. NJ), 20 mL in capacity, were used as carriers in the quantitative carrier tests (QCT) for bactericidal, sporicidal, mycobactericidal, and fungicidal activities (ASTM, 2000). Glass Petri dishes were used as

carriers in the virucidal activity.

### **PROCEDURE FOR MANUAL REUSE STRESS**

Three plastic containers, each separately containing the test lots, were set up in parallel in the laboratory. The stressing was carried out according to the procedure described before (Sattar *et al.*, 2002). In contrast to the previous study, this investigation used 20 L of each lot of the test disinfectant. The cumulative bioburden was adjusted to give at least  $10^4$  CFU/mL of test solution per day.

As specified in the U.S. EPA (1984) protocol, each bath with the test microbicide received enough bovine serum to give a final serum concentration of 2%. Every day each bath also received 880 glass beads contaminated with each of the three types of vegetative bacteria and 200 penicylinders coated separately with one of the two types of spores. On the first day, each bath received the carriers contaminated with *S. aureus* and *B. subtilis*. The carriers used on the following day were contaminated with *P. aeruginosa* and *C. sporogenes* and those for the third day were contaminated with *S. choleraesuis* and *B. subtilis*. The cycle was repeated for the remaining days of stress.

In addition to the stress from the contaminated carriers, a complete set of inhalation therapy equipment consisting of one flexible, clear plastic CF cuffed tracheal tube 10.02 mm OD/7.5 mm ID, one flexible tubing 1.83 m (6 ft.) in length, one 2.0 L capacity breathing bag, one face mask (C.H. Medical Ltd) and two plastic, 22-mm bifurcate Y-connector, was subjected to three cycles of processing in each microbicide bath on each one of the 14 days of stress. The above equipment set was submitted to a cycle of cleaning with a detergent (Sparkleen 7 for manual washing), a water rinse and a soak in the corresponding disinfectant lots for 30 minutes. Three cycles were run each day. Each morning, before the first challenge, the hydrogen peroxide ( $H_2O_2$ ) concentration and the pH from the test lots were recorded. The  $H_2O_2$  content was determined by iodometric titration according to the Test Procedure, Virox No. 1 FT, "Peroxide Concentration Test" (Virox Technologies Inc., 2000) and a pH meter (Metrohm 632, Swiss) was used to determine the pH.

### **TESTING FOR MICROBICIDAL ACTIVITY**

QCT-1 (Springthorpe & Sattar, 2003) used in this evaluation meets the requirements of the Canadian General Standards Board for testing microbicides to be used on environmental surfaces and medical devices (CGSBA, 1997) and it is an accepted standard of ASTM International (2000). The method is designed to assess the sporicidal, bactericidal, mycobactericidal and fungicidal activities of liquid chemicals. It uses the inside bottom surface of glass vials as the carrier for the challenge microorganism.

Ten  $\mu$ L of the test microbial suspension, without any added soil load, was dried in each carrier and the dried inoculum was then overlaid with 1 mL of the disinfectant sample to be tested. The carriers are held for the required contact time at the desired temperature. The inoculum was eluted and the needed dilutions of the eluate were made and separately passed through membrane filters. The filters were placed on suitable recovery media and incubated, colonies counted and  $\log_{10}$  reductions calculated as described below. Control carriers were used in the same manner as test carriers except saline solution was applied to the dried inoculum instead of the disinfectant.

The virucidal activity was determined according to the ASTM E1053/97 Method. Virus

suspension (200 µL) was placed into the middle of a glass Petri dish and spread with a glass rod. The inoculum was left to dry and then exposed to 2 mL of the test formulation for a contact time of 5 minutes. Earle's balanced salt solution (EBSS) was used for the control samples. At the end of the contact period, 200 µL of the virus-disinfectant mixture were transferred into 1.8 mL of neutralizer to stop the reaction. A 1.2 mL volume of the neutralized samples was layered onto a 5 mL column of Sephadex LH-20. Serial dilutions from the eluates were performed and used for plaque assay.

### **Recovery Media and Detection of Viable Organisms**

For the sporicidal testing with *B. subtilis* and for the bactericidal testing with *S. choleraesuis*, *S. aureus* and *P. aeruginosa*, the filters were placed on TSA plates (Quelab), incubated at 37°C, monitored, and the colony forming units (CFU) recorded at 24-hour intervals for a total of 5 days. For *C. sporogenes*, the filters were placed on FAA plates (Quelab), incubated at 36°C, monitored, and the CFU recorded at 48 hours, and every 24 hour interval thereafter for a total of 5 days. For mycobactericidal testing using *M. terrae*, the filters were placed on 7H11 agar (Difco), incubated at 37°C, monitored, and the CFU recorded at weekly intervals for a total of 4 weeks. For fungicidal testing with *T. mentagrophytes*, the filters were placed on Sabouraud's dextrose agar (SDA; Quelab) and incubated at 28°C, monitored, and the CFU recorded at 3 days, and every 24 hour interval thereafter for a total of 10 days.

### **Plaque assay for the virus**

Monolayers of Vero cells in 12-well cell culture plates were used. The growth medium was removed, and each well received 100 µL of the samples to be evaluated. The plates were kept for 60 minutes at 37°C in a 5% CO<sub>2</sub> atmosphere to allow for virus adsorption. The monolayers were then overlaid with 2 mL of 2 X Eagle's Minimum Essential Medium supplemented with N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid (HEPES), L-glutamine, nonessential amino acids, and 2% FBS, 30mmol/L MgCl<sub>2</sub> and 1.8 % agar. Once the overlay became solidified the plates were incubated for 40 hours in a 5 % CO<sub>2</sub> atmosphere at 37°C. The cells were fixed in 3.7 % formaldehyde in normal saline and stained with 0.1 % solution of crystal violet, and the PFU counted.

### **Cytotoxicity and Interference with Plaque Formation**

This assay was performed to determine the effect of the detoxified test product on cell monolayers and the plaque forming ability of the test virus. 1.2 mL of a 1:10 and 1:100 dilutions of the test product in neutralizer were passed through the Sephadex column to remove cytotoxicity, the filtrates were then placed into three wells each of a 12-well cell culture plate while the other six wells received neutralizer, which was also passed through the column, and EBSS, respectively, as controls. The plate was incubated for 30 minutes. The monolayers were observed under an inverted microscope for signs of toxicity of the test product. In the absence of any apparent cytotoxicity, the monolayers were then washed once with 1 mL of EBSS, the virus was added to each well and allowed to adsorb for 60 minutes. Each cell monolayer was then overlaid with a semisolid overlay and the plates held at 37°C for the development of virus plaques.

### **Microbicide Neutralization Control in Virus**

This was carried out to determine if the neutralization of the sample, followed by detoxification, was sufficient to render it ineffective against the test virus. The test virus (200  $\mu\text{L}$ ) was added to 1.8 mL of the neutralized sample and the mixture was then passed through a Sephadex column. The same amount of virus was added to 1.8 mL of the neutralizer control. The virus eluates were inoculated onto cell monolayer, followed by adsorption for 1 hour and subsequent addition of overlay medium and incubation.

### **Neutralizer, Microbial Diluent and Filter Rinse**

In the four remainder microbial groups, Lethen Broth (with 0.1% sodium thiosulphate pentahydrate) was used as the neutralizer and 0.85 % saline solution was used to make the dilutions and to rinse the membrane filters and the filter holder unit.

### **Product Performance Criteria**

Ten test and three control carriers were used in each QCT-1 test. Three glass Petri dishes were used as carriers for each control and test samples in the virucidal activity. The results are reported as  $\log_{10}$  reductions in viability in reference to the controls. For a sample to be regarded as bactericidal, sporicidal or mycobactericidal, it was necessary to get a reduction in the viability titre of the test organism  $\geq 6 \log_{10}$  under the conditions of the test;  $\geq 5 \log_{10}$  reduction was needed for fungicidal activity and  $\geq 4 \log_{10}$  for virucidal activity (Springthorpe & Sattar, 2003).

### **Neutralizer Efficacy**

The efficiency of the neutralizing system was tested. A countable number of CFU/PFU of the test organism was added to a portion of Lethen broth + disinfectant solution and after a contact time of 5 minutes the mixture was assayed for viable organisms. Lethen broth alone acted as control. Recovery of comparable numbers of viable organisms from test and control solutions would indicate successful neutralization.

## **RESULTS**

### **Sporicidal activity**

Table 1 gives the results of the sporicidal tests. All three lots of the product showed sporicidal activity against *B. subtilis* and *C. sporogenes*, with a reduction in the viability titre of  $>6 \log_{10}$  in a contact time of 6 hours at 20°C.

### **Bactericidal activity**

Table 2 shows the results of the bactericidal activity. The stressed disinfectant displayed bactericidal activity against the three vegetative bacteria. A reduction in the viability titre of  $>6 \log_{10}$  in a contact time of 5 minutes was obtained.

**Table 1. Sporidical activity of the stressed formulation after a contact of six hours at 20°C.**

Lot No.	Date of expt.	CFU per control carrier	CFU per test carrier	Log <sub>10</sub> reduction
<i>Bacillus subtilis</i>				
3575	04/07/04	2.79 x 10 <sup>6</sup>	0	6.45
3576	04/07/04	2.79 x 10 <sup>6</sup>	0	6.45
3577	04/07/04	2.79 x 10 <sup>6</sup>	0	6.45
<i>Clostridium sporogenes</i>				
3575	05/26/04	3.02 x 10 <sup>6</sup>	0	6.48
3576	05/26/04	3.02 x 10 <sup>6</sup>	0	6.48
3577	05/26/04	3.02 x 10 <sup>6</sup>	0	6.48

**Table 2. Bactericidal activity of the stressed formulation after a contact of 5 minutes.**

Lot No.	Date of expt.	CFU per control carrier	CFU per test carrier	Log <sub>10</sub> reduction
<i>S. aureus</i>				
3575	08/12/04	5.48 x 10 <sup>6</sup>	0	6.73
3576	08/12/04	5.48 x 10 <sup>6</sup>	0	6.73
3577	08/12/04	5.48 x 10 <sup>6</sup>	0	6.73
<i>P. aeruginosa</i>				
3575	08/24/04	1.49 x 10 <sup>7</sup>	0	7.17
3576	08/24/04	1.49 x 10 <sup>7</sup>	0	7.17
3577	08/24/04	1.49 x 10 <sup>7</sup>	0	7.17
<i>S. choleraesuis</i>				
3575	08/25/04	8.28 x 10 <sup>6</sup>	0	6.91
3576	08/25/04	8.28 x 10 <sup>6</sup>	0	6.91
3577	08/25/04	8.28 x 10 <sup>6</sup>	0	6.91

### **Mycobactericidal activity**

As summarized in Table 3, all three lots of the product showed mycobactericidal activity, with a reduction in the viability titre of >6 log<sub>10</sub> in a contact time of 5 minutes.

### **Fungicidal activity**

As shown in Table 4, all three lots of the product also showed fungicidal activity of >6 log<sub>10</sub> in a contact time of 5 minutes, higher than the product performance criterion of 5 log<sub>10</sub>.

**Table 3. Mycobactericidal activity of the stressed formulation after a contact of 5 minutes.**

Lot No.	CFU/Control Carrier	CFU/Test Carrier	Log <sub>10</sub> Reduction
3575	3.07 x 10 <sup>6</sup>	0	6.49
3576	3.07 x 10 <sup>6</sup>	0	6.49
3577	3.07 x 10 <sup>6</sup>	0	6.49

**Table 4. Fungicidal activity of the stressed formulation after a contact time of 5 minutes**

Lot No.	Date of expt.	CFU/control carriers	CFU/test carrier	Log <sub>10</sub> reduction
3575	04/16/04	1.32 x 10 <sup>6</sup>	0	6.12
3576	04/16/04	1.32 x 10 <sup>6</sup>	0	6.12
3577	04/16/04	1.32 x 10 <sup>6</sup>	0	6.12

### Virucidal activity

The results for virucidal activity are given in Table 5. All three lots of 'Prevention' showed virucidal activity with a reduction in the viability titre of >4 log<sub>10</sub> in a contact time of 5 minutes.

**Table 5. Virucidal activity of the stressed formulation after a contact time of 5 minute.**

Lot No.	Date of the expt.	PFU/Control	PFU/Test	Log <sub>10</sub> Reduction
3575	04/09/09	1.35 x 10 <sup>4</sup>	0	4.13
3576	04/09/09	1.35 x 10 <sup>4</sup>	0	4.13
3577	04/09/09	1.35 x 10 <sup>4</sup>	0	4.13

### Cytotoxicity of the test product

A 1:10 dilution of the product in the neutralizer, followed by gel filtration, showed no apparent toxicity for the cell line used for the study.

### Interference with Plaque Formation

Pre-exposure of the cell monolayer to a 1:10 dilution of the test product in the neutralizer, followed by gel filtration, did not interfere with the plaque formation by the test virus.

### Neutralizer Efficacy

The results given in Table 6 show that the neutralizer used in this study was able to

effectively arrest the sporicidal, mycobactericidal, fungicidal and bactericidal activities of the test disinfectant at the end of the contact time.

### Neutralization of Virucidal Activity

The neutralization of the test product at the end of the contact time, followed by gel filtration, was sufficient to arrest its virucidal activity. The results are given Table 6.

**Table 6. Effectiveness of product neutralization.**

Test organism	Number of colonies on plates after exposure to neutralizer and test solution	Number of colonies on plate after exposure to neutralizer
<i>B. subtilis</i>	65, 77	63, 61
<i>C. sporogenes</i>	108, 100	79, 81
<i>M. terrae</i>	69, 77	59, 62
<i>T. mentagrophytes</i>	52, 54	43, 36
<i>S. aureus</i>	57, 54	58, 57
<i>P. aeruginosa</i>	81, 75	93, 92
<i>S. choleraesuis</i>	90, 91	94, 96
Poliovirus	20, 23, 24	19, 22, 23

### Hydrogen peroxide levels and pH

The hydrogen peroxide concentration and the pH were monitored after 7 and 14 days of stress and they did not show any significant change (Table 7).

**Table 7. H<sub>2</sub>O<sub>2</sub> concentration and pH of the disinfectant solutions during stress.**

Lot No.	Days of stress	pH	% H <sub>2</sub> O <sub>2</sub>
3575	7	2.51	1.73
3576	7	2.53	1.73
3577	7	2.55	1.73
3575	14	2.43	1.67
3576	14	2.45	1.67
3577	14	2.45	1.67

### CONCLUSIONS

All three lots of disinfectant 'Prevention' maintained their sporicidal, bactericidal, mycobactericidal, fungicidal and virucidal activities after 14 days of simulated reuse.

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