RNA VIRAL PROPHYLAXIS: PROBLEMS AND POTENTIAL SOLUTIONS

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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

Over 80% of the newly emerging infectious diseases are caused by RNA viruses. Major global problems associated with the development of vaccines against the RNA virus are their high genetic and antigenic diversity. Hence, effective control of epidemics with newly emerging RNA viruses require improved vaccines which are either specific to the new strain or broadly effective even when new viral strains emerge. The main focus of this dissertation is to develop epidemic vaccines using these two approaches. Using a newly emerged swine enteric virus called porcine epidemic diarrhea virus (PEDV) as a model, our first goal was to develop a quick and easy method for rapid response vaccines with potential applicability to a range of RNA viruses. We hypothesized that the methods which can disrupt genomic RNA without impacting the structural integrity of the virus would result in attenuated vaccine with minimum replication in the host while inducing immune responses. As hypothesized, developed rapid response PEDV vaccine induced complete protection against the virulent challenge virus, while vaccine viral shedding was not detected in vaccinated pigs. To address the second problem of rapid viral evolution leading to vaccines becoming obsolete, we used swine influenza virus (SIV) as a model to develop and test a universal vaccine composed of peptides encoding conserved antigenic epitopes which are present in most influenza A viruses. Importantly, a novel amphiphilic invertible polymer (AIP) was used to address the well-recognized problem of poor antigenicity of peptides. We hypothesized that peptides encoding conserved epitopes when conjugated with an AIP will induce strong immune responses and protect against challenge virus. While the conserved epitopes were previously tested by others in mice, we were the first to test a combination of these epitopes in pigs. Pigs vaccinated with the peptide polymer vaccine mounted strong antibody responses against the epitopes indicating that the delivery system was effective.

However, protection against replication of the challenge virus was delayed. In summary, the methods developed and tested in this body of work significantly contribute to the area of emergency response management in infectious disease outbreaks.

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LIST OF ABBREVIATIONS

1H NMR	proton Nuclear Magnetic Resonance Spectroscopy
Ab	Antibodies
ADRDL	Animal Disease Research and Diagnostic Laboratory
AF	Allele Frequency,
AIP	Amphiphilic Invertible Polymer
AIV	Avian Influenza Virus
APCs	Antigen Presenting Cells
bp	base pair
BSA	Bovine Serum Albumin
CMI	Cell-Mediated Immunity
CPE	Cytopathic Effect
CTLs	Cytotoxic T Lymphocytes
DMEM	Dulbecco's Modified Eagle’s Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DPC	Day Post-Challenge
DPV	Day Post-Vaccination
ELISA	Enzyme-Linked Immunosorbent Assays
ER	Endoplasmic Reticulum
ERGIC	ER-Golgi Intermediate Compartment
FFN	Fluorescent Focus Neutralization
FTIR	Fourier-Transform Infrared Spectroscopy
GISRS	Global Influenza Surveillance and Response System

GPC.....Gel Permeation Chromatography

HA.....Hemagglutinin

HE .....Hematoxylin and Eosin

HI .....Hemagglutination Inhibition

HISAT.....Hierarchical Indexing for Spliced Alignment of Transcripts

HP .....Histopathological

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

IAV .....Influenza A viruses

IBC.....Institutional Biosafety Committee

IFN .....Interferon

Ig .....Immunoglobulins

IHC.....Immunohistochemical

IIV .....Inactivated Influenza Virus

IL.....Interleukin

INDEL.....Insertion and Deletion of Bases

Kd.....Kilo-dalton

LAIV .....Live Attenuated Influenza Vaccines

MDCK.....Madin-Darby Canine Kidney

MHC .....Major Histocompatibility Complex

MOI.....Multiplicity of Infection

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

NA.....Neuraminidase

NDSU.....North Dakota State University

NEP .....Nuclear Export Protein

NK cells .....Natural Killer cells

nm .....nanometer

NS2 .....Nonstructural Protein 2

NVSL .....National Veterinary Services Laboratory

OD.....Optical Density

pAPN.....porcine Aminopeptidase N

PBS .....Phosphate Buffered Saline

PBST.....PBS with Tween

PCR.....Polymerase Chain Reaction

PCV2.....Porcine Circovirus Strain 2

PDCoV.....Porcine Deltacoronavirus

PEDV .....Porcine Epidemic Diarrhea Virus

PEG_{600}.....Polyethylene Glycol (molecular weight 600 g/mol)

PFU .....Plaque Forming Units

ppm .....parts per million

PRCV .....Porcine Respiratory Corona Virus

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

$PTHF_{650}$.....Polytetrahydrofuran (molecular weight 650 g/mol)

qPCR.....quantitative (real-time) PCR

qRT-PCR.....Reverse Transcriptase qPCR

RNA .....Ribonucleic Acid

RNP.....Ribonucleoprotein

ROC .....Receiver Operating Characteristics

RTC.....Replication and Transcription Complex

S/P .....Signal to Positive ratios

SARS.....Severe Acute Respiratory Syndrome

SBARE.....State Board of Agricultural Research

SDSU .....South Dakota State University

sg .....segmented

SIV .....Swine Influenza Virus

SNP .....Single Nucleotide Polymorphism

TCID50 .....Tissue Culture Infective Dose50 assay

TCR.....T-Cell Receptor

TGEV .....Transmissible Gastroenteritis Coronavirus

Th cells.....T helper cells

Ti .....Transition Mutation

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

TPB .....Tryptose Phosphate Broth

Tv .....Transversion Mutation

US/USA/U. S .....United States of America

USDA.....US Department of Agriculture

UTR.....Untranslated

V/N.....Virus Neutralization

w/v.....weight by volume

WHO .....World Health Organization

γ.....gamma

CHAPTER 1. LITERATURE REVIEW

Overview of RNA viruses and their infections

Unlike other microorganisms, viruses are non-cellular, protein shells packed with either RNA or DNA as genetic material. RNA viruses (viruses with RNA genome) are more prone to mutation and genetically diverse than DNA viruses due to the inherent instability of RNA molecules and low fidelity of RNA dependent RNA polymerase (Choi 2012). This rapid variation in RNA viruses results into emerging of a new virus strain that may lead to failure in prevention strategies, causing a variety of the emerging and re-emerging infections (Figure 1). They are also more likely to cause acute infections with high mortality and morbidity rates, while a majority of DNA viruses cause chronic, lifelong infections. The more notorious infections caused by RNA viruses in the human population include the severe acute respiratory syndrome (SARS), the 2009 pandemic swine influenza, avian influenza (AIV) and Ebola hemorrhagic fever. In swine production, porcine epidemic diarrhea virus (PEDV), swine influenza virus (SIV), Transmissible gastroenteritis coronavirus (TGEV), Porcine Respiratory Corona Virus (PRCV), Porcine deltacoronavirus (PDCoV) are among those that caused recent epidemics (Singh et al. 2018).

PEDV is an economically important enteric swine coronavirus, which causes severe diarrhea in all age groups, and over 90% mortality in neonatal piglets (Jung and Saif 2015). In 2013, it emerged in the US, spreading rapidly in the swine population. In just ten months, roughly one-quarter of the US swine neonatal population was lost, leading to a loss of $540 million to the US swine industry (Schulz and Tonsor 2015). SIV is another most economically important respiratory swine pathogen, affecting all stages of production causing huge economic losses. It is also a very important zoonotic human pathogen and the cause of several epidemic

and pandemics (Vincent et al. 2008). In this study, PEDV and SIV were used as models to address problems associated with vaccine development against RNA viruses i.e. their high antigenic variability and rapid evolution leading to emergences of new strains (Stephenson 1985, Carrasco-Hernandez et al. 2017).

Virus life cycle

Understanding the virus life cycle is crucial for developing better antiviral agents, therapies and prevention strategy. The virus life cycle starts with the attachment of the virus attachment proteins (ligand of the virion) with the receptor on the plasma membrane of the host cell (Figure 1) (Murphy et al. 1999). The virion ligand plays important role in determining the role in inducing protective host immune response and virus tissue tropism. Following the attachment, the virus can enter the cell by one of two main mechanisms: 1. Fusion and 2. Receptor-mediated endocytosis (Murphy et al. 1999). In the fusion entry mechanism, the virus envelope fused with the host cell cytoplasmic membrane and the viral nucleocapsid (viral genome coated with viral proteins) gets released into the host cytoplasm. The nucleocapsid is then transported to the site of viral genome replication (i.e., cytoplasm for most RNA virus, and nucleus for most DNA viruses). In the endocytosis-based virus entry, the host cytoplasmic membrane invaginates the entire virus forming an endoplasmic vesicle, which further fuses with endosome (acid prelysosomal vesicle). Acid environment of the endosome triggers the change on the viral proteins which leads to the release of the viral genome into the cytoplasm. Based on the replication strategy of the virus, the virus genome then transported to the viral genome replication site.

Once the viral genome reaches the replication site, viral enzymes/host factors make several copies of viral genome copies for the progeny virions. Some genome copies are

transcribed into viral mRNA (messenger RNA). The viral mRNA then gets translated by the host cell's ribosome to viral structure proteins and enzymes required for virus genome replication and assembly. The translated viral structural proteins interact with the viral genome and form viral particles (in case of non-enveloped viruses) or nucleocapsid (in case of the enveloped virus).

After assembly of the virion, most of the non-enveloped viruses start accumulating inside the nucleus or cytoplasm and eventually release when cell lyses whereas enveloped viruses bud either from the host plasma membrane or from the membrane of the cellular organelle (nucleus, Golgi apparatus, or endoplasmic reticulum). Viruses are budding out of inner cellular organelle transported to the cell surface via vesicle and released by exocytosis.

In this study, both model viruses are enveloped. Their replication mechanism is briefly explained in their respective sections of this chapter.

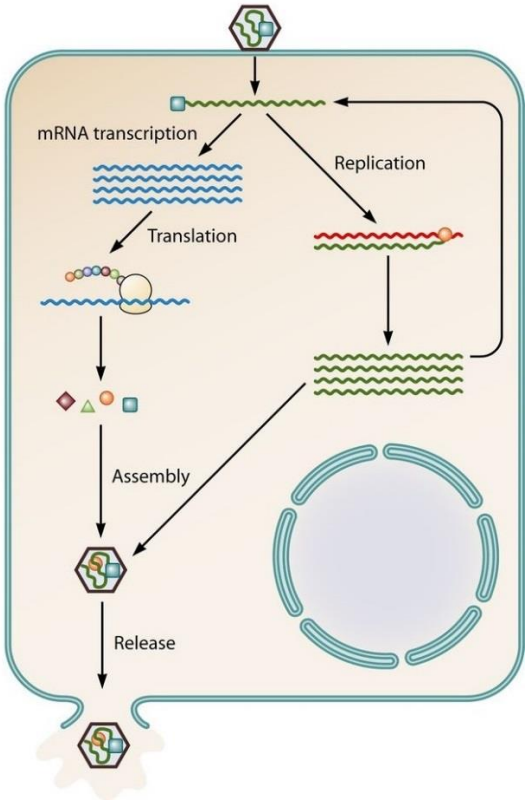


Figure 1. General feature of the viral life cycle (Li, Wei, et al. 2013)

Immune response against viruses

To counter the viral infections the animal body developed a variety of nonspecific (innate) and specific (acquired) immune responses (Figure 2, Table 1). The innate immune response is the first line of defense against pathogens. It lacks specificity and memory but acts quickly upon the infection. It consists of physical barriers such as mucus and collectins, and immune cells (monocytes, natural killer cells, mast cells, and granulocytes) which do not require previous pathogen exposure to carry out defense function. Monocytes and neutrophils are phagocytes which destroy the pathogen by internalization. Figure 3 gives a visualization representation of different innate immune cells and their function. Whereas the adaptive immune response is the second line of defense which is highly specific and includes memory but is relatively slower in response than innate immunity during first exposure to the pathogen. However, due to previous memory, the response during a second exposure to the same pathogen is stronger and quicker. This phenomenon is known as anamnesis and forms the basis of vaccination. Depending on the mechanism and molecules involved, the host immune response can be divided into humoral and cell-mediated immunity (CMI) (Klimpel 1996, Louten 2016) (Figure 2, Table 2). There are two different types of immune cells involves in the adaptive immune system: 1) T-cells or T-lymphocytes, which originates in bone marrow and mature in the thymus, and part of CMI; 2) B-cells or B-lymphocytes, which originates and mature in bone marrow, and part of humoral immunity (Chaudhry 2014).

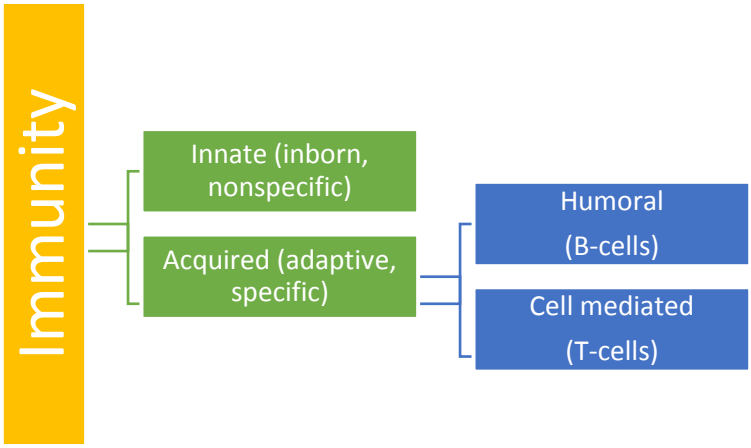


Figure 2. The different branches of the host immune response elicited against the virus infections.

Table 1. The differences between adaptive and innate immunity.

	Adaptive immunity	Innate immunity
Definition	The aspect of immunity which targets a specific antigen (foreign substance). It involves the production of specific antibodies against an antigen	Initial immune response directed against all types of antigens
Synonyms	Acquired or specific immunity	Inborn or nonspecific immunity
Activation	Active prior by exposure to any microbe or antigen	Active before exposure to any microbe or antigen
The main cell involved	B and T lymphocytes	Natural killer (NK) cells, monocytes, macrophages
Specificity	Yes	No
Lag phase	Present; response takes a few days	Absent; Response is immediate
Memory	Present; Amplified response in 2nd exposure of the same pathogen	Absent: same response as in 1st exposure

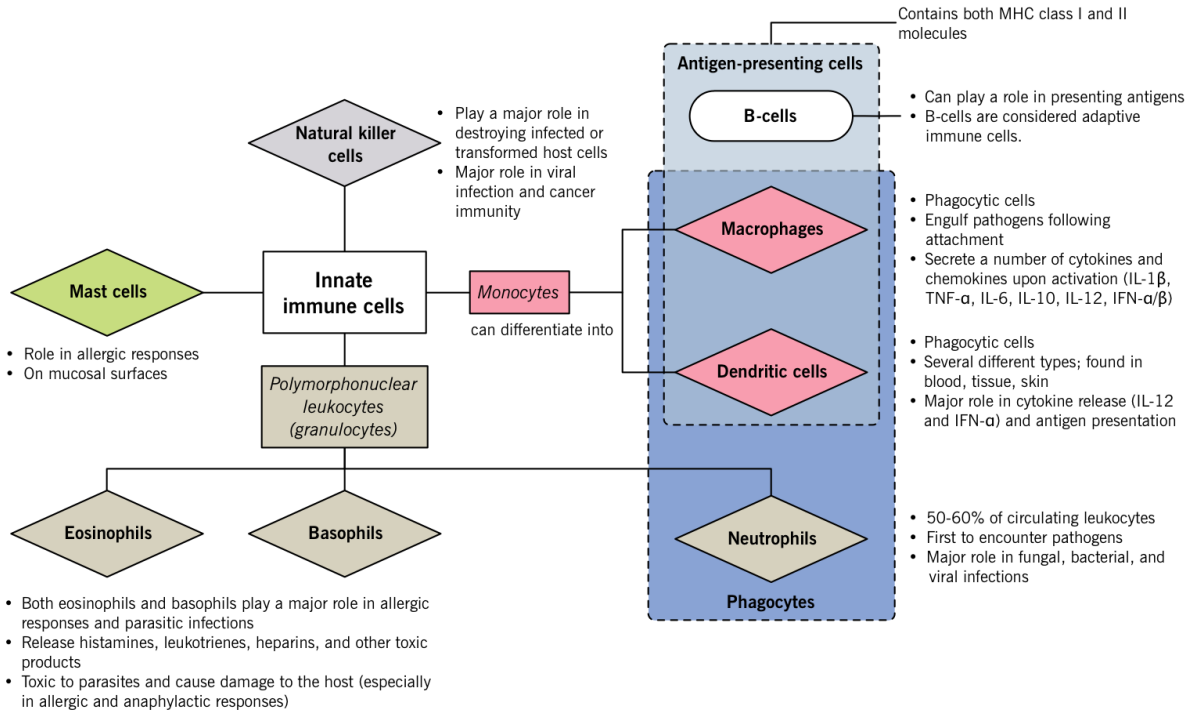


Figure 3. Different cells of innate immune system and antigen presenting cells (Chaudhry 2014).

Table 2. The differences between humoral and cell-mediated immunity (Bozhilova 2018).

	Humoral immunity	Cell-mediated immunity
Definition	The aspect of immunity mediated by protein macromolecules (antibodies) found in the extracellular body fluid	The aspect of immunity that identifies and destroys infected cells using immune cells
Target pathogens	It protects against the extracellular pathogens: extracellular bacteria, circulating virus	It protects against the intracellular pathogens: viruses, fungi, intracellular bacteria
Main cell types	The main cells involved in the humoral immune response are the B-lymphocytes (B-cells). These cells are generated and mature in the bone marrow	The main cells involved in the cell-mediated immune response are the T-lymphocytes (T-cells). These cells are generated in the bone marrow and mature in the thymus
Mode of action	Activated plasma B-cells secretes antibodies	Activated T-cells secretes cytokines

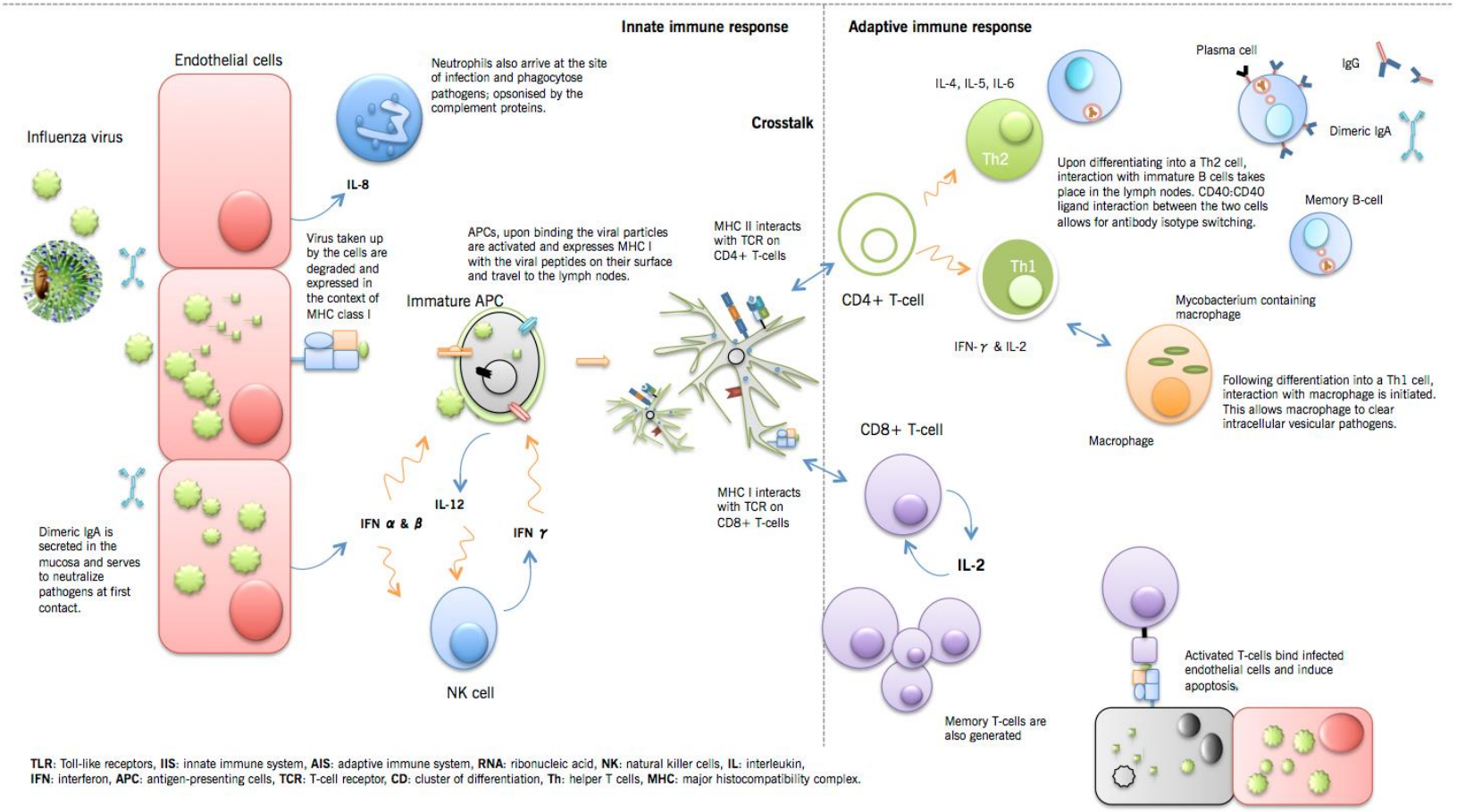
Monocytes and B-cells are the professional antigen presenting cells (APCs) which internalize and process the antigen to be present on the major histocompatibility complex (MHC)

to T-cells. MHC is a cell surface molecule that mediates the interaction of the immune cells with other immune cells or body cells. There is two class of MHCs are expressed by cells: MHC class I and MHC class II molecules. Key differences between them are summarized in table 3. APCs expresses both class of MHC molecules (Chaudhry 2014).

Table 3. The differences between MHC class I and class II molecule.

	MHC class I	MHC class II
Presence	Present on the surface of all nucleated cells	Only present on the surface of lymphoid tissue cells
Antigen processing	Cytosolic protein	Lysosomal, endosomal or extracellular pathogens
Receptor cells	Cytotoxic T cells or CD8+ T cells	T helper cells or CD4+ T cells

Antigen-presenting cells (APCs) plays a vital role in crosstalk between innate and adaptive immune response (Figure 4). During infection, APCs uptakes the antigen and process it into small peptides which then expressed on the surfaces of APCs in the context of MHC-I or MHC-II molecules. Once APCs gets mature via expressing MHC and antigen peptide on its surface, they travel to the lymph nodes and present processed antigen to T-cells (Kindt et al. 2007).



TLR: Toll-like receptors, **IIS**: innate immune system, **AIS**: adaptive immune system, **RNA**: ribonucleic acid, **NK**: natural killer cells, **IL**: interleukin, **IFN**: interferon, **APC**: antigen-presenting cells, **TCR**: T-cell receptor, **CD**: cluster of differentiation, **Th**: helper T cells, **MHC**: major histocompatibility complex.

Figure 4. Crosstalk between innate and adaptive immune response (Chaudhry 2014).

T-cells of the adaptive CMI have a unique receptor for antigen, known as TCR (T-cell receptor), which recognize a specific antigenic peptide sequence (known as T-cell epitopes) bound with the MHC molecules on APCs and the infected cells. This interaction between TCR and MHC activation and proliferation of T-cells (Chaudhry 2014). T-cells are divided into two major type based on their functions (Romagnani 1992). 1) CD8+ or cytotoxic T lymphocytes (CTLs), which kills virus infected host cell. CTLs recognized antigen T-cell epitope in the context of MHC class I molecule. Once CTLs get activates by APCs, they travel to site of infection and recognize and binds to the infected cells via TCR-MHC-I bond. This interaction of CTLs TCR and infected cell MHC-I leads to release of various cytokines triggering apoptosis of the infected cells. 2) CD4+ or T helper cells (Th cells), which secretes cytokines to help other T-cells, B-cells and macrophages. Th cells recognize the peptide sequence present in context of MHC II molecule. There are two major Th cells: Th1 and Th2 cells. Th1 plays a vital role in CMI by activating macrophage and natural killer (NK) cell activation via secretion of interleukin (IL)-2 and interferon (IFN)-ɣ. Whereas Th2 cells play a vital role in humoral immunity by B-cell activation and antibody production (Romagnani 1992, Chaudhry 2014).

Humoral immunity is mediated by antibodies (specific for a viral antigen) contained in body fluids (known as humor). Antibodies are produced by B lymphocytes when stimulated by viral antigens produced by virally-infected cells. Antibodies also are known as an immunoglobulins (Ig) consist of 5 classes: IgG, IgA, IgM, IgE, and IgD; among which IgG, IgM, and IgA have shown antiviral activity (Kindt et al. 2007). IgG and IgM antibodies are present in serum, and IgA is responsible for antiviral activity when a virus infects mucosal surfaces. These antibodies neutralize viruses by blocking virus-host interaction hence preventing the virus entry

to host cells (Louten 2016, Klimpel 1996). Hence stimulating a strong antibody response is usually critical to the effectiveness of vaccines.

Protection against viruses is primarily mediated by neutralizing antibodies, which host acquire as lifelong immunity after first infection. However, to escape the host immune response viruses evolve by changing the antigenic site targeted by neutralizing antibodies, which leads to generation of new virus variants. These new variants also maintain some shared epitopes with the parental strains. When host immune gets exposed to the new virus variant, two different immune response happens to ensure successful protection: 1) activation of memory B-cell response against shared epitopes, 2) activation of naïve B-cell response against novel epitopes (Kim et al. 2009). However, in several viral infection such as Influenza virus, Human Bocavirus, Dengue virus, Zika virus and Human immunodeficiency virus, the latter response fails to be triggered, which leads to either poor immune response hence mild infection or antibody dependent enhancement of infection hence severe infection. This phenomenon is called ‘original antigenic sin’ (Figure 5) (Vatti et al. 2017, Kim et al. 2009).

Knowledge about how a given virus interacts with the three arms of the immune system and which type of response is critical for protection of the host is key to the development of an effective vaccine against the virus (Louten 2016, Klimpel 1996).

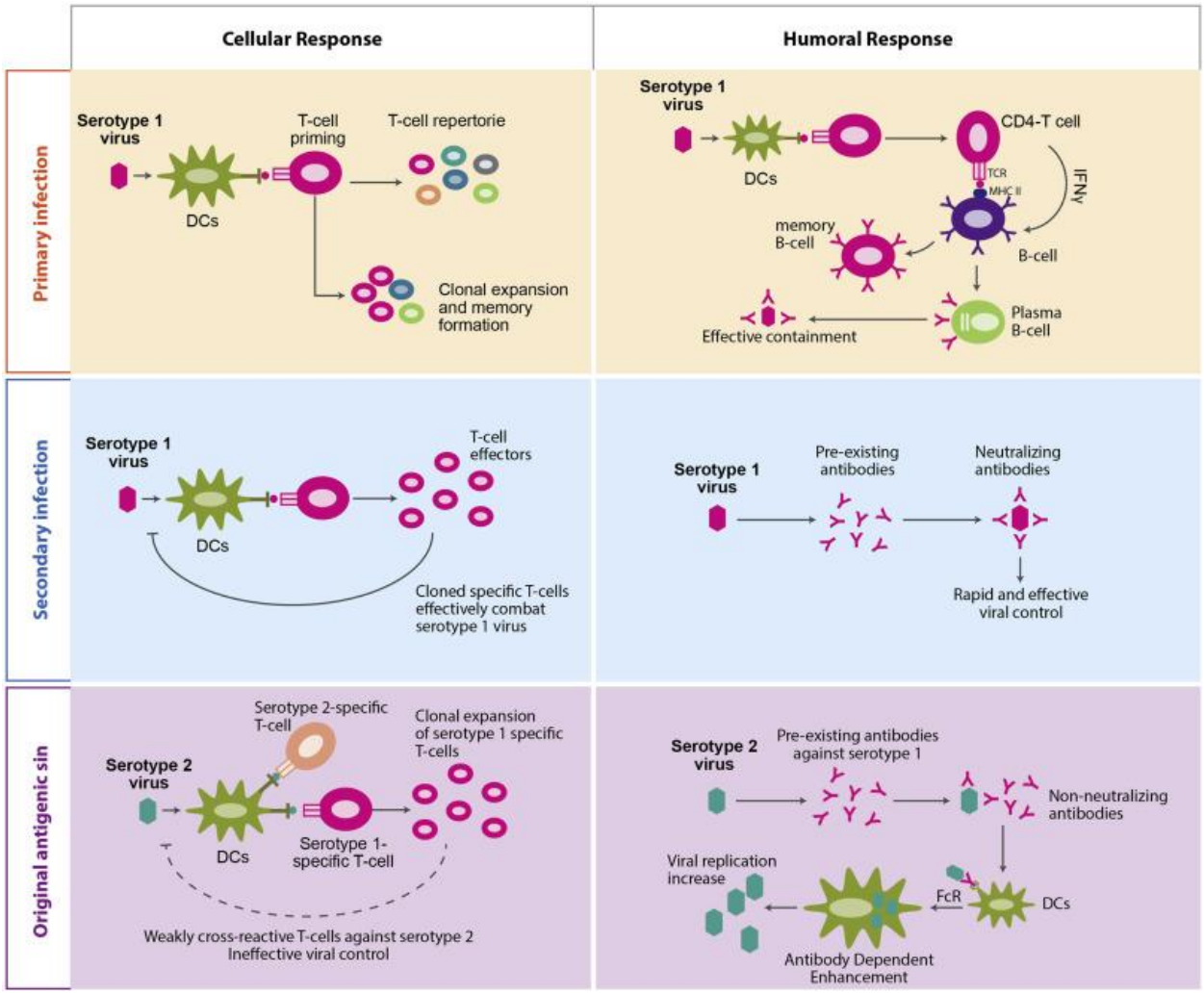


Figure 5. Conceptual mechanism of "original antigenic sin" (Vatti et al. 2017). Reprinted by permission from Elsevier Journal of Autoimmunity, Original antigenic sin: A comprehensive review, Anup Vatti, Diana M. Monsalve, Yovana Pacheco, Christopher Chang, Juan-Manuel Anaya, M. Eric Gershwin, 2017.

Vaccines against viral infections

Available vaccines against most of the RNA viruses provides incomplete protection due to the high rate of genetic and antigenic variation, and the emergence of new strains (Figlerowicz et al. 2003). Most of the current commercial RNA viral vaccine contain whole viral particles, either in an inactivated form or attenuated form. In term of effectiveness, inactivated vaccines are less effective than attenuated vaccines, whereas in term of safety attenuated vaccines have a risk

of reverting to the virulent virus (Table 4) (Stephenson 1985). Other most common vaccine types in use is recombinant DNA vaccine, which is mostly safe but requires thorough knowledge of genetic sequences, protective antigens and effective epitopes. The major development issue with these (attenuated, inactivated and recombinant) vaccines is length of time required for their development. Whereas for controlling epidemics the development time should be shorter. Thus, for successful emergency preparedness plan, there is need a for universal vaccines which can protect against all strains of the virus eliciting both humoral and cellular immune response or a rapid response vaccine which can easy to develop within a short period, providing the combined efficacy and safety of attenuated and inactivated vaccine respectively.

Table 4. The differences between attenuated and inactivated vaccines.

Attenuated Vaccine	Inactivated Vaccine
Live organism capable of replication	Killed organism
Antibody and cell-mediated immunity.CD 4+ and CD 8+ Stimulation	Mostly antibody mediated. Mainly CD 4+
Can potentially revert to virulence. Not Safe	Very Safe
Longer duration of immunity. Sometimes life-long	The short duration of immunity
Cannot be used in pregnant animals	Can be used in pregnant animals

Universal problems associated with vaccination against RNA viruses

The major two problems associated with vaccination against RNA viruses are 1) Rapid emergence and evolution of new strains (Carrasco-Hernandez et al. 2017); 2) Genetic and antigenic variability of RNA virus (Stephenson 1985).

Rapid emergence and evolution of new strains: Strikingly, over 80% of newly emerging infectious diseases are caused by RNA viruses (Figure 6) (Woolhouse and Gaunt 2007). RNA

viruses become of special concern due to interspecies transmission ability. RNA viruses achieve high probability to infect new host species via their faster evolution rate and their shorter generation times (Carrasco-Hernandez et al. 2017). The rapid evolution of RNA viruses occurs because of their error-prone replication cycle, which build up mutation rate in RNA viruses up to 5 times higher than DNA viruses and up to 6 times higher than their cellular host (Holmes 2009). Figure 7 shows the difference between the substitution rate (mutation rate) of DNA and RNA viruses. This higher mutation rate able RNA viruses to transmit from their natural reservoir and infect other species. Thus, RNA viruses represent the most common pathogen behind new human diseases (with rate of 2 to 3 novel viruses per year) (Rosenberg 2015). The table 5 show examples of zoonotic RNA viruses and reservoirs.

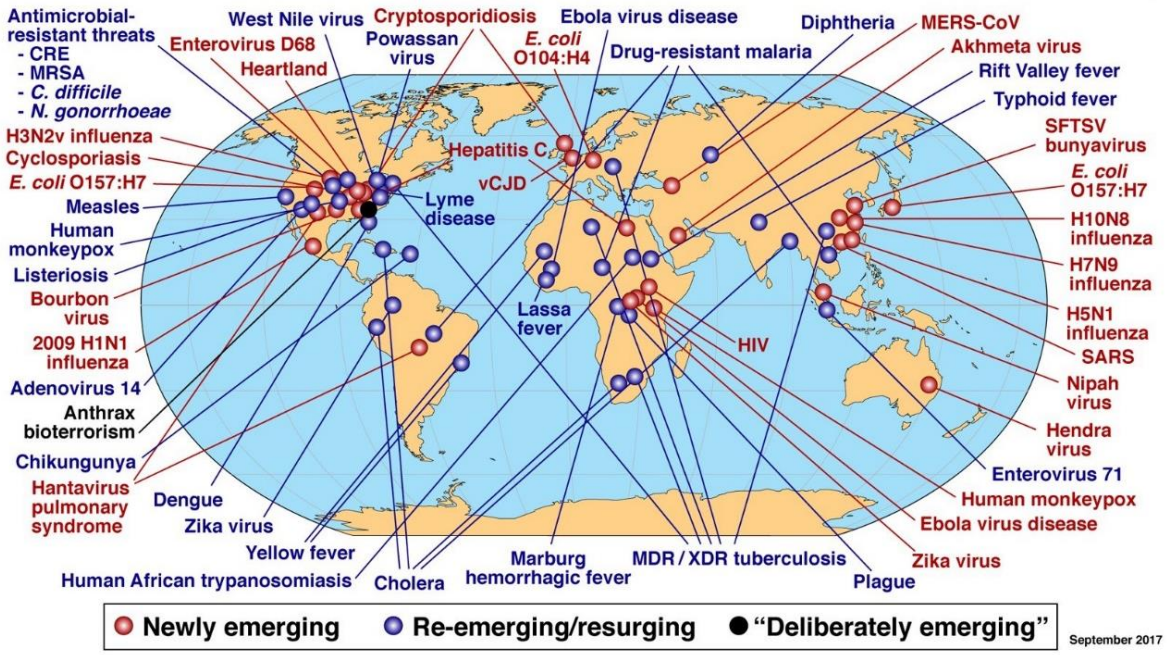


Figure 6. Global examples of emerging and re-emerging infectious diseases (https://www.niaid.nih.gov).

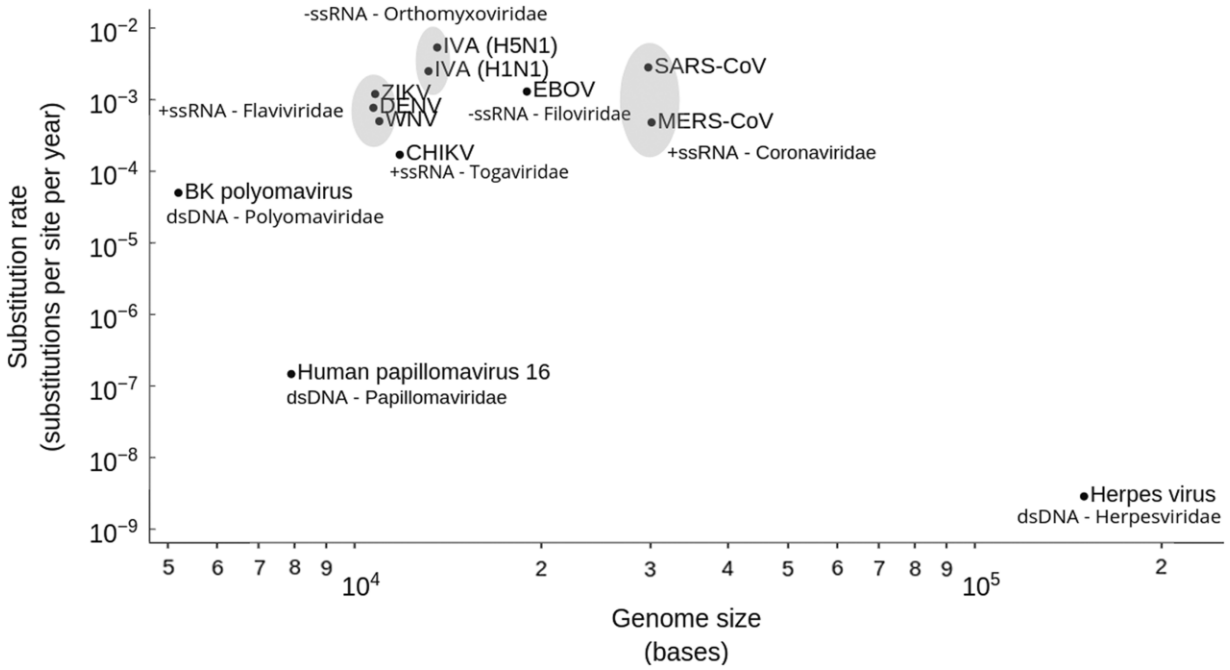


Figure 7. Genome size against substitution rate of DNA and RNA viruses (Carrasco-Hernandez et al. 2017). Reprinted from ILAR Journal, Are RNA Viruses Candidate Agents for the Next Global Pandemic? A Review, Carrasco-Hernandez, R; Jácome, Rodrigo, Copyright (2017), with permission from Oxford University Press.

Table 5. Examples of zoonotic RNA viruses, their reservoirs, and reported routes of transmission (Carrasco-Hernandez et al. 2017).

Group	Virus	Known or suspected reservoir(s)	Reported route(s) of zoonotic transmission
(+) Single-stranded	Foot-and-mouth disease virus	Wild and domestic bovines	Shared water sources
	Chikungunya virus	Nonhuman primates	Mosquito vectors
	West Nile virus	Birds	Mosquito vectors
	SARS coronavirus	Bats	Direct contact during wildlife trading/butchering, respiratory droplet transmission
(−) Single-stranded	Influenza A virus	Birds/swine	Aerosols and direct contact with reservoirs
	Ebola virus	Fruit and insectivorous bats	Direct contact (hunting or butchering)
	Nipah virus	Fruit bats	Contaminated fruit
Double-stranded	Colorado tick fever virus	Squirrels and chipmunks	Tick vector (Dermacentor andersoni)
	Banna virus	Unknown (isolated from mammals)	Mosquito vectors
Reverse transcribing	Primate T-lymphotropic viruses	Primates	Living in close contact with infected nonhuman primates

Many of the above-mentioned viral infections have the potential to cause zoonotic and reverse zoonotic transmission, raising a public health concern (Best 2011). To limit the initial infection and spread of epidemic from these emerging or re-emerging infections, emergency/pandemic preparedness plans (Figure 8) are formulated. They consist of five major steps: disease surveillance using diagnostics, communication networks, maintenance of community services, medical care, and supply and delivery of vaccines and/ or drugs (Azziz-Baumgartner et al. 2009). Therefore, the effectiveness of pandemic preparedness plans depends

on the availability of reliable first- response diagnostics for surveillance and vaccines to limit the extent of spread. Previous efforts in our laboratory resulted in the development of novel methods to rapidly produce diagnostic antigens and develop serological diagnostic tests to detect newly emerging viruses, such as PEDV (Song et al. 2016). In chapter 3, our efforts to decrease testing time and effort for serological tests for PEDV and adapt them to a high-through put format are described.

Figure 8. Different components of the pandemic preparedness plan.

In this thesis, methods to rapidly develop effective and safe vaccines against newly emerging RNA viruses are targeted. Currently available standard methods for vaccine development are limited by the long length of time required for development and licensing. This delay in vaccine production creates a huge gap between vaccine availability and rapid disease transmission and spread, resulting in huge socio-economic losses (Smith, Lipsitch, and Almond 2011, Noah and Fidas 2000, Jennings et al. 2008, Ozawa et al. 2017). Thus, improved technology to develop emergency vaccines for RNA viruses is urgently required (Singh et al.

2018) . This need is addressed in chapter 2 of this thesis using PEDV as a model of an emerging RNA viral infection. The major objectives were to achieve a relatively simple method with a short lead development time and a combination of the advantages of inactivated and attenuated vaccines, namely high safety and efficacy margins.

Genetic and antigenic variability: The rapid mutation rate of RNA viruses also contributes to the rapid change in the antigenic properties of the viruses, which can lead to 'original antigenic sin' response (Kim et al. 2009). The phenomenon of such antigenic changes due to mutation known as antigenic drift. The other major phenomenon is antigenic shift, which occurs when two different segmented RNA virus strain infects the same host cells and new virus strains are evolved via recombination or reassortment of the segment genes (Flint et al. 2000). Both antigenic drift and antigenic shift plays an important role in influenza virus epidemic (Janke 2014). In chapter 4 of this dissertation, the problem of need of single universal vaccine against influenza virus is addressed. The major objectives of study were to develop a single multi-epitope based universal vaccine against SIV, which will be highly effective and safe.

Porcine Epidemic Diarrhea Virus (PEDV)

Classification

Porcine Epidemic Diarrhea Virus is a swine, enteric coronavirus which causes severe liquid diarrhea, dehydration, and vomiting in the pigs (Jung and Saif 2015, Gallien et al. 2018). PEDV can infect all ages of pigs but cause high mortality (more than 90%) in the suckling piglet population (Gallien et al. 2018, Lee 2015). PEDV is a single-stranded, positive-sense RNA virus belonging to the Coronaviridae family. The Coronaviridae family divided into four genera: Alphacoronavirus, Betacoronavirus, Gammacoronavirus, and Deltacoronavirus. PEDV is a member of the genera Alphacoronavirus (Gerdts and Zakhartchouk 2017, Jung and Saif 2015).

Economic impact

The first report of PEDV in the US swine population appeared in May 2013, after which it spread through the North America (USDA-APHIS 2019). Economically, 2013 PEDV epidemic was characterized as supply-impacting disease (reduction in the pork products supply due to morbidity and mortality) not demand inhibiting (hesitant to consume pork products due to fear of zoonosis) (Schulz and Tonsor 2015). The severity of PEDV infection varies widely and mostly depends on the age, immunological and epidemiological status of the herd. It is more severe in the neonatal piglets with 80%-100% morbidity and mortality (Song and Park 2012, Schulz and Tonsor 2015). It is estimated that during this epidemic there was 0.31 decrease in the number of pigs saved per litter, which was maximum from September 2013 to August 2014, and roughly over one-quarter of piglet get lost (Schulz and Tonsor 2015). The PEDV outbreak also impacted the swine breeding decisions. The farrowing intentions, which are primary intentions of the hog supply, declined during the PEDV outbreak period (from September 2013 to August 2014). The decline in the farrowing further tightened the supply of the hogs into the market (Schulz and Tonsor 2015, Diersen 2004).

As PEDV having no impact on human health, there was no noticeable effect on the consumer demand of pork was observed during 2013 epidemic (Schulz and Tonsor 2015). However, due to short supply of pigs, pork price increased by 10.29%. Aggregately, it is estimated that in 2014, due to 3% reduction in pig supply there was $481 million decrease in annual return to the pork industry (Paarlberg 2014).

Genome and virus structure

Porcine Epidemic Diarrhea Virus is an enveloped virus of icosahedral shape (Jung and Saif 2015, Pensaert and De Bouck 1978). The virion envelop is a host-cell derived lipid bilayer

containing three surface-associated proteins: spike (S), membrane (M), and envelope (E). Inside the virion envelope, the nucleocapsid (NP) protein assembles into a viral capsid enclosing the PEDV genome (Figure 9). The PEDV genome is approximately 28kb long, single-stranded positive-sense RNA with 5' cap and 3' polyadenylated tail. It contains a 5' untranslated (UTR) and 7 open reading frames (Figure 9) (Song and Park 2012, Lee 2015, Jung and Saif 2015, Kocherhans et al. 2001).

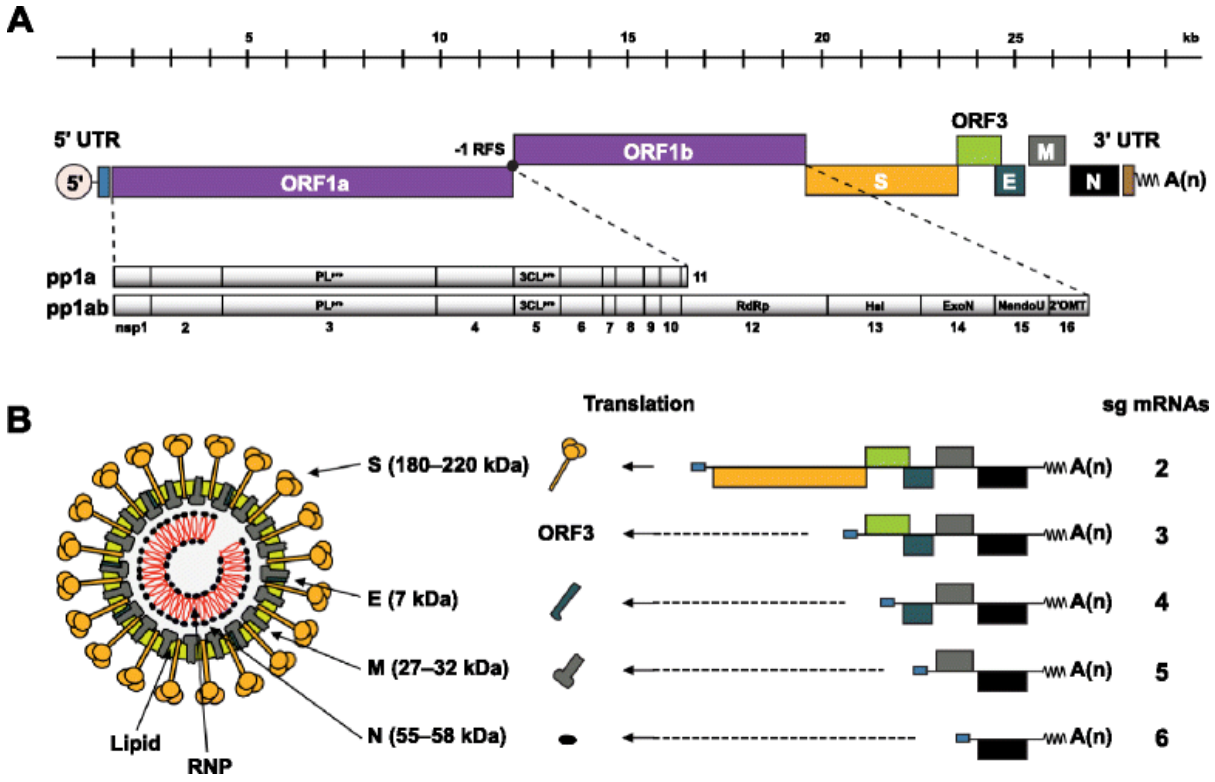


Figure 9. Schematic representations of PEDV genome organization and virion structure. A) The structure of PEDV genomic RNA. The 5'-capped and 3'-polyadenylated genomes of approximately 28 kb is shown at the top. B) Model of PEDV structure (Lee 2015).

Virus replication

PEDV infects and replicates efficiently in the porcine small intestinal enterocytes. The small intestinal enterocytes express the porcine aminopeptidase N (pAPN) on its surface that has been identified as the cellular receptor for the PEDV (Li, Ge, and Li 2007, Nam and Lee 2010).

The N-terminal region of the PEDV spike (S) protein S1 domain binds to the pAPN receptor on the enterocytes followed by the fusion of the virus envelope and host cell membrane initiating virus internalization and finally releasing the viral genome into the host cell cytosol (Lee, Cha, and Lee 2011, Lee 2015). The viral genome gets uncoiled from the RNP via releasing from the N protein and immediately translate to yield the polyprotein pp1a and pp1ab. These polyproteins are then cleaved into 16 nonstructural proteins (nsps) composing replication and transcription complex (RTC). The RTC first transcribe the genomic positive-sense RNA into full-length minus-strand RNA and then into segmented-length (Fosgerau and Hoffmann) minus-strand RNA. The full-length minus-strand RNA and sg minus-strand RNA are then used to synthesize full-length genomic positive-sense RNA and sg messenger RNAs (mRNA). Each sg mRNA then translated into the viral proteins. The envelop surface-associated proteins: S, E, and M get inserted into the endoplasmic reticulum (ER) and then anchored to ER-Golgi intermediate compartment (ERGIC). Meanwhile, N protein interacts with the newly synthesized genomic RNA and form helical RNP complex. The RNP complex and S, E and M protein anchored to the ERGIC interact with each other and leads to budding into of progeny virus into the golgi apparatus. The progeny viruses are then released via exocytosis-like fusion of smooth-walled vesicles with the cell membrane (Figure 10) (Lee 2015, Gerdts and Zakhartchouk 2017).

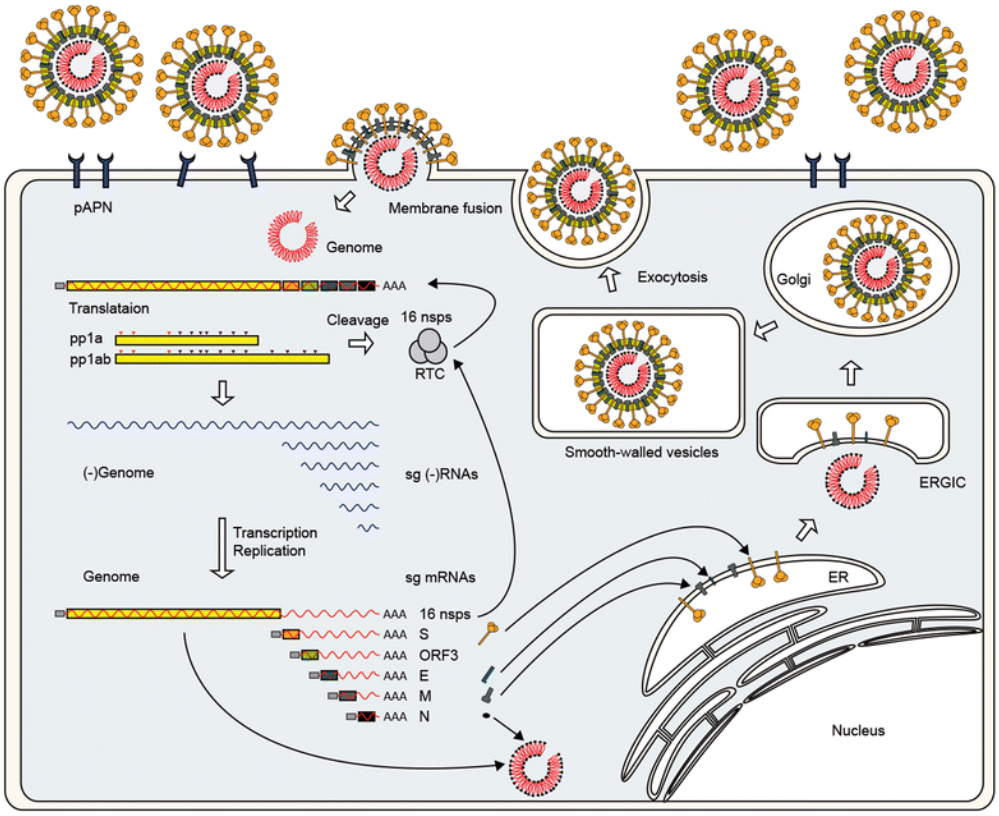


Figure 10. PEDV life cycle (Lee 2015).

Transmission and molecular epidemiology in the US

PEDV primarily transmitted from infected to healthy pigs by direct or indirect fecal-oral route (Jung and Saif 2015). PEDV can enter in a healthy pig population via several PEDV-contaminated sources such as transportation (food, pigs, equipment etc.), people (pig owner, visitors, practitioner), or wild animals and birds (Dee et al. 2014, Lowe et al. 2014).

PEDV undergoes into an evolutionary process by accumulating mutations or by recombination necessary for viral fitness and survival (Kim, Langmead, and Salzberg 2015). These genetic changes have been used to determine diversity and relationship between the global PEDV isolates using whole-genome or individual gene phylogenetic analysis(Lee 2015, Chen et al.). The full-length S gene and its S1 domain are widely used for investigating genetic

relatedness and molecular epidemiology (Lee 2015); based on which there are two genetically different groups: genogroup 1 (G1, classical) and genogroup 2 (G2, field epidemic and pandemic). Each genogroup further divided into subgroups 1a and 1b, and 2a and 2b. The G1a comprise the prototype PEDV strain CV777, cell culture-adapted strains and vaccine strains, whereas G1b includes new isolates. In G2 group PEDV field isolates, most of the S genes are nine nucleotides (3 amino acid) longer than its homology gene of the prototype CV777. The G2 PEDV strains are termed as S INDEL strains due to the presence of insertions and a deletion in their S gene compared to the sequence of CV777 (Lee 2015).

In the 1970s, the PEDV was first reported in the United Kingdom (Wood 1977), and later spread to other European countries. After a decade, the first PEDV outbreak in Asia was reported (Lee 2015). It remained exotic in the United States until its sudden emergence in the 2013 when it and causes losses of one-quarter of the suckling piglet population within the 10 months (Stevenson et al. 2013). Genetic and phylogenetic analyses of these emergent US strains revealed their genetic closeness with the AH2012 Chinese strain of the G2b lineage with S INDEL genetic signature (Liu et al. 2013). Other PEDV strains emergent in the US in January of 2014, without S INDEL genetic signature and their phylogenetic analyses revealed their closeness with novel Chinese strains of G1b subgroup lineage (Wang et al. 2014).

Current vaccines against PEDV and their limitations

Vaccines are the fundamental strategical tool for control and eradication of PED during endemic or epidemic outbreaks (Lee 2015). The success of PEDV vaccine depends mostly on its ability to stimulate high titer of PEDV-specific neutralizing mucosal IgA antibodies in the intestinal mucosa, and lactogenic immunity (protection by transfer of the maternal antibodies to the suckling piglets from the vaccinated dam via colostrum and milk) (Park and Lee 2009, Lee

2015). Despite the availability and use of inactivated and attenuated PEDV vaccines in Asian countries for several years, the efficacy and safety of PEDV vaccines is highly questionable. These vaccines increase the chance of piglet survival, however do not significantly reduce the virus shedding in the feces and morbidity rate (Lee 2015).

There are currently two commercial vaccines are available in the US against PEDV under condition license: PED RNA (also known as iPEDV plus) by Harrisvaccines^{TM}, and PED vaccine by Zoetis. Both vaccines are given intramuscularly twice with a three-week interval. The immunity produced by these vaccine decrease mortality but do not provide full protection from the challenge virus (Gerdts and Zakhartchouk 2017, Mogler MA 2014). Two major factors associated with low to moderate effectiveness of PEDV are: 1) antigenic, genetic variation between the vaccine and field epidemic strain; 2) route of administration (Lee 2015). The best route of administration for induction of mucosal gut and lactogenic immunity is oral not intramuscularly (Bohl et al. 1972). Thus, there is need for PEDV vaccines which are antigenic close to the field strains and can be administered via oral route.

In chapter 2 of this study, two different rapid-response vaccine development approaches are demonstrated. The method developed provides full protection against PEDV in a swine challenge model while satisfying all the required criteria for a rapid response vaccine.

Virus quantification and antibody detection methods

The effective and accurate detection and diagnosis of the emerging viral infection is another crucial part of pandemic preparedness plan. The success of vaccination depends on whether it elicits strong neutralizing antibody responses (Pankaj et al. 2018). Enzyme-linked immunosorbent assays (ELISA) is the most common serological method used to detect PEDV or PEDV specific antibodies. However, in the veterinary diagnostics the level of protective

antibody response, elicit by vaccine or previous virus exposure, against new virus strains is determined by virus neutralization (V/N) tests or fluorescent focus neutralization (FFN) tests (Diel et al. 2016). Both V/N and FFN test measures the level of the neutralization antibodies in the test serum sample, which blocks virus-host cell interaction via masking the virus receptor to the host cell. The accuracy of V/N and FFN depends on two factors: 1) accurate virus stock titer used in the tests; 2) accuracy in detecting the level of inhibition of virus replication and cytopathic effect (CPE) (Pankaj et al. 2018).

For PEDV, the plaque assay is the traditional gold standard method for virus titration, which measure the level of infectious virus particles in terms of plaque forming unit (pfu). Another routine tradition method for tittering PEDV infectious particles is the tissue culture infective dose50 ($TCID^{50}$) assay.

Although the traditional methods V/N, $TCID_{50}$ and plaque assay are widely acceptable, they are time-consuming and labor extensive. The $TCID_{50}$ and V/N assay involve the manual examination of the numerous wells of the 96-well plates under the light microscope, and plaque assay involves the manual counting of the plaques in the Petri plates. In the laboratories with high volumes of testing samples as is likely to occur during epidemic surveillance, manual visualization assessment become tedious, and over the long period of the time can lead to health issues and increase operator errors (Pankaj et al. 2018). In chapter 3 of this study, we describe the development and testing of a convenient colorimetry-based assay to assess the PEDV virus titer and PEDV specific neutralizing antibodies, which can be used to assess post-vaccination neutralizing antibody responses in a high throughput format.

Swine Influenza Virus

Swine Influenza virus is another RNA virus with very high antigenic and genetic diversity. It is an economically important zoonotic pathogen of the swine industry, first recognized clinically in swine in 1918 in the US, coinciding with human pandemic influenza or Spanish flu. SIV infection in swine exhibits clinical symptoms of the acute respiratory disease similar to human influenza, characterized by fever, coughing, sneezing, inactivity, decreased food intake, respiratory distress, and nasal discharge. The severity of infection depends on both host factors and virus strain (Influenzavirus 2009, Vincent, Lager, and Anderson 2014).

Swine respiratory epithelial cells have receptors for both avian and mammalian influenza viruses. Hence they can serve as a 'mixing vessel' for influenza viruses (Scholtissek 1990). Infection of a host by two or more strains at the same time can result in the emergence of new influenza strains with the potential to cause pandemics such as the 2009 outbreak with the H1N1 virus (Haß et al. 2011). While vaccines against influenza viruses are of sub-optimal efficacy in both swine and humans, the control of influenza in swine remains critical in preventing influenza epidemics.

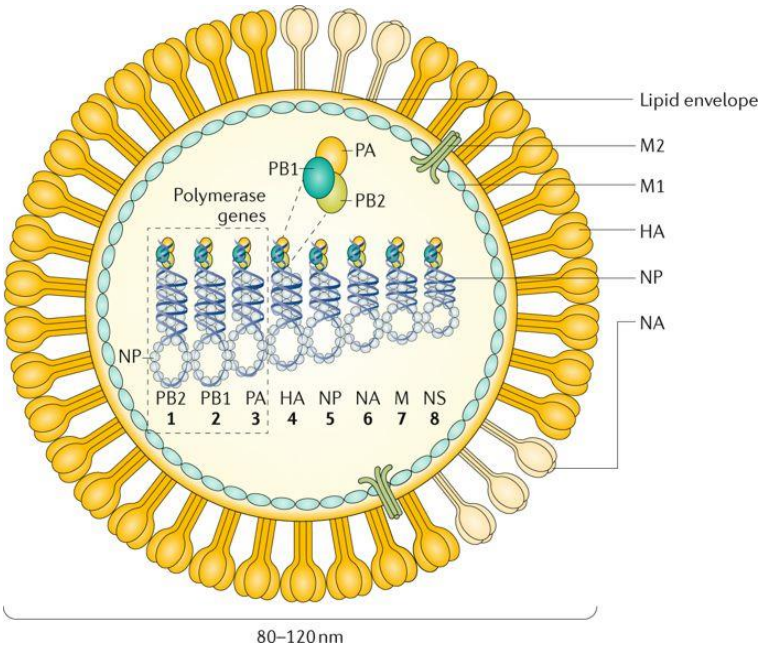


Figure 11. Influenza A structure (Krammer et al. 2018). Reprinted by permission from Springer Nature Reviews Disease Primers, Influenza, Florian Krammer *et al*, 2018.

Classification

Influenza viruses are segmented, single-stranded, negative-sense RNA viruses belong to the *Orthomyxoviridae* family. The *Orthomyxoviridae* family consists of seven different genera: *Alphainfluenzavirus*, *Betainfluenzavirus*, *Deltainfluenzavirus*, *Gammainfluenzavirus*, *Isavirus*, *Quaranjavirus* and *Thogotovirus.* SIV is a member of Influenza A virus of genus *Alphainfluenzavirus.* Other important members of the *Orthomyxoviridae* family are Influenza B virus of *Betainfluenzavirus* genus, Influenza D virus of *Deltainfluenzavirus* genus, Influenza C virus of *Gammainfluenzavirus* genus. Influenza A virus further classified into subtypes based on the surface antigenic glycoproteins hemagglutinin (HA) and neuraminidase (NA). To the date, there are 18 identified HA and 11 NA subtypes (ICTV 2018). The phylogenetic relation between different HA protein of Influenzas A and B virus is shown in Figure 12.

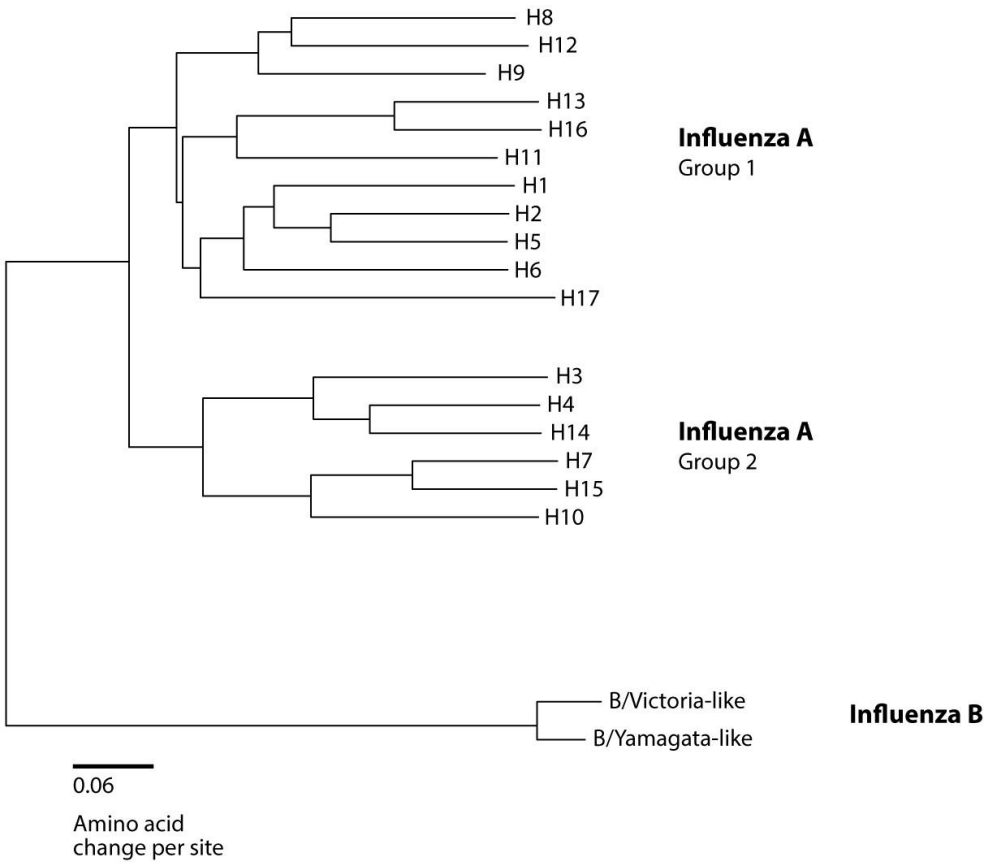


Figure 12. Rooted phylogenetic tree is based on amino acid sequences of hemagglutinin segments from influenza A and B viruses (Pica and Palese 2013). Republished with permission of Annual review of medicine, from "Toward a universal influenza virus vaccine: prospects and challenges.", Natalie Pica and Peter Palese, 2013; permission conveyed through Copyright Clearance Center, Inc.

Genomic and virus structure

Influenza A viruses are enveloped viruses, either spherical or filamentous in shape. The virion envelop is a host-cell derived lipid bilayer studded spikes of glycoprotein HA and NA, and integral matrix 2 (M2) ion channel. The envelope and its integral proteins (HA, NA and M2) overlay viral capsid composed of matrix 1 (M1) protein (Fig 6). Inside the virion capsid, there is nuclear export protein (NEP), also known as nonstructural protein 2 (NS2) and ribonucleoprotein (RNP) complex. The RNP complex consists of viral-RNA segments coated with nucleoprotein (NP) and the heterotrimeric RNA-dependent RNA polymerase (consists of PB1, PB2, and PA).

Influenza A virus consists of 8 different RNA segments transcribing more than 13 different proteins. (Bouvier and Palese 2008).

The HA glycoprotein is the most important protein of the Influenza virus, serve two most vital role in viral infection: 1) it contains viral ligand to host cell receptor for viral entry; 2) host immune response elicits neutralizing antibodies against it, because of which most of licensed vaccines target HA glycoprotein. The HA molecule includes two subunits HA1 and HA2 proteins (Figure 13 b) (Pica and Palese 2013). Functionally, HA molecule can divide into two domains: head domain mainly composed of HA1, and stalk domain composed of part of HA1 and HA2 (Figure 13 a) (Pica and Palese 2013). The head domain of the HA molecule contains the epitopes which elicits neutralizing antibody responses via preventing virus attachment to the host cell, however due to constant immune pressure the degree of sequences variation is high (Pica and Palese 2013, Nachbagauer and Krammer 2017). Whereas, the stalk domain of the HA molecules elicits both neutralizing antibody response via preventing membrane fusion. Unlikely head domain, its sequences have high degree of conservation, thus making it target for developing broad protecting universal vaccine candidate (Nachbagauer and Krammer 2017, Gilbert 2013).

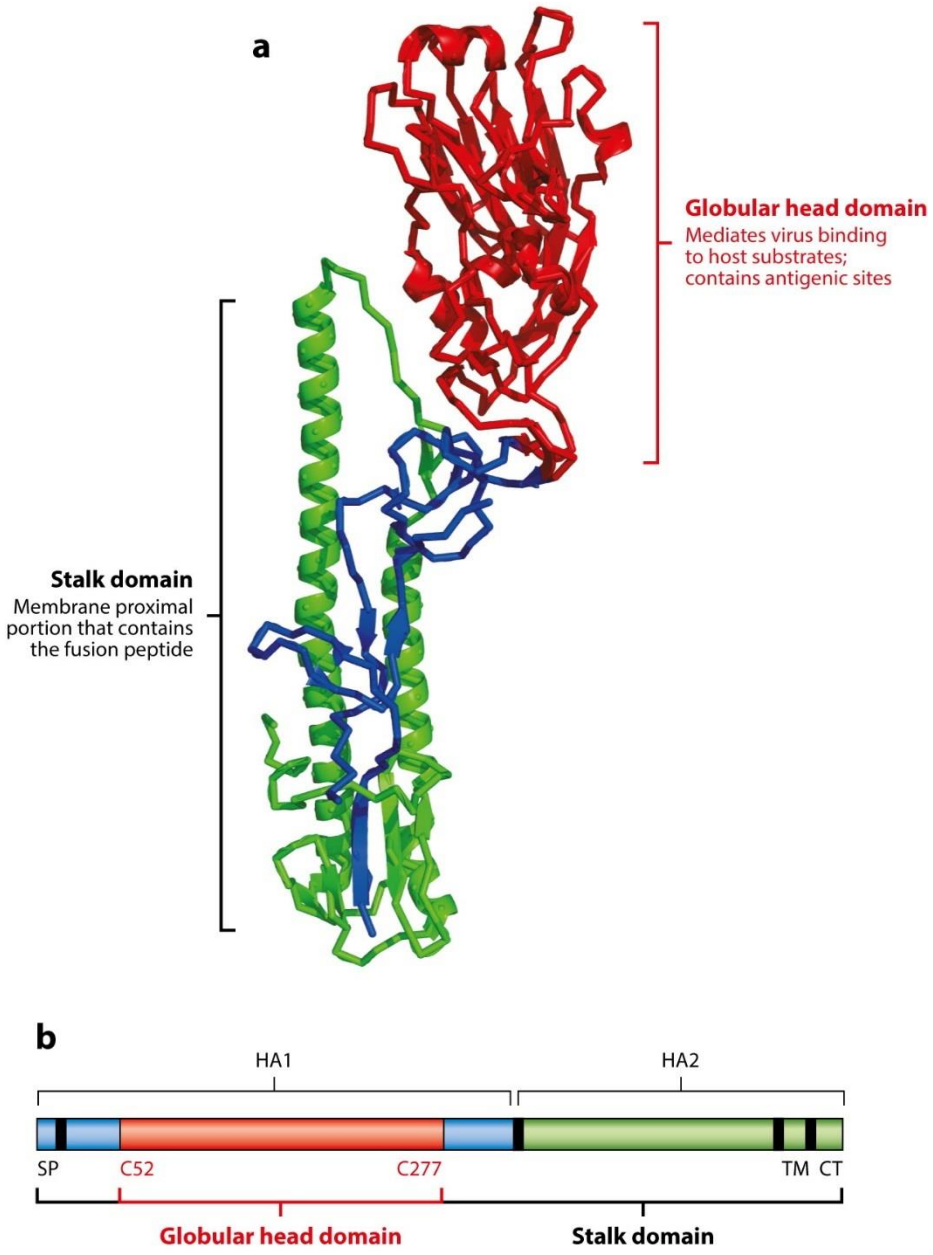


Figure 13. Ribbon diagram and schematic representation of hemagglutinin monomer (H1 subtype). (a) The HA1 subunit is depicted in red (globular head domain) and blue (stalk domain), and the HA2 subunit in green. The head domain (red) contains the receptor-binding site and the five predicted antigenic sites; the fusion peptide is located within the HA2 portion of the stalk (green) (Pica and Palese 2013). Republished with permission of Annual review of medicine, from Toward a universal influenza virus vaccine: prospects and challenges, Natalie Pica and Peter Palese, 2013; permission conveyed through Copyright Clearance Center, Inc.

Virus replication

Upon the influenza virus entry into a naïve host, the HA glycoprotein of virus bind to the N-acetylneuraminidase (sialic acids) present on the host cell surface. Following the binding of influenza virus HA protein to the sialic acid of the host cell, the virus is endocytosed by the host cell. As the pH drops in the endosome, the HA protein changes its conformation exposing the HA2 fusion peptide that facilities the merging of virus envelop with host endosomal membrane (Stegmann 2000). At the same time, the hydrogen ions from the endosome are pumped into the virion via M2 ion channel, decreasing internal virion pH which leads to disrupting the protein-protein interaction and eventually releases of viral RNPs from the viral matrix into the cellular cytoplasm (Pinto et al. 2011). The released viral RNPs then trafficked from host cell cytoplasm to the host cell nucleus (Cros and Palese 2003, Martin and Helenius 1991). Inside the nucleus, the negative sense viral RNA transcribed into mRNA (messenger RNA) by viral RNA-dependent RNA-polymerase (a part of the RNP complex). Some of the viral mRNA act as a template for viral RNA genome; other viral mRNA gets 5' cap via 'cap-snatching' mechanism of PB1 and PB2 protein of influenza virus. Once capped, viral mRNAs get exported out of the nucleus where they get translated into viral protein by host cell ribosomes (Bouvier and Palese 2008). The synthesized enveloped proteins HA, NA, and M2, get processed by Golgi apparatus and subsequently exported into the host cell membrane for viral assembly. The newly formed RNPs bounds to M1 protein and get exported out of the nucleus and trafficked to the host cell membrane where M1 protein interacted with cytoplasmic tails of HA and NA and facilitates virus budding (Fiers et al. 2009, Imai and Kawaoka 2012).

Protective immunity against SIV

Protective immunity against influenza infection involves both the humoral and cell-mediated immunity (Vincent et al. 2008). Antibodies play a significant role in preventing infection by preventing the binding of the HA to its receptors and other non-neutralizing mechanisms (Bikour, Cornaglia, and Elazhary 1996). Protection against viral infection is directly correlated with the Hemagglutination inhibition (HI) titers in the serum of an individual animal. Unfortunately, cross protection is only found between closely related or homologous virus strains. Given the vast genetic and antigenic diversity of influenza viruses, eliciting broadly protective antibody responses by vaccination has been the holy grail of vaccinology. Effective mucosal immunity or level of IgA antibodies in the respiratory tract is more important than the IgG antibodies level in the serum for preventing initial infection. Thus, the success of the controlling the future SIV infections and novel SIV subtypes emergence heavily depends on vaccines inducing higher HI titers in both sera, as well in mucosal surfaces against the challenge virus (Vincent et al. 2008).

T-cells based cell-mediated immunity (CMI) play a vital role in controlling and clearing influenza virus infection, hence protects against severe illness or deadly outcomes. Unlikely, homosubtypic protection by B-cells of humoral immunity, CD4+ and CD8+ T-cells of CMI may confer heterosubtypic immunity (Mohn et al. 2018). Thus, vaccine providing both humoral and CMI is highly preferred.

Current vaccines against SIV and their limitations

The first inactivated influenza vaccine became commercially in 1994, and since then it is widely in use (Vincent et al. 2008). Currently, there are two types of human seasonal vaccine widely available: inactivated influenza virus (IIV) and live attenuated influenza vaccines (LAIV)

(WHO 2019). Both IIV and LAIV contains 3 (trivalent) or 4 (quadrivalent) different virus, among which one is pandemic A(H1N1) strain, one seasonal strain and 1 or 2 influenza B lineage viruses (WHO 2019). The composition both IIV and LAIV is recommended by World Health Organization which update its information based on worldwide surveillance by the Global Influenza Surveillance and Response System (GISRS) (WHO 2019). Both IIV and LAIV vaccines have pro and cons too. Where IIV elicits more HI titer of IgG and IgA than LAIV, but it does not stimulate CMI because it is killed or inactivated. On other hand, LAIV elicits both humoral and CMI as it mimic influenza infection, thus also seem to superior than IIV (Mohn et al. 2018, Spitaels, Roose, and Saelens 2016). However, unlike human influenza, SIV is not seasonal. The number of variants which are circulating in the swine population which make available bivalent or trivalent killed vaccine ineffective against the more distantly related strains. Moreover, the current vaccines require annual boosters to maintain immune memory. In 2006, it was reported that approximately 20% of swine farms are using autogenous SIV vaccine rather than the commercial vaccine. Autogenous vaccines are inactivated influenza vaccines prepared from the virus cultures isolated from the herd and only to be used in a herd of origin under the veterinarian's supervision (Vincent et al. 2008). The two major problems in the control and prevention of SIV infection are; 1) the SIV is changing faster speed than the conventional vaccine development; and 2) there is a need for a single vaccine (universal vaccine) that can provide long-lasting cross-protection among the SIV variants (Vincent et al. 2008).

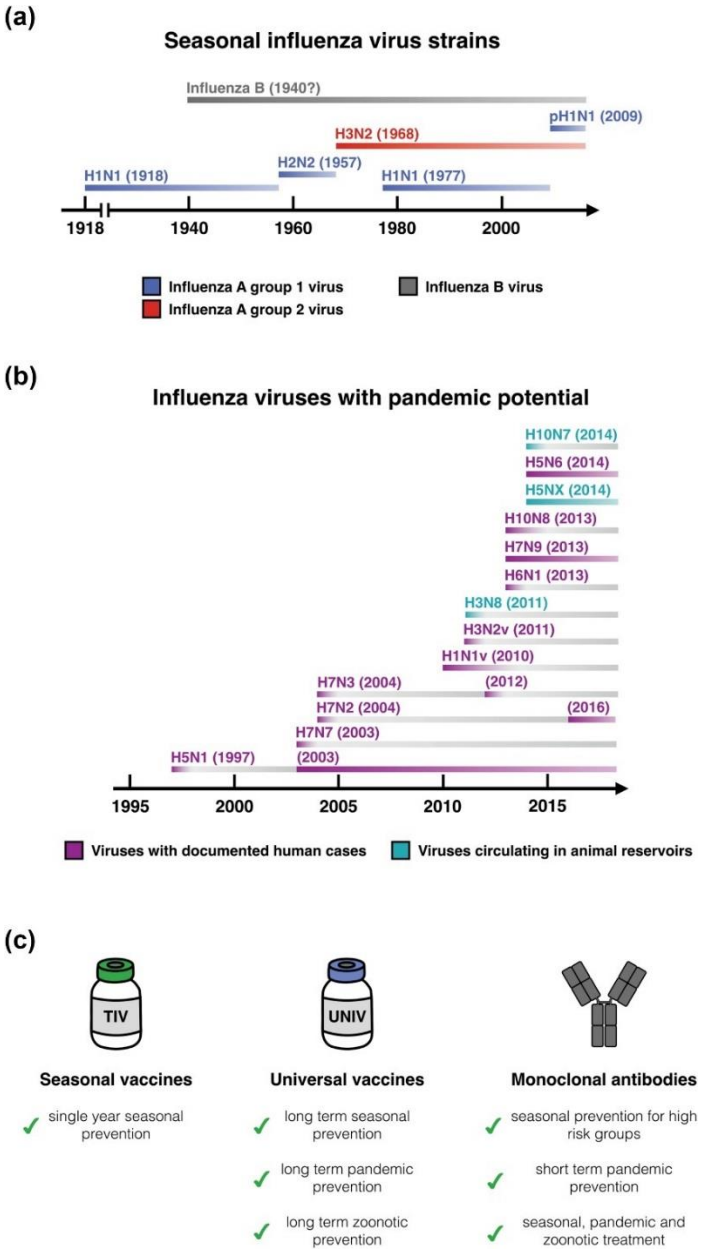


Figure 14. Influenza virus circulation. (a) Two subtypes of influenza A, H1N1 (group 1, blue) and H3N2 (group 2, red) are currently circulating alongside influenza B viruses in the human population. (b) A large number of different subtypes of influenza viruses have been discovered in animals (mainly avian species). This graph shows viruses with documented human zoonotic cases (purple) and viruses of concern that widely circulate in animal reservoirs (teal). The bars are shown in color for times when human cases or outbreaks were reported. If a virus disappeared from surveillance, the bar is shown in grey. (c) Overview of the benefits of vaccines and biological agents for influenza virus infection prophylaxis and therapy (Nachbagauer and Krammer 2017). Reprinted from Clinical Microbiology and Infection, Volume 23, Issue 4, R. Nachbagauer, F. Krammer, Universal influenza virus vaccines and therapeutic antibodies, 222-228, Copyright (2017), with permission from Elsevier.

A major breakthrough in the challenge associated with the development of broadly protective influenza vaccines occurred with the discovery that certain regions of the HA protein are highly conserved in structure, sequence and function. The development of single cell cloning techniques enabled the discovery of these conserved regions or epitopes, first for HIV and then for influenza (Haynes et al. 2012, Wrammert et al. 2008). Among the discovered epitopes, the matrix 2 (M2e) epitope, HA1 stalk domain and HA2 fusion protein are well characterized and have demonstrated broad cross-protection against several influenza strain in the mice models (Jang and Seong 2014, Nachbagauer and Krammer 2017, Krammer and Palese 2014).

The M2 protein of the SIV is a transmembrane, homo-tetrameric protein ion channel which is involved in the release of RNP following virus entry (Lamb 2001). The M2e-based universal vaccine are based on the 18-24 amino acid region of the extracellular domain of the M2 protein (Neirynck et al. 1999). The M2e-peptide based protection was first demonstrated using 14C2, a monoclonal antibody (mAb) against M2e peptide, which reduced the plaque size formation in vitro (Zebedee and Lamb 1988), and reduced virus titer by 100-fold in the lung tissue in the model challenge model (Treanor et al. 1990). Since then M2e-based vaccine has been tested widely in the different animal models (Pica and Palese 2013).

Both HA1 and HA2 epitopes are part of HA molecules, which plays a vital role in elicits neutralizing immune response against various influenzas virus strains. It is hypothesized that HA1 based vaccine protect interfering with the viral attachment to the cellular receptor, whereas HA2 based vaccines elicit immune response provide broad protection via blocking viral envelop and host cell membrane fusion (Jang and Seong 2014). The HA2 based vaccine are still under preclinical development (Gilbert 2013).

While epitopes (which are small peptides) are highly specific and elicit broad protection, they are week immunogens and vulnerable to degrade faster (Fosgerau and Hoffmann 2015). To address this problem associated with small peptides, in chapter 4 of this study, a novel amphiphilic invertible polymer (AIP) is used to enhance the vaccine delivery and immune response by acting as an adjuvant. The epitope-based vaccine also holds promise to serve as rapid response vaccines, as peptides can be rapidly synthesized when the sequence is known. With the knowledge of sequence of the virus and using the power of bioinformatic we can predict the conserved epitopes of the virus, which can be synthesized quickly and use as vaccine (Song et al. 2016).

Objectives for this dissertation

Objective I to develop methods for developing rapid response vaccine against RNA viruses during epidemics.

Hypothesis I: Methods which can disrupt the integrity of genomic viral RNA, while retaining structural integrity, will result in an attenuated viral vaccine with minimal to no replication in the host, while inducing strong antibody and CMI immunity.

Objective II to develop convenient diagnostic methods for rapid quantification of virus and neutralizing antibodies against it.

Hypothesis II: Method which can reduce labor and time to assess PEDV-induced cytopathic effects (CPE) and with applicability to virus TCID50 and virus neutralization assays.

Objective III to develop and test the epitope-based vaccine for broad protection against SIV in swine challenge model.

Hypothesis III: Peptides encoding epitopes which are highly conserved among various strains of influenza viruses, when conjugated with an amphiphilic invertible polymer

(PEG600PTHF650) will induce strong immune responses and protect against challenge influenza virus.

CHAPTER 2. A RAPID-RESPONSE VACCINE AGAINST PORCINE EPIDEMIC DIARRHEA VIRUS[1]

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Abstract

Effective and safe rapid response vaccines and diagnostics are critical to the success of pandemic preparedness plans, especially with the recent increase in emerging infections. A 2013 epidemic caused by porcine epidemic diarrhea virus (PEDV), an enteric swine coronavirus with over a 90% mortality rate in neonatal piglets, was a typical outbreak situation with heavy economic consequences. Using PEDV as a model, we herein describe a novel rapid-response vaccine method with high efficacy, safety and potential as a platform for RNA viruses. The relatively quick and uncomplicated method consisted of heat treatment of PEDV virions at 44^{o}C for 10 mins to reversibly unfold structural proteins, followed by exposure to RNase to fragment the genome. As targeted, treated virions were structurally intact but exhibited diminished replication in vitro. Vaccination of weanling piglets elicited strong antibody responses.

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

Vaccinated pigs were completely protected against challenge with virulent virus based on fecal viral shedding and intestinal pathology. The vaccine was safe as vaccinated pigs did not shed the vaccine virus in fecal matter or develop intestinal lesions prior to challenge. Thus, the described method has significant promise in terms of efficacy, safety and rapidness of development; ideal characteristics for epidemic vaccines.

Introduction

The number of newly emerging and re-emerging infectious agents has increased several folds over the last few decades, probably due to changing practices in travel, trade and farming (Mackey et al. 2014). In the last 25 years, over 15 emerging or re-emerging viral infections have affected swine operations in the U.S, averaging at 1.6 new viral diseases/ year. Some of these emerging infections such as influenza are transmissible to humans and thus have public health consequences. The timely availability of vaccines and diagnostics are critical to curtailing the initial spread of infection and for the success of pandemic-preparedness plans. Based on the lessons learned from SARS and other global pandemics, there is increasing recognition for need for alternate methods and change in policies for rapid-response or epidemic vaccines. The major differences between epidemic or rapid, first -response vaccines and other vaccines are the requirement for short lead development times, ease of scaling up and deployment, a high safety margin, and reasonable efficacy and stability (Finlay, See, and Brunham 2004, Plotkin 2017, Yamey et al. 2017).

In both human and veterinary medicine, RNA viruses constitute over 80% of the newly emerging viral infections (Woolhouse and Gaunt 2007). With a few exceptions like measles, yellow fever and rubella current vaccines against a majority of RNA viruses tend to provide incomplete protection. The high rates of genetic and antigenic diversity and the rapid evolution

in RNA viruses render the gaining of a clear understanding of the molecular correlates of vaccine-mediated protection a challenge (Figlerowicz et al. 2003). A majority of current commercial RNA viral vaccines contain whole virus particles in an inactivated or attenuated form. Generally, inactivated vaccines are less effective than attenuated vaccines for RNA viruses. The more effective, attenuated vaccines run the risk of reverting to virulence (Stephenson 1985) or recombining with field strains. Th development of conventional attenuated vaccines or recombinant vaccines may involve a relatively long lead development time. Hence an ideal rapid-response vaccine development method would combine the safety and efficacy advantages of inactivated and attenuated vaccines respectively and can be easily adapted to newly emerging strains.

Porcine epidemic diarrhea virus [PEDV] is an economically important swine coronavirus, which causes severe diarrhea and over 90% mortality in neonatal piglets. The emergence of PEDV in the U.S represented a typical epidemic, where the virus spread rapidly in the naïve U.S swine population, affecting all the major swine producing states and resulting losses to the tune of $540 million to the industry and severe mortality in the production swine population (Schulz and Tonsor 2015). Similar to other RNA viruses, current inactivated and attenuated vaccines against PEDV are of suboptimal efficacy in Asian countries where the virus has been endemic for several years (Song, Moon, and Kang 2015). Using PEDV as a model, we have developed a novel approach for rapid-response vaccine development in this study. Our approach targeted the development of a relatively simple process to diminish the replicative abilities of the virus while maintaining structural integrity so as to achieve high vaccine safety and efficacy margins respectively.

Previously published data showed that the SARS coronavirus capsid is metastable and can be reversibly denatured by changes in temperature or pH, with unfolding commencing at 35^{o}C and complete denaturation occurring at 55^{o}C (Wang et al. 2004). Hence, in this study, our approach consisted of exposing PEDV virions to 44^{o}C to unfold the capsid, followed by fragmentation or digestion of the genome with RNase and subsequent refolding of the capsid at 25^{o}C. Virions subjected to this treatment had intact structures when viewed by electron microscopy and were detected only after amplification by three serial passages in cells. Gamma-irradiated PEDV virions were used as an inactivated control vaccine (Ramamoorthy et al. 2006, Seo 2015). Confirming our hypothesis, piglets vaccinated with the heat and RNase treated virions were completely protected against challenge with the virulent virus, while those receiving the irradiated vaccine were not. Both vaccines tested had a very high safety margin as no vaccine virus replication was detected in vaccinated animals, nor were clinical signs or intestinal pathology noted. Thus, the described approach represents a new, uncomplicated but highly safe and effective approach for rapid-response vaccine development. The described methods can potentially be adapted as a platform for other RNA viruses with some optimization for each virus.

Materials and methods

Ethics statement

All experimentation was carried out in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee (IACUC) of S. Dakota State University with oversight by the North Dakota State University's IACUC (Protocol 15-013A) and Institutional Biosafety Committee (IBC) (Protocol – B13008).

Cells and viruses

Porcine epidemic diarrhea virus (PEDV) strain PEDV CO2013 (National Veterinary Services Laboratory [NVSL], Ames, IA) was cultured using Vero 76 cells (ATCC® CRL-1587) as previously described (Song et al. 2016), using a multiplicity index (MOI) of 0.1 in infection media containing 10 µg/ml Trypsin (Sigma-Aldrich). The stock virus obtained was titrated three times to obtain the mean 50% tissue culture infectious dose [$TCID_{50}$] using the Spearman and Karber formula (Kärber 1931).

Vaccine preparation

To optimize the temperature, time of incubation, and dose of RNase treatment, the virus stock was resuspended to $1x10^5$ $TCID_{50}$/ml in infection media (pH 7.2). Diluted virus culture was exposed to temperatures ranging from 37ºC to 60ºC for 10mins for unfolding, followed by incubation at 25ºC for 30 mins for refolding, and then moved to 4^0C for 1hr, as previously described for the SARS coronavirus (Wang et al. 2004). Cultures were visualized by electron microscopy to ensure structural integrity. A temperature of 44^0C for 10 mins was selected for unfolding. Similarly, to fragment the genomic RNA, varying combinations of concentrations of RNase A (Ameresco) and RNase T (Thermo Scientific) were tested by adding them to the unfolded virus cultures, followed by incubation for 5, 4, 3 or 2 hrs at 44^0C. Treated cultures were then exposed to 25ºC for 30 mins for refolding and cooled down on ice for 1hr. The final optimized protocol consisted of exposing the virus culture to 44^0C for 10 mins, followed by 0.1mg/ml of RNase A and 1µl/ml of RNase T1 (equivalent to 10 units/ml RNase A or 1000 units/ml of RNase T1, incubation at 44^0C for 4hrs, exposure to 25ºC for 30 mins and cooling down on ice for 1hr before storage at -80^0C for further testing.

To prepare an inactivated control vaccine, $1x10^5$ $TCID_{50}$/ml of PEDV was irradiated in a Cesium-137 source gamma (γ) irradiator at time points of 8 h to 24 h at 753 rad/min. An effective dose of 24 h (1,084,320 rad), was used to prepare the irradiated vaccine, after validation as described above.

Electron Microscopy

The ultrastructure of treated and untreated viruses were visualized by standard negative staining methods as described before (Booth, Avila-Sakar, and Cheng 2011) and examined with a JEOL JEM-100CX II transmission electron microscope.

Viral Amplification Test

To determine the effect of the treatments on viral viability, cultures were passaged three times as described above and replicating viruses visualized by an immunofluorescence assay (IFA), performed essentially as described previously (Song et al. 2016). Fixed cultures in 8 well chamber slides (Nunc) were stained with 100 µl of 1:500 polyclonal swine anti-PEDV sera (NVSL) and anti-swine IgG fluorescein-conjugated secondary Ab (KPL). Slides were examined with a fluorescent microscope for green cytoplasmic fluorescence characteristic of RNA viral replication.

Swine Vaccination and challenge

Twenty-four, 2-3-week-old piglets which were negative for PEDV by PCR and serology were divided into 3 groups; Group 1 - an unvaccinated control group (N=8) (2ml of PBS intramuscular and oral route each), Group 2 - RNase and Heat treated PEDV vaccine group (PEDV-VAC) group (N=8) (2ml of 10^5 $TCID_{50}$/ml, intramuscular and oral route each) and Group 3- irradiated PEDV vaccine group (N=8) (2ml of 10^5 $TCID_{50}$/ml, intramuscular and oral route each). Piglets were boosted by the same route and dose at DPV 14 and 28. On DPV 43,

small intestine, heart, liver and spleen were collected 2 piglets from each group (N=2/group) to assess vaccine safety. The remaining piglets (N=6/group) were challenged orally with 1ml of 10^3 $TCID_{50}$/ml of virulent PEDV CO2013. Post-challenge, the piglets were observed daily for clinical signs of PED.

All piglets were euthanized one-week post challenge (DPC) or at DPV 49 and three sections of the small intestine (duodenum, jejunum and ileum) were collected for histopathological (HP) and immunohistochemical (IHC) analysis. Serum was collected from all piglets on DPV 0, 14, 28, 43 and 49 to measure binding and neutralizing Ab responses. Fecal swabs were collected at DPV 7, 21, 38 and 42 from all piglets to measure shedding of the vaccine virus by qPCR. Fecal swabs were collected on DPV 45 and 49 (DPC day 3 and 7) from all piglets to measure protection against shedding of the challenge virus by qPCR.

PEDV- specific Ab responses

Nucleoprotein and spike protein-specific IgG responses in pigs were measured by indirect ELISAs as previously described (Okda et al. 2015). Pre-validated standardized operating procedures were followed at Animal Disease Research and Diagnostic Laboratory (ADRDL), SDSU. and assay formats were pre-validated, using serum samples from animals of known serological status. were followed for sample analysis. Results were calculated as signal to positive (S/P) ratios as follows: S/P=optical density (OD) of the sample – OD of buffer/OD of positive control – OD of the buffer.

Fluorescent focus neutralization assay

To assess the neutralizing Ab responses elicited by vaccination, a pre-validated fluorescent focus neutralization **(**FFN) assay was used as previously described (Okda et al.

2015), following the standard operating procedures of the ADRDL, SDSU. The end point was defined as a 90% reduction of foci compared to the controls.

qPCR for vaccine and challenge virus shedding

Fecal viral shedding was assessed by a qRT-PCR performed by the NDSU Veterinary Diagnostic Laboratory, using pre-validated standard operating procedures and a commercial kit called the Swine Enteric PCR Panel (Thermo Fisher).

Histology

Formaldehyde fixed tissue samples were stained with hematoxylin and eosin (HE) or a PEDV N protein-specific monoclonal Ab (SD6-29) for immunohistochemistry (IHC) following the standard operating procedures of the SDSU Veterinary Diagnostic Laboratory. Scores to measure atrophic enteritis characteristic of PED were assigned as follows: 0= negative, 2= mild, 4 = moderate, 6 = severe. Sections with crypt hypertrophy were assigned an additional 2 points. Antigen detection in enterocytes by IHC was semi-quantitatively scored based on the following criteria: 0= negative, 2= positive, ≤10%, 4 = positive, 11-50%, 6 = positive, >50% (Fig 5). The consistency of fecal matter during necropsy was assigned scores as follows: Formed Feces=0, Semi-formed feces = 3, Liquid feces = 6. Scores were recorded in a blinded fashion by a board-certified veterinary pathologist. Data was analyzed for statistical significance by the Student's T test.

Deep sequencing of heat and RNase treated PEDV virions

Heat and RNase treated s and untreated PEDV virus obtained from infected of Vero cells were purified by ultra-centrifugation. To detect genetic differences by deep sequencing, unpackaged RNA and DNA were removed by a RNase and DNase cocktail before viral RNA was purified with the Qiamp Viral RNA isolation kit (Qiagen). Deep sequencing and analysis

were conducted by a commercial vendor (BGI Genomic). The cDNA library was prepared using random hexamers, sequenced using HiSeq 4000 PE100 platform (Illumina Inc., USA) and raw reads (100bp) were obtained. Raw reads were filtered using SOAPnuke to get "Clean reads" by removing the reads with adaptors, reads with more than 5% of unknown bases (N), and low-quality reads (Chen et al. 2018). Clean reads were mapped to reference PEDV genome (GenBank: KF267450.1) using HISAT (Hierarchical Indexing for Spliced Alignment of Transcripts) (Kim, Langmead, and Salzberg 2015) and analyzed using the Genome Analysis Toolkit (GATK) to call SNP (single nucleotide polymorphism) and INDEL (insertion and deletion of bases) (McKenna et al. 2010). Only SNPs with a quality score above the threshold (Qpred>20) and with a SNP frequency of over 85% were included in the consensus sequences (S1 sequence file). The consensus sequences of the treated and untreated samples were aligned with Clustal Omega (McWilliam et al. 2013). Detected changes were annotated to include the locations and proteins affected (Table 2). Clean reads were mapped to the reference genome using BOWTIE2 to detect differentially expressed genes. Gene expression levels were calculated with RSEM version 1.2.12 (Li and Dewey 2011). Differentially expressed genes were identified by the possionDis, EBSeq software for samples without replicates (Leng et al. 2013).

Statistical analysis

All statistical analysis was performed using Microsoft Excel 2016 and SPSS software (IBM, USA). Spike protein-specific Ab responses, virus neutralizing titers, histology scores and qPCR data were analyzed by the Student's T-test. Differences between groups was considered significant at the level of $p<0.05$.

Results

Treatment with heat and RNase diminishes viral replication while maintaining structural integrity

To achieve the targeted outcomes of maintaining structural integrity while achieving diminished viral replication leading to attenuation, rather than complete inactivation, PEDV virus cultures were first exposed to temperatures ranging from 37°C to 60°C for 10 mins and visualized by electron microscopy. Intact structures were detected at all temperatures tested. However, increasing numbers of misshapen and fragmented virions were detected at 50°C and above. Cultures treated at 37°C and 45°C remained viable as viral replication was visible by immunofluorescence (IFA) in infected Vero cells using a PEDV-specific antibody, without any amplification by serial passaging. Virus was detected after the 1st passage in the cultures treated at 50°C. Virus cultures treated at 55°C and 60°C were not amplified even after four serial passages in Vero cells, indicating that complete inactivation occurred at these temperatures. Hence a temperature of 44°C for 10 mins was chosen for reversible unfolding of the viral capsid (Figure 15 C & D) without completely inactivating the virus. Similarly, the reduction in viral replication was proportional to the dose and time of exposure to RNase [data not shown]. A dose of 10 units of RNase A and 1000 units of RNase T with an exposure time of 4hrs was chosen as optimal for the final vaccine preparation. Following this treatment protocol, viral replication was detected only in the 3rd passage in Vero cells (Figure 16 B).

For the gamma (γ) irradiated virus culture which was used a control vaccine, typical icosahedral structures were seen in electron microscopy after 23hrs of exposure to radiation. However, the corona-like layer containing the protective spike antigens appeared to be damaged (Figure 15 E & F). At this dose of radiation, the virus was not detected by the IFA with a PEDV-

specific Ab at the third serial passage in cell culture (Figure 16 C). Hence, a final dose of 24 h (1,084,320 rad) was selected to prepare the inactivated control vaccine.

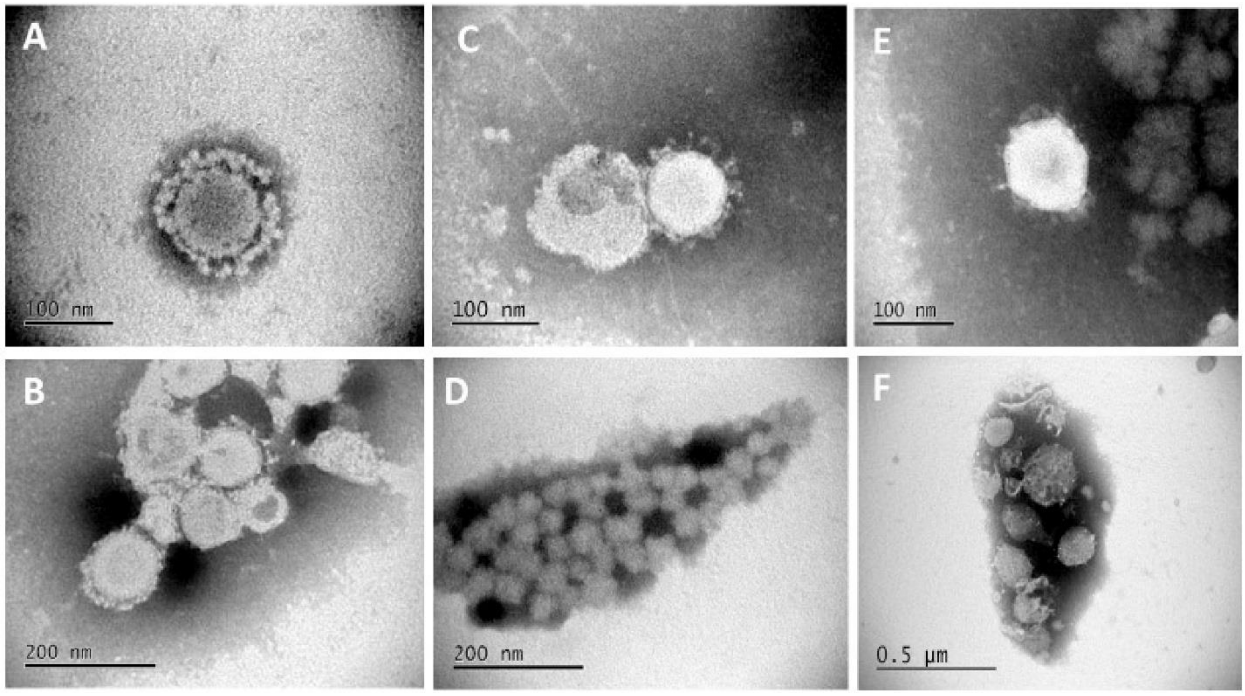

Figure 15. Electron micrographs of untreated and treated PEDV. Micrographs show the characteristic corona-like structure formed by the immunogenic spike protein embedded in the virus envelop of the icosahedral virus particle. A. Single virion of untreated PEDV B. Cluster of untreated PEDV virions C. Single virion of heat and RNase treated PEDV D. Cluster of heat and RNase treated PEDV E. Single virion of irradiated PEDV F. Cluster of irradiated PEDV virions.

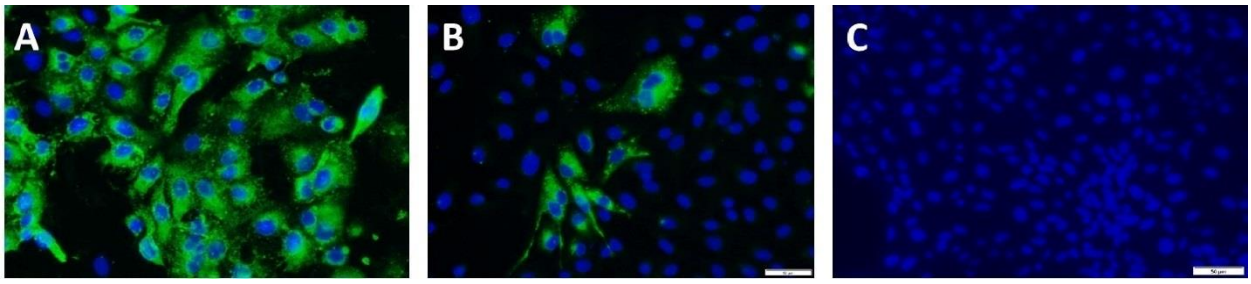

Figure 16. Amplification test for viral inactivation. Immunofluorescence images of vaccine viruses at the 3rd passage. Green cytoplasmic fluorescence is indicative of viral replication and blue fluorescence localizes to the nucleus of the infected Vero cells. Images were obtained by staining with a PEDV-specific polyclonal antibody. A. Untreated PEDV B. Heat and RNase treated PEDV at the 3rd passage showing minimal replication C. Irradiated PEDV at the 3rd passage.

Vaccination of pigs with the heat and RNase treated virions elicits a strong protective antibody (Ab) response

To assess vaccine safety and efficacy, 3 groups of 8 pigs each were immunized with the either the heat and RNase treated virions, irradiated virions or remained unvaccinated. Following two boosters, the pigs were challenged with virulent PEDV virus and euthanized after a week's observation. Vaccine efficacy and safety was measured by virus-specific Ab responses, reduction in fecal viral shedding and intestinal pathology in vaccinated pigs, compared to the controls.

Measurement of Ab responses against the PEDV spike and nucleocapsid proteins (NP) by ELISA (Okda et al. 2015) showed that animals vaccinated with the heat and RNase treated virions mounted strong Ab responses against the protective PEDV spike antigen but not the immunogenic but non-protective nucleocapsid protein, prior to the challenge. The Ab titers increased following the booster vaccinations on DPV 14 and 28 (Figure 17 A, B). A strong anamnestic response against the nucleoprotein was detected in the heat and RNase treated PEDV vaccine group but not the irradiated vaccine group, after challenge. In pigs immunized with the irradiated vaccine, Ab responses to both viral antigens were low. The optical density values for the ELISAs were significantly different between the groups (Figure 17 B).

Measurement of virus neutralizing antibodies by a fluorescent focus inhibition test (FFN) showed a trend which was similar to that of the spike protein-specific Abs. Strong virus neutralizing Ab responses, were detected in animals vaccinated with the heat and RNase treated virions but not in the pigs which received the irradiated viral vaccine. The differences between the groups was statistically significant (Figure 17 C). The spike protein-specific Ab and virus neutralizing Ab levels were strongly correlated in the heat and RNase treated PEDV vaccinated

pigs, with a correlation coefficient of 95.11%. As expected, the unvaccinated control pigs remained sero-negative for the duration of the study.

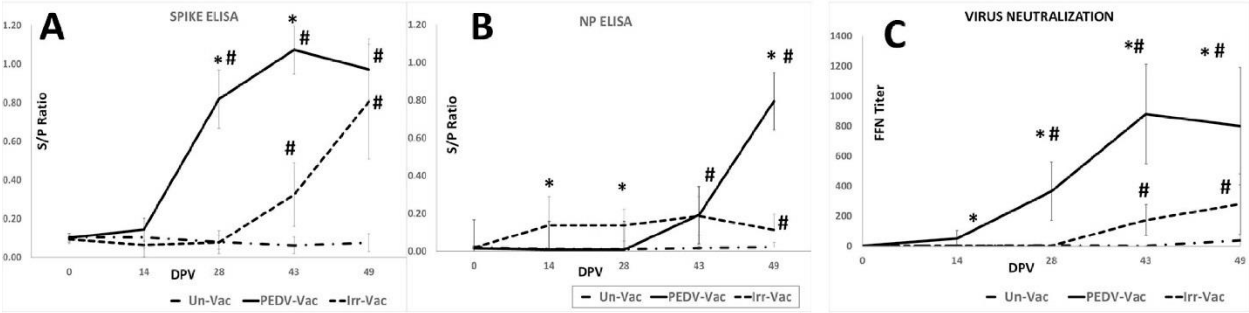

Figure 17. Serological responses to vaccination. A. Antibody responses to the PEDV spike protein as assessed by ELISA B. Antibody responses to the PEDV nucleoprotein as assessed by ELISA C. Virus neutralizing antibody responses as assessed by a fluorescent focus neutralization (FFN) assay. X axis – Days post vaccination, Y axis – ELISA OD value expressed as a signal to positive control ratio, Hashed line with dots – Unvaccinated controls, Solid line – Heat and RNase treated vaccine, Hashed line – Irradiated vaccine.

Vaccination induces sterilizing immunity against virulent viral challenge

To assess the efficacy of the vaccine in preventing replication of the challenge virus in immunized animals, shedding of the challenge viral RNA in fecal matter was assessed by a PEDV-specific qPCR on days 0, 3 and 7 post-challenge. All experimental animals were qPCR negative on day 0 post-challenge (DPC). At DPC 3 and 7, challenge viral RNA was not detected in any of the pigs vaccinated with the heat and RNase treated PEDV vaccine (Fig 4A & B), while 4 of the 6 pigs administered the irradiated vaccine were positive by qPCR on DPC3. All 6 pigs turned positive by DPC7 (Figure 18 A & B). The Ct values were significantly different between the two vaccine groups at DPC3 and DCP7 at $p \leq 0.05$. As expected, viral RNA was detected in the fecal matter of all unvaccinated pigs on both sample collection days. The average Ct values in unvaccinated pigs increased during the week-long post-challenge observation period, with values decreasing from 29.29 to 27.86 between DPC 3 and 7 respectively. There were no significant differences in fecal qPCR values between the unvaccinated controls and pigs

administered the irradiated vaccine at both the time points tested, indicating that the irradiated vaccine did not provide protection against viral replication in the host.

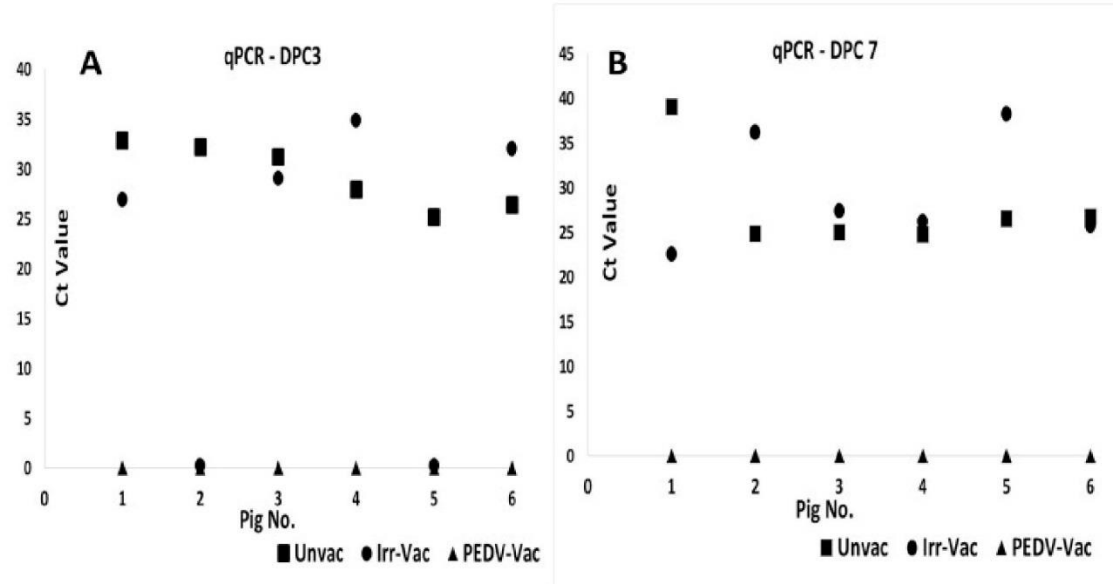

Figure 18. Post-challenge fecal viral loads. Viral RNA detected in the fecal matter of experimental pigs on day 3 and day 7 post-challenge depicted as Ct values from a PEDV-specific qPCR. A - Day 3 post-challenge, B - Day 7 post-challenge. X- Axis – Pig numbers. Y axis – Ct Values, Triangles – Heat and RNase vaccine, Circles – Irradiated vaccine, Squares- Unvaccinated pigs. N=6 pigs/ group (2 pigs/ group were sacrificed prior to challenge to assess vaccine safety). The heat and RNase treated PEDV group was significantly different from the irradiated vaccine group and untreated controls. No significant differences were found between the irradiated vaccine group and unvaccinated pigs. $p \leq 0.05$ by a Student' T test.

Vaccination protects against intestinal pathology

Examination of the gastro-intestinal tissue of the experimental animals by histology and immunohistochemistry (IHC) to assess the efficacy of the vaccines in preventing intestinal damage showed that the heat and RNase treated PEDV vaccine completely protected vaccinated pigs against the development of microscopic lesions following challenge. Four of the 6 animals in the unvaccinated and irradiated vaccine groups showed characteristic microscopic intestinal lesions of atrophic enteropathy and crypt hyperplasia in all 3 intestinal sections examined; namely duodenum, jejunum and ileum (Figure 19 D, E, F, G). Viral antigen was also detected in

the enterocytes in all three sections using a PEDV-specific monoclonal Ab-based immunohistochemistry assay (Figure 19 H, I, J). There were no significant differences between the 3 sections, indicating the entire small intestine was affected. The total microscopic score, including the histopathology and immunohistochemistry scores was 17 for the unvaccinated animals and 42 for the pigs immunized with the irradiated vaccine, and 0 for the animals vaccinated with the heat and RNase treated virions (Table 6). The difference between the two vaccine groups, as well as the difference between the vaccinated and unvaccinated animals was statistically significant, indicating that the irradiated vaccine actually enhanced intestinal pathology. Five of the 6 unvaccinated pigs, 1 out 6 vaccinated pigs from both vaccinated groups had semi-formed stools at necropsy. The total necropsy scores, which included both the fecal and histology scores, were highly significantly different between the two vaccine groups but not between the unvaccinated group and the irradiated vaccine group (Table 6). Although shedding of the challenge viral RNA was detected by qPCR in the fecal matter of all unvaccinated pigs, significant clinical signs of PED such as acute diarrhea, pyrexia or vomiting were not observed in the experimental animals during the post-challenge observation period, perhaps because severe clinical PED is manifested only in neonatal piglets.

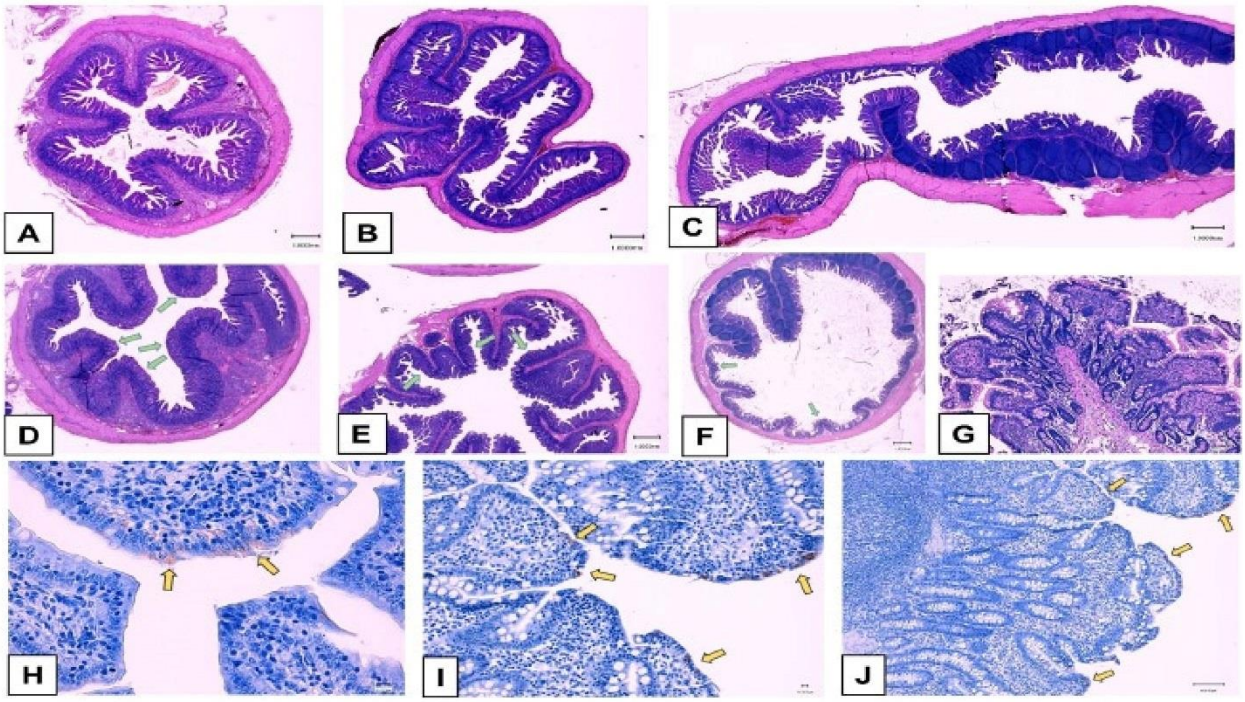

Figure 19. Post-challenge histopathology of small intestines. A-G – Hematoxylin and Eosin stained sections at 10X magnification. A-C – Healthy pigs. A- Duodenum, B-Jejunum, C-Ileum, D-G – Unvaccinated, PEDV challenged pigs D- Duodenum E -Jejunum F- Ileum G- Ileum (100x). Green arrows indicate areas of villus atrophy and crypt hyperplasia H-J - Immunohistochemistry of unvaccinated challenged pigs. Sections were stained by a PEDV-specific antibody. Yellow arrows indicate viral antigen localized to enterocytes. H- Jejunum (400X) I-ileum (200X) J- Duodenum(100X).

Table 6. Microscopic lesion scores

Vaccine Efficacy					
Unvaccinated	8 (4/6)	9 (4/6)	17 (4/6)	15 (5/6)	32 (6/6)
RNase + Heat treated PEDV/ Challenged	0 (0/6)	0 (0/6)	0 (0/6) ($p=0.02$)	3 (1/6) ($p=0.05$)	3 (1/6) ($p=0.0009$)
Irradiated PEDV / Challenged	26 (4/6)	16 (4/6)	42 (4/6) ($p_a=0.017$) ($p_b=0.002$)	3 (1/6) ($p_a=0.009$) ($p_b=0.0.05$)	45 (5/6) ($p_a=0.147$) ($p_b=0.0009$)
Vaccine Safety					
RNase + Heat treated PEDV/ Unchallenged	0 (0/2)	0 (0/2)	0 (0/2)	0 (0/2)	0 (0/2)
Irradiated PEDV / Unchallenged	0 (0/2)	0 (0/2)	0 (0/2)	0 (0/2)	0 (0/2)

Total number of pigs = 8, No. of pigs sacrificed for vaccine safety assessment prior to challenge N= 2, No. of pigs sacrificed at day 7 post challenge N = 6. % - Total atrophic enteritis score for the ileum, jejunum, duodenum where 0= negative, 2= mild, 4 = moderate, 6 = severe, 2= sections with crypt hypertrophy. & - Total immunohistochemistry (IHC) for the Ileum, jejunum, duodenum where 0= negative, 2= positive, ≤10%, 4 = positive, 11-50%, 6 = positive, >50%. *Fecal score at necropsy– Formed Feces=0, Semi-formed feces = 3, Liquid feces = 6. pa = Statistical significance when compared to the unvaccinated group, pb - Statistical significance when compared to the RNase and heat treated PEDV vaccinate group.

The irradiated and heat and RNase treated vaccines are safe

Post-vaccination safety was assessed by observation of animals for manifestation of clinical signs of PED after vaccination, shedding of the vaccine viral RNA in feces and necropsy of 2 animals per group prior to challenge to assess intestinal pathology. No clinical signs of PED such as pyrexia, diarrhea, vomiting or weight loss were observed in vaccinated pigs after either the primary or booster vaccines. The vaccine viral RNA was not detected by qPCR in the fecal matter of any of the vaccinated pigs at 7 days after the primary vaccination or at one week after the boosters. All animals remained PCR negative until the day of challenge. Therefore, although

the heat and RNase treated PEDV virions were detected by amplification after 3 serial passages in Vero cells, replication of the vaccine virus in the host appeared to be curtailed by its immune system. In the 2 pigs euthanized from each group prior to challenge, stools were fully formed at necropsy, (Table 6). No microscopic lesions or viral antigen were detected in the small intestine sections, heart, spleen and liver of the 2 animals necropsied from each group prior to challenge (Table 6). Representative images of the duodenum, jejunum and ileum are depicted in Figure 19.

Heat and RNase treatment results in genomic mutations.

To identify possible mutations that could explain the highly effective attenuation observed with the heat and RNase treatment, treated virions were subjected to deep sequencing. A total of 59.42 and 24.44 MB of raw reads were obtained by RNA seq for the treated and untreated samples respectively, while the clean reads after trimming were 26.94 and 19.53GB respectively. The Qphred20 values for the clean reads were 96.69 and 98.49 for the untreated and tread samples respectively, indicating satisfactory quality of the data obtained. Single nucleotide polymorphisms (SNPs) and insertions or deletions (INDELS) were detected in the polyprotein, spike and envelope proteins of heat and RNase treated virions, when compared to the untreated virions. Two point mutations in NSP3 and NSP9 respectively, and one frame shift mutation affecting the last 9 amino acids of NSP13 were detected in the polyprotein (Table 7). Three non-synonymous mutations each were detected in the S1 and S2 regions of the immunogenic spike protein respectively. In addition, insertions and deletions were detected in the S1 region. The N terminal signal peptide region of the spike protein had a 2 amino acid deletion and one non-synonymous change at position 355, changing the sequence from IGEN to K—N. A conservative in-frame insertion was detected at position 355 in the S1 region, changing the amino acid sequence from L----AT to LKKKGAT. A single SNP was detected in the envelop protein at

position 62, with a transition from S to F (Table 7). Differential gene expression analysis showed that four viral genes were significantly upregulated in the treated sample when compared to the untreated control; namely envelope (11.34 fold), ORF3 (5.0 fold), spike (4.7 fold), M (4.1 fold), and polyprotein (1.6 fold).

Table 7. SNPs and INDELS

Pos	R	Un-Trt	Trt	Con-sequence	Residue Change	Gene	AF	Type
Poly-protein								
4982	C	C	T	NS	S1564F	PP-NSP3	1.0	Ti
12156	TC	TC	CG	NS	R3956G	PP-NSP9	0.99	Ti
20203	A	A	-	Frame-shift	P6640-LALGGTVAIK to VGTWWYCSY.	PP-NSP13	0.99	
Spike								
20796	TTGGTG	TTGGTG	-	NS & Del	P55- IGEN to K--N	S-N term	1.0	
21307	T	T	C	S	_	S-S1	1.0	Ti
21698	-	-	AAGAAGAAAGGT	In-frame insertion, conservative	P355 L----AT to LKKKGAT	S-S1	0.86	
21761	C	C	T	NS	L377F	S-S1	1.0	Ti
22541	T	C	C	NS	F637L	S-S1	1.0	Ti
23300	G	G	C	NS	G890R	S-S2	1.0	Tv
24395	G	G	T	NS	D1211Y	S-S2	1.0	Tv
24796	G	T	T	NS	Q1388H	S-S2	1.0	Tv
Envelop								
25638	C	C	T	NS	S62F	Envelop	1.0	Ti

Pos: position on the consensus sequence of the treated vaccine virus, R: nucleotide in the reference genome, Un-Trt: SNP in the un-treated PEDV, Trt: SNP on the treated PEDV, NS-Non-Synonymous, S -Synonymous, PP-Polyprotein, S- Spike, AF: allele frequency, Ti: transition mutation, Tv: transversion mutation.

Discussion

The recognition that new technology and policies for rapid-response vaccines (Finlay, See, and Brunham 2004, Plotkin 2017, Wong and Qiu 2018, Yamey et al. 2017) and diagnostics (Perkins et al. 2017, Song et al. 2016) are critical for emergency management is one of the important lessons learnt from the recent global pandemics such as SARS and Ebola. Despite the need, only a few other approaches for rapid-response vaccine development are described in literature. One approach consists of exploiting pre-existing immunity to non-cognate, chronic pathogens, such as cytomegaloviruses. Incorporation of CD4+T cell epitopes from the chronic pathogen in vaccine formulations against the new agent improved vaccine-induced Ab responses (Hills et al. 2016). Two other successful experimental approaches include enzymatic, cell-free, gene assembly to generate synthetic influenza viral particles (Dormitzer et al. 2013) and using dendrimers to deliver mRNA-replicons encoding protective antigens (Chahal et al. 2016). While elegant, all of these approaches require a thorough knowledge of protective antigens, genetic sequences and well-characterized epitopes to be effective. This knowledge is not always available for newly emerging pathogens. Recombinant DNA methods can also be more complex and require a longer development time, depending on success with expressing the required targets. Hence, the approach described in this study has the advantage that it circumvents the above-mentioned bottle necks. However, a potential limitation is it requires culturable virus.

Chemical methods for inactivation of viruses, such as formaldehyde and beta-propiolactone have long been in commercial use. They are rapid and convenient. However, the efficacy of inactivated vaccines is questionable; especially against a majority of RNA viruses including PEDV (Song, Moon, and Kang 2015), likely due to irreversible denaturation of protective antigens and changes in the antigenicity of critical epitopes during chemical exposure.

More recently, H2O2 was used to rapidly inactivate influenza and lymphocytic choriomeningitis viruses (Amanna, Raue, and Slifka 2012). However, H2O2, can also oxidize amino acids (Finnegan et al. 2010) and the H2O2 platform could predominantly target cell mediated immune responses (Walker, Raue, and Slifka 2012). While irradiation has commonly been used for inactivation of viruses, several irradiation-based vaccines have been described with varying degrees success (Seo 2015). In a previous study, we were able to successfully develop an inactivated vaccine Neospora caninum using gamma-irradiated techyzoites (Ramamoorthy et al. 2006). While irradiation can also affect antigenic structures depending on the conditions on irradiation (Alsharifi and David 2017), gamma-irradiation was our preferred method of choice to prepare a control vaccine for this study as it was more likely to maintain a virus-like-particle like structure while achieving complete inactivation.

The exact corelates of cell mediated immune protection against PEDV are as yet unknown but the importance of spike protein-specific antibodies for protection against PEDV is well established (Song et al. 2016). The contribution of the nucleoprotein (NP) antigen to protection is as yet uncharacterized but strong Ab responses to the NP can be detected in acute in PEDV infections when the virus is replicating actively. The lack of strong Ab responses to the NP in vaccinated pigs in this study is likely due to viral replication being absent or minimal in the host. Since viable virus was detected by amplification in cell culture after the heat and RNase treatment, it is likely that host innate immunity was sufficient to prevent active vaccine viral replication. Cell mediated immune responses to vaccination were not studied due to technical difficulties associated with reviving immune cells after transport. However, since our approach was very successful in preventing PEDV associated lesions in challenged pigs, it is very likely that cell mediated immunity was not compromised by the process used.

While characterizing the exact physical interactions involved in the heat and RNase treatment is not within the scope of this study, our finding that exposure of PEDV to temperatures below 50oC did not affect structure was similar to those of Wang et.al, (Wang et al. 2004), who showed that the SARS coronavirus structure is metastable and can be reversibly denatured by exposure to varying physical conditions such pH and temperature (Darnell and Taylor 2006). It is possible that the RNase can access the viral genome via the ion-channel E protein, a viroporin which spans the capsid to form transmembrane pores of about 1-2nm diameter (Wetherill et al. 2012). Besides ions, viroporins are known to permit the trafficking of small molecules (Nieva, Madan, and Carrasco 2012), and interact with viral glycoproteins (Bour, Perrin, and Strebel 1999). In the relaxed state induced by the gentle heat, it is possible that RNase, which is also metastable and has a diameter of 3-4nm (Ramm, Whitlow, and Mayer 1985) can access the viral genome within the capsid to degrade the viral genomic RNA.

Similar to our findings with the irradiated vaccine, a dendritic cell targeted spike protein-based subunit vaccine against PEDV exacerbated intestinal pathology in vaccinated pigs despite stimulating strong CD4+/CD8+ T cell responses (Subramaniam et al. 2018). Therefore, the higher efficacy of the heat and RNase treated PEDV compared to irradiated PEDV could be attributed to effective priming and differences in antigen presentation in-vivo. Alternately, mutations caused by repair of the RNA genome after fragmentation by RNase treatment could have led to the highly effective attenuation achieved in this study. Insertions and deletions in the spike protein, especially the S1 region, are common in PEDV and are known to influence pathogenicity and immunogenicity of the isolates (Vlasova et al. 2014, Sun et al. 2018). It is possible that the two INDELS located in the spike protein of treated virions as detected by deep sequencing (Table 7), positively influenced the immunogenicity and level of attenuation of the

vaccine virus. The core neutralizing epitope of the PEDV spike protein has been localized to amino acid positions 503-568 (Chang et al. 2002). The SNPs identified in the spike protein of the vaccine virions did not map to these residues. However, other detected SNPs could have positively influenced immunogenicity. Interestingly, a comparison of differential viral gene expression between untreated and treated virions showed that the levels of the ORF3, whose deletion has been associated with improved tissue culture adaptation (Li, Li, et al. 2013), was upregulated by about 5-fold. The exact functions of the various non-structural proteins encoded by the replication associated polyprotein are as yet fully uncharacterized. The mutations detected in the polyprotein (Table 7) could promote the upregulation of gene expression detected in the treated virions. While more detailed studies are required to confirm these hypotheses, they are not within the scope of this manuscript. A limitation of the described method is that the mutations induced by treatment and repair are unpredictable. However, maintaining consistency in the treatment process is very likely to ensure reproducibility of vaccine efficacy and safety using our approach.

The pigs in this study showed mild clinical symptoms, fecal shedding and histological lesions, which can be due to age of the pigs as wells as the higher cell-culture passage of challenge virus (Jung and Saif 2015). As document in several studies, PEDV infectivity is age-dependent, in neonatal piglets PEDV causes severe diseases and high morality (80-100%) whereas in pigs older than 3 weeks severity and mortality is much lower (1-3%) and usually recover over a week (Madson et al. 2014, Shibata et al. 2000). The infectivity of PEDV also depends on the virulence of the PEDV strains, in this case attenuation of the PEDV virulence might have induced through the high cell-culture passage (Lawrence et al. 2014, Song et al. 2003, Sato et al. 2011).

The route of immunization is also a major factor in inducing successful immune response for protection at the initial site of infection (Joo et al. 2010, Bumann et al. 2010). For successful protective immune response mucosal pathogen such as PEDV require tailoring mucosal immunity, which can be done by either oral immunization or by systemic immunization such as intramuscular or subcutaneous route (Belyakov and Ahlers 2009, John 2001, Suzich et al. 1995). However, systemic immunization for inducing protective mucosal immunity is still controversial and available PEDV vaccine injected intramuscular didn't confer fully protection against the challenge virus (Bumann et al. 2010, Joo et al. 2010, Crawford et al. 2016, Schwartz TJ 2015). On other hand, oral immunization holds greater potential for generating the protective gut immune response, but its main challenge is need of effective delivery system which can protect antigen form the hard environment of the gut (Belyakov and Ahlers 2009, Zhang, Wang, and Wang 2015). Due to constrain resources in this study we immunization piglets with both oral and intramuscular route to induced better and fully protection against the challenge virus.

While ideal for PEDV vaccine studies, studying vaccine efficacy in pregnant sows is expensive and procedurally tedious. Hence the weanling piglet model used in this study has been advocated and is frequently used as a surrogate to pregnant sow models for vaccine testing (Crawford et al. 2016, Schwartz TJ 2015). While PEDV was not present in the U.S prior to 2013, it was endemic in other parts of the world. Both attenuated and inactivated vaccines are routinely deployed in the field in endemic countries (Lee 2015). However, data accumulated over time shows that PEDV vaccines are of questionable efficacy, have a low duration of immunity and are ineffective in preventing outbreaks in vaccinated herds or the emergence of new strains (Gerdts and Zakhartchouk 2017, Lee 2015, Song, Moon, and Kang 2015). In North America, a S-protein based subunit vaccine (iPED plus, Harris Vaccines Inc.) and inactivated vaccines produced by

Zoetis and VIDO-Intervac were conditionally licensed. However, their efficacy has also been questioned by independent studies (Crawford et al. 2016, Schwartz TJ 2015). Producers continue the practice of feeding back virus infected fecal matter or minced intestines from infected piglets to sows, in an attempt to induce more effective immunity against PEDV (Crawford et al. 2016, Schwartz TJ 2015). The use of autogenous vaccines, where a custom vaccine tailored to each herd is prepared using a sample provided from the production unit, is also popular for economically important infectious diseases for which commercial vaccines are ineffective (Attia, Schmerold, and Honel 2013, Saegerman et al. 2007, Sandbulte et al. 2015). Both the feedback and autogenous vaccine approaches are, once again, associated with significant safety and efficacy issues. Further, a number of experimental PEDV vaccines ranging from inactivated, attenuated, subunit and vectored vaccines, tested in the piglet model, are described in literature (Chang, Hsu, et al. 2018, Chang, Chang, et al. 2018, Hain et al. 2016, Lee et al. 2018, Lin et al. 2019, Liu et al. 2019, Mogler, Gander, and Harris 2014, Opriessnig et al. 2017, Subramaniam et al. 2018). While most of the cited studies report a reduction in viral shedding and clinical signs, to the best of our knowledge, sterilizing immunity as achieved in this study was not encountered in any of these studies. Besides being an effective rapid-response vaccine approach, since it also has a very high safety margin, the heat and RNase treatment approach can potentially provide a much-needed improvement in PEDV vaccines. The approach can also have applications in the veterinary autogenous vaccine industry. Therefore, in comparison to existing commercial and experimental PEDV vaccines the primary advantages of the described proof-of-concept method are convenience, a short development time, high safety and efficacy; all hall-marks of epidemic vaccines. Our future goals include testing the heat and RNase treated vaccine in pregnant sows,

adapting our method to other RNA viruses and improving oral and respiratory mucosal vaccine delivery systems for rapid response vaccines.

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CHAPTER 3. A CONVENIENT COLORIMETRIC ASSAY FOR THE QUANTIFICATION OF PORCINE EPIDEMIC DIARRHEA VIRUS AND NEUTRALIZING ANTIBODIES [2]

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Abstract

Neonatal enteritis caused by the porcine epidemic diarrhea virus (PEDV) is an important cause of high mortality and economic losses to the swine industry. Virus neutralization (V/N) assays are commonly requested in diagnostic laboratories for the assessment of protective antibodies. However, the visual assessment of viral cytopathic effects by operators to determine antibody titers or for viral quantification is a tedious, subjective and time-consuming process, especially when high volume testing is involved. To improve the ease of testing, a colorimetric virus neutralization and $TCID_{50}$ assays were developed and validated in this study using (3-(4,5-dimethylthiazol-2-yl) Tr-2,5-diphenyltetrazolium- bromide) (MTT), a colorimetric agent which

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[2] Singh, Pankaj, **Gagandeep Singh**, Jenna Karsky, Eric Nelson, and Sheela Ramamoorthy. "A convenient colorimetric assay for the quantification of porcine epidemic diarrhea virus and neutralizing antibodies." Journal of virological methods 262 (2018): 32-37. The material in this chapter was co-authored by Gagandeep Singh, Pankaj Singh and Sheela Ramamoorthy. Gagandeep Singh and Pankaj Singh had primary responsibility for conducting experiments, and analysis the samples. Gagandeep Singh was the primary developer of the conclusions that are advanced here. Gagandeep Singh also drafted and revised all versions of this chapter. Sheela Ramamoorthy served as proofreader and checked the math in the statistical analysis conducted by Gagandeep Singh.

measures cell viability. The respective conventional assays were used as the gold standards. An OD cut off value of ≤0.53, selected by receiver operating characteristics analysis, could distinguish between wells with and without CPE accurately. Performance and reproducibility parameters of the colorimetric assays were comparable to the conventional assays. The described methods can reduce testing time in diagnostic laboratories, while significantly improving current protocols.

Introduction

The porcine epidemic diarrhea virus (PEDV) is a highly contagious viral infection of neonatal pigs, characterized by severe diarrhea, vomiting and dehydration. Mortality rates can be as high as 100% in neonatal piglets born to naïve sows (Wang et al. 2014). A member of the alphacoronavirus family, PEDV has been prevalent in Asian countries for over thirty years. It was first detected in the U.S. in 2013 (Chen et al.). Rapid spread of the virus in a naïve population, in conjunction with the high mortality rate resulted in the loss of about 7 million piglets in the first year alone (Schulz and Tonsor 2015). While PEDV has become enzootic in the U.S. now, it continues to pose a significant economic problem to the pork industry. Therefore, the effective detection and diagnosis of PEDV plays a key role in the control of the disease.

Enzyme linked immunosorbent assays (ELISA) are commonly used for the serological detection of PEDV. However, the quantification of protective antibody responses is often undertaken to determine vaccine-induced protection or herd level immunity against new or related strains, and is assessed by virus neutralization (V/N) tests or fluorescent focus neutralization (FFN) tests in veterinary diagnostic labs(Diel et al. 2016). Both V/N and FFN tests measure the extent of inhibition of viral replication or cytopathic effect (CPE) by the test serum, which in turn depends on the accurate titration of virus stocks used in the assay.

Quantitative reverse transcription PCRs are available for PEDV and used to measure viral nucleic acid content. However, conventional tissue culture infective dose$_{50}$ ($TCID_{50}$) assays are required for the measurement of infective virus. Plaque assays are useful for the quantification of coronaviruses and to distinguish between strains by plaque morphology. However, they are not used frequently for PEDV, perhaps because laboratory culture of PEDV is tedious due to the strict trypsin requirements. Trypsin is required for the cleavage of receptor-bound PEDV S protein and entry into cells (Park, Cruz, and Shin 2011). In laboratories with a high volume of testing, the manual and visual evaluation of both the $TCID_{50}$, V/N or plaque neutralization assays are tedious to perform, as they involve the manual examination of numerous wells of 96 well plates or counting of plaques in Petri plates by the operators. Visual assessment of numerous 96 well plates can cause eye and neck strain over long periods of time and thus pose a health hazard. Variation can also be introduced due to differences between operators in scoring the wells as positive or negative, introducing subjectivity in testing.

The goal of this study is to develop a convenient assay to assess PEDV-induced cytopathic effects (CPE)and with applicability to virus $TCID_{50}$ and V/N assays. We have used MTT (3-(4,5-dimethylthiazol-2-yl) Tr-2,5-diphenyltetrazolium- bromide), a colorimetric agent which measures cell viability. Loss of cell viability is characteristic of virus induced CPE and can be measured by MTT. The reduction of MTT to formazan by cellular dehydrogenases is indicative of the mitochondrial and other enzymatic activity in healthy cells and can be measured as an optical density value with an enzyme linked immunosorbent assay (ELISA) reader (Mosmann 1983). Therefore, PEDV-infected cells showing CPE are expected to be less efficient in formazan production when compared to healthy cells. Thus, this study is the first description of a colorimetric assay format for a virus neutralization test for a coronavirus. By employing a

receiver operating characteristics (ROC) analysis-based cut-off value to distinguish between wells with and without CPE, the optical density (OD) value output from an ELISA reader could be easily used to score wells, reducing the subjectivity and time involved in visual reading. The improved colorimetric methods had a high level of agreement with conventional assays and can significantly reduce the labor involved in the serological testing of PEDV.

Materials and Methods

Virus culture

Laboratory culture of PEDV strain CO 2013 (National Veterinary Services Laboratory (NVSL), Ames, IA) was achieved using a monolayer of Vero cells grown to confluence in T-75 flasks, at a multiplicity index (MOI) of 0.1. After infection, the flasks were incubated at 37°C in 5% CO_2 incubator. Growth media contained Dulbecco's Modified Eagle's Medium (DMEM) (Corning, Manassas, VA), 10 µg/ml Trypsin (Sigma-Aldrich, St. Louis, MO), 7% Tryptose phosphate broth (TPB) (Gibco BRL, Grand Island, NY) and 100 units/mL of penicillin and 100 µg/mL of streptomycin (GE Healthcare Cell Culture, Logan, UT). After 3h the virus inoculum was removed, and fresh growth media was added to the cells. Fresh trypsin was added to the flask every 24h until the cytopathic effect could be seen at approximately 72h. The virus culture was harvested with three consecutive freeze-thaw cycles and centrifuged at 2415 x g for 30 mins at 4°C. The supernatant was distributed in 1ml aliquots and stored at -80 °C until further use.

Plaque assay for PEDV quantification

Vero cells were seeded in 6 well plates (Corning) and incubated at 37ºC in a CO_2 incubator until a complete monolayer was formed. Log dilutions of the PEDV virus culture from 10^{-1} to 10^{-6} were prepared in infection media as described above. The monolayers were infected with the 1ml of diluted culture after washing twice with Hanks Balanced Salt Solution (HBSS)

(Corning, Manassas, VA). One well remained as an uninfected cell control. The plates were incubated for 3h at 37ºC in a CO_2 incubator. The viral inoculum was removed, and cells were washed once with HBSS. A 1% solution of low melting agarose (Thermofisher, Waltham, MA) mixed with 2X infection media was overlaid onto the infected monolayers, such that the final concentration of the agarose was 0.5% and the infection media was 1X. After solidification of the agarose, the plates were incubated in a CO_2 incubator at 37ºC for 72h. For fixing, 2ml of 10% buffered formalin (Thermofisher, Waltham, MA), pH 7.0, was added to the each well and incubated for 4h. Agarose overlays were removed by tapping, and wells were stained with 0.1% crystal violet (Thermo Scientific, Waltham, MA). The virus stock was independently titrated five times by the plaque assay. Plaques were enumerated by two independent operators. As no plaques were visible in wells infected with the 10^{-6} dilution circular, countable plaques which were visible in the wells infected with the 10^{-4} and 10^{-5} logarithmic dilutions were used to calculate the plaque forming units (PFU) by standard methods (Okda et al. 2015).

Virus titration by the conventional $TCID_{50}$ method

The stored aliquots of the PEDV virus culture were titrated by the $TCID_{50}$ method three times, in duplicate, to obtain a total of 6 readings. The assay was set up by the standard method (Schumacher et al. 2016) using logarithmic dilutions of the culture ranging from -1 to -8, in 96 well plates. Six wells were used for each dilution. The process for virus culture was essentially as described above, except 96 well plates were used and 100µl of each log dilution was plated per well. Cell controls incubated with growth media to rule out any other causes of toxicity and undiluted virus controls were included in each plate. The presence of CPE in each well was scored by visual microscopic examination, by two independent operators and titers calculated by the Spearman and Karber method (Ramakrishnan 2016).

Colorimetric MTT_{50} assay

On completion of the visual reading, the supernatant in each well was removed by aspiration. A solution of 0.5mg/ml (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide) (Thermo Scientific, Waltham, MA) (MTT) was prepared in sterile phosphate buffered saline (PBS), pH 7.0, and added to each well in 100µl volumes. The plates were incubated in a CO_2 incubator for 4h to reduce MTT into formazan. The supernatant from each well was carefully removed without disturbing the cells by aspiration. To solubilize the intracellular formazan, 100µl dimethyl sulfoxide (DMSO) (Thermo Scientific, Waltham, MA), was added to the each well, mixed well by vigorous pipetting, and incubated for 5 mins at 37^{o}C. Plates were read at 570 nm in an ELISA microplate reader (Biotek, Winooski, VT) to obtain optical density (OD) readings.

Receiver operating characteristics (ROC) analysis

To determine the most optimal OD cut-off value to distinguish between wells with and without virus-induced CPE, the readings obtained by visual assessment for the $TCID_{50}$ assay were used as the gold standard for analysis, since the $TCID_{50}$ is well-established as a standard method for viral quantification.

Dichotomized values of the visual readings from $TCID_{50}$ assays were analyzed against the OD value read outs obtained from the ELISA plate reader, after addition of MTT to the plates. The sensitivity (Y axis) was plotted against the 100-specificity (X axis) for different cut-off points of the OD values, to obtain the predicted sensitivity and specificity at each point. The area under the curve was used to determine the accuracy of distinguishing between positive and negative wells, with 100% values representing perfect discrimination. The sensitivity and specificity of differentiating wells with and without CPE, and the positive and negative

predicative values at the software generated OD cutoff value off were obtained using a commercial statistical software package (MedCalc software, MedCalc Inc., Ostend, Belgium). Agreement between wells scored visually and by the OD value was assessed by the Kappa statistic.

Conventional and colorimetric virus neutralization (V/N) assays

A standard V/N assay format (Chen et al. 2016) was optimized using control sera purchased from the National Veterinary Services Laboratory (NVSL), Ames, IA. Briefly, 10 doubling dilutions of the positive and negative control sera, starting at a 1:2 dilution, was prepared in DMEM and incubated with an equal volume (50 µl) of 10^3 $TCID_{50}$ of the PEDV virus stock in 96 well U bottom plates for one hour at 37°C. After washing the wells twice with HBSS, the serum and PEDV mixture was transferred to 70% confluent Vero cells monolayers. The plates were incubated at 37ºC in a CO_2 incubator for 3h. The inoculum was removed after the initial incubation and replaced with infection media, as described in the virus culture method. Six wells each were maintained as either uninfected controls or virus controls. After incubation for 48h, the plates were read visually to assess inhibition of CPE by the test serum. A 90% inhibition of viral replication was considered the end point to assign the V/N titer (Hansen et al. 2015).

Thereafter, to adapt the assay to the colorimetric format, MTT was added to the plates as described above. Optical density values corresponding to the visual readings were obtained by reading the plates in an ELISA reader. For the colorimetric assay, wells were scored as positive or negative based on the OD cutoff value previously determined by Receiver Operating Characteristics (ROC) analysis. The colorimetric V/N titer was determined as the lowest dilution at which the wells had an OD value ≤ the cut off value. The agreement between the conventional

and colorimetric tests were assessed by comparison of the titers obtained by the two methods. To assess reproducibility in terms of inter and intraassay variation, the positive and negative controls were titrated by the conventional and colorimetric V/N assays in triplicate on two separate occasions indicated as replicate 1 and 2 with values from 1-6 in Table 1. Sera from pigs infected with the other commonly prevalent viruses like porcine circovirus strain 2 (PCV2), porcine reproductive and respiratory disease syndrome virus (PRRSV) were used to test for specificity.

Sera were selected randomly from six PEDV-suspect farms, submitted to the serology section of the South Dakota Animal Disease Research and Diagnostic Laboratory (SDSU-ADRDL) to validate the colorimetric V/N assay on field samples. The samples were assessed using a fully standardized PEDV N protein-based ELISA protocol, routinely used at the S. Dakota State University Veterinary Diagnostic Laboratory (Okda et al.). Seropositive (N=35) and negative sera (N=15) (total N=50) were randomly selected based on the ELISA. Reactivity of the selected samples to PEDV further confirmed by a standard immuno-fluorescence assay as described before (Song et al. 2016). The selected samples were tested by both the conventional and colorimetric V/N assays in 2 independent assays.

All experimental protocols were carried out in compliance with the Institutional Biosafety Committee of North Dakota State University and South Dakota State University.

Data analysis

The agreement between the $TCID_{50}$ assay, plaque assay and MTT_{50} was assessed by the Spearman's rank coefficient or kappa statistic. The agreement between the conventional V/N tests and the MTT V/N assay was assessed by the Spearman's rank coefficient. The $\log_2$ values of the V/N titers were analyzed by a Student's T test (Chen et al. 2016). All data analysis was

carried out using commercial statistical software (MedCalc software, MedCalc Inc., Ostend, Belgium) and p values <0.05 were considered significant.

Results

To ensure accuracy initial quantification of the virus stock was first measured both by plaque assay, where growth of the virus could be visualized as plaques, and $TCID_{50}$, where virus replication could be visualized as CPE. As expected, wells with a higher concentration of virus showed a complete loss of the cell sheet in the plaque assay. Distinct, countable plaques were detected in the 10^{-4} and 10^{-5} dilutions (Figure 20). The average titer of the virus stock based on the plaque assay was $10^{6.05 \pm 0.43}$ plaque forming units (PFU) while the mean titer obtained by $TCID_{50}$ titrations was $10^{4.55 \pm 0.21}$. The agreement between the $TCID_{50}$ and the plaque assay as measured by the Spearman's rank coefficient was 0.975 with a p value of 0.005. The 95% confidence interval for rho was 0.660 to 0.998. The cell controls remained uninfected while the virus controls showed clear CPE, for the duration of the incubation. As expected for the MTT_{50} assay, the OD values obtained after addition of MTT were low for wells with higher concentrations of virus, and vice versa, reflective of low cell viability due to viral infection. The uninfected cell controls had an average OD value of 1.10. The relationship between decreasing concentrations of virus in the logarithmic viral dilutions and increase in the OD value on the MTT assay was linear with the equation $y = 0.1496x + 0.0821$, $R^2 = 0.916$, indicating specific detection of viral replication by the MTT assay (Figure 21).

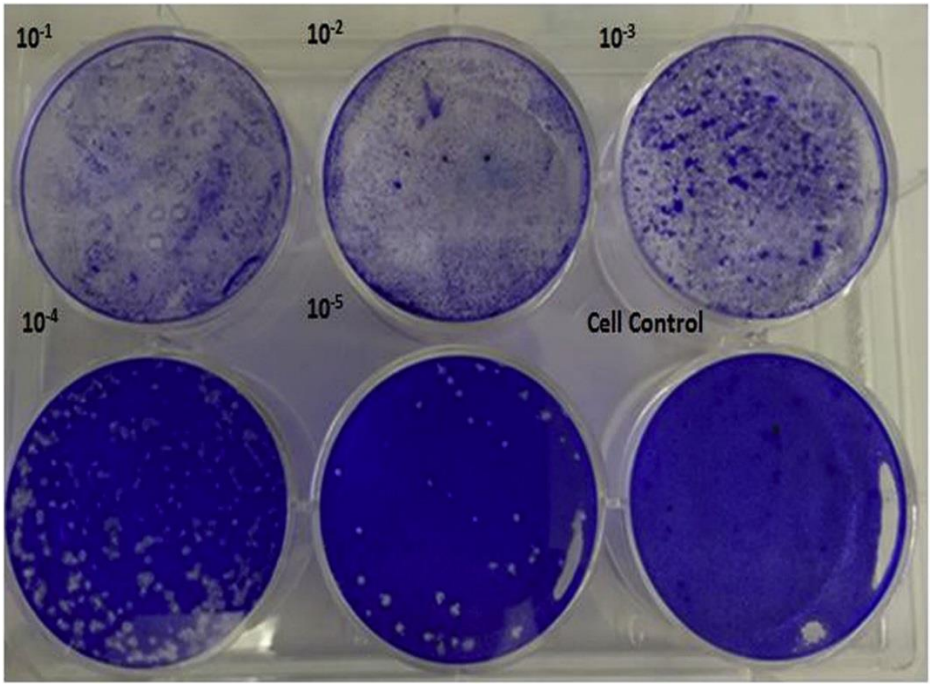

Figure 20. Plaque assay of the PEDV virus culture. Representative image of the PEDV plaque assay showing a complete loss of the cell sheet in the lower dilutions and distinct viral plaques in the 10^{-4} and 10^{-5} dilutions. The cell control shows an intact cell sheet.

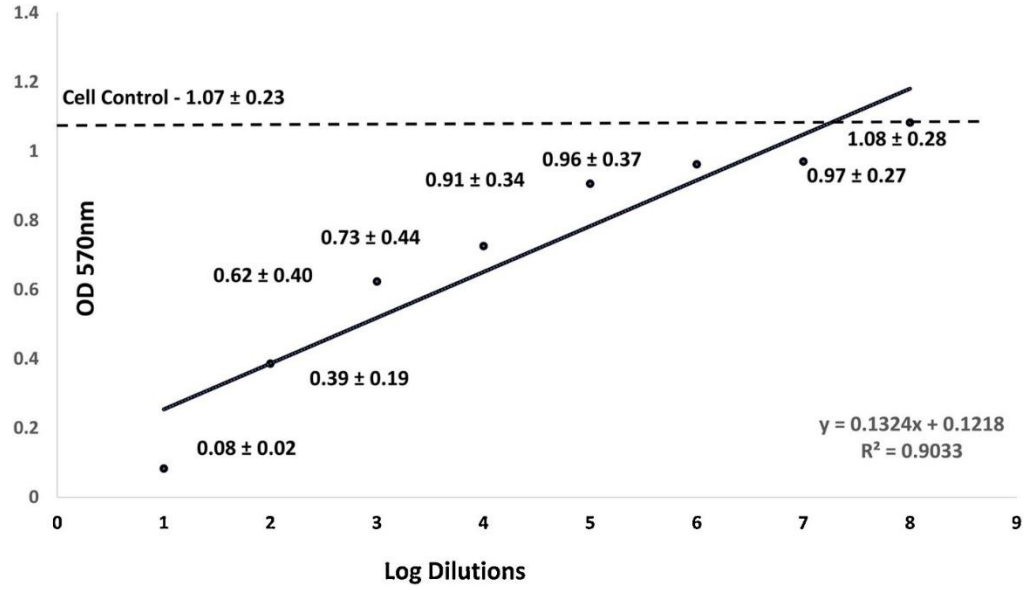

Figure 21. Linear relationship between the logarithmic viral dilutions and OD values. The average OD values for each logarithmic dilution obtained from the readout of the MTT assay plotted against the logarithmic dilution factor. The relationship was linear between the 10−1 and 10-5 log dilutions. The dashed horizontal line indicates the average OD value of the cell controls.

Using dichotomized values from the visual reading as the gold standard for ROC analysis, an OD value of ≤ 0.53 was selected as the optimal cutoff to distinguish between wells with and without CPE. The area under the curve was 0.933 with a Z statistic p value of <0.0001 (Figure 22). The area under the curve represents the accuracy of the test, with a value of 1.0 representing a perfect test. The p value is the statistical probability that the test can accurately distinguish between positive and negative samples. At this software generated cutoff, the positive and negative predictive values were both 90% respectively and the specificity was 95%, while the sensitivity was 81%. The kappa statistic for the agreement between the visual and MTT read outs was 0.83, with a 95% confidence interval of 0.780 to 0.874. These values indicated a good agreement between the tests.

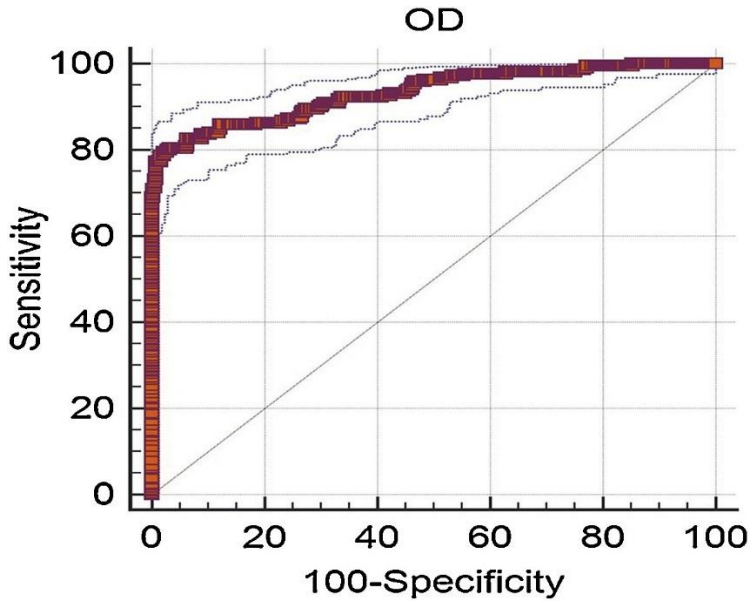

Figure 22. Receiver Operating Characteristics (ROC) analysis. Dichotomized values for the visual readouts from 96 well plates of the $TCID^{50}$ assays were compared with the OD values obtained from the MTT assay using ROC analysis tool in the MedCalc Inc. software, to obtain a cut-off value to distinguish between positive and negative wells. The true positive rate (sensitivity) and the false positive rate (100-specificity) were plotted at the Y and X axis respectively at different cutoff points for the OD values. The curve obtained is indicative of the sensitivity and specificity of the assay at a given cutoff point. The top left corner, representing the area under the curve (AUC) or accuracy of the test, was 0.931. At the selected cutoff of ≥ 0.53 the specificity was 95%, while the sensitivity was 81% with a p value of <0.0001.

When samples were tested by both the conventional V/N and MTT V/N assays determine agreement between the tests, the conventional V/N assay performed as expected since the commercial positive control sample showed high V/N titers while the negative control did not (Table 8). Following visual assessment of the 96 well plates, MTT was added to the wells and OD values obtained as the read out from the ELISA plate reader. The colorimetric V/N titer was determined as the lowest dilution at which the wells had an OD value ≤ the cut off value of 0.53. There was complete agreement between the conventional and MTT V/N assays across the triplicate testing in two replicate assays, indicating high reproducibility with no measured inter or intra assay variation between the two compared tests (Table 8). There was no statistically significant difference between the groups as assessed by a Student's T test ($p=0.71$). Two-fold differences were noted for the positive control sample between two titrations of replicate 2. This difference is generally accepted to be within the detection limits for V/N assays (Stephenson et al. 2009) or can be attributed to operator error but did not affect agreement between the colorimetric and conventional V/N tests. Similarly, high levels of consistency between the conventional and colorimetric V/N's was evident in the field samples tested with only 4 samples showing a two-fold inter assay variation (Table 9). The negative field samples tested had a titer of 1:2 or > 1:2 in both assays. To test non-specific samples by the V/N assay, serum samples from porcine circovirus strain 2 (PCV2), porcine reproductive and respiratory syndrome virus, porcine respiratory coronavirus (PRCV), transmissible gastro-enteritis virus (TGEV) or porcine delta coronavirus (PDCoV) infected pigs were tested by conventional and colorimetric V/N assays. The PCV2 and PRRSV specific sera were obtained from a previous study (Ramamoorthy et al. 2011) . A pooled sample of sera (N=5) collected at 28 days post infection

was tested in duplicate. The PRCV, PDCoV or TGEV- specific sera were gnotobiotic pig derived antisera provided by Dr. Linda Saif, Ohio State University and were also tested in duplicate. Similar to other studies (Lin et al. 2014), detectable neutralization of PEDV with the non-specific controls was not observed in this study.

Table 8. Inter and intra-assay variation of the colorimetric PEDV virus neutralization assay*

	Replicate 1#			**Replicate 2#**		
	1	**2**	**3**	**4**	**5**	**6**
Negative						
Visual	1:2	>1:2	>1:2	>1:2	>1:2	>1:2
Colorimetric*	1:2	>1:2	>1:2	>1:2	>1:2	>1:2
Positive						
Visual	<1:1024	<1:1024	<1:1024	1:256	1:512	<1:1024
Colorimetric	<1:1024	<1:1024	<1:1024	1:256	1:512	<1:1024

*A cut-off value of ≤ 0.53 OD was used to distinguish between positive and negative wells for the colorimetric assay. # Two independent assays with triplicate values each.

Table 9. Assessment of field samples by the conventional and colorimetric V/ N assays*

Sample	Replicate 1		Replicate 2	
	Colorimetric	Conventional	Colorimetric	Conventional
1	1:32	1:16	1:32	1:16
2	1:16	1:16	1:16	1:16
3	1:8	1:8	1:16	1:16
4	1:64	1:64	1:128	1:128
5	1:64	1:64	1:64	1:64
6	1:32	1:32	1:64	1:64
7	1:16	1:16	1:8	1:8
8	1:8	1:8	1:8	1:8
9	1:32	1:32	1:32	1:32
10	1:64	1:64	1:64	1:64
11	1:8	1:8	1:16	1:8
12	1:16	1:8	1:8	1:8
13	1:32	1:32	1:32	1:32

*A cut-off value of ≤ 0.53 was used to distinguish between positive and negative wells

Table 9. Assessment of field samples by the conventional and colorimetric V/ N assays* (continued)

Sample	Replicate 1		Replicate 2	
	Colorimetric	Conventional	Colorimetric	Conventional
14	1:16	1:16	1:16	1:16
15	1:32	1:32	1:16	1:16
16	1:128	1:128	1:64	1:128
17	1:128	1:128	1:64	1:128
18	1:64	1:64	1:64	1:64
19	1:128	1:128	1:128	1:128
20	1:32	1:32	1:32	1:32
21	1:32	1:32	1:32	1:32
22	1:64	1:32	1:32	1:32
23	1:256	1:128	1:128	1:128
24	1:128	1:128	1:128	1:128
25	1:64	1:64	1:64	1:64
26	1:128	1:128	1:128	1:128
27	1:256	1:256	1:256	1:256
28	1:64	1:64	1:128	1:128
29	1:128	1:128	1:128	1:128
30	1:64	1:64	1:128	1:128
31	1:256	1:256	1:256	1:256
32	1:256	1:256	1:256	1:256
33	1:256	1:256	1:256	1:256
34	1:512	1:512	1:1024	1:1024
35	1:32	1:16	1:32	1:32

*A cut-off value of ≤ 0.53 was used to distinguish between positive and negative wells

Discussion

While quantitative PCR based methods are convenient and commonly used for the detection of active PEDV infections, virus quantification methods such as TCID50 titrations are required for measurement of infective virus and has application in the study of pathogenesis, immunity, vaccine development, and the isolation and propagation of field strains. Similarly, while ELISA's (Song et al. 2016) are useful in assessing the serological status of animals, the

measurement of protective, neutralizing antibody responses are the method of choice for determining vaccine efficacy and the level of protection or cross-protection in a herd. Colorimetric assays, based on MTT, have been previously developed for the titration of other viruses such as parvoviruses (Heldt et al. 2006) and picornaviruses (Andersson et al. 2005). However, this study is the first description of adaption of the method for V/N assays and for the quantification of PEDV, a coronavirus. The primary advantage of the colorimetric assay is that it can be easily adapted to a high throughput format for use in veterinary diagnostic labs. It also eliminates the subjectivity, labor and time involved in visual examination of TCID50 or virus neutralization assays for PEDV.

Culture of PEDV in Vero cells typically results in the production of virus stocks with titers that range from about 103 to 106 TCID50, with the titers increasing over passages (Chen et al. 2014, Hofmann and Wyler 1989). Similar to previously described findings, a titer 104.5 TCID50 was obtained for the virus culture used for optimization of the MTT50 colorimetric assay. Few other published studies describe the use of the plaque method for PEDV quantification, probably because of the tedious nature and longer turnaround time of the assay (Hofmann and Wyler 1989, Oka et al. 2014, Zhang et al. 2015). In this study, a titer of 106 PFU was obtained in the plaque assay for the same culture, which deviates from the general rule of thumb suggested by ATCC that PFU= 0.7x TCID50 (ATCC 2012). However, virus titrations are prone to biological variation, in addition to the inherent limitations of serial dilution methods and possible differences between operators in the assessment of CPE or plaque enumeration. Hence, replication of assays was carried out to ensure accuracy. The differences in the methodology for TCID50 and plaque assays such as the agar overlay, staining to visualize plaques and the acceptable limits for countable plaques are other sources of variation between TCID50 and

plaque assays. Therefore, variation between TCID50 and plaque assays may deviate from the rule of thumb for different viruses and culture systems. Similar to another study, comparing a fluorescent focus assay with the plaque assay for PEDV (Cruz and Shin 2007), the agreement between TCID50 and plaque assays for PEDV in this study was excellent, with a correlation coefficient of 0.975.

Two other studies describe the successful use of MTT to colorimetrically quantify viral CPE, even reducing virus titration time for parvoviruses from 7 to 2 days (Andersson et al. 2005, Heldt et al. 2006). In these studies, the OD value to corresponding to the 50% end point was either applied directly (Andersson et al. 2005) or calculated as 50% of the OD of the negative control (Heldt et al. 2006). Similar to Heldt et. al, (Heldt et al. 2006) where half the mean value of the negative controls was used as the cutoff to distinguish positive and negative wells, in this study, the mean OD of the uninfected cell controls was 1.1 and the selected cut off value of 0.53 was approximately half the mean value of the uninfected cell controls. However, unlike Andersson et. al, (Andersson et al. 2005) who used half the mean OD of the 50% end point as their cut-off value, using 0.35 or half of the average OD value of the 50% end point (0.7) in this study as the cut-off would have reduced the sensitivity and specificity of the assay significantly. To improve accuracy, and obtain a standardized OD cut off value with reliable sensitivity and specificity parameters, that could be applied across test batches in the same laboratory, we used ROC analysis as the tool of choice. The selected OD cut-off value to distinguish between wells with and without CPE, when applied to scoring wells and the calculation of MTT50 values using the Spearman and Karber formula, produced excellent agreement between the conventical and MTT-based assays. While the agreement between the colorimetric and conventional tests was also high in the other two studies, detailed statistical analysis was not available in these

publications to compare our approach of using ROC analysis versus directly applying an OD value to obtain the 50% end point.

With improved understanding of the mechanisms of action of MTT, it is now known that MTT reduction occurs not only in the mitochondrion but also by the activity of cellular oxidoreductases throughout a cell (Berridge, Herst, and Tan 2005). In addition, oxidative stress, glycolysis and factors which influence metabolism or energy also influence MTT reduction. Since MTT measures several cellular enzymatic activities simultaneously, its reduction can be significantly influenced by changes in experimental parameters (Stepanenko and Dmitrenko 2015). Further, comparison of the MTT and trypan blue assays for measuring adenovirus replication showed differences between the two assays at early time points (Tollefson et al. 1996, van den Hengel et al. 2011), with reduction of MTT occurring even before the onset of cell death (Tollefson et al. 1996) . Hence, it is important to validate of the cut-off point with the specific culture conditions of each laboratory to avoid inter-laboratory variation and potential under/overestimation of cell viability.

Virus neutralization tests for PEDV often used in the field to determine the level of protective antibodies, as a follow up for vaccination or in herds where animals show clinical signs (Okda et al. 2015) and are also tedious to assess visually, especially for high volume testing. This study is the first to describe methods for a colorimetric V/N tests for PEDV which can significantly reduce the labor effort in performing this test. Conventional virus neutralization tests for PEDV are well established (Chen et al. 2016, Cruz and Shin 2007, Okda et al. 2015) and the primary goal of this study was to compare the conventional and colorimetric V/N's. Obtaining large numbers of quality samples from experimentally infected animals at the time this study was conducted was a challenge as PEDV was relatively new in the U.S. However, the

design of this study meets the WHO guidelines stating that analytical validation is considered sufficient for incremental or adjunct tests (OIE 2017).

In general, it is accepted that there is good agreement between spike-protein specific Ab levels and V/N titers (Paudel et al. 2014) but it is not clear if there is a direct correlation between the PEDV-specific IgG levels as assessed by the N-protein ELISA used in this study (Okda et al. 2015) and V/N titers. However, the PEDV N protein ELISA is a reliable tool to distinguish between and select positive and negative samples for assay validation. In addition to the ELISA, samples were also screened by an indirect immunofluorescence assay (IFA). Only samples with similar results on the ELISA and IFA were selected for testing in this study. As expected no cross-neutralization was detected in the non-specific control sera tested. Antigenic cross-reactivity, but not cross-neutralization, of PEDV and other related swine coronaviruses such as the transmissible gastro-enteritis virus (TGEV), porcine respiratory and enteric coronavirus (PRCV) and porcine delta-coronavirus (PDCoV) has been previously demonstrated (Lin et al. 2015).

Therefore, the performance of the newly developed colorimetric assays was comparable to the conventional assays for the quantification of PEDV virus cultures and neutralizing antibodies. The availability of an OD cut-off value to distinguish between wells with and without CPE can eliminate visual plate reading by operators to significantly improve the turnaround time in diagnostic laboratories with a high volume of testing for PEDV virus isolations and V/N tests.

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CHAPTER 4. AN AMPHIPHILIC INVERTIBLE POLYMER AS A DELIVERY VEHICLE FOR A M2E-HA2-HA1 PEPTIDE VACCINE AGAINST AN INFLUENZA A VIRUS IN PIGS[3]

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Abstract

Influenza A viruses (IAVs) are a group of genetically diverse and economically important zoonotic pathogens. Despite decades of research, effective and broadly protective vaccines are yet to be developed. Recent breakthroughs in epitope-based immunization for influenza viruses identify certain conserved regions of the HA2 and M2e proteins as capable of inducing broad protection against multiple influenza strains. The M2e and HA2 peptides have been evaluated in mice but not as a combination in pigs, which play an important role in the transmission and evolution of IAV. Peptides are inherently weak immunogens; and effective delivery of peptide antigens is challenging. To enhance the delivery and immunogenicity of peptide-based vaccines,

[3] The material in this chapter was co-authored by Gagandeep Singh, Oksana Zholobko, Andriy Voronov and Sheela Ramamoorthy. Gagandeep Singh had primary responsibility for preparing antigen, preparing vaccines candidates, conducting cell culture and animal experiments and analysis the collected samples from the animals. Oksana Zholobko had primary responsibility for preparing polymer and micelles assemblies. Gagandeep Singh was the primary developer of the conclusions that are advanced here. Gagandeep Singh also drafted and revised all versions of this chapter. Sheela Ramamoorthy and Andriy Voronov served as proofreader and checked the math in the statistical analysis conducted by Gagandeep Singh.

the conserved M2e and HA2 and a strain-specific HA1 epitope of Influenza A (H1N1) pdm09 were expressed as a chain in a bacterial expression system and entrapped in a novel amphiphilic invertible polymer made from polyethyelene glycol (PEG, molecular weight 600 g/mol) and polytetrahydrofuran (PTHF, molecular weight 650 g/mol), $PEG_{600}PTHF_{650}$. Piglets vaccinated with polymeric peptide vaccine mounted significantly stronger antibody responses against the peptide construct when compared to piglets immunized with the multi-epitope peptide alone. When vaccinated pigs were challenged with Influenza A (H1N1) pdm09, viral shedding in nasal secretions and lung lesion scores were significantly reduced when compared to the unvaccinated controls and pigs vaccinated with the peptide alone at six days post-challenge. Thus, the combination of the $PEG_{600}PTHF_{650}$ polymer and trimeric peptide construct enhanced delivery of the peptide antigen, acted as an adjuvant in stimulating strong antibody responses, reduced the effects of viral infection in vaccinated pigs.

Introduction

Influenza A viruses (IAV) of the *Orthomyxoviridae* family are important zoonotic pathogens. Genetic and antigenic variation associated with IAV renders the succesful development of broadly-protective human and swine vaccines against IAV a long-standing challenge. Pigs serve as "mixing vessels" for human and avian influenza viruses, supporting the emergence of new influenza virus strains (Imai and Kawaoka 2012, Ma et al. 2009). Thus, vaccines that provide effective and broad protection against several strains of influenza virus in pigs would be very valuable for animal and public health.

Recently, vaccines containing certain conserved antigenic epitopes of influenza viruses were shown to elicit broad protection against a number of genetically diverse strains in mouse models (De Filette et al. 2005, De Filette, Fiers, et al. 2006, De Filette, Ramne, et al. 2006,

Huleatt et al. 2008, Fiers et al. 2009, Du, Zhou, and Jiang 2010, Zhao et al. 2010, Wen et al. 2016, Ameghi et al. 2016, Wu et al. 2012). The extracellular N-terminal domain of the M2 protein (M2e) is a 23 amino acid peptide which is highly conserved in all influenza A viruses (Staneková and Varečková 2010). M2e- based peptide vaccines were shown to provide heterogenetic immunity against IAV in mice, but were not as effective in swine models (Opriessnig et al. 2018, Heinen et al. 2002, Wen et al. 2016). Similarly, the fusion peptide located in the HA2 protein is highly conserved among the different influenza virus strains (Staneková and Varečková 2010, Chun et al. 2008, Daniels et al. 1985) and provided broad protection in mice, but has not been tested in swine (Stanekova et al. 2011, Wang et al. 2010). Nor has a combination of the HA2 and M2e peptides been tested in pigs (De Filette et al. 2005, De Filette, Fiers, et al. 2006, De Filette, Ramne, et al. 2006, Huleatt et al. 2008, Fiers et al. 2009, Du, Zhou, and Jiang 2010, Zhao et al. 2010, Wen et al. 2016, Ameghi et al. 2016, Wu et al. 2012). In this study, we test the hypothesis that a polypeptide encoding a combination of the M2e and HA2 conserved epitopes with one type specific epitope would be effective in preventing IAV infection in pigs (Staneková and Varečková 2010).

While highly specific, small peptides are weak immunogens and vulnerable to degradation (Fosgerau and Hoffmann 2015), necessitating the development of improved systems the delivery of peptide vaccine. Polymers are well-established as substances that can enhance vaccine delivery, reduce dosage, and act as adjuvants, to broaden vaccine-induced immune protection. In addition, self-assembled polymeric architectures can increase the duration of immunity due to slow and sustained release of the antigen over time (Moghimi and Hunter 2000, Newman, Todd, and Balusubramanian 1998, Greenland and Letvin 2007). Amphiphilic polymers which enable customizing of peptide antigen delivery by varying the macromolecular structure

and also act as adjuvants provide significant value to the development of peptide vaccines (Adams, Haughney, and Mallapragada 2015). We have previously synthesized a library of amphiphilic invertible polymers (AIPs) which self-assemble into polymeric micelles as AIP concentration increases, both in polar and nonpolar solvents, and can rapidly switch their conformation in response to changes in the environmental polarity, thus facilitating the micellar inversion (Voronov et al. 2006, Voronov et al. 2008). The AIP conformational inversion is a promising tool for rapid and controlled self-assembly in applications that require simultaneous utility in polar and nonpolar media, e.g., in drug delivery systems. In our previous studies, the incorporation of two different peptides into micellar assemblies of AIP, made from polyethylene glycol (PEG, molecular weight 600 g/mol) and polytetrahydrofuran (PTHF, molecular weight 650 g/mol), $PEG_{600}PTHF_{650,}$ were characterized and described in detail (Kohut et al. 2017). The micellar inversion of this AIP loaded with peptides was also demonstrated (Pan et al. 2018).

In this study, the three selected influenza A virus epitopes described above (conserved M2e, HA2, and type-specific HA1) were expressed as a chain in a bacterial expression system and incorporated into the $PEG_{600}PTHF_{650}$ micellar assemblies. The efficacy of the AIP micellar assemblies as a peptide antigen delivery system was evaluated in vitro and in pigs. The data presented below demonstrates that the $PEG_{600}PTHF_{650}$ micellar assemblies are effective in delivering the peptide cargo to cells, and act as an adjuvant in stimulating strong antibody responses against the delivered antigen in vaccinated pigs.

Materials and methods

All experiments described below were carried out in compliance with the Institutional Biosafety Committee (IBC) and Institutional Animal Care and Use Committee (IACUC)

regulations of North Dakota State University (NDSU) and South Dakota State University (SDSU).

Cells and viruses

To prepare the virus stock culture for both the challenge of vaccinated pigs and for the hemagglutination inhibition (HI) assay, Influenza A (H1N1) pdm09 virus was cultured using Madin-Darby Canine Kidney (MDCK) cells as previously described (Organization 2011). After 48h of incubation, virus particles were harvested by three freeze-thaw cycles, followed by clarification at 10,000xg for 10 mins at 4°C to remove cellular debris. The titer was assessed using the tissue culture infectious dose 50% [$TCID_{50}$] assay and the Reed–Muench formula (Reed and Muench 1938).

Preparation of the peptide antigen

The coding sequence for the previously characterized conserved peptides HA2 and M2e (Neirynck et al. 1999, Hashem et al. 2010) and a strain-specific HA1 epitope (Horváth et al. 1998), were commercially synthesized (Integrated DNA Technologies, USA) as a chain with glycine-serine linkers and BamHI and NcoI restriction sites on the ends. The synthesized DNA with the sequence MGSSHHHHHHSSGLVPRGSHMASMTGGQQMGRGSSLLTEVETPTRSEWECRC SDSSGSGSGSRGLFGAIAGFIEGGWGGGGSGGGGSATGLRNIPSIQSRLEHHHHHH was inserted into the pET28a (+) (MilliporeSigma, USA) bacterial protein expression vector. The vaccine antigen was prepared by bacterially expressing the peptide encoding three IAV epitopes. The expressed recombinant peptide was purified by using Ni-NTA affinity chromatography under native conditions and characterized by a western blot using an anti-M2e peptide-specific

monoclonal antibody. The purified M2e-HA1-HA2 peptide was dissolved in dimethyl sulfoxide (DMSO) and stored in aliquots at -80^{o}C until further use.

Amphiphilic invertible polymer (AIP) synthesis

The AIP, $PEG_{600}PTHF_{650}$, was synthesized as previously reported from PEG (molecular weight 600 g/mol) and PTHF (molecular weight 650 g/mol) using a polycondensation reaction (Voronov et al. 2008, Hevus, Kohut, and Voronov 2011). Chemical composition of $PEG_{600}PTHF_{650}$ was confirmed by FTIR and ^{1}H NMR spectroscopy. Average molecular weights and the corresponding polydispersity index of the AIP was measured using gel permeation chromatography (GPC) (data not shown).

Cellular cytotoxicity of $PEG_{600}PTHF_{650}$ micellar assemblies

To ensure the $PEG_{600}PTHF_{650}$ is not toxic to cells, cytotoxicity of polymer micellar assemblies was assessed in vitro using the MTT (3-(4,5-dimethylthiazol-2-yl) Tr-2,5-diphenyltetrazolium- bromide) assay (Mosmann 1983). Micellar assemblies were prepared using different concentrations of $PEG_{600}PTHF_{650}$ (0.05, 0.2, 0.5, 0.75 and 1.0 w/v%) by the thin film technique using DMSO as solvent (Kohut et al. 2017). One hundred µl/well of each prepared solution was added into 96 well cell culture plates (VWR, USA) containing monolayers of Vero cells and incubated for 8h at 37^{o}C in a CO_2 incubator. After incubation, the solution from the wells was removed and the wells were washed three times with Hank's balanced salt solution (HBSS). The MTT was dissolved to 0.5mg/ml in sterile PBS, and 100µl was added to each well. Plates were incubated in a CO_2 incubator for 4h to reduce MTT into formazan. The supernatant from each well was carefully removed by aspiration without disturbing the cells. To solubilize the intracellular formazan, 100µl of DMSO was added to each well, mixed well by vigorous pipetting, and incubated for 5 mins. Plates were read at 570 nm in microplate reader.

Interaction between $PEG_{600}PTHF_{650}$ micellar assemblies and M2e-HA1-HA2 peptide

To demonstrate the interaction between $PEG_{600}PTHF_{650}$ micellar assemblies and M2e-HA1-HA2 peptide, ^{1}H NMR spectra were recorded on an AVANCE III HDTM 400 high-performance digital NMR spectrometer at 400MHz and 22.5°C. Proton spectra were collected for 0.5 w/v% PEG_{600}-$PTHF_{650}$ or polymer-peptide micellar assemblies containing 0.005 w/v% peptide added to 0.5 w/v% $PEG_{600}PTHF_{650}$ formed in deuterated water by thin film technique [27]. The spectra were obtained for polymer samples with or without the peptide and referenced to a 3-(Trimethylsilyl) propionic-2,2,3,3-d4 acid sodium salt (TMSP) signal as an internal standard.

Relative antigen loading capacity

The peptide loading capacity of the $PEG_{600}PTHF_{650}$ micellar assemblies was assessed by a whole cell enzyme-linked immunosorbent assay (ELISA), capable of detecting intracellular peptide. Polymer-peptide micellar assemblies were prepared using different concentrations of $PEG_{600}PTHF_{650}$ (0.05, 0.2, 0.5, 0.75, and 1.0 w/v%) and a constant M2e-HA1-HA2 peptide concentration of 0.5 µg/µl. One hundred µl/well of prepared assemblies were added into 96 well cell culture plates (VWR, USA) containing a monolayer of Vero cells, and incubated for 8h at 37ºC in a CO_2 incubator. The peptide alone or wells with no treatment were used as controls. After 8h incubation, the solution from wells was removed, and wells were washed three times with phosphate buffered saline with tween (PBST). To each well, 100µl of anti-M2e monoclonal primary antibody diluted 1:100 in blocking buffer (PBST +2% Bovine serum albumin, BSA) was added and incubated for 1h at 37ºC with 5% CO_2 followed by washing. 100µl of HRPO conjugated anti-mouse IgG secondary antibody (KPL, USA) was added and incubated for 1h at 37ºC with 5% CO_2. After washing, the reaction was developed with 3,3′,5,5′-

tetramethylbenzidine substrate (TMB) (KPL, USA) and stopped after 15 minutes with 1M Hydrochloric acid. The optical density values were read at 450 nm using a microplate reader (BioTek Instruments, Winooski, VT).

In-vitro peptide delivery by Immuno-Fluorescence Assay (IFA)

The effectiveness of the $PEG_{600}PTHF_{650}$ micelles in delivering the M2e-HA2-HA1 peptide into the cells was observed by an immunofluorescence (IFA) assay. Polymer-peptide assemblies were prepared using 1w/v% of $PEG_{600}PTHF_{650}$ and 0.5 µg/µl M2e-HA2-HA1. 100 µl/well of prepared solution were added into an 8-well Nunc® Lab-Tek™ chamber slide system containing a Vero cell monlayer and incubated for 1, 2, 4, and 8h at 37ºC with 5% CO_2. Wells with no treatment or wells incubated with peptide alone were used as controls. After incubation, the solution was aspirated from the wells and wells were washed three times with HBSS. To detect intracellular antigen, cells were fixed using chilled acetone: methanol (1:1). Following overnight fixation, the chamber slides were washed three times using PBST and 100 µl of 1:100 anti-M2e monoclonal antibody diluted in blocking buffer was added to each well,incubated at 37^0C for 1h and washed with PBST. 100 µl of 1:500 anti-mouse IgG fluorescein-conjugated secondary antibody (KPL, USA) was added to each well and incubated at 37^0C for 1h. Washed slides were mounted with 50% glycerol, followed by fluorescent microscopic examination.

Vaccine formulation

Twenty-four, 3-week old, SIV negative piglets of both sexes were assigned to four groups as follows: Group I – Unvaccinated control (N=7), Group II –$PEG_{600}PTHF_{650}$/M2e-HA1-HA2 peptide micelles (SIV-VAC) (N=7), Group III – M2e-HA1-HA2 peptide only (N=5) or Group IV- $PEG_{600}PTHF_{650}$ polymer assemblies only (N=5). For each vaccine dose, 0.8 ml of M2e-HA1-HA2 peptide (1 mg/ml) dissolved in DMSO was added to a 30ml Pyrex glass vial

containing 40mg of $PEG_{600}PTHF_{650}$ and mixed well by vortexing. A thin film was prepared and subsequently hydrated with 4ml of DMEM to form $PEG_{600}PTHF_{650}$/ M2e-HA1-HA2 micellar assemblies. For each animal in the M2e-HA1-HA2 peptide control group, a thin film was obtained from 0.8 ml of M2e-HA1-HA2 peptide (1mg/ml) dissolved in DMSO and hydrated with 4ml of DMEM to prepare the M2e-HA1-HA2 peptide solution. Similarly, for each animal in the $PEG_{600}PTHF_{650}$ control group, a thin film was obtained from 40mg of $PEG_{600}PTHF_{650}$ dissolved in DMSO hydrated with 4ml of DMEM to prepare 1w/v% of the $PEG_{600}PTHF_{650}$ solution. Therefore, the effective vaccine dose for each piglet was 0.8mg (0.2 µg/µl) of M2e-HA1-HA2 peptide antigen and 1 w/v% of $PEG_{600}PTHF_{650}$ polymer per dose.

Swine immunization and challenge

At the day of vaccination (DPV 0), piglets in each group were treated with 4ml of innocula (2ml intransal and 2ml subcutaneous) as described above. At the second and third week post-vaccination (DPV 14 and DPV 20, respectively) piglets were boosted with the same doses and routes. At DPV 35 (0 day post-challenge or DPC 0), two pigs from group I and group II were sacrificed prior to challenge with the virulent virus, to assess vaccine safety. All remaining pigs were challenged intranasally with $10^{5.5}$ $TCID_{50}$/ml of Influenza A (H1N1) pdm09 culture. All piglets were euthanized at 41 DPV (DPC 6) for necropsy. Pathology procedures were carried out as described below. Serum was collected from all piglets on DPV 0, 14, 20, 35 and 41 to detect antibodies to the peptide by ELISA. Nasal swabs were collected from all piglets at DPV 35, 38 and 41 (or DPC 0, 3 and 6) and tested by qPCR for shedding of the challenge virus.

Clinical observation and pathological examination

Piglets were observed every day post-challenge for clinical signs of SIV including fever, nasal discharge, coughing, anorexia, and lethargy. Weight and temperatures were measured every day post-challenge.

Pathological evaluation and scoring was carried out in a blinded fashion by a board-certified veterinary pathologist. Heart, liver, spleen, kidney and lymph node tissues were collected from two pigs each euthanized prior to challenge from the vaccine group and unvaccinated control group to assess vaccine safety (Opriessnig et al.). Lung sections were prepared from the right and left cranial, medial and caudal lobes and accessory lobes. Hematoxylin and eosin-stained tissue sections were observed for microscopic changes indicating viral infection. In addition, the lung sections were stained with the anti-M2e monoclonal antibody to determine localization of the M2e-HA1-HA2 peptide by immunohistochemistry (IHC).

For the remaining animals sacrificed on the 6th day after challenge, protection against the development of gross lesions due to virulent viral challenge was assessed as the percentage of the tissue affected in each of the six lung regions listed above. The total percentage of lungs affected for the 5 pigs/group is shown in Table 8. Similarly, microscopic lesions were assessed using hematoxylin and eosin stained sections, as previously described, with some modifications (Halbur et al. 1995, Gauger et al. 2012). Briefly, bronchial/bronchiolar epithelial changes, and/or bronchitis and bronchiolitis were assessed as a percentage value for each of the six lung sections examined using the following scoring matrix: 25% airways affected =1, 26-50% airways affected =2, 51-75% airways affected =3, 76-100% airways affected = 4. Interstitial pneumonia (IP) was scored as 0 = none, 1 = mild, focal to multifocal IP, 2 = moderate, locally extensive to multifocal

IP, 3 = moderate, multifocal to coalescing IP, 4 = severe, coalescing to diffuse. Peribronchiolar lymphocytic cuffing was scored as 0 = none, 1 = minimal, loosely formed, 2 = mild, loosely formed, 3 = moderate, well formed, 4 = severe, thick, well-formed cuffs. Total values were calculated as a sum for the five pigs per group (Table 10).

Lung sections were stained with an SIV specific monoclonal antibody for IHC and scored as weak =1, moderate = 2, strong = 3. The sum of the number of sections positive for antigen and the scores for each group is listed in Table 1. Consolidated total lesion scores were calculated as the sum of the gross, microscopic and IHC scores per group. The Mann-Whitney U test was applied to determine whether there were significant differences between groups at $p \leq 0.05$.

Antibody responses to the M2e-HA1-HA2 peptide

Sera collected from the experimental pigs were assessed for antibody responses against the M2e-HA1-HA2 peptide using an indirect ELISA. Briefly, 96-microwell ELISA plates (Corning, USA) were coated with 100µl/well of 1:200 recombinant M2e-HA1-HA2 peptide (0.3 mg/ml in water) diluted in carbonate coating buffer (pH 9.6), and incubated overnight at room temperature, followed by five washes with PBST. Plates were blocked with 200µl/well of blocking buffer (2% BSA in 1X PBST) for 2h at 37 °C. After blocking, plates were washed five times using 1X PBST. To each well, 50µl of 1:50 serum diluted in PBST was added in duplicate and incubated for 2h at 37 °C. After washing five times with PBST, 50 µl/well of a 1:2500 diluted anti-swine IgG peroxidase-conjugated secondary antibody (KPL, USA) was added, and the plates were incubated at 37 °C for 1h. After washing five times, 50 µl/well of TMB substrate (KPL, USA) was added to plates and incubated in the dark for 15 minutes at room temperature to catalyze the reaction. Finally, 50 µl/well of 1 M HCl was added to stop the reaction. The OD

readings were obtained at 450 nm using a microplate reader (BioTek Instruments, Winooski, VT).

Hemagglutination inhibition (HAI) assay

The HAI assay was performed using 0.5% chicken RBCs and four hemagglutinating units of Influenza A (H1N1) pdm09 as per World Health Organization (Organization 2011).

Detection of Challenge Virus Shedding by qPCR

Virus shedding in nasal secretions was assessed by a diagnostic one-step qRT-PCR using a commercial kit; the Path-ID RT-PCR Kit (Thermo Fisher, USA). The assay was performed in duplicate by NDSU VDL, as per the manufacturer's instructions, following standardized operating procedures.

Statistical analysis

The antibody titers, HAI titer and the viral load in nasal secretions were compared by Student's T-test using Microsoft Excel 2016. The histology scores were compared by Mann-Whitney U-test using SPSS software (IBM, USA). Data analysis were considered significant at $p<0.05$.

Results

The peptide antigen interacts with the exterior of the micellar assemblies

The structure of PEG_{600}-$PTHF_{650}$ macromolecules contains 6 proton sites a, b, c, d, e and f, with d and f localizing to the hydrophobic PTHF fragments (Figure 23, Panel 1). Proton spectra collected from solutions of 0.5 w/v% PEG_{600}-$PTHF_{650}$ alone or 5 w/v% polymer and 0.005% peptide assemblies in deuterated water showed that addition of peptide to the PEG_{600}-$PTHF_{650}$ micellar solution led to a shift of the signals for the hydrophilic PEG protons a and c (Figure 23 2a and 2c). The broadening of the signals (half-height width increases from 2.8 Hz to

6.5 Hz for protons c) implied that the mobility of the PEG fragments became limited at those locations, presumably due to their interaction with M2e-HA1-HA2 molecules. The exterior of the polymer-peptide assemblies appeared to be more tightly packed as evidenced by signal broadening, due to the association of the polar fragments of the peptide at these zones, which is in agreement with previous studies for $PEG_{600}PTHF_{650}$ and two model peptides [27]. A slight shift of the signals of protons a and c toward lower ppm values indicated that the polarity in the micellar exterior became lower after polymer interaction with M2e-HA1-HA2, when compared with those of the micelles with no incorporated peptide molecules. The finding can be explained by the replacement of polar water molecules with the less polar hydrophilic fragments of M2e-HA1-HA2 upon peptide incorporation. After adding the M2e-HA1-HA2 peptide, the signals for protons b and e shifted upfield, implying changes in the microenvironmental polarity of the area where the protons b and e were localized. ^{1}H shift of the protons b and e corresponding to methylene group in the α position of the carbonyl groups in the PTHF moieties and succinic acid moieties respectively, indicated that these protons are transferred into a less polar micellar core (Figure 23 2b and 2e). Addition of the peptide to the 0.5 w/v% solution of $PEG_{600}PTHF_{650}$ did not lead to chemical shifts of the signals of protons d and f attributed to the hydrophobic PTHF fragments (Figure 23 2d and 2f). Hence, it can be concluded that the M2e-HA1-HA2 molecules are preferentially localized into the exterior of the $PEG_{600}PTHF_{650}$ micellar assemblies.

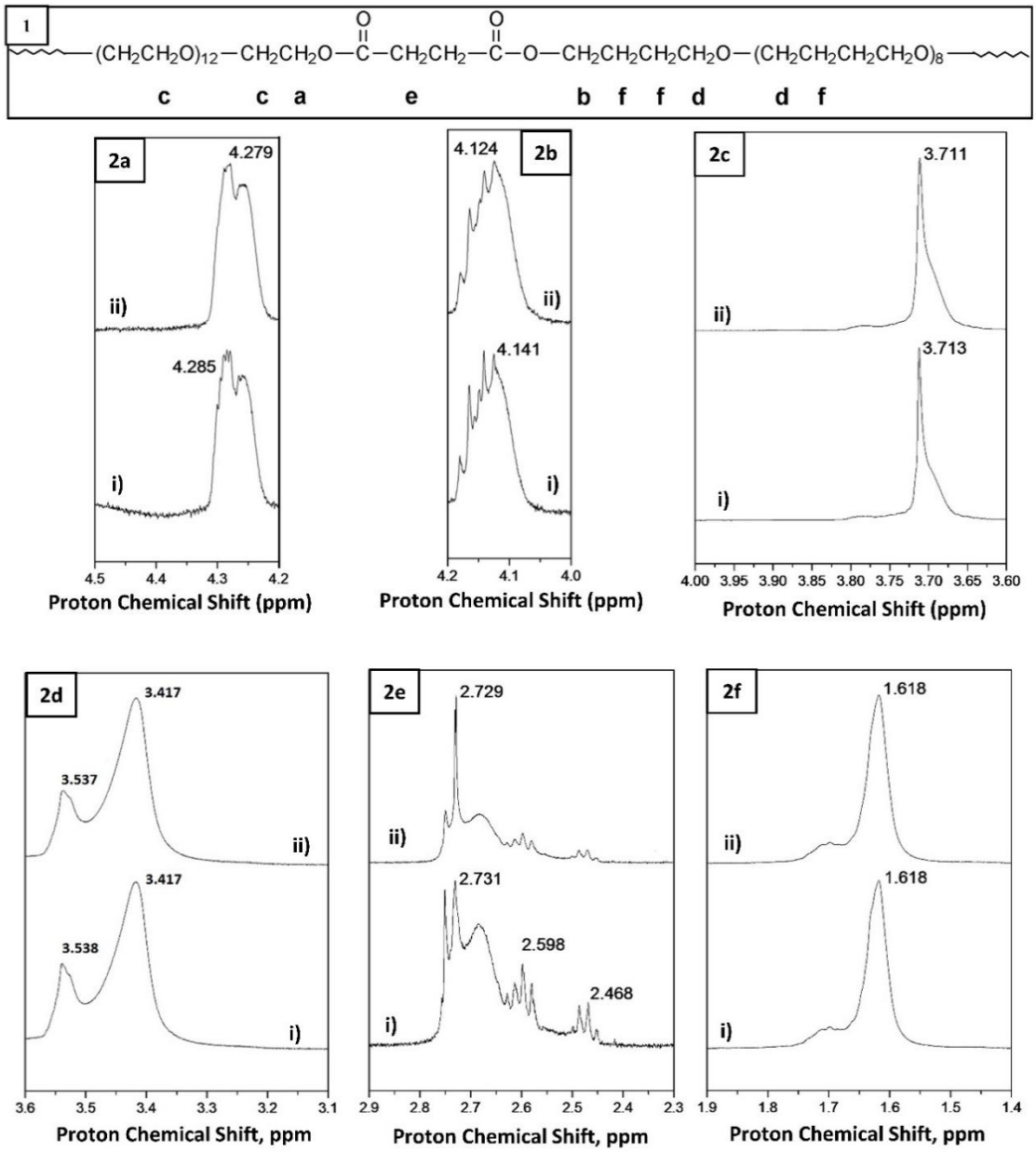

Figure 23. ^{1}H NMR spectra of the $PEG_{600}PTHF_{650}$ solution (0.5 w/v%) in D_2O. Panel 1- The chemical structure of $PEG_{600}PTHF_{650}$ with protons labeled a-f. Panel 2a through 2f correspond to the peaks representing the protons a - f depicted in panel 1 respectively. X-axis – proton chemical shift measured as ppm (parts per million). i) Spectrum of the 0.5 w/v% $PEG_{600}PTHF_{650}$ solution alone (ii) Shift in spectrum after the addition of M2e-HA1-HA2 peptide to the 0.5 w/v% $PEG_{600}PTHF_{650}$ solution.

Micellar assemblies formed by 1 w/v% $PEG_{600}PTHF_{650}$ are efficient in peptide delivery

The purified M2e-HA1-HA2 peptide was detected at the expected molecular weight of 11Kd by a M2e-specific monoclonal antibody provided by Dr. Eileen Thacker, Iowa State University. No significant cytotoxicity was detected at any of the tested concentrations of 0.05, 0.2, 0.5, 0.75 and 1.0 w/v% $PEG_{600}PTHF_{650}$ by the MTT assay compared to untreated cells (data not shown). Further assesment of the antigen loading capcity at the same concentrations of polymer showed that the 1 w/v% concentration of $PEG_{600}PTHF_{650}$ had a significantly higher antigen loading capacity than the next lower dilution of 0.75 w/v% $PEG_{600}PTHF_{650}$ and the other dilutions tested (Figure 24). As there was also no significant cytotoxicity at the 1 w/v% $PEG_{600}PTHF_{650}$, this concentration was used for further testing and vaccine formulation (Figure 24).

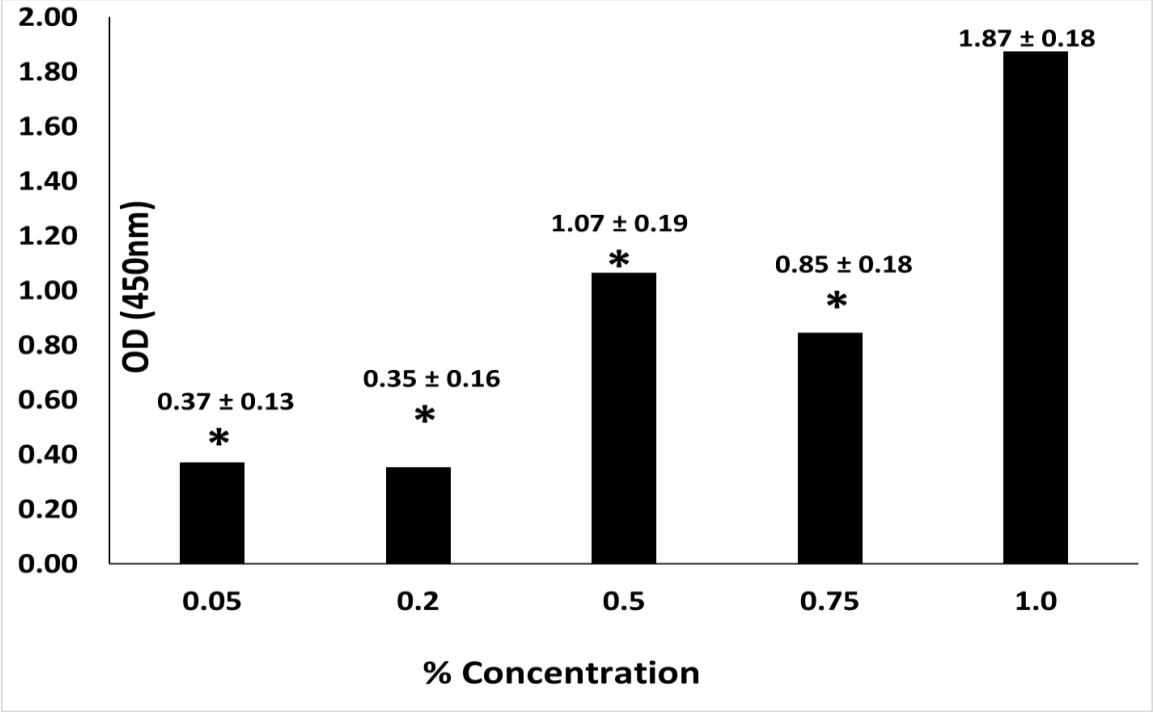

Figure 24. Antigen loading capacity of $PEG_{600}PTHF_{650}$ micellar assemblies. Intracellular delivery of peptide antigen as measured by an antigen detection ELISA using an M2e peptide-specific monoclonal antibody. Y-Axis: optical density (OD) at 450 nm, Y axis – w/v% concentration of $PEG_{600}PTHF_{650}$. Vero cells monolayers incubated with micellar assemblies prepared with 0.05, 0.2, 0.5, 0.75, and 1.0 w/v% of $PEG_{600}PTHF_{650}$ and a constant concentration of 0.5 µg/µl of the M2e-HA1-HA2 peptide for 8h. Intracellular delivery of antigen was detected by ELISA using a M2e peptide-specific monoclonal detecting antibody after 24hrs. * - significantly different from micellar assemblies prepared from 1.0 w/v% polymer ($P<0.05$).

When the effectiveness of intracellular delivery of the incorporated peptide was assessed by an immunofluorescence assay (IFA), the control recombinant M2e-HA1-HA2 peptide could not penetrate cells by itself as it is highly hydrophobic in nature and, thus, poorly water-soluble. It could only be internalized with the help of delivery vehicle. With an increase in incubation time from1h to 8h, the $PEG_{600}PTHF_{650}$ micellar assemblies at the selected 1 w/v% concentration level delivered proportionately more M2e-HA1-HA2 peptide into the cells as determined by an increasing fluorescent signal by IFA (Figure 25).

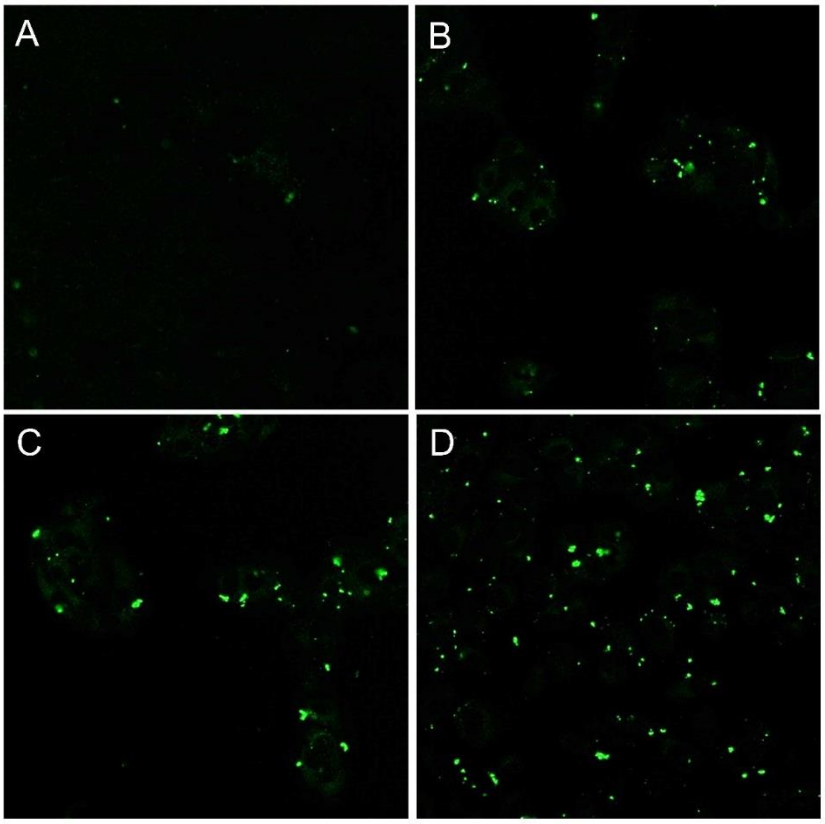

Figure 25. Intracellular delivery of M2e-HA1-HA2. Vero cell monolayers were incubated with micellar assemblies prepared with 1.0 w/v% of $PEG_{600}PTHF_{650}$ and 0.5 µg/µl of M2e-HA1-HA2 peptide for 1 h (A), 2 h (B), 4 h (C) and 8 h (D) and assessed by IFA using a M2e peptide-specific monoclonal antibody. Increasing green fluorescence is indicative increasing accumulation of intracellular peptide over time.

Vaccination induces strong antibody responses against the peptide antigen

Piglets vaccinated with $PEG_{600}PTHF_{650}$/M2e-HA1-HA2 micelles and M2e-HA1-HA2 peptide alone mounted strong antibody responses against the M2e-HA1-HA2 peptide. The antibody titers increased with the boosters on DPV 20 and 35. The mean optical density values remained significantly different ($P<0.05$) from pigs vaccinated with PBS and $PEG_{600}PTHF_{650}$ micelles alone for the duration of the study. Piglets vaccinated with $PEG_{600}PTHF_{650}$/M2e-HA1-HA2 micellar assemblies mounted significantly stronger antibody responses when compared to

M2e-HA1-HA2 peptide alone on DPV 20 and DPV 35 indicating that $PEG_{600}PTHF_{650}$ acts as an adjuvant by enhancing antibody responses (Figure 26).

The hemaglutination inhibtion (HAI) titers for all piglets for the duration of the study were <40, with no significant differences between groups [data not shown], suggesting that the antibodies generated against the M2e-HA1-HA2 peptide were not neutralizing in nature.

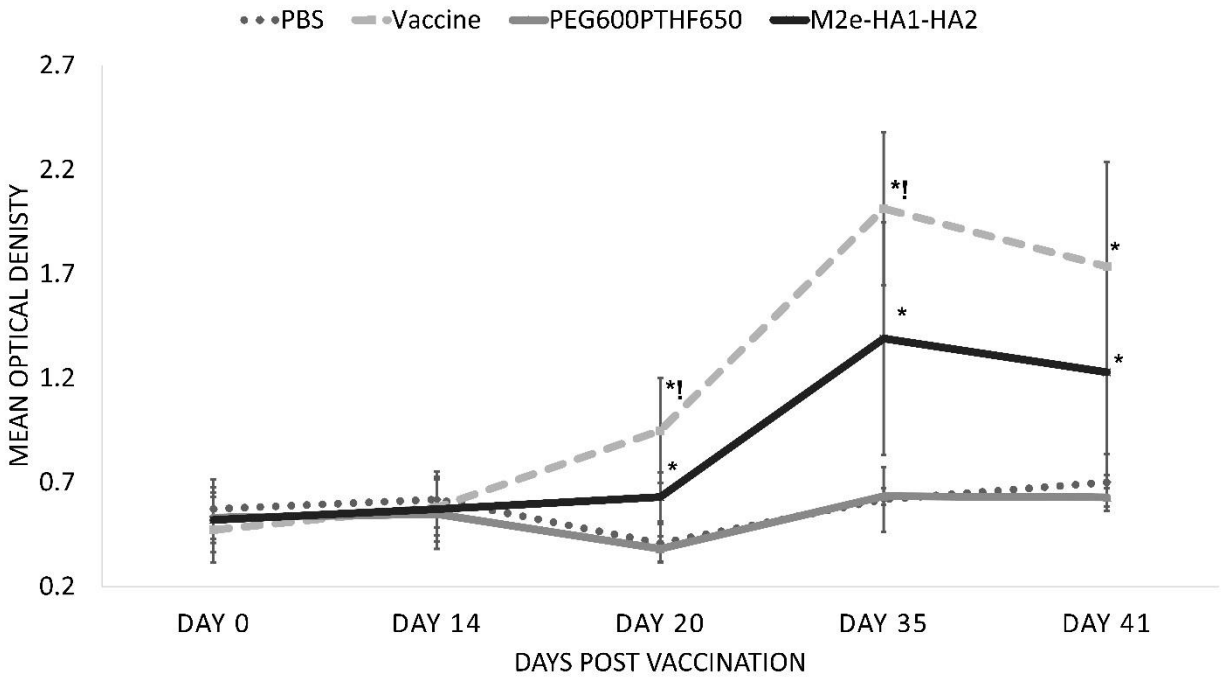

Figure 26. Antibody response in vaccinated pigs. Antibody response against M2e-HA1-HA2 peptide measured as ELISA OD values. Y-axis - mean OD reading (450nm) for each group, x-axis - days post vaccination (DPV). An asterisk (*) symbol represents the groups were statistically different ($p<0.05$) from the PBS group at the respective days post vaccination (DPV). An exclamation (!) symbol represents $PEG_{600}PTHF_{650}$/M2e-HA1-HA2 micellar assemblies vaccinated group is significantly different ($P<0.05$) from M2e-HA1-HA2 peptide group at the respective timepoint.

Vaccination reduces lung pathology

No clinical signs of IAV infection such as pyrexia, respiratory distress or body weight loss was observed in any of the piglets throughout the study. One of the five vaccinated pigs did not develop any gross or microscopic lesions (Table 10). The unvaccinated pigs had a total

microscopic lesion score of 111.00 while the vaccinated pigs had a score of 69.00 (Table 10). While the gross and microscopic lesion scores for the vaccinated and control pigs immunized with the peptide alone were lesser than those of the unvaccinated pigs, these differences were not statistically significant. However, the amount of viral antigen detected by IHC was significantly different between the unvaccinated and vaccinated pigs. The total consolidated lesion score for the unvaccinated group (191.00) was significantly different from that of the vaccinated pigs (97.00). While the total scores for the peptide (141.00) and polymer (130.00) groups were considerably lower than those of the unvaccinated pigs but higher than the vaccinated pigs (97.00), statistical significance was not detected by the Mann Whitney U test (Table 10).

Table 10. Lesion scores at necropsy

Group	Gross Lesion Score/ No of positive animals	Microscopic Lesions Score/ No of positive animals	Immunohisto-Chemistry Score/ No of positive animals	Total Lesion Score
PBS	24.000 ± 4.658 (5/5)	111.000 ± 11.692 (5/5)	56.000 ± 5.070 (5/5)	191.000 ± 19.967
$PEG_{600}PTHF_{650}$	15.000 ± 1.732 (5/5)	90.000 ± 2.550 (5/5)	26.000 ± 1.095 [a] (5/5)	131.000 ± 1.924
M2e-HA1-HA2 Peptide	19.000 ± 3.701 (5/5)	97.000 ± 13.390 (5/5)	24.000 ± 3.421 [a] (5/5)	140.000 ± 20.162
Vaccine ($PEG_{600}PTHF_{650}$/ M2e-HA1-HA2)	18.000 ± 2.966 (4/5)	69.000 ± 6.229 (4/5)	17.000 ± 3.130 [a] (4/5)	97.000 ± 11.760 [a]

Gross lesion scores – Total percentage of lungs affected (N= 5 pigs/group). Microscopic lesion scores – Sum of the percentage of each lung section affected (N= 5 pigs/group, 6 lung sections per pig), scored as follows: Bronchial/bronchiolar epithelial changes, and/or bronchitis and bronchiolitis - Scoring -25% airways affected =1, 26-50% airways affected =2 =, 51-75% airways affected =3, 76-100% airways affected = 4. Interstitial pneumonia (IP) - Scoring - 0 = none, 1 = mild, focal to multifocal IP, 2 = moderate, locally extensive to multifocal IP, 3 = moderate, multifocal to coalescing IP, 4 = severe, coalescing to diffuse. Peribronchiolar lymphocytic cuffing - 0 = none, 1 = minimal, loosely formed, 2 = mild, loosely formed, 3 = moderate, well formed, 4 = severe, thick, well-formed cuffs. Immunohistochemistry (IHC) scores – Sum of the number of sections positive for antigen as detected by a SIV specific monoclonal antibody and IHC score (N= 5 pigs/group, 6 lung sections per pig), Scoring - weak =1, moderate = 2, strong = 3. Total lesion scores – Sum of the gross, microscopic and IHC scores. a- significantly different from the PBS group, b- significantly different from the $PEG_{600}PTHF_{650}$ group, c- significantly different from the M2e-HA1-HA2 peptide group. Mann-Whitney U test at $p \leq 0.05$.

Vaccination induces delayed but significant reduction of viral shedding

Protection against nasal shedding of the challenge virus by vaccination as measured with a matrix gene-specific qPCR unexpectedly showed that viral loads in pigs vaccinated with the peptide alone or the polymer-peptide vaccine were significantly higher than the PBS control group, at 3 days post challenge (DPC 3). However, values for the pigs administered the polymer alone were similar to those of the PBS control group, indicating that early enhancement of viral

replication was due to the peptide vaccine contruct and not the AIP-based delivery system. Three days later, on the 6th day post challenge, the trend reversed to where nasal shedding of the challenge virus was significantly lower in the pigs administered the vaccine and peptide alone compared to unvaccinated pigs, while the viral loads continued to increase in pigs administered PBS or the polymer alone. The difference in viral loads between DPC 3 and DPC 6 in the pigs administered either the peptide alone or the polymer-peptide vaccine were statistically different, indicating that infleunza-specific protection induced by vaccination was delayed but robust, resulting in a significant reduction in challenge viral shedding during the 3 days period (Figure 27).

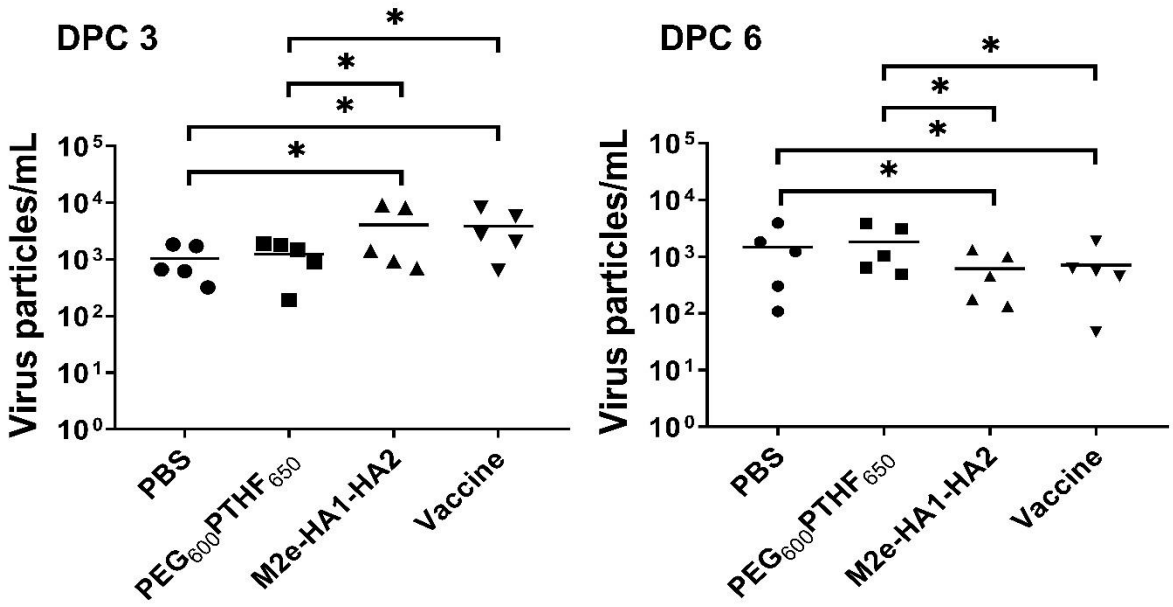

Figure 27. Detection of viral load in nasal secretions. The challenge pH1N1 viral particles in nasal secretions swabs for each treatment group on day 3 and 6 post-challenge (DPC) were determined by qRT-PCR. X-Axis – Groups, Y-axis – Mean viral particles/ml in individual pig. An asterisk (*) symbol represents the groups were statistically different ($p<0.05$) from each other. Horizontal bars represent the mean viral particles/ml in the group.

The $PEG_{600}PTHF_{650}$ peptide vaccine was safe

No untoward clinical signs were observed in vaccinated animals prior to challenge. Similarly, no gross or microscopic lesions were observed in the vaccinated piglets euthanized prior to challenge, indicating the $PEG_{600}PTHF_{650}$/M2e-HA1-HA2 vaccine did not cause any side effects. Localization of the M2e-HA1-HA2 peptide by immunohistochemistry using the M2e peptide-specific monoclonal antibody showed antigen-specific staining in the alveolar septa, alveolar spaces and perivascular areas of the lung tissue and lymph node sections. Representative images are depicted in Figure 28. This observation suggests that $PEG_{600}PTHF_{650}$/M2e-HA1-HA2 complexes were taken up by antigen presenting cells and transported to the germinal center in lymph nodes. Antigen specific staining was absent in the PBS control pigs sacrificed prior to challenge. However, this experiment was not conducted on pigs vacccinated with peptide alone thus quantifying the amount of peptide uptaken by cells in vivo was not feasible.

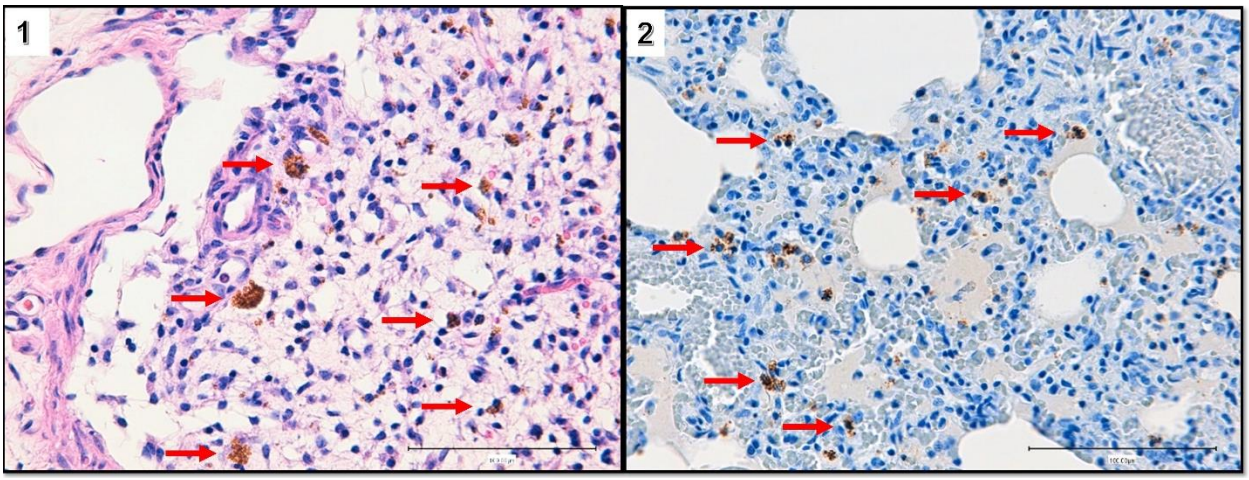

Figure 28. Localization of vaccine antigen in pigs vaccinated with $PEG_{600}PTHF_{650}$/M2e-HA1-HA2. 1- Lymph node, 2-Lung. Arrows indicate brown staining of the peptide antigen detected by a M2e-specific monoclonal antibody. No antigen was detected in unvaccinated pigs (images not shown).

Discussion

The discovery that the highly conserved M2e and HA2 epitopes can confer broad protection against influenza viruses was a major break through in the development of universal vaccines against influenza viruses (Du, Zhou, and Jiang 2010). These epitopes have been tested extensively and succesfully in mice; individually or in conjunction with other immunogenic proteins and peptides (De Filette et al. 2005, De Filette, Ramne, et al. 2006, Fiers et al. 2009, Adar et al. 2009, Du, Zhou, and Jiang 2010, Staneková and Varečková 2010, El Bakkouri et al. 2011). However, in pig models the M2e peptide failed to reduce challenge virus shedding and ameliorate disease outcomes (De Filette et al. 2005, De Filette, Ramne, et al. 2006, Huleatt et al. 2008, Chun et al. 2008, Fiers et al. 2009, Adar et al. 2009, Du, Zhou, and Jiang 2010, Staneková and Varečková 2010, El Bakkouri et al. 2011, Heinen et al. 2002, Opriessnig et al. 2018). A combination of the M2e and HA2 epitopes, together with a H1N1strain-specific HA1 epitope, as used in this study, has not been tested before in pigs to determine if there are syngergistic protective effects. Similarly, this study addresses the need for effective delivery systems for peptide antigens which are inherently poor immunogens (Chun et al. 2008, Feng et al. 2006) but have great promise in inducing epitope-specific, broad coverage. Our results support our hypothesis that the micellar assemblies prepared from amphiphilic invertible polymers (AIPs), represented in this study by $PEG_{600}PTHF_{650}$, effectively deliver the peptide vaccine cargo and act as an adjuvant in stimulating a strong humoral immune response against the delivered peptide.

While several conserved influenza epitopes have been identified, the M2e and HA2 epitopes are the most widely tested (De Filette, Fiers, et al. 2006, De Filette, Ramne, et al. 2006, Du, Zhou, and Jiang 2010, Zhao et al. 2010, Wen et al. 2016, Horváth et al. 1998). The HA2 epitope is an 11 amino acid conserved sequence in the N-terminal of the HA2 subunit of HA

protein. In mice models, vaccination with the HA2 peptide provided complete protection against IAV; whereas, this has not been studied yet in pig models (Bommakanti et al. 2010, Steel et al. 2010). The M2e epitope is a 23 amino acid long, highly conserved peptide chain from the M2 protein. M2e-based vaccine was also completely protective against multiple IAV strains in mice models (De Filette, Fiers, et al. 2006, De Filette, Ramne, et al. 2006, Zhao et al. 2010, Wen et al. 2016), whereas, in ferrets (Fan et al. 2004) and chicken (Zhang et al. 2011, Reese et al. 2013) M2e vaccination was only able to reduce the virus shedding and pathological symptoms. However, in pigs M2e-based vaccines resulted in enhanced challenge viral infection (Heinen et al. 2002, Opriessnig et al. 2018). The strain-specific HA1 epitope used in this study was untested in swine but reduced viral shedding and pathology in a mouse model (Horváth et al. 1998). While the experimental conditions in our study do not exactly match those referenced above, the early enhancement of viral replication seen in the peptide-immunized animals was similar to observations in other studies cited above. Previous studies in mice suggest that the enhancement of infection in mice immunized with a chimeric peptide encoding the M2e and HA2 epitopes occurs via Fc region-based antibody dependent enhancement of infection of macrophages (Ameghi et al. , Dutry et al. 2011).

However, unlike other studies, vaccination had a delayed but significantly clearing effect between day 3 and day 6 post-challenge as evidenced by the reduction in viral shedding in vaccinated animals. Had the observation period been continued beyond 6 days, it is likely that data would show that vaccinated animals had succesfully cleared the infection. However, the study was terminated at day 6 post-challenge because of the acute nature of SIV infections which decreases the probability of detecting the virus in the nasal secretions by qRT-PCR beyond 6 days (Goodell et al. 2013, Janke 2014). The clearing effect is most likely due to cell mediated

immunity or non-neutralizing mechanisms of antibody mediated protection. While the detailed characterization of these mechanisms is not within the scope of this study, our hypothesis that a combination of the AIP packaged epitopes will improve protection against influenza infection is well supported by the similar trends in the pathology and viral load data showing lower values for vaccinated animals. While the protection seen this study can also be attributed to differences in the vaccine construct, dose, route of vaccination and infection, challenge virus strain and culture conditions used (Mozdzanowska et al. 2007, Price et al. 2018, Schepens, De Vlieger, and Saelens 2018, Kim et al. 2017, Wolf et al. 2011). The low level of virus infectivity, immunopathology and no clinical signs can be due to route of challenge virus inoculation, as intranasal inocuation reduce the virus amount reaches the lungs compare to the intratracheal route of inoculation (Janke 2014, Landolt et al. 2003, De Vleeschauwer et al. 2009). Further improvements in design, possibly by the addition of other B or T cell epitopes, could further improve the early immune responses and viral clearance.

Several approaches such as linking epitopes with immunogenic peptides or proteins, using multiple copies of epitopes, creating virus-like particles or using amphiphilic polymers (De Filette, Ramne, et al. 2006, Huleatt et al. 2008, De Filette et al. 2005, Du, Zhou, and Jiang 2010, De Filette, Fiers, et al. 2006, Stanekova et al. 2011, Adams, Haughney, and Mallapragada 2015) have been previously used to improve the weak immunogenicity of peptide antigens (Feng et al. 2006, Chun et al. 2008). Amphiphilic polymers have several advantages; they can form micelles and micellar assemblies that can load antigen in a controllable manner, can be used for controlled antigen release and are generally immunologically safe (Shakya and Nandakumar 2013). Efficient entrapment and delivery of the hydrophobic drug , curcumin, into cancerous breast carcinoma and osteosarcoma cells as a potential treatment for breast and bone cancer

respectively, was previously demonstrated for the AIP macromolecules used as the vaccine delivery vehicle in this study (Hevus et al. 2012). Interestingly, unlike previous studies (Kohut et al. 2017, Pan et al. 2018), the peptide molecules are preferentially localized within the PEG exterior of the $PEG_{600}PTHF_{650}$ micellar assemblies, which can be attributed to the higher molecular weight of the M2e-HA1-HA2 peptide (11 Kd, compared to the 1.6-1.9 Kd for peptides studied in (Kohut et al. 2017, Pan et al. 2018)), differences in peptide sequence and conformation of the molecules. While there is no previously published data on the possible biological mechanisms of action of the AIP, it is evident that incorporation of M2e-HA1-HA2 peptide in aqueous solution into micellar assemblies resulted in protection and effective delivery of the antigen vaccination. While the peptide alone was not uptaken by Vero cells due to its hydrophobic nature, the uptaken of peptide incorporated with the micellar assemblies by the Vero cells (in vitro) and by lung epithelial cell (in vivo) clearly provide evidence that the micellar assemblies enhanced the bioavailability and delivery of the M2e-HA1-HA2 peptide into cells in vitro and in vivo. Since the M2e-HA1-HA2 peptide was found in the lymph nodes, the AIP micellar assemblies most likely faciltiated peptide uptake by antigen presenting cells. The adjuvant effects in enhancing antibody mediated immunity are clearly substantiated by the significantly higher peptide-specific antibody titers in piglets vaccinated with $PEG_{600}PTHF_{650}$/M2e-HA1-HA2 micellar assemblies compared to control piglets vaccinated with M2e-HA1-HA2 peptide alone. Further, as no toxicity was noted in vitro or in vivo, the described AIP-based vaccine formulation has significant promise as a peptide antigen delivery system, especially to stimulate strong antibody responses for effective protection. The AIP's efficacy in delivering hydrophilic peptides or other complex peptides with diverse physical properties remains to be tested.

M2e antibodies can prevent the release of viral RNA genome from the endosome by preventing ion channel activity of the M2 protein. HA2 antibodies are reported to bind with the fusion peptide of HA2 protein hence preventing the fusion of the viral envelope with the endosomal membrane (Imai et al. 1998). Hence, anti-M2e and HA2 antibodies are non-neutralizing and likely do not prevent virus attachment and initial infection (Staneková and Varečková 2010, Heinen et al. 2002, Chun et al. 2008, Ameghi et al. 2016) but likely act via other mechanisms which are not fully understood. Studies in mice suggest that anti-M2e and anti-HA2 antibodies could reduce the viral replication by eliminating infected cells by antibody dependent cell-mediated phagocytosis (El Bakkouri et al. 2011). While characterization of cell mediated or innate immune mechanisms was not undertaken in this study, similar mechanisms were likely involved in this study, as animals vaccinated with $PEG_{600}PTHF_{650}$/M2e-HA1-HA2 micellar assemblies had fewer lung lesions compared to the control groups in the absence of neutralizing antibody responses. The HA1 epitope-specific antibodies could be expected to bind to the cleavage site of precursor HA0 protein thus prevent the formation of HA1-HA2 mature protein (Horváth et al. 1998) and can be expected to have a neutralizing effect. Also, HA1 epitope-specific antibodies showed cpability to mediating cytotoxity of the infected cells via FcγR dependence mechanism (DiLillo et al. 2014). While we did not measure the levels of HA1-specific antibodies in this study, it is likely that the magnitude of the antibody reponse to this epitope at pre-challenge sample collection time point was below the detection threshold of the HI assay used. Other limitations of this study are that the the level of IgA antibodies were not determined and protection was assessed only against the Influenza A (H1N1) pdm09 strain.

In conclusion, the use of multiple epitopes and the adjuvant effects of the micellar assemblies from AIP macromolecules were effective in enhancing epitope-based immunization

approaches against influenza viruses. A more detailed characterization of the mechanisms by which the $PEG_{600}PTHF_{650}$ assemblies modulate immunity will help to fully exploit its use as a delivery system and adjuvant.

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CHAPTER 5. SUMMARY

Despite the advances in the medical field, the number of newly emerging and re-emerging infectious diseases are surging. Out of these newly emerging infectious diseases, over 80% are caused by RNA virus. Lack of previous exposure and immunity against these viruses leads to their rapid spread resulting in epidemics. To effectively control the quick spread of the agent during such epidemics there is an urgent need for better technology for rapid-response diagnostics for disease surveillance and vaccines for prevention. However, due to the high genetic variability and antigenic diversity of RNA viruses, existing vaccines become obsolete whenever a new strain or serotype emerges. The primary contribution of this thesis is the development of novel approaches to develop rapid-response or epidemic vaccines and high throughput serological methods which can be conveniently deployed in an outbreak.

In our first objective of this study to develop a relatively uncomplicated method to rapidly attenuated newly emerging viruses for use as vaccine candidates, the method developed employed heat and enzymatic digestion to successfully integrate the advantages of both attenuated and inactivated vaccines, namely, efficacy and safety, into a simple but unique process. The integrity of genomic viral RNA of the rapidly-attenuated viruses was intact while structural integrity of the vaccine antigen was retained. The PEDV vaccine developed by this method had superior efficacy and safety when compared to current licensed vaccines. With the developed method less than two weeks were required to prepare the vaccine (time also includes in-vitro testing for replication and structural integrity). Both of these characteristics (short-time vaccine development, and high safety and efficacy) are important for epidemic vaccines. Our future goals also include testing the method against other RNA viruses. Preliminary results from testing the rapid-attenuation method on swine influenza viruses showed similar levels of

protection and safety. As PEDV infection greatly impact the suckling piglets (80-100% mortality), our future goal for PEDV vaccine is to test it in pregnant sows to assess its safety and efficacy in stimulating lactogenic immunity which will protect suckling piglets from the PEDV challenge virus and infection.

Our second objective was to develop convenient diagnostic method for rapid quantification of virus and neutralizing antibodies against it. Using a colorimetric dye which is usually used to measure cell viability, we were able to replace the labor- and time-consuming step of visual reading of 96 well teat plates with a digital output that could easily help the operator distinguish between positive and negative samples. The newly developed colorimetric assay performance was accurate and reliable. The method can be used in diagnostic laboratories which usually receive serum samples from outbreaks for disease surveillance or where high-throughput testing is routinely carried out.

In our last and third objective we tested an alternative method for rapid-response vaccine development, based on the premise that using segments of the viral proteins (epitopes) which are conserved among different stains of the same virus as a vaccine will protect against the newly emerging strain, precluding the need to make a new vaccine every time a new variant emerged. While this concept is not new, conserved epitopes are usually delivered as peptides which are inherently weak immunogens. The primary impact of our work was to develop a unique delivery system for the conserved peptides to achieve effective delivery and a strong immune response. As anticipated, the unique amphiphilic invertible polymer ($PEG_{600}PTHF_{650}$) delivery system conjugated to the selected conserved peptides, elicited strong antibody responses to the peptides in vaccinated pigs, indicating that the polymer was effective as an adjuvant and peptide delivery vehicle. However, protection against the challenge virus was delayed, indicating that alternate

peptide epitope targets need to be identified and incorporated. More experiments are needed to characterize the mechanisms by which $PEG_{600}PTHF_{650}$ delivers the antigens and modulates the immunity.

In conclusion, this dissertation provides significant advances in the areas of A) emergency vaccine development B) high-throughput serological diagnostics and C) peptide antigen delivery; all of which are play an important role in the prevention of infectious diseases.

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