TEST REPORT

KR-2006-014- ECW01-C

Virus Disinfectant Test

Summary of the Experiment

○ **Test:** Virucidal Activity Test

○ **Test No:**

○ **Product Name** VSP-A62_salt, VSP-A62_HOCl activator /Derivative model: ITASH iClean IC-S

○ **Client**

Affiliation :

Address :

○ **Institute**

Affiliation : (ISO13485:2016)

Address :

Written : / Ph.D. **Sign**

Approved : /Ph.D. Director

Sign **date**

* This test report is a result limited to the sample and sample name provided by the client, and does not guarantee the quality on the overall product.
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July 02, 2020

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1. Summary

This test was conducted to measure the efficacy of the virus-killing of the disinfectant presented by LTD. The SARS-CoV-2 (Severe acute respiratory syndrome-related coronavirus) virus was used as a test virus, and the sample (liquid) was mixed with the virus culture solution and contacted for a period of time, and then the test was conducted by confirming the activity of the virus. The activity of the virus was confirmed by infecting the host cell with the virus and then measuring by a 50% tissue culture infectious dose assay ($TCID_{50}$). As a result of treating the , LTD. disinfectant (VSP-A62_salt), (VSP-A62_HOCl activator) for 30 seconds, they showed that 99.98% and 99.99% of COVID-19 virus reduction (Virucidal) effect, respectively.

2. Outline of the test

2.1 Test schedule

Test start date: June 13, 2020

Test end date: June 26, 2020

2.2 Scope of test

This test method aims at demonstrating the virucidal action of a test substance containing a virus existing in a floating (liquid) state. With reference to the Ministry of Environment and the Ministry of Food and Drug Safety, the sterilizing and disinfectant test for the COVID-19 virus was conducted following the test guide for virus external antiseptics modified by .

3. Materials and Equipment

3.1 Test materials

The sample was provided by the client ., LTD. disinfectant (VSP-A62_salt), (VSP-A62_HOCl activator)

Fig1. CO., LTD. Disinfectant (VSP-A62_salt), (VSP-A62_HOCl activator)

3.2 Culture media and reagents

(1) Dulbecco's Modified Eagle Medium (DMEM), Hyclone, US

(2) Dulbecco's Phosphate buffered saline (PBS), Invitrogen, US

(3) Fetal bovine serum (FBS), Gibco, US

(4) Trypsin-EDTA (0.25% Trypsin), Gibco, US

(5) Penicillin-Streptomycin, Gibco, US

(6) Ethyl Alcohol (EtOH), Deoksan Pharmaceutical, South Korea

(7) Hydrochloric Acid (HCl), Daejung, South Korea

(8) Formaldehyde (HCHO), Deoksan Pharmaceutical, South Korea

(9) Crystal Violet, JUNSEI, Japan

3.3 Equipment and facility

(1) Biological safety cabinet (sterile worktable), Thermo scientific, US

(2) The optical microscope, OPTINITY, China

(3) Centrifuge (LABOGENE1248), Zyrozen, South Korea

(4) Refrigerator (4°C), Samsung Electronics, South Korea

(5) Freezer (-20°C), Samsung Electronics, South Korea

(6) cryogenic freezer (-80°C), Thermo scientific, US

(7) Constant temperature carbon dioxide gas incubator (37°C) BB15,

Thermo Scientific, US

(8) Vortex mixer KMC-1300V, Vision Science, South Korea

(9) Dry oven HM-28, Hanil Science, South Korea

(10) LN2 Tank (Locator JR Plus), Thermo scientific, US

(11) Water bath, Korea Science, South Korea

(12) Multi-well plate reader, Epoch, US

(13) PE6000, Mettler Instrument, US

(14) BSL-3 (No. KCDC-09-3-01)

4. Methods

4.1 Host cell line and culture

The cell line Vero-E6 is isolated from renal epithelial cells extracted from African green

monkeys. Since SARS-CoV-2 can be cultured and causes virus-infected cell lesions (Cytopathic effect), Vero-E6 is used as a host cell in this test for measuring the viral titer.

4.2 Virus

COVID-19 (SARS-CoV-2)

- The Corona Virus COVID-19 (SARS-CoV-2) first emerged in Wuhan, China in December 2019, and currently, on May 21, 2020, there are over 4.8 million people infected worldwide. Besides, over 310,000 people died from COVID-19, and it is still spreading seriously in the US and South America, etc.

- COVID-19 is included in the beta-corona classification to have positive single-strand RNA as the genome, and it is a spherical form of the virus with envelope.

- On March 11, 2020, the WHO declared a pandemic on this virus, and there is no medicine or vaccine in the present. The resistance to the disinfectant is in mid-grade, but the spreading power is very high to have a serious impact globally.

Severe acute respiratory syndrome-related coronavirus (SARS-CoV-2)

- Classification: Coronaviridae family, Betacoronavirus
- Virus genome: ss-RNA
- envelope: Yes
- Resistance: middle
- Titer: 1.43 x 10^6 $TCID_{50}$/mL

4.3 Virucidal test by disinfectant

4.3.1 Cell cytotoxicity

After dissolving by Chapter 9 or ISO 10993-12 Sample preparation and reference

materials of ``Common Standards for Biological Safety of Medical Devices-24-(

')", the eluate in the colloidal state is not filtered. It is tested according to ISO 10993-5 or Chapter 2 of the "Common Criteria for Biological Safety of Medical Devices (. Notice 2006-32)". Cytotoxicity is usually evaluated according to the test method by direct contact method.

It was carried out according to the cytotoxicity test guideline (document number , Crystal violet method) of this test institution.

① The cell is cultured in the 96 well plates, and the sample is performed with 10 times serial dilution to be added to the cell culture medium. Here, one column is left for use as the negative control group.

② The cell is cultured in the cell incubator during the designated time (About 3 days) according to the test plan.

③ Optical microscope is used for a visual check on the cell state, and 8 channel multi pipette is used to insert 50 μl of crystal violet solution into all wells.

④ The plate is left at room temperature for 30 minutes to wash the plate cleanly in flowing water.

⑤ The plate is dried appropriately to measure the absorbance with the ELISA reader at 575nm wavelength.

4.3.2 Virucidal Test

This test was conducted based on ASTM E1052-11, the virus killing test in the sample stock solution. Also, to neutralize the cytotoxicity of the disinfectant itself, the neutralizing agent (10% FBS) suggested in the "Sterilization Disinfectant Efficacy Test Method Data Collection (NIER-GP2018-170)" was used.

① One day before the test, prepare Vero-E6 cells in a 96 well plate.

② Sample stock solution and SARS-CoV-2 virus were mixed and reacted at room temperature for 30 seconds, 1 minute, and 5 minutes.

③ Neutralizing agent (10% FBS) was added and allowed to stand at room temperature for 10~20 minutes, and then diluted 10 times. To reduce cytotoxicity, a gel filtration method (E-1482) is proposed, but it was omitted in consideration of the virus risk, and the cytotoxicity was evaluated based on the step dilution without cytotoxicity. For example, in 10^{-1}, cytotoxicity was observed, but in 10^{-2}, it did not show cytotoxicity, and when the virus was killed and all cells were healthy, it was evaluated that the virus was killed in 10^{0},10^{-1}.

④ Each diluent was infected with Vero-E6 cells, and cultured at 5% CO_2 at 37°C. At this time, normal physiological saline was used as a control.

⑤ After 3 days of culture, the cytopathic effect (CPE) was observed under a microscope.

⑥ Crystal violet staining reagent was treated with cells and stained at room temperature for 30 minutes.

⑦ The titer of the virus was calculated by counting the number of stained wells.

4.4 Data reading and calculation

4.4.1 Cytotoxicity Test

The results of the cytotoxicity test are judged to have no cytotoxicity when the absorbance (value) is 50% or more based on the absorbance (100%) of the cell negative control.

4.4.2 Virucidal Test by disinfectant

To evaluate the virus killing efficacy, each diluent was inoculated into a host cell, and virus titers of the control group and the test group were measured after 3 days.

The number of wells stained with Crystal violet dyeing reagent was counted to

calculate the titer by Reed & Muench method. Virus titers were calculated according to 4.4.3 and reduction rates were determined according to 4.4.4.

4.4.3 Calculate viral titer

The virus titers can be confirmed by observing the morphological changes (CPE) of cultured cells caused by virus growth for a while. The virus infectious value is obtained by inoculating, cultivating, and observing the cultured cells seeded in a plurality of incubators by preparing a 10^n dilution series of the virus solution. After the CPE observation for a certain period (four days post-infection), the virus infection value ($TCID_{50}$) is calculated according to ICH Q5A (R1), which is indicated by taking the commercial log value.

The number of wells determined to be positive is cumulatively calculated from the high diluent side to obtain the cumulative positive rate (%) of each diluent.

$TCID_{50}$: $N=10^{[(A-50)/(A-B)]-(a)}$

How to calculate viral titer

1) Calculate the cumulative for the number of well which had decided to be positive from high diluted solution and obtain the cumulated positivity rate (%) of each diluted solution.
2) Obtain 50% of cumulative positivity rate and cumulative positivity rate of high diluted solution is called as A; cumulative positivity rate of low diluted solution is called as B, and the natural logarithm value of the diluted solution with A obtained is called as a.
3) Obtain the viral titer according to the following formula.

However, if overall well became negative even for the diluted solution having the lowest magnification, assume that overall well become positive in the diluted solution that is one step lower than that diluted solution and then calculate; add a sign of inequality to obtained

value and then write down. And make the valid number to have 2 digits by rounding the 3rd number of calculated value for the valid digit number of viral titer.

4.4.4 How to calculate the viral reduction factor (Ri)

Regarding the combustion process, the viral reduction factor (Ri) can be calculated with a natural logarithm for the ratio of viral titer in the test solution, whether the sample underwent a combustion process or not for test solution. However, in case of a reduction of viral titer in the test solution is less than 10^1 ($\log_{10}$= natural logarithm value 1), it is not determined as the reduction of viral titer and not used for calculation of viral clearance factor.

How to calculate the viral reduction factor (Ri)

- Viral titer appeared in the experimental group before the combustion: 10^A
  The total amount of test solution before the combustion: V^A
  - → The viral titer of test solution before the combustion $V^A \times 10^A = N_A$
- Viral titer appeared in the experimental group after the combustion: 10^B
  The total amount of test solution after the combustion: V^B
  - → The viral titer of test solution after the combustion $V^B \times 10^B = N_B$

Viral titer (Ri) of test solution is

$$10^{Ri} = V^A \times 10^A / V^B \times 10^B = N_A / B_A$$

$$Ri = \log_{10}(N_A / B_A) = \log_{10} N_A - \log_{10} N_B$$

5. Results

5.1 Cytotoxicity by disinfectant

As a result of treating the sample stock solution into Vero-E6 cells, the sample of the

stock solution showed cytotoxicity. As a result of confirming the cytotoxicity by diluting the sample 10 times, no toxicity was observed in Vero-E6 cells.

Table 1. Cytotoxicity test results

Cell	The highest dilution factor that does not show host cell toxicity
Vero-E6	10^{-1}

5.2 Disinfectant test

The initial virus titer of SARS-CoV-2 for the disinfectant test is 6.16 $\log_{10}$ $TCID_{50}$/ml.PBS was used as a control to evaluate the efficacy of the disinfectant in this test. After mixing the virus and the control (PBS) and neutralizing it after 30 seconds, the virus titer was calculated through cell infection, and the titer of the control group was 6.50 $\log_{10}TCID_{50}$/ml. After mixing the requested disinfectant (VSP-A62_salt), and (VSP-A62_HOCl activator) and the virus and neutralizing it after 30 seconds, the virus titers were calculated and the titer of the test groups were (VSP-A62_salt), 2.75 $\log_{10}TCID_{50}$/ml and (VSP-A62_HOCl activator) 2.50 $\log_{10}$ $TCID_{50}$/ml, respectively. Therefore, the reduction rate of SARS-CoV-2 by disinfectant (VSP-A62_salt) and (VSP-A62_HOCl activator) and were confirmed to be 3.75 and 4.00 after 30 seconds, respectively. As a result of processing SARS-CoV-2 in the sample disinfectant solution for 30 seconds, the virus killing efficacy of (VSP-A62_salt)99.98% and (VSP-A62_HOCl activator)99.99% were confirmed.

Table 2. Virus titer calculation

(unit: $log_{10}TCID_{50}/ml$)

Disinfectant	Treatment	Virus titer	Control (PBS)	Test
(VSP-A62_salt)	30 sec	6.16	6.50	2.75
	1 min	6.16	6.50	2.75
	5 min	6.16	6.50	2.75
(VSP-A62_HOCl activator)	30 sec	6.16	6.50	2.50
	1 min	6.16	6.50	2.50
	5 min	6.16	6.50	2.50

Table 3. Virus reduction rate

Disinfectant	Treatment	Log reduction (LR)
(VSP-A62_salt)	30 sec	3.50
	1 min	3.75
	5 min	4.00
(VSP-A62_HOCl activator)	30 sec	4.00
	1 min	4.00
	5 min	4.00

$LR = L_U - L_T$

L_U : Virus titer of the control (untreated)

L_T : Virus titer of the test (treated)

Table 4. Disinfectant test results

Disinfectant	Virus	Treatment	Virus reduction (log)	Virus reduction (%)
(VSP-A62_salt)	SARS-CoV-2	30 sec	≥3.50	99.97%
		1 min	≥3.75	99.98%

		5 min	≥4.00	99.99%
(VSP-A62_HOCl activator)	SARS-CoV-2	30 sec	≥4.00	99.99%
		1 min	≥4.00	99.99%
		5 min	≥4.00	99.99%

* Interpretation of results

Log reduction	Percent (%) reduction
≥1	≥90 %
≥2	≥99 %
≥3	≥99.9 %
≥4	≥99.99 %
≥5	≥99.999 %

6. Conclusion

The SARS-CoV-2 (Severe acute respiratory syndrome-related coronavirus) virus reduction effect (virucidal) for . . LTD. Disinfectants (VSP-A62_salt) and (VSP-A62_HOCl activator) under guideline test conditions were 99.98% and 99.99% after 30 seconds of sample treatment, respectively.

7. References

(1) ASTM E1052-11, Standard Test Method to Assess the Activity of Microbicides against Viruses in Suspension

(2) Schmidt, N. J. et. Al., Diagnostic Procedures for Viral, Rickettsial and Chlamydial infection, 7th edition, Am. Pub. Hlth. Assoc., Washington, DC, 1995.

(3) BS EN 14476:2013 A1:2015, Chemical disinfectants and antiseptics – Quantitative suspension test for the evaluation of virucidal activity in the medical area

(4) Test method for the evaluation of virucidal efficacy of three common liquid surface disinfectants on a simulation environmental surface. Appl Microbiol, 28(1974), pp.748-752

(5) In vitro evaluation of antiviral and virucidal activity of a high molecular weight hyaluronic acid. Virology Journal 8, Article number:141(2011)

(6) Virucidal and Neutralizing Activity Tests for Antiviral Substances and Antibodies 10.21769/BioProtoc.2855 Vol 8, Iss 10, May 20, 2018

(7) Guidelines for disinfectants for external use (non-pharmaceutical products) Effectiveness Evaluation Act 2014.8. Food and Drug Safety Evaluation Institute

(8) Sterilization. Disinfectant Efficacy Test Method Data Collection 2018. 12. National Institute of Environmental Science

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