OBJECTIVE

The objective of this study was to evaluate the virucidal efficacy of a test substance for registration of a product as a virucide. The test procedure was to simulate the way in which the product is intended to be used. This method is in compliance with the requirements of and may be submitted to Health Canada.

SUMMARY OF RESULTS

Test Substance:	One Spray Concentrate, Lot # 8250111001 and Lot # 8250111901
Dilution:	0.32 oz/128 oz defined as 0.25% test substance + 99.75% 400 ppm AOAC Synthetic Hard Water
Virus:	Porcine Epidemic Diarrhea Virus, Strain Colorado 2013 Isolate, Obtained from the National Veterinary Services Laboratories (NVSL), Ames, IA
Exposure Time:	2 minutes
Exposure Temperature:	Room temperature (22.0°C)
Exposure Humidity:	24.35%
Organic Soil Load:	0% fetal bovine serum
Efficacy Result:	Two lots of One Spray Concentrate (Lot # 8250111001 and Lot # 8250111901) met the performance requirements specified in the study protocol. The results indicate **a ≥3 $\log_{10}$ reduction in titer** of Porcine Epidemic Diarrhea Virus under these test conditions as required by Health Canada.

TEST SYSTEM

1. Virus
   The Colorado 2013 Isolate strain of Porcine Epidemic Diarrhea Virus used for this study was obtained from the National Veterinary Services Laboratories (NVSL), Ames, IA. The stock virus was prepared by collecting the supernatant culture fluid from 75-100% infected culture cells. The cells were disrupted and cell debris removed by centrifugation at approximately 2000 RPM for five minutes at approximately 4°C. The supernatant was removed, aliquoted, and the high titer stock virus was stored at ≤-70°C until the day of use. On the day of use, an aliquot of stock virus (ATS Labs Lot PED-54) was removed, thawed and maintained at a refrigerated temperature until used in the assay. The stock virus culture contained 0% fetal bovine serum as the organic soil load. The stock virus tested demonstrated cytopathic effects (CPE) typical of Porcine Epidemic Diarrhea Virus on Vero 76 cells.

2. Indicator Cell Cultures
Cultures of Vero 76 cells were originally obtained from the American Type Culture Collection, Manassas, VA (ATCC CRL-1587). The cells were propagated by Analytical Lab Group-Midwest personnel. The cells were seeded into multiwell cell culture plates and maintained at 36-38°C in a humidified atmosphere of 5-7% CO_2. On the day of testing, the cells were observed as having proper cell integrity and confluency, and therefore, were acceptable for use in this study.

All cell culture documentation was retained for the cell cultures used in the assay with respect to source, passage number, growth characteristics, seeding densities and the general condition of the cells.

3. Test Medium
The test medium used in this study was Minimum Essential Medium (MEM) supplemented with 2 μg/mL TPCK-trypsin, 10% (v/v) tryptose phosphate broth, 10 μg/mL gentamicin, 100 units/mL penicillin, and 2.5 μg/mL amphotericin B.

TEST METHOD

1. Preparation of Test Substance
Two lots of One Spray Concentrate (Lot # 8250111001 and Lot # 8250111901) were tested at an equivalent dilution of 0.32 oz/128 oz dilution defined as 0.25% test substance + 99.75% 400 ppm AOAC Synthetic Hard Water (1.00 mL product + 399.0 mL water) as requested by the Sponsor. The test substance was in solution as determined by visual observation and used on the day of preparation. The prepared test substance was at the exposure temperature prior to use.

The 400 ppm AOAC Synthetic Hard Water was prepared using 4.2 mL of Solution I and 4.0 mL of Solution II. The total volume of hard water was brought to approximately 1 liter using sterile deionized water. The 400 ppm hard water was prepared, titrated (at 387 ppm) and used on the day of testing.

2. Preparation of Virus Films
Films of virus were prepared by spreading 200 μL of virus inoculum uniformly over the bottoms of three separate 100 x 15 mm sterile glass petri dishes (without touching the sides of the petri dish). The virus films were dried at 20.0°C in a relative humidity of 30% until visibly dry (20 minutes).

3. Preparation of Sephadex Gel Filtration Columns
To reduce the cytotoxic level of the virus-test substance mixture prior to assay of virus, and/or to reduce the virucidal level of the test substance, virus was separated from the test substance by filtration through Sephadex LH-20 gel. On the day of testing, Sephadex columns were prepared by centrifuging the prepared Sephadex gel in sterile syringes for three minutes to clear the void volume. The columns were then ready to be used in the assay.

4. Input Virus Control (TABLE 1)
   On the day of testing, the stock virus utilized in the assay was titered by 10-fold serial dilution and assayed for infectivity to determine the starting titer of the virus. The results of this control are for informational purposes only.

5. Treatment of Virus Films with the Test Substance (TABLE 1)
   For each lot of test substance, one dried virus film was individually exposed to a 2.00 mL aliquot of the use dilution of the test substance and held covered for 2 minutes at room temperature (22.0°C) and 24.35% relative humidity. The virus films were completely covered with the test substance. Just prior to the end of the exposure time, the plates were individually scraped with a cell scraper to resuspend the contents and at the end of the exposure time the virus-test substance mixtures were immediately passed through individual Sephadex columns utilizing the syringe plungers in order to detoxify the mixtures. The filtrates (10^{-1} dilution) were then titered by 10-fold serial dilution and assayed for infectivity and/or cytotoxicity.

6. Treatment of Dried Virus Control Film (TABLE 1)
   One virus film was prepared as previously described (paragraph 2). The virus control film was exposed to 2.00 mL of test medium in lieu of the test substance and held covered for 2 minutes at room temperature (22.0°C) and 24.35% relative humidity. Just prior to the end of the exposure time, the virus control was scraped with a cell scraper and at the end of the exposure time the virus mixture was immediately passed through a Sephadex column in the same manner as the test virus (paragraph 5). The filtrate (10^{-1} dilution) was then titered by 10-fold serial dilution and assayed for infectivity.

7. Cytotoxicity Controls (TABLE 2)
   A 2.00 mL aliquot of the use dilution of each lot of the test substance was filtered through an idnividual Sephadex column and the filtrate was diluted serially in medium and inoculated into Vero 76 cell cultures. Cytotoxicity of the Vero 76 cell cultures was scored at the same time as the virus-test substance and virus control cultures.

8. Assay of Non-Virucidal Level of Test Substance (Neutralization Control) (TABLE 3)
   Each dilution of the neutralized test substance (cytotoxicity control dilutions) was challenged with an aliquot of low titer stock virus to determine the dilution(s) of test substance at which virucidal activity, if any, was retained. Dilutions that showed virucidal activity were not considered in determining reduction of the virus by the test substance.

   Using the cytotoxicity control dilutions prepared above, an additional set of indicator cell cultures was inoculated with a 200 µL aliquot of each dilution in quadruplicate. A 100 µL aliquot of low titer stock virus (approximately 500 infectious units) was inoculated into each cell culture well and the indicator cell cultures were incubated along with the test and virus control plates.

9. <u>Infectivity Assays</u>
The Vero 76 cell line, which exhibits cytopathic effect (CPE) in the presence of Porcine Epidemic Diarrhea Virus, was used as the indicator cell line in the infectivity assays. Cells in multiwell culture dishes were inoculated in quadruplicate with 200 µL of the dilutions prepared from test and control groups. The input virus control was inoculated in duplicate. Uninfected indicator cell cultures (cell controls) were inoculated with test medium alone. The cultures were incubated at 36-38°C (37.0°C) in a humidified atmosphere of 5-7% CO_2 (6.0% CO_2) in sterile disposable cell culture labware. The cultures were scored periodically for seven days for the absence or presence of CPE, cytotoxicity, and for viability.

10. <u>Statistical Methods</u>: Not applicable

PLANNED PROTOCOL CHANGES

Protocol Amendments:
No protocol amendments were required for this study.

Protocol Deviations:
No protocol deviations occurred during this study.

DATA ANALYSIS

Calculation of Titers

Viral and cytotoxicity titers will be expressed as -$\log_{10}$ of the 50 percent titration endpoint for infectivity ($TCID_{50}$) or cytotoxicity (TCD_{50}), respectively, as calculated by the method of Spearman Karber.

Per Volume Inoculated ($TCID_{50}$/volume inoculated):

$$-\text{Log of 1st dilution inoculated} - \left[\left(\left(\frac{\text{Sum of \% mortality at each dilution}}{100}\right) - 0.5\right) \times (\text{logarithm of dilution})\right]$$

Per Carrier ($TCID_{50}$/carrier):

(Antilog of $TCID_{50}$*) x (volume inoculated per carrier/ volume inoculated per well) = Y

Log_{10} of Y = the $TCID_{50}$/carrier (Example: $10^{5.50}$ or 5.50 Log_{10})

*$TCID_{50}$ value calculated based on the volume inoculated per well

Calculation of Log Reduction

The following calculation will be used to calculate the log reduction per volume inoculated per well and the log reduction per carrier.

Dried Virus Control Log_{10} $TCID_{50}$ – Test Substance Log_{10} $TCID_{50}$ = Log Reduction

Calculation of Infectious Units

$$\left(\frac{\text{input virus titer}}{\text{dilution of test virus used for neutralization control}}\right)\left(\frac{\text{low titer virus inoculation volume}}{\text{input virus inoculation volume}}\right) = \sim \text{infectious units}$$

Example: Titer of the input virus: $10^{5.50}$ ($TCID_{50}$ of $10^{6.00}$), 1:1,000 dilution made from stock virus for use in the neutralization control, 100 µL/well of low titer virus inoculated and 250 µL/well of input virus inoculated)

($10^{5.50}$ / $10^{3.00}$) (100 µL / 250 µL) = ~126 infectious units

STUDY ACCEPTANCE CRITERIA

Health Canada Submission

A valid test requires 1) that at least 4.8 $\log_{10}$ of infectivity per carrier be recovered from the dried virus control film; 2) that a ≥3 $\log_{10}$ reduction in titer must be demonstrated; 3) if cytotoxicity is evident, at least a 3 $\log_{10}$ reduction in titer must be demonstrated beyond the cytotoxic level. Similarly, the log reduction will also take into consideration the level of neutralization; 4) that the cell controls be negative for infectivity. An efficacious product does not need to demonstrate complete inactivation at all dilutions.

RECORD RETENTION

Study Specific Documents

All of the original raw data developed exclusively for this study shall be archived at Analytical Lab Group-Midwest following the record retention policy outlined in the internal SOP ALS-0032. These original data include, but are not limited to, the following:

1. All handwritten raw data for control and test substances including, but not limited to, notebooks, data forms and calculations.
2. Any protocol amendments/deviation notifications.
3. All measured data used in formulating the final report.
4. Memoranda, specifications, and other study specific correspondence relating to interpretation and evaluation of data, other than those documents contained in the final study report.
5. Original signed protocol.
6. Certified copy of the final study report.
7. Study-specific SOP deviations made during the study.

Test Substance Retention

The test substance will be discarded following study completion. It is the responsibility of the Sponsor to retain a sample of the test substance.

REFERENCES

1. ASTM E1053-20, Standard Practice to Assess Virucidal Activity of Chemicals Intended for Disinfection of Inanimate, Nonporous Environmental Surfaces, ASTM International, West Conshohocken, PA, 2020, www.astm.org.
2. American Society of Testing and Materials (ASTM). Standard Practice for Use of Gel Filtration Columns for Cytotoxicity Reduction and Neutralization, E1482-12 (Reapproved 2017).
3. U.S. Environmental Protection Agency, Office of Chemical Safety and Pollution Prevention, Product Performance Test Guidelines, OCSPP 810.2000: General Considerations for Testing Public Health Antimicrobial Pesticides – Guidance for Efficacy Testing. February 2018.
4. U.S. Environmental Protection Agency, Office of Chemical Safety and Pollution Prevention, Product Performance Test Guidelines, OCSPP 810.2200: Disinfectants for Use on Environmental Surfaces – Guidance for Efficacy Testing. February 2018.
5. Diagnostic Procedures for Viral, Rickettsial, and Chlamydial Infections. Lennette, E.H., Lennette, D.A. and Lennette, E.T. editors. Seventh edition, 1995.
6. Blackwell, J.H., and J.H.S. Chen. 1970. Effects of various germicidal chemicals on HEP-2 cell culture and Herpes simplex virus. J. AOAC 53:1229-1236.
7. Health Canada, April 2020. Guidance Document - Disinfectant Drugs.
8. Health Canada, April 2020. Guidance Document - Safety and Efficacy Requirements for Surface Disinfectant Drugs.
9. Association of Official Analytical Chemists (AOAC) Official Method 960.09, Germicidal and Detergent Sanitizing Action of Disinfectants Method [Preparation of Synthetic Hard Water]. In Official Methods of Analysis of the AOAC, 2013 Edition.
10. OECD Environment, Health and Safety Publications, Series on Testing Assessment No. 187 and Series on Biocides No. 6, Guidance Document on Quantitative Methods for Evaluating the Activity of Microbicides used on Hard Non-Porous Surfaces, June 21, 2013.
11. U.S. Environmental Protection Agency, Office of Chemical Safety and Pollution Prevention, Product Performance Test Guidelines, Series 810 Guidelines FAQ, August 2019.
12. U.S. Environmental Protection Agency, Office of Pesticide Programs SOP Number: MB-30-02, Preparation of Hard Water and Other Diluents for Preparation of Antimicrobial Products, August 2019.

STUDY RESULTS

Results of tests with two lots of One Spray Concentrate (Lot # 8250111001 and Lot # 8250111901), diluted 0.32 oz/128 oz defined as 0.25% test substance + 99.75% 400 ppm AOAC Synthetic Hard Water, exposed to Porcine Epidemic Diarrhea Virus in the presence of a 0% fetal bovine serum organic soil load at room temperature (22.0°C) and 24.35% relative humidity for 2 minutes are shown in Tables 1-3. All cell controls were negative for test virus infectivity.

The titer of the input virus control was 6.50 $\log_{10}$/200 µL. The titer of the dried virus control was 5.50 $\log_{10}$/200 µL (5.50 $\log_{10}$/carrier). Following exposure, test virus infectivity was not detected in the virus-test substance mixture for either lot at any dilution tested [≤0.50 $\log_{10}$/200 µL (≤0.50 $\log_{10}$/carrier)]. Test substance cytotoxicity was not observed in either lot at any dilution tested (≤0.50 $\log_{10}$/200 µL). The neutralization control (non-virucidal level of the test substance) indicates that the test substance was neutralized at ≤0.50 $\log_{10}$/200 µL for both lots.

Taking the cytotoxicity and neutralization control results into consideration, the reduction in viral titer, per volume inoculated per well and per carrier, was ≥5.00 $\log_{10}$ for both lots.

STUDY CONCLUSION

Under the conditions of this investigation and in the presence of a 0% fetal bovine serum organic soil load, One Spray Concentrate, diluted 0.32 oz/128 oz defined as 0.25% test substance + 99.75% 400 ppm AOAC Synthetic Hard Water, demonstrated a ≥3 $\log_{10}$ reduction in titer of Porcine Epidemic Diarrhea Virus following a 2 minute exposure time at room temperature (22.0°C) and 24.35% relative humidity as required by Health Canada.

In the opinion of the Study Director, there were no circumstances that may have adversely affected the quality or integrity of the data.

The use of the Analytical Lab Group-Midwest name, logo or any other representation of Analytical Lab Group-Midwest without the written approval of Analytical Lab Group-Midwest is prohibited. In addition, Analytical Lab Group-Midwest may not be referred to in any form of promotional materials, press releases, advertising or similar materials (whether by print, broadcast, communication or electronic means) without the expressed written permission of Analytical Lab Group-Midwest.

TABLE 1: Virus Controls and Test Results

Effects of One Spray Concentrate (Lot # 8250111001 and Lot # 8250111901) Following a 2 Minute Exposure to Porcine Epidemic Diarrhea Virus Dried on an Inanimate Surface

Dilution	Input Virus Control	Dried Virus Control	Porcine Epidemic Diarrhea Virus + Lot # 8250111001	Porcine Epidemic Diarrhea Virus + Lot # 8250111901
Cell Control	0 0	0 0 0 0	0 0 0 0	0 0 0 0
10^{-1}	+ +	+ + + +	0 0 0 0	0 0 0 0
10^{-2}	+ +	+ + + +	0 0 0 0	0 0 0 0
10^{-3}	+ +	+ + + +	0 0 0 0	0 0 0 0
10^{-4}	+ +	+ + + +	0 0 0 0	0 0 0 0
10^{-5}	+ +	+ + + +	0 0 0 0	0 0 0 0
10^{-6}	+ +	0 0 0 0	0 0 0 0	0 0 0 0
10^{-7}	0 0	NT	NT	NT
$TCID_{50}$/200 µL	$10^{6.50}$	$10^{5.50}$	$\leq 10^{0.50}$	$\leq 10^{0.50}$
$TCID_{50}$/carrier	NA	$10^{5.50}$	$\leq 10^{0.50}$	$\leq 10^{0.50}$

(+) = Positive for the presence of test virus
(0) = No test virus recovered and/or no cytotoxicity present
(NA) = Not applicable
(NT) = Not tested

TABLE 2: Cytotoxicity Control Results

Cytotoxicity of One Spray Concentrate on Vero 76 Cell Cultures

Dilution	Cytotoxicity Control Lot # 8250111001	Cytotoxicity Control Lot # 8250111901
Cell Control	0 0 0 0	0 0 0 0
10^{-1}	0 0 0 0	0 0 0 0
10^{-2}	0 0 0 0	0 0 0 0
10^{-3}	0 0 0 0	0 0 0 0
10^{-4}	0 0 0 0	0 0 0 0
10^{-5}	0 0 0 0	0 0 0 0
10^{-6}	0 0 0 0	0 0 0 0
TCD_{50}/200 µL	$\leq 10^{0.50}$	$\leq 10^{0.50}$

(0) = No test virus recovered and/or no cytotoxicity present

TABLE 3: Neutralization Control Results

Non-Virucidal Level of the Test Substance (Neutralization Control)

Dilution	Test Virus + Cytotoxicity Control Lot # 8250111001	Test Virus + Cytotoxicity Control Lot # 8250111901
Cell Control	0 0 0 0	0 0 0 0
10^{-1}	+ + + +	+ + + +
10^{-2}	+ + + +	+ + + +
10^{-3}	+ + + +	+ + + +
10^{-4}	+ + + +	+ + + +
10^{-5}	+ + + +	+ + + +
10^{-6}	+ + + +	+ + + +

(+) = Positive for the presence of test virus after low titer stock virus added (neutralization control)
(0) = No test virus recovered and/or no cytotoxicity present

Results of the non-virucidal level control indicate that the test substance was neutralized at a $TCID_{50}$/200 µL of ≤0.50 log_{10} for both lots.