Final report

Project "Quantification of the in vitro antiviral activity of disinfectants on SARS-CoV-2"

Submission date: August 24, 2020

1. BACKGROUND

The company **99Technologies S.A.** from Switzerland requested the evaluation of the **99T DISINFECTION SYSTEM** which uses a 99MB micronebulizer machine with a 99S disinfectant solution (Hydrogen peroxide (H2O2): ~ 6.6%, 0.006% silver cations and coformulants. The technology uses a micronebulization system that generates microdroplets loaded with the disinfectant. The system was transferred to the BSL-3 laboratory of the Immunovirology Group of the **Universidad de Antioquia**, and it was used to determine the antiviral potential against the SARS-CoV-2 Virus, isolated at the University of Antioquia.

1. METHODOLOGY

Materials and reagents

The antiviral activity of the **99T DISINFECTION SYSTEM** (Photo 1) was analyzed; For this analysis, the SARS-CoV-2 virus isolated in the Immunovirology Group of the **Universidad de Antioquia** in the VERO E6 cell line was used. The VERO E6 cells were kept in DMEM culture medium, supplemented with 5% FBS (Fetal bovine serum), in a 5% CO2 atmosphere and at a temperature of 37ºC. The titer of the SARS-CoV-2 virus isolated in the laboratory was determined using the plaque assay and TCID50 (Tissue Culture Infectious Doses 50) in VERO E6 cells, following a protocol previously described in the literature (1). The titer obtained was $4.21x10^6$ PFU (plaque-forming units) / mL.

1. Fan H.H., et al., Repurposing of clinically approved drugs for treatment of coronavirus disease 2019 in a 2019 - novel coronavirus (2019-nCoV) related coronavirus model. Chin. Med. J. 2020;6. doi: 10. 1097/CM9. 0000000000000797.

Virucidal Activity Assay

On the day of the assay, 100uL of the SARS-CoV-2 virus with a viral titer of $4.21x10^6$ PFU / mL was added to a well of a 6-well plate. In parallel, the **99T DISINFECTION SYSTEM** was located on the floor of the BSL3 laboratory and by means of a laser, the outlet nozzle of the equipment containing the disinfectant was directed into the biosafety cabin where the virus was found (2 meters away) (Photo 2). Chemical indicators were placed inside the biosafety cabin, in order to control and confirm the entry of the micro drops to the interior of the cabin; these indicators change color on contact with the disinfectant components (Photo 3). The spray time of the equipment was 19 minutes and the post contact time was 35 minutes. In parallel, a virus control was included, which contained culture medium and virus, without exposure to the **99T DISINFECTION SYSTEM**. In addition, a cytotoxicity control was included, which consists of culture medium only, exposed to the **99T DISINFECTION SYSTEM**, without virus; this control allows to determine the cytotoxicity produced by the disinfectant on the cell culture. Subsequently, the virus exposed or not to the **99T DISINFECTION SYSTEM** was titrated, using the TCID50 technique and plaque assay (1).

The TCID50 was determined by a cell viability analysis using the MTT assay, which is based on the metabolic reduction of 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazole bromide performed by the succinate dehydrogenase mitochondrial enzyme in a blue colored compound (formazan), allowing to determine the mitochondrial functionality of treated cells. This method has been widely used to

measure survival, cell proliferation, and antiviral activity; thus, the amount of formazan is directly proportional to cell viability (2). Initially, cells were seeded at a density of $1x10^5$ cells / well in a 96-well plate in 200 µL of DMEM with 10% FBS and cultured for 24 hours at 37°C with 5% CO2, prior to the antiviral experiment. Then, cells were infected with base 10 dilutions of the virus obtained in the previous step, in triplicate and for 1 hour. At the end of this time, the remaining virus that failed to enter the cell was discarded and fresh DMEM culture medium with 5% FBS was added. Forty-eight hours after infection, the supernatant was removed and the MTT solution (0.5 mg / mL) was added. After 2 hours of incubation, 130 µL/well of DMSO were added. The plates were left in agitation for 15 minutes and were finally read on a spectrophotometer at 550nm. Each experimental condition was evaluated in triplicate in 2 independent experiments (n=6). The calculation of the virus titer is obtained by the Reed and Muench method (3).

2. Shen L., et al. High-throughput screening and identification of potent broad spectrum inhibitors of coronaviruses. J. Virol. 2019;93(12).

3. Reed, L.J.; Muench, H. A simple method of estimating fifty percent endpoints. Am. J. Hyg. 1938, 27, 493–497.

Additionally, the highest dilution in which a difference of more than 20% in cell viability was observed comparing the virus exposed to the disinfectant versus the control virus, and in which the cytotoxicity of the disinfectant was less than 20%, was used to determine the virus titer in a plaque assay.

The plaque assay is a technique considered as a gold standard to determine the viral titer; therefore, it is the technique of choice to efficiently check the reduction in the viral titer caused by the disinfectant in this type of experiments.

Finally, the statistical analyzes for all the tests are shown as the average with the respective standard deviation in each dilution. Parametric or non-parametric statistical tests were performed, as appropriate, to find differences between the conditions of each experiment. A value of $p < 0.05$ was considered statistically significant.

3. RESULTS

Figure 1 shows the percentage of live cells after being infected with SARS-CoV-2 exposed to the **99T DISINFECTION SYSTEM** or without exposure to the disinfectant (Virus Control). The use of the disinfectant succeeded in reducing the cytotoxicity of the virus, producing an increase in the percentage of cell viability from the 10^{-3} dilution, with an average of 58%; cell viability remained above 99.5% in subsequent dilutions (Figure 1). In contrast, in the control virus, the percentage of cell viability was 31%, 35%, 46% and 63%, in the dilutions 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}, respectively; with statistically significant differences between the virus control and Virus+Disinfectant, at the 10^{-4} dilution ($p<0.05$). Therefore, it is clear that cell viability measured by MTT technique, was superior when cells were infected with SARS-CoV-2 virus previously exposed to the disinfectant; suggesting a virucidal effect, which becomes evident with the decrease of the cytopathic effect of the virus.

Additionally, it can be observed that cell viability, at 10^{-4} and higher dilutions, is on average 91% in the cytotoxicity control. This suggests that the cytotoxicity of the disinfectant is not affecting the reading or interpretation of the results observed in the cells infected with the virus and exposed to the disinfectant.

Subsequently, from the data of the cell viability assay measured by MTT, it is possible to calculate the titer of the virus as TCID50. It was found that the control

virus had a viral titer of $10^{-3,956}$ (Figure 2); while, for the virus exposed to the **99T DISINFECTION SYSTEM**, the titer of the virus was $10^{-2,99}$ (Figure 2); suggesting a decrease in the titer of the virus exposed to the disinfectant. .

To confirm this reduction, the plaque assay was performed, starting from the supernatant of the 10^{-4} dilution of the previous test; dilution chosen according to the criteria mentioned in the methodology section of this report.

When the 10^{-4} dilution was used, it can be observed that in the control virus (cells infected with virus, none exposed to the disinfectant) the calculation of the viral titer by plaque forming units (PFU)/mL was in average $1.5x10^{8}$. In contrast, the titer of the virus in the condition of Virus+Disinfectant (cells infected with virus exposed to the disinfectant), was $3.75x10^{5}$, indicating a reduction of 99.75% in the viral titer (Figure 3); in other words, the **99T DISINFECTION SYSTEM** inactivated 99.75% of the SARS-CoV-2 infectious viral particles under the conditions described above.

4. CONCLUSION

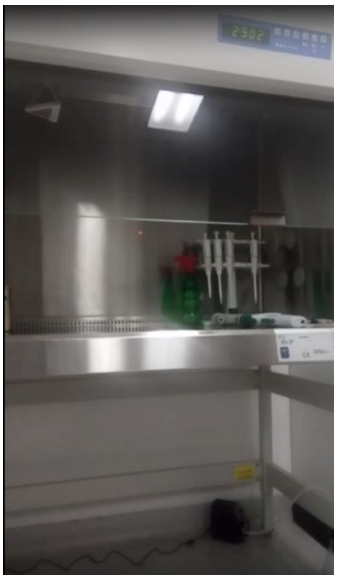
We can conclude that the 99T Disinfection System, during a contact time of 35 minutes with the virus in solution, inactivated 99.75% of the SARS-CoV-2 infectious virus.

Photograph 1.

99T DISINFECTION SYSTEM; which uses a 99MB micronebulizer machine with a 99S disinfectant solution (Hydrogen peroxide (H2O2): ~ 6.6%, 0.006% silver cations and coformulants.

A.

B.

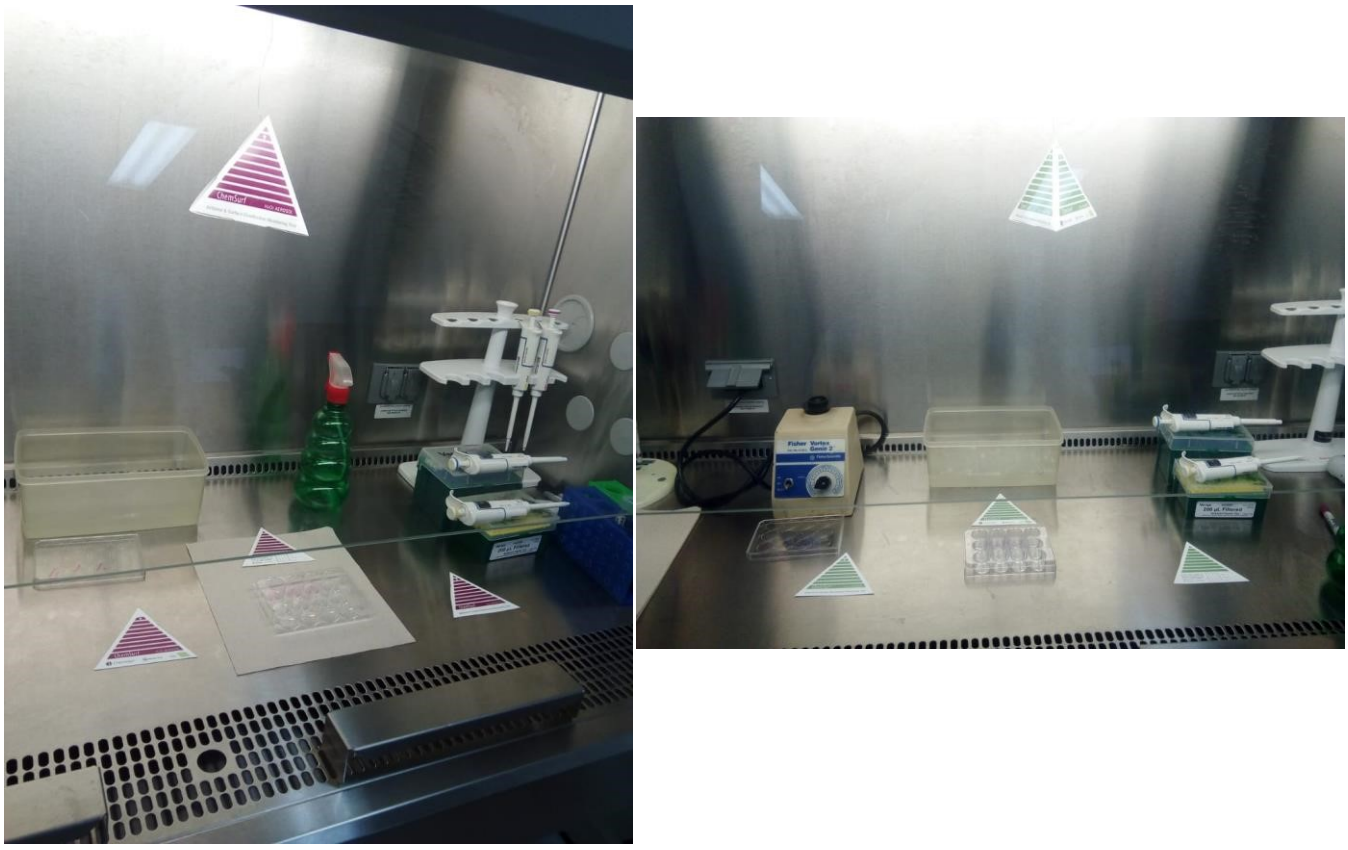


C.

Photograph 2.

Location of the 99T DISINFECTION SYSTEM.

The position of the micronebuilization equipment is observed at the BSL3 biosafety room (A); in addition, the red dot of the laser is observed inside the biosafety booth, indicating the direction in which the nozzle of the micronebuilization equipment was directed (B and C).



Photograph 3.

Chemical indicators located inside the biosafety cabinet; before (left) and after (right) the spraying with the 99T DISINFECTION SYSTEM. The color change is observed due to contact with the disinfectant 99S.

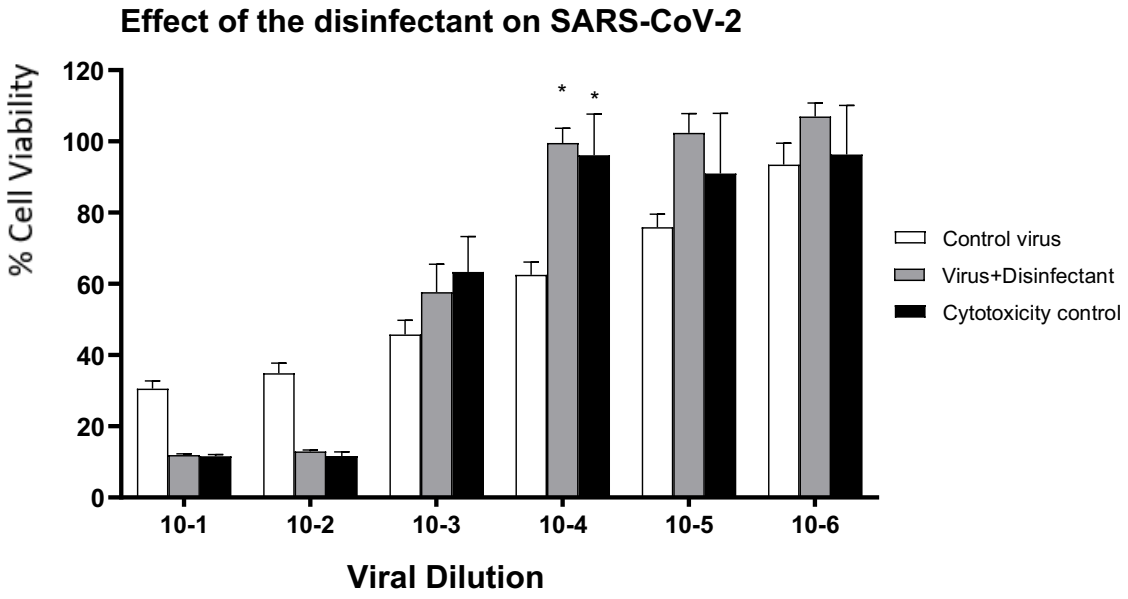


Figure 1.

Cell viability test showing the percentage of live cells, after 48 hours of infection with SARS-CoV-2 virus dilutions, previously exposed or not to the **99T DISINFECTION SYSTEM**. A cytotoxicity control was included in the experiment, which contained only culture medium exposed to the disinfectant. The graph shows the average of each measurement and the standard deviation. Two experiments were carried out with 4 replicates each. *Statistically significant difference ($p<0.05$) between the Virus+Disinfectant, the cytotoxicity control and Virus control.

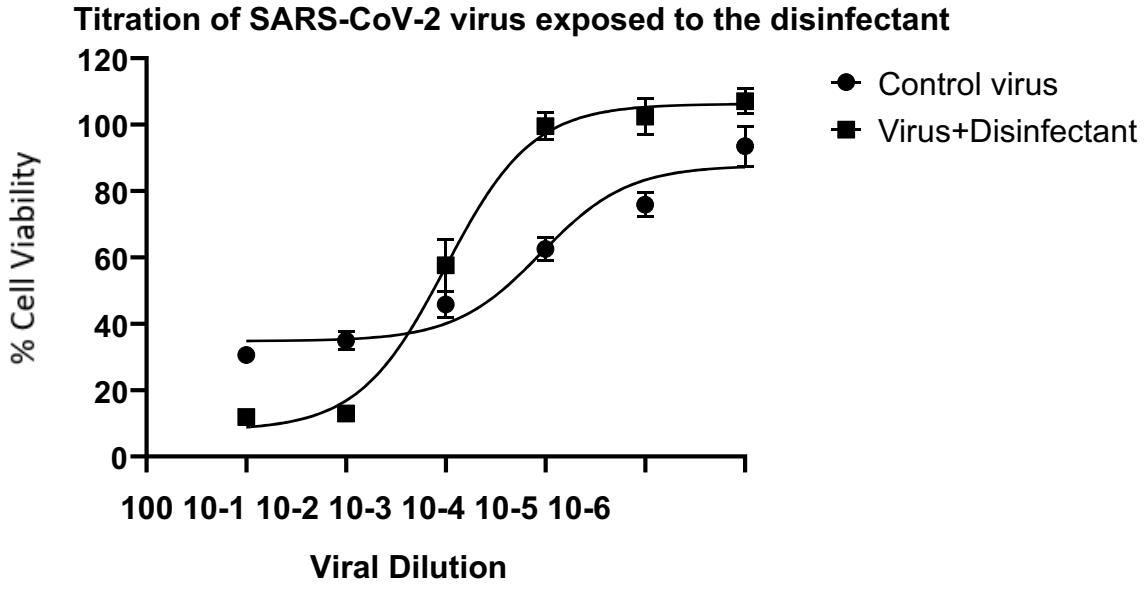


Figure 2.

Cellular viability assay from which the TCDI50 virus titer is obtained. The graph shows the percentage of live cells and the different viral dilutions that allow to define the TCID50 for each condition. The curve of the viral titer is observed in the condition of the virus control and Virus+disinfectant.

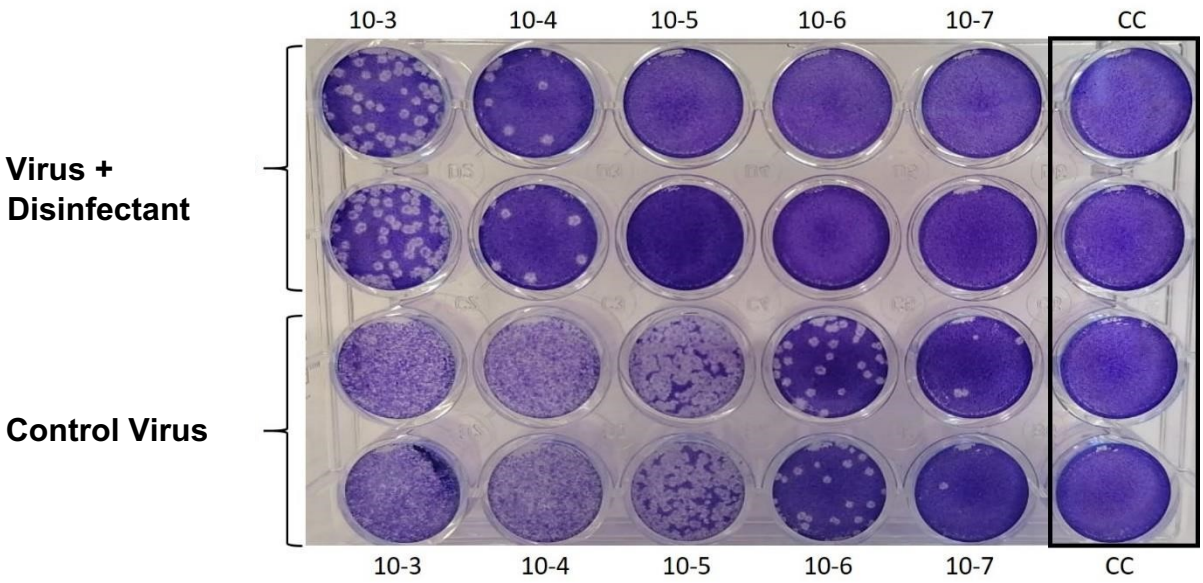


Figure 3.

Plaque assay in which the plaque formed by SARS-CoV2 obtained from the supernatant of the virus control and the virus exposed to the **99T Disinfection System** in the anti-viral test are shown. Dilutions from 10^{-3} to 10^{-7} are observed in the condition of Virus+Disinfectant and in the Virus Control. The result is expressed in plate-forming units (PFU)/mL. CC: control of cells without infection and without exposure to disinfectant.

Report prepared and reviewed by: Wildeman Zapata Builes. Associated Researcher.

Report reviewed by: Juan Carlos Hernández López. Associated Researcher.

Report reviewed by: María Teresa Rugeles López. Coordinator of the Immunovirology Group.

Contact information: Immunovirology Group.

Universidad de Antioquia. Calle 62 # 52-59, torre 2, laboratorio 532. Medellín. Colombia.

Tel: 2196482. E-mail: maria.rugeles@udea.edu.co.