Development of a Vero cell platform for rVSV vector production

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Abstract

Viral vector-based vaccines are receiving increased attention, especially in light of the recent Ebola virus epidemic in West Africa and the COVID-19 pandemic. Their cell culture-based manufacturing, however, is lengthy and cumbersome, mostly using conventional production technologies. This work aims at contributing to the field of vaccine bioprocess engineering by further developing a cell culture platform to establish more efficient, scalable and cost-effective manufacturing technologies.

The main subject is the Vero cell line. We highlight its significance in the context of viral vaccine production by reviewing the prevailing literature on Vero cell bioprocess development. As a result, our analysis leads to a call for further research activities in this field to study and establish advanced technologies.

Applied to the recombinant vesicular stomatitis virus (rVSV) vaccine platform, and in particular the Ebola virus disease vaccine rVSV-ZEBOV, we study bioreactors for adherent Vero cell processes. Based on small-scale optimization studies, we develop microcarrier and fixed-bed bioreactors for serum-free rVSV-ZEBOV production. Further, using newly developed analytical assay techniques, we compare critical process and product characteristics, such as yield of infectious particles, cell specific productivities as well as ratio of total to infectious particles, and determine the optimal time of harvest.

Even though microcarrier and fixed-bed bioreactors are considered superior with regard to scalability when compared to the current rVSV-ZEBOV process employing roller bottles, suspension cell cultures are favored even more. Therefore, we intensified efforts to further develop a Vero suspension cell line as a platform for rVSV production. Following the adaptation to a commercially available medium, which can reduce the risk of lot-to-lot variants and potentially make the platform more amenable to work under standard conditions, we demonstrate the applicability of this system by developing bioreactor processes for three rVSV-vectored vaccine and vaccine candidates.

In conclusion, this work presents advancements in the field of bioprocess development for urgently needed vaccines. We show that the herein presented bioprocesses using adherent and suspension adapted Vero cells can serve as highly efficient systems for accelerated and scalable manufacturing of rVSV-vectored vaccine candidates.

Résumé

Les vaccins à base de vecteurs viraux ont reçu une attention accrue, en particulier à la lumière de la récente épidémie de virus Ebola en Afrique de l'Ouest et de la pandémie de COVID-19. Leur fabrication basée sur la culture cellulaire est, cependant longue et fastidieuse, utilisant principalement des technologies de production conventionnelles. Ce travail vise à contribuer au domaine de l'ingénierie des bioprocédés pour la fabrication de vaccins, en développant une plateforme de culture cellulaire et des technologies de fabrication plus efficaces, évolutives et rentables.

Le sujet principal est centré sur la lignée cellulaire Vero. En premier, nous soulignons son importance dans le contexte de la production de vaccins viraux à travers l'examen de la littérature en vigueur sur le développement de bioprocédés utilisant des cellules Vero. Les résultats de notre analyse appellent à démarrer de nouvelles activités de recherche pour étudier et mettre en place des technologies de pointe utilisant cette lignée cellulaire.

Appliqués à la plateforme de vaccination utilisant le virus recombinant de la stomatite vésiculaire (rVSV), et en particulier au vaccin contre la maladie à virus Ebola rVSV-ZEBOV, nous avons étudié les bioréacteurs pour les processus cellulaires Vero en cultures adhérentes. Sur la base des résultats d'études d'optimisation à petite échelle, nous avons développé des cultures avec support microporteurs et des bioréacteurs à lit fixe pour la production de rVSV-ZEBOV utilisant des milieux sans sérum. En outre, en utilisant des techniques d'analyse analytiques nouvellement développées, nous avons comparé les caractéristiques critiques du processus et du produit, telles que le rendement en particules infectieuses, les productivités spécifiques aux cellules ainsi que le ratio du nombre de particules totales au nombre de particules infectieuses, et nous avons déterminé le temps optimal de la récolte du produit viral.

Même si les supports microporteurs et les bioréacteurs à lit fixe sont considérés comme des procédés avancés par rapport au procédé actuel de production de rVSV-ZEBOV qui utilise des flacons agités, le mode de culture utilisant des cellules en suspension serait encore plus favorable d'un point de vue de mise à l'échelle. Par conséquent, nous avons intensifié nos efforts pour accélérer le développer d'une lignée cellulaire Vero en suspension et établir ce système comme plate-forme pour la production de vaccins utilisant le système rVSV. Suite

à l'adaptation à un milieu de culture commercialement disponible, afin de réduire le risque de variabilité d'un lot de milieu à un autre et potentiellement rendre la plateforme plus à même de fonctionner dans des conditions standards, nous avons démontrer l'applicabilité de ce système en opérant des productions en bioréacteur pour trois vecteurs rVSV correspondants a un vaccin et des candidats vaccins.

En conclusion, ce travail présente des avancées remarquables dans le domaine du développement de bioprocédés pour les vaccins pour répondre aux urgences des pandémies. Nous avons montré que les bioprocédés développés utilisant des cellules Vero adhérentes et adaptées en suspension sont des systèmes hautement efficaces pour la fabrication accélérée et évolutive de candidats vaccins à vecteur rVSV.

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Contribution of Authors

The present thesis consists of four manuscripts - three published (Chapter 1, 2 and 3) and one to be submitted (Chapter 4). I am the first or co-first author of all four manuscripts. The contributions of all authors to each manuscript are listed below.

Chapter 1:	**Vero cell upstream bioprocess development for the production of viral vectors and vaccines**
Published in	*Biotechnology Advances* 2020, 44 (Elsevier)
Authors:	**Sascha Kiesslich**, Amine A. Kamen
Contributions:	Conceptualization: **SK**, AAK; Writing - original draft: **SK**; Writing – review & editing: **SK**, AAK; Supervision: AAK, Funding acquisition: AAK

Chapter 2:	**Titration methods for rVSV-based vaccine manufacturing**
Published in	*MethodsX* 2020, 7 (Elsevier)
Authors:	Jean-François Gélinas, **Sascha Kiesslich**, Rénald Gilbert, Amine A. Kamen
Contributions:	JFG and **SK** contributed equally (co-first authors). Conceptualization: **SK**, JFG, AAK; Investigation: **SK**, JFG; Formal analysis: **SK**, JFG; Writing - original draft: **SK**, JFG; Writing – review & editing: **SK**, JFG, RG, AAK; Supervision: AAK, Funding acquisition: AAK

Chapter 3:	**Serum-free production of rVSV-ZEBOV in Vero cells: microcarrier bioreactor versus scale-XTM hydro fixed-bed**
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Chapter 4:	**Bioreactor production of rVSV-based vectors in Vero cell suspension cultures**
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Authors:	**Sascha Kiesslich**, Gyoung Nyoun Kim, Chun Fang Shen, C. Yong Kang, Amine A. Kamen
Contributions:	Conceptualization: **SK**, GNK, CYK, AAK; Investigation: **SK,** CFS, GNK; Formal analysis: **SK**; Writing - original draft: **SK**, GNK; Writing – review & editing: **SK**, GNK, CFS, CYK, AAK; Supervision: CYK, AAK, Funding acquisition: AAK

Contribution to the Original Knowledge

The study in chapter 1 reviews the prevailing literature on Vero cell bioprocess development for the production of viral vectors and vaccines with the aim to assess the recent advances in bioprocess development. The need for further research activities is critically underlined and bottlenecks to improve the Vero cell platform by taking advantage of recent developments in the cell culture engineering field are described.

The study in chapter 2 describes titration tools elaborated to assess the titre of rVSV-ZEBOV productions in a companion paper (Gélinas et al., 2019). A streamlined Median Tissue Culture Infectious Dose ($TCID_{50}$) assay to determine the infectious titer of this vaccine was established. In addition, a digital polymerase chain reaction (dPCR) assay to assess the total number of viral particles present in cell-free culture supernatants of rVSV productions was developed. These assays can be used to titre rVSV-ZEBOV samples and characterize the ratio of total particles to infectious units for monitoring process robustness and product quality attributes and can be used to titre samples generated in the production of further rVSV vectors. These analytical methods were essential to the studies of chapters 3 and 4 and have also been applied to two other studies where I contributed as a co-author (Gélinas et al., 2019; Mangion et al., 2020).

The study in chapter 3 investigates rVSV-ZEBOV production in adherent Vero cells. As a highly relevant vaccine against Ebola virus disease, this virus is manufactured using a conventional roller bottle process. However, this process is lengthy and cumbersome, thus giving the motivation to develop a new and more efficient bioprocess. At first, rVSV-ZEBOV infection kinetics were optimized in 6-well plates. Here, we determined optimal conditions with regard to multiplicity of infection and temperature during the viral replication phase. Next, we transferred the knowledge and developed a microcarrier process at the bioreactor scale. Bioreactors are more scalable to large manufacturing scales compared to roller bottle processes and in addition, there is better control over process parameters contributing to a more defined process which is essential in the context of production of human vaccines. Following, a process in a novel fixed-bed bioreactor was developed and compared to the microcarrier bioreactor. Importantly, the findings of this study could be rapidly applied to

the current manufacturing process of rVSV-ZEBOV in Vero cells to optimize the bioprocess and to ultimately lower the cost for Ebola virus vaccine manufacturing.

The study in chapter 4 continues the story of chapter 3. Here, instead of adherent growing Vero cells, suspension adapted cultures of this cell line were used. Suspension cultures are considered superior with regard to process scalability, since the cells do not have to be detached and re-attached from and to surfaces. Recently, a collaborator was successful in the adaptation of the Vero cell line to grow in suspension and provided us with this cell line (Shen et al., 2019). However, the use of in-house developed media, in this case IHM03, prevents other research groups from conducting studies using this system when media supply is not assured. Therefore, adaptation efforts in this work looked at the use of different media to establish a process using commercial media that could reduce the risk of lot-to-lot variations. As a result, we successfully adapted the suspension Vero cell line to grow in MDXK medium. Next, in shake flask at small scale we studied rVSV-ZEBOV replication in IHM03 and MDXK. We determined optimal conditions with regard to multiplicity of infection and studied the effect of cell density at the time of infection. More, we applied the process conditions to two other rVSV-based vaccine candidates against HIV and COVID-19, respectively. Thereafter, we developed bioreactor processes for all three rVSV strains and compared virus production in the two different media. Further, we compare the results to our previous study of rVSV-ZEBOV production in two adherent Vero cell bioreactor processes. Our findings suggest that the production of rVSV-ZEBOV is superior in suspension culture compared to previously developed microcarrier and fixed-bed bioreactors. Due to the better scalability, the suspension Vero system can serve as a viable alternative to the current Ebola virus disease vaccine manufacturing using roller bottles. Moreover, in the context of the current COVID-19 pandemic, this work shows relevant advancement in the field of bioprocess development for urgently needed vector-based vaccine candidates. We show that the herein presented bioprocess using suspension adapted Vero cells can serve as a highly efficient system for accelerated and scalable manufacturing of the COVID-19 vaccine candidate. Further, the quality of the produced viruses in terms of the ratio total particles to infectious particles is far superior, potentially leading to facilitated downstream processes and ultimately very economical manufacturing.

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List of Acronyms

CCID	cell culture infectious dose
COVID-19	coronavirus disease 2019
DIP	Defective interfering particle
DMEM	Dulbecco′s Modified Eagle′s Medium
dPCR	digital polymerase chain reaction
EVD	Ebola virus disease
FBS	Fetal Bovine Serum
FDA	US Food and Drug Administration
GFP	green fluorescent protein
HEK	Human Embryonic Kidney cells
HIV	human immunodeficiency viruses
hpi	hours post-infection
LV	lentiviral vector
MOI	multiplicity of infection
PAT	process analytical technology
PET	polyethylene terephthalate
PFU	plaque forming units
qPCR	quantitative polymerase chain reaction
rVSV	recombinant vesicular stomatitis virus
$TCID_{50}$	Median Tissue Culture Infectious Dose
TOH	time of harvest
TOI	time of infection
ZEBOV	Zaire Ebola virus

Preface

Cell culture systems have been established as an alternative to *in ovo* and *in vivo* viral vaccine production. In contrast to conventional strategies to produce vaccines, cell culture-derived vaccines have several advantages. Benefits include rapid manufacturing technologies, independency of the supply of chicken eggs as well as minimization of cross-contamination and allergy reactions due to the use of defined substrates free of animal sources.

Several cell lines have been used as manufacturing platforms such as BHK-21, HEK-293, MDCK and Vero. However, the Vero cell line represents the most widely used continuous cell line for the production of viral vaccines over the last 40 years. This includes the development and production of vaccines against dengue fever, influenza, Japanese encephalitis, polio, rabies, rotavirus and smallpox. Moreover, the WHO established a Vero cell bank meeting appropriate production requirements (Ammerman et al., 2008; Barrett et al., 2009).

In recent years, research has focused on the screening for serum-free media (Petiot et al., 2010b), metabolic analysis (Petiot et al., 2010c) and bioprocess optimization using microcarriers (Mattos et al., 2015; Thomassen et al., 2014). However, vaccine production processes using adherent Vero cells still suffer from limitations such as low virus productivity and limited cell growth.

Viral vaccine manufacturing processes commonly employ adherent cell lines. When adapting cells to grow in suspension, there is a potential to develop unwanted properties such as limited growth performance, low cell-specific yields or tumorigenic features. However, there is great economic potential of suspension cell lines in the vaccine manufacturing industry. Suspension cultures generally have the advantage of better process scalability. In contrast to adherent cell cultures, the steps of detachment from and re-attachment to surfaces (e. g. microcarriers or roller bottles) are being omitted when passaging the cells or scaling-up the process. This facilitates bioprocess development and can also lead to higher cell densities (Kluge et al., 2015).

Cell line adaptation to suspension growth can be difficult, but it has been demonstrated to be feasible for many important producer cell lines. For example, the CHO (Chinese Hamster Ovary) cell line, the HEK (Human Embryonic Kidney) cell line and many others have

been successfully adapted to grow in suspension culture. It has been reported that Vero cells were adapted to grow in suspension, capable of being used as a substrate for propagation of several viruses in a serum-free environment (Paillet et al., 2011, 2009; Thomassen et al., 2012b). Even though these reports showed promising results, no further studies by these authors investigated the potential of Vero cells in suspension. More recently, a research group published a work describing a successfully suspension adapted Vero cell line capable of producing rVSV (Shen et al., 2019).

The vesicular stomatitis virus (VSV) can be used as an effective replication-competent vaccine platform, inducing both cellular and humoral immunity. VSV infections cause acute disease in cattle, horse and swine, but for humans, infections are mostly asymptomatic. Therefore, recombinant VSV (rVSV) can be used to safely deliver and express foreign antigens.

Several strains of VSV have been propagated in Vero cells (Paillet et al., 2009; Stojdl et al., 2003; Wu et al., 2008). As an example of rVSV being used as a vaccine, a pseudotyped rVSV vector expressing the Ebola virus glycoprotein (rVSV-ZEBOV) has been demonstrated to be safe to administer to humans (Agnandji et al., 2016). Recently, this Ebola virus has been approved by the EMA and FDA and is available under the tradename Ervebo.

The hypothesis of my thesis is that through cell culture engineering, bioprocess optimization and analytical tool development, limitations of low rVSV productivity of the Vero cell culture system can be overcome and more scalable processes can be developed. Based on this, the three main research objectives of my project were 1) Development of analytical tools to quantify rVSV; 2) Process optimization and scale-up of rVSV-ZEBOV production; and 3) Development of a suspension Vero cell bioreactor process.

The following first chapter serves as the introduction to the thesis work and reviewed the prevailing literature on Vero cell bioprocess development for the production of viral vectors and vaccines with the aim to assess the recent advances in bioprocess development. The need for further research activities was critically underlined and bottlenecks to improve the Vero cell platform by taking advantage of recent developments in the cell culture engineering field were described.

Chapter 1

Vero cell upstream bioprocess development for the production of viral vectors and vaccines

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Abstract

The Vero cell line is considered the most used continuous cell line for the production of viral vectors and vaccines. Historically, it is the first cell line that was approved by the WHO for the production of human vaccines. Comprehensive experimental data on the production of many viruses using the Vero cell line can be found in the literature. However, the vast majority of these processes is relying on the microcarrier technology. While this system is established for the large-scale manufacturing of viral vaccine, it is still quite complex and labor intensive. Moreover, scale-up remains difficult and is limited by the surface area given by the carriers. To overcome these and other drawbacks and to establish more efficient manufacturing processes, it is a priority to further develop the Vero cell platform by applying novel bioprocess technologies. Especially in times like the current COVID-19 pandemic, advanced and scalable platform technologies could provide more efficient and cost-effective solutions to meet the global vaccine demand.

Herein, we review the prevailing literature on Vero cell bioprocess development for the production of viral vectors and vaccines with the aim to assess the recent advances in bioprocess development. We critically underline the need for further research activities and describe bottlenecks to improve the Vero cell platform by taking advantage of recent developments in the cell culture engineering field.

Keywords

Vero, Cell culture, Process development, Virus production, Optimization, Vaccines, Bioreactor, Microcarrier, Suspension culture

Abbreviations

CCID, cell culture infectious dose; MOI, multiplicity of infection; PAT, process analytical technology; PFU, plaque forming units; TOH, time of harvest; TOI, time of infection

Introduction

Vaccines are considered to be the most effective way to prevent and control infectious disease propagation. One of the main sources of infectious diseases are viruses. Research in the discovery of new vaccines or the development and improvement of existing vaccines against viral diseases is currently a world-wide high priority. Within this field, the cell culture-based production of viral vectors and vaccines is gaining increasing attention owing to the trend of moving away from established manufacturing strategies, such as production in chicken eggs or primary cell lines. Advantages of cell culture-based production processes include the independency from the supply of chicken eggs along with a minimization of cross-contamination or allergy reactions. Besides, the use of defined and serum-free cell culture media allows for more consistent processes. Further, cell cultures can be adapted to manufacturing processes involving bioreactors that are scalable, need less space and where process analytical technology can be deployed to monitor production and to control and maintain the process within defined parameters. Moreover, this allows for a more rapid manufacturing, especially during times of pandemics when vaccines are needed urgently (Aubrit et al., 2015).

Notably, the Vero cell line was the first continuous cell line (CCL) to be approved by the WHO for the manufacturing of viral vaccines for human use under specified regulatory guidelines (World Health Organization, 1987a, 1987b). Vero cells are considered non-tumorigenic below a certain passage number and safe to use as a substrate for vaccines. A summary of the establishment of Vero as a vaccine producing cell line and the creation of the WHO approved cell bank can be found elsewhere (Barrett et al., 2009).

This cell line has been established from cells extracted from the kidney of an African green monkey in 1962 (Yasumura and Kawakita, 1963). Several sub cell lines, such as Vero 81, Vero 76 or Vero E6, have been derived thereafter. Later genome analysis showed that the cells originated from a female of the species *Chlorocebus sabaeus* (Osada et al., 2014). The Vero cell line is a continuous cell line, hence it can be passaged indefinitely allowing extensive cell characterization and the creation of large cell banks, a valuable advantage over primary cell lines with limited passage capacities (e.g. chicken embryo fibroblasts). Vero cells are grown adherently, are interferon expression deficient (Emeny and Morgan, 1979)

and can be adapted to grow in serum-free conditions (Merten et al., 1994). They are widely used in many research areas, particularly virology, bacteriology, parasitology and toxicology (Ammerman et al., 2008).

Vero cells are susceptible to many viruses. One of the reasons for this is considered to be its interferon expression deficiency. The cells do not secret the signal peptide interferon upon infection with viruses and therefore the anti-viral defense mechanism of the cell is impaired (Emeny and Morgan, 1979). Examples of viruses that can be propagated in this cell line are listed in **Table 1**.

Historically, this broad range of susceptibility led to the development of vaccines that are based on the corresponding disease-causing virus produced in Vero cells, including vaccine types such as whole inactivated virus vaccines or live attenuated virus vaccines. This feature makes the Vero cell line an attractive host for the development of new vaccines against viral diseases. Several processes have been developed for the production of a number of vaccines and vaccine candidates using Vero cells, as can be found in the literature (**Table 2**). Importantly, several Vero cell-based vaccines have been marketed to date as shown in **Table 3**. Furthermore, with respect to the ongoing COVID-19 pandemic, three inactivated SARS-CoV-2-based vaccine candidates produced in Vero cells are currently in clinical trials (ChiCTR2000031809, ChiCTR2000032459, NCT04383574) (World Health Organization, 2020a).

Despite the wide regulatory acceptance and use of the Vero cell line for vaccine production, it is important to note that simian endogenous retrovirus sequences were detected in the genome sequence of Vero cells (Fukumoto et al., 2016; Ma et al., 2011; Sakuma et al., 2018). Safety in vaccine production is crucial and needs to be taken extremely seriously, and in particular the presence of endogenous retroviruses in pharmaceutical cell substrates is an important but technically difficult issue to control. However, an effective solution for adventitious viral detection could be a modified high-throughput RNA-sequencing method to ensure that the final vaccine product is safe for use in humans (Cheval et al., 2019).

Table 1: Examples of viruses that can be propagated in Vero cells.

Group	Family	Genus	Species	Envelope	Reference
I: dsDNA	*Adenoviridae*	*Mastadenovirus*	*Human adenovirus (HAdV)*	no	(Rhim et al., 1969)
	Herpesviridae	*Simplexvirus*	*Herpes simplex virus (HSV)*	yes	(Rhim et al., 1969)
	Herpesviridae	*Varicellovirus*	*Varicella zoster virus (VZV)*	yes	(Rhim et al., 1969)
	Poxviridae	*Orthopoxvirus*	*Vaccinia virus (VACV)*	yes	(Rhim et al., 1969)
	Poxviridae	*Capripoxvirus*	*Sheeppox virus (SPV)*	yes	(Trabelsi et al., 2014)
III: dsRNA	*Reoviridae*	*Orthoreovirus*	*Reovirus*	no	(Berry et al., 1999)
	Reoviridae	*Rotavirus*	*Rotavirus (RV)*	no	(Wu et al., 2017)
IV: +ssRNA	*Coronaviridae*	*Betacoronavirus*	*Middle East respiratory syndrome-related coronavirus (MERS-CoV)*	yes	(Chan et al., 2013)
	Coronaviridae	*Betacoronavirus*	*Severe acute respiratory syndrome coronaviruses (SARS-Cov and SARS-Cov-2)*	yes	(Ma et al., 2020; Spruth et al., 2006)
	Flaviviridae	*Flavivirus*	*Dengue virus (DENV)*	yes	(Liu et al., 2008)
	Flaviviridae	*Flavivirus*	*Japanese encephalitis virus (JEV)*	yes	(Wu and Huang, 2000)
	Flaviviridae	*Flavivirus*	*Yellow fever virus (YFV)*	yes	(Souza et al., 2009)
	Flaviviridae	*Flavivirus*	*West Nile virus (WNV)*	yes	(Lim et al., 2008)
	Flaviviridae	*Flavivirus*	*Zika virus (ZIKV)*	yes	(Nikolay et al., 2018)
	Matonaviridae	*Rubivirus*	*Rubella virus (RuV)*	yes	(Aubrit et al., 2015)
	Picornaviridae	*Enterovirus*	*Enterovirus A (EV-A71)*	no	(Wu et al., 2004)
	Picornaviridae	*Enterovirus*	*Enterovirus C/Poliovirus*	no	(Rhim et al., 1969)
	Picornaviridae	*Hepatovirus*	*Hepatovirus A (HAV)*	no	(Sun et al., 2004)
	Togaviridae	*Alphavirus*	*Chikungunya virus (CHIKV)*	yes	(Tiwari et al., 2009)
	Togaviridae	*Alphavirus*	*Ross River virus (RRV)*	yes	(Kistner et al., 2007)
V: -ssRNA	*Hantaviridae*	*Orthohantavirus*	*Hantaan orthohantavirus (HTNV)*	yes	(Choi et al., 2003)
	Orthomyxoviridae	*Alphainfluenzavirus*	*Influenza virus A*	yes	(Kistner et al., 1998)
	Orthomyxoviridae	*Betainfluenzavirus*	*Influenza virus B*	yes	(Kistner et al., 1998)
	Paramyxoviridae	*Morbillivirus*	*Measles morbillivirus (MeV)*	yes	(Rhim et al., 1969)
	Paramyxoviridae	*Morbillivirus*	*Peste des Petits ruminants virus (PPR)*	yes	(Silva et al., 2008)
	Paramyxoviridae	*Orthoavulavirus*	*newcastle disease virus (NDV)*	yes	(Rhim et al., 1969)
	Paramyxoviridae	*Orthorubulavirus*	*Mumps orthorubulavirus (MuV)*	yes	(Rhim et al., 1969)
	Pneumoviridae	*Orthopneumovirus*	*Respiratory syncytial virus (RSV)*	yes	(Rhim et al., 1969)
	Rhabdoviridae	*Lyssavirus*	*Rabies virus*	yes	(Mendonca et al., 1993)
	Rhabdoviridae	*Vesiculovirus*	*Vesicular stomatitis virus (VSV)*	yes	(Rhim et al., 1969)

Apart from Vero, other CCLs are also being used (MDCK) or considered (e.g. HEK-293, PER.C6, CAP, AGE1.CR, EB66) for the production of viral vaccines. However, the long-standing experience with the Vero cell line and its acceptance by regulatory authorities, continue to make it the substrate of choice for many vaccine manufacturers (Barrett et al., 2009; Genzel, 2015). Moreover, in direct comparison with other cell lines, Vero has shown superior virus productivity for a range of viruses, including dengue virus , enterovirus type

71, Japanese encephalitis virus, measles virus and Peste des Petits ruminants virus (Grein et al., 2017; Liu et al., 2008; Silva et al., 2008; Wu et al., 2004; Wu and Huang, 2000). For influenza virus, however, MDCK cells demonstrated superior virus production than Vero cells (Genzel et al., 2010).

Table 2: Examples of studies reporting potential Vero cell-based vaccines.

Disease	Type	Reference
Chikungunya fever	Inactivated	(Tiwari et al., 2009)
Dengue fever	chimeric, attenuated, live	(Blaney et al., 2007)
Hand-foot-and-mouth disease (EV-A71)	Inactivated	(Wu et al., 2015)
Hemorrhagic fever with renal syndrome (Hantaan virus)	Inactivated	(Choi et al., 2003)
Ross river fever	Inactivated	(Kistner et al., 2007)
SARS	Inactivated	(Spruth et al., 2006)
West Nile encephalitis	attenuated, live	(Monath et al., 2006)
West Nile encephalitis	Inactivated	(Lim et al., 2008)
Yellow fever	Inactivated	(Pereira et al., 2015)

Table 3: Examples of marketed Vero cell-based vaccines.

Disease	Vaccine	Type
Ebola virus disease	Ervebo®	rVSVΔG-ZEBOV-GP-based, live
Influenza (pandemic)	Vepacel®	whole virion, inactivated
Influenza (seasonal)	Preflucel®	trivalent, split, inactivated
Japanese encephalitis	IMOJEV®	recombinant chimeric, attenuated, live
Japanese encephalitis	Ixiaro®	attenuated, inactivated
Poliomyelitis	IMOVAX Polio®	trivalent, whole virion, inactivated
Poliomyelitis	OPV®	attenuated, live
Rabies	VERORAB®	whole virion, inactivated
Rotavirus gastroenteritis	RotaRIX®	monovalent, attenuated, live
Rotavirus gastroenteritis	RotaTeq®	pentavalent, reassortant, live
Smallpox	ACAM2000®	vaccinia-based, live

One example of a novel viral vaccine platform that can be applied to the Vero cell line is based on the vesicular stomatitis virus (VSV). Using recombinant strains of this virus (rVSV),

where the native surface glycoprotein (VSV-G) has been replaced by the surface glycoprotein of another virus, antigens of other viruses can be administered to humans in a safer way. This is especially interesting for cases where the disease-causing virus does not replicate well in cell culture or biosafety standards and protocols are too stringent to allow for efficient development of large-scale bioprocesses. As an example of this novel recombinant vaccine platform, the recently approved Ebola virus disease vaccine is based on an rVSV which expresses the Zaire Ebola virus glycoprotein (rVSV-ZEBOV) and is produced in Vero cells (Monath et al., 2019). In addition, vaccines against the acquired immune deficiency syndrome (rVSV-HIV), against Marburg virus disease (rVSV-MARV) or Lassa fever (rVSV-LASV), are currently in development (Geisbert and Feldmann, 2011; Racine et al., 2017).

However, cell culture-based viral vaccine manufacturing is challenged with the requirement to produce large quantities of virus at a relatively low cost to meet the global demand, particularly in low-income countries. Doses for marketed whole virus vaccines can be in the range of 10^6 to 10^8 viral particles, for instance 1×10^6 $CCID_{50}$ (RotaRIX®), 4.0-6.3×10^6 pfu (IMOJEV®) or 7.2×10^7 pfu (Ervebo®). Hence, it is necessary to develop vaccine manufacturing processes with a high yield. Bioprocess development and optimization strategies have been proven to intensify production, decreasing manufacturing time and cost.

More recently, Vero cells are also investigated as cell substrate for the production of certain oncolytic viruses (Grein et al., 2017). In contrast to infectious disease preventing vaccines, viral oncolytic therapy fights cancer by selectively targeting and killing cancer cells through oncolytic virus replication. Examples of oncolytic viruses that could potentially be produced in Vero cells are strains of HAdV, HSV, MeV, reovirus, VACV, VSV (Twumasi-Boateng et al., 2018). The main challenge in the production of oncolytic viruses is the even greater demand of functional viral particles per treatment compared to the amount needed per vaccine dose. The demand can be compared to that from gene therapy applications, and is generally more than 1000 times higher than for vaccination (Weiss et al., 2012). For MeV based systems, for example, even 10^7 - 10^8 times more MeV particles are required for oncolytic therapy treatment of one patient than for one vaccine dose (Grein et al., 2017). Therefore, reaching higher production yields is even more crucial for the implementation and establishment of this new treatment technology.

In the following, this work focusses on the use of the Vero cell line as a platform for the production of viral vectors and vaccines. The literature of Vero cell upstream bioprocess development is reviewed with respect to current trends and strategies to intensify virus production.

Cell line engineering to increase cell-specific virus productivity

Independently of the cultivation mode, increasing the cell specific virus productivity is required to improve the Vero cell line as a substrate. In recent years, RNAi and CRISPR screens have been conducted to search for target genes that upon knockdown or knockout would increase the production of viruses in cell culture. These studies use RNAi or CRISPR libraries that are targeted towards the human gene sequence. Libraries that are designed for the Vero cell line do not exist to this date and impede whole genome-wide studies of this cell line. One of the obstacles is the lack of an annotated genome. A draft sequence of the Vero genome has been published (Osada et al., 2014), but detailed genomic information is still not available.

An alternative method to search for gene sequences of interest in the Vero cell genome is to refer to the corresponding sequences in the *Chlorocebus sabaeus* genome (NCBI assembly accession: GCF_000409795.2) (Warren et al., 2015). However, difficulties in genetic engineering approaches arise since many changes in the Vero cell genome occurred with respect to its ancestral origin, hence an annotated whole genome of the Vero cell line is desired. For example, similar efforts have been made in this context for the CHO-K1 cell line, where genetic engineering tools such as CRISPR can be readily applied (NCBI assembly accession: GCF_000223135.1) (Schmieder et al., 2018; Shin and Lee, 2020).

Despite these obstacles, a study investigated the potential of genetic engineering applied to the Vero cell line to reduce the manufacturing cost of viral vaccines (van der Sanden et al., 2016). A primary genome wide RNAi screen was performed in a human cell line. Upon gene knockdown, poliovirus production was shown to be increased. The top gene hits were then validated in Vero cells and a significant increase of viral titer was reported. These exciting results indicated the potential on cell-based vaccine manufacturing and resulted in follow-up studies (Murray et al., 2017; Wu et al., 2017). Similar to the original work, these studies

investigated the application of gene knockdown or knockout for the increase of production of other viruses in Vero cells. All these studies were conducted at small scale in tissue culture well-plates. When the work was scaled-up to T-flasks and microcarrier cultures and applied to a different Vero cell line, the results could not be repeated (Hoeksema et al., 2018). The authors highlighted the problems they were facing with regard to the availability of genomic information of the Vero cell line. The RNAi and CRISPR constructs targeting the Vero cell line in the work by van der Sanden et al. were designed using the human gene sequences, while Hoeksema et al. designed gRNAs with regard to the draft Vero genome (Osada et al., 2014). Nevertheless, it was only possible to validate gene target hits that were identified by using a RNAi library targeting the human gene sequence.

To further study the application of these novel genetic engineering technologies to the Vero cell line, tools need to be developed to carry out such screens that are tailored to the Vero genome. Moreover, to study the apparent problem of the difference of knockdown versus knockout effects, a fully annotated Vero genome would enable the application of transcriptomics as well as next-generation sequencing and gene editing tools. In addition, different Vero strains are being used and an annotated genome is needed to compare those and explain the difference in results of experiments in those cell lines.

Bioprocess development for the production of viruses

In general, the cell culture-based process for virus production can be divided into two parts, the upstream and the downstream process. At first, cells are cultivated to generate enough substrate during the cell expansion phase. The cells are then infected to initiate the virus replication phase. Once the virus production has been completed and a peak in virus concentration has been reached, the downstream process begins by harvesting the culture. Typically, this is followed by steps of clarification, virus inactivation, virus purification and final product formulation (Barrett et al., 2017).

To reduce the overall manufacturing cost, the goal from an upstream bioprocess development standpoint is to establish a process with optimized viral productivity. Given this, a guiding principle is that the more cells are available as substrate, the more virus can be produced. Furthermore, the aim is to keep the cells in the best physiological state for virus

production. Therefore, the main goal of upstream process development is to generate as much cell material as possible while maintaining the cells at a state of optimal virus productivity. This can be achieved by optimizing the time of infection (TOI) and process control is responsible for maintaining optimal conditions important for cell growth and viability. Examples of process parameters are temperature, pH, dissolved oxygen, osmolality, shear stress and nutrient supply (Tapia et al., 2016).

Virus production begins with the infection of the culture which is carried out by addition of a quantified virus stock solution at a pre-determined multiplicity of infection (MOI), which is the ratio of virus particles per cell at the TOI. The virus attachment and entry into the cell is a critical step. It depends on specific properties of cell-virus interaction, and additional process changes might be required, such as lowering the pH, reducing the temperature or changing the agitation rate (Frazatti-Gallina et al., 2001; Trabelsi et al., 2005).

Then, during the replication phase, the host cell's machinery is taken over for the production of viral particles and optimal process conditions are still essential to yield the best quality product possible. Once the intracellular viral replication is complete, cellular resources like nucleic acids, amino acids and cell membrane will be depleted, ultimately leading to cell death and concluding the upstream process part (Ursache et al., 2015).

Vero cells are typically grown adherently. Therefore, adapted bioprocesses for adherent cultures need to be implemented. The initial stages in developing an adherent process are usually performed in cell culture well plates and T-flasks. These cultures are incubated in static incubators with control over temperature, CO_2-level and humidity. When passaging, cells need to be detached from the surface which is typically done using enzymatic solutions such as trypsin. After detachment, cells are centrifuged, resuspended in fresh medium and seeded in a new vessel. These small-scale cultures can be observed under the microscope to examine the cells and monitor the cell growth. Besides, exchanging the medium is easy and multiple conditions can be tested at the same time for optimization purposes.

To increase the number of cells, scale-out by increasing the surface area employs larger T-flasks, multilayer systems or roller bottles. However, at larger scales or when handling multiple vessels simultaneously, cell passaging and virus harvest can become quite laborious. In addition, options for process monitoring and control are very limited. Nevertheless, these technologies are still in use for the production of well-established

vaccines or part of the seed train for the generation of cell substrate for large-scale systems (Gallo–Ramírez et al., 2015).

In recent years, novel fixed-bed bioreactors systems have been developed to increase the available surface area for adherent cell growth even more and to tackle process development challenges, i.e. scale-up. Here, cells adhere to a densely packed support matrix. The culture liquid is then pumped through the fixed-bed to provide the cells with oxygen and nutrients. As a result, cells are not in contact with gas bubbles that occur during sparging as in stirred-tank bioreactors. Shear stress is reduced and negligible within the fixed-bed as compared to agitation-induced shear stress from impellers. Perfusion application requires no cell retention system and eliminates the risk of fouling of such filters.

There are many types of fixed-bed bioreactor systems, and no system has prevailed so far. For example, Vero cells have been successfully cultivated and used for virus production on packed Fibra-Cel® disks (Knop and Harrell, 2007), in a packed-bed BelloCell oscillating bioreactor (Toriniwa and Komiya, 2007), iCellis® bioreactor (Rajendran et al., 2014) and scale-X™ hydro fixed-bed bioreactors (Berrie et al., 2020; Kiesslich et al., 2020).

Scale-up to generate large manufacturing lots in fixed-bed bioreactors has been demonstrated for adenoviral and lentiviral vector production in HEK293 cells using the commercial sized iCellis® 500 (Leinonen et al., 2019; Lesch et al., 2015), indicating that this technology presents a feasible option for adherent cell-based processes.

Microcarrier process development

Cell expansion on microcarriers

The vast majority of adherent Vero cell bioprocesses in agitated systems is carried out using the microcarrier technology. Here, cells are attached to beads that are designed to be maintained in suspension in the agitated culture. There are many types of microcarriers, which are mainly divided into solid and macroporous ones and differing in their surface properties. Several studies have compared different types of microcarriers for the propagation of Vero cells (Arifin et al., 2010; Berry et al., 1999; Ng et al., 1996; Rourou et al., 2009a; Souza et al., 2005; White and Ades, 1990; Yokomizo et al., 2004). In reality, the use of Cytodex 1 microcarriers has prevailed in the last decade (Genzel et al., 2010; Grein et al.,

2018; Rourou et al., 2014; Sousa et al., 2019; Thomassen et al., 2013b). Cytodex 1 microcarriers are solid, dextran-based microcarriers displaying a charged surface that are on average 180 µm in diameter. Nevertheless, novel microcarriers for the growth for Vero cells are being studied (Kurokawa and Sato, 2011; Sun et al., 2015).

Cell attachment to microcarriers during the inoculation of the culture has been studied under different conditions such as cell to carrier ratio (Mendonça and Pereira, 1995; Souza et al., 2005; Yokomizo et al., 2004), media components (Mendonça et al., 1999; Souza et al., 2005), stirring speed (Ng et al., 1996) or reduced volume (Mattos et al., 2015). Recently, advanced PAT has been used to observe the attachment process (Grein et al., 2018). On the contrary, cell detachment from microcarriers *in situ* has been studied for the purpose of scale-up and seed-train improvement (Rourou et al., 2013; Sousa et al., 2019).

The microcarrier technology can be carried out in stirred-tank bioreactors. One advantage of bioreactors is that the process parameters can be scaled up to larger sized bioreactors. In contrast to scale-out, using consecutively larger vessels until the final production stage is expected to reduce operational cost. Moreover, the usage of bioreactors for animal cell culture has been established with many different configurations available and is suitable for large-scale operation. In addition, scale-down approaches can be used to carry out process optimization experiments on a smaller and more cost-effective scale, which in turn can be directly translated to improve the large scale-process (Gallo–Ramírez et al., 2015).

Additionally, stirred-tank bioreactors provide the option of applying a wide range of process analytical technology and process control strategies. A key point is that controlled mixing at a constant rate keeps the culture homogeneous. Cells, nutrients and dissolved gases are distributed evenly, minimizing local limitations of any component and allowing for accurate measurement of process parameters throughout (e.g. temperature, pH, dissolved oxygen). In addition to the online monitoring of the culture, bioreactors allow for sampling of small volumes for offline analyses without disturbing the culture (e.g. cell count, metabolite concentration).

Process scale-up in bioreactors using microcarriers has been established. The first large scale process involving Vero cells growing on microcarriers was developed for the production of a whole inactivated poliovirus vaccine almost 40 years ago (Montagnon et al.,

1981) and culture systems for virus production of up to 6000 L have been developed thus far (Barrett et al., 2017). The development of a seed train for scale-up has been described for final volumes of 20 L, 200 L, 500 L and up to 1200 L (Kistner et al., 1998; Sousa et al., 2019; Sugawara et al., 2002; Wu et al., 2015).

Especially at these large scales, hydrodynamic shear forces on the cells associated with the agitation rate can have a significant effect on the cells. Therefore, the effect of stirring speed on the growth of Vero cells grown on microcarriers has been investigated (Ng et al., 1996; Souza et al., 2005; Wu and Huang, 2000). In general, agitation needs to be high enough to keep microcarriers suspended in culture and to ensure homogenous mixing as well as sufficient gas transfer. At the same time, stirring should be maintained at a minimum since high agitation rates can impair cell growth significantly due to shear stress. Further, it was found that surface aeration can improve cell growth as compared to sparging due to reduced shear stress. However, poloxamer-based shear protectant agents can protect Vero cells, which can be especially important when sparging is required due to a higher demand of oxygen (Rourou et al., 2009a, 2007). In this context, one study looked at the use of two impellers instead of one as well as a reduced dissolved oxygen set point. As a result, there was less formation of foam and aggregates and the required amount of sparging was decreased, ultimately reducing the shear stress on the cells (Mattos et al., 2015).

The cell density in adherent processes is limited, amongst other things, by the surface area, which is determined by the total number of microcarriers. The use of different concentrations of Cytodex 1 microcarriers has been studied, with concentrations ranging from 1.5 to 10 g/L. While on the one hand higher microcarrier concentrations consistently lead to higher cell densities, technical challenges such as oxygen and nutrient limitations occur on the other hand. Additionally, a higher frequency of direct bead to bead interactions can impair cell growth (Mattos et al., 2015; Mendonça and Pereira, 1998, 1995; Trabelsi et al., 2006; Wu et al., 2004). For example, in batch mode, cell densities of 2.1×10^6 cells/mL, 2.6×10^6 cells/mL and 3.6×10^6 cells/mL have been reached on Cytodex 1 at microcarrier concentrations of 2 g/L, 3 g/L and 6 g/L, respectively (Kiesslich et al., 2020; Rourou et al., 2007; Trabelsi et al., 2006).

In contrast, a process in perfusion mode can lead to higher cell densities than batch mode. During perfusion, fresh medium is constantly added to the culture and spent medium is

removed at the same rate. This can be applied to a microcarrier processes by implementing retention systems such as spin filters. In this case, microcarriers can be separated from the culture liquid easier compared to single cells in suspension cell cultures. Direct comparison between batch and perfusion mode has been studied extensively and demonstrated higher Vero cell densities for perfusion cultures, as it can, for example, sustain sufficient substrate levels for prolonged time and reduce amounts of inhibiting by-products (Mendonça and Pereira, 1998, 1995; Rourou et al., 2007; Sousa et al., 2019; Thomassen et al., 2014; Trabelsi et al., 2006, 2005). Maximum cell densities of 2.1×10^6 cells/mL, 4.7×10^6 cells/mL and 7.8×10^6 cells/mL have been reported for perfusion processes on Cytodex 1 at a microcarrier concentration of 2 g/L, 3 g/L and 10 g/L, respectively (Mendonça and Pereira, 1998; Sun et al., 2015; Trabelsi et al., 2005). In recirculation mode, where the outlet medium of a perfused culture is pumped into the feeding container, even higher cell densities of 5.5×10^6 cells/mL and 10.1×10^6 cells/mL have been reported at a Cytodex 1 concentration of 3 g/L and 6 g/L, respectively (Rourou et al., 2009a; Trabelsi et al., 2006).

Providing the cells with the right amount of nutrients is a complex task, especially at high cell densities. On one hand, studies have been carried out to compare the effect of different kinds of media on Vero cell growth (Arifin et al., 2010; Chen et al., 2011; Frazatti-Gallina et al., 2001). In particular, Vero cell growth was compared between serum-containing and serum-free conditions on microcarriers (Butler et al., 2000; Frazatti-Gallina et al., 2001; Mendonça et al., 1999; Merten et al., 1994; Quesney et al., 2003, 2001). Additionally, the development and improvement of serum-free medium for Vero cells has been described (Butler et al., 2000; Petiot et al., 2010b; Rourou et al., 2009b). Overall, the use of serum-free media has prevailed and is preferred by vaccine manufacturers due to regulatory concerns regarding the risk of contamination. Additionally, the undefined nature and lot-to-lot variation of serum make it an unattractive substrate in the pharmaceutical industry.

On the other hand, when looking more closely at the metabolic level, studies have investigated the use of different substrates and feeding strategies to increase Vero cell growth and viability (Mendonça et al., 2002; Mendonça and Pereira, 1998; Nahapetian et al., 1986; Petiot et al., 2010c; Trabelsi et al., 2006, 2005). The main metabolites in Vero cell culture are glucose, glutamine, glutamate, lactate and ammonia. Analyzing their consumption and formation, respectively, contributed to the evaluation of process

development of bioprocesses at different scales (Thomassen et al., 2013b), different culture modes (Thomassen et al., 2014) or between different bioreactor systems (Kiesslich et al., 2020; Thomassen et al., 2012a). Further, it can be used to ensure that the cell growth rate is not impaired by limiting or inhibiting levels of metabolites, for example by feeding additional nutrients or adjusting the perfusion rate depending on residual substrate concentration (Sun et al., 2015).

Moreover, a method was developed to correlate glucose and lactate concentrations to measurements of near-infrared spectroscopy in cell culture media during bioreactor process. The study made use of optic fiber probes for *in situ* monitoring which could advance feeding strategies even further (Petiot et al., 2010a). Similarly, the online monitoring of the cell density has been studied recently. Here, dielectric spectroscopy can be used to measure cell growth and death in bioreactor cultures (El-Wajgali et al., 2013; Grein et al., 2018, 2017; Petiot et al., 2012).

Virus production in microcarrier cultures

Next to intensifying the cell expansion phase to obtain a high quantity and quality of cell substrate, establishing a microcarrier process that yields in a viral titer that is as high as possible is crucial. Similar to the previous section, different types of microcarriers have been studied for virus production as well. In direct comparison, it depends on the virus which type of microcarrier leads to a higher titer in Vero. For example, the use of porous microcarriers yielded in a slightly higher titer for rabies virus production (Yokomizo et al., 2004). In contrast, and despite higher cell densities, lower titers were produced on porous carriers for reovirus compared to production on solid ones (Berry et al., 1999). However, the use of non-porous Cytodex 1 has also prevailed in the virus production phase, which can be linked to its dominant use in the cell expansion phase as described above. In addition, the microcarrier concentration can be optimized specifically for the virus production phase, as shown for enterovirus type 71(Wu et al., 2004).

Especially for enveloped viruses, production in bioreactors can be affected by shear forces due to agitation and sparging. For instance, Grein et al. found that measles virus is highly sensitive to shear stress in bioreactors. Under certain conditions, sparging and agitation reduced viral titers 1000-fold. Here, as a solution, headspace aeration was able to

provide enough oxygen to the Vero cell culture while reducing shear-induced impact from sparging (Grein et al., 2019).

Reduced agitation rates, such as intermittent stirring, were found to improve reovirus production when applied during the initial virus infection phase, promoting virus-cell attachment (Berry et al., 1999). Nevertheless, this strategy can also decrease Vero cell growth, which is likely caused by a resulting oxygen limitation due to insufficient mixing, as found in a yellow fever virus study (Souza et al., 2009). Another successful strategy to enhance virus-cell attachment for reovirus und yellow fever virus was to reduce the working volume temporarily during the beginning of infection (Berry et al., 1999; Mattos et al., 2015; Souza et al., 2009).

Reducing the process temperature when entering the virus production phase has been a common strategy in Vero processes. Typically, rabies virus (Rourou et al., 2009a, 2007; Trabelsi et al., 2006, 2005) and rVSV (Kiesslich et al., 2020) production is carried out at 34 °C, whereas poliovirus (Thomassen et al., 2014, 2013a) favors production at 32.5 °C and measles virus (Weiss et al., 2015) and enterovirus type 71 (Liu et al., 2018, 2007; Wu et al., 2004) prefer 32 °C.

In contrast, establishing the optimal MOI is a highly virus-specific issue and needs to be determined case-by-case in preliminary experiments. However, a general strategy is to choose an MOI with the best trade-off between high final titer, short production timeline and conservation of virus stock (Kiesslich et al., 2020; Thomassen et al., 2013a; Yuk et al., 2006).

In this context, the TOI is another critical aspect. Typically seen as the time between cell seeding and cell infection, it corresponds to the cell density at infection, and as a general principle many processes are infected during the late exponential growth phase of the cells.

As described above, the cell expansion phase generally aims at providing a high cell density environment, assuming that with more cells being available as substrate, more virus can be produced. However, the condition of the cell substrate needs to be taken into account and the TOI carefully be selected. For example, it was found that in poliovirus processes, higher Vero cell densities lead to lower cell specific poliovirus D-antigen levels, a measure of the immunogenicity of the produced virus quantified by ELISA and indicator of product quality. Despite, the overall D-antigen yield was still increased at higher cell density (Thomassen et al., 2014). This cell density effect has also been observed for a chimeric virus

production in Vero (Yuk et al., 2006). Trabelsi et al. even found that for the RM-65 sheep pox virus, infection at the time of cell seeding resulted in the highest Vero cell specific virus productivity (Trabelsi et al., 2014).

Nevertheless, reaching high cell densities is a widely used approach and in particular perfusion processes have been explored extensively. While perfusion during the cell expansion phase can increase the cell density by retaining cells within the system, retaining virus is rather difficult due to its smaller size. Hence, perfusion mode during the virus production phase leads to a continuous harvest of the product. In the case of enterovirus type 71, perfusion with continuous harvest increased production yields by more than 7-14 fold compared to batch mode in Vero cells (Liu et al., 2018). Similarly, for measles and rabies virus production, continuous harvest during perfusion improved overall production yields compared to batch mode, although viral titers were not significantly different (Trabelsi et al., 2006; Weiss et al., 2015)

While continuously supplying fresh and complete growth medium during perfusion is one option, another strategy is to feed only selected nutrients to the culture to improve virus production. For example, feeding of nutrients during the virus replication phase has been shown to increase the Vero cell specific poliovirus production (Merten et al., 1997). In a measles virus production process, feeding with galactose and glutamine increased virus production (Mendonça et al., 2002). Moreover, feeding of glucose and glutamine even improved titers 30-fold in the case of enterovirus type 71 (Wu et al., 2004). In addition to supplying optimized amounts of nutrients to the cell, the metabolic state of the cells can play an important role with regard to high cell specific virus productivities. One study found that a higher cellular energy status in terms of ATP and adenylate concentrations is beneficial for reovirus production in Vero cells (Burgener et al., 2006).

At last, determining the optimal time of harvest (TOH) is a critical bioprocess challenge. The TOH could be determined in preliminary experiments and then assumed to be consistent in subsequent production runs. In biological systems, however, individual runs can vary quite significantly, and processes are consequently monitored continuously regarding indicative parameters such us cell density, nutrient consumption, metabolic byproduct formation or cytopathic effect to conclude when to harvest. To monitor the progression of virus replication in Vero cells growing on microcarriers more sophisticated, dielectric

spectroscopy was successfully used in one study. The researchers were able to predict the optimal TOH based on online measurements for measles virus production (Grein et al., 2018, 2017).

In recent years, modelling of microcarrier Vero cell bioprocesses has been studied with the goal to better understand the process and improve virus production (Abbate et al., 2018; Jiang et al., 2019; Ursache et al., 2015). For example, these models can analyze different combinations of MOI and TOI and predict their impact on viral yield. Further, they can be used to study cell metabolism and infection kinetics. Such approaches can be very useful to identify process control strategies, and to compare and optimize processes. Challenges in this field are the integration of large amounts of data associated with complex culture medium composition. However, modern computational technologies becoming more accessible could enable the use of artificial intelligence and can assist with real-time process evaluation.

Suspension cultures

Perfusion culture mode might be easier applied to adherent cultures on microcarriers or in fixed-beds, but those systems face the problem that surface area is the growth limiting factor (Genzel, 2015). In suspension cultures, cells do not run into this limitation. In addition, scale-up is carried out much easier since no trypsinization is required and suspension cultures of other mammalian cell lines such as HEK293 or CHO are established. Existing technology can be used and is readily available. Further, it is assumed that limited available surface area or cell multilayers can impair cell growth even after virus infection and reduce virus productivity (Thomassen et al., 2014).

However, adaptation of the Vero cell line to grow in suspension culture is a very challenging process. Litwin first reported Vero cells growing in suspension culture in serum-free medium as suspended aggregates (Litwin, 1992). However, cell aggregates are not the optimal condition because of arising diffusional barriers for substrates, metabolites and products. Hence, a single-cell suspension culture is much preferred (Dee and Shuler, 1997).

Paillet et al. reported the successful adaptation of Vero cells to grow in single cell suspension culture. Bioreactor processes in batch and perfusion mode were developed and

the production of viruses was demonstrated in those systems (Paillet et al., 2009). However, this system was not further exploited. A decade later, new efforts were made and two other lab groups described the adaptation of Vero cells to grow in serum-free suspension cultures (Rourou et al., 2019; Shen et al., 2019). Shen et al. demonstrated the operation of batch and perfusion bioreactors, including biomass monitoring via capacitance, as well as rVSV production in this system.

Although these reports indicate successful progress, more research needs to be done to improve the Vero suspension system. Low cell growth rates with doubling times of more than 40 hours and the frequent formation of aggregates remain important issues. Media development will certainly play an important role, highlighted by the fact that no commercial media but only in-house media were able to support Vero suspension cultures (Rourou et al., 2019; Shen et al., 2019). For example, cell adhesion is heavily dependent on the concentration of the bivalent cation of calcium in the cell surrounding environment and thus media with reduced concentrations could decrease cell aggregation. In addition, finding the optimal amount of growth factors is crucial to increase growth rate.

In a study using DNA microarrays to identify genes involved in attachment of HeLa cells, it was found that an increase of expression of the *SIAT7E* gene, encoding a type II membrane glycosylating sialyltransferase, will lead to reduced adhesion, while reduced expression of the *LAMA4* gene, which is responsible for expressing the glycoprotein laminin α4, will also result in reduced adhesion (Jaluria et al., 2007). Similarly, MDCK cells were transfected with a plasmid expressing the *SIAT7E* gene to adapt the cells to grow in suspension (Chu et al., 2009). Another study also indicates that the same strategy can be applied to Vero cells (Mehrbod et al., 2015). Upon knock-down of the gene *PTEN*, encoding a lipid phosphtase, by siRNAs, HEK293T cells also showed a loss of adhesion (Mise-Omata et al., 2005). However, based on experience from our lab, applying these strategies did not result in a working suspension Vero cell line and we assume that this challenge is too complex to be solved by singular genetic modifications.

Altogether, the development of high cell density processes and continuous operation could make the suspension cell system a successful alternative. Despite the establishment of the microcarrier and the fixed-bed bioreactor technologies, suspension cell lines remain the optimal substrate for virus production (Vlecken et al., 2013).

Conclusion

The microcarrier technology that has been industrialized presents a robust platform for the production of viruses using the Vero cell line. However, there are several challenges this platform is facing in order to achieve higher virus productivities. Compared to bioprocesses with other animal cell lines, Vero cell densities are still relatively low and generally do not exceed 10^7 cells/mL. More in-depth research is therefore required to analyze the state of Vero cells at high cell concentrations. Novel omics technologies such as metabolomics, transcriptomics and proteomics can aid to develop strategies to provide Vero cells with the optimal environment for growth and virus production at high cell densities. In addition to this, sophisticated feeding strategies are required. Here, online monitoring of substrate and metabolite concentrations combined with direct control via feeding or perfusion rate adjustment can intensify processes.

Another critical process parameter is the TOH. Online monitoring systems using dielectric spectroscopy have demonstrated their usefulness and should be explored in more detail to optimize TOH for different viruses. In general, viruses are sensitive to culture conditions such as temperature and shear stress, and harvest at exact peak production has the potential to reduce process losses significantly. Further, modelling approaches that integrate large amounts of data and artificial intelligence are becoming more accessible with modern computational technologies and can assist with real-time process evaluation.

Successful process scale-up still requires a high level of technical expertise and know-how. Linear scalable systems such as fixed-bed bioreactor systems or highly scalable suspension cell cultures could make processes more robust, streamlined and cost-effective.

Increasing the cell specific productivity independently of the cultivation mode, could be achieved by applying novel tools within the field of genomics and genetic engineering. However, these efforts are impaired by the fact that limited genomic data is available. Hence, the full annotation of the Vero genome is necessary.

When facing sudden outbreaks of viral diseases, processes rely mostly on the standard model and only a few virus specific parameters are tested. Hence it is of great interest to further develop the Vero cell platform for accelerated production now so that it can be readily applied and provide sufficient material when novel viruses emerge, and diseases

spread for which there is currently no vaccine available. Recent reports indicate trends toward preparedness, but research activities need to be intensified. Advances in Vero genomics and further development of the suspension cell line as a platform for the high yield production of viral vectors and vaccine could therefore become significant contributions.

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Preface to Chapter 2

The previous chapter has highlighted recent advances in bioprocess development using the Vero cell line for the production of viral vectors and vaccines. With regard to the overall objective of my PhD, to further develop the Vero cell line as an efficient and scalable platform for viral vector manufacturing, rVSV was chosen as a model virus. Before tackling the bioprocess development challenges outlined in chapter 1, analytical techniques needed to be developed to assess potential progress in bioprocess development in terms of improved virus productivity.

To determine virus production yields, many different analytical techniques are available to measure virus concentration. Depending on the assay, it is possible to quantify the number of infectious viral particles (e.g. tissue culture infectious dose assays, plaque assays, laser force cytology), the number of viral genomes (e.g. quantitative PCR, digital PCR), the number of viral antigens (e.g. enzyme-linked immunosorbent assay, hemagglutination assay for influenza, single radial immunodiffusion assay) or the number of total viral particles (transmission electron microscopy, tunable resistive pulse sensing, flow cytometry, high-performance liquid chromatography). Each method has their own advantages and constraints, which can be labor intensity, time to perform the assay, the requirement for highly advanced instruments or the need of specialized assay ingredients (e.g. antiserum).

In the following chapter, two methods are described to quantify the concentration of rVSV: a Tissue Culture Infectious Dose ($TCID_{50}$) assay to determine the infectious titer and a digital polymerase chain reaction (dPCR) assay to assess the total number of viral particles. The $TCID_{50}$ assay is a standard method in virology. It relies on basic end-point dilution assay and the determined infectious particle concentration of the virus is a very important value for viral vaccine development. The advantage is its robustness and simplicity. It can be performed in most microbiology laboratories and results are comparable between different operators. However, it takes up to seven days to receive results. In contrast, the dPCR method is much faster and results can be obtained within eight hours. The assay determines the total number of viral genomes, which can be used to estimate the number of total viral particles. Together, $TCID_{50}$ and dPCR are used to characterize the ratio of total particles to infectious units for monitoring process robustness and product quality attributes.

Chapter 2

Titration methods for rVSV-based vaccine manufacturing

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Abstract

The recombinant Vesicular Stomatitis Virus (rVSV) is an emerging platform for viral vector-based vaccines. Promising results have been reported in clinical trials for the rVSV-ZEBOV vaccine for Ebola virus disease prevention. In this study, we describe the titration tools elaborated to assess the titre of rVSV-ZEBOV production.

- A streamlined Median Tissue Culture Infectious Dose ($TCID_{50}$) assay to determine the infectious titer of this vaccine was established.
- A digital polymerase chain reaction (dPCR) assay to assess the total number of viral particles present in cell-free culture supernatants of rVSV productions was developed.
- These assays are used to titre rVSV-ZEBOV samples and characterize the ratio of total particles to infectious units for monitoring process robustness and product quality attributes and can be used to titre samples generated in the production of further rVSV vectors.

Keywords

rVSV-ZEBOV, Ebola, rVSV-HIV, HIV, dPCR, $TCID_{50}$

Background

The recombinant Vesicular Stomatitis Virus (rVSV) platform is a replication-competent vaccine that has been shown to generate both cell-mediated and humoral immunity to expressed foreign antigens (Garbutt et al., 2004) and is being developed to target several infectious diseases and cancers (Zemp et al., 2018). One of these targets, the Ebola virus disease, is an urgent international priority, hence there is intensive research activity surrounding the development of a safe and efficacious vaccine. Recently, the Ebola virus vaccine Ervebo has been approved by the FDA (US Food And Drug Administration, 2019), prequalified by the World Health Organization and granted a conditional marketing by the European Commission (Mahase, 2019). This vaccine is based on the rVSV-ZEBOV, an

attenuated, replication-competent rVSV pseudotyped with the Ebola Zaire glycoprotein (Jones et al., 2005).

Replication-competent vaccine titres can be quantified by a functional titre, an enumeration of the infectious virus particles, and/or by a particle titre, an enumeration of the number of particles present in a volume of vaccine. The combination of a functional assay with a total particle assay through their ratio (functional particles over total particles) is a critical quality attribute for the characterization of the candidate rVSV-based vaccine as defective interfering rVSV particles have been shown to modulate virulence (Cave et al., 1985).

The endpoint dilution assay ($TCID_{50}$) (Ramsburg et al., 2004) is a measure of the functional titre of samples as it quantifies the number of transducing particles required to produce a cytopathic effect in 50 % of inoculated tissue culture cells. $TCID_{50}$ and plaque-forming units (pfu) are reliable techniques and routinely used to titre vaccine preparations (Monath et al., 2019).

The total particle count is usually established using quantitative polymerase chain reaction (qPCR). Specific fluorescent labelling is used to measure the progress of PCR in real time and allows for quantification of the DNA template. A standard curve generated from a serial dilution of DNA standard of known concentration is used to evaluate the qPCR result. In contrast, the digital PCR (dPCR) method is a relatively novel tool that can be used to obtain a total particle count without the need for a standard curve (Coudray-Meunier et al., 2015; Pinheiro-de-Oliveira et al., 2018). Here, around 20,000 water-oil emulsion droplets are generated from one sample and during the PCR step, specific DNA amplification is carried out within each droplet. The method takes advantage of this template DNA separation to partition the sample and to enable individual droplet analysis following the PCR. By counting positive and negative reactions, the method determines the value of viral genomes per mL. Because of this absolute quantification, no standard curve is required, and the workflow is simplified (Hindson et al., 2011). Recently, dPCR has been directly compared to qPCR indicating higher precision and reproducibility in the case of dPCR (Taylor et al., 2017). Further, dPCR has shown better intra- and inter-assay precision than qPCR without the issues related to the use of plasmids as DNA reference material in the case of quantification

of the number of total genomes for rAAV vectors (Furuta-Hanawa et al., 2019; Lock et al., 2014).

Here we describe a $TCID_{50}$ method as well as a dPCR method for titration of rVSV samples. Certain aspects that can affect the variability of these assays are explored and the dPCR method is tested against three different variants of rVSV vectors.

Method details

Cells

HEK 293A cells (Graham et al., 1977) (American Type Culture Collection, Manassas, VA, USA) were maintained in cell culture dishes (Greiner Bio-One, Kremsmünster, Austria), in a humidified incubator (Thermo Fisher Scientific, Waltham, MA, USA) 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 500 × g for 5 minutes, resuspended in fresh medium and seeded at a 1:10 dilution.

Viruses

The generation of rVSV-EboGP B6 (rVSV-ZEBOV) (Gélinas et al., 2019; Wong and Qiu, 2016) and rVSV-GFP (Elahi et al., 2019) samples has been described previously. Sample generation for rVSV-EboGP B6-NL4.3Env/SIVtm (rVSV-HIV) will be described in detail in upcoming publications. Briefly, the genome plasmid used for the production of rVSV-ZEBOV was modified to include the Env sequence of NL4.3 with an SIV transmembrane domain. The generation of the lentiviral vector (LV) used to validate the rVSV primers has been described previously (Manceur et al., 2017).

Median Tissue Culture Infectious Dose ($TCID_{50}$)

The endpoint dilution assay (Reed and Muench, 1938) was used as a measure of the functional titre of virus samples as it quantifies the number of transducing particles required to produce a cytopathic effect in 50% of inoculated tissue culture cells. rVSV titration on HEK

293A was previously shown to give similar results as titration on Vero cells (Elahi et al., 2019). HEK 293A cells were seeded at ~5 × 10^4 live cells per mL in 96 well plates (Greiner Bio-One, Kremsmünster, Austria), referred to as the $TCID_{50}$ plates, with 100 µL per well one day before initiating the titration. Virus dilution series of twelve times 1:5 were prepared in separate 96 V bottom well plates (Sarstedt, Nümbrecht, Germany), referred to as the dilution plates, using 50 µL in 200 µL of HyClone HyCell TransFx H. Eight dilution series were performed per dilution plate. Using a multichannel pipette, 20 µL per well of each individual dilution series were transferred into each of the eight rows of a single $TCID_{50}$ plate. The $TCID_{50}$ plates were incubated at 37 °C and 5% CO_2. Cytopathic effect was observed after at least 7 days incubation and scored by standard light microscopy. The $TCID_{50}$/mL value was calculated by the Spearman & Kärber algorithm (Kärber, 1931; Spearman, 1908) as described previously (Hierholzer and Killington, 1996).

Digital Polymerase Chain Reaction (dPCR)

To estimate the total number of viral particles present in cell-free culture supernatants, the copy number of viral genomes was assessed by dPCR. Viral RNA was extracted using the High Pure Viral Nucleic Acid Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. The input-volume was kept at 200 µL for all samples, and purified RNA was eluted into 50 µL elution buffer. This kit has been shown to have better recovery rate than others in a side-by-side comparison of five different nucleic acid extraction kits on a different DNA virus (Hepatitis B virus) possibly because of the use of a proteolytic enzyme in addition to the chemical denaturants (Read, 2001). The viral RNA was then reverse transcribed using the iScript Select cDNA synthesis kit (Bio-Rad Laboratories) according to the manufacturer's instructions and using gene-specific primers targeted towards the viral L-protein (polymerase) amplifying a 114 base pair fragment. The primer sequence for the forward primer was [5′-CTGCTGTCCGGAATCAGGTT-3′] and for the reverse primer [5′-GCCGTCTCCACAACTCAAGA-3′] (Integrated DNA Technologies, Inc., Coralville, IA, USA). The cDNA reaction mix consisted of 4 µL of 5x iScript reaction mix, 0.5 µL of each primer at a concentration of 10 µM, 2 µL of GSP enhancer solution, 2 µL of purified RNA from the extraction, 1 µL of reverse transcriptase, and 10 µL of purified and nuclease-free water. The dPCR was performed using the QX200™ Droplet Digital™ PCR System with the EvaGreen

Supermix (Bio-Rad Laboratories) following the manufacturer's instructions. Briefly, the dPCR reaction mix consisted of 10 µL EvaGreen Supermix, 0.5 µL of each primer at a concentration of 10 µM, 5 µL of cDNA from the reverse transcription step, and 4 µL of purified and nuclease-free water. Using the droplet generator and the corresponding cartridges, the 20 µL reaction mix was combined with 65 µL of EvaGreen droplet generation oil to generate droplets. The droplets were then transferred to dPCR 96-well plates where the dPCR reaction took place. Thermocycling conditions were as follows: initial denaturation at 95 °C for 5 minutes followed by 35 cycles at 95 °C for 30 s, 57 °C for 60 s, and 72 °C for 30 s, followed by a final extension at 72 °C for 5 minutes. The annealing temperature of 57 °C was established in a preliminary temperature gradient experiment. An internal virus reference standard, consisting of a new vial taken from a viral seed stock generated as described previously (Gélinas et al., 2019), was run in every assay alongside to ensure repeatability of the whole assay including RNA extraction. In addition, a non-template control was included in each run to rule out the contamination of the PCR reagents. The analysis of the droplet read after dPCR-amplification resulted in a value which, after correction for the dilution factor, gave the number of copies of viral genomes, which contain the selected sequence, present in the original sample with the unit of VG/mL.

To demonstrate the trueness of the assay, the plasmid pATX.V2.Full, which was used to generate the rVSV-ZEBOV (Gélinas et al., 2019), was analyzed by ddPCR using the protocol above. The resulting gene copy number was compared to the gene copy number determined via spectrophotometry. The amount of DNA in the plasmid sample was determined using a ND-2000 spectrophotometer (NanoDrop, Thermo Fisher Scientific). The plasmid copy number was then calculated using the formula:

$$number\ of\ copies\ (molecules) = \frac{X\ ng \times 6.022 \times 10^{23}\ \frac{molecules}{mol}}{N \times 10^{9} \frac{ng}{g} \times 650\ \frac{g}{mol}}$$

where X = amount of DNA in the plasmid sample, N = length of the pATX.V2.B6.Full plasmid (14086 base pairs), and 650 g/mol = average mass per base pairs.

To show specificity of the dPCR method towards rVSV, a lentiviral vector (LV) was titrated using the method developed for rVSV. Viral RNA of the LV was extracted using the same method as for rVSV while reverse transcription was performed using a random primer mix supplied with the cDNA kit. dPCR was performed in triplicates using the same cDNA. The LV-specific primer sequences for the dPCR were: forward primer [5′-GTCCTTTCCATGGCTGCTC -3′] and reverse primer [5′-GCCGTCTCCACAACTCAAGA-3′] (Integrated DNA Technologies).

Statistical analysis

Mean and standard deviations, represented as error bars in figures, as well as unpaired Welch's t-tests were calculated using Prism 8.2.0 (GraphPad, La Jolla, CA, USA). Statistical power was evaluated using the G*Power 3.1.9.2 software (University of Düsseldorf, Düsseldorf, Germany) (Faul et al., 2007) using an *a priori* t test to determine the required sample size to observe the difference between two independent means with an α error probability of 0.05.

Results

Variability of titration using $TCID_{50}$

To evaluate the repeatability of rVSV-ZEBOV titration using this method, twelve parallel $TCID_{50}$ evaluations of a single sample of an rVSV-ZEBOV seed stock were performed using the same procedure, operator, measuring system, operating conditions and location. The functional titre of that production batch was evaluated to be 1.23×10^7 $TCID_{50}$/mL (standard deviation: 4.88×10^6) (**Figure 1A**). The intermediate precision was also assessed by titration of the same sample on twelve different days over months (**Figure 1B**). As expected, intermediate precision was shown to be more variable than the assay's repeatability with an average titre of 1.43×10^7 $TCID_{50}$/mL and a standard deviation of 9.10×10^6. When performing an unpaired Welch's test between the results from the replicate titrations and the repeat titrations, a significant difference ($p<0.0499$) was found between the variances. The intermediate precision of this assay has been reported before in the titration of filovirus where the range was approximately 1.5 log (Smither et al., 2013). Hence, each of the samples

presented in the same figure should be titrated on the same day to avoid the added impact of interday variability. To further reduce variability, an automated process could be developed and would limit operator variability.

The repeatability of virus production was also assessed to determine its impact on the evaluation of the titre of a sample as well as the number of replicates necessary to have sufficient power to observe statistical significance in production experiments where different parameters are evaluated. The production of rVSV-ZEBOV was evaluated in multiple independent infections using two 6 well plates seeded with HEK 293SF cells and again using the same procedure, operator, measuring system, operating conditions and location. These were infected with rVSV-ZEBOV at a multiplicity of infection (MOI) of 0.001 and left to incubate with agitation for 2 days at 34 °C. The functional viral titre for each of the 12 independent cultures, as determined by $TCID_{50}$, is shown in **Figure 1C** (mean of twelve wells: 3.13×10^7 $TCID_{50}$/mL, standard deviation: 1.59×10^7). Using these data, to model future studies incorporating three replicates, statistical power analysis demonstrated that a minimum of a 2.26-fold increase in functional titre would be necessary to observe a statistical difference with 80% power using triplicates and accounting for the variability of the $TCID_{50}$ assay if performed for all samples on the same day.

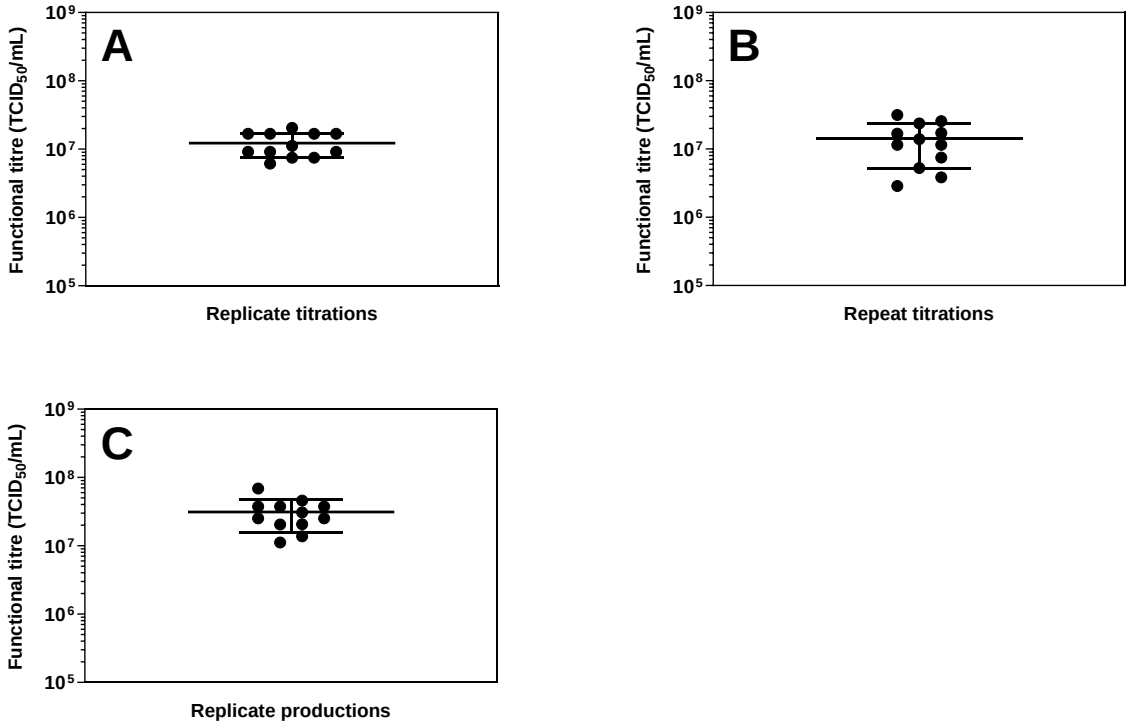

Figure 1. Production and titration variability using $TCID_{50}$. Functional titres were measured by $TCID_{50}$. Bars represent the mean of the twelve samples ± standard deviation. A) Titration repeatability. Independent titration by $TCID_{50}$ in 12 replicates on the same day of a single production sample. B) Titration intermediate precision. Independent titrations by $TCID_{50}$ repeated on 12 separate days for aliquots of the same production. C) Production repeatability. Production yields for 12 independent infections with rVSV-ZEBOV at MOI 0.001 of two 6 well plates containing 1×10^6 cells/mL in 2 mL per well.

Variability of titration using dPCR

For any given sample to be analyzed by dPCR, the cDNA needs to be diluted appropriately prior to the run for the purpose of achieving clear peak resolution of dPCR events and so that the resulting dPCR signal falls within the linear dynamic range of analysis for accurate measurements according to the manufacturer's instructions. A histogram of the dPCR analysis of a dilution series of a cDNA sample extracted from rVSV-ZEBOV is shown in **Figure 2**. As expected, the least diluted samples showed almost only positive events due to the abundance of gene copies. The more the sample got diluted, the less positive and the more negative events occurred. The most diluted sample showed only a few positive events and mostly negative events. The histogram from the sample with 1:3,200 dilution (sample C03) is individually shown in **Figure 3** and shows a clear peak resolution of dPCR events without

significant amount of rain. Positive events typically peaked at around 20,000 to 25,000, whereas negative events peaked between 5,000 and 8,000.

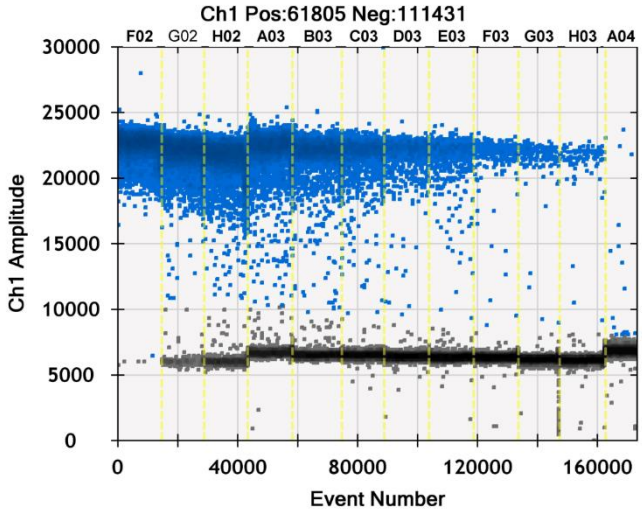

Figure 2. Histogram of dPCR analysis of a dilution series of cDNA extracted from rVSV-ZEBOV. The extracted cDNA was diluted in a 1:2 dilution series starting from 1:100 (sample F02) to 1:102,400 sample (H03). Sample A04 consisted of a non-template control. Channel 1 amplitude is given in arbitrary fluorescent units. Positive events are marked in blue, negative events in grey.

To demonstrate that the developed dPCR method is measuring the correct amount of gene copies, a plasmid containing the VSV polymerase gene sequence was analyzed via dPCR and the gene copy number was verified via spectrophotometry. The amount of DNA in the plasmid sample was measured by spectrophotometry and was 371 ng/µL. Using the formula stated in the methods, the resulting number of gene copies was determined to be 2.44×10^{13} per mL. In comparison, the number of gene copies determined via dPCR was 3.48×10^{13} per mL, indicating an appropriate correlation between the two methods.

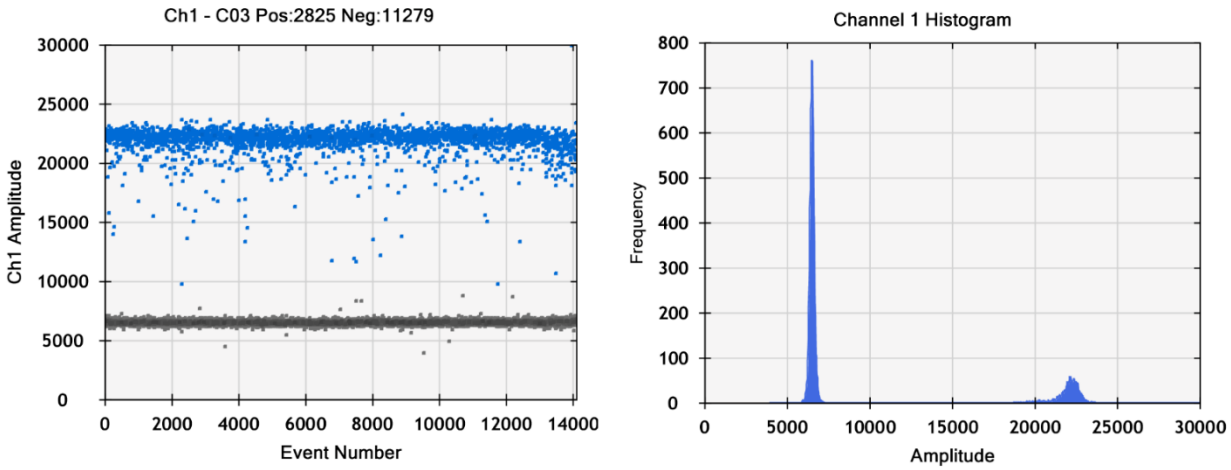

Figure 3. Histogram of dPCR analysis of rVSV-ZEBOV. This exemplary histogram shows the data of an rVSV-ZEBOV sample at a cDNA dilution resulting in a clear peak resolution of dPCR events. Channel 1 amplitude is given in arbitrary fluorescent units. Positive events (blue) typically peaked around 20,000 to 25,000 and negative events (grey) peaked between 5,000 and 8,000.

To estimate the repeatability of the dPCR assay, the number of viral genomes in the rVSV-ZEBOV seed stock was quantified in 3 different approaches. To evaluate the total variability of the assay, twelve separate RNA extractions were performed on the same sample. Each extraction was then reverse transcribed into cDNA and analyzed by dPCR. The average titre was 6.01×10^9 VG/mL (standard deviation: 2.06×10^9) (**Figure 4A**).

To evaluate which step led to the most variability, one RNA extraction sample from the previous experiment was used and, reverse transcription was carried out 12 times resulting in 12 separate cDNA batches. Each of these cDNA batches was analyzed individually by dPCR. Here, the average titre was 9.61×10^9 VG/mL (standard deviation: 4.62×10^8) (**Figure 4B**). When performing an unpaired Welch's test between the results from the replicate RNA extraction and the replicate reverse transcription, a significant difference ($p<0.0001$) was found between the variances, indicating that the RNA extraction step leads to a significantly greater variance compared to the RNA transcription step with regard to the final dPCR titer. Finally, from one of the cDNA batches, twelve dPCR were performed separately. The average titre was 1.05×10^{10} VG/mL (standard deviation: 5.23×10^8) (**Figure 4C**). An unpaired Welch's test between the results from the replicate reverse transcription and the replicate dPCR from the same cDNA found no significant difference between the variances ($p=0.6884$). Hence, the main source of error of the dPCR assay was found to be the step of viral RNA

extraction. Whereas reverse transcription and dPCR are one step reactions, the RNA extraction involved multiple steps including viral particle disruption, RNA binding to the membrane, inhibitor removal, washing and elution of the extracted RNA. Besides, viral RNA is relatively unstable compared to DNA.

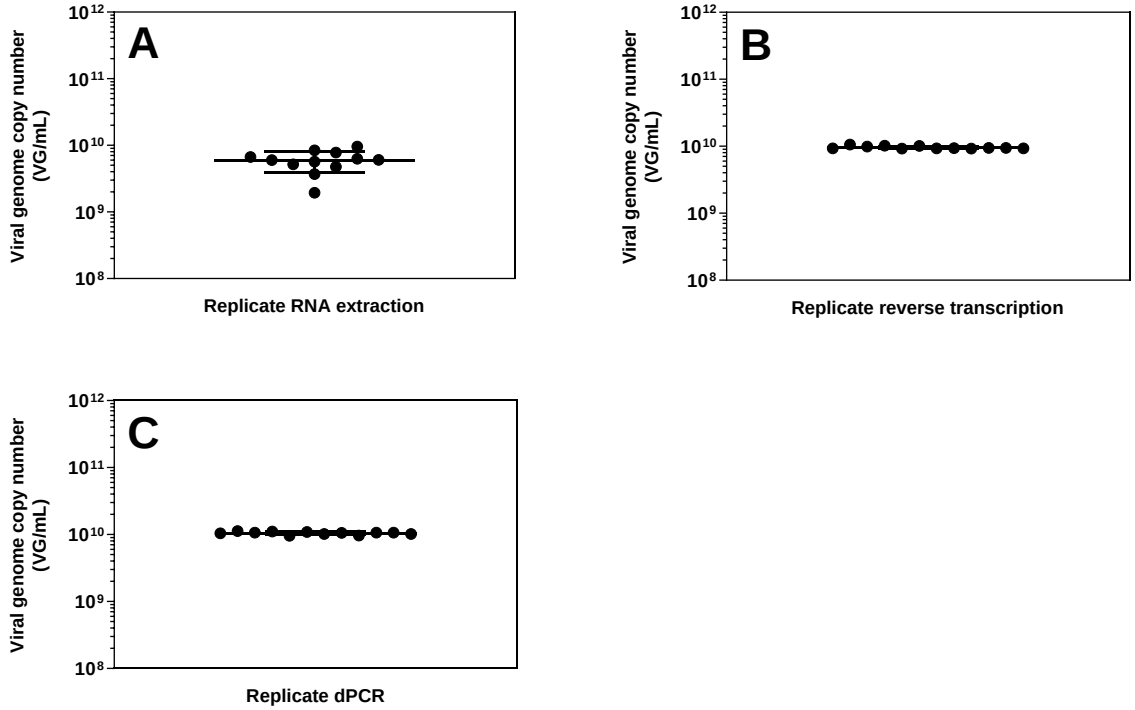

Figure 4. Titration variability of rVSV-ZEBOV using dPCR. Viral genome copy number was measured by dPCR. Bars represent the mean of the twelve samples ± standard deviation. A) Total assay variability. dPCR analysis of the same sample with 12 independent RNA extractions. B) Combined reverse transcription and dPCR variability. dPCR analysis of the same RNA extract with 12 independent reverse transcriptions. C) dPCR variability. dPCR analysis was performed 12 times of the same cDNA.

Titration using dPCR for other strains of rVSV

In addition to the titration of rVSV-ZEBOV, the method was further tested by titrating two other significant rVSV strains: rVSV-GFP and rVSV-HIV. rVSV-HIV is currently being studied as a promising vaccine candidate against human immunodeficiency virus infection where the glycoprotein of VSV has been replaced by HIV's Env glycoprotein (Racine et al., 2017). rVSV-GFP is taking advantage of GFP expression to study, for example, the viral life cycle, but still expresses the wild-type glycoprotein (VSV-G) (Elahi et al., 2019; Stojdl et al., 2003). $TCID_{50}$ was performed in triplicate on a single rVSV-HIV and rVSV-GFP sample. The

functional titres of these samples were 8.07 $\times 10^7$ (standard deviation: 3.78 $\times 10^7$) and 4.03 $\times 10^9$ (standard deviation: 1.28 $\times 10^9$) respectively. Viral RNA from rVSV-HIV and rVSV-GFP was extracted and reverse transcribed as before. In both cases, the dPCR was performed in triplicates using the same cDNA. The genomic titre of the sample of rVSV-HIV was determined to be 2.67 × 10^{10} VG/mL (standard deviation: 3.06 × 10^8) and 1.86 × 10^{10} VG/mL (standard deviation: 9.24 × 10^8) for the rVSV-GFP sample.

To show specificity of the dPCR method towards rVSV, a lentiviral vector (LV) was titrated using the method developed for rVSV. LV showed no PCR amplification when using rVSV-specific primers for the dPCR step. As a control, LV showed PCR amplification when using LV-specific primers. In contrast, cDNA of rVSV-ZEBOV generated as in **Figure 2** did not show dPCR amplification using these LV-specific primers.

Conclusion

In this work, we described an assay to determine the functional titre of rVSV vectors ($TCID_{50}$) as well as an assay to determine the number of viral genomes (dPCR). The later can be used to estimate the total number of viral particles. Together, these assays provide reliable methods to determine significant values in viral vaccine bioprocess development.

Depending on the rVSV strain, the $TCID_{50}$ assay can require up to seven days before reading of the cytopathic effect. Immunoperoxidase staining could help improve and speed up the process of distinguishing positive and negative wells. It is however important to note that, because of a strong cytopathic effect in the case of rVSV-ZEBOV, positive wells show no surviving cells after a few days while in negative wells cells are completely intact. An alternative method to quantify the infectious viral titer of VSV has been recently published which uses laser force cytology (Hebert et al., 2018). This method has the advantage of reducing the time required until the titer can be determined. It still needs to be demonstrated that this method can be applied to other recombinant strains of VSV with similar reduction in assay time. However, the benefit of the $TCID_{50}$ assay is its simplicity which can be carried out in standard equipped laboratories without the need for complex instruments.

In contrast, the dPCR assay can be completed within one day and can, therefore, be used as a preliminary estimation of the viral titre if necessary. Nevertheless, like other vectors,

rVSV typically produces a variety defective interfering particles during infection (Frensing, 2015; Meier et al., 1984). In this study, only one methodology was used to determine each, the infectious and the total particle count, respectively, to get analytical data that can be used in the development of the upstream bioprocess. To more accurately determine the number and molecular diversity of the defective interfering particles that are created during the rVSV-ZEBOV vaccine production process, further analytical methods would be required. This would allow for additional insights into the biological complexity of rVSV production, further enabling bioprocess improvements. For example, the dPCR method developed in this work targets only those particles which contain the sequence of the VSV polymerase gene. The method does not consider defective particles lacking this gene sequence. A physicochemical approach to determine the number of total particles, for example via HPLC, could be a valuable extension of the analytical methods tool box for rVSV total viral particle quantification.

Despite these limitations, the developed method to estimate the number of total particles can still generate very useful data. For example, following the production step, dPCR would allow the confirmation of a successful production of vectors as well as the number of total viral particles which is useful to proceed with the downstream processing of viral vectors with an appropriate concentration step. Since both the number of infectious particles and total particles are expected to change throughout the purification process, the dPCR assay can be a sufficient quantification method enabling continuation to the next step with the benefit of reducing the required time for process analytics in between the upstream and downstream steps. We further demonstrated that these assays are applicable to rVSV-ZEBOV, a very relevant candidate vaccine and that the dPCR can be used for other recombinant strains of VSV. Different pseudotyped rVSV are currently being investigated as vaccine candidates, and further, as possible oncolytic viruses. In particular, rVSV-HIV and rVSV-GFP were successfully titrated using the same dPCR method as used for rVSV-ZEBOV. Since the primers used in the dPCR assay are targeting a sequence of the VSV polymerase (L-gene), this assay can serve as a universal method to determine the number of viral genomes of other rVSV as long as the L-protein sequence remains wild-type. It has been further demonstrated that the developed dPCR method is specific for rVSV vectors and does not result in dPCR amplification of other viral vectors.

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Declaration of Competing Interest

The Authors confirm that there are no conflicts of interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at https://doi:10.1016/j.mex.2020.100806.

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Preface to Chapter 3

Chapter 3 of this thesis is studying bioprocess development of adherent Vero cell cultures. As outlined in chapter 1, traditional viral vaccine manufacturing processes are still using roller bottle processes. This technology has several drawbacks, for example limited scalability, risk of contamination and higher labor intensity. Even the recently approved Ebola virus vaccine (rVSV-ZEBOV) is using this process. To advance the knowledge in manufacturing technology, the next chapter is developing bioreactor processes to propose a new and more efficient way of rVSV-ZEBOV manufacturing and to overcome process limitations. Using the analytical methods developed in chapter 2, the first experiments are studying process parameters such as multiplicity of infection and temperature at small scale in tissue culture plates of rVSV-ZEBOV replication. Since a working suspension culture adapted Vero cell line was not readily available at this time, the production is scale-up and two different production systems for adherent Vero cell culture, i.e. microcarrier and fixed-bed bioreactors, are compared.

Chapter 3

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 target 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.

Keywords

rVSV-ZEBOV, Vero cell, Serum-free, Bioreactor, Microcarrier, Fixed-bed

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

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 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 hours 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 6,000 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 with 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.

Materials and methods

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 (Human Health Therapeutics, 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).

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 hours 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.

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 minutes at 1200 × g to remove cellular debris, aliquoted and stored at −80 °C until further analysis.

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., 2009a). 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 minutes 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.

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 hours, 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 hours 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.

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).

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'-GCCGTCTCCACAACTCAAGA-3') (Integrated DNA Technologies, Inc., USA) as described in MethodsX (Gélinas et al., 2020). 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.

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 minutes to inactivate the virus prior to metabolite analysis.

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).

Results and discussion

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 (**Figure 5**). 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.

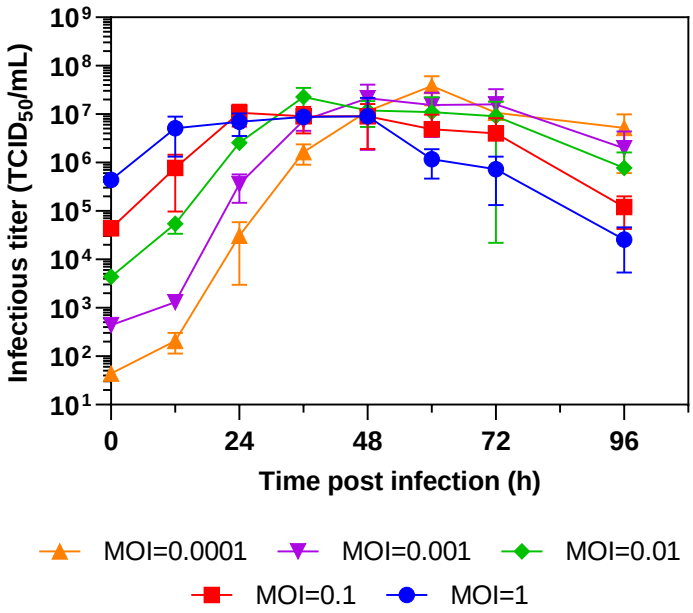

Figure 5. rVSV-ZEBOV infection of Vero cells at different MOIs ranging from 0.0001 to 1 in 6-well plates. Infectious viral titers expressed as $TCID_{50}$/mL are plotted against the time post-infection. Bars represent the mean of the three independent samples ± standard deviation.

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. **Figure 6** 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.

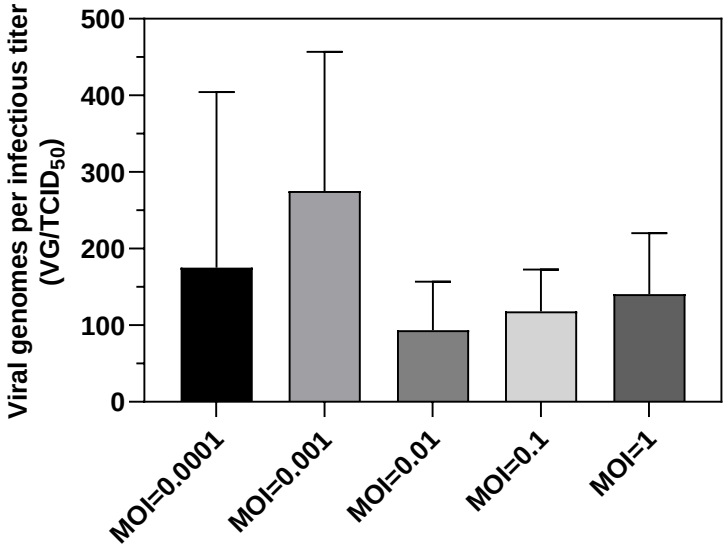

Figure 6. Viral genome copy number (VG/mL) per infectious viral titer ($TCID_{50}$/mL) at peak titers for each MOI investigated. Ratios were determined for each MOI at their respective peak of infectious titer in the 6-well experiment, i.e. 60, 48, 36, 24 and 12 hpi for MOI of 0.0001, 0.001, 0.01, 0.1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

Focusing on the run performed at MOI of 0.01, **Figure 7** compares the accumulation of infectious viral particles and of viral genomes throughout the production run. As already shown in **Figure 5**, 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.

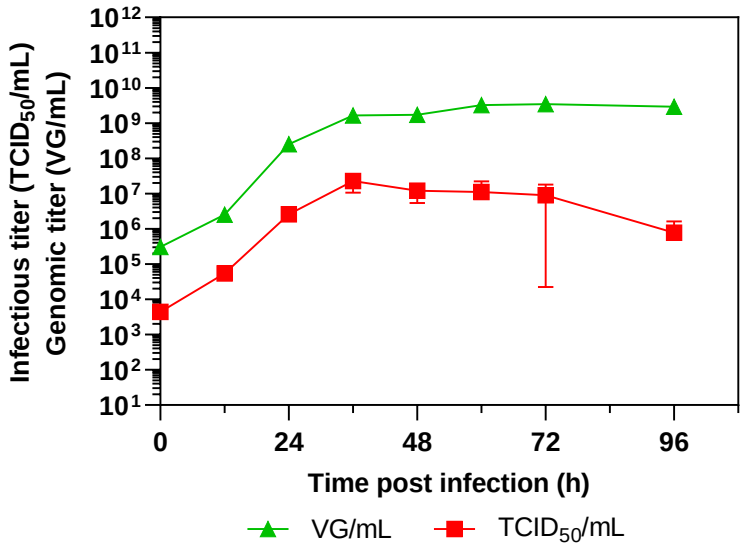

Figure 7. rVSV-ZEBOV infection of Vero cells at MOI of 0.01 in 6-well plates comparing infectious titers and genomic titers. Infectious viral titers expressed as $TCID_{50}$/mL and the number of viral genomes expressed as VG/mL are plotted against the time post-infection. Bars represent the mean of the three independent samples ± standard deviation, however, the standard deviation of the genomic titer is too low to be visible on the logarithmic scale.

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.

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. **Figure 8** 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.

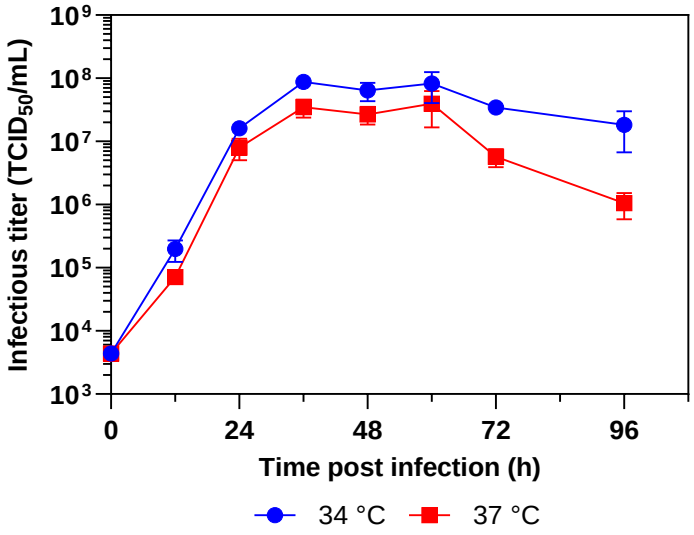

Figure 8. rVSV-ZEBOV infection of Vero cells at different temperatures in 6-well plates. Cells were infected with rVSV-ZEBOV at an MOI of 0.01 and virus production was carried out at either 34 °C or 37 °C. Infectious viral titers expressed as $TCID_{50}$/mL are plotted against the time post-infection. 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.

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 **Figure 9**, 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).

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 regard 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.

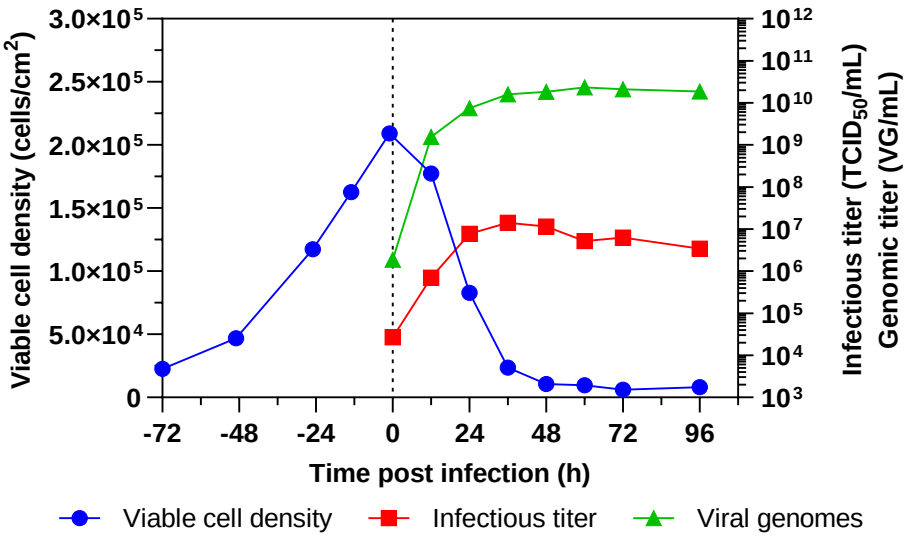

Figure 9. Vero cell growth and rVSV-ZEBOV production in a 1 L bioreactor with temperature reduction to 34 °C during the virus production phase. Cells were grown on Cytodex 1 microcarriers and infected with rVSV-ZEBOV at an MOI of 0.01 after 3 days of cultivation.

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.

Figure 10 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.

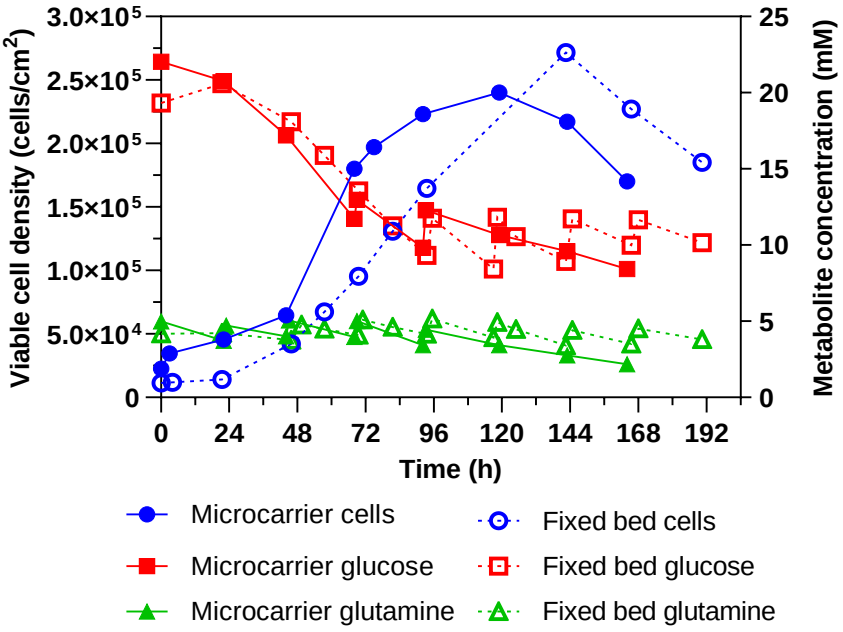

Figure 10. Vero cell growth was compared between a microcarrier bioreactor and the scale-X™ hydro fixed-bed bioreactor. Cell growth in the fixed-bed is lagging behind the microcarrier process due to a lower cell seeding density. Concentrations of the main metabolic substrates indicate similar growth kinetics in the two systems.

At the end of the fixed-bed bioreactor cultivation (192 h), a cell distribution analysis was carried out. As shown in **Figure 11**, Vero cells were homogeneously distributed within the fixed-bed in both, axial and radial direction. The overall cell count average between all 18 samples resulted in 265,226 ± 60,138 cells/cm². However, the cell count determined from the sample strip at this time point was only 185,185 cells/cm² indicating a slight underestimation of the cell count by the sample strip method.

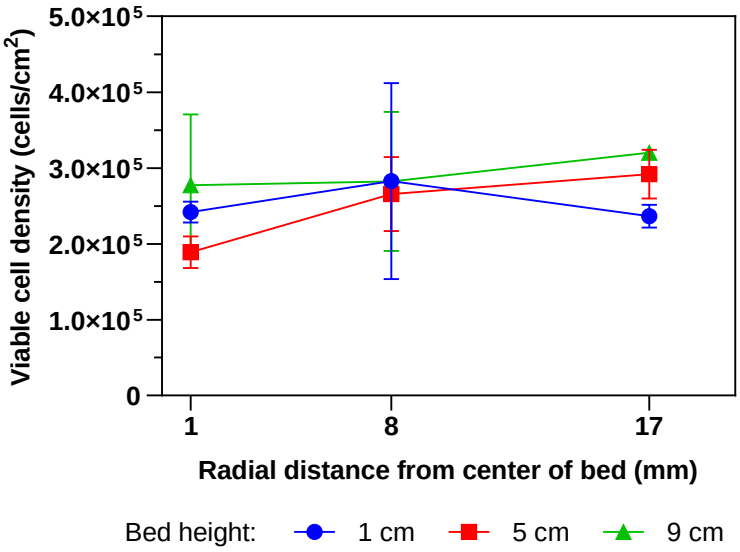

Figure 11. Vero cell distribution within the fixed-bed cell support layers. From each of the 2 layers, 9 samples were taken at different axial (bed height) and radial positions. Error bars indicate standard deviation between the two layers.

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 **Figure 9**), cells were seeded at similar cell densities in the fixed-bed (22,708 cells/cm²) compared to the previous microcarrier bioreactor (22,727 cells/cm²) 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/cm² at the time of infection (**Figure 12**), where the microcarrier cell density was at 209,091 cells/cm². As seen for the microcarrier bioprocess at 34 °C, the cell density did not continue to increase 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 hours 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 regard 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.

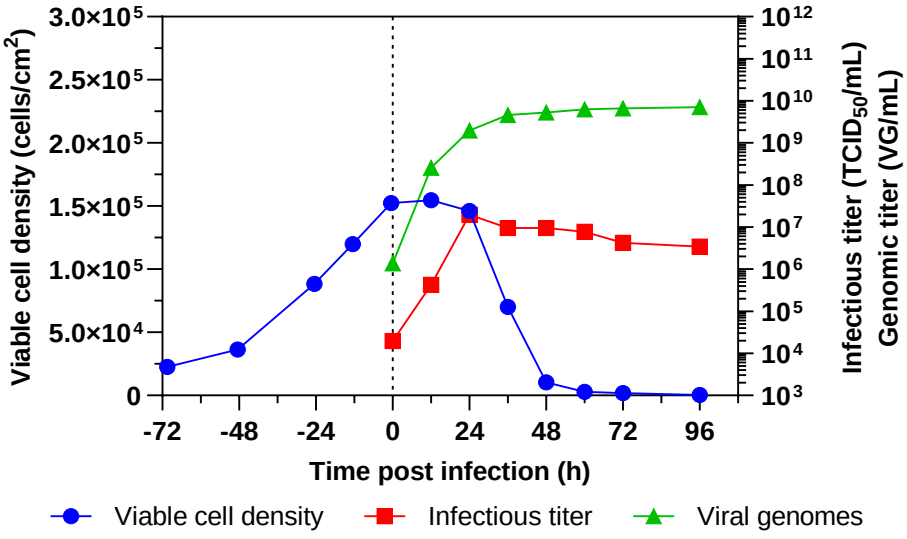

Figure 12. Vero cell growth and rVSV-ZEBOV production in the scale-X™ hydro fixed-bed bioreactor. Cells were infected with rVSV-ZEBOV at an MOI of 0.01 after 3 days of cultivation. The temperature was lowered to 34 °C during the virus production phase.

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. **Figure 13** 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.

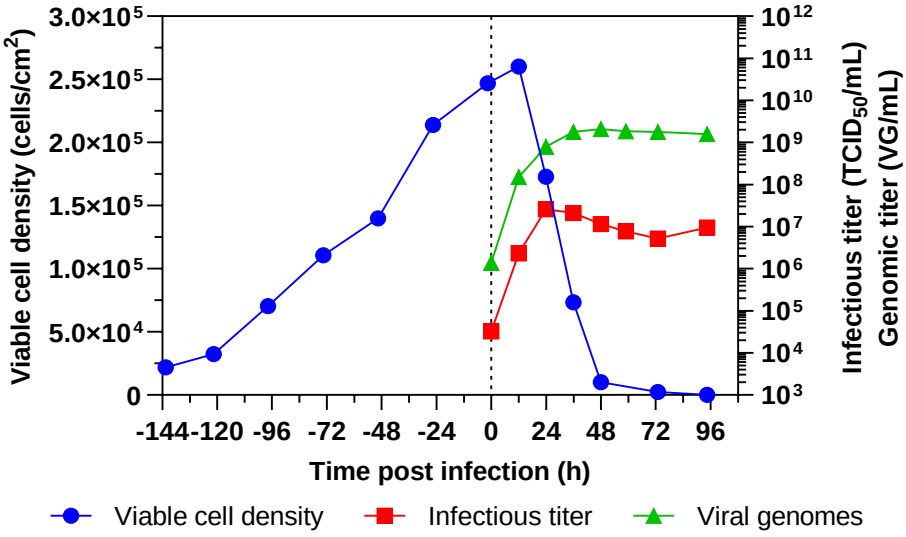

Figure 13. Vero cell growth and rVSV-ZEBOV production in the scale-X™ hydro fixed-bed bioreactor with extended cell growth phase and two media exchanges. Cells were infected with rVSV-ZEBOV at an MOI of 0.01 after 6 days of cultivation. The temperature was lowered to 34 °C during the virus production phase. A complete medium exchange was performed on day 3 and again on day 6, immediately before infection.

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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fixed-bed bior- eactor system free of charge. Pratik Gajjar, from Univercells contributed to the first bioreactor set up and operation. Univercells had no input in the redaction of this manuscript.

Appendix A. Supplementary data

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Preface to Chapter 4

The final chapter of this thesis is studying bioprocess development of suspension adapted Vero cell cultures for rVSV production. This work is a direct continuation of the previous chapter. As described in the first chapter, suspension cell culture systems are considered the optimal substrate for virus production. Adaptation of the Vero cell line to grow in suspension culture has a long history and only recently studies proposed a working suspension cell line. Despite intense adaptation efforts in our laboratory, a working cell bank was not established. Therefore, we obtained a Vero suspension cell line from a collaborator.

At first, small scale experiments are looking at important process parameters such as multiplicity of infection and cell density. In addition, the use of different media is compared. rVSV-ZEBOV production is scaled-up to the bioreactor and productions between previously described microcarrier and fixed-bed bioreactors are compared. In addition, the bioreactor process is transferred to an rVSV-based vaccine candidate against HIV.

In the context of the recent COVID-19 pandemic, we received another strain or rVSV which expresses the SARS-CoV-2-S protein on its surface and is currently in pre-clinical trials to assess the safety and efficacy as a COVID-19 vaccine candidate. Based on the process of rVSV-ZEBOV, we demonstrate the applicability of this system to the production of an urgently needed vaccine candidate.

Chapter 4

Bioreactor production of rVSV-based vectors in Vero cell suspension cultures

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Abstract

The Vero cell line is the most used continuous cell line in viral vaccine manufacturing. This adherent cell culture platform requires the use of surfaces to support cell growth, typically roller bottles or microcarriers. We have recently compared the production of rVSV-ZEBOV on Vero cells between microcarrier and fixed-bed bioreactors. However, suspension cultures are considered superior with regard to process scalability. Therefore, we further explore the Vero suspension system for rVSV-vectored vaccine production. Previously, this suspension cell line was only able to be cultivated in proprietary medium. Here, we show the adaptation and bioreactor cultivation in a commercial medium. Following small scale optimization and screening studies, we demonstrate the bioreactor production of highly relevant vaccines and vaccine candidates against Ebola virus disease, HIV and COVID-19 in the Vero suspension system. rVSV-ZEBOV, rVSV-HIV and $rVSV_{Ind}$-*msp*-S_F-*Gtc* can replicate to high titers in the bioreactor, reaching 3.87×10^7 $TCID_{50}$/mL, 2.12×10^7 $TCID_{50}$/mL and 3.59×10^9 $TCID_{50}$/mL, respectively. Further, we compare cell specific productivities, and take an in-depth look at the quality of the produced viruses by determining the ratio of total viral particles to infectious viral particles.

Keywords

Vero suspension culture, Viral vaccine bioprocess, Bioreactor production, rVSV-ZEBOV HIV, COVID-19

Abbreviations

COVID-19, coronavirus disease 2019; DIP, defective interfering particle; HIV, human immunodeficiency virus; hpi, hours post-infection; MOI, multiplicity of infection; TOI, time of infection; rVSV, recombinant vesicular stomatitis virus

Introduction

Recombinant vectored vaccines produced in cell culture are receiving increased attention in the fight against infectious diseases. More and more vaccines are available that are based on this technology and research efforts to develop new vaccines or to improve current manufacturing processes have intensified over the last years (Ura et al., 2020). One such system is based on the recombinant vesicular stomatitis virus (rVSV). In addition to its use as a vaccine vector, VSV has been used extensively in many areas of research, for example as an oncolytic virus or as a gene delivery tool (Lichty et al., 2004; Munis et al., 2020).

VSV is a replication-competent virus with a single-stranded, negative-sense RNA genome. The native glycoprotein, VSV-G, is responsible for viral entry into the cell. When genetically engineered to express the glycoprotein of another virus instead, rVSV can be used as a vaccine vector by delivering foreign antigens (Munis et al., 2020). The advantage of such a vectored vaccine is the increased safety during manufacturing, since the production of live-attenuated or inactivated vaccines of highly pathogenic viruses (e.g. HIV, Ebola) would require stringent biosafety standards. The recent success story of the EMA and FDA-approved Ebola vaccine rVSV-ZEBOV showcases the potential of the rVSV platform (Henao-Restrepo et al., 2017). rVSV-ZEBOV is a replication competent virus in which VSV-G was replaced by a Zaire Ebolavirus glycoprotein (ZEBOV), which is the main antigen of the Ebolavirus. Several rVSV-based vaccines are in development, for example against measles, Lassa fever and Middle East respiratory syndrome (MERS) (Henao-Restrepo et al., 2017; Kiesslich and Kamen, 2020; Munis et al., 2020).

In the light of the progress achieved with rVSV-ZEBOV, three novel rVSV constructs have been described recently, which carry different glycoproteins of the Human Immunodeficiency Virus (HIV) (Mangion et al., 2020). These HIV-vaccine candidates were produced in adherent Vero cells in tissue culture plates and it was demonstrated that they induced an HIV gp140-specific antibody response when administered to mice. The rVSV-B6-A74Env(PN6)/SIVtm construct was selected for further studies in non-human primates.

In the current race for a COVID-19 vaccine, recombinant vectored vaccines produced in cell culture are amongst the most promising (Ura et al., 2020). For example, ChAdOx1 nCoV-19, developed by the University of Oxford, is based on a chimpanzee adenovirus-vectored

vaccine expressing the SARS-CoV-2 spike protein (Folegatti et al., 2020) and its safety, efficacy, and immunogenicity is being assessed in a phase III clinical trial (NCT04516746). Further, the Oxford–AstraZeneca COVID-19 vaccine has been authorized by several national agencies, for example in the United Kingdom, and has been given WHO Emergency Use Listing (EUL) (World Health Organization, 2021).

Besides, several rVSV-based COVID-19 vaccine candidates expressing the SARS-CoV-2 spike protein are being evaluated in preclinical trials (University of Manitoba, Canada; University of Western Ontario, Canada; Aurobindo Pharma, India; Israel Institute for Biological Research/Weizmann Institute of Science, Israel; FBRI SRC VB VECTOR, Russia) and a phase I clinical trial (Merck Sharp & Dohme/IAVI; NCT04569786) (World Health Organization, 2020b). The COVID-19 vaccine candidate $rVSV_{Ind}$-*msp*-S_F-*Gtc*, is a temperature-sensitive construct. It is based on a recombinant VSV_{Ind}(GML), and shows reduced cytopathic effect *in vitro* at 37 °C, but replicates well at 31 °C (Kim et al., 2015). This attenuation was used as a strategy to further increase the safety of rVSV for its use as a human vaccine. $rVSV_{Ind}$-*msp*-S_F-*Gtc* is expressing the SARS-CoV-2 spike protein gene, the honeybee melittin signal peptide gene and the VSV-G protein transmembrane domain gene.

Currently, the rVSV-ZEBOV vaccine is manufactured under serum-free conditions in adherent Vero cells using the roller bottle technology (Monath et al., 2019). To improve manufacturing cost-effectiveness, more scalable bioprocesses involving microcarrier bioreactors and fixed-bed bioreactors have been studied recently (Kiesslich et al., 2020). However, these adherent cell processes still have scale-up limitations, for example the cell expansion steps during the seed train operation require cell detachment from and re-attachment to surfaces, usually involving enzymatic solutions such as trypsin. Suspension cell systems are considered superior with regard to process scale-up, since the transfer of cells to successively larger bioreactor vessels is straightforward.

Adherently growing Vero cells are the most used continuous cell line in viral vaccine manufacturing. For example, vaccines against Ebola, influenza, Japanese encephalitis, polio, rabies, rotavirus and smallpox are available on the market and vaccines against other infectious diseases are under development, using this cell line. The many advantages of this cell line are its broad susceptibility to many viruses, the long-term experience in cell culture

and the regulatory portfolio associated with vaccine manufacturing organization and health authorities worldwide (Kiesslich and Kamen, 2020).

Adaptation of the Vero cell line to grow in suspension culture to significantly improve this cell culture manufacturing platform has been of interest for many years (Litwin, 1992; Paillet et al., 2009). Lately, studies have reported the successful adaptation using proprietary media (Rourou et al., 2019; Shen et al., 2019). Shen et al. showed that Vero cells can grow in suspension culture in serum-free batch and perfusion bioreactors, and successfully applied their system to the production of rVSV-GFP, which uses the native glycoprotein VSV-G for viral entry into the cell.

In this work, we further explore the Vero suspension system described previously (Shen et al., 2019), and demonstrate its applicability to relevant rVSV-based vaccine candidates. Using rVSV-ZEBOV as a model for rVSV, we focus on small scale experiments to optimize the multiplicity of infection (MOI) and investigate effects of different cell densities. Next, we compare the production of rVSV-ZEBOV in this system to the production in Vero cells that were adapted to grow in suspension culture in a commercially available medium. In addition, we show the production of newly developed candidate vaccines against HIV (rVSV-HIV) and COVID-19 ($rVSV_{Ind}$-*msp*-S_F-*Gtc*). Based on these results, we demonstrate production in batch bioreactor for all three rVSV variants.

Materials & Methods

Cell line and culture media

The suspension adapted Vero cell line was provided by the National Research Council of Canada, Montreal, Canada, and its adaptation process has been described previously (Shen et al., 2019). For routine passaging, the cells from the late exponential growth phase were harvested by centrifugation for 5 minutes at 500 g and the cell pellet was resuspended in fresh medium to a seeding cell density of 2.5–5 × 10^5 cells/mL in 125 mL polycarbonate shake flasks (TriForest Enterprises, USA) and maintained at 37 °C, 135 rpm and 5% CO_2 in an humified Multitron orbital shaker (Infors HT, Switzerland). The cells were cultivated in 20 mL working volume of either IHM03 medium, provided by the National Research Council

of Canada, Montreal, Canada, or in MDXK medium (Xell AG, Germany), supplemented with 4 mM GlutaMAX (Thermo Fisher Scientific, USA).

Viruses

Origin and viral seed stock amplification of rVSV-ZEBOV and rVSV-B6-A74Env(PN6)/SIVtm (hereafter referred to as rVSV-HIV) have been described previously (Kiesslich et al., 2020) (Mangion et al., 2020). The viral seed stock of rVSV-ZEBOV had a titer of 1.49 × 107 TCID50/mL and 2.80 × 109 VG/mL, whereas rVSV-HIV had a titer of 7.14 × 106 TCID50/mL and 3.13 × 1010 VG/mL, respectively

The recombinant COVID-19 vaccine candidate rVSV$_{Ind}$-*msp*-S$_F$-*Gtc* was constructed as follows: Codon-optimized full-length spike protein gene of SARS-CoV-2 (GenBank: JX869059.2) was purchased from Genscript USA Inc (Pscataway, NJ, USA), cloned into an avirulent Indiana serotype of vesicular stomatitis virus [VSV$_{Ind}$(GML)] as has been described previously (Kim et al., 2015). The honeybee melittin signal peptide (*msp*) was inserted at the NH_2-terminus and the transmembrane domain and cytoplasmic tail (Gtc) of the S protein was substituted by the Gtc of VSV$_{Ind}$ G protein at the COOH-terminus. In addition, the VSV transgenic sequences were added in front of the spike protein gene in order to provide the transcription terminal signal, polyadenylation signal, and the transcription re-initiation signal. The modified SARS-CoV-2 spike protein gene was inserted into the G and L gene junction of the VSV$_{Ind}$(GML) at *Pme I* and *Mlu I* sites. Recombinant rVSV$_{Ind}$-*msp*-S$_F$-*Gtc* virus was recovered by VSV reverse genetics as has been described previously (Kim et al., 2015). The recombinant virus was purified by three consecutive plaque picking, and a stock virus was prepared by infecting BHK_{21} cells. The viral seed stock of rVSVInd-msp-SF-Gtc had a titer of 1.43 × 108 TCID50/mL and 4.64 × 109 VG/mL.

Shake flask virus studies

For virus infection studies in shake flasks, suspension adapted Vero cells from the late exponential growth phase were harvested, if required pooled, and seeded at the indicated cell density in fresh medium. Vero cells were infected with rVSV at the indicated MOI and the temperature was shifted to either 34 °C (rVSV-ZEBOV, rVSV-HIV) or 31 °C (rVSV$_{Ind}$-*msp*-S$_F$-

Gtc). Samples for virus titration were centrifuged for 5 minutes at 1200 × g to remove cellular debris, aliquoted and stored at −80 °C.

Bioreactor cultures

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. MDXK medium was supplemented 4 mM L-glutamine (GE Healthcare, USA) instead of GlutaMAX to enable monitoring of L-glutamine consumption. Vero cell seed cultures were grown in progressively larger polycarbonate shake flasks (TriForest Enterprises, USA), harvested by centrifugation and resuspended in fresh medium before inoculation. The bioreactor was inoculated at a cell density of 2.5×10^5 cells/mL in 850 mL working volume. The culture was agitated at 100 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 through the sparger 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. Samples for metabolite analysis and virus titration were centrifuged for 5 minutes at 1200 × g to remove cellular debris, aliquoted and stored at −80 °C.

For virus production, Vero cells were infected with rVSV at an MOI of 0.01 once the desired cell density was reached and the temperature was shifted to 34 °C or 31 °C, respectively, during the virus production phase. 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.

Analytical Methods

Vero cell concentration and viability were determined via the Vi-CELL XR cell counter (Beckman Coulter, USA). The Median Tissue Culture Infectious Dose ($TCID_{50}$) assay and digital PCR assay used in this work to quantify the infectious titer and the number of viral genomes, respectively, have been described previously (Gélinas et al., 2020; Kiesslich et al.,

2020). For rVSV$_{Ind}$-*msp*-S$_F$-*Gtc*, the TCID$_{50}$ plates were incubated at 31 °C, due to the temperature sensitivity of this construct.

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.

Results and Discussion

Cell growth of Vero cells in suspension cultures

Screening for commercial media

Suspension adapted Vero cells grew well in shake flasks in IHM03 medium up to a cell density of around 2 × 10^6 cells/mL and with doubling times of around 48 h as previously reported (Shen et al., 2019). IHM03 is an in-house medium developed and produced in small batches by the National Research Council, Montreal, Canada, supporting the growth and virus production of Vero suspension cultures. As reported by Shen et al. and Rourou et al., media composition is critical for successfully generating a suspension adapted Vero cell line and no commercial media was able to support Vero cell adaptation so far (Rourou et al., 2019; Shen et al., 2019). Despite these recently reported adaptation successes with in-house media, establishing a process using commercial media could reduce the risk of lot-to-lot variations and would make the platform more amenable to work under standard conditions when media supply is assured.

Therefore, efforts have been dedicated to assessing different commercial media. After 20 passages of adaptation in shake flasks, with gradual media replacement during the first five passages, a Vero cell line was obtained that was able to grow in MDXK medium (Xell AG) supplemented with 4 mM Glutamax and which exhibited cell doubling times of around 48-72 h (data not shown). Other commercial media that were tested, but did not support growth of Vero cells in suspension culture included VP-SFM, OptiPRO, FreeStyle 293 (Thermo Fisher

Scientific, USA), HyClone HyCell TransFx-H (GE Healthcare, USA), HEK GM (Xell AG, Germany) and ProVeroTM -1 serum free medium (SFM) (Lonza, Switzerland).

Despite slightly slower growth rates in MDXK than in IHM03 in shake flasks, the cells were able to grow to similar cell densities in batch shake flasks with less formation of cell aggregates. Compared to other mammalian suspension cell lines, like derivatives of HEK293 or CHO with cell doubling times of 24 h, there is still great potential to develop media that can support similar growth rates. With a suspension Vero cell line available, further research can use novel analytical techniques such as transcriptomics and metabolomics to facility media development specifically geared towards this cell line.

Cell growth in batch bioreactor

The suspension adapted Vero cells showed similar growth in IHM03 medium in 1L batch bioreactors as previously reported, reaching 1.78 × 10^6 cells/mL after 6 days (Shen et al., 2019). Cell viability was above 99 % during the whole run. The doubling time was around 51 hours for the entire batch process duration between cell seeding and peak in maximum cell density, and around 40 hours for the exponential cell growth phase taking place between cell seeding up until 96 hours. During the exponential growth phase, the cell growth rate was 0.0174 h^{-1} (**Figure 14** A). The substrates glucose and glutamine were almost depleted at the end of the culture. Ammonia was produced throughout and stayed below concentrations of 4 mM. Lactate production reached a concentration of 24.48 mM after 96 hours, but declined thereafter which can be explained by an uptake of lactate by the cells as previously reported (Quesney et al., 2003).

Cell growths in MDXK medium was slightly slower and only reached 1.45 × 10^6 cells/mL after 7 days. In MDXK, the doubling time was around 65 hours for the entire batch process duration between cell seeding and peak in maximum cell density. During the exponential cell growth phase between cell seeding up until 96 hours, the cell doubling time was 52 hours, resulting in a cell growth rate of 0.0132 h^{-1} (**Figure 14** B). However, in contrast to the shake flask experiments, Vero cells in MDXK medium showed a higher degree of aggregate formation in the bioreactor compared to cells in IHM03. This could have led to an underestimation of the cell count, which is also indicated by a higher glucose consumption rate in MDXK. Cell viability again was above 99 % throughout the process. As opposed to the

previous run, glucose and glutamine were depleted earlier and required feeding, adjusting glucose to 2 g/L and glutamine to 2 mM once daily starting on day 2 for glutamine and day 6 for glucose. Ammonia production was similar than before, never exceeding concentrations of 4 mM. Lactate production, however, was considerably higher and surpassed 50 mM at the end of the culture. Lactate was not consumed by the cells in MDXK medium indicating significant differences in the cell metabolism in the two media.

A drawback of the current procedure for cell passaging and bioreactor seed preparation is the need for centrifugation. Due to long cell doubling times, it is necessary to exchange spent medium at the end of each passage instead of diluting the culture with fresh medium. Further, the resuspension of the cell pellet after centrifugation breaks apart the majority of loosely aggregated cell clumps. While this procedure works well for small shake flask cultures, scale-up to larger volumes such as those in bioreactor vessels at the manufacturing stage can be challenging. Further studies will investigate the use of perfusion technologies to achieve higher cell densities, so that culture dilution can be applied instead of centrifugation when passaging. In addition, media optimization will be carried out to increase cell growth rates and to reduce cell adherence and aggregation.

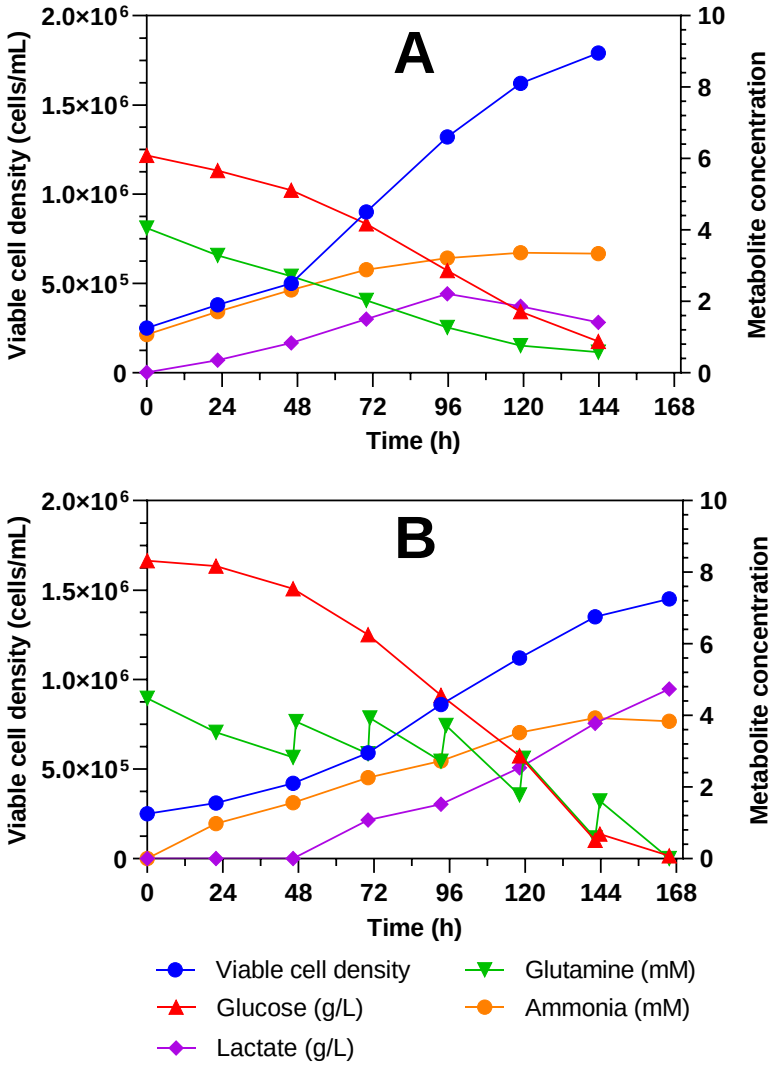

Figure 14. Cell growth of suspension adapted Vero cells in IHM03 (A) and MDXK (B) medium in a 1 L bioreactor in batch mode. The concentration of the main metabolic substrates (glucose, L-glutamine) and by-products (lactate, ammonia) are given in mM or g/L.

rVSV-ZEBOV production in shake flask

rVSV-ZEBOV production experiments were initially carried out at a smaller scale in shake flasks to test multiple conditions simultaneously. In particular, the effects of varying multiplicities of infection (MOI), which is the ratio of infectious particles to the number of cells at the time of infection (TOI), as well as infections at different cell densities and in different growth media were screened.

During the late stages of the cultivation when cell densities exceeded 1×10^6 cells/mL, cells started to adhere to the surface of the shake flask and cell aggregates were formed, which made it quite difficult to accurately determine the cell count. Therefore, initial experiments to investigate the infection kinetics of rVSV-ZEBOV in suspension adapted Vero cells, were carried out by seeding a single cell culture in fresh medium at a cell density of 1×10^6 cells/mL and cells were infected immediately thereafter.

Previous studies of rVSV-ZEBOV in adherent Vero cells and suspension cultures of HEK293 cells have shown that infection at a reduced temperature of 34 °C led to higher infectious titers compared to 37 °C (Kiesslich et al., 2020)(Gélinas et al., 2019). Based on these studies, the temperature was lowered to 34 °C after infection in all experiments of this work involving rVSV-ZEBOV.

Multiplicity of infection

Vero cells grown in IHM03 were infected with rVSV-ZEBOV at different MOIs and samples were taken every 12 hours to determine infection kinetics (**Figure 15**). For the selected range of MOI, peak production of rVSV-ZEBOV occurred between 24 and 36 hours post infection (hpi) and the titers were in the same range with 1.10×10^7 $TCID_{50}$/mL, 1.05×10^7 $TCID_{50}$/mL and 1.58×10^7 $TCID_{50}$/mL at an MOI of 0.001, 0.01 and 0.1, respectively. In all cases, the infectivity declined after the maximum titer had been reached. Similar kinetics have been observed for adherent growing Vero cells, however, the titers were more than eight times higher in adherent cell experiments in 6-well tissue culture plates, for example 8.79×10^7 $TCID_{50}$/mL at an MOI of 0.01 at 36 hpi (Kiesslich et al., 2020). Further, the cell density at the time of infection was more than three times higher for the suspension cultures than for the adherent cell cultures in the reported study, indicating even lower virus production per cell. Compared to rVSV-ZEBOV studies in suspension cultures of HEK293-SF, where cells were also seeded at 1×10^6 cells/mL, infected at an MOI of 0.001 and incubated at 34 °C, titers were almost ten times higher in HEK293-SF at 48 hpi with 1.00×10^8 $TCID_{50}$/mL (Gélinas et al., 2019). It is to mention that adherent Vero as well as HEK239-SF cells were cultivated in commercially available media, optimized for cell growth and production.

rVSV infections of adherent Vero cells typically lead to a very distinct cytopathic effect, where cells become round shaped and eventually lift off from the surface. Of note, since suspension cells are already round shaped and not attached to a surface, the cytopathic effect induced by rVSV infections was less noticeable in the early stages of infection and only became more apparent when the cell diameter increased due to viral replication and when cells started to die from lysis caused by viral release.

Nevertheless, based on these experiments and in accordance with our previous work, it was decided to continue all subsequent rVSV experiments at an MOI of 0.01.

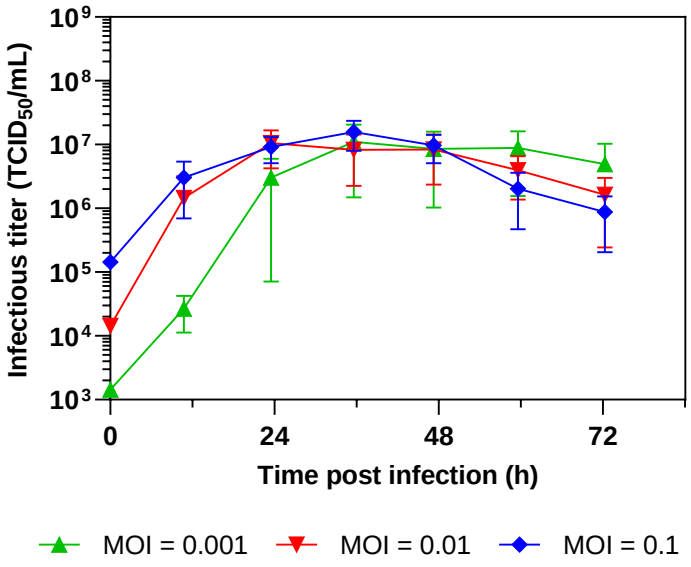

Figure 15. rVSV-ZEBOV infection of suspension adapted Vero cells at different MOIs ranging from 0.001 to 0.1 in 125 mL shake flask in IHM03 medium at a cell density of 1 × 10^6 cells/mL. Infectious viral titers expressed as $TCID_{50}$/mL are plotted against the time post-infection. Bars represent the mean of the samples from three independent shake flask replicates ± standard deviation.

Cell density

rVSV-ZEBOV replication in Vero cells grown in IHM03 and seeded at different cell densities was investigated to evaluate the effect of varying cell densities and to assess if the production yield was affected by the media capacity at the time of infection. **Figure 16** shows rVSV-ZEBOV infection at 1 × 10^6 cells/mL, 2 × 10^6 cells/mL and 4 × 10^6 cells/mL. The viral infection kinetics were slower compared to the previous experiments and infectious titers peaked at 48 hpi for all cases, indicating considerable variation between experiments when comparing the data to the experiment presented in **Figure 15**. Additionally, maximum

infectious titers were around three times higher for the run at 1×10^6 cells/mL, reaching 3.28×10^7 $TCID_{50}$/mL. To reduce variations and improve repeatability, cells from similar passages should be used. Further, cell density estimations, especially when the cultures contain aggregates, need to be carried out very careful since small errors can affect MOI calculations significantly.

Throughout the time curse of the experiment, titers were even higher at 2×10^6 cells/mL, but the maximum infectious titer was not significantly elevated at 48 hpi. For the run with a seeding cell density of 4×10^6 cells/mL, infectious titers of rVSV-ZEBOV were significantly higher, reaching 1.32×10^8 $TCID_{50}$/mL, exceeding infectious titers obtained from adherent Vero studies in 6-well plates (Kiesslich et al., 2020). A similar study carried out in HEK293-SF obtained two to three times higher titers with 2.92×10^8 $TCID_{50}$/mL and 2.36×10^8 $TCID_{50}$/mL, at 2.5×10^6 cells/mL and 5×10^6 cells/mL, respectively, compared to infection at 1×10^6 cells/mL (Gélinas et al., 2019). Furthermore, for rVSV-GFP produced in suspension cell cultures of Vero cells at different cell densities in a similar experiment, 3.8 times higher virus titers were obtained at 2.5×10^6 cells/mL compared to 0.87×10^6 cells/mL (Shen et al., 2019). But a further increase in cell density from 2.5×10^6 cells/mL to 5×10^6 cells/mL did not result in higher infectious titers, comparable to the results obtained in HEK293-SF. This cell density effect has also been observed for the production of other viruses. For example during poliovirus production, a higher cell density lead to lower cell specific poliovirus D-antigen levels (Thomassen et al., 2014).

These results indicate that suspension cultures of Vero cells could be a viable alternative if high cell density processes of suspension cultures can be achieved at larger scale, but further research is necessary to investigate effects of high cell density in more detail. One advantage though is that suspension cultures are not limited by the surface area, as is the case for adherent cell cultures using microcarriers, roller bottles or fixed-bed bioreactors, which is the prevalent mode of virus production in Vero cells. Nevertheless, these results need to be carefully evaluated since it is not feasible to seed bioreactors in fresh medium at high cell density. In this context, shake flask studies in HEK293-SF cells found that if the cultures were grown to high cell densities and infected without medium exchange, the infectious titer of rVSV-ZEBOV could not be enhanced by increasing the cell density above 2×10^6 cells/mL (Gélinas et al., 2019).

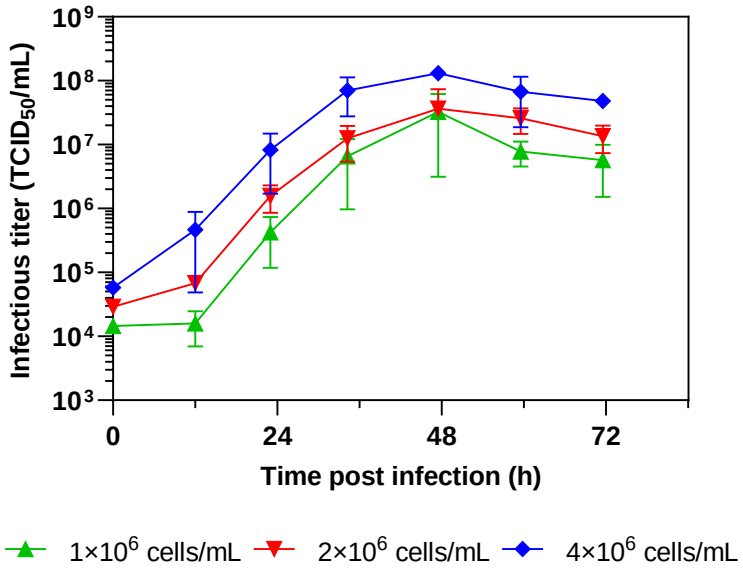

Figure 16. rVSV-ZEBOV infection of suspension adapted Vero cells at different cell densities ranging from 1×10^6 to 4×10^6 cells/mL in 125 mL shake flask in IHM03 medium. Infectious viral titers expressed as TCID50/mL are plotted against the time post-infection. Bars represent the mean of the samples from three independent shake flask replicates ± standard deviation.

rVSV-ZEBOV, rVSV-HIV, and rVSV$_{Ind}$-*msp*-S$_F$-*Gtc* shake flask production in different media

Next, the kinetics of three variants, namely rVSV-ZEBOV, rVSV-HIV, and rVSV$_{Ind}$-*msp*-S$_F$-*Gtc* were compared and the effect of different media on rVSV production were studied. Based on temperature study results of rVSV-ZEBOV infections in Vero cells, rVSV-HIV infections were carried out at 34 °C in a recent study and this condition was adopted for this work as well (Mangion et al., 2020). The rVSV$_{Ind}$-*msp*-S$_F$-*Gtc* construct, however, is temperature sensitive and therefore all infections were carried out at 31 °C.

The IHM03 and MDXK adapted cell lines were infected with rVSV to compare the virus production capacities of both media. Despite higher titers obtained at higher cell densities (**Figure 16**), these experiments were carried out at a seeding cell density of 1×10^6 cells/mL to avoid cell aggregation. **Figure 17** shows rVSV-ZEBOV (A), rVSV-HIV (B) and rVSV$_{Ind}$-*msp*-S$_F$-*Gtc* (C) replication in the MDXK adapted cell line at an MOI of 0.01 in comparison to the corresponding experiment conducted on IHM03.

rVSV-ZEBOV replication in MDXK medium reached slightly higher titers than in IHM03 medium (**Figure 17** A). Where the infectious titer reached a maximum at 24 hpi in IHM03, the titer in MDXK was with 2.63×10^7 $TCID_{50}$/mL around 2.5 times higher. Otherwise, almost identical infection kinetics were observed.

rVSV-HIV replicated better in IHM03 with a higher maximum titer of 9.59×10^6 $TCID_{50}$/mL reached in a shorter period of time (24 hpi) compared to 2.08×10^6 $TCID_{50}$/mL in MDXK after 36 hpi (**Figure 17** B). Titers of rVSV-HIV were lower in both media than rVSV-ZEBOV and the infectivity declined faster than for rVSV-ZEBOV. Besides, rVSV-HIV produced in adherent Vero cells reached up to 3.91×10^7 $TCID_{50}$/mL at MOI of 0.01. However, the peak was reached significantly later at 96 hpi (Mangion et al., 2020).

In contrast to these two strains, $rVSV_{Ind}$-*msp*-S_F-*Gtc* reached significantly higher titers (**Figure 17** C). In MDXK, 5.19×10^8 $TCID_{50}$/mL were reached at 36 hpi. In IHM03, almost two-fold higher titers with 1.17×10^9 $TCID_{50}$/mL were reached. However, it took additional 24 hours to reach this number, which aligns with the beginning of the replication phase being delayed. In addition to higher infectious titers compared to rVSV-ZEBOV and rVSV-HIV, the infectivity of viral particles did not decline significantly over the following sample time points.

Differences in replication kinetics between the three rVSV-variants can be attributed in part to the use of different glycoproteins. The Ebola virus glycoprotein (Moller-tank and Maury, 2015), the HIV envelope glycoprotein (Klasse, 2012) and the SARS-CoV-2 spike protein (S) (Hoffmann et al., 2020), which are responsible for cell entry of the corresponding rVSV used in this work, all use different types of cell receptors and entry mechanisms. Further, the formation of these proteins during intracellular replication of the virus and the final assembly of the viral particles are unique. These mechanisms have their own rate limiting steps and efficiencies, resulting in different amounts of functional rVSV particles and different ratios of infectious to total particles.

Higher infectious titers of $rVSV_{Ind}$-*msp*-S_F-*Gtc* in Vero cells could also be linked more specifically to the use of different transmembrane domains. Whereas rVSV-ZEBOV and rVSV-HIV use Ebola GP or SIV transmembrane domains (Mangion et al., 2020), $rVSV_{Ind}$-*msp*-S_F-*Gtc* is expressing the native VSV-G protein transmembrane domain gene. It has been shown that

the stem region of the VSV-G glycoprotein was important for efficient virus assembly, and viruses with shortened sequences were replicated up 20-fold less (Robison and Whitt, 2000). More research investigating the use of different transmembrane domains with the same extracellular domain could reveal interesting aspects on virus replication rates and identify new targets to improve the rVSV platform. Nevertheless, it might be more appropriate to compare replication of $rVSV_{Ind}$-*msp*-S_F-*Gtc* to rVSV-GFP production, which also uses the native VSV-G protein transmembrane domain, and where titers of up to 8.93×10^9 $TCID_{50}$/mL have been obtained in shake flask experiments.

Besides, the lower process temperature of 31 °C is likely affecting the infectivity. For example, the infectivity of this strain was not declining over the following 36 h after the peak titer had been reached thus potentially stabilizing infectivity and preventing virus degradation. Though for rVSV-ZEBOV, an optimal production temperature of 34 °C was determined in adherent Vero cells (Kiesslich et al., 2020), $rVSV_{Ind}$-*msp*-S_F-*Gtc* is based on VSV_{Ind}(GML) which was adapted to replicate well at the lower temperature of 31 °C (**Figure 17** C).

Another reason for differences in production titers between the three rVSV variants could be linked to defective interfering particles (DIPs) and the quality of the viral seed stocks (Ziegler and Botten, 2020). The ratios of total viral particles to infectious particles for rVSV-ZEBOV, rVSV-HIV and $rVSV_{Ind}$-*msp*-S_F-*Gtc* are 188 VG/$TCID_{50}$, 4384 VG/$TCID_{50}$, and 32 VG/$TCID_{50}$, respectively. Especially the high ratio of VG/$TCID_{50}$ of the rVSV-HIV stock indicates a lower quality of this virus stock and could have led to DIPs influencing the production process.

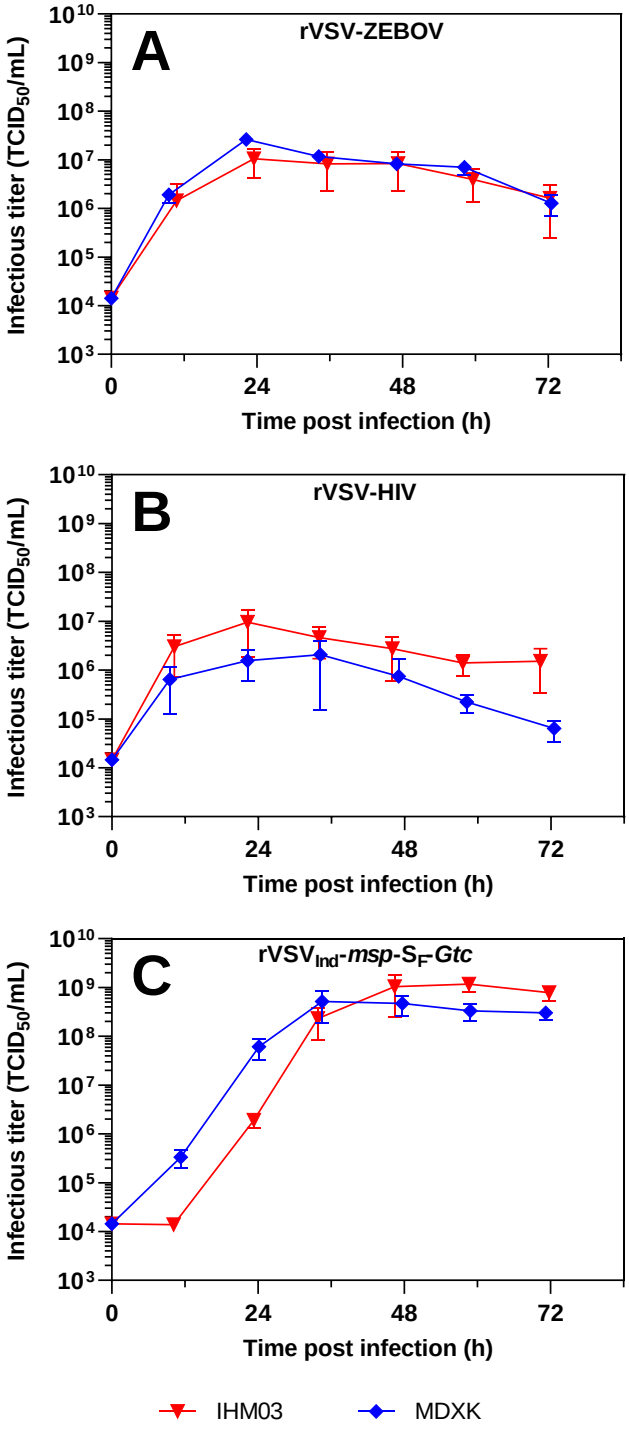

Figure 17. rVSV-ZEBOV (A), rVSV-HIV (B) and rVSV$_{Ind}$-*msp*-S$_F$-*Gtc* (C) infections of suspension adapted Vero cells cultivated in either IHM03 or MDXK medium at an MOI of 0.01, in 125 mL shake flask at a cell density of 1×10^6 cells/mL. The rVSV-ZEBOV and rVSV-HIV infections were carried out at 34 °C, whereas the rVSV$_{Ind}$-*msp*-S$_F$-*Gtc* infection was carried out at 31 °C. Infectious viral titers expressed as TCID$_{50}$/mL are plotted against the time post-infection. Bars represent the mean of the samples from three independent shake flask replicates ± standard deviation.

Bioreactor processes of suspension adapted Vero cells

Bioreactor production of rVSV-ZEBOV

Two bioreactors of Vero cells were infected at a cell density of 1.37×10^6 cells/mL and 1.02×10^6 cells/mL after cells grew for 4 days in IHM03 and MDXK medium, respectively (**Figure 18**). The cell growth phase corresponded well to the data shown in **Figure 14**. Despite a lower cell density at the TOI, maximum infectious titers where with 3.87×10^7 $TCID_{50}$/mL more than one log higher than in IHM03, where only 3.55×10^6 $TCID_{50}$/mL were obtained. In addition, replication was faster in MDXK, were peak production occurred at 24 hpi compared to 36 hpi, respectively. The MDXK bioreactor also exhibited an almost 15-times higher cell specific productivity with 37.9 $TCID_{50}$/cell compared to 2.6 $TCID_{50}$/cell. Moreover, the ratio of total viral particles to infectious particles was lower in MDXK (282 VG/$TCID_{50}$) than in IHM03 (817 VG/$TCID_{50}$), further indicating a better quality of the final product if harvested at the time of peak infectious titer. As a result, MDXK medium appears better suited for rVSV-ZEBOV production in the bioreactor than IHM03. As shown in **Figure 14**, the main substrates glucose and glutamine were not at limiting concentrations at the TOI. However, differences in media compositions such as nutrient profile and concentration, could have affected rVSV-ZEBOV production and other metabolites which were not quantified could have been at limiting or inhibiting concentrations.

Compared to the shake flask experiments, rVSV-ZEBOV replication in MDXK medium reached similar maximum infectious titer, indicating a successful scale-up. For IHM03, titers were three times lower than in shake flask and the peak was reached 12 hours later.

Further, in comparison to adherent Vero bioreactor productions of rVSV-ZEBOV, the production using suspension adapted Vero cells in MDXK appears elevated. The infectious titer and the cell specific productivities were slightly higher compared the production in a microcarrier bioreactor (1.42×10^7 $TCID_{50}$/mL, 7.6 $TCID_{50}$/cell) and a fixed-bed bioreactor (2.59×10^7 $TCID_{50}$/mL, 11.2 $TCID_{50}$/cell). The ratio of total viral particles to infectious particles was four times lower than in the microcarrier but nine times higher compared to the fixed-bed process (Kiesslich et al., 2020). Overall, the suspension Vero system resembles

a viable alternative to the current Vero manufacturing system carried out in roller bottles for this Ebola virus disease vaccine (Monath et al., 2019).

When set side by side to a suspension bioreactor production of rVSV-ZEBOV in HEK293-SF, where a maximum of 1.19×10^8 $TCID_{50}$/mL was reached, production in Vero cells in MDXK was 3 times lower. However, it can be expected that future media development, bioprocess and cell line engineering of suspension Vero can lead to significantly higher titers comparable to HEK293-SF (Gélinas et al., 2019).

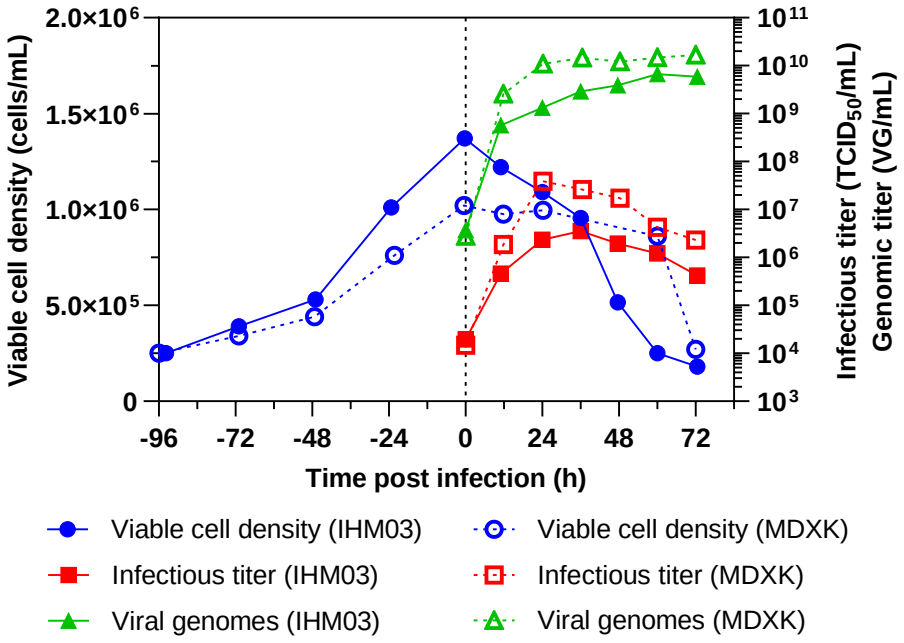

Figure 18. Comparison of the production of rVSV-ZEBOV in suspension adapted Vero cells cultivated in either IHM03 or MDXK medium in a 1 L bioreactor in batch mode. Infectious viral titers expressed as $TCID_{50}$/mL and the number of viral genomes in VG/mL are plotted against the time post-infection.

Bioreactor production of rVSV-HIV

In the next experiment, two bioreactors were prepared as before, and Vero cell growth phase in IHM03 and MDXK medium was consistent with the data from **Figure 14** and **Figure 18**. The two cultures were infected with rVSV-HIV after 4 days (**Figure 19**). In contrast to the previous experiment, virus production was favoured in IHM03 over MDXK. In IHM03, rVSV-HIV reached a maximum titer of 2.12×10^7 $TCID_{50}$/mL, which was 25-times higher than in MDXK. However, production kinetics of viral genomes were almost identical. This is supported by a lower ratio of 143 VG/$TCID_{50}$ (IHM03) compared to 7041 VG/$TCID_{50}$

(MDXK). Additionally, the fact that the cell specific productivity in MDXK was 0.9 $TCID_{50}$/cell implies that the rVSV-HIV/MDXK system failed to scale-up and is not an adequate production system.

Besides, the shake flask experiment (**Figure 17** B) already indicated the suitability of IHM03 for rVSV-HIV replication. Moreover, bioreactor production of rVSV-HIV in IHM03 exceeded titers from the smaller scale, whereas bioreactor titers in MDXK subsided the small scale.

Differences between the two media were already seen for bioreactor production of rVSV-ZEBOV. Here, IHM03 appears to be favoured for rVSV-HIV production. However, the production of total rVSV-HIV particles, estimated from the number of viral genomes, is comparable between the two media. Therefore, one reason of a higher titer of rVSV-HIV in IHM03 could be that this medium is better suited to stabilize the HIV glycoprotein and to maintain the viral infectivity.

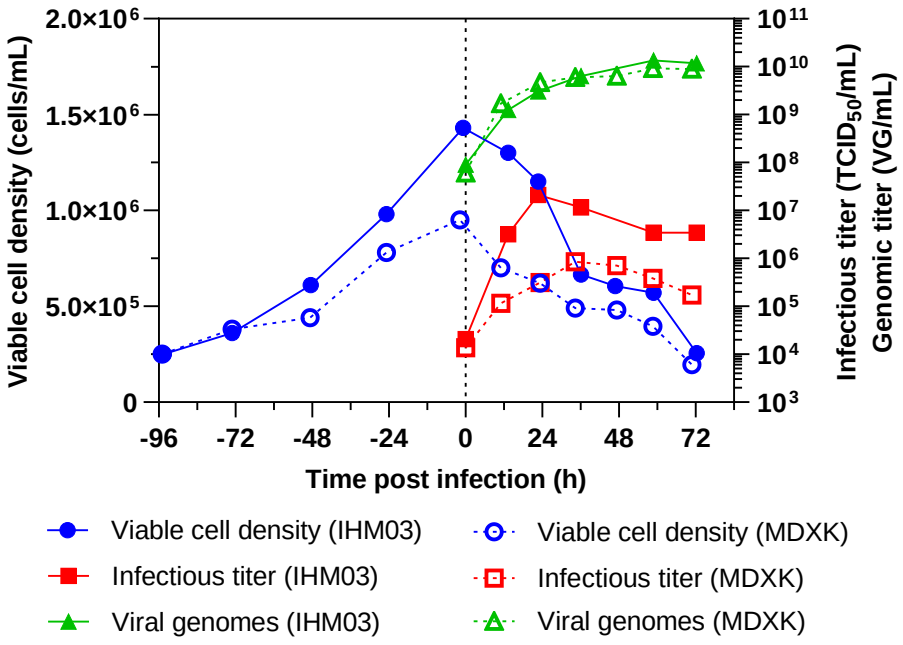

Figure 19. Comparison of the production of rVSV-HIV in suspension adapted Vero cells cultivated in either IHM03 or MDXK medium in a 1 L bioreactor in batch mode. Infectious viral titers expressed as $TCID_{50}$/mL and the number of viral genomes in VG/mL are plotted against the time post-infection.

Bioreactor production of rVSV$_{Ind}$-msp-S$_F$-Gtc

Finally, rVSV$_{Ind}$-*msp*-S$_F$-*Gtc* production in bioreactors of Vero suspension cell cultures was studied (**Figure 20**). Again, the cell growth phase was comparable to the previous runs, and cells were infected after 4 days. Infectious titers of rVSV$_{Ind}$-*msp*-S$_F$-*Gtc* peaked in both media at 48 hpi. The beginning of the replication phase was delayed in IHM03, as already seen in the shake flask experiments. Nevertheless, titers were similar with 2.38×10^9 TCID$_{50}$/mL in IHM03 and 3.59×10^9 TCID$_{50}$/mL in MDXK. Thus, results from the shake flask experiment were exceeded by two-fold and seven-fold, respectively. The quality in terms of total viral particles to infectious particles was with 3.0 VG/TCID$_{50}$ comparable in IHM03 to that in MDXK, where this value was 6.0 VG/TCID$_{50}$. In addition, the cell specific productivity was in the same range in MDXK compared to IHM03, with 3670 TCID$_{50}$/cell and 1803 TCID$_{50}$/cell, respectively.

In comparison, rVSV-ZEBOV and rVSV-HIV productions in the bioreactor peaked earlier. However, the number of viral genomes continued to increase in those experiments even when the infectious titer declined. Therefore, the rate of viral degradation is higher than the viral production rate after the corresponding peak was reached in the case of these two strains, potentially due to the higher process temperature of 34 °C versus 31 °C and its impact on viral stability.

Overall, the scale-up of rVSV$_{Ind}$-*msp*-S$_F$-*Gtc* production to the bioreactor was successful, exceeding small scale results. In addition, this strain appears to replicate to much higher titers, with a superior cell specific productivity and an improved ratio of VG/TCID$_{50}$ as compared to rVSV-ZEBOV and rVSV-HIV. **Table 4** shows a summary of the six bioreactor runs, comparing results for the three strains in two different culture media.

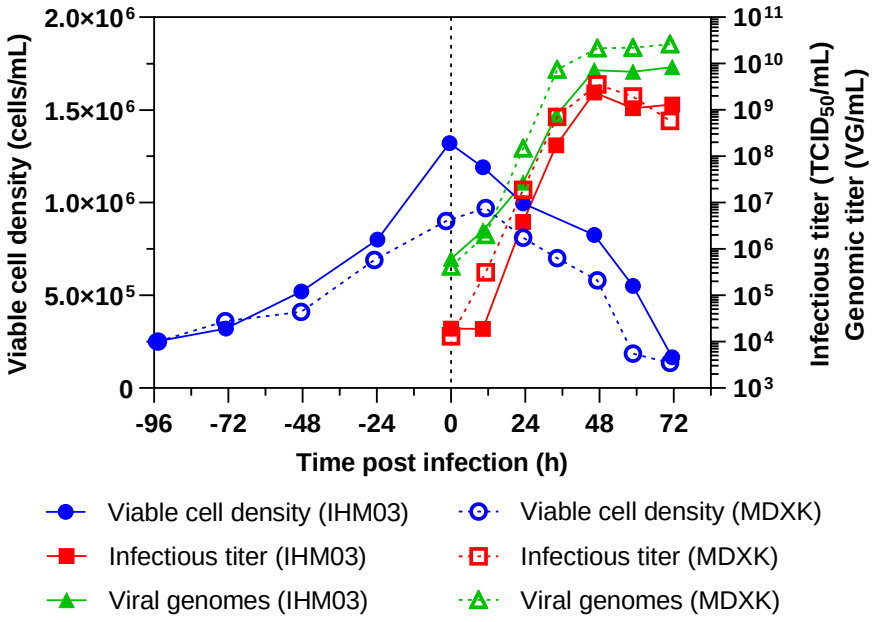

Figure 20. Comparison of the production of rVSV$_{Ind}$-*msp*-S$_F$-*Gtc* in suspension adapted Vero cells cultivated in either IHM03 or MDXK medium in a 1 L bioreactor in batch mode. Infectious viral titers expressed as TCID$_{50}$/mL and the number of viral genomes in VG/mL are plotted against the time post-infection.

Table 4: Comparison of bioreactor productions of rVSV-ZEBOV, rVSV-HIV and rVSV$_{Ind}$-*msp*-S$_F$-*Gtc*

	rVSV-ZEBOV		rVSV-HIV		rVSV$_{Ind}$-*msp*-S$_F$-*Gtc*	
Medium	IHM03	MDXK	IHM03	MDXK	IHM03	MDXK
Peak infectious titer	36 hpi	24 hpi	24 hpi	36 hpi	48 hpi	48 hpi
Infectious titer (TCID$_{50}$/mL)	3.55×10^6	3.87×10^7	2.12×10^7	8.45×10^5	2.38×10^9	3.59×10^9
Genomic titer (VG/mL)	2.9×10^9	1.09×10^{10}	3.30×10^9	5.95×10^9	7.15×10^9	2.13×10^{10}
Ratio VG/TCID$_{50}$	817	282	143	7041	3.0	6.0
Cell specific titer (TCID$_{50}$/cell)	3	38	15	1	1803	3670

Conclusion

In this work, we have demonstrated the feasibility and applicability of suspension adapted Vero cell cultures for the production of highly relevant rVSV-based vaccines and vaccine candidates. For three rVSV strains, namely rVSV-ZEBOV, rVSV-HIV and rVSV$_{Ind}$-*msp*-S$_F$-*Gtc*, production was successfully scaled-up to the bioreactor scale. Further, proof-of-concept is provided that rVSV-ZEBOV and rVSV$_{Ind}$-*msp*-S$_F$-*Gtc* can be produced in commercially available media in suspension adapted Vero cells.

Previously, the suspension adapted Vero cell line was tested for tumorigenicity at passage 163 (Shen et al., 2019). Since the experiments with the Vero cell line in commercial MDXK medium were carried out at a higher passage number, this cell line would also require testing for tumorigenicity to ensure its safety as a substrate for the production of vaccines.

Process parameters developed in suspension Vero and previously in adherent Vero cells for rVSV-ZEBOV (Kiesslich et al., 2020), have been shown to be applicable to other strains. This is an important observation as rVSV-ZEBOV can thus serve as a model virus for other rVSV strains. More, this can be of significant value for the production of future rVSV-based vaccine candidates against emerging infectious diseases.

Moreover, the production of rVSV-ZEBOV was shown to be elevated compared to previously developed adherent processes in microcarrier and fixed-bed bioreactors (Kiesslich et al., 2020). Due to the better scalability, the suspension Vero system can serve as a viable alternative to the current Ebola virus disease vaccine manufacturing using roller bottles.

Production of rVSV-ZEBOV was leading to higher infectious titers in suspension cultures of HEK293-SF (Gélinas et al., 2019). However, this system used commercially available media and bioprocesses developed with years of experience. The commercially available MDXK medium has only been on the market for a short period of time. Hence, there is great potential for optimization of Vero suspension media and bioprocesses specifically for virus production.

In the context of the current COVID-19 pandemic, this work shows relevant advancement in the field of bioprocess development for urgently needed vector-based vaccine candidates. Given that rVSV$_{Ind}$-*msp*-S$_F$-*Gtc* grows to titers that are around 100-fold higher than titers of

rVSV-ZEBOV in the same system, and given that rVSV-ZEBOV produced in conventional roller bottle processes has been approved as a vaccine candidate by regulatory affairs, the herein presented bioprocess using suspension adapted Vero cells can serve as a highly efficient system for accelerated and scalable manufacturing of a COVID-19 vaccine candidate. Further, the quality of the produced viruses in terms of the ratio total particles to infectious particles is better, potentially leading to facilitated downstream processes and ultimately very economical manufacturing.

In the future, fed-batch and perfusion processes should be developed for high cell density bioreactors. As indicated in shake flask experiments and already demonstrated for VSV-GFP (Shen et al., 2019), these can be approaches to further push the boundaries and to increase virus productivities of the suspension Vero system.

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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Author contributions

Sascha Kiesslich: Conceptualization, Investigation, Formal analysis, Writing - Original Draft, Writing - Review & Editing. **Gyoung Nyoun Kim:** Conceptualization, Investigation, Writing - Original Draft, Writing - Review & Editing. **Chun Fang Shen:** Investigation, Writing - Review & Editing. **C. Yong Kang:** Conceptualization, Writing - Review & Editing, Supervision. **Amine A. Kamen:** Conceptualization, Writing - Review & Editing, Supervision, Funding acquisition.

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General Discussion & Conclusion

The work presented in this thesis has contributed to the field of vaccine bioprocess engineering with the main objective of this work being the development of the Vero cell line as an efficient platform for rVSV vector manufacturing through cell culture engineering and bioprocess optimization, to overcome limitations of low virus productivity and to develop more scalable processes.

Vaccines are biologically or genetically engineered antigens. They can consist of proteins, genetic material, killed or weakened pathogens. Vaccines are intended to stimulate the immune system of the person that was vaccinated to produce specific antibodies against a pathogen. This antigenic action of the vaccine induces an immune response in the vaccinated person through so-called active vaccination, which consists not only of antibodies but also of specialized T helper cells. The achieved immunity protects the person against the respective disease. Depending on the pathogen, this protection can last for a number of years, even for a lifetime.

Viral vaccines, which protect against infectious diseases caused by viral infections, can be produced in different ways, for example using chicken eggs, primary cell lines or continuous cell lines as substrate. What is common is that the substrate is infected with a defined amount of a viral seed stock. The virus then replicates within the cells and after a certain period of time, when the virus has been multiplied by a significant amount with respect to the inoculation amount, it is harvested, purified and formulated to constitute the vaccine (Aubrit et al., 2015).

Traditionally, viral vaccines have been produced in chicken eggs and primary cell lines. In addition to ethical concerns of using these substrates, there are many advantages to use continuous cell lines instead, for example the minimization of cross-contamination and allergy reactions, as well as independency from the supply of chicken eggs. However, there are several challenges of this technology, including the high cost of vaccine production, the need to establish standardized manufacturing platforms and stringent regulatory guidelines to ensure vaccine safety (Rodrigues et al., 2015).

Together, cell culture systems are a very complex technology which requires keeping up with current trends and training of highly skilled personnel. Innovation progresses rather

slow in this field. For example, the majority of influenza vaccine doses is still produced in chicken eggs (Sharon et al., 2020). Besides, only a few cell lines have been established as viral vaccine producer cell lines and are accepted by regulatory agencies worldwide (Genzel, 2015). When continuous cell lines are being used, the manufacturing involves typically only conventional process technologies, such as decade old adherent cell cultures employing roller bottles or microcarriers.

In this context, the Vero cell line is the most used continuous cell line for the production of viral vaccines and historically the first that was approved by the WHO. This cell line is the main subject of this work and the motivation was to develop improved viral vector production technologies for an already established and by regulatory agencies accepted cell line that have an immediate impact on creating more efficient and cost-effective manufacturing processes. Better cost-effectiveness could be achieved for example through processes with higher cell density which, assuming higher viral yields, could lower capital investments into the production facility by using smaller bioreactor systems. Since vaccines are considered the single most effective way to prevent infectious diseases, advancements in the field of bioprocess development research can contribute to lowering the prices for vaccines and making them available worldwide.

Chapter 1 of this thesis is a literature research study and reviewed the prevailing literature on Vero cell bioprocess development for the production of viral vectors and vaccines. We aimed to assess recent advances in bioprocess development and to describe the bottlenecks to improve the Vero cell platform.

In this chapter, we illustrate the importance of the Vero cell line. After a brief history of this cell line, we give examples of past and recent viral vaccines that have been produced using this cell substrate. We discuss trends in cell line engineering to increase the cell-specific virus productivity, for example by using novel technologies such as CRISPR, and point out that future work needs to focus on the full annotation of the Vero genome to enable a breakthrough of these technologies when applied to the Vero cell line.

The main aspect of this work, however, focusses on bioprocess development. In detail, we analyze the prevailing literature of microcarrier bioreactors using adherent Vero cell cultures. We emphasize which advances in bioprocess development were able to improve

the cell expansion phase to yield high cell density processes and what has been done to achieve enhanced virus production processes.

As a conclusion, we critically underline the need for further research activities in this field. In the light that successful microcarrier process scale-up still requires a high level of technical expertise and know-how, especially linear scalable systems such as fixed-bed bioreactor systems or highly scalable suspension cell cultures could make processes more robust, streamlined and cost-effective. Overall, this review gives the scientific community a good overview of recent progress in the field of Vero cell upstream bioprocess development and highlights implications for the manufacturing of viral vectors and vaccines caused by current technological trends as well as global health care needs.

With regard to the overall objective of my PhD, we chose rVSV as a model virus. Following the first chapter, which highlighted recent advances in bioprocess development using the Vero cell line, and before tackling the challenges outlined therein, it was crucial to develop analytical techniques to assess potential progress in bioprocess development in terms of improved rVSV productivity.

Since rVSV is gaining momentum as a vectored vaccine platform (Munis et al., 2020), highly accurate and dependable analytical techniques are required to ensure its product quality and safety. In addition, sophisticated quantification methods are important to monitor production processes to guarantee batch to batch consistency. Further, refined analytical techniques are invaluable in facilitating bioprocess development, for example by reducing the time-to-market for a new product or by assuring regulatory agencies that newly developed processes are sound and reliable.

Ideally, such analytical methods should be inexpensive, rapid, highly accurate and dependable over a long period of time. Moreover, they need to lead to comparable results when carried out by different operators or at different institutions.

For highly complex analytes such as viruses, a combination of analytical techniques is necessary to cover their range of characteristics. For instance, measurements of the number of infectious particles, number of total particles, number of viral genomes and number of viral antigens should be considered.

Chapter 2 of this thesis describes two analytical methods. The first one is a cell culture-based end-point dilution assay to quantify the number of viral particles in a given sample,

called Median Tissue Culture Infectious Dose ($TCID_{50}$) assay. This method is widely used due to its robustness and simplicity. In this work, we apply the $TCID_{50}$ assay to quantify the infectious particle count of rVSV-ZEBOV as a representative of the rVSV platform. Additionally, we analyze production and titration variability with statistical tests. Within a larger collaboration, this method has already been successfully used in different lab groups and contributed to three published journal articles (Gélinas et al., 2019; Kiesslich et al., 2020; Mangion et al., 2020). Moreover, we have demonstrated that this assay can be applied to other strains of rVSV, such as rVSV-HIV (Mangion et al., 2020) and $rVSV_{Ind}$-*msp*-S_F-*Gtc,* a COVID-19 vaccine candidate studied in chapter 4 of this thesis.

The second method developed in this work is a digital polymerase chain reaction (dPCR) assay to assess the total number of viral particles. This assay determines the total number of viral genomes, which we have already used to estimate the number of total viral particles in three published journal articles (Gélinas et al., 2019; Kiesslich et al., 2020; Mangion et al., 2020) as well as in the manuscript of chapter 4 of this work. More, in chapter 2 we investigate the titration variability of each assay steps, including RNA extraction, cDNA reverse transcription and digital PCR by applying statistical tests. Similarly to the $TCID_{50}$ assay, the dPCR assay can be used as a universal method to quantify the total number of genomes for any given rVSV under the condition that it contains the wild-type polymerase gene, as demonstrated in our work for rVSV-ZEBOV, rVSV-GFP, rVSV-HIV and $rVSV_{Ind}$-*msp*-S_F-*Gtc*.

Altogether, $TCID_{50}$ and dPCR can not only be used to determine their respective process characteristics, but in combination they can serve as a tool to characterize the ratio of total particles to infectious units for sophisticated monitoring process robustness and product quality attributes. We have demonstrated that these techniques can be applied to several strains of rVSV, making them highly appropriate as standard quantification method for the rVSV vaccine platform.

In the future, it would be of interest to develop a faster method for the quantification of infectious viral particles, to be able to analyze samples at-line and not off-line with delays of several days. Further, it would be valuable to extend the analytical toolbox for rVSV total viral particle quantification by developing a physicochemical method, for example using an HPLC approach. A universal assay to enumerate the number of viral antigens, for instance in

a monoclonal antibody-based ELISA, cannot be applied to the rVSV platform as a whole but would have to be developed individually for each strain expressing a different antigen.

With the tools developed in chapter 2, the bioprocess challenges outlined in chapter 1 were tackled by developing bioreactor processes for the Vero cell line and applying them to the rVSV-vaccine platform.

In chapter 3, we develop adherent Vero cell bioreactor processes for rVSV-ZEBOV, a representative of the rVSV platform and vaccine against Ebola virus disease. At first, small-scale experiments were used to determine optimal process parameters such as MOI and temperature during the virus production phase. Then, the process was scaled-up to two bioreactor systems, microcarrier and fixed-bed. We compare both systems in terms of the yield of infectious particles, cell specific productivities as well as ratio of total to infectious particles, Next to identifying these critical process and product characteristics, the optimal time of harvest is determined, were viral titer and quality are at their respective peak.

As pointed out in chapter 1, these more scalable processes, as compared to the current mode employing roller bottles, can be used to improve virus productivity, therefore directly addressing the objectives of the thesis work.

Nevertheless, suspension cell culture bioreactors are considered superior in terms of scalability when compared to microcarrier and fixed-bed bioreactors. Therefore, we further developed a Vero suspension cell line, which was provided by a collaborator, as a platform for rVSV production. Previously, this cell line has been used to produce rVSV-GFP in proprietary medium (Shen et al., 2019). To reduce the risk of lot-to-lot variants and potentially make the platform more amenable to work under standard conditions, the cell line was successfully adapted to grow in a commercially available medium. In bench-scale bioreactors, we demonstrate the applicability of the Vero suspension system by developing processes for rVSV-ZEBOV, rVSV-HIV and $rVSV_{Ind}$-*msp*-S_F-*Gtc* production.

These three strains of rVSV are vaccine and vaccine candidates against Ebola virus disease, HIV and COVID-19, respectively. These infectious diseases are responsible for some of the most prominent epidemics and pandemics in recent history. Finding effective vaccines against such diseases is one of the greatest challenges this generation has to face.

The work presented in this thesis contributed to the advancement of knowledge in the field of vaccine manufacturing. Overall, significant progress was made that can have direct and immediate impact within the field. For example, the $TCID_{50}$ and dPCR assay, developed for rVSV quantification, can be used for a wide range of rVSVs. Such a universal assay can be a very valuable tool considering that rVSV is becoming an important vaccine platform. Further, process optimization of rVSV-ZEBOV production was carried out in adherent Vero cells, contributing to the goal of achieving a more efficient bioprocess. It was also demonstrated that rVSV-ZEBOV can be produced in scalable bioreactors, and this scale-up approach has many advantages over scale-out. In particular, the bioprocesses developed herein can alleviate production bottlenecks due to their higher scalability and productivity, leading to more cost-effective production.

In addition, other research groups are also developing rVSV-based vaccine candidates, for example against Marburg virus disease (rVSV-MARV) or Lassa fever (rVSV-LASV) (Geisbert and Feldmann, 2011) which could also be produced in scalable microcarrier, fixed bed or suspension cell bioreactors of Vero cell cultures as developed in this thesis.

Furthermore, the development and combination of two platform technologies, the Vero cell system and the rVSV-based vaccine system, is of significant value for the fight against future infectious diseases. The use of established platforms will accelerate vaccine manufacturing, providing life-saving health care in a more rapid manner. In addition, it can lower the drug prices, making vaccines available worldwide, which is especially important in low-income countries.

Building on the work of this thesis, future research activities are necessary to further improve the Vero cell production system and to establish it as a platform that is accepted by industrial and regulatory players as the standard in viral vector manufacturing.

For instance, Vero suspension cell densities in batch mode have not exceeded concentrations of 2×10^6 cells/mL. Increasing the cell density can increase virus production yields, since more substrate is available. Media development can investigate improvements in formulation to sustain higher cell densities, and in particular for the Vero cell suspension culture, higher cell growth rates. Fed-batch or perfusion technologies can be used to achieve processes with higher cell densities, as has been shown for suspension Vero cell cultures in a recent study (Shen et al., 2019). Nevertheless, more research is necessary to screen for

feeding agents and to optimize parameters such as perfusion or feed flow rate. Advanced analytical technologies, such as online monitoring of substrate and metabolite concentrations combined with direct control via feeding or perfusion rate adjustment can help to intensify these processes. Higher cell densities have been obtained for adherent Vero cell cultures, i.e. 3.6×10^6 cells/mL in a batch and even up to 10×10^6 cells/mL in perfusion microcarrier bioreactors as previously reported (Trabelsi et al., 2006). However, for adherent cell processes, the cell density is mainly limited by the surface area, therefore these processes face different challenges to further increase the cell density.

Another aspect, future research should be focused on, is to increase the cell growth rate and cell specific virus productivity. Here, advanced analytical technologies can perform in-depth studies such as metabolomics, transcriptomics and proteomics to improve cell growth and virus production phase conditions. Extensive characterization studies can for example directly compare cell growth of adherent Vero strains and suspension adapted Vero strains to determine targeted improvement strategies. Further, large-scale CRISPR screens can be conducted with the goal to screen for factors that either increase cell growth rate or virus productivity. However, in the context of knockout screens, more efforts with regard to genome annotation are required.

Finally, this work investigated the Vero cell system by using rVSV as a model virus. When for example studying the Vero suspension cell platform or fixed-bed bioreactors using adherent Vero cells, the efficient production of other relevant viruses needs to be demonstrated. Applying advanced and more efficient bioprocess technologies to the manufacturing of established vaccines against infectious diseases such as polio and rabies virus can decrease costs and, for instance in the case of seasonal influenza, reduce production timelines.

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