**Final report**

**Project: " *In vitro* quantification of antiviral activity of disinfectants on SARS-CoV-2 (Covid-19)" Presentation date: July 27th, 2020**

## 1. BACKGROUND

The company Basic Farm S.A.S. sent a disinfecting solution, called VIRUKILL (Code S-V-K; **manufacuered and suppiled by ICA International Chemicals, South Africa**), to the Immunovirology Group of the Antioquia University, which was used to determine the antiviral potential against the SARS-CoV-2 (Covid-19) virus, isolated in Antioquia University.

## 2. METHODOLOGY.

### Materials and Reactives

The antiviral activity of the disinfectant VIRUKILL (Code S-V-K) was analyzed; for this analysis a SARS-CoV-2 (Covid-19) virus isolated in the Immunovirology Group of Antioquia University was used in VERO E6 cell line. VERO E6 cells were maintained in DMEM culture medium, supplemented with 5% FBS (Fetal Bovine Serum), in a $CO_2$ atmosphere at 5% and temperature of 37°C. The title of the SARS-CoV-2 virus isolated in the laboratory was determined using the plating technique and TCID50 (*Tissue Culture Infectious Doses* 50) in VERO E6 cells, following a protocol previously described in the literature (1). The obtained title was $4.21x10^6$ PFU (plaque forming units) / ml.

1. Fan HH, 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.

**Viricidal Activity Assay**

The day of the test, 100 µL of disinfectant (diluted 1:100) was added to a tube containing 50 µL of SARS-CoV-2 virus with a viral titer of $4.21x10^6$ PFU/ml. The evaluated contact time was 5 and 10 minutes. At the end of time, 1000 µL of DMEM culture medium without FBS were added. In parallel, it was included a virus control, which includes culture medium (100 µL) and 50 µL of the virus, without disinfectant. Furthermore, a cytotoxicity control was included, adding 100 µL of disinfectant with 50 µL of culture medium, without virus; this control allows to determine the cytotoxicity produced by the disinfectant on cell culture. Subsequently, in order to decrease disinfectant cytotoxicity, all conditions were added to a filter (Millipore, Schwalbach, Germany) which was centrifuged for 10 min at 4000 x g. Then, the concentrated virus was titrated using the TCID50 and plaque assay technique (1).

TCID50 was determined by cell viability analysis using the MTT assay, which is based on the metabolic reduction of Bromide of 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazole made by the mitochondrial enzyme succinate-dehydrogenase in a blue 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; in this way, the amount of formazan is directly proportional to cell viability (2). Initially, cells were cultured at density of $1x10^5$ cells / well in a 96-well plate in 200 µL DMEM with 10% FBS and cultivated for 24 hours at 37°C with 5% CO2, prior to carrying out the antiviral experiment. The cells were then infected with 10 base dilutions of the virus obtained in the previous step, in triplicate and for 1 hour. At the end of this time, the remainder virus that could not enter to cells was discarded and cells were added with fresh DMEM culture medium with 5% FBS. Forty-eight hours after infection, supernatant was removed and MTT solution was added (0.5 mg / mL). After 2 h incubation, 130 µL / well of DMSO was added. The plates

were agitated for 15 minutes and finally read on a spectrophotometer at 550 nm. Each experimental condition was evaluated in triplicate in 2 independent experiments (n = 6). Calculation of the virus titer it is obtained by 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, LJ; 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 greater than 20% in cell viability comparing virus exposed to disinfectant versus virus control, and in which the cytotoxicity of the disinfectant was less than 20%, were used to determine the virus titer in a plate assay.

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

Finally, statistical analysis for all trials are shown as the mean with respective standard deviation at each dilution and comparisons between each condition. Parametric statistical or not parametric tests were performed, as appropriate, to find differences between conditions of each experiment. A value of $p < 0.05$ was considered statistically significant.

**3. RESULTS**

Figure 1 shows living cells percentage after being infected with SARS-CoV-2 exposed to VIRUKILL (S-V-K) disinfectant or without exposure to disinfectant (Control Virus) for 5 and 10 minutes. The use of the disinfectant managed to reduce the cytotoxicity of the virus, producing an increase in percentage of cell viability from dilution $10^{-2}$, for two times evaluated, with an average of 87% and greater than 100%, for 5 and 10 minutes, respectively. In the other dilutions the cell viability reached 100% for both evaluated times. In contrast, in virus control, percentage of cell viability was 24%, 25%, 34% and 51%, in dilutions $10^{-1}$, $10^{-2}$, $10^{-3}$ and $10^{-4}$, respectively; with statistically significant differences between virus control and Virus + Disinfectant, in dilutions $10^{-2}$, $10^{-3}$ and $10^{-4}$ ($p < 0.05$). Therefore, it is clear that cell viability measured by MTT technique was superior when cells were previously infected with the SARS-CoV-2 virus exposed to the disinfectant; suggesting a viricidal effect, which is evident with diminishing cytopathic effect of the virus.

Additionally, it can be observed that cell viability, in dilution $10^{-2}$ and higher dilutions, is greater than 93% in cytotoxicity control. This suggests that cytotoxicity of disinfectant is not affecting the reading or interpretation of results observed in cells infected with virus exposed to disinfectant.

Subsequently, from the data of cell viability assay measured by MTT, it is possible to calculate virus titer by TCID50. Control showed that virus had a viral titer of $10^{-4,329}$; whereas for virus exposed to disinfectant for 5 and 10 minutes, the virus titer was $10^{-1,876}$ and $10^{-1,077}$ respectively; suggesting a decrease in the titer of the virus exposed to disinfectant (Figure 2).

To confirm this reduction, plate test was performed, starting from the $10^{-3}$ dilution supernatant from the previous trial, in 5 minutes contact condition; dilution chosen according to the criteria mentioned in the Methodology section of this report.

In virus control (cells infected with virus not exposed to disinfectant) the viral titer calculation by plaque forming units (PFU) / ml was on average $1.55x10^{8}$, while the virus titer in the condition of Virus + Disinfectant (Cells infected with virus exposed to disinfectant), was less than $1x10^{3}$ (reciprocal of dilution $10^{-3}$), indicating a reduction of more than 4 logs in viral titer (Figure 3); in other words, the VIRUKILL ( Code S-V-K) disinfectant inactivated more tan 99.99% of infectious viral particles, confirming its viricidal effect in 5 minutes.

**4. CONCLUSION**

We can conclude that disinfectant VIRUKILL (Code S-V-K) (Diluted 1: 100), for contact time of 5 minutes with virus suspension, inactivated more than 99.99% of infectious SARS-CoV-2 viruses.

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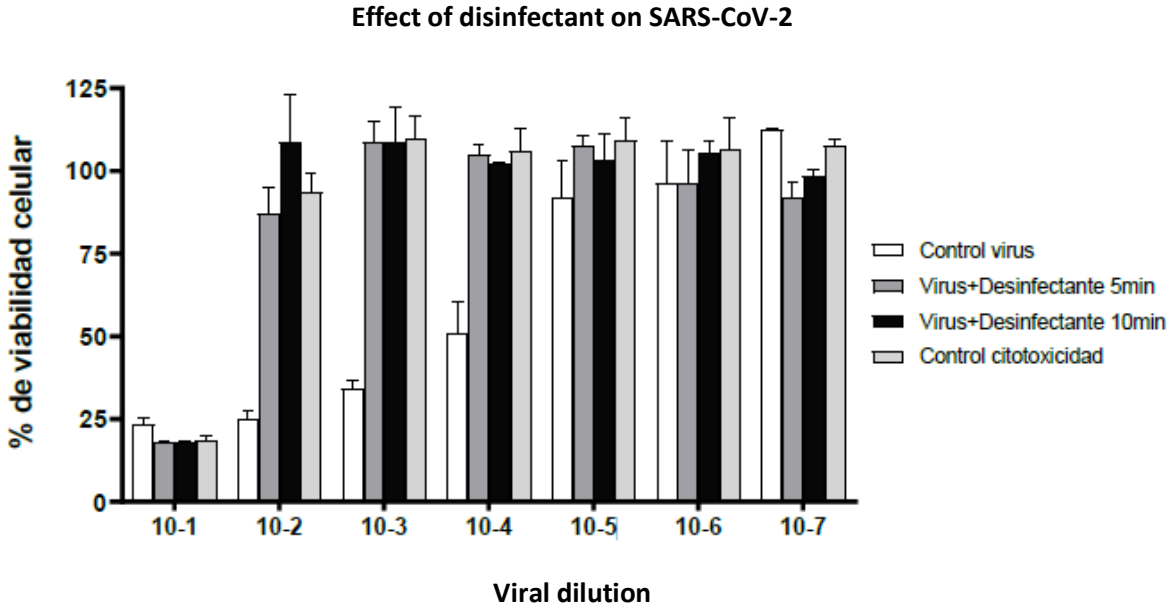


**Figure 1.**

Cell viability test showing percentage of living cells, after 48 hours of infection with SARS-CoV-2 virus dilutions of previously exposed or not to disinfectant VIRUKILL (Code S-V-K) for 5 and 10 minutes. It was included in the experiment a cytotoxicity control, which contained only culture medium exposed to disinfectant VIRUKILL (Code S-V-K). The graph shows the mean of each measurement and standard deviation. 2 experiments were performed with 3 replicates each one.

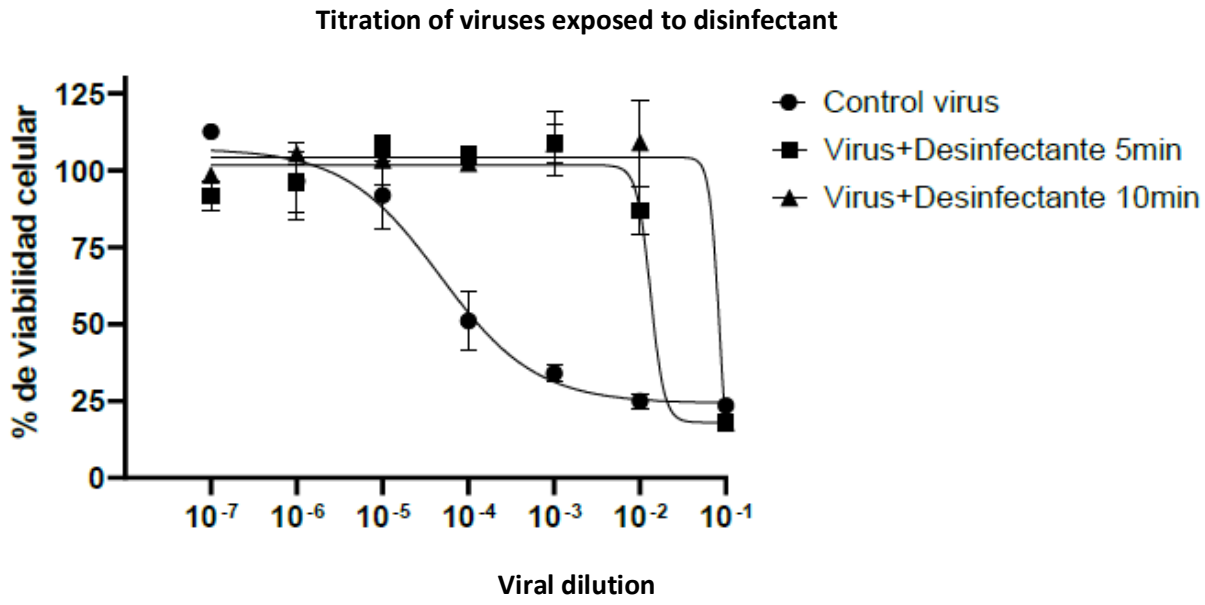


**Figure 2.**

Cell viability test from which virus title is obtained by TCDI50. The graph shows the percentage of living cells and the different viral dilutions allowing to define TCID50 for each condition. Curve of viral titer (virus control) and virus exposed to disinfectant for 5 and 10 min (Virus + Disinfectant) can be observed.

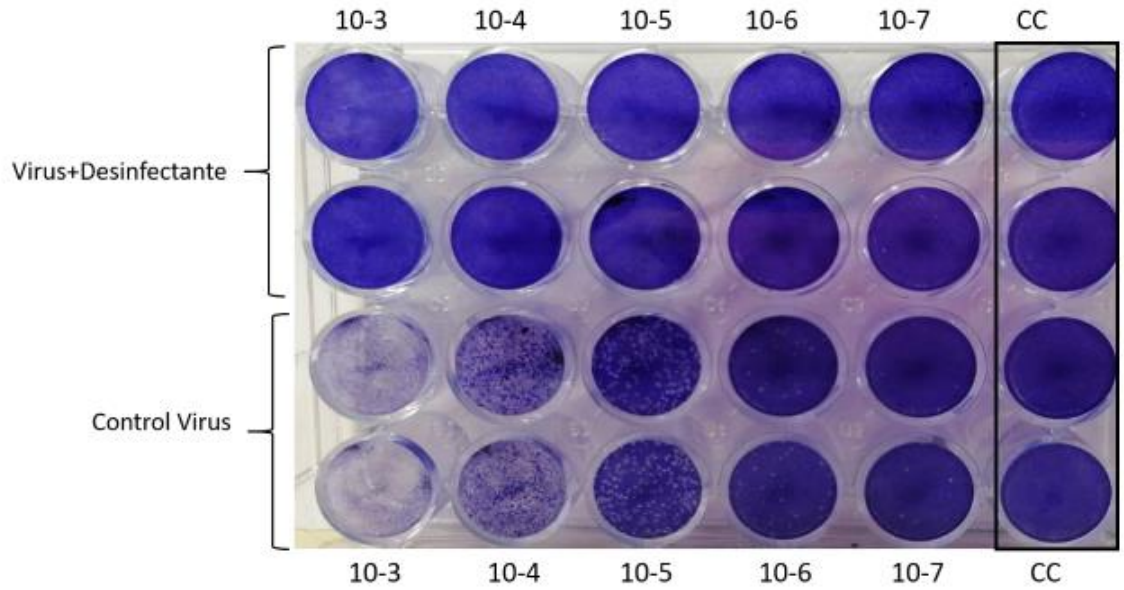


**Figure 3.**

Plating test showing the plates formed by SARS-CoV-2 obtained from the virus control supernatant and virus exposed to disinfectant in the antiviral assay. Dilutions from $10^{-3}$ to $10^{-7}$ are observed. The result is expressed in plaque forming units (PFU) / ml. CC: control of uninfected cells and without exposure to disinfectant.

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