RESEARCH REPORT
SWINE HEALTH

Title: PCVAD Induced Immune Dysfunction-**NPB# 07-208**

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Industrial Summary:
This project included a team of PCVAD researchers from KSU, ISU and SDSU who utilized a single set of experimentally infected pigs to address several important questions related to pathobiology, immunology and immunodiagnostics of PCVAD. The four principal project objectives included:

1. Characterization of the virological and immunological response of vaccinated or naïve pigs following PCV2b and/or PCV2b-PRRS infection.
2. Development of a quantitative ELISA for assessment of active and passive antibody levels.
3. Development of a seroassay to differentiate infected from vaccinated animals (DIVA).
4. Development of a monoclonal antibody panel for the differentiation of PCV genotypes and diagnostic tools.

Results:
Objective 1:

- Experimental co-infection (dual challenge) with PCV2b and PRRS can result in clinical disease and death in conventional animals.
- PCV2b or PRRS infections alone do not result in significant clinical disease or death.
- PRRS infection potentiates PCV2b replication.
- PCV2b infection does not enhance PRRS replication.
- Temporal coincidence of PCV2b and PRRS co-infection contributes significantly to the mortality of animals.
- Vaccination of animals with a heterologous PCV2a subunit vaccine generates neutralizing antibodies and a protective immune response in vaccinated animals.
- Vaccination results in no apparent viremia following single or dual challenge, whereas non-vaccinated animals are viremic for at least 42 days post challenge.

Objective 2:

- A quantitative PCV2b ELISA was developed and then updated to use a fluorescent-microsphere immunoassay (MIA) using Luminex technology.
- PCV2 antibody quantitation results using the MIA compared favorably to the IFA gold standard.
- A quantitative MIA assay for PRRS was developed.
- MIA assays proved to be more rapid than IFA.
- The MIA assay for PCV2 and PRRS (multiplex assay) is currently undergoing validation testing using the control and infection sera generated in the animal study from Objective 1.
- Multiplex MIA are run as single sample against multiple pathogens in a single test system. Once fully developed diagnostic sero-testing should be more cost effective.
- Multiplex MIA as a herd profiling tool can be developed and used to monitor antibody levels to multiple pathogens to make informed management decisions.

Objective 3:

- A DIVA (differentiate infected from vaccinated animals) ELISA was developed to enable the discrimination between subunit vaccinated animals and naturally infected animals.
- A second DIVA ELISA based on PCV2b-ORF2 capsid reactivity is currently under development. This DIVA ELISA should be useable for animals vaccinated with baculovirus subunit vaccines as well as conventional whole virus vaccines.
- Both DIVA tests are based on the control and infection sera generated in the animal study from Objective 1.

Objective 4:

- Monoclonal antibodies (MAb) to PCV2 were developed allowing for the differentiation of PCV1 and PCV2.
- The MAbs developed for this project will be useful tools for future development of diagnostic assays
- The MAb are available to all swine disease researchers and diagnostic labs as important tools for current diagnostics and future diagnostic test development.

The tools and knowledge from this collaborative project are being integrated into disease management programs. The multi-university collaborative effort efficiently leveraged the intellectual and scientific resources of three leading institutions. The savings cost and time are a consequence of the incorporation of a single group of experimentally infected pigs combined with the collaboration of a multidisciplinary team of virologists, immunologists and diagnosticians.

Scientific Abstract:

Objective 1: Pathobiology and Immunology of naïve or vaccinated pigs following PCV2b or PCV2b/PRRS challenge.

Forty-nine conventional pigs with low maternal indirect fluorescent antibody (IFA) titers to porcine circovirus 2b (PCV2b) were divided into seven groups, four challenge control groups and three vaccine groups. Respective control/vaccine groups were subsequently challenged with either porcine reproductive and respiratory virus (PRRSV), PRRS/PCV2b or PCV2b via the intranasal route. One of the remaining non-vaccinate groups and the remaining vaccinate group were left as negative controls. The non-vaccinated group that was challenged with the dual challenge (PRRSV/PCV2b) had a mortality rate of 43%. There was no mortality in any of the other groups. All of the animals challenged with PCV2b generated significant antibody titers to PCV2b as determined by IFA and SN. A differential qPCR assay detected PCV2b in the serum of all of the animals in non-vaccinated groups challenged with PCV2b. The geometric mean copy number of PCV2b in the serum of the dual challenged group was significantly greater than that of the group challenged with only PCV2b. Animals that died in the dual challenge group had 20-100 times more virus than the PCV2b only challenge group. While the PRRSV potentiated the PCV2b infection, there was not an increase in the amount of PRRSV detected by PCR in the serum of the dual challenged group when compared to the PRRSV only challenged group. No PCV2b virus was detected in either the vaccinated single or dual challenge groups, indicating that the PCV2a based subunit vaccine was capable of inducing apparent sterilizing immunity in the face of a moderately lethal heterologous dual challenge. This challenge model system appears to replicate overt clinical disease in conventional pigs and demonstrates the synergism of a PRRSV/PCV2b dual challenge on the replication of PCV2b *in vivo*.

Cellular response following vaccination and/or challenge.

PBMC data has been somewhat problematic due to the large number of data points collected, the lymphocyte proliferation data is being still analyzed. It has been determined that the impact of PCV2b, PRRSV infection, or vaccination does not result in a robust response by lymphocytes to either the poke weed mitogen or PCV2 antigen. The results determined to date indicate that there are subtle differences between groups with the majority of differences occurring at day 14 of the trial. On day 14, the presence of PRRSV did appear to have a minor impact on the ability of cells from the PCV2 vaccinated and infected pigs to respond to the PCV2 antigen. By days 28 and 42, there was little response by the various cell populations to the stimulants. Further analysis of the data is needed to assess more closely the impact of infection and vaccination on the specific immune response by the lymphocyte populations.

B-cell epitope mapping.

Open reading frame 2 (ORF2) of porcine circovirus type 2 (PCV2) codes for the 233 amino acid capsid protein (CP). Baculovirus-based vaccines that express ORF2 are protective against experimental challenge and natural infection. The goal of this study was to identify regions in CP preferentially recognized by sera from experimentally infected and vaccinated pigs and pigs diagnosed with porcine circovirus-associated disease (PCVAD), including porcine multi-systemic wasting syndrome (PMWS) or porcine dermatitis and nephropathy syndrome (PDNS). The approach was to react sera with CP polypeptide fragments followed by finer mapping studies using overlapping oligopeptides that covered amino acids 141-200. The results showed that vaccinated pigs and a subset of pigs experimentally infected with PCV2 recognized only the largest CP(43-233) polypeptide fragment. Another subset of experimentally infected pigs and sera from pigs with PDNS showed strong reactivity against a CP oligopeptide, 169-STIDYFQPNNKR-180. Alanine scanning identified Y-173, F-174, Q-175 and K-179 as important for antibody recognition. The results from this study support the hypothesis that PCV2 modulation of immunity is involved in disease progression. The differences in the recognition of CP(169-180) and other polypeptides provide opportunities to devise diagnostic tests for the surveillance of vaccination, infection and disease.

Objective 2: Development of a Quantitative ELISA.

The ability to use purified capsid protein also allowed for the transition of the assay from an ELISA format to a Fluorescent-microsphere immunoassay (MIA) using Luminex technology. This system allows for more rapid analysis and is paving the way for the development of an assay that will allow for testing for the presence of multiple types of antibodies and antigens at one time. The results from the PCV2b MIA assay are very similar to the ones from the IFA and SN assay. The MIA assay offers the potential of "Multiplexing" or testing antibody levels to multiple pathogens in a single serum sample. This type of assay would allow producers to antibody profile their herd to monitor passive antibody levels, vaccine response, and the circulation of various pathogens within the herd. Preliminary development of a multiplex for PCV2 and PRRS has been completed and correlates well with IFA and ELISA results. The MIA assay for PCV2 and PRRS is currently undergoing validation testing and will be offered as a diagnostic test at KSVDL.

Objective 3: Development of a DIVA ELISA.
The DIVA ELISA is based on the bacterial expression of PCV2b ORF1 and ORF2. Animals vaccinated with a PCV2 ORF2 subunit vaccine produce antibodies only to the ORF2 protein while PCV2 infected animals generate antibodies to both ORF1 and ORF2. Using a traditional ELISA approach, ORF1 and ORF2 polypeptides were used as detection antigens. Initial results demonstrated lack of reaction to ORF1 in vaccinated animals and reaction to ORF1 and 2 in infected pigs. In addition to the ORF1/ORF2 DIVA ELISA, a second DIVA ELISA is currently under development. That assay is based on different reactivity patterns of infected or vaccinated pigs with polypeptides of the ORF 2 capsid protein. In theory, a DIVA ELISA based only on ORF2 reactivity would be useable for baculovirus subunit vaccines as well as conventional whole virus vaccines.

Objective 4 Development of PCV2-specific monoclonal antibodies.
Mice were immunized with recombinant N protein and their splenocytes were used to prepare hybridomas using traditional methodology. Hybridoma screening was done by IFA using PCV2 virus infected PK15 cells and 14 MAbs were obtained. Interestingly, epitope mapping of these MAbs showed that all of these MAbs recognized the N-terminal epitope region, amino acid 49-69 of the N protein. MAbs SD65-16, SD16-10 and SD 72-10 were subcloned and maintained for further study. MAbs against the C-terminal region of the N protein were obtained by immunizing mice with synthetic peptides that cover the amino acid region of 71-90, 119-138, or 171-190. Following fusion, seven MAbs were obtained from the initial hybridoma screen, of which three MAbs, SD39-29, SD2-21, and SD18-43 were subcloned and maintained. Each of these clones recognizes one of the C-terminal peptides. MAbs 72-10 and 36-29 had high titer in ELISA and IFA, were expanded and a large quantity of MAbs were produced. IFA and ELISA results showed that these MAbs can recognize both 2a and 2b genotypes, but did not recognize PCV1. To generate the PCV2b specific MAbs, mice were immunized with two PCV2b N protein specific peptides, 86-SNPRSVPF and 69-VDMMRFNINDFLPPGGGSNPRSV. Unfortunately, mice immunized with these two synthetic peptides did not produce any PCV2 specific MAb.

Phage Display Swine monoclonal antibodies.
Production of antibodies through phage display was initiated. Primers recognizing two heavy chains and 3 kappa and lambda light chains were produced. These amplified approximately 400 base long distinct sequences whose authenticity was confirmed by cloning and sequencing 12 randomly picked clones. The clones were sequenced by colleagues at the University of Arizona and were determined to be similar to published sequences from pigs. Of six kappa fragments sequenced (130 amino acids long), the range of identity was between 89.8 and 64.7% at the amino acid level. As expected the primer pairs sampled the variability of the B-cell immune repertoire.

Future work will involve fusing the cloned antigen binding sites with phage coat protein genes. The fused phage then infects the bacteria and inserts its genome containing the antigen binding site construct into the bacterial genome. The bacterial enzymes and machinery replicates the different proteins and genome of the phage. Phage proteins and antibodies replicate separately and assemble at the inner membrane, or periplasmic space, of the

bacteria. Since the phage is not lytic, the bacteria secrete the bacteriophage containing antibody in the phage's protein coat. The secreted phages constitute the Antibody Phage Display Library. Each of these phages displays a different antigen combining site on its coat protein and has the potential to bind a specific antigen. At this point, the secreted phages will be screened against PCV2b antigens. This process results in the selection of specific avid antibodies to PCV2b antigens.

Project Introduction:
Viral infection of swine with PCV2 manifests itself in a variety of clinical forms which have been grouped together as PCV associated disease (PCVAD). Clinical presentations of PCVAD include multisystemic disease accompanied by weight loss, porcine dermatitis and nephropathy syndrome (PDNS), and respiratory disease including pneumonia (Kim and Chae, 2004; Chae, 2005; Segalés et al., 2005). Accompanying these clinical manifestations are the depletion of immunologically important T and B lymphocytes (Nielsen et al., 2003). The hallmark lesions associated with Postweaning Multi-systemic Wasting Syndrome (PMWS) are the depletion of the B and T cells and the macrophage infiltration of lymphoid tissue (Allan and Ellis, 2000). Previous studies have demonstrated increased apoptosis of peripheral blood mononuclear cells (PBMC) when infected with PCV (Yu et al ., 2007). However, despite this dysregulation of lymphocytes, PCV2 infected pigs have significant levels of anti-PCV2 antibodies. While high titers of anti-PCV2 antibodies are present, these antibodies do not have the same capacity as antibodies produced due to vaccination to neutralize PCV2 *in vitro*. This failure of the immune system to produce corresponding levels of neutralizing antibody is likely to be at least in part responsible for the development of clinical disease and subsequent loss of livestock due to PCVAD. Therefore, development of rapid assays to determine not only the sero-status of animals, but also their vaccine status, is critical for predicting the clinical outcome of animals and herds.

Project Objectives:
The primary focus of this project was on the pathobiology and immunology of PCVAD. The experimental model for studying PCVAD in conventional animals was confirmed to successfully replicate many of the clinical symptoms associated with PCVAD in the field. Samples collected from experimental groups of animals in the experiment allowed for characterization of virology of the infectious process and of the humoral and cellular immune response to infection. Additionally, the samples also allowed for the development and characterization of new diagnostic tests and reagents. See below for specific project goals.

1. Determine the Pathobiology and Immunological Basis for PCVAD.
The functional properties of cellular and humoral responses in the PCVAD dual challenge experimental model were measured. Those same parameters were used to evaluate heterologous protection in pigs vaccinated with a commercial PCV2 vaccine. We hypothesized that the antibody response during natural infection is non-protective and the response to vaccination should lead to high levels of neutralizing antibody and protection. The specificity and virus neutralizing capacity of sera from pigs that developed PCVAD was evaluated. Antibody reactivity to linear epitopes on the capsid protein were also determined through the use of PEPSCAN (Mahé et al., 2000).

2. Development of Quantitative PCV2 ELISA
At present, there are no ELISA-based assays that provide a quantitative measure of PCV2-specific antibody (Nawagitgul et al., 2002). The approach under this objective was to clone and express recombinant PCV2 capsid proteins which were then coated onto ELISA plates. Quantitation results achieved by using the standard ELISA were compared to the IFA gold standard.

In addition to a quantitative ELISA a microsphere-immuno-assay (MIA) using Luminex technology was developed through a collaborative relationship with Dr. Susan Wong of the Wadsworth Institute of the New York Public Heath Lab. PCV2 antibody quantitation results using MIA were compared to the IFA gold standard and found to be highly similar. A unique feature of MIA antibody tests is that different proteins can be linked

to different colored microspheres and antibodies in sera to these different proteins can be quantified simultaneously from as single test well; this is known as multiplexing. MIA a multiplex assay for multiple swine pathogens allows Herd Profiling to be a reality.

3. Development of a DIVA ELISA Test

A companion DIVA ELISA test is important for monitoring vaccination status and compliance. The experimental approach is to express recombinant polypeptides derived from the polymerase protein, which are not part of the vaccine formulation and not found in PCV1. Using an ELISA-based format, we identified a polymerase polypeptide that was recognized by PCV2-infected pigs, but not vaccinated pigs.

4. Generation of Genotype-Specific Monoclonal Antibodies (MAb)

Antibodies that are specific to PCV2a and PCV2b are needed for the rapid detection and identification of the different PCV2 viruses. Under this objective, attempts were made to generate genotype-specific MAb antibodies using classical methods and by screening phage display libraries prepared from pigs.

Objective 1--Materials and Methods:

Animals. The pigs were purchased from a PCV and PRRS free herd. The conventional sows were screened for the presence of PCV2 antibody by IFA. Litters from sows with low PCV2 titers were selected and serum from three piglets in each litter was tested to confirm low levels or no PCV2 antibodies. Piglets were weaned at 3 weeks of age and then transferred to the research facility at Iowa State University.

Housing and feeding. The animals were randomly assigned to seven different groups of seven animals. The groups were then randomly selected for the different treatments. The animals were housed with seven to fourteen animals per room dependent on the group assignment. The housing arrangements can be found in Table 1. The rooms were identical in size and environmental controls.

Experimental design, vaccination, and inoculation. The experimental design is summarized in Table 1. Following arrival and dispersement, animals in vaccine groups were vaccinated at 3 and 6 weeks of age with a commercially available PCV2 vaccine (Circumvent PCV, Intervet, Inc., Millsboro, DE). Both rounds of vaccination were done intramuscularly in the neck. The animals were challenged with virus two weeks after the second vaccination. The challenge inoculum was delivered through the intranasal route using 3 mL/nare.

PCV2b inoculum. The PCV2b inoculum was originally isolated from a field case by the Kansas State Veterinary Diagnostic Laboratory (KSVDL). A 5% tissue homogenate was prepared using normal methods and was then heat treated for thirty minutes at 57˚C to inactivate PRRS and other pathogens that may be present. The homogenate was tested for porcine parvovirus and porcine Teshovirus via PCR at KAVDL and shown to be negative. A total of 3.7 log10 tissue culture infective dose 50% ($TCID_{50}$) of PCV2b was delivered intranasally in the PCV2b single challenge and the PCV2b/PRRSV dual challenge groups.

PRRSV inoculum. The PRRSV was isolated on MARC cells from the same field case as the PCV2b inoculum. The PRRSV was passed a total of four times on MARC cells prior to use as inocula. The PRRSV component of the challenges contained 5.6 log 10 $TCID_{50}$ of virus.

Serology. All of the animals were bled at the time of the first and second vaccination, challenge, and then weekly throughout the course of the study. Sera were processed and collected using normal procedures and stored at -70^{o}C for future antibody and virus testing.

Serially diluted serum samples were assayed for PCV2 antibodies using an indirect fluorescent antibody assay (IFA) in a 96-well format. The IFA antigen was obtained by infecting ST cells with a

standardized stock of PCV2b virus. IFA assay endpoints were calculated as the reciprocal of the last serum dilution that gave a positive IFA response when viewed with a fluorescent microscope.

PCV2 neutralizing antibody levels were determined using a 96-well microtiter system with ST cells as the substrate and a standardized PCV2b stock as the indicator virus. Serial dilutions of serum were mixed with a constant quantity of PCV2b virus (50-300 $TCID_{50}$), incubated for 1 hour at 37^{o}C and inoculated into 4 replicate wells of one day old ST cells in 96 well plates. Cultures were incubated 3 days at 37^{o}C and the presence of virus was determined by a fluorescent antibody test using a monoclonal antibody specific to PCV2 capsid as the indicator. Serum neutralization titers were based on 50% inhibition of the indicator virus and 50% endpoints were then determined by the method of Spearman and Karber.

PRRS antibody was detected by a commercially available PRRVS enzyme-linked immunosorbent assay (ELISA) (HerdChek PRRSV antibody test kit; Idexx Laboratories, Inc., Westbrook, MA). Optical density readings of greater than 0.4 at 405 nm were set as the positive threshold ELISA value.

Viral nucleic acid quantification. Viral nucleic acid was extracted from sera collected on day 21 of the study and all subsequent bleeds using the MagMax Total RNA Isolation Kit (Applied Biosystems/Ambion, Inc. Austin, TX). PRRSV RT-qPCR was performed with Tetracore PRRSV reagents (Tetracore, Inc. Rockville, MD). The PCV2 qPCR assay was developed at the Kansas State University Veterinary Diagnostic Laboratory and is able to differentiate PCV2a from PCV2b.

Peripheral blood mononuclear cells (PMBC) collection and processing. In addition to directly measuring the antibody response through IFA and SN assays the immune response of PCV challenged animals was assayed using a lymphocyte proliferation assay. Serums from pigs in all groups excluding Group #2 were used in this assay. Blood for the proliferation assay was collected on days 0, 14, 28, and 42 of the trial. Peripheral blood monocytes (PBMC) were isolated from the blood of seven pigs in each group by venipuncture into BD Vacutainer blood collection tubes containing heparin as described previously (Yu et al., 2009) The tubes were then centrifuged and the cell layer at the plasma and gel interface in each tube was collected. The PMBCs consisted of mononuclear cells, primarily lymphocytes. Red blood cells were lysed and the isolated PBMCs suspended in PBS. Cell numbers were determined by a Beckman cell counter and adjusted with PBS 10 1 x 10^{7} cells/mL for staining. Cells were stained with CFSE prior to stimulation to allow measurement of proliferation as described previously (Yu 20009). The CFSE stained cells were counted and adjusted to a concentration of 2 x 10^{6} cell/mL, and 100 uL of the cells was added to each well of round bottomed 96-well plates. Cell stimulation was achieved by adding pokeweed mitogen (PWM), PCV2 virus, or medium as a non-stimulating control. The inoculated plates were incubated at 37C in an incubator with 5% CO2 for five days. After five days of stimulation, cells were stained with CD4 and/or CD8 antibodies to identify T lymphocyte populations or CD21 for B cells. The stained cells were analyzed for proliferation and lymphocyte populations using two or three color analysis by flow cytometry at the Iowa State University Cell Facility. Data acquired from flow cytometry was analyzed using ModFit LT software and a stimulation index was calculated as described previously (Yu et al., 2009).

Objective 1--Results:

The dual challenge system used in this study consisted of PCV2b and PRRSV isolated from a single field case received at the Kansas State Diagnostic Laboratory and was successful in causing clinical PCVAD in conventional animals in a controlled experimental environment. The group of unvaccinated animals that received the PCV2b/PRRSV challenge had a 43% mortality rate (Table 1). There was no depletion of lymphoid cells in the lymphoid tissue of the surviving animals in this group at the conclusion of the study. However, PCV2 antigen was detected in the lesions of these animals. There were no clinical or histological lesions found in the vaccinated animals that received the dual challenge.

Table 1.

Group Information		Vaccination (Days -35 and -14)	Challenge (Day 34)		PCV2 Viremia	Mortality
			PCV2b	PRRSV		
1	Control	None	-	-	No	No
2	Vac/Control	Yes	-	-	No	No
3	PCV 2	None	+	-	1,300	No
4	Vac/PCV2	Yes	+	-	No	No
5	PCV2/PRRSV	None	+	+	50,000	43%
6	Vac/PCV2/PRRSV	Yes	+	+	No	No
7	PRRS	None	-	+	No	No

Initially a majority of animals had low levels of passive antibodies to PCV2b. The PCV2b antibody titer of non-vaccinated animals decayed to non-detectable levels by the time of challenge. The titers of the animals in the vaccine groups increased following the initial vaccination and were significantly higher ($P < 0.005$) than non-vaccinated animals by the time of challenge (Figure 1). Following challenge there was not a significant increase in the antibody titer of the animals in the vaccinated groups throughout the remainder of the study. The non-vaccinated animals that were challenged with PCV2b generated very high levels of IFA antibody to PCV2b by 21 days post challenge (Figure 1). The antibody levels of these animals were similar to the antibody levels in the vaccinate groups. There was no significant difference in the geometric mean titers of the animals challenged with PCV2b and PRRS as compared to the animals challenged with only the PCV2b. The PCV2b titers of pigs in both of these groups remained high through the conclusion of the study. Animals in the PRRSV and Control groups did not generate antibodies to PCV2b at any point during the course of the study.

The ability of serums to neutralize PCV2b *in vitro* was similar in pattern to the IFA data. Vaccinated animals generated low levels of neutralizing antibodies following initial vaccination. The titer of neutralizing antibodies increased following the second vaccination. The level of neutralizing antibodies remained high whether or not the animals were challenged with PCV2b. There did appear to be a decline in the amount of neutralizing antibodies present in the sera of vaccinated animals at the conclusion of the study (Figure 1). However this decline was not statistically significant.

Animals in the PCV and PCV/PRRSV challenge groups also developed PCV2b neutralizing antibodies. These antibodies were detected 21 days after challenge. The concentration of these antibodies increased over the next two weeks before leveling off. The peak titer of neutralizing antibodies in the challenge groups was significantly lower than the amount of neutralizing antibodies present in the vaccinated animals (Figure 1).

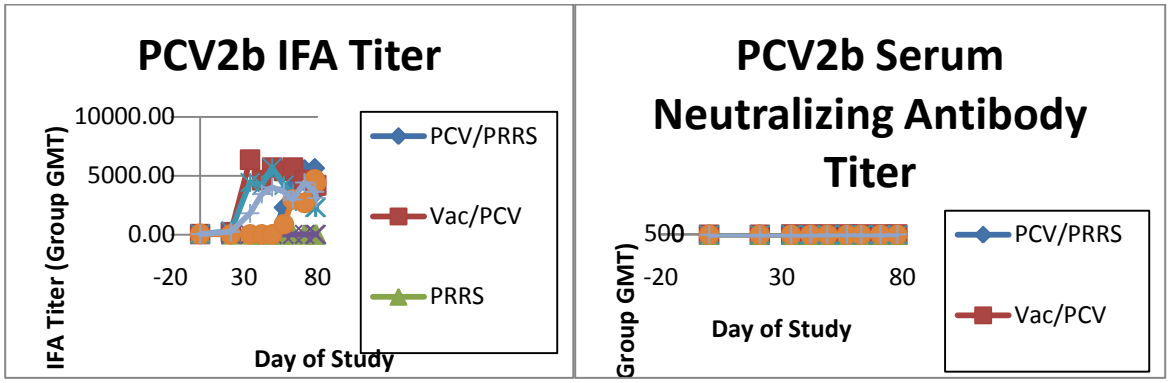


Figure 1. IFA and SN response at the time of first vaccination and 6 weeks post challenge.

Prior to challenge all of the groups were negative for PRRSV RNA. The Control, Vac/Control, PCV2b, and Vac/PCV2b groups were negative for the presence of PRRSV RNA in serum (Figure 2) throughout the study. Following challenge, PRRSV RNA was detected in the serum of all animals in the PRRSV, PCV2b/PRRSV, and Vac/PCV2b/PRRSV groups. The peak of PRRSV RNA concentration was eight days following challenge and declined after that time point. There was no detectable PRRSV RNA in the PRRSV and Vac/PCV2b/PRRS groups 35 days following challenge, however there were still appreciable amounts of PRRSV RNA remaining in two of the five surviving animals in the PCV2b/PRRSV group at that time. The PRRSV RNA did not return to undetectable levels in this group until 42 days after challenge.

There was no detectable PCV2 DNA in any of the animals prior to challenge. PCV2 DNA was first detected in animals 14 days after challenge. PCV2 DNA was not detected in all of the groups challenged with PCV2b; the animals in the Vac/PCV2b/PRRSV and Vac/PCV2b groups remained free of PCV2b DNA throughout the course of the study (Figure 2). PCV2b nucleic acid was not detected in any of the animals that did not receive a PCV2b in the challenge inocula. The animals in groups that were not vaccinated, yet challenged, with the PCV2b virus, had detectable levels of PCV2 DNA beginning 14 days after challenge. These animals continued to test PCR positive for PCV2 throughout the remainder of the study. The amount of PCV nucleic acid present in the serum peaked 21 days after challenge and decayed over time from the peak levels. At the peak of PCV viral nucleic acid concentration the dual challenged animals had 50 times more PCV nucleic acid circulating than the animals challenged solely with PCV2b. There was also a delayed clearance of PRRSV nucleic acids in two of the dual challenged animals when compared to the animals challenged with PRRSV alone.

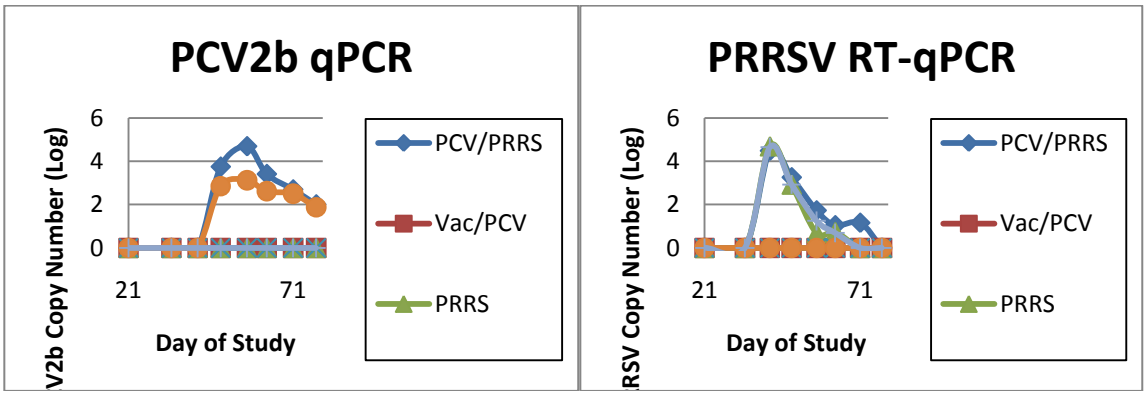
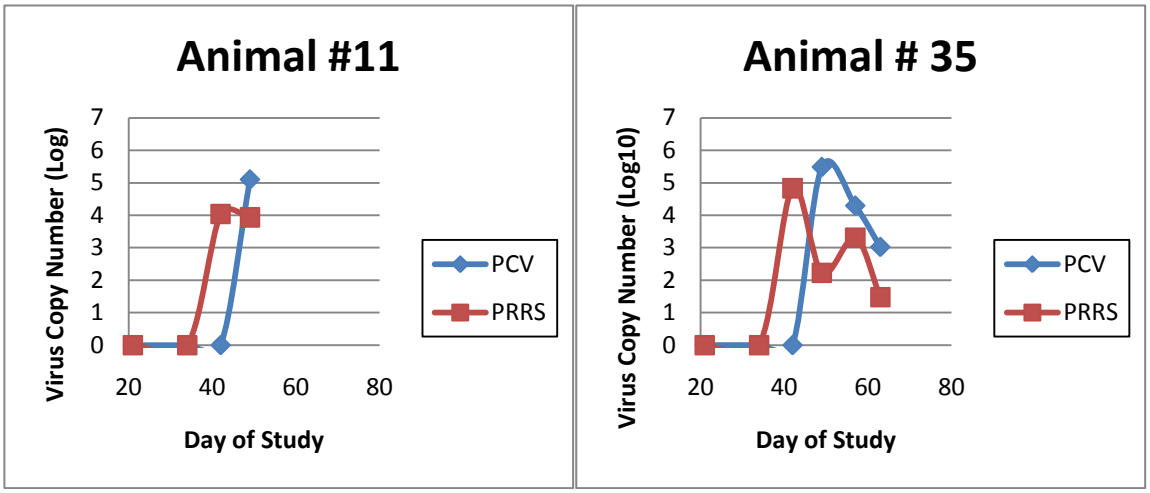


Figure 2. PCV2b and PRRS viremia following challenge as determined by real time PCR.

Discussion:
Conventional pigs can be used in an experimental model system to replicate PCVAD when co-infected with PCV2b and PRRSV. The infection model used in this experiment resulted in clinical PCVAD and death in 43% of the dual challenged non-vaccinated animals. However, infection with PCV2b or PRRSV alone was not sufficient to cause either clinical disease or death; there is clearly a synergistic effect between the two viruses.

Animals infected with both PCV2b and PRRSV had higher concentrations of PCV2b virus present in their serum than animals only infected with PCV2b. The PRRSV infection potentiates the replication of PCV2b virus *in vivo*. There was not a corresponding increase in the amount of PRRSV isolated from the sera of dual challenged animals. The PRRVS was detected one week earlier than PCV2 in challenged animals (Day 7 vs. Day 15). The presence of the PRRSV could be affecting the animal's ability to mount an immune response to the PCV2b, allowing for a more severe infection.
The PCV2b infection by itself affects animal's immune response. While animals infected with PCV2b produce large amounts of antibodies that recognize wild type PCV2b in an IFA assay, they do not produce high levels of PCV2b neutralizing antibodies when compared to vaccinated animals. This reduction in the concentration of neutralizing antibodies is not dependent on the presence of PRRSV. Animals infected with PCV2b alone have the same reduction in PCV2b neutralizing antibodies as the dual infected animals.

Generally the replication of PRRSV precedes the replication of PCV2b with the peak concentration of PRRSV being 7 days after challenge and the highest concentration of PCV2b at 21 days or later following infection. However, in animals that died during the course of the study, the PCV2b viral load was higher earlier, and there was a greater amount of circulating PRRSV in these animals (Figure 3). This temporal coincidence of PCV2b and PRRSV appears to be a significant contributing factor to the morbidity and mortality of animals infected with both PCV2b and PRRSV. This suggests that when high levels of PRRSV viremia occur at the same time as high levels of PCV2b infection there is an increase in the severity of disease to the point of causing death. Further exploration of this temporal effect could lead to better understanding of the immune impairment caused by PCV2b and its downstream effect on herd health.



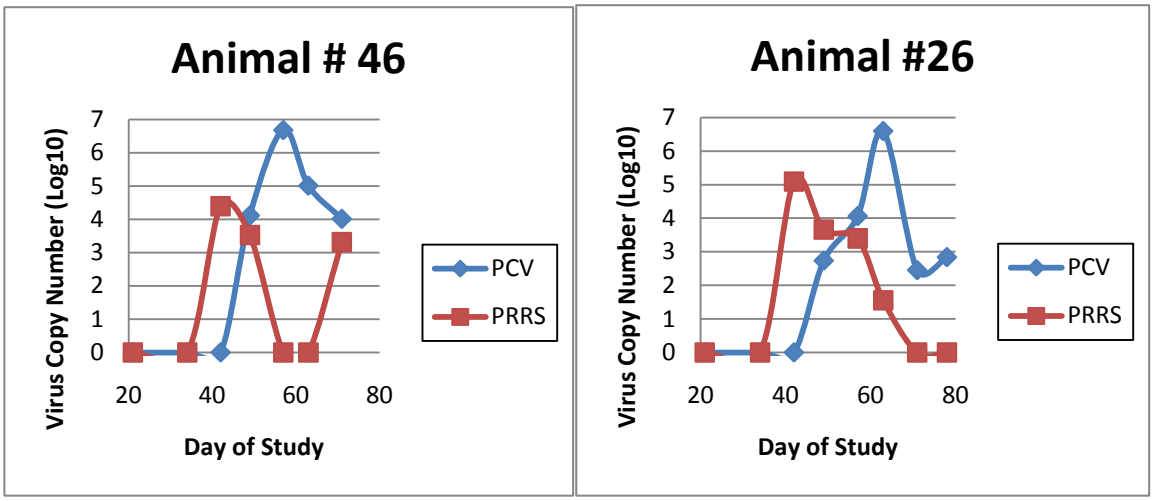


Figure 3. Temporal relationship between PCV2b and PRRS viremia in animals that died or survived dual infection.

Analyzing the PBMC data has been somewhat problematic due to the large number of data points collected, the lymphocyte proliferation data is still being analyzed. It has been determined that the impact of PCV2b, PRRSV infection, or vaccination does not result in a robust response by lymphocytes to either the PWM or PCV2 antigen. The results determined to date indicate that there are subtle differences between groups with the majority of differences occurring at day 14 of the trial. By days 28 and 42, there was little response by the various cell populations to the stimulants. On day 14, the presence of PRRSV did appear to have a minor impact on the ability of cells from the PCV2 vaccinated and infected pigs to respond to the PCV2 antigen. Further analysis of the data is needed to assess more closely the impact of infection and vaccination on the specific immune response by the lymphocyte populations.

B-cell epitope mapping. Results reported here were part of this NPB grant and an NRI grant to look at and characterize the immune response to B-cell epitopes following vaccination and/or infection. Open reading frame 2 (ORF2) of porcine circovirus type 2 (PCV2) codes for the 233 amino acid capsid protein (CP). Baculovirus-based vaccines that express ORF2 are protective against experimental challenge and natural infection. The goal of this study was to identify regions in CP preferentially recognized by sera from experimentally infected and vaccinated pigs, and pigs diagnosed with porcine circovirus-associated disease (PCVAD), including porcine multi-systemic wasting syndrome (PMWS) or porcine dermatitis and nephropathy syndrome (PDNS). The approach was to react sera with CP polypeptide fragments followed by finer mapping studies using overlapping oligopeptides that covered amino acids 141-200. The results showed that vaccinated pigs and a subset of pigs experimentally infected with PCV2 recognized only the largest CP(43-233) polypeptide fragment. Another subset of experimentally infected pigs, and sera from pigs with PDNS showed strong reactivity against a CP oligopeptide, 169-STIDYFQPNNKR-180. Alanine scanning identified Y-173, F-174, Q-175 and K-179 as important for antibody recognition. The results from this study support the hypothesis that PCV2 modulation of immunity is involved in disease progression. The differences in the recognition of CP(169-180) and other polypeptides provide opportunities to devise diagnostic tests for the managment of vaccination, infection and disease.

Objective 2—Methods and materials: Development of a quantitative assay for the determination of anti-PCV2 antibodies was based on the bacterial expression of histadine tagged PCV2b capsid protein followed by purification of the capsid protein. Purified PCV2b capsid protein has the benefit of eliminating non-specific antibody binding due to the presence of cellular or non-PCV2b related proteins. In addition to a standard ELISA, a Fluorescent-microsphere immunoassay (MIA) using Luminex technology was also developed.

Briefly, bacterially expressed PCV2 capsid protein is purified and then covalently linked to specifically dyed fluorescent microspheres. Swine serum is incubated with these microspheres followed by incubation with an anti-swine secondary antibody that was tagged with a flurophor. The detector then identified the specific microsphere passing the detector and quantitated the fluorescence from the secondary antibody associated with the microsphere.

Objective 2—Results and discussion:
Due to the inherent advantages of MIA over ELISA, development of the later was discontinued early in the project with the major effort going to MIA. The ability to use purified capsid protein also allowed for the transition of the assay from an ELISA format to a Fluorescent-microsphere immunoassay (MIA) using Luminex technology. This system allows for more rapid analysis and is paving the way for the development of an assay that will allow for the testing for the presence of multiple types of antibodies and antigens at one time. The results from the PCV2b MIA assay (Figure 4) are very similar to the ones from the IFA and SN assay.

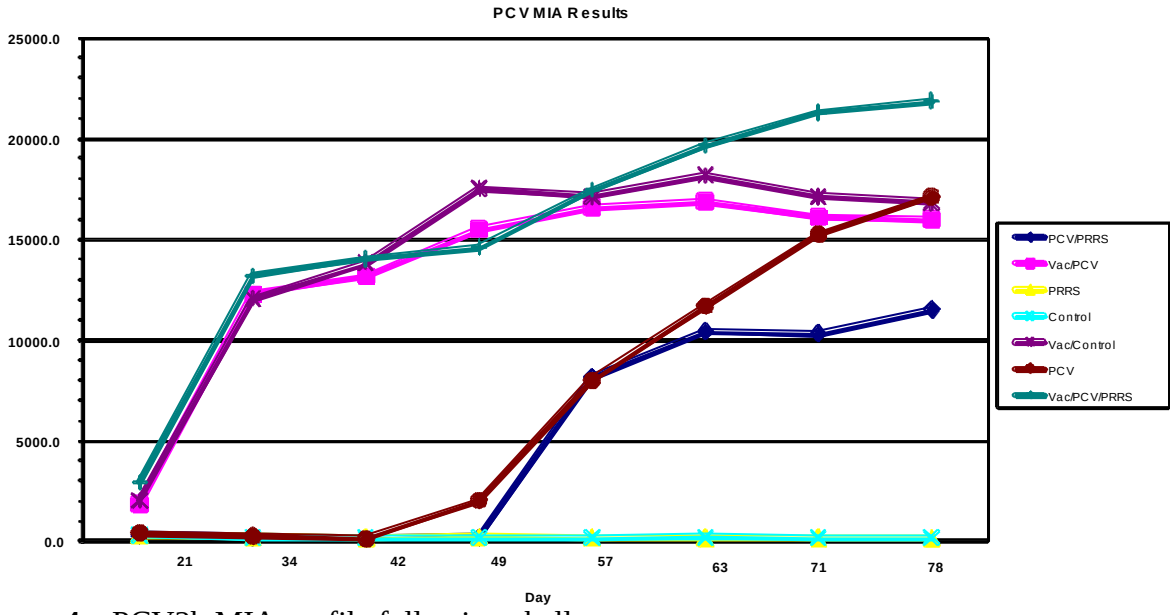


Figure 4. PCV2b MIA profile following challenge.

The MIA assay offers the potential of "Multiplexing" or testing antibody levels to multiple pathogens in a single serum sample. This type of assay would allow producers to antibody profile their herd to monitor passive antibody levels, vaccine response, and the circulation of various pathogens within the herd. Preliminary data from a Multiplex for PCV2 and PRRS is shown in figure 5. The MIA assay for PCV2 and PRRS is currently undergoing validation testing and will be offered as a diagnostic test at KSVDL.

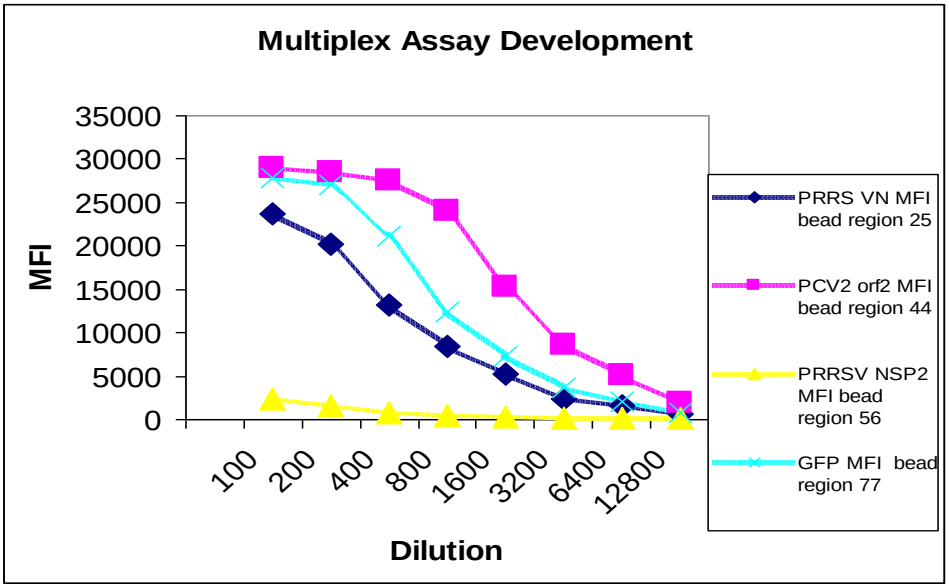


Figure 5. Preliminary MIA results of PCV2b and PRRS Multiplex.

Objective 3—Methods and materials:
In addition to a quantitative ELISA a DIVA ELISA was developed. Standard methodology for the ELISA Protocol is: 100uL of 4ug/mL of PCV2 ORF1 and PCV2 ORF2 (truncated to remove N-term NLS sequence) was coated in every other column respectively onto a Costar EIA/RIA 96 well plate and incubated 12hr at 4^{o}C. Plates were then washed 3X with PBS-Tween 20 (0.05% Tween 20 into PBS). 100uL of 5% goat serum in PBS (PBS-GS) was added to each well to block and incubated at 37oC for 1hr. After washing, serum was added at an initial 1:10 dilution in PBS-GS followed by serial 2-fold dilutions leaving the last row with PBS-GS as a background standard. Plates were incubated and washed, then reacted with an anti-swine HRP antibody diluted 1:2000. After incubation and washing, reactivity was detected by the chromagenic substrate ABTS. Plates were read at an absorbance of 405nm. Serum tested in the above format include: negative sera from CDCD pig, Positive sera from a pig diagnosed with PDNS from a farm in northern Kansas and test sera (Pigs 6,19,26, and 4) from the NPB funded study. Serum samples used in the DIVA ELISA were obtained at 28 days after the last vaccination.

Objective 3—Results:
The DIVA ELISA (Figure 6) is based on the bacterial expression of PCV2b ORF1 and ORF2. Vaccinated animals produce antibodies only to the ORF2 protein (Pig 6, Lane 2) while PCV2 infected animals generate antibodies to both ORF1 and ORF2 (Positive, Lanes 1 and 2). In addition to the ORF1/ORF2 DIVA ELISA, a second DIVA ELISA is currently under development. This assay is based on different reactivity patterns of infected or vaccinated pigs with polypeptides of the ORF 2 capsid protein. In theory, a DIVA ELISA based only on ORF2 reactivity would be useable for baculovirus subunit vaccines as well as conventional whole virus vaccines.

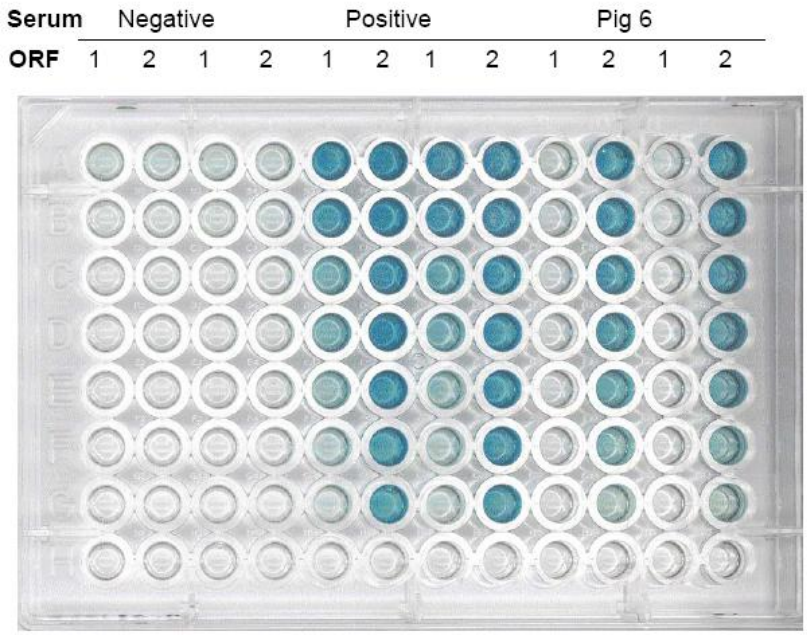


Figure 6. DIVA ELISA based on ORF1 and ORF2 reactivity.

Objective 4—Methods and materials:
DNA encoding the N protein (with N-terminal 41 amino acid truncation) was amplified from DNA prepared from the PCV2b strain SD 06-11, and cloned into the pET-28(a) protein expression vector. The N protein was expressed in *E. coli* as a 6-His tagged fusion protein and purified using Ni-NTA columns (Qiagen). Peptides from the identified B cell epitopes of N protein were synthesized (Table 2). BALB/c mice were immunized with purified recombinant N protein or synthetic peptides at two-week intervals for eight weeks. Splenocytes from immunized mice were fused with NS-1 myeloma cells and cultured on 24 well plates. Cell culture supernatants from wells containing hybridoma colonies were initially screened by IFA in virus infected PK15 cells. Hybridoma cells from positive wells were subcloned, expanded and cell culture supernatant were retested. Ascites were obtained by injecting hybridoma cells into the mouse abdomen. The isotypes of MAbs were determined using an IsoStrip Kit (Serotech)

For IFA, infected PK15 cells were fixed with 2% paraformaldehyde at 5 days after infection, and then permeabilized with PBS containing 3% normal goat sera and 0.2% saponin (blocking buffer) for 15 minutes. MAbs were diluted 1:500 in blocking buffer and incubated with cells for 1 h at room temperature. Following extensive wash, the cells were incubated with a goat anti-mouse antibody conjugated to FITC (Jackson Laboratories). After incubation, the cells were washed and mounted onto microscope slides. Samples were analyzed by fluorescent microscopy.

For ELISA, recombinant PCV2b N protein were coated onto 96-well immunlon II plates (Dynatech) in coating buffer (pH 7.4) overnight at 4 ^{0}C. After washing, plates were incubated with serially diluted MAbs for 1 h at 37 ^{0}C, washed and then incubated with HRP-goat anti-mouse IgG/IgM/IgA for 1 h at 37 ^{0}C. Plates were developed using ABTS, and results were quantified by reading at 405 nm with an EL800 microplate reader (BioTek Instruments Inc., Winooski, VT) controlled by XChek Software (IDEXX Laboratories).

Objective 4—Results and discussion:
Initially, mice were immunized with recombinant N protein (Fig. 7A). Hybridoma screening by IFA using PCV2 virus infected PK15 cells obtained 14 MAbs. Interestingly, epitope mapping of these MAbs showed that all of these MAbs recognized the N-terminal epitope region, amino acid 49-69 of the N protein. We subcloned and maintained three such MAbs, including SD65-16, SD16-10 and SD 72-10. To obtain the MAbs against the C-terminal region of the N protein, we immunized the mice with synthetic peptides that cover the amino acid

region of 71-90, 119-138, or 171-190. Seven MAbs were obtained in initial hybridoma screen, and we subcloned and maintained three MAbs, SD39-29, SD2-21, and SD18-43. Each of these clones recognizes one of the C-terminal peptide. MAb isotyping results of these MAbs is shown in Table 2. MAbs 72-10 and 36-29 (Fig. 7) had higher titer in ELISA and IFA, which were expanded and a large quantity of MAbs were produced. We further tested whether these MAbs have the ability to differentiate PCV2a and PCV2b using IFA and ELISA. The result showed that these MAbs can recognize both 2a and 2b genotypes, but did not recognize PCV1. To generate the PCV2b specific MAbs, we immunized the mice with two PCV2b N protein specific peptides, 86-SNPRSVPF and 69-VDMMRFNINDFLPPGGGSNPRSV. Unfortunately, mice immunized with these two synthetic peptides did not produce any PCV2 specific MAb.

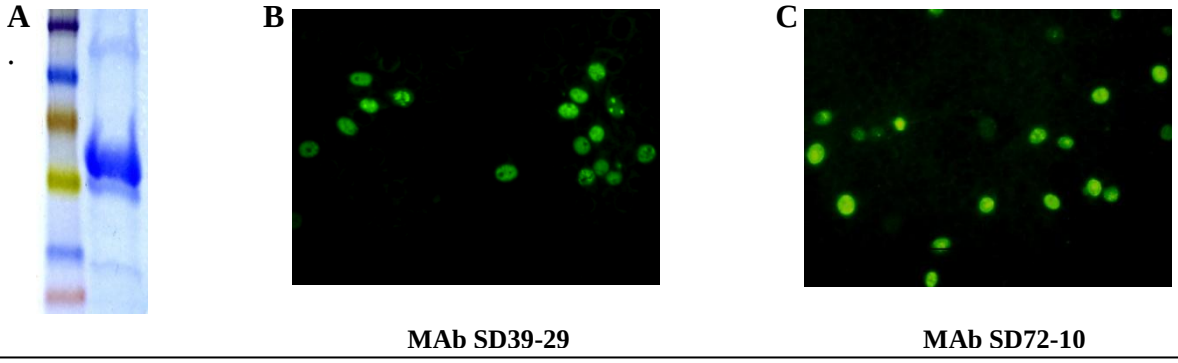


Figure 7. Production of PCV2 specific monoclonal antibodies. **4A.** SDS-PAGE gel showing purified recombinant N protein from *E. coli*; **4B and 4C.** Immunofluorescent staining of PCV2 infected PK-15 cells using specific MAbs.

Table 2

Monoclonal antibody reactivity to different peptide region of PCV2 N protein

MAb	Isotype	Peptide Reactivity			
		49-69	71-90	119-138	171-190
SD16-10	IgM	+	-	-	-
SD65-16	IgG3	+	-	-	-
SD72-10	IgG3	+	-	-	-
SD2-21	IgG1	-	+	-	-
SD18-43	IgG1	-	-	-	+
SD39-29	IgG1	-	-	+	-

The monoclonal antibodies produced during the course of this grant are key basic reagents for study of the fundamental biology of the viral proteins, and protein interactions between the virus and the host, which will give insight into better understanding protective immunity for future development of vaccines and anti-viral drugs.

The MAbs and proteins developed for this project will be useful tools for future development of diagnostic assays to specifically identify PCV2. These MAbs will serve as widely shared materials and be integrated into the swine disease research infrastructure. They are available to all swine disease researchers as important tools in current PCV2 diagnostics and future diagnostic test development.

Phage Display Swine monoclonal antibodies:
Production of antibodies through phage display was initiated. Primers recognizing two heavy chains and 3 kappa and lambda light chains were produced. These amplified approximately 400 base long distinct sequences whose authenticity was confirmed by cloning and sequencing 12 randomly picked clones. The clones were sequenced by colleagues at the University of Arizona and were determined to be similar to published sequences from pigs. Of six kappa fragments sequenced (130 amino acids long), the range of identity was between 89.8 and 64.7% at the amino acid level. As expected the primer pairs sampled the variability of the B-cell immune repertoire.

Future work will involve fusing the cloned antigen binding sites with phage coat protein genes. The fused phage then infects the bacteria and inserts its genome containing the antigen binding site construct into the bacterial genome. The bacterial enzymes and machinery replicates the different proteins and genome of the phage. Phage proteins and antibodies replicate separately and assemble at the inner membrane, or periplasmic space, of the bacteria. Since the phage is not lytic, the bacteria secrete the bacteriophage containing antibody in the phage's protein coat. The secreted phages constitute the Antibody Phage Display Library. Each of these phages displays a different antigen combining site on its coat protein and has the potential to bind a specific antigen. At this point, the secreted phages are screened against PCV2b antigens. This process results in the selection of specific avid antibodies to PCV2b antigens.

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