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**EVALUATION OF THE EFFECTIVENESS OF AN
ACCELERATED HYDROGEN PEROXIDE-BASED
FORMULATION (AHP-5) AGAINST A FELINE
CALICIVIRUS (A SURROGATE FOR NORWALK
VIRUS)**

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OBJECTIVE OF THE STUDY

The objective of this study was to evaluate the activity of a formulation based on accelerated hydrogen peroxide (AHP-5) against a feline calicivirus, using protocol # E1053-97 of ASTM International (ASTM 1997).

MATERIALS AND METHODS

The Test Product

One lot (3783) of AHP-5 was shipped to us directly by the Sponsor. Upon receipt, it was stored at room temperature in an area with controlled access. The product was tested at a dilution of 1:32 and 1:64 in hard water with 200 ppm hardness as CaCO₃. The product performance criterion was arbitrarily set at a minimum $\geq 3\log_{10}$ reduction in virus infectivity.

The Challenge Virus

Feline Calicivirus: The feline calicivirus used in this study was the F9 strain (ATCC #VR-782). The virus was grown and plaque assayed in Crandall'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 Eagle 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 virus suspension was inoculated onto an 18-24 hour-old cell monolayer, in a 75-cm²-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 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 plaque forming units (PFU)/mL.

Organic load

For inoculation of carriers, the test virus was first suspended in bovine serum (Gibco BRL Life Technologies) at a final concentration of 5%

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.

Calicivirus 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 (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 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 90 minutes. Each cell monolayer was then overlaid with an agar 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 90 minutes and subsequent addition of overlay medium and incubation.

Plaque Assay

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 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 90 min in a CO_2 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% CO_2 atmosphere at 37°C . 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 feline Calicivirus: As seen in Table 1, a 1/32 and 1/64 dilution of the product was able to bring about a $>4 \log_{10}$ reduction in the viability titre of the virus in a contact time of 5 minutes at ambient temperature, indicating good virucidal activity against this virus.

Table 1: The activity of AHP against Feline Calicivirus

Date of experiment	Lot number	Dilution	contact time	PFU/control carrier	PFU/test carrier	Log ₁₀ Reduction
26/07/04	3783	1:32	5 minutes	3.33×10^4	0	4.52
26/07/04	3783	1:64	5 minutes	3.33×10^4	0	4.52

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 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

FCV is generally accepted as a surrogate for noroviruses (Norwalk virus) because noroviruses cannot be grown and quantitated in the lab. FCV belongs to the same family (Caliciviridae) as noroviruses and thus share the same physiochemical characteristics, genomic organization.

Under the test conditions reported here, Virox Accelerated Hydrogen Peroxide (AHP-5) at a dilution of 1:32 and 1:64 was able to bring about a $>4 \log_{10}$ reduction in the viability titre of the Feline Calicivirus. 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.