

**Final Report submitted to Virox Technologies Inc.
Oakville, Ontario**

**ASSESSMENT OF THE BACTERICIDAL ACTIVITIES
OF ACCEL HYDROTHERAPY PLUS**

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

E. mail: ssattar@uottawa.ca

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A. OBJECTIVE

The main objective of this study was to evaluate the bactericidal activities of Accel Hydrotherapy Plus using a quantitative carrier test method (Springthorpe and Sattar, 2005) and its effectiveness as a sanitizer using a suspension test method. The first tier of the quantitative carrier test (QCT-1) used to assess the bactericidal activities of the formulation was ASTM International's protocol #E-2111 (ASTM 2000) which incorporates all important elements specified in the Canadian General Standard Board's document number CAN/CGSB-2.161-97 entitled *Assessment of Efficacy of Antimicrobial Agents for use on Environmental Surfaces and Medical Devices* (CGSB 1997).

B. MATERIALS AND METHODS

The Product:

Three separate lots of Accel Hydrotherapy Plus 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.

Carriers:

The inside bottom surface of glass vials (Galaxy Co., Newfield, New Jersey) was used as the carrier surface for the quantitative carrier test.

Soil Load:

For inoculation of the carriers, all the test organisms 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. This soil load mixture contained a level of protein roughly equal to that in 5% bovine serum. No soil load was used in the sanitizer test.

Neutralizer, Microbial Diluent and Filter Rinse:

Lethen Broth (with 0.1% sodium thiosulphate 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.

Standard Hard Water:

Water with 400 ppm as calcium carbonate (CaCO₃) was used as the diluent for the product in the quantitative carrier test while water with 200 ppm hardness was used in the sanitizer test (AOAC 1990).

Test Organisms:

The organisms used and their specific strain number, where available, are given below:

1. *Staphylococcus aureus* (ATCC 6538)
2. *Pseudomonas aeruginosa* (ATCC 15442)
3. *Salmonella choleraesuis* (ATCC 10708)
4. Vancomycin-Resistant *Enterococcus* (clinical isolate)
5. Methicillin-Resistant *Staphylococcus aureus* (clinical isolate)
6. *Trichophyton mentagrophytes* (ATCC 9533)

a) **Bacteria:** Stock suspensions of all the bacteria were prepared by culturing them in tryptic soy broth (TSB; Difco) for 24 hours at 37°C.

b) *Trichophyton mentagrophytes* (ATCC 9533): A stock suspension of the conidia was obtained by inoculating the center of a Sabouraud Dextrose 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 gauze to remove the hyphae

C. TESTS TO ASSESS THE MICROBICIDAL ACTIVITIES:

1. Quantitative Carrier Test:

QCT-1 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 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 microbicidal 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 test method, therefore, eliminates the deficiencies associated with the AOAC Use-Dilution Test (Springthorpe and Sattar 2005).

2. The Suspension Test:

The test was carried out by adding 100 μL of the bacterial suspension without soil load to 900 μL of the test product in a 2 mL capacity cryovial, vortexed to mix and allowed to sit for the required contact time at room temperature. At the end of the contact time, the reaction mixture received 9.0 mL of the neutralizer and vortexed. This mixture was passed through a membrane filter and the vial was rinsed 2X with 10.0 mL of saline. The membrane filtration technique used was the same as that in the quantitative carrier test for bactericidal activity.

Recovery Media and Detection of Viable Organisms:

The control suspensions and the test samples were passed through 47 mm diameter membrane filters (Millipore; 0.22 μm pore diameter). The filters were placed on TSA plates, incubated at 37°C, and the colony forming units (CFU) recorded at 24 hour intervals for a total of 5 days. For *T. mentagrophytes*, the filters were placed on Sabouraud dextrose agar and incubated at 28°C, monitored, and the CFU recorded at 4 days, and every 24 hour interval thereafter for a total of 10 days

Controls:

For the quantitative carrier test for fungicidal and 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. In the suspension test; 100 μL of bacterial suspension was added to 9.0 mL of saline instead of the disinfectant.

Neutralization Verification:

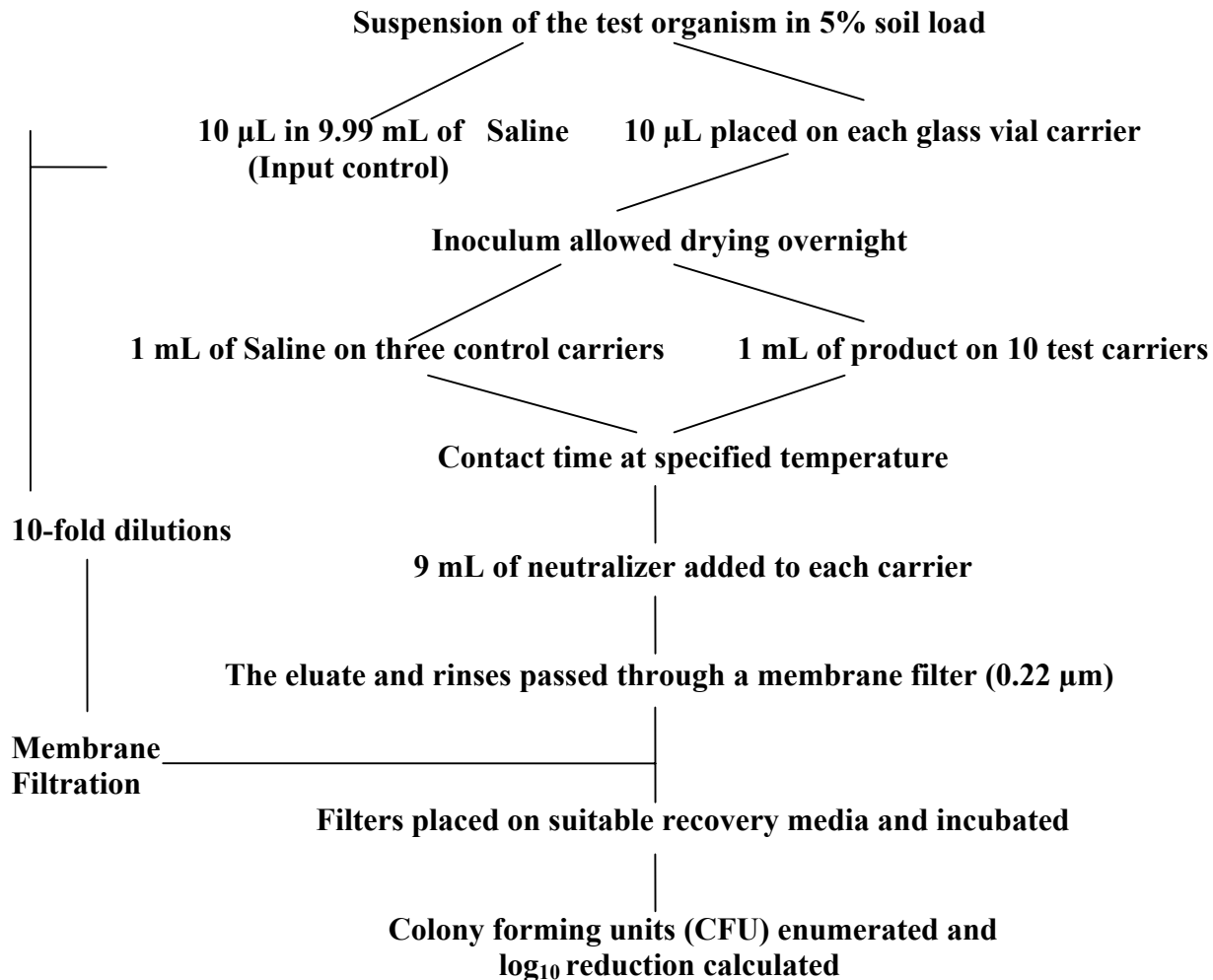
One part of the use-dilution of the product was mixed with 9 parts of the neutralizer. The test organism was added to the neutralized solution to give an estimated 20-100 CFU. The

neutralizer alone was used as the control solution. At the end of a contact time of 5 minutes at 20°C, the mixture was passed through a membrane filter to capture the bacteria. The filters were placed on the appropriate recovery medium. The plates were incubated for 24 hours at 37°C 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 carrier vial and the last lot of rinse passed through the membrane filter.

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.

PRODUCT PERFORMANCE CRITERIA

In each bactericidal and fungicidal test, 10 test carriers and 3 control carriers were used. The results are reported as \log_{10} reductions in viability in reference to the control carriers. Under the conditions of this test, for the product to be considered microbicidal, it was expected to reduce the viability of the test organisms by a minimum of 6 \log_{10} . The number of repeats in the suspension test was six. The test also included three control repeats; for the product to be considered bactericidal, it was expected to reduce the viability titre of each test organism by at least 4 \log_{10} under the conditions of this test.

D. RESULTS

Activity of the product against *Staphylococcus aureus* (Carrier Test Method): Table 1 summarizes the results of tests against *S. aureus*. All three lots of the product were able to bring about a $>6\log_{10}$ reduction in the viability titre of *S. aureus* 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 Accel Hydrotherapy Plus against *Staphylococcus aureus* in a Contact Time of 5 Minutes

Lot Number	Date of Experiment	CFU/Control Carrier	CFU/Test Carrier	Log ₁₀ Reduction
5001	08/11/05	5.05×10^6	0	6.70
5002	08/11/05	5.05×10^6	0	6.70
5003	08/11/05	5.05×10^6	0	6.70

Activity of the product against *Staphylococcus aureus* (Suspension Test Method): Table 2 summarizes the results of the suspension test. All three lots were able to bring about a 5 \log_{10} reduction in the viability titre of *S. aureus* in a contact time of 30 seconds at room temperature, indicating bactericidal activity against this organism.

Table 2: The activity of a 1:128 Dilution of Accel Hydrotherapy Plus against *Staphylococcus aureus* in a Contact Tim of 30 Seconds

Lot Number	Date of Experiment	CFU/Control	CFU/Test	Log ₁₀ Reduction
5001	28/11/05	1.00×10^5	0	5.01
5002	28/11/05	1.00×10^5	0	5.01
5003	28/11/05	1.00×10^5	0	5.01

Activity of the product against *Pseudomonas aeruginosa* (Carrier Test Method): Table 3 summarizes the results of tests against *P. aeruginosa*. All three lots of the product were able to bring about a $>7 \log_{10}$ reduction in the viability titre of *P. aeruginosa* in a contact time of 5 minutes at room temperature, indicating bactericidal activity against this organism.

Table 3: The activity of a 1:16 Dilution of Accel Hydrotherapy plus against *Pseudomonas aeruginosa* in a Contact Time of 5 Minutes

Lot Number	Date of Experiment	CFU/Control Carrier	CFU/Test Carrier	Log ₁₀ Reduction
5001	10/11/05	1.17 x 10 ⁷	0	7.04
5002	10/11/05	1.17 x 10 ⁷	0	7.04
5003	10/11/05	1.17 x 10 ⁷	0	7.04

Activity of the product against *Pseudomonas aeruginosa* (Suspension Test Method): Table 4 summarizes the results of the suspension test. All three lots were able to bring about a >4 log₁₀ reduction in the viability titre of *P. aeruginosa* in a contact time of 30 seconds at room temperature indicating bactericidal activity against this organism.

Table 4: The activity of a 1:128 Dilution of Accel Hydrotherapy Plus against *Pseudomonas aeruginosa* in a Contact Time of 30 Seconds

Lot Number	Date of Experiment	CFU/Control	CFU/Test	Log ₁₀ Reduction
5001	28/11/05	8.57 x 10 ⁴	0	4.93
5002	28/11/05	8.57 x 10 ⁴	0	4.93
5003	28/11/05	8.57 x 10 ⁴	0	4.93

Activity of the product against *Salmonella choleraesuis* (Carrier Test Method): Table 5 summarizes the results of tests against *Salmonella choleraesuis*. All three lots of the product were able to bring about a >6 log₁₀ reduction in the viability titre *Salmonella choleraesuis* in a contact time of 5 minutes at room temperature indicating bactericidal activity against this organism.

Table 5: The Activity of a 1:16 Dilution Accel Hydrotherapy Plus against *Salmonella choleraesuis* in a Contact Time of 5 Minutes

Lot Number	Date of Experiment	CFU/Control Carrier	CFU/Test Carrier	Log ₁₀ Reduction
5001	10/11/05	3.43 x 10 ⁶	0	6.44
5002	10/11/05	3.43 x 10 ⁶	0	6.44
5003	10/11/05	3.43 x 10 ⁶	0	6.44

Activity of the product against VRE (Carrier Test Method): Table 6 summarizes the results of tests against VRE. All three lots of the product were able to bring about a >5 log₁₀ reduction in the viability titre of in a contact time of 5 minutes at room temperature indicating bactericidal activity against this organism .

Table 6: The activity of a 1:16 Dilution Accel Hydrotherapy Plus against VRE in a Contact Time of 5 Minutes

Lot Number	Date of Experiment	CFU/Control Carrier	CFU/Test Carrier	Log ₁₀ Reduction
5719	11/07/06	7.77 x 10 ⁶	0	6.89
5593	11/07/06	7.77 x 10 ⁶	0	6.89
5594	11/07/06	7.77 x 10 ⁶	0	6.89

Activity of the product against VRE (Suspension Test Method): Table 7 summarizes the results of the suspension test. All three lots were able to bring about a >4 log₁₀ reduction in the viability titre of VRE in a contact time of 30 seconds at room temperature indicating bactericidal activity against this organism.

Table 7: The activity of a 1:128 Dilution of Accel Hydrotherapy plus against VRE in 30 Seconds

Lot Number	Date of Experiment	CFU/control	CFU/test	Log ₁₀ Reduction
5001	26/11/05	8.33 x 10 ⁴	0	4.92
5002	26/11/05	8.33 x 10 ⁴	0	4.92
5003	26/11/05	8.33 x 10 ⁴	0	4.92

Activity of the product against MRSA (Carrier Test Method): Table 8 summarizes the results of tests against MRSA. All three lots of the product were able to bring about a >6log₁₀ reduction in the viability titre of in a contact time of 5 minutes at room temperature indicating bactericidal activity against this organism.

Table 8: The activity of 1:16 Dilution of Accel Hydrotherapy Plus against MRSA in a Contact Time of 5 Minutes

Lot Number	Date of Experiment	CFU/control carrier	CFU/test Carrier	Log ₁₀ Reduction
5001	13/12/05	4.37 x 10 ⁶	0	6.44
5002	13/12/05	4.37 x 10 ⁶	0	6.44
5003	13/12/05	4.37 x 10 ⁶	0	6.44

Activity of the product against MRSA (Suspension Test Method): Table 9 summarizes the results of the suspension test. All three lots were able to bring about a >4log₁₀ reduction in the viability titre of MRSA in a contact time of 30 seconds at room temperature indicating bactericidal activity against this organism.

Table 9: The activity of a 1:128 Dilution of Accel Hydrotherapy Plus against MRSA in a Contact Time of 30 Seconds

Lot Number	Date of Experiment	CFU/control	CFU/test	Log ₁₀ Reduction
5001	29/11/05	1.10 x 10 ⁵	0	5.04
5002	29/11/05	1.10 x 10 ⁵	0	5.04
5003	29/11/05	1.10 x 10 ⁵	0	5.04

Activity of the product against *Trichophyton mentagrophytes* (Carrier Test Method): Table 10 summarizes the results of test against *T. mentagrophytes*. All three lots of the product were able to bring about a $>5\log_{10}$ reduction in the viability titre in a contact time of 5 minutes at room temperature indicating fungicidal activity against this organism.

Table 10: The activity of a 1:16 Dilution Accel Hydrotherapy Plus against *T. mentagrophytes* in a Contact Time of 5 Minutes

Lot Number	Date of Experiment	CFU/Control Carrier	Average CFU Test Carrier	Log ₁₀ Reduction
5001	14/11/05	3.20×10^5	0	5.50
5002	14/11/05	3.20×10^5	0	5.50
5003	14/11/05	3.20×10^5	0	5.50

Neutralization Verification Results to Arrest Activity of Accel Hydrotherapy Plus: Table 10 summarizes the results of the neutralization test of the product. The absence of any significant difference in the number of colonies of the test organism in the test and control samples was taken to mean that a 1:10 dilution of the product in the neutralizer was sufficient to arrest its bactericidal activity.

Table 11: Neutralization Verification of Accel Hydrotherapy Plus

Test Organism	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>P. aeruginosa</i>	40/42	66/73
<i>S. choleraesuis</i>	24/26	52/56
<i>S. aureus</i>	15/24	27/31
VRE	64/53	52/55
MRSA	24/28	27/28

E. CONCLUDING REMARKS

All three lots of the formulation tested were able to meet the product performance criteria under the conditions of the testing carried out in this study.

F. REFERENCES

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