

Final report submitted to
Virox Technologies, Inc.

**EVALUATION OF THE EFFECTIVENESS OF
ACCELERATED HYDROGEN PEROXIDE (AHP)
AGAINST HUMAN RHINOVIRUS TYPE 14, A FELINE
CALICIVIRUS AND A HUMAN ROTAVIRUS**

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OBJECTIVE OF THE STUDY

The objective of this study was to evaluate the virucidal activity of an formulation based on accelerated hydrogen peroxide (AHP) against human rhinovirus type-14, a feline calicivirus and the Wa strain human rotavirus using a quantitative carrier test developed at CREM, which is now a standard of ASTM International (#E2197-02).

MATERIALS AND METHODS

The Test Product

Three lots of the product were shipped directly to us by the Sponsor. Upon receipt here the samples were stored at room temperature in an area with controlled access. The product was tested at a 1:16 dilution in water with a standard hardness of 400 ppm as CaCO₃. The product effectiveness criterion was set at a minimum $\geq 3 \log_{10}$ reduction in virus infectivity.

The Challenge Viruses

Human rhinovirus: Human rhinovirus type-14 (ATCC VR-1059) was used in this study. The virus was grown and plaque assayed in the HeLa-T4⁺ line of human cervical cancer cells transfected with the human CD⁴ receptor gene. Dr. K. Wright of this department kindly provided a seed culture of this cell line to us. She had originally received the cells from Dr. R. Axel of the U.S. National Institutes of Health, Rockville, MD. The cells were grown in minimal essential medium (MEM; GIBCO-BRL Cat # 41600-016) with the appropriate supplements and 10% fetal bovine serum (FBS) in 75 cm² flasks at 37°C.

For preparing the virus pool, 100 µL of the viral suspension was inoculated onto a confluent cell monolayer in a 75-cm² culture flask. The inoculum was spread evenly over the monolayer by gentle rocking of the flask. The flask was then kept at 33°C for 60 minutes to allow for virus adsorption. Supplemented MEM with 2% FBS was added to the inoculated monolayer and the flask incubated at 33°C for 2 days by which time nearly 75% of the cell monolayer showed viral cytopathology. The virus was separated from the cells by three rapid freeze-thaw cycles followed by centrifugation at 2,000 rpm for 10 minutes. The supernatant, which contained the virus, was aspirated, dispensed in aliquots of 200 µL and stored at -80°C. The viral titer was determined by a plaque assay method and was found to be about 2.3×10^8 plaque forming units (PFU)/mL.

Human rotavirus: The human rotavirus used in this study was the Wa strain (ATCC VR-2018). The virus was grown and plaque assayed in the MA-104 cell line of monkey kidney epithelial cells. The cells were grown in MEM in the presence of 25 mM HEPES, L-glutamine, antibiotics, and 7% FBS in 75 cm² flasks at 37°C.

For preparing the virus pool, 100 µL of the viral suspension was inoculated onto a confluent cell monolayer that was washed twice with EBSS (Earle balanced salt solution), in a 75- cm² culture flask. The inoculum was spread evenly over the monolayer by gentle rocking of the flask. The flask was kept at 37°C for 60 minutes to allow for virus adsorption. Supplemented MEM with 5 µg/mL 1:250 trypsin (Gibco) was added to the inoculated monolayer and the flask incubated at 37°C for 3 days by which time nearly 85% of the cell monolayer showed viral cytopathology. The virus was separated from the cells by three rapid freeze-thaw cycles followed by centrifugation at 2,000 rpm for 10 minutes. The supernatant, which contained the virus, was

aspirated and pooled together from 10 flasks and concentrated by ultra centrifugation at 24,000 rpm for 2 hrs at 4°C. The supernatant was discarded and the pellet resuspended in EBSS. The viral titer was determined by a plaque assay method and was found to be about 3×10^7 PFU/mL.

Feline Calicivirus: The feline calicivirus used in this study was the F9 strain (ATCC VR-782). The virus was grown and plaque assayed in Crandell's feline kidney (CrFK) cell line (ATCC # CCL-94). A seed culture of these cells and the virus were kindly provided to us by Dr. Sabah Bidawid of Health Canada, Ottawa. The cells were grown in MEM in the presence of L-glutamine, antibiotics, and 10% FBS in 75 cm² flasks at 37°C.

For preparing the virus pool, 200 µL of the virus suspension was inoculated onto an 18-24-hour-old cell monolayer, in a 75-cm² culture flask. The inoculum was spread evenly over the monolayer by gentle rocking of the flask. The flask was kept at 37°C for 90 minutes to allow for virus adsorption. Supplemented MEM with 2% FBS was added to the inoculated monolayer and the flask incubated at 37°C for 18-24 hrs by which time nearly 90% of the cell monolayer showed virus-induced destruction. The virus was separated from the cells by two rapid freeze-thaw cycles followed by centrifugation at 2,000 rpm for 10 minutes. The supernatant, which contained the virus, was aspirated, dispensed in aliquots of 200 µL and stored at -80°C. The viral titer was determined by a plaque assay method and was found to be about 2×10^9 PFU/mL.

Soil load

The test viruses were first suspended in a tripartite soil load in the following ratios: 25 µL of 5% bovine serum albumin, 100 µL of 0.4% mucin and 35 µL of 5% Tryptone were added to 340 µL of the virus suspension. The soil load mixture contains a level of protein roughly equal to that in 5% serum.

Carrier Test

The test method used in this study is based on ASTM standard #E-2197-02 and meets with the requirements of the Canadian General Standard Board's national standard (document number CAN/CGSB-2.161-M97 entitled *Assessment of Efficacy of Antimicrobial Agents for Use on Environmental Surfaces and Medical Devices*). The hard surface carriers were 1 cm diameter disks of brushed and magnetized stainless steel.

One disk each was placed in a well of a 12-well cell culture plate and received 10 µL of the test virus in soil load. After the inoculum had dried, the disk was transferred, with the inoculated side up, to the inside bottom surface of a Teflon vial. The carriers were either exposed to 50 µL of EBSS or the test product for the corresponding contact time at ambient temperature ($23 \pm 2^\circ\text{C}$). At the end of the contact time, 950 µL of a neutralizer (Lethen broth+1% sodium thiosulphate) was added to both the test and control vials. The vials were vortexed and the eluates were transferred into sterile labeled dilution vials. The control and test eluates were serially diluted and inoculated into cell culture monolayers for virus plaque assays. The PFU were determined and log₁₀ reductions calculated.

Toxicity and Interference with Plaque Formation:

To determine the effect of the diluted test product on cell monolayers and the plaque-forming ability of the test virus, 100 μL of a 1/20 dilution of the test product in neutralizer was placed into three wells of a 12-well cell culture plate while the other six wells received the neutralizer and EBSS, respectively, as controls and allowed to incubate for 30 minutes. The cells 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 EBSS and virus, diluted to give countable plaques/well, was added to each well. The virus was allowed to adsorb for 60 minutes. Each cell monolayer was then overlaid with an agar overlay and the plates held at the appropriate incubation temperature for the development of virus plaques.

Germicide Neutralization Control:

To determine if the dilution of the product at the end of the contact time was sufficient to render it ineffective against the test virus, 100 μL of the test virus was added to 900 μL of a 1/20 dilution of the test product in neutralizer. The same amount of virus was also added to 900 μL of the neutralizer as a control. The tubes were allowed to stand for 5 minutes and they were then inoculated into cell monolayer for virus plaque formation.

Plaque Assays

Monolayers for all plaque assays were put up in 12-well cell culture plates (Corning cat #08-757-16B). The cells were dispensed at a density of approximately 1×10^6 cells/well to allow for formation of confluent monolayers within 24-72 hours. Each assay included three wells as cell controls and each dilution of the sample tested was inoculated into at least three wells. At the end of the required incubation period for plaque assay, each monolayer received 2 mL of a 3.7% solution of formaldehyde in saline as a fixative and virus inactivator for at least four hours or overnight. The fixative and the agar overlay were then removed from each plate and each well received 2 mL of a 0.1 % aqueous solution of crystal violet to stain the cells. Following a contact time of about five minutes, the stain was aspirated; the wells washed in tap water and plates allowed to dry to determine the plaque counts.

Rhinovirus: HeLa-T4⁺ monolayers were grown as described earlier. Confluent monolayers of cells were trypsinized and dispensed into 12-well plates. The cells were dispensed at a density to allow for the formation of confluent monolayers within 72 hours. The growth medium from each plate was aspirated and 100 μL of the appropriate dilution of the test virus suspension was then dispensed directly onto each monolayer. Each dilution was titrated in triplicate. The plates were incubated for 60 minutes at 33°C in a 5% CO₂ atmosphere to allow for virus adsorption. Each monolayer was overlaid with 2 mL of an overlay medium containing supplemented MEM, 2% FBS, DEAE-Dextran, 5'-bromo-2'-deoxyuridine, 26 mM MgCl₂ and purified agar (Oxoid L28). The ratio of the agar and the supplemented medium was 1:1. Once the overlay had solidified, the plates were held for 3 days in a 5% CO₂ atmosphere at 33°C.

Rotavirus: The growth medium from each plate was aspirated and the monolayers washed twice with EBSS. 100 μL of the appropriate dilution of the test virus suspension was then dispensed directly onto each monolayer. Each dilution was titrated in triplicate. The plates were incubated for 60 minutes at 37°C in a 5% CO₂ atmosphere to allow for virus adsorption. Each

monolayer was overlaid with 2 mL of an overlay medium containing MEM supplemented with HEPES, L-glutamine, non-essential amino acids (NEAA), 5 µg/mL (1-250 trypsin) (Gibco), and agarose type II (Sigma). The ratio of the agarose and the supplemented medium was 1:1. Once the overlay had solidified, the plates were held for 72 hrs in a 5% CO₂ atmosphere at 37°C.

Feline Calicivirus: CrFK cells were grown as described earlier. Confluent monolayers of cells were trypsinized and dispensed into 12-well plates. The cells were dispensed at a density to allow for the formation of confluent monolayers within 24 hours. The growth medium from each plate was aspirated and 100 mL of the appropriate dilutions of viral suspension was then dispensed directly onto the monolayer. Each dilution was titrated in triplicate. The plates were incubated for 90 min in a CO₂ incubator after which a 2 mL overlay, consisting of supplemented 2X MEM and a 1.2% agarose type II (Sigma Cat # A-6877) in a 1:1 ratio. Once the overlay had solidified, the plates were held for 24-30 hrs in a 5% CO₂ atmosphere at 37°C.

RESULTS AND DISCUSSION

The Activity of AHP against Human Rotavirus (Wa): In these tests, the control carriers were found to have an average of 1.73×10^5 PFU. All test carriers were found to negative for any detectable PFU. Therefore, as shown in Table 1, the product was able to bring about a $>5 \log_{10}$ reduction in the viability titre of the rotavirus in a contact time of 5 minutes, indicating virucidal activity against this organism.

Table 1. **Activity of AHP formulation against human rotavirus (Wa)**

| Date of Experiment | Lot Number | Dilution | Contact time | Log ₁₀ Reduction |
|--------------------|------------|----------|--------------|-----------------------------|
| 6/10/03 | 3387 | 1:16 | 5 min | 5.24 |
| 6/10/03 | 3388 | 1:16 | 5 min | 5.24 |
| 6/10/03 | 3389 | 1:16 | 5 min | 5.24 |

Activity of AHP against Human Rhinovirus Type 14: In these tests, the control carriers were found to have an average of 1.27×10^5 PFU. All test carriers were found to negative for any detectable PFU. Therefore, as can be seen from Table 2, the product was able to bring about a $>5 \log_{10}$ reduction in the viability titre of the rhinovirus in a contact time of 5 minutes, indicating virucidal activity against this organism.

Table 2. **Activity of AHP formulation against human rhinovirus type 14.**

| Date of Experiment | Lot Number | Dilution | Contact time | Log ₁₀ Reduction |
|--------------------|------------|----------|--------------|-----------------------------|
| 25/11/03 | 3387 | 1:16 | 5 min | 5.10 |
| 25/11/03 | 3388 | 1:16 | 5 min | 5.10 |
| 25/11/03 | 3389 | 1:16 | 5 min | 5.10 |

Activity of AHP against Feline Calicivirus (F9): In these tests, the control carriers were found to have an average of 3.20×10^5 PFU. There were approximately 100 PFU on the test

carriers. Therefore, as can be seen from Table 3, the product was able to bring about a $>3 \log_{10}$ reduction in the viability titre of the calicivirus in a contact time of 5 minutes, indicating virucidal activity against this organism.

Table 3. **Activity of AHP formulation against the feline calicivirus (F9)**

| Date of Experiment | Lot Number | Dilution | Contact time | Log ₁₀ Reduction |
|--------------------|------------|----------|--------------|-----------------------------|
| 29/09/03 | 3387 | 1:16 | 5 min | 3.68 |
| 29/09/03 | 3388 | 1:16 | 5 min | 3.68 |
| 29/09/03 | 3389 | 1:16 | 5 min | 3.68 |

Interference with Plaque Formation: Pre-exposure of the cell monolayers to a 1:20 dilution of the test product or a neutralizer did not interfere with the plaque formation by the viruses tested in the study.

Dilution of the Product to Arrest its Virucidal Activity: Adding the virus separately to a 1:20 dilution of the product in the neutralizer did not result in any loss in its infectivity, indicating that the dilution of the test product in the neutralizer at the end of the contact time was sufficient to arrest its virucidal activity.

DISCUSSION AND CONCLUSION

The test protocol used in this study was a fully quantitative carrier test based on an international standard (ASTM 2002). Necessary controls were included to ensure that (a) the virucidal activity of the test formulation was effectively arrested at the end of the contact time, (b) the neutralizer process itself did not affect the viability of the virus and (c) the sub-cytotoxic level of the test formulation did not interfere with the ability of the virus to infect and form plaques in the host cells.

Under the test conditions reported here, AHP was able to bring about a $>3 \log_{10}$ reduction in the viability titre of the three viruses tested.

References:

ASTM International: **Standard Quantitative Disk Carrier Test Method for Determining the Bactericidal, Virucidal, Fungicidal, Mycobactericidal and Sporocidal Activities of Liquid Chemical Germicides: E 2197-02.** ASTM, West Conshohocken, PA.