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Prepared for:
PH+ Cleanee Inc.

Test Report

Influenza A Virus Inactivation Analysis

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1. Aim of the test

To investigate the antiviral efficacy of alkaline electrolytic water using *influenza A virus* (H1N1).

2. Client

Company: PH+ Cleanee Inc.

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3. Testing organization

Virology Division, Department of Virology,

Kitasato Research Center for Environmental Science

Address: 1-15-1 Kitasato, Minami, Sagamihara, Kanagawa, 252-0329, Japan

4. Test sample and condition

Test sample: Alkaline electrolytic water "JJJee"

Measured value; 12.7 (measured by glass electrode pH meter, HORIBA D-52)

Exposure time: 15 seconds at room temperature.

5. Test virus

Influenza A virus (A/PR/8/34, H1N1, ATCC VR-1469)

6. Preparation of the test virus

Influenza A virus was inoculated into the allantoic cavity of embryonated chicken eggs. These eggs were incubated at 37 ℃. After 2 days, the virus multiplying in the allantoic fluid was harvested and purified by the sucrose density gradient centrifugation method.

7. Methods

1) Test procedure

To examine virucidal effect of "JJJee", 100 μL of virus suspension was added into 900 μL of the sample, and this mixture was kept at room temperature for 15 seconds. After exposure to the sample, the mixture was diluted with 100-fold in phosphate buffered saline (PBS) to avoid the virucidal activity of the sample. Then, 100 μL of the diluted mixture was immediately serially diluted with 900 μL of PBS (1:10) to measure for virus titer. Additionally, control reaction was carried out by using PBS instead of sample.

2) Measurement of infectivity titer

Viral infectivity titers were determined by plaque assay. MDCK monolayer cell cultures were inoculated with 0.1 mL of the viral suspension which was 10-fold serially diluted with PBS. The

cultures were incubated for 1 hour at 37 ℃ in the humidified atmosphere with 5% CO_2 to allow the virus to be adsorbed. After 1-hour incubation, 1.5 mL of the medium (Minimum Essential Medium containing 100 μg/mL DEAE-dextran, 0.42% bovine serum albumin, 2 μg/mL acetylated trypsin and 1% agarose) was added to the each well. After 2-day incubation at 37 ℃ in the humidified atmosphere with 5% CO_2, the cells were fixed with 2 mL of 4% formaldehyde for 1 hour at room temperature. After the formaldehyde solution and the medium layer were removed, the attached cells were stained with crystal violet solution. Once the cells were washed with tap water and air dried, the number of plaques was counted and the virus infectivity titers (PFU/test piece) were calculated based on the amount of the virus recovery suspension (10 mL). These PFU values were [$\log_{10}$] transformed to display Log Reduction Values (LRV). Then the LRVs were transformed to display reduction ratio (%).

8. Test results

The viral inactivation efficacy of "JJJee" supplied by PH+ Cleanee Inc, is summarized in Table 1 Figure 1.

As shown in the Table 1, when the *influenza A virus* was exposed to PBS (control) for 15 seconds at room temperature, virus infectivity titer (8.5×10^6 PFU/mL) was scarcely changed to initial viral infectivity. On the other hand, when the virus was exposed to "JJJee" for 15 seconds at room temperature, initial virus infectivity titer was decreased to 1.7×10^2 PFU/mL (LRV; 4.6 and reduction ratio; > 99.99 %).

9. Comments

In the present investigation, the antiviral efficacy against *influenza A virus* by "JJJee" was evaluated. The U.S. EPA (Environmental Protection Agency) recommends 4-$\log_{10}$ reduction of viral infectivity titer or at least 3-$\log_{10}$ reduction when cytotoxicity is present against the cells used for virus infection.

In this test, it appears to be effective against *influenza A virus* which indicates more than 4.0 $\log_{10}$ reduction of virus infectivity titer within 15 seconds.

References:

1) Antimicrobials Division U.S. EPA, Confirmatory Virucidal Effectiveness Test, Using Feline Calicivirus As Surrogate for Norovirus

Table 1. Virucidal efficacy of "JJJee" against *influenza A virus* (H1N1).

Test sample	Exposure time		LRV [a)]	Reduction ratio [b)]
	0 (initial)	15seconds		
JJJee		1.7×10^2	4.6	99.997
PBS (control)	8.5×10^6	8.0×10^6	0.0	

Test virus: *Influenza A virus* (H1N1, A/PR/8/34, ATCC VR-1469)

Units: PFU/mL

Detection limit: 1.0×10^1 PFU/mL

a) Difference in infectivity titer between initial and after exposure: Log_{10} (Initial viral infectivity titer / infectivity titer after exposure)

b) Calculation formula: $(1 - 1/10^{LRV}) \times 100$ (%)

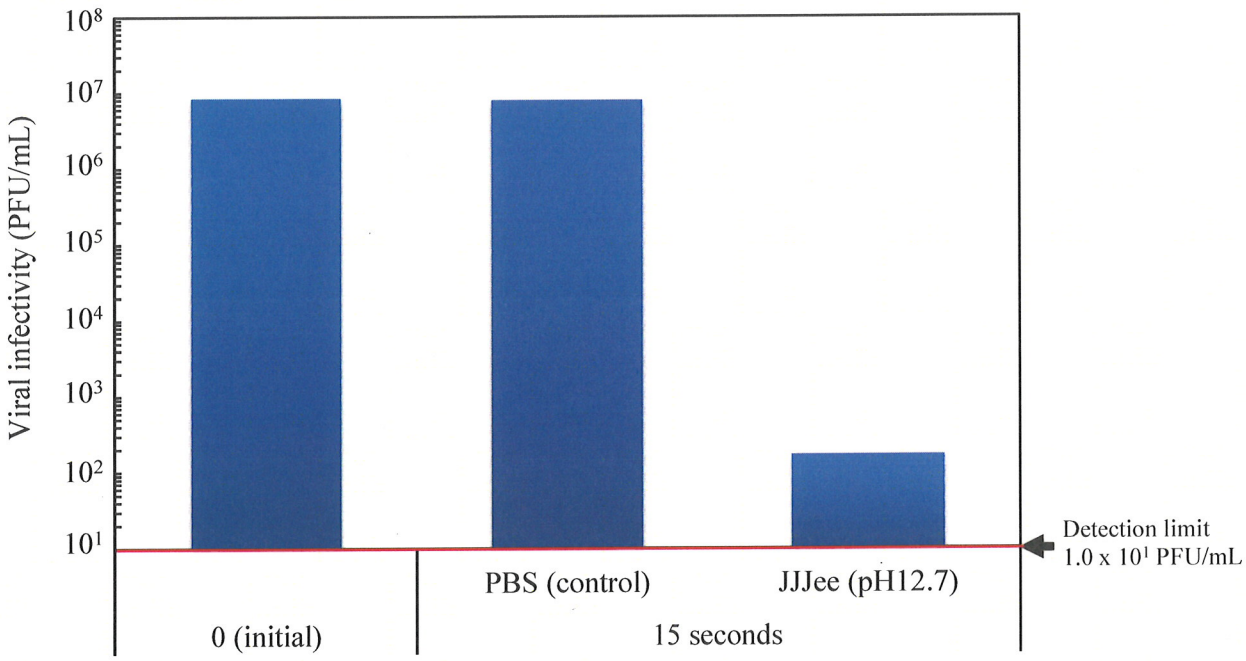

Figure 1. Virucidal efficacy of "JJJee" against *influenza A virus* (H1N1)