Development of Virus-like particles (VLPs) Based Vaccines Against Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) and Porcine Epidemic Diarrhea Virus (PEDV)

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Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) and porcine epidemic diarrhea virus (PEDV) are two of the most prevalent swine pathogens that have impacted the global swine industry for decades. Both are RNA viruses with increasing heterogeneity over the years, making a vaccine solution ever so challenging. Modified live-attenuated vaccines (MLVs) have been the most common approach, but the long-term safety regarding their potential for pathogenic reversion still needs to be addressed. Subunit based vaccines have been the focus of numerous development studies around the world with renewed interest in their promising prospects in both safety and efficacy.

Our lab has developed a unique approach to use hepatitis B virus core capsid protein (HBcAg) as a vaccine delivery vehicle for either PRRSV or PEDV viral epitope antigens. Recombinantly produced HBcAg forms an icosahedral capsid virus-like particle (VLP) that has 240 repeats in a single assembled particle. By inserting different epitope antigens from these porcine pathogens into the particle, we can achieve repetitive antigen presentation to the host's immune system by taking advantage of the polymeric nature of VLP.

The first animal study evaluated the efficacy of 4 VLP based vaccine candidates against PRRSV in mice. These 4 vaccines incorporated 2 B-cell epitopes (^{61}QAAIEVYEPGRS^{72} and ^{89}ELGFVVPPGLSS^{100}) and 2 T-cell epitopes (^{117}LAALICFVIRLAKNC^{131} and ^{149}KGRLYRWRSPVIIEK^{163}) from PRRSV structural proteins GP3 and GP5 respectively. Candidate GP3-4 was able to stimulate a significant viral neutralizing response in mouse sera against two PRRSV strains, one being heterologous, demonstrating its potential of cross-protection against PRRSV.

The second animal study took an optimized VLP vaccine candidate against PEDV from previous development studies in mice, and assessed its efficacy through a comprehensive pregnant gilt vaccination and neonatal piglet challenge model. The vaccine candidate incorporated B-cell epitope ^{748}YSNIGVCK^{755} from the PEDV spike protein. It was able to elicit significant viral neutralization antibody titer in gilt milk at 3 days post-farrowing (DPF), and provided nursing piglets with clinical relief in terms of morbidity, viral shedding, small intestinal lesions, and 10 days post-challenge (DPC) survival rate.

Development of Virus-like particles (VLPs) Based Vaccines Against Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) and Porcine Epidemic Diarrhea Virus (PEDV)

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General Audience Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) and porcine epidemic diarrhea virus (PEDV) are two pathogens that infect pigs, resulting in immense economic losses to the global pork production industry every year. Both viruses have large diversity with various strains due to mutations that have occurred over the years. This makes vaccine development that aims at combating the pathogens even more challenging.

One common vaccine strategy has been immunizing animals with modified live viruses with decreased pathogenicity. Naturally, long term safety of this option has been a concern. A much safer vaccine approach that is purely protein based has attracted renewed interest around the world. Protein based vaccines lack genetic materials from the viruses and are not able to replicate inside the host.

Our lab has developed a platform that uses protein-based particles (VLPs) originated from the hepatitis B virus (HBV), and incorporates short pieces of proteins from either PRRSV or PEDV to train host's immune system to recognize these pathogens, and hopefully to prevent future infection.

For the first animal study, we tested 4 VLP vaccine candidates against PRRSV in mice and discovered that mouse serum from one candidate GP3-4 was able to prevent infection of 2 distinct PRRSV strains in petri dishes, paving the way for further development.

For the second animal study, we took an optimized VLP vaccine candidate against PEDV from previous mouse studies, and evaluated its performance in pigs. We immunized pregnant mother pigs with the vaccine before they gave birth, then experimentally infected newborn piglets with the virus. Piglets from the vaccinated mothers showed improved clinical signs and faster recovery from the infection.

Dedication

I would like to dedicate this to my wife Miaotian Xie, my parents Fangya Cheng, Jianmin Lu, and my in laws, Lunping Wang, Daogui Xie. Without their support for the past four and half years, this would not have been possible.

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I would like to thank Dr. Sherrie Clark-Deener for being such a pillar for the entire pig trial, and all the volunteers who worked so tirelessly to help us complete the study.

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Attribution

Chapter 3: Hepatitis B core antigen based vaccine incorporated with both B-cell and T-cell epitopes demonstrates cross-neutralization against heterologous Porcine Reproductive and Respiratory Syndrome Virus

Frank Gillam provided guidance on cloning, expression, purification, formulation, animal study, and ELISA.

Qian M. Cao helped with *in vitro* splenocyte re-stimulation.

Amy Rizzo provided trainings for the mouse study, and performed the final intracardiac blood collection.

Xiang-Jin Meng is the PI for Qian M. Cao.

Chenming Zhang is the PI for Yi Lu.

Chapter 4: Virus-like particle vaccine with B-cell epitope from porcine epidemic diarrhea virus (PEDV) incorporated into hepatitis B virus core capsid provides clinical alleviation against PEDV in neonatal piglets through lactogenic immunity

Sherrie Clark-Deener performed every major element of the pig study.

Frank Gillam designed and produced the cell line of the vaccine for evaluation.

C. Lynn Heffron helped with blood draw, final necropsy for the pig study.

Debin Tian helped with the viral propagation for the pig study.

Harini Sooryanarain helped with blood draw, and Vero cell maintenance for the pig study.

Tanya LeRoith performed piglet necropsy, tissue preparation, and histology analysis.

Jessica Zoghby, Mallori Henshaw, and Steven Waldrop helped with animal monitoring and piglet necropsy.

Jeremy Pittman provided and transported the gilts in the pig study.

Xiang-Jin Meng is the PI for C. Lynn Heffron, Debin Tian, and Harini Sooryanarain.

Chenming Zhang is the PI for Yi Lu.

Chapter 1: Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) and porcine epidemic diarrhea virus (PEDV) are two of the most prevalent and problematic swine pathogens that have impacted the global swine industry for decades [1, 2]. In the United States alone, the price tag on PRRSV in terms of economic loss was about $664 million, according to a 2011 assessment [3], while a 2014 analysis indicated PEDV's financial impact to be between $900 million and $1.8 billion [4]. Both viruses are RNA viruses, their genetic heterogeneity sustained numerous outbreaks globally with increasing phylogenetic diversity [5, 6], making the task of developing a potent vaccine that provides broad cross-protection incredibly challenging.

Modified live-attenuated vaccines (MLVs) have been the most efficacious solutions in terms combating these two pathogens. However, this vaccine strategy uses live viruses. While the efficacy is relatively robust, long term safety has been a concern due to MLV's risk of pathogenic reversion for both PRRSV [7] and PEDV [8]. In addition, the commercially available MLV against PRRSV falls short on providing heterologous protection [9].

There is renewed interest in subunit vaccine development for both PRRSV [10] and PEDV [8] due to its superior safety from the lack of viral genetic materials. However, vaccine efficacy still needs to be addressed for individual subunit vaccine candidate [11-13].

The unique approach that we took was utilizing a virus-like particle (VLP) based vaccine delivery vehicle, the hepatitis B virus core capsid protein (HBcAg). We

incorporated different viral epitope antigens from either PRRSV or PEDV into the HBcAg backbone and expressed the chimeric VLPs recombinantly in *E. coli*. The assembled icosahedral capsid of HBcAg is composed of 240 monomeric units when the triangulation number is 4 [14]. As a result, every chimeric VLP also includes 240 copies of the viral epitope antigen that we inserted into the HBcAg backbone. Through repetitive antigen presentation, this approach has the potential to stimulate potent immune response against either PRRSV or PEDV.

Both PRRSV and PEDV are enveloped viruses with large structural proteins protruded from the viral surface. Those structural proteins are critical in terms of viral attachment and entry during infection, as a result, major targets for eliciting neutralizing antibodies in the host [15-20].

Numerous epitopes have been previously identified in those viral proteins. For the VLP vaccine against PRRSV, we used 2 B-cell epitopes ^{61}QAAIEVYEPGRS^{72} and ^{89}ELGFVVPPGLSS^{100} from the GP3 protein of PRRSV [21], and 2 T-cell epitopes ^{117}LAALICFVIRLAKNC^{131} and ^{149}KGRLYRWRSPVIIEK^{163} from the GP5 protein of PRRSV [22]. For the VLP vaccine against PEDV, we used a B-cell epitope ^{748}YSNIGVCK^{755} from the spike protein of PEDV [23].

For the VLP vaccine against PRRSV, we tested 4 vaccine candidates that have viral epitopes inserted at different sites of the HBcAg in mice. We evaluated their efficacy both in terms of humoral immune response and cell-mediated immune response. We also assessed the vaccines' cross-protection through serum viral neutralization assay against two PRRSV strains, one homologous and one heterologous. The results are detailed in Chapter 3.

For the VLP vaccine against PEDV, we took the optimized product from previous development studies in mice [24, 25], and assessed its immunogenicity in a comprehensive pregnant gilt vaccination and piglet challenge model. The results are detailed in Chapter 4.

Finally, Chapter 5 summarizes our findings from the PRRSV mouse trial and the PEDV pig trial, and lays out our path forward in the future.

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Chapter 2: Literature Review

Current Status of PRRSV Vaccines: Successes and Challenges

Since the emergence in the 1980s, porcine reproductive and respiratory syndrome virus (PRRSV) has been the target of intense vaccine research in the hope of eradicating the disease. However, due to the nature of RNA viruses, rapid mutation and genetic heterogeneity make the ultimate goal more than trivial. In this review, we will examine the two major approaches in PRRSV vaccine development, live vaccines and non-live vaccines. In addition, we will specifically look at efforts on virus-like particle (VLP) based vaccine development.

Virus Overview

PRRSV belongs to the *Arteriviridae* family[1], with a genome size of about 15 kb [2]. It has at least 10 open reading frames (ORFs), which code for structural proteins GP2, GP3, GP4, GP5, matrix (M) and nucleocapsid (N), and other regulatory proteins [3]. The ectodomains of the structural proteins have been the targets of extensive research in terms of identifying antigenic regions or epitopes that are capable of inducing viral neutralizing antibodies in host [4].Viral infection of PRRSV of susceptible porcine alveolar macrophages (PAMs) takes place in three major steps involving different viral structural proteins and receptors on the surface of PAMs. First is the relatively non-specific interaction between the M protein and heparin sulfate. Second is the higher affinity viral attachment between GP5 and sialoadhesin. Third is viral entry through the interaction between the virion and CD163 [3]. CD163 also associates with the virus inside the

endosome, facilitating the uncoating process inside PAMs [2]. PRRSV can be transmitted through physical contact in pigs, also via semen from infected boars [5]. Clinical signs from PRRSV include lethargy, anorexia, pneumonia, late-term abortions, and weak-born piglets [6].

Live Vaccines

Killed virus-based vaccines have the superiority in safety, however, efficacy for this approach has been shown to be insufficient to protect pigs from viral challenge [7]. Commercially available modified live-attenuated vaccine (MLV) is successful in providing protection against homologous strains, while facing short falls in heterologous protection [8]. Safety of MLV based vaccines has been a concern since live viruses have the potential for pathogenic reversion [9]. Vaccine derived isolates have been shown to cause diseases in pigs [10]. However, PRRSV-MLV vaccine is still currently the most effective measure with respect to offering limited cross-protection [9].

To improve PRRSV-MLV's efficacy even more in the face of PRRSV's rapid mutation and heterogeneity, codon pairs de-optimization and DNA shuffling are two methods being used. Synthetic attenuated virus engineering (SAVE) is used to de-optimize codon pairs to decrease translation efficiency of viral proteins, in turn, attenuating the virus and creating strain specific MLV [9]. The advantage of this attenuation process is its pace. It allows researchers to create attenuated strains for vaccine purpose as new PRRSV strains emerge in the fields. On the other hand, DNA shuffling creates chimeras by molecularly breeding genes for major PRRSV structural proteins like GP5 and GP3 from various heterologous field strains, the products of which had been tested for their ability to entice viral neutralizing antibody response in pigs [9]. Two of the neutralizing epitopes

(^{61}QAAIEVYEPGRS^{72} and ^{89}ELGFVVPPGLSS^{100}) in our VLP-based vaccine design, which are covered in detail in Chapter 3, are identified through this method [11].

Another class of live vaccines for PRRSV is replicating viral vectors. Different recombinant viral vectors are used as vehicles to deliver genetic material that codes for PRRSV antigens to host. Since the viral vectors are capable of replication, a sustained expression of PRRSV antigens is expected. However, only partial protection is observed in pigs with low levels of neutralizing antibodies [12].

Non-Live Vaccines

Inactivated vaccines present an attractive alternative to achieve safety. However, comparing with live vaccines, most inactivated vaccines came short on efficacy and cross-protection. PRRSV infects PAMS through binding of sialic-acid on the viral surface with sialoadhesin receptor that's expressed on PAM [13]. Sialoadhesin also mediates endocytosis of the virus into PAM [14]. The PRRSV viron has a nucleocapsid within a lipid envelope. The envelope has several proteins embedded: a membrane protein M, glycoproteins, GP2, GP3, GP4, GP5, and a small envelope protein E [15]. The central strategy for many non-infectious vaccines focuses on priming host with these viral proteins to stimulate both viral neutralizing humoral immune response and viral specific cell-mediated immune response to achieve protection against PRRSV [16].

Recombinant based systems are used to express the viral proteins. Similar to the utilization of replicating viral vectors, non-replicating viral vectors are designed to express either individual major PRRSV structural proteins or fusion proteins. This method has generated both targeted B-cell and T-cell responses. However, it was only able to provide

limited and insufficient protection against PRRSV in pigs. It achieved clinical relief but not prevention for viral infection [16].

Transgenic plant has also been used to express either individual PRRSV structural proteins [17] or fusion protein that includes *E. coli* heat-labile enterotoxin B subunit to enhance immunogenicity [18]. However, with the limited studies that have been conducted in pigs, efficacy of plant-based vaccine has been confined to clinical relief as well [18]. Although it is a very economical approach, vaccine dosage control and quality characterization are just few concerns that are associated with plant-based vaccines [19].

DNA vaccines use plasmid DNA that encodes major PRRSV viral proteins like GP5. However, it was insufficient to inhibit viral shedding in the respiratory tract in pigs post-challenge [20]. A DNA vaccine that co-expresses GP3, GP5, along with IFN-α/γ demonstrated homologous protection but missing heterologous challenge [21]. It is still unclear regarding the general efficacy of DNA vaccines against PRRSV [16].

Nanoparticle based vaccine development is a relatively new but promising approach to combat a wide range of non-infectious and infectious diseases. Due to its sub-micrometer size, it can be utilized to reach draining lymph nodes more efficiently to stimulate innate response that is critical for subsequent adaptive immune response [22]. Innate response against PRRSV has shown to be weak, and has been partially attributed as the cause of persistent viral infection in swine [12]. With the potential to address this major hurdle in the battle against PRRSV, nanoparticles could be an attractive solution.

Virus-like particle (VLP) is one major class of the nanoparticle-based vaccine approach. A VLP is comprised of viral proteins that form a viral capsid. Produced recombinantly, VLPs are free of viral genetic material, hence the superior safety [22]. The

spatial organization of the capsid allows for repetitive antigen display due to its polymeric nature. Through genetic engineering, PRRSV specific epitope antigens can be introduced into each of the monomeric viral protein that forms the capsid, in turn, eliciting specific immune responses through either direct presentation or cross-presentation by antigen presenting cells (APCs) [22]. VLP based approach has been used for PRRSV vaccine development in several studies.

Chimeric VLPs that are composed of M1, NA protein from H1N1 influenza virus and GP5 protein from PRRSV has been developed by infecting *Sprodoptera frugiperda* Sf9 cells with recombinant baculovirus (rbV) [23]. Immunogenicity study in mice showed comparable immune response with inactivated PRRSV. Further pig study was not conducted [23]. Another study constructed VLPs with HA, M1 proteins from H3N2 influenza virus and GP5 from PRRSV by using a similar coinfection method also demonstrated comparable immunogenicity in mice with inactivated PRRSV [24].

VLP containing five PRRSV viral surface proteins GP5, GP4, GP3, GP2a. and M has been developed and tested in pigs. It was insufficient against heterologous challenge [25]. By expressing whole viral proteins as VLP vaccine, potential adverse effect like glycan shielding of neutralizing epitope could interfere with effective immune response against PRRSV [8]. Incorporation of viral epitopes into VLPs hence can potentially facilitate a more specific response.

A bivalent vaccine against both PRRSV and porcine circovirus type 2 (PCV2) has been studied by inserting a GP5 B cell epitope (^{37}SHIQLIYNL^{45}) into PCV2 capsid protein. The VLP was expressed in *E. coli* and immunogenicity study in mice showed comparable response with inactivated PRRSV-PCV2. No pig study was conducted [26].

Development of VLP-based PRRSV vaccine is very much ongoing. Preliminary data have been primarily limited to mice studies. However, further development is warranted considering VLP's promising safety feature.

Looking forward for PRRSV Vaccine Development

To develop a successful vaccine against PRRSV, the solution needs to be satisfactory on two fronts, safety and efficacy. Currently, MLV vaccines have the best efficacy regarding cross-protection. However, safety is a major concern for this approach. Commercially available MLV vaccines are only recommended for usage in PRRS-positive swine herds [9]. In addition, heterologous protection from MLV vaccines still has large room to improve.

On the other hand, inactivated vaccines provide assurance on safety, but lack the same level of potency. With that being said, many studies are still underway, with incomplete clinical data from pigs to make determinative evaluations on those vaccines' efficacy.

It has been reported that a neutralizing antibody titer of 1:32 is required to prevent PRRSV infection in swine [27]. A successful vaccine, regardless of its form or type, needs to be able to produce a comparable anamnestic immune response while addressing any safety concern for usage in naïve herds.

Current Status of PEDV Vaccines: Successes and Challenges

With a later occurrence in the United States when compared to PRRSV, porcine epidemic diarrhea virus (PEDV) has shown to be just as intractable ever since its first emergence in 2013. It costed immense economic losses in the swine industry [28]. The enteric viral shedding of PEDV and the primary route of transmission from fecal to oral make PEDV extremely difficult to contain and obliterate. Since its 1st cited cases in Europe in the 1970s, various vaccine strategies have been employed to combat PEDV. We will look at both modified live-attenuated vaccine (MLV) and killed virus-based approaches, in addition, more recent tactics like subunit and DNA vaccines.

Virus Overview

PEDV belongs to the *Coronaviridae* family, with a genome size of about 28 kb, and at least 7 ORFs that code for structural proteins like the spike (S), membrane (M), envelope (E), nucleocapsid (N), and other regulatory proteins [29]. PEDV targets villous epithelial cells in pig small intestine, which express porcine aminopeptidase N (pAPN) as a major receptor for PEDV [29]. The spike protein (S) from PEDV plays a key role in both viral attachment and entry [30], with S1 being the receptor binding domain and S2 being the membrane fusion domain [31]. The main transmission route for PEDV is fecal-to-oral [32]. Neonatal piglets are most vulnerable to PEDV with almost 100% mortality in the age group between 1 to 3 days old [29]. Clinical signs from PEDV infection include villi atrophy, distended GI tract, watery diarrhea, vomit, lethargy, and dehydration [33].

Modified live-attenuated vaccine (MLV)

Modified live-attenuated vaccines (MLVs) are usually more effective due to their ability to replicate and continuously stimulate host's immune system when compared to other alternatives. In the United States, the first commercially approved vaccine against PEDV utilizes viral replicating particles derived from the Venezuela equine encephalitis virus with packaged PEDV genes [34]. A three-dose regime of naïve sows improved litter mortality [34].

Cell adapted CV777 strain from high number of cell passage also demonstrated ability to lower morbidity and viral shedding in 11-day old piglets when challenged with the same but virulent strain of CV777 21 days post-vaccination [35]. Attenuated strain KPEDV-9 through cell passage alleviated pathogenicity of the same virulent strain in 4-day old neonatal piglets [36]. Vaccination of pregnant sows with attenuated DR-13 strain improved mortality in 3-day old piglets when challenged with the virulent DR-13 strain [37].

However, reversion to virulence is often a concern for MLVs [34]. In addition, cross-protection was not investigated for most of the cited studies since viral challenges were performed with the same virulent strain that was used for attenuation.

Killed Virus

Killed virus-based approach uses inactivated PEDV whole viruses to prime host in the hope of sufficient protection during an actual infection event in the future. Commercially available option from Zoetis in the United States is one example. It was tested to induce neutralizing antibodies against PEDV in pregnant sows and reduce mortality in neonatal piglets [34, 38].

In Korea, a study has been done with binary ethylenimine (BEI) inactivated PEDV strain KNU-14112. Intramuscular immunization of pregnant sows with this inactivated virus increased survival of 6-day old piglets when challenged with the virulent form of KNU-14112 [39].

On the other hand, efficacy of killed virus-based vaccine has often been a concern while it is relatively safe. Study has shown that naïve sows that immunized with the killed virus-based vaccine from Zoetis did not stack up to previously exposed sows when it came down to piglet protection. Five-day old piglets from previously exposed sows showed little clinical signs from PEDV when challenged, while the piglets from vaccinated naïve sows suffered 100% mortality in 15 days, given the challenge dose was relatively high [40]. In China, variant PEDV strains arose in farms where the sows had been vaccinated with inactivated CV777 [41], casting doubt on both the efficacy and cross-protection from killed virus-based vaccines.

DNA Vaccines

DNA vaccines use plasmid DNA as vaccine delivery vehicles have been studied as well. PEDV gene codes for the spike protein was inserted into eukaryotic expression plasmid pIRES and injected intramuscularly into 6-week old mice. The subjects showed PEDV specific cellular and humoral immune responses [42]. However, this strategy was not evaluated in pigs hence there were no viral challenge data available.

DNA vaccines use bacteria that contain plasmid vectors as a delivery method have also been explored. Gene codes for the S1 domain of the PEDV spike protein was cloned into eukaryotic expression plasmid pVAXD. *Salmonella typhimurium* cells were then transformed with the plasmid before oral administration to 20-day old piglets. Pig sera

exhibited certain viral neutralizing ability *in vitro* but no viral challenge was performed [43].

DNA vaccine could be a viable vaccine option against PEDV, however, due to the limited data on pregnant sow and lactogenic immunity in nursing piglets, much more remains to be seen before its potential could be assessed more extensively.

Subunit Vaccine

With the protein-based nature of subunit vaccine, it is inherently much safer than traditional viral based vaccine methods. However, development of a subunit vaccine that can mount a sufficient immune response in sow and subsequently in neonatal piglets to fend off PEDV remains a challenge.

Transgenic tobacco plant that expresses spike protein epitope has been studied as an option to induce mucosal and systemic responses when it is orally delivered to mice. The epitope gene was 504-base pair long. Mouse sera and fecal extracts both exhibited some viral neutralizing ability *in vitro* [44]. The study was preliminary since there was no experimentation done in pigs. As a result, the effectiveness of this vaccine is still unclear.

There were three pig studies that examined the efficacy of vaccines that used recombinantly expressed S1 domain of the PEDV spike protein. One in Korea [45], one in Canada[46], and one in the United States [38]. The study in Korea expressed the S1 protein from PEDV genogroup G2a strain KNU-0801 using a porcine cell line PK-15. A 3-dose regime was administered to pregnant sows intramuscularly before farrowing. Four to five-day old piglets were challenged with a PEDV dose of 10^5 $TCID_{50}$. Sow serum and colostrum showed viral neutralizing ability *in vitro*. The vaccine was able to alleviate some clinical signs of PEDV although not as effective when compared to piglets from sows

inoculated with live PEDV vaccine. Viral shedding was not quantified using qPCR. While all piglets from different vaccine groups survived from the viral challenge, there were only 2 piglets in each group [45]. This evidently puts the statistical robustness of this study into question.

The other subunit vaccine which was done in Canada used recombinantly expressed S1 protein from HEK293T cells. A pregnant sow was immunized with it three times intramuscularly prior to farrowing. Eight 4-day old piglets were then challenged with 2013 Colorado PEDV strain, which is a genotype G2b strain [47], at a dose of 3×10^2 $TCID_{50}$ per piglet. The lactogenic immunity was able to increase piglet survival 6 days post-challenge but did little to reduce clinical signs. Given the low challenge dose, this vaccine did not provide the piglets with complete protection against PEDV [46].

The S1 protein-based vaccine study in the U. S. used recombinantly expressed S1 protein of the PEDV MN strain [48] from insect cell line BTI-TN5B1-4. A pregnant sow received two doses of the vaccine prior to farrowing. Killed PEDV vaccine (Zoetis) was used in the study as a positive control. Ten 5-day old piglets were then challenged with the PEDV 2013 Colorado strain at a dose of 10^5 $TCID_{50}$ per piglet. The S1 protein-based vaccine helped with intestinal lesion post challenge, but was not significantly different from the Zoetis control [38].

In conclusion, all three S1 protein-based vaccines failed to demonstrate complete protection from PEDV in neonatal piglets. However, the clinical data generated in these studies are extremely valuable with their complete modeling of pregnant sow vaccination and neonatal piglet viral challenge for future vaccine development efforts.

Looking forward for PEDV Vaccine Development

A vaccine candidate that can provide complete clinical protection against PEDV for neonatal piglets is yet to be developed. With the safety concern of MLVs and limited efficacy of killed virus-based option, subunit vaccine stays as an attractive alternative as along as it is able to mount a potent immune response in sows and piglets.

Efforts to utilize viral capsid's polymeric structure to achieve repetitive antigen presentation have been taken in the form of developing virus-like particle (VLP) based vaccine against PEDV. Its potential to stimulate robust immune response from viral particles without the safety concern of a replicable viral genome makes it uniquely suited as a vaccine delivery vehicle for infectious diseases and more recently, cancers [49]. VLP that incorporates viral epitope ^{748}YSNIGVCK^{755} from the PEDV spike protein, using the hepatis B virus core antigen (HBcAg) as a particle backbone, demonstrated extremely high level of antigen specific and viral neutralizing responses in a mouse model [50, 51]. Evaluation of the effectiveness of this platform in a pig model with a comprehensive pregnant gilt immunization and subsequent piglet challenge experimental design is covered in detail in Chapter 4.

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Chapter 3: Hepatitis B core antigen based vaccine incorporated with both B-cell and T-cell epitopes demonstrates cross-neutralization against heterologous Porcine Reproductive and Respiratory Syndrome Virus

Hepatitis B core antigen based vaccine incorporated with both B-cell and T-cell epitopes demonstrates cross-neutralization against heterologous Porcine Reproductive and Respiratory Syndrome Virus

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Abstract

The U.S. swine industry have been bearing the financial impact of Porcine Reproductive and Respiratory Syndrome (PRRS) for decades. Absent of a safe and efficacious vaccine to combat PRRS virus's genetic heterogeneity, it remains a costly disease on pig farms across the country. We have developed virus-like-particle (VLP) based vaccines that incorporate 4 PRRSV epitopes in the hepatitis B core antigen (HBcAg) backbone. Administration of the vaccines in female BALB/C mice resulted in extremely significant PRRSV epitope specific antibody response. One vaccine candidate GP3-4 was able to mount a significant viral neutralizing response against both parental PRRSV strain VR2385 and heterologous PRRSV strain NADC20, showing a promising potential for cross-protection against PRRSV.

Introduction

Porcine Reproductive and Respiratory Syndrome (PRRS) is a disease that first arose in late 1980s in North America, with the causative agent being the PRRS virus (PRRSV) [1]. In the past decades, PRRS has become one of the most infectious swine diseases, with vast economic impact on the swine production industry. A 2011 assessment estimated the financial burden that PRRS imposed on the U.S. swine industry was about $664 million per annum, draining $1.8 million everyday out of the 60,000 pork producers' pocket [2, 3]. Of course, with today's globalized economy, drawbacks are rarely confined by sovereign borders. The U.S. is a major agriculture exporter, selling 20 percent of its commercial pork overseas [4]. Pork demand abroad has increased overtime. Recently, the demand was boosted largely by the latest outbreak of African Swine Fever in Europe and Asia. The

increased demand on U.S. pork has also driven up its price [5]. Since the economic impact of PRRS is largely attributed to productivity loss, every pound of pork lost due to PRRS will now incur higher erosion into producers' revenue.

Currently, an efficacious vaccine that is also highly safe is yet to be developed, considering the existing attenuated and inactivated vaccines against PRRSV have shortcomings in both efficacy and safety [1]. Commercially available modified live virus (MLV) based vaccine is successful in providing protection against homologous strains, while facing short falls in heterologous protection [6]. Safety of MLV based vaccines has been a concern since live viruses have the potential for pathogenic reversion [7]. Vaccine derived isolates have been shown to cause diseases in pigs [8]. However, PRRSV-MLV vaccine is still currently the most efficacious vaccine with respect to offering limited cross-protection [7].

The PRRSV virion has a nucleocapsid within a lipid envelope. The envelope has several proteins embedded: a membrane protein M, glycoproteins, GP2, GP3, GP4, GP5, and a small envelope protein E [1]. PRRSV infects pig alveolar macrophages (PAMs) through interaction with several of these viral proteins, M, GP2, GP3, GP4, and GP5 [9-11]. The central strategy for many subunit vaccines focuses on priming hosts with these viral proteins to entice both viral neutralizing humoral response and viral specific cell-mediated response to achieve protection against PRRSV [12].

Subunit vaccines present an attractive alternative to achieve safety. However, comparing with live vaccines, most subunit vaccines come short on efficacy and cross-protection. To potentially address this, we used virus-like-particle (VLP) based approach. A VLP is comprised of viral proteins that self-assemble into a structure similar to that of a

viral capsid. Produced recombinantly, VLPs are free of viral genetic material, hence the superior safety [13]. The spatial organization of the capsid allows for repetitive antigen display due to its polymeric nature. Through genetic engineering, PRRSV specific antigens or epitopes can be introduced into each of the monomeric viral protein that forms the capsid, in turn, eliciting specific immune responses through either direct presentation or cross-presentation by antigen presenting cells (APCs) [13]. One of the VLPs that have been studied extensively and employed in a myriad of vaccine applications is the nucleocapsid of hepatitis B virus, hepatitis B core antigen (HBcAg) [14-18]. We constructed VLPs (Fig.1) that utilize HBcAg as a backbone carrier for different PRRS viral epitopes to entice specific immunological responses, producing antibodies that target these epitopes which would potentially block PRRSV's entry into susceptible cells, training the immune system to combat subsequent infection of the virus.

Gene shuffled GP3 epitopes, gsGP31 and gsGP33 (^{61}QAAIEVYEPGRS^{72} and ^{89}ELGFVVPPGLSS^{100}), were two epitopes that have been identified in a chimera PRRSV strain GP3TS22, which has increased cross-neutralizing ability [19]. The GP3 gene from the chimera strain was bred from six different PRRSV strains (VR2385, VR2430, MN184B, JXA1, FL12, and NADC20) with VR2385 as the parental backbone [19]. Since production of virus-specific IFN-γ and neutralizing antibodies have been correlated to the protection against PRRSV [20-22], GP5T1 and GP5T2 (^{117}LAALICFVIRLAKNC^{131} and ^{149}KGRLYRWRSPVIIEK^{163}) were two identified immunodominant T cell epitopes [23] included in our vaccine designs as well. Poly-histidine (His) tag is attached to the N terminus of all the constructs in order for the vaccines to be purified by immobilized metal affinity chromatography (IMAC).

In this preliminary mouse study, we evaluated the efficacy of all four vaccine candidates in terms of both humoral and cellular immune response, and also serum viral neutralizing ability against two type 2 (North American) PRRSV strains.

Methods

Cells

DNA sequences that code for different vaccine candidates (IDT) were cloned into the multiple cloning site (MCS) in pET-28(+) plasmid (Novagen, Madison, WI). T7 Express Competent *E. coli* (NEB) was then transformed with individual plasmid construct through heat shock. Cell line integrity was confirmed using Sanger sequencing before cryo storage at -80 ˚C.

Vaccine Expression

Cells were grown in 2.8 L PYREX Fernbach flasks (Corning, Corning, NY) with 2×YT media and 30 μg/mL kanamycin at 37 °C, shaking at 200 rpm. 0.2 % (v/v) overnight culture that grown under the same condition was used as inoculum. Optical density at 600 nm (OD_{600}) was monitored throughout cell growth. Once OD_{600} reached 0.6-0.8, culture was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Incubator temperature was then decreased to 28 °C for overnight expression. Cells were pelleted through centrifugation the next day morning and stored at -20 °C for further processing.

Purification

Purification of the vaccines followed steps from previous work [24]. Briefly, cell pellet was sonicated in lysis buffer (20 mM Na_2HPO_4, 500 mM NaCl, 2% Triton X-100). Inclusion bodies were then washed once with lysis buffer and solubilized overnight with

solubilization buffer (20 mM Na_2HPO_4, 50 mM NaCl, 6 M urea, 0.9% sarkosyl). Solubilized protein was subsequently purified through columns that were packed with diethylaminoethyl (DEAE) Sepharose Fast Flow anion exchange chromatography resin (GE Healthcare, Marlborough, MA), and then immobilized metal ion affinity chromatography (IMAC) packed with IMAC Sepharose 6 Fast Flow resin (GE Healthcare) charged with nickel ions. IMAC eluate was buffer exchanged into 1× PBS with 0.9% sarkosyl at pH 7.4. Afterwards, stepwise dialysis was performed to decrease sarkosyl concentration to 0.45%, 0.23%, and finally 0% to facilitate protein folding and VLP formation. Unassembled protein was removed using Amicon ultracentrifugal filter with 100 kDa MWCO (Millipore, Danvers, MA).

Formulation

Sterile filtration was performed using 30 mm, 0.10 µm syringe filter (Celltreat, Pepperell, MA). Individual dose was formulated in a 0.5 mL PBS mixture designed for subcutaneous injection in mice. Each dose contained 20 µg vaccine, along with 20 µg CpG ODN (Alpha Diagnostic, San Antonio, TX), and 100 µg murabutide (InvivoGen, San Diego, CA) as adjuvants. Negative control doses included only adjuvants in PBS. All injections were formulated the night before administration and stored at 4 °C.

VLP Assembly Characterization

Dynamic light scattering (DLS) was performed to confirm particle size of formed VLP with Zetasizer Nano (Malvern, Malvern, UK). With preliminary confirmation, transmission electron microscopy (TEM) was further used to characterize particle assembly. Analyte was applied on a copper grid for a few seconds before blotted off with filter paper. After drying, a drop of purified deionized (DI) water was applied to wash the

grid before blotted off with filter paper. Then, 2% phosphotungstic acid (PTA) at pH 7 was used as negative staining agent before blotted off with filter paper. Once the grid was dried, it was examined by JEM 1400 (JEOL, Peabody, MA) under 300,000× magnification.

SDS-PAGE

NuPAGE 4-12% Bis-Tris Protein Gel (Invitrogen, Carlsbad, CA) was used to characterize protein sample purity and target protein molecular weight. Each well on the gel was loaded with 10 µL sample mixture that underwent 20 minutes heat treatment at 75 °C and consisted of 2 µL protein sample, 2.5 µL 4× NuPAGE LDS Sample buffer (Invitrogen), 1 µL 10× NuPAGE Sample Reducing agent (Invitrogen), and 4.5 µL purified DI water. Loaded gel was run with 1× NuPAGE MES SDS Running Buffer (Invitrogen) at 200 V for 35 minutes. Afterwards, 5-minute water wash was repeated 3 times before gel staining with SimplyBlue SafeStain (Invitrogen) for 1 hour. Overnight de-stain in purified DI water took place before imaging with ChemiDoc Imaging System (Bio-Rad, Hercules, CA). Image Lab (Bio-Rad) was then used to determine product purity (>98% for all purified vaccines) and target protein molecular weight through densitometry analysis and comparison with Precision Plus Protein Standards (Bio-Rad) respectively.

Western Blot

Vaccine identity was characterized by western blot, using anti-His6× antibody to probe cell lysate samples. NuPAGE 4-12% Bis-Tris Protein Gel (Invitrogen) was run with Precision Plus Protein WesternC Standards (Bio-Rad) loaded before blotting using Trans-Blot Turbo Blotting System (Bio-Rad) for 7 minutes. Blotted 0.2 µm nitrocellulose membrane (Bio-Rad) was washed in TBS for 5 minutes. It was then blocked in blocking

solution (TBS-T with 0.05% Tween 20 and 5% nonfat milk) for 1 hour, followed by 2 times TBS-T wash for 5 minutes. Blocking solution was also used for subsequent antibody dilutions. The membrane was incubated in 6×-His Tag Monoclonal Antibody (Invitrogen) with 1:3,000 dilution for 1 hour, then in Goat Anti-Mouse IgG-HRP Conjugate (Millipore) (1:5,000 dilution) and Precision Protein StrepTactin-HRP Conjugate (Bio-Rad) (1:10,000 dilution) for 1 hour. 3 times TBS-T wash for 5 minutes were performed after each incubation. A final wash with TBS was performed before Clarity Max Western ECL Substrate (Bio-Rad) application for signal development, which was captured immediately by ChemiDoc Imaging System (Bio-Rad).

Endotoxin Detection

All purified vaccines were tested for endotoxin levels (<1 EU/mL) with Pierce Chromogenic Endotoxin Quant Kit (Thermo Scientific, Waltham, MA) following manufacturer's instructions.

Animals

All animal related procedures were approved by Virginia Tech Institutional Animal Care and Use Committee (IACUC protocol 18-036). 4-6 weeks old female BALB/C mice (Charles River, Wilmington, MA) were divided into 6 groups (4 vaccine candidates + ΔHBcAg+ PBS) with 8 mice in each group. After a week of acclimation, all mice received 3 subcutaneous injections corresponding to each group with 2 weeks in between each administration. They were bled through submandibular collection before each injection. 2 weeks after the final injection, all mice were bled through intracardiac collection and then euthanized. Spleen tissue was harvested postmortem. Animal health was checked by weekly weight monitoring throughout the study.

ELISA

Epitope specific IgG titer in mouse serum was quantified using indirect ELISA. Mice sera were prepared from centrifugation (2,000 *g* for 10 minutes at 4 °C) of clotted whole blood and stored at -20 °C until analysis. Either epitope gsGP31 or gsGP33 was inserted recombinantly at the C terminus of an ELISA fusion protein (EFP) that was described in previous work [25]. Nunc MaxiSorp flat-bottom 96-well plate (Invitrogen) was coated with either EFP-gsGP31 or EFP-gsGP33 at 10 μg/mL in ELISA Coating Buffer (BioLegend, San Diego, CA) overnight at 4 °C. Plate was washed (4 times wash with BioLegend ELISA Wash Buffer) before blocking with ELISA Assay Diluent (BioLegend) for 1 hour. Mice sera were added in 1:25 serial dilutions for a 2-hour incubation. Goat Anti-Mouse IgG-HRP Conjugate (Millipore) (1:10,000 dilution) was then used as the detection antibody for 1-hour incubation. Plate was washed before and after each incubation step. Substrate was prepared from TMB Substrate Set (BioLegend) right before application. A 20-minute incubation in the dark took place before Stop Solution (BioLegend) was added. Plate was read by Synergy HTX Multi-Mode Reader (BioTek, Winooski, VT) at 450 nm with background absorbance at 570 nm subtracted. Titer was calculated as the highest dilution at which the test groups' absorbance is no longer statistically significant from the PBS negative control group that was run on each plate with the minimal 1:25 dilution.

Virus Neutralization (VN) Assay

The PRRS virus neutralizing activity of mice sera was evaluated by South Dakota State University's Animal Disease Research & Diagnostic Lab through a fluorescent focus assay (FFA) [26]. Briefly, heat inactivated mice sera at 1:2 serial dilutions with a starting dilution at 1:4 were mixed with 2,000 $TCID_{50}$/ mL of either parental PRRSV strain VR2385 or

heterologous strain NADC20 for a 1-hour incubation at 37 °C, before transferring to a 96-well plate with MARC-145 cells for a 24-hour incubation. Plate was fixed with 80% acetone. Fluorescein conjugated anti-PRRSV monoclonal antibody SDOW-17 (1:100 dilution) was used to detect infected cells. Virus neutralizing titer was reported as the highest dilution at which at least 90% reduction in the number of fluorescent foci was observed versus assay control.

In vitro Splenocyte Re-Stimulation

Mice splenocytes were re-stimulated *in vitro* by either PRRSV or VLP vaccines to characterize viral/antigen specific IFN-γ response. Spleen tissue from each mouse was stored in 10 mL RPMI 1640 media (Gibco, Waltham, MA) supplemented with 10% FBS (Gibco) and 1× Antibiotic-Antimycotic (Gibco) at 4 °C upon collection. It was then filtered through a 70 μm Falcon Cell Strainer (Corning). Red blood cells were lysed using 2 mL 1× eBioscience RBC Lysis Buffer (Invitrogen) after splenocytes were pelleted through an 8-minute 350 *g* centrifugation cycle at 4 °C. Lysis reaction was stopped after 5 minutes by adding 10 mL of the same media for spleen storage. After another centrifugation cycle with the same setting, cell pellet was resuspended in media to a cell concentration of 1×10^7/mL. 1.5×10^6 cells were plated in each well on a Falcon polystyrene round bottom 96-well microplate (Corning). Either 4.2 μg VLP vaccine corresponding to respective mice group or 1×10^5 $TCID_{50}$ VR2385 mixed with same amount of killed VR2385 was used as the stimulant for a 3-day *in vitro* cell stimulation at 37 °C with 5% CO_2. 2 μg Concanavalin A (ConA) was used in positive control wells. Cell culture supernatant was then collected to quantify IFN-γ level by using Mouse IFN-gamma Quantikine ELISA Kit (R&D Systems, Minneapolis, MN) according to manufacturer's instructions.

Statistical Analysis

Prism 6 (GraphPad, San Diego, CA) was used to perform all statistical analysis on obtained data. Multiple comparisons were used to determine statistically significant differences between individual groups after ANOVA with a significance level (α) of 0.05.

Results

Vaccine Production and Characterization

Gene sequences for all vaccines (Fig.1) were successfully integrated into *E. coli* cell lines through pET-28(+) plasmids. Sanger sequencing confirmed sequence integrity for all candidates. Vaccine expression was confirmed in terms of both molecular weight and identity through SDS PAGE and Western blot (Fig.2) respectively. Target molecular weight for each construct is 19 kDa (ΔHBcAg), 22 kDa (GP3-1), and 25 kDa (GP3-2, GP3-3, GP3-4). Product purity was above 98% for all candidates after chromatographic based purification process. Dynamic light scattering offered preliminary confirmation of VLP size of around 30 nm (data not shown) for all candidates. Particle assembly was then visualized through TEM (Fig.3). Due to the vaccines' recombinant nature from a bacterial host, lipopolysaccharides (LPS) level was confirmed to be below 1 EU/mL (data not shown) before administration to mice.

Epitope Specific IgG Response

All vaccines that carried the two gene shuffled GP3 epitopes gsGP31 and gsGP33 elicited strong and highly significant ($p \leq 0.0001$) epitope specific IgG response in mice after 3 injections when comparing with the empty HBcAg backbone (Fig.4). The only exception was GP3-3, which activated lower anti-gsGP31 response when comparing with rest of the

vaccine candidates, although still significant from ΔHBcAg ($p \leq 0.01$). There was a significant difference ($p \leq 0.05$) between GP3-2 and GP3-3 in anti-gsGP31 IgG titer.

When examining the kinetics of epitope specific IgG response, titer gradually increased through the course of vaccinations (Fig.5). Serum samples from two weeks after each vaccination were evaluated. Using GP3-4 as an example, it was able to elicit significantly higher (between $p \leq 0.0001$ and $p \leq 0.05$) epitope specific titers with the 1st and 2nd injections, but not with the 3rd injection. The mean of anti-gsGP31 for GP3-4 increased 97% after the 1st injection, 55% after the 2nd injection. The mean of anti-gsGP33 for GP3-4 increased 100% after the 1st injection, 75% after the 2nd injection. GP3-3 stimulated numerically lower anti-gsGP31 response through the course of vaccinations when comparing with the rest of the candidates, albeit the difference was statistically insignificant.

Virus Neutralization

Mouse serum was tested against two different type 2 PRRSV strains for its virus neutralizing (VN) ability. For the parental strain VR2385 (Fig.6A), which was the backbone strain that the two gene shuffled GP3 epitopes are based from, sera from mice vaccinated with GP3-4 generated significantly higher ($p \leq 0.01$) virus neutralizing response when comparing with either of the two negative control groups (ΔHBcAg and PBS) with a mean VN titer of 32. For the heterologous strain NADC20 (Fig.6B), all groups generated higher VN titer including the two negative control groups. Numerically, GP3-4 remained to be the group that generated the highest VN response with a mean titer of 128. However, due to the higher titer across all groups including the negative controls, VN titer for GP3-

4 was only significantly higher ($p \leq 0.01$) than ΔHBcAg, but not PBS. GP3-3 also generated a significantly higher ($p \leq 0.05$) response when comparing with ΔHBcAg.

Cellular Response Through IFN-γ Quantification

Antigen and viral specific cellular responses were characterized through the quantification of IFN-γ in cell culture supernatant from *in vitro* splenocytes re-stimulation. For antigen specific response, GP3-1 was able to elicit extremely high level of IFN-γ *in vitro* with a mean of 5,048 pg/mL by splenocytes from mice immunized with GP3-1 (Fig.7). The rest of the groups did not yield any detectable IFN-γ response. For viral re-stimulation with VR2385, none of the groups yielded conclusive results (data not shown).

Discussion

While subunit vaccines present an attractive alternative from live virus vaccines in terms of safety, concerns on long term efficacy and cross protection need to be addressed. The design of presenting PRRSV epitopes on HBcAg VLP takes advantage of the spatial organization of the capsid structure, which allows repetitive antigen display due to its polymeric nature and generation of epitope specific immune responses through either direct presentation or cross-presentation by antigen presenting cells (APCs) [13].

Our previous work has demonstrated strong epitope specific humoral response in mice against Porcine Epidemic Diarrhea Virus (PEDV) by using HBcAg VLP as a vaccine delivery vehicle [24, 25]. In this study, we have further demonstrated HBcAg's ability to elicit highly specific antibodies in vaccinated mice (Fig.4). By inserting the gene shuffled GP3 epitopes into the immunodominant region (MIR) of HBcAg, highly significant (between $p \leq 0.0001$ and $p \leq 0.01$) GP3 epitope specific IgG titers were detected in mice at

the end of the study when comparing with the negative control of empty HBcAg backbone. In terms of anti-gsGP31 IgG titer, GP3-3 provided us with some insights regarding the antigen presentation efficiency of HBcAg's MIR. The difference in design between GP3-3 and the rest of the candidates was the incorporation of both the GP5 T cell epitopes and the GP3 B cell epitopes in the MIR (Fig.1). By inserting 4 pieces of foreign peptides in the MIR, it could have altered the epitope presentation in the spike region of HBcAg, negatively impacting host recognition of the 3rd epitope immediately following GP5T2 since GP3-3 generated a significantly lower ($p \leq 0.05$) anti-gsGP31 response compared to GP3-2 (Fig. 4A). However, anti-gsGP33 IgG response was not affected by this design since GP3-3 generated equally significant ($p \leq 0.0001$) titer compared to the rest of the candidates (Fig. 4B).

By evaluating the kinetics of antigen specific humoral response throughout the course of the animal study (Fig. 5), it was discovered that epitope specific IgG titers increased significantly (between $p \leq 0.0001$ and $p \leq 0.05$) following the 1st and 2nd injection. However, for the 3rd and final injection, titers plateaued. While booster doses are essential for subunit vaccines to mount sustainable and long-term protection of the host against pathogens [27-29], this provides the potential to decrease immunization regimen for our vaccine from 3 doses to 2. However, this study did not include an arm that examines the effects of a 2-dose regimen, which could ease up both labor and financial burden for future vaccine implementation on pig farms. As with the Human Papillomavirus (HPV) VLP based subunit vaccine, fewer even single dose regimen has been an area of interest for further evaluation in terms of long term efficacy when comparing with the current 3-dose program [30].

With the aim to induce cross protection in host by our vaccine candidates, we assessed virus neutralizing ability of mice sera against both the parental PRRSV strain VR2385 and heterologous strain NADC20. GP3-4 generated the highest VN titer against both strains, and is most significant ($p \leq 0.01$) when comparing with the negative control groups (Fig.6). It shows the promising potential of GP3-4's ability to grant host with broad protection against PRRSV strains with its strategic design of GP3 B-cell epitopes in the MIR and GP5 T-cell epitopes in the C terminus of HBcAg. However, there is definitely room for improvement in terms of mounting a more significant VN response against the heterologous strain when compared to PBS control. It is also worth noting that multiple comparisons (MCs) were used to detect statistically significant differences between two groups instead of two sample t-test. MCs take into consideration the variability across experiment by pooling standard deviations (SDs) from all groups not just the two groups in comparison, at the same time, adjusting for individual test-wise significant level (α) so that the experiment-wise α does not balloon. MC is a much more stringent test here especially when the SDs for both GP3-4 and PBS groups were zero.

Interestingly, GP3-3 was able to generate a significant ($p \leq 0.05$) VN response against NADC20 compared to ΔHBcAg, while being the group with the lowest titer with a mean of 8 against VR2385 (Fig.6). Recalling that GP3-3 had the least significant ($p \leq 0.01$) anti-gsGP31 IgG but equally significant ($p \leq 0.0001$) anti-gsGP33 IgG (Fig.4), anti-gsGP33 could have played an important role when it comes to neutralization against NADC20. The epitope gsGP33 does have a proline residue at a. a. position 96 of GP3, which is identical to NADC20, while VR2385 instead has a serine residue at this position [19].

With the understanding of the role that viral specific cell mediated response plays during PRRSV infection [31], we incorporated 2 GP5 T cell epitopes into candidates GP3-2, GP3-3, and GP3-4, expecting differences in response due to the location of the T cell epitope on the HBcAg backbone and in turn their presentation to the host. We evaluated both viral and antigen specific IFN-γ response by re-stimulating mice splenocytes *in vitro*. While viral re-stimulation did not yield any conclusive results, candidate GP3-1 was able to re-stimulate an extremely high level of IFN-γ by the splenocytes from mice vaccinated with GP3-1 (Fig.7), despite the fact that GP3-1 does not contain GP5T1 and GP5T2. Although both of the epitopes included in GP3-1 cover peptide positions on GP3 that have been designated as B cell epitopes [32], the elicitation of strong IFN-γ casts new light on their ability to induce cell mediated response. The lack of IFN-γ response from the rest of the groups could be attributed to the differences in epitope presentation by the VLPs. GP3-2, GP3-3, GP3-4 could potentially presented the epitopes in structural ways which were unable to entice splenocytes from respective groups *in vitro*. Given the fact that IFN-γ is the major anti-viral cytokine secreted by T helper cells [33], we did not evaluate other pro-inflammatory cytokines during splenocytes re-stimulation. Since a strong VN response would require both B cell and T cell mediation [34], we have decided to select GP3-4, which induced the highest VN titer against both VR2385 and NADC20 (Fig.6), to be the most promising candidate for future evaluation in porcine study.

In summary, all our vaccine candidates were able to elicit significant epitope specific antibody response from mice, further validating the concept of using HBcAg as an effective vaccine delivery vehicle. GP3-4 was able to generate the highest and most significant VN response against both VR2385 and NADC20. With that being said, future

modifications can be explored in terms of incorporating additional PRRSV epitopes to broaden GP3-4's viral neutralizing ability against heterologous strains.

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Figures

Fig.1. Schematic diagrams of vaccine designs.

(A) Truncated HBcAg with poly-histidine (His 6×) tag attached to the N-terminus that enables purification by immobilized metal affinity chromatography (IMAC). (B-E) Vaccine designs with epitopes (gsGP31, gsGP33, GP5T1, GP5T2) inserted at various sites of HBcAg, namely the immunodominant region (MIR) between a. a. 78 and 79, the N-terminus, and the C-terminus.

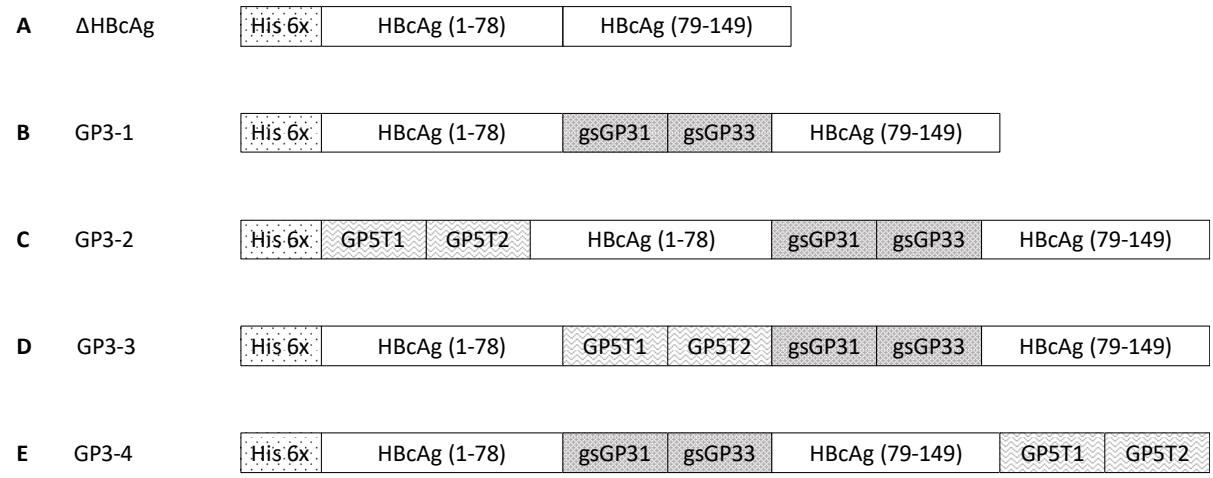


Fig.2. Protein characterization images.

(A) SDS PAGE of cell lysate samples. (B) SDS PAGE of purified samples. (C) Western blot on lysate samples using anti-His6× as the primary antibody for target protein detection. The lanes are in the order of (1) Protein standard with molecular weight annotated on the left in units of kDa, (2) ΔHBcAg, (3) GP3-1, (4) GP3-2, (5) GP3-3, (6) GP3-4.

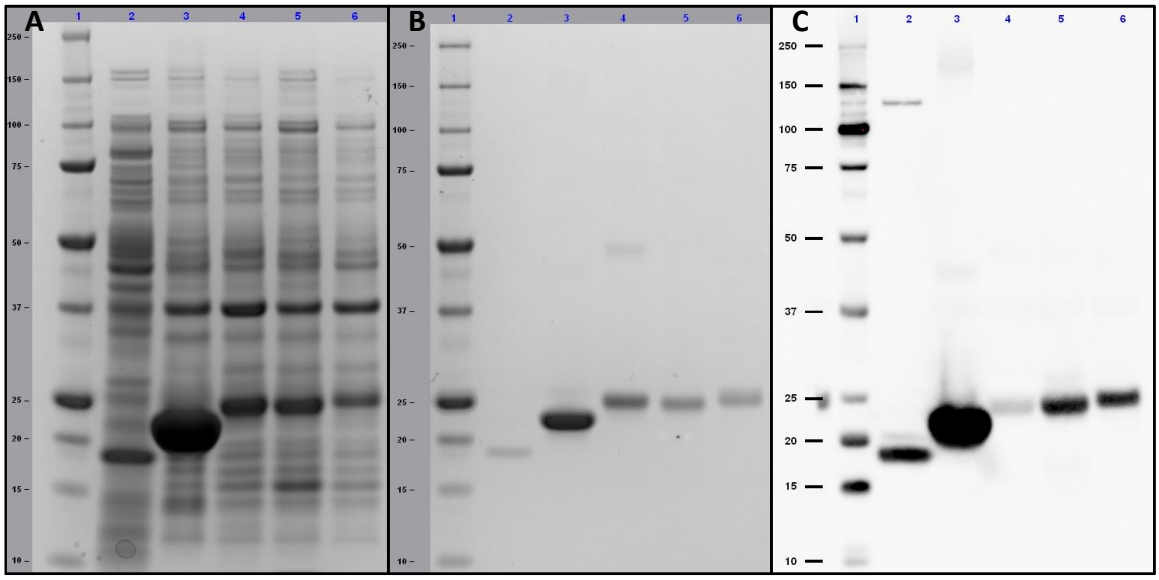


Fig.3. TEM images of purified vaccine samples.

(A) GP3-1. (B) GP3-2. (C) GP3-3. (D) GP3-4. Concentration for each candidate varies since this step precedes final formulation. Images were taken with a maximum magnification of 300,000×. Post imaging close-up is displayed on the upper left corner for each candidate.

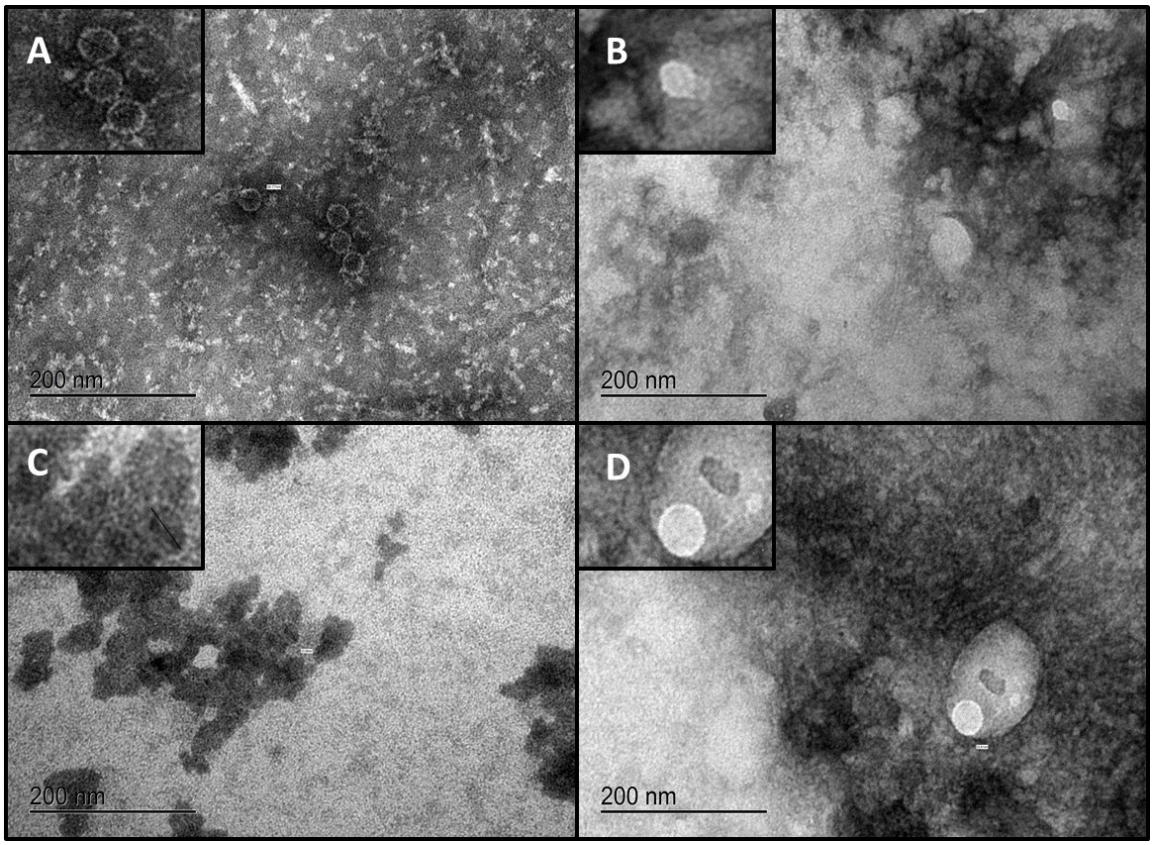


Fig.4. Epitope specific antibody titers.

Serum samples collected at the end (day 41 and 42) of the animal study were tested in ELISA plates which were coated with respective B-cell epitopes, (A) gsGP31 and (B) gsGP33. Titer was calculated as the highest dilution at which the test groups' absorbance is no longer statistically significant from the PBS negative control group. Each vaccine group is compared with ΔHBcAg group for statistical significance through Tukey's multiple comparisons post ANOVA. Error bars indicate ±SD for each group. *p* values are represented as **p*≤0.05, ***p*≤0.01, ****p*≤0.001, *****p*≤0.0001.

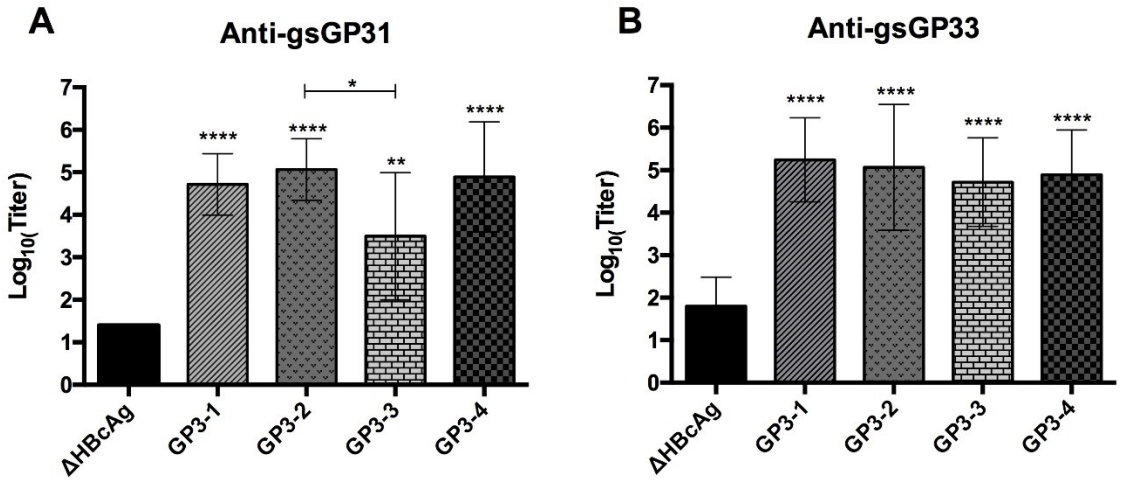


Fig.5. Kinetics of epitope specific antibody titers.

Serum samples collected throughout the course of the animal study were tested on ELISA plates which were coated with respective B-cell epitopes, (A) gsGP31 and (B) gsGP33. Titer was calculated as the highest dilution at which the test groups' absorbance is no longer statistically significant from the PBS negative control group at corresponding time point. Day -2 was before any vaccination. Day 12 was two weeks after the 1st injection. Day 26 was two weeks after the 2nd injection. Day 41&42 was two weeks after the 3rd and final injection. Each group of 8 mice were divided into two sub-groups of 4 mice for blood collection on either day 41 or day 42 so that all specimen could be collected and processed in a timely manner on each day. Statistical significance is tested using Tukey's multiple comparisons post ANOVA across different time points within same test group. GP3-4 is presented here as an example. Error bars indicate ±SD for each group. *p* values are represented as *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

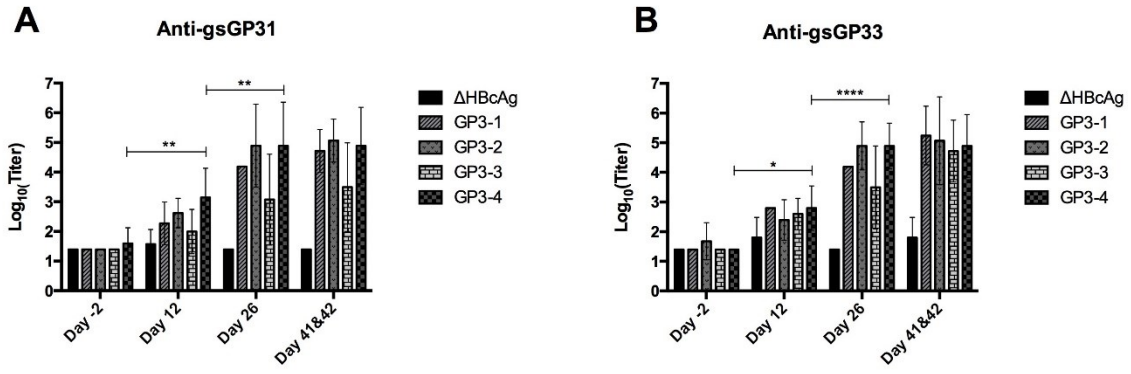


Fig.6. Virus neutralization (VN) titers against two type 2 PRRSV strains.

(A) parental strain VR2385, (B) heterologous strain NADC20. Serum samples collected at the end (day 41&42) of the animal study were tested. Titer was calculated as the highest dilution at which 90% or greater reduction in the number of fluorescent foci was observed versus assay control. Statistical significance is tested using Dunnett's multiple comparisons post ANOVA with either ΔHBcAg or PBS as the control group. Error bars indicate ±SD for each group. *p* values are represented as **p*≤0.05, ***p*≤0.01, ****p*≤0.001, *****p*≤0.0001.

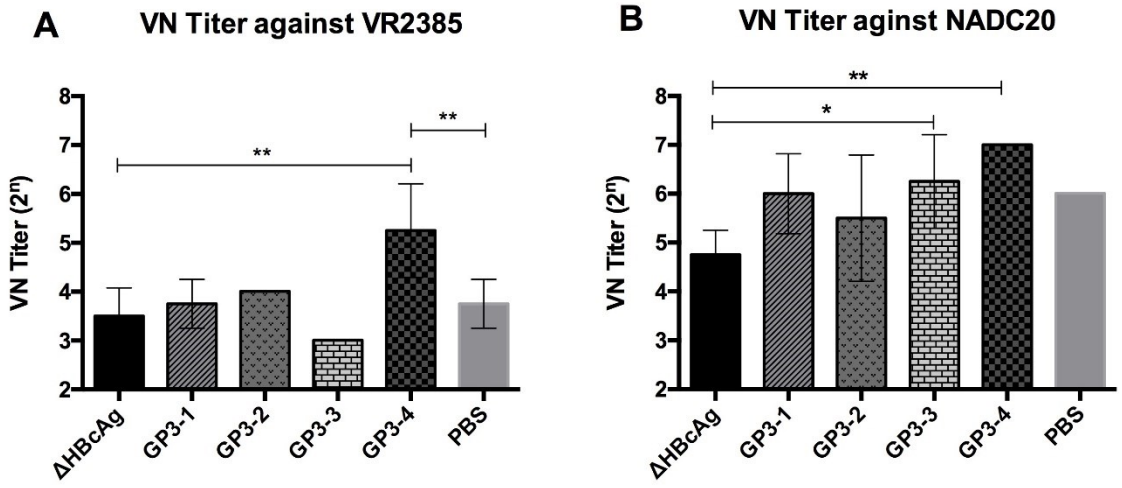
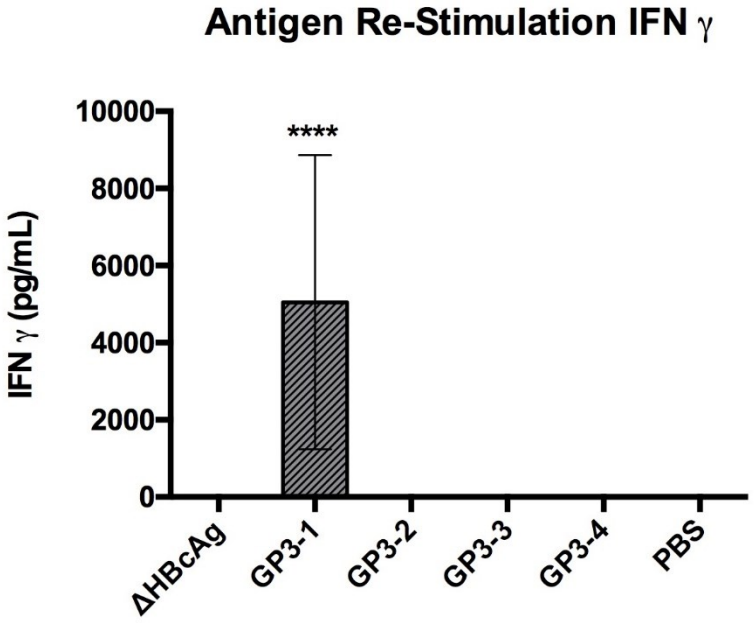


Fig.7. Antigen specific IFN-γ.

It was quantified for cell culture supernatant that was collected 72 hours post stimulation using ELISA. 1.5×10^6 mouse splenocytes were plated in each well on a round bottom 96-well plate. Groups GP3-1, GP3-2, GP3-3, and GP3-4 were all stimulated with their respective VLP vaccines. Groups ΔHBcAg and PBS were stimulated with GP3-3.



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Chapter 4: Virus-like particle vaccine with B-cell epitope from porcine epidemic diarrhea virus (PEDV) incorporated into hepatitis B virus core capsid provides clinical alleviation against PEDV in neonatal piglets through lactogenic immunity

Virus-like particle vaccine with B-cell epitope from porcine epidemic diarrhea virus (PEDV) incorporated into hepatitis B virus core capsid provides clinical alleviation against PEDV in neonatal piglets through lactogenic immunity

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Keywords

Virus-like particle (VLP); Hepatitis B virus core antigen (HBcAg); Vaccine; Porcine epidemic diarrhea virus (PEDV); Epitope; Viral Neutralization; Pigs.

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Abstract

Porcine epidemic diarrhea virus (PEDV) has had a negative economic impact on the global swine industry for decades since its first emergence in the 1970s in Europe. In 2013, PEDV emerged for the first time in the United States, causing immense economic losses to the swine industry. Efforts to protect U.S. swine herds from PEDV infection and limit PEDV transmission through vaccination had only limited success so far. Following our previous success in our virus-like particle (VLP) based vaccine in mouse model, in this study we determined the immunogenicity and protective efficacy of a VLP-based vaccine containing B-cell epitope ^{748}YSNIGVCK^{755} from the spike protein of PEDV incorporated into the hepatitis B virus core capsid, in a comprehensive pregnant gilt vaccination and piglet challenge model. The results showed that the vaccine was able to induce significant virus neutralization response in gilt milk, and provide alleviation of clinical signs for piglets experimentally infected with PEDV. Piglets from pregnant gilt that was vaccinated with the VLP vaccine had faster recovery from the clinical disease, less small intestinal lesions, and higher survival rate at 10 days post-challenge (DPC).

Introduction

Since its first emergence in Europe in the 1970s [1] and its introduction in 2013 into the United States [2], porcine epidemic diarrhea virus (PEDV) has shown to be highly transmittable with worldwide outbreaks and increasing genetic diversity [3], causing immense economic losses to the global swine industry [4]. The current available vaccines had only limited success against PEDV so far. Modified live-attenuated vaccines (MLVs) tend to have better efficacy, but long-term safety of MLVs remains a concern [5]. Killed

virus-based vaccines often offer only partial protection in neonatal piglets [6] with uncertainty in their cross-protection ability against heterologous strains [7].

In order to develop a safe, efficacious vaccine against PEDV, there is a renewed interest in the subunit vaccine approach [5]. Several studies have examined the possibility of using S1 domain from the PEDV spike protein as a potential vaccine, but none was able to provide neonatal piglets with a complete protection from clinical disease [8-10]. The spike protein of PEDV has been the target for vaccine development, because its large structural projections of 18-23 nm [3] is critical for viral attachment and entry during PEDV infection, and a major antigen for eliciting neutralizing antibodies in pigs [11-13].

Instead of using the complete domains of the PEDV spike protein as candidate vaccines, we took a unique approach by incorporating only the short previously-identified B-cell epitope ^{748}YSNIGVCK^{755} on the spike protein [14] into the hepatitis B virus capsid protein (HBcAg) recombinantly in order to form a virus-like particle (VLP). It is well known that VLPs are more potent vaccine candidates than subunit antigens, since VLPs resemble infectious viruses. HBcAg has been studied extensively for its application as chimeric VLP vaccines in combating infectious diseases [15-17], and more recently, cancer [18, 19]. Each HBcAg VLP is composed of 240 monomeric units when the assembled icosahedral capsid has a triangulation number of 4 [20]. By inserting two copies of the 8-amino acid epitope into the HBcAg backbone (Fig. 1A), we can present this PEDV epitope antigen 480 times on just a single VLP particle, thereby with the potential of eliciting potent humoral immune response against PEDV.

This chimeric VLP vaccine platform has been tested in mice through our previous work, where significantly high virus neutralization (VN) antibody titer was induced in

immunized mouse sera [21, 22]. Two a.a. substitutions with cysteine were implemented in the HBcAg backbone at D29 and R127 for this design (Fig. 1A), which had been proven to improve VLP particle stability [23] and vaccine efficacy [22] due to the formation of additional disulfide linkage.

The newborn piglets are most vulnerable to PEDV infection with almost 100% mortality rate in 1-3-day old piglets [3], because neonatal piglets would not have sufficient time to develop immunity against PEDV from direct vaccination, even if an effective vaccine were available. Therefore, our goal is to stimulate robust PEDV-specific viral neutralizing antibody response first in pregnant gilts, then through lactogenic immunity, passively transferred to neonatal piglets and protect them from subsequent PEDV infection. Chemokine ligand 28 (CCL28) was included as an additional adjuvant for one of the test groups (Table 1), considering its ability to enhance lymphocyte migration to the mammary gland [24, 25] in the hope of maximal antibody transfer.

In this piglet challenge study, we evaluated the efficacy of the VLP-based vaccine candidate, both in terms of its ability to stimulate systemic and lactogenic VN responses in pregnant gilts, and more importantly, in piglets against PEDV.

Methods

Plasmid and Cells

The DNA sequence of the PEDV spike protein B-cell epitope (5'-TACTCTAACATCGGTGTTTGCAAA-3') was synthesized by IDT (Coralville, IA), and cloned into the mutated HBcAg backbone including D29C and R127C substitutions on a pET-28a(+) plasmid (Novagen, Madison, WI) using an overlap extension PCR. Plasmid containing the vaccine design was then transformed into T7 Express Competent *E. coli*

(NEB, Ipswich, MA). Sanger sequencing was used to verify cell line integrity post transformation and before long term cryo storage at -80 °C.

Vaccine Antigen Expression

Cells were grown in 2.8 L shake flasks with 2×YT media and 30 µg/mL kanamycin at 37 °C, shaking at 200 rpm, after inoculation of 0.2% (v/v) overnight culture that was grown under the same condition. The culture was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) once the OD_{600} reached 0.6-0.8. The incubation temperature was then lowered to 28 °C for overnight protein expression. Cells were pelleted by centrifugation the next morning before storage at -20 °C for further processing.

Purification

Vaccine antigen purification followed a similar procedure as described earlier [22]. Briefly, inclusion bodies were solubilized using solubilization buffer (20 mM Na_2HPO_4, 50 mM NaCl, 2 M urea, 0.9% sarkosyl) after cell lysis. Solubilized protein was then loaded onto a column packed with diethylaminoethyl (DEAE) Sepharose Fast Flow anion exchange chromatography resin (GE Healthcare, Marlborough, MA), followed by an immobilized metal ion affinity chromatography (IMAC) packed with IMAC Sepharose 6 Fast Flow resin (GE Healthcare). $NiSO_4$ (200mM) was used to charge the IMAC column. IMAC eluate was buffer exchanged into 1× PBS with 0.9% sarkosyl at pH 7.4, followed by stepwise dialysis to gradually decrease sarkosyl concentration to 0% to aid VLP formation. Unassembled protein was eliminated using Amicon ultracentrifugal filter with 100 kDa MWCO (Millipore, Danvers, MA).

VLP Assembly

VLP formation was confirmed first with dynamic light scattering (DLS) using Zetasizer Nano (Malvern, Malvern, UK). Subsequently, transmission electron microscopy (TEM) was used to further characterize VLP particle assembly. Uranyl acetate (2%) was used to negatively stain the analyte before examination with JEM 1400 (JEOL, Peabody, MA) under 300,000× magnification.

SDS PAGE

Protein purity and target protein molecular weight (MW) were characterized using NuPAGE 4-12% Bis-Tris Protein Gel (Invitrogen, Carlsbad, CA). Samples were treated at 75 °C for 20 minutes before loading onto the gel. Loaded gel was run at constant voltage of 200 V for 35 minutes in 1× NuPAGE MES SDS Running Buffer (Invitrogen). Gel was then stained with SimplyBlue SafeStain (Invitrogen) for 1 hour after 3 times 5-minute water wash. De-stain took place overnight before imaging with ChemiDoc Imaging System (Bio-Rad, Hercules, CA). Image Lab (Bio-Rad) was used to confirm product purity and verify target protein's MW. Precision Plus Protein Standards (Bio-Rad) were used as the reference for MW.

Western Blot Analysis

Anti-6×-His tag antibody was used to probe the target vaccine protein in both cell lysate and purified vaccine samples using Western blot. Following the SDS PAGE procedure as described with Precision Plus Protein WesternC Standards (Bio-Rad) loaded as the reference, proteins from the gel were then blotted onto a 0.2 μm nitrocellulose membrane (Bio-Rad) using Trans-Blot Turbo Blotting System (Bio-Rad). The membrane was first washed with TBS and then blocked in the blocking solution (TBS-T with 0.05% Tween 20

and 5% nonfat milk) for 1 hour. After washing, the membrane was incubated in 6×-His Tag Monoclonal Antibody (Invitrogen) (1:3,000 dilution) for 1 hour, then in Goat Anti-Mouse IgG-HRP Conjugate (Millipore) (1:5,000 dilution) and Precision Protein StrepTactin-HRP Conjugate (Bio-Rad) (1:10,000 dilution) for 1 hour. Between and after each incubation, the membrane was washed with TBS-T three times, each for 5 minutes. Signal was then developed with Clarity Max Western ECL Substrate (Bio-Rad) and captured by ChemiDoc Imaging System (Bio-Rad).

Endotoxin Detection

Endotoxin level was determined to be below 1 EU/mL for the purified vaccine using Chromogenic Endotoxin Quant Kit (Thermo Scientific, Waltham, MA) following manufacturer's instructions.

Vaccine Formulation

Syringe filters with 30 mm diameter and 0.10 µm pore size (Celltreat, Pepperell, MA) were used for sterile filtration of each vaccine dose. Every individual dose was formulated in a 2 mL PBS mixture for intramuscular injection in pigs. The first 3 injections included squalene-based oil-in-water adjuvant AddaVax (InvivoGen, San Diego, CA) for all 3 groups, 200 µg of VLP for groups 2 and 3, 150 µg of recombinant CCL28 protein (R&D Systems, Minneapolis, MN) for group 3. The 4^{th} and final injection included 600 µg of VLP for groups 2 and 3, 50 µg CCL28 for group 3, and did not include AddaVax for all 3 groups. Formulation for each injection is detailed in Table 1 as well. All injections were prepared the night before administration and stored at 4 °C.

Challenge Virus

Vero (African green monkey kidney) cells cultured in DMEM (Gibco, Waltham, MA) supplemented with 10% FBS (Gibco) and 1× Antibiotic-Antimycotic (Gibco) were used to propagate the PEDV 2013 Colorado strain (commercially purchased from the National Veterinary Services Laboratories, Ames, IA). After virus inoculation, cells were cultured in MEM (Gibco) supplemented with 1× Antibiotic-Antimycotic (Gibco), 0.3% tryptose phosphate broth (Gibco), 0.02% yeast extract (Sigma Aldrich, St. Louis, MO), and 2 μg/mL trypsin (Gibco). After 4 days of incubation at 37 °C with 5% CO_2, the propagated viruses were collected through 3 cycles of freeze and thaw. Cell lysate along with culture supernatant was stored at -80 °C after centrifugation at 3,000×g for 10 minutes at 4 °C.

To determine the infectious titer of the propagated virus, 100 μL serially-diluted viral stock (from 10^{-1} to 10^{-5}) was added into each well on a 96-well plate containing confluent monolayers of Vero cells. Three days after incubating at 37 °C with 5% CO_2, cells were fixed with methanol. Immunofluorescence assay (IFA) was done with mouse anti- PEDV N IgG (Medgene Labs, Sioux Falls, SD) and anti-mouse IgG-Alexafluor 594 both at a dilution of 1:500. The primary and secondary antibody incubations were both done at 37 °C for 1 hour. Wells with fluorescent foci were considered positive. Infectious viral titer was calculated using the Reed-Muench method [26]. The virus stock was diluted to a titer of 10^5 $TCID_{50}$/2 mL for the piglet challenge study.

Experimental Design for Animal Study

All procedures pertaining to the animal study were approved by Virginia Tech Institutional Animal Care and Use Committee (IACUC protocol 19-092). Six White/Landrace cross genetic breed pregnant gilts were obtained from a farm that was negative for both PEDV

and PRRSV (porcine reproductive and respiratory syndrome virus). They were divided into 3 groups as described in Table 1 with 2 gilts in each group. All gilts received the first 3 intramuscular injections of vaccines which were 2 weeks apart starting at 6 weeks before farrowing. A final booster injection was administered 1 week before farrowing. Blood samples were collected via jugular venipuncture before each injection and one additional time at 3 days before farrowing. All gilts were induced to farrow on the same day. Colostrum samples were collected on the day of farrowing. Milk samples were collected on day 3 post-farrowing (DPF). Additional piglets were euthanized at 3 DPF so that each group had 10 piglets (5 piglets/gilt). The 30 remaining piglets were then challenged orally with PEDV 2013 Colorado stain at 4 DPF at a dose of 10^5 $TCID_{50}$/ piglet. Fecal swab materials were collected from each piglet daily for up to 5 days post-challenge (DPC). All animals were monitored daily for clinical signs (activity, body condition, and diarrhea) until 9 DPC. Clinical scores ranging from 1 to 3, with 1 being normal, and 3 being severely ill were recorded. If a score of 3 was reached for any of the three categories (activity, body condition, and diarrhea), then the animal was euthanized and necropsy was performed. All surviving piglets were euthanized for final necropsy at 10 DPC.

Virus Neutralization (VN) Assay

PEDV neutralizing activity in pig serum and colostrum/milk was evaluated by South Dakota State University's Animal Disease Research & Diagnostic Lab through a fluorescent focus neutralization assay (FFN) [27]. Briefly, for pig serum, heat inactivated samples were 1:2 serially-diluted with 1:20 as a starting dilution before mixing with PEDV 2013 Colorado strain at a concentration of 100 foci forming units/100 µL for a 1-hour incubation at 37 °C. Then the mixture was added to a plate containing confluent

monolayers of Vero cells for a 2-hour initial incubation followed by a 24-hour incubation at 37 °C with a wash step in between. The plate was then fixed with 80% acetone and stained with FITC-conjugated mAb SD6-29 to probe the infected cells. Virus neutralizing titer was reported as the highest dilution at which at least 90% reduction in the number of fluorescent foci was observed versus assay control. For pig colostrum/milk, 5 mg/mL rennet (Sigma Aldrich) was added to samples before a 30-minute incubation at 37 °C. Coagulant was sedimented by centrifugation at 2,000×g for 15 minutes at 4 °C. The resulting whey sample was then tested by the same procedure for pig serum described above.

Quantitative PCR

Piglet fecal swab materials were tested for PEDV RNA load up to 5 DPC by South Dakota State University's Animal Disease Research & Diagnostic Lab using a quantitative PCR (qPCR) assay [28]. Briefly, 7 µL of extracted RNA sample from each fecal swab was mixed with 18 µL assay master mix before the initial reverse transcription step (15 minutes at 48 °C followed by 2 minutes at 95 °C). Thirty-eight amplification cycles (5 seconds at 95 °C followed by 40 seconds at 60 °C) were then performed with PEDV positive control set at ≤ 38 cycles. Cycle threshold (Ct) for each sample was then reported.

Gross Pathology and Histology

Gross small intestine pathology and colon content were evaluated at the time of piglet necropsy. A score between 1 to 3 was assigned. For small intestine gross pathology, 1 is normal, 2 is either thin walled or gas-distended small intestine, while 3 is both thin walled and gas-distended small intestine. For colon fecal content, 1 is solid or pasty feces, 2 is

semi watery feces, while 3 is watery feces with no solid content. Small intestine tissue was also collected during necropsy and fixed in formalin for histological analysis. Hematoxylin & eosin (H&E) slides were subsequently prepared from fixed and sectioned tissues. Villous length (v) and crypt depth (c) were measured at 10 different sites on each jejunum slide section from each piglet by a board-certified veterinary pathologist who was blinded to different treatment groups. The average v to c ratio was calculated. A lower v to c ratio indicates more severe intestinal lesion while a higher v to c ratio shows better small intestinal health.

Statistical Analysis

Prism 6 (GraphPad, San Diego, CA) was used to perform all statistical analysis on data collected from this study. Tukey's multiple comparisons were used to determine statistically significant differences between individual groups after ANOVA with a significance level (α) of 0.05.

Results

VLP Vaccine Production and Characterization

The DNA sequence of the vaccine construct (Fig. 1A) was verified by Sanger sequencing. An expected protein of 21 kDa was revealed at the correct MW on both SDS PAGE (Fig. 1B) and Western blot (Fig. 1C). The product purity was above 93% for the purified vaccine. This included only the monomer band at 21 kDa. If the dimer band at 42 kDa had been included as well, the product purity would have been even higher. The VLP particle assembly was visualized by TEM (Fig. 1D). Before administration to animals, the endotoxin level for the purified vaccine was measured and found to be below 1 EU/mL.

Virus Neutralization

The viral naturalization (VN) titer from gilt sera remained below the detection limit (<1:20 dilution) 4 days after the 4th and final injection for all groups (data not shown). However, VN titer gradually increased post-farrowing in gilt colostrum and milk for the Vaccine group (Group 2). With a mean VN titer of 113 three days post farrowing, it was significantly higher ($p \leq 0.05$) than both the PBS control and Vaccine + CCL28 group (Fig. 2).

Clinical Evaluations

Once the piglets were challenged with PEDV, they were evaluated daily up to 9 DPC across 3 clinical sign categories (activity, body condition, and diarrhea) with a score from 1 to 3, 1 being normal, and 3 being severely ill (Fig. 3). For activity, the clinical sign started to develop for all 3 groups at 2 DPC. The Vaccine group (Group 2) had significantly lower ($p \leq 0.05$) score when compared to the PBS control at 8 DPC, indicating improved activity and alertness in the vaccinated animals (Fig. 3A). Clinical signs for body condition (spinous processes and hook bone visibility) gradually developed through the initial 5 DPC for all groups. The Vaccine group showed significant improvement ($p \leq 0.05$) at 8 DPC and 9 DPC when compared to the PBS control (Fig. 3B). Both the PBS control and the Vaccine group started to developed semi-watery diarrhea at 1 DPC while the Vaccine + CCL28 group had a slightly delayed clinical manifestation starting at 2 DPC ($p \leq 0.01$). Piglets in the Vaccine group started to improve in fecal health at 5 DPC, while the PBS control and the Vaccine +CCL28 group did not start the recovery process until 7 DPC (Fig. 3C). ANOVA and subsequent multiple comparisons were not performed for the overall 9-day diarrhea scores, with the exception for 1 DPC, since fecal scoring could not be carried out

due to the lack of defecation at the time of evaluation for various groups on different days. However, with continuous improvement in diarrhea scores starting at 5 DPC, the Vaccine group had numerically lower mean scores than both the PBS control and the Vaccine + CCL28 group starting at 6 DPC, indicating a faster recovery.

No statistical significance was detected across three groups for viral RNA loads at 1, 3, and 5 DPC due to the large intra-group variability (Fig. 4). At 1 DPC, the Vaccine + CCL28 group had a numerically higher mean Ct value, indicating a lower viral RNA level. At 3 DPC, the Vaccine group had a numerically higher mean Ct value than the other two groups.

The survival rate at 10 DPC for the PBS control was 30%, while both the Vaccine group and the Vaccine + CCL28 group had a survival rate of 60% (Fig. 5).

Gross Pathology and Histology

Although no statistical significance was detected among the 3 groups for either small intestine gross pathology or colon fecal content, the PBS control group exhibited numerically higher average score in both categories when compared to the other 2 test groups (Fig. 6).

For histological analysis, the Vaccine group had a significantly higher ($p \leq 0.05$) jejunum villous length to crypt depth ratio when compared to the PBS control (Fig. 7B), signifying a less severe small intestinal lesion for piglets in the Vaccine group. Different degrees of lesion were visualized in representative microscopy images of jejunum H&E slides from the 3 groups (Fig. 7A).

Discussion

Subunit vaccine has its appeal in terms of safety due to the lack of viral genetic materials, but, like all the other alternative vaccine approaches, the issue of vaccine efficacy is critically important and still needs to be addressed. Our unique approach in this study was to take advantage of HBcAg's polymeric viral capsid structure for repetitive PEDV-specific antigen presentation, in turn, eliciting robust immunogenicity in the host.

Our previous studies in mice during the development of this VLP-based vaccine demonstrated a strong virus neutralization (VN) ability *in vitro* by mouse sera [21, 22]. The high VN titer from potential candidates (mean VN titer around 370 two weeks after 3rd injection) provided a strong justification for this pig study.

When the initially planned 3-dose immunization regime of pregnant gilts did not produce detectable PEDV neutralizing antibody response in sera for the 2 test groups (Group 2 and Group 3), we then administered a 4th injection to all the gilts with higher amount of VLP vaccine (600 μg) for each gilt in both of the test groups 1 week before farrowing (Table 1). We removed AddaVax in the formulation for the 4th injection to eliminate any potential interference of the adjuvant on the VLP structural integrity. At 3 DPF, which was 10 days after the 4th injection, the Vaccine group (Group 2) had a mean VN titer of 113 in pig milk, which is significantly higher ($p \leq 0.05$) than both the PBS control and the Vaccine + CCL28 group (Fig. 2). The continuation of relatively flat VN response up till the day of farrowing (0 DPF), combing with the observation of steady decline of VN titer in sow milk post-farrowing in previous subunit PEDV vaccine study [10], led us to believe that the 4th injection with higher vaccine dose had an effect in boosting the VN response. Both live and killed virus-based vaccines had exhibited differences in VN

response between 20-week old pigs and 8-week old pigs when young pigs were used as a surrogate model in another PEDV vaccine study [6]. Dosage dependency was one of the most likely causes. The vaccine dose to body weight ratio difference between BALB/C mice and pregnant gilts could provide an explanation for the large differences in VN response when compared to our previous mouse studies, not ignoring species specific characteristics.

Our initial vaccine dosage design of 200 μg per injection was largely comparable with other subunit PEDV vaccine studies in sows [8-10]. However, considering there are 2 repeats of short spike protein epitope ^{748}YSNIGVCK^{755} on every HBcAg monomer (21 kD), we expected more efficient PEDV-specific antigen delivery with repetitive epitope presentation on every single VLP.

The fact that the VN response in milk peaked at 3 DPF provided useful insight on the timing of sow immunization regime, having the final booster dose close to farrowing could sustain a higher antibody titer in milk and last for longer to enhance lactogenic immunity in nursing piglets.

Th higher VN titer from the Vaccine group increased clinical protection of neonatal piglets from PEDV. In terms of clinical relief, piglets from the Vaccine group experienced faster recovery ($p \leq 0.05$) starting at 8 DPC in both activity and body condition when compared to the PBS control and the Vaccine + CCL28 group (Fig. 3A-B). Albeit the lack of statistical analysis for the overall 9-day diarrhea scores, the Vaccine group had more prompt clinical improvement in diarrhea starting at 5 DPC and better fecal health towards the end of the challenge study (Fig. 3C).

For viral shedding quantification, it was difficult to control the variability within each group largely due to both the sensitivity of the qPCR assay and inconsistency of the amount of fecal swab material collected on each individual animal. However, the numerically higher mean Ct values from the Vaccine + CCL28 group at 1 DPC and the Vaccine group at 3 DPC both provided corroboration for two statistically significant events (Fig. 4). First, the delayed clinically manifestation of diarrhea to 2 DPC for the Vaccine + CCL28 group (Fig. 3C) agrees with the lower viral shedding in piglet feces at 1 DPC. Second, the higher VN titer (Fig. 2) from the Vaccine group likely contributed to the lower viral RNA load at 3 DPC when all 3 groups developed some degree of diarrhea.

One of the major small intestinal tissue lesion markers is villous length to crypt depth ratio, with higher ratio associated with healthier tissue. This is largely due to the fact that villous epithelial cells in the small intestine are PEDV's main infection target [3, 29]. The higher VN titer from the Vaccine group likely was able to block PEDV's infection in the small intestine more effectively than the Vaccine + CCL28 group and the PBS control, as a result, significantly less severe ($p \leq 0.05$) villi atrophy in the jejunum (Fig. 7).

CCL28 is a critical chemokine that mediates maternal immunity in pregnant sows [30]. It attracts IgA plasma cells and facilitates their migration to the mammary gland during pregnancy and lactation [24, 25]. Our original intention by including CCL28 as an additional adjuvant in Group 3 was to maximize the availability of antibody secreting cells (ASCs) in the mammary gland, in turn, boosting lactogenic immunity against PEDV in nursing piglets. However, the Vaccine + CCL28 did not generate higher VN titer when compared to the PBS control on either 0 DPF or 3 DPF (Fig. 2). Interestingly, the Vaccine + CCL28 group had a delayed clinical manifestation of diarrhea ($p \leq 0.01$) when compared

to the PBS control (Fig. 3C), at the same time, numerically lower mean gross pathology scores in both categories when compared to the PBS control (Fig. 6). Moreover, it had the same 10 DPC survival rate of 60% as the Vaccine group, while the PBS control had a 10 DPC survival rate of 30% (Fig. 5). Aside from chemotaxis of ASCs, CCL28 also mediates proinflammatory responses through the mitogen-activated protein kinase (MAPK) signaling pathway [31]. Cell-mediated immune response could be a potential cause of the clinical relief observed from CCL28. However, cell-mediated immunity was not a focus for this study.

In summary, our VLP-based vaccine was able to stimulate significant PEDV neutralizing response in gilt milk, as a result, provided neonatal piglets clinical alleviation with improvement in morbidity, viral shedding, small intestinal lesion, and survival. This preliminary pig study paved the way for additional development in terms of optimal vaccine dosage design and adjuvant selection in the near future.

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Figures

Fig.1. Vaccine design and characterizations.

(A) Schematic diagrams of vaccine design. Mutated HBcAg includes D29C and R127C substitutions (mHBcAg) with poly-histidine (6×-His) tag attached to the N-terminus that enables purification by immobilized metal affinity chromatography (IMAC). B-cell epitope ^{748}YSNIGVCK^{755} from PEDV spike protein is inserted at both the mHBcAg immunodominant region (MIR) between a. a. 79 and 80, and the N-terminus of the His tag. (B) SDS PAGE of cell lysate sample (lane 2) and purified sample (lane 3). Protein standard is in lane 1. MW unit is kDa. (C) Western blot on lysate sample (lane 2) and purified sample (lane 3) using anti-6×-His as the primary antibody for target protein detection. Protein standard is in lane 1. MW unit is kDa. (D) TEM image of purified vaccine sample stained with 2% uranyl acetate. The image was taken with a maximum magnification of 300,000×. Red arrows point to the virus-like particles (VLPs).

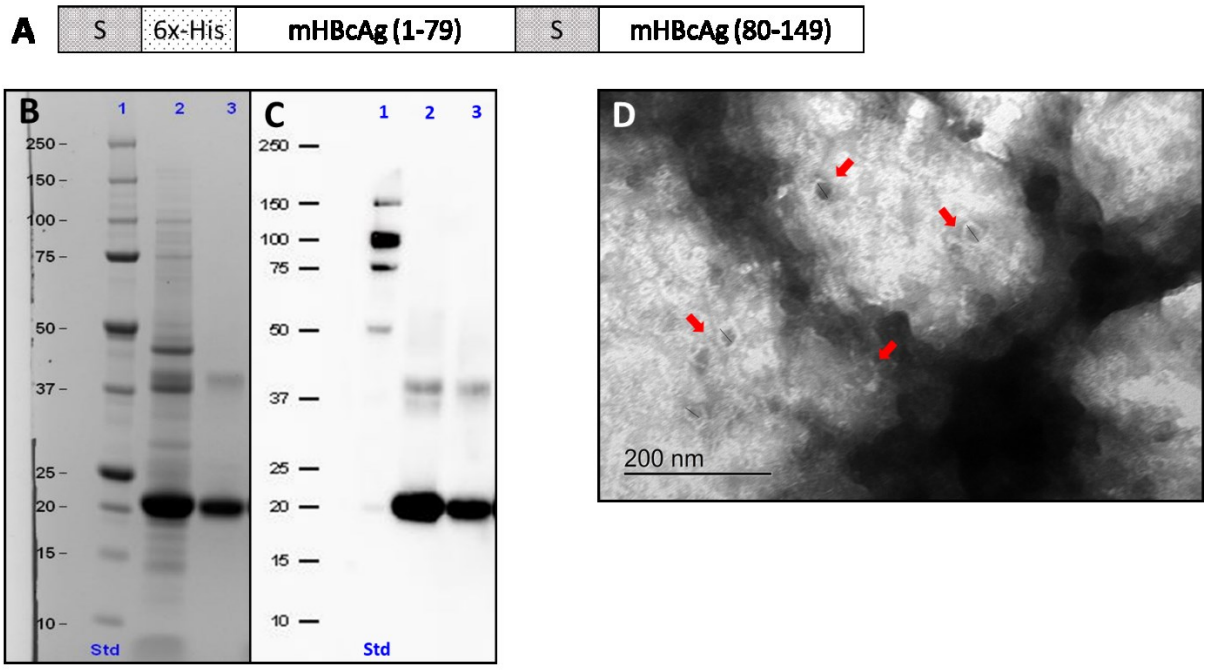


Fig.2. Virus neutralization (VN) antibody titers against PEDV 2013 Colorado strain. Colostrum samples collected on the day of farrowing (0 DPF) and milks samples collected 3 days post-farrowing (3 DPF) from all 6 gilts were tested. Antibody titer was calculated as the highest dilution at which 90% or greater reduction in the number of fluorescent foci was observed versus assay control. Statistical significance is tested using Tukey's multiple comparisons post ANOVA within each day. Error bars indicate ±SD for each group on each day. *p* values are represented as *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

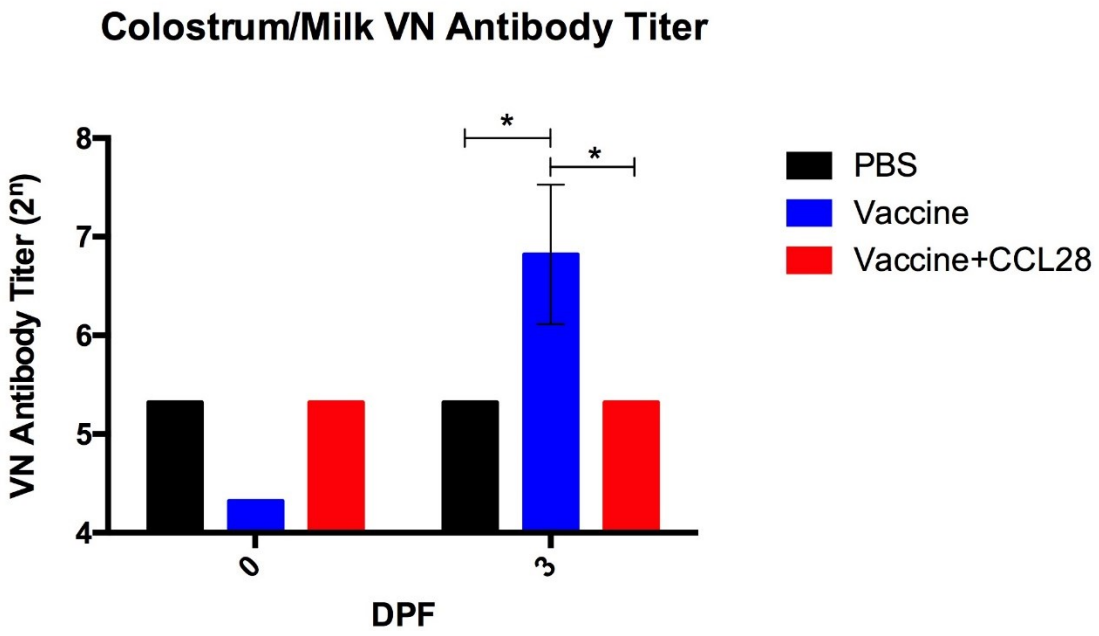


Fig.3. Clinical signs monitored up to 9 days post-challenge (DPC).

Each piglet was monitored daily for clinical signs in terms of activity, body condition, and diarrhea and given a score from 1 to 3. (A) Activity scores. 1, normal, bright, and alert. 2, dull, depressed, and lethargic. 3, recumbent, unresponsive. (B) Body condition scores. 1, undetectable spinous processes and hook bones. 2, spinous processes and hook bones were slightly felt. 3, spinous processes and hook bones were easily felt and visible. Statistical significance is tested using Tukey's multiple comparisons post ANOVA within each day. Error bars indicate ±SD for each group on each day. *p* values are represented as *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$. Statistical significance between Vaccine group (Group 2) and PBS control (Group 1) is represented on (A) and (B). (C) Diarrhea scores. 1, normal to pasty feces. 2, semi-liquid diarrhea with some solid content. 3, liquid diarrhea with no solid content. ANOVA and multiple comparisons were not performed for the overall 9-day diarrhea scores due to missing scores for different groups on different days since it could not be judged if piglet did not defecate at the time of scoring. ANOVA and multiple comparisons were carried out for 1 DPC diarrhea scores. Statistical significance between the Vaccine + CCL28 group and the PBS control is represented on (C).

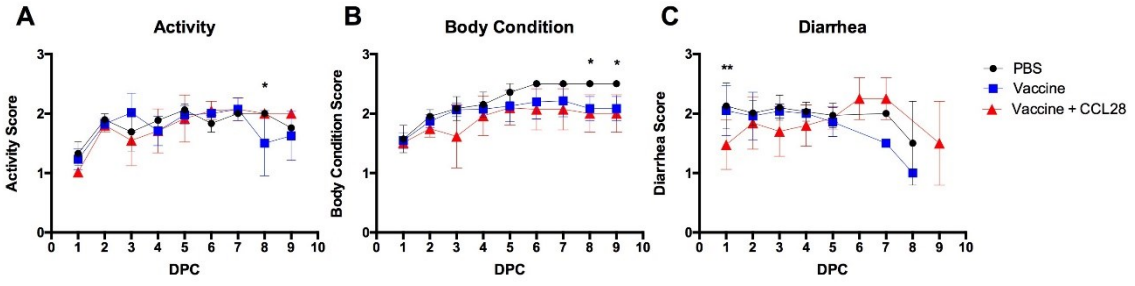


Fig.4. Viral RNA load in piglet feces.

Fecal swab materials collected from all surviving piglets from 1, 3, and 5 days post-challenge (DPC) were tested for PEDV viral RNA load through qPCR. RNA load is reported as cycle threshold (Ct). Statistical significance is tested using Tukey's multiple comparisons post ANOVA within each day. Error bars indicate ±SD for each group on each day.

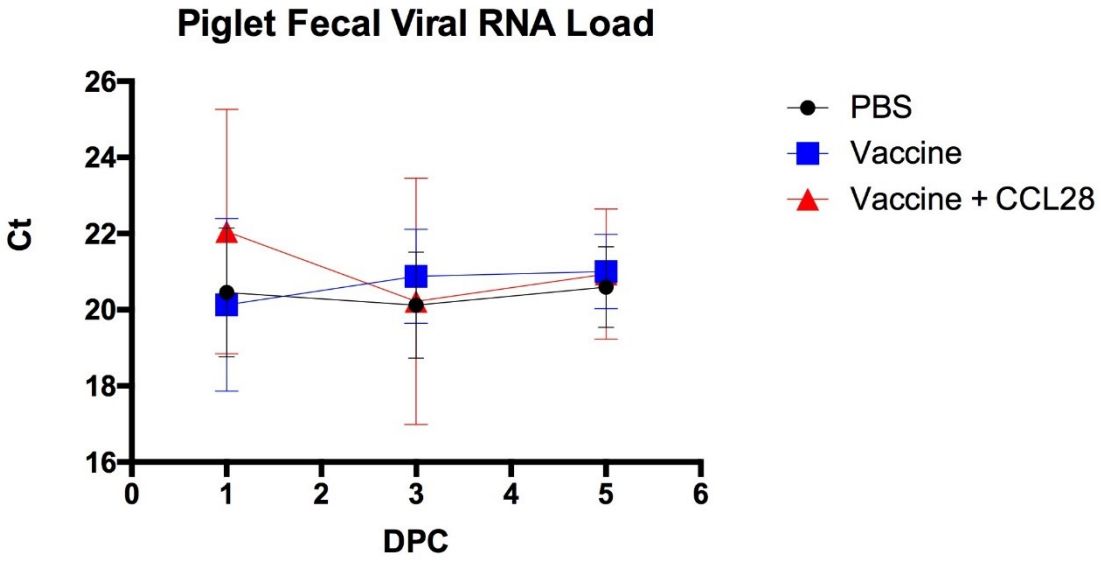


Fig.5. Piglet survival.

10 days post-challenge (DPC) was the end of the study and the day of final necropsy.

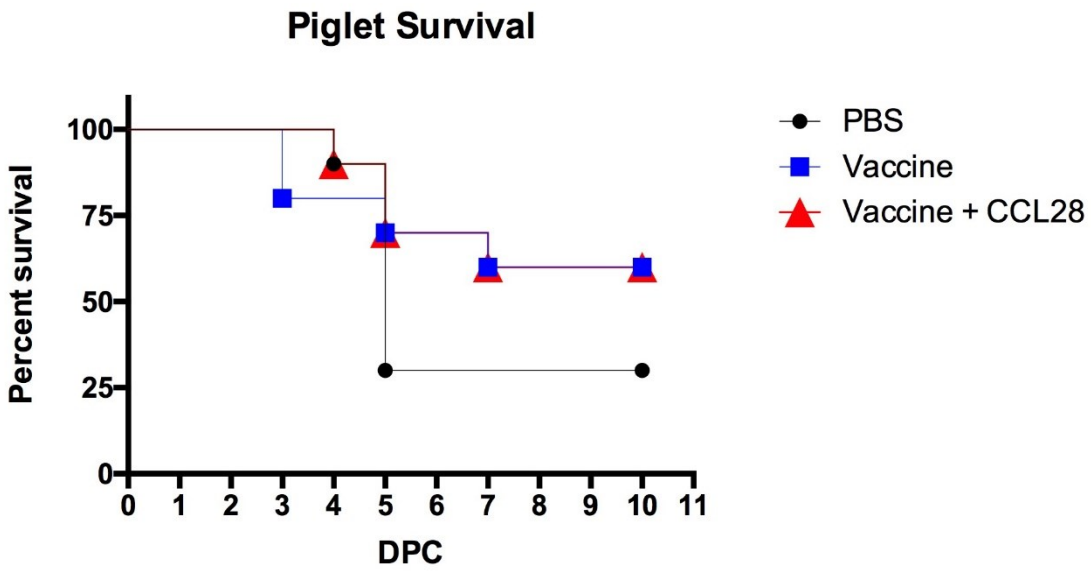


Fig.6. Gross pathology evaluation for piglets.

Small intestine gross pathology and colon fecal content were scored from 1 to 3 at the time of necropsy post either euthanasia or natural death. (A) Small intestine gross pathology scores. 1, normal. 2, either thin walled or gas-distended small intestine. 3, both thin walled and gas-distended small intestine. (B) Colon fecal content scores. 1, solid or pasty feces. 2, semi watery feces. 3, watery feces with no solid content. Statistical significance is tested using Tukey's multiple comparisons post ANOVA. Error bars indicate ±SD for each group.

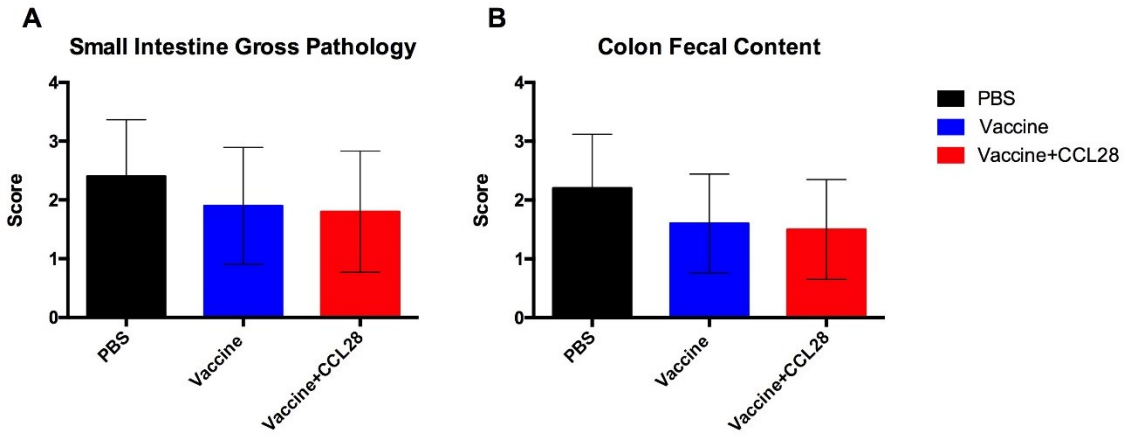
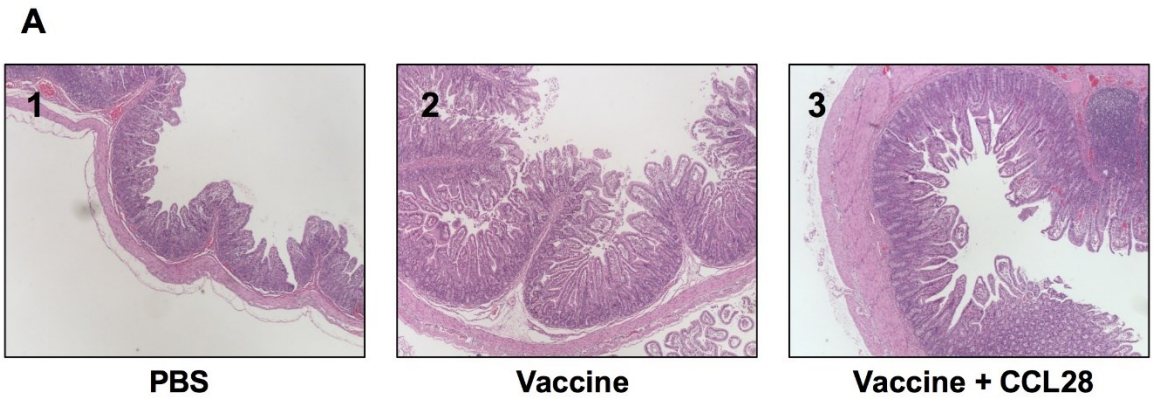
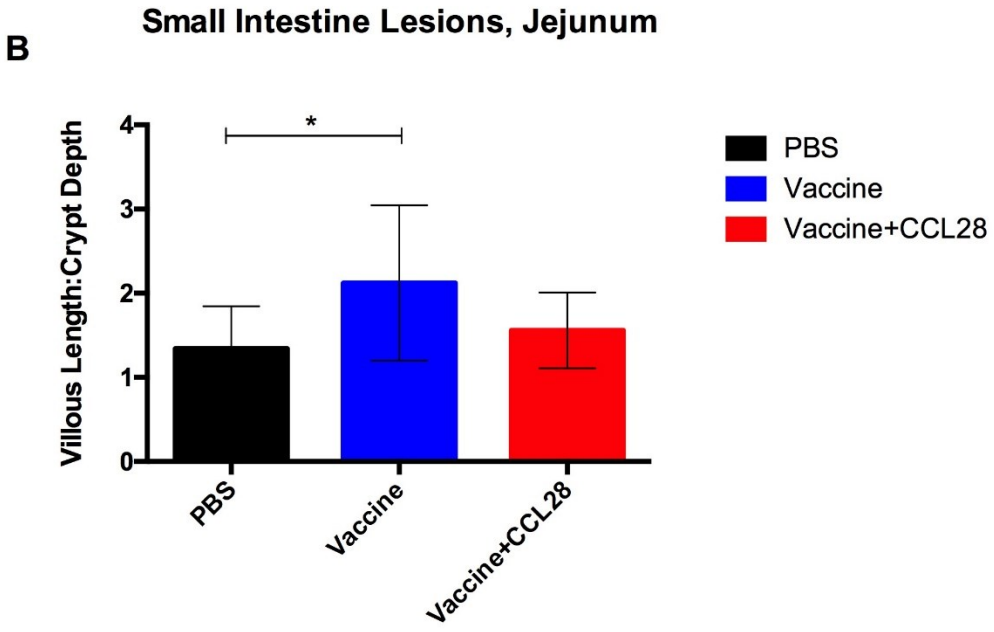


Fig.7. Small intestine lesions.

Jejunum from each piglet was fixed in formalin and hematoxylin & eosin (H&E) slide was subsequently prepared. (A) Microscopy images of one representative H&E slide of sectioned jejunum from each group. (B) Villous length (v) and crypt depth (c) was measured at 10 different sites on each jejunum slide section from each piglet, then the average v to c ratio was calculated. Statistical significance is tested using Tukey's multiple comparisons post ANOVA. Error bars indicate ±SD for each group. *p* values are represented as *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.





Table

Table 1 Immunization schedule for pregnant gilts

Group	Weeks prior to farrowing			
	6 weeks[a]	4 weeks[a]	2 weeks[a]	1 week[b]
1	PBS	PBS	PBS	PBS
2	Vaccine	Vaccine	Vaccine	Vaccine
3	Vaccine + CCL28	Vaccine + CCL28	Vaccine + CCL28	Vaccine + CCL28

[a] All injections in all 3 groups were formulated with 1 mL of AddaVax. 200 μg VLP vaccine was included in Group 2 and Group 3. 150 μg CCL28 was included in Group 3.

[b] All injections in all 3 groups were formulated without AddaVax. 600 μg VLP vaccine was included in Group 2 and Group 3. 50 μg CCL28 was included in Group 3.

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Chapter 5: Conclusions

Our VLP-based PRRSV vaccine once again proved its ability to elicit significant epitope-specific IgG response in mice, demonstrating the promise of HBcAg-based vaccine against PRRSV. Moreover, we were able to test its cross-neutralizing potential by testing immunized mice sera against two PRRSV strains with one being heterologous. While vaccine candidate GP3-4 was able to stimulate highly significant VN titer against homologous strain VR2385 when compared to both negative controls, its significance against heterologous strain NADC20 had room for improvement when compared to the PBS control. To improve the cross-protection of our vaccine candidate further, our potential next step is to incorporate other epitope antigens from additional PRRSV structural proteins into the HBcAg backbone, and then evaluate them in mouse model. Our ultimate goal is to assess the optimized VLP-based vaccine in pigs through a viral challenge study.

The kinetics of epitope-specific IgG response from our 3-dosage vaccine regime also revealed useful information to optimize a both effective and efficient immunization schedule which we can test in mice first, with subsequent implementation in pigs. This could be achieved by including a 2-dose vaccine arm in our next animal study.

Following our success of our VLP-based PEDV vaccine in mouse model, we were able assess its immunogenicity in pigs through a comprehensive pregnant gilt vaccination and piglet challenge model. The vaccine was able to stimulate significant VN titer in pig milk at 3 DPF, which resulted in clinical alleviation for piglets in terms of morbidity, viral shedding, small intestinal lesions, and 10 DPC survival rate.

Although significant, the VN antibody titer was not as high as what we were able to observe from mouse sera following a 3-dose vaccine regime. On the other hand, we were not able to stimulate systemic VN response from pig sera with the initially planned 3-dose administration schedule. By increasing dosage one week before farrowing, we were able to gain useful insight in terms of dosage optimization and injection scheduling for additional development of this vaccine in the future. Additionally, selecting an effective adjuvant will also be critical for a successful vaccine performance.