Process development for production of Newcastle Disease Virus-vectored vaccines using suspension Vero cells

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Abstract

As seen with the devastating COVID-19 pandemic, infectious diseases remain a global concern. Consequently, there have been many efforts placed on preparing for pandemics and developing vaccine platforms. Viral vectored vaccines are especially promising, having been approved for use against Ebola and SARS-CoV-2. In this category, the Newcastle Disease Virus (NDV) is an avian virus which shows great potential as a viral vector for veterinary and human vaccines. Currently, NDV is mostly produced in embryonated chicken eggs, which lack the control over parameters and scalability that production in cell culture can offer. As such, this work sets out to establish the basis for producing NDV using suspension Vero cells, therefore contributing to the important field of vaccine manufacturing by improving the scalability of NDV production and expanding the toolbox of viral vectors available to the market.

NDV has prominent applications for oncolytic therapy and vaccination. We highlight its potential as a viral vector by reviewing the advantages of NDV as a platform for vaccines and compiling the pre-clinical and clinical studies for human and veterinary vaccines using NDV as a vector. Importantly, our literature review identifies the gap in data for NDV manufacturing, pointing towards the necessity of process development studies. We also draw comparisons to progress achieved in the production of similar viral vectors, laying out a roadmap for what could be done for NDV and, ultimately, identifying process intensification as an important aspect.

Aiming to fill this gap on NDV research, we adapt this virus to production in suspension cell culture, using the HEK293 and Vero cell lines. We use viral constructs that contain green fluorescent protein (NDV-GFP) or full-length SARS-CoV-2 Spike protein (NDV-FLS) as models to evaluate upstream parameters for viral production, including multiplicity of infection (MOI), temperature, trypsin concentration and time of harvest. On the analytical level, we develop a tissue culture infectious dose 50% assay (TCID50) and a digital droplet polymerase chain reaction assay (ddPCR), which quantify infectious and total viral particles, respectively. We apply these efforts to 1 L batch bioreactor runs, demonstrating the feasibility of the bioprocesses that were developed.

In conclusion, this work advances the field of vaccine bioprocessing, which is critical in responding to and preventing pandemics. We establish key aspects of a production process for NDV and elucidate the next steps required to consolidate this viral vector as a vaccine platform, so that it can quickly be adapted to target emerging viruses.

Résumé

Comme constaté pendant la pandémie dévastatrice de COVID-19, les maladies infectieuses restent une préoccupation mondiale. Par conséquent, de nombreux efforts ont été déployés pour la préparation aux pandémies et le développement des plateformes vaccinales. Les vaccins à base de vecteurs viraux sont particulièrement prometteurs, ayant déjà été approuvés pour l'utilisation contre l'Ebola et le SARS-CoV-2. Dans cette catégorie, le virus de la maladie de Newcastle (NDV) est un virus aviaire qui présente un grand potentiel en tant que vecteur viral pour les vaccins vétérinaires et humains. Actuellement, le NDV est principalement produit dans des œufs de poule embryonnés, une méthode qui n'offre ni le contrôle des paramètres, ni le potentiel de mise en échelle offert par la production en culture cellulaire. En tant que tel, ce travail vise à établir la base de la production de NDV en utilisant des cellules Vero en suspension. Ce travail contribue ainsi au domaine important de la fabrication de vaccins, en améliorant la mise a l'échelle de la production de NDV et en élargissant la boîte à outils de vecteurs viraux disponibles sur le marché.

Le NDV a des applications importantes dans la thérapie oncolytique et la vaccination. Nous mettons en évidence son potentiel en tant que vecteur viral en décrivant les avantages du NDV en tant que plateforme pour les vaccins et en compilant les études précliniques et cliniques pour les vaccins humains et vétérinaires qui utilisent ce vecteur. Notre revue de la littérature identifie le manque de données pour la fabrication de NDV, soulignant la nécessité d'études de développement de procédés. Nous établissons également des comparaisons avec le progrès réalisé dans la production de vecteurs viraux similaires, établissant une feuille de route pour ce qui pourrait être fait pour le NDV et en identifiant l'intensification du procédé comme un aspect important.

Dans le but de combler cette lacune dans la recherche sur le NDV, nous adaptons ce virus à la production en culture cellulaire en suspension, en utilisant les lignées cellulaires HEK293 et Vero. Nous utilisons des constructions virales contenant la protéine fluorescente verte (NDV-GFP) ou la protéine SARS-CoV-2 Spike pleine longueur (NDV-FLS) comme modèles pour évaluer les paramètres de production virale, y compris la multiplicité d'infection (MOI), la température, la concentration de trypsine, et le moment de la récolte. Sur le plan analytique, nous développons un essai de dose infectieuse pour 50 % de la culture tissulaire ($TCID_{50}$) et un essai de réaction en chaîne par polymérase numérique en gouttelettes (ddPCR), qui quantifient, respectivement, les

particules virales infectieuses et totales. Nous appliquons ces efforts à des bioréacteurs de 1 L, démontrant la faisabilité des bioprocédés qui ont été développés.

En conclusion, ce travail fait progresser le domaine des bioprocédés pour la production de vaccins, qui est essentiel pour la prévention et la réponse aux pandémies. Nous établissons les aspects clés d'un processus de production de NDV et élucidons les prochaines étapes nécessaires pour consolider ce vecteur viral en tant que plateforme vaccinale, afin qu'il puisse être rapidement adapté pour cibler les virus émergents.

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Contribution of Authors

The present Master's thesis is formed by two published manuscripts (Chapter 1 and Chapter 2). I am the first author of all manuscripts and each author's contributions are listed below.

Chapter 1: **Development and scalable production of Newcastle Disease Virus-vectored vaccines for human and veterinary use**

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Authors: **Julia P. C. Fulber** and Amine A. Kamen

Contributions: **JPCF:** writing—original draft preparation, writing—review and editing. AAK: supervision, funding acquisition, writing—review and editing.

Chapter 2: **Process Development for Newcastle Disease Virus-Vectored Vaccines in Serum-Free Vero Cell Suspension Cultures**

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Contributions: **JPCF:** conceptualization, investigation, formal analysis, writing—original draft preparation, writing—review and editing. OF: conceptualization, investigation, writing—review and editing. SK: conceptualization, investigation, writing—review and editing. ZY: investigation ($TCID_{50}$). SD: investigation (fluorescence microscopy). LS and SKW: resources (construction and characterization of NDV-GFP and NDV-FLS). AAK: conceptualization, supervision, funding acquisition, writing—review and editing.

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List of Acronyms

COVID-19	Coronavirus disease 2019
DMEM	Dulbecco's Modified Eagle's Medium
ddPCR	Digital droplet polymerase chain reaction
EBOV	Ebola virus
ECEs	Embryonated chicken eggs
EID_{50}	Embryo infectious dose 50%
FBS	Fetal bovine serum
FFU	Focus-forming units
GFP	Green fluorescent protein
HA	Hemagglutinin
HEK	Human embryonic kidney cells
HIV	Human immunodeficiency viruses
HPAIV	Highly pathogenic avian influenza virus
hpi	Hours post-infection
MDCK	Madin-Darby canine kidney cells
MOI	Multiplicity of infection
NDV	Newcastle disease virus
PFU	Plaque-forming units
PCR	Polymerase chain reaction
SARS-CoV	Severe acute respiratory syndrome coronavirus
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
$TCID_{50}$	Median tissue culture infectious dose 50%
VLP	Virus-like particle
VP-SFM	VP – Serum Free Medium

Chapter 1

Development and scalable production of Newcastle Disease Virus-vectored vaccines for human and veterinary use

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Abstract

The COVID-19 pandemic has highlighted the need for efficient vaccine platforms that can rapidly be developed and manufactured in large-scale to immunize the population against emerging viruses. Viral vectored-vaccines are prominent vaccine platforms that have been approved for use against Ebola virus and SARS-CoV-2. The Newcastle Disease Virus is a promising viral vector, as an avian paramyxovirus that infects poultry but is safe for use in humans and other animals. NDV has been extensively studied as not only an oncolytic virus but also a vector for human and veterinary vaccines, with currently ongoing clinical trials for use against SARS-CoV-2. However, there is a gap in NDV research when it comes to process development and scalable manufacturing, which are critical for future approved vaccines. In this review, we summarize the advantages of NDV as a viral vector, describe the steps and limitations to generating recombinant NDV constructs, review the advances in human and veterinary vaccine candidates in pre-clinical and clinical tests, and elaborate on production in embryonated chicken eggs and cell culture. Mainly, we discuss the existing data on NDV propagation from a process development perspective and provide prospects for the next steps necessary to potentially achieve large-scale NDV-vectored vaccine manufacturing.

Keywords

Newcastle Disease Virus; viral vaccine bioprocess; bioreactor production; vaccine production platform; clinical trials; COVID-19; SARS-CoV-2; Vero suspension culture.

Introduction

Infectious diseases have been an important issue throughout human history, with epidemics affecting entire populations, as well as causing losses in livestock (Kim & Samal, 2016). Recently, due to the COVID-19 pandemic, the world turned its attention to viral diseases and vaccine manufacturing. The pipeline to develop and manufacture new vaccines became a global concern, leading to significantly more resources being leveraged into this field (Annas & Zamri-Saad, 2021). With this increased demand comes a need for the development and optimization of vaccine platforms – technologies that can rapidly be adapted to target an emerging disease, with minimal changes to the manufacturing process (Fulber et al., 2021).

In this context, vaccines based on RNA and viral vectors have shown to be promising options for vaccine platforms. Both avoid the costly biosafety level 3 manipulation of the target pandemic

pathogen and are quickly adaptable by modifying the RNA sequence used or the antigen expressed on the viral vector backbone. RNA vaccines stand out for their fast development and ease of production, comprising a straightforward chemical synthesis, with the approved Moderna and Pfizer-BioNTech vaccines playing an essential role in the race for immunization against SARS-CoV-2 worldwide (Kon et al., 2022). However, their lack of stability leads to the challenging requirement of frozen storage (lower than – 20 °C), which poses issues with transportation as well as hindering access in low-income countries and remote regions which lack the cold chain infrastructure (Crommelin, Anchordoquy, et al., 2021). Live virus vaccines, on the other hand, are commonly freeze dried to be stored at 2 to 8 °C (Crommelin, Volkin, et al., 2021).

Viral vectored vaccines represent a promising alternative, with several approved vaccines including: the adenovirus-vectored vaccines used against SARS-CoV-2 (Oxford-Astrazeneca, Johnson & Johnson, Gamaleya and CanSino) (Samaranayake et al., 2021) and the vectors used against Ebola virus (Tomori & Kolawole, 2021) (adenovirus (European Medicines Agency (EMA), 2020b), modified Vaccinia Ankara (Crommelin, Volkin, et al., 2021; European Medicines Agency (EMA), 2020a), and vesicular stomatitis virus (Henao-Restrepo et al., 2017)). This type of vaccine is highly versatile, with a wide range of human and non-human viruses being studied as vector candidates (Pinschewer, 2017) and the possibility to genetically engineer each vector to modify the surface proteins, generate chimeric strains and select for the desired characteristics, including thermostability (Yoshida et al., 2019; Zhang et al., 2021). This allows researchers to optimize the balance between immunogenicity and safety by modulating virulence and evading pre-existing immunity. It is also possible to design unique vaccination strategies such as co-expressing different antigens for a more robust response or even generating a bivalent vector that provides immunization against multiple pathogens (Bello et al., 2020; Choi, 2017).

Newcastle Disease Virus (NDV) is an avian virus that has been extensively researched as an oncolytic virus, with a long history of clinical trials for this application (Burman et al., 2020). Due to host range restriction, it is not pathogenic in humans, which avoids the issue of pre-existing immunity in the population (Kim & Samal, 2016). As such, it is an ideal candidate for a vaccine vector in terms of safety and immunogenicity and has been implemented in several studies targeting human and veterinary diseases (Bello et al., 2020). These vaccine candidates currently rely on the well-established production process in embryonated chicken eggs (ECEs), which can

be a cost-effective way of using existing facilities that produce influenza vaccines to produce large quantities of doses (Sun, Leist, et al., 2020). However, there are very few studies exploring the production of NDV in cell culture, which could be scaled up to bioreactor production facilities. Cell culture-based processes provide several advantages over production in eggs: they avoid issues with allergens, eliminate dependence on chicken egg supply, and allow greater control over each operation parameter, which leads to more reproducibility, scalability and optimization of the process (Fulber et al., 2021; Silva et al., 2021).

This review elaborates on the potential of NDV as a viral vector based on its fundamental biology, outlines the recommendations and limitations for designing recombinant NDV constructs and summarizes the history of developed vaccine candidates for human and veterinary use, including the strains and routes of administration used, as well as highlighting innovative strategies. Lastly, we discuss what has been done in terms of viral propagation and process development, and how this could potentially be leveraged for scalable manufacturing in bioreactors.

Characteristics of NDV as a viral vector

The Newcastle Disease Virus is an avian paramyxovirus that stands out as a promising viral vector based on many advantageous characteristics. Although NDV poses a concern in the poultry industry for the neurological and respiratory disease it can cause among chicken (Kim & Samal, 2016), this virus typically does not lead to disease in humans (Bukreyev et al., 2005). Only a few cases of conjunctivitis have been reported among those who work closely with poultry or virus samples (Mustaffa-Babjee et al., 1976; Nelson et al., 1952; Shimkin, 1946). This avoids the issue of pre-existing immunity in the population that can arise when using widely spread human viruses as viral vectors (Pinschewer, 2017), such as highly seroprevalent adenoviruses (Fausther-Bovendo & Kobinger, 2014). Additionally, NDV replicates efficiently in the respiratory tract, enabling it to be a vector for intranasal vaccines (Bukreyev et al., 2005; DiNapoli et al., 2007; DiNapoli et al., 2010; Gallo et al., 2021; Lara-Puente et al., 2021; Manoharan et al., 2018; Park et al., 2021; Tcheou et al., 2021; Warner et al., 2021). This type of vaccine generates both mucosal and systemic immunity against the target disease, which is especially useful for vaccines against highly contagious respiratory diseases, including SARS-CoV-2 (Annas & Zamri-Saad, 2021; Gallo et al., 2021).

Another key aspect of this viral vector is safety. NDV strains are classified into three different pathotypes, based on their level of virulence in chicken: lentogenic, mesogenic and velogenic. Lentogenic strains, such as LaSota or B1, show the lowest virulence in chicken (Kim & Samal, 2016) and have an extensive documented history of being safe in humans, as has been shown in clinical trials using NDV as an oncolytic agent (Freeman et al., 2006; Schirrmacher, 2016). More detailed information on such trials can be found in the most recent reviews on the oncolytic application of NDV (Burman et al., 2020; Meng et al., 2021; Schirrmacher et al., 2019). Mesogenic and velogenic strains, on the other hand, are typically not used as vaccine vectors due to their virulence in chicken (Kim & Samal, 2016) and their status as a "Select Agent" in the United States (Yoshida et al., 2019). As an RNA virus with cytoplasmic replication, NDV also poses very low risk when it comes to the chance of recombination with the host's DNA, and it has been suggested to lack gene exchange with other viruses (Schirrmacher, 2016).

Aside from safety and efficacy, NDV also offers versatility when it comes to production processes. This virus has been produced extensively in embryonated chicken eggs (Lara-Puente et al., 2021; Santry et al., 2018) and has also shown the capacity of infecting continuous cell lines such as HEK293 (Fulber et al., 2021), Vero (Bukreyev et al., 2005; Fulber et al., 2021; Yurchenko et al., 2019), DF-1 (Bukreyev et al., 2005; DiNapoli et al., 2007; Santry et al., 2018), MDCK (Park et al., 2006) and HeLa (Zhan et al., 2020). Although there are very few studies focusing on process development for NDV, it shows the potential to be produced in either egg-based or cell culture-based processes, depending on which production facilities are available and which strategies are adopted.

Designing and generating recombinant NDV

NDV contains an RNA genome which is single-stranded, negative-sense and non-segmented, ranging from 15,186 to 15,198 nucleotides in length (Schirrmacher, 2017). The genome comprises six transcriptional units, encoding: a nucleocapsid protein (N), a phosphoprotein (P), a matrix protein (M), a fusion protein (F), a hemagglutinin-neuraminidase protein (HN), and a large polymerase protein (L) (Kim & Samal, 2016). Additionally, the RNA of the P gene can be edited to generate the V or W proteins, which are non-structural and generally associated with modulating the avian host's immune response (Yang et al., 2021). Techniques for generating recombinant NDV constructs are already well established, with protocols following the general steps of:

antigenomic plasmid construction, transfection, rescue and amplification (Vijayakumar & Zamarin, 2020).

It is important to consider the limitations and recommendations for recombinant NDV when designing the antigenome plasmid. As a non-segmented genome, large increases in genome length can impair virus replication, and it has been suggested that the maximum transgene size tolerated by NDV is around 3 kb (Cardenas-Garcia & Afonso, 2017; Gao et al., 2008) or 5 kb (Schirrmacher et al., 2019; Warner et al., 2021). NDV constructs bearing genes encoding the SARS-CoV S protein (DiNapoli et al., 2007) and the SARS-CoV-2 S protein (Warner et al., 2021), both around 3.8 kb in size, have been successfully generated, demonstrating this vector's capacity for inserts in this size range. NDV has also been shown to tolerate multiple transgenes (Chellappa et al., 2021; Cho et al., 2018; H. Hu et al., 2017; Khattar et al., 2015; Kim & Samal, 2017; Kortekaas, de Boer, et al., 2010; Murr, Karger, et al., 2020; Pühler et al., 2008; Roy Chowdhury et al., 2019; Viktorova et al., 2018; Zhang et al., 2019) using different co-expression strategies (He et al., 2020). Each foreign gene must be flanked by untranslated regions (UTRs) of NDV genes called gene start (GS) and gene end (GE) sequences (Figure 1). The level of transgene expression varies according to the GS and GE, with the highest expression achieved using UTRs from the M and F genes (Kim & Samal, 2010). Any GS or GE-like sequences within the transgene should be removed through silent mutagenesis (Ayllon et al., 2013).

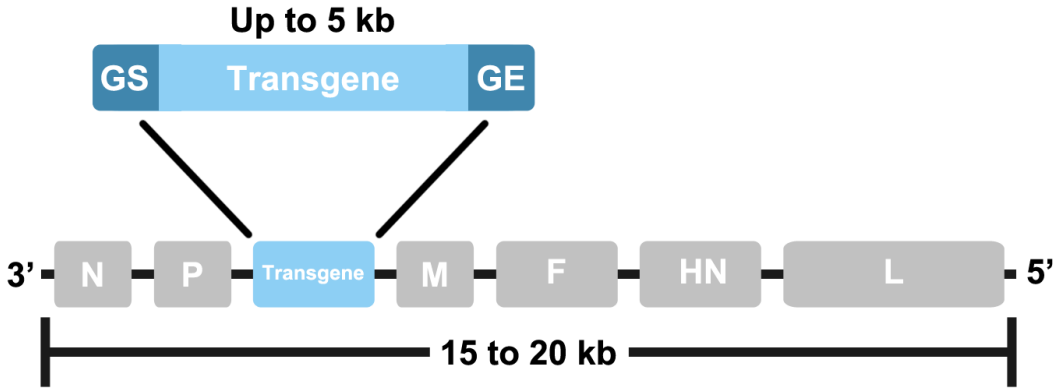

Figure 1: A representation of the NDV single-stranded negative-sense RNA genome including the six native transcriptional units. Transgenes are typically inserted between the P and M genes and flanked by NDV gene start (GS) and gene end (GE) sequences. The total genome length must be an even multiple of six ("rule of six") to ensure complete encapsidation.

Transgenes are typically inserted in the optimal site between the P and M genes (Figure 1), although other sites can also be used (Zhao et al., 2015), and must follow the "rule of six", which determines that the genome length should be an even multiple of six. This is important for efficient virus replication, as each nucleocapsid protein monomer covers around six nucleotides, so this rule ensures that the nucleotide sequence can be completely encapsidated (Schirrmacher, 2017).

For rescue, the antigenome plasmid containing T7 promoter and terminator sequences must be co-transfected with plasmids expressing the N, P and L genes (Vijayakumar & Zamarin, 2020). The T7 DNA-dependent RNA polymerase is commonly introduced by: (i) infecting the cell with a recombinant virus, such as Modified Vaccinia Ankara; (ii) using a stable cell line expressing this polymerase; or (iii) introducing an additional plasmid expressing the T7 polymerase (Molouki & Peeters, 2017). After rescue, the virus is typically amplified in embryonated chicken eggs (Vijayakumar & Zamarin, 2020) or permissive cell lines (Santry et al., 2018). Once amplified, the infectious stock of recombinant virus is ready to be used for further production by infecting embryonated chicken eggs or cell culture.

NDV-vectored vaccines

Vaccines for human use

NDV has been explored as a vector for human vaccines over the past two decades, with nearly thirty published studies assessing these vaccine candidates in animals (Table 1). Other reviews have listed these studies previously (Choi, 2017; Hu et al., 2020; Kim & Samal, 2016), and an updated table is provided below.

Table 1: NDV-vectored vaccine candidates for human use in chronological order (publication date).

Vaccine type	Pathogen	Disease	Antigen	Animal model	Production platform *	Route *	Dose *	Reference
Live B1 strain	Influenza A H1N1	Respiratory infection	HA	Mouse	ECEs	iv, ip	two doses; 3×10^7 PFU	(Nakaya et al., 2001)
Live LaSota or BC strain	HPIV3	Respiratory infection	HN	African green monkey; rhesus monkey	ECEs, DF-1 cell line	in + it	two doses; 3×10^6 PFU	(Bukreyev et al., 2005)
Live B1 strain	HRSV	Respiratory infection	F	Mouse	ECEs	in	one dose; 5×10^5 PFU	(Martinez-Sobrido et al., 2006)

Live BC or LaSota/VF strain	SARS-CoV	Respiratory infection	S, S1	African green monkey	ECEs, DF-1 cell line	in + it	one or two doses; 2×10^7 PFU	(DiNapoli et al., 2007)
Live LaSota strain	HPAIV H5N1	Respiratory infection	HA	African green monkey	DF-1 cell line	in	two doses; 2×10^7 PFU	(DiNapoli et al., 2007)
Live B1 strain	HIV	AIDS	Gag	Mouse	ECEs, Vero cell line	in	two doses; 5×10^5 PFU prime and 10^6 PFU boost	(Carnero et al., 2009)
Live LaSota or BC strain	EBOV	Ebola virus disease	GP	Rhesus monkey	DF-1 cell line	in + it	two doses; 10^7 PFU	(DiNapoli et al., 2010)
Live LaSota strain	HIV	AIDS	Gag	Mouse	ECEs	in	two doses; 5×10^5 PFU prime and 10^6 PFU boost	(Maamary et al., 2011)
Live LaSota/VF strain	*Borrelia burgdorferi*	Lyme	BmpA, OspC	Hamster	ECEs	in, im, ip	one or two doses; 10^6 PFU	(Xiao et al., 2011)
Live LaSota strain	HIV	AIDS	Env (gp160)	Guinea pig	ECEs	in, im, in + im	two or three doses; 3×10^5 PFU (in) or 5×10^5 PFU (im)	(Khattar et al., 2011)
Live LaSota strain	NiV	Encephalitis	F, G	Mouse, pig	ECEs	im	two doses; 10^8 EID_{50} (mice) or 2×10^9 EID_{50} (pigs)	(Kong et al., 2012)
Live LaSota or Lasota/BC strain	NoV	Gastroenteritis	VP1	Mouse	ECEs	in	one dose; 10^6 EID_{50}	(Kim et al., 2014)
Live LaSota strain	HIV	AIDS	Env (gp160) / Gag (p55)	Guinea pig, mouse	ECEs	in	two doses; 2×10^5 PFU (guinea pigs) or 4×10^4 PFU (mice)	(Khattar et al., 2015)
Live LaSota strain	HIV	AIDS	Env (gp160)	Guinea pig	ECEs	in	four doses of 2×10^5 PFU or two doses of 2×10^5 PFU followed by two doses of recombinant protein (gp120 or gp140)	(Khattar et al., 2016)
Live LaSota strain	Poliovirus	Poliomyelitis	P1/3CD	Guinea pig	ECEs	in	two doses; 10^5 PFU	(Viktorova et al., 2018)
Live chimeric NDV strain	EBOV	Ebola virus disease	GP	Guinea pig	ECEs	in	two doses; 2×10^6 $TCID_{50}$	(Yoshida et al., 2019)
Live LaSota strain	JEV	Encephalitis	E, NS1	Mouse	ECEs	in	one dose; 10^6 EID_{50}	(Nath et al., 2020)
Live LaSota strain	SARS-CoV-2	COVID-19	S, S-F chimera	Mouse	ECEs	in	two doses; 10 or 50 μg	(Sun, Leist, et al., 2020)
Inactivated LaSota strain	SARS-CoV-2	COVID-19	S, S-F chimera	Mouse, hamster	ECEs	im	two doses; 5 or 10 μg	(Sun, McCroskery, et al., 2020)

Live B1 strain	SARS-CoV-2	COVID-19	S	Mouse, hamster	ECEs	in	one or two doses; 10^4 PFU (mice) or 10^6 PFU (hamster)	(Park et al., 2021)
Live or inactivated LaSota strain	SARS-CoV-2	COVID-19	HXP-S	Pig	ECEs	in, im, in + im	two doses of 10^7, 3×10^7, 10^8 or 3×10^8 EID_{50} (live); two doses of 10^8 EID_{50} (inactivated)	(Lara-Puente et al., 2021)
Live or inactivated LaSota strain	SARS-CoV-2	COVID-19	HXP-S	Hamster, mouse	ECEs	in, im, in + im	two doses of 1.0, 0.3, 0.1, 0.03 or 0.03 μg (im, inactivated, hamsters); two doses of 10^6 EID_{50} (in, live, hamsters); two doses of 10^4, 10^5 or 10^6 EID_{50} (in prime and im boost, live, mice); two doses of 1 μg (im, inactivated, mice)	(Sun et al., 2021)
Live LaSota strain	SARS-CoV-2	COVID-19	HXP-S	Rat	ECEs	in, im, in + im	two doses; 7.4×10^8 EID_{50}	(Tcheou et al., 2021)
Live LaSota strain	SARS-CoV-2	COVID-19	S, truncated S	Hamster	ECEs	in	one or two doses; 10^7 PFU	(Warner et al., 2021)
Live LaSota strain	EBOV	Ebola virus disease	GP	Mouse	ECEs	in	two doses of 10^6 PFU; one dose of 10^6 PFU and one dose of adenovirus-vectored vaccine	(Zhao et al., 2021)
Inactivated VG/GA strain	SARS-CoV-2	COVID-19	RBD	Mouse	ECEs	im	two doses of 1, 5 or 10 μg	(Jung et al., 2022)

* ECEs: embryonated chicken eggs, iv: intravenous, ip: intraperitoneal, in: intranasal, it: intratracheal, im: intramuscular, PFU: plaque-forming units, $TCID_{50}$: tissue culture infectious dose 50%, EID_{50}: embryo infectious dose 50%.

NDV-vectored vaccine candidates have been developed for a range of pathogens, including HIV, EBOV and, predominantly, respiratory viruses such as Influenza, SARS-CoV and SARS-CoV-2 (Table 1). Most of the vaccine candidates employed recombinant lentogenic NDV strains as an intranasal live vectored vaccine. The LaSota strain is predominantly used, although other lentogenic strains such as Hitchner B1 and VG/GA, as well as the mesogenic strain Beaudette C (BC), are also present. Interestingly, a few vaccine candidates use chimeric NDV strains, such as the LaSota/VF. This strain is based on a LaSota backbone with the BC strain F protein cleavage sequence, which slightly increases the virulence in birds but allows the virus to replicate in cell culture without the need for added trypsin (DiNapoli et al., 2007). In other studies, the mesogenic BC strain was modified by exchanging the ectodomains in the surface glycoproteins F and HN by their equivalents from the LaSota strain (Kim et al., 2014) or avian paramyxovirus 3 (APMV-3) (Yoshida et al., 2019) to reduce virulence and increase safety.

Due to host-range restriction, NDV was shown to have attenuated replication in primates, while still generating sufficient mucosal immunity as a respiratory virus (Bukreyev et al., 2005), demonstrating both safety and immunogenicity. As such, many studies targeting respiratory diseases implemented the intranasal route of inoculation, including most of the recently developed vaccines targeting SARS-CoV-2 (Lara-Puente et al., 2021; Park et al., 2021; Sun, Leist, et al., 2020; Sun et al., 2021; Tcheou et al., 2021; Warner et al., 2021). Combinations of intranasal and intramuscular doses were also assessed for several vaccine candidates (Khattar et al., 2011; Lara-Puente et al., 2021; Sun et al., 2021; Tcheou et al., 2021). Inactivated NDV-vectored vaccines, on the other hand, were administered exclusively by the intramuscular route, as the inactivated virus can no longer replicate in the mucosal passages (Jung et al., 2022; Lara-Puente et al., 2021; Sun et al., 2021; Sun, McCroskery, et al., 2020).

A few studies used multiple antigens to target the pathogen of concern. Notably, an NDV vector co-expressing the poliovirus P1 and 3CD proteins resulted in the formation of poliovirus viral-like particles (VLPs) in the host cells upon vaccination, which means antigens are presented in a form most similar to the native pathogen while still being safer than live poliovirus vaccines (Kong et al., 2012; Viktorova et al., 2018). The replication of NDV also serves as a natural adjuvant, increasing the immunogenicity of the vaccine. In another study, NDV was used to co-express the HIV Env and Gag proteins, testing several different orders and positions in the genome (Khattar et al., 2015). Most constructs in this study also generated VLPs, enhancing the immune response and providing a promising vaccination platform for HIV. Other studies evaluated the effect of multiple antigens by co-infecting animals with different NDV constructs expressing each antigen separately (Nath et al., 2020; Xiao et al., 2011), although this approach was less efficient than inoculating a single construct in a study for JEV vaccines (Nath et al., 2020). This strategy could avoid issues with slow NDV propagation due to co-expression of multiple transgenes but would require the production of different NDV vectors for the same vaccine, similar to Influenza vaccines containing multiple strains (Silva et al., 2021).

Certain studies combined different vaccination approaches for a heterologous immunization strategy. One study used an NDV vector expressing the HIV Env (gp160) protein for a priming dose and boosted with purified recombinant proteins (gp120 or gp140), which was the most efficient regimen of vaccination tested (Khattar et al., 2016). This mixed regimen induced higher

magnitude of immune response than the regimen with only NDV-vectored doses, while also providing a longer lasting immune memory in comparison to the purified protein-only regimen. As such, the NDV-vectored prime was important for long-term immunity, while the protein boost enhanced immunogenicity. Another study explored combining different vectors by priming with an adenovirus-vectored vaccine and boosting with an NDV-vectored vaccine, or vice-versa (Zhao et al., 2021). This heterologous regimen induced a more potent and robust response than the homologous alternatives, potentially due to avoiding pre-existing immunity to each vector.

NDV-vectored vaccines have great potential to be used against pandemic diseases, having shown efficient protection against EBOV (Yoshida et al., 2019; DiNapoli et al., 2010; Zhao et al., 2021), SARS-CoV (DiNapoli et al., 2007) and SARS-CoV-2 (Sun et al., 2020; Park et al., 2021; Warner et al., 2021; Tcheou et al., 2021; Lara-Puente et al., 2021; Sun et al., 2020; Sun et al., 2021; Jung et al., 2022). Intranasal vaccines against EBOV were shown to induce neutralizing antibody responses in monkeys (DiNapoli et al., 2010), guinea pigs (Yoshida et al., 2019) and mice (Zhao et al., 2021), although the latter study found more robust responses when mixing adenovirus and NDV-vectored doses as compared to a homologous NDV-vectored regimen. An intranasal vaccine against SARS-CoV (DiNapoli et al., 2007) also generated a protective antibody response in monkeys and highlighted the need for two doses, as one dose provided insufficient immunogenicity. A similar result was found in a study against SARS-CoV-2 in hamsters (Warner et al., 2021), in which two doses induced a protective neutralizing response while the single-dose regimen did not significantly reduce viral loads upon infection. Inactivated NDV-vectored SARS-CoV-2 vaccines have also been successful, inducing higher neutralizing responses in mice than a purified protein vaccine (Jung et al., 2022) and significantly reducing viral loads in a hamster model (Sun et al., 2020).

Notably, some NDV-vectored SARS-CoV-2 vaccines provided potent protective immunity and reduced viral loads to undetectable amounts on day 4 or 5 post infection, but they did not induce sterilizing immunity, as there were still detectable amounts of virus on day 2 post infection that could potentially lead to shedding (Park et al., 2021; Warner et al., 2021; Sun et al., 2020). However, it is considered that widely-available vaccines capable of reducing disease severity are highly beneficial in a pandemic, even if sterilizing immunity is not achieved (Sun et al., 2020).

Although there are numerous NDV-vectored vaccine candidates tested in animal models (Table 1), only a select few proceeded to clinical trials. The increased demand for vaccines throughout the COVID-19 pandemic advanced the field, leading to a series of clinical trials for two vaccine candidates (Table 2). A research group based in the Icahn School of Medicine at Mount Sinai (USA) engineered the HexaPro-S (HXP-S) version of the SARS-CoV-2 S protein, which is stabilized in its pre-fusion conformation and anchored in the NDV membrane by containing domains from the NDV F protein (Lara-Puente et al., 2021). Using the LaSota strain backbone expressing the HXP-S antigen, two vaccine candidates were generated: a live version (Sun, Leist, et al., 2020) ("Patria"), in Phase II clinical trials in Mexico, and an inactivated version (Sun, McCroskery, et al., 2020), in Phase I/II clinical trials in Thailand ("HXP-GPOVac"), Vietnam ("COVIVAC") and Brazil ("ButanVac") (Sun et al., 2021). These vaccines are produced in embryonated chicken eggs (ECEs) in GMP-certified facilities in each country, taking advantage of the existing infrastructure for production of influenza vaccines. The inactivated version uses beta-propiolactone (BPL) for inactivation and CpG 1018 as an adjuvant, while the live version has no adjuvant addition, as the replicative virus is considered self-adjuvanted (Sun et al., 2021).

Table 2: Recombinant NDV-vectored vaccine candidate in clinical trials for human use.

Responsible group	Vaccine type	Pathogen	Disease	Antigen	Phase	Route *	Dose *	Reference
Icahn School of Medicine at Mount Sinai, USA	Live LaSota strain	SARS-CoV-2	COVID-19	HXP-S	I	in, im, in + im	3.3×10^8 EID_{50} 1×10^9 EID_{50}	NCT05181709
Laboratorio Avi-Mex, Mexico	Live LaSota strain	SARS-CoV-2	COVID-19	HXP-S	I/II	im,in + im	10^8 EID_{50}	NCT04871737 (Ponce-de-León et al., 2022) NCT05205746
Institute of Vaccines and Medical Biologicals, Vietnam	Inactivated LaSota strain	SARS-CoV-2	COVID-19	HXP-S	I/II	im	1, 3 or 10 µg	NCT04830800
Butantan Institute, Brazil	Inactivated LaSota strain	SARS-CoV-2	COVID-19	HXP-S	I/II	im	1, 3 or 10 µg	NCT04993209
Mahidol University, Thailand	Inactivated LaSota strain	SARS-CoV-2	COVID-19	HXP-S	I/II	im	1, 3 or 10 µg	NCT04764422 (Pitisuttithum et al., 2022)

* in: intranasal, im: intramuscular, EID_{50}: embryo infectious dose 50%

Interim results for Phase I clinical trials of the NDV-vectored vaccine against SARS-CoV-2 have been reported using the live version in Mexico (Ponce-de-León et al., 2022) and the inactivated version in Thailand (Pitisuttithum et al., 2022). The results showed both versions of

the vaccine to be safe in humans. The live version was sufficiently immunogenic at the highest dose tested (10^8 EID_{50}) and all formulations were safe. Interestingly, adequate immunogenicity was only achieved when doses were administered twice intramuscularly or intramuscularly followed by intranasally. The exclusively intranasal regime was found to generate cellular immunity but lacked a robust systemic antibody response. As such, the high dose formulations with IM-IM and IN-IM routes were chosen for the Phase II trials (Ponce-de-León et al., 2022). As for the inactivated vaccine candidate, the immunogenicity was proportional to the dosage, and was not considerably affected by the use of the adjuvant CpG 1018. The mid-dose (3 μg) was considered sufficiently immunogenic and was chosen for the Phase II trials, with and without CpG 1018 (Pitisuttithum et al., 2022).

Vaccines for veterinary use

NDV has been extensively explored as a vector for veterinary vaccines, with over sixty published studies (Table A1) on vaccine candidates over the past two decades mainly targeting use in poultry or cattle. Other review papers have listed and summarized some of these studies (Bello et al., 2020; Choi, 2017; Hu et al., 2020; Kim & Samal, 2016, 2019), and an up-to-date table is provided in the supplementary material (Table A1).

Importantly, as virulent NDV strains cause severe disease in chicken and economic loss, the lentogenic strains are often used to vaccinate chicken for protection against virulent NDV. As such, several studies implemented a bivalent vaccine approach for poultry in which NDV expresses antigens of another avian virus to immunize chicken against both diseases, including highly pathogenic influenza virus (HPAIV) (Cho et al., 2018; Ge et al., 2007; Kim & Samal, 2017; Nayak et al., 2009; Park et al., 2006; Veits et al., 2006), avian reovirus (ARV) (Saikia et al., 2019), infectious laryngotracheitis virus (ILTV) (Kanabagatte Basavarajappa et al., 2014; Zhao et al., 2014) and fowl adenovirus serotype 2 (FAdV-4) (Tian et al., 2020). Notably, certain bivalent vaccines against NDV and HPAIV have been licensed for use in poultry (Choi, 2017; Kim & Samal, 2019). Bivalent NDV vaccines have also been developed for ducks against duck Tembusu virus (DTMUV) (Sun et al., 2018) and HPAIV H5N1 (Ferreira et al., 2012); for geese against goose parvovirus (GPV) (Wang et al., 2015) and goose astrovirus (GoAstV) (D. Xu et al., 2019); and for turkeys against avian metapneumovirus (AMPV) (H. Hu et al., 2017).

Similar to vaccine candidates for human use, several NDV vaccines expressing multiple antigens have been developed for veterinary use, either by co-expressing the antigens within the same vector (Chellappa et al., 2021; Cho et al., 2018; H. Hu et al., 2017; Kim & Samal, 2017; Kortekaas, de Boer, et al., 2010; Murr, Karger, et al., 2020; Roy Chowdhury et al., 2019; Zhang et al., 2019) or by inoculating different vectors, each expressing a different antigen, in the same formulation (Ge et al., 2015; Kanabagatte Basavarajappa et al., 2014; Kong et al., 2012; Olbert et al., 2016). For the latter, two studies found that a formulation with only one vector/antigen yielded better results and sufficient protection (Ge et al., 2015; Kanabagatte Basavarajappa et al., 2014), while another study found adequate immune responses regardless of inoculating one or multiple vectors (Kong et al., 2012).

NDV strains have also been engineered to generate chimeric or modified strains with novel characteristics. Thermostability is a key characteristic for poultry immunization through spraying or drinking water, while also relieving difficulties associated with cold chain requirements (Zhang et al., 2021). To address this, thermostable NDV strains have been isolated and modified to be used as vectors in avian influenza vaccines (Xu et al., 2020; Zhang et al., 2021). Another concern in poultry immunization is pre-existing immunity to NDV (Hu et al., 2020), which led to the development of chimeric NDV strains in which the native surface glycoproteins (F and HN) were substituted by the corresponding genes from another virus, namely APMV-8 (Steglich et al., 2013) or APMV-2 (Kim et al., 2017; Liu et al., 2018). Chimeric strains have also been developed to modulate virulence by substituting the F and HN proteins of the mesogenic BC strain (partly or completely) with the corresponding proteins from the lentogenic LaSota strain, or by modifying basic residues in the F protein cleavage site (Manoharan et al., 2018; Yadav et al., 2018).

Manufacturing of NDV-vectored vaccines

Workflow for viral vector production

Viral vectors are typically produced in ECEs or cell culture, from which they are harvested and purified for vaccine formulation (Figure 2). NDV has been extensively produced in ECEs for poultry vaccination and for pre-clinical studies, with only a few studies propagating this virus in cell lines (Table 1 and Table A1) and, to our knowledge, only four studies producing it in lab-scale bioreactors (Arifin, 2011; Arifin et al., 2010; Fulber et al., 2021; Jaafar, 2009).

For production in ECEs (Figure 2A), eggs must be acquired and incubated at 37 °C prior to inoculation. They are infected between 9 to 11 days old and are incubated for another 24 hours for viral production. ECEs containing dead embryos before 24 hours of incubation are discarded, while embryos that die after that timepoint are stored at 4 to 8 °C for several hours before collecting the allantoic fluid to harvest NDV (Al-Ziaydi et al., 2020).

For production in cell culture (Figure 2B), bioreactors are inoculated with defined medium and the chosen cell line for the cell growth phase. Critical operation parameters such as pH, oxygen concentration, agitation and temperature are kept constant throughout the entire run. Once the required cell density is reached, cells are infected with NDV to initiate the viral production phase. For batch productions, the bioreactor is interrupted for harvest when the known peak production timepoint is reached, typically between 24 to 48 hours post infection (hpi) (Arifin et al., 2010; Fulber et al., 2021).

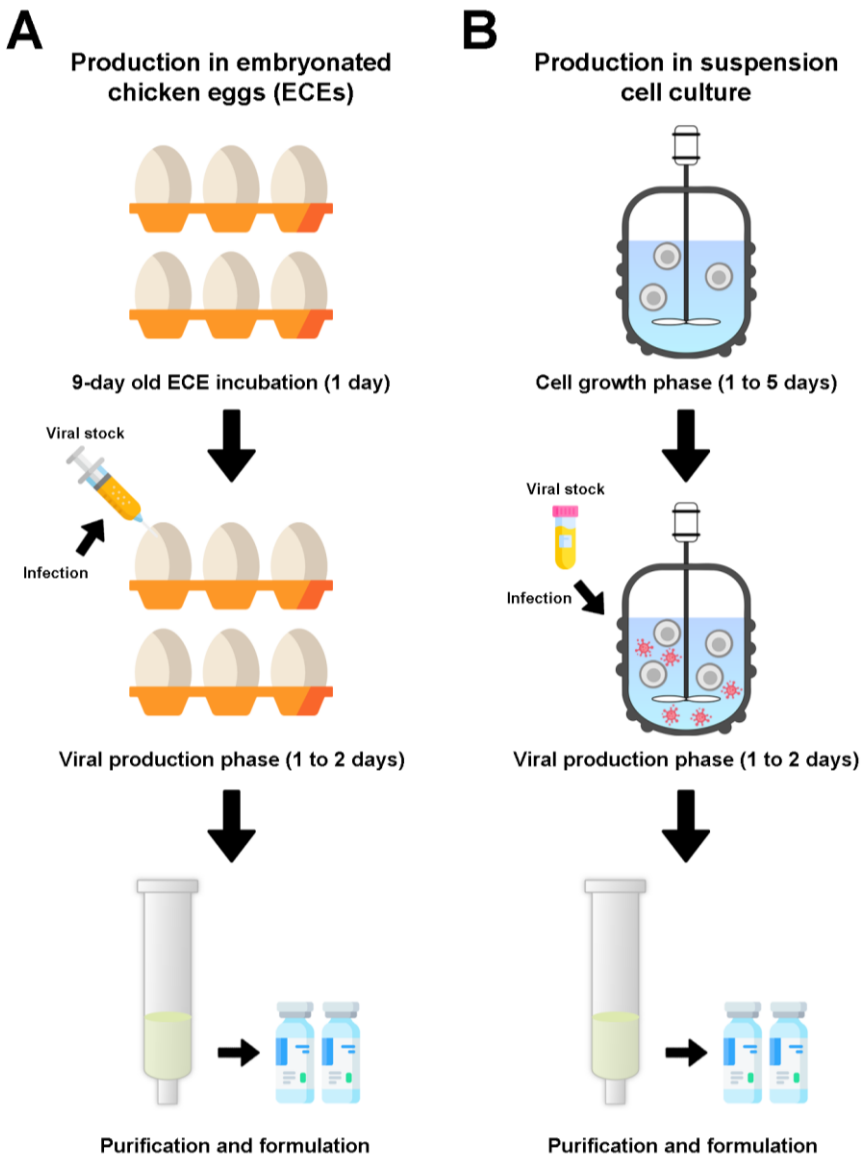

Figure 2: Overview of production processes for viral-vectored vaccines in (A) embryonated chicken eggs (ECEs) and (B) suspension cell cultures in stirred-tank bioreactors.

The processes also differ in terms of waste treatment: infected ECEs result in solid waste that is typically incinerated (Chen et al., 2020), while reusable bioreactors use methods such as cleaning/sterilization in place and chemical treatment of liquid waste, and single-use bioreactors are disposed of through chemical or physical treatments (Sharma et al., 2022).

Parameters for NDV production in cell culture

When using lentogenic NDV strains to infect cell lines for propagation, an exogenous protease must be provided to cleave the F protein and activate infection (DiNapoli et al., 2007). This can

be done by providing allantoic fluid at a concentration of 5 to 10% (Bukreyev et al., 2005; Dey et al., 2017; Kim et al., 2014), or by adding TPCK-treated trypsin (DiNapoli et al., 2007; Fulber et al., 2021), as is done for influenza (Le Ru et al., 2010). Trypsin is more suitable than allantoic fluid due to the variability regarding undefined animal products in cell culture. This is the same reason why serum-free media is preferred over the use of fetal bovine serum (FBS) in industry (Kiesslich & Kamen, 2020). Alternatively, there are strains which can replicate without trypsin addition, including mesogenic strains like BC and R2B or strains with a modified F cleavage site like LaSota/VF (DiNapoli et al., 2007). The optimal trypsin concentration to produce recombinant NDV LaSota in suspension Vero cells was found to be 1 µg/mL (Fulber et al., 2021), although NDV constructs expressing certain antigens can become self-sufficient for viral entry and no longer require trypsin (Ge et al., 2011).

Another key parameter is the multiplicity of infection (MOI), which has been optimized in suspension Vero cells using serum-free media. Our past research has shown that MOIs of 0.1, 0.01 and 0.001 resulted in similar peak production titers (around 10^8 $TCID_{50}$/mL), while production at an MOI of 0.0001 was approximately 100-fold lower (Fulber et al., 2021). Replication of NDV in DF-1 and adherent Vero cells is usually achieved with an MOI of 0.01 (Chellappa et al., 2021; Dey et al., 2017; Khattar et al., 2011; Kim et al., 2014; Saikia et al., 2019; Xiao et al., 2011), which is also within this range. A study using adherent Vero cells with microcarriers in media containing FBS showed similar titers of around 4×10^7 $TCID_{50}$/mL for MOI 2 and 0.2 (Arifin et al., 2010). When infecting BHK-21 cells, an MOI of 5 resulted in a peak of around 10^7 EID_{50}/mL at 24 hpi, while an MOI of 0.01 caused a delay in that peak to 96 hpi (Ge et al., 2011). Thus, NDV seems to have wide range of MOIs that achieve adequate production and the optimal MOI can depend on other process and culture parameters, although 0.01 seems to be a suitable MOI for most conditions.

NDV can be adapted to a cell line by serial passaging, which selects for more efficient replication. This facilitates the next infection and can lead to a higher yield, with an observed increase of around 500-fold in lentogenic strains after 4 passages in suspension Vero cells and 13-fold in suspension HEK293 (Fulber et al., 2021). Another study found an increase of 5 to 25-fold in lentogenic strains and 6 to 10-fold in mesogenic strains after 8 passages in adherent Vero cells (Yurchenko et al., 2019).

Recombinant lentogenic NDV production in several cell lines resulted in titers around 10^7 and 10^8 infectious units per mL between 30 and 48 hpi, including suspension Vero cells (Fulber et al., 2021) and adherent cells: Vero (Arifin et al., 2010; Dey et al., 2017), DF-1 (Kim et al., 2014; Yoshida et al., 2019), BHK-21 (Ge et al., 2011) and MDCK (Park et al., 2006). The highest titers reported were around 10^9 PFU/mL in DF-1 (Khattar et al., 2011), 5 x 10^8 PFU/mL in MDCK (Park et al., 2006) and 2.37 × 10^8 $TCID_{50}$/mL in suspension Vero cells (Fulber et al., 2021). Production of the mesogenic strain R2B in adherent Vero cells were between 6 × 10^8 and 6 × 10^7 $TCID_{50}$/mL (Chellappa et al., 2021; Debnath et al., 2020; Saikia et al., 2019). The production in ECEs is similar but still overall higher, with titers around 10^8 and 10^9 infectious units per mL (Carnero et al., 2009; Ge et al., 2011; Murr, Karger, et al., 2020; Park et al., 2006; Veits et al., 2006; Yoshida et al., 2019).

NDV production in lab-scale bioreactors

Although there is still a small number of studies available, a few successful productions of NDV in bioreactors have been published. It is important to note that serum-free media is preferred for industrial bioprocesses, as the use of FBS implies an undefined composition and lot-to-lot variability (Kiesslich & Kamen, 2020). Additionally, suspension cell lines are preferred over adherent cells, as they are not limited by the surface area available, resulting in a more straightforward scale-up and homogenization of cultures. It is still possible to use adherent cells in a stirred-tank bioreactor by using microcarriers, which are beads that the cell can attach to whereas the microcarriers themselves remain suspended and stirred in the media (Kiesslich et al., 2020). Adherent cells can also be used in other bioreactor models such as fixed-bed and wave, but all methods are ultimately limited by surface area (Kiesslich & Kamen, 2020).

In 2010, a pioneer work reported the production of the lentogenic F strain in adherent Vero cells using microcarriers in a 2 L bioreactor scale, reaching a peak production of 4.79 × 10^7 $TCID_{50}$/mL (Arifin et al., 2010). Although the production was sufficient, the process developed has limited scalability due to the use of serum and adherent cell culture. The same authors tested DF-1 cells in the same microcarrier and stirred-tank bioreactor system, but the resulting titer was low (1.03 × 10^3 $TCID_{50}$/mL) (Arifin, 2011).

In 2021, our group published 1 L bioreactor productions of NDV constructs based on the LaSota strain (Fulber et al., 2021) using a recently developed suspension Vero cell line (Shen et

al., 2019) in commercial serum-free media. Adequate production comparable to ECEs was achieved for all constructs, with a peak titer of 2.37×10^8 $TCID_{50}$/mL for NDV-GFP and 3.16×10^7 $TCID_{50}$/mL for the COVID-19 vaccine candidate NDV-FLS (Fulber et al., 2021). This work established the basis for the upstream and analytics of a cell culture-based process for NDV production, showing the potential for a scalable process.

Downstream processing and formulation

Aside from the upstream production in cell culture, it is also important to establish downstream protocols and formulation. Although it is possible to inject the harvested allantoic fluid from eggs directly into animals for experiments (Nath et al., 2020), a few studies implemented sucrose gradient centrifugation to purify NDV (Khattar et al., 2011; Sun, Leist, et al., 2020; Sun, McCroskery, et al., 2020; Warner et al., 2021). However, this is not the ideal method for industrialization, as it can be impractical and lack reproducibility. Chromatography-based purification methods would be more appropriate for industrial applications, due to high scalability and reproducibility (Segura et al., 2011). Chromatography purification protocols have been developed for other enveloped viruses, such as lentivirus (Moreira et al., 2021), and could potentially be developed for NDV as well.

Formulation is also an essential aspect that must be studied for NDV. Vaccines with low stability might require storage and transportation in temperatures below - 20 °C, which poses a major bottleneck related to cold chain infrastructure (Crommelin, Anchordoquy, et al., 2021). A few studies have implemented lyophilized versions of their vaccine candidates (Fakri et al., 2021; Warner et al., 2021), which represents a promising way of simplifying transportation. The lyophilization of a COVID-19 vaccine candidate did not significantly reduce the virus infectivity and allowed for storage at 4 °C (Warner et al., 2021), which greatly decreases the burden on storage. Further optimization of formulation strategies for NDV and stability studies for the existing vaccine candidates are extremely important to prepare for large-scale manufacturing and distribution.

Conclusions

In a time where vaccines are in high demand, it is important to establish vaccine platforms with rapid development and scalable manufacturing. NDV is a promising viral vector for vaccines with well-established recombinant technology, a long history of safety in humans and animals, and

extensive pre-clinical research. With the progression of clinical trials for NDV-vectored vaccines in humans, it is important more than ever to fill the gap of process development for this virus by testing and optimizing scalable production methods for NDV using cell culture in bioreactors. The basis for a cell culture-based NDV production process has been established, but improvements in the upstream and downstream processing are critical, especially when it comes to purification, formulation and process intensification.

The results achieved in batch bioreactor productions of NDV were comparable to those in ECEs but could potentially be further optimized through process intensification. Different modes of operation, such as fed-batch and perfusion, could reduce by-products and replenish nutrients in the media, potentially allowing cells to reach a higher cell density and higher titers of viral production due to a more favorable metabolic state (Silva et al., 2021). Perfusion could be particularly beneficial for NDV, seeing as this virus has been shown to lose infectivity over time in bioreactors (Fulber et al., 2021). This indicates viral degradation in the bioreactor due to unfavorable temperatures and shear stress from agitation, which could be avoided by using perfusion to continuously harvest the virus and reduce the retention time in the vessel, as has been done for the VSV (Shen et al., 2019). This can also potentially be integrated with purification for a continuous or semi-continuous manufacturing process (Silva et al., 2021).

Further developments in these areas are necessary so that NDV-vectored vaccines that are approved in the future can be produced sufficiently in large-scale without major transportation and cold chain constraints.

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Appendix A

Table A1: NDV-vectored vaccine candidates for veterinary use in chronological order (publication date).

Vaccine type	Pathogen	Disease	Antigen expressed	Animal model	Production platform *	Route *	Dose *	Reference

Live B1 Strain	HPAIV H7N2	Respiratory infection	HA	Chicken	ECEs	io	one dose of 1×10^6 EID_{50}	(Swayne et al., 2003)
Live B1 strain	SIV	Simian AIDS	Gag	Mouse	ECEs	iv, ip, in	one or two doses of 5×10^7 PFU	(Nakaya et al., 2004)
Live LaSota strain	IBDV	Infectious bursal disease	VP2	Chicken	ECEs	io	one or two doses of 1×10^4 EID_{50}	(Huang et al., 2004)
Live Clone 30 strain	HPAIV H5N1	Respiratory infection	HA	Chicken	ECEs	on	one dose of 10^6 EID_{50}	(Veits et al., 2006)
Live B1 Strain	HPAIV H7N2	Respiratory infection	HA	Chicken	ECEs, MDCK cell line	io	one or two doses of 5×10^5 to 1.25×10^6 mean EID_{50}	(Park et al., 2006)
Live LaSota strain	HPAIV H5N1	Flu (respiratory infection)	HA	Chicken, mouse	ECEs	on (chicken), ip (mice)	one dose of 10^6 EID50 (chicken), two doses of 10^8 EID_{50} (mice)	(Ge et al., 2007)
Live LaSota strain	HPAIV H7N1	Respiratory infection	HA	Chicken	ECEs	in	one dose of 1×10^6 EID_{50}	(Schröer et al., 2009)
Live LaSota strain	HPAIV H5N1	Respiratory infection	HA	Chicken	ECEs	on	one dose of 1×10^6 EID_{50}	(Nayak et al., 2009)
Live LaSota strain	HPAIV H5N2	Respiratory infection	HA	Chicken	ECEs	io	one dose of 6×10^4, 6×10^5, 6×10^6 or 6×10^7 mean EID_{50}	(Sarfati-Mizrahi et al., 2010)
Live LaSota strain	RVFV	Rift Valley fever	Gn	Calf	ECEs	in, im	one dose of 2×10^6 $TCID_{50}$ (in), one dose of 3×10^7 $TCID_{50}$ (im)	(Kortekaas, Dekker, et al., 2010)
Live LaSota strain	BHV-1	Respiratory infection	gD	Calf	ECEs	in + it	one dose of 1.5×10^7 PFU	(Khattar et al., 2010)
Live LaSota strain	RVFV	Rift Valley fever	Gn/Gc	Mouse, lamb	ECEs	im	two doses of 2×10^5 $TCID_{50}$ (mice), two doses of 2×10^7 $TCID_{50}$ (lambs)	(Kortekaas, de Boer, et al., 2010)
Live Clone 30 strain	HPAIV H6N2	Respiratory infection	HA	Chicken, turkey	ECEs	on	one dose of 10^6 EID_{50} (chicken) or 10^7 EID_{50} (turkeys)	(Schröer et al., 2011)
Live LaSota/VF strain	*Borrelia burgdorferi*	Lyme	BmpA, OspC	Hamster	ECEs	in, im, ip	one or two doses of 1×10^6 PFU	(Xiao et al., 2011)
Live LaSota strain	RV	Rabies	G	Mouse, cat, dog	ECEs	im	one dose of 2×10^8 EID_{50}, 2×10^7 EID_{50} or 2×10^6 EID_{50} (mice); three doses of 6×10^9 EID_{50} (cats); three doses of 6 × 109 EID50, 2×10^9 EID_{50} or 2×10^8 EID_{50} (dogs)	(Ge et al., 2011)
Live LaSota strain	AMPV	Respiratory infection	G	Turkey	ECEs	in, io	one or two doses of 10^6 $TCID_{50}$	(Hu et al., 2011)

Live LaSota strain	NiV	Encephalitis	F, G	Mouse, pig	ECEs	im	two doses of 1×10^8 EID_{50} (mice), two doses of 2×10^9 EID_{50} (pigs)	(Kong et al., 2012)
Live LaSota strain	HPAIV H7N9	Respiratory infection	HA	Mouse	ECEs	in	two doses of 10^4 or 10^6 FFU	(Goff et al., 2013)
Live chimeric NDV	HPAIV H5N1	Respiratory infection	HA	Chicken	ECEs	on	one dose of 10^6 $TCID_{50}$	(Steglich et al., 2013)
Live LaSota strain	IBV	Respiratory infection	S2	Chicken	ECEs	io	one dose of 10^6 EID_{50} followed by one dose of an attenuated IBV vaccine	(Toro et al., 2013)
Live LaSota strain	HPAIV H5N1	Respiratory infection	HA	Duck	ECEs	io	two doses of 10^6 EID_{50}	(Ferreira et al., 2012)
Live LaC30L strain	IBDV	Infectious bursal disease	VP2	Chicken embryo	ECEs	in ovo	one dose of 3×10^2, 3×10^3, 3×10^4 or 3×10^5 EID_{50}	(Ge et al., 2014)
Live LaSota strain	ILTV	Respiratory infection	gB, gC, gD	Chicken	ECEs	on	two doses of 2×10^5 $TCID_{50}$	(Kanabagatte Basavarajappa et al., 2014)
Live LaSota strain	ILTV	Respiratory infection	gB, gD	Chicken	ECEs	in + io	one dose of 1×10^6 $TCID_{50}$	(Zhao et al., 2014)
Live NA strain	GPV	Derzsy's disease (goose hepatitis)	VP3	Gosling	ECEs	sc	two doses of 10^6 EID_{50}	(Wang et al., 2015)
Live LaSota strain	CDV	Canine distemper	F, HN	Mink	ECEs	im	two doses of 10^9 EID_{50}	(Ge et al., 2015)
Live LaSota strain	H7N9, H5N1	Respiratory infection	HA	Chicken	ECEs	im, on	two doses of 5×10^6 PFU	(Liu et al., 2015)
Live LaSota strain	VSV	Vesicular stomatitis	G	Mouse	ECEs	im	two doses of 1×10^7 $TCID_{50}$	(Zhang et al., 2016)
Live LaSota strain	HPAIV H9N2	Respiratory infection	HA	Chicken	ECEs	on, im	two doses of 10^7 FFU	(Nagy et al., 2016)
Live LaSota strain	WNV	West Nile fever	PrM/E	Mouse, horse, chicken, duck, goose	ECEs	im, in, oral	two doses of 1×10^8 EID_{50} (im) (mice); two doses of 2×10^9 EID_{50} (im) (horses); two doses of 1×10^8 EID_{50} (im) or 1×10^{10} EID_{50} (oral) (chicken); two doses of 5×10^8 EID_{50} (im, in, or oral) (geese)	(Wang et al., 2016)
Live LaSota strain	BEFV	Bovine ephemeral fever	G	Mouse, calf	ECEs	im	one dose of 1×10^6 $TCID_{50}$ (mice), two doses 8×10^7 $TCID_{50}$ (calves)	(Zhang et al., 2017)
Live Clone 30 strain	PaBV-4, CnBV-2	Proventricular dilatation disease	N/P	Cockatiel, canary	ECEs	im	one dose of 8×10^5 FFU to 1×10^6 FFU (cockatiel) or 4×10^6 FFU (canary)	(Olbert et al., 2016)

Live chimeric NDV	HPAIV H5N1	Respiratory infection	HA	Chicken	ECEs	on	two doses of 10^6 PFU/mL	(Kim et al., 2017)
Live LaSota strain	IBV	Respiratory infection	S1	Chicken	ECEs	on	one or two doses of 10^6 PFU	(Zhao et al., 2017)
Live LaSota strain	AMPV	Respiratory infection	F/G	Turkey	ECEs	in, io	one dose of 10^6 $TCID_{50}$	(H. Hu et al., 2017)
Live LaSota strain or chimeric NDV	HPAIV H5N1	Respiratory infection	HA, HA/NA, HA/M1, HA/NS1	Chicken	ECEs	on	one dose of 10^6 PFU/mL or two doses: prime with chimeric NDV construct, boost with LaSota construct (10^6 PFU/mL)	(Kim & Samal, 2017)
Live F strain	IBDV	Infectious bursal disease	VP2	Chicken	ECEs, Vero cell line	in	two doses of 10^5, 10^6 or 10^7 EID_{50}	(Dey et al., 2017)
Live LaSota strain	MERS-CoV	Respiratory infection	S	Mouse, camel	ECEs	im	two doses of 10^8 EID_{50} (mouse) or 1×10^9 EID_{50} (camel)	(Liu et al., 2017)
Live or inactivated LaSota strain	HPAIV H5N2	Respiratory infection	HA	Chicken	ECEs	im, spraying	two doses of 5×10^6 $TCID_{50}$ (im live) or 10^7 $TCID_{50}$ (im inactivated); around 10^6 $TCID_{50}$ (spraying)	(Ma et al., 2017)
Live LX strain	HPAIV H7N9	Respiratory infection	HA	Chicken	ECEs	in	two doses of 5×10^6 EID_{50}	(Z. Hu et al., 2017)
Live attenuated GM strain	DTMUV	Duck Tembusu virus disease	PrM/E	Duck	ECEs	sc	two doses of 10^6 EID_{50}	(Sun et al., 2018)
Live LaSota strain or chimeric NDV	SIV	Simian AIDS	Env (gp160)	Guinea pig	ECEs	in	two doses of 10^5 $TCID_{50}$	(Manoharan et al., 2018)
Live chimeric NDV	HPAIV H9N2	Respiratory infection	HA	Chicken	ECEs	on	one dose of 10^6 EID_{50}	(Liu et al., 2018)
Live LaSota strain or chimeric NDV	HPAIV H5N2	Respiratory infection	HA/NA	Chicken	ECEs	in	two doses: prime with chimeric NDV construct (1×10^5 PFU), boost with LaSota construct (2×10^5 PFU)	(Cho et al., 2018)
Live rAI4 strain	HPAIV H7N9	Respiratory infection	HA	Chicken	ECEs	in, io	one dose of 10^6 EID_{50}	(Shi et al., 2018)
Live NA Strain	HPAIV H9N2	Respiratory infection	HA	Chicken	ECEs	on	one or two doses of 10^6 EID_{50}	(X. Xu et al., 2019)
Live LaSota strain	IBV	Respiratory infection	S	Chicken	ECEs	on	one or two doses of 1×10^6 PFU	(Abozeid et al., 2019)
Live LaSota strain or chimeric NDV	HPAIV H7N8	Respiratory infection	HA, HA/NA	Chicken, turkey	ECEs	in	two doses: prime with chimeric NDV construct (1×10^5 PFU), boost with LaSota construct (2×10^5 PFU)	(Roy Chowdhury et al., 2019)

Live R2B strain	ARV	Viral arthritis/tenosynovitis	σC	Chicken	ECEs	oral + in, im	two doses of 1×10^5 EID_{50}	(Saikia et al., 2019)
Live SH12 strain	GoAstV	Visceral gout	Cap	Gosling	ECEs	on	one dose of 1×10^7 $TCID_{50}$	(D. Xu et al., 2019)
Live LaSota strain	CSFV	Classical swine fever	E2, E^{rns}	Pig	ECEs	in	two doses of 10^3 $TCID_{50}$	(Kumar et al., 2019)
Live LaSota strain	IBV	Respiratory infection	S1 (multi-epitope)	Chicken	ECEs	on	one dose of 1×10^6 EID_{50}	(Tan et al., 2019)
Live LaSota strain	PRRSV	Porcine reproductive and respiratory syndrome	GP5, GP3/GP5	Piglet	ECEs	im	two doses of 4×10^8 EID_{50}	(Zhang et al., 2019)
Live TS09-C (thermostable) strain	HPAIV H5N1	Respiratory infection	HA, HA1	Chicken	ECEs	in, io	two doses of 10^6 $TCID_{50}$	(Xu et al., 2020)
Live LaSota strain	FAdV-4	Hepatitis-hydropericardium syndrome	Fiber 2	Chicken	ECEs	im	one dose of 10^7 EID_{50}	(Tian et al., 2020)
Live Clone 30 strain	PPRV	Peste des petits ruminants (PPR)	H	Goat	CEFs	sc	one or two doses of 6×10^6 $TCID_{50}$/mL	(Murr, Hoffmann, et al., 2020)
Live R2B strain or chimeric NDV	RV	Rabies	G	Mouse	ECEs	im	two doses of 2×10^6 $TCID_{50}$	(Debnath et al., 2020)
Live HR09 (thermostable) strain	HPAIV H9N2	Respiratory infection	HA, chimeric HA	Chicken	ECEs	on	one dose of 10^6 EID_{50}	(Zhang et al., 2021)
Live LaSota strain	IBV	Respiratory infection	N (multi-epitope)	Chicken	ECEs	on	one dose of 10^6 EID_{50}	(Qin et al., 2021)
Live LaSota strain	PPRV	Peste des petits ruminants (PPR)	H	Sheep, goat	ECEs	im	two doses of 1×10^8, 5×10^8 or 3×10^9 EID_{50}	(Fakri et al., 2021)
Live R2B strain	CIAV	Chicken infectious anaemia	VP1/VP2	Chicken	Vero cell line	on	three doses of 1×10^6 $TCID_{50}$/mL	(Chellappa et al., 2021)
Live K148/08 strain	HPAIV H5N6	Respiratory infection	HA	Chicken, duck	ECEs	on, spray	two doses of 10^7 EID_{50}	(Lee et al., 2022)

* ECEs: embryonated chicken eggs, CEFs: chicken embryo fibroblasts, io: intraocular, iv: intravenous, ip: intraperitoneal, in: intranasal, on: oculonasal, im: intramuscular, it: intratracheal, sc: subcutaneous, EID_{50}: embryo infectious dose 50%, PFU: plaque-forming units, $TCID_{50}$: tissue culture infectious dose 50%, FFU: focus-forming units.

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Preface to Chapter 2

Chapter 1 points to the importance of establishing vaccine platforms to prepare for pandemics and summarizes the potential of NDV as a viral vector to fulfill this purpose. Although there has been extensive research testing NDV as a vector for oncolytic treatments or vaccines, the review identifies the gap in NDV research when it comes to process development.

It is essential to develop and optimize scalable methods of producing, purifying, storing and distributing NDV-vectored vaccines to establish this vector as a reliable vaccine platform. In this context, the past chapter summarizes the published data on NDV propagation and highlights key findings from the work in chapter 2, which is one of the few published studies to evaluate parameters for production of NDV in bioreactors.

Chapter 2 further elaborates on these findings, with a focus on developing potentially scalable methods and processes. This chapter elaborates on the development of analytical assays, which are critical tools for quality control and assessment of a production process. Next, upstream parameters for NDV production are evaluated at the small-scale to define the optimal conditions for NDV replication in suspension cell culture. Once the cell line, MOI, trypsin concentration, temperature and time of harvest for production are defined, the process is demonstrated at the lab scale using 1 L bioreactors.

Chapter 2

Process Development for Newcastle Disease Virus-Vectored Vaccines in Serum-Free Vero Cell Suspension Cultures

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Abstract

The ongoing COVID-19 pandemic drew global attention to infectious diseases, attracting numerous resources for development of pandemic preparedness plans and vaccine platforms — technologies with robust manufacturing processes that can quickly be pivoted to target emerging diseases. Newcastle Disease Virus (NDV) has been studied as a viral vector for human and veterinary vaccines, but its production relies heavily on embryonated chicken eggs, with very few studies producing NDV in cell culture. Here, NDV is produced in suspension Vero cells, and analytical assays ($TCID_{50}$ and ddPCR) are developed to quantify infectious and total viral titer. NDV-GFP and NDV-FLS (SARS-CoV-2 full-length spike protein) constructs were adapted to replicate in Vero and HEK293 suspension cultures using serum-free media, while fine-tuning parameters such as MOI, temperature, and trypsin concentration. Shake flask productions with Vero cells resulted in infectious titers of 1.07×10^8 $TCID_{50}$/mL for NDV-GFP and 1.33×10^8 $TCID_{50}$/mL for NDV-FLS. Production in 1 L batch bioreactors also resulted in high titers in culture supernatants, reaching 2.37×10^8 $TCID_{50}$/mL for NDV-GFP and 3.16×10^7 $TCID_{50}$/mL for NDV-FLS. This shows effective NDV production in cell culture, building the basis for a scalable vectored-vaccine manufacturing process that can be applied to different targets.

Keywords

Newcastle Disease Virus; Vero suspension culture; viral vaccine bioprocess; bioreactor production; vaccine production platform; COVID-19; SARS-CoV-2

Introduction

Infectious diseases are present throughout history, emerging and reemerging as decades pass (Samaranayake et al., 2021). In the most recent years, the world has seen outbreaks of H1N1 influenza, severe acute respiratory syndrome coronavirus (SARS-CoV), human immunodeficiency virus (HIV) (Kim & Samal, 2016) and, notably, SARS-CoV-2 (Ndwandwe & Wiysonge, 2021). Vaccines have been a key player in containing the spread and reducing the mortality of bacterial and viral pathogens, taking part in national and global immunization strategies that have led to eradication of smallpox and near eradication of polio (Orenstein & Ahmed, 2017).

Recombinant viral vectors have become an important platform for vaccination, with growing interest in a variety of possible vectors. Viral vector vaccines have been approved against Ebola (Tomori & Kolawole, 2021)—using adenovirus (European Medicines Agency (EMA), 2020b),

modified Vaccinia Ankara (Crommelin, Volkin, et al., 2021; European Medicines Agency (EMA), 2020a), and vesicular stomatitis virus (Henao-Restrepo et al., 2017) as vectors—and against SARS-CoV-2, using adenovirus as a vector (Johnson & Johnson, Gamaleya, Oxford-Astrazeneca and CanSino) (Samaranayake et al., 2021; World Health Organization (WHO), 2021). There are also examples of approved viral vector vaccines for veterinary use, using vectors such as poxviruses, herpesvirus of turkeys (HVT) (Mebatsion, 2021) and adenovirus (Fernandez-Sainz et al., 2017; Pedersen et al., 2016). This technology fits the concept of platform-based vaccines, in which the viral vector is a backbone that can be modified to express and carry different antigens to quickly adapt the vaccine to target other pathogens, including for emerging outbreaks. By developing a platform-based vaccine and establishing a production process for it, both the product and process can be adapted to other targets with minimal changes. Thus, the time to develop, scale up and, consequently, deliver the vaccine can be greatly reduced, making this a promising approach for pandemic preparedness (Adalja et al., 2019).

In this context, the Newcastle Disease Virus (NDV) is an avian paramyxovirus which is non-pathogenic in humans (Kim & Samal, 2016). NDV is classified into three types of strains: velogenic, mesogenic or lentogenic, based on virulence and pathogenicity in avian species (Schirrmacher, 2017). Lentogenic strains, such as LaSota and B1, are avirulent in both birds and humans, making them the strains of choice for viral vector studies. NDV expresses a fusion protein (F) in its precursor form (F_0), which must be cleaved by host cell proteases for viral entry (Kim & Samal, 2016). In cell culture, trypsin can be added at the moment of infection to activate the virus (Wen et al., 2013), as it is done for production of influenza (Le Ru et al., 2010). As an avian virus, there is a lack of pre-existing immunity against NDV among humans, as well as remarkable safety, which has been documented in clinical trials for oncolytic treatments using this virus (Kim & Samal, 2016). Another advantage of using this vector for vaccines includes operation at biosafety level 2, rather than working directly with the target pathogens, which can require costly operation at biosafety level 3 (Sun, Leist, et al., 2020). Additionally, there are well established methods to generate recombinant NDV constructs bearing protective key antigens from other viral pathogens. These aspects make NDV a promising vaccine vector that has already been explored in vaccine candidates against H1N1 influenza, SARS-CoV, HIV, among others (Kim & Samal, 2016).

Despite having garnered interest as a viral vector for vaccination and for cancer therapy (Schirrmacher, 2017), there are still few studies on the development of production processes for NDV. Typically, this virus is produced in embryonated chicken eggs and collected in the allantoic fluid. Although this is a cost-effective method that can take advantage of existing manufacturing structures for influenza (Sun, Leist, et al., 2020), it also presents disadvantages when compared to cell culture technologies and their potential large-scale production under controlled operational conditions in bioreactors. Virus production in cell culture allows for greater control over several parameters, leading to less variation between batches and the capacity to further optimize each aspect. It may also avoid issues with allergens and the dependency on chicken egg supply (Kiesslich & Kamen, 2020; Milián & Kamen, 2015). As such, developing a cell culture-based NDV production process would be highly valuable in the pursuit of establishing a reliable vaccine production platform that allows for quick adaptation to emerging pathogens.

The COVID-19 pandemic highlighted the importance of mass vaccination and the race for fast implementation. Notably, vaccines were developed and approved within months, rather than the usual timespan of years (Ndwandwe & Wiysonge, 2021), which was partly due to extensive previous research on the technologies used. These vaccines were key to reduce cases and deaths (Christie et al., 2021), as well as to recover economies (Khalfaoui et al., 2021), in contrast to the 2009 H1N1 pandemic, in which vaccines were only widely available after the main onset of the pandemic (Rambhia et al., 2010). This further showcases the importance of using vaccine platforms to implement vaccination early on in a pandemic, maximizing the positive contribution of vaccines and avoiding the peak of cases and deaths.

In this work, we set out to develop a novel cell-based production process for two NDV constructs: NDV-GFP and the vaccine candidate NDV-FLS, which expresses the full-length SARS-CoV-2 spike protein. We first adapted both constructs to HEK293 and Vero cells, and then evaluated several infection parameters. In shake flasks, viral production kinetics were compared for different multiplicities of infection (MOI) and a Design of Experiment (DoE) was performed to analyze the effects of temperature, trypsin concentration and trypsin addition. The best conditions were then implemented for batch bioreactor productions of both viruses. For the analytics, we established two assays for viral quantification: median tissue culture infectious dose ($TCID_{50}$) for infectious viral titer and digital droplet PCR (ddPCR) for genomic/total viral titer. As

such, this is an innovative work in exploring and establishing these essential aspects for robust production and quality assessments of NDV in cell culture.

Materials and methods

Cell lines and culture media

The Vero cell line adapted to suspension was provided by the National Research Council of Canada (NRC), and the adaptation was described in a previous work (Shen et al., 2019). For routine passaging, cells were centrifuged at 800 × *g* for 5 min and resuspended in fresh media with a seeding density of 3–6 × 10^5 cells/mL. Cell cultures were maintained at 37 °C, 135 rpm and 5% CO_2 in humified Multitron orbital shakers (Infors HT, Bottmingen, Switzerland). Cells were cultured in MDXK medium (Xell AG, Bielefeld, Germany), supplemented with 4 mM GlutaMAX (Thermo Fisher Scientific, Waltham, MA, USA), at a working volume of 20 mL, 25 mL, 50 mL or 100–200 mL in polycarbonate shake flasks of volume 125 mL, 250 mL, 500 mL or 1 L (TriForest Enterprises, Irvine, CA, USA), respectively.

HEK293 suspension cells are originated from HEK293SF (clone 293SF-3F6) cells, which derive from a GMP-grade master cell bank (Côté et al., 1998). The cells were cultured in HEK GM medium (Xell AG, Bielefeld, Germany), supplemented with 4 mM GlutaMAX. Routine passaging and incubation in shakers was the same as for suspension Vero cells.

Adherent Vero cells (ATCC CCL-81.5) were routinely passaged by washing with PBS without calcium and magnesium (WISENT Inc., Saint-Jean-Baptiste, QC, Canada), detaching with TrypLE™ Express Enzyme (Gibco, Gaithersburg, MD, USA) and adding VP Serum-Free Medium (VP-SFM) (Gibco, Gaithersburg, MD, USA) with 4 mM GlutaMAX and 1% Penicillin-Streptomycin solution (WISENT Inc., Saint-Jean-Baptiste, QC, Canada) to collect. Once collected, cells are pelleted at 300× *g* for 5 min and resuspended in VP-SFM to remove TrypLE. Cells are plated onto T-175 flasks or 150 mm plates, at 5–10 × 10^6 cells and are passaged every 2–3 days.

Adherent HEK293 (HEK293A, ATCC CRL-1573 (Graham et al., 1977)) cells were routinely passaged in the same way as adherent Vero cells, but using Dulbecco's Modified Eagle's Medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, USA) with 10% Fetal Bovine Serum (FBS) (Gibco, Gaithersburg, MD, USA) and 1% Penicillin-Streptomycin solution instead of VP-SFM.

Virus adaptation

The engineering and rescue of the Newcastle Disease Virus constructs NDV-GFP and NDV-FLS were described in another publication (Warner et al., 2021). Briefly, the gene of interest (encoding green fluorescent protein or human codon-optimized full-length spike from SARS-CoV-2, respectively) was inserted between the P and M genes of the NDV (LaSota strain) genome. These viruses were initially produced in allantoic fluid and passaged for adaptation to Vero and HEK293 cells. Passages consisted of infecting cells, harvesting the virus produced and using it to reinfect cells for the next passage.

In Vero cells, for passages 1 and 2, adherent Vero cell cultures in T-25 flasks with VP-SFM media and 4 mM GlutaMAX were infected at a confluency of 80–90% and an MOI of 0.5 with TPCK-treated trypsin (MilliporeSigma, Oakville, ON, Canada) to a final concentration of 1 µg/mL. The supernatant was collected at 24 hpi. From passage 3 onwards, suspension Vero cells were seeded at 1×10^6 cells/mL in 25 mL MDXK media with 4 mM GlutaMAX in 250 mL shake flasks. The cells were immediately infected at an MOI of 0.01 with 1 µg/mL trypsin. At 36 hpi, the culture was centrifuged at 800 × *g* for 5 min to collect the supernatant, which was stored at −80 °C.

In HEK293 cells, for all passages, suspension cells were seeded at 1×10^6 cells/mL in 25 mL Xell HEK GM media with 4 mM GlutaMAX in 250 mL shake flasks. The cells were immediately infected at an MOI of 0.01 with 1 µg/mL trypsin. At 36 hpi, the culture was centrifuged at 800× *g* for 5 min to collect the supernatant, which was stored at −80 °C.

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

For routine quantification, adherent Vero cells were seeded on 96-well plates with 15,000 cells in 100 µL of media (VP-SFM) per well. For media and cell line comparison during $TCID_{50}$ development, adherent HEK293 cells were used with DMEM. When using DMEM, BSA 2.5 µg/mL was added instead of FBS, to avoid trypsin activity inhibition. The following day, the media was aspirated and replaced by 100 µL of media containing 1 µg/mL trypsin and a serial dilution of the virus (1:5 or 1:10). After 4 and 7 days of incubation at 37 °C with 5% CO_2, wells were analyzed on a standard light microscope for cytopathic effect (CPE), consisting of rounded cells, a disrupted monolayer and/or clumps. The number of CPE-positive wells in each column was used to quantify

the experiment by the Spearman and Kärber algorithm (Hierholzer & Killington, 1996; Kärber, 1931; SPEARMAN, 1908).

The assay with 1:5 dilutions (Coefficient of Variation: 34.57%) was chosen for all the $TCID_{50}$ development and for samples which were below the range of detection of the 1:10 dilutions (<3.16 × 10^2 $TCID_{50}$/mL). The assay with 1:10 dilutions (Coefficient of Variation: 34.69%) was chosen for all samples from shake flask experiments and bioreactors.

For comparison of CPE readings and Alamar blue readings, CPE was read first on the microscope before addition of the dye. The cell viability reagent Alamar blue (Invitrogen, Waltham, MA, USA) was diluted 1:10 in PBS without calcium and magnesium, and 100 µL of the dilution was added to each well, as described previously (Mo et al., 2008). Plates were incubated at 37 °C with 5% CO_2 and the absorbance was analyzed after 4 h. The absorbances at 570 nm and 600 nm were measured, and the absorbance at 600 nm was subtracted from the absorbance at 570 nm ($ABS_{570nm} - ABS_{600nm}$) to obtain the normalized value. Cut-off values were determined in a way that none of the wells in the (non-infected) negative control would be considered infected.

For comparison with fluorescence readings, a triplicate of an NDV-GFP sample was used for $TCID_{50}$ and plates were read both by CPE, using a standard light microscope, and by fluorescence, using a plate reader with the excitation at 485/20 nm and emission at 528/20 nm.

After classifying the wells as positive through the cell viability (Alamar blue) or the fluorescence, the viral titer was determined by the Spearman and Kärber algorithm (Hierholzer & Killington, 1996; Kärber, 1931; SPEARMAN, 1908), in the same way as when reading CPE.

For fluorescent microscope imaging, the $TCID_{50}$ plates infected with NDV-GFP samples were observed on day 7 on Olympus IX-83 microscope using a 10× objective lens. Images were processed on ImageJ to merge bright-field images with green fluorescence channel images.

One-way ANOVA with the Tukey method was performed to determine statistical significance when comparing titration between different cell lines and different reading methods.

Polymerase chain reaction (PCR)

The Q5 High Fidelity Polymerase (New England Biolabs, Ipswich, MA, USA) was used with primers targeting the L gene (polymerase) of NDV: NDV-L F [5′-

ATATGTTCTGACTCCTGCCC-3′] and NDV-L R [5′-TCTAGTCGCTTGATCTCTGC-3′]. PCR was performed according to the manufacturer's instructions, with the following thermocycler program: initial denaturation (1 min at 98 °C), followed by 30 cycles of the steps: 10 s at 98 °C, 30 s at the annealing temperature, 30 s at 72 °C. Next, the final elongation step happens for 2 min at 72 °C. The same NDV-GFP and NDV-FLS cDNA samples were used for PCR with different annealing temperatures: 56 °C, 57.6 °C, 59.2 °C and 60 °C. The amplified bands were visualized in a 2.5% agarose gel with SYBR Safe DNA gel stain (Thermofisher, Waltham, MA, USA).

Digital droplet polymerase chain reaction (ddPCR)

For routine quantification, RNA extraction was done for 20 µL of supernatant samples diluted with 180 µL PBS (without calcium and magnesium) using the High Pure Viral Nucleic Acid kit (Roche, Basel, Switzerland). During assay development, different dilutions of the sample were also tested: 1× (no dilution—200 µL sample), 4× (50 µL sample with 150 µL PBS) and 10× (20 µL sample with 180 µL PBS). Next, 2 µL of the extracted RNA was used with the iScript Select cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA) to generate cDNA using random RT-PCR primers. Then, the cDNA was diluted (between 1:10 to 1:10000) to target the linear range of ddPCR. 5 µL of the template dilution was used with the QX200 ddPCR kit (Bio-Rad Laboratories, Hercules, CA, USA), using the EvaGreen master mix and the same primers listed for PCR. The manufacturer's instructions were followed to prepare the reaction and generate droplets. As for the thermocycler program: after initial denaturation (5 min at 95 °C), 34 cycles of the following steps were repeated: 30 s at 95 °C, 1 min at 59 °C, 30 s at 72 °C. Then, the final elongation step happened for 5 min at 72 °C.

Droplets are analyzed individually in the droplet reader and the copies/µL of each sample is given. This output is corrected for the dilution and volumes used to determine the viral genomes/mL of the original sample with the following calculation:

$$\text{Viral genomes/mL} = I \times J \times K \times (L/M) \times (O/N)/P \times Q \times 1000,$$

in which: I = Copies/µL (ddPCR output); J = volume of the ddPCR reaction; K = dilution of the cDNA template; L = volume of RT-PCR reaction; M = volume of the cDNA dilution added in the ddPCR reaction; N = volume of RNA added in the RT-PCR reaction; O = elution volume for RNA extraction; P = initial sample volume used for the RNA extraction; Q = dilution of the sample in RNA extraction.

Design of experiment (DoE) for infection parameters

A two-level full factorial design was done with triplicates of each condition to screen 3 parameters at infection: trypsin concentration (from 1 to 5 µg/mL), trypsin addition (no repeated addition or addition at 24 h) and temperature (from 34 to 37 °C). To start the experiment, cultures of suspension Vero cells were centrifuged at 800 × g for 5 min and seeded at 1×10^6 cells/mL in 30 mL MDXK media with 4 mM GlutaMAX in 250 mL shake flasks. The flasks were immediately infected with NDV-FLS at an MOI of 0.01 using the chosen DoE parameters. For trypsin addition at 24 h, trypsin was added to a final concentration of 1 or 5 µg/mL, according to the initial trypsin concentration assigned to each flask. Viral samples were taken at 30 hpi by centrifuging at 800 × g for 5 min and aliquoting the supernatant (storage at −80 °C). Samples were quantified by TCID50 and analyzed with the Design Expert 13 software (Stat-Ease Inc., Minneapolis, MN, USA) using base 10 log-transformed values. Statistical significance was determined through ANOVA, followed by several residual analyses and diagnostics to confirm the quality of the model.

Multiplicity of infection (MOI) optimization

4 different multiplicities of infection (MOI) were evaluated: 0.1, 0.01, 0.001 and 0.0001 IVP/cell. Cultures of suspension Vero cells were centrifuged at 800× *g* for 5 min and seeded at 1×10^6 cells/mL in 25 mL MDXK media with 4 mM GlutaMAX in 250 mL shake flasks. Immediately, cells were infected with NDV-FLS at the corresponding MOIs with 1 µg/mL trypsin, in triplicates. Shake flasks were incubated at 37 °C, 135 rpm and 5% CO_2 and samples were taken every 12 h after infection. For sampling, 0.8 mL culture of each flask was taken and spun down at 800× *g* for 5 min to aliquot the supernatant (storage at −80 °C). Viral samples were quantified by $TCID_{50}$ and triplicates were averaged to plot the viral production kinetics of each MOI. The peak values of viral production for each MOI were compared in a one-way ANOVA with the Tukey method to investigate statistical significance.

Bioreactors

For 1 L bioreactors, a culture of 650 to 850 mL was seeded at 2.5×10^5 cells/mL in MDXK with 4 mM glutamine. The bioreactors (Applikon Biotechnology, Delft, The Netherlands) were assembled with a marine impeller for stirring and sensors for dissolved oxygen (DO) concentration, temperature and pH. The following parameters were controlled in the system: pH

at 7.2, temperature at 37 °C, DO at 40–50% and stirring at 100 rpm. DO was maintained by a constant airflow in the headspace of 10 mL/min, along with pure oxygen sparging when necessary. The pH was regulated by addition of CO_2 in the headspace or injection of $NaHCO_3$ (90 g/L) (Sigma, USA).

Samples were taken every 24 h to monitor cell growth using the Vi-CELL XR Cell Viability Analyzer (Beckman Coulter Life Sciences, Brea, CA, USA). Glutamine was injected at 1 mM daily. For virus production, cells were infected at an MOI of 0.01 IVP/cell with 1 µg/mL trypsin on day 4 or 5 of culture, when cell density was approximately 8×10^5 cells/mL. After infection, samples were taken every 12 h and the supernatant was obtained by centrifuging at 800 × *g* for 5 min (storage at −80 °C).

Results

Development and optimization of analytical assays

Cell line assessment for $TCID_{50}$ set-up

Adherent Vero and HEK293 cells were compared in order to choose the most adequate cell line for the development of a $TCID_{50}$ assay, to quantify infectious viral particles in culture supernatants. When using NDV-GFP and NDV-FLS viral samples to infect both cell lines in $TCID_{50}$ assays, evident differences were observed upon visual inspection of cytopathic effect, as well as in the measured titers and fluorescence detection (Figure 3). These $TCID_{50}$ plates were incubated for 7 days after infection, and analyzed on days 4 and 7.

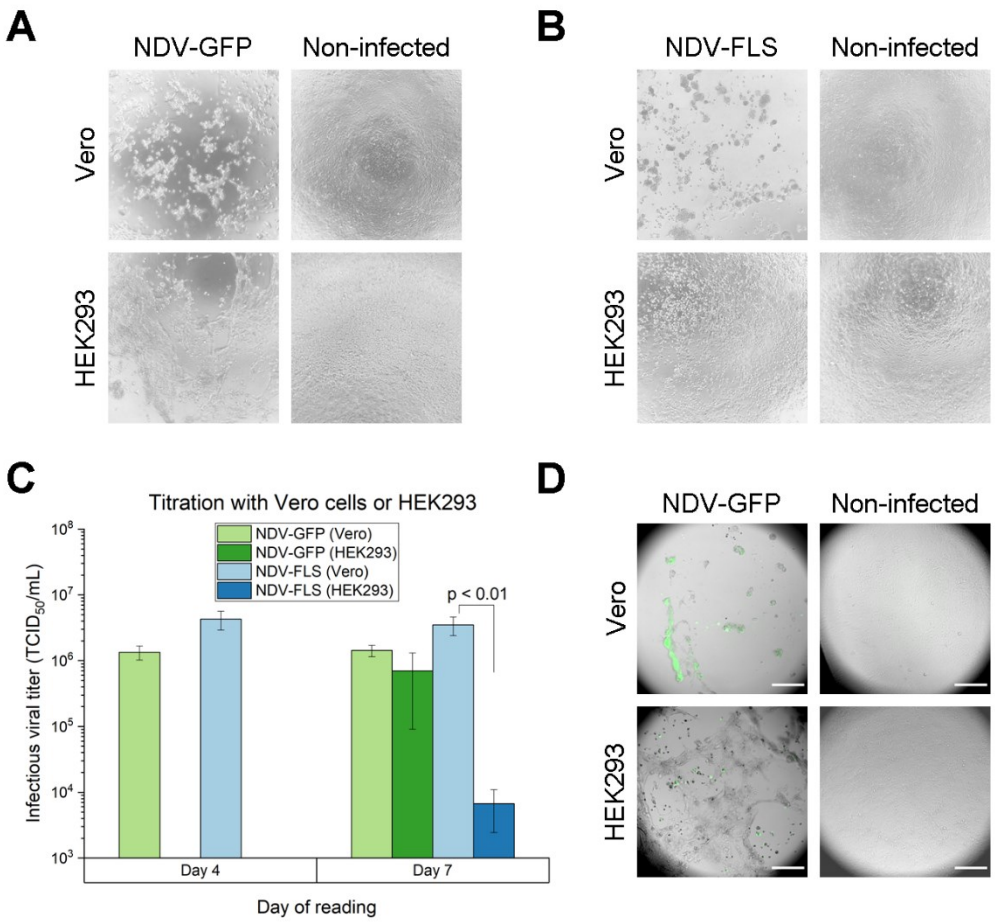

Figure 3: Comparison between Vero cells in VP-SFM or HEK293 in DMEM for quantification by $TCID_{50}$ of NDV-GFP and NDV-FLS viruses. (A,B) Cytopathic effect seen in Vero and HEK293 adherent cells after 7 days of incubation with the two different viruses: NDV-GFP (A) and NDV-FLS (B). The comparisons show an infected well in $TCID_{50}$ and a negative control well, with no virus. (C) Infectious titers obtained by quantifying the same NDV-GFP and NDV-FLS viral samples using Vero or HEK293 cells for $TCID_{50}$. Error bars correspond to the average of triplicate plates ± standard deviation. Statistically significant differences are labeled with the corresponding p-value. (D) Fluorescence detection in $TCID_{50}$ wells infected with NDV-GFP in Vero or HEK293 cells, compared with the respective non-infected negative controls. The images show a merge of the green fluorescence channel (GFP) with the bright-field image (cells). Scale bar corresponds to 250 µm.

When comparing the cytopathic effect generated on each cell line by the same virus inoculum, NDV-GFP induced a strong effect in Vero and HEK293, forming large clumps of non-viable cells and completely disrupting the cell monolayer after 7 days of incubation (Figure 3A). However, with NDV-FLS, the cytopathic effect was more evident in Vero cells than in HEK293 (Figure 3B). Like NDV-GFP, it caused the formation of aggregates and disrupted the Vero cell monolayer, but in HEK293 the cytopathic effect was limited to individual rounded cells, with very rare clumping. Even in Vero cells, NDV-FLS showed a slightly smaller cytopathic effect when compared to NDV-GFP (Figure S1), forming smaller aggregates that were still clearly distinguishable from the

non-infected cells. When comparing different media for adherent Vero, cytopathic effect for both viruses was more noticeable when using VP-SFM (Figure S1). DF-1 cells were also tested in the $TCID_{50}$ assay, but cytopathic effect was not discernible compared to non-infected cells, even after 7 days of incubation.

For both viruses, 4 days of incubation in HEK293 was not enough to allow for quantification, as the cytopathic effect was still difficult to distinguish from non-infected cells. This was only possible on day 7 of incubation (Figure 3C). With Vero cells, on the other hand, cytopathic effect was already distinguishable on day 4, allowing for an earlier quantification. When comparing the quantification for the same viral sample in the different cell lines on day 7, NDV-GFP had no significant differences, but the titer for NDV-FLS obtained with Vero cells was significantly higher ($p < 0.01$) than with HEK293. This was in line with the more subtle cytopathic effect observed with NDV-FLS in HEK293, which resulted in a more difficult reading and apparent lower titers. Since both constructs came from egg-derived aliquots with similar yielding passages, the titers observed when quantifying with Vero cells were more adequate, with both constructs resulting in similar titers.

Lastly, the $TCID_{50}$ plates infected with NDV-GFP were imaged under an inverted confocal fluorescence microscope. In Vero cells, the aggregates seen in the cytopathic effect were paired with strong fluorescence (Figure 3D). In HEK293, however, there was less fluorescence, even when abundant cytopathic effect was present. Although NDV-GFP showed signs of infection in both cell lines, GFP production was higher in Vero cells.

When analyzing all three aspects (cytopathic effect, titers and fluorescence), Vero cells seemed to be more suitable for NDV titration than HEK293 cells, with distinguishable cytopathic effect, higher titer and fluorescence, aside from allowing quantification within a shorter period of time. Thus, adherent Vero cells were chosen as the most appropriate cell line for the $TCID_{50}$ assay and were used in all subsequent quantifications.

Quantification of NDV infectious particles through fluorescence measurements and viability-based assays

The next step in $TCID_{50}$ development was to use a plate reader to test alternative methods of reading, which do not require subjectively analyzing cytopathic effect on a microscope. For NDV-GFP, the green fluorescence was read on a plate reader to determine the infected wells and calculate the infectious titer (Figure 4A). When quantifying the same sample by cytopathic effect or by fluorescence, there was no statistically significant difference between the two methods, both on day 4 and day 7 ($p = 0.5653$ and $p = 0.8301$, respectively). This showed that fluorescence can also be used for quantification and that the virus infected the cells, simultaneously expressing detectable GFP. Most wells with cytopathic effect also showed fluorescence on days 4 and 7 (95.48% and 98.92%, respectively).

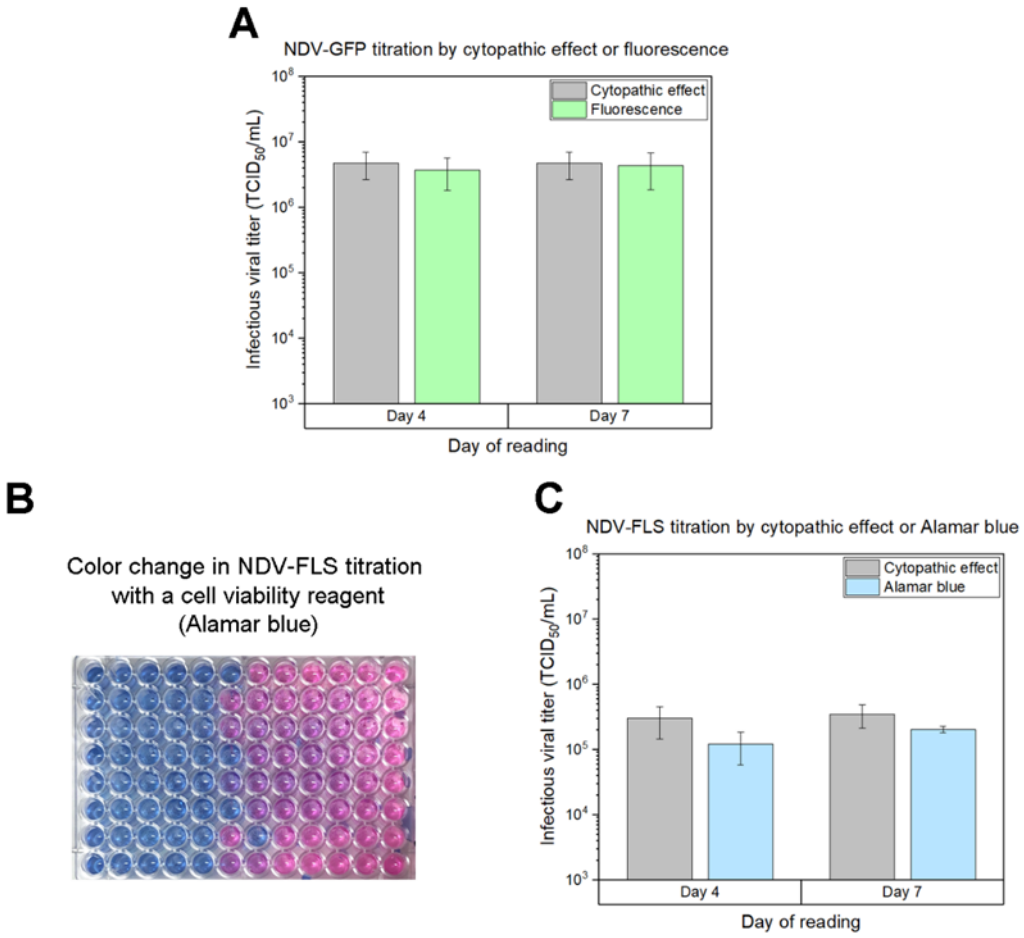

Figure 4: Different titration assays for NDV infectious particle determination. (A) Titration of the same sample of NDV-GFP in triplicate quantified by CPE and by fluorescence. Error bars correspond to the average of triplicate plates ± standard deviation. (B) $TCID_{50}$ plate (on day 7) after 4 h of incubation with a cell viability reagent (Alamar blue).

Blue wells corresponded to infected/dead cells (low viability) while pink wells corresponded to non-infected/healthy cells (high viability). (C) Titration of the same sample of NDV-FLS in triplicates quantified by CPE and by the cell viability reagent Alamar blue. Error bars correspond to the average of triplicate plates ± standard deviation.

Since fluorescence can only be used to quantify NDV constructs bearing the GFP coding sequence, a reading method based on cell viability was also evaluated. For $TCID_{50}$ calculations, the plates were incubated with a cell viability reagent (Alamar blue), resulting in infected wells that remained blue while the non-infected ones, containing healthy cells, became red/pink (Figure 4B). The infectious titer of the same NDV-FLS sample was quantified by cytopathic effect observation on the microscope and by cell viability staining, resulting in similar titers and no statistically significant differences between both methods on day 4 and day 7 ($p = 0.1395$ and $p = 0.1478$, respectively) (Figure 4C).

ddPCR-based quantification of NDV

A quantification assay based on digital droplet PCR (ddPCR) was developed to measure total viral particles. First, different annealing temperatures were tested by PCR to confirm specificity, using NDV-GFP and NDV-FLS samples (Figure 5A). For all temperatures tested with both viruses, the expected amplification product was observed, without presence of non-specific bands.

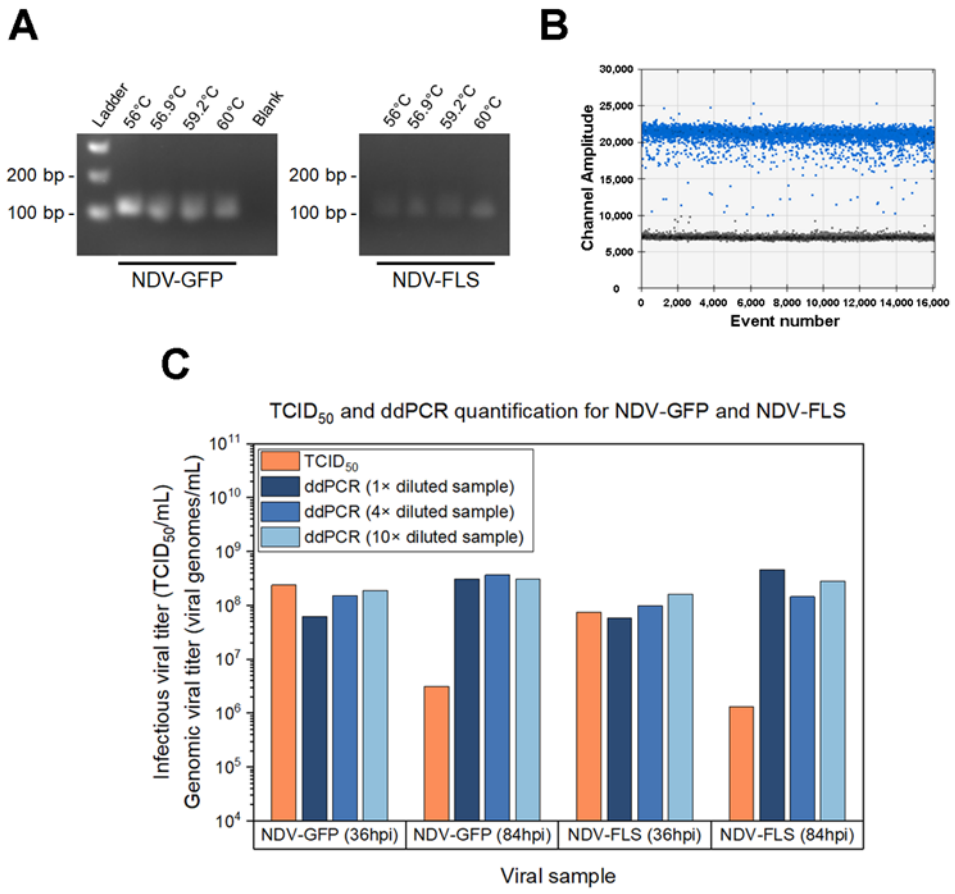

Figure 5: Development of a digital droplet PCR (ddPCR) assay for quantification of NDV. (A) Agarose DNA gel to verify PCR reactions at different annealing temperatures with primers designed for ddPCR, targeting the NDV-L (polymerase) gene on an NDV-GFP and an NDV-FLS sample. The expected band is 117 bp. (B) Plot showing positive (blue) and negative (dark grey) events in ddPCR. (C) Comparison between each sample's infectious titer ($TCID_{50}$/mL) with the genomic titer (viral genomes/mL) quantified by ddPCR. For ddPCR, different dilutions of the viral sample in RNA extraction were used: a non-diluted sample (1×), a 4 times diluted sample (4×) and a 10 times diluted sample (10×). Sample dilutions were taken into account in the calculation of final titers. Samples of NDV-FLS and NDV-GFP at a peak production time point (36 h post infection) and late time point (84 h post infection) were used.

Next, the chosen primers were used for ddPCR, with the selected annealing temperature of 59 °C. Individually partitioned events were clearly defined as positive or negative (Figure 5B), indicating proper functioning of the assay. When performing ddPCR on viral samples from peak production time points (36 hpi), the genomic titer was similar or higher than the infectious titer quantified by $TCID_{50}$ (Figure 5C). For later time points (84 hpi), the genomic titer was notably higher than the infectious titer, as the infectious titer decreased, and the genomic titer remained constant. Out of the three sample dilutions for the viral supernatant tested in the RNA extraction

step, the 10× dilution was selected for the genomic quantification of NDV in subsequent experiments.

Evaluation of NDV infection and production parameters

The two viral constructs, which were initially produced in eggs and contained in allantoic fluid, were serially passaged in Vero and HEK293 cell lines for adaptation (Figure 6A,B).

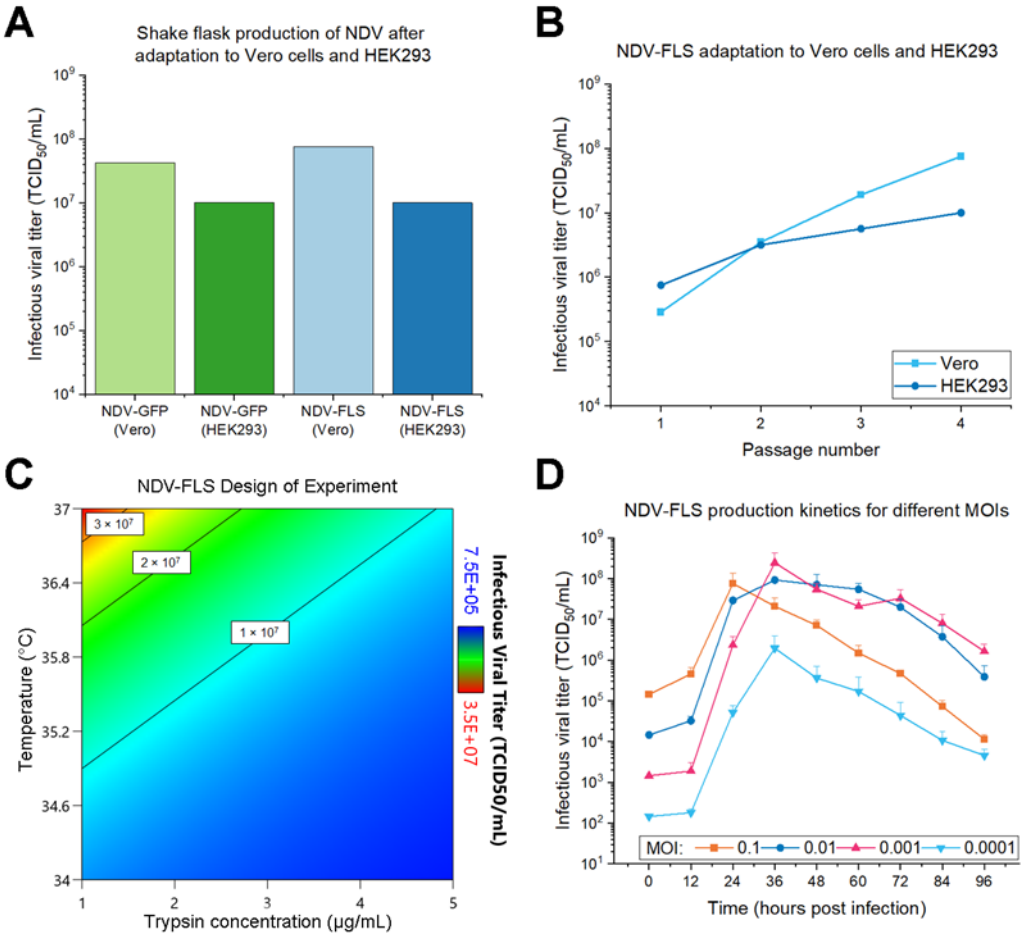

Figure 6: Optimization of infection parameters in small scale shake flask productions. (A) Infectious viral titers achieved in the fourth round of infection (passage 4) of each NDV construct in the two cell lines evaluated. (B) Serial passaging of NDV-FLS in Vero and HEK293 cells for viral adaptation. The first two passages in Vero were conducted in adherent cells with MOI = 0.5. Passages 3 and 4 were conducted in suspension cells with MOI = 0.01. For HEK293, suspension cells infected at MOI = 0.01 were used in all the passages. (C) Design of experiment (DoE) modeling for production of NDV-FLS, showing the highest viral titer produced at 37 °C with 1 µg/mL trypsin added to the culture media. (D) Viral production kinetics for NDV-FLS using the MOIs 0.1 to 0.0001, with the highest titer achieved of around 1.00×10^8 $TCID_{50}$/mL. Time is shown as hours post infection (hpi). Error bars correspond to the average titer calculated from shake flask triplicates + standard deviation.

For both NDV-GFP and NDV-FLS, higher infectious titers were achieved in Vero than in HEK293 cells after adaptation. Viral production in Vero cells at passage 4 was 4.22×10^7

$TCID_{50}$/mL for NDV-GFP and 7.50×10^7 $TCID_{50}$/mL for NDV-FLS, while production in HEK293 reached a maximum of 1.00×10^7 $TCID_{50}$/mL for both viruses (Figure 6A). As shown for NDV-FLS (Figure 6B), both cell lines started with productions lower than 3×10^6 $TCID_{50}$/mL at passage 1 and showed increased viral titers as passages progressed. This increase throughout adaptation was higher in Vero cells (over 250-fold) than in HEK293 (less than 20-fold). After passage 4, subsequent passaging for NDV-FLS or NDV-GFP did not increase the titer levels. Thus, suspension Vero cells were selected for NDV production and further optimizations.

Next, a two-level full factorial design of experiment was done to determine parameters for infection with NDV-FLS (Figure 6C). Temperature ($p < 0.0001$) and trypsin concentration at infection ($p = 0.0004$) impacted infectious titers significantly, with the best condition being 1 µg/mL of trypsin and incubation at 37 °C. The third parameter, however, which was trypsin addition at 24 h post infection vs. no repeated addition, showed no statistically significant difference ($p = 0.3271$). As such, the best conditions were used for the next experiments, with no repeated trypsin addition.

Additionally, different MOIs were tested for NDV-FLS, ranging from 0.1 to 0.0001 (Figure 6D). The lowest MOI (0.0001) had the lowest peak of viral production (1.96×10^6 $TCID_{50}$/mL), while the other 3 MOIs (0.1–0.001) all reached similar peaks around 1.00×10^8 $TCID_{50}$/mL, with no significant differences ($p = 0.178$). As expected, the highest MOI showed the earliest peak, at 24 hpi, while the next two MOIs (0.01 and 0.001) peaked at 36 hpi. Despite having an earlier peak, the infectious titer with MOI 0.1 dropped considerably as time progressed to 96 hpi, declining to similar titers as those reached at the lowest MOI. Although the MOIs 0.01 and 0.001 also showed a loss in infectious titer after the peak, the losses were the smallest when compared to other MOIs. Therefore, the MOI 0.01 was chosen for the following viral productions, with a high peak of production and adequate stability. Upon applying the selected conditions to the NDV-GFP construct, the titer of 1.07×10^8 $TCID_{50}$/mL was obtained in shake flasks.

Production in bioreactors

After parameter optimization in shake flasks, the next aim was to produce the viruses in suspension Vero cells using stirred tank bioreactors. A 1 L batch bioreactor was performed for production of NDV-GFP (Figure 7A) and NDV-FLS (Figure 7B). Infectious titers quantified for

both viruses showed the ability of the system to reach peaks in the orders of 10^8 and 10^7 $TCID_{50}$/mL, respectively.

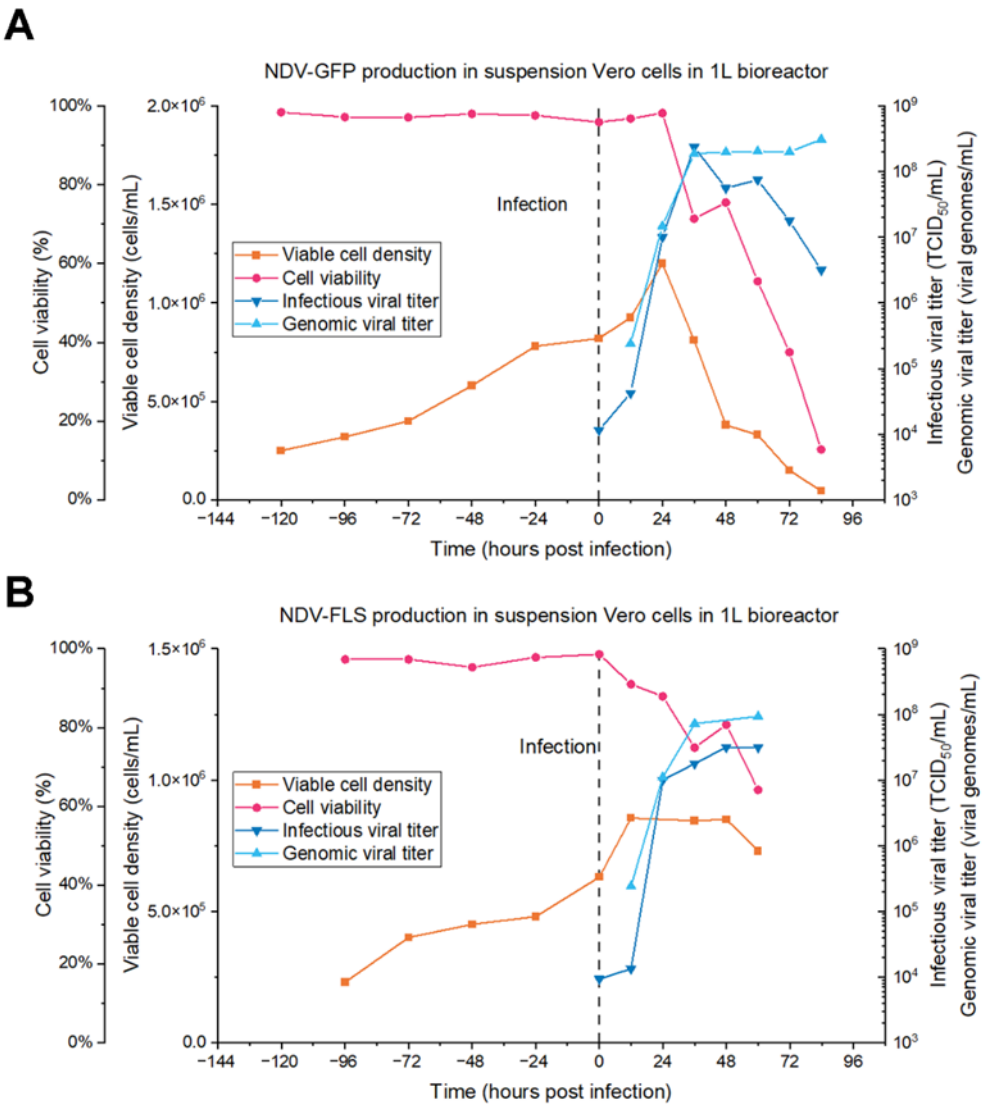

Figure 7: Batch bioreactor production of NDV-GFP (A) and NDV-FLS (B) at the 1 L scale. Offline measurements were taken by regular sampling. Infectious viral titers were quantified by $TCID_{50}$ and total/genomic viral titers were quantified by ddPCR. The time of infection is indicated by a black dashed line in the figure.

For NDV-GFP (Figure 7A), the infectious titers peaked at 36 hpi, reaching 2.37×10^8 $TCID_{50}$/mL, after which values decreased over time, dropping to 3.16×10^6 $TCID_{50}$/mL at 84 hpi. The total viral titer, on the other hand, remained constant after the peak production, at around 2.00×10^8 VGs/mL. The virus also affected cell viability, as seen with the considerable drop to below 80% observed at 36 hpi, that reached below 20% by the end of the bioreactor run at 84 hpi.

For NDV-FLS (Figure 7B), the peak production was 3.16×10^7 $TCID_{50}$/mL at 48 hpi, which remained constant until 60 hpi. The genomic titer was higher than the infectious titer, plateauing at around 1×10^8 VGs/mL from the peak production onwards. A decrease in cell viability was observed post infection, dropping to lower than 65% at 60 hpi.

The online measurements for bioreactor productions of NDV showed that pH, temperature and DO were maintained constant during the cell growth and virus production phases (Figure 8) through effective control strategies, including the addition of oxygen.

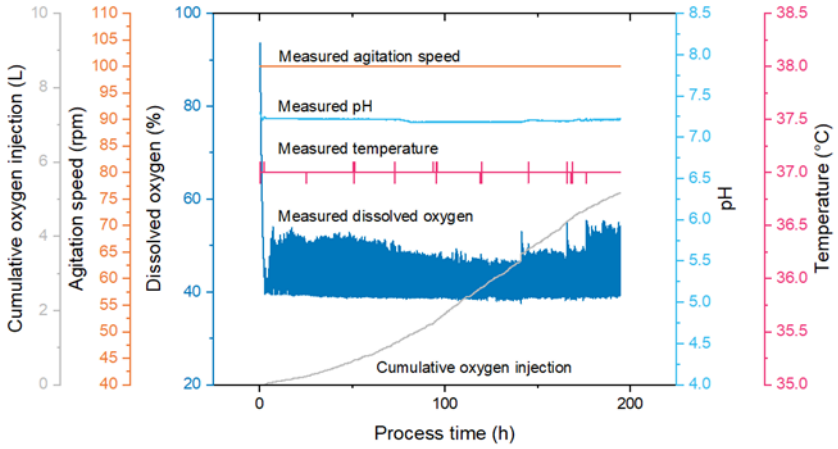

Figure 8: Online bioreactor measurements recorded throughout a batch bioreactor production of NDV-FLS at the 1 L scale.

Discussion

NDV is a promising viral vector for vaccine development that has been studied for its potential application against several human diseases, and it is still commonly produced in embryonated chicken eggs (Kim & Samal, 2016). In this study, we set out to provide an alternative for NDV production by developing the foundation for a cell culture-based production process. Bioprocesses for vaccine manufacturing are composed of an upstream phase, a downstream phase, and the analytics used throughout the entire process to quantify the production and optimizations. Here, we developed analytical assays and evaluated upstream process parameters by testing cell lines for production, adapting the virus to suspension cell cultures and comparing several infection conditions. After this evaluation, we applied the selected parameters to produce NDV in 1 L scale bioreactors.

MDCK and Vero cells are well established systems for viral vaccine production, but a range of other continuous cell lines (CCLs) have also been studied for this purpose, including HEK293. While HEK293 shows promise as a cell line that has been adapted for suspension and grows to high densities in serum-free media (Petiot et al., 2015), manufacturers tend to prefer processes using established cells for faster licensing (Genzel, 2015). Vero cells have a long history of proven safety, being the first CCL approved for viral vaccine production for human use. From a process perspective, these cells are commonly used with adherent cell culture technologies, such as microcarriers or fixed bed bioreactors, which are labor intensive and limited by surface area, resulting in a difficult scale up (Kiesslich & Kamen, 2020). However, recent advances in adapting Vero cells to suspension have been successful (Shen et al., 2019), as these suspension Vero cells have been shown to work for virus production using stirred tank bioreactors in batch (Kiesslich et al., 2021) and perfusion (Shen et al., 2019) modes. These cells have also been adapted to grow in the serum-free commercially available MDXK medium, after screening with several other media (Kiesslich et al., 2021). Recently, this cell line's genome has been sequenced through de novo assembly and annotated, facilitating future genome editing approaches (Sène et al., 2021). Furthermore, Vero cells are interferon-deficient (Sène et al., 2021), making them susceptible to a wide range of viruses that have achieved high productivity when produced in Vero cells (Kiesslich & Kamen, 2020).

In this study, suspension Vero cells showed the additional ability of yielding higher viral titers for both NDV-GFP and NDV-FLS constructs, which was in line with the more evident CPE and intensity of fluorescence observed in adherent Vero cells when compared to HEK293. Serial passaging of NDV in Vero cells led to an increase in titer after four passages, similar to what has been shown for other strains of NDV (Yurchenko et al., 2019), in which the number of passages required for such an increase varied for each strain. This increase is expected, as the viruses were originally collected in allantoic fluid, and viral adaptation to cell culture may select for viruses with more efficient replication in the new host cell. Further characterization of the viruses adapted to these cell lines could be important to evaluate if there were changes to safety, efficacy and abundance of recombinant protein on the viral surface when compared to the virus produced in eggs.

After defining suspension Vero as the cell line of choice for NDV production, a DoE revealed that the highest NDV-FLS titers were obtained when infecting at 37 °C with 1 µg/mL trypsin, and that repeated trypsin addition had no significant effect. VSV titers are influenced by the temperature in the production phase, and each construct has an optimal temperature (Kiesslich et al., 2021). As the LaSota strain of NDV is not thermostable (Liu et al., 2019), similarly to VSV, a lower temperature could have resulted in higher viral titers. However, a production temperature of 37 °C led to significantly higher titers than 34 °C, ruling out the use of low temperatures for these NDV constructs. This may be in line with the 37 °C incubation step that is typically implemented when producing NDV in embryonated eggs (Al-Ziaydi et al., 2020; Sun, Leist, et al., 2020). As for trypsin, the concentrations tested were 1 and 5 µg/mL, which are values reported in the literature for NDV experiments (Liu et al., 2019; Nan et al., 2021). In our study, the highest NDV titers were achieved with the lowest trypsin concentration, which is similar to what has been observed for influenza virus (Le Ru et al., 2010). Vero cells are known to produce trypsin inhibitors (Genzel et al., 2010), and multiple additions of trypsin have been described as having a positive effect (Kaverin & Webster, 1995) or no effect (Genzel et al., 2010) on the multi-cycle production of influenza in this cell line. For NDV, we found that repeated trypsin addition had no apparent effect on the viral titer produced, which prompted us to add trypsin only at the moment of infection.

A range of MOIs (0.1–0.0001) that encompasses the MOIs used for NDV in previous works [37,39,42] was also evaluated. With the exception of the lowest one tested, all MOIs reached a similar peak of approximately 1×10^8 $TCID_{50}$/mL. The viral production peak was 24 hpi for the highest MOI (0.1), and shifted to a later time point (36 hpi) with lower MOIs. However, this higher MOI showed a greater and earlier loss of infectivity than the next two MOIs assayed (0.01 and 0.001). For the 0.01 MOI, the titer remained relatively constant until 60 hpi, and was still higher than the 0.1 MOI by the end of the experiment at 96 hpi. Such stability is important for a robust process, as it is more likely to result in an adequate yield even if production kinetics shift due to variations in the process. The 0.01 MOI was chosen for the process, since an MOI 10 times lower still yielded similar results, and thus possible volume errors when adding the virus at 0.01 MOI would still lead to a reliable production. Overall, from the first passage in Vero cells to the last shake flask optimization experiment, the produced NDV-FLS titers increased by almost 320-fold, from 2.87×10^5 $TCID_{50}$/mL to 9.17×10^7 $TCID_{50}$/mL, indicating that the selection of culture and infection parameters was adequate.

Aside from the cell lines and infection parameters used, analytics are also an essential part of the production process that should not be overlooked. The virus being produced must be characterized and quantified throughout several steps of manufacturing to generate crucial data for process development and for regulatory approval (Thompson et al., 2013). As a replicative viral vector (Kim & Samal, 2016), NDV can be quantified regarding the replication-competent particles—also known as functional or infectious titer—and regarding the total particles, which may or may not be functional. The ratio between these two titers is indicative of quality and can be used to assess different time points or conditions of the process (Gélinas et al., 2020). As such, when developing a process, it is important to establish reliable and scalable analytical methods to increase feasibility of implementing this process in large scale, consequently improving the chances of quickly achieving mass vaccination for a new pathogen of concern.

In this study, not only have we developed assays for each type of quantification, but we have also established methods of reading the $TCID_{50}$ assay amenable to automation. NDV-GFP was quantified by reading fluorescence on a plate reader, while other constructs, such as NDV-FLS, can also be quantified on a plate reader when paired with a reagent that detects viability. Alamar blue is a blue dye based on resazurin, which changes to a red color when reduced to resorufin in metabolically active cells, indicating cell health (Rampersad, 2012). Both fluorescence and viability were shown to be comparable to CPE quantification, resulting in valid methods of reading $TCID_{50}$. As these methods rely on plate readers, and not visual inspection, they are non-subjective and can be automated for use in industry or for standardization across collaborating institutions and facilities. Therefore, the availability of these tools makes the assay more feasible for high throughput processes and industrial application. Antibody-based assays, such as an immunofluorescence assay (IFA) (Sun, Leist, et al., 2020; Vijayakumar & Zamarin, 2020), could also be of interest, as they can be targeted to quantify only viruses that contain the protein required for immunization, which could be important in vaccine manufacturing. This specificity, however, means having to adapt the assay with a different antibody for each new construct, which could slow down the development of new vaccines using the platform. Therefore, $TCID_{50}$ and ddPCR assays were chosen, as they can be used for any NDV construct.

After establishing the infection parameters at small scale and the analytical assays, we set out to produce NDV in batch mode in 1 L stirred tank bioreactors. For NDV-GFP, the peak titer

produced was $2.37 \pm 0.82 \times 10^8$ $TCID_{50}$/mL at 36 hpi, which is similar to the highest titer observed in shake flasks ($1.07 \pm 0.37 \times 10^8$ $TCID_{50}$/mL). As for NDV-FLS, the peak production was $3.16 \pm 1.09 \times 10^7$ $TCID_{50}$/mL at 48 hpi, which is similar to the value at 36 hpi ($1.78 \pm 0.62 \times 10^7$ $TCID_{50}$/mL) when considering the analytical error. This is lower than the highest values achieved with this MOI in shake flasks ($9.17 \pm 1.44 \times 10^7$ $TCID_{50}$/mL), which can occur when scaling up to bioreactors because of differences in stirring and many other factors. Both productions are comparable to the titers produced in embryonated eggs, which is in the order of 10^8 FFU/mL (Sun, Leist, et al., 2020) and 10^7 PFU/mL (Bukreyev et al., 2005), indicating that the bioreactor-based process developed in this study is a valuable substitute for existing egg-based productions.

Furthermore, process intensification could increase the quantity of infective particles harvested, using technologies such as fed-batch or perfusion (Silva et al., 2021). The lower infectious titers observed at later time points in bioreactors and shake flasks suggest that NDV could be a good candidate for production in perfusion mode, as the viruses could be continuously harvested before suffering a loss in infectivity due to temperature and shear stress in the bioreactor.

Therefore, we have successfully developed the upstream process and analytical methods for suspension Vero cell-based production of NDV, using the constructs NDV-GFP and NDV-FLS as models. Future steps include establishing a scalable purification protocol and testing different bioreactor production modes, such as fed-batch and perfusion, to move towards a complete process based on continuous manufacturing.

Supplementary materials

The following are available online at https://www.mdpi.com/article/10.3390/vaccines9111335/s1, Figure S1: Cytopathic effect seen in TCID50 when using different media with Vero cells.

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General Discussion and Conclusions

This present work contributes to pandemic preparedness and the important research field of vaccine bioprocessing. The main objective of this project is establishing the basis for NDV production in cell culture to boost its potential as a vaccine platform for emerging pathogens to overcome its dependence on chicken egg-based production processes. As such, this study contributes to enabling future large-scale production of NDV as an oncolytic or vaccine vector, for human or veterinary use.

Infectious diseases remain a serious threat worldwide, as has been evidenced by the COVID-19 pandemic. The spread of a viral infection across nations has caused countries to close their borders, issue lockdowns and close businesses in an attempt to contain further transmission and, consequently, the rising number of hospitalizations and death. Vaccination has been shown to be essential in lowering the number of cases and protecting the population. As such, the world has seen a race to develop, approve and produce vaccines in a short time span (Warner et al., 2021).

Vaccines are a safe form of immunizing populations without the need of exposure to the disease. They comprise antigens packaged in a formulation of adjuvants and/or stabilizers, designed to provide the ideal balance between safety and immunogenicity to generate a robust and long-lasting immune response. There are several ways of presenting such antigens, such as using the original pathogen in an attenuated or inactivated form. It is also possible to eliminate the use of the pathogen of interest altogether by using nucleic acids (DNA or RNA) bearing the sequence to an antigen or by expressing the antigen on a viral vector backbone. Vaccine platforms are the concept of developing these technologies and establishing production processes in a way that they can rapidly be adapted to face emerging viruses, with minimal changes (Adalja et al., 2019).

Viral vectors are viruses that can be presented in a way that is safe to the species it will be used for, whether that is humans, livestock, domestic animals or wildlife. Viral vectored-vaccines can be made to express a variety of antigens and be formulated in a replication competent or incompetent manner. Adenovirus, modified Vaccinia Ankara virus and vesicular stomatitis virus have been approved for use as viral vectors in certain vaccines (Samaranayake et al., 2021; Tomori & Kolawole, 2021) and there is a wide variety of viral vectors undergoing research, including Newcastle disease virus.

Aside from the conceptualization and development of these vaccine candidates, it is also critical to invest efforts in the manufacturing aspect. To be able to achieve the global production scale these vaccines might require, it is important to research and optimize each step of viral production. Viruses are typically produced in embryonated chicken eggs or in cell culture, and these bioprocesses are divided into: upstream, comprising cell growth and viral production; downstream, comprising purification and formulation; and analytics, comprising quality control and quantitative assays that take place throughout the entire process (Silva et al., 2021).

Although production in eggs can be cost-effective, especially for countries which already have facilities in place for influenza vaccine production, there are many advantages to using cell culture in bioreactors. The content of embryonated eggs cannot be defined as precisely as culture medium, and they do not contain the numerous probes and control loops that bioreactors do. As such, bioreactor productions can lead to less lot variability, a greater potential for optimization and scale-up, and eliminate the dependency on chicken egg supply, a known bottleneck for vaccine production (Milián & Kamen, 2015; Silva et al., 2021).

The Newcastle disease virus (NDV) is an avian virus that has been extensively studied as a viral vector for vaccines and oncolytic treatments. NDV is an enveloped virus (Figure 9A) which typically replicates in cells by budding (Figure 9B) (Schirrmacher & Fournier, 2009). This virus has shown great potential in oncolytic clinical trials for its safety and effectivity, along with promising pre-clinical trials for its use as a vaccine vector (Hu et al., 2020). Despite all the interest it has garnered, this virus still relies on embryonated chicken eggs for production, and the availability of studies on NDV production in bioreactors is extremely limited.

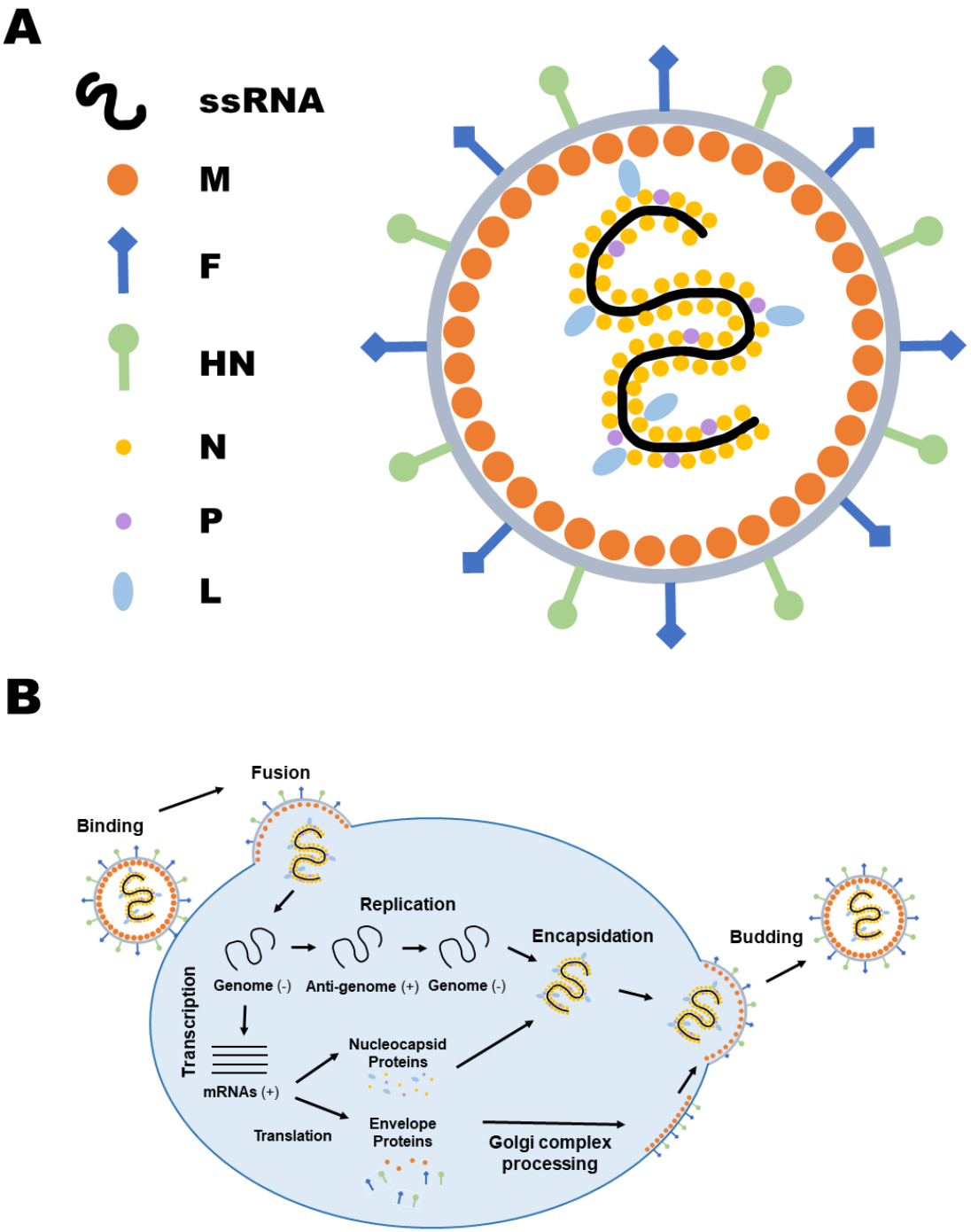

Figure 9: Newcastle Disease Virus structure (A) and replication cycle (B), in which: ssRNA = single stranded RNA, M = matrix protein, F = fusion protein, HN = hemagglutinin-neuroaminidase, N = nucleoprotein, P = phosphoprotein, L = polymerase protein, mRNAs = messenger RNAs.

Chapter 1 of this thesis is a literature review on NDV as a viral vector, its applications and the next steps to enable widespread use of NDV-vectored vaccines once they are approved. To

emphasize the potential and importance of this vector, we elaborate on the advantages of using NDV, provide a guide on the design of NDV constructs and thoroughly review the pre-clinical and clinical studies using NDV-vectored vaccines, highlighting the technological innovations and interesting mechanisms that have been developed.

The main goal of the review, however, was to compile and compare the limited information that exists on the production of NDV and lay a roadmap for the points that should be addressed. After discussing the basic workflow for processes in eggs and in bioreactors, we identify important parameters for NDV propagation in cell culture such as MOI, cell line for production and addition of an exogenous protease for viral entry. We compare values that can be found in the literature and elaborate on the few studies that have used bioreactors for NDV production.

Importantly, the literature research points to a critical gap in the development of production processes for NDV. Aside from the findings in this thesis, the only bioreactor productions found in the literature involved adherent cells, which have reduced scalability due to their limitation by surface area. For downstream processing, the literature was limited to a few studies implementing sucrose gradient centrifugation for viral purification and lyophilization for formulation. Therefore, there is still a great need to research all steps of the manufacturing process before NDV can be produced sufficiently for the global market of human vaccines.

Chapter 2 further elaborates on some data mentioned in chapter 1. This chapter comprises a research paper with the aim of establishing the required fundamental aspects to produce NDV in cell culture. In this study, we focus on the development of analytical assays, assessment of upstream parameters and validation of the process in lab-scale bioreactors.

Developing adequate analytical tools is essential to any bioprocess, especially when it comes to complex products such as viruses. Aside from accurately quantifying the viral particles and assessing the critical characteristics, these assays should be reproducible and practical. Here, we developed a tissue culture infectious dose 50% ($TCID_{50}$) assay and a digital droplet PCR (ddPCR) assay, to quantify infectious particles and total particles, respectively. Both assays performed well with multiple NDV constructs, which fits the notion of developing a vaccine platform that can be adapted for use against other diseases without the need for changes in each analytical assay.

Next, we evaluated suspension cell lines for production, namely HEK293 and Vero cells. Suspension cells are preferred over adherent cells for bioreactor productions, as their scalability is not limited to the surface area. This thesis builds on previous work in which a suspension Vero cell line was developed (Shen et al., 2019), adapted to commercial media and implemented in a platform for VSV production (Kiesslich et al., 2021). After adapting NDV for production in these cells, we defined the Vero cell line as the best option for production.

Once the cell line was defined, we set out to identify critical upstream parameters for NDV production and assess the optimal conditions for each of them. We established the best MOI, trypsin concentration, temperature and time of harvest, and then proceeded to implement these conditions in 1 L bioreactor runs to produce NDV-GFP and NDV-FLS, a vaccine candidate against SARS-CoV-2. Altogether, we established the basis for the upstream and analytics of a cell culture-based production process for NDV.

Therefore, the work described in this thesis has contributed significantly to the field by providing the first steps to critical research that must be done to establish a vaccine platform using NDV. The review portion of the thesis provides a relevant guide to using NDV as a vaccine vector, from construct design to production processes. The tables listing pre-clinical and clinical studies are resources to easily identify studies of interest, while the manufacturing section provides a comprehensive summary of the available data and identifies critical aspects for production processes that must be addressed. From a more practical aspect, the research paper provides assays that can be adopted by other research groups and institutions, as well as an evaluation of several parameters that are critical for NDV production.

As more NDV-vectored vaccine candidates are developed and the SARS-CoV-2 vaccines advance through clinical trials (Ponce-de-León et al., 2022), the demand for NDV will grow and the development of scalable and reliable manufacturing processes will become critical. As such, this study has a direct impact on advancing the potential large-scale use of this vector. Future work involving purification, formulation and process intensification studies will be essential in establishing the NDV platform for vaccines, contributing to a growing variety of platforms to respond to future pandemics.

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