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Studies on transmission of porcine circovirus type 2 (PCV2)

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Studies on transmission of porcine circovirus type 2 (PCV2)

by

Michelle Grabosch

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTERS OF SCIENCE

Major: Immunobiology

Program of Study Committee:
Tanja Opriessnig, Major Professor
Rodney Butch Baker
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Iowa State University
Ames, Iowa
2012

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DEDICATION

I dedicate this thesis to my husband Shane Grabosch for having patience while I worked towards this degree.
# TABLE OF CONTENTS

CHAPTER 1. INTRODUCTION ...........................................................................................................1
   Introduction ..................................................................................................................................1
   Thesis Organization ..................................................................................................................4

CHAPTER 2. LITERATURE REVIEW ..............................................................................................5
   Taxonomy and Organization ......................................................................................................5
   Disease Manifestations ..............................................................................................................6
   PCV2 Transmission ...................................................................................................................9
   Prevention Through Biosecurity and Farm Management ........................................................11
   Prevention Through Vaccination ............................................................................................13

CHAPTER 3. A LIVE-ATTENUATED AND AN INACTIVATED CHIMERIC PCV1-2 VACCINE ARE BOTH EFFECTIVE AT INDUCING A HUMERAL IMMUNE RESPONSE AND REDUCING PCV2 VIREMIA AND INTRAUTERINE INFECTION IN BREEDING AGE FEMALES ....................................24
   Abstract ...................................................................................................................................24
   Introduction ..............................................................................................................................25
   Material and Methods ..............................................................................................................28
   Results ......................................................................................................................................34
   Discussion ..................................................................................................................................36
   Conclusion ...............................................................................................................................40
   Acknowledgments ....................................................................................................................40
   References ..............................................................................................................................41
   Tables and Figures ...................................................................................................................45

CHAPTER 4. VACCINATION WITH INACTIVATED OR LIVE-ATTENUATED CHIMERIC PCV1-2 RESULTS IN DECREASED VIREMIA IN CHALLENGE-EXPOSED PIGS AND MAY REDUCE TRANSMISSION OF PCV2 ........................................51
   Abstract ...................................................................................................................................51
CHAPTER 1. INTRODUCTION

1. Statement of the problem and objectives

Porcine circovirus (PCV) is a single stranded, covalently closed, circular, DNA virus that was first identified as a cell culture contamination (Tischer et al., 1974). Subsequently, research studies have shown that PCV was widespread in the pig population and non-pathogenic (Tischer et al., 1986; Allan et al., 1995; Tischer et al., 1995). In 1991, a severe wasting syndrome in pigs was observed in Canada (Harding and Clark, 1997), and in the United States and Europe shortly thereafter (Daft et al., 1996; LeCann et al., 1997). PCV was found associated with the new syndrome (Ellis et al., 1998), but was shown to exhibit less than 80% sequence homology with the previously described PCV, indicating a new genotype (Meehan et al., 1998). Today, PCV is divided into two distinct genotypes: the non-pathogenic PK-15 cell line associated PCV type 1 (PCV1), and the pathogenic disease causing PCV type 2 (PCV2) (Meehan et al., 1998). Retrospective studies have since shown that PCV2 antigen was identified as early as 1962 in tissues from a pig in Northern Germany (Jacobsen et al., 2009), and PCV2-associated microscopic lesions were identified in archived tissues as early as 1985 in Spain (Rodríguez-Arrioja et al., 2003). PCV2 can be further subdivided into PCV2a and PCV2b (Olvera et al., 2006), and is present in all major pork producing countries.

Since the early 1990’s, PCV2 has been associated with a number of disease manifestations including post-weaning multisystemic wasting syndrome (PMWS), respiratory disease, porcine dermatitis and nephropathy syndrome (PDNS), reproductive failure, enteritis, and neuropathy (Chae, 2005). The combination of all the above described disease manifestations is today known as porcine circovirus associated disease (PCVAD).
(American Association of Swine Veterinarians, 2006) which has been shown to have a major impact on the swine industry. Specifically, PCVAD has been shown to cause morbidity rates of 12.5% to 59.9% (depending on production phase and farm size) (USDA, 2006) often resulting in decreased average daily gain, increased time to market, and increased mortality rates from 3.3% to greater than 6.5% (British Pig Executive, 2006).

PCV2 has been shown to be shed in high quantities through feces, nasal secretions, oral secretions, and urine (Shibata et al., 2003; Chung et al., 2005; Segalés et al., 2005; Caprioli et al., 2006). Presence of PCV2 in these secretions and excretions has been shown to facilitate horizontal transmission when PCV2 naïve contact animals were comingled with PCV2-infected pigs (Bolin et al., 2001; Patterson et al., 2010a). In addition, the ability of PCV2 to cross the zona pellucida and infect oocytes (Bielanski et al., 2004; Mateusen et al., 2004), the ability of PCV2 to cross the placenta and infect fetuses (Nielsen et al., 2004; Park et al., 2005; Madson et al., 2009b), and the ability of PCV2 to spread intrauterine and infect fetuses (Pensaert et al., 2004) potentially allows for vertical transmission. In support of this, in several experimental inoculation studies using pregnant breeding animals, vertical transmission was achieved (Nielsen et al., 2004; Park et al., 2005; Madson et al., 2009b). All of the above studies add to the body of evidence that PCV2 is not only widespread, but can also be transmitted through many routes.

Due to the ubiquitous nature of PCV2 and the economic impact of PCVAD, several vaccines have been developed to combat this virus. There are currently four vaccines commercially available in North America: 1) Ingelvac® CircoFLEX™ (Boehringer Ingelheim Vetmedica Inc.) is a subunit vaccine that is based on the PCV2 capsid protein expressed in baculovirus and is licensed for use as a single dose vaccine in pigs 3 weeks and
older in the United States. 2) Circumvent\textsuperscript{TM} PCV (Merck Animal Health) is also a subunit vaccine based on the PCV2 capsid protein expressed in baculovirus and is licensed as a two dose vaccine in pigs 3 weeks and older in the United States. 3) Fostera\textsuperscript{TM} PCV (Pfizer Animal Health Inc.) is a chimeric PCV1-2 vaccine with the PCV2 capsid gene expressed in the backbone of PCV1 and is licensed for use as a single dose vaccine in pigs 3 weeks and older in the United States. 4) Circovac\textsuperscript{®} (Merial Inc.) is an inactivated PCV2 vaccine licensed for use as a single dose vaccine in 3 week old pigs and as a two dose vaccine in healthy breeding age females. The Circovac\textsuperscript{®} vaccine is currently not available in the United States, but is available in Canada and Mexico. In addition, several experimental live PCV2 vaccines have been developed. Live vaccines are currently not commercially available, but could be advantageous as they could induce a strong cellular immune response and thus potentially providing better protection against PCVAD compared to inactivated vaccines.

PCV2 is spread vertically from the dam to the fetus and has been found associated with reproductive failure and reduced litter sizes. However, currently available vaccines are not licensed to protect against vertical PCV2 transmission and reproductive failure. Therefore, the main objective of our first study was to determine if inactivated commercial or experimental live PCV2 vaccines given in one or two dose administrations could minimize or even prevent PCV2 transmission from the dam to the fetus.

PCV2 is also spread horizontally among pigs leading to PCV2 viremia and potentially PCVAD, and has been shown to be spread to naïve contact animals between 1 and 42 days after challenge of a source population (Bolin et al., 2001). However, no study had yet determined if chronically PCV2 infected pigs can transmit PCV2 horizontally to naïve contact animals. PCV2 vaccination has been shown to be effective in preventing or
minimizing PCV2 infection and PCVAD in growing pigs, and vaccinating nursery and
grower age pigs have been monitored for 21 days (Opriessnig et al., 2009; Shen et al., 2010b;
Sinha et al., 2010; Liu et al., 2011; Beach et al., 2010; Xujie et al., 2011), however no study
had yet monitored the long-term efficacy of vaccination. Therefore, the main objectives of
our second study were to determine if PCV2 is transmissible to naïve contact pigs 140 days
after initial challenge and if PCV2 vaccination is beneficial in this scenario.

2. Thesis Organization

The present thesis has been prepared in an alternate manuscript format. The thesis
contains an introduction, a literature review, two separate scientific manuscripts, and a
conclusion. References for the introduction, literature review, and the conclusion are cited at
the end of the thesis in the references cited section.

The first manuscript describes the effect of PCV2 vaccination on breeding age
females and intrauterine transmission of PCV2 and is currently in preparation for publication.
The second manuscript describes the transmission of PCV2 to PCV2 naïve contact animals
and the long-term efficacy of PCV2 vaccination in growing age pigs is currently in
preparation for publication.
CHAPTER 2. LITERATURE REVIEW

1. Taxonomy and organization

Porcine circovirus (PCV) was first identified in 1974 as a contaminant of the continuous porcine kidney cell line PK-15 ATCC CCL-33 (Tischer et al., 1974) and was later shown to be non-pathogenic in swine (Tischer et al., 1986; Allan et al., 1995). In 1998, a new genotype of PCV was identified in swine in North America and Europe in association with wasting disease (Morozov et al., 1998; Allan et al., 1998b; Allan et al., 2000). The new PCV was designated as PCV type 2 (PCV2) and the original genotype was designated as PCV type 1 (PCV1) (Meehan et al., 1998; Allan et al., 1998a). Together, PCVs are classified in the family Circoviridae and the genus Circovirus (Todd et al., 2005).

PCVs consist of two major open reading frames (ORFs), though 11 potential ORFs exist in the PCV genome (Hamel et al., 1998). ORF1 encodes for the viral replicase protein and ORF2 encodes for the viral capsid protein (Mankertz et al., 1998; Nawagitgul et al., 2000; Cheung, 2003). A third ORF, ORF3, plays a role in apoptosis (Liu et al., 2006b) and, more recently, has been indicated to play a role in systemic spread of infection by recruitment of macrophages and initiating early release of PCV2 from infected cells (Karuppannan et al., 2011). Mutation of ORF3 has been shown to decreased PCV2 viremia in mice (Karuppannan et al., 2009) and pigs (Juhan et al., 2009); however no differences in gross or microscopic lesions were noted (Karuppannan et al., 2009; Juhan et al., 2010).

Since first recognized in 1998, several subtypes of PCV2 have been identified. The two main subtypes include PCV2a, which until approximately 2005, was the only subtype found in North America, and PCV2b which has become the most prevalent subtype
worldwide (Gagnon et al., 2007; Allan et al., 2007; Patterson et al., 2010a). PCV2a and
PCV2b differ in genome length (PCV2a has 1768 nucleotides and PCV2b has 1767
nucleotides) (Olvera et al., 2006) and in their sequence with the most recognizable difference
found in a 6 amino acid stretch known as the signature motif located in ORF2 (Cheung et al.,
2007). Initial results from the field indicated that PCV2b was more pathogenic than PCV2a;
however experimental studies were unable to confirm this under controlled conditions
(Opriessnig et al., 2007b; Fort et al., 2008; Harding et al., 2010).

2. Disease manifestations

PCV2 was first associated with a chronic wasting syndrome in young pigs (Ellis et
al., 1998), termed post-weaning multisystemic wasting syndrome (PMWS), originally
described in 1991 (Harding and Clark, 1997). Since this time, PCV2 has been associated with
a series of diseases currently termed porcine circovirus associated disease (PCVAD). This
includes PMWS or systemic disease, porcine dermatitis and nephropathy syndrome (PDNS),
respiratory disease, enteritis, reproductive failure, and possibly neuropathy (Gillespie et al.,
2009).

2.1. Post-weaning multisystemic wasting syndrome (PMWS) or systemic disease

Clinical symptoms in PMWS affected pigs include progressive weight loss, jaundice,
lethargy, unthriftiness, and diarrhea (Harding et al., 1998). In addition, other less common
symptoms such as lameness, sudden death, heart failure, and intestinal torsion have also been
reported. Consistent gross lesions found in affected pigs include generalized
lymphadenopathy and often lungs are mottled tan and fail to collapse. Frequently seen
microscopic lesions in lymphoid tissues include depletion of lymphoid follicles with histiocytic replacement. Macrophages can contain intracytoplasmic inclusion bodies (Sorden, 2000). Additional microscopic lesions can include lymphohistiocytic to granulomatous inflammation in other organ systems such as lungs, liver, intestines, and kidneys (Sorden, 2000). Typically, PCV2 antigen can be demonstrated in high levels in tissues with immunohistochemistry (IHC) or in-situ hybridization (ISH) (Opriessnig et al., 2007a).

PMWS can lead to mortality rates around 10% or higher in herds (Harding et al., 1997) and most commonly affects animals between 7 and 16 weeks of age in North America and between 5 and 12 weeks of age in Europe (Gillespie et al., 2009).

2.2. Porcine dermatitis and nephropathy syndrome (PDNS)

PDNS was first associated with PCV2 in 2000 and is commonly characterized by raised purple skin lesions with black centers located on the hind quarters, fever, and lethargy (Rosell et al., 2000). At necropsy, animals with PDNS will have enlarged kidneys with petechial hemorrhages (Rosell et al., 2000) which is thought to be due to deposition of a large amount of antigen:antibody complexes throughout the vasculature. Microscopic lesions consist of vasculitis and glomerulonephritis (Rosell et al., 2000). Pigs are most commonly affected with PDNS between the ages of 12 and 16 weeks (Gresham et al., 2000).

2.3. PCV2-associated respiratory disease

PCV2-associated respiratory disease is often associated with the porcine respiratory disease complex (PRDC) (Harms et al., 2002). Common symptoms include sneezing, respiratory distress, nasal discharge, lethargy, decreased growth rate, and fever.
Histopathologically, animals with PCV2-associated respiratory disease have bronchointerstitial pneumonia, marked cellular infiltrates composed mainly of macrophages, necrotizing bronchiolitis, and peribronchiolar fibrosis (Harms et al., 2002; Chae, 2005). PCV2-associated respiratory disease usually affects pigs between 8 and 26 weeks of age.

2.4. PCV2-associated enteritis

PCV2-associated enteritis was first reported in 2004 (Kim et al., 2004b). Clinical signs include reduced growth rates, diarrhea, unthriftiness, and increased mortality rates. Gross lesions include thickening of the mucosa in the ileum and enlarged mesenteric lymph nodes (Kim et al., 2004b). Histopathologically, PCV2-associated enteritis is characterized by infiltration of macrophages and mononuclear cells in the mucosa and submucosa of the small and large intestines, granulomatous enteritis, and lymphoid depletion with histocytic replacement of Peyer’s patches (Jensen et al., 2006). To confirm PCV2 involvement, IHC on the intestines can be used to detect antigen in macrophages (Kim et al., 2004a). Enteritis with PCV2 involvement often affects animals between 8 and 16 weeks of age (Gillespie et al., 2009).

2.5. PCV2-associated reproductive failure

PCV2-associated reproductive failure was first noted in 1999 in Canada and is characterized by abortions and increased numbers of still-borns and mummified fetuses as well as weak-born fetuses in full term litters (West et al., 1999; Madson et al., 2009b). Common histopathological lesions include myocardial necrosis with infiltration of macrophages in still-born and mummified fetuses. Abundant PCV2 antigen can typically be
found in the myocardium and tonsils of affected animals by IHC (Madson et al., 2009c). This manifestation is most commonly associated with gilts or young parity sows, thus affecting start-up operations more than stable operations (Mikami et al, 2005).

2.6. PCV2-associated neuropathy

An association of PCV with congenital tremors was first reported in 1994 when sows were infected with a PCV detected in a fetus with congenital tremors and farrowed litters that all had congenital tremors (Hines et al., 1994). Since this first link between PCV and congenital tremors, this disease manifestation has been reproduced in newborn piglets (Choi et al., 2002), but in some studies, when tissues from fetuses with congenital tremors were tested, no PCV2 was detected (Kennedy et al., 2003; Ha et al., 2005). Microscopic lesions associated with congenital tremors include lymphohistiocytic vasculitis in brain tissues, meningitis, and encephalitis (Correa et al., 2007).

3. PCV2 Transmission

PCV2 is widespread and present in nearly all major pork producing countries (Patterson et al., 2010a). Transmission of PCV2 has been shown to occur by both horizontal and vertical routes.

3.1. Horizontal Transmission

PCV2 DNA has been detected in tonsillar and fecal swabs (Bolin et al., 2001; Caprioli et al., 2006), in nasal and fecal swabs (Bolin et al., 2001; Harms et al., 2001; Shibata
et al., 2003; Patterson et al., 2010b), tracheobronchial swabs, and urinary swabs (Segalés et al., 2005). In addition, viable PCV2 has been recovered from nasal and fecal swabs (Magar et al., 2000; Krakowka et al., 2000). Samples in these studies were collected between 1 and 70 days post inoculation (dpi), and PCV2 DNA or viable PCV2 could be detected in a variety of time points (Shibata et al., 2003; Patterson et al., 2010b). PCV2 DNA has also been shown to be shed in the colostrum of sows (Shibata et al., 2006). A study on the effect of disease status on PCV2 shedding indicated that more DNA was detected in feces of nursery pigs suffering from PCVAD compared to clinically healthy pigs (McIntosh et al., 2008). All these studies demonstrate that PCV2 can be transmitted through a wide variety of secretions and excretions for extended periods of time at varying quantities.

3.2. Vertical Transmission

a. PCV2 infection in boars. PCV2 can be demonstrated in testes and accessory glands of boars (Opriessnig et al., 2006). In addition, several studies have detected PCV2 in semen from infected boars (Larochelle et al., 2000; Kim et al., 2003; McIntosh et al., 2006; Madson et al., 2008). PCV2 can be shed for periods ranging from 6 days (Madson et al., 2008) to 27 weeks (McIntosh et al., 2006) in serum and semen from experimentally infected or naturally infected boars. In addition, PCV2 is more often detected in the non-cell fraction of semen and in the seminal fluid than the sperm fraction (Kim et al., 2003). The effect of PCV2 infection on semen quality has also been studied and PCV2 DNA was not associated with changes in semen morphology (McIntosh et al., 2006; Madson et al., 2008).
b. PCV2 infection in sows. PCV2 can infect breeding age females resulting in subclinical infections (Madson et al., 2011). While sows often do not show clinical signs of disease, if PCV2 viremia occurs during pregnancy PCV2 can cross the placenta and infect the fetuses. Under field conditions, natural infection of pregnant sows with PCV2 has resulted in late term abortions characterized by increased numbers of mummified fetuses which all had abundant PCV2 antigen in the myocardium (West et al., 1999) as well as increased mortality rates in live-born piglets (Calsamiglia et al., 2007). Pregnant sows that have been experimentally infected intranasally with PCV2 had increased numbers of still-borns and mummified fetuses (Park et al., 2005). In addition, reproductive failure was experimentally reproduced by spiking semen with PCV2 and artificially inseminating naïve sows (Madson et al., 2009b).

c. PCV2 infection in fetuses. After intranasal PCV2 infection of sows, fetuses had detectable PCV2 antigen and DNA in lymphoid tissues and other organs indicating PCV2 replication (Park et al., 2005). In fetuses, PCV2 targets myocardial tissues for replication leading to vasculitis and cardiac failure in some cases. PCV2 antigen is found in myocardial tissues and tonsils of still-born and mummified fetuses (Madson et al., 2009c). In one study, healthy live-born piglets in the United States and Mexico were tested for presence of PCV2 viremia and PCV2-specific antibodies prior to colostrum uptake, and 39.9% and 21.4% respectively were positive (Shen et al., 2010a) indicating that vertical transmission of PCV2 is not a rare event.

4. Prevention through biosecurity and farm management
4.1. Biosecurity and farm management

Several publications have listed ideal biosecurity and farm management practices for decreasing the spread of disease (Moore, 1992; Madec et al., 1999; Stokes et al., 2010). The following summarizes the most important farm management and biosecurity practices that can minimize any virus spread including PCV2: introduction of a quarantine period, following strict all-in all-out practices with proper cleaning and disinfecting between groups, only using semen from boars of known health status for artificial insemination (AI), minimizing visitors to a facility, disinfecting trailers prior to shipment, and removing sick animals quickly. In addition, dead animals should be removed from the facility in a timely manner and necropsied for evaluation of cause of death.

4.2. PCV2 disinfectants

The effect of disinfectants on PCV2 viability has been evaluated by several research groups. An in-vitro study determined that potassium peroxomonosulfate, sodium hypochlorite, and sodium hydroxide, but not formalin were able to inactivate PCV2 (Kim et al., 2009a). In other in-vitro studies, products containing aldehydes or quaternary ammonium, but not iodine or phenolytic compounds were able to reduce PCV2 viability (Royer et al., 2001; Martin et al., 2008). When four disinfectants with known in-vitro capabilities to inactivate PCV2 were applied under field conditions, all four were capable of decreasing but not totally eliminating PCV2 DNA (Patterson et al., 2011). PCV2 naïve animals remained PCV2 negative after being exposed to the residual DNA (Patterson et al., 2011). These studies indicate that PCV2 is relatively stable, but that the use of certain disinfectants could decrease the viability of PCV2 to a non-infectious level.
5. Prevention through vaccination

Since PCV2 was first associated with clinical disease, several experimental PCV2 vaccines have been developed. This section will describe the development of experimental vaccines, commercially available vaccines, and studies on vaccine efficacy. In 2003, the documentation of the development of several candidate PCV2 vaccines began. Initial publications showed the PCV2 capsid protein (ORF2) as the most immunogenic portion of the viral genome (Blanchard et al., 2003; Nawagitgul et al., 2000), which has become a main target for PCV2 vaccine production.

5.1. Vaccine types

Several types of PCV2 vaccines have been developed and tested in both the swine and mouse model. These include DNA vaccines, subunit vaccines, chimeric vaccines, and divalent vaccines.

a. DNA vaccines. In 2004, a PCV2 isolate was attenuated by passaging it 120 times in cell culture (Fenaux et al., 2004b). Comparison of the genomes of the original virus and the virus after the 120\textsuperscript{th} passage showed two point mutations in the capsid protein which increased viral growth \textit{in-vitro}, but caused attenuation \textit{in-vivo} (Fenaux et al., 2004b) indicating the virus could potentially be used as an attenuated-live vaccine. When the PCV2 capsid protein was expressed in a DNA vector, it induced PCV2-specific antibodies in the mouse model (Kamstrup et al., 2004). In addition, expression of the PCV2 capsid protein bound to ubiquitin induced higher levels of T-helper 1 cellular response and PCV2-specific
antibodies in vaccinated mice in comparison to mice vaccinated with the PCV2 capsid alone (Fu et al., 2011), indicating that binding ubiquitin to a PCV2 vaccine could initiate a stronger immune response. Vaccination with small hairpin RNAs (shRNAs) has also been shown to decrease the levels of PCV2 DNA and protein synthesis in vaccinated challenged mice (Liu et al., 2006a) and vaccination with porcine parvovirus-like particles carrying PCV2 immunodominant epitopes was capable of eliciting a humeral immune response (Pan et al., 2008).

Despite ORF2 being considered the immunogenic portion of PCV2, several studies have attempted to utilize other ORFs of PCV2 to determine their potential as vaccine candidates. Using the prime-boost theory in which one vector is utilized initially to prime the immune response and boosted by vaccination with a second vector to produce higher levels of T-cell responses, the immune system of mice was primed with the naked modified vaccinia virus ankara vector and then immunized with any combination of the three ORFs of PCV2 cloned into the vector (Aravindaram et al., 2009). Increasing levels of T-helper 1 cells were found in vaccinated mice, especially in mice that had been vaccinated with the combination of ORF2 and ORF3 or with all three ORFs (Aravindaram et al., 2009). In another study, vaccination of mice with plasmids expressing ORF2 along with either ORF1 or ORF3 resulted in development of humeral (neutralizing antibodies) and cellular (T-helper 1) immune responses to challenge in both groups (Shen et al., 2009). Finally, vaccination of mice with DNA encoding one of six ORFs gave different cytokine profiles based on the ORF used (An et al., 2008). For example, vaccination with ORF3 induced a lethal amount of tumor necrosis factor alpha (TNF-α) in several vaccinated mice, vaccination with ORF2 induced high levels of interleukin-10 (IL-10, related to humeral immunity and T-helper 2
responses), and vaccination with ORF1 induced high levels of interferon gamma (IFN-γ) and IL-13 (related to T-helper 1 responses) (An et al., 2008). These studies could give researchers an idea on which ORFs to use to stimulate a specific immune response against PCV2 infections, though similar studies would need to be carried out in the swine model to verify these results.

**b. Subunit vaccines.** Several vaccines have been developed by cloning a PCV2 protein into a vector such as adenovirus or baculovirus, thereby developing subunit vaccines. In 2003, vaccination with PCV2 ORF2 expressed in a baculovirus vector induced a more effective immune response than DNA vaccination with ORF1 of PCV2 and ORF2 of PCV2 in the swine model (Blanchard et al., 2003). A vaccine based on the PCV2 capsid protein expressed in an adenovirus vector has been shown to prevent PCVAD and decrease lesions and viremia in comparison to control groups (Wang et al., 2007). In addition, vaccination with the PCV2 capsid protein expressed in a baculovirus vector induced PCV2 specific lymphocyte responses in vaccinated pigs (Fan et al., 2007), and a baculovirus-based PCV2 capsid protein vaccine expressed in a Trichoplusia ni larvae induced PCV2 protection by reducing PCV2 viremia and shedding in challenged pigs (Perez-Martin et al., 2010). A vaccine based on the PCV2 capsid protein cloned into the *Bordetella bronchiseptica* aroA vector led to development of a PCV2-specific antibody response and a decrease of PCV2 DNA in lymph nodes of vaccinated mice and pigs (Kim et al., 2009b). These are examples of the development of candidate subunit vaccines for induction of a PCV2-specific immune response in pigs. Currently, there are three commercially available subunit vaccines available that are highly effective against PCV2 in pigs.
Subunit vaccines in the adenovirus vector have also been evaluated in the mouse model. The shRNAs expressed in an adenovirus vector decreased PCV2 replication both \textit{in-vitro} and \textit{in-vivo} in the mouse model (Feng et al., 2008). Interestingly, linking IFN-γ with the PCV2 capsid protein through a hydrophobic peptide in an adenovirus vector led to stronger PCV2-specific antibody responses in comparison to mice that had been immunized with the PCV2 capsid protein expressed in the adenovirus vector without IFN-γ (Genmei et al., 2011). This indicates that IFN-γ could help elicit a stronger immune response and could be used to increase the efficacy of the vaccine. In one study, the capsid protein of PCV2 was cloned into an adenovirus vector and used to immunize mice which led to development of PCV2-specific antibodies (Wang et al., 2006). Finally, expression of the PCV2 capsid protein in the \textit{Lactococcus lactis} vector used for oral vaccination of mice resulted in a strong PCV2-specific antibody response (Wang et al., 2008) indicating this could be a possible oral vaccine candidate. Similar to the DNA vaccines, studies done in mice would need to be verified in the swine model.

c. \textit{Chimeric vaccines.} Chimeric vaccines, using the capsid protein of one PCV and the backbone of another, have also been created for protection against PCVAD. In 2003, chimeric PCV1-2 (capsid protein of PCV2 cloned into the backbone of PCV1) and PCV2-1 (capsid protein of PCV1 cloned into the backbone of PCV2) DNA clones were developed and tested in the pig model (Fenaux et al., 2003). PCV1-2 chimeric DNA clones, but not PCV2-1 chimeric DNA clones, elicited PCV2-specific antibodies and had significantly less severe gross and microscopic lesions in comparison to animals inoculated with PCV2 infectious DNA clones (Fenaux et al., 2003). Similar to the results seen in the pig studies, a
PCV1-2 DNA clone has also been shown to induce humeral immunity in mice (Yi et al., 2008). To further determine the ability of PCV1-2 to induce protective immunity in the swine model, pigs were subsequently challenged with PCV2 after vaccination (Fenaux et al., 2004a). Vaccinated pigs had minimal microscopic lesions, decreased PCV2 DNA load in lymph nodes, and no detectable PCV2 viremia, which was in contrast to non-vaccinated, challenged animals (Fenaux et al., 2004a). Intramuscular vaccination of pigs with a chimeric PCV1-2 live vaccine led to decreased PCV2 DNA in lymph nodes and lesions after challenge in comparison to non-vaccinated challenged pigs (Cheng et al., 2009) indicating the live chimeric vaccine could be effective. Although a live-attenuated PCV1-2 vaccine has been shown to spread to naïve contact animals, it provided protection to vaccinated and contact animals in a PCV2 and co-infecting pathogens triple challenge model (Opriessnig et al., 2011a). Tagging a live chimeric PCV1-2 vaccine with an epitope tag also induced an immune response, and allowed for verification of vaccination through antibodies to both PCV2 and the tag (Beach et al., 2011). This vaccine has the potential to serve as a marked attenuated-live vaccine as detection of seroconversion to the tag could demonstrate vaccine compliance in the presence of passively-acquired antibodies against PCV2.

d. Divalent vaccines. Vaccines can be used to provide protective immunity against not only PCV2 but to co-infecting pathogens simultaneously. Pigs vaccinated with a divalent vaccine containing inactivated PCV2 and porcine reproductive and respiratory syndrome virus (PRRSV), developed antibodies to both viruses and were protected from challenge (Jin et al., 2010). A similar study in mice expressing ORF2 of PCV2 and GP5 of PRRSV in an adenovirus vector as a candidate vaccine elicited antibody responses to both viruses (Wang et
Expression of the PCV2 capsid protein in the pseudorabies (PRV) vector (Song et al., 2007) and expression of a portion of PCV2 ORF1 fused with a portion of PCV2 ORF2 in the PRV vector (Ju et al., 2005) elicited a strong humeral immune response to both viruses in pigs, though animals were not subjected to challenge. Similarly, expression of the PCV2 ORF1 and ORF2 fused protein in a PRV vector induced strong antibody responses to both viruses in mice, and provided protection from disease in a PRV challenge (Ju et al., 2005). These candidate vaccines could provide protection not only to PCV2 infection, but to two important swine pathogens simultaneously.

5.2. Commercially available PCV2 vaccines

In response to the severe PCVAD outbreaks in North America during 2005-2006 several commercial PCV2 vaccines became available. Currently available globally are three subunit vaccines where the ORF2 of PCV2a is expressed in a baculovirus vector (Ingelvac® CircoFLEX™, Circumvent™ PCV, and Porcilis® PCV), one inactivated chimeric vaccine with ORF2 of PCV2a and ORF1 of PCV1 (Fostera™ PCV, formerly known as Suvaxyn® PCV), and one inactivated PCV2 vaccine (Circovac®), which is not available in the United States but is licensed for use in Canada and Mexico (table 1).
Table 1. Globally most widely utilized PCV2 vaccines.

<table>
<thead>
<tr>
<th>Vaccine Name</th>
<th>Company</th>
<th>Vaccine Type</th>
<th>Age at Vaccination</th>
<th>Dosage</th>
<th>Protection</th>
</tr>
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<tbody>
<tr>
<td>Ingelvac® CircoFLEX™</td>
<td>Boehringer Ingelheim Vetmedica Inc.</td>
<td>Subunit</td>
<td>3 weeks</td>
<td>1</td>
<td>2 weeks post-vaccination for 17 weeks</td>
</tr>
<tr>
<td>Circumvent™ PCV</td>
<td>Merck Animal Health</td>
<td>Subunit</td>
<td>3 weeks</td>
<td>2</td>
<td>Not available</td>
</tr>
<tr>
<td>Porcilis® PCV</td>
<td></td>
<td></td>
<td>3 days</td>
<td>1</td>
<td>2 weeks post-vaccination for 22 weeks</td>
</tr>
<tr>
<td>Fostera™ PCV</td>
<td>Pfizer Animal Health</td>
<td>Chimeric</td>
<td>3 weeks</td>
<td>1</td>
<td>2 weeks post-vaccination for 4 months</td>
</tr>
<tr>
<td>Circovac®</td>
<td>Merial Inc.</td>
<td>PCV2</td>
<td></td>
<td>1</td>
<td>2 weeks post-vaccination for 14 weeks 5 weeks post-parturition</td>
</tr>
</tbody>
</table>

5.3. PCV2 vaccine efficacy under experimental conditions

a. Naïve pigs. Since co-infections are common in the field, several recent studies have evaluated the efficacy of commercially available vaccines in a dual or triple challenge model to mimic field conditions. Suvaxyn® PCV decreased viremia and lesions in a dual challenge model (Opriessnig et al., 2008a) and both commercial (Suvaxyn® PCV, Ingelvac® CircoFLEX™, and Circumvent™ PCV) and experimental vaccines were effective in a triple challenge model at decreasing viremia and lesions (Opriessnig et al., 2009; Shen et al., 2010b). Additionally, one group investigated the effect of PRRSV infection at the time of vaccination and determined that three commercially available vaccines (Suvaxyn® PCV by
Fort Dodge Animal Health, Ingelvac® CircoFLEX™, and Circumvent™ PCV) were efficacious regardless of PRRSV infection at the time of vaccination (Sinha et al., 2010). More recently, questions as to whether or not neonatal piglets are able to mount an effective immune response to vaccination have arisen. In a recent study, two commercially available vaccines (Suvaxyn® PCV and Ingelvac® CircoFLEX™) elicited immune responses and decrease viremia in piglets vaccinated at 5 days and 21 days of age (O'Neill et al., 2011) indicating young animals are able to mount an immune response.

b. Conventional pigs. All of the studies in the above section were done in animals naïve to this virus, however since PCV2 is ubiquitous in nature, maternal antibodies are common in the field. In Germany, the use of Porcilis® PCV was tested in both a two dose administration and a one dose administration. Decreased mortality and improved growth performance was demonstrated for both vaccine administration protocols, though some side effects were noted in the two dose administration (Beek, 2008). Vaccination of pigs with a single dose of Porcilis® PCV resulted in strong cellular and humeral immune responses associated with decreased PCV2 viremia and shedding in another study (Fort et al., 2009). Many practitioners questioned the efficacy of PCV2 vaccination in pigs with passively acquired antibodies, and several studies were conducted to clarify this. PCV2 vaccination with Suvaxyn® PCV was highly effective in the presence of maternal antibodies (Opriessnig et al., 2008b). Similarly, vaccination with Porcilis® PCV was effective in the presence of maternal immunity and provided protection against different isolates from different geographical regions (Fort et al., 2008). Piglets born to sows that had been vaccinated with either Circovac® or Ingelvac® CircoFLEX™ and that were subsequently vaccinated with the same vaccine as their dam had similar antibody responses and were protected against
PCV2 challenge in comparison to naïve piglets that were vaccinated (Opriessnig et al., 2010a). These experimental studies indicate that maternal antibodies do not have negative impacts on PCV2 vaccine efficacy.

c. Breeding age animals. Vaccination or infection of sows with PCV2 increased anti-PCV2 levels in the colostrum; however, little research has been done on the effect of vaccination on vertical transmission and the effect of vaccination on boars. Sows vaccinated with Ingelvac® CircoFLEX™ and challenged with PCV2 had decreased PCV2 viremia, but vaccination did not prevent transmission of PCV2 to the fetuses in-utero (Madson et al., 2009a; Madson et al., 2009c). Under field conditions, vaccination of sows with the Circovac® vaccine in Polish herds led to increasingly favorable returns to estrus and decreased rates of abortion (Pejsak et al., 2009). Vaccination of boars led to decreased length of recurring infections (Alberti et al., 2011), and vaccination with Suvaxyn® PCV significantly decreased PCV2 shedding in semen of experimentally infected boars (Opriessnig et al., 2011b). Vaccination of boars with a commercially available tissue homogenate vaccine (CircoPrime, Komipharm International Company Ltd.) also decreased quantity of viral shedding in semen of experimentally infected boars (Seo et al., 2011). These studies indicate that vaccination of breeding age animals could be used to decrease, but not prevent vertical PCV2 transmission.

5.4. PCV2 vaccine efficacy under field conditions.
Vaccine efficacy in all phases of production is commonly evaluated by comparing average daily gain, feed conversion ratios, mortality rates, and morbidity rates between vaccinated and non-vaccinated pigs.

\textit{a. Suckling pigs.} As mentioned in the previous section, vaccination of breeding age females provides improved passive protection to piglets during the suckling phase. In the field, piglets are often vaccinated at 3 weeks of age which does not provide protection during the suckling phase. To account for this, vaccination of sows with Circovac® resulted in increased health and performance of piglets in comparison to non-vaccinated sow litters (Joisel et al., 2008). These results were confirmed by a different study where again decreased mortality of piglets prior to weaning was seen in response to sow vaccination (Pejsak et al., 2010).

\textit{b. Nursery pigs.} Vaccinating piglets with several commercially available vaccines (Circovac®, Ingelvac® CircoFLEX™, Porcilis® PCV) led to increased average daily gain and decreased mortality rates in comparison to non-vaccinated pigs (Liber et al., 2011; Fraile et al., 2011; Pejsak et al., 2010). Interestingly, nursery pigs vaccinated with Circumvent™ PCV had decreased average daily gain compared to those vaccinated with Ingelvac® CircoFLEX™ (Potter et al., 2009). It is important for producers to keep in mind that vaccinating sows may enhance passively acquired antibody levels for piglets and decreased mortality rates in the suckling phase; however, vaccinating piglets in the nursery phase can lead to better production performances and decreased mortality rates when maternally derived antibodies wane.
c. Grow-finish pigs. PCV2 vaccination has been shown to decrease mortality and morbidity rates and increase average daily gain and feed conversion ratios (Jacela et al., 2007a; Jacela et al., 2007b; Horlen et al., 2008; Takahagi et al., 2009; Paphavasit et al., 2009; Richthofen et al., 2009; Arnold et al., 2010; Cardinal, 2010; Haugegaard et al., 2010; Martelli et al., 2011; Jacela et al., 2011; Venegas-Vargas et al., 2011; Young et al., 2011). In addition, two dose vaccination at 5 and 7 weeks rather than 9 and 11 weeks was more effective (Jacela et al., 2007a), though vaccination at this later age is uncommon. A meta-analysis of all field cases of PCV2 vaccination showed decreased mortality and increased average daily gain with average daily gain differences dependent on PRRSV status of the herd (Kristensen et al., 2011), and vaccination with Porcilis® PCV or Ingelvac® CircoFLEX™ performance results were dependent on the PCV2 subtype that infected the animals (Takahagi et al., 2009). In addition, pigs vaccinated with Ingelvac® CircoFLEX™ had decreased levels of PRDC (Bischoff et al., 2009; Fachinger et al., 2008). Vaccination with Ingelvac® CircoFLEX™ or Suvaxyn® PCV decreased PCVAD symptoms present at several farms (Kixmøller et al., 2008; Desrosiers et al., 2009; Segalés et al., 2009). Vaccination with Circumvent™ PCV improved overall herd performance and decreased losses in animals concurrently infected with PRRSV (Shelton et al., 2009). In addition, one study showed that other factors such as gender, birth weight, and vaccine strategy had additional effects on performance in association with PCV2 vaccination (Bergstrom et al., 2009). In summary, producers should keep in mind the differences between vaccines on different phases of production when deciding which vaccine to use in their herds.
CHAPTER 3: A LIVE-ATTENUATED AND AN INACTIVATED CHIMERIC PCV1-2 VACCINE ARE BOTH EFFECTIVE AT INDUCING A HUMERAL IMMUNE RESPONSE AND REDUCING PCV2 VIREMIA AND INTRAUTERINE INFECTION IN BREEDING AGE FEMALES

A paper prepared for publication

Michelle Hemann, Nathan M. Beach, Xiang-Jin Meng, Chong Wang, Patrick G. Halbur, and Tanja Opriessnig

Abstract

The objective of this study was to determine the efficacy of inactivated (one or two dose) and live-attenuated chimeric PCV1-2 vaccines in sows using the PCV2-spiked semen model. Thirty-five sows were randomly divided into six groups: negative and positive controls, one dose inactivated PCV1-2 vaccine challenged (1-VAC-PCV2), two dose inactivated PCV1-2 vaccine challenged (2-VAC-PCV2), one dose live-attenuated PCV1-2 vaccine unchallenged (1-LIVE-VAC), and one dose live-attenuated PCV1-2 vaccine challenged (1-LIVE-VAC-PCV2). The inactivated PCV1-2 vaccine induced higher levels of PCV2-specific antibodies in dams. All vaccination strategies provided good protection against PCV2 viremia in dams, whereas the majority of the unvaccinated sows were viremic. Four of the 35 dams became pregnant: a negative control, a positive control, a 2-VAC-PCV2 sow, and a 1-LIVE-VAC-PCV2 sow. PCV2 DNA was detected in 100%, 67% and 29% of the fetuses obtained from the positive control, inactivated vaccinated or live-attenuated vaccinated dams. PCV2 antigen
in hearts was only detectable in the positive control litter (23% of the fetuses). PCV1-2 DNA was detected in 29% of the fetuses in the litter from the 1-LIVE-VAC-PCV2 dam. Under the conditions of this study, both vaccines protected against PCV2 viremia in breeding age animals; however, vertical transmission was not prevented.

Keywords: Porcine circovirus; Breeding animals; Vaccination; Live-attenuated chimeric vaccine; Inactivated vaccine.

Introduction

Porcine circovirus (PCV) is a member of the Circoviridae family in the genus Circovirus. It is a non-enveloped, single stranded DNA virus with a circular genome. The genome of PCV2 contains two major open reading frames (ORFs): ORF1 encodes for a protein essential for viral replication, and ORF2 encodes for the capsid protein (1,2). Two main types of PCV have been identified: PCV type 1 (PCV1) and PCV type 2 (PCV2) which share approximately 83% nucleotide sequence identity in ORF1 but only 67% identity in ORF2 (3).

PCV1 was first identified as a contaminate of a continuous porcine kidney cell line (PK-15) in 1974 (4,5). Despite being widespread in the pig population, PCV1 has been shown to be non-pathogenic in pigs (6,7). However, PCV2 is associated with a group of diseases collectively called porcine circovirus associated disease (PCVAD) including PCV2-associated reproductive failure in mature animals (8).

PCV2-associated reproductive failure is typically characterized by increased numbers of abortions, mummified and stillborn fetuses and weakborn piglets (9). Confirmation of
PCV2 as the causative agent of reproductive failure is done by identification of myocardial fibrosis, lymphoplasmacytic myocarditis, and association of PCV2 antigen with the fetal heart lesions by immunohistochemistry (IHC) (10). Piglet serum or fetal thoracic fluid may also be positive for PCV2 DNA or PCV2-specific antibodies (10).

It has been shown that a chimeric PCV1-2 strain with the capsid gene of PCV2 in the backbone of the non-pathogenic PCV1 was non-pathogenic under experimental conditions. The inactivated commercial PCV2 vaccine “Fostera™ PCV” was recently reintroduced to the global market and is based on the PCV1-2 chimera. The non-pathogenic chimeric PCV1-2 virus may also have potential for use as a live-attenuated chimeric PCV2 vaccine (11,12).

PCV2 can be further divided into at least three subtypes. The most important subtypes which are prevalent worldwide include PCV2a which was the predominant strain in the pig population before 2000, and PCV2b which has replaced PCV2a in most herds and is currently the predominant PCV2 genotype in North America (13,14). One of the main differences between PCV2a and PCVb is in the signature motif in ORF2. Comparative pathogenicity studies among the two PCV2 subtypes side by side in experimentally-infected pigs have failed to show any differences in virulence (15,16).

Vaccination against PCV2 has been shown to be highly effective in decreasing losses associated with PCVAD. There are currently several types of commercial vaccines available including an inactivated PCV2 vaccine for use in dams or piglets (Circovac®, Merial, Inc.), two subunit vaccines based on PCV2-ORF2 expressed in baculovirus for use in growing pigs (Circumvent™ PCV, Intervet, Inc. and Ingelvac® CircoFLEX™, Boehringer Ingelheim, Vetmedica, Inc.), and an inactivated chimeric PCV1-2 vaccine for use in growing pigs (Fostera™ PCV, Pfizer Animal Health Inc, formerly Suvaxyn® PCV2 One Dose™ from
Fort Dodge Animal Health). All these vaccines are based on PCV2a with differences in dosage and recommended timing for use of these products (8).

All commercially available PCV2 vaccines are inactivated or subunit vaccines.

Another type of vaccine currently in the experimental stage of development is a live-attenuated PCV2 vaccine based on a chimeric PCV1-2. One concern with any live-attenuated vaccine is the development of vaccine virus viremia in immunized pigs and spread of the vaccine virus among pigs and herds. It has been previously shown that a live-attenuated chimeric PCV1-2 vaccine prevented viremia and decreased macroscopic and microscopic lesions caused by PCV2 infection (12). Interestingly, recently a chimeric PCV1-2 was recovered from clinically healthy pigs on Canadian farms with no signs of PCVAD (17). Another concern with a live-attenuated chimeric vaccine based on the PCV1 backbone is the disease causing potential of PCV1. It has been previously shown that PCV1 is associated with congenital tremors in newborn fetuses (Hines, RK and Lukert, PD, 1994: Porcine circovirus as a cause of congenital tremors in newborn pigs. Proc Am Assoc Swine Pract. Chicago, IL. 25:344-345), but other authors failed to reproduce these initial findings (18,19). Recently, PCV1 has been associated with hemorrhages in lung tissues of fetuses experimentally inoculated with the PK-15 cell-derived PCV1 isolate but not a field isolate of PCV1 (20).

PCV2 is known to be shed in oral, nasal, and fecal excretions (21,22,23), and has recently been shown to be shed in boar semen without damaging sperm morphology (24). It has also been shown that insemination of naïve dams with semen containing low levels of PCV2 DNA did not result in virus transmission (viremia, seroconversion) or reproductive
failure (25); however, insemination with semen spiked with high levels of PCV2 was capable of inducing reproductive failure in naïve dams (10).

The objective of this study was to determine efficacy of a commercial inactivated (administered as one or two dose) vaccine and an experimental live-attenuated chimeric PCV1-2 vaccine to prevent viremia in sows using the PCV2-spiked semen model. Four of the 35 sows became pregnant including two vaccinated sows (two dose inactivated and one dose live-attenuated PCV1-2 vaccine), a negative control and a positive control sow, and their litters were utilized to further determine the efficacy of sow vaccination in preventing or reducing vertical transmission.

Materials and Methods

Animals and housing

Thirty-five dams, ranging in age from 792 to 2,389 days (Table 1), were obtained from a herd confirmed to be free of PCV2 by serology and PCR testing. The dams were transported over a three day interval approximately 250 km to Iowa State University in Ames, Iowa and housed in groups of 1 to 6 depending on room size. Each room was equipped with one nipple drinker and sows were fed daily with a pelleted feed ration which contained whey but was free of other animal proteins and antibiotics (Nature’s Made, Heartland Co-op, Cambridge, IA). Fifteen of the 35 dams were pregnant on arrival (between 15-116 days of gestation) and the pregnancies were terminated in all dams less than 52 days into gestation by using 2 ml cloprostenol sodium (Estrumate®, Intervet/Schering-Plough Animal Health). The dams were also given 3 ml ceftiofur (Excede® Pfizer Animal Health).
Experimental design

The experimental protocol was approved by the Iowa State University Institutional Animal Care and Use Committee. The experimental design is summarized in Table I. After arrival, the dams were blocked by age and randomly assigned to groups and rooms. After an acclimation period of approximately 3 months, 12/35 dams were vaccinated with one 2 ml dose of an experimental live-attenuated chimeric PCV1-2 vaccine (1-LIVE-VAC, and 1-LIVE-VAC-PCV2), 6/35 dams were vaccinated with one 2 ml dose of an inactivated chimeric PCV1-2 vaccine (1-VAC-PCV2), 6/35 dams were vaccinated with two 1 ml doses of an inactivated chimeric PCV1-2 vaccine three weeks apart (2-VAC-PCV2), and 11/35 dams remained unvaccinated as controls. Estrus cycles were synchronized for all dams as previously described (10) followed by artificial insemination 35 days post one dose inactivated chimeric or live vaccination or 14 days post two dose inactivated chimeric vaccination. Extended semen obtained from 9 boars all of the same breed and confirmed to be PCV2 negative was used for artificial insemination. Each dam in the negative control group and in the 1-LIVE-VAC group received 80 ml of PCV2 free semen. All dams in the other groups were inseminated with 75 ml of semen spiked with 5 ml of PCV2b immediately before insemination. Inseminations were repeated in 24 h intervals for 3 days. Dams were monitored for signs of estrus, and if any recycled they were re-inseminated using 80 ml PCV2 free semen in 24 h intervals for 3 days. After vaccination, the dams were bled weekly until necropsy at 105 days post inoculation (DPI). All dams tested negative for specific antibodies against porcine respiratory and reproductive syndrome virus and porcine parvovirus prior to initiation of the study and at termination of the study (data not shown). At necropsy all fetuses were removed from the uterus, euthanized, and samples were collected.
Vaccination

Dams in the 1-VAC-PCV2 group were vaccinated with 2 ml of Suvaxyn® PCV2 (Fort Dodge Animal Health Inc.; now reformulated and known as “Fostera™ PCV” from Pfizer Animal Health Inc.), dams in the 2-VAC-PCV2 group were vaccinated with two 1 ml doses of Suvaxyn® PCV2 three weeks apart, and dams in the 1-LIVE-VAC and 1-LIVE-VAC-PCV2 groups were vaccinated with one 2 ml dose of an experimental live-attenuated chimeric PCV1-2 vaccine at a dose of $10^4$ median tissue culture infective dose (TCID$_{50}$) as described (29).

Clinical observation

All dams were examined daily for signs of illness such as lethargy, respiratory disease, inappetence, and lameness as well as signs of return to estrus.

Inoculation

The PCV2b isolate NC-16845 (16) used for the inoculation was propagated in PCV1-free PK-15 cells to an infectious titer of $10^{4.5}$ TCID$_{50}$. Five weeks after arrival and vaccination, semen was spiked with 5 ml PCV2b and used to artificially inseminate 24/35 of the dams (1-VAC-PCV2; 2-VAC-PCV2, 1-LIVE- VAC-PCV2, positive controls).

Sample collection

Blood was collected from dams in 8.5 ml serum separator tubes (BD vacutainer®, BD Biosciences) on a weekly basis from the time of vaccination until necropsy at DPI 105
(except on DPI 63). At necropsy, fetal blood was collected from all live fetuses and fetal thoracic fluid was collected from all dead or mummified fetuses if possible. The blood was centrifuged at 3220 × g for 10 min at 4°C and the serum was aliquoted into 5 ml polystyrene round bottom tubes (Fisher Scientific, Inc.) and stored at -20°C until testing.

**Serology**

All serum samples were tested for PCV2-specific IgG antibodies using an indirect PCV2 ORF2-based ELISA as previously described (26). The results were expressed as sample-to-positive (S/P) ratio. Samples were considered to be negative if the S/P ratio was less than 0.2 and positive if the S/P ratio was greater than or equal to 0.2. In addition, all serum samples from dams at DPI 0 were tested for PCV2-specific neutralizing antibodies using a fluorescence focus neutralization (FFN) assay (27). Virus neutralizing titers are expressed as the highest serum dilution resulting in a 90% reduction in virus replication compared to the virus control.

**PCV2 viremia detection**

All serum samples or fetal thoracic fluid samples were tested for the presence and quantity of PCV2 DNA by a quantitative PCV2 PCR. If no serum or fetal thoracic fluid could be collected from a fetus, PCR was run on tissue homogenates. PCV2 viral DNA was extracted from the samples using the MagMax™ Viral Isolation Kit (Applied Biosystems, Carlsbad, CA) on the KingFisher Flex System (ThermoFisher Scientific, Pittsburgh, PA). PCV2 DNA detection to verify and quantify the presence of PCV2 in all samples was done by a quantitative real-time PCR using the same primers and probe at the same concentrations.
as previously described (28) using the TaqMan® Fast Universal PCR Master Mix (Applied Biosystems, Foster City, CA). The total PCR reaction volume including 2.5 μl of DNA extract was 25 μl. The thermal cycle conditions were 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 1 min. Samples were considered negative if no signal was observed during the 40 amplification cycles.

**PCV1-2 PCR**

PCV1-2 DNA detection was done to verify the presence of any PCV1-2 using the same DNA extracts as for the PCV2 PCR. DNA quantification was done by quantitative real-time PCR using the same primers and probes at the same concentrations as previously described (29) using the TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA). The total PCR reaction volume was 25 μl. The thermal cycle conditions were 50ºC for 2 min, 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 sec, 55ºC for 30 sec, and 60ºC for 1 min. The sensitivity of the PCV1-2a real-time PCR was 8.13×10⁴ copies/ml (204 copies per reaction). Samples were considered negative if no signal was observed during the 40 amplification cycles.

**Necropsy**

All dams were humanely euthanized by intravenous pentobarbital sodium overdose (Fatal Plus ®, Vortech Pharmaceuticals, LTD, Dearborn, MI) and necropsied at DPI 105. The extent of total macroscopic lung lesions (ranging from 0% to 100%) was scored by a veterinary pathologist in a blinded fashion as previously described (30). Fetuses were surgically removed from the uterus immediately after euthanasia of the dam. Fetal blood was
collected from all live fetuses, and the piglets were then humanely euthanized by intravenous pentobarbital sodium overdose (Vortech Pharmaceuticals, LTD) and necropsied. If blood collection was not possible (mummified or dead fetuses), fetal thoracic fluid was collected if available. Fetal heart tissues were collected at necropsy, a section stored fresh at -20°C until further testing and a section fixed in 10% neutral buffered formalin and then routinely processed for histological examination as previously described (10).

**Immunohistochemistry**

Immunohistochemistry (IHC) for detection of PCV2-specific antigen was performed on fetal hearts using a rabbit polyclonal antiserum (31). Antigen scoring was performed by a veterinary pathologist blinded to treatment groups and scores were reported from 0 (no antigen detected) to 3 (abundant PCV2 antigen) as previously described (32).

**Statistical analysis**

Statistical analysis of the data was performed using the SAS and JMP® softwares version 9.0.0 (SAS Institute, Cary, NC). Summary statistics were calculated for all groups to assess the overall quality of the data including normality. Real-time PCR results and FFN results were log_{10} transformed prior to statistical analysis. Continuous repeated measured data (PCV2 viremia, ELISA S/P ratios) were assessed with the repeated measures analysis of variance (ANOVA) method. In each repeated measures ANOVA analysis, group, time and their interaction were fixed effects whereas animal was the subject of repeated measures. Differences in response among groups were assessed using F-tests by time. Non-repeated measurements (FFN results) were assessed using one-way ANOVA. If an ANOVA F-test
was significant (p-value<0.05), then pairwise t-tests with the Tukey’s adjustment were used to assess specific group differences.

**Results**

**Clinical observation**

One negative control dam and one 1-LIVE-VAC-PCV2 dam developed severe lameness and were euthanized at DPI 84. The overall conception rate of the sows was 11.4% (4/35 sows) after insemination during the first estrus cycle following synchronization and 37.1% (13/35) after 16 sows were rebred during subsequent estrus cycles.

**Reproductive parameters**

Of the four sows that were pregnant after initial insemination using PCV2-spiked semen, the litter compositions were as follows: two mummies and 12 live fetuses (1-LIVE-VAC-PCV2), four mummies and 11 live fetuses (2-VAC-PCV2), zero mummies and 10 live fetuses (negative control dam) and one mummy and 13 live fetuses (positive control dam). Fetuses that were not full term at necropsy (pregnant upon second insemination after returning to estrus) were excluded from analysis.

**Seroconversion to PCV2**

Negative control dams remained negative for PCV2-specific antibodies throughout the study (Figure I; Table II). All 1-VAC-PCV2 and 2-VAC-PCV2 dams had seroconverted to PCV2 prior to the first blood collection at DPI -7 (Table II). Five of six 1-LIVE-VAC and 5/6 1-LIVE-VAC-PCV2 dams had seroconverted by DPI 7, respectively. There was a
significant group by time interaction (p<0.001). There was no significant difference in antibody levels between the 1-VAC-PCV2 and 2-VAC-PCV2 groups at any time points except at DPI -7 (Figure I). Also, there was no significant difference in antibody levels at any time points between the 1-LIVE-VAC and 1-LIVE-VAC-PCV2 groups (Figure I). Dams vaccinated with the inactivated vaccine had significantly higher (p<0.05) S/P ratios as detected by ELISA compared to those vaccinated with the live-attenuated vaccine at every DPI throughout the course of the study. Positive controls had similar S/P ratios as the LIVE-VAC and 1-LIVE-VAC-PCV2 groups from DPI 28 through DPI 105 (Figure I). All 39 fetuses from all four litters were negative for PCV2-specific antibodies at derivation (data not shown).

Neutralizing antibodies

At the day of challenge, the log$_{10}$ transformed FFN titers were 2.71±0.08 for 1-VAC-PCV2, 2.96±0.24 for 2-VAC-PCV2, 1.57±0.17 for 1-LIVE-VAC, 1.52±0.13 for 1-LIVE-VAC-PCV2, 0.28±0.17 for the negative control group, and 0.28±0.18 for the positive control group. The inactivated chimeric PCV1-2 vaccine induced significantly higher (p<0.05) levels of neutralizing antibodies compared to the live-attenuated vaccine prior to challenge.

PCV2 viremia

Negative control dams as well as the 1-LIVE-VAC dams remained negative for PCV2 viremia throughout the study (Table III). There was a significant group by time interaction (p<0.001). Specifically, positive control dams developed PCV2 viremia by DPI 14 and had significantly higher (p<0.05) levels of viremia from DPI 14 through DPI 56 when
compared to vaccinated groups (Figure II). Challenged, vaccinated dams had detectable PCV2 DNA at varying DPIs during the study (Table III) without significant differences among the vaccinated groups at any time point (Figure II). The prevalence and concentration of PCV2 DNA detected in the fetuses are summarized in Table IV.

**PCV1-2 viremia**

All dams remained negative for PCV1-2 DNA throughout the course of the study. Three of 14 fetuses from the 1-LIVE-VAC-PCV2 litter were positive for PCV1-2 DNA with an average $\log_{10}$ transformed mean amount PCV1-2 DNA of 6.45±0.31 per ml. All other piglets were negative for PCV1-2 DNA (data not shown).

**Macrosopic lesions**

Lungs, lymph nodes, and the reproductive tract of the dams were examined for gross lesions and all were macroscopically normal.

**Immunohistochemistry**

All 10 piglets from the negative control dam and all 29 piglets from the 2-VAC-PCV2 and 1-LIVE-VAC-PCV2 dams were negative for PCV2 antigen in heart tissues. Low-to-abundant amounts of PCV2 antigen were detected in 3 of 13 fetuses from the positive control dam.

**Discussion**
Evidence from the field supports an association of PCV2 with reproductive failure. Although limited experimental trials have been conducted in breeding age animals, sows developed PCV2 viremia when inseminated with PCV2-positive semen (10). In the current study, sows were inseminated with PCV2 spiked semen and viremia was detected in the positive control group further confirming that PCV2 is infectious when administered via artificial insemination. Very limited evidence is available in the literature on the efficacy of use of inactivated PCV2 vaccines in breeding age animals indicating that PCV2-vaccinated dams still may deliver PCV2 positive piglets (33), potentially an important source of PCV2 transmission in pig production systems.

Currently there are three commercial vaccines on the market approved for use in healthy pigs (three weeks of age or older), and one vaccine is approved for use in both dams and growing pigs. All of these commercial vaccines are inactivated. Experimental and field trial evidence clearly demonstrates that use of these vaccines reduces losses associated with PCVAD in growing pigs (34,35). In the current study, for the first time an experimental attenuated live PCV1-2 vaccine was utilized in breeding age animals, and compared to two different dose regimens of the inactivated version of the vaccine. The results indicate that the use of the three different vaccination protocols all decreased PCV2 viremia and increased anti-PCV2-specific antibody production in dams when compared to the positive control group regardless of inactivation of the vaccine virus.

The exact mechanism for the decrease of PCV2 viremia in vaccinated animals is unknown but likely due to development of a combination of cellular and humoral immune responses. Interestingly, the groups vaccinated with the inactivated chimeric PCV1-2 vaccine (1-VAC-PCV2 and 2-VAC-PCV2) developed detectable PCV2-specific antibody levels
earlier and maintained higher levels throughout the study compared to those vaccinated with the live-attenuated chimeric vaccine (1-LIVE-VAC and 1-LIVE-VAC-PCV2). In contrast to these results in breeding age animals, a previous study comparing several commercial and experimental vaccines in young pigs found no significant differences between any of the commercial vaccines (29).

In Canada, PCV1-2 was recently isolated from three different pigs in three different herds vaccinated with a commercial PCV2 vaccine based on an inactivated chimeric PCV1-2 (17). Several studies have shown that the PCV1-2 infectious DNA clones and the live-attenuated PCV1-2 vaccine are capable of inducing an antibody response, but are attenuated in pigs and not capable of producing any characteristic lesions of PCV2 infection (11,12,36). In the current study, the 1-LIVE-VAC dams had no evidence of PCV2 or PCV1-2 viremia; however, they did develop a PCV2-specific antibody response. The 1-LIVE-VAC-PCV2 dams developed PCV2 viremia at selected time points at low levels which quickly resolved. Overall, the results are similar to previous studies where some PCV1-2 vaccinated animals developed viremia while others did not; however, as in this study, the majority of the animals seroconverted and had no or minimal lesions characteristic of PCV2 infection (12,36). These results further confirm that the live PCV1-2 chimeric vaccine is capable of inducing a humeral immune response and remains attenuated.

In this study, only 11.4% (4/35) of the sows became pregnant during the first artificial insemination and only 37.1% (13/35) sows became pregnant overall. Low reproductive rates are not uncommon in older parity sows. In addition, PCV2 has been implicated in early embryonic death (37) and re-absorption leading to irregular or regular return to estrus. Several sows arrived at the research facility with varying stages of pregnancy which were
purposely terminated, potentially causing complications in their ability to conceive during the subsequent artificial insemination attempts. However, previously, using the same insemination technique and PCV2 dose, conception rates of 88.9% (8/9) (38) and 66.7% (6/9) (10) were observed.

Among the pregnant animals, PCV2-positive piglets were detected in all three challenged sows regardless of vaccination status further confirming that dam vaccination does not prevent vertical PCV2 transmission. The fact that sows can still deliver PCV2-positive piglets causes some to question the value of vaccination in breeding age females; however, while the vaccines did not entirely prevent PCV2 spread to fetuses in the current study, vaccination did decrease the overall PCV2 load in the dam and fetuses.

The vaccination protocols in the pregnant sows (1-LIVE-VAC-PCV2 and 2-VAC-PCV2) resulted in a decrease in PCV2 DNA detection in piglets (28.6% and 66.7%, respectively) when compared to the positive control group (100%) indicating both types of the PCV1-2 chimeric vaccine are efficacious in breeding age animals. However, due to the low numbers of pregnancies in this study, these results should be interpreted with caution. In fetuses, PCV2 antigen detection was performed on fetal heart tissues since it was previously shown that PCV2 antigen in fetuses was found predominantly in the heart and tonsil of piglets (33,38). PCV2 antigen was only detected in the positive control piglets in the current study. This is further confirmation that both vaccines are efficacious in decreasing levels of PCV2.

To further evaluate the safety of the live-attenuated PCV1-2 vaccine, all fetuses were tested for presence of PCV1-2 DNA. Four of 14 piglets from the 1-LIVE-VAC-PCV2 dam were positive for PCV1-2 DNA at birth; however, no lesions were noted and no PCV2
antigen was detected in the fetal myocardium. As only fetal heart tissues were investigated in the current study, the tissue tropism of PCV1-2 is unknown, and PCV1 has been previously identified in fetal lung tissues (20), PCV1-2, although unlikely, could have been present elsewhere in the fetal tissues. As a PCV1-2 chimeric virus was identified in Canadian swine herds, the risks of introduction of a live-attenuated PCV2 vaccine for use in breeding age females needs to be seriously considered before approval.

**Conclusion**

To our knowledge this is the first study investigating the use of a live PCV2 vaccine in breeding animals. While the inactivated chimeric PCV1-2 vaccine was capable of inducing higher levels of PCV2-specific antibodies throughout the course of this study when compared to the live-attenuated experimental chimeric vaccine, no significant differences among vaccinated groups in levels and duration of PCV2 viremia were found. All vaccines were capable of reducing viremia in dams and despite some cross-placental transmission of PCV2, the piglets obtained from the two vaccinated dams had lower levels of PCV2 in comparison to those obtained from the non-vaccinated dam. Live-vaccine virus viremia was detected in selected dams and piglets at low levels which was not associated with myocardial lesions or antigen suggesting that the vaccine may be safe and effective for broader use in breeding herds.

**Acknowledgements**

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References


Table I

Experimental design.

<table>
<thead>
<tr>
<th>Group</th>
<th>Animals</th>
<th>Mean age in days (95% CI)</th>
<th>PCV2 Vaccine</th>
<th>PCV2 inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative Control</td>
<td>5</td>
<td>1,048 (1,290; 806)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Positive Control</td>
<td>6</td>
<td>1,174 (1,421; 928)</td>
<td>-</td>
<td>- Spiked semen</td>
</tr>
<tr>
<td>2-VAC-PCV2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6</td>
<td>1,247 (1,654; 841)</td>
<td>YES</td>
<td>YES Spiked semen</td>
</tr>
<tr>
<td>1-VAC-PCV2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6</td>
<td>1,291 (1,836; 746)</td>
<td>YES</td>
<td>- Spiked semen</td>
</tr>
<tr>
<td>1-LIVE-PCV2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6</td>
<td>1,462 (1,991; 935)</td>
<td>YES</td>
<td>- Spiked semen</td>
</tr>
<tr>
<td>1-LIVE-VAC&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6</td>
<td>1,363 (1,719; 1,008)</td>
<td>YES</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup>Dams vaccinated with Suvaxyn® PCV.

<sup>b</sup>Dams vaccinated with an experimental live-attenuated chimeric PCV1-2 vaccine.
Table II

Group prevalence of PCV2-specific antibodies in serum. Groups that contain seropositive pigs are shaded in grey. Vaccines were administered at -35 days post inoculation and in the case of the 2 dose product a second dose was given at -14 days post inoculation. Dams were challenged at 0 days post inoculation with PCV2 free or PCV2 spiked semen.

<table>
<thead>
<tr>
<th>Group</th>
<th>Days post inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-7</td>
</tr>
<tr>
<td>1-LIVE-VAC</td>
<td>1/6</td>
</tr>
<tr>
<td>1-LIVE-VAC-PCV2</td>
<td>2/6</td>
</tr>
<tr>
<td>Negative controls</td>
<td>0/5</td>
</tr>
<tr>
<td>Positive controls</td>
<td>0/6</td>
</tr>
</tbody>
</table>

\(^a\)One negative control dam and a 1-LIVE-VAC-PCV2 dam developed severe lameness and were euthanized at DPI 84.
Table III

Group prevalence of PCV2 DNA in serum. Groups that contain viremic pigs are shaded in grey. Vaccines were administered at -35 days post inoculation and in the case of the two dose product a second dose was given at -14 days post inoculation. Dams were challenged at 0 days post inoculation with PCV2 free or PCV2 spiked semen.

<table>
<thead>
<tr>
<th>Group</th>
<th>Days post inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1-VAC-PCV2</td>
<td>0/6</td>
</tr>
<tr>
<td>2-VAC-PCV2</td>
<td>0/6</td>
</tr>
<tr>
<td>1-LIVE-VAC</td>
<td>0/6</td>
</tr>
<tr>
<td>1-LIVE-VAC-PCV2</td>
<td>0/6</td>
</tr>
<tr>
<td>Negative controls</td>
<td>0/5</td>
</tr>
<tr>
<td>Positive controls</td>
<td>0/6</td>
</tr>
</tbody>
</table>

*One negative control dam and a 1-LIVE-VAC-PCV2 dam developed severe lameness and were euthanized at DPI 84.
Table IV

Mean group amount of $\log_{10}$ PCV2 DNA and prevalence in serum or fetal thoracic fluid of piglets born to dams vaccinated or unvaccinated and infected with PCV2. A sample with no threshold cycle ($C_T$) value during the 40 amplification cycles was considered negative.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PCV2 DNA</th>
<th>Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-VAC-PCV2</td>
<td>3.09±0.67</td>
<td>10/15</td>
</tr>
<tr>
<td>1-LIVE-VAC-PCV2</td>
<td>1.65±0.74</td>
<td>4/14</td>
</tr>
<tr>
<td>Negative Control</td>
<td>0.00±0.00</td>
<td>0/10</td>
</tr>
<tr>
<td>Positive Control</td>
<td>6.72±0.57</td>
<td>13/13</td>
</tr>
</tbody>
</table>
Figure I

Mean group PCV2 ELISA sample-to-positive (S/P) ratios in serum at different days post inoculation. An S/P ratio equal or greater than 0.2 was considered positive. Groups with different letters (A,B,C) on a certain day post inoculation have significantly (p<0.05) different group S/P ratios.
**Figure II**

Mean group amount of \(\log_{10}\) PCV2 DNA in serum at different days post inoculation. A sample with no threshold cycle (\(C_T\)) value during the 40 amplification cycles was considered negative. Asterisks indicate a significant (\(p<0.05\)) difference between positive controls and all other PCV2 challenged groups (1-LIVE-VAC-PCV2, 1-VAC-PCV2, 2-VAC-PCV2) on that day post inoculation.
CHAPTER 4: VACCINATION WITH INACTIVATED OR LIVE-ATTENUATED CHIMERIC PCV1-2 RESULTS IN DECREASED VIREMIA IN CHALLENGE-EXPOSED PIGS AND MAY REDUCE TRANSMISSION OF PCV2

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Abstract

The objectives were to determine transmissibility of PCV2 to naïve contact pigs 140 days after infection of resident pigs and the benefit of vaccination with live-attenuated or inactivated chimeric PCV2 vaccines on chronic PCV2 infection. Twelve 6-week old PCV2 naïve pigs were randomly divided into four groups of three pigs: negative controls, positive controls, and pigs vaccinated with either a live-attenuated or inactivated chimeric PCV1–2 vaccine. All animals were bled weekly and tested for anti-PCV2 antibodies and PCV2 and PCV1–2 DNA and all groups except negative controls were challenged at 10 weeks. Two pigs vaccinated with the live PCV2 vaccine were PCV1–2 viremic at a single observation point. Both vaccine regimens induced an anti-PCV2 antibody response which was detected sooner and reached a higher level with the commercial inactivated vaccine. Both vaccines significantly decreased the concentration and duration of PCV2 viremia compared to the positive controls. PCV2 DNA was detected in lymphoid tissues of 1/3 pigs in the live-attenuated vaccine group and 3/3 positive control pigs. Three, 2-week old, PCV2 naïve
contact pigs were comingled with each group at 168 days post-vaccination or 140 days post-challenge. After seven days of co-housing, the resident pigs were removed and the contact pigs remained for six weeks. Evidence of chimeric PCV1–2 vaccine or PCV2 challenge virus transmission to naïve contact pigs was lacking in all groups. The results of this study suggest that 140-day closure of a small pig population in a controlled environment may result in stabilization and elimination of PCV2.

Keywords: Porcine circovirus type 2, vaccination, transmission

1. Introduction

Porcine circovirus type 2 (PCV2) is a ubiquitous virus found in most pork producing regions (Patterson and Opriessnig, 2010) and has been identified as the main cause of a series of diseases collectively called porcine circovirus associated disease (PCVAD). PCVAD has been implicated in high production losses and most commonly manifests as post-weaning multisystemic wasting syndrome, reproductive failure, enteritis, or respiratory disease (Opriessnig et al., 2007; Gillespie et al., 2009).

In order to understand and control PCVAD, knowledge of the transmission of PCV2 and proper disinfection protocols are important. A substantial amount of work has been done testing the efficacy of cleaning and disinfection protocols for PCV2 in research facilities and transportation vehicles (Martín et al., 2008; Patterson et al., 2011a) and PCV2 was found to be stable in the environment and resistant to most disinfectants (Patterson and Opriessnig, 2010). Moreover, PCV2 can be detected for extend periods of time in serum and tissues of pigs after experimental infections (Opriessnig et al., 2010b; Patterson et al., 2011b). The
often long duration of PCV2 viremia in certain pigs raises questions and concerns about the related risk of PCV2 transmission to PCV2 negative cohorts.

Several commercial PCV2 vaccines have been developed to reduce or prevent PCVAD in pigs. Two of these vaccines are subunit vaccines based on PCV2 capsid protein expressed in baculovirus, one is an inactivated PCV2 vaccine, and one is an inactivated chimeric PCV1–2 vaccine. The chimeric PCV1–2 vaccine is produced with the ORF2 capsid gene of the PCV2a cloned into the genomic backbone of the non-pathogenic PCV1 (Fenaux et al., 2003, 2004). A reformulated version of the chimeric PCV1–2 vaccine (Suvaxyn® PCV, Fort Dodge Animal Health Inc.) re-entered the market in August 2011 under a new brand name (Fostera™ PCV, Pfizer Animal Health Inc.).

The objectives of this study were (1) to determine the ability of PCV2 to be transmitted to naïve pigs 140 days after infection of the resident animals and (2) to determine if vaccination with a commercial inactivated PCV1–2 vaccine or an experimental live-attenuated PCV1–2 vaccine is safe and beneficial in this scenario.

2. Materials and methods

2.1. Pig source and arrival

Twelve, 6-week-old, pigs (‘‘residents’’) were obtained from a herd confirmed to be free of PCV2 and porcine reproductive and respiratory syndrome virus (PRRSV) as determined by routine serology. Twelve 2-week-old pigs (‘‘contacts’’) from the same herd as the first group of pigs were weaned and transported to the isolation facility to serve as contact pigs. The pigs were transported 168 days apart (Fig. 1) to a Biosafety Level 2 (BSL-2) animal facility at Iowa State University, Ames, IA.
2.2. Animals housing

The resident pigs were randomly assigned to one of three groups and were kept in four separate 2 m x 2.5 m rooms. Each room had one pen and was equipped with one nipple drinker and pigs were fed daily with a pelleted feed ration that contained whey but was free of other animal proteins and antibiotics (Nature’s Made; Heartland Co-op; Cambridge, IA). On day R154 (=154 days after the resident pigs were vaccinated), the resident pigs were moved into larger rooms which contained two pens separated by a gate with vertical bars that allowed for nose-to-nose contact. The resident pigs were kept in one of the two pens. On day R168, three contact pigs were placed in a separate pen (=arrival of contact pigs or C0) with nose-to-nose contact with the resident pigs.

2.3. Experimental design

The experimental protocol was approved by the Iowa State University Institutional Animal Care and Use Committee and the timeline for the experiment is summarized in Fig. 1. Six-week-old PCV2 naïve resident pigs were either vaccinated with an experimental live-attenuated PCV1–2 vaccine (n = 3), vaccinated with a commercial inactivated PCV1–2 vaccine (n = 3), or remained unvaccinated (n = 6). A sample size of three was chosen as it is the smallest number considered reasonable for statistical analysis and because the limited availability of PCV2-free pigs and funding prevented the use of larger group sizes. On day R28, when the resident pigs were 10 weeks old, 9 of the 12 pigs were inoculated intranasally (2 ml) and intramuscularly (1 ml) with PCV2b. All pigs were bled once a week until necropsy and the serum samples were tested for the presence of PCV2 DNA, chimeric
PCV1–2 vaccine virus DNA and anti-PCV2 antibodies. At day R168 (=140 days post challenge) or C0, three PCV2 naïve contact pigs were moved into each room. Similarly to the resident pigs, blood was collected from the contact pigs weekly. Necropsy was conducted on day R175 for the 12 resident pigs and on day C49 for the 12 contact pigs. Macroscopic and microscopic lesions were compared between groups and lymphoid tissues were assessed for presence of PCV2 antigen and DNA.

2.4. Clinical observations, vaccination, inoculation, and sample collections

All pigs were examined daily for signs of clinical disease such as lethargy, respiratory disease, inappetence and lameness. On day R0, resident pigs in the inactivated vaccine group were vaccinated with 2 ml of Suvaxyn® PCV (now reformulated and known as ‘‘Fostera™ PCV’’ from Pfizer Animal Health Inc.) based on a chimeric PCV1–2a. The resident pigs in the live-attenuated vaccine group were vaccinated with 2 ml of an experimental live-attenuated PCV2 vaccine based on a chimeric PCV1–2b (Beach et al., 2010) that has been shown to decrease viremia, microscopic lesions, and PCV2 antigen (Opriessnig et al., 2011). Vaccination was done by intramuscular injection into the right neck area. On day R28, vaccinated and positive control pigs were challenged with 2 ml (intra-nasally) and 1 ml (intramuscularly) of a PCV2b virus stock inoculum. The PCV2 isolate NC-16845 (Opriessnig et al., 2008) used for the challenge was based on an infectious clone as described (Opriessnig et al., 2008) and further propagated in PK-15 cells to an infectious titer of $10^{4.5}$ 50% tissue culture infective dose (TCID$_{50}$) per ml. The weekly collected blood samples from resident and contact were centrifuged at 3220 x g for 10 min at 4°C, and the serum was aliquoted into 5 ml polystyrene round bottom tubes and stored at -20°C until testing. Lymph
node samples (tracheobronchial lymph nodes, mesenteric lymph nodes, mediastinal lymph nodes, superficial inguinal lymph nodes) were collected from each pig at necropsy and stored at -80°C until testing.

2.5. Laboratory methods used

2.5.1. Serology

All serum samples were tested for anti-PCV2 antibodies using an indirect PCV2-ORF2-based ELISA as previously described (Nawagitgul et al., 2002). The results were expressed as sample-to-positive (S/P) ratio. Samples were considered to be negative if the S/P ratio was less than 0.2, and positive if the S/P ratio was greater than or equal to 0.2. In addition, all serum samples from resident pigs obtained on day R28 (day of PCV2 challenge) were tested for PCV2-specific neutralizing antibodies using a fluorescence focus neutralization (FFN) assay (Pogranichniy et al., 2000). Virus neutralizing titers were expressed as the highest serum dilution in which 50% of virus is neutralized compared to the control virus.

2.5.2. PCV2 and PCV1–2 DNA detection

All serum samples and pooled lymph nodes were tested for the presence and quantity of PCV2 DNA by a quantitative real-time PCR (Opriessnig et al., 2003). Total DNA was extracted from serum samples using the MagMax™ Viral Isolation Kit (Applied Biosystems, Life Technologies, Carlsbad, CA) on the KingFisher Flex System (ThermoFisher Scientific, Pittsburgh, PA). Total DNA was extracted from lymph node homogenates using the QIAamp® DNA Mini Kit (Qiagen, Valencia, CA). In addition, the sera and lymphoid tissues
were also tested for chimeric PCV1–2 vaccine virus DNA by real-time PCR as described (Shen et al., 2010). A sample was considered negative if no threshold cycle (CT) was detected in 40 amplification cycles.

2.6. Post mortem examination

2.6.1. Necropsy

All pigs were humanely euthanized by intravenous pentobarbital sodium overdose (Fatal Plus®, Vortech Pharmaceuticals, Ltd., Dearborn, MI) and necropsied at day R175 (31 weeks of age, resident pigs) or at day C49 (9 weeks of age, contact pigs), respectively. The total extent of macroscopic lung lesions (ranging from 0% to 100%) was scored subjectively by a veterinary pathologist (TO) as previously described (Halbur et al., 1995). Lymph nodes were scored from 0 (normal) to 4 (enlarged, 3 times normal size) (Opriessnig et al., 2004). Sections of tracheobronchial lymph nodes, mesenteric lymph nodes, mediastinal lymph nodes, superficial inguinal lymph nodes, tonsil, thymus, spleen, kidney, liver, heart, small intestine, colon and lungs were collected at necropsy and fixed in 10% neutral buffered formalin. Tissues were then routinely processed for histological examination, embedded in paraffin and stained with hematoxylin and eosin. Sections of all lymph nodes were also collected in separate bags for PCR analysis as described under Section 2.4.

2.6.2. Histopathology

Microscopic lesions were examined and scored by a veterinary pathologist (TO) blinded to the treatment groups as described (Opriessnig et al., 2004). Briefly, lung tissues were scored for the severity of interstitial pneumonia ranging from 0 (normal) to 6 (severe
Sections of thymus, kidney, liver, heart, small intestine, and colon were scored for the severity of lymphohistiocytic inflammation ranging from 0 (none) to 3 (severe). Sections of lymphoid tissues (lymph nodes, tonsil and spleen) were scored for lymphoid depletion ranging from 0 (none) to 3 (severe) and for lymphohistiocytic inflammation and replacement of follicles ranging from 0 (none) to 3 (severe).

2.6.3. Immunohistochemistry

Immunohistochemical detection of PCV2-specific antigen was performed on selected formalin-fixed sections of lymphoid tissues using a rabbit polyclonal antiserum (Sorden et al., 1999). Antigen scoring was performed by a veterinary pathologist (TO) blinded to treatment groups and scores were reported from 0 (no antigen detected) to 3 (more than 50% of cells contained PCV2 antigen) as previously described (Opriessnig et al., 2004).

2.7. Statistical analysis

Statistical analysis of the data was performed using the JMP® software version 9.0.0 (SAS Institute, Cary, NC). Summary statistics were calculated for all groups to assess the overall quality of the data including normality. One-way analysis of variance (ANOVA) was used to evaluate the differences among treatment groups. If differences in group means were observed then Tukey–Kramer test was used for each pair-wise comparison. A P-value of less than 0.05 was set as a statistically significant level throughout this study. Real-time PCR results (PCV2 DNA copies per ml of serum) and FFN titers were log10 transformed prior to statistical analysis. All group means were calculated using results from all animals in each group with negative results reported as 0 for the statistical analysis.
3. Results

3.1. Clinical observations

One positive control pig developed lameness 63 days after PCV2 challenge, was treated with 1 ml ceftiofur (Excede®, Pfizer Inc., New York City, NY) and subsequently recovered. All other animals remained clinically healthy.

3.2. Seroconversion to PCV2 and neutralizing antibodies

3.2.1. Anti PCV2-IgG

All resident negative controls (Fig. 2) and all contact pigs remained seronegative for PCV2 for the duration of the study. All animals vaccinated with the inactivated PCV2 vaccine seroconverted to PCV2 by day R21 and remained seropositive for the remainder of the study. All animals vaccinated with the live-attenuated PCV2 vaccine seroconverted between days R28 and R42 and remained seropositive for the remainder of the study. All positive control pigs seroconverted to PCV2 by day R49 (which corresponds to 21 days after PCV2 challenge) and remained seropositive for the remainder of the study. In addition to earlier seroconversion, the pigs vaccinated with the inactivated PCV2 vaccine had significantly (P < 0.05) higher mean group anti-PCV2 IgG S/P ratios than those vaccinated with the live-attenuated PCV2 vaccine from day R21 through R49 (Fig. 2).

3.2.2. Neutralizing antibodies

The pigs vaccinated with the inactivated PCV2 vaccine had significantly (P < 0.05) higher concentrations of neutralizing antibodies at day R21 than pigs vaccinated with the
live-attenuated PCV2 vaccine (2.71 ± 0.00 versus 1.61 ± 0.26) at which time positive and negative control pigs had no detectable anti-PCV2 neutralizing antibodies.

3.3. PCV2 and PCV1–2 DNA detection in sera and lymphoid tissues

3.3.1. PCV2

All resident negative controls and all contact pigs remained negative for PCV2 DNA for the duration of the study (data not shown). Positive control animals became PCV2 viremic at day R35 (7 days post PCV2 challenge) and remained viremic until day R147 (119 days post PCV2 challenge) and had significantly (P < 0.05) higher concentration of DNA from PCV2 in serum compared to all other groups (Fig. 3). The group mean amount of log10 PCV2 DNA was significantly (P < 0.05) lower for both vaccinated groups compared to the positive control group on days R42, R63, R70, R77 and R84. In both vaccinated groups, PCV2 DNA was detected sporadically throughout the study (Fig. 3). At necropsy, PCV2 DNA in lymphoid tissues was detected in 3/3 positive controls and 1/3 pigs in the live-attenuated vaccine group. PCV2 DNA was not detected in any of the lymphoid tissues of the other resident or contact pigs (data not shown).

3.3.2. PCV1–2

Two of three animals vaccinated with the live-attenuated PCV2 vaccine were positive for PCV1–2 DNA in serum on days R21 and R28 after vaccination, respectively. All other animals (residents and contacts) were negative for PCV1–2 DNA at all time points tested. PCV1–2 DNA was not detected in any of the lymphoid tissues.
3.4. Macroscopic and microscopic lesions and amount of PCV2 antigen

No macroscopic lesions were noted on any animals at necropsy. No microscopic lesions were observed in the lymphoid tissues of pigs (residents and contacts) in any of the treatment groups. Low amounts of PCV2 antigen (score 1) were detected in lymphoid tissues and tonsil of two of three resident positive control pigs. In addition, individual resident pigs from all treatment groups had mild interstitial pneumonia (score 1) characterized by increased numbers of lymphocytes and macrophages in alveolar septa.

4. Discussion

The current study demonstrated that a small closed group of growing pigs exposed to PCV2 140 days earlier did not transmit PCV2 to naïve contact pigs suggesting that under the conditions of the study, closure of the population to new animal entries for 140 days may stabilize population immunity resulting in elimination of infectious PCV2. It should be noted that the risk of re-exposure from the environment (PCV2 present in the rooms) was minimized in the current study by moving the population into disinfected rooms at 128 days post challenge. This work needs to be repeated under field conditions to increase sample size and better simulate population infection dynamics where not all pigs in the population are infected on the same day and co-infections are common.

Several studies have shown that acutely infected pigs can transmit PCV2 to naïve contact animals (Bolin et al., 2001; Patterson et al., 2011b), but to the authors’ knowledge the transmissibility of PCV2 from long-term infected pigs to naïve animals has not been examined to date. In our earlier study in growing pigs, four of six animals inoculated with PCV2a were positive for PCV2 DNA at 140 days after challenge; however, viremia was
intermittent from 63 to 140 days post challenge (Opriessnig et al., 2010b). Similar results were seen in the current study where PCV2 viremia in unvaccinated, PCV2 challenged pigs was intermittent between 77 and 119 days after infection; however, no evidence of viremia was detected after day 119 post challenge. One difference that could account for the discrepancy in length of detection of viremia between studies (140 days versus 119 days post challenge) is that different DNA extraction methods were used in the two studies with potential differences in sensitivity. Another possibility for the overall shorter viremia length in the positive control pigs in the current study could be the use of different PCV2 challenge isolates. In the previous study a PCV2a isolate was used for challenge (Opriessnig et al., 2010b) while a PCV2b isolate was used in this current study. Additionally, due to limited availability of PCV2 free pigs of different ages, 2-week-old contact pigs were used and were brought in nose-to-nose contact with the older resident pigs. The age difference could be important for overall contact time and could have affected the infection rate in this study.

Interestingly, in the current study, PCV2 DNA was detected in lymphoid tissues of all positive controls but only in one of six vaccinated pigs (attenuated-live vaccine group) at the time of necropsy perhaps indicating that vaccination could reduce chronic infection. Most published experiments investigating PCV2 vaccine efficacy have been terminated 21 days after infection with PCV2 (Fenaux et al., 2004; Fort et al., 2009; Shen et al., 2010; Opriessnig et al., 2010a, 2011; Xujie et al., 2011). One of the goals of this study was to determine the efficacy of an inactivated and a live-attenuated PCV1–2 vaccine in reducing viremia and lesions in pigs over a longer period of time. Similar to previous experiments, both vaccines substantially reduced detectable PCV2 viremia. Interestingly, all animals vaccinated with the inactivated PCV2 vaccine developed higher concentrations of anti-PCV2
IgG more quickly post-vaccination than those vaccinated with the live-attenuated vaccine, whereas in a previous study no significant differences in mean group anti-PCV2 S/P ratios were observed between the two vaccinated groups (Shen et al., 2010). After day 49 (21 days post PCV2 challenge), both vaccinated groups in this study continued to have intermittent, sporadic viremia; however, all vaccinated pigs were negative for PCV2 DNA after day 126.

In the current study, PCV1–2 vaccine viremia was not detected in any of the pigs vaccinated with the inactivated commercial vaccine. However, we did detect a low concentration of PCV1–2 DNA at days R21 and R28 in two of three animals after vaccination with the live-attenuated PCV2 vaccine which is consistent with previous reports (Opriessnig et al., 2011). PCV1–2 vaccine viremia was not detected in any contact pigs throughout the study indicating that vaccine virus was not transmissible 168 days after vaccination.

PCV2-associated microscopic lesions were not observed in this study, and this was not unexpected since PCV2-associated lesions typically develop around 21 days after PCV2 infection in experimentally infected pigs and begin to resolve approximately two weeks later (Opriessnig et al., 2010b). Since the time of necropsy in this study was at 147 days after PCV2 infection, as expected, only minimal lesions were observed and no significant differences were found between groups. Fecal and nasal viral excretion of PCV2 were not measured in this study, but could prove useful in a future study. Although the results from this pilot study are encouraging for application of herd closure, which is essentially closure of the population to new animal entries, to reduce PCV2 transmission and PCVAD, more work needs to be conducted using larger numbers of pigs under field conditions to verify the data from this pilot study.
5. Conclusions

Under the study conditions, PCV2 virus was not transmitted to naïve pigs that had nose-to-nose contact with pigs that had been challenged 140 days earlier implying that closure of a pig population may result in stabilization and elimination of PCV2. The attenuated and live vaccines performed similar in reducing the duration and concentration of PCV2 viremia in vaccinated pigs. In addition, live PCV2 vaccine virus was not transmitted to naïve pigs with nose-to-nose contact to vaccinated pigs 168 days after vaccination.

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Fig. 1. Experimental design indicating the timeline in days for each event for resident pigs (indicated by an “R”) and contact pigs (indicated by a “C”).
Fig. 2. Mean group amount of anti-PCV2 IgG (± SEM) in serum of resident pigs vaccinated with an inactivated or a live PCV1–2 vaccine and in positive and negative control groups. The results are expressed as ELISA sample to positive (S/P) ratio. A sample was considered negative if the S/P ratio was less than 0.20. *Indicates significantly (P < 0.05) different group mean S/P ratios between the two vaccinated groups.
**Fig. 3.** PCV2 DNA concentration presented as mean group log10 PCV2 DNA (± SEM) in serum samples and prevalence of PCV2 DNA positive resident pigs vaccinated with an inactivated or a live PCV1–2 vaccine and in positive control animals after PCV2 challenge at day 28. The prevalence was combined for the two vaccinated groups. A sample was considered to be negative if the no signal was detected in the 40 cycles.
CHAPTER 5: GENERAL CONCLUSIONS

Porcine circovirus (PCV) type 2 (PCV2) has been associated with a series of disease manifestations in swine including respiratory disease and wasting syndrome in growing pigs and reproductive failure in breeding herds (Gillespie et al., 2009). In addition, PCV2 is transmitted by both horizontal and vertical routes. Horizontal transmission has been shown to occur through presence of viable PCV2 in secretions and excretions (Patterson et al., 2010a). Vertical transmission has been shown to occur in-utero from the dam to the fetus (Madson et al., 2011).

Several commercial and experimental vaccines have been developed in an attempt to prevent PCV-associated disease (PCVAD) in animals. PCV2 infection of dams via artificial insemination of spiked semen has been shown to result in dam viremia, fetal infections, and increased numbers of mummified and still-born fetuses (Madson et al., 2009b). Despite the fact that commercially available vaccines for use in breeding animals are currently not available in the United States, vaccination of dams with a single dose of Ingelvac® CircoFLEX™ prior to artificial insemination with spiked semen was not effective in preventing vertical transmission of PCV2 (Madson et al., 2009a). The previous work only used one product in a one dose application. In growing pigs, minor differences between one and two dose administrations in preventing PCV2 infection have been detected (Shen et al., 2010b) and this may be similar in sows and their fetuses. Additionally, no group had yet determined the safety and efficacy of an experimental live vaccine in breeding age females. Thus, the main objectives of our first study were to determine if different PCV2 vaccines and dose administrations could decrease or prevent PCV2 infection in sows. In addition, we
wanted to determine if any vaccination regimen was able to decrease or even prevent vertical transmission of PCV2.

In experimentally infected growing pigs, PCV2 viremia is detectable for up to 140 days (Opriessnig et al., 2010b), has been shown to be shed for up to 70 days post-infection in some secretions (Shibata et al., 2003; Patterson et al., 2010b) and was transmissible when infected pigs were comingled with naïve contact animals 42 days post-challenge (Bolin et al., 2001). Despite several studies showing presence of PCV2 in secretions and successful horizontal transmission of PCV2 between infected and naïve contact pigs, no study had yet determined if chronically PCV2 infected animals can transmit PCV2 to naïve contact pigs and if vaccination is beneficial in this scenario. Additionally, the safety of an experimental live vaccine, and its ability to prevent PCV2 transmission to contact pigs, has not been evaluated for more than 21 days post challenge (Opriessnig et al., 2011a; Beach et al., 2010). Therefore, the main objectives of our second study were to determine if PCV2 could be spread to naïve contact animals 140 days after PCV2 challenge, if vaccination with an inactivated or experimental-live vaccine is beneficial, and if the live vaccine is transmitted 168 days post-vaccination to naïve pigs.

The results from this thesis indicate that vaccination of pigs with an attenuated live chimeric PCV1-2 vaccine induced significantly lower levels of PCV2-specific antibodies and had delayed development in comparison to the inactivated adjuvanted vaccine version. Vaccine viremia was not detected in any of our dams, but low levels of PCV1-2 were detected in a few fetuses. Similarly, vaccination of growing pigs with the attenuated live chimeric PCV2 vaccine resulted in PCV1-2 viremia in individual vaccinated animals at single days indicating that the live vaccine could be safe for use in breeding and finishing.
herds. In addition, vaccination of sows and growing pigs prior to PCV2 challenge led to decreased PCV2 viremia which was independent of vaccine type used (live or inactivated) or number of doses administered, indicating that despite lower levels of PCV2-specific antibodies with the attenuated live chimeric PCV2 vaccine, both vaccines were effective. In addition, vaccination with the attenuated live chimeric PCV1-2 vaccine led to decreased prevalence of PCV2 DNA in piglets born to vaccinated animals in comparison to the inactivated chimeric PCV1-2 vaccine.

The results of the first study also further verify that dam vaccination prior to challenge does not prevent *in-utero* spread of PCV2 to fetuses, but resulted in reduced levels of PCV2 viremia in sows and fetuses. Additionally, by closing an infected swine population for 140 days, under the conditions of the second study, the animal immunity was stabilized, preventing the spread of PCV2 to the contact animals in all groups. Unfortunately, the infectivity of PCV2 potentially present in the environment could not be determined as the PCV2 infected pigs were moved to clean rooms shortly before comingling with the contact pigs.

While these studies are able to provide evidence of decreased viremia in vaccinated animals, both areas need to be studied further to further determine the result of vaccination on persistence and spread of PCV2 among animal populations. A reproductive study with more pregnant dams could provide important confirmation that PCV2 vaccination can indeed decrease reproductive failure. A growing pig study with larger sample sizes and without moving resident animals prior to the addition of contact animals could provide more clarity on the effect of closing a pig population for a long period (140 days) after PCV2 exposure on the viability of PCV2 in chronically infected pigs and the environment. They could both add
important knowledge for the management of swine herds and could lead to better
management/prevention practices for decreasing PCV2 infections in breeding and finishing
herds.
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