RESEARCH REPORT
SWINE HEALTH

Title: Determination of the virulence of an emerging porcine parvovirus (PPV2) and its possible interaction with PCV2 in PCVAD – **NPB - #13-004**

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Date: January 14, 2016

Industry Summary

The objective of this study was to characterize a novel PPV previously designated as PPV2 in pigs and to determine the interaction of PPV2 and PCV2. Colostrum-deprived pigs experimentally infected with PPV2 did not show any clinical signs or lesions despite developing PPV2 viremia and having high tissue levels of PPV2 DNA. Similarly, conventional pigs infected with PPV2 showed no clinical signs and had no macroscopic or microscopic lesions. When pigs were co-infected with PCV2 and PPV2, coinfected pigs had more severe microscopic lesions compared to singular-PCV2-infected pigs. The results of this study indicate that PPV2-PCV2 coinfection can result in enhanced disease and lesions consistent with PCVAD and intervention strategies to control PPV2 infection in pigs may help control PCVAD.

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Keywords: PCV2; PPV2; interaction; pathogenicity; pigs.

Scientific Abstract

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Introduction

During a porcine circovirus type 2 (PCV2) vaccine failure investigation in two different swine production systems we identified PCV2d and a novel porcine parvovirus (PPV) designated PPV type 2 (PPV2) (Opriessnig et al., 2013).

Similar to circoviruses, PPVs are small, non-enveloped DNA viruses. The best known parvovirus in pigs is PPV1 which is also known as classical PPV and can be associated with reproductive failure in breeding herds. Under experimental conditions, PPV1 has also been shown to enhance PCV2 replication. Similar to PCV2, PPV1 has a strong cellular tropism for mitotically active tissues like lymph nodes or heart muscle (Allan and Ellis, 2000; Oraveerakul et al., 1993). The first experimental study demonstrating the potentiating effect of another pathogen on PCV2 was conducted in 1999 (Ellis et al., 1999) with PPV1 and PCV2. Several groups have since demonstrated that pigs dually-inoculated with PCV2 and PPV1 develop more severe disease and lesions than pigs infected with PCV2 alone (Allan et al., 1999; Hasslung et al., 2005; Opriessnig et al., 2004; Ostanello et al., 2005).

Less information is available for other recognized parvoviruses in pigs including PPV2. In a study conducted in China, a peak in PPV2 viremia was detected 2–3 weeks prior to onset of severe respiratory disease associated with PRRSV and PCV2; however, a definitive relationship between presence and level of PPV2 DNA and severity of the outbreak was not confirmed (Wang et al., 2010). In a Hungarian study investigating randomly collected samples, PPV2 was detected in 5.4% (6/111) of serum samples investigated (Cságola et al., 2012). In our US study, the prevalence of PPV2 was determined to be 43.1% (31/72) in production system A and 70% (42/60) in production system B with an overall PPV2 prevalence of 55.3% (73/132). There was a difference in prevalence and PPV2 viral load between the farms (A, 1.20 ± 1.66 log10 copies per ml; B, 2.37 ± 1.90 PCV2 log10 copies per ml, $p < 0.001$). Of note, the prevalence of PPV2 was higher in the younger age groups in Farm B where PCVAD was also observed earlier at an earlier age. Finally, in a retrospective study using a total of 586 serum samples and 164 lung homogenates collected from 1996 to 2013 in the USA and Canada, all samples were tested for PPV1, PPV2 and PCV2 (Opriessnig et al., 2014). Overall, PPV2 had the highest prevalence rates in sera (35.2%) and tissues (42.7%)**.** Concurrent infection of PCV2 and PPV occurred in 14.3% (84/586) of the serum samples and in 49.4% (81/164) of the tissue samples. Moreover, the prevalence of PPV1 or PPV2 DNA was significantly higher in tissues containing high amounts of PCV2 DNA compared to non-PCVAD cases (Opriessnig et al., 2014).

These combined data possible indicate that PPV2 is involved in PCVAD and may be another important cofactor in enhancing PCV2 replication; however, studies under controlled conditions are needed to further confirm this.

Objectives

Objective 1: To characterize the virulence of PPV2 in the pig model by determining clinical disease, macroscopic and microscopic lesions, length of viremia and anti-PPV2 response.

Objective 2: To investigate the possible interaction of PPV2 and PCV2.

Materials and Methods

Animals, housing, and experimental design. The experimental protocols were approved by the Iowa State University Institutional Animal Care and Use Committee and the Iowa State University Biosafety Committee (Approval numbers:12-I-0039-A; 11-12-7467-S, 1-14-7692-S , 14-I-0001-A). The study was done in three different parts.

PPV2 pathogenesis in young pigs and stock inoculum production. Initially, four colostrum-deprived snatch-farrowed pigs were used to create an inoculum stock for future projects and to also determine pathogenicity in young pigs. In brief, colostrum-deprived pigs were purchased at birth and infected with PPV2 PCR-positive tissue homogenate from field cases negative for PCV2 and other known PPVs including PPV1, PPV3, PPV4, PPV5 and PPV6 at 3 days of age. For the inoculation, the two selected tissue homogenates were filtered using a 0.45 µm filter and heat inactivated for 30 min at 56°C. After heat inactivation antimicrobials

(Penicillin-Streptomycin-Antimycotic, 1:100) were added for 30 min at room temperature to eliminate any bacteria in the preparation. The PPV2 PCR cycle threshold (C_T) was 25.4 and 29.6. Inoculation was done by a combination of using intramuscular and oro-nasal routes using approximately 2 ml of the tissue homogenate per pig. Blood samples were collected from all pigs prior to inoculation and every three days after challenge. Fecal swabs were collected daily. Samples were stored at -80°C until testing. All samples were tested for presence of PPV2 and PCV2 DNA. At necropsy, tissues were collected for histopathology and also for long-term storage and possible future use at -80°C.

Conventional pig studies to investigate possible interaction of PPV2 and PCV2. Forty-seven, 2-week-old, colostrum-fed, arbitrarily selected crossbred pigs were bought from a herd free of major swine pathogens such as PRRSV, influenza A virus and *Mycoplasma hyopneumoniae* and with low levels of antibodies to PCV2 in a portion of the dams. Selected litters were pre-screened to confirmed absence of PCV2 antibodies in the dams. At arrival at the research facility the pigs were randomly assigned to one of seven groups with 4 to 8 pigs in each group. The experimental design to test concurrent PCV2 and PPV2 infection is outlined in Table 1 and the experimental design to test the effect of differences in the sequence of infection of pigs with PCV2 and PPV2 is outlined in Table 2.

Table 1: Experimental design to test concurrent PCV2 and PPV2 infection.

Group	**Pig#**	**Inoculation** 3 weeks of age 0 dpi	**Necropsy** 6 weeks of age 21 dpi
PPV2	8	PPV2	8
Coinfected group	8	PPV2 and PCV2	8
PCV2	7	PCV2	8
Negative controls	4	-	4

Table 2: Experimental design to test the effect of different order of PCV2 and PPV2 infection.

Group	**Pig #**	**Inoculation 1** 3 weeks of age 0 dpi	**Inoculation 2** 5 weeks of age 14 dpi	**Necropsy** 8 weeks of age 35 days after inoculation 1 21 days after inoculation 2
PPV2(PCV2)	8	PPV2	PCV2	8
PCV2(PPV2)	8	PCV2	PPV2	8
Negative controls	4	-	-	4

Inoculation. Each conventional pig received 0.5 ml Excede (Zoetis) into the right neck by the intramuscular route. Pigs infected with PCV2 (Tables 1 and 2) received 4 ml from a PCV2b inoculum stock a dose of $10^{4.5}$ TCID50 intranasally. Similarly, pigs infected with PPV2 (Tables 1 and 2) received PPV2 positive tissue homogenate intramuscularly (1 ml) and intranasally (2 ml; Table 1). For the final PPV2 inoculum, 20 ml of serum from a CD pig described above was filtered with a 0.45µl filter, heat inactivated at 56°C for 30 min, incubated at room temperature with antimicrobials (Penicillin-Streptomycin-Antimycotic, 1:100) for 30 min and diluted with PBS for a final volume of 100 ml. The final inoculum stock was divided into four tubes and frozen at -80°C until usage. The final PPV2 C_T value of the PPV2 inoculum stock was 20.1.

Sample collection. Blood, nasal swabs and fecal swabs were collected once a week from all pigs in all groups and serum and swabs were stored at -80C until usage.

Necropsy. All pigs were humanely euthanized by intravenous pentobarbital sodium overdose (Fatal Plus®, Vortech Pharmaceuticals, LTD, Dearborn, MI, USA) and necropsied according to Table 1. The extent of macroscopic lung lesions ranging from 0 to 100% was scored as described previously (Halbur *et al.*, 1995). The sizes 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. The presence and amount of PCV2 antigen in tissues was determined by immunohistochemical stains (Sorden et al., 1999). An overall PCV2 lymphoid score was calculated for each pig (Opriessnig et al, 2004). In brief, a scoring system for each lymphoid tissue ranging from 0 to 9 (lymphoid depletion score 0–3; granulomatous inflammation score 0–3; PCV2-IHC score 0–3) was used. The scores (lesions and PCV2-IHC) of the seven lymphoid tissues ([lymph node pool] × 5, spleen, and tonsil) were added together and divided by 7. The lymph node pool consisted of superficial inguinal, external iliac, mediastinal, tracheobronchilar lymph node, and mesenteric lymph nodes. Pigs were grouped into four categories on the basis of overall microscopic lymphoid lesion scores: I (normal; score = 0), II (mild; score = 1–3), III (moderate; score = 4–6), and IV (severe; score = 7–9). A pig was diagnosed with PCVAD if the mean lymphoid microscopic lesion severity score was in category IV (Opriessnig et al., 2004). In addition, fresh lung and superficial inguinal lymph node were collected in separate bags and stored at -80°C.

Serology. Serum samples were tested by a commercial available PCV2 ELISA (Synbiotics, France). An *in-house* ELISA for PPV2 was developed by expressing VP2 in an *E. coli* vector. However, due to the lack of a confirmed positive and negative control, validation of this assay could not be completed and results are not reported.

PCR. Total nucleic acids were extracted from serum samples, nasal swabs or fecal swabs 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 and swabs were tested for the presence of PCV2 DNA and PPV2 by a quantitative real-time PCR assay using primer-probe combinations as described (Opriessnig et al., 2003). Samples were considered negative when no signal was observed within the 40 amplification cycles. For PPV2, a real-time PCR assay as previously described was used (Xiao et al., 2012).

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

A. PPV2 pathogenesis in young pigs and stock inoculum production

Clinical signs were not observed in any of the pigs and all pigs remained active and had a good appetite until termination of the study. The first two pigs were PCR positive on serum at dpi 3 (34.3-38.9), positive on day 7 (17.3-19.6) and DNA in serum decreased by 10 dpi (33.0-38.2) and which point the pigs were necropsied. One pig was also positive on fecal samples on dpi 6 and 7. For the second set of pigs, fecal samples were negative for PPV2 for the duration of the trial (7 days) but serum samples were positive on day 3, day 6 and 7 (C_T 16.4 and 17.4). At that point the two pigs were necropsied. At necropsy tissues were collected in separate bags, tested by PCR and stored at -80°C for further usage. All tissues were negative for PCV2 and PPV1, PPV3, PPV4, PPV5 and PPV6 by PCR. The PPV2 PCR results on tissues obtained at necropsy for the two pigs necropsied at peak viremia are summarized in Table 3. There was no evidence of microscopic lesions in the tissues from any of the CD pigs.

Table 3: PPV2 DNA C_T values in different tissues obtained from pigs experimentally infected with PPV2 7 days earlier.

	Brain	Heart	Kidney	Liver	Lung	Small intestines	Lymph nodes	Bone marrow
Pig A	26.1	26.0	21.9	24.3	24.7	22.1	20.3	25.6
Pig B	25.8	25.5	25.0	22.9	22.7	21.3	18.4	24.0

Overall, the data indicate that PPV2 viremia lasts around 7 days and fecal shedding can occur during peak viremia. Macroscopic or microscopic lesions were not present in the pigs examined.

B. Conventional pig study.

Clinical observation and average daily weight gain. Signs of illness were not recognized in the pigs during the duration of the study. The average daily weight gain was not different between groups (data not shown).

Antibody levels. All pigs were negative for anti-PCV2 IgG antibodies at inoculation. PPV2-inoculated pigs and negative control pigs remained PCV2 seronegative throughout the study. The prevalence of antibody positive pigs is summarized in Table 4.

Table 4: Prevalence of PCV2 antibody positive pigs in the different treatment groups.

Group	D0	D7	D14	D21	D28	D35
PPV2	0/8	0/8	0/8	0/8		
Coinfected group	0/8	0/8	0/8	**1/8**		
PCV2	0/7	0/7	0/7	**2/7**		
Negative controls	0/8	0/8	0/8	0/8	0/4	0/4
PPV2(PCV2)	0/8	0/8	0/8	0/8	0/8	0/8
PCV2(PPV2)	0/8	0/8	0/8	**1/8**	**5/8**	**6/8**

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. PCV2 DNA was also not detected in PPV2 pigs. In the PCV2 group, 5/7 were PCV2 viremic at dpi 7 and in the coinfected group 7/8 pigs were viremic at dpi 7. All other samples (serum, fecal swabs, nasal swabs) were positive for PCV2 DNA on all days tested.

Prevalence and amount of PPV2 DNA in serum, nasal swabs, and fecal swabs. PPV2 DNA was not detected in any serum sample, nasal swab, or fecal swab collected from the negative control pigs. PPV2 DNA was not detected in PCV2 pigs and PCV2 DNA was not detected in PPV2 pigs. By 7 dpi, 7/8 PPV2 pigs and 4/8 coinfected pigs were PPV2 viremic. In addition, in the PPV2 group 5/8 nasal swabs and 6/8 fecal swabs were PPV2 DNA positive on dpi 7 whereas in the coinfected group only 1/8 fecal swabs were positive. PPV2 DNA was also detected at dpi 14 (2/8 PPV2 pigs and 1/8 coinfected pigs) and dpi 21 (1/8 PPV2 pig and 1/8 coinfected pig).

Gross lesions: Macroscopic lesions were characterized by mild to severe enlargement of lymph nodes in the majority of the pigs regardless of infection status.

Microscopic lesions and PCV2 antigen levels in tissues: Microscopic lesions were not present in lymphoid tissues of the negative controls or single PPV2 infected pigs and PCV2 antigen was also not detected in these pigs. At dpi 21, moderate PCV2-associated lymphoid lesions were seen in 3/7 PCV2 pigs and in 5/8 of the coinfected pig. In addition, in the coinfected group, 2/8 pigs had microscopic PCVAD (severe lymphoid lesions associated with high levels of PCV2 antigen). Mean group lymphoid lesions scores were 0.0±0.0 for the negative controls, 0.1±0.1 for the PPV2 group, 3.0±0.6 for the PCV2 group and 5.3±0.8 for the coinfected group at dpi 21. While the PCV2(PPV2) and PPV2(PCV2) had significantly more severe lesions compared to

negative controls they were not different from each other. Mean group lymphoid lesions scores were 0.0±0.0 for the negative controls, 2.8±0.3 for the PCV2(PPV2) group and 2.1±0.6 for the PPV2(PCV2) group at dpi 35.

Discussion

PCV2 is an important viral pathogen of pigs and associated with a variety of clinical manifestations commonly summarized as PCVAD. Due to its ubiquitous distribution and relative resistance to thermal or chemical inactivation, most pig herds are subclinically infected with PCV2. PCV2 commonly needs a trigger to increase replication to levels that become pathogenic for its host. Recognized triggers that have been proven to increase PCV2 replication leading to enhanced lesions and clinical PCVAD include PRRSV, PPV1, *Mycoplasma hypneumoniae* and others. Due to increased usage of molecular tools such as sequencing over the last decade novel and previously unrecognized viruses have been discovered in pigs. The role of these pathogens is not always clear as *in vitro* propagation and experimental challenge trials can be expensive and difficult to perform.

To investigate the importance of PPV2 on the outcome of PCV2 infection, CD and conventional pigs were experimentally infected. The results indicate that PPV2 infection by itself results in short viremia of about 7 days and PPV2 fecal shedding occurs during peak viremia. However, clinical signs associated with PPV2 were not seen under the study conditions.

When pigs were concurrently infected with PPV2 and PCV2, PCV2 associated lesions were significantly upregulated and individual coinfected pigs developed microscopic lesions consistent with PCVAD which was not seen in pigs singularly infected with PCV2. Therefore, PPV2 is a trigger of PCVAD and PCV2/PPV2 coinfections likely account for the increased incidence and severity of PCVAD occurring in some production systems. Future studies should focus on control of PPV2 in order to minimize its impact on other viruses including PCV2.

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