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Serum-free production of rVSV-ZEBOV in Vero cells: Microcarrier bioreactor versus scale-X™ hydro fixed-bed

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ABSTRACT

Ebola virus disease outbreaks have repeatedly occurred on the African continent over the last decades, with more serious outbreaks in recent years. Being highly transmissible and associated to high fatality rates, it constitutes a serious threat to public health. Vaccination, however, may allow for efficient control of its propagation. The most promising Ebola vaccine candidate to date, rVSV-ZEBOV, relies on a recombinant vesicular stomatitis virus construct, in which the native viral glycoprotein is replaced by the glycoprotein of Ebola virus (Zaire). However, its cell-based manufacturing process is still lengthy and cumbersome, thus urging the implementation of a new and more efficient bioprocess.

To address these issues, serum-free production of rVSV-ZEBOV in Vero cells has been studied with the aim to test an alternative upstream process. Until viable options of suspension cell culture are available, Vero cell cultures still rely on adherent bioprocesses and have thus been developed in this work. Particularly, a bioprocess developed with standard microcarrier bioreactor technology was successfully transferred to the novel single-use scale-X™ hydro fixed-bed.

1. Introduction

The recent 2014–2016 Ebola virus disease (EVD) outbreak in West Africa has caused the death of more than 11,000 people and marks the most devastating EVD outbreak to date (World Health Organization, 2016). Another severe outbreak in the Democratic Republic of the Congo is currently ongoing (World Health Organization, 2019a), and, consequently, a Public Health Emergency of International Concern (PHEIC) has been declared by the World Health Organization (WHO) (World Health Organization, 2019b). Among many research efforts to develop a vaccine against this disease, the recombinant vesicular stomatitis virus (rVSV) genetically engineered to express the glycoprotein of a Zaire Ebolavirus (rVSV-ZEBOV) is one of the most promising candidates. Originally developed by the Public Health Agency of Canada (Regules et al., 2015), this replication-competent virus has been demonstrated to be safe to administer to humans (Agnandji et al., 2016). Further, the interim results of a ring vaccination phase 3 efficacy trial carried out in Guinea have indicated very high protective efficacy of this vaccine candidate (Henao-Restrepo et al., 2015). The final analysis was conducted in a follow-up study, supporting the previous results (Henao-Restrepo et al., 2017).

With a potential vaccine against the EVD on the horizon, the need for a robust, efficient and affordable manufacturing process is increasing. However, information on the replication of rVSV-ZEBOV in cell culture and on bioprocess development efforts is limited. Recent studies investigated the production of rVSV-GFP (Elahi et al., 2019) and rVSV-ZEBOV (Gélinas et al., 2019) using human cell lines in serum-free suspension cultures. However, one study indicated the advantage of using Vero cells over other mammalian or avian cell lines regarding the infection efficiency of rVSV expressing the Ebola Reston glycoprotein (Takada et al., 1997). Furthermore, the Vero cell line has a long history of being used for the production of viral vaccines with over 40 years of experience. This includes the development and production of vaccines against dengue fever, influenza, Japanese encephalitis, polio, rabies, rotavirus and smallpox (Ammerman et al., 2008; Barrett et al., 2009).

Despite a favorable regulatory environment for using the Vero cell line, the current method for large-scale production of rVSV-ZEBOV employs roller bottles to grow Vero cells under animal-component free

Abbreviations: dPCR, digital polymerase chain reaction; EVD, Ebola virus disease; HEK, Human Embryonic Kidney cells; Hpi, hours post-infection; MOI, multiplicity of infection; PET, polyethylene terephthalate; PFU, plaque forming units; rVSV, recombinant vesicular stomatitis virus; rVSV-ZEBOV, rVSV genetically engineered to express the glycoprotein of a Zaire Ebolavirus

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conditions (Monath et al., 2019). Owing to the known limitations of a roller bottle manufacturing process, such as scalability or labor intensity (Merten, 2015), the present study was investigating alternative methods such as bioreactors for the production of rVSV-ZEBOV in Vero cells.

Although recent advances have been made in the suspension adaptation of Vero cells (Shen et al., 2019), doubling times remain above 40 h and suspension cultures are still complex and laborious. Therefore, Vero cell bioprocess development predominantly relies on adherent cultures. In this context, the use of microcarriers has been widely adopted for the production of viruses (Mattos et al., 2015; Rourou et al., 2007; Thomassen et al., 2014; Trabelsi et al., 2014) and scalability of up to 6000 L has been demonstrated (Barrett et al., 2009). Still, the scale-up of such a complex technology can be challenging. In addition, to meet the oxygen demand of the cells at larger scales, sparging is required and can cause increased foaming and shear stress on the cells. Besides, the impellers of stirred-tank bioreactors are introducing additional shear stress (Merten, 2015).

These burdens can be overcome by the application of packed or fixed-bed bioreactors. Recently, several commercial bioreactors of varying configurations have been developed and used for viral vector production. Examples include the basket-type BioFlo (Eppendorf) employing Fibra-Cel® disks (McCarron et al., 2019) or the iCellis bioreactor (Pall) using polyethylene terephthalate (PET) microfiber macrocarriers (Emmerling et al., 2016; Lesch et al., 2015; Powers et al., 2016; Valkama et al., 2018). In addition, the scale-X™ system from Univercells offers single-use, fixed-bed bioreactors of different sizes, ranging from bench-top (2.4 m^2 of surface area) to industrial scale (600 m^2 and above). The support matrix for cell growth consists of spiral-wound, non-woven PET layers. In contrast to conventional microcarrier systems, these reactors are characterized by low-shear stress that the cells are exposed to due to linear velocity of culture liquid, steric protection inside the carriers, as well as the inexistence of abrasion between carriers (Ozturk and Hu, 2005).

The aim of this work was to investigate specific process parameters for the production of rVSV-ZEBOV in Vero cells, such as MOI, temperature and harvest time. The findings were then applied to the development of a microcarrier bioprocess. Additionally, this work compares results of a microcarrier run to the results obtained by producing rVSV-ZEBOV in the scale-X™ hydro fixed-bed bioreactor.

2. Materials and methods

2.1. Cell lines and culture media

The adherent Vero cell line originated from ATCC CCL-81.5. Vero cells were maintained in static culture using VP-SFM medium (Thermo Fisher Scientific, USA) supplemented with 4 mM GlutaMAX (Thermo Fisher Scientific) at 37 °C and 5 % CO_2 in a humidified incubator (Infors HT, Switzerland). Cells were passaged twice weekly using TrypLE Express (Thermo Fisher Scientific) as dissociation reagent. HEK 293A cells used in this study were kindly provided by the National Research Council of Canada, Montreal, QC, Canada. The cells were cultivated in Dulbecco's Modified Eagle's medium (DMEM) (Wisent, Canada) supplemented with 4 mM L-glutamine (GE Healthcare, USA) and 5 % fetal bovine serum (FBS) (Corning, USA) without antibiotics. Both Vero and HEK 293A cell concentration and viability from static cultures were determined via the Vi-CELL XR cell counter (Beckman Coulter, USA).

2.2. Virus

Original rescue and initial amplification of rVSV-ZEBOV has been described previously (Gélinas et al., 2019). The viral seed stock used in this work was generated in adherent HEK 293A. In brief, HEK 293A cells were infected with rVSV-ZEBOV originating from the initial amplification at a multiplicity of infection (MOI) of 0.01. The supernatant was harvested 48 h post-infection (hpi), aliquoted and stored at −80 °C. For each subsequent experiment, a new aliquot of this rVSV-ZEBOV stock was used to avoid freeze-thaw of the virus.

2.3. 6-well plate virus studies

To study the infection kinetics of rVSV-ZEBOV in Vero cells, preliminary experiments were performed in 6-well plate format (Sarstedt, Germany). Cells were seeded at around 30,000 cells per well and infected the next day with rVSV-ZEBOV at different MOIs. Further, the influence of a temperature shift to 34 °C during the virus production phase was investigated. Samples taken from the cell culture supernatant were centrifuged for 5 min at 1200 × *g* to remove cellular debris, aliquoted and stored at −80 °C until further analysis.

2.4. Microcarrier cultures in bioreactors

Bioreactor cultures were performed in a 1 L bioreactor (Applikon Biotechnology, The Netherlands) equipped with a marine impeller, pH sensor, temperature sensor, and dissolved oxygen (DO) concentration sensor. Cytodex 1 microcarriers (GE Healthcare) were prepared according to the manufacturer's instructions. The microcarrier concentration in the bioreactor was 2 g/L. The medium VP-SFM was supplemented with 4 mM L-glutamine (GE Healthcare) to enable monitoring of L-glutamine consumption as well as with 0.1 % poloxamer 188 (Sigma, Germany) which was shown to reduce shear stress and to improve Vero cell growth on microcarriers (Kilburn and Webb, 1968; Rourou et al., 2009). Vero cells were collected from static culture and the bioreactor was inoculated at a cell density of 2 × 10^5 cells/mL in 850 mL working volume. The culture was agitated at 90 rpm and kept at 37 °C. The DO concentration was kept at 50 % air-saturation by continuous surface aeration of 5 mL/min air and injection of pure oxygen when required. The pH was set to 7.2 and regulated by injection of CO_2 into the headspace or addition of $NaHCO_3$ (90 g/L) (Sigma, USA). Samples were taken once or twice daily, depending on the progress of the culture, to subsequently determine viable cell density, metabolite concentration and virus titer. To determine the number of viable cells grown on microcarriers, nuclei were counted as described elsewhere (Trabelsi et al., 2005). Samples for metabolite analysis and virus titration were centrifuged for 5 min at 1200 × *g* to remove cellular debris, aliquoted and stored at −80 °C. The glucose concentration was estimated once daily and if required adjusted to 2 g/L by feeding glucose (Sigma) concentrate (180 g/L). In addition, L-glutamine was maintained at a minimum concentration of 2 mM. For virus production, Vero cells were infected with rVSV-ZBOV at an MOI of 0.01 once the desired cell density was reached and the temperature was shifted to 34 °C during the virus production phase.

2.5. Fixed-bed bioreactor cultivation

Fixed-bed cultivations were carried out in the single-use scale-X™ hydro bioreactor (Univercells, Gosselies, Belgium) with an available surface area of 24,000 cm^2. The system was equipped with a pH sensor, temperature sensor, and DO concentration sensor. The medium VP-SFM was supplemented with 4 mM L-glutamine but no poloxamer 188 was added as no significant shear stress on the cells was expected according to the manufacturer. Vero cells were collected from static culture and the fixed-bed was inoculated at a cell density of around 22,700 cells/cm^2 to be consistent with the seeding density per area of the microcarrier bioreactors. The initial working volume was 700 mL during the cell attachment phase. After 4 h, a bottle containing 2000 mL of fresh media was connected to the system and recirculation of culture liquid between the fixed-bed unit and the media bottle was set to 10 mL/min.

The culture was maintained at 37 °C, dissolved oxygen was set to 50 % air-saturation by continuous surface aeration of 30 mL/min air and regulated by injection of pure oxygen when required. The pH was kept

between 7.2 and 7.4, regulated by injection of CO_2 into the headspace or addition of NaOH (0.5 M) (VWR, USA). Sampling, feeding and virus infection was done as described for the microcarrier bioreactor. Additionally, recirculation of the culture liquid between the fixed-bed unit and the media bottle was stopped right before virus infection and turned back on again after 4 h to enhance cell-virus interaction during the initial infection phase.

To determine the number of viable cells grown during fixed-bed bioreactor cultivations, a sample carrier was aseptically extracted from the fixed-bed and processed according to the manufacturer's instructions. In brief, cells growing on the sample carrier were lysed using the Reagent A100 lysis buffer (Chemometec, Denmark), nuclei were stained and counted using a Neubauer improved hemocytometer (BRAND GmbH & Co. KG, Germany). When the medium exchange was carried out, the whole medium was replaced with pre-heated fresh medium, supplemented as before.

To evaluate the overall cell distribution and the cell homogeneity within the fixed bed, a cell distribution analysis was performed. At the end of the cultivation, the fixed bed was dismantled and from each of the two spiral wound cell support layers, 9 squares of approximately 1 cm^2 were cut out. Here, samples were taken from 3 different bed heights (i.e. 1 cm, 5 cm and 9 cm starting from the bottom of the bed). Further, at each bed height, samples were taken from 3 different vertical locations of the bed layer (i.e. 20 cm, 72 cm and 155 cm starting from the outside end of the spiral wound layer) corresponding to different radial distances of the wound fixed bed. Then, cells were counted as described above for the sample carriers.

2.6. Median tissue culture infectious dose ($TCID_{50}$)

The $TCID_{50}$ assay was used to determine the infectious titer of virus samples as follows: on day 1, HEK 293A cells were seeded into 96-well plates at 15,000 cells/well. On day 2, the medium was aspirated and a serial dilution of the virus sample in fresh growth medium was added to the plate. After at least 7 days, the cells were assessed for cytopathic effect via microscope observation. The $TCID_{50}$/mL value was calculated according to the method by Spearman–Kärber (Kärber, 1931). An internal virus reference standard was run in every assay alongside to ensure reproducibility of the assay. rVSV titration on HEK 293A was previously shown to give similar results as titration on Vero cells (Elahi et al., 2019).

2.7. Digital PCR

The number of viral genomes was quantified by digital PCR (dPCR) using the QX200™ ddPCR™ system (Bio-Rad, USA) with specific primers (5'-CTGCTGTCCGGAATCAGGTT-3' and 5'-GCCGTCTCCACAACTCA AGA-3') (Integrated DNA Technologies, Inc., USA) as described in MethodsX (in press). The resulting genomic titer was expressed in the number of viral genomes (VG/mL) and can be related to the number of viral particles containing a genome.

2.8. Metabolite analysis

During cell culture cultivations, the glucose concentration was estimated using the D-Fructose/D-Glucose Assay Kit (Megazyme, Ireland). More extensive metabolite analysis was performed offline via Bioprofile 400 (Nova Biomedical, USA) from samples that were stored at −80 °C. In addition, these samples were incubated at 65 °C for 5 min to inactivate the virus prior to metabolite analysis.

2.9. Statistical analysis

Where stated, statistically significant differences between two group means were determined by a Wilcoxon matched-pairs signed rank test using Prism software (version 8, GraphPad, La Jolla, CA, USA).

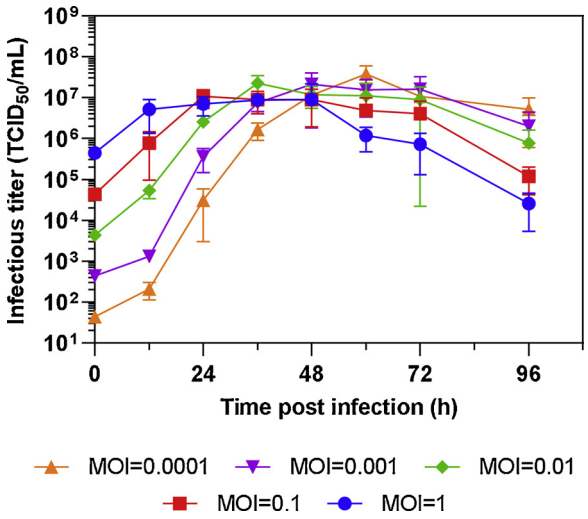

Fig. 1. rVSV-ZEBOV infection of Vero cells at different MOIs ranging from 0.0001 to 1 in 6-well plates. Infectious viral titers expressed as $TCID_{50}$/mL are plotted against the time post-infection. Bars represent the mean of the three independent samples ± standard deviation.

3. Results and discussion

3.1. rVSV-ZEBOV infection kinetics in Vero cells in 6-well plates

To first study the effect of MOI on rVSV-ZEBOV production, Vero cells grown in triplicate 6-well plates were infected with rVSV-ZEBOV at different MOIs. rVSV-ZEBOV production in Vero cells initially occurred exponentially (Fig. 1). It was further observed that the higher the initial MOI, the shorter the time it took to reach the maximum titer. Although in all cases maximum titers in the same range have been obtained, the highest overall titers observed were 2.28×10^7 $TCID_{50}$/mL at an MOI of 0.01 after 36 hpi and 3.82×10^7 $TCID_{50}$/mL at an MOI of 0.0001 after 60 hpi. Once the maximum titer had been reached, infectivity declined. This could be attributed to the instability of the virus at production temperature (37 °C). Similarly, it has been indicated that VSV-GFP produced in BHK21 cells lost infectivity over time when exposed to this temperature (Zimmer et al., 2013).

Compared to similar MOI screening experiments for the production of rVSV-ZEBOV in HEK 293SF cells, titers are slightly lower but similar production kinetics with regard to varying MOIs have been observed (Gélinas et al., 2019). Nevertheless, the said study used suspension cell cultures with a more than 10 times higher initial cell density as well as a lower production temperature of 34 °C.

Besides a high infectious viral titer, another important aspect of any bioprocess development is to increase the viral yield. When comparing the initial amount of virus used to infect the cells with the maximum resulting titer, the fold-increase was around 21 and 242 at MOI 1 and 0.1, respectively. In contrast, the fold-increase was superior at lower MOIs with around 5182, 48,235 and 867,424 at MOI 0.01, 0.001 and 0.0001, respectively.

Besides viral titer and viral yield, the quality in terms of infectivity per particle of the product is another critical aspect. With this in mind, the ratio of the viral genome copy number (VG/mL) to the infectious viral titer ($TCID_{50}$/mL) was used to determine the quality of the product, considering the number of viral genomes is correlated to the total (infectious and non-infectious) number of viral particles containing a genome. In this case, a lower ratio of VG/$TCID_{50}$ is desired since it indicates a better quality of the viral product in terms of infectivity per particle. Fig. 2 shows, for the previous experiment, the ratio of VG/$TCID_{50}$ for each MOI at their respective peak of infectious titer. With 94 VG/$TCID_{50}$, the most infectious viral particles were produced at an MOI of 0.01.

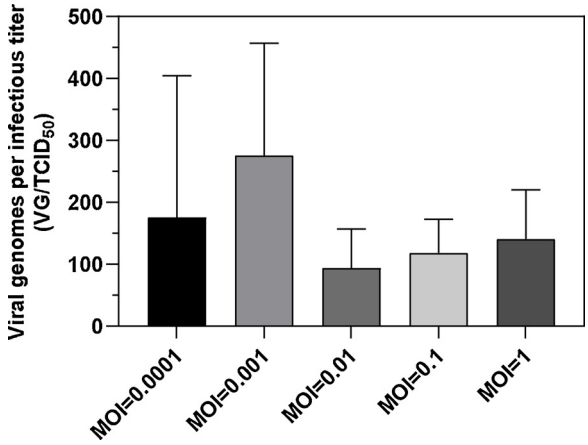

Fig. 2. Viral genome copy number (VG/mL) per infectious viral titer ($TCID_{50}$/mL) at peak titers for each MOI investigated. Ratios were determined for each MOI at their respective peak of infectious titer in the 6-well experiment, i.e. 60, 48, 36, 24 and 12 hpi for MOI of 0.0001, 0.001, 0.01, 0.1 and 1, respectively. Bars represent the mean of the three independent sample ratios ± standard deviation.

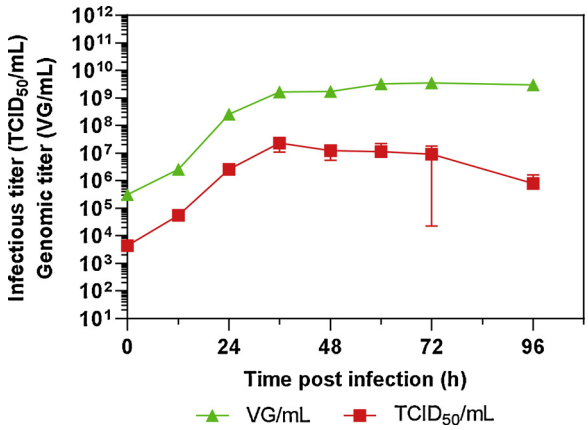

Fig. 3. rVSV-ZEBOV infection of Vero cells at MOI of 0.01 in 6-well plates comparing infectious titers and genomic titers. Infectious viral titers expressed as $TCID_{50}$/mL and the number of viral genomes expressed as VG/mL are plotted against the time post-infection. Bars represent the mean of the three independent samples ± standard deviation, however, the standard deviation of the genomic titer is too low to be visible on the logarithmic scale.

Focusing on the run performed at MOI of 0.01, Fig. 3 compares the accumulation of infectious viral particles and of viral genomes throughout the production run. As already shown in Fig. 1, the infectious viral titer reached a plateau after which it started to decline. In contrast, the number of viral genomes did not decline after reaching a plateau. Since the number of viral genomes can be linked to the number of viral particles containing a genome, this effect can be attributed to a loss of viral infectivity over time whereas the total viral particles count remained constant. This results in a lower quality of the product during the late stage of production as a higher ratio of infectious to total particles is desired. Consequently, the virus should be harvested right at the time of peak production to avoid losing infectivity of the final product.

Based on the data of this preliminary experiment, it was decided to use an MOI of 0.01 for all subsequent experiments. Despite a much higher fold increase in the infectious viral titer at an MOI of 0.0001, the better quality of the product (higher ratio of infectious to total viral particles) and the shorter production timeline (peak production at 36 hpi compared to 60 hpi) were crucial.

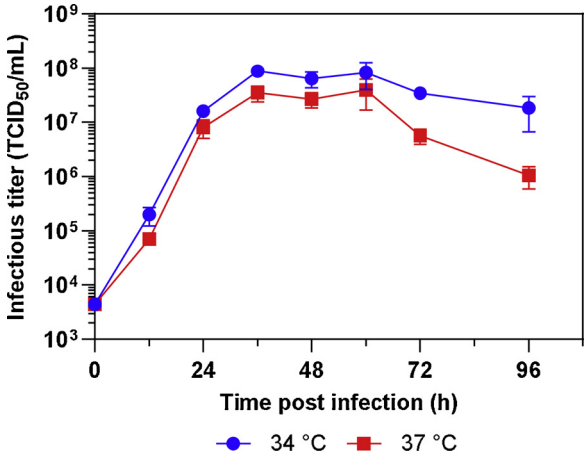

Fig. 4. rVSV-ZEBOV infection of Vero cells at different temperatures in 6-well plates. Cells were infected with rVSV-ZEBOV at an MOI of 0.01 and virus production was carried out at either 34 °C or 37 °C. Infectious viral titers expressed as $TCID_{50}$/mL are plotted against the time post-infection. Bars represent the mean of the three independent samples ± standard deviation, however, the standard deviation of some values is too low to be visible on the logarithmic scale. There was a statistically significant difference ($p = 0.0156$) between the two group means as determined by a Wilcoxon matched-pairs signed rank test.

3.2. Reduced temperature during virus production increases titer in 6-well plates

Next, the influence of a temperature shift on rVSV-ZEBOV production was studied in 6-well plates. Lowering the temperature of the culture to 34 °C at the time of infection has been reported to increase infectious titers of rVSV-GFP and rVSV-ZEBOV in HEK 293SF cells (Elahi et al., 2019; Gélinas et al., 2019). Hence, this temperature shift was compared to a parallel virus production at 37 °C. Fig. 4 shows the progression of rVSV-ZEBOV production over time. The maximum titer of 8.79×10^7 $TCID_{50}$/mL was reached at 36 hpi at 34 °C. Compared to the parallel production at 37 °C with otherwise identical conditions, this represents a 2.5-fold increase at this time point. There was a statistically significant difference ($p = 0.0156$) between the two group means as determined by a Wilcoxon matched-pairs signed rank test. In agreement, a 3.3-fold and 6-fold increase during production of rVSV-GFP and rVSV-ZEBOV, respectively, have been reported (Elahi et al., 2019; Gélinas et al., 2019). Of interest, the infectious titer did not decline as rapidly as at 37 °C, possibly indicating higher virus stability at lower temperatures. Moreover, the ratio of viral genomes to the infectious titer was 20.5 ± 2.5 VG/$TCID_{50}$ at 34 °C and 54.7 ± 8.1 VG/$TCID_{50}$ at 37 °C, and therefore more favorable at the lower temperature.

3.3. Microcarrier bioprocess of rVSV-ZEBOV production in Vero cells

To evaluate the scalability potential of rVSV-ZEBOV production in adherent Vero cells and to have better control over process parameters, the process was operated at bioreactor-scale. Thus, a bioprocess using microcarriers was operated with similar cell growth conditions as previously described (Rourou et al., 2007).

Vero cells were infected with rVSV-ZEBOV after 3 days of initiating the culture. The temperature was lowered to 34 °C just before infecting the culture, following the results of the previous 6-well plate temperature study.

As shown in Fig. 5, the Vero cell density did not continue to increase after virus infection. A slower cell growth rate at 34 °C can be linked to the preferred growth temperature of 37 °C of Vero cells. Moreover, VSV infections typically lead to the rounding of cells, cell detachment from surfaces and, finally, to cell death decreasing the viable cell density as observed during the virus production phase (Lichty et al., 2004).

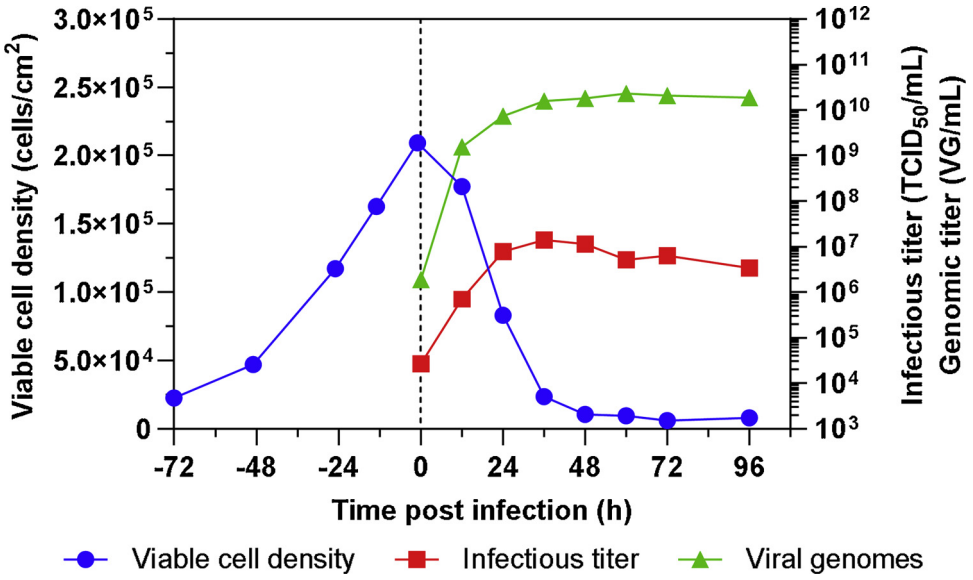

Fig. 5. Vero cell growth and rVSV-ZEBOV production in a 1 L bioreactor with temperature reduction to 34 °C during the virus production phase. Cells were grown on Cytodex 1 microcarriers and infected with rVSV-ZEBOV at an MOI of 0.01 after 3 days of cultivation.

Virus production peaked at 36 hpi, reaching 1.42 × 10^7 $TCID_{50}$/mL. As seen before, the infectious titer declined thereafter. Compared to the 6-well plate experiments, similar kinetics were obtained. However, 6-times lower infectious titers were reached. In addition, the ratio of viral genomes to infectious particles at peak production was, with a value of 1130 VG/$TCID_{50}$, more than 55-fold higher than at 34 °C in 6-well plates. Taken together these observations suggest that, while more total viral particles were produced in the bioreactor at 34 °C, a significant fraction of the virus is either defective or unstable under these bioprocess conditions. Further, with regards to the maximum cell density, a ratio of 7.6 $TCID_{50}$/cell and 8557 VG/cell has been obtained at peak production, supporting the argument that the productivity of infectious particles per cell could be improved.

3.4. Comparing Vero cell growth on microcarriers versus fixed-bed

The scale-X™ hydro is a novel fixed-bed bioreactor system from Univercells with the purpose of creating a high cell density environment that could be exploited for, amongst other things, viral vector production. First, Vero cell growth alone in the fixed-bed bioreactor was compared to its cultivation on microcarriers. In both cases, the same supplemented serum-free medium was used, omitting only the poloxamer 188 in the case of the fixed-bed. In addition, the bioprocesses were maintained at the same parameters (pH, DO, temperature, working volume to surface area ratio) to achieve comparable results.

Fig. 6 shows the growth profile of Vero cells in these two bioreactors as well as the metabolite concentration of the main substrates. Despite a lower initial cell seeding density in the case of the fixed-bed bioreactor (11,458 cells/cm^2) compared to the microcarrier process (22,700 cells/cm^2), similar cell growth patterns were obtained, reaching maximum cell densities of 240,000 cells/cm^2 on microcarriers and 271,605 cells/cm^2 in the fixed-bed, which is in agreement with prior data obtained in similar fixed-bed bioreactors (Valkama et al., 2018) and likewise consistent with data from microcarrier cultivations mentioned in the literature (Mendonça et al., 2002; Rourou et al., 2007). Besides, the consumption of glucose and L-glutamine was comparable, underlining similar cell growth kinetics.

At the end of the fixed bed bioreactor cultivation (192 h), a cell distribution analysis was carried out. As shown in Fig. 7, Vero cells were homogeneously distributed within the fixed bed in both, axial and radial direction. The overall cell count average between all 18 samples

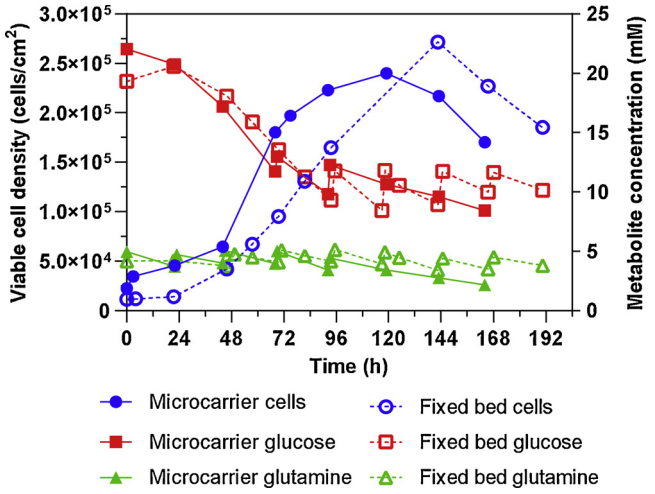

Fig. 6. Vero cell growth was compared between a microcarrier bioreactor and the scale-X™ hydro fixed-bed bioreactor. Cell growth in the fixed-bed is lagging behind the microcarrier process due to a lower cell seeding density. Concentrations of the main metabolic substrates indicate similar growth kinetics in the two systems.

resulted in 265,226 ± 60,138 cells/cm^2. However, the cell count determined from the sample strip at this time point was only 185,185 cells/cm^2 indicating a slight underestimation of the cell count by the sample strip method.

3.5. Fixed-bed bioreactor production of rVSV-ZEBOV in Vero cells

To compare rVSV-ZEBOV production in Vero cells in the scale-X™ hydro to the microcarrier bioreactor run at 34 °C (see Fig. 5), cells were seeded at similar cell densities in the fixed-bed (22,708 cells/cm^2) compared to the previous microcarrier bioreactor (22,727 cells/cm^2) and the bioprocess was carried out in a similar way, maintaining process parameters at the same setpoints. Cells were infected with rVSV-ZEBOV at an MOI of 0.01 after the same cell cultivation time of 3 days. Cell growth was slightly slower in the fixed-bed, reaching 152,263 cells/cm^2 at the time of infection (Fig. 8), where the microcarrier cell density was at 209,091 cells/cm^2. As seen for the microcarrier bioprocess at 34 °C, the cell density did not continue to increase

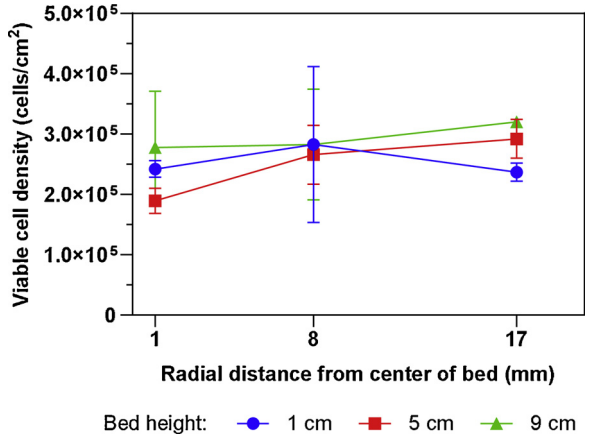

Fig. 7. Vero cell distribution within the fixed bed cell support layers. From each of the 2 layers, 9 samples were taken at different axial (bed height) and radial positions. Error bars indicate standard deviation between the two layers.

after infection, however, it did not decline as sharply in the fixed-bed bioreactor for the first 24 hpi.

The maximum infectious titer of 1.95×10^7 $TCID_{50}$/mL was reached after 24 hpi, indicating faster reaction kinetics while infectious viral titers were comparable. Faster reaction kinetics can be due to the fact that the cells were more tightly packed within the fixed bed compared to the microcarrier system, making it potentially more likely for a virus to attach to a cell within this space. In addition, for the first 4 h after infection, the recirculation of the culture liquid between the fixed-bed unit and the media bottle was suspended. This led to a 2.8-fold higher volumetric concentration of the virus during this time compared to the microcarrier bioreactor, while maintaining the same MOI, further increasing the likelihood of virus-to-cell attachment.

Moreover, at this time point, the ratio of viral genomes to the infectious titer was only 101 VG/$TCID_{50}$ implying a better quality of the produced virus compared to 1130 VG/$TCID_{50}$ at the peak production during the microcarrier process. Nevertheless, this also indicates a lower titer in total viral particles produced. With regards to the maximum cell density, a ratio of 14.4 $TCID_{50}$/cell and 1459 VG/cell has been obtained at peak production in the fixed bed bioreactor. This represents a 1.9-fold increase of the cell-specific productivity of infectious particles compared to the microcarrier system, but also a 5.9-fold decrease in the productivity of total viral particles per cell.

In the following experiment, another scale-X™ hydro fixed-bed bioreactor was inoculated with Vero cells as previously described. The aim was to investigate if a higher cell density at the time of infection would impact virus production. Hence, the whole medium was exchanged after 3 days and again after 6 days, to promote Vero cell growth during prolonged cultivation time. On day 6 of the process, cells were infected right after the medium exchange with the same MOI of 0.01 as before. Fig. 9 shows the profile of Vero cell growth and virus production of this experiment. Vero cell numbers continued to increase for an additional 12 hpi after which cell density sharply declined. Like the previous run, rVSV-ZEBOV production peaked after 24 hpi, reaching 2.59×10^7 $TCID_{50}$/mL. Although maximum infectious titers were not significantly increased compared to the previous run, the ratio of viral genomes to the infectious titer was with 31 VG/$TCID_{50}$ even lower than before and comparable to those obtained at 34 °C in 6-well plates. Whether this improvement in product quality can be attributed to the higher cell density or the medium exchanges needs to be verified in future experiments. However, the cell specific productivity of infectious particles was with 11.2 $TCID_{50}$/cell 1.3-fold lower than in the previous fixed bed bioreactor run.

In direct comparison to rVSV-ZEBOV production in HEK 293SF suspension cultures, where a bioreactor production led to 1.19×10^8 $TCID_{50}$/mL, production in Vero cells resulted in lower infectious yields. In the case of HEK 293SF cultures, a different media was selected, likely contributing to the different results. In addition, cells continued to grow for the first 24 hpi despite a temperature shift to 34 °C. Moreover, the cell specific yield was only 11.2 $TCID_{50}$/cell in Vero compared to 103 in HEK 293SF. Similarly, the genomic titer fell short with 7.90×10^8 VG/mL in Vero at 24 hpi, compared to 5.95×10^9 VG/mL in HEK 293SF at 36 hpi, at their respective peak of infectious titer (Gélinas et al., 2019).

In either case, this fixed-bed run produced 2412 vaccine dose equivalents, considering that each vaccine dose corresponds to 2×10^7 PFU (Henao-Restrepo et al., 2017). Infectious viral titers are lower compared to the roller bottle manufacturing process of this vaccine candidate, where titers of up to 8–9 log10 PFU/mL have been reported (Monath et al., 2019). In contrast to this process, however, which employs the parallel usage of around 300–400 roller bottles, bioprocess operation using bioreactors could reduce the amount of manual labor and hence the risk of contamination.

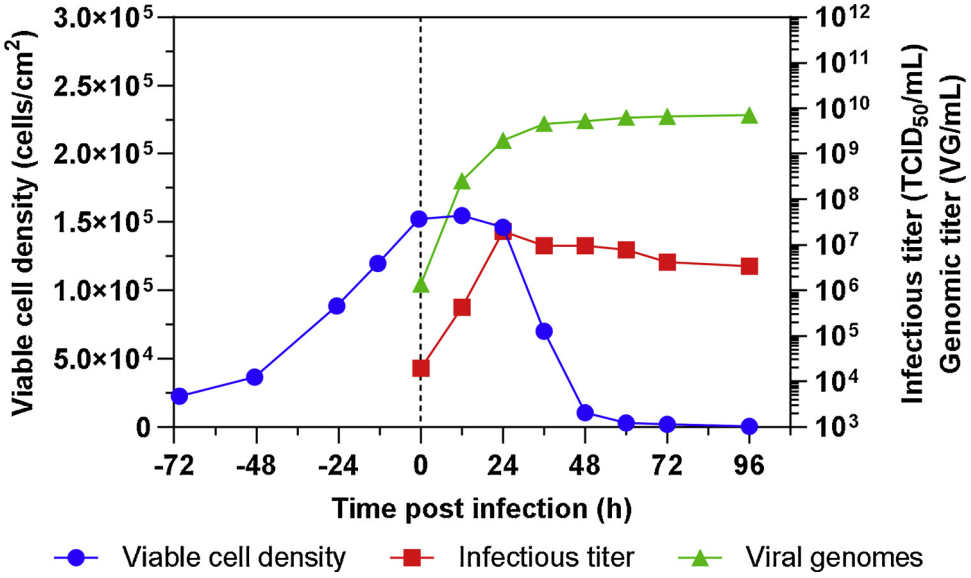

Fig. 8. Vero cell growth and rVSV-ZEBOV production in the scale-X™ hydro fixed-bed bioreactor. Cells were infected with rVSV-ZEBOV at an MOI of 0.01 after 3 days of cultivation. The temperature was lowered to 34 °C during the virus production phase.

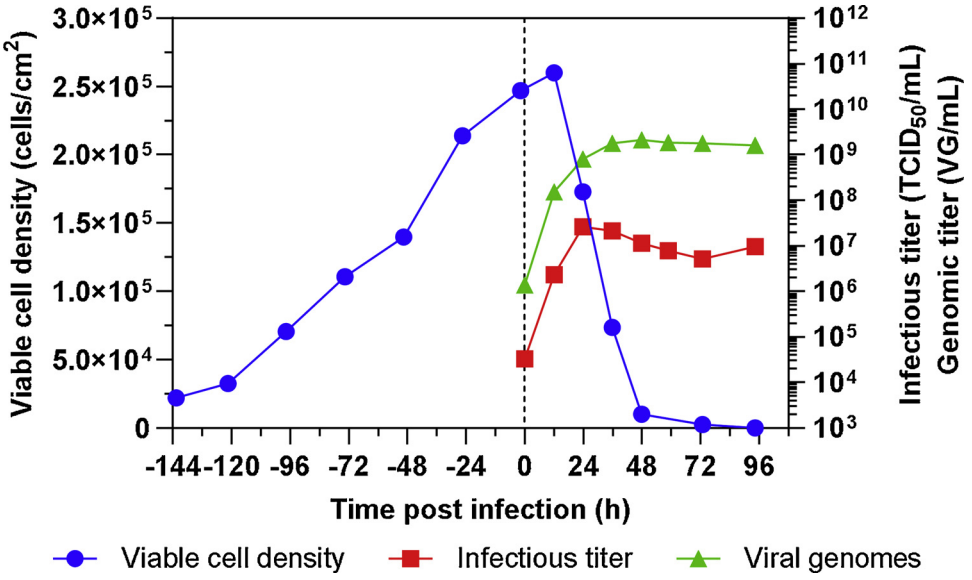

Fig. 9. Vero cell growth and rVSV-ZEBOV production in the scale-X™ hydro fixed-bed bioreactor with extended cell growth phase and two media exchanges. Cells were infected with rVSV-ZEBOV at an MOI of 0.01 after 6 days of cultivation. The temperature was lowered to 34 °C during the virus production phase. A complete medium exchange was performed on day 3 and again on day 6, immediately before infection.

4. Conclusion

In this study, rVSV-ZEBOV production in Vero cells was investigated. At first, experiments in 6-well plate format were used to determine process parameters such as MOI and temperature during the virus production phase. To determine the best conditions for optimized titer and yield, a cell-based assay ($TCID_{50}$) and a droplet PCR assay were utilized to scrutinize the infectious viral titer and the number of total viral particles. Following this optimization study, the bioprocess was developed in a microcarrier bioreactor and additionally in a novel fixed-bed bioreactor system.

Based on the 6-well plate experiments, it was found that using a MOI range of 0.01 to 0.0001 resulted in the best combinations of high infectious titer and viral yield. Moreover, to achieve efficient production timelines and to obtain the best quality product possible, the viral product should be harvested as early as possible, ideally at the peak of the infectious titer. In addition, the results provide further indication that production at 34 °C enables higher maximum infectious titers as shown previously (Gélinas et al., 2019), as well as a lower ratio of VGs to infectious units of the product.

Scale-up of the bioprocess to bench-scale using microcarrier technology resulted in slightly lower infectious viral titers compared to the 6-well plates with otherwise consistent viral kinetics. The scale-X™ hydro fixed-bed bioreactor produced similar infectious titers than the microcarrier run but achieved in shorter production timelines. Additionally, the fixed-bed bioreactor produced rVSV-ZEBOV at a lower ratio of viral genomes to the infectious titer and a higher cell-specific productivity of infectious viral particles.

Importantly, the findings of this study could be rapidly applied to current manufacturing processes of rVSV-ZEBOV in Vero cells to optimize the bioprocess and to ultimately lower the cost for Ebola virus vaccine manufacturing.

Future experiments should explore the potential of bioprocess improvements such as fed-batch and perfusion cultures for both, microcarrier and fixed-bed processes. Further, scalability of this process within the scale-X™ portfolio needs to be demonstrated.

CRediT authorship contribution statement

Sascha Kiesslich: Conceptualization, Investigation, Formal analysis, Writing - original draft, Writing - review & editing. **José Pedro Vila-Chã Losa:** Investigation, Writing - review & editing. **Jean-François Gélinas:** Conceptualization, Writing - review & editing. **Amine A. Kamen:** Conceptualization, Writing - review & editing, Supervision, Funding acquisition.

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.

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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.jbiotec.2020.01.015.

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