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Short communication

Evaluation of formulations to improve SARS-CoV-2 viability and thermostability after lyophilisation

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ABSTRACT

In the context of the COVID-19 pandemic, virus collections such as EVA-GLOBAL play a key role in the supply of viruses and related products for research. Freeze-drying techniques for viruses represent a method of choice for the preservation of strains and their distribution without the need for a demanding cold chain. Here, we describe an optimised lyophilisation protocol usable for SARS-CoV-2 strains that improves preservation and thermostability. We show that sucrose used as an adjuvant represents a simple and efficient stabilizer providing increased protection for long-term preservation and shipment of the virus under different climatic conditions.

A novel Coronavirus called Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2) emerged in late 2019 in China and spread worldwide causing COVID-19 (coronavirus disease 2019) pandemic. In this context of public health emergency, there was a need to gather as much information as possible to understand the biology of the virus and develop appropriate control methods.

The European Virus Archive (EVA-GLOBAL) is a non-profit consortium of virology laboratories aiming to provide the scientific community with easy access to reference virus material. EVA-GLOBAL has provided a strong support to the international response during COVID-19 outbreak, by rapidly offering relevant clinical strains and molecular diagnostic tools (EVA Zika Workgroup et al., 2020). As a core partner of EVA, we developed methods for long term preservation and worldwide distribution of viral material (Pastorino et al., 2015; Romette et al., 2018). Lyophilisation preserves virus infectivity for several years (Adams, 2007) and allows cost effective transportation at ambient temperature (Hansen et al., 2015; Malenovská, 2014). To improve the stability during lyophilisation and storage, appropriate stabilizing substances (Kang et al., 2010) are required to limit viral degradation under environmental stresses that could occur both in the freezing and the sublimation stages (Zhai et al., 2004).

Here, we present a robust freeze-drying method for preservation of infectious SARS-CoV-2 strains. We describe our standard lyophilisation protocol and evaluated the ability of four common chemical stabilizers (sucrose, sorbitol, trehalose and polyethylene glycol (PEG), (Chen et al., 2020; Pastorino et al., 2015)) to preserve the infectivity of SARS-CoV-2 viability at different temperatures and time points.

The SARS-CoV-2 clinical strain used in the present study is called 2020/FR/702 (Lineage B.1; GB: MT777677) and is available to the scientific community via EVA-GLOBAL project (www.european-virus-archive.com, ref: 001V-03967).

The virus was first propagated on Vero E6 cells (ATCC#CRL-1586). Virus stock was diluted at an MOI of 0.01 to infect Vero E6 cells. After overnight incubation at 37 °C, the medium was changed, and incubation was continued for 24 h. Supernatant was collected, clarified by spinning at 1500× g for 10 min, supplemented with 25 mM HEPES (Sigma), and stored at −80 °C before lyophilisation.

The day of the freeze-drying run, viral supernatant was mixed with the stabilizing substances to obtain four solutions of 0.2 M sucrose, 2% sorbitol, 10% trehalose (Sigma, France) or 1% PEG 8000 (Fischer Scientific) respectively. Viral supernatant supplemented with cell culture medium (MEM + 5% FBS) was used as control.

Lyophilisation was carried out in a BSL3 laboratory using a CHRIST gamma 1–16 LSC freeze-dryer. For each of the five aforementioned mixture, a 200 µL volume was dispensed in each glass vial. The cap was applied loosely to each vial, which was frozen on a cooling plate (previously stored at −80 °C) and transferred to the freeze-drying cabinet pre-cooled at −25 °C.

Freeze-drying process was initiated by vacuuming the cabinet. When the barometric pressure gauge reached 1.03 mBar, the temperature was gradually increased during 4 h until it reached −10 °C. The freeze drying process continued inside the cabinet until the barometric pressure was 0.006 mBar. The temperature was gradually raised to +20 °C during 6 h.

The process was achieved by pressing the caps into vials whilst they

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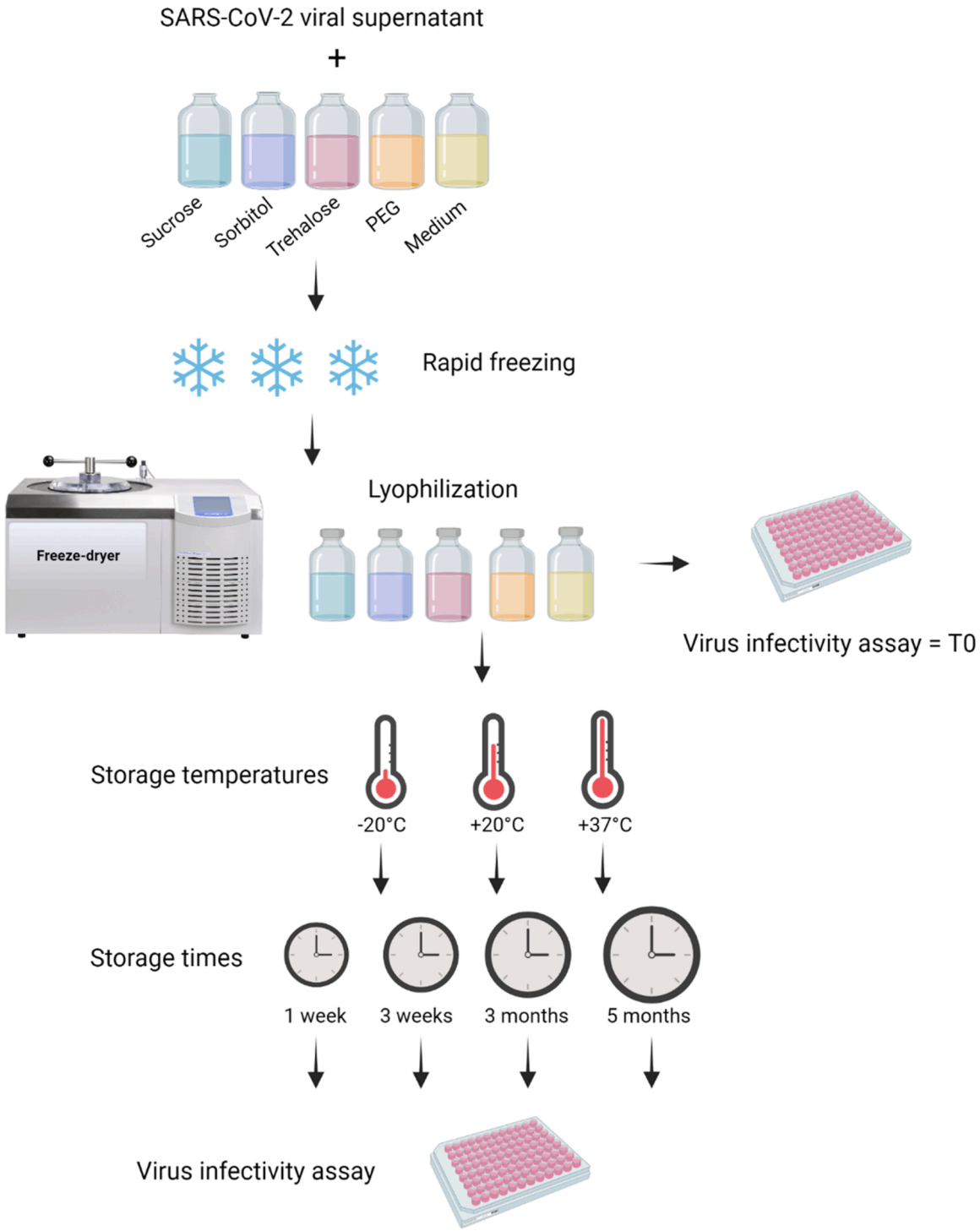

Fig. 1. Graphical representation of the experimental process. Created with BioRender.com.

were still under vacuum. Each cap was crimped with a metal capsule on the silicone cap. Resulting vials were sealed and thus suitable for safe inspection and transportation.

Consequently, separate freeze-dried virus vials were exposed to different storage temperatures in a light-protected environment for various times (Fig. 1). Vials were then rehydrated with 200 µL of sterile distilled water before virus titration as described below. Each conditions (Stabilizers and Temperature) was tested in duplicate.

Virus infectivity was measured using a 50% tissue-culture infectivity dose ($TCID_{50}$). Briefly, Vero E6 cells at 90% confluence were infected with 150 µL of tenfold serial dilutions of the virus sample in six replicates and incubated for 4 days at 37 °C under 5% CO_2. Cytopathic effect (CPE) was read using an inverted microscope, and infectivity was expressed as $TCID_{50}$/mL on the basis of the Karber formula (REED and MUENCH, 1938). All the experiments were performed in BSL3 facilities.

Firstly, the infectivity of SARS-CoV-2 was quantified just after lyophilisation (T0) for each formulation and was compared with the frozen virus in order to evaluate the loss of infectivity after lyophilisation (supplemental Table 1). Regardless the nature of stabilizer, the loss of infectivity was constantly lower than one log. The assays revealed the protective property of our lyophilisation protocol on SARS-CoV-2.

Freeze-dried virus vials were stored at three different temperatures:

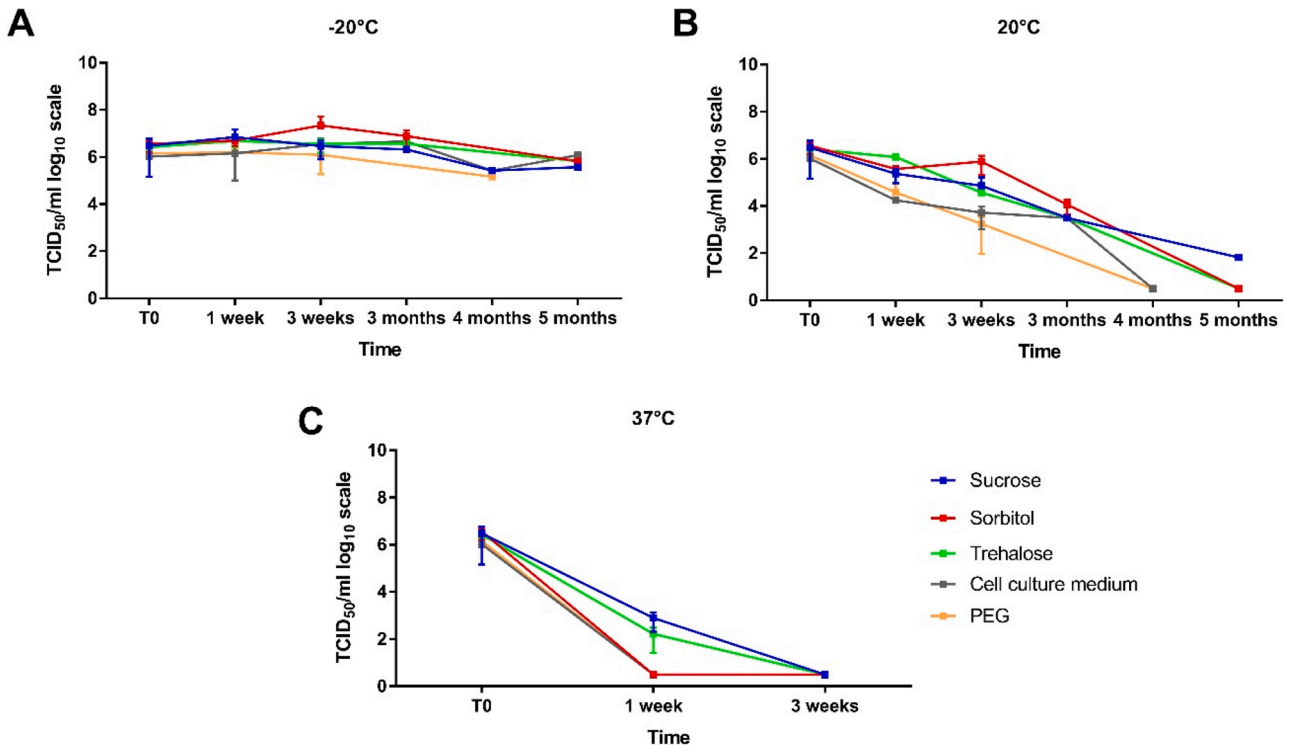

Fig. 2. SARS-CoV-2 infectivity overtime after lyophilisation with different stabilizers.
A. SARS-CoV-2 infectivity at −20 °C. B. SARS-CoV-2 infectivity at 20 °C (Room temperature). C. SARS-CoV-2 infectivity at 37 °C. Data represent mean ± SD of a quadruplicate. Titers are expressed as $TCID_{50}$/mL.

−20 °C, room temperature: +20 °C, and +37 °C. The viability of the freeze-dried SARS-CoV-2 was evaluated at different time points: one week, 3 weeks, 3 months and 5 months and compared to the T0.

We observed a relative thermostability of the SARS-CoV-2 up to 5 months under cold chain conditions (−20 °C) for sucrose, sorbitol and trehalose (Fig. 2A). However, the titre of freeze-dried SARS-CoV-2 seemed to decrease slowly, around one log, over time when associated with PEG as previously described (Carpenter et al., 1993; Hansen et al., 2015).

At room conditions (19 °C–21 °C temperature range and 45%–55% relative humidity), the titre of SARS-CoV-2 remained quite stable for one week except with PEG and in the absence of stabilizing substance (two logs decrease). Between three weeks and five months of storage at room temperature, the virus infectivity of the freeze-dried SARS-CoV-2 diminished drastically, independently of sample formulation. By month five, all formulations resulted in an undetectable infectious titre, except with the sucrose (Fig. 2B). Indeed, virus combined with sucrose remained detectable with a low infectious titer of 6.67E + 01 $TCID_{50}$/mL, after five months at room temperature, allowing virus recovery. This observation also demonstrated that all stabilizing substances tested didn't protect the virus from lyophilisation anymore.

We then explored the stability of freeze-drying formulations under unfavourable conditions such as high temperatures. For this purpose, we stored the vial at 37 °C. We were able to recover replicative virus when associated to sucrose or trehalose with a 3 log reduction of the infectious titre at one week (Fig. 2C). In contrast, at the same time point, when associated with PEG, sorbitol or without stabilizing substance, the infectivity of the virus was totally impaired with no detectable infectious titre. At three weeks, they were no more replicative virus in all conditions (Fig. 2C) making unnecessary to test others time points.

For a long-term storage at −20 °C, all conditions have shown similar results meaning that at this temperature there is no need to stabilizer formulation optimization. However, at standard room conditions which is 19 °C – 21 °C temperature with 45%–55% relative humidity, as recommended for indoor living spaces by the American Society of Heating, Refrigeration and Air Conditioning Engineers, there is substantial differences. Only sucrose allows the recovery of infectious replicating viral particles after a five months storage. Sucrose was also associated with a substantial protection for the storage at unfavorable conditions such as high temperatures as previously shown by Stewart et al. for Adenovirus (Stewart et al., 2014). This sucrose protective effect is still unknown but one common explanation could be a vitrification of the aqueous phase which generate a glassy state. The viral particle are held inside the glass in a form of "stasis" where they are more efficiently protected from the environmental conditions (Leopold et al., 1994). Moreover for a technical point of view, the sucrose "glassy state", results in a more compact powder after lyophilisation, making a more accurate and easy resuspension before propagation.

Sucrose seems to be the most suitable stabilizing substance for the SARS-CoV-2 lyophilisation. In the framework of EVA-GLOBAL collection developed at Unité des virus émergents (UVE) lab, sucrose is used as additive substance for the lyophilisation of various categories of viruses. As previously demonstrated (Bovarnick et al., 1950; Howell and Miller, 1983; Tannock et al., 1987), sucrose represents a powerful stabilizer leading to a better protection for long term preservation and transportation of viruses, which are essential in the development of a viral collection and its worldwide distribution. This work is a useful contribution regarding SARS-CoV-2 stability. Indeed, we demonstrated that our protocol is perfectly suitable for cold chain free shipment whatever climatic conditions encountered during the transport.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2021.114252.

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