RESEARCH REPORT
SWINE HEALTH

Title: Effect of PCV2 vaccination on chronic PCV2 infection and determination of infectivity of PCV2 present in chronically infected pigs – **NPB #10-167**

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Industry Summary:

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 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 closure of a small pig population in a controlled environment may result in stabilization and elimination of PCV2.

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Scientific 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 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 closure of a small pig population in a controlled environment may result in stabilization and elimination of PCV2.

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., 2011b) 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., 2011c). The often long duration of PCV2 viremia in certain pigs pose the question on the viability and infectivity of PCV2 in these cases and 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; Fenaux et al., 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).

Objectives

a) Determine the effect of chronic PCV2 infection on shedding and infectivity of PCV2.
b) Determine if killed or live vaccines prevent chronic PCV2 infection.

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 to a Biosafety Level 2 (BSL-2) animal facility at Iowa State University, Ames, Iowa.

2.2. Animals housing

The resident pigs were randomly assigned to one of three groups and were kept in four separate 2 × 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 and an additional nipple drinker. 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. Six-week-old PCV2 naïve resident pigs were either vaccinated with an experimental live-attenuated PCV2 vaccine (n=3), vaccinated with a commercial inactivated PCV2 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 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 PCV2b 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 3,220 × g for 10 min at 4ºC, and the serum was aliquoted into 5 ml polystyrene round bottom tubes and stored at -20ºC 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 -80ºC until testing.

2.5. Laboratory methods used

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 is 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.

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^{TM} 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 (C_T) was detected in 40 amplification cycles.

2.6. Post mortem examination.

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 3 (enlarged, 4 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 the section 2.4.

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 diffuse). 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).

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 percent 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 $\log_{10}$ 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.

Results

3.1. Clinical observations

One positive control pig developed lameness 63 days post-challenge (dpc), 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

Anti PCV2-IgG. All resident negative controls 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.

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

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. The group mean amount of $\log_{10}$ 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. 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).

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.

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. 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.

In contrast to the present study, off site segregated early (13 days of age) weaning (SEW) of pigs shortly after a previously PCV2-naïve herd was determined to be breaking with PCVAD was ineffective in deriving PCV2-free piglets from a population that contained PCV2 viremic sows (Patterson et al., 2011a). Although we utilized the same disinfection protocols in the current experiment as were used in the SEW experiment, the risk of re-exposure from the environment (PCV2 present in the rooms) was also minimized in the current study by

moving the population into disinfected rooms at 128 days post challenge versus 13 days after weaning shortly after natural exposure to PCV2.

In the current study, the ability to successfully transmit PCV2 from known infected to naïve pigs140 days after challenge was investigated. Previously, several studies have shown that acutely infected pigs can transmit PCV2 to naïve contact animals (Bolin et al., 2001; Patterson et al., 2011c), 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 to 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. 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; Opriessnig et al., 2011; Xujie et al., 2011). One of the goals of this study was to determine the efficacy of an inactivated and a live-attenuated PCV2 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.

Under the study conditions, PCV2 challenge virus was not transmitted to naïve pigs with nose-to-nose contact with challenged pigs 140 days after vaccination 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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