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Research Article

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Application and comparison of lyophilisation protocols to enhance stable long-term storage of filovirus pseudotypes for use in antibody neutralisation tests

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Abstract

Aims: Filoviruses encompass highly pathogenic viruses placing significant public health burden on countries affected. Efforts for improved diagnostics and surveillance are needed. The requirement for high-containment can be circumvented by using pseudotype viruses (PV), which can be handled safely, in tropism, drug screening, vaccine evaluation, and serosurveillance studies. We assessed the stability and functionality after long-term storage of lyophilised filovirus pseudotypes for use in neutralisation assays.

Methods and results: We generated a panel of filovirus lentiviral pseudotypes followed by lyophilisation and storage in different conditions. Next, we reconstituted and tested PVs in infection experiments and pseudotype neutralisation assays where possible. Lyophilised Ebola and Marburg PVs retained production titres for at least two years when stored at +4°C or less. Lyophilised Ebola PVs performed similarly to non-lyophilised PVs in neutralisation assays after reconstitution. When stored at high temperatures (+37°C), lyophilised PVs did not retain titres after 1-month storage, however, when lyophilised using pilot-scale facilities EBOV PVs retained titres and performed as standard in neutralisation assays after on 1-month storage at 37°C.

Conclusions: Filovirus PVs are amenable to lyophilisation and can be stored for at least 2 years in a household fridge to be used in antibody assays. Lyophilisation performed in the right conditions would allow transportation at room temperature, even in warmer climates.

Significance and impact of study

Lyophilisation allows reagents to be transported more efficiently as well as reducing costs for a future serological kit in low-resource countries. This technology can be applied to emerging viruses of public health importance.

Keywords: lyophilisation, pseudotypes, filovirus, neutralisation assay, serosurveillance

Introduction

Filoviruses have been responsible for several serious disease outbreaks within resource-limited countries, which have posed challenges for the implementation of appropriate public health measures. These sporadic Ebola and Marburg virus outbreaks culminated in the large epidemic in West Africa in 2013–2016, highlighting the need for better serosurveillance, diagnostics, containment measures, treatments, and vaccines (Languon and Quaye 2019).

The gold standard for diagnostics of filoviruses is viral RNA detection via RT-qPCR, which has high sensitivity and specificity but requires user expertise and expensive equipment (Weidmann et al. 2004, Broadhurst et al. 2016, Cherpillod et al. 2016). Several approaches for point-of-care use are being evaluated. Some of these platforms such as RT-PCR based GeneXpert require minimal training and no sample pretreatment (Semper et al. 2016, Vuren et al. 2016, Raftery et al. 2018). Portable lateral flow devices for antigen detection are also being evaluated as more affordable options. These exhibit varying degrees of sensitivity, which would have to be addressed before being rolled out (Phan et al. 2016, Makiala et al. 2019, Wonderly et al. 2019). More recently, genomic approaches, including next-generation sequencing platforms have been employed for diagnostics, as well as monitoring geographical spread and adaptations as an epidemic progresses (Gire et al. 2014, Gardy and Loman 2018, Deng et al. 2020). They have the advantage of detecting as yet unidentified pathogens as well as avoiding "signature-erosion" where mutations occur in primer targets resulting in false negative or positive results (Sozhamannan et al. 2015, Deng et al. 2020).

Serological evaluation is also important to map the geographical distribution of pathogens as well as assessing vaccine responses and community impact. Ebola virus (EBOV) serological surveys have been conducted more frequently due to the fact most of the human outbreaks are caused by EBOV (Mulangu et al. 2018, Brook et al. 2019). However, individuals with antibodies against the Marburg virus have been detected in locations in West and Central Africa with no previous history of Marburg virus outbreaks (Steffen et al. 2020). In

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August 2021 the first case of Marburg virus disease (MVD) was confirmed in Guinea (WHO). After a rapid public health response, further cases have not been reported and in September 2021 the end of the outbreak was declared. Serological studies could be very useful should an unidentified disease outbreak occur, as unusual locations can hinder efforts to identify such outbreaks, such as in the large EBOV outbreak in West Africa.

Serosurveillance of bats is equally important considering they are potential reservoirs for EBOV, raising the possibility of zoonotic spillover events (Laing et al. 2018, Nys et al. 2018). In the past decade, filovirus RNA has been detected in bats in Europe. These were designated as a new genus, *cuevavirus*, with a sole species, Lloviu (LLOV) virus, which recently re-emerged in bats in Hungary. Vesicular stomatitis virus (VSV) particles pseudotyped with the LLOV surface glycoprotein (GP) were shown to infect human cells *in vitro* (Negredo et al. 2011, Maruyama et al. 2014, Kemenesi et al. 2018, De Arellano et al. 2019); and recently isolation of infectious LLOV from an asymptomatic Schreiber's bat was achieved. Monkey and human cell lines were permissive to LLOV (Kemenesi et al. 2022). Consequently, monitoring the distribution of different filovirus species in animals is important as they may pose the potential for a future spillover into humans.

Pseudotypes are chimeric non-replicative viruses encoding a reporter gene and bearing the GP of interest on its envelope. The use of pseudotyped virus particles has several advantages when studying highly pathogenic viruses, as they can be handled in low-containment facilities, often yield high production titres permitting upscaled use, can be multiplexed for assaying different viruses, and can be adapted for high-throughput screening. In addition, there is a range of reporter genes that can be incorporated, as well as being highly sensitive in neutralisation assays (Wright et al. 2008, Scott et al. 2012, Mather et al. 2013, Long et al. 2015, Temperton et al. 2015, Ferrara and Temperton 2018) exhibiting strong correlation to the native study virus (Konduru et al. 2018). They have been used in tropism (Goldstein et al. 2018), vaccine evaluation (Ewer et al. 2016), antiviral screening (Xiao et al. 2018) studies amongst others.

Most of the assays and methods described so far require high-power (−70/80°C) freezers for virus storage and expensive transportation requirements to maintain a cold chain to other laboratories or in-field facilities. Alternative methods, involving more modest temperature requirements for reagents would be advantageous for accessibility and cost. One solution to reduce those costs would be to use lyophilised reagents whenever possible, especially if these are to be sent to and used in tropical regions with high temperature and humidity. Lyophilisation or freeze-drying has been used in production of pharmaceutical products, such as vaccines, to avoid the need for cold chain transportation and to increase shelf life of reagents (Kraan et al. 2014). Lyophilisation usually consists of two steps: freezing of the sample followed by drying in a low-pressure environment, whereby frozen water in the sample sublimates in the first drying step (primary drying) and unfrozen water evaporates in the second drying step (secondary drying). The secondary drying step is performed at a higher temperature (20–40°C) to eliminate moisture (Wang 2000, Nireesha et al. 2013, Kraan et al. 2014). For most of the current proof-of-concept study described here, only the primary drying step was performed using a standard laboratory freeze-dryer. In addition, an independent proof-of-concept study using a pilot scale freeze-drier was conducted at Intravacc (The Netherlands), incorporating an additional drying step to determine the reproducibility of the methodology and test the potential benefits of the extra drying procedure.

Cryoprotectants are routinely added to samples prior to freeze drying in order to protect the integrity of the substance being lyophilised. These excipient formulations suitable for freeze-drying are commonly prepared with sugars such as sucrose, trehalose, and sorbitol dissolved in various buffer solutions (Wang 2000) and have been applied to the freeze-drying of viruses, including recombinant adenoviruses and lentiviruses (Shin et al. 2010). We previously assessed the use of sucrose as a cryoprotectant for lyophilising pseudotyped viruses (PVs of influenza, rabies, and Marburg viruses), followed by storage for up to 1 month at different temperatures and humidity conditions (Mather et al. 2014). PV titres were shown to be maintained in infectivity assays after resuspension of lyophilised pellets. Marburg virus PV titre recovery was near 100% in temperatures up to +20°C after 1-month storage at +20°C, as well as influenza and rabies PVs. In addition, reconstitution of the pellets with either cell culture medium or distilled water made no significant difference in these tests. Reconstituted influenza and rabies PVs continued to perform well in antibody neutralisation assays, where convalescent sera were available (Mather et al. 2014).

In this study, we aimed to assess stability (including air transport) of lyophilised filovirus PVs after significantly extended storage periods and, in addition, to test functionality in neutralisation assays.

Materials and methods

Cells

HEK293T/17 cells were maintained in Dulbecco's Modified Eagle Medium DMEM supplemented with 10% (v/v) foetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin (Pan Biotech), hereby referred to as 'complete medium'. Cells were kept at 37°C and 5% CO_2.

Plasmids and pseudotype production

The pseudotypes used in this study were EBOV (Makona C15 Genebank accession number KJ660346), LLOV (Genebank accession number JF828358), and RAVV (Genebank accession number DQ447649). GP genes were encoded in the pCAGGS expression vector.

PVs were generated in T75 flasks (Thermo Scientific) by 3-plasmid transfection protocol: 1 μg GP (300 ng GP for EBOV), 1.5 μg pCSFLW luciferase reporter (Demaison et al. 2002), 1 μg p8.91 HIV-1 gag-pol (Zufferey et al. 1997), using polyethylenimine (PEI; Sigma) at a ratio of 1:10 DNA: PEI in producer HEK293T/17 (ATCC CRL-11268) cells. Supernatants were harvested 48 h post transfection and kept at −80°C.

Lyophilisation reagents and equipment

Sucrose (Sigma–Aldrich 84097–250 G) was used as a cryoprotectant during lyophilisation. A stock solution was prepared to the desired final concentration in Dulbecco's Phosphate-Buffered Saline (Pan Biotech).

Low surface-tension polypropylene 1.5 mL microtubes (Simport, Canada T330-7LST) were used to prepare and lyophilise PV samples.

Unless indicated otherwise, lyophilisation was carried out in a FreeZone 2.5 L freeze-dryer (Labconco, USA), connected to a vacuum pump (Rotary Vane 7 739 402), except additional EBOV samples, which were prepared in Sucrose-DPBS cryoproctectant in the Viral Pseudotype Unit (VPU) in Kent, frozen by placing into at a −80°C freezer and shipped on dry ice to Intravacc (Bilthoven, The Netherlands) for lyophilisation in a Telstar Lyobeta freeze dryer, which includes additional pellet drying which reduces moisture retention, routinely used at Intravacc for commercial vaccine preparation. This allowed for the comparison between simple lab-based and pilot scale lyophilisation processes.

Convalescent serum samples for antibody studies were obtained from the National Institute of Biological Standards and Control (NIBSC code 15/262).

Lyophilisation and storage of PVs

PV supernatant of known Relative Light Units (RLU) mL $^{-1}$ titre was mixed with 1 mol L^{-1} Sucrose-DPBS solution in to a total volume of 200 μL at a 1:1 (v/v) ratio in a low-surface tension microtube, vortexed to mix contents, centrifuged briefly, and placed at −80°C overnight. A needle-pierced lid was placed on top of each tube before freeze-drying to let vapour escape during pressure change in the lyophilisation process. The lyophilisation cycle was run overnight at −40°C to −50°C with pressure dropping to <0.033 mBar (3.3 Pa).

After lyophilisation, pierced lids were removed and the low-surface tension tubes' own lids were closed before the freeze-dried samples were placed in experimental storage.

These storage conditions were: −20°C, +4°C, ambient temperature ∼+22.5°C, +37°C (<25% humidity)-, and + 37°C (90% humidity). Temperature and humidity were monitored regularly in the different storage containers with a Fisherbrand Traceable Jumbo Thermo-Humidity Meter (Fisher Scientific 11 536 973).

Reconstitution of lyophilised pellets was carried out in 100 μL of complete medium before titration or use in neutralisation assays.

For the lyophilisation performed at Intravacc, EBOV PV supernatant was mixed with 1 mol L^{-1} Sucrose-DPBS solution at a 1:1 (v/v) ratio, placed at −80°C overnight and shipped by air on dry ice, remaining frozen on receipt ∼24 h later. EBOV samples lyophilised at VPU were also sent in the same shipment box, and stored at −80°C at Intravacc, then returned with the newly lyophilised samples to assess whether the journey had an impact on titre retention.

The materials used by Intravacc were as follows: glass vials (APG Packaging 1 003 201), autoclaved in-house before use. Rubber stoppers (APG Packaging 1 008 739), in-house dried overnight at 105°C. Samples were thawed at RT. Forty glass vials were filled with 200 μL of PV + Sucrose and half-stoppered before loading in the Telstar Lyobeta freeze dryer. The sample vials were surrounded with empty vials in a metal fork.

After freeze-drying, the vials were fully stoppered in the freeze dryer, still under a pressure of 20 μbar. Subsequently, the vials were capped sealed with an aluminium cap.

A summary of the lyophilisation cycle is provided (Table 1) including the temperatures monitored during the cycle.

All lyophilised samples were transported back to the VPU by air. Lyophilised EBOV PV samples were then stored at −20°C, +4°C, +22.5°C, +37°C (<25%), and +37°C (90%) for an initial 1-month period, and some for 6 months, at +22.5°C and +37°C (<25%) to compare titre retention between lab and pilot scale lyophilisation processes.

Infectivity and neutralisation assays

Infectivity and neutralisation assays were performed as previously described (Temperton et al. 2007, Wright et al. 2008, Ferrara and Temperton 2018). Briefly, PV supernatant was added (100 μL/well) to a white, flat-bottom, sterile Nunc 96-well microplate (Thermo Scientific 10 072 151) and serially diluted 2-fold. Target cells (2 × 10^4/well in 50 μL) were added and incubated for 48 h at 37°C, 5% CO_2. After 48 h, the media was removed and discarded; Bright-Glo reagent (Promega) was added to the plate and incubated at room temperature for 5 min before measuring luminescence on a GloMax 96 luminometer (Promega).

In antibody neutralisation assays, a 2-fold serial dilution of serum was conducted in duplicate, in white, flat-bottom, sterile Nunc 96-well microplates, at a starting dilution of 1:40 and incubated with ∼100 000 RLU of PV at 37°C, 5% CO_2 for 1 h to allow the antibodies to bind to the GP. A PV only control (0% neutralisation equivalent) with no serum and cell only wells (100% neutralisation equivalent) was also set up. Target cells (2 × 10^4/well) were added and incubated for 48 h at 37°C, 5% CO_2. After 48 h, the plate was read as previously described.

The data were normalised to the percentage reduction in luminescence according to the average Relative Light Units (RLU) of the cell only (100% neutralisation) and PV only (0% neutralisation) controls and fitted into a non-linear regression model (log [inhibitor] vs. normalised response—variable slope) to interpolate the inhibitory concentrations at 50% (IC_{50}), that is, the reciprocal of the dilution at which 50% of PV cell entry was inhibited.

Titre recovery of lyophilised PVs was calculated in comparison to their unlyophilised counterparts.

Statistical analyses

All graphs and statistical analyses were performed using Graphpad Prism. The Mann–Whitney test was used to compare the titre difference between two groups assuming a non parametric distribution.

Results

PV production titres

Typical titres of ∼1 × 10^8 RLU mL^{-1} were measured for EBOV and LLOV PV supernatants, and ∼1 × 10^{10} RLU mL^{-1} for RAVV PVs (Fig. 1). Some of these PV supernatants were lyophilised, and the remaining stock used as unlyophilised positive controls, as well as for comparison to calculate titre retention in further experiments.

Long-term storage of lyophilised PVs utilising a standard laboratory freeze-drier

Filovirus PVs lyophilised at the VPU earlier in the study were stored for up to 2 years under various conditions. EBOV PVs retained 90% of their titre when reconstituted after being stored at +22.5°C for 1 month (Fig. 2a), then titres decreased to background levels between 1 and 6 months (Fig. 2b). At

Table 1. Lyophilisation protocol performed at Intravacc.

Freezing Temp (°C)	Primary drying Time (h)	Secondary drying Temp (°C)	Time (h)	Pressure (μbar)	Temp (°C)	Time (h)	Pressure (μbar)
−50	-*	−45	0.5	20	25.5	24	20
−50	2	−45**	96	20	25.5	24	20
		−45**	2	20	4	0.5	20
					4	99 ***	20

* Shelf preparation, prior to loading of the vials
** Pressure rise test (PIM):
Max loops: 10
Extra drying time: 2 h
Allowed pressure rise: 5 μbar
Test time: 60 seconds
*** Storage of the vials at 4°C until the freeze dryer was stopped manually

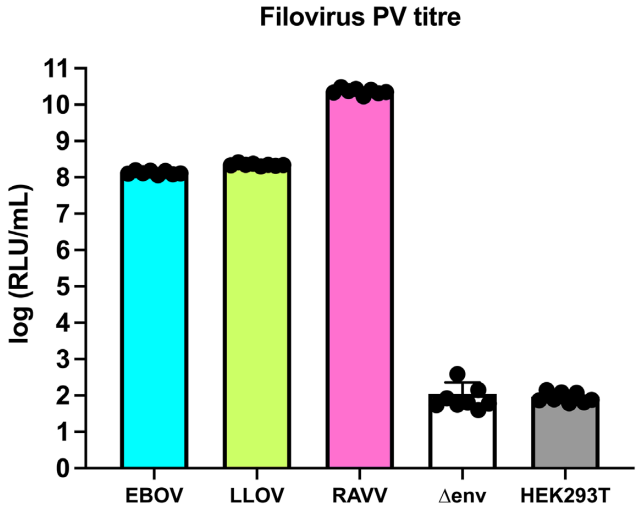

Figure 1. Generation of EBOV, LLOV, and RAVV filovirus PVs. displaying the GPs of EBOV and RAVV. Transduction titres, as measured by PV-mediated luciferase enzymatic activity, are expressed as the (log) mean RLU mL^{-1} ± SD from at least three independent experiments. The titre of lentiviral particles bearing no GP (Δenv) and background luminescence from uninfected cells (HEK293T) are also shown.

higher temperatures, titres decreased to background levels within 6 months (Fig. 2b). Impressively, EBOV PVs retained ∼100% of their titre when reconstituted after being stored at −20°C and +4°C for 2 years (Fig. 2e). EBOV PVs stored at +37°C in dry (<25% humidity) and humid (90% humidity) conditions for 1 month did not generate any functional titres (Fig. 2).

For RAVV (Marburg virus) PVs, >90% of original titres were retained when reconstituted after being stored at −20°C and +4°C for up to 2 years (Fig. 3). They retained 93.9% of their titre when reconstituted after being stored at +22.5°C for 1 month (Fig. 3a), then titre recovery decreased to 69.5% between 1 and 6months (Fig. 3b). At higher temperatures, titres decreased to background level within 6 months (Fig. 3b).

RAVV PVs stored at +37°C in dry (<25% humidity) and humid (90% humidity) conditions did not generate any functional titres (Fig. 3), although titres were slightly higher (∼10^4 RLU mL^{-1}) than background level when stored for only 1 month at +37°C (<25% humidity) (Fig. 3a).

Last, LLOV PVs (from the third filovirus genus) were lyophilised and stored for up to 1.5 years under different conditions. LLOV PVs retained ∼90% of their titre when reconstituted after being stored at −20°C and +4°C for up to 1.5 years (Fig. 4). LLOV PVs retained 84.9% of their titre when reconstituted after being stored at +22.5°C for 1 month (Fig. 4a), then titres decreased to background level between 1 and 6 months (Fig. 4b). PVs stored at +37°C in dry (<25% humidity) and humid (90% humidity) conditions did not generate any functional titres after storage (Fig. 4).

Short-term storage of lyophilised PVs comparing a standard laboratory freeze-drier to pilot scale facilities

A 1-month storage stability assessment was performed. EBOV PVs lyophilised (Labconco freeze dryer) at the VPU were shipped to The Netherlands, kept refrigerated for 2 weeks, then shipped back to the VPU, retained titres after a further 1 month's storage at −20°C and +22.5°C above 90%, however at higher temperatures titres were dropped to background levels (Fig. 5a—blue). A temperature of +4°C was not tested, as retention at this temperature did not differ greatly from samples stored at −20°C in previous lyophilisation tests.

By contrast, EBOV PV samples lyophilised (Lyobeta pilot scale freeze dryer) at Intravacc withstood storage at high temperatures of +37°C in dry or humid conditions for at least a month (Fig. 5a—green), with 86.1% of titre recovered after being stored at +37°C (<25% humidity) and 87% of titre recovered after being stored at +37°C (90% humidity). This represents a significant increase ($P < 0.01$) in stability and recovery when compared to the EBOV PV samples lyophilised in the VPU Labconco freeze-dryer (Fig. 5a).

Samples lyophilised at Intravacc stored at −20°C, ambient temperature (+22.5°C) and +37°C (<25% humidity) were further assessed after 6 months (Fig. 5b). For PV samples stored at lower temperatures (−20°C and +22.5°C) titre retention was above 89.4%, however for samples stored at +37°C (<25% humidity) PV titres had decreased to background (HEK293T) levels (Fig. 5b).

Pseudotype neutralisation assay (PVNA) with lyophilised samples

Following demonstration that lyophilised, stored, and reconstituted PVs retained ability to transduce target cells, EBOV samples were then tested for biological functionality in antibody neutralisation assays.

EBOV PVs lyophilised in Intravacc's Telstar Lyobeta freeze dryer then stored at −20°C, +37°C (<25%) and +37°C (90%) were reconstituted to be used as input in Pseudotype Virus Neutralisation Assay (PVNAs) (Fig. 6a) against pooled convalescent EBOV serum (WHO standard NIBSC 15/262) to

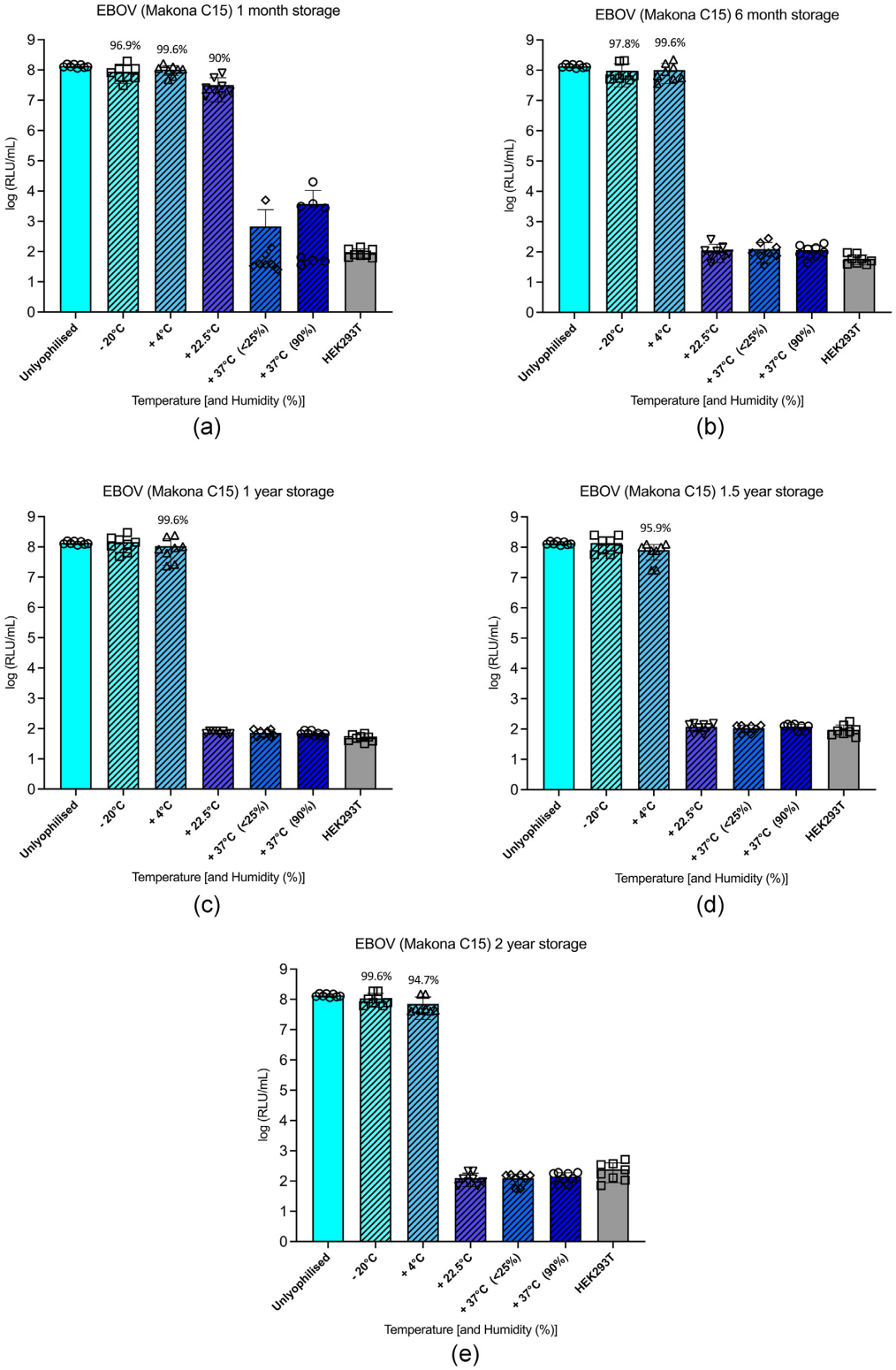

Figure 2. Infectivity assay following long-term storage of lyophilised EBOV PVs. Lyophilised PVs were stored at different temperatures and humidity conditions (<25% or 90%) for (a) 1 month, (b) 6 months, (c) 1 year, (d) 1.5 years, and (e) 2 years. Unlyophilised EBOV PVs were positive controls for the assay, as well as a parameter for comparison to calculate titre retention after lyophilisation, storage and reconstitution. Transduction titres are expressed as the (log) mean RLU/mL ± SD from at least two independent experiments and % titre retention for functional titres are displayed on top of each bar if <100%, for those samples with measureable titre. Background luminescence in uninfected cells (HEK293T) is also shown.

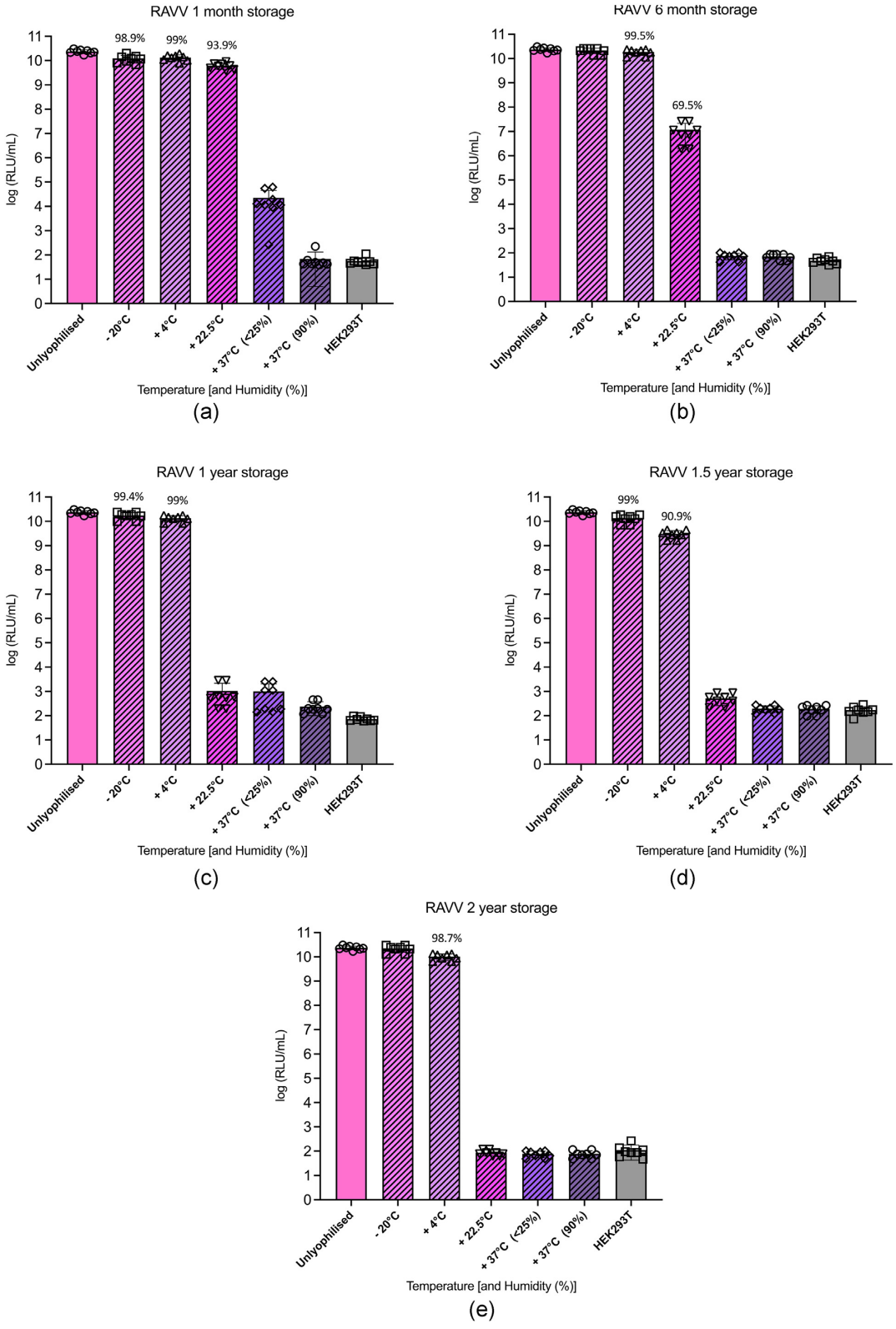

Figure 3. Infectivity assay following long-term storage of lyophilised RAVV PVs. Lyophilised PVs were stored at different temperatures and humidity conditions (<25% or 90%) for (a) 1 month, (b) 6 months, (c) 1 year, (d) 1.5 years, and (e) 2 years. Unlyophilised RAVV PVs were positive controls for the assay, as well as a parameter for comparison to calculate titre retention after lyophilisation, storage and reconstitution. Transduction titres are expressed as the (log) mean RLU/mL ± SD from at least two independent experiments and % titre retention for functional titres are displayed on top of each bar if <100%, for those samples with measureable titre. Background luminescence in uninfected cells (HEK293T) is also shown.

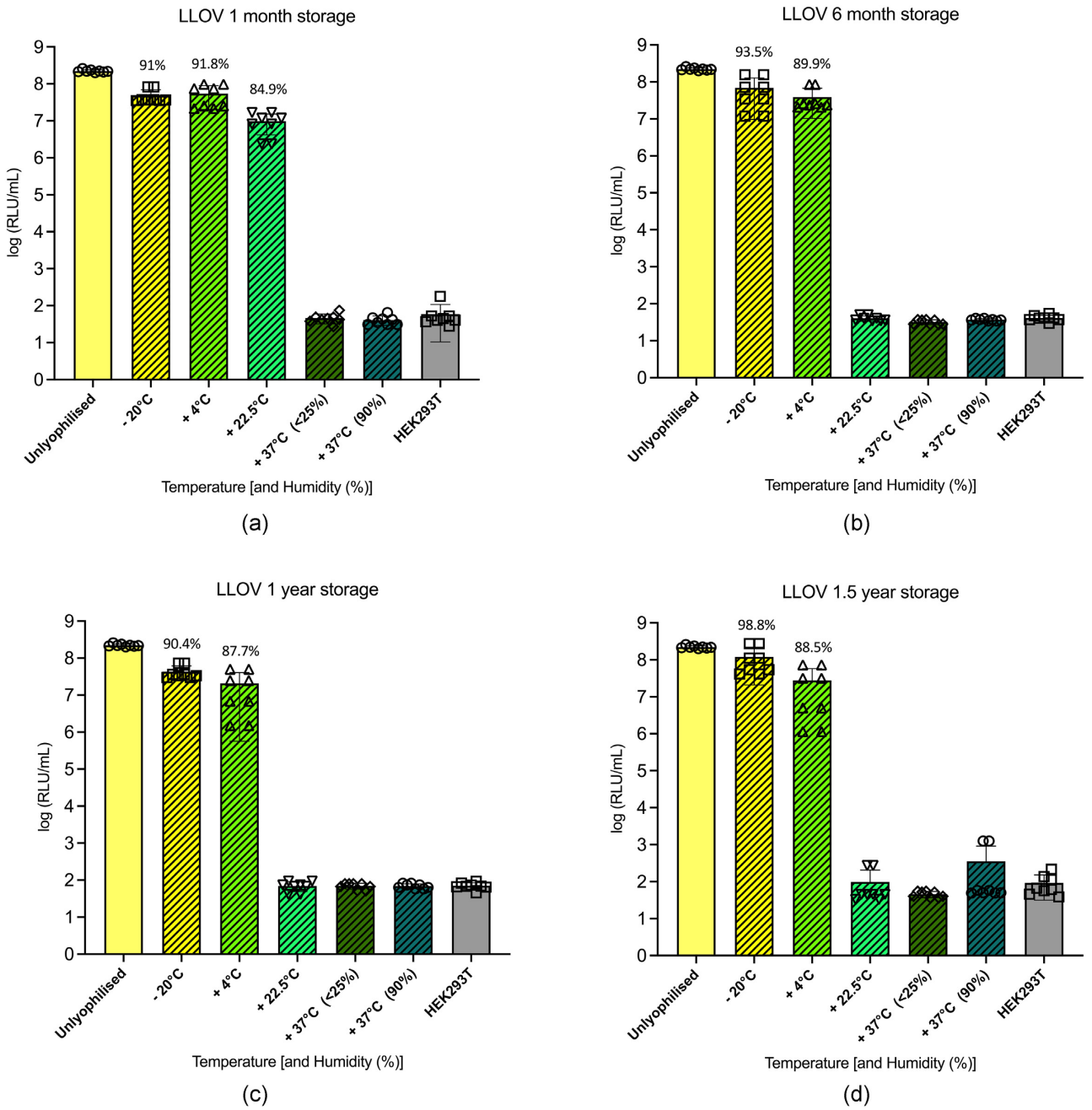

Figure 4. Infectivity assay following long-term storage of lyophilised LLOV PVs. Lyophilised PVs were stored at different temperatures and humidity conditions (<25% or 90%) for (a) 1 month, (b) 6 months, (c) 1 year, and (d) 1.5 years. Unlyophilised LLOV PVs were positive controls for the assay, as well as a parameter for comparison to calculate titre retention after lyophilisation, storage, and reconstitution. Transduction titres are expressed as the (log) mean RLU/mL ± SD from at least two independent experiments and % titre retention for functional titres is displayed on top of each bar if <100%, for those samples with measureable titre. Background luminescence in uninfected cells (HEK293T) is also shown.

compare performance with earlier unlyophilised PVNA data. EBOV PVs lyophilised in the Labconco freeze-drier that had been stored for 1.5 years at 4°C then reconstituted and used as the PV input in PVNA (Fig. 6b).

PVs that had been lyophilised using both freeze drying systems were neutralised by antibodies present in the convalescent test serum as evidenced by the reduction in luciferase activity (Fig. 6). IC_{50} values seen were comparable to those previously observed for unlyophilised PVs .

Discussion

The need to improve diagnostics and serological tests for emerging diseases was illustrated during the large EBOV outbreak in West Africa in 2013–2016 (Gatherer 2014, Formella and Gatherer 2016, Murphy 2019), and more recently during EBOV outbreaks in the DRC and Guinea with over 50 people affected as well as the first case of MARV reported in West Africa in 2021 (Centers for Disease Control and Prevention). Other emerging diseases such as measles in the DRC,

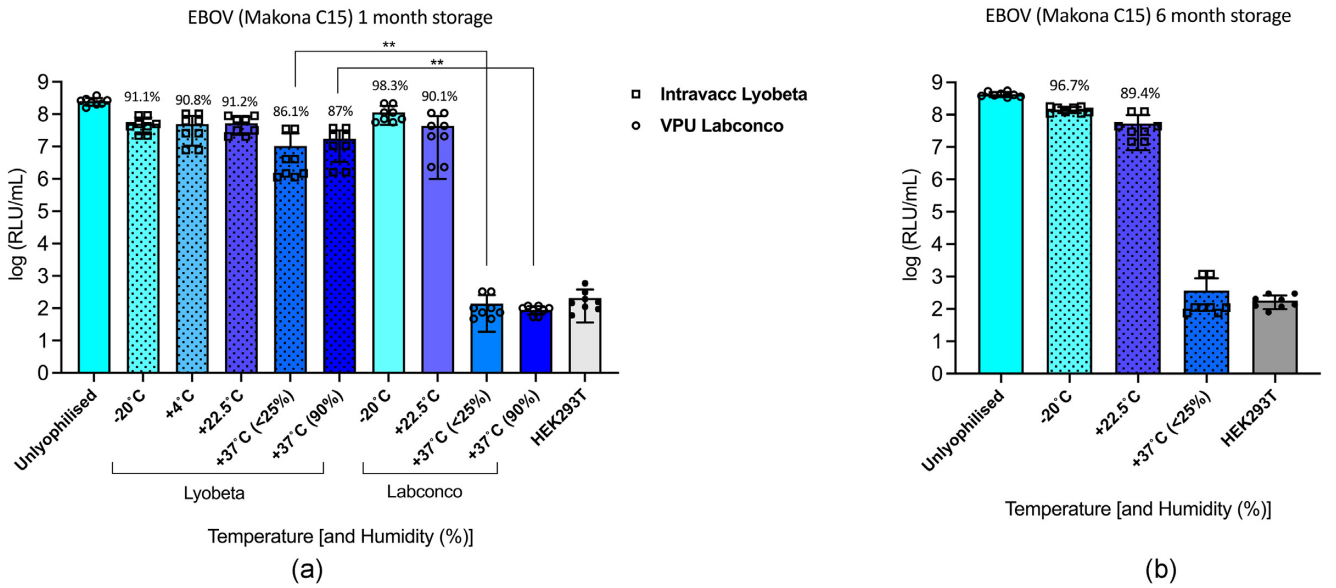

Figure 5. Infectivity assay following 1 and 6-month storage of EBOV PVs lyophilised at Intravacc. PVs were lyophilised at Intravacc (green) or at the (VPU; blue) and stored at different temperatures and humidity conditions (<25% or 90%) for (a) 1 month and (b) 6 months. Unlyophilised EBOV PVs were employed as positive controls for the test, as well as a parameter for comparison to calculate titre retention after lyophilisation, storage, and reconstitution. Titre recovery (%) is expressed on top of each bar. $^{**}P < 0.01$ (Mann–Whitney test). Background luminescence in uninfected cells (HEK293T) is also shown.

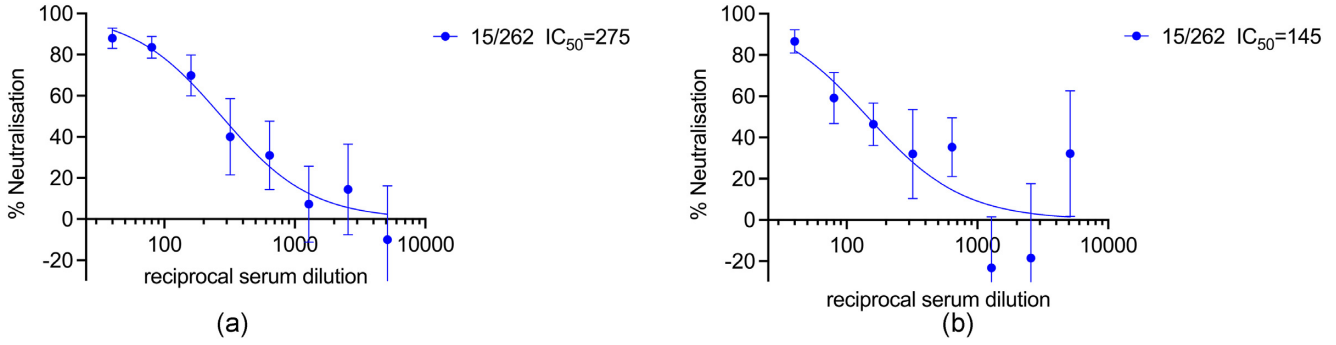

Figure 6. Neutralisation assay using reconstituted lyophilised EBOV PVs. WHO standard NIBSC 15.262 tested against EBOV PVs lyophilised with (a) Telstar Lyobeta that had been stored for 1 month at -20˚C, +37˚C (<25% humidity), +37C (90% humidity); and (b) Labconco stored for 1.5 years at + 4˚C. Human serum from a healthy donor (Sigma) was used as a negative control. Decrease in target cell luminescence (mean ± SEM) from duplicates in at least two independent experiments.

concurrent with EBOV, and therefore increasing the burden on health services, as well as the ongoing SARS-CoV-2 outbreak with over 659 million confirmed cases globally (WHO-8/01/2023) have also stressed the need for research in emerging diseases.

Although RT-PCR based assays are the gold standard for diagnostic testing of filoviruses and other viruses such as SARS-CoV-2 (Clark et al. 2018, Osterdahl et al. 2020), these require the presence of virus genetic material in patient blood or tissues. Serology looks for the antibodies in humans and animals to reveal the imprint of infection, even in individuals that did not exhibit clinical signs. Seroepidemiological studies can reveal geographical distribution, zoonotic spillover and can be used retrospectively to detect historical infections (Mather et al. 2013, Ewer et al. 2016, Kinsley et al. 2016, Luczkowiak et al. 2016). A major issue with conducting neutralising antibody studies however is the use of native virus in the assays, and the need to handle potentially dangerous pathogens in high containment facilities. This particularly impacts the ability for field laboratory work. The application of PVs to such studies may provide a suitable alternative, as long as the essential reagents can be provided in a stable form. A PV-based neutralising antibody assay that could differentiate between genera and even species of filoviruses and a complementary ELISA would be highly desirable to provide epidemiological data, as well as monitoring outbreaks when cross-reactivity might be an issue and next generation sequencing is not available. Importantly, PVs have the advantage of only requiring low containment facilities (BSL 1–2), and being amenable to multiplexing (Ewer et al. 2014, Carnell et al. 2015). Like native virus neutralisation assays, PVNAs require a suitable target cell line exhibiting the virus-specific receptor but only need 1–2 days to obtain results.

The majority of emerging virus outbreaks, such as those caused by filoviruses has occurred in low-resource countries. In order to provide assay reagents to laboratories in these

regions there is often a need for cold-store transportation and storage to prevent deterioration. Lyophilisation has been utilised as a means to address this issue and permit the transport of certain reagents and vaccines in ambient conditions (Kraan et al. 2014, Bjelošević et al. 2018, Bjelošević et al. 2020, Wong et al. 2020).

The possibility of using lyophilisation for short-term storage of PVs for antibody detection was explored for influenza, rabies, and Marburg viruses (Mather et al. 2014). Generally, PVs retained titres after storage at lower temperatures of −80°C up to room temperature (20°C) when stored for up to 1 month. However, at +37°C in dry or humid conditions, PV titres decreased ∼100-fold, though still retaining functionality due to their initial high titre. Reconstituted lyophilised influenza and rabies PVs also continued to perform in PVNAs. Marburg PVs were not tested in PVNAs due to lack of available anti-sera.

In the current study, we undertook a more comprehensive analysis of the application of lyophilisation to PVs with subsequent stability and functionality testing. This involved investigating different lyophilisation equipment and protocols, long-term storage under a range of conditions. Reconstituted PVs were tested in titration and neutralisation assays. We compared a standard laboratory freeze-dryer (Labconco) to a pilot scale freeze-dryer (Telstar Lyobeta). The disaccharide sucrose was used as a cryoprotectant for freeze-drying, as successful use had been established previously (Nireesha et al. 2013, Kraan et al. 2014, Mather et al. 2014).

All PVs were assessed in infectivity assays to calculate titre retention. The availability of convalescent patient antisera enabled the functionality of lyophilised and non-lyophilised EBOV PVs to be compared in neutralisation tests (n.b. no specific antisera was available for other filovirus genera).

All PVs utilised in this study had a lentiviral core. Initial PV titres for *ebolavirus* (EBOV) and *cuevavirus* (LLOV) PVs were $\sim 1 \times 10^8$ RLU mL^{-1} was obtained, whereas. For *marburgvirus* (RAVV) PVs was $\sim 1 \times 10^{10}$ RLU mL^{-1} was observed (Fig. 1), consistent with those generated in our previous study (Mather et al. 2014).

Following production, PVs were then lyophilised using a standard laboratory freeze dryer (Labconco), placed under a range of storage conditions then sampled and titrated at various time intervals over a 2 year period. At higher temperatures, we observed a large drop in transduction titres after only one-month storage at +37°C in dry and humid conditions (Figs. 2–4a).

For assessment of long-term storage and stability of lyophilised samples, PVs were generated then mixed with Sucrose-DPBS cryoprotectant solution to a final concentration of 0.5 mol L^{-1} before freeze drying. All three genera of lyophilised filovirus PVs followed a similar trend after long-term storage.

All lyophilised PVs had titre recovery above 85.9% when stored in a household fridge at +4°C for 1.5 years (Fig. 2-4). Furthermore, EBOV (Fig. 2e) and RAVV (Fig. 3e) −80°C PVs had titre recoveries of 94.7% and 98.7%, respectively, after being stored at +4°C for 2 years. These are particularly encouraging results, as avoiding the need for high-powered freezers would expand the number of labs in low-resource countries, such as those involved in the recent filovirus outbreaks, being able to employ PVs for research and assays. For samples stored at ambient temperature (+22.5°C), the decrease in titre recovery between 1 and 6 months was not investigated further, assuming that fridge storage could be achieved within a month, following transport and delivery.

We hypothesised that the rapid decrease in titres at +37°C could be due to the residual moisture remaining after lyophilisation without employing further pellet drying (Nireesha et al. 2013). Consequently, we sent frozen EBOV PV samples in sucrose/DPBS (final concentration 0.5 mol L^{-1}; on dry ice) to be lyophilised using a pilot-scale freeze-dryer facility (Telstar Lyobeta) at Intravacc including a secondary drying step. Samples were then transported back (on dry ice) for analysis at the VPU. EBOV PVs lyophilised at Intravacc had titre recovery of 86.1% and 87% even after being stored for a month at +37°C in dry and humid conditions (Fig. 5a—green). The samples for treatment at Intravacc were accompanied by a set that had been lyophilised at the VPU (see data in Fig. 5a—blue), which were temporarily stored at −80°C in the Netherlands then returned to the VPU on dry ice. By contrast, these samples had a drastic drop in titres after a month's storage at +37°C. Samples lyophilised at Intravacc and then stored at +37°C (<25% humidity) only began to lose titre after between 1 and 6 months of storage (Fig. 5). Overall, these are very encouraging results as PVs lyophilised using pilot scale equipment will retain a functional titre even when stored at harsher, warmer conditions. This suggests lyophilised PVs could be transported at room temperature to warmer tropical countries.

Finally, to assess the performance of lyophilised PVs in PVNAs, EBOV PVs lyophilised at Intravacc, then stored at various temperatures, or lyophilised at the VPU and stored at +4°C for 1.5 years, performed similarly to unlyophilised PVs (Fig. 6). They both were able to detect neutralising antibodies in the convalescent serum. End point titres reported by the NIBSC at the time were 164 (median estimate) for lentiviral based pseudotype assay and 160 for whole virion (Makona 14) in fluorescence reduction neutralisation assay (Wilkinson et al. 2015).

Other filovirus genera were not assessed in PVNAs in this study due to the lack of specific convalescent sera; however, they could be tested in PVNAs against monoclonal antibodies with neutralising activity in the future, as proof-of-principle.

It would also be useful to assess performance of filovirus VSV core PVs in lyophilisation studies as VSV is widely used as a PV core for filoviruses (Takada et al. 1997, Maruyama et al. 2014, Ilinykh et al. 2016, Ruedas et al. 2017, Salata et al. 2019).

Overall, these results are very promising for a future serological kit that could be transported at ambient temperature and would last at least 2 years in a household fridge. The lyophilisation of mammalian cells has been explored but so far has proved elusive, although there has been some success in lyophilisation and reconstitution of platelets (Wolkers et al. 2002), or somatic cells that have been lyophilised then used in nuclear transfer experiments (Loi et al. 2008). Even though lyophilisation of mammalian cells for later propagation in culture has not been successful so far due to the integrity of the cell membrane being compromised and the resulting damage (Zhang et al. 2017), more recently, cells have been transported at ambient temperature using a low-melting agarose method (Wheatley and Wheatley 2019). In some cases, good recovery was obtained for up to three weeks at 20°C–22°C.

These data demonstrates that PVs lyophilised using either lab-based or pilot-scale systems are functional in antibody neutralisation assays, after short-term storage in tropical

conditions or after several years in standard refrigeration. This important finding would permit the widespread employment of PV-based antibody assays in countries that have historically suffered from filovirus outbreaks.

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Conflict of interest

No conflict of interest declared.

Author contribution

M.M. and S.S. contributed to the conception and design of the study. M.M conducted the majority of experiments, data collection and analysis, and wrote the first draft of the manuscript. .PS., R.H., and I.P. were responsible for the pilot scale lyophilisation.

All authors contributed to the study progression with advice throughout, as well as manuscript revision, reading, and approved the submitted version.

Data availability

The raw data that support the findings of this study are available from the corresponding author upon reasonable request.

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