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Efficacy of a subunit vaccine against *Actinobacillus pleuropneumoniae* in an endemically infected swine herd

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Summary

Objective: To evaluate lung lesions at slaughter after three-dose vaccination with a subunit *Actinobacillus pleuropneumoniae* vaccine containing ApxI, ApxII, ApxIII, and an outer membrane protein.

Materials and methods: A total of 430 newborn piglets in a herd endemically infected with *A pleuropneumoniae* were assigned to control and treatment groups by block randomization at litter level. Pigs vaccinated at 6, 10, and 14 weeks of age and unvaccinated controls were housed in three rooms containing 12 pens each, with treated and control groups mixed in pens. Individual pig data included average daily gain (ADG), number of antimicrobial treatments, and clinical disease. Sera of vaccinated (n = 5) and control pigs (n = 6) collected at 6, 10, 14, 18, and 23 weeks of age were tested for *A pleuropneumoniae* antibodies using the complement fixation test and ELISAs for Apx toxins and outer membrane protein. At slaughter, lungs were examined for gross and microscopic lesions and cultured for *A pleuropneumoniae*. Differences in ADG and occurrence of *A pleuropneumoniae*-related lesions were compared between treatment groups using multilevel mixed models with room and pen as random effects.

Results: *Actinobacillus pleuropneumoniae*-related lesions and ADG did not differ between control and treatment groups. Maternal immunity against *A pleuropneumoniae* was detected until 10 weeks of age. Moderate vaccine titres were observed after the third vaccination.

Implications: Maternal antibody may interfere with the response to subunit *A pleuropneumoniae* vaccines. Postponing vaccination until 10 to 14 weeks of age might be advisable in herds with high maternal immunity.

Keywords: swine, *Actinobacillus pleuropneumoniae*, vaccine, clinical trial, lung lesions

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Resumen - Eficacia de una vacuna de subunidades contra *Actinobacillus pleuropneumoniae* en un hato de cerdos infectado endémicamente

Objetivo: Evaluar las lesiones pulmonares en el rastro después de una vacunación con tres dosis de una vacuna de *Actinobacillus pleuropneumoniae* de subunidades conteniendo ApxI, ApxII, ApxIII, y una proteína de membrana externa.

Materiales y métodos: Un total de 430 lechones recién nacidos en un hato infectado endémicamente con *A pleuropneumoniae* fueron asignados a grupos de tratamiento y control por bloque al azar a nivel camada. Los cerdos vacunados a las 6, 10, y 14 semanas de edad y los controles no vacunados se alojaron en tres cuartos que contenían 12 corrales cada uno, con grupos control y tratamiento mezclados en los corrales. Los datos de cerdos individuales incluyeron ganancia diaria promedio (ADG por sus siglas en inglés), número de tratamientos antimicrobianos, y enfermedad clínica. Los sueros de cerdos vacunados (n = 5) y control (n = 6) recolectados a las 6, 10, 14, 18, y 23 semanas de edad fueron examinados en busca de anticuerpos contra *A pleuropneumoniae* utilizando la prueba de fijación de complemento y ELISAs en busca de toxinas de Apx y proteína de membrana externa. En el rastro, se examinaron los pulmones en busca de lesiones macro y microscópicas y se cultivaron en busca de *A pleuropneumoniae*. Las diferencias en ADG y en la incidencia de lesiones relacionadas con el *A pleuropneumoniae* se compararon entre grupos de tratamiento utilizando modelos mixtos de niveles múltiples teniendo el cuarto y el corral como efectos al azar.

Resultados: Las lesiones relacionadas con el *A pleuropneumoniae* y el ADG no difirieron entre grupos de tratamiento y control. La inmunidad materna contra

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el *A pleuropneumoniae* se detectó hasta las 10 semanas de edad. Títulos moderados de vacunación se observaron después de la tercera vacunación.

Implicaciones: Los anticuerpos maternos pueden interferir con la respuesta a las vacunas de subunidades de *A pleuropneumoniae*. Posponer la vacunación hasta las 10 ó 14 semanas de edad puede ser aconsejable en hatos con alta inmunidad materna.

Résumé - Efficacité d'un vaccin sous-unitaire contre *Actinobacillus pleuropneumoniae* dans un troupeau infecté de manière endémique

Objectif: Évaluer les lésions pulmonaires au moment de l'abattage après administration de trois doses de vaccin sous-unitaire contre *Actinobacillus pleuropneumoniae* contenant Apx1, ApxII, ApxIII, et une protéine de la membrane externe.

Matériels et méthodes: Un total de 430 porcelets nouveau-nés provenant d'un troupeau au prise avec une infection endémique à *A pleuropneumoniae* ont été assignés à des groupes témoins ou traités par randomisation en bloc au niveau de la portée. Les porcs vaccinés à 6, 10, et 14 semaines d'âge et les témoins non-vaccinés ont été logés dans trois chambres contenant 12 enclos chacune, avec les groupes traités et témoins mélangés dans les enclos. Les données individuelles par porc incluaient le gain quotidien moyen (ADG), le nombre de traitement antimicrobien, et la présence de maladie clinique. Des échantillons de sérum provenant de porcs vaccinés (n = 5) et témoins (n = 6) prélevés chez des animaux âgés de 6, 10, 14, 18, et 23 semaines ont été éprouvés pour la présence d'anticorps contre *A pleuropneumoniae* à l'aide d'un test de fixation du complément et par ELISA pour les toxines Apx et la protéine de la membrane externe. Après l'abattage, les poumons étaient examinés pour la présence de lésions macroscopiques et microscopiques et mis en culture pour détecter *A pleuropneumoniae*. Les différences d'ADG et la fréquence de lésions associées à *A pleuropneumoniae* étaient comparées entre les groupes de traitement à l'aide d'un modèle mixte à plusieurs niveaux avec la chambre et l'enclos comme effets aléatoires.

Résultats: Il n'y avait pas de différence entre les groupes témoins et traités quant aux lésions reliés à *A pleuropneumoniae* et l'ADG. L'immunité maternelle contre *A pleuropneumoniae* a été détectée jusqu'à l'âge de 10 semaines. Des titres vaccinaux modérés ont été notés après la troisième vaccination.

Implications: Les anticorps maternels peuvent interférer avec la réponse aux vaccins sous-unitaires contre *A pleuropneumoniae*. Retarder la vaccination des animaux jusqu'à ce qu'ils atteignent l'âge de 10 à 14 semaines serait recommandable dans les troupeaux où l'immunité maternelle est forte.

Porcine pleuropneumonia caused by *Actinobacillus pleuropneumoniae* is one of the most important bacterial diseases affecting the swine industry worldwide. It results in serious economic losses, especially in finishing pig units.[1] Fifteen serovars of *A pleuropneumoniae* are currently recognised,[2] with most serovars able to cause the same disease. However, serovars show variations in virulence, partly attributable to differences in Apx toxin profiles.[3-6] Acute *A pleuropneumoniae* outbreaks are characterized by severe clinical signs, including high fever, respiratory distress, cyanosis, and death within 1 or 2 days.[1] Chronically infected pigs may show less severe clinical signs, such as coughing and poor growth rate. Pigs that recover may become carriers, ie, remain colonized without clinical signs.

Vaccination strategies to control and prevent disease caused by *A pleuropneumoniae* have been used with variable results.[7,8] Vaccines containing whole cells reduce mortality after infection with homologous serovars, but do not protect against heterologous serovars.[8] Subunit vaccines containing Apx toxins and outer membrane protein (OMP) reportedly provide partial heterologous protection and reduce clinical signs and lung-lesion scores.[9,10] Although both types of vaccines provide some level of protection (homologous, heterologous, or both), they do not prevent colonization. Many factors are associated with the pathogenesis of disease caused by *A pleuropneumoniae* and protection against it, such as OMP (including transferrin-binding proteins), lipopolysaccharide, and fimbriae.[11,12] Therefore, variation in protective efficacy of the existing vaccines is expected.

Vaccination of pigs at 6 and 10 weeks of age is recommended by vaccine manufacturers for protection against *A pleuropneumoniae* infection. However, vaccination at this early age is a concern due to interference of maternally derived antibodies.[13-15] A third vaccination might help to overcome this effect. Considering the limitations of the currently available vaccines and recommended vaccination protocols by vaccine manufacturers, we evaluated a new vaccination program that includes an additional vaccination at 14 weeks of age (extra-label use). Assessment of the effectiveness of this new protocol was based on reduction of *A pleuropneumoniae*-related lesions and improvement in production parameters in a swine herd endemically infected with *A pleuropneumoniae*.

Materials and methods

Animals and housing

Four hundred and thirty (430) pigs in a farrow-to-finish herd on a Dutch research farm were selected. This herd had been endemically infected with *A pleuropneumoniae* serovar 2 during the previous 3 years. No *A pleuropneumoniae* vaccines had been administered before the study was initiated. At birth, piglets were ear-tagged with a unique identification number and were randomly assigned to vaccinated and control groups by block randomization at litter level. Piglets were weaned at an average age of 4 weeks. From weaning to finishing, pigs from both groups were housed in three mechanically ventilated, thermostatically controlled rooms that each contained 12 pens with partially slatted floors. Each pen was equipped with one long trough with feeding places for 12 pigs, and one drinker. Liquid feed was provided three times daily. A nursery diet was fed at weaning, and grower and finisher diets were introduced when pigs were approximately 10 and 16 weeks of age, respectively. Pigs were randomly assigned to pens, with treatment groups mixed in each pen. Daily care was provided by animal caretakers blinded to treatment groups. All pigs were managed according to the official guidelines of the Dutch government.[16]

Vaccination scheme

Pigs in the vaccinated group were vaccinated intramuscularly at 6, 10, and 14 weeks of age with Porcilis APP vaccine (Intervet International BV, Boxmeer, The Netherlands), a subunit vaccine containing an OMP and ApxI, ApxII, and ApxIII toxins.

Except for the additional dose at 14 weeks of age, vaccine was administered according to the instructions of the manufacturer. Pigs in the control group were not vaccinated.

Blood collection and serological testing

At 6 weeks of age, five pigs from the vaccinated group and six from the control group were randomly selected for serial blood sampling at 6, 10, 14, 18, and 23 weeks of age. All sera were tested for antibodies against *A pleuropneumoniae* using ApxI, ApxII, ApxIII, ApxIV, and OMP ELISA tests (Intervet International BV) and by the complement fixation test (CFT) for *A pleuropneumoniae* serovars 2 and 9.

ELISA tests were performed at the R&D Service Laboratory (Intervet International BV). Microtitre plates were coated with purified ApxI, ApxII, ApxIII, ApxIV, or OMP antigen, respectively, and were incubated with positive and negative standards, reference sera, and samples to be tested. For all ELISA tests except ApxIV ELISA, sera were tested using two dilutions, 1:100 and 1:1000. The optical density (OD) of each sample was measured at 450 nm. For each antigen, antibody titre was calculated relative to a negative standard serum and by using the correlation between dilutions of a positive standard serum and the corresponding OD values. By extrapolation from a regression equation, the titre of each tested sample was determined. Results were reported as $\log_2$ titres.

For the ApxIV ELISA, a commercial test kit was used (Chekit APP-ApxIV; Idexx Europe BV, Schiphol-Rijk, The Netherlands). The ODs of the positive standard serum and the samples were corrected by subtracting the OD of the negative standard serum. The corrected OD of each sample was expressed as a percentage of the corrected OD of the positive standard serum. Samples with ODs that were < 30%, ≥ 30% to < 40%, and ≥ 40% of the positive standard were considered negative, suspect, and positive, respectively.

The CFT was performed at the R&D Laboratory of the Animal Health Service (GD) at Deventer, The Netherlands. Titres ≥ 1:10 were considered positive.

Data collection

Individual pig data, including weight gain, clinical signs (eg, coughing, diarrhea, lameness), and treatment with antimicrobials were collected until the end of the study. Pigs were examined by the farm veterinarian on alternate weeks, and were individually weighed on an electronic scale accurate to 0.1 kg at the start of the finishing period and at slaughter. All clinical data were recorded by animal caretakers using a check-off list of clinical signs. Ill pigs were treated according to farm treatment protocols and remained in their pens for treatment. Group medications were applied only after consulting with the farm veterinarian. Feed conversion ratio (FCR) was calculated at pen level, but as pigs from both groups were housed in the same pens, FCR was not compared between treatment groups.

Pigs that reached market weight were sent to the packing plant in three weekly batches. In order to have access to abnormal lungs, the study investigators were responsible for retaining these lungs during slaughter. All abnormal lungs were collected, with the exception of those tightly adherent to the thoracic cavity, which were categorized as "lungs with missing parts" and classified as lungs with pleuritis. Lungs collected at slaughter were immediately submitted to the Central Veterinary Institute of Wageningen UR (Lelystad, The Netherlands) and carefully examined for macroscopic lesions, with pleuritis and pneumonia lesions recorded. Stage (acute, chronic) and location of lesions (cranial, cardiac, or caudal lobe, or interlobular) were classified, and extent of pleuritis, pneumonia, and pleuropneumonia, or other lung lesions, were graphically recorded. Lungs with necrotizing pleuropneumonia or focal abscesses with pleuritis in the same lobe were classified as *A pleuropneumoniae*-related lesions.

A representative number of lungs with macroscopic lesions of pleuritis, pneumonia, or both, were examined histologically and by bacterial culture for *A pleuropneumoniae* (control group, n = 65; vaccinated group, n = 40).

Bacteriological culture procedures

Bacteriological culture procedures were performed at the Central Veterinary Institute of Wageningen UR (Lelystad, The Netherlands), using the method described by Velthuis et al[17] for isolation of *A pleuropneumoniae*. Briefly, the surface of each lung specimen was decontaminated for 6 to 8 seconds in boiling water and cut aseptically into sections of 1 cm^3. Each sample was sealed in an individual polyethylene bag and blended in a Stomacher-80 Lab blender (Laméris Laboratorium BV, Breukelen, The Netherlands). Glycerol was added to a final concentration of 15% and samples were stored at -70°C. This material was cultured on heart infusion agar plates (Biotrading Benelux BV, Mijdrecht, The Netherlands) containing 5% sheep blood and 0.1% nicotinamide adenine dinucleotide (NAD) to enable V-factor (NAD)-dependent bacteria to grow. Plates were incubated at 37°C under microaerophilic conditions for 48 to 72 hours. Bacterial isolates were identified by standard methods for biochemical characterization. Agglutination with serotype-specific hyperimmune rabbit antiserum was performed to confirm *A pleuropneumoniae* serotypes.[18]

Statistical analysis

In order to perform statistical analysis at the individual pig level, we applied a split-plot design, ie, half of the pigs were vaccinated. Because power calculations for split-plot designs are not available, the power calculation was based on the assumption of proportions of pleuropneumonia in two separate groups. A prevalence of 30% pleuropneumonia was found in this herd during pilot studies. A sample size of 238 pigs was calculated with 80% power to detect a difference between treatment groups, assuming 30% pleuropneumonia in the control group and 15% in the vaccinated group and using a 95% confidence interval. The sample size was increased to 430 pigs to compensate for the expected underestimation of vaccination efficacy associated with use of a split-plot design, and to fit with management of the herd.

The outcome variables of interest were average daily gain (ADG) and occurrence of *A pleuropneumoniae*-related lesions at slaughter (ie, pigs with or without *A pleuropneumoniae*-related lesions). We calculated ADG for the finishing period of individual pigs using the difference between weight at the beginning of the finishing period and live weight at slaughter, divided by days to market, excluding culled pigs. The extent of recorded pleuritis and pneumonic lesions was calculated as the proportion of the perimeter or surface of the whole lung that the lesions represented. Descriptive analysis was performed in SPSS (SPSS version 12; SPSS Inc, Chicago, Illinois) followed by the generalized mixed model

in MLwiN (MLwiN version 2; Centre for Multilevel Modelling, The Bristol Institute of Public Affairs, Bristol, UK). A multilevel linear mixed model and multilevel logistic mixed model were used, respectively, to evaluate differences in ADG (Model 1) and *A pleuropneumoniae*-related lesions (Model 2) between vaccinated and control groups. As both treatment groups were housed in the same rooms and pens, pigs from the same pen or the same room were more alike than pigs from different pens or rooms. To correct for the effects of the hierarchical data, pen and room were treated as random effects (level two and level three variables, respectively). The predictor variables analyzed for their association with these outcome variables were treatment (control and vaccinated groups), batch of slaughtered pigs, occurrence of *A pleuropneumoniae*-related lesions (for Model 1), occurrence of antimicrobial treatments during the study (yes or no), time of antimicrobial treatment (no treatment or treatment 1, 2, or 3 months before the slaughter date), duration of antimicrobial treatment, body weight at the beginning of the finishing period, and gender. The likelihood ratio test was used as a criterion to include the predictor variables of Model 1, and the Wald test was used to assess the statistical significance of each parameter in Models 1 and 2. The "batch of slaughtered pigs" variable was added to both models to control for confounding. First-order interaction terms between the predictor variables in the final model were tested for significance. The level of significance was determined at $P = .05$. Assumptions of normality, homogeneity of variance, and independence of the residuals for the generalized mixed model were tested.

Results

Data for 26 pigs were excluded from the generalized mixed model analysis: 13 pigs were culled, seven lost their ear tags during transportation to the packing plant, and six were excluded because of missing data.

Treatment for clinical illness

Almost 45% of pigs in the vaccinated group and 49% in the control group were treated for clinical illnesses, but only three pigs from the vaccinated group and five from the control group were diagnosed with respiratory disease during the study period. Diarrhea related to the porcine intestinal adenomatosis complex was the most common diagnosis. Either pigs were treated individually with injectable antibiotics or the whole pen was treated with antibiotic in drinking water, as required (Table 1). Antimicrobial treatments included tylosin for diarrhea, oxytetracycline for respiratory signs, penicillin-streptomycin or ampicillin for lameness, and ampicillin for all other signs. No pigs died during the study. However, 13 pigs (three control and 10 vaccinated) were culled before they reached market weight. Of these, one control and two vaccinated pigs were culled because of respiratory problems.

ADG

There was no difference between vaccinated and control pigs in means for weight at the beginning of the finishing period, live weight at slaughter, days to market, or ADG (Table 2). The final multilevel linear mixed model for ADG, with pen and room as random effects, included the following variables as fixed effects: treatment group, batch of slaughtered pigs, occurrence of *A pleuropneumoniae*-related lesions, and time of antimicrobial treatment. In Model 1, the estimated ADG of vaccinated pigs was numerically higher than that of the control pigs by 8 g per day, but this difference was not statistically significant ($P = .11$). The estimated ADG of pigs without *A pleuropneumoniae*-related lesions was also numerically higher (by 0.46 g per day) than that of the pigs with lesions, but this difference was also not statistically significant ($P = .57$). The ADG of pigs that became ill early in the finishing period (pigs that were treated 3 months before slaughter date), corrected for effects of other variables, was 32 g per day lower than that of pigs without clinical illness, regardless of treatment group ($P < .01$).

Lung lesions

No significant differences were found in prevalence of pleuritis, lung lesions, or both, or in the extent of pleuritis or pneumonic lesions. Lung lesions were found in 123 of 198 vaccinated pigs (62%) and in 145 of 206 control pigs (70%). Overall pleuritis lesions (ie, in pigs with and without other pneumonic lesions) were found in 115 of 198 vaccinated pigs (58%) and in 135 of 206 control pigs (66%). Pleuritis without other lesions occurred in 73 of 198 vaccinated pigs (37%) and in 86 of 206 control pigs (42%). Prevalence of *A pleuropneumoniae*-related lesions was 11% in vaccinated pigs and 14% in control pigs. Pleuritis without other pneumonic lesions occurred in 20 of 198 vaccinated pigs (10%) and in 20 of 206 control pigs (10%). The extent of pleuritis, expressed as the proportion of affected areas of the lung perimeter, was 15.1% in the vaccinated group and 16.2% in the control group. Pneumonic lesions without pleuritis occurred in 8 of 198 vaccinated pigs (4%) and in 10 of 206 control pigs (5%). The extent of pneumonic lesions was 1.3% in the vaccinated group and 1.7% in the control group. *Actinobacillus pleuropneumoniae* was isolated from 19 of 40 samples from the vaccinated group (48%) and from 24 of 65 samples (37%) from the control group. All *A pleuropneumoniae* isolates were confirmed to be serovar 2. *Pasteurella multocida* and *Streptococcus suis* were also cultured from some lung samples.

The final multilevel logistic mixed model for presence of *A pleuropneumoniae*-related lesions, with pen and room as random effects, included the following variables as fixed effects: treatment group, batch of slaughtered pigs, and time of antimicrobial treatment. Growth rate was highest in the first delivery batch (Batch 1) (Model 1; $P < .001$), but Batch 1 also included the highest percentage of pigs with *A pleuropneumoniae*-related lesions (Model 2; $P < .001$). In Model 2, there was a nonsignificant odds ratio of 1.2 for the association of control group and *A pleuropneumoniae*-related lesions ($P = .64$). When overall lung lesions was used as an outcome variable in the same final multilevel mixed model, there was a nonsignificant odds ratio of 1.5 ($P = .06$) for association of control group and overall lung lesions (data not shown). Neither the estimated extent of pleuritis nor the estimated extent of pneumonic lesions was significantly different between control and vaccinated groups ($P > .05$).

Serological test results

Control pigs. Median $\log_2$ antibody titers in the ApxI, ApxII, ApxIII, and OMP ELISAs were 8, 12, 9, and 8, respectively, at 6 weeks of age, and 8, 9, 7, and 7, respectively at 10 weeks of age. Median $\log_2$ titers remained stable until pigs were 18 weeks of age, then modestly increased to 12, 14, 11, and 11, respectively, for ApxI, ApxII, ApxIII, and OMP ELISAs at 23 weeks of age. All control pigs were seropositive by the ApxIV ELISA until 14 weeks of age, but by 23 weeks of age, all except two of the six pigs were seronegative. Median reciprocal CFT titer measured at 6 weeks

Table 1: Clinical signs observed among 404 pigs either vaccinated for *Actinobacillus pleuropneumoniae* or not vaccinated in a herd endemically infected with *A pleuropneumoniae**

	No. of ill pigs							
Clinical signs	**By batch number**				**By month before slaughter when treated**			
Vaccinated pigs (n = 198)								
	1 (n = 38)	2 (n = 70)	3 (n = 90)	**Total**	Month 3	Month 2	Month 1	**Total**
Diarrhea	12	23	43	78	2	71	5	78
Respiratory signs	1	2	1	4	1	2	1	4
Lameness	2	4	9	15	6	8	1	15
Other signs†	1	0	2	3	2	1	0	3
Control pigs (n = 206)								
	1 (n = 37)	2 (n = 86)	3 (n = 83)	**Total**	Month 3	Month 2	Month 1	**Total**
Diarrhea	12	32	35	79	1	72	6	79
Respiratory signs	1	1	1	3	0	3	0	3
Lameness	2	2	7	11	9	1	1	11
Other signs†	0	1	1	2	1	0	1	2

* Pigs were vaccinated intramuscularly at 6, 10, and 14 weeks of age with Porcilis APP vaccine (Intervet International BV, Boxmeer, The Netherlands). Vaccinated and control (nonvaccinated) pigs were housed together from weaning to the end of the finishing period. Pigs were treated with antimicrobials as required during the growing-finishing period and were marketed in three weekly batches.

† Other signs included poor growth, tail-biting lesions, and abscesses.

Table 2: Production parameters in groups of pigs either vaccinated and or not vaccinated with an *Actinobacillus pleuropneumoniae* subunit vaccine containing ApxI, ApxII, ApxIII, and an outer membrane protein*

Parameter	**Vaccinated (n = 198)**		**Control (n = 206)**	
	Mean	**SD**	**Mean**	**SD**
Initial weight (kg)	22.40	3.79	22.97	4.00
Live weight at slaughter (kg)	111.22	6.79	111.10	8.09
Days to market	119	8	119	7
Average daily gain (g)	747.31	82.46	743.31	95.79

* Pigs were vaccinated intramuscularly at 6, 10, and 14 weeks of age with Porcilis APP vaccine (Intervet International BV, Boxmeer, The Netherlands). Production parameters did not differ by treatment group (multilevel linear mixed model; $P > .05$).

of age was < 40, increasing to 160 and 320 at 10 and 14 weeks of age, respectively, declining to 80 at 18 weeks of age, and again increasing to 1280 at 23 weeks.

Vaccinated pigs. When pigs were vaccinated at 6 weeks of age, colostrum-derived antibodies were still high (median $\log_2$ titers in the ApxI, ApxII, ApxIII, and OMP ELISAs were 9, 12, 8, and 8, respectively). Titers had declined to 7, 10, 7, and 7, respectively, in the samples collected when the pigs were 10 weeks old. Median $\log_2$ titers in the samples collected after the second *A pleuropneumoniae* vaccination, when the pigs were 14 weeks of age, were 9, 8, 9, and 9, respectively, for the ApxI, ApxII, ApxIII, and OMP ELISAs. In samples collected after the third vaccination, when the pigs were 18 weeks old, median $\log_2$ titers were 11, 10, 10, and 11, respectively, for the ApxI, ApxII, ApxIII, and OMP ELISAs. In the samples collected when the pigs were 23 weeks of age, median $\log_2$ titers had increased to 13, >14, >14, and >14, respectively, for the ApxI, ApxII, ApxIII, and OMP ELISAs.

Serological results for the ApxIV ELISA in vaccinated pigs were similar to those of the control pigs until they were 14 weeks of age. At 18 weeks of age, only one vaccinated pig still remained positive, but all five were seropositive by 23 weeks of age. Median reciprocal CFT titer was < 40 at 6 weeks of age and steadily increased thereafter (median titers were 80, 320, 320, and 1280, respectively, at 10, 14, 18, and 23 weeks of age).

Discussion

Well-designed randomized trials to evaluate vaccine efficacy in the field are rare. This split-plot trial was a well-designed study that allowed statistical analysis to be performed at pig level, as the vaccinated pigs and nonvaccinated control pigs were mixed together in pens and rooms. However, when a split-plot design is used, there is a risk of underestimating vaccine efficacy. Given the assumed difference that was expected from the power calculation, ie, 30% and 15% prevalence of pleuropneumonia in the control and vaccinated groups, respectively, we

must conclude that this difference was not observed in this study. However, the overall actual prevalence in both groups (14% and 11%) was lower than the prevalence observed in earlier pilot studies (30%) in the same herd, which was the basis of the power calculation. Why the overall prevalence of pleuropneumonia in this study was low cannot be fully explained. The sudden decline in the incidence of pleuropneumonia coincided with the vaccination trial, suggesting that vaccination may have contributed to the lower incidence of pleuropneumonia in the unvaccinated group, possibly by reducing exposure of these pigs to *A pleuropneumoniae*.

No significant difference in ADG between vaccinated and control groups was found when the effects of batch, *A pleuropneumoniae*-related lesions, and time of antimicrobial treatment were controlled for. However, several other investigators report different results in pigs vaccinated with an *A pleuropneumoniae*-subunit vaccine. In the studies of Martelli et al,[19] Valks et al,[20] and Ridremont,[21] there was a tendency for higher ADG in pigs vaccinated using a two-dose vaccination protocol, while the study of Pommier et al[22] showed a significantly higher ADG in vaccinated pigs. Wongnarkpet et al[23] found that growth rate in pigs vaccinated with *Mycoplasma hyopneumoniae* and *A pleuropneumoniae* vaccines was higher only in the final stage of the finishing period (16 weeks of age to slaughter). This may explain why ADG calculated for the whole finishing period in our study did not differ between vaccinated and control pigs. It is also possible that infected pigs can compensate for lower ADG early in the finishing period, ie, growth rate may increase after the period of infection.[24] Nevertheless, lowest growth rate, compared to that of pigs showing no clinical illness, occurred in pigs that were treated 3 months before slaughter, early in the finishing period, and this was a significant difference.

The use of a three-dose protocol for the *A pleuropneumoniae* subunit vaccine had no significant effect on the incidence of abnormal lung lesions. A high percentage of abnormal lungs were found in both vaccinated and control groups. Lium et al[25] reported a lower prevalence of pleuropneumonia and fewer necrotic lung lesions in vaccinated pigs, although pleuropneumonia and pleuritis with lung abscesses, assumed to be related to *A pleuropneumoniae*, were rarely found in either group. Until recently, the protective mechanism of *A pleuropneumoniae* vaccines was not fully understood. Several *A pleuropneumoniae* virulence factors, including Apx toxins, capsular polysaccharide, OMPs, transferrin binding proteins, lipopolysaccharide, and fimbriae, are involved in antibody protection against disease.[3,4,6,11] Moreover, factors involved with the adhesion capacity of the bacterium also play a role in protection.[12] Any other factors involved with disease protection still need to be studied. Because the protective mechanism is not fully understood, the ideal vaccine that can protect pigs against infection cannot be described.

In this study, diarrhea occurred once in almost 50% of the pigs in both groups, beginning early in the finishing period. Diarrhea has been identified as a risk factor for respiratory disease.[26,27] Diarrhea may influence the pig's immune system, interfering with the protective effect of the subunit vaccine. On the other hand, diarrhea may have increased the risk for pleuritis lesions related to respiratory diseases other than *A pleuropneumoniae*, as overall lung lesions in both groups were high, while lung lesions related to *A pleuropneumoniae* were low. Growth rate was highest in Batch 1 pigs, which also had more pleuropneumonia lesions. It was assumed, on the basis of serological results, that the pigs in this study were infected late in the finishing period (after 18 weeks). It is possible that in the pigs in Batches 2 and 3, which had lower ADG, lung lesions had time to heal.

The bacteriological results and historical record of the herd indicated that pigs in this study were infected with *A pleuropneumoniae* serovar 2, which produces ApxII and ApxIII toxins but not ApxI toxin. The positive ApxI ELISA tests may have been a response to a similar toxin produced by other bacteria, for example, Pasteurellaceae species that are closely related to *A pleuropneumoniae*.[5]

Although only a limited number of pigs were tested serologically, consecutive serological data showed that when pigs were vaccinated the first time, at 6 weeks of age, all had high ELISA titers to OMP, ApxI, ApxII, and ApxIII antigens. Titres were lower at 10 weeks of age (at the time of the second vaccination), remained stable until 14 weeks of age (at the time of the third vaccination), and had increased by 18 weeks of age. A similar pattern of these responses were reported by Bak et al.[28] The high antibody titres in 6-week-old pigs were likely maternally derived and clearly interfered with the first and possibly with the second vaccination, since no antibody responses to the four protein antigens in the vaccine were detected. Tumamao et al[29] showed that Porcillis APP vaccine induced high antibody titres against ApxI, ApxII, ApxIII and OMP in pigs with low levels of maternally derived antibody. In our study, it is likely that maternally derived antibodies still interfered with vaccination at 10 and 14 weeks of age, because in vaccinated animals, antibody levels had increased only modestly in samples collected after the pigs were 14 weeks of age. Results of the ApxIV ELISA also demonstrated that maternally derived antibodies still persisted until the pigs were 14 weeks of age. Administering the second and third vaccinations later, eg, at 10 and 14 weeks of age, might have resulted in better vaccine efficacy in this endemically infected herd in the face of high levels of maternally derived antibodies.

Implications

- Maternally derived antibodies that persist until pigs are 10 to 14 weeks old may interfere with the antibody response to subunit *A pleuropneumoniae* vaccines.
- Postponement of vaccination to 10 or 14 weeks of age might be advisable in herds with high levels of maternally derived antibodies.

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