Effect of vaccination against porcine circovirus type 2 (PCV2) on ejaculate characteristics and the shedding of virus in boar semen

by

Kyle A. Alberti

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Mark J. Estienne, Chair
Allen F. Harper
Xiang-Jin Meng

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ABSTRACT

Research has demonstrated that porcine circovirus type 2 (PCV2) can be shed into boar semen, raising the possibility that artificial insemination may be an important route by which disease associated with PCV2 is transmitted.   The objective of this experiment was to determine the effect of vaccination against PCV2 on ejaculate characteristics and PCV2-specific antibody titers in serum of PCV2-positive boars viremia and viral shedding in semen. Semen and blood samples were collected weekly from week 0 to week 8. After collections at week 0, boars were vaccinated with a commercial vaccine against PCV2 (n = 5) (Suvaxyn PCV2 One dose; Fort Dodge Animal Health, Fort Dodge, IA) or served as controls and received 2 ml 0.9% saline (n = 5).   Sperm concentrations and characteristics of sperm motility were assessed using a computer-assisted sperm analysis system (Hamilton Thorne Research, Beverly, MA) and sperm morphology was evaluated after staining using light microscopy. The PCV2 antibody titers were determined in serum using an ELISA (Iowa State Veterinary Diagnostic Laboratory; Ames, IA). The genomic copy number of PCV2 DNA in serum and semen was determined by PCR (Iowa State Veterinary Diagnostic Laboratory; Ames, IA).   There were no effects of treatment or treatment by week on semen characteristics ($P > 0.05$).   An effect of treatment by week was detected for serum antibody titers ($P < 0.01$).   Compared with controls, antibody titers in vaccinated boars tended to be greater at week 0 (1.13 ± 0.05 titer/ml vs 1.01 ± 0.05 titer/ml; $P = 0.09$) and were greater at week 2 (1.15 ± 0.05 titer/ml vs 1.01 ± 0.05 titer/ml; $P < 0.05$) but lesser at week 7 (1.01 ± 0.05 titer/ml vs 1.23 ± 0.05 titer/ml; $P < 0.01$) and tended to be lesser at week

8 (1.05 $\pm$ 0.05 titer/ml vs 1.17 $\pm$ 0.05 titer/ml; $P = 0.07$). There were no effects of treatment, week, or treatment by week for serum genomic copy number of PCV2 DNA ($P > 0.1$). An effect of week was detected for semen genomic copy number of PCV2 DNA ($P < 0.04$). During week 3, PCV2 genomic copy number was at its greatest numerical value, however, semen PCV2 genomic copy number was at its lowest point. This was followed by an increase in semen PCV2 genomic copy number during week 7. This increase could be related to the increase in viral shedding in the serum. In summary, vaccination against PCV2 can lower antibody titers when given post-infection and has no effect on indicators of semen fertility. Vaccination also can decrease the length of reoccurring infection by decreasing the length of viral shedding in serum.

ACKNOWLEDGMENTS

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I.

INTRODUCTION

Porcine circovirus type 2 (PCV2) is a problem to the swine industry and can lead to significant negative impacts on the profitability of pork production. In 2005, the prevalence of PCV2 in countries with antibody results ranged from 30% to as high as 93% in Mexico (Ramamoorthy and Meng, 2008). PCV2 can lead to wasting in pigs after weaning and death in the nursery. Syndromes associated with PCV2 are known as porcine circovirus associated diseases (PCVAD) and include: postweaning multisystemic wasting syndrome, PCV2-associated enteritis, PCV2-associated pneumonia, PCV2-reproductive failure, porcine dermatitis and nephropathy syndrome, and PCV2-associated neuropathy.

In the swine industry, artificial insemination (AI) is widely practiced as a means of breeding sows and gilts. Benefits of AI include more extensive use of genetically superior boars and a decrease in the number of boars kept for breeding. As of 2001 it was estimated that approximately 60% of swine herds in the United States utilized AI following an upward trend in Europe where greater than 90% of swine herds use AI (Madson et al., 2009a). This extensive use has risks which include the shedding of bacteria and viruses into semen and possible infection in females mated by AI. Indeed, research has shown that PCV2 can be shed into semen and can cause disease in inseminated females. Recently, vaccines have been developed against PCV2 and are currently in use in the swine industry. In this thesis, the effect of vaccination against PCV2 on serum antibodies and PCV2 genomic copies in serum and semen, in previously PCV2-infected boars, was examined.

CHAPTER II.

REVIEW OF LITERATURE

Porcine Circovirus Type 2

Introduction

Porcine circovirus (PCV) belongs to the family *Circoviridae* and is a small, non-enveloped DNA virus. The PCV was first discovered as a contaminant of the porcine kidney PK-15 cell line and was later named PCV1 (Tischer et al., 1974). This variant was shown to be nonpathogenic though it was demonstrated to infect the lungs and spleen (Allan et al., 1995). The PCV2 variant was first described in 1998 when the virus was associated with a new syndrome in pigs, later termed postweaning multisystemic wasting syndrome (PMWS), though the presence of PCV2 can be traced back to 1969 in Belgium (Allan et al., 1998; Opriessnig et al., 2007). The PCV2a and PCV2b subtypes have circular genomes that are 1768 base pairs and 1767 base pairs, respectively, in size. The genomes are single stranded with only two known functional genes, the rep and capsid genes (Hamel et al., 1998; Mankertz et al., 2004). The PCV2a is found predominantly in North America, whereas PCV2b is found in Europe, Asia, and North America. Currently PCV2b is the predominant subtype in swine herds worldwide. It has been reported that there is no difference in pathogenesis between the PCV2a and PCV2b subtypes (Gagnon et al., 2007; Gillespie et al., 2009).

Effects of PCV2 on the swine industry

Based on price data from April 1, 2010 the average net live price for market hogs was $53.76/cwt with a range of $45.89/cwt to $57.00/cwt. This average price would result in a value of $134.40 for the average hog sold at market (USDA Market News Service, 2010). At this level, a producer is making a small profit per hog sold. During an outbreak, porcine circovirus

associated diseases (PCVAD) have cost producers on average three to four dollars per pig, with the highest losses approaching $20 per pig (Gillespie et al., 2009). A loss of three to four dollars per hog is significant and makes PCVAD a significant threat to the profitability of pork production. Specific effects of PCV2 outbreaks, including increased mortality, will be addressed later. This disease has led to changes in management and the development of vaccination programs focused on limiting deleterious effects on the swine industry (Gillespie et al., 2009).

Porcine circovirus associated diseases

Postweaning multisystemic wasting syndrome in pigs is now known as PCVAD. A position statement by the American Association of Swine Veterinarians (AASV) stated that the name PCVAD was adopted to recognize the variability in clinical symptoms that are observed in pigs exposed to PCV2 (AASV, 2006a). Porcine circovirus associated diseases are inclusive of all syndromes currently associated with PCV2 infection. According to the AASV case definition, PCVAD can be subclinical or have several different clinical manifestations. These include: multisystemic disease with weight loss, high mortality, respiratory disease, porcine dermatitis and nephropathy syndrome, enteric signs including diarrhea, and reproductive disorders including abortions, stillbirths, and fetal mummification. Histopathological findings in infected pigs include: depletion of lymphoid follicles in lymphoid tissues, disseminated granulomatous inflammation in one or more tissues, detection of PCV2 within lesions, or the detection of PCV2 antigen in fetal myocarditis lesions for diagnosis of PCV2 associated reproductive disease (AASV, 2006b).

Postweaning multisystemic wasting syndrome

Postweaning multisystemic wasting syndrome is the most prominent clinical sign of PCVAD. This syndrome initially affects nursery pigs at approximately 4 to 12 weeks of age

with symptoms persisting into the grower phase of production. The acute phase of this syndrome can persist in a herd for an extended period of time. Results from a case herd suggest that increased postweaning mortality can persist in the nursery for up to 16 months following initial clinical disease signs and peak at approximately 9 months from initial clinical signs compared to pre-outbreak postweaning mortality (Harding et al., 1998; Allan and Ellis, 2000). The most common clinical signs associated with PMWS are: wasting/unthriftiness, dyspnea, enlarged lymph nodes, diarrhea, pallor, and jaundice. Jaundice is the least frequently occurring of the clinical signs (Harding and Clark, 1997). Gross pathologic findings at necropsy that are associated with PMWS include: moderate pallor of the skin, marked enlargement of all lymph nodes, a yellowish-orange liver with mild to moderate molting, either zero to grossly enlarged lesions on the kidneys, enlarged spleen, fluid filled intestines, and ulcers and gastric wall edema in the stomach. Also, the lungs appear diffusely noncollapsed, palpably firm and rubbery. In severe cases, scattered, large reddish-brown areas in the lungs are also noticeable (Harding and Clark, 1997). The mortality associated with PMWS is usually around 10%, but can be as high as 50%. The range is usually 4 to 20% (Gillespie et al., 2009). Due to the prolonged clinical course of this disease and the decrease in performance and potential returns, roughly 70 to 80% (as reviewed by Segales and Domingo, 2002) of pigs that exhibited clinical signs of PCVAD are euthanized (Gillespie et al., 2009).

PCV2-associated enteritis

Another syndrome associated with PCV2 infection is PCV2-associated enteritis which resembles infection with *Lawsonia intracellularis* and affects pigs aged 2 to 4 months. Distinguishing the two infections is easy histopathologically, however, the infections have some common features including depletion and necrosis of Peyer's patches and mononuclear cell

infiltration of the lamina propria. Clinically, the pigs exhibit diarrhea, wasting, unthriftiness, and jaundice. Evidence of the PCV2 infection can be seen in superficial and coagulative necrosis of the ileum, colon, or both. The PCV2-associated enteritis is differentiated histopathologically from *Lawsonia intracellularis* infection by a lack of evidence of bacterial infection in the latter (Jensen et al., 2006). In PCV2-associated enteritis, histopathological lesions include granulomatous inflammation and lymphoid depletion in the Peyer's patches of the small and large intestines (Kim et al., 2004).   The severity of these lesions is directly correlated with the amount of viral antigen present (Jensen et al., 2006).

PCV2-associated pneumonia

Porcine respiratory disease complex (PRDC) is a concern, particularly in indoor swine production systems where close contact of swine with each other is inherent. This can lead to a spread of several respiratory pathogens including swine influenza (SIV), porcine reproductive and respiratory syndrome virus (PRRSV), porcine respiratory coronavirus, and *Mycoplasma hyopneumoniae*. These pathogens have been shown to be present in respiratory disease cases of swine (Harms et al., 2002). Coinfection with PCV2 and PCV2 alone have been shown to be a cause of PRDC and proliferative and necrotizing pneumonia (Harms et al., 2002; Szeredi and Szentirmai, 2008). PRDC affects pigs from 8 to 26 weeks of age and has been shown to be associated with these pathogens. This disease is characterized clinically by a decreased rate of growth, decreased feed conversion efficiency, anorexia, fever, cough, and dyspnea. It is also possible that a diagnosis of PCV2 associated systemic infection can be made in conjunction with PCV2-associated pneumonia. Histopathological findings in cases of PCV2-associated pneumonia include granulomatous bronchointerstitial pneumonia with mild to severe necrotizing and ulcerative bronchiolitis and bronchiolar fibrosis. Also, abundant PCV2 antigen in the lesions

is present. It is possible for lesions associated with PCV2 to resemble lesions induced by SIV and porcine respiratory coronavirus (Opriessnig et al., 2007). Proliferative and necrotizing pneumonia is characterized by severe acute pneumonia and intra-alveolar and interlobular edema. Also degeneration and necrosis of bronchiolar epithelial cells have been observed. Pathological findings include firm, non-collapsed lungs and enlargement of the mediastinal lymph node (Szeredi and Szentirmai, 2008).

PCV2-associated reproductive failure

Reproductive failure associated with PCV2 infection was first reported in Canada in 1999 when PCV2 antigen was discovered in myocardial and liver tissue from an aborted pig fetus (West et al., 1999). Clinical signs of the disease might include an increase in the number of abortions, stillbirths, fetal mummifications and an increase in pre-weaning mortality. This disease appears to affect gilts and swine operations with new populations of animals. Lesions commonly associated with this disease are nonsuppurative to necrotizing or fibrosing myocarditis associated with considerable PCV2 antigen (Mikami et al., 2009).

Fetal age appears to play a role in the ability of PCV2 to replicate in the fetus. Sanchez et al. (2001), inoculated fetuses in utero at either day 57, 75, or 92 of gestation. Fetuses were killed 21 days following inoculation with PCV2. Virus replication was significantly higher in fetuses inoculated on day 57 of gestation compared to fetuses inoculated on day 75 or day 92 of gestation. Those fetuses that were inoculated at day 75 or 92 of gestation did not exhibit any gross abnormalities, which was in contrast to the fetuses inoculated at day 57 of gestation. Edema, enlarged livers and congestion along with internal bleeding were observed in all fetuses inoculated at day 57 of gestation. The results of this study also support the heart as the primary

site of PCV2 replication as the number of antigen positive cells was significantly higher in cardiac tissue than in the liver, spleen, kidneys, lungs or lymph nodes.

PCV2 can also cause reproductive problems when fetuses are infected close to the time of farrowing. In a study by Johnson et al. (2002), pregnant sows were inoculated intramuscularly with PCV2 at 86, 92, and 93 days of gestation and only 18 of 37 pigs born alive survived to weaning. One sow lost her entire litter prior to weaning and had three stillborn pigs, two mummified fetuses, five pigs that died within 24 hrs after farrowing, and two pigs that died by day 17 post-farrowing. Although these results show that infection of late term fetuses can cause reproductive problems, data from field cases suggests that reproductive failure associated with PCV2 infection is rare and that most breeding herds are apparently immune, however vaccination could be warranted if replacement animals come from outside the herd (Opriessnig et al., 2007). A case report by Pittman (2008) showed an increase in stillborn and mummified fetuses farrowed by gilts obtained through a new on farm gilt development program compared to gilts obtained from an external source. Gilts from the on farm development program tested positive for PCV2, specifically PCV2b, and negative for PRRSV and also showed clinical signs of PCVAD. Fetal myocardial and lung tissue also tested positive for PCV2 confirming PCV2 as the sole pathogen responsible for the increase in reproductive failure. There is variability among sources of replacement gilts with regard to susceptibility to reproductive failure associated with PCV2 (West et al., 1999; Josephson and Charbonneau, 2001).

Porcine dermatitis and nephropathy syndrome

The first association between porcine dermatitis and nephropathy syndrome (PDNS) and PCV2 infection was reported in 2000 (Rosell et al., 2000). Clinical manifestations of PDNS are skin lesions, fever, and lethargy. The lesions are red to purple discolorations of the skin, which

may or may not be elevated, and occur most prominently on the perineum and back legs of affected pigs (Choi and Chae, 2001). The PDNS can be fatal as quickly as three days after development of lesions, affecting pigs from 5 to 12 weeks of age. Animals that survive typically have the skin lesions regress in two to three weeks after initial appearance (Thibault et al., 1998; Gillespie et al., 2009). At necropsy the kidneys are enlarged with the cortex of the kidneys being pale. Multiple red circular hemorrhagic cortical foci approximately 2 to 4 mm in diameter are present in the kidneys, and lymph nodes are enlarged, which is common with PCV2 infection. The most severe lesions are severe, fibrinoid, necrotizing vasculitis which affected the dermis, subcutis, renal pelvis and medulla of the kidneys (Choi and Chae, 2001). The development of PDNS is aided by coinfection with porcine reproductive and respiratory syndrome virus (PRRSV) (Choi and Chae, 2001), *Pasteurella multocida* (Lainson et al., 2002), along with many other bacteria (Thomson et al., 2002). A more recent study showed that PDNS-like lesions can be experimentally reproduced with PRRSV and torque teno virus and occur without PCV2 coinfection (Krakowka et al., 2008).

PCV2-associated neuropathy

Porcine circovirus type 2 has been associated with congenital tremors affecting newborn piglets. Congenital tremors were shown to be associated with deficient myelin in the brain and spinal cord (Christensen and Christensen, 1956). This lead to the hypothesis that oligodendrocytes were the primary cell type infected with PCV2, as this cell type is associated with the insulation of axons in the brain. Interestingly, in a study by Stevenson et al. (2001), a large number of PCV infected neurons were found throughout the brain and spinal cord. These infected cells were more numerous and widespread throughout the brain in PCV2-infected pigs suffering from congenital tremors than PCV2-infected pigs which were not suffering from

tremors. These results suggest that PCV2 could potentially have another, as of yet unknown, mechanism leading to congenital tremors in piglets which could be in addition to, or separate from, myelin deficiency.

Some more recent studies have demonstrated PCV2 infection is associated with cerebellar vasculitis. This was evidenced by hemorrhages distributed in the meninges and parenchyma in affected pigs from six to eight weeks of age (Seeliger et al., 2007). Clinical neurological signs included apathy, ataxia, paddling, and opisthotonus. Microscopic findings demonstrated large amounts of PCV2 antigen staining in the cerebellar vessels and leptomeninges along with cerebellar hemorrhages and lymphohistiocytic meningitis, both of which were associated with lymphohistiocytic vasculitis and suppurative meningitis (Correa et al., 2007). The role of PCV2 in the development of vasculitis in the brain is still not clear; however this could potentially be a new type of PCV2 infection that has yet to be well-described (Gillespie et al., 2009).

Cell targets of PCV2

PCV2 exhibits pluripotency in the cell types it targets, infecting cells of epithelial, endothelial and myeloid origin. Vincent et al. (2003) reported that PCV2 harbors in the dendritic cells of both monocyte and bone marrow origin but PCV2 does not replicate in dendritic cells. The researchers concluded that the presence of PCV2 antigen in the cytoplasm of dendritic cells was a result of endocytosis of the viral particle. The dendritic cell, as a result of its immune functions, might act as a reservoir for PCV2, as opposed to being infected by PCV2. The long clinical course of PCVAD and the persistent nature of PCV2 infection support the results of this study.

Another study investigated infection and replication in five different cell types including a porcine endothelial cell line, primary porcine aortic endothelial cells, gut epithelial cells, monocyte-derived dendritic cells and fibrocytes. This was the first study to examine fibrocytes with respect to PCV2 infection. The endothelial cell line showed an infection percentage of greater than 90% at seven days post infection. The aortic endothelial cells (29.7%) and gut epithelial cells (41%) showed an infection percentage that was less than the endothelial cell line at seven days post infection. The fibrocytes exhibited the lowest infection percentage at 7 days post infection and was less than 20%. The dendritic cells had a high antigen positivity (97.5%), however this is not believed to be due to infection because UV-inactivated viruses had a similar positivity percentage. This strongly suggests endocytosis as the reason for the high positivity percentage seen in dendritic cells. Also, dendritic cells were the only cell type tested which did not have a detectable level of the rep protein expression and rep protein is required for production of new virons or increasing levels of titers associated with new virus production (Steiner et al., 2008). Fibrocytes are of myeloid origin and function in wound healing (Bucala et al., 1994), antigen presentation (Balmelli et al., 2005), and innate immune responses (Balmelli et al., 2008). These cells did not have a detectable level of the rep protein until 14 days post infection, whereas the endothelial and epithelial cells had detectable rep protein beginning as early as day two post infection and no later than day three post infection. Fibrocytes did, however, have the highest percentage of cells positive for rep protein (11%) compared to less than 3% in the endothelial and epithelial cell groups (Steiner et al., 2008). The difference in the ability of PCV2 to infect and replicate in fibrocytes but not in dendritic cells suggests that there is some mechanism or cell surface difference which allows active PCV2 to enter fibrocytes and initiate replication.

10

The recent interest in xenotransplantation, especially from pigs, has promoted interest in whether PCV2 can infect human cells. Hattermann et al. (2004) tested whether PCV1 and PCV2 can infect and replicate in several different human cell lines. PCV1 and PCV2 were able to infect Cheng liver, 293, Hep2, Hela, and RH cells and those cells also expressed viral antigen. PCV2 induced a cytopathological effect, with loss of PCV2 DNA, two weeks after initial infection. A test was then done to determine if PCV1 and PCV2 could replicate in these cell lines. It was found that PCV1 and PCV2 could replicate in Hep2 and 293 cells, but electron microscopy found no viral particles in these cell lines. This suggests that the positive results of the test were from naked DNA due to the dead cells. Based on these results infection with PCV1 and PCV2 is non-productive and that transmission to humans under natural conditions has a low probability of occurring.

Breed and gender differences in susceptibility to PCV2 lesions

The breed of pig can have an influence on the susceptibility of piglets to PCV2 associated lesions. Landrace swine in particular seem to have an increased susceptibility to PCV2 associated lesions. Opriessnig et al. (2006) used Landrace, Large White, and Duroc swine from a single source and measured viremia duration, amount of PCV2 genomic DNA copies, and anti-PCV2 antibody production. The researchers found no significant differences for these measurements among the breeds suggesting that all the breeds used for this study were equally susceptible to PCV2 infection. Despite the similarities among breeds in these measurements of infection, the observance of clinical disease and the presence of microscopic lesions were significantly different among the breeds. Clinical disease was observed by mild respiratory distress and was measured by mean respiratory scores. Landrace pigs had higher mean respiratory scores during post inoculation day seven through post inoculation day 14, but these

values were not significantly different from the other two breeds. Landrace pigs at post inoculation day 21 had significantly more severe lymphoid depletion scores in the tonsils and at post inoculation day 35 in the lymph node pool when compared with the scores for the Duroc and Large White swine. No Duroc or Large White pigs had microscopic lesions consistent with PMWS, however three (15.8%) of the Landrace pigs exhibited these lesions. This suggests that Landrace pigs have a predisposition to progress from subclinical PCV2 infection to a clinical case of PCVAD.

In a later study comparing Landrace and Pietrain pigs for their susceptibility to PCV2-associated lesions, Opriessnig et al. (2009) found Landrace pigs more susceptible. Pietrain lesion scores were strictly in the normal to mild categories with approximately 65% in the normal category, whereas approximately 31% of the Landrace pigs had scores in the moderate to severe categories. Interestingly, in this study the pigs were exposed to PCV2 at 21 weeks of age, which is late in the production cycle and most likely past the time at which most pigs receive a natural exposure to the virus. At this older age, it would seem that the pigs would have increased resistance to PCV2 and not show lesions as severe as those in younger animals. This was not the case, however, with the Landrace pigs, which suggests that timing of infection has an important role in the severity of the expression of disease and lesions associated with PCVAD (Opriessnig et al., 2009). It is obvious that genetics has a role in susceptibility to PCVAD based on the observation that Landrace swine are more susceptible to PCVAD compared to Large White, Duroc, or Pietrain swine, however, the exact mechanism behind this difference is still unclear.

Evidence has been reported which suggests an effect of gender on susceptibility to PCVAD. Corrégé et al. (2001) found that 38% of the castrated male pigs showed susceptibility to PMWS compared to 29% of female pigs. This difference was perhaps due to the castration

procedure and possible secondary infections that could have been acquired after castration. Rodriguez-Arrioja at al. (2002) also observed a difference in the mortality rates between the males (both intact and castrated) and females, and castration was suggested as a possible risk factor leading to secondary infection though the data for this was not published.

Management practices to protect against PCV2

There are several important management practices that can help to limit PCVAD. The first is appropriate management of animals when PCV2 is known to be a problem on the farm. This includes all in/all out management and limiting pig to pig contact especially between different age groups, minimizing stress, keeping the facilities clean, and maintaining a high level of nutrition (Opriessnig et al., 2007). There are several risk factors for PCVAD which include coinfection with porcine parvovirus (PPV) or PRRSV, more pigs per pen in the nursery, and greater than 15% cross-fostering (Rose et al., 2003). Interestingly, the use of on farm collection of semen and natural mating increased the likelihood of PCVAD as opposed to purchasing semen from an outside source. This is thought to be due to the type of housing of boars on farm compared to the type of housing at a collection facility. Boars at a collection facility are housed individually, whereas boars are often housed with pregnant sows on farm which increases the chance of within-herd circulation of PCV2 (Rose et al., 2003). Based on evidence from 149 herds in France, common factors that decreased the risk for PCVAD were empty periods of five or more days between groups of pigs, regular treatment for external parasites, keeping gestating sows in pens versus crates, and producing replacement gilts in house (Rose et al., 2003).

Another important practice is the use of disinfectants for cleaning buildings and transport vehicles between groups of pigs. In research settings, several disinfectants have been shown to reduce the infectivity of PCV2 following a 10 min exposure period. The disinfectants that were

selected are considered to be in common use on swine operations or in research laboratories. The disinfectants that significantly reduced the infectivity of PCV2 in vitro were Tek-Trol (Bio-Tek Industries Inc, Atlanta, GA), Fulsan (Fuller Brush Company, Great Bend, KS), 1-Stroke Environ (Steris Corporation, Road Mentor, OH), Clorox bleach (Clorox Company, Oakland, CA), Roccal D Plus (Pharmacia and Upjohn, Peapack, NJ), sodium hydroxide, and Virkon S (Antec International, Sudbury, Suffolk, UK). Ethanol, Nolvasan (Fort Dodge Labs, Fort Dodge, IA), DC&R (Hess and Clark Inc, Ashland, OH), and Weladol (Pitman Moore, Mundelein, IL) did not reduce the infectivity of PCV2 in vitro (Royer et al., 2001). The swine research facility at Iowa State University, which has housed animals known to be infected with PCV2, uses the following protocol to disinfect its facilities. Following removal of the animals, a degreaser detergent product is applied to cover the room and pens. This product is applied with a foamer at a dilution of 1:64. This is allowed to sit for 10 minutes, after which the room and pens are rinsed off with hot water using a pressure washer. Once rinsed clean, Virkon S (Antec International, Sudbury, Suffolk, UK), at a dilution of 1:30, is applied and sits for 10 minutes. This is the step where decontamination occurs. The room and pens are then rinsed clean with hot water. Prior to new animals being moved into the room, the room is fogged with Clidox-S (US Pharamacal Com LLC, Erie, Co) at a dilution that is one part base, five parts water, and one part activator followed by allowing the room to dry. The room is then rinsed with water 6 to 12 hours later to decrease corrosion and then is allowed to dry prior to being refilled with pigs. This protocol has been shown to be highly effective at disinfecting the swine research facility at Iowa State University as indicated by the lack of transmission of the disease to animals entering the facility (Opriessnig et al., 2007).

The last good management practice is the prevention of coinfections, which have been shown to play a role in the progression from PCV2 infection to PCVAD. The control and treatment of coinfections in pigs with PCVAD can decrease the severity of the coinfection and even improve the prognosis for the affected pig (Opriessnig et al., 2007). Torque teno virus (TTV) infection can lead to the development of PMWS if the pig is coinfected with PCV2. In a research study by Ellis et al. (2008), half of the pigs that were exposed initially to TTV and then seven days later exposed to PCV2 developed PMWS as early as 18 days following PCV2 infection. Pigs that were singularly infected with either TTV or PCV2 did not develop clinical signs of PMWS. Pigs that were previously infected with PCV2 and then infected with TTV seven days later also did not develop a clinical case of PMWS. The order of infection clearly plays a role in development of PMWS though the mechanism behind this remains unclear. Porcine reproductive and respiratory syndrome virus is another virus for which coinfection can lead to a more progressed disease state. Between 14 and 21 days post-infection with both PCV2 and PRRSV there were significantly higher mean respiratory scores compared to either uninoculated controls, only PCV2 infected, or only PRRSV infected groups. The respiratory scores increased beginning at day four post-infection until day 20 post-infection. Lung lesions and interstitial pneumonia were noted as being similar, though more severe than in the PRRSV-only infected pigs (Harms et al, 2001). A more recent study found that coinfection with SIV, PRRSV, and *M. hyopneumoniae* with PCV2 lead to more severe tissue damage and greater evidence of systemic disease compared to PCV2 infection alone. This effect was greatest during the early to late nursery phase which fits with the timing of PMWS seen in the field (Dorr et al., 2007).

PCV2 vaccines

There are several PCV2 vaccines currently on the market. One of the first was CIRCOVAC® (Merial Com, Lyon, France), which is an inactivated PCV2 vaccine with oil adjuvant and is licensed for use in breeding age animals. This vaccine is primarily used in Europe and Canada and is designed to be administered as two intramuscular injections given three to four weeks apart. The last injection must be given at least two weeks before breeding and sows should be revaccinated at subsequent pregnancies (Opriessnig et al., 2007; Cardinal and Jones, 2008). This protocol was effective in reducing PCV2 circulation and shedding during the first weeks of life of piglets born to the sows vaccinated using CIRCOVAC® (Charreyre et al., 2005). CIRCOVAC® also reduced piglet mortality in PRRSV negative groups of sows from 5.8% (unvaccinated) to 4.0% (vaccinated) during one field trial at a farrow to finish unit (Cardinal and Jones, 2008).

Another available vaccine is Ingelvac® CIRCOFLEX™ (Boehringer Ingelheim Vetmedica Inc, St. Joseph, MO), which is a capsid-based subunit vaccine expressed in inactivated baculovirus. This vaccine is designed to be given as a single intramuscular dose to piglets less than three weeks of age (Desrosier et al., 2007; Gillespie et al., 2009). In one field trial at a farrow to finish unit, piglets vaccinated with this vaccine had reduced mortality (3.3%) compared to unvaccinated piglets (9.7%) (Cardinal and Jones, 2008).

A third vaccine which is available is marketed under two different names. In the United States and Canada this vaccine is marketed as Circumvent PCV (Intervet Inc, Millsboro, DE). In Europe and Asia this vaccine is marketed as Porcilis PCV (Intervet Inc). This vaccine is also a capsid-based subunit vaccine which is expressed in a baculovirus. Vaccination is intramuscularly to piglets three weeks of age or older, but the treatment protocol differs

depending on which of the two market names is used. Circumvent PCV is given intramuscularly as two doses three weeks apart whereas Porcilis PCV is only given as one dose (Gillespie et al., 2009).   Field trials on 21 different Canadian farms that included 35,000 pigs showed a 77.5% decrease in mortality of vaccinated pigs compared to unvaccinated pigs (de Grau et al., 2007a).

The vaccine used in the study discussed later was Suvaxyn® PCV2 One Dose™ (Fort Dodge Animal Health, Fort Dodge, IA), which was the first PCV2 vaccine approved for commercial use in the United States by the United States Department of Agriculture (USDA) (Opriessnig et al., 2007). This vaccine is a killed virus that is chimeric, in that the vaccine is part PCV1 and part PCV2. This chimeric virus was developed to have the PCV2 capsid and the PCV1 genome. The chimeric virus was termed PCV1-2 because of the immunogenic ORF2 capsid protein of PCV2 that induces the immune response in pigs. It was found that this chimeric virus was attenuated in pigs, which is due to the nonpathogenic PCV1 genome (Fenaux et al, 2003). Fenaux et al. (2003) found that the PCV1-2 chimeric virus caused only mild lesions in the lymph nodes of two of seven pigs and the lesion rate was not significantly different from that in pigs treated with the PCV1 virus. The PCV2 virus caused lesions in the lymph nodes which were classified as moderate to severe in eight of eight pigs. The researchers concluded that lesions in the two pigs in the PCV1-2 group were likely non-specific due to the lack of microscopic lesions at necropsy and were not considered to have been caused by the PCV1-2 (Fenaux et al, 2003). Fenaux et al. (2004) performed a study in which pigs were vaccinated with the chimeric PCV1-2 which led to the development of protective immunity against a PCV2 challenge. It was also demonstrated that pigs could be vaccinated effectively by an intramuscular route, as all 12 pigs developed PCV2 antibodies prior to PCV2 challenge. After PCV2 challenge, viremia was detected in nine of 12 unvaccinated pigs, but none was detected in

the vaccinated pigs. Antigen for PCV2 was detected in the lymph nodes (75%), tonsil (67%), and spleen (42%) in unvaccinated pigs, whereas only 1 of 12 pigs (8%) in the vaccinated group had detectable PCV2 antigen in the lymph nodes. These organs are significant in the function of the immune system and it is the infection of lymphoid tissues, which make up these organs, that leads to the pathogenesis of PCVAD. This shows that the PCV1-2 vaccine is capable of significantly reducing the amount of PCV2 virus in the lymphoid tissues and protects against the development of PCVAD (Fenaux et al., 2004).

Conclusion

PCV2 causes PCVAD which is inclusive of several syndromes that affect pigs from the farrowing to the nursery to the grower-finisher phase of production. These syndromes can cause economic hardship on producers by negatively impacting growth performance of pigs, with mortality being as high as 50%, and leading to reproductive problems in the breeding herd. Porcine circovirus type 2 is known to affect immune system cells and this is one of the main problems leading to issues with coinfections. There are management practices which can help control the disease once a farm is infected and limit the effect of PCV2 on overall production. These practices include using appropriate disinfectants, vaccination programs, all-in/all-out production, and identifying infected animals. The vaccines that have been developed can help to limit the horizontal and vertical transmission of disease. This leads to a decrease in mortality, increases in average daily gain, and decreases in the number of cull animals (de Grau et al., 2007b)

Artificial Insemination

Introduction

Artificial insemination (AI) has revolutionized swine breeding operations. In North America, it was estimated that close to 60% of swine herds were using AI as of 2001. This followed the trend in Europe, where estimates were 90% or greater (Madson et al., 2009a). Use of AI offers numerous advantages over natural mating, including time and labor savings. Once collected, a boar ejaculate can be diluted in a semen extender, creating multiple insemination doses that can be used to breed several sows and gilts. Thus, fewer boars are necessary and as a consequence, feed, veterinary, and housing costs are reduced. Moreover, AI allows more extensive use of genetically superior boars, increasing the rate of genetic improvement within a herd.

In the modern swine industry, semen is typically collected once or twice each week from boars housed at isolated sites called "studs" and trained to mount an artificial sow. Ejaculates are then diluted and multiple insemination doses are transported and stored for use at commercial swine units. It has been demonstrated that PCV2 can be present in semen and thus there is the potential for horizontal disease transfer from boars to sows (West et al., 1999). Standard AI methods use a catheter and semen is deposited in the cervix which is also the site of semen deposition during natural mating. Artificial insemination is performed two or three times following the display of standing estrus, which on average lasts approximately two days (Soede et al., 1995). Multiple inseminations maximize the likelihood of depositing spermatozoa 0 to 24 h before ovulation, which maximizes fertility (Soede et al., 1995; Martinez et al., 2005). The AI doses each contain 2.5 to 4 billion spermatozoa in a volume of 70 to 100 ml (Martinez et al., 2005).

Potential Infections from AI

There are a number of bacteria and viruses that can be present in boar semen (Maes et al., 2008). These microbes may not necessarily cause clinical symptoms in the boar, but can potentially lead to disease in the sow and perhaps the future offspring. This presents a serious problem for producers, as limiting the spread of these pathogens is a primary goal. The majority of bacteria present in boar semen are Gram negative and are from the family Enterobacteriaceae (Maes et al., 2008). Antimicrobials are used in extended semen with the primary objective being to prevent bacterial growth during storage. Antimicrobials, however, are not effective against all bacteria and therefore some bacteria shed into semen may not be susceptible to a particular antimicrobial. This presents a possible risk of transmission to the sow. Pathogenic bacteria that have been detected in boar semen and linked to disease in the sow include *Brucellosis, Chlamydia, Leptospirosis, Mycoplasma,* and *Tuberculosis*. Most cause reproductive tract infections or infertility in the boar and can lead to reproductive failure in the sow including abortions and possible infertility (Maes et al., 2008).

Conditions during the storage, handling, and utilization of fresh boar semen provide a favorable environment for the preservation and spread of viral pathogens in swine. Antiviral agents that render semen virus free have not been adopted for use in the swine AI industry (Guerin and Pozzi, 2005). However, other approaches have been implemented that have led to specific viral pathogen-free AI centers. These approaches have mainly relied on monitoring boars for specific virus and viral antibodies. These centers have rejected or removed animals that test positive for these specific viruses (Guerin and Pozzi, 2005). Viruses that have been detected in boar semen include: foot and mouth disease virus, swine vesicular viruses, classical swine fever virus, Pseudorabies virus, PRRSV, PCV2, PPV, Japanese B-encephalitis virus,

African swine fever virus, Rubulavirus, picornaviruses, enteroviruses, porcine retroviruses, and bovine viral diarrhea virus. Not all of these viruses have been shown to cause disease in swine, however the presence of these viruses in semen warrants mentioning (Guerin and Pozzi, 2005; Maes et al., 2008).

PCV2 in semen

The DNA from the two subtypes of PCV2 has been detected in boar semen using nested and real-time quantitative PCR (Kim et al., 2001; Pal et al., 2008). Madson et al. (2008) used real time quantitative PCR to determine the shedding pattern of PCV2a and PCV2b up to 90 day post inoculation. The researchers inoculated six boars with PCV2a and six boars with PCV2b and an additional three boars were used as negative controls. Blood and semen were collected at variable intervals beginning three days before inoculation. During the first week post-inoculation samples were collected three times, beginning on day 2. Samples were collected taken twice a week for the next three weeks. Following the fourth week samples were collected once a week, for a period of nine weeks, until the end of the study. In the group that was inoculated with PCV2a, initial detection of PCV2 DNA in serum was on day two post inoculation in one boar and in all boars by day six post inoculation. The length of time PCV2 DNA was detected in serum, ranged from 18 days to 43 days. The last detection of PCV2a DNA in serum, in all boars, occurred on day 48 post inoculation. The initial detection of PCV2b in serum was day 6 post inoculation and all boars tested positive by day 16 post inoculation. PCV2b DNA was detected in serum from 8 days to 84 days among individual boars, and one boar tested positive at the end of the study at day 90 post inoculation. The control boars did not test positive for PCV2a or PCV2b DNA in serum or semen during the entire course of the study. The PCV2a DNA was detected in semen in all boars inoculated with PCV2a, with individual

boars testing positive for as few as one collection up to testing positive for a period of 49 days, which spanned multiple collections. Peak shedding in this group occurred between day 16 and day 23 post inoculation. There was no intermittent shedding in semen, meaning that once shedding ceased it did not restart again during the course of the study. The PCV2b DNA was detected in semen from five of six boars inoculated with PCV2b. The first detection of shedding in semen occurred at day nine post inoculation and the last was at day 90 post inoculation which was the last day of the study. The period of shedding in semen ranged from 0 to 77 days. The PCV2 DNA was detected in the lymph nodes, testes, epididymis, bulbourethral gland, seminal vesicle, and prostate in all boars from the PCV2a and PCV2b groups. At necropsy, the boars inoculated with PCV2a and PCV2b showed characteristic signs of PCV2 infection including enlarged lymph nodes, tan lungs, and mild lymphoid depletion. Interstitial pneumonia was also observed in several of the boars from both PCV2 inoculated groups. This shows that the presence of PCV2 DNA in semen and the length of time shedding occurs is largely on an individual basis and presents a means of transmission of the virus from boar to sow (Madson et al., 2008).

Since PCV2 was demonstrated to be shed into semen, the logical next step was to find out if the PCV2 in the semen was infectious and could cause disease in PCV2 negative animals. Another study by Madson et al. (2009a) showed that PCV2 DNA in semen was infectious. This was accomplished by using a bioassay in which 12 four week old pigs (gender not stated) were divided into four groups of three pigs each. These groups were inoculated intraperitoneally with either PCV2 negative semen, PCV2a positive semen, PCV2b positive semen, or PCV2 live virus. The pigs inoculated with PCV2 negative semen remained negative for PCV2 antibodies and PCV2 DNA for the duration of the study. One pig in the group inoculated with PCV2a positive

semen first tested positive for anti-PCV2 IgG antibodies at day 28 post inoculation. All pigs in this group tested positive for PCV2 antibodies by day 49 post inoculation. The PCV2b group had 2 pigs test positive for PCV2 antibodies by day 49 post inoculation with the first positive at day 42 post inoculation. All pigs in the PCV2 live virus group tested positive for PCV2 antibodies by day 14 post inoculation and remained positive for the remainder of the study. The PCV2 DNA was detected by PCR in serum samples beginning at day 21 post inoculation in the PCV2a group with all three pigs positive by day 35 post inoculation. The PCV2b group had similar results to the PCV2a group for detection of PCV2 DNA in serum. All three pigs in the PCV2 live virus group tested positive for PCV2 DNA at day seven post inoculation and all three remained positive for the duration of the study. In a second experiment three groups of three PCV2 negative gilts each, received AI with either PCV2 DNA negative semen, PCV2a DNA positive semen, or PCV2b DNA positive semen. Interestingly, none of the gilts in any of the groups seroconverted to PCV2 positive during the study and there was no evidence of gross lesions in the gilts or farrowed piglets at necropsy. These results are not believed to be due to the lack of infectivity of PCV2 in semen, but rather the dose which was used in this experiment. Indeed, the amount of PCV2 DNA was decreased due to extending of the semen for AI, though the authors provided no details as to the magnitude of the decrease. The raw semen was shown to be infectious in the bioassay described by Madson et al. (2009a) and mentioned above. It was suggested by the researchers that a low amount of PCV2 in semen, due to extending and mixing with ejaculates from other boars, as might occur at a commercial stud, may be a lesser risk of infection compared to other routes. Nevertheless this does not eliminate the possibility of AI as a possible route of PCV2 transmission (Madson et al., 2009a).

Reproductive failure was observed in sows when they were inseminated with PCV2 spiked semen (Madson et al., 2009b). In this study, three groups of three sows were bred by AI with either PCV2 negative semen, PCV2a positive semen, or PCV2b positive semen. The positive semen was created by adding 5 ml of either PCV2a or PCV2b for the two different groups, added to 80-ml insemination doses. The PCV2a and PCV2b had infectious titers of $10^{4.4}$ and $10^{4.2}$ TCID$_{50}$ (50% tissue culture infectivity dose) per ml respectively, which corresponds to the number of infectious DNA clones in the 5 ml spike. The negative control sows farrowed a total of 31 pigs (29 live and 2 stillborns). None of the three sows in the PCV2a group farrowed. Pregnancy status was not determined until the twelfth week post-mating, therefore it could not be determined if sows from the PCV2a group had established pregnancies and aborted pregnancy early in the study or failed to get pregnant at the start of the study. The PCV2b treated sows farrowed 35 pigs (8 live, 2 stillborn, and 25 mummified fetuses). Fetal death for the mummified fetuses was estimated to have occurred between 42 and 105 days of gestation. All PCV2a and PCV2b treated sows were positive for anti-PCV2 IgG antibodies on day 112 post inoculation. Serum samples from PCV2a-treated and PCV2b-treated sows were positive beginning at day seven and day 14 post inoculation, respectively. The two stillborn fetuses from the PCV2b treated sows had mild to moderate abdominal distension, enlarged hearts, and mild interlobular pulmonary edema. These characteristics were not present in the two stillborn fetuses from the negative control sows. It is unknown why the PCV2a treated sows failed to become pregnant, though the authors gave possible reasons which included the small sample size, the PCV2a genotype, and/or a slight difference in the inocula titer. In the field, if increased amounts of stillborn or mummified fetuses are farrowed, then possible PCV2 infection should be considered (Madson et al., 2009b).

Conclusion

Artificial insemination is a possible route of transmission of several diseases from boar to sow including PCV2. Management techniques have been employed in various stages of the pork production system to limit PCV2. The results suggest that PCV2 could be transmitted through AI by the use of infected semen, however, the amount of PCV2 in semen appears to be important in terms of the ability to transmit infection from boar to sow. The duration of shedding of PCV2 in semen varies among boars, but in some cases could be sufficient to cause infection in a herd. Therefore, vaccination of boars might be a possible management practice that could be employed to aid in limiting horizontal transmission of PCV2 to sows. However, it is unclear how vaccinating boars currently infected with PCV2 against PCV2 might affect shedding of the virus into semen or the antibody titers in serum.

CHAPTER III.

EFFECT OF VACCINATION AGAINST PORCINE CIRCOVIRUS TYPE 2 (PCV2) ON EJACULATE CHARACTERISTICS AND THE SHEDDING OF VIRUS IN BOAR SEMEN

Introduction

Porcine circovirus (PCV) belongs to the family *Circoviridae* and is a small, non-enveloped DNA virus that was first discovered as a contaminant of the porcine kidney PK-15 cell line and was later named PCV type 1 (PCV1) (Tischer et al., 1974). PCV1 was nonpathogenic though it was demonstrated to infect pigs (Allan et al., 1995). The PCV type 2 (PCV2) variant was first described in 1998 when the virus was associated with a new syndrome in pigs, later termed postweaning multisystemic wasting syndrome (PMWS), although the presence of PCV2 can be traced as far back as 1969 in Belgium (Allan et al., 1998; Opriessnig et al., 2007).

There are currently at least two known subtypes of PCV2. The PCV2a, is found predominantly in North America whereas PCV2b is found in Europe, Asia, and North America. Currently PCV2b is the most prominent subtype worldwide. The two genotypes are not well understood but it has been reported that there is no difference between the two in pathogenesis (Gagnon et al, 2007; Gillespie et al, 2009).

An outbreak of porcine circovirus associated diseases (PCVAD) costs producers on average three to four dollars per pig with the highest losses approaching 20 dollars per pig (Gillespie et al., 2009). Thus, PCVAD is an economically important disease. There is a critical need for research determining mechanisms by which the virus is spread among swine and management strategies to mitigate effects of the disease. Artificial Insemination (AI) is the predominant method of breeding swine in the United States and there exists potential for PCV2 to be spread from boar semen to commercial sow operations. Using nested and real-time

quantitative PCR, DNA from the two subtypes of PCV2 has been detected in boar semen (Kim et al., 2001; Pal et al., 2008). The objective of the experiment reported herein was to determine the effect of vaccination against PCV2 on ejaculate characteristics and antibody titers in serum of PCV2-positive boars viremia and viral shedding in semen.

Materials and Methods

Animals

The experiment was conducted at the Virginia Tech-Tidewater Agricultural Research and Extension Center in Suffolk, VA, USA, and the protocol was approved by the Institutional Animal Care and Use Committee of Virginia Polytechnic Institute and State University.

Ten American Landrace boars, donated by Smithfield Premium Genetics (Roanoke Rapids, NC), were housed in a curtain-sided barn and kept in individual pens (4.5 m^2) with a combination of solid concrete and steel rod flooring. Boars had ad libitum access to water and were fed 2.27 kg of a fortified corn and soybean meal based diet daily that met or exceeded NRC (1998) recommendations for the various nutrients. At approximately seven months of age, boars were trained to mount an artificial sow for semen collection. All animals were determined, by nested PCR, to be PCV2-positive due to natural infection, prior to the start of the sampling.

Vaccination

Boars served as sham-vaccinated controls (n = 5) or were vaccinated against PCV2 (n = 5) following the week 0 sample collection. Boars were vaccinated intramuscularly (I.M.) with 2 ml of a killed vaccine (Suvaxyn® PCV2 One Dose™; Fort Dodge Animal Health, Fort Dodge,

IA) (Opriessnig et al, 2007). Sham-vaccinated controls received 2 ml of 0.9% sterile saline solution I.M.

Sampling

Ejaculates were collected once weekly for 8 weeks following the week 0 collection. Collection was done by the gloved-hand technique and semen was filtered (US BAG; Minitube of America, Inc., Verona, WI, USA) to remove the gel fraction of the ejaculate. Gel-free volume and gel weight were determined gravimetrically using a top loading balance (Acculab; Minitube of America, Inc.).

Characterization of Sperm Motility and Morphology

Samples of semen were diluted 1:5 in Androhep-Lite (Minitube of American, Inc.) and were then loaded into a Leja standard count four-chamber slide (IMV USA, Maple Grove, MN, USA) and sperm concentration and characteristics of sperm motility were determined using a computer-assisted sperm analysis system (CASA; Integrated Visual Optical System, Version 12; Hamilton Thorne Research, Beverly, MA, USA). The following characteristics of sperm motility were determined: percentages of motile and progressively motile sperm, path velocity corresponding to the average velocity of the smoothed cell path (VAP), progressive velocity, defined as the average velocity measured in a straight line from the beginning to the end of track (VSL), track speed, defined as the average velocity measured over the actual point-to-point track followed by the cell (VCL), amplitude of lateral head displacement corresponding to the mean width of the head oscillation as the sperm cell swam (ALH), frequency with which the sperm track crossed the sperm path (BCF), straightness (average value of the ratio VSL/VAP) which

measured the departure of the sperm cell path from a straight line, linearity (average value of the ratio VSL/VCL) which measured the departure of the cell track from a straight line, average value of the ratio of minor to major axis of all sperm heads (Elong), and the average size in μm^2 of all sperm heads (Area) (Hamilton Thorne, 2008).

The percentage of spermatozoa with normal morphology was determined using a light microscope (1000x) after semen samples were eosin-stained and dried. Abnormalities included abnormal heads, abnormal tails, detached heads, bent tails, proximal droplets, distal droplets, or other. Other was defined as not meeting the criteria for the other classifications. A total of 100 spermatozoa from each weekly ejaculate were evaluated by a single investigator without knowledge of the treatment groups of boars from which the samples were obtained.

Serology for Detection of PCV2 Antibody

Blood samples were collected via jugular venipuncture and serum was harvested following centrifugation. Blood was sampled once weekly for 8 weeks following the week 0 collection. All serum samples were tested for PCV2 antibody at the Iowa State University Veterinary Diagnostic Laboratory (Iowa State University, Ames, IA). Measurement of antibody titers in serum was determined by an ORF2-based PCV2 ELISA, which measured the presence of anti-PCV2 IgG antibodies as previously described (Nawagitgul et al., 2002). Serum samples with a sample to positive ratio (s/p) equal to or greater than 0.2 were considered positive.

Detection and Quantification of PCV2 DNA in serum and semen samples

All semen and serum samples were tested for the presence and amount of PCV2 genomic DNA by quantitative real-time PCR at the Iowa State University Veterinary Diagnostic

Laboratory (Iowa State University, Ames, IA). Results were expressed as genomic copy number per ml as previously described (Opriessnig et al., 2003).

Statistical Analysis

Data were analyzed using the GLM procedure of SAS (SAS Institute, Inc.; Cary, NC). Data were subjected to analysis of variance for a repeated measures design using a model that included treatment, week, and the treatment by week interaction as possible sources of variation. Treatment was tested using boar within treatment as the error term. Week was tested against the treatment by week interaction. Individual means were compared using the P-DIFF option of the GLM procedure.

Results

Semen Characteristics

Group means, standard errors, and p-values for the effects of treatment, week, and treatment by week are summarized for the semen analyses in Table 1. Many of the semen characteristics were affected by week ($P < 0.05$), however there were no effects of treatment or treatment by week for any semen characteristic ($P > 0.05$).

Table 2 describes the sperm morphology for the boars in the two different groups including group means, standard errors, and p-values for the effects of treatment, week, and treatment by week. There were no effects of treatment or treatment by week interaction on sperm morphology ($P > 0.05$). The percentages of normal spermatozoa and spermatozoa with bent tails were affected by week ($P < 0.01$), but other morphological classifications were not affected ($P > 0.05$).

Serum Antibodies

There was an effect of treatment by week (P <0.01) for serum antibody titers expressed in sample to positive (s/p) ratio, as is shown in Figure 1. For week 0, the vaccinated group tended ($P = 0.09$) to have higher serum antibody titers compared to the control group. In week 1 the two groups were not significantly different (P = 0.48). During week 2, the vaccinated group had higher ($P = 0.04$) serum antibody titers compared to the control group. During weeks 3 through 6 the two groups were not different from each other (week 3, $P = 0.95$; week 4, $P = 0.51$; week 5, $P = 0.85$; week 6, $P = 0.23$). The vaccinated group had lower ($P < 0.01$) serum antibody titers at week 7 compared to the control group. During week 8, the vaccinated group tended ($P = 0.07$) to have lower serum antibody titers compared to the control group.

PCV2 Viral Load in Serum

There were no statistically significant treatment, week, or treatment by week effects for serum genomic copy number (P > 0.1). Figure 2 shows serum genomic copy number for PCV2 for each week for the vaccinated and control groups. The data suggests that reoccurrence of infection began during week 3 in both the vaccinated and control group. Based on the time when the genomic copy number values returned to the pre-week 3 values, the length of the infection was three to five weeks in the control group. The length of the reoccurrence of infection was only one week for the vaccinated group. During week 3 there was an increase in genomic copy number in both groups and the genomic copy number was numerically greater in the control group. Compared with controls, serum genomic copy number in vaccinated boars was numerically lower at week 4. These differences, however, were not statistically significant ($P >$ 0.05).

Serum samples from two boars, both in the vaccinated group, had very high PCR titers (2512 and 5012 genomic copies per ml), even though serum samples collected from the same animals the week before and after were negative for PCV2. Further confirmation of these high genomic titer levels from the two samples using a virus isolation assay were negative for PCV2, indicating that these two samples were probably false-positive by PCR, which could be due to testing error or sample contamination. Therefore, these two data points were not included in the statistical analysis.

PCV2 Viral Load in Semen

There were effects of week ($P < 0.05$) but no effects of treatment or treatment by week ($P > 0.7$) for PCV2 genomic copy number in semen. The effect of week is illustrated in Figure 3 which shows overall PCV2 genomic copy number in semen for PCV2 for the vaccinated and control groups.

Discussion

Antiviral agents that render semen free of viruses have not been adopted for use in the swine AI industry. This is significant, because conditions during the storage, handling, and utilization of fresh boar semen provide a favorable environment for the preservation and spread of viral pathogens in swine (Guerin and Pozzi, 2005). Strategies have been implemented that have resulted in specific viral pathogen-free AI centers (Dees and Deen, 2001). These approaches have relied mainly on monitoring boars, by testing serum and semen for specific viruses such as PRRSV (Dees and Deen, 2001; Guerin and Pozzi, 2005; Madson et al., 2008). It

has been reported that PCV2 can be shed into semen as DNA from both subtypes of PCV2 have been detected in ejaculates of boars (Kim et al, 2001; Pal et al, 2008).

At the start of the experiment reported here, all boars were classified as positive for natural PCV2 infection, but prior to vaccination, the serum IgG antibodies to PCV2 were lower in the control group than in the vaccinated group. Following vaccination, serum IgG antibodies gradually decreased in the vaccinated group, but gradually increased in the non-vaccinated controls. Coupled with data of PCV2 genomic copies in serum, which appeared to show a heightened reoccurrence of infection, the rise in antibodies in unvaccinated controls may represent the immune response to new PCV2 infection. Vaccination appeared to decrease the length of reoccurring infection, suggesting a beneficial effect of vaccination even in naturally infected pigs. Also the decrease of the serum IgG antibodies to PCV2 in the vaccinated group suggests that there is likely no new infection in the vaccinated pigs. Possible explanations for the sharp decrease in the control group viral load during week 5 and then increase during week 6 could be testing error or the relatively small sample size in this experiment.

Madson et al. (2008) showed that shedding of PCV2 into semen was not intermittent and that once shedding ceased it did not restart for the duration of the study. These same investigators also reported that the length of time PCV2 was shed into semen varied among individuals. The PCV2 DNA in semen has been shown to be infectious through a bioassay; however, this level of dose of PCV2 was not able to cause infection in inseminated gilts. Route of exposure could play a role in PCV2 transmission as in the bioassay inoculation was intraperitoneally with PCV2 positive raw semen whereas with AI the route of exposure would be intrauterine with raw semen diluted in extender (Madson et al, 2009a). Dose appears to be a key factor concerning the infectivity of PCV2 in semen as extender would decease the amount of

PCV2 compared to a raw ejaculate. When semen spiked with PCV2 was used, all gilts exposed were determined to be positive for PCV2 antibodies and reproductive failure was observed when these gilts carried to term. An increase in stillborn pigs and mummified fetuses, characteristics of reproductive failure, were observed in the groups with a high PCV2 titer in the insemination dose (Madson et al, 2009b).

Interestingly, in the current experiment, the period of highest serum PCV2 genomic copies (week 3), was the period of lowest PCV2 shedding into semen. When the serum genomic copies decreased following week 3, the shedding into semen increased. A possible explanation for this is that new viral particles were being released from cells known to harbor PCV2, such as the aortic endothelial cells, gut epithelial cells, and dendritic cells (Vincent et al., 2003; Steiner et al., 2008). Perhaps new viral particles were localizing in other systemic tissues, such as the reproductive tract, thus leading to increased shedding into semen.

In the current study various semen characteristics were evaluated, including characteristics of sperm motility as determined using a CASA. Measures of sperm motion have been shown to be important correlates to fertility. Moreover VCL was shown to be the most significant predictor of fertility (Larsen et al., 2000). The VAP and VCL have been shown to be positively correlated with litter size (Holt et al., 1997). Other CASA parameters, such as VSL, STR, and ALH, have been previously reported to be predictors of cervical mucus penetration, though in swine this is not necessarily an issue as sperm are deposited in the uterus and therefore do not encounter cervical mucus (Mortimer, 1994). That there were no effects of treatment or treatment by week on semen characteristics including morphology suggests that vaccination had no negative impact on predictors of fertility in boars.

Conclusion

Data from this research provides evidence that vaccinating PCV2 positive boars with a commercially-available PCV2 vaccine possibly prevents reoccurring PCV2 infection or decreases the length of reoccurring infection. Therefore, vaccination of pigs already infected by PCV2 is still of value. It has yet to be conclusively shown if PCV2 shed into semen is a route of transmission of disease and new infection in sows. Nevertheless, AI could be a possible route of transmission. Therefore, vaccinating boars against PCV2 could limit exposure of sows and is warranted. Vaccination also has no negative effects on predictors of boar fertility. Negative effects of PCV2 on reproduction in sows have been demonstrated (Madson et al., 2009b), therefore, vaccination of AI boars against PCV2 is a practice which could reduce the risk of PCV2 transmission and the development of disease in the breeding herd.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the current manuscript.

Figure Legends

Fig. 1. Antibody titers (s/p ratio, sample to positive ratio) for serum IgG in the control boars (n = 5) and boars vaccinated against PCV2 (n = 5) as measured by ELISA. Values are LSMeans ± SE. Blood samples were collected weekly for nine weeks via jugular venipuncture. There was a treatment by week interaction ($P < 0.01$). Compared with controls, antibody titers in vaccinated boars tended to be greater at week 0 ($P = 0.09$) and were greater at week 2 ($P = 0.04$). Compared with controls, antibody titers in vaccinated boars were lower at week 7 ($P < 0.01$) and tended to be lower at week 8 ($P = 0.07$).

Fig. 2. PCV2 genomic copy number in serum of control boars (n = 5) and boars vaccinated against PCV2 (n = 5). Blood samples were collected weekly for nine weeks. The results are displayed as PCV2 genomic copies per ml of sample. Compared with controls, serum viral load in vaccinated boars was numerically lower at week 4, though, there were no statistically significant effects ($P > 0.05$) of treatment or treatment by week.

Fig 3. Overall mean PCV2 genomic copy number in semen of control and PCV2-vaccinated boars (n = 10). There was a significant effect of week. Data points with different letters (a,b,c) differ ($P < 0.05$).
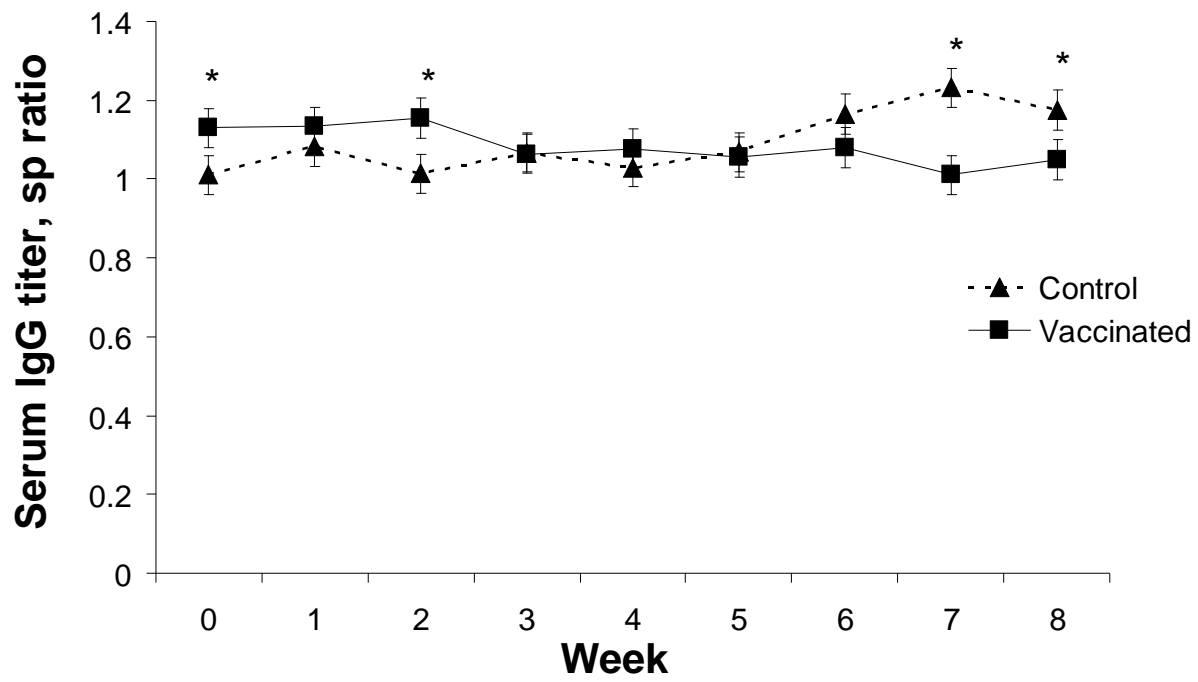
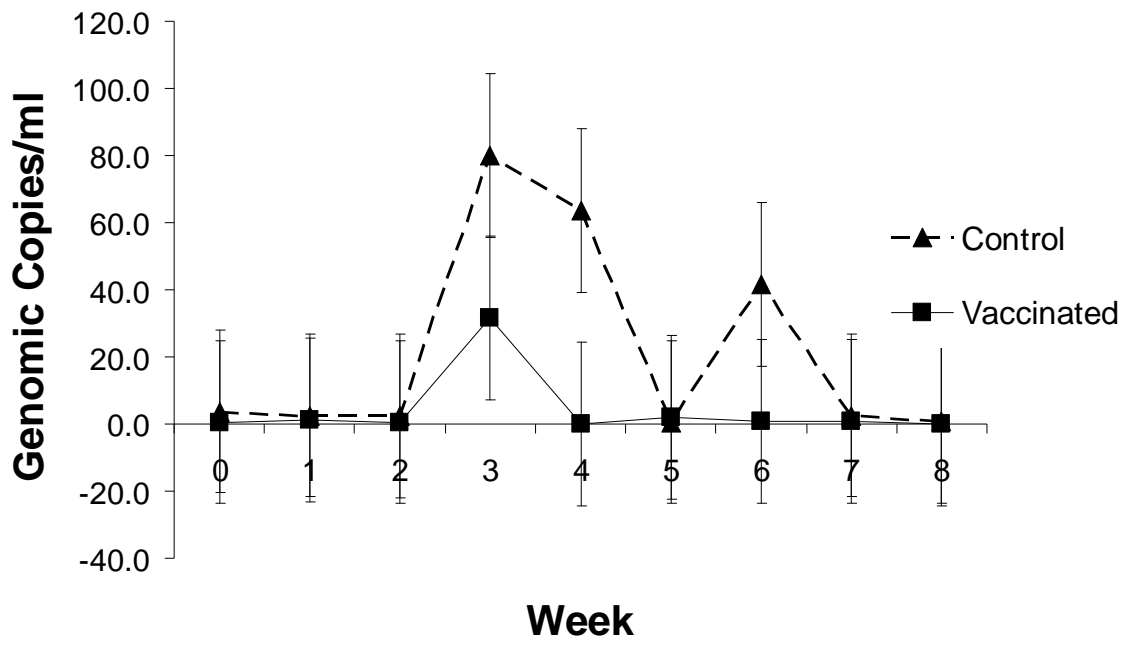**Figure 1**

PCV2 Genomic Copies in Serum
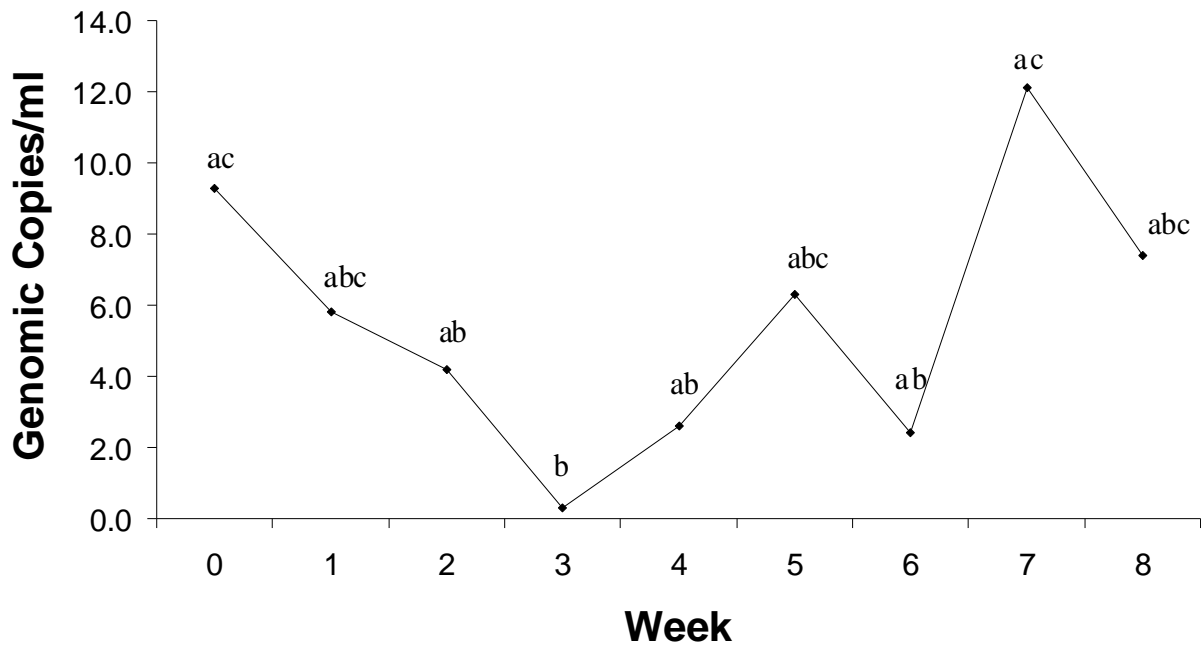
Figure 2

PCV2 Genomic Copies in Semen

Figure 3

Table 1

Semen characteristics in control boars (n = 5) and boars vaccinated against PCV2 (n = 5) that were collected weekly for nine weeks. Values are LSMeans.

					P- values	
Item	Control	Vaccinated	SE	Treatment	Week	Treatment x Week
Volume, mL	249.4	226.4	35.4	0.65	0.69	0.12
Gel, g	55.7	50.6	7.0	0.62	0.18	0.63
Sperm Concentration, million/mL	317.1	381.1	41.8	0.31	<0.01	0.95
Total Sperm Cells, billions	75.2	77.3	6.5	0.82	0.12	0.52
AI Doses[1]	12.0	12.4	1.1	0.80	0.12	0.57
Motility, %	96.2	96.3	0.6	0.86	<0.01	0.92
Progressive Motility, %	69.6	67.8	2.8	0.65	0.03	0.64
VAP[2], μm/sec	91.7	92.8	3.2	0.81	<0.01	0.64
VSL[3], μm/sec	69.4	69.0	2.7	0.90	<0.01	0.71
VCL[4], μm/sec	160.0	168.0	5.8	0.35	<0.01	0.64
ALH[5], μm	7.4	7.5	0.2	0.69	<0.01	0.91
BCF[6], Hz	34.4	35.0	0.6	0.52	<0.01	0.88
STR[7], %	73.9	72.3	1.2	0.34	0.09	0.37
LIN[8], %	44.3	41.8	1.3	0.18	0.11	0.41
Elong[9], %	46.9	47.9	0.4	0.08	0.41	0.07
Area[10], μm sq	12.6	12.8	0.4	0.72	0.14	0.59

[1]Six billion sperm cells/AI dose.

[2]VAP= Path velocity of the smoothed cell path.

[3]VSL= Average velocity measured in a straight line from the beginning to the end of track.

[4]VCL= Average velocity measured over the actual point to point track followed by the cell.

[5]ALH= Amplitude of lateral head displacement corresponding to the mean width of the head oscillation as the sperm swam.

[6]BCF= Frequency with which the sperm track crossed the sperm path (i.e., frequency of sperm head crossing the sperm average path in either direction).

[7]STR= Average value of the ratio VSL/VAP; Measured the departure of the cell path from a straight line.

[8]LIN= Average value of the ratio VSL/VCL; Measured the departure of the cell track from a straight line.

[9]Elong= Average value of the ratio of minor to major axis of all sperm heads.

[10]Area= Average size of all sperm heads.

Table 2

Sperm morphology in control boars (n = 5) and boars vaccinated against PCV2 (n = 5) that were collected weekly for nine weeks. Values are LSMeans.

Item	Control	Vaccinated	SE	*P*- values		
				Treatment	Week	Treatment x Week
Normal, %	88.6	85.1	2.6	0.37	<0.01	0.95
Head Abnormality, %	0.4	0.9	0.02	0.09	0.07	0.71
Tail Abnormality, %	1.0	2.4	1.0	0.37	0.30	0.47
Detached Head, %	6.0	7.2	1.0	0.43	0.27	0.49
Bent Tail, %	3.7	3.9	0.8	0.87	<0.01	0.86
Proximal Droplet, %	0	0.1	0.02	0.24	0.73	0.47
Distal Droplet, %	0	0	0.02	0.34	0.50	0.44
Other, %[1]	0.3	0.4	0.2	0.54	0.32	0.13

[1]Other was defined as not meeting the criteria for the other classifications.

Literature Cited

Allan, G.M., McNeilly, F., Cassidy, J.P., Reilly, G.A.C., Adair, B., Ellis, W.A., McNulty, M.S., 1995. Pathogenesis of porcine circovirus; experimental infections of colostrum deprived piglets and examination of pig foetal material. Vet. Microbiol. 44, 49-64.

Allan, G.M., McNeilly, F., Kennedy, S., Daft, B., Clarke, E.G., Ellis, J.A., Haines, D.M., Meehan, B.M., Adair, B.M., 1998. Isolation of porcine circovirus-like viruses from pigs with a wasting disease in the USA and Europe. J. Vet. Diagn. Invest. 10, 3-10.

Allan, G.M., Ellis, J.A., 2000. Porcine circovirus: a review. J. Vet. Diagn. Invest. 12, 3-14.

American Association of Swine Veterinarians (AASV), 2006a. AASV board approves porcine circovirus position statement. Available at: http://www.aasp.org/news/story.php?id=2131. Accessed January 6, 2010.

American Association of Swine Veterinarians (AASV), 2006b. Porcine circovirus associated disease (PCVAD) case definition. Available at: http://www.aasp.org/aasv/position-PCVAD.htm. Accessed January 6, 2010.

Balmelli, C., Ruggli, N., McCullough, K., Summerfield, A., 2005. Fibrocytes are potent stimulators of anti-virus cytotoxic T cells. J. Leukocyte Biol. 77, 923-933.

Balmelli, C., Alves, M.P., Steiner, E., Zingg, D., Peduto, N., Ruggli, N., Gerber, H., McCullough, K., Summerfield, A., 2008. Responsiveness of fibrocytes to toll-like receptor danger signals. Immunobiol. 212, 693-699.

Bucala R., Spiegel, L.A., Chesney, J., Hogan, M., Cerami, A., 1994. Circulating fibrocytes define a new leukocyte subpopulation that mediates tissue repair. Mol. Med. 1, 71-81.

Cardinal, F., Jones, B., 2008. PCVAD vaccine results in grower-finisher units: practical evaluation and considerations. Adv. In Pork Prod. 19, 197-203.

Charreyre, C., Beseme, S., Brun, A., Boblot, M., Joisel, F., Lapostolle, B., Sierra, P., Vaganay, A., 2005. Vaccination strategies for the control of circoviral diseases in pigs. Proc. Intern. Conf. Anim. Circoviruses and Associated Diseases 26-30.

Choi, C., Chae, C., 2001. Colocalization of porcine reproductive and respiratory syndrome virus and porcine circovirus 2 in porcine dermatitis and nephropathy syndrome by double-labeling technique. Vet Pathol. 38, 436-441.

Christensen E., Christensen, N.O., 1956. Studies on trembling in newborn pigs. Nord Veterinarmed. 8, 921–943.

Correa, A.M., Zlotowski, P., de Barcellos, D.E., da Cruz, C.E., Driemeier, D., 2007. Brain lesions in pigs affected with postweaning multisystemic wasting syndrome. J. Vet. Diagn. Invest. 19, 109–112

Corrégé I, Pirouelle D., Gaudré, D., Le Tiran, M., 2001. A study of the influence of various animal husbandry criteria on the occurrence of PMWS (postweaning multisystemic wasting syndrome) in an experimental pig. J. Recherche Porcine Fr. 33, 283–290.

Dee, S., Deen, J., 2001. Establishment of a PRRS virus ELISA-negative boar population using previously exposed boars. Vet. Rec. 149, 678-680.

Desrosier, R., Clark, E., Tremblay, D., Tremblay, R., Polson, D., 2007. Preliminary results with Ingelvac CircoFLEX to protect multiple ages of Quebec pigs against PCVAD. Proc. Am. Assoc. Swine Pract. 38, 143-145.

Dorr, P.M., Baker, R.B., Almond, G.W., Wayne, S.R., Gebreyes, W.A., 2007. Epidemiologic assessment of porcine circovirus type 2 coinfection with other pathogens in swine. Am. Vet. Med. Assoc. 230, 244-250.

Ellis, J.A., Allan, G., Krakowka, S., 2008. Effect of coinfection with genogroup 1 porcine torque teno virus on porcine circovirus type 2–associated postweaning multisystemic wasting syndrome in gnotobiotic pigs. Am. J. Vet. Res. 69, 1608-1614.

Estienne, M.J., Harper, A.F., Day, J.L., 2007. Characteristics of sperm motility in boar semen diluted in different extenders and stored for seven days at 18 degrees C. Reprod. Biol. 7, 221-231.

Fenaux, M, Opriessnig, T., Halbur, P.G., Meng, X.J., 2003. Immunogenicity and pathogenicity of chimeric infectious DNA clones of pathogenic porcine circovirus type 2 (PCV2) and nonpathogenic PCV1 in weanling pigs. Virology 77, 11232-11243.

Fenaux, M, Opriessnig, T., Halbur, P.G., Elvinger, F., Meng, X.J., 2004. A chimeric porcine circovirus (PCV) with the immunogenic capsid gene of the pathogenic PCV type 2 (PCV2) cloned into the genomic backbone of the nonpathogenic PCV1 induces protective immunity against PCV2 infection in pigs. Virology 78, 6297-6303.

Gagnon, C.A., Tremblay, D., Tijssen, P., Venne, M.H., Houde, A., Elahi, S.M., 2007. The emergence of porcine circovirus 2b genotype (PCV-2b) in swine in Canada. Canadian Vet. J. 48, 811-819.

Gillespie, J., Opriessnig, T., Meng, X.J., Pelzer, K., Buechner-Maxwell, V., 2009. Porcine circovirus type 2 and porcine circovirus-associated disease. J. Vet. Intern. Med. 23, 1151-1163.

de Grau, A.F., Jorgensen, J., Thacker, B., Francisco, C., Wilson, W., Schlueter, R., Eggen, A., 2007a. Field performance of a conditionally licensed vaccine: the Canadian experience. Proc. Am. Assoc. Swine Vet. 38, 159-161.

de Grau, A.F., Thacker, B., Francisco, C., Wilson, W., Schlueter, R., Eggen, A., 2007b. Field trials to assess the performance of a conditionally licensed PCV2 vaccine in Canada. Proc. Intern. Symposium Emerging and Re-Emerging Pig Diseases 5, 120.

Guerin, B., Pozzi, N., 2005. Viruses in boar semen: detection and clinical as well as epidemiological consequences regarding disease transmission by artificial insemination. Theriogenology 63, 556-572.

Hamel, A.L., Lin, L.L., Nayar, G.P., 1998. Nucleotide sequence of porcine circovirus associated with postweaning multisystemic wasting syndrome in pigs. Virology 72, 5262-5267.

Hamilton Thorne, 2008. Technical guide for IVOS, TOX IVOS, CEROS. Hamilton Thorne, Inc, Chapter 1 pg 7-8.

Harding, J.C.S., Clark, G., 1997. Recognizing and diagnosing postweaning multisystemic wasting syndrome (PMWS). J. Swine Health Prod. 5, 201-203.

Harding, J.C.S., Clark, E.G., Strokappe, J.H., Willson, P.I., Ellis, J.A., 1998. Postweaning multisystemic wasting syndrome: epidemiology and clinical presentation. J. Swine Health Prod. 6, 249-254.

Harms, P.A., Sorden, S.D., Halbur, P.G., Bolin, S.R., Lager, K.M., Morozov, I., Paul, P.S., 2001. Experimental reproduction of severe disease in cd/cd pigs concurrently infected with type 2 porcine circovirus and porcine reproductive and respiratory syndrome virus. Vet. Pathol. 38, 528-539.

Harms, P.A., Halbur, P.G., Sorden, S.D., 2002. Three cases of porcine respiratory disease complex associated with porcine circovirus type 2 infection. J. Swine Health Prod. 10, 27-30.

Hattermann, K., Roedner, C., Schmitt, C., Finsterbusch, T., Steinfeldt, T., Mankertz, A., 2004. Infections studies on human cell lines with porcine circovirus type 1 and porcine circovirus type 2. Xenotransplantation 11, 284-294.

Holt, C., Holt, W.V., Moore, H.D.M., Reed, H.C.B., Curnock, R.M., 1997. Objectively measured boar sperm motility parameters correlate with the outcomes of on-farm inseminations: results of two fertility trials. J. Andrology 18, 312-323.

Jensen, T.K., Vigre, H., Svensmark, B., Bille-Hansen, V., 2006. Distinction between porcine circovirus type 2 enteritis and porcine proliferative enteropathy caused by *Lawsonia intracellularis*. J. Comp. Path. 135, 176-182.

Kim, J., Han, D.U., Choi, C., Chae, C., 2001. Differentiation of porcine circovirus (PCV)-1 and PCV-2 in boar semen using a multiplex nested polymerase chain reaction. J. Virol. Methods 98, 25-31.

Kim, J., Ha, Y., Jung, K., Choi, C., Chae, C., 2004. Enteritis associated with porcine circovirus 2 in pigs. Canadian J. Vet. Res. 68, 218-221.

Krakowka, S., Hartunian, C., Hamberg, A., Shoup, D., Rings, M., Zhang, Y., Allan, G., Ellis, J.A., 2008. Evaluation of induction of porcine dermatitis and nephropathy syndrome in gnotobiotic pigs with negative results for porcine circovirus type 2. Am. J. Vet. Res. 69, 1615-1622.

Lainson, F.A., Aitchison, K.D., Donachie, W., Thomson, J.R., 2002. Typing of *pasteurella multocida* isolated from pigs with and without porcine dermatitis and nephropathy syndrome. Clin. Microbiol. 40, 588-593.

Larsen, L., Scheike, T., Jensen, T.K., Bonde, J.P., Ernst, E., Hjollund, N.H., Zhou, Y., Skakkebaek, N.E., Giwercman, A., Danish First Pregnancy Planner Study Team, 2000. Computer-assisted semen analysis parameters as predictors for fertility of men from the general population. Hum. Reprod. 15, 1562-1567.

Madson, D.M., Ramamoorthy, S., Kuster, C., Pal, N., Meng, X.J., Halbur, P.G., Opriessnig, T., 2008. Characterization of shedding patterns of porcine circovirus types 2a and 2b in experimentally inoculated mature boars. J. Vet. Diagn. Invest. 20, 725-734.

Madson, D.M., Ramamoorthy, S., Kuster, C., Pal, N., Meng, X.J., Halbur, P.G., Opriessnig, T., 2009a. Infectivity of porcine circovirus type 2 DNA in semen from experimentally-infected boars. Vet Res. 40, 10.

Madson, D.M., Patterson, A.R., Ramamoorthy, S., Pal, N., Meng, X.J., Opriessnig, T., 2009b. Reproductive failure experimentally induced in sows via artificial insemination with semen spiked with porcine circovirus type 2. Vet. Pathol. 46, 707-716.

Maes, D., Nauwynck, H., Rijsselaere, T., Mateusen, B., Vyt, P., de Kruif, A., Van Soom, A., 2008. Diseases in swine transmitted by artificial insemination: An overview. Theriogenology 70, 1337-1345.

Mankertz, A., Caliskan, R., Hattermann, K., Hillenbrand, B., Kurzendoerfer, P., Mueller, B., Schmitt, C., Steinfeldt, T., Finsterbusch, T., 2004. Molecular biology of porcine circovirus: analyses of gene expression and viral replication. Vet. Microbiol. 98, 81-88.

Martinez, E.A., Vazquez, J.M., Roca, J., Cuello, C., Gil, M.A., Parrilla, I., Vazquez, J.L., 2005. An update on reproductive technologies with potential short-term application in pig production. Reprod. Dom. Anim. 40, 300-309.

Mikami, O., Nakajima, H., Kawashima, K., Yoshii, M., Nakajima, Y., 2005. Nonsuppurative myocarditis caused by porcine circovirus type 2 in a weak born piglet. J. Vet. Med. Sci. 67, 735-738.

Mortimer, D., 1994. Practical Laboratory Andrology. Oxford University Press, New York, pg. 1-393.

Nawagitgul, P., Harms, P.A., Morozov, I., Thacker, B.J., Sorden, S.D., Lekcharoensuk, C., Paul, P.S., 2002. Modified indirect porcine circovirus (PCV) type 2-based and recombinant capsid protein (ORF2)-based enzyme-linked immunosorbent assays for detection of antibodies to PCV. Clin. Diagn. Lab. Immun. 9, 33-40.

Opriessnig, T., Yu, S., Gallup, J.M., Evans, R.B., Fenaux, M., Pallares, F., Thacker, E.L., Brockus, C.W., Ackermann, M.R., Thomas, P., Meng, X.J., Halbur, P.G., 2003. Effect of vaccination with selective bacterins on conventional pigs infected with type 2 porcine circovirus. Vet. Pathol. 40, 521-529.

Opriessnig, T., Fenaux, M., Thomas, P., Hoogland, M.J., Rothschild, M.F., Meng, X.J., Halbur, P.G., 2006. Evidence of breed-dependent differences in susceptibility to porcine circovirus type-2 associated disease and lesions. Vet Pathol. 43, 281-293.

Opriessnig, T., Meng, X.J., Halbur, P.G., 2007. Porcine circovirus type 2-associated disease: update on current terminology, clinical manifestations, pathogenesis, diagnosis, and intervention strategies. J. Vet. Diagn. Invest. 19, 591-615.

Opriessnig, T., Patterson, A.R., Madson, D.M., Pal, N., Rothschild, M., Kuhar, D., Lunney, J.K., Juhan, N.M., Meng, X.J., Halbur, P.G., 2009. Difference in severity of porcine circovirus type two-induced pathological lesions between landrace and pietrain pigs. J. Anim. Sci. 87, 1582-1590.

Pal, N., Huang, Y.W., Madson, D.M., Kuster, C., Meng, X.J., Halbur, P.G., Opriessnig, T., 2008. Development and validation of a duplex real-time PCR assay for the simultaneous detection and quantification of porcine circovirus type 2 and an internal control on porcine semen samples. J. Virol. Methods 149, 217-225.

Rodríguez-Arrioja, G.M., Segalés, J., Calsamiglia, M., Resendes, A.R., Balasch, M., Plana-Durán, J., Casal, J., Domingo, M., 2002. Dynamics of porcine circovirus type 2 infection in a herd of pigs with postweaning multisystemic wasting syndrome. Am. J. Vet. Res. 63, 354-357.

Ramamoorthy, S., Meng, X.J., 2008. Porcine circoviruses: a minuscule yet mammoth paradox. Anim. Health Res. Rev. 10, 1-20.

Rose, N., Larour, G., Le Diguerher, G., Eveno, E., Jolly, J.P., Blanchard, P., Oger, A., Le Dimna, M., Jestin, A., Madec, F., 2003. Risk factors for porcine post-weaning multisystemic

wasting syndrome (PMWS) in 149 French farrow-to-finish herds. Preventative Vet. Med. 61, 209-225.

Rosell, C., Segalés, J., Folch, J.M., Rodríguez-Arrioja, G.M., Domingo, M., Ramos-Vara, J.A., Duran, C.O., Balasch, M., Plana-Duran, J., 2000. Identification of porcine circovirus in tissues of pigs with porcine dermatitis and nephropathy syndrome. Vet Rec. 146, 40-43.

Royer, R.L., Nawagitgul, P., Halbur, P.G., Paul, P.S., 2001. Susceptibility of porcine circovirus type 2 to commercial and laboratory disinfectants. J. Swine Health Prod. 9, 281-284.

Sanchez, R.E., Nauwyncka, H.J., McNeilly, F., Allan, G.M., Pensaert, M.B., 2001. Porcine circovirus 2 infection in swine foetuses inoculated at different stages of gestation. Vet. Microbiol. 83, 169-176.

Seeliger, F.A., Brügmann, M.L., Kruger, L., Greiser-Wilke, I., Verspohl, J., Segalés, J., Baumgärtner, W., 2007. Porcine circovirus type 2-associated cerebellar vasculitis in postweaning multisystemic wasting syndrome (PMWS)-affected pigs. Vet. Pathol. 44, 621-634.

Segales, J., Domingo, M., 2002. Postweaning multisystemic wasting syndrome (PMWS) in pigs. a review. Vet. Q. 24, 109-124.

Soede, N.M., Wetzels, C.C.H., Zondag, W., de Koning, M.A.I., Kemp, B., 1995. Effects of time of insemination relative to ovulation, as determined by ultrasonography, on fertilization rate and accessory sperm count in sows. J. Reprod. Fertility 104, 99-106.

Steiner, E., Balmelli, C., Herrmann, B., Summerfield, A., McCullough, K., 2008. Porcine circovirus type 2 displays pluripotency in cell targeting. Virology 378, 311-322.

Stevenson, G.W., Kiupel, M., Mittal, S.K., Choi, J., Latimer, K.S., Kanitz, C.L., 2001. Tissue distribution and genetic typing of porcine circoviruses in pigs with naturally occurring congenital tremors. J. Vet. Diagn. Invest. 13, 57–62.

Szeredi, L., Szentirmai, C., 2008. Proliferative and necrotizing pneumonia and severe vascular lesions in pigs naturally infected with porcine circovirus type 2. Acta Veterinaria Hungarica 56, 101-109.

Thibault, S., Drolet, R., Germain, M.C., D'Allaire, S., Larochelle, R., Magar, R., 1998. Cutaneous and Systemic Necrotizing Vasculitis in Swine. Vet. Pathol. 35, 108-116.

Thomson, J.R., Higgins, R.J., Smith, W.J., Done, S.H., 2002. Porcine dermatitis and nephropathy syndrome. clinical and pathological features of cases in the united kingdom (1993–1998). J. Vet. Med. Assoc. 49, 430-437.

Tischer, I., Rasch, R., Tochtermann, G., 1974. Characterization of papovavirus- and picornavirus-like particles in permanent pig kidney cell lines. Zentralblatt Fuer Bakteriologie (Originale A) 226, 153-167.

Vincent, I.E., Carrasco, C.P., Herrmann, B., Meehan, B.M., Allan, G.M., Summerfield, A., McCullough, K.C., 2003. Dendritic cells harbor infectious porcine circovirus type 2 in the absence of apparent cell modulation or replication of the virus. Virology 77, 13288-13300.

West, K.H., Bystrom, J.M., Wojnarowicz, C., Shantz, N., Jacobson, M., Allan, G.M., Haines, D.M., Clark, E.G., Krakowka, S., McNeilly, F., Konoby, C., Martin, K., Ellis, J.A., 1999. Myocarditis and abortion associated with intrauterine infection of sows with porcine circovirus 2. J. Vet. Diagn. Invest. 11, 530–532.