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M&R Chemicals

**EVALUATION OF THE EFFECTIVENESS OF VIROX  
AHP 5 CONCENTRATE AGAINST HEPATITIS A VIRUS,  
HUMAN RHINO VIRUS TYPE 14, HUMAN  
ADENOVIRUS TYPE 4, CANINE PARVOVIRUS AND A  
HUMAN ROTAVIRUS USING THE CARRIER TEST  
METHOD.**

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## OBJECTIVE OF THE STUDY

The objective of this study was to evaluate the virucidal activity of Virox AHP 5 concentrate against human rhinovirus type-14, hepatitis A virus, human adenovirus type 4, a canine parvovirus and the Wa strain human rotavirus using a carrier test method.

## SITE OF THE STUDY

The study was conducted at the Centre for Research on Environmental Microbiology (CREM), Faculty of Medicine, University of Ottawa, Ottawa, Canada. Our laboratory used standard practices for quality control and quality assurance. The facilities here are equipped to handle infectious agents at biosafety levels 2 and 3. Everyone working here is properly trained first and is thoroughly briefed on the safe handling of infectious agents and hazardous chemicals. We have been routinely conducting research on human health-related environmental microbiology for the past 27 years and, apart from peer-reviewed research, we also occasionally undertake contract-funded investigations on the activity of chemical germicides against vegetative bacteria, bacterial spores, mycobacteria, viruses, fungi and protozoa.

## MATERIALS AND METHODS

### The Test Product

Three lots of the product (#015,#016 and #017) were shipped directly by the Sponsor and upon receipt, they were stored at room temperature in an area with controlled access. They were tested as originally formulated and its dilutions were made using 200 ppm hard water.

### The Challenge Viruses

**Hepatitis A virus (HAV):** It was grown and quantitated in monolayer cultures of FRhK-4 cells that had previously been confirmed to allow for the growth and plaques formation by HAV. The cells were grown in minimum essential medium (MEM; GIBCO-BRL Cat # 11575-032) with the appropriate supplements and 10% fetal bovine serum (FBS) in 75cm<sup>2</sup> flasks in a CO<sub>2</sub> incubator at 37°C. 100µL of the viral suspension was inoculated onto a confluent monolayer. The virus was allowed to spread by gentle rocking of the flask. The flask was kept at 37°C for 90 min. to allow for virus adsorption after which supplemented maintenance medium (MEM with 2% FBS) was added. The flask was incubated for 8 days at 37°C. by which time nearly 75% of the monolayer showed virus-induced degeneration. The virus was separated from the cell debris by three rapid freeze-thawing cycles followed by gentle centrifugation at 1,000 rpm for 10 min. The supernatant, which contained the virus, was removed from the cell debris and dispensed in aliquots of 200 µL and stored at -80°C.

**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<sup>+</sup> line of human cervical cancer cells transfected with the human CD<sup>4</sup> receptor gene. A seed culture of these cells was kindly provided to us by Dr. K. Wright of this department. She had originally received the cells from Dr. R. Axel

of NIH, Rockville, MD. The cells were grown in MEM with the appropriate supplements and 10% FBS in 75 cm<sup>2</sup> 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 cm<sup>2</sup> cell 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 1,000 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 (see below) and was found to be about 2.3 X 10<sup>8</sup> 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 MA-104 cell line of monkey kidney epithelial cells. A seed culture of these cells was kindly provided to us by Mr. Tim Cusack of Reckitt and Colman, Montvale, N.J. The cells were grown in MEM (GIBCO-BRL Cat # 41600-016) in the presence of 25 mM HEPES, L-glutamine, antibiotics, and 7% FBS in 75 cm<sup>2</sup> 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, in a 75 cm<sup>2</sup> cell 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 5µg/mL 1-250 trypsin (Nutritional Biochemicals Co.) 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 1,000 rpm for 10 minutes. The supernatant, which contained the virus, was aspirated, dispensed in aliquots of 200 µL and stored at -80°C.

***Human adenovirus:*** Human adenovirus type-4 (ATCC VR-4 ), strain RI-67, was used in this study. The virus was grown in the 293 cell line of human lung fibroblast and plaque assayed on Vero cells of monkey kidney origin. The cells were grown in MEM with the appropriate supplements and 7% FBS in 75 cm<sup>2</sup> flasks at 37°C.

For preparing the virus pool, 200 µL of the viral suspension was inoculated onto a subconfluent cell monolayer in a 75 cm<sup>2</sup> cell culture flask. The inoculum was spread evenly over the monolayer by gentle rocking of the flask. The flask was then 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 3-4 days by which time nearly 80%-90% of the cell monolayer had rounded up. The cells are scraped and pelleted at 1100 rpm for 10 minutes. The supernatant was removed and the pellet was resuspended in a medium with 2% FBS. The virus was separated from the cells by three rapid freeze-thaw cycles followed by centrifugation at 1,000 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 (see below) and was found to be about 2.3 X 10<sup>7</sup> plaque forming units (PFU)/mL.

**Canine parvovirus:** The canine parvovirus used in this study was the Cornell strain (ATCC VR-2017). The virus was grown and plaque assayed in the A72 line of canine tumor cells. A seed culture of these cells and virus was kindly provided to us by Ms. Karen Ramm of Viomed Laboratories Inc, Minneapolis, MN. The cells were grown in MEM (GIBCO-BRL Cat # 41600-016) in the presence of L-glutamine, antibiotics, and 10% FBS in 75 cm<sup>2</sup> flasks at 37°C.

For preparing the virus pool, 200 µL of the viral suspension was inoculated onto a 18-24 hour-old cell monolayer, in a 75 cm<sup>2</sup> cell culture flask containing 1.8mL MEM with 5% FBS. 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% FBS was added to the inoculated monolayer and the flask incubated at 37°C for 4-5 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 5 minutes. The supernatant, which contained the virus, was aspirated and pooled together from 10 flasks and concentrated by pelleting at 25,000 rpm for 4 hrs at 4°C. The supernatant was discarded and the pellet resuspended in 2 mL MEM containing 5% FBS. The viral titer was determined by a plaque assay method (see below) and was found to be about  $1 \times 10^7$  plaque forming units (PFU)/mL.

### **Organic load**

For rotavirus, it was tryptose phosphate broth (Difco). A 10-fold concentrate of it was prepared and filter sterilized and one part of it was added to 9 part of the rotavirus suspension. Fetal bovine serum was used as the organic load for hepatitis A virus, the canine parvovirus and adenovirus 4 at a final concentration of 5%. Mucin was used as the organic load for the rhinovirus and a 10-fold concentration of it was prepared and filtered sterilized. One part of it was then added to 9 part of the rhinovirus suspension to give a final concentration of 5 mg/mL.

### **Carrier Test**

The test method used in this study is based on 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*).

Stainless steel disks (1 cm in diameter) were used as carriers and each disk received 10 µL of the test virus in the presence of the required organic load. After the inoculum had been allowed to dry, each disk was placed in a sterile vial. It was either exposed to 25 µL of buffer (EBSS) or the test product for the required contact time at the specified temperature, after which 975 µL of an eluent/diluent was added and the vial vortexed to recover the inoculum. The control and test eluates were serially diluted and inoculated into cell cultures for virus plaque assays. The plaque forming units (PFU) were determined and  $\log_{10}$  calculated.

### **Toxicity and Neutralization Control**

The following two controls were included :

1. To determine the effect of the diluted test product on the cell monolayer and the plaque forming ability of the test virus.

2. 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.

We placed 100  $\mu$ L of a 1/100 dilution of the test product into six wells of a twelve-well plate while the other six wells received Earle's balanced salt solution (EBSS) as control and allowed for 30 minutes of adsorption. Then, virus, diluted to give countable plaques/well, was added to each well. The virus was allowed to adsorb for 60-90 minutes at 37°C. Each monolayer was then overlaid and the plates incubated at the appropriate temperature for the development of the virus plaques

We added 100  $\mu$ L of the test virus to 900  $\mu$ L of a 1/100 dilution of the test product. The same amount of virus was also added to 900  $\mu$ L of EBSS to act as a control. The tubes were allowed to stand for 5 minutes and they were then inoculated onto cell monolayer for virus plaque formation.

### **Plaque Assays**

Monolayers for all plaque assays were put in 12-well cell culture plates (Corning cat #08-757-16B). The cells were dispensed at a density (approximately  $1 \times 10^6$  cells/well) to allow for formation of confluent monolayers within 24-48 hour. In the case of the parvovirus, which required rapidly dividing cells for its replication, the monolayers used for plaque assay were 3-4 hours old after seeding. 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 three hours. 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 well washed in tap water and plates allowed to dry to determine the plaque counts.

**Rhinovirus:** HeLa-T4<sup>+</sup> 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  $\mu$ L 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% CO<sub>2</sub> 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 MgCl<sub>2</sub> 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% CO<sub>2</sub> atmosphere at 33°C.

**Rotavirus:** The growth medium from each plate was aspirated and the monolayers washed twice with EBSS. 100 $\mu$ L 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% CO<sub>2</sub> 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), 5µg/mL 1-250 trypsin (Nutritional Biochemicals Co. Cleveland, Ohio), 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 72hrs in a 5% CO<sub>2</sub> atmosphere at 37°C..

***Hepatitis A virus:*** 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 CO<sub>2</sub> incubator after which a 2mL overlay, consisting of 2X MEM and a 1.2% agarose type II (Sigma Cat # A-6877) in a 1:1 ratio, was added and allowed to solidify for 25 min..

***Canine Parvovirus:*** Trypsinized cells from 2-4 day old cultures in 75 cm<sup>2</sup> flasks were seeded in 2mL amount in 12 well plates. After the cells had attached (2-4 hrs). The growth medium from each plate was aspirated and 100µL 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 60 min. in a CO<sub>2</sub> incubator after which a 2mL overlay medium consisting of equal part of McCoy's and L-15 medium containing inhibitor free 5% FBS and 1% methyl cellulose (Fisher, 4000 centipoises) were added and inoculated plates were incubated at 37°C in an atmosphere of 5% humidified CO<sub>2</sub> and air.

***Adenovirus:*** Confluent monolayers of vero cells were trypsinized and dispensed into 12-well plates (Corning Cat #08-757-16B). The cells were dispensed at a density (approximately 3.5 x10<sup>5</sup> cells/well) to allow for the formation of confluent monolayers within 24-48 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 90 minutes at 37°C in a an atmosphere of 5% humidified CO<sub>2</sub> to allow for virus adsorption. Each monolayer was overlaid with 2 mL of an overlay medium containing supplemented 2X MEM, 2% FBS, DEAE Dextran, 10 mM MgCl<sub>2</sub> and 0.76% agarose type II (Sigma Cat # A-6877) The ratio of the agar and the supplemented medium was 1:1. Once the overlay had solidified, inoculated plates were incubated at 37°C in an atmosphere of 5% humidified CO<sub>2</sub> and air. After a standard 8 day period incubation, plates were fixed for 2-4 hours with 3.7% formaldehyde and then stained with 0.1% aqueous crystal violet.

## RESULTS AND DISCUSSION

***Activity of Virox AHP 5 Concentrate against Human rotavirus Type Wa:*** As can be seen from Table 1, full strength as well as a 1/16 dilution of the product was able to bring about a >4 log<sub>10</sub> reduction in the viability titre of the rotavirus in a contact time of 10 minutes at 20±1°C, indicating virucidal activity against this organism.

**Table 1 The Activity of Virox AHP 5 Concentrate Against Rotavirus**

<b>Date of expt.</b>	<b>Lot number</b>	<b>Dilution</b>	<b>Contact time (minutes)</b>	<b>PFU/control carrier</b>	<b>PFU/test Carrier</b>	<b>Log Reduction</b>
27/7/98	#015	full strength	10	$1.47 \times 10^4$	0	4.17
27/7/98	#016	full strength	10	$1.47 \times 10^4$	0	4.17
27/7/98	#015	1/16	10	$1.47 \times 10^4$	0	4.17
4/8/98	#016	1/16	10	$1.69 \times 10^4$	0	4.23
4/8/98	#017	1/16	10	$1.69 \times 10^4$	0	4.23

**Activity of Virox AHP 5 Concentrate against Hepatitis A Virus:** As can be seen from Table 2, The product at full strength was able to bring about a  $>4 \log_{10}$  reduction in the viability titre of HAV in a contact time of 10 minutes at  $20 \pm 1^\circ\text{C}$ , indicating virucidal activity against this virus. Also a 1/2 dilution was virucidal at a contact time 30 minutes. However a 1/16 dilution of the product was not able to bring about a  $>3 \log_{10}$  reduction in the viability titre of hepatitis A virus in a contact time of 10, 20 and 30 minutes at  $20 \pm 1^\circ\text{C}$ .

**Table 2 The Activity of Virox AHP 5 Concentrate Against Hepatitis A Virus**

<b>Date of expt</b>	<b>Lot number</b>	<b>Dilution</b>	<b>Contact time (minutes)</b>	<b>PFU/control carrier</b>	<b>PFU/test carrier</b>	<b>Log Reduction</b>
16/7/98	#015	full strength	10	$5 \times 10^4$	0	4.7
16/7/98	#015	1/16	10	$5 \times 10^4$	$4 \times 10^4$	0.02
21/7/98	#016	full strength	10	$1.39 \times 10^5$	0	5.14
21/7/98	#017	full strength	10	$1.39 \times 10^5$	0	5.14
21/7/98	#016	1/16	20	$1.39 \times 10^5$	$3.2 \times 10^4$	0.64
28/7/98	#016	1/16	30	$1.33 \times 10^5$	$1.15 \times 10^4$	1.09
28/7/98	#016	1/2	10	$1.33 \times 10^5$	$6.05 \times 10^2$	2.51
16/9/98	#016	1/2	30	$1.2 \times 10^5$	0	5.1
16/9/98	#017	1/2	30	$1.2 \times 10^5$	0	5.1
30/9/98	#015	1/2	30	$1.1 \times 10^5$	0	5.04

**Activity of Virox AHP 5 Concentrate against the Rhinovirus:** As summarized in Table 3, a 1/16 dilution of the product was able to bring about a  $>4 \log_{10}$  reduction in the viability titre of the rhinovirus in a contact time of 10 minutes at  $20 \pm 1^\circ\text{C}$ , indicating virucidal activity against this organism

**Table 3 The Activity of Virox AHP 5 Concentrate Against Rhinovirus type 14**

Date of expt	Lot number	contact time (minutes)	PFU/control carrier	PFU/test carrier	Log Reduction
24/7/98	#015	10	$9.2 \times 10^4$	0	4.96
30/7/98	#016	10	$7.43 \times 10^4$	0	4.87
30/7/98	#017	10	$7.43 \times 10^4$	0	4.87

**Activity of Virox AHP 5 Concentrate Against the Adenovirus:** A 1/16 dilution of the product was able to bring about a  $>4 \log_{10}$  reduction in the viability titre of the adenovirus in a contact time of 30 minutes at  $20 \pm 1^\circ\text{C}$ , indicating virucidal activity against this organism (Table 4).

**Table 4 The activity of Virox AHP 5 Concentrate against Adenovirus type 4**

Date of expt	Lot number	contact time (minutes)	PFU/control carrier	PFU/test carrier	Log Reduction
8/1/99	#015	10	$1.27 \times 10^4$	$2.15 \times 10^3$	0.77
8/1/99/	#016	10	$1.27 \times 10^4$	$1.53 \times 10^3$	0.92
8/1/99	#015	30	$1.27 \times 10^4$	0	4.10
8/1/99	#016	30	$1.27 \times 10^4$	0	4.10
8/1/99	#017	30	$1.27 \times 10^4$	0	4.10

**Activity of Virox AHP 5 Concentrate against the canine parvovirus:** As in Table 5, a 1/16 dilution of the product was able to bring about a  $>3 \log_{10}$  reduction in the viability titre of the parvovirus in a contact time of 30 minutes at  $20 \pm 1^\circ\text{C}$ , indicating virucidal activity against this organism. A  $\frac{1}{2}$  dilution of the product was able to do the same in a contact time of 10 minutes.

**Table 5. The activity of Virox AHP 5 Concentrate against Canine parvovirus**

<b>Date of expt</b>	<b>Lot number</b>	<b>Dilution</b>	<b>contact time</b>	<b>PFU/control carrier</b>	<b>PFU/test carrier</b>	<b>Log Reduction</b>
9/12/98	#015	1/16	10 minutes	8.9 x10 <sup>3</sup>	1.2 x 10 <sup>3</sup>	0.86
9/12/98	#015	1/16	30 minutes	8.9 x10 <sup>3</sup>	0	3.95
9/12/98	#015	1/2	10 minutes	8.9 x10 <sup>3</sup>	0	3.95
15/12/98	#015	1/16	30 minutes	5.9x10 <sup>3</sup>	0	3.77
15/12/98	#016	1/16	30 minutes	5.9 x10 <sup>3</sup>	0	3.77
15/12/98	#017	1/16	30 minutes	5.9 x10 <sup>3</sup>	0	3.77

***Cytotoxicity of the Test Product:*** At a dilution of 1:100 in EBSS, the product showed no apparent toxicity on the five cell lines used for the study.

***Interference with Plaque Formation:*** Pre-exposure of the cell monolayers to a 1:100 dilution of the test product in EBSS did not interfere with the plaque formation by any of the viruses tested in the study. Any interference by the residual amounts of the product would have resulted in significantly lower numbers of plaques in the monolayers pre-treated with its dilution when compared to the the number of plaques in the control monolayers.

***Dilution of the Product to Arrest its Virucidal Activity:*** Adding the viruses to a 1:100 dilution of the product in EBSS did not result in any loss in their infectivity, which indicates that the dilution of the test product at the end of the contact time was sufficient to arrest its virucidal activity.

## **CONCLUDING REMARKS**

As can seen from the summary table (Table 6) given below, the viruses showed some differences in their susceptibility to the product.

**Table 6. Summary of results for the Virucidal Activity of Virox AHP 5 Concentrate**

<b>Virus</b>	<b>Organic load</b>	<b>Product dilution</b>	<b>Contact time (minutes)</b>	<b>&gt;3 log<sub>10</sub> reduction in titer</b>
Rotavirus	Tryptose PO <sub>4</sub> broth	Full strength	10	Yes
Rotavirus	Tryptose PO <sub>4</sub> broth	1/16	10	Yes
Hepatitis A	Fetal bovine serum	Full strength	10	Yes
Hepatitis A	Fetal bovine serum	1/2	10	No
Hepatitis A	Fetal bovine serum	1/2	30	Yes
Hepatitis A	Fetal bovine serum	1/16	10	No
Hepatitis A	Fetal bovine serum	1/16	30	No
Rhinovirus 14	Mucin	1/16	10	Yes
Adenovirus 4	Fetal bovine serum	1/16	10	No
Adenovirus 4	Fetal bovine serum	1/16	30	Yes
Parvovirus	Fetal bovine serum	1/2	10	Yes
Parvovirus	Fetal bovine serum	1/16	10	No
Parvovirus	Fetal bovine serum	1/16	30	Yes