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**Final Report submitted to Virox Technologies
Mississauga, Ontario**

**ASSESSMENT OF THE GERMICIDAL
ACTIVITY OF A MODIFIED FORMULATION
BASED ON 7% ACCELERATED HYDROGEN
PEROXIDE (AHP)**

Syed A. Sattar, Ph.D.
Professor of Microbiology and Director
Centre for Research on Environmental Microbiology (CREM)
Faculty of Medicine, University of Ottawa
Ottawa, Ontario, Canada
K1H 8M5

*Phone: (613) 562-5800 ext. 8314; Fax: (613) 562-5452
E-mail: ssattar@uottawa.ca*

This study was conducted with the technical assistance of
Ms. Sola Adegbunrin, M.Sc. and Ms. Teresa Burke

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01/12/01 14:01 FAX 613 562 5452

MICROBIOL & IMMUN

003

2

TABLE OF CONTENTS

	Page No.
A. OBJECTIVE	3
B. MATERIALS AND METHODS	3
The Product	3
Carriers	3
Soil Load	3
Neutralizer, Microbial Diluent and Filter Rinse	3
Test Organisms	3
1. <i>Bacillus subtilis</i> (ATCC 19659)	3
2. <i>Clostridium sporogenes</i> (ATCC) 7955	3
3. <i>Mycobacterium terrae</i> (ATCC 15755)	3
4. <i>Trichophyton mentagrophytes</i> (ATCC 9533)	3
C. THE QUANTITATIVE CARRIER TEST	4
The Method for Testing Bactericidal Activities	4
Recovery Media and Detection of Viable Organisms	6
Controls	6
D. PRODUCT PERFORMANCE CRITERIA	6
E. RESULTS	7
Activity of Modified AHP against <i>Bacillus subtilis</i>	7
Activity of Modified AHP against <i>Clostridium sporogenes</i>	7
Activity of Modified AHP the against <i>Mycobacterium terrae</i>	8
Activity of Modified AHP the against <i>Trichophyton mentagrophytes</i>	8
F. DISCUSSION AND CONCLUDING REMARKS	8
G. LITERATURE CITED	8

Final Report on the Germicidal Activity of Modified Virox AHP, January, 2001

01/12/01 14:01 FAX 613 562 5452

MICROBIOL & IMMU

004

3

A. OBJECTIVE

The objective of this study was to determine if the product is sporicidal, fungicidal and mycobactericidal at full strength when tested using a quantitative carrier test protocol.

B. MATERIALS AND METHODS

The Product: Three lots of the product, as supplied by the sponsor, were tested in this study. Once received here, they were stored at room temperature in a place where only authorized personnel had access.

Carriers: Glass vials were used as hard surface carriers for sporicidal, mycobactericidal and fungicidal tests.

Silk suture loops are not used as carriers in our laboratory because they are extremely difficult to work with in a standardized test method. We also do not consider them representative or relevant as carriers for materials currently subjected to disinfection by liquid chemicals. Therefore, silk suture loops were not used in this study.

Neutralizer, Microbial Diluent and Filter Rinse: Lethen Broth (with 0.1% sodium thiosulphate pentahydrate) was used as the neutralizer. It was also used to rinse the membrane filters and the filter holder unit. Phosphate buffer (PB), at pH 7.2, was used as diluent and final filter rinse in sporicidal test; this was replaced with normal saline (0.85% NaCl) in mycobactericidal and fungicidal tests. Both of these solutions worked well in rinsing out the froth created by the Lethen broth.

Soil Load: For inoculation of the carriers, all test organisms were first suspended in bovine serum at a final concentration of 5%.

Test Organisms: The organisms used and their specific strains are given below:

1. *Bacillus subtilis* (ATCC 19659): *B. subtilis* spores were grown aerobically in a 1:10 dilution of Columbia broth (Difco), with manganese, for 72 hours at 37°C. To yield a concentration of 10^9 spores/mL, the spore suspension was centrifuged, washed and resuspended in sterile distilled, deionized water.

2. *Clostridium sporogenes* (ATCC 7955): *C. sporogenes* spores were grown anaerobically in undiluted Columbia broth for 5 days at 30°C. To yield a concentration of 10^9 spores/mL, the spore suspension was centrifuged, washed and resuspended with sterile distilled, deionized water.

3. *Mycobacterium terrae* (ATCC 15755): *M. terrae* was grown in 7H9 broth (Difco) containing glycerol but no antibiotics. The bacterial suspension was centrifuged at 2,500 rpm for 15 minutes and the pellet resuspended to give approximately 2.5×10^8 cells/mL (No. 8 McFarland Standard).

4. *Trichophyton mentagrophytes* (ATCC 9533): A stock suspension of the conidia was obtained by inoculating the center of a Mycobiotic Agar plate 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 to remove the hyphae. (AOAC, 1990)

Final Report on the Germicidal Activity of Modified Virox AHP, January, 2001

C. THE TEST METHODOLOGY

The quantitative carrier test used in this evaluation has been designed to: (a) permit the determination of the exact number of colony forming units (CFU) placed on each carrier and the CFU remaining after the drying of the inoculum, (b) avoid wash-off of any cells of the test organism, (c) allow complete recovery of the inoculum from the carrier surface, (d) arrest the test product's activity by dilution immediately at the end of the contact time, (e) capture all the cells of the test organism on a membrane filter before and after exposure to the test product, (f) removal of any residual germicidal activity by a thorough rinsing of the membrane filter, (g) allow a ratio of 1:100 between the volume of the test microbial inoculum and the volume of the product being evaluated, (h) incorporation of glass inserts to eliminate any false-positive results due to the generation of micro-aerosols in the carriers and (i) give a precise determination of \log_{10} reduction in CFU of the test organism after exposure to the product under test.

This new test method, therefore, eliminates the deficiencies associated with the AOAC Use-Dilution Test (AOAC, 1990) while meeting the Canadian General Standards Board's requirements for germicide test methodology (CGSB, 1997). This method has recently been accepted as a standard of the American Society for Testing and Materials (ASTM, 2000).

The Basic Procedure: The Flow Chart outlines the methodology used for the germicide test.

Recovery Media and Detection of Viable Organisms: For sporicidal testing with *B. subtilis*, the filters were placed on trypticase soy agar (TSA) plates, incubated at 37°C, monitored, and colony forming units (CFU) recorded at 24 hour intervals for a total of 5 days. For *C. sporogenes*, the filters were placed on fastidious anaerobic agar (FAA), incubated at 30°C, monitored, and 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, incubated at 37°C, monitored, and CFU recorded at weekly intervals for a total of 4 weeks. For fungicidal testing with *T. mentagrophytes*, the filters were placed on Sabouraud agar and incubated at 28°C, monitored, and CFU recorded at 4 days, and every 24 hour interval thereafter for a total of 10 days.

Controls: Control carriers were used in the same manner as test carriers except phosphate buffer was applied to the dried inoculum instead of disinfectant for the sporicidal and bactericidal tests, and sterile saline is applied to the dried inoculum instead of disinfectant for the mycobactericidal and fungicidal tests.

D. PRODUCT PERFORMANCE CRITERIA

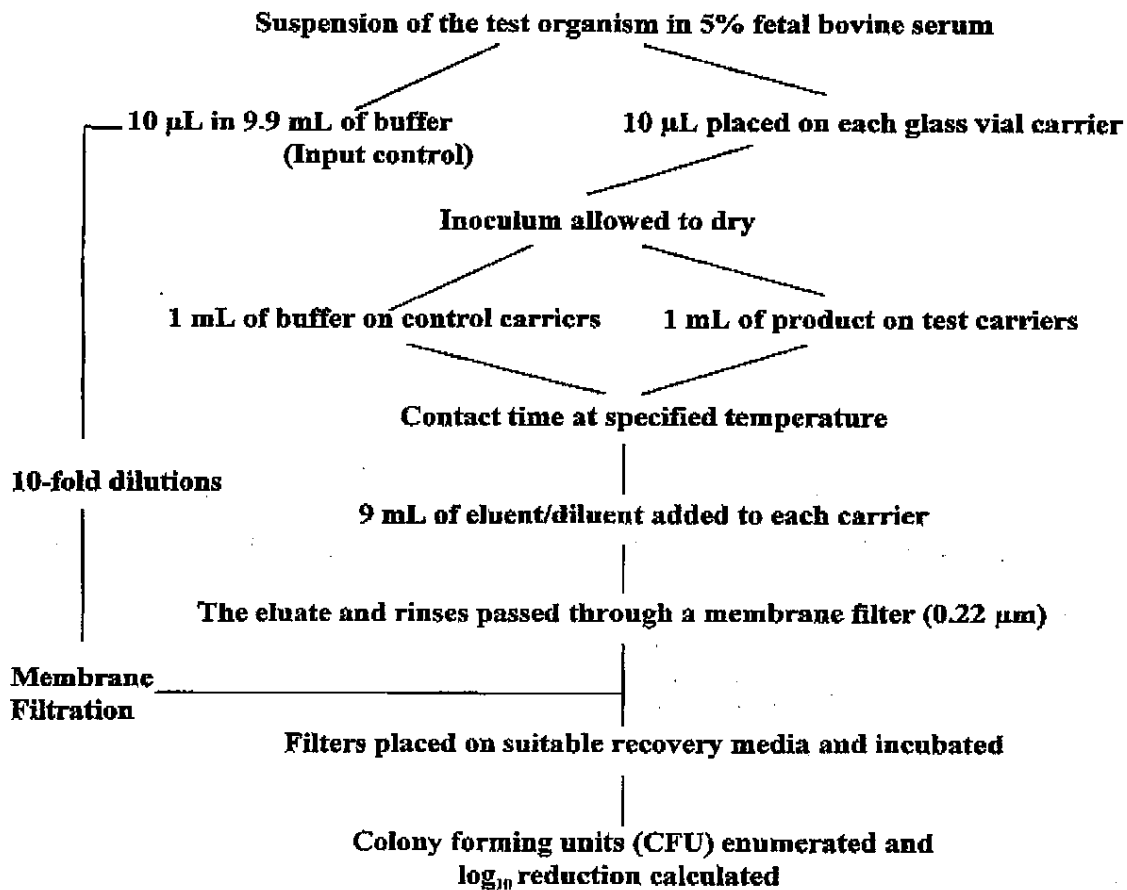
The number of test carriers ranged between 6-10. Three control carriers were incorporated in each test. The results are reported as \log_{10} reductions in viability in reference with the control carriers. For a product to be considered sporicidal or mycobactericidal, it is expected to reduce the titer of all the test organisms by a minimum of 6 \log_{10} (at least 1 million-fold) under the conditions of this test. A minimum of 5 \log_{10} was expected for the fungicidal activity

01/12/01 14:03 FAX 613 562 5452

MICROBIOL & IMMU

006

5

FLOW CHART***BASIC METHOD FOR TESTING GERMICIDES USING THE QUANTITATIVE CARRIER TEST**

*This basic procedure was used for testing sporicidal, mycobactericidal and fungicidal activities of the test product(s).

E. RESULTS

Activity of Modified AHP against the spores of *Bacillus subtilis*: Table 1 summarizes the results of the testing against the spores of *B. subtilis*. The product was able to bring about a >7 \log_{10} reduction in the titre of the spores in a contact time of 6 hours at $20\pm 1^\circ\text{C}$, indicating activity against this organism in our test protocol.

Table 1. Activity of full-strength Modified AHP against the spores of *Bacillus subtilis*.

Lot Number	Date of Expt.	Contact Time	Number of Carriers	CFU/Control Carrier	CFU/Test Carrier	Log Reduction
2319	April. 4, 2000	6 hours	6	1.01×10^7	1	7.03
2319	April. 6, 2000	6 hours	6	1.23×10^7	0	7.09
2435	July 13, 2000	6 hours	10	2.21×10^7	0	7.34
2436	July 13, 2000	6 hours	10	2.21×10^7	0	7.34

Activity of Modified AHP against the spores of *Clostridium sporogenes*: As shown in Table 2, the product was able to bring about a >7 \log_{10} reduction in the titre of *C. sporogenes* spores in a contact time of 6 hours at $20\pm 1^\circ\text{C}$, indicating activity against this organism.

Table 2. Activity of full-strength Modified AHP against the spores of *C. sporogenes*

Lot Number	Date of Expt	Contact Time	Number of Carriers	CFU/Control Carrier	CFU/Test Carrier	Log Reduction
2319	April. 12, 2000	6 hours	6	5.33×10^7	0	7.73
2435	July.07, 2000	6 hours	10	1.68×10^8	0	8.23
2436	July.07, 2000	6 hours	10	1.68×10^8	0	8.23

Activity of Modified AHP against *Mycobacterium terrae*: The product proved to be mycobactericidal by reducing the titer of *M. terrae* by >6 \log_{10} in 20 minutes at $20\pm 1^\circ\text{C}$, as summarized in Table 3.

Table 3. Activity of full-strength Modified AHP on *Mycobacterium terrae*

Lot Number	Date Of Expt	Contact Time	Number of Carriers	CFU/Control Carrier	CFU/Test Carrier	\log_{10} Reduction
2319	March.13, 2000	20 min	10	1.13×10^6	1	6.05
2319	March 15, 2000	20 min	10	1.63×10^6	0	6.21
2435	August.03, 2000	20 min	10	1.83×10^6	0	6.26
2436	August.08, 2000	20 min	10	8.33×10^6	0	6.92

Activity of Modified AHP against the spores of *T. mentagrophytes*: As can be seen from Table 4, the product was able to bring about a $>5 \log_{10}$ reduction in the viability titre of *T. mentagrophytes* conidia in a contact time of 5, 10 and 20 minutes respectively at $20 \pm 1^\circ\text{C}$, indicating fungicidal activity against this organism.

Table 4. Activity of full-strength Modified AHP against the conidia of *T. mentagrophytes*

Lot Number	Date Of Expt	Contact Time	Number of Carriers	CFU/Control Carrier	CFU/Test Carrier	Log Reduction
2319	April.11, 2000	5 min	6	6.33×10^5	0	5.80
2319	April.11, 2000	10 min	6	6.33×10^5	0	5.80
2319	April.11, 2000	20 min	6	6.33×10^5	0	5.80
2435	July. 07, 2000	20 min	10	1.14×10^6	0	6.05
2436	July.07, 2000	20 min	10	1.14×10^6	0	6.05

F. DISCUSSION AND CONCLUDING REMARKS

This study used a fully quantitative hard surface carrier test to determine the germicidal activity of Modified 7% AHP. All three lots of the product tested proved to be fungicidal, sporicidal and mycobactericidal. The procedure, as reported here, met the testing requirements of the Canadian General Standards Board (1990) except that it used the flat surface of a glass vial instead of penicylinders.

F. LITERATURE CITED

- AOAC (1990); *Official Methods of Analysis of the AOAC*. AOAC, Washington, D.C.
 Am. Soc. Testing & Materials (2000). Standard Quantitative Carrier Test Method to Evaluate the Bactericidal, Fungicidal, Mycobactericidal and Sporicidal Activities of Liquid Chemical Germicides. ASTM, West Conshohocken, PA. (Approved in Oct., 2000).
 Canadian General Standards Board (1997); *Assessment of Efficacy of Antimicrobial Agents for Use on Environmental Surfaces and Medical Devices*. Document #: CAN/CGSB-2.161-M91. CGSB, Ottawa, Canada.