ORIGINAL RESEARCH

Comparison of antibody production, lymphocyte stimulation, and protection induced by four commercial *Mycoplasma hyopneumoniae* bacterins

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Summary

Objectives: To compare the ability of four commercial *Mycoplasma hyopneumoniae* bacterins to stimulate humoral and cellular immune responses against a mycoplasmal membrane preparation and the level of protection induced against experimental challenge.

Methods: Five groups (n=10) of 2-week-old crossbred pigs with no antibodies to *M. hyopneumoniae* were vaccinated with one of four commercial *M. hyopneumoniae* bacterins or with saline at 3 and 5 weeks of age. Lymphocyte stimulation assays and serum antibody concentrations were measured on –7, 28, and 45 post vaccination days (PVD). The pigs were challenged with *M. hyopneumoniae* strain 11 on PVD 51 and necropsied on PVD 78.

Results: Lymphocyte stimulation and the antibody titers induced by the four bacterins differed significantly among the five experimental groups. In addition, the number of pigs seroconverting in response to vaccination varied among groups. Immunoblot analysis demonstrated differences in the number of *M. hyopneumoniae* proteins that induced antibodies. The group mean percent of pneumonic lungs did not differ among the vaccinated pigs.

Implications: The inconsistent immune response observed in this study may be an important source of susceptible populations of pigs. All vaccines evaluated induced some protection from clinical pneumonia; however, the variation in antibody concentrations and the protection induced among pigs was considerable and may be important in herd management.

Pneumonia caused by *Mycoplasma hyopneumoniae* is a worldwide problem affecting the majority of swine herds in the United States. Mycoplasma pneumonia usually presents as a chronic disease with high morbidity and low mortality. Vaccination-induced, protective immunity against *M. hyopneumoniae* has been demonstrated experimentally by challenge studies, and vaccination induces both cellular and humoral immunity.[1] In various animal species, including cattle and mice, locally produced mucosal antibodies have been shown to provide a degree of protection against mycoplasmal pneumonia.[2,3] However, passive immunization with serum containing antibodies to *M. hyopneumoniae* also provided protection against the development of pneumonia, suggesting that circulating antibodies are also capable of providing some degree of protection.[4]

Studies of *Mycoplasma pulmonis* infections in mice have demonstrated that cell-mediated immunity (CMI) plays an important role in providing protective immunity.[5] Two studies of the CMI responses in pigs have shown inconsistent in vitro stimulation of lymphocytes by mycoplasmal antigens after experimental challenge or vaccination with *M. hyopneumoniae*.[1,6] In both studies, there was significant variation among individual animals, although the degree of stimulation in peripheral blood lymphocytes was greater in vaccinated pigs compared to nonvaccinated control pigs. Conversely, suppression of T-cell responses by thymectomy and treatment with antithymocyte serum resulted in decreased severity of microscopic pneumonic lesions after challenge, although the multiplication of *M. hyopneumoniae* was enhanced compared to immunocompetent swine, suggesting that the CMI may both help and hinder the development of mycoplasma pneumonia.[7]

The objective of this controlled study was to compare four commercial *M. hyopneumoniae* vaccines to stimulate both the humoral and CMI components of the immune system against a specific mycoplasmal membrane preparation, and to evaluate the degree of protection against an experimental challenge.

Materials and methods

Experimental animals and design

Fifty 2-week-old crossbred pigs were procured from a herd in which the sows had low serum antibody concentrations against *M. hyopneumoniae* as measured by ELISA. Pigs were randomly assigned to five treatment groups with stratification by weight and sex.

During the vaccination phase of the experiment, the pigs were randomly divided into six pens and housed in one room. One week prior to challenge, the pigs were moved by pen to an isolation facility. The pigs in groups A, B, C, and D were immunized according to the manufacturers' guidelines (Table 1). Subcutaneous (SC) injections were administered in the flank and intramuscular (IM) injections in the neck muscle behind

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the ear. The controls were injected with 2 mL of saline in the neck muscle.

Blood samples were collected for lymphocyte stimulation assays and serum antibody testing on postvaccination days (PVD) -7, 28, and 45. On PVD 51, all pigs were inoculated endotracheally with a derivative of *M hyopneumoniae* strain 11 (10^5 CCU per mL) as previously described.[8] All pigs were observed daily for a period of 15 minutes for coughing. Pigs were weighed on PVD -1 and 51, and at necropsy, 27 days after challenge. At necropsy, pigs were anesthetized with sodium pentobarbital administered intravenously and exsanguinated. The lungs were evaluated and sketched using diagrams of the dorsal and ventral surfaces as previously described by Neurand, et al.[9] The proportion of lung area with pneumonic lesions was determined using a Zeiss SEM-IPS image analyzing system.[10]

Lymphocyte isolation

Peripheral blood monocytes (PBMC) were isolated from heparinized blood by differential centrifugation of diluted blood over Histopaque (Sigma, St. Louis, MO).[11] The PBMCs were resuspended at a concentration of 1×10^6 cells per mL in media. Viability was greater than 98% as determined by the trypan blue dye exclusion test.

Lymphocyte-stimulation test

The assay used was a modification of the assay described by Messier, et al.[1] Briefly, 100 mL of the PBMC cell suspension was dispensed into each well of a flat-bottomed 96-well microtitration plate. Cultures were tested in triplicate and incubated for 5 days. Eighteen hours before the plates were harvested, 1 mCi of 3[H] thymidine was added to each well. Cells were harvested, and the results were expressed as a stimulation index (SI): SI = average cpm of the three wells in stimulated cultures divided by the average cpm in control cultures. A SI of ≥3 was considered a positive stimulation.

Enzyme-linked immunosorbent assay

All serum samples were assayed for antibodies to *M hyopneumoniae* by ELISA as previously described.[12] Known positive and negative sera were included as controls in each plate. Readings > 2 SD above the mean value of the negative control were considered positive. For this experiment, the positive:negative cut-off value was 0.260.

Antigen for electrophoresis

Mycoplasma hyopneumoniae organisms were grown in modified Friis broth containing 8% swine serum at 37°C for 4 days on a shaker. The cells were harvested and washed with PBS. The protein content was estimated by the BCA protein assay method (Pierce, Rockford, Illinois). The antigen was subjected to freezing and thawing five to six times followed by solubilization with 1 mg of SDS for each 1 mg protein. The antigen preparation was used for SDS-polyacrylamide gel electrophoresis (PAGE) analysis.

Polyacrylamide gel electrophoresis

The *M hyopneumoniae* antigen preparation was mixed with an equal volume of a denaturation mixture. The antigen was subjected to SDS-PAGE analysis (12% polyacrylamide) using a discontinuous buffer system. Modified Friis media and 1:20 dilution of swine serum were included as negative controls. Following electrophoresis, the gel was stained with Fast Stain (Zoion Research Inc., Allston, Massachusetts) to visualize the protein bands. For immunoblot analysis, the gels were transblotted to nitrocellulose membrane under constant current (200 mA) for 2 hours.
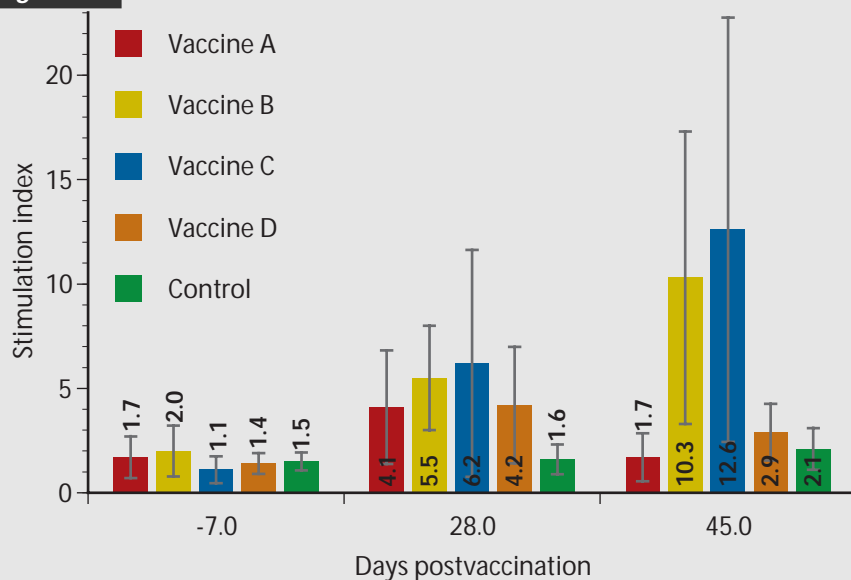
Immunoblot analysis

Transblotted nitrocellulose membranes were blocked with PBS containing 0.05% Tween 80 and 5% nonfat dry milk. Following standard washing procedures, the nitrocellulose membrane that contained the *M hyopneumoniae* protein bands was cut into strips, and each strip was incubated for 90 minutes at 37°C with a pool of sera (1:50 dilution) obtained from all pigs in each treatment group on PVD 45. The membranes were washed followed by incubation with peroxidase-conjugated anti-swine IgG (1:80,000 dilution) for 1 hour at 37°C. After washing, 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase membrane dye substrate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Maryland) was added. The bands that were visible on the membrane were recorded and photographed. The relative migration of the bands which showed antibodies was measured by a caliper. The molecular weight of these bands was evaluated based on relative migration of a molecular weight standard.
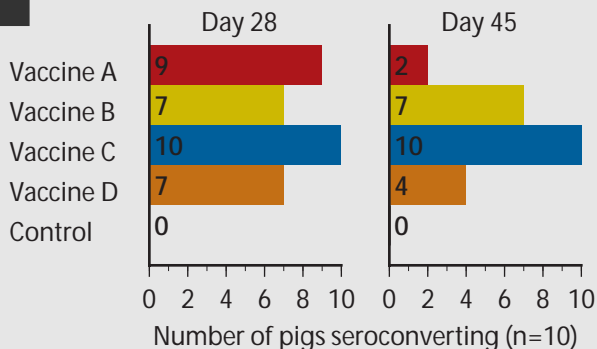
Statistical analysis

ANOVA was used to detect differences among the treatment groups. When the ANOVA *P* value was <.05, between-group comparisons were done by least significant difference. Regression analysis was performed to evaluate the relationships of serum antibody levels, lymphocyte stimulation indices, and the severity of pneumonia after challenge.
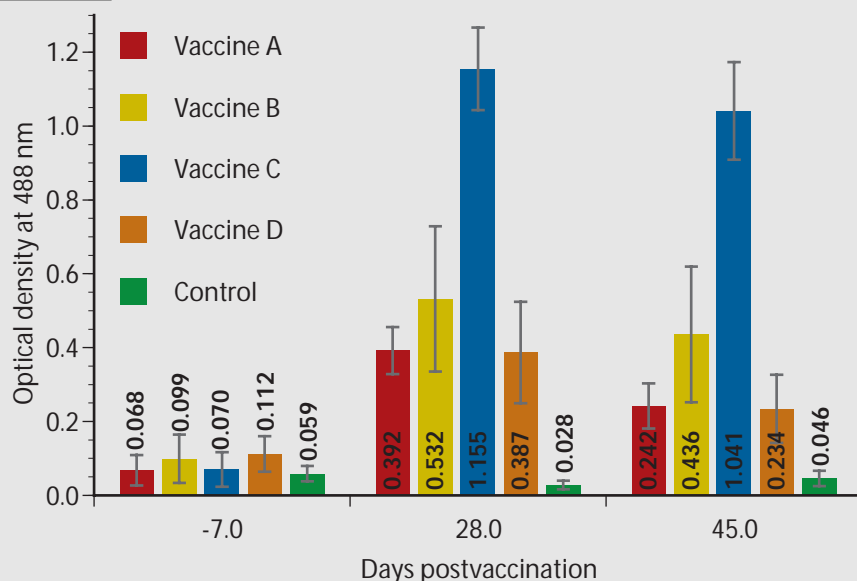
Results

Overall, vaccination induced a mycoplasmal membrane-specific, cell-mediated immune response as determined by the lymphocyte stimulation test (Figure 1). At PVD 28, the SI of the vaccinated groups ranged from 4.1–6.2, whereas the SI for the control group was only 1.6. At PVD 45, the group mean SIs for the vaccinated groups ranged from 1.7–12.6 and the control group mean SI was 2.1. However, even with the high variability, significant differences were detected between the groups (*P*<.05). Groups B and C had significantly higher SIs than Groups A, D, and the control group.

Figure 1

Mean (± 95% CI) stimulation indices for peripheral blood monocytes before and after vaccination with four commercial *M. hyopneumoniae* bacterins.

Figure 2

Number of pigs seroconverting *M. hyopneumoniae* after vaccination with four commercial *M. hyopneumoniae* bacterins..

Figure 3

Mean (± 95% CI) *M. hyopneumoniae* serum antibody levels as measured by ELISA before and after vaccination with four commercial *M. hyopneumoniae* bacterins.

The number of pigs that seroconverted in response to each vaccine (Figure 2) at 28 and 45 PVD varied between groups with an increased number of pigs failing to maintain measurable systemic antibodies at 45 PVD (Figure 3). All but one pig (Group B, ODV =.285) were classified as seronegative at vaccination and the group mean ODVs were well below the cut-off. Group differences in ODV were detected at both 28 and 45 PVD. At both times, Group C was significantly greater than the other three vaccine groups and the control group ($P<.05$). The mean ODVs of all groups declined between 28 and 45 PVD for the vaccine groups while the control group essentially stayed the same (i.e., it increased only 0.014). However, the decrease in ODV was not significantly different among the four vaccine groups.
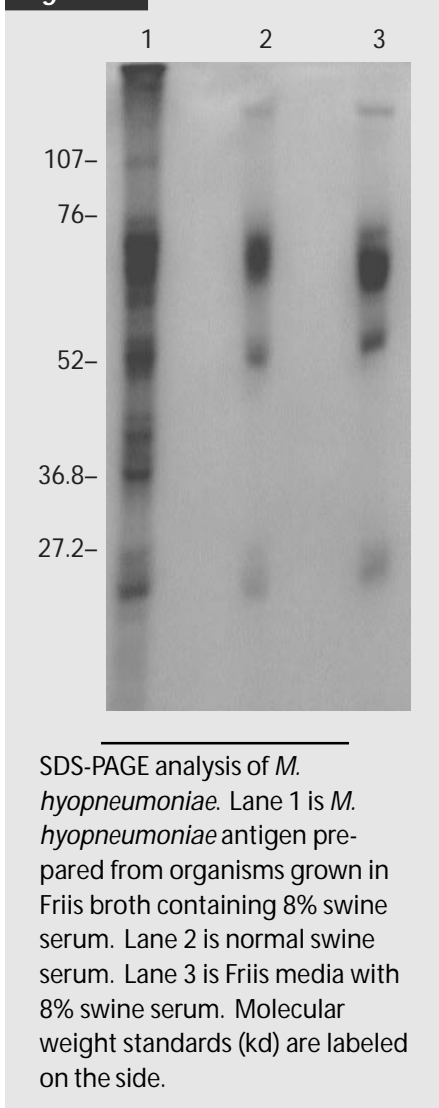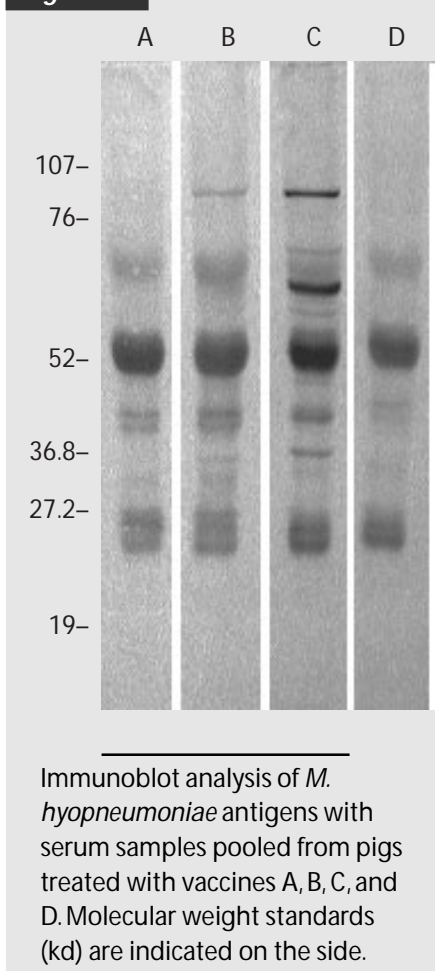
A total of 31 proteins were observed by SDS-PAGE analysis of the *M hyopneumoniae* antigen preparation (Figures 4–5). Several differences among the vaccine groups were apparent. Vaccine C induced antibodies to the greatest number of proteins (15 total). Vaccine B induced antibodies to 11 proteins, and Vaccines A and D induced antibodies to eight proteins.

Growth

During the vaccination period, all pigs remained healthy, and no differences in weight gains were detected among the groups. After challenge, weight gains were similar among the groups. Overall, average weight of all pigs on PVD -1 was 10.45±1.01 lb (2.2 ± 0.46 kg); PVD 51, 68.53±7.88 lb (31.15 ± 3.58 kg) ; and at necropsy, 103.03 ± 10.80 lb (46.83 ± 4.91 kg).

Health

Coughing was first observed 8 days after challenge. There were no differences among the five groups with respect to the number of days of coughing per pig, or the first day that coughing was observed. Overall, coughing was observed in 33 pigs. Two pigs in Group C died at 14 and 15 days after challenge. Pathological examinations indicated that one pig had lesions suggestive of a viral myocarditis, and the other pig had viral encephalitis. No infectious agents were isolated from either pigs, and no other pigs exhibited similar clinical signs.

Figure 4

SDS-PAGE analysis of *M. hyopneumoniae.* Lane 1 is *M. hyopneumoniae* antigen prepared from organisms grown in Friis broth containing 8% swine serum. Lane 2 is normal swine serum. Lane 3 is Friis media with 8% swine serum. Molecular weight standards (kd) are labeled on the side.

Figure 5

Immunoblot analysis of *M. hyopneumoniae* antigens with serum samples pooled from pigs treated with vaccines A, B, C, and D. Molecular weight standards (kd) are indicated on the side.

cal pneumonia is generally incomplete. Immunization with *M hyopneumoniae* vaccines induces serum antibodies, but provides limited protection against mycoplasmal infection and does not prevent colonization by *M hyopneumoniae* in the respiratory tract. In our study, the percentage of lung surface exhibiting pneumonic lesions was significantly reduced ($P<.05$) by all four vaccines compared to the control group; however, there was considerable individual pig variation within each group.

Since the purpose of vaccination is to induce specific and long-lasting immunity, the ability of vaccines to stimulate T and B lymphocytes is an important consideration. In this study, we attempted to further define the vaccine-induced immune responses by evaluating humoral immune responses by ELISA and immunoblot analysis, and cellular responses by lymphocyte stimulation.

Differences were observed in the ability of the four vaccines to induce a proliferative response by lymphocytes to a mycoplasmal membrane preparation. While many of the pigs in each vaccine group had circulating lymphocytes that were responsive to a mycoplasmal membrane antigen prepar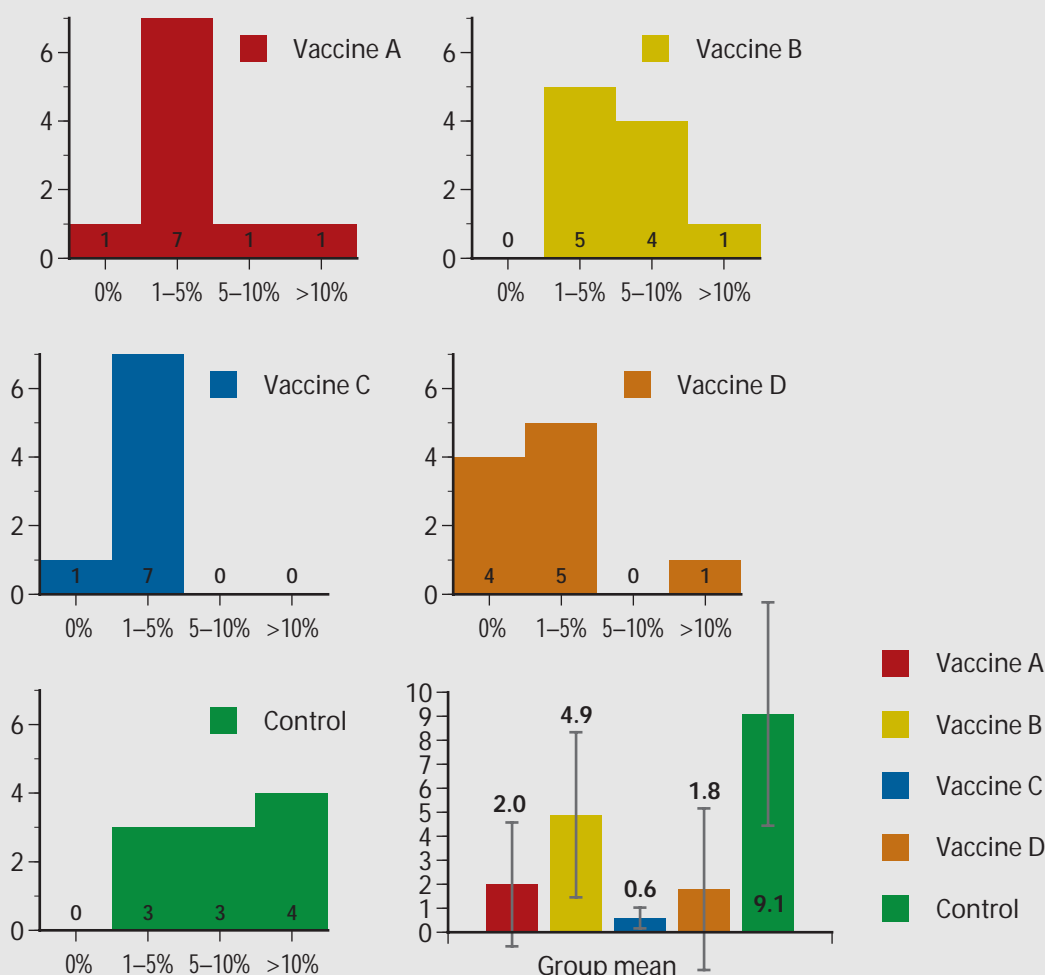ation in culture, no correlation was found between the quantity of lymphocyte responsiveness and the degree of protection against *M hyopneumoniae* challenge on an individual pig basis. Our failure to observe a statistical difference in SI among groups was due to the high degree of variability among the pigs within the treatment groups. However, pigs vaccinated with C had the highest group mean lymphocyte response to the mycoplasma membrane preparation and the lowest percentage of pneumonic lung lesions. Conversely, Group B had a high group mean lymphocyte stimulation index, but also had the highest percentage of pneumonia lesions among the four vaccinated groups.

The lack of correlation between lymphocyte stimulation in response to the mycoplasmal membrane antigen and protection from challenge may be due to a number of factors including:

- failure of the vaccines to elicit a predictable CMI response,
- lack of response *in vitro* to the antigen preparation used, or
- failure of systemic lymphocytes to respond to the antigen, while mucosal lymphocytes may have been sensitized.

Lymphocytes involved in local, mucosal immunity are not normally present in the systemic circulation, residing instead in the mucosal lymphoid tissues. To measure local immune responses, lymph nodes and bronchial associated lymphoid tissue would need to be evaluated.

All pigs in the control group had pneumonic lesions, and the percentage of pneumonia lesions ranged from 2.0%–20.2% (Figure 6). All vaccine groups had significantly fewer pneumonic lesions than the controls and the group mean percent pneumonia for the vaccinated pigs ranged from 0.6%–4.9%, while the control group mean was 9.1%. Differences among the four vaccine groups were not significant..

No significant relationships between the severity of pneumonia and the SI at PVD 28 and 45 were found. Significant relationships between the ELISA ODV's at PVD 28 and 45 and the severity of pneumonia were apparent if the control group was included in the analysis. Among vaccinated pigs, regardless of the vaccine group, no significant relationships were detected.

Discussion

Mycoplasma hyopneumoniae vaccines are typically bacterins consisting of either whole-organism and/or membrane preparations. Protective immunity induced by vaccination against *M hyopneumoniae* has been demonstrated experimentally; however, protection against clini-

Figure 6

Percentage of lung surface exhibiting pneumonia as determined by image analysis in pigs vaccinated with *Mycoplasma hyopneumoniae* bacterins and subsequently challenged with *M. hyopneumoniae*
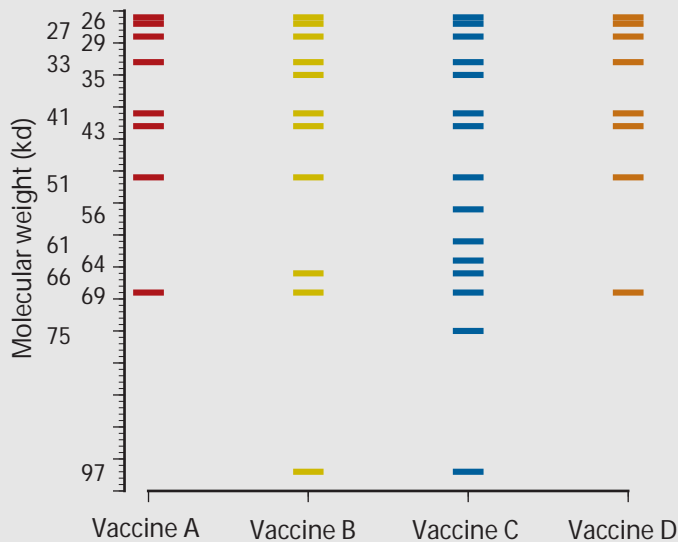
Significant differences in the group mean systemic antibody concentrations, as measured by ELISA, were detected, and considerable individual-pig variation within each vaccine group was apparent. Only vaccine C induced seroconversion in all pigs, and the antibody concentrations induced by vaccine C were significantly higher than those induced by the other vaccines. In Groups A, B, and D, 30%–50% of the pigs failed to develop and maintain *M hyopneumoniae* antibody concentrations. This inconsistent immune response may be an important cause of subpopulations of pigs that are more susceptible to infection and clinical pneumonia induced by *M hyopneumoniae*. Antibody concentrations appeared to be more closely correlated with the percentage of pneumonic lung lesions on a group basis; however, there was no correlation on an individual animal basis if the control group was not included in the analysis. In all groups, with the exception of Group C, several pigs with low antibody concentrations had minimal lung lesions and pigs with apparently adequate antibody titers had <5% pneumonic lesions.

A possible explanation for the discrepancy in the relationship between antibody concentrations and severity of pneumonia was provided by the immunoblot analysis (Figure 7) that showed differences among the

vaccines with respect to the antigen-antibody reactions that were visible on the blots.

Immunoblot analysis showed several potentially important differences among the vaccine groups. Sera were pooled from all pigs within each vaccine group and assessed for the presence of antibodies to specific *M hyopneumoniae* proteins. The development of antibodies to a greater number of *M hyopneumoniae* membrane proteins in the sera of Group C pigs compared to the other groups may explain the comparatively high degree of protection provided by vaccine C against challenge. Sera from the pigs vaccinated with C contained antibodies to proteins in the region thought to contain the surface lipoprotein P65, which may be an important *M hyopneumoniae* immunogen.[13] A report by Young and Ross[14] indicated that convalescent serum from swine with experimental *M hyopneumoniae* pneumonia recognized 65kd, 50kd, and 44kd antigens, thought to be lipoproteins, by SDS-PAGE and immunoblot. Production of antibodies against these surface proteins may be a determinant of vaccine efficacy.

Vaccines B and C produced antibodies to a 97 kd protein (P97). Previous studies have shown that this protein facilitates adherence of *M*

Figure 7

Molecular weight (kd)

| | Vaccine A | Vaccine B | Vaccine C | Vaccine D |

Estimated molecular weights of antigens detected by immunoblot analysis using pooled sera from pigs vaccinated with commercial *M. hyopneumoniae* bacterins.

- Variable numbers of pigs seroconverting in response to vaccination may be a source of susceptible populations of pigs.
- Measurements of systemic cellular and humoral immune responses did not necessarily predict the degree of protection against experimental challenge suggesting local, mucosal immunity may be important in protection.

Acknowledgements

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hyopneumoniae to respiratory tract cilia.[15] Monoclonal antibodies produced against the P97 prevented adherence of *M hyopneumoniae* to swine cilia in vitro.[13]

Unexpectedly, the antibody concentrations against *M hyopneumoniae* declined between 28 and 45 PVD. This decrease was observed in all four vaccine groups. Currently, the duration of immunity induced by *M hyopneumoniae* bacterins is generally unknown. This rapid rate of antibody decline may play an important role in vaccine failure.

The swine industry still recognizes enzootic pneumonia to be an economically significant problem. With the emergence of porcine respiratory and reproductive syndrome virus (PRRSV), the incidence and severity of pneumonia due to *M hyopneumoniae* has apparently increased.[14] Vaccination in combination with management strategies are often necessary to control the impact of this disease. This study showed that all of the commercial bacterins evaluated induced some protection from clinical pneumonia under experimental conditions; however, there was considerable pig-to-pig variation in the amount of protection. In this study, we only investigated the systemic cellular and humoral responses induced by the vaccines. These measurements did not necessarily predict the degree of protection against experimental challenge. The role of local, mucosal immunity in protection against enzootic pneumonia may be more important and should be evaluated in future vaccine studies.

Implications

- All *Mycoplasma hyopneumoniae* vaccines evaluated in this trial induced some protection against experimental challenge.