# Vaccine Development Against Porcine Epidemic Diarrhea Virus Utilizing the Hepatitis B Virus Core Antigen Protein

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## Abstract (Academic)

Porcine epidemic diarrhea Virus (PEDV) is the causative agent for porcine epidemic diarrhea, a disease manifesting itself clinically with watery diarrhea, depression, and death. The mortality rate of PEDV amongst suckling pigs can be as high as 100%, causing severe economic loss in the agriculture industry. The first reports of PEDV came from the mid 1970's, but its existence was later shown to have started around 1971. Since then, several modified live virus (MLV) and killed virus (KV) vaccines have been developed, but there is also a strong interest in the development of a recombinant alternative. In this report, several vaccine candidates were expressed in *E. coli*, purified, and tested *in vivo* for efficacy. The overall goal was to develop an effective, recombinant vaccine for PEDV that could be used in the swine industry.

In the first experiment, five vaccine candidates were expressed, purified, and tested for immune system activation *in vivo*. Four of these vaccines contained individual B-cell epitopes from PEDV located in the major immunodominant region (MIR) of the Hepatitis B virus core antigen (HBcAg) in an effort to elucidate the importance of these amino acid motifs. The fifth and final candidate contained all four of the epitope motifs in an effort to make a multivalent recombinant vaccine. Each candidate was able to elicit an immune

response to the inserted antigen(s), but only two were able to provide virus neutralization.

With this information, the vaccines were redesigned in an attempt to improve the immune response to the vaccines and allow for a more efficient presentation of inserted epitopes to the immune cells. During the second attempt, an interaction in the proteins caused the elimination of the immune response to one of the known B-cell epitopes, YSNIGVCK, which subsequently eliminated the virus neutralization capabilities of the vaccines. A third experiment was therefore completed, with two new vaccines designed to emphasize the previously eliminated epitope. As hypothesized, the generation of antibodies to the YSNIGVCK during the third experiment correlated with an improved ability to neutralize PEDV *in vitro*.

Through this process, several design aspects of the HBcAg protein have been revealed, allowing increased efficiency on a per dosage basis. Furthermore, it was demonstrated that there is a hierarchy amongst the B-cell epitopes from PEDV regarding virus neutralization. Finally, mutations to the HBcAg backbone protein can help increase the immune response to the inserted antigen targets.

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## Abstract (General Audience)

Porcine epidemic diarrhea Virus (PEDV) is a virus effecting swine. It is the cause of disease that manifests with symptoms ranging from depression, to severe dehydration and death. Young piglets are particularly susceptible to the virus, which can reach mortality rates of 100%. Presence of the virus on a swine farm can therefore cause severe economic losses. Treatments currently exist for PEDV, but are mostly generated from the virus itself. There has recently been renewed interest in a vaccine that is made from a different source, which might help eliminate some of the side effects of those that currently exist on the market.

This project outlines three experiments performed in animals. During the first experiment, a structural protein from the Hepatitis B virus was genetically altered to include important structural portions of PEDV. This new protein is generated in *E. coli* and purified. After purification, the protein assembles into a virus-like particle (VLP). VLPs are structural proteins of existing viruses that are expressed and assembled to mimic the virus. By doing so, the immune system recognizes the protein as a potential threat, and launches a response in the form of antibodies. Manipulations of the VLPs as describe herein allow the new vaccine to generate antibodies toward other diseases such as PEDV. Although all five of the vaccines used in the first experiment were able to

generate appropriate antibodies, only two of them were effective at preventing PEDV from entering susceptible cells (virus neutralization).

A second experiment, with three newly designed vaccines was therefore performed. This experiment, like the first, was successful in producing antibodies to several of the included PEDV protein sections, but none were able to neutralize the virus. These results led to a third experiment, during which further design improvements were made to the basic vaccine structure in an attempt to increase the neutralization capabilities of the vaccines. The results from the third experiment indicated that several changes to the vaccine increased the immune response to the structural portions of PEDV, providing a better overall vaccine candidate. This also led to the conclusion that one specific sequence from PEDV has a better ability to neutralize the virus than the other sections.

# Dedication

This whole thing is dedicated to Anne Elizabeth Gillam.

Carter Gillam and Dorothy Gillam did not help in any way, but I'm very much glad they were here for the process.

I suppose my parents, Scott Gillam and Mary Gillam, had something to do with me being here as well.

What can I say about my brothers, Ingram Gillam and Tom Gillam?

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## CHAPTER I

# Chapter I: Introduction

Porcine Epidemic Diarrhea Virus (PEDV) is a member of the *Coronaviridae* (CV) family in the *Nidovirales* order and was first reported in 1977 [1]. This initial report detailed outbreaks of diarrhea on four separate farms in Belgium, leading to an initial diagnosis of transmissible gastroenteritis virus (TGEV). Transmission electron microscopy images showed viral particles in stool samples of infected pigs that were morphologically similar to previously identified porcine coronaviruses TGEV and hemagglutinating encephalomyelitis virus (HEV). A direct immunofluorescence test routinely done on pigs infected with these two other CVs delivered negative results, however, leading to the conclusion that there may be a new diarrheal coronavirus [2, 3]. This marks the first use of the phrase “Porcine Epidemic Diarrhea” as a descriptor for the new virus. These viral particles were isolated *in vitro* and designated as CV777, the parent strain of PEDV. These newly generated samples were then able to induce the symptoms of the disease in orally inoculated pigs, indicating successful isolation of the virus [2]. Biological and physiochemical properties of a German strain of the new virus further implicated PEDV as existing within the CV family [4]. Eventually, sequencing of the PEDV genome identified several open reading

frames (ORFs) of similarity between it and other CVs including TGEV, helping officially designate PEDV as an alphacoronavirus (formerly Group 1 CV) [5, 6].

Coronaviruses are pleomorphic, but have a size range typically between 60 – 220 nm, with projections on the surface of about 20 nm [7, 8]. They are enveloped, with a genome consisting of single stranded, positive sense, polyadenylated RNA. The sequencing of six coronavirus species indicates a genomic size range between approximately 27,000 nt to approximately 31,000 nt, placing them at the largest for RNA viruses [9]. There are four genera of *Coronavirinae* including *Alphacoronavirus*, *Betacoronavirus*, *Deltacoronavirus*, and *Gammacoronavirus*. These genera were previously organized into numbered groups, but their classification has changed recently.

The genome for PEDV is approximately 28 kb in size, and contains a 5' untranslated region (UTR), a 3' UTR and a number of open reading frames (ORFs) [9]. This includes a large ORF 1, which consists of a majority of the genome. ORF 1 is separated into ORFs 1a and 1b, which encode non-structural proteins used to enhance replication [10]. 3' downstream from 1a and 1b are the ORFs for the structural proteins in the following order: spike (S) protein, envelope (E) protein, membrane (M) glycoprotein, ORF 3, and nucleocapsid (N) protein. ORF 3 has been predicted to be an ion channel protein important for viral reproduction, while the other proteins are structural [11]. The S proteins in most

coronaviruses undergo posttranslational cleavage into an S1 and an S2 portion, a phenomenon that does not occur in PEDV due to a lack of cleavage site [12]. However, there is a conventional split of the S protein into two domains based on the cleavage site of similar CVs. This split occurs around the $785^{th}$ nucleotide, and divides the S protein into the S1 domain and the S2 domain.

The recent PEDV panzootic occurred during 2010 – 2013, and was associated with mutations in the ORF 3, E, and M proteins of the genome in some of the new strains [13-15]. It is thought that these mutations have led to an increase in virulence and thus a very serious outbreak. The mortality rate and symptoms of PEDV tends to be mild in adult pigs. However mortality and symptoms are highly dependent on the age of the animal upon infection. The infection of suckling piglets less than two weeks old is associated with a high rate of extreme clinical signs and mortality increases dramatically in neonates up to 100% [16-19]. The cause of high mortality rates in newborns is dehydration and the loss of already underdeveloped absorptive cells in the small intestine [20]. Several vaccinations are currently on the market for PEDV, and are for the most part based on the modified live virus (MLV) and killed virus (KV) formats. Though they are effective preventative treatments, their use on farms can cause severe side effects and their use does not necessarily protect the most susceptible suckling piglets [21, 22]. The focus of this research is therefore to design,

express, purify and test a recombinant alternative to current vaccination strategies for PEDV.

This dissertation breaks down a vaccine development process performed for PEDV using the HBcAg backbone protein. Chapter II introduces the PEDV virus, and reviews some of the complications involved in vaccination toward PEDV. Chapter III details an animal trial during which five vaccine candidates were tested in two separate application techniques for immune response in mice. Chapter IV outlines improvements made and tested in the vaccine design, including evidence that there is a hierarchy in epitope sequence significance for virus neutralization. Chapter V outlines the prospective details of a pig trial to test the final design of the PEDV vaccine in a neonatal challenge experiment. Finally, Chapter VI will outline the overall conclusions reached with the data presented within.

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# Chapter II: Literature Review

## History of PEDV

### *Outbreaks and Research of PEDV*

Although first reported in 1977, Porcine Epidemic Diarrhea Virus (PEDV) was retroactively identified as starting in the UK, Belgium, and China in the early 1970's [1-4]. For several years after the initial article describing what was then called the "coronavirus-like agent (CVLA)" was published, records of PEDV outbreaks were reported sparsely due to the lack of an efficient diagnostic tool [5]. Diagnoses at the time relied wholly on morphological similarities of PEDV to both Transmissible Gastroenteritis Virus (TGEV) and Hemagglutinating Encephalomyelitis Virus (HEV), requiring there to be a lack of immunohistological activation by well-established diagnostic assays for either of the well-known TGEV or HEV [2]. The epidemiology of PEDV has been reviewed several times recently, but will be briefly covered for geographical and historical context with research and vaccine development [6-8].

Incidents of the virus continued to spread throughout Europe and China during the 1980's, though the outbreaks are reported infrequently. The disease had made it more Eastern in Europe to Hungary and Germany by 1981 [9, 10]. It was first reported in Canada in an article published in 1980 and again in 1985, indicating that the virus had crossed the Atlantic Ocean [11, 12]. However many

other citations have indicated that the disease did not reach North America until much later, indicating that these original diagnoses may have been in error. The first articles on PEDV outbreaks in Japan were published from 1982 to 1985, again indicating that the virus had spread over seas [13, 14]. Though not initially reported, the first outbreak in South Korea occurred in 1987 [15]. For the rest of the decade, reports of outbreaks seemed isolated and scarce [16, 17]. Throughout this period, PEDV research tended to focus more on diagnosis and culturing of the virus [3, 5, 9, 10, 18, 19]. No publications were found to indicate that a vaccine had been developed during this decade.

In the years 1992 - 1993, the disease appeared again in countries previously hit with outbreaks including England, Spain, South Korea, China and Japan [16, 17, 20-22]. Meanwhile, two teams were able to resolve the sequence of multiple reading frames from the British and Belgian isolates (Br1/87 and CV777 respectively) in 1994 [23]. This allowed phylogenetic comparisons to be made, and presented more solid evidence that PEDV was closely related to TGEV and Human Coronavirus (HCV 299E) based on the sequence of the proteins and genomic RNA [23, 24]. Genetic variation between the two PEDV strains was also seen, indicating mutation of the virus as outbreaks occurred. Sporadic outbreaks continued, but were largely of little concern for the rest of the 1990’s as they were fairly localized enzootics without significant loss to industry as a whole [25, 26]. PEDV research throughout this decade again largely

focused on identification and diagnosis of the disease [23, 27-30]. However, research into vaccination strategies for TGEV and other porcine coronaviruses were very active throughout [31-36]. Vaccination against PEDV using attenuated, high passage strains of virus isolates CV777, Strain S, P-5V and KPEDV-9 were tested from 1993 through the end of the decade [37-40]. At this point in history, all vaccine candidates tested were attenuated strains or modified live virus vaccines (MLVs).

Outbreaks during the first decade of the 2000s were similar. The geographic locations previously prone to outbreaks continued to experience enzootic conditions with few notable differences. However, during this decade there was a large shift in research from merely reporting of disease outbreaks to reports of vaccinations in an attempt at preventing the disease. The full genome of PEDV was solved in 2001, and epidemiology of the disease began to improve [41]. Retroactive genetic analysis of small outbreaks in Korea and China from this decade initiated a process of grouping PEDV strains to further clarify strain variability by classification into the G1 and G2 genotypes [42-44]. There were genetic mutations in the ORF 3, E, and M protein regions compared to the original and attenuated strains. In particular, the case was made that the ORF 3 mutations may increase the virulence of the new groups of PEDV strains. Meanwhile, several neutralizing epitopes were discovered in the spike protein allowing for the creation of possible subunit vaccines and a new attenuated

strain, DR13 was produced and tested *in vivo* for oral administration [45-48]. The total overall academic interest in the virus retained the awareness of the disease, but without much urgency. Reports of novel strategies of viral prevention included the inoculation of cows to provide lactogenic immunity, administration of exogenous Ig from chicken egg yolk, and recombinant expression of single chain variable fragments corresponding to epitopes of PEDV expressed on the surface of *Lactobacillus* [49-51]. Interest in vaccinations using recombinant subunit strategies began during this decade as well, with a focus on the S-protein [52].

A new epizootic outbreak of PEDV demonstrating the phylogenetic differences from previous domestic and foreign strains occurred in China beginning in 2010 [53]. Although it was after the fact, these differences included those outlined by genetic analysis of PEDV outbreaks through the 2000 – 2010 decade. The virus reached the United States in the Spring of 2013 and quickly spread across borders to Mexico and Canada [54, 55]. Similar variants were found in Korea, though they could have originated from there in the previous decade [56]. There was a strongly renewed interest in vaccination against PEDV after this outbreak began. In the time between the most recent outbreak began in 2010 and the time of this writing, there have been an equal number of publications citing vaccine candidates as there were in the three decades prior. These publications include MLV, recombinant subunit, and nucleotide based vaccines [57-65]. There also seems to be interest in using virus like particles

(VLPs) in a recombinant strategy for PEDV vaccination [66]. Several of these new vaccines are or have been commercially available, though to varying degrees of efficacy and with possible side effects including viral shedding [67, 68].

## *Phylogeny of PEDV*

Phylogenetic analysis of PEDV has been discussed at length in several previous publications [6-8, 69-72]. In this dissertation, phylogeny will be discussed as a basis for choosing peptide sequences from PEDV for use in recombinant vaccination strategies. A phylogenetic tree was created (**Fig. 1**) based on the genetic sequence of the S protein for PEDV. The tree was created by MEGA 7.0 software using the distance-based neighbor-joining method. Strains were chosen to include the widest geographical base as well as popular MLV strains used in previous vaccine candidates. The strains have been separated into the G1 and G2 genotypes, with further subdivisions of 1a, 1b, 2a, and 2b based on spike protein sequence [73, 74]. The majority of the strains discovered in the USA during the 2010 panzootic are classified as G2 based on this phylogenetic tree.

Figure 1 also shows four short, linear B-Cell epitope sequences from PEDV in the context of a phylogenetic tree. The first conserved sequence shown is YSNIGVCK. Two other conserved sequences, GPRLQPY and WAFYVR,

located 3' downstream of YSNIGVCK in the S protein and within the M protein respectively are shown as well. Conservation of these three motifs is sustained across the G1 and the G2 genotypes, and across all of the strains selected. This includes the original strains such as CV777 and DR13, the INDEL strains, as well as the more virulent strains such as CO13. A different B-Cell epitope, that designated as (X)Q(X)GVKI in Figure 1, is variable amongst strains included within this phylogenetic tree where "(X)" denotes a residue in the sequence that is not conserved. The majority of the G2 genotype strains contain the sequence SQSGVKI, but there are strains related more closely to the G1 genotype that have variable sequences in this motif. There is not much of a pattern throughout the G1 genotype phylogeny for this particular motif.

## Transmission and Infection of PEDV

### *Transmission of PEDV*

Transmission of PEDV primarily occurs through the fecal-oral route. Mice have been eliminated as a vector for PEDV; and although bats have been implicated as possible carriers, it has not been demonstrated experimentally [75, 76]. There are no known other carriers or vectors of the virus to implicate in the spreading of the disease. Horizontal infection by oral exposure to similar aged older pigs occurs at days 14 – 16 post inoculation [77]. PEDV can survive in manure for up to 9 months, with evidence that the virus is able to replicate further

within manure storage facilities and outside of the host [78]. It was recently concluded that as little as 1 g of infected piglet feces could contaminate 500 tons of dry feed to an extent where it would cause further transmission [79].

The largest method of transmission for PEDV is the fecal oral route, including possible transmission involving feed. One such instance was thought to be spray dried porcine plasma, a common additive in feed for weaning piglets, which came under attack during the most recent PEDV outbreak [55]. Follow-up studies, performed after this initial finding, have shown that PEDV is unable to survive the spray-drying process [80-82]. However PEDV can survive in some dry feed ingredients including meat and bone meal, even maintaining infectious titers for up to 30 days [83]. Additionally, evidence was found of viral shedding in sow's milk, which has also been seen in porcine circovirus type 2 [53, 84].

Air samples collected from a facility housing infected pigs during an acute outbreak of PEDV in the US tested positive for genomic material from PEDV [85]. These air samples were then collected and used to inoculate seronegative pigs successfully for PEDV, indicating that airborne spreading of the disease may be possible. The genomic material found from the outbreak was found as far as ten miles away from the facility in which the outbreak occurred. Though this material was not used for a subsequent inoculation, it may be possible for long distance travel of infective material to occur.

Incubation time for PEDV varies with age and strain where the original strain, CV777, can take up to 8 days before viral shedding is detected [86]. The age of the pig at infection should therefore be of great concern in any vaccination strategy, as suckling piglets have been the primary source of the economic loss from outbreaks [87]. Newborn piglets (<7 days) develop symptoms to PEDV including fecal shedding within 24 hours of infection and die at a high rate within 3 – 4 days [88]. Fecal shedding of PEDV typically begins in weaned pigs (> 2 weeks old) 24 – 48 hours later [89-91]. An isolated strain of PEDV was previously used to orally inoculate multiple cohorts of pigs at varying ages [92]. All piglets 7 days old and younger developed symptoms within one day and died within four days of inoculation. Three out of five of the 2-week-old piglets developed severe diarrhea within four days of inoculation, but recovered. Several 4-week-old piglets developed mild symptoms. All 8- and 12-week-old piglets showed no symptoms at all. It has been hypothesized that this phenomenon is due to a lack of activity in natural killer (NK) cells and IFN-γ in the intestines of the suckling pigs, indicating an immature innate immune system and an inability to fend off viral infection [91]. Another hypothesis proposed was that a lower rate of proliferation of cells in the intestinal crypts of the suckling pigs causes a decreased turnover in enterocytes, allowing earlier detection of viral infection through symptomatic displays [90].

### *Pathogenesis*

Upon inoculation of pigs via the oral or nasal route, the virions travel to the stomach where they survive in low pH conditions and are able to remain infectious through the digestion process. The trypsin rich lower intestine cleaves the S protein of the PEDV virions allowing them to better bind to cellular receptors for infection and replication [93, 94]. The lower intestine is also rich in the most susceptible cells to PEDV. Similar to both TGEV and human coronavirus HCoV-229E, PEDV targets the aminopeptidase N (APN, CD13) receptors, a multifunctional enzyme found in multiple tissue types [95, 96]. This has been confirmed multiple times using porcine cells susceptible to the virus as well as transgenic expression of porcine APNs in genetically modified cell lines and whole animals of other species [97-100]. However, it was later demonstrated that the APNs may not be *required* for PEDV to infect susceptible cells, helping explain some reports demonstrating how the virus can infect the APN deficient cells in the large intestine [101-103].

Upon infection of cells, PEDV undergoes replication culminating in cytolysis of the infected cell [104]. The enveloped virus is internalized by direct membrane fusion, and the genomic RNA is spread throughout the cytosol. Similar to other *Coronaviridae*, autophagy is induced in the cell and PEDV is able to avoid innate responses to viral pathogen associated molecular patterns (PAMPs) by replicating within the autophagosome-like double membrane vesicles [105]. During replication within the cell, PEDV is able to control the

mitogen-activated protein kinase (MAPK) or extracellular signaling-regulated kinase (ERK) signaling cascade in order to increase its replication [106]. PEDV is then able to perpetuate the S-phase of the cell cycle through the N or M protein, and prolong the favorable environment for replication [107, 108]. After replication is sufficiently complete, apoptosis of the cell is induced via the mitochondrial apoptosis-inducing factor (AIF) [109].

Successful replication and apoptosis of PEDV in intestinal epithelial cells of pigs causes enteritis. In young piglets (<7 days) the replication causes malabsorptive diarrhea induced by decreased efficacy of brush border membrane-bound digestive enzymes and leaky junctions in the small intestine [110, 111]. The symptoms are severe enough in young piglets to result in a death rate of nearly 100% upon exposure due to dehydration. This is not true for adult pigs, which may be due to the delayed infection and more mature microvilli.

### *Modulation of the Interferon Response*

Interferon (IFN) regulation by PEDV has been covered in depth by Zhang and Yoo, but will be covered briefly in this dissertation [112]. Upon recognition of PAMPs, one of the innate immune response's first lines of defense is to secrete Type I IFNs (IFN-α and IFN-β) [113]. The role of the Type I IFNs is to establish an antiviral state through transcription factors in surrounding cells by increasing temperature and slowing cell growth, thus limiting the spread of virions. However,

PEDV has demonstrated an ability to act as a Type I IFN antagonist through multiple means. An *in vitro* experiment showed that PEDV can prevent the ubiquitination of proteins in the IFN activating cascade including papain-like protease [114]. PEDV is also able to degrade CREB-binding protein (CBP) using non-structural protein 1 (nsp1), again suppressing the release of Type I IFNs [115]. Additionally, non-structural protein 5 (nsp5) has shown proteolytic activity toward the nuclear transcription factor kappa B (NF-κB) essential modulator (NEMO) protein [116]. The nucleocapsid (N) protein from PEDV has shown the ability to interfere with Type I IFN production through its direct interaction with the upstream cascade protein TANK binding kinase 1 (TBK1) [117]. Finally, PEDV is able to inhibit the IFN-β promoter stimulator 1 (IPS-1) protein in the retinoic acid-inducible gene I (RIG-1) pathway of dsRNA recognition and subsequent chemokine production [118]. Though some of these pathways may be slightly redundant, together they act as potent IFN modulators allowing PEDV to bypass a potent innate immune response.

Studies have shown that PEDV is also able to up regulate Interluekin-8 (IL-8) during S-phase elongation [107]. By doing so, the virus causes an increase in chemotaxis of, and phagocytosis by, immune cells. This process delays apoptosis and allows yet another method of action for the down regulation of Type I IFNs [107, 108, 119]. What is not clear is which protein from the virus is

primarily responsible for the mechanism of action, though the M, N, and E proteins have all been implicated as playing some role [108].

Although no mechanism of action has yet been determined, there is evidence PEDV is also able to down regulate Type III IFNs (IFN-λ) [120]. A change in gut microbiota during PEDV infection indicated that there was an effect of PEDV on dysbiosys [120]. Zhang and Yoo have hypothesized that this change could be due to the down regulation of Type III IFNs by the virus, citing a study by Baldridge, et al demonstrating an effect of Type III IFNs on commensal bacteria and Norovirus [112, 121]. Type III IFNs have a separate activation cascade from the Type I IFNs, but induce a similar downstream immune response. Intestinal epithelial cells have demonstrated an increased susceptibility *in vivo* to rotavirus in mice with fewer Type III IFN receptors, whereas the same was not true for Type I IFN receptors [122]. *In vitro* results have recently indicated that PEDV may also be preferentially inhibited by Type III IFNs, similar to other mesenteric viruses [123].

## *S-INDEL Strains in the United States*

There are several US PEDV strains that have been identified with insertions/deletions (INDELs) in the genome coding for the S-protein. These strains, labeled as S-INDEL PEDV strains, emerged around April of 2013 coinciding with the transmission of the more virulent strains in the US and abroad

[124]. Initial reports varied widely in the prognoses of the S-INDEL PEDV strains, with some reporting reduced clinical symptoms in the US but others in Europe reporting high rates of mortality in suckling piglets [125-127]. The S-INDEL PEDV strain Iowa106 was later shown to have a reduced mortality rate upon exposure compared to that of the original US PEDV PC21A strain [124]. This finding has been corroborated by other groups [128, 129].

Although the S-INDEL PEDV strains may have a reduced pathogenicity, they may be more difficult to neutralize compared to original strains. Okda et al demonstrated that the injection of mice with a recombinant S-protein could generate monoclonal antibodies (mAbs) to specific regions of the S-protein [130]. These mAbs were purified and mapped on the S-protein of original PEDV strain CO13 as well as S-INDEL strain Iowa106. Each strain contains the same amino acid sequences coding for the regions of the S-protein identified as mAb attachment regions. However, when the mAbs were tested for whole virus (WV) ELISA and viral neutralization, there were stark differences. In each case, the neutralization titers to the S-INDEL virus were reduced compared to that of the original strain. For one of the mAbs, there was a complete lack of WV ELISA titer to the S-INDEL strain only [130].

## Current Treatments

### *Common Practices*

Primers detailing purposeful infection of a swineherd to PEDV have been published, proposing that the feeding of infective material to the older pigs may be able to provide some herd immunity [131]. Inclusion of fresh fecal samples from nursing piglets at the onset of disease in feed allows an effective dose to the relatively asymptomatic animals orally. Although cost-effective, this strategy by nature requires that a farm be exposed to PEDV. Vaccine development has therefore been pursued throughout the past couple of decades. Table 1 shows a number of vaccine designs that have been tested in the past. The vast majority of the treatments studied are MLV and KV vaccines from newly mutated species.

Though several of the vaccine candidates listed in Table 1 were previously or are currently available on the market in one or more countries, they have generally been regarded as providing moderate protection as determined by both viral shedding post inoculation and suckling piglet mortality [67, 68]. Most of the outlined vaccine candidates were based on PEDV strains within the G1 genotype, meaning that they are more closely related to CV777 based on their S protein gene sequence than to the more recent (and more virulent) strains seen during the 2012 panzootic. However, it seems unlikely that mutations in the epitopes and therefore genealogy are the cause for the lack of efficacy on farms. It seems more likely, then, that there are other immunological factors that are preventing the efficacy of current vaccine candidates. From an economic standpoint, it can be argued that the hypoimmunoglobulinemia of newborn piglets

and subsequent mortality is of the most importance for vaccination toward PEDV. A high level of protection for suckling piglets has not been seen in IM administered vaccinations for PEDV in seronegative sows, but *can* be seen upon oral inoculation [48].

## *Recombinant Vaccines*

In the case of recombinant vaccines for PEDV, several epitope motifs have been identified as having B-Cell activity including some with neutralization activity as well [132, 133]. Focus of the vaccine candidates outlined in Table 1, excluding the KLV and MLV candidates, lies solely on the S protein from PEDV. Many of those focused on the S1 portion for the S protein, conventionally divided from the S2 domain at aa 785. When expressed, the S1 portion of the S protein contains the CO-26K equivalent (COE) region (aa 499 – 638). This domain has been shown to produce Abs with neutralizing capabilities, though it is not a linear epitope and thus cannot easily be defined [47]. However, Suo et al included both the S1 as well as the entire S protein in their vaccination strategy, with the conclusion that the full recombinant protein performed better than the S1 domain alone [57]. In fact, another group repeated this conclusion that the full length S protein is preferable to the S1 section alone on more than one occasion [134, 135]. This is an interesting finding, since the S1 domain includes the COE as well as the linear epitopes $^{748}$YSNIGVCK$^{755}$, and $^{764}$SQYGQVKI$^{771}$. Inclusion of the S2 domain in a recombinant protein would add the linear epitope

$^{1368}$GPRLQPY$^{1374}$, but it does not seem likely that this addition would contribute significantly to the neutralizing properties of the Abs generated [130]. Likely, this phenomenon is due to the disparity between the conventional division of the two S protein domains, as some groups indicate the split occurs at aa 730, whereas others have it at aa 785. This disparity may be of significant importance as it contains two known neutralizing epitopes. In fact, this region seems to be of particular importance in regard to virus neutralization [61].

## Lactogenic Immunity in Pigs

There is little to no transfer of immunoglobulin to the fetus during pregnancy in pigs due to an epitheliochorial placenta in the species [136]. Piglets are therefore born immunoglobulinemic. When combined with the speed at which the virus can destroy the microvilli, a lack of secretory IgA specific to the virus requires the presence of neutralizing antibodies in the colostrum and milk if the suckling piglets are to survive. A previous report has shown that subunit vaccines were capable of inducing neutralizing antibodies toward TGEV in the sera of seronegative sows, but lactogenic immunity failed [137]. When the same group performed the experiment on sows having previously been exposed to an attenuated live virus vaccine through oral/intranasal administration, the subunit vaccine was able to provide protection to the suckling piglets [138]. This same phenomenon was reported for PEDV using a commercially available KV vaccine [48, 139]. Vaccination of a pregnant, seropositive sow induced a strong immune

response in the form of secretory IgA (sIgA) in milk and was able to reduce the mortality rate to 0% for 10 days post challenge. The mortality rate using the same vaccine rises to 70% when the sow is seronegative. Previous exposure to PEDV followed by vaccination during pregnancy seems to be critical to the efficacy of vaccination, likely due to the origin of the sIgA in the milk.

The colostrum from lactating sows has been reported as being comprised of serum-derived immunoglobulins at varying proportions based on isotype. For IgG, IgM and IgA, the amount of immunoglobulins derived from serum were reported as being ~100%, 70 – 100%, and 40% respectively [140]. The rest were derived from immune cells within the mammary gland (MG) itself. This proportion shifts down to ~30%, <10%, and <10% respectively, in milk. Therefore upon the change from colostrum to milk, a *vast* majority of sIgA in milk comes from within the MG. The ratio of IgA per total Ig subclass increases with the switch from colostrum to milk as well, making it the more important immunoglobulin for viral neutralization in the suckling piglets' gut during lactation.

Therefore to achieve lactogenic immunity to PEDV, a vaccination strategy should focus on the production of IgA in the MG upon milk production. IgA+ plasmablasts migrate based on several chemokines, adhesion molecules, hormones, and cytokines. There is evidence that lymphocytes in the MG during late stage pregnancy may have migrated from either the upper respiratory tract

or the gut into the mammary gland, where they then produce IgA for lactogenic immunity [141, 142]. The migration to the MG is done based on levels of vascular adhesion molecule 1 (VCAM-1) and the α4 integrin expressions. The resulting gut-mammary gland (gut-MG) axis could be an important factor in immunization toward PEDV. In the case of seropositive sows, vaccination for PEDV during this crucial migratory period may increase the number of PEDV-specific lymphocytes in the gut leading to an increase in the MG prior to farrowing. The immune response may therefore lead to an increase in lymphocytes capable of secreting neutralizing antibodies from the MG into the milk, providing enough of them to reduce the mortality rate of the suckling piglets dramatically.

One possible exploitation of the gut-MG axis in a vaccination strategy could be to recruit lymphocytes to the epithelial tissues of the seronegative pregnant sow during vaccination. Chemokine (C-C motif) ligand 25 (CCL25) has been associated with lymphocyte migration to the small intestinal epithelia through its interaction with Chemokine (C-C motif) receptor 9 expressed by T cells of the immune system [143-145]. This interaction leads to a chemotaxis of the cells into the small intestine. It may therefore be possible that a vaccination formulation could increase the level of systemic CCL25, or possibly a separate CCR9 agonist, and thus the number of lymphocytes in the gut epithelia specific to neutralizing peptide sequences [146]. The goal of this formulation would be to

mimic the immune response of a sow previously exposed to PEDV via the traditional oral route.

An alternative could be to increase the amount of circulating CCL28, an agonist for CCR10. CCL28 was also once known as the mucosae-associated epithelial chemokine (MEC) [147]. In sows, the expression of CCL28 has been found in both the gut and the MG, a possible benefit for PEDV sIgA production in the MG during the last stages of pregnancy [148]. CCR10 is a receptor that is commonly expressed on circulating and mucosal epithelial IgA secreting cells [149]. In both cases, the goal of these chemokines would be to recruit the lymphocytes to the gut epithelia and mammary gland of the pregnant sow [150]. The increase in antigen-specific lymphocytes in the gut should lead to an increase of the same lymphocytes in the MG via migration during the late stages of pregnancy and increase the sIgA in the colostrum and milk.

In the case of sufficient IgA production in the MG, the bottle neck for lactogenic immunity may then be the secretion of the IgA into the milk [151]. The polymeric immunoglobulin receptor (pIgR) protein is a transmembrane protein responsible for the transcytosis of IgA into the extracellular secretions of an epithelial cell. During transcytosis the pIgR protein is cleaved and remains bound to the dimeric IgA (dIgA), possibly as a means of protection [151]. Another protection strategy for PEDV may therefore be to increase the secretion of IgA

from mucosal surfaces through pIgR stimulation. Expression of pIgR on mucosal cell surfaces can be up-regulated by the cytokines IFN-γ, TNF, IL-4, and IL-1 [152]. Additionally, the pIgR expression can be increased through the stimulation of TLR 3 and TLR 4, with the latter allowing increased expression without increased inflammation [153]. Each mechanism results in the translocation of transcription factors to the nucleus of the cell, and an increase in pIgR expression. These transcription factors include Signal Transducer and Activator of Transcription (STAT), Interferon Regulatory Factor (IRF), and Nuclear Factor-κB (NF-κB) [152]. However, an observational study in humans indicated that there was a decrease in all of the indicated cytokines in healthy pregnant vs non-pregnant women, and that preeclampsia is associated with an increase in each of these cytokines indicating that there may be a benefit to reducing the concentrations of these pIgR stimulators in healthy pregnancies [154]. It may therefore be important to include a TLR 3 or TLR 4 agonist in the formulation of a vaccine reliant on lactogenic immunity. An increase in pIgR translation in sows should increase the overall amount of secretory IgA in the mammary gland [155]. By priming this system simultaneously as inducing immunity toward PEDV, it may be possible to increase the amount of secretory IgA in the colostrum and milk, increasing lactogenic immunity. However, there is a possibility that the administration of the vaccine may be done too early during pregnancy that the immediately effects of a pIgR up regulation would have an effect.

## Virus-Like Particles

Recombinant subunit vaccines have often had issues with immunogenicity without the addition of an adjuvant [156]. In order for the dendritic cells of the immune system to be activated into full antigen presenting status, there must typically be an additional trigger of the innate immune system. The goal of these formulations is therefore to increase the immune response to the antigen enough to allow efficacy without causing side effects. VLPs are considered to be an excellent option in this regard, as the VLP by design closely mimics the structure of a virus, including the presence of pathogen-associated molecular patterns (PAMPs). In regard to safety, VLPs contain none of the DNAs that cause viral infection, but are able to maintain their ability to act as a self-adjuvant.

There is evidence of successful vaccination using VLPs in several different animal types using several different expression systems [157-162]. VLPs for two highly mutagenic viruses that attack the mucosa, HIV and Influenza virus, have been explored in humans and in mice [163, 164]. Through the use of VLPs, vaccination strategies can present a more conserved, but less immunogenic antigen from these viruses in a way that induces a strong immune response. This precedence includes VLP use in pigs, who saw an enhanced Ig response when VLP based vaccines were used [165]. VLPs have been tested for their ability to generate a mucosal immune response with positive results through intranasal inoculation including antigen specific IgA and IgG induction [164, 166].

In regard to CVs, VLPs are considered to be an optimal platform for mucosal immunity due to their size distribution and their ability to be taken up by M-cells in Peyer's Patches in the gut [167]. However, intranasal administration on a commercial farm may not be feasible.

## HBcAg

The positive sensed strand of the Hepatitis B Virus (HBV) DNA contains two ORFs of structural significance [168]. The first is the ORF for the viral nucleocapsid or Hepatitis B virus core antigen (HBcAg). The second is ORF for the outer membrane of the virus or surface structure (HBsAg) [169]. Discovery of the HBsAg, and specifically, the region 5' upstream containing the preS1 and the preS2 regions was important in vaccine design for the disease because there are amino acid sequences within these regions that code for significant neutralizing epitopes [170-175].

The HBcAg is a 183 AA protein that has the ability to self-assemble into the capsid structure measuring at 24 to 31 nm in diameter without any viral DNA [176]. The protein can also be expressed successfully in *E. coli* with the *lac* operon [177-179]. Through genetic manipulation, this protein was used as a carrier protein to increase the immunogenicity of the pre-S epitopes and successfully vaccinate against HBV [180-182].

Of the three popular insertion sites (the N-terminus, the truncated C-terminus, and the loop region), it was found that insertion of the Pre-S epitopes into the loop region had the highest immunogenic effect. This could be because the deletion of or insertion into the loop region of the HBcAg disrupts an identified epitope for the protein itself, which reduces the antibodies generated for the core protein [183]. Insertion around amino acid 80, a proline residue, can be done with self-assembly for up to 46 amino acids [184]. Insertion at the N-terminus had had the second highest immunogenic effect, while insertion into the truncated C-terminus showed only partial surface presentation for the short to medium epitopes studied. All three sites were found not to interfere with the self-assembly process of the chimeric proteins when these guidelines were followed.

HBcAg has been confirmed to be immunogenic via an intranasal inoculation in humans as well as mice [185, 186]. It was determined that an intranasal administration route was superior over other inoculation sites in mice for the generation of IgA in multiple mucosal sites (pulmonary and vaginal) [187]. Additionally, the intranasal administration in mice was found to generate a serum antibody profile that was not significantly different from peritoneal injection. Unfortunately, oral vaccination may not be possible due to the inability of the HBcAg protein to survive in low pH conditions [188].

Effective activity and antigen presentation through the use of the HBcAg protein requires that the expression and purification process does not alter the ability of the proteins to self-assemble. Individual HBcAg proteins form homodimers which, in turn, form multimers that eventually assemble into the VLP [184]. Each monomer contains two long 42 Å alpha helices, with a hairpin loop in between [184]. The alpha helices as well as disulfide bonds were hypothesized to be responsible for a majority of the monomer-monomer interaction required to create each dimer. Although both are considered important for particle stability, neither are required for VLP self-assembly [160, 189]. There are two major geometries in VLP assembly: T = 3 containing 180 subunits (90 dimers) or T = 4 containing 240 subunits (120 dimers) [190].

Upon assembly, HBcAg VLPs have exposed regions at amino acids 78-83 as well as 127-133, making them appropriate targets for epitope insertion [191]. However, as stated earlier, the N-terminus and C-terminus are also available places for epitope insertion. Insertion of an epitope onto the N-terminus of HBcAg requires a pre-core link of amino acids for successful self-assembly [192]. This pre-core linker should include the 0 through -4 amino acids of the HBcAg only. Inclusion of more amino acids means possibly including the -7 cysteine residue, which has been shown to prevent self-assembly [193]. A C-terminally truncated version of the HBcAg at the Pro144 amino acid has been shown to be

able to accept and display insertions on the surface of the VLP without preventing self-assembly [194].

Interaction between the peptide inserts upon dimerization should be carefully considered [195]. If any physicochemical interactions were strong enough to offset the interactions between the two HBcAg proteins, dimerization and VLP self-assembly could be dissuaded [191]. Preventative interactions include hydrophobicity, β-strand index, and steric hindrance, all of which can be prevented through dimerization with a native protein or inclusion of a flexible insert [195, 196].

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## Tables and Figures

Figure 1 - A phylogenetic tree of multiple PEDV strains showing the relationships between species.

Specific species were chosen based on geography, with a focus on covering as many different countries as possible in addition to strains located within the USA. Other strains were chosen based on their use as MLV or KV vaccine candidates. The evolutionary history was inferred using the Neighbor-Joining method [197]. The optimal tree with the sum of branch length = 0.27982360 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site [198]. The analysis involved 56 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 5160 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [199]. Included with the phylogenetic tree is the genotype classification as well as an indication of the conservation of four important small, linear epitopes throughout the phylogeny. * = (X)Q(X)GQVKI and ** = SQSGQVKI

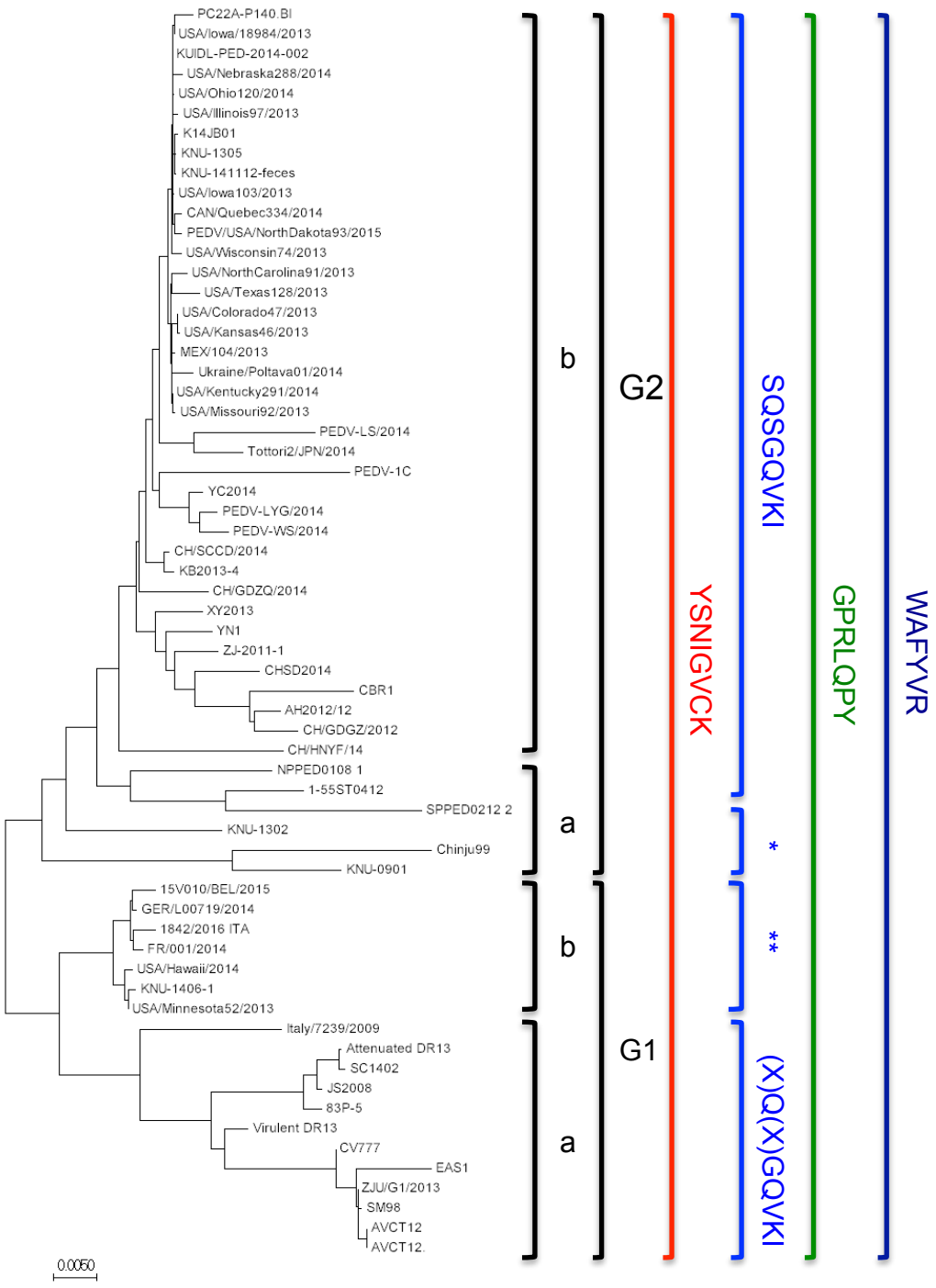
PC22A-P140.BI
USA/Iowa/18984/2013
KUIDL-PED-2014-002
USA/Nebraska288/2014
USA/Ohio120/2014
USA/Illinois97/2013
K14JB01
KNU-1305
KNU-141112-feces
USA/Iowa103/2013
CAN/Quebec334/2014
PEDV/USA/NorthDakota93/2015
USA/Wisconsin74/2013
USA/NorthCarolina91/2013
USA/Texas128/2013
USA/Colorado47/2013
USA/Kansas46/2013
MEX/104/2013
Ukraine/Poltava01/2014
USA/Kentucky291/2014
USA/Missouri92/2013
PEDV-LS/2014
Tottori2/JPN/2014
PEDV-1C
YC2014
PEDV-LYG/2014
PEDV-WS/2014
CH/SCCD/2014
KB2013-4
CH/GDZQ/2014
XY2013
YN1
ZJ-2011-1
CHSD2014
CBR1
AH2012/12
CH/GDGZ/2012
CH/HNYF/14
NPPED0108 1
1-55ST0412
SPPED0212 2
KNU-1302
Chinju99
KNU-0901
15V010/BEL/2015
GER/L00719/2014
1842/2016 ITA
FR/001/2014
USA/Hawaii/2014
KNU-1406-1
USA/Minnesota52/2013
Italy/7239/2009
Attenuated DR13
SC1402
JS2008
83P-5
Virulent DR13
CV777
EAS1
ZJU/G1/2013
SM98
AVCT12
AVCT12.
0.0050
b
a
b
a
G2
G1
YSNIGVCK
SQSGQVKI
*
**
(X)Q(X)GQVKI
GPRLQPY
WAFYVR

Table 1 - A timeline of vaccination candidates for PEDV.

Included is the strain used, if available, as well as the date of publication. Important notes were also included to give further information on the nature of the study performed.

| Vaccine Type | Strain | Of Note From the Study | Year | Citation |
|---|---|---|---|---|
| Inactivated Live Virus | Strain S | ~80% protection rate | 1993 | [71, 200, 201] |
| Attenuated Live Virus | CV777 | ~85% protection rate | 1994 | [201, 202] |
| Inactivated Live Virus | CV777 | Bivalent with TGEV; used commercially in China | 1995 | [71] |
| Attenuated Live Virus | CV777 | ~95% protection rate | 1998 | [40, 71] |
| Attenuated Live Virus | P-5V | Commercially available in Japan | 1998 | [203, 204] |
| Attenuated Live Virus | CV777 | Bivalent with TGEV; used commercially in China | 1999 | [205] |
| Attenuated Live Virus | KPEDV-9 | Survival rate was double that of control piglets upon challenge | 1999 | [49] |
| Exogenous Ig from Chicken Egg Yolk | KPEDV-9 | ~60% Survival, Whole virus titer | 2000 | [206] |
| Exogenous Ig from Cow Colostrum | Z94P5 | VN titers reported | 2001 | [207] |
| Recombinant COE Protein | Brl/87 | Transgenic plant expressed COE protein | 2003 | [52] |
| Viral Antigen on *Lactobacillus* Surface | kPEDV-9 | Nucleocapsid protein antigens | 2007 | [51] |
| Attenuated Live Virus | DR13 | Oral Delivery; Viral shedding in feces | 2007 | [208] |
| Single Chain Variable Fragments Expressed in *E. coli* | SM98P | In vitro only | 2009 | [209] |
| Attenuated Live virus | CV777 | Trivalent with TGEV, PRoV | 2010 | [71] |
| Viral Antigen on *Lactobacillus* Surface | KPEDV-9 | Nucleocapsid protein antigens as well as S1 protein antigens | 2012 | [210] |
| DNA Plasmid Vaccine | CV777 | Included IL-18 Plasmid in one test group; full S protein performed better compared to S1 only | 2012 | [57] |
| RNA Particle | Unknown | Truncated S-protein vaccine; licensed as iPED®; conditionally | 2013 | [58, 139] |

| | | | | |
|---|---|---|---|---|
| | | approved for commercial use | | |
| RNA Particle | Unknown | Contains more of the S-protein; licensed as iPED+® | 2014 | [58, 139] |
| Recombinant S1 Protein | KNU-0801 | Combined with Fc chain of Human Ig; Challenge performed with SM98-1 | 2014 | [60] |
| Attenuated Live Virus | Unknown | Attenuated from a US strain | 2014 | [58] |
| Attenuated Live Virus | CO/13 | Attenuated from a US strain | 2015 | [61] |
| Exogenous Ig from Chicken Egg Yolk | KNU-0801 | Offered some protection, viral shedding still prevalent | 2015 | [134] |
| Attenuated Live Virus | KNU141112/2014 | Genotype 2b attenuated virus | 2016 | [62] |
| Recombinant Subunit Vaccine | CO/13* | Multiple expression systems of the S1 domain. Mammalian system used in challenge. | 2016 | [63] |
| DNA Plasmid Vaccine | CV777 | Bivalent S protein plasmids for TGEV and PEDV expressed in *Salmonella typhimurium.* | 2016 | [64] |
| Recombinant parapoxvirus Expressing Spike Protein | CO13 | Intramuscular administration in young pigs without oral challenge | 2016 | [211] |
| Recombinant Subunit Vaccine | MN | S Protein fused to a single-chain-variable-fragment targeting Langerhans cells | 2017 | [65] |
| Recombinant Virus Like Particles | AH2012 | Combination of S, M and E proteins formed virus like particles; not tested in pigs | 2017 | [66] |
| Attenuated Live Virus | PC22A | Attenuated from a US strain | 2017 | [212] |
| *Strain was not completely identifiable, and guesses were made based on other information | | | | |

# Chapter III: Hepatitis B virus core antigen based novel vaccine against porcine epidemic diarrhea virus.

# Hepatitis B virus core antigen based novel vaccine against porcine epidemic diarrhea virus

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## Abstract

Porcine epidemic diarrhea Virus (PEDV) is the causative agent of porcine epidemic diarrhea, which is a devastating viral disease and causes severe economic loss to the swine industry. Current vaccine options for PEDV include modified live viruses and killed live viruses. Though these vaccines have shown efficacy, some have side effects including viral shedding. This report details an *E. coli* based expression and purification process of multiple vaccine candidates for PEDV using Hepatitis B virus core antigen (HBcAg) as a backbone protein. Short linear peptide sequences from PEDV were inserted into the immunodominant region of HBcAg in a novel recombinant vaccine design against PEDV. These peptide sequences were successfully inserted individually as well as all together in a multivalent strategy. Each vaccine candidate was tested *in vivo* in an intranasal as well as an intraperitoneal administration. Although each candidate was able to elicit a strong immunogenic response specific for the inserted peptide sequences, only two out of five of the test candidates demonstrated an ability to elicit an immune response capable of virus neutralization when delivered via intraperitoneal administration in mice.

**Keywords**: HBcAg; PEDV; Vaccine; VLP; Purification

Abbreviations:

PEDV, Porcine Epidemic Diarrhea Virus; EFP, ELISA Fusion Protein; Ab, Antibody; HBcAg, Hepatitis B virus core antigen; VLP, Virus-like Particle; VN, Virus Neutralization; PS, Polystyrene; GST, Glutathione-S-Transferase; AEX, Anion Exchange; IMAC, Immobilized Metal Affinity Chromatography; IPTG, Isopropyl β-D-1-thiogalactopyranoside; DLS, dynamic light scattering

## Introduction

Porcine epidemic diarrhea Virus (PEDV) is a member of the *Alphacoronavirus* genus in the Coronaviridae (CV) family in the Nidovirales order. The genome of PEDV is approximately 28 kb in length and incudes 5' and 3' untranslated regions and 7 known open reading frames (ORFs) in the order of ORF1a, ORF1b, spike (S) protein, ORF3, envelope (E) protein, membrane (M) glycoprotein, and nucleocaspid (N) protein [1]. PEDV was first discovered in Europe in the 1970s and spread to Asia in the 1980s and 1990s [1, 2]. PEDV was detected for the first time in North America in 2013 and caused severe disease and substantial economic losses [3, 4]. The symptoms of PEDV are watery diarrhea, depression, and anorexia. The high mortality rate of PEDV in suckling piglets is caused by the destruction of susceptible microvilli in the small intestine [5]. The fecal-oral route of infection has the highest rate of transmission, although evidence suggests that airborne aerosolized virus may also be infectious [6]. Even though vaccines currently exist for PEDV in killed virus (KV) and modified live virus (MLV) formats, there are side effects from their administration. A recombinant vaccine strategy may therefore be more appropriate for PEDV in agriculture.

Hepatitis B virus core antigen (HBcAg) is the nucleocapsid protein from the Hepatitis B virus. The native form of the protein is 183 amino acids (AA) in length and has the ability to self-assemble into the nucleocapsid structure with a diameter around 30 nm [7, 8]. The full protein consists of two domains, only the

first of which is required for self-assembly of the protein [9, 10]. Recombinant versions of the protein typically consist of only the first 149 AA and are abbreviated as ΔHBcAg. Genetic manipulation of ΔHBcAg has been demonstrated previously in vaccine designs, some of which have been approved for use in humans in the US [11-14]. Short linear epitopes from other viruses are often inserted at AA P80, which can be done with successful self-assembly and subsequent foreign antigen presentation provided that the peptide insertion does not cause sever steric hindrance through secondary or tertiary structure. This location within the protein has been shown to induce the highest immune response and is considered to be the major immunodominant region (MIR) of the protein [15, 16]. Other tags, such as histidine tags have successfully been inserted at both the N-terminus and the C-terminus of the protein without adverse effects on the assembly of the virus-like particle (VLP) [17-20].

A vaccination strategy against PEDV should induce a strong immune response toward the disease with a focus on mucosal stimulation in order to provide the lactogenic immunity needed. Although Cholera Toxin (CT) is generally regarded as the strongest mucosal immune response generator, it has drawbacks as an adjuvant due to its toxicity. It was recently proposed that the mechanism for CT's success as an adjuvant may lie in it's interaction between dendritic cells (DCs) and the nod-like receptor (NLR) NOD2 [21]. The NLR family and, specifically, the NOD2 receptors are B cell receptors that recognize bacteria

pathogen associated patterns (PAMPS), which instigate several protein cascades to increase the immune response [22-24]. Murabutide is a NOD2 agonist and has been shown to enhance the immunogenicity of HBsAg, a protein closely related to HBcAg [25]. It has also been shown to enhance the immunogenicity of the Norwalk Virus VLP, in an intranasal administration of a vaccine candidate [26]. CpG class C motifs are bacterial DNA motifs that are known to interact with the toll like receptor 9 (TLR9) to induce Th1 cell type switching and increase antibody (Ab) production in both humans and mice [27, 28]. CpGs have been used in intranasal administrations at high concentrations without any toxic effects on mice [29-31]. Ideally, the inclusion of both CpG ODN and murabutide in a vaccine's formulation should allow it to have the same or similar mucosal immune response as CT.

Several peptide sequences have been previously identified as fairly conserved B-cell epitopes in PEDV. There are four of these epitopes located in the spike (S) protein of the virus, though only three of them are short linear peptides [1]. These peptide sequences are $^{744}$YSNIGVCK$^{752}$, $^{756}$SQYGQVKI$^{771}$, and $^{1371}$GPRLQPY$^{1377}$, hereby designated in this publication as S1, S2, and S3 respectively [32, 33]. A conserved linear B-cell epitope was also identified in the membrane (M) protein of the virus ($^{195}$WAFYVR$^{200}$), designated as M in this publication [34]. These four sequences were used in the genetic engineering of a

subunit vaccine candidate in an effort to create a more universal vaccination strategy against PEDV.

In this study, five vaccine candidates (**Fig. 1**) using the HBcAg protein as a backbone were produced and tested *in vivo*. These vaccine candidates were delivered via both intranasal and intraperitoneal administrations in an effort to elicit the strongest mucosal immune response possible.

## Materials and Methods

### *Plasmid Construction*

DNA fragments (IDT, Coralville, IA) coding an optimized sequence for the truncated HBcAg with the inserts outlined above were amplified using PCR with primers that added the *Nhe I* and *Hind III* cut sites to the 5' and 3' ends, respectively [35]. The amplified fragments as well as the vector plasmid pET28a (+) (Novagen, Madison, WI) were digested using the *Nhe I* and *Hind III* restriction enzymes according to vendor instructions (NEB, Ipswitch, MA) [36, 37]. A 5:1 stoichiometric ratio of plasmid to fragment was ligated using T4 DNA ligase according to vendor instructions (NEB). The subsequent plasmid DNA was transformed into T7 Express BL21(DE3) *E. coli* cells (NEB) using the heat shock method.

### *Expression*

Overnight starter cultures were used to inoculate 1 L batches of 2×YT media supplemented with 30 μg/mL kanamycin at 0.2% v/v. The *E. coli* cultures were allowed to incubate in a shaker at 37 °C at 200 RPM until the $OD_{600}$ reached 0.7 AU. 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) was used to induce protein production, which was allowed to incubate for an additional 6 h. Two of the constructs: pHBcAgS2 and pHBcAgM1 would not overexpress under these conditions. Therefore, these proteins were expressed at 28 °C overnight using 1 mM IPTG after reaching an $OD_{600}$ of 0.7 AU. All resultant cultures were centrifuged at 8,000 × *g* for 10 min to collect the cell pellets. Cell pellets were immediately frozen until purification.

### *Purification*

Cell pellets were re-suspended in lysis buffer (20 mM $NaH_2PO_4$, 500 mM NaCl, 10 mM Imidazole, and 2% Triton X-100 at pH 7.8) at a 1:10 w/w ratio. Cell disruption was performed on ice using a Sonic Dismembrator Model 500 (Fisher Scientific, Pittsburgh, PA), with 60 cycles of 7 sec on/5 sec off. The amplitude for sonication was set at 30%. The lysate was then centrifuged at 10,000 × *g* for 10 min to separate the insoluble and soluble portions.

Except for ΔHBcAg, the proteins were located in the insoluble portion of the cell lysate. Inclusion bodies (IBs) were washed one time with cell lysis buffer and centrifuged again at 10,000 × *g* for 10 min. The IBs were solubilized using a

1:1 v/v of solubilization buffer (20 mM $NaH_2PO_4$, 500 mM NaCl, 10 mM Imidazole, and 0.9% Sarkosyl at pH 7.8) and allowed to mix overnight at room temperature [38]. Solubilized IBs were then centrifuged at 10,000 × *g* for 10 min, and the supernatant was kept.

Lysates (soluble for ΔHBcAg protein, insoluble for chimeric proteins) containing the proteins of interest (POIs) were treated with 15% w/w ammonium sulfate for a total final concentration of 1.14 M ammonium sulfate. The reaction was allowed to incubate for at least 2 h with mixing at room temperature. The precipitates were then centrifuged at 10,000 × *g* for 10 min. All POIs were found in the solid portion of the reaction. All precipitated proteins were then re-dissolved in a 1:1 v/v ratio of solubilization buffer.

Immobilized metal affinity chromatography (IMAC) was performed using IMAC 6 Sepharose Fast Flow (GE Healthcare, Marlborough, MA) resin. The column was rinsed with 5 column volumes (CVs) of water, followed by 2 CVs of 200 mM $NiSO_4$ solution to charge the resin with $Ni^{2+}$ ions. The column was again flushed with 5 CVs of water and equilibrated with 5 CVs of solubilization buffer. Sample was loaded onto the column at 2 mg protein per mL of resin. Loading was followed by another 7 CVs wash with solubilization buffer. Finally, an isocratic step elution was performed with IMAC elution buffer (20 mM $NaH_2PO_4$, 500 mM NaCl, 300 mM Imidazole, and 0.9% Sarkosyl at pH 7.8).

Following IMAC purification, step-wise dialysis was performed in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, and 1.8 mM $KH_2PO_4$ at pH 7.8) at pH 7.8 with various concentrations of sarkosyl. Each dialysis step was performed using Spectralpor® dialysis membrane tubing (Spectrum Laboratories, Rancho Dominguez, CA) with a molecular weight cut off of 6 – 8 kDa. The step-wise buffer exchange contained 0.45%, 0.1% and 0% sarkosyl, respectively. The proteins were then taken through a polishing step using anion exchange chromatography (AEX) utilizing DEAE Sepharose FF resin (GE Healthcare). The resin was equilibrated using 5 CVs of PBS, followed by loading of the sample. The column was then washed with an additional 5 CVs of PBS. Elution was carried out using AEX elution buffer (20 mM $NaH_2PO_4$, 1 M NaCl at pH 8.2).

### *Formulation*

Formulation was performed within two days of each inoculation using Spin X UF desalting tubes with a molecular weight cut off (MWCO) of 5 kDa (Corning, Corning, NY). In each case, the VLPs were concentrated to >0.526 mg/mL, then diafiltered with PBS. Samples were sterile filtered through 30 mm, 0.10 µm sterile syringe filters (Celltreat, Pepperell, MA) and diluted to the desired concentration with sterile PBS. Each protein sample was formulated with 10 µL of Murabutide and 2 µL of CpG ODN at a concentration of 10 mg/mL and 5 mg/mL respectively. All vaccines were then diluted to their final volumes to result in a 1 mL dose for IP administration and a 50 µL dose for IN administration. Final vaccines should

have a total of 20 μg protein, 100 μg murabutide, and 20 μg of CpG ODN. Two additional formulations were performed using the same ΔHBcAg and HBcAg+S1S2S3M materials without the addition of the adjuvants. All candidates were then held at 4 °C [10].

### *TEM*

Transmission electron microscopy (TEM) was performed on pure protein samples at a concentration of 0.2 mg/mL. Samples were allowed to set on formvar coated copper grids for a few seconds until the sample was blotted with filter paper. The samples were then stained with 2% phosphotungstic acid at pH 7, again for a few seconds before the stain was blotted with filter paper [39]. The resulting sample was then viewed using a JEM-1400 (JEOL, Peabody, MA) electron microscope at 200,000× magnification.

### *Protein Detection*

Protein samples were prepared for gel loading by dilution using 18 MΩ water with appropriate amounts of NuPAGE Reducing Agent (10×) (Invitrogen, Carlsbad, CA) and NuPAGE LDS Buffer (4×) (Invitrogen) to a total loading volume of 10 μL per well. Each dilution was held at 80 °C for 20 min before being cooled to 4 °C. Samples were loaded onto NuPAGE™ 4 – 12% Bis-Tris PAGE gels (Invitrogen) along with a PrecisionPlus™ protein ladder (Bio-Rad, Hercules, CA) and run at 200 V for 35 min. Gels were washed with 18 MΩ water three times for five minutes each and stained with SimplyBlue™ Safestain (Invitrogen)

for one hour prior to an overnight rinse in 18 MΩ water. Gels were imaged using a Chemidoc XR Imager (Bio-Rad). Densitometry measurements for protein purity were done using Image Lab software (Bio-Rad). A bicinchoninic acid (BCA) assay (Thermofisher, Waltham, MA) was performed for total protein measurements by manufacturer's instructions.

### *Western Blot*

Western blot samples were prepared identically to the NuPAGE samples, but with a Precision Plus WesternC protein ladder (Bio-Rad). NuPAGE™ 4 – 12% Bis-Tris PAGE gels (Invitrogen) were blotted using a Transblot® Turbo™ instrument (Bio-Rad) onto nitrocellulose membranes. The membranes were then blocked using 5% non-fat dry milk in Tris-Buffered Saline with 0.05% Tween 20 (TBST). Membranes were rinsed three times in TBST for 5 min each and treated with primary Ab solution containing a 1:15,000 dilution of mouse anti-his tag Ab in blocking buffer for 1 h. The membrane was subsequently washed three times with TBST and treated with a secondary Ab solution containing 1:12,000 of HRP conjugated goat anti-mouse Ab (Millipore, Billerica, MA) and HRP conjugated anti-strep (Bio-Rad) for sample and ladder chemiluminescence, respectively. A final three rinses with TBST were performed followed by detection using Clarity® ECL substrate (Bio-Rad).

### *Endotoxin Testing*

A Pierce® LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific) was used to test levels of endotoxins according to manufacturer's instructions. Each purification process was tested for endotoxin levels after the AEX chromatography step.

### *Animals*

Seven-week old germ free balb/c mice (Charles River) were used to determine the immunogenicity of the vaccine candidates. Each treatment group consisted of 8 mice total. Mice were acclimated for 6 days prior to a Day -1 blood draw using the submandibular collection method. Additional blood samples were collected on days 13, 27, and 42, when the mice were sacrificed via exsanguination. Fresh fecal samples were collected during each blood draw on days -1, 13, 27 and 42. Intranasal and intraperitoneal vaccinations were given on days 0, 14, and 28. Mice were weighed on a regular basis to determine overall health, with a 15% weight-loss as the cut off weight for removal from the study.

Blood samples were kept at 4 °C for 1 h prior to centrifugation. Serum was collected and frozen at -20 °C until ready for testing [40]. Fecal pellets were collected and suspended at 1:10 w/v in Superblock™ PBS based buffer containing a proprietary blocking protein and the antimicrobial agent Kathon® [41, 42]. The sample was vortexed until solids appeared to be resuspended, and then the samples were centrifuged at 10,000 × *g* for 10 min to eliminate solids. Samples were then frozen at -20 °C until testing.

*ELISA*

ELISA detection of individual peptides was performed using newly created proteins containing each peptide insert at the C-terminus. These proteins, referred to as ELISA fusion proteins (EFPs), contain a polystyrene (PS) binding tag RAFIASRRIRRP on the N-terminus of glutathione-S-transferase (GST) [43]. The GST protein is followed by a series of glycine and serine residues for flexibility. At the C-terminal end of each EFP, PEDV epitope peptides were attached genetically. Each protein (EFP-S1, EFP-S2, EFP-S3, EFP-M) was expressed and purified using a Glutathione Sepharose 4B resin (GE Healthcare). The final result was high concentrations of pure proteins containing a freely available and customizable peptide region. For this study, the attached peptides coded for B-cell epitopes to PEDV. Each purified protein was diluted to 10 μg/mL in coating buffer (50 mM $Na_2CO_3$, 50 mM $NaHCO_3$, pH 9.6) for ELISA detection. For detection of antibodies to ΔHBcAg, purified and assembled VLPs were diluted to 10 μg/mL in coating buffer.

Microlon 600 flat bottom plates were filled with 100 μL of coating solution and allowed to incubate at 37 °C for >2 h. Plates were washed 3 times each with 200 μL of water per well, and blocked overnight in blocking buffer. Blocking buffer for the PS binding peptides was Pierce™ Protein-Free (PBS) Blocking buffer (Thermo Fisher). ELISA assays for ΔHBcAg were blocked with PBS + 5% nonfat dry milk. Between each subsequent step, plates were washed with PBS-T three

times. Serum or fecal samples were added to each well at a total volume of 100 µL of diluted sample and allowed to incubate at RT for 1 h. Plates were then washed and treated with 100 µL of secondary Ab solution at a dilution of 1:10,000 and 1:8,000 in blocking buffer for goat anti-mouse IgG HRP (Millipore, Billerica, MA) and goat anti-mouse IgA unlabeled (Southern Biotech, Birmingham, AL) respectively. Plates were incubated at RT for 1 h and washed again. IgA plates were treated with a tertiary Ab solution at a 1:4,000 dilution of rabbit anti-goat IgG HRP (Southern Biotech) for 1 h and washed. Finally, 100 µL of TMB One Component Microwell Substrate (SouthernBiotech) was added to each well and incubated for ≤10 min. The reaction was then stopped using 0.05% sulfuric acid, and plates were read at 450 nm using a Biotek Synergy HTX plate reader (Winooski, VT).

Each ELISA plate was arranged to include negative control samples from a group of mice receiving sterile PBS injections only for absorbance comparison. For each plate, this included three minimally diluted (1:25) samples corresponding to the appropriate sample day from the PBS injected group per plate. Absorbance values from the test samples were compared to the negative control values. The inverse of the predicted dilution at which the absorbance is no longer statistically significant (average + 3 × standard deviation) from the PBS negative controls was reported as the titer. Titers are reported as the $\log_{10}()$ of the original value calculated. Each plate also contained samples from the test

groups at the -1 DPI sample point. In every case, the PBS negative control group samples had higher $OD_{450}$ measurements, so they were chosen as the comparator.

### *PEDV Virus Neutralization (VN) Test*

A U.S. PEDV prototype strain cell culture isolate USA/IN19338/2013 was isolated and propagated in Vero cells (ATCC CCL-81) as previously described [44]. A stock of this virus isolate at the $7^{th}$ passage in cell culture with an infectious titer of $1.6 \times 10^6$ $TCID_{50}$/mL was 1:400 diluted, giving rise to virus with a titer of $4 \times 10^3$ $TCID_{50}$/mL, and then used for virus neutralization test in this study.

Serum and fecal samples were tested for PEDV neutralizing Ab following the previously described procedures [45]. Briefly, serum and fecal samples were inactivated at 56 °C for 30 min and then 2-fold serially diluted from 1:4 to 1:512. Equal volume of PEDV isolate was mixed with the diluted serum or fecal samples and incubated for 1 h at 37 °C with 5% $CO_2$. Each serum or fecal sample was tested in duplicate. Vero cell monolayers grown in 96-well plates were washed once with post-inoculation media (minimum essential medium supplemented with 0.3% tryptose phosphate broth, 0.02% yeast extract, and 5 μg/mL trypsin 250, 100 unit/mL penicillin, 100 μg/mL streptomycin, 0.05 mg/mL gentamicin, and 0.25 μg/mL amphotericin B). Then 100 μL of the serum-virus mixture (containing 200 $TCID_{50}$ of virus) was transferred to prewashed Vero cell monolayers. After 1 h

incubation at 37 °C, the cells were washed twice with post-inoculation media and incubated with 100 µL/well of such media for 48 h at 37°C with 5% $CO_2$. Cells were fixed with cold 80% acetone and stained with PEDV N protein-specific monoclonal Ab SD6-29 conjugated to FITC (Medgene, Brookings, South Dakota) at a 1:100 dilution for 40-60 min. The staining was examined under a fluorescent microscope. The reciprocal of the highest serum and/or fecal dilution resulting in >90% reduction of staining as compared to the negative serum control was defined as the VN titer of the serum and/or fecal sample. A VN titer of ≥8 was considered positive.

### *Statistics*

JMP® Pro 12 software (SAS, Cary, NC) was used in all statistical analysis. All samples were run in a nested experimental design using administration, peptide/protein, and group, respectively. Individual ANOVA contrasts were performed to determine statistical significance between individual groups.

## Results

### *Expression and Purification*

Expression, purification and characterization of each vaccine candidate were performed prior to *in vivo* testing. The overexpression of each protein was accomplished in the insoluble portion of the cell lysates requiring an extra solubilization step (**Fig. 2A**). The estimated molecular weight of ΔHBcAg is 18.3

kDa as seen in lane 2 of **Fig. 2A**, making it slightly smaller than the other proteins. Those constructs with a single insert are expected to have a molecular weight closer to 19 kDa as seen in lanes 3 – 6 of **Fig. 2A**. The fifth construct, HBcAg + S1S2S3M is the largest protein and is expected to have a higher molecular weight near 22 kDa as seen in lane 7 of **Fig. 2A**. Expressions of each protein were achieved at a concentration of approximately 2 mg/mL and as approximately 60% of the total protein content as measured by BCA assay and densitometry respectively. The presence of each protein in the insoluble lysate was confirmed by Western blot analysis using anti-histidine6x Abs prior to purification (**Fig. 2C)**. All proteins were then purified, as can be seen in **Fig. 2B**. One of the candidates, HBcAg+M may have a smaller molecular weight impurity as seen in lane 6 of **Fig. 2B**. The protein, however, is above 96% pure by densitometry analysis indicating that it is safe for *in vivo* experiments. Dynamic light scattering (DLS) analysis of the AEX fractions (data not shown) each showed a particle size distribution centering between 30 and 40 nm. These measurements are as predicted, indicating that the VLPs are forming. Successful refolding and assembly of the proteins was then confirmed by TEM analysis (**Fig. 3**). In each case, the VLPs formed by the vaccine candidates (**Fig. 3B-F**) emulate that of the native protein (**Fig. 3A**). Finally, endotoxin measurements for safety in animal trials were performed after the AEX chromatography step. The highest value measured was 3 EU/mL, which was below the acceptable value for vaccination in humans as reported previously (data not shown) [46].

### *Intranasal Immune Response*

ELISA analysis of serum samples was performed for ΔHBcAg first, as a measure of overall immune response. Ab titers to ΔHBcAg in the IN group showed a poor Serum IgG response as well as serum IgA and fecal IgA responses (**Fig. 4A**, **4C**, **4E**, respectively). These data indicate that the IN administration resulted in a poor overall immune response. The highest serum IgG response came from the ΔHBcAg groups as expected, but the rest of the vaccines delivered by IN demonstrated very little response (**Fig. 4A**). The same pattern is true in the fecal samples, where there is only a significant difference between the ΔHBcAg group and all other vaccine candidates (**Fig. 4E**). There was no statistical significance between any of the vaccine candidate groups for serum IgA titers to the backbone protein (**Fig. 4C**). This result may be due to how low the overall serum IgA titers were in the intranasally administered groups. The titers to the ΔHBcAg coated plates were low enough in the IN groups that other analysis of the serum samples was not performed.

### *Intraperitoneal Immune Response*

Mice groups having received vaccinations through an IP administration were also tested for Abs specific to ΔHBcAg first. All vaccine candidates delivered by IP generated a strong Ab response to the ΔHBcAg protein. Serum IgG titers to histidine tagged ΔHBcAg increased with each booster (**Fig. 4B**). The mice treated with the HBcAg+S1S2S3M candidate showed a higher IgG titer

at the end of the experiment, whereas none of the other groups were found to be significantly different. Serum IgA titers to the ΔHBcAg coated plates also increased with each booster (**Fig. 4D**). Mice treated with ΔHBcAg, HBcAg+S1, and HBcAg+S1S2S3M produced the highest titers. The difference in serum IgA concentration between mice treated with HBcAg+S1 and HBcAg+S1S2S3M was not significant, but it is significant between groups treated with ΔHBcAg and HBcAg+S1S2S3M. Fecal IgA titers to ΔHBcAg demonstrated the production of IgA antibodies secreted into the intestines, with the ΔHBcAg treated mice having the highest values (**Fig. 4F**). Groups treated with HBcAg+S1 and HBcAg+S1S2S3M had similar fecal IgA titers, and were both higher than the other groups. Presence of antibodies in the feces indicated that the vaccination strategy was able to produce secretory IgA responses to the vaccine candidates, and may aid in prevention of the disease. It should be noted, however, that fecal IgA titers are not as highly correlated with intestinal secretions as serum IgA [47].

Each vaccine, when delivered in an IP injection, was also able to produce antibodies specific to the inserted PEDV epitopes as seen by ELISA analysis in **Figure 5**. In all cases, the immune response to each vaccine candidate was as anticipated. Only those mice treated with vaccines containing the S1 epitope showed reactivity to the EFP-S1 coated plates (**Fig. 5A**). The same is true for EFP-S2, EFP-S3 and EFP-M coated plates as well (**Fig. 5B – D** respectively). Together, these data indicate that there is very little cross-reactivity from each

vaccine candidate to the other epitopes. Interestingly, the HBcAg+S1S2S3M group demonstrated a statistically similar immune response to each individual epitope coating as compared to each corresponding individually inserted vaccine candidate. This indicates that the presence of multiple epitopes in the HBcAg+S1S2S3M protein did not reduce the Ab response to individual epitopes despite having been administered at the same dosage.

Immune responses to the inserted epitopes were also measured using a VN assay. Data in **Fig. 6** indicates only the HBcAg+S1 and the HBcAg+S1S23M vaccine candidates showed a significantly higher VN titer than the PBS negative control. It is worth noting that high doses of PEDV given to pigs have previously demonstrated a VN titer using the same assay to be around 32 [45].

## Discussion

Contemporary strategies in vaccination primarily focus on immune system identification and elimination upon exposure to the virus. This is usually done by Ab identification, where antibodies generated through vaccination can help mobilize elements of the immune system for swift elimination of pathogens. With PEDV, however, the most susceptible subjects (young piglets) have been damaged to too great of an extent during infection in the epithelia. It is therefore critical to stop the virus from entering the mesenteric epithelia, requiring virus neutralization by secreted immunoglobulins in the intestine. For suckling piglets, this requires the transfer of immunoglobulins through colostrum and milk.

Although it wasn't successful in this case, an IN administration may be preferable to help induce a virus-specific secretory IgA response in the lactating sow during the last ten days of gestation. This strategy could increase the amount of virus-specific IgA in the colostrum and milk to provide the lactogenic protection to the suckling piglet. The lack of immune response in the IN administered groups is likely due to a low contact time between the vaccine and the epithelia tissue. The introduction of a polymer, such as chitosan, in the formulation of the vaccines could allow the VLPs to adhere to the epithelial surface for a longer amount of time. An increase in contact time would then promote the internalization of the proteins and possibly increase the immune response [27, 48].

One important finding was that there was no difference in neutralizing Ab concentration between the monovalent HBcAg+S1 vaccine candidate and the multivalent HBcAg+S1S2S3M vaccine candidate. Individual epitope titers as measured by ELISA indicated that the HBcAg+S1S2S3M vaccine candidate was able to produce comparable specific antibody levels as the vaccine candidates containing the single epitopes. Given only the ELISA data, one might be tempted to conclude that the HBcAg+S1S2S3M vaccine candidate could be much more effective compared to the other proteins. The ELISA data indicates that HBcAg+S1S2S3M induced a higher overall immune response because of its ability to induce an equal epitope-specific titer in mice at the same dosage. Inclusion of multiple epitopes could also have the added benefit of providing a

more diverse immune response as well, allowing possible protection from multiple strains of the virus if mutations were to occur in the epitope regions. Neutralization data, however, indicates that there is a more complicated phenomenon.

A second important finding was that only two of the candidates were able to successfully neutralize virus using the assay as described. This may be due to the amount of virus that is used in the assay's execution, or the genotype of the virus itself. It is hypothesized that Abs corresponding to the S1 epitope are better at neutralizing the virus than Abs to the other epitopes. In this experiment, each individual vaccine candidate had a significantly higher ELISA titer to the epitope insertion than the negative controls, but there was a lack of VN response in the majority of the vaccine candidates. This discrepancy may be due to something as simple as conformational changes in the neutralizing motifs based on viral genotypes [49]. Another possible mechanism for prevention of neutralization may simply be efficacy. Monoclonal antibodies (mAbs) made and purified from the injection of the spike protein from PEDV have indicated that mAbs containing high titers to portions of the protein containing the YSNIGVCK (S1) motif have a much higher virus neutralization capability than mAbs with high titers to peptide sequences containing either the SQYGQVKI (S2) or the GPRLQPY (S3) motifs [50]. Though most mAbs produced in that study indicated that there was some neutralization activity, the reduced VN titers may indicate that they are less

suitable targets in a subunit vaccination strategy for PEDV. The relatively high virus titers used in the VN testing during this experiment may have simply overwhelmed antibody populations with lower utility in neutralization.

It is important to note that mice treated with HBcAg+S2 cannot be included in the data to support this hypothesis. The HBcAg+S2 vaccine was originally designed to have the neutralizing antibodies from the CV777 strain originally found in Denmark. However, many of the strains in the recent (2013) outbreak in the US are phylogenetically distant from the original strain when arranged based on the genome coding for the spike protein [51, 52]. Indeed, sequence data from Indiana based strains during the 2013 outbreak such as that used for the assay confirms that the neutralizing “S2” epitope sequence is actually $^{756}$SQSGQVKI$^{771}$ instead of $^{756}$SQYGQVKI$^{771}$ as was used in the vaccine design. This oversight can be easily changed, possibly allowing for a more effective multivalent vaccine candidate.

It was interesting that the HBcAg+S1S2S3M vaccine candidate was able to neutralize the same amount of PEDV on a per dose basis as the HBcAg+S1 candidate. With four epitopes inserted into the MIR of the ΔHBcAg, it could be hypothesized that the S1 epitope would have a 1:4 chance of interaction with a B-cell receptor. Perhaps the inclusion of multiple B-cell epitopes in the MIR increases the pathogen associated molecular patterns (PAMPS) recognized by

the immune system, and therefore increases the overall immunogenicity of the VLP. This phenomenon may be of use in the design of future vaccine candidates. If the inclusion of multiple conserved B-cell epitopes raises the overall immunogenicity of the vaccine without depleting its ability to produce neutralizing antibodies, then perhaps this strategy could be employed to produce a vaccine that infers protection to both suckling piglets as well as adult pigs.

One possible pitfall to using the HBcAg backbone protein might be the prevalence of swine hepatitis B virus (SHBV). Recent evidence suggests this prevalence may be as high as 25% in agricultural pigs [53]. However, another study has indicated that the presence of anti-HBcAg Abs in sows may be closer to 12% [54]. There is therefore concern that the presence of these antibodies in a pregnant sow may cause the elimination of the vaccine through previous immunity, reducing its efficacy. One possible strategy to combat this phenomenon may be through simple redesign of the vaccine. The elimination of key amino acids in the sequence of the backbone have been shown to reduce immunity to the HBcAg portion of the protein and enhance the immune response to the insert [55]. It may therefore be possible to modify the backbone protein, maintaining vaccine efficacy while reducing the exposed epitopes from HBcAg.

## Conclusions

The preceding data indicate that the HBcAg VLP platform shows promise as an effective tool in a vaccine strategy toward PEDV. Neutralization of a strain

of PEDV from the more recent outbreak corroborates previous identification of the YSNIGVCK motif from the S-protein in PEDV. ELISA titers to three other B-cell epitopes indicate that though they are able to produce significant Abs, the Abs produced may have limited neutralization of high titers of virus. There seemed to be no disadvantage to the inclusion of multiple epitopes in the MIR of HBcAg on viral neutralization, indicating that the multivalent strategy might allow for an effective vaccine used for herd immunity as well as lactogenic protection.

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## Figures

Figure 1 - Each vaccine candidate is based off of the ΔHBcAg protein.

Foreign epitopes have been inserted into the major immunodominant region (MIR) in each case after AA79. Each construct was named as follows: (A) ΔHBcAg, (B) HBcAg+S1, (C) HBcAg+S2), (D) HBcAg+S3, (E) HBcAg+M, (F) HBcAg+S1S2S3M.

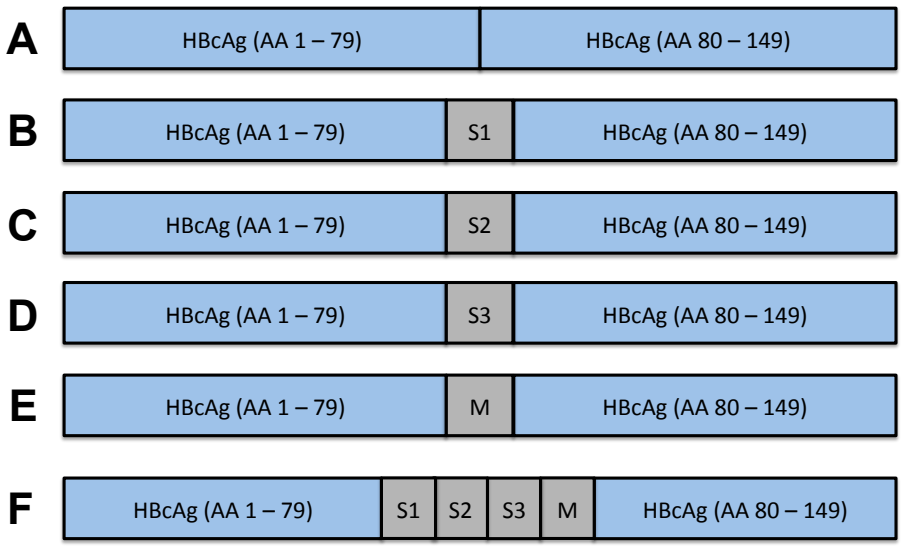


Figure 2 - Purification of each construct was performed and tested using NuPAGE gels.

In each case, the lanes are ordered as follows: (1) Protein Ladder, (2) ΔHBcAg, (3) HBcAg+S1, (4) HBcAg+S2), (5) HBcAg+S3, (6) HBcAg+M, (7) HBcAg+S1S2S3M. (A) Cell lysate samples indicated successful expression of each protein construct. (B) Elimination of impurities prior to formulation as seen by SimplyBlue™ Safestain staining. (C) A Western blot performed on cell lysates using anti-His6x conjugated antibodies for detection.

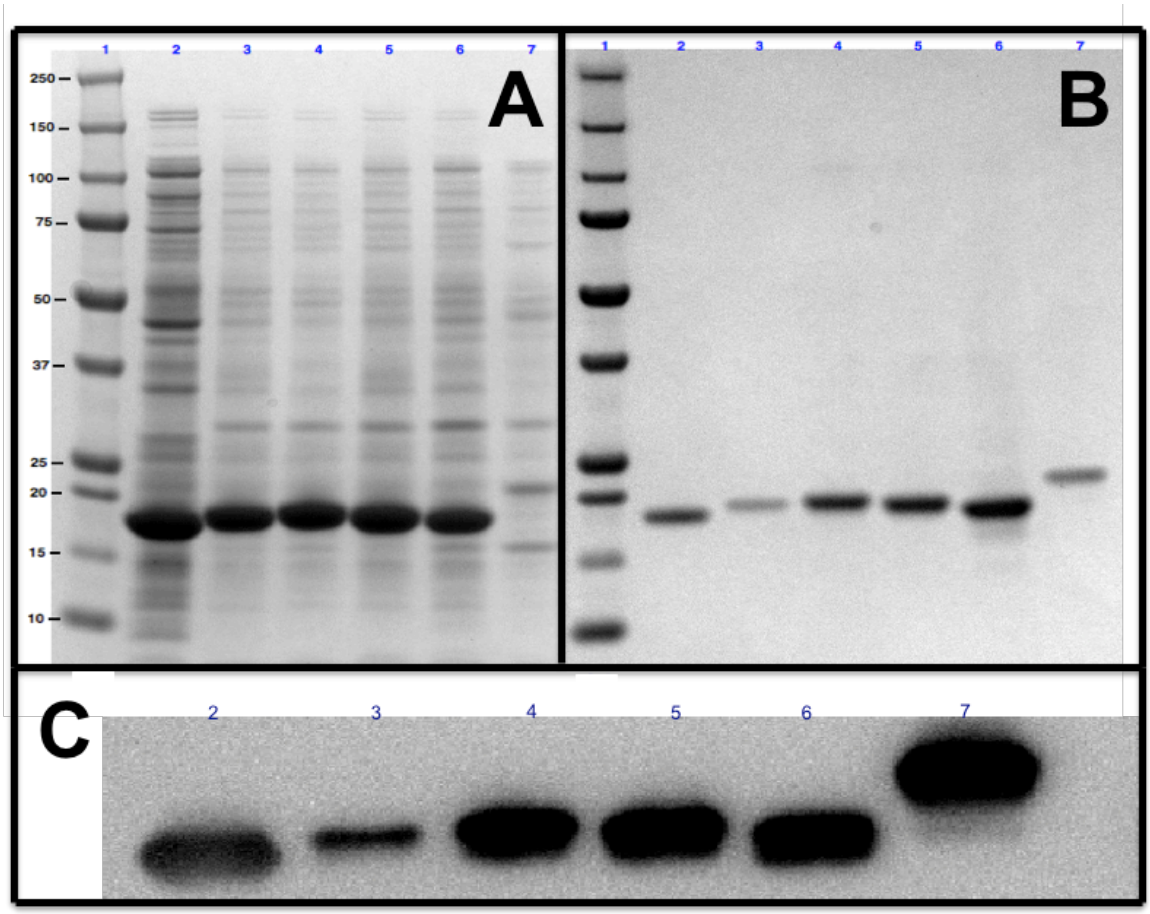


Figure 3 - Transmission electron microscopy was used to elucidate the assembly of each vaccine candidate.

(A) ΔHBcAg, (B) HBcAg+S1, (C) HBcAg+S2), (D) HBcAg+S3, (E) HBcAg+M, and (F) HBcAg+S1S2S3M.

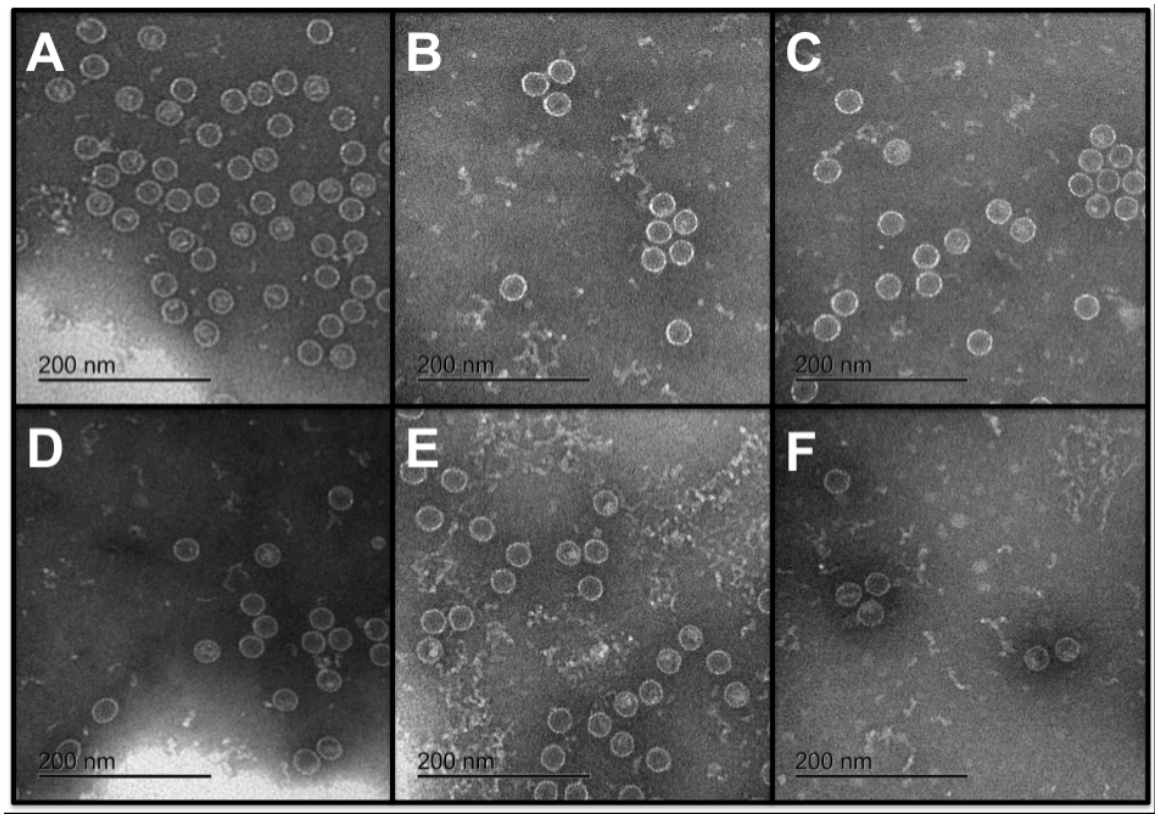


Figure 4 - Preliminary analysis of immune response was performed using an ELISA assay for antibodies to ΔHBcAg.

(A) Serum IgG Titers for IN Administration, (B) Serum IgG Titers for IP Administration, (C) Serum IgA titers for IN Administration, (D) Serum IgA Titers for IP Administration, (E) Fecal IgA titers for IN Administration, and (F) Fecal IgA titers for IP Administration. Each value is compared to a PBS negative control group on each plate. Error bars represent the standard error of the measurements. (* = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$)

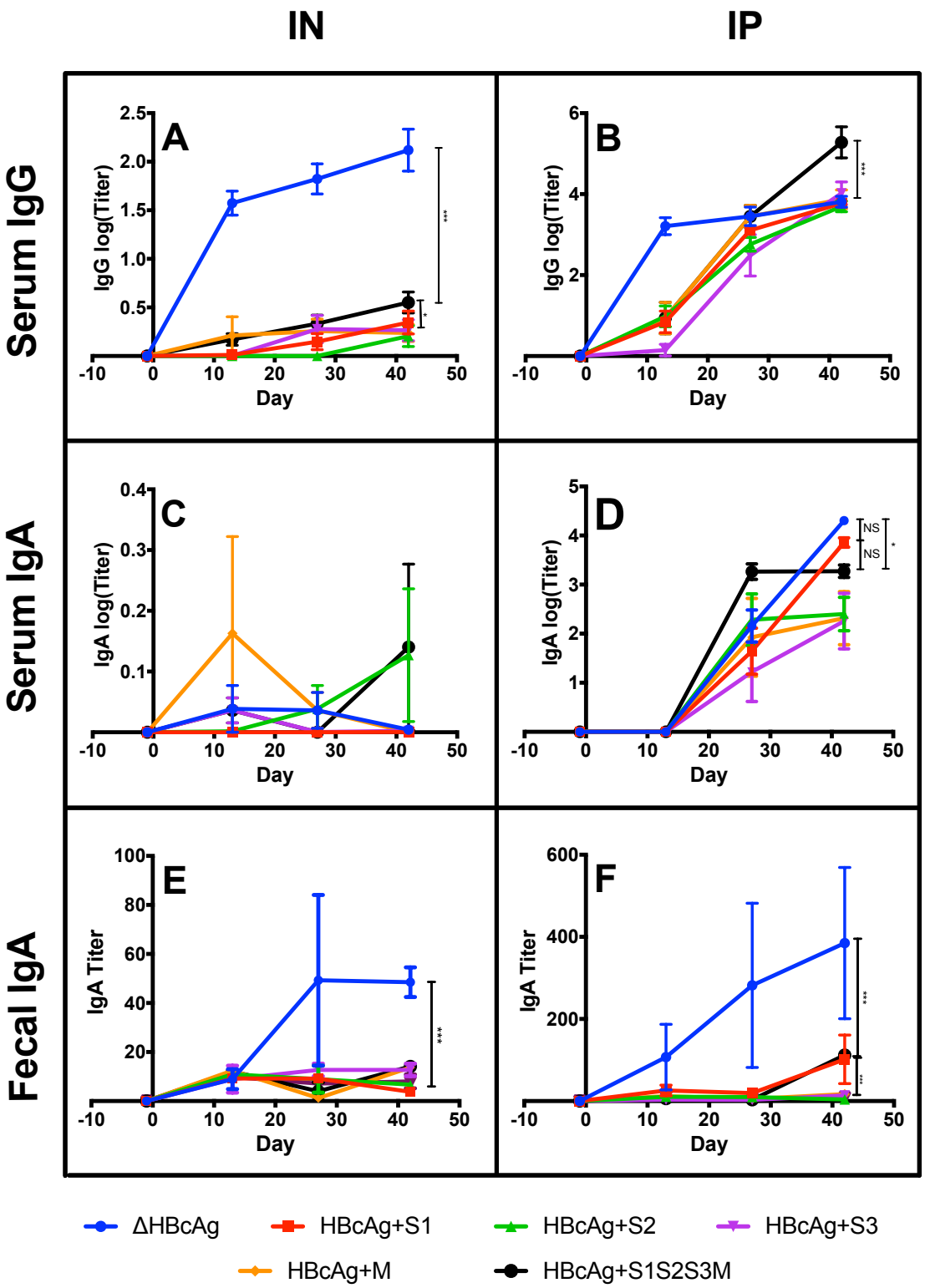

IN
IP
Serum IgG
Serum IgA
Fecal IgA
A
B
C
D
E
F
IgG log(Titer)
IgA log(Titer)
IgA Titer
Day
NS
ΔHBcAg
HBcAg+S1
HBcAg+S2
HBcAg+S3
HBcAg+M
HBcAg+S1S2S3M

Figure 5 - ELISA titers to individual epitopes.

ELISA was performed on samples from 42 days post inoculation with EFP proteins containing the individual epitopes as coating proteins to test the immune response to the inserted epitopes. All samples were tested for an immune response to each epitope to ensure there was no cross-reactivity. Coating peptides are as follows: (A) EFP-S1, (B) EFP-S2, (C) EFP-S3, and (D) EFP-M. Each value is compared to a PBS negative control group on each plate. Error bars represent the standard error of the measurements. (* = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$)

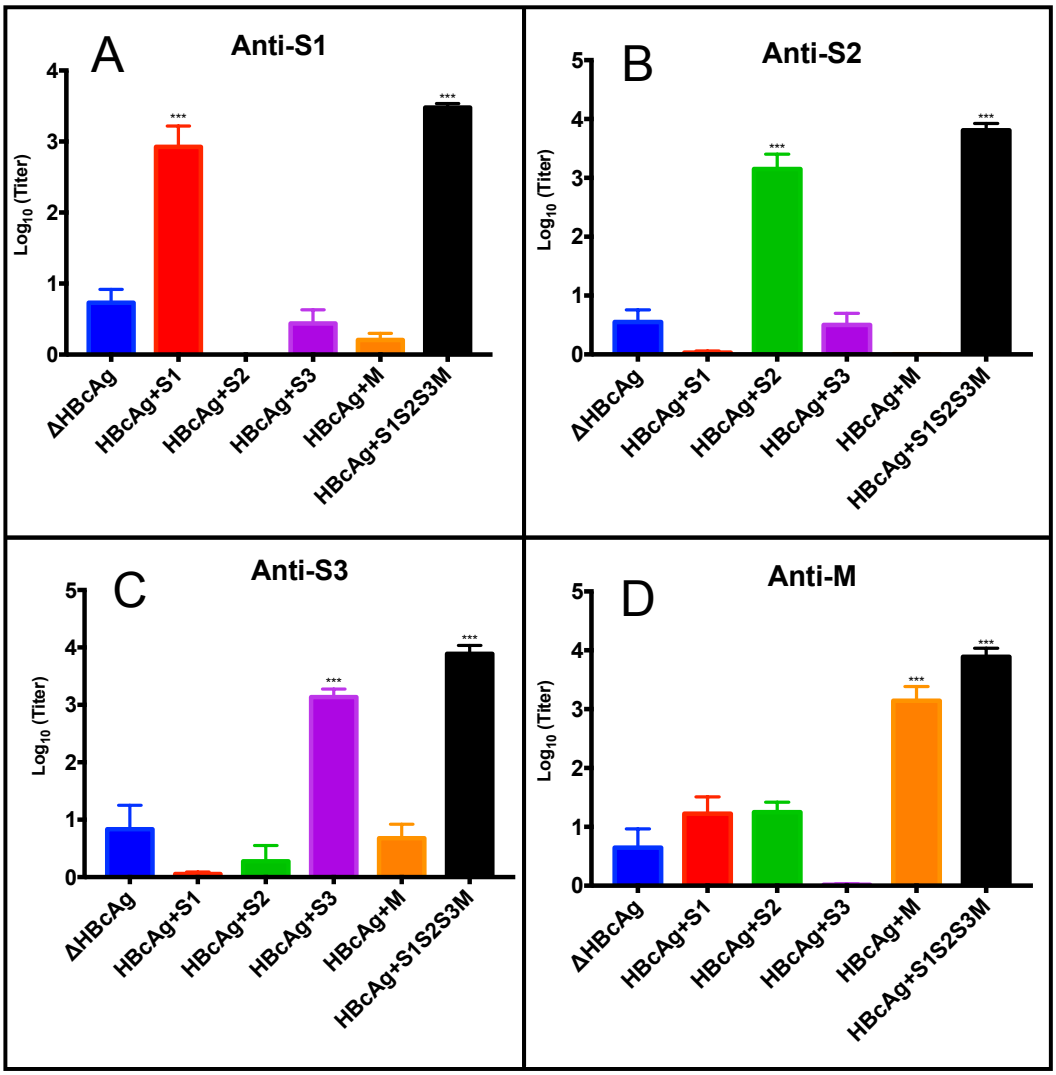
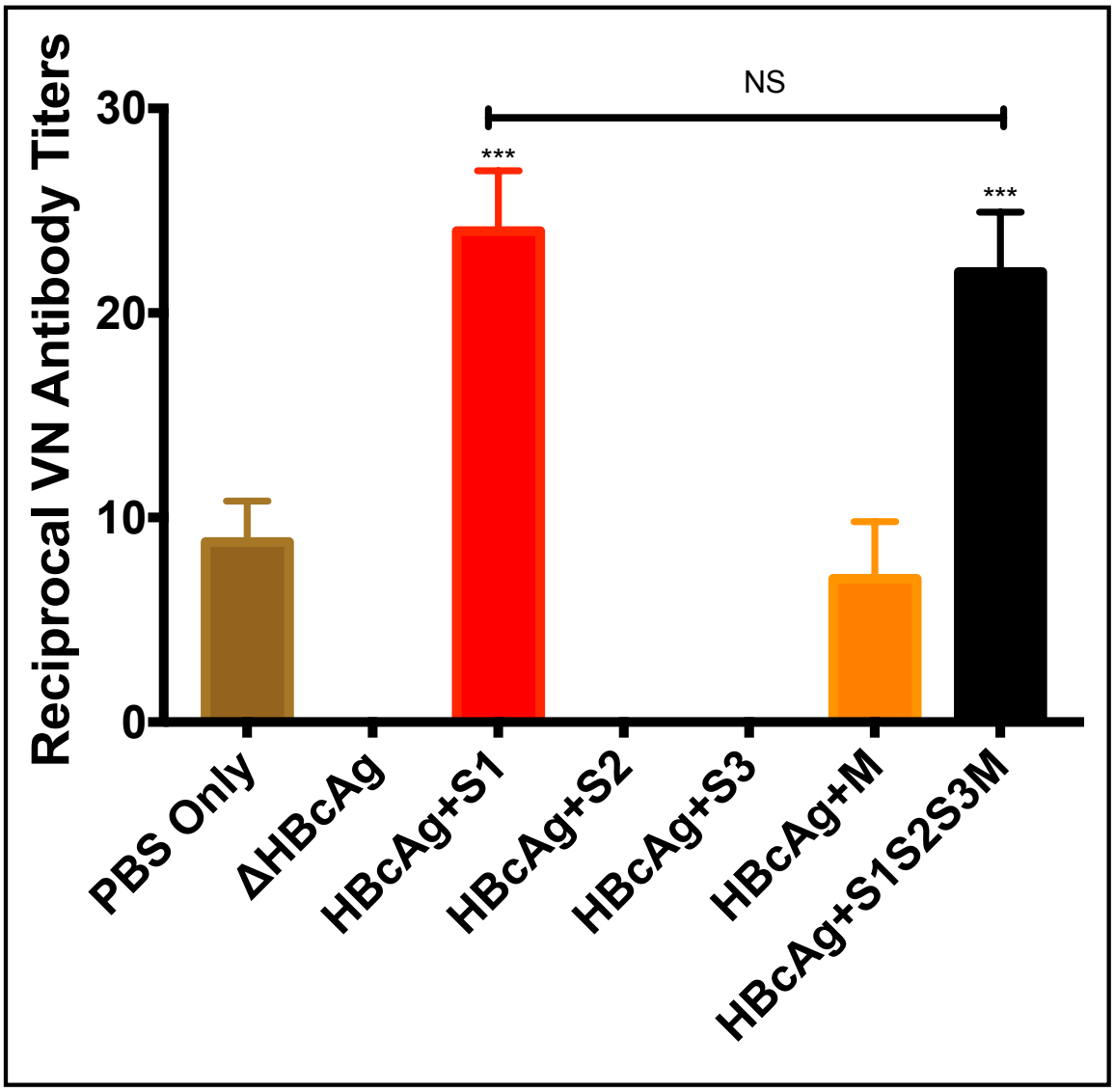



Figure 6 - A virus neutralization assay was run on samples from the 42nd day post inoculation.

Error bars represent the standard error of the measurements. (* = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001)


Reciprocal VN Antibody Titers
30
20
10
0
NS
***
***
PBS Only
ΔHBcAg
HBcAg+S1
HBcAg+S2
HBcAg+S3
HBcAg+M
HBcAg+S1S2S3M

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# Chapter IV: Design improvements on a novel vaccination strategy for porcine epidemic diarrhea Virus utilizing the Hepatitis B virus core antigen for increased virus neutralization.

**Design improvements on a novel vaccination strategy for porcine epidemic diarrhea Virus utilizing the Hepatitis B virus core antigen for increased virus neutralization.**

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Abstract:

Porcine epidemic diarrhea virus (PEDV) is a member of the *Alphacoronaviridae* genus within the *Coronaviridae* family. It is the causative agent of porcine epidemic diarrhea, a disease that can have mortality rates as high as 100% in suckling piglets. PEDV causes severe economic loss, and has been in existence for decades. A panzootic starting in 2010 renewed interest in the development of a recombinant vaccine strategy toward PEDV. This report details several design changes made to a Hepatitis B virus core antigen (HBcAg)-based recombinant vaccine strategy, and their effect *in vivo*. Initially, several multivalent vaccine candidates were able to elicit antibodies specific to three out of four B-cell epitopes inserted into the chimeric proteins. However, a lack of virus neutralization led to a redesign of the vaccines. The focus of the newly redesigned vaccines was to elicit a strong immune response to the YSNIGVCK amino acid motif from PEDV. Those candidates that were able to demonstrate a strong antibody response to the YSNIGVCK peptide were correlated with an increased ability to neutralize the CO strain of PEDV. This report also presents evidence that placement of epitope inserts and a mutation in the HBcAg backbone protein can increase the overall immune response to the inserted epitopes.

Importance:

This report details multiple recombinant vaccinations for PEDV utilizing the HBcAg VLP chimeric protein format. Several design strategies were used and presented to allow the maximal efficiency in antigen presentation to the immune cells within the body. Previous evidence suggested that the motif YSNIGVCK might be of more importance in virus neutralization when compared to other B-cell epitopes from PEDV. The data presented demonstrates much stronger evidence that this is indeed the case, and perhaps this epitope should be considered of high priority in future recombinant vaccine design.

**Keywords**: HBcAg, PEDV, Vaccine, VLP, Purification

**Abbreviations**: PEDV, Porcine Epidemic Diarrhea Virus; EFP, ELISA Fusion Protein; Ab, Antibody; mAb, monoclonal antibody; HBcAg, Hepatitis B virus core antigen; VLP, Virus-like Particle; MIR, Major Immunodominant Region; VN, Virus Neutralization; GST, Glutathione-S-Transferase; AEX, Anion Exchange; IMAC, Immobilized Metal Affinity Chromatography; IPTG, Isopropyl β-D-1-thiogalactopyranoside; ORF, Open Reading Frame; S, Spike; E, Envelope; M, Membrane; N, Nucleocapsid; MLV, Modified Live Virus; KV, Killed Virus; aa, Amino Acid; COE, CO-26K equivalent;

## Introduction

Porcine epidemic diarrhea virus (PEDV) is a mesenteric virus that causes severe diarrhea, dehydration, and depression [1, 2]. PEDV is a species within the *Alphacoronavirus* genus (previously the type I *Coronaviridae*) in the *Coronaviridae* family of the *Nidovirales* order [3, 4]. Instances of PEDV began in the early 1970's, but outbreaks had been relatively tame, small, and sporadic until a panzootic occurred beginning in 2010. A mutation in the virus led to an increased virulence and caused severe worldwide economical losses as well as the introduction of the disease into the North America and the United States in 2013 [5-9].

The typical genome for all *Coronaviridae* contains a 5' and a 3' untranslated region (UTR), and contains a range of open reading frames (ORFs) in between [10]. PEDV has an approximately 28 kb genome and seven ORFs. The ORFs are arranged 5' to 3' in the order of ORF1a, ORF1b, spike (S) protein, envelope (E) protein, membrane (M) glycoprotein, ORF3, and nucleocapsid (N) protein. For most coronaviruses, the S protein undergoes posttranslational modifications including cleavage into an S1 and an S2 domain [11]. This is not true for PEDV, but there is a conventional separation of the protein into these domains around amino acids (aa) 781/782. Increased virulence is associated with mutations in the ORF3, E, and M proteins of the genome in new strains of PEDV emerging in 2010 - 2013 [12-14]. Although several modified live virus

(MLV) and killed virus (KV) vaccines exist on multiple markets, some of these vaccines are associated with side effects including viral shedding and reversion to an active state for MLV vaccines. There has therefore been an increased interest in recombinant vaccine strategies toward PEDV.

Recent research in recombinant vaccination against PEDV has predominantly focused on the S protein [15-18]. Many of these strategies have focused on the S1 domain of the S protein (aa 1 – ~785, depending on species). The S1 domain contains a region of importance known as the CO-26K equivalent (COE) region, a peptide sequence spanning aa 503 – 715 known to be similar to a neutralizing epitope from transmissible gastroenteritis virus (TGEV) [19]. However, this region is non-linear and large. There are two other known, small, and linear B-cell epitopes within the S1 domain corresponding to the aa sequences $^{748}$YSNIGVCK$^{755}$, and $^{764}$SQYGQVKI$^{771}$ from the original CV777 strain [20-22]. An additional small B-cell epitope was discovered within the S2 domain corresponding to the aa sequence $^{1368}$GPRLQPY$^{1374}$ [20]. A fourth short, linear B-cell epitope is located in the M protein corresponding to the sequence $^{195}$WAFYVR$^{200}$ [23]. In one experiment, a virus like particle (VLP) was able to assemble and induce an immune response in mice using the S, M, and E proteins [24].

Hepatitis B virus core antigen (HBcAg) is a popular virus-like particle (VLP) used in a chimeric form for antigen presentation of foreign epitopes. Upon expression and purification, VLPs can be successfully assembled *in vitro.* HBcAg is typically divided into two separate sections for the sake of chimeric protein design. The change from the N-terminal to the C-terminal section occurs around Proline-79, a loop section previously identified as the major immunodominant region (MIR) [25]. Insertion of foreign epitopes in this section will have the highest immunogenic response. An N-terminal insertion of amino acids into HBcAg has been shown to have efficient antigen presentation as long as there is a long enough linker present between the native start codon and the antigen to allow the N-terminal amino acids to form crude spike structures on the surface of the VLP [26, 27]. C-Terminal insertion of epitopes has also been shown to produce antibodies specific to the inserted peptide sequence, though to a lesser degree [25, 28].

Previous work by this lab used HBcAg as a backbone protein in a novel PEDV vaccination strategy. In the preliminary experiment, five treatment vaccines were designed including four candidates with each of the four ($^{748}$YSNIGVCK$^{755}$, $^{764}$SQYGQVKI$^{771}$, $^{1368}$GPRLQPY$^{1374}$, $^{195}$WAFYVR$^{200}$) linear B-cell epitopes inserted individually into the MIR. A fifth candidate had all four epitopes together in a linear peptide sequence inserted into the MIR of the HBcAg backbone protein [29]. The resultant epitope Ab titers as measured by

ELISA where not correlated with virus neutralization aside from that of the $^{748}$YSNIGVCK$^{755}$ motif. It was therefore hypothesized that this motif may be better able to neutralize the virus, a property that could be of importance in future vaccine design.

In an experiment performed by Okda et al, whole virus from the CO13 strain was used to generate a series of monoclonal antibodies (mAbs) to PEDV which were tested for their ability to neutralize the virus *in vitro* [22]. They were able to narrow down the mAbs' active sites to relatively short peptides, four of which were located in the S2 domain and corresponded to the sequences $^{744}$CTEPVLVYSNIGVCK$^{759}$, $^{747}$PVLVYSNIGVCKSGSIGYVPSQSGQVKI$^{771}$, $^{756}$VCKSGSIGYVPSQSGQ$^{771}$, and $^{1371}$GPRLQPY$^{1377}$. Three out of four of these sequences contained the previously identified B-cell epitopes to PEDV. One sequence, $^{756}$VCKSGSIGYVPSQSGQ$^{771}$, contained only a small portion of two of the epitopes with a fairly conserved spacer in between. Interestingly, the mAbs identified as subclass IgG1 and IgG3 with a titer response to the $^{744}$CTEPVLVYSNIGVCK$^{759}$ and the $^{747}$PVLVYSNIGVCKSGSIGYVPSQSGQVKI$^{771}$ motifs showed a remarkably higher ability to neutralize the CO13 strain of PEDV as measured by two separate assays.

This report focuses on several design changes testing multivalence, structural mutations, and epitope placement. All constructs used in this report are outlined in Fig. 1A. In the first experiment, the vaccines were designed to induce an immune response to multiple B-cell epitopes in a single dose. During the second experiment, the focus of the re-design was to induce a maximal reaction to the $^{748}$YSNIGVCK$^{755}$ motif by separating the epitopes between domains of the backbone protein.

It was recently discovered that the elimination of some residues in the MIR, 79-Pro-Iso-Ser, could increase the immune response to the inserted epitope in lieu of an immune response to the HBcAg backbone protein [30]. In this report, this modification is denoted as an “s” prior to the epitope insertions as in “HBcAg+sXX.” Insertion of foreign epitopes does not interfere with VLP assembly [31]. However, it may be possible that the addition of some flexible, water-soluble amino acids could aid in epitope presentation. For this report, addition of three serine amino acids between PEDV epitopes is denoted with an “m” as in “HBcAg+mXX.”

Modifications have been made to the VLP backbone away from the MIR as well. During normal assembly of HBcAg-based proteins, dimers form, followed by higher ordered structures. Upon successful assembly, two 61-cys residues of a dimer are able to form a disulfide bond. Though the cys residues are not

required for assembly, they are important in the speed at which the VLPs form and in the strength of the resultant VLP [32]. The mutation of two residues within the HBcAg sequence to cysteine (D29C and R127C) followed by the successful assembly of the mutated HBcAg VLPs (Fig. 1B) causes the creation of a network of additional disulfide covalent bonds between pentamers and hexamers in the assembled product (additional disulfide bonds shown in red in Fig. 1C and Fig. 1D) [33]. For the purposes of this report, this modification is denoted as an additional “m” prior to the protein name as in “mHBcAg.” Successful execution of these mutations followed by assembly has demonstrated an increase in the strength of the assembled VLPs without morphological change, but their immunogenic properties are uncertain [33]. All vaccines were tested for *in vivo* immunogenicity and PEDV neutralization, with the goal of finding a vaccine candidate that was superior to others.

## Results

### *Expression and Purification*

Overexpression of each protein of interest (POI) resulted in the presence of insoluble inclusion bodies (IBs) with the exception of ΔHBcAg. After solubilization, POI concentrations were approximately 2 mg/mL and constituted approximately 60 – 65% of the total protein (data not shown). Each protein went through the same purification process. Final, successfully purified proteins can be seen in Fig. 2A and Fig. 2B for the first experiment and second experiment

respectively in reduced Nupage gels stained by SimplyBlue™ SafeStain. Purification is considered a success if the densitometry reading of the final product is >96%, indicating that the vaccine is safe for animal use [29]. The estimated molecular weight of ΔHBcAg is 18.3 kDa. Constructs containing single or multiple epitopes should appear higher, at approximately 19 kDa and 23 kDa respectively. Vaccine candidates with added amino acids between epitopes are expected to be slightly higher in molecular weight than 23 kDa in a reduced sample gel.

For the non-reduced samples, multiple bands were seen at multiple sizes, with distinctly darker bands appearing at the monomer and dimer (~18.3 kDa and ~36 kDa respectively for ΔHBcAg) ranges of size. The presence of dimers in a non-reduced PAGE preparation indicates there is a good rate of disulfide bond formation between the $^{61}$C residues in monomers [32]. The mutated HBcAg backbone constructs (mHBcAg+ms4E, and mHBcAg+SSM) both had a much lighter band at both the monomeric and the dimeric size range under non-reducing sample preparations, while reducing conditions demonstrated a single, monomeric sized protein alone. These gels therefore indicate that a lack of a reducing agent in each case eliminated the lower ordered monomers and dimers from the sample when the mutations were made. These results would be expected if the network of disulfide bonds were forming between multiple dimers

as indicated in Fig. 1C and Fig. 1D. Overall, the gels indicate a successful purification and assembly scheme.

The protein expression was confirmed by western blot analysis of crude lysate samples using anti-his6x polyclonal antibodies (Abs) (Fig. 2C and Fig. 2D). In each case, dark bands appear at sizes corresponding to their respective purified NuPAGE analyses. Fig. 2C also appears to have lighter bands above the monomer range of sizes. These are likely dimers, as their pattern of size mimics the configuration of the monomers and the non-reduced NuPAGE gels seen in Fig. 2A. All purified and assembled VLPs were tested for assembly using DLS (data not shown) and verified with TEM (Fig. 2E). TEM pictures indicate that each construct was successfully assembled and each test vaccine emulated the native VLP.

### *Epitope Antibody Generation*

During the first animal trial, there was a strong immune response seen to three out of four of the individual epitope peptides as seen in Fig. 3A - 3D for both HBcAg+s4E as well as mHBcAg+ms4E. It is important to note that the responses seen to the YSNIGVCK motif were minimal (Fig. 3A), while the immune response to the GPRLQPY motif was quite strong (Fig. 3C). The lack of response to the YSNIGVCK motif may indicate that there is an interaction taking place between amino acids in this section of the vaccine candidates that allows the masking of the epitope from B-cell interaction [34]. An initial hypothesis was

made implicating either hydrophobicity or secondary structure as the mechanism behind the interaction of the YSNIGVCK motif. To further elucidate this interaction, hydrophobicity of each epitope insert was measure by its GRAVY score and are shown in Fig. 3E. These scores indicate that although the most hydrophilic motif (GPRLQPY) had the highest immune response, the most hydrophobic motif (WAFYVR) did not have the lowest immune response. It therefore appears that the interaction is not purely hydrophobic.

There was no statistical difference between any of the test groups for an immune response to the WAFYVR epitope, although all three were statistically different than the ΔHBcAg treated group. Although there was no difference between mHBcAg+ms4E and the HBcAg+s4E groups, there was an increased immune response in both the mHBcAg+ms4E and HBcAg+s4E treated groups compared to the HBcAg+ms4E group for both the SQSGQVKI and the GPRLQPY epitopes (Fig. 3B and Fig. 3C respectively). The lower immune response of the HBcAg+ms4E group compared to the HBcAg+s4E indicates that there may be a distinct disadvantage to the addition of flexible amino acids between epitopes in the MIR of HBcAg. However, the increased immune response of the mHBcAg+ms4E treated group indicates this disadvantage can be overcome by mutation of the HBcAg backbone to include an extra network of covalent disulfide bonds created by the additional cysteine residues upon assembly.

During the second experiment, the SQSGQVKI epitope was dropped from the design due to its lack of conservation across the PEDV phylogenetic tree. One vaccine candidate, HBcAg+SE contains only the YSNIGVCK motif in the MIR of HBcAg without any modification to the backbone protein. It should be noted that this exact vaccine candidate was used previously by this group, and demonstrated PEDV VN neutralization [29]. Three adjuvant schemes were used on this same vaccine candidate to test their efficacy. These three formulations included a group without adjuvant, a group with alum alone, and a group with MPLA in addition to alum. The two groups receiving the same vaccine, but containing alum as an adjuvant, showed little or no immune response to the YSNIGVCK peptide epitope inserted into the MIR as previously done (Fig. 4A). Because the formulations of all three vaccines were done using the same product, it appears as though the addition of alum to the formulation was detrimental to the application. None of these three vaccine candidates demonstrated an immune response to the GPRLQPY nor the WAFYVR epitopes, as expected since they did not include those sequences in their design.

Alum was also included in the formulation of both the HBcAg+SSM, and the mHBcAg+SSM vaccine candidates. However, one of these candidates, the mHBcAg+SSM protein, was able to elicit a response to all three individual epitopes included in its design (Fig. 4A – 4C). This data again indicates that the

additional disulfide bonds between the added cysteine residues formed upon assembly of the mHBcAg+SSM VLPs had a large impact on the immunogenicity towards the individual peptide epitopes in the presence of alum.

### *Backbone Peptide Antibody Generation*

There is a great concern in the recombinant production of mosaic protein-based vaccines that pre-existing Abs to the backbone protein may cause the elimination of the vaccine prior to its interaction with the necessary immune cells. The Ab titers toward the ΔHBcAg protein itself were therefore tested in conjunction with the individual epitope inserts. During both trials, there was significant antibody production to the native protein (Fig. 5A and 5B). There is no statistical difference in the Ab generation between vaccine candidates in the initial mouse trial (Fig. 5A). There was also no significant difference during the second mouse trial between the HBcAg+SE groups and the ΔHBcAg treated group, an interesting finding given that the response to the inserted epitope was non-existent. However, there was statistical significance in the groups treated with HBcAg+SSM and mHBcAg+SSM where they had lower Ab responses to the backbone protein compared to the other candidates and the ΔHBcAg treated group (Fig. 5B). This finding suggests the placement of a PEDV epitope (GPRLQPY) N-terminal to the histidine tag was able to reduce the Ab response to the backbone protein. Furthermore, there was a significant difference between the HBcAg+SSM and the mHBcAg+SSM treated groups, indicating that the

added disulfide bonds of the mutated candidate were able to further reduce the immune response to the histidine tagged backbone protein (Fig. 5B).

In an effort to determine the cause of this difference, ELISA plates were coated with an unrelated protein, the *Acinetobacter* phage-derived VLP AP205, which was expressed and purified from the same plasmid (pET-28a) as the PEDV vaccine candidates. The protein therefore has the same histidine tag sequence on its N-terminus, including the region between the tag and the protein's original start codon. An ELISA analysis demonstrating specificity toward this otherwise unrelated protein should indicate a specificity of the Abs to the N-terminal tag alone. Interestingly, the ELISA results show Ab titers having the same pattern as to the ΔHBcAg. This finding indicates that the histidine tag itself is available to the immune cells and is perhaps somewhat to blame for Ab titers to the ΔHBcAg protein (Fig. 5B and 5C). This finding is not surprising, since the tag is N-terminal to the HBcAg itself and this portion of the protein can form crude spikes upon assembly [26, 27]. Figure 5C shows that there is no significant difference between treatment groups from the initial experiment in Ab titer toward the histidine tag. However, Figure 5D indicates again that groups treated with HBcAg+SSM and mHBcAg+SSM had lower Ab responses to the histidine tag than the other groups. Again, the mHBcAg+SSM treated group had a significantly lower Ab titer to the histidine tag than even the HBcAg+SSM treated group (Fig. 5D). Together, these data indicate that the histidine tag on the N-terminus of the

vaccine candidates may play a large role in Ab recognition to the backbone protein by eliciting an immune response to the tag itself. Insertion of a target epitope N-terminally to a N-terminal histidine tag in HBcAg-based vaccines may therefore block recognition of the tag by the immune system.

### *Virus Neutralization (VN)*

The response of the murine serum to the virus itself was also determined. The CO strain was therefore used in a viral neutralization assay to determine the ability of the generated Abs to prevent virus binding to susceptible cells. Figure 6A shows the VN titers from the preliminary experiment. Although there is statistical significance between the three test groups (HBcAg+4E, HBcAg+ms4E, and mHBcAg+ms4E) and the negative controls (ΔHBcAg, and PBS Only), the numbers remain relatively low. This is true especially if the numbers are compared to the second experiment (Fig. 6B). The averages of the two treatment groups (HBcAg+SE and mHBcAg+SSM+Alum) previously demonstrating a high titer to the YSNIGVCK motif also demonstrated a high ability to neutralize virus.

## Discussion

Initially, the goal of this project was to improve upon vaccine designs made and tested previously for PEDV. Past evidence had indicated that the insertion of all four B-cell epitopes into the MIR of HBcAg did not reduce nor enhance viral neutralization when compared to the YSNIGVCK epitope alone. Keeping the virus neutralization in mind, the hope was to improve upon the

multivalent vaccine by increasing the immune response while maintaining an overall advantage with regard to herd immunity. This was to be accomplished by the modification of the backbone protein in an effort to reduce the immune response to ΔHBcAg as well as include some flexible peptide linker sequences in an effort to increase epitope exposure. If the designs had gone as anticipated, an overall increase in efficacy of the vaccines should have been seen. However, ELISA results indicate that the YSNIGVCK motif was hidden from the immune system, meaning that the viral neutralization ability was nullified. It is hypothesized that this is due to secondary structure, as GRAVY scores indicate that a more hydrophobic epitope, WAFYVR, was still exposed to immune cells. Theoretical evidence for this hypothesis includes the elimination of the $^{79}$P residue, which may have previously prevented the secondary structural interaction from taking place. Any additional flexibility produced by the inclusion of the serine peptides between epitopes for added flexibility in the MIR seemed to allow even further interactions between epitopes, as indicated by the reduced overall immune response to the individual epitope peptides. This may be due to an increased interaction between amino acid residues within the immunogenic region, or with the backbone protein. Despite the elimination of the YSNIGVCK Abs, there was a very strong immune response to the other epitopes in two of the new vaccine candidates, with the GPRLQPY epitope seemingly taking precedence.

One important finding during this initial trial was the consistent improvement in epitope titer in the mHBcAg+ms4E group when compared to the HBcAg+ms4E group. Each of the MIRs for these two proteins is coded the exact same, as is the rest of the protein with the exception of the D29C and R127C mutations in the mHBcAg+ms4E vaccine. It is hypothesized that an improvement in the strength of the assembled VLPs may increase their half-life in the body, allowing them more time to interact with the immune system in the assembled form.

With the preliminary data in mind, the focus of the second experiment was two-fold: to test the use of alum and MPLA as an adjuvant using a vaccine candidate known to induce an VN immune response, and attempt two redesigns to try to further increase the immune response with a focus on the YSNIGVCK epitope for virus neutralization. The non-adjuvanted HBcAg+SE treatment group saw a positive immune response while the other two formulations saw a complete lack of immune response to the MIR region and the epitopes therein. Since all three HBcAg+SE groups received the same vaccine with different formulations, it is hypothesized that the inclusion of alum in this case has eliminated any immune response to the individual epitopes in the MIR. The use of MPLA as a pIgR agonist was therefore not successfully measured, due to a complete lack of response. However, each of these treatment groups was capable of inducing a response to the ΔHBcAg protein. It is therefore

hypothesized that the assembled VLPs were either broken up by the combined formulation, or the alum was too successful in its ability to maintain the vaccine at the site of injection. In the case of the first hypothesis, the resultant antigens would be individual ΔHBcAg proteins, dimers, or higher ordered structures as antigens. In the case of the second hypothesis, there may be reduced ability for the VLPs to drain into the lymph nodes until after they have been degraded into the same lowered ordered structures (dimers, etc.).

Further evidence for these hypotheses comes from the two redesigned vaccine candidates (HBcAg+SSM and mHBcAg+SSM). The sole difference between the HBcAg+SSM and mHBcAg+SSM candidates was the D29C and R127C mutations in the backbone protein. Again, since the added network of covalent disulfide bonds exists in the assembled VLPs of the mHBcAg+SSM vaccine, it is hypothesized that stability is the mechanism behind its success. If, as hypothesized above, the alum is causing the degradation of the VLPs prior to their exposure to the immune cells, then it could be that strengthening the assembled VLPs allows them exposure in spite of the presence of alum. Should this theory hold true however, then it may be expected that there should be a reduced immune response to the backbone protein, since the immune system would have less access to the disassembled protein structures of the mutated VLPs. Though the results from the second trial (HBcAg+SSM and mHBcAg+SSM) indicate that this is indeed the case, the results from the initial

trial (HBcAg+4E and mHBcAg+4E) do not. Titers to the ΔHBcAg protein cannot be separated in regard to Ab titers to the histidine tag vs titers to the backbone itself however, since both are included in the ΔHBcAg coating protein.

It is therefore hypothesized that the lack of difference in Ab titers to the ΔHBcAg protein-coated plates may be attributed to the histidine tag. This hypothesis is substantiated by the Ab titers to the AP205-coated plates. If the N-terminus forms spikes in an assembled VLP, the presence of an epitope N-terminal to the histidine tag could block the interaction between the tag and the immune cells. The added half-life of the mHBcAg+SSM VLPs could allow the continuation of that blocking until a majority of the VLPs have been endocytosed, as opposed to the mHBcAg+4E VLPs, which would likely have exposed histidine tags in the assembled form. The lack of degradation of the mHBcAg+SSM may have therefore enhanced the efficiency of the vaccine by blocking the histidine tag allowing a higher epitope presentation per dosage. Additionally, the added covalent structure may prevent established Abs to HBcAg from eliminating the VLPs from the bloodstream prior to their interaction with immunocytes through the blockage of the histidine tag. This lack of immune response to the HbcAg backbone may provide a further benefit in that patients previously exposed to the HBcAg protein through vaccination or viral infection would still be able to receive the vaccine with equal efficacy. However, a systemic immune response is not the

desirable outcome in a vaccination strategy towards PEDV. Therefore VN titers must be considered as well.

In both the HBcAg+SSM and mHBcAg+SSM vaccines for the second trial, YSNIGVCK is the only epitope inserted into the MIR of HBcAg. Both candidates also contain two other epitopes, GPRLQPY and WAFYVR in the N-terminus and C-Terminus Respectively. The goal of their placement was to keep the “focus” of the immune system on the YSNIGVCK motif while allowing recognition of two other conserved epitopes. By having this design, the vaccines may have been able to simultaneously neutralize virus while maintaining multivalence. One of the vaccine candidates, the mHBcAg+SSM, induced a statistically similar immune response to the HBcAg+SE vaccine while also inducing an immune response to the GPRLQPY and the WAFYVR epitopes at the same dosage. The non-mutated redesigned vaccine, the HBcAg+SSM candidate, was unable to induce an immune response as mentioned.

The VN titers from the initial experiment came back negative for all groups tested. Previous results have indicated that two similarly designed vaccines, one containing all four epitopes, and one containing only the YSNIGVCK epitope were capable of viral neutralization at a high virus titer [29]. These two vaccine candidates were also the only treatments capable of inducing an immune response to the YSNIGVCK motif, but all others were capable of inducing an

immune response to their respective epitope insertions. During the experiments outlined in this report, we again demonstrated that only vaccines capable of inducing a response to the YSNIGVCK epitope were capable of virus neutralization.

The hypothesis generated from these data is therefore that the three motifs: SQSGQVKI, GPRLQPY, and WAFYVR may be of less use in viral neutralization compared to the YSNIGVCK motif. This includes the epitope GPRLQPY, which has repeatedly been shown to induce a VN titer. Data generated by Okda et al, who demonstrated that portions of the S protein from PEDV containing the YSNIGVCK motif had considerably higher VN capabilities compared to portions containing the GPRLQPY motif, corroborates this hypothesis [22]. This phenomenon was essentially reproduced herein, though in a recombinant VLP format. In a recombinant vaccine model, this could have significant consequences on the design.

## Conclusions

The data presented in this report indicate that design changes in a well-known vaccine format can have large consequences on the immune response. Two mutations in the backbone HBcAg protein corresponding to D29C and R127C appear to increase the immune response to the MIR insertion. Additionally, placing an inserted epitope N-terminal to a histidine tag on the HBcAg protein can shield the immune response to the tag itself, and lessen the

immune recognition of the backbone protein. Finally, the YSNIGVCK motif from PEDV seems to be of more clinical significance in a virus neutralization strategy.

## Materials and Methods

### *Plasmid Construction*

Plasmid construction was performed using the pET-28a (+) plasmid (Novagen, Madison, WI) and λ-cloning. Briefly, genes coding for the optimized sequences (IDT, Coralville, IA) for each protein were amplified using phosphorylated reverse primers to include an annealing sequence to the plasmid corresponding to the *Nhe I* restriction enzyme cut site in the target plasmid. Upon successful amplification of each gene, λ-exonuclease was used to digest the fragments to create forward ssDNA megaprimers. These were then used to amplify the pET-28a plasmid, now containing the gene of interest (GOI). An enzyme cocktail was then used to digest any unreacted plasmid, while phosphorylating the new linear plasmids with GOIs and circularizing them. All enzymes used were purchased from New England Biolabs (Ipswitch, MA), and were used according to manufacturer's instructions. Each circular plasmid was transformed into BL21 (DE3) E. Coli T7 express® (NEB) cells using the heat shock method. A diagram and description of all constructs are given in **Fig. 1A**. Each construct was verified by Sanger sequencing after transformation.

### *Expression*

Overnight cultures of BL21 (DE3) *E. coli* cells grown in LB media supplemented with 30 μg/mL kanamycin were used to inoculate 1 L batches of 2×YT media supplemented with the same antibiotics at a rate of 0.2% v/v. The *E. coli* cultures were allowed to incubate at 28°C at 200 RPM until the $OD_{600}$ reached 0.7 AU. Protein production was induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG), which was allowed to incubate overnight. The resulting cells were centrifuged at 8,000 × *g* for 10 min to collect all solids left in the media. Cell pellets were stored at -20°C until purification began.

### *Purification*

All cell pellets were weighed and re-suspended in lysis buffer (20 mM $NaH_2PO_4$, 50 mM NaCl, and 2% Triton X-100 at pH 7.4) at a 1:10 w/w ratio. Sonication was performed using a Sonic Dismembrator Model 500 (Fisher Scientific, Pittsburgh, PA), with 60 cycles of 7 sec on/5 sec off on ice. The amplitude of the sonication was set at 30%. Resulting lysates were then centrifuged at 10,000 × *g* for 10 min to separate the insoluble and soluble portions.

With the exception of ΔHBcAg, the proteins were located in the insoluble portion of the cell lysate. IBs were then washed one more time with cell lysis buffer prior to solubilization. For ΔHBcAg expressions, the soluble fraction was precipitated using 15% ammonium sulfate (1.14 M). IBs or precipitates were

solubilized in an equal amount of solubilization buffer (20 mM $NaH_2PO_4$, 50 mM NaCl, 6 M Urea and 0.9% Sarkosyl at pH 6.0) to Lysis Buffer. The proteins were then loaded onto an anion exchange chromatography (AEX) column utilizing DEAE Sepharose FF resin (GE Healthcare, Marlborough, MA) at 2 mg/mL resin. The resin was equilibrated using 5 column volumes (CVs) of solubilization buffer, followed by the sample load. The column was then washed with an additional 5 CVs of solubilization buffer. Elution was carried out in an isocratic step with 100% AEX elution buffer (20 mM $NaH_2PO_4$, 500 mM NaCl, 6 M Urea, 0.9% Sarkosyl at pH 6.0).

Eluents were pH adjusted to pH 7.8 and loaded onto an immobilized metal affinity chromatography (IMAC) column packed with IMAC 6 Sepharose Fast Flow (GE Healthcare) resin at 2 mg/mL resin. The resin was previously equilibrated by rinsing with 5 CVs of water, followed by 2 CVs of 200 mM $NiSO_4$ solution for nickel chelation, followed by another 5 CV rinse with water, and finally 5 CVs of IMAC EQ Buffer (20 mM $NaH_2PO_4$, 500 mM NaCl, 10 mM Imidazole, 6 M Urea, 0.9% Sarkosyl at pH 7.8). Sample loading was followed by a wash with 7 CVs of IMAC EQ Buffer. Elution was performed in a single isocratic step with 100% IMAC elution buffer (20 mM $NaH_2PO_4$, 500 mM NaCl, 300 mM Imidazole, and 0.9% Sarkosyl at pH 7.8).

Following IMAC purification, step-wise dialysis was performed using Spectralpor® dialysis membrane tubing (Spectrum Laboratories, Rancho Dominguez, CA) with a molecular weight cut off of 6 – 8 kDa. Each dialysis step was performed in PBS at pH 7.8 with additives. These additives were, in order: 0.9% Sarkosyl, 0.45% Sarkosyl, 0.01% Sarkosyl, 0% Sarkosyl (PBS only). Each vaccine candidate was then ultrafiltered/diafiltered (UF/DF) into PBS buffer using Spin X UF tubes with a molecular weight cut off of 100 kDa (Corning, Corning, NY).

### *Formulation*

Samples in PBS were sterile filtered through 30 mm, 0.10 µm syringe filters (Celltreat, Pepperell, MA) followed by adjuvant addition and dilution with sterile PBS such that each dosage of 0.5 mL contained 20 µg of VLPs. Final vaccines for the first experiment should have a total of 20 µg protein in PBS at pH 7.8 with 100 ug per dose of Murabutide (Invivogen, San Diego, CA) and 100 ug per dose of Chitosan (Invivogen) [35]. Candidates formulated for the second experiment had a total of 20 µg protein in PBS at pH 7.8 with 100 µg Alhydrogel (Invivogen). Vaccine candidates were then held for <8 hours at 4°C until ready for use [36].

### *TEM*

Assembled proteins stored in PBS were diluted slowly with water to a total concentration of 200 µg/mL. Formvar coated copper grids were treated with

protein samples for a few seconds until the grid was blotted with filter paper and allowed to dry. The samples were then stained with 2% phosphotungstic acid at pH 7, again for a few seconds before the stain was blotted with filter paper and allowed to dry [37]. Images were taken using a JEM-1400 (JEOL, Peabody, MA) microscope at a maximum of 300,000x.

### *Protein Visualization*

Protein samples were prepared for analysis by adding water, NuPage Reducing Agent (10×) (Invitrogen, Carlsbad, CA), and NuPage LDS Buffer (4×) (Invitrogen) for a total of 10 µL per well. Non-reduced samples were prepared similarly, substituting 1 µL of water for the reducing agent. Each sample was put into a NuPage™ 4 – 12 % Bis-Tris PAGE gel (Invitrogen) along with a PrecisionPlus™ protein ladder (Biorad, Hercules, CA). Gels were run in MES buffer (Invitrogen) and run at 200 V for 35 min. After electrophoresis, gels were rinsed three times with 18 MΩ water for 5 minutes each. Gels were then treated with SimplyBlue™ Safestain (Invitrogen) for at least one hour, and then rinsed with water overnight. Images of stained gels were taken using a Chemidoc Touch Imager (BioRad). Densitometry measurements were proformed using ImageLab software (BioRad). Densitometry readings of >96% were considered to be pure enough to proceed with animal trials.

### *Western Blot*

Western blots were performed using the same protocol as reduced PAGE samples, but with a Precision Plus WesternC protein ladder (Biorad). Each Gel was blotted onto a nitrocellulose membrane using a Transblot® Turbo™ instrument (Biorad) according to manufacturers instructions. Membranes were blocked for at least one hour using 5% non-fat dry milk in TBST. A 3x wash was performed between each step consisting of three five-minute rinses in TBST. Each membrane was initially treated with a primary antibody solution containing a 1:10,000 dilution of unlabeled mouse anti-histag antibody (Thermo) in blocking buffer for one hour. The membranes were then treated with a secondary antibody solution containing 1:20,000 of HRP conjugated goat anti-mouse antibody (Millipore, Billerica, MA) and 1:50,000 HRP conjugated antistrep (Bio-Rad) for sample and ladder chemiluminescence, respectively. Detection was done using a Chemidoc Touch instrument (Biorad) with Clarity® ECL substrate (Biorad).

### *Endotoxin Testing*

Endotoxin levels were tested after removal of detergent and chaotropic agents. Tests were performed using a Pierce® LAL Chromogenic Endotoxin Quantitation Kit (Thermo). The kit was used according to manufacturer's instructions, with a high endotoxin cut off level of 5 EU/mL.

### *Animals*

Germ free balb/c mice from Charles River were used for *in vivo* testing of the vaccine candidates. 7 – 8 week old mice were allowed to acclimate for 13

days prior to a Day -1 blood draw. Vaccine candidates were administered intraperitoneally on day 0. Blood samples were collected on days 13, 27, and finally on day 42. Fecal samples were collected during each blood draw on days -1, 13, 27 and 42. Vaccination boosters were given on days 14, and 28.

Collected blood samples were incubated at 4°C for 2 hours and centrifuged. Fecal pellets were collected and suspended at 1:10 wt/vol in Superblock™ PBS based buffer containing a proprietary blocking protein and the antimicrobial agent Kathon® [38, 39]. Samples were aliquoted, if required, and frozen at -20°C until testing [40].

### *Peptide ELISA*

ELISAs for specific epitopes were performed using an ELISA fusion protein (EFP) previously designed for this purpose. A binding tag RAFIASRRIRRP was genetically attached to the N-terminus of Glutathione-S-Transferase (GST) in the pET28a plasmid as described previously [29, 41]. Purification of the peptides was done in one step using a Glutathione Sepharose 4B (GE Healthcare) column. Other proteins used in coating were purified and diluted to 10 µg/mL in coating buffer (50 mM $Na_2CO_3$, 50 mM $NaHCO_3$, pH 9.6).

Microlon 600 flat bottom plates (Grenier, Monroe, NC) were coated with 100 µL of of protein coating solution. Plates were then allowed to incubate at 37°C for >2 hours. Coated plates were washed 3x with 200 µL per well of 18 MΩ

water prior to blocking overnight in blocking buffer (TBS + 5% Nonfat dry milk) at 4°C. Plates were washed 3x with TBST between subsequent steps. Samples were diluted in blocking buffer serially 1:5, with a starting dilution of 1:25 and 1:10 for serum and fecal samples, respectively. A total volume of 100 µL of diluted sample per well was incubated at RT for 1 hour. Plates were then treated with 100 µL of secondary antibody solution for 1 hour diluted into dilution buffer (TBST + 5% Nonfat dry milk). Dilution schemes were 1:10,000 and 1:8,000 in for goat anti-mouse IgG HRP (Millipore, Billerica, MA) and goat anti-mouse IgA unlabeled (Southern Biotech, Birmingham, AL) respectively. Plates testing for IgA were treated with 100 µL of a tertiary rabbit anti-goat IgG HRP (Southern Biotech) antibody at a dilution of 1:4,000 in dilution buffer for 1 hour. Finally, 100 uL of TMB One Component Microwell Substrate (SouthernBiotech) was added to each well and incubated for ≤10 minutes. The reaction was observed and stopped using 0.05% sulfuric acid. Plates were read at 450 nm using a Biotek Synergy HTX plate reader (Winooski, VT).

ELISA plates were arranged with multiple samples, including at least three of the minimally diluted (1:25) negative PBS controls. These three controls were designated as a zero antibody response, and were used in calculation of ELISA titers. The negative PBS control samples on each plate were measured for absorbance at 450 nm and their average and standard deviation were calculated. Each test sample dilution on the plate was measured and plotted. The titer was

defined as the predicted concentration within the dilution range at which the test sample's absorbance was that of the negative control sample average plus three standard deviations. ELISA results therefore do not report the values of the PBS negative control group, as these are defined as zero values for consistency between different ELISA plates.

### *PEDV Virus Neutralization (VN) Test*

PEDV VN titers were measured as described previously [42]. Briefly, serum samples were heat-treated and diluted 1:10 in minimal essential medium (MEM) with 1.5 µg/mL osyl phenylalanyl chloromethyl ketone (TPCK) trypsin. A serial, 2-fold dilution was then performed in 96-well plates with the MEM/TPCK-trypsin diluent. Cell culture adapted PEDV was then added at 100 foci forming units (FFU)/100 uL and incubated for 1 hour at 37°C. The resulting mixture was then added to confluent monolayers of Vero-76 cells and incubated for 2 hours at 37°C. These new wells were then washed with MEM/TPCK-trypsin and allowed to incubate for 20 – 24 hours. Plates were fixed with acetone and stained with fluorescein isothiocyanate (FITC) conjugated mAb SD6-29 for visualization. End point titers were determined as the lowest concentration resulting in a 90% or greater reduction in fluorescence relative to controls.

### *Statistics*

JMP® Pro 12 software (SAS, Cary, NC) was used in all statistical analysis. All samples were run in a nested experimental design using

administration, peptide/protein, and group, respectively. Individual ANOVA contrasts were performed to determine statistical significance between individual groups.

## Acknowledgement

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We also wish to thank Aaron Singrey, Eric Nelson, and the rest of the team at the Animal Disease Research and Diagnostic Laboratory in the Veterinary and Biomedical Sciences Department at South Dakota State University.

## Figures

Figure 1 – Vaccine and assembly diagrams

(A) A schematic diagram of each vaccine tested throughout this report. Amino acid sequences are reported for the MIR, the N-terminus, and the C-terminus of the HBcAg protein. Additional mutations are reported as seen. (B) A rendering of a fully assembled HBcAg VLP without any mutations to the protein. (C) A fully formed HBcAg VLP with the added disulfide bonds formed with the D29C and R127C mutations added. The new disulfide bonds are highlighted in red. (D) A close up of a pentamer of dimers within the fully assembled HBcAg VLP with added disulfide bonds highlighted in red.

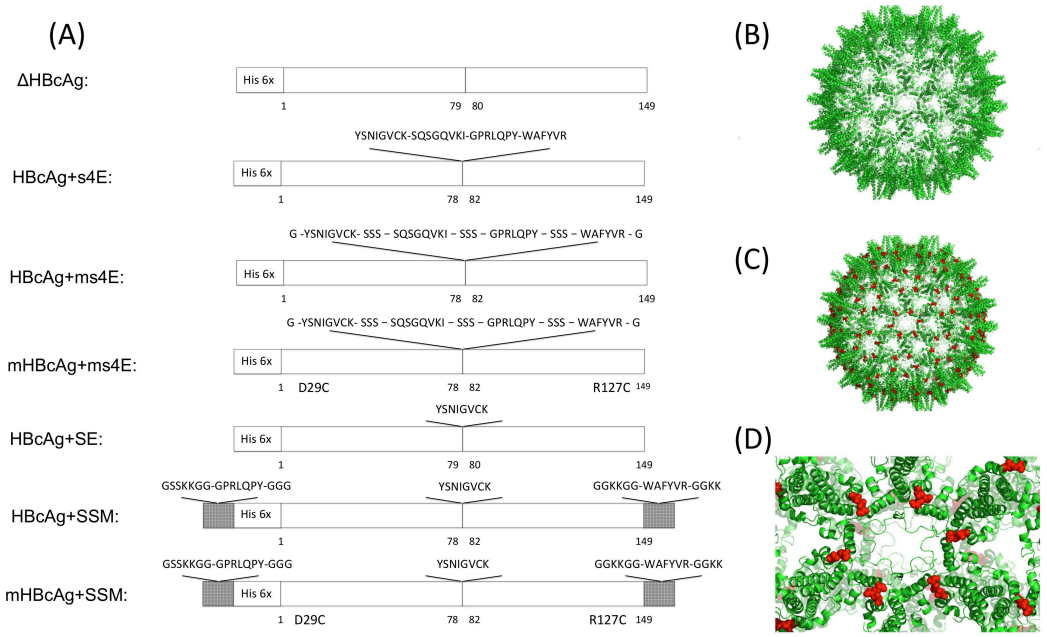


Figure 2 – Purification and assembly of vaccine candidates.

(A) A reduced and non-reduced NuPAGE gel showing the purified and assembled VLPs from the primary experiment. (B) A reduced and non-reduced NuPAGE gel showing the purified and assembled VLPs from the secondary experiment. (C) A Western blot of cell lysates from the preliminary experiment using α-His6x antibodies, indicating that the highly expressed proteins have the correct sequences. (D) A Western blot of cell lysates from the secondary experiment using α-His6x antibodies, indicating that the highly expressed proteins have the correct sequences. (E) TEM images of each vaccine candidate prior to each animal trial.

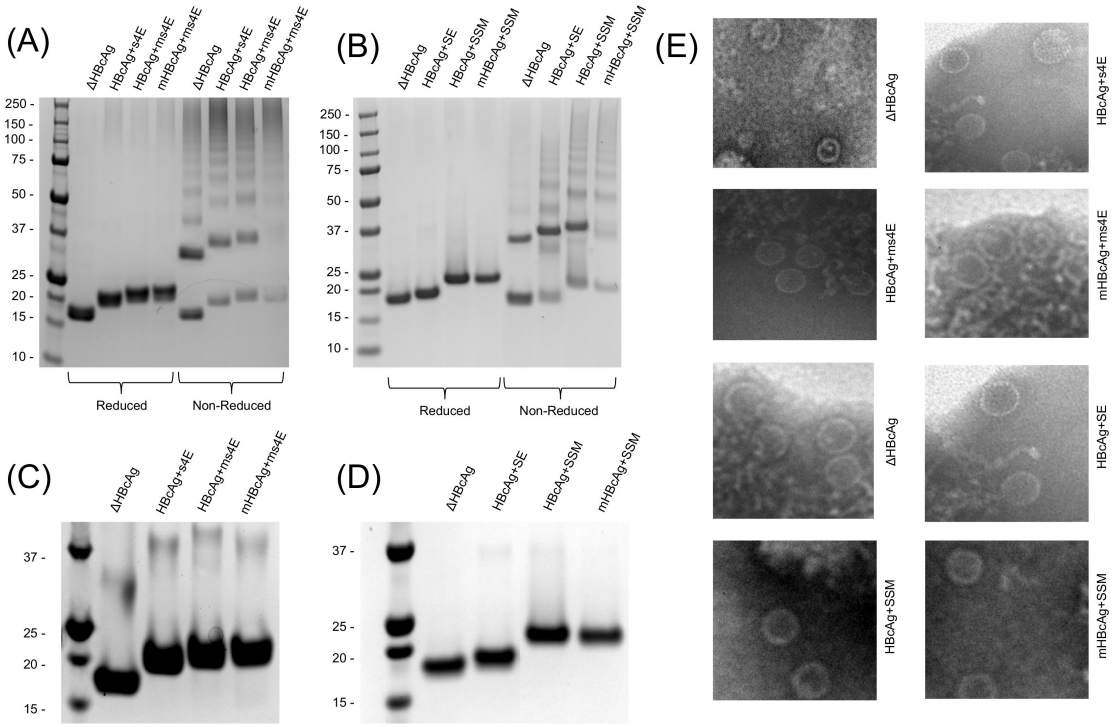


Figure 3 – ELISA titers to individual epitopes of preliminary experiment.

(A) Antibody responses from the preliminary experiment as measured by ELISA titers corresponding to a plate coated with EFP-YSNIGVCK. (B) Antibody responses from the preliminary experiment as measured by ELISA titers corresponding to a plate coated with EFP-SQSGQVKI. (C) Antibody responses from the preliminary experiment as measured by ELISA titers corresponding to a plate coated with EFP-GPRLQPY. (D) Antibody responses from the preliminary experiment as measured by ELISA titers corresponding to a plate coated with EFP-WAFYVR. (E) GRAVY scores were estimated for each epitope insert in an effort to determine if hydrophilicity of the sequence correlated with immune response. Each individual group titer is compared to the ΔHBcAg negative control (* = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$).

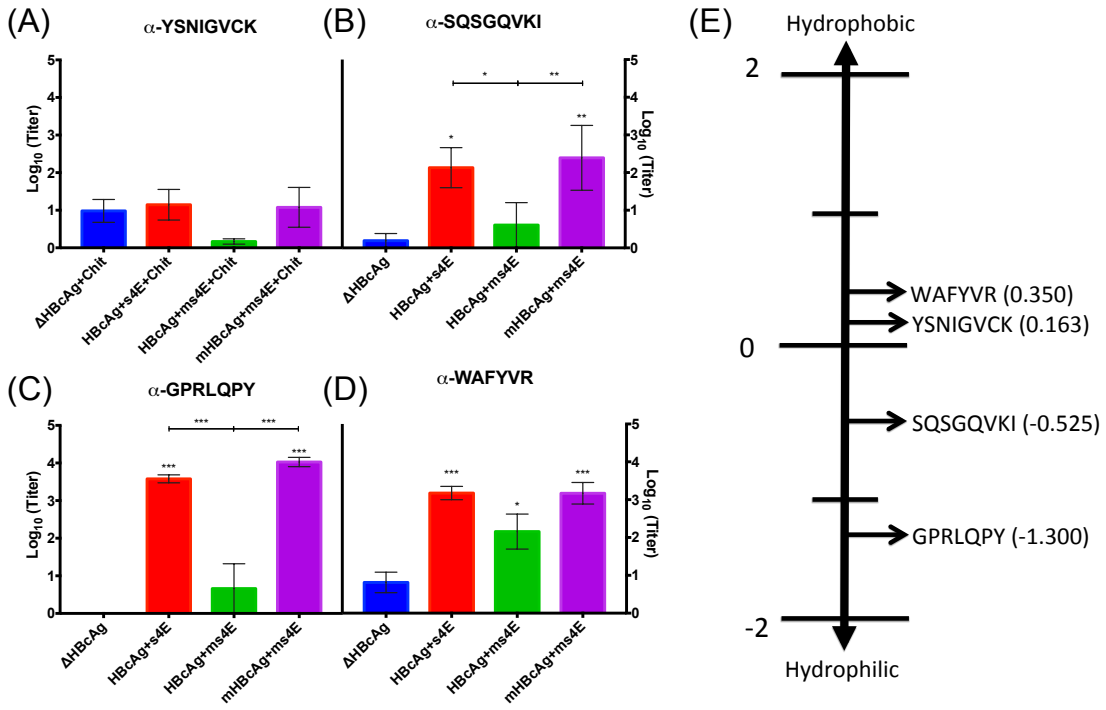



Figure 4– ELISA titers to individual epitopes of secondary experiment.

(A) Antibody responses from the secondary experiment as measured by ELISA titers corresponding to a plate coated with EFP-YSNIGVCK. (B) Antibody responses from the secondary experiment as measured by ELISA titers corresponding to a plate coated with EFP-GPRLQPY. (C) Antibody responses from the secondary experiment as measured by ELISA titers corresponding to a plate coated with EFP-WAFYVR. Each individual group titer is compared to the ΔHBcAg negative control (* = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001).

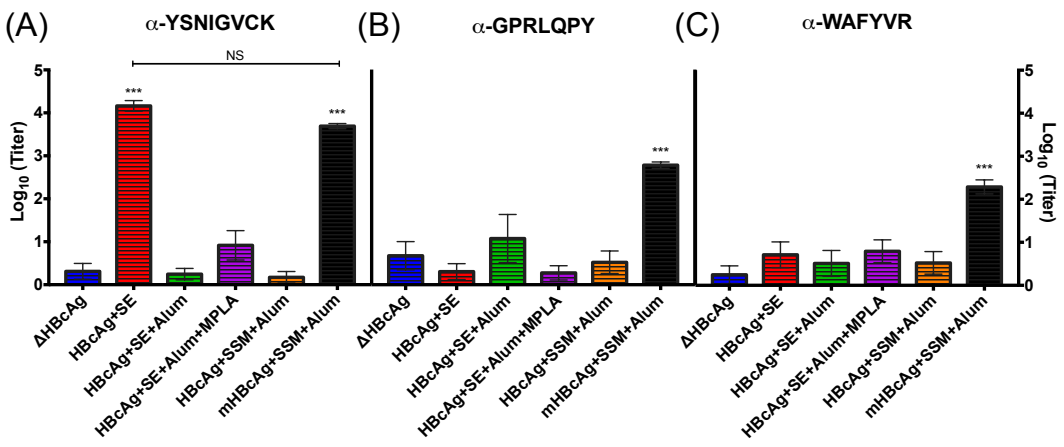

(A)
α-YSNIGVCK
(B)
α-GPRLQPY
(C)
α-WAFYVR
NS
***
Log10 (Titer)
ΔHBcAg
HBcAg+SE
HBcAg+SE+Alum
HBcAg+SE+Alum+MPLA
HBcAg+SSM+Alum
mHBcAg+SSM+Alum

Figure 5 – ELISA titers to the backbone protein and histidine tag.

(A) Antibody responses from the preliminary experiment as measured by ELISA titers corresponding to a plate coated with ΔHBcAg protein. (B) Antibody responses from the secondary experiment as measured by ELISA titers corresponding to a plate coated with ΔHBcAg protein. (C) Antibody responses from the preliminary experiment as measured by ELISA titers corresponding to a plate coated with the AP205 protein containing the same N-terminal histidine tag as the vaccine candidates. (D) Antibody responses from the secondary experiment as measured by ELISA titers corresponding to a plate coated with the AP205 protein containing the same N-terminal histidine tag as the vaccine candidates. Each individual group titer is compared to the ΔHBcAg negative control (* = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$).

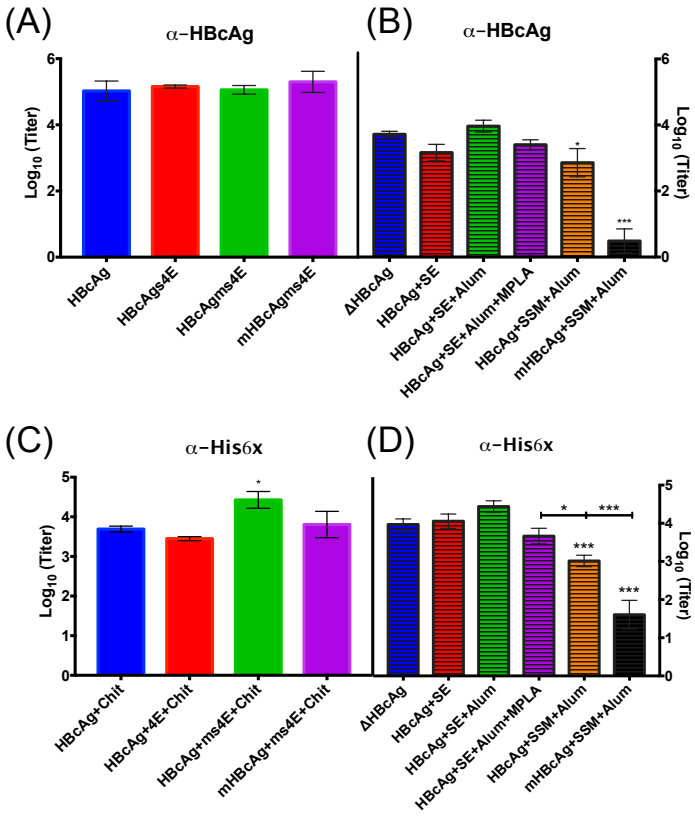
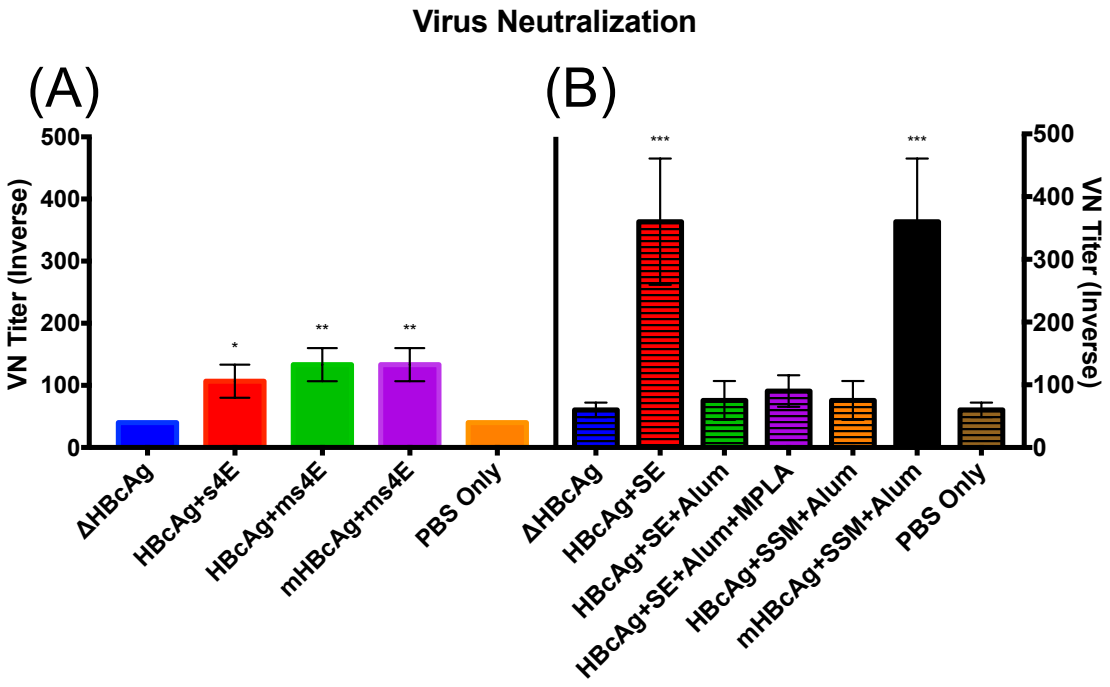



Figure 6 – Virus neutralization of the generated antibodies.

(A) Antibodies generated during the primary experiment were tested for their ability to neutralize PEDV virus using an FFA assay. (B) Antibodies generated during the secondary experiment were tested for their ability to neutralize PEDV virus using an FFA assay. Each individual group titer is compared to both the ΔHBcAg, and PBS negative controls (* = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001).


Virus Neutralization
(A)
(B)
VN Titer (Inverse)
500
400
300
200
100
0
*
**
**
***
***
ΔHBcAg
HBcAg+s4E
HBcAg+ms4E
mHBcAg+ms4E
PBS Only
ΔHBcAg
HBcAg+SE
HBcAg+SE+Alum
HBcAg+SE+Alum+MPLA
HBcAg+SSM+Alum
mHBcAg+SSM+Alum
PBS Only

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## Chapter VI: General conclusions and future directions.

Although there is an approved killed virus (KV) vaccine for porcine epidemic diarrhea virus (PEDV) commercially available in the US, there is still an interest in a recombinant alternative. The Hepatitis B virus core antigen (HBcAg) as a backbone protein, when used for the insertion of foreign epitopes, allows the customization of the protein to cater to any virus with known epitopes. The very nature of PEDV presents several challenges in the design of a vaccination strategy. Traditional vaccination requires a systemic identification of antigens specific to the target virus, which are then recognized upon infection to allow for rapid elimination by the immune system. In the case of adult pigs with PEDV, this strategy may or may not work. Since the symptoms and mortality rate in the adult pigs are relatively tame, they are not the primary targets for vaccination. The focus of any vaccination strategy for PEDV must be on the survival of the suckling piglets. It is therefore *critical* that the epitopes used in a chimeric protein vaccination are able to efficiently neutralize the virus *and* generate lactogenic immunity if the suckling piglets are to survive.

The three experiments performed within this report all indicated that there are modifications in the HBcAg protein that can be made to improve the vaccine efficacy specific to PEDV, but perhaps to other viruses as well. The second experiment performed demonstrated that a vaccine design eliminating the YSNIGVCK motif from presentation to the immunocytes upon intraperitoneal

injection in mice led to the generation of antibodies incapable of virus neutralization. When the YSNIGVCK motif was efficiently presented during the third experiment, the vaccines were able to produce antibodies with virus neutralization as seen in the HBcAg+S1/HBcAg+S1S2S3M candidates from the first trial and the HBcAg+SE/mHBcAg+SSM candidates from the third trial. Overall, these data indicate that the YSNIGVCK motif is crucial in a recombinant vaccine strategy for PEDV utilizing the short neutralizing epitopes.

On two occasions (mHBcAg+ms4E and mHBcAg+SSM), there was a mutation in the HBcAg backbone protein to include two cysteine residues. Upon assembly, this addition was able to improve the immune response significantly compared to the exact same vaccines without the mutations. It is hypothesized that the increased covalent bonds formed during assembly of the mHBcAg vaccines allows the VLPs a longer half-life in the body, which leads to a more efficient antigen presentation. The use of the mutation in combination with a strategically placed epitope N-terminal to the histidine tag (mHBcAg+SSM) decreased the immune response to the native protein to nearly zero. The data generated from these two design changes indicate that it may be possible to use the HBcAg backbone protein in subjects previously exposed to Hepatitis B Virus, or viruses similar to Hepatitis B Virus without previously established antibodies eliminating the vaccine from the system without efficacy.

The obvious next step to proceed in this research is to perform a neonatal piglet challenge experiment. Since the goal of vaccination is to provide the suckling piglets with lactogenic immunity from the sow, that must be the ultimate test. Additionally, there may need to be some improvements made to the purification process during scale up that could help economically, although these improvements would likely contribute little to the overall vaccine's efficacy.