Title: Evaluation of pathogenesis of concurrent SIV and PCV2 infection in CD/CD pigs – NPB 08-148

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Institute: Purdue University

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Industry Summary: The objectives of the project were to determine if 1) SIV infection (H1N1, not pandemic H1N1) could initiate clinically significant porcine circovirus associated respiratory disease (PCVAD-respiratory) or the severe form of systemic PCVAD (PCVAD systemic) in pigs subclinically infected with PCV2b; and, 2) a pre-existing, subclinical PCV2b infection would have any effect on the duration or severity of the SIV infection. In order to test these hypotheses, we chose a PCV2-SIV co-infection model using caesarean-derived, colostrum-deprived (CD/CD) pigs to compare the clinical, serological, virological and pathological parameters. Pigs were housed by experimental group in separate rooms in a biosafety level 2 isolation facility. Pigs inoculated intranasally on day 1 with PCV2b, followed 17 days later by an intra-tracheal inoculation of SIV. Appropriate control groups (sham-inoculated, PCV2b only and SIV only) were used. PCV2b infection was confirmed by serum PCR and serology. SIV infection was confirmed by identifying virus shed in nasal secretions. Under the conditions of this study, subclinical PCV2b infection plus SIV infection resulted in increased severity of clinical respiratory signs, an increased amount of SIV shed in nasal secretions of dual-infected pigs, and shedding was sustained for 9 days longer than the SIV-only group. SIV initiated the severe form of PCVAD in 20% of the dual-infected pigs, but did not increase the PCV2b load in nasal secretions or tissues of pigs without the severe form of the disease. These results indicate that there are reciprocal effects between SIV and PCV2b when the PCV2b infection precedes SIV by approximately 17 days, and, at least in part, help to explain anecdotal observations of increased duration and severity of disease in field cases of SIV in PCV2-infected herds.

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Abstract

The objective of this project were to investigate the pathogenesis of an H1N1 (not pandemic H1N1) swine influenza infection in pigs subclinically infected with porcine circovirus type 2b (PCV2b). Forty caesarean-derived, colostrum-deprived (CD/CD) pigs were randomly assigned to four groups, 10 pigs per group. Group 1 pigs served as sham-inoculated controls. Group 2 pigs were inoculated with SIV, Group 3 pigs were inoculated with PCV2b and SIV, and Group 4 pigs were inoculated with PCV2b. Pigs were inoculated intranasally with PCV2b at 4 weeks of age (study day 0) and SIV pigs were inoculated intra-tracheally at 6.5 weeks of age (study day 17). Dual-infected pigs had increased (p < 0.05) clinical respiratory scores (dyspnea and cough), shed increased amount of SIV in nasal secretions (p < 0.05), and had a prolonged period of SIV shedding when compared to SIV-only inoculated pigs. Macroscopic and microscopic lung and lymph node lesions were not significantly different between the dual-infected group and the SIV group. Group mean...
genomic copies of SIV or PCV2b were not increased in lung tissue, and PCV2b was not increased in lymph nodes, of dual-infected animals. Two of ten (20%) dual-infected pigs developed lesions of the severe form of PCVAD, characterized by hydrothorax, diffuse pulmonary interlobular edema, ascites and mesocolonic edema. In these two pigs, lymphoid depletion was severe in all lymphoid tissues (lymph nodes, spleen, tonsil), and was associated with increased amount of PCV2 antigen as determined by immunohistochemistry. Under the conditions of this study, subclinical PCV2 infection potentiated the amount and duration of SIV shedding and the severity of clinical signs, when SIV infection occurred at 17 days post-PCV2b infection and, SIV infection was capable of triggering the severe form of PCVAD in 20% of the animals.

Introduction

Porcine circovirus-2 (PCV2) continues to be one of the principal viral pathogens affecting the United States swine herd, and PCV2 infection is wide-spread in swine populations in most pork-producing countries. Although PCV2 infection alone has been caused to result in post-weaning multisystemic wasting syndrome (PMWS; PCV2-associated systemic disease (PCVAD)) (Allan et al., 1998; Bolín et al., 2001; Allan et al., 1999; Ellis et al., 1998), other clinicopathological forms of PCVAD are more commonly recognized, including PCV2-associated pneumonia, PCV2-associated enteritis, PCV2-associated respiratory disease (PCVAD associated respiratory disease (PCVAD)) in pigs subclinically infected with PCV2 and SIV infection will result in increased severity of one or both diseases in pigs subclinically infected with PCV2; and, 2) if pre-existing, subclinical PCV2 infection would have any effects on the duration or severity of the SIV infection. In order to test these hypotheses, we chose a PCV2-SIV co-infection model in caesarean-derived, colostrum-deprived (CD/CD) pigs to compare the clinical, serological, virological and pathological parameters.

Objectives: We hypothesize that concurrent PCV2 and SIV infection will result in increased severity of one or both diseases in affected pigs. We propose to determine 1) if SIV (H1N1, not pandemic H1N1) infection can initiate clinically significant porcine circovirus associated respiratory disease (PCVAD-respiratory) in pigs subclinically infected with PCV2; and, 2) if pre-
existing, sub-clinical PCV2 infection effects the duration or severity of a subsequent SIV (H1N1) infection. Specifically, we propose to:

1. Compare the severity of clinical signs, gross and microscopic lesions and amount of PCV2 and SIV antigen (by immunohistochemistry) in pigs inoculated with PCV2 alone, SIV alone, or PCV2 + SIV.
2. Compare the amount of SIV and PCV2 nucleic acids in oronasal secretions between pigs inoculated with PCV2 alone, SIV alone, or PCV2 + SIV.
3. Compare the amount of PCV2 and SIV nucleic acids in sera, tissues and nasal swabs by quantitative real time PCR.

Materials and Methods

Animals: All animal procedures were approved in advance by the Purdue Animal Care and Use Committee. Caesarean-derived, colostrum-deprived (CD/CD) crossbreed piglets were purchased from a high health commercial herd known to be free of the majority of swine pathogens. Sows were negative serologically (by ELISA) for PRRS virus and porcine parvovirus (PPV), and had no detectable PRRS virus or PCV2 virus in serum, as determined by quantitative real time PCR. Piglets were reared individually to 3 weeks of age on raised stainless steel decks in the biosafety level 2 livestock infectious disease isolation facility at Purdue University, with 12h light-dark cycle and HEPA-filtered ventilation. At 17-21 days of age, they were weaned onto a meal diet appropriate for the age. At 4 weeks of age, 40 piglets were randomly assigned to one of four treatment groups, 10 piglets per group. Each group was housed in a separate animal isolation room (same facility), on stainless steel decks, with 12h light-dark cycle and HEPA-filtered ventilation. Standard meal diet (appropriate for the stage of production) and water were provided ad libitum for the duration of the study. At the time of group assignment, all piglets were seronegative (by ELISA) to PRRS virus, PCV2, SIV and PPV, and did not have detectable circulating PRRS or PCV2 virus (as determined by quantitative real time PCR).

Virus Inoculums: An infectious clone of PCV2b isolate ADDL PP 10069 was propagated on PK-15 cells free of PCV1 and PCV2 viruses. This infectious clone was previously shown to cause minimal morbidity and no mortality when inoculated intramuscularly in CD/CD pigs, but did result in infection (viremia detected by PCR, seroconversion to PCV2) and microscopic lymph node lesions of granulomatous inflammation with detectable PCV2 antigen (Pogranichiy and Lenz, unpublished data). Pigs in groups 1 and 2 were inoculated intranasally on day 0 with 5 ml (2.5 ml per nostril) of virus suspension containing 10^{15} TCID_{50} of PCV2. The H1N1 SIV isolate (A/Swine/PA07-7967/IN) was propagated on MDCK cells. On study day 17, pigs in groups 2 and 3 were sedated (see below for details) and inoculated intratracheally with 2.5 ml of virus suspension containing 10^{4.5} TCID_{50} SIV H1N1.

Experimental Design: A PCV2 + SIV co-infection model was used. Forty, 4-week-old, CD/CD pigs, negative for PRRS virus, PCV2, SIV, PPV and M. hyopneumoniae (by serology and serum PCR) on study day 0, were randomly assigned (10 pigs per group) to one of four treatment groups: Group 1, sham inoculated, negative control group; Group 2, SIV only; Group 3, PCV2b + SIV; Group 4, PCV2b only. On study day 0, pigs in groups 1 and 2 were inoculated intranasally with 5 ml (2.5 ml per nostril) of virus suspension containing 10^{15} TCID_{50} of PCV2b ADDL PP 10069. Pigs in groups 3 and 4 were sham-inoculated with an equal volume of sterile media. On study day 17, pigs in groups 2 and 3 were sedated with a combination of tiletamine/zolazepam/ketamine/xylazine (50 mg/ml each, final concentration; dosed at 0.2 ml/lb body weight, intramuscularly), and inoculated intratracheally with 2.5 ml of virus suspension containing 10^{4.5} TCID_{50} SIV H1N1 (A/Swine/PA07-7967/IN). Pigs in groups 1 and 4 were sham-inoculated with an equal volume of sterile media. To measure shedding of SIV and PCV2, nasal swabs were collected from all pigs on study days 17, 19, 21, 23, 26, 32, 39 and 45 (respectively, days 0, 2, 4, 6, 9, 15, 22 and 28 post-SIV inoculation). To assess seroconversion and viral load in serum, blood was collected from all pigs on study days -3, 10, 18, 21, 26, 32 and 45. Four pigs from each group were randomly selected for sacrifice on study day 21 (4 days post-SIV) and necropsied. The remaining pigs were sacrificed on study day 45 (28 days post-SIV). Gross lesions were photographed and recorded, and specimens of target tissues (lung, lymph node, kidney, liver, spleen, ileum) were collected for quantitative PCR, histopathology and immunohistochemistry, and virus isolation.

Serology: Blood was collected at birth (umbilical cord) from each pig, and via anterior vena cava venapuncture, on study days -14, -3, 10, 18, 21, 26, 32 and 45. Serum was tested for SIV (HI and ELISA for H1N1 and H3N2), PCV2 (IFA and
ELISA) and *M. hyopneumoniae* (ELISA) according to protocols in place at the Animal Disease Diagnostic Laboratory, Purdue University.

**Clinical Assessment:** Beginning on study day 0, all pigs were monitored and scored daily for lethargy, coughing, dyspnea, wasting and icterus. Each category was scored 1-4, 1 being normal and 4 being severe. For statistical analysis, the daily observation score was the sum of the daily observations for lethargy, coughing, dyspnea, wasting and icterus, and the daily respiratory score was the sum of the daily observations for coughing and dyspnea.

**Gross Pathology:** Target organs of SIV and PCV2 (lung, lymph nodes - inguinal, iliac, mesenteric, tracheobronchial, mediastinal; tonsil, thymus, kidney, ileum, colon and liver) were evaluated at each sacrifice date for gross lesions. Lymph node enlargement was estimated on a scale of 0 (normal) to 3 (3 times normal size) (Opriessnig et al., 2004). Lungs were photographed, and the images were subjected to stereologic analysis (Gundersen, et al., 1988) to determine the percentage of lung affected by pneumonia. Specimens of lung (all lobes), lymph nodes (tracheobronchial, inguinal, cervical, iliac and mesenteric), spleen, tonsil, thymus, kidney, liver, ileum, and colon were collected into 10% buffered-neutral formalin and processed for histopathology.

**Histopathology and Immunohistochemistry:** All tissue sections were coded to facilitate unbiased (i.e., blinded) evaluation and scoring by a board-certified veterinary pathologist (SDL). Microscopic lung lesions (necrotizing bronchiolitis, septal inflammation, proprial and peribronchiolar inflammation, type 2 pneumocyte hyperplasia, alveolar exudate) were scored on a scale of 0 (no lesion) to 6 (severe, diffuse) and lymph node lesions (lymphoid depletion, loss of follicle architecture and granulomatous/histiocytic inflammation) were scored from 0 (no lesion) to 3 (severe) (Opriessnig, et al., 2004). For immunohistochemical detection of PCV2 antigen, a rabbit polyclonal antibody (Iowa State University Veterinary Medical Research Institute) was reacted with formalin-fixed, paraffin embedded sections of lymph node using an avidin-biotin complex procedure (Sorden, et al., 1999). Amount of PCV2 antigen was estimated based on a scale of 0 (no antigen) to 3 (> 50% of follicles contain PCV2 antigen) (Opriessnig, et al., 2004; Opriessnig, et al., 2007). SIV antigen was detected using a monoclonal mouse antibody (Biodesign, Inc.) on sections of lung. The amount of SIV antigen was estimated using a scale of 0 (no detectable antigen), 1 (<10% of bronchioles affected), 2 (10-50% of bronchioles contain antigen) or 3 (>50% of bronchioles contain antigen). Immunohistochemical procedures were performed on an automated stainer (DAKO Autostainer, Carpinteria, CA)

**Quantitative RT-PCR:** Viral load in serum, nasal secretions, lung, lymph node pool (inguinal, sublumbar, mesenteric, tracheobronchial, and cervical) was determined by quantitative real-time PCR. Specimens were frozen at −80 °C until processed for PCR. 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 of pooled lung or lymph node samples. Real-time PCR with standard curve for PCV2 (Opriessnig, et al., 2003) and SIV H1N1 (Richt, et al., 2004) were used to quantify the amount of virus genomic copies in each sample.

**Virus Isolation:** 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 (Tischer, et al., 1987) and presence of the virus confirmed by immunofluorescence assays using specific PCV2 antibodies. SIV was isolated from lung homogenate as previously described.(WHO, 2005)

**Statistical Analysis:** Clinical scores and pathology lesion scores (gross and microscopic) were analyzed by non-parametric Kruskal-Wallis analysis of variance. Where indicated, differences were further analyzed by pair-wise analysis of groups using a Tukey post-hoc test. Continuous parametric data (serology, viral load in nasal swabs, lung, lymph nodes and serum, animal weights,) was analyzed by analysis of variance (ANOVA) using SAS software (SAS 9.1.3, SAS Institute, Cary, NC). Incidence data (clinical disease, gross and microscopic lesions) was evaluated with a Fisher’s exact test. Significance for all analyses was set at p < 0.05.

**Results**

**Clinical Assessment:** Clinical signs were not observed in any group prior to day 17 of the study. Signs of cough and/or dyspnea (clinical respiratory signs) were detected in SIV-inoculated pigs on day 18 (1 day post-SIV) and on subsequent
days in SIV-inoculated and PCV2b-infected, SIV-inoculated groups. Clinical respiratory scores in dual-infected pigs were 2-3 fold higher (statistically significant, p < 0.05) than in SIV-infected pigs on days 5-8 post-SIV (Fig. 1). There were no significant differences in the duration of clinical respiratory signs between these two groups. Clinical respiratory scores in both SIV-inoculated groups (SIV and PCV2b+SIV) were significantly greater (p < 0.05) than that in the PCV2b inoculated group. Clinical respiratory scores in PCV2b-infected pigs were slight and affected only a few pigs in the group. There was no detectable icterus in any PCV2b-infected pigs. PCV2b-infected pigs (Group 4) had reduced average daily weight gain beginning week 2 of the study through week 5 (Fig. 2), and this reduction was statistically significant at weeks 2, 4 and 5 (p < 0.05). SIV-infected pigs (groups 2 and 3) had reduced average daily weight gain during the week following SIV inoculation, and corresponding to the period of peak clinical respiratory signs and disease due to SIV.

**Figure 1.** Clinical respiratory scores (cough + dyspnea) at time points following SIV inoculation (d17). Prior to this date, no clinical signs were observed in PCV2b inoculated pigs. No clinical respiratory signs were detected after day 29.

**Figure 2.** Different superscripts indicate statistically significant (p < 0.05) differences at each time point. Data presented as group mean average daily gain per week ± SD.

**Serology:** At study day 0, all pigs were seronegative for PCV2 (by ELISA and IFA), SIV (by ELISA and HI), PPV (ELISA), *Mycoplasma hyopneumoniae* (ELISA) and porcine respiratory coronavirus (ELISA), and had no circulating PRRS virus or PCV2 as determined by quantitative real-time PCR. All pigs remained seronegative for *M. hyopneumoniae*, PPV and PRC throughout the study. Pigs in groups 1 (sham-inoculated control) and 4 (PCV2b only) remained seronegative to SIV throughout the study, and pigs in groups 1 and group 2 (SIV only) remained seronegative to PCV2 throughout the study. There were no statistically significant differences in SIV HI titers or in PCV2 IFA titers between the PCV2b + SIV infected group and the respective SIV-infected or PCV2b-infected group at any time point during the study (data not shown).

**Viral Load in Nasal Secretions Serum and Tissues:** The PCV2b+SIV infected group shed increased amount of SIV in nasal secretions when compared to the SIV infected group at days 2 and 6 post-SIV (p < 0.05), and the PCV2b+SIV group continued to shed virus out to day 15 post-SIV (3/6 pigs at day 9 post-SIV and 2/6 pigs at day 15 post-SIV). For the SIV group, virus in nasal secretions was only detected out to day 6 post-SIV inoculation. There were no statistically
significant increases in the amount of PCV2 shed in nasal secretions of the PCV2b+SIV infected group or the PCV2b infected group at any time point (Fig. 4). No statistically significant differences in the amount of PCV2 in serum (Fig. 5) or lung or lymph node pool (Fig. 6) were detected in the PCV2b+SIV infected group or the PCV2b infected group at any time point, and no differences in the amount of SIV in lung in the PCV2b+SIV infected group when compared to the SIV group at day 4 post-SIV (data not shown).

**Figure 3.** Amount of SIV in nasal secretions. Different superscripts indicate statistically significant (p < 0.05) differences at each time point. Data presented as group mean genome copies per ml media ± SD.

**Figure 4.** Amount of PCV2 in nasal secretions. No differences between the PCV2b-SIV group and the PCV2b group at any time point. Data presented as log10 of the group mean/ml media ± SD.

**Figure 5.** Amount of PCV2 in serum. There were no statistically significant differences between the PCV2b-SIV group and the PCV2b group at any time point. Data presented as log10 of the group mean/ml serum ± SD.
**Gross Pathology:** At day 4 after SIV inoculation (21 days post-PCV2b inoculation), macroscopic lung and lymph node lesions were identified in the three inoculated groups (4/4 pigs in the PCV2b + SIV group, 4/4 in the SIV group, and 3/4 in the PCV2b group). The estimated percentage of lung tissue affected by pneumonia was similar in the PCV2b+SIV and SIV only groups (Table 1.). Lesions in these groups were typical of SIV infection, being lobular in distribution, sharply demarcated from adjacent, non-pneumonic lung, and affecting primarily the dependent regions of the apical and cardiac lobes. Affected tissue was dark red-purple, slightly firm and slightly depressed (Fig. 7). On section, lesions were typically peribronchial, and bronchi contained clear to slightly opaque, catarrhal exudate. Lung lesions in PCV2 inoculated pigs at 4 days were minimal, with few identifiable areas of light grey parenchyma. Lymphadenopathy was identified in 4/4 pigs in each of the PCV2b-inoculated groups. In the SIV inoculated group, the tracheobronchial lymph nodes were enlarged in all 4 pigs, but other lymph nodes were grossly normal. Statistically, there were no differences among the three inoculated groups (Table 1). At the end of the study (45 days post-PCV2b, 28 days post-SIV), macroscopic lung lesions were minimal, consisting of few small foci of lobular atelectasis in the apical and cardiac lobes of pigs in the SIV and PCV2b+SIV groups only, and minimal lymphadenopathy in the PCV2b+SIV and PCV2b groups (data not shown).

**Figure 6.** Amount of PCV2 in lung and lymph node at each sacrifice day. There were no statistically significant differences between the PCV2b-SIV group and the PCV2b group at either time point. Data presented as log10 of the group mean/g tissue ± SD.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Percentage of Lung with Pneumonia&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Gross Lymphadenopathy&lt;sup&gt;2&lt;/sup&gt;</th>
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<tr>
<td>PCV2b and SIV</td>
<td>4</td>
<td>39.30 ± 18.36&lt;sup&gt;b3&lt;/sup&gt;</td>
<td>1.35 ± 0.34&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>SIV</td>
<td>4</td>
<td>33.38 ± 8.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.50 ± 0.35&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>PCV2b</td>
<td>4</td>
<td>0.93 ± 0.67&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.10 ± 0.77&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Sham Inoculated</td>
<td>4</td>
<td>0.00 ± 0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.00 ± 0.00&lt;sup&gt;a&lt;/sup&gt;</td>
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<sup>1</sup>Total amount (0-100%) of entire lung affected with pneumonia. 
<sup>2</sup>Score range from 0 (normal) to 3 (three times normal size). 
<sup>3</sup>Different superscripts in each column indicate significantly (P < 0.05) different values in mean scores among treatment groups.
Histopathology: At day 4 post-SIV (day 21 post-PCV2b), microscopic lung lesions in the SIV-inoculated groups and PCV2b + SIV inoculated groups were not statistically different (Table 2), and the majority of lesions were directly attributable to SIV infection. Lesions consisted of mild to moderate, multifocal necrotizing bronchiolitis with associated bronchointerstitial pneumonia (peribronchiolar and septal inflammation) and intra-alveolar infiltrates of neutrophils, macrophages and proteinaceous debris. Type 2 pneumocyte hyperplasia was mild in SIV-inoculated pigs, and mild to moderate in PCV2b + SIV-inoculated pigs, but there was no statistically significant difference between these two groups. At day 28 post-SIV (day 45 post-PCV2b), lung lesions were minimal, representing residual peribronchiolar and proprial lymphoplasmacytic inflammation and mild type 2 pneumocyte hyperplasia. There were no statistically significant differences in the amount of PCV2 or SIV antigen in lungs at either time point, based on IHC.

Microscopic lymph node lesions are summarized in Table 3. At day 4 post-SIV (day 21 post-PCV2b), lymphoid depletion and granulomatous inflammation was present in one or more lymph nodes in PCV2b-inoculated and PCV2b+SIV inoculated pigs. Lymphoid depletion consisted of decreased cellularity in lymphoid follicles and paracortex, loss of follicle architecture and variably sized collections of epithelioid macrophages replacing follicles (Fig. 8, plates A-D). The severity of these lesions varied between lymph nodes within each group, such that there were no statistically significant differences in the overall lymph node lesion scores between PCV2b and PCV2b+SIV groups. Similar variation in the amount of PCV2 antigen detected by IHC resulted in no statistically difference between PCV2b and PCV2b+SIV groups. Lymph node lesions were mild and affected only a few pigs in each group at day 28 post-SIV (day 45 post-PCV2b).

Microscopic lesions attributable to PCV2b infection in other target organs were mild, consisting of small lymphohistiocytic to lymphoplasmacytic inflammatory foci in the portal tracts of the liver and peritubular and
perivascular interstitium of the kidney, and small foci of granulomatous inflammation in the tonsil and spleen of several pigs. The paucity of these lesions, and the small number of animals affected, precluded further analysis.

**Severe PCVAD:** Two of ten pigs in the PCV2b + SIV inoculated group developed lesions/clinical signs consistent with the severe systemic form of PCVAD. One pig (#23), a scheduled sacrifice on day 4 post-SIV, had diffuse expansion of pulmonary interlobular septa by clear, slightly gelatinous edema fluid (Fig. 7D), serous pleural and pericardial effusion, ascites and mesocolonic edema. The second pig (#8), failed to gain weight days 3-5 post-SIV, developed increased respiratory clinical signs (primarily dyspnea), and was humanely euthanized on day 8 post-SIV (day 25 post-PCV2b). This animal had similar edematous expansion of pulmonary interlobular septa, serous pleural and pericardial effusion, and mesocolonic edema. Microscopically, pulmonary lesion scores in these pigs were not different from other pigs in this group sacrificed at day 4-post SIV, with the exception of increased amounts of interlobular, interstitial and intra-alveolar edema and fibrin exudate. However, there was a 1-2 log increase in the amount of PCV2 antigen detected (by quantitative real-time PCR, data not shown) in lung from these two pigs, compared to the other pigs sacrificed at day 4 post-SIV. Lymphoid depletion was severe in lymph nodes (Fig. 8F), spleen and tonsil, granulomatous inflammation was moderate to marked, and there was abundant PCV2 antigen demonstrated by IHC (Fig. 8E) in all lymphoid organs from these two pigs.
Table 2. Microscopic lung lesion scores in pigs at 4 days post-SIV inoculation (21 days post-PCV2b inoculation) and at 28 days post-SIV inoculation (45 days post-PCV2b inoculation). Data represents: Incidence (group mean[mean of affected animals]). Group 1 – Sham inoculated; Group 2 – SIV only; Group 3 – PCV2b-SIV; Group 4 – PCV2b only.

<table>
<thead>
<tr>
<th>Group</th>
<th>Necrotizing bronchiolitis</th>
<th>Inflammation in Lamina Propria</th>
<th>Peribronchiolar inflammation</th>
<th>Septal inflammation</th>
<th>Type 2 Pneumocyte Hyperplasia</th>
<th>Alveolar exudate</th>
<th>PCV2 Antigen (IHC)</th>
<th>SIV Antigen (IHC)</th>
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<td>21 days post-PCV2b</td>
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<td>4 days post-SIV</td>
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<td>1 (n=4)</td>
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<td>2/4 (0.5[1.0])a</td>
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<td>2 (n=4)</td>
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<td>4/4 (2.8[2.8])b</td>
<td>4/4 (3.2[3.2])b</td>
<td>4/4 (0.6[0.6])ab</td>
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<td>28 days post-SIV</td>
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<tr>
<td>1 (n=6)</td>
<td>0/6 (0.0[0.0])a</td>
<td>1/6 (0.1[0.3])a</td>
<td>4/6 (0.2[0.2])</td>
<td>0/6 (0.0[0.0])a</td>
<td>0/6 (0.0[0.0])a</td>
<td>0/6 (0.0[0.0])a</td>
<td>0/6 (0.0[0.0])a</td>
<td>0/6 (0.0[0.0])a</td>
</tr>
<tr>
<td>2 (n=5)</td>
<td>0/5 (0.0[0.0])a</td>
<td>2/5 (0.1[0.3])a</td>
<td>4/5 (0.8[1.0])</td>
<td>0/5 (0.0[0.0])a</td>
<td>0/5 (0.0[0.0])a</td>
<td>0/5 (0.0[0.0])a</td>
<td>0/5 (0.0[0.0])a</td>
<td>0/5 (0.0[0.0])a</td>
</tr>
<tr>
<td>3 (n=5)</td>
<td>0/5 (0.0[0.0])a</td>
<td>1/5 (0.3[1.3])a</td>
<td>5/5 (2.1[2.1])</td>
<td>2/5 (0.2[0.5])a</td>
<td>3/5 (0.6[1.0])</td>
<td>0/5 (0.0[0.0])a</td>
<td>2/5 (0.4[1.0])a</td>
<td>0/5 (0.0[0.0])a</td>
</tr>
<tr>
<td>4 (n=6)</td>
<td>0/6 (0.0[0.0])a</td>
<td>2/6 (0.2[0.5])a</td>
<td>6/6 (1.9[1.9])</td>
<td>4/6 (0.4[0.7])a</td>
<td>6/6 (0.8[0.8])</td>
<td>0/6 (0.0[0.0])a</td>
<td>2/6 (0.3[1.0])a</td>
<td>0/6 (0.0[0.0])a</td>
</tr>
</tbody>
</table>

† Different superscripts (a, b, c) denote significantly different (p < 0.05) mean group lesion scores within a column at each day post-inoculation.

Table 3. Microscopic lymph node lesion scores in pigs at 4 days post-SIV inoculation (21 days post-PCV2b inoculation) and at 28 days post-SIV inoculation (45 days post-PCV2b inoculation). Data represents: Incidence (group mean[mean of affected animals]). Group 1 – Sham inoculated; Group 2 – SIV only; Group 3 – PCV2b-SIV; Group 4 – PCV2b only.

<table>
<thead>
<tr>
<th>Group</th>
<th>Lymphoid depletion</th>
<th>Granulomatous Inflammation</th>
<th>PCV2 Antigen (IHC)</th>
<th>Lymphoid Depletion</th>
<th>Granulomatous Inflammation</th>
<th>PCV2 Antigen (IHC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>21 days post-PCV2b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 days post-SIV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (n=4)</td>
<td>0/4 (0.0[0.0])a</td>
<td>0/4 (0.0[0.0])a</td>
<td>0/4 (0.0[0.0])a</td>
<td>0/4 (0.0[0.0])a</td>
<td>0/4 (0.0[0.0])a</td>
<td>0/4 (0.0[0.0])a</td>
</tr>
<tr>
<td>2 (n=4)</td>
<td>0/4 (0.0[0.0])a</td>
<td>0/4 (0.0[0.0])a</td>
<td>0/4 (0.0[0.0])a</td>
<td>0/4 (0.0[0.0])a</td>
<td>0/4 (0.0[0.0])a</td>
<td>0/4 (0.0[0.0])a</td>
</tr>
<tr>
<td>3 (n=4)</td>
<td>2/4 (1.0[2.0])a</td>
<td>4/4 (1.5[1.5])b</td>
<td>3/4 (1.3[1.7])b</td>
<td>4/4 (1.8[1.8])b</td>
<td>4/4 (1.1[1.1])b</td>
<td>4/4 (1.3[1.3])b</td>
</tr>
<tr>
<td>4 (n=4)</td>
<td>3/4 (1.0[1.3])a</td>
<td>4/4 (1.5[1.5])b</td>
<td>4/4 (1.8[1.8])b</td>
<td>4/4 (1.1[1.1])b</td>
<td>4/4 (1.3[1.3])b</td>
<td>4/4 (1.1[1.1])b</td>
</tr>
<tr>
<td>45 days post-PCV2b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>28 days post-SIV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (n=6)</td>
<td>0/6 (0.0[0.0])a</td>
<td>0/6 (0.0[0.0])a</td>
<td>0/6 (0.0[0.0])a</td>
<td>0/6 (0.0[0.0])a</td>
<td>0/6 (0.0[0.0])a</td>
<td>0/6 (0.0[0.0])a</td>
</tr>
<tr>
<td>2 (n=5)</td>
<td>1/5 (0.1[0.5])a</td>
<td>0/5 (0.0[0.0])a</td>
<td>0/5 (0.0[0.0])a</td>
<td>2/5 (0.1[0.3])b</td>
<td>0/5 (0.0[0.0])a</td>
<td>0/5 (0.0[0.0])b</td>
</tr>
<tr>
<td>3 (n=5)</td>
<td>2/5 (0.4[1.0])a</td>
<td>3/5 (0.8[1.3])a</td>
<td>0/5 (0.0[0.0])a</td>
<td>5/5 (1.0[1.0])a</td>
<td>5/5 (0.7[0.7])a</td>
<td>2/5 (0.4[1.0])a</td>
</tr>
<tr>
<td>4 (n=6)</td>
<td>3/6 (0.3[1.0])a</td>
<td>3/6 (0.7[1.3])a</td>
<td>1/6 (0.2[1.0])a</td>
<td>4/6 (0.7[1.0])a</td>
<td>5/6 (0.6[0.7])a</td>
<td>2/6 (0.3[1.0])a</td>
</tr>
</tbody>
</table>

* Iliac, inguinal, mesenteric and cervical lymph nodes.
† Different superscripts (a, b, c) denote significantly different (p < 0.05) mean group lesion scores within a column at each day post-inoculation.
Figure 8. Photomicrographs of tracheobronchial lymph nodes from a PCV2b + SIV infected pig (A and B); a PCV2b only infected pig (C and D); and, from a PCV2b + SIV infected pig with severe PCVAD (E and F). There is diffuse, marked lymphoid depletion and granulomatous inflammation in the PCV2b + SIV infected pig with severe PCVAD, associated with abundant PCV2 antigen (plates E and F), moderate lymphoid depletion and granulomatous inflammation in the PCV2b only infected pig (plates C and D) and minimal lymphoid depletion in the PCV2 + SIV infected pig without severe disease.
Discussion

A CD/CD pig model was used in order to insure naïve pigs devoid of maternally derived colostral antibodies that might prevent or ameliorate the induction of experimental infections or confound interpretation of experimental results. Our model, in conjunction with biosecurity measures put in place when the animals arrived, resulted in all pigs being seronegative for PCV2 SIV, PPV, PRRS virus and *Mycoplasma hyopneumoniae* at the start of the study (and remaining seronegative to PPV, PRRS virus and *M. hyopneumoniae* throughout the study). In addition, serum PCR determined that all pigs lacked circulating PCV2 at the start of the study, and lacked circulating PRRS viral nucleic acids at the beginning and end of the study. Pigs singularly infected with PCV2b or SIV did not seroconvert to the heterologous virus at any time during the study, indicating no cross-contamination between infected groups.

The hypothesis underlying this research was that SIV infection, in pigs subclinically infected with PCV2, would result in increased severity of one or both diseases in affected pigs. The specific objectives were to determine 1) if SIV (H1N1, not pandemic H1N1) infection could initiate clinically significant porcine circovirus associated respiratory disease (PCVAD-respiratory or PCVAD systemic) in pigs subclinically infected with PCV2; and, 2) if pre-existing, subclinical PCV2 infection would have any effects on the duration or severity of the SIV infection.

Clinical signs in the PCV2b + SIV group were directly attributable to the SIV infection, and morbidity in this group was 2-3 fold more severe in this group when compared to the SIV-inoculated group, as measured by clinical respiratory signs (cough and dyspnea). Also, SIV was shed in greater quantities in nasal secretions, and for a longer time period (out to 15 days post-SIV inoculation) in the PCV2b + SIV group compared to the SIV group. These observations suggest that there was potentiation of SIV replication in pigs sub-clinically infected with PCV2b. These results are in contrast to reports of concurrent experimental infection of pigs with porcine respiratory coronavirus (PRC) and SIV, where there was no measurable increase in morbidity (as determined by clinical signs, duration of viral shedding and average daily weight gain), but a decreased replication of SIV in target tissues and nasal secretions (Lanza, et al., 1992; Van Reeth, et al., 1994). Concurrent PRRS virus and SIV infection in conventional SPF pigs resulted in increased duration of clinical signs and decreased weight gain in dual-infected pigs, and delayed the onset of SIV shedding in nasal secretions (Van Reeth, 1996; Van Reeth, 2001). Similar experiments in CD/CD pigs detected no differences (Van Reeth, 2001).

In contrast to the observed effects of subclinical infection on SIV pathogenicity, the reciprocal effects of SIV on PCV2 pathogenicity in subclinically infected CD/CD pigs was limited to the induction of severe systemic PCVAD in 2/10 (20%) dual-infected pigs. In these two pigs, the observed gross (serous pleural (hydrothorax) and pericardial effusions, pulmonary interstitial and interlobular edema, mesocolonic edema) and microscopic (severe lymphoid depletion in multiple lymphoid tissues associated with increased PCV2 antigen) lesions are consistent with those reported from field cases of severe systemic PCVAD, as well as those reported in several experimental co-infection models. With the exception of these two pigs, the SIV infection had no measurable effect on the amount of PCV2 shed in nasal secretions, the amount of circulating PCV2 virus, clinical signs or average daily weight gain, or gross or microscopic pathology attributable to PCV2 infection in the remaining PCV2+SIV infected pigs. These results (in pigs without severe disease) are similar to those reported in CD/CD pigs infected with PCV2 and inoculated 7 days later with SIV (H1N1) (Wei, et al., 2010). Also, in the current study, severe PCVAD was reproduced with SIV inoculation at day 17 following PCV2, whereas severe PCVAD was not reproduced in the study reported by Wei, et al. (2010). These results differ from those reported for an experimental co-infection with PCV2 and *M. hyopneumoniae*, where dual infection (*M. hyopneumoniae* at 4 weeks, followed by PCV2 at 6 weeks) resulted in increased severity of cough and dyspnea, reduced weight gain, increased severity of gross lung lesions and microscopic lung and lymph node lesions attributable to PCV2 infection (Opriessnig, et al., 2004). It appears that the sequence of infections, and perhaps the timing of a subsequent SIV infection following PCV2 infection, likely play a role in the pathogenesis of these combined infections with PCV2 and other swine respiratory pathogens.
Under the conditions of the current study, we conclude that 1) a pre-existing, subclinical PCV2b infection results in increased severity of a subsequent H1N1 SIV infection, with increased amounts of SIV shed in nasal secretions and a prolonged period of nasal SIV shedding; 2) there is no reciprocal increase in the amount of PCV2b shed in nasal secretions, circulating in serum, or proliferating in tissue in dual-infected pigs; and, 3) that SIV can trigger the severe form of PCVAD, although the percentage of animals affected is no greater than that reported for other co-infections known to trigger the severe form of PCVAD.

References


