

SWINE HEALTH

Title: The “Kansas Cluster” of severe PMWS cases: characterization of a novel PCV2 as the possible etiological agent. **NPB #06-073**

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Industry Summary.

The proposal was submitted in response to a request for proposals to investigate PCV2 associated disease. The three objectives were (1) analyze PCV2 genetic sequences from herds experiencing an outbreak of severe PMWS in late 2005, (2) identify novel co-factors associated with severe disease, (3) determine if the PCV2 isolates from the Kansas Cluster cause severe PMWS. A fourth objective was added later to look at the effectiveness of commercial vaccines. The approach focused on affected pigs from herds experiencing PMWS and PDNS in Kansas. DNA sequencing was used to characterize the PCV2 viruses involved in the outbreak. PCR and virus isolation were used to identify other viruses, which might function as co-factors. Tissue homogenates from affected pigs were used to reproduce the disease. And finally, a random blind study was incorporated to evaluate the effectiveness of PCV2 vaccines. The results from this study demonstrate that **(1) The recent outbreak of PCVAD is associated with the emergence of the PCV2b genotype.** Severe PCVAD in a group of Kansas herds was a disease problem not previously encountered in this region. Interestingly, the clinical disease pattern was present in herds negative for PRRSV, a cofactor frequently linked to PCVAD. The PCV2b genotype was tracked to approximately 70 PCVAD affected herds. The original source of the virus was never identified. **(2) Several differential diagnostic assays were developed that can distinguish PCV2a from PCV2b.** We incorporated sequence differences between PCV2a and PCV2b to develop genotype specific assays. These assays are being used by the Kansas State Veterinary Diagnostic Laboratory to track infections of PCV2a and b in the field. The assays are available for use by other diagnostic laboratories. **(3) A variety cofactors are implicated in the etiology of disease.** Over the course of the study we identified a “zoo” of bacteria and viruses in PCVAD pigs. The importance of cofactors in the control of the disease remains unknown. It appears that the effectiveness of PCV2 vaccines means that cofactors, for the present time, are not as important. **(4) Vaccines are effective in controlling the disease.** The results from field studies demonstrated that vaccination reduces mortality, morbidity and virus load; while increasing PCV2 antibody levels and significantly improving weight gain. These studies place a clear economic benefit to vaccination. Vaccines are also helpful in re-defining the disease.

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Scientific Abstract

In Kansas, the first clinical recognition of severe porcine circovirus associated disease (PCVAD) occurred in November 2005, affecting four separate finisher operations located in the same geographic region of the northeastern part of Kansas. All farms had a history of good health with minimal incidence of common pathogens such as swine influenza, proliferative enteritis, and *Mycoplasma*. Two farms were positive for PRRSV. The first indication of a problem was increased morbidity and mortality during finishing with mortality increasing from 3-5% to as much as 15%. Affected pigs exhibited clinical signs typical of porcine multisystemic disease (MSD, formally known as PMWS) and PDNS. In pigs with MSD, the lymphocytes in germinal centers were consistently replaced by large macrophage-like cells and occasional multinucleated giant cells. Immunohistochemical staining revealed abundant PCV2 antigen in affected lymphoid tissues. In three of the four herds, there were affected pigs with rectal prolapse and weakness in the rear legs. In the brain and spinal cord there was non-suppurative inflammation, but without detectable PCV2 antigen staining. Whole genome PCV2 sequences were obtained by PCR amplification of lung and lymph node homogenates from 12 affected pigs from the four farms. All sequences formed a single group, which were approximately 99.5% identical to a representative 1998 French PCV2b isolate, AF055393. A fifth farm located in the same general area was investigated as fitting within the same PCVAD cluster. However, histopathology showed no evidence of PCVAD. Two PCV2 sequences were obtained and identified as PCV2a-like isolates with only about 94% identity to the PCV2b isolates from neighboring PCVAD farms. We sought to determine if the boar stud, which supplied three of the four farms with semen, could be the source of the PCV2b virus. Based on the differences between the PCV2a and PCV2b viruses, we developed steady state and real time PCR assays to distinguish PCV2a from PCV2b. Seven boars, culled because of poor semen production, were necropsied. Gross and microscopic analysis confirmed that all boars were negative for PCVAD. PCR amplification of lung and lymph node homogenates identified only the presence of PCV2a. These results indicated that the boar stud was not a likely source of PCV2b.

PCR amplification of RNA from tissues of PCVAD-affected animals identified a porcine teschovirus (PTV), which was closely related to a group of PTV-6 isolates. Other viruses were also identified, including PRRSV, adenovirus, parvovirus, influenza virus, reovirus and other members of the PTV group. Disease was reproduced in CD/CD pigs with a combination of PCV2 and PRRSV.

A randomized controlled clinical trial was conducted to evaluate, under field conditions, the effects of a commercial PCV2 vaccine in a herd with a history of PCVAD. A total of 485 commercial, cross-bred, growing pigs were randomly assigned within litter to receive either PCV2 vaccination or serve as unvaccinated control pigs. PCV2 vaccination reduced finisher mortality, increased finisher pig growth rate, and resulted in fewer lightweight pigs at market. For vaccinated pigs, overall mortality was reduced by 50% and finisher growth rate increased by 9.3%. At market, PCV2 vaccinated pigs were 8.8 kg heavier in the same number of days to market compared to unvaccinated control pigs. In serum from unvaccinated pigs, PCV2 antibody titers increased and PCV2 DNA was detected indicating active PCV2 infection. PCV2 DNA levels were significantly reduced following vaccination. These results indicate that PCV2 vaccination is an effective tool to aid in the control of PCV2 infection.

Introduction

Porcine circovirus type-2 (PCV2) is a small, non-enveloped, DNA virus with a single-stranded circular genome assigned to the family Circoviridae. PCV2 is recognized as the primary etiological agent associated with a group of complex, multi-factorial diseases classified as porcine circovirus associated disease (PCVAD), of which porcine multisystemic disease (MSD) and porcine dermatitis and nephropathy syndrome (PDNS) are major syndromes. Porcine multisystemic disease was described in 1997 as a new disease seen in several high-health herds in Western Canada and has become a growing concern in the swine industry throughout the world. Porcine multisystemic disease primarily affects growing pigs between 8 to 13 weeks of age. Clinical signs include weight loss, emaciation, tachypnea, dyspnea, and jaundice. Diarrhea, coughing, and central nervous system disorders are observed less frequently. Common postmortem findings include a diffuse lymphadenopathy, with the superficial inguinal lymph nodes notably enlarged. Additionally, the lungs are non-collapsed, firm, and show varying degrees of mottling, with more severe cases showing large patches of dark red to brown lobules, secondary to alveolar hemorrhage. Other less common findings include edema of the cecum and mild petechiation of the proximal spiral colon mucosal surface. The hallmark histological lesion of porcine multisystemic disease is loss of B cell follicles and T cell areas in secondary lymphoid organs including lymph nodes, Peyer's patches, tonsils and spleen. Lymphocytes are replaced with large histiocytic cells and multinucleated syncytial cells. The lungs show varying degrees of patchy to diffuse interstitial pneumonia ranging from lymphocytic-histiocytic in early cases to granulomatous in more severe cases. Another unique lung lesion is partial to complete airway epithelial sloughing and replacement by fibroplasia and infiltration of lymphohistiocytic cells.

Although it is now recognized in many pig-producing countries, the etiology of PDNS remains unclear. PCV2 has been implicated as the causative agent, but the simple presence of PCV2 in most PDNS animals is not conclusive proof of pathogenesis. However, the pathological changes of PDNS are consistent with an immune-complex disorder, and it has been hypothesized that extremely high PCV2 serum antibody titers and immune complex deposition are a significant factor for disease. Common findings in PDNS pigs include multifocal erythematous lesions secondary to a necrotizing vasculitis. Renal lesions include gross enlargement of the kidney with cortical petechia and necrotizing glomerulitis.

Phylogenetic analyses of PCV2 ORF2 gene sequences show branches that correspond to different geographic regions. The genomic sequences of recent interest are those that are identified as PCV2a and PCV2b. The terminology PCV2a and PCV2b is based upon whether the isolated virus is more similar to historical North American isolates, 'a', or European "French" isolates, 'b'. Based on restriction endonuclease digestion, most PCV2 genotypes in the U.S. can be further described by the RFLP patterns 422 (PCV2-422) and 321 (PCV2-321); which, with some exceptions, correspond to PCV2a and PCV2b respectively. Historically, PCVAD cases in the U.S. have been associated with viruses possessing the 422 genotype. Recent cases of PCVAD in Canada are linked to the emergence of the 321 genotype, which shows close similarities to certain PCV2b isolates, such as a 1998 French isolate (GenBank AF055393).

The interactions between PCV2 and other factors, including infectious agents are well documented. Swine influenza virus (SIV), porcine parvovirus (PPV) and porcine reproductive and respiratory syndrome virus (PRRSV) have been identified as cofactors in the development of porcine multisystemic disease. Additionally, bacteria such as *Mycoplasma hyopneumonia* have been shown to increase the severity of symptoms in a manner similar to PPV and PRRSV. It is likely that there are numerous other infectious cofactors that are involved in the etiology of PCVAD.

In this study, we identified four herds within the same geographical region of Kansas that were experiencing severe PCVAD. The term "severe" was used as a means to describe this outbreak as not only meeting the criteria of Sorden's definition of porcine multisystemic disease, but also exhibiting a dramatic increase in the morbidity and mortality of growing pigs. Furthermore, severe PCVAD was observed in both PRRSV negative and positive herds, and included some clinical signs not typically associated with porcine multisystemic disease or PDNS. The purpose of this study was to characterize the clinical, pathological, and virological features of an outbreak of severe PCVAD in Kansas including the genotypic properties of PCV2 isolates

recovered from affected and non-affected pigs, possible sources of PCV2 in a production system, and the identity of other viral cofactors.

In the past year, there have been several new developments regarding PCVAD, including the introduction of several commercial vaccines. However, there are few data on their effectiveness under field conditions. One purpose of this study was to conduct blind, randomized, well controlled clinical field trials to evaluate the effects of PCV2 vaccination on mortality and growth rate in a herds infected with PCV2 and a history of PCVAD.

Objectives

Objective 1 Analyze PCV2 genetic sequences from herds with severe PMWS. The purpose was to determine if severe PCVAD is associated with a particular genotype.

Objective 2 Identify cofactors involved in severe PMWS. The purpose was to determine if PCVAD pigs possess other infectious agents, which might function as co-factors in the disease process.

Objective 3. Determine if the PCV2 isolates from the Kansas Cluster can cause severe PMWS. This objective was directed at testing the disease potential of PCV2 isolates alone and when combined with other co-factors.

Objective 4. Evaluate the effectiveness of commercial vaccines in the field.

Materials and Methods

Methods for Kansas Cluster Analysis

Clinical History. In Kansas, the first clinical recognition of severe PCVAD occurred in November 2005, affecting 4 separate finisher operations located in the northeastern part of the state. For the purpose of convenience, we labeled these sites Farms A, B, C and D. Farms A and B were PRRSV positive, multi-site farrow to finish operations. Farms C and D were PRRS negative, and served as nursery and finisher sites for weaned pigs from Farm E, a PRRSV negative sow farm. The PRRS status of each farm was determined by routine monitoring for the presence of PRRSV antibody. Farms A and E received replacement gilts as weaned piglets from Farm F, a PRRSV negative multiplier, and semen from Farm G. Thus, the genetic source of Farms A, C, and D was the same. Farm B received animals from a different boar stud and gilt multiplier. All farms were considered to be of overall good health with minimal incidence of common pathogens such as swine influenza, proliferative enteritis, and *Mycoplasma* prior to the study investigation. For the initial investigation, approximately 20 pigs were sampled per site.

Virus isolation. The isolation of PCV2 was performed on monolayers of rapidly dividing ST cells (approximately 50% confluent). Cells were maintained in minimal essential medium (MEM) supplemented with 7% fetal bovine serum (FBS) and antibiotics (penicillin, streptomycin, Amphotericin B (fungizone), and ciprofloxacin). Monolayers were incubated with different dilutions of clarified homogenates prepared from lung, lymph node and kidney. After three days, the media containing the virus was removed and stored at -80°C . To detect the presence of virus, monolayers were fixed in 80% aqueous acetone and stained with FITC-labeled porcine anti-PCV2 antibody (VMRD). Cells were incubated with antibody for one hour, extensively washed with PBS, counterstained with Evans Blue, and then viewed under a fluorescence microscope. For the isolation of other viruses, the same clarified homogenates were placed on confluent monolayers of ST cells and PK-15 cells with cultures monitored over several days for the appearance of cytopathic effect (CPE).

Immunohistochemistry. Immunohistochemistry (IHC) for the detection of PCV2 antigen was performed on thin sections prepared from paraffin-embedded formalin fixed tissues. Antibody staining of deparaffinized tissues was performed by the Kansas State Veterinary Diagnostic Laboratory using an automated procedure. Briefly, PCV2 antigen was detected using a mouse monoclonal antibody (VMR1), diluted 1:300 in PBS, followed by a DAB staining kit (Ventana). Slides were counterstained with Myer's Hematoxylin followed by treatment with bluing reagent.

Whole PCV2 genome sequencing and phylogenetic analysis. One gram of lung and lymph node tissue was homogenized in 10ml of MEM and tissue debris removed by centrifugation. Total DNA was isolated from

the clarified medium using a QIAamp DNA blood mini kit (Qiagen). PCR and DNA sequencing of whole PCV2 genomes was performed according to published protocols. Two overlapping segments were amplified using primer pairs CV1F 1336-5'AGGGCTGTGGCCTTTGTTAC / CV2R 536-5'TCTTCCAATCACGCTTCTGC, respectively, and CV3F 453-5'TGGTGACCGTTGCAGAGCAG / CV4R 1525-5'TGGGCGGTGGACATGATGAG, respectively. The reaction mixture included the DNA template, 1x PCR buffer, 1.25 mM MgCl₂, 0.2 mM of each dNTP, 0.2 uM each primer and 2.5 U Taq polymerase. PCR conditions were one cycle at 95°C for four min followed by 40 cycles of 93°C for 45 sec, 50°C for 45 sec, and 72°C for 90 sec. At the end, the reaction was incubated at 72°C for 10 min. DNA sequences were determined using the PCR products, the relevant primer sets, and a sequencing kit from Beckman-Coulter. Analyses were performed on a Beckman-Coulter CEQ 8000 sequencer. Prior to sequencing, the PCR products were cleaned up using a Wizard SV Gel and PCR Clean-Up System (Promega). Analysis of the sequence chromatograms and assembly of the sequences were performed using several computer-based programs, including 4-Peaks, Vector NTI, Clustal-X, and Biosoft Gene Jockey II. Phylogenetic trees were constructed using the whole 1768 nucleotide genome for each isolate. Sequences were aligned using Clustal-X. The unrooted phylogram was generated by a neighbor joining method using Mega.¹⁷

Selective PCR amplification of PCV2-422 and 321 genotypes. For the detection of PCV2 in clinical samples, we took advantage of the differences between 321 and 422 isolates to prepare genotype-specific primers. The original 422-specific PCR-specific assay was performed according to Kim, et al.¹⁸ This approach incorporated the forward and reverse primers AF-PCV2 939-5'CAGTTCGTCACC and AR-422 1485-5'GGGGGACCAACAAAATCTC, respectively. Amplification of 321 isolates utilized the same forward primer and a 321-specific reverse primer, AR-321 1485-5'GGGGCTCAAACCCCGCTC. DNA was amplified using 35 cycles at 94°C for 30 sec, 60°C for 30 sec, and 72°C for 60 sec, followed by a 10 min extension at 72°C. The PCR products were electrophoresed on a 2% agarose gel and visualized under UV light after staining with ethidium bromide. Each run included positive and negative controls.

Detection and identification of porcine teschovirus (PTV). PCR detection of teschovirus RNA was performed as described in the literature. Total RNA was extracted from tissue homogenates composed of lung, lymph node and spinal cord using a Qiagen RNAeasy kit. RT-PCR was performed using a Qiagen One Step RT-PCR kit, according the manufacturer's recommendations. Forward and reverse primers were PTV-F 5'GTGGCGACAGGGTACAGAAGAG and PTV-R 5'GGCCAGCCGCGACCCTGTCTAG, respectively. The PCR reaction conditions were 40 cycles at 94°C for 60 sec, 56°C for 60 sec and 72°C for 60 sec, followed by a 10 min extension at 72°C. Using this assay, the recovery of a 163 bp PCR product identified the virus as PTV, while the recovery of a 183 bp product also indicated the presence of the porcine enterovirus, PEV-8. To further subtype the PTV, a variable region encoding the C-terminal end of the 1C protein and N-terminal portion of the 1D protein was amplified by PCR and sequenced. The PCR reaction incorporated the degenerate forward and reverse primers, 2163F 5-GGCAATGCAAGGYCATTATGT and 2746R 5'AGCCRCAYCTCADRTARGETGGCATG, respectively. Amplification conditions were the same as described for porcine teschovirus PCR. The DNA sequence was compared to PTV sequences deposited on GenBank.

Methods for vaccine study

Swine – Four hundred eighty-five weaned, cross-bred (TR4^a x C22^b) swine were used. Pigs were randomly selected from litters of 65 sows ranging in parity from 1 to 8 with a mean of 3.4. Pigs were divided into 6 weaning groups based on date of birth and randomly assigned by litter and gender into a vaccinated (n = 235) and a control (n = 250) population. Within each group, vaccinated and control animals were commingled with one another in approximately equal proportions. Each weaning group was housed in separate nursery rooms in pens ranging in size from 15 to 30 animals each for approximately 7 weeks post-weaning for the nursery phase. For the finishing phase, the 6 weaning groups were realigned into 4 finishing groups with approximately equal numbers of control and vaccinated animals within each group. Three of the finishing groups were housed in separate dirt floor, single room, hoop barn buildings of approximately 180 animals. The fourth finishing group was housed in a modified open front confinement building with pigs randomly assigned to pens of 20 to 25 individuals. The finishing phase was 18 weeks, at which time all pigs within a group were marketed on the same day. Pigs were routinely vaccinated in accordance with the farm's previous vaccination protocol, which was an *Erysipelas rhusiopathia* and *Mycoplasma hyopnumoniae* combination product administered at 7 to 8 weeks of age.

PCV2 Vaccine – A commercially available killed baculovirus-expressed capsid protein derived vaccine in process for full licensure was obtained and administered according to the label instructions.^c

Experimental Design – A blind randomized controlled clinical trial study design was used to evaluate the effects of PCV2 vaccination and gender on mortality and growth performance. Prior to cross fostering, pigs were individually identified with numbered ear tags that were all the same color. Piglets were subject to a single cross fostering between 4 and 24 hours of age with the objective of equalizing the number of piglets across litters and to more evenly match the number of piglets with the sow's ability to nurse them. Pigs were randomly assigned within each birth litter and by gender within litter to receive either the PCV2 vaccine or serve as unvaccinated controls. Over the course of the study, farm staff and research data collection personnel were blinded as to vaccination status of each pig.

Pigs were weighed and either vaccinated intramuscularly on the right side of the neck or left unvaccinated as controls at either 4 (n=436 pigs) or 11 (n=49 pigs) days after weaning. A second dose of vaccine was administered 3 weeks after the first dose in accordance with the manufacturer's directions. Control unvaccinated pigs were handled in a similar manner as vaccinated pigs with the exception of administration of the vaccine. All pigs were subsequently weighed just prior to entering the finisher phase (63 to 70 days of age) and at the day prior to marketing. ADG for each phase and overall was calculated for each pig that was marketed. The ADG was obtained by dividing the weight gain by the number of days from vaccination to the end of the phase for the nursery or until marketed for the overall phase or from the end of the nursery until marketed for the finisher phase.

Mortality was calculated as the number of pigs that died in each treatment category divided by the number of pigs initially placed on test in each treatment category. Dead and electively culled animals were collected throughout the duration of the trial and pigs without severe post-mortem autolysis were necropsied. The standard set of tissues evaluated included: submandibular lymph node, superficial inguinal lymph node, tonsil, lung, heart, liver, spleen, ileum, spiral colon, stomach, kidney, brain, and spinal cord.

PCV2 serology-Serum samples from a sub sample of pigs were assayed for PCV2 antibodies using a 96-well format IFA assay. The IFA antigen was obtained by infecting ST cells with a low passage wild-type PCV2 virus stock. After three days, the cells were fixed in 80% acetone and plates stored at -20° C until use. Serial 1:2 dilutions of each sample, beginning at a 1:20 dilution, were prepared in PBS and plates incubated for one hr at 37° C. Plates were washed 3 times and bound pig antibody detected with FITC-conjugated goat anti-pig IgG (Jackson Laboratories), diluted 1:400 in PBS. After one hr incubation at 37° C, plates were washed and viewed under a fluorescence microscope. The titration endpoint was calculated as the reciprocal of the last serum dilution that gave a positive result.

PCV2 viral DNA detection- PCV2 DNA was measured using a real time SYBR Green PCR method. Total DNA was extracted from serum using a QIA Amp DNA Mini-Kit (Qiagen). DNA extractions were performed according to manufacturer's instructions. For PCR, the forward primer, dPCV2-G1f (5'-GCC AGA ATT CAA CCT TAA CCT TTC-3') and reverse primer, dPCV2-G1r (5'-GCG GTG GAC ATG ATG AGA TT-3') were used at a 250 nM concentration in a 25 µl reaction mixture containing 12.5 µl of 2X Power SYBR Green PCR Master Mix (Applied Biosystems), 8.0 µl of sterile water, 1.5 µl of 25 mM MgCl₂ and 2.5 µl of sample DNA. A PCV2b genomic plasmid control cloned into plasmid vector PCR 2.1 (Invitrogen) was used to generate a standard curve for the SYBR Green PCR method. Plasmid standards, ranging from 4 x 10⁸ templates per reaction to 4 x 10³ templates per reaction, were prepared in 10 mM Tris pH 8.0 and run in triplicate with the vaccine study sample templates. Reactions were prepared in a MicroAmp Fast Optical Reaction Plate (Applied Biosystems), sealed with Optical Adhesive Covers (Applied Biosystems) and centrifuged in a micro plate rotor at 3000 RPM for five minutes. The reaction plate was subjected to amplification and dissociation analysis on the ABI 7500 Fast Real Time PCR System (Applied Biosystems). Amplification was performed by an initial denaturing/activation step of 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 58°C for 40 seconds and 72°C for 40 seconds with the SYBR Green fluorescence recorded at the 72°C extension step of each cycle. Dissociation curve data was collected following amplification with one cycle of 95°C for 15 seconds, 60°C for one minute and data collected during a ramp back to a final 95°C hold. Ct data and dissociation temperature analysis were done using a 7500 Sequence Detection Software Version 1.3.1 (Applied Biosystems). The Ct values were calculate from a threshold of 0.099, determined using a manual background between cycles 3 and 12. Dissociation curve data was used to verify PCV2 positive SYBR Green PCR amplicons by a single 1st derivative peak between 76°C and 78°C. A sample meeting both a positive Ct value and a verified dissociation temperature was considered to be a positive sample. Comparative

quantitative data between samples was estimated using the six point standard curve generated from the PCV2 plasmid clone. Comparative quantitative data results were reported as \log_{10} PCV2 templates per reaction.

Statistical Analysis – Pig was used as the experimental unit for all analysis of weaning, mortality, growth rate, market and quantitative PCR data. The analysis was performed using a mixed model analysis of variance. With the exception of mortality and proportion greater than the minimum market weight, all analysis was performed using the MIXED procedure in SAS version 9.1.^d The fixed effects of the statistical model included the effects of PCV2 vaccination (control or vaccine), gender (gilt or barrow), and their interaction. The random effects included group and litter. Due to inability of the statistical model to converge, the effects of litter were removed from the model for parity and age at weaning. For mortality and proportion above the minimum market weight the analysis was performed using the GLIMMIX procedure. Fixed effects were the same as for the MIXED procedure analysis and the random effect of group included in the model. Data are reported as least-square means and all standard errors reported are pooled standard errors of the mean. Values of $P < 0.05$ were considered significant and all confidence intervals are 95%.

Results

Objective 1. Analysis of PCV2 genetic sequences from herds with severe PMWS.

A detailed analysis of the outbreak in the first four affected herds in Kansas is described in detail in Horlen et al., 2007 (JSHAP). Over the course of the study, we identified the PCV2b genotype in affected pigs from approximately 70 herds. In summary, whole genome sequences from the original four herds were used to construct phylogenetic trees. As shown in Fig. 1, 12 PCVAD-associated sequences formed a single group, which was located near a 1998 French PCV2b isolate, AF055393 (approximately 99.5% nucleotide identity). Sequences from a given farm tended to cluster together. A fifth farm located in the same area was investigated as possibly fitting within the same PCVAD cluster; however, even though the pigs were PCR-positive for PCV2, histopathology showed no evidence of PCVAD and the resulting sequences, identified as 804-SF and 804-SP in Fig.1, localized near the historical North American strains. The 12 Kansas PCVAD sequences were only about 94% identical to the North American group of isolates. These data indicate that the PCVAD-associated PCV2 isolates clearly belonged to the PCV2b genotype. The emergence of PCV2b in the U.S. and its association with PCVAD has been confirmed by others.

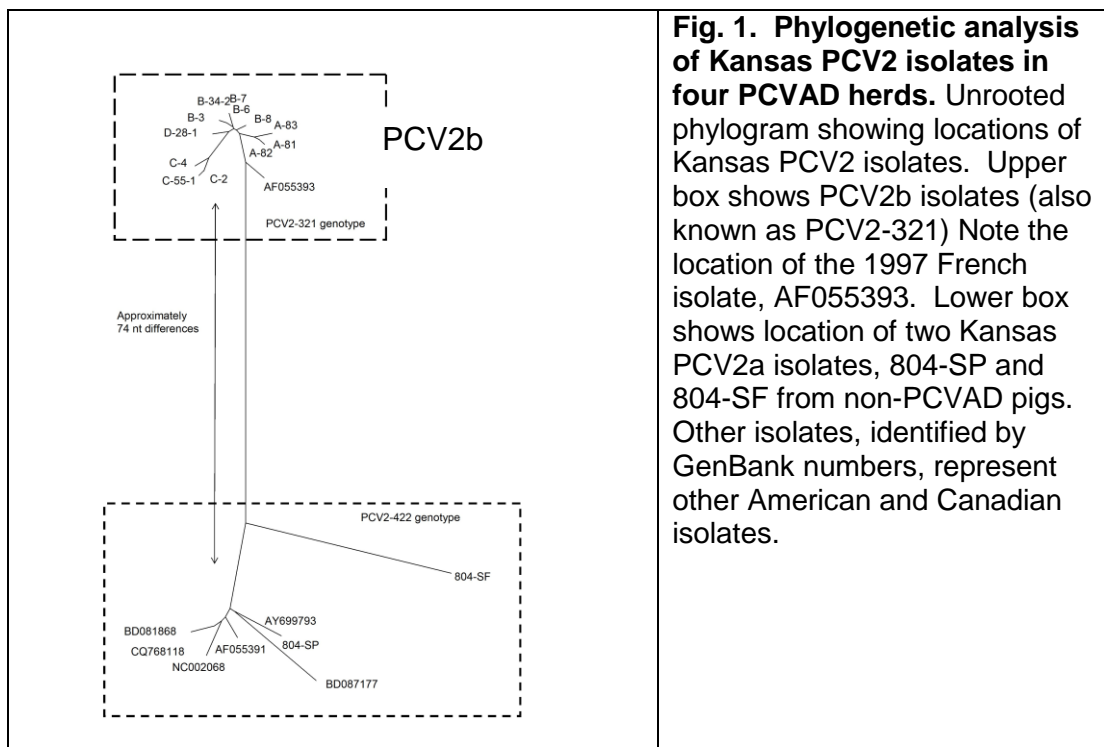


Fig. 1. Phylogenetic analysis of Kansas PCV2 isolates in four PCVAD herds. Unrooted phylogram showing locations of Kansas PCV2 isolates. Upper box shows PCV2b isolates (also known as PCV2-321) Note the location of the 1997 French isolate, AF055393. Lower box shows location of two Kansas PCV2a isolates, 804-SP and 804-SF from non-PCVAD pigs. Other isolates, identified by GenBank numbers, represent other American and Canadian isolates.

Development of genotype-specific diagnostics tests and the analysis of pigs with co-infections of PCV2a and PCV2bv. Both gel-based and real time PCR assays were developed to rapidly identify PCV2a and PCV2b in diagnostic samples. The gel-based assay incorporated primers that exploited the sequence differences between PCV2a and PCV2b (see Fig. 2) and described in Horlen et al., (2007, JSHAP). The extensive analysis of plasmid DNA templates demonstrated the specificity and sensitivity of the assay system (data not shown). We used the gel assay for the analysis of PCV2a and PCV2b PCV2 isolates obtained from PCVAD pigs. All isolates were positive for PCV2b. Interestingly, five of the isolates were positive for both PCV2a and b sequences (Fig. 2D). In the analysis of 90 diagnostic samples from PCVAD pigs, all were positive for PCV2b and 24 of those double positive for a and b genotypes. The gel-based assay was used to determine if the boar stud, which supplied 3 of the 4 farms in Kansas, was the source of the PCV2b virus. The PCR results, presented in Fig. 2B, showed that six of the seven boars were positive for PCV2a only. The PCR products were sequenced to confirm the presence of only PCV2a DNA. The source of the PCV2b virus in the original Kansas outbreak was never identified.

We also developed real time PCR tests, including SYBR Green and TaqMan assays. For the SYBR Green assay, total DNA was extracted from serum using a QIA Amp DNA Mini-Kit (Qiagen). Plasmid standards, ranging from 4×10^8 templates to 4×10^3 templates per reaction, were added to the reaction buffer. PCR amplification and dissociation analysis were performed on an ABI 7500 Fast Real Time PCR System (Applied Biosystems). Examples of standard and dissociation curves are shown in Fig. 3. The genotype-specific assays are being offered by the Kansas Veterinary Diagnostic Laboratory and available to any user.

A

	AF-PCV2 Forward $\xrightarrow{\hspace{1.5cm}}$	$\xleftarrow{\hspace{1.5cm}}$	PCV2a -Reverse
	AF050391-422 939-CAGTTCGTCACC. . . .GAGATTTTGTGGTCCCC-1485		*** * * * ****
	AF050393-321 939-CAGTTCGTCACC. . . .GAGCGGGGGTTTGAGCCCC-1485		PCV2b-Reverse

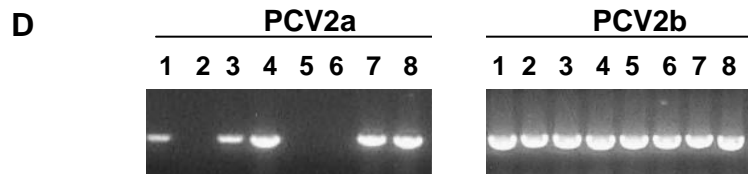
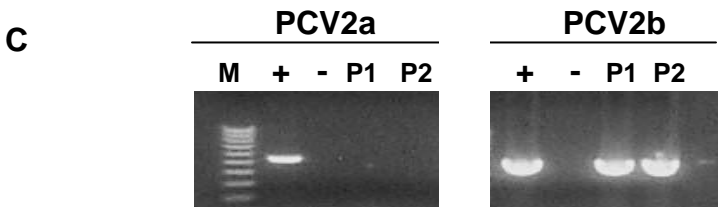
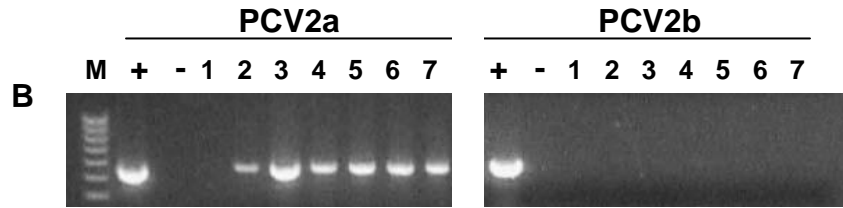


Fig. 2. PCR identification of PCV2a and PCV2b genotypes. (A) Primers used to amplify 321 and 422 genotypes. (B) PCR results of 7 boars supplying semen to 3 of the 4 farms in the original Kansas outbreak. (C) PCR results for two pooled sets containing lymph node and lung tissues from 6 pigs with severe PCVAD (3 pigs per set). (D) PCV2 screening of 9 virus isolates from pigs with severe PCVAD.

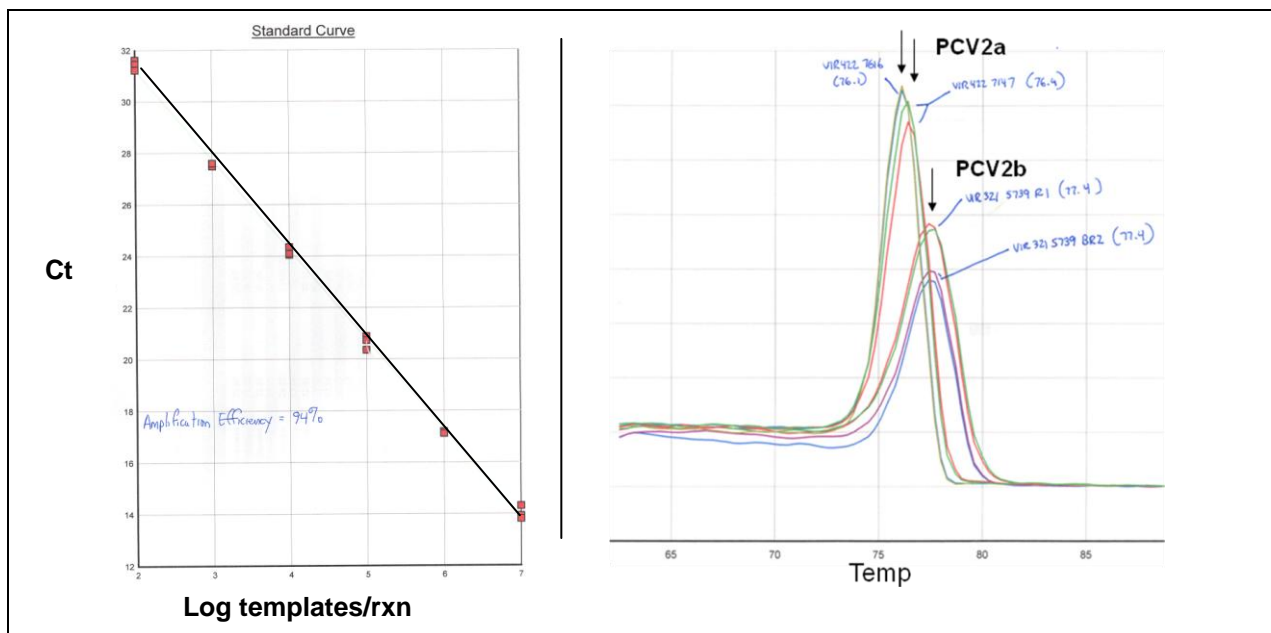


Fig. 3. SYBR Green real time PCR for the detection of PCV2a and PCV2b isolates. The left figure shows the standard curve for PCR reactions spiked with different amounts of a plasmid containing a PCV2b template. The result shows a linear curve between 10^2 and 10^7 templates/rxn. The right figure shows the different melting curves for selected PCV2a and PCV2b isolates.

Objective 2. Identification of cofactors involved in PCVAD

The hypothesis under this objective was that other co-factors, such as viruses or bacteria, are working in conjunction with PCV2 to cause PCVAD. We have identified several potential co-factor viruses, including a variety of porcine teschoviruses, porcine enterovirus, adenoviruses, parvovirus, reovirus, influenza virus and as expected, PRRSV. Several bacteria were also identified. In summary, the PCVAD-affected pig is a “zoo” of infectious disease agents. The unique aspect of the Kansas Cluster is that many of the PCVAD herds were PRRS free. However, when PCV2 was combined with PRRSV the outcome was more severe, with larger numbers of affected animals. Several of the PCVAD pigs had neurological lesions that were not associated with the presence of PCV2 antigen. One possibility was that lesions were the result of a porcine enterovirus (PEV) or porcine teschovirus (PTV). A PTV was isolated on ST cells from a PCVAD pig. To further characterize this isolate, we prepared a set of degenerate PCR primers designed to amplify the structural region of several PTV serotypes. The PCR product was sequenced and then compared with known PTV sequences from GenBank. The phylogenetic tree, presented in Fig. 4, showed that this PTV sequence was the most closely related to a group viruses belonging to the PTV-6 serotype. The contribution of PTV-6 and other teschoviruses to neurological lesions in PCVAD pigs remains unknown and deserves further study.

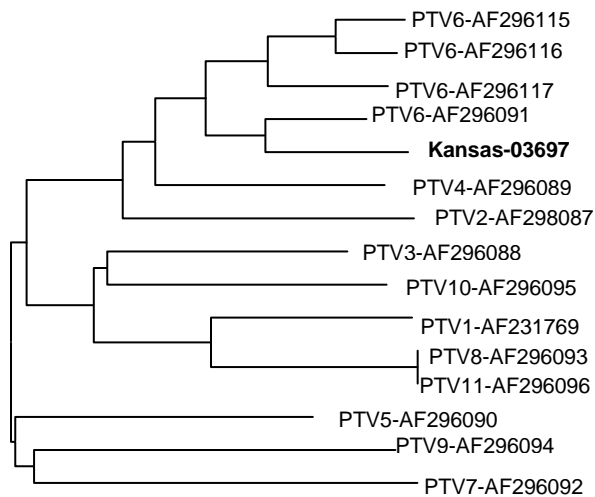


Fig. 4. Phylogenetic Characterization of the Kansas PTV Isolate.

Unrooted phylogram showing location of porcine teschovirus (PTV) isolate 36972 in relation to sequences of known serotypes. The PTV reference sequences are identified by GenBank numbers.

Objective 3. Determine if the PCV2 isolates from the Kansas Cluster can cause severe PMWS.

Originally, we intended to perform these studies in commercially available pigs. One problem was the relatively high prevalence of PCV2 in pigs available for experimentation. As an alternative, we obtained 5 PCV2 negative CD/CD pigs. Three pigs were challenged with a clarified homogenate from a PCV2b/PRRSV affected pig and two pigs challenged with homogenate after heat inactivation to kill PRRSV and other heat sensitive agents. All dual infected pigs seroconverted to PCV2b and PRRSV and showed signs of disease, including one pig with “blue” ears. Pigs receiving the heat-treated homogenate seroconverted to PCV2, but not PRRSV and remained healthy. We then incorporated 12 CD/CD pigs to determine the minimal amount of virus needed to establish a productive infection. Unfortunately, several of the pigs seroconverted prior to challenge and the experiment terminated. One outcome of this experiment is the development of PCV2 infection and disease models.

Objective 4. Evaluate the effectiveness of commercial vaccines.

Vaccine Study #1

A more detailed description of the vaccine study can be found in a manuscript by Horlen et al. (2007), which has been accepted for publication in JAVMA. This study was conducted on an isolated, 300-sow, PRRSV free, farrow-to-finish farm in northeast Kansas. The farm had a documented history of PCVAD and a pattern of mortality was consistent with that reported on other regional farms experiencing severe PCVAD. Four hundred eighty-five weaned, cross-bred (TR4^a x C22^b) pigs were randomly selected assigned by litter and gender into a vaccinated (n = 235) and a control (n = 250) population. Vaccinated and control animals were commingled with one another in approximately equal proportions. Pigs were weighed, bled and either vaccinated or left unvaccinated. A second dose of vaccine was administered 3 weeks after the first dose in accordance with the manufacture’s directions. Pigs were weighed again just prior to entering the finisher phase (63 to 70 days of age) and at the day prior to marketing. Average daily gain (ADG) for each phase and overall was calculated for each pig marketed.

During the nursery phase, no differences in mortality were noted between treatment group. However, within the finisher phase, vaccinated pigs had lower mortality ($P < 0.002$; 16.7 ± 4.6 % for control pigs vs. 6.1 ± 2.3 % for vaccinates). For the finisher phase, ADG was significantly increased ($P < 0.001$) in vaccinated pigs (1.91 lbs) vs. control controls (1.74 lbs). The pattern of distribution of market weight was similar for control and vaccinated pigs. However, the mean of the vaccinated pigs was shifted to the right by 8.8 kg resulting in a significantly lower ($P = 0.003$) percentage of vaccinated compared to control pigs that were below the minimum market weight goal of 111.4 kg (245 lbs). A subset of 52 pigs were randomly selected (31 controls and 21 vaccinates) and blood samples collected at 3 weeks (time of vaccination), 6 weeks (time of booster), 9 weeks (entry into the finisher) and 17 weeks (mid-finish) of age. By 9 weeks of age, 11 of the 31 unvaccinated pigs

were seronegative. In contrast, all vaccinated pigs were seropositive with titers ranging from 640 to 20,480. At 17 weeks, PCV2 antibody titers in the unvaccinated group were dramatically elevated, an indication of active infection. In the vaccinated group, pigs remained seropositive with titers similar to the 9 week measurements. Real time PCR measurement of PCV2 DNA in serum showed that the control pigs had more ($P < 0.01$) PCV2 DNA than pigs from the unvaccinated group. In the unvaccinated group, the estimated PCV2 SYBR Green PCR values ranged from undetectable to a value of $4.5 \log_{10}$ templates/rxn ($>30,000$ templates/rxn), with a mean value of 2.6 ± 0.14 (≈ 400 templates/rxn). In contrast, the distribution for the vaccinated pigs was more narrow and shifted to the left with estimated PCV2 DNA concentrations ranging between 0.1 and $1.9 \log_{10}$ templates/rxn (mean = 1.3 ± 0.17 or ≈ 400 templates/rxn). The results from the melting curve analysis of PCR products showed that pigs were infected with the PCV2b subtype, the same virus identified prior to the initiation of the study.

Vaccine study #2

The second study was performed on a PRRS positive Kansas commercial farm with a history of PCVAD. Pigs, 1,470, were randomly allotted into control or two vaccine treatments. Pigs were vaccinated with either a commercial two dose product or a second commercial one dose product. Vaccines were administered in nursery according to label instructions. Treatment pigs were commingled within the same pens. In this study, there was no significant effect of vaccination on mortality (11% control; 8% one dose, 8% 2 dose). However, similar to the previous study there was a significant increase in ADG for the vaccinated pigs (1.53 lb for control; 1.58 lb for the one dose vaccine, and 1.68 lb for the 2 dose vaccine). As shown in Fig. 5 there was a shift in the weight distribution to the right for the vaccinated pigs with the largest shift for pigs vaccinated with the two dose product. This study confirmed the capacity of PCV2 vaccination to improve performance.

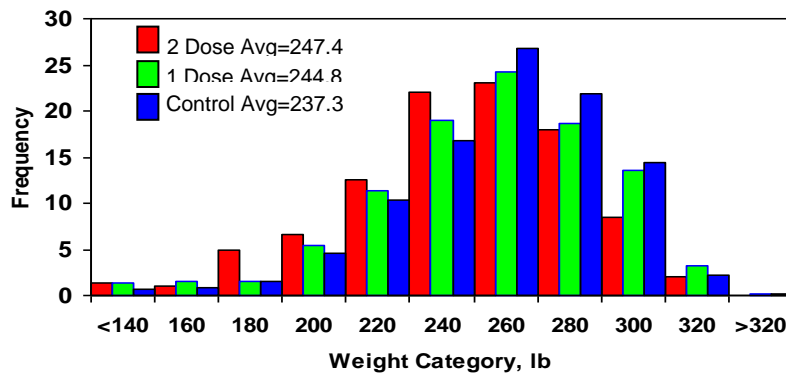


Fig. 5. Weight distribution following vaccination.

Discussion

Severe PCVAD in a group of Kansas herds was a disease problem not previously encountered in this region. The diagnosis of severe PCVAD was made based on the presence of PDNS and porcine multisystemic disease with finisher mortality sometimes reaching 15%. This clinical disease pattern was present in herds negative for PRRSV, a cofactor frequently linked to PCVAD. Molecular analysis of PCV2 sequences from severely affected pigs identified the presence of a PCV2 that was nearly identical to a 1998 French isolate (AF055393) and clearly distinct from historical U.S. PCV2 isolates previously reported in the literature. Since we have no historical information on PCV2 sequences in the four Kansas herds prior to the first appearance of PCVAD, we cannot determine the temporal association between the introduction of PCV2b and the current PCVAD outbreak. Interestingly, the data that has emerged from the study of the Kansas herds paints a picture similar to reports from Canada that link PCV2-321 with the increased incidence and severity of PCVAD. For example, the Animal Health Laboratory at the University of Guelph in Ontario reported only 1 case of PCV2-321-associated PCVAD in 2004, whereas 135 cases were reported in 2005. Furthermore, the increased incidence of PCV2-321 is associated with an overall increase of PCVAD in Canada. However, the degree of similarity between Kansas PCV2b and Canadian PCV2-321 is currently unknown. Reasons for the association of PCV2b with increased severity of clinical disease are unclear.

Although the focus of this paper is on the association of PCV2b with severe PCVAD, PCV2b may also be present in pigs not affected by disease. Since three of the four affected farms received semen from the same source, we originally believed that the boar stud was the most logical source of the PCV2b genotype. However, analysis of a small number of healthy boars showed only the PCV2a genotype. This does not rule out the possibility that boars possess the PCV2b virus and further studies are needed in this area. It is interesting to note that six of the seven boars studied were PCR positive for PCV2a. However, the ability of the boars to shed PCV2 in semen was not studied. Both PCV2a and PCV2b genotypes were found in isolates from PCVAD affected pigs, suggesting that pigs can be infected simultaneously with both genotypes. The presence of the two viruses within a single animal raises the intriguing possibility that the two PCV2 genotypes may undergo recombination and we have found a recombination virus in a diagnostic lab submission (Hesse et al- submitted for publication).

PCV2 antigen has been found associated with brain and spinal cord lesions in PCVAD pigs exhibiting neurological signs. However, in this study we failed to identify PCV2 in association with the central nervous system lesions from the Kansas PCVAD pigs. A search for teschoviruses in one PCVAD affected pig yielded an isolate that was closely related to PTV-6. Although PTV-6 is a serogroup 1 enterovirus, it is not known to be associated with enteroviral encephalomyelitis or Talfan disease as PTV serogroup 1, serotype 1 (PEV-1) is. The rare detection of PEV-8 in clinical samples by PCR was judged to not be important in this instance. Additionally, isolations carried out on PK-15 cells resulted in no other viruses that were consistently detected amongst PCVAD pigs. Overall, the contribution of tescho- and enteroviruses to PCVAD remains unclear. Multiple viruses have been implicated in PCVAD, and PTV may be only one of several contributing co-factors.

The data from the first vaccine study clearly demonstrated that vaccination led to significant reductions in mortality, increased finisher pig growth rate, and fewer light weight pigs compared to the unvaccinated control pigs. We were unable to detect an interaction between PCV2 vaccination and gender. This indicates that the magnitude of impact of PCV2 vaccination was similar regardless of gender. While there was clearly no interaction between vaccine and gender for growth rate, for finishing mortality the relative risk reduction for PCV2 vaccination was 76% for gilts while in barrows it was 46%. This suggests that vaccination may be more protective in gilts than in barrows. Regardless of vaccination status, barrows had higher mortality rates overall and during the finishing phase. Studies have confirmed that compared with gilts, males have a slightly lower chance of survival before weaning, and this strong association of gender with mortality may result a potential confounder when population studies are conducted. In newborn pigs, these differences may be attributable to higher testosterone and cortisol levels in males; however, no explanation is available for such an effect in older pigs following castration.

While the cumulative finisher mortality rate of unvaccinated pigs in this trial was higher than prior to the study, the weekly pattern of mortality in the unvaccinated pigs was similar to pre-trial data with the greatest differences between treatment groups occurring during weeks 6 to 14 of the finishing phase. Further support for this pattern is provided by the significant differences in mortality that were noted to occur within the finisher phase, while no significant differences were observed while pigs were in the nursery. Explanations of this observation would include time of infection by PCV2, loss of protective passive immunity to PCV2, stress associated with finishing, the occurrence of a necessary co-infecting agent during finish that results in an overall decrease in protective immunity, or that PCV2 exhibits delayed clinical signs after prolonged infection. In this study, nursery pigs were all seropositive at 3 weeks of age. The detectable levels of PCV2 antibody likely reflect the transfer of passive maternal antibody during lactation. By the time the unvaccinated pigs had reached the finisher, antibody titers had markedly decreased with approximately 30% of pigs with undetectable levels of antibody. Therefore, in this particular herd it appears that the level of passive immunity in pigs after leaving the nursery had sufficiently decayed to the point that pigs become susceptible to infection during finishing. One effect of the vaccine was to maintain elevated levels of PCV2 antibody.

The effects of PCV2 vaccination on increasing growth rate were easily seen. Growth rate was increased by 9.3% in vaccinated pigs compared to unvaccinated pigs. This was over twice the difference in growth rate between genders and resulted in 8.8 kg heavier pigs at a similar age. Further analysis of the ADG data indicates that vaccinated pigs would reach a comparable market weight approximately 11 days sooner than unvaccinated pigs. Since facility costs are typically the second largest cost of production on swine farms, the

increased growth rate could lead to a significant reduction in facility costs and increased economic return. Similar to the mortality rate, effects of the increases in growth rate due to vaccination were during the finisher phase while there were no differences in growth rate during the nursery phase. While one of the clinical outcomes of PCVAD is weight loss in a subpopulation of pigs, we are unaware of any other studies that have characterized the impact of PCV2 infection on the overall growth rate. Therefore, PCV2 vaccination may have a significant economic impact on performance parameters other than mortality.

The initial outbreak of acute PCVAD appeared in the study herd in early 2006, with increased mortality, including several pigs diagnosed with PDNS and PMWS was associated with the appearance of the PCV2b subtype. However, after the initiation of the vaccine study, these classical PCVAD clinical signs largely abated, with a small number of unvaccinated pigs developing PDNS and only mild signs of PMWS. The principal source of mortality was attributed to bacteria-associated infections. The change in the clinical disease pattern raised the possibility that pigs were not productively infected with PCV2, perhaps a result of elevated herd immunity acquired following the initial outbreak of acute PCVAD. The analysis of PCV2 antibody in a subset of pigs showed that all of the unvaccinated control pigs had seroconverted in the finisher, and by 17 weeks of age were producing large quantities of anti-PCV2 antibody. Elevated PCV2 antibody responses suggested that pigs were productively infected with PCV2. Interestingly, in spite of the elevated antibody response, 11 of the 19 pigs with antibody titers greater than 40,000 either died or failed to reach market weight (data not shown). These data raise the possibility that the immune response elicited during natural infection is defective, perhaps a consequence of the immune dysregulation caused by the virus. Another approach for determining the infection status was the use of PCR to measure virus load in serum. Published studies using quantitative PCR methods have linked PCVAD to the quantity of PCV2 DNA circulating in the blood. The presence of an active PCV2 infection in the unvaccinated finishing pigs was confirmed using real time PCR to estimate the amount of PCV2 DNA in the serum. The mean value for unvaccinated pigs was 2.6 log₁₀ templates compared to an approximate 10 fold decrease in the amount of PCV2 DNA in the group of vaccinated pigs (mean of 1.2). These data confirmed that the herd was actively infected with PCV2 and the vaccine reduced the level of infection. The presence of an active PCV2 infection in unvaccinated pigs combined with a decrease in viremia and mortality in vaccinated pigs demonstrate that the clinical disease signs associated with morbidity and mortality were the result of PCV2 infection. In addition to PMWS and PDNS, there are several disease syndromes associated with PCV2 infection. In this study, most succumbed from non-PMWS or non-PDNS associated causes. The clinical disease syndromes responsible for the death of pigs may represent a manifestation of PCVAD distinct from PCVAD associated syndromes previously reported in the literature. However, similar to PCVAD, the disease pattern may result from a generalized state of immune suppression caused by PCV2.

Although we did not measure rates of PCV2 shedding or transmission, we believe the reductions in viremia indicate that there may be significant herd level effects of vaccination. Thus, vaccination may be a useful tool to reduce overall viral shedding and inhibit pig to pig viral transmission. Since PCV2 virus is relatively resistant to thermal and chemical inactivation²³ reducing the environmental viral load from the pig may be a key factor in future control strategies.

Analysis of the PCV2 genome and proteins indicate that the capsid protein, which is coded by open reading frame 2 (ORF2), interacts with the cell receptor on host cells and stimulates protective immunity. Current PCV2 vaccines are largely based on stimulating anti-capsid immunity. Phylogenetic analyses of PCV2 ORF2 gene sequences show branches that correspond to different geographic regions of both North America and Europe. The genomic sequences of interest are those identified as PCV2a and PCV2b. Historically, PCVAD cases in the U.S. have been associated with PCV2a viruses. However, recent cases of PCVAD in the U.S. and Canada are linked to the emergence of the PCV2b genotype, which show close similarities to certain European isolates. Although, most swine herds appear to be infected with PCV2, unique differences in the clinical manifestation of field cases of PCVAD have led to the hypothesis that different PCV2 genotypes may differ in virulence. Recent research evaluating the microscopic lesions of PCVAD and amount of PCV2 antigen identified by IHC has provided the first evidence that even highly homologous PCV2 isolates may vary significantly in virulence. Current PCV2 vaccines are targeted against the more historical PCV2a isolate, while the majority of PCVAD cases in Kansas have been associated with the PCV2b genotype. This is important because, given the high prevalence of PCV2a seropositive healthy pigs within the U.S. swine population and

the severe clinical disease associated with PCV2b infection, naturally occurring cross-protective immunity is not predicted. This trial presented the first known opportunity to test a commercial vaccine against known PCV2b infection and associated disease. The decrease in mortality combined with a decrease in virus load after vaccination demonstrate the presence a significant level of cross-protection.

Conclusions and Implications

1. The recent outbreak in PCVAD is associated with the emergence of PCV2b. Severe PCVAD in a group of Kansas herds was a disease problem not previously encountered in this region. Clinical disease pattern was present in herds negative for PRRSV, a cofactor frequently linked to PCVAD. The PCV2b genotype was tracked to approximately 70 PCVAD affected herds. The original source of the virus was never identified.

2. Several differential diagnostic assays were developed that can distinguish PCV2a from PCV2b. We incorporated sequence differences between PCV2a and PCV2b to develop genotype specific assays. These assays are being used by the Kansas State Veterinary Diagnostic Laboratory to track infections in the field. The assays are available for use by other diagnostic laboratories.

3. A variety cofactors are implicated in the etiology of disease. Over the course of the study we identified a “zoo” of bacteria and viruses. The importance of cofactors in the control of the disease remain unknown. It appears that the effectiveness of PCV2 vaccines means that cofactors for the present time are not important.

4. Vaccines are effective in controlling the disease. The results from field studies demonstrated that vaccination reduces mortality, morbidity and virus load; while increasing PCV2 antibody levels and significantly improving weight gain. These types of studies place a clear economic benefit to vaccination. Vaccines are also proving helpful in re-defining the disease.

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Can Porcine Circovirus Type 2 (PCV2) vaccination be used to aid elimination of PCV2 from infected swine
herds? S. S. Dritz, B.E. Straw, R.O. Bates, R.D. Hesse, J.C. Nietfeld, S.C. Henry, and L.M. Tokach.
National Pork Board. \$102,600.

Porcine Circovirus Type 2 elimination study: Baseline diagnostic data. S.S. Dritz, K. Horlen, R.D. Hesse, R.R.R.
Rowland, J.C. Nietfeld, B.E. Straw, R.O. Bates, S.C. Henry, and L.M. Tokach. 2007 to 2008. Ag Experiment
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