Comparative Medicine
Copyright 2010
by the American Association for Laboratory Animal Science

Vol 60, No 1
February 2010
Pages 45–50

Infection of Cesarean-Derived Colostrum-Deprived Pigs with Porcine Circovirus Type 2 and Swine Influenza Virus

Huiling Wei, Stephen D Lenz, William G Van Alstine, Gregory W Stevenson,[†] Ingeborg M Langohr,[‡] and Roman M Pogranichniy[*]

Porcine circovirus type 2 (PCV2) and swine influenza virus (SIV) are important pathogens for porcine respiratory disease complex, which is economically significant worldwide. The pathogenesis of PCV2–SIV coinfection is unknown. In this study, we focused on establishing a challenge model for PCV2 to determine whether SIV influences PCV2 replication and increases the severity of PCV2-associated disease. Cesarean-derived colostrum-deprived pigs were inoculated intratracheally with cell culture medium only (negative control group), PCV2 only, or PCV2 followed 1 wk later with SIV H1N1. Two pigs from each group were necropsied at 12, 21, 28, and 35 d after inoculation. Coinfection with SIV did not increase the number of PCV2 genomic copies in serum or target tissues or the severity of microscopic lesions associated with PCV2 in lung or lymph node. The antibody titer to PCV2 did not differ significantly between PCV2–SIV- and PCV2-infected groups. In conclusion, SIV H1N1 did not influence PCV2 replication in dually infected pigs in this study.

Abbreviations: PCV2, porcine circovirus type 2; PRDC, Porcine respiratory disease complex; SIV, Swine influenza virus.

Porcine respiratory disease complex (PRDC) is an economically significant problem characterized by slow growth, poor food utilization, lethargy, anorexia, fever, cough, and dyspnea in pigs 16 to 22 wk of age.[14,38] PRDC is associated with complex sequential or concurrent infections with multiple viral or bacterial respiratory pathogens.[6,8,17,29] Field investigations and case-trend analyses demonstrate that porcine circovirus type 2 (PCV2) plays a role in PRDC.[12,17,24]

PCV2 belongs to the family *Circoviridae*, which contains the smallest nonenveloped, single-stranded, circular DNA viruses.[21,39] In the late 1990s, a pathogenic circovirus designated PCV2 was isolated, which differed from the nonpathogenic PCV1.[2,21] PCV2 is considered ubiquitous and can be detected in both diseased and clinically healthy pigs.[1] Infection induces various degrees of lymphoid depletion and immune suppression, demonstrated by experimentally infecting pigs with PCV2 infectious DNA clones.[10] However, the factors that contribute to the pathogenicity of PCV2 remain unknown.[24] Generally, infection with PCV2 alone is limited in its ability to induce the full spectrum of symptoms associated with PRDC; the role of PCV2 in PRDC always involves interaction or synergism with other respiratory pathogens.[4,17]

Swine influenza virus (SIV) is a common pathogen associated with PRDC.[6,8] SIV is an enveloped, negative-sense, segmented RNA virus belonging to the family *Orthomyxoviridae*.[36] SIV infects the epithelium of the respiratory tract of pigs, causing an acute infection with clinical signs of cough, fever, lethargy, and anorexia beginning 1 to 2 d after experimental infection and lasting for 3 to 4 d.[14,44] High morbidity and low mortality are quite common in uncomplicated disease, but mortality usually is high when other infectious agents are present along with SIV.[36] Together with PCV2, SIV frequently is found in pigs with clinical signs of PRDC. At one farm, mortality reached as high as 10% in pigs coinfected with PCV2 and SIV, and 5% of the coinfected pigs failed to reach market weight.[15] In a cross-sectional study, SIV infection was 11 times more likely to occur in PCV2-positive pigs compared with PCV2-negative pigs.[8] Field studies on pigs with PRDC conducted in different years showed a 1.9% to 13% rate of coinfection with PCV2 and SIV.[6,9,15,17,28,29] Clinical evidence suggests that SIV acts synergistically with PCV2 to cause PRDC. However, the pathogenesis of PCV2–SIV coinfection is unknown.

In this study, our goal was to establish a challenge model for PCV2 and PCV2–SIV and to determine whether SIV influences PCV2 replication and increases the severity of PRDC. Throughout the study, microscopic lesions attributable to PCV2 and PCV2 viral load in serum, nasal swab, lung, and lymph node did not differ between PCV2- and PCV2–SIV-inoculated pigs. On the basis of these findings, we conclude that SIV H1N1 did not influence PCV2 replication in dually infected animals.

Materials and Methods

Experimental design. Cesarean-derived colostrum-deprived Large White pigs were obtained from Struve Labs (Manning, IA) and were randomly assigned to 3 groups of 8 pigs each after they were delivered to the livestock infectious disease isolation facility at Purdue University (West Lafayette, IN), which is a Biosafety Level 2 animal housing facility with negative-pressure HEPA-

Received: 28 Aug 2009. Revision requested: 28 Sep 2009. Accepted: 09 Nov 2009.
Department of Comparative Pathobiology, Purdue University, West Lafayette, Indiana.
[*]Corresponding author. Email: rmp@purdue.edu
Current affiliation: [†]Department of Veterinary Diagnostic and Production Medicine, Iowa State University, Ames, Iowa; [‡]Diagnostic Center for Population and Animal Health, Michigan State University, Lansing, Michigan.

filtered ventilation. The experimental protocol was approved by the Purdue Animal Care and Use Committee. All the rooms and equipment in the isolation facility were disinfected twice (1% Virkon S, Pharmacal Research Laboratories, Waterbury, CT) followed by thorough washing before the delivery of the pigs. Each group of pigs was housed together on raised wire decks in a single, isolated room equipped with a nipple drinker and a self-feeder. Feed that was neither autoclaved nor irradiated was delivered by Struve Labs. The pigs were kept at 75 to 79 °F on a 12:12-h light:dark cycle. Before the study, the pigs were negative serologically (by ELISA) and by quantitative real-time PCR to most PRDC-related pathogens: PCV2, SIV, porcine reproductive and respiratory virus, and *M. hyopneumoniae*. At the end of the experiment, all pigs remained negative for porcine reproductive and respiratory virus and *M. hyopneumoniae.* The respective experimentally inoculated groups were positive for PCV2 and SIV (by serologic assay), and PCV2-inoculated groups were positive by real-time PCR for the presence of circovirus DNA in serum; negative control animals remained negative for SIV and PCV2 throughout the study.

One week after delivery (study day 0), pigs (age, 7 wk) were sedated by intramuscular injection (0.2 mL/12 lb. body weight) of a mixture of telazol, ketamine, and xylazine (final concentration of each drug, 50 mg/mL). Sedated animals were placed in sternal recumbency, the jaws were opened manually, the larynx was sprayed lightly with topical anesthetic (Cetacine, Cetylite Industries, Pennsauken, NJ), and a lighted oral speculum was placed in the mouth to facilitate insertion of a semirigid catheter into the proximal trachea, through which the virus suspension was to be deposited into the trachea. Pigs in groups 1 (5 female, 3 male) and 2 (3 female, 5 male) each were inoculated with 3 mL PCV2 genotype 1 (strain ADDLPP 10069, 1.6×10^5 $TCID_{50}$/mL), whereas those in group 3 (2 female, 6 male) received 3 mL culture medium only. The following week (study day 7), pigs (age, 8 wk) again were anesthetized and inoculated with PCV2 as previously (groups 1 and 2) or culture medium (group 3); group 1 animals also received 3 mL SIV H1N1 (A/Swine/A07-7967/IN, 2×10^7 pfu/mL). Pigs were kept under strict Biosafety Level 2 conditions to avoid cross-contamination among groups. In particular, appropriate personal protective equipment (coveralls, respirator [N95] masks, hairnets, latex gloves, and boots) and a designated room-entry order (group 3 > group 2 > group 1) were used by all personnel. People who had been in contact with other animals during the previous 24 h were required to shower before entering the pig isolators.

On the day of necropsy, pigs were euthanized by intraperitoneal injection (1 mL/10 lb. body weight; Beuthanasia D Special, Schering–Plough Animal Health, Union, NJ) and, after a surgical plane of anesthesia was achieved (that is, lateral recumbency and loss of palpebral, swallow, and gag reflexes), exsanguination by severing the axillary artery.

Clinical evaluation. Clinical signs (behavior, dyspnea, and cough) were monitored and scored (1, normal; 2, mild change; 3, marked change) daily.

Serologic assays. ***Immunofluorescent and hemagglutination inhibition assays.*** Blood for serum samples was collected from jugular veins or at necropsy on study days 0, 7, 12, 21, 28, and 35. PCV2 antibody titer was assessed by immunofluorescent assay as previously described.[31] Briefly 4-fold dilutions (starting at 1:20) of serum samples were applied to 96-well titer plates prepared by infecting PK15 cells with PCV2 and fixing infected cells to the bottoms of the wells by using 80% aqueous acetone. Antigen–antibody reaction was visualized by staining cells with fluorescein-conjugated goat-antiswine PCV2 antibody (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Antibody titers for individual samples were determined as the reciprocal of the highest dilution that showed specific fluorescence. The presence of SIV antibody in serum samples was tested by hemagglutination inhibition assay as described previously.[45]

Virus isolation and real-time PCR assay. Nasal swabs were collected on study days 7, 10, 12, 15, 17, 19, and 21. Inguinal, sublumbar, mesenteric, tracheobronchial, and submandibular lymph nodes and lung collected at necropsy (days 12, 21, 28, and 35; 2 pigs from each group per day) were frozen at –80 °C until processing. Viral DNA and RNA were extracted (QiaAmp DNA Mini Kit and QiaAmp Viral RNA Mini Kit, respectively; Qiagen, Santa Clarita, CA) from serum, nasal swabs, and tissue homogenate from pooled lung or lymph node samples. Real-time PCR for PCV2[26] and SIV H1N1[32] were used to quantify the viral genome copies in each sample. Freshly prepared pooled lung or lymph node tissue homogenate was used for virus isolation for both PCV2 and SIV. For PCV2, tissue homogenate was inoculated into a PK15 cell suspension and cultured for 3 d; infected cells were treated with 300 mM D-glucosamine, incubated 24 h longer,[40] and used in immunofluorescent assays. SIV was isolated from lung homogenate as previously described.[45]

Histopathology. On study days 12, 21, 28, and 35 d, 2 pigs from each group were weighed, euthanized, and necropsied and a mean group body weight determined by using the weights of the 2 representative pigs euthanized.

Lung and lymph node samples from all pigs were fixed in 10% neutral buffered formalin, processed, and embedded in paraffin, and sections were stained with hematoxylin and eosin (Animal Disease Diagnostic Laboratory, West Lafayette, IN). Lung and lymph node lesions attributable to PCV2 infection were evaluated by a board-certified veterinary pathologist, and scored by using a semiquantitative scoring system. Airway lesions in lung epithelial (necrosis, hypertrophy, hyperplasia, intraepithelial leukocytes, periglandular inflammation) were scored as: 0, absent; 1, mild (0% to 10% of the airways affected); 2, moderate (11% to 30% of airways affected); 3, marked (31% to 50% of airways affected); and 4, severe (more than 50% of the airways affected). Pulmonary inflammation was categorized as suppurative, lymphoplasmacytic or histiocytic; each category of inflammation was scored as: 0, absent; 1, mild; 2, moderate; and 3, marked. Individual airway lesion and inflammation scores were summed for each animal to give an overall score for the pulmonary alterations for each animal. Lymph node lesions (lymphoid depletion, lymphohistiocytic or granulomatous lymphadenitis) were scored as: 0, absent; 1, mild; 2, moderate; and 3, marked. Scores for lymphoid depletion and inflammation were summed for each animal to give an overall score for lymph node alterations for each animal.

In situ hybridization for PCV2. PCV2 nucleic acid in lung and lymph nodes was detected by in situ hybridization as previously described[13,31] with some modifications. After deparaffinization and rehydration, tissue sections on slides were prehybridized at 42 °C for 1 h. Hybridization was performed overnight at 42 °C with digoxigenin-labeled oligoprobes specific for the PCV2 genes encoding the replicase and capsid proteins.[18] Antidigoxigenin antibody conjugated with alkaline phosphatase (dilution 1:500)

and the substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Roche Diagnostics, Indianapolis, IN) were applied to visualize the presence of PCV2 nucleic acid.

Statistical analysis. Differences in body weight and PCV2 viral load in nasal swabs, lung, and lymph nodes were evaluated by comparing the means with one-way ANOVA (Prism 4.0, GraphPad Software, La Jolla, CA). Any statistical values $P < 0.5$ was considered statistically significant in this study. Clinical and pathologic scores and PCV2 nucleic acid signals by in situ hybridization were analyzed by using the Wilcoxon rank-sum test (SAS 9.1.3, SAS Institute, Cary, NC).

Results

Clinical evaluation. The negative control group (group 3) remained healthy throughout the study. Pigs inoculated with only PCV2 (group 2) showed mild and transient respiratory disease without coughing. Pigs dually infected with PCV2 and SIV (group 1) exhibited mild to moderate respiratory signs of PRDC characterized by increased respiratory rate, lethargy, and occasional coughing. Clinical signs for behavior and coughing did not differ significantly between PCV2- and PCV2–SIV-groups (data not shown), but respiratory scores were significantly ($P < 0.05$) higher for PCV2–SIV-infected pigs than those in the PCV2 group from days 9 to 23 (Figure 1). Respiratory disease lasted 4 times longer in the PCV2–SIV group than in PCV2 group. The group mean body weight of the 2 pigs at necropsy did not differ between groups (Figure 2).

Serologic assays. ***Immunofluorescence and hemagglutination inhibition assays.*** All pigs were serologically negative for PCV2 and SIV before inoculation. On day 12, 6 of the 8 pigs in the PCV2 group and 3 of the 8 pigs in the PCV2–SIV group had seroconverted to PCV2. By day 21, 7 of the 8 PCV2–SIV-infected pigs and all 8 pigs in the PCV2 group had developed detectable PCV2 antibody responses. The mean antibody titer to PCV2 did not differ between the 2 groups throughout the study (Figure 3). Antibodies to SIV were first detected in all pigs in PCV2–SIV group on day 21 after SIV infection.

Virus isolation and PCR. Negative-control pigs (group 3) remained negative for both PCV2 and SIV throughout the study. On day 7, all 8 pigs in the PCV2 group and 4 of the 8 comprising the PCV2–SIV group were viremic for PCV2. All pigs in both groups were viremic by day 12, and the viremia of the pigs in both groups persisted for the entire 35 d of the study. PCV2 shedding was detected in all nasal swabs (obtained on days 7, 10, 12, 15, 17, 19, and 21) from all the pigs in both the PCV2–SIV and PCV2 groups. The PCV2–SIV and PCV2 groups did not differ significantly in the mean number of PCV2 genomic copies in serum, pooled lung, pooled lymph node, and nasal swabs (Figure 4).

Real-time PCR detected SIV in nasal swabs collected from PCV2–SIV-infected pigs on days 10 and 12 (3 and 5 d after SIV inoculation) and in pooled lung homogenates on day 12. In addition, SIV was isolated from the lungs of PCV2–SIV-infected pigs euthanized on day 12. On day 35 (study end), PCV2 was isolated from all lymph node samples, 6 of 8 lung samples from PCV2–SIV-infected pigs, and from 5 of 8 lungs from pigs in the PCV2 group.

Histopathology. In the PCV2–SIV group, gross lung lesions were lobular in distribution; were sharply demarcated from adjacent, nonpneumonic lung; and primarily affected the dependent regions of the apical and cardiac lobes. Affected tissue was dark red-purple, slightly firm, and slightly depressed. On section, lesions typically were peribronchial, and bronchi contained clear to slightly opaque, catarrhal exudates. Pigs in the PCV2 and negative-control groups lacked gross lung lesions. Tracheobronchial lymph nodes were enlarged in pigs in both the SIV–PCV2 and PCV2 groups.

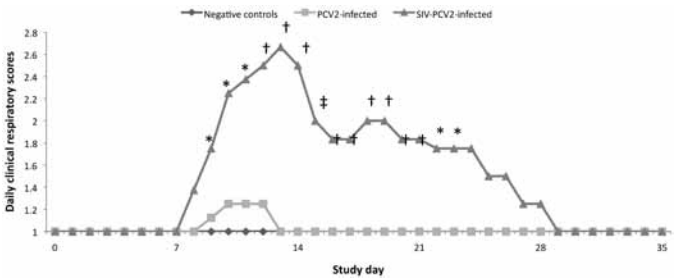

Figure 1. Mean daily clinical respiratory scores from day 1 to study end. *, $P < 0.05$; between values for negative-control and SIV–PCV2-infected groups.

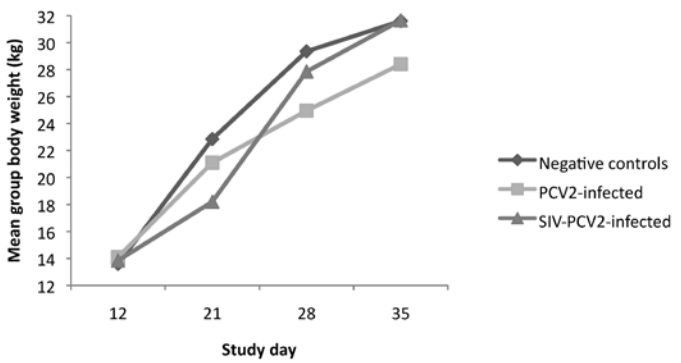

Figure 2. Mean body weight of the 2 pigs from each group euthanized on days 12, 21, 28, and 35.

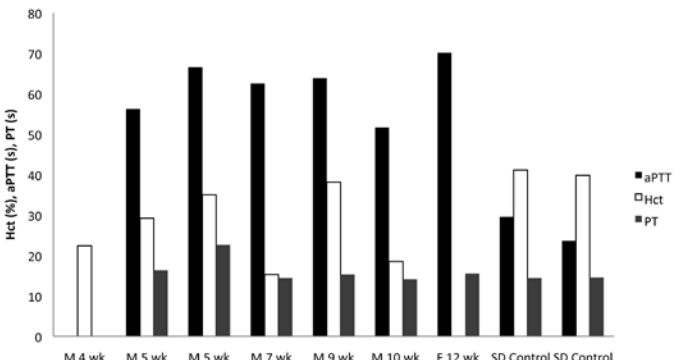

Figure 3. Mean serum PCV2 antibody titer measured by immunofluorescent assay.

Microscopic lesions attributable to PCV2 infection consisted of lymphoid depletion, lymphohistiocytic or granulomatous lymphadenitis, and interstitial pneumonia. According to semiquantitative scoring, the microscopic lesions associated with PCV2 in lymphoid tissues (Figure 5) and lungs (Figure 6) were not significantly different between PCV2–SIV and PCV2 groups (data not shown). The intensity of PCV2 signal by in situ hybridization of lung and lymph node also did not differ significantly between the 2 groups (data not shown).

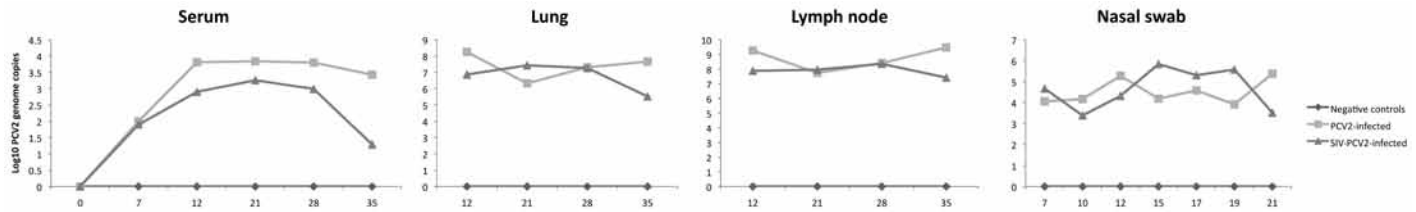

Figure 4. Group mean PCV2 viral load in serum, lung, lymph node, and nasal swabs.

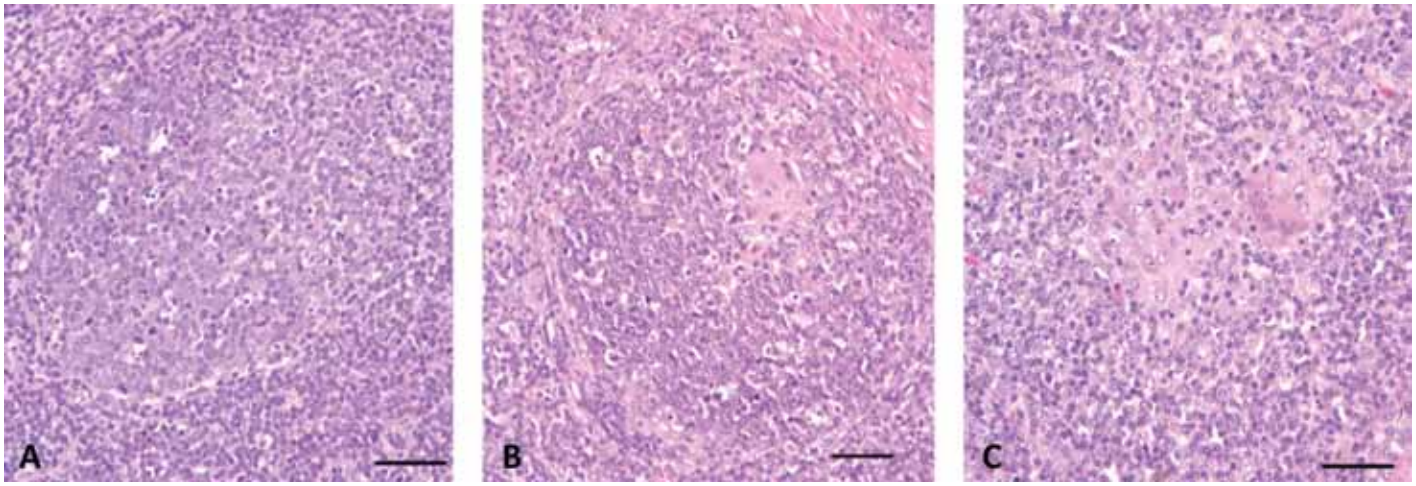

Figure 5. Lymph node, day 28. (A) Representative section from the negative-control group shows a normal lymphoid follicle. Representative sections from (B) SIV–PCV2-infected and (C) PCV2-infected pigs show similar lesions, with mild lymphoid depletion and few histiocytes and multinucleated giant cells infiltrating lymph node follicles. Hematoxylin and eosin stain; bar, 50 µm.

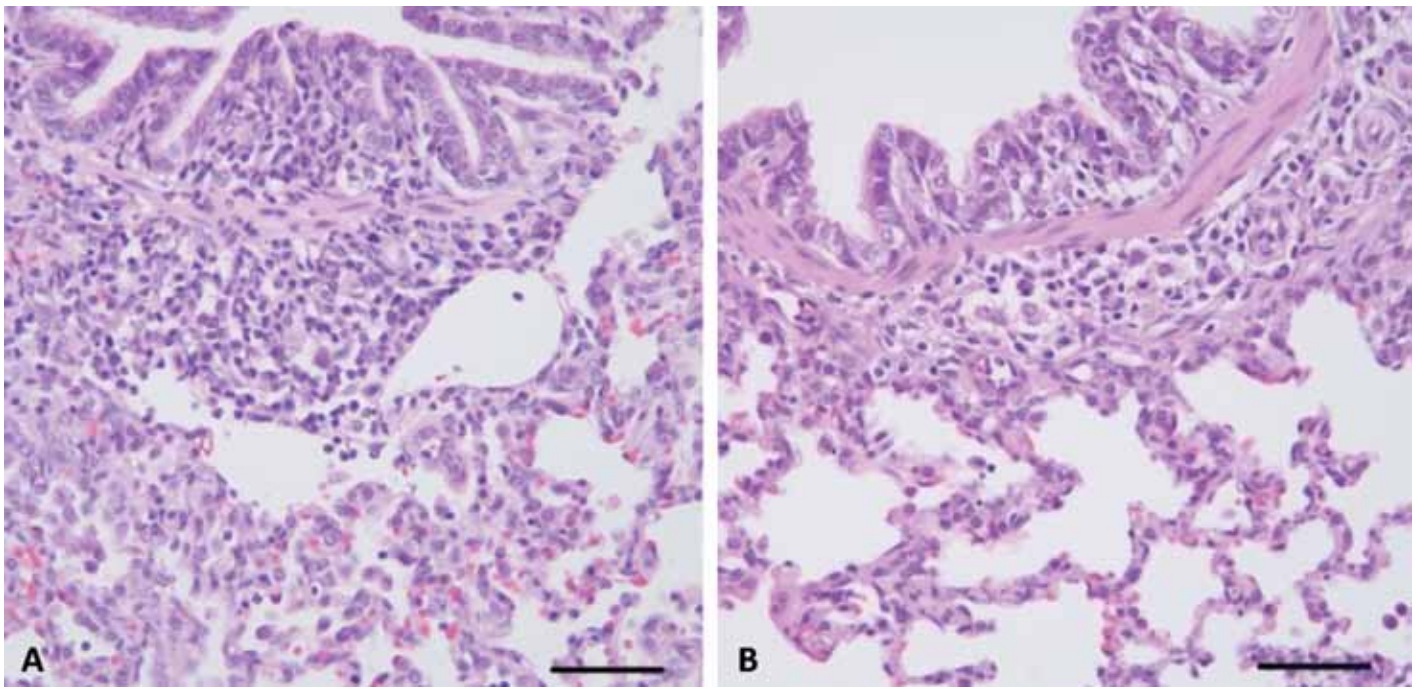

Figure 6. Lung, day 35. Representative sections from (A) SIV–PCV2-infected and (B) PCV2-infected pigs. Both sections show moderate peribronchiolar lymphoplasmacytic inflammation and mild mononuclear leukocyte infiltrates in the alveolar septa, consistent with interstitial pneumonia. Hematoxylin and eosin stain; bar, 100 µm.

Discussion

Field case studies have shown that coinfection of pigs with SIV and PCV2 is associated with severe PRDC.[15,17] In the present study, infection with PCV2 only caused mild respiratory signs, whereas PCV2–SIV-infected pigs showed severe and prolonged disease. This difference might reflect synergism between SIV and PCV2. However, scoring of lesions in lung and lymph nodes and the PCV2 viral load in lung, lymph node, and nasal swabs were not significantly different between the PCV2- and PCV2–SIV-infected groups. Therefore, the data of this study indicate that the H1N1 strain of SIV does not enhance the replication of PCV2, and there is limited synergism between the 2 viruses. There may be multiple known or unknown factors complicating the clinical manifestation of PCV2–SIV coinfections in the field.

The SIV strain in the conditions of this study is weakly pathogenic. Due to insufficient replication, SIV may not have induced sufficient cytokines (including such as IFNα, IL6, and TNFα) or monocyte-attracting chemokines to potentiate PCV2 replication in the lung.[42,43] The low pathogenicity of the SIV strain used may have limited pulmonary cell death and subsequent cellular regeneration, resulting in fewer actively replicating cells in lung than typical during severely pathogenic swine influenza. This decrease would be significant because PCV2 DNA synthesis requires cellular enzymes that are expressed during the S-phase growth of actively replicating cells.[40] The lack of these enzymes to enhance replication of PCV2 might account for the failure of SIV to potentiate PCV2 replication in our model.

The timing of coinfection might also be important in the induction of PRDC.[25] In PCV2 infection, viral load has been correlated with disease severity.[3,23,34] Perhaps with a longer interval between inoculation with PCV2 and SIV the pigs might have developed a PCV2 viral load high enough to interact synergistically with SIV. In addition, the age of pigs when they are infected with PCV2 may influence disease development.[31,41] A meta-analysis of experimental infections with PCV2 found that age younger than 3 wk at inoculation showed strong positive correlation with the development of disease.[41] Because of the terms of our contract with the vendor, we could not obtain cesarean-derived colostrum-deprived pigs younger than 5 wk; future studies likely should involve pigs 3 wk of age and younger. Although disease severity is inversely correlated to the magnitude of the PCV2 antibody response,[22,30] the cause of the weak immune response in severely diseased pigs is unknown. All pigs in the present study developed strong humoral immunity to PCV2 (Figure 3) and lesions typical of PCV2-associated disease, except wasting. Animal husbandry also plays a role in the development of disease.[24]

The results of this study suggest that coinfections of SIV and PCV2 in severe cases of PRDC are underestimated by the routine diagnostic testing done in the field. Infection with PCV2 is ubiquitous in both clinically healthy and sick pigs all over the world.[29,33] Infection with SIV is also common. Serologic surveillance for SIV reveals a prevalence ranging from 20% to 100% in pig populations of different ages in different countries.[5-7,11,16,19,20,27,35,37] Therefore, the likelihood of the 2 viruses coinfecting the same pig population is high. In contrast, the reported rate of concurrent SIV-PCV2 during PRDC in the field is 1.9% to 13%.[6,9,15,17,28,29] In the present study, SIV was cleared rapidly but PCV2 was not. Perhaps similarly in field cases of PRDC, the SIV infection often has cleared before routine diagnostic testing is done on clinically ill animals. Coinfection with PCV2 and SIV remains a likely common cause of PRDC; however, given the many interacting variables of infection including strains of viruses, age at infection, relative timing of infection, and immune reactivity of individual pigs, additional studies are needed to model and study the disease.

In addition, to obtain deeper understanding of the pathogenesis of concurrent infection with PCV2 and SIV, comprehensive epidemiologic studies should be done by measuring and monitoring important parameters including pig age, the approximate time of infection by PCV2 and other common respiratory viruses, viral titers, vaccinations, and immune response—all of which may influence the disease outcome. The rate and consequences of PCV2–SIV coinfection differs from pig to pig and from herd to herd, and these different parameters should be explored further to understand PRDC disease development in the field and to help develop models for future study.

Acknowledgments

The authors thank the staffs of the Virology and Serology Section (Animal Disease Diagnostic Laboratory) and Veterinary Lab Animal Care (Purdue University, West Lafayette, IN) for their help during this study. The study was supported by School of Veterinary Medicine Internal Funding.

References

1. **Allan GM, Ellis JA.** 2000. Porcine circoviruses: a review. J Vet Diagn Invest **12:**3–14.
2. **Allan GM, McNeilly F, Kennedy S, Daft B, Clarke EG, Ellis JA, Haines DM, Meehan BM, Adair BM.** 1998. Isolation of porcine circovirus-like viruses from pigs with a wasting disease in the USA and Europe. J Vet Diagn Invest **10:**3–10.
3. **Brunborg IM, Moldal T, Jonassen CM.** 2004. Quantitation of porcine circovirus type 2 isolated from serum, plasma, and tissue samples of healthy pigs and pigs with postweaning multisystemic wasting syndrome using a *Taq*Man-based real-time PCR. J Virol Methods **122:**171–178.
4. **Chae C.** 2005. A review of porcine circovirus 2-associated syndromes and diseases. Vet J **169:**326–336.
5. **Choi YK, Goyal SM, Joo HS.** 2002. Prevalence of swine influenza virus subtypes on swine farms in the United States. Arch Virol **147:**1209–1220.
6. **Choi YK, Goyal SM, Joo HS.** 2003. Retrospective analysis of etiologic agents associated with respiratory diseases in pigs. Can Vet J **44:**735–737.
7. **Damrongwatanapokin S, Parchariyanon S, Pinyochon W.** Serological study of swine influenza virus h1n1 infection in pigs of Thailand. Fourth International Symposium on Emerging and Reemerging Pig Diseases. 29 Jun–02 Jul 2003. Rome, Italy.
8. **Dorr PM, Baker RB, Almond GW, Wayne SR, Gebreyes WA.** 2007. Epidemiologic assessment of porcine circovirus type 2 coinfection with other pathogens in swine. J Am Vet Med Assoc **230:**244–250.
9. **Drolet R, Larochelle R, Morin M, Delisle B, Magar R.** 2003. Detection rates of porcine reproductive and respiratory syndrome virus, porcine circovirus type 2, and swine influenza virus in porcine proliferative and necrotizing pneumonia. Vet Pathol **40:**143–148.
10. **Fenaux M, Halbur PG, Haqshenas G, Royer R, Thomas P, Nawagitgul P, Gill M, Toth TE, Meng XJ.** 2002. Cloned genomic DNA of type 2 porcine circovirus is infectious when injected directly into the liver and lymph nodes of pigs: characterization of clinical disease, virus distribution, and pathologic lesions. J Virol **76:**541–551.
11. **Golinar I, Valenak Z.** Swine influenza in slovene herds. Fourth International Symposium on Emerging and Reemerging Pig Diseases. 29 Jun–02 Jul 2003. Rome, Italy.
12. **Grau-Roma L, Segales J.** 2007. Detection of porcine reproductive and respiratory syndrome virus, porcine circovirus type 2, swine

influenza virus and Aujeszky disease virus in cases of porcine proliferative and necrotizing pneumonia (PNP) in Spain. Vet Microbiol **119:**144–151.

13. **Gregory CR, Latimer KS, Niagro FD, Campagnoli RP, Steffens WL, Ritchie BW.** 1996. Detection of eastern equine encephalomyelitis virus RNA in formalin-fixed, paraffin-embedded tissues using DNA in situ hybridization. J Vet Diagn Invest **8:**151–155.
14. **Halbur PG.** 1997. Porcine respiratory disease complex. Proceedings of the North Carolina Healthy Hogs Seminar. 5 Nov 1997. Greenville, NC.
15. **Harms PA, Halbur PG, Sorden SD.** 2002. Three cases of porcine respiratory disease complex associated with porcine circovirus type 2 infection. Journal of Swine Health and Production **10:**27–30.
16. **Jung T, Choi C, Chung HK, Kim J, Cho WS, Jung K, Chae C.** 2002. Herd-level seroprevalence of swine influenza virus in Korea. Prev Vet Med **53:**311–314.
17. **Kim J, Chung HK, Chae C.** 2003. Association of porcine circovirus 2 with porcine respiratory disease complex. Vet J **166:**251–256.
18. **Langohr IM, Stevenson GW, Nelson EA, Lenz SD, HogenEsch H, Wei H, Pogranichniy RM. 2009.** Vascular lesions in Pigs experimentally infected with porcine circovirus type2b. Vet Pathol in Press.
19. **Li H, Xin X, Yang H, Li Y, Qin Y, Cai X, Chen H, Yu K, Bi Y, Tong G.** Serological and virologic surveillance for swine influenza virus infections among pigs over large areas in China in 1998. 2002. Fourth International Symposium on Emerging and Reemerging Pig Diseases. 29 Jun–02 Jul 2003. Rome, Italy.
20. **Maldonado J, Van Reeth K, Riera P, Sitja M, Saubi N, Espuna E, Artigas C.** 2006. Evidence of the concurrent circulation of H1N2, H1N1, and H3N2 influenza A viruses in densely populated pig areas in Spain. Vet J **172:**377–381.
21. **Meehan BM, McNeilly F, Todd D, Kennedy S, Jewhurst VA, Ellis JA, Hassard LE, Clark EG, Haines DM, Allan GM.** 1998. Characterization of novel circovirus DNAs associated with wasting syndromes in pigs. J Gen Virol **79:**2171–2179.
22. **Okuda Y, Ono M, Yazawa S, Shibata I.** 2003. Experimental reproduction of postweaning multisystemic wasting syndrome in cesarean-derived, colostrum-deprived piglets inoculated with porcine circovirus type 2 (PCV2): investigation of quantitative PCV2 distribution and antibody responses. J Vet Diagn Invest **15:**107–114.
23. **Opriessnig T, McKeown NE, Zhou EM, Meng XJ, Halbur PG.** 2006. Genetic and experimental comparison of porcine circovirus type 2 (PCV2) isolates from cases with and without PCV2-associated lesions provides evidence for differences in virulence. J Gen Virol **87:**2923–2932.
24. **Opriessnig T, Meng XJ, Halbur PG.** 2007. Porcine circovirus type 2 associated disease: update on current terminology, clinical manifestations, pathogenesis, diagnosis, and intervention strategies. J Vet Diagn Invest **19:**591–615.
25. **Opriessnig T, Thacker EL, Yu S, Fenaux M, Meng XJ, Halbur PG.** 2004. Experimental reproduction of postweaning multisystemic wasting syndrome in pigs by dual infection with *Mycoplasma hyopneumoniae* and porcine circovirus type 2. Vet Pathol **41:**624–640.
26. **Opriessnig T, Yu S, Gallup JM, Evans RB, Fenaux M, Pallares F, Thacker EL, Brockus CW, Ackermann MR, Thomas P, Meng XJ, Halbur PG.** 2003. Effect of vaccination with selective bacterins on conventional pigs infected with type 2 porcine circovirus. Vet Pathol **40:**521–529.
27. **Ouchi A, Nerome K, Kanegae Y, Ishida M, Nerome R, Hayashi K, Hashimoto T, Kaji M, Kaji Y, Inaba Y.** 1996. Large outbreak of swine influenza in southern Japan caused by reassortant (H1N2) influenza viruses: its epizootic background and characterization of the causative viruses. J Gen Virol **77:**1751–1759.
28. **Pallarés FJ, Halbur PG, Opriessnig T, Sorden SD, Villar D, Janke BH, Yaeger MJ, Larson DJ, Schwartz KJ, Yoon KJ, Hoffman LJ.** 2002. Porcine circovirus type 2 (PCV-2) coinfections in US field cases of postweaning multisystemic wasting syndrome (PMWS). J Vet Diagn Invest **14:**515–519.
29. **Palzer A, Ritzmann M, Wolf G, Heinritzi K.** 2008. Associations between pathogens in healthy pigs and pigs with pneumonia. Vet Rec **162:**267–271.
30. **Perez-Martin E, Grau-Roma L, Argilaguet JM, Nofrarias M, Escribano JM, Gomez-Sebastian S, Segales J, Rodriguez F.** 2008. Development of two *Trichoplusia ni* larvae-derived ELISAs for the detection of antibodies against replicase and capsid proteins of porcine circovirus type 2 in domestic pigs. J Virol Methods **154:**167–174.
31. **Pogranichnyy RM, Yoon KJ, Harms PA, Swenson SL, Zimmerman JJ, Sorden SD.** 2000. Characterization of immune response of young pigs to porcine circovirus type 2 infection. Viral Immunol **13:**143–153.
32. **Richt JA, Lager KM, Clouser DF, Spackman E, Suarez DL, Yoon KJ.** 2004. Real-time reverse transcription–polymerase chain reaction assays for the detection and differentiation of North American swine influenza viruses. J Vet Diagn Invest **16:**367–373.
33. **Segales J, Allan GM, Domingo M.** 2005. Porcine circovirus diseases. Anim Health Res Rev **6:**119–142.
34. **Segales J, Calsamiglia M, Olvera A, Sibila M, Badiella L, Domingo M.** 2005. Quantification of porcine circovirus type 2 (PCV2) DNA in serum and tonsillar, nasal, tracheobronchial, urinary, and faecal swabs of pigs with and without postweaning multisystemic wasting syndrome (PMWS). Vet Microbiol **111:**223–229.
35. **Shieh HK, Chang PC, Chen TH, Li KP, Chan CH.** 2008. Surveillance of avian and swine influenza in the swine population in Taiwan, 2004. J Microbiol Immunol Infect **41:**231–242.
36. **Straw BE, Zimmerman JJ, D'Allaire S, Taylor DJ.** 2006. Diseases of swine. Ames (IA): Blackwell Publishing Professional.
37. **Teodoroff TA, Pecoraro MR, Baumeister E, Janke BH, Machuca M, Cappuccio JA, Perfumo CJ.** Serological and immunohistochemical studies of influenza virus in fattening pigs in Argentina. Fourth International Symposium on Emerging and Reemerging Pig Diseases. 29 Jun–02 Jul 2003. Rome, Italy.
38. **Thacker EL.** 2001. Porcine respiratory disease complex—what is it and why does it remain a problem? Pig J **48:**66–70.
39. **Tischer I, Gelderblom H, Vettermann W, Koch MA.** 1982. A very small porcine virus with circular single-stranded DNA. Nature **295:**64–66.
40. **Tischer I, Peters D, Rasch R, Pociuli S.** 1987. Replication of porcine circovirus: induction by glucosamine and cell-cycle dependence. Arch Virol **96:**39–57.
41. **Tomas A, Fernandes LT, Valero O, Segales J.** 2008. A meta-analysis on experimental infections with porcine circovirus type 2 (PCV2). Vet Microbiol **132:**260–273.
42. **Van Reeth K.** 2000. Cytokines in the pathogenesis of influenza. Vet Microbiol **74:**109–116.
43. **Van Reeth K, Labarque G, Nauwynck H, Pensaert M.** 1999. Differential production of proinflammatory cytokines in the pig lung during different respiratory virus infections: correlations with pathogenicity. Res Vet Sci **67:**47–52.
44. **van Reeth K, Nauwynck H.** 2000. Proinflammatory cytokines and viral respiratory disease in pigs. Vet Res **31:**187–213.
45. **World Health Organization**. 2005. WHO manual on animal influenza diagnosis and surveillance. Geneva (Switzerland): World Health Organization.