STUDY REPORT

Study Title
Non-GLP ASTM E1053: Standard Practice to Assess Virucidal Activity of Chemicals Intended for Disinfection of Inanimate, Non-porous Environmental Surfaces

Product Identity
Hypochlorous Acid

Test Microorganism
Canine parvovirus, Cornell strain, ATCC VR-2017

Study Identification Number
NG21893

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Study Completion Date
17NOV2023

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STUDY REPORT SUMMARY

General Study Information

Study Title:	Non-GLP ASTM E1053: Standard Practice to Assess Virucidal Activity of Chemicals Intended for Disinfection of Inanimate, Non-porous Environmental Surfaces
Study Identification Number:	NG21893

Test System

Test Microorganism:	Canine parvovirus, Cornell strain, ATCC VR-2017
Host Cell:	A-72 cells (ATCC CRL-1542)

Test Substance:	Hypochlorous Acid
Test Substance Receipt Date:	17OCT2023

Test Parameters

Test Substance Application:	Liquid application (2.0 ml)
Organic Soil Load:	No additional soil load supplementation: virus tested as propagated (7.5% fetal bovine serum)
Number of Replicates per Lot:	One
Contact Times:	1 minute, 2 minutes and 5 minutes
Exposure Temperature:	Ambient room temperature (See "Results" for details)
Neutralization Method:	Filtrate (10^{-1} dilution) passed through two Sephadex LH-20 gel filtration columns. Followed by serial 10-fold dilution using Eagle's Minimum Essential Medium (EMEM) supplemented with 7.5% fetal bovine serum (FBS); then the 10^{-2} dilution passed through an additional Sephadex LH-20 gel filtration column (see "Summary of Test Procedure" for details).

Study Dates

Experimental Start Date/Time:	31OCT2023 / 1104
Experimental Termination Date/Time:	07NOV2023 / 1607
Study Completion Date:	17NOV2023

SUMMARY OF TEST PROCEDURE

- Stock virus was thawed and was not supplemented with an organic soil load.
- Sterile Petri dishes (100 mm x 15 mm) were used as the test carriers. For each lot of substance assayed, one carrier was inoculated with a 0.200 ml volume of virus suspension. The appropriate number of plate recovery control carriers were also prepared.
- The inoculated carriers were dried at the appropriate temperature and relative humidity to lessen the level of virus inactivation due to drying.
- The test substance was prepared according to the Study Sponsor's instructions as requested and applied to each test carrier via liquid application using a pipette. For pipette delivery products, a 2.0 ml volume was applied per carrier.
- The treated carriers were held covered for the Study Sponsor's specified contact times at the Study Sponsor specified exposure temperature. Just prior to the completion of each contact time, a sterile cell scraper was used to re-suspend each viral film and the solution was immediately transferred into a gel filtration column. The syringe plunger was used to pass the contents of the re-suspended test carrier through the column. Serial 10–fold dilutions (e.g. 0.1 ml filtrate + 0.9 ml test media) of the filtrate (10^{-1} dilution) were prepared to the appropriate dilution. To aid in reducing test substance cytotoxicity, the 10^{-1} dilution was passed through a second, individual gel filtration column and then 10^{-2} dilution was passed through an individual gel filtration column following .
- The plate recovery control carrier was held covered for the contact time then harvested and neutralized in the same manner as the test.
- Following neutralization of test and control carriers, the viral suspensions were quantified to determine the levels of infectious virus using standard cell culture techniques (e.g. $TCID_{50}$).
- The inoculated cell culture plates were incubated for the period most suitable for the virus-host cell system (e.g. ~7 days).
- On the final day of incubation, a hemagglutination assay was performed using porcine red blood cells . The appropriate calculations were performed (e.g. Spearman-Kärber) to determine viral titers and levels of test substance cytotoxicity, where applicable.
- The log_{10} and percent reductions in viral titer were calculated for viral films exposed to the test product relative to the titer obtained for the study control carrier(s).

<u>Study Notes</u>

- Study Sponsor was on site 31OCT2023 to prepare the test substance and observe testing.
- The use of additional gel filtration columns was determined based on the non-GLP pre-test cytotoxicity and neutralization screen and utilized in testing to aid in reducing test substance cytotoxicity (see "Summary of Test Procedure" for details).

SUCCESS CRITERIA

- The following measures are met to ensure the acceptability of virucidal efficacy data:
  - A minimum of 4.80 log_{10} to infective units/control carrier is recovered from each plate recovery control film(s).
  - The virus titer control demonstrates obvious and/or typical cytopathic effects on the monolayers unless a detection method other than cytopathic effect is used.
  - Comparable levels of infective units must be recovered from the neutralized test substance and neutralization control substance.
  - Quantification of the test and control parameters is conducted at a minimum of four determinations per dilution.

Note: Although the test method does not specify a product performance criterion, for registration as a hard surface disinfectant, the U.S. EPA requires a ≥3.00 log_{10} reduction in viral titer as compared to the corresponding plate recovery control.

CALCULATIONS AND STATISTICAL ANALYSIS

- The $TCID_{50}$ (Tissue Culture Infectivity Dose) represents the endpoint dilution where 50% of the cell cultures exhibit cytopathic effects due to infection by the test virus. The endpoint dilution at which 50% of the host cell monolayers exhibit cytotoxicity is termed the Tissue Culture Dose (TCD_{50}). The $TCID_{50}$ and TCD_{50} were determined using the Spearman-Kärber method and calculated as follows:

  Negative logarithm of endpoint titer = [– Log of first dilution inoculated] – [((sum of % mortality at each dilution/100) – 0.5) x Logarithm of dilution]

  The result of this calculation is expressed as $TCID_{50}$/0.1 ml (or volume of dilution inoculated) for the test, virus control, and neutralization control and TCD_{50}/0.1 ml (or volume of dilution inoculated) for the cytotoxicity control.

- Calculation of the Log_{10} Reduction

  The log_{10} reduction in viral titer was calculated as follows:

  Plate Recovery Control Log_{10} $TCID_{50}$ – Virus-Test Substance Log_{10} $TCID_{50}$

- Calculation of the Percent Reduction

  The percent reduction in viral titer was calculated as follows:

  Percent Reduction = 1- (C/B) x 100, where:
  B = Average $TCID_{50}$ of virus in control suspensions.
  C = Average $TCID_{50}$ of virus in virus-test suspensions.

- The presence of any test substance cytotoxicity was taken into account when calculating the log and percent reductions in viral titer.

- If multiple virus control and test replicates were performed, the average $TCID_{50}$ of each parameter was calculated and the average result used to calculate the log reductions in viral titer.

Statistical Analysis
Not applicable.

Methods for the Control of Bias
Not applicable.

RESULTS

Table 1: Virus Inoculum Titer Control and Time Zero Results

		Virus Inoculum Titer
Cell Control		0 0 0 0
Dilution	10^{-2}	+ + + +
	10^{-3}	+ + + +
	10^{-4}	+ + + +
	10^{-5}	+ + + +
	10^{-6}	+ + 0 0
	10^{-7}	0 0 0 0
$TCID_{50}$ per 0.1 ml		6.00 log_{10}

Table 2: Plate Recovery Control Results

		Recovery Control
		Contact Time – 5 Minutes
Dilution	10^{-2}	+ + + +
	10^{-3}	+ + + +
	10^{-4}	+ + + +
	10^{-5}	+ + + +
	10^{-6}	0 0 + 0
	10^{-7}	0 0 0 0
$TCID_{50}$ per 0.1 ml		5.75 log_{10}
$TCID_{50}$ per Carrier		6.05 log_{10}

Table 3: Test Results -- Hypochlorous Acid

		Test Results		
Contact time		1 minute	2 minutes	5 minutes
Dilution	10^{-1}	+ + 0 0	0 0 0 +	0 + 0 0
	10^{-2}	0 0 0 0	0 0 0 0	0 0 0 0
	10^{-3}	0 0 0 0	0 0 0 0	0 0 0 0
	10^{-4}	0 0 0 0	0 0 0 0	0 0 0 0
	10^{-5}	0 0 0 0	0 0 0 0	0 0 0 0
	10^{-6}	0 0 0 0	0 0 0 0	0 0 0 0
$TCID_{50}$ per 0.1 ml		1.00 log_{10}	0.75 log_{10}	0.75 log_{10}
$TCID_{50}$ per Carrier		1.30 log_{10}	1.05 log_{10}	1.05 log_{10}
Log_{10} Reduction		4.75 log_{10}	5.00 log_{10}	5.00 log_{10}
Percent Reduction		99.998%	99.999%	99.999%

Key: + = Virus recovered; 0 = Virus not recovered and/or no cytotoxicity observed; T = Cytotoxicity observed; N/A = not applicable

RESULTS (cont.)

Table 4: Test Substance Cytotoxicity Control & Neutralization Control Results

		Cytotoxicity Control	Neutralization Controls	
		Hypochlorous Acid	Hypochlorous Acid	Control Substance
Dilution	10^{-1}	0 0 0 0	+ + + +	+ + + +
	10^{-2}	0 0 0 0	+ + + +	+ + + +
	10^{-3}	0 0 0 0	+ + + +	+ + + +
TCD_{50} per 0.1 ml		≤ 0.50 $\log_{10}$	N/A	N/A
$TCID_{50}$ per 0.1 ml		N/A	≤ 0.50 $\log_{10}$	≤ 0.50 $\log_{10}$

The test substance and control substance demonstrated comparable levels of infective units recovered in the Neutralization Control.

Table 5: Testing Conditions

Parameter	Duration	Temperature (°C)	Relative Humidity (%)
		Start / End	Start / End
Carrier Inoculation	N/A	22.2	27.6
Carrier Drying	14 minutes	20.0 / 20.0	30 / 30
Recovery Control	5 minutes	22.2 / 22.2	26.5 / 26.3
Treatment: Hypochlorous Acid	1 minute	22.1 / 22.1	26.9 / 26.9
	2 minutes	22.2 / 22.2	26.8 / 26.7
	5 minutes	22.3 / 22.2	26.4 / 26.5
Incubation of Assay Plates	~7 days	36.0 / 36.0	6.0[1] / 6.1[1]

Key: + = Virus recovered; O = Virus not recovered and/or no cytotoxicity observed; T = Cytotoxicity observed; N/A = not applicable, , 1 = Incubation reported as % CO_2

STUDY CONCLUSION

The purpose of the study was to determine the virucidal efficacy of Hypochlorous Acid against Canine parvovirus, Cornell strain, ATCC VR-2017 with no additional organic soil supplementation, at contact times of 1 minute, 2 minutes and 5 minutes and an exposure temperature of room temperature and then neutralized.

The Plate Recovery Control demonstrated a viral titer of 5.75 $\log_{10}$ $TCID_{50}$ per 0.1 ml and 6.05 $\log_{10}$ $TCID_{50}$ per carrier.

Taking the cytotoxicity and neutralization control results into consideration, the evaluated test substance, Hypochlorous Acid demonstrated the following:

- A 4.75 $\log_{10}$ reduction (99.998%) in viral titer as compared to the titer of the corresponding plate recovery control at 1 minute.
- A 5.00 $\log_{10}$ reduction (99.999%) in viral titer as compared to the titer of the corresponding plate recovery control at 2 minutes.
- A 5.00 $\log_{10}$ reduction (99.999%) in viral titer as compared to the titer of the corresponding plate recovery control at 5 minutes.

No test substance cytotoxic effects to the host monolayer were observed at ≤ 0.50 $\log_{10}$ TCD_{50} per 0.1 ml for Hypochlorous Acid.

The Test Substance Neutralization Control demonstrated that Hypochlorous Acid was neutralized at ≤ 0.50 $\log_{10}$ $TCID_{50}$ per 0.1 ml.

The test substances(s) will be disposed of 30 days after the completion of this study, unless otherwise requested by the Study Sponsor.

The results of this study apply to the tested substances(s) only. Extrapolation of findings to related materials is the responsibility of the Sponsor.