RATIONALLY RECONSTRUCTED AND ATTENUATED VACCINES FOR EPIDEMIC RESPONSE

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Title

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Most of the recent viral outbreaks were caused by highly mutating RNA and single stranded DNA viruses. The availability of safe and effective rapid response vaccines early on in an epidemic situation, along with good vaccine delivery systems, is critical for pandemic response plans. Additionally, immunodominance patterns in the host response to epitopes in vaccine antigens can complicate immune responses to vaccines.

In this thesis, using porcine circovirus type 2 (PCV2) as a model, we have focused on changing viral immunodominance patterns to rationally improve vaccine efficacy. We hypothesized that rational alteration of the immunodominant decoy epitope would remove nonprotective antibody response and improve the overall quality of neutralizing antibodies. As hypothesized, the antibody response to the target immunodominant epitopes were abrogated in the vaccinated pigs, and they were protected upon with the challenge of a heterologous strain PCV2d.

To ensure the safety of the rationally restructured PCV2 vaccine, we have developed novel strategy to ensure suicidal replication of the vaccine virus in vivo. We hypothesized that recoding serine and leucine codons of the PCV2 capsid gene will increase the probability of accumulating stop mutations during viral replication. As expected, immunized pigs with the suicidal vaccine, protected them against PCV2d heterologous challenge. Furthermore, subjecting the suicidal vaccine construct to in vitro immune pressure with sub-neutralizing serum, resulted in an accumulation of stop mutations and abortive replication.

Finally, using porcine epidemic diarrhea virus (PEDV) as a model, we have developed an effective oral delivery system for a rapid response vaccine. Treatment of PEDV with heat to denature the capsid, followed by RNase to fragment the RNA genome, resulted in a minimally replicative vaccine which was highly effective in weanling piglets. Here, we determined treatment conditions to either completely inactivate or rapidly attenuate PEDV. To improve oral delivery of the vaccine to sows, biodegradable niosome formulation composed of edible lipid, cholesterol, and charge stabilizer was optimized. The antigen loading capacity of the niosome was over 80% with minimal cellular cytotoxicity. In summary, the methods described in this thesis have addressed three major gaps in vaccinology and have broad applicability in the field.

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I want to thank my wife, Humiera Begum, who has sacrificed many things for my studies, motivated me, and provided unlimited mental support. In addition, I am obliged to my parents and all the family members for supporting me throughout the journey.

Finally, I thank the almighty GOD, who has made all the things possible.

DEDICATION

This dissertation is dedicated

To my Parents:

MD Abdul Gafur & Rowshan Ara Begum

To My Wife:

Humiera Begum

To my Parent in law:

Late Khalil Ahmed & Gulshan Ara Begum

To My Brothers:

AGM Rafiuzzaman, AGM Rafiquzzaman & AGM Rasheduzzaman

To my Nieces:

Orchid, Gerbera & Ruellia

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Ab.....Antibody

APCs .....Antigen Presenting Cells

ASF .....African Swine Fever

BCG .....Bacillus Calmette-Guérin

BPL .....Beta Propiolactone

BSA.....Bovine Serum Albumin

CEPI.....Coalition for Epidemic Preparedness Innovations

CMI.....Cell Mediated Immunity

CpG.....5'-C-phosphate-G-3'

DCP.....Diacetyl Phosphate

DIVA.....Differentiate the Infected and Vaccinated Animal

DMEM .....Dulbecco's Modified Eagle's Medium

DMSO.....Dimethyl Sulfoxide

DNA.....Deoxynucleic Acid

DPC.....Days Post Challenge

DPV.....Days Post Vaccine

E.M.....Electron Microscopy

EC50 .....Effective Concentration 50

ELISA .....Enzyme linked Immunosorbant assay

FDA.....Food and Drug Administration

FFN .....Fluorescence Focus Neutralization

FFU .....Fluorescent Focus Units

H&E .....Hematoxylin and Eosin

HBSS.....Hank’s balanced salt solution

HIV .....Human Immunodeficiency Virus

HPLC .....High Performance Liquid Chromatography

HPO.....Horseradish Peroxidase

IACUC .....Institutional Animal Care and Use Committee

IBC.....Institutional Biosafety Committee

IFA .....Immune Fluorescence Assay

IFN .....Interferon

Ig .....Immunoglobulin

IHC.....Immunohistochemistry

IKK .....Inhibitor κß kinase

IRF .....Interferon Regulating Factos

ISCOM.....Immunostimulating complex

MAMPs.....Microbe Associated Molecular Patterns

MARS .....Middle East Respiratory Syndrome

MEM.....Minimum Essential Medium

MHC .....Major Histocompatibility Complex

MLV.....Modified Live Vaccine

mRNA.....Messenger RNA

MTT .....3-(4,5-dimethylthiazol-2-yl) Tr-2,5-diphenyltetrazolium- bromide

NA.....Neutralizing antibody

NF- κß .....Necrotic Factor kappa beta

NGS.....Next Generation Sequencing

NISV .....Non-Ionic Surfactant Vehicles

NK.....Natural Killer

NVSL .....National Veterinary Services Laboratory

OPV.....Oral Polio Vaccine

ORF.....Open Reading Frame

PAM.....Point Accepted Mutation

PAMPs .....Pathogen Associated Molecular Patterns

PBS .....Phosphate Buffer Saline

PBST .....Phosphate Buffered Saline- Tween

PCR.....Polymerage Chain Reaction

PCV2.....Porcine Circovirus type 2

PCV2d.....Porcine circovirus type 2d

PCVAD .....Porcine Circovirus Associated Diseases

PEDV .....Porcine Epidemic Diarrhea Virus

PEG.....Polyethylene Glycol

PLGA .....poly D L-lacti-co-glycolic acid (PEG)

PMWS.....Post-weaning multi-syatemic wasting syndrome

PRRs .....Pattern Recognizing Receptors

PRRSV.....Porcine Respiratory and Reproductive Syndrome Virus

PTA.....Phosphor Tungstic Acid

RNA .....Ribonucleic Acid

rPCV2-Vac.....recombinant PCV2-Vaccine

RU .....Response Unit

SARS-CoV.....Severe Aquired Respiratory Syndrome Coronavirus

SARS-CoV2.....Severe Aquired Respiratory Syndrome Coronavirus 2

sPCV2-Vac .....Suicidal PCV2 Vaccine

SPR .....Surface Plasmon Resonance

SRS .....Surface-antigen Related Sequence

ssDNA.....Single-Stranded DNA

TCID .....Tissue Culture Infectious Dose

Th .....T helper

TLRs .....Toll-like Receptors

TMB.....3,3′,5,5′-tetramethylbenzidine substrate

UpA.....5'-U-phosphate-A-3'

UTR.....Untranslated Region

UV.....Ultra Violet

VLP .....Virus Like Particle

LIST OF APPENDIX TABLES

LIST OF APPENDIX FIGURES

CHAPTER 1: LITERATURE REVIEW

Introduction

Over the past decades, the number of emerging and re-emerging diseases has increased for both humans and animals. Recent influenza, ebola, zika, and ongoing coronavirus are the best examples of human epidemics and pandemics. On the other hand, more than 15 emerging or re-emerging viral infections in the swine population have struck in the world in the last 25 years. Swine coronavirus, named as porcine epidemic diarrhea virus (PEDV) and porcine circovirus type 2, are the best examples of swine emerging and re-emerging viral infection. At the same time, some of these swine infectious diseases can transmit to humans, which raises public health concerns (Best, 2011). Most of these emerging viral diseases are caused by RNA viruses which cover more than 80% of the newly emerging viral diseases for both the human and animal world (Woolhouse & Gaunt, 2007). The RNA viruses tend to emerge as new strains and show a genetic and antigenic variability by a high mutation rate. Additional to the RNA virus, small single-stranded DNA virus, like PCV2, has a very high mutation rate. Therefore, although the vaccines are available against these viruses, protections are suboptimal and need improvement. Besides the genetic and antigenic variability, another reason for suboptimal protection against viruses is immunodominance, and the ideal example of suboptimal protection due to immunodominance is PCV2 vaccination. Moreover, there are safety issues with the live attenuated vaccines, which require rapid and effective attenuation strategies. To limit the damages by an epidemic or pandemic situation, early diagnosis, surveillance, biosecurity measures, and vaccine availability

is critical (2004; 2007; Y. Song, Singh, Nelson, & Ramamoorthy, 2016). Therefore, a rapidly developed, first response vaccine strategy should be in the emergency preparedness plan for the emerging virus like PEDV. Furthermore, as PEDV is an emerging viral disease that infects the gastrointestinal tract and is spread by the fecal-oral route, an oral vaccine delivery system is necessary. The current oral delivery methods are not optimal, and a well-developed oral vaccine delivery system is also required. Hence, this thesis will highlight the three global gaps in knowledge of vaccinology, firstly about immunodominance, secondly on rapid response and rapid attenuation, and finally on the rapid response vaccine with improved oral vaccine delivery system. Therefore, the following sections will deliver the vital background and gaps in knowledge regarding these three topics.

History and public health significance of vaccination

A vaccine is a protein or nucleic acid encoding a protein that gives acquired protection against infectious pathogens, mostly viruses, and bacteria. Commonly, vaccines are containing killed or inactivated pathogens, live or attenuated organisms, or selected immunogenic proteins from a pathogen formulated to train the host's immune system to fight against that particular pathogen. Unlike other medicines which are therapeutic, the primary purpose of the vaccine is to give protection from future infection (prophylactic) rather than cure established diseases. Therefore, vaccines are integral for preventive healthcare. However, therapeutic vaccines are available for some diseases like cancer (Guo et al., 2013; Hollingsworth & Jansen, 2019; Melief, van Hall, Arens, Ossendorp, & van der Burg, 2015).

The importance of vaccination and the concept of herd immunity are the centers of global attention, following the SARS-CoV2 pandemic, which was preceded by the Ebola and Zika outbreaks. In parallel, within the veterinary field, avian influenza had led to the loss of more than 50 million poultry flocks in 2014 (Ramos, MacLachlan, & Melton, 2017). In the event of the 2013-2015 PEDV epidemic, nearly 10 million piglets had died in the US (C. Lee, 2015). The unprecedented halting of "normal" lifestyles and activities, the impact on health care systems, the economy, and above all, the loss of life has re-established that vaccines are the single most important public health measure to protect against infectious diseases in human and veterinary medicine. Vaccines save more than 2-3 million lives annually worldwide and because of the mass vaccination program, the deadly disease smallpox has been eradicated, and other deadly diseases, polio, are nearly eradicated (Delany, Rappuoli, & De Gregorio, 2014). Many other deadly diseases are at different stages towards eradication which may be categorized as under control (cholera), elimination of disease (neonatal tetanus), elimination of infections (measles, poliomyelitis) (Dowdle, 1998; Schlipköter & Flahault, 2010). Therefore, a routine vaccination program is likely to be the most successful intervention in preventing infectious diseases. The different stages for disease eradication are listed in table 1.1.

Table 1.1: Stages towards disease eradication (Dowdle, 1998; Schlipköter & Flahault, 2010).

Stage	Definition	Example
"Control or Reduction	Incidence, prevalence, morbidity, or mortality to a locally acceptable level due to deliberate efforts; continued intervention measures are required to maintain the reduction.	Example: diarrhoeal diseases.
Elimination of disease:	Reduction to zero of the incidence of a specified disease in a defined geographical area as a result of deliberate efforts; continued intervention measures are required	neonatal tetanus.
Elimination of infections	Reduction to zero of the incidence of infection caused by a specific agent in a defined geographical area due to deliberate efforts; continued measures to prevent re-establishment of transmission are required.	measles, poliomyelitis;
Eradication:	Permanent reduction to zero of the worldwide incidence of infection caused by a specific agent resulted from deliberate efforts; intervention measures are no longer needed.	smallpox;
Extinction:	The specific infectious agent no longer exists in nature or in the laboratory.	None.

Source: Dowdle WR. The principles of disease elimination and eradication.38 MMWR Morb Mort Wkly Rep. 1999;48(SU01:23-7. Schlipköter, U. and A. Flahault, *Communicable diseases: achievements and challenges for public health.* Public Health Reviews, 2010. **32**(1): p. 90-119

Successful routine vaccination has significantly reduced the incidence of infectious diseases, which caused high mortality and morbidity in the early and mid 20th centuries (Slifka & Amanna, 2014). In addition to the routine vaccine, lab research workers and international travelers are advised to take some region-specific vaccines to prevent unexpected illness during their travel and outbreaks when they return to their native country. Nevertheless, the number of vaccine "success stories" is minimal compared to infectious pathogens, against which we do not

have any effective vaccines, or the protections are suboptimal since the discovery of the vaccines over the last two centuries. Table 1.2 provides a list of vaccine success stories.

Table 1.2: Widespread use of vaccines in the United States has eliminated or almost eliminated infectious diseases that were once terrifying household names.

Diseases	Baseline 20th Century Pre-Vaccine Annual Cases	2008 Cases	Percent Decrease (%)
Measles	503,282	55	99.9
Diphtheria	175,885	0	100
Mumps	152,209	454	95.7
Pertusis	147,271	10,735	92.7
Smallpox	48,164	0	100
Rubella	47,745	11	99.9
Haemophilus influenzae type b invasive	20,000	30	99.9
Polio	16,316	0	100
Tetanus	1,314	19	98.6

Source: Morbidity and Mortality Weekly Report, Centers for Disease Control and Prevention, 4/2/99, 12/25/09, 3/12/10

Host immune responses to antigens

Vaccines introduce the pathogenic agent to our immune system and activate the natural immune defense systems. As the vaccine is the inactive or attenuated form of the pathogen, it should not produce any disease. Instead, it primes the immune system against that pathogen so that the immune system retains a memory response for that agent. Therefore, in the event of actual infection, the pathogen is recognized immediately. Ideally, an effective immune response will block its propagation inside the body and clears it from the system, eventually giving protection. A vaccine can initiate both the humoral or antibody-mediated immune system and cell-mediated immune system. Usually, when a vaccine is administered, or a virus has infected for the 1st time, it will be internalized by the macrophages or antigen-presenting cells (dendritic

cells). Then the virus will be processed and presented to helper T-cells which will then activate both B-cells and cytotoxic T-cells. Figure 1.1 shows a general description of how vaccines initiate immune responses. The cross-talk between the innate and adaptive immune responses are crucial for effective vaccine-mediated immunity against the virus.

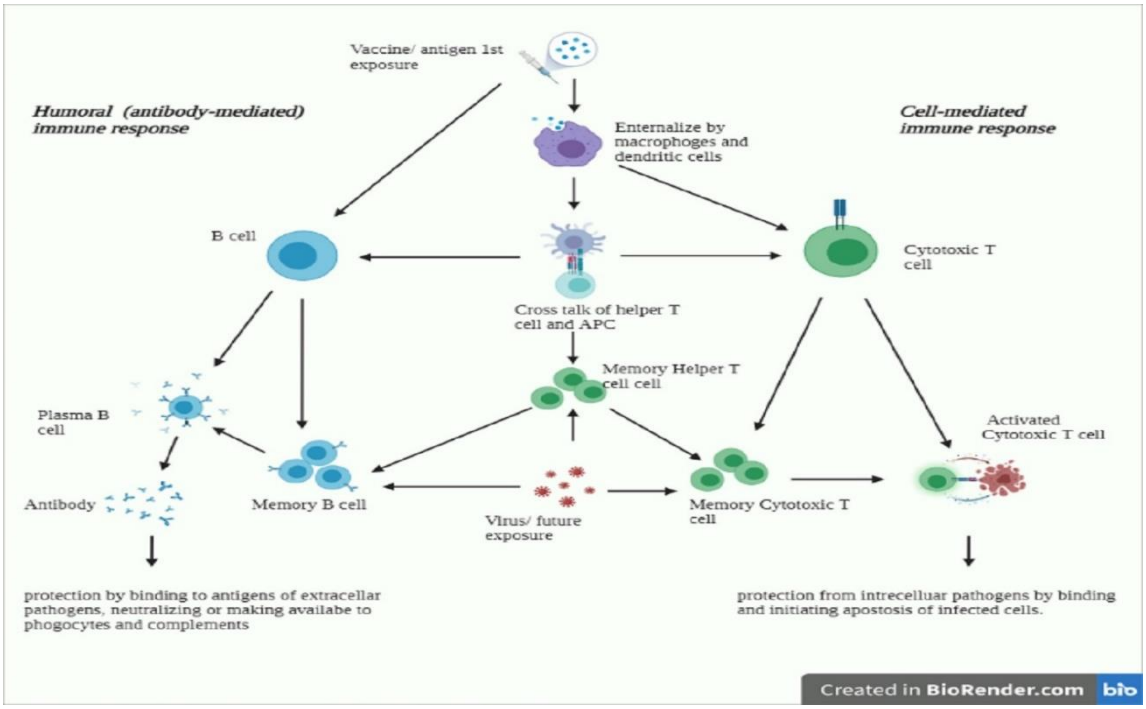

Figure 1.1: Activation of immune system by vaccine/pathogen. Prepared from BioRender.com

Innate immunity mechanisms

Innate immunity is the host's 1st line defense system regardless of the pathogen types, and mainly performed by phagocytes, including macrophages and dendritic cells (Akira, Uematsu, & Takeuchi, 2006). Innate immunity is a non-specific but fast-acting immune response. The epithelial and mucosal barriers protect against microbial invasion. The innate immune system recognizes molecular structures of the microbes or viruses that are not present in

the host cells. These structures are called pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs) (Abbas, Litchmann, & Pillai, 2016; Akira et al., 2006) (Table 3). The innate immune system poses a range of receptors called pattern recognizing receptors (PRRs) to recognize the PAMPs. The PRRs are mainly proteins that are highly expressed in the innate immune cells, including dendritic cells, macrophages, NK. cells, epithelial cells, etc (Kumar, Kawai, & Akira, 2011; Takeda, Kaisho, & Akira, 2003), and many PRRs are the toll-like receptors (TLRs). A list of PRRs/ TLRs and their targeting PAMPs has given in table 1.3.

Table 1.3: TLR recognition of microbial component (Akira et al., 2006).

Microbial Components	Species	TLR Usage
Bacteria		
LPS	Gram-negative bacteria	TLR4
Diacyl lipopeptides	*Mycoplasma*	TLR6/TLR2
Triacyl lipopeptides	Bacteria and mycobacteria	TLR1/TLR2
LTA	Group B *Streptococcus*	TLR6/TLR2
PG	Gram-positive bacteria	TLR2
Porins	*Neisseria*	TLR2
Lipoarabinomannan	Mycobacteria	TLR2
Flagellin	Flagellated bacteria	TLR5
CpG-DNA	Bacteria and mycobacteria	TLR9
ND	Uropathogenic bacteria	TLR11
Fungus		
Zymosan	*Saccharomyces cerevisiae*	TLR6/TLR2
Phospholipomannan	*Candida albicans*	TLR2
Mannan	*Candida albicans*	TLR4
Glucuronoxylomannan	*Cryptococcus neoformans*	TLR2 and TLR4
Parasites		
tGPI-mutin	*Trypanosoma*	TLR2
Glycoinositolphospholipids	*Trypanosoma*	TLR4
Hemozoin	*Plasmodium*	TLR9
Profilin-like molecule	*Toxoplasma gondii*	TLR11
Viruses		
DNA	Viruses	TLR9
dsRNA	Viruses	TLR3
ssRNA	RNA viruses	TLR7 and TLR8
Envelope proteins	RSV, MMTV	TLR4
Hemagglutinin protein	Measles virus	TLR2
ND	HCMV, HSV1	TLR2
Host		
Heat-shock protein 60, 70		TLR4
Fibrinogen		TLR4

ND = not determined. See text for references.

Source: Reprinted from Akira, S., S. Uematsu, and O. Takeuchi, *Pathogen recognition and innate immunity*. Cell, 2006. 124(4): p. 783-801, permission taken from the copyright clearance center

There are compartmentalized TLRs and sensors to recognize the invading or internalized viruses. For example, TLR2/6, TLR4, Ctype lectins are located on the cell membrane and recognize viral protein and coat, TLR3, TLR9, TLR7/8 are localized in the endosome and

recognized the viral nucleic acid, RLR, RIG-I, MDA5, IFI16, cGAS, etc. are located on cytoplasm and detects viral nucleic acid, MAVS located on mitochondria and detects viral nucleic acids (Carty et al., 2021; Yoo, Kim, Hufford, & Braciale, 2013). When the TLRs or other sensors sense any viral components (proteins or nucleic acids), it activates the inhibitor κß kinase (IKK), which activates NF- κß and interferon regulating factors (IRF). Once NF κß and IRF are activated, it enters the nucleus and initiates the synthesis of interferon and other antiviral cytokines (Akira et al., 2006; Aoshi, Koyama, Kobiyama, Akira, & Ishii, 2011; Carty et al., 2021; Kawai & Akira, 2006; Pichlmair & Reis e Sousa, 2007; Yoo et al., 2013). A visual diagram of viral recognition by the innate immune system is shown in figure 1.2.

Though the various types of vaccines are discussed in greater detail in the upcoming sections, a strong and long-lasting adaptive immunity can be achieved by an ideal vaccine, if that is capable of inducing an appropriate innate immune response. However, in some natural infections caused by virulent viruses, immune dysregulation can be initiated by the PAMPs. The best example of this immune dysregulation is the cytokine storm and proinflammatory condition induced in influenza and coronaviral infections. Typically, the attenuation of natural variants to make the attenuated vaccine, focused on reducing the pathogenicity, espicially capability of dysregulation of the host immune system. Thus, an appropriate immune response from an ideal vaccine can be achieved by exposing the host to PAMP's to induce optimal innate immunity, at the same time ensuring no hyperreactivity by the immune system and uncompromised safety by

the inadequate attenuation. These are great challenges for vaccine development and are the focus of the research described in this thesis.

Adaptive immunity to viral infection

It is also called the acquired immunity. Compared to innate immunity, adaptive immunity is specific and slower to be triggered for the 1st time encounter with a virus and is critical for viral clearance. T-cells and B-cells mainly mediate adaptive immunity, where T-cells confers the cell-mediated immunity and B-cells confers humoral immunity. After the internalization of the virus by antigen-presenting cells, the viral antigen is processed and presented to T cells at lymph nodes. The viral antigens are loaded on a type of membrane protein named the major histocompatibility complex (MHC) and presented on T-cells. There are two types of MHC molecules, MHC-I and MHC-II, depending on the type of cells (APC or infected cells) presenting the antigen. The viral antigens processed in the endosome of antigen presenting cells (APCs) and loaded on MHC-II and presented to helper $CD4^+$ T-Cell to activate $CD4^+$ T-cells. These helper $CD4^+$ T-cells can be subdivided into three types based on their function and interaction as Th1, Th2, and Th17 helper $CD4^+$ T-cells. Th1 interacts with $CD8^+$Tcells and activates them for phagocytosis of infected cells and also plays a role in B-cell activation. Th2 cells mainly interact with B-cells and activate B cells for clonal expansion. Plasma B cells secrete antibodies for protection and while memory B cells ensure future protection. Antibodies are also called immunoglobulins (IgG). There are mainly five classes, and these are IgG, IgM, IgD. IgA and IgE. IgM. IgG is mainly found in serum, and IgA is produced at the mucosal

surface. IgM, IgG, and IgA are mainly involved in viral immunity (Kindt, Goldsby, Osborne, & Kuby, 2007). Another type of helper $CD4^+$ T-cells, Th17 T-cells, mainly fights against fungal infections(Abbas et al., 2016; Chaudhry, 2014). T-cells consist of two major types named $CD8^+$ T-cells and $CD4^+$ T-cells. Antigen processed in infected cells is presented in the context of MHC-I molecules to $CD8^+$ T-cells or cytotoxic T-cells, which confers the cell-mediated immunity (CMI) (Abbas et al., 2016). Once activated, $CD8^+$ T-cells travel to the site of infection, recognize the infected cells, and trigger cell death (Chaudhry, 2014). Figure 1.3 shows the cellular component of adaptive immunity.

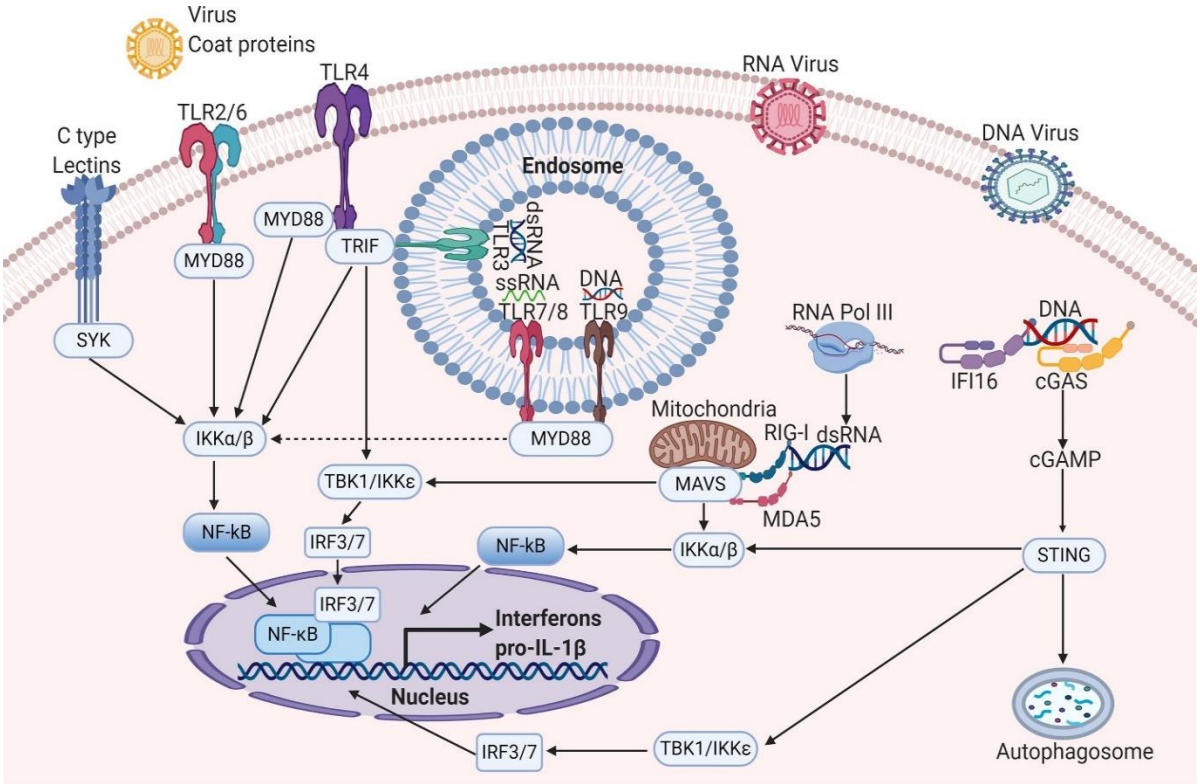

Figure 1.2: Sensing of viruses using different PRRs (Carty, Guy, & Bowie, 2021).
Source: Reprinted from Carty, M., C. Guy, and A.G. Bowie, *Detection of Viral Infections by Innate Immunity*. Biochem Pharmacol, 2021. 183: p. 114316, permission waived by the terms of the "Creative Commons CC-BY" license, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Vaccines can induce both humoral and cell-mediated immunity, like a natural viral infection. However, the rational design for an effective vaccine depends on the mechanisms of pathogenesis of the virus. Thus, to develop a rational, effective vaccine, the preferential targeting of a selected immune compartment is often critical and poses a significant challenge. Chapter 2 of this thesis addresses vaccine design to counter the effects of immunodominance in protein epitopes, while chapter 3 addresses rapid-attenuation strategy, which ensures minimal vaccine viral replication to achieve a balance between effective priming and safety, while chapter 4 described strategies to target effective gastric mucosal immunity for enteric viruses.

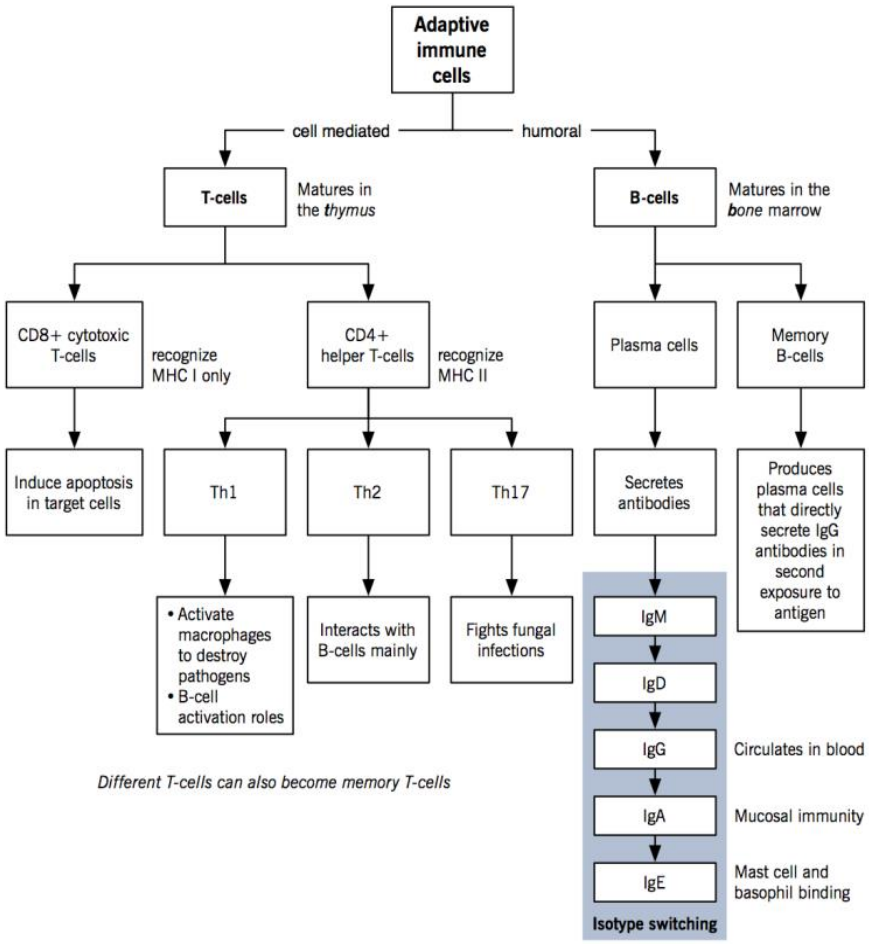

Figure 1.3: Cells of adaptive immunity. Adapted from Chaudhry et al. 2014 (Chaudhry, 2014)

Types of vaccines

In this thesis, different vaccination strategies will be discussed. Three vaccination strategies have been tested on animal and in-vitro models for their efficacy. Therefore a brief decription of different vaccine technologies has discussed below-

Inactivated vaccine

Inactivated vaccines contain the whole viral particle and are mainly killed versions of the virus. The virus may be killed by chemicals, heat, UV, or gamma radiation. Inactivated vaccines are safer than a live attenuated vaccines as the virus cannot replicate into the host. Thus it cannot

mutate or revert to virulent strains. However, disadvantages of the inactivated vaccines are that the antigenic components of the virus may get damaged by the inactivation process (Karch & Burkhard, 2016) and inactivated vaccines have a shorter duration of immunity and hence require periodical booster vaccinations (Pulendran & Ahmed, 2011). A few examples of inactivated vaccines are typhoid, cholera, plague, pertussis, polio, rabies, hepatitis A, etc. (Delany et al., 2014).

Live attenuated vaccine

Live attenuated vaccines are also whole viral particles and the weakened form of infective viruses so that they can't produce diseases but can induce immunity. Compared to inactivated vaccines, attenuated vaccines induce robust and lengthy immunity to the host (Pulendran & Ahmed, 2011; Tretyakova et al., 2013; Zou et al., 2018). The general procedure for attenuation requires series of passaging of the virus around 50-150 times in a non-definitive host. This process takes more than a year to obtain a completely attenuated vaccine strain. For the effectiveness of the attenuated vaccine, prolonged exposure of the viral antigen and antigen presentation in the host system by the attenuated vaccine replication is necessary as this closely resembled the natural infection. However, there is a risk of reversion of the vaccine virus to virulence, and thus, attenuated vaccines tend to pose a higher risk than inactivated vaccines (Nan et al., 2017; Shimizu et al., 2004). Furthermore, it has been found that the attenuated vaccines which remain in circulation can also recombine with the field strains and become more virulent (C. Wang et al., 2010; Wenhui et al., 2012). **A major focus of the research presented in this**

thesis is the development of rapid attenuation methods, which result in minimal vaccine viral replication and rapid clearance from the host, without compromising immune responses. Although there are some safety issues, it's widely used, and few examples of attenuated vaccines are smallpox, rabies, tuberculosis (BCG), yellow fever, oral polio vaccine (OPV), measle, rubella, etc (Delany et al., 2014). Genetic engineering can produce a modified live vaccine (MLV) by altering a particular epitope or deletion of the virulence-associated genes, also referred to as recombinant live vaccine. A majority of current commercial vaccines are either conventional attenuated vaccines or MLV's. While MLVs and attenuated vaccines are more effective than the inactivated vaccine, especially for the RNA virus, attenuation strategy, the time required to develop vaccine safety is critical. Most of the available vaccines are safe but not able to give protection at the desire threshold level, and are the major focus of the research described in this thesis.

Subunit vaccines

Subunit vaccines contain only the antigenic components of the virus instead of the whole virus. They are mainly protein antigens of the virus and can be produced by recombinant DNA technology. Subunit vaccines are gaining popularity as they are safe when compared to attenuated vaccines as they don't contain whole viruses, the risk of antigen denaturation is less than in the inactivated vaccine, and they are relatively easier to produce (Karch & Burkhard, 2016). However, the single viral antigen may not induce adequate protection. Additionally,

subunit vaccines require strong adjuvants to improve immunogenicity (Baxter, 2007; Moyle & Toth, 2013; Vartak & Sucheck, 2016).

Nucleic acid vaccine

Nucleic acid vaccines involve a transfer of the genetic code for selected viral antigens in plasmid DNA, or mRNA, or via viral replicons. The nucleic acids are then uptaken by cells to express the viral antigens and trigger humoral and cell-mediated immunity (Rauch, Jasny, Schmidt, & Petsch, 2018). Nucleic acid vaccines can be divided into DNA vaccines and mRNA vaccines.

DNA vaccines

DNA vaccines are mainly produced by large-scale bacterial plasmid production systems and purified to be used as a plasmid DNA vaccine (Josefsberg & Buckland, 2012). As the host cells will produce the antigens upon vaccination, the viral antigen will be in its native form7. Thus, these viral proteins can be uptaken by theAPCs, thus inducing both MHC class I and MHC class II T-cell responses, which will induce antibody responses (Michael James Francis, 2018). However, the main disadvantages of DNA vaccines are the low transfection efficiency in the host cellular system. There are chances of extracellular degradation of low abundance plasmids even before entering the cell. Even to start the mechanism of action, the plasmid needs to cross the barrier of the nuclear membrane (Rauch et al., 2018; Wallis, Shenton, & Carlisle, 2019; Zanta, Belguise-Valladier, & Behr, 1999). The main advantages are the ease and rapidity of production, and multiple antigens encoded plasmid can be designed and coupled with different

adjuvants as immunostimulatory factors (Suschak, Williams, & Schmaljohn, 2017; Wallis et al., 2019). However, several studies have shown that the plasmid DNA of the DNA vaccine tends to persist in the injection site for a long time (Rauch et al., 2018), sometimes more than two years (Armengol, Ruiz, & Orduz, 2004). Therefore, the FDA has a stringent regulation for the DNA vaccine for human use, and none of them have been approved yet, although few are under clinical trial time (Rauch et al., 2018). Thus, only veterinary DNA vacancie has been approved and licensed for commercial use (Michael James Francis, 2018; Wallis et al., 2019).

mRNA vaccines

Due to the above-mentioned issues of the DNA vaccines, mRNA vaccines were considered as an alternative technology. The instability of RNA and lack of strong immune responses were long-standing roadblocks to the development of RNA or mRNA vaccines for a long time (Pardi, Hogan, Porter, & Weissman, 2018; Wallis et al., 2019). Many experimental methods have been explored in the last several years to address these issues, and mRNA vaccines have gained popularity as a successful strategy in the current pandemic. Compared to the DNA vaccine, the mRNA vaccine doesn't require crossing the nuclear membrane to initiate the translation. There are mainly two types of mRNA vaccines named non-replicating mRNA and self-amplifying mRNA vaccines (Rauch et al., 2018). The non-replicating mRNA vaccines are the simple version of the mRNA vaccine, which contains a 5' cap and 3' UTR for translation. Therefore the non-replicating mRNA can not replicate in the host. On the other hand, in the self-amplifying mRNA, the alphavirus genome is reconstructed such that the structural proteins of the

alphavirus are replaced by the target vaccine antigens and viral polymerases mRNA production. So the self-amplifying mRNA can produce larger amounts of mRNA but can not produce the active alphavirus as the structural genes are deleted. Thus self-amplifying mRNA vaccines induce stronger immunity than non-replicating mRNA vaccines (Pardi et al., 2018; Rauch et al., 2018). mRNA vaccines can be prepared by direct cell-free in vitro enzymatic transcriptions from plasmid DNA. The plasmid DNA can be produced and purified from the *E.coli* system and then linearized enzymatically to make the template for in vitro transcription using phage-dependent RNA polymerase. Finally, the mRNA is purified using HPLC to be used as a vaccine, and this purification step is crucial for the vaccine yield (Pardi et al., 2018; Rauch et al., 2018). A brief diagram showing the mode of action of DNA and mRNA vaccine is shown in figure 1.4. However, like the DNA and subunit vaccines, the mRNA vaccines also involve immunization with selected viral antigens. Still, undoubtedly these are the enormous technological advancement for improving the ease of production and viral antigen administration.

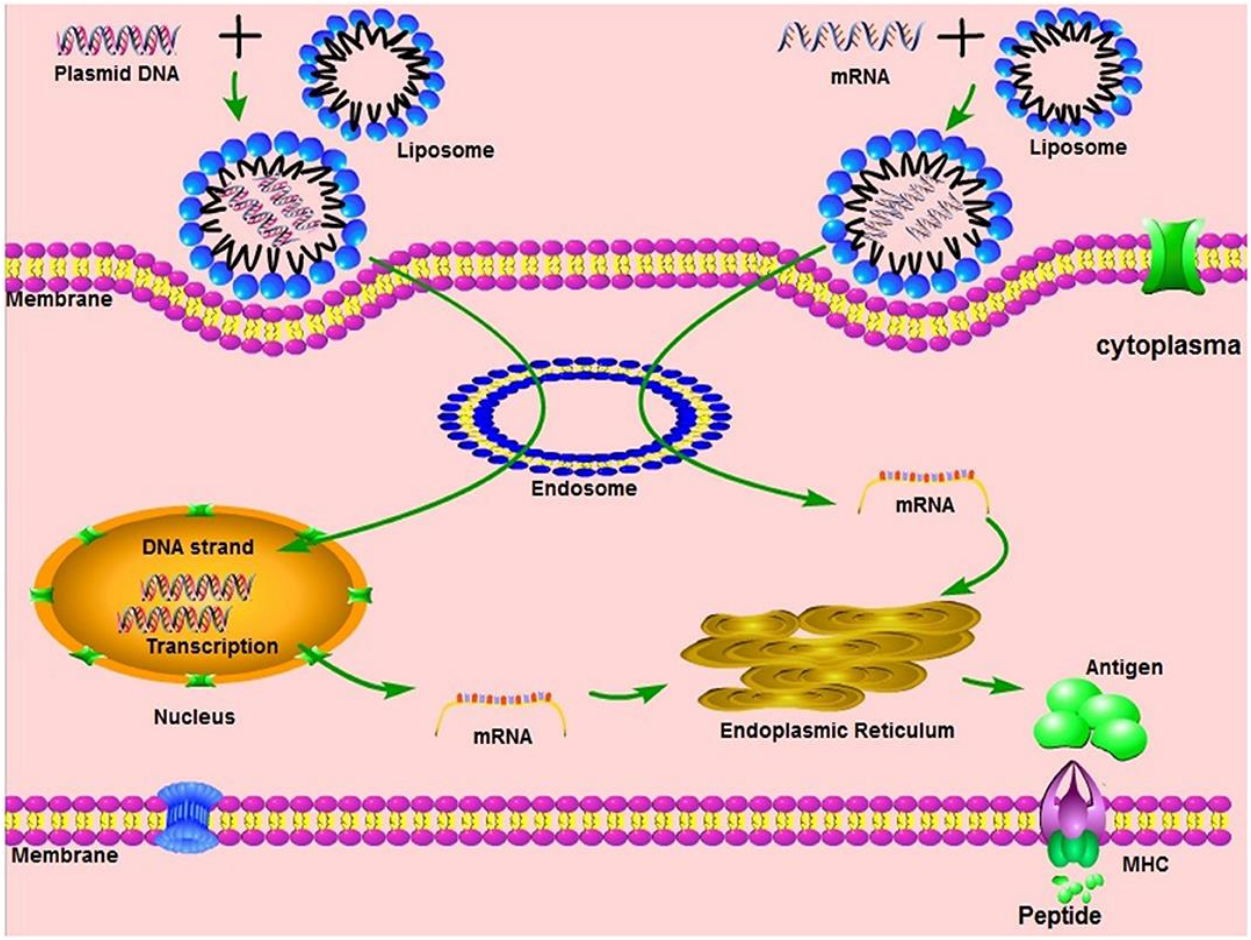

Figure 1.4: Mode of action of the nucleic acid vaccine (C. Zhang, Maruggi, Shan, & Li, 2019). **Source:** reprinted from Zhang, C., Maruggi, G., Shan, H., & Li, J. (2019). Advances in mRNA Vaccines for Infectious Diseases. *Front Immunol*, *10*, 594. doi:10.3389/fimmu.2019.00594 . Permission waived by the terms of the "Creative Commons CC-BY" license, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Vectored vaccines

An alternate strategy is to use an unrelated virus to express target protective antigens in the host. The virus vectors are usually weakened and genetically modified so they cannot cause disease. Weakened adenovirus, vaccinia and canary pox vectors are commonly used as vectors for human vaccines (Callaway, 2020), and consist of two types: non-replicating and replicating

viral vaccines. Replicating viral vectors can replicate into the host system and to produce the target antigen for the duration of replication (weakened measles viral vector). On the other hand, the non-replicating viral vector cannot replicate in the host system (adenovirus viral vector) (Callaway, 2020; Rauch et al., 2018). The oxford university and AstraZeneca based covid-19 vaccine is based on the adenoviral vector.

Vaccine delivery systems

Although the main focus of the vaccine development is aiming for the protective and immunogenic properties of the viral antigens to inducing the pathogen-specific immune response, the vaccine delivery platforms and adjuvant used in the formulation can have a significant effect on the quality and magnitude of the immune response, especially for peptides encoding specific immunogenic epitopes. Nanoparticle-based delivery systems significantly improve the efficacy of vaccines (Pati et al., 2018). Moreover, nanoparticles can be formulated to activate specific immune pathways, boost antigen processing, and eventually enhance the vaccine's overall immunogenicity (Zhao et al., 2014). Processes like conjugation, adsorption, entrapment, or admixture can be used to target antigens to various biological locations by incorporating targeting ligands (figure 1.5). Common materials used for nanoparticle formulation include lipids, inorganic compounds, polymers, virus-like particles (VLP), immunostimulating complexes (ISCOM), etc. (Pati et al., 2018; Zhao et al., 2014). Nanoparticle formulations can be used to supplement target strategies for the development of the vaccine (figure 1.6).

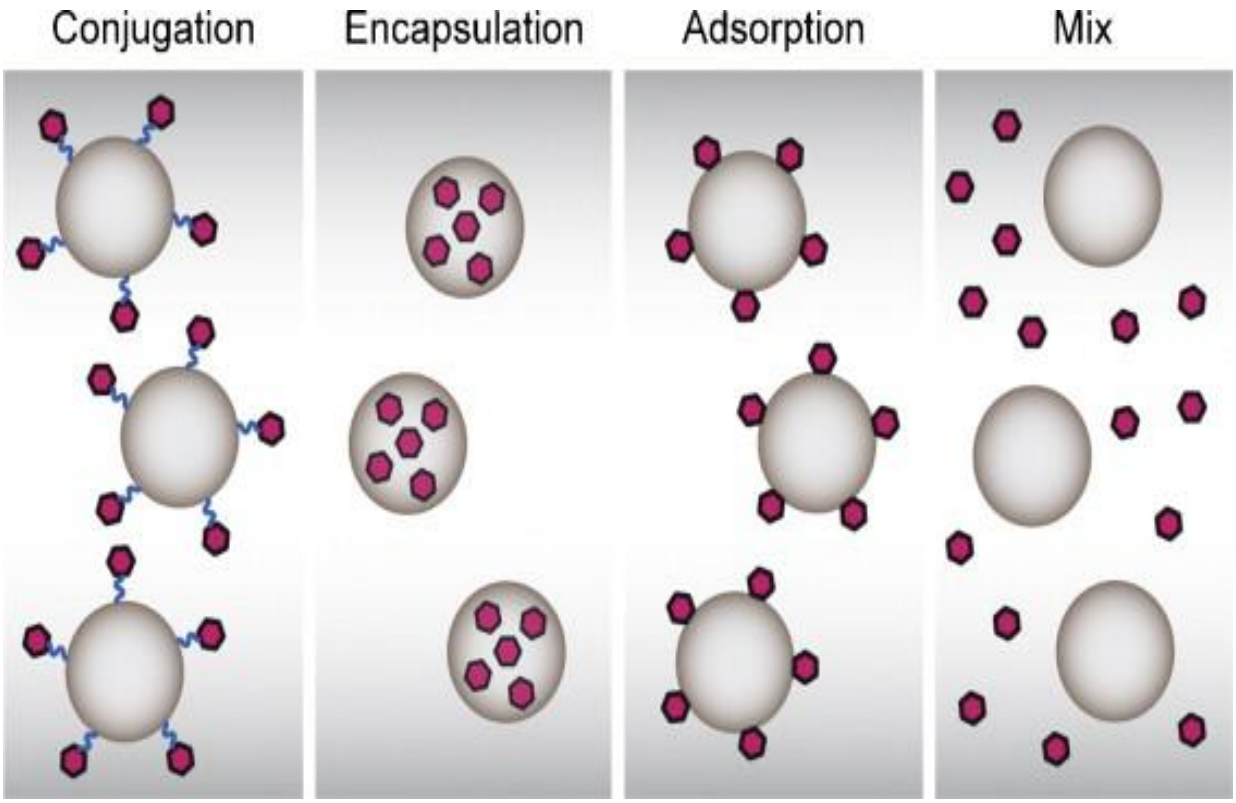

Figure 1.5: Interaction of viral antigen with nanoparticle (Zhao et al., 2014).
Source: Reprinted from Zhao, L., Seth, A., Wibowo, N., Zhao, C.-X., Mitter, N., Yu, C., & Middelberg, A. P. (2014). Nanoparticle vaccines. *Vaccine*, *32*(3), 327-337, permission taken from the copyright clearance center and elsevier publisher.

In order to achive the goals of this dissertation, which are targeted modification of the immunodominance patterns (chapter 1) to develop novel and broadly appliable strategies for rapid attenuation of viruses, and achiehiving minimal vaccine viral replication (chapters 2 and 3), we have used two economicallly important swine viral apthogens, porcine circovirus type 2 (PCV2) and porcine epidemic diarrhea virus (PEDV) as model organisms and provide context for the research conducted.

Antigen	Nanocarrier used	Disease
AGAINST BACTERIAL INFECTION		
Antigenic protein	Poly(D,L-lactic-co-glycolic acid) nanospheres	Anthrax
DNA encoding T cell epitopes of Esat-6 and FL	Chitosan Nanoparticle	Tuberculosis
Mycobacterium lipids	Chitosan Nanoparticle	Tuberculosis
Polysaccharides	Liposomes	Pneumonia
Bacterial toxic and parasitic protein	Liposomes	Cholera and Malaria
Fusion protein	Liposomes	*Helicobacter pylori* infection
Antigenic protein	Nanoemulsion	Cystic fibrosis
Antigenic protein	Nanoemulsion	Anthrax
Mycobacterium fusion protein	Liposome	Tuberculosis
AGAINST VIRAL INFECTION		
Antigenic protein	Chitosan Nanoparticles	Hepatitis B
Viral protein	Gold Nanoparticles	Foot and mouth disease
Membrane protein	Gold Nanoparticles	Influenza
Viral plasmid DNA	Gold Nanoparticles	HIV
Tetanus toxoid	Poly(D,L-lactic-co-glycolic acid) nanospheres	Tetanus
Hepatitis B surface antigen	Poly(D,L-lactic-co-glycolic acid) nanospheres	Hepatitis B
Hepatitis B surface antigen	Alginate coated chitosan Nanoparticle	Hepatitis B
Live virus vaccine	Chitosan Nanoparticles	Newcastle disease
Capsid protein	VLPs	Norwalk virus infection
Capsid protein	VLPs	Norwalk virus infection
Influenza virus structural protein	VLPs	Influenza
Nucleocapsid protein	VLPs	Hepatitis
Fusion protein	VLPs	Human papilloma virus
Multiple proteins	VLPs	Rotavirus
Virus proteins	VLPs	Blue tongue virus
Enveloped single protein	VLPs	HIV
Viral protein	Polypeptide Nanoparticles	Corona virus for Severe acute respiratory syndrome (SARS)
AGAINST PARASITIC INFECTION		
Merozoite surface protein	Iron oxide Nanoparticles	Malaria
Epitope of *Plasmodium berghei* circumsporozoite protein.	Polypeptide Nanoparticles	Rhodent mamarial parasitic infection
Surface protein from *Eimeria falciformis* sporozoites	ISCOMs	Diarrhea

Figure 1.6: List of antigens used nanoparticles for delivery (Pati, Shevtsov, & Sonawane, 2018)
Source: Adapted from Pati, R., Shevtsov, M., & Sonawane, A. (2018). Nanoparticle vaccines against infectious diseases. *Frontiers in immunology*, *9*, 2224, Permission waived by the terms of the "Creative Commons CC-BY" license, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Porcine circovirus type 2

Porcine circovirus type 2 is a small DNA virus, approximately 17nm diameter in size with a 1.8 kb (figure 1.8) single-stranded, circular DNA genome (Tischer, Gelderblom, Vettermann, & Koch, 1982). It was first identified as a causative agent for the post-weaning multisystemic wasting syndrome (PMWS) (Allan et al., 1998; Ellis et al., 1998; Meehan et al., 1998). Subsequently, PCV2 was associated with several other disease manifestations, including porcine dermatitis and nephropathy syndrome (PDNS), porcine respiratory diseases complex (PRDC), reproductive failure, and enteric disease, collective known by the term porcine circovirus-associated diseases (PCVAD) (Hu et al., 2017; Kim, Chung, & Chae, 2003; Opriessnig, Meng, & Halbur, 2007). PCV2 is an icosahedral, non-enveloped virus with a T1 symmetry. It is composed of just one structural protein, of which 60 subunits assemble to form the capsid (figure 1.7)(Khayat et al., 2011). Major open reading frames of PCV2 are ORF1 and ORF2, flanking the origin of replication. ORF1 encodes replicase, and ORF2 encodes the capsid protein (Lekcharoensuk et al., 2004; Nawagitgul et al., 2000; Shang et al., 2009). PCV2 forms a unique model for epitope based vaccine studies as the 203 amino acid long protein is necessary and sufficient for protection. It is also the target for serological diagnostic assays (Nathan M Beach & Meng, 2012; Blanchard et al., 2003; Huang, Lu, Wei, Guo, & Liu, 2011; Sun et al., 2010).

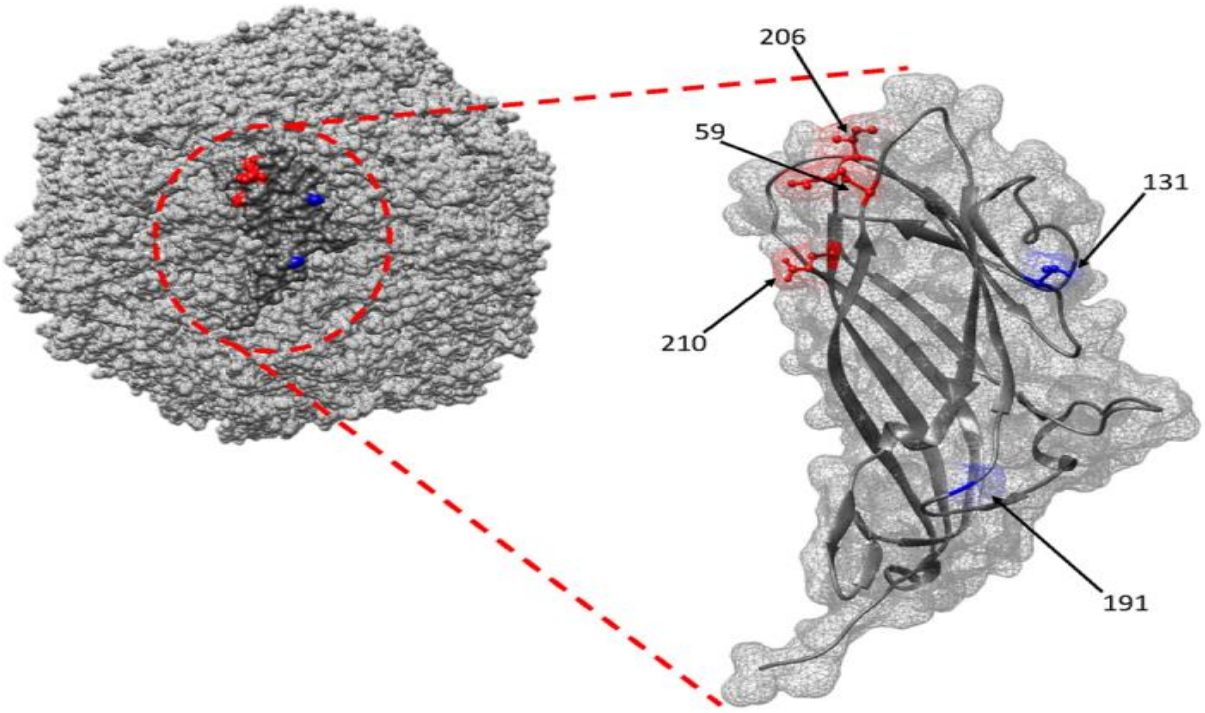

Figure 1.7: Quaternary and tertiary structure of the PCV2 capsid protein (Franzo, Tucciarone, Cecchinato, & Drigo, 2016).
Source: Permission waived by the terms of the "Creative Commons CC-BY" license, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

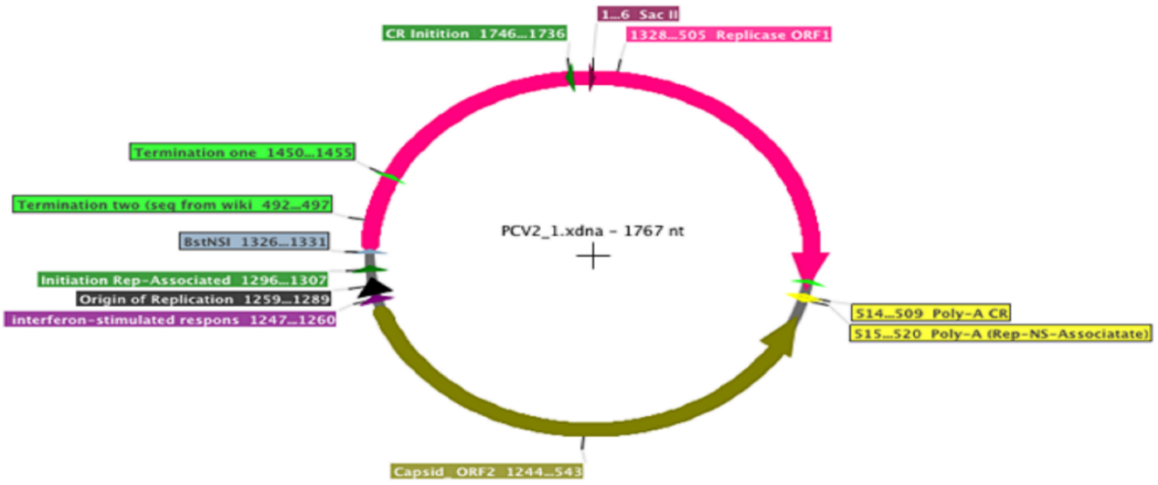

Figure 1.8: Genomic Organization of PCV2

The commercial vaccines for PCV2 were introduced in the US in 2006. They are highly effective in controlling the clinical signs in production pigs and eventually reducing he economic burden because of PCV2 infection. These are mainly subunit or inactivated vaccines based on the PCV2a (Z. Afghah, B. Webb, X.-J. Meng, & S. Ramamoorthy, 2017). However, the PCV2a was replaced by subtype PCV2b within few years. The new subtype showed more severe PCVAD when it was initially identified in the field. While PCV2a vaccines provide significant cross-protections against PCV2b clinically, this can't prevent the replication of transmission of PCV2a or PCV2b in the infected herds (Nathan M Beach & Meng, 2012; Opriessnig et al., 2013). These observations suggest that current PCV2 vaccines exert selection pressure on the virus and may influence viral evolution. This observation is further validated by replacing PCV2b with the PCV2d in the following few years. Multiple PCV2 subtypes 2a, 2b, 2c, 2d, 2e, 2f have been identified and co-circulating in production swine herds (Bao et al., 2018; López et al., 2018). Recently, two more new PCV types, PCV3, and PCV4 have been identified by metagenomic sequencing methods, and PCV3 has been spread worldwide quickly (Nguyen et al., 2020; Ouyang et al., 2019; Palinski et al., 2017; H. H. Zhang et al., 2020). Viral evolution from the vaccine immune pressure is not new, and previously identified for other viruses like canine parvoviruses (Zhou, Zeng, Zhang, & Li, 2017), marek's disease virus (Read et al., 2015), influenza virus (Escorcia et al., 2008; C.-W. Lee, Senne, & Suarez, 2004; Su et al., 2020), and rotavirus (Dóró et al., 2014; Patton, 2012). Therefore, the current definitions of an effective vaccine and its measures solely on protecting against clinical infection are questionable and need

to be changed. To achieve eventual eradication of the viral diseases, an effective vaccine with sensible efficacy and strategies to gain sterilizing immunity is essential, which can trim the viral evolution. In this research we have targeted two strategies; A) diminish of the immunodominance hierarchy to prevent immune subversion by the virus, which in turn would result in effective priming coupled with clearance of the attenuated vaccine B) development of minimally replicative attenuated vaccines with the objective of raising the threshold of protection compared to currently available inactivated vaccines.

Immunodominance as a confounding factor in vaccine development

Although whole protein is required as an antigen to elicit good vaccine-mediated protection by the B or T cell response, in practice, the antigen contains several epitopes (only 8-12 amino acid long peptide) that engages with T and B cell to include the adaptive immunity (Sanchez-Trincado, Gomez-Perosanz, & Reche, 2017). Each epitope may differ in its specific physical properties and how it engages with the immune system**.** For many viruses, including PCV2, a strong antibody response can be detected at 7-10 days of infection of the host (Ilha et al., 2020; Yoon et al., 1995). However, this early response does not protect the host from the virus and the development of disease. The neutralizing antibody is generally detected at the late stage of the infection, around 21-28 days of infection to host, by the time the virus established the disease in the host (ROMAN M POGRANICHNYY et al., 2000). Therefore, the early antibody responses towards the immunodominant epitopes are usually non-protective, while the

delayed antibody responses towards the subdominant epitopes are protective and of lesser magnitude. Thus, effectively subverting the host immune response.

Immunodominance is a kind of immune response by the host, where the host immune system prefers to recognize only a few antigenic peptides, while MHC class II co-expressed thousands of distinct antigenic peptides (Akram & Inman, 2012). Furthermore, the decoy epitopes are generally adjacent to the neutralizing epitopes and can interfere spatially with B cell stimulation and binding of the neutralizing antibody. This elegant mechanism is also known as deceptive imprinting and constitutes a significant concern for developing an effective vaccine (Nara, 1998; Nara & Garrity, 1998; Tobin et al., 2008).

At least four linear immunodominant regions have been identified within the capsid protein of PCV2 (Lekcharoensuk et al., 2004; Dominique Mahe et al., 2000; Shang et al., 2009) and are located at the amino acid position as indicated by boxes in figure 1.10 (Ilha et al., 2020). Our lab has identified three decoy epitopes within these immunodominant regions (Ilha et al., 2020). These decoy epitopes or non-neutralizing epitopes could decrease the induction or activity of antibodies against an adjacent neutralizing epitope (Thaa, Sinhadri, Tielesch, Krause, & Veit, 2013). Additionally, we have shown that vaccinated pigs tend to produce antibodies towards the decoy epitopes, especially towards the decoy epitopes 166-181 position (Ilha et al., 2020; Worsfold et al., 2015). Changing this unwanted immunodominance pattern may improve the quality of antibody binding response that can effectively eliminate the vaccine virus from the system with uncompromising immune response. To check this premise, we have mutated the

identified decoy epitopes and successfully tested the efficacy of our rationally designed PCV2 capsid antigen as attenuated modified live vaccine in pigs (Chapter 2). The approach used in this research to quickly track the immunodominant, decoy B cell epitopes and then rationally reconstruct vaccine antigen. This rationally reconstructed vaccine antigen has a broad applicability in improving the vaccine efficacy for those that can prevent clinical signs but not the viral evolution and for those viruses that are traditionally vaccine-resistant.

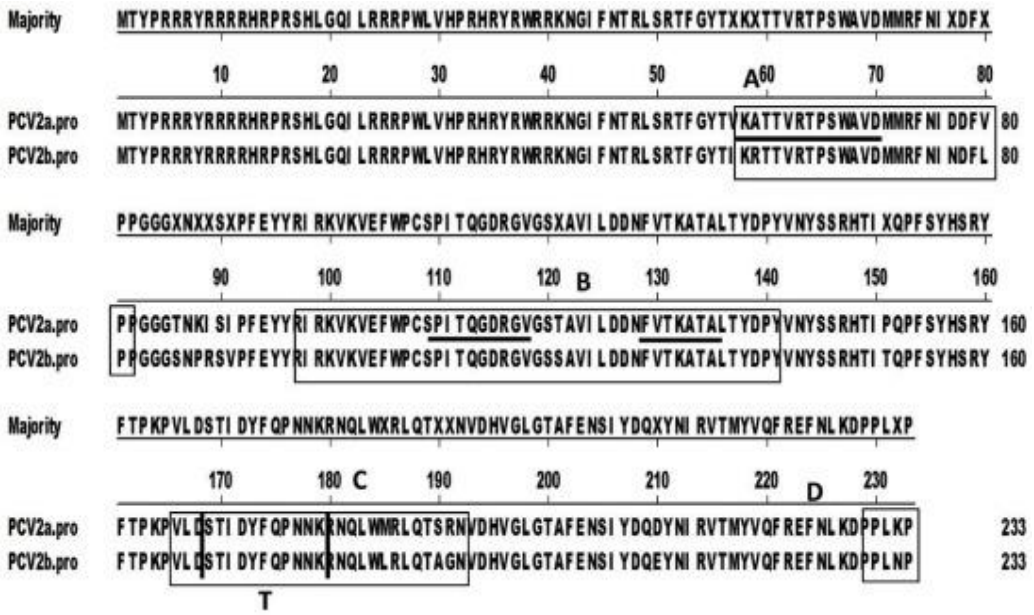

Figure 1.9: Immunodominant regions of PCV2 capsid protein (Ilha, Nara, & Ramamoorthy, 2020).
Source: Adapted from Ilha, M., Nara, P., & Ramamoorthy, S. (2020). Early antibody responses map to non-protective, PCV2 capsid protein epitopes. *Virology, 540*, 23-29, permission taken from the copyright clearance center and elsevier publisher.

Attenuation of rapidly mutating viruses by directed suicidal replication

Although vaccine efficacy is the essential parameter in assessing the success of a vaccine development approach, vaccine safety is a paramount factor. Re-direction of the immune

response described in chapter 2 is a highly effective strategy; however, an additional safety feature to minimize the viral vaccine replication without compromising the immune response would ensure the safety of the modified live PCV2 vaccine with a great prospect in the field. High mutation rates in RNA viruses are attributed to the poor proofreading ability of the viral RNA polymerases. Mutational robustness is a phenomenon by which a virus can bring a genetic variation while maintaining the same phenotype by balancing tolerating the deleterious mutations and benefiting from beneficial mutations (Montville, Froissart, Remold, Tenaillon, & Turner, 2005). One of the biggest problems of vaccinology is the high genetic and antigenic variability characteristic of these rapidly mutating viruses. Moreover, although live vaccines induce a more robust immune response than other vaccines, reversion to virulence may happen from the MLV due to mutation. In some cases, both recombination with the field strain and reversion to the original virulent strain have been reported for some MLVs (Nan et al., 2017; C. Wang et al., 2010; Wenhui et al., 2012). In general, RNA viruses have a higher mutation rate compared to DNA virus. However, a DNA virus, PCV2, and other single-stranded DNA viruses have mutation rates that are similar to RNA viruses (Sanjuán, 2010). While the mechanism of the high mutation rate of small ssDNA virus is not wholly understood, this high mutation rate is a big concern for the vaccine safety of MLV as well as causing the evolution of new strains from both wild-type virus as well as MLVs. Hence, we have taken advantage of this high mutation rate to develop a unique vaccine development strategy. Therefore, to attenuate the PCV2 for developing the modified live vaccine, we have re-write all the serine and leucine amino acids in

a manner so that a single mutation of the codons would increases the chances of generating a stop signal and abortive replication (in vivo suicidal replication) of the vaccine virus in vaccinated pigs. While testing the suicidal PCV2-MLV in weanling piglet vaccination and challenge, it confirms the vaccine safety as it rapidly cleared from the vaccinated pigs without compromising the protection against the challenge virus, as described in details in chapter 3. This strategy targeting suicidal replication of live viral vaccines can be advantageous in situations where MLVs are effective. Still, safety is a concern, and for viruses for which reverse genetics systems are available.

In addition to adequate protection and safety, eradication of the virus is an ultimate goal of vaccination. In veterinary medicine, eradication is achieved by mass vaccination with vaccines which are enabled to differentiate the infected and vaccinated animals (DIVA). The PCV2 vaccines described in chapters 2 and 3, also engineered to express a foreign epitope to check the aim that only vaccinated pigs will have antibodies against this epitope, not the naturally infected pigs. Thus, the vaccinated and infected pigs can be differentiated by using a serological method, and the infected pigs can be removed by a systemic vaccination program. Therefore, the developed vaccine can drive the way for the eventual eradication of the PCV2 in the swineherd and significantly improve the swine health.

Rapid-response, orally delivered vaccines

Although highly effective, both of the previously described vaccine development strategies require that a reliable reverse genetics system is available for the virus in question, that

the virus is well characterized genetically and antigenically, and that the essential and non-essential genes are identified. However, in the case newly emerging viruses which can spread rampantly in naïve populations, the development and characterization of infectious clones can be challenging and long winded, especially for complex RNA viruses. Rapid-response vaccines and diagnostics are critical and indispensable components of a pandemic preparedness plan. However, traditional methods of virus attenuation can take months to years to develop an effective vaccine to pass the different trials and get licensed to be used. In the case of the pandemic situation, this delay of vaccine availability causes the huge spread of the virus and causes severe damage to the host population (Jennings, Monto, Chan, Szucs, & Nicholson, 2008; Noah & Fidas, 2000; Smith, Lipsitch, & Almond, 2011).Despite the glaring need, there are very few established platforms for rapid-attenuation which are broadly applicable to emerging viruses.

Porcine epidemic diarrhea virus (PEDV) is a positive-sense single-stranded RNA virus and a member of the genus alphacoronavirus in family coronaviridae and order Nidovirales (Gerdts & Zakhartchouk, 2017; Jung & Saif, 2015). PEDV has a genome size of around 28kb, with a 5'-cap and 3'-polyA tail (figure 1.11)(Pensaert & De Bouck, 1978). PEDV causes very high mortality in suckling piglets of up to 80-100% (T. Schwartz, Rademacher, Gimenez-Lirola, Sun, & Zimmerman, 2015; D. Song & Park, 2012). Although PEDV was reported in Europe and Asia about 30 years ago, it first arrived in the US in 2013 and swept through the production swine population, causing widespread economic losses (Jung, Saif, & Wang, 2020). The previous finding on coronavirus showed that envelope protein is reversibly denatured when

subjected to gently heating (Y. Wang et al., 2004). Using this information our lab has found that heating the PEDV at 44˚C for 10 mins, followed by exposure to a cocktail of RNAse's treatment, results in fragmentation of the genome without compromising the structure, especially that of the immunogenic spike protein (G. Singh et al., 2019). With a different extent of the heat and RNAse treatment, we have found out that rapid inactivation or rapid-attenuation can be achieved without compromising the immunogenicity. The heat and RNase treated vaccine showed a very high level of efficacy when tested in weanling piglets. The vaccine was also minimally replicative as it was rapidly cleared from vaccinated pigs and hence was very safe (G. Singh et al., 2019). However, PEDV is mainly devastating for the suckling piglets, whose immune system is not mature. Thus, the protection needs to become from a maternal antibody from milk, which can be achieved by inducing lactogenic immunity and can protect the piglets from the deadly virus. Therefore, an effective PEDV vaccine should be effective in inducing lactogenic immunity in pregnant sows characterized by secretory IgA antibodies in milk (Crouch et al., 2000; Langel, Paim, Lager, Vlasova, & Saif, 2016). Although the oral route of vaccination is most effective for PEDV (Bohl, Gupta, Olquin, & Saif, 1972; Chattha, Roth, & Saif, 2015), current PEDV vaccines are inactive vaccines and administrated by intramuscular. Thus, lactogenic immunity is not strong enough to give full protection from the deadly virus, and currently, it's only 40-60% (Won, Lim, Noh, Yoon, & Yoo, 2020).

Oral vaccines have several advantages for gastrointestinal infections and would be a preferred route of administration for emergency vaccines since they are needle-free. However,

oral vaccines have to face an adverse gastrointestinal tract environment and the very prone to degradation, especially for the inactivated vaccines. Consequently, in chapter 4, in vitro characterization of a novel vaccine delivery system has performed. This is completely biodegradable, non-toxic, and designed to protect the heat and Rnase treated PEDV vaccine from the gastric environment by encapsulating and delivering the vaccine into the enterocyte.

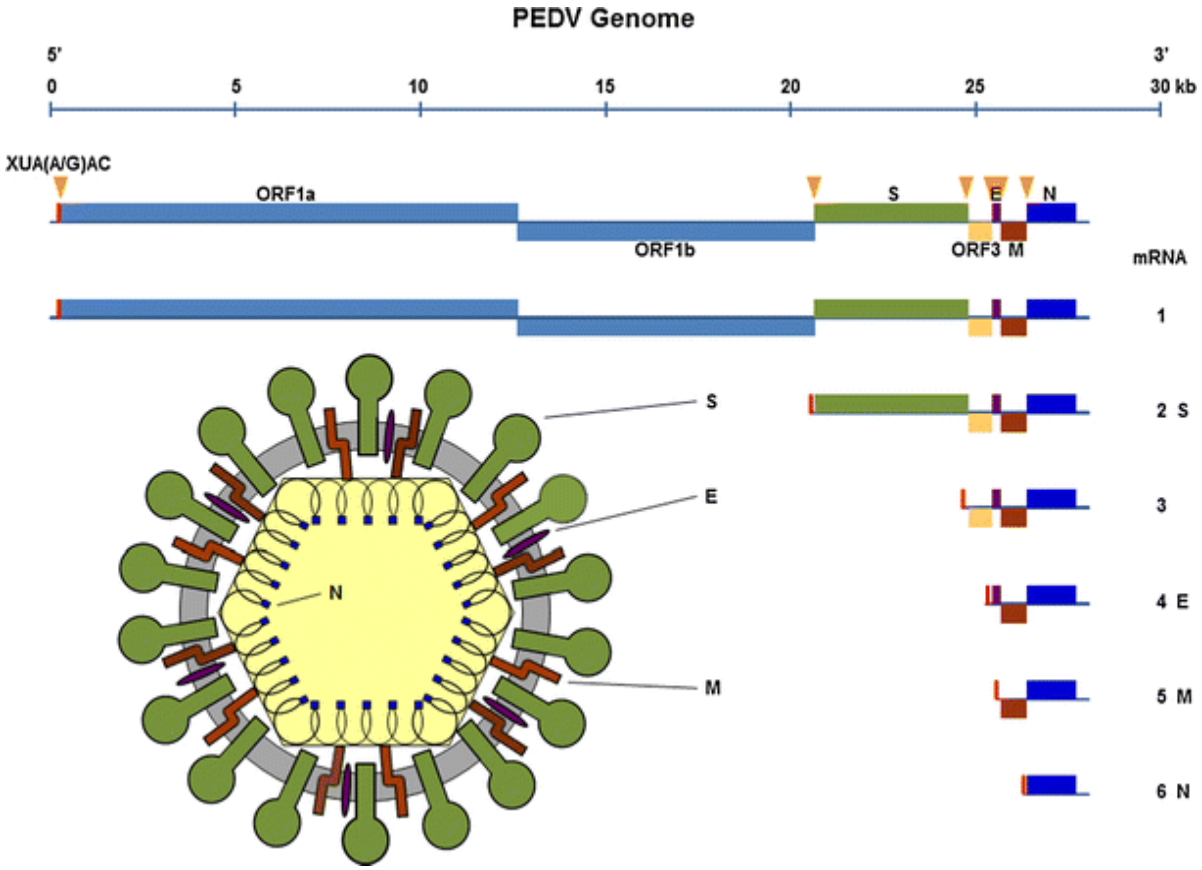

Figure 1.10: Schematic representation of PEDV genome and virus (D. Song & Park, 2012). **Source:** Reprinted from Song, D., & Park, B. (2012). Porcine epidemic diarrhoea virus: a comprehensive review of molecular epidemiology, diagnosis, and vaccines. *Virus genes, 44*(2), 167-175 permission taken from the copyright clearance center and springer nature.

Finally, a critical need in vaccinology has been addressed in this thesis, which is the need for improved technologies for the rapid attenuation of viruses for vaccine development.

Additionally, by which vaccines can achieve both efficacy and safety while being an effective vaccine candidate. Data presented in chapter 2 about refocusing the immune response from immunodominance decoy epitope to protective antigens can be used to achieve the above-mentioned objectives. At the same time, the data of chapter 3, besides the efficacy, shows a proof of concept about a novel codon recoding strategy to result in rapid attenuation of the vaccine virus by in vivo suicidal replication, which enhances the vaccine safety. Chapter 4 was based on previously established platforms for epidemic vaccine development by targeting novel oral vaccine delivery technology, which can potentially enhance lactogenic immunity for neonatal protection in long term goal. Thus, the objectives of this dissertation are:

Objectives of dissertation

Objective I

Improve the efficacy of PCV2 vaccines by refocusing the immune response towards protective epitopes of the capsid protein in a live attenuated PCV2 vaccine

Hypothesis I

Mutation of selected decoy epitopes will diminish the non-protective immunodominant antibody response and improve vaccine efficacy

Objective II

Improve the safety of the live attenuated PCV2 vaccine by directed suicidal replication

Hypothesis II

Recoding of leucine and serine codons to increase the chances of accumulating stop mutation during vaccine viral replication will attenuate PCV2.

Objective III

To develop a quick oral vaccine delivery system for rapid response vaccine, to deliver at enterocyte with improved antigen loading capacity.

Hypothesis III

A niosome formulation composed of edible lipids, cholesterol, and the charge stabilizer, the viral antigen will be packaged effectively and deliver vaccine antigen to enterocytes.

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CHAPTER 2: TARGETED ALTERATION OF ANTIBODY-BASED IMMUNODOMINANCE ENHANCES THE HETEROSUBTYPIC IMMUNITY OF AN EXPERIMENTAL PCV2 VACCINE[1]

Abstract

Despite the availability of commercial vaccines which can effectively prevent clinical signs, porcine circovirus type 2 (PCV2) continues to remain an economically important swine virus, as strain drift, followed by displacement of new subtypes, occurs periodically. We had previously determined that the early antibody responses to the PCV2 capsid protein in infected pigs map to immunodominant but non-protective, linear B cell epitopes. In this study, two of the previously identified immunodominant epitopes were mutated in the backbone of a PCV2b infectious clone, to rationally restructure the immunogenic capsid protein. The rescued virus was used to immunize 3-week-old weanling piglets, followed by challenge with a virulent heterologous PCV2d strain. As expected, immunodominant antibody responses to the targeted epitopes were abrogated in vaccinated pigs, while a broadening of the virus neutralization responses was detected. Vaccinated pigs were completely protected against challenge viral

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replication, had reduced microscopic lesions in lymphoid organs and gained significantly more body weight when compared to unvaccinated pigs. Thus, the experimental PCV2 vaccine developed was highly effective against challenge, and, if adopted commercially, can potentially slow down or eliminate new strain creation.

Keywords: vaccine; porcine circovirus; PCV2; decoy epitope; antibody; virus neutralization; mutation

Introduction

Porcine circovirus type 2 (PCV2) is a small, single-stranded DNA virus which belongs to the *circoviridae* family. It is an economically important swine virus which causes post-weaning multi-systemic wasting syndrome (PMWS) and lymphadenopathy in weanling piglets, along with a range of clinical signs including jaundice, nephropathy, reproductive and respiratory disorders, collectively known as porcine circovirus associated diseases or PCVAD (Afghah, Webb, Meng, & Ramamoorthy, 2017). Several commercial vaccines against PCV2 are available and commonly deployed in the field. They are very effective in preventing clinical signs of PCV2 and in reducing economic losses associated with PCV2 infection. However, they do not prevent transmission or shedding of PCV2. Most of the commercial vaccines continue to target the first discovered PCV2 subtype: PCV2a. Commercial vaccines contain either the whole inactivated virus, inactivated chimeric PCV1-2a virus preparations or subunits of the PCV2a capsid protein. After the introduction of commercial vaccines, the initially predominating field subtype, designated PCV2a, was replaced by PCV2b and, more recently, by PCV2d. While

serological cross-reactivity between the subtypes and cross-protection due to vaccination is observed, it is possible that selection pressure induced by immunity developed against commercial vaccines could be driving viral evolution in the field (Afghah et al., 2017; Karuppannan & Opriessnig, 2017; Ssemadaali, Ilha, & Ramamoorthy, 2015).

The approximately 1700bp PCV2 genome encodes just two major proteins: the replicase and capsid proteins. The capsid protein is considered to be both necessary and sufficient for the prevention of PCV2, as the subunit vaccination with the capsid protein alone is effective at preventing the clinical signs of PCVAD. While the cell mediated immune response to PCV2 is not well studied, neutralizing antibody responses targeted to the capsid protein are considered to be critical for protection against PCV2 (Afghah et al., 2017; Karuppannan & Opriessnig, 2017). Strong binding Ab responses to PCV2 can be detected as early as 7 days post-infection in naturally or experimentally infected pigs. However, neutralizing Ab responses, which correlate with a reduction in viremia, are not detected until later in the course of infection (Pogranichnyy et al., 2000). Immuno-dominance, the phenomenon by which the immune system preferentially mounts responses to selected antigens, or epitopes within antigens, is an effective immuno-subversion mechanism for pathogens, and a well-established confounding factor in the development of effective vaccines (Nara, 1999). The delayed production of neutralizing Ab responses, coupled with the periodical emergence of new PCV2 subtypes following vaccination suggests that antibody based immunodominance plays an important role in PCV2 pathogenesis and vaccine mediated protection. Currently, PCV2 vaccines are extensively deployed in pork

production units. Based on diagnostic case submissions, it is estimated that a large percentage of production pigs also harbor the virus (Afghah et al., 2017). It has been suggested that lack of vaccine compliance due to improper storage or administration, and not a lack of cross-protection, is responsible for the shift in genotypes (Dvorak, Yang, Haley, Sharma, & Murtaugh, 2016). Therefore, the availability of tools to monitor vaccine compliance in the field can advance the control of PCV2 significantly.

In a previous study, we had explored the hypothesis that the early Ab responses in PCV2 infected pigs would be directed towards non-protective epitopes in the PCV2 capsid protein. Using sequential anti-sera collected from infected pigs, and a panel of overlapping peptides spanning the PCV2 capsid protein, we identified three new linear immunodominant—but non-protective—regions of the PCV2 capsid protein (Ilha, Nara, & Ramamoorthy, 2020). We also confirmed the presence of a previously identified immuno-dominant decoy epitope (Ilha et al., 2020; Trible et al., 2011; Trible et al., 2012). In addition, we found that a majority of the Abs produced by vaccination mapped to the non-protective immunodominant epitopes identified in the study. Hence, the primary objective of this study was to introduce mutations in two of the previously identified non-protective epitopes to alter immunodominance patterns and evaluate the performance of the modified recombinant virus as a vaccine. The secondary objective of this study was to develop a marker vaccine against PCV2 by introducing an immunogenic foreign peptide in the vaccine construct, to enable monitoring of vaccine compliance.

Vaccination of pigs with the restructured PCV2b vaccine (rPCV2-Vac) encoding a marker, and challenge with the currently predominating heterologous PCV2d strain resulted in improved heterosubtypic virus neutralization responses, protection against tissue pathology, lack of viremia due to the challenge virus, improved weight gain and Ab responses specific to the marker. The strategy described in this manuscript provides insights into the mechanisms of vaccine-mediated protection against PCV2 with long-term implications for improving the control and prevention of PCV2.

Materials and methods

Cells and viruses

The PCV1 free porcine kidney cell line, PK-15N (005-TDV, National Veterinary Services Laboratory, Ames, IA, USA), was used to culture all PCV2 strains. An infectious clone of PCV2b strain 41513 (GenBank accession number KR816332) was used as the backbone to develop the restructured PCV2 vaccine. An infectious clone of a heterologous PCV2d strain (GenBank accession number JX535296.1) was used to prepare the challenge virus (Kolyvushko, Rakibuzzaman, Pillatzki, Webb, & Ramamoorthy, 2019). For virus neutralization assays, PCV2a (AF264042.1), PCV2b (EU340258.1) and PCV2d (JX535296.1) infectious clones were used to generate virus stocks by transfection as described below.

Cloning of the vaccine construct

Using the infectious clone of PCV2b 41,513 as the backbone, two previously identified linear immuno-dominant, but non-protective epitopes in the immunogenic PCV2 capsid protein

(Ilha et al., 2020; Trible et al., 2011) were mutated. The capsid gene segment encoding the desired mutations was commercially synthesized and cloned into the backbone of PCV2b 41,513 by restriction digestion. To minimize the risk of producing a lethal mutation, selected amino acids in the linear decoy epitopes were replaced with other amino acids with a low penalty score on a point accepted mutation (PAM) matrix (Schwartz & Dayhoff, 1979); Epitope A124 ILDDNFVTKATALTYDPY 141 (Ilha et al., 2020) was modified to 124 ILDDNFVNKSTALTYDPY 141 and epitope B166 VLDSTIDYFQPNNKR 180 (Trible et al., 2011) was modified to 166 VLDSTIDYFNPNNSR 180 (Table S1, Figures S1 and S2). The replacement of a threonine (T) with an asparagine (N) residue in epitope A resulted in the introduction of a putative N-linked glycosylation sequon (NxS) (Table S1). Epitope B naturally contained a predicted N-linked glycosylation site (Table S1) and was not altered for glycosylation properties. All mutations were validated by sequencing (Eurofin Genomic, Louisville, KY, USA). The vaccine construct is henceforth referred to as the re-structured PCV2 vaccine (rPCV2-Vac) throughout the manuscript.

Insertion of a marker to enable the monitoring of vaccine compliance

To enable the monitoring of vaccine compliance using a serological assay, the vaccine construct was tagged with an immunogenic marker. *Neospora caninum* is an apicomplexan parasite which has not been detected in pigs (Donahoe, Lindsay, Krockenberger, Phalen, & Slapeta, 2015). A highly immunogenic segment of 18 amino acid length selected from the surface antigen-1 related sequence 2 (SRS2) protein (AAD04844.1) of *N. caninum* was selected

following the in-silico prediction of antigenicity (Lasergene 11, Protean 13, DNASTAR, Madison, WI, USA) (Figure 2.1). The selected sequence was subjected to a protein blast to rule out possible serological cross reactivity with other swine related proteins. Amino acids 324 QSSEKRDGEQVNKGKPP 348 of the SRS2 protein, with an antigenicity index score of 1.7 (Figure 2.1), was inserted into 5′ end of the capsid gene of the rPCV2-Vac construct described above, as a separate transcriptional unit (Figure S2), using the Q5 mutagenesis kit (New England Biologicals, Ipswich, MA, USA), according to the manufacturer's instructions.

Preparation of PCV2 virus cultures

The vaccine and challenge virus cultures, as well as the virus cultures required for the virus neutralization assay, were prepared by transfection of PK-15 cells (Fenaux, Opriessnig, Halbur, & Meng, 2003), with some modifications. Briefly, the PCV2 genome was excised from the shuttle plasmid by restriction digestion and re-circularized with DNA ligase, unless dimerized infectious clones were available. For transfection,12 µg of viral genomic DNA or plasmids containing the dimerized infectious clones (Fenaux et al., 2002; Kolyvushko et al., 2019) were diluted in Opti-MEM, mixed with 36 µL of TransIT-2020 (Mirus Bio, Madison, WI, USA), and incubated at room temperature for 30 min. After the incubation period, the mixture was overlaid on cell culture flasks (25 cm^2, Corning, Tewksbury, MA, USA) containing 50% confluent monolayers of PK-15 cells and incubated at 37 °C in a CO_2 incubator for 3h, followed by addition of Dulbecco's Modified Eagle's Medium (DMEM) with 2% fetal bovine serum and 1X penicillin-streptomycin. The flasks were frozen and thawed three times after 72 h of

incubation. The rescued viruses were titrated by the ^{TCID50} method. The stock cultures were stored at −80 °C until used.

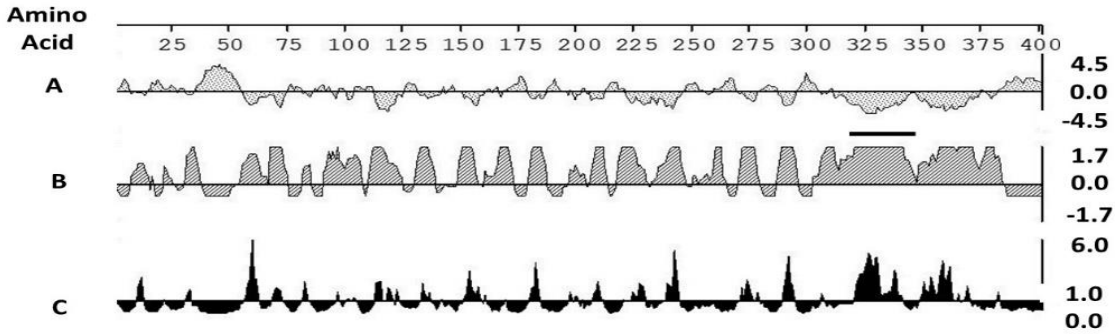

Figure 2.1: Selection of an immunogenic marker.
Evaluation of the antigenicity of the *Neospora caninum* SRS2 protein by: A. Kyle Doolittle hydropathy plot—a negative value indicates hydrophilic residues; B. Jameson Wolfe antigenicity index; C. Emini surface probability plot. B and C: the height of the vertical bar is proportional to the predicted antigenicity of the sequence. A positive value indicates higher immunogenicity. Horizontal bar: amino acid positions in the sequence selected for analysis. Solid dark line: peptide sequence 324 QSSEKRDGEQVNKGKPP 348 selected as the marker in the vaccine construct.

Immunofluorescence assay

As PCV2 does not produce cytopathic effects, replication of the PCV2 strains was visualized by an IFA as previously described (Fenaux et al., 2003). Briefly, 50% confluent PK-15 monolayers grown in eight well chamber slides were either transfected as described above or infected with the virus cultures. After 72 h of incubation in a CO_2 incubator, the cells were fixed with a 1:1 mixture of methanol and acetone. The fixed cell sheets were stained with a PCV2 specific monoclonal antibody (Rural Technologies, USA) or *Neospora caninum* specific polyclonal antibody, followed by detection with a FITC-conjugated secondary antibody (KPL, SeraCare, Milford, MA, USA), and counter-staining with DAPI (Life Technologies, Carlsbad,

CA, USA). The stained cells were evaluated for apple green nuclear fluorescence indicative of PCV2 replication or expression of the SRS2 marker tag.

In-vitro vaccine stability

The rPCV2-Vac cultures rescued by transfection of PK-15 cells were serially passaged three times in PK-15 cells. Virus titers were compared against the wildtype virus. The construct was sequenced to verify the stability of the mutations.

Vaccination and challenge of piglets

All procedures pertaining to animal experimentation were carried out with the approval and oversight of the Institutional Animal Care and Use Committee (IACUC) and Institutional Biosafety Committee (IBC) regulations of N. Dakota (NDSU) and S. Dakota State Universities (SDSU). Twenty-seven 3–4-week-old piglets, which were serologically and PCR negative for PCV2 and other major swine pathogens, such as PRRSV, SIV and *Mycoplasma sp*., were divided into three groups of 9 pigs each. Group I was administered PBS, group II were administered a commercial, inactivated PCV2 vaccine as per label instructions (2 mL, intramuscular), and group III were inoculated with the rPCV2-Vac at $10^4 TCID_{50}$/mL, 2 mL intramuscular and 2 mL intranasally. Although the exact details regarding the antigen dose and formulation of the commercial vaccine are not publicly available, a commercial vaccine was selected as a control to represent current industry standards. The vaccine used consisted of a PCV1-2a chimeric virus, wherein the PCV2a capsid gene was cloned in the backbone of the non-pathogenic PCV1 (Fenaux, Opriessnig, Halbur, Elvinger, & Meng, 2004), followed by inactivation and formulated

with squalene as an adjuvant (Fostera ® PCV MetaStim ®, Zoetis, Inc, Parsippany, NJ, USA). Vaccinated pigs were boosted with the same dose and route on day 14 post-vaccination (DPV). On DPV 28 post or day 0 post-challenge (DPC), all study animals were challenged with a heterologous PCV2d strain at 10^4TCID$_{50}$, 2 mL intramuscular and 2 mL intranasally. Two pigs per group were sacrificed prior to challenge to assess vaccine safety. Pigs were monitored daily for signs of porcine circovirus associated diseases (PCVAD), such as wasting, respiratory distress, jaundice, inappetence or diarrhea. Body weights were assessed on DPC 0, 9 and 21 (Figure S3). Serum samples were collected on day 0, and every 2 weeks thereafter to assess Ab responses. All animals were humanely euthanized on DPC 21 for evaluation of pathological lesions as described below.

Anti-PCV2 IgG responses

The measurement of binding IgG responses to PCV2 in vaccinated pigs was achieved with a commercial PCV2 ELISA kit (Ingezim Circovirus IgG kit, Ingenasa, Madrid, Spain), at the Iowa State University Veterinary Diagnostic Laboratory, following their standard operating procedures and the manufacturer's instructions. Sample to positive control (S/P) ratios produced as the assay output were used for further analysis of the data.

Virus neutralizing antibody responses

Functional antibody responses against the homologous PCV2b subtype and heterologous PCV2a and PCV2d subtypes were measured by a rapid fluorescence focus neutralization (FFN) assay, essentially as described before (Ilha et al., 2020), except that the virus cultures were

adjusted to 30–40 fluorescent focus units (FFU)/100 µL for consistent enumeration. Virus replication was assessed by an IFA, as described above. Four replicate values of the DPV 28 sera were obtained and used for analysis. The titers were expressed as the percentage reduction in viral replication compared to the virus only control, which was not treated with serum.

Antibody responses to the mutated epitopes

The abrogation of the immunodominant Ab response to the selected epitopes in vaccinated pigs was assessed by surface plasmon resonance on a Reichert SR7500DC instrument (Reichert Technologies, Buffalo, NY, USA). Biotinylated peptides encoding the wildtype peptide sequences of epitopes A and B described above were commercially synthesized (Biomatik, Wilmington, DE, USA). Pooled sera collected at DPV 28 from the three treatment groups and from archived sera collected from PCV2b infected pigs (Beach, Ramamoorthy, Opriessnig, Wu, & Meng, 2010) (provided by X. J. Meng, Virginia Tech, Blacksburg, VA, USA) were used to purify IgG using a commercial kit (Melon gel IgG purification kit, Thermo Fisher, Waltham, MA, USA). The biotinylated peptides were immobilized on streptavidin coated carboxymethyl dextran sensor chips (Reichert Technologies, USA) by injecting 0.16 µg/µL peptide solution over the sensor chip at a flow rate of 25 µL/min. After an increase of about 300 µRU was observed, indicating immobilization of each peptide had occurred, the purified IgGs for the experimental groups were injected over the flow cells at a concentration of 20 µM in phosphate buffered saline with 0.005% Tween 20 (PBST), at a flow rate of 25 µL/min for 240 s. Binding of the IgGs to the peptides was assessed by the response in µ response units (µRU).

Antibody responses to the marker

The selected peptide from the N. caninum SRS2 protein was cloned into a bacterial expression vector (pETSumo Thermo Fisher Scientific, USA) using the Q5 site directed mutagenesis kit (New England Biologicals, Ipswich, MA, USA). The protein was expressed with a HIS tag and purified with by nickel affinity chromatography (His-spin protein miniprep, Zymo research, Irvine, CA, USA), following the manufacturer's instructions. The identity of the purified protein was verified by Western blotting with an anti-HIS tag specific monoclonal Ab (Figure S3). The purified protein was used to coat ELISA plates, followed by washing with PBST and blocking (General block with 2% BSA, Immuno Chemistry Technologies, Bloomington, MN, USA) for 2 h at 37 °C. The blocked plates were washed with PBST. A 1:50 dilution of the test anti-sera was diluted in PBS with 2% BSA, added to the wells, and incubated for 2 h. The plates were then reacted with a 1:5000 dilution of anti-swine IgG conjugated to HPO (KPL, SeraCare, Milford, MA, USA), followed by addition of TMB substrate. The reaction was stopped with 1M HCl and plate was read at 450 nm in an ELISA plate reader.

Measurement of vaccine viral replication by qPCR

Replication of the rPCV2-Vac virus following immunization was quantified by a TaqMan quantitative PCR (qPCR), using a SRS2 marker specific primer and probe combination and serum collected on DPV 0.14 and 28. Samples were assessed in duplicate. Viral DNA was extracted using the QiaAmp DNA mini Kit (Qiagen, Valencia, CA, USA) according to manufacturer's instruction. Primer pairs with sequences of

5′-AAGTGGGAGGTTTGCCTTTGT-3′ and 5′-ATGGCCCAATCCTCGGAGAA-3′ and a probe with a sequence of 5′-TACCTGTTCCCCGTCGCGT-3′ were used. Briefly, 2.0 µL of extracted DNA, 0.4 µM of primers, 0.1 µM probe and a Tm of 67 °C were used in combination with the QuantiFast Probe PCR Kit (Qiagen, USA) and cycled in a qPCR thermocycler (CFX96 Touch, Bio-Rad, Hercules, CA, USA). The obtained Ct values were converted to log copy numbers using a standard curve generated with plasmid DNA encoding the SRS2 peptide marker. The specificity of the assay was evaluated using the infectious clones for the wildtype PCV2b and heterologous PCV2a and PCV2d. The lowest limit of detection of the assay was 2000 genomic copies per mL of serum.

Detection of challenge viral replication

A qPCR assay specific to the PCV2d subtype was designed after analysis of PCV2a, PCV2b and PCV2d sequences to identify regions unique to PCV2d (Figure S1). The sequences of the primers used were 5′-GGCCTACATGGTCTACATTTCCAGT-3′ and 5′-GGTACTTTACCCCGAAACCTGTC-3′, and the probe sequence was 5′-TGGGTTGGAAGTAATCGATTGTCCTATCA-3′ (Biosearch Technologies, Novato, CA, USA). The specificity of the assay for PCV2d was evaluated by testing for the absence of detection with PCV2a and PCV2b. A standard curve was generated using cloned PCV2d genomic DNA and the lowest limit of reliable detection determined as 3000 genomic copies per mL of serum. To quantify the challenge virus loads in serum, post-challenge sera collected at DPC 9 and DPC 21 were assessed essentially as described above.

Assessment of pathological lesions

Evaluation of tissue pathology was carried out essentially as described previously (Kolyvushko et al., 2019). Macroscopic evaluation of the major organs for gross lesions in the major organs was conducted by assessing lungs for the presence of lesions scored as the percentage of lung parenchyma affected from 1%–100%. Inguinal lymph node enlargement was scored from 0–3, where 0 was no enlargement, 1, 2 and 3 were two, three or four times the normal size. Sections of the major organs including the lung, liver, kidney, spleen ileum, tonsils, tracheobronchial and mesenteric lymph nodes were fixed in 10% buffered formalin for 48 h and then transferred to 70% ethanol for sectioning. Slides were examined by hematoxylin and eosin (H&E) staining for microscopic lesions and immunohistochemistry (IHC) to detect viral antigen, following the standard operating procedures of the Iowa State University Veterinary Diagnostic Laboratory. The slides were assigned scores ranging from 1–4 in a blinded fashion by a board-certified veterinary pathologist as follows; 1 = single follicle or focus staining, 2 = rare to scattered staining, 3 = moderate staining, 4 = strong widespread staining.

Statistical analysis

A significance level of $p < 0.05$ was used for all statistical analysis. Analysis was conducted using the Minitab19 software (Minitab, State College, PA, USA) or Microsoft excel. Where data were not normally distributed, non-parametric analysis was used. Serological and qPCR data were analyzed by a Student's t test. The lesion scores and body weight data were

analyzed by the Mann–Whitney U test. The consolidated values, statistical significance and standard deviation are represented in the figures.

Results

The rPCV2-Vac was successfully rescued and expressed the marker peptide

The reverse genetics approaches used to mutate the selected immunodominant linear B cell epitopes in the PCV2 capsid protein (Ilha et al., 2020) enabled the successful rescue of the recombinant rPCV2-Vac virus (Figure 2.2A). Introduction of the mutations did not affect detection of the recombinant PCV2 virus by polyclonal antibodies. Expression of the marker peptide was clearly detected by a *Neospora caninum* specific antibody (Figure 2.2B).

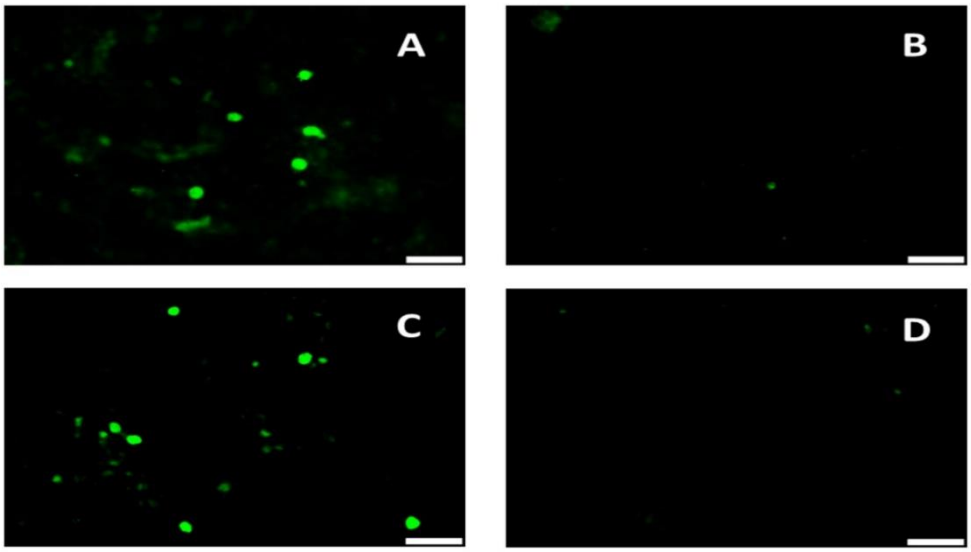

Figure 2.2: Rescue of the recombinant rPCV2-Vac.
(A) PK-15 cells transfected with the rPCV2-Vac construct and stained with a PCV2 specific polyclonal antibody (C) PK-15 cells transfected with the rPCV2-Vac construct and stained with a Neospora caninum specific polyclonal antibody. (B) and (D) Un-transfected PK-15 cells stained with the PCV2 or Neospora caninum antibodies respectively. Apple green florescence is indicative of a specific signal. White bar–Scale of 50 µM.

The rPCV2-Vac Induces Binding Antibody Responses in Vaccinated Pigs

Measurement of anti-PCV2 IgG responses in the study animals using a commercial PCV2 ELISA kit showed an increase in titers after 14 DPV in both the vaccine groups, with the differences between rPCV2-Vac and unvaccinated control group being significantly different at DPV 28 and DPC 09. Although a direct comparison between rPCV2-Vac and the commercial control cannot be drawn due to differences in vaccine formulation, the magnitude of the IgG response to the commercial vaccine remained consistently higher than that of the rPCV2-Vac. Antibody responses in the unvaccinated controls remained low until DPC 9, after which significant differences were not noted between the groups at DPC 21 (Figure 2.3).

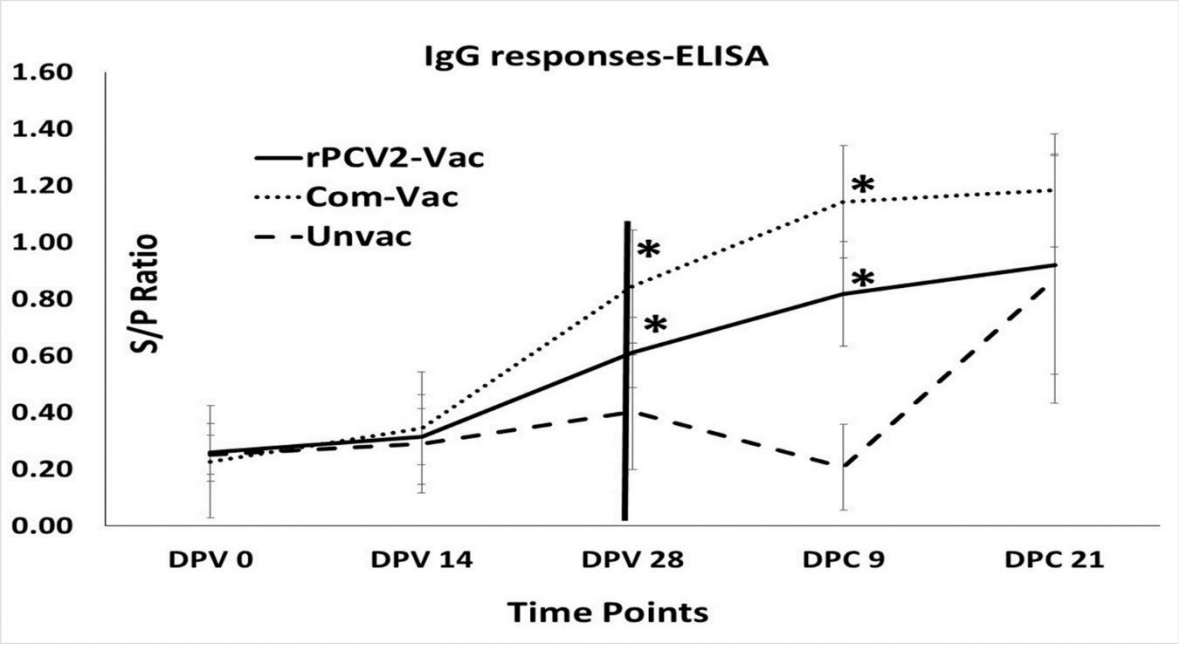

Figure 2.3: Anti-PCV2 IgG responses.
Mean signal to positive (S/P) ratios of sera collected on days 0, 14 and 28 post vaccination (DPV) and on days 9- and 21-days post-challenge (DPC), as measured by a PCV2 specific commercial ELISA. X axis: time points of serum collection, Y axis: sample to positive (S/P) ratio, dotted line: commercial vaccine, solid line: rPCV2-Vac, dashed line: unvaccinated. Error bars indicate the standard deviation, * significantly different from the unvaccinated control, $p \leq 0.05$, Students t test. Significant differences were not detected between the rPCV2-Vac and commercial vaccine.

The rPCV2-Vac elicits broad virus neutralization responses

Virus neutralizing responses were measured against the homologous PCV2b subtype, as well as heterologous PCV2a and PCV2d subtypes, using a rapid fluorescence focus reduction assay. Despite the fact that the commercial vaccine has an adjuvant and has undergone extensive dose optimization, neutralization responses elicited by the rPCV2-Vac against the PCV2a subtype were comparable in kinetics and magnitude to that of the commercial vaccine, which contains the PCV2a capsid antigen. Similarly, neutralizing responses against the currently predominant PCV2d subtype in the rPCV2-Vac group were higher than that of commercial

vaccine by DPV14, with the difference becoming statistically significant at DPV28. Neutralizing responses elicited by the rPCV2-Vac against its homologous PCV2b strain were robust. However, the commercial vaccine was significantly less effective than rPCV2-Vac in neutralizing PCV2b (Figure 2.4).

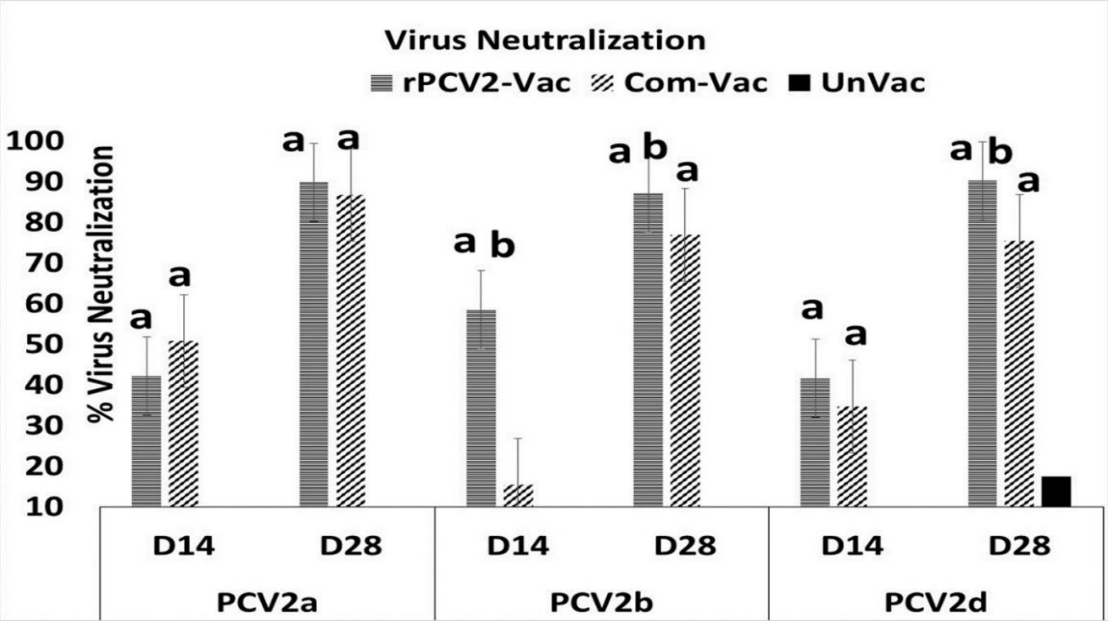

Figure 2.4: Virus neutralization responses.
Mean virus neutralization titers measured by a rapid fluorescent focus reduction assay. Values for days 14 and 28 post vaccination (DPV) are depicted. No significant activity was detected in the sera of the unvaccinated control group or at day 0. X axis-virus neutralization titers against PCV2a, PCV2b or PCV2d. Y axis–% virus neutralization, horizontal lines: rPCV2-Vac, slanted lines: commercial vaccine, solid bar: unvaccinated. Error bars indicate the standard deviation, a-significantly different from the unvaccinated control, b-significantly different from the commercial vaccine group, $p \leq 0.05$, Students t test.

Surface plasmon resonance (SPR) analysis of epitope binding

Antibody responses to epitope A and B were not detected in the serum of rPCV2-Vac immunized pigs by a qualitative SPR analysis, while the responses in pigs infected with the wildtype virus were strong. For epitope A, the response in pigs administered the rPCV2-Vac was

similar to that of the unvaccinated pigs. The response in the pigs administered the commercial vaccine was of a lesser magnitude than that of the pigs infected with the wildtype virus. In the case of epitope B, strong responses were noted pigs infected with the wildtype virus, but the differences between the other three groups were not significant (Figure 2.5).

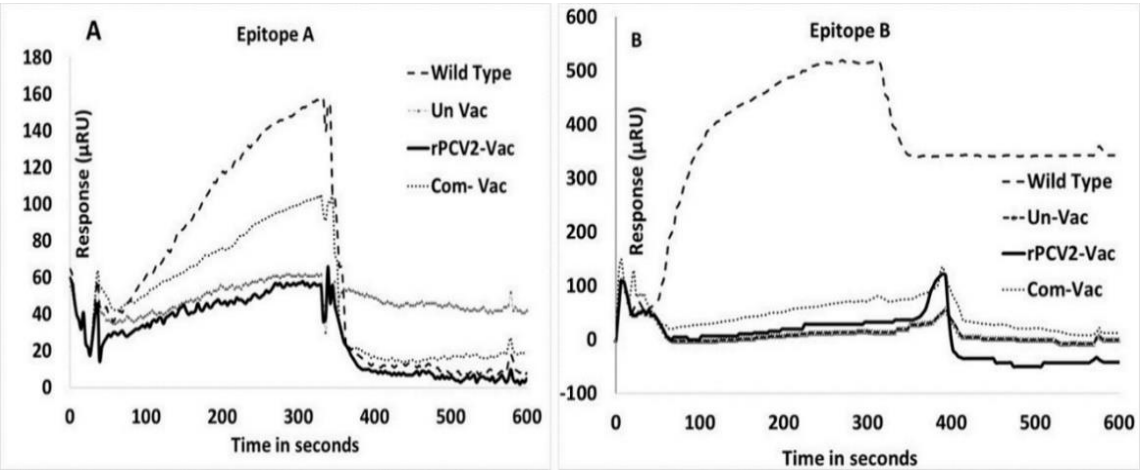

Figure 2.5: Antibody responses to the mutated epitopes.
Loss of immunodominant effects due to mutation of epitopes (A) and (B) as assessed qualitatively by surface plasmon resonance. 20 µM of purified IgG was tested for all experimental antisera. X axis—Time in seconds, Y axis—Response measured in µRU (µ response units). (A). Responses to a peptide encoding the wildtype epitope (A,B). Responses to a peptide encoding wildtype epitope (B). Slashed line-anti-serum to the wildtype virus, dotted line: anti-serum to the commercial vaccine, solid line-anti-serum to the rPCV2-Vac, connected triangles: anti-serum from the unvaccinated group.

Measurement of the marker specific ab responses

Assessment of the antibody responses to the marker by an ELISA specific to the peptide selected from the N. caninum SRS2 protein showed that pigs vaccinated with the rPCV2-Vac mounted detectable Abs responses to the marker by DPV14, with the responses becoming significantly different from not only the unvaccinated control group but also the commercial

vaccine by DPV 28. The unvaccinated pigs and pigs administered the commercial vaccine did not mount significant antibody responses to the marker (Figure 2.6).

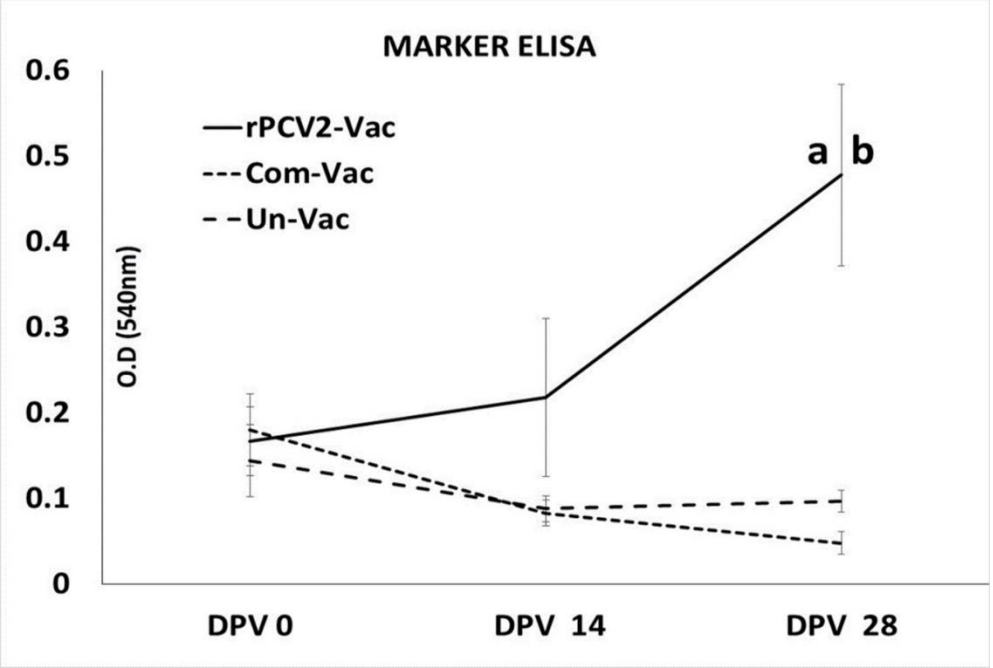

Figure 2.6: Antibody responses to the marker.
Mean optical density values of sera collected on days 0, 14 and 28 post vaccination (DPV) (N = 9), as measured by an ELISA specific to an antigenic peptide selected from the Neospora caninum SRS2 protein. X axis: time points of serum collection, Y axis: mean optical density (O.D) value, solid line: rPCV2-Vac, dotted line: commercial vaccine, dashed line: unvaccinated group. Error bars indicate the standard deviation, a: significantly different from the unvaccinated control, b: significantly different from the commercial vaccine group; $p \leq 0.05$; Students t test.

Vaccination Protects Against Challenge Viral Replication:

Replication of the heterologous PCV2d challenge virus was not detected in the sera either of the vaccine groups at DPC 9 or DPC 21. Robust challenge viral replication was detected in the unvaccinated pigs, with the viral titers increasing by about 1 log between day 9 and day 21 post-challenge. The values for both vaccine groups were significantly different from the unvaccinated control group at both the time points tested (Figure 2.7).

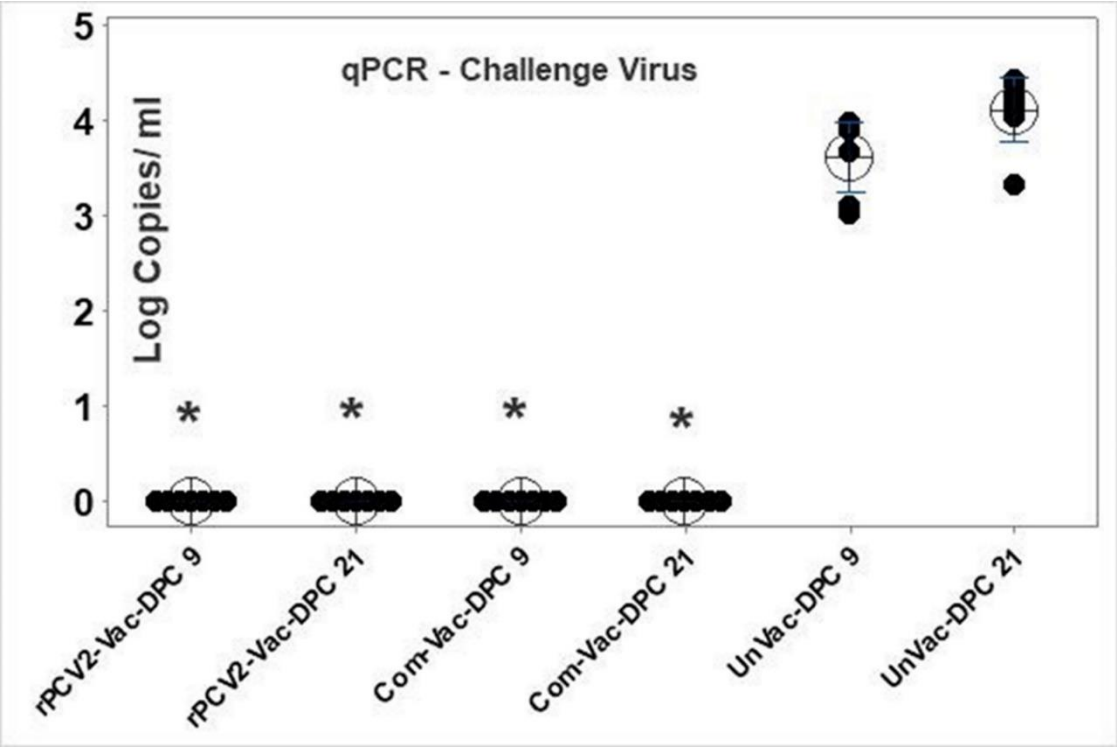

Figure 2.7: Replication of the challenge virus.
Quantification of the heterologous PCV2d challenge virus at 9 and 21 days post challenge by a PCV2d subtype specific qPCR. X axis: experimental groups and time of serum collection, Y axis: log10 viral copy numbers per mL of serum. Interval bars: 95% confidence interval of the means. * Significantly different from the unvaccinated control group; $p \leq 0.05$; Students t test.

In contrast to wildtype PCV2 viruses, which can be easily detected by qPCR by DPC 9 (Figure 2.7), viremia due to the rPCV2-Vac virus was not detected by the SRS2 tag-specific qPCR assay in the sera of any of the vaccinated pigs at DPV14. The rPCV2-Vac virus was detected at low levels in the serum of only one out of nine pigs at DPV 28, indicating that the rPCV2-Vac was attenuated in vivo. Sequencing of the rPCV2-Vac genome from the viremic pig confirmed the presence of the mutations in the two epitopes and the presence of the SRS2 marker, indicating the vaccine remained stable in the host. Similarly, sequencing of the rPCV2-

Vac genome after three passages in cell culture showed that the mutated and inserted sequences were intact, suggesting that the vaccine was genetically stable in vitro.

Protection against gross and histological lesions

Except for the lungs, gross lesions were not observed in any of the other major organs for all experimentally challenged pigs. For the lymph nodes, the microscopic lesion scores (consisting of the sum of the H&E and IHC scores), were significantly lower for the rPCV2-Vac group than those of the commercial vaccine group and the unvaccinated group (Figure 2. 8A) with only two out of seven pigs showed mild changes, while six of seven the pigs in the control groups showed histiocytic infiltration and lymphoid depletion. Microscopic lesions were not detected in the spleen (Figure 2.8B) liver and heart. The microscopic lesion scores of the ileum and tonsils (Figure 2.8C,D) of the rPCV2-Vac group were also significantly lower than that of the control groups. The pulmonary lesion scores in the rPCV2-Vac group were lower than that of the controls but the difference was not statistically significant (Figure 2.8E). The overall lesion scores for the rPCV2-Vac was highly significantly different from the control groups (Figure 2.8F), while the scores of the commercial vaccine group was similar to that of the unvaccinated group. Significant gross or microscopic lesions were not observed in the pigs sacrificed prior to challenge (two pigs per group) to assess vaccine safety. There were no significant differences in the lesion scores between the experimental groups, indicating that the rPCV2-Vac was both attenuated and safe.

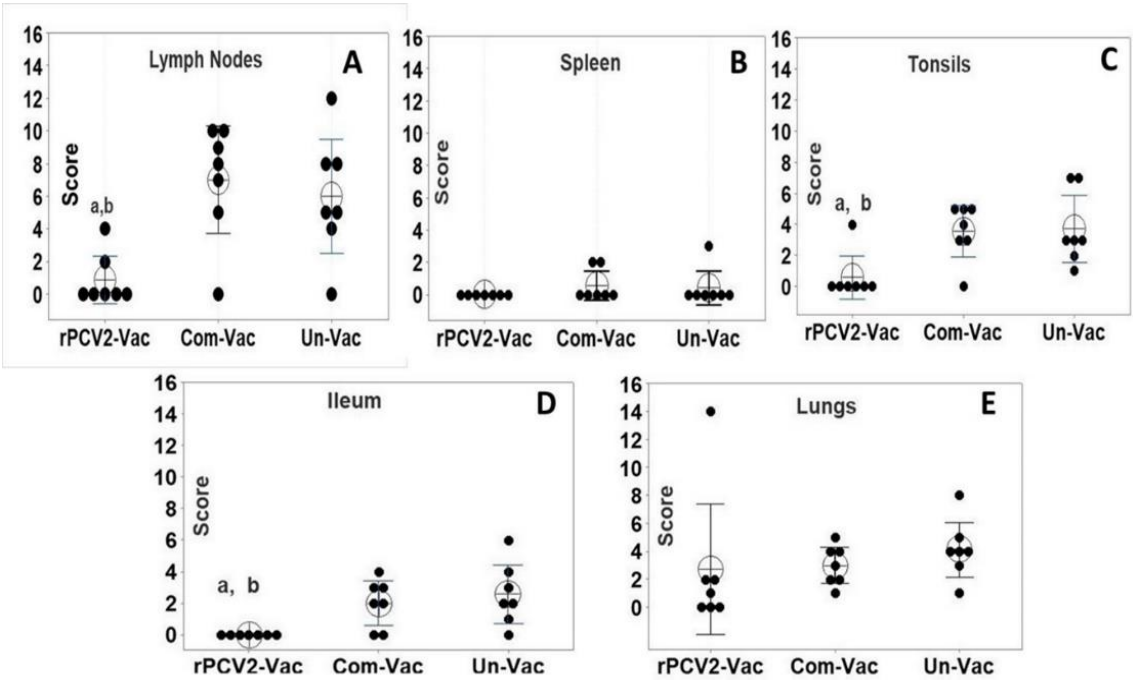

Figure 2.8: Lesion scores in tissues.
Assessment of lesion scores resulting from viral challenge is represented as the sum of the scores for each tissue (A–E). Gross lung lesions were scored from 0%–100% to represent the % area of affected lung. Microscopic lesions were scored with a scale of 1–4; where 1 = single follicle or focus staining 2 = rare to scattered staining, 3 = moderate staining 4 = strong widespread staining. X axis: groups, Y axis: scores, dots: values for the individual pigs, horizontal bar with the large circle: group mean, bars: 95% confidence interval of the means, a: Significantly different from the unvaccinated control, b: Significantly different from the commercial vaccine group, ($p < 0.05$) by a Mann–Whitney U test.

Vaccination protects against weight loss due to challenge

As is commonly encountered in experimental models, severe clinical signs of PCVAD were not observed in any of the experimental groups during the 21 days post-challenge observation period. However, the post-challenge weight gain in both vaccination groups were significantly higher than the unvaccinated control group at DPC 21 (Figure S3B), but not at DPC 14 (Figure S3A). There were no significant differences between the two vaccine groups during the post-challenge observation period (Figure S3).

Discussion

The phenomenon of "original antigenic sin" or ability to elicit memory responses to antigens and specific epitopes is critical to the success of vaccination. On the other hand, the preferential clonal expansion to immuno-dominant but non-protective epitopes encountered by the host on challenge, coupled with minor sequence variation leading to escape variants, is an elegant immuno-subversion strategy we term "deceptive imprinting". Strategies to counter deceptive imprinting in vaccine design include "dampening" the response to the immuno-dominant non-protective epitopes (Nara, 1999). The immune refocusing strategy has been successfully applied to several viruses, such as human immunodeficiency virus (HIV) (Barnett et al., 2001; Jeffs, Shotton, Balfe, & McKeating, 2002), influenza (Nara et al., 2010; Zost, Wu, Hensley, & Wilson, 2019) and dengue virus (Frei et al., 2018), among others. Unlike structurally complex pathogens, where protection is mediated by multiple antigens, the requirement for a single protective antigen makes PCV2 both a simple and elegant model for studying the effects of immunodominance on vaccine design. In this study we explored the hypothesis that alteration of the immunodominance properties of the PCV2 capsid protein will enhance rational vaccine design and result in significant protection against challenge.

The PCV2 capsid protein contains four major immunodominant regions (Mahe et al., 2000). Within these regions, four putative immunodominant non-protective linear B cell epitopes were identified (Ilha et al., 2020; Trible et al., 2011). As the PCV2 capsid protein is relatively small (233 amino acids), and incapable of tolerating large sequence changes, only two of the

identified decoy epitopes were selected for mutation in this study. It was previously demonstrated that mutation of an immunodominant HIV-1 epitope located in proximity to a neutralizing epitope can direct the response towards the neutralizing epitopes, possibly due to alteration of steric constraints (Garrity et al., 1997). As both epitope A and B were flanked by putative neutralizing epitopes (Ilha et al., 2020) they were selected for analysis. To minimize the risk of introducing lethal mutations, we elected not to delete residues, but rather replace them with other residues with a low penalty score on a point accepted mutation (PAM) matrix (Barker & Dayhoff, 1979; Schwartz & Dayhoff, 1979), and were able to successfully rescue the recombinant virus harboring mutations in the selected epitopes (Figure 2.2).

As anticipated, the introduced changes to the amino acid sequences of the PCV2 capsid protein resulted in the loss of immunodominance of epitope A and B as assessed by SPR (Figure 2.5). As paratopes which bind rapidly to their epitopes receive stronger stimulatory signals and can influence the magnitude of clonal expansion during the affinity maturation stage (Nayak, Agarwal, Nakra, & Rao, 1999; Rajewsky, 1996), an assessment of the affinity kinetics of the Abs generated in this study to their cognate peptides or to peptides encoding the mutations could not be carried out due to a shortage of samples, and only a qualitative measurement was obtained by SPR (Figure 2.5). Interestingly, antibody responses to epitope B were not detected in pigs administered the commercial PCV2 vaccine. It has been previously suggested that vaccination with fully assembled viral particles does not induce strong Ab responses to epitope B while vaccination with monomers of the subunit does (Trible et al., 2011). Further, MHC-II processing

for the same antigen is known to differ between endogenous and exogenous antigens which may be introduced by infection or vaccination respectively (Bonifaz, Arzate, & Moreno, 1999; Kittlesen et al., 1993). A limitation of this study is that only linear epitopes were targeted.

Several other factors, such as glycosylation, hypervariability, proximity to MHC-II epitopes or other neutralizing epitopes, could also potentially influence the outcomes of this study. While a detailed experimental characterization of the above listed parameters is not within the scope of the study, they are discussed below. Hyper-glycosylation is a strategy which has been previously used to dampen the Ab response to immunodominant epitopes (Trujillo, Kumpula-McWhirter, Hotzel, Gonzalez, & Cheevers, 2004). While not the primary strategy targeted in this study, the alteration in glycosylation patterns as described in the method section could have influenced the outcomes of this study. As immunodominance is influenced by the successful competition for the recruitment of antigen specific T cells in early infection, the presence of a helper T cell epitopes overlapping or adjacent to a B cell epitope can influence the strength of the Ab response elicited, (Agarwal & Rao, 1997). Epitope A contained a predicted (Propred MHC-II server) (Singh & Raghava, 2001), but non-conserved, MHC-II epitope 124 ILDDNFVT31 (Constans, Ssemadaali, Kolyvushko, & Ramamoorthy, 2015), which was altered by the mutation of the residue T to an N. Two conserved predicted MHC-II epitopes, 161 FTPKPVL167 and 174 FQPNNKRNQL184 overlapped with epitope B (Constans et al., 2015). The second predicted MHC-II epitope within epitope B was also altered by the mutations

introduced. It is possible that mutation of these T helper epitopes could have enhanced the loss of immunodominance of Epitopes A and B.

Hypervariability is a common property of decoy epitopes (Nara, 1999), and is an effective immuno-subversion mechanism. However, Epitope A and B were conserved between the first discovered PCV2a and PCV2b subtypes (Table S1, Figure S1). Only residue 131 in epitope A and residue 169 in epitope B varied between the newly evolved PCV2d challenge strain and the previously existing PCV2a and 2b subtypes (Table S1, Figure S1). For influenza, it has been suggested that the reduced vaccine efficacy observed for the H3N2 component of the polyvalent vaccine could result from the reinforcement of persistent and preferential strain specific memory (deceptive imprinting) to the H1 subtype and B type by annual vaccination, leading to competition between the polyvalent antigens (Lee, Shim, & You, 2018). Therefore, prior exposure to the unmodified epitopes A and B by infection with PCV2a or 2b, or by vaccination, could diminish protection against the newly evolved PCV2d subtype in the field(Seo, Park, Han, & Chae, 2014; Zhai et al., 2011). While direct comparisons of the rPCV2-Vac to the commercial control vaccine are avoided as the commercial vaccine is extensively standardized for optimal dosage which can differ from the experimental vaccine, is inactivated and contains an adjuvant, in this study, the rPCV2-Vac was significantly more effective at inducing neutralizing Ab responses against the heterologous PCV2d subtype (Figure 2.4).

While vaccine viral replication was not detected at 14 days post-vaccination, it is possible that replication of the experimental vaccine virus could have been detected if sampling was done

at time points prior to day 14. However, the fact that vaccine virus was not detected at day 14 while wildtype viruses increase in titers at 14 days post-infection supports the conclusion that rPCV2-Vac was attenuated. The broadened virus neutralization responses elicited by vaccination with rPCV2-Vac (Figure 2.4) correlated with the significant reduction in tissue pathology caused by early challenge viral replication and localization to the sites of predilection (Figure 2.8). The reduced lesion scores in lymphoid organs, which are the primary sites of predilection for PCV2, indicate the rPCV2-Vac was highly effective in curtailing local infection as well as systemic dissemination. Overall, the data supports the conclusion that rPCV2-Vac was more effective in neutralizing heterologous subtypes than the PCV2a based commercial vaccine, while acknowledging that the observed effects could be due to differences in the nature of the treatments, as the commercial vaccine is an inactivated preparation and the rPCV2-Vac is a live virus (Figure 2 4).

The exact mechanisms by which a low-level of exposure to protective antigens is successful in eliciting good vaccine efficacy is not fully understood. However, initial priming of the immune response is known to be critical in influencing the quality of the response. Recent studies in cancer immunotherapy have shown that low doses of antigen, rather than high doses, preferentially primed high avidity CD4+T cells, which in turn stimulated both antibody (Trible et al.) responses and cytotoxic T cell responses effectively, instead of skewing the response towards one arm of the immune system (Lovgren et al., 2012). While it is possible that the insertion of the SRS peptide marker at the 5′end of the capsid gene could have influenced outcomes, the

presence of the tag itself if unlikely to provide PCV2-specifc immunity or enhance protection. It has been reported that PCV2 can be detected in the nasal secretions in the absence of viremia, post-challenge (Fort et al., 2008). However, a limitation of this study is that shedding of the challenge virus in nasal secretions or fecal matter was not measured. With the reasonably strong performance of current PCV2 vaccines in the field, the availability of an enhanced vaccine could pave the way for the eventual eradication of the virus (Afghah et al., 2017). Successful disease eradication efforts in veterinary medicine typically employ a stamping out strategy, wherein infected animals can be differentiated from vaccinated animals using serological assays and then removed from the herd in a systematic manner (Francis, 2018). Detection of antibody responses to the SRS2 peptide will only provide information regarding whether an animal is vaccinated and will not enable differentiation of animals which can get infected after they receive the vaccine. However, availability of the SRS2 tag enables the monitoring of vaccine compliance in the field (Figure 2.2 and 2.6). With additional dose optimization and possible commercialization, the improved efficacy parameters of the rPCV2-Vac could reduce or eliminate the emergence of new PCV2 subtypes, and significantly advance current control measures for PCV2.

Conclusions

Thus, targeted modification of the selected non-protective immunodominant epitopes in the PCV2 capsid protein resulted in broadened virus neutralization responses against newly evolved heterologous PCV2 strains, prevented replication of the challenge virus and development of tissue pathology in vaccinated and challenged pigs. The described approach can

potentially have a broad application in rationalizing vaccine design for agents with delayed virus neutralizing antibody responses.

Patents

The submitted work is protected by a provisional patent application, in compliance with the regulations of North Dakota State University.

Supplementary materials

The following are available online at https://www.mdpi.com/2076-393X/8/3/506/s1 **Figure S1:** Multiple sequence alignment of the PCV2 capsid protein, **Figure S2:** Map of the rPCV2-Vac construct, **Figure S3:** Post-challenge weight gain, Table S1: Amino acid sequences of Epitope A and B.

Author contributions

Data acquisition, analysis, writing and editing, A.R., methodology, data acquisition, O.K., data acquisition, analysis, G.S., conceptualization, review and editing, P.N., data acquisition, analysis, review, E.L., data acquisition, review, P.P., data acquisition, review, A.P., conceptualization, funding acquisition, project management, writing-review, editing, S.R. All authors have read and agree to the published version of the manuscript.

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Conflicts of interest

The authors declare no conflict of interest. The funding agencies played no role in design, data collection, interpretation or publication of this work.

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CHAPTER 3: RAPID ATTENUATION OF A HIGHLY MUTATING VIRUS BY DIRECTED SUICIDAL REPLICATION: PCV2B AS A MODEL[2]

Abstract

With the increasing number of newly emerging infections, the development of improved strategies to shorten the lead development time for attenuated vaccine candidates has become critical. Porcine circovirus 2 (PCV2) is a small DNA virus that is economically important as the causative agent for the postweaning multisystemic wasting syndrome (PMWS), in weanling piglets. Although a DNA virus, PCV2 has a mutation rate that is similar to that of RNA viruses, leading to the frequent emergence of new subtypes in the field despite the availability of standard vaccines. Using PCV2 as a model, in this study, we have explored a strategy for rapid attenuation of viruses that harnesses high viral mutation rates to target the premature termination of the viral translation during viral replication. By rationally recoding the serine and leucine codons in the PCV2 capsid protein, the chances of accumulating stop mutations during viral replication was increased in a directed manner. The PCV2 vaccine candidate with recoded serine and leucine codons was successfully rescued by transfection. When tested in a piglet model, the test vaccine elicited strong neutralizing antibody responses. Vaccinated pigs were completely

[2] The material in this chapter was co-authored by AGM Rakibuzzaman, Pablo Piñeyro, Angela Pillatzki, and Sheela Ramamoorthy. AGM Rakibuzzaman had primary responsibility for conducting experiments, preparing vaccines candidates, and analysis the collected samples from the animals. AGM Rakibuzzaman was the primary developer of the conclusions that are advanced here. AGM Rakibuzzaman also drafted and revised all versions of this chapter. Sheela Ramamoorthy served as a proofreader and checked the math in the statistical analysis conducted by AGM Rakibuzzaman.

protected against challenge with a heterologous PCV2d strain and had reduced lesion scores compared to pigs administered a commercial vaccine. Importantly, the test vaccine virus was cleared in vaccinated pigs within two weeks of exposure and did not cause tissue pathology in vaccinated pigs, indicating that it was both attenuated and safe. Furthermore, exposure of the suicidal PCV2 vaccine construct to immune pressure in vitro using sub-neutralizing antibodies resulted in the accumulation of stop codons, as expected. This study is the first to demonstrate an effective and safe rapid-attenuation strategy for rapidly mutating single-stranded DNA viruses, with broad applicability to other animal viruses.

Key words: Rapid attenuation, PCV2, suicidal replication, high mutation rate

Introduction

"Vaccine" is the highest mooted word right now in the world because of its urgency and necessity to save lives. A vaccine is the most effective weapon to protect the human and animal from viral infections. In recent years, a couple of viral outbreaks have happened in both the human and animal worlds. RNA viruses are mainly responsible for most of these outbreaks (Woolhouse & Gaunt, 2007). The reason behind the dominance in the emerging outbreaks is its high mutation rate. Due to the high mutation rate, it poses a variety of genetic and antigenic determinants. Thus, previously available vaccines become ineffective for reemerging viruses. Similar to the RNA virus, small single-stranded DNA (ssDNA) like PCV2 also has a very high mutation rate in the range of RNA virus (Correa-Fiz et al., 2020; Firth, Charleston, Duffy, Shapiro, & Holmes, 2009; Franzo, Cortey, Segalés, Hughes, & Drigo, 2016). To control

damages by an emerging or reemerging virus like RNA viruses or highly mutating viruses, early diagnosis, surveillance, biosecurity measures, and vaccine availability is critical (Knobler et al., 2004; Mack, Choffnes, Sparling, Hamburg, & Lemon, 2007; Song, Singh, Nelson, & Ramamoorthy, 2016). Therefore, a rapidly developed, attenuated vaccine is required for the emerging RNA or ssDNA viruses like PCV2.

PCV2 is a very small DNA virus with about a 1.8 kb genome (17 nm in diameter) icosahedral, non-enveloped, with a single-stranded DNA genome (Tischer, Gelderblom, Vettermann, & Koch, 1982). It was first identified as a cause for the post-weaning multi-systemic wasting syndrome (PMWS) in a swine herd (Clark, 1996) and responsible for a variety of type of diseases, collectively called porcine circovirus associate disease (PCVAD); which causes a substantial economic loss at swine industry (Hu et al., 2017). Vaccines are available for PCV2 and it significantly reduces the clinical signs of the diseases. Even though the vaccines' availability for the last 15 years, - PCV2 is evolving periodically with new strains, and the USA's common subtypes are PCV2d (Afghah, Webb, Meng, & Ramamoorthy, 2017; Karuppannan & Opriessnig, 2017). Recently, two more variants of porcine circovirus have been identified as PCV3 and PCV4 (Klaumann et al., 2018; Phan et al., 2016; Zhang et al., 2020). Therefore, the vaccine protection is suboptimal and its anticipated that its contributions towards the viral evolution by inducing the vaccine selection pressure (Bao et al., 2018; López et al., 2018). Thus, a safe and effective vaccine with rapid attenuation capability is required.

To protect the animal world from a viral infection, various technology has been used to develop a vaccine. Attenuation by genetic recoding (Gonçalves-Carneiro & Bieniasz, 2021), especially by codon deoptimizations (Van Leuven et al., 2021), and by codon optimization to trigger in vivo attenuation by suicidal replication (Moratorio et al., 2017) are previously available methods for rapid attenuation. Inducing in vivo suicidal replication strategy involves recoding the genetic information that does not change protein sequences. However, it shifts the genetic information towards close to termination of protein synthesis in vivo. Therefore, when a virus is making any mutations in vivo, it increases the chances of producing stop codons or deleterious mutations, thereby attenuating the virus. This is a very new technique and effective against highly mutating viruses, and people have shown its effectiveness for RNA viruses only (Moratorio et al., 2017). The advantages of this method are it can be used for rapid attenuation of the virus and its complete safe without compromising the immunogenicity. As this method used the high mutation rate of the virus, and PCV2 is highest mutating virus among the DNA viruses, in this article, for the first time, we have explored this latest strategy for a highly mutating DNA virus porcine circovirus type 2 (PCV2) as an experimental model.

In this study e, we have redesigned the serine and leucine codons of ORF2 such that any mutations in these changed codons will increase the chances of nonsense mutations by creating stop codon and eventually terminate the protein synthesis or at least induces mutations into these regions which will be resulting changes of proteins and attenuating the virus. The primary objective of this study was to assess whether the approach of destabilizing of serine and leucine

codons would be applicable to DNA viruses with high mutation rates, using PCV2 as a model. The secondary objective of the study was to determine whether redesigning the leucine and serine codon induces accumulation of stop codons under the immune selection pressure by imposed by sub-neutralizing antibody pressure in vitro.

Materials and methods

Cells and viruses

PK-15 cell line free of PCV1 has been used to culture the PCV2 virus. We have used an infectious clone of PCV2b strain 41513 (GenBank ID – KR816332) within the backbone of pBluescript SK II to develop the vaccine virus. PCV2a (AF264042.1), PCV2b (EU340258.1), and PCV2d (JX535296.1) were used for virus neutralization assay. The heterologous strain of PCV2d (GenBank ID- JX535296.1) was used as a challenge virus culture (Kolyvushko, Rakibuzzaman, Pillatzki, Webb, & Ramamoorthy, 2019).

Cloning and mutations

The infectious clone of PCV2b strain 41513 (accession number KR816332) was used as a backbone for the vaccine virus. For preparing the attenuated virus, we have redesigned all the serine and leucine codons of the ORF2 from BstB1 to MscI cutting site and commercially synthesized from a company (Eurofins Genomic, Louisville, KY, USA) and had replaced with the wild type ORF2 of PCV2b 41513 and named as sPCV2-Vac hereafter. The codon changes were done as mentioned in table 3.1 and schematic position of the amino acids on ORF2 has

shown in figure 3.1. The sPCV2-Vac was rescued by transfection of PK-15 cells and viral replication in infected cells visualized with an immunofluorescence assay (IFA).

Table 3.1: Redesigning the serine and leucine codons.

Amino acids	Codons in Wild type	Codons in sPCV2-Vac	Target Stop codon
Leucine	CTC	TTA	TAA or TGA
Leucine	CTC	TTA	TAA or TGA
Leucine	CTA	TTA	TAA or TGA
Leucine	CTA	TTG	TAG
Leucine	CTG	TTG	TAG
Leucine	CTG	TTA	TAA or TGA
Leucine	CTT	TTG	TAG
Serine	TCC	TCA	TGA or TAA
Serine	TCC	TCG	TAG
Serine	AGT	TCA	TGA or TAA
Serine	TCT	TCA	TGA or TGA

Insertion of DIVA (Differentiation of Vaccinated and Infected Animals) marker

An immunological selection marker was added to the vaccine construct to distinguish between the vaccinated and naturally infected animal. An apicomplexan parasite Neospora Caninum, which is not detected and causes any diseases in pigs, has been selected to find the marker. The marker was adapted from our previous publication of the PCV2 immunodominance vaccine (A. Rakibuzzaman et al., 2020). Briefly, the surface antigen-1 related sequence 2 (SRS2) protein of N. Caninum (AAD04844.1) was analyzed by Protein 13 (DNASTAR, Madison, WI, USA) to identify the 18 amino acid long highly immunogenic sequence. The immunogenic

peptide sequence of 324 QSSEKRDGEQVNKGKPP 348 of the SRS2 protein was inserted at the 3' end of the ORF2 gene of the vaccine construct by site-directed mutagenesis using Q5 mutagenesis kit (New England Biologicals, Ipswich, MA, USA), according to the manufacturer's instructions.

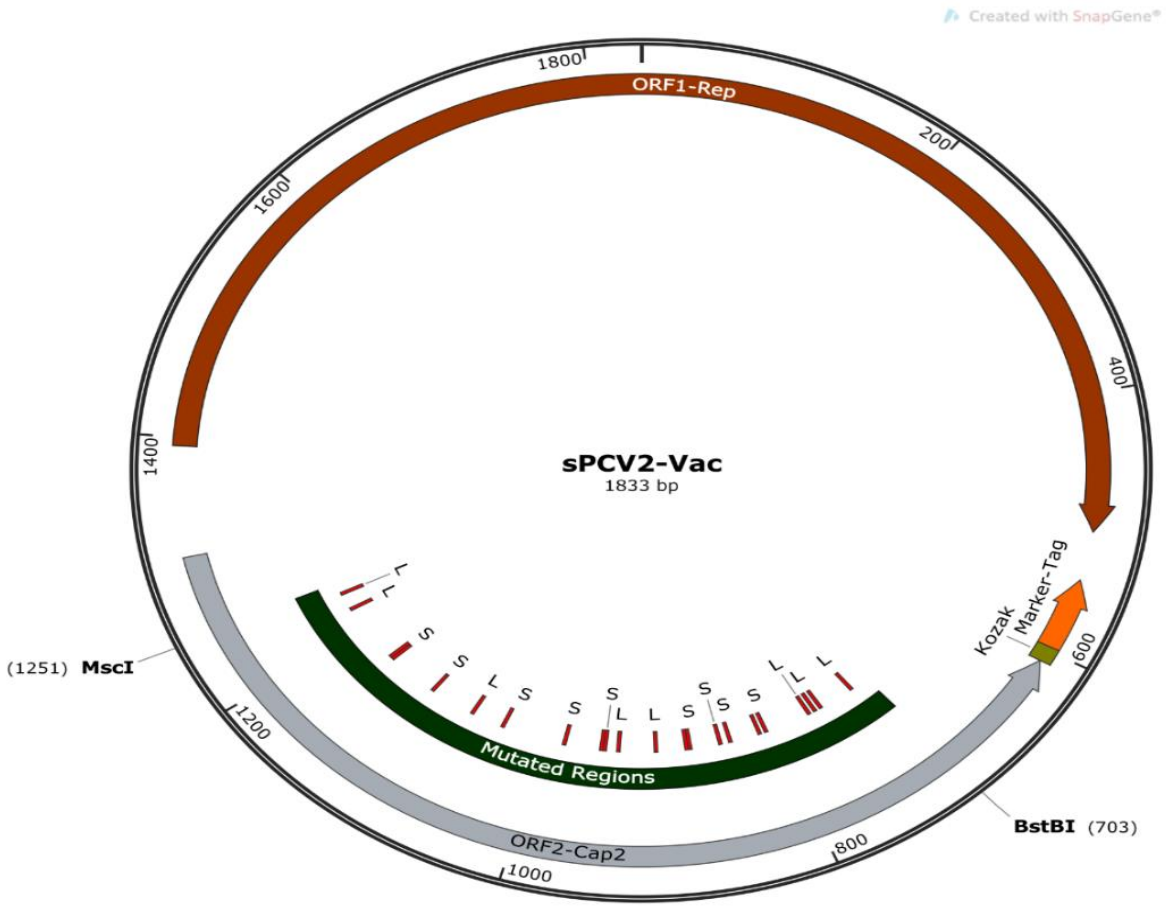

Figure 3.1: Schematic plasmid map of modified PCV2.
The red bar shows the position of the serine and leucine, while L is for leucine and S is for Serine.

Vaccine and challenge virus preparations

The mutated infectious clones (sPCV2-Vac, PCV2d, PCV2a) were used to transfect PK15 N (PK15 cells free of PCV1 contamination) (NVSL labs) to prepare the virus cultures for

vaccine viruses and virus cultures for neutralization assays. TransIT-2020 (Mirus Bio, Madison, WI, USA) was used for transfections, according to the manufacturer's instructions and described at (Kolyvushko et al., 2019; A. Rakibuzzaman et al., 2020). Briefly, 12.0 µg of infectious clone DNA were diluted with minimum essential media (DMEM) (Corning, Tewksbury, MA, USA), then 36.0 µlof TransIT-2020 was added to the diluted DNA and incubated at room temperature 25-30 minutes. During the incubation time, one T25 flask (Bio-lite) (Thermo-Scientific, Waltman, MA, USA) and one 8-well chamber slide (Corning, Tewksbury, MA, USA) of the PK-15 cell line were washed with Gibco's Hank's balanced salt solution (HBSS) (Thermo-Scientific, Waltman, MA, USA). After the incubation period, 100.0 ul and 900.0 ul of transfection mixture were added to a single well of the chamber slide and the T25 flask, respectively, and incubated at 37°C in a CO_2 incubator for 3 hours. Transfection media was removed after incubation, and 2% FBS MEM, 1X antibiotic/antimitotic (Thermo-Scientific, Waltman, MA, USA) was added as infection media before incubating at 37°C in a CO_2 incubator. After 48 hours of incubation, the T25 flask was frozen at -80°C.

Immunofluorescence assay

Immunoreactivity of the cloned PCV2b capsid protein to anti-PCV2b anti-serum was verified by an indirect immunofluorescence assay (IFA) as described at (A. Rakibuzzaman et al., 2020). Briefly, the transfected/ infected cell sheet of the 8-well chamber slide was fixed using a 1:1 mixture of methanol: acetone. The fixed cell sheets were stained with a monoclonal antibody to PCV2b or a polyclonal antibody to *Neospora Caninum*, followed by a FITC-conjugated anti-

swine or anti-rabbit IgG secondary antibody (KPL, SeraCare, Milford, MA, USA), and counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Life Technologies, Carlsbad, CA, USA). The stained cells were viewed using a fluorescent microscope for evidence of PCV2 replication or expression of *N. Caninum* SRS2 marker. The frozen T25 flask was thawed and frozen three times to rescue the virus and check the infectivity by IFA, as stated above. Preparation of the challenge virus was conducted similarly as stated above using a PCV2d infectious clone.

Animal study design

Approximately 3-4-week-old piglets from a PCV2 PCR negative herd were randomly assigned to one of three groups, Group I -Expt sPCV2-Vac (N=9), Group II – one 2.0 ml-IM dose Merial (N=9), Group III- unvaccinated control (N=9). 10^4 $TCID_{50,}$ 2ml Intranasal (i/n), 2ml intramuscular (i/m), serum was collected on day 0, every two weeks thereafter, on the day of the challenge, ten days post challenged, and at necropsy. Vaccinated pigs were boosted with the same dose of each vaccine to respective groups. Two animals from each group were euthanized prior to the challenge to assess vaccine safety. All animals were challenged with PCV2d (10^4 $TCID_{50,}$ 2ml i/m, and 2ml i/n) on day 28 post-vaccination. All animals were euthanized 20 or 21 days after the challenge to assess gross and histological lesions. All procedures pertaining to animal experimentation were carried out with the approval and oversight of the Institutional Animal Care and Use Committee (IACUC) and Institutional Biosafety Committee (IBC) regulations of N. Dakota (NDSU) and S. Dakota State Universities (SDSU).

Anti-PCV2 IgG response

The serum Anti-PCV2 capsid IgG response was measured at the Veterinary Diagnostic Lab at Iowa State University using a commercial PCV2 ELISA kit (Ingezim Circovirus IgG kit, Ingenasa, Madrid, Spain) following manufacturer instruction and inhouse standard operating procedures.

Neutralizing antibody response

The titer of neutralizing antibody (NA) against PCV2a, 2b, and 2d were assessed by the fluorescence focus neutralization (FFN) assay as described in (Kolyvushko et al., 2019; A. Rakibuzzaman et al., 2020) Briefly, all the virus (PCV2a, 2b, and 2d) cultures were adjusted to have 30-40 fluorescent focus unit (FFU/100 ul). The tested serum samples were inactivated at 56°C for 30 min, serially diluted at 1:128 with MEM. The diluted serum was mixed with an equal volume of each PCV2 virus and incubated for one h at 37°C. During the incubation, 30 to50% monolayer confluent Pk-15 cells were washed 2x with HBSS. Following the incubation, the serum-virus mixture was added to the cells, followed by incubating at 37°C in a CO_2 incubator for 48 hours. After the 48 hour incubation period, a similar IFA protocol was performed as mentioned above. Serum NA titer was determined as the highest dilution at which there was a 90% or greater reduction in virus replication compared with the virus control.

Antibody response to DIVA marker

Antibody response to the immunogenic marker was determined as described in our previous publication (A. Rakibuzzaman et al., 2020). Briefly, the immunogenic marker's

nucleotide sequence was cloned and expressed into the pET-Sumo vector with his tag and peptide purified with affinity chromatography. The expressed peptide was used to coat the plate, and ELISA was performed as described in (A. Rakibuzzaman et al., 2020).

Determination of viral load

The viral vaccine load was determined by a quantitative real-time PCR targeting the SRS2 specific TaqMan probe. Serum from day 14 and 28 post-vaccination was used to extract DNA using the QiaAmp DNA mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instruction. The sequence of the primer are Fw-5' CCATGCCCTGAATTTCCATA-3' , Rv-5'- CCCCACTTAACCCTTAATGA-3' and probe 5′-TACCTGTTCCCCGTCGCGT-3′. The PCR amplification was performed as described in (A. Rakibuzzaman et al., 2020). Briefly, the reaction mixture was prepared as 2.0 µL of extracted DNA, 0.4 µM of primers, 0.1 µM probe in a 25 ul reaction volume with the QuantiFast Probe PCR Kit (Qiagen, USA). A Tm of 65°C was used in a qPCR thermocycler (CFX96 Touch, Bio-Rad, Hercules, CA, USA).

The viral challenge load was quantified from post-challenge serums of day9 and 21 by qPCR as described in our previous publication using the same primer-probe (A. Rakibuzzaman et al., 2020). Briefly, primers and probes were designed from the PCV2d specific regions. The reaction mixture was prepared as 2.0 µL of extracted DNA, 0.4 µM of primers, 0.1 µM probe in a 25 ul reaction volume with the QuantiFast Probe PCR Kit (Qiagen, USA). A Tm of 67°C was used in a qPCR thermocycler (CFX96 Touch, Bio-Rad, Hercules, CA, USA).

Necropsy and histopathology

The animal study and necropsy were conducted at the Veterinary Diagnostic Lab at SDSU. At necropsy, lung, liver, heart, spleen, kidney, ileum, and tonsil samples were collected, and dissected tissue samples were fixed with 10% neutral buffered formalin. Histopathology was examined at the Veterinary Diagnostic Lab at Iowa State University. Briefly, tissues were examined microscopically by hematoxylin and eosin staining and by immunohistochemistry (IHC) staining with a PCV2-specific antibody. Microscopic and gross lesion scores were assigned in a blinded fashion by board certified veterinary pathologists. Gross lung lesions were scored as the percentage of lung parenchyma involved. Enlargement of inguinal lymph nodes was assigned scores of 0-3 as follows, 0 = no enlargement, 1= two times the normal size, 2= three times the normal size. and 3= four times the normal size. Viral antigen in IHC slides were scored from 1-4 as follows: 1 = single follicle or focus staining, 2 = rare to scattered staining, 3= moderate staining, 4 = strong widespread staining.

In vitro immune pressure study

The selective immune pressure study was conducted according to described at (Zhao, Ma, Dong, & Cui, 2012) with little modification. Briefly, both the wild-type and sPCV2-Vac virus culture was adjusted to 10^4 $TCID_{50}$ /ml. Previously stored serum from PCV2b immunized DPI 28, was diluted 1000 times to achieve 25% reduction in fluorescent foci by FFN assay as mentioned above. Then the selected serum dilutions were prepared with 2% FBS, MEM, 1X antibiotic/antimitotic, and passage both of the viruses separately up to 5 times. Briefly, 500.0 ul

of diluted serum in MEM were mixed with an equal amount of 10^4 $TCID_{50}$ /mL of virus and incubated at 37°C for 1 hour. For control without serum, only MEM was used instead of serum dilution. After the incubation, the virus serum mixtures were toped onto the monolayer of the PK15 cell line and incubated for 2-3 hours. After the incubation periods, the 2.0mL infection media 2%FBS were added onto the top of the cells. The process was continued up to 5 passages, and samples were preserved at -80C.

Deep sequencing of mutation analysis

A sample pool was made for each virus culture from passage 3 to 5. Then viral DNA was extracted using the Qiagen Viral RNA extraction kit (Qiagen, Valencia, CA, USA) according to the manufacturer protocol. Before the viral DNA extraction, the pooled samples were treated with DNase (Thermo-Scientific, Waltman, MA, USA) to remove any plasmid or cellular DNA. After the DNA extraction, a PCR was done with low cycle (25 cycle) amplification with Phusion PCR mix (New England Biologicals, Ipswich, MA, USA) to amplify the whole virus about 1.8kb. The PCR was performed with Sac II forward and reverse primers to amplify the PCV2 whole genome. Primers are SacII F-5'- GAA CCG CGG GCT GGC TGA ACT TTT GAA AGT-3', SacII Rev- 5' – GCA CCG CGG AAA TTT CTG ACA AAC GTT ACA-3'.

The PCR purified samples were sent to a company (CD-genomics, New York, NY, USA) for deep sequencing and bioinformatic analysis. Briefly, sequencing libraries were generated using NEBNextR Ultra™ DNA Library Prep Kit for Illumina (New England Biologicals, Ipswich, MA, USA) following the manufacturer's recommendations, and index codes were

added to attribute sequences to each sample. The DNA sample was fragmented by sonication to a size of 350bp, then DNA fragments were end-polished, A-tailed, and ligated with the full-length adaptor for Illumina sequencing with further PCR amplification. At last, PCR products were purified (AMPure XP system), and libraries were analyzed for size distribution by Agilent2100 Bioanalyzer and quantified using real-time PCR. The whole genome was sequenced using Illumina PE150. The reads were curated to remove poor-quality reads and adapter sequences using trim_galore software. Clean reads were mapped to the wild-type and sPCV2 sequences, respectively, using BWA toolkit. The GATK tool was used to identify single nucleotide polymorphisms (SNPs) and INDELS (insertion or deletion of bases). Only SNPs with a Qpred > 20 quality score above the threshold and with an SNP frequency of over 85% were included in assembling the consensus sequences. The consensus sequences of the treated and untreated samples were compared by alignment with GATK to obtain changes that could be attributed to the treatment. Detected changes were annotated to include the locations based on the reference sequences and are presented in Table 3.2.

Statistical analysis

A significance level of $p < 0.05$ was used for all statistical analysis. Analysis was conducted using the Microsoft excel. Serological and qPCR data were analyzed by a Student's t test. The lesion scores and body weight data were analyzed by the Mann–Whitney U test. The consolidated values, statistical significance and standard deviation are represented in the figures.

Results

The constructed virus was rescued successfully and expressed the DIVA marker peptide

The commercially synthetic gene was cloned successfully into the viral genome by restriction digestion and ligation. The recombinant virus was successfully rescued after the transfection to PK-15 cell lines and was detected with a PCV2 specific polyclonal antibody (Figure 3.2). The peptide marker was also detected using *Neospora caninum* specific antibody by an immunofluorescence assay as described in our previous publication (data not shown here) (A. Rakibuzzaman et al., 2020).

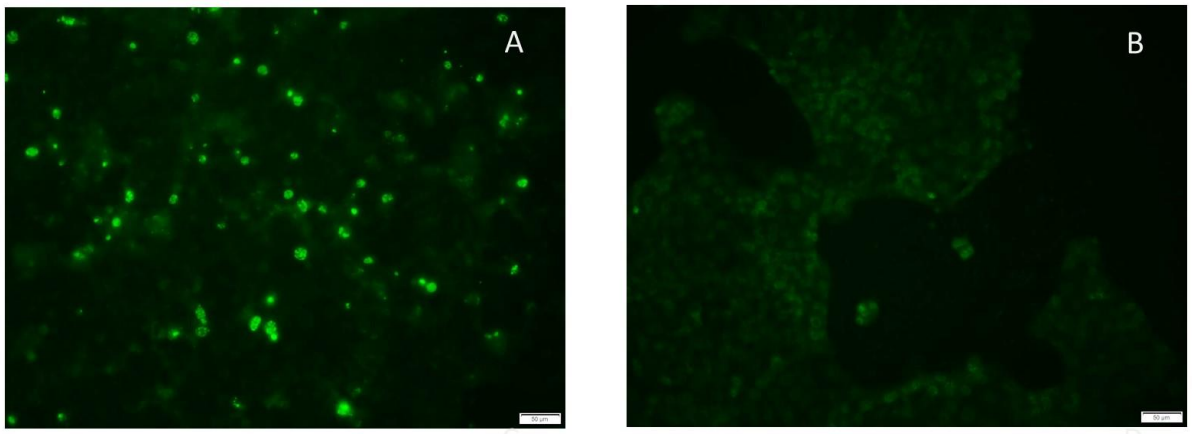

Figure 3.2: Rescue of the vaccine virus.
A. PK-15 monolayer cell line transfected with the recombinant sPCV2-Vac virus and showing the apple-green fluorescence of the viable virus stained with an anti-PCV2 polyclonal antibody. B, cell control stained with an anti-PCV2 antibody.

Antibody responses against PCV2 virus were developed in vaccinated pigs

Serum collected from the pigs at different days of post-vaccination was subjected to an ELISA using the commercial PCV2 ELISA kit to determine Ab responses. The results show a slight elevation of serum IgG against PCV2 on day 14 on vaccinated groups; however, it

becomes significant on day 28 post-vaccination compared to the unvaccinated group. However, it's not directly comparable with the commercial vaccine because of the formulation, adjuvant used in the commercial one, and it is showing significantly higher antibody responses on day 28, post-challenge 9, and 21. The antibody response on the unvaccinated groups elevated at day 21 post-challenged and was significantly lower at day 28 vaccination and day 09 of post-challenge (figure 3.3).

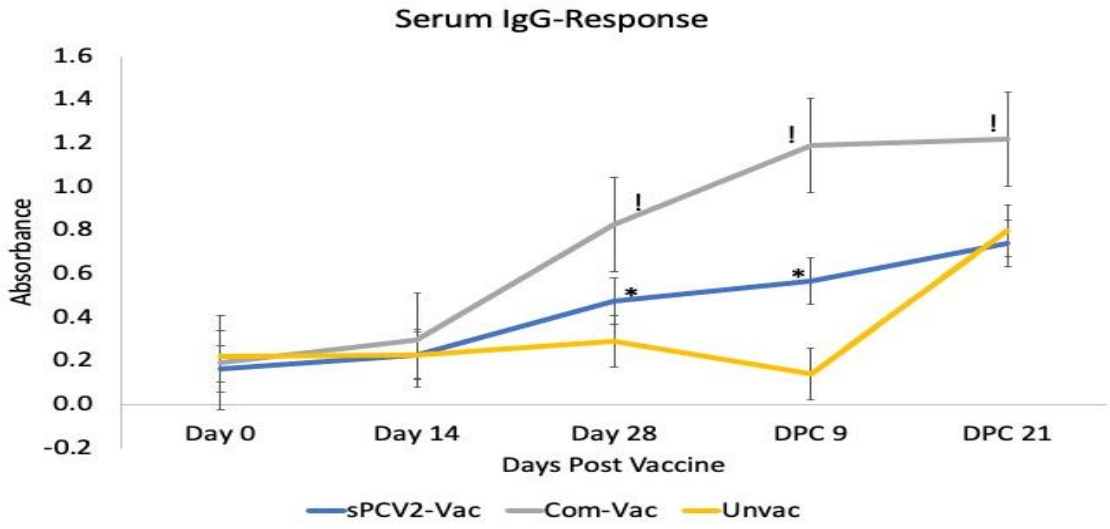

Figure 3.3: Anti-PCv2 antibody response.
Figure Shows the mean PCV2 Cap-specific antibodies in pigs of different groups. Serums from Day 0, 14, 28 of post-vaccination and day 09, and 21 of post-challenge (DPC) were accessed by commercial anti-PCV2b ELISA kit. X-axis: time points of serum collection, Y-axis: sample to positive (S/P) ratio, two replicate were used to calculate each mean, Error bars indicate the standard deviation, ! and * are significantly different from the unvaccinated control, $p \leq 0.05$, Students *t*-test n=9 for DPV0 to DPV 28, n= 7 for DPC 09 and DPC 21.

sPCV2-Vac induces heterologous virus-neutralizing antibody

We measured the virus neutralization antibody against the heterologous PCV2a and PCV2d as well as homologous PCV2b by fluorescence focus neutralization assay. sPCV2-Vac

induces a strong neutralizing antibody against all of the strains. Although the commercial vaccine had undergone dose optimization and adjuvant, sPCV2-Vac has a significantly higher neutralizing antibody against homologous PCV2b and heterologous PCV2d. The unvaccinated control group shows minimal protection against the viruses (figure 3.4).

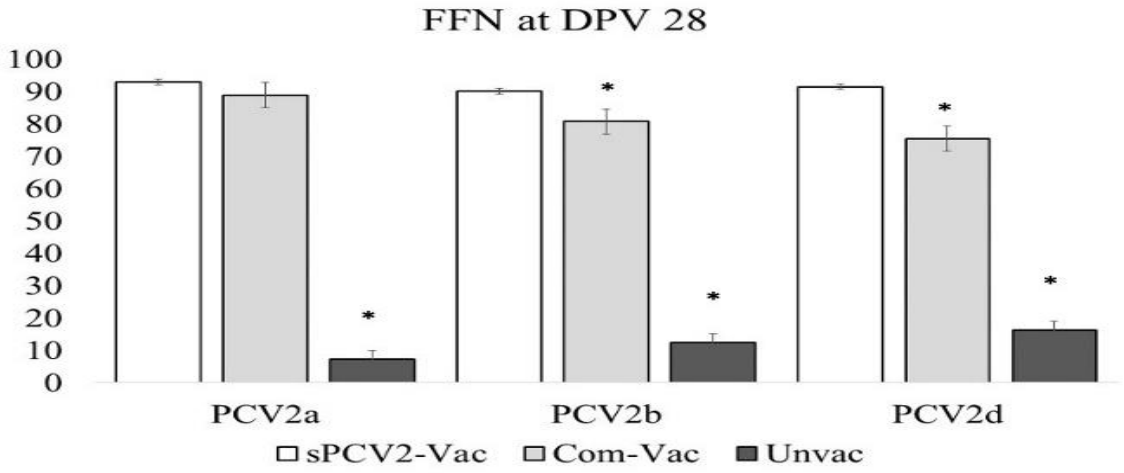

Figure 3.4: Virus neutralization assay.
Virus neutralizing antibodies measured by a fluorescent focus neutralization assay using days post-vaccination 28 pre-challenge sera. X-axis— PCV2 subtypes used in the assay. Y-axis mean % reduction in fluorescent foci compared to the untreated virus culture. Error bars indicate the standard deviation, *-significantly different from the unvaccinated control and commercial vaccine groups, $p \leq 0.05$, Students t-test.

Antibody response against DIVA marker

The antibody response towards the DIVA marker was accessed by an in-house developed ELISA specific to the marker peptide. Purified SRS2 target peptide thorough pET-Sumo expression was used as a capture antigen. Data shows that the commercial vaccine and the unvaccinated group did not induce any responses against the marker peptide. sPCV2-Vac vaccinated pigs induced a strong antibody response against the marker peptide and spiked at day

14 post vaccinated. However, the antibody response of sPCV2-Vac for marker peptide has boosted up and significantly higher than both commercial and unvaccinated groups at day post-vaccine 28 (figure 3.5).

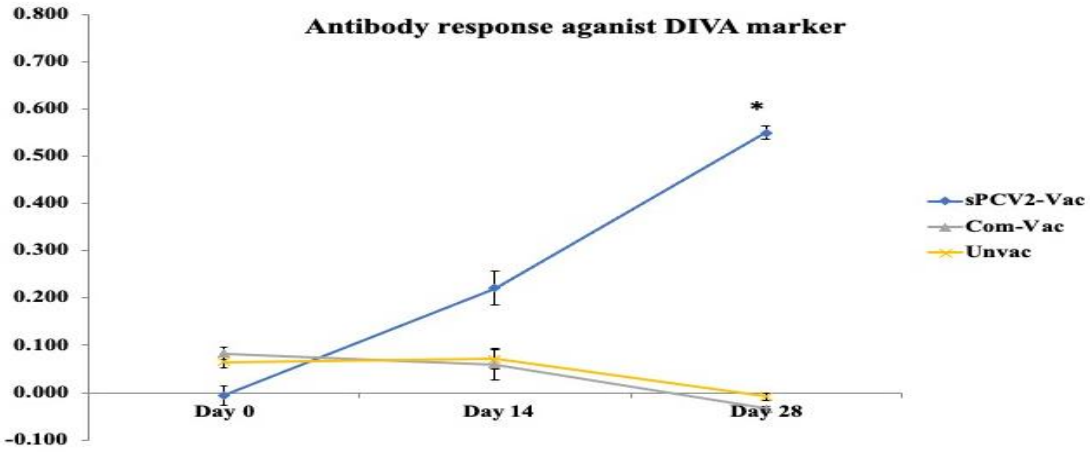

Figure 3.5: Antibody response against DIVA marker.
Antibody response measured by ELISA using the SRS2 peptide as capture antigen and showing mean anti-SRS2 antibodies in pigs of different groups. In-house ELISA accessed serum from post-vaccination days 0, 14, 28 of post-vaccination. X-axis: time points of serum collection, Y-axis: mean value, Error bars indicate the standard error mean, and * are significantly different from the unvaccinated control, $p \leq 0.01$, Students *t*-test.

Protection against challenge virus

The piglets of the vaccinated groups were protected from heterologous challenge virus PCV2d. The virus was not detected on the DPC 09 in any of the vaccinated groups. However, a slight replication of the virus was detected at day post-challenge 21 in both vaccinated groups. The viral replication into the unvaccinated groups was significantly higher in both post-challenged days 09 and day 21 compared to other groups in the study (figure 3.6).

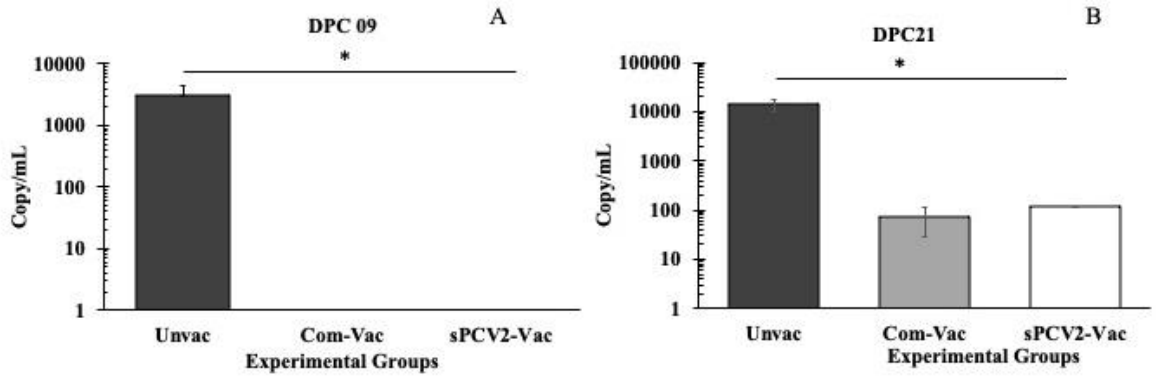

Figure 3.6: Replication of challenged virus PCV2d in different groups.
Serum samples from post-challenge day 09 and 21 were assessed by a PCV2d specific qPCR. The X-axis shows the groups, and the y-axis shows the viral copy number per mL of serum. A, shows the viral copy number at day 09 post-challenge, and B, shows the viral copy number at day 21 post-challenge. * are significantly different from the unvaccinated control, $p \leq 0.01$, Students *t*-test.

On the other hand, the vaccine virus sPCV2-Vac was not detected at 14 days post-vaccination when measured with an SRS2 specific qPCR. However, sPCV2-Vac was detected at a very low label into only one pig out of 9 pigs of the group at 28 days post-vaccination, even after giving a booster dose at day 14 post-vaccination (data not shown). This indicates the vaccine virus gets attenuated in vivo and cleared from the system, although not compromising with neutralizing antibody production.

sPCV2-Vac protects pigs from pathological lesions

Although the antibody response was lower than that of the commercial vaccine, the lesion scores (the combination of H&E and IHC scores) were significantly lower sPCV2-Vac group compared to the commercial vaccine group and unvaccinated group. For the sPCV2-Vac group, lesion scores were not seen in major organs except the lung, which was comparable to the

commercial vaccine. In lymph nodes, tonsils, and ilium, the commercial vaccine and unvaccinated group show significantly higher lesion scores than sPCV2-Vac (Figure- 2.7 A, B, and D). Although few lesion scores were found in the lungs for both sPCV2-Vac and commercial vaccine, only the sPCV2-Vac shows significantly lower scores than the unvaccinated groups. However, no lesion scores were seen in the spleen, liver, and heart in any groups, and thus data are not shown here. No lesions scores were found from the vaccinated pigs of sPCV2-Vac at Day 28 postvaccination to access the vaccine safety, indicating that the sPCV2-Vac was very safe (data not shown).

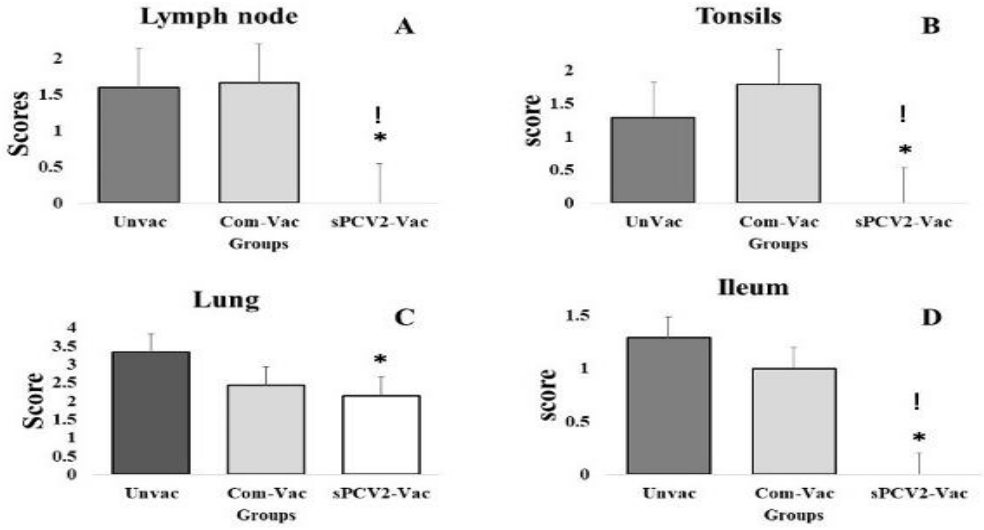

Figure 3.7: Microscopic lesions scores.
Samples collected from the study pigs were examined for microscopic lesions by H&E staining and antigen load by IHC immunohistochemistry with PCV2 antibody. Figures show the average lesions score of different organs of challenged pigs at DPC 21 during necropsy. In the figure, the x-axis depicts different groups, and the y-axis shows the average lesion scores. The bar shows the average lesion score of respective tissues. A, B, C, and D represent Lymph node lesions, Tonsils, Lung, and Ileum, respectively.

Bodyweight reduction after heterologous challenge

We had monitored the daily weight gain after the challenge with heterologous PCV2d virus. Results show that the weight gain in both vaccinated groups was significantly higher compared to the unvaccinated control at day 21 post-challenge. There was not a significant change in body weight among the vaccinated groups (Figure 3.8).

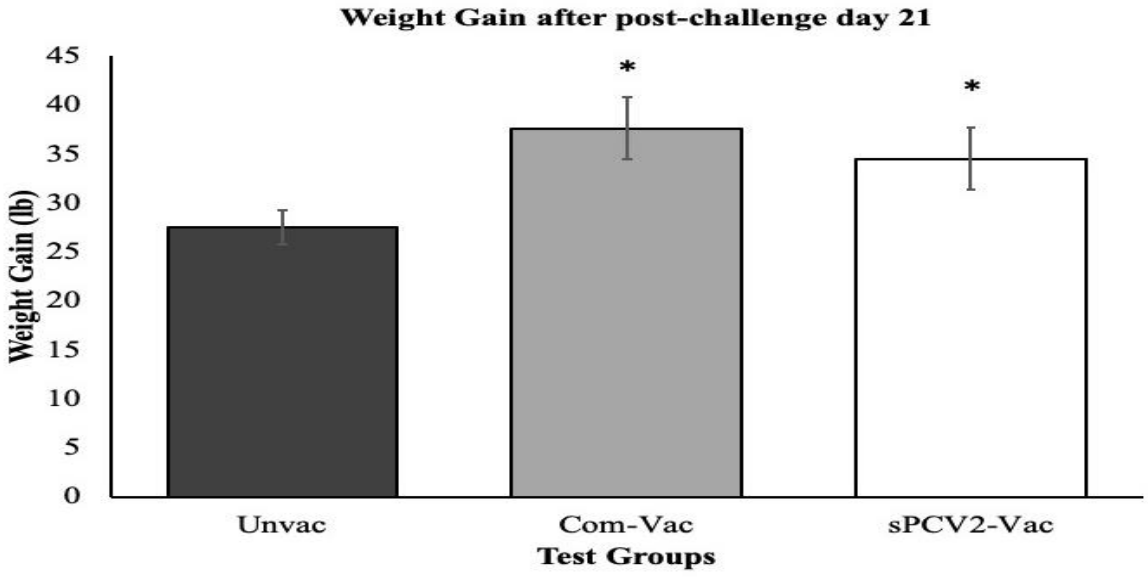

Figure 3.8: Weight gain after the challenge.
Y-axis showing the weight gain in-lb and X-axis represents the groups. * are significantly different from the unvaccinated control, $p \leq 0.05$, Students *t*-test.

Serum immune pressure attenuates sPCV2-Vac by induces stop mutations in cell culture

To check if the minimal selective immune pressure causes the attenuation or not, we had treated both of the wild-type and sPCV2-Vac viruses with serum antibodies, up to passage five, and checked the through immune fluorescence assay (IFA). The IFA pictures of the selective immune pressure experiment show that the replicative virus foci are going down over the

passage, indicating the attenuation of the sPCV2-Vac. In contrast, the wild-type virus foci got increased over the passages (data not shown). This finding indicates that sPCV2-Vac can be attenuated in cell culture over the passages and seeks further investigations towards the reasons for attenuation in cell culture.

To investigate the reasons for attenuation in cell culture for both with serum treatment, we conducted a next-generation sequencing from the pooled samples of the different passages of selective immune pressure treatment. The result shows that the sPCV2-Vac stop mutation at least five targeted codons, whereas there was no generation of the stop codons in the wild-type virus under the serum treatment. Specifically, we have seen the leucine to stop codon in the 23rd, 49th, 80th position and serine to stop codon in the 50^{th} and 90^{th} position. Additional to the stop mutation generation, according to the PAM 250 matrix, we have seen some deleterious mutations to the target and non-targeted regions of the sPCV2-Vac candidate. On the other hand, we have seen only one transversion mutation for the wild type, which converts to valine from leucine (Table 3.2).

Table 3.2: Effect of immune selection pressure.

Initial codon	Codon change	Mutation/ position #	Frequency of detection	Type	PAM 250 Score	Entropy	Fitness
sPCV2-Vac							
TTA	TGA	L 23 STOP*	7204/7204	Tv			
CGC	GGC	R 24 G	7122/7122	Tv	-3	0.54	-0.3
TTG	TGG	L 29 W	7269/7269	Tv	-2	0.18	-0.39
TAC	GAC	Y 36 D	1973/7080	Tv	-4	0.18	-0.39
TGG	AGG	W 38 R	1849/7076	Tv	2	0.16	-0.30
TTA	TGA	L 49 STOP*	1314/7118	Tv			
TCA	GCA	S 50 G	7145/7149	Tv	1	0.99	-0.18
TCA	TGA	S 50 STOP*	7321/7321	Tv			
ATC	ACC	I 57 T	1196/6743	Ti	-1	1.07	-0.27
CGA	CCA	R 59 P	1198/6757	Tv	0	2.1	0.34
TCG	TGG	S 66 W	6800/6800	Tv	-2	0.47	-0.31
AAT	ACT	N 77 T	492/5391	Tv	0	1.74	-0.11
TTG	TAG	L 80 Stop*	5091/5092	Tv			
CCC	GCC	P 81 A	4612/5057	Tv	1	0.35	-0.28
GGA	CGA	G 83 R	410/5189	Tv	-3	0.47	-0.40
TCA	TAA	S 90 Stop*	4593/4593	Tv			
PCV2b Wildtype							
CTA	GTA	L 167 V	7372/7391	Tv	2	0.69	-0.25

Shaded text – target serine and leucine codons which were mutated

*Modified serine or leucine codons which mutated to stop codons

PAM250 score – 0- equivalent substitutions which occur at a frequency predicted by chance, >0 - favorable substitutions which occur more frequently than predicted by chance, <0-unfavorable substitutions which occur less frequently than predicted by chance

Ti – Transition, Tv – Transversion

Entropy score- Shannon's sequence entropy score from DeMaSk. Higher values indicate residues with a greater tendency towards substitution, Low values indicate conserved residues with high fitness consequences when substituted

Fitness score - 0 – No fitness change, <0– loss of fitness, >0 – gain of fitness.

Discussion

Although most of the mutations are deleterious for the viruses, especially for the RNA viruses (Cuevas, Domingo-Calap, & Sanjuán, 2012), they tend to have mutations to evolve new phenotypes to evade host immune responses. Organisms maintain the mutational robustness, by maintain the consistency of the phenotype over genetic variations and make balances between

tolerating the deleterious mutations and gaining the beneficial mutations (Montville, Froissart, Remold, Tenaillon, & Turner, 2005). RNA viruses and ssDNA viruses have low mutational robustness and high mutation rate (Sanjuán, 2010). It's believed that the high mutation rate of the RNA virus is due to a lack of proofreading activity of the RNA polymerase. Therefore, the RNA viruses are getting benefited from this lack of proofreading activity by evolving to new strain via random mutations caused by RNA polymerase activity. Small ssDNA viruses like PCV2 also have a very high mutation rate (10^{-3} to 10^{-6}) within the range of the RNA virus (Correa-Fiz et al., 2020; Firth et al., 2009; Franzo et al., 2016). In general, to survive, viruses must block host immunity by making proteins that interfere with the host immune pathway or have to escape the immunity system by evolving to a new variant. Although, big RNA viruses can use both strategy, the simple and small RNA virus or ssDNA viruses mostly relies on the strategy to evolve into new strains to avoid host immunity (Correa-Fiz et al., 2020). Therefore, as expected, small ssDNA virus-like PCV2 is evolving to new strains, and in the last few years, multiple versions of PCV have emerged in the swine population. Although it's not clear exactly why PCV2 has a very high mutation rate in the range of RNA viruses, but it's proposed that low mutational robustness can drive towards high mutation rates for ssDNA and RNA viruses (Sanjuán, 2010). Additionally, it has discussed in many previous publication that the vaccine selection pressure, natural selection, selective immune pressure, and international pig trade might have responsible for the higher diversity of the PCV2 by mutations (Correa-Fiz, Franzo, Llorens, Segalés, & Kekarainen, 2018; Lv et al., 2020; Wang et al., 2019). Hence, its anticipated that it continues to

evolve into a new subtype of the virus because of its high mutation rate. The suicidal replication strategy by genetic recoding uses the benefit of high mutation rate of the virus. In this method, we shift the targeted codons closer to nonsense codons, where the stop codons will be apart by only one mutation. So, genetic recoding increases the chances of having nonsense mutations into the genome and eventually will attenuate the virus in vivo. As the strategy is based on the high mutation rate of the virus, in this study, we have successfully used it against the highly mutating ssDNA virus PCV2.

Other genetic recoding strategies to attenuate the virus is including but not limited to codon deoptimization, reducing codon pair bias, and CpG/UpA dinucleotide biases (Atkinson, Witteveldt, Evans, & Simmonds, 2014; Coleman et al., 2008; Van Leuven et al., 2021). In reducing codon pair bias strategy, poliovirus was successfully attenuated while targeting the underrepresented codon pairs by introducing 631 mutations in the gene of around 2.5 kb (Coleman et al., 2008). Similar to reducing codon pair bias, codon deoptimization and CpG/UpA dinucleotide biases require the introduction of a high number of mutations. In contrast to these strategies, the suicidal replication genetic recoding targets only two amino acids of a gene with high codon redundancy. Our strategy has altered only the Leucine and Serine codons of ORF2 towards the neighboring codon of the nonsense codon. Therefore, the total number of mutations covers only less than 4% of the total gene size and therefore can minimize or avoid the effect of codon pair bias or codon deoptimization or CpG/UpA dinucleotide biases (Atkinson et al., 2014; Coleman et al., 2008; Van Leuven et al., 2021). Certainly, there are still chances that the

neighboring codon of stop codon might be underrepresented into the target host; therefore, we cannot rule out a little effect of these strategies towards attenuation. Another reason for selecting the serine and leucine codons in their codon redundancy, both of the amino acid has at least six codons, and 2 are neighbors of the stop codon (Moratorio et al., 2017).

As we targeted for a safe and rapid attenuated vaccine, clearing the vaccine virus from pig serum on day 14 of post-vaccination indicates the test vaccine was safe. However, clearance of vaccine viruses at day 14 limits the scope of the study to understand the reason for in vivo attenuation. Therefore, not collecting the serums before Day 14 was a drawback of the study. While it's not feasible to repeat pig study with a limited budget, we have designed an in vitro study to understand the reason for the attenuation. However, as it's not possible to create the in vivo environment in vitro, we have introduced selective immune pressure using the protective neutralizing serum from the study. Here we have tried to identify the frequency of the generation of mutations induced by the neutralizing serum as selective immune pressure. For the RNA viruses, it has been shown that genetic recoding by suicidal replication group shows a significantly higher amount of mutations and, specifically, generation of the stop mutation (Moratorio et al., 2017). Similarly, we also have a significantly higher frequency of generation of stop codons in targeted leucine and serine codons in the sPCV2-Vac than the wild-type virus. Besides the stop codon generation, we have also identified multiple point mutations in the target codons. Although Moratorio et al. did not mention any non-target mutations in their report (Moratorio et al., 2017), we have identified few point mutations towards the non-target regions

in the sPCV2-Vac strains (Table 3.2). While checking the point mutations on the point accepted mutation matrix 250 (PAM 250) (Pearson, 1990), we found that most of the mutations are unfavorable with a negative value. Therefore, as expected, the generation of the stop mutation and other unfavorable point mutations have contributed towards the attenuation. However the other gene ORF1 was not checked as no changes were introduced on that gene.

Even though, direct comparison of our test vaccine with the commercial vaccine is not possible, as a commercial vaccine is optimized with dose and adjuvant. Additionally, due to the unavailability of commercial live vaccines, we have used the inactivated vaccine. In comparison, our test vaccine shows a lower serum antibody response against the PCV2 virus (figure 3.3). However, instead of a lower magnitude of antibody response, our test vaccine has shown better heterologous neutralizing antibodies compared to commercial vaccines. Although it's not clear how the test vaccine shows the protection against heterologous strain, a possible reason might be the production of truncated protein during translation and nonviable or defective viral genomes during replication as a result of suicidal replication. The truncated protein could have given better protection by initiating effective activation of the innate immune system (Dabaghian, Latifi, Tebianian, Dabaghian, & Ebrahimi, 2015; Kovac et al., 2011). On the other hand, the produced nonviable genome might be acting as a natural adjuvant and induce the immune system (Yount, Kraus, Horvath, Moran, & López, 2006). In addition, the defective viral genome in vivo can trigger antiviral immunity by inducing a critical danger signal (Tapia et al., 2013).

There are strong antibody responses towards the marker peptide that was inserted at the 3' of the ORF2, although it was inserted at 5' of the ORF2 in our previous publication (A. Rakibuzzaman et al., 2020). Therefore, as described in our previous publication, this marker will helps to differentiate between the vaccinated and infected animals and eventually help eradicate the virus from the swine population by stamping out strategy in a systemic manner (Francis, 2018; A. Rakibuzzaman et al., 2020).

Conclusion

In summary, we have produced a rapid, safe, and effective in vivo attenuated vaccine for the highly mutating PCV2 virus, which is better compared to the commercial vaccine used in the study. Because the strategy used in the vaccine preparation, it will minimize the chances of viral evolution from the vaccine virus. However, a further detailed study is needed to understand the exact mechanisms involved in virus attenuation, induction of heterologous protective antibodies with frequent sample collection.

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Conflicts of interest

The authors declare no conflict of interest. The funding agencies played no role in design, data collection, interpretation or publication of this work.

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CHAPTER 4: IN VITRO CHARACTERIZATION OF A RAPID-RESPONSE PEDV VACCINE WITH AN EFFICIENT ORAL DELIVERY SYSTEM[3]

Abstract

The world has faced multiple epidemics and pandemics in past decades in both the human and animal world. RNA viruses are primarily responsible for these outbreaks. There is a need for a standard rapid-response vaccine method that can be used for emergency preparedness, to prevent the initial spread of the virus and disease. Using the porcine epidemic diarrhoea virus (PEDV) as a model, we have developed a novel technology for rapid response vaccines for RNA viruses. By heating PEDV to 44˚C for 10 min to reversibly unfold the viral capsid, followed by the addition of RNase to degrade the RNA genome, viral replication was diminished while immunogenic structures were preserved. Vaccination of weanling piglets induced sterilizing immunity, combined with a high safety margin as the vaccine virus was rapidly cleared in vaccinated pigs. However, as PEDV mainly affects newborn pigs, and protection is mediated by maternal transfer of antibodies, the goal of this study was to optimize an improved oral vaccine delivery system with the long-term goal of improving lactogenic immunity in vaccinated sows. As commercial vaccines need to be either completely inactivated or attenuated to avoid

[3] The material in this chapter was co-authored by AGM Rakibuzzaman, and Sheela Ramamoorthy. AGM Rakibuzzaman had primary responsibility for conducting experiments, preparing vaccines candidates, and analysis the collected samples from the animals. AGM Rakibuzzaman was the primary developer of the conclusions that are advanced here. AGM Rakibuzzaman also drafted and revised all versions of this chapter. Sheela Ramamoorthy served as a proofreader and checked the math in the statistical analysis conducted by AGM Rakibuzzaman.

incomplete inactivation, the endpoints for complete inactivation and attenuation were established at 5 hours and 4 hours, respectively. The oral delivery system (niosomes) composed of biodegradable ingredients, namely non-ionic surfactants, cholesterol, and a charge stabilizer, was optimized. The niosome formulation had an antigen loading capacity of over 80%. At an Effective Concentration 50 (EC50) value of 500.0 µg/mL, the cytotoxicity of the niosome preparation was negligible. The developed oral vaccine delivery method is completely biodegradable, non-toxic, requiring minimal time for preparation with a high entrapment efficiency and low cytotoxicity. Therefore, it has broad applicability for orally delivered vaccines, considering that oral delivery is the preferred method for vaccine delivery in epidemic or pandemic situations due to the ease of administration.

Key Words: Rapid response vaccine, PEDV, Niosome, Oral vaccine delivery

Introduction

In the past two decades, multiple infectious diseases have stricken and killed many populations in both the human and animal worlds. Most of these are caused by multiple viral diseases, especially RNA viruses. The flaming example of the emerging diseases is the current covid-19 pandemic, and millions of people have already died because of it. Other notable examples of these human viral infections are the Ebola virus, Middle East Respiratory Syndrome (MARS) virus, Swine Influenza virus, and Zika virus, which have also caused outbreaks that had been declared an epidemic (Lowe et al., 2018; Roychoudhury et al., 2020). However, it's believed that most of these viruses have been jumped from animal to human through zoonotic

transmission (Bender, Hueston, & Osterholm, 2006). The best example for the animal emerging and re-emerging viral diseases are the porcine epidemic diarrhoeal virus (PEDV) (C.-M. Lin, Saif, Marthaler, & Wang, 2016), porcine circovirus type 2 (PCV2) (Patterson & Opriessnig, 2010), porcine respiratory and reproductive syndrome virus (PRRSV) (W.-H. Lin et al., 2020; Nathues et al., 2016; Tian et al., 2007), African swine fever (ASF) (Gallardo et al., 2015), etc. In 2013 PEDV emerged in the U.S started in April, spread to most of the swine-producing states in a few months, and caused losses of several million U.S dollars to the industry within two years (Jung & Saif, 2015). Despite the rapid increase in the number of newly emerging viruses in human and animal health, there are very few effective and safe platforms for the development of attenuated, rapid-response vaccines, which are critical for emergency/pandemic preparedness plans.

Developing effective vaccines for RNA viruses is complicated because of the rapid emergence and evolution of new strains (Carrasco-Hernandez, Jácome, López Vidal, & Ponce de León, 2017), and genetic and antigenic variability of the RNA viruses (Stephenson, 1985), with PRRSV in swine and HIV in humans being classic examples (Figlerowicz, Alejska, Kurzyńska-Kokorniak, & Figlerowicz, 2003). Traditional methods of vaccine development require a long time for development, production, and licensing. This lengthy process allows the virus to transmit the disease and spread rapidly, and cause significant socio-economic losses (Jennings, Monto, Chan, Szucs, & Nicholson, 2008; Noah & Fidas, 2000; Smith, Lipsitch, & Almond, 2011). This work provides proof of concept for a novel strategy for the rapid attenuation or

inactivation of PEDV from our previous publication (G. Singh et al., 2019), which can be applied to any other emerging RNA viruses.

PEDV virus is a swine coronavirus that mainly targets swine enterocytes and causes severe diarrhea, especially with newborn piglets. The last PEDV epidemic in the U.S. killed one-quarter of the neonatal swine population (Schulz & Tonsor, 2015). As the neonatal piglets don't have a strong immunity system or time to develop adaptive immunity by vaccination, strong lactogenic IgA antibodies in sows are critical for protecting the suckling piglets. Lactogenic IgA antibody and gut-associated mucosal immunity can be achieved by targeting intestinal enterocytes through the oral route of administration. Although some oral vaccines against PEDV are available, the protection of suckling piglets are not optimal (Crawford, Lager, Kulshreshtha, Miller, & Faaberg, 2016; Langel, Paim, Lager, Vlasova, & Saif, 2016; D. Song, Moon, & Kang, 2015). Therefore, improving current oral immunization methods can improve lactogenic immunity against PEDV.

Traditional methods for developing live attenuated vaccines are time-consuming and will not fit with the rapid-time frame needed for the first response vaccine for epidemic/pandemic emergencies. There are many oral delivery techniques available nowadays. For live attenuated vaccines, some vaccine candidates may successfully pass the gastric environment and replicate in host cells to trigger the immunity system (Embregts & Forlenza, 2016). Drug encapsulation via different polymers or liposomes is a popular medium for oral delivery. Similar strategies are used for live attenuated vaccines. Liposomes can deliver DNA efficiently due to the net negative

charges of the DNA but cannot efficiently be used for protein delivery as proteins are not negatively charged. On the other hand, niosomes or non-ionic surfactant vehicles (NISV) can easily uptake any molecules and are easy to prepare compared to liposomes.

Considering the gap in standard methods for developing first response emergency vaccines and an effective oral vaccine delivery system, we have developed a unique method to rapidly develop attenuated or inactivated vaccines based on our previous publication (G. Singh et al., 2019). We have treated the viruses with heat to unfold the viral capsid and then RNase treatment to degrade the genetic material to attenuate or inactivate the virus. In this study we have find out the endpoint of the attenuated or inactivated vaccines and have combined the heat and RNAse treatment method for rapid-response vaccine development with a niosome based oral delivery system and demonstrated effective packaging of the vaccine virus in the niosome. Potentially, the methods developed have broad application to other RNA viruses and enteric pathogens.

Methods and materials

Cell and viruses

Vero C1008 (ATCC 1586) was used to culture PEDV. The PEDV virus strain PEDV CO2013 (National Veterinary Services Laboratory (NVSL), AMES, IA) was used to infect Vero cells to prepare the virus culture. For culturing the virus, virus growth media was prepared with 1X Dulbecco's modified eagle medium (Corning, Corning, NY, USA), 1x antibiotic antimitotic (Gibco/ Thermo-Scientific, Waltman, MA, USA), 7% of tryptose phosphate broth (Invitrogen/ /

Thermo-Scientific, Waltman, MA, USA), and 100 ug/ml porcine trypsin (USA). The virus culture prepared was titrated three times to obtain the TCID50 using the Reed-Muench formula (Reed & Muench, 1938), and 1ml aliquots of the virus culture were kept at -80˚C for future use.

Complete inactivation of PEDV

The previously developed heat and RNAse treatment protocol for rapid-response vaccine development (G. Singh et al., 2019), was modified to achieve complete inactivation. Briefly, the virus culture was resuspended to a titer of $2x10^5$ $TCID_{50}$/mL. To unfold the viral capsid, the culture was placed in a water bath at 44˚C for 10 min. RNase A (Amresco, Solon, OH, USA) and RNase T1 (Thermo Scientific, Waltham, MA USA) were added to the virus culture at a final concentration of 200µg/ml and 2000 units/ml, respectively. The virus culture was incubated at 44˚C for 3,4,5 and 6 hours to arrive at the endpoint for inactivation. The cultures were then placed at 4˚C for 1 hour to refold. The heat and RNase treated virus cultures from the various time points were used to infect semi-confluent Vero cells in a 6-well plate and 8-well chamber slide. The treated viruses and an untreated control were passaged three times in Vero cells to ensure inactivation, as assessed by an immunofluorescence assay (IFA).

Immunofluorescence assay

An IFA was conducted as described previously (Okda et al., 2015; Y. Song, Singh, Nelson, & Ramamoorthy, 2016) to visualize the viral replication to ensure inactivation. Briefly, the infected cell culture was fixed 1:1 acetone and methanol for a two hours and then stained

with polyclonal swine anti-PEDV sera and checked under a fluorescence microscope for cytoplasmic fluorescence; the characteristics of replication of RNA virus.

Rapid-attenuation of PEDV by heat and RNAse treatment

To rapidly attenuate PEDV by heat and RNase treatment, virions were subjected to the treatment in such a way that they could be rescued after three serial passages in cell culture, based on the premise that RNA damage due to treatment and repair during viral replication in cells would lead to the accumulation of non-lethal mutations or deletions which would result in viral attenuation. The heat and RNase treated virus was passaged three times in Vero cells to rescue virions with repaired genomes. To purify attenuated isolates, a plaque assay of the rescued virus was performed. Vero cells were grown 95-100% in a cell culture dish. Cells were washed 2X with hank's balanced salt solution (HBSS) (Corning, Corning, NY, USA). Logarithmic dilution of the rescued virus culture was added to the wells of the 6-well plate and incubated at 37˚C for 3 hours. After the incubation, the virus culture was removed completely, and 0.6% of ultra-pure Agar (Invitrogen, Waltham, MA USA) was mixed with the 2x infection media as 1:1 and layered on the top of the cell sheet. The culture dish was allowed to solidify in the hood for 10 minutes and kept at 37C for 48-72 hours for plaque formation. Six clearly separated plaques were picked with the tip of the pipette tips and placed on 500ul of 1x infection media. Then it was vortexed vigorously to release the virus from the agar to the media. The PEDV isolates were passaged three times on the Vero cells to ensure viability. Viral RNA was extracted from 4

plaques and will be submitted for next-generation sequencing to map possible attenuating mutations.

Oral delivery formulation

To package the vaccine virus for enhanced oral vaccination, Brij 93 (Spectrum Chemical, Gardena, CA, USA), cholesterol (M.P. Biomedical LLC, Solon, OH, USA), and Diacetyl Phosphate (DCP) (Sigma-Aldrich, St. Louis, MO, USA) were used to prepare the base niosomes, as described by Pardakhty et al. and Hassan et al. (Hassan, Brewer, Alexander, & Jennings, 1996; Pardakhty, Varshosaz, & Rouholamini, 2007). Briefly, a total of 300µM of Brij93, cholesterol, and DCP were tested in different molar ratios, like 7:3:1 (C1), 6:4:1 (C2), 5:5:1 (C3), and 4:6:1 (C4). The ingredients were appropriately weighted and mixed in a glass vial. 15ml of chloroform (VWR, Randor, PA, USA) was used to dissolve all components by vortexing vigorously and heated to 130˚C in a fume hood until complete evaporation of chloroform to form a thin film. Then 5 ml of PBS, preheated to 37˚C, was added to the vial and mixed vigorously to make a milk-like solution which was incubated at 60˚C for 2 hours to form the niosome.

Entrapment of viral antigen

The entrapment of the viral antigens was conducted as described in Hassan et al. 1996 (Hassan et al., 1996) with slight modification. Briefly, the viral antigen was mixed at a ratio of 1:9 with niosomes and then froze at liquid nitrogen for 1 minute and completely thawed at 37C in a water bath. The freeze and thaw process was repeated five times to complete the viral entrapment.

Loading capacity of niosome

The antigen loading capacity was determined by separating the free and entrapped viral antigen with a 0.22 µm filter followed by a modified ninhydrin assay as described by Bradford et al. (Bradford, 1976). Samples were filtered with a 0.22 µm filter (VWR, Randor, PA, USA) to separate the free viral antigen and entrapped viral antigen and dried on a heat block at 110˚C. Samples were dissolved with 150.0µL of 13.5 N NaOH and hydrolyzed by heating at 110˚C for 20 min. NaOH was inactivated with 250µL of glacial acetic acid. 500µL of 2% ninhydrin solution (Sigma-Aldrich, St. Louis, MO, USA) was added, mixed vigorously, and heated on a heat block for 20 min at 110˚C. Occasional vortexing during incubation was performed. After the incubation with ninhydrin, the samples were allowed to come to room temperature. A total of 50µL of this solution was transferred to a 96-well plate, and 150µL of 50% isopropyl alcohol was added to each well. After mixing by pipetting, the absorbance was read at 570nm using an ELISA reader. The protein concentration was measured using a standard curve of doubling dilutions of a BSA solution. The encapsulation efficiency was measured by using the following formula:

Efficiency = ((total protein content- protein content on the flow through (free antigen))/ total protein content)) x 100

Cellular cytotoxicity assay

The Vero cell line was used to assess the cytotoxicity of the prepared niosome. The EC50 of the prepared niosome was obtained using a (3-(4,5-dimethylthiazol-2-yl) Tr-2,5-

diphenyltetrazolium- bromide) (MTT) (Thermo Scientific, Waltham, MA) based assay. Briefly, cells were seeded in a 96-well plate and allowed to grow overnight in a CO2 incubator. Different concentrations of the prepared niosome (5000 – 500 µg/mL) were added and incubated for 6 hours. After the incubation period, 20ul of 5.0 mg/ml of MTT solution was added to each well and incubated for 4 hours. After the 4 hours incubation, media was aspirated carefully by not disturbing the cell sheet, and 100ul of dimethyl sulfoxide (DMSO) (Thermo Scientific, Waltham, MA) was added into each well and incubated for 10 min. Wells were mixed to dissolve the formazan. Absorbance was measured at 570 nm in an ELISA reader (Biotek, Winooski, VT). Controls included Vero cells incubated with growth media to represent O.D. values for 100% cell survival control, only growth media and 0.1% of Triton X-100 (Invitrogen, Thermo Scientific, Waltham, MA) treated cells to represent controls with 100% cell death. Finally, the cell viability was calculated by using the following formula, and EC50 was determined at the 50% end point.

Cell viability = (absorbance of sample / absorbance of cell control) x 100

Light microscopy

A light microscopy was done to visualize the niosomes size under 100x magnification, using Olympus BX-61 (Olympus, Waltham, MA, USA) microscope.

Electron microscopy

To visualize the structural integrity of the treated virus and the size of the niosome, 3,4, and 5-hour heat-treated virus were subjected to analysis under electron microscopy by standard

negative staining method (Booth, Avila-Sakar, & Cheng, 2011). Briefly, samples were added to UV-activated grids and incubated for 10 min for absorption. Then, excess samples were soaked by touching with a filter paper and drying it for 30-60s, followed by staining with 0.1% phosphor tungstic acid (PTA). Stained grids were examined at the NDSU electron microcopy center with a JEOL JEM-100CX II transmission electron microscope.

Statistical analysis

A significance level of $p < 0.05$ was used for all statistical analysis. Analysis was conducted using the Microsoft excel. Antigen loading capacity was analyzed by a Student's t test. The consolidated values, statistical significance and standard deviation are represented in the figures.

Results

Heat and RNAse treatment can effectively inactivate PEDV

Exposure of the PEDV virus culture to heat and RNAse treatment at 3,4,5 and 6 hours showed that the virus was incompletely inactivated at 3 and 4 hrs as it could be rescued at passage 2 and passage 3, respectively. However, the virus was completely inactivated at the 5hr and 6 hr time points. (figure 4.1).

Heat and RNAse treatment can be used to isolate attenuated PEDV variants

To isolate attenuated PEDV strains carrying attenuating mutations due to damage and repair of the RNA genome due to heat and RNAse treatment, the treated virus culture was subjected to a plaque assay using passage 3 of the 4-hour time point. Multiple single plaques

(figure 4.2) were isolated and are being analyzed for mutations, insertions or deletions by next-generation sequencing

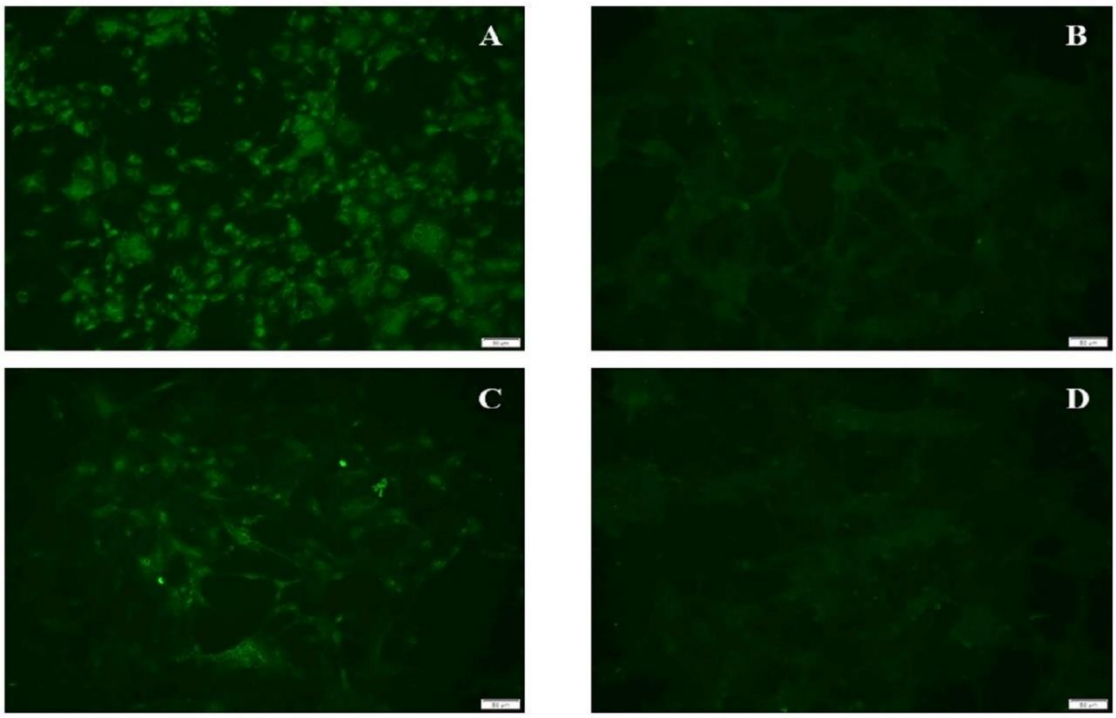

Figure 4.1: Immune fluorescence assay (IFA) of treated PEDV virus.
Virus infected cells were stained with anti-PEDV polyclonal antibody. Green cytoplasmic fluorescence indicates viral replication. A. virus with out treatment, B. cell control, C.4 hour treatment after passage 3, D. 5 hour treatment at passage 3

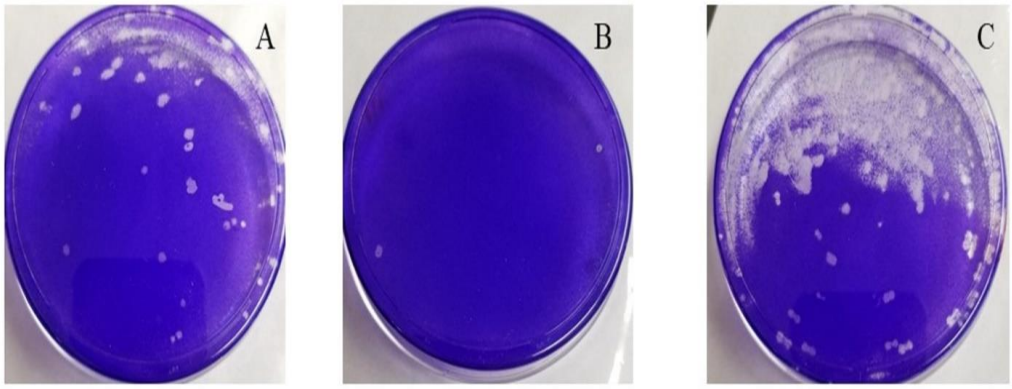

Figure 4.2: Plaque assay to isolate single strain.
The plaques were observed under microscope and picked before the staining. A crytal violet staining were performed after the fixation of the cells with formaldehyde. A. 4 hour heat and Rnase treated sample from passage 3; B. Cell control; C. virus control.

Heat and RNAse treatment does not affect structural integrity

Examination of the treated virus cultures from the 4 and 6 hour time points indicated that the viral spike protein was intact (figure 4.3), although the virus was not rescued even after 3 passages by IFA for the 5 hour time point. These indicate although the virus was inactivated, the outer spike protein of the virus was not destroyed.

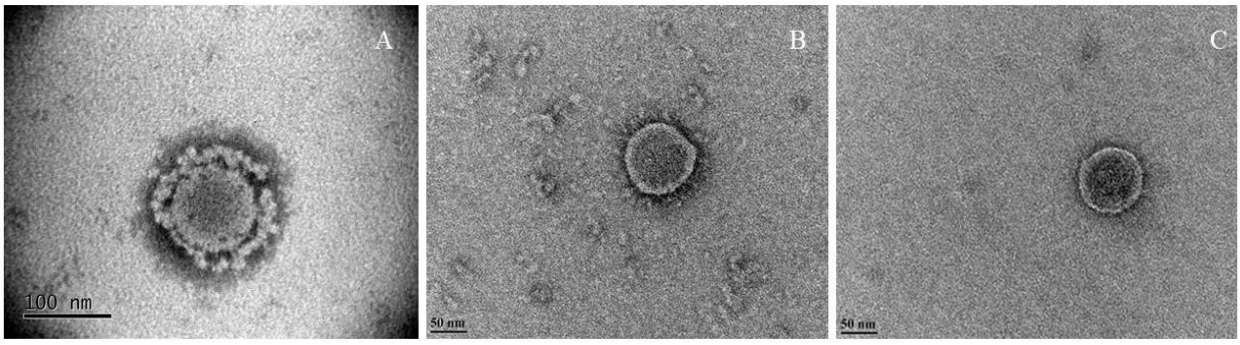

Figure 4.3: Electron microscopy of PEDV viruses.
A. Untreated virus; B. Heat for 4 hour at 44˚C; C. Heat for 5 hours at 44˚C

Niosomes are efficient in antigen entrapment

The heat and hand-shaking method produced variably sized niosomes. Based on light and electron microscopy, the size range of the niosomes prepared by this method was from 200nm to 4000 nm. No significant differences in size were seen between the varying molar ratio combinations used to optimize the preparation of the niosomes (figure 4.4). However, the majority of the size was around 2000 nm, which was also confirmed by electron microscopy (figure 4.2E). We found that the 6:4:1 (C2) formulation had a loading capacity of over 80%, with the maximum entrapment efficiency (figure 4.5). Therefore, niosome prepared from the C2 molar ratio was selected for further use.

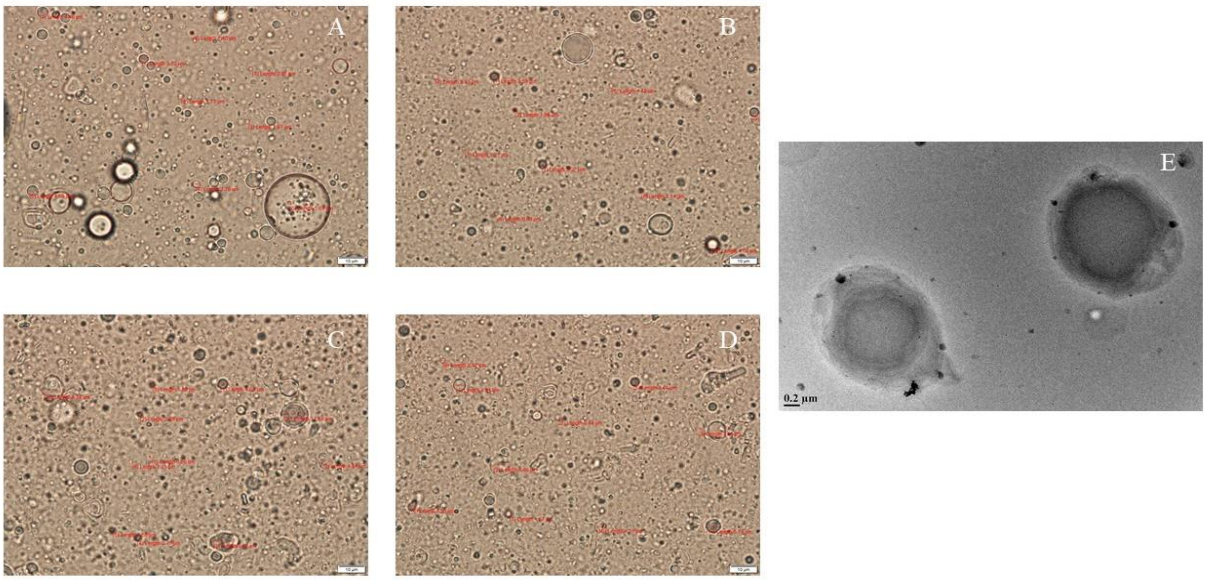

Figure 4.4: Microscopy of nisome to determine the sizes.
A-D light microscopy for the molar concentration of C1 to C4 respectively. E. Electron microscopy of the C2 concentration.

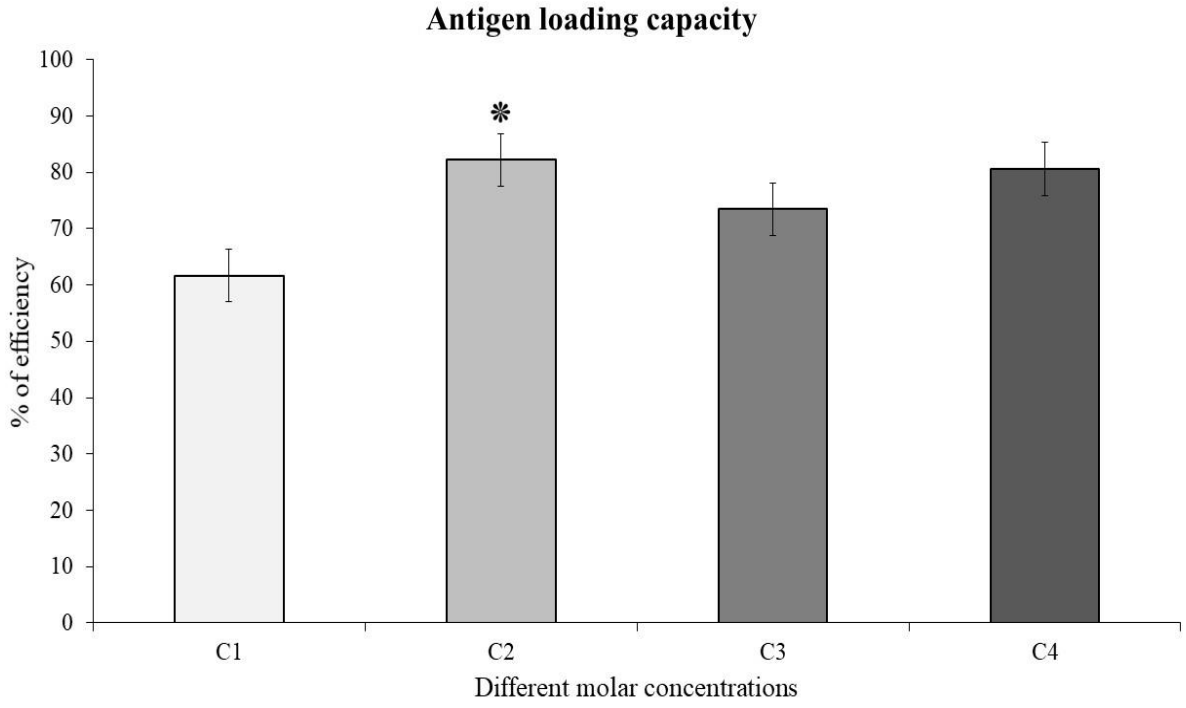

Figure 4.5: Entrapment efficiency of prepared niosome.
X-axis— different molar concentration used. Y-axis % of antigen loading capacity. Error bars indicate the standard deviation, *-significantly different from the C1 and C2, $p \leq 0.05$, Students t-test

Niosomes are moderately cytotoxic in vitro

To determine the cellular cytotoxic effect of the prepared niosome C2, we have measured the EC50 of the preparation by an MTT based cellular viability assay. We have found a greater value of EC50, which is about 500 µg/mL for our prepared niosome. However, at 1000 µg/mL of niosome, the in vitro cell viability is reduced at only 20%.

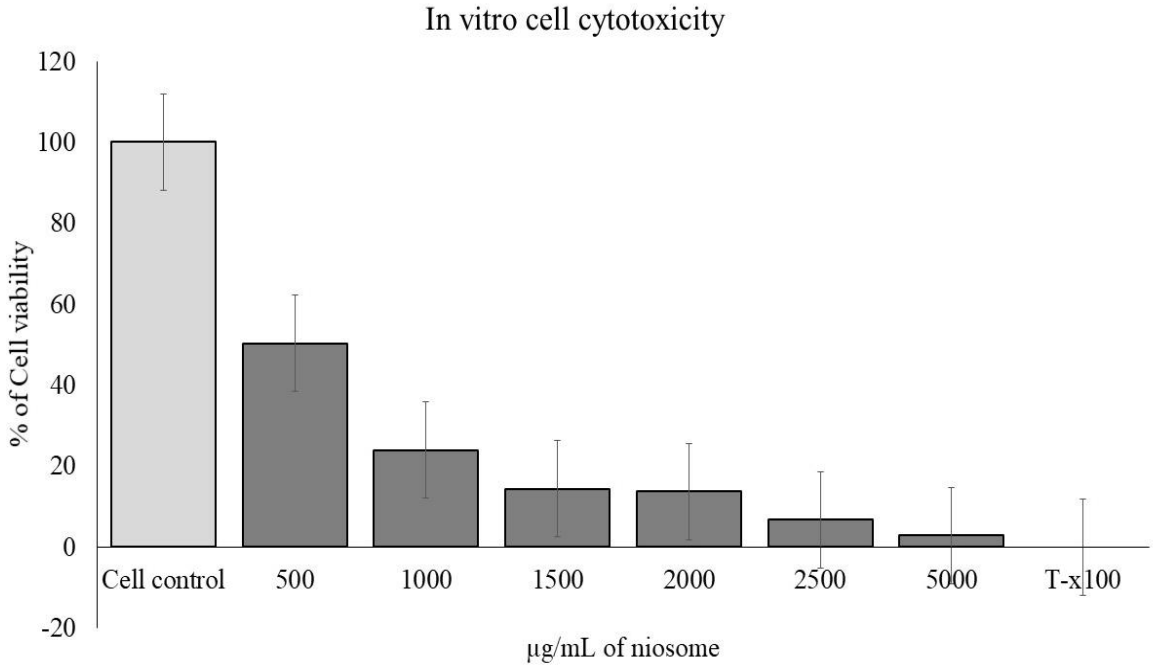

Figure 4.6: Cytotoxicity of prepared niosome.
X-axis- different concentrations of niosome used. Y-axis % of cell viability. Error bars indicate the standard error.

Discussion

The recent outbreaks of SARS-CoV in 2003-2004, H1N1 in 2009, MERS in 2012, ebola in 2013-16, and zika virus in 2015-16 showed the critical importance pandemic preparedness plans (Finlay, See, & Brunham, 2004; Wong & Qiu, 2018). To meet the need for rapid response

vaccines, improved methods for first-response vaccine development are important (Finlay et al., 2004). The coalition for epidemic preparedness innovations (CEPI) was established in 2016 (Gouglas, Christodoulou, Plotkin, & Hatchett, 2019; Gouglas et al., 2018). One of its main goals is to develop vaccines for 11 different infectious pathogens for emergency preparedness since its formation (Gouglas et al., 2019). However, unfortunately, the traditional approaches for vaccine development are not designed for rapidity, scalability, stability and ease of use in epidemics (Finlay et al., 2004). Since the formation of the CEPI, only a limited number of rapid response vaccine has reported, and one of them is the rapid response subunit vaccine (Wijesundara et al., 2020). Other methods for rapid response vaccine described by others involve including CD4 T-cell epitope in vaccines (Hills et al., 2016), synthetic viral particle (Dormitzer et al., 2013), and dendrimer-RNA nanoparticle vaccines (Chahal et al., 2016). One most prominent drawback of DNA vaccines is their long-lasting stability in the host; sometimes, they may stay up to 60 days (Rauch, Jasny, Schmidt, & Petsch, 2018). mRNA vaccines are also popular but can not induce proper immunity by stand-alone and always require adjuvant. Additionally, initial induction of type I IFN may reduce the antigen production from the vaccine by phosphorylation of eukaryotic translation initiation factor 2α (Rauch et al., 2018). Furthermore, inflaetory respnses may be seen within few hours by the self-replicating mRNA vaccine by upregulation of the IFN-stimutated genes (Pepini et al., 2017) . Therefore, although innovative, these technologies require in-depth knowledge about the epitopes, protein, and genomic sequences. Indeed, all of this knowledge will not be available for the new emerging viruses and will take time to identify the

target epitopes for the re-emerging viruses. Therefore, the heat and RNAse method described here is straightforward compared to others. Additionally, detailed viral genomic or proteomic knowledge, which may not be available for emerging or re-emerging viruses, is not required for the described method of attenuation or inactivation. However, a culturable virus is required by this method and might be a limitation.

Common attenuation methods of the vaccines include serial passaging (Antia, Ahmed, & Bull, 2021; Jordan & Sandig, 2014; Mayr & Munz, 1964), the introduction of deletions, or targeted modifications of virulence genes by recombinant technologies (Antia et al., 2021; Lauring, Jones, & Andino, 2010). By serial passaging, the vaccine virus is adapted to grow in a different host so that the virus grows very poorly in the original host. However, this old conventional method is haphazard as the mechanisms of attenuation are left to chance (Bull, 2015). Furthermore, a mixture of genetic variants may be present in the final product of the vaccine and maybe a significant safety concern (Depledge et al., 2014). To overcome these drawbacks of conventional methods, recombinant DNA technology is used to create modified live attenuated vaccines based on directed attenuation, to alter viral immune evasion, growth rates, and potentially improve immunity (Antia et al., 2021). However, the success of MLV's depend on exact knowledge of virulence and immune mechanisms, ability to rescue the desired mutants; a process which can be time-consuming and therefore may not be suitable for emergency preparedness. In contrast, the heat and RNAse treatment method is rapid, and will result in several potentially attenuated variants due to random rearrangement of the viral

genome; which can be isolated by plaque assay and quickly characterized by next-generation sequencing (Bull, 2015). In our previous works we also have got a mixture of PEDV variants and that induces the attenuation (G. Singh et al., 2019). Therefore, in this work to isolate the individual strain that has the potentials of attenuation by genome mapping, we have completed the plaque assay after the heat and RNase treatment. Those isolated strained will be subjected to NGS sequencing for future use.

Current methods of the inactivation of viruses for inactivated vaccines are chemical methods and gamma irradiation. Formaldehyde and beta propiolactone (BPL) are the most commonly used chemicals for the inactivation of the virus (Bonnafous et al., 2014; C. Fan et al., 2017; Y.-C. Fan, Chiu, Chen, Chang, & Chiou, 2015; Perrin & Morgeaux, 1995). Both of the chemicals interact with viral antigens like protein and nucleic acids and inactivate the virus. Therefore, these methods can reduce antigen availability to the antigen-presenting cell and reduce immunogenicity (Furuya et al., 2010; Tano et al., 2007). Gamma-irradiation is also frequently used to inactivate viruses and other pathogens (Ramamoorthy et al., 2006). However, depending on the irradiation conditions, this method can also affect the antigenic structures (Alsharifi & David, 2017). Coronaviruses are metastable and can be reversibly denatured under different physical conditions (Wang et al., 2004). We have demonstrated that PEDV viral structures are stable at 50˚C (G. Singh et al., 2019), as demonstrated by E.M. pictures in this study.

There are several molecules available to be used for oral delivery. Polymers like poly-ethylene glycol (PEG), poly D L-lacti-co-glycolic acid (PEG) PLGA) etc., Polysaccharides based carriers like chitosan, a lipid-based delivery system like liposome, and non-ionic surfactant vehicle (NISV) as niosome are common. (Pati, Shevtsov, & Sonawane, 2018; Zhao et al., 2014). Polymers might have bio-adhesin properties and can serve as an easy adjuvant, but their derivatives and residues in the host system as well as the poor biodegradability issue, have limited their practical use in drug application (Liechty, Kryscio, Slaughter, & Peppas, 2010). Additionally, a thorough knowledge of the chemistry of the polymer for the binding capacity, encapsulation, chain conformation, solubility, etc. are required, which will require optimization and may be time consuming. Thus, polymers may not apply for the rapid response vaccine delivery for epidemic situations. Liposomes are effective for the delivery of DNAs and other nucleic acids but not for proteins. On the other hand, niosomes are made of a non-ionic surfactant and cholesterol, which is completely biodegradable, non-toxic, and very easy to prepare (Kazi et al., 2010). Additionally, niosomes can deliver various types of drugs as they have a hydrophilic, amphiphilic, and lipophilic moiety in their structure (Singh, Biswas, Shukla, & Maiti, 2019). The use of niosome in some food industries ensures its safety and biodegradability (Debnath & Kumar, 2015). Furthermore, the preparation of niosome requires very little time and thus the best candidate for the rapid response vaccine delivery system and used in this study. The niosomes prepared in this study varied in size from 400nm to 3000nm. While, depending on the method, type of surfactant, and cholesterol ratio used, the niosome size

can vary from 10nm to 5000nm (Durak et al., 2020). Our result has matched with the previous finding using similar components (figure 4.4) (Pardakhty et al., 2007). However, to get a smaller size of niosome, additional steps like sonication, manual extrusion, etc., can help. (Obeid, Gebril, Tate, Mullen, & Ferro, 2017). Microfluidazation is a popular method to have a smaller niosome below 100nm (10-60 nm usually) and is used for small chemical drugs and siRNAs (Ge, Wei, He, & Yuan, 2019; Obeid, Khadra, Mullen, Tate, & Ferro, 2017). However, such a small-sized niosome will not be suitable for our purpose, as our viral particle size is around 100nm-150nm.

We have used a modified ninhydrin assay to measure the antigen loading capacity because this method is not affected by lipids. Other methods like BCA can be used when successful separation of the niosomes is possible by high-speed centrifugation. Using filtration with a 0.22um filter, we separated the free antigen from the entrapped antigens and determined that the entrapment efficiency was over 80%. In a previous finding using the same type of niosome, the antigen loading capacity was around 40-50% (Ge et al., 2019; Pardakhty et al., 2007). Therefore, the antigen loading capacity has improved potentially from the previous finding. However, one limitation with this filtration method would be the chances of losing some entrapped antigen with sizes below 220 nm. Therefore, this filtration method can not be applicable for the nanoparticle niosomes that are produced by the microfluidazation or mechanical extrusion method, where the niosome size is below 100nm.

In vitro cell cytotoxicity is not typical for niosome based delivery systems. Most of the published paper of niosome with DCP has not reported cell cytotoxicity assay (Baillie, Florence,

Hume, Muirhead, & Rogerson, 1985; Pardakhty et al., 2007; Zhang et al., 2016). DCP is a vital molecule for niosome formation, where its negative charge prevents aggregation of the niosome and increases the preparation's stability (Gianasi, Cociancich, Uchegbu, Florence, & Duncan, 1997; Obeid, Gebril, et al., 2017). Only limited studies have shown the cell viability report for charged niosome with DCP, and its EC50 is around up to 323.6 µg/ml of lipid (Obeid, Gebril, et al., 2017; Obeid, Khadra, et al., 2017). Our finding is similar to Obeid et al., where we found our preparation shows EC50 at 500.0 ug/ml of lipid concentration.

Conclusion

Overall, our heat and RNase treatment method enables the rapid development of inactivated and attenuated vaccines for RNA viruses. The oral vaccine encapsulation technology is efficient at entrapping up to 80% of the target vaccine. Both the vaccine and oral niosome preparation can be prepared within a couple of days. Therefore, this study provides proof of principle for a unique rapid response vaccine platform that can be used for emergency preparedness in the event of an epidemic or pandemic to prevent the initial spread of the virus.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest**.**

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CHAPTER 5: GENERAL CONCLUSION AND FUTURE DIRECTION

Over the past decade, despite the notable advancement in the field of vaccinology, the number of emerging disease outbreaks has increased dramatically. Recent outbreaks in the human and veterinary field and the death toll associated with these outbreaks emphasized the need for improved strategies for vaccination, diagnosis, and treatment of viral infections. Due to the high mutation rates of the RNA virus and small single-stranded DNA viruses, these pathogens can adapt to a wide host range and breach the host immune system by immune-subversion due to multiple genetic and antigenic variants. Therefore, highly mutable viruses are commonly resistant to the available vaccine and frequently emerge or re-emerge to cause outbreaks. Imunodominance is a common immune evasion strategy for viruses, including PCV2. The virus induces the host to produce non-neutralizing antibodies that cannot clear the virus from the host. As many available vaccines do not factor in the immunodominance properties of epitopes, they are suboptimal, and possibly contribute to the viral evolution and re-emerging of the diseases with a new strain. Therefore, besides the effectiveness of the vaccine, safety and sterile immunity to prevent viral evolution are great concerns in vaccinology. Thus the primary objective of this thesis to develop novel and practical strategies to rapidly develop attenuated vaccines for emergency preparedness.

In our first objective, to re-focus the immune response towards the protective antigen, we have altered the immunodominant decoy epitopes in the PCV2 capsid protein, which in turn abrogated the early non-neutralizing antibody response and eventually improved the response of

the protective antibody. Therefore, the vaccinated pigs were protected from the viral challenge. Because of the early production of the neutralizing antibody, the viral vaccine was cleared quickly from the vaccinated pigs indicating the vaccine was safe. This method can be applied rapidly within a couple of months, where an infectious viral clone is available, and for those vaccines, where viral protection is suboptimal. Additionally, this technique will be highly effective for those viruses, where virus neutralization antibody production is delayed by mounting of early non-neutralizing antibody.

Our second objective to harness the high mutation rate of viruses to rapidly attenuate viral vaccines and ensure a high safety margin. The developed approach to rationally recode the viral gene to increase the probability of having a mutation to form a stop codon is both elegant and simple, thus eventually terminating the protein synthesis during replication in vivo. The rational recoding of serine and leucine amino acids of the PCV2 capsid protein in our study resulted in a very safe but effective attenuated vaccine, which did not compromise immunity. This method is very rapid for viruses with functional infectious clones. Complete vaccine preparation can be achieved within a couple of months, starting from the recoding, synthesis to making the modified live vaccine. Thus, this rapid method will be very effective for any virus with a high mutation rate, including RNA or DNA viruses. This study's future goal is to test the strategy with other RNA viruses (like PRRSV) and the newly emerging PCV3 virus, which have spread worldwide within a couple of years.

Finally, optimized a previously established, novel but simple heat and RNase method to rapidly attenuated RNA viruses to determine the time points for either attenuation or complete inactivation. To improve the oral delivery of the rapid response vaccine, we have developed a completely biodegradable oral delivery system composed of edible ingredients, which is easy to formulate and can encapsulate over 80% of the total antigen payload. As the oral delivery system is not toxic and biodegradable, peptide ligand to target enterocytes can be added to the formulation to further improve the delivery system to target antigen delivery into a specific type of cells. For example, amino-peptidase N is the putative receptor for PEDV, and its ligand is NGR. By incorporating NGR peptides in the formulation, the antigen encapsulated in the niosome can be directly targeted to intestinal enterocytes and induce lactogenic IgA antibodies via the gut-mammary axis stimulation. Additionally, bile salts can be added to the formulation system to protect the vaccine vehicles from the adverse gastrointestinal environment. Our future goal with this delivery system is to add NGR peptide and bile salt into the niosome preparation and check its efficacy in the swine animal model by vaccinating the pregnant sows and then checking the IgA production into the milk for the protection of the piglets. Both the vaccine preparation method and oral delivery system preparation method are straightforward and quick. Vaccine preparation can be completed within a couple of weeks; thus, this method is very suitable as a rapid response vaccine for epidemic situations.

In short, this thesis addresses three major areas in the field of vaccinology A) attenuation and rational design of vaccine antigens by alteration of immunodominance properties B)

improving the safety of attenuated vaccines by directed suicidal replication C) improving oral delivery of rapid-response vaccines for enteric, neonatal infections with the long term goal of improving lactogenic protection by secretory IgA.

APPENDIX. SUPPLEMENTARY INFORMATION OF CHAPTER 2

Table A1: Amino acid sequences of Epitope A and B.

Subtype	Epitope A	Epitope B
PCV2a (AF264042.1)	124ILDDNFVTKATALTYDPY141	166VLDSTIDYFQPNNKR180
PCV2b (KR816332)	124 ILDDNFVTKATALTYDPY 141	166 VLDSTIDYFQPNNKR 180
rPCV2-Vac	124 ILDDNFVNKSTALTYDPY 141	166 VLDSTIDYFNPNNSR 180
PCV2d (JX535296.1)	124ILDDNFVTKANALTYDPY141	166VLDRTIDYFQPNNKR180

Shadowed residues-mismatches from the PCV2b vaccine (KR816332) backbone, Residues in a larger font size–residues mutated in the rPCV2-Vac, Underlined residues–putative glycosylation sites (NetNGlyc 1.0 Server, DTU Bioinformatics, Department of Bio and Health Informatics, Copenhagen, Denmark).

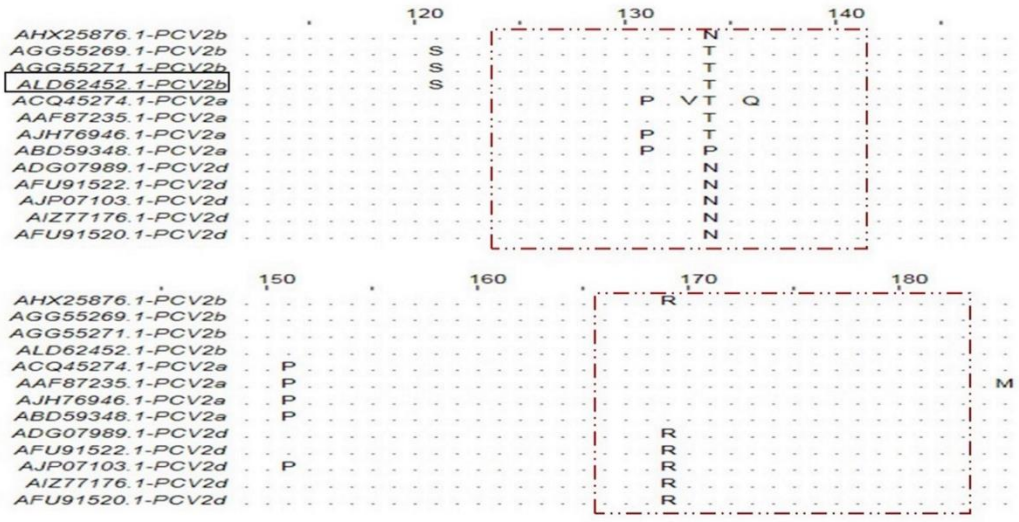

Figure A1: Multiple sequence alignment of the PCV2 capsid protein.
Selected amino acid sequences of the PCV2 capsid protein representing the major circulating subtypes PCV2a, b and d, generated using the Jal View 2.4 software. Conserved residues are indicated by dots. Boxes represent epitope A and B. The boxed accession number pertains to the rPCV2-Vac backbone.

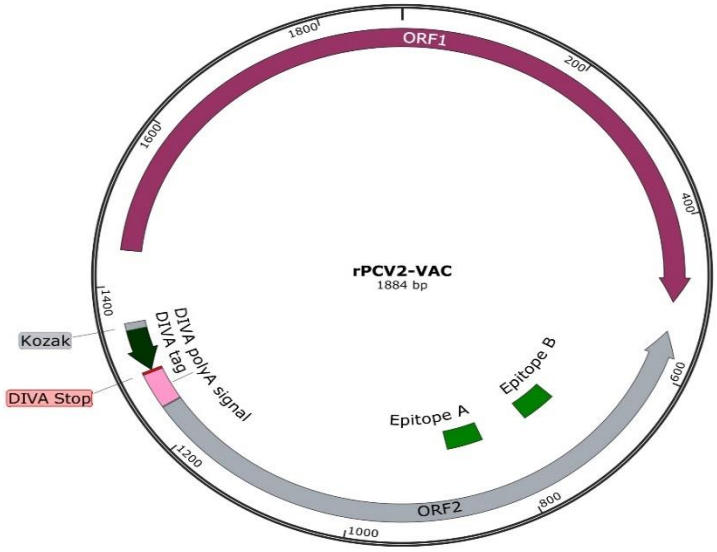

Figure A2: Map of the rPCV2-Vac construct.
Diagrammatic representation of the PCV2b infectious clone showing the PCV2b genome, major open reading frames, location of Epitope A and B and the insertion site of the DIVA tag as an independent transcriptional unit in the 5' end of the capsid gene (ORF2).

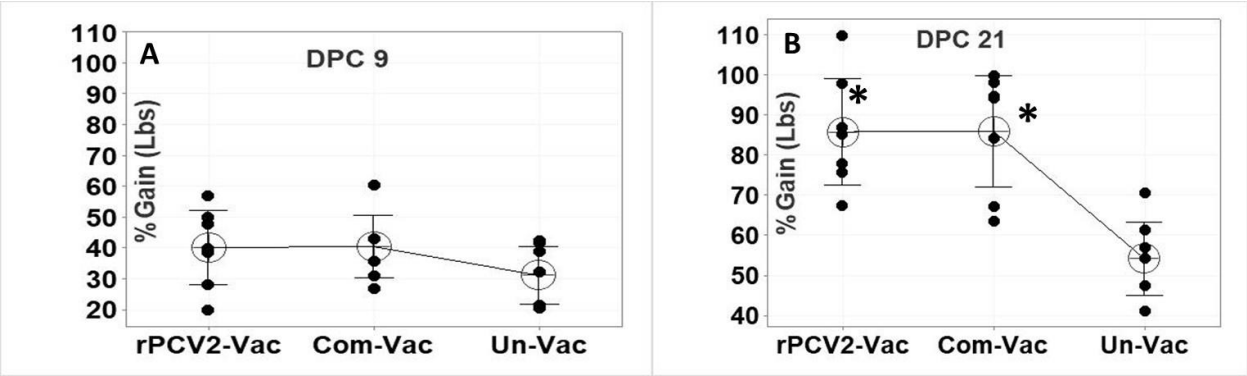

Figure A3: Post-challenge weight gain.
The effects of heterologous viral challenge on the weight gain of vaccinated pigs is depicted as the % weight gain in pounds. A. Weight gain of the experimental pigs on DPC 9, B – Weight gain of the experimental pigs on DPC 21. X axis – groups, Y axis - % weight gain in pounds, horizontal bar with the large circle – group mean, bars – 95% confidence interval of the means, Solid line – Mean connect line, *-significantly different from the PBS group, @ *-significantly different from the commercial vaccine group, ($p < 0.05$) by the Mann Whitney U test.