

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

**EVALUATION OF THE EFFECTIVENESS OF
ACCEL HYDROTHERAPY PLUS AGAINST
POLIOVIRUS**

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 or 8192; *Fax:* (613) 562-5452
E-Mail: ssattar@uottawa.ca

February, 2006

OBJECTIVE OF THE STUDY

The objective of this study was to evaluate the activity of ACCEL HYDROTHERAPY PLUS (a formulation based on accelerated hydrogen peroxide) against poliovirus type 1 (Sabin), using protocol #E1053 of ASTM International (2002).

MATERIALS AND METHODS

The Test Product

Three lots (5001, 5002 and 5003) of the test formulation were shipped to us directly by the Sponsor. Upon receipt, they were stored at room temperature in an area with controlled access. The product was tested at full strength. The product performance criterion was a $\geq 3 \log_{10}$ reduction in virus infectivity titre under the conditions of this test.

The Challenge Virus

The Sabin Vaccine Strain of Poliovirus Type 1 (ATCC VR-192): A stock of the virus was prepared by infecting a monolayer of Vero cells in 75 cm² flasks. The virus was allowed to adsorb to cells for 60 minutes at 37°C and the infected monolayer kept in minimum essential medium, without any antibiotics or fetal bovine serum (FBS), until approximately 75% of the monolayer had been affected by the virus cytopathic effects. The culture was then frozen (-20°C) and thawed three times and the suspension was centrifuged at 1,000x g for 10 minutes to remove cellular debris. The supernatant was used as the virus pool.

Soil Load

For inoculation of carriers, the test virus was first suspended in FBS (Invitrogen Corp., Toronto, ON) at a final concentration of 5%.

Test Method

The test method used in this study was ASTM International's *Standard Method for Efficacy of Virucidal Agents Intended for Inanimate Environmental Surfaces* (ASTM 2002).

The poliovirus suspension (0.2 mL) was spread over the surface of a sterile glass Petri dish with a pipette tip and allowed to air dry for about 20-25 minutes at ambient temperature. The dried virus films were then exposed to 2.0 mL of the disinfectant for the required exposure time at room temperature (23±1°C). Ten seconds before the end of the contact time, the inoculum was scraped with a rubber policeman and remained in suspension until the end of the contact time. At the end of the contact time, the virus-disinfectant mixture was swirled gently to mix in the Petri dish and 0.2 mL from the mixture was transferred into 1.8 mL of neutralizer (Lethen broth+1% sodium thiosulphate pentahydrate). A control experiment was run in parallel and treated in the same manner except that 2.0 mL of Earle balanced salt solution (EBSS) was used in place of the disinfectant. To remove any cytotoxicity in the neutralized mixture, the neutralized samples were passed through a column of Sephadex LH-20 as described in the ASTM method E-1482 (ASTM 2004). The filtrates were transferred into sterile labeled dilution vials. The control and test

filtrates were serially diluted and inoculated into cell culture monolayer for virus plaque assays. Plaque forming units (PFU) were determined and \log_{10} reductions calculated.

Cytotoxicity and Interference with Plaque Formation

To determine the effect of the detoxified test product on cell monolayers and the plaque-forming ability of the test virus, 1.2 mL of a 1/10 and 1/100 dilution of the test product in neutralizer were first passed through the Sephadex column to remove cytotoxicity. The filtrates were then placed into three wells each of a 12-well cell culture plate while the other six wells received neutralizer which was also passed through the column and EBSS, respectively, as controls and allowed to incubate for 30 minutes. The monolayers 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. 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 37°C for the development of virus plaques.

Microbicide Neutralization Control

This was to determine if the neutralization of the sample, followed by detoxification, was sufficient to render it ineffective against the test virus. The test virus (200 μ L) was added to 1.8 mL of the neutralized sample (in the ratio of 1:9). The mixture was then passed through a Sephadex column. The same amount of virus was added to 1.8 mL of the neutralizer control. The virus eluates were then inoculated onto cell monolayer, followed by adsorption for 60 minutes and subsequent addition of overlay medium and incubation.

Plaque Assays

Confluent monolayer of Vero cells were trypsinized and dispensed into 12-well cell culture plates (Corning cat # 08-757-16B) for all plaque assays. The cells were dispensed at a density (approximately 1×10^6 cells/well) to allow for formation of confluent monolayer within 24-48 hour. Each assay included three wells as cell controls and each dilution of the sample tested was inoculated into at least three wells.

The growth medium from each plate was aspirated and 100 μ 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% CO₂ atmosphere to allow for virus adsorption. Each monolayer was overlaid with 2 mL of an overlay medium containing 2X MEM supplemented with HEPES, L-glutamine, non-essential amino acids (NEAA), and 2% FBS, 26 mM MgCl₂ and Noble Difco Agar. The ratio of the agar and the supplemented medium was 1:1. Once the overlay had solidified, the plates were held for 30 hrs in a 5% CO₂ atmosphere at 37°C.

At the end of the required incubation period for the plaque assay, 2 mL of a 3.7% solution of formaldehyde in saline was added to each well and the plates were left for three to four hours to fix the cells and inactivate the virus. 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 the plates allowed to dry to determine the plaque counts.

RESULTS AND DISCUSSION

Activity of the Test Formulation against the Poliovirus: As seen in the table below, all the three lots of the test product at a dilution 1:16 were able to bring about a $>5 \log_{10}$ reduction in the viability titre of the virus in a contact time of 5 minute at ambient temperature, indicating virucidal activity against this virus.

Activity of the product against the poliovirus in a contact time of 5 minutes

Date of experiment	Lot number	Dilution	PFU/control carrier	PFU/test carrier	Log ₁₀ Reduction
15/11/05	5001	1:16	1.30×10^5	0	5.12
15/11/05	5002	1:16	1.30×10^5	0	5.12
15/11/05	5003	1:16	1.30×10^5	0	5.12

Cytotoxicity of the Test Product: A 1:10 dilution of the product in the neutralizer, followed by gel filtration, showed no apparent toxicity for the cell line used for the study.

Interference with Plaque Formation: Pre-exposure of the cell monolayer to a 1:10 dilution of the test product in the neutralizer, followed by gel filtration, did not interfere with the plaque formation by the virus tested in the study. Any interference by the residual amounts of the product would have resulted in significantly lower numbers of plaques in the monolayer pre-treated with its dilution when compared to the number of plaques in the control monolayer.

Neutralization of the Product to arrest Virucidal Activity: Adding the virus separately to a 1:10 dilution of the product in the neutralizer followed by gel filtration did not result in any loss in its infectivity, which indicates that the neutralization of the test product at the end of the contact time, followed by gel filtration, was sufficient to arrest its virucidal activity.

CONCLUDING REMARKS

Under the test conditions reported here, all three lots of ACCEL-HYDROTHERAPY-PLUS at a dilution of 1:16 were able to bring about a $>5 \log_{10}$ reduction in the viability titre of the poliovirus at a contact time of 5 minutes. Pre-exposure of the cell monolayer to a 1:10 dilution of the detoxified test product or a neutralizer did not interfere with the plaque formation by the virus tested in the study.

LITERATURE CITED

- ASTM International (2002): Standard Test Method for Efficacy of Virucidal Agents Intended for Inanimate Environmental Surfaces. Document #E 1053. ASTM International, West Conshohocken, PA.
- ASTM International (2004): Standard Test Method for Neutralization of Virucidal Agents in Virucidal Efficacy Evaluations. Document #E 1482. ASTM International, West Conshohocken, PA.