RESEARCH REPORT
SWINE HEALTH

Title: Determination of the virulence of a mutant PCV2 (PCV2d) recently identified in cases of apparent vaccine failure and the ability of commercial PCV2a vaccines to protect against PCV2d
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Industry Summary:

PCV2d (formerly known as mutant or mPCV2) emerged in the U.S. in 2012 and important questions on virulence of this novel strain and vaccine protection by currently used vaccines needed to be answered. Two different studies were conducted. Initially the pathogenesis of PCV2d was evaluated by comparing PCV2d to PCV2a and PCV2b in caesarian-derived, colostrum-deprived (CDCD) pigs (Aim 1). In a follow-up study the efficacy of PCV2a-based commercial vaccines to protect against PCV2d challenge was investigated in the conventional pig model (Aim 2). For Aim 1, a total of 29, two-week-old CDCD pigs were assigned to one of 4 treatment groups. At 3 weeks of age, the pigs were experimentally inoculated with saline, PCV2a, PCV2b or PCV2d. All pigs were necropsied 21 days post infection (dpi). Gross lesions were limited to visible icterus and loss of body condition in a portion of the PCV2d pigs. The amount of PCV2 DNA was significantly higher in pigs inoculated with PCV2d compared to those infected with PCV2b in sera at 7 dpi and fecal swabs at 14 dpi. The results indicated that all PCV2 isolates were capable of inducing severe lesions and disease in the CDCD pig model, and there was no overall significant difference in virulence. For Aim 2, 50-naturally PCV2b-infected 2-week-old pigs were divided into five treatment groups with 10 pigs each. Pigs were unvaccinated (positive and negative controls) or vaccinated at 3 (BIVI, Zoetis, Merck) and at 5 weeks of age (Merck). At 11 weeks of age, all pigs except the negative controls were challenged with PCV2d. The experiment was terminated 21 days after challenge. Under the conditions of this study, vaccinated pigs were protected against PCV2d viremia and lesions whereas non-vaccinated pigs were not. The results indicate that commercial vaccines are effective in protecting conventional pigs against emerging PCV2d strains despite the presence of an ongoing PCV2b infection and passively-acquired anti-PCV2 antibodies at the time of vaccine administration.

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Scientific Abstract

PCV2d (formerly known as mutant or mPCV2) emerged in the U.S. in 2012 and important questions on virulence of this novel strain and vaccine protection by currently used vaccines needed to be answered. Two different studies were conducted. Initially the pathogenesis of PCV2d was evaluated by comparing PCV2d to PCV2a and PCV2b in caesarian-derived, colostrum-deprived (CDCD) pigs (Aim 1). In a follow-up study the efficacy of PCV2a-based commercial vaccines to protect against PCV2d challenge was investigated in the conventional pig model (Aim 2). For Aim 1, a total of 29, two-week-old CDCD pigs were assigned to one of 4 treatment groups. At 3 weeks of age, the pigs were experimentally inoculated with saline, PCV2a, PCV2b or PCV2d. All pigs were necropsied 21 days post infection (dpi). Gross lesions were limited to visible icterus and loss of body condition in a portion of the PCV2d pigs. The amount of PCV2 DNA was significantly higher in pigs inoculated with PCV2d compared to those infected with PCV2b in sera at 7 dpi and fecal swabs at 14 dpi. The results indicated that all PCV2 isolates were capable of inducing severe lesions and disease in the CDCD pig model, and there was no overall significant difference in virulence. For Aim 2, 50-naturally PCV2b-infected 2-week-old pigs were divided into five treatment groups with 10 pigs each. Pigs were unvaccinated (positive and negative controls) or vaccinated at 3 (BIVI, Zoetis, Merck) and at 5 weeks of age (Merck). At 11 weeks of age, all pigs except the negative controls were challenged with PCV2d. The experiment was terminated 21 days after challenge. Under the conditions of this study, vaccinated pigs were protected against PCV2d viremia and lesions whereas non-vaccinated pigs were not. The results indicate that commercial vaccines are effective in protecting conventional pigs against emerging PCV2d strains despite the presence of an ongoing PCV2b infection and passively-acquired anti-PCV2 antibodies at the time of vaccine administration.

Introduction

Porcine circovirus (PCV) type 2 (PCV2) is currently one of the most important viruses affecting the global pork industry. PCV2 was first recognized as a pathogen in weanling pigs of high health status in the late 1990s (Harding et al., 1997) and has since been identified in all major swine producing regions of the world. During 2005 and 2006, a newly recognized PCV2 genotype, PCV2b, spread rapidly across North American swine production regions devastating the swine industry with high mortality and morbidity (Cheung et al., 2007). A global shift in the prevalence of PCV2 genotype from the previously dominant PCV2 genotype PCV2a to PCV2b was also identified around the time of the severe PCV associated disease (PCVAD) outbreaks in North America (Cheung et al., 2007; Gagnon et al., 2007; Patterson et al., 2010). PCV2c isolates have only been identified in archived Danish samples (Dupont et al., 2008) and are of no relevance today.

During an investigation into cases of apparent PCV2 vaccine failure during May 2012 the presence of a mutant PCV2 in these groups of pigs (Xiao et al., 2012) later designated as PCV2d was confirmed. The PCV2d strain found in these cases is very similar to strains reported in China (Guo et al., 2010). A similar PCV2d strain with 234 amino acids expressed by ORF2 was found to be more virulent in the pig model compared to PCV2a and PCV2b isolates (Guo *et al.*, 2012). The finding of the PCV2d in U.S. cases of perceived vaccine failure case was unexpected.

In a recent large study conducted by our group in 2010-2011, the PCV2 status of 1675 serum samples and 100 lung tissues collected from pigs on 61 sites in the U.S. (with one to five barns on each site and a total of 105 barns) was investigated (Shen et al., 2012). In particular, 185 putative amino acid sequences of the PCV2 Cap were obtained, aligned and compared and the PCV2d sequence was not present in these samples (Shen et al., 2012). This may indicate that PCV2d emerged in North America sometime during late 2011 or 2012. The first objective of this study was to determine the virulence of PCV2d in a conventional U.S. pig model by comparing it to PCV2a and PCV2b isolates we have used in our model in the past.

The PCV2b genotype, first recognized in the U.S. in 2006 (Cheung et al., 2007; Gagnon et al., 2007), now predominates in the global pig population (Patterson et al., 2010). While commercially available PCV2 vaccines in the U.S. are all based on the PCV2a genotype, cross-protection between PCV2 genotypes has been demonstrated experimentally (Opriessnig et al., 2007; Fort et al., 2008) and this is further supported by vaccine efficacy data obtained under field conditions (Kixmöller et al., 2008; Horlen et al., 2008; Fraile et al., 2012). The second objective of this study was to determine if currently available commercial vaccines, which are all PCV2a based, are protective against PCV2d.

Objectives

- Aim 1: To determine the virulence of PCV2d in a conventional U.S. pig model by comparing it to PCV2a and PCV2b isolates we have used in our model in the past.
- Aim 2: To determine if currently available commercial vaccines, which are all PCV2a based, are protective against PCV2d

Materials and Methods

Generation of infectious virus DNA clones. The full length infectious DNA clone of the PCV2d isolate Jx535296 (Opriessnig *et al.*, 2013) was amplified from DNA extracted from a lung homogenate of a 2012 Iowa pig. The PCR product was blunt ligated to the pCR® Blunt II-TOPO® vector using the Zero Blunt® TOPO® PCR Cloning Kit (Life Technologies, Carlsbad, CA, USA), per the manufacturer's instructions.

Production of the infectious virus stocks. A PK-15 cell line was used to produce and titrate infectious virus stocks for this study. Virus production was analyzed by IFA using a monoclonal Mouse anti-PCV2 capsid primary antibody (Rural Technologies, Brookings, SD, USA) and a Goat anti-mouse FITC-labeled monoclonal antibody (KPL, Gaithersburg, MD, USA).

Animals, housing, and experimental design. The experimental protocols were approved by the Iowa State University Institutional Animal Care and Use Committee.

Aim 1: Twenty-nine CDCD pigs were purchased at 2 weeks of age and arbitrarily assigned to one of 4 groups and rooms with 7-8 pigs in each group. The pigs were housed in raised plastic decks equipped with one nipple drinker and one self-feeder. Virus challenge was done at 3 weeks of age. Each group was challenged with a different PCV2 strain (PCV2a 40895; PCV2b NC16845; PCV2d Jx535296) by a combination of intramuscular and intranasal routes. At 6 weeks of age corresponding to 21 dpi, all pigs were humanely euthanized and a necropsy was conducted. All pigs were weighed at arrival at 2 weeks of age and at the time of necropsy at 6 weeks of age. The average daily weight gain was calculated and compared among groups. All pigs were examined daily for signs of illness including lethargy, respiratory signs, inappetance, icterus, and lameness. Blood was collected from all pigs prior to challenge, and again at dpi 7, 14 and 21 and serum was stored at -80°C until testing. In addition, nasal and fecal samples were collected weekly from each pig.

Aim 2: Fifty, 2-week-old, colostrum-fed, arbitrarily selected crossbred pigs were randomly assigned to one of five groups with 10 pigs in each group. At 3 weeks of age, BIVI-VAC, Zoetis-VAC, and Merck-VAC pigs were vaccinated according to the instructions of the manufacturers. In brief, the pigs were vaccinated intramuscularly in the right neck at 3 weeks of age with 1 ml of Ingelvac CircoFLEX® (Boehringer Ingelheim Vetmedica), 2 ml of Fostera™ PCV (Zoetis Inc), or 2 ml of Circumvent® PCV (Merck Inc). The Merck-VAC group was revaccinated at 5 weeks of age with 2 ml of the vaccine. At 5 weeks of age, the Merck-VAC pigs were revaccinated as recommended by the vaccine manufacturer. Negative and positive control pigs were not vaccinated. At 10 weeks of age, all pigs were transferred to the Iowa State University research facility. At arrival, negative controls were placed in a separate room and the remaining pigs were arbitrarily assigned to one of two rooms with four pens in each room. Each pen was equipped with one nipple drinker and one self-feeder. Challenge with PCV2d was done intranasally using 5 ml of the virus stock at 11 weeks of age and all pigs were humanely euthanized and necropsied at 14 weeks of age.

Necropsy. All pigs were humanely euthanized by intravenous pentobarbital sodium overdose (Fatal Plus®, Vortech Pharmaceuticals, LTD, Dearborn, MI, USA) and necropsied at 21 dpi. The extent of macroscopic lung lesions ranging from 0 to 100% was scored as described previously (Halbur *et al.*, 1995). The size of superficial inguinal lymph nodes were compared among groups as described (Opriessnig *et al.*, 2004). Sections of lymph nodes (superficial inguinal, external iliac, mediastinal, tracheobronchial, and mesenteric), tonsil, heart, thymus, kidney, colon, spleen, liver, small (ileum) and large (spiral colon) intestines were fixed in 10% neutral-buffered formalin, and routinely processed for histological examination. In addition, lung and superficial inguinal lymph node were collected in separate bags and stored at -80°C for further PCR testing.

Serology. Serum samples were tested by an ORF2-based PCV2 IgG ELISA as previously described (Nawagitgul *et al.*, 2002)and were considered positive if the S/P ratio was 0.2 or greater. Samples with S/P ratios between 0.1 and 0.2 were considered suspect.

PCV2 PCR. Total nucleic acids were extracted from serum samples, nasal swabs, fecal swabs, lung homogenates, or superficial inguinal lymph node homogenates using the MagMaxTM Pathogen RNA/DNA Kit (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) and an automated DNA/RNA extraction system (Thermo Scientific Kingfisher® Flex, Thermo Fisher Scientific, Pittsburgh, PA, USA) according to the instructions of the manufacturer. All serum samples, lung homogenates and superficial inguinal lymph node homogenates were tested for the presence of PCV2 DNA by quantitative real-time PCR assays using primer-probe combinations as described (Opriessnig et al., 2003; Shen et al., 2010). Samples were considered negative when no signal was observed within the 40 amplification cycles. Five serial dilutions of a PCV2 genomic DNA clone (10^5 to 10^9 copies/ml) were used to generate a standard curve with a correlation coefficient of more than 0.99 Opriessnig 2003.

Statistical analysis. For data analysis, JMP® software version 10.0.2 (SAS Institute, Cary, NC, USA) was used. Summary statistics were calculated for all the groups to assess the overall quality of the data set including normality. Statistical analysis of the data was performed by one-way analysis of variance (ANOVA) for continuous data ($\log_{10}$ transformed PCR data, ELISA data, average daily weight gain and macroscopic lung scores). A p-value of less than 0.05 was set as the statistically significant level. Pairwise test using Tukey's adjustment was subsequently performed to determine which differences among groups were statistically different. Real-time PCR results (copies per ml of serum) were $\log_{10}$ transformed prior to statistical analysis.

Results

Aim 1:

Clinical observation and average daily weight gain. Signs of illness were not recognized in the pigs in the 21 day duration of the study except for the last day where visible icterus and loss of body condition were observed in 3/8 PCV2d pigs. The average daily weight gain was not different between groups (data not shown).

Anti-PCV2 antibody levels. All pigs were negative for anti-PCV2 IgG antibodies at inoculation and the majority of the pigs remained negative throughout the study due to the short 21-day duration of the study. One of eight PCV2d pigs did seroconvert to PCV2 by 21 dpi, and 3/7 PCV2a pigs in addition to 1/7 PCV2b pigs had sample-to-positive (S/P) ratios within 0.1 to 0.2 and were considered suspect positive (data not shown).

Prevalence and amount of PCV2 DNA in serum, nasal swabs, and fecal swabs. PCV2 DNA was not detected in any serum sample, nasal swab, or fecal swab collected from the negative control pigs. In serum samples at 7 dpi, PCV2d infected pigs had significantly higher amounts of PCV2 DNA compared to PCV2b infected pigs. Moreover, the prevalence of PCV2 DNA positive pigs was 6/7 for PCV2a, 4/7 for PCV2b, and 8/8 for mPCV2d at 7 dpi. Thereafter all pigs in all infected groups were viremic and there was no difference among PCV2a, PCV2b and PCV2d groups. In nasal swabs, PCV2 DNA was first detected in 3/8 PCV2d pigs at 7 dpi and in the other groups by dpi 14. In fecal swabs, PCV2 DNA was detected by 7 dpi, in 2/7 PCV2a pigs, 3/7 PCV2b pigs and 4/8 PCV2d pigs. Significantly higher amounts of PCV2 DNA were excreted by PCV2d infected pigs compared to PCV2b infected pigs at 14 dpi.

Gross lesions: Macroscopic lesions were characterized by mild to severe enlargement of lymph nodes in the majority of the pigs regardless of infection status. Mild-to-severe icterus, severe thymic atrophy, fatty liver, and serous atrophy of fat was present in 3/8 PCV2d pigs.

Microscopic lesions, PCV2 antigen in tissues, and PCVAD prevalence: Microscopic lesions were not present in lymphoid tissues of the negative controls and PCV2 antigen was also not detected in these pigs. The majority of the PCV2 infected pigs, regardless of PCV2 subtype, had severe lymphoid depletion of follicles and

mild-to-moderate histiocytic replacement of follicles in multiple lymphoid tissues often associated with high amounts of PCV2 antigen. An overall lymphoid lesions score of 7, 8, or 9 which is consistent with PCVAD was evident in 3/7 PCV2a pigs, in 4/7 PCV2b pigs and in 4/8 PCV2d pigs. In 3/8 PCV2d pigs hepatic lesions were severe and diffuse (score 3) with moderate hepatocyte degeneration in 3/8 PCV2d pigs.

Aim 2:

Clinical observation and average daily weight gain. No remarkable clinical signs were noted and there was no difference in average daily weight gain among groups.

Anti-PCV2 antibody levels. At -63 days post challenge (dpc), all but one of the pigs had ELISA S/P ratios above the cutoff consistent with the presence of passively-acquired antibodies. Pigs in the Merck-VAC group, which received a booster dose at 5 weeks of age, had significantly higher levels of anti-PCV2 IgG antibodies at 0 and 7 dpc compared to all other groups. At dpc 0, 5/10 negative controls, 8/10 positive controls, 7/10 BIVI-VAC, 4/10 Zoetis-VAC, and 0/10 Merck-VAC pigs had no detectable anti-PCV2 antibodies. At 21 dpc, there were no significant differences in group mean ELISA S/P ratios.

Prevalence and amount of PCV2 DNA in serum. At -63 dpc, each group had 1 to 3 PCV2 DNA-positive pigs indicating an active field infection as expected. The PCV2 type was determined to be PCV2b by differential PCR and ORF2 sequencing. By 0 dpc, PCV2b DNA was detected in 6/10 negative controls, 5/10 positive controls, 0/10 BIVI-VAC pigs, 2/10 Zoetis-VAC pigs, and 0/10 Merck-VAC pigs. After PCV2d challenge, the PCV2 DNA prevalence increased in negative and positive controls to 70-100%, whereas in vaccinated pigs the PCV2 DNA was detected only in 0-40% of the pigs in each group. The main PCV2 genotype detected after challenge was PCV2b; however, PCV2d was detected in serum of one BIVI-VAC pig at 7 dpi and in serum samples of three positive control pigs at 14 and 21 dpc. By 21 dpc, the percent reduction for the amount of PCV2 DNA compared to the positive controls was 93.5% for BIVI-VAC, 84.6% for Zoetis-VAC, and 71.8% for Merck-VAC.

Gross lesions. The lymph nodes appeared mild-to-moderately enlarged in all pigs regardless of treatment status.

Microscopic lesions and PCV2 antigen and DNA in tissues. Lesions consistent with PCVAD (severe lymphoid depletion and granulomatous inflammation of the majority of the lymphoid tissues examined) were identified in 3/10 positive control pigs (overall lymphoid lesion scores: 8, 8, and 9). These pigs also had mild lymphohistocytic interstitial nephritis. The prevalence of PCV2 IHC positive pigs was 8/10 in negative controls, 9/10 in positive controls, 1/10 in BIVI-VAC pigs, 1/10 in Zoetis-VAC pigs, and 0/10 in Merck-VAC pigs. The prevalence of PCV2d DNA positive pigs using pooled lung and lymph node homogenate was 0/10 in negative controls, 10/10 in positive controls, 4/10 in BIVI-VAC pigs, 3/10 in Zoetis-VAC pigs, and 0/10 in Merck-VAC pigs.

Discussion

PCV2d (formerly known as mutant or mPCV2) was initially identified in the U.S. in 2012 and frequently associated with perceived PCV2 vaccine failures. To investigate the importance of PCV2d, a pathogenicity study was conducted initially in which PCV2d was compared to traditional U.S. PCV2a and PCV2b strains. The main finding in that study was the lack of marked differences in virulence between PCV2a, PCV2b, and PCV2d strains. All three viruses were capable of producing PCVAD in young CDCD pigs. While this is in line with previous observations indicating that differences in virulence between PCV2a and PCV2b genotypes are lacking (Opriessnig et al., 2008), this is in contrast to a Chinese study which suggested that mPCV2 (now known as PCV2d) is more virulent (Guo et al., 2012). In that study, conventional pigs were utilized and infected with cell culture propagated PCV2 isolates recovered from field cases.

No significant differences in virulence among distinct classical and emerging PCV2 isolates using a CDCD pig model. All isolates utilized were capable of inducing severe disease in young pigs. Limitations of the study include the naïve immune system of CDCD pigs and the young age at infection, which may have

contributed to artificial acceleration of clinical disease in this model, and the short duration of the study which may have prevented a more pronounced antibody response.

The second objective of this study was to investigate the efficacy of current commercial PCV2a-based vaccines used in the United States in a model that mimics field conditions i.e. the presence of passively acquired antibodies at the time of vaccination, natural PCV2 infection during the nursery stage, and challenge with a recent emergent PCV2d virus in the early finishing stage.

Under the conditions of this study, which was designed to closely mimic field conditions, commercial PCV2a vaccines were able to protect 11-week old pigs from the effects of infection with an emerging PCV2d strain despite the presence of passively acquired anti-PCV2 antibodies and natural infection with PCV2b at or near the time of vaccination.

High levels of PCV2d DNA were detected in serum of individual positive control pigs, and PCV2d DNA was also detected in tissue homogenates of all pigs with the exception of negative controls and Merck-VAC indicating that the PCV2d strain was capable of replicating in a portion of the pigs despite vaccination. PCVAD and reduced weight gain were solely observed in the positive controls and not in the negative controls in which only the field PCV2b strain could be detected.

In summary, evidence for significant differences in virulence between PCV2a, PCV2b and PCV2d variants is lacking and indications are that the current commercial vaccines are efficacious in reducing PCVAD caused by all of these isolates.

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