A PORCINE CIRCOVIRUS VACCINE WITH ENHANCED CAPABILITIES

A Thesis
Submitted to the Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Science

By

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In Partial Fulfillment of the Requirements
for the Degree of
MASTER OF SCIENCE

Major Department:
Veterinary and Microbiological Sciences

April 2016

Fargo, North Dakota

North Dakota State University
Graduate School

Title

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

MASTER OF SCIENCE

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ABSTRACT

Porcine circovirus type 2 (PCV2) is a pathogen of swine. Vaccines against PCV2 are available, although none are capable of differentiating infected from vaccinated animals (DIVA). Positive and negative DIVA markers were introduced in the vaccine constructs. Decoy epitopes were modified by site directed mutagenesis to avoid possible subversion of host immunity and achieve a rationalized vaccine design. Immunization of pigs with the modified vaccines, followed by challenge with a virulent field strain showed that the efficacy of the vaccine was comparable to a commercial vaccine. The average weight gain was significantly higher group vaccinated with experimental construct if compared to the group that received commercial vaccine. An appropriate response to the positive and negative DIVA tags was detected. Therefore, the strategy used in this study is the first to enable a DIVA capable vaccine and accompanying immuno-assay, while using an epitope based approach to target improved immunogenicity.

ACKNOWLEDGEMENTS

First of all I would like to acknowledge my advisor Dr. Sheela Ramamoorthy for accepting me as a student and providing me with the opportunity to work on this project. I would also like to express my acknowledgement to Dr. Yunfeng Song and Dr. Pankaj Singh. They both played an integral role in crucial parts of this work. I would like to thank my committee members Dr. Brett Webb and Dr. Sangita Sinha for their critical input in to this work. Our collaborators from South Dakota State University Dr. Angela Pilatzki, Dr. Michele Mucciante, Ms. Amanda Zubke, Dr. Eric Nelson, Mr. Steven Lawson and Dr. Diel Diego who did a tremendous job of conducting animal experiment for this study and supporting us along the way. The animal facility in the South Dakota State University was crucial for implementation of this study. I would also like to thank our other collaborators, Dr. Peter Nara, Biological Mimetics for the guidance with the technical aspects of the projects and Dr. Marcia Ilha, University of Georgia, for assistance with epitope mapping, Courses from Biochemical department at NDSU such as recombinant DNA technology by Dr. Stuart Haring and methods of biochemical research by Dr. Christopher Colbert provided me with both theoretical and practical knowledge to conduct this research.

I would like to thank the Fulbright organization for providing me with the opportunity to study at NDSU. The Department of Virology as well as the entire ESC "Institute of biology" helped prepare me well to face most of the challenges during my graduate studies.

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Porcine Circovirus

Introduction

Porcine circovirus strain 1 (PCV1) was first described in 1974 as a contaminant virus in a pig kidney cell line (PK-15) (Tischer et al., 1974). Porcine circovirus type 2 (PCV2) was first discovered in 1998 from the cases of post-weaning multi-systemic wasting syndrome (PMWS) (Ellis et al., 1998). Since then PCV2 has been identified as the causative agent of several associated clinical syndromes, now collectively designated as the porcine circovirus associated diseases (PCVAD).

Post-weaning multi-systemic wasting syndrome (PMWS) was first described by Clark et al (Clark, 1997). It affected pigs at about 5-6 weeks of age. The main clinical signs of PMWS were wasting and dyspnea occasionally jaundice and reduced weight gain when compared to healthy pigs. It can also be accompanied with a loss of appetite and lead to emaciation and weakness. Lymphoid depletion, histiocytic replacement and enlargement of lymph nodes were noted as the hall-mark lesions of PMWS. The architecture of the lymph node is often disrupted (Opriessnig et al., 2007; Segalés, 2012). Mechanisms of lymph node depletion remains to be examined in detail but it was suggested that inhibition of cell proliferation leads to the observed depletion (Mandrioli et al., 2004). Inflammation of the liver, spleen, lung and intracellular inclusion bodies localized in the cytoplasm are commonly detected. The inclusion bodies were proven to be clusters of PCV particles (Harding and Clark, 1997). The lesions in the lymphoid system leads to immune suppression and increased risk of opportunistic infections.

As described below, PCV2 was later found to induce other clinical manifestations that were not included in PMWS. So the more general classification as PCVAD was introduced

(Opriessnig et al., 2007; Segalés, 2012). The interest in this pathogen have been growing ever since (Figure 1).

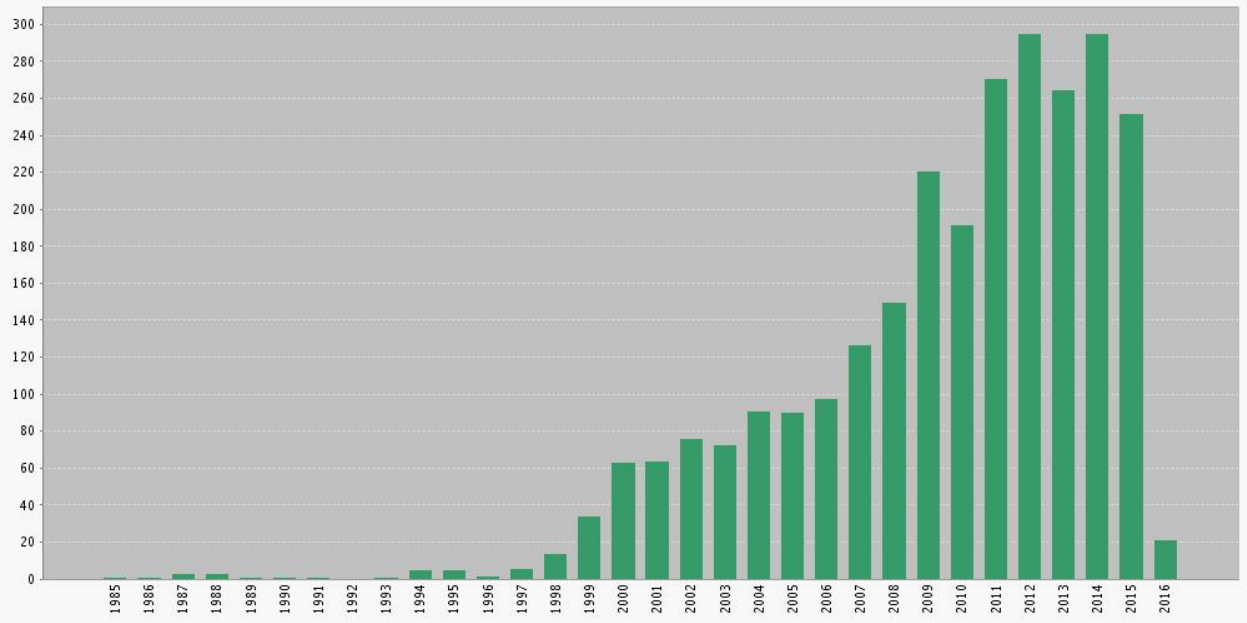

Figure 1. Absolute number of academic publications on the topics related to porcine circovirus. Considerable increase in the number if publications is observed after year 1998, when PCV was associated with Post Weaning Multi-systemic Wasting Syndrome (PMWS). Produced with Web of Science (Thomson Reuters, NY).

The other manifestations of PCVAD incudes respiratory signs, reproductive disorders, enteric disease, diarrhea and the porcine dermatitis and nephropathy syndrome (PDNS) (Meng, 2013).On a histological level, PCV2 associated enteric disease presents itself as inflammation of the enteric tract (Jensen et al., 2006). Enteric disease associated with PCVAD develops at 8-16 weeks. Respiratory signs caused by PCV2 fall under the umbrella of the porcine respiratory disease complex (PRDC), which usually involves coinfections with other viruses and bacteria. Respiratory signs of PCVAD are usually observed at 8-16 weeks of age also. Signs can include dyspnea or difficulty in breathing and cough, which can further reduced weigh gain due to anorexia (Bolin et al., 2001). Reproductive disorders caused by PCV2 include increased probability of abortions, stillbirth and pre-weaning mortalities (Mikami et al., 2005). Porcine

Dermatitis and Nephropathy Syndrome (PDNS) can also be one of the signs of PCV2 infection. It manifests as an appearance of purplish red and slightly raised blotches of different shapes and sizes on the various parts of animal body, and sometimes pigs have difficulty breathing or appear depressed and reluctant to eat (An et al., 2007). Hence, if unchecked, PCVAD can lead to a catastrophic decreases in the weight gain of production pigs and impose a significant pressure on the pork industry.

PCV2 – Virus Classification, Structure

Porcine circovirus type 2 is one of the smallest known viruses. Its non-enveloped icosahedral capsid is only 17 nm in diameter. The genome of PCV2 virus is a single stranded circular covalently closed molecule of DNA (ssDNA) about 1767 bases long; however different strains vary in exact length by a few base pairs. The circular nature of their genomes led to the naming the family *Circoviridae*. A number of other viruses are also classified in the *Circoviridae* family, such as Psittacine Beak and Feather Disease Virus, porcine circovirus type 1, pigeon circovirus, canary circovirus, goose circovirus and chicken anemia virus. While the chicken anemia virus belongs to the *Gyrovirus* genus, the other members are grouped under the Circovirus genus (Olvera et al., 2007). PCV1 and PCV2 was shown to infect human cell culture, but they were not found in the humans (Hattermann et al., 2004). Torque teno virus (TTV) that is present in healthy humans was for previously considered as a circovirus, but it was later reclassified.

The genome of the virus is ambisense in that two main ORFs are on the opposite strands of the double stranded replicative form of the DNA. The origin of replication is located between the two major ORFs and it is presumed that it forms a hairpin loop (Mankertz et al., 1997). The two major viral proteins include the capsid protein (Cap) and the replicase protein (Rep),

encoded by the open reading frames (ORFs) ORF2 and ORF1 respectively. Other minor proteins such as ORF3, 4 and 5 whose function is to modify host's cellular machinery and modulate immune response have also been reported (Liu et al., 2006, 2007; He et al., 2013; Lv et al., 2015). Sequence identity between PCV1 and PCV2 ORF1 (rep) is 83%, while for ORF2 (cap) is 67% (Constans et al., 2015a).The capsid is composed of 60 capsomeres and can be described as having T=1 symmetry.

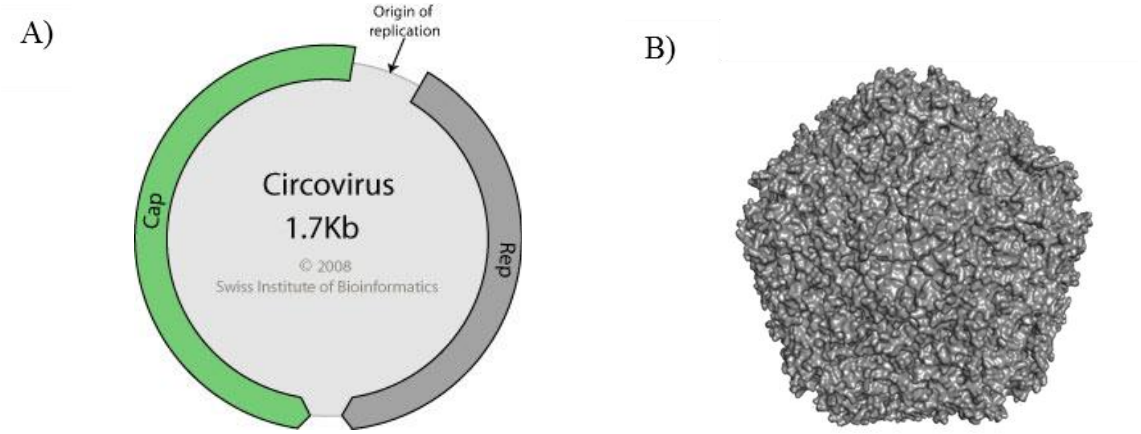

Figure 2. PCV genome and capsid. Schematic representation of the genome of PCV2 (A) that consists of two major ORFs. ORF1 encodes the replicase, ORF2 encodes the capsid protein. This ORF runs in an antiparallel manner. The origin of replication is located between the ORFs. Two dimensional representation of 3-dimentional model of Porcine circovirus capsid (Khayat,R. et al., 2011).

The primary cell line for culturing PCV2 is PK15 epithelial cells from porcine kidney. There is no obvious cytopathic effect from viral infection; thus all operations that involve detection of virus infected cells are based on indirect immunofluorescence (IFA) or immunohistochemical assays. The viral genome can also replicate in a number of human cell lines upon transfection, however it is not clear if there is productive infection (Hattermann et al., 2004).

Transmission and Global Distribution of PCV2

Porcine circovirus is transmitted both vertically and horizontally. Horizontal transmission occurs through direct contact (Bolin et al., 2001), while vertical transmission occurs from the mother to the fetus. Infected animals are PCR positive from nasal swabs, oropharyngeal swab, and feces (Shibata et al., 2003). Vertical transmission was shown to occur *in-utero* from PCV2 positive sows (West et al., 1999). Transmission via semen is also a possibility as viral DNA has been identified in semen of boars at 35 days post infection (Larochelle et al., 2000).

PWMS and PCVAD is a global epizootic disease of swine. The virus is very infectious (Patterson and Opriessnig, 2010; Patterson et al., 2011) and can readily be spread from farm to farm. Hence, PCV2 is detected in a large proportion of farms globally. Since infection with PCV2 is not always manifested clinically, it can be subclinical. In the U.S about 54% out of 61 sites were PCV2 positive (Shen et al., 2012). It was estimated that that PCVAD costs the EU swine industry €562-900 million per year (Alarcon et al., 2013). By other estimates PCVAD can lead to loss of $6 per weanling pig and $17 for each mating sow.

Molecular Epidemiology Of PCV2

Currently, there are 4 circulating PCV2 subtypes; PCV2a, b, c and d (or mPCV2b) (Figure 3 and 4). Until about 2008, the predominant subtype in the U.S was PCV2a. Following the introduction of commercial vaccines in 2006, a type-switching event resulted in PCV2b becoming the predominant subtype in the U.S, indicating that current commercial vaccines could induce selection pressure (Ssemadaali et al., 2015).

PCV2a was the first described PCV2 subtype in the U.S. PCV2b emerged later (Opriessnig et al., 2013) and was associated with increased pathogenicity compared to PCV2a. PCV2c was identified in Denmark and is not highly prevalent or of remarkable pathogenicity

(Dupont et al., 2008).The latest of such newly emergent strains is PCV2d also referred to as the mutant PCV2b or mPCV2b in literature (Guo et al., 2010; Opriessnig et al., 2014a) (Figure 3 and 4). Current vaccines in the U.S. are all based on PCV2a and they are still effective in prevention of clinical manifestation in herds that are infected with PCV2b (Opriessnig et al., 2014b).

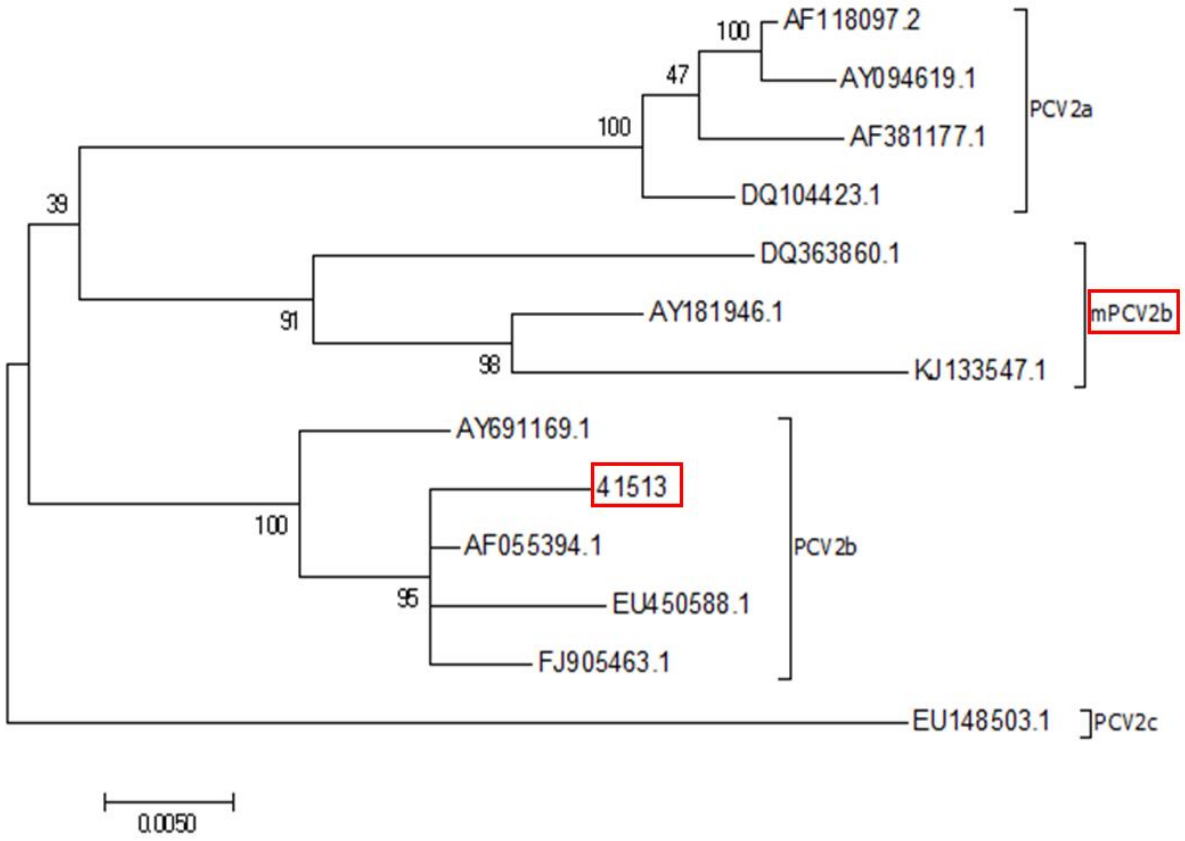

Figure 3. Phylogenetic representation of PCV2 subtypes. The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model (Jukes, 1969) . The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Total of 13 whole genome nucleic acid sequences 12 of which were previously described and PCV2b strain 41513 were analyzed. Nucleic acid sequence of PCV2b strain 41513 was used as backbone for the vaccine developed in this study, while mPCV2b strain was used as a challenge culture.

However, the chance of emergence of a vaccine tolerant or more pathogenic, or more transmissible strain is present when multiple strains co-circulate in herds and infect the same

animal (Firth et al., 2009). Eradication of PCV2 from agricultural farms will reduce the possibility of viral evolution and the need to update vaccines and diagnostics.

Vaccination against PCV2 is effective in prevention of clinical signs but does not prevent shedding of the virus or subclinical infection. Continuous circulation of virus under the selective pressure of vaccine- induced immunity could lead to the emergence of new strains that will have subtle antigenic difference from vaccine strain, leading to isolated cases of vaccine escape (Constans et al., 2015a). Indeed, it was estimated that mutation rate for PCV is among the highest for viruses that have DNA genomes, at 1.2×10^{-3} substitutions/site/year (Firth et al., 2009). New emergent strains can vary in their biological properties, and they might have increased pathogenicity (Horlen, 2007).

AF118097.2	PCV2a	M	T	Y	P	R	R	R	Y	R	R	R	R	H	R	P	R	S	H	L	G	Q	I	L	R	R	R	P	W	L	V	[30]
41513	PCV2b	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[30]
KJ133547.1	mPCV2b	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[30]
EU148503.1	PCV2c	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	H	.	.	.	.	.	.	.	.	.	.	[30]
AF118097.2	PCV2a	H	P	R	H	R	Y	R	W	R	R	K	N	G	I	F	N	T	R	L	S	R	T	F	G	Y	T	V	K	R	T	[60]
41513	PCV2b	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	[60]
KJ133547.1	mPCV2b	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	K	.	[60]
EU148503.1	PCV2c	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	S	.	V	.	.	.	N	A	S	[60]
AF118097.2	PCV2a	T	V	T	T	P	S	W	A	V	D	M	M	R	F	K	L	D	D	F	V	P	P	G	G	G	T	N	K	I	S	[90]
41513	PCV2b	.	.	R	.	.	.	.	.	.	.	.	.	.	.	N	I	N	A	.	L	.	.	.	.	.	S	.	P	R	.	[90]
KJ133547.1	mPCV2b	.	.	R	.	.	.	.	N	.	.	.	.	.	.	N	I	N	.	.	L	.	.	.	.	.	S	.	P	L	T	[90]
EU148503.1	PCV2c	Q	.	S	P	.	.	.	.	.	.	.	.	.	.	N	I	N	Q	.	L	.	.	.	.	.	S	.	P	L	T	[90]
AF118097.2	PCV2a	I	P	F	E	Y	Y	R	I	R	K	V	K	V	E	F	W	P	C	S	P	I	T	Q	G	D	R	G	V	G	S	[120]
41513	PCV2b	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[120]
KJ133547.1	mPCV2b	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[120]
EU148503.1	PCV2c	V	.	.	.	.	.	.	.	.	.	.	.	.	.	F	A	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[120]
AF118097.2	PCV2a	T	A	V	I	L	D	D	N	F	V	P	K	A	N	A	L	T	Y	D	P	Y	V	N	Y	S	S	R	H	T	I	[150]
41513	PCV2b	S	.	.	.	.	.	.	.	.	T	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[150]
KJ133547.1	mPCV2b	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[150]
EU148503.1	PCV2c	.	.	.	.	.	N	.	.	.	T	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[150]
AF118097.2	PCV2a	P	Q	P	F	S	Y	H	S	R	Y	F	T	P	K	P	V	L	D	S	T	I	D	Y	F	Q	P	N	N	K	R	[180]
41513	PCV2b	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[180]
KJ133547.1	mPCV2b	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	[180]
EU148503.1	PCV2c	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[180]
AF118097.2	PCV2a	N	Q	L	W	L	R	L	Q	T	S	R	N	V	D	H	V	G	L	G	T	A	F	E	N	S	K	Y	D	Q	D	[210]
41513	PCV2b	.	.	.	.	.	.	.	.	A	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	G	[210]
KJ133547.1	mPCV2b	.	.	.	.	.	.	.	.	T	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	[210]
EU148503.1	PCV2c	.	.	.	.	M	.	.	.	T	G	.	.	.	.	.	.	.	.	H	.	.	Q	.	.	T	N	A	.	A		[210]
AF118097.2	PCV2a	Y	N	I	R	V	T	M	Y	V	Q	F	R	E	F	N	L	K	D	P	P	L	N	P	*	[234]						
41513	PCV2b	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[234]							
KJ133547.1	mPCV2b	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	K	[234]							
EU148503.1	PCV2c	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	K	[234]							

Figure 4. Alignment of amino acid residue of ORF2 strains of different strains of PCV2. In the figure presented difference in amino acid residue composition between PCV2 strains is presented. PCV2b has a motif which distinguishes it from PCV2a located in the region between residues 85 and 102 (Cheung, 2009); while mPCV2b has additional changes at several positions such as 8, 53, 59, 63, 68, 78, 90, 134, 169, 210, 215, and the addition of a lysine residue in position 234.

Immune Response to Pcv2 Infection

Cell Mediated Immunity

Pathogens have an intricate relationship with the host's immune system. PCV2 is not an exception to this rule. The two major arms of the immune response include cell mediated immunity (CMI) and humoral or antibody-mediated immunity. While the CMI against PCV2 is not very well characterized, PCV2 is known to infect dendritic cells (DCs), and persist in them without viral replication, induction of apoptosis, or antigen presentation. Since DCs are one of the most important MHC class II antigen presenting cells, their functional inactivation can be one of the mechanisms for immune evasion for the virus (Vincent et al., 2003). In addition, it was shown that PCV2 infected peripheral blood mononuclear cells (PBMC) showed reduced response to mitogens, ability of PBMS to produce INF- γ, IL-2 and IL-4 (Darwich et al., 2003). Infection of natural interferon producing cells (NIPCs) by PCV2 reduced their ability to produce IFN-α and TNF-α, thus preventing maturation of DCs (Vincent et al., 2007). The genome of PCV2 contains a number of interferon-stimulated response element (ISRE) like sequences. The role of these sequences was examined by site directed mutagenesis of the sequence, followed by infection of pigs with the mutated PCV2 alone or in a co-infection with the porcine reproductive and respiratory syndrome virus (PRRSV). The results indicated that the ISRE influenced PCV2 pathogenesis (Ramamoorthy et al., 2011).

The viral protein encoded by ORF3 was shown to induce apoptosis *in-vitro* through activation of caspace-3 and caspase-8 (Liu et al., 2005), and *in-vivo* in BALB/c mice. However, pigs that were inoculated with PCV2 that has a null mutation in ORF3 did not show reduction in lymphocyte count, nor did this virus showed histopathological effect on the lymph node (Liu et al., 2006). Similarly, in another *in-vivo* study on swine there were no significant differences in

either histological and gross lesions between wild type and ORF3 null mutant virus (Juhan et al., 2010). As of now the role of ORF3 remains to be interrogated. Furthermore, the ORF4 protein was implicated in the negative regulation of ORF3 expression, thus down-regulating cellular apoptosis pathways (Gao et al., 2014). In this study mutants of PCV2 were created that had mutations either in the start or stop codon of ORF4. The results off this study were explained by competitive inhibition of transcription (Lapidot and Pilpel, 2006) or through codon pair bias (Kunec and Osterrieder, 2016) since there is no evidence that ORF4 product itself interacts with molecular machinery to inhibit the ORF3 expression. Thus, despite its small genome size, PCV2 possesses a repertoire of effects to modulate host's immune response.

Humoral Immunity

In PCV2 infected animals, specific antibodies can be detected as early as 7 days post-infection (DPI), with a maturing of the response between DPI 14-21(Beach et al., 2010, 2011). At least 3 proteins of PCV2 are known to induce seroconversion: ORF1, 2, 3 (Peng et al., 2016). Neutralizing antibodies to PCV2 are observed on DPI 14 (Meerts et al., 2006). The best characterized predictor of clinical outcomes in PCV2-infected animals is the presence of neutralizing antibodies IgG to the capsid protein (Blanchard et al., 2003). Maternal antibodies can confer titer dependent protection against PCV2 infection, however such passive immunity does not confer absolute protection (McKeown et al., 2005; Ostanello et al., 2005). While controversial, it is largely accepted that maternal antibodies do not interfere with the development of vaccine induced immunity (Fort et al., 2008; Opriessnig et al., 2008a). Thus the induction of humoral immune responses against the capsid protein has been the primary focus vaccine development against PCV2. Additional information about antibody responses towards PCV2 is referenced throughout this thesis

Current PCV2 Vaccines

Today, a number of commercial PCV2 vaccines are used in veterinary practice. They are all efficient in prevention of clinical manifestations of PCVAD. Existing vaccines are based on varying compositions of viral antigens. Whole inactivated PCV2 viral particles are used as an antigen in Circovac (Merial S.A.S., Lyon, France)(Ishikawa et al., 2008). Recombinant, baculovirus-expressed capsid protein is used for the formulation of the Ingelvac CircoFLEX (Boehringer Ingelheim, Ingelheim, Germany) (Roof et al., 2010), Circumvent PCV2 (Intervet/Schering-Plough Animal Health, Millsboro, USA)(De Grau et al., 2007) and Porcilis PCV2 (Intervet International BV, Boxmeer Netherlands)(Ruiz et al., 2008) vaccines. The Suvaxyn PCV2 vaccine (Fort Dodge Animal Health Limited, Southampton, United Kingdom) consists of a unique formulation of a chimeric PCV1-2 virus with the replicase gene from non-pathogenic PCV1 and capsid gene from PCV2 (Connor et al.; Fenaux et al., 2004).

Typically, piglets should be vaccinated at 3 weeks of age, and sows should be immunized with two doses three weeks prior to farrowing. Revaccination for sows is also recommended before each gestation. The ability of immunized sows to transfer immunity via the colostrum to the piglets is important for protection. Immunization with the Circovac vaccine, which contains killed virus, is recommended twice with 3 weeks of time between the initial immunization and booster. Ingelvac CircoFLEX is unique in that it requires a single dose vaccination at three weeks, with the advantage that immunity is mounted earlier and labor cost is reduced. The Circumvent PCV2 and Porcilis PCV2 vaccines allow users to choose between single and double dose vaccination, although double dose is recommended. Circumvent PCV2 is unique in that it allows primary immunization as early as 3 days of age. Two dose schemes are recommended if piglets have a high level of maternal antibodies (European Medicines Agency, 2009).

A meta-analysis study conducted by Kristensen *et al*. summed up the results of 66 trials that tested vaccine efficacy against PCV2 (Kristensen et al., 2011). This meta-analysis indicates that all vaccines significantly increase average daily gain (ADG) when compared to unvaccinated animals. However, there is no significant difference in efficacy between individual vaccines. This study also revealed that presence of porcine reproductive and respiratory syndrome virus (PRRSV) in the herd reduce the effect of PCV2 vaccination; herds without PRRSV showed higher values of average daily weight gain (ADG). The presence or absence of PRRSV had no significant effect on the levels of mortality reduction due to vaccination of piglets in the trials (Kristensen et al., 2011). Vaccinated finishing pigs (post nursing period) showed an ADG of 41.5g, while the AFG in nursing pigs was only 10.6 g. This finding can be explained by the fact that in nursing pig a large part of protection is conferred by the maternal IgM and IgA antibody that is transferred in the milk. All vaccines studied reduced neonatal mortality by about 4-5%, thus improving cost effectiveness. Authors also mention that in individual trials only a small group of animals are vaccinated, while in case of commercial practice all animals are vaccinated, leading to increased protection due to herd immunity (Kristensen et al., 2011).

While current vaccines are effective in reducing clinical signs by stimulating immune responses to the capsid protein (Kristensen et al., 2011), they do not induce "sterile" immunity or, therefore, prevent viral evolution. Additionally, there are no accompanying Differentiation of Infected from Vaccinated Animal (DIVA) assays for any of the commercially available vaccines, a key requirement for monitoring vaccine compliance or for future disease eradication efforts. The primary difference between a DIVA capable vaccine and regular vaccine is that the DIVA vaccine includes differential markers that are absent in wild type pathogen, e.g. deletion of a

viral antigen or insertion of a foreign antigen. An accompanying ELISA test will either not detect antibodies in the former case or detect antibodies to the foreign antigen in the latter case. Vaccines with the capability of differentiating vaccinated and infected animals enable the removal of infected animals from the population to eventually reach a disease free status. For example, the pseudorabies virus was eliminated due to utilization of marker vaccines that had glycoprotein E (gE) excluded from vaccine strain of PRV (Bech-Nielsen et al., 1995; Bouma, 2005). Similarly, brucellosis is largely eliminated in the U.S due to systematic vaccination with the DIVA-enabled *Brucella abortus* RB51 strain in combination with the euthanasia of infected animals as detected by the DIVA immunoassay assay (Pappas et al., 2006). Therefore, the development of a DIVA-capable vaccine involves the addition of negative or positive identification markers to the vaccine, and an accompanying DIVA assay, usually an enzyme-linked immunosorbent assay (ELISA).

Current PCV2 vaccines do not have DIVA capabilities. Thus, they do not enable the removal of infected animals to produce PCV2 free farms. Even though vaccines are effective in reducing clinical signs, the PCV2 virus is still circulating as subclinical infections. Such circulation under constant selective pressure by vaccination may lead to the evolution of mutants which can escape vaccine induced immune responses (Ssemadaali et al., 2015).

Epitope Analysis Of The PCV2 Capsid Protein

The PCV2 capsid protein is typically 233 amino acid long. Four major immunogenic regions have been identified by several researchers in the PCV2 capsid protein (Mahé et al., 2000; Truong et al., 2001; Lekcharoensuk et al., 2004) (Figure 5). When PCV2 viral particles were incubated with heparin, heparan sulfate, and chondroitin sulfate B, the glycosaminoglycans treated virus had reduced growth titers, when tested on 3D4/31 cells, indicating that a receptor-

ligand interaction had occurred. Therefore, glycosaminoglycans are believed to be a potential receptor for PCV2. Further, PCV2 infects a broad range of cells and a heparan sulfate-binding motif XBBXBX (where B is a basic amino acid and X is a hydrophobic residue) is present in the PCV2 capsid. The putative receptor binding site was identified as spanning amino acid residues 98-103, which are conserved among PCV strains (Meerts et al., 2006;Misinzo et al., 2006). It can be assumed that antibodies against this region will possess neutralizing properties as they can potentially block the virus-receptor interaction.

The initial examination of immunogenic epitopes on the PCV2 capsid protein was conducted with the goal of differentiating it from PCV1.The study by Mahe *et.al.* revealed that the major antigenic regions of PCV2 Cap spanned residues 69-83, 117-131 and 169-183 (Mahé et al., 2000). Interestingly, in another study, the prevalence of sero-positive animals with PMWS to residues 117-131 increased from week 8-10 to week 16-19 from 38 to 69% indicated that this region is a marker for antibody responses in later stages of PCV2 infection or disease. It was also shown that 26% of the 406 animals studied from PMWS-free herds had detectible titers against the same epitope. Testing for antigen specificity by western blotting confirmed the specificity of the reaction with PCV2 (Truong et al., 2001). A number of shorter PCV2 capsid specific epitopes were identified using a randomly generated phage library. However their neutralizing activity was not explored (Ge et al., 2013). In another study, analysis of linear epitopes identified residues 233 and 156 as important for virus neutralization (Lekcharoensuk et al., 2004; Shang et al., 2009). Bioinformatics prediction of B and T cell epitopes in the various PCV2 subtypes indicated that very subtle changes of 1-2 amino acids could affect the antigenic profile of the PCV2 capsid protein (Constans et al., 2015a).

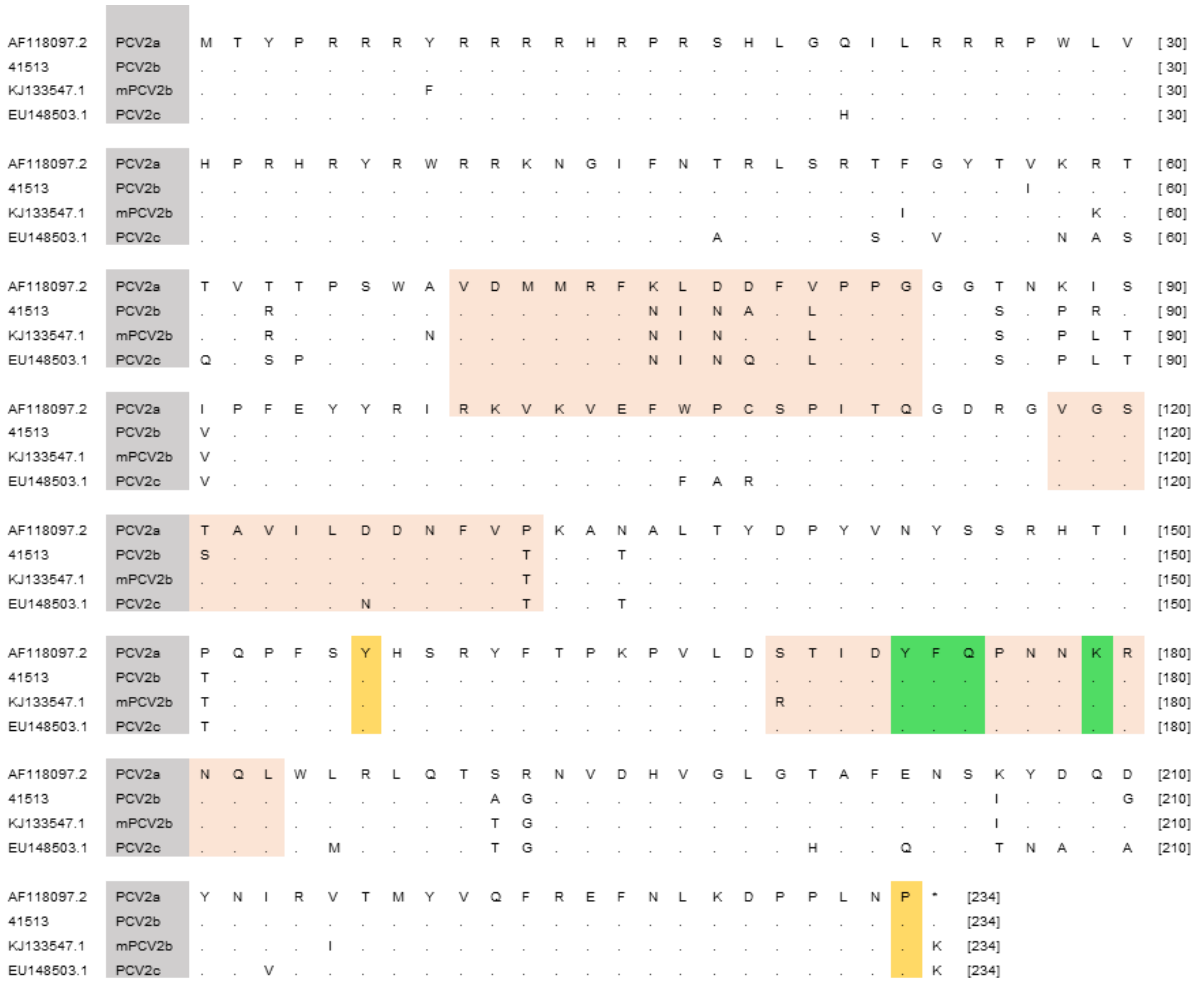

Figure 5. Previously described epitopes. Amino acids highlighted in pink immunogenic regions identified by Mahe (Mahé et al., 2000). Amino acid residues highlighted in gold were identified by Shang (Shang et al., 2009). Epitopes identified by Trible *et. al.* as the most important amino acids in epitope 169-180 (Trible et al., 2011) are in emerald green.

Antigenic epitopes that are highly immunogenic, but do not play a protective role in the neutralization of viral infectivity, are known as decoy epitopes. The presence of decoy epitopes in protective antigens is a mechanism of immune evasion that also diminishes vaccine efficacy. In 2011, Trible *et. al.* identified the immunogenic region 169-180 of the capsid protein as a possible decoy epitope because pigs showing clinical signs of PMWS had high levels of antibodies to this region while pigs that did not show clinical signs did not (Trible et al., 2011). Truncation of the ORF2 protein followed by alanine scanning, a technique where each amino

acid residue in the span of 169-180 was sequentially substituted to alanine, resulted in the identification of residues 173,174,175 and 179 as key players. The identified amino acid residues are conserved among the PCV2 strains, indicating that they may possess an important biological function (Constans et al., 2015a).

In the same study it was shown that at 3 week of age, the response towards peptide 169-180 is almost as high as to the ORF2 antigen lacking the nuclear localization signal (residues 40-233), but at 16 weeks of age this immune response is significantly diminished. In later functional studies, pigs were immunized with the ORF2 antigen (residues 40-233) or 169-180 peptide and challenged with virulent PCV2 virus (Trible et al., 2012). Pigs immunized with the 169-180 peptide had total antibodies titers similar to that of the group that was immunized with the ORF2 antigen (40-233). Surprisingly the serum from pigs that were vaccinated with peptide 169-180, was similar to non-immunized pigs in terms of neutralizing activity. Moreover, pigs that were immunized with 169-180 peptide had viral loads similar to the unvaccinated controls, while pigs immunized with the ORF2 antigen (residues 40-233) had no detectable viral loads. Interestingly, upon challenge with the virulent virus, the level of antibodies specific to the 169-180 peptide increased in pigs that were immunized with 169-180 peptide and non-immunized group, while in group that was immunized with ORF2 antigen (40-233) level of antibodies specific to 169-180 peptide remained low. The pigs immunized with the oligopeptide 169-180 had the highest immuno-histochemisty (IHC) scores for post-challenge, PCV2 antigen detection in their lungs. The scores were even higher than non-immunized pigs (Trible et al., 2012). In totality, these results suggest that amino acids residues 169-180 of the ORF2 protein, while able to induce strong antibody responses, are not able to induce protective immunity. Amino acid residues 173,

174, 175,179 were particularly important, as was shown by alanine scanning and the fact that these residues are conserved among PCV2 strains.

Immune Evasion Of Antibody Responses

Protective epitopes are the epitopes which upon binding to corresponding antibodies lead to neutralization of viral particles and therefore viral infectivity. Protective epitopes are most often located near the virus receptor recognition site and are conserved (Köhler et al., 1994). Viruses have evolved to avoid the immune response by directing the immune response away from protective epitopes towards non-protective but immuno-dominant epitopes also called decoy epitopes. Decoy epitopes are defined as highly immunogenic epitopes, but their binding to the corresponding antibody does not lead to viral inactivation, and can potentially lead to antibody mediated immune evasion (Carme et al., 2009; Di Lorenzo et al., 2011; Diamond, 2003). Decoy epitopes are also often more variable than protective epitopes, as this allows for pathogen to more easily avoid the immune response.

Based on the knowledge that initial repertoire of antibodies produced in the response to HIV-1 infection remains mostly unchanged later in infection, even when the virus undergoes antigenic variation Köhler et al. proposed the term deceptive imprinting (Köhler et al., 1994) to describe the phenomenon described above. Deceptive imprinting can be described as an emerging area of knowledge in host-pathogen interactions. The host's immune response has a bias to the first immuno-dominant epitopes encountered. However, the pathogen can diverge under the pressure of host's immune response to alter its antigenic properties. The evolution of the pathogen can occur inside the same host, as part of clinical or subclinical infections, as the case for HIV or caprine arthritis and encephalitis virus (CAEV) respectively, or in a non-species specific host as in the case of the influenza virus (Bianchi et al., 2005). Realization of the role

that deceptive imprinting plays in infection has led to the development of immune refocusing to channel the immune response towards protective immunity and avoid the immune bias towards non-protective correlates.

Immune refocusing is therefore an approach where the rationality of vaccine design can be achieved through manipulation of epitopes (Tobin et al., 2008). For example, if epitopes that are variable throughout the serotypes of pathogen are excluded from vaccine, then animals will develop immune response only to epitopes that is conserved across the serotypes. In the same manner changes can be introduced in decoy epitopes in the vaccine construct, so that deceptive imprinting is avoided at time of challenge with the agent, so that the animal will presumably have higher titers of protective rather than non-neutralizing antibodies.

For example, in the case of foot-and-mouth disease virus (FMDV) the main protective antigen is a capsid (VP1), which is highly variable. Analysis of hypervariable regions helped to identify conserved regions that would be most suitable for vaccine development leading to the development of effective vaccines (Tulasiram and Suryanarayana, 1998; Mayr et al., 1999; Cheevers et al., 2003; Lech et al., 2013). One important consideration for the rational design of vaccines is the surface localization of epitopes. In the native form of the antigen, some of the epitopes may not be exposed to the outside environment due to three dimensional folding. To identify whether a particular epitope is exposed or not, two approaches are available. The first is the examination of the three dimensional structure of the protein. This is often difficult because the structure of many viral antigens remained to be solved. Another approach would be to directly screen for antibodies to particular epitopes using sera from animals exposed to the agent. Linear epitopes can be mapped by PEPSCAN technology, while conformational epitopes can be

mapped by the interrogation of antigenic properties of truncated proteins with monoclonal antibodies.

Objectives of This Thesis

The primary goal of the current study is to enhance the functionality of existing PCV2 vaccines by simultaneously improving their immunogenicity and introducing a DIVA capability. We have identified additional decoy epitopes and tested the premise that mutation of the identified decoy epitopes will result in enhanced antibody mediated immunity, when pigs are immunized with the altered proteins. Increasing the threshold of protection could likely prevent or reduce the evolution of new viral variants. The disease eradication potential of an improved vaccine will not only will relieve the farms of the burden of constant vaccination but also improve the life quality of the animals, so that we can enjoy bacon in our sandwiches with a slightly reduced sense of guilt. If eradicated, PCV2 would not have the chance to evolve into serotypes that would not be affected by vaccine induced immunity. Finally, to address the gap in the unavailability of DIVA PCV2 vaccines and enhance the performance of the improved vaccine, we have introduced a positive and negative DIVA marker in the rationally re-designed vaccine. This study is the first to describe an epitope- based approach to immunization against PCV2 in swine, while further validating the ”immune-refocusing” approach to vaccine development (Tobin et al., 2008).

IMMUNOGENICITY OF A PORCINE CIRCOVIRUS STRAIN 2 CAPSID PROTEIN WITH MUTATED IMMUNO-DOMINANT DECOY EPITOPES AND DIVA CAPABILITIES [1]

Abstract

Porcine circovirus type 2 (PCV2) belongs to the Circoviridae family and is an important pathogen for swine industry as cause of porcine circovirus associated diseases (PCVAD). While several effective commercial vaccines against PCV2 are available, none of them are capable of differentiating infected and vaccinated animals (DIVA). Moreover, all target the PCV2a strain that has been replaced by PCV2b in the U.S since 2007. Furthermore, while current vaccines are effective, new virus variants continue to emerge, indicating that vaccine induced selection pressure could trigger viral evolution in the field.

To develop a rationally designed, improved PCV2 vaccine with the ability to differentiate vaccinated and infected animals (DIVA), positive and negative DIVA markers were introduced in the vaccine constructs. In addition, decoy B cell epitopes that can divert the protective antibody responses towards non-protective regions of the vaccine antigen, were modified by site directed mutagenesis to avoid possible subversion of host immunity and achieve a rationalized vaccine design. Immunization of pigs with the modified vaccines, followed by challenge with a

[1] The material in this chapter was co-authored by Oleksandr Kolyvushko, Brett Webb, Angela Pilatzki, Marcia Ilha, Peter Nara, Diel Diego, Steve Lawson, Eric Nelson, and Sheela Ramamoorthy. Oleksandr Kolyvushko had primary responsibility for design and preparation of vaccine samples, establishing assay required for this work and analysis of vaccine efficacy and DIVA capabilities. Oleksandr Kolyvushko was one of the primary developer of the conclusions that are advanced here. Oleksandr Kolyvushko also drafted and revised all versions of this chapter. Sheela Ramamoorthy served as proofreader and was responsible for the statistical analysis conducted and experimental design. Brett Webb, Angela Pilatzki, Marcia Ilha, Peter Nara, Diel Diego, Steve Lawson And Eric Nelson were crucial for animal study.

virulent field strain showed that the efficacy of the vaccine was comparable to a commercial vaccine. The average weight gain was significantly higher than that observed with the commercial vaccine. An appropriate response to the positive and negative DIVA tags was detected. Therefore, the strategy used in this study is the first to enable a DIVA capable vaccine and accompanying immuno-assay, while using an epitope based approach to target improved immunogenicity.

Introduction

Porcine circovirus type two (PCV2) is a small virus with a single stranded circular DNA genome. It is the causative agent of porcine circovirus associated disease (PCVAD), an important disease of swine in agricultural settings across the world (Alarcon et al., 2013). Postweaning multisystemic wasting syndrome (PMWS) is the primary manifestation of PCVAD in weanling piglets (Clark, 1997). Clinical signs of PMWS include wasting, dyspnea and jaundice. Other syndromes caused by PCV2 include the porcine respiratory disease complex (PRDC), dermatitis and nephropathy syndrome (PDNS), reproductive disorders, lymphadenopathy, and enteritis. PCV2 is widely present on commercial farms as well as in wild swine population (Larochelle et al., 2000; Reiner et al., 2010; Schmoll et al., 2008; Vicente et al., 2004).

The PCV2 infectious viral particle consists of 60 subunits of the capsid protein encoded by the viral open reading frame 2 (ORF2). While little is known about cell mediated immunity to PCV2, neutralizing antibodies to the capsid protein (Cap) are a reliable predictor of protective immunity in the case of PCV2 infection (Meerts et al., 2006). Binding as well as neutralizing antibody in infected or vaccinated animals can be detected as early as 14-21 days post infection (DPI) (Beach et al., 2010, 2011; Meerts et al., 2006).

Currently, there are 4 circulating PCV2 subtypes; PCV2a, b, c and d. Until about 2008, the predominant subtype in the U.S was PCV2a. Following the introduction of commercial vaccines in 2006, a type-switching event resulted in PCV2b becoming the predominant subtype in the U.S, indicating that current commercial vaccines could induce selection pressure (Ssemadaali et al., 2015). However, a number of commercial PCV2 vaccines that effectively reduce or prevent clinical signs are available. They primarily target the PCV2a capsid protein and they are effective in preventing or reducing clinical manifestations of PCVAD (Kristensen et al., 2011) through formation of immune response to capsid protein. However, they do not prevent viral evolution. Additionally, there are no accompanying Differentiation of Infected from Vaccinated Animal (DIVA) assays for any of the commercially available vaccines, which is a key requirement for monitoring vaccine compliance or for future disease eradication efforts. The DIVA strategy usually consists of a vaccine, and accompanying assay, usually an ELISA. Difference between a DIVA capable vaccine and regular vaccine is that DIVA vaccines include differential markers that are absent in wild type pathogen. Presence or absence to differential markers will be detected with accompanying ELISA test, and allow the differentiation of vaccinated from infected animals to enable the removal of infected animals from the population and disease eradication.

Currently PCV2 imposes a huge financial burden on the industry and it decreases life quality of animals. Continuous viral circulation in animal farms may lead to emergence of new strains, potentially with higher pathogenicity. Epitope analysis of the PCV2 capsid protein by our group and others indicate the presence of 4 major immunodominant regions (Mahé et al., 2000). Recently, Trible et al., described a decoy epitope at positions 169-181 on the viral capsid protein (Trible et al., 2011). When taken in conjunction with the finding that neutralizing antibody

responses against PCV2 are only detected about 3 weeks post infection, in this study, we have explored the hypothesis that additional decoy epitopes may be present on the PCV2 capsid protein. In addition we have tested the premise that mutation of the identified decoy epitopes will result in enhanced antibody mediated immunity, post-immunization with the altered proteins. Finally, to address the gap in the unavailability of DIVA vaccines, we have introduced a positive and a negative DIVA marker in the rationally re-designed vaccine. This study is the first to describe an epitope based approach to immunization against PCV2 while further validating the ”immune-refocusing” approach to vaccine development (Tobin et al., 2008). With the changes to decoy epitopes suggested in this study the resulting vaccine will presumably improve immunogenicity thus reducing the probability of negative clinical outcomes. Having a vaccine with a corresponding DIVA assay, will allow assessment of vaccine compliance, and detection of the vaccine escape cases. The DIVA capability will allow for early isolation of vaccine escape cases, thus reducing viral circulation and reducing potential for emergence of new strains of PCV2. Combination of a DIVA capable vaccine and appropriate DIVA test will potentially lead to rational protocols for the eradication of PCV2 from animal farms.

Material and Methods

Mapping of neutralizing and decoy epitopes in the PCV2 capsid protein

Two complimentary approaches were used to map the protective and decoy epitopes on the PCV2 capsid protein. For the first approach, based on the premise that early antibody responses will target non-neutralizing epitopes while later responses will target the protective epitopes. Archived sera collected at days post infection (DPI) 7 and 28 from PCV2 infected animals (Ramamoorthy et al., 2011) were used to perform a PepScan analysis of the PCV2a capsid protein. Overlapping 12-mer peptides with a 9 residue overlap, spanning the entire capsid

protein were purchased from a commercial source (Mimotopes, Victoria, Australia). To perform the peptide ELISAs, 100µl of a 10ug/ml solution of biotinylated peptides were bound to streptavidin coated ELISA plates. After blocking with 2% BSA and skimmed milk powder, 100 µl of a 1:50 dilution of serum was reacted with the peptides in duplicate. Specific reactivity was detected by anti-swine IgG labeled with Horseradish peroxidase (HRPO) conjugate at a 1:2000 dilution. The average of the lowest quartile OD values from each data set plus two standard deviations was subtracted from the final readings. Each corrected value was then expressed as a signal/negative [S/N] ratio with the corresponding value from the negative control data set. Values above an S/N ratio of 1 were considered positive.

Mapping of neutralizing and decoy epitopes in the PCV2 capsid protein by a fluorescent focus inhibition assay

To confirm the findings of the PEPSCAN analysis using the second approach, pools of equal quantities of 5 peptides, spanning the previously defined immunogenic regions (Lekcharoensuk et al., 2004; Mahé et al., 2000) were used in peptide binding fluorescent focus inhibition assay to map which epitopes are protective (responsible for binding with neutralizing antibodies) and which may be decoy epitopes. Serum collected at DPI 28 because it would represent a mature immune response. Briefly, 10 ul of each of 5-6 selected peptides [1mg/ml] was pooled. 10 ul of each pool was incubated with 1:2 dilution of heat inactivated pooled DPI 28 PCV2a anti-serum for 60 mins at 37ºC. PCV2a strain 40895 with a titer of $10^{4.5}$ $TCID_{50}$/ml (Tissue culture infectious dose 50%) was diluted 1:10 in DMEM. Equal volumes of diluted virus and peptide blocked antiserum were incubated at 37ºC for 60 mins before layering on PK-15 monolayers in 8 well chamber slides. Supernatants were removed after 3 hours and replaced with 2% DMEM. Slides were stained with a PCV2 monoclonal antibody and anti-mouse FITC after

36 hours. The number of PCV2 specific fluorescent foci in each well was counted as blinded evaluations by two individuals in two independent experiments with 4 replicates for each peptide pool. The mean value is presented as the percentage change in the treatment group when compared to the unblocked PCV2 antiserum collected at DPI 28. A non-specific swine influenza virus (SIV) peptide and negative DPI 0 serum pool were used as controls.

Cloning of the PCV2b capsid protein with mutated decoy epitopes

Using the ORF2 sequence of a previously cloned and sequenced PCV2b genome (Constans et al., 2015a), codon optimized template DNA was commercially synthesized (Eurofins Genomics, Louisville, KY) for cloning and bacterial expression. To introduce a negative DIVA marker, the nuclear localization signal (NLS) (1-39 amino acid residues) was omitted from the template DNA. To prepare the construct (designated as construct A), the coding sequence was modified to incorporate 5 single amino acid changes located in potential decoy regions Fig 6 and 7)

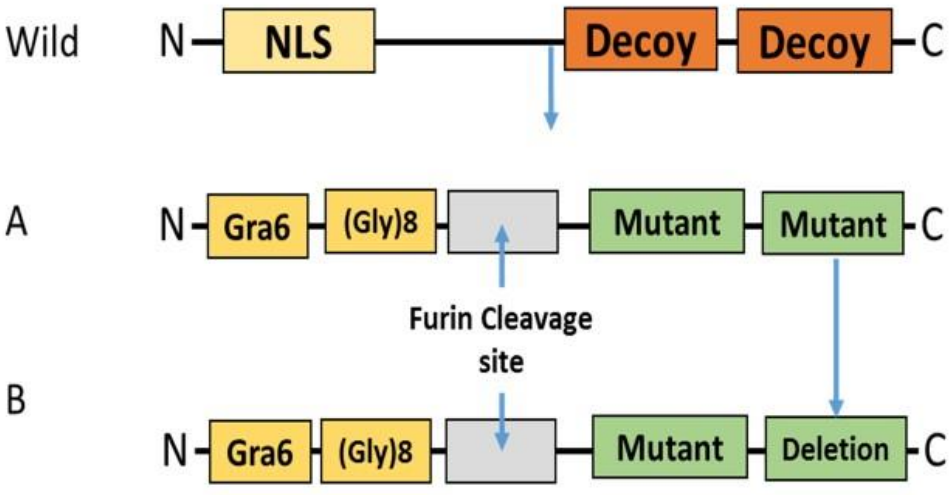

Figure 6. Construction of the vaccine candidates**.** Schematic representation of the construction of vaccine candidates constructs A and B. NLS- Nuclear localization signal. Gra6- 20aa long epitope tag from Gra6 protein. (Gly)8- is a 8 glycine linker.

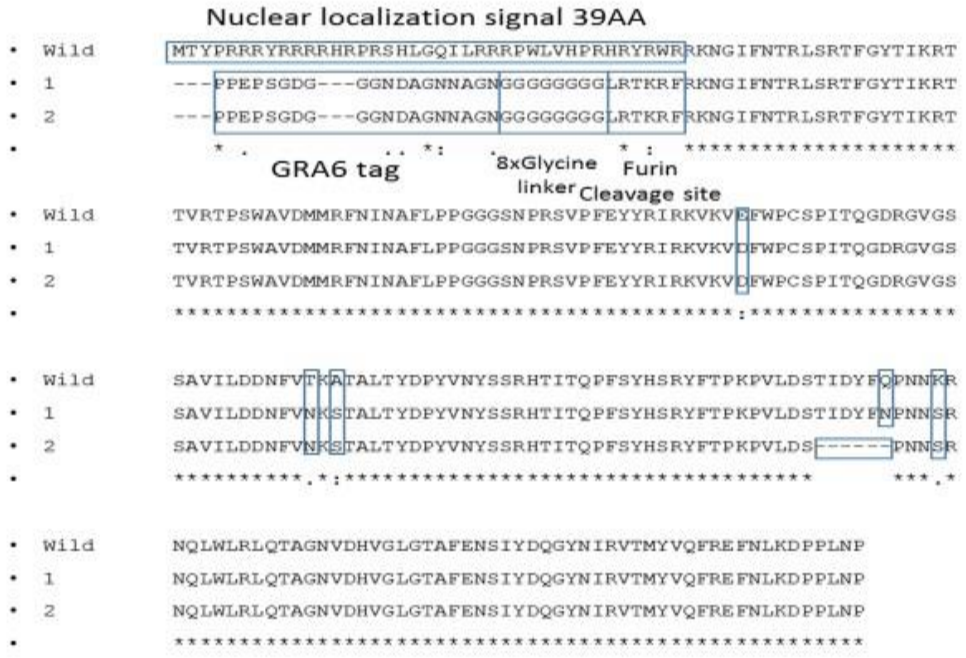

Figure 7. Alignment of wild type ORF2 with constructs A and B. Changes that were introduced are highlighted with a blue box. NLS was replaced with GRA-6 tag, following by 8xGlycine linker and furin cleavage site. Construct 2 has 5 additional single amino acid changes: E104D, T131N, A133S, Q175N, and K179S. The commercially synthesized sequence containing restriction sites for XhoI and EcoRI (NEB, Ipswich, MA) was digested with the appropriate enzymes ((NEB, Ipswich, MA). and directionally cloned into a pET 28 vector (Novagen Darmstadt, Germany) to produce pre-construct A.

A second construct (designated as construct B), that in addition to the four amino acid changes, six other amino acid residues (positions 170-175) within a decoy epitope previously identified by Trible et al (Trible et al., 2011) deleted, was prepared by overlap extension polymerase chain reaction (SOE-PCR) from construct A. The SOE-PCR was performed in two steps. Step one started with initial denaturation at 95°C for 5 min, followed by 25 cycles of 95°C for 1 min, 55°C for 30 secs, 72°C for 30 sec, final extention 72°C for 2 min with Phusion High-Fidelity DNA Polymerase master mix (NEB, Ipswich, MA). Step one was carried out in two separate reactions, each of them had corresponding pair of primers 1-2 and 3-4 (Primer sequences were as follows: 1) 3'- GACTGAATTCCGTAAAAACGGTA-5' 2) 3'-

GAGTTGTTCGGAGAGTCCAG-5’ 3) 3’- CTGGACTCTCCGAACAACTC-5’ 4) 3’-GCTACTCGAGTTACGGGTTCAGCG-5’). The amplified products were subjected to gel electrophoresis to verify the correct size and extracted from the gel with QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The second step of SOE-PCR was performed in similar conditions as step one, except with 30 instead 25 cycles using primer 1 and 4. SOE-PCR reactions were performed using Phusion High-Fidelity DNA Polymerase (NEB, Ipswich, MA). Both sequences A and B were directionally cloned in to pET28a (Novagen Darmstadt, Germany) bacterial expression vector. The integrity of the construct was verified by sequencing and restriction digestion.

Introduction of a positive DIVA marker

Constructs A and B were then modified by inserting a positive DIVA marker. The Q5 Site-Directed Mutagenesis Kit (NEB, Ipswich, MA) was used according to manufacturer’s instructions to introduce a DNA sequence that encoded a 20 amino acid long peptide from the GRA6, protein of *Neospora caninum*, an apicomplexan parasite which does not infect swine (Figure 3) together with a Glycine linker ((Gly) x8) and a furin proteolysis site. In short, a two-step PCR reaction was used. Initial denaturation 98°C for 2 min, followed by 98°C for 2 min and 25 cycles of 72°C for 30 sec and 72°C for 140 sec, final extension step was 72°C for 2 min. The following primer were used in final concentration of 0.5mM: Forward: 3’-CGTTATTGCCGGCATCATTGCCGCCACCGTCACCAGATGGTTCC GGAGGGAATTCGGATCCGCG-5’, reverse: 3’- CGGGCAACGGTGGCGGCGGTGGT GGCGGTGGCCTGCGCACTAAACGTTTTCGTAAAAACGGTATC -5’ Site direct mutagenesis PCR reaction was carried out with Phusion High-Fidelity DNA Polymerase (NEB, Ipswich, MA).

One µl of PCR reaction mix was used in KLD (*Kinase-Ligase*-DpnI) reaction following the manufacturer's instructions. The reaction mix consisted of 1µl of PCR product, and 5µl of 2x KLD buffer, and 1 µl of KLD enzyme mix, and was incubated for 10 minutes at room temperature. This KLD reaction mix was used to transform competent Top-10 cells (Thermo Fisher Scientific, Waltham, MA). The accuracy of the sequence of constructs A and B was confirmed by sequencing (Eurofins Genomics, Louisville, KY).

Expression and purification of the construct A and B

For bacterial expression, BL21 (DE3) (Thermo Fisher Scientific, Waltham, MA) cells were transformed with plasmids encoding constructs A and B according to the manufacturer's instructions. Freshly transformed cells were grown overnight in 2000 ml of LB with 50 µg/ml of kanamycin. An overnight culture was used to inoculate the expression culture. Bacterial expression was induced when the OD_{630} reached 0.4 with 1 mM IPTG. Cells were collected at 6 hours post induction by centrifugation at 6000 g at 4°C for 15 minutes with Avanti® J-26 XP Centrifuge (Beckman Coulter, Brea, CA) with a fixed angle rotor J-LITE® JLA-16.250 (Beckman Coulter, Brea, CA).

The cell pellets were lysed in a binding buffer (8M Urea, pH=8.0, Imidazole 10mM, 100mM sodium phosphate monobasic, 10mM TRIS) with sonication on ice at 30% amplitude until the solution became transparent. The total cell lysate was incubated with 2 ml of Ni-NTA (Qiagen, Hilden, Germany) agarose beads for 1 hour with rotation at RT and applied to a gravity flow column. Agarose beads on the column were washed with 10 column volumes of wash buffer (pH=6.3, 50mM Imidazole, 100mM sodium phosphate monobasic, 10mM TRIS). Specific protein was eluted with 5 column volumes of Elution buffer (pH=4.5, 250mM imidazole, 100mM sodium phosphate monobasic, 10mM TRIS), 2 ml fractions were collected. The eluted

fractions were precipitated with 9 volumes of ethanol that was cooled down to -20°C. The mixture was incubated overnight at -20°C. Precipitated protein was pelleted with centrifugation at 10000 RCF for 30 minutes at 4°C with Avanti® J-26 XP Centrifuge (Beckman Coulter) with a fixed angle rotor J-LITE® JLA-16.250 (Beckman Coulter). Supernatant was carefully discarded and pellet was air dried. When pellet became dry it was re-suspended with 1x Tris-Glycine-SDS Running Buffer (Boston BioProducts). Purified proteins were stored at -80°C for vaccine formulation and were analyzed by western blotting, with anti-PCV2 and anti-*N. caninum* antibodies.

Validation of the vaccine constructs by Western blotting

SDS-PAGE was performed using a 12% polyacrylamide gel, in a Mini-PROTEAN Electrophoresis System (Bio-Rad, Hercules, CA). Prior to transfer, a PVDF membrane was submerged in methyl alcohol, and then incubated in transfer buffer (Boston BioProducts). Trans-Blot SD semi-dry transfer cell (Bio-Rad, Hercules, CA) was ran at 10V for 30 for transfer. Following the transfer, the membrane was washed 3 times with PBST. The membrane was blocked for 1 hour with 3% dry non-fat milk in PBST on a rotary shaker at RT. Primary antibodies was added at dilution of 1:250 in PBST, and incubated for 1 hour with gentle rotation. The membrane was washed 4 times with PBST at 5 minute each. Secondary HRPO labeled antibody was added at dilution of 1:1000 in PBST and incubated for 1 hour. The membrane was washed 4 times with PBST for 5 minute each and developed using TMB (Thermo Fisher Scientific, Waltham, MA).

Cloning and expression of the DIVA markers as capture antigens for the DIVA ELISA

For use as capture antigen for the DIVA ELISA, the NLS region of the ORF2 and *N. caninum* derived GRA6 tag were cloned into plasmid pET28 A Briefly, primers 3’- GCT

AGAATTCACCTACCCGCGTCGT-5' and 3'- CGATCTCGAGTTAACGACGCCA ACGGTAACGGT-5' were used to amplify the 117bp NLS sequence, Cloning into the pET-28a-sumo vector was achieved using EcoRI and XhoI (NEB, Ipswich, MA) restriction enzymes. The DNA sequence from GRA6 gene that represents amino acid residues spanning from 179 to 197of the 232 long protein were codon optimized and cloned into the pET-28a-sumo bacterial expression vector using Q5 Site-Directed Mutagenesis Kit and primers 3'- GCAA TGATGCCGGCAATAACGCGGGCAACCTCGAGCACCAC-5'and 3'-CGCCACCG TCACCAGATGGTTCCGGAGGGAATTCGGATCC-5', as described earlier. Both constructs were subjected to restriction digestion and sequences were verified .Verification of protein expression by western blotting was carried out as described above with a swine anti-PCV2 serum for the NLS construct and a rabbit, polyclonal anti-*N. caninum* serum (provided by Dr. David Lindsay, Virginia Tech) for the GRA6 protein. Purification of the diagnostic proteins was carried out essentially as described above, except that the native purification system was used for the Gra6-sumo protein. Native purification was performed with a His-Spin Protein Miniprep (Zymo Research, Irvine, CA) following the manufacturer's instructions.

Preparation of the PCV2 challenge culture

The cloned genome of mPCV2b strain (JX535296.1) was kindly provided as a monomeric copy in the TA cloning vector pCR2.1 (Thermo Fisher Scientific, Waltham, MA) by Dr. X.J. Meng, Virginia Tech and Dr. Tanja Opriessnig, The University of Edinburgh. The genome was dimerized as previously described into the plasmid pBlueScript II SK (+) (Fenaux et al., 2002), such that two tandem copies of the genome were inserted . Expression of the recombinant virus was verified by an immunofluorescence assay, as previously described

(Constans et al., 2015a). The titer of resulting virus culture was obtained as a $TCID_{50}$ value in PK15 cells using Immunofluorescence assays.

Formulation of the vaccine

After adjusting concentration of the purified proteins for construct A and B to 1mg/ml, the proteins were individually mixed with an adjuvant MONTANIDE ISA 201 VG (Seppic) (Montanide, Fairfield, NJ) following the manufacturer's instructions. In short, the protein solution was mixed 1:1 with adjuvant solution, for 2 hours at 31°C, with gentle shaking. The water-in-oil-in-water emulsion was transferred to 4°C right away until further use

Testing of the vaccine in pigs

Thirty-one five week old pigs were randomly divided in to 5 groups as follows: Group 1- unvaccinated controls (N=7), Group2 - Positive controls were administered a commercial vaccine following label instructions (Merial, Duluth, GA) (N=7) , Group 3– Con A (2ml dose, 1ml i/m, 1ml S/C) (N=7), Group 4 – Con B (2ml dose, 1ml I/M, 1ml S/C) (N=7) Group 5 – Negative controls (N=3). Animals were boosted on DPV 18 by the same dose and route. Serum samples were collected on days post vaccination (DPV) 0, 11, 18, 25, 32, 42 and 53. At DPV 32 all animals were challenged with 4ml of 10^5 $TCID_{50}$/ml mPCV2d virus. Temperature and weights were measured at the day of challenge (DPV32), at DPV 38, 42, 47, and at the end of the experiment. At DPV 53 animals were euthanized. Gross lesions were assessed at necropsy. Tissues were fixed in 10% buffered formalin for 48 hours then transferred to 70% ethanol before being sectioned and routinely processed. 5µm thick sections of lung, tonsil, liver, kidney, spleen, tracheobronchial lymph node, mesenteric lymph node and thymus were prepared. Immunohistochemistry (IHC) was performed at the South Dakota State University Animal

Disease Research and Diagnostic Laboratory.The amount and in intensity of staining was scored on a 1-4 scale by a board certified pathologist who was blinded to the treatments.

ELISA for the detection of binding antibody responses

Antigens were diluted to final concentration of 400 ng/ml in carbonate coating buffer. 50 ul was added to the 96 well high binding plates and incubated overnight. The following proteins were used as antigens on the ELISA: the complete ORF2 protein was prepared by *in-vitro* transcription and translation TnT® Quick Coupled Transcription/Translation System (Promega, Madison, Wisconsin, USA), the DIVA marker antigens for the NLS and GRA-6. Plates were washed 4 times with PBST and blocked with 100 ul of general block (ImmunoChemistry Technologies, Bloomington, MN) for 2 hours with shaking at 37°C. Plates were washed 4 times with PBST. The test serum was added at a dilution of 1:50 and incubated for 2 hours. Plates were washed 4 times with PBST. Secondary anti-swine HRPO labeled antibodies were added at dilution 1:5000 and incubated for 1 hour. Plates were washed 4 times with PBST and developed using 50ul of SureBlue TMB (KPL, Gaithersburg, Maryland). The reaction was terminated with 50µl of 1M HCl. OD_{450} was measured with ELx800 Absorbance Reader (Biotek, Winooski, VT).

Fluorescent focus inhibition assays to detect virus neutralization

Virus cultures for PCV2b strain 41513 (accession no. KR816332) (Constans et al., 2015b) was rescued from the infectious clone as described above. The titer of the culture was adjusted to $10^{3.7}$ TCID50/ml. Equal volumes (50 µl) of pooled pre-challenge serum (DPV 32) from each group and homologous PCV2b virus culture was incubated for 1 hour at 37 °C. The virus-serum solution was layered on PCV2 negative PK-15 cells and incubated for 1hr at 37ºC, in four replicates. Four wells were infected with virus alone. The inoculum was removed, cells were washed twice and left in DMEM with 2% FBS. After 36 hours post inoculation cells were

fixed and stained with PCV2 specific antibodies as described above in the IFA section. The number of PCV2 specific foci in each well was enumerated by two independent operators. The reduction in the number of foci in wells with serum, indicating virus neutralization, was calculated as percentage reduction in number of PCV2 specific fluorescent foci compared to the virus controls.

Detection of viral loads by qPCR

Quantification of the viral loads in the pre and post-challenge sera collected at day 10 and day 17 post-challenge was assessed by qPCR. Total genomic DNA was extracted from the test sera with the Qia Amp DNA mini kit (Qiagen, Valencia, CA) and eluted into 100 µl of H_2O. The qPCR reaction was essentially performed as described earlier (Opriessnig et al., 2003) with following modifications: only 1ul of extracted DNA from serum was used for qPCR. A primer concentration of 0.4 µM, probe concentration of 0.1uM, and Tm of 65°C was used with the QuantiFast Probe PCR Kit (Qiagen, Hilden, Germany) and CFX96 Touch (Bio-Rad, Hercules, CA) qPCR thermocycler.

Statistical Analysis

Statistical analysis of data was performed in Minitab software (Minitab, State College PA) or on Microsoft Excel. Date collected was analyzed using multivariate analysis of variance (ANOVA) with significance value of $p \leq 0.05$. The Tukey method was applied for multiple comparisons. Differences in weight gain and viral loads were assessed by a Student T test.

Results

Mapping of neutralizing and decoy epitopes in the PCV2 capsid protein by PEPSCAN analysis

Epitope mapping indicated that the initial (DPI 7) immune response targets peptides 19-22, 33-38, 45-46 (Fig. 1). These results indicate the presence of immune-dominant and likely

non-protective epitopes at these locations due to the strong immune response to these regions in early post-infection sera with low neutralizing antibody levels. On the other hand, the mature immune response (DPI28) shifted towards peptides 21-25, 33-44, 56-59, and 72-75, indicating the presence of neutralizing epitopes in these regions.

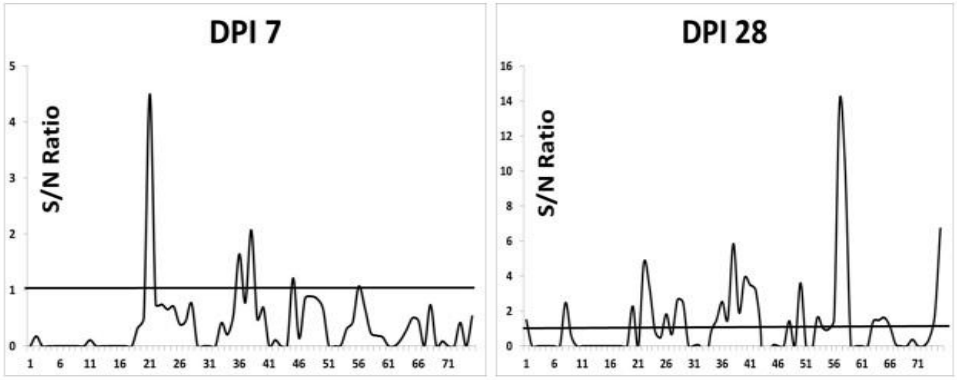

Figure 8. PepScan analysis of the PCV2a capsid protein. PepScan analysis of the PCV2a capsid protein. Was performed using pooled sera from PCV2a infected pigs collected at DPI 7 and 28 against a PCV2 ORF2 12-mer peptide library. Average corrected values are expressed as a signal/negative [S/N] ratio with the corresponding value from the negative control data set. Values above an S/N ratio of 1 [solid bar] were considered positive.

Mapping of Neutralizing And Decoy Epitopes In The Pcv2 Capsid Protein By A Fluorescent Focus Inhibition Assay

To further confirm which epitopes are responsible for protection, peptides were incubated with serum from DPI 28, which had a virus neutralization titer of 1:68. Peptide binding fluorescent focus inhibition assay revealed that peptides 33-38, and 45-46 resulted in a reduction of the number of specific fluorescent foci by about 50 and 15 percent respectively, while peptide 19-22 had no appreciable effect. Therefore, the regions spanned by the peptides mentioned above were hypothesized to contain immunodominant, non-neutralizing epitopes.

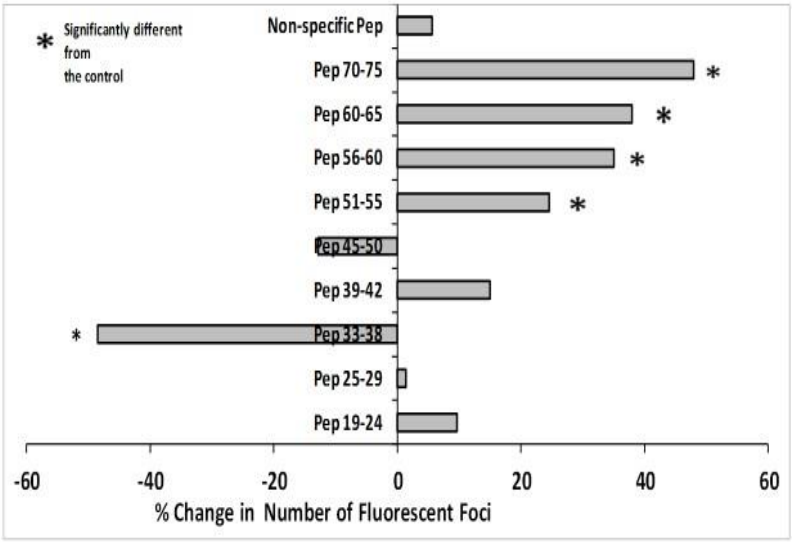

Figure 9. Peptide binding fluorescent focus inhibition assay to map protective and decoy epitopes. Pools of 4-6 peptides were incubated with DPI 28 PCV2 antiserum. Blocked antiserum was challenged with live virus. Blocking of neutralization activity was assessed as the number of PCV2 specific fluorescent foci in cells incubated with the blocked serum/virus mixture. Each well was blindly evaluated by two individuals in two independent experiments with 4 replicates for each peptide pool. The mean value is presented as the percentage change in the treatment group when compared to the unblocked DPI 28 PCV2 antiserum, with a decrease in the number of foci indicating decoy properties of that region and vice versa

Cloning and Expression Of The Pcv2b Capsid Protein With Mutated Decoy Epitopes

Sequencing of the cloned plasmid DNA encoding Con A and B indicated that desired mutations were intact, that the amino acid residues 170-175 were deleted in construct B, and that the introduction of the GRA-6 tag with linker ((Gly) x8) and furin proteolysis site was successful. Western blot analysis (Fig 4) indicated that desired constructs reacted with both anti-PCV and anti-Neospora antibodies, suggesting that GRA-6 tag and PCV2 part of protein both expressed correctly and d appropriate antigenic properties.

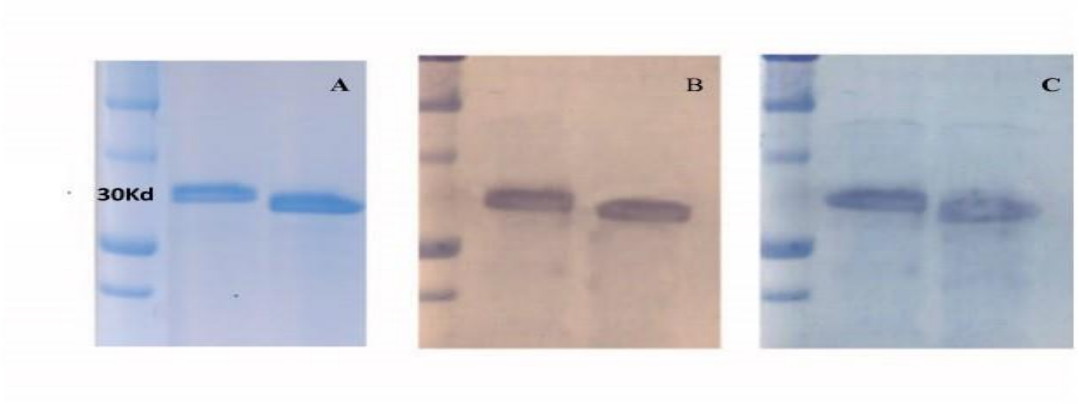

Figure 10. Analysis of proteins A and B. SDS-PAGE (A), Western blot with anti-PCV2 (B) and anti-Neospora(C) polyclonal antibodies. Both proteins A and B reacted well with both anti-PCV2 and anti-Neospora antibodies with molecular weight about 30 kDa as expected.

Expression of the DIVA antigens

The bacterial expression constructs for the NLS and GRA-6 had the expected sequence. As expected, western blotting of the purified NLS protein the PCV2 antiserum showed the presence of an approximately 20 kDa band in both cases. Similarly, the purified GRA6 protein reacted specifically with the anti-*N. Caninum* antiserum at the expected molecular weight of approximately 20Kd (Figure 11).

Preparation of the Pcv2 Challenge Culture

The dimerized infectious clone of mPCV2b was created and confirmed by sequencing. The immunofluorescence assay showed the expression of apple green nuclear fluorescence typical of PCV2 infection (Figure 12). The virus culture with a titer of $1x10^5$ $TCID_{50}$ was obtained and used to challenge the vaccinated pigs.

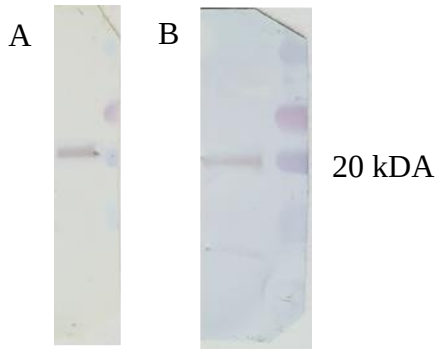

Figure 11. Expression of the DIVA antigens. Western blot image of A) purified Gra6 protein reacted with rabbit anti-*neospora* serum. B) Purified NLS protein reacted with swine anti-PCV2 serum. Both proteins were detected at the expected molecular weight of approximately 20 kDa

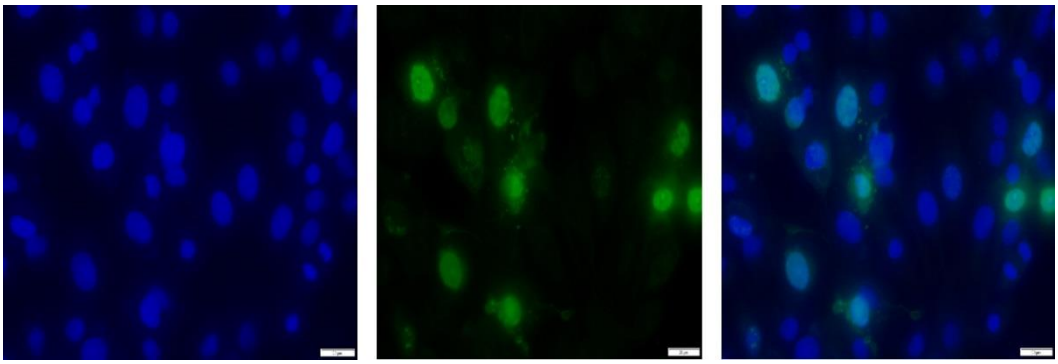

Figure 12. Immunofluorescent analysis of cells transfected with the dimerized infectious clone of mPCV2b. From left to right, cell nucleus stained with DAPI, anti-PCV2 antibodies stained cells showing recombinant viral particles and nuclear fluorescence, overlay of the DAPI and antibody stained images showing nuclear fluorescence specific to PCV2.

Average Weight Gain Measurements

The average weight gain between the day of challenge and necropsy for group 1, 2, 3, 4 was 25.3, 26.0, 26.8, and 31.5lb respectively (Fig 9). The pigs in group 4 gained an average of 5.5lb more compared to the other groups. The difference in weight gain between pigs administered Con B and the unvaccinated pigs was statistically significant with a p=0.04.

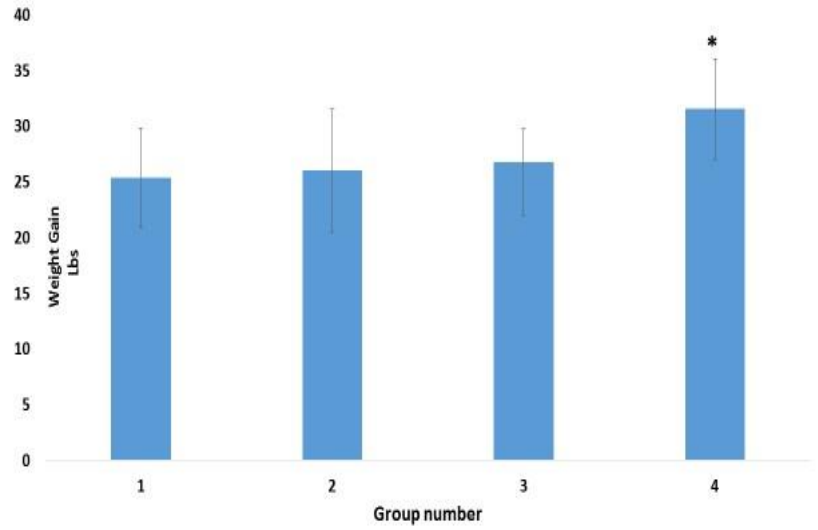

Figure 13. Average weight gain in the treatment groups. The weight of each pig was measured in pounds on the day of challenge and necropsy. Mean values of the weight gain in pigs from each group (N=7) and the standard deviations are depicted. * $p \leq 0.05$ as determined by a Student T test.

Bodily Condition Measurements

Of animals varied from slightly thin to slightly overweight but most of the animals had ideal score of 3(Cutler, 2014). The unvaccinated animals experienced a slight loss of bodily condition 5 days after challenge but this loss was not significant, indicating that the challenge strain was not virulent enough to cause severe disease. The vaccinated animals did not show differences in bodily condition after challenge, except that animals in the ConA group showed a slight loss in bodily condition 10 days after challenge. The differences between groups was not significant (Figure 14).

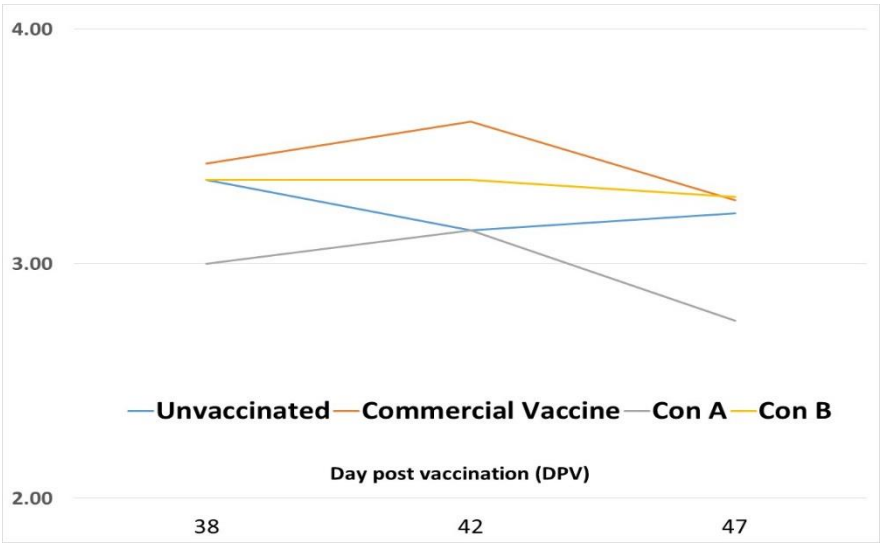

Figure 14. Bodily condition scores (BCS). A score of 3 is considered to be optimal. BCS was estimated by operators on the 5 grade scale, based on the shape of the animal and visibility of the backbone and hips, with 5 being round overweight pig and 1 being emaciated animal with hips and backbone visible.

Bodily Temperature Measurements

The temperature of all animals ranged from103-104°F at day of challenge and was within the normal body temperature range of 101-104^{o}F. Following challenge, temperature measurements were carried out every five days. There were no significant differences in body temperatures between the unvaccinated and vaccinated animals, except for 1 animal in group 3 with a temperature of **(**>105°F) at DPV 38 and 42 and one animal in group 4 with 107°F at DPV38. A drop of temperature, which was within the range for normal temperatures, was detected in all groups on the day of necropsy (Figure 15).

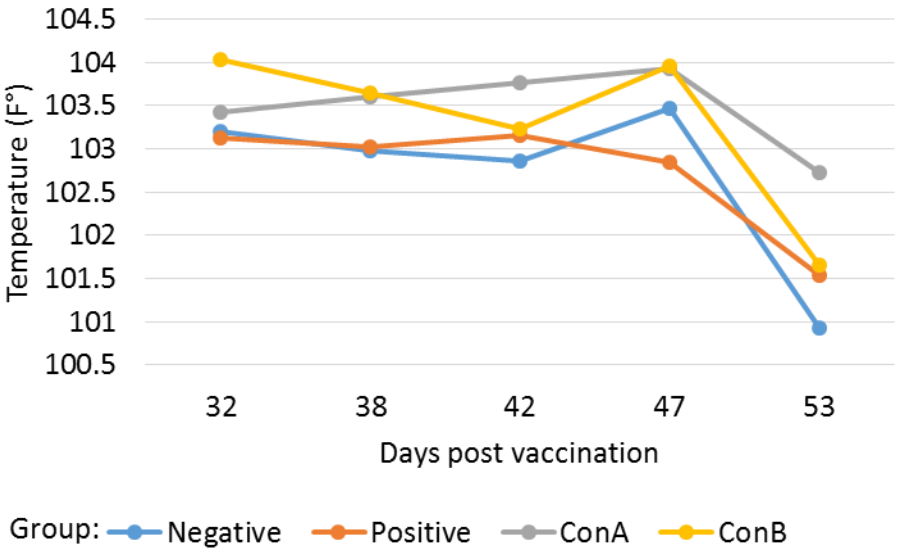

Figure 15. Bodily temperature measurement. The temperature of each pig (n=7) in each group was measured prior to challenge and every five days (DPV 38, 42, 47) post-challenge until necropsy on DPV 53. The mean temperature for each group is presented. Group 1 – Unvaccinated, Group 2 – Commercial vaccine, Group 3- Construct A, Group 4 – Construct B.

Gross Pathology

On necropsy, two pigs from the unvaccinated group were found to have a moderate enlargement of the mesenteric lymph while one of them had a patchy atelectasis affecting approximately 10% of right cranial and middle lung lobes. No other significant changes were noted in the organs of the pigs in the other groups.

Elisa for the Detection of Diva Antigens

As expected, animals vaccinated with constructs A and B mounted an anamnestic antibody response to the positive marker antigen, GRA6 following the booster at DPV 18, while the response of pigs in the groups not immunized with GRA6 (Groups 1 and 2) remained below the baseline. The response to the GRA6 antigen increased for the duration of the experiment in groups 3 and 4. For the negative NLS marker, as expected, the unvaccinated animals and animals vaccinated with Con A and B did not mount an anamnestic response on challenge, while animals

which received the commercial vaccine showed a sharp increase in titers. Similarly, the unvaccinated animals showed an increase in titer after the challenge, when they were first exposed to the negative marker. Reactivity to both antigens was detected at the start of the experiment when maternal antibody levels were high (Figure 16

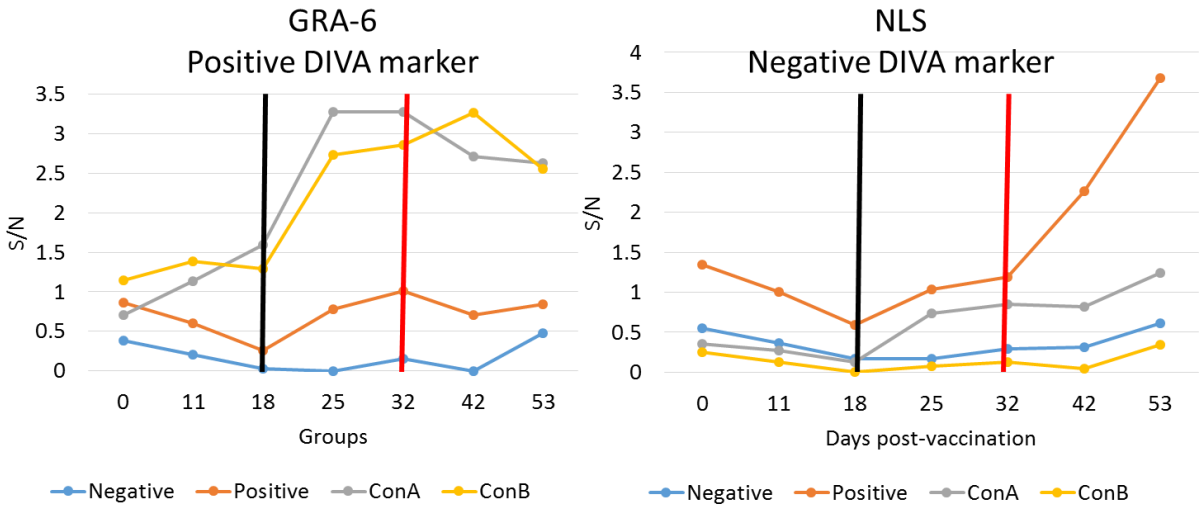

Figure 16. Detection of antibody responses to the DIVA antigens. Antibody responses to the positive (GRA6) and negative (NLS) markers were measured by ELISA for all four test groups (N=7 each). The mean value of two replicate measurements is depicted. Black vertical bar- day of booster, red vertical bar – day of challenge.

Detection of Binding Antibody Responses to the Capsid Protein

Baseline antibody responses to the capsid protein were detected at day 0 in all groups due to the presence of maternal antibodies. The maternal antibody responses declined by DPV18. As expected, following the booster vaccination, an anamnestic response was mounted in all vaccinated groups, with the difference being most apparent in groups which received Con A and B. A further increase was noted in the vaccinated animals post challenge, while the unvaccinated animals showed an increase in PCV2-ORF2 specific antibodies a week after the challenge (Figure 17).

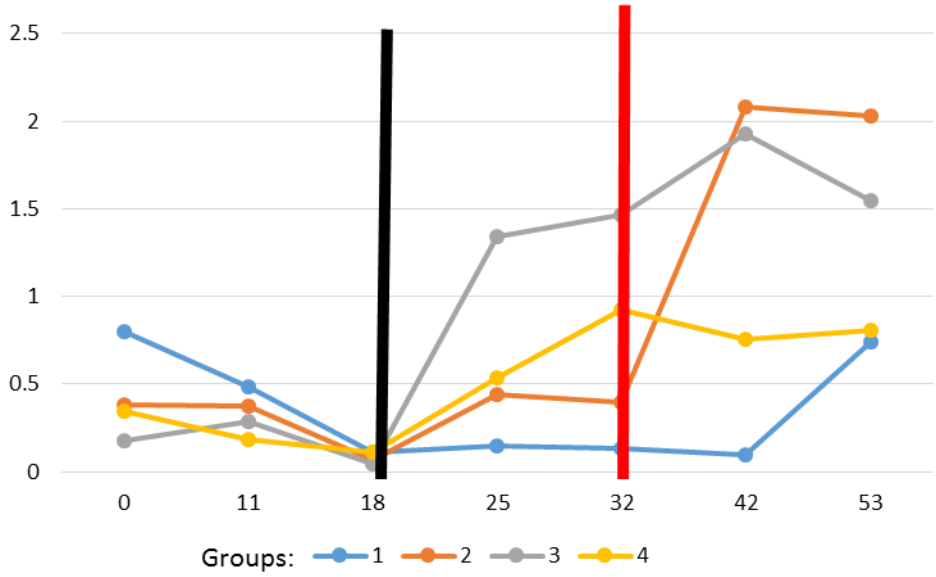

Figure 17. Antibody responses to the PCV2 ORF2 antigen. Antibody responses to the complete PCV2 ORF2 antigen was measured by ELISA for all four test groups (N=7 each). The mean value of two replicate measurements is depicted. Black vertical bar- day of booster, red vertical bar – day of challenge.

Quantification of Viral Loads

Detection of PCV2 DNA by quantitative real time PCR showed that all animals were negative prior to the challenge. All groups were negative prior to challenge. In negative control inoculation group 6/7 animals were positive after 10 days post challenge, and 7/7 were positive at 17 days post challenge, with average of $10^{4.3}$ and $10^{5.1}$ genomes per ml at day post challenge 10 and 17 correspondingly. Group vaccinated with commercial vaccine had only 2/7 animals positive at both time points, but interestingly while animal #14 showed constant viral load at days post challenge 10 and 17, animals #11 and #13 were positive at a single time point each: #11 at DPI42 and #13 at DPI 39. Animals vaccinated with construct A and B were all positive for PCV2 genomes at both time points (fig 7.). The only statistically significant difference was observed between groups vaccinated with Commercial vaccine group if compared to any other

group. There was no statistically significant difference in genome loads between mock vaccinated groups and groups immunized with construct A and B.

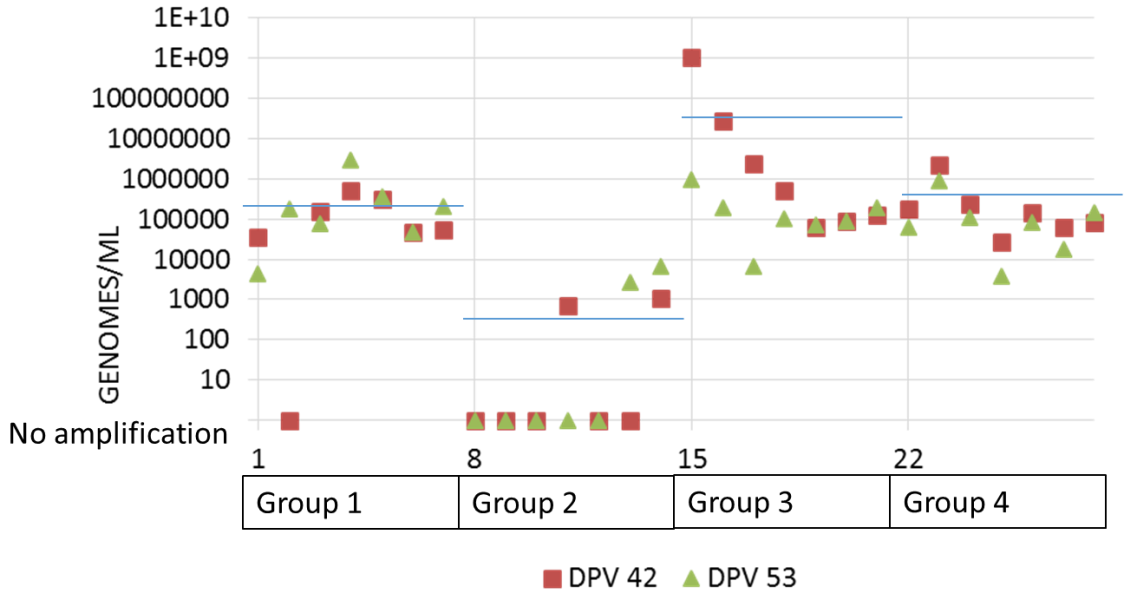

Figure 18. Detection of viral load by qPCR. The viral loads in the sera of the test animals at 10 and 17 days' post challenge in all four groups (N=7 each) were measured by qPCR. The mean of duplicate Ct values, converted into genome copy per ml is depicted. Blue bar indicates average value for the group.

Virus Neutralization Assay

The ability of pre-challenge sera from the test animals to neutralize the viral strain which is homologous to the vaccine constructs (PCV2b strain 41513) as measured by a fluorescent focus neutralization (FFN) assay, showed that the commercial vaccine induced a percentage FFN reduction of 69% while the Con A and B groups had a reduction of 51 and 52% respectively. Sera from the unvaccinated pigs showed no reduction, as expected. All groups, except 3 and 4, were significantly different from one another (Figure 18).

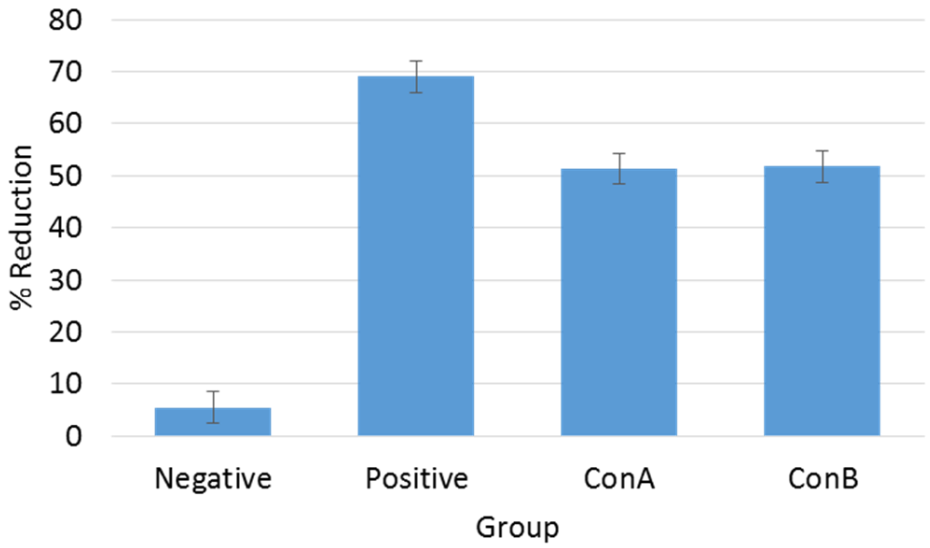

Figure 19. Virus neutralization assay. Pooled sera from all pigs (N=7) in each of the 4 test groups; namely, Group 1 – Unvaccinated, Group 2 – commercial vaccine, Group 3- construct A, Group 4 – construct B, assessed for virus neutralizing ability by a fluorescent focus inhibition assay. The homologous virus strain PCV2b 41513 was used for neutralization. The mean percentage reduction in the number of fluorescent foci in treatment groups compared to the virus control is depicted. Bars with similar letters are not significantly different.

Discussion and Future Directions

Previously published studies on the identification of protective epitopes of PCV2 suggest that residues 132 and 146 (Truong et al., 2001) and 157 and 186 (Lekcharoensuk et al., 2004) are neutralizing in nature. Our analysis did not confirmed this findings but rather showed that residues 98-103 are responsible for neutralization, and that changes introduced in the current study to a capsid protein did not disrupt the neutralizing epitope identified in this study.

While little published information is available regarding decoy epitopes on the PCV2 capsid protein, the region 169-180,was identified as a decoy epitope by Tribe et.al., (Trible et al., 2012) and spanned peptides 56-59. In this study, this region showed a low initial antibody response on PEPSCAN analysis and increased immune response by DPI 28 (Fig9) indicating a discrepancy between our findings and those of Trible et al. However, the differences can be

explained by variations in the immune response of individual animals used in the study, or strain specific differences. Based on the PEPSCAN analysis and FFN assays, the strongest decoy activity was predicted for peptides 33-38 which corresponds to amino-acid residues 97-123. Interestingly, this region also contains a putative receptor binding region (residues 98-103) (Meerts et al., 2006; Misinzo et al., 2006). However, decoy epitopes are commonly present adjacent to conserved receptor binding sites as steric interference due to antigen antibody binding can prevent the binding of neutralizing antibodies to the receptor binding site (Köhler et al., 1994). While a limitation of this study is the exact residues involved in the decoy effect were not finely mapped, it is likely that the residues within the 97-123 region, which do not form a part of the receptor binding site, namely 104-123 are involved in the decoy activity. Hence, based on published information and our own analysis, six amino-acid substitutions were implemented: E104D, T131N, A133S, Q175N, and K179S to modify the putative decoy epitopes. In addition, construct B, would also have deletion of amino-acid residues 170-175, that were not shown to be a decoy epitope in our epitope analysis, but were responsible for increase in antibody production in a later stages of infection and were previously identified as decoy (Trible et al., 2012).

Since the putative neutralizing epitopes are located in the N term region of the capsid protein, a furin cleavage site was introduced between the ORF2 protein and the positive DIVA marker, so that antigen processing would not be interfered with. While specific cleavage could not be demonstrated *in vitro*, possibly due to interference with the buffer by the protein constituents such as trace amounts of urea, the furin cleavage site has previously been active *in-vivo* in swine (Drews et al., 1995). The selection of the positive marker was done on the basis of following criteria: it should not be found in swine, sequence and serological data should be

available, also it would be desired to have antibodies specific to selected protein, to be able to confirm its antigenic properties after obtaining of heterologous proteins. Selected epitope of` GRA6 of *N.caninum* satisfied all the conditions in that, *N.caninum* have not been found in swine, it's sequence and antigenic properties have been described (Baszler et al., 2008; Ramamoorthy et al., 2007), and we had polyclonal serum at our disposal for this pathogen. A possible reason for cross-reactivity noted in the commercial vaccine group is that the *Toxoplasma gondii* which is sometimes found in swine producing farms has a homologous gene although there is no published information that the *N. caninum* GRA6 protein and the *T.gondii* GRA6 protein cross-react serologically due to some degree of amino acid similarity.

As an alternate strategy, the NLS of the ORF2 protein was removed, as it is known to contain a B cell epitope (Lekcharoensuk et al., 2004) but this did not seem to contribute to the protective response in our analysis. In group 2, the only group that should have been in contact with NLS prior to challenge, a very strong response was detectable after the challenge. Immune response to the NLS, was detected in groups 2 and 3 at DPV25 at lower levels. In groups 1 and 4 the trend of increasing immune response to NLS became evident only 21 days after challenge (DPV53), but the response was not strong enough to classify animals as positive. A trend of increased response to NLS in group 3 from DPV18 to DPV25 and may indicate that some animals had contact with wild PCV2. In fact, only 2 animals out of 7 in group 3 show increase in titers of anti-NLS response. Further optimization of the DIVA ELISAs to improve sensitivity and specificity will increase the utility of these markers to differentiate infected and vaccinated animals.

While loss of bodily condition and poor weight gain is a hall mark sign of PMWS in the field, existing experimental animal models for PCV2 research do not accurately reproduce these

signs (Opriessnig et al., 2008b, 2013)**.** The mPCV2b strain used as the challenge strain in this study is known to be more virulent under field conditions. However, similar to the scenario when PCV2b emerged and in this study, the increased pathogenicity was not demonstrated in experimental models (Guo et al., 2011). Previous characterization of the mPCV2b strain in pigs showed that the virus replicated to $\log10^{4\pm 3.21}$ copies/ml in infected animals at 10 weeks (Opriessnig et al., 2013). However, in this study, very low levels of replication were detected at 10 and 18 days' post challenge, cofounding the interpretation of results for the efficacy of Con A and B in viral clearance. The dose of 10^5 $TCID_{50}$ used in this study is standard for PCV2 challenge studies (Fenaux et al., 2002; Ramamoorthy et al., 2011). The low levels of viral replication are therefore unlikely to be due to a dose effect. However, the examination of microscopic lesions between groups may shed a clearer light on vaccine efficacy.

In this study, the lack of strong viral replication in the unvaccinated controls renders the interpretation of viral clearance data difficult. While the commercial vaccine appeared to induce immunity in all but two pigs, the level of viral load detection in the ConA and B and unvaccinated controls was also extremely low at few hundred viral particles, which would be below the 35% margin of error for a $TCID_{50}$ assay that only detects live virus (Roldão et al., 2009). Moreover, DNA copy number does not always correlate exactly with the detection of live virus; genome copy numbers generally provide for an over-estimation, especially in *in vivo* studies (Gustafsson et al., 2012; Kim et al., 2014).

Binding antibody response to the PCV2 capsid protein were appropriately detected in all groups, following an anamnestic pattern. In confirmation of the hypothesis that amino acid residues 160-178 (peptides 56-59) map to an immune-dominant region (Fig 1), pigs exposed to Con A in which this region was deleted showed a lower total IgG response to the ORF2 when

compared to the other groups. The mPCV2b strain has an additional K in the C terminus of the ORF2 where neutralizing epitopes are located (Opriessnig et al., 2014a). However, as one of the properties of decoy epitopes is to confer subtle serological differences, which can translate to neutralizing properties, between strains, hypothetically, a vaccine with changed decoy epitopes should induce wider protection against different serotypes. Although the V/N titers were less than those generated by immunization with the commercial vaccine, pigs immunized with ConA and B had significant V/N titers for the homologous PCV2b strain 41513. The exact parameters by which a vaccine induces sterilizing immunity is not well understood. It is possible that additional 20% reduction as measured by the FFN assay could be sufficient to overcome the threshold of balance between pathogen and immune system that would translate in to protection *in-vivo*. Virus neutralization titers against other heterologous virus strains remain to be tested.

In conclusion, the successful addition of the DIVA markers provides an enhanced capability to existing PCV2 vaccines, which is likely to improve vaccine compliance and monitoring capability in the field level. Moreover, the developed vaccines are the only DIVA capable vaccines available in the U.S and are likely targets for deployment in a national PCV2 eradication plan. While more sophisticated testing at the epitope level is required to demonstrate the efficacy of the immune-refocusing strategy used in this study, the performance of the developed vaccines was comparable to the commercial vaccine, especially for weight gain parameters for ConB vaccinated pigs which was significantly higher than the commercial vaccine.

While the more sophisticated testing at the epitope level is required to demonstrate the efficacy of the immune-refocusing strategy used in this study, the performance of the developed vaccines was only marginally lower compared to the commercial vaccine. However, as the

details of vaccine formulation for the commercial vaccine are unknown, an objective comparison cannot be drawn. Additional testing with a more virulent challenge strain and increased number of animals is the focus of the next phase of vaccine testing.

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