Effect of vaccination on the potentiation of porcine reproductive and respiratory syndrome virus (PRRSV)-induced pneumonia by *Mycoplasma hyopneumoniae*

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Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) and *Mycoplasma hyopneumoniae* are frequently isolated pathogens from pigs with respiratory disease. A previous study conducted in our laboratory found that infection with *M. hyopneumoniae* increased the duration and severity of respiratory disease induced by PRRSV. The purpose of this experiment was to determine whether vaccination against *M. hyopneumoniae* and/or PRRSV decreased the enhancement of PRRSV-induced pneumonia. Both *M. hyopneumoniae* bacterin and PRRSV vaccine decreased the severity of clinical respiratory disease. Infection or vaccination with PRRSV appeared to decrease the efficacy of the *M. hyopneumoniae* bacterin. Vaccination with *M. hyopneumoniae* bacterin decreased the potentiation of PRRSV-induced pneumonia observed in the dual infected pigs. However, PRRSV vaccination in combination with *M. hyopneumoniae* bacterin eliminated this benefit and the amount of pneumonia induced by PRRSV increased. PRRSV vaccine alone did not decrease the potentiation of PRRSV pneumonia by *M. hyopneumoniae*. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Mycoplasma hyopneumoniae; PRRSV; Vaccination

1. Introduction

Porcine respiratory disease complex (PRDC) is an economically significant respiratory disorder characterized by slow growth, decreased feed efficiency, lethargy, anorexia, fever, cough and dyspnea. Porcine reproductive and respiratory syndrome virus (PRRSV) and *Mycoplasma hyopneumoniae* are two of the most common pathogens isolated from pigs exhibiting signs consistent with PRDC [1]. *M. hyopneumoniae*, the causative agent of enzootic pneumonia, induces a mild, chronic pneumonia commonly complicated by opportunistic infections with other bacteria [2]. PRRSV is perhaps the most important respiratory and reproduc-

tive pathogen in the swine industry. Recent research conducted in our laboratory found that *M. hyopneumoniae* potentiated the pneumonia induced by PRRSV [3]. The model, which used dual infections of PRRSV and *M. hyopneumoniae*, demonstrated that *M. hyopneumoniae* significantly prolonged and increased the severity of PRRSV-induced pneumonia. No increase in macroscopic mycoplasmal pneumonia was observed, although visible lung lesions were more severe early in the course of the disease. Microscopic lesions typical of *M. hyopneumoniae* were more extensive in dual infected pigs at all stages of infection.

Vaccines against *M. hyopneumoniae* and PRRSV are commonly used in the US as aids for controlling swine respiratory disease. Current *M. hyopneumoniae* vaccines are bacterins administered either intramuscularly or subcutaneously. A previous study conducted in our laboratory demonstrated that *M. hyopneumoniae* vac-

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Table 1
Experimental design summarizing vaccination against and infection with either *Mycoplasma hyopneumoniae* and/or porcine reproductive and respiratory syndrome virus (PRRSV)

Vaccine	Challenge inoculum			
	Sham inoculation	PRRSV and *M. hyopneumoniae*	PRRSV	*M. hyopneumoniae*
M. hyopneumoniae	0[a]	13[b]	0	0
PRRSV	0	13	0	0
Both	7	13	13	13
None	7	13	13	13

[a] Number of pigs/group.
[b] Three or six pigs from each group of pigs were necropsied at 10 DPI and the remaining pigs were necropsied at 38 DPI.

cines provide protection against experimental *M. hyopneumoniae* challenge, although pneumonia was not completely eliminated and colonization was only slightly reduced [4]. Currently, there are several modified live virus PRRSV vaccines commercially available for use in young pigs. The objective of this experiment was to determine whether vaccination against *M. hyopneumoniae* and/or PRRSV decreased the potentiation of PRRSV-induced pneumonia by *M. hyopneumoniae* observed in our previous study.

2. Materials and methods

2.1. Experimental animals

One hundred and eighteen cross-bred pigs were obtained from a commercial herd which had no serological evidence of exposure to either PRRSV or *M. hyopneumoniae*. The pigs were 10–12 days of age and randomly assigned to one of ten vaccination/challenge groups. The pigs were housed in individual isolation rooms and fed a commercial feed containing no antibiotics. The experimental design is summarized in Table 1. The study was conducted in accordance with guidelines provided by the Iowa State University Institutional Committee on Animal Care and Use.

2.2. Vaccines

Pigs in the designated groups received either a *M. hyopneumoniae* vaccine (M + Pac™, Schering-Plough Animal Health, Omaha, NE) at 3 and 5 weeks of age and/or a modified live PRRSV vaccine (Resp-PRRSRepro®, Boehringer Ingelheim/NOBL Laboratories, Ames, IA) at 4 weeks of age according to label directions.

2.3. Inocula and challenge

The pigs were 7-weeks-old when challenged (designated as 0 day post infection — DPI). A tissue homogenate containing a derivative of *M. hyopneumoniae* strain 11 (10^5 color changing units (CCU) per ml) was administered intratracheally to pigs in the appropriate groups at a dilution of 1:100 in 10 ml mycoplasmal Friis media in the morning [4]. An inoculating dose of 1×10^5 $TCID_{50}$ of the high virulence PRRSV strain ATCC-VR2385 in a 2 ml volume was administered intranasally to pigs in the appropriate groups in the afternoon of the same day [5].

2.4. Clinical evaluation

Pigs were evaluated daily for a period of 15 min for clinical signs, including appetite (abdominal fill), cough, tachypnea, dyspnea, or behavioral changes for 22 days following challenge. A daily clinical respiratory score was assessed from 0 to 22 DPI using a previously described system [6]. Rectal temperatures were measured daily from 0–10 DPI and every other day from 12 to 22 DPI. Pigs were weighed at 0 DPI and at necropsy.

2.5. Necropsy

Pigs were necropsied at either 10 or 38 DPI. The right rib cage was reflected and a portion of lung was aseptically collected for *M. hyopneumoniae* and PRRSV isolation. The lungs were removed and evaluated for macroscopic lesions. The bronchi were swabbed for bacterial and *M. hyopneumoniae* isolation. The lungs were lavaged with 50 ml of minimal essential medium (MEM) containing antibiotics (9 µg of gentamicin/ml, 100 U of penicillin G/ml, and 100 µg of streptomycin/ml) [7]. Lesions consistent with mycoplasmal pneumonia (dark red-to-purple consolidated areas) were sketched on a standard lung diagram. The proportion of lung surface with lesions was determined from the diagram using a Zeiss SEM-IPS image analyzing system [8]. In contrast to mycoplasma-induced lesions, PRRSV infected lungs were characterized by parenchyma that was mottled tan and rubbery and failed to collapse. The lung lesions were scored using a

previously developed system based on the approximate volume that each lobe contributes to the entire lung: the right cranial lobe, right middle lobe, cranial part of the left cranial lobe, and caudal part of the left cranial lobe contribute 10% each of the total lung volume, the accessory lobe contributes 5%, and the right and left caudal lobes each contribute 27.5% [5]. These scores were then used to calculate a total lung lesion score based on the relative contribution of each lobe.

Tissue sections were taken from all lung lobes and frozen for flourescent antibody assay (FA) or fixed in 10% neutral buffered formalin and routinely processed and embedded in paraffin in an automated tissue processor for histopathologic examination. Lung sections were examined and given a score (0–4) based on the severity of the peribronchiolar and perivascular lymphoid cuffing and nodule formation consistent with *M. hyopneumoniae*-induced pneumonia lesions. PRRSV-induced pneumonia lesions were scored (0–6) based on the severity of interstitial pneumonia as previously described [3]. A direct immunofluorescence (FA) procedure was used for detection of *M. hyopneumoniae* antigen as previously described [9].

2.6. PRRSV and M. hyopneumoniae isolation

PRRSV isolation was performed using BAL fluid obtained at necropsy. Virus isolation was then performed using an established protocol [10]. Monolayers of cells were stained with an anti-PRRSV monoclonal antibody SDOW-17 (South Dakota State University, Brookings, SD) followed by fluorescein isothiocyanate (FITC)-conjugated antimouse immunoglobulin and viewed with a fluorescence microscope for evidence of specific viral antigens [10]. Isolation of *M. hyopneumoniae* was performed from lung sections as previously described [8]. Mycoplasma-appearing colonies were specifically identified using epi-immunofluorescence with conjugate prepared from pig antisera to *M. hyopneumoniae* strain 11 [11].

2.7. Serology

Blood was collected periodically throughout the trial to evaluate antibody production. Sera were tested for antibodies against PRRSV with a commercially available enzyme-linked immunofluorescent assay (ELISA; HerdChek: PRRS, IDEXX Laboratories, Inc., Westbrook, ME) following the procedures described by the manufacturer. Samples were considered positive if the calculated sample to positive control (S/P) ratio was 0.4 or greater. *M. hyopneumoniae* antibody titers were determined by ELISA as previously described [12]. Known positive and negative sera were included as controls in each plate. Readings > 2 standard deviations (SD) above the mean optical density (OD) value

Table 2
Clinical respiratory scores and number of days average rectal temperatures were greater than 104 F in pigs vaccinated against *Mycoplasma hyopneumoniae* and/or PRRSV followed by challenge with PRRSV and/or *M. hyopneumoniae*[a]

Group No.	Vaccine	Challenge	1–10 days post PRRSV challenge		12–22 days post PRRSV challenge	
			Respiratory and clinical score[b]	No. of days rectal temperature > 104 F	Respiratory and clinical score	No. of days rectal temperature > 104 F
1	P & M[c]	None	0.0 ± 0.0[d]	1.0 ± 0.8[c]	0.0 ± 0.0[e]	0.3 ± 0.5[a,b]
2	None	None	0.0 ± 0.0[d]	0.7 ± 1.0[c]	0.0 ± 0.0[e]	0.5 ± 0.6[a,b]
3	M	P & M	17.2 ± 2.5[b]	5.7 ± 2.8[a]	7.9 ± 3.1[b]	1.6 ± 1.1[a]
4	P	P & M	13.3 ± 2.4[c]	5.0 ± 2.6[a]	4.3 ± 0.8[d]	0.4 ± 0.8[a,b]
5	P & M	P & M	12.7 ± 2.6[c]	3.2 ± 1.2[b]	5.3 ± 1.8[c,d]	0.3 ± 0.5[a,b]
6	None	P & M	21.7 ± 2.9[a]	6.5 ± 2.5[a]	9.6 ± 1.4[a]	1.6 ± 0.5[a]
7	P & M	M	0.0 ± 0.0[d]	1.7 ± 1.6[b,c]	0.0 ± 0.0[e]	0 ± 0[b]
8	None	M	0.0 ± 0.0[d]	0.8 ± 0.8[c]	0.0 ± 0.0[e]	0.4 ± 0.5[a,b]
9	P & M	P	16.2 ± 3.2[b]	5.2 ± 2.2[a]	6.4 ± 0.8[b,c]	1.0 ± 1.0[a,b]
10	None	P	21.7 ± 2.5[a]	6.3 ± 1.9[a]	5.6 ± 1.9[c,d]	1.1 ± 0.9[a,b]

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

[b] Presented as group mean ± standard deviation. Scoring: 0 = normal; 1 = mild dyspnea and/or tachypnea when stressed; 2 = mild dyspnea and/or tachypnea when at rest; 3 = moderate dyspnea and/or tachypnea when stressed; 4 = moderate dyspnea and/or tachypnea when at rest; 5 = severe dyspnea and/or tachypnea when stressed; 6 = severe dyspnea and/or tachypnea when at rest.

[c] P = PRRSV, M = *M. hyopneumoniae*.

Table 3

Macroscopic lung lesions in pigs vaccinated against *Mycoplasma hyopneumoniae* and/or PRRSV followed by challenge with PRRSV and/or *M. hyopneumoniae*. Data presented as group mean ± standard deviation[a]

Group no.	Vaccine	Challenge	Day 10 post challenge		Day 38 post challenge	
			M. hyopneumoniae[b]	PRRSV[c]	*M. hyopneumoniae*	PRRSV
1	P & M[d]	None	0.0 ± 0.0[d]	0.0 ± 0.0[c,d]	0.1 ± 0.2[b]	0.0 ± 0.0[b]
2	None	None	0.02 ± 0.03[d]	0.0 ± 0.0[c,d]	0.1 ± 0.1[b]	0.0 ± 0.0[b]
3	M	P & M	7.9 ± 5.0[a]	24.2 ± 10.9[a,b]	6.6 ± 15.6[a]	3.4 ± 2.1[b]
4	P	P & M	4.7 ± 3.0[a,b,c]	16.3 ± 13.2[a,b,c]	12.2 ± 8.7[a]	17.5 ± 8.6[a]
5	P & M	P & M	3.2 ± 2.8[c,d]	16.0 ± 11.1[a,b,c]	4.4 ± 3.1[a]	16.9 ± 18.4[a]
6	None	P & M	6.7 ± 5.1[a,b]	31.3 ± 20.9[a]	15.2 ± 12.8[a]	16.2 ± 8.1[a]
7	P & M	M	1.6 ± 1.7[c,d]	0.0 ± 0.0[c]	5.6 ± 7.3[a]	0.0 ± 0.0[b]
8	None	M	2.1 ± 3.2[c,d]	0.0 ± 0.0[c]	9.2 ± 5.5[a]	0.0 ± 0.0[b]
9	P & M	P	0.3 ± 0.5[d]	11.8 ± 11.0[a,b,c]	0.1 ± 0.1[b]	0.9 ± 2.7[b]
10	None	P	1.4 ± 2.5[c,d]	25.6 ± 18.7[a,b]	0.2 ± 0.2[b]	0.2 ± 0.6[b]

[a] Within each column, values with different bold superscripts are significantly different by least significant difference ($p < 0.05$).

[b] Percentage of lung exhibiting *M. hyopneumoniae*-induced pneumonia as determined by lesion sketches and image analysis.

[c] Percentage of lung exhibiting PRRSV-induced pneumonia as estimated by visual observation.

[d] P = PRRSV, M = *M. hyopneumoniae*.

of the negative control were considered positive (OD = 0.200).

2.8. M. hyopneumoniae-specific antibodies in bronchoalveolar lavage fluid

M. hyopneumoniae-specific antibodies were measured using an ELISA. Microtiter plates (Immulon II, Dyantech, Chantilly, VA) were coated with a membrane preparation from *M. hyopneumoniae* clone 232-2A3, isolated from the lung of a pig inoculated with *M. hyopneumoniae* strain 11 [13]. The BAL fluid was tested undiluted. In order to determine the isotype of *M. hyopneumoniae*-specific antibodies produced in BAL, peroxidase-labeled goat anti-swine IgA (Bethyl Laboratories, Montgomery, TX), IgG and IgM (Kirkegaard & Perry, Gaithersburg, MD), all heavy chain specific, were used. Optical density (OD) was determined at 405 nm with an ELISA reader.

2.9. Statistics

Data were subjected to analysis of variance (ANOVA). If the *P* value from the ANOVA was less than or equal to 0.05, pairwise comparisons of the different treatment groups were performed by least significant difference at the *P* < 0.05 rejection level.

3. Results

3.1. Clinical disease

Results of the clinical respiratory scores and rectal temperatures are summarized in Table 2. All groups inoculated with PRRSV displayed symptoms of respiratory disease consistent with PRRSV pneumonia including labored and accentuated abdominal breathing (dyspnea) and increased rate of breathing (tachypnea). Coughing was observed in all groups inoculated with *M. hyopneumoniae*. At 1–10 DPI, non-vaccinated pigs challenged with either PRRSV alone (group 10) or in conjunction with *M. hyopneumoniae* (group 6) had the most severe respiratory disease. Vaccination of dual infected pigs with the PRRSV vaccine (groups 4 and 5) significantly decreased, but did not eliminate respiratory signs. Vaccination against *M. hyopneumoniae* (group 3) only resulted in a slight, but significant decrease in PRRSV-induced respiratory disease in dual infected pigs. Pigs vaccinated against both *M. hyopneumoniae* and PRRSV and challenged with PRRSV alone (group 9) exhibited a similar decrease in respiratory disease. Respiratory signs decreased between 12 and 22 DPI, although the scores of the non-vaccinated, dually infected pigs (group 6) remained significantly higher than all other groups through day 22.

Rectal temperature data is summarized in Table 2. In the first 10 DPI, pigs challenged with PRRSV had significantly higher rectal temperatures than the non-challenged pigs or pigs challenged with only *M. hyopneumoniae*. The average temperatures in all PRRSV infected groups were equivalent with the exception of group 5, vaccinated against both *M. hyopneumoniae* and PRRSV, which had a lower average temperature score. Rectal temperatures on 12–22 DPI were similar to 1–10 DPI and remained high in the pigs inoculated with PRRSV. However, there was a great deal of overlap between the various vaccine and challenge groups by 12–22 DPI due to the large variation in temperatures between individual pigs.

Table 4

Microscopic lung lesion scores of pigs vaccinated against *Mycoplasma hyopneumoniae* and/or PRRSV followed by challenge with PRRSV and/or *M. hyopneumoniae*. Data presented as group mean $\pm$ standard deviation[a]

Group no.	Vaccine	Challenge	Day 10 post challenge		Day 38 post challenge	
			M. hyopneumoniae[b]	PRRSV[c]	*M. hyopneumoniae*	PRRSV
1	P & M[d]	None	1.0 ± 1.4[d,e]	1.5 ± 0.7[d,e]	2.0 ± 0.0[c]	1.0 ± 0.0[d]
2	None	None	0.7 ± 0.6[d,e]	0.3 ± 0.6[e]	1.0 ± 0.0[d]	1.0 ± 0.0[d]
3	M	P & M	1.3 ± 0.5[c,d]	3.0 ± 1.1[b,c]	3.0 ± 0.0[b]	1.1 ± 0.4[d]
4	P	P & M	1.7 ± 0.8[b,c]	2.5 ± 1.4[c,d]	3.4 ± 0.5[a,b]	2.3 ± 0.8[b,c]
5	P & M	P & M	2.2 ± 0.8[a,b]	2.5 ± 0.5[c,d]	3.4 ± 0.8[a,b]	1.9 ± 0.7[c]
6	None	P & M	1.7 ± 0.5[b,c]	3.5 ± 0.8[a,b]	3.7 ± 0.5[a]	2.9 ± 0.4[a]
7	P & M	M	2.5 ± 0.8[a]	2.7 ± 0.5[b,c,d]	3.3 ± 0.8[a,b]	2.2 ± 0.4[b,c]
8	None	M	1.0 ± 0.6[c,d]	0.5 ± 0.5[e]	3.6 ± 0.5[a]	0.9 ± 0.4[d]
9	P & M	P	1.3 ± 0.5[c,d]	3.5 ± 0.8[a,b]	1.7 ± 0.5[c]	3.0 ± 0.6[a]
10	None	P	0.0 ± 0.0[e]	4.0 ± 0.0[a]	1.4 ± 0.5[c,d]	2.7 ± 0.5[a,b]

[a] Within each column, values with different bold superscripts are significantly different by least significant difference ($p < 0.05$).

[b] *M. hyopneumoniae*: Score based on the severity of peribronchiolar and perivascular cuffing and lymphoid nodule formation. 0 = no microscopic lesions; 1 = mild; 2 = moderate; 3 = moderate to severe; 4 = severe.

[c] PRRSV: Score based on the severity of interstitial pneumonia. 0 = no microscopic lesions; 1 = mild pneumonia; 2 = mild diffuse pneumonia; 3 = moderate multifocal pneumonia; 4 = moderate diffuse pneumonia; 5 = severe multifocal pneumonia; 6 = severe diffuse pneumonia.

[d] P = PRRSV, M = *M. hyopneumoniae*.

3.2. Macroscopic lung lesions

Complete necropsies were performed on all pigs and all organ systems were examined. The estimated percentages of lung tissue with visible PRRSV and/or *M. hyopneumoniae* pneumonia are summarized in Table 3. Several pigs in the non-challenged control group and the PRRSV-only challenged groups had small pneumonic lesions similar to those observed with mycoplasmal pneumonia. No evidence of *M. hyopneumoniae* was found by culture or FA in any of the pigs not inoculated with *M. hyopneumoniae*. However, eight of the non-challenged pigs were culture positive for *Mycoplasma flocculare*, which is considered non-pathogenic in pigs. In addition, *Haemophilus parasuis*, *Actinobacillus suis* and *Bordetella bronchiseptica* were isolated from several of the pigs. Infection with these pathogens could have resulted in the mild pneumonia lesions observed at necropsy.

At 10 DPI, nonvaccinated pigs and those vaccinated with either mycoplasma or PRRSV vaccine alone followed by challenge with both PRRSV and *M. hyopneumoniae* (groups 3, 4, and 6) had the greatest percentage of lung lesions consistent with *M. hyopneumoniae* infection. In addition, the percentage of lesions in dual infected groups 3 and 6 was significantly greater than pigs infected with *M. hyopneumoniae* only (groups 7 and 8).

No differences were observed in the percentage of PRRSV-induced lesions in any of the PRRSV challenged groups at 10 DPI. There was a trend for the percentage of pneumonic lesions to be lower in PRRSV-vaccinated pigs, but no statistically significant differences were observed.

At 38 DPI, no differences in the percentage of *M. hyopneumoniae*-induced pneumonia were observed between any of the *M. hyopneumoniae* challenged groups. There was a trend for the *M. hyopneumoniae* lesions to be more severe in the dual infected groups that were not vaccinated for *M. hyopneumoniae*. PRRSV-induced pneumonia was still present in all of the dual infected groups. As in our earlier study, the percentage of pneumonia typical of PRRSV was minimal in the groups infected with PRRSV only. There was no difference in the percentage of PRRSV-induced pneumonia between any of the dual infected groups with the exception of the group vaccinated for *M. hyopneumoniae* (group 3), where the percentage of PRRSV-induced pneumonia was equivalent to that observed in the non-challenged controls and the PRRSV only challenged groups (groups 9 and 10).

3.3. Microscopic lesions

Microscopic lesion scores are summarized in Table 4. At 10 DPI, microscopic lesions consistent with *M. hyopneumoniae* infection were most severe in the group vaccinated for both *M. hyopneumoniae* and PRRSV followed by either dual infected (group 5) or *M. hyopneumoniae* (group 7) infected groups. All other mycoplasma challenged groups had less severe and similar microscopic lesion scores. There were mild microscopic changes consistent with mycoplasmal pneumonia in the groups which were not challenged with *M. hyopneumoniae*. However, these changes appear to be non-specific and may be related to the other bacterial agents isolated from the pigs.

PRRSV-induced microscopic lesions at 10 DPI were

most severe in the non-vaccinated pigs (groups 6 and 10) and the pigs vaccinated against both mycoplasma and PRRSV and infected only with PRRSV (group 9). All other PRRSV challenged and/or PRRSV-vaccinated pigs had similar levels of microscopic lesions consistent with PRRSV-induced interstitial pneumonia. There were mild microscopic changes of interstitial pneumonia in the groups which were not challenged with PRRSV. However, no evidence of viral contamination was observed in any of these pigs as determined by virus isolation and PRRSV ELISA.

At 38 DPI, both groups which were not vaccinated against mycoplasma (groups 6 and 8) had significantly higher average microscopic lesion scores consistent with mycoplasma infection than the dual infected group which received only the mycoplasma vaccine (group 3). Again, the non-challenged groups of pigs had low levels of microscopic lesions suggestive of *M. hyopneumoniae*.

PRRSV-induced microscopic lesions at 38 DPI were most severe in the non-vaccinated, dual infected group (group 6) and both of the groups challenged only with PRRSV (groups 9 and 10). The dual infected group vaccinated against mycoplasma (group 3) had similar microscopic lesions to the groups which had not received PRRSV (groups 1, 2 and 8).

3.4. PRRSV and M. hyopneumoniae isolation

M. hyopneumoniae was isolated only from the groups inoculated with *M. hyopneumoniae*. PRRSV was isolated from PRRSV vaccinated non-challenged groups (groups 1 and 7) as well as all groups challenged with wildtype PRRSV. Neither vaccine was effective in reducing the recovery rate of PRRSV at either 10 and 38 DPI, although the number of pigs from which PRRSV was isolated in the BAL at the second necropsy decreased from 91.1% (41 of 45) of the pigs to 27.5% (14 of 51). Interestingly, pigs vaccinated with both vaccines followed by challenge with only *M. hyopneumoniae* (group 7) had the second highest number of pigs from which PRRSV was isolated with all pigs positive on day 10 DPI and 50% of the pigs on day 38 DPI.

3.5. Serology

At challenge, all pigs vaccinated against *M. hyopneumoniae* were seropositive (OD > 0.200) and all non-vaccinated pigs were negative. Groups which received both *M. hyopneumoniae* and PRRSV vaccines (groups 1, 5, 7 and 9) had significantly higher *M. hyopneumoniae* antibody titers than the group which received only *M. hyopneumoniae* vaccine (group 3; data not shown). Similar results were found 2 weeks following challenge. At the final necropsy, the group which

received only *M. hyopneumoniae* vaccine followed by dual infection (group 3) and the group which received both PRRSV and *M. hyopneumoniae* vaccines and infected only with *M. hyopneumoniae* (group 7) had the highest levels of *M. hyopneumoniae* antibodies. Group 5 which received both vaccines and challenged with both *M. hyopneumoniae* and PRRSV had significantly lower *M. hyopneumoniae* antibody levels at necropsy than the other groups vaccinated against and challenged with *M. hyopneumoniae*. The pigs in the two non-vaccinated, non-challenged groups remained negative throughout the experiment.

All pigs which received PRRSV vaccine developed antibodies against PRRSV (S/P > 0.4). At the final necropsy, all pigs vaccinated or challenged with PRRSV were seropositive for antibodies, while all non-vaccinated or non-challenged pigs were seronegative.

3.6. M. hyopneumoniae-specific antibodies in bronchoalveolar lavage (BAL) fluid

At 10 DPI, all groups of pigs vaccinated against mycoplasmal pneumonia had significantly higher levels of *M. hyopneumoniae*-specific IgG antibodies than the non-vaccinated pigs (data not shown). There were minimal differences between groups in IgA levels at 10 DPI due to the wide variation between pigs and the low levels of antibodies present in the BAL. There was a non-specific response of IgM antibodies to *M. hyopneumoniae*.

At 38 DPI, IgG levels remained elevated in the mycoplasma vaccinated and challenged group. All other groups had similar levels of IgG antibodies against *M. hyopneumoniae*. IgA levels were greatest in the vaccinated/challenged groups (groups 3, 5 and 7). The IgA levels were similar in all remaining groups.

4. Discussion

This experiment reproduced the potentiating effect of *M. hyopneumoniae* on PRRSV-induced pneumonia observed in our previous study [3]. PRRSV-induced pneumonia remained significantly worse at 38 DPI in the dual infected pigs in this experiment as compared to pigs inoculated with PRRSV alone. Similar to our initial study, there was no increase in pneumonic lesions attributed to *M. hyopneumoniae* at 38 days DPI in dual infected pigs, although there was a trend that suggests that *M. hyopneumoniae*-induced pneumonia is more severe in pigs infected with PRRSV. The pneumonic lesions consistent with *M. hyopneumoniae* were slightly but significantly increased both macroscopically and microscopically at 10 DPI in pigs infected with both PRRSV and *M. hyopneumoniae* in our first

study. Interestingly, in this second experiment, the percentage of mycoplasma pneumonia at 10 DPI was clearly increased when pigs were also infected with PRRSV. These findings suggest that PRRSV infection hastens the onset of acute mycoplasmosis.

Vaccination against *M. hyopneumoniae* and PRRSV are commonly utilized as intervention strategies for the control of swine respiratory disease. Timing of these vaccines is variable according to the vaccination schedule of the individual farm. However, the label directions on the mycoplasma vaccine recommends vaccinating pigs at approximately 1 and 3 weeks of age. The PRRSV vaccine label recommended administration of a single vaccination between 3 and 18 weeks of age. In this study, mycoplasma vaccine administered alone decreased the potentiation of the PRRSV induced pneumonia at 38 DPI in dually infected pigs (group 3). All other dual infected groups exhibited *M. hyopneumoniae* potentiation of PRRSV-induced pneumonia at 38 DPI. Of particular importance was the finding that dual infected pigs that were previously vaccinated against PRRSV only (group 4), or both PRRSV and *M. hyopneumoniae* (group 5) had a level of PRRSV-induced pneumonia at 38 DPI equivalent to non-vaccinated pigs (group 6). The severity of *M. hyopneumoniae*-induced pneumonia lesions observed in pigs vaccinated against both *M. hyopneumoniae* and PRRSV and challenged with *M. hyopneumoniae* only (group 7) was greater than anticipated based on observations from previous experiments using this vaccine [4]. Due to the large number of pigs used in this trial, we did not include a group vaccinated against and challenged with only *M. hyopneumoniae*. In previous trials, the group average percentage of lung lesion following experimental *M. hyopneumoniae* challenge was always less than 2% in vaccinated pigs [4]. Apparently, exposing pigs to PRRSV, either through infection or use of a modified live vaccine, may potentially decrease the efficacy of mycoplasma vaccines. This finding could explain some of the reported failures of commercial mycoplasma vaccines in the field, which in our experimental setting have been shown to be uniformly effective.

Vaccination against PRRSV decreased, but did not eliminate the clinical respiratory disease associated with PRRSV infection. No decrease in PRRSV-induced macroscopic or microscopic lesions were observed in PRRSV vaccinated/challenged pigs. Furthermore, the PRRSV vaccine used in this experiment provided no protection against the potentiation of PRRSV pneumonia induced by *M. hyopneumoniae*. In this experiment we tested only one of four commercial PRRSV vaccines. The strain of PRRSV used to infect pigs in this study (VR2385) appears to differ from the parent strain of the PRRSV vaccine (VR2332) [5,14]. Vaccine strain differences my be im-

portant in a PRRSV vaccines ability to protect against the various strains of PRRSV. An experiment conducted in Europe demonstrated that vaccination with a PRRSV vaccine derived from an American strain of PRRSV afforded minimal cross protection against challenge with various European strains of PRRSV [15]. Similarly, there appeared to be a lack of protection provided by the PRRSV vaccine against pneumonia and respiratory disease induced by the challenge strain of PRRSV used in this study. This suggests that the various strains of PRRSV may have unique immunological characteristics which may preclude cross protection between differing strains of PRRSV.

Dual infection resulted in a significant increase in *M. hyopneumoniae*-induced lesions at 10 DPI compared to the groups receiving only *M. hyopneumoniae*. Vaccination against either PRRSV or *M. hyopneumoniae* did not appear to protect against the pneumonia induced by *M. hyopneumoniae* at this early stage. A possible explanation for the increased mycoplasma pneumonia in PRRSV infected pigs may be that infection of macrophages by PRRSV, which has been shown to compromise their phagocytic capabilities [16], results in increased colonization by mycoplasmal organisms in the early stages of infection. An additional explanation may be that the inflammation induced by the acute PRRSV-induced pneumonia may facilitate the inflammatory reaction induced by the mycoplasmal organisms, thus increasing the rate of lymphocyte proliferation and macrophage infiltration associated with *M. hyopneumoniae*. However, PRRSV infection did not significantly increase lesions induced by *M. hyopneumoniae* in the later stages of disease (38 DPI). These results suggest that the duration of PRRSV's effect on the respiratory systems ability to control the colonization and spread of *M. hyopneumoniae* is limited.

PRRSV was isolated from the BAL of all groups vaccinated and/or challenged with PRRSV. This was not unexpected as the PRRSV vaccine used in this study is a modified live virus vaccine. In all groups, the number of pigs with PRRSV isolated at 38 DPI was greatly decreased. However, the non-vaccinated, dual challenged group had the largest number of pigs with PRRSV isolated from the BAL at 38 DPI. One mechanism that *M. hyopneumoniae* may use to potentiate PRRSV-induced pneumonia is by the continued attraction and/or activation of susceptible pulmonary alveolar macrophages. Group 7, vaccinated for both *M. hyopneumoniae* and PRRSV and challenged with *M. hyopneumoniae*, had the second highest number of pigs (50%) from which the virus was isolated in the BAL. This suggests that *M. hyopneumoniae* may act to decrease the clearance of both high and low virulence strains of PRRSV and may contribute to the persist-

ence reported with PRRSV, either the wild-type or vaccine strains [17].

Serological data and the presence of *M. hyopneumoniae*-specific antibodies in the BAL support a previous report that PRRSV infection is not systemically immunosuppressive as measured by antibody production [18]. Pigs vaccinated against either PRRSV or *M. hyopneumoniae* developed serum antibodies as expected. Because *M. hyopneumoniae* is a mucosal pathogen that adheres only to the ciliated epithelial cells of the respiratory tract, *M. hyopneumoniae*-specific antibodies in the BAL may be important in protecting against the development and the resolution of clinical disease. To assess the impact of PRRSV infection on the local immune response, we measured *M. hyopneumoniae*-specific antibodies in the BAL to determine if PRRSV infection resulted in a decrease in antibody levels at the local mucosal level. All groups which received mycoplasma vaccine had measurable *M. hyopneumoniae*-specific antibodies in the BAL by 10 DPI. Both *M. hyopneumoniae*-specific IgG and IgA antibodies were increased in groups vaccinated and challenged with *M. hyopneumoniae*. Infection or vaccination with PRRSV did not appear to affect the production of *M. hyopneumoniae*-specific antibodies in the BAL. Thus, decreased local or systemic antibody production does not appear to be an explanation for the decreased efficacy of the mycoplasma vaccine in PRRSV vaccinated or infected pigs.

The findings of this study confirm that *M. hyopneumoniae* plays an important role in the increased duration and severity of PRRSV-induced pneumonia. The mechanism by which *M. hyopneumoniae* potentiates PRRSV remains unknown, as does the role PRRSV plays in the increased severity of the mycoplasmal pneumonia early in the course of infection. Based on the results of this study, it appears that vaccinating against *M. hyopneumoniae* may decrease, but not eliminate the potentiation of PRRSV-induced pneumonia by *M. hyopneumoniae*. PRRSV vaccination or challenge appeared to decrease the efficacy of *M. hyopneumoniae* vaccination, which provides a potential reason for *M. hyopneumoniae* vaccine failure in the field. The mechanism by which PRRSV effects *M. hyopneumoniae* vaccination is unknown at this time as there was no difference in either the local or systemic immunological parameters measured in this study. Current mycoplasma vaccines do not prevent colonization of the mycoplasma organisms. Therefore, when the respiratory system is infected with *M. hyopneumoniae* and PRRSV, both may affect the local immune system resulting in the increased pneumonia and inflammation associated with the porcine respiratory disease complex (PRDC).

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