Vaccine efficacy and immune response to swine influenza virus challenge in pigs infected with porcine reproductive and respiratory syndrome virus at the time of SIV vaccination

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1. Introduction

Porcine respiratory disease complex (PRDC), characterized by pneumonia and reduced growth performance, is an economically significant respiratory disorder of nursery and finishing pigs and remains a challenge to the swine industry worldwide. Multiple agents have been reported to be associated with PRDC including porcine reproductive and respiratory syndrome virus (PRRSV), Mycoplasma hyopneumoniae (MHYO), swine influenza virus (SIV), Pasteurella multocida and porcine circovirus type 2 (PCV2) (Thacker, 2001). However, a previous study indicated SIV and PRRSV to be the primary etiological agents associated with respiratory disease of pigs in the mid-western region of the U.S. (Choi et al., 2003).

In general, SIV-specific passive immunity from vaccinated sows persists until 8–12 weeks of age (Loeffen et al., 2003). As a result, SIV typically infects pigs in the late nursery and early finishing stages with the loss of passive immunity (Loeffen et al., 2003). To protect pigs from SIV-induced disease, active immunity is induced through the use of SIV vaccines. Current SIV vaccines in the U.S. are inactivated vaccines that are approved for use in pigs 3 weeks of age or older and require a second injection administered 2–3 weeks following the first vaccination. With the exception of antigenic mismatch between the vaccine and challenge strains, SIV vaccination in seronegative pigs appears to be effective in experimental studies (Haesebrouck and Pensaert, 1986; Kitikoon et al., 2006). However, diminished SIV vaccine efficacy in the field has been reported with SIV disease outbreaks occurring in vaccinated farms.
PRRSV, as opposed to SIV, can be transmitted transplacentally during gestation in addition to direct physical contact after birth (Albina, 1997; Prieto et al., 1997). Accordingly, pigs from PRRSV-infected sows, become a source of infection to pigs in subsequent production stages, especially at the late nursery and early finishing stages. Thus, pigs are frequently infected with PRRSV at the time of SIV vaccination making the influence of PRRSV infection on SIV vaccine efficacy a concern to swine producers and veterinarians. However, data of the impact of PRRSV infection on SIV vaccination efficacy is minimal.

PRRSV and SIV co-infection studies have yielded conflicting results with regard to clinical disease interactions, ranging from enhancement (Van Reeth et al., 1996) to minimal disease overall (Pol et al., 1997). Perhaps more importantly, PRRSV impact on swine vaccines indicates that the timing of PRRSV infection may influence vaccination outcome. A study performed in our lab found that the presence of PRRSV either from a MLV vaccine or infection during or within 2 weeks of inactivated MHYO vaccination significantly decreased the MHYO vaccine efficacy in reducing the percentage of MHYO associated lung pneumonia (Thacker et al., 2000b). In contrast, another study demonstrated that administration of a MLV PRRSV vaccine to MHYO-free pigs at 7 days prior to vaccination with MHYO inactivated vaccine did not interfere with the MHYO vaccine efficacy or immune response to MHYO infection (Boettcher et al., 2002). Two additional studies have also documented the negative impact of PRRSV infection 2 days (Li and Yang, 2003) or 7 days prior to the administration of a MLV classical swine fever vaccine (Suradhat et al., 2006). However, no studies have been conducted to investigate the impact of PRRSV infection on SIV vaccine efficacy. Therefore, the objective of the study reported here was to investigate the influence of PRRSV infection on the efficacy of an inactivated SIV vaccine using a virulent strain of North American PRRSV. The impact of the PRRSV infection on SIV vaccine efficacy was evaluated using clinical disease symptoms, macroscopic and microscopic lung lesions, virus isolation and immunological parameters. The PRRSV infection was placed between SIV vaccines based on the earlier studies (Thacker et al., 2000b). In addition, the impact of PRRSV infection 2 weeks following SIV vaccination was assessed to provide increased understanding of the temporal relationship between PRRSV infection and SIV vaccine efficacy. Understanding the impact of PRRSV infection on SIV vaccines provides important information regarding both the immunology of PRRSV infection and the development of successful intervention strategies to control important respiratory pathogens such as influenza in pigs.

2. Materials and methods

2.1. Experimental design

2.1.1. Animals

Ninety-six 8–12-day-old crossbred pigs, obtained from a commercial herd serologically negative for PRRSV, MHYO and SIV, were used in the study. Pigs were assigned to groups with stratification by arrival weight to 8 groups of 12 pigs. Throughout the study, pigs were housed in identical isolation rooms based on their challenge status. Pigs were provided feed and water ad libitum throughout the trials. The experimental design is summarized in Table 1. All study procedures and animal care activities were conducted in accordance with the guidelines and under the supervision of the Iowa State University (ISU) Institutional Committee on Animal Care and Use.

2.1.2. Vaccine and challenge inocula

Pigs were vaccinated with a commercial inactivated bivalent SIV vaccine (End-FLUence™-2, Intervet Inc., Millsboro, DE) containing a newer classic H1N1 and clade 1 H3N2 (Richt et al., 2003) viruses according to label directions at 3 and 5 weeks of age. A virulent strain of North American PRRSV, VR-2385, was administered intranasally (1 ml/nostril) to pigs in the appropriate groups at a dose of 10^5.6 TCID50/ml at either 4 or 7 weeks of age, as previously described (Halbur et al., 1995). A virulent classic H1N1 strain (A/Swine/IA/40776/92) was administered intratracheally to pigs of the appropriate groups at a dose of 10^5.3 TCID50/ml (4 ml) at 7 weeks of age (Thacker et al., 2001). PRRSV and SIV were propagated and titrated in MARC-145 and Madin–Darby canine kidney (MDCK) cells, respectively. Viruses used for challenge and for serological tests were below the 5th cell culture passage. The day of SIV inoculation was designated as 0 days post-infection (DPI).

2.2. Laboratory investigations

2.2.1. Clinical evaluation and production parameters

Clinical signs including cough, respiratory rate and rectal temperature were evaluated daily from 1 day prior to challenge until 7 DPI. Each pig was assigned a daily respiratory score (0–3) for respiratory distress associated with SIV. Scoring: 0 = normal; 1 = mild dyspnea at rest; 2 = moderate dyspnea and/or tachypnea at rest; 3 = severe dyspnea and tachypnea with distinct abdominal breathing (Kitikoon et al., 2006). Pigs with rectal temperatures ≥40°C were considered to be febrile. Pigs were weighed upon arrival, prior to challenge, and at necropsy to evaluate weight gain.

2.2.2. Macroscopic and microscopic lesions

One-half of the pigs in each group were necropsied at 7 DPI and the remaining pigs were necropsied at 28 DPI. Lesions consistent with SIV pneumonia (dark red-to-purple lobular consolidation) were sketched onto a standard diagram and assessed for percentage of lung surface exhibiting lesions as determined from a diagram using a Zeiss SEM-IPS image analysis system as previously described (Thacker et al., 2001). PRRSV lesions were scored as a percentage of affected lungs as previously described (Halbur et al., 1995). Bronchial swabs were obtained from each pig and cultured for swine respiratory bacteria using standard microbiologic procedures.

Tissue samples were collected from each lung lobe, fixed in 10% neutral buffered formalin, processed and
Table 1
Experimental design.

<table>
<thead>
<tr>
<th>Groups</th>
<th>SIV vaccine(^a)</th>
<th>Challenged with:</th>
<th>No. of pigs necropsied at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SIV</td>
<td>PRRSV</td>
</tr>
<tr>
<td>NEG</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>V[S7]</td>
<td>Yes</td>
<td>Yes (7(^c))</td>
<td>No</td>
</tr>
<tr>
<td>S[7]</td>
<td>No</td>
<td>Yes (7)</td>
<td>No</td>
</tr>
<tr>
<td>V[P4][S7]</td>
<td>Yes</td>
<td>Yes (7)</td>
<td>Yes (4)</td>
</tr>
<tr>
<td>V[S7][P7]</td>
<td>Yes</td>
<td>Yes (7)</td>
<td>Yes (7)</td>
</tr>
<tr>
<td>S[7][P7]</td>
<td>No</td>
<td>No</td>
<td>Yes (7)</td>
</tr>
<tr>
<td>P[7]</td>
<td>No</td>
<td>No</td>
<td>Yes (7)</td>
</tr>
</tbody>
</table>

\(^a\) Pigs received SIV vaccination at 3 and 5 weeks of age.
\(^b\) DPI, days post-infection.
\(^c\) Weeks of age that the pigs were challenged with SIV or PRRSV.

The viral titer in each nasal swab was expressed as log 10 TCID\(_{50}\) per milliliter.

2.2.3. SIV isolation

Nasal swabs were collected and placed in infecting medium on –1, 2, 5, 7 and 12 DPI to evaluate the level of SIV shedding. Virus isolation was performed followed by SIV detection using immunocytochemistry (ICC) staining as previously described (Kitikoon et al., 2006). Briefly, 10-fold serial dilutions of the swab samples were prepared in minimum essential medium (Invitrogen, Carlsbad, CA) supplemented with 2 mM L-glutamine, 8% bovine albumin fraction V solution (Invitrogen, Carlsbad, CA), 50 μg/ml of gentamycin (Sigma–Aldrich, St. Louis, MO) and 1 μg/ml (Sigma–Aldrich, St. Louis, MO). The diluted samples were then inoculated onto MDCK cells in a 96-well tissue culture plate and incubated at 37 °C with 5% CO\(_2\) for 48 h. Cells were fixed with 4% phosphate-buffered formalin and washed with 0.5% Tween-20 in phosphate buffered saline (PBS). An anti-influenza A nucleoprotein monoclonal antibody (clone HB-65, ATCC, Rockville, MD) was used as the primary antibody followed by rabbit anti-mouse IgG conjugated horseradish peroxidase (Dako Cytomation, Carpinteria, CA) as the secondary antibody. The color was developed using the chromogen aminothyl carbazole substrate (Sigma, St. Louis, MO). Each assay included mock-infected negative control cells and positive control cells infected with SIV with a known titer. The viral titer in each nasal swab was expressed as log 10 TCID\(_{50}\) per milliliter.

2.2.4. Serology

Serum samples were collected prior to each vaccination (–28 and –14 DPI), prior to challenge (–1 DPI) and at 6 and 27 DPI. Sera were tested for SIV antibodies by hemagglutination-inhibition (HI) assay. The HI assay was performed according to the standard protocol routinely performed at ISU-Veterinary Diagnostic Laboratory (Yoon et al., 2004) using 0.5% rooster erythrocytes for hemagglutination and 8 hemagglutination (HA) units per 50 μl of the challenge virus strain A/Swine/IA/40776/92 (H1N1). Pigs were considered to have positive HI antibody titers when the HI titer is ≥40. Antibodies against PRRSV were measured prior to the first vaccination and at 6 and 27 DPI using a commercially available enzyme-linked immunosorbant assay (ELISA) (Herdchek: PRRS, IDEXX Laboratories, Inc., Westbrook, ME) according to the manufacturer’s protocol. Samples were considered positive for PRRSV if the calculated sample to positive control (S/P) ratio was equal to or greater than 0.4.

2.2.5. SIV-specific lymphoproliferation

Peripheral blood mononuclear cells (PBMC) were collected in heparinized blood collection tubes and isolated by differential centrifugation. Samples were collected prior to each vaccination (–28 and –14 DPI), prior to challenge (–1 DPI) and at 6 and 27 DPI. Proliferation of PBMCs was measured by \(^3\)H-thymidine incorporation as described previously (Thacker et al., 2000a). Data were expressed as stimulation index obtained by dividing the \(^3\)H-thymidine incorporation (CPM) of SIV-stimulated PBMC by the CPM of non-stimulated control PBMC from the same pig and the average stimulation indexes of each group from different time points were compared.

2.2.6. SIV-specific antibodies in lower airways

Bronchoalveolar lavage (BAL) fluids were collected at each necropsy. To collect BAL, the lungs were lavaged with 50 ml of collecting solution (sterile PBS with 1% BSA, 300 U/ml penicillin and 300 mg/ml streptomycin). Samples were stored at –20 °C prior to testing. The BAL fluids were incubated at 37 °C for 1 h with an equal amount of 10 mM dithiothreitol (DTT) to disrupt mucus present in the fluids. ELISA assays for SIV-specific antibodies in the lower respiratory tract was performed as previously described (Kitikoon et al., 2006). In brief, inactivated challenge virus was diluted to a hemagglutination (HA) concentration of 100 HA units/50 μl. Immulon-2HB 96-well plates (Dynex, Chantilly, VA) were coated with 100 μl of SIV antigen and incubated at room temperature.
overnight. Plates were blocked for 1 h with 100 μl of 10% BSA in PBS and washed 3 times with 0.05% Tween-20 in PBS (PBS–T). The assay was performed on each BAL sample in triplicate. Negative controls (DTT with equal amount of PBS solution) were included on each plate. Plates were incubated with peroxidase-labeled goat anti-swine IgG (Kirkegaard and Perry, Gaithersburg, MD) or peroxidase-labeled goat anti-swine IgA (Bethyl, TX) at 37 °C for 1 h and ABTS/peroxidase was used as the substrate (Kirkegaard and Perry, Gaithersburg, MD). Antibody levels were reported as the mean optical density (OD) and the mean OD of each treatment group was compared.

2.3. Statistical analysis

Macroscopic and microscopic lesion scores, log 10 nasal swab titers, log 2 transformations of HI reciprocal titers and ELISA OD readings were analyzed using analysis of variance (ANOVA) (JMP, SAS Institute, Cary, NC). Statistical differences between groups were considered significant when *p* ≤ 0.05. Response variables shown to have a significant effect by treatment group were subjected to pairwise comparisons using the Tukey–Kramer test.

3. Results

3.1. Clinical disease

SIV vaccination did not prevent SIV-induced clinical disease as all SIV-challenged groups had increased rectal temperatures at 1 DPI (Table 2). Pigs vaccinated in the absence of PRRSV (group VS[7]) had fever for an average of 1 day post-SIV inoculation while the pigs infected with PRRSV between SIV vaccinations (group VP[4]S[7]) did not differ from groups VS[7]P[7] and S[7]. Pigs vaccinated for SIV in the absence of PRRSV (groups VS[7]), and VS[7]P[7] had SIV lung lesions similar to NEG pigs, indicating effective vaccine protection against SIV if pigs became infected with PRRSV following SIV vaccination. By 28 DPI, there were no differences in the percentage of pneumonia consistent with SIV, independent of PRRSV infection or vaccination status.

All groups that were challenged with PRRSV had significant PRRSV-induced lesions at 7 DPI (Table 3). Interestingly, at 28 DPI the severity of PRRSV-associated lesions was significantly greater in the unvaccinated dual infected pigs and pigs vaccinated for SIV in the face of PRRSV challenge (groups S[7]P[7] and VP[4]S[7]) compared to the VS[7]P[7] group.

Microscopic lesions (Table 3) consistent with SIV infection at 7 DPI were prominent in groups S[7], VP[4]S[7], and S[7]P[7] and the IHC test confirmed the

<table>
<thead>
<tr>
<th>Table 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Summary of clinical observations following infection with SIV and/or PRRSV. Data expressed as group average of original data ± SEM.*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of pigs cough</th>
<th>Respiratory score*</th>
<th>Days febrile</th>
<th>Gain per day*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEG</td>
<td>0/12</td>
<td>0.0 ± 0.0</td>
<td>0 ± 0.4</td>
<td>674 ± 26</td>
</tr>
<tr>
<td>VS[7]</td>
<td>0/12</td>
<td>0.0 ± 0.0</td>
<td>1 ± 0.2</td>
<td>642 ± 16</td>
</tr>
<tr>
<td>S[7]</td>
<td>2/12</td>
<td>0.3 ± 0.1</td>
<td>2 ± 0.2</td>
<td>629 ± 18</td>
</tr>
<tr>
<td>VP[4]S[7]</td>
<td>2/12</td>
<td>0.2 ± 0.1</td>
<td>2 ± 0.5</td>
<td>551 ± 37</td>
</tr>
<tr>
<td>V</td>
<td>0/12</td>
<td>0.0 ± 0.0</td>
<td>0 ± 0.2</td>
<td>669 ± 38</td>
</tr>
<tr>
<td>VS[7]P[7]</td>
<td>2/12</td>
<td>1.8 ± 0.1</td>
<td>3 ± 0.6</td>
<td>571 ± 23</td>
</tr>
<tr>
<td>S[7]P[7]</td>
<td>0/12</td>
<td>1.9 ± 0.2</td>
<td>4 ± 0.5</td>
<td>577 ± 19</td>
</tr>
<tr>
<td>P[7]</td>
<td>1/12</td>
<td>1.3 ± 0.1</td>
<td>4 ± 0.5</td>
<td>631 ± 22</td>
</tr>
</tbody>
</table>

* Within each column, values with different superscripts are significantly different by least significant difference (*p* < 0.05).

* Average daily score from 1 to 7 days post-SIV and/or PRRSV infection.

* Proportion of days (out of 7 total) that each pig’s rectal temperature was >104 °F.

* Daily weight gain (g) from –1 to 28 days post-SIV and/or PRRSV infection.

Single infection with SIV or PRRSV at 7 weeks of age also did not significantly reduce weight gain compared to the NEG group. In contrast, all groups infected with both pathogens, independent of vaccination status, had significantly reduced weight gain. The SIV vaccinated pigs that were challenged with PRRSV between vaccines (VP[4]S[7]) had the lowest rate of gain, although it was not significantly different than the other groups infected with both pathogens.

Coughing was minimal throughout the trial period, but was observed in 2 pigs from each of groups S[7], VP[4]S[7] and VS[7]P[7] and 1 pig from group P[7]. The average daily respiratory scores from 1 to 7 DPI were significantly higher in co-infected groups, independent of SIV vaccination status, compared to all other groups. Based on clinical signs, pigs infected with both SIV and PRRSV at 7 weeks of age with or without SIV vaccination exhibited the most clinical disease.

3.2. Macroscopic and microscopic lesions

The estimated percentage of lung tissue with visible SIV and/or PRRSV-induced pneumonia (macroscopic lesions) is summarized in Table 3. At 7 DPI, pigs co-infected with PRRSV and SIV demonstrated the highest percentage of SIV-induced pneumonia. The severity of lung lesions consistent with SIV in the pigs vaccinated concurrently with PRRSV infection (group VP[4]S[7]) did not differ from groups VS[7]P[7] and S[7]. Pigs vaccinated for SIV in the absence of PRRSV (groups VS[7]), and VS[7]P[7] had SIV lung lesions similar to NEG pigs, indicating effective vaccine protection against SIV if pigs became infected with PRRSV following SIV vaccination. By 28 DPI, there were no differences in the percentage of pneumonia consistent with SIV, independent of PRRSV infection or vaccination status.
presence of SIV antigen in all of those groups. Pigs in groups VS[7] and VS[7]P[7], had similar microscopic lesion scores to groups SIV and the NEG. By 28 DPI, the lesions associated with SIV infection had resolved in all groups.

Interstitial pneumonia associated with PRRSV infection at 7 DPI was significantly greater in pigs co-infected at 7 weeks of age independent of their vaccination status (groups VS[7]P[7] and S[7]P[7]) compared to the NEG group. Viral antigen detection by IHC confirmed the presence of PRRSV antigen only in pigs challenged with PRRSV. At 28 DPI, microscopic lesions consistent with PRRSV infection in group S[7]P[7] remained significantly greater than the NEG control group while the lesions in SIV-vaccinated group (VS[7]P[7]) and PRRSV infected group (P[7]) had resolved.

### 3.3. SIV isolation

Nasal swabs were collected to evaluate virus excretion in relation to SIV vaccination (Fig. 1). Group S[7] shed significantly more virus at both 2 and 5 DPI than any other group. Pigs co-infected with SIV and PRRSV (group S[7]P[7]) did not have prolonged SIV shedding, although the levels of virus were greater at 5 DPI compared to VS[7], VS[7]P[7] and S[7]. At 2 DPI, group VP[4]S[7] shed significantly higher levels of virus compared to the other SIV-vaccinated groups, VS[7] and VS[7]P[7]. In addition, pigs in group VP[4]S[7] shed virus for a longer period compared to all other groups, as at 7 DPI a low amount of virus was detected while all other groups were virus negative. No virus was isolated from nasal swabs collected from non-SIV infected groups at any time throughout the trial. By 12 DPI, all groups were negative for virus (data not shown).

#### 3.4. Serology

Two weeks following the first vaccination all SIV vaccinated pigs had low HI titers while all other groups remained seronegative (Fig. 2). At 2 weeks after the second vaccination and prior to challenge with SIV and/or PRRSV (at 7 weeks of age), all SIV vaccinated pigs had HI antibody titers greater than 40. Following SIV challenge, all vaccinated groups had similar HI antibody levels and the levels were significantly greater than any of the nonvaccinated pigs, independent of challenge status. The

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**Table 3**

Average percentage of macroscopic lesions, microscopic scores and immunohistochemistry (IHC) test in pigs vaccinated against SIV followed by challenge with SIV and/or PRRSV. Data expressed as group averaged original data ± SEM.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Day 7 post-challenge microscopic scores</th>
<th>Day 28 post-challenge microscopic scores</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SIV&lt;sup&gt;a&lt;/sup&gt;</td>
<td>PRRSV&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>NEG</td>
<td>0.2 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0 ± 0.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>VS[7]</td>
<td>0.2 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.5 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>S[7]</td>
<td>4.6 ± 1.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.5 ± 1.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>VP[4]S[7]</td>
<td>2.7 ± 1.4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>9.2 ± 2.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>V</td>
<td>0.1 ± 0.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0 ± 0.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>VS[7]P[7]</td>
<td>1.2 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>42.5 ± 3.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>S[7]P[7]</td>
<td>7.0 ± 1.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>35.7 ± 5.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>P[7]</td>
<td>0.2 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>36.3 ± 5.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Within each column, values with different superscripts are significantly different by least significant difference (p < 0.05).*

<sup>a</sup> Percentage of lung exhibiting SIV-induced pneumonia as determined by lesion sketches and image analysis.

<sup>b</sup> Percentage of lung exhibiting PRRSV-induced pneumonia as estimated by visual observation.

<sup>c</sup> SIV: score based on severity of bronchiolar epithelial necrosis. 0 = no. microscopic lesions; 1 = <30% airways affected; 2 = 30–70% of airways affected; 3 = >70% airways affected. Symbol in brackets indicate the presence of SIV antigen detected by IHC.

<sup>d</sup> PRRSV: score based on severity of interstitial pneumonia. 0 = no. microscopic lesions; 1 = mild multifocal pneumonia; 2 = moderate diffuse pneumonia; 3 = severe multifocal pneumonia. Symbol in brackets indicate the presence of PRRSV antigen detected by IHC.

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Fig. 1. SIV titers in nasal swabs collected at 2, 5 and 7 days post-infection (DPI) from vaccinated, SIV-challenged at 7 weeks of age (weeks) pigs (VS[7]), nonvaccinated, SIV-challenged at 7 weeks pigs (S[7]), vaccinated, SIV-challenged at 7 weeks, PRRSV-challenged at 4 weeks pigs (VP[4]S[7]), vaccinated, SIV-challenged at 7 weeks, PRRSV-challenged at 7 weeks pigs (VS[7]P[7]) and nonvaccinated, SIV and PRRSV-challenged at 7 weeks pigs (S[7]P[7]). Data expressed as group average ± SEM with different letters (a, b and c) were statistically different (p < 0.0001). The results of non-SIV-challenged groups are not included.
pigs in the NEG and PRRSV infected (group P[7]) remained HI-antibody negative for SIV throughout the trial (data not shown). Overall, vaccination in the face of PRRSV or PRRSV co-infected with SIV did not impact the serum HI antibody response to SIV vaccination or infection.

Serum samples collected prior to vaccination were negative for both PRRSV and SIV antibodies. At 6 DPI, less than 50% of pigs infected with PRRSV at 7 weeks of age seroconverted (S/P ratio > 0.4) while group VP[4]S[7] which was infected with PRRSV at 4 weeks of age over 50% of the pigs had developed antibodies (data not shown). By 27 DPI all pigs infected with PRRSV were antibody positive confirming PRRSV infection in all PRRSV challenged pigs. The NEG and non-PRRSV infected groups remained seronegative to PRRSV throughout the study.

3.5. Lymphocyte proliferation

There were no significant differences observed in lymphocyte proliferation to SIV antigen (Fig. 3) between groups at any time point with the exception of 6 DPI when pigs in group VP[4]S[7] had significantly increased lymphocyte proliferation compared to all groups except VS[7]. Interestingly, lymphocyte proliferation levels in the PRRSV and SIV co-infected groups increased from the time of PRRSV infection until 3–4 weeks following the infection as compared to either nonchallenged or pigs infected with only SIV or PRRSV. However, these differences were not statistically significant. Lymphocyte proliferation in response to the SIV challenge antigen was not detected in any of the non-SIV-challenged pigs (groups V, P[7] and NEG).

3.6. SIV-specific antibodies in lower airways

An isotype-specific ELISA was used to measure the antibody response to the SIV challenge antigen in BAL fluids. IgA was the dominant SIV-specific antibody at both 7 and 28 DPI in the BAL fluids (Fig. 4). At 7 DPI, the level of IgA antibodies specific to the SIV challenge antigen was significantly higher (p < 0.0001) in all the vaccinated, SIV-challenged groups independent of PRRSV infection compared to the nonvaccinated, SIV-challenged groups. At 28 DPI, all SIV-challenged pigs, independent of vaccination status, had significantly higher levels of SIV-specific IgA antibodies in the BAL compared to nonchallenged pigs or pigs infected with PRRSV only.
4. Discussion

The goal of this study was to assess the impact of PRRSV infection on SIV vaccine efficacy. Pigs were vaccinated at 3 and 5 weeks of age with a commercial bivalent SIV vaccine containing inactivated classic H1N1 and clade 1 H3N2 viruses. The effect of PRRSV on vaccine efficacy was evaluated by infecting pigs with a virulent isolate of PRRSV (North American strain, VR-2385) either between the SIV vaccinations at 4 weeks of age, or 2 weeks following the second vaccination, at 7 weeks of age with a virulent heterologous classic H1N1 strain A/Swine/IA/40776/92. This protocol was used to enable evaluation of the temporal relationship between SIV vaccine efficacy and PRRSV infection. The timing was determined by our earlier study of PRRSV and MHYO vaccines as well as the relationship of infection expected under field conditions. Control groups were included to monitor SIV vaccine efficacy in the absence of PRRSV as well as positive control groups consisting of SIV and/or PRRSV challenged groups to assess clinical disease severity.

This SIV vaccine-challenge model was conducted in high health status pigs from a herd that was free of all major respiratory pathogens (M. hyopneumoniae (MHYO), PRRSV and SIV). As a result of the high health status of the pigs, the SIV-associated clinical disease has been shown in our earlier studies to be reduced compared to SIV studies with pigs infected with other pathogens such as MHYO. The severity of lesions and disease in this study were similar to those of our previous SIV-inactivated vaccine study (Kitikoon et al., 2006). In addition, similar to the earlier study that determined that SIV vaccination, while reducing the severity of clinical disease did not prevent infection or all SIV-induced clinical disease as vaccinated pigs were febrile 24 h following infection. Infection with PRRSV between the SIV vaccines resulted in a slight prolongation of fever, and increased coughing and clinical signs similar to the nonvaccinated pigs challenged only with SIV. Vaccination did not reduce weight gain, while pigs infected with SIV and PRRSV, independent of vaccination status, had reduced weight gains, similar to earlier findings (Kay et al., 1994; Van Reeth et al., 2001) and mimicking what might occur in field situations where co-infections frequently occur.

Reduced macroscopic and microscopic lesions consistent with SIV infection are the primary parameters indicative of SIV vaccine efficiency. Here we demonstrated that infection with PRRSV between SIV vaccinations reduced vaccine efficacy based on increased macroscopic and microscopic lesions associated with SIV. This was further confirmed by higher levels of SIV antigen detected by IHC compared to vaccinated pigs infected only with SIV. Of the vaccinated groups, only the pigs vaccinated in the presence of PRRSV were IHC positive for SIV. Dual infection with SIV and PRRSV appeared to enhance lesions consistent with SIV acutely, but the presence of PRRSV did not prolong the SIV-induced pneumonia. In contrast, SIV appeared to increase the severity and duration of PRRSV lung lesions, as co-infected pigs had prolonged PRRSV-induced pneumonia based on the macroscopic lesions at 28 DPI. In addition, SIV vaccinated pigs infected with PRRSV between vaccinations had PRRSV-associated lesions similar to the nonvaccinated co-infected pigs at 28 DPI. In contrast, in the absence of PRRSV, SIV vaccination reduced the PRRSV-associated lesions to levels that were similar to the negative control group at 28 DPI. This demonstrates the importance of effective SIV vaccination in reducing disease induced by co-infections of PRRSV and SIV.

A potential mechanism for the disease enhancement of PRRSV by SIV may be explained by the pathogenesis of SIV infection. SIV primarily infects airway epithelial cells resulting in cell necrosis, production of inflammatory mediators and rapid infiltration of phagocytic cells (Van Reeth et al., 2002a,b). These phagocytic cells which include pulmonary alveolar macrophages (PAM) are susceptible to PRRSV infection and the primary cell type to support PRRSV replication (Chang et al., 2005; Rossow et al., 1996). It is possible that SIV infection increases the inflammation and thus the target cells for the initial PRRSV infection resulting in increased and prolonged pneumonia. PRRSV levels were
not quantified in the respiratory tract in this study as the primary interest was the impact of PRRSV infection on SIV vaccine efficacy, not the mechanism of disease.

Reduced viral shedding is a important parameter for efficacious SIV vaccines. Normally, SIV is rapidly cleared from the pigs respiratory tract following infection and viral shedding is undetectable by 5–7 DPI (Brown et al., 1993). In this study, no virus was recovered beyond 5 DPI with the exception of one pig in group VP[4]S[7]. Co-infection of pigs with SIV and PRRSV at 7 weeks of age did not appear to enhance or extend nasal shedding of SIV. In fact, SIV was not detected at the acute stage of infection and at the later time points, the amount of virus in the nasal cavity was significantly reduced. Co-infection studies with other viruses have reported inhibition of one virus over another. An example is the inhibition of hepatitis B virus (HBV) replication when co-infected with hepatitis D virus (HDV) or with triple infection with hepatitis C and D (Jardi et al., 2001). It was suggested there that the inhibitory effect of these hepatitis viruses could be related to the host DNA-dependent RNA polymerase II used by HBV being inhibited by a large delta antigen (Modahl and Lai, 2000). In this study, there was insufficient data to explain the mechanism by which PRRSV appeared to limit SIV replication. This study, determined that PRRSV infection at the time of SIV vaccination reduced vaccine efficacy against SIV excretion in the early stage of SIV infection (2 DPI). The level of virus shedding at that time was increased which may explain the significant increase in the severity of the SIV-associated macroscopic and microscopic lung lesions at 7 DPI compared to pigs vaccinated in the absence of PRRSV. This is an important finding as one of the goals of a SIV vaccine is to reduce the amount of virus shed during an SIV outbreak in addition to decreasing the severity of clinical disease.

Previous studies of PRRSV and SIV co-infections have yielded conflicting results on the severity of clinical disease, ranging from enhanced disease (Van Reeth et al., 1996) to minimal disease (Pol et al., 1997). The variation in these study results suggest that disease severity is dependent on the virulence of the viruses and on the time interval between PRRSV and SIV infections (Van Reeth et al., 2001). Significant differences in clinical respiratory and macroscopic lung lesions have been demonstrated between the European (Lelystad virus) and the North American PRRSV isolates (VR-2332) (Halbur et al., 1995; Murtaugh et al., 1995). The European PRRSV isolate and a low-virulence U.S. isolate both induced mild fever with macroscopic lung lesions of less than 10% compared to several more virulent U.S. isolates that caused severe clinical signs and greater than 50% of the lung tissue had pneumonia (Halbur et al., 1995, 1996). This may explain why Pol et al. (1997) found no increase in clinical disease in pigs infected with both SIV and the European Lelystad viruses. In earlier studies, source and immune status of the pigs was found to affect the clinical outcome of dual PRRSV and SIV infection as milder clinical disease was observed in caesarean-derived colostrum-deprived pigs compared to conventional pigs that were seronegative to PRRSV and SIV (Van Reeth et al., 2001). In the study reported here, we demonstrated increased clinical respiratory disease in conventional pigs co-infected with SIV and PRRSV at 7 weeks of age independent of their SIV vaccination status. One potential explanation for these differences is of the virulence of the isolate of PRRSV used in this study.

Previous findings by van Reeth and Nauwynck (2000) and Van Reeth et al. (2002a,b) demonstrated increased levels of interferon-alpha (IFN-α), tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) in the BAL of SIV-infected pigs. All 3 cytokines are essential for activating phagocytic cells resulting in the induction of a specific adaptive immune response (reviewed in van Reeth and Nauwynck (2000)). In addition, they are pyrogenic in nature and thus are highly correlated with the clinical signs of fever and fatigue typically associated with SIV infection. In contrast, PRRSV infection has been shown to down regulate IFN-α (Albina et al., 1998) and TNF-α but prolong production of IL-1. Accordingly, these cytokines produced during the acute phase of SIV and PRRSV co-infection may act in synergy to induce lung inflammation and enhance clinical disease. We did not assess these cytokines in the study reported here, but their production may be an explanation for the results observed in our study and should be studied further.

While the mechanism by which PRRSV decreases vaccine efficacy is currently not known, previous in vitro and in vivo studies have suggested an immunosuppressant effect by the virus due to increased IL-10 levels (Suradhat and Thana-wongnuwech, 2003; Suradhat et al., 2003). In vivo experiments demonstrated that PRRSV infection decreased classical swine fever (CSFV) specific antibodies following CSFV vaccination (Li and Yang, 2003). In contrast, antibody levels following MHYO vaccination were increased in the face of PRRSV infection (Thacker et al., 2000b). In our study, all SIV vaccinated and challenged groups appeared to have serum HI antibody responses, independent of PRRSV infection at the time of vaccination. This indicates that the first SIV vaccination, which was free of PRRSV infection in all groups, was able to successfully prime an active antibody response to SIV. A possible explanation for these findings is the determination that PRRSV infection induces a polyclonal expansion of B cells (Butler et al., 2007) which may result in increased production of antibodies with a lower affinity to the virus and thus reduced vaccine efficacy and increased disease in the presence of antibodies.

In addition to the systemic antibody response, PRRSV infection appeared to have little impact on the local antibody response to SIV challenge. SIV-specific IgA antibody responses in BAL fluids of SIV vaccinated and challenged pigs were significantly higher than the non-vaccinated, challenged pigs independent of PRRSV status at either the time of vaccination or at the later challenge date. While both PRRSV and SIV replicate in the lung, the difference in cell tropism of the two viruses may have minimized the effect on the antibody response to SIV. PRRSV however, seemed to increase the SIV-specific lymphocyte proliferation in PBMCs collected at 4 weeks after each PRRSV inoculation. These findings contradict previous findings by De Bruin et al. (2000) where PRRSV infection prior to PRV vaccination shortened the time course and reduced the level of lymphocyte proliferation following vaccination and PRV challenge. The capability of
PRV to infect, replicate and impair the function of PAMs (Iglesias et al., 1989a,b, 1992) may be one explanation to this finding. PRV vaccines are made up of modified live viruses, while SIV vaccines consist of inactivated viruses in an adjuvant. In addition, SIV infects respiratory epithelial cells and while potentially infecting PAMs, viral replication is restricted (Seo et al., 2004). As a result an early proinflammatory cytokine production of short duration is induced by the SIV-infected PAMs resulting in a rapid and effective immune response and control of SIV-induced disease (Rodgers and Mims, 1981; Seo et al., 2004; Van Reeth and Adair, 1997).

In summary, SIV vaccination in the absence of PRRSV significantly reduced pneumonia and SIV shedding following SIV challenge with a heterologous virus of the same subtype. The presence of PRRSV between vaccinations, while reducing vaccine efficacy, did not negatively impact either the systemic or local antibody response to either SIV vaccination or challenge. However, control of SIV at the acute stage of infection appeared to be slightly compromised as demonstrated by increased levels of virus. Overall, the results of the study described here provide a possible explanation for some of the SIV vaccine failures reported in the field. The presence of PRRSV should be considered when implementing vaccination strategies for controlling SIV, MHYO and potentially other pathogens. The impact of PRRSV infection on vaccine efficacy is considered when implementing vaccination strategies for controlling SIV, MHYO and potentially other pathogens. The presence of PRRSV between vaccinations, while reducing vaccine efficacy, did not negatively impact either the systemic or local antibody response to either SIV vaccination or challenge. However, control of SIV at the acute stage of infection appeared to be slightly compromised as demonstrated by increased levels of virus. Overall, the results of the study described here provide a possible explanation for some of the SIV vaccine failures reported in the field. The presence of PRRSV should be considered when implementing vaccination strategies for controlling SIV, MHYO and potentially other pathogens. The impact of PRRSV infection on vaccine efficacy is especially important during gilt acclimatization and in nursery pigs, common times for vaccinating pigs against disease. Further information on the impact of PRRSV on vaccine efficacy and its effect on the immune system is needed to develop successful intervention strategies against other pathogens in the presence of PRRSV.

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