RESEARCH REPORT
SWINE HEALTH

Title: Transmission of PCV2: Comparison of shedding patterns between PCV2a and PCV2b, evaluation of routes of transmission (fecal, oral, nasal, mechanical) and understanding the roles of spray-dried plasma and transport vehicles. – **NPB #07-212**

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Industry Summary:

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Temporal evidence correlated the emergence of PCV2b in the U.S. in 2005 with more frequent and severe porcine circovirus associated disease (PCVAD) cases. Information on the quantity and duration of PCV2b DNA in various biological samples is vital to understanding the virulence of this isolate. As PCV2 is known to be present in blood, the recent outbreaks have raised concern about the spread of PCV2 in spray-dried plasma protein products in the feed. This has led some producers and practitioners to discontinue the use of plasma protein on their farms. As research makes a stronger case for fecal-oral transmission of PCV2, producers have also become concerned over introduction of new PCV2 strains by means of transport vehicles. In order to determine if PCV2b was present in the US prior to 2005, tissue samples collected on different farms in the Midwest from pigs (n=81) suspected to have clinical PCVAD during 2002 and 2003 were analyzed. To determine shedding patterns, 4 groups of 9-week-old, conventional SPF pigs were inoculated as follows: Group 1 (n=7) negative controls, Group 2 (n=6) inoculated with virulent PCV2a isolate, Group 3 (n=6) inoculated with PCV2b, and Group 4 (n=6) inoculated with a less virulent PCV2a isolate. Blood samples were collected weekly for two weeks following infection. To determine the quantity and infectivity of PCV2b, five pigs were inoculated with PCV2b isolate NC-16845 at three weeks of age (positive control). Twenty-eight PCV2 naïve pigs either remained un-inoculated or were inoculated by various routes (intraperitoneal, intranasal, oral, intramuscularly or oral gavage) with either pooled nasal, oral, or fecal samples or with a contaminated needle collected from the positive control pigs. Blood samples were collected weekly for 42 days following infection to determine viremia and the serological response. To determine whether spray-dried plasma is infectious, twelve three-week-old, colostrum-fed, crossbred, specific-pathogen-free (SPF) conventional pigs were divided into groups of three and placed into separate rooms. Pigs were left un-inoculated (NEG), inoculated intraperitoneally with a reconstituted spray-dried plasma product (SDP-IP), inoculated intraperitoneally with PCV2 infected plasma (POS) or inoculated with reconstituted SDP via oral gavage (SDP-OG). Blood samples were collected weekly following inoculation for 7 weeks and tested for the presence of anti-PCV2-IgG antibodies and PCV2 DNA. To determine the efficacy of various trailer disinfection methods, three model trailers were designed and manufactured by Eby Trailers. The models were constructed with identical materials found in full-size trailers. The trailers were contaminated with feces collected at the time of euthanasia from a pig exhibiting clinical signs of PCVAD. Trailers which were disinfected (all trailers excluding positive controls) were power-washed and rinsed similar to commercial washing procedures. After washing, the following disinfectants were applied per manufacturer's instructions: (1) Synergize, (2) Virkon, (3) Quatricide, and (4) Virkon followed by Bleach. Two naïve pigs were placed into each trailer for a total of 2 hours followed by placement into separate rooms and monitoring by ELISA and PCR for evidence of PCV2 seroconversion and viral replication for 42 days. While the differences in clinical signs apparent under

field conditions in 2004 and 2005 may be correlated with the emergence of PCV2b in the United States, consistent differences between the amount of PCV2a and PCV2b shed in nasal, oral and fecal routes were not noted. Secondly, PCV2b was recovered from only two of 81 samples from PCVAD cases submitted between 2002 and 2003. This data suggests that PCV2b was not the predominant strain prior to the severe PCVAD cases seen in 2005. In the current study, peak shedding of PCV2b in experimentally infected pigs in oral, nasal and fecal samples appeared at DPI 16, 16 and 19 respectively. Intraperitoneal inoculation with contaminated fecal, nasal and oral excretions resulted in viremia and seroconversion in all animals by 28 and 42 days post inoculation (DPI), respectively. Intranasal inoculation of nasal secretions resulted in seroconversion and viremia in all animals by DPI 35 and 28, respectively. Feces fed to naïve animals resulted in viremia and seroconversion in 2 of 4 animals by DPI 35 and 42, respectively. Viremia and microscopic lesions were noted in one animal inoculated with a contaminated needle; however, seroconversion was not detected. The current study provided evidence that spray-dried plasma is indeed infectious as evidenced by seroconversion and viremia following intraperitoneal injection and oral gavage. Finally, to mimic commercial transportation conditions, three model trailers were designed and manufactured by a commercial company to possess the best features for virus survival (seams, gates, etc.). Results of the study indicated that in all disinfection protocols were equally able to prevent seroconversion and viremia in naïve animals for 49 days.

Scientific Abstract:

Temporal evidence correlated the emergence of PCV2b in the U.S. in 2005 with more frequent and severe porcine circovirus associated disease (PCVAD) cases. Information on the quantity and duration of PCV2b DNA in various biological samples is vital to understanding the virulence of this isolate. As PCV2 is known to be present in blood, the recent outbreaks have raised concern about the spread of PCV2 in spray-dried plasma protein products in the feed. This has led some producers and practitioners to discontinue the use of plasma protein on their farms. As research makes a stronger case for fecal-oral transmission of PCV2, producers have also become concerned over introduction of new PCV2 strains by means of transport vehicles. In order to determine if PCV2b was present in the US prior to 2005, tissue samples collected on different farms in the Midwest from pigs (n=81) suspected to have clinical PCVAD during 2002 and 2003 were analyzed. DNA was extracted and analyzed by a previously described quantitative PCV2a /PCV2b differential real-time PCR. To determine shedding patterns, 4 groups of 9-week-old, conventional SPF pigs were inoculated as follows: Group 1 (n=7) negative controls, Group 2 (n=6) inoculated with ISU 40895 as a representative of PCV2a, Group 3 (n=6) inoculated with NC 16845 as a representative of PCV2b, and Group 4 (n=6) inoculated with ISU 4838 as a representative of PCV2a. Each pig was inoculated intramuscularly (2ml) and intranasally (3ml) with inoculum prepared at $10^{4.0}$ 50% tissue culture infectious dose. Blood samples were collected weekly for two weeks following infection. DNA extraction from serum samples was performed using the QIAamp DNA Mini kit (Qiagen) followed by quantification of PCV2 genomic DNA copy numbers by a previously described real-time PCR. To determine the quantity and infectivity of PCV2b, five pigs were inoculated with 3ml intranasally and 2ml intramuscularly of $10^{4.0}$ 50% tissue culture infectious dose ($TCID_{50}$) per ml of PCV2b isolate NC-16845 at three weeks of age (positive control). Twenty-eight PCV2 naïve pigs either remained un-inoculated or were inoculated by various routes (intraperitoneal, intranasal, oral, intramuscularly or oral gavage) with either pooled nasal, oral, or fecal samples or with a contaminated needle collected from the positive control pigs. Blood samples were collected weekly for 42 days following infection to determine viremia and the serological response. To determine whether spray-dried plasma is infectious, twelve three-week-old, colostrum-fed, crossbred, specific-pathogen-free (SPF) conventional pigs were divided into groups of three and placed into separate rooms. Pigs were left un-inoculated (NEG), inoculated intraperitoneally with a reconstituted spray-dried plasma product (SDP-IP), inoculated intraperitoneally with PCV2 infected plasma (POS) or inoculated with reconstituted SDP via oral gavage (SDP-OG). Blood samples were collected weekly following inoculation for 7 weeks and tested for the presence of anti-PCV2-IgG antibodies and PCV2 DNA. To determine the efficacy of various trailer disinfection methods, three model trailers were designed and manufactured by Eby Trailers. The models were constructed with identical materials found in full-size trailers. The trailers were contaminated with feces collected at the time of euthanasia from a colostrum-fed, crossbred, specific-pathogen-free (SPF) conventional pig was inoculated with 3ml intranasally and 2ml intramuscularly of $10^{4.0}$ 50% tissue culture infectious dose ($TCID_{50}$) per ml of PCV2b isolate NC-16845 at 3 weeks of age as part of a separate trial which developed clinical signs of PCVAD at 35 days post infection. Trailers which were disinfected (all trailers excluding positive controls) were power-washed and rinsed similar to commercial washing procedures. After washing, the following disinfectants were applied per manufacturer's instructions: (1) Synergize, (2) Virkon, (3) Quatricide, and (4) Virkon followed by Bleach in an incomplete block design with four replicates. Two naïve pigs were placed into each trailer for a total of 2 hours followed by placement into separate rooms and monitoring by

ELISA and PCR for evidence of PCV2 seroconversion and viral replication for 42 days. While the differences in clinical signs apparent under field conditions in 2004 and 2005 may be correlated with the emergence of PCV2b in the United States, consistent differences between the amount of PCV2a and PCV2b shed in nasal, oral and fecal routes were not noted. Secondly, PCV2b was recovered from only two of 81 samples from PCVAD cases submitted between 2002 and 2003. This data suggests that PCV2b was not the predominant strain prior to the severe PCVAD cases seen in 2005. In the current study, peak shedding of PCV2b in experimentally infected pigs in oral, nasal and fecal samples appeared at DPI 16, 16 and 19 respectively. Intraperitoneal inoculation with contaminated fecal, nasal and oral excretions resulted in viremia and seroconversion in all animals by 28 and 42 days post inoculation (DPI), respectively. Intranasal inoculation of nasal secretions resulted in seroconversion and viremia in all animals by DPI 35 and 28, respectively. Feces fed to naïve animals resulted in viremia and seroconversion in 2 of 4 animals by DPI 35 and 42, respectively. Viremia and microscopic lesions were noted in one animal inoculated with a contaminated needle; however, seroconversion was not detected. The current study provided evidence that spray-dried plasma is indeed infectious as evidenced by seroconversion and viremia in naïve animals following intraperitoneal injection and oral gavage. Finally, to mimic commercial transportation conditions, three model trailers were designed and manufactured by a commercial company to possess the best features for virus survival (seams, gates, etc.). Results of the study indicated that in all disinfection protocols were equally able to prevent seroconversion and viremia in naïve animals for 49 days.

Introduction

Porcine circovirus type 2 (PCV2) is a small, nonenveloped, single stranded DNA virus which emerged in the 1990's as a swine pathogen. While the majority of the swine population worldwide is seropositive for PCV2, severe clinical disease manifested as respiratory, reproductive, enteric or systemic disease occurs in 5-30% of infected populations (Opriessnig et al., 2007). Based on sequence analyses of PCV2 strains, isolates can be divided into five genotypes PCV2a-e. Porcine circovirus type 2a (PCV2a), type 2b (PCV2b) and type 2c (PCV2c) have been well documented (Segales et al., 2008) while PCV2d and PCV2e are unique to China to date (Wang et al., 2009).

Temporal evidence correlated the emergence of PCV2b in the U.S. in 2005 with more frequent and severe porcine circovirus associated disease (PCVAD) cases. Specifically, a phylogenic analysis (Cheung et al., 2007) evaluated eight PCV2 sequences from herds in Kansas, North Carolina and Iowa experiencing an acute onset of high mortality in 2005, 18 US PCV2 sequences obtained from GenBank (all available sequences as of May 16, 2006) and 107 sequences reported previously by Olvera *et al*. in 2007 (Olvera et al., 2007). The results of this analysis revealed the presence of PCV2b isolates in the U.S for the first time. Similarly, in a 2007 case study of several swine production operations experiencing severe clinical disease in the US, whole genomic sequencing revealed PCV2 isolates were most similar (99.5% sequence homology) to a 1998 French PCV2b isolate AF055393 (Horlen et al., 2007). Currently it is unknown whether the rapid spread of PCV2-asscoiated disease in the North American swine population in 2005 was associated with the introduction of PCV2b into North America or an increase in virulence of an already existing strain.

Information on the quantity and duration of PCV2b DNA in various biological samples is vital to understanding the virulence of this isolate. However, to the author's knowledge only information for PCV2a exists. Specifically, PCV2 DNA has been detected in fecal and tonsillar swabs from DPI 1 – 21 (excluding DPI 6) (Caprioli et al., 2006). In the study, colostrum deprived, five week old pigs were experimentally infected with a PCV2 strain (GenBank # AF055392) which has 99% homology with a well characterized PCV2a strain (GenBank# AF264042) (Opriessnig et al., 2008). In another study, blood samples were collected at 2-3 week intervals from conventionally-raised, naturally-PCV2 infected pigs to determine the onset of viremia and seroconversion. In this population, PCV2 viremia and anti-PCV2-IgG antibodies were detected at 6 weeks of age through 25 weeks of age; the earliest and latest sampling point, respectively (Carasova et al., 2007). Similarly, in a longitudinal case-control study in naturally PCVAD affected farms in Denmark and Spain, PCV2 DNA and anti-PCV2-IgG antibodies were found in serum samples, nasal swabs and fecal swabs obtained from piglets at 1 week of age until time of necropsy (between 9-21 weeks of age) (Grau-Roma et al., 2009). In this study, the amount of PCV2 DNA present in serum was significantly correlated with the amount present in nasal and fecal swabs (Grau-Roma et al., 2009). PCV2 was also detected in oropharyngeal and nasal swabs, feces and serum in PCV2 experimentally infected animals from DPI 1 to the termination of the study at DPI 70 (Shibata et al., 2003). Information on the quantitative amount of PCV2 present in tracheobronchial, fecal, nasal,

tonsillar or urinary swabs has been reported; however, samples were taken from swine case submissions to a diagnostic laboratory, differential PCV2a/PCV2b testing was not reported and in vivo infectivity of the virus was not assessed (Segalés et al., 2005).

A limited number of studies have investigated the transmissibility of PCV2a. In one study, naïve pigs were placed in the same pen as experimentally PCV2a inoculated colostrum-deprived-cesarean-derived pigs. PCV2 DNA was detected in oropharyngeal and nasal swabs and feces from the naïve animals three days after inoculation of the experimental animals (Shibata et al., 2003). In a study which modeled the time-dependent transmission rate of PCV2 by placing naïve pigs in direct contact with experimentally infected pigs for 2 days, it was shown that PCV2a has a latency period of 8 days and a mean disease generation time of 18.4 days (Andraud et al., 2009).

As PCV2 is known to be present in blood, the recent outbreaks have raised concern about the spread of PCV2 in spray-dried plasma protein products in the feed. This has led some producers and practitioners to discontinue the use of plasma protein on their farms (Personal communication, Dr. Steve Dritz, Consulting Veterinarian and Nutritionist, Kansas State University). Evidence of the value of incorporating spray-dried plasma into the diet of weanling pigs to improve feed intake and growth performance has been well documented (Hansen et al., 1993; Lawrence et al., 2004; Zhao et al., 2007). However, the spray-drying process may not eliminate all possible bacterial and viral contaminates. PCV2, which is a small circular DNA virus, has been shown to be extremely resistant as characterized by a 1-log reduction following dry-heat treatment of freeze-dried PCV2 at 120°C for 30 minutes (Welch et al., 2006) and resistance to a 15 minute, 70°C heat treatment in cell culture (O'Dea et al., 2008). Although resistant, a 2008 publication reported that commercially manufactured spray-dried plasma protein (SDPP) containing 2.47×10^5 genomic copies/ml fed to weaning pigs did not result in seroconversion or viremia (Pujols et al., 2008). However, the pigs used in this study had maternally derived PCV2 antibody prior to initiation of the trial (Pujols et al., 2008). Similarly, in a study in which pigs were fed a ration containing 8% spray-dried porcine plasma (SDPP), subsequent blood samples were PCR and ELISA negative. The authors concluded that feeding SDPP did not result in transmission of PCV2 (Polo et al., 2007). Conversely, a study in which mice were fed a ration with 10% SDPP blood samples taken from 1 - 10 weeks post-inoculation were PCR positive for PCV2 viral DNA; an antibody response was also noted (Song et al., 2007). This study indicates that SDPP may be infectious to pigs.

As research makes a stronger case for fecal-oral transmission of PCV2, producers have become concerned over introduction of new PCV2 strains by means of transport vehicles. Field studies indicate that after disinfection, 100% of swabs taken on trailer surfaces were positive for PCV2 DNA as determined by PCR (personal communication, Dr. Mark Wagner, Fairmont Veterinary Clinic).

Information on the infection dynamics and transmission of porcine circovirus type 2b (PCV2b) is lacking. Without this vital information, development of biosecurity policies and other prevention strategies are substantially limited.

Objectives

1. Determination if PCV2b was present in North American prior to 2004 and 2005
2. Determination if there are differences in PCV2a and PCV2b shedding patterns (route, amount)
3. Determination of the routes of PCV2 transmission (fecal, oral, nasal, mechanical/needles)
4. Determination of the role of spray dried plasma protein products in PCV2 transmission
5. Determine the role of transport vehicles in PCV2 transmission and evaluate 4 different disinfection protocols

Materials and Methods

Objective 1: In order to determine if PCV2b was present in the US prior to 2005, tissue samples collected on different farms in the Midwest from pigs (n=81) suspected to have clinical PCVAD during 2002 and 2003 were analyzed. DNA was extracted and analyzed by a previously described quantitative PCV2a /PCV2b differential real-time PCR.

Objective 2: To determine shedding patterns, 4 groups of 9-week-old, conventional SPF pigs were inoculated as follows: Group 1 (n=7) negative controls, Group 2 (n=6) inoculated with ISU 40895 as a representative of PCV2a, Group 3 (n=6) inoculated with NC 16845 as a representative of PCV2b, and Group 4 (n=6) inoculated with ISU 4838 as a representative of PCV2a. Each pig was inoculated intramuscularly (2ml) and intranasally (3ml) with inoculum prepared at $10^{4.0}$ 50% tissue culture infectious dose. Blood samples were collected weekly for two weeks following infection. DNA extraction from serum samples was performed using the QIAamp DNA Mini kit (Qiagen) followed by quantification of PCV2 genomic DNA copy numbers by a previously described real-time PCR.[12] Continuous data was analyzed using a non-parametric ANOVA at each DPI followed by Wilcoxon pair-wise testing; *P* values < 0.05 were considered significantly different.

Objective 3: Five pigs were inoculated with 3ml intranasally and 2ml intramuscularly of $10^{4.0}$ 50% tissue culture infectious dose ($TCID_{50}$) per ml of PCV2b isolate NC-16845 at three weeks of age (positive control). Twenty-eight PCV2 naïve pigs either remained un-inoculated or were inoculated by various routes (intraperitoneal, intranasal, oral, intramuscularly or oral gavage) with either pooled nasal, oral, or fecal samples or with a contaminated needle collected from the positive control pigs.

Objective 4: At three weeks of age, nine colostrum-fed, crossbred, specific-pathogen-free (SPF) conventional pigs were divided into groups of three and placed into separate rooms. Pigs were left un-inoculated (NEG), inoculated intraperitoneally with a reconstituted spray-dried plasma product (Spray-Dried Product; SDP) or inoculated intraperitoneally with PCV2 infected plasma (POS). Blood samples were collected weekly following inoculation for 7 weeks and tested for the presence of anti-PCV2-IgG antibodies and PCV2 DNA. The inoculum was prepared as follows: A colostrum-fed, crossbred, specific-pathogen-free (SPF) conventional pig was inoculated with 3ml intranasally and 2ml intramuscularly of $10^{4.0}$ 50% tissue culture infectious dose ($TCID_{50}$) per ml of PCV2b isolate NC-16845 at 3 weeks of age as part of a separate trial. At 35 days post infection the pig was euthanized with an overdose of pentobarbital (Fatal Plus®, Vortech Pharmaceutical, Dearborn, MI) due to development of clinical signs consistent with PCVAD including severe dyspnea, diarrhea, and loss of condition. Following euthanasia, blood was collected in jars containing 14,300 Units of Heparin (Hospira, Inc., Lake Forest, IL) per liter of blood. The plasma was immediately centrifugated at 1700 *x g* for 10 minutes in 50 ml centrifuge tubes and stored at 4°C. A portion of the collected plasma was tested for the presence of PCV2 DNA by quantitative PCV2 PCR resulting in 1.1×10^3 genomic copies/ml. The diagnosis of PCVAD was further confirmed by the presence of intense PCV2 antigen staining by immunohistochemistry in the lungs, intestine and lymphoid tissues. Additional immunohistochemistry tests for Transmissible Gastroenteritis Virus, Rotavirus, and *Lawsonia intracellularis* were negative. Additional tests for porcine respiratory and reproductive virus, swine influenza virus, and *Mycoplasma hyopneumoniae* were negative. Three hundred milliliters of the collected plasma was spray-dryed using a benchtop spray dryer (Yamato Model ADL310, Yamato Scientific Co., LTD, Tokyo, Japan). A 0.4mm nozzle was used with the following parameters: T_{inlet} (inlet air temperature) of 67°C, aspiration rate of 0.6 m^3/min, T_{outlet} (outlet temperature) of 166°C, and an 820 ml/hr sample flow rate under 0.1 MPa of pressure. The resulting spray-dried plasma product was reconstituted in sterile saline to a concentration of 0.33g/ml. Pigs in the SDP group were inoculated with 3 ml of the reconstituted spray-dried plasma product. Pigs in the POS group were inoculated with 3 ml of the plasma collected from the pig diagnosed with PCVAD. Pigs in the NEG group were sham inoculated with 3 ml of sterile saline. To ensure that the spray dryer was not contaminated with PCV2 prior to the initiation of the run, it was disinfected with Virkon®S according to the manufactures recommendations (Dupont™, Pharmacal Research Laboratories, Inc., Naugatuck, CT). Ten swabs were then taken from various components of the apparatus, placed in sterile saline and were confirmed to be PCV2 negative by PCR analysis.

Objective 5: Three model trailers were designed and manufactured by Eby Trailers. The models were constructed with identical materials found in full-size trailers. The trailers were contaminated with feces collected at the time of euthanasia from a colostrum-fed, crossbred, specific-pathogen-free (SPF) conventional pig was inoculated with 3ml intranasally and 2ml intramuscularly of $10^{4.0}$ 50% tissue culture infectious dose ($TCID_{50}$) per ml of PCV2b isolate NC-16845 at 3 weeks of age as part of a separate trial which developed

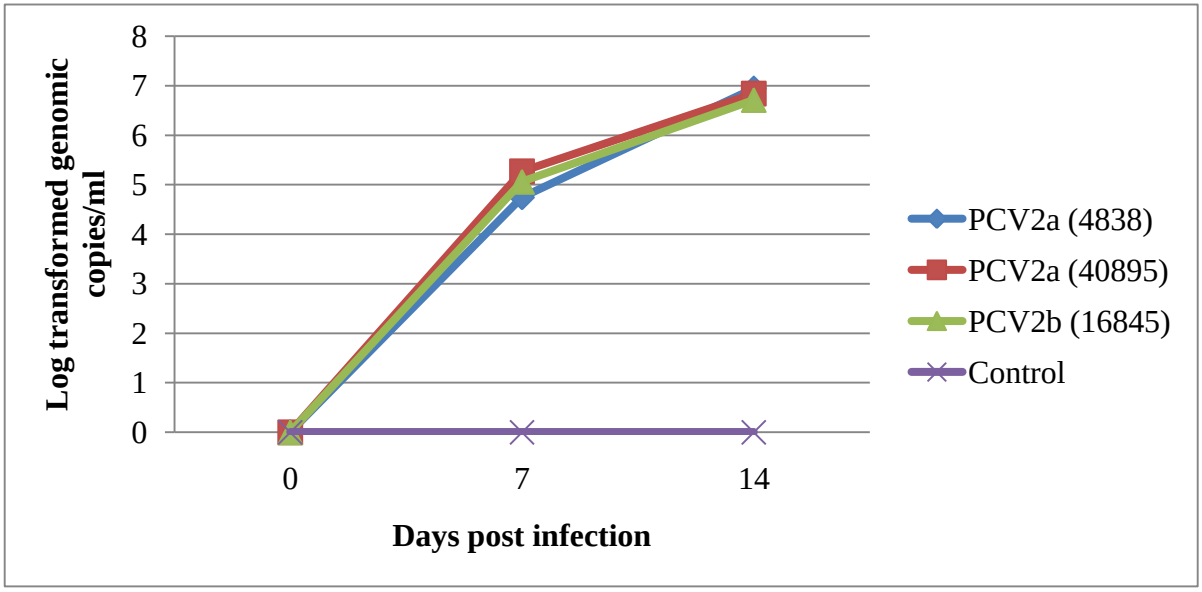
clinical signs of PCVAD at 35 days post infection. The collected feces was smeared on the wall and floor of the trailer and allowed to sit for 10 minutes. Trailers which were disinfected (all trailers excluding positive controls) were power-washed and rinsed similar to commercial washing procedures. After washing, the following disinfectants were applied per manufacturer's instructions: (1) Synergize, (2) Virkon, (3) Quatricide, and (4) bleach followed by Synergize in an incomplete block design with four replicates. The surface of the trailer was swabbed (6 swabs in total for each trailer) prior to contamination, following contamination, following the wash step and following the disinfection step. The swabs were tested by PCR for the presence of PCV2 DNA. As PCV2 is slow replicating and non-cytopathogenic, two naïve pigs were placed into each trailer for a total of 2 hours followed by moving into separate rooms and monitoring by ELISA and PCR for evidence of PCV2 seroconversion and replication.

Results

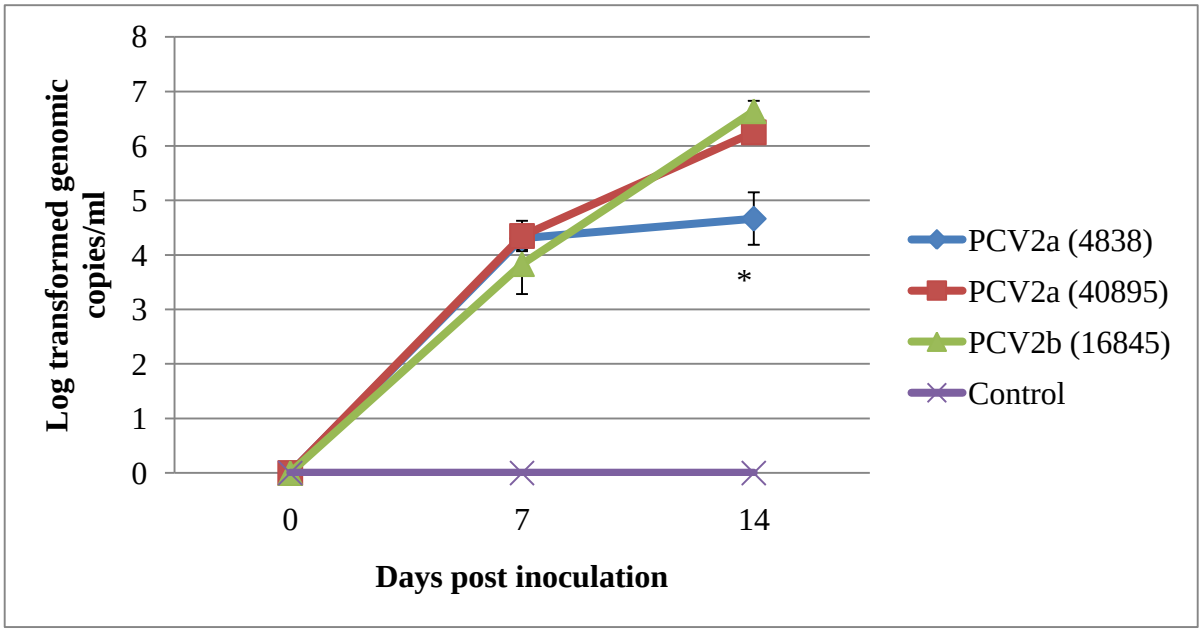
Objective 1: PCV2a was detected in 77/81 field samples; PCV1 was not detected in any of the tissue cases collected during the PCV2 surveillance study. PCV2b was detected in tissues in two cases. The first was tissue from a 107-day-old pig from a group of 2000 head in which 25% were affected by wasting, pallor, respiratory disease, diarrhea, icterus and anorexia. PCR on lung tissue was positive for H1N1. Immunohistochemistry for PRRSV and PCV2 were positive. The second case was tissue from a 60-day-old pig from a group of 1300 head in which 30 head were affected and 45 had died from disease characterized by wasting, pallor, respiratory disease, diarrhea, anorexia and lethargy. Immunohistochemistry for PRRSV was positive in the lung; however, PCV2 was not detected in the lymphoid tissues. The table below summaries selected data from the case submissions.

# of submitted cases	***PCV2 IHC score***	***PCV2a genomic copies/ml (±SE)***	***Average animal age (days)***
10	**1+**	**4.73 ± 0.54**	**95.9 ± 10.1**
18	**2+**	**6.74 ± 0.44***	**96.5 ± 7.5**
29	**3+**	**7.92 ± 0.23***	**86.3 ± 4.4**
24	**negative**	**4.20 ± 0.25**	**77.0 ± 8.8**

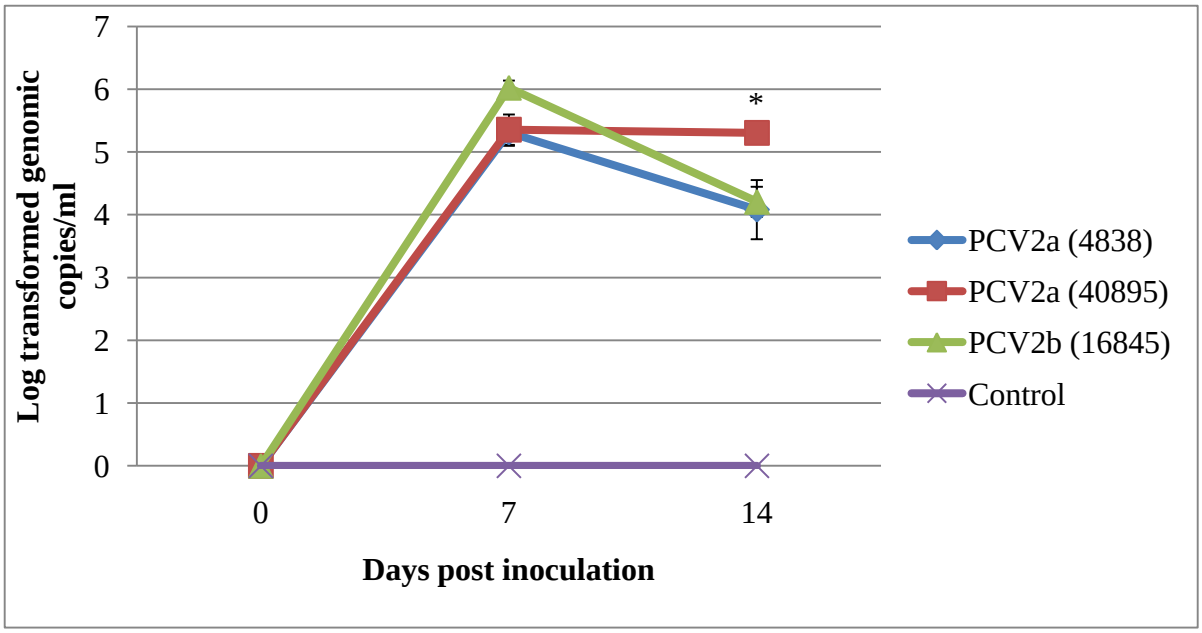
Objective 2:



A. Fecal swab results



B. Oral swab results. Asterisk (*) indicates a significant difference.



C. Nasal swab results. Asterisk (*) indicates a significant difference.

Objective 3:

Table 1 Number of anti-PCV2 IgG positive animals over total number of animals following intramuscular inoculation with a contaminated needle (Needle); intraperitoneal inoculation with feces, nasal secretions or oral secretions (Fecal IP, Nasal IP, oral IP respectively); oral gavage with oral secretions (Oral); oral inoculation with pooled fecal material (Fecal); or animals which were non-inoculated (Neg). Animals were considered positive if the sample-to-positive ratio was greater than 0.2.

Sample*	DPI					**Time to Seroconversion**
	14	**21**	**28**	**35**	**42**	**Mean days ± Std. err**
Fecal IP	0/3	1/3	3/3	3/3	3/3	25.7 ± 2.3
Fecal	0/4	0/4	1/4	1/4	2/4	35.0 ± 7.0
Nasal IP	0/3	1/3	1/3	3/3	3/3	30.3 ± 4.7
Nasal	0/4	1/4	2/4	4/4	4/4	29.8 ±3.4
Needle	0/4	0/4	0/4	0/4	0/4	NA
Negative	0/3	0/3	0/3	0/3	0/3	NA
Oral†	0/4	0/3	0/3	0/3	0/3	NA
Oral IP	0/3	0/3	1/3	2/3	3/3	35.0 ± 4.0

*Samples (oral, fecal, nasal) were taken at 21 days post inoculation from five pigs experimentally infected with PCV2b. The contaminated needle sample was taken at 25 days post inoculation.

†One animal in this group died of Mulberry Heart Disease

Table 2. Number of PCV2 viremic animals over total number of animals following intramuscular inoculation with a contaminated needle (Needle); intraperitoneal inoculation with feces, nasal secretions or oral secretions (Fecal IP, Nasal IP, oral IP respectively); oral gavage with oral secretions (Oral); oral inoculation with pooled fecal material (Fecal); or animals which were non-inoculated (Neg). Group mean $\log_{10}$ genomic copies/ml ± std. err of PCV2 at days post inoculation (DPI) 42 and time to viremia (days ± std. err) are provided.

	DPI							Time to viremia	DPI 42
Sample*	**0**	**7**	**14**	**21**	**28**	**35**	**42**	**(days ± std. err)**	**($\log_{10}$ genomic copies/ml ± std. err)**
Fecal IP	0/3	2/3	2/3	3/3	3/3	3/3	3/3	11.7±4.7	4.37±0.48
Feces	0/4	0/4	0/4	1/4	1/4	2/4	4/4	35.0±4.9	4.41±0.53
Nasal	0/4	2/4	2/4	3/4	4/4	4/4	4/4	15.8±5.3	4.60±0.44
Nasal IP	0/3	1/3	1/3	2/3	3/3	3/3	3/3	18.7±6.2	4.58±0.48
Needle	0/4	0/4	0/4	0/4	0/4	0/4	1/4	42.0±0.0	5.07±0.00
Negative	0/3	0/3	0/3	0/3	0/3	0/3	0/3	NA	NA
Oral†	0/4	0/3	0/3	0/3	0/3	0/3	0/3	NA	NA
Oral IP	0/3	0/3	0/3	3/3	2/3	3/3	3/3	21.0±0.0	4.69±0.23

*Samples (oral, fecal, nasal) were taken at 21 days post inoculation from five pigs experimentally infected with PCV2b. The contaminated needle sample was taken at 25 days post inoculation.
†One animal in this group died of Mulberry Heart Disease

Table 3. Microscopic lesions following intramuscular inoculation with a contaminated needle (Needle); intraperitoneal inoculation with feces, nasal secretions or oral secretions (Fecal IP, Nasal IP, oral IP respectively); oral gavage with oral secretions (Oral); oral inoculation with pooled fecal material (Fecal); or animals which were non-inoculated (Neg). Group mean ± std. error for each group at time of necropsy (42 days post inoculation) is provided.

Group	Overall lymphoid lesion score*	Lung score†
Oral IP	3.72±0.63	0.33±0.33
Fecal IP	0.72±0.72	0.00±0.00
Nasal IP	3.67±1.35	0.67±0.67
Oral	0.00±0.00	0.00±0.00
Fecal	1.42±0.48	0.50±0.29
Nasal	2.08±1.20	0.75±0.25
Needle	0.83±0.83	0.75±0.25
Negative control	0.00±0.00	0.00±0.00
Positive control	0.17±0.17	0.75±0.48

* An overall microscopic lymphoid lesions score which accounts for lymphoid depletion, histiocytic inflammation, and PCV2-antigen present in lymphoid tissues was calculated for each pig and ranged from 0=normal to 9=severe.

† Lung sections were scored for the presence and severity of interstitial pneumonia ranging from 0 (normal) to 4 (severe interstitial pneumonia).

Figure 1. Anti-PCV2-IgG sample-to-positive ratios for individual pigs (A-E) challenged with PCV2b at days post inoculation (DPI) 0.

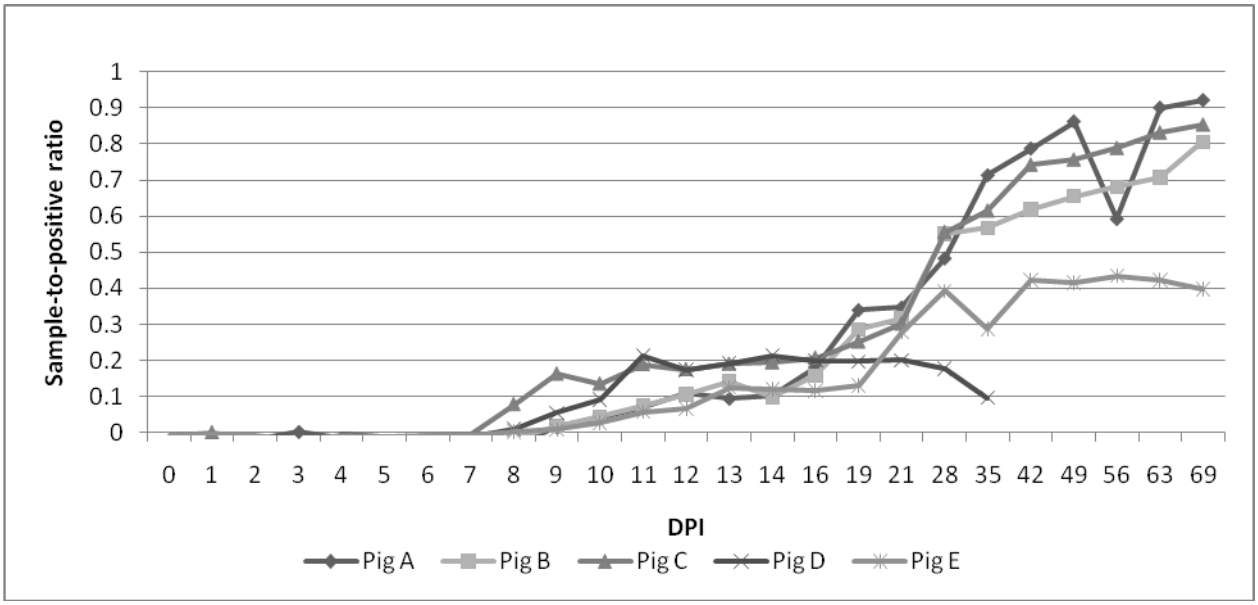
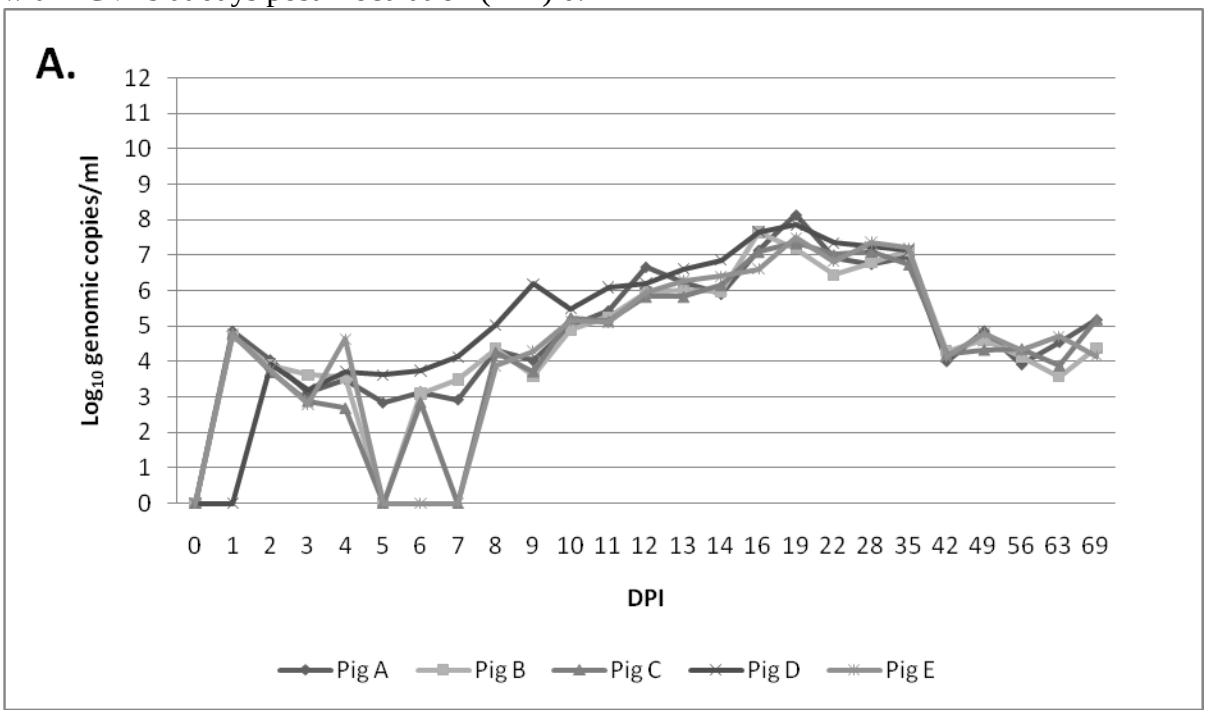
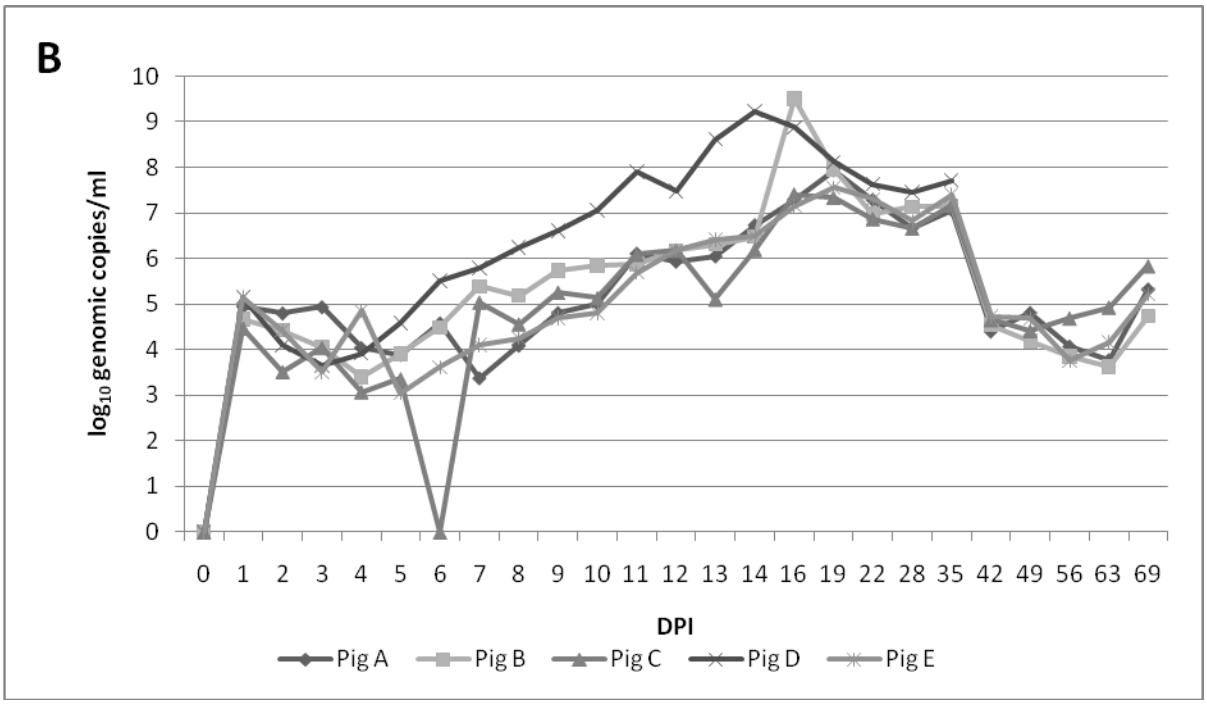
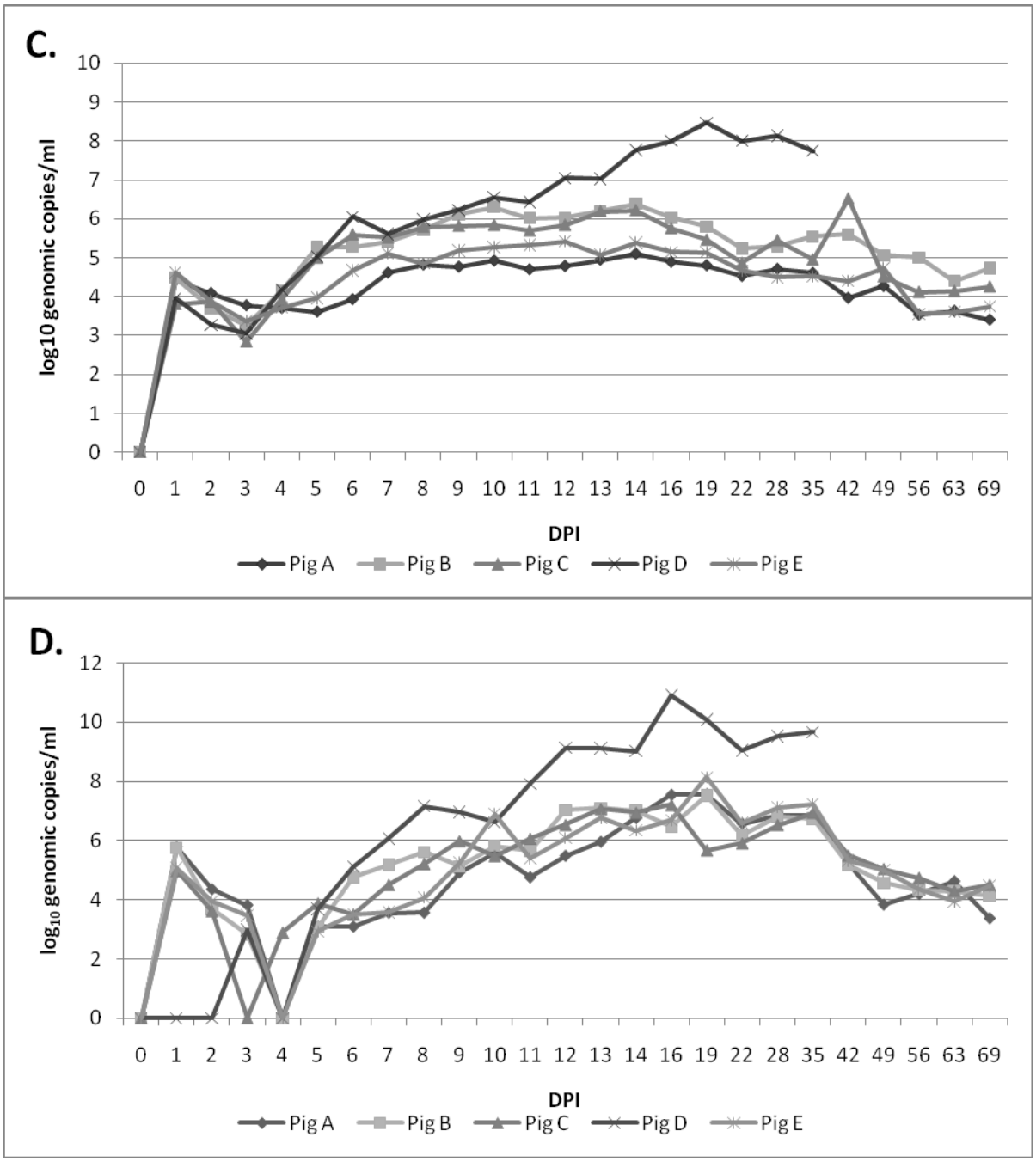


Figure 2. Log_{10} genomic copies/ml of PCV2 shed in oral fluid (Figure A), nasal secretions (Figure B), serum (Figure C) or feces (Figure D). Samples were collected from each individual pig (A-E) following challenge with PCV2b at days post inoculation (DPI) 0.






C.
log10 genomic copies/ml
0 1 2 3 4 5 6 7 8 9 10
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 16 19 22 28 35 42 49 56 63 69
DPI
Pig A
Pig B
Pig C
Pig D
Pig E

D.
log10 genomic copies/ml
0 2 4 6 8 10 12
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 16 19 22 28 35 42 49 56 63 69
DPI
Pig A
Pig B
Pig C
Pig D
Pig E

Objective 4:

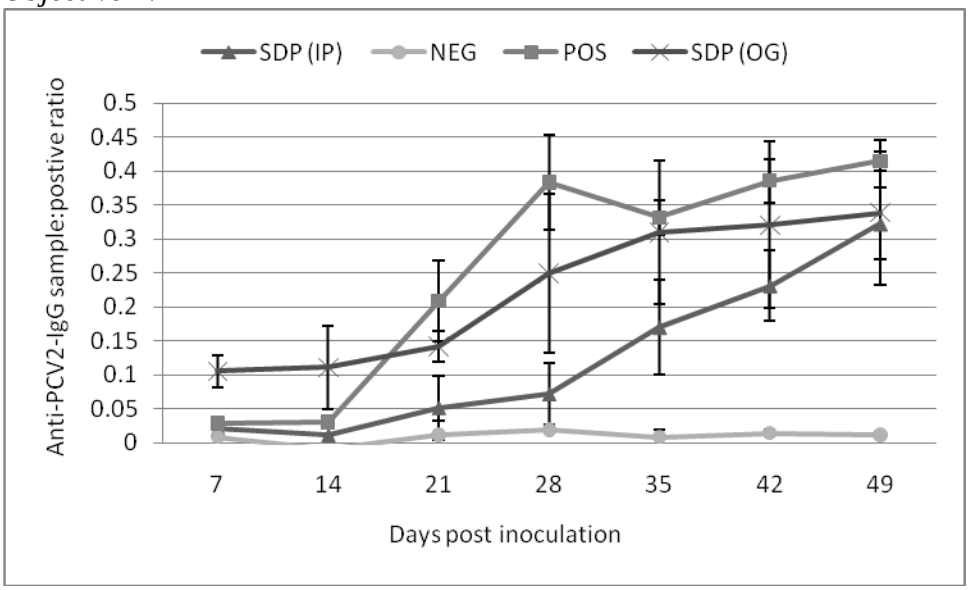


Figure 1. Anti-PCV2-IgG sample-to-positive ratios for pigs inoculated with spray dried plasma (SDP) by the intraperitoneal route (IP) or by oral gauge (OG), inoculated intraperitoneally with PCV2 contaminated plasma (POS) or inoculated intraperitoneally with sterile saline (NEG).

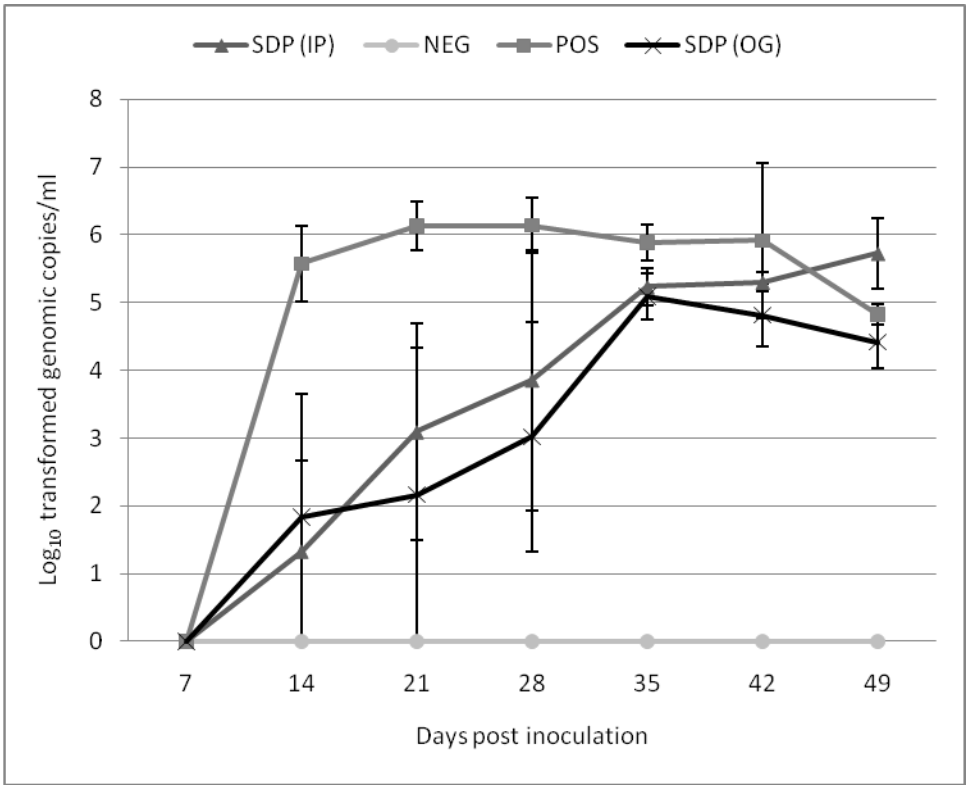


Figure 2. Log_{10} genomic copies/ml of PCV2 in pigs inoculated with spray dried plasma (SDP) by the intraperitoneal route (IP) or by oral gauge (OG), inoculated intraperitoneally with PCV2 contaminated plasma (POS) or inoculated intraperitoneally with sterile saline (NEG).

Table 1. Microscopic lesions from pigs inoculated with spray dried plasma (SDP) by the intraperitoneal route (IP) or by oral gauge (OG), inoculated intraperitoneally with PCV2 contaminated plasma (POS) or inoculated intraperitoneally with sterile saline (NEG). An overall microscopic lymphoid lesions score which accounts for lymphoid depletion, histiocytic inflammation, and PCV2-antigen present in lymphoid tissues was calculated for

each pig and ranged from 0=normal to 9=severe. Lung sections were scored for the presence and severity of interstitial pneumonia ranging from 0 (normal) to 4 (severe interstitial pneumonia).

Group	Lung Score Mean ± std. err	Lymphoid Tissue Score Mean ± std. err
SDP (IP)	2.00±0.58	3.67±2.81
SDP (OG)	1.00±0.00	2.67±1.20
POS	3.00±0.00	3.29±0.22
NEG	2.67±0.33	0.00±0.00

Objective 5:

Table 1. Anti-PCV2-IgG sample-to-positive (S:P) ratios and log_{10} genomic copies/ml of PCV2 on day post inoculation (DPI) 49 for pigs exposed to trailers which remained uncontaminated (NEG); were contaminated only (POS); were contaminated, washed and disinfected with Virkon® (VIRK); were contaminated, washed and disinfected with Quatricide® (QUAT); were contaminated, washed and disinfected with Synergize® (SYN); or were contaminated, washed and disinfected with Virkon® and Bleach® (VIRK+B). Sample-to-positive ratios greater than 0.2 are considered positive.

		DPI 49	
Treatment	Replicate	S:P Ratio	Log_{10} genomic copies/ml
NEG	1	0.07	0.00
POS	1	0.50	5.47
POS	2	0.25	4.18
POS	3	0.01	5.87
POS	4	0.20	5.90
QUAT	1	0.34	0.00
QUAT	2	0.01	0.00
SYN	3	0.10	0.00
SYN	4	0.44	0.00
VIRK	2	0.10	0.00
VIRK	3	0.44	0.00
VIRK+B	4	0.05	0.00

Discussion

While the differences in clinical signs apparent under field conditions in 2004 and 2005 may be correlated with the emergence of PCV2b in the United States, differences between PCV2a and PCV2b strains have not been reproduced under experimental conditions using a conventional pig model (Opriessnig et al., 2008). Additional evidence provided by this study further supports this theory. First, consistent differences between the amount of PCV2a and PCV2b shed in nasal, oral and fecal routes were not noted. Specifically, a significantly greater amount of PCV2a was recovered in nasal swabs compared to PCV2b. However, this difference was not replicated in either fecal or oral swabs. In reference to the results of this study it should be noted that it is more challenging statistically to prove evidence of a difference, especially with a limited sample size. Secondly, PCV2b was recovered from only two of 81 samples from PCVAD cases submitted between 2002 and 2003. This data suggests that PCV2b was not the predominant strain prior to the severe PCVAD cases seen in 2005.

To further investigate the PCV2b strain, this study quantitatively examined the shedding patterns of PCV2b in experimentally-infected, conventional, SPF pigs and determined whether nasal, fecal and oral

samples from these pigs are infectious to naïve animals. Peak viremia was detected in positive control pigs at DPI 14 with a mean ± standard error of 6.17 ± 0.47 $\log_{10}$ genomic copies/ml. This is similar to previous reports of experimental infection of SPF pigs with PCV2a (GenBank accession #AF027217) in which the highest number of animals were positive by conventional PCR at 2 to 3 weeks post infection (Magar et al., 2000). However, in populations of naturally infected pigs peak viremia has been shown to occur later. Specifically, in a study by Carasova et al., peak viremia was detected at 10 weeks of age with a maximum value of 7.85 $\log_{10}$ genomic copies/ml in samples from pigs in a conventional herd with previously diagnosed cases of PCVAD (Carasova et al., 2007). The differences in time to peak viremia are likely due to protection from maternal antibodies in the naturally infected pigs as the majority of herds are seropositive (Opriessnig et al., 2007). The differences in quantity of virus between the studies are likely less significant as there can be systematic differences in quantities reported between laboratories (Hjulsager et al., 2009). PCV2b DNA was detected in oral, nasal and fecal swabs and in serum from the majority of the positive control animals from DPI 1 until the termination of the study at DPI 69. Similarly, Shibata et al. reported that CDCD pigs inoculated with PCV2a had PCR positive fecal swabs from DPI 1 to 70 (Shibata et al., 2003). Following initial detection in the current study, the amount of PCV2b DNA decreased between DPI 2 – 7 before resuming an increasing trend at DPI 8. This is similar to a previous study in which PCV2 DNA was detected in 10/12 and 11/12 tonsillar and fecal swabs, respectively, by PCR at DPI 1 but in 0/12 and 1/12 tonsillar and fecal swabs, respectively, at DPI 6 (Caprioli et al., 2006). This trend was attributed to the direct detection of the PCV2 inoculum (Caprioli et al., 2006). However, in the previously mentioned study, PCV2 DNA was not detected in the serum until DPI 9 (samples were taken on DPI 2, 3, and 6) (Caprioli et al., 2006). In contrast, pigs in the current study were viremic at DPI 1 indicating that the DNA detected may be consistent with viremia. Additionally, a previous longitudinal study has indicated that shedding of PCV2 DNA in fecal and nasal swabs is intermittent in naturally-infected, viremic animals (Grau-Roma et al., 2009). Intermittent shedding in fecal samples was also shown following experimental PCV2 infection of cesarean-derived, colostrum-deprived (CDCD) pigs (Bolin et al., 2001).

In the current study, peak shedding in oral, nasal and fecal samples appeared at DPI 16, 16 and 19 respectively. No significant differences were apparent among group mean log transformed genomic copies/ml of PCV2 among the groups at the time of peak shedding. In subsequent time points, amounts of virus remained fairly stable with a downward trend until the removal of the PCVAD affected pig (Pig D) at DPI 35. When the PCVAD pig was removed from the group, the amount of PCV2b DNA decreased in all sample types collected from the remaining pen mates. This provides evidence to support the intuitive practice of removing or segregating sick animals from pen mates to reduce the viral load in the pen. Decreased viral load in the environment has been previously associated with herds without PCVAD (Larochelle et al., 2003).

To determine whether PCV2b was infectious by various routes, naïve animals were inoculated either by intraperitoneal, intranasal, oral or oral gavage with either pooled nasal, oral, or fecal samples collected from the positive control pigs. Intraperiotoneal inoculation with contaminated fecal, nasal and oral excretions resulted in viremia and seroconversion in naïve pigs. Nasal secretions inoculated directly into the nares and fecal material fed to naïve animals was also infectious resulting in seroconversion of all four animals by DPI 35. Feces fed to naïve animals resulted in transmission to 2 of 4 animals by DPI 42. Additionally, viremia and microscopic lesions were noted in one of the animals inoculated with a contaminated needle indicating that PCV2 may be spread among animals during vaccination. The lack of seroconversion in the animal inoculated with the contaminated needle is likely due to the termination of the study at DPI 42. Had the study been continued for an additional period of time, seroconversion likely would have occurred based on the seroconversion trends in the other groups.

While the results in the study are consistent with true infection of naïve animals with the Nasal IP, Fecal IP, Oral IP, Fecal and Nasal groups, the possibility of room contamination exists. However, animals in the negative control room, which was maintained in exactly the same conditions as experimental rooms, did not seroconvert, did not become viremic and did not have microscopic lesions consistent with PCV2. Additionally, extensive steps were taken to prevent cross-contamination among rooms including the use of multiple sampling teams whenever possible. If entering multiple rooms on the same day was absolutely necessary, the number of rooms entered was limited to 2, a shower was taken between rooms and different face masks, gloves and

coveralls were worn in each room. Additionally, sequence analysis of the PCV2 recovered from the pig had 100% homology to the isolated used for inoculation.

As all animals were housed together, it is possible that transmission within the room occurred once one animal became infected. Previous work has shown that the mean time for a newly infected animal to infect a susceptible animal is approximately 18 days when naïve animals are placed into the same pen as experimentally infected animals (Andraud et al., 2008). Based on this data, it is highly probable that within the fecal, oral and nasal IP groups at least one pig was infected (primary infection) by the original inoculation and subsequent pigs were infected from interaction with this animal (secondary infection). While ideally each pig would have been housed individually to perform replicates of the experiment, the conditions of the trial closely mimic field situations and answer the question of whether a group of animals can be infected by a particular route.

Mean time to viremia and seroconversion were calculated and statistically evaluated to determine if there were differences in the time required for various samples (nasal, oral or fecal) to infect pigs. Trends in the data suggest that the nasal route may be more effective than either the fecal and oral route. Additionally, in the positive control animals at DPI 69, a significantly higher amount of PCV2 DNA was detected in nasal swabs in comparison to fecal swabs and serum. This also may indicate that contact with nasal secretions may be a more important route than fecal-oral, especially in persistently infected animals. However, the main objective of the study was to determine simply whether the samples were infectious. As such, sample size per group was too small to gain statistical validation of differences in time to infection.

One potential mechanism of spread of PCV2b is through spray-dried plasma products. The most common spray-drying process uses a co-current flow of heated air and atomized spray in an open-cycle system to evaporate moisture from an aqueous or organic solution (Buchi training papers - spray drying; Buchi Labortechnik AG, 1997-2002). While inlet temperatures of 240°C are achieved during this process, the time in which the product is exposed to this temperature is short. This design enables drying of plasma products without the destruction of various proteins (Borg et al. 2008). Therefore, when spray dried plasma products are incorporated into the diets of weanling pigs, IgG and other proteins promote immune modulation of pro- and anti-inflammatory cytokines occurs resulting in improvements in growth parameters (Moreto et al., 2009). This same process which allows proteins to remain stable, may allow extremely resistant viruses such as PCV2 to retain infectivity.

Spray-dried products are generally considered free of infectious agents (Borg et al., 2002). Specifically, a study in which bovine plasma was spiked with pseudorabies virus (PRV) and porcine respiratory and reproductive syndrome virus (PRRSV), spray-dried and incorporated into the diet of weanling pigs did not result in seroconversion (Polo et al., 2005). However, in contrast to PRRSV and PRV, PCV2 has been shown to be extremely resistant. Additionally, a study where irradiation of feed-grade plasma was used to decrease the bacterial concentration of the spray-dried product, increased average daily gain (ADG) and average daily feed intake (ADFI) were noted in animals fed an irradiated feed-grade spray-dried animal plasma product in comparison to controls (DeRouchey et al., 2004). This suggests that bacteria are present within the spray-dried plasma product.

A 2008 publication reported that commercially manufactured spray-dried plasma protein (SDPP) containing 2.47×10^5 genomic copies/ml fed to weaning pigs did not result in seroconversion or viremia (Pujols et al., 2008). Differences between this trial and the current study are that PCV2 maternal antibodies were present in the animals prior to their use in the trial and the spray-dried plasma was incorporated into the feed. The current study provided evidence that spray-dried plasma is indeed infectious as evidenced by seroconversion and viremia following intraperitoneal injection and oral gavage.

Finally, trailers are a high risk factor for transmission of viruses due to the frequency of use and the variable efficacy of cleaning/disinfection methods. To mimic commercial transportation conditions, three model trailers were designed and manufactured by a commercial company. Trailers were specifically engineered to possess the worse features for cleaning and the best features for virus survival (seams, gates, etc.). The trailers were than contaminated with feces collected from a PCVAD affected pig. To maintain a constant level of contamination between trailers and replicates, the same amount of feces was used in all cases. Disinfection was conducted according to manufacturer's recommendations. To further mimic commercial

conditions, the pigs were hauled in the model trailers for 2 hours. Results of the study indicated that in all disinfection protocols were equally able to prevent seroconversion and viremia in naïve animals for 49 days.

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