Replicon vaccines to combat avian infectious diseases

REPLICAID

Jerôme D.G. Comes

Propositions

1. The *cis*-acting elements within the capsid gene enable the adjustability of flavivirus replicon vaccines in chickens.

2. Synthetically formulated replicon RNA vaccines are a valuable backup during the development of virus-like replicon particle vaccines.

3. Publishing a scientific paper resembles posting a photo on Instagram.

4. Neglecting valorization of inventions in academia impedes the development of innovations in industry.

5. Knowledge clips to inform the general public are withholding facts.

6. Nutriscore misleads consumers.

Propositions belonging to the thesis, entitled

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Jerôme D.G. Comes
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REPLICAID

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REPLICAID

Replicon vaccines to combat avian infectious diseases

Jerôme D.G. Comes

Thesis

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Pro optimo hominum et animalium prosperitate

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Chapter 1

Introduction

Poultry Industry

Livestock products are a vital source of protein for many people worldwide. To satisfy the growing demands for animal products, intensive livestock farming has become increasingly prevalent. In contrast to the 1960s, when plant-based proteins were the primary dietary protein source, animal-based proteins now constitute half of our protein intake *(Ranganathan et al., 2016)*. Among all the animal-based protein sources, poultry meat products are consumed the most. Poultry is defined as any domesticated bird such as chickens, ducks, turkeys, and quails which are reared for their meat (broilers) and eggs (layers). The highly efficient productivity of these avian species has contributed to the substantial share of poultry in global consumption (*Marangon & Busani, 2007*). For instance, the global consumption of poultry meat in 2022 reached an estimated 133 million tonnes with a projected increase of over 5% expected over the next five years as depicted in **Figure 1A**. A similar trend is observed in the European Union, where poultry meat consumption continues to rise. Notably, the global poultry market has been dominated by two countries, the United States of America and China, supplying more than 50% of the total poultry meat and egg products in the last 15 years (*FAO, 2023*) (**Figures 1B & C**). However, the high market demand for poultry products brings significant challenges, particularly in safeguarding animal health and well-being (*Coker et al., 2011; Gržinić et al., 2023; Schuck-Paim, 2020; Siegel & Honaker, 2014*).

Avian infectious diseases

The intensive farming of poultry broilers and layers causes increased susceptibility to viral infections affecting, among others, the respiratory and gastrointestinal tracts (*Coker et al., 2011; Gržinić et al., 2023; Schuck-Paim, 2020; Siegel & Honaker, 2014*). Furthermore, intensive farming also increases the risk of viral transmission by direct or indirect contact with domesticated or wild birds and farmworkers, thereby increasing the likelihood of zoonotic events. The emergence of zoonotic diseases can have a substantial social and economic impact in densely populated areas and extensive livestock industries, respectively (*Klous et al., 2016; Kuiken et al., 2011*). This section discusses examples of the most prevalent and economically important avian viral diseases.

Avian Influenza Virus

The prime example of a viral infection with zoonotic potential is avian influenza, a respiratory disease caused by the avian influenza A virus (AIV; *Orthomyxoviridae* family) (**Figure 2**). The influenza virion is composed of three major antigenic surface proteins: haemagglutinin (HA), neuraminidase (NA), and matrix protein (M2) These three proteins are embedded in the viral envelope that surrounds the negative-sense, single-stranded segmented RNA genome. The virion itself contains intracellular proteins such as RNA polymerase proteins (PB1, PB2, and PA), matrix protein (M1), nucleocapsid protein (NP), and nuclear export protein (NEP). The antigenically variable structural proteins HA and NA play a crucial role in the development of an AIV vaccine. HA facilitates viral entry by recognizing the sialic acid-containing glycoproteins on the surface of respiratory and intestinal epithelial cells (*Nicholls, 2006; Wan & Perez, 2007; H.*

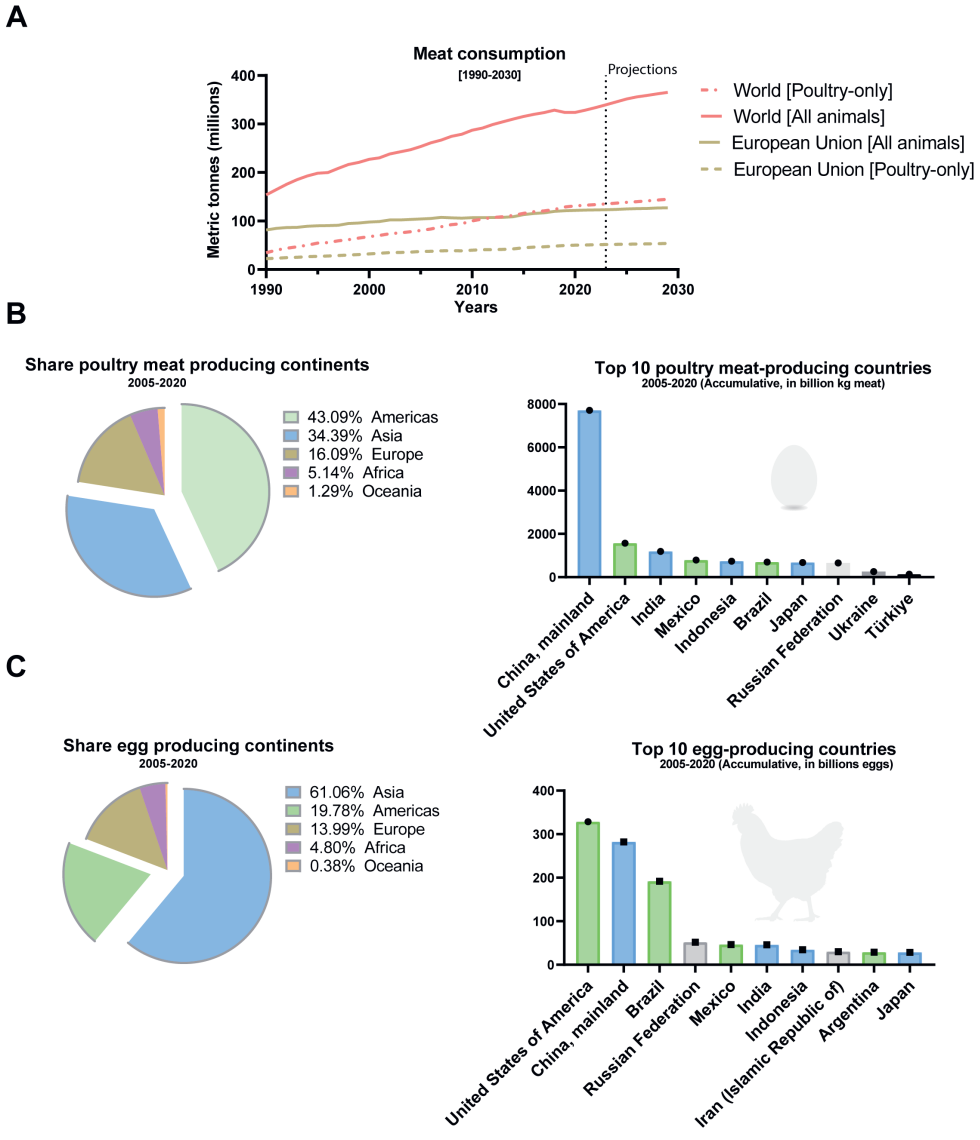

Figure 1. Overview on the global meat consumption and the share of the five continents and top 10 countries in the poultry-meat and egg production. (A) The total and poultry-specific meat consumption (in billion kg) from 1990 to 2023 with a seven-year prognosis taking into account the average population increase worldwide. **(B)** The share of each continent in the production of meat **(left)** and the top 10 countries that produced the most poultry meat (accumulative) in the last 15 years **(right)**. **(C)** The share of each continent in the production of eggs **(left)** and the top 10 countries that produced the most eggs (accumulative) in the last 15 years **(right)**. Data was obtained from the Food and Agriculture Organization (FAO) of the United Nations, accessed on 6 March 2023.

Zeng et al., 2013), while NA is involved in virus egress by cleaving off the sialic acid residues, thereby preventing virus aggregation and re-infection of already infected cells *(Dou et al., 2018*). The substantial variation in the amino acid sequence of these two glycoproteins HA and NA is due to the error-prone replication machinery leading to the accumulation of mutations in the viral genome. These mutations slightly alter the antigenic properties of HA and NA, enabling the virus to escape pre-existing humoral and cellular immune responses (*van de Sandt et al., 2012*). This process of continuous mutation and selection, known as antigenic drift, is mainly responsible for seasonal flu epidemics but may also be involved in the virus crossing species barriers (zoonosis) (*Flaherty, 2012*). Additionally, during a co-infection of two (or more) influenza viruses in the same host cell, a reassortment of influenza's segmented genomes

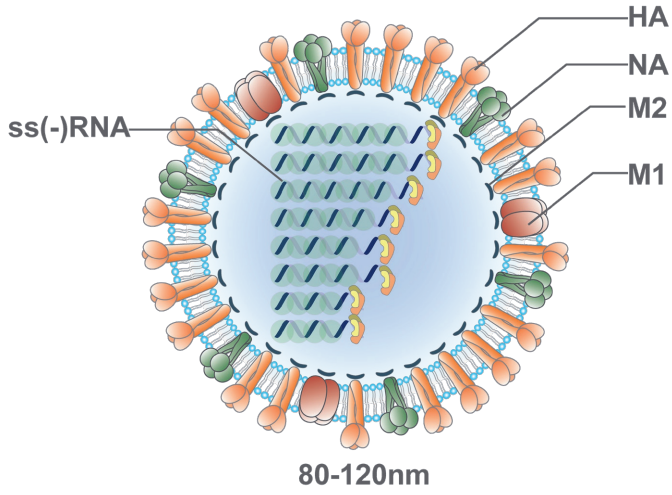

Figure 2. Schematic representation of the avian influenza A virus (AIV). Haemagglutinin protein (HA); neuraminidase protein (NA); nuclear export protein (NEP); nucleocapsid protein (NP); matrix proteins (M1 & M2); RNA polymerase complex consisting of three subunits: PB1, PB2, and PA; negative sense (-), single stranded (ss) RNA.

can occur, resulting in an abrupt change in viral properties. This phenomenon, known as antigenic shift, is the primary cause of flu pandemics (*C. Li & Chen, 2014; Shu et al., 1996*). An example of such a zoonotic event occurred in 1997 when an avian influenza virus (H5N1) successfully crossed species from wild waterbirds to poultry and further to mammals including humans (*Bridges et al., 2002; Koopmans et al., 2004; Peiris, 2009; Y. Yang et al., 2007*). In response, the U.S. Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO) initiated surveillance of influenza A virus infections in 2003. Their monitoring efforts revealed virus spread across 60 wild bird species, the culling of millions of poultry, and the loss of 457 human lives (*World Health Organization, 2023*). These high poultry losses severely impacted the economy of low- and middle income countries such as Latin America or South Asia, which significantly contribute to global poultry production (**Figures 1B & C**). The significant economic impact and the numerous

poultry deaths serve as the primary driver for preventing influenza disease and thus the development of an effective, but most importantly, rapidly adaptable vaccine (*Burns et al., 2009*).

Infectious bronchitis virus

Another economically important virus in poultry is the positive-sense, single-stranded RNA virus named infectious bronchitis virus (IBV) (**Figure 3**). The enveloped IBV virion consists of four virus-specific proteins: spike protein (S), membrane protein (M), envelope protein (E), and nucleoprotein (N). IBV, a member of the *Coronaviridae* family, is a major pathogen primarily affecting chickens but can also cause acute respiratory disease in other domesticated birds such as turkeys, ducks, and waterfowls (*Cavanagh, 2007*). Infections with IBV negatively impact the egg-laying performance of layers and meat production of broilers (*Awad et al., 2014*). In these hosts, the immune responses against IBV are primarily directed toward the spike (S) glycoprotein as the major antigen for the induction of neutralizing antibodies (*Du et al., 2021; Eldemery et al., 2017*). Like the influenza HA protein, the coronavirus S protein is a class I fusion protein that recognizes receptors specifically located in the upper respiratory tract (*Rahman et al., 2009*). Co-infections with IBV pose a high risk of recombination, leading to new viral variants, as observed for human-infecting coronaviruses (*Mardani et al., 2010; Xu et al., 2019*). Similar to avian influenza, the genetic variability of IBV serotypes originates from the error-prone replication machinery (*Boni et al., 2020; Lai & Cavanagh, 1997; Moreno et al., 2017*).

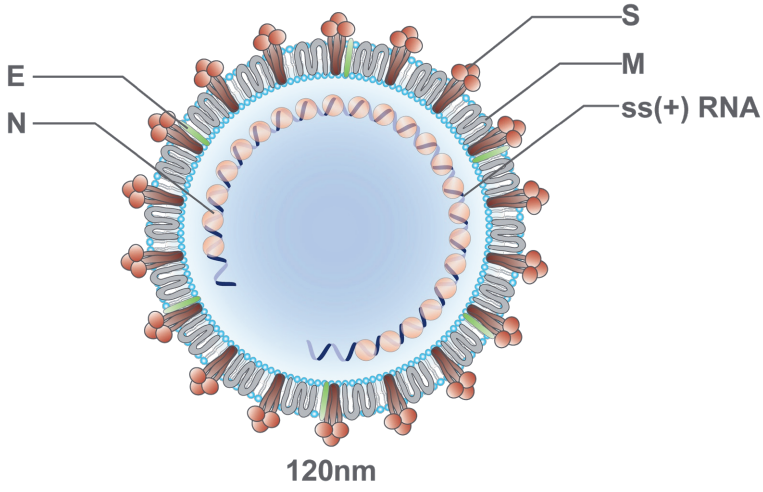

Figure 3. Schematic representation of the infectious bronchitis virus (IBV). Envelope protein (E); nucleocapsid protein (N); spike protein (S); membrane protein (M); positive-sense (+), single-stranded (ss) RNA.

Infectious bursal disease virus

Infectious bursal disease virus (IBDV), also known as the Gumboro disease virus, exemplifies a non-respiratory pathogen with a large economic impact on the poultry industry (*van den Berg et al., 2000*). This virus, belonging to the *Birnaviridae* family, has a double-stranded, segmented

RNA genome encoding the five major viral proteins: the RNA-dependent RNA polymerase (VP1), structural proteins (VP2 and VP3), viral protease (VP4) and viral release protein (VP5) (*Tacken et al., 2002; Y. Wu et al., 2009*). The typical adaptive immune response against IBDV targets the major antigenic determinant VP2 and to a lesser extent VP3 (*Palka et al., 2021*) (**Figure 4**). IBDV can be classified into two serogroups with serotype I, being pathogenic to chickens (species*: Gallus gallus*) whereas serotype II is non-pathogenic (*Ismail et al., 1988*). Within serotype I, IBDV strains can be further subdivided based on the clinical outcome of the disease into classical (attenuated virulent, mild virulent, or virulent), variant, and very virulent strains (*Pikuła et al., 2018; Pikuła & Lisowska, 2022*). Classical strains originate from the first emergence of IBDV near the town of Gumboro (United States) in 1957. Variant strains originate from an antigenic variant that escaped the immune protection against classical IBDV strains and emerged in the late 1980s (*Jackwood & Saif, 1987*). As the name suggests, the very virulent strain can cause up to 100% mortality in chickens, while the classical or variant strains cause mortality in only 0-15% of the infections and are therefore categorized as non- or low-virulent (*Brandt et al., 2001; Ignjatovic et al., 2004*). Most of the variation in clinical outcomes of serotype I strains is due to the high mutation rate of the replication machinery. Similar to AIV, co-infections can lead to the generation of new IBDV variants through genomic segment shuffling (*Pikuła et al., 2018*). Although non-virulent IBDV strains do not cause high mortality rates, infected birds display clear immunosuppressive phenotypes. These compromised birds show an increased risk of secondary viral or bacterial infections and exhibit reduced responsiveness to subsequent vaccinations such as against IBV and AIV (*Pejkovski et al., 1979; Spackman et al., 2017*).

Conventional poultry vaccines

In light of the socio-economic impact of viral outbreaks in the modern poultry industry, the reduction of morbidity and mortality caused by viral and bacterial pathogens is prioritized. The implementation of a vaccination regime, with an average of 15-20 vaccine administrations per chicken during their production cycle (*Cserep, 2008*), has proven to be an effective control measure for many global viral diseases such AIV, IBV, and IBDV (*Abdul-Cader et al., 2018; Cserep, 2008*). The use of vaccines in the poultry industry dates back to 1929, when the first United States Department of Agriculture (USDA)-licensed fowl pox vaccine was introduced (*Espeseth & Lasher, 2010*). However, current strategies extend beyond vaccination alone and include preventive measures such as minimizing the contact between poultry and humans, wild birds, or insects, as well as ensuring adequate hygiene measures. Integrating these preventive measures can pose challenges and be costly for large poultry farms. Additionally, the current regulation mandated by the European Union (EU) prohibits the export of poultry to other non-EU countries due to the high risk of AIV in poultry products, despite the availability of protective avian influenza vaccines (*Cardador et al., 2019; Johnson, 2015*). Altogether, this stresses the importance of an effective and globally implemented vaccination strategy in the poultry industry. Throughout the years, several types of vaccines have been developed and registered for the immunization of poultry, such as live-attenuated virus (LAV) vaccines, inactivated virus vaccines, subunit vaccines, immunocomplex vaccines, and recombinant-vectored (RV) vaccines (**Figure 5**) (*Bhuiyan et al., 2021; de Wit, Sjaak, 2011; Müller et al., 2012*).

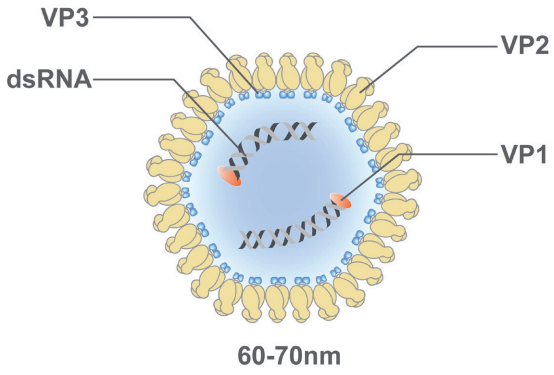

Figure 4. Schematic representation of the Infectious Bursal Disease virus (IBDV). RNA-dependent-RNA polymerase (VP1), viral capsid proteins (VP2 and VP3) , double-stranded RNA (dsRNA).

The traditional market for poultry vaccination focuses predominantly on inactivated virus vaccines or LAVs due to their low cost-of-goods and straightforward developmental process. However, the inactivated virus vaccines require a relatively high dose of inactivated viral antigens to elicit proper protection in poultry. Since inactivated vaccines do not replicate or spread, the presence of an adjuvant or the administration of a second dose might be required to confer protective immunity to hosts, whereas LAV vaccines often do not require adjuvants. LAVs, whether naturally or intentionally attenuated, retain the ability to replicate within the host and can provide protective immunity after a single dose (*S. Chen et al., 2021; Jang et al., 2018*). Nonetheless, the use of attenuated viruses raises safety concerns due to the replicative nature and the generation of viral progeny. Deviating from the suggested vaccine regimen by lowering the vaccine dose or changing the delivery methods can result in vaccine strains circulating for prolonged periods in the vaccinated flock, posing significant safety risks (*Blacker et al., 2011*). For instance, the production of IBV vaccine lots resulted in viral quasi-species as a result of the error-prone replication of IBV. These quasi-species can still proliferate within the host, increasing the risk of the escape of a more fit viral variant and compromising vaccine effectiveness (*Legnardi et al., 2020; McKinley et al., 2008*). Similarly, in the case of IBDV, early vaccination strategies involving LAVs have demonstrated that, depending on the selected vaccine strain, different degrees of attenuation were observed, causing persistent immunosuppressive side effects in chickens and affecting subsequent heterologous immunizations (*Müller et al., 2012*). Given the increased viral fitness or adverse side effects, the use of specific LAVs requires thorough evaluation. More recently, there has been a shift towards RV vaccines advanced in the poultry industry. Currently, the marketed RV vaccines are based on the herpesvirus of turkeys (HVT; *Herpesviridae* family) or Fowlpox virus (FPV, *Poxviridae* family). These vectors can be modified to express up to two heterologous viral antigens making them feasible for multivalent vaccine formulation. It has been demonstrated that the F-gene of Newcastle disease virus (NDV; *Paramyxoviridae* family), the VP2-gene of IBDV, the HA-gene of AIV, as well as the glycoprotein (g) of avian

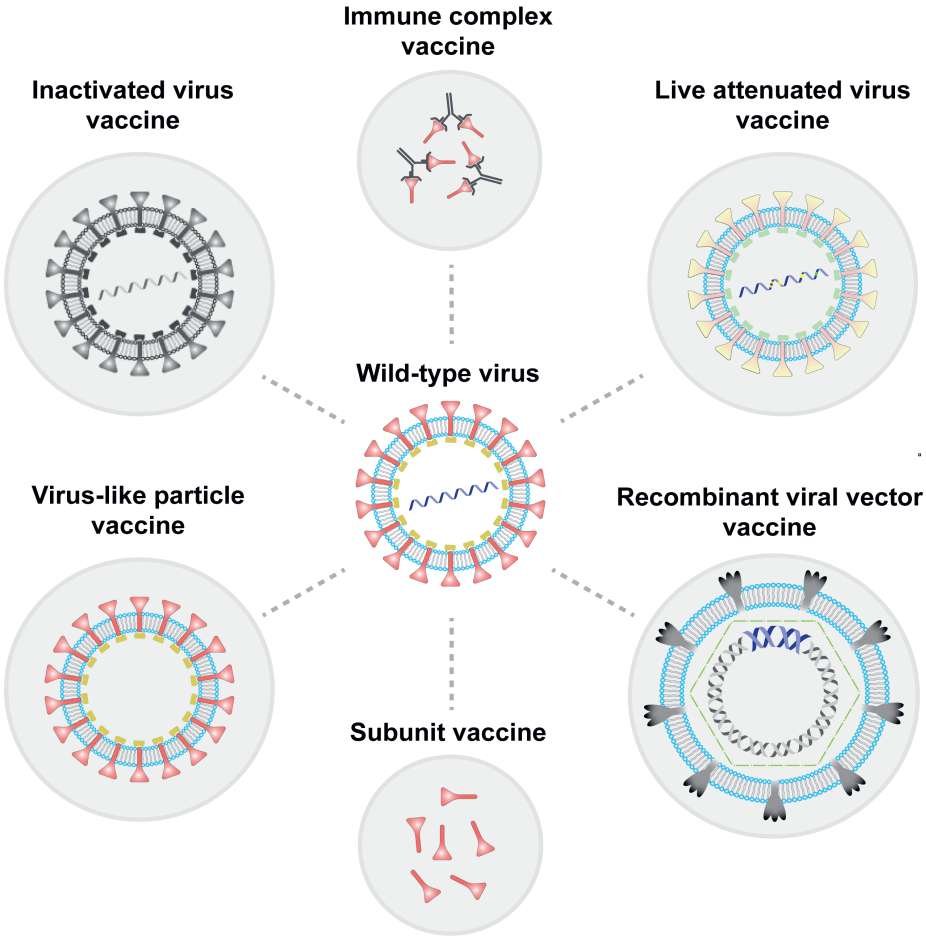

Figure 5. Overview of current vaccines used in the poultry industry. An immune complex vaccine is a combined vaccine that consists of a subunit, inactivated, or live attenuated virus vaccine coated with neutralizing antibodies. An attenuated virus vaccine is a live virus with reduced virulence and disease-causing capabilities. A recombinant viral vector vaccine is a live virus that is genetically engineered to express additional genes from a heterologous pathogen. A subunit vaccine contains (parts of) purified antigenic proteins of the wildtype virus. A virus-like particle vaccine mimics the wildtype virus' outer shell but is not infectious. An inactivated virus vaccine is produced by chemical or physical inactivation of the wildtype virus (*Pollard et al., 2021*).

infectious laryngotracheitis virus (ILTV; *Orthoherpesviridae* family) could be introduced into RV vaccines and subsequently be used in the protecting against the corresponding pathogens (*Soejoedono et al., 2012; Vagnozzi et al., 2012; van Hulten et al., 2021*). Moreover, RV played a large role in reshaping the understanding of the correlates of protection (COP) in poultry (*Suarez & Pantin-Jackwood, 2017*). Traditionally, the COP in poultry was generally defined by the presence of a high neutralizing antibody (nAb) titer against the antigen of interest. However, increased insights and understanding of the cellular immune system together with

the development of cell-based assays, highlighted the importance of the different aspects of the immune system. RV vaccines demonstrated that despite the generally lower nAb titer, it could still protect against disease, and a reduction in viral shedding was observed in poultry (*Palya et al., 2012; Suarez & Pantin-Jackwood, 2017*). As expected, the induction of both humoral and cellular immunity played a significant role in this and contributed to a renewed view of the major COP in viral poultry diseases (*Ingrao et al., 2017, 2018; van Hulten et al., 2021*). A major drawback of the HVT RV vaccines is the laborious construction of the viral vectors. For example, a homologous recombination event within an HVT-adapted cell line is required to introduce the foreign viral transgenes into the HVT backbone. This tedious process typically depends on a random recombination event and requires additional verification of the constructed vector. Moreover, herpes-based RV vaccines result in persistent infection, thereby hindering the repeated use of the same type of vaccine in earlier immunized individuals. If a booster vaccination is required, a non-HVT-based vaccine should therefore be administered. A brief overview of the currently authorized poultry vaccines in Europe against viral infectious diseases is listed in **Table 1**.

Table 1. Overview of the approved poultry vaccines in the European Union protecting against IBV, AIV or IBDV. i.m.: intramuscular, s.c.: subcutaneous. Data was obtained from the European Medicines Agency (EMA) accessed on 22 February 2023

Type of vaccine	Licensed product (example)	Manufacturer	Antigen	Administration route	Reference
Inactivated whole virus vaccine	Nobilis® Influenza H5N2	MSD Animal Health	• AIV	• i.m. • s.c.	Philippa et al., 2007 Ellis et al., 2004
(Attenuated) live virus vaccine	Nobilis® IB Primo QX	MSD Animal Health	• IBV	• Drink water • Spray	Laconi et al., 2020
	Nobilis® IB 4-91	MSD Animal Health	• IBV	• Drink water • Spray • Eye-drop	Terregino et al., 2008
	Gumbohatch®	HIPRA	• IBDV	• *in ovo* • s.c.	no data available
Recombinant virus vector vaccine	Vaxxitek® HVT+IBD	Boehringer Ingelheim Animal Health	• MDV • IBDV	• *in ovo* • s.c.	Le Gros et al., 2009 Rautenschlein et al., 2009
	Innovax® ND-IBD	MSD Animal Health	• MDV • NDV • IBDV	• *in ovo* • s.c.	Ferreira et al., 2020 van Hulten et al., 2021b
	Ultifend™ ND IBD	Ceva Veterinary Biologicals	• MDV • NDV • IBDV	• *in ovo* • s.c.	no data available

Nucleic acid-based vaccines represent a newer class of vaccines that have not yet been widely adopted for poultry vaccination. While the EMA provided full market authorization for a single nucleic acid vaccine for use in Atlantic salmon (*Dalmo, 2018*), no other veterinary animals in the EU are currently vaccinated using nucleic acid-based vaccines. These innovative vaccines encode the target antigen via synthetic genetic material such as DNA (plasmid) or messenger RNA (mRNA) (*F. Qin et al., 2021*). Upon administration of these vaccines, the genetic material is delivered to the host cells, instructing them to produce the desired protein(s). Although nucleic vaccines are an emerging technology, the ongoing coronavirus disease 2019 (COVID-19) pandemic has revolutionized the licensing procedure for mRNA vaccines, making them commercially attractive. As a result, the second marketed COVID-19 vaccine was based on nucleoside-modified mRNA encoding the full-length, pre-fusion stabilized Spike protein of the SARS-CoV-2 virus formulated in lipid nanoparticles (LNPs) (*Abu-Raddad et al., 2021; Bar-On et al., 2021; Hall et al., 2021; Lamb, 2021*). These mRNA vaccines relied on a complete synthetic manufacturing process that could be rapidly mass-produced for the first clinical trial. This resulted in the first Food and Drug Administration (FDA)-approved mRNA vaccine for human use in 2021, eight months after the initial trials (*P. Ball, 2021*). The success of mRNA vaccine technology in responding to newly emerging viruses with pandemic potential within 65 days after the online access to the genetic sequence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2; family *Coronaviridae*) (*Scudellari, 2020*), resulted in a positive outlook for repurposing mRNA-LNP vaccine technology for use in the veterinary sector (*Le et al., 2022; Verbeke et al., 2021*). However, it should be noted that multiple intramuscular booster administrations required by current coronavirus mRNA vaccines in humans are not favorable in the poultry industry due to the relatively high costs per dose and the labor-intensive nature of vaccination. Fortunately, a potential new mRNA vaccine technology based on self-amplifying mRNA, the so-called replicons, holds promise for overcoming these challenges. Replicon vaccines provide prolonged expression upon single dose administration making them highly valuable for the poultry sector.

Replicon vaccines

Replicons are commonly, but not exclusively, derived from positive-strand RNA virus genomes belonging to the *Togaviridae* or *Flaviviridae* family *(Comes, Pijlman, et al., 2023; Hikke & Pijlman, 2017*). Wildtype RNA viruses, encode proteins that facilitate the replication and encapsidation of the viral genome. In the case of replicons, the structural genes are replaced with a gene of interest while the nonstructural genes, so-called self-amplifying genes, are maintained. This enables the replicon RNA to be amplified within the host cells and the production of high levels of heterologous protein, without the ability to form an infectious virus (**Figure 6**). Replicon technology offers several advantages. Firstly, replicon RNA, similar to mRNA, can be entirely manufactured synthetically. Moreover, since replicon RNAs are unable to produce viral progeny, a low biocontainment applies making replicon RNA safe-by-design. Lastly, the target antigen can be easily synthesized and inserted in the replicon backbone, making it a "plug-and-play" system. One well-established example of a replicon system is based on the alphavirus Venezuelan equine encephalitis virus (VEEV). VEEV is a positive-sense, single-

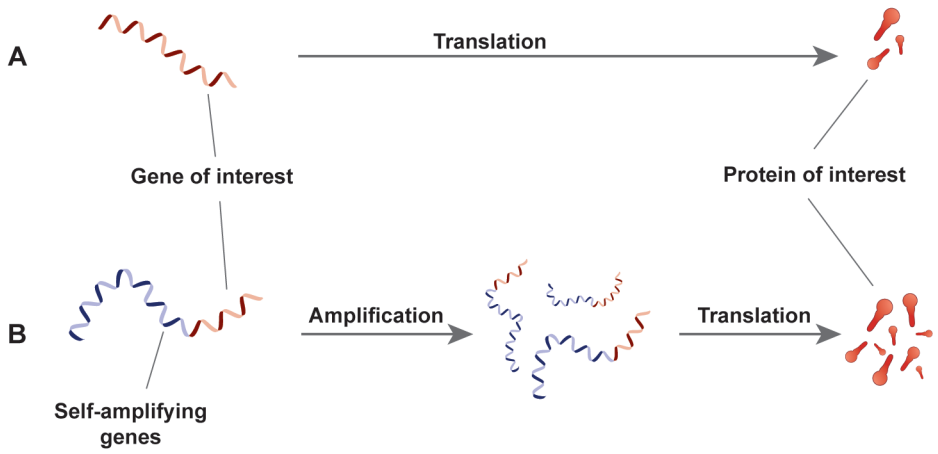

Figure 6. Comparison of (A) conventional mRNA and (B) self-amplifying mRNA vaccines for transgene delivery. Upon administration to the host, the conventional mRNA vaccine, encoding for the gene of interest, is translated into the protein of interest. In contrast, self-amplifying mRNA encodes not only the gene of interest but also the essential genes for self-amplification of the replicon RNA. Consequently, translation of the self-amplifying mRNA leads to elevated proteins of interest levels within the target host when compared to conventional mRNA.

strand RNA virus that can infect various vertebrate hosts (*Azar et al., 2020*). The alphavirus genome encodes four nonstructural proteins (nsP) and five structural proteins under the control of a separate, subgenomic promoter (26S) (**Figure 6A**), which, when working with replicons, are replaced with the gene of interest. Upon delivery to the cell, the presence of the 26S promoter in the alphavirus replicon allows for very high levels of transgene expression (*Liljeström & Garoff, 1991; Pushko et al., 1997*). To facilitate the delivery of replicon RNA to the host, three approaches are often described: The first approach involves the administration of naked replicon RNA by means of injection or electroporation. However, this method is hindered by low delivery efficiency and is susceptible to RNA degradation (*Huysmans et al., 2019*). Another approach is the use of non-viral carriers such as synthetically-formulated lipid nanoparticles (LNPs). LNPs have unique properties including target-specific delivery, increased drug retention, and adjuvating effects that can boost vaccine efficacy. Despite being novel and costly, this entirely synthetic delivery approach holds great promise for future veterinary vaccines (*Hou et al., 2021; Pollock et al., 2022*). The third approach involves the use of virus-like replicon particles (VRPs). This extensively-studied viral carrier mimics the wildtype virus outer shell and follows the normal route of infection of a wildtype virus. VRPs are produced by in trans delivery of the replicon mRNA and the structural (helpers) genes to the same cell. Alphavirus helper constructs are typically delivered in a split-helper manner to reduce the risk of recombination that may otherwise yield replication-competent viruses (*Kamrud et al., 2010*) (**Figure 6A**). VRPs have been the subject of many promising studies in the protection against various infectious diseases as well as in the field of oncology in a wide range of animal species (*Hubby et al., 2007; Slovin et al., 2013; Wecker et al., 2012*). However, for poultry, the rapid onset and high level of transgene expression achieved using the VEEV replicon

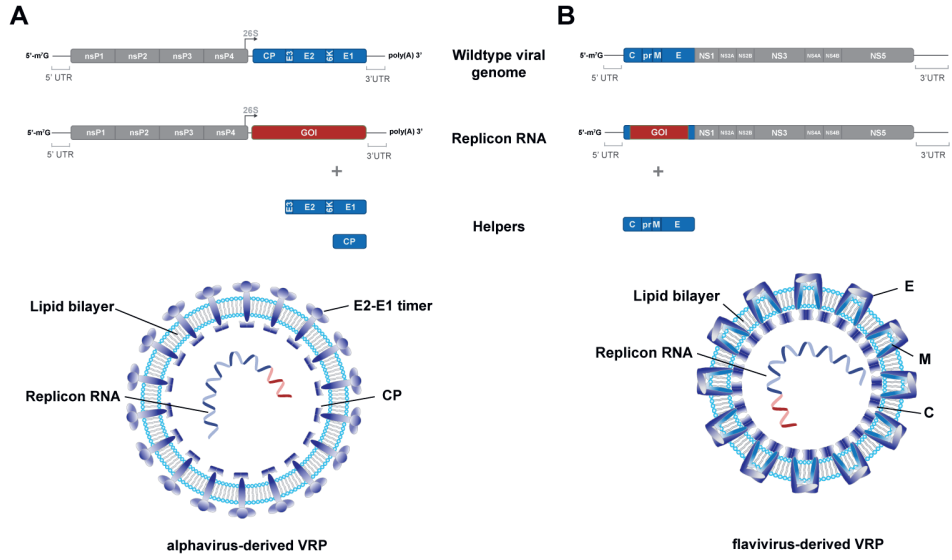

Figure 7. Schematic overview of the wildtype viral genome and replicon organization of alpha- and flaviviruses. (A) a wildtype alphavirus genome encoding for the viral nonstructural proteins (nsP1-4) and a subgenomic 26S promoter controlling a separate open reading frame (ORF). This ORF carries the viral structural gene cassette encoding for the capsid (CP) and envelope glycoproteins (E3, E2, E2, 6K, and E1). For the design of an alphavirus-derived replicon, the structural gene cassette is replaced by a gene of interest (GOI). For the production of virus-like replicon particles (VRPs), capsid, and the viral envelope genes are co-delivered on separate helpers to a production cell line. **(B)** A wildtype flavivirus genome encoding a single polyprotein encoding the viral structural proteins (C-prM-E) and nonstructural proteins (NS1-5). For the design of a flavivirus-derived replicon, the structural gene cassette is substituted by an in-frame placement of the GOI. For the production of flavivirus-derived VRPs, the structural gene cassette is in-trans supplemented to a production cell line.

system did not induce the required protective immunity (*Schultz-Cherry et al., 2000; Sylte et al., 2007*), emphasizing the need for an alternative replicon platform specifically for poultry.

Tembusu (TMUV) virus-based replicon platform

Tembusu virus (TMUV), a member of the *Flaviviridae* family, emerged during a significant outbreak of an unidentified infectious disease in 2010, affecting predominantly ducks within the Chinese poultry industry. This outbreak had a profound economic impact, causing a sharp decline in egg production, high fever, and retarded growth in newly hatched ducklings, affecting over 10 million ducks (*P. Yan et al., 2011*). Later the causing agent was identified as a novel duck-infecting TMUV virus. TMUV was first isolated from *Culex tritaeniorhynchus* mosquitoes in 1955 in Malaysia, which likely serves as a vector for viral transmission. Afterward, cases of TMUV were sporadically reported in various locations across Southeast Asia, not only affecting ducks but also other avian species, including chickens, geese, sparrows, and pigeons (*Cui et al., 2022; Hamel et al., 2021; Lei et al., 2017*). Although other members of the *Flaviviridae* family such as yellow fever virus (YFV), dengue virus (DENV), West Nile virus (WNV), and Japanese

encephalitis virus (JEV) are known to cause disease in humans, no reports of pathology in humans or other mammals have been documented so far, probably due to the high sensitivity of this virus to mammalian interferon (*Ruangrung et al., 2021; Y. Tang et al., 2013; J. Wang et al., 2016*). TMUV has an 11-kb, single-stranded, positive-sense RNA genome with a ^{7}G-methylated cap at its 5'end and highly structured 5' and 3' untranslated regions. The genome translates into a polyprotein that is processed into seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) and three structural proteins (C, prM, and E; **Figure 7B**). Although alphaviruses can infect a wide range of mammalian and bird species (*Reeves et al., 1958; Weaver et al., 2004*), the natural tropism of TMUV to exclusively infect avian hosts allows for specialized and host-specific anti-viral responses necessary to induce a proper immune response in poultry. Previous research has already considered duck-derived TMUV strain CQW1 as a valuable tool for the investigation of novel TMUV outbreaks and developed a TUMV-based replicon system (*He et al., 2019; X. Wang et al., 2021*). However, the feasibility of a TMUV-based replicon system as an alternative vaccine platform in poultry has never been assessed before.

Scope of this thesis

This thesis focuses on the construction and evaluation of an avian-adapted replicon system as a safe and effective vaccine platform for protecting poultry against avian infectious diseases, including AIV, IBDV, and IBV.

Chapter II lays the foundation for a novel replicon platform by constructing an infectious clone of the TMUV WU2016 strain using a reverse genetics approach. Next, the propagation of this TMUV infectious clone is studied in cell culture and compared to the well-studied Malaysian TMUV isolate (MM1775). Moreover, the TMUV replicon is adapted to enable the expression of a broad-range of reporter and (viral) transgenes, and the duration of expression is analyzed *in vitro*.

In **Chapter III** the performance of the newly constructed TMUV replicon is compared to the benchmark VEEV replicon. Furthermore, two new TMUV replicon capsid deletion variants are generated to investigate *in vitro* how the timing and transgene expression levels can be modulated.

In **Chapter IV** a DNA-encoded replicon RNA (DREP) based on the TMUV isolate Perak virus is constructed. The expression kinetics of the TMUV DREP is demonstrated in both mammalian and avian cells. To confirm the application of both TMUV replicon RNA and DREP, an AIV and IBD vaccination trial of the TMUV replicon and the capsid mutants is conducted in comparison to the benchmark VEEV replicon for which the self-amplifying RNA was formulated in LNPs. After testing this non-viral carrier, research continued in developing a TMUV-based viral carrier to encapsidate the replicon RNA.

As such, in **Chapter V** a wide range of (in)vertebrate cells are screened to find a suitable packaging cell line.Ultimately, the mammalian cell line HEK293T is selected for the future production of TMUV VRPs. The TMUV structural gene cassette under the control of a tetracycline-inducible promoter is introduced in the HEK293T genome via a lentiviral

transduction system. A monoclonal packaging cell line is then generated and further evaluated in relation to the replicon packaging efficiency.

Regarding RNA vaccines, the occurrence of the COVID-19 pandemic accelerated the development of all facets of nucleic acid vaccines. An overview of novel vaccine delivery strategies, possibilities for multivalent vaccine formulation, and administration routes, in combination with a thorough safety evaluation of self-amplifying RNAs beyond poultry is provided in **Chapter VI.**

Finally, all the findings presented throughout this thesis are discussed in a broader context in **Chapter VII** taking into account the latest advancements in the field of self-amplifying RNA, mucosal vaccination, and humoral and cellular immunity.

I

Chapter 2

Infectious clone of a contemporary Tembusu virus and replicons expressing reporter genes or heterologous antigens from poultry viruses

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Abstract

The novel mosquito-borne Tembusu virus (TMUV, family *Flaviviridae*) was discovered as the cause of a severe outbreak of egg-drop syndrome affecting ducks in Southeast Asia in 2010. TMUV infection can also lead to high mortality in various additional avian species such as geese, pigeons, and chickens. This study describes the construction of an infectious cDNA clone of a contemporary duck-isolate (TMUV WU2016). The virus recovered after transfection of BHK-21 cells shows enhanced virus replication compared to the mosquito-derived MM1775 strain. Next, the WU2016 cDNA clone was modified to create a SP6-driven, self-amplifying mRNA (replicon) capable of expressing a range of different reporter genes (Renilla luciferase, mScarlet, mCherry, and GFP) and viral (glyco)proteins of avian influenza virus (AIV; family *Orthomyxoviridae*), infectious bursal disease virus (IDBV; family *Bunyaviridae*) and infectious bronchitis virus (IBV; family *Coronaviridae*). The current study demonstrates the flexibility of the TMUV replicon system, to produce different heterologous proteins over an extended period of time and its potential use as a platform technology for novel poultry vaccines.

1. Introduction

Recombinant viral vectors are very powerful tools for gene delivery into eukaryotic cells. These vectors are often applied as human or veterinary vaccine platforms and used in the gene therapy field. The different strategies developed over the years have mostly focused on plasmid-based expression vectors, or large dsDNA viruses such as adeno-, pox- or herpesviruses. More recently, positive-stranded RNA viruses also gained interest for the application of these viruses as heterologous gene expression platforms (*Ballesteros-Briones et al., 2020; Lundstrom, 2021; Spencer et al., 2021*). The RNA of positive-stranded RNA viruses serves as a messenger RNA (mRNA) that can be directly translated into transfected cells. Several members of the *Togaviridae* family such as Venezuelan equine encephalitis virus (VEEV) and Semliki Forest virus (SFV) and of the *Flaviviridae* family such as West Nile virus strain, Kunjin (KUNV) have been engineered as efficient viral 'replicon' vectors for high-level transgene expression for applications in gene therapy, recombinant protein production or as vaccine platform technology against emerging viral diseases (*de Alwis et al., 2021; Hoang-Le et al., 2008; Khromykh et al., 1998; Komdeur et al., 2021; Pijlman et al., 2006*). By removing the structural and maintaining the nonstructural genes, efficient RNA replication and translation from the replicon is achieved without the formation of infectious viral progeny. Tembusu virus (TMUV) is an emerging epornitic flavivirus that was initially isolated from *Culex tritaeniorhynchus* vector mosquitoes in 1955 in Malaysia (*W. Zhang et al., 2017*). TMUV remained obscure until the major outbreak of the disease in ducks in 2010 in China (*Cao et al., 2011*), which had a significant economic impact on the poultry industry. Since then, TMUV has been widely detected in other avian species such as chickens, pigeons, and sparrows (*Benzarti et al., 2019*). Some studies have reported TMUV-specific antibodies in humans (*Pulmanausahakul et al., 2022; Y. Tang et al., 2013*), but flavivirus serodiagnosis is complicated due to extensive cross-reactivity with other species within the Flavivirus genus (*Chan et al., 2022; Endale et al., 2021*). Experimental infections suggest that TMUV is highly sensitive to interferon and cannot replicate well in primates (*H.-J. Wang et al., 2016*). The TMUV genome is approximately 11 kb in size and encodes a single polyprotein that is post-translationally processed into the three structural proteins capsid (C), premembrane (prM), and envelope (E) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) involved in viral replication and modulation of host responses. The nonstructural proteins NS3 (helicase) and NS5 (RNA-dependent RNA polymerase) are part of the replication complex responsible for the synthesis of the double-stranded (ds) RNA replication intermediate and positive-sense viral progeny RNA. Other functions of the nonstructural proteins include the proteolytical processing of the polyprotein by the protease NS2B-NS3, capping of the viral RNA genome by the methyltransferase activity of NS5, and interfering with the host interferon pathway (*J. Wang et al., 2016; W. Zhang et al., 2020; P. Zhou et al., 2022*). The structural proteins C, prM, and E are involved in the packaging of the viral RNA genome into virions. Although many of the TMUV virus-encoded proteins functions have been studied to various extents (*He et al., 2019, 2021*), the use of the TMUV replicon system for the expression of heterologous virus antigens has not been described. To obtain a better understanding of the replication process of TMUV and investigate the

possibilities for transgene expression, this paper shows a reverse genetic approach to generate a full-length cDNA clone and a series of SP6-driven non-infectious subgenomic replicons of the contemporary TMUV isolate WU2016. Furthermore, this study demonstrates the long-lasting expression of reporter genes and the production of poultry-specific viral antigens by the TMUV replicon in BHK-21 cells.

2. Material and methods

Construction of the TMUV WU2016 infectious cDNA clone

Viral RNA was extracted by using TRIzol reagent (Invitrogen, catalog number (*CN*) :*15596026*) and reverse transcribed by SuperScript II (Invitrogen) using random hexanucleotides. PCR primers were designed to create overlapping subgenomic fragments (F1 to F6, **Figure 1A**) that were subsequently cloned into pJET1.2 and sequenced by Sanger sequencing (Macrogen). Primer sequences were based on the closely related DF-2 DTMUV isolate (GenBank: KJ489355.1) (**Table 1**). The individual fragments were designed to allow for the assembly of the TMUV WU2016 cDNA clone using restriction cloning. F1 was extended by inserting an SP6 promoter sequence upstream of the 5' untranslated region (UTR) to allow for *in vitro* RNA transcription of the viral genome. F6 was extended by including a hepatitis delta virus (HDV) ribozyme after the last nt of the 3'UTR to ensure a native 3' terminus and the *PacI* restriction site (primer 13 & 14, **Table 1**). The copy-control pCC1BAC vector (Epicentre Biotechnologies, *CN: V008675*) (*Göertz et al., 2019*) was used for the assembly of the individual fragments via ligation cloning with *EcoRI* and *AatII* (Fragment 1), *AatII* and *NheI* (fragment 2), *NheI* and *BbvCI* (fragment 3), *BbvCI* and *NsiI* (Fragment 4), *NsiI* and *BamHI* (fragment 5) and *BamHI* and *PacI* (fragment 6). The TMUV WU2016 infectious cDNA clone or intermediates were transformed into copy-control EPI300 *E.coli* (Lucigen, *CN:C300C105*) and induced using CopyControl Solution (Lucigen, CN:C300C105). DNA was isolated using the Nucleobond Xtra Midi Kit (Macherey-Nagel, *CN: MN740412*) and checked for quantity and quality using the spectrophotometer ND-1000 (Nanodrop). The complete sequence was confirmed by Sanger sequencing of intermediate clones and by Oxford Nanopore sequencing of the entire cDNA clone (Plasmidsaurus) and submitted to GenBank (accession no. OQ920272).

Construction of TMUV subgenomic replicons expressing reporter genes and heterologous poultry virus antigens

A synthetic DNA fragment was designed containing the SP6 promoter preceding the 5'UTR and the first 60 nt of the coding region of the capsid (C) gene required for translation initiation and cyclization of the viral genome (*Khromykh & Westaway, 1997*). The last 22 amino acids of the envelope (E) protein, nonstructural gene cassette, 3'UTR, and HDVr were amplified using PCR (primer 14 & 15, **Table 1**) from the TMUV WU2016 cDNA and ligated to the synthetic DNA fragment (**Figure 1A**). The unique restriction sites *AscI* and *AvrII* were added for insertion of the reporter genes; GFP, mScarlet, mCherry, and Renilla luciferase (Rluc) into the subgenomic

Table 1. List and sequence of primers used for the TMUV WU2016 infectious clone and replicon construction.

No.	Fragment	Sequence (5' - 3')
1	SP6-F1-fw	atttaggtgacactatagagaagttcatctgtgtgaact
2	F1-rv	agcataagttgccttggg
3	F2-fw	tcgaccaaagccactaaatatc
4	F2-rv	tgctgctgtcatcaaactg
5	F3-fw	ctgagagccgtgtttgaag
6	F3-rv	tccgactatctatgacccg
7	F4-fw	gacaaagaaggacaggtg
8	F4-rv	cctcaaggtctggaacatct
9	F5-fw	agctacaacatttctgactcc
10	F5-rv	atggctgacaacctgttc
11	F6-fw	atcgtggcaagatggatg
12	F6-rv	agactctgtgttctaccacc
13	F6-ext-fw*	caagctgtaactctaggggaa
14	F6-ext-rv*	agaaagatgcggcccttaattaaac
15	E22-fw	ttggcgcgccaacgcctaggctccatttctatgacttttctagcc

*used to extend the F6 amplicon with HDVr element and *PacI* site

replicon. The reporter genes were inserted between the remaining capsid gene and the retained 66 nt of the E gene that encodes the signal sequence to ensure translocation into the endoplasmic reticulum of the downstream NS1 protein. The reporter genes were followed by the sequence for the ribosomal skipping element 2A of the foot-and-mouth disease virus (FMDV). The construct TMUVrep-HA was obtained by inserting the codon-optimized hemagglutinin (HA) high pathogenic avian influenza A virus (AIV; *Orthomyxoviridae* family) H5N1 A/turkey/Turkey/01/05) gene isolated from an expression plasmid kindly provided by MSD Animal Health. For the TMUVrep-Spike construct, the spike (S) protein of infectious bronchitis virus (IBV; *Coronaviridae* family) strain Ma5 (Serotype Massachusetts) was obtained as a synthetic gene fragment (GeneArt, Thermo Fisher Scientific). For the TMUVrep-pVP2 construct, the precursor VP2 (pVP2) gene of infectious bursal disease virus (IBDV; *Birnaviridae* family) strain Faragher 52/70 was isolated using high-fidelity PCR amplification with Q5 High-Fidelity DNA Polymerase (NEB, *CN: M0491L*) from a template provided by MSD Animal Health (Boxmeer) and sequence verified using sanger sequencing (Macrogen). All the viral antigens were inserted into the compatible *AscI*/*AvrII* restriction sites of the TMUVrep construct.

In vitro RNA transcription and RNA purification

Plasmids were purified using the endotoxin-free Nucleobond Midiprep kit (Macherey-Nagel, *CN: MN740412*) and linearized using *PacI*. Capped, *in vitro* transcribed viral or

replicon RNA was synthesized using SP6 polymerase (New England Biolabs, *CN: M0207S*) and 500 ng of the unpurified linearized plasmid DNA in a total volume of 40 µL. The total reaction mixture was incubated for 2 h at 37°C and afterward treated for 30 min at 37°C with RNAse-free DNAse (Promega, *CN: M6101*). The RNA was purified using the RNeasy Micro kit (Qiagen, *CN: 74004*) according to the manufacturer's instructions. Both the quantity and quality of the *in vitro* transcribed RNA were analyzed using the spectrophotometer ND-1000 (Nanodrop) and via conventional electrophoresis using a 1% agarose gel in tris-acetate-EDTA (TAE) buffer for 15 min at 150 V.

Cells and virus preparation

Baby hamster kidney cells (BHK-21; clone 13, ECACC 85011433) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, *CN: 41966052*) supplemented with 10% fetal bovine serum (FBS; Gibco, *CN:10270106*) and 100 $U \cdot mL^{-1}$ penicillin-streptomycin (Gibco, *CN: 15140122*). Cells were cultured at 37°C under 5% CO_2. A sub-confluent 6-well plate containing BHK-21 cells was transfected with approximately 10 micrograms *in vitro* transcribed RNA of the TMUV WU2016 infectious clone using Lipofectamine 2000 (Invitrogen, *CN: 11668019*). The supernatant of the transfected cells was used to infect healthy BHK-21 cells for the generation of the passage 1 (P1) virus stock. P5 virus stocks of TMUV MM1775 (Genbank accession no. JX477685; EVAg Ref-SKU 001v-EVA135) were propagated on BHK-21 cells. The viral titer was determined on chicken embryonic fibroblast (DF-1) cells using the end-point dilution assay (EPDA) and expressed as median tissue culture infectious dose per milliliter ($TCID_{50} \cdot mL^{-1}$) according to the Reed–Muench method. Virus samples were 10-fold serially diluted, mixed with DF-1 cells, and distributed on 60-well microtiter plates. The plates were incubated for 5-7 days. The scoring of the plates was done by the observation of cytopathic effect (CPE) using brightfield microscopy.

Cell electroporation

Electroporation of TMUV replicon RNA into BHK-21 cells was performed using the Gene Pulser Xcell (Bio-Rad Laboratories). A total of 8 x 10^6 BHK-21 cells were harvested and resuspended in 1 mL Dulbecco's phosphate-buffered saline (DPBS; Gibco, *CN: 14190144*). Next, 10 µg of purified replicon RNA was added to the resuspended BHK-21 cells and transferred to a 0.4 cm cuvette (Bio-Rad Laboratories, *CN: 1652088*). Subsequently, the cuvette was pulsed twice (850 V/25 µF) and cells were resuspended in 10 mL supplemented DMEM. Hereafter, cells were processed in plates according to the application and incubated at 37°C under 5% CO_2.

Viral growth kinetics

The viral growth kinetics were studied in BHK-21 cells. Cells were seeded in a 6-well plate with a concentration of $1x10^6$ cells·mL^{-1} and inoculated at a multiplicity of infection (MOI) of 0.01 $TCID_{50}$ per cell. After 2 h of incubation, the supernatant was removed and the cells were washed once with DPBS. DMEM+HEPES (Gibco, *CN: 10564011*) supplemented with 10% FBS (Gibco, *CN:10270106*) and 100 U·mL^{-1} penicillin-streptomycin (Gibco, *CN: 15140122)* was added to the infected cells and incubated at 37°C under 5% CO_2. The supernatants were collected every 24 h for 5 days and stored at -80°C prior to titration by EPDA on DF-1 cells.

Indirect immunofluorescence assay

An indirect immunofluorescence assay (IFA) was performed to detect the presence of viral proteins or viral double-stranded RNA (dsRNA) in infected or replicon-transformed cells. A monolayer of BHK-21 cells was washed with DPBS and fixed with 4% paraformaldehyde (Thermo Fisher Scientific, *CN: J61899.AP*) in DPBS at RT for 5 min. The cells were washed and permeabilized using 0.1% sodium dodecyl sulfate (SDS) in DPBS at RT for 10 min. Next, the monolayer was blocked by DBPS supplemented with 5% FBS at 37°C for 1 h and incubated at 37°C for 1 h with primary antibodies pan-flavivirus α-NS1 (1:50, 4G4; mouse), pan-flavivirus α-E (1:100, 4G2; mouse), α-dsRNA (1:32, 3G1.1; mouse), serum α-HA (1:500, strain H5N1; chicken), α-VP2 (1:500, R63; mouse) (*Snyder et al., 1988*), or α-S (1:50, strain M41 IBV; mouse) diluted in DPBS containing 5 % FBS. Hereafter, cells were incubated at 37°C for 1 h with a secondary α-mouse IgG conjugated with Alexa Fluor 488 (goat; 1:2000; Invitrogen, *CN: A32723*), Alexa Fluor 546 (goat, 1:2000; Invitrogen, *CN: A11030*), or α-chicken IgY conjugated with Alexa Fluor 488 (goat; 1:2000; Invitrogen, *CN: A11039*) antibody in DPBS containing 5% FBS. Cells were then stained with Hoechst (1:100; Thermo Fisher Scientific, *CN: 11594876*) in DPBS for 5 min. Images were acquired using an inverted fluorescence microscope (Axio observer Z1; Zeiss).

3. Results

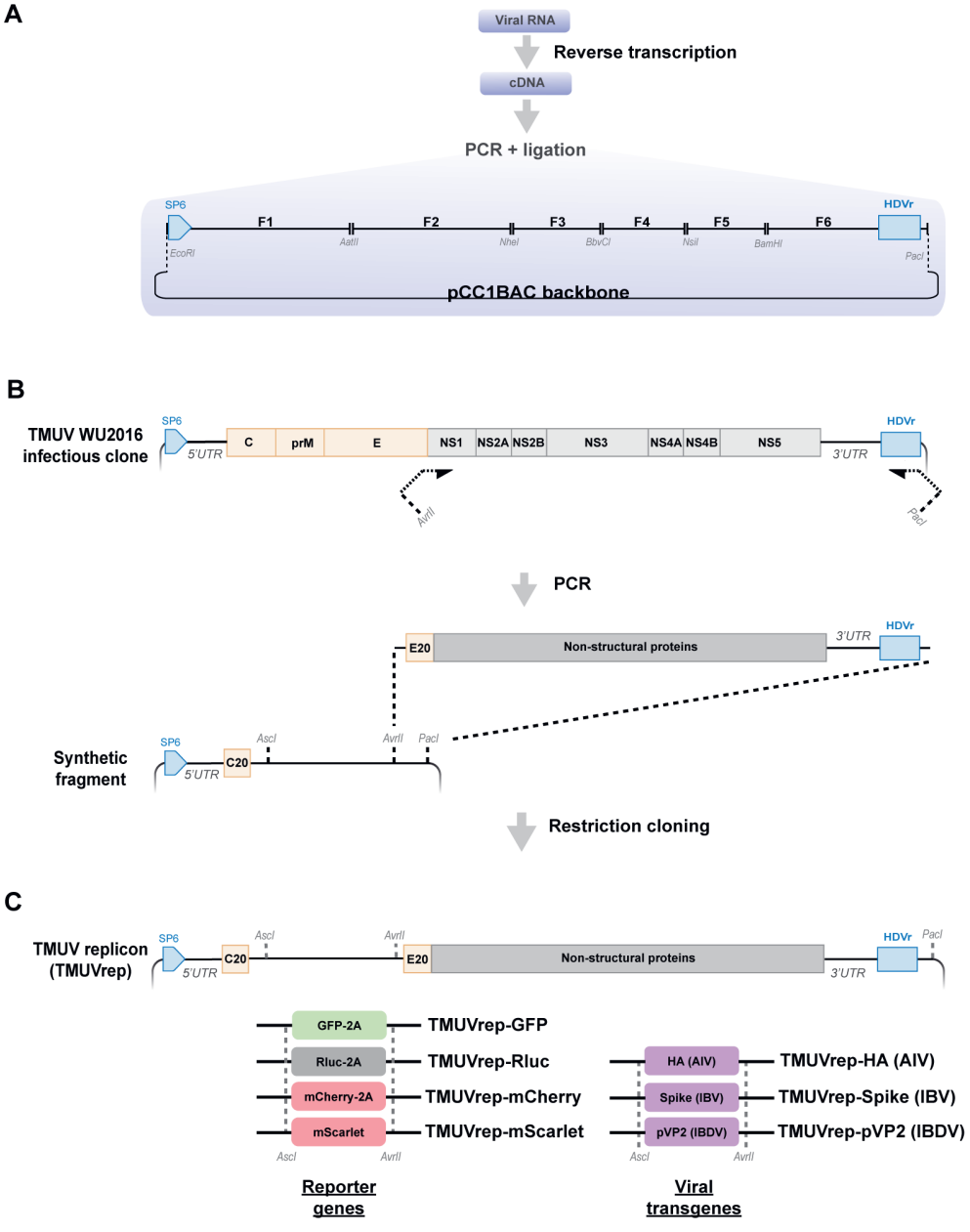
Construction of the TMUV WU2016 infectious clone and sub-genomic replicons

To create an infectious cDNA clone of duck Tembusu virus isolate WU2016, the RNA was isolated and used for RT-PCR amplification (**Figure 1A**). The six overlapping PCR fragments were cloned, sequenced, and then assembled in a single-copy pCC1BAC backbone by restriction cloning (**Figure 1B**). The subgenomic replicon (TMUVrep) was constructed by deleting part of the coding sequence of the structural genes from the TMUV WU2016 cDNA, retaining only the first 60 nucleotides of the capsid coding sequence (C_{20}) and the last 66 nucleotides of the envelope protein coding sequence (E_{22}). C_{20} is necessary to initiate the translation of the polyprotein and for cyclization of the replicon RNA (*Khromykh et al., 2001*), whereas E22 encodes the signal sequence required for proper translocation of the nonstructural protein 1 (NS1) into the endoplasmic reticulum (ER) (*Khromykh & Westaway, 1997*). To study the function of the constructs to replicate and express heterologous proteins, the replicon was subsequently modified by cloning the reporter genes (e.g. GFP, Rluc, mScarlet, and mCherry) or viral transgenes HA, (AIV) Spike (IBV) or pVP2 (IBDV) in frame with the ORF (**Figure 1C**).

Characterization of the TMUV WU2016 infectious clone

An infectious virus was generated by transfection of capped, *in vitro* transcribed TMUV WU2016 RNA into BHK-21 cells. In order to analyze the growth kinetics of TMUV WU2016 and compare this to the prototypical TMUV MM1775 isolate, BHK-21 cells were infected at an MOI of 0.01 $TCID_{50}{\cdot}mL^{-1}$ and samples were taken every day for the duration of 4 days (**Figure 2A)**. The highest titer was detected at 72 hpi (1.33×10^8 $TCID_{50}{\cdot}mL^{-1}$) and 48 hpi (6.08×10^6 $TCID_{50}{\cdot}mL^{-1}$) for TMUV WU2016 and MM1775, respectively. Clear cytopathic effect in BHK-21 cells was observed at 96 hours post-infection (hpi) (**Figure 2B**). Comparative sequence analysis of TMUV WU2016 showed an 89.1% nucleotide sequence and 97.0% protein sequence homology to the TMUV MM1775 isolate. The TMUV WU2016 most closely relates to the DTMUV DF-2 isolate with 99.9% nucleotide and 99.9% protein homology (**Figure 2C**).

►**Figure 1. Schematic representation of the TMUV genome, TMUV WU2016 infectious cDNA clone, and TMUV replicon. (A)** Purified TMUV WU2016 RNA was used as a template in a reverse transcriptase reaction for the generation of cDNA. Next, the cDNA was amplified into 6 fragments (F1 to F6) using TMUV-specific primers and ligated into a SP6-containing pCC1BAC backbone creating the **(B)** TMUV WU2016 infectious cDNA clone. The TMUV genome consists of a single open reading frame flanked by 5' and 3' untranslated regions (UTR). The genome translates into a single polyprotein consisting of three structural proteins capsid (C), premembrane (prM), envelope (E) protein, and five nonstructural proteins (NS1-5). The TMUV WU2016 infectious cDNA clone served as a template for the construction of the **(C)** subgenomic replicon RNA by PCR amplifying the last 22 amino acids of E protein, nonstructural proteins, 3'UTR, and hepatitis delta virus ribozyme (HDVr) and ligating it into a synthetic fragment encoding the SP6, 5'UTR and first 20 amino acids of the C protein. Lastly, the TMUV replicon was modified via AscI and AvrII restriction digestion to express the green fluorescent protein (GFP), Renilla luciferase (Rluc), or mCherry flanked by foot-and-mouth disease virus 2A elements or mScarlet reporter proteins. For the expression of viral transgenes, the hemagglutinin (HA) of avian influenza A virus (AIV), the Spike of infectious bronchitis virus (IBV), or the nucleocapsid (pVP2) gene of infectious bursal disease virus (IBDV) was inserted into the TMUV replicon.


A
Viral RNA
Reverse transcription
cDNA
PCR + ligation
SP6
F1
F2
F3
F4
F5
F6
HDVr
EcoRI
AatII
NheI
BbvCI
NsiI
BamHI
PacI
pCC1BAC backbone
B
TMUV WU2016 infectious clone
SP6
5'UTR
C
prM
E
NS1
NS2A
NS2B
NS3
NS4A
NS4B
NS5
3'UTR
HDVr
AvrII
PacI
PCR
E20
Non-structural proteins
3'UTR
HDVr
Synthetic fragment
SP6
5'UTR
C20
AscI
AvrII
PacI
Restriction cloning
C
TMUV replicon (TMUVrep)
SP6
5'UTR
C20
AscI
AvrII
E20
Non-structural proteins
3'UTR
HDVr
PacI
GFP-2A
Rluc-2A
mCherry-2A
mScarlet
TMUVrep-GFP
TMUVrep-Rluc
TMUVrep-mCherry
TMUVrep-mScarlet
AscI
AvrII
Reporter genes
HA (AIV)
Spike (IBV)
pVP2 (IBDV)
TMUVrep-HA (AIV)
TMUVrep-Spike (IBV)
TMUVrep-pVP2 (IBDV)
AscI
AvrII
Viral transgenes

Characterization of the TMUV replicon

To verify the functionality of the TMUV replicon, capped *in vitro* transcribed RNA was produced from the TMUVrep-GFP cDNA and electroporated in BHK-21 cells, which were analyzed using (immuno)fluorescence microscopy 48 h post-electroporation (hpe). As a control, BHK-21 cells were infected with TMUV WU2016 at an MOI of 0.01 $TCID_{50}{\cdot}mL^{-1}$. The TMUV E and NS1 proteins were detected using monoclonal antibodies (mAb) 4G2 and 4G4, respectively. Additionally, the presence of double-stranded RNA (dsRNA) replication intermediates within the flavivirus RNA replication complex was detected using mAb 3G1.1. NS1 and dsRNA are detected in cells electroporated with TMUVrep-GFP replicon RNA or infected with TMUV WU2016 (**Figure 3**). NS1 was detected in the cytoplasm, where a perinuclear localization was observed. Similarly, dsRNA intermediates were detected in the perinuclear region for both the TMUV replicon and the TMUV WU2016 infectious clone. As expected, the TMUV envelope protein could only be detected in the samples infected with the TMUV WU2016 infectious clone, whereas GFP expression could only be detected in cells electroporated with TMUVrep-GFP. In order to analyze the expression and timing of reporter protein expression, capped, *in vitro* transcribed RNA of TMUVrep-GFP, TMUVrep-mScarlet, TMUVrep-mCherry, or TMUVrep-Rluc was electroporated into BHK-21 cells (**Figure 4A**). Transgene expression was measured over

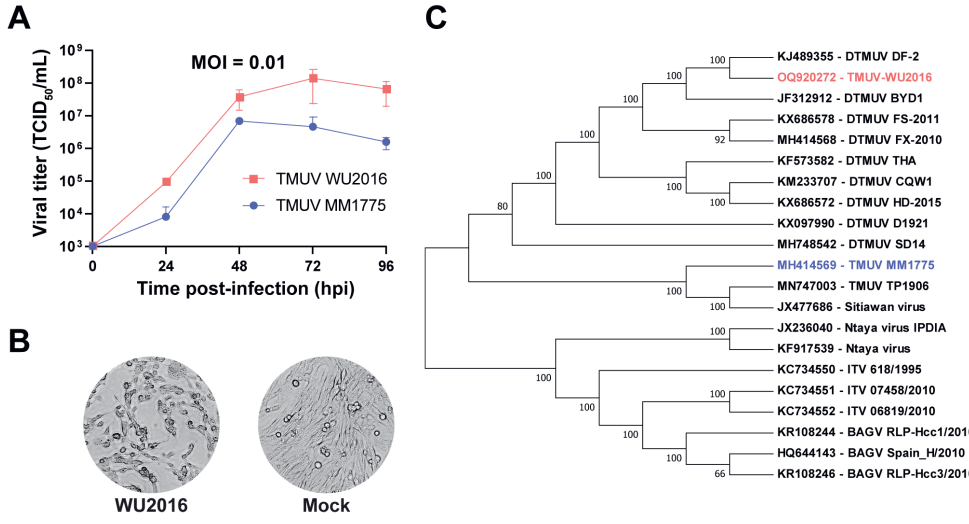


Figure 2. Viral growth kinetics of TMUV WU2016 compared to the TMUV MM1775 isolate. (A) BHK-21 cells were infected with TMUV WU2016 or TMUV MM1775 isolate at a multiplicity of infection (MOI) of 0.01 median tissue culture infective dose per milliliter ($TCID_{50}{\cdot}mL^{-1}$). Samples were taken every 24 hours for up to 4 days and virus titers were determined by end-point dilution assay and expressed in $TCID_{50}{\cdot}mL^{-1}$. Error bars indicate the standard error of the mean titers of three biological replicates. **(B)** Virus-induced cytopathic effect TMUV WU2016 compared to mock-infected BHK-21 cells at 48 hours post-infection (hpi). **(C)** Phylogeny of TMUV WU2016 (*red*) and TMUV MM1773 (*blue*) compared to other flaviviruses belonging to the Ntaya serocomplex. The phylogenetic tree was constructed using the maximum likelihood method based on the general time reversible model (GTR+G+I) of the complete genome sequences. Evolutionary analyses were conducted in MEGA7 software. The reliability of the analysis was calculated using 100 bootstrap replications. The reference nucleotide sequences of the corresponding viruses were obtained from the GenBank database.

four consecutive days. The fluorescent reporter proteins (GFP and mScarlet) were analyzed using fluorescence microscopy and showed the highest fluorescence expression between 48- and 72 hours post-electroporation (hpe). This was also confirmed for TMUVrep-eGFP-transformed cells using flow cytometry analysis (**Figure 4B**). Moreover, a strictly cytoplasmic localization of mScarlet was observed in the absence of the FMDV-2A site while this is not seen for GFP and mCherry, both of which are dispersedly distributed in the entire cell (**Figure 4C**). The Rluc activity was detected from cellular lysate prepared from BHK-21 cells and peaked at 48 hpe (**Figure 4D**). Next, the TMUV replicons encoding the HA (AIV), Spike (IBV), and pVP2 (IBDV) genes were tested for the expression and localization of the viral (glyco)proteins. The signal sequence encoded by the two glycoproteins directs both HA or Spike glycoproteins into the ER for transport to the plasma membrane. In contrast, the capsid protein pVP2 has a cytosolic localization therefore it is separated from the viral polyprotein by incorporation of FMDV-2A sites (**Figure 5A**). To confirm the expression and processing of the viral (glyco)proteins, capped, *in vitro* transcribed replicon RNA was electroporated in BHK-21 cells, and cells were analyzed 72 hpe using

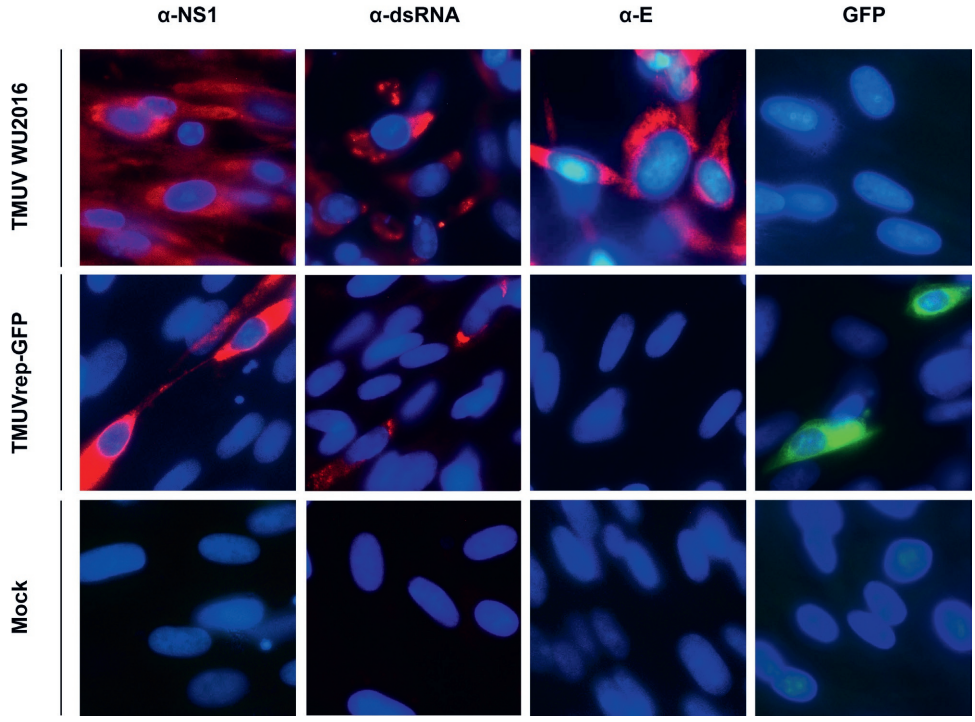


Figure 3. Cellular localization of viral structural, nonstructural, or reporter proteins in TMUV WU2016-infected or TMUVrep-GFP-transfected BHK-21 cells. An indirect immunofluorescence assay (IFA) was performed on BHK-21 cells 48 hours post-infection with TMUV WU2016 at an MOI 0.1 median tissue culture infectious dose per mililiter or post-electroporation with capped, *in vitro* transcribed TMUVrep-GFP replicon RNA, detecting the production of TMUV envelope (E) glycoprotein, nonstructural protein 1 (NS1), or double-stranded RNA (dsRNA) replication intermediate. Primary antibodies: α-E (4G2; mouse), α-NS1 (4G4; mouse), or α-dsRNA (3G1.1; mouse) Secondary antibody: Alexa Fluor 546 conjugated α-mouse IgG (goat). Nuclei were counterstained using Hoechst.

an indirect immunofluorescence assay. Successful expression was detected for all TMUV replicon-expressed (glyco)proteins. Both glycoproteins Spike and HA showed a perinuclear localization while a more dispersed signal for the cytosolic pVP2 was detected (**Figure 5B**).

4. Discussion

In this manuscript, we constructed and characterized a full-length infectious cDNA clone

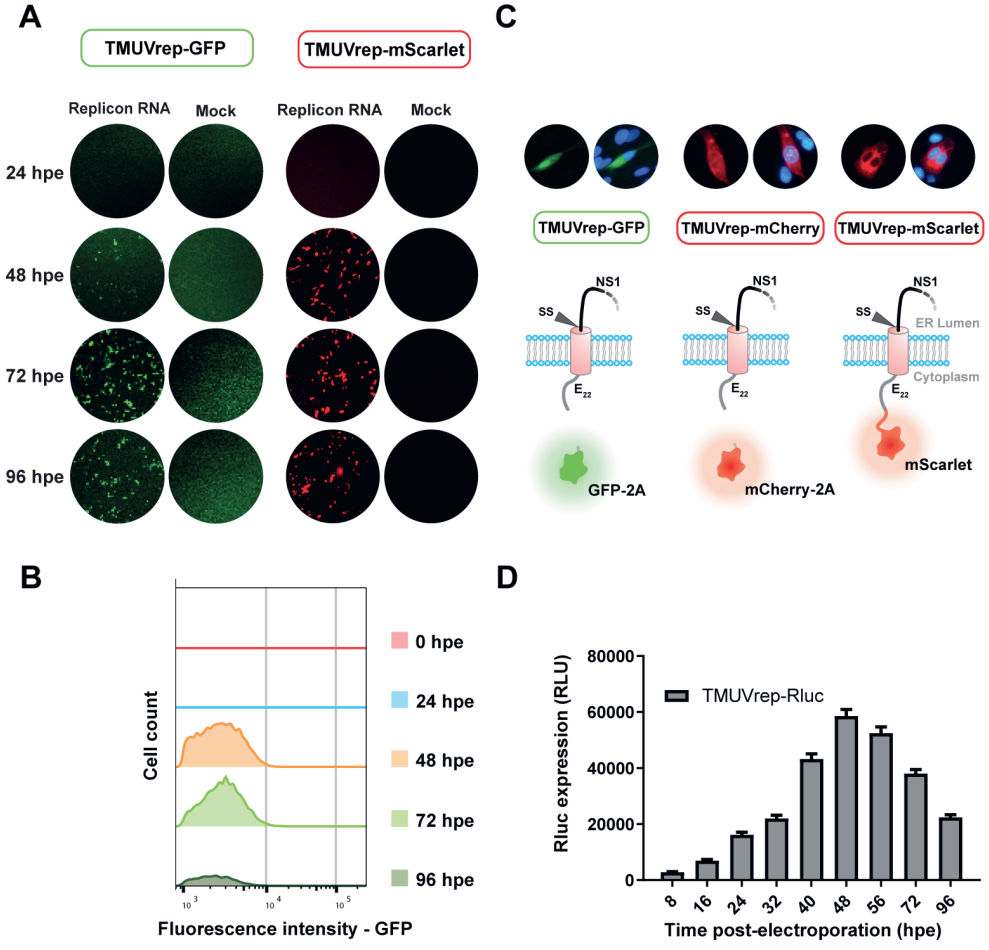


Figure 4. TMUV replicon-driven reporter gene expression in BHK-21 cells over time. The GFP and mScarlet expression from BHK-21 cells electroporated with capped, *in vitro* transcribed replicon RNA (TMUVrep-GFP or TMUVrep-mScarlet) or DPBS (mock) was determined using **(A)** fluorescence microscopy or, in case of TMUVrep-GFP, by **(B)** flow cytometry at 0, 24, 48, 72 and 96 hours post electroporation (hpe). **(C)** The cellular localization of the fluorescent reporter proteins GFP (TMUVrep-GFP), mCherry (TMUVrep-mCherry), and mScarlet (TMUVrep-mScarlet), and the effect of the foot-and-mouth disease virus 2A element were displayed in BHK-21 cells after 48 hpe. **(D)** Additionally, the expression of Renilla luciferase (Rluc) from capped, in vitro transcribed replicon RNA (TMUVrep-Rluc) was analyzed using luminometry up to 96 hpe. The luminescence of Rluc was expressed in relative light units (RLU) and was measured from two independent electroporation, normalized against mock electroporated cells. The data was plotted as mean ± SEM.

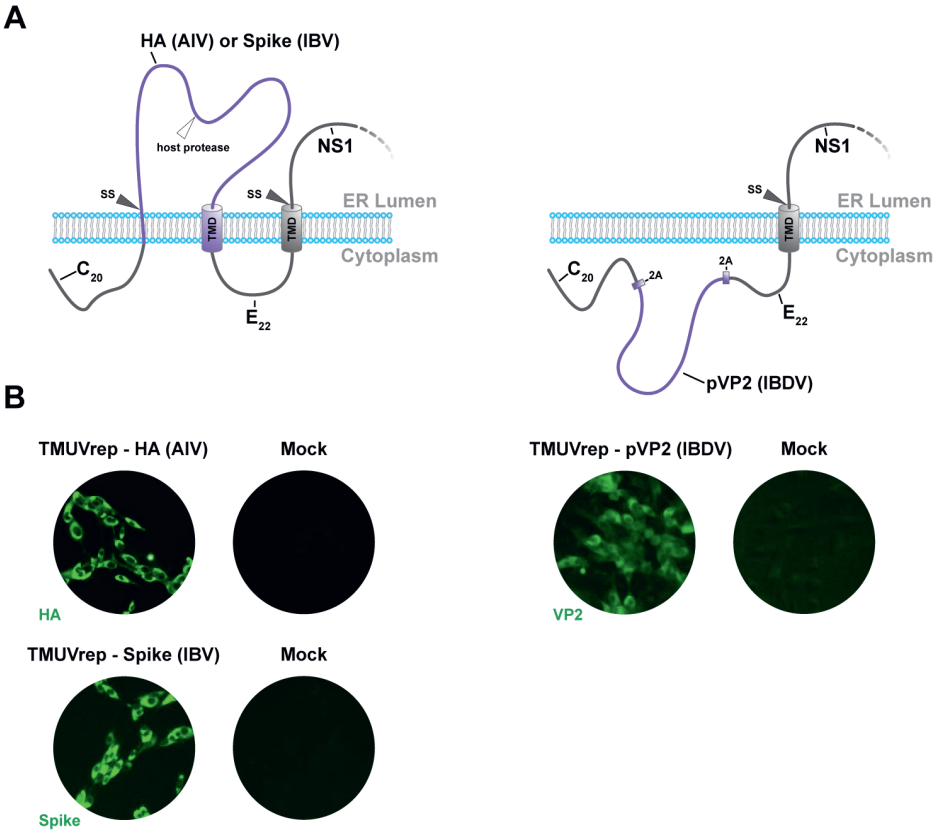


Figure 5. The expression of viral (glyco)proteins using the TMUV replicon in BHK-21 cells. **(A)** Predicted membrane topology from the polyprotein expression of TMUVrep-HA and TMUVrep-Spike (left) shows a luminal (ER) localization, while TMUVrep-pVP2 (right) displays a cytoplasmic localization. C_{20}: first 20 amino acids of the capsid protein; SS: signal sequence; TMD: trans-membrane domain; E_{22}: 3' terminal 22 amino acids of the envelope (E) protein; NS1: nonstructural protein 1; 2A: foot-and-mouth disease virus 2A element. **(B)** Detection of hemagglutinin (HA; AIV), Spike (S; IBV), and nucleocapsid (pVP2; IBDV) proteins by indirect immunofluorescence assay in BHK-21 cells electroporated with TMUVrep-HA, TMUVrep-Spike, or TMUVrep-pVP2, respectively. Primary antibodies: α-HA (chicken), α-Spike (mouse), and α-VP2 (mouse). Secondary antibodies: Alexa Fluor 488-conjugated α-chicken IgY (goat) or α-mouse IgG (goat).

and replicons based on the TMUV WU2016 isolate as a valuable tool to express high levels of heterologous (viral) proteins. The construction of flavivirus cDNA clones and replicons often results in genetic instability and toxicity during plasmid propagation within bacterial hosts as was described previously for WNV (*Yamshchikov et al., 2001*), ZIKV (*Münster et al., 2018*), JEV (*Yamshchikov et al., 2001*), and DENV (*D. Li et al., 2011*). Although current prediction tools can point toward cryptic prokaryotic sites in the flavivirus genome, strategies for counteracting the toxicity resulted in highly variable outcomes (*D. Li et al., 2011*). It is known that not only the common viral elements such as the cytomegalovirus (CMV) promoter but also flavivirus-specific elements (e.g. the flavivirus E coding sequence) carry cryptic promoters toxic to bacteria (*Pu et al., 2011*). To overcome genetic instability, applying a yeast

cloning system or inserting an intron within cryptic promoter sites could reduce the toxic viral byproduct (*Aubry et al., 2015*). Although these introns do not impact the flavivirus protein coding sequence, introns can, however, disrupt higher-order RNA structures vital to efficient flavivirus replication. We circumvented problems with genetic instability by cloning the TMUV WU2016 cDNA in an inducible single-copy bacterial artificial chromosome (BAC) backbone (*Mutso et al., 2017*). The pCC1BAC vector is a so-called copy control plasmid typically maintained in the *E. coli* DH10β strain as a single-copy plasmid, but larger amounts of plasmid DNA can be obtained by L-arabinose induction of the *E. coli* strain EPI300. Indeed, no large genomic deletion or rearrangements were detected in the TMUV WU2016 cDNA when maintained in the *E. coli* DH10β strain, and the infectious virus could be successfully recovered upon transfection of capped, *in vitro* transcribed RNA. Future strategies could include permutating the flavivirus genome and preserving any conserved secondary and tertiary RNA motifs while reducing or completely abrogating the transcriptional activity of the cryptic bacterial promoter without affecting viral replication (*Fros et al., 2017*). Furthermore, the infectious virus was successfully recovered from BHK-21 cells transfected with *in vitro* transcribed RNA. The viral growth kinetics of duck-derived TMUV WU2016 were compared to that of mosquito-derived TMUV MM1775 in BHK-21 cells. TMUV WU2016 replicated faster and achieved higher overall viral titers than MM1775. The peak titers of TMUV MM1775 at 48 and 72 hpi were ~5 to 30-fold lower than those of TMUV WU2016. It was previously reported that another duck-derived TMUV strain CWQ1 led to higher peak titers than the mosquito-derived MM1775 isolate. The study described that favorable replication of the MM1775 strain in mosquito cells was most likely caused by a variable stem-loop structure in the 3'UTR of the viral genome (*Mao et al., 2022*). However, similar to CWQ1, WU2016 shared high levels of sequence homology of the 3' UTR with the CQW1 strains (97.59%) in contrast to the MM1775 strain (92.59%) thereby likely explaining the enhanced growth kinetics compared to MM1775. To study the TMUV infectious cDNA clone as the basis for a potential vaccine platform technology, TMUV replicons were successfully engineered to express different reporter genes and viral transgenes. We tested the transgene expression of the WU2016-based TMUV replicon and showed a long-lasting protein production of up to 96 hpe in BHK-21 cells, without considerable cytopathic effect. It was demonstrated that the transgene expression peaked at 48 hpe confirming the results of an earlier study (*He et al., 2019*). The apparent discrepancy between the peak expression of fluorescence reporter genes (i.e. GFP and mScarlet) and bioluminescent reporter genes (i.e. Rluc) could be explained by the reporter protein half-lives since luminescence assays are a more precise tool for measuring dynamic changes in expression levels (*Thorne et al., 2010*). Moreover, the presence of the FMDV-2A sites did not impair reporter protein function since both GFP and mCherry expression was detected. However, the absence of the FMDV-2A element restricts the reporter protein dispersion to the cytoplasm as was seen for mScarlet. Additionally, we demonstrated that the TMUV replicon was capable of expressing viral (glyco)proteins of AIV (HA), IBV (Spike), and IBDV (pVP2). HA was detected in the perinuclear region within electroporated cells, indicating that this viral glycoprotein was correctly routed to the cell surface as previously described for a reporter Influenza A virus (*dos Anjos*

Borges et al., 2020). Indeed, HA was also detected at the cell surface. For the TMUV replicon expressing pVP2, a cytosolic distribution was observed which is in agreement with the literature. It has been described that during the individual expression of either precursor or mature VP2 protein, the signal is located in the cytoplasm of insect, avian, and mammalian cells while in mammalian and avian cells aggregates of mature VP2 can be detected (*Y. Qin et al., 2017*). In conclusion, we have successfully created an infectious clone and replicon system based on the TMUV WU2016 isolate that efficiently replicates in cell culture and expresses transgenes, including the viral (glyco)proteins of key poultry diseases such as AIV, IBV, and IBDV. Our results suggest that this platform may have the potential to be used in vaccination against these poultry diseases. As we continue to characterize this system, we will explore its efficacy *in vivo* using various delivery methods. Additionally, our work builds on prior research towards replicon RNA vaccines, which underscores the importance of developing effective and readily adaptable vaccines to combat emerging and re-emerging diseases (*Kent et al., 2008; Komdeur et al., 2021; Langereis et al., 2021; Pijlman et al., 2006*).

Chapter 3

Evaluation of transgene expression and cytopathic effects of Tembusu virus versus Venezuelan equine encephalitis virus replicon systems

Abstract

Efficient and versatile platforms for heterologous gene expression are crucial in biomedical research, biotechnology, and vaccine development. Self-amplifying RNAs (replicons) are promising tools typically derived from positive-sense RNA genomes to enable robust and long-lasting transgene expression without the production of infectious viral progeny. While the alphavirus Venezuelan equine encephalitis virus (VEEV; family *Togaviridae*) replicon system has been extensively studied with demonstrated vaccine efficacy in several animal species, an alternative flavivirus-based replicon platform, based on the duck Tembusu virus (TMUV; family *Flaviviridae*), is under development for heterologous gene expression in poultry. In this study, we directly compared the established VEEV replicon system to the novel, bird-adapted TMUV replicon system in terms of the reporter gene expression level and duration in different mammalian and avian cell lines. Despite a later onset of reporter gene expression by the TMUV replicon system, an overall higher reporter protein production was observed compared to the VEEV replicon system. Alternatively, we constructed two TMUV replicon capsid variants and investigated the impact of the included *cis*-acting elements within the capsid gene on the transgene kinetics and the virus-induced cytopathic effect. We showcased the tuneability of the TMUV replicon system, achieving expression levels equivalent to those of the VEEV replicon system. As a future application, we successfully achieved the expression of avian influenza hemagglutinin glycoprotein using all three TMUV replicon variants. Overall, the findings highlight the potential of an avian-adapted TMUV replicon system as a suitable, alternative, and effective platform for heterologous gene expression in both mammalian and avian cells.

1. Introduction

The development of efficient and versatile platforms for heterologous gene expression has become the foundation of biomedical research, biotechnology, and vaccine development. Self-amplifying RNAs, also known as 'replicons' are useful tools to achieve robust and long-lasting gene expression *in vitro* and *in vivo* (*Galanis et al., 2001; Manservigi et al., 2010; Nogueira et al., 2013; van den Pol & Davis, 2013*). These replicon RNAs are derived from negative- or positive-strand RNA viruses by conserving the nonstructural 'replicase' genes and replacing the viral structural genes to prevent the formation of viral progeny (*Almazán et al., 2013; Berger Rentsch & Zimmer, 2011; Kalhoro et al., 2009; Langereis et al., 2021; Oreshkova et al., 2021*). Among these replicon systems, a well-studied and highly effective replicon system is based on the Venezuelan equine encephalitis virus (VEEV), a member of the *Togaviridae* family. VEEV replicon RNA has successfully expressed a wide variety of immunogens including advanced cancers antigens (*Morse et al., 2010*), Ebola virus antigens (*Herbert et al., 2013*), swine influenza antigens (*R. L. Vander Veen et al., 2012*) in various species. The strength of the VEEV replicon platform lies in the fact that the heterologous gene is encoded on a separate mRNA that is under the control of a separate subgenomic (26S) promoter, leading to very high levels of transgene expression. However, the high levels of alphavirus RNA replication and expression of alphavirus replicase proteins can also induce a significant cytopathic effect (CPE) (*Frolov & Schlesinger, 1994; D. Y. Kim et al., 2014*). Unlike alphaviruses, flaviviruses lack a subgenomic promoter. Instead, they translate both their structural - capsid (C), premembrane (prM), and envelope (E) - and nonstructural proteins 1 to 5 (NS1 to NS5) as a single polyprotein, which is post-translationally processed by both viral and cellular proteases. For the construction of a flavivirus-based replicon system, these structural genes are replaced by the gene of interest that is cloned in-frame with the flavivirus nonstructural polyprotein (*Comes, Poniman, et al., 2023*). Currently, several flavivirus-based replicon platforms have been developed derived from yellow fever virus 17D (YFV) (*Oreshkova et al., 2021*), West Nile virus (WNV) (*Khromykh & Westaway, 1997*), dengue virus (DENV) (*Kato & Hishiki, 2016*), Tick-borne encephalitis virus (TBEV) (*Gehrke et al., 2005*), and Tembusu virus (TMUV) (*He et al., 2019*). What these flaviviruses replicon systems have in common, is the requirement of *cis*-acting RNA elements encoded within the C gene and a signal sequence in the E protein to facilitate efficient viral RNA replication and ER translocation of downstream NS1 protein, respectively. The first 20 amino acids of C-protein (C_{20}) and the last 22 amino acids of E-protein (E_{22}) are therefore retained in the replicon design (*Pijlman et al., 2006*). The absence of specific *cis*-acting RNA elements in the 5' terminal region of the flavivirus genome among which the 5' cyclization sequence (5'CS) and downstream of 5'CS pseudoknot (DCS-PK) have been reported to negatively impact genomic replication of flaviviruses and thus a potential target for antiviral or vaccine strategies (*He et al., 2019; Khromykh et al., 2001; Z.-Y. Liu et al., 2013; Patkar et al., 2007*). Furthermore, the capsid protein itself is known to interact with numerous host proteins and plays a structural role in the formation of infectious particles (*Freire et al., 2013; Mori et al., 2005b; Samuel et al., 2016; Schrauf et al., 2008; M. R. Yang et al., 2008*). However, how *cis*-acting RNA elements or the capsid proteins impact heterologous gene expression and cytopathicity of the flavivirus replicon is not well understood.

In this study, we described a direct comparison between the established VEEV TC-83 replicon vaccine platform and the newly described, bird-adapted, TMUV WU2016 replicon system to express several report genes and the avian influenza virus (AIV) hemagglutinin (HA) glycoprotein. Moreover, we assess the cytopathic effect (CPE) of both replicon systems on mammalian and avian cells and study the effect of *cis*-acting RNA elements within the TMUV capsid gene on replicon expression kinetics and CPE levels. Collectively, our findings highlight the suitability and adaptability of the avian-adapted TMUV replicon system as an alternative platform for transgene expression in mammalian and avian cells.

2. Material and methods

Cells

Adherent vertebrate cell lines derived from chicken embryonic fibroblasts (DF-1; ATCC CRL-3586) and baby hamster kidney cells (BHK-21; clone 13 ECACC 84011433) were cultured in completed Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 $U \cdot mL^{-1}$ penicillin and 100 $\mu g \cdot mL^{-1}$ streptomycin (Gibco). The suspension-adapted cell lines derived from duck embryonic stem cells (EB66; Valneva) were cultured in serum-free Ex-cell growth II medium (SAFC Biosciences) supplemented with 40 mg/L gentamycin, 2.5 mg/L natamycin, and 0.24 % (v/v) antifoam. Adherent cells were maintained in T-flasks (Greiner) while suspension cells were maintained in single-use shaker flaks (Greiner) at 37°C under 5% CO_2.

TMUV and VEEV replicon constructs

The construction of replicon vectors TMUVrep-C_{20}-eGFP, TMUVrep-C_{20}-Rluc, and TMUVrep-C_{20}-mScarlet was described in **Chapter 2** (*Comes, Poniman, et al., 2023*). For the construction of TMUVrep-C_{38} or C_{109}-eGFP replicons, the first 20 codons of the C-coding region of TMUVrep-C_{20}-eGFP replicon construct was replaced via restriction cloning (*KasI* and *AscI*; New England Biolabs) by a synthetic DNA gene (Thermo Fisher Scientific) coding for the first 38 or 109 codons of the capsid gene. For the construction of the TMUVrep-C_{38}-HA and TMUVrep-C_{109}-HA, the HA gene of TMUVrep-C_{20}-HA (*Comes, Poniman, et al., 2023*), was isolated using *AscI* and *AvrII* and inserted into the aforementioned replicon constructs. The plasmids encoding for the T7-driven VEEV replicon were fully synthesized by Integrated DNA technologies and modified using restriction cloning (*AscI* and *PacI*) to insert the GFP, mScarlet, Renilla luciferase (Rluc) or HA genes behind the 26S subgenomic promotor (*Hooper et al., 2009; Langereis et al., 2021*).

In vitro RNA transcription and purification

Replicon-encoding DNA plasmids were purified using the endotoxin-free Nucleobond Midiprep kit (Macherey-Nagel) and linearized using *PacI* (TMUV-derived replicons) or NotI (VEEV-derived replicons) restriction enzymes. Capped, *in vitro* transcribed replicon RNAs were generated by using 2.5 micrograms of linearized plasmid DNA in an SP6- or T7-polymerase reaction (both New England Biolabs) for TMUV and VEEV replicon constructs, respectively. The reaction was incubated for 2 h at 37°C and then treated for 30 min at 37°C with RNAse-free DNAse (Promega). Replicon RNA was purified using the RNeasy Midiprep kit (Qiagen) according to the manufacturer's RNA Cleanup protocol. Both quantity and quality of the *in vitro* transcribed RNA were analyzed using the spectrophotometer ND-1000 (Nanodrop) and via conventional electrophoresis using a 1% agarose gel in tris-acetate-EDTA (TAE) buffer for 15 min at 150V. The replicon RNA was stored at -80°C for further use.

Transfection and electroporation

One day prior to the experiment, 2 x 10^5 DF-1 cells were seeded in 24-well plates. Next, cells were transfected with *in vitro* transcribed replicon RNA using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturers' protocol. After 4 hours, the medium was replaced with supplemented DMEM and incubated at 37°C under 5% CO_2. For adherent BHK-21 cells, electroporation using the Gene Pulser Xcell (Bio-Rad Laboratories) was performed. A total of 8 x 10^6 BHK-21 cells were harvested and resuspended in 1 mL Dulbecco's phosphate-buffered saline (DPBS; Gibco). Next, 10 µg of purified replicon RNA was added to the resuspended BHK-21 cells and transferred to a 0.4 cm cuvette (Bio-Rad Laboratories). Subsequently, the cuvette was pulsed twice (850 V/25 µF) and cells were resuspended in 10 mL supplemented DMEM. Hereafter, cells were processed in plates according to the application and incubated at 37°C and 5% CO_2. Suspension EB66 cells were electroporated using the BTX electroporator (ECM830 Electro Square Porator; BTX). These cells were resuspended to a concentration of 2 x 10^8 cells·mL^{-1} in OptiPRO® SFM (Gibco) containing 25 mM HEPES (Gibco), MEM non-essential amino acids solution (Gibco), and 2mM L-Glutamine (Gibco). Next, 30 µg purified replicon RNA was added to 0.6 mL resuspended cells and transferred to a 0.4 cm BTX electroporation cuvette plus (BTX). Hereafter, EB66 cells were electroporated by providing four 400 µs pulses at 650 V. After electroporation, cells were processed in flasks or plates according to the application and incubated at 37°C under 5% CO_2.

Indirect immunofluorescence assay

An indirect immunofluorescence assay was performed to detect the expression of the viral glycoprotein HA in replicon-containing cells. A monolayer of BHK-21 cells was washed with DPBS and fixed with 4% paraformaldehyde in DPBS at room temperature (RT) for 5 min. The cells were washed and permeabilized using 0.1% sodium dodecyl sulfate (SDS) in DPBS at RT for 10 min. Next, the monolayer was blocked by 5% FBS in DPBS at 37°C for 1 h and incubated at 37°C with α-HA (strain H5N1; chicken) antibody diluted in 5% FBS in DPBS for 1 h. Hereafter, cells were incubated with a secondary α-chicken IgG conjugated with Alexa Fluor 568 antibody (goat, 1:2000; Invitrogen) in 5% FBS in DPBS at 37°C for 1 h. Cells were then counterstained with Hoechst (1:100; Thermo Fisher Scientific) in DPBS for 5 min. Images were acquired using the Axio Observer Z1 fluorescence microscope (Zeiss).

Renilla luciferase activity assay

To assess the Rluc activity, 24-well plates were seeded with 1 x 10^5 cells/well of electroporated BHK-21 or transfected DF-1 cells and incubated for up to 4 days. The culture supernatant was aspirated and cells were lysed by 1x passive lysis buffer (Promega) for 20 min incubation at RT. The lysate was cleared by centrifugation at 12.000 x g and pipetted into an opaque 96-well plate. Next, pre-made Rluc buffer was automatically injected using a FLUOstar Optima microplate reader (BMG Labtech) and measured for 8 min (10 intervals). Samples were measured in triplicate and normalized against the lysate of non-electroporated or non-transfected healthy cells. To determine the total expression (area under the curve, AUC), values were processed using the trapezoid rule. Student's *t*-test was performed using GraphPad Prism (V9.5.0.) to assess the statistical significance ($p \leq 0.05$ was considered statistically significant).

Viral cytotoxicity assay

To assess the cytopathic effect (CPE) of the TMUV and VEEV replicon RNA on BHK-21 cells, 1.5 x 10^4 electroporated cells/well were plated in a 96-well plate (Greiner). The cells were analyzed every 24 h up to 4 days post electroporation to evaluate the CPE using the Viral ToxGlo assay (Promega) according to the manufacturer's protocol. The luminescence was measured using the FLUOstar OPTIMA microplate reader (BMG Labtech). The cytotoxicity was determined by the average relative light units (RLU) of two biological replicates and three technical replicates normalized against the average RLU of healthy, mock electroporated cells.

Western blot analysis

Hemagglutinin (HA) protein samples produced by BHK-21 cells were collected in DPBS (Gibco) and incubated at 95°C for 15 min with a loading buffer containing β-mercaptoethanol. After centrifugation at 13.000 x g for 1 min, samples were separated on 8-16% Mini-PROTEAN precast protein gels (Bio-Rad Laboratories). Hereafter, the proteins were semi-dry blotted using the Power Blotter system (Invitrogen) on PVDF membranes and blocked for 1 h at RT using DPBS-T (0.05% Tween-20) containing 1% skimmed milk. Next, the membranes were immunostained with primary monoclonal antibody α-GAPDH (mouse, 1:2500; Sigma-Aldrich) or polyclonal α-HA (chicken, 1:500; in kind provided by MSD Animal Health) diluted in DPBS-T for 1 h at RT. Membranes were washed three times with DPBS-T for 5 min and incubated with alkaline phosphatase (AP)-conjugated secondary antibodies: α-chicken IgY-AP (goat, 1:2000; Sigma-Aldrich) or α-mouse-AP (goat, 1:2000; Sigma-Aldrich) diluted in DPBS-T. After 1 h at RT, the membranes were washed three times for 5 mins and incubated with AP-buffer (100 mM NaCl, 100 mM Tris-Cl (pH 9.5), 50 mM $MgCl_2$, 1% Tween-20) for 15 min at RT. Lastly, the AP-buffer was replaced with NBT-BCIP (Sigma-Aldrich) diluted in AP-buffer to develop the membranes.

Flow cytometry analysis

eGFP or mScarlet expression were measured using the SH800S Cell sorter (Sony) or Accuri C6 (BD) respectively. For flow cytometry analysis, cells were collected via trypsinization, washed with DPBS, and resuspended in FACS buffer (DPBS supplemented with 1% BSA and 1mM EDTA). Forward scatter (FSC) and back/side scatter (BSC/SSC) were used to exclude debris, abnormalities, and doublets. To determine the mean fluorescence intensity (MFI) of the highest 5% of GFP-positive cells, a gating strategy was implemented that excluded 99% of non-fluorescent mock cells. For the flow cytometry analysis a total of 100.000 events per sample were measured.

Secondary RNA structure prediction

Models of the secondary RNA structures within the 5' untranslated region (5'UTR) and capsid region of the TMUV WU2016 genome (Genbank accession no.: OQ920272) were predicted using the webserver RNAalifold (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAalifold.cgi) and RNAstructure 6.3 and verified using the phylogenetically related TMUV isolates aligned in Clustal X V2.0. The secondary RNA structures were visualized and modelled in VARNA (*Darty et al., 2009*). The secondary structures that were selected are based on the minimum free energy prediction not taking into account weaker GU pairs at the end of helices. For long-distance RNA interaction such as 5' upstream AUG region (5'UAR), 5' downstream AUG region (5'DAR), and 5' cyclization signal (5'CS) the prediction was generated using the Mfold web server (http://www.unafold.org/mfold/applications/rna-folding-form.php) (*Zuker, 2003*) in which the 5'UTR and 3'UTR were separated by a poly(A) stretch of 100 nt (*Khromykh et al., 2001*).

3. Results

Comparison of reporter transgene expression between TMUV replicon RNA and VEEV replicon RNA

Previously, a TMUV replicon was constructed based on the TMUV WU2016 infectious cDNA clone (*Comes, Poniman, et al., 2023*). To compare the expression kinetics of the TMUV replicon to the benchmark VEEV replicon in mammalian and avian cell lines, both replicon RNAs were modified to express the Rluc protein (**Figure 1A**).

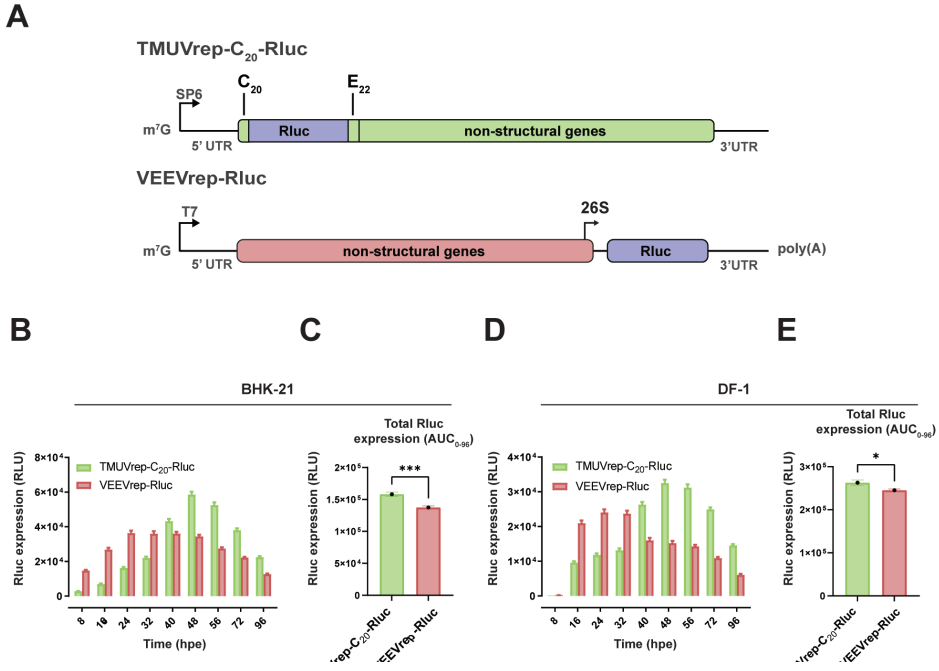


Figure 1. Comparison of Renilla luciferase (Rluc) expression between the TMUV and VEEV replicon RNA in mammalian and avian cell lines. (A) Schematic diagram of the SP6 promoter-driven TMUV replicon construct and T7 promoter-driven VEEV replicon construct encoding the Rluc protein. C_{20}: first 20 amino acids of capsid; E_{22}: last 22 amino acids of envelope protein; UTR: untranslated region; Poly(A): polyadenylated tail; 26S: 26S promoter are indicated. Rluc activity expressed in relative light units (RLU) of TMUVrep-C_{20}-Rluc or VEEVrep-Rluc electroporated **(B)** BHK-21 cells or transfected **(D)** DF-1 cells over 96-hour period. (**C** & **E**) The total (0-96 h) Rluc expression was calculated by approximating the area under the curve (AUC)f into trapezoids and summing their areas. Two independent experiments are presented as means and SEM, with significance defined by $p \leq 0.05$ (*) and $p \leq 0.0005$ (***) in an unpaired Student's *t*-test. hpe = hours post electroporation, hpt = hours post-transfection.

Replicon RNA was electroporated into BHK-21 cells or transfected into DF-1 cells, which were sampled over multiple time points in a 96-hour period to quantify the Rluc activity as a measure of gene expression (**Figures 1B and 1D**). It was observed that the VEEV replicon RNA resulted in an early onset - 8 hours post electroporation (hpe) for BHK-21 cells and 8 hours post-transfection (hpt) for DF-1 cells of Rluc expression, while the TMUV-mediated expression was lower at these earlier time points in BHK-21 (**Figure 1B**) and DF-1 (**Figure 1D**). Despite the later onset of Rluc expression by TMUV, the total Rluc activity over 96-hours period was significantly higher than VEEV replicon RNA expressed Rluc in BHK-21 (**Figure 1C**) and in DF-1 cells (**Figure 1E**).

Next, fluorescent reporter gene expression of TMUV replicon RNA was compared to VEEV replicon RNA among different cell lines. (**Figure 2**). Replicon RNA of the corresponding constructs was electroporated in EB66 and BHK-21 or transfected in DF-1 cells. Expression of GFP or mScarlet by TMUV was observed from 48 hpe onwards in EB66 and BHK-21 cells, respectively (**Figure 2B & C**). For VEEV replicon RNA, an early onset for GFP and mScarlet expression was observed from 24 hpe onwards in EB66 and BHK-21, respectively. In DF-1, however, the heterologous gene expression from both TMUV replicon RNA and VEEV replicon RNA was already observed at 24 hpt (**Figure 2D**), which is in correspondence to the earlier data on Rluc activity.

A notable detachment of the monolayer and distinct morphological changes were observed in BHK-21 (**Figure 2E**) and DF-1 (**Figure S1**) cells harboring VEEV replicon RNA when compared to cells containing TMUV replicon RNA. These phenotypical changes in cells harboring VEEV replicon RNA likely contributed to the decrease in the number of fluorescent cells throughout the assay. Although a similar decline in fluorescent cells was observed in EB66 harboring VEEV replicon RNA, assessing the morphological changes in these non-adherent cells proved to be more challenging (**Figure S1**).
To quantify the mScarlet expression levels of TMUV replicon- or VEEV replicon-containing BHK-21 cells, cells were harvested at 48 hpe and subjected to flow cytometry (**Figure 2F**). Consistent with the findings from the fluorescence microscopy, it was observed that the cumulative expression of individual cells, determined by analyzing the mean fluorescence intensity (MFI) of the top 5% highest expressing cells, was higher in VEEV replicon RNA electroporated cells compared to the TMUV replicon RNA.

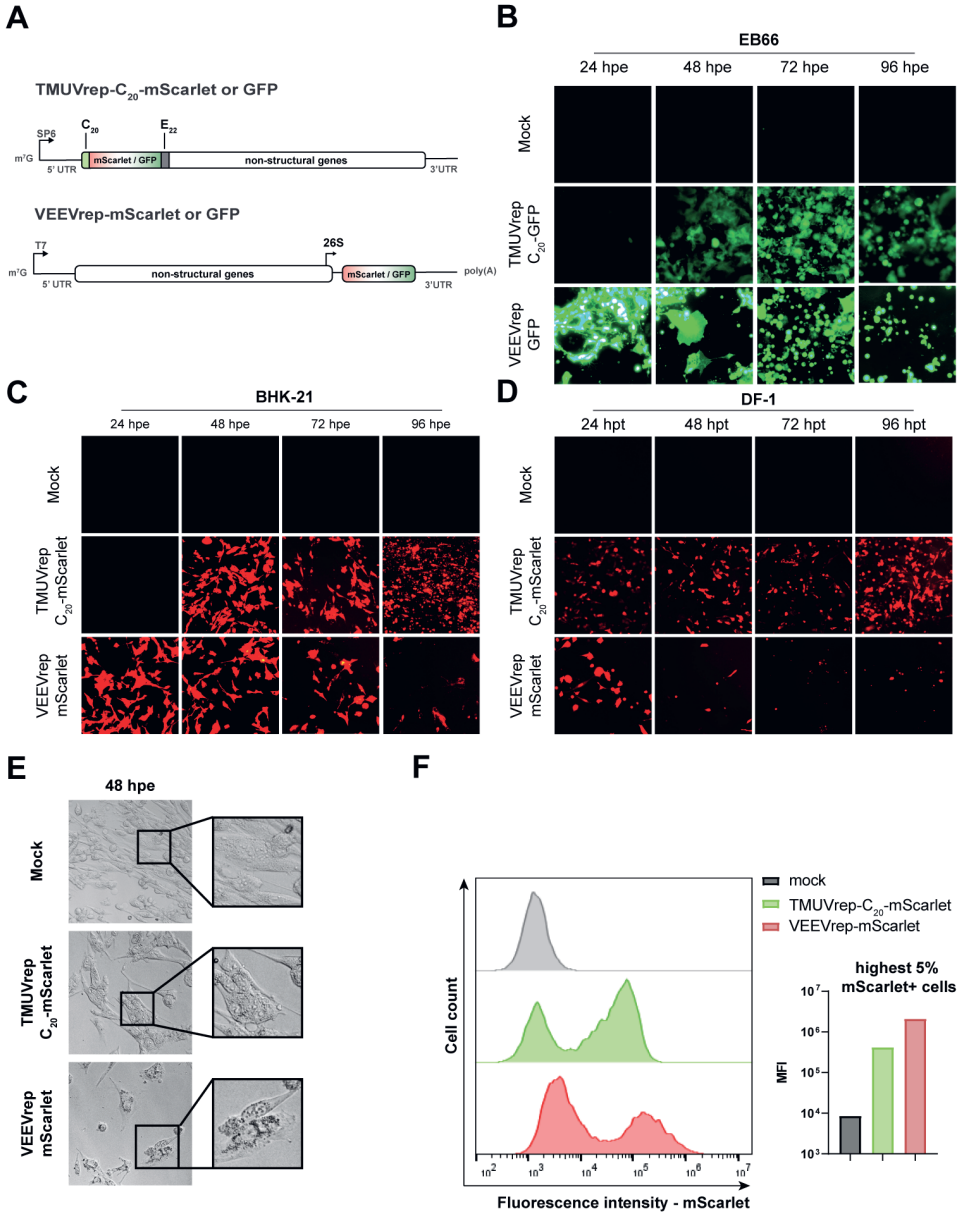
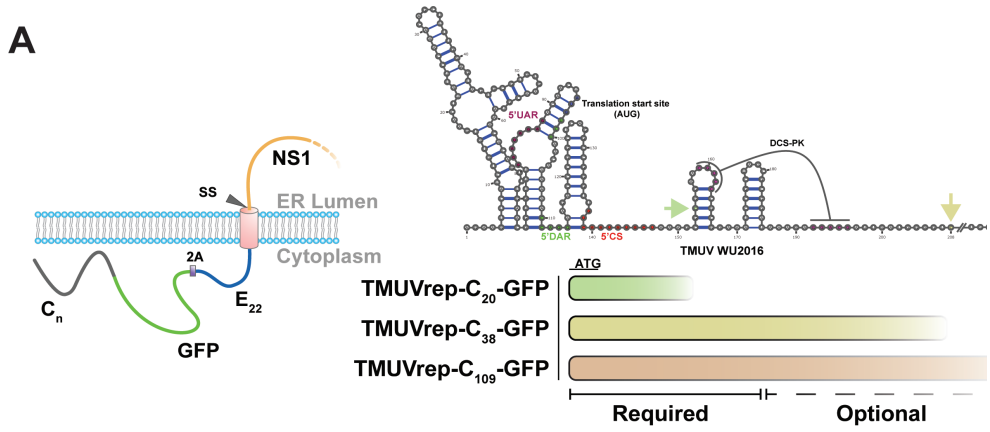


Figure 2. Comparison of fluorescence reporter gene expression between the TMUV and VEEV replicon RNA. (A) Schematic overview of the TMUV and VEEV replicon constructs encoding mScarlet (TMUVrep-C_{20}-mScarlet or VEEVrep-Scarlet) or GFP (TMUVrep-C_{20}-GFP or VEEVrep-GFP). Replicon RNA of the corresponding constructs were electroporated in **(B)** EB66, **(C)** BHK-21 cells, or transfected in **(D)** DF-1 and subsequently analyzed using fluorescence microscopy over a 96 h time course **(E)** Brightfield images of mock, TMUVrep-C_{20}-mScarlet, or VEEVrep-mScarlet electroporated BHK-21 cells. **(F)** After 48 hours post electroporation (hpe), BHK-21 cells harboring TMUVrep-C_{20}-mSarlet or VEEVrep-mScarlet were collected and measured with flow cytometry. Mean fluorescence intensity (MFI) was determined for the highest 5% fluorescent cells. hpt = hours post transfection.

Effects of *cis*-acting elements within the capsid gene of the TMUV replicon.

In earlier studies, the functional flexibility of the TMUV virus capsid gene was evaluated, highlighting the consequences of including a truncated capsid-coding region and its positive implications of putative *cis*-acting elements on viral replication (*He et al., 2019*). To investigate the presence of the *cis*-acting elements in the TMUV WU2016-based replicon and to assess their role in the dynamics of transgene expression, two additional TMUV replicon variants were constructed that encode the first 38 (TMUVrep-C_{38}-GFP) or 109 (TMUVrep-C_{109}-GFP) codons of the capsid gene (**Figure 3**). It was demonstrated that, in correspondence with earlier reports, all analogous patterns of cis-acting elements including the 5'AUR, 5'DAR, 5'CS, and DCS-PK were identified in the TMUVrep-C_{38}-GFP and TMUVrep-C_{109}-GFP variants (**Figure 3A**). The TMUVrep-C_{20}-GFP replicon, however, lacked the DCS-PK which resulted in an alternate expression kinetics observed during the fluorescence microscopy analysis of BHK-21 cells electroporated with the different TMUV replicon variants (**Figure 3B**). Interestingly, in cells containing TMUVrep-C_{38}-GFP or TMUVrep-C_{109}-GFP replicon RNA an earlier onset of transgene expression was observed compared to the TMUVrep-C_{20}-GFP replicon RNA. Moreover, the GFP accumulation measured in samples expressing TMUVrep-C_{109}-GFP replicon RNA was noticeably higher than TMUVrep-C_{20}-GFP and TMUVrep-C_{38}-GFP. Furthermore, the effect of the DCS-PK also became apparent when comparing the viral-induced cytopathic effect (CPE) of the various replicon RNA variants (**Figure 3C**). No observable difference was detected between the TMUVrep-C_{20}-GFP and TMUVrep-C_{38}-GFP in the first 24 hpe, however, after 48 hpe a noticeably stronger CPE was seen for TMUVrep-C_{38}-GFP. In contrast, VEEVrep-GFP evidently showed a more intense CPE from 24 hpe onwards.

During the direct comparison of TMUV replicon variants, a pronounced sub-nucleolar localization of the GFP signal became apparent in TMUVrep-C_{109}-GFP electroporated cells (**Figure 4A**). The application of a Hidden Markov Model analysis revealed a strong nuclear localization signal (NLS) at the C-terminus (position 103-109) of the capsid protein along with a weaker NLS consisting of three basic residues at position 29-32 (**Figure 4B**). Moreover, an additional nucleolar localization signal (NoLS) was identified in the last 16 amino acids of the mature capsid protein, hence the aberrant localization within nuclear bodies inside the nucleus. TMUVrep-C_{38}-GFP lacks the strong C-terminal NLS and NoLS, however, a faint nuclear localization remained visible in the electroporated cells. (*Nguyen Ba et al., 2009; Scott et al., 2011*).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TMUVrep-C_{109}-GFP
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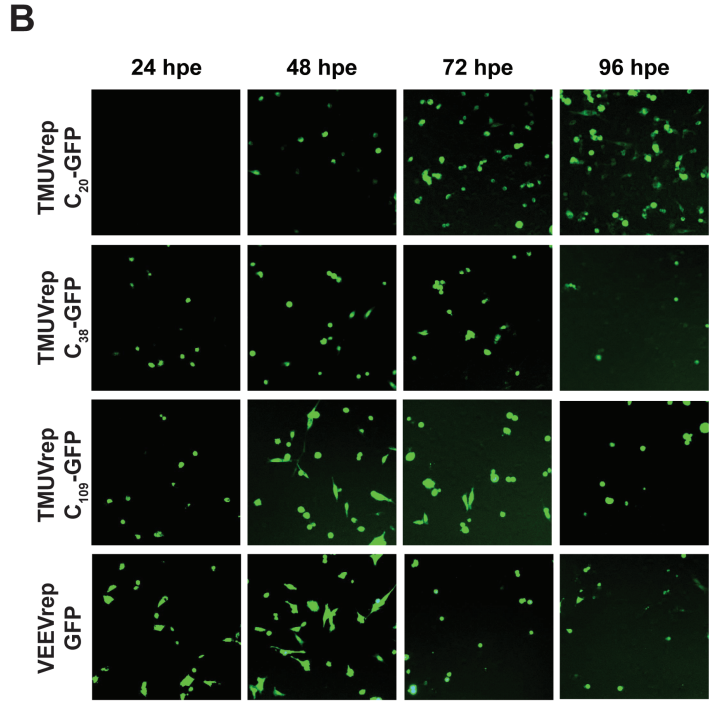
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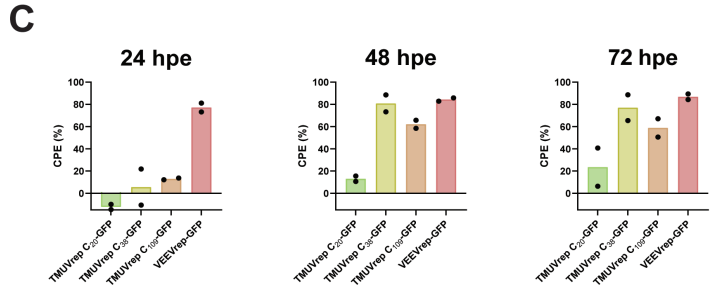
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TMUVrep C_{20}-GFP
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TMUVrep C_{109}-GFP
VEEVrep-GFP

◄Figure 3. The effect of *cis*-acting elements encoded within capsid gene on the heterologous protein expression and cytopathicity of the TMUV replicon. (A) Prediction of conserved secondary RNA structures using RNAstructure 6.3 and RNAalifold amongst different TMUV isolates within the capsid gene. Secondary structures were drawn using RNAalifold and VARNA software package (*Darty et al., 2009*). The following cis-acting elements were identified: 5' upstream of AUG region (5'UAR), 5' downstream of AUG region (5'DAR), 5' cyclization sequence (5'CS), and downstream of 5'CS pseudoknot (DCS-PK). Three different TMUV replicon RNAs were constructed to encode the first 20 (C_{20}), 38 (C_{38}) or 109 (C_{109}) amino acids of the capsid gene. **(B)** Fluorescence images and **(C)** comparative cytopathic effect analysis based on cellular ATP consumption as a surrogate marker of host cell viability of BHK-21 cells electroporated with TMUVrep-C_{20}-GFP, TMUVrep-C_{38}-GFP, TMUVrep-C_{109}-GFP, and VEEVrep-GFP replicon RNA. hpe = hours post electroporation.

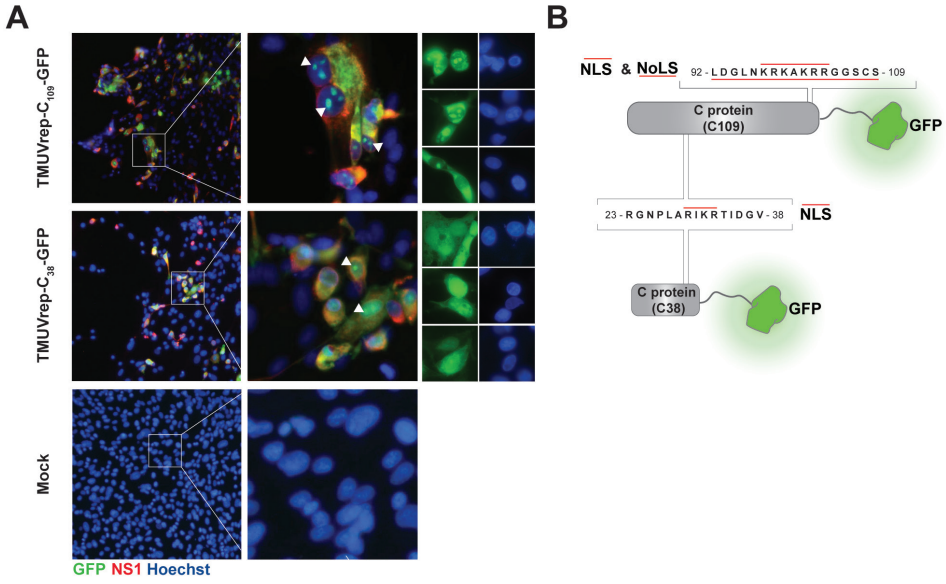

Figure 4. Nuclear localization of the TMUV (mature) capsid protein. (A) BHK-21 cells were electroporated with TMUVrep-C_{109}-GFP, TMUVrep-C_{38}-GFP replicon RNA, or DPBS and immuno-stained with α-NS1 (4G4; mouse) antibody and Alexa Fluor 546-conjugated α-mouse IgG (goat). **(B)** Schematic representation of the nuclear localization signal (NLS; overstruck in red) prediction using the Hidden Markov Model (*Nguyen Ba et al., 2009*) or nucleolar localization signal (NoLS; underlined in red) prediction (*Scott et al., 2011*) in C_{109} and C_{38}-GFP fusion protein expressed by TMUVrep-C_{109}-GFP and TMUVrep-C_{38}-GFP replicon, respectively. Hoechst was used to counterstain the nuclei.

To obtain a more accurate comparison between the protein expression levels between the TMUV replicon capsid variants and VEEV replicon, a flow cytometry analysis was conducted (**Figure 5**). BHK-21 cells were electroporated with the respective replicon RNAs, and samples

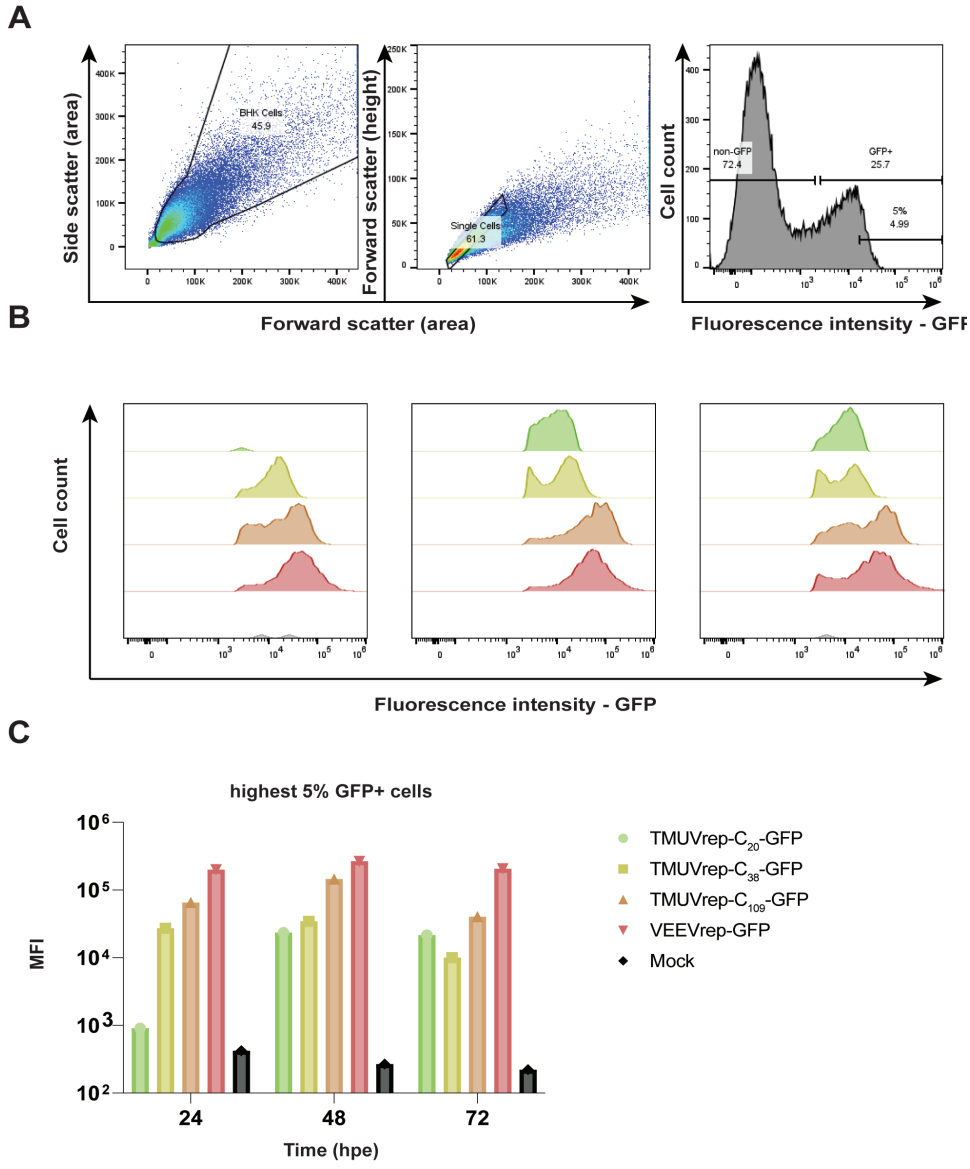

Figure 5. Semi quantitative comparison of GFP expression levels between the TMUV replicon RNA variants and VEEV replicon RNA using flow cytometry. (A) Fluorescent, single BHK-21 cells were gated according to a standardized method using the Sony-SH800 flow cytometer. **(B)** BHK-21 cells were electroporated using TMUVrep-C_{20}-GFP, TMUVrep-C_{38}-GFP, TMUVrep-C_{109}-GFP, or VEEVrep-GFP and analyzed over a 96-hour period. Y-axis displays the percentage of the maximum event count **(C)** The mean fluorescence intensity (MFI) was determined based on the highest 5% of GFP positive (GFP+) cells. hpe = hours post electroporation.

were taken every 24 h for four consecutive days. Subsequently, fluorescent single cells were gated and subjected to semiquantitative measurement of GFP expression (**Figure 5A**). Consistent with earlier fluorescence microscopy findings, TMUVrep-C_{20}-GFP exhibited barely detectable levels of GFP expression at 24 hpe (**Figure 5B**). In contrast, robust GFP signals were detected from 24 hpe onwards in cells electroporated with TMUVrep-C_{38}-GFP, TMUVrep-C_{109}-GFP, and VEEVrep-GFP replicon RNA. Moreover, the analysis confirmed that for the cumulative expression of the 5% highest expressing cells, VEEVrep-GFP outperformed the different TMUV replicon RNA variants (**Figure 5C**). However, a noticeably higher MFI was measured for TMUVrep-C_{109}-GFP compared to the TMUVrep-C_{20}-GFP and TMUCrep-C_{38}-GFP replicon variants.

Comparison of viral glycoprotein expression between the TMUV replicon variants.

To assess the TMUV replicons as a novel vaccine platform, the expression of the avian influenza glycoprotein hemagglutinin (HA) was compared among the replicons with different capsid lengths. The HA gene was cloned into the TMUV and VEEV replicon constructs (**Figure 6A**) and the replicon RNA was electroporated into BHK-21 cells. At 72 hpe, cells were stained for the presence of HA using an indirect immunofluorescence assay (**Figure 6B**).

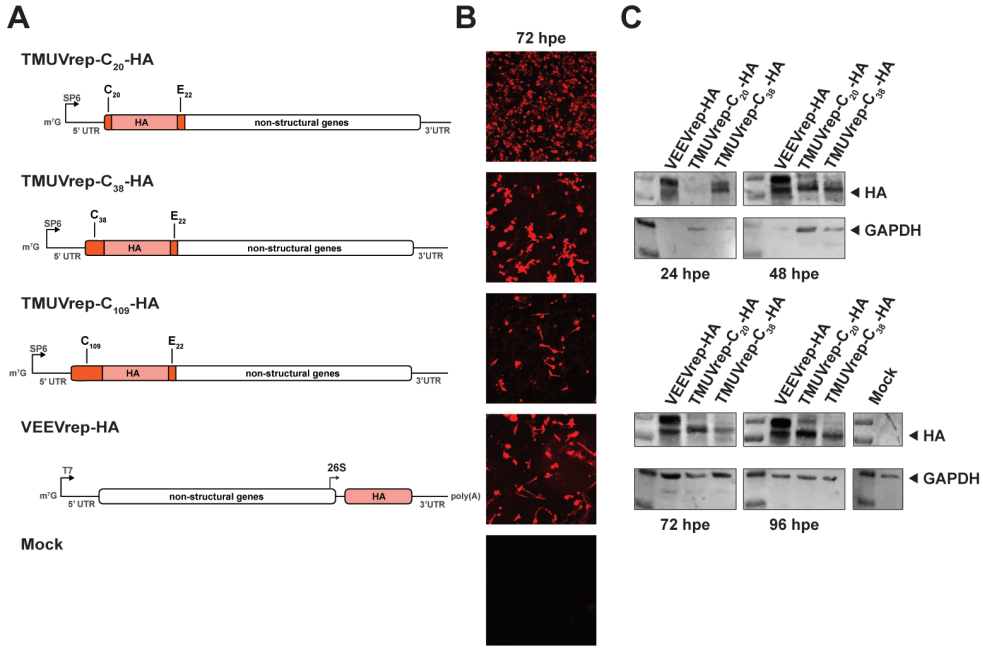

Figure 6. Comparison of avian influenza A virus hemagglutinin (HA) expression by VEEV replicon and TMUV replicons encoding different truncated capsid genes. (A) Schematic representation of the various TMUV and VEEV replicon construct encoding the AIV HA gene **(B)** Indirect immunofluorescence analysis of BHK-21 cells containing replicon RNA at 72 hours post electroporation (hpe). **(C)** Western blot detection of HA of replicon-expressed HA over a 96-hour period. Primary antibody: serum α-HA (chicken) and α-GAPDH (mouse). Secondary antibodies are Alexa Fluor 568 or alkaline phosphatase-conjugated α-chicken IgY (goat).

The expression of HA was detected in all cells electroporated with replicon RNA. This was in correspondence with earlier results on the expression of TMUVrep-C_{20}-HA (*Comes, Poniman, et al., 2023*). When the cell lysates were subject to a western blot, the HA glycoprotein migrated at the height of ~75 kDa. Similar to Rluc and mScarlet expression, the TMUVrep-C_{20}-HA-mediated HA expression was only detected from 48 hpe onwards while HA expression from TMUVrep-C_{38}-HA and VEEVrep-HA was already detected at 24 hpe.

4. Discussion

The VEEV-based replicon system is the most well-studied replicon platform, typically used as a tool for heterologous protein production or vaccination. A significant drawback of the VEEV replicon system is that the very high levels of alphavirus RNA replication and expression of both heterologous and viral replicase proteins often result in virus-induced CPE in many vertebrate cells (*Garmashova et al., 2007; Petrakova et al., 2005; Schlesinger & Dubensky, 1999*). In a previous study, we developed a bird-adapted TMUV replicon platform and showcased its robust and sustained gene expression in different cell lines (*Comes, Poniman, et al., 2023*). In this study, we created two additional replicons with extended capsid lengths and compared these to the established VEEV replicon platform. Our findings revealed that despite the later onset of reporter gene expression, the TMUVrep-C_{20}-GFP replicon RNA produced a significantly higher total amount of heterologous protein than VEEV replicon RNA. This difference could be attributed to the inherent low cytopathicity of the TMUV-based replicon system, similar to other flaviviruses such as WNV and TBEV (*Gehrke et al., 2005; Khromykh & Westaway, 1997; Pijlman et al., 2006; Varnavski & Khromykh, 1999*). Despite being described as less cytopathic compared to old-world alphaviruses, like Sindbis and chikungunya virus (*Garmashova et al., 2007; Petrakova et al., 2005; Sawicki & Sawicki, 1980*), VEEV still induced a more pronounced CPE than other flaviviruses (*Gehrke et al., 2005*). Our results demonstrated that VEEV replicon altered the cell morphology, increased cellular ATP consumption, and reduced the number of fluorescent cells compared to the TMUV replicon RNA. While the host transcriptional shutoff, a key factor in VEEV-mediated CPE, is mainly determined by the function of the capsid protein, it is not encoded on the VEEV replicon (*Garmashova et al., 2007*). Another, novel factor that might contribute to CPE in vertebrate cells is the host translational shut-off by the alphavirus replicase protein, nsP2 (*Treffers et al., 2023*). Comparing the TMUV replicon capsid variants with the VEEV replicon, it became apparent that only TMUVrep-C_{38}-GFP demonstrated an earlier onset of gene expression while TMUVrep-C_{109}-GFP demonstrated both an earlier onset and a higher GFP accumulation over time. Notably, these differences compared to TMUVrep-C_{20}-GFP came at the cost of a higher cytopathic effect. Previous studies already demonstrated the functional flexibility of the TMUV capsid protein by looking at the expression and replication kinetics (*He et al., 2021*). To our knowledge, this is the first report that directly compares the heterologous gene expression and virus-induced cytopathic effect of TMUV replicons with different capsid lengths. The variation in the onset and expression levels among different TMUV replicons capsid variants may be attributed to the presence of secondary RNA structures within the capsid gene. While the capsid protein sequence is not highly conserved amongst members of the

Flaviviridae family, the secondary RNA structures in this region are (*Z.-Y. Liu et al., 2013*). In this study, we identified *cis*-acting elements within the TMUV WU2016 capsid gene that play a role in the earlier onset of heterologous gene expression in both TMUVrep-C_{38}-GFP and TMUVrpe-C_{109}-GFP compared to TMUVrep-C_{20}-GFP (*He et al., 2020*). Specifically, the presence of the DCS-PK structure in both replicon variants appears to enhance viral RNA replication by presumably regulating genome cyclization, a mechanism observed in many other flaviviruses (*Z.-Y. Liu et al., 2013; Mazeaud et al., 2018*). However, further investigation is needed to determine whether the viral RNA levels increase when the first 38 or 109 amino acids are encoded by the TMUV replicon RNA, or whether translation of the polyprotein is enhanced. Notably, the disparity in GFP accumulation between TMUVrep-C_{38}-GFP and TMUVrep-C_{109}-GFP cannot solely be explained by the presence of *cis*-acting elements, as no other putative RNA structures were predicted downstream of DCS-PK. Therefore, we postulate that the effect of a higher GFP accumulation for the TMUVrep-C_{109}-GFP can potentially be attributed to the mature capsid protein. Particularly, since we also demonstrated a prominent difference in the localization of the capsid-GFP fusion protein in nuclear bodies within the nucleus. It is therefore possible that the capsid protein might (directly or indirectly) interact on a protein level. The mature capsid proteins encompass essential structural elements, such as hydrophobic α-helixes, which may facilitate interactions with (internal) biological membranes or binding of viral RNA (*He et al., 2020*). The distinct cellular translocation of the mature capsid protein could be attributed to the presence of an NLS and NoLS sequence near the C-terminus of the protein (*Sangiambut et al., 2008b*). Moreover, the presence of additional phosphorylatable residues in the C-terminus of the mature capsid protein may affect its localization or modulate its function (*X. Zhang et al., 2021*). Overall, the multifaceted properties of capsid make it a central player in various aspects of controlling viral replication such as interfering with RNA silencing (*Samuel et al., 2016*), inhibiting cell apoptosis (*Urbanowski & Hobman, 2013*), interacting with other host proteins (*Tsuda et al., 2006*) or promoting viral RNA replication (*Mori et al., 2005b*). Collectively, our data highlights that despite VEEV replicon RNA producing an early onset of a very high level of transgene expression, the long-lasting expression characteristic of the TMUV replicon RNA poses a valuable alternative replicon platform. Additionally, by encoding different capsid coding regions, the TMUV replicon expression can be tailored to achieve the desired onset and level of heterologous protein expression. As such, this avian-adapted TMUV replicon system provides promising prospects as a vaccine platform technology in avian species.

5. Acknowledgments

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Chapter 4

Bird-adapted self-amplifying mRNA vaccines in lipid nanoparticles generate seroconversion against infectious bursal disease virus in chickens

Abstract

Each year, millions of poultry die as a result of highly pathogenic avian influenza A virus (AIV) and infectious bursal disease virus (IBDV) infections. Conventional vaccines based on inactivated or live-attenuated viruses are useful tools for disease prevention and control, yet, they often fall short in terms of safety, efficacy, and development times. Therefore, versatile vaccine platforms are crucial to protect poultry from emerging viral pathogens. Self-amplifying (replicon) RNA vaccines offer a well-defined and scalable option for the protection of both animals and humans. The best-studied replicon platform, based on the Venezuelan equine encephalitis virus (VEEV; family *Togaviridae*) TC-83 vaccine strain, however, displays limited efficacy in poultry, warranting the exploration of alternative, avian-adapted, replicon platforms. In this study, we engineered a DNA-launched RNA replicon based on a duck Tembusu virus (TMUV; family *Flaviviridae*) and successfully expressed the AIV haemagglutinin (HA) glycoproteins and the IBDV capsid protein (pVP2). To assess the immune responses elicited by the TMUV replicon system in chickens, a prime-boost vaccination trial was conducted using lipid nanoparticle (LNP)-formulated replicon RNA and DREP encoding the viral (glyco)proteins of AIV or IBDV. While TMUV replicon RNA was unable to induce a humoral response against AIV, it significantly promoted the IBDV-specific seroconversion in vaccinated chickens, in contrast to VEEV replicon RNA, which showed no significant humoral response. In conclusion, this study highlights the potential of an avian-adapted, TMUV-derived replicon platform as a novel vaccine technology to combat emerging poultry diseases.

1. Introduction

The development of new efficacious vaccine platforms is crucial to protect chickens against novel emerging viral pathogens that annually cause the death of millions of poultry worldwide. During the 1997 avian influenza virus (AIV) H5N1 pandemic, it became clear that solely human vaccination against zoonotic influenza viruses was not sufficient to prevent new pandemics from arising (*Baz et al., 2013; Shortridge, 2005; Swayne, 2012b; van der Velden et al., 2014; X. Zeng et al., 2018*). Since then, not only vaccination of poultry against AIV H5N1 was implemented, but also routine vaccination against other avian-specific pathogens gained more interest as a potent control measure to prevent disease worldwide (*H. Chen & Bu, 2009; Yee et al., 2009*). Although conventional (inactivated or live-attenuated) vaccines were a step forward in the prevention and control of diseases in birds, the vaccines often performed sub-optimally in terms of safety, efficacy, or development times (*Marangon & Busani, 2007; D. Wu et al., 2015*). As such, still, millions of poultry are culled each year due to the sudden occurrence of highly pathogenic AIV or very virulent infectious bursal disease virus (IBDV). To mitigate morbidity, mortality, and the zoonotic spillover of poultry pathogens to humans, an effective and versatile vaccine platform technology is required.

Conventional vaccines are less suitable for emergency vaccination during an outbreak, because of biosafety regulations, difficulties during pathogen cultivation, or less control over batch-to-batch variations. The first step in creating a more easily adaptable vaccine platform was the registration of recombinant live viral vector vaccines. A commercially available vector for poultry is the genetically modified version of the meleagrid herpesvirus 1, commonly known as turkey herpesvirus or herpesvirus of turkey (HVT). This recombinant HVT vaccine can be modified relatively easily and can encode multiple antigens of interest rendering it a suitable platform for multivalent vaccination (*D. Li et al., 2011; L. Liu et al., 2019; N. Tang et al., 2018; van Hulten et al., 2021*). Nevertheless, scaling up the production of this cell-associated recombinant live virus is challenging due to the requirement of primary chicken embryo fibroblast cells for virus propagation, which restricts its suitability as a platform for rapid response vaccines (*Śmietanka et al., 2019; Zai et al., 2022*).

Interestingly, the recent coronavirus disease 2019 (COVID-19) pandemic has accelerated the use of nucleic acid-based messenger (m)RNA vaccines, which are also attractive for the poultry industry as they are generally well-defined, scalable, and most importantly, quickly adaptable. Current mRNA vaccines for human use require a substantial dose (30 micrograms for BioNtech/Pfizer's Comirnaty, 100 micrograms for Moderna's Spikevax) plus a booster vaccination in order to provide protective immunity (*Haveri et al., 2022; Moraga-Llop, 2023*). However, smaller doses have been reported for self-amplifying mRNA vaccines, the so-called replicons (*Bloom et al., 2021*). These replicon formulations are often based on the replication genes of Venezuelan equine encephalitis virus (VEEV; family *Togaviridae*; genus Alphavirus) TC-83 vaccine strain or Kunjin virus (KUNV; family *Flaviviridae*; genus Flavivirus) (*Langereis et al., 2021; Pijlman et al., 2006; R. Vander Veen et al., 2009; Varnavski & Khromykh, 1999*). To generate replicon vaccines, the wildtype viral genome is modified by replacing the viral

structural genes for a gene of interest. Since only the replicase genes are maintained, the replicon RNA can self-amplify in the cytoplasm but cannot propagate or spread to other cells. The process of active RNA amplification in cells receiving the replicon may eliminate the need for an adjuvant while a strong immune response is induced (*Erasmus et al., 2020; Langereis et al., 2021; R. L. Vander Veen et al., 2012*). Despite this self-adjuvating effect of replicon vaccines, the VEEV replicon platform does not always induce a protective immune response in poultry (*Schultz-Cherry et al., 2000; Sylte et al., 2007*). Consequently, there is a niche for an alternative, preferably a poultry-adapted, replicon-based platform. A suitable virus candidate to develop an alternative replicon for poultry is the Tembusu virus (TMUV), a member of the *Flaviviridae* family. TMUV has been isolated from a wide range of bird species such as ducks, geese, chickens, and pigeons, and thus shares hosts with the avian influenza virus (*Hamel et al., 2021*).

In this study, we constructed a DNA-launched replicon (DREP) platform based on the TMUV isolate Perak and assessed the expression of hemagglutinin (HA) glycoprotein of AIV and nucleocapsid protein (VP2) of IBDV. Furthermore, we describe the immunization of chickens with TMUV- versus VEEV-based replicons containing the viral structural transgenes of AIV or IBDV. The replicon RNA and DREP vectors were formulated in lipid nanoparticles (LNP) and administered in a homologous prime-boost setting to 1-day-old specific pathogen-free (SPF) chickens. The humoral responses in chickens against HA and VP2 were followed over time and compared to those induced by commercial vaccines Vaccine M or Innovax-ND-IBD® (both MSD Animal Health), respectively. We conclude that a synthetically produced, TMUV-based replicon system has the potential to vaccinate poultry against emerging viral pathogens.

2. Material and methods

Cells

Baby Hamster kidney cells (BHK-21, CCL-10) were cultured at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS) and 100 $U \cdot mL^{-1}$ penicillin-streptomycin (Gibco).

Plasmids

The constructed TMUV DREP was based on the Tembusu isolate Perak (Genbank accession no.: KX097989) and synthesized in a single-copy pCC1BAC backbone (Genscript). VEEV DREP was based on the attenuated VEEV TC-83 strain and synthesized in a high-copy pUC57 backbone (Genscript) (*Hooper et al., 2009*). Both DREP constructs were designed with cytomegalovirus (CMV) promoters upstream of the replicon sequence. To ensure the formation of a native 3' untranslated region (UTR) in the TMUV replicon RNA, similar to that described in (*Comes, Poniman, et al., 2023*), a Hepatitis delta virus (HDV) ribozyme and simian virus 40 (SV40)-poly(A) signal were included downstream of the 3'UTR terminus. To insert the viral transgenes of HA (AIV) or pVP2 (IBDV) in TMUV and VEEV DREP, unique *AscI-AvrII* and *AscI-PacI*

restriction sites were incorporated in each replicon, respectively. All plasmids were purified using the endotoxin-free Nucleobond Midiprep kit (Macherey-Nagel) and the DNA concentration was determined using the spectrophotometer ND-1000 (Nanodrop). Plasmids were stored for further use at -80°C. For the evaluation of TMUV and VEEV DREP constructs, sub-confluent BHK-21 cells were transfected using Lipofectamine 2000 reagent (Invitrogen) in Opti-MEM (Gibco) according to the manufacturer's protocol. After 4 h, the monolayer was washed with Dulbecco's phosphate-buffered saline (DPBS; Gibco) and fresh supplemented medium was added. Cells were incubated at 37°C with 5% CO_2 prior to the indirect immunofluorescence assays.

Secondary RNA structure prediction

Models of the secondary RNA structures within the 5'UTR and capsid region of the TMUV WU2016 genome (Genbank accession no.: OQ920272) were predicted using the webserver RNAalifold (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAalifold.cgi) and RNAstructure 6.3 and verified using the phylogenetically related TMUV isolates aligned in Clustal X V2.0. The secondary RNA structures were visualized and modeled in VARNA (*Darty et al., 2009*). The secondary structures that were selected are based on the minimum free energy prediction not taking into account weaker GU pairs at the end of helices. For long-distance RNA interaction such as 5' upstream AUG region (5'UAR), 5' downstream AUG region (5'DAR), and 5' cyclization signal (5'CS) the prediction was generated using the Mfold web server (http://www.unafold.org/mfold/applications/rna-folding-form.php) (*Zuker, 2003*) in which the 5'UTR and 3'UTR were separated by a poly(A) stretch of 100 nt (*Khromykh et al., 2001*).

In vitro replicon RNA transcription, purification, and electroporation

The construction of the SP6-driven TMUVrep-C_{20} or TMUVrep-C_{38} replicon, encoding the first 20 or 38 N-terminal amino acids of the capsid protein, respectively, has been described in (*Comes, Poniman, et al., 2023*) and **Chapter 3**. Replicon-encoding DNA plasmids were purified using the endotoxin-free Nucleobond Midiprep kit (Macherey-Nagel) and linearized using *PacI* (TMUV-derived replicons) or *NotI* (VEEV-derived replicons) restriction enzymes. Capped in vitro transcribed replicon RNAs were generated by using 2.5 micrograms of linearized plasmid DNA in an SP6- or a T7-polymerase reaction (both from New England Biolabs) for TMUV and VEEV replicon constructs, respectively. The reaction was incubated for 2 h at 37°C and thereafter incubated for 30 min at 37°C with RNAse-free DNAse (Promega). Replicon RNA was purified using the RNeasy Midiprep kit (Qiagen) according to the manufacturer's RNA Cleanup protocol. Both the quantity and quality of the in vitro transcribed RNA were analyzed using the spectrophotometer ND-1000 (Nanodrop) and via conventional electrophoresis using a 1% agarose gel in tris-acetate-EDTA (TAE) buffer for 15 min at 150V. The generated replicon RNA was stored at -80°C until further use. For the evaluation of replicon RNA, BHK-21 cells were electroporated using the Gene Pulser Xcell (Bio-Rad Laboratories). Purified replicon RNA was mixed with 6 x 10^6 resuspended BHK-21 cells in DPBS and pulsed twice (850 V/25 µF) in a 0.4 cm electroporation cuvette (Bio-Rad Laboratories). After electroporation, cells were recovered in 10 mL supplemented medium and incubated at 37°C under 5% CO_2.

Replicon formulation into lipid nanoparticles

The purified replicon RNA and DNA-encoded replicon plasmids were formulated into LNPs. First, a mixture of ionizable lipids, cholesterol, distearoylphosphatidylcholine (DSCP), and polyethylene glycol (PEG) C14 was dissolved at a molar ratio of 58:30:10:2 to a final lipid concentration of 22.34 mM in absolute ethanol. The lipid mixture was then emulsified (N/P ratio 6) with a citrate buffer (pH 5) containing the purified DNA or RNA stock using the NanoAssemblr microfluidic system (Precision Nanosystems). Next, the resulting LNPs were dialyzed against ddH_2O for 3-6 h at RT and subsequently against TRIS-G buffer (10mM TRIS, 70mM NaCl, 5% sucrose) for 12-18 h at 2-8°C. The dialyzed LNPs were then filter sterilized using a double-layer filter (Acrodisc PF syringe filter, 0.8/0.2 µm/25 mm; Thermo Fisher Scientific). Following this, the LNP particle size (Z-average) and polydispersity (pdi) were measured by dynamic light scattering (DLS). Additionally, the concentration and formulation efficiency were determined by an LNP disruption assay specific for either DNA or RNA. For the DNA:LNP disruption assay, 10 µL of each DNA:LNP formulation was treated using 4% (v/v) Triton-X-100 in DPBS and incubated at 37°C for 10 min. Hereafter, the disrupted LNP were mixed with 0.2% SybrGold in DPBS solution, incubated for 60 seconds while agitating at 500 rpm, and measured using the ClarioStar spectrofluorometer (ex.: 485 nm, em.: 530 nm; BMG Labtech). A plasmid DNA standard was used to determine the DNA concentration. For the RNA:LNP disruption assay, 10 µL of each RNA:LNP formulation was treated using 4% (v/v) Triton-X-100 in DEPC-treated ddH2O and incubated at 37°C for 10 min. The released RNA was separated using conventional gel-electrophoresis (1% agarose in tris-acetate-EDTA buffer) for 30 min at 150V. Quantitative and qualitative analysis of the released RNA was accomplished using an in-house replicon RNA standard and Image Lab software (Bio-Rad Laboratories).

Coomassie Brilliant Blue staining and western blotting

Cell fractions and precipitated supernatant fractions were dissolved in DPBS and incubated at 95°C for 5-10 min with a loading buffer containing β-mercaptoethanol. Protein samples were then separated using an 8-16% Mini-PROTEAN TGX Precast Protein gel (Bio-Rad Laboratories). After electrophoresis, proteins were visualized by Coomassie brilliant blue (CBB) staining or transferred to a polyvinylidene difluoride membrane (PVDF; Invitrogen) for immunodetection. Membranes were blocked overnight at 4°C with 5-10 mL DPBS-T containing 1% skimmed milk (blocking buffer). Then, membranes were incubated for 1 h at room temperature with diluted convalescent α-HA serum (chicken, 1:500) or α-VP2 (mouse, R63)(*k*) antibodies in blocking buffer. Thereafter, secondary antibodies α-chicken IgY conjugated with alkaline phosphatase (AP; goat, 1:2500; Sigma-Aldrich) or α-mouse IgG-AP (goat, 1:2500; Sigma-Aldrich) were added and incubated for 1 h at RT. Membranes were washed and proteins were visualized by incubation of 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP; Sigma-Aldrich) staining in 5 ml AP-buffer. To remove the N-glycosylated groups from the glycoproteins, cell lysates were treated using peptide-N-glycosidase F (PNGase F; New England Biolabs) according to the manufacturer's recommendation.

Indirect immunofluorescence assay

Indirect immunofluorescence assays were performed to detect the presence of viral (glyco) proteins in replicon-transfected cells. A monolayer of BHK-21 cells was washed using DPBS and fixed using 4% paraformaldehyde in DPBS at RT for 10 min. The cells were washed and permeabilized using 0.1% sodium dodecyl sulfate (SDS) in DPBS at RT for 10 min. Next, the monolayer was blocked by 5% FBS in DPBS at 37°C for 1 h and incubated at 37°C for 1 h with primary antibody diluted in 5% FBS in DPBS; for α-VP2 (mouse, 1:1000), α-HA serum (chicken, 1:500). Hereafter, cells were incubated with a secondary α-mouse or α-chicken IgG conjugated with Alexa fluor 546 (goat, 1:2000; Invitrogen) antibody in 5% FBS in DPBS at 37°C for 1 h. Cells were then stained with Hoechst (1:100; Thermo Fisher Scientific) in DPBS for 5 min. Photos were acquired using the Axio Observer Z1 fluorescence microscope (Zeiss).

Chicken vaccination and sampling

One-day-old, specific-pathogen-free (SPF) chickens (white leghorn layers) were divided into six groups with 10 chickens in each group. The chickens assigned to each group were tagged and placed in a housing isolator with water and feed *ad libitum*. Day-old chickens were vaccinated via the intramuscular route with 10 µg or 3 µg LNP-formulated replicon RNA or DREP, respectively. At T=3 weeks, an additional booster injection of 10 µg (LNP:RNA) or 3 µg (LNP:DREP) was administered. One-day-old chickens belonging to the positive control group were vaccinated subcutaneously in the neck with Vaccine M or Innovax-ND-IBD® (both MSD Animal Health, Boxmeer). The mock group received no vaccination. Blood samples (1.1 mL) were collected from the left- and right-wing vein of all individuals in each group at T=3, 5, 7, 10, and 13 weeks post-vaccination. Serum was collected using Bio-One Vacuette Z Serum clot activator tubes (Greiner). Clotting of the blood was achieved by inverting the tubes and incubation at 4°C for 30 min. Subsequently, the tubes were centrifuged at 3.000 x g at 20°C for 10 min and then heat-inactivated at 56°C for 30 min. The serum samples were stored at -20°C.

Serology

HA- and VP2-specific antibodies from the collected serum were detected with a commercially available antibody ELISA kit (ID Screen Influenza H5 indirect or ID Screen IBD VP2; IDvet) or reversed competition ELISA kit (IDscreen Influenza H5 antibody competition; IDvet) following the standard protocol provided by the manufacturer. Hemagglutination inhibition (HI) assays were used to differentiate HA-specific antibodies against the H5N1 subtype according to the World Health Organization guidelines (*World Health Organization, 2002*). An unpaired, two-tailed *t*-test (Mann-Whitney) was used to assess the statistical significance of the antibody readouts detected in the indirect ELISAs or HI assay ($p \leq 0.05$ was considered statistically significant).

3. Results

Design and construction of a DNA-launched replicon construct

The design of the DREP was based on the Tembusu virus isolate Perak (Genbank accession nr: KX097989), which is a virus isolated from ducks (*Homonnay et al., 2014*). TMUV Perak shows a high nucleotide (92%) and protein (99%) sequence identity to the previously described TMUV WU2016 (Genbank accession no.: OQ920272). Whole genome analysis shows that the TMUV Perak clusters together with other duck-derived TMUV isolates. A more distant relationship is observed for mosquito and chicken-derived isolates such as TMUV MM1775 and Sitiawan virus (STWV), respectively **(Figure 1A)**. (*Kumar et al., 2016; Nei & Kumar, 2000*) For the TMUV DREP platform, the design contained only the first 20 amino acids of the capsid protein and the last 22 amino acids of the envelope protein **(Figure 1B)**, similar to the RNA replicon design (*Comes, Poniman, et al., 2023*). The DREP constructs were transfected into BHK-21 cells and the expression of viral transgenes was visualized using an indirect immunofluorescence assay (**Figures 1C & 1D**). The expression of pVP2 (**Figure 1C**) and HA (**Figure 1D**) from TMUV DREPs was detectable by IFA despite the lower transfection efficiency compared to the benchmark VEEV DREP or the TMUV replicon RNA. These differences in expression efficiency might be explained by the significantly larger size of the TMUV DREP (pCC1BAC, 11.6 kbp + TMUV replicon, ~ 10.5 kbp ≈ 22 kbp) compared to the VEEV DREP (pUC57, 3.1 kb + VEEV replicon ~ 9.5 kbp ≈ 12.6 kbp).

Viral (glyco)protein translocation and glycosylation status

To investigate whether the viral (glyco)proteins expressed from the different replicons were correctly processed intracellularly, BHK-21 cells were transfected with replicon RNA of either the TMUVrep-C_{20}-pVP2, VEEVrep-pVP2, TMUVrep-C_{20}-HA, or VEEVrep-HA to determine the localization of the pVP2 and HA proteins. Since the pVP2 protein of wildtype IBDV is autoproteolytically processed in the cytoplasm (*Méndez et al., 2017*), it is expected that pVP2 accumulates in a similar location when expressed from the replicon RNA (**Figure 2A**). The AIV membrane protein HA naturally encodes both a signal sequence (SS) and a transmembrane domain (TMD) targeting the protein to the ER and subsequently via the Golgi to the plasma membrane (**Figure 2A**). The intracellular localization of pVP2 and HA was determined by an indirect immunofluorescence assay using α-VP2 and α-HA antibodies (**Figure 2B**). The pVP2 protein was detected in clusters dispersed throughout the cytoplasm, and only in SDS-permeabilized cells, whereas HA could be detected both in the presence and absence of SDS. This indicates that the pVP2 protein remained inside the cells and that the HA protein was displayed on the outside of the plasma membrane, as expected. To investigate whether the viral (glyco)proteins showed the correct molecular mass and whether HA was glycosylated, cell lysates of replicon RNA-transfected BHK-21 cells were analyzed on a western blot **(Figures 2C-E)**. The pVP2 protein migrated at the expected molecular mass of ~ 48 kDa

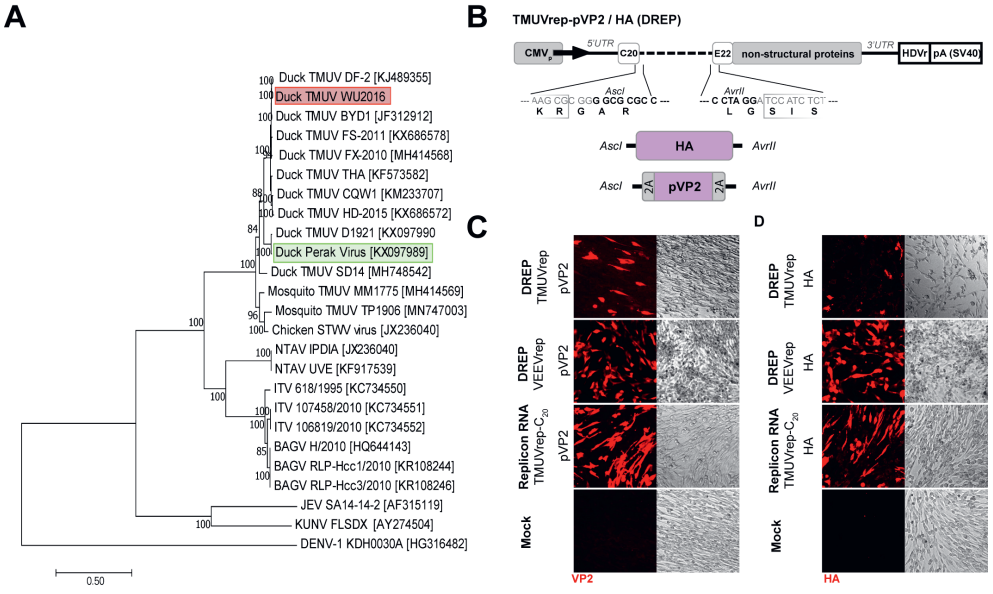


Figure 1. Construction and validation of the TMUV DNA-launched replicon (DREP). (A) Phylogenetic analysis of the TMUV isolate Perak based on a multiple sequence alignment of the complete flavivirus genomes retrieved from Genbank. The tree was constructed using the maximum likelihood method based on the general time reversible model. A discrete gamma (+G) distribution was used to model evolutionary rate differences among sites. The tree with the highest likelihood is shown in which the percentage indicates the constructed trees were the included isolates clustered together (100 replicates) (*Kumar et al., 2016; Nei & Kumar, 2000*). **(B)** Design of the TMUV Perak DREP is similar to the replicon RNA construct. The *AscI* and *AvrII* cloning sites (bold) within the DREP construct allow for in-frame insertion of the avian influenza virus haemagglutinin (HA) and infectious bursal disease virus nucleocapsid protein (pVP2) flanked by foot-and-mouth disease virus (FMDV) 2A elements. The RNA transcription is under control of the human cytomegalovirus promoter (CMV_p) starting from the 5' untranslated region (5'UTR). To ensure that the highly conserved 3' untranslated region (UTR) contained an authentic nucleotide end, a Hepatitis δ virus-like ribozyme (HDVr) followed by the simian virus 40 (SV40) polyadenylation signal (pA) was inserted downstream of the last nucleotide of the 3'UTR. C_{20}: first 20 amino acids of capsid; E22: last 22 amino acids of envelope protein are indicated. BHK-21 cells transfected with TMUV replicon RNA, TMUV DREP or VEEV DREP expressing the viral (glyco) proteins **(C)** pVP2) or **(D)** HA were detected using an indirect immunofluorescence assay. Primary antibodies: α-HA convalescent sera (chicken), monoclonal α-VP2 IgG (mouse). Secondary antibody: Alexa Fluor 546-conjugated α-chicken IgY (goat) or α-mouse IgG (goat).

(**Figure 2C**). An additional faint band of 55 kDa was observed, which could result from incomplete ribosome skipping by FMDV-2A (**Figure 2A**) (*Furler et al., 2001; Minskaia et al., 2013*). For the detection of HA glycosylation, the cell lysates of transfected cells with either the TMUV or VEEV replicon RNA were treated with peptide-N-glycosidase F (PNGase F) to remove putative N-linked oligosaccharides (**Figure 2D**). The expression of HA from either replicon RNAs was detected at a molecular mass of ~ 70-100 kDa. In the PNGase F-treated samples, both detected

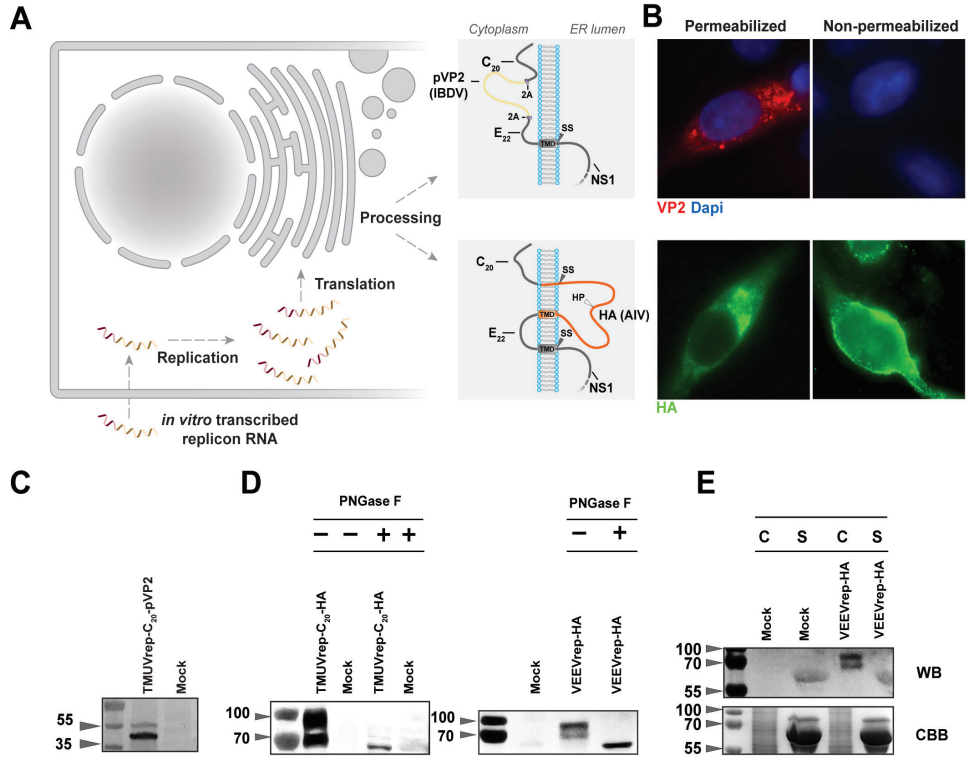


Figure 2. Cellular location and processing of viral (glyco)proteins expressed using replicon RNA in BHK-21 cells. (**A**) A schematic overview of the replication and translation of in vitro transcribed replicon RNA and the membrane topology of the viral (glyco)proteins in the cytoplasm or the lumen of the endoplasmic reticulum (ER). C_{20}: first 20 amino acids of capsid (C) protein, E_{22}: last 22 amino acids of envelope (E) protein, 2A: foot-and-mouth disease virus element 2A, HP: trypsin-like protease cleavage site, SS: signal peptide cleavage sites, TMD: trans-membrane domain, NS1: viral nonstructural protein 1. (**B**) Indirect immunofluorescence assay of transfected BHK-21 cell in the presence (*left*) or absence (*right*) of 0.1% SDS for the localization of viral (glyco)proteins. (**C**) Western blot analysis of transfected BHK-21 cell lysate for the detection of pVP2. (**D**) Lysate from BHK-21 cells transfected with TMUV replicon RNA (left) or VEEV replicon RNA (right) were analyzed using western blot for the presence of N-glycosylation of HA (**E**) Western blot (WB; top panel) and Coomassie brilliant Blue (CBB; bottom panel) of HA detection in both the cellular fraction (*c*) and concentrated supernatant (*s*) of transfected BHK-21 cells. Primary antibodies: α-HA convalescent chicken sera and monoclonal α-VP2 IgG (mouse). Secondary antibodies for immunofluorescence assay: Alexa Fluor 488 or 546-conjugated α-chicken IgY (goat) or α-mouse IgG (goat) respectively. Secondary antibodies for western blot: alkaline phosphatase-conjugated α-chicken IgY (goat) or α-mouse IgG (goat). Cell nuclei were counterstained using Hoechst. Protein sizes in kDa are indicated on the left.

protein bands shifted to a single protein band at a molecular mass of ~ 60kDa, suggesting that HA was indeed *N*-glycosylated. To confirm the tethering of the HA protein in the plasma membrane, the (concentrated) supernatant was screened for the presence of any soluble HA protein (**Figure 2E**), which confirmed that no HA protein could be detected in the supernatant.

Formulation of DREP and Replicon RNA into lipid nanoparticles

Replicon RNA or DREP was formulated in LNPs for the *in vivo* delivery to chickens. Both VEEV and TMUV replicon RNAs and DREPs were purified and formulated using the NanoAssemblr Ignite microfluidic system to mix an organic phase of lipids in ethanol with an aqueous phase containing the nucleic acids into LNPs (**Figure 3A**). These LNPs were then analyzed using transmission electron microscopy (TEM) and dynamic light scattering (DLS) to characterize the LNP shape and size (**Figures 3B-D**). Particles were detected ranging from 50 to 600 nm in diameter (**Figure 3B**). Although no significant difference was detected in the mean particle size between the various formulations, LNPs sized larger than 300 nm were only observed when LNPs contained a nucleic acid cargo (**Figure 3C**). The analysis of LNPs using DLS yielded an average size of 150 nm with a similar diversity in particle size and a comparable trend in size distribution as for empty LNPs (**Figure 3D**). For 1-day-old chickens to be vaccinated with 10 µg or 3 µg of LNP:RNA or LNP:DREP, respectively, a minimal concentration of 50 ng/µL formulated vaccine should be achieved to minimize the injection volume. To accurately determine the vaccination dose and quality of the formulated replicon RNA, an LNP:RNA disruption assay was performed (**Figure 3E**). This assay showed that a concentration of 94-140 ng of replicon RNA/µL of the formulated vaccine was realized and that the quality of replicon RNA was similar to before the formulation. Furthermore, it was shown that a formulation efficiency of more than 50% was obtained in all formulations (**Table 1**).

RNA replicon and DREP vaccination of chickens against IBDV

To test the ability of TMUV replicons to induce an antigen-specific immune response in chickens, the LNP:RNAs and LNP:DREPs encoding either HA (**Figure 4**) or pVP2 (**Figures 5 & 6**) were used in a prime-boost vaccination study. Each experimental group consisted of ten chickens, while the positive control (HVT-ND-H5) and unvaccinated (Mock) groups included five chickens each. At T=0 weeks, prime vaccination with 10 µg of LNP:RNA or 3 µg LNP:DREP encoding the HA protein was conducted by injection into the right leg of day-old chickens. At T=3 weeks, a booster injection of 10 µg or 3 µg was administered, respectively (**Figure 4A**). Serum was obtained at timepoints T=3, 5, and 7 weeks post prime vaccination (ppv), and the antibody response was detected by reversed competition ELISA, indirect ELISA (**Figures 4B-E**), or hemagglutinin inhibition assay (**Figure S1**). In all the sera and at all time points, no significant response against HA passed the manufacturer's threshold for seroconversion in the reversed competition ELISA, irrespective of whether the chickens were vaccinated with TMUV or VEEV replicon RNA (**Figure 4B**), whereas the positive control showed seroconversion at all time points. Only one chicken in the group vaccinated with VEEVrep-H5 replicon RNA showed both a positive seroconversion for the reversed competition ELISA (**Figure 4B**) and a positive response in the HI test (**Figure S1**) at 7 weeks ppv. Similar to the reversed competition assay, no significant HA-specific antibody response was detected in the sera of TMUV replicon RNA vaccinated chickens using an

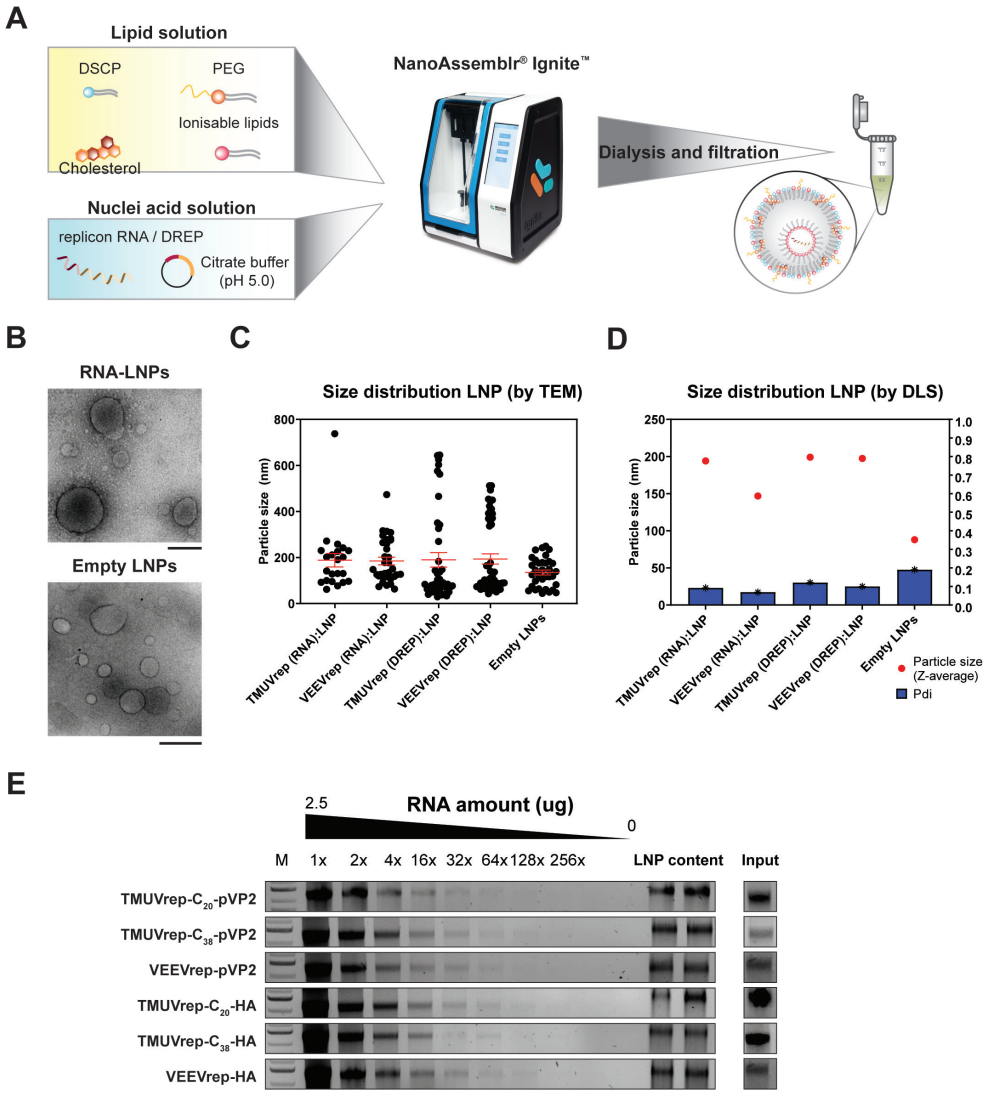


Figure 3. Replicon RNA and DREP formulation in lipid nanoparticles. (A) Schematic overview of the formulation of lipid nanoparticles (LNPs) using the NanoAssemblr Ignite microfluidic system by mixing an aqueous phase, containing replicon RNA in a citrate buffer (pH 5.0) and an organic phase, containing ionizable lipids, cholesterol, distearoylphosphatidylcholine (DSCP), and polyethylene glycol (PEG). **(B)** Empty and RNA-containing LNPs were analyzed by transmission electron microscopy (TEM) and the size distribution determined via **(C)** semiquantitative analysis from the acquired TEM images or **(D)** dynamic light scattering (DLS). For the measurements using DLS, the average particle size (Z-average; *left axis*) and size polydispersity index (Pdi; *right axis*) were visualized in the graph. **(E)** An LNP disruption assay was performed to determine the quantity and quality of the formulated LNPs against a replicon RNA standard visualized via conventional gel electrophoresis. The dilution factors of the RNA standard (1x-256x) are indicated above the images. 'Input' resembles unpurified, unformulated *in vitro*-transcribed RNA. Error bars represent standard error. Scale bars represent 200 nm.

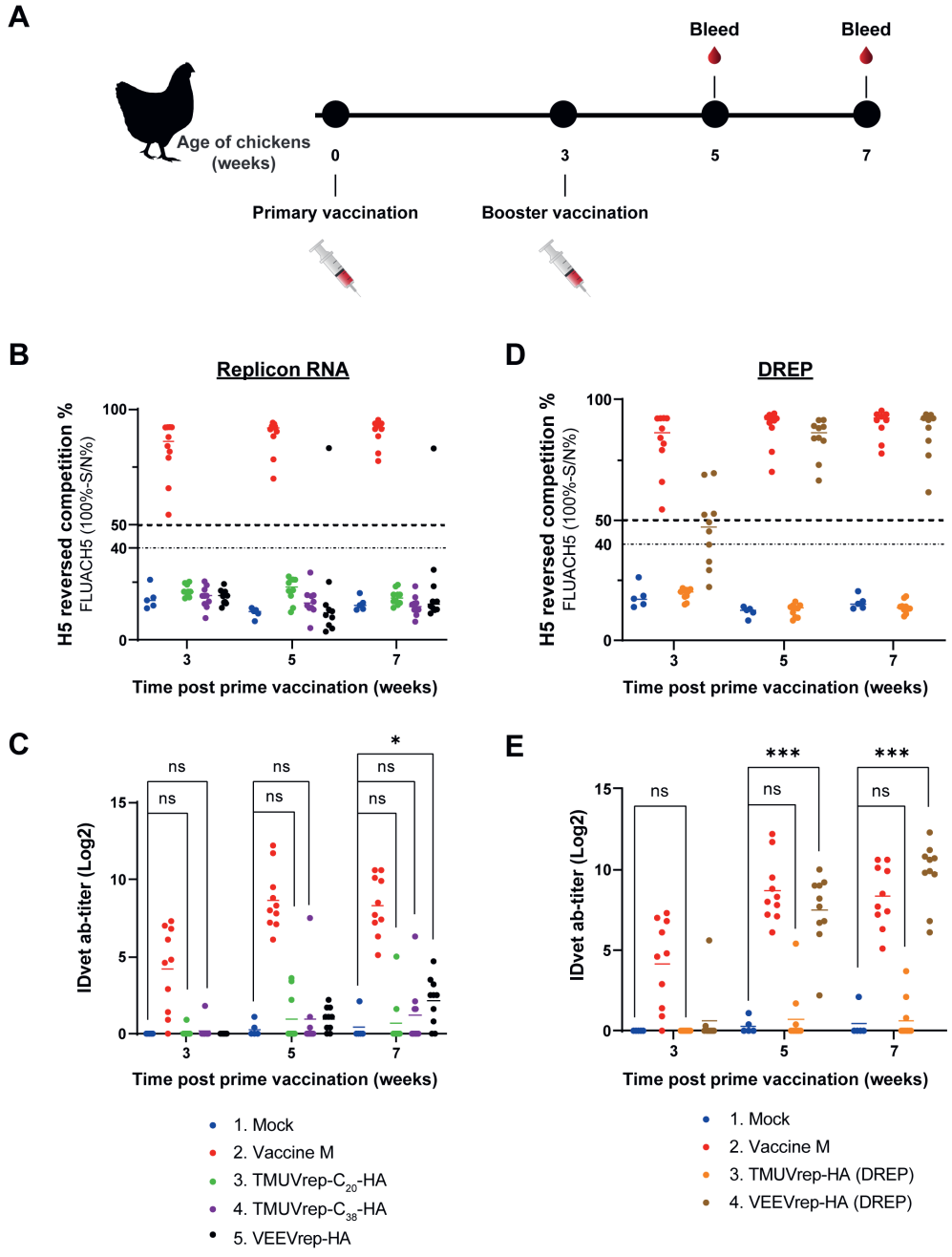
Table 1. Formulation efficiency of the replicon RNA formulation in LNPs.

	Input (mg)	Conc. RNA in LNP	Tot. Vol. LNP formulation (mL)	Tot. RNA in LNP formulation (mg)	Encapsidation efficiency (%)
HA encoding replicons					
TMUVrep-C_{20}-HA	0.75	108	3.5	0.38	50.5
TMUVrep-C_{38}-HA	0.68	94	4	0.38	55.0
VEEVrep-HA	0.82	138	4	0.55	67.5
pVP2 encoding replicons					
TMUVrep-C_{20}-pVP2	0.75	140	4	0.56	74.6
TMUVrep-C_{38}-pVP2	0.73	129	4	0.52	70.7
VEEVrep-pVP2	0.75	132	4	0.53	70.6

indirect ELISA (**Figure 4C**). Only at T=7 weeks ppv, the detection of α-HA antibodies in the VEEV replicon RNA vaccinated group were significantly higher than in the mock group.

Chickens were also vaccinated with DREP:LNP formulations. In this experiment, immunization with TMUV DREP did not result in a detectable antibody response determined by the reversed competition ELISA nor the indirect ELISA, (**Figures 4D & E**). In contrast, the VEEV DREP vaccinated chickens showed the initiation of seroconversion in the reversed competition ELISA after a single vaccination at T = 3 weeks ppv (**Figures 4D & 4E**). After the booster vaccination, the antibody response induced by VEEV DREP vaccinated chickens became markedly higher, surpassing the antibody titers induced by the Vaccine M vaccinated control group.

Another immunization trial was performed to test the function of the TMUV in comparison to VEEV replicons in eliciting an immune response against VP2 (IBDV). Sample groups were set up similarly as for the HA study, except that the positive control was immunized using Innovax-ND-IBD® (MSD Animal Health). At T=0 weeks, the prime vaccination of 10 µg of LNP:RNA (**Figure 5**) or 3 µg LNP:DREP (**Figure 6**) was injected into the right leg of day-old chickens. At T=3 weeks ppv, an additional booster injection (10 µg for LNP:RNAs or 3 µg for LNP:DREPs) was administered (**Figures 5A or 6A**). Following this prime vaccination, no significant increase in antibody titers was detected at 3 weeks ppv (T=3 weeks) with LNP:RNA (**Figure 5B**). However, two weeks after the booster vaccination (T=5 weeks) seroconversion was detected in both TMUVrep-C_{20}-pVP2 and TMUVrep-C_{38}-pVP2 vaccinated groups, which clearly differed from the VEEVrep-pVP2 vaccinated group ($p \leq 0.05$) which showed no humoral immune response at this time point. Unfortunately, due to a technical failure in two isolators, 80% of the chickens assigned to the positive control group and 100% of chickens assigned to the TMUVrep-C_{20}-pVP2 replicon RNA group were lost during this experiment. For the LNP:DREP-vaccinated chickens a different response profile was observed (**Figure 6B**). The presence of antibodies in VEEVrep-pVP2 DREP-vaccinated chickens was detected as early as 3 weeks ppv (T=3 weeks; **Figure 6B**). Although both were not significantly different from the mock ($p = 0.178$), a noticeably higher antibody titer was detected in the VEEVrep-pVP2 DREP-vaccinated group compared to the TMUVrep-pVP2 DREP-vaccinated group ($p = 0.0325$) at T=3 weeks (**Figure 6C**). Following the booster vaccination, a progressive increase in antibody titers was observed in VEEVrep-pVP2 DREP-vaccinated chickens for up to 13 weeks ppv, approaching the antibody titers observed in the positive control group. In contrast,


A
Bleed
Bleed
Age of chickens (weeks)
0
3
5
7
Primary vaccination
Booster vaccination
B
Replicon RNA
H5 reversed competition %
FLUACH5 (100%-S/N%)
Time post prime vaccination (weeks)
D
DREP
H5 reversed competition %
FLUACH5 (100%-S/N%)
Time post prime vaccination (weeks)
C
IDvet ab-titer (Log2)
ns
*
Time post prime vaccination (weeks)
1. Mock
2. Vaccine M
3. TMUVrep-C20-HA
4. TMUVrep-C38-HA
5. VEEVrep-HA
E
IDvet ab-titer (Log2)
ns
***
Time post prime vaccination (weeks)
1. Mock
2. Vaccine M
3. TMUVrep-HA (DREP)
4. VEEVrep-HA (DREP)

◄ **Figure 4. Immunogenicity of SPF-chickens using LNP-formulated replicon RNAs (*left*) and DREPs (*right*) expressing HA (AIV) antigen. (A)** Timeline of immunizations and key points. (**B**) Competitive and (**C**) indirect ELISA assay detecting the presence of α-HA in the serum of replicon RNA, Vaccine M, or mock vaccinated chickens. (**D**) Competitive and (**E**) indirect ELISA assay detecting the presence of α-HA in the serum of DREP, Vaccine M, or mock vaccinated chickens. Dotted lines in the competitive ELISA graphs (40-50%) correspond with an "uncertain" competition percentage (100%-S/N%) according to the manufacturers protocol. Data equal or above 50% are considered positive while data equal or less than 40% are considered negative. Arithmetic mean values are indicated by the colored line in each group. Statistical significance was determined compared to the mock group using the Mann-Whitney *U*-test (* = $p \leq 0.05$, ** = $p \leq 0.01$ *** = $p \leq 0.001$, **** = $p \leq 0.0001$, ns = no significant difference).

TMUV DREP-vaccinated chickens demonstrated a slower but still significant difference in α-VP2 antibody titers starting from ten weeks ppv (T=10 weeks). Throughout the VP2 immunization trial, an overall increase in antibody titer readouts was observed for all groups and was particularly notable in mock, VEEVrep-pVP2 replicon RNA- and VEEVrep-pVP2 (DREP)-vaccinated groups. This increase often coincided with the rise in plasma viscosity as the chickens aged also resulting in a higher background (*Robertson & Maxwell, 1996*).

4. Discussion

In the wake of the success of mRNA vaccination, self-amplifying replicon vaccines are gaining renewed attention as a highly versatile and safe vaccination alternative for a range of animal species. It has been demonstrated that the well-studied VEEV replicon platform provided protective immunity against SARS-CoV-2 or Rabies virus in mice (*Langereis et al., 2021; H. Zhang et al., 2020*); Ebolavirus in non-human primates (*Herbert et al., 2013*); and influenza A virus or PEDV in swine (*Crawford et al., 2016; Erdman et al., 2010*). However, the VEEV-based replicon vaccine underperformed in mounting a protective immune response in young chickens (*Schultz-Cherry et al., 2000*). In our research, our aim was to develop a bird-adapted TMUV replicon platform that induced a potent antibody response against common poultry infections such as AIV and IBDV. We demonstrated a DNA-launched replicons can be successfully used to express viral antigens *in vitro*. For the expression of pVP2 of IBDV, the FMDV-2A ribosomal skipping elements released the pVP2 from the TMUV polyprotein and demonstrated a molecular mass and intracellular localization in line with what has been described for a wildtype IBDV infection (*Chevalier et al., 2002; Irigoyen et al., 2012; Maraver et al., 2003*). The AIV HA expressed from the TMUV replicon was displayed on the plasma membrane similar to other heterologously expressed HA proteins (*Hsu et al., 2016*). Immunoblotting of the cell lysate showed two distinct protein bands at ~ 70 and ~ 100 kDa. Heterogeneity in glycosylation patterns and protein processing have been observed amongst HA proteins (*Cruz et al., 2018; Koroleva et al., 2020; C. Li et al., 2010*), suggesting that HA is N-glycosylated similar to a wildtype avian influenza infection (*Mishin et al., 2005*).

After successful *in vitro* expression of viral (glyco)proteins, the formulation of both replicon modalities (RNA and DREP constructs of TMUV and VEEV) in LNPs was performed in

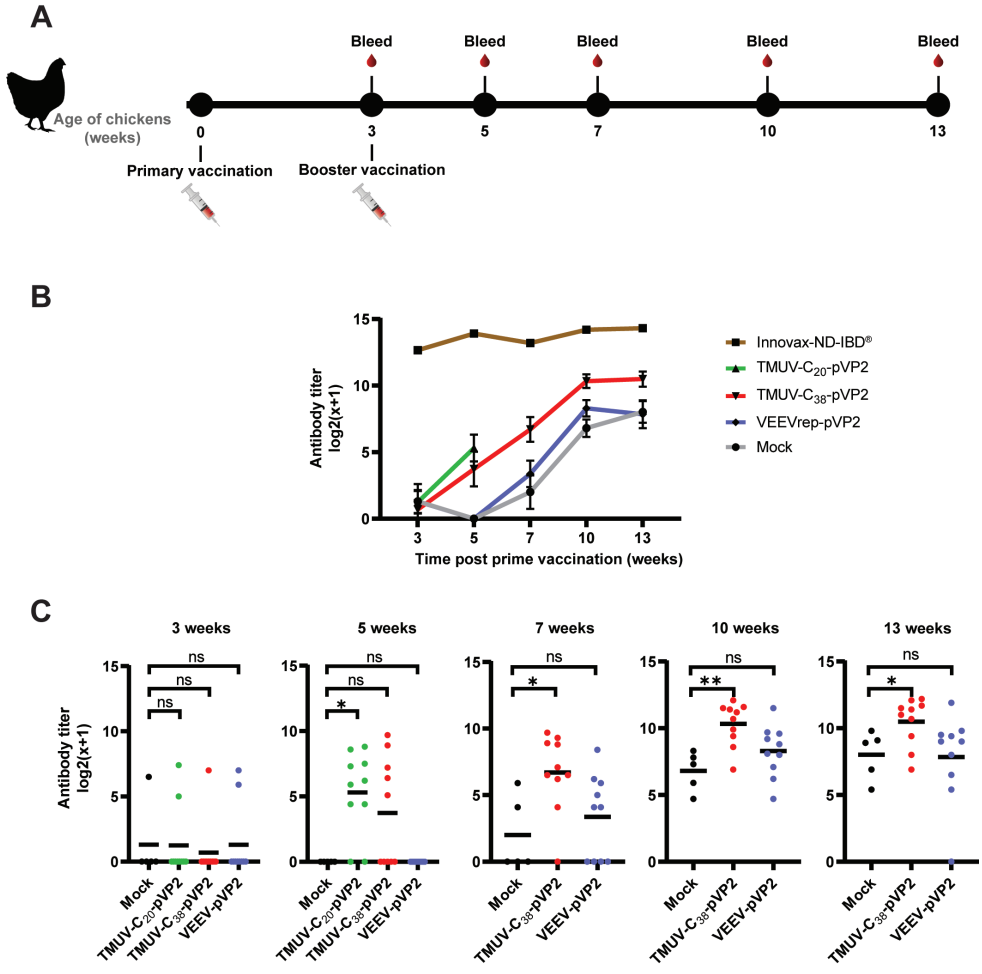


Figure 5. Immunogenicity of SPF-chickens vaccinated using LNP-formulated replicon RNAs expressing pVP2 (IBDV) antigen. (A) Timeline of immunizations and key points **(B-C)** Indirect ELISA detecting α-VP2 antibodies in groups vaccinated with Innovax-ND-IBD® (brown), TMUVrep-C_{20}-pVP2 (green), TMUVrep-C_{38}-pVP2 (red), VEEVrep-pVP2 in (blue), mock (black). Arithmetic mean values are indicated by the black line in each group. Statistical significance was determined compared to the mock group using the Mann-Whitney *U*-test (* = $p \leq 0.05$, ** = $p \leq 0.01$, ns = no significant difference).

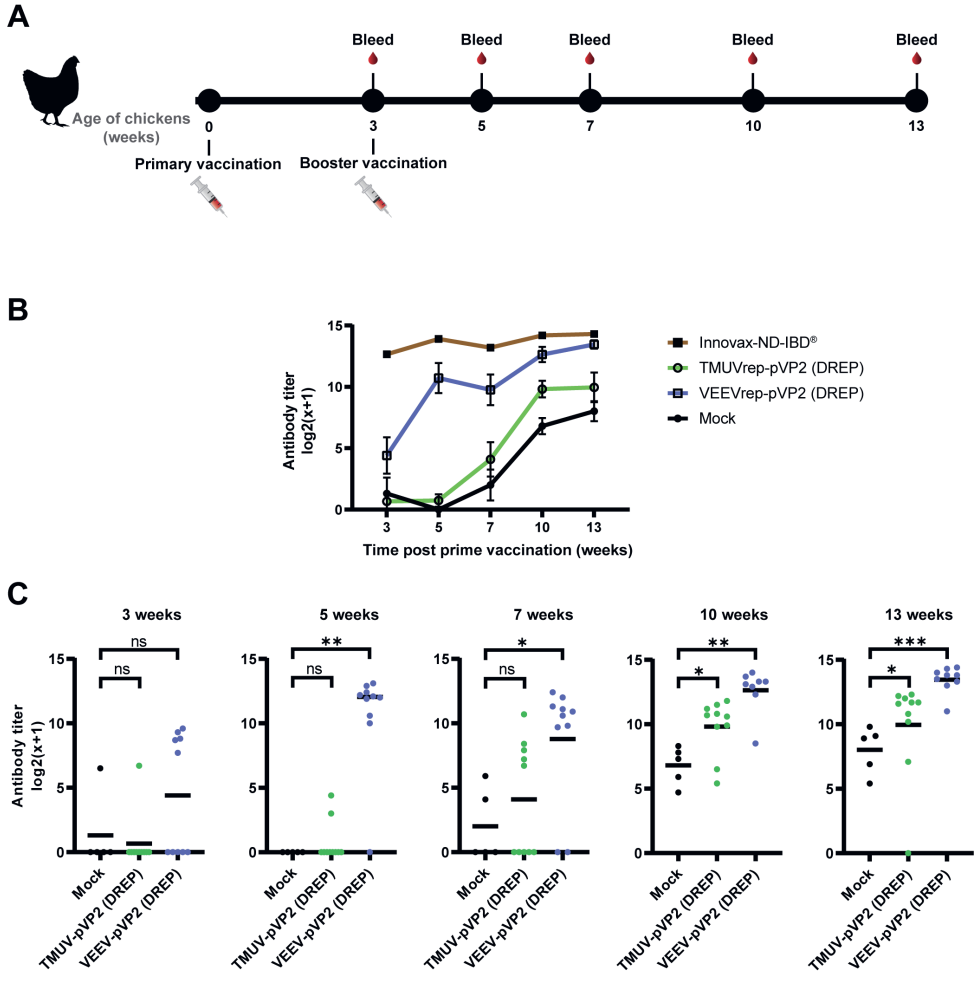


Figure 6. Immunogenicity of SPF-chickens vaccinated using LNP-formulated DREPs expressing pVP2 (IBDV) antigen. (A) Timeline of immunizations and key points **(B-C)** Indirect ELISA detecting α-VP2 IgY in chicken sera in groups vaccinated with Innovax-ND-IBD® (brown), TMUVrep-C_{20}-pVP2 DREP (green), VEEVrep-pVP2 in (blue), mock (black). Arithmetic mean values are indicated by the black line in each group. Statistical significance was determined compared to the mock group using the Mann-Whitney *U*-test (* = $p \leq 0.05$, ** = $p \leq 0.01$ *** = $p \leq 0.001$, ns = no significant difference).

preparation for the animal study. The formulated LNPs displayed a variable size ranging from small (~ 30 nm) to very large (~ 700 nm) measured via digital image analysis and DLS. Although DLS is used to measure the hydrodynamic radius and not the actual particle size, it should be noted that size variation was expected between both measuring techniques. Additionally, the analyses only provided a snapshot of the current size homogeneity of the LNPs while these might have temporal dynamics or be affected by the sample preparation for analysis (*Jakubek et al., 2023*). The understanding of the impact of LNP particle size is reported to be an important parameter for enabling potent LNP vaccines. A study in mice reporting an average particle size of around 100 nm was ideal for the consistent production of high antibody titers (*Hassett et al., 2021*). Since this is the first study considering LNP-formulated RNA or DNA in chickens, further validation of the optimal size homogeneity of the LNPs should be performed.

We demonstrated that a prime-boost vaccination trial in SPF layers using LNP-formulated TMUV replicon RNA induced higher antibody titers against the pVP2 of IBDV than for VEEV replicon RNA expressing the same antigen. It is expected, that the slower but robust transgene expression in addition to the minimal CPE of the TMUV replicon RNA in vitro, contributes to a more sustained response in chickens compared to the VEEV replicon RNA (*Gehrke et al., 2005; He et al., 2021; Pijlman et al., 2006*). Notably, TMUVrep-C_{38}-pVP2 was significantly different from the mock at 7 weeks until the end of the study (13 weeks), whereas VEEV replicon RNA was not. Interestingly, TMUVrep-C_{20}-pVP2 was already significant at week 5, suggesting that both TMUV replicon variants outperformed VEEV replicon.

In contrast to the successful induction of IBDV VP2-specific antibodies in the VP2 study, neither TMUV nor VEEV replicon RNA vaccinated groups showed significant induction of HA-specific antibodies in both reversed competition and indirect ELISA at week 3 and 5 ppv in the HA study. Despite much lower titers than the positive control, a few individual responders in the replicon RNA vaccinated groups showed the first indication of seroconversion beyond week 3, with the VEEV replicon RNA vaccinated group showing a significantly higher induction of HA-specific antibodies compared to the mock vaccinated group at week 7. Whether these low antibody titers confer protection during a homologous or heterologous challenge remains untested. Since no studies have been performed with LNP-formulated replicon RNA in chickens, it is still unresolved if the induction of α-HA antibodies requires a higher dose or longer incubation times before seroconversion is observed. Different studies tested the administration of various LNP-formulated replicon RNA doses ranging from 0.5 $\mu g \cdot kg^{-1}$ to 500 $\mu g \cdot kg^{-1}$ (*Geall et al., 2012; McKay et al., 2020; Melo et al., 2019*), which is in line with, the in this study, estimated 200 $\mu g \cdot kg^{-1}$. However, it should be noted that these studies only evaluated the delivery of VEEV replicon RNA in mice and not in chickens.

Of note, however, VEEV DREP in both the pVP2 and HA study showed an early onset (T=3) of antibody response after a single vaccination. Intriguingly, the VEEV DREP vaccinated group in the HA study even outcompeted the positive control at week 7, while the HVT-vectored vaccines used to immunize chickens maintain the ability to spread from cell to cell and therefore

persist much longer than most RNA and DNA vaccines (*Ingrao et al., 2017*). In contrast to the VEEV DREP, the TMUV-based DREP only induced a significant response in the pVP2 study after the administration of the booster. Whether the slower seroconversion of the TMUV DREP vaccinated animals had to do with the inherent different expression kinetics compared to VEEV replicon remains untested. Another reason why the constructed TMUV DREP efficacy tested both *in vitro* as well as *in vivo* differs compared to the VEEV DREP, could be explained by the difference in molar mass between the constructs (TMUV DREP = 13.6 x 10^6 $gmol^{-1}$ vs VEEV DREP = 7.8 x 10^6 $g \cdot mol^{-1}$). In this study, only 3 µg of DREP was administered in each dose without considering the normalization for the DREP size. This would mean that the TMUV DREP dose per chicken is 43% less than for VEEV DREP-vaccinated chickens. Whether DREP vaccination in general, conveys a longer protection compared to replicon RNA has not been evaluated. However, it is known that conventional plasmid DNA as well as replicon-encoding DNA plasmids can be maintained in cells for months (*Gehrke et al., 2005; Morris-Downes et al., 2001; Pietschmann et al., 2001*). Furthermore, it was expected that from both replicon modalities, the replicon RNA might show an earlier onset of antigen production than its DNA counterpart. The required nuclear delivery to initiate RNA transcription compared to the direct translation of *in vitro* transcribed replicon RNA in the cytoplasm, might explain the delayed generation of an antibody response of TMUV DREP compared to the TMUV replicon RNA in the VP2 vaccination study. As a next step, performing an optimal dose finding in combination with a virus challenge study would be critical to identify the requirements to elicit a protective immune response in chickens. Overall, the successful implementation of the bird-adapted flavivirus replicon system demonstrated in this study holds promise for driving future research and fostering the development of innovative vaccines against avian viral diseases.

5. Acknowledgments

The authors are thankful to Jelmer Vroom from the Wageningen Electron Microscopy Centre for the electron microscopy analysis of LNPs and Marleen Henkens, Corinne Geertsema, Frans Manders, Christiaan Vossen, and Martin Piest for their technical assistance. We thank Mateusz Walczak, Linda van Oosten, Tessy Hick, and Jelke Fros for their helpful discussions.

6. Supplemental data

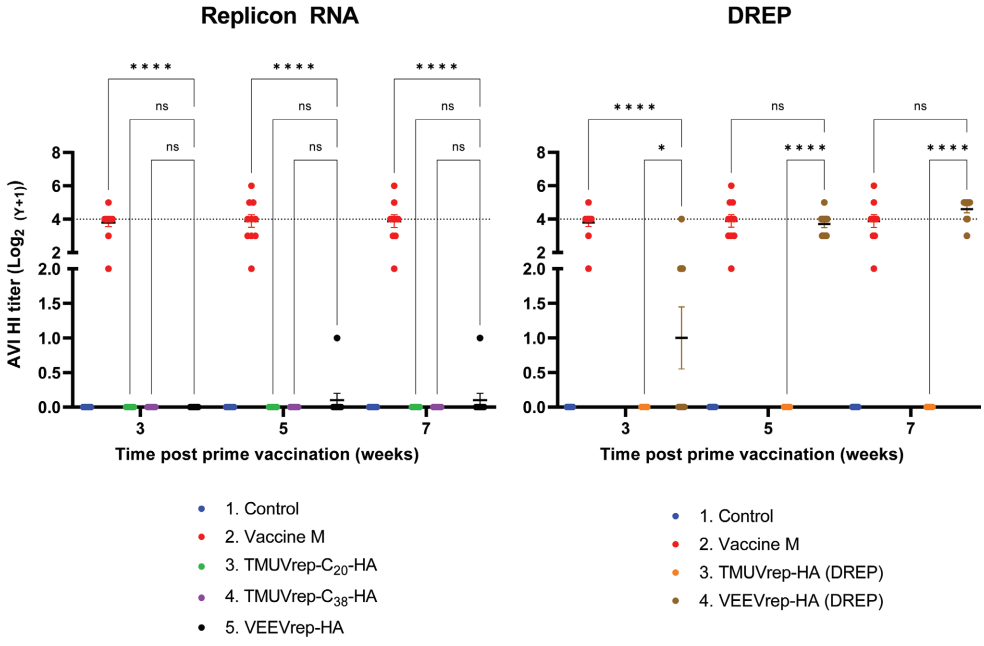


Figure S1. **Heterologous (AIV H5N6) haemagglutinin inhibition test. (Left)** HI titer of replicon RNA vaccinated or **(Right)** DREP vaccinated chickens The dotted line represents the suggested titer for protection against AIV H5N6. Arithmetic mean values are indicated by the colored line in each group. Error bars represent standard error. Statistical significance was determined using the Mann-Whitney U-test (* = $p \leq$ 0.05, **** = $p \leq 0.0001$, ns = no significant difference).

IV

Chapter 5

The development of a doxycycline-inducible packaging cell line for the production of Tembusu-derived virus-like replicon particles

Abstract

Virus-like replicon particles (VRPs) are useful delivery vehicles for self-amplifying (replicon) RNA to vaccinate livestock against emerging viral pathogens. VRPs are produced by co-transfecting cells with a packaging plasmid encoding the viral structural genes along with replicon RNA. In this study, the objective was to establish a stable packaging cell line capable of large-scale production of flavivirus-derived VRPs. We screened several mammalian-, avian- and mosquito-derived cell lines using the duck-derived Tembusu virus (TMUV; family *Flaviviridae*) WU2016 isolate to identify a suitable packaging cell line to produce VRPs. Our results demonstrate that TMUV WU2016 replicated to high viral titers in both human (HEK293T), avian (DF-1 and EB66), and mosquito (Chao ball, Aag2, and C6/36) cell lines. We transduced HEK293T cells using a recombinant lentiviral vector to introduce a doxycycline-inducible structural gene cassette, encoding for the TMUV capsid, premembrane, and envelope proteins. Following a novel approach, incorporating FACS-mediated clonal cell selection and RNA electroporation, a monoclonal packaging cell line capable of efficiently producing VRPs was generated. The infectivity of the produced VRPs was assessed in naive chicken cells and insights into potential factors influencing VRP production were obtained with the help of transmission electron microscopy. Overall, our findings demonstrated the development of a straightforward pipeline for the development of a clonal packaging cell line using lentiviral transduction for the efficient production of flavivirus-derived VRPs.

1. Introduction

During the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2, family *Coronaviridae*) pandemic, highly versatile mRNA vaccines emerged as a new-generation vaccine platform. Notably, two out of three FDA-approved COVID-19 vaccines were lipid nanoparticle (LNP)-formulated mRNA vaccines. These synthetically produced mRNA vaccines are easily adapted, scalable, and safe-by-design (*Pardi et al., 2018; Pourseif et al., 2022*). However, despite the recent advancements in mRNA vaccine design, storage, and delivery techniques, challenges regarding vaccine efficacy and the requirement for prime-boost regimens still remain (*Comes, Pijlman, et al., 2023; Rosa et al., 2021; C. Zeng et al., 2020*).

A potential solution to mitigate these challenges is the use of self-amplifying mRNA, commonly known as 'replicons'. These replicons are typically derived from positive-sense, single-stranded RNA viruses of the *Togaviridae* or *Flaviviridae* families. Replicons encode, next to their heterologous gene of interest, the genes necessary for the self-amplification of the mRNA, resulting in high-level and durable transgene expression (*de Alwis et al., 2021*). Replicon RNA exhibits the unique ability to be packaged into synthetic non-viral (e.g. lipid nanoparticles, liposomes, cationic polymers, etc.) as well as viral carriers, the latter known as virus-like replicon particles (VRPs) (*Erasmus et al., 2020; Jawalagatti et al., 2022; Y. Li et al., 2020*). VRPs effectively deliver replicon RNA to the target cells, and in general elicit a robust immunogenic response against the selected candidate antigen without conferring antivector immunity (*Aberle et al., 2005; Kamrud et al., 2008; MacDonald & Johnston, 2000; Uematsu et al., 2012; Walczak et al., 2011; White et al., 2007; H.-Q. Zhang et al., 2022*). VRPs are traditionally produced in cells by *trans*-complementation of replicon RNA with the viral structural genes (helpers). The alphavirus Venezuelan equine encephalitis virus (VEEV) replicon has been widely explored for its application for VRP-based vaccines. VEEV VRPs have been used to immunize small mammals and non-human primates against a wide range of infectious diseases and cancers. Furthermore, the VEEV replicon technology entered clinical phase I/II in the protection against SARS-CoV-2 and for the treatment of carcinoembryonic antigen-based malignancies in humans (*Hooper et al., 2009; Langereis et al., 2021; Reap, Watson, et al., 2007; Scholte et al., 2019; Spengler et al., 2022; Y.-N. Zhang et al., 2020*). However, immunization using VEEV VRPs or replicon RNA in poultry was less successful (*Schultz-Cherry et al., 2000; Sylte et al., 2007*) (**Chapter 4**). It is possible that vector-specific cytopathicity of VEEV nonstructural proteins adversely affects the efficacy of the VEEV replicon platform in poultry (*Atasheva et al., 2008; Garmashova et al., 2007; M. L. Li & Stollar, 2004; Polo et al., 1999*).

In contrast to alphavirus replicons, those derived from flaviviruses have a less cytopathic nature (**Chapters 3 and 4**). Flaviviruses are positive-sense, single-stranded RNA viruses with a genome length of approximately 11-kb encoding a single open reading frame. Post-translation processing yields three structural proteins: capsid (C), premembrane (prM), and envelope (E) protein and seven nonstructural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (*Pierson & Diamond, 2020; Schmaljohn & McClain, 1996*).

Flavivirus-based replicon RNA retains at least the initial 20 codons of the capsid gene - containing structured *cis*-acting RNA elements – to allow for genome replication and translation initiation, and the last 22 codons of the E gene for translocation of the nonstructural protein NS1 into the ER (*He et al., 2021; Khromykh & Westaway, 1997*). Previously, we constructed a poultry-adapted replicon derived from the Tembusu virus (TMUV; family *Flaviviridae*). TMUV has been detected in diverse avian species, including ducks, geese, chickens, and pigeons (*Hamel et al., 2021*). It was demonstrated that the TMUV replicon RNA successfully induced a humoral response against a replicon-expressed viral antigen in chickens (**Chapters 1, 3, and 4**). However, since the replicon RNA was delivered using lipid nanoparticles, no TMUV VRPs were evaluated.

Similar to alphavirus-derived VRPs, flavivirus-derived VRPs also require trans-complementation with the complete (CprME) structural cassette (*Harvey et al., 2004; Khromykh et al., 1998; Lücke et al., 2022; Qing et al., 2010*). However, in contrast to alphavirus VRPs, various complementation strategies have been described to produce flaviviruses VRPs, such as transient expression of the structural cassette using a DNA vector or the bicistronic expression vectors based on alphavirus replicons (*Khromykh, Varnavski, & Westaway, 1998b; Lücke et al., 2022b; Scholle et al., 2004a; Varnavski & Khromykh, 1999*). However, large-scale VRP production requires a substantial amount of purified DNA and *in vitro* transcribed RNA to be delivered into the same cells. A stable packaging cell line can potentially mitigate these complications and make VRP production more efficient. Several flavivirus-based packaging cell lines have successfully been established and demonstrated the production of various VRPs (W. *Li et al., 2017; Lücke et al., 2022*), yet limited research has been conducted on selecting the most suitable cell line for flavivirus-specific VRP production (*Gehrke et al., 2003; He et al., 2019; W. Li et al., 2017*).

In this study, we investigate and compare the growth kinetics of TMUV MM1775 and WU2016 isolates on several vertebrate and invertebrate cell lines to identify the most suitable cell line for VRP production. A stable, doxycycline-inducible monoclonal packaging cell line was generated using lentiviral transduction with the aim to produce higher VRP titers compared to the complementation of structural proteins via typical co-transfection.

2. Material and methods

Cells and Virus preparation

Vertebrate cell lines derived from human embryonic kidney (HEK293T; ATCC CRL-3216), chicken embryonic fibroblasts (DF-1; ATCC CRL-3586), and baby hamster kidney fibroblast cells (BHK-21; Clone 13, ECACC 84011433) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) complemented with 10% (v/v) fetal bovine serum (FBS; Thermo Fisher Scientific), 100 $U \cdot mL^{-1}$ penicillin and 100 $\mu g \cdot mL^{-1}$ streptomycin (Gibco; hereafter called complete DMEM). The suspension cell line derived from duck embryonic stem cells (EB66; Valneva) was cultured in serum-free Ex-cell growth II medium (SAFC Biosciences)

complemented with 100 U·mL^{-1} penicillin and 100 µg·mL^{-1} streptomycin. To determine the cell viability and the pH of the suspension cultures, the NucleoCounter NC-100 (ChemoMetec) and 766-Laboratory pH meter (Knick International) were used, respectively. The invertebrate cell lines derived from *Culex pipiens* (Cpip), *Aedes albopictus* (C6/36), *Culex tarsalis* (Chao Ball), and *Aedes aegypti* (Aag2) were cultured in Leibovitz's L-15 medium complemented with L-glutamine (Sigma-Aldrich), 10% (v/v) FBS, 100 U·mL^{-1} penicillin and 100 µg·mL^{-1} streptomycin. All vertebrate cells were maintained at 37°C under 5% CO_2 and invertebrate cells at 28°C without CO_2. TMUV WU2016 and TMUV MM1775 passage 1 stocks were generated as previously described (**Chapter 2**). To determine virus growth kinetics, 6-well plates were seeded at sub-confluent densities and infected in triplicate at a multiplicity of infection (MOI) of 0.01 or 0.1 median tissue culture infectious dose per milliliter $TCID_{50}$·mL^{-1} for vertebrate and invertebrate cells, respectively. After 2 h of incubation, the supernatant was removed and the cells were washed once with Dulbecco's phosphate-buffered saline (DPBS; Gibco). Complete DMEM+HEPES (Gibco) was added to infected cells and the virus was collected every 24 h and stored at -80°C. The virus was titrated on DF-1 cells by end-point dilution assay (EPDA) in 60-well microtiter plates (Greiner). The plates were incubated and scored by the observation of cytopathic effect (CPE) using brightfield microscopy after 5-7 days.

Plasmid construction

Lentiviral expression vectors were created based on the third-generation inducible transfer plasmid pCW57.1 (a gift from David Root laboratory; Addgene no.: 41393). The TMUV WU2016 (accession no.: OQ920272) structural genes were isolated by PCR amplification of the infectious cDNA clone described in **Chapter 2** using TMUV-CprME-F (5' atggcgcgccatgtctaacaaaaaaccaggaa 3') and TMUV-CprME-R (5' gattaattaacttaggcattgacatttactgc 3') primers. The isolated PCR fragment was cloned into the Gateway entry vector pENTR 11 (Invitrogen), sequenced, and then transferred into pCW57.1 backbone via Gateway cloning which resulted in pCW-CprME. A GFP-control transfer vector (pCW-GFP) was constructed for validation of the lentiviral transduction and induction. pCW-CprME or pCW-GFP were transfected in BHK-21 to evaluate the functioning and responsiveness to doxycycline (Sigma-Aldrich). BHK-21 cells were induced 24 hours after transfection by adding doxycycline at various concentrations (ranging from 0 to 500 ng·mL^{-1}) to complete DMEM. For the construction of the VEEV replicon-derived helper (VEEVrep-CprME) or DNA-helper (pCMV-CprME), the CprME cassette isolated using PCR amplification using the previous TMUV-CprME-F forward and reverse primer (5' gtcgcggccgcttaggcattgacatttactgcca 3'). The isolated fragments were cloned into the T7-driven VEEV replicon RNA vector (described in **Chapter 3**) or pEGFP-N1 (Novopro) using *AscI* and *PacI* restriction digestion, sequenced and used as a template for *in vitro* RNA transcription reaction or directly used for Lipofectamine 2000 transfection in BHK-21 cells, respectively.

Preparation of lentiviral stocks and transduction of cells

Recombinant VSV-G pseudotyped lentiviral particles were produced by introducing a three-vector packaging system via calcium chloride ($CaCl_2$) transfection in HEK-293T cells. The phCMV-VSV-G (envelope plasmid), psPAX (packaging plasmid), and the (constructed)

transfer plasmids (e.g. pCW57.1-CprME or pLOV-eGFP) were added in a 1.2:1.1:1 (µg) ratio to HEPES buffered saline (HBS) and 0.3M $CaCl_2$ solution. After 2 min incubation at 37°C, the solution was added in a dropwise manner to a 60% confluent T25-flask containing completed DMEM. After 16 h, the media was replaced with DMEM containing 5% FBS and incubated at 37°C. After a 48-hour incubation period, the lentiviral particles were harvested by centrifugation at 900 x g for 15 minutes. Following this, the supernatant was filtered through a 0.45 µm filter, divided into aliquots, and then stored at -80°C. Lentiviral transductions were performed by incubating HEK293T cells with 0.5 mL cleared supernatant. After two days, transduced cells were subjected to puromycin selection (0.5 $\mu g \cdot mL^{-1}$). After two passages, resistant cells were subjected to fluorescence-activated cell sorting (FACS) for clonal selection. A confluent 25 cm^2 T-flask containing puromycin-resistant cells was collected using trypsinization, washed twice using DPBS, and resuspended in a chilled FACS-buffer (DPBS, 1%BSA, and 0.5 mM EDTA) to a concentration of 2 x 10^6 $cells \cdot mL^{-1}$. Uninduced cells were sorted based on fluorescence (pLOV-GFP-transduced cells) or randomly (pCW57.1-transduced cells) using the SH800S Cell Sorter (Sony) with a 100 µm sorting chip (Sony). Subsequently. single cells were sorted using the single-cell 3-drop sort protocol and deposited in a 96-well cell culture plate containing 200 µL of prewarmed DMEM complemented by 20% FBS, fungizone (2.5 $\mu g \cdot mL^{-1}$), and penicillin-streptomycin (100 $U \cdot mL^{-1}$ penicillin and 100 $\mu g \cdot mL^{-1}$ streptomycin). Cells were clonally expanded for 1-2 weeks at 37°C under 5% CO_2. Flow cytometry data was analyzed using FlowJo software (TreeStar, Ashland, OR, USA).

Indirect immunofluorescence assay

HEK293T cells were washed using DPBS and fixed with 4% paraformaldehyde in PBS for 5 min at RT. Afterward, cells were washed and permeabilized using 0.1% sodium dodecyl sulfate (SDS; Sigma-Aldrich) in DPBS for 10 min at RT. Next, the monolayer was blocked using 5% FBS in DPBS for 1 h at 37°C and subsequently incubated using the primary antibody pan-flavi α-E (mouse; Sigma-Aldrich, MAB10216) diluted in DPBS containing 5% FBS for 1 h at 37°C. Hereafter, cells were washed and incubated with the secondary antibody Alexa Fluor 488- or 568-conjugated α-mouse IgG (goat; Abcam) in DPBS containing 5% FBS at 37°C for 1 h. Cells were then stained with Hoechst (Thermo Fisher Scientific) in DPBS for 5 min. Photos were acquired using the Observer Z1 fluorescence microscope (Zeiss).

In vitro RNA transcription

The TMUV replicon or VEEV replicon plasmids were purified using the endotoxin-free Nucleobond Midiprep kit (Macherey-Nagel) and linearized using *PacI* (TMUV-derived replicon) or *NotI* (VEEV-derived replicon restriction enzyme (New England Biolabs). Capped *in vitro* transcribed replicon RNA was generated by using 2.5 micrograms of linearized plasmid DNA in an SP6- or a T7-polymerase reaction (both from New England Biolabs) and incubated for 2 h at 37°C in the presence of RNAse inhibitor (Promega). The quality of the capped RNA was assessed via conventional gel electrophoresis using 1% agarose gel in tris-acetate-EDTA (TAE) buffer for 15 min at 150 V.

RNA transfection and electroporation

Transfection of HEK293T cells was performed in 6-well plates seeded with 5 x 10^5 cells/well. After 24 h, the culture medium was replaced with Opti-MEM (Gibco), and cells were transfected with Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's protocol. After 4 h, the transfection mixture was replaced with complete DMEM, and cells were incubated for the duration of the assay at 37°C with 5% CO_2. For the electroporation of monoclonal HEK293T packaging cells with replicon RNA, cells were grown to approximately 80% confluency and harvested using trypsinization. The cells were washed and resuspended at 5 x 10^6 cells·mL^{-1} in DPBS. Next, 0.4 mL of resuspended cells were mixed with capped, *in vitro* transcribed RNA and transferred to a 0.4-cm cuvette (Bio-Rad Laboratories). The electroporation was carried out using the Gene Pulser Xcell (Bio-Rad Laboratories) at 230 V with a single, square-wave pulse of 4 ms. Transformed cells were recovered for two minutes at room temperature and subsequently seeded in a 25cm^2 T-flask in complete DMEM in the absence or presence of doxycycline. Electroporation of the EB66 and BHK suspension cells was performed using the BTX electroporator (ECM830 Electro Square Porator, Thermo Fisher Scientific). First cells were harvested, washed, and resuspended at 1.25-2.0 x 10^8 cells·mL^{-1} in DPBS. Hereafter, 0.6 mL of concentrated cells were mixed with 30 µg of capped, *in vitro* transcribed RNA and transferred into 0.4 cm gap width BTX electroporation Cuvette Plus (BTX) and pulsed (EB66: voltage: 580 V, pulse duration: 400 µs, pulse interval: 1 s, pulses: 4; BHK: voltage 650 V, pulse duration: 400 µs, pulse interval: 1s, pulses: 4). Electroporated cells were immediately transferred to shaker flasks containing pre-warmed OptiPRO SFM medium (Gibco) complemented with 1 M HEPES (Gibco), 50 g/L L-Glutamine and non-essential amino acids (Gibco) and incubated for the duration of the experiment at 37°C under 5% CO_2.

RNA isolation and RT-qPCR

The detection of viral copies was evaluated using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Several monoclonal HEK293T packaging cell lines were electroporated using *in vitro* transcribed replicon RNA. After 72 h, electroporated cells were collected via centrifugation at 900 x g for 5 minutes and the viral RNA was extracted from the cleared supernatant using TRIzol (Invitrogen) according to the manufacturer's protocol. Both the quantity and quality of the extracted RNA were analyzed using the spectrophotometer ND-1000 (Nanodrop). Following the isolation, the RNA was reverse transcribed using the SuperScript III First-strand synthesis Supermix (Invitrogen) and amplified using the SYBR Green Supermix (Bio-Rad Laboratories) with specific primers targeting the NS3 gene of TMUV WU2016 (forward primer: 5' gagcgccattgacaaacg 3', reverse primer: 5' gtatgctccctctttactgc 3'). An in-house RNA standard was prepared by serially diluting purified capped, *in vitro* transcribed replicon RNA with a known concentration. The RT-qPCR was run on the CFX-96 touch PCR detection system (Bio-Rad Laboratories) in opaque 96-well plates (Bio-Rad Laboratories). Data was analyzed using CFX Real-Time analysis software (version 4.0; Bio-Rad Laboratories).

3. Results

Tembusu virus growth kinetics in (in)vertebrate cell lines

To select a suitable cell line for the production of VRPs, the growth characteristics of TMUV were assessed on various vertebrate and invertebrate cell lines. One-step growth curves of the duck-derived WU2016 and the mosquito-derived MM1775 TMUV isolates were conducted on baby hamster kidney fibroblast cells (mammalian; BHK-21), human kidney epithelial cells (mammalian; HEK293T) and primary chicken embryonic fibroblasts cells (avian; DF-1) (**Figure 1A**). The highest viral titer was observed for the TMUV WU2016 isolate between 48 and 72 hours post-infection (hpi) in all three cell lines. HEK293T cells showed the highest viral titer of 1.4 x 10^9 $TCID_{50}$·mL^{-1} for TMUV WU2016 at 72 hpi, whereas BHK-21 and DF-1 cells reached 1.3 x 10^8 $TCID_{50}$·mL^{-1} and 1.9 x10^9 $TCID_{50}$·mL^{-1} for TMUV WU2016, respectively. Although all cell lines showed clear cytopathic effect (CPE) at 96 hpi, the CPE in BHK-21 cells was already observed at 72 hpi (**Figure 1B**).

Next, two invertebrate cell lines derived from the mosquitoes *Culex pipiens* (Cpip; **Figure 2A**) and *Aedes albopictus* (C6/36, **Figure 2B**) were evaluated for their susceptibility to the TMUV virus isolates. For TMUV WU2016, two additional cell lines derived from *Culex tarsalis* (Chao Ball, **Figure 2C**) and *Aedes aegypti* (Aag2, **Figure 2D**) were screened. Despite the low viral yields for both TMUV isolated in Cpip cells, viral titers of C6/36, Chao Ball, and Aag2 cells peaked at 1.4 x 10^8, 3 x 10^8, and 9.5 x 10^7 $TCID_{50}$·mL^{-1}, respectively. In addition to these viral yields, clear CPE was observed in C6/36 cells (**Figure 2B**), which might relate to the absence of a functional antiviral RNAi response (*Brackney et al., 2010*).

As an alternative, suspension cell lines are attractive for biomanufacturing due to scalability and high efficiency in (attenuated) virus propagation. Suspension BHK-21, EB66 (duck), and CCX E10 (quail) were infected with TMUV MM1775 at an MOI of 0.01 $TCID_{50}$·mL^{-1} (**Figure 3A**). Similar to the adherent BHK-21 cells, the viral titers of TMUV MM1775 propagated in BHK suspension cells peaked at 48 hpi. However, a sudden drop in virus titer was then observed, which coincided with a decrease in cell viability (**Figure 3B**) and an increase in culture acidity (**Figure 3C**). In contrast, the EB66 and CCX E10 cells did not exhibit a substantial drop in viral titers. TMUV in EB66 cells reached titers up to 2 x 10^8 $TCID_{50}$·mL^{-1} without a decline in cell viability. CCX E10 cells did not yield high viral titers for TMUV MM1775 isolate within the timeframe of 96 hpi. Both EB66 and CCX E10 cells continued to proliferate during the established TMUV infection, whereas BHK-21 suspension cells stopped dividing from 48 hpi onwards (**Figure 3B**). No change in the culture pH was observed for both avian cell lines (**Figure 3C**) which is important to retain full infectivity of the produced viral particles (*Baloch et al., 2019*).

Virus-like replicon particle production in BHK cells

VRP production was first tested in BHK-21 cells. Capped, *in vitro* transcribed TMUV-C_{20}-Scarlet replicon RNA was co-transfected with a replicon RNA helper (VEEVrep-CprME) or

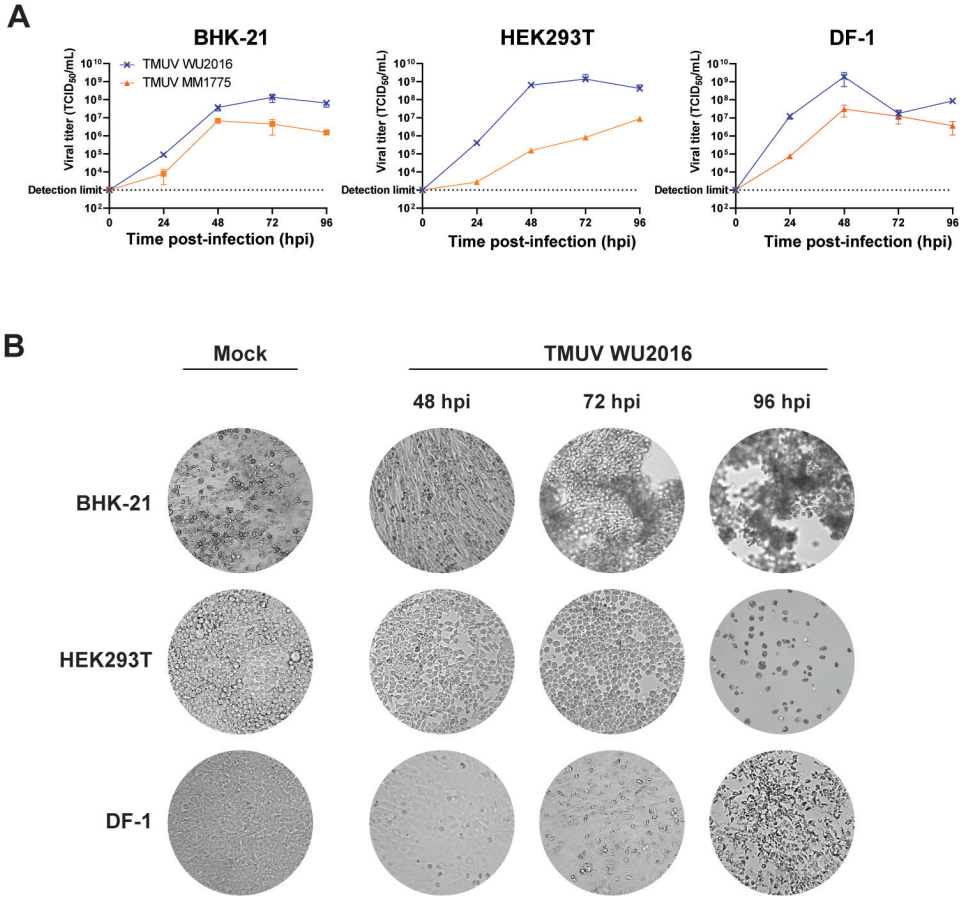


Figure 1. One-step growth curves of duck-derived TMUV WU2016 and mosquito-derived TMUV MM1775 on adherent vertebrate cells. (A) BHK-21 (*left*), HEK293T (*middle*) and DF-1 (*right*) were infected with TMUV WU2016 or MM1775 at a multiplicity of infection of 0.01 median tissue culture infectious dose per milliliter ($TCID_{50}$·mL^{-1}). The viral titers were determined by an end-point dilution assay at 24, 48, 72 and 96 hours post infection (hpi). Mean viral titers with standard errors are indicated in each graph with a lower detection limit of 10^3 $TCID_{50}$·mL^{-1}. All infections were performed in triplicate. **(B)** BHK-21, HEK293T and DF-1 cells infected with TMUV WU2016 were visualized by bright field microscopy.

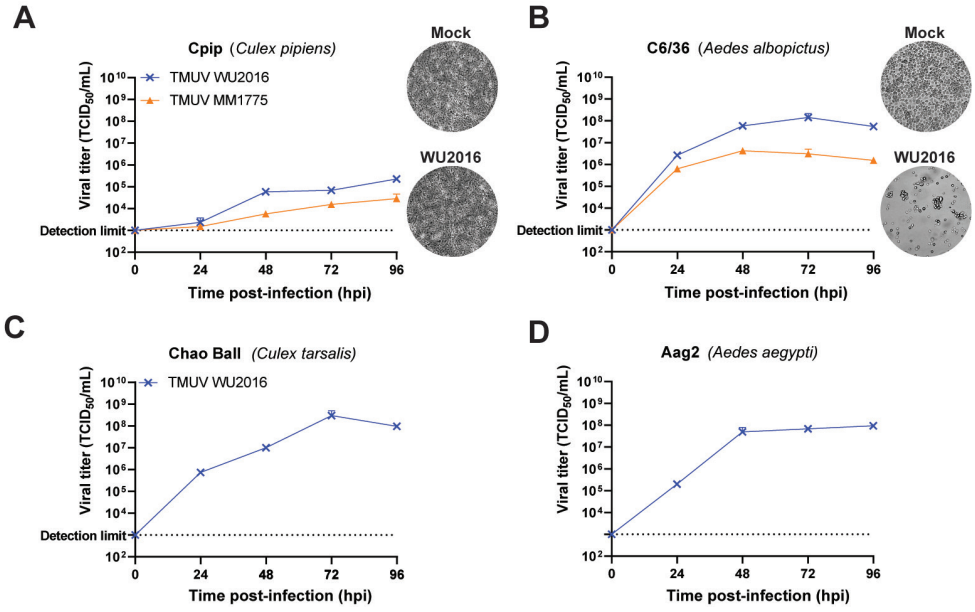


Figure 2. One-step growth curves of duck-derived TMUV WU2016 and mosquito-derived TMUV MM1775 on invertebrate cells. (A) Mosquito cell lines Cpip (*Culex pipiens*) and **(B)** C6/36 (*Aedes albopictus*) were infected with TMUV WU2016 or MM1775 at a multiplicity of infection (MOI) of 0.1 median tissue culture infectious dose per milliliter ($TCID_{50}$·mL^{-1}). Cell viability at 96 hours post infection (hpi) for the TMUV WU2016-infected samples were observed using bright field microscopy and visualized next to the corresponding graph. **(C)** Chao Ball (*Culex tarsalis*) and **(D)** Aag2 (*Aedes aegypti*) cell lines were infected with TMUV WU2016 at an MOI of 0.1 $TCID_{50}$·mL^{-1}. All viral titers were determined by an end-point dilution assay (EPDA) at 24, 48, 72 and 96 hpi. Mean titers with standard errors are indicated in each graph with a lower detection limit of 10^3 $TCID_{50}$·mL^{-1} indicated with a dotted line. All infections were performed in triplicate.

DNA helper (pCMV-CprME) into BHK-21 cells (**Figure 4A**). Both replicon RNA helper and DNA helper approaches successfully produced VRPS as was demonstrated from the detection of mScarlet fluorescence in naive BHK-21 cells infected with the undiluted culture fluid collected from co-transfected cells (**Figure 4B**). However, substantial CPE was observed in BHK-21 cells (co-)-transfected with the VEEV RNA helper (**Figure 4B**). The additional CPE could be attributed to the host translational shutoff by the nonstructural protein 2 encoded by the VEEV replicon RNA favoring alphavirus replication (*Khromykh et al., 1998*). An additional factor contributing to the low VRP production (< 100 VRPs·mL^{-1}), is the inefficient co-delivery of two replicon RNAs into a single cell, as was seen from the limited number of cells co-expressing both envelope and mScarlet protein in the immunostaining (**Figure 4C**).

Virus-like replicon particle production in HEK293T cells

Having demonstrated that the highest viral titers for TMUV MM1775 and WU2016 isolates were detected in HEK293T cells, we suggest that these cells could be suitable for VRP

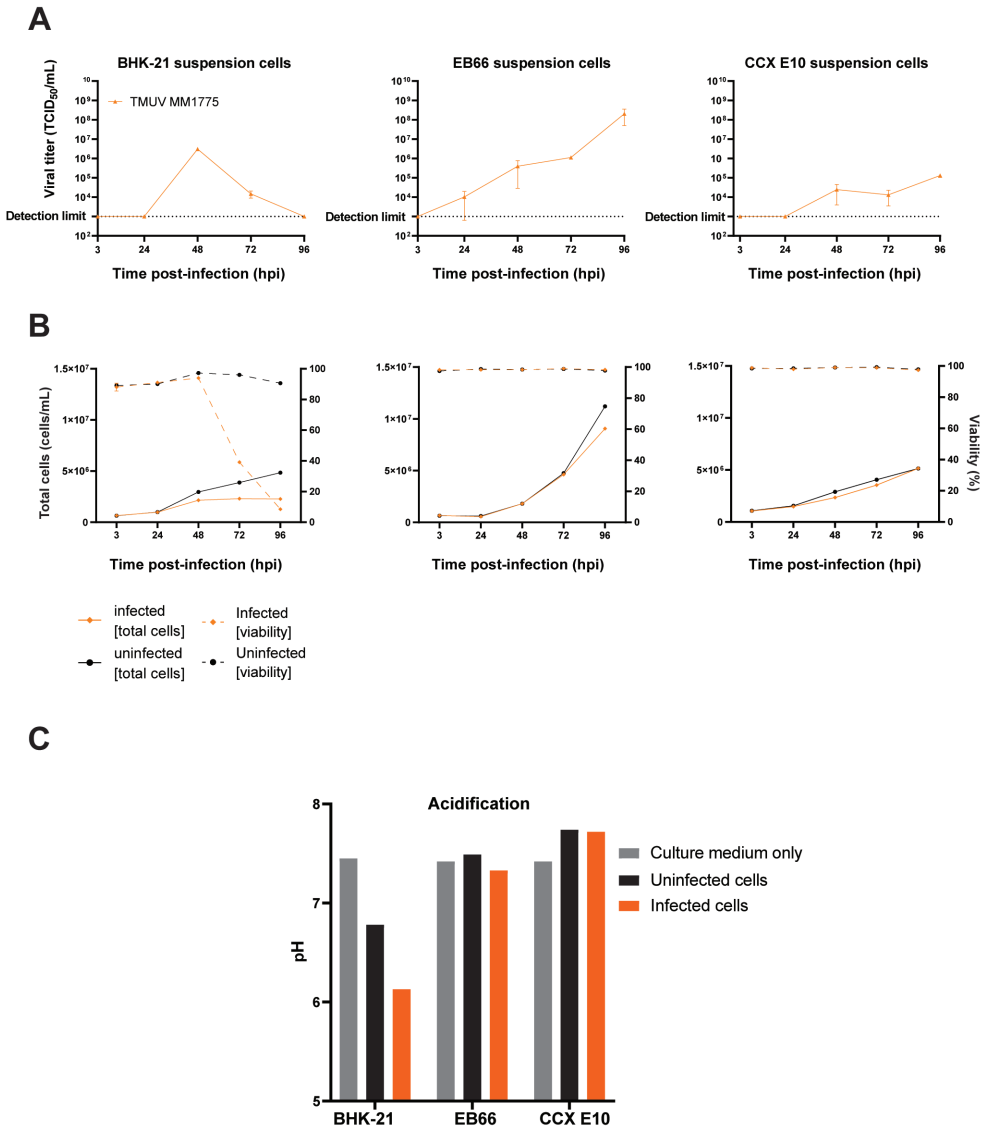


Figure 3. One-step growth curves, cell density and cell viability of mammalian BHK-21 and avian EB66 and CCX E10 suspension cells infected with TMUV MM1775 virus. (A) The cell density and viability of BHK-21, EB66 and CCX E10 cell lines infected with TMUV MM1775 at an MOI of 0.01 median tissue culture infectious dose per milliliter ($TCID_{50} \cdot mL^{-1}$) was assessed using NucleoCounter® NC-100® (ChemoMetec) throught the 96-hours time period. **(B)** Virus supernatant of TMUV MM1775 infected cells were titrated on DF-1 cells and scored 5-7 days post infection to determine the viral titer. **(C)** The acidification of the (infected) culture media was determine using a pH-probe. All viral titers were determined by an end-point dilution assay (EPDA) at 24, 48, 72 and 96 hours post infection (hpi). Mean viral titers with standard errors are indicated in each graph with a lower detection limit of 10^3 $TCID_{50} \cdot mL^{-1}$ indicated with a dotted line. All infections were performed in duplicate.

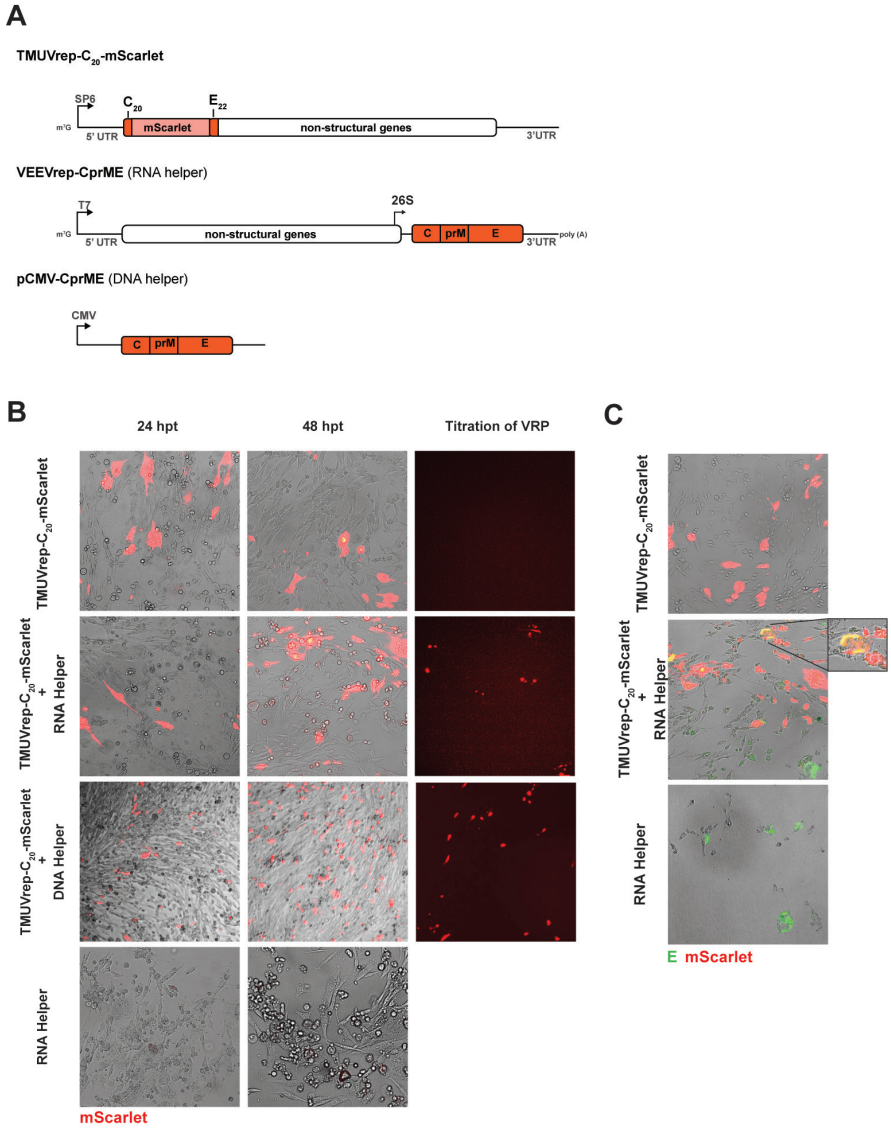


Figure 4. The production TMUV VRPs via the co-transfection of a VEEV-based replicon RNA (VEEVrep-CprME) or CMV-driven DNA (pCMV-CprME) helper construct together with the TMUV replicon RNA in BHK-21 cells. (A) Schematic representation of the SP6-driven TMUV replicon encoding the mScarlet gene (TMUVrep-C_{20}-Scarlet) and the T7-driven VEEV replicon RNA (VEEVrep-CprME) or the CMV-driven DNA helper (pCMV-CprME) expressing the TMUV structural proteins. **(B)** (Fluorescent) Microscopic images showing the detection of mScarlet after the (co-)transfection of TMUVrep-C_{20}-Scarlet and VEEV replicon RNA - or DNA helper in BHK-21 cells. At 48 hours post transfection (hpt), the supernatant of transfected BHK-21 cells was collected and used to infect naive BHK-21 cells. **(C)** Indirect immunofluorescence assay of the (co-) transfected cells at 48 hpt for the detection of envelope (E) after removal of the culture supernatant containing VRPs. Primary antibody: pan-flavi α-E (4G2; mouse). Secondary antibody: Alexa Fluor 488-conjugated α-mouse IgG (goat).

production via trans complementation of the TMUV structural proteins using a DNA helper (**Figure 5**). HEK293T cells were co-transfected using the replicons TMUVrep-C_{20}-GFP, TMUVrep-C_{38}-GFP, or TMUVrep-C_{109}-GFP, previously described in **Chapter 3**, along with pCMV-CprME (**Figure 5A**). Despite the relatively low transfection efficiency observed at 24 hours post-transfection (hpt), the GFP expression confirmed the functionality of the TMUV replicon variants in HEK293T cells (**Figure 5B**). The onset of reporter gene expression at 24 hpt and the GFP intensity was similar to what has been described in BHK-21 cells in **Chapter 3.** From 48 hpt onwards, the co-expression of the TMUV structural proteins resulted in the observation of clear GFP-positive 'plaques' for the constructs TMUVrep-C_{38}-GFP and TMUVrep-C_{109}-GFPs. These plaques were indicative of the reinfection by VRPs of neighboring cells since the number and size of these plaques increased over time. Next, the supernatant of co-transfected cells was collected and titrated to determine the highest VRP release titer (**Figure 5C**). At 72 hpt, the highest VRP titer of 7.3 x 10^3 RP·mL^{-1} was detected in HEK293T co-transfected with TMUVrep-C_{109}-GFP replicon RNA.

To enhance VRP production on HEK293T cells, a more efficient packaging strategy is necessary. Co-transfections using RNA or DNA helpers often result in low transfection efficiencies due to the requirement of delivering the replicon and helper constructs to the same cell (**Figures 4C and 5C**). Hence, a cell line with the stably integrated structural gene cassette of TMUV is preferred. Since the HEK293T cells are commonly used for recombinant protein production and are easily transduced (*E. Tan et al., 2021; Thomas & Smart, 2005*), a lentiviral strategy was designed (**Figure 6**). To avoid unwanted cytotoxicity resulting from the presence of the TMUV structural proteins, an inducible "all-in-one" lentiviral expression vector was used (**Figure 6A**). The structural genes were amplified via PCR and cloned into the pCW57.1 expression plasmid to generate pCW-CprME. As a visual control to track the transfection efficiency, the structural genes were replaced by a GFP reporter gene resulting in the pCW-GFP construct. The constitutively expressed reversed tetracycline-transactivator (rtTA) binds to the tetracycline response element (TRE_{tight}) upon the addition of doxycycline, facilitating the expression of the transgene. In the absence of doxycycline, rtTA undergoes a conformational change which prevents the binding to the TRE_{tight} element and the expression of the transgene. Firstly, BHK-21 cells were transfected with pCW-GFP and cultured in the presence of varying concentrations of doxycycline ranging from 0 to 500 ng·mL^{-1} (**Figure 6B**). The expression of GFP was first detected at 24 hpt and 2 hours post-induction (h_i) in the presence of 50 ng·mL^{-1} doxycycline. The highest GFP expression was achieved with an excess (≥ 100ng·mL^{-1}) of doxycycline supplied in the culture medium. Additionally, a complete suppression of GFP expression was seen in the absence of doxycycline, suggesting that the induction is tightly controlled. Next, BHK-21 cells were transfected with pCW-CprME to express TMUV structural proteins and after 24 hpt, 200 ng·mL^{-1} of doxycycline was supplied to the culture medium (**Figure 6C**). After 24 h_i, an immunofluorescence assay was performed on BHK-21 cells transfected with pCW-CprME. The induced cells clearly showed the expression of TMUV E protein in the presence of doxycycline. In order to establish a stable packaging cell line for the efficient production of TMUV VRPs,

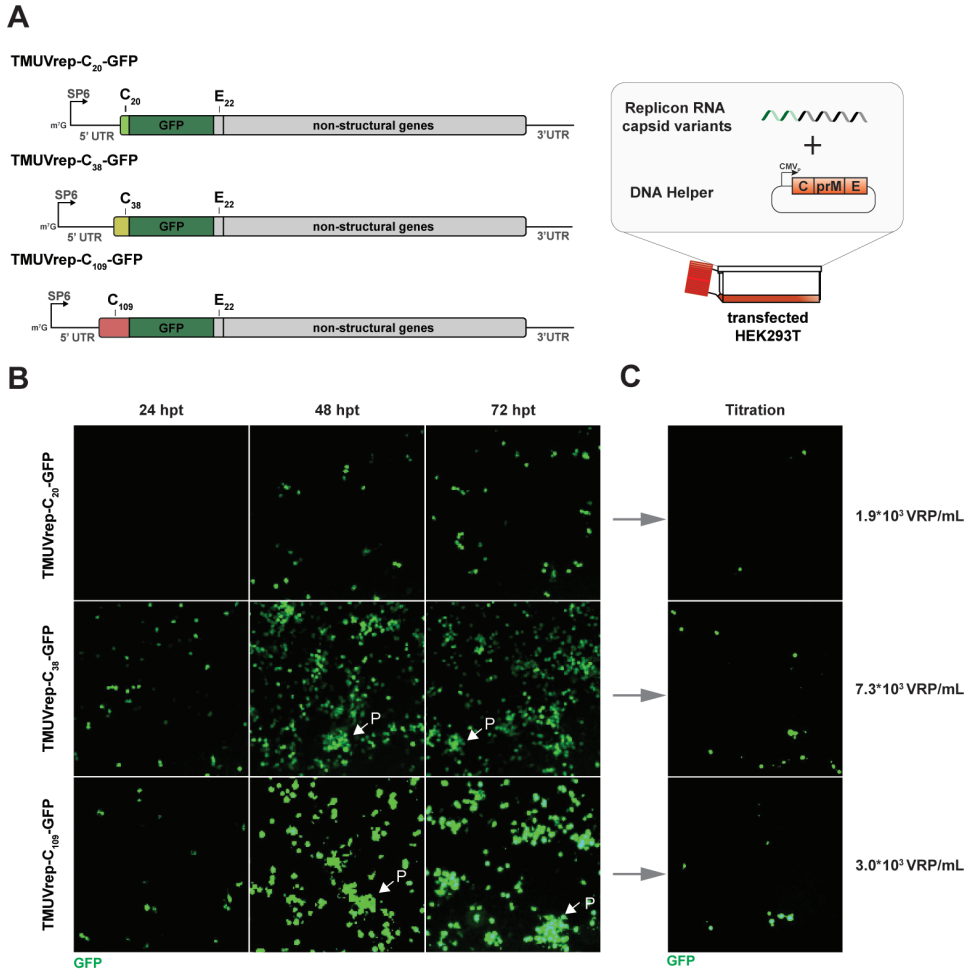


Figure 5. GFP expression of TMUV replicon capsid variants and the transient production of VRP by HEK293T cells. (A) Overview of the various TMUV replicon capsid variants and schematic representation of the trans-complementation of structural proteins by a DNA helper – expressing C-prM-E under control of a cytomegalovirus promoter (CMV_p) for the production of virus-like replicon particles (VRPs) by HEK293T cells. **(B)** Fluorescence images of co-transfected HEK293T cells expressing GFP expressed by the TMUV replicon capsid variants. White arrows indicate the formation of GFP 'plaques (P)' as a result from the production of VRPs. **(C)** Fluorescence images of a two-fold VRP titration on DF-1 cells expressing replicon-encoded GFP. The associated VRP titer of the supernatant at 72 hpt was expressed in replicon particles (RP)·mL^{-1} and indicated to the right.

HEK293T cells were co-transfected with a packaging plasmid (psPAX), an envelope plasmid (phCMV-VSV-G), and the constructed pCW-CprME (**Figure 7A**). Additionally, a visual control plasmid (pLOV-GFP), constitutively expressing GFP, was included to follow the transduction without the need for induction while pCW-GFP served as an induction control. After 48 hpt, the supernatant containing the recombinant lentiviral particles was harvested and used to transduce naive HEK293T (**Figure 7B**). After incubation for two days, cells were split and

either cultured in the presence of doxycycline - to check expression levels and transduction efficiency - or selected using puromycin to establish a stable cell line. After 72 h_i, the induced cells were subjected to an indirect immunofluorescence assay to detect the E protein (**Figure 7B**). Approximately 5-10% of the cells showed the presence of envelope protein in the case of pCW-CprME or GFP protein in the case of pCW-GFP or pLOV-GFP. However, following the induction of the puromycin-selected cells, the population of cells harboring the inducible gene cassette increased, and more than 50% of the cells showed the synthesis of either the envelope or GFP protein indicating the successful generation of a polyclonal packaging cell line (**Figure 7C**).

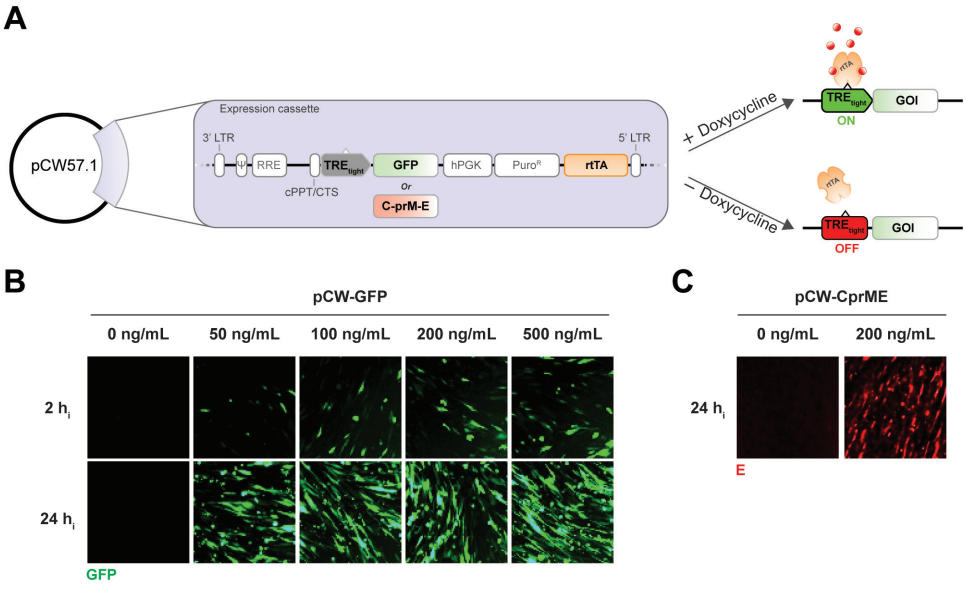


Figure 6. Schematic representation of the lentiviral transfer vector and its responsiveness to doxycycline. **(A)** The transfer vector based on the pCW57.1 vector (in kind provided by David Root) is an "all-in-one" TET-on plasmid system that is compatible with the 3rd generation lentiviral vector system containing the lentiviral packaging signal sequence (Ψ), Rev responsive element (RRE), central polypurin tract (cPPT/CTS) and the following TETon elements: tetracycline response element (TREtight), transgene (GFP or CprME), and a human phosphoglycerate kinese promotor (hPGK) controlling the expression of the puromyicin resistance gene (PuroR) and reverse teetracycline transactivator (rtTA). All the latter elements are flanked by 3' and 5' long terminal repeats (LTR) for packaging into recombinant lentiviral particles. The transgene transcripton from the pCW57.1 expression cassette can be controlled by the addition (+) or removal (-) of doxycyline from the culture medium. **(B)** The functionality of the TET-on system was tested by transfecting pCW-GFP to BHK-21 cells and supplying 0 to 500 ng·mL^{-1} doxycycline to the culture medium. **(C)** Fluorescence images of an indirect immunofluorescence assay detecting the envelope protein expressed by pCW-CprME in BHK-21 cells in the presence of 200 ng·mL^{-1} of doxycycline for 24 h. Primary antibody: pan-flavi α-E protein (4G2; mouse). Secondary antibody: Alexa Fluor 546-conjugated α-mouse IgG (goat). h_i = hours post induction.

In order to establish a stable, monoclonal packaging cell line, the heterogenous population of transduced cells was subjected to fluorescence-activated cell sorting (**Figure 8A**). As no induction was applied during the clonal selection, the process involved random selection of single cells without knowing individual CprME expression levels. The gating strategy for single-cell selection was based on the pLOV-GFP transduced cells, prioritizing the selection of live cells (**Figure 8B**). For the positive control pLOV-GFP, 98% of the cells

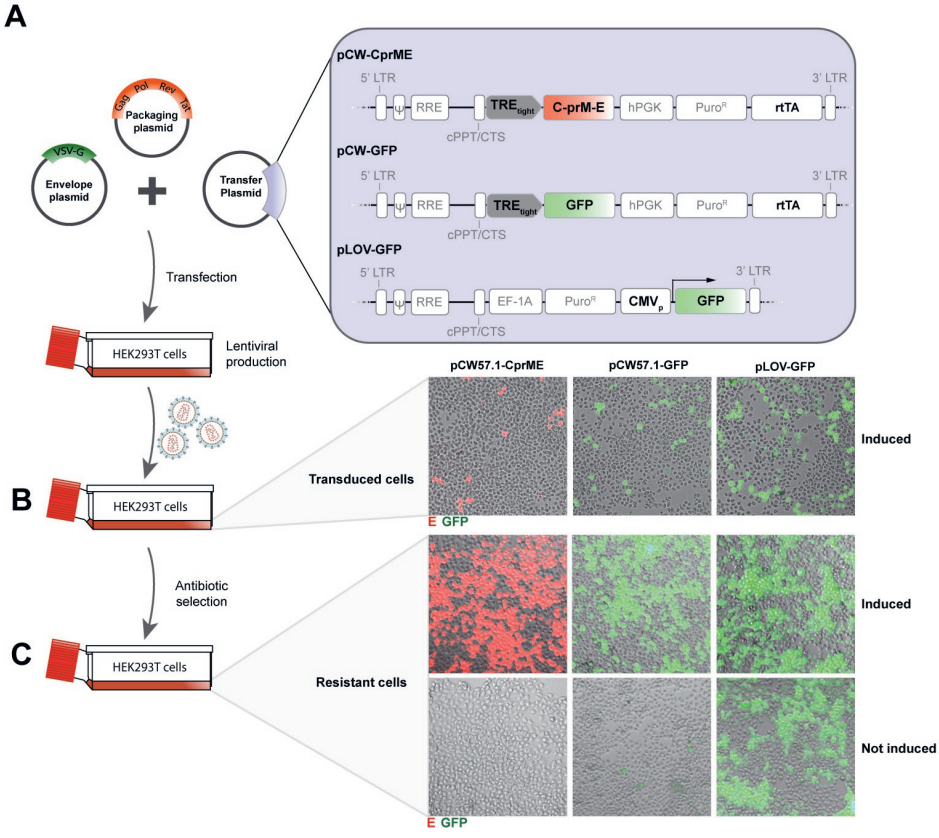
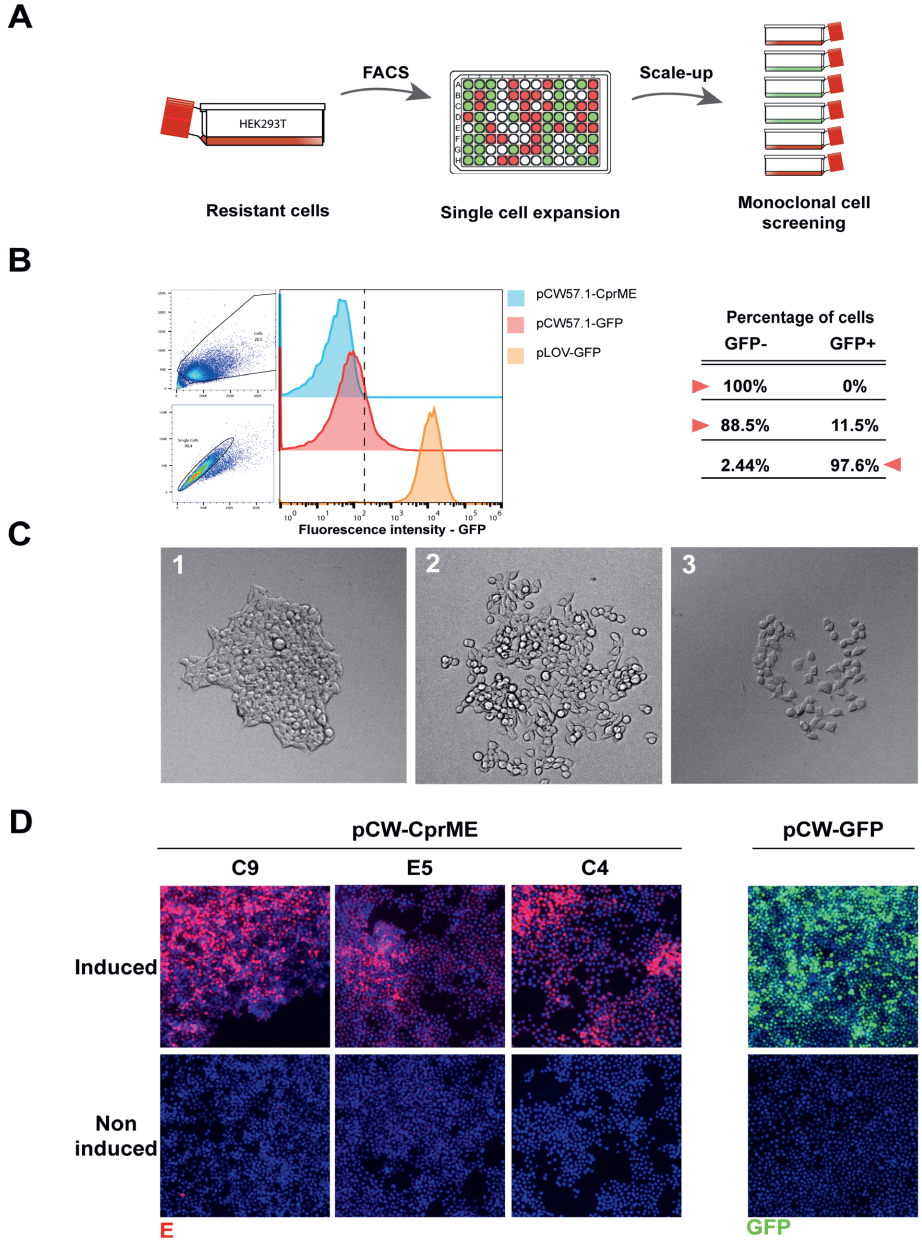


Figure 7. Generation of an inducible TMUV-packaging cell line using lentivirus transduction. (A) Schematic overview of the lentiviral packaging plasmid, envelope plasmid, and the constructed inducible transfer plasmids encoding CprME (pCW-CprME) or GFP (pCW-GFP). As a visual control to track the transduction, an additional transfer plasmid (pLOV-GFP) constitutively expressing GFP under the control of a cytomegalovirus promoter (CMVp) was used. The three vector lentiviral system was introduced into HEK293T cells by $CaCl_2$ transfection. At 48 hours post-transfection, the supernatant containing recombinant lentiviruses was used to **(B)** infect naive HEK293T cells. At 2 days post-transduction, cells were split, induced using doxycycline for 72 h, and subsequently analyzed using immunodetection to visualize cells expressing the envelope (E) protein. The transduced cells were subjected to antibiotic selection and **(C)** the resistant cells were culture in the absence or presence of doxycycline and immunostained to detect the E protein. Primary antibody: pan-flavi α-E protein (4G2; mouse). Secondary antibody: Alexa Fluor 546-conjugated α-IgG (goat).

showed expression of GFP as anticipated. From the pool of gated single cells, non-induced pCW-CprME and pCW-GFP cells were randomly selected, sorted in 96-well plates, and proliferated to sub-confluent densities. Notably, not all clonal cells resulted in the same visual phenotype as healthy HEK293T cells. Aberrant cells exhibiting excessive proliferation, atypical morphology, or increased cell dissociation were excluded during the initial screening process (**Figure 8C**). After two passages, clonal cell lines were screened for E protein expression upon induction with doxycycline (**Figure 8D**). Three selected cell lines, C9, E5, and C4 exhibited a typical cell morphology, robust E gene expression upon induction with doxycycline, and complete suppression of E gene expression in the absence of doxycycline. Furthermore, western blot analysis of the cell lysates of the selected clones demonstrated the prolonged synthesis of the E protein up to 96 hours of induction (**Figure 8E**). Altogether, these results confirmed the establishment of several monoclonal inducible packaging cell lines.

To investigate the potential of monoclonal packaging cells to enhance the overall viral replicon particle (VRP) yield, capped, in vitro-transcribed TMUV replicon RNA was electroporated into monoclonal C3 and C9 cells and immediately induced with doxycycline (200 $ng \cdot mL^{-1}$) (**Figure 9**). Previously, the highest VRP release titer was achieved between 48-72 hpt for both TMUVrep-C_{38}-GFP and TMUVrep-C_{109}-GFP through the trans-supplementation of a DNA helper (**Figure 4**). Consequently, only these two replicon RNA capsid variants were electroporated in the clonal packaging cell lines C4 and C9 and cultured in the presence of doxycycline in the culture medium (**Figure 9B**). As expected, the absence of doxycycline resulted in no observable increase in the number of GFP-expressing cells which coincided with the lack of RNA copies in the supernatant (**Figure S1**). Conversely, in the presence of doxycycline, the cells electroporated with TMUVrep-C_{38}-GFP or TMUVrep-C_{109}-GFP replicon RNA showed an increase in the number of GFP-producing cells over time. Interestingly, a difference in the total number of GFP-expressing cells was observed between TMUVrep-C_{38}-GFP and TMUVrep-C_{109}-


A
HEK293T
FACS
Scale-up
Resistant cells
Single cell expansion
Monoclonal cell screening
B
pCW57.1-CprME
pCW57.1-GFP
pLOV-GFP
Fluorescence intensity - GFP
Percentage of cells
GFP-
GFP+
100%
0%
88.5%
11.5%
2.44%
97.6%
C
1
2
3
D
pCW-CprME
pCW-GFP
C9
E5
C4
Induced
Non induced
E
GFP
E
C9CprME
E5CprME
C4CprME
PC
NC
Induced
hi
24
48
72
96
96
E (55 kDa)

◄Figure 8. Clonal selection and expansion of lentiviral-transduced TMUV packaging cells. (A) Experimental strategy for fluorescence-activated cell sorting (FACS)-mediated clonal selection from a pool of pCW-CprME-transduced packaging cells. **(B)** Gating strategy of transduced HEK293T cells. The percentage of cells expressing GFP (GFP+) was calculated relative to the total number of gated single cells. Red arrow indicates the pool of cells selected for single-cell sorting. **(C)** Brightfield images of proliferated single-cell clones were acquired one week post-sorting. Sorted cells reveal diverse cell morphologies such as *[1]* distinct high density cell clusters with abnormal proliferation rates, others showed *[2]* increased rounding and detachment from the cell monolayer, while certain cells *[3]* maintained a regular phenotype akin to that of healthy HEK293T cells **(D)** Fluorescence images of an immunostaining performed on (non-) induced clonal cell lines C9, E5, and C4 for the detection of envelope (E) protein. pCW-GFP-transduced cells served as an induction control by the expression of GFP. **(E)** Western blot analysis of E protein expression in clonal cell lines C9, E5 and E5 over 96-hour induction period (h_i) in the presence (+) or absence (-) of doxycycline (200 ng·mL^{-1}) in the culture medium. As a positive control (PC) the pCMV-CprME was introduced in regular HEK293T cells constitutively expressing the E protein. Regular HEK293T cells were included as a negative control (NC).

GFP replicon RNA electroporated cells (**Figure 9B**). At 96 hours post electroporation (hpe), a limited number of cells electroporated with TMUVrep-C_{109}-GFP synthesized GFP while nearly all cells produced GFP when TMUVrep-C_{38}-GFP was introduced. VRP titers, however, were very similar and ranged from 3.9 x 10^4 to 7.9 x 10^4 VRPs·mL^{-1} observed at 72 hpe (**Figure 9C**). Interestingly, a higher number of RNA copies was detected in the supernatant of TMUVrep-C_{38}-GFP transfected cells compared to the TMUVrep-C_{109}-GFP transfected cells (**Figure S1**).

Although VRP production was observed in HEK293T cells, it remains unclear why the VRP titers are markedly lower than the viral titers in a wildtype TMUV infection. To study this in more detail, HEK293T packaging cells electroporated with TMUV replicon RNA were analyzed using the transmission electron microscope (TEM; **Figure 10**). Patches of organized protein aggregates, enclosed within a membrane and positioned closely to the ER, were observed within the cell (**Figure 10A-1**). Conversely, these aggregates could not be detected in non-induced packaging cells, as well as in healthy or TMUV WU2016-infected cells (**Figure S2**). Furthermore, the detection of virions in the ER was clearly visible in TMUW WU2016-infected cells while no distinct particles were detected within the ER of VRP-producing packaging cells. When analyzing the size of the individual VRP-like structures within these aggregates (**Figure 10-A1**) or the ER (**Figure 10-B3**), an average size of between ~25 nm and ~38 nm was observed, respectively (**Figure 10C**). In the culture medium collected from VRP- or TMUV WU2016-producing cells, the particle size was on average ~25 nm and ~35 nm, respectively.

4. Discussion

During the COVID-19 pandemic, the interest in vaccine platform technologies gained ground. The mRNA vaccines, consisting of the more conventional mRNA and the novel, self-amplifying RNA (replicon) vaccines took center stage in this vaccine revolution (*Anderluzzi et al., 2022; Künzli et al., 2022; Luisi et al., 2020; Palmer et al., 2022, 2023*). Despite the major development around conventional mRNA vaccines and the numerous trials involving fully synthetic carriers such as LNPs, replicon RNA can benefit from both

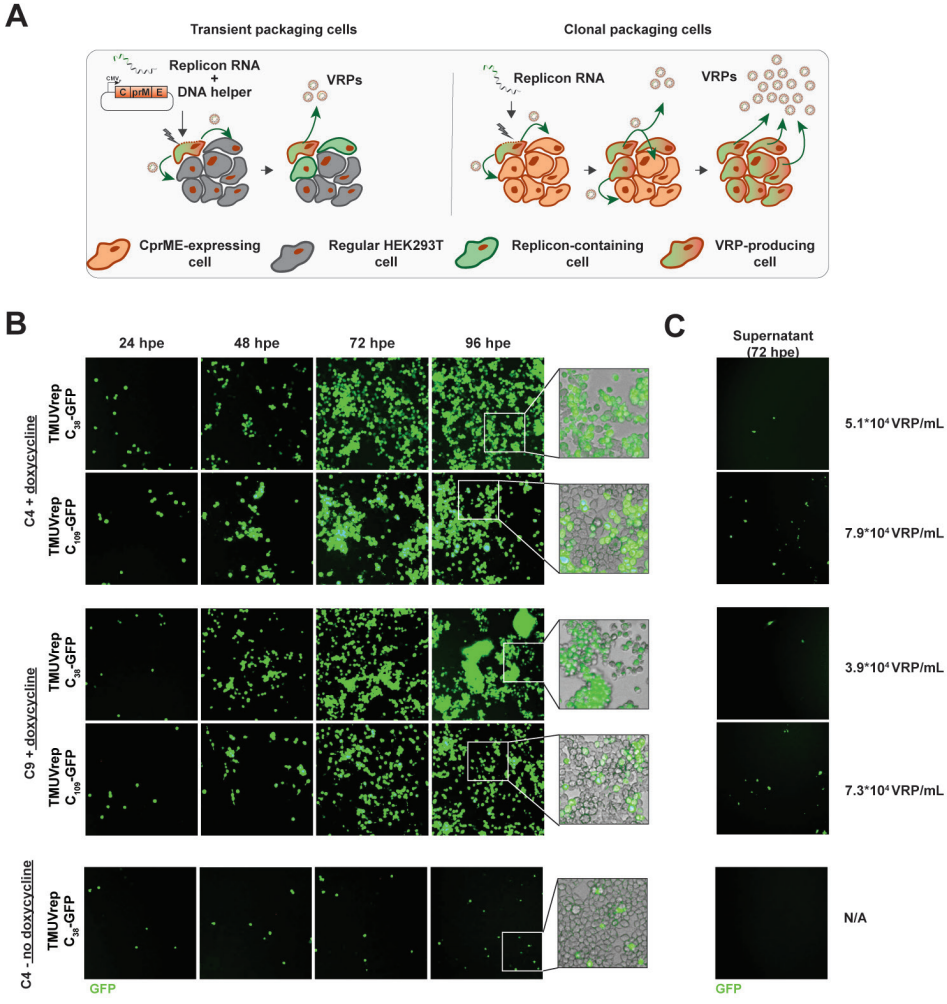


Figure 9. VRP production by introducing TMUV replicon RNA into the monoclonal packaging cells via electroporation. (A) Schematic representation illustrating the difference between the transient production of VRPs or using a clonal packaging cell line. Upon electroporation of TMUV replicon RNA into the monoclonal packaging cells, the produced VRP can infect naive, neighboring packaging cells thereby enhancing the number of cells producing VRP, while the production of VRPs via transient expression of the structural proteins will be limited to cells only co-transfected with the DNA helper and replicon RNA. **(B)** Fluorescence images of doxycycline-induced (+ doxycycline) or non-induced (- no doxycycline) C4 and C9 packaging cells electroporated with TMUVrep-C_{38}-GFP or TMUVrep-C_{109}-GFP replicon RNA visualized over a 96-hour period. Merged images showcased the difference in the number of GFP-expressing cells across the different TMUV replicon capsid variants. **(C)** Fluorescence images of DF-1 cells infected with the culture supernatant collected at 72 hours post-electroporation (hpe) to assess the VRP release titers expressed in VRP·mL^{-1}. Non-induced C4 packaging cells electroporated with TMUVrep-C_{38}-GFP replicon RNA served as a negative control.

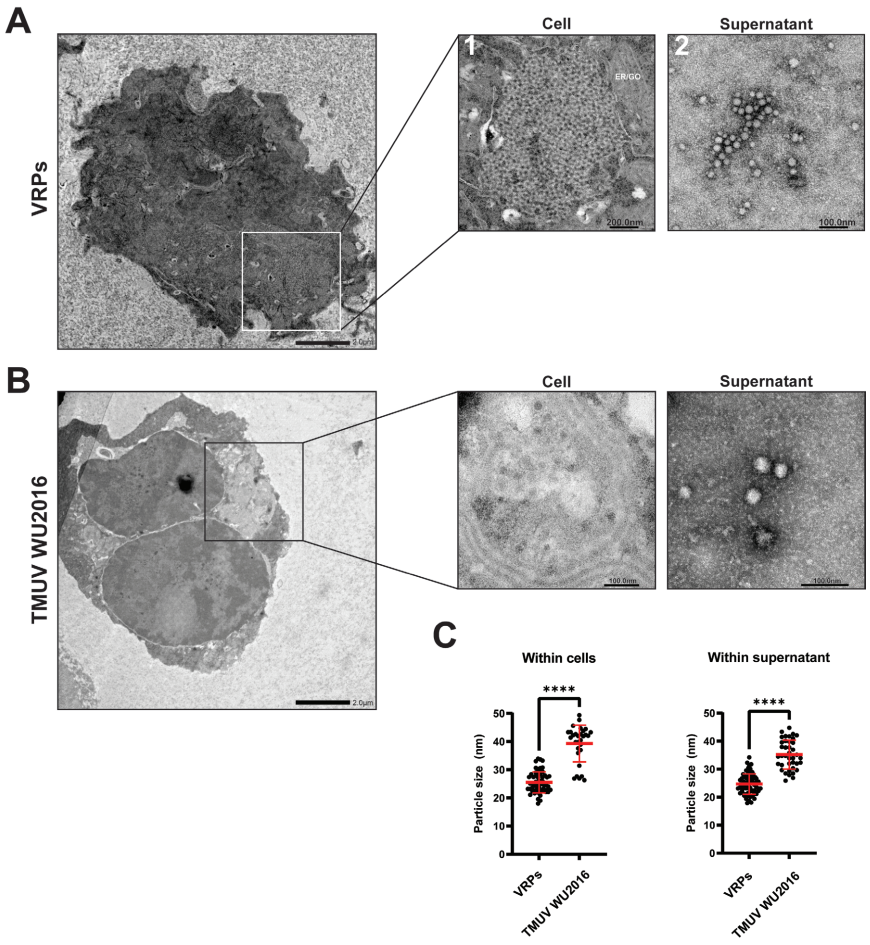


Figure 10. Transmission electron microscopy (TEM) images of HEK293T cells producing TMUV VRPs or wildtype TMUV virions. (A) The clonal HEK293T packaging *[1]* cell electroporated with TMUV replicon RNA showed dense protein aggregates within the cytosol and secretion of virus-like replicon particles (VRPs) in the *[2]* culture supernatant. **(B)** HEK293T *[3]* cell infected at a multiplicity of infection of 0.1 median tissue culture infectious dose per milliter ($TCID_{50} \cdot mL^{-1}$) with TMUV WU2016 isolate showed virions within the ER and secreted virions in the *[4]* culture supernatant. **(C)** The particle size (in nm) distribution was determined using ImageJ analysis of the acquired TEM photos. Indicated statistics showed the p-value ≤ 0.0001 in an unpaired student's t-test.

non-viral and viral formulation (*Blakney, Ip, et al., 2021; Comes, Pijlman, et al., 2023; Erasmus et al., 2020; Langereis et al., 2021; McCullough et al., 2014; Velders et al., 2001*).

In this chapter, we continued the development of the TMUV replicon platform by performing a viral growth analysis using the infectious clones of mosquito-derived TMUV MM1775 and duck-derived TMUV WU2016 in vertebrate and invertebrate cells to select the best cell line for VRP production. During the initial screening, the TMUV MM1775 isolate demonstrated overall lower viral titers than the TMUV WU2016 isolate in adherent vertebrate cells. It has been demonstrated by others, that the mosquito isolate replicates differently in vertebrate cells than duck-derived TMUV isolates (*X. Wang et al., 2021; D. Yan et al., 2018*). In contrast to the previous studies, our duck-isolated TMUV WU2016 outperformed the mosquito-derived TMUV MM1775 in the cell line derived from its natural vector *Culex pipiens* (Cpip) and the commonly used *Aedes aegypti* (C6/36) cells. In general, the titer of both TMUV MM1775 and TMUV WU2016 was lower in invertebrate cells than in vertebrate cells. Hence, vertebrate cells were considered more suitable for the selection of a packaging cell line. BHK-21 cells have been evaluated frequently both in the propagation, but also as a VRP helper cell line for flaviviruses (*Arias-Arias & Mora-Rodríguez, 2021; C. Feng et al., 2020; Lv et al., 2019; Mandl et al., 2001*). Since the viral titers of TMUV MM1775 and WU2016 produced by BHK-21 in our study compared with what is known from the literature (*Hu et al., 2023; Liang et al., 2016; Lv et al., 2019; Mao et al., 2022; D. Yan et al., 2022*), an initial evaluation of VRP production in BHK-21 was performed. Similar to studies describing the production of Kunjin or TMUV VRPs by trans-complementing the structural proteins using a Semliki forest virus-derived replicon in BHK-21 cells (*He et al., 2019; Khromykh et al., 1998*), our VEEV replicon-derived helper demonstrated limited transfection efficiency due to the cytopathic nature of the alphavirus replicons. Additionally, attempts to generate VRPs in BHK-21 cells through the trans-complementation of structural proteins using a DNA helper did not yield the expected results reported for TMUV by others (*He et al., 2019*).

During the evaluation of other vertebrate cell lines both avian DF-1 and EB66 cells but also the mammalian HEK293T cells demonstrated remarkably higher titers than the BHK-21 cells. Despite the high viral titers by DF-1 cells in this study, these cells do not meet the industrial standards yet due to their low proliferation capacity and absence of suspension growth (*Lin et al., 2019*). EB66 cells, however, are used for the large-scale production of modified vaccinia virus Ankara (MVA) vaccines but also for the propagation of Zika, yellow fever virus 17D, and inactivated TMUV virus vaccines (*Léon et al., 2016; Nikolay et al., 2018; Z. Yang et al., 2020*). Although TMUV titers in EB66 cells typically peak between 36-42 hpi (*Z. Yang et al., 2020*), we demonstrated that in these cells the TMUV MM1775 yields increased up to 96 hpi without a noticeable effect on the cell viability, cell proliferation, or acidification of the medium. Unfortunately, due to the high-cost Valneva research license to evaluate EB66 cells further, the viral growth kinetics of TMUV WU2016 and the production of VRPs could not be investigated on these cells.

Ultimately, HEK293T cells were selected, as these cells maintained their high viral titers even in the presence of observable cytopathic effects. HEK293 cells are commonly used as a platform for the propagation or production of flavivirus VLP vaccines (*Powers et al., 2022; Queiroz et al., 2018*), adenovirus-based COVID-19 vaccines (*Joe et al., 2022*), and studying permissive to persistent flavivirus infections (*Mlera et al., 2015, 2016; Sempere & Arias, 2019*). Contrary to earlier reports of TMUV sensitivity to human interferon (Ma et al., 2019) and the moderate susceptibility to TMUV infection reported for HEK293T cells (*Ruangrung et al., 2021*), our experiments demonstrate an early onset of virus propagation and high virus titers at 72 hpi. Additionally, the TMUV replicon RNA was efficiently translated, and the accumulation of GFP was readily detected. In the VRP production assay, we demonstrated that HEK293T cells were capable of producing VRPs upon trans-complementation of a DNA helper. Surprisingly, a noticeable difference was observed between the viral release titer of cells electroporated with TMUVrep-C_{38}- or TMUVrep-C_{109}-GFP compared to those electroporated with TMUVrep-C_{20}-GFP. It is unknown whether the mature capsid synthesized from the TMUVrep-C_{109}-GFP replicon RNA and the DCS-PK RNA element in the capsid gene contributes to the enhanced production or release of VRPs. The production of the full-size capsid protein production has been reported to prevent early-phase cell apoptosis during flavivirus infection (*Urbanowski & Hobman, 2013*) and enhance virus production (*Ishida et al., 2019; Mori et al., 2005a; Sangiambut et al., 2008a*). However, it should be noted that due to the low GFP fluorescence intensity in cells infected with TMUVrep-C_{20}- and TMUVrep-C_{38}-GFP VRPs, it was more challenging to score single GFP-positive cells compared to cells infected with TMUVrep-C_{109}-GFP VRPs.

To further improve the VRP production yield, an alternative for transient and constitutive expression of the structural gene cassette was explored. By the use of lentiviral-mediated transduction of the inducible transfer plasmid in HEK293T and subsequent single-cell sorting using flow cytometry, multiple monoclonal packaging cell lines were established. Inducible expression of the envelope gene was confirmed using indirect immunofluorescence assay and western blot analysis in all clonal cell lines, but the number of VRPs secreted in the supernatant differed between the two clonal cell lines and among the replicon RNA variants. Interestingly, the difference in VRP titer between the TMUVrep-C_{38}-GFP and TMUVrep-C_{109}-GFP was not reflected by the number of genome copies in the supernatant despite detecting generally more replicon RNA in the monoclonal cell line C4 compared to C9. Whether this is a limitation of the limited visibility of individual cells expressing GFP mediated from the TMUVrep-C_{38}-GFP remains untested.

Despite the increase of VRP titers produced by stable cell lines compared to transient expression of TMUV structural genes, other reports demonstrate even higher VRP titers produced with stable packaging cells (*Garg et al., 2017; Harvey et al., 2004*). To assess this difference between VRP production by packaging cells and cells producing wildtype TMUV virus, the clonal packaging cells were analyzed using the TEM. Interestingly, clonal cells electroporated with TMUV replicon RNA and induced with doxycycline showed the presence of large protein aggregates surrounded by a membrane. In the absence of doxycycline, these aggregates could not be detected. It is possible that the early and separate expression of C-prM-E under control

of the highly active, doxycycline-inducible TRE_{tight} promoter, disturbs a normally highly coordinated viral process that ultimately results in a low amount of C-prM-E processing by the NS2B-NS3 protease. As a consequence, the unprocessed virions might accumulate. Despite the equal size of the VRPs in the supernatant compared to the VRP-like structures of these aggregates within the cell, it remains unknown whether these VRP-like structures contain replicon RNA. Moreover, there seems no effect of the expression of different capsid gene variants (e.g. TMUVrep-C_{109}-GFP) on the formation of aggregates in these cells (**Figure S2**). Identifying whether these aggregates indeed contain viral proteins and are infectious could partly be answered by immunogold labelling against any of the TMUV structural proteins. Next to the formation of aggregates, a difference in particle size was observed between the virions in the supernatant of wildtype TMUV WU2016 infected cells compared to the VRPs in the supernatant of packaging cells. However, it was confirmed that the smaller VRPs were infectious and contained replicon RNA. Size differences between wildtype virions and VRPs have been described earlier, demonstrating the structural flexibility of VRPs containing either flavivirus replicon RNA (*Khromykh et al., 1998*), alphavirus RNA genomes (*Barrett et al., 1984; Johnston et al., 1975; Nanda et al., 2009*) or coronavirus replicon RNA (*L. Tian et al., 2022*).

Our findings demonstrated that TMUV WU2016 can be propagated in a series of different (in)vertebrate cell lines although notable differences in CPE can be observed. While we successfully generated a clonal HEK293T packaging cell line and demonstrated the TMUV VRP production, further efforts are needed to optimize VRP production to develop a robust packaging cell line for future VRP vaccines.

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6. Supplemental data

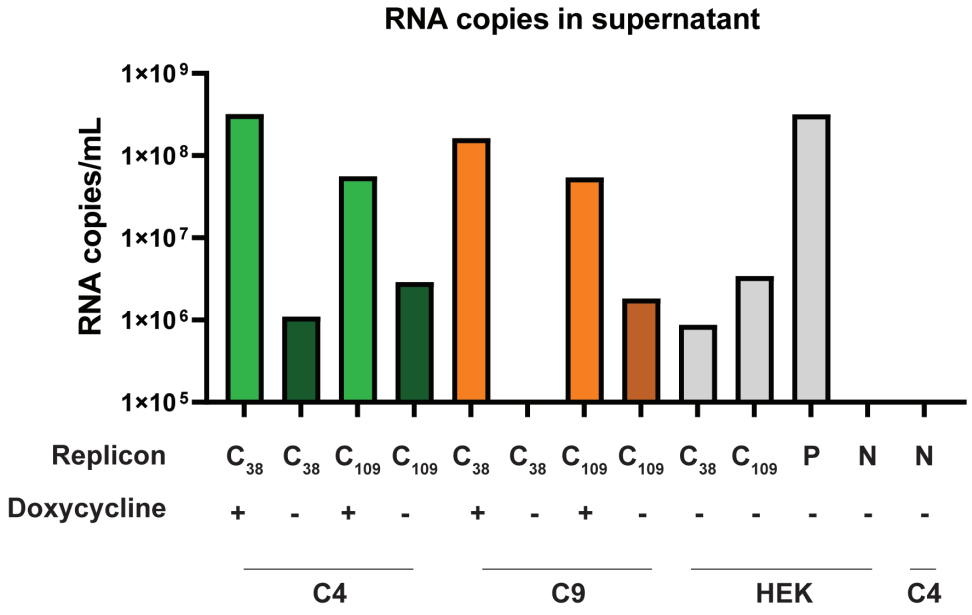

Figure S1. RT-qPCR analysis for the detection of genome copies in the supernatant of VRP producing packaging cells. TMUVrep-C_{38}-GFP (C_{38}), TMUVrep-C_{109}-GFP (C_{109}) replicon RNA were electroporated in monoclonal packaging cells C4, C9 or regular HEK293T (HEK) cells and culture in the absence (-) or presence (+) of doxycycline. As a positive control (P), regular HEK293T cells were infected with TMUV WU2016 at an MOI of 0.01 $TCID_{50}$·mL^{-1}. As a negative control (N), regular HEK293T cells or monoclonal packaging cell C4 were electroporated using PBS. After 72 h, the supernatant was collected and RNA isolated using TRIzol and used for RT-qPCR. Specific primers for nonstructural protein 3 gene were used to assess the amount of genome copies in the culture supernatant.

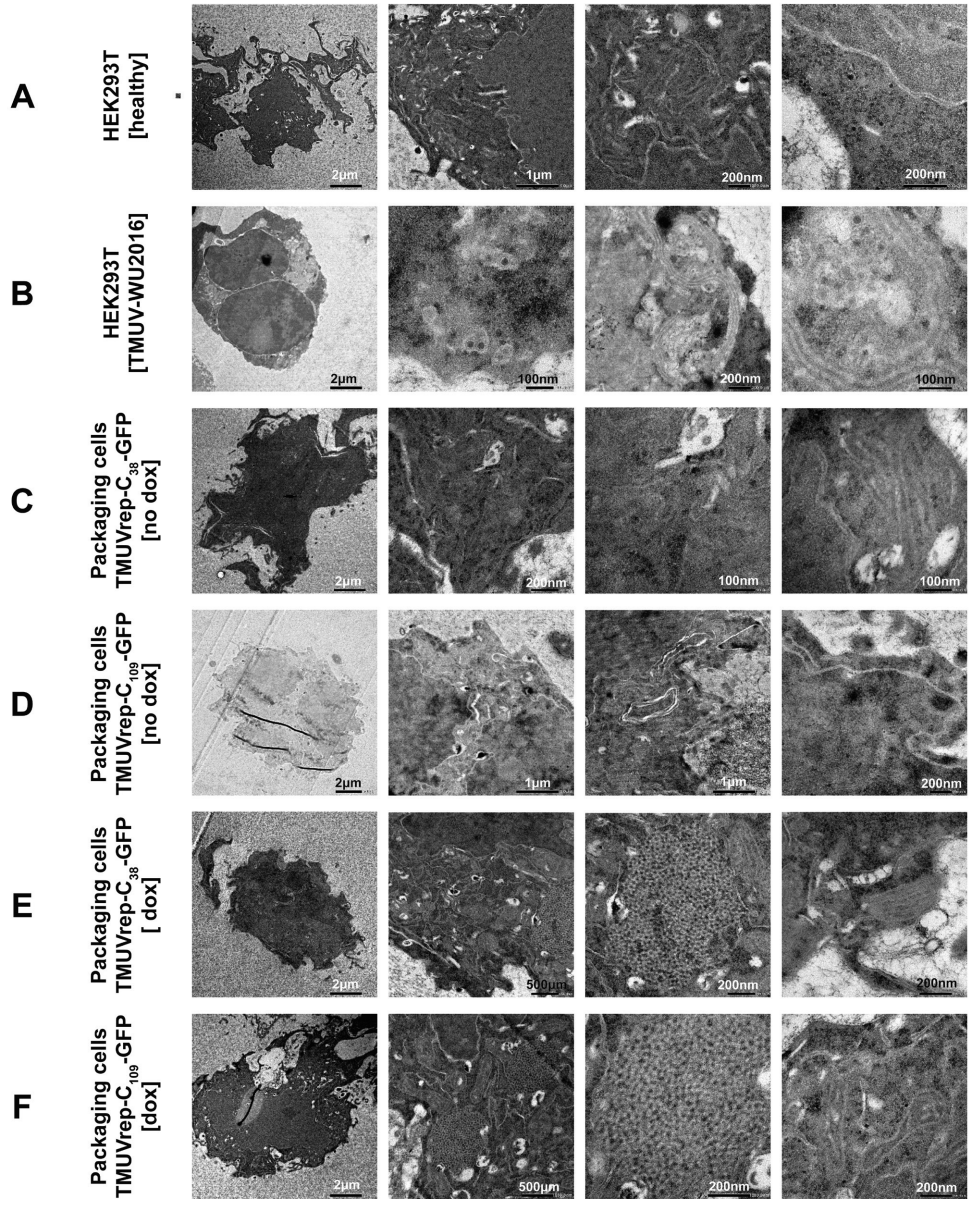

Figure S2 Transmission electron microscopy images of packaging cells producing VRPs. (A) Naive HEK293, **(B)** TMUV WU2016 (MOI 0.01 $TCID_{50}{\cdot}mL^{-1}$) infected HEK293T cells and TMUVrep-C_{38}-GFP or TMUVrep-C_{109}-GFP electroporated packaging cells cultured in the (**C-D**) absence or (**E-F**) presence of doxycycline (dox).

V

Chapter 6

Rise of the RNA machines – self-amplification in mRNA vaccine design

This chapter has been published in a slightly modified version as:

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Trends Biotechnol, https://doi.org/10.1016/j.tibtech.2023.05.007

Abstract

mRNA vaccines have won the race for early COVID-19 vaccine approval, yet improvements are necessary to retain this leading role in combating infectious diseases. A next generation of self-amplifying mRNAs, also known as replicons, form an ideal vaccine platform. Replicons induce potent humoral and cellular responses with few adverse effects upon a minimal, single-dose immunization. Delivery of replicons is achieved with virus-like replicon particles (VRPs), or in nonviral vehicles such as liposomes or lipid nanoparticles. Here, we discuss innovative advances, including multivalent, mucosal, and therapeutic replicon vaccines, and highlight novelties in replicon design. As soon as essential safety evaluations have been resolved, this promising vaccine concept can transform into a widely applied clinical platform technology taking center stage in pandemic preparedness.

The mRNA vaccine landscape – what is on the horizon?

Synthetic mRNA vaccines made their grand entrance during the coronavirus disease 2019 (COVID19) pandemic after many years of fundamental and preclinical research. Given their rapid development, cell-free manufacturing (see **Supplementary - Glossary**) and high clinical efficacy, mRNA vaccines outcompeted conventional live-attenuated, inactivated, and protein-based subunit vaccines in the race for early vaccine approval (*Bio, 2021*). However, the massive roll-out of mRNA vaccines also revealed challenges in balancing the high administration dose with adverse effects, the requirement of prime–boost vaccinations, and the necessity of cold-chain storage. These challenges can be overcome by the next generation of mRNA vaccines based on self-amplifying mRNA, also known as replicon RNA (*de Alwis et al., 2021; Vogel et al., 2018; Voigt et al., 2022*).

Whereas mRNA vaccines encode a protein of interest, replicons have been engineered as a molecular chassis encoding the gene of interest (GOI; transgene) and all essential elements allowing self-amplification of the replicon RNA. The rapid amplification of replicon RNA in target cells increases the expression of the protein of interest (e.g., a viral (glyco)protein) (**Figure 1**) and induces a protective immune response at a markedly lower initial RNA dose than conventional mRNA vaccines (*de Alwis et al., 2021; Vogel et al., 2018*). The self-amplifying replicon genes have been derived from a wide variety of positive-stranded RNA viruses. In this review, we focus on alpha- and flavivirus-based replicons as they are best studied for both human and veterinary applications (*Hikke & Pijlman, 2017; Lundstrom, 2016*). Because the viral structural genes have been replaced by a transgene, the replicon RNA cannot spread in the environment, which is a key difference with chimeric or recombinant virus vaccines (**Supplementary - Box 1**) (*Kamrud et al., 2010; Suzuki et al., 2014*). The replicon technology has several key advantages over traditional vaccines. First, as the transgene is synthetically derived and the replicon cannot spread, replicon manufacturing processes have low biocontainment restrictions and application is safe-by-design (*Meechan & Potts, 2020*). Second, the transgene can simply be inserted into the 'plug-and-play' replicon (e.g., the commercially available Simplicon plasmid of Merck/Sigma–Aldrich), allowing rapid application in case of emerging infectious disease outbreaks. Finally, due to the self-amplifying character of the replicon vaccine, both humoral and cellular immune responses are triggered, which promises induction of protective immunity with a single low-dose immunization (*de Alwis et al., 2021; Erasmus et al., 2020; Vogel et al., 2018*). Despite a thorough understanding of the biology and the benefits of replicon technology, commercial application of alphavirus- and flavivirus-based replicons has only just commenced.

Here, we provide an overview of current challenges in replicon formulation and delivery and the safety considerations that need to be overcome. We discuss future opportunities for mucosal and therapeutic vaccination as well as novel advances in replicon design that can be exploited to transform this promising vaccine concept into a widely applied clinical platform technology.

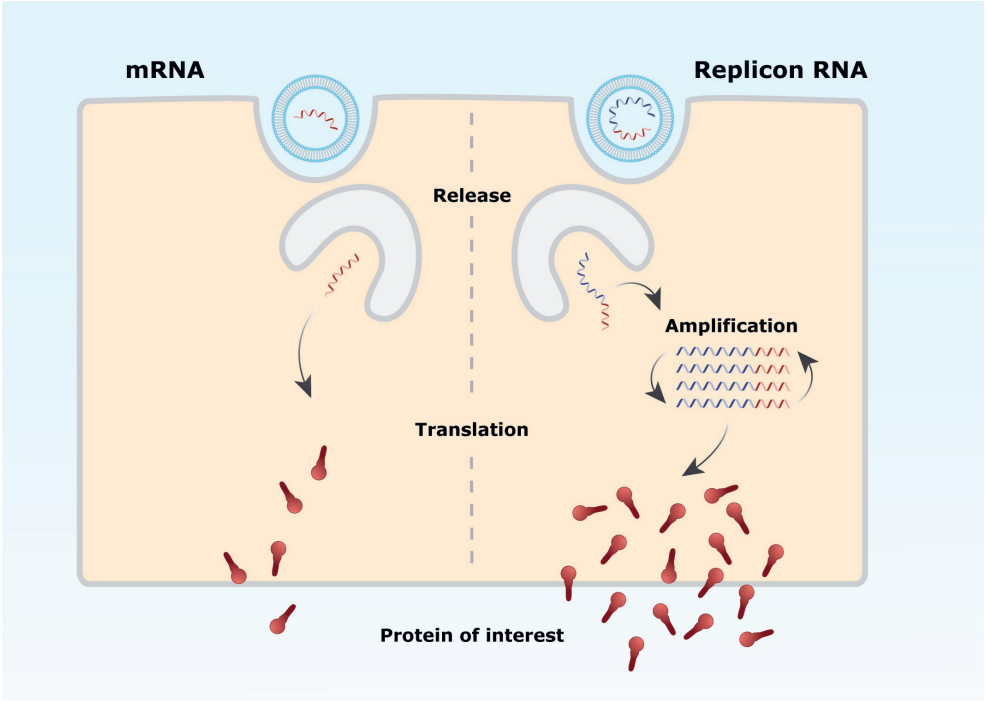

Figure 1. Schematic representation of the protein of interest expression induced by a conventional mRNA and a replicon vaccine. Once released in the cell, the mRNA is translated to produce the protein of interest. In contrast to mRNA, replicon RNA encodes alongside the protein of interest, self-amplifying genes (depicted in blue) that amplify the replicon RNA. This intracellular amplification will subsequently result in higher expression levels of the protein of interest.

Developments in replicon vaccine formulation and delivery

The most straightforward replicon formulation is based on naked delivery of the nucleic acids to the target cell. Early studies showed that direct injection of a low dose (10 μg) of naked RNA was sufficient to establish potent cellular and humoral immune responses in mice (*Zhou et al., 1994*). However, throughout the years, studies continued to report the susceptibility of naked RNA to nucleases and thus their underperformance in clinical applications (*Démoulins et al., 2021; Diken et al., 2011*). While the replicon RNA amplifies itself and mediates the production of the protein of interest reaching 15–20% of total cell protein, the high sensitivity to RNases and poor capacity for internalization into the host cell, compromise the full capabilities of the naked replicon RNA (*Geall et al., 2012; Kamrud et al., 2010*). Hence, several more stable modalities have been developed and are still being optimized, such as VRPs, as well as nonviral delivery systems, such as liposomes and lipid nanoparticles.

VRPs

Replicon RNA is packaged into VRPs by the viral structural proteins expressed in trans from helper RNAs (**Figure 2A**). The natural tropism of these particles, which are structurally similar

to a virus, enables the efficient delivery of the replicon to dendritic cells (direct priming) or target cells that indirectly prime antigen-presenting cells, resulting in the induction of a robust immune response (*Huckriede et al., 2004; Rayner et al., 2002*). Although the genetic cargo of the VRP consists of replicon RNA, which is limited to a single round of transduction, there is an anticipated risk of recombination between the replicon RNA and the trans-provided helper RNA resulting in the generation of replication competent virus (RCV) during manufacturing (*Weiss & Schlesinger, 1991*). RCV is an undesirable contamination in clinical products. To eliminate the risk for RCV formation, split-helper systems are used for VRP production with one helper RNA encoding the capsid protein and the other helper RNA the envelope glycoproteins. Both helpers lack subgenomic promoter sequences to reduce homologous overlap with the replicon. At least two independent, nonhomologous recombination events would be required to generate RCVs, but this has never been observed (*Pushko et al., 1997*).

Another potential concern for VRP delivery is antivector immunity induced after repetitive application of the highly immunogenic VRPs. Antivector immunity reduces the efficiency of vaccines as a consequence of antibody binding to the delivery vehicle. These expectations were based on interfering antivector responses in the adenovirus and vaccinia virus vector field (*Barouch et al., 2004; Sharpe et al., 2001*). Although vaccination with alphavirus or flavivirus VRPs can trigger vector-immune responses, these did not adversely influence the vaccine efficiency after repetitive vaccinations with the same VRP (*Aberle et al.,*

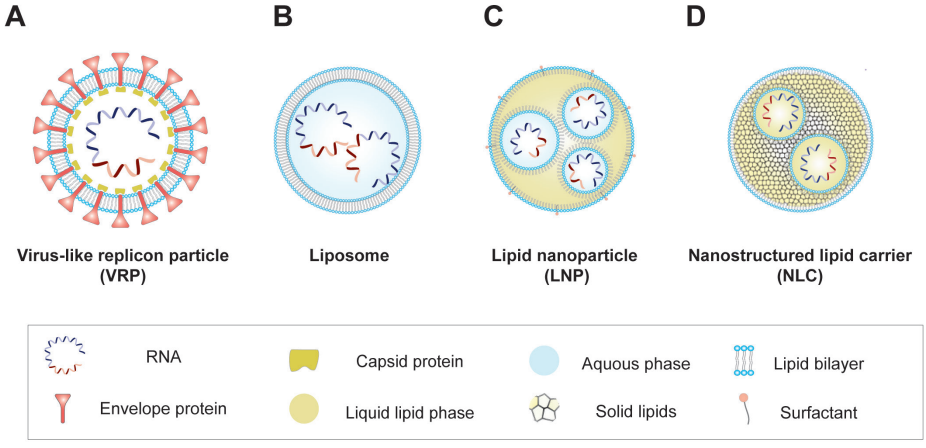

Figure 2. Schematic overview of replicon delivery vehicles. (A) In trans co-expression of replicon RNA and helper RNAs in a mammalian production cell line enables encapsulation of replicon RNA in virus-like replicon particles. Delivery vehicles can also be based on nonviral carriers that encapsulate the replicon RNA in **(B)** liposomes, **(C)** lipid nanoparticles, or **(D)** nanostructured lipid carriers in a cell-free manufacturing process. The delivery vehicles protect the replicon RNA and allow efficient delivery to target cells upon immunization.

2005; Erasmus et al., 2020; Uematsu et al., 2012; Walczak et al., 2011; White et al., 2007). Another study showed that five repetitive vaccinations with the same VRP did not cause a decrease in serum antibody levels against a subsequent vaccination encoding a new protein of interest (*Uematsu et al., 2012*). These results demonstrate that VRPs can repeatedly be applied against diverse antigens in a platform technology strategy without excessive interference of antivector immunity. However, to fully exclude antivector immunity, nonviral and lipid-based carriers for replicon RNA delivery can be considered.

Liposomes, lipid nanoparticles, and other nonviral carriers

Besides the VRP system, several alternative nucleic acid delivery methods based on chemical formulations have been developed and optimized over the years; namely, liposomes, liquid lipid nanoparticles (LNPs), and solid lipid nanoparticles (SNPs). These synthetic formulations improve vaccine stability, allow efficient replicon delivery, and rely on manufacturing processes without (mammalian) cell substrates. Most of these synthetic carriers are also extensively used in the pharmaceutical industry for the delivery of antibodies, peptides, or contrast substances (*Musielak et al., 2022*).

Liposomes consist of a charged lipid bilayer with an aqueous core that can capture hydrophilic molecules such as DNA and RNA (*Tenchov et al., 2021*) (**Figure 2B**). The surface of liposome complexes can easily be adapted with other moieties, such as polyethylene glycol (PEG)–lipid conjugates or small molecules (e.g., antibodies), to facilitate tissue-specific vaccine delivery. Although most phospholipids used in liposomes spontaneously self-assemble when exposed to water, the ability to scale up the production procedure, the efficient trapping of RNA, and the flexibility in liposomes size are confined (*Tenchov et al., 2021*). Early-generation cationic liposomes used in the formulation of RNA might be challenged by the fact that RNA is sometimes exposed on the outside of the carrier compromising both the stability and toxicity of the vaccine (*Xue et al., 2015*). In contrast to VRPs, the bare lipid exterior lacks immunogenic proteins, which prevents unfavorable antivector immunity (*Chen & Huang, 2005*).

A more flexible delivery platform for nucleic acids are LNPs, which are composed of a single layer of lipids combined with surfactants (**Figure 2C**). The core is not required to be aqueous, but can consist of liquid lipids (e.g., LNPs), solid lipids (e.g., SNPs), or a combination known as nanostructured-lipid carriers (NLCs). Similar to liposomes, the lipid membrane of LNPs allows for additional modification to either the surface as well as the drug cargo itself (*Buschmann et al., 2021; Xue et al., 2015*). For example, in 1998 the implementation of ionizable lipids revolutionized the LNP characteristics and reduced innate immunogenicity towards the exterior lipid molecules used in early-generation lipid carriers. In contrast to liposomes, these newer-generation LNPs require a carefully controlled manufacturing process that first captures the RNA at low pH while an additional step neutralizes the lipid charge for effective in vivo delivery. As a result, a more precise cargo formulation, broader application due to the variability in core composition, and better control over LNP size are achieved. These LNPs are taken up by antigen-presenting cells via receptor-mediated endocytosis.

Subsequently, the pH drop within the endosome results in protonation of the ionizable lipids and facilitates membrane fusion of the LNP and release of the RNA into the cytosol. The delivery of the replicon RNA formulated in LNPs does however require precise and extensive testing as was observed in a study comparing the intradermal delivery of LNP-encapsulated replicon RNA and naked replicon RNA in vivo. Upon injection of a high-dose replicon RNA:LNP complex in mice, a short-lived peak in innate immune responses resulted in a lower replicon-mediated protein expression compared to an injection with a low-dose replicon RNA:LNP complex. This highlights that highly efficient delivery of the LNP-formulated RNA to the cells can sometimes induce a strong local innate immune response that counteracts the protein expression via mRNA translational blockage (*Huysmans et al., 2019*). To better control the delivery of the replicon RNA, the use of SNPs or NLCs may be exploited. Both are developed to overcome the weaknesses of liposomes and LNPs, such as stability, large-scale production, and drug release. The hybrid composition of liquid and solid lipids in NLCs (**Figure 2D**) makes these superior to SNPs. The increased drug loading capacity, the prolonged drug release and ability to stockpile vaccines in preparation for a pandemic makes the NLCs a valuable delivery method (*Gerhardt et al., 2021; Ghasemiyeh & Mohammadi-Samani, 2018; Mishra et al., 2018*).

DNA-launched replicons

In the early race for nucleic acid vaccines, the primary focus was on DNA instead of RNA. In the case of replicon vaccines, a prerequisite of the in vitro transcription of replicon RNA is a DNA template. Since the DNA template can also be delivered directly to the cell itself, thereby surpassing the need of an in vitro RNA reaction, DNA-launched replicon (DREP) constructs were designed. A DREP is typically a circular double-stranded DNA molecule with a strong promoter (e.g., human cytomegalovirus immediate early promoter) enabling RNA polymerase II-driven transcription of replicon RNA in the nuclei of transfected cells (*Agapov et al., 1998; Berglund et al., 1998; Varnavski & Khromykh, 1999*) (**Figure 3**). The replicon RNA is then transported to the cytoplasm as a capped mRNA and is translated to initiate self-amplification, similar to the direct delivery of in vitro transcribed replicon RNA (*Kallen & Theß, 2014*). A DREP shows less susceptibility to nucleases, is intrinsically more stable, and simplifies storage requirements in the logistic pipeline. However, the delivery of naked plasmid DNA to the nucleus is more challenging than the delivery of mRNA to the cytoplasm (*Gómez-Aguado et al., 2020; Johansson et al., 2012*). Nonetheless, combining advanced physical delivery techniques such as electroporation can aid the delivery of DNA to the nuclei and result in similar levels of immune response elicited by naked replicon RNA. Electroporation is mostly optimized for intradermal delivery of vaccines, which somewhat restricts their application (*Ljungberg & Liljeström, 2014*); however, DNA can efficiently be delivered by LNPs both *in vitro* and *in vivo*. It was observed that both protein of interest production and immunogenicity of LNP-delivered DNA was higher compared with naked DNA (*Mucker et al., 2020*). To improve nuclear DNA delivery, specific sequence elements can facilitate recognition by transcription factors and aid the translocation of the DNA via nuclear pores (*Dean et al., 1999*). A potential safety concern of DNA vaccines is the theoretical risk of DNA vector integration into the host genome or adverse (health) effects of prokaryotic elements. Fortunately, several

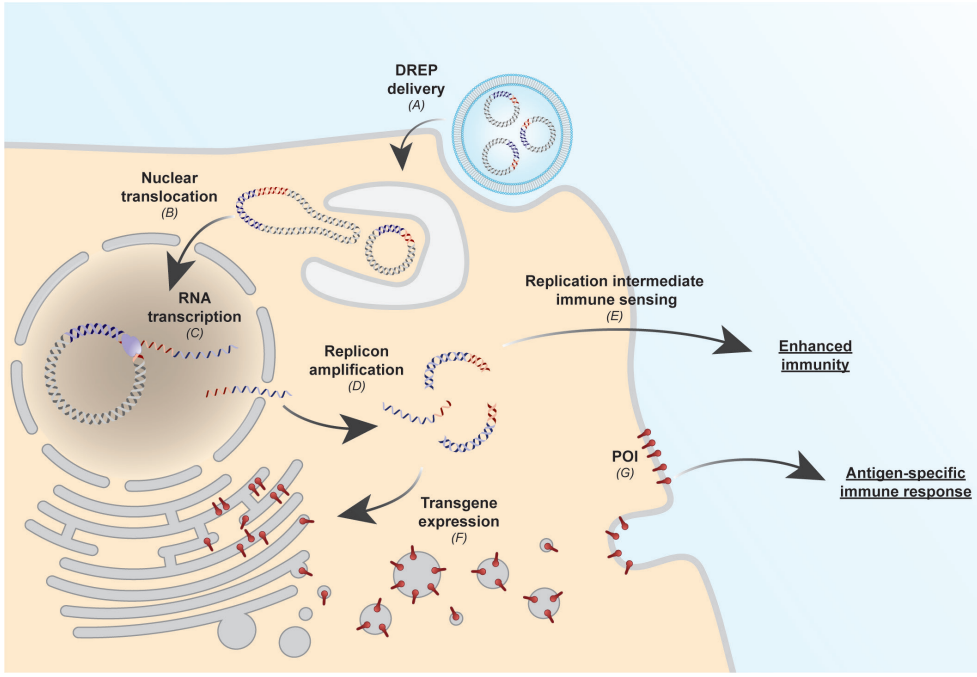

Figure 3. Schematic overview of heterologous gene expression using a liposomal-delivered DNA-launched RNA replicon (DREP). (A) Upon liposomal delivery to a cell, **(B)** the DREP migrates to the nucleus where **(C)** it serves as a template for the RNA polymerase II-mediated transcription of replicon RNA. **(D)** Subsequently, the replicon RNA is transported to the cytoplasm where the self-amplification, mediated by replicase proteins, occurs. **(E)** During amplification, cellular sensors recognize amplification intermediates (double-stranded RNA), enhancing host immunity. **(F)** At the same time, translation of the replicon RNA produces the protein of interest (POI). **(G)** This will induce an antigen-specific immune response.

studies have shown that the risk of plasmid integration is negligible under a variety of experimental conditions (*Ledwith et al., 2000; Manam et al., 2000; Nichols et al., 1995*). Provided that incidents of integration described in literature are rare, actual integration might depend on factors such as the route of administration, the cell type, the expressed antigen, and the presence of prokaryotic elements (*Doerfler, 2021, p. 2003; Ledwith et al., 2000; Würtele et al., 2003*). In contrast to RNA vaccines, DNA vaccines typically require a prokaryotic host for plasmid propagation; therefore, functional elements such as a replication origin and an antibiotic resistance gene are encoded on the DNA vector. Research shows that the prokaryotic sequence elements could affect expression in the eukaryotic cells or cause DNA vector instability (*Williams et al., 2009*). To tackle this, several strategies could be implemented in the DNA vector design, such as the use of ministring/minicircle DNA (*Nafissi et al., 2014*).

Safety considerations that challenge vaccine registration

Widespread commercial application of replicon vaccines can have an impact on pathogen control strategies, but to reach full potential of the replicon technology, registration as a vaccine platform is required. The vaccine platform concept relies on licensing a single

replicon vector, a standardized delivery method, and a well-defined process for inserting the transgene to generate different replicon vaccines which do not require extensive re-evaluation as part of the authorization procedure. Indeed, the United States Department of Agriculture (USDA) already registered alphavirus-based Venezuelan equine encephalitis virus (VEEV; vaccine strain TC83) replicon particles as veterinary platform technology (product code 9PP0.00), allowing rapid delivery of customized replicon vaccine for farmers (*Animal and Plant Health Inspection Service & Veterinary Services, 2023*). EU regulations on veterinary platform technologies are currently in development to help increase the availability of vaccines (*EMA, 2021*). Confidence in replicon platform registration in the veterinary field will help establishing similar procedures for the human vaccine field. Recently, the first human replicon vaccine was granted an Emergency Use Approval by Indian regulators (Central Drugs Standard Control Organization; CDSCO). This CDSCO-approved VEEV-TC83-based replicon vaccine expresses a severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike gene and is encapsulated in LNPs (license number MF/BIO/22/000064 under PD/Vacc-06) (*Dauer, 1955; Erasmus et al., 2020; Hawman et al., 2022, 2022*).

Global authorization of alphavirus- and flavivirus-based replicon vaccines is expected now that others have paved the way. The USDA-approved VEEV-TC83 replicon vaccine already showed successful utilization in the immunization of millions of animals against a swine coronavirus – porcine epidemic diarrhea virus (*Vander Veen et al., 2012*). Furthermore, a live-attenuated, chimeric flavivirus vector vaccine platform (ChimeriVax) is commercially available in Australia, Thailand, Mexico, the Philippines, and Brazil (*Condit et al., 2016*). In this chimeric yellow fever virus (YFV) vectored vaccine, the YFV envelope gene is replaced by a heterologous flavivirus gene to induce protective immunity against Japanese encephalitis virus and dengue virus (DENV) in humans (*Monath et al., 2002*). Whereas the Chimerivax live-attenuated vaccines still encode structural genes, replicon vaccines do not encode the structural alphavirus or flavivirus genes, cannot spread in the environment, and consequently are anticipated safer. Despite these recent successes, there are still challenges ahead for global authorization of alphavirus and flavivirus replicon vaccines.

At present, the main challenges involved in the global authorization are potential safety concerns regarding the replicative character of these vaccines. As for all self-amplifying vaccines, concerns have been raised over adverse events in vulnerable individuals. For example, replicon vaccines could persist in immunocompromised individuals as clearance may be less efficient. Another group of consideration is pregnant women, especially when using replicon vectors derived from viruses that cause congenital infections (e.g., VEEV and YFV) (*Charlier et al., 2017; Platt et al., 2018*). Nevertheless, it is expected that replicon RNA will not disseminate to the fetus, because the most common route of replicon vaccine administration, intramuscular injection, is associated with local distributions at the injection site. Intravenous injections can cause systemic distribution, but the placenta will hinder passage of the replicon (*Colmenero et al., 2001; Morris-Downes et al., 2001*). Even if the replicon spreads to the fetus, it is not expected to affect the development of the fetus as was shown for an attenuated YFV17D vector

vaccine (*Kum et al., 2018; Silva et al., 202*0). However, additional preclinical and clinical studies are required to safeguard the implementation of replicon vaccines in vulnerable individuals.

Lastly, the ability of replicon vaccines to recombine with circulating viruses must be considered. This potential safety risk is based on reported genetic interactions between traditional live-attenuated vaccines and viruses in nature (*Becher et al., 2001; Camus-Bouclainville et al., 2011; Chong et al., 2010; Kew et al., 2004; Lee et al., 2012; Moussatché et al., 2008; Wenhui et al., 2012*). Moreover, several recombination events have been described for alphaviruses of which the most intriguing has been the ancient recombination event between two distinct alphaviruses resulting in the pathogenic western equine encephalitis virus (*Hahn et al., 1988*). Although genetic interactions between flaviviruses have been described as well, these are less likely to occur (*McGee et al., 2011; Twiddy & Holmes, 2003*). Nevertheless, even if the incidence of recombination is considered low, the consequences of such an event need to be evaluated prior to the widespread distribution of replicon vaccines.

Revolutionizing replicon technology

A diverse spectrum of replicon vaccines has been developed, yet still new innovative advancements and applications arise. Here, we provide some examples of recent and current developments that can revolutionize the replicon vaccine field.

Multivalent replicon vaccines

Multivalent vaccines are useful tools to control and prevent the spread of co-circulating or seasonal pathogens as a greater number of protective antigens are presented to the immune system. In the case of multivalent replicon vaccines, a single replicon can encode multiple antigens, or multiple replicons each expressing a different antigen are mixed in one vaccine formulation. Both designs have shown protection against a mixture of seasonal influenza strains, and cocirculating Lassa and Ebola viruses (*Magini et al., 2016; Pushko et al., 2001*). Although multivalent replicon vaccines can be rapidly developed and formulated, optimizing the composition of the individual target antigens can be laborious and may require extensive testing. This is particularly important when cross reactivity against different virus isolates of a virus is observed and antibody-dependent enhancement leads to adverse effects in the vaccinated individual. For example, incomplete protection of a tetravalent live-attenuated DENV vaccine against all DENV subtypes resulted in an increased replication of non-neutralized DENV subtypes (*Shukla et al., 2020*). If antigen ratios are less important and a platform technology is in place, multivalent replicon vaccine formulations can be a great solution to effectively combat dynamic virus outbreaks, for example, SARS-CoV-2 with novel variants continuously emerging.

Replicons targeting the mucosa

It becomes increasingly clear that local immune responses in mucosal tissue are important in the protection against a wide range of pathogens (*Morens et al., 2023*). Since the majority of viral pathogens enter the host via the mucosal surfaces such as the respiratory, gastrointestinal,

or urogenital tract, mucosal vaccination deserves more attention. In the ongoing SARS-CoV-2 pandemic, vaccines are delivered via direct intramuscular injection and are mostly focused on systemic cellular and humoral immunity without conferring sufficient mucosal immunity (*Bleier et al., 2021; Singanayagam et al., 2021*). As such, the full potential of reducing viral entry and shedding is not obtained.

In the past, the focus was on mucosal delivery of mostly live-attenuated vaccines as these showed an effective immune response for an array of pathogens including poliovirus (*Dauer, 1955*), influenza A virus (*Belshe et al., 1998*), and rotavirus (*Bernasconi et al., 2016*). However, the associated risk of reversion to virulence with live-attenuated vaccines, and the lack of well-defined mucosal adjuvants, held back the exploration of the mucosal administration route. The latter is mainly what self-adjuvating replicon vaccines may be able to tackle. In a recent study on intranasal vaccination with therapeutic VEEV-TC83 replicon particles, effective delivery of the replicon RNA and a long-lasting expression of the proteins of interest (anti-SARS-CoV-2 neutralizing antibodies) were able to prevent the onset of viral infection (*J. Q. Li et al., 2021*). On top of that, recent advances in liposome formulation and LNP encapsulation for RNA vaccines, and the propensity of these molecules to be taken up by the mucosal tissue, creates new opportunities for replicon RNA application (*M. Li et al., 2017; Phua et al., 2014*). These add to the capabilities of mucosal vaccines to be more easily mass delivered via noninvasive methods, for instance via nasal sprays in humans, or via water and feed for livestock (*Walsh et al., 2020*).

Therapeutic application of replicon vectors

In addition to the versatile application of alphavirus- and flavivirus-based replicon vectors as vaccine platforms, replicon vectors have also been utilized in cancer immunotherapy. These therapeutic replicon vaccines express tumor antigens to activate tumor-specific T cells and antibody responses. The antigen target can be a general tumor factor such as carcinoembryonic antigen, or a specific tumor-associated protein such as the human papillomavirus E6 and E7 antigens (*Morse et al., 2010; van de Wall et al., 2018*). Expression of the antigens by replicon vectors demonstrated promising immune modulation and efficient tumor regression in recent clinical trials (*Crosby et al., 2019, 2020; Komdeur et al., 2021; Morse et al., 2010; van de Wall et al., 2018*). These encouraging clinical results support additional studies to enhance immune responses and develop novel delivery strategies, for example, intratumoral injection of LNP-encapsulated replicon RNA (*Y. Li et al., 2020*).

The future of replicons and replicons of the future

The replicon vector technology is constantly advancing. One recent development is the establishment of a bipartite replicon vector system (*Beissert et al., 2020*). Whereas a monopartite replicon encodes the self-amplifying genes and the transgene on one RNA strand, the bipartite replicon expresses the protein of interest from a trans-amplifying RNA. This carries a safety advantage for proteins of interest that may otherwise package the replicon vector in a monopartite system, for example, glycoprotein of rabies virus (*Zhang et al., 2020*). Furthermore, this modular system allows pre-production of the replicon

vector and fast, on demand production of the variable RNA for the protein of interest. New strategies are explored to improve the efficiency of the replicon vector by reducing the replicon-induced interferon activation, which inhibits translation. Elements such as the vaccinia virus E3L protein (Simplicon expression plasmid of Merck/Sigma–Aldrich) and Middle East respiratory syndrome coronavirus ORF4a protein have been inserted in the VEEV-TC83-based replicon to evade innate immune recognition (*Blakney et al., 2021*). It was demonstrated that these elements can abate the nonlinear dose dependency and enhance immunogenicity. However, putative recombination events involving such a replicon may potentially generate viruses that are more pathogenic than the wild-type virus the replicon was derived from, and thus strict safety testing is required before deliberate release of such replicons in the environment is allowed. We also foresee developments in a few other areas. First, there is a drive for miniaturization of the replicon to reduce the length of the mRNA molecule. The replicons used most widely are based on the alphavirus replicases, which consist of several interacting multifunctional proteins encoded by a relatively long 8 – 9 kb gene cassette. A shorter replicon RNA molecule will ease manufacturing, increase RNA delivery efficiency, and reduce administration dose and possibly production cost. Arguably, not all functions within the alphavirus replicase are required for RNA self-amplification (*Tan et al., 2023*). Through rational design in combination with directed evolution experiments, smaller replicases may be generated that outperform the canonical alphavirus replicase. To take this further, in theory an RNA-dependent RNA polymerase (RdRp) in combination with the essential 5′ and 3′ noncoding regions at the replicon RNA termini is sufficient for self-amplification. Viral genomes with RdRps sized ~2 kb are present in nature (*Wolf et al., 2018*) and these may inspire future replicon design.

Second, *de novo* designed replicons may in the future not be subject to current genetically modified organism (GMO) legislation. With the implementation of artificial intelligence in the design of proteins, we expect it will be possible to engineer small RdRps (based on sequence information of all viral RdRp in public databases) using deep learning algorithms (*Dauparas et al., 2022*). Various *de novo* assembled proteins have been realized, demonstrating advantages in protein size and stability compared to natural proteins (*Korendovych & DeGrado, 2020*). In a similar way, novel synthetic RdRps not derived from, or associated with, any particular virus family may be engineered. If successful, a replicon encoding such RdRp plus a transgene (e.g., coronavirus spike gene) will have a similar legal status as the currently licensed mRNA vaccines. In other words, these sophisticated ‘RNA machines’ will not be subject to GMO legislation and/or registration procedures that may now restrict, or at least delay, market authorization of alphavirus- and flavivirus-based replicons.

Lastly, circular RNAs (circRNAs) are a novel recent direction in the mRNA vaccine field (*Bai et al., 2023; Meganck et al., 2018; Wesselhoeft et al., 2018*). As these circRNAs lack free ends susceptible to exonuclease degradation, they have an increased stability compared to capped, polyadenylated, linear mRNA vectors. Furthermore, circRNA in eukaryotic cell lines led to increased protein expression levels and improved protein production half-life compared to the linear mRNA counterpart (*Wesselhoeft et al., 2018*). Although the delivery of circRNA vectors in mice

was promising, optimization was shown to be essential for specific tissues (*Meganck et al., 2018*). To improve RNA stability, the development of replicon circRNA vaccines can be explored.

Concluding remarks

From all recent advances in the delivery, efficacy, and safety of nucleic acid vaccines, a clear role emerges for self-amplifying mRNA vaccines in the battle against current and future infectious disease outbreaks, and in the rapidly growing field of cancer immunotherapy. The most attractive feature is that replicons encode their own replication machinery to boost their copy numbers directly after administration in target cells. The self-amplifying nature dramatically lowers the required initial mRNA dose and consequently reduces adverse effects in patients. However, the current application of replicon technology barely touches the surface of what the system is capable of (see **Supplementary - Outstanding questions**). Our view is that it is possible to revise or simplify regulatory procedures to license replicon vaccine platforms without extensive safety re-evaluations every time the GOI changes. The paradigm shift in the vaccine field from protein to mRNA will definitely help to mature replicon technology in general and promote the further improvement of these sophisticated RNA machines.

Supplementary - Glossary

Antibody-dependent enhancement: the increased severity of disease as a result of pre-existing, non-neutralizing antibodies against the pathogen. Carcinoembryonic antigen: a protein found in a wide range of cells that is often associated with certain tumors and fetal development when detected at elevated levels.

Cell-free manufacturing: the production of biological compounds (e.g., RNA, DNA, or proteins) without the interference of a living cell.

Circular RNA (circRNA): novel type of mRNA vectors that are synthetically derived by ligation of in vitro transcribed linear RNA fragments.

Helper RNA: in vitro transcribed RNA that encodes and supplements structural proteins to the replicon mRNA, allowing encapsulation of the mRNA in VRPs.

Intramuscular injection: administration of a biological compound directly into specifically selected muscles.

Intravenous injection: administration of a biological compound directly into the vein.

Ministring DNA: a small linear, covalently closed plasmid DNA without any undesirable bacterial sequences. Multivalent vaccine: a vaccine that aims to induce protective immunity against various pathogens or serotypes.

Nanostructured-lipid carrier (NLC): LNP that consists of solid lipids, liquid lipids, surfactants, water, and the active pharmaceutical ingredients.

Plug-and-play principle (replicon): straightforward and effortless exchange of genetic elements of an existing platform technology (often from a fully synthetic origin).

Replication-competent virus (RCV): a propagation-competent virus that originates from a propagation-defective viral vector (replicon) that acquired structural genes for propagation via an RNA recombination event.

Replicon RNA: an mRNA molecule that encodes the genetic elements necessary for the self-amplification and transient protein of interest expression.

RNA-dependent RNA polymerase (RdRp): a versatile enzyme that catalyzes genomic RNA replication and transcription.

Safe-by-design principle (replicon): a genetic design that is self-limiting and cannot escape the host cell.

Supplementary - Box 1. Mode of action of (viral) nucleic acid vaccines

mRNA

The structure of in vitro transcribed mRNA closely resembles cellular mRNA, and in general consists of a 5′-terminal 7-methylguanosine cap analog; a 5′ and 3′ untranslated region (UTR); the gene of interest (GOI); and a polyadenosine (poly-A) tail (**Figure IA**). To optimize the mRNA for vaccination purposes, modifications have been applied to increase the vector stability, translation efficacy, or immunogenicity. Since these nucleic acid vaccines encode solely the GOI, they are unable to replicate in, or spread to, neighboring cells.

Replicon RNA

Replicon RNA resembles in vitro transcribed mRNA, but additionally encodes viral replicase genes. These genes allow the rapid amplification of the mRNA and thereby increase the production of the GOI in comparison to non-amplifying mRNAs. The self-amplifying viral genes originated from viruses, for example, alphaviruses and flaviviruses (**Figure IB**). Alphavirus-based replicons contain a separated open-reading frame (ORF) upstream of the GOI that encodes all the replicase proteins. In contrast, in flavivirus-based replicons, these replicase proteins are encoded in a single ORF downstream of the GOI. Since replicon RNA does not encode all alphavirus or flavivirus structural proteins, the RNA is propagation defective. As a result, replicon RNA vaccines are, similar to the non-amplifying mRNA vaccines, categorized as synthetic nucleic acid vaccines.

Chimeric virus

Similar to replicon RNA vaccines, chimeric virus vaccines are used to express protective antigens of a heterologous viral pathogen. The genomes of chimeric viruses encode their own replicase, but the structural genes are (partly) substituted for genes of a heterologous virus to induce protective immune responses against these structural proteins upon vaccination. Since both viral structural and replicase genes are maintained, viral propagation is observed in contrast to the non-propagative character of replicon vaccines. Therefore, chimeric virus vaccines are categorized as live-virus vaccines (**Figure IC**). Recent chimeric alphavirus vaccine research focused on the development of a safe chimeric alphavirus vaccine platform based on the insect-restricted Eilat virus. The Eilat virus structural genes were replaced with the genes of highly pathogenic alphaviruses, such as VEEV, eastern equine encephalitis virus, and chikungunya virus (*Erasmus et al., 2017*). For flaviviruses, the production of a chimeric vaccine was successfully demonstrated for the well-studied and attenuated YFV 17D strain. This strain was modified to express the structural genes of the heterologous West Nile virus, DENV, and Japanese encephalitis virus (JEV). The YFV 17D vaccine vector for DENV or JEV structural genes has been approved for human application (*Arroyo et al., 2004; Biedenbender et al., 2011; Guirakhoo et al., 2006; Monath et al., 2003*).

Recombinant virus

Similar to chimeric virus vaccines, recombinant virus vaccines are based on modified viral genomes to express an exogenous GOI (**Figure ID**). However, in contrast to chimeric virus vaccines, the recombinant viruses encode all the genes for viral replication and encapsidation/transmission. Despite the many applications of the alphavirus-based replicon platform, the cytopathic nature of the complete alphavirus genome makes them less favorable as recombinant virus vaccines. In contrast, recombinant flavivirus vaccines have been considered feasible in their use as an immunotherapy or for the protection against bacterial or viral infectious diseases (*McAllister et al., 2000; Nogueira et al., 2013; Sanchez-Felipe et al., 2021*). Notwithstanding, other viruses such as vesicular stomatitis virus, adenovirus or measles virus have been described more frequently in their use as recombinant viral vectors and pose a suitable alternative (*Cross et al., 2022; Fischer et al., 2021; Mateo et al., 2021*). Similarly to chimeric viruses, recombinant-vectored viruses are categorized as live-virus vaccines.

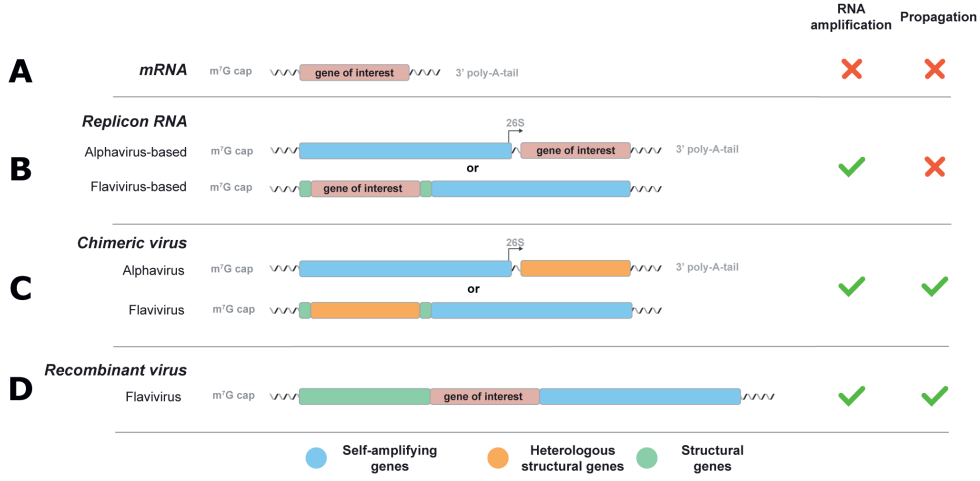

Figure I. Schematic overview of (viral) nucleic acid vectors. (A) An mRNA molecule contains the coding sequence of the gene of interest (GOI) flanked by a 5 m7G cap analog and a 3' poly-A-tail. Similar to the mRNA vector, **(B)** virus-based replicons encode the GOI but also self-amplification genes, allowing RNA amplification. Both mRNA and replicon vectors are propagation deficient as they do not encode a complete structural gene cassette. This is in contrast to **(C)** chimeric and **(D)** recombinant virus vectors that encode a complete (heterologous) structural gene cassette. These genes facilitate viral propagation, not limiting transduction of the viral vector to a single cell.

VI

Chapter 7

General Discussion

The importance of RNA vaccines in combatting emerging diseases

As the global population continues to surge and welfare increases, placing ever-growing demands on the worldwide food supply chain, intensive livestock husbandry emerges as a pivotal source of animal protein. The numerous animals involved in this supply chain concurrently increase the contact between livestock and other individuals providing ideal environments for novel viruses to emerge. The past COVID-19 pandemic showed that intensive contact between humans and livestock can result in a great global threat from emerging pathogens such as SARS-CoV-2. Although the initial spill-over event that led to the virus pandemic remains unsure, the animal origin of SARS-CoV-2 is very probable (*W. J. Liu et al., 2023; Lytras et al., 2021*). Other viruses with a similar pandemic potential can be found in the poultry industry and often relate to gastrointestinal or respiratory viruses such as the avian influenza virus (AIV) and infectious bronchitis virus (IBV), respectively. Although not all viruses are a direct threat to human health (e.g. zoonosis), the impact on poultry health is the main reason for concern (*Trapp & Rautenschlein, 2022*).

With the annual mortality of 50 million chickens in Europe as a result of highly pathogenic avian influenza (HPAI) alone, the need for an effective vaccination platform becomes clear (*Adlhoch et al., 2023*). Most of the diseases in poultry are caused by naturally occurring pathogens circulating among wild birds that travel long distances. As such, they contribute to the constant evolution of viruses among uninfected domesticated birds (*de Wit, Sjaak, 2011; Graziosi et al., 2022; H. Tian et al., 2015*).

Human vaccines, similar to veterinary vaccines, are constantly being developed to prevent the disease-causing effects of viruses and other pathogens. Since past pandemics often greatly impacted society, there are numerous incentives for the rapid development of novel vaccine types (*Kudlay & Svistunov, 2022*). As seen for the COVID-19 pandemic conventional vaccines such as inactivated or live-attenuated virus vaccines, were a prudent first choice due to their well-established production pipeline (*Lazarus et al., 2022; Nouailles et al., 2023; Tanriover et al., 2021; Y. Wang et al., 2021; Z. Wu et al., 2022*). However, the concerns regarding the limited vaccine efficacy and safety coupled with the rapid emergence of new variants of concern demonstrate the need for a modular platform to take center stage in COVID-19 disease control (*Grubaugh et al., 2019; Lim et al., 2021*). As discussed in this thesis, RNA vaccines are a novel vaccine type, that is easily adapted, well-defined, and scalable. Moreover, these mRNA vaccines showed great effectiveness in the protection against coronavirus disease. As a result of this efficacy, novel RNA vaccines also gained the interest of the veterinary industry. Despite past considerations of these RNA vaccines to combat important veterinary diseases (*Borrego et al., 2013; Crawford et al., 2016; Gerdts & Zakhartchouk, 2017; Pulido et al., 2010*) only conditional licenses have been approved. The swift progress made in the RNA vaccine field as a result of COVID-19 offered promising prospects for the licensure of future vaccines (*Le et al., 2022; J. Li et al., 2022*). The certain bottlenecks that hold back the broad application of

these RNA vaccines for veterinary application are the requirement for a high dose, multiple booster administration, and the high cost. This is where self-amplifying RNA vaccines represent a promising alternative (*Bloom et al., 2021; de Alwis et al., 2021; Pollock et al., 2022*).

In this concluding chapter, I discuss why we need a more elaborate understanding of the adaptive immune system for the development of an effective vaccination strategy. Moreover, I emphasize the value of how and where vaccines are being administered, and to conclude, I provide an outlook for future RNA vaccines in the veterinary sector, exploring the potential of AI in the design of RNA vaccines and discussing its impact on the approval of novel vaccines.

Immunity beyond antibodies

In the pursuit of developing an effective universal vaccine technology, it is crucial to recognize that the role of immunity extends beyond the scope of humoral responses alone, particularly the antibody response. While antibody responses have traditionally been considered the primary indicator for protective immunity due to well-established, cost-effective, and easily interpretable serological assays, the role of the cellular immune system is often underestimated (*Moss, 2022*). Understanding the intricate interplay between these two branches of the adaptive immune system and how they relate to protection against a specific pathogen is crucial when designing a universal vaccine platform, challenging the prevailing belief that a neutralizing antibody response alone is the primary inducer of immunity (*Khoury et al., 2021; Shrotri et al., 2021; Zajac & Harrington, 2014*).

The importance of cellular immunity became increasingly evident from the combined global research efforts amid the SARS-CoV-2 pandemic where the complex interaction of both the innate and adaptive immune system was carefully studied (*Khoury et al., 2021; Moss, 2022*). Several studies indicated that a positive clinical outcome was observed despite the absence of detectable levels of neutralizing antibodies either after vaccination or natural infection, which can be attributed to a robust cellular response (*Iannetta et al., 2021; Kornek et al., 2022; McMahan et al., 2021; Wyllie et al., 2021*). Moreover, studies with recombinant viral vector vaccines in chickens demonstrated good protection even in the absence of detectable levels of antibodies during the first initial weeks post-vaccination (*Ingrao et al., 2018*), further indicating the important role of the cellular immune system. Despite our research efforts in developing a new replicon platform with the aim to provide protective immunity in chickens, we only assessed the seroconversion in young chickens vaccinated with LNP-formulated replicon RNA (**Chapter 4**), while the core strength of the replicon RNA platform technology may predominantly lie in the cellular response (*Blakney, Ip, et al., 2021; McCafferty et al., 2022*). As such, before delving into the role of replicon RNA in the induction of cellular immunity, it is essential to first understand the role of innate immunity and how they can affect the outcome of the adaptive immune response.

Self-amplifying RNA and innate immunity: less is more?

When administering replicon vaccines to the host, the RNA will either be taken up by scavenging immune cells or end up in the cytosol of local epithelial and muscle cells. In these cells, the first response is the innate immune response activated by encountering pattern recognition receptors (PRRs) that recognize this non-self RNA as pathogen-associated molecular patterns (PAMPs). The primary aim of innate immunity is to sense invading pathogens and activate immune cells that bridge the innate and adaptive immune systems (*Alberts et al., 2017*). Replicon RNAs are adept at activating multiple innate signaling pathways due to the presence of dsRNA replication intermediates that are formed during the self-amplification of the replicon RNA. The dsRNA may be recognized by toll-like receptors (TLR) such as TLR-3, Retinoic acid-inducible gene I (RIG-I)-like receptors (RLR), Melanoma differentiation-associated gene-5 (MDA-5), and oligoadenylate synthetase receptor family (OAS), which senses specific RNAs as a pathogen-associated molecular pattern (PAMP) (*Ho et al., 2022; Nasirudeen et al., 2011; Nikonov et al., 2013*). Chickens have lost the single-stranded (ss) RNA sensor RIG-I during evolution, possibly explaining the increased sensitivity of chickens to AIV and other ssRNA viruses compared to different avian species that retained the RIG-I receptors (*Barber et al., 2010, 2010; S. Chen et al., 2013; Karpala et al., 2011; Zou et al., 2009*). Whether or not the lack of chicken RIG-I also impacts the response to other viruses such as alpha- or flaviviruses has not been investigated. Interestingly, a study investigating the interferon response upon alphavirus infection demonstrated that human cells expressing only RIG-I induced more interferon as a result of VEEV replication than cells only expressing MDA-5 (*Akhrymuk et al., 2016*). Whether this effect is similar in chickens and can explain the lack of effective immune stimulation upon vaccination with VEEV replicon RNA remains unknown. Similar to alphaviruses, TMUV is primarily detected by MDA-5 (*Z. Wu et al., 2022*), making it less likely that the lack of RIG-I is the primary reason why the VEEV replicon system only partially protects chickens (*Schultz-Cherry et al., 2000*). The slower onset of heterologous protein expression (**Chapter 3**) and most likely delayed production of dsRNA intermediates, however, might lower the innate immune reaction elicited in chickens in response to TMUV replicon vaccination (*Näslund et al., 2011*).

The sensing of PAMPs triggers intracellular signaling cascades leading to the expression and release of type I interferon (IFN), a group of cytokines crucial for an immediate response to cope with the infection or in this case, the effects of the replicon RNA. Additionally, IFN signals neighboring cells to induce an antiviral state, blocking foreign mRNA translation and activating the transcription of numerous antiviral effector proteins (e.g. interferon-stimulated genes), and may ultimately lead to the death of the infected cell (*Dalskov et al., 2023; Linares-Fernández et al., 2020*). Another mode of action of IFN is to enhance viral antigen presentation by the infected cell and activate cells, such as dendritic cells (DCs), that transmit these danger signals to the effector cells of the adaptive immune system (*Le Bon & Tough, 2002; Y. Liu et al., 2022; Näslund et al., 2011; Zhu & Fernandez-Sesma, 2020*). Not only the direct effects of IFN but also replicon amplification and expression might shape the innate immune response, as was demonstrated by the various transgene expression levels and degree of replicon-induced cytopathicity of cells (CPE) defective in the production

of type I interferons such as BHK-21 cells used in this study. Whether this is the direct consequence of the amount of dsRNA replication intermediates or the presence of viral non-strucutral proteins, or the indirect effect from the high abundance of heterologous protein, or a combination of both, remains to be tested (*Aliahmad et al., 2023; Beissert et al., 2017; Pan et al., 2021*). In our study, depending on the type of replicon platform (VEEV vs TMUV) and the length of the TMUV capsid coding sequence ($C_{20,}$ C_{38}, or C_{109}), various levels of replicon-induced CPE were observed (**Chapters 2 and 3**). It did become apparent that when the expression level of the TMUV replicon was enhanced by incorporating the DCS-PK (present in C_{38} and C_{109} but lacking in C_{20}), it was accompanied by an increase in CPE. Whether the latter is the result of enhanced expression levels or an effect of the number of replicon copies within these cells, as was observed when the DCS-PK structures in flavivirus were affected (*He et al., 2021; Z.-Y. Liu et al., 2013*), remains to be tested.

Another important factor prompting the IFN response might be the administration of high doses of conventional mRNA vaccines. These high doses might lead to excessive IFN responses that block the translation of the RNA vaccines and ultimately reduce the efficacy of vaccine-induced protective immunity (*Alameh & Weissman, 2022; S. Davis & Watson, 1996; Linares-Fernández et al., 2020*). This dual-edged sword makes the design of effective mRNA vaccines without side effects very challenging. To mitigate the excessive IFN response, the potency of conventional RNA vaccines can be enhanced through the incorporation of modified bases (i.e. pseudouridine), avoidance of highly stable secondary RNA structures, and the use of high production temperatures to prevent dsRNA intermediates in the vaccine formulation that are recognized by cellular PRRs (*Anderson et al., 2010; Leppek et al., 2022; Morais et al., 2021; Nance & Meier, 2021*).

While not all of these modifications can be incorporated into the replicon design, replicon RNA can be administered at a low dose, holding promise for reducing the risk of an immediate IFN-induced translational shutdown. Alternatively, the replicon RNA could be administered as a DREP, which alters the innate response by avoiding the use of non-self RNA, but trigger different TLR and cyclic GMP-AMP synthase receptors recognizing (bacterial) plasmid DNA (*Fu et al., 2020; Häcker et al., 2002*). Previous studies demonstrated that mice vaccinated with DREPs exhibited a lower IFN response compared to those vaccinated with replicon RNA (*Ljungberg et al., 2007; Näslund et al., 2011*). This finding could elucidate why seroconversion was not observed in chickens receiving VEEVrep-pVP2 replicon RNA but only in chickens receiving VEEVrep-pVP2 DREP. The VEEV DREP might be inherently less immunogenic, as it possibly induces a milder immediate interferon response compared to the high amounts of VEEV replicon RNA delivered in the cytosol (*Ljungberg et al., 2007*). Although no direct comparison between VEEV DREP and VEEV replicon RNA was presented in this thesis, less cytopathic effect were observed for the VEEV DREP (**Chapter 4**) compared to the VEEV RNA (**Chapter 3**) in vitro which can be indicative that the kind of vaccine modality also plays a large role in determining the immune response, which was similar as demonstrated in other research comparing these different replicon modalities (*Berglund et al., 1998; Ljungberg et al., 2007*). Altogether, this might indicate that the gradual transcription of VEEV replicon RNA from a

DNA vector may temper the initial innate responses resulting in lower amounts of apoptosis, higher heterologous gene expression, and ultimately enhance seroconversion in a later phase.

Additionally, and in contrast to conventional mRNA vaccines, replicon RNAs encode their own viral-derived nonstructural proteins, highly specialized in manipulating the host's immune response, with the primary goal of facilitating self-amplification and translation of the encoded genes. In flavivirus replicons, many reports describe the antagonistic function of these nonstructural proteins in evading the host's innate immunity in which for example NS1 of WNV can inhibit TLR-3 signaling, a PRR recognizing dsRNA (*J. R. Wilson et al., 2008*), NS2 of KUNV can inhibit phosphorylation of STAT-1 and 2: transcription activators for downstream interferon stimulating genes (*W. J. Liu et al., 2005*), Zika NS3 can antagonize the RIG-I and MDA-5 mediated innate immunity (*Riedl et al., 2019*), and NS5 of TBEV can directly bind to the interferon receptor preventing the JAK-STAT-signaling (*Best, 2017; Best et al., 2005*). Wildtype VEEV also harbors antagonistic viral proteins that can interfere with the host's innate immune responses. However, many of these functions are performed by the capsid protein, which is not encoded by the VEEV replicon RNA (*Atasheva et al., 2008; Lundberg et al., 2017*). Whether the absence of the capsid protein causes an increased susceptibility of the VEEV replicon to the chicken's antiviral response remains unclear. The reason behind selectively affecting the VEEV replicon RNA but not the VEEV DREP might be attributed to the absence of an interferon-induced translational shutoff. This could be due to the comparatively lower quantities of DNA delivered to the cell, in contrast to the higher amounts of foreign replicon RNA, or the potentially slower initiation of VEEV replicon transcription from the DNA vector.

Self-amplifying RNA in cellular Immunity

Once the optimal dosage of replicon vaccines is established, they will trigger a robust innate immune response and deliver the essential costimulatory signals to evoke a response tailored to the specific type of pathogen. For instance in the case of obligate intracellular pathogens such as viruses, the adaptive immune system shifts towards a predominantly T-helper (Th) 1 response, while in the case of an obligate extracellular pathogen (e.g. helminths), a Th2-phenotype is favored. Inactivated viral and subunit vaccines lack the ability to enter or replicate inside the cell, and, without proper adjuvants, typically yield a Th2-biased response (*Hui & Hashimoto, 2008; Luan et al., 2022*), whereas RNA vaccines, which act within cells, are known to trigger a more robust Th1-biased response (*Aberle et al., 2005; Ganneru et al., 2021; McKay et al., 2020; Voigt et al., 2022*). In the case of Th2 responses, the immune system focuses mostly on the production of high serum levels of neutralizing antibodies, and Th1-mediated immunity is inhibited (*Chaplin, 2010; Spellberg & Edwards, 2001*).

In the following section, I will briefly outline a conceptual framework that elucidates the adaptive immune response orchestrated by LNP-formulated replicon RNA vaccination against AIV (**Figure 1**). Firstly, upon internalization of the LNPs by the cells at the site of injection (e.g epithelial cells, muscle cells, but also immune cells), the LNP undergoes degradation by the endosome, releasing the replicon RNA into the cytosol (*Paunovska et al., 2022*). Next, the

replicon RNA will be amplified and subsequently recognized as non-self dsRNA by cytosolic PRRs, triggering a signaling cascade that results in the predominant production of type-I IFN (*N. Li et al., 2020*). While a portion of the replicon-positive, antigen-producing cells undergo apoptosis, releasing numerous PAMPS, these signals are detected by antigen-presenting cells (APCs), including dendritic cells (DCs), which engage in the process of scavenging antigen-producing cells. Once APCs are stimulated, they interact with T-lymphocytes through the presentation of co-stimulatory signals and secretion of proinflammatory cytokines along with displaying (captured) antigens produced by the replicon. These antigens will be displayed in two distinct ways: DCs will process the engulfed antigens directly and process them into peptides that are presented on MHC class II molecules to activate naive CD4+ helper T-lymphocytes, or alternatively, cross-present the antigens on MHC class-I molecules to CD8+ cytotoxic T-lymphocytes (CTLs). Especially these activated antigen-specific CD4+T lymphocytes play a pivotal role in mediating the antibody responses by activating B-cell, promoting

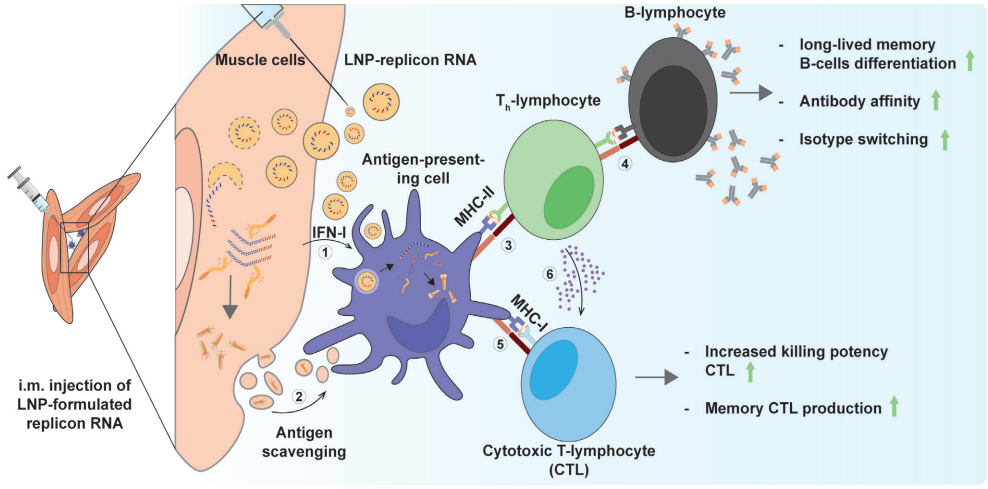

Figure 1. Mechanistic model on how replicon RNA induces both humoral and cellular immune responses. Upon intramuscular injection (i.m.) of LNP-formulated replicon RNA, the LNPs will be internalized in a wide variety of cells such as epithelial, muscle, and immune cells (e.g. dendritic cells). Within the cell's cytosol, the replicon RNA cargo is released, undergoing translation and replication. The latter results in the formation of dsRNA replication intermediates, recognized as pathogen-associated molecular pattern (PAMP) by cellular pattern recognition receptors (PRR) leading to the production of type-I interferon (IFN-I) and thus pro-inflammatory microenvironment **(1)**. Simultaneously, the replicon-encoded candidate antigen is translated and scavenged by antigen presenting cells (APC) **(2)**. The scavenged antigen will be processed and presented on MHC-class II complex **(3)**. Upon recognition of the MHC/antigen complex by the Th-lymphocyte and additional co-stimulatory signals from the APC, the T-cell becomes activated and can performs its effector function. Activated Th-lymphocyte will recognize naive B-lymphocytes that present a similar combination of MHC/antigen complex **(4)**, subsequently activating the B-lymphocyte to differentiate into long-lived memory cells, stimulate antibody affinity maturation, and isotype switching. These all contribute to a robust humoral response. APCs also (cross)present the scavenged antigen on MHC-I molecules along with co-stimulatory signals to CD8+ T-lymphocytes **(5)**. These activated CD8+ T-lymphocytes develop into cytotoxic T-lymphocytes (CTLs). Th-lymphocytes **(6)** will increase the CTL's killing potency and stimulate the differentiation into memory CTLs.

isotype switching, enhancing antibody affinity maturation through somatic hypermutation, and instigating the production of memory B-cells. An additional function of these CD4+ helper T lymphocytes is to further enhance the killing potency of the activated CTLs, thus contributing to enhancing cellular immunity (*Elliott et al., 2022; Englezou et al., 2018; Heine et al., 2021; Hershkovitz et al., 2008; Natarajan et al., 1999; van Montfoort et al., 2014*).

Although most viruses initially enter the host as an extracellular pathogen and require a robust humoral response to neutralize the initial virus particles, the obligate intracellular characteristics of these pathogens require CTLs to directly kill infected cells. The coordinated interplay between these cellular and humoral responses prompted by these replicon RNAs enhances the potency and breadth of mounted adaptive immune responses.

An important difference between the humoral and cellular responses seen during a wildtype virus infection compared to an RNA vaccine-induced response is the wide array of available epitopes derived from virus-encoded proteins. While most vaccine designs tend to emphasize encoding the major external viral antigens to neutralize the target pathogen, they may inadvertently overlook the potential of targeting intracellular antigens or viral nonstructural proteins, which are often conserved and elicit cross-protective cellular responses (*Geers et al., 2021; McCaffrey, 2022; Tarke et al., 2021*). This has been well-documented for AIV (**Figure 2**), where CD8+ T-cells exhibit specific responses targeting highly conserved proteins shared among different subtypes such as the RNA polymerase (PB1), nucleocapsid protein (NP) or other matrix proteins (MP) (*Noisumdaeng et al., 2021; Seo & Webster, 2001; Singh et al., 201*0).

In the pursuit of designing future replicon RNA vaccines, it may be worthwhile to move beyond solely expressing crucial surface (glyco)proteins such as HA, which primarily aims to confer protection via neutralizing antibodies (**Chapter 4**). Instead, a multivalent approach including multiple (conserved) antigens such as HA and NP could be considered (**Figure 2**). The comprehensive design could be complemented by mapping specific T-cell antigen epitopes that hold the potential to confer protection. Additionally, the development of robust assays to assess the host's cellular immune status becomes imperative. In this way, the immune system might provide cross-protection against heterosubtypic influenza as a result of annual drift variants, where viruses might escape recognition by virus-specific antibodies (*Duvvuri et al., 2014; Gotch et al., 1987; Kees & Krammer, 1984; Noisumdaeng et al., 2021; Rimmelzwaan et al., 2007; Stanekov & Varekov, 2010; Townsend & Skehel, 1984; Yewdell et al., 1985*).

Ultimately, this synergistic effect of both robust humoral and cellular immunity, not only aids in preventing new infections and clearing established infections but also advances the development of a more universally applicable vaccine platform (*Speiser & Bachmann, 2020*).

Mucosal route of vaccine administration

It has become evident that immune responses elicited by a vaccine are not solely determined by its type but also by the site of administration. Traditionally, vaccines were mostly administered

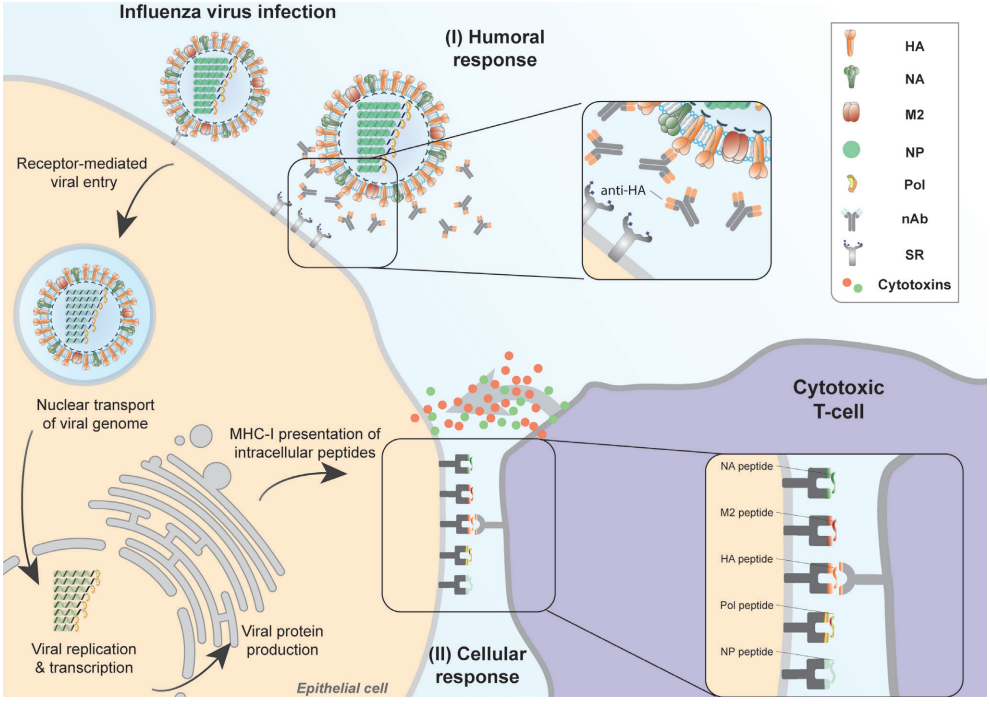

Figure 2. Schematic overview of humoral and cellular immune responses against (A) influenza virus and (B) multivalent replicon RNA. HA = hemagglutinin, NA = neuraminidase, M2 = Matrix protein 2, NP = nucleocapsid protein, Pol = RNA-dependent RNA polymerase complex, nAB = neutralizing antibody, Sia = sialic acid-linked receptor.

via parenteral routes, such as epidermal, intramuscular, or subcutaneous injections, with the primary focus on the seroconversion and the detection of IgG (IgY in chickens) in the blood (*Zuckerman, 2000*). Despite the myriad of immune cells present in the skin, recent research focused on the first point of pathogen contact: the mucosal surfaces (*Nochi et al., 2018; Russell et al., 2020*). The mucosal surfaces are characterized by dedicated mucosal immune properties such as specific humoral (IgA-specific) or cellular (resident CD4+ and CD8+ specific T cells) components, which are recognized as crucial in preventing viral pathogen entry.

Despite the limited number of mucosal vaccines licensed for human use (*Donlan & Petri, 2020; Hird & Grassly, 2012; Jelinek & Kollaritsch, 2008; S.-H. Kim & Jang, 2014; Surman et al., 2014; Ward & Bernstein, 2009*), the veterinary sector has more commonly adapted mucosal vaccination, especially in hatcheries, due to its more readily employed mass-delivery systems (*H. L. Wilson et al., 2020b*). Traditionally, poultry vaccines were primary administered via parenteral routes, resulting in a robust systemic immunity but facing challenges in eliciting local, mucosal responses. Most mucosal vaccination includes inactivated whole-cell preparation with adjuvants such as enterotoxins, TLR ligands, or interleukins with specific mucoadhesive properties to confer a robust response (*Nochi et al., 2018; T. Wang et al., 2020*). However, these adjuvants often come with undesired adverse effects and still require a high antigenic dose or multiple

booster vaccination to achieve protective immunity (*Pavot et al., 2012; Spickler & Roth, 2003*). This is, in contrast to live (recombinant) vaccines which retain the ability to replicate within the host and thus the intrinsic self-adjuvating properties, overcoming the challenges of the mucosal immune barrier. Self-amplifying RNA vaccines, similar to live (recombinant) vaccines, also demonstrate this self-adjuvating effect, and thus provide a suitable alternative for this difficult immune site. During the COVID-19 pandemic, mucosal administration of replicon vaccines demonstrated protective immunity against a SARS-CoV-2 in cats (*Langereis et al., 2021*), while conventional RNA vaccines faced limitations in adjuvating effect, immunotolerance at certain mucosal sites, and degradative properties of RNase (*Hameed et al., 2022*).

In chickens, the mucosal immune system closely resembles that of mammals, including the bronchus-associated lymphoid tissue (BALT), skin-associated lymphoid tissue (SALT), and gut-associated lymphoid tissue (GALT). Chickens also possess specific immune sites such as the nasal-associated lymphoid tissue (NALT) and the head-associated lymphoid tissue (HALT) consisting of the conjunctiva-associated lymphoid tissue (CALT) and Harderian gland (**Figure 3**) (*Nochi et al., 2018*).

Among these, the CALT is the first lymphoid tissue to come into contact with pathogens. Although these lymphoid tissues mature differently during embryonic development, lymphocytes, and germinal centers can be observed in the CALT in 2-week-old chickens (*Fix & Arp, 1991; Kang et al., 2013; Maslak & Reynolds, 1995*). Although these lymphoid tissues mature differently during embryonic development, lymphocytes, and germinal centers can be observed in the CALT in 2-week-old chickens. Notably, the CALT exhibits a close association with M-cells, cells specialized in the uptake of antigens and microorganisms, and efficient in the transport of lipid nanoparticles to the underlying immune cells (*Illum, 2007; Peek et al., 2008; van Ginkel et al., 2012*). In our study, we demonstrated that after a parenteral prime-boost vaccination of lipid nanoparticle-formulated TMUV replicon RNA, chickens induced a humoral response against the VP2 capsid protein (**Chapter 4**). However, it remains unknown whether the antibody response against VP2 is systemic and whether a local IgA response would also be elicited. In the absence of a mucosal IgA response, trans-nasal or spray administration of the LNP-formulated replicon vaccine to chickens would be interesting to explore, testing for the presence of both systemic and mucosal adaptive immune responses, thus providing a less invasive and mass administration route for poultry vaccination using replicon RNA. Previous research has demonstrated that ocularly administered adenovirus vectors or attenuated IBV vaccines in chickens increased the localization of T- and B-lymphocytes in CALT and provided robust mucosal IgA and systemic IgG responses after prime-boost vaccination (*Orr-Burks et al., 2014; van Ginkel et al., 2012*). Another of advantage of focusing on IgA antibodies is the limited interference during early life immunization due to the presence of very low amount of maternally derived IgA antibodies (MDA) in the plasma of young chicks (*Hamal et al., 2006*).

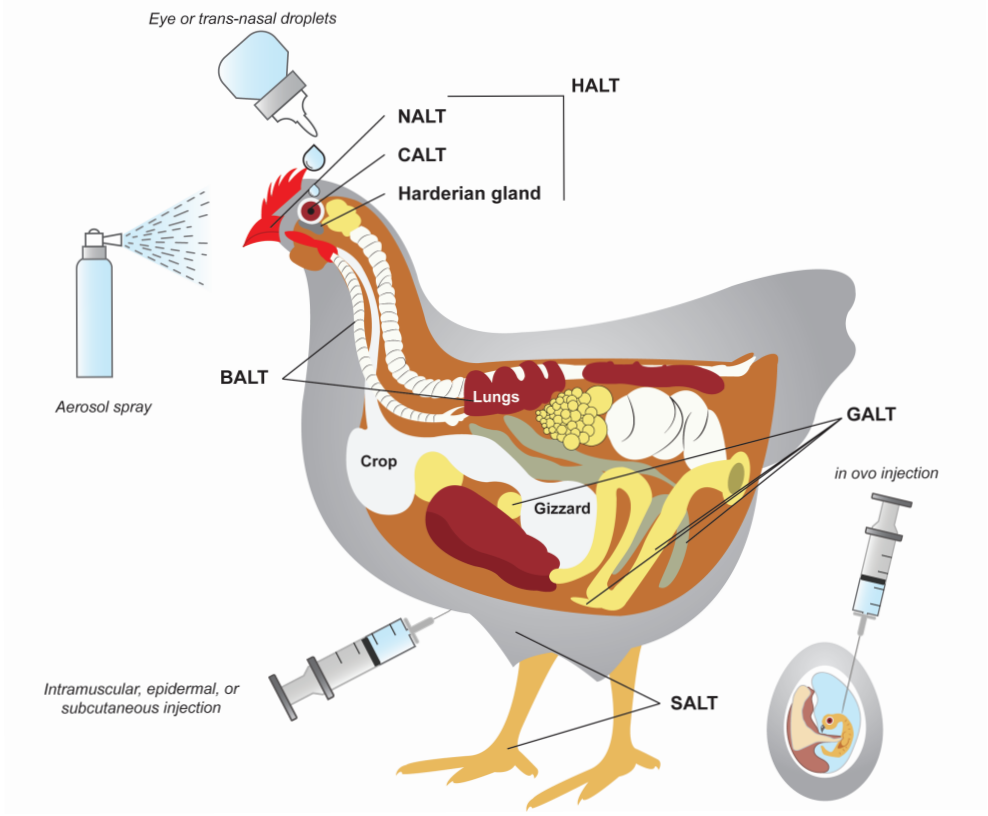

Figure 3. Schematic overview of the common immunization routes in chickens and the associated lymphoid tissues. Nasal-associated lymphoid tissue (NALT), conjunctiva-associated lymphoid tissue (CALT), head-associated lymphoid tissue (HALT), bronchus-associated lymphoid tissue (BALT), gut-associated lymphoid tissue (GALT), and skin-associated lymphoid tissue (SALT).

The (future) role of artificial intelligence in vaccine platform technology

The impact of artificial intelligence (AI) on various facets of modern life is becoming increasingly apparent. Currently, AI has found diverse applications including the implementation as a linguistics model and for visual content creation but its primary characterization lies in the development of computer systems that process extensive datasets to create algorithms and models that mimic human cognitive behavior often utilizing neural networks or deep learning techniques. A less recognized implementation of AI is its involvement in bioinformatics where it plays a crucial role in analyses of complex data sets related to genomics, proteomics, and immunology (*McCaffrey, 2022*).

In the context of mRNA vaccine development, AI emerges as a valuable tool in designing and optimizing vaccines. For the current SARS-CoV-2 mRNA vaccine, deep-learning trained

models were used to optimize the mRNA molecule half-life and stabilize the encoded Spike protein to enhance expression *(H. Zhang et al., 2023*). Despite the apparent simplicity of the 1273-codon-containing mRNA vaccine, it can be encoded by more than a septuagintillion (10^{693}) different mRNA sequences, albeit not all efficiently translated. The same applies to whole virus vaccines, where it was demonstrated that by altering the dinucleotide bias using bioinformatic analysis of conserved sequences of related flaviviruses, the viral growth kinetics can be attenuated rendering a safe alternative approach for creating live-attenuated vaccines (*Fros et al., 2021; Kunec & Osterrieder, 2016*).

Another interesting application of AI dives into the more immunological aspect of vaccine design. Deep learning algorithms have been trained with immunological phenotyping of patients' immune cells, and cytokine responses but also data from structural biology methods such as X-ray crystallography, cryo-electron microscopy, or nuclear magnetic resonance, that visualized antigen-antibody and antigen-MHC complexes (*D. A. S. Davis et al., 2022; McCaffrey, 2022; Mueller et al., 2022; Sahoo et al., 2021*). Especially the latter could be of high value in future replicon vaccine design. Careful consideration in the selection of (parts of) the target transgene can result in the precise induction of the adaptive immune system. Despite specific epitope requirements for either humoral or cellular immune responses, most conventional vaccine designs solely copy-paste the major immunogenic antigen in the hope of inducing an effective immune response (*Purcell et al., 2007*). Leveraging the current computational power can be used to train deep learning algorithms. This approach facilitates the design and manipulation to enhance their selectivity for antigen processing in specific MHC-complexes, leading to a more targeted and precise induction of the adaptive immune system (*Ahmad et al., 2016; Kapingidza et al., 2020; Tahir et al., 2021*). A bioinformatic approach for designing specific B and T cell epitopes has already been demonstrated in a subunit vaccine, resulting in a multi-peptide SARS-CoV-2 vaccine that elicited both humoral and cellular immune responses (*Y. Feng et al., 2021*) Although most of these bioinformatically optimized vaccines are administered as peptide-based vaccines, a similar approach can be applied to replicon platforms by encoding multiple of these optimized peptide sequences under separate promoters (*Pushko et al., 2001; Reap, Dryga, et al., 2007; M. Wang et al., 2018*).

Feasibility-by-design

Vaccines have long been at the forefront of safeguarding human health against a multitude of diseases, particularly those with zoonotic origins (*Dehove, 2010; Taylor et al., 2001*). Remarkably, many vaccines developed for human use have demonstrated the potential not only to protect humans but also other non-human species, most notably evident with the SARS-CoV-2 mRNA vaccines (*Kalnin et al., 2021; Langereis et al., 2021; McMahan et al., 2021; Nouailles et al., 2023*). Despite the existence of robust RNA vaccine pipelines available nowadays, the utilization of these vaccines beyond their intended target groups remains largely untapped, primarily due to the high costs, cold storage requirements, and time-consuming registration process. Therefore, the feasibility of RNA vaccines for their use in veterinary vaccination is discussed in the following section looking beyond costs as the principal limiting factor.

While in this thesis I frequently referred to self-amplifying RNA as a platform technology, it is noteworthy, at present, only the United States has conditionally licensed the use of (self-amplifying) RNA vaccines based on VEEV as a platform technology for treating swine Influenza, porcine epidemic diarrhea virus, rabies, and other animal pathogens. The authorization is granted as long as these vaccines share the same fundamental safety characteristics and thus comply with the regulation concerning platform technology (*Canadian food inspection agency, 2018; U.S. department of agriculture, 2023*). However, in Europe, conventional RNA and self-amplifying RNA vaccines do not yet benefit from the platform status within the European Union. A new legislative proposal is currently under review to authorize the use of vaccine platform technologies as an immunological veterinary medicinal product, which includes a wide variety of vaccine types such as recombinant live (viral) vectors, virus-like particles, and nucleic acids vaccines (*European medicines agency, 2021; Francis, 2022*). The impending authorization of platform technologies by the European Medicin Agency (EMA) will accelerate forthcoming market approvals including the derivates of the already approved RNA vaccines, as long as the same backbone carrier is used.

RNA vaccines, in particular, will benefit from a platform technology status, as the production solely relies on enzymatic transcription reaction, not completely and directly reliant on biological systems (e.g., eggs, animals or mammalian cell culture). Furthermore, the individual reaction constituents are fully defined with no involvement of live pathogens, simplifying the biosafety evaluations (*Rosa et al., 2021; World health organization, 2020; C. Zhang et al., 2019*). Moreover, the recent strides in addressing the logistic challenges, such as cold-storage requirements, and physical stability of RNA vaccines, have greatly improved. While the early developmental phases of LNP-formulated RNA vaccines only demonstrated a shelf-life of up to 30 days when refrigerated or hours at room temperature (*Crommelin et al., 2021; Tinari, 2021*), second-generation formulations have focused more on enhancing physical and chemical stability. These formulations demonstrated the potential of extending the shelf-lives to 24 weeks under refrigeration or 12 weeks at room temperature without compromising the vaccine efficacy, achieved through innovative techniques like lyophilization (e.g freeze-drying) (*R. L. Ball et al., 2017; Hong et al., 2021; Muramatsu et al., 2022*). Additionally, alternative non-viral carriers such as nanostructured lipid carriers (NLC), have demonstrated even greater physical and chemical stability than LNPs with shelf-lives up to 8 months at room temperature. This durability permits RNA vaccine stockpiling and the novel carrier for the point-of-care mixing of nucleic vaccines (*Gerhardt et al., 2022*). Other non-viral carriers and their ease in administration concerning RNA vaccine feasibility have been evaluated in more detail in **Chapter 6**. Arguably, the most pivotal determinant for vaccine feasibility is cost. However, the perception of cost is notably subjective. From a manufactures standpoint, novel RNA vaccines are relatively costly due to their high risk of failure during the initial research phases when pipelines are being developed. The development of COVID-19 RNA vaccines developed for human use necessitated substantial financial investment in discovery, preclinical and clinical evaluation. Notably, the most substantial contributor to the developmental cost of human RNA vaccines in humans is the stringent regulatory requirements and safety assessments

involved in these clinical phases, accounting for at least 20-60% of the total development cost (*Adams & Brantner, 2006; Moore et al., 2020; Wong et al., 2014*) and almost 50% of the development time (*Francis, 2020; Martin et al., 2017; Warimwe et al., 2021*) (**Figure 4**). In contrast, the veterinary vaccine development process centers around field trials that result in less stringent regulatory and preclinical trial requirements saving both time and cost in the vaccine development (*Meeusen et al., 2007*). Most importantly, veterinary vaccines can be evaluated in the target species thereby minimizing the risk of failure in later stages.

Therefore, implementing an already-established RNA technology with a known manufacturing process and excessive safety evaluation performed in humans would be a waste of valuable (financial) resources and confer substantial advantages to the veterinary vaccine sector. Despite the smaller veterinary vaccine market, the linear cost profile – characterized by its high variable costs due to the raw materials and relatively low fixed costs due to the small-scale production facilities- renders RNA vaccines an attractive alternative for small markets or as an emergency

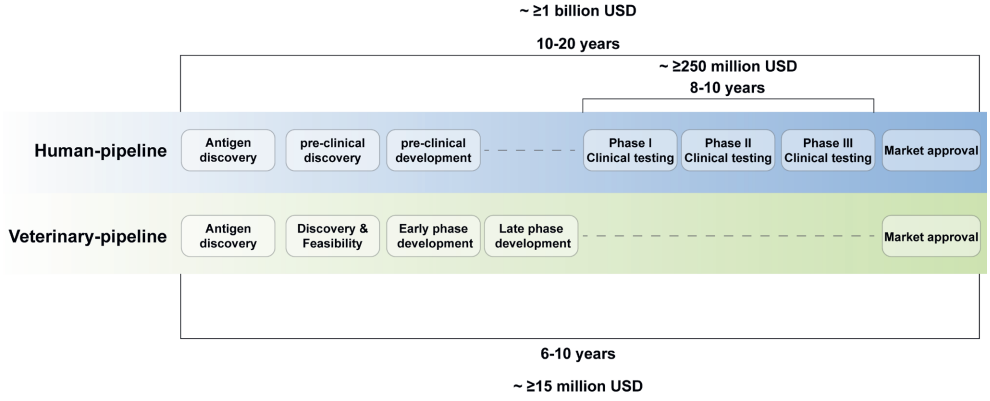

Figure 4. Human and veterinary vaccine development pipeline showing the estimated costs and lead times from the initial antigen discovery phase to the final market approval. The data used for this representation are derived from *Frances (2020)*, *Martin et al. (2017)*, and *Warimwe et al. (2021)*.

vaccine pipeline in response to newly emerging diseases (*Kis et al., 2020; Newall et al., 2023*). From a consumer perspective, such as an average Dutch poultry farmer, buying RNA vaccines for an entire hatchery of around 5,000 chickens becomes a substantial investment (*van Horne, 2020; R. van Leeuwen, personal communication, May 24, 2023*). Nevertheless, from a One-Health perspective, the vaccination of either humans or animals that reduces infectious disease morbidity and mortality, even with the current expense of effective RNA vaccines, outweighs the costs linked to disease treatment and healthcare expenses. This was demonstrated in the case of smallpox eradication, where a merely 100 million USD investment in an eradication vaccination program yielded a total cost savings of 1.35 billion USD annually (*Fenner et al., 1988*). Different from smallpox, which typically affects humans, controlling AIV via a worldwide eradication program has been described before but is challenged by the wide variety of local

variants and a permanent AIV reservoir in wild birds (*Marangon et al., 2008*). Although other countries already implemented local vaccination of poultry to combat AIV (*Swayne, 2012a*), the Netherlands, despite the sharp rise in the cost of controlling bird flu and the constant risk of zoonotic events, remains cautious about implementing a vaccination eradication program due to the expected negative impact on the export of poultry products (*Rekenkamer, 2023*).

In a broader context, in the face of newly emerging threats, the most important factor is the implementation of an adaptable technology, like RNA vaccines, capable of keeping pace with the evolving pathogenic threat. The advancements outlined here, share a common denominator which is to speed up the process of developing new vaccine variants based on established technologies in which the primary focus can be relayed to vaccine efficacy testing to maintain the health of both the animals as well as those responsible for their care.

Conclusion & future directions

In the rapidly evolving field of vaccinology, the search for effective vaccines to combat disease-causing pathogens and reduce the burden of illnesses on the host is a constant pursuit.

This thesis was dedicated to delving into the development of a novel self-amplifying RNA technology with the specific aim to fill a niche in the poultry-vaccine portfolio. The ultimate goal was to create a platform that is safe-by-design, effective in poultry, and versatile in case of newly emerging pathogens. The insight gained from the research outlined in **Chapters 2 and 3** on the novel bird-adapted TMUV replicon provided valuable information about the onset of expression, the total amount of heterologous protein production but also the level of viral-induced CPE in cells. Additionally, it was demonstrated that the TMUV replicon harbored great flexibility in the capsid-coding region, enhancing the adjustability of the replicon platform. By contrasting these distinctive characteristics with those of the established VEEV replicon platform, a plausible explanation emerged for the observed low efficacy of VEEV replicon RNA in chickens.

In **Chapter 4** we presented the first preliminary data on the vaccination of layers using LNP-formulated TMUV replicon RNA. We demonstrated that this completely synthetic vaccine was able to successfully seroconvert layers against IBDV. However, it also revealed that the sort of replicon delivery modality (e.g. DREP) could significantly impact vaccine efficacy. Further investigation into the presence and duration of replicon RNA levels in the host, especially in comparison to DNA-launched replicon RNA, holds promise in optimizing future vaccine delivery strategies. Additionally, conducting a challenge study to assess whether the reported presence or absence of a humoral response against IBDV or AIV respectively, could still protect the chickens against future infections is crucial. This aligns with the importance of elucidating the adaptive immune system, particularly the cellular immunity discussed in this chapter. Developing host-specific cellular immune assays will enhance our comprehension of how the vaccine-mediated immune response correlates with that of a wildtype infection. Beyond the successful vaccination of the TMUV replicon RNA formulated in a synthetic carrier, the strength of replicon RNA lies in the encapsidation into a viral-derived carrier.

Chapter 5 demonstrated the successful generation of a helper cell line capable of producing virus-like replicon particles. The next step would be to evaluate these VRPs in a challenge study in chickens, comparing their efficacy to the established VEEV VRPs. This evaluation will provide crucial insights into the wide range of modalities that can be harnessed as a suitable vaccine platform for this host. Looking ahead, the scope of research could extend to other avian species to create an even more versatile bird-adapted replicon platform technology

VII

Chapter R

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Summary

Summary

The poultry industry plays a pivotal role in supplying the ever-growing global demand for animal-based food products. However, it faces a continuous challenge posed by the emergence of infectious diseases such as avian influenza virus (AIV; *Orthomyxoviridae* family), infectious bronchitis virus (IBV; *Coronaviridae* family), and infectious bursal disease virus (IBDV; *Birnaviridae* family). Safeguarding poultry well-being becomes not only a socio-economic imperative but also a crucial aspect of public health. To achieve this, alongside adhering to standard hygienic protocols in hatcheries, the implementation of prophylactic measures is important. These preventive measures decrease the likelihood of novel viral variants to emerge. In case novel variants do arise, a quickly adaptable, safe, and effective vaccine platform technology could reduce disease burden and prevent the culling of millions of poultry every year. One such platform technology is the self-amplifying RNA, also known as 'replicon'. These replicon vaccines are typically derived from positive-sense, single-stranded RNA viruses such as the Kunjin virus (KUNV; family *Flaviviridae*) or Venezuelan equine encephalitis virus (VEEV; family *Togaviridae*). The RNA genome of these viruses is modified by replacing the viral structural genes with a heterologous gene of interest while maintaining the viral replicase genes for replicon RNA amplification. In contrast to conventional mRNA vaccines, replicons induce long-lasting expression upon administering a low dose which can result in fewer side effects. Despite the success of the VEEV replicon vaccines in both human and veterinary trials, their application to poultry vaccination has also shown limitations. Studies in chickens have shown that a VEEV replicon vaccine only provides partial protection against viral challenges, underscoring the need for a novel replicon platform to effectively protect against and prevent viral diseases in this growing poultry industry.

Such an alternative replicon platform could be based on the duck-derived Tembusu virus (TMUV; family *Flaviviridae*), because of the bird-adapted and non-cytophatic characteristics. To investigate this, an infectious cDNA clone based on the contemporary TMUV WU2016 isolate, which is closely related to other duck-derived TMUV isolates, was constructed using reverse genetics to study the virus replication kinetics. The infectious clone derived from TMUV WU2016 showed better viral propagation compared to the prototypical, mosquito-derived TMUV MM1775 isolate in various cell lines. Two similar TMUV replicon system were designed that could either be used indirectly by first an *in vitro* RNA transcription reaction prior to its delivery to the target cell or directly be delivered as as a DNA-launched replicon (DREP). The replicon system was constructed by replacing the TMUV structural genes with transgenes encoding for fluorescent or luminescent proteins, as well as viral (glyco)proteins of specific poultry diseases. Using fluorescent and luminescent reporter proteins, the expression kinetics and cytopathic effect (CPE) were assessed in vitro. It was revealed that TMUV replicon-mediated expression of reporter transgenes lasted up to 4 days post-electroporation with a minimal viral-induced cytopathic effect in baby hamster kidney fibroblast (BHK-21) cells. Additionally, a comprehensive analysis of the expression, glycosylation, and cellular localization successfully demonstrated that TMUV

replicon system could produce haemagglutinin (HA; AIV), spike (S; IBV), or nucleocapsid protein (VP2; IBDV), laying grounds for a new self-amplifying vaccine platform in poultry.

To obtain a better understanding of the difference between the novel bird-adapted TMUV replicon system and the well-established VEEV replicon system, a head-to-head comparison was performed revealing the replicon expression kinetics in both mammalian and avian cell lines. In contrast to VEEV, the TMUV replicon demonstrated a slow onset, but overall higher cumulative heterologous gene expression in the absence of a noticeable CPE. Additionally, two more TMUV replicons with extended capsid (C) gene lengths were constructed, which included additional *cis*-acting RNA elements that were absent in the TMUV replicon containing the minimal capsid gene (minimal replicon). It was demonstrated that these RNA elements enhanced the TMUV replicon heterologous protein expression. The TMUV replicon capsid variants demonstrated an early onset and overall similar or higher accumulation of heterologous protein production at the cost of an increased CPE compared to the minimal replicon variant. These results demonstrated the versatility of the TMUV replicon system in the onset and level of transgene expression, and, most importantly, revealed key differences in expression kinetics compared to the VEEV replicon system.

In succession to the previous comparison between TMUV and VEEV replicon platforms, which displayed a distinctively different expression profile in vitro, these platform technologies were further evaluated in vivo with the immunization of 1-day-old chickens using lipid nanoparticle-formulated replicon RNA or DREP. At three weeks post-prime vaccination, the chickens were boosted after which serum was collected for 13 weeks post-prime vaccination. An antibody response was measured against IBDV in TMUV replicon RNA and DREP-vaccinated chickens, but no significant humoral response against AIV was detected. Intriguingly, the VEEV DREP-vaccinated chickens induced an early and robust humoral response against HA and VP2, whereas no substantial seroconversion was observed in chickens vaccinated with VEEV replicon RNA. It was concluded that both replicon RNA and DREP modalities of the bird-adapted TMUV replicon platform were suitable for the vaccination of chickens against IBDV. However, whether the elicited humoral response conferred protective immunity against IBDV in birds vaccinated with the TMUV replicon should be investigated in another vaccination challenge trial. Further research is warranted to investigate the absence of humoral response against TMUV-expressed HA and elucidate the differing impact of the replicon modalities on seroconversion in chickens.

As an alternative to the synthetic lipid nanoparticle used in the previous immunization study, our research extended towards the development of a virus-derived carrier: virus-like replicon particles (VRPs). VRPs are efficient delivery vehicles that are typically produced by complementing cells with the viral structural genes along with the replicon RNA. To identify the most suitable cell line for the production of VRPs (helper cell line), several mammalian-, avian- and mosquito-derived cell lines were infected with TMUV WU2016 in comparison to the prototypical mosquito-derived TMUV MM1775 strain. Both virus isolates replicated to high viral titers in human (HEK293T), chicken (DF-1), duck (EB66), and mosquito (Chao ball,

Aag2, and C6/36) cell lines. In proceeding to produce VRPs, HEK293T cells were selected as the most suitable packaging cell line. A monoclonal inducible packaging cell line expressing the TMUV structural proteins was successfully established using lentiviral transduction and fluorescent-activated cell sorting. Using electroporation, the replicon RNA was introduced into the monoclonal cells and this resulted in the efficient production of VRPs. Challenged by the relatively low TMUV VRP titer, potential factors influencing the VRP yields were investigated using transmission electron microscopy. This revealed that protein aggregates accumulated within the induced monoclonal packaging cells, while absent within non-induced packaging cells. Although the individual particles within these aggregates resembled a similar size to the secreted VRPs in the supernatant, it remains uncertain whether these intracellular aggregates genuinely resemble viral proteins and if they directly relate to the low VRP yield.

To conclude, the experimental results outlined in this thesis contributed to the development of a novel, bird-adapted TMUV replicon platform. Substantial expression differences from the existing VEEV replicon were demonstrated *in vitro* and *in vivo.* The TMUV replicon, whether administered as RNA or DNA-launched, effectively elicited specific humoral responses in chickens. Moreover, the comprehensive analysis of the TMUV propagation in different cell lines, coupled with the establishment of a monoclonal packaging cell line contributes to the establishment of a complete repertoire of replicon modalities that hold potential for vaccination of poultry.

Samenvatting

De pluimvee-industrie speelt een cruciale rol bij het voorzien in de voortdurend groeiende en wereldwijde vraag naar dierlijke producten. Echter, deze industrie wordt constant geconfronteerd met de opkomst van besmettelijke ziekten zoals veroorzaakt door het vogelgriepvirus (AIV; virusfamilie *Orthomyxoviridae*), infectieus bronchitis virus (IBV; familie *Coronaviridae*) en het infectieus bursitis virus (IBDV; virusfamilie *Birnaviridae*). Het waarborgen van het welzijn van pluimvee is, buiten het zijn van een sociaal-economische noodzaak, ook een cruciaal aspect voor de volksgezondheid. Om dit dierlijk welzijn te kunnen garanderen volgen kippenboeren standaard hygiëneprotocollen en maken ze gebruik van profylactische maatregelen. Deze preventieve maatregelen verlagen ook de kans op nieuwe virusvarianten. Mochten deze varianten toch ontstaan dan kan een snel aanpasbare, veilige en effectieve platformtechnologie de ziekte en dood van jaarlijks miljoenen pluimveedieren voorkomen. Een van deze platformtechnologieën is het zelf-vermeerderend RNA, ook wel bekend als replicon. Deze replicon-vaccins zijn vaak afgeleid van positief-enkelstrengse RNA-virussen, zoals het Kunjin-virus (KUNV; virusfamilie *Flaviviridae*) of het Venezolaans paarden hersenontstekingsvirus (VEEV; virusfamilie *Togaviridae*). Het RNA-genoom van deze virussen wordt aangepast door de virale structurele genen te vervangen door een gen van interesse, terwijl de virale replicatiegenen behouden blijven voor de vermeerdering van het replicon RNA. In tegenstelling tot conventionele mRNA-vaccins, zorgen replicons voor langdurige eiwitproductie bij vaccineren met een lage dosis RNA, wat kan resulteren in minder bijwerkingen. Ondanks het succes van VEEV replicon vaccins in zowel menselijke als veterinaire studies, heeft de toepassing bij pluimveevaccinatie beperkingen laten zien. Studies in kippen hebben aangetoond dat dit replicon vaccin slechts gedeeltelijke bescherming bood tegen pathogene viral infecties na het vaccineren met VEEV replicons. Dit benadrukt de behoefte aan een nieuw repliconplatform dat in staat is om deze groeiende pluimveesector effectief te beschermen tegen virale ziektes.

Een dergelijk alternatief repliconplatform is gebaseerd op het, van eenden-afkomstige, Tembusu-virus (TMUV; virusfamilie *Flaviviridae*) dat gekenmerkt wordt door de adaptatie aan vogels en zijn niet-cytopatische eigenschappen. Om dit platform te ontwikkelen, hebben we een infectieuze cDNA-kloon geconstrueerd op basis van het TMUV WU2016-isolaat, dat lijkt op vele andere uit eend geïsoleerde isolaten, om zo de replicatiekinetiek te bestuderen. De infectieuze TMUV WU2016 kloon liet betere viral replicatie zien in verscheidene cellijnen dan het prototypische, uit muggen geïsoleerde, TMUV MM1775-isolaat. Vervolgens zijn twee gelijke TMUV repliconsystemen ontworpen die ofwel indirect gebruikt konden worden door eerst een *in vitro* RNA transcriptiereactie uit te voeren alvorens ze aan de doelcel werden toegediend, of direct toegediend als een DNA-gelanceerd replicon RNA (DNA-launched replicon vector; DREP). Om het replicon systeem te construeren hebben we de TMUV-infectieuze kloon aangepast door de virale structurele genen te vervangen door genen die coderen voor verschillende fluorescerende, luminescente of virale (glyco) eiwitten van specifieke kippenziektes. Met behulp van deze marker-eiwitten hebben we de expressiekinetiek en het cytopathisch effect (CPE) van het TMUV

replicon in babyhamster nier-fibroblast cellen (baby hamster kidney fibroblast cells, BHK-21) geëvalueerd. Daarnaast is aangetoond dat de TMUV-replicon markertransgenen tot vier dagen na elektroporatie van het replicon in BHK-21 cellen tot expressie komen en dat daarbij een minimaal cytopathisch effect wordt gedetecteerd. Bovendien hebben we met een uitgebreide analyse, waarbij we kijken naar de expressie, glycosylering, en lokalisatie, aangetoond dat het TMUV-replicon systeem uitermate geschikt is voor de expressie van hemagglutinine (hemagglutinin, HA; AIV), peplomeer (spike, S; IBV) of eiwitmantel (nucleocapsid, VP2; IBDV), wat een robuuste basis legt voor een nieuw zelf vermeerderend vaccinplatform in pluimvee.

Om een beter inzicht te krijgen in het verschil tussen het nieuwe, op vogels aangepaste TMUV repliconsysteem en het bestaande VEEV repliconsysteem, hebben we een rechtstreekse vergelijking uitgevoerd waarin we hebben gekeken naar de replicon expressie kinetiek in zowel zoogdier- als vogelcellijnen. In tegenstelling tot VEEV, kwam de expressie van het TMUV replicon traag op gang, maar resulteerde uiteindelijk in een hogere cumulatieve expressie van de heterologe reportergenen zonder duidelijke aanwezigheid van CPE. Verder hebben we twee extra TMUV-replicons geconstrueerd waarbij de eiwitmantel (capsid, C) genen verlengt zijn in vergelijking met het al bestaande replicon waarbij alleen het minimale capside gen is gecodeerd (minimale replicon). Deze verlengde replicons bevatten aanvullende, in *cis*-werkende, RNA-elementen waarmee de heterologe genexpressie van het TMUV-replicon kan worden verbeterd. De gegenereerde TMUV replicon capsidevarianten vertoonden een vroege marker eiwitsynthese in combinatie met een gelijke of zelfs hogere totale marker eiwitproductie. Dit alles ten koste van een verhoogde CPE niveau vergeleken met het minimale replicon. Deze resultaten dragen bij aan het verder karakteriseren van het TMUV replicon systeem, zoals de aanpasbaarheid van dit systeem met betrekking tot de aanvang alsmede de totale hoeveelheid genexpressie, maar vooral de verschillen in expressiekinetiek vergeleken met het VEEV repliconsysteem.

Na het karakteriseren van de verschillen tussen het TMUV en het VEEV repliconsysteem, waarbij kenmerkende expressieprofielen zijn geobserveerd *in vitro*, hebben we het onderzoek voortgezet met de evaluatie van deze platformtechnologieën *in vivo* door één-dag-oude kuikens te vaccineren met lipide nanodeeltjes die replicon-RNA of DREP bevatten. In de derde week na de eerste vaccinatie werden de kuikens opnieuw gevaccineerd met hetzelfde vaccin, waarna bloedmonsters werden genomen tot en met de 13de week na de eerste vaccinatie. Ondanks de aanwezigheid van antilichamen tegen IBDV in kuikens gevaccineerd met TMUV replicon RNA en DREP, is er geen significante humorale reactie tegen AIV gedetecteerd. Intrigerend genoeg is in kuikens gevaccineerd met VEEV DREP een vroegtijdige en krachtige antilichaamreactie gedetecteerd tegen HA en VP2, terwijl dit niet het geval is voor kuikens gevaccineerd met VEEV replicon RNA. Desalniettemin kan geconcludeerd worden dat zowel de replicon-RNA als de DREP entiteit van het op vogels afgestemde TMUV-replicon-platform geschikt zijn voor het vaccineren van kuikens tegen IBDV. Echter, om zeker te weten of de door TMUV-opgewekte immunereactie tegen IBDV genoeg is om de kippen daadwerkelijk te beschermen, zijn additionele virus "challenge" noodzakelijk. Daarnaast is verder onderzoek nodig om de oorzaak van de afwezige humorale reactie tegen het HA-antigeen,

tot expressie gebracht door het TMUV-replicon, te identificeren en het verschil tussen de verschillende replicon-entiteiten in kaart te brengen met betrekking tot seroconversie bij kuikens.

Als alternatief voor de synthetische lipide deeltjes die in de vorige immunisatiestudie werden gebruikt, werd ons onderzoek uitgebreid met het maken van een drager afkomstig van een virus: de zogenaamde virus-achtige replicon deeltjes (virus-like replicon particles, VRPs). VRPs zijn efficiënte dragers die normaliter worden geproduceerd door cellen te voorzien van zowel de virale structurele genen als het replicon RNA. Om de meest geschikte cellijn voor de productie van deze VRPs (helper cellijn) te identificeren, hebben we verschillende cellijnen die afkomstig zijn van zoogdieren, vogels en muggen geïnfecteerd met TMUV WU2016 en vergeleken met de prototypische en van muggen afkomstige TMUV MM1775 stam. Beide virusstammen hebben geleid tot hoge virale titers in humane (HEK293T), kippen (DF-1), eenden (EB66) en muggen (Chao ball, Aag2 en C6/36) cellijnen. Uiteindelijk zijn de HEK293T-cellen geselecteerd als de meest geschikte cellijn om VRPs mee te produceren. Vervolgens is door middel van lentivirale transductie en fluorescentie-geactiveerde celsortering een monoclonale, induceerbare helpercellijn gemaakt. Daarnaast is door middel van elektroporatie het replicon-RNA in de monoclonale helpercellen geïntroduceerd en is de efficiënte productie van VRPs in gang gezet en aangetoond. Vanwege de relatief lage TMUV VRP titer, hebben we geprobeerd om potentiële factoren die invloed hebben op de VRP productie door middel van transmissie-elektronenmicroscopie in kaart te brengene. Hiermee is ontdekt dat in de geïnduceerde monoclonale helpercellen eiwitaggregaten ophoopte die afwezig waren in niet-geïnduceerde helpercellen. Ondanks dat deze deeltjes in de eiwitaggragaten van vergelijkbare grootte waren als de VRPs in het supernatant, is het onbekend of deze aggregaten daadwerkelijk virale eiwitten bevatten en of dit gecorreleerd kan worden aan de lagere VRP opbrengst.

Kortom, de experimentele resultaten die in deze scriptie worden gepresenteerd hebben als doel bij te dragen aan de ontwikkeling van een nieuw, op vogels-aangepast, TMUV repliconplatform. We hebben aangetoond dat er aanzienlijke verschillen in expressie te zien zijn ten opzichte van het bestaande VEEV-replicon zowel *in vitro* als *in vivo*. Het TMUV replicon, of het nu als RNA of DNA-vector werd toegediend, resulteerde in een specifiek humorale immuunreactie bij kippen. Daarnaast heeft de uitgebreide analyse van de groeikinitiek van TMUV in verschillende cellijnen, in combinatie met de maken van een monoclonale helpercellijn, bijgedragen aan het vaststellen van een volledig repertoire van replicon-modaliteiten dat een groot potentieel biedt voor de toekomstige vaccinatie van pluimvee.

Chapter A

Appendices

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About the author

Jerôme Davine Gerardus Comes was born on January 28th, 1994 in Maastricht. After completing primary school in Munstergeleen, where he spent most of his childhood, he attended high school at Trevianum, where he developed a growing interest in biology. In 2012, he graduated and subsequently moved to Ede to pursue a BSc in biotechnology at Wageningen University. During his undergraduate studies, he conducted a thesis at the Laboratory of Virology on a case study focusing on Schmallenberg virus under the supervision of Dr. Richard Kormelink.

Due to his strong affinity with animal health, Jerôme continued his academic career with a MSc in Medical Biotechnology. For his MSc thesis, he conducted research at the Laboratory of Cell Biology and Immunology, working on the development of an immunotherapeutic model against melanoma in dogs. His project involved the attenuating a *Salmonella typhimurium* strain using CRISPR-cas9 under the supervision of Dr Edwin Tijhaar.

After completing his MSc thesis, Jerôme was presented with a remarkable opportunity in the United States. He temporarily paused his hobby and student job as a private driver for EasyWay B.V. to undertake a MSc Internship at Protein Sciences Corporation. During his internship, he focused on optimizing the vector stability of the first licensed recombinant influenza vaccine Flublok® under the supervision of Dr Manon Cox and Dr Wafaa Mahmoud.

In 2018, Jerôme graduated with honors, earning a Masters degree from Wageningen University and continued his career in the veterinary sector at MSD Animal Health in Boxmeer. In his role, he was involved in the development of Quality Control tests for a poultry vaccine site located in Spain. In 2019, Jerome started his PhD candidacy at the Laboratory of Virology in collaboration with MSD Animal Health under the supervision of Dr Gorben Pijlman and Dr Marielle van Hulten. His researched focused on the development of a novel replicon RNA vaccine to combat avian infectious diseases, known as REPLICAID. The outcomes of his research are documented in this PhD thesis. During his doctoral studies, he gained special interest in the Business development of life sciences and immunology during post-graduate courses at the Vrije Universiteit (VU) van Amsterdam and Erasmus Medical Center (Erasmus MC) in Rotterdam. Currently, Jerôme holds the position of a project leader at MSD Animal Health at the department of Biotechnology Solutions.

Educational Statement of the graduate school Experimental Plant Sciences

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review/project proposal (10.5 ECTS)

- Rise of the RNA machines – self-amplification in mRNA vaccine design
- Replicon vaccines to combat avian infectious diseases

Post-graduate courses (5.9 ECTS)

- Basic statistics; PE&RC (2020)
- Advanced immunology; Erasmus postgraduate school of Molecular Medicine (2023)

Laboratory training and working visits (3 ECTS)

- Work visit animal trial RNA vaccines in poultry; MSD Animal Health (2021, 2022)

Invited review of journal manuscripts (2 ECTS)

- Journal of Virology: dimerization of Dengue virus E-subunits impacts antibody function and domain focus (2020)
- Frontiers in Immunology: decreased virulence of duck Tembusu virus harbouring a mutant non-structural protein 2A with impaired interaction with STING and IFN-B induction (2021)

Deficiency, refresh, brush-up courses (3 ECTS)

- Business management in life sciences; Amsterdam VU (2020)

Competence, skills and career-oriented activities (4.1)

- Basics in LaTeX; WGS (2020)
- - Adobe Indesign; WGS (2020)
- - Teaching and supervising thesis students; WGS (2020)
- - Reviewing scientific manuscript; WGS (2021)
- - Scientific publishing; WGS (2021)
- - Scientific integrity; WGS (2022)
- - Scientific writing; WGS (2022)

PE&RC Annual meetings, seminars and PE&RC weekend/retreat (0.6 ECTS)

- PE&RC Midterm weekend (2021)

Discussion groups/local seminars or scientific meetings (5.7 ECTS)

- DAVS (2019, 2021, 2022, 2023)
- DARN (2019-2023)
- International virtual seminars on Arbovirus biology (2020-2023)
- Vaccine symposium (2022)
- COGEM symposium (2023)

International symposia, workshops and conferences (11.3 ECTS)

- American society of virology annual meeting; poster presentation; Madison, USA (2022)
- 10th International symposium on Avian viral respiratory diseases; poster presentation; Utrecht, the Netherlands (2022)
- Dutch annual virology symposium poster presentation; Amsterdam the Netherlands (2023)
- European conference of virology; oral presentation; Gdansk, Poland (2023)

Societally relevant exposure (0.3 ECTS)

- Masterclass replicon vaccines at MSD animal health (2019)

Lecturing/supervision of practicals/tutorials (4.5 ECTS)

- Molecular virology (2019)
- Cell biology and health (2020)
- Immunotechnology (2020, 2021, 2022)

BSc/MSc thesis supervision (3 ECTS)

- Designing Tembusu (TMUV) replicon helper construct and producing TMUV viral replicon particle
- The expression of AIV H5 or H9 launched from a TMUV RNA replicon
- Development of a Tembusu virus derived replicon vaccine for use in poultry
- CpG and UpA optimization of Tembusu virus replicon
- Evaluation of the Tembusu virus replicon system in BHK and EB66 cell lines and chicken
- The generation of Tembusu replicon (particles) expressing H5 or H9 of avian influenza virus
- The SARS-CoV-2 outbreak and current vaccine development
- Evaluation of the total expression and cytotoxicity of the alphavirus- versus the flavivirus-based RNA replicons in transfected cells
- Generation and characterization of DNA based perak-virus replicon vaccines for poultry and generation of stable packaging cell lines through Lentiviral transduction
- Insights into the Tembusu virus growth curves and Tembusu virus-based RNA replicon expression patterns in various cell lines
- Development of an infectious clone of the Tembusu virus replicon

The research (REPLICAID) described in this thesis was conducted at the Laboratory of Virology at Wageningen University and Research (Wageningen, the Netherlands) and was financially funded by Merck Sharpe & Dohme B.V. Animal Health (MSD AH; Boxmeer, the Netherlands).

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