Department of Molecular Medicine

UNIVERSITY OF PADOVA

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VIRUCIDAL EFFECTIVENESS REPORT

Quantitative suspension test for the evaluation of the virucidal activity against the *SARS-CoV-2* virus

PRODUCT:

SANITY SYSTEM device
for environment sanitization (air and surfaces) with Ozone technology

CUSTOMER

Sanity System Italia Srl. Address: Via delle Industrie, 13/C- 35010 Limena (PD)
VAT and tax code 04954700284

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[HANDWRITTEN SIGNATURES AND STAMP OF THE UNIVERSITY OF PADOVA]

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1. PURPOSE

The following report has the purpose of defining in a clear and detailed way the methods of execution and the results of the study carried out to assess the virucidal activity on surfaces of equipment using ozone.

2. TERMS AND DEFINITIONS

Virucidal or antiviral activity: the ability of a product to reduce the number of infecting viral particles through experimental procedures including precise and defined test conditions.

Plaque Forming Units (PFUs): number of infecting viral particles per ml.

ID_{50}: dose infecting 50% of the viral suspension or the dilution of the viral suspension which induces 50% of the viral cytopathic effect (CPE) in cell cultures.

Viral cytopathic effect (CPE): morphological alteration of cells and/or their destruction due to the multiplication of the virus.

Inactivation of viruses: reduction of the infectivity of a virus against the product under investigation.

3. INTRODUCTION

The test method used to verify the viral inhibition activity (virucidal activity) against the SARS-CoV-2 virus of the "Sanity System" device (test product) was performed at the Department of Molecular Medicine (University of Padua). All the tests were carried out in the Biosafety Level 3 Laboratory (BSL3).

The virucidal activity was tested using the SARS-CoV-2 strain. All the tests were carried out in the Biosafety Level 3 Laboratory (BSL3).

4. SAMPLE CHARACTERISATION

Product: "Sanity System" device

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Product description: Sanity System, mod. SANYMED is a professional sanitization device used to eliminate bacteria, moulds, viruses and, in general, the microbial load, in addition to pollutants and odours.

Storage conditions: room temperature

Equipment instructions: see annex

5. TEST CONDITIONS

Test temperature: Performed at +20 °C ± 1 °C.

Contact time: according to the manufacturer's indications

Analysis period: Test start date: 16-08-2020 -:- Test end date: 30-09-2020

6. MATERIALS AND REAGENTS

Test microorganisms:

SARS-CoV2

Cell line:

VERO E 6 (ATCC CCL-81)*

*ATCC (American Type Culture Collections)

Viral stock suspension

Each viral suspension was prepared and amplified on a large scale in monolayer cell cultures. After infection and multiplication of the virus, cell debris was removed by double centrifugation at low speed (2,500 rpm for 10 minutes). The supernatant containing the virus was removed to determine the viral titre. It was divided into portions with a known titre (2ml of volume) inside Eppendorf tubes and stored at -80 °C in the freezer.

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Cell cultures

VERO E6 cells, cells of epithelial origin taken from monkey kidney (continuous line).

Carriers

35mm diameter AISI 316 stainless steel discs were used, previously sterilized in an autoclave.

CULTURE MEDIA AND REAGENTS

Reagents must be pure for analysis and/or suitable for microbiological applications.

Cell culture medium

Each cell line is kept in a thermostat at 37 °C with 5% (v/v) of CO_2 in DMEM (Dulbecco's Modified Eagle Medium) medium supplemented with 10% (v/v) of foetal bovine serum (FBS) and 1% (w/v) of penicillin-streptomycin (pen-strep).

Phosphate Buffered Saline (PBS)

Solution containing: 8g of NaCl, 0.2g of KCl, 2.89g of Na_2HPO_4 12 H_2O, 0.20g of KH_2PO_4 in 1,000ml of distilled H_2O.

7. EQUIPMENT

- Inverted microscope for the observation of cell cultures
- Stopwatch
- Vortex mixer
- Centrifuge
- CO_2 incubator (5% v/v) able to maintain the temperature at 37 °C ± 1 °C.
- Vertical laminar flow hood "BioHazard", class II
- Freezers

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8. PRELIMINARY TESTS

PREPARATION OF THE VIRAL SUSPENSION - VIRAL TITRE

0.2ml of viral suspension (stock solution) + 1.8ml of serum-free DMEM were mixed, and serial dilutions from 10^{-2} to 10^{-9} (1:10 dilutions) were prepared.

250µl of each dilution was transferred to 24-well plates containing the cell monolayer at confluence (> 90%) after aspiration of the culture medium. Each dilution of the viral suspension was plated in sextuple. 12 wells were left uninoculated, (cell line control). After 1 hour of incubation at 37 °C (viral adsorption time), the inoculum was removed, a wash with PBS was performed and 500µl of DMEM supplemented with 2% (v/v) of FBS and 0.75 % (v/v) of carboxymethylcellulose was added.

Thermostat incubation conditions

Infections were incubated with 5% (v/v) of CO_2 at 37 °C ± 1 °C and observed under inverted microscope to detect the lysis plaque formation caused by the cytopathic effect (CPE) of the viral suspension. After fixation with formaldehyde and staining with a crystal violet solution, the plaques in the wells with the calculated dilution were counted under an inverted microscope.

The CPE (quantitative test) results of each dilution are expressed with a percentage of positive results ranging from 100% to 0%, and recorded as "0" for no CPE and from "1" (25% CPE) to "4" (100% CPE) depending on the degree of cell damage.

Viral titre was calculated using the Spaerman-Karber method (ID_{50} evaluation).

9. INACTIVATION CHECK - TEST WITH FORMALDEHYDE

In order to check the validity of the system, 2ml of viral suspension was mixed with 8ml of PBS and 10ml of formaldehyde solution at

1.4% (w/v). Immediately after a contact of the duration of 30 minutes and 60 minutes, 0.2ml of this solution was mixed with 1.8ml of DMEM + 2% of FBS on ice.

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Serial dilutions from 10^{-2} to 10^{-6} (1:10 dilutions) were performed with PBS + 2% FBS. For each dilution, 250µl was distributed in 6 wells of the 24-well microplate and placed in the incubator at 37 °C for 1 hour. After 1 hour of incubation at 37 °C (viral adsorption time), the inoculum was removed, a wash with PBS was performed and 500µl of DMEM supplemented with 2% (v/v) of FBS and 0.75% (v/v) of carboxymethylcellulose was added.

The cell culture was placed in an incubator at 5% (v/v) of CO_2 at 37 °C ± 1 °C and observed under an inverted microscope for the detection of the cytopathic effect (CPE) of the viral suspension. After fixation and staining with a crystal violet/methanol solution, the plaques in the wells with the calculated dilution were counted. The CPE (quantitative test) results of each dilution are expressed with a percentage of positive results ranging from 100% to 0%, and recorded as "0" for no CPE and from "1" (25% CPE) to "4" (100% CPE) depending on the degree of cell damage.

Viral titre was calculated using the Spaerman-Karber method (ID_{50} evaluation).

Cytotoxicity of the formaldehyde test solution

1ml of formaldehyde at 1.4% (w/v) has been added to 1ml of PBS. From this dilution, serial dilutions from 10^{-2} to 10^{-4} (1:10 dilutions) were prepared by taking 0.2ml of the obtained mixture + 1.8ml of serum-free DMEM.

0.1ml of each dilution was plated in sextuple in confluence monolayer cell cultures (> 90%). The mixture was not added to 6 wells (cell line control). After 1 hour at 37 °C ± 1 °C, 100µl of DMEM + 10% of FBS was added, and the cell culture was placed in an incubator at 37 °C ± 1 °C with 5% of CO_2 and then constantly observed under the inverted microscope for the next 9 days for the detection of the cytopathic effect (CPE), caused by the cytotoxic action of the formaldehyde solution.

10. VIRUCIDAL ACTIVITY TEST OF THE DEVICES

PREPARATION OF THE VIRAL SUSPENSION

For the preparation of the test suspension, see paragraph 8 - PRELIMINARY TESTS.

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EXPOSURE STAGE

Carriers preparation

Before being used, the carriers were sterilised in an autoclave at 121 °C for 5 minutes. The carriers were placed inside empty Petri dishes. In both control and ozone-exposed carriers, 50µl of viral suspension was placed, subsequently well distributed through the use of a loop. The suspension was left to dry under a laminar flow hood.

Control carriers

The control carriers were kept in the laboratory with the lid of the Petri dish closed.

Ozone-exposed carriers

The carriers to be exposed to ozone were placed in the supplied case, with the lid open, in the opposite position to the location of the test device, exposing them to the action of the Sanity System equipment.

RECOVERY STAGE

Carriers recovery

All carriers (exposed and control) were eluted with 1ml of culture medium. Then, the carriers were subject to scraping for 1 minute. Serial dilutions from 10^{-2} to 10^{-9} (1:10 dilution) were prepared.

Plating and incubation

250µl of each dilution was transferred to 24-well plates containing the cell monolayer at confluence (> 90%) after aspiration of the culture medium. Each dilution of the viral suspension was plated in sextuple. 12 wells were left uninoculated, (cell line control). After 1 hour of incubation at 37 °C (viral adsorption time), the inoculum was removed, a wash with PBS

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was performed and 500µl of DMEM supplemented with 2% (v/v) of FBS and 0.75 % (v/v) of carboxymethylcellulose was added.

11. RESULTS EXPRESSION CALCULATION

Infectivity titre determination (ID_{50}).

The infecting activity was determined with the Spaerman - Karber method, which uses the following formula to calculate the ID_{50} value:

$$-\log_{10} ID_{50} = -(x_0) - \{[R/100] - 0.5\} \times \log_{10} \text{dilution factor}$$

Where:

x_0 = $\log_{10}$ of the lowest dilution with 100% of positive reaction

(CPE) R = total (%) of positive cultures

The virucidal activity test is valid when the following condition occurs in the preliminary tests: the viral test suspension must have a viral concentration able to determine the reduction of at least 4lg of the initial viral titre: **$ID_{50} = 10^7$/ml.**

12. RESULTS AND VIRUCIDAL ACTIVITY

Tests have been performed using the P1 and P2 programmes. The results obtained are contained in Table 1.

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Programme	*Test*	*Initial viral titre**	*Viral titre after treatment*	*%Reduction*
P1	1	*6.5*	*0.5*	*92.3*
	2	*6.6*	*0.4*	*93.9*
P2	1	*7.0*	*0.02*	*99.7*
	2	*7.0*	*0.01*	*99.9*

Table 1 - Effects of the treatment with Sanity System. The reduction values of the viral load are expressed both in logarithmic units and in percentage terms; P1, programme l, P2, programme 2.

Considering the results obtained, the tests were repeated in the same conditions using the P2 Programme only, whose results are described in Table 2.

Programme	*Test*	*Initial viral titre**	*Viral titre after treatment*	*%Reduction*
P2	3	*7.0*	*0.01*	*99.9*
	4	*7.0*	*0.02*	*99.7*
	5	*7.0*	*0.05*	*99.3*

Table 2 - Effects of the treatment with Sanity System. The reduction values of the viral load are expressed both in logarithmic units and in percentage terms.

*The data express the logarithmic value of the Plaque Forming Units (PFU/ml) on 1ml of test viral suspension

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15. CONCLUSION

The results obtained show that the Sanity System device (ozone-based technology) has an effective virucidal action against SARS-CoV-2, with a reduction in viral load of over 99% with the P2 programme.

16. REFERENCES

- EN 17272:2020 EUROPEAN STANDARD - Chemical disinfectants and antiseptics - Methods of airborne room disinfection by automated process
- EN 14476:2019 EUROPEAN STANDARD - Chemical disinfectants and antiseptics - Quantitative suspension test for the evaluation of virucidal activity in the medical area
- ISO/IEC 17025:2017 General requirements for the competence of testing and calibration laboratories
- ISO 15189:2012 Medical laboratories - Requirements for quality and competence