UNIVERSIDAD
DE ANTIOQUIA
1803

FACULTAD DE MEDICINA
Grupo Inmunovirología

Medellín, 1 de agosto de 2020

INFORME DE RESULTADOS

El Grupo Inmunovirología de la Universidad de Antioquia se permite INFORMAR que:

El análisis de la actividad antiviral contra el virus SARS-CoV-2 (Cepa de virus aislada en la Universidad de Antioquia) realizado al **Desinfectante Diox-oro Solución Líquida** de la empresa **Biostec S.A.S** a una concentración de 100 ppm y por un tiempo de contacto de 60 segundos con el virus en solución, produjo el siguiente resultado:

El **Desinfectante Diox-oro Solución Líquida** tuvo un efecto antiviral significativo, inactivando mas del 99.99% de las partículas virales infecciosas, confirmando su efecto viricida en las condiciones descritas.

El grupo de investigación sólo se hace responsable de las evaluaciones realizadas con el producto enviado al laboratorio y bajo las condiciones establecidas en el informe final entregado a la empresa. El grupo NO se hace responsable por el uso indebido de esta información, por los cambios a la formulación inicial o a las condiciones de la evaluación, que realice la empresa.

Atentamente,

María Teresa Rugeles López. MSci; DSci.
Coordinadora Grupo Inmunovirología
Facultad de Medicina, Universidad de Antioquia
e-mail: maria.rugeles@udea.edu.co

Medellín, August 1, 2020

REPORT ON RESULTS

The Immunovirology Group of Universidad de Antioquia is pleased to REPORT that:

The analysis of antiviral activity against SARS-CoV-2 (a strain isolated by the University of Antioquia) using **Biostec S.A.S.'s** disinfectant **Diox-oro** at a concentration of 100 ppm in contact with the virus solution for 60 seconds, produced the following results:

The Diox-oro Liquid Disinfectant solution had a significant antiviral effect by deactivating over 99.99% of the infectious virus particles, thus confirming its antiviral effect under the foregoing conditions.

The research group is responsible only for the tests performed with the product sent to the laboratory and under the conditions described in the final report delivered to the Company. The Group ASSUMES NO LIABILITY for the improper use of this information, for changes to the initial formulation, or for the conditions of the tests carried by the Company.

Sincerely,

Maria Teresa Rugeles Lopez. MSci; DSci.
Coordinator – Immunovirology Group
School of Medicine, University of Antioquia
maria.rugeles@udea.edu.co

GLORIA MARIA MEJIA R.
TRADUCTORA E INTERPRETE OFICIAL
Inglés-Español / Español-Inglés
Resolución Nº 1699 Minjusticia 1991

NOTARÍA 17 MEDELLÍN

FIRMA REGISTRADA

Como Notario Diecisiete del Círculo de Medellín, doy testimonio que la firma de:

MEJIA RAMIREZ GLORIA MARIA

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MARIA LUISA LONDOÑO ARRUBLA

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DECLARACIÓN JURAMENTADA

Yo, GLORIA MARÍA MEJÍA RAMIREZ, Traductora e Intérprete Oficial ante el Ministerio de Justicia de la República de Colombia, mediante la Resolución 1699, certifico que traduje el siguiente documento del español al inglés:

- **Informe de Resultados del Grupo de Inmunovirología de la Universidad de Antioquia respecto del análisis de la actividad antiviral contra el virus SARS-CoV-2 realizado al Desinfectante Diox-oro Solución Líquida de la empresa Biostec S.A.S. Fecha: 1° de agosto de 2020.**

Doy fe que traduje fielmente lo anotado, retengo una copia del documento arriba anotado en mi oficina y adjunto copia de mi Resolución como Traductora Oficial.

Gloria María Mejía R.
Cédula de Ciudadanía No. 42'874.299

GLORIA MARIA MEJIA R.
TRADUCTORA E INTERPRETE OFICIAL
Inglés-Español / Español-Inglés
Resolución Nº 1699 Minjusticia 1991

19 de agosto de 2020

AFFIDAVIT

I, GLORIA MARÍA MEJÍA RAMIREZ, Certified Translator and Interpreter before the Ministry of Justice and Education of the Republic of Colombia, by Resolution 1699, do hereby certify that I have translated the following document from English to Spanish:

- **Report on Results of the Immunovirology Group of University of Antioquia regarding the analysis of antiviral activity against SARS-CoV-2 using Diox-oro Liquid Disinfectant of Biostec S.A.S. Date: August 1, 2020.**

I give faith that I have translated the above mentioned document, I keep a copy of the foregoing at my office, and hereby provide a copy of my Resolution as a Certified Translator.

Gloria María Mejía R.
Colombian I.D. Card No. 42.874.299

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August 19, 2020

NOTARÍA 17 MEDELLÍN

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MARIA LUISA LONDOÑO ARRUBLA
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Final report

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

Submission date: August 5, 2020

1. BACKGROUND

The company **Biostec SAS** sent a sample of 20 g Diox-gold tablets (Photo 1) to the Immunovirology Group of the University of Antioquia, which were prepared in water to produce a disinfectant solution, called

Diox-oro Disinfectant Liquid Solution (50 ppm and 100 ppm); saying

disinfectant was used to determine the antiviral potential against the SARSCoV-2 Virus, isolated at the University of Antioquia.

2. METHODOLOGY.

Materials and REACTIVES

The antiviral activity of the **Diox-oro Disinfectant Liquid Solution (50 ppm and 100 ppm);** For this analysis, the SARS-CoV-2 virus isolated in the Immunovirology Group of the UdeA 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 an atmosphere of CO_{two} at 5% and at a temperature of 37ºC. The titer of the SARS-CoV-2 virus isolated in the laboratory was determined using the plating technique and TCID50 (English, *Tissue Culture Infectious Doses* 50) in VERO E6 cells, following a protocol previously described in the literature (1). The title obtained 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 2019novel coronavirus (2019-nCoV) related coronavirus model. Chin. Med. J. 2020; 6. doi: 10.1097 / CM9.0000000000000797.

Viricidal Activity Assay

On the day of the test, 100uL of **Diox-oro Disinfectant Liquid Solution (50 ppm and 100 ppm)** were added to a tube containing 50uL of the SARS-CoV-2 virus with a viral titer of $4.21x10_6$ PFU / mL. The contact time evaluated was 1 minute for the 100 ppm concentration and 30 seconds for the 50 ppm concentration. At the end of the time, 1000uL of DMEM culture medium without FBS were added. In parallel, a virus control was included, which contains culture medium (100uL) and 50uL of the virus, without the disinfectant. Furthermore, a cytotoxicity control was included, which consists of 100uL of the disinfectant with 50uL of culture medium, without virus; This control makes it possible to determine the cytotoxicity produced by the disinfectant on the cell culture. Subsequently, in order to reduce the cytotoxicity of the disinfectant, all the conditions were added to a filter (Millipore, Schwalbach, Germany) which was centrifuged for 10 min at 4000 x g. Then the concentrated virus was titled,

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 by the mitochondrial enzyme succinate dehydrogenase. in a compound of blue color (formazan), allowing to determine the mitochondrial functionality of the 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% $CO_{two,}$ prior to the antiviral experiment. Then, the cells were infected with dilutions in base 10 of the virus obtained in the previous step, in triplicate and for 1h. After this time, the

The remnant of the virus that did not enter the cell was discarded and the cells were added fresh DMEM culture medium with 5% FBS. Forty-eight hours after infection, the supernatant was removed and the MTT solution (0.5 mg / mL) was added. After 2h of incubation, 130 µL / well of DMSO were added. The plates were left shaking for 15 minutes and 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, 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 of more than 20% in cell viability was observed comparing the virus exposed to the disinfectant versus the virus control, and in which the cytotoxicity of the disinfectant was less than 20%, were used to determine the virus titer in a plating assay.

The plating test is a technique considered as *gold standard* to determine the viral title; therefore, it is the technique of choice to efficiently check the reduction in viral titer caused by the disinfectant in this type of experiment.

Finally, the statistical analyzes for all the tests are shown as the mean with the respective standard deviation in each dilution. Parametric or non-parametric statistical tests were performed, depending on the case, 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 living cells after being infected.
with SARS-CoV-2 exposed to **Diox-oro Disinfectant Liquid Solution (50 ppm)** or without exposure to the disinfectant (Control Virus) for 30 seconds. The use of the disinfectant managed to reduce the cytotoxicity of the virus, producing an increase in the percentage of cell viability, with an average of 42%, 54% and 81% in the 10- dilutions. two, 10^{-3} and 10^{-4}, respectively; while, for the virus control, the average viability was 30%, 42% and 55%, for the same dilutions mentioned (Figure 1). However, no statistically significant differences were observed with respect to the control without disinfectant ($p > 0.05$).

Additionally, it can be observed that cell viability, at the 10- one and dilutions higher than this, is on average higher than 86% in the cytotoxicity control (Figure 1). This suggests that the cytotoxicity of **Dioxoro Disinfectant Liquid Solution (50 ppm)** it is not affecting the reading or interpretation of the result observed in cells infected with the virus exposed to the disinfectant.

Subsequently, from the data of the cell viability assay measured by MTT, it is possible to calculate the virus titer by TCID50. The virus control was found to have a viral titer of $10^{-4,220}$ (Figure 2); whereas, for the exposed virus
to the **Diox-oro Disinfectant Liquid Solution (50 ppm)** for 30 seconds, the title
viral was $10^{-3,171}$, suggesting a slight decrease in the titer of the exposed virus
to the **Diox-oro Disinfectant Liquid Solution (50 ppm).** To confirm this
reduction, the plating test was carried out, starting from the supernatant of the 10^{-4} from the previous trial; dilution chosen according to the criteria mentioned in the methodology section of this report.

In the virus control (cells infected with virus Not exposed to the disinfectant) the calculation of the viral titer by plaque-forming units (PFU) / mL was on average $4x10^{8}$, while the virus titer in the Virus + Disinfectant condition (Cells infected with virus exposed to the disinfectant), was $1x10^{8}$, indicating a reduction in viral titer (Figure 3); in other words, the **Dioxoro Disinfectant Liquid Solution (50 ppm)** inactivated 75% of the infectious viral particles, in 30 seconds.

Regarding the evaluation of the viricidal activity of the **Diox-gold Disinfectant Liquid Solution (100 ppm)** During a contact time of 60 seconds, we can observe in Figure 4, that the use of the disinfectant managed to reduce the cytotoxicity of the virus, producing an increase in the percentage of cell viability, with an average of over 89% in the first 4 dilutions. In contrast, in the virus control, the percentage of cell viability was 25%, 35%, 45%, and 54%, in the 10^{-one}, 10^{-two}, 10^{-3} and 10^{-4}, respectively; with statistically significant differences between the virus control and the Virus + Disinfectant, in dilutions 10^{-one}, 10^{-two}, 10^{-3} and 10^{-4} ($p < 0.05$) (Figure 4). Therefore, it is clear that the cell viability measured by the MTT technique was higher when the cells were infected with the SARS-CoV-2 virus previously exposed to the disinfectant; suggesting a viricidal effect, which becomes evident with the decrease in the cytopathic effect of the virus.

Additionally, it can be observed that cell viability, at the 10^{-one} and dilutions higher than this, is on average 100% in the cytotoxicity control. This suggests that the cytotoxicity of the disinfectant is not affecting the reading or interpretation of the result observed in cells infected with the virus exposed to the disinfectant.

Subsequently, from the data of the cell viability assay measured by MTT, it is possible to calculate the virus titer by TCID50. The virus control was found to have a viral titer of $10^{-4,180}$ (Figure 5); whereas, for the exposed virus

to the **Diox-gold Disinfectant Liquid Solution (100 ppm)** for 60 seconds, I don't know

was able to determine the exact value of the viral titer by this method, because it inhibits the cytopathic effect of the virus from the 10- dilution one; but if it can be indicated that the virus titer is less than 10^{-one} at the contact time of 60 seconds (Figure

4); suggesting a decrease in the titer of the virus exposed to the **Dioxoro Disinfectant Liquid Solution (100 ppm).** To confirm this reduction, the plating test was carried out, from the supernatant of the 10^{-3} from the previous trial; dilution chosen according to the criteria mentioned in the methodology section of this report.

In the virus control (cells infected with virus Not exposed to the disinfectant) the calculation of the viral titer by plaque-forming units (PFU) / mL was on average $1.15x10^{9}$, while the virus titer in the Virus + Disinfectant condition (Cells infected with virus exposed to the disinfectant), was less than $1x10^{3}$

(reciprocal of dilution 10^{-3}), indicating a reduction of more than 4 logarithms in the viral titer (Figure 6); in other words, the **Diox-gold Disinfectant Liquid Solution (100 ppm)** it inactivated more than 99.99% infectious viral particles, in 60 seconds.

4. CONCLUSION

We can conclude that the **Diox-oro Disinfectant Liquid Solution at 50 ppm,**

during a contact time of 30 seconds with the virus in suspension, it inactivated 75% of the infectious SARS-CoV-2 viruses. However the **Disinfectant**

Diox-gold Liquid Solution at 100 ppm, for a contact time of 60

seconds, it had a significant antiviral effect, inactivating more than 99.99% of the infectious viral particles, confirming its virucidal effect under these conditions.

Photograph 1.

Diox-gold tablets of 20 grams.

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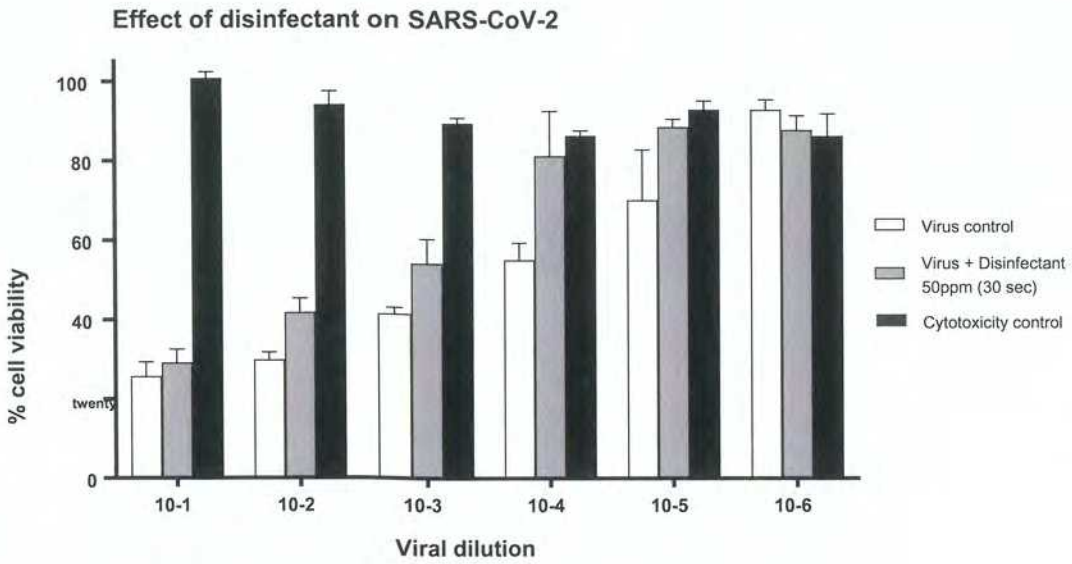

Figure 1.

Cell viability assay in which the percentage of living cells is shown, after 48h of infection with the dilutions of the SARS-CoV-2 virus, previously exposed or not to **Diox-oro Disinfectant Liquid Solution (50 ppm)** for 30 seconds. A cytotoxicity control was included in the experiment, which contained only culture medium exposed to the disinfectant. The graph shows the mean of each measurement and the standard deviation. 2 experiments were performed with 3 replicates each.

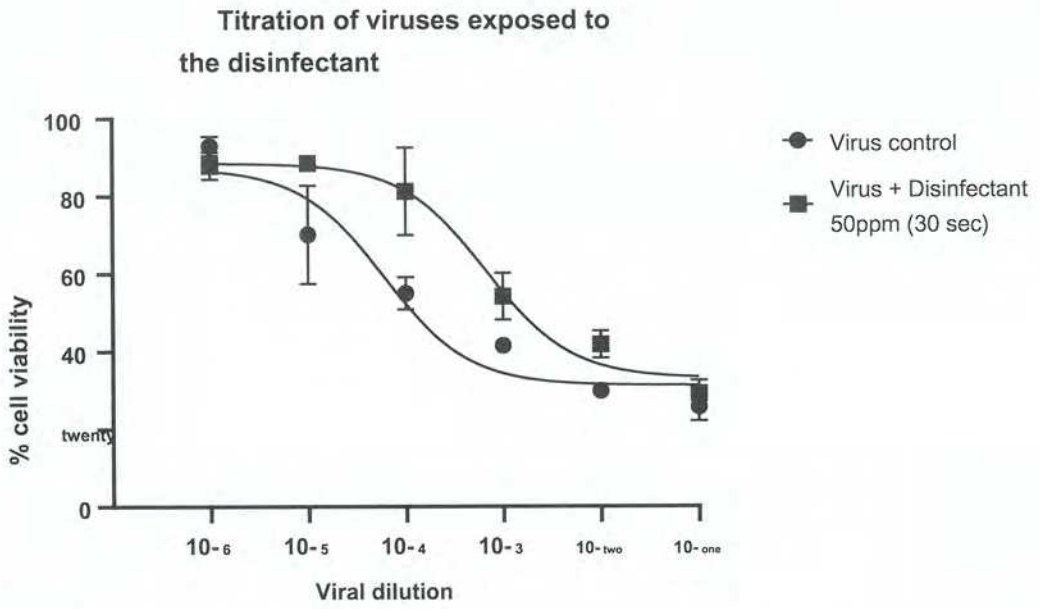

Figure 2.

Cell viability assay from which the virus Titer is obtained by TCDI50. The graph shows the percentage of live cells and the different viral dilutions that allow defining the TCID50 for each condition. The viral titer curve is observed in the condition of the virus control and Virus + disinfectant at 50 ppm for 30 seconds of exposure.

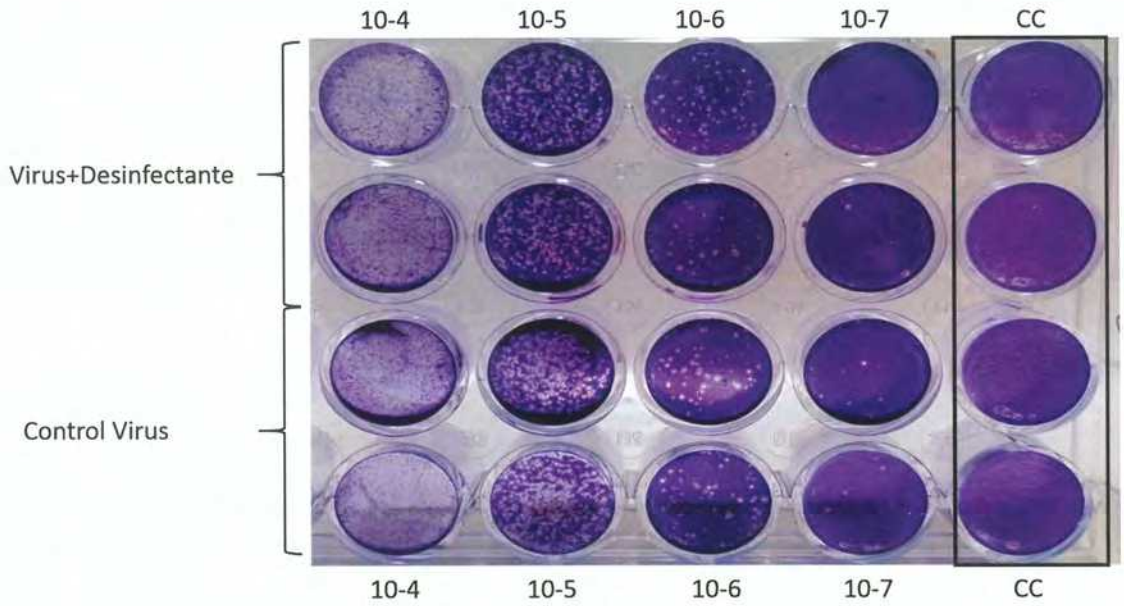

Figure 3.

Plaque assay in which the plaques formed by SARS-CoV2 obtained from the virus control supernatant and from the virus exposed to the

Diox-oro Disinfectant Liquid Solution (50 ppm) for 30 seconds in the test

antiviral. Dilutions from 10^{-4} up to 10^{-7} in the Virus + Disinfectant condition and in the Virus Control. The result is expressed in plaque-forming units (PFU) / mL. CC: control of cells without infection and without exposure to the disinfectant.

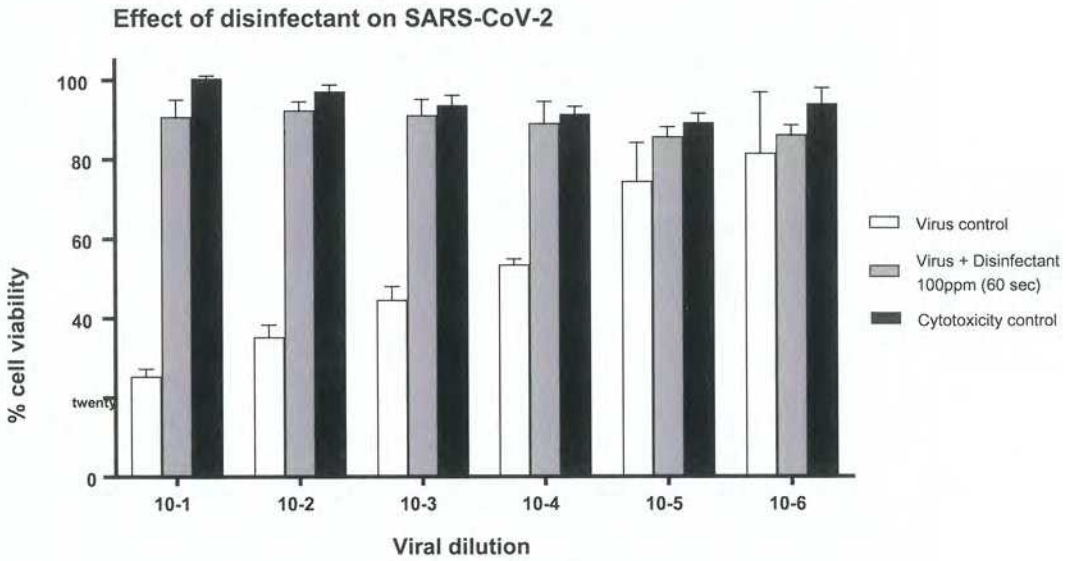

Figure 4.

Cell viability assay in which the percentage of living cells is shown, after 48h of infection with the dilutions of the SARS-CoV-2 virus, previously exposed or not to **Diox-gold Disinfectant Liquid Solution (100 ppm)** for 30 seconds. A cytotoxicity control was included in the experiment, which contained only culture medium exposed to the disinfectant. The graph shows the mean of each measurement and the standard deviation. 2 experiments were performed with 3 replicates each.

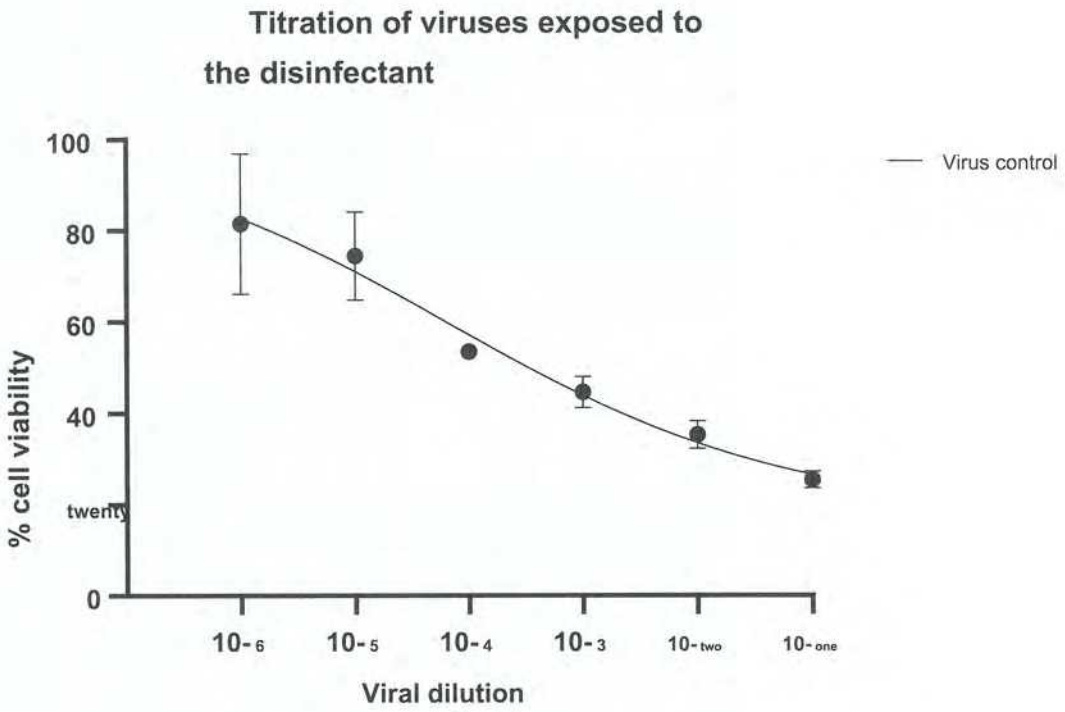

Figure 5.

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

could not be graphed, because the **Diox-gold Disinfectant Liquid Solution (100 ppm)** inhibits the cytopathic effect of the virus from dilution 10-one, which does not allow to obtain the viral title.

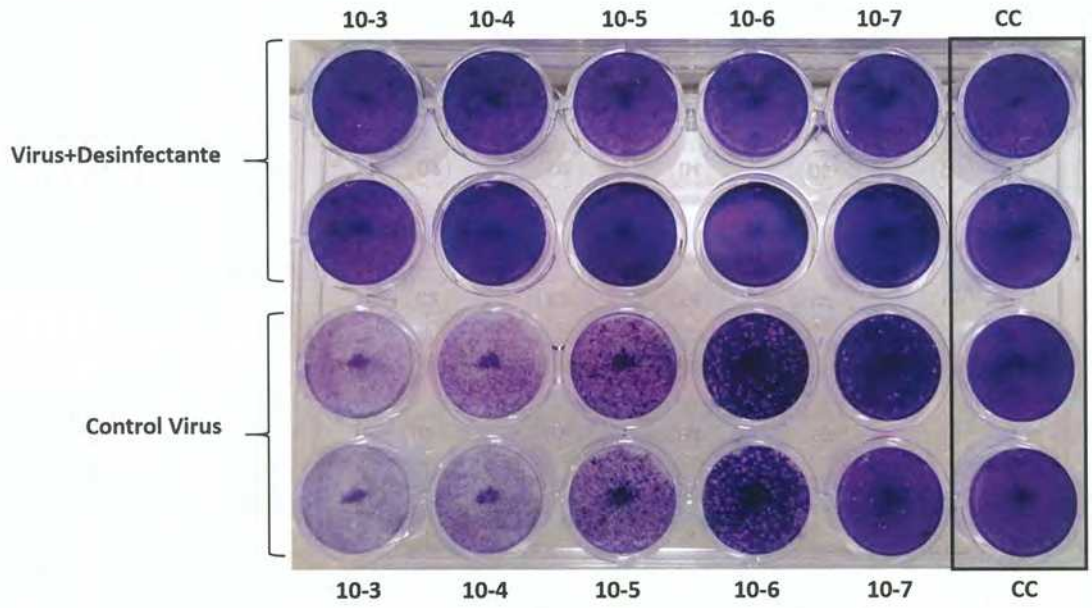

Figure 6.

Plaque assay in which the plaques formed by SARS-CoV2 obtained from the virus control supernatant and from the virus exposed to the

Diox-gold Disinfectant Liquid Solution (100 ppm) for 60 seconds in the antiviral assay. The dilutions from 10^{-3} up to 10^{-7} in the Virus + Disinfectant condition and in the Virus Control. The result is expressed in plaque-forming units (PFU) / mL. CC: control of cells without infecting and without exposing to the disinfectant.

Report prepared and reviewed by: Wildeman Zapata Builes. Research associate. Report reviewed by: Juan Carlos Hernández López. Research associate. Report reviewed by: María Teresa Rugeles López. Coordinator of the Immunovirology Group.

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