RESEARCHREPORT
SWINE HEALTH

Title: Evaluation of a new vaccine approach against mycoplasma pneumonia.
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I. Abstract:

Mycoplasma hyopneumoniae is a common pathogen associated with the porcine respiratory disease complex (PRDC). The ISU Veterinary Diagnostic Laboratory has seen a three-fold increase in cases with mycoplasmal pneumonia, often from vaccinated herds. Vaccination and management strategies are currently the best methods to control mycoplasmal pneumonia in swine herds, but these strategies often fail. Enhancement of antigen-specific immune responses will lead to improved vaccines. The objective of this study is to investigate the potential of a new immune enhancing protein (ESAT-6) to enhance antigen-specific immune responses and provide protection against *M. hyopneumoniae* experimental challenge following vaccination. This protein will be incorporated into a DNA vaccine format with three different *M. hyopneumoniae* antigens. We used 66 pigs divided into 11 groups. There were 7 DNA vaccines tested (1 group per vaccine) plus 2 groups of vaccine combinations and 2 control groups. Pigs were inoculated with DNA vaccines using a new Bioject 2000 needleless injection device, boosted three weeks later and challenged three weeks following the booster. Twenty-eight days following challenge with *M. hyopneumoniae*, pigs were necropsied and percent lung lesions assessed. Additionally, serum antibody responses to each antigen were followed throughout the study. Our results indicated that DNA vaccines have potential for protecting against mycoplasma pneumonia in pigs. In addition, we showed that ESAT-6 fusions with individual antigens enhanced the efficacy of the vaccine. Further studies identifying additional antigens will be necessary before a fully effective vaccine can be developed.

II. Introduction:

M. hyopneumoniae infection of swine has been universally established as among the most important diseases in the swine industry. Economic losses attributed to mycoplasma pneumonia in the U.S. swine industry easily exceeds $2 per pig or approximately $200 million annually. Loss occurs as a consequence of reduced average daily gain and efficiency of feed utilization, prophylactic and therapeutic

interventions, and mortality. Prevalence of lesions of enzootic pneumonia in swine at slaughter has been very high (>70%) for many years and does not seem to have abated over the past 40 years. During recent years, swine practitioners and diagnosticians have noted an increased occurrence of severe pneumonia, referred to as Porcine Respiratory Disease Complex (PRDC). *M. hyopneumoniae* is an integral component of PRDC in association with other swine pathogens such as PRRSV and swine influenza virus (SIV). In combination with other respiratory pathogens, i.e., PRRSV, however, pneumonia due to the second pathogen is significantly enhanced. Vaccination against *M. hyopneumoniae* does not prevent colonization of the swine respiratory tract or protect sufficiently against disease, *nor does it obviate the potentiating role of M. hyopneumoniae in dual infection with other infectious pathogens*. In the absence of effective intervention strategies to reduce disease, improved vaccines are essential if we are to reduce economic losses due to mycoplasma pneumonia.

For many years, subunit vaccines have gained popularity because they eliminate many of the concerns associated with the use of modified live vaccines in the field. They are more stable and tend to cause fewer adverse reactions. Subunit vaccines have not been universally accepted, however, because of the difficulty in provoking the appropriate immune responses to elicit protection. For effective immunity against pathogens, both a humoral antibody (type 2) and a cell-mediated immune (type 1) response is required. Until recent advances in vaccine development, however, there has been no practical way to enhance type 1 responses. Thus, the prominent response in most vaccines is a type 2 humoral response, which may explain their failure to provide adequate protection. Dr. Minion's group has developed a novel approach to subunit vaccine development that enhances antigen-specific type 1 immune responses. This approach has been tested with purified proteins and in a DNA vaccine format in a mouse model. Although this approach has not been tested in swine, there is compelling evidence that it could revolutionize vaccine development for the swine industry.

III. Objectives:

The **long range goal** for this laboratory is to develop fully effective vaccines for mycoplasmal diseases in pigs. With this goal in mind, we have identified and characterized three different *M. hyopneumoniae* proteins (P97, P102, and P71) that produce type 2 immune responses in pigs. In addition, there is evidence that purified P97 will provide partial protection against mycoplasma pneumonia when used as a conventional vaccine inducing type 2 immunity. To accomplish the goal of enhanced type 1 immunity against these proteins, we tested the efficacy of ESAT-6 in DNA vaccines based on P97, P71 and P102 antigens in pigs. We expected that enhanced type 1 immunity against the P97, P102 and P71 antigens of *M. hyopneumoniae* would generate a more protective vaccine against *M. hyopneumoniae* challenge.

IV. Procedures:

All plasmids needed for this study were constructed and are described in Table 1. DNA was prepared for the vaccination experiments using the endotoxin-free giga-prep kits from Qiagen, Inc. This technique resulted in endotoxin-free DNA preparations of high purity suitable for vaccination.

Vaccination and challenge: Sixty-six*M. hyopneumoniae*-free, male, castrated pigs were obtained at 8-12 days of age and randomly assigned to 9 groups of 7 pigs with stratification by weight. Pigs were acclimated for 1 week prior to vaccination. Vaccine groups consisted of 7 groups receiving individual plasmid vaccines, one non-vaccinated-challenged control group, and one non-vaccinated, non-challenged control group (Table 1). Each group received 750 µg of plasmid DNA injected intramuscularly. Three weeks later, an identical booster dose was given. *M. hyopneumoniae* (strain 11)

challenge inoculum was administered intratracheally at a dilution of 1:100 to pigs three weeks post booster vaccination.

Table 1. VR1020 Plasmid and Experimental Group Descriptions

Plasmid No.	Group No.	Gene(s) Cloned	Vaccine
417	1	ESAT-6	Plasmid 417
419	2	P97	Plasmid 419
420	3	ESAT-6, P97	Plasmid 420
423	4	P102	Plasmid 423
424	5	ESAT-6, P102	Plasmid 424
421	6	P71	Plasmid 421
422	7	ESAT-6, P71	Plasmid 422
	10		Non-vaccinated, challenged
	11		Non-vaccinated, non-challenged

Clinical evaluation: Pigs were evaluated daily for a period of 15 minutes for clinical signs including cough or behavioral changes. Rectal temperatures were measured daily for 3 days following each vaccination. All pigs were weighed periodically and at necropsy to evaluate growth. Mycoplasma serum antibody levels were measured weekly and at necropsy.

Necropsy: Pigs were euthanized by pentobarbital overdose followed by exsanguination 28 days following challenge. Lung lavage was taken and mycoplasma-specific secretory antibody assessed by ELISA. Lung lesions were sketched on a standard diagram and assessed for the proportion of lung surface exhibiting lesions. Tissues were collected and processed for histopathological and immunohistochemical examination.

Statistics: Analysis of variance was used to detect significant differences among treatment groups. Pairwise comparisons was performed using Tukey's test. A non-parametric ANOVA (Kruskal-Wallis test) was used for non-normally distributed data ore when group variances are dissimilar; pairwise comparisons was done using the Wilcoxon sum test.

V. Results:

We completed the construction of all of the plasmids needed for these studies including site-directed mutagenesis of the P102 gene sequence. These plasmids used the VR1020 plasmid backbone and are described in Table 1 below. For plasmids 420, 424, 422, the genes were cloned as fusions with ESAT-6 sequences at the N-terminus. The construction for each plasmid was confirmed by DNA sequencing.

Table 1. VR1020 Plasmid and Experimental Group Descriptions

Plasmid No.	Group No.	Gene(s) Cloned

419	2	P97
420	3	ESAT-6, P97
423	4	P102
424	5	ESAT-6, P102
421	6	P71
422	7	ESAT-6, P71

Each of the plasmids was prepared using the Qiagen Giga-prep kit. The endotoxin levels in each DNA preparation was uniformly less than 1 µg per mg DNA. At this level, the pig would be unresponsive to the level of endotoxin in the vaccine preparation.

Sixty two mycoplasma and PRRSV-free pigs were purchased and randomly assigned into 9 groups, 7 pigs per group. The assignment was based upon weight in order to distribute the weight evenly across the groups. The groups were as follows: group 1, non-vaccinated, challenged control group, saline only; group 2, P71 vaccinates (plasmid 421); group 3, EsP71 fusion vaccinates (plasmid 422); group 4, P97 vaccinates (plasmid 419); group 5, EsP97 vaccinates (plasmid 420); group 6, P102 vaccinates (plasmid 423); group 7, EsP102 vaccinates (plasmid 424); group 8, P71+P97+P102 vaccinates (plasmids 419, 421, 423); group 9, EsP71+EsP97+EsP102 vaccinates (plasmids 420, 422, 424).

Pigs were vaccinated with 750 µg plasmid DNA IM using the Bioject needle-less delivery system. Vaccinations occurred on day 0 and day 21. Throughout the experiment, pigs were monitored each day for clinical signs including cough and behavioral changes. All pigs were weighed periodically and rectal temperatures obtained. On day 42, the pigs were challenged with *M. hyopneumoniae* per our standard challenge model. Twenty-eight days later, the pigs were sacrificed and lungs scored for gross lesions. Serum samples were taken to measure anti-mycoplasma antibody levels.

Figure 2 shows the results of the vaccine trial. Lung lesion scores were reduced in animals vaccinated with P97, EsP97 and EsP102 and with the combined plasmids. The greatest reduction occurred in animals receiving the EsP97 vaccine. Due to large variances in the animal population, it was not possible to establish statistical significance with the low numbers of pigs per group. These results do support our hypothesis that ESAT-6 fusions with mycoplasma proteins enhances the vaccine efficacy. In these studies, we tested DNA vaccines. In other studies, we have shown enhanced immune responses with purified proteins as well. Further studies should be undertaken with ESAT-6 to test other potential antigens as components of a multi-subunit vaccine.

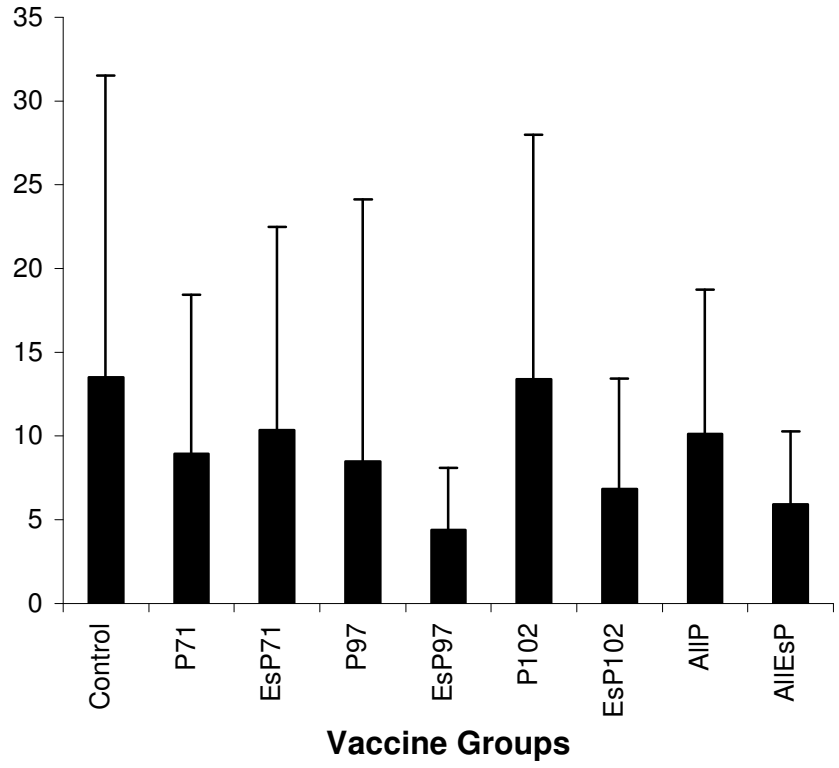

Figure 2. Mean percent lung lesions in DNA vaccinated pigs. Pigs (8 per group) were immunized with 750 µg purified plasmid DNA at day 0 and day 21. The plasmids were constructs based on VR1020 with or without Es sequences. On day 42, pigs were challenged with virulent *Mhyo* intratracheally. On day 77, pigs were sacrificed and lung lesion scores determined. Control, saline; AllP, all three constructs, P71, P97 and P102; AllEsP, all three constructs, EsP71, EsP97, and EsP102. Data is given as mean + standard deviation.