Production of rVSV-ZEBOV in serum-free suspension culture of HEK 293SF cells

Jean-François Gélinas [a], Hiva Azizi [b,1], Sascha Kiesslich [a,1], Stéphane Lanthier [c], Jannie Perdersen [b], Parminder S. Chahal [c], Sven Ansorge [c], Gary Kobinger [b], Rénald Gilbert [a,c], Amine A. Kamen [a,*]

[a] Department of Bioengineering, McGill University, Montreal, QC, Canada
[b] Centre de Recherche en Infectiologie, Centre Hospitalier Universitaire de Québec, Université Laval, Quebec City, QC, Canada
[c] Human Health Therapeutics, National Research Council Canada, Montreal, QC, Canada

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ABSTRACT

Ebola virus disease is an urgent international priority. Promising results for several vaccine candidates have been reported in non-human primate studies and clinical trials with the most promising being the rVSV-ZEBOV vaccine. In this study, we sought to produce rVSV-ZEBOV in HEK 293SF cells in suspension and serum-free media. The purpose of this study was to establish a process using the HEK 293SF production platform, optimise the production titre, demonstrate scalability and the efficiency of the generated material to elicit an immune reaction in an animal model. Critical process parameters were evaluated to maximize production yield and process robustness and the following operating conditions: $1-2 \times 10^6$ cells/mL grown in HyClone HyCell TransFx-H media infected at an MOI of 0.001 with a temperature shift to 34 °C during the production phase and a harvest of the product after 48 h. Using these conditions, scalability in a 3.5 L controlled bioreactor was shown reaching a titre of 1.19×10^8 TCID$_{50}$/mL at the peak of production, the equivalent of 4165 doses of vaccine per litre. The produced virus was shown to be thermostable in the culture media and, when concentrated, purified and administered to mice, demonstrated the ability to induce a ZEBOV-specific immune response.

1. Introduction

Ebola virus disease is an urgent international priority. The 2014–2015 Ebola virus disease outbreak resulted in 28,616 confirmed, probable and suspected cases and 11,310 deaths [1]. The development of a safe and efficacious vaccine would provide an important public health tool, in addition to the primary strategy to stop the virus transmission which currently consists of identifying and isolating contacts and the use of appropriate personal protective equipment. Promising results for several vaccine candidates have been reported in non-human primate studies and clinical trials [2]. These have used different recombinant adenoviruses, modified vaccinia Ankara viruses and recombinant vesicular stomatitis viruses (rVSV). Two of these vaccines have been approved, a recombinant adenovirus-based vaccine, in China [3,4] and a heterologous rVSV and adenovirus prime-boost vaccine in Russia [5].

The most promising of the Ebola virus vaccine candidates (rVSV-ZEBOV), currently in advanced phase III clinical trials, is based on an attenuated, replication-competent rVSV, originally developed by the National Microbiology Laboratory, Public Health Agency of Canada [6]. The Vesicular Stomatitis Virus (VSV) is a cytopathic virus member of the Rhabdoviridae family, which also includes the rabies virus. It was first identified in livestock cases of vesicular stomatitis, a vesicle-inducing disease similar to foot-and-mouth disease [7]. However, unlike foot-and-mouth disease, VSV is infectious in humans [8], especially in high-risk occupational exposure situations such as laboratory workers, farmers and veterinarians [9,10]. In human hosts, the infection is usually asymptomatic or associated with a mild febrile illness lasting 2–5 days [10]. There is, therefore, a very low level of pre-existing immunity to the virus among the general human population except in some rural communities [11].

The rVSV platform is used for the design of -replication-competent vaccines that have- been shown to generate both cell-mediated and humoral immunity to expressed foreign antigens [12]. rVSV-ZEBOV induces replication of viral particles similar to VSV, but it is genetically engineered to replace the VSV glycopro-

tein with the glycoprotein from a Zaire strain of the Ebola virus (ZEBOV). The ZEBOV glycoprotein is responsible for receptor binding and membrane fusion with the host target cells and the induction of antibody expression [13]. The vaccine causes a transient systemic infection after a single injection and produces a rapid immune response against the Ebola virus surface protein [14,15]. It has been shown to be safe to administer to humans [14,16–19] and, importantly, it has shown protective efficacy against Ebola virus in a human phase III clinical trial [20]. In the 2018 outbreak, according to a preliminary report, the estimated vaccine efficacy was 97.5% [21]. The rVSV platform is also being used to develop a -human -immunodeficiency - vaccine [22,23].

Production of rVSV is usually done in BHK-21 or Vero cells using media containing serum. Table 1 lists the parameters used in other studies to produce rVSV and shows the general usage of serum and adherent cells. It is important to note that the disparities in the observed titres are mostly dependent on the pseudotype used, the wildtype G glycoprotein leading to much higher titres, and both the technique and cells used to do the titration. In this study, we sought to produce rVSV-ZEBOV in HEK 293SF cells in suspension in serum-free media. The HEK 293SF (clone 293SF-3F6) cell line has previously been used in the production of vaccine candidates [24–26] and other viral vectors [27–31]. Production of viral vectors in this cell line has been shown to be scalable in bioreactors up to 45 L and 70 L culture volumes [25,32]. The use of these cells would, therefore, allow larger scale and cost-effective production of high-quality vaccines for the rVSV platform. The purpose of this study is to establish an initial production process for rVSV-ZEBOV in HEK 293SF cells, select optimimal production conditions, evaluate the scalability in a 3.5 L stirred-tank bioreactor and assess the efficiency of the generated material to elicit an immune reaction in an animal model.

2. Methods

2.1. Cells

HEK 293SF (clone 293SF-3F6) [33] is a cell line derived from the HEK 293S cell line adapted for suspension culture [34], which was adapted to grow in a serum-free medium. These cells were used for vector production and were grown in polycarbonate vented-cap shake flasks (Corning, Corning, NY, USA). They were maintained on an orbital shaker platform (Infor's HT, Montréal, QC, Canada) at 110 revolutions per minute (rpm) in a humidified incubator (Thermo Fisher Scientific, Waltham, MA, USA) at 5% CO_2 and 37 °C in HyClone HyCell TransFx-H, a medium free of animal-derived components (GE Healthcare, Chicago, IL, USA) without serum or antibiotics. This medium was supplemented with 4 mM L-glutamine (GE Healthcare) and with 0.1% poloxamer 188 (MilliporeSigma, Etobicoke, ON, Canada). Cell growth was monitored by determining live cell density using 0.2% trypan blue dye (Thermo Fisher Scientific) exclusion in a TC20 Automated Cell Counter (Bio-Rad, Hercules, CA, USA), or manually using a hemocytometer (Reichert, Buffalo, NY, USA). Cells were passaged twice a week by diluting to 2.0×10^5 live cells per mL in fresh media.

HEK 293A cells [35] (American Type Culture Collection, Manassas, VA, USA) were used for analytical assays. They were maintained in cell culture dishes (Greiner Bio-One, Kremsmünster, Austria), in a humidified incubator at 5% CO_2 and 37 °C in Dulbecco's Modified Eagle's Medium (DMEM) (Thermo Fisher Scientific), supplemented with 2 mM L-glutamine and 5% Fetal Bovine Serum (FBS) (GE Healthcare) without antibiotics. Cells were passaged twice a week. The confluent cells were detached using a cell scraper, centrifuged at 500g for 5 min, resuspended in fresh medium and seeded at a 1:10 dilution.

Table 1
Published parameters of production of rVSV.

Study	Virus	Cells	Media	Container	Cell density	MOI	Titres
[43]	rVSV(MΔ51)-M3	HEK 293	DMEM, 10% FBS, PS	10-layer cell factory	$7.5 \times 10^4/cm^2$	0.01	2.0–9.1×10^8 PFU/mL on BHK-21
[40]	VSV-GFP	SF-BMAdR and 293SF-3F6	PRO293s-CDM, 6 mM glutamine and SFM4Transfx-293, 4 mM glutamine or IHM03	125 mL, 250 mL and 1 L shaker flasks	$1.0 \times 10^7/mL$	0.1	2.0×10^{10} TCID$_{50}$/mL on HEK 293A
[12]	rVSV-ZEBOVGP rVSV-MARVGP, rVSV-LASVGPC	VeroE6 and Jurkat	DMEM, 2% FBS	12-well plate	80% confluence or 1×10^6/well	10	$\sim 10^9$ TCID$_{50}$/mL on VeroE6
[44]	VSV-IFNβ	BHK-21	DMEM, 10% FBS, PS, amphotericin B	6 well plate	1×10^6/well	10	$\sim 10^9$ PFU/mL on BHK-21
[45]	rVSV-EncG$_4$-G$_6$	Vero	VP-SFM, 2% L-Glutamine, 1% PS	N/A	N/A	N/A	N/A PFU/mL on Vero
[46]	rVSV-SHIV 89.6P Env	BHK	Serum-free DMEM	N/A	N/A	N/A	2×10^7 to 8×10^8 PFU/mL on N/A
[47]	VSVrwt, VSV-CT1, VSV-CT9, VSV-HA	BHK	DMEM, 5% FBS	N/A	N/A	N/A	N/A PFU/mL on BHK
[38]	VSV-GFP	suspension adapted Vero cells	IHM03	125 mL shake flask and 3.5 L bioreactor	1.0–6.8×10^6/well	0.1	2.3×10^9 to 1×10^{10} TCID$_{50}$/mL on HEK 293A
[48]	rVSV N1	BHK-21	MEM, 10% FBS, 2 mM GlutaMAX	96 well plate	1 cell/well	5	N/A PFU/mL on BHK-21
[49]	G-pseudotyped rVSV-ΔG	BHK-21 clone WI-2	DMEM, 5% FBS	10 cm dish	~85% confluency	≤ 0.1	N/A PFU/mL on HeLa and BHK-21

BHK: Baby Hamster Kidney cells, DMEM: Dulbecco's Modified Eagle's Medium, FBS: Fetal bovine serum, FCS: Fetal calf serum, HEK: Human Embryonic Kidney cells, MEM: Minimum Essential Media, MOI: Multiplicity of infection, N/A: Data not available as it was not stated in the publication, rVSV: recombinant vesicular stomatitis virus, PS: Penicillin-Streptomycin.

2.2. Virus

The generation of the original stock is described in the Supplemental Methods. The seed stock used in all experiment was generated by infection of a 2L flask containing 600 mL HEK 293SF cells at 1×10^6 cells/mL with rVSV-ZEBOV from the original stock at a multiplicity of infection (MOI) of 0.001. This production, harvested after 48 h, had a viral genome titre of 7.47×10^9 viral genomes (VG)/mL and a functional titre of 1.23×10^7 Median Tissue Culture Infectious Dose (TCID$_{50}$)/mL. The virus stock was aliquoted in small volumes. All the experiments were performed in 6 well plates (Corning) with 2 mL media per well except for the bioreactor experiment. Unless stated otherwise, the standard conditions were 1×10^6 live cells per mL in HyCell TransFx-H infected with the seed stock at an MOI of 0.001 and incubated in a 34 °C humidified incubator with 5% CO_2 until harvested after 48 h. These conditions were then modified to test each parameter.

2.3. Variation of production parameters

A common batch of cells grown to a concentration of 1.56×10^6 cells/mL was used in experiments where MOI, incubation temperature, production media or cell density were modified. The required number of cells per condition were spun down in conical flasks at 500g for 5 min and resuspended in fresh media to reach concentrations of 1.0×10^6 or the indicated concentration when this was the parameter evaluated. For production in different media, a selection of media readily available in the laboratory was used: BalanCD (Irvine Scientific), DMEM, Dulbecco's Phosphate-Buffered Saline (DPBS) (GE Healthcare), FreeStyle 293 (Thermo Fisher Scientific) and VP-SFM (Thermo Fisher Scientific) in addition to the HyClone HyCell TransFx-H used in all other conditions. Cells were then seeded in triplicates per condition in 6 well plates with 2 mL per well. Cells were then infected with rVSV-ZEBOV at either an MOI of 0.001 or the indicated MOI when this was the parameter evaluated. The cells were then left to incubate with agitation for 2 days at 34 °C or at the indicated temperature when this was the parameter evaluated.

In order to determine if cells could be grown to high concentration and then infected, cells were seeded in 50 mL of medium in a 250 mL flask at the standard cell density for passage of 2×10^5 cells/mL. Daily, for five days, the cells of the initial flask were counted, and 4×10^6 cells were removed and placed into a new 125 mL flask with a 20 mL total volume of medium for a new seed density of 2×10^5 cells/mL. On the fifth day, all flasks, now all at different cell concentration, were infected with rVSV-ZEBOV at an MOI of 0.001 and left to incubate with agitation for 2 days at 34 °C.

2.4. Titration

The methods for the TCID$_{50}$ (performed on HEK 293A) and dPCR assays are described elsewhere (to be published). To prevent the effect of interday variability, all titres shown in the same figure were obtained from samples which were titrated on the same day [36]. Throughout, the TCID$_{50}$ value shown for the titres at the time of infection was calculated from the titre of the vial used to infect the culture rather than from samples taken after infecting the cells and this is reflected by using open symbols in the figures. The infectious virus particle to viral genome ratio (TCID$_{50}$/VG) was derived from the ratio of the TCID$_{50}$ titre to the dPCR titre.

2.5. Bioreactor production

Similar bioreactor setups to the one used in this study have been described previously [30,37,38]. The culture was conducted in a controlled 3.5 L CF-3000 (type SG) (working volume 2.7 L)

bioreactor (Chemap AG, Mannedorf, Switzerland) equipped with three surface baffles. Agitation with two pitched blade impellers was kept constant at 80 rpm and the temperature was controlled at 37 °C during the cell growth phase and then at 34 °C after infection with a water jacket. The pH (Ingold pH probe, Andover, MA or Mettler pH probe) was maintained at 7.0 by addition of CO_2 via surface aeration. The culture was surface-aerated, and the dissolved oxygen concentration was kept at 40% of air saturation, measured using a InPro 6800 probe (Mettler Toledo, Billerica, MA, USA), by controlling the fraction of nitrogen and oxygen in the gas inlet. The bioreactor was also equipped with a Biomass Monitor 220 probe (Aber instruments Ltd., Aberystwyth, UK) to measure cellular biomass. Data acquisition was performed using FIX MMI software (Intellution, Norwood, MA, USA). Cells were seeded at 2.0×10^5 cells/mL and samples were taken every 24 h to measure cell density progression. The culture was infected after reaching the targeted cell density ($1–2 \times 10^6$ cells/mL) with rVSV-ZEBOV at an MOI 0.001. Samples were taken every twelve hours for 72 h of production. Metabolite analysis was performed on a Cedex Bio Analyzer (Roche).

2.6. Exposition of aliquots to different temperatures

For temperature exposition of virus aliquots, temperatures of −80 °C and −20 °C were maintained using freezers (Thermo Fischer Scientific and White Consolidated Industries, Cleveland, OH, USA), 4 °C using a refrigerator (W.C. Wood, Guelph, ON, Canada), 25 °C by leaving samples at room temperature, 34 °C and 37 °C by leaving samples in incubators and 42 °C by putting samples in a heated water bath (VWR, Mont-Royal, QC, Canada). The same −80 °C freezer and a 37 °C water bath (Thermo Fisher Scientific) were used for freeze–thaw experiments. Thirty tubes containing 1 mL of rVSV-ZEBOV were exposed in triplicates to a different number of freeze–thaw cycles by incubating them at 37 °C for 3–5 min (until all visible ice had melted) and then left at −80 °C for 10–15 min (until the whole tube had frozen over).

2.7. Production and purification for the animal study

HEK 293SF cells at a cell density of 1.88×10^6 cells/mL were infected with rVSV-ZEBOV at MOI 0.01 in two 2L flasks with a working volume of 600 mL each. The cell culture was harvested two days post infection and frozen at −80 °C was treated with 10 U Benzonase (MilliporeSigma) per mL of cell culture supplemented with 2 mM of $MgCl_2$ (VWR) for 1 h at 37 °C to digest free DNA and RNA. The rVSV-ZEBOV produced was recovered in the supernatant by pelleting cellular debris by centrifugation at 3500g for 20 min at 4 °C. The resulting supernatant was filtered using a two-step process going first through a 63 mm fiberglass pre-filter (Corning) and then a 0.45 μm filter (MilliporeSigma). The filtrate was then concentrated 40 times in volume using the KrosFlo® Research Iii Tangential Flow Filtration System (KR2i) (Spectrum, Rancho Domingez, CA, USA). A hollow fibre cartridge (750 kDa MWCO; Spectrum Laboratories, Rancho Dominguez, CA) was used for tangential flow filtration (TFF). The concentrated virus was then purified on an Optiprep (iodixanol) density gradient medium (MilliporeSigma), by underlaying 5 mL and 8 mL layers containing 13% and 35% Iodixanol solutions (made in 0.9% NaCl, 10 mM Tris at pH 7.2 (BioShop, Burlington, ON, Canada), respectively, in Optiseal ultracentrifuge tubes (Beckman Coulter, Brea, CA, USA). The virus was partially purified by separating it from other cellular contaminants using rotor 70Ti in an Optima L-80XP (Beckman Coulter) ultracentrifuge. After 3.5 h of centrifugation at 30,000 rpm, 1 mL fractions were collected from the bottom of the tube using a pump at 1.5 mL/min. The refractive index of each fraction was measured and the fractions with a value between 1.3657 and 1.3811 were

pooled for a total of 6 mL. This was followed by diafiltration with 7 volumes against the injection buffer consisting of 10 mM Tris at a pH of 7.2 supplemented with 4% sucrose (Bioshop). The sample was concentrated further to a final volume of 3.5 mL. Human serum albumin (MilliporeSigma) was then added at a final concentration of 2.5 g/L to ensure stability of the purified virus in the injection buffer when frozen.

2.8. Mice study

Female, 6- to 8-week-old C57BL/6 mice (Charles River, Wilmington, MA, USA) were used in accordance with the Canadian Council on Animal Care guidelines and all protocols were approved by the Animal Care Ethics Committee of the Université Laval. Groups of 7 mice were bled via saphenous vein one day prior to immunization and 4 weeks after immunization. Mice were immunised intramuscularly with one dose of 50 μL of virus preparation diluted in injection buffer to a concentration of 1×10^6 TCID$_{50}$/mL. Sera was isolated and kept frozen until used.

2.9. Humoral immune response (ELISA assay)

Half area 96-well flat-bottom high-binding polystyrene microtiter plates (Corning, Corning, NY, USA) were coated with 2 μg/ml EBOV glycoprotein recombinant protein (Sino Biological, Beijing, China), overnight at 4 °C. Plates were blocked for 1 h with blocking buffer (KPL Milk Diluent/Blocking Concentrate Kit, SeraCare, Milford, MA, USA) at 37 °C. KPL diluent buffer was used to prepare 1:200 dilutions of sera and added to each well, then incubated for 2 h at room temperature. The plates were washed 4 times with PBS (Corning) containing 0.1%Tween 20 (VWR). Then, a goat anti-mouse IgG-HRP conjugated secondary antibody (Tonbo Bioscience, San Diego, CA, USA) at a 1:2000 dilution was added and incubated for 1 h at room temperature. The ELISA plate was then washed 4 times with PBS-Tween 20, 0.1%. Finally, the horseradish peroxidase substrate (SeraCare) was added and the signal was left to develop by incubating for 30 min at 37 °C. Reactions were stopped by addition of 1% sodium dodecyl sulfate (SDS) (MilliporeSigma). The signals were then measured using a Synergy HTX microplate reader (BioTek, Winooski, VT, USA). The data are reported as the optical density at 405 nm.

2.10. Statistical analysis

Where stated, statistically significant differences between two group means were determined by an unpaired t-test using Prism 8.1.1.330 (GraphPad, La Jolla, CA, USA). For comparing multiple groups one-way an ANOVA was calculated and reported as F(k−1, N–k) = F value, p = p value, where k is the number of groups and N the number of data points. This was followed by Dunnett's post-test, to compare each of a number of treatments with a single control. Calculated p values of <0.05 were deemed a significant difference and, when relevant, indicated with two stars (**) for a p value < 0.01 and three stars (***) for a p value < 0.001. Error bars in figures represent the standard deviation. Statistical power was evaluated using the G*Power 3.1.9.2 software (University of Düsseldorf, Düsseldorf, Germany) [39] which demonstrated that a minimum of a 2.259-fold increase in functional titre would be necessary to observe a statistical difference with 80% power using triplicates.

3. Results and discussion

3.1. Infection kinetics

Before any experiment could be performed, it was essential to determine when the peak production of rVSV-ZEBOV was happen-

ing in standard conditions and what MOI should be used. HEK 293SF cells are routinely used to produce lentiviral vectors and this is done using the HyClone HyCell TransFx-H medium without serum or antibiotics at 37 °C with agitation [30]. An incubation temperature of 34 °C was selected based on previous experience with rVSV expressing the green fluorescent protein [40]. A cell concentration of 1×10^6 cells/mL was used as a starting point pending further evaluation of the impact of cell density on production.

MOI refers to the number of virions that are added per cell during infection. To evaluate the impact of MOI and time of harvest, rVSV-ZEBOV infections were performed at MOIs of 10, 1, 0.1, 0.01, 0.001 or 0.0001 and samples were harvested every 12 h. Fig. 1A shows the results of the titration of these samples. The optimal harvest time was determined to be between 48 and 60 h post-infection with best amplification of functional titres for MOI values of 0.01 to 0.0001. In these conditions, an average titre of 7.71×10^7 TCID$_{50}$/mL was reached. This number compares favourably to the 2×10^7 plaque-forming units per dose needed to vaccinate humans [20]. A large operation window in terms of MOI and time of harvest was also reported for the current production protocol of the rVSV-ZEBOV vaccine [41].

This peak titre is lower and comes at a later time point than what was observed (3.90×10^8 at 24 h) for production of VSV-GFP in the same cells [40], but this is most likely explained by the presence of the more potent native VSV glycoprotein on VSV-GFP instead of the Ebola glycoprotein here. The slower kinetics of the present virus can also be observed by the longer time required to read TCID$_{50}$ titration plates (7 days versus 3 days for VSV-GFP) [40].

3.2. Temperature of production

It was previously shown that the temperature used during the production of rVSV can have a considerable impact on virus production [40]. To assess this, triplicate rVSV-ZEBOV productions were performed at either 34 °C or 37 °C. Fig. 1B shows the results of the TCID$_{50}$ titration of these samples with a 6-fold increase in functional titre at 48 h when production is done at 34 °C instead of 37 °C. There was a statistically significant difference (p = 0.0253) between the two group means as determined by paired two-tailed t-test.

To better understand the impact of the temperature during production, the number of viral genomes was titrated by dPCR for samples at the selected optimal harvest time (48 h post-infection). At that time point, infectious viral titers were 1.00×10^8 TCID$_{50}$/mL and 1.62×10^7 TCID$_{50}$/mL at 34 °C and 37 °C, respectively. In addition to a higher infectious titer, production at 34 °C also results in a higher TCID$_{50}$/VG ratio. The number of viral genomes reached 8.46×10^9 VG/mL and 3.06×10^9 VG/mL at 34 °C and 37 °C, respectively, resulting in corresponding ratios of 1/84 TCID$_{50}$/VG and 1/189 TCID$_{50}$/VG. A higher ratio of TCID$_{50}$/VG indicates a higher infectivity of the produced viruses compared to the total (infectious and non-infectious) viral particles containing a genome and is therefore desired. The incubation temperature for further production was, therefore, kept at 34 °C.

3.3. Media selection during production

Next, we wanted to confirm that the medium used to grow HEK 293SF, HyClone HyCell TransFx-H, offered a good performance during production compared to other commercially available media. Fig. 1C shows the results of the TCID$_{50}$ titration of production in these media. In this experiment, HyClone HyCell TransFx-H was equivalent to VP-SFM, DMEM and BalanCD and these all outperformed FreeStyle 293. It is important to note that the cells were maintained in HyClone HyCell TransFx-H before this experiment.
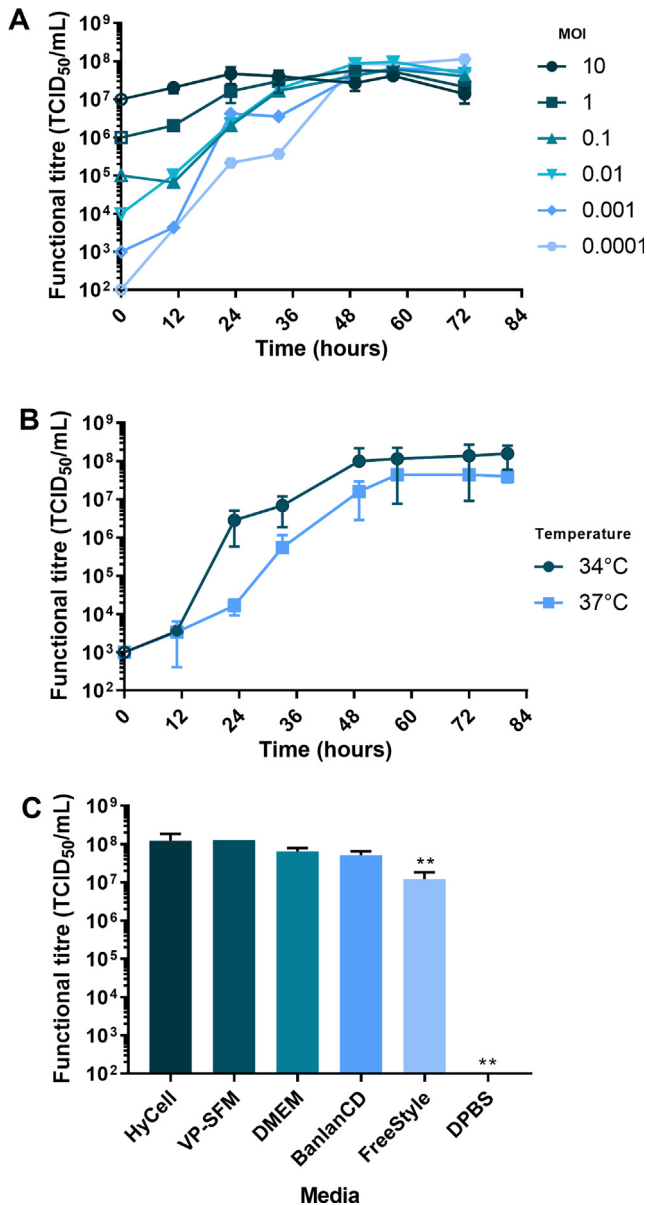
Fig. 1. Effect of initial MOI, incubation temperature and cell culture media on functional titre. Functional titres were measured by $TCID_{50}$. Bars represent the mean of triplicate production studies ± standard deviation. Samples were harvested at indicated time points (about every 12 h) or at 48 h in the case of the last panel. (A) Effect of MOI. Production yields for 3 independent infections with rVSV-ZEBOV at MOI 10, 1, 0.1, 0.01, 0.001 and 0.0001 in 6 well plates with 1×10^6 cells/mL in 2 mL per well. (B) Yields of triplicate infections with rVSV-ZEBOV at an MOI of 0.001 at 34 °C and 37 °C. There was a statistically significant difference (p = 0.0253) between the two group means as determined by paired two-tailed t-test. (C) Yields of rVSV-ZEBOV production in various media. There were some statistically significant differences between group means as determined by one-way ANOVA (F(5,11) = 8.589, p = 0.0016) followed by Dunnett's post-test and indicated with two stars (**) for a p value < 0.01 (0.0039 and 0.0018 respectively).

This might confound the results of this experiment with cells having sufficient reserves for a 48 h incubation. To this effect, it is interesting to note the comparable performance (not statistically significant different to HyClone HyCell TransFx-H) of DMEM, a relatively nutrient poor medium when not supplemented with serum. The inferior performance of FreeStyle 293 might be due to the media change or the presence of components in the medium that act as inhibitors.

3.4. Cell density

A cell concentration of 1×10^6 cells/mL was initially selected for virus production. One effective way to increase functional titres per mL is to increase the cell density at infection. In order to determine if cells could be grown to higher concentrations and then infected, cells were left to grow to different cell concentrations and then infected with rVSV-ZEBOV. This experiment, repeated twice, showed a breaking point at 2×10^6 cells/mL after which point functional titres dramatically diminished (Fig. 2A). A similar effect has been previously observed in the production of other vectors where lower specific yields and overall production were apparent above certain cell concentrations [42] and is generally refered to as the "cell density effect". This effect might be attributed to very different metabolic requirements over the cell growth phase versus the infection/viral production phase.

To evaluate the impact of different initial cell densities not affected by their cell growth phase, HEK 293SF cells at 1.56×10^6 cells/mL were centrifuged and seeded in triplicate wells containing concentrations of 5×10^5, 1×10^6, 2.5×10^6, 5×10^6, 1×10^7 and 2×10^7 cells/mL and then infected with rVSV-ZEBOV. Fig. 2B shows the results of the titration of samples taken through this infection and -there was a direct correlation between increases in cell density and functional titres obtained until the 48 h post-infection which shows that cells in the right growth phase can support production even if at much higher concentration during the virus production phase. Past the 48 h time point, a slowly declining plateau was reached, and similar functional titres were obtained for all cell densities.

3.5. Production in a stirred-tank bioreactor

Production of an adenovirus-vectored tuberculosis vaccine candidate in HEK 293SF cells has been shown to be scalable in bioreactors from this 3.5 L scale up to 60 and 200 L (45 and 70 L culture volume) [25,32]. In order to evaluate the scalability of the production of rVSV-ZEBOV in serum-free suspension HEK 293SF, a 3.5 L scale bioreactor was set up. The cell concentration raised from 2×10^5 to 1.16×10^6 cells/mL at the time of infection (Fig. 3A). At this point, the temperature was lowered to 34 °C for the production phase as this temperature was shown to be optimal in flask production. The cells were then infected with rVSV-ZEBOV at an MOI 0.001. Cell concentration increased sharply in the first 24 h after virus infection to reach 2.56×10^6 cells/mL and then stabilised at around 1.8×10^6 cells/mL during the production phase. The viability profile after infection showed an expected decline, reaching 32% at harvest time, three days post infection. This late harvest time was chosen to confirm the 48 h post-infection harvest time point previously established in flasks and observe the impact on titres of this prolonged production. Production was found to be directly scalable from shake flasks to the bioreactor, with comparable production profiles. Metabolites (Fig. 3B) and bioreactor parameters (Fig. 3C) behaved as expected for such a bioreactor run. In particular, no limitation of glucose is observed at this rather low cell density. The accumulation of Lactate Dehydrogenase (LDH), an indicator of cell lysis in the media, reflects the observed decline in cell viability at day 4. The on-line monitoring of the capacitance probe signal, an indication of the total biovolume of the culture; and the input gas molar fraction of oxygen (yO_2), that is directly proportional to the oxygen uptake rate of the culture under the set operating conditions provide a highly reliable production kinetics of the rVSV that will be used to document the robustness of the process and support further scale-up.

Functional rVSV-ZEBOV titre reached a peak of 1.19×10^8 $TCID_{50}$/mL at 36 h post-infection (specific production of 103 $TCID_{50}$/cell, based on cell count at infection point) followed by a
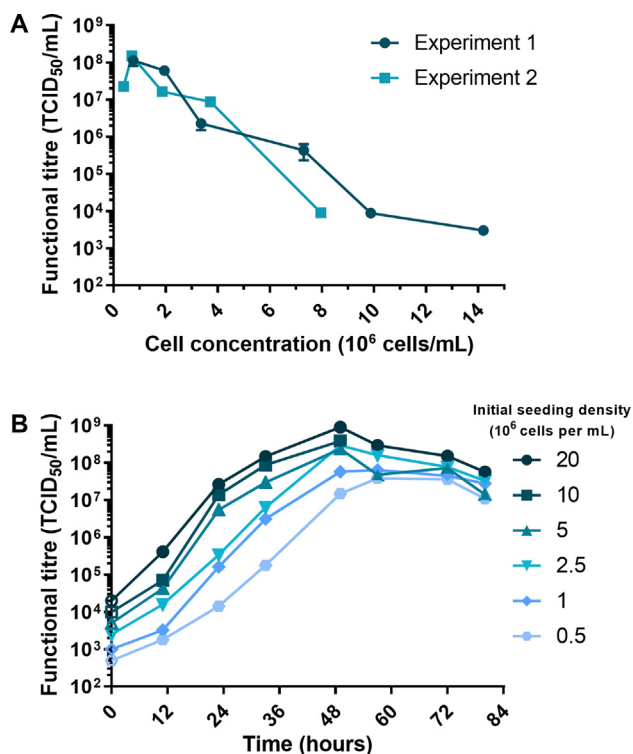
Fig. 2. Effect of cell density during production on functional titres. Functional titres were measured by TCID$_{50}$ (A) Production yields for two series of independent infections with rVSV-ZEBOV of cells grown to different cell concentration in 2 mL per well. Samples were harvested at 48 h. (B) Production yields for 3 independent infections with rVSV-ZEBOV at MOI 0.001 in 6 well plates with 5×10^5, 1×10^6, 2.5×10^6, 5×10^6, 1×10^7 or 2×10^7 resuspended cells/mL (from a common centrifuged seed stock originally at 1.56×10^6 cells/mL) in 2 mL per well. Samples were harvested at indicated time points (about every 12 h).

declining plateau ending at 1.59×10^7 TCID$_{50}$/mL at the time of harvest. In contrast, the number of viral genomes reached 5.95×10^9 VG/mL at 36 h post-infection but continued to increase throughout the remaining production phase, reaching 1.16×10^9 VG/mL at the time of harvest. The ratio of infectious viral particles to viral genomes was 1/50 TCID$_{50}$/VG at 36 h post-infection. During the remaining production phase, this ratio steadily declined until it reached 1/727 TCID$_{50}$/VG at the time of harvest. As mentioned previously, a higher ratio of TCID$_{50}$/VG is desired since it indicates a better quality of the viral product in terms of infectivity per particle. Here, it can be observed that the produced virus loses infectivity over time while the number of viral particles containing a genome increases. It remains to be established if this change in ratio is due to the degradation of previously produced viruses, to the production of non-infectious viruses after a certain point or to a combination of these factors.

3.6. Exposition to different temperatures

The sensitivity of rVSV to different temperatures is critical but poorly characterized. To assess the impact of temperature in the production media during downstream processes, 21 tubes containing 1 mL of rVSV-ZEBOV were exposed in triplicates to various temperatures. After 4 h, 1 day, 2 days, 1 week and 1 month, samples from each tube were harvested for TCID$_{50}$ and dPCR analysis (except frozen tubes which were only harvested after 1 month). Fig. 4A shows the impact of exposure to these various temperatures on the TCID$_{50}$ functional titres of the virus present in the tubes. Beginning at 48 h, exposure to

42 °C had a statistically significant negative impact (p = 0.0192) compared to exposure to 4 °C, as determined by one-way ANOVA (F(4,10) = 3.639, p = 0.0444) followed by Dunnett's post-test. After 1 week of exposition, there were statistically significant differences between group means (one-way ANOVA (F(4,10) = 4.792, p = 0.0203) followed by Dunnett's post-test) for temperatures 34 °C (p = 0.0250) and above when compared to 4 °C. It took two weeks to observe a statistically significant difference (p = 0.0075) between a sample left in the refrigerator and at room temperature (p = 0.0075) (one-way ANOVA (F(3,7) = 15.62, p = 0.0017). Finally, after nearly a month, no statistically significant difference was found in functional titre between samples stored at 4 °C (p = 0.6279) or frozen at –20 °C (0.3706) and samples frozen at –80 °C (one-way ANOVA (F(4,8) = 3.957, p = 0.0456) followed by Dunnett's post-test).

A viral genome titration was performed by dPCR on all samples after they were kept at the range of temperatures listed for 29 days. All dPCR titres were similar at this time point with an average of 6.65×10^9 VG/mL and a standard deviation of 5.485×10^8. This remains similar to the original viral genome of the samples of 7.47×10^9 VG/mL. This shows a loss of functional titre due to the exposure to these temperatures while the viral genome titre stays relatively constant. Downstream processing of the production could, therefore, be performed at room temperature and over multiple days without the need to freeze the virus if the media composition stays relatively the same throughout the process.

3.7. Impact of freeze-thaw cycles on titre

Viruses are very sensitive to freeze-thaw cycles without cryoprotectant. Nevertheless, stability through five freeze-thaw cycles was previously demonstrated for the current production protocol of the rVSV-ZEBOV vaccine [41]. The following experiment assessed the impact of freeze-thaw cycles during downstream processing of viruses produced in HEK 293SF using HyClone HyCell TransFx-H. Thirty tubes containing 1 mL of rVSV-ZEBOV were exposed in triplicates to a different number of freeze-thaw cycles. Fig. 4B shows the impact of exposure to increasing numbers of freeze-thaw cycles on the TCID$_{50}$ titres of the virus present in the tubes. This impact was not significant. This could be because the virus is stable when exposed to these drastic temperature changes or because HyClone HyCell TransFx-H acts as a sufficient cryoprotectant.

3.8. Efficiency at generating an immune response

To evaluate the immunogenicity of viruses produced in the established conditions, 1.2L rVSV-ZEBOV was produced. This production was concentrated/purified to a final concentration of 1.3×10^9 TCID$_{50}$/mL (1.65×10^{11} VG/mL) in 3.5 mL by TFF and iodixanol density gradient. While the concentration/purification process needs to be optimised because of extensive loss, this purified product would have been sufficient to produce 159 doses of the 2×10^7 plaque-forming units required for human vaccination [20].

Mice were injected intramuscularly with 50 μL of vaccine containing 1×10^6 TCID$_{50}$ rVSV-ZEBOV diluted storage/injection buffer (10 mM Tris in PBS with 4% sucrose and 2.5 g/L human serum albumin). Anti-Ebola glycoprotein antibodies were measured by ELISA and the results (Fig. 5) demonstrate the presence of Ebola glycoprotein-specific antibodies in these mice 4 weeks after immunisation.
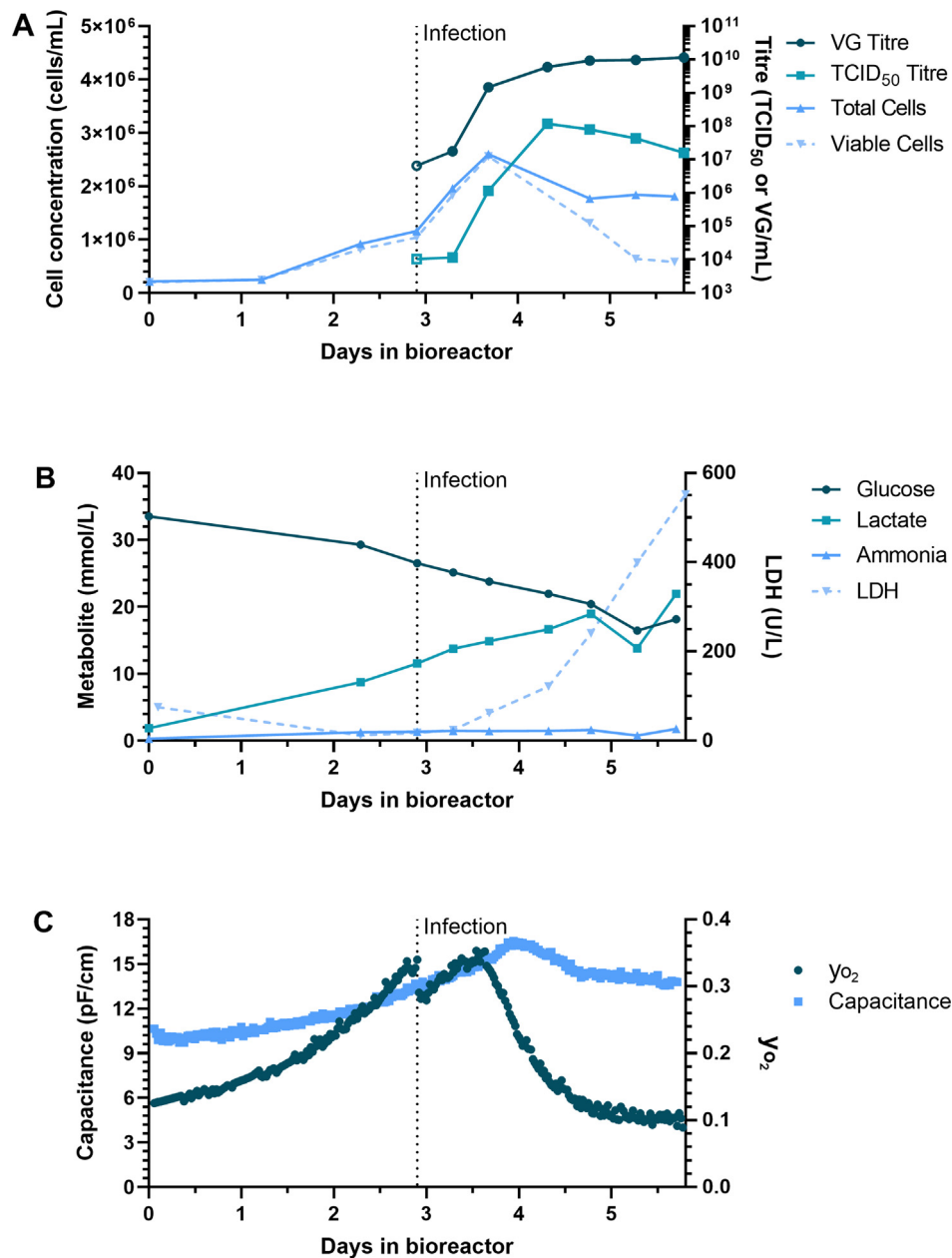
Fig. 3. Production of rVSV-ZEBOV in a bioreactor. Samples were harvested at indicated time points (about every 12 h from the time of infection). (A) Titres and cell counts. Cell concentration was evaluated manually using a hemocytometer, viability by trypan blue dye exclusion, functional viral titres by $TCID_{50}$ and viral genome titres by dPCR. The value shown for both titres at the time of infection was calculated from the titres of the vial used to infect the culture rather than titrated from a bioreactor sample. (B) Metabolite analysis. Glucose, lactate, ammonia and lactate dehydrogenase (LDH) evaluated by a Cedex Bio Analyzer. C) Online measurments performed during the culture. Data is reported on the graph every 30 min for yO_2 (input gas molar fraction of oxygen) and capacitance.

4. Conclusions

In the current study, rVSV-ZEBOV was produced in HEK 293SF cells in suspension without serum. First, a robust titration protocol was established, showing minimal variability between similar samples. Then, through various experiments, the ideal conditions were established to be $1–2 \times 10^6$ cells/mL grown in HyClone HyCell TransFx-H media infected at an MOI of 0.001 with a temperature shift to 34 °C and harvested after 48 h. Using these conditions, scalability of production in a 3.5 L bioreactor was shown reaching a titre of 1.19×10^8 $TCID_{50}$/mL at the peak of production, the equivalent of more than 4000 doses of vaccine per litre. The very promising results generated in this feasibility study provide the bases for further process intensification work to take advantage of the HEK 293SF manufacturing platform potential to produce at higher cell densities through use of perfusion or fed-batch process as demonstrated for other vectors [37]. The produced rVSV, in the original media, is then stable at 25 °C for at least 2 days without a major change to the titre allowing downstream processing to be done over multiple days. Alternatively, due to its resistance to freeze-thaw cycles in the production medium, the virus could also be frozen as it remains stable for at least a month. A concentration process was performed, but a full purification process remains to be developed. When administered to mice, rVSV-ZEBOV produced in suspension HEK 293SF demonstrated the ability to induce a ZEBOV-specific immune response.
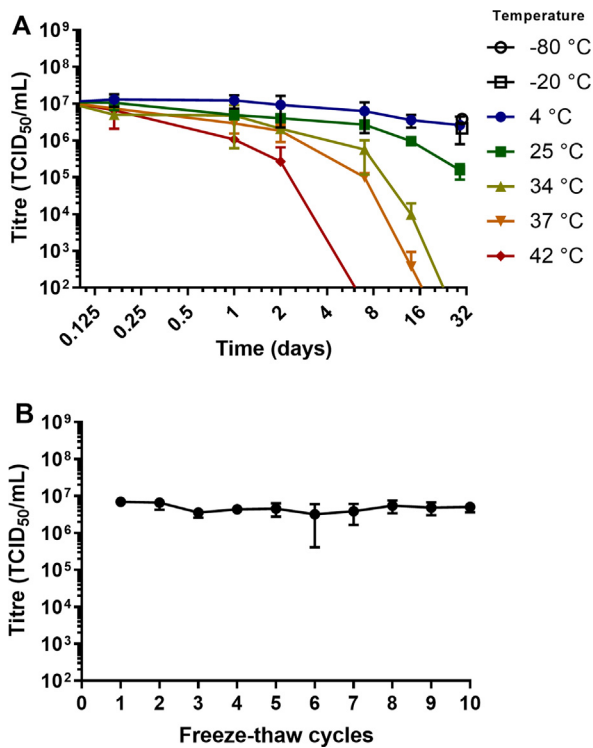
Fig. 4. Effect of temperature on functional titres of rVSV-ZEBOV. Functional titres were measured by $TCID_{50}$. Bars represent the mean of triplicate samples ± standard deviation. (A) Samples exposed to different temperatures for increasing amounts of time. Titres falling below the limit of detection of the assay are shown as an intercept of the axis. (B) Samples exposed to increasing numbers of freeze-thaw cycles.
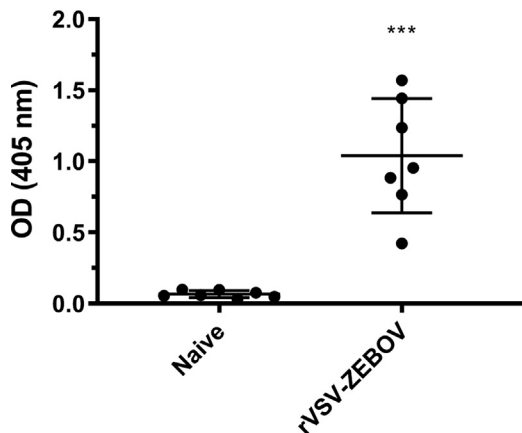

Fig. 5. Mouse total IgG binding antibody titers against Ebola GP at 4 weeks post immunisation. An ELISA plate was coated with recombinant EBOV GP protein to a final concentration of 2 μg/mL in PBS. 1:200 dilution of sera from immunised mice or pre-immune sera was used to detect the total IgG antibodies against Ebola GP antigen. Error bars represent the standard deviation among the seven mice. There was a statistically significant difference (p < 0.0001) between the two group means as determined by paired two-tailed t-test.

Currently the rVSV-ZEBOV vaccine is distributed at −65 °C. There is, therefore, an equally important formulation study that needs to be performed to retain the functionality of the candidate rVSV-ZEBOV vaccine after purification under standard storing conditions to enable efficient distribution or, alternatively, the development of lyophilisation could reach this goal.

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.

Acknowledgment

Author Contributions

JP performed the plasmid cloning and the viral rescue described in the supplementary methods. JFG, RG and AAK designed the cell culture experiments and JFG performed them. JFG and SK designed the dPCR protocol and SK performed this assay. JFG and SL performed the bioreactor production. PSC designed and supervised the purification process. HA designed and performed the animal studies. SA, GK, RG and AAK provided material and financial support. JFG wrote the manuscript and all authors revised the final version.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.09.044.

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