RESEARCH REPORT
SWINE HEALTH

Title: Etiology of severe form of PMWS – NPB #06-094

Investigator: Kelly M. Lager, DVM, PhD

Institution: National Animal Disease Center USDA-ARS, Ames, Iowa

Date submitted: September 27, 2008

Industry Summary

The objectives of the project "Etiology of severe form of PMWS" were 1) Investigate the etiology of an apparent emerging disease complex known as "severe form of Post-weaning Multisystemic Wasting Syndrome (PMWS)," and 2) Evaluate the feasibility of applying DNA-microarray technology for use in veterinary diagnostics. For objective 1, tissue samples collected from field cases of the severe form of PMWS were tested for viruses. As would be expected, a variety of swine viruses were detected in the sick pigs; however, one virus, porcine circovirus type 2 (PCV2), was found in all sampled pigs. Genetic analysis revealed the pigs were infected with a European-like PCV2, this was the first time the European-like virus had been found in the United States. How this virus came to the United States, and to what extent it played a role in the severe form of PMWS was not clear. To investigate further, germ-free pigs were inoculated with the European-like PCV2 and North American-like PCV2 viruses, both of these virus types were found in the same farm in one case. Under the conditions of the germ-free pig experiments, the effect on pigs was similar for both viruses. From an experimental perspective, there was no clear indication as to the clinical significance of the European-like PCV2 appearing in the United States. Additional study is required to determine if the European-like PCV2 is clinically distinct from the North American PCV2, a distinction that might contribute to the emergence of the new virus in the United States. For objective 2, a new technology designed to detect all known virus families was used for diagnostic purposes for some of the field cases described above. In addition to viruses detected by traditional methods, a novel swine virus was detecetd. Efforts are underway to fully characterize this virus at a genetic level as well as to determine if the virus is a pathogen in swine, or just another interesting virus that has been detected in pigs.

III. Scientific Abstract:

The objectives of the project "Etiology of severe form of PMWS" were 1) Investigate the etiology of an apparent emerging disease complex known as "severe form of Post-weaning Multisystemic Wasting Syndrome (PMWS)," and 2) Evaluate the feasibility of applying DNA-microarray technology for use in veterinary diagnostics. For objective 1, tissue samples collected from field cases of the severe form of PMWS were tested for viruses. As would be expected, a variety of swine viruses were detected in the sick pigs; however, one virus, porcine circovirus type 2 (PCV2), was found in all sampled pigs. Genetic analysis revealed the pigs were infected with a European-like PCV2, this was the first time the European-like virus had been found in the United States. How this virus came to the United States, and to what extent it played a role in the severe form of PMWS was not clear. To investigate further, germ-free pigs were inoculated with the European-like PCV2 and North American-like PCV2 viruses, both of these virus types were found in the same farm in one case. Under the conditions of the germ-free pig experiments, the effect on pigs was similar for both viruses with no clear indication as to the clinical significance of the European-like PCV2 appearing in the United States. Additional study is required to determine if the European-like PCV2 is distinct from the North American PCV2 and this would contribute

to the emergence of the new virus in the US. For objective 2, a new technology designed to detect all known virus families was applied for diagnostic purposes for some of the field cases described above. In addition to viruses detected by traditional methods, a novel swine virus was detecetd. Efforts are underway to fully characterize this virus at a genetic level as well as to determine if the virus is a pathogen in swine, or just another interesting virus that has been detected in pigs.

IV. Introduction

The emergence of new diseases is a very active process. For example, from 1980 to 2005 thirty-five new diseases affecting man have been described, an average of one every 8 months[1]. Likewise, the emergence of new swine diseases is dynamic as can be seen when looking at what has happened in the last 20 years. Two new swine viruses, porcine reproductive and respiratory syndrome virus (PRRSV)[2] and porcine circovirus type 2 (PCV2)[3], have emerged and 2 known viruses mutated to produce swine influenza A H3N2 virus (H3N2 SIV)[4] and porcine respiratory coronavirus (PRCV)[5]. All four viruses are responsible for significant economic loss. In addition to these viruses, there are newly discovered porcine viruses (porcine species of rubulavirus [Blue-Eye virus][6], sapovirus[7], norovirus[8], torovirus[9], hepevirus[10], annellovirus[11], and alphavirus[12]) that may or may not be associated with disease in swine. Lastly, there are the zoonotic viruses from the *Paramyxoviridae* family, porcine rubulavirus (Menangle virus[13]) and henipaviruses (Hendra virus[14] and Nipah virus[15]) that can cause severe disease in swine and humans. Although recognition of some of these new viruses may be related to the improvement of diagnostic methods that have just "discovered" old viruses, clearly, PRRSV, PCV2, PRCV, and H3N2 SIV are new viruses that have truly emerged in North America. Based on this history, it seems reasonable to assume there will be new swine pathogens in the future. A case in point was the swine health crisis in North America known as the severe form of postweaning multisystemic wasting syndrome (PMWS). It was first recognized in Canada during the winter of 2004/2005 as a syndrome characterized by pigs becoming anorexic and listless 4-6 weeks after they were placed in finishers. Affected pigs progressed to a state of cachexia with many of them dying. Diagnostic investigations found the usual pathogens, but this syndrome had a clinical character different than the diseases attributed to these pathogens. These differences fueled speculation that a new pathogen had emerged, or there had been a mutation in an "old" pathogen that led to this apparent new syndrome. Initial diagnostic investigations consistently identified PCV2 in these affected herds indicating this virus may have had some role in this syndrome. Based on a consistent finding of PCV2 in affected pigs, this syndrome became known as severe PMWS. Not surprisingly, this assumption was met with much doubt since PCV2 is a ubiquitous virus in North America, and essentially all affected herds were PCV2 positive prior to the epidemic. In the second half of 2005 epidemics of disease resembling those seen in Quebec were observed in finishing barns in the United States. The initial outbreaks were reported in Kansas and North Carolina and the epidemic spread to most swine dense regions in the United States. Anecdotal reports described mortality rates surpassing 50% in some finisher barns. As in Quebec, diagnostic investigations were identifying many known pathogens including PCV2. Investigations at NADC studying tissues collected from affected swine in the United States revealed for the first time in North America a European-like genotype now known as PCV2b[16]. These viruses had a very high homology and are distinct from the North American PCV2 genotype (known as PCV2a). Investigations into the Canadian cases also consistently found PCV2b in affected herds. The finding in North America of an apparent new PCV2 genotype associated with a newly described syndrome implied this virus had some causal effect for the syndrome, and it was different from the North American genotype. This report describes studies designed to evaluate this possibility.

V. Objectives:

1. Investigate the etiology of an apparent emerging disease complex known as "severe form of Post-weaning Multisystemic Wasting Syndrome (PMWS)."
2. Evaluate the feasibility of applying DNA-microarray technology for use in veterinary diagnostics.

VI. Materials and Methods:

Objective 1. A study was conducted to compare the pathogenic effects of the PCV2a and PCV2b genotypes using germ-free pigs and virus derived from infectious clones. Germ-free pigs were derived and maintained in sterile isolators for the duration of the experiment. Twenty-eight pigs were used with 4 pigs per group/isolator. At 7 days-of-age, a 1 ml volume of three different doses of PCV2a challenge virus was given to pigs by the oronasal route; Isolator 1 received

undiluted stock virus, Isolator 2 a 100-fold dilution and Isolator 3 a 10,000-fold dilution of stock virus. Isolators 1, 2 and 3 will be referred to as PCV2a-H (high dose), PCV2a-M (medium dose) and PCV2a-L (low dose), respectively. Similarly, pigs in Isolators 4, 5, and 6 were inoculated with 1 ml of undiluted, a 100-fold dilution, or a 10,000-fold dilution of PCV2b challenge stock virus, respectively. Isolators 4, 5, and 6 were labeled PCV2b-H (high dose), PCV2b-M (medium dose) and PCV2b-L (low dose), respectively. Pigs in Isolator 7, the negative control isolator, received 1 ml of cell-culture medium only. One-half of the pigs were scheduled for necropsy at 28 days-post-inoculation (dpi) and the other half at 41 dpi. Blood samples were collected in EDTA tubes at 14, 19, 22, 26, 29, 33, 36, and 41 dpi to test for viremia by real-time PCR [17] and PCV2 specific antibody by ELISA [18]. At necropsy, a bronchoalveolar (lung) lavage (BAL) was collected, and sections of lung, liver and assorted lymph nodes were collected for formalin fixation and fresh tissue storage at -80^0 C.

Lesion examination

Macroscopic lesions were evaluated and scored, ranging from (-) to (+++), with (-) being normal and (+++) being most severe and diffuse [19,20]. Tissue sections were formalin-fixed and processed in a routine fashion. Microscopic lesions were examined and scored by a pathologist blinded to the treatment groups using the same scoring system for macroscopic lesions. Immunohistochemical detection of PCV2-specific antigen was performed on formalin-fixed and paraffin-embedded lymphoid tissue sections of superficial inguinal lymph nodes and livers of selected pigs using a rabbit polyclonal antiserum [21].

Bacterial and viral detection assays

Gel-based PCR was used to test individual pig samples for viruses capable of transplacental infection which included: porcine reproductive and respiratory syndrome virus (PRRSV) [22], bovine viral diarrhea virus (BVDV) [23], porcine parvovirus (PPV) [24], PCV2 [25] and porcine hepatitis E virus (PHEV) [26]. At weekly intervals and at necropsy, rectal swabs were collected from each pig and grouped together by isolator. The swabs were tested for aerobic and anaerobic bacteria using routine microbiologic methods. Rectal swabs were not used for any virus detection. Real time PCR was performed on necropsy serum samples to verify PCV2 viremia and quantification of virus. Real time PCR was also performed on BAL fluid (BALF) samples for quantification and comparison between dose groups. DNA was extracted from serum samples taken at necropsy using a commercial DNA isolation kit (QIAmp® DNA Blood Mini Kit, Qiagen, Valencia, California). PCR was performed as described previously [17].

Serology

Necropsy serum samples were tested for the presence of antibody to select viruses. A commercially available ELISA (HerdChek PRRS 2XR, Idexx Laboratories, Inc. Westbrook, Maine) was used to test for PRRSV antibody. A hemagglutination inhibition test was used for PPV antibody [27]. An ELISA for PCV2 was performed using recombinant ORF2 capsid protein of PCV2 [18], and samples were considered to be positive if the calculated sample-to-positive (S/P) ratio was 0.2 or greater, and suspect with S/P ratios between 0.12 – 0.2. Complete blood cell counts (CBC) were performed on whole blood samples to count the number of two specific white blood cell populations, the lymphocytes and neutrophils.

Statistical analysis

Macroscopic and microscopic lesion scores, CBCs, and $\log_{10}$ transformed PCV2 PCR genomic copies/ml were analyzed using analysis of variance (ANOVA) with a p-value ≤ 0.05 considered significant (JMP, SAS Institute, Cary, NC). Response variables shown to have a significant effect by dose group were subjected to pair-wise comparisons using the Tukey-Kramer test.

Virus and cell culture

PCV2a or PCV2b virions were recovered from the same pig in one of the 2005 United States outbreaks described above [16]. The sequenced viral genomes were deposited in GenBank (accession numbers DQ629114 and DQ629115 for PCV2a and PCV2b, respectively). Each viral genome was inserted into the EcoR1 restriction enzyme site of the Bluescript plasmid (Strategene). After the PCV2 genome was excised from the Bluescript plasmid and circularized by ligation, the ligated DNA mixture was transfected into PK15 cells which had been seeded at approximately 60-80% confluency as previously described [28]. The ligated DNA mixture was replaced with fresh growth medium after 5 hours. After 1 week, the transfected cultures were frozen and thawed 3 times to prepare viral stocks. All inoculums were passed through a 0.45 micron filter and the titer of each virus stock was determined on fresh PK15 monolayer cells by

immunohistochemical (IHC) staining as previously described [9] with each foci of stained cell(s) reported as an IHC unit. The number of genomic copies in challenge viruses was determined using a previously reported real-time PCR assay with modifications [17].

Objective 2. To test the feasibility of a new diagnostic concept, selected samples collected from the field that have or were suspected of having viruses in them were submitted for analysis by DNA microarray technology. The samples were submitted to the laboratory of Dr. Wang, Washington University, St. Louis, MO. The goals of this study were to determine normal background "noise" for swine in this assay, and to determine if the assay could detect common swine viruses.

VII. Results:

Objective 1:

PCV2 virus

Titration of the PCV2a undiluted stock virus was 8.3×10^4 IHC units/ml and 13.1 $\log_{10}$ genomic copies/ml. Titer of PCV2b undiluted stock virus was 8.5×10^3 IHC units/ml and 12.0 $\log_{10}$ genomic copies/ml. PCV2 recovered from PCV2a and PCV2b challenged pigs was identical to the respective challenge virus.

Clinical signs, lesions, and mortality

All control pigs were normal in appearance and behavior for the duration of the study. No macroscopic lesions were observed at necropsy. No microscopic lesions were found in any tissues examined. Some of the pigs challenged with PCV2a and PCV2b virus were clinically affected. One PCV2a-H, three PCV2a-M, and one PCV2a-L pigs, and two PCV2b-H, three PCV2b-M, and one PCV2b-L pigs either died or were euthanized prior to scheduled necropsy times. The remaining 13 pigs were alive and normal in appearance at the scheduled necropsy time. However, some of these pigs had macroscopic lesions and most of them had microscropic lesions in one or more tissues. In general, all clinically-affected PCV2a and PCV2b pigs exhibited similar clinical signs and lesions with some individual variation. The first clinical signs observed were anorexia and listlessness that was frequently followed by dyspnea within a few hours. The dyspnea rapidly became severe and was followed by death within 12 hours if the pigs were not euthanized. The most frequently observed macroscopic lesions in the pigs that succumbed to infection consisted of pneumonia with interlobular edema, enlarged tracheobronchial lymph nodes, peritoneal and thoracic effusions that were cloudy and icteric, and gelatinous edema around the kidneys, base of the heart, and the mesentery of the spiral colon. Less frequently, hemorrhages were seen along the renal cortico-medullary junction and the liver was discolored. Some PCV2a and PCV2b infected pigs were normal in appearance at necropsy, but had similar macroscopic lesions, although less severe, when compared to clinically-affected pigs. Microscopic lesions were found in a variety of tissues; the most significant lesions were interstitial pneumonia, a loss of distinct lymphoid follicles with depletion of lymphocytes in all lymph nodes examined (Fig. 2a), and severe vacuolar degeneration and necrosis of hepatocytes with multifocal areas of hemorrhage (Fig. 2b). Abundant PCV2 antigen was observed in tissues of the affected pigs (Figures 2a and 2b).

Bacterial and viral analysis

No aerobic or anaerobic bacteria were isolated from the rectal swabs collected from any of the pigs in either experiment. All samples collected from control pigs in both experiments tested negative for PCV2 by real-time PCR. In addition, all control pigs tested negative for PPV, PRRSV, PHEV nucleic acid.

Based on the quantitative PCR assay, all PCV2a and PCV2b -inoculated pigs developed a viremia post challenge. At 14 dpi all PCV2a pigs were viremic with genomic copies ranging from 3.3 to 8.7 $\log_{10}$/ml of plasma. At necropsy, titers ranged from 5.7 to 12.6 $\log_{10}$ genomic copies/ml of plasma. BALF titers of the infected pigs at necropsy ranged from 7.4 to 12.6 $\log_{10}$ genomic copies/ml. For PCV2b pigs, at 14 dpi, all PCV2b-M and PCV2b-H pigs were viremic with genomic copies ranging from 5.9 to 8.8 $\log_{10}$/ml of plasma. PCV2 nucleic acid was not detectable in any of the PCV2b-L pigs until 19 dpi. At necropsy, all PCV2b pigs were viremic with titers ranging from 5.6 to 10.5 $\log_{10}$ genomic copies/ml of plasma. All PCV2a and PCV2b pigs tested negative for PPV, PRRSV, PHEV and BVDV nucleic acid.

Serology.

All samples collected from control pigs in both experiments tested negative by PCV2 ELISA (data not shown). In addition, all control and inoculated pigs tested negative for PPV and PRRSV antibody.

At the time of euthanasia only 6 of the 13 non-clinical pigs in experiment 2 developed antibody to PCV2; one PCV2b-H pig became positive by 26 dpi and one PCV2b -M pig by 41 dpi. One PCV2b -L pig became suspect positive by 41 dpi. Two PCV2a -H pigs became positive by 26 dpi and a third PCV2a-H pig seroconverted by 28 dpi. Only one PCV2a-L pig became positive at 41 dpi.

In PCV2-infected pigs a reduction in the lymphocyte population was detected at the 14 dpi bleeding that lasted until the end of the study. In contrast to the decrease in lymphocytes, there was an increase in the neutrophil population detected about a week later that was more pronounced in clinically-affected pigs. There was a direct relationship between the increasing neutrophil count and severity of sick pigs with the sicker a pig became the more likely it had a significant increase in neutrophil counts.

Objective 2:
The assay utilized by Dr. Wang could detect PRRSV, PCV2, swine influenza virus (but not subtype the influenza viruses) in nucleic acids extracted from diagnostic samples. In addition, the technology may have identified a novel virus.

VIII. Discussion:

In the study reported here 100-fold virus dilutions were used to evaluate a dose-dependent clinical effect of PCV2a and PCV2b in germ-free pigs. The dilutions were selected with the anticipation of demonstrating clinical differences; thus the highest dose of inoculum (undiluted stock) was given followed with 100 and 10,000 fold dilutions. Back titration of the challenge viruses indicated there was about a 10 fold difference in the IHC titer of the undiluted stock of PCV2a and PCV2b challenge viruses. The 10 fold difference between PCV2a and PCV2b challenge virus was confirmed with quantitative PCR (Table 1). Although the quantitative PCR assay that we use may give us an accurate count of genomic copies, it does not give us any insight into how many infectious units are in the challenge dose. Moreover, we did not thoroughly rinse the cell culture following the transfection step resulting in a virus preparation that contains not only nascent virus, but may include any excess DNA that was used in the transfection process. Although the PCV2b-L challenge dose per pig contained less than one cell culture infectious unit (about 0.85 IHC units), it is clear the challenge inoculum contained at least one pig infectious dose (PID) since the pigs did become infected. How many PID of virus were in the PCV2b-L inoculum is not known, but we can presume the PCV2b-H dose contained at least 10,000 PID. Assuming pigs are equally susceptible to PCV2a challenge virus, the PCV2a-H inoculum contained at least 100,000 PID/ml. The relationship between a PID unit and an IHC unit is unknown due to the low sensitivity of the IHC assay for quantitative purposes. A major factor contributing to low sensitivity is the relatively low percentage of PK-15 cells used in the IHC assay that are permissive for PCV2 growth. Perhaps a recently described PK-15 cell line that has been selectively cloned for enhanced replication of PCV2 would increase the sensitivity of the IHC assay [28].

Based on serum virus load, during the early stages of infection there were significant differences between both low-dose challenge groups and their respective medium- and high-dose groups. This apparent difference was not recognized during the later stages of infection. However, there was a trend for such a response based on the onset of clinical disease at 21dpi in the PCV2a and PCV2b high and medium dose groups compared to the onset of clinical disease in the PCV2a-L and PCV2b-L groups at 34 and 37 dpi, respectively. Likewise, 4 of the 6 pigs that seroconverted by necropsy time were in the high dose challenge group. Although there may be dose-dependent differences as to when clinical disease begins, the character of the disease was similar among all treatment groups. Since the PCV2a and PCV2b groups received a different dose of challenge virus, we cannot make a direct comparison of the pathogenicity of these viruses. However, since the severity and magnitude of clinical disease in the latter stages of the experiment were similar between the two groups, it seems there were minimal differences between the viruses under the conditions of this study.

It has been proposed the potential of PCV2 to cause disease can be predicted from the quantity of detectable virus in serum or tissue by quantitative PCR. Conventional pigs with serum values greater than 10^7 genomic copies per milliliter are more likely to experience severe PCVAD/PMWS compared to pigs with values at 10^5 genomic copies or less [29,30]. Interestingly, this relationship was observed in this study in which animals that were clinically affected had on average a greater virus load than pigs that appeared clinically normal at the time of necropsy. Moreover, when the clinically normal pigs were separated into two groups, one that had seroconverted and one that did not, there is a significant difference in the serum virus load at 36 and 41dpi, and when all necropsy samples were compared. Pigs that seroconverted had fewer genomic copies in their serum versus those that did not seroconvert. A similar relationship was found in the BALF. In the studies reported here, the virus load in germ-free pigs is considerably greater than what has been reported in conventional pigs experimentally-infected with PCV2. Some of this difference may be attributed to different assays and methodology, but we believe most of the difference is due to the unique physiology of the germ-free pig. In the case of

PCV2 infection, it is clear that germ-free pigs exacerbate any clinical signs that might be seen in like-infected conventional pigs, thus it is likely the virus load would be higher in germ-free pigs as well.

Under these experimental conditions, severe disease followed by death was induced by experimental infection of germ-free pigs with PCV2a and PCV2b derived from a 2005 U.S. case of the high mortality disease syndrome in finishing swine. Our observations are in contrast to previous germ-free pig studies in which little, if any, clinical disease was observed following infection with only a PCV2a isolate [31]. However, a coinfection of PCV2a with PPV [32], or an immune stimulation event (keyhole limpet hemocyanin in incomplete Freund's adjuvant) in conjunction with PCV2a infection [33] induced clinical signs and mortality in germ-free pigs that were similar to what we observed. Those studies led to the hypothesis that clinical disease following PCV2 infection is dependent on a co-factor; e.g. a viral infection or immunostimulation [33]. In a recent report severe disease was induced in gnotobiotic pigs following a coinfection of PCV2a with torque teno virus (TTV), a newly discovered virus of swine [34]. Collectively, the clinical disease and lesions found in pigs that were coinfected or had immune stimulation were very similar to what we observed in the present animal study.

Except for TTV, we did not detect any infectious agents other than PCV2 challenge virus in PCV2-inoculated pigs. The pigs did not knowingly receive any immunostimulating event, and were maintained in a sterile isolator for the duration of the experiment. Although TTV was detected in several of the isolator pigs at the beginning of the study indicating they had a congenital TTV infection, there was no relationship between clinical disease and a congenital TTV infection in pigs given PCV2 challenge virus. Since the viruses used in this study were derived from cloned PCV2a and PCV2b DNA, we presume the clinical effects were attributed to the rescued viruses used in the experiments and not extraneous viruses.

It is possible that some aspect of the routine husbandry for raising germ-free pigs provides an immunostimulating event, and thus the necessary co-factor to induce clinical disease. However, based on previous germ-free pig experiments, no significant up-regulation of the immune system has been recognized in germ-free pigs that did not receive colonizing bacteria or a PRRSV infection [35]. Since the hypothesis for PCV2 inducing disease is based on synergism between PCV2 and a cofactor, by definition no clinical event would be detected in germ-free pigs in which the cofactor was present in the absence of PCV2 infection. The milk diet prepared from cow's milk that has been formulated for small mammals might be considered immunostimulatory to the pig, thus providing a cofactor for the PCV2-infected pig. We believe the probability of the milk diet acting as an immunostimulation event is very low based on previous germ-free pig experiences described above and unpublished observations.

Results from this animal study demonstrate the PCV2b virus is pathogenic in a germ-free pig model. Collectively, the germ-free-pig lesions mimic what has been reported in North American field cases following the onset of the high mortality syndrome in 2004/2005. This observation supports the role of PCV2 in this disease. However, this experimental work does not explain why an apparent new PCV2 genogroup (PCV2b) to North America seems to have spread through high-density pig areas like an emerging infectious agent [16, 36, 37]. Likewise, it is not clear why some experimentally-infected pigs die and some appear unaffected by the same virus challenge. Additional studies are necessary to determine if these two viruses are representative of PCV2a and PCV2b viruses circulating in the pig populations, and the clinical significance of the congenital TTV infections.

Results from Objective 2 demonstrate the pan-viral detection assay could detect some swine viruses in diagnostic samples, however, the sensitivity of the test for each virus has not be determined. As part of these studies a novel porcine virus was detected. We are in the preliminary stages of characterizing this virus and trying to determine the prevalence of it in US swine.

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Table 1: Quantity of virus inoculum used in each of 2 experiments
based on immunohistochemistry units/ml and real-time PCR genomic copies/ml

Experiment	PCV2 Dose Group	IHC units/ml	PCR PCV2 copies/ml [a]
1	PCV2a-H	8.3×10^4	13.1 [c]
	PCV2a-M	---	11.2
	PCV2a-L	---	9.2
1	PCV2b-H	8.5×10^3	12.0
	PCV2b-M	---	10.0
	PCV2b-L	---	8.1

a – log base 10 values, b – not tested, c – virus and plasmid quantification.