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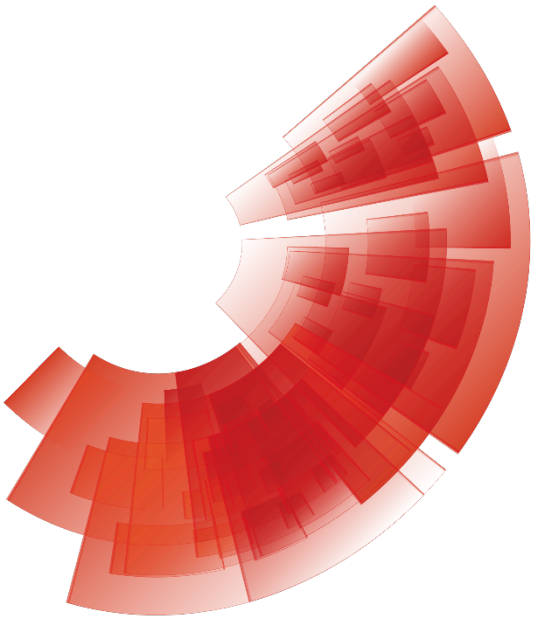

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REPORT

MEASUREMENT OF ANTIVIRAL ACTIVITY ON NON-POROUS SURFACES ACCORDING TO THE ISO 21702:2019 ON SURFORMA LAMINATES

MEDICAL AND INDUSTRIAL BIOTECHNOLOGY LABORATORY

Laboratory registered in the Health Regulatory Authority (ERS), number E150638 as Clinical Analysis Laboratory and member of the COVID-19 Laboratories National Network, certified by the National Health Institute Doutor Ricardo Jorge (INSA.) as of July 13th, 2020.

PORTO, JUNE 2021

Measurement of Antiviral Activity on Surfaces

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Measurement of SURFORMA Laminates Antiviral Activity

Background

The International Organization for Standardization developed the ISO 21702:2019 norm, "*Measurement of antiviral activity on plastics and other non-porous surfaces,*" intending to standardize the measurement of antiviral activity on plastic treated surfaces and non-porous ones (1).

The Medical and Industrial Biotechnology Laboratory (**LaBMI**) is a laboratory integrated into the COVID-19 Laboratories National Network certified, as of July 13th, 2020, by the National Health Institute Doutor Ricardo Jorge (INSA.) registered in the Health Regulatory Authority (ERS), number E150638 as Clinical Analysis Laboratory.

Objectives

To determine the antiviral capacity of SURFORMA antiviral laminates.

Aim

To provide a quantitative analysis of the antiviral capacity of different SURFORMA laminates.

Materials and biologics

- MultiSkan Ascent, Thermo Fisher, Massachusetts, USA;
- XL Core Microscope, Thermo Fisher, Massachusetts, USA;
- PCHbi Incubator, MCO-170AIC-PA;
- qTOWER^3 touch, Analytik Jena GmbH, Germany;
- Lab-Aid 824 Nucleic Acid Extraction System, ZeeSan, China;

- MTT [(3-(4,5-Dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide;
- LDH, Lactate dehydrogenase
- DMSO (Dimethyl sulfoxide);
- DMEM (*Dulbecco's Modified Eagle's Medium*);
- Antibiotic and antimycotic mixture;
- VTM (universal viral transport medium);
- FBS (foetal bovine serum);
- SCDLP medium – *Soybean Casein Digest Broth with Lecithin Polyoxyethylene Sorbitan Monooleate* (Cleaning medium);

- CaCo2 cells – *Colorectal adenocarcinoma*;
- SARS-CoV-2 clinical isolate.

Methods

Application of **ISO 21702:2019**, with the following stages in mind:

M1 Virus expansion and measurement of strength;
M2 Cytotoxic effect of (SCDLP) cleaning medium in host cells;
M3 Virus sensitivity to cleaning medium;
M4 Kinetic analysis of SURFORMA antiviral laminates antiviral activity (0 minutes, 10 minutes, 30 minutes, and 4 hours);

Beyond the applied measures of mentioned ISO 21702:2019, the following analyses were additionally performed:
M5 Measurement of viral load through PCR (polymerase chain reaction) in quantitative real-time (qRT-PCR) after 0 minutes, 10 minutes, 30 minutes, and 4 hours.

Results

M1 Virus expansion and measurement of strength

The SARS-CoV-2 virus was expanded in a CaCo-2 cell line. Soon after, the virus was inoculated in the cell line in 96 well flat-bottom microplates with a density of $2x10^5$ cells/mL in a DMEM media supplemented with 10% FBS and antibiotics until it reached a subconfluent state. At this stage, the CaCo-2 were infected with clinical strains of SARS-CoV-2 obtained after universal viral transport medium (VTM) filtration at 0,2 µm. Incubation was performed in an incubator with 5% CO_2 at 37ºC for 3 to 4 days.

Measurement of SARS-CoV-2 strength was determined through PCR in quantitative real-time (qRT-PCR). RNA was extracted in an automatic system, and afterwards, SARS-CoV-2 was quantified by analysing the nucleocapsid gene (N gene). SARS-CoV-2 aliquots were prepared with an approximate quantity of $1x10^6$/mL.

M2 Cytotoxic effect of (SCDLP) cleaning medium in host cells

SURFORMA antiviral laminates will be inoculated with the virus. Removal of the virus from the surface will be promoted with an agent that should not interfere with the virus and host cell activity. Another property of this cleaning solution should be the non-interference in the effects on viral viability of SURFORMA antiviral laminates. According to the norm, the first medium that must meet the mentioned expectations should be the SCDLP medium.

Our results indicate this medium does not interfere with the cellular activities of SARS-CoV-2 cell hosts (CaCo-2) when treated with SCDLP (Figure 1).

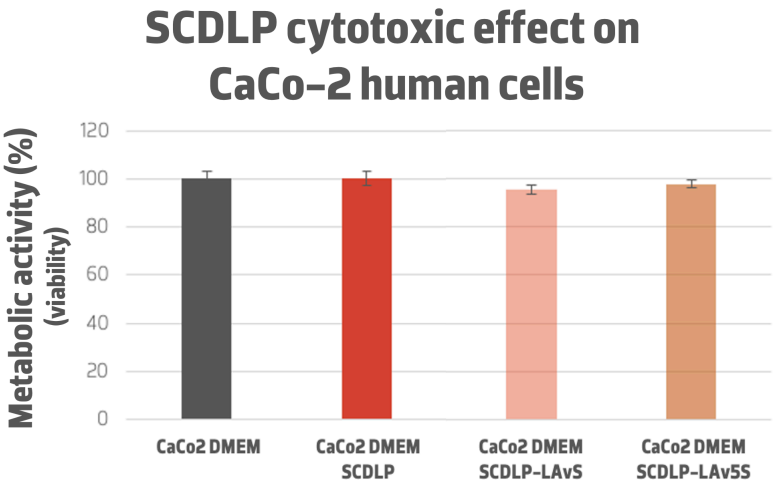


Figure 1: Cytotoxic effect of SCDLP medium on CaCo2. DMEM – cell growth medium. SCDLP – Virus removal medium from SURFORMA antiviral laminates. SAvL – SURFORMA antiviral laminates. SAv5L – SURFORMA antiviral laminates aged 5 years.

In the previous image, treatments were performed for 1 hour. After that period, cells were cleaned and incubated with a new medium at 37ºC for 24 hours. The cellular activity was measured through the MTT test. Results were quantified in a +/- standard error rate relative to cells grown without treatments.

The treatments were:

a) Cells grown in complete medium (CaCo2 DMEM);
b) Cells grown in complete medium treated with SCDLP medium non-exposed to surfaces (CaCo2 DMEM SCDLP);
c) Cells grown in complete medium treated with SCDLP medium exposed to the SURFORMA antiviral laminate (CaCo2 DMEM SCDLP-SAvL);
d) Cells grown in complete medium treated with SCDLP medium exposed to the SURFORMA antiviral laminate with a 5-year ageing period (CaCo2 DMEM SCDLP-SAv5L).

Explanation

The SCDLP medium is a suitable virus cleaning medium from antiviral surfaces, like SURFORMA laminates. These results indicate that the medium shows no toxicity to CaCo-2 human cells neither to the SURFORMA laminates.

M3 Virus sensitivity to cleaning medium

Lastly, to validate the use of SCDLP as a cleaning medium for SURFORMA antiviral laminates, its influence on viral activity still needs to be tested. According to the **ISO 21702:2019** norm, a demonstration showing that SCDLP cannot impact virus activity has to be proven. Figure 2 shows that SCDLP is a neutral medium that does not impact viral activity negatively.

This experiment also predicts that SURFORMA antiviral laminates might impact viral activity either with or without a 5-year ageing period (SAvL and SAv5L). The MTT method assesses the metabolism of cells hosting the virus – a viability measure.

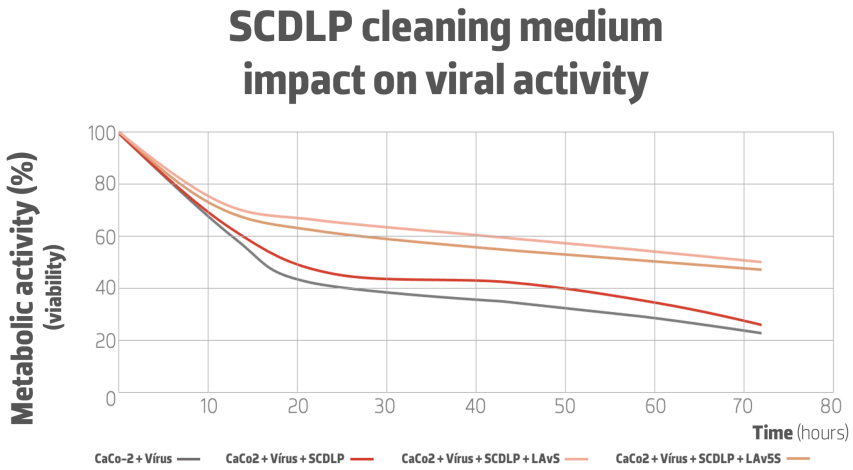


Figure 2: SCDLP cleaning medium impact on viral activity
SCDLP – cleaning medium. SCDLP-SAvL – SCDLP medium previously exposed to SURFORMA antiviral laminates. SCDLP-SAv5L – SCDLP medium previously exposed to SURFORMA antiviral laminates after a 5-year equivalent ageing period treatment.

In the graph, there is a more noticeable metabolic activity (viability) in cells inoculated with the virus in experiments where surfaces were previously cleaned.

Explanation
The grey graph shows the decaying viability pattern from (CaCo-2) cells infected by the SARS-CoV-2 virus.
The viability of (CaCo-2) cells infected by SARS-CoV-2 increases when the cleaning medium previously exposed to SURFORMA antiviral laminates is present. Since the SDCLP cleaning medium does not impact cellular activity on its own, antiviral activity can only be affected by treatments from SURFORMA antiviral laminates.

M4 Kinetic analysis of SURFORMA antiviral laminates antiviral activity

2 SURFORMA laminates were analysed: i) SURFORMA antiviral laminate (SAvL) and ii) SURFORMA antiviral laminate with a 5-year equivalent aging period treatment (SAv5L). Droplets containing approximately 10^5 viral particles were inoculated in each laminate at different endpoints: 0, 10, 30, and 240 minutes.

The surfaces were cleaned afterwards with SCDLP to collect the virus. This solution was filtered in a 0,22 µm filter and used to inject the viruses in crops with a density of $2x10^5$ cells/mL grown in 96 well microplates as previously described. Incubation was performed in an incubator with 5% CO_2 at 37ºC for a period of 3 to 4 days or until confluence was reached. After this period, viral load (number of particles) was measured by quantifying the N gene from SARS-CoV-2 through qRT-PCR (quantitative PCR).

The **rate of viral inactivation** from surfaces can be observed in Table 1. This rate was calculated based on the variation in inoculated viral load and the one detected through qRT-PCR after each endpoint (ΔCt parameter). The endpoints analysed in an experimental period of 4 hours were 0, 10, 30, and 240 minutes.

Table 1: Rate of viral inactivation from surfaces

	Tempo 0	10 min	30 min	240 min (4h)
SURFORMA antiviral laminate	**44,8 % ± 17,5%**	**65,5 % ± 10,8%**	**84,0 % ± 4,1%**	**98,8 % ± 2,0%**
SURFORMA antiviral laminate aged 5 years	24,7 % ± 8,5%	57,4 % ± 9,8%	52,2 % ± 9,6%	98,3 % ± 1,7%

The surface which showed a better viral inactivation rate for all tested endpoints was the SURFORMA antiviral laminate without the 5-year ageing period.

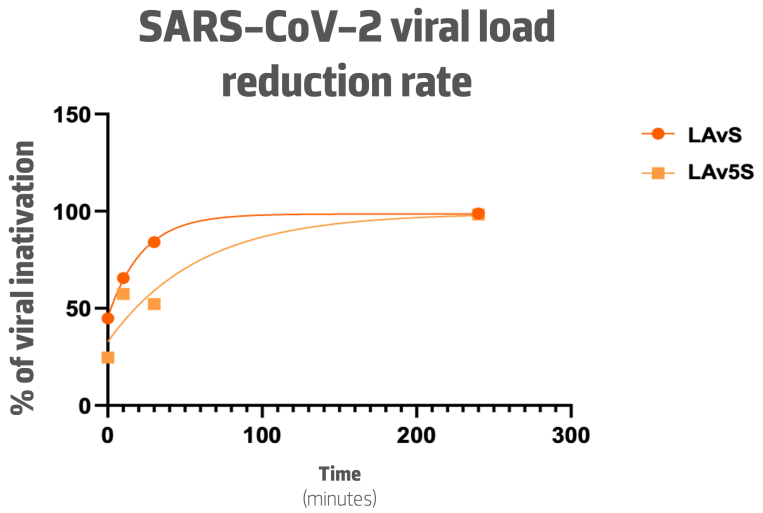


Figure 3: Inactivation curve of virus inoculated in SURFORMA antiviral laminates with and without a 5-year ageing period (SAvL and SAv5L), in the 0 minutes, 10 minutes, 30 minutes, and 240 minutes time period (4 hours).

Explanation

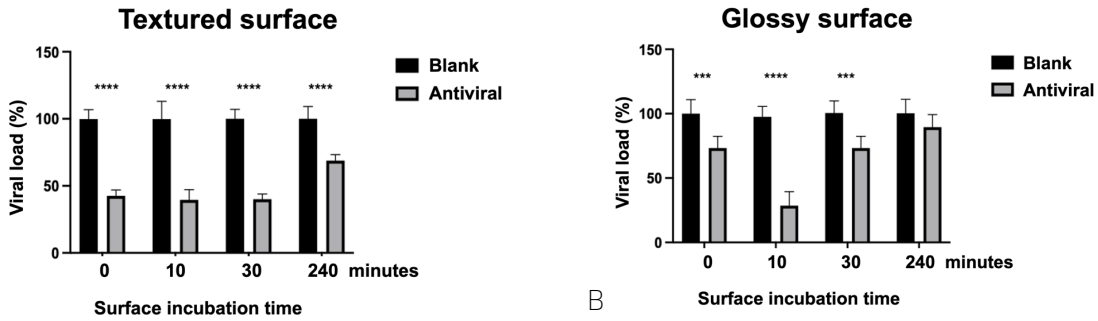
The graph shows that at 4 hours, both SURFORMA laminates can achieve an almost total inactivation of viral particles (98 to 99%). However, non-aged SURFORMA laminates are efficient quicker as antivirals for SARS-CoV-2.

M5 Measurement of viral load through PCR (polymerase chain reaction) in quantitative real time (qRT-PCR) after 0 minutes, 10 minutes, 30 minutes, and 4 hours

Considering the recommended methodology by several health authorities like the World Health Organization for **diagnosing SARS-CoV-2** is the method known as quantitative real-time PCR or qRT-PCR (3), the number of viral particles (viral load) observed through qRT-PCR was also measured after the inoculation of SURFORMA antiviral laminates with 5000 viable viral particles.

This time, non-aged antiviral laminates were used with two types of finish: textured SURFOMA antiviral laminates (TAvL) and with a glossy finish (GAvL).

SURFORMA antiviral laminates were inoculated with 5000 particles of SARS-CoV-2. After an exposure period, viral load was measured through qRT-PCR. The laminates exposure period to SARS-CoV-2 was 0 minutes, 10 minutes, 30 minutes, and 240 minutes after that inoculation. Results were quantified regarding SURFORMA laminates with no antiviral treatment (Figure 4).



A B

Figure 4: A – Anti-SARS-CoV-2 effect from SURFORMA antiviral **textured laminates**. B – Anti-SARS-CoV-2 effect from SURFORMA antiviral **glossy laminates**.
Statistics: t-student test. Significance level (95%): ***: p<0.001; ****: p<0.0001

Explanation

The graph shows that the textured surface presents a highly significant viral load decrease on all initial (0 minutes, 10 minutes, and 30 minutes) and later (4 hours) endpoints. Despite a reduction in viral load, the glossy surface shows an also significant but lower efficiency.

Discussion

The survivability of respiratory viruses on surfaces is known. Respiratory viruses can reach surfaces through aqueous droplets. It is also known that when the droplet containing the respiratory viruses dries, viruses quickly become inactivated (2). Rate of activation depends on factors like viral strain, type of surface, size of the droplet, climate, and environmental conditions such as sun exposure, temperature, humidity, if it is a closed or open environment, frequency of surface hygienization / disinfection and its porosity / impermeability (4).

Therefore, an assessment of 3 surfaces was conducted according to the recommended methodological parameters in the ISO 21702:2019 norm (Measurement of antiviral activity on plastics and other non-porous surfaces), chosen because it matched the coatings on these surfaces since they are non-porous materials (Figure5).

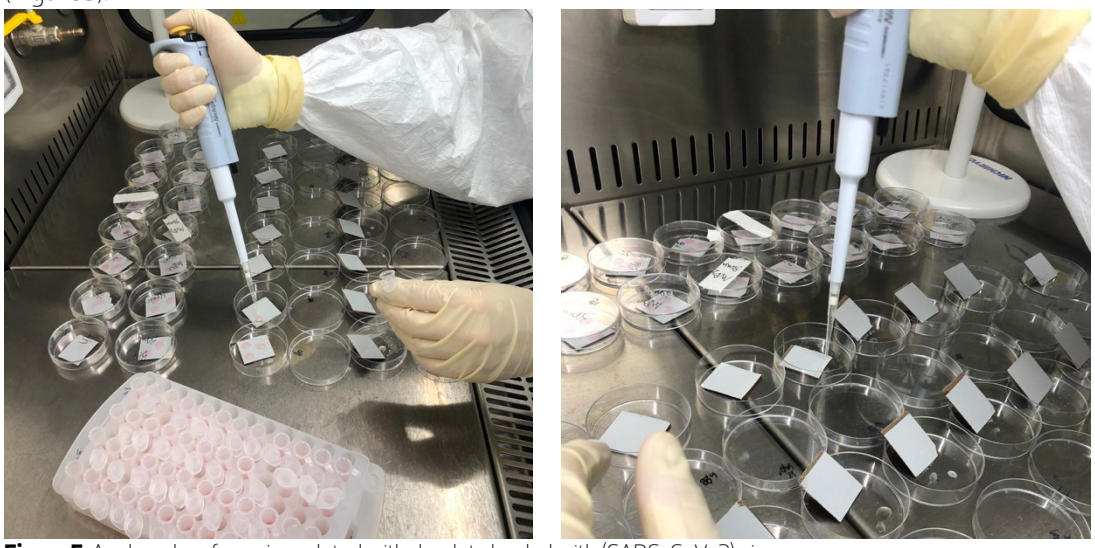


Figure 5: Analysed surfaces inoculated with droplets loaded with (SARS-CoV-2) virus.

Within this norm, **adaptations were made** to adjust to the objectives of surface application. Instead of being assessed multiple times or at 24 hours, the endpoints were measured in a maximum period of 4 hours to do an **accurate assessment of the decaying process of the virus biologic activity;** another adaptation was the measurement method of viral particles strength, since this was performed through **qRT-PCR** for the SARS-CoV-2 N gene (considered the most sensitive gene for viral load quantification purposes).
In conclusion, every **SURFORMA** antiviral laminates meet the **biosecurity/low toxicity** criteria (previously assessed in the first research). Furthermore, the cleaning mediums proposed by the norm do not interfere with cellular (M2) or viral activity (M3). In this context, surfaces that showed better viral inactivation to all the tested endpoints (M4/M5) were the non-aged, textured laminates. Overall, all end up converging to a near-total inactivation at 4 hours.

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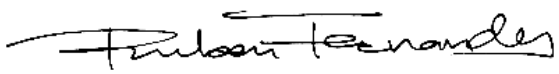

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