RESEARCH REPORT
SWINE HEALTH

Title: Evaluation of a new etiological agent of PMWS/PDNS in conventional pigs.
NPB #06-088

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Industry Summary:
Porcine circovirus-associated disease (PCVAD) is considered a multifactorial disease since a variety of cofactors, including infectious agents, seem to be necessary for full expression of clinical disease. In order to investigate the role of ruminant pestiviruses in PCVAD that has been frequently detected from field cases, two studies were conducted. Porcine circovirus 2-1a (PCV2-1), cytopathic type 1 bovine viral diarrhea virus (cpBVDV) strain NADL and a field strain of BVDV, were inoculated intramuscularly and intranasally into cesarean-derived, colostrum-deprived pigs either alone or in combination in two different experiments. In this study we were able to demonstrate that PCV2 is essential for developing PCVAD clinical signs and disease. Vaccination against BVDV with Aluminum Hydroxide adjuvant in combination of with non pathogenic cpBVDV NADL strain of the virus did not initiated PCV2 virus replication as was observed in a previous study. However, vaccination against BVDV virus lowers the number of infected cells with BVDV and PCV2 virus in the tissues of infected pigs with PCV2-1a inoculums. Results from this study will help veterinarians and producers better understand the role of a newly mutated strain of PCV2 virus during infection in swine.
The role of BVDV-like porcine field strains of the virus remains an important issue that needs immediate attention BEFORE this virus change into a more virulent form.
It needs to be determined if inoculation with the noncythopatic BVDV strain of the virus adapted to a porcine cell line in combination with PCV2 in different time points or by itself will cause disease. This research area is not well investigated and needs immediate attention.

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Scientific Abstract:

Porcine circovirus-associated disease (PCVAD) is considered a multifactorial disease since a variety of cofactors, including infectious agents, seem to be necessary for full expression of clinical disease. In order to investigate the role of ruminant pestiviruses in PCVAD, porcine circovirus 2-1 (PCV2-1), field strain of BVDV and a cytopathic type 1 bovine viral diarrhea virus (BVDV) strain NADL, were inoculated intramuscularly and intranasally into cesarean-derived, colostrum-deprived pigs either alone or in combination. Most of the animals remained healthy throughout the study. Antibodies against PCV2 and cpBVDV strain NADL were first detected by 14 and 18 days, respectively. The majority of the pigs inoculated with PCV2-1 that were euthanized at 14 and 35 days post infection developed gross and microscopic lesions of classic PCVAD. Clinical signs were seen in a single animal inoculated only with PCV2-1. This pig had growth retardation over 12 days followed by acute respiratory distress leading to death 30 days post infection. We were able to reproduce PCVAD-like clinical signs (wasting, respiratory signs), gross lesions in this animal consisted of marked hydrothorax, pulmonary edema, and mild pneumonia lesions (lymphoid depletion, granulomatous inflammation, lymphohistiocytic infiltrate in kidney (with inclusions), liver, esophagus, and heart; lung with necrotizing vasculitis, marked edema, and fibrinous pleuritis; acute vasculitis and thrombosis in tubaric branches of ovarian artery) with PCV2 present in the lesions. Experimental reproduction of clinical signs and lesions typical of the more virulent form of PCVAD in one of the pigs inoculated with PCV2-1 but since mortality was low it does not exclude that other co-factors(like ncpBVDV) have to be present in the inoculums fully express the disease.

Introduction:

Porcine circovirus 2 (PCV2)–associated disease (PCVAD) is considered a multifactorial disease since a variety of co-factors, including infectious agents, seem to be necessary for full expression of clinical disease. Postweaning multisystemic wasting syndrome (PMWS) is an emerging problem of swine in many countries. The syndrome was first recognized in Canada in 1991 as a protracted disease, which is characterized by progressive weight loss in pigs 4 to 16 weeks of age and high case fatality rate. Affected pigs commonly show severe respiratory signs such as tachypnea and dyspnea, and less consistently, icterus and diarrhea Consistent gross lesions include interstitial pneumonia and lymphadenopathy . The most common microscopic lesions are a lymphocytic to granulomatous interstitial pneumonia and, in lymphoid organs, depletion of lymphocytes in T and B cell-dependent areas with replacement by macrophages and also substantial intra-lesional PCV2. Other less common microscopic changes include lymphocytic granulomatous hepatitis, nephritis, and enteritis

Porcine circovirus type 2 (PCV2), which is genetically and antigenically distinct from non-pathogenic PCV type 1 (PCV1), has been consistently demonstrated in pigs affected by PMWS, and is thus postulated as the causative agent . PCV2 antigens or nucleic acids are commonly detected in lesions in tonsil, lymph nodes, spleen, Peyer's patches, bone marrow, kidney, liver and lung . Many field isolates of PCV2 have been demonstrated to be infectious to naïve swine based on development of viremia and seroconversion . In addition, PCV2 infection has been demonstrated to induce varying degrees of lymphoid depletion . However, clinical signs of PMWS have not been consistently reproduced by singular infection with PCV2 under experimental conditions even though lesions were still present. Additional infectious factors are necessary to have disease fully develop in the pigs. Co-infection with PRRS, PPV and/or *Myocoplasma hyopneumoniae* (*M. hyo.*) may lead to development of PMWS. The Purdue ADDL has been receiving many submissions from outbreaks of PMWS in finishing swine associated with high mortality and PDNS. Dr. Stevenson, a pathologist in ADDL, recognized lesions in cases of virulent PMWS that had large amounts of intralesional PCV2, but that were of a unique distribution and character not previously reported in PMWS. This suggested a new tissue tropism that might indicate presence of a unique infectious agent (virus). Subsequently, in younger pigs, lesions suggestive of a viral infection were observed with a distribution identical to the aforementioned "unique" PMWS lesion – but without features of PMWS and without intra-lesional PCV2 antigen. PCR tests on tissues from these younger pigs were negative for PCV2, PPV, PRRSV and other common viral swine pathogens that have been associated

with PMWS. It was suspected that these nursery pigs were infected with the putative "unknown" epidemic viral initiator of virulent PMWS/PDNS. Subsequently, ADDL virologist Dr. Pogranichniy, demonstrated a virus in tissues from the younger pigs by electron microscopy, PCR and virus isolation. The same virus was also demonstrated in the aforementioned cases of PMWS/PDNS that had unique PMWS lesions.

It is likely that this newly identified virus (Purdue ADDL PPPLV06-BVDV like virus) is the putative initiator that is responsible for the recent North American outbreak of virulent PMWS – PDNS. We have detected previously unrecognized ruminant pestiviruses (bovine viral diarrhea virus – BVDV) along with PCV2 in tissues of severely diseased pigs without concurrent infection with other common porcine pathogens. We suspect our isolate is an evolved virulent mutant that is spreading in the swine population. We have identified the same virus in additional field cases of PMWS/PDNS originating from several states. In a previous experiment, gross and microscopic lesions typical of those observed in the more severe form of PCVAD were reproduced in one of four pigs vaccinated for BVDV and inoculated with filtered tissue homogenate known to contain PCV2-1 and BVDV type 1-like viruses obtained from a diseased pig.

Objectives:

To determine whether a novel virus, PPPLV06(BVDV type 1-like), is an initiator that along with PCV2 causes PMWS/PDNS and to determine the relative contribution of each virus to the pathogenesis of PMWS/PDNS.

We seek to do three things in the NPB-funded study:

1. To determine whether a pestivirus (a modify live virus -MLV BVDV1), that is closely related to PPPLV06, is an initiator that along with PCV2 causes the virulent form of PMWS and to determine the relative contribution of each virus to the pathogenesis of virulent PMWS.
    a. Compare the presence and severity of expected lesions of virulent PMWS in target tissues between pigs inoculated with PCV2 alone, MLV BVDV1 alone or PCV2 + MLV BVDV1.
    b. Compare the location of PCV2 and MLV BVDV1 in selected tissues between pigs inoculated with PCV2 alone, MLV BVDV1 alone or PCV2 + MLV BVDV1.
    c. Compare the amount of PCV2, as determined by quantitative real time PCR, in serum and tissues of pigs inoculated with PCV2 alone or with PCV2 + MLV BVDV1.
2. To determine whether prior vaccination with an Al-hydroxide adjuvanted killed BVDV vaccine administered prior to inoculation with MLV BVDV1 and PCV2 will augment or prevent PMWS.
3. To determine whether passage of PPPLV06 in CDCD pigs, with serial bleeding and qPCR to determine optimal time for viral harvest, will result in an inoculum with an amount of pestiviral DNA equivalent to at least 10^4 $TCID_{50}$ of BVDV1.
4.

Materials & Methods:

Study Master Schedule (~40 pigs)
PCV2-1A with titer 10^4 $TCID_{50}$/ml (New PCV2 strain in US)
Cytopathic strain of BVDV NADL with titer 10^5 $TCID_{50}$/ml- NOT PATHOGENIC IN PIGS was chosen from previous study to determine its role in pathogenesis together with PCV2 virus
Al Hydroxide adjuvant was used in the killed BVDV vaccine in the study.

I.

Date	Pig Age (weeks)	Study Day (a)	Groups, Treatments and Number of Animals per Group							
			1	2	3	4	5	6	7	8
			PCV2	MLVBVDV	PCV2 + MLV BVDV	Vaccine + MLV BVDV + PCV2	Negative Control	NI-FTH	NI-FTH	NI-FTH
			6	6	8	8	8	1-2	1-2	1-2
Wed, Aug 29, 07	0	-23	Cesarean section at Whiteshire Hamroc							
Tuesday, Sept 4, 2007	1	-16								
Tuesday, Sept 11, 2007	2	--9								
Friday, Sept 14, 2007	3	0	Bleeding	Bleeding	Bleeding	Bleeding & vaccination	Bleeding	Bleeding & inoculation of NI-FTH		
Monday Sept 14,07								Bleeding		
Wednesday Sept 17,07								Bleeding		
Friday Sept 21, 2007								Bleeding & euthanasia		
Monday Sept 24, 2007	7.5	10	Bleeding	Bleeding & inoculation of BVDV	Bleeding & inoculation of BVDV	Bleeding & inoculation of BVDV	Bleeding		Bleeding & inoculation of NI-FTH	
Sep 26, 07									Bleeding	
Friday Sept 28, 2007	8	14	Bleeding & inoculation of PCV2	Bleeding	Bleeding & inoculation of PCV2 of all 8 Necropsy 2	Bleeding & inoculation of PCV2 of all 8 Necropsy 2	Bleeding of all 8 Necropsy 2		Bleeding	
Mon Oct 1, 2007									Bleeding & euthanasia	
Tuesday Oct 2, 2007	8.5	17	Bleeding of all 6 Necropsy	Bleeding of all 6 Necropsy 2	Bleeding of all 6 Necropsy 2	Bleeding of all 6 Necropsy 2	Bleeding of all 6 Necropsy 2			

			2							
Oct 3, 2007										Bleeding & inoculation of NI-FTH
Oct 5, 2007										Bleeding
Monday Oct 8, 2007										Bleeding
Wed Oct 10, 2007										Bleeding & euthanasia
Friday Oct 12, 2007	10	28	Bleeding of all 4 Necropsy 2	Bleeding of all 4 Necropsy 2	Bleeding of all 4 Necropsy 2	Bleeding of all 4 Necropsy 2	Bleeding of all 4 Necropsy 2			
Friday Nov 2, 2007	13	49	Bleeding & necropsy 2	Bleeding & necropsy 2	Bleeding & necropsy 2	Bleeding & necropsy 2	Bleeding & necropsy 2			

The experimental design is delineated in Table 1. At 3 weeks of age, forty-six conventionally reared, cesarean-derived, colostrum-deprived pigs were randomly assigned into groups of 8 (two groups) or 10 animals (three groups). Animals in groups 4 and 5 were dually inoculated with a cytopathic strain of BVDV type 1 (cpBVDV-NADL, 2 ml intranasally + 2 ml intramuscularly, $10^{5.5}$ $TCID_{50}$/ml) and with PCV2-1 (2 ml intranasally + 2 ml intramuscularly, $10^{4.5}$ $TCID_{50}$/ml). Inoculation of BVDV preceded that of PCV2-1 by 4 days. Pigs in group 5 were also vaccinated with a killed BVDV product, thus receiving a similar vaccination protocol as the one used in the previous study.[4] Group 3 was inoculated with cpBVDV-NADL only, group 2 was inoculated with PCV2-1 only, and group 1 was sham inoculated (negative control). The study was terminated 5 weeks after inoculation of PCV2-1. Pigs were kept under strict biosecurity conditions and were monitored daily for clinical signs. Animals were euthanatized at 0, 4, 14, and 35 days post PCV2-1 inoculation. Serum samples were collected on the same days. At necropsy, samples of selected tissues were collected from each pig and preserved at -80°C and in 10% buffered formalin for virus detection and histopathologic examination, respectively. *In situ* hybridization (ISH) was performed for PCV2 on sections of multiple tissues, and quantitative real time PCR assays for PCV2 and pestiviruses were done on tissues and serum. Additionally, ELISA and immunofluorescence assay for serum antibodies to PCV2 and BVDV were completed for each pig.

Results: Report your research results by objective.

a. Compare the presence and severity of expected lesions of virulent PMWS in target tissues between pigs inoculated with PCV2 alone, MLV BVDV1 alone or PCV2 + MLV BVDV1.

We observed that PCV2 alone can induce lesions that are similar to those observed in the field described for PCVAD. An important fact is that lymphadenopathy and mesenteric edema ***were not observed in this*** *CDCD pigs challenge study and probably was attributed to field strain of BVD virus since in this study we used cpBVDV NADL strain of the virus only. No neurological clinical signs were observed in any pigs from the study in contrast to the previous studies.*

b. Compare the location of PCV2 and MLV BVDV1 in selected tissues between pigs inoculated with PCV2 alone, MLV BVDV1 alone or PCV2 + MLV BVDV1.

BVDV NADL strain and PCV2-1a virus were found in multiple tissues of the PCV2 infected pigs: tonsil, lymph nodes, spleen, ileum, brain and spinal cord. Figures 7; 10 and 11 below show BVDV, PCV2 detection in the treatment groups.

c. Compare the amount of PCV2, as determined by quantitative real time PCR, in serum and tissues of pigs inoculated with PCV2 alone or with PCV2 + MLV BVDV1.

PCV2 and BVDV viruses were detected in multiple tissues by real time PCR. The data is presented in the figures 7; 10 and 11 below.

e. To determine whether prior vaccination with an Al-hydroxide adjuvanted killed BVDV vaccine administered prior to inoculation with MLV BVDV1 and PCV2 will augment or prevent PMWS.

Prior to vaccination, BVDV virus did not influence PCV2 replication but significantly reduced the amount of BVD virus in the selected tissues.

Most of the animals remained healthy throughout the study. Clinical signs of PCVAD were seen in a single animal inoculated only with PCV2-1 (Fig. 1). This pig failed to grow over two weeks followed by acute respiratory distress and death 30 days post infection. Gross lesions in this animal consisted of marked hydrothorax, pulmonary edema, and mild pneumonia (Fig. 2). These alterations were histologically associated with multisystemic acute vasculitis and minimal inflammation of multiple tissues (Fig. 3). Remaining pigs inoculated with PCV2-1 and euthanized at 14 and 35 days PI developed gross and microscopic lesions of classic PCVAD, namely multisystemic lymphohistiocytic or granulomatous inflammation (Fig. 3-6). Antibodies to PCV2 and cpBVDV-NADL were first detected at 14 and 18 days post-inoculation, respectively (Fig. 7). The number of PCV2 genomic copies (Fig.7- 8), serologic response and lesion severity at 35 days PI did not significantly differ between pigs inoculated with PCV2-1 only and with both PCV2-1 and cpBVDV-NADL.
Field strain of porcine BVDV-like virus was propagated in CDCD pigs and was monitored for titer increase in the serum and in the white blood cells (buffy coat). The virus was detected but the titer did not increase in these infected animals.

Figure 1. PCV2-1 inoculated pigs. One of the animals is unthrifty, pale, and has rough hair coat

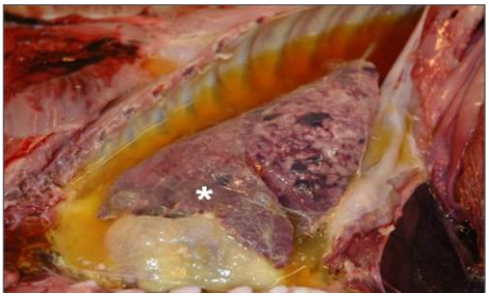

Figure 2. PCV2-1 inoculated pig. Marked hydrothorax and non-collapsing lungs with evidence of pneumonia.

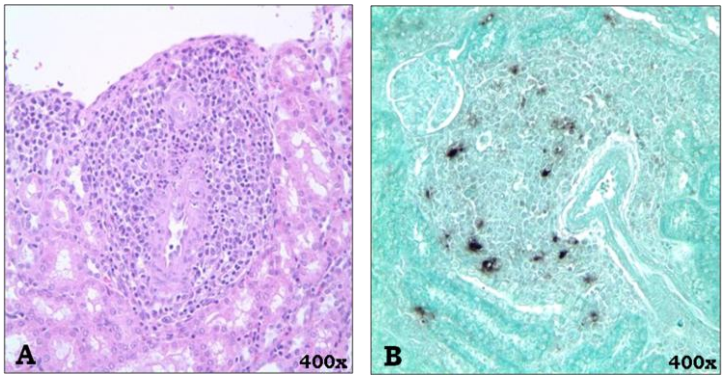

Fig. 3, PCV2-1 and BVDV inoculated pigs. Kidney with perivascular and interstitial lymphohistiocytic infiltrate with intralesional PCV2 nucleic acid. A, H&E; B, *in situ* hybridization for PCV2.

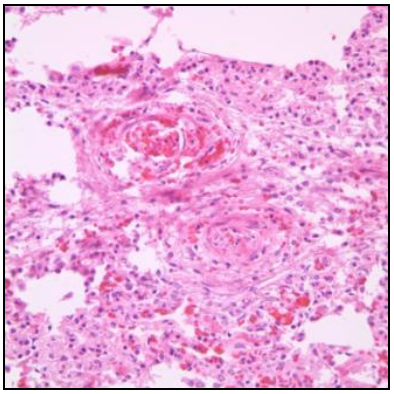

Fig. 4. Acute pulmonary vasculitis from PCV2 only inoculated group, 35 DPI, 400x

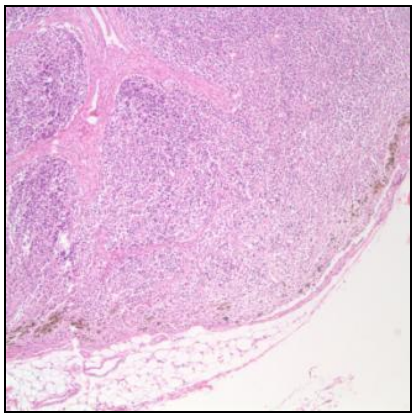

Fig. 5. Lymphoid depletion submandibular LN, H&E, 100x

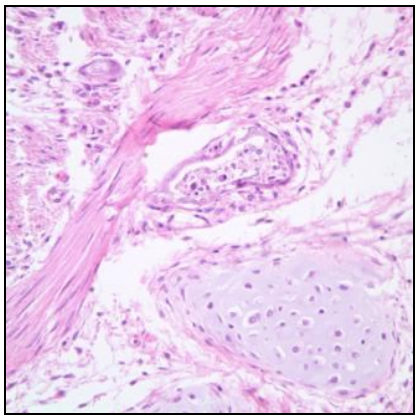

Fig. 6. Bronchial gland epithelial degeneration/necrosis PCV2 only inoculated group, 35 DPI, 400x

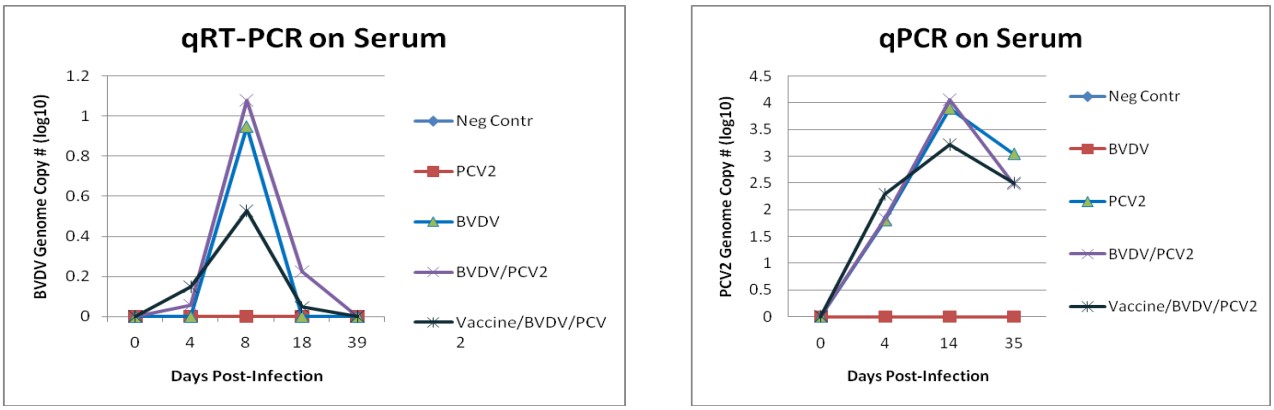

Fig. 7. PCV2-1 and/or BVDV inoculated pigs. Levels of PCV2 (A) and BVDV (B) viremia.

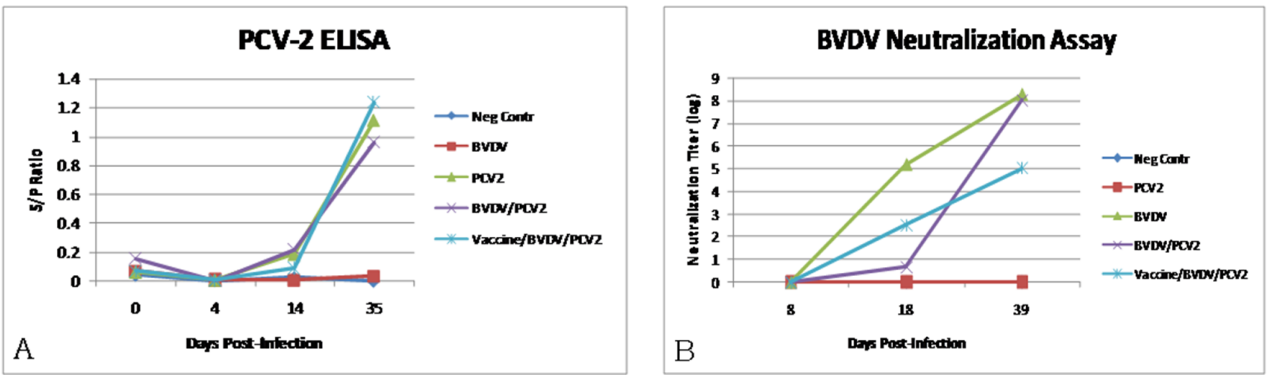

Fig. 8 PCV2-1 and/or BVDV inoculated pigs. Titer of antibodies to PCV2 (A) and BVDV (B).

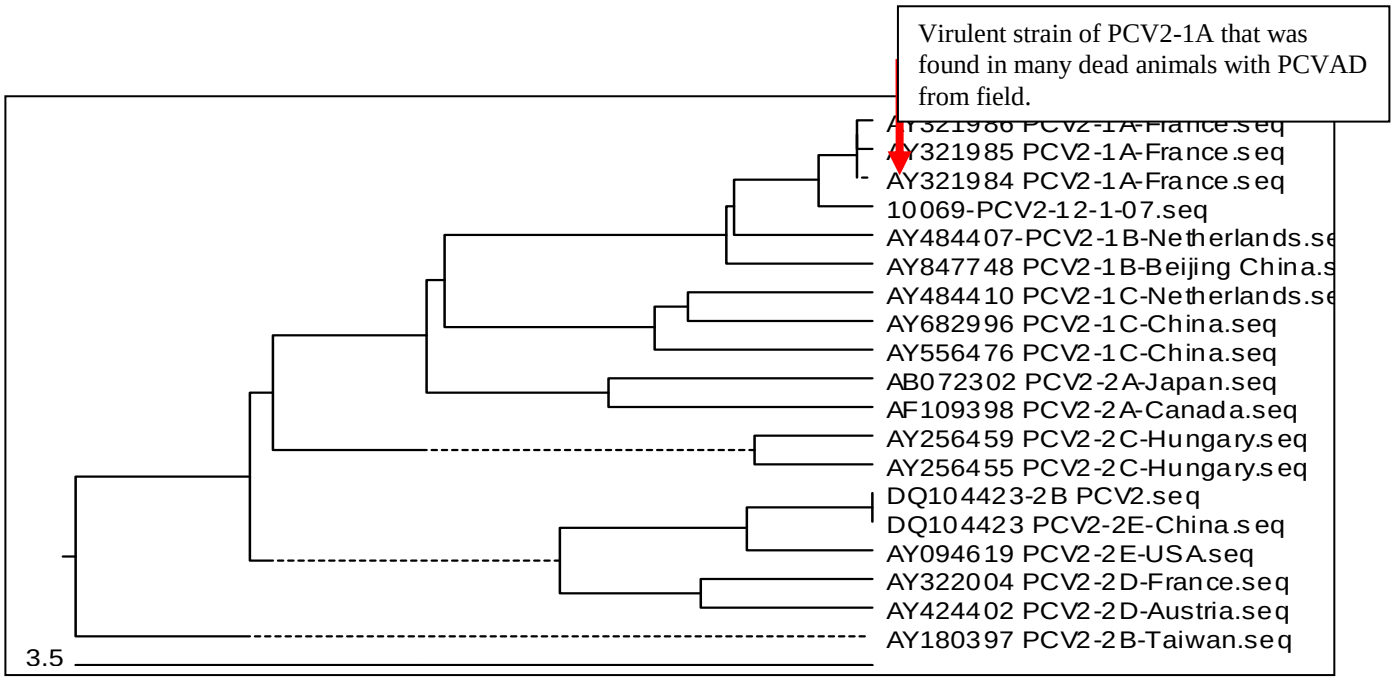

Fig. 9 Phyolgenetic tree of the PCV2 viruses that are available in Genebank and comparison to newly introduce PCV2-1a virus in US that was used in the studv.

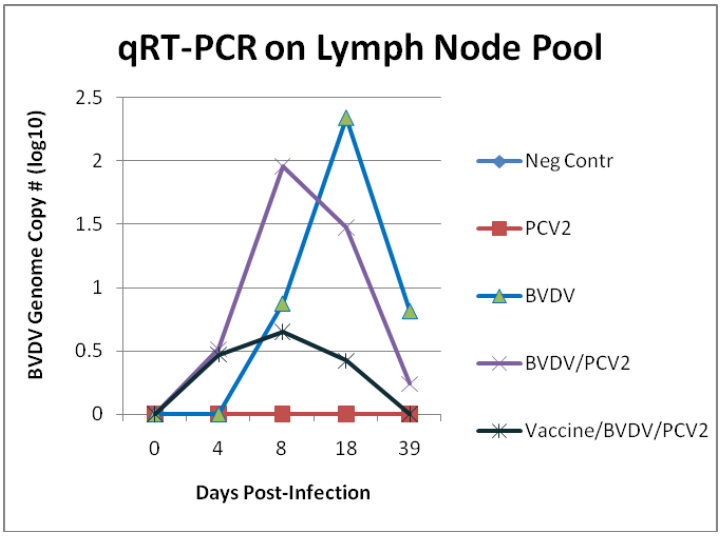

Fig. 10a. Detection of BVD virus in different lymph nodes in different study groups. Negative control group remain negative during all study.

Discussion:

Clinical signs and lesions, typical of those recently observed in North American swine with a more severe form of PCVAD, were experimentally reproduced in one of the PCV2-1 inoculated pigs. This supports the theory that the introduction of a new strain of the virus PCV2-1a or previously unrecognized pathogen in the North American pig population in 2005 accounts for the severe form of PCVAD.
In the previous study, one of the vaccinated plus PCV2-1a/pestivirus-infected pigs developed severe disease and mortality, with lesions resembling the more virulent form of PCVAD. It was speculated that the ruminant pestivirus could represent a novel triggering agent in the PCVAD syndrome and that vaccination apparently intensified the severity of the disease through one or more mechanisms. The current study shows, however, that PCV2-1a alone is capable of inducing disease, suggesting that the cytopathic NADL strain of BVDV is not an additional co-factor of PCVAD and it possible that pig BVDV virus are important factor in disease process The morbidity and mortality in this study was much lower than that reported during the recent outbreaks of PCVAD (8% vs. up to 50%). It is possible that the outcome would have been different if a porcine non-cytopathic strain of BVDV, younger pigs and different inoculation times had been used.

Prior to vaccination, BVDV virus did not influence PCV2 replication but significantly reduced the amount of BVD virus in the selected tissues. An interesting observation was made in that the vaccinated group for BVDV virus had much less **PCV2 replicating virus in the tissues compared to PCV2-only infected group** or PCV2/BVDV infected group. The following Fig. 11 shows some of the data.

It is an indication that rapidly dividing cells are necessary for PCV2 virus replication and since BVDV virus was blocked in the vaccinated group, that lower amount of PCV2 and BVDV in the spleen and tonsil means that our original hypothesis has merit even though in this study we did not produce sick pigs in the BVDV group as it has been observed in the field.

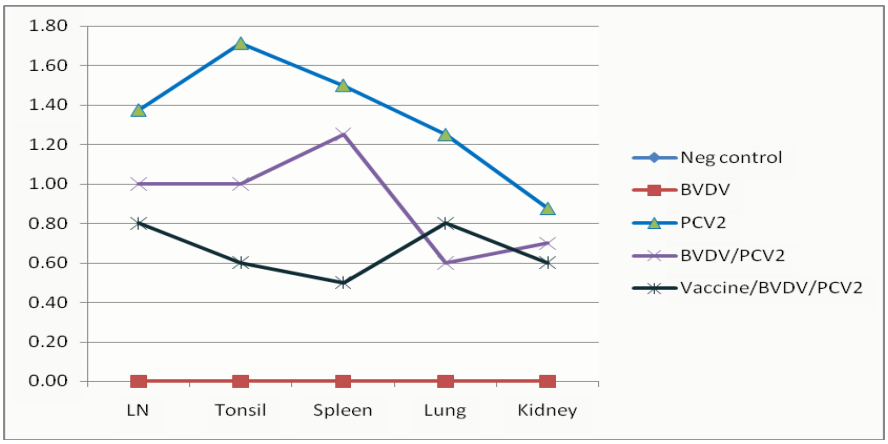

Fig. 11 In situ hybridization for PCV2 virus where amount of PCV2 virus was determined in the following scale:
0 = 0 positive cells/400x
1 = 1-9 positive cells/400x
2 = 10-19 positive cells/400x
3 = >20 positive cells/400x

SUMMARY AND FUTURE STUDY PROSPECTIVE

In this study we were able to demonstrate that PCV2 is essential for developing PCVAD clinical signs and gross/histo lesions. Vaccination against BVDV with Aluminum Hydroxide adjuvant in combination of with non pathogenic MLV cpBVDV NADL strain of the virus did not initiated PCV2 virus replication as it was observed in a previous study. However, vaccination against BVDV virus

lowers number of infected cells with PCV2 virus in the tissues of infected pigs with PCV2-1a inoculums.
The role of BVDV porcine field strain of virus remains an important issue. It needs to be determined if inoculation with the noncythopatic strain of the virus adapted to a porcine cell line in combination with PCV2 in different time points or by itself will cause disease.
It needs to be determined if inoculation of PCV2-1 virus earlier and inoculation of porcine BVDV-like viruses later during disease progress will make a significant difference on PCVAD pathogenesis during dual infection which we hope to determine in later studies if support from NPB is approved.

Acknowledgement
This project was funded by the National Pork Checkoff