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**EVALUATION OF THE VIRUCIDAL ACTIVITY OF AN  
ACCELERATED HYDROGEN PEROXIDE PRODUCT  
(AHP-5) AGAINST HUMAN CORONAVIRUS 229E**

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Signature

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Date

## OBJECTIVE OF THE STUDY

The objective of this study was to evaluate the virucidal activity of AHP-5 on human coronavirus 229E using a quantitative carrier test developed in our laboratory, which is now a standard of ASTM International (E-2197-02).

## MATERIALS AND METHODS

### The Test Product

Three lots of the product were shipped to us directly by the Sponsor. Upon receipt, they were stored at room temperature in a secure area with controlled access. The product effectiveness criterion was arbitrarily set at a minimum  $\geq 3 \log_{10}$  reduction in virus infectivity.

### The Challenge Virus

**Human Coronavirus:** The human coronavirus used in this study was the 229E strain (ATCC #VR-740). The virus was grown and plaque assayed in L-132 cell line (ATCC #CCL-5). The cells were originally derived from human embryonic lung tissue. 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 36±1°C.

For preparing the virus pool, 200 µL of the virus suspension was inoculated into a 24-48 hour-old 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 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. The virus was harvested 48-50 hours post-adsorption before CPE was visible and 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 500 µL and stored at -80°C. The viral titer was determined by a plaque assay method and was found to be about 4 X 10<sup>6</sup> PFU/mL.

### Soil load

The test virus was first suspended in a tripartite soil load: 25 µL of bovine serum albumin, 100 µL of mucin and 35 µL of 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 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*).

One stainless steel disk (1 cm in diameter) carrier each was placed in a well of a 12-well cell culture plate. Each disk then received 10 µL of the test virus in soil load. After the inoculum had dried, each disk was placed with the inoculated side up, on the inside bottom surface of a Teflon vial. The carriers were either exposed to 50 µL of Earle balanced salt solution (EBSS) or the test product for the corresponding contact time at ambient temperature (23±2°C. At the end of the

contact time, 950 µL of neutralizer (Lethen broth) 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 monolayer for virus plaque assays. The PFU were determined and log<sub>10</sub> reductions calculated.

#### **Toxicity and Interference with Plaque Formation:**

To determine the effect of the diluted test product on the cell monolayer and the plaque forming ability of the test virus, 100 µL of a 1/20 and 1/200 dilutions of the test product in neutralizer were placed into three wells each of a twelve-well plate while the other six wells received neutralizer/EBSS as control and allowed to incubate for 30 minutes. The cells were observed under the microscope for signs of toxicity of the test product. The cells 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 at 33°C. Each cell monolayer was then overlaid and the plates incubated at the appropriate temperature for the development of the virus plaques.

#### **Germicide Neutralization Control:**

To determine if the neutralizer sample 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 and 1/200 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 Assay**

L-132 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 48-72 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% 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 6 days in a 5% CO<sub>2</sub> atmosphere at 33°C. They were then fixed and stained as described above and the plaques counted.

## **RESULTS**

**Activity of AHP-5 against Human Coronavirus 229E:** As can be seen from Table 1, at a dilution of 1:16, AHP-5 was able to bring about a >4 log<sub>10</sub> reduction in the viability titre of the coronavirus in a contact time of 5 minutes indicating good virucidal activity against this virus.

**Table 1: Activity of AHP-5 against Human Corona virus 229E**

<b>Lot Number</b>	<b>Contact time</b>	<b>PFU/Control Carrier</b>	<b>PFU/Test Carrier</b>	<b>Log<sub>10</sub> Reduction</b>
3783	5 minutes	1.27 x 10 <sup>4</sup>	None detected	4.09
3815	5 minutes	1.27 x 10 <sup>4</sup>	None detected	4.09
3816	5 minutes	1.27 x 10 <sup>4</sup>	None detected	4.09

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

**Dilution of the Product to Arrest its Virucidal Activity:** Adding the virus separately to a detoxified 1:200 dilution of the product in the neutralizer did not result in any loss in its infectivity, indicating that the 1/200 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 (Sattar et al. 2003) 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-5 at a dilution of 1:16 was able to bring about a >4 log<sub>10</sub> reduction in the viability titre of human coronavirus 229E in a contact time of 5 minutes.

## **LITERATURE CITED**

- ASTM International (2002): Standard Quantitative Disk Carrier Test Method for Determining the Bactericidal, Virucidal, Fungicidal, Mycobactericidal and Sporocidal Activities of Liquid Chemical Germicides, Document #E 2197-02. ASTM International, West Conshohocken, PA.
- Sattar, S.A., Springthorpe, V.S., Adegbonrin O., Zafer, A.A. & Busa M. (2003). A Disc-based quantitative carrier test method to assess the virucidal activity of chemical germicides. J.