RESEARCH REPORT
SWINE HEALTH

Title: Evaluation of live vaccine strains of *Actinobacillus pleuropneumoniae* - **NPB #:04-058**

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II. Abstract:

Actinobacillus pleuropneumoniae(Apl) is the causative agent of porcine pleuropneumonia, a highly contagious, and frequently fatal respiratory tract disease in swine. Current vaccines do not adequately protect against disease. We have characterized 4 mutant strains of *A. pleuropneumoniae* which could be developed into a live attenuated vaccine for prevention of porcine pleuropneumoniae. The mutant strains, in addition to not producing the ApxII hemolysin, contained mutations in the *napA*, *hlyX*, *fur*, or *tatA* genes. These vaccine strains were administered by an intranasal route of administration. Vaccination with any of the mutants reduced clinical symptoms, hemorrhage and fibrinous exudates in or on the lungs, and reduced the numbers of wild-type *A. pleuropneumoniae* recovered from the lungs at necropsy. We were unable to prevent colonization with the wild type serotype 1 strain 4074mm-AP. These results indicate that administration of these live attenuated strains by an intranasal route of administration elicits protective immunity. Further modifications are planned to enhance the efficacy of these attenuated live vaccines.

III. Introduction:

Actinobacillus pleuropneumoniae(Apl) is the causative agent of porcine pleuropneumonia, a highly contagious, and frequently fatal respiratory tract disease in swine (5, 8, 19, 23). *Apl* is a Gram negative bacterium belonging to the family *Pasteurellaceae*. Multiple serotypes have been identified. Both acute and chronic forms of porcine pleuropneumonia exist (8, 23). Acute porcine pleuropneumonia is characterized by fever, cough, extensive hemorrhage, fibrinous exudation, and multi-focal areas of necrosis in the lung and pleural cavity (23). The chronic form of the disease is characterized by well-encapsulated necrotic lesions in the lungs. Carrier pigs harbor *Apl* in their nasal cavities, tonsils, and in infected lung lesions (21, 22, 38). In addition to losses due to mortality, infection with *Apl* results in increased use of medication, reduced feed conversion, lower weaning rates, and reduced market value of animals (8, 37). Thus, there is a significant effect on the swine industry in most pig-rearing countries. Elimination of the chronic form of the disease should have a significant impact on the control of the disease. In an experimental model of *Apl* transmission, Velthius et al. (42) showed that transmission of *Apl* from one pig to another is correlated with the presence of *Apl* in the nasal cavities or tonsils of carrier pigs.

Improved husbandry, including vaccination with either bacterins or subunit vaccines, is currently used to control *Apl* infections in swine (8); however, the disease still occurs with a disturbing frequency. Bacterins, which are protective against the homologous serotype, do not induce immunity against acute disease with heterologous serotypes and do not prevent chronic infections or the subclinical carrier state (7, 10, 12, 16, 18, 24, 25, 26, 27, 28). Live attenuated strains, which either do not secrete the RTX hemolysin/cytotoxins or produce little or no capsule, have been developed; however, only capsule deficient strains protected against challenge with the heterologous strain (2, 11, 13, 14, 29, 35, 36). Several subunit vaccines, containing cell-free extracts, capsular polysaccharide, outer membrane proteins, hemolysins, lipopolysaccharides, and transferrin binding proteins have been developed (1, 3, 4, 6, 9, 10, 13a, 17, 20, 40, 41, 42). These vaccines may reduce clinical disease, but lung lesions do occur (6, 39). For example, vaccination with the 105 kDa serotype 1 hemolysin prevents mortality but not colonization when pigs are challenged with the homologous *Apl* strain (6). These vaccines often do not protect against challenge with herterologous serotypes. However, Prideaux et al. (29), using a live vaccine serotype 7 strain that does not produce ApxII, were able to protect pigs challenged with a serotype 1 strain. This study did not evaluate the ability of the vaccine strain to prevent colonization with the wild type strain. These observations suggest that with increased knowledge about *Apl* infections, it will be possible to produce an effective vaccine that prevents the chronic form of this disease and also provides protection against the mortality associated with acute disease.

IV. Objectives:

We have isolated four transposon-generated mutants (30-34) of *A. pleuropneumoniae (Apl)* UWP36N, a strain that contains a deletion in the ApxII gene (15). These mutants produce no or reduced levels of ApxI, an RTX hemolysin/cytotoxin produced by *Apl* that is responsible for much of the damage to lung tissue and mortality associated with this disease. These mutants contain mutations in the following genes: *napA, hlyX*, *tatA*, and *fur*. Our goal is to determine whether these mutants or UWP36N can be used as a live vaccine strain to prevent colonization of pigs when challenged with fully virulent wild-type *Apl* strains. We hypothesized that intranasal and intramuscular vaccination of young pigs with these live vaccine strains will prevent colonization of the vaccinated pigs with a fully virulent strain belonging to a different serotype.

V. Materials and Methods:

The mutants that were evaluated are described in Table 1. The vaccination-challenge protocol is outlined in Table 2. Pigs were given a minimum of a three-day acclimation period, and the initial infection (vaccination) was performed when the pigs were seven-to eight weeks of age. Each experimental group of five pigs was exposed by intranasal infection to *A. pleuropneumoniae* containing one of the following mutations: *apxIIA, apxIIA napA, apxIIA hlyX, apxIIA fur,* or *apxIIA tatA*. The unvaccinated control group was age-matched to the experimental pigs. For the intranasal infection, 0.7 ml of the organism suspended in sterile PBS solution for the vaccinated and/or challenged pigs or sterile PBS for the unvaccinated pigs was introduced into the pig's nostrils. For the intranasal infection with the mutants, approximately 10^6 CFU was administered to each pig. For challenge with the wild type strain 4074mm-AP, approximately 10^9 CFU was administered to each pig. For the intramuscular injection with the mutants, approximately 10^6 CFU was given per pig. The following parameters were monitored to assess the efficacy of the vaccine strains: clinical signs, gross and microscopic changes in the lungs, and the presence of *A. pleuropneumoniae* in the lungs. Each pig was necropsied to grade lung lesions and collect tissue samples for histopathology and bacterial culture. For isolation of *Apl* from lung lavage fluid, dilutions of the lung lavage fluid were plated on Trypticase soy agar plus yeast extract, NAD, 4ug/ml of vancomycin to inhibit Gram-positive bacteria, and 0.75 ug/ml gentamicin to prevent growth of other Gram-negative bacteria. All potential *Apl* strains were confirmed by Gram-reaction, antibiotic resistance, hemolysis reaction, production of urease, mannitol fermentation, oxidase reaction, and requirement of NAD for growth.

Table 1. Characteristics of the Vaccine Strains.

Strain	Genotype	Hemolysin Phenotype	Comment
UWP36N	)*apxIIA*	$ApxI^{+}$, $ApxII^{-}$	This strain and derivatives of it do not produce the ApxII hemolysin. It does produce the ApxI hemolysin.
UWP36N-#1	)*apxIIA napA*	$ApxI^{-}$, $ApxII^{-}$	*napA* encodes a periplasmic nitrate reductase.
UWP36N-#60	)*apxIIA hlyX*	$ApxI^{variable}$, $ApxII^{-}$	*hlyX* encodes a regulatory protein which controls the transition from aerobic to anaerobic growth. This strain often over produces ApxI.
UWP36N-#206	)*apxIIA tatA*	$ApxI^{-}$, $ApxII^{-}$	*tatA* encodes a protein required for transport of multi-subunit proteins out of the bacterial cell.
UWP36N-#215	)*apxIIAfur*	$ApxI^{-}$, $ApxII^{-}$	*fur* encodes a regulatory protein that in the presence of iron represses expression of many proteins that are produced in response to iron limitation.

Table 2. Vaccination and Challenge Protocol

Day 0	Intranasal infection with a mutant at 10^6 CFU/pig or with PBS
Day 10	Intramuscular "boost" with a mutant at 10^6 CFU/pig or with PBS
Day 30	Intranasal challenge with the serotype 1 strain 4074mm-AP at 10^9 CFU/pig
Day 34	Euthanasia/necropsy

VI. Results:

In preliminary studies, we found that the optimal dosage of the serotype 1 strain 4074mm-AP to reproducibly cause disease when administered intranasally to approximately 11 week old pigs was approximately 10^9 CFU/pig. We evaluated the ability of UWP36N, UWP36N-#1 (*napA*), UWP36N-#60 (*hlyX*), UWP36N-#206 (*tatA*), and UWP36N-#215 (*fur*) to protect against disease when challenged with 10^9 CFU of Apl 4074mm-AP. After challenge with 4074mm-AP, the pigs were monitored for symptoms of porcine pleuropneumoniae. At necropsy, the lungs were evaluated for pathological lesions and samples were obtained for histopathological analysis. We also determined the number of bacteria present in the lung lavage fluid from the challenged pigs at necropsy.

Clinical Scores for each group of 5 pigs are given in Figure 1. All pigs in the vaccinated groups had fewer clinical signs than pigs in the three unvaccinated groups (Clinical Scores = 9.9 ± 5.2, 7.1 ± 0.9, and 6.7 ± 1.7). The clinical signs observed in the three unvaccinated groups (total of 15 pigs) included weight loss, (8 pigs), depression/lethargy (15 pigs), vomiting (5 pigs), labored breathing (14 pigs), discharge from nose and/or mouth (11 pigs, and cough (1 pig). Two of the pigs were euthanized at approximately 30 hours post challenge with 4074mm-AP because of the severity of their symptoms. Pigs vaccinated with mutants UWP36N, UWP36N-#60 and UWP36N-#215 had no or minimal signs of clinical disease (Clinical Scores <1.0). Pigs (total of 10 pigs) vaccinated with mutant UWP36N-#206 had weight loss (4 pigs), depression/lethargy (2 pigs), and/or labored breathing (2 pigs) (Clinical Scores = 1.2 ± 1.3 and 2.4 ± 2.1). The five pigs vaccinated with UWP36N-#1, which is devoid of both ApxI and ApxII hemolysin production, showed signs of depression/lethargy (4 pigs), labored breathing (2 pigs), and cough (1 pig) (Clinical Score = 2.6 ± 3.6) . These observations indicate that vaccination with any of the mutant strains protected against clinical signs when the pigs were challenged with the wild-type strain 4074mm-AP; vaccination with either UWP36N-#60 (*hlyX*) or UWP36N-#215 (*fur*) was the most protective. Both of these mutants produced the ApxI hemolysin.

At necropsy, the lungs of each pig were examined for the presence of fibrinous exudate and areas of hemorrhage. Scores were assigned based on the percentage of the lung affected by fibrinous exudate or hemorrhage. Specifically, a grid was superimposed over digital photographs of the dorsal and ventral sides of the lung, the number of squares covering the lungs and the number of squares with either fibrinous exudate or hemorrhage were counted, and the proportion of the lung that was affected was calculated. The fibrinous exudate score is show in Figure 2 and the hemorrhagic lesion score is shown in Figure 3 for each group of 5 pigs. For both fibrinous exudate and hemorrhage, all pigs in the vaccinated groups, except for the UWP36N-#1 group, had lower scores (<0.05 for fibrinous exudate and 0.03 for hemorrhage) than the pigs in the three unvaccinated groups (0.35, 0.16, and 0.10 for fibrinous exudates and 0.14, 0.06, and 0.08 for hemorrhage). Three of five pigs vaccinated with UWP36N-#1 had significant areas of fibrinous exudate (scores of 0.63, 0.11, and 0.10) and two of five pigs had significant areas of hemorrhage (scores of 0.20 and 0.15). The two pigs in the first PBS (unvaccinated) group that were euthanized within 30 hours of challenge with the wild type strain had significant quantities of blood-tinged fluid (>750 and >500 mls) in the pleural cavities. Figure 4 shows the gross appearance of lungs from PBS (unvaccinated) group 1, UWP36N, UWP36N-#60, and UWP36N-#206. Similar results were obtained for UWP36N-#215 in the second vaccine trial. These observations indicate that vaccination with any of the mutant strains protected against clinical signs when the pigs were challenged with the wild-type strain 4074mm-AP; vaccination with either UWP36N-#60 (*hlyX*) or UWP36N-#206 (*tatA*) was the most protective. Both of these mutants produced the ApxI hemolysin.

Representative samples were taken from a consistent location in each lobe and processed for histopathological analysis. The histological diagnoses were converted to scores based on the severity of fibrin depostion, hemorrhage, pleural inflammatory infiltrates, parenchymal inflammatory infiltrates, necrosis, and edema. The overall histopathology scores are given in Figure 5. All pigs in the vaccinated groups, except for

the UWP36N-#1 group, had lower histopathology scores (<28) than the pigs in the three unvaccinated groups (119, 71.8, and 55.4). Figure 6 shows the microscopic changes observed in the lungs of pigs vaccinated with UWP36N as compared to the lungs of an unvaccinated pig from vaccine challenge experiment 1. In summary, pigs vaccinated with UWP36N, UWP36N-#60, UWP36N-#206, or UWP36N-#215 had minimal histopathological changes in the lungs after challenge with the virulent serotype 1 strain 4074mm-AP.

In our preliminary experiments, the vaccine strain appeared to prevent colonization of the wild type strain. In the current experiments, the vaccine strain was not always recovered from the tonsils at necropsy and did not consistently prevent colonization with the challenge strain (data not shown). However, the wild type strain was recovered at lower numbers in the vaccinated pigs than in the unvaccinated pigs (data not shown). We also determined the numbers of *Apl* present per 100 ml of lung lavage fluid. Figure 7 shows the quantity of bacteria recovered. Vaccination of the pigs resulted in reduced numbers of *Apl* in the lungs of challenged pigs, especially those pigs vaccinated with the mutants UWP36N, UWP36N-#60, and UWP36N-#206 compared to the unvaccinated pigs.

VII. Discussion.

In summary, we are excited that the vaccine strains are protecting pigs from clinical disease when challenged with a wild-type strain. The intranasal route of vaccine administration appears to be an easy to use route of administration for these live vaccine strains. The most protective strains were those that produced ApxI confirming the role of this specific virulence factor in porcine pleuropneumonia, specifically UWP36N, UWP36N-#60, UWP36N-#206, and UWP36N-#215. UWP36N-#1 which did not produce either ApxI or ApxII was the least protective. Additionally, in other experiments, we have found that UWP36N-#1 and UWP36N-#206 do not cause clinical disease when administered at 10^9 CFU/pig. Both of these strains should be further evaluated for use as a live attenuated vaccine strain.

VIII. Lay Interpretation:

Even though improved husbandry has reduced the level of porcine pleuropneumonia caused by *Apl,* this disease is still a significant problem for the swine industry. Current vaccines do not adequately protect against disease. We have characterized 4 mutant strains of *A. pleuropneumoniae* which could be developed into a live attenuated vaccine for prevention of porcine pleuropneumoniae. These strains would be administered by intranasally. The intranasal route of administration of the vaccine is easy, elicits protective immunity, and does not damage muscular tissue. We were unable to prevent colonization with the wild type serotype 1 strain 4074mm-AP. However, vaccination did reduce the number of wild type bacteria in the lungs compared to unvaccinated pigs. Further modifications are planned to enhance the efficacy of these attenuated live vaccines.

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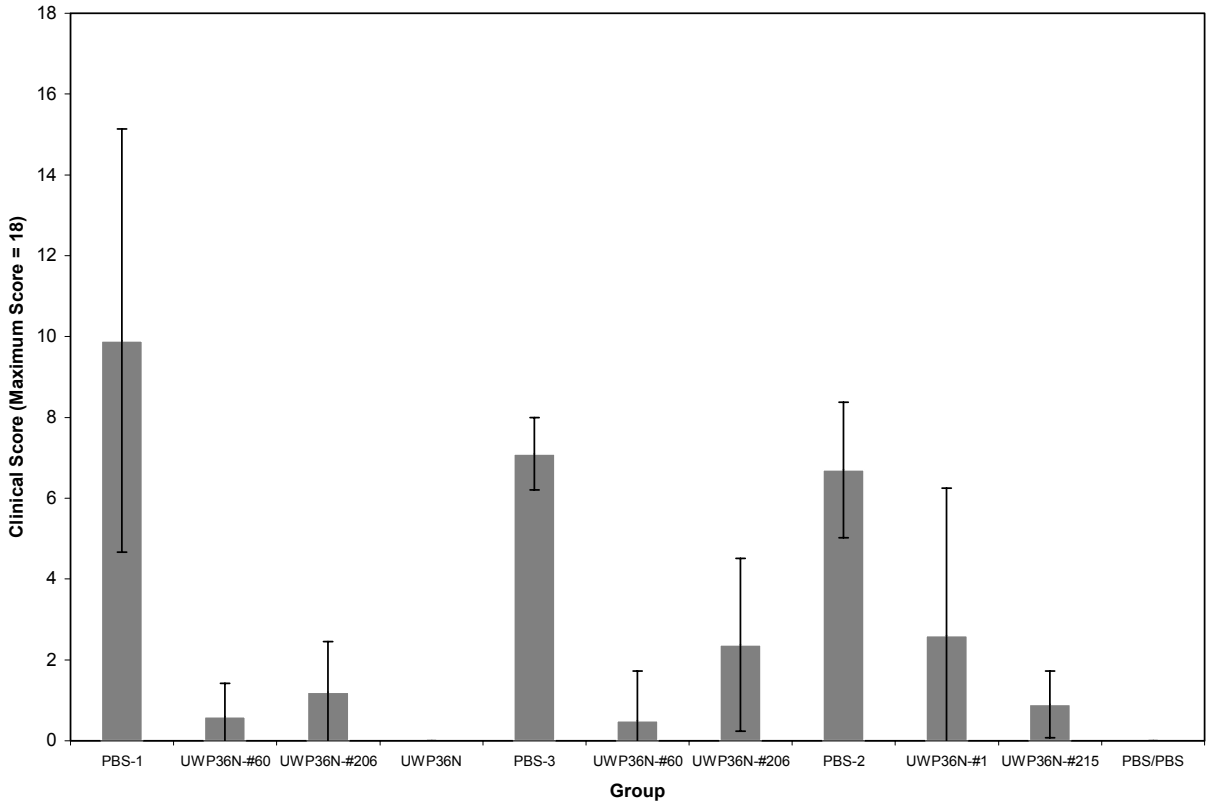

Figure 1.

Clinical Scores. Each pig was given a clinical score each day after challenge with 4074mm-ap. Gain of less than 0.5 kg/day was scored as 0.5, loss of weight was scored as 1, vomiting as 0.5, depression or lethargy as 1, labored breathing or dyspnea as 1, cough as 1, and presence of discharge from the nose or mouth as 1. If a pig was euthanized for humane reasons, it was given a score of 6 for the day of euthanasia and all subsequent days. Scores from days 1-3 were added to obtain a final score. The maximum score that could be obtained is 18. Scores are the average score for each group of pigs.

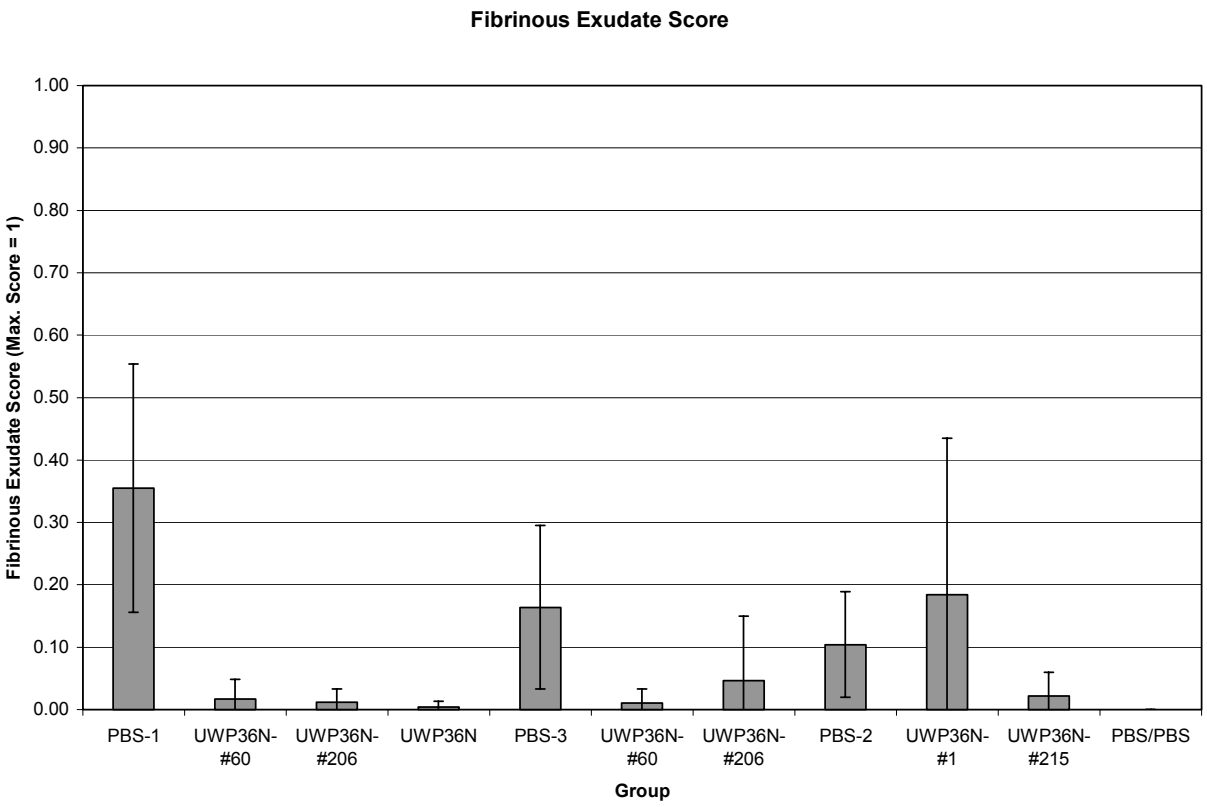

Figure 2. Fibrinous exudate scores. Scores are the average ratio of squares with fibrinous exudate to total number of squares for each lung for each group of pigs.

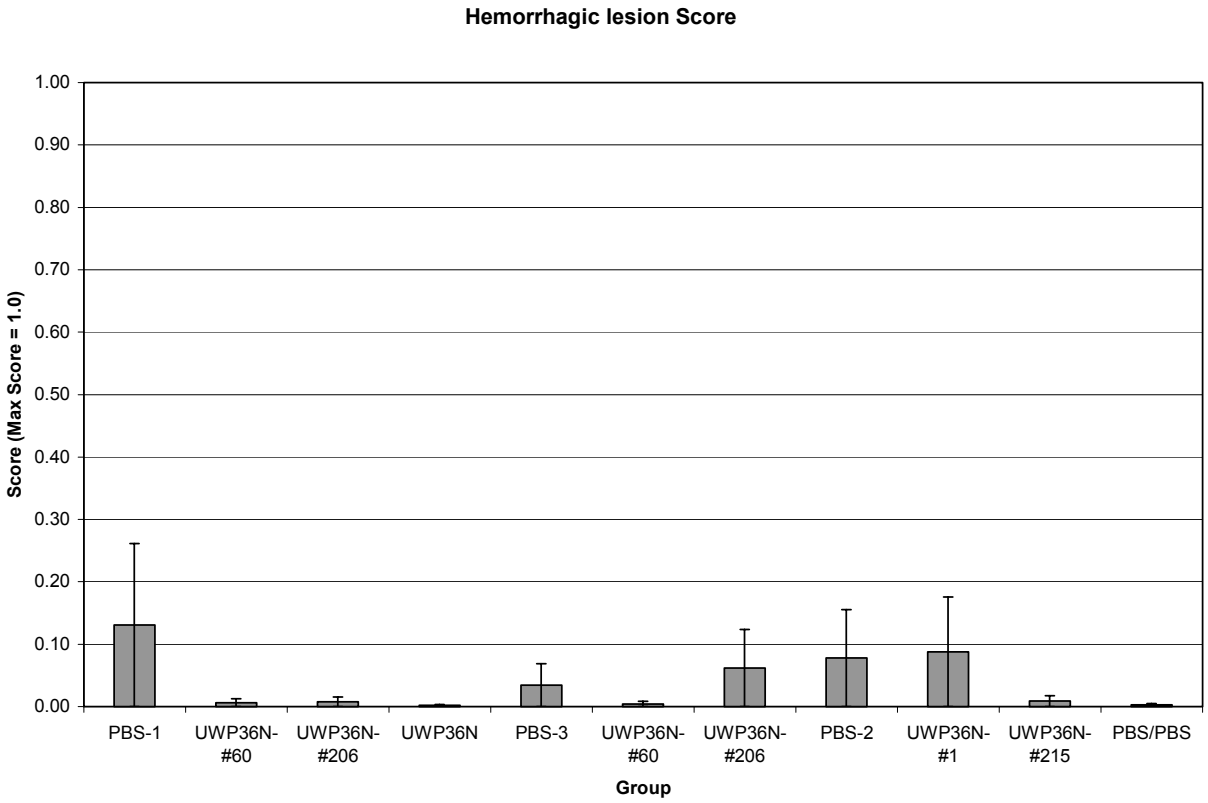

Figure 3. Hemorrhagic lesion exudate scores. Scores are the average ratio of squares with hemmorhagic lesions to total number of squares for each lung for each group of pigs.

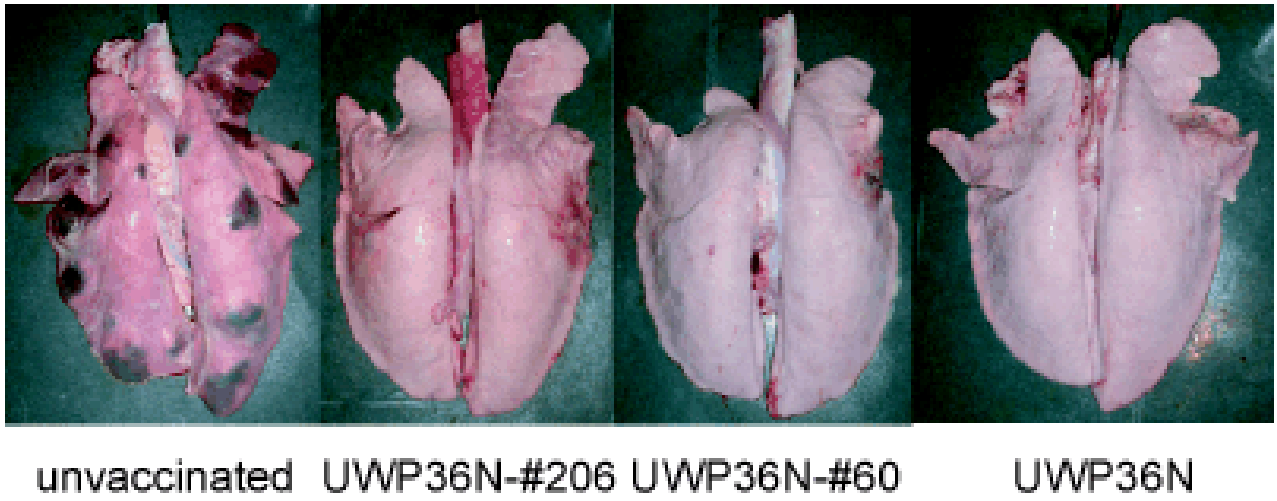

Figure 4. Gross appearance of representative lungs of pigs in vaccine challenge experiment 1.

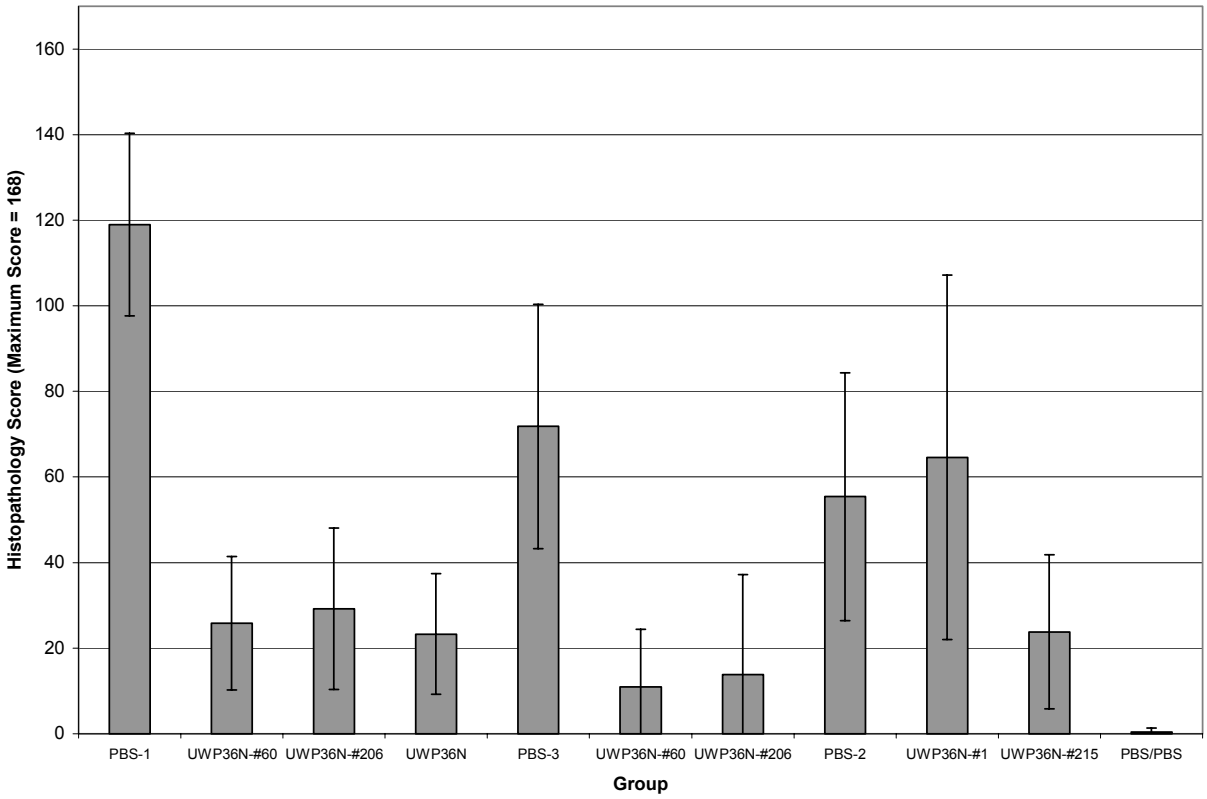

Figure 5. Histopathology scores. Representative sections were taken from each lobe of the lung and prepared for histopathological analysis. The histological diagnoses were converted to scores based on the severity of fibrin depostion, hemorrhage, pleural inflammatory infiltrates, parenchymal inflammatory infiltrates, necrosis, and edema. The severity of each histopathological observation was graded on a scale of 0 to 4, with 0 = within normal limits, 1 = minimal, 2 = mild, 3 = moderate, and 4 = severe. When all observations for each lung lobe were added together, a maximum score of 168 could be obtained.

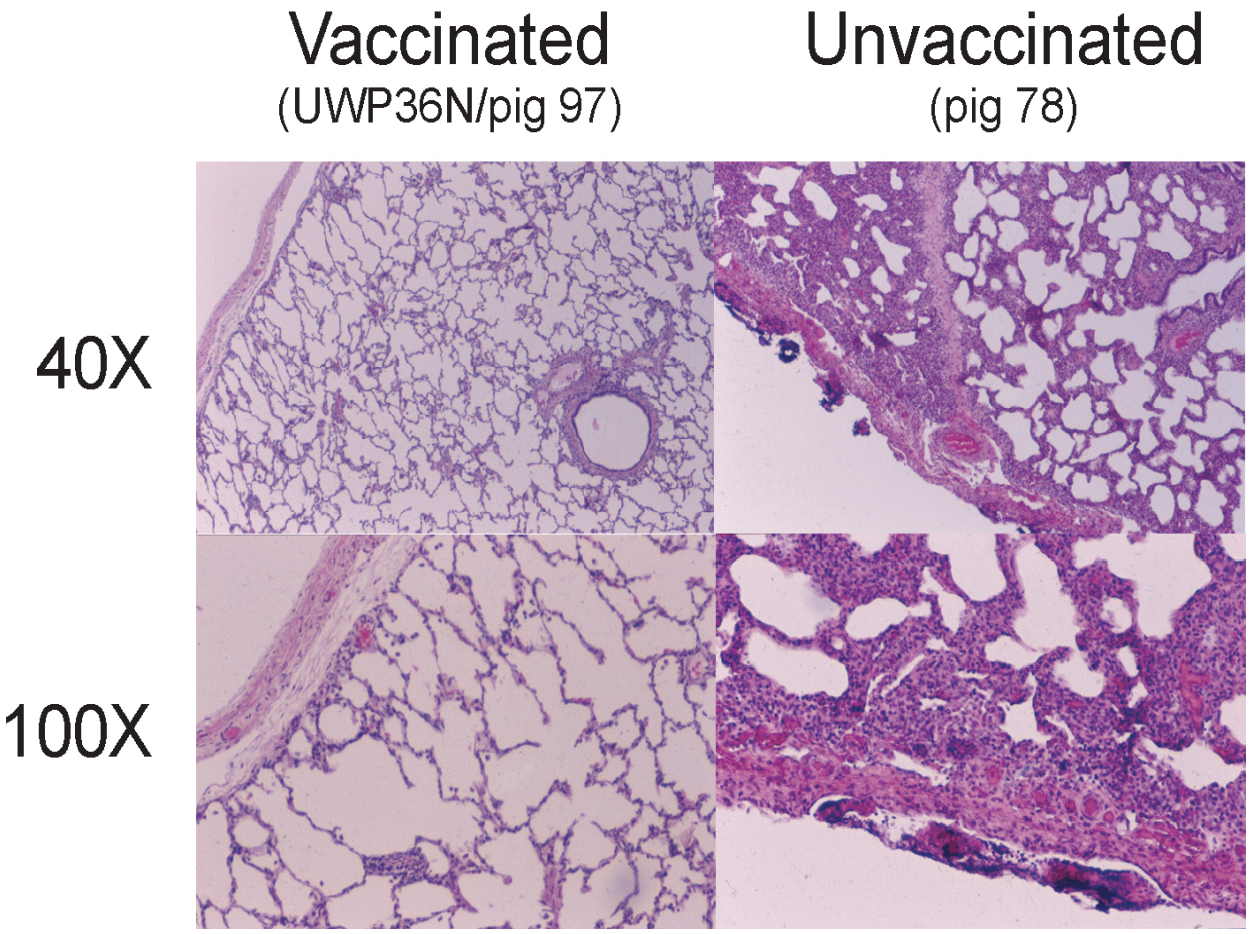

Figure 6. Microscopic appearance of lungs from a pig vaccinated with UWP36N and with unvaccinated pig 78 at 40X and 100X magnification. The histopathological changes observed in the left cranial cranial lobe of pig 78 were moderate fibrino-suppurative necrotizing pleuritis, interlobular dilated empty lymphatics, mild fibrino-suppurative bronchitiolitis, and a moderate peribronchiolar mononuclear with occasional PMNs interstitial infiltrate usually with intraalveolar macrophages. The histopathological changes observed in the right caudal lobe of pig 97 are dilated empty pleural & interlobular lymphatics and a mild mononuclear interstitial infiltrate.

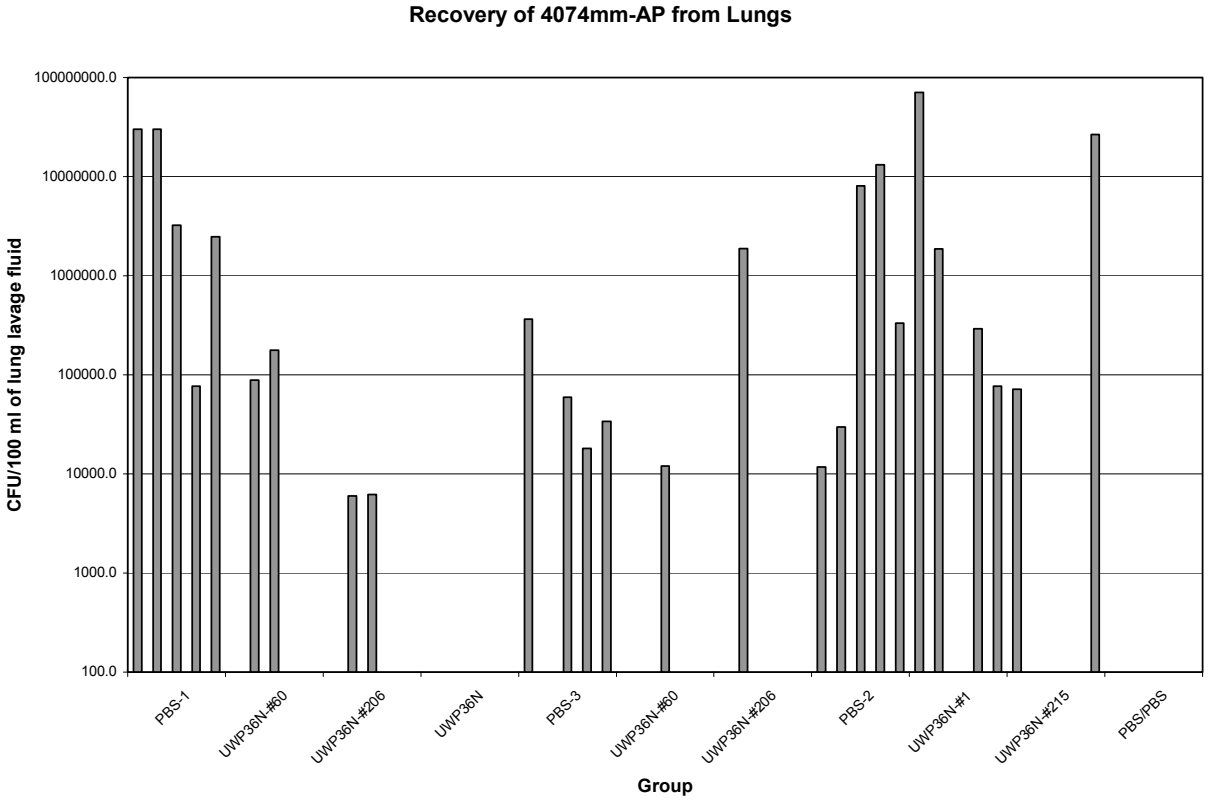

Figure 7. Quantitative recovery of A. pleuropneumoniae 4074mm-AP from the lungs of vaccinated and challenged pigs. The lungs were lavaged with sterile PBS, the lavage fluid was quantitatively plated on selective media for *A. pleuropneumonia*e as described in the Materials and Methods.