

Final Report submitted to
Virox Technologies Inc., Mississauga, Ontario

**POTENTIAL OF VIROX STF (7%) FOR REUSE AS A
HIGH-LEVEL DISINFECTANT: EVALUATION OF ITS
MYCOBACTERICIDAL, FUNGICIDAL AND
SPORICIDAL ACTIVITIES USING A FOURTEEN-DAY
STRESS PROTOCOL**

Syed A. Sattar, M.Sc., Dip. Bact., M.S., Ph.D., RM (CCM)

Professor and Principal Investigator
Department of Microbiology and Immunology
Faculty of Medicine, University of Ottawa
Ottawa, Ontario, Canada
K1H 8M5

Phone: (613) 562-5800 ext.8314

Fax: (613) 562-5452

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

February 2001

TABLE OF CONTENTS

	Page No.
Executive Summary	4
A. Background and Introduction	5
B. Objective of Study	5
C. Site of Study	5
D. Materials and Methods	5
Disinfectants	5
Challenge Organisms	6
1. <i>Pseudomonas aeruginosa</i> (ATCC 15442)	6
2. <i>Staphylococcus aureus</i> (ATCC 6538)	6
3. <i>Salmonella choleraesuis</i> (ATCC 10708)	6
4. <i>Bacillus subtilis</i> (ATCC 19659)	6
5. <i>Clostridium sporogenes</i> (ATCC 7955)	6
6. <i>Mycobacterium terrae</i> (15755)	6
7. <i>Trichophyton mentagrophytes</i> (ATCC 9533)	6
Soil Load	6
Carriers	6
E. Procedure For Manual Use Reuse Stress	7
F. Microbiological Tests to Assess the Germicidal Activity of The Stressed Solutions	8
Methodology	8
Flow Chart of Basic Method	9
Recovery Media and Detection of Viable Organisms	9
Controls	9
Neutralizer, Microbial Diluents and Filter Rinse	10
Product Performance Criteria	10
G. Quality Control	10
H. Results	11
Recovery of Bacteria from Contaminated Carriers	11
Activity of the Stressed STF on <i>B. subtilis</i>	11
Activity of the Stressed STF on <i>C. sporogenes</i>	11
Activity of the Stressed STF on <i>M. terrae</i>	12
Activity of the Stressed STF on <i>T. mentagrophytes</i>	12

Neutralizer Efficacy	12
Settling Plates Results	13
I. Conclusion	13
K. Literature Cited	14
Appendix 1	15
Appendix 11	18

EXECUTIVE SUMMARY

This study at the Centre for Research on Environmental Microbiology (CREM), University of Ottawa, assessed the suitability of Virox STF; a 7% stabilized solution of hydrogen peroxide, in the manual disinfection of semi-critical medical devices for a 14-day reuse claim.

As specified in the U.S. Environmental Protection Agency (EPA)- approved stress test protocol, the disinfectant solutions under study were challenged each day by adding carriers contaminated with microorganisms dried on them. The carriers contaminated with vegetative non-spore forming bacteria *Salmonella choleraesuis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, and spores of *Bacillus subtilis* and *Clostridium sporogenes* were added alternately to containers with the disinfection solution each day. In addition to the carriers with the dried microorganisms, two complete sets of inhalation equipment (two sections of corrugated rubber tubing, each 3 to 4 feet in length, one 3-liter breathing bag, one face mask, one endotracheal tube and five “Y” connector per set), subjected to minimal pretreatment cycles, were added to each batch of disinfectant solution being stressed during each of the three cycles per day. Three lots of the disinfectant were evaluated in this study.

The evaluation of the germicidal activities of the stressed disinfectant for a high level disinfectant claim was carried out by determining if the stressed disinfectant was sporicidal, fungicidal and mycobactericidal at full strength using a quantitative carrier test, which is now a standard of the American Society for Testing and Materials.

The findings of this study indicate that Virox STF 7% remains fungicidal, mycobactericidal and sporicidal even after a fourteen day stress period.

A. BACKGROUND AND INTRODUCTION

Because of the heat-sensitive nature of many semi-critical medical devices, chemical germicides are used either in manual or automated systems for their decontamination between patients.

Manual disinfection tends to expose hospital staff to toxic disinfectants (Fleisher, 1991) and this is particularly true for glutaraldehyde-based formulations (Foss & Monagan, 1992). Improper reprocessing of such medical devices can also increase the risk of disease transmission (Bond, 1991; Spach *et al.*, 1993) and exposure of patients to disinfectant residues in the device (Vesley *et al.*, 1992). In view of this, more and more hospitals/clinics are looking for safer yet equally effective substitutes for glutaraldehyde-based disinfectants for manual as well as machine reprocessing of medical devices.

Properly formulated hydrogen peroxide-based products can be fast-acting and broad-spectrum germicides while being safer for humans and the environment. Therefore, they show considerable promise as substitutes for glutaraldehyde, a common active ingredient in most of the medical devices disinfectants in use today.

In earlier studies, glutaraldehyde-based products have demonstrated variable germicidal activity after 7 days of reuse (Mbithi *et al.*, 1993). There is also mounting concern on the human and environmental safety of glutaraldehyde (NICNAS, 1994). In this study, Virox STF, a 7% stabilized hydrogen peroxide solution with broad-spectrum antimicrobial activity, was evaluated as a possible substitute for glutaraldehyde.

B. OBJECTIVE OF THE STUDY

The main objective of this study was to test the germicidal effectiveness of Virox STF for reuse through a 14-day stress protocol approved for this purpose by the U.S. Environmental Protection Agency (EPA). The quantitative carrier test used to assess the germicidal activity of samples of the stressed solutions is now a standard of the American Society for Testing Materials (ASTM, 2000)

C. SITE OF STUDY

The study was conducted at the Centre for Research on Environmental Microbiology (CREM), University of Ottawa. There is a QA/QC unit here as part of EPA requirements. Our laboratory was audited by the EPA and was found to be in compliance with the Good Laboratory Practices (GLP) Guidelines. As required by the University, all members of the laboratory staff at CREM are properly trained in the safe handling of infectious agents and hazardous chemicals.

D. MATERIALS AND METHODS

1. Disinfectants:

The sponsor supplied us with Virox STF in 20 L white plastic pails. The solutions were clear but with a pale yellow color and slight odor. The disinfectant was stored at room temperature in an area with controlled access. The study commenced on September 28, 2000 and was completed in November 2000.

The product is already registered with Health Canada as a high level disinfectant. Earlier studies in our laboratory found the product to be bactericidal, virucidal and fungicidal in 5 minutes, mycobactericidal in 20 minutes, and sporicidal in 6 hours at 20°C.

2. The Challenge Organisms:

Standard strains of *Pseudomonas aeruginosa* (ATCC 15442), *Staphylococcus aureus* (ATCC 6538), *Salmonella choleraesuis* (ATCC 10708), *Mycobacterium terrae* (ATCC 15755), *Trichophyton mentagrophytes* (ATCC 9533), *Bacillus subtilis* (ATCC 19659) and *Clostridium sporogenes* (ATCC 7955), already available in our laboratory, were used in this study. They were cultured as follows:

a) *Pseudomonas aeruginosa* (ATCC 15442), *Staphylococcus aureus* (ATCC 6538) and *Salmonella choleraesuis* (ATCC 10708): Stock suspensions of the three vegetative bacteria were prepared by culturing them in tryptic soy broth (TSB) for 24 hours at 37°C.

b) *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 re-suspended in sterile distilled, deionized water

c) *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 re-suspended with sterile distilled, deionized water.

d) *Mycobacterium terrae* (ATCC 15755): The mycobacterium was grown in Middlebrook 7H9 broth with ADC enrichment and glycerol, in vented plug seal capped tissue culture flasks. The test suspension was prepared from stocks grown for 21 days. The cell suspension was washed 3 times by centrifugation at 2,500 rpm for 15 minutes and re-suspended in sterile distilled water. The final stock suspension was prepared by re-suspending the bacterial pellets in sterile bijoux bottles containing glass beads to approximately 10^8 cells/mL. The stock solution was stored at 4°C.

e) *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 gauze to remove the hyphae.

3. Soil Load:

To provide a greater stress to the test solution used in this study, fetal bovine serum at a final concentration of 2% was used to simulate organic soil loading. Such serum is non-inhibitory for the bacteria under study and is universally accepted as a soil load in testing the germicidal activity of liquid chemical disinfectants. Also, the addition of the contaminated carriers as bioburden and the soaking of several items of respiratory equipment over the 14-days stress cycle simulated the stress the product may face under reuse.

4. Carriers:

For the manual contamination of the disinfectant reuse bath, glass beads, 6 mm in diameter, were used as carriers for the vegetative bacteria, and stainless steel penicylinders were used as

carriers for the spore-formers.

Glass vials were used as hard surface carriers for sporicidal, mycobactericidal and fungicidal quantitative carrier tests (ASTM, 2000).

E. PROCEDURE FOR MANUAL REUSE STRESS:

The procedure is described in detail in the standard operating procedure given in Appendix 1. Samples from three different lots of the test disinfectant were challenged for the reuse testing. Each test solution consisted of 10 litres of undiluted test product.

Disinfectant solutions under study were challenged each day by adding carriers contaminated with microorganisms dried on them. Carriers contaminated with vegetative non-spore forming bacteria, *Salmonella choleraesuis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, and spores of *Bacillus subtilis* and *Clostridium sporogenes* were added alternately to the containers with disinfection solution each day. A minimum cumulative bioburden provided a stress of at least 10^4 dried carrier viable microorganisms/mL of test solution/day.

The surface area of 4.28 6-mm glass beads is equivalent to the surface area of one penicylinder. To provide a margin of safety, the surface area of 4.4 6-mm glass beads was used as equivalent to the surface area of 1 penicylinder.

In addition to the microbial bio-burden dried on the carriers, a complete set of inhalation equipment was added to each batch of disinfectant solution being stressed. Each set was subjected to a minimal pretreatment cycle consisting of cleaning with a detergent (Fisher brand Sparkleen7 for manual washing), a water rinse, and a soak in the test disinfectant for 30 minutes. The number of cycles run each day was 3.

Chemical determination of the concentration of the active ingredient in the test solution using wet chemistry method and use of high range peroxide test strips were tested daily to ensure that the hydrogen peroxide concentration in the bath was above the minimum effective level. The pH of each bath was determined using a pH meter (Accument Fisher Scientific) and recorded daily prior to the first challenge during the simulated use study. (Chart of results are presented in Appendix 11)

The numbers of viable organisms on the carriers used in the stress test were determined once on each of the challenge organisms by individually adding three inoculated carriers to 10 mL tubes of PB+1% Tween 80. The tubes were vortexed thoroughly. The eluates were serially diluted in phosphate buffer (pH 7.2) and appropriate dilutions were membrane filtered and the filters placed on tryptic soy agar (TSA) plates for the vegetative bacteria and *B. subtilis*. All plates were incubated at 37°C for 24-48 hours. Plates of fastidious anaerobic agar (FAA) were used for the recovery of *C. sporogenes* and incubated at 30°C.

Gram stains were performed on colonies isolated from one set of the carrier counts for each challenge organisms to further confirm their identity by morphology and staining characteristics.

F. MICROBIOLOGICAL TESTS TO ASSESS THE GERMICIDAL ACTIVITY OF THE STRESSED SOLUTIONS:

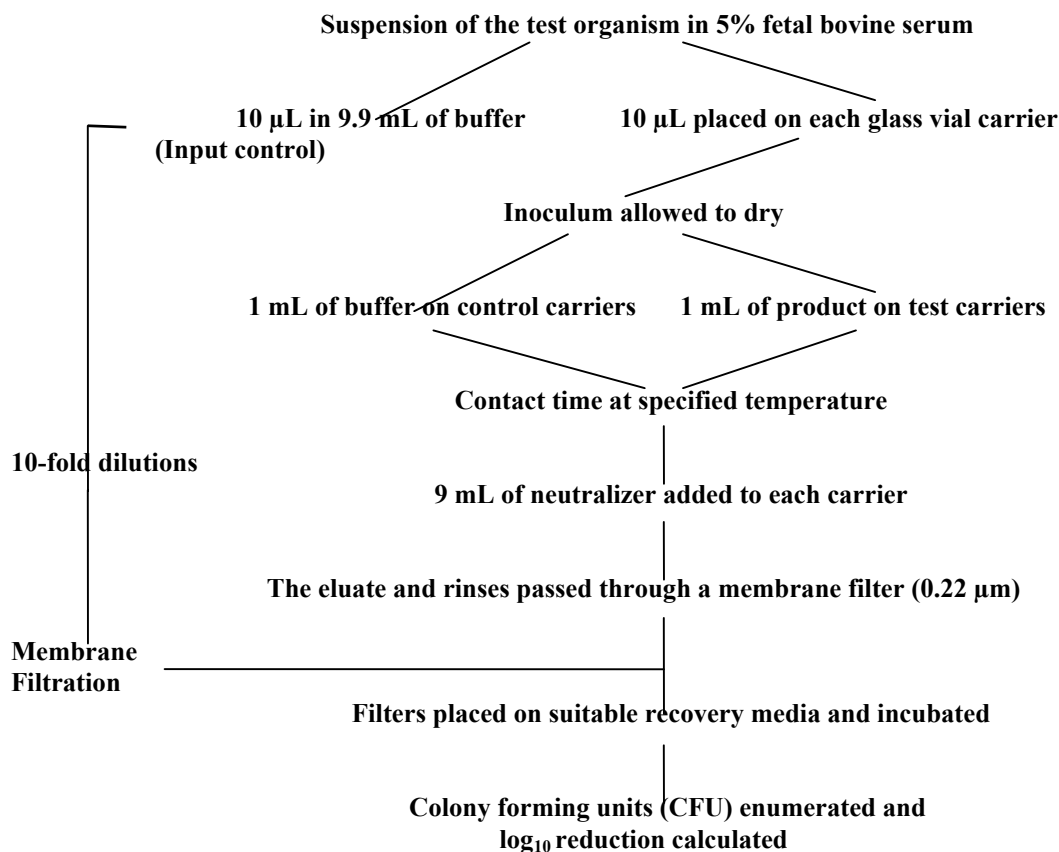
1. The Test Methodology

The quantitative carrier tests used in this evaluation have 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). As stated above, it is now an accepted standard of ASTM.

The Flow Chart outlines the methodology used for the germicide test.

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).

Recovery Media and Detection of Viable Organisms:

For the sporicidal testing with *B. subtilis*, the filters were placed on trypticase soy agar (TSA) plates, 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 fastidious anaerobic agar (FAA), incubated at 30°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, 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 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:

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

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 the diluent and filter rinse in the sporicidal test; this was replaced with normal saline (0.85% NaCl) in the mycobactericidal and fungicidal tests. (It worked well in rinsing out the froth created by the Lethen broth).

2. Product Performance Criteria

In each 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. For a product to be considered sporicidal or mycobactericidal, it was expected to reduce the viability titre 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} reduction was expected for fungicidal activity.

G. QUALITY CONTROL:

The following testing was included as a part of routine quality control:

- Lethen broth+0.1% sodium thiosulphate pentahydrate solution used as neutralizer was tested to demonstrate that it does not promote the growth or cause the death of the test organisms in the length of time the cells were exposed to them.
- The efficiency of the neutralizing system was tested by adding a known number of CFU of the test bacteria to a portion of Lethen broth+0.1% sodium thiosulphate pentahydrate/Virox STF in the ratio (1 mL Virox: 9 mL neutralizer) and then determining the population after 10 minutes (time required for disinfection). This was to ascertain that the neutralizer is able to render the samples free of germicidal activity.
- The air in the microbiology laboratory where the study was conducted was monitored by exposing settling plates of TSA in designated areas as outlined in our quality control manual.

H. RESULTS

As can be seen from Table 1, an average of 1.84×10^4 CFU/mL of the bacteria was loaded daily: 8.96×10^3 of vegetative bacteria and 9.48×10^3 of the spore-forming bacteria.

Table 1: Recovery of the Challenge Bacteria from Contaminated Carriers:

Organism	Carrier Type	CFU Recovered	Number of carriers	Total CFU/mL of bacteria loaded
<i>P. aeruginosa</i>	6 mm Pyrex glass beads	1.76×10^5	880	1.55×10^4
<i>S. aureus</i>	6 mm Pyrex glass beads	7.2×10^4	880	6.37×10^3
<i>S. choleraesuis</i>	6 mm Pyrex glass beads	5.7×10^4	880	5.01×10^5
<i>C. sporogenes</i>	Stainless steel penicylinder	1.03×10^5	200	2.06×10^3
<i>B. subtilis</i>	Stainless steel penicylinder	8.47×10^5	200	1.69×10^4

Activity of Stressed STF against the spores of *Bacillus subtilis*: Table 2 summarizes the results of testing against *B. subtilis*. The product was able to bring about a $>7 \log_{10}$ reduction in the viability titre of *B. subtilis* spores in a contact time of 6 hours at $20 \pm 1^\circ\text{C}$, indicating sporicidal activity against this organism in our test protocol.

Table 2. The Activity of Full-Strength Stressed STF Against the Spores of *Bacillus subtilis*.

Lot Number	Date of Experiment	Contact Time	Number of Carriers	CFU/control Carriers	CFU/test Carrier	Log ₁₀ Reduction
2484	12/10/00	6 hours	10	2.18×10^7	0	7.34
2485	12/10/00	6 hours	10	2.18×10^7	0	7.34
2486	12/10/00	6 hours	10	2.18×10^7	0	7.34

Activity of Stressed STF against the Spores of *Clostridium sporogenes*: As can be seen from Table 3, the product was able to bring about a $>7 \log_{10}$ reduction in the viability titre of *C. sporogenes* spores in a contact time of 6 hours at $20 \pm 1^\circ\text{C}$, indicating sporicidal activity against this organism.

Table 3. The Activity of Full-Strength Stressed STF Against the Spores of *C. sporogenes*.

Lot Number	Date of Experiment	Contact Time	Number of Carriers	CFU/control Carriers	CFU/test Carrier	Log ₁₀ Reduction
2484	12/10/00	6 hours	10	6.34×10^7	0	7.80
2485	12/10/00	6 hours	10	6.34×10^7	0	7.80
2486	12/10/00	6 hours	10	6.34×10^7	0	7.80

Activity of Stressed STF Against *Mycobacterium terrae*: The product proved to be mycobactericidal by reducing the titre of *M. terrae* by $>6 \log_{10}$, as summarized in Table 4.

Table 4. The Activity of Full-Strength Stressed STF Against *Mycobacterium terrae*.

Lot Number	Date of Experiment	Contact Time	Number of Carriers	CFU/control Carriers	CFU/test Carrier	Log ₁₀ Reduction
2484	23/11/00	35 min	8	4.70×10^6	1	6.58
2485	23/11/00	35 min	8	4.70×10^6	0	6.58
2486	23/11/00	35 min	8	4.70×10^6	0	6.58

Activity of Stressed STF Against the Conidia of *T. mentagrophytes*: As can be seen from Table 5, 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 20 minutes at $20 \pm 1^\circ\text{C}$, indicating fungicidal activity against this organism.

Table 5. The Activity of Full-Strength Stressed STF Against the Conidia of *T. mentagrophytes*.

Lot Number	Date of Experiment	Contact Time	Number of Carriers	CFU/control Carriers	CFU/test Carrier	Log ₁₀ Reduction
2484	3/11/00	20 min	10	4.70×10^6	0	6.67
2485	3/11/00	20 min	10	4.70×10^6	8	6.48
2486	3/11/00	20 min	10	4.70×10^6	1	6.59

Neutralizer Efficacy: Table 6 is a summary of the data, which demonstrates that the neutralization solution used in this study was able to arrest the germicidal activity of the test product at the end of the contact time.

Table 6. Effectiveness of the Neutralizer in Arresting the Activity of the Test Solution

Test Organism	Number of colonies on plates after exposure to neutralizer and test solution	Number of colonies on plates after exposure to Neutralizer
<i>Bacillus subtilis</i>	37, 44	47, 49
<i>Clostridium sporogenes</i>	78, 66	64, 61
<i>Mycobacterium terrae</i>	47, 50	19, 16
<i>Trichophyton mentagrophytes</i>	69, 74	107, 100

Settling Plates Results: As can be seen from the data presented in Table 7, the level of aerobic bacterial contamination in the air of the laboratory was quite low.

Table 7. Number of Bacterial Colonies on Plates Exposed to Air at Selected Locations in the Laboratory during one Period of Study

Site	Colonies on the Plates of Trypticase-Soy Agar
A. Bench Top	2
B. Bench Top*	1
C. Fume hood*	0
D. Work Station 1	1
E. Work Station 2	1
F. Work Station 3	5
G. Work Station 4	0

*Most of the study work was carried out in these areas.

I. CONCLUSION

Virox STF, a 7% stabilized solution of hydrogen peroxide tested in this study, was found to retain its mycobactericidal, fungicidal and sporicidal activities in spite of stressing to simulate fourteen days of reuse.

J. LITERATURE CITED

- American Society for Testing and Materials (2000). Document # E-2111-00, ASTM, West Conshohocken, PA.
- AOAC (1990); Official Methods of Analysis of the AOAC. AOAC, Washington, D.C.
- Bond, W.W. (1991). Disinfection and endoscopy: Microbial considerations. *J. Gastroenterol. & Hepatol.* 6: 31-36.
- 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.
- Fleisher, D.E. (1991). Disinfection and endoscopy: Procedures and staff safety. *J. Gastroenterol. & Hepatol.* 6: 41-43.
- Foss, D. and D. Monagan (1992). A national survey of physicians' and nurses' attitudes toward endoscope cleaning and the potential for cross-contamination. *Gastroenterol. Nursing.* Pages 59-65, Oct., 1992.
- Mbithi, J.N., Springthorpe, V.S., Sattar, S.A. & Paquette, M. (1993). Bactericidal, virucidal & mycobactericidal activities of reused alkaline glutaraldehyde in an endoscopy unit. *J. Clin. Microbiol.* 31:2988-2995.
- Spach, D. H. *et al.* (1993). Transmission of infection by gastrointestinal endoscopy. *Ann. Intern. Med.* 118: 117-128.
- Vesley, D. *et al.* (1992). Significant factors in the disinfection and sterilization of flexible endoscopes. *Am. J. Infect. Control* 20: 291-300.

APPENDIX I

STANDARD OPERATING PROCEDURE

Title: **FOURTEEN DAY USE-RE-USE MANUAL STRESSING**

Prepared by: _Sola Adegbunrin_____ Date: _August, 31, 2000_____

Approved by: _____ Date: _____

1.0 PURPOSE

The procedure is designed to simulate the organic and inorganic loading that occurs when a disinfectant solution is repeatedly reused.

2.0 APPLICATION

Applies to all manual re-use stressing for disinfecting and sterilizing products

3.0 REFERENCES

The standard operating procedure conforms to the EPA "Re-use Test Protocol Specifications" documents

4.0 ASSOCIATED SOPs

SOP: Growing cultures for filtration tests

SOP: Media Preparation

SOP: Hydrogen Peroxide Titration

5.0 PROCEDURE

5.1 Materials:

1. Three lots of test disinfectants
2. Medical equipment for each 10litres of disinfectant
 - Two sections of corrugated rubber tubing
 - One 3litres-breathing bag
 - Facemask
 - Endotracheal tube
 - "Y" connectors
3. Carriers (stainless steel penicylinders and 6mm glass beads)
4. Media
5. Reagents
6. Dilution fluids
7. Equipments
8. Microorganisms:

The test bacteria used in the AOAC Use Dilution Test are required by the EPA document for stressing the disinfectant solution.

1. *Salmonella choleraesuis* ATCC 10708
2. *Staphylococcus aureus* ATCC 6538
3. *Pseudomonas aeruginosa* ATCC 15442

The test spores used in the AOAC Sporicidal Test are required by the EPA document for stressing the disinfectant/sterilant solution.

1. *Bacillus subtilis*, ATCC 19659
2. *Clostridium sporogenes*, ATCC 3584

5.2 Method:

EXPERIMENTAL DESIGN

Three samples of the test disinfectant (three different lots or batches) will be challenged for the reuse testing. The test disinfectant will be prepared according to the manufacture's directions. 10litres per batch of the disinfectant will be stressed.

Disinfectant solutions under study will be challenged each day by adding carriers contaminated with microorganisms dried on them. Carriers contaminated with vegetative non-spore forming bacteria. *Salmonella choleraesuis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* will be added alternately to the containers with the disinfection solution per day. Carriers contaminated with viable spores of *Bacillus subtilis* and *Clostridium sporogenes* will be added alternately to the containers of solution being stressed to provide an added bioburden when reuse sterilant claim is being supported. A minimum cumulative bioburden will provide a stress of at least 10^4 dried carrier viable microorganisms per ml of test solution per day.

Glass beads, 6mm in diameter are used as carriers for vegetative bacteria and stainless steel penicylinders are used as carriers for spore forming bacteria. The surface area of 4.28 6mm glass beads is equivalent to the surface area of one penicylinder. To provide a margin of safety, the surface area of 4.4 6mm glass beads will be used as equivalent to the surface area of 1 penicylinder.

In addition to microorganisms dried on carriers, medical equipments listed in the list of materials will be added to each batch of disinfectant solution being stressed during each cycle per day. Each set of the medical equipment will be subjected to minimal treatment cycle consisting of cleaning with a detergent (Fisher brand Sparkleen7 detergent for manual washing), a water rinse, and soaking in the test disinfectant. The number of cycles run each day will be 3 for disinfecting and sterilizing claim.

Chemical determination of the content the active ingredient in the test formulation will be performed daily. The PH of each test solution will be recorded before the first challenge each day.

CONTROLS:

1. Carrier counts:

Carrier counts will be done once on each of the challenge organisms used for the stressing by individually adding three inoculated carriers to 10ml tubes of PB+1% Tween 80. The tubes will be vortexed thoroughly. The carriers will be removed and the eluate will be serially diluted in PB and appropriate dilutions filtered and filters placed on recovery media. All plates will be incubated at 37°C for 24-48 hours.

2. Confirmation of the challenge bacteria:

Gram stains will be performed on an isolated colony from each set of the carrier counts for each challenge bacteria.

5.2.1 CARRIER PREPARATION

5.2.1.1 Glass Beads for Contamination With Vegetative Bacteria

5.2.1.1.a Soak glass beads overnight in 1N NaOH

5.2.1.1.b Rinse with tap water, then with deionized water until PH of water is neutral

5.2.1.1.c Place beads into covered beakers containing double distilled water

5.2.1.1.d Steam sterilize beakers at 121C

5.2.1.1.e Allow to cool

5.2.1.1.f Decant water

5.2.1.1.g Add 24-hour culture of a 10^4 CFU/mL of any of the three vegetative bacteria grown in Trypticase Soya Broth (TSB) to cover the beads

5.2.1.2 Stainless Steel Penicylinders for Contamination with Spore forming Bacteria

5.2.1.2.a Soak penicylinders in 0.1% Triton-X for one minute

5.2.1.2.b Rinse four times with deionized water

5.2.1.2.c Sterilize at 121°C for 20minutes

5.2.1.2.d Dry in hot air oven for two hours at 180°C

5.2.1.2.e Suspend carriers in a beaker containing a 10^4 -cfu/mL spore of *B. subtilis* or *C. sporogenes*

- 5.2.2.1. Allow bacteria to remain in contact with carriers for 15 minutes
- 5.2.2.2. Decant culture
- 5.2.2.3. Dry carriers in an incubator for approximately 45 minutes at 37°C
- 5.2.2.4 Measure the PH and % hydrogen peroxide of the test disinfectant
- 5.2.2.5 put each 880 contaminated glass beads with one of the vegetative bacteria into each of the three mesh bags.
- 5.2.2.6 put each mesh bag into each batch 10litres of the test disinfectant once daily
- 5.2.2.7 Allow to remain in the disinfectant for one hour
- 5.2.2.8 Remove the bags, decontaminate the beads and reprocess for the next use
- 5.2.2.9 Add medical instruments, which have gone through a cycle of washing and rinsing into each batch of

Test disinfectants for 30 minutes three times daily (12am, 2pm and 3pm)

1. put each dried 200 penicylinders contaminated with *Bacillus subtilis* or *Clostridium sporogenes* into three mesh bags
2. Put each mesh bag into each batch of 10litres of the test disinfectant once daily (pm)
3. Allow to remain overnight (remove before the cycles are started the next day)

14-Day Cycle Addition of Bioburden

Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Vegetative Bacteria	SA	PA	SC	SA	PA	SC	SA	PA	SC	SA	PA	SC	SA	PA
Bacteria Spore	BS	CS	BS	CS	BS	CS	BS	CS	BS	CS	BS	CS	BS	CS

PA: *Pseudomonas aeruginosa*. SA: *Staphylococcus aureus*,

SC: *Salmonella choleraesuis*. CS: *Clostridium sporogenes*, BS: *Bacillus subtilis*

A Typical Day Cycle

9:00.am	10:30.am	11:30.am	12:00.am	2:00.pm	3:00.pm	3:45.pm
* Remove spore carriers from bath. Prepare vegetative carriers and dry for 45 minutes. Measure the active ingredient by titration and by test strips Measure pH.	Measure PH of disinfectant. Add Vegetative Carriers to disinfectant baths.	Remove Carriers from disinfectant.	1 ST Cycle of medical equipment processing.	2 nd Cycle of medical equipment processing. At 2:30 pm Prepare spore carriers. Dry for 45 minutes.	3rd Cycle of medical equipment processing.	Add spore Carriers to disinfectant & leave overnight.

* Applies only from Day 2 of the 14 day cycle

Complete Cycle of Medical Equipment Processing

1. Manual wash with Fisher brand Sparkleen7 detergent.
2. Rinse in Water.
3. Soak in Disinfectant for 30 minutes.

APPENDIX 11

Table 8: VIROX STF RESULT CHART

Day	Organism loaded	pH			Strip			Titration (%H ₂ O ₂)		
		Lot-A	Lot-B	Lot-C	Lot-A	Lot-B	Lot-C	Lot-A	Lot-B	Lot-C
	Product as Received	2.84	2.85	2.88	Pass	Pass	Pass	7.9	8.0	8.0
	Product with 2% serum	2.87	2.85	2.89	Pass	Pass	Pass	7.9	8.0	7.9
1	SA BS	2.90	2.88	2.87	Pass	Pass	Pass	7.8	7.7	7.8
2	PA CS	2.86	2.85	2.82	Pass	Pass	Pass	7.7	7.7	7.4
3	SC BS	2.88	2.86	2.85	Pass	Pass	Pass	7.5	7.5	7.7
4	SA BS	2.90	2.89	2.87	Pass	Pass	Pass	7.3	7.3	7.4
5	PA CS	2.87	2.87	2.87	Pass	Pass	Pass	7.8	7.5	7.5
6	SC BS	2.83	2.84	2.83	Pass	Pass	Pass	7.5	7.6	7.6
7	SA BS	2.93	2.91	2.89	Pass	Pass	Pass	7.6	7.4	7.5
8	PA CS	2.87	2.87	2.86	Pass	Pass	Pass	7.0	7.0	7.3
9	SC BS	2.97	2.95	2.92	Pass	Pass	Pass	7.0	7.0	7.2
10	SA BS	2.97	2.95	2.94	Pass	Pass	Pass	6.9	7.0	7.0
11	PA CS	2.97	2.95	2.91	Pass	Pass	Pass	6.9	6.8	6.9
12	SC BS	2.99	2.97	2.95	Pass	Pass	Pass	7.0	6.9	7.0
13	SA BS	2.97	2.94	2.94	Pass	Pass	Pass	6.8	6.9	6.8
14	PA CS	2.98	2.90	2.89	Pass	Pass	Pass	6.9	6.9	6.8

Lot A: 2484

Lot B: 2485

Lot C: 2486

PA: *Pseudomonas aeruginosa*SA: *Staphylococcus aureus*SC: *Salmonella choleraesuis*CS: *Clostridium sporogenes*BS: *Bacillus subtilis*