

Final Report submitted to Virox Technologies Inc.
Oakville, Ontario

**ASSESSMENT OF THE MICROBICIDAL ACTIVITY
OF AN ACCELERATED HYDROGEN PEROXIDE-
BASED FORMULATION (AHP-5) AGAINST VRE AND
MRSA**

Syed A. Sattar, Ph.D.

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



Signature

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A. INTRODUCTION

This study on the microbicidal activity of Virox AHP-5 incorporated testing against two different types of antibiotic-resistant bacteria. The evaluation was conducted using a quantitative carrier test developed in our laboratory, which is now a standard (E-2111) of ASTM International (ASTM, 2000). The test method used also incorporates all the important elements specified in the standard of the Canadian General Standards Board (CGSB 1997).

B. OBJECTIVE

The main objective of this study was to determine the bactericidal activities of the product at a dilution of 1:16 using a quantitative carrier test method.

C. MATERIALS AND METHODS

The Product:

Three separate lots of AHP-5 were provided for testing in this study. Upon arrival in our laboratory, the bottles were stored at room temperature in a place with restricted access. The product was tested at a dilution of 1:16 in hard water with 200 ppm as CaCO₃.

Carriers:

The inside bottom surface of glass vials (Galaxy Co., Newfield, NJ) was used as the carrier surface.

Soil Load:

The test bacteria were first suspended in a tripartite soil load: 25 µL of bovine serum albumin, 100 µL of mucin and 35 µL of tryptone were added to 340 µL of the bacterial suspension. The soil load mixture contains a level of protein roughly equal to that in 5% serum

Neutralizer, Microbial Diluent and Filter Rinse:

Lethen Broth (with 1% sodium thiosulfate pentahydrate) was used as the neutralizer and to rinse the membrane filters and the filter holder unit. Normal saline was used to make dilutions of the bacterial suspensions and as the final rinse of the carrier vials and the filter holder unit to aid in rinsing off the froth created by the Lethen broth.

Test Organisms:

Seed culture of **Methicilin-Resistant *Staphylococcus aureus* (MRSA, ATCC 29247)** and **Vancomycin-Resistant *Enterococcus* (VRE; ATCC 51299)** were received through the courtesy of Dr. Frank Chan of the Children's Hospital of Eastern Ontario, Ottawa, ON.

Stock suspensions of the two vegetative bacteria were prepared by culturing them in tryptic soy broth (TSB; Difco) for 24 hours at 37°C.

D. THE TEST METHODOLOGY

Quantitative Carrier Test:

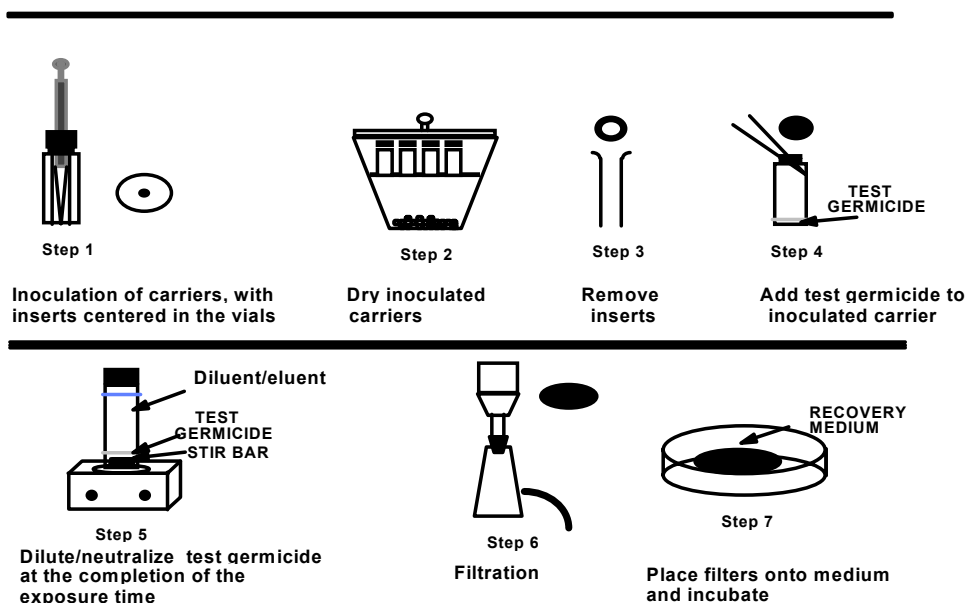
The quantitative carrier test used in this evaluation has been designed to: (a) permit the determination of the exact number of colony forming units (CFU) or plaque forming units (PFU) placed

on each carrier and the CFU/PFU remaining after the drying of the inoculum, (b) avoid wash-off of any 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) in the case bactericidal tests, capture all the bacterial 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). As stated above, it is now an accepted standard of ASTM

The Method for Testing Bactericidal Activity: The general equipment and procedure for testing are given in Figure 1 and Flow Chart 1, respectively.

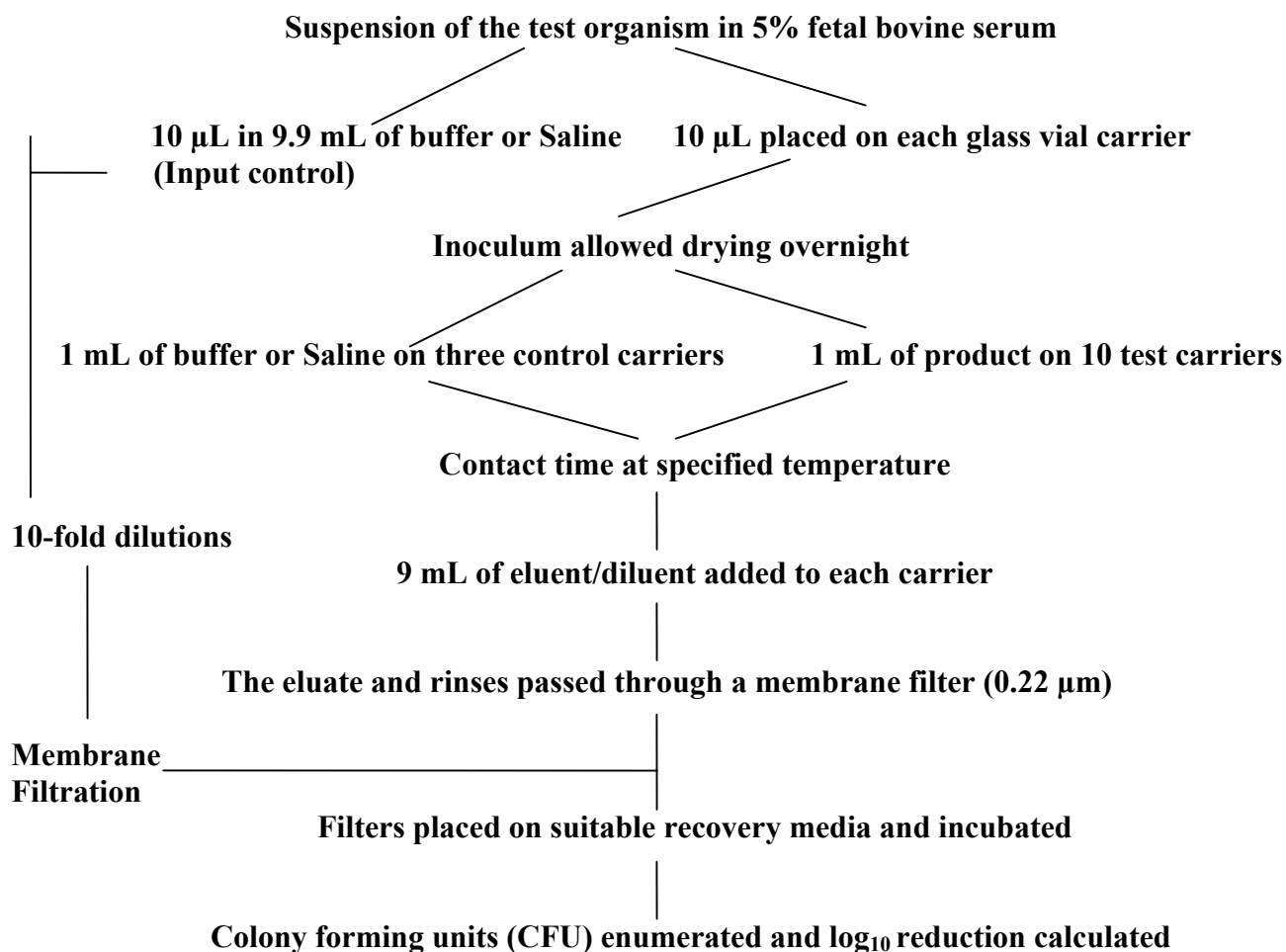
FIGURE 1.

GENERAL STEPS FOR THE QUANTITATIVE CARRIER TEST



FLOW CHART 1

THE BASIC QUANTITATIVE CARRIER METHOD FOR TESTING THE BACTERICIDAL ACTIVITIES OF LIQUID CHEMICAL GERMICIDES



The test involved drying a microbial suspension on a hard surface carrier and covering the dried inoculum with the use-dilution of the disinfectant for the specified contact time at room temperature. At the end of the contact time, an eluent/rinse was used to recover the reaction mixture from the carrier and the eluate was passed through a membrane filter (0.22µm pore diameter) to capture the test organism. The filters were then placed on plates of suitable recovery agar medium and incubated to allow viable organisms to form visible colonies. The numbers of colony forming units (CFU) were recorded and the level of inactivation of the test organism was calculated.

Recovery Media and Detection of Viable Organisms:

For bactericidal testing using, VRE and MRSA, the filters were placed on TSA plates, incubated at 37°C, monitored, and the CFU recorded at 24 hour intervals for a total of 5 days.

Controls:

For the quantitative carrier test for bactericidal activity, control carriers were used in the same manner as test carriers except that normal saline was applied to the dried inoculum instead of the test product.

Neutralization Verification:

Bactericidal Test:

One part of the use-dilution of the product was mixed with 9 parts of the neutralizer. The test organism was added to the neutralized solution. The neutralizer alone was used as the control solution. At the end of a contact time of 5 minutes at room temperature, the mixture was passed through a membrane filter to capture the bacteria. The filters were placed on TSA plates the appropriate recovery medium. The plates were incubated and the colonies counted.

The time of 5 minutes was selected in these experiments because it is the maximum delay that may occur between the initial dilution of the product in the vial and the last lot of rinse passed through the membrane filter.

E. PRODUCT PERFORMANCE CRITERIA

The number of test carriers in the bactericidal test was 6. Each test also included three control carriers. The results are reported as log₁₀ reductions in viability in reference to the control carriers.

For a product to be considered bactericidal it was expected to reduce the viability titre of each test organism by at least 5 log₁₀ under the conditions of this test.

F. RESULTS

Activity of the product against MRSA: Table 1 summarizes the results of the QCT I tests. All three lots were able to bring about a >6-log₁₀ reduction in the viability titre of MRSA in a contact time of 5 minutes at room temperature indicating bactericidal activity against this organism.

Table 1: The activity of a 1:16 dilution of AHP-5 against Methicilin Resistant *S. aureus*

Lot Number	Date of Experiment	Contact Time (minutes)	CFU/Control Carrier	CFU/test Carrier	Log ₁₀ Reduction
3783	19/08/04	5	7.27 X 10 ⁶	0	6.83
3815	19/08/04	5	7.27 X 10 ⁶	0	6.83
3816	19/08/04	5	7.27 X 10 ⁶	0	6.83

Activity of the product against VRE: Table 2 summarizes the results of the QCT I tests. All three lots were able to bring about a >5-log₁₀ reduction in the viability titre of VRE in a contact time of 5 minutes at room temperature indicating bactericidal activity against this organism.

Table 2: The activity of a 1:16 dilution of AHP-5 against Vancomycin-Resistant *Enterococcus*

Lot Number	Date of Experiment	Contact Time (minutes)	CFU/Control Carrier	CFU/test Carrier	Log ₁₀ Reduction
3783	1/09/04	5	3.74 X 10 ⁵	0	5.74
3815	1/09/04	5	3.74 X 10 ⁵	0	5.74
3816	1/09/04	5	3.74 X 10 ⁵	0	5.74

Neutralization to arrest microbicidal activity: Table 3 summarizes the result of the neutralization test. The absence of any significant difference in the number of colonies of the test organism in the test and control was taken to mean that a 1:10 dilution of the product in the neutralizer was sufficient to arrest its germicidal activity.

Table 2: Neutralization Verification of AHP-5

Test Organism	Product dilution used in testing	Number of colonies on plates after exposure to a 10-fold dilution of the test solution in the neutralizer	Number of colonies on plates after exposure to the neutralizer
<i>MRSA</i>	1:16	101/98	83/85
<i>VRE</i>	1:16	115/131	115/112

G. CONCLUDING REMARKS

The formulation tested was able to meet the product performance criteria under the conditions of the testing carried out in this study.

H. REFERENCES

American Society for Testing and Materials (2000); Document # E-2111-00, ASTM, West Conshohocken, PA.

Canadian General Standards Board (1991); Assessment of Efficacy of Antimicrobial Agents for Use on Environmental Surfaces and Medical Devices. Document; #CAN/CGSB-2.161-M91. CGSB, Ottawa, Canada.