

Final report submitted to Virox Technologies, Inc.

**EVALUATION OF THE EFFECTIVENESS OF A 7%
ACCELERATED HYDROGEN PEROXIDE-BASED
FORMULATION AGAINST CANINE PARVOVIRUS**

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OBJECTIVE OF THE STUDY

The objective of this study was to evaluate the activity of a formulation based on 7% accelerated hydrogen peroxide (AHP) against canine parvovirus, using protocol # E1053-97 of ASTM International (ASTM 1997).

MATERIALS AND METHODS

The Test Product

Three lots (3387, 3388 and 3389) of AHP5 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 a dilution of 1:16 in hard water with 200 ppm hard as CaCO_3 . The product performance criterion was arbitrarily set at a minimum $\geq 3\log_{10}$ reduction in virus infectivity.

The Challenge Virus

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 the virus were kindly provided to us by Ms. Karen Ramm of Viromed Laboratories Inc., Minneapolis, MN. The cells were grown in Eagle's minimal essential medium (MEM; GIBCO-BRL Cat # 41600-016) in the presence of L-glutamine, antibiotics, and 10% fetal bovine serum (FBS) in 75 cm² 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 cm² 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 dispensed in aliquots of 1 mL and stored at -80°C. The viral titer was determined by a plaque assay method and was found to be about 2×10^5 plaque forming units (PFU)/mL.

Organic load

No added soil load was used in this testing because the virus pool already contained about 5% FBS.

Test Method

The test method used in this study is ASTM International's Standard Method for Efficacy of Virucidal Agents Intended for inanimate Environmental Surfaces.

Parvovirus 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 15-20 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 ($23\pm 1^\circ\text{C}$). Thirty 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 Lethen broth + 1% sodium thiosulphate. A control experiment was run in parallel and treated in the same manner except that 2.0 mL of 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 E1482-92 (ASTM 1992). 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. The 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 a semisolid overlay and the plates held at 37°C for the development of virus plaques.

Germicide 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 μL) 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 1 hour and subsequent addition of overlay medium and incubation.

Plaque Assay

Canine Parvovirus: Trypsinized cells from 2-4 day old cultures in 75 cm^2 flasks were seeded in 2 mL volumes in each well of a 12-well cell culture plate. The plates were incubated for 2-4 hrs for the cells to attach. The growth medium from each plate was aspirated and 100 μL 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 5% CO_2 incubator after which a 2 mL 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 CO₂ for seven days. The plates were then fixed for at least 3 hours with 3.7% formaldehyde and stained with 0.1% aqueous crystal violet.

RESULTS AND DISCUSSION

Activity of AHP against the canine parvovirus: As seen in Table 1, a 1/16 dilution of the product was able to bring about a $>4 \log_{10}$ reduction in the viability titre of the parvovirus in a contact time of 5 minutes at ambient temperature, indicating good virucidal activity against this organism.

Table 1: The activity of AHP against Canine parvovirus

Date of experiment	Lot number	contact time	PFU/control carrier	PFU/test carrier	Log ₁₀ Reduction
5/02/04	3387	5 minutes	9.60 x10 ⁴	0	4.90
5/02/04	3388	5 minutes	9.60 x10 ⁴	0	4.90
5/02/04	3389	5 minutes	9.60 x10 ⁴	0	4.90

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 the number of plaques in the control monolayers.

Neutralization of the Product to Arrest its Virucidal Activity: Adding the viruses separately to a 1:10 dilution of the product in the neutralizer followed by gel filtration did not result in any loss in their 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, Virox 7% Accelerated Hydrogen Peroxide (AHP) at a dilution of 1:16 was able to bring about a $>4 \log_{10}$ reduction in the viability titre of the canine parvovirus. 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 (1997): Standard Test Method for Efficacy of Virucidal Agents Intended for Inanimate Environmental Surfaces. Document #E 1053-97. ASTM International, West Conshohocken, PA.

ASTM International (1992): Standard Test Method for Neutralization of Virucidal Agents in Virucidal Efficacy Evaluations. Document #E 1482-92. ASTM International, West Conshohocken, PA.