Title: Vaccination of pigs with alphavirus replicon particles expressing PRRSV ORF 3, 4, 5 and 6 - **NPB** #08-207

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Industry Summary:
The objective of this project was to evaluate alphavirus replicon particles (RP) expressing various PRRSV proteins as vaccine candidates. Previous work has identified the minor structural glycoproteins (GP2, GP3, and GP4) and the major structural components (GP5 and M) as vaccine targets for use in recombinant vaccine platforms. This project focused on GP3, GP4, GP5, and M as experimental vaccine antigens.

The genes encoding the GP3, GP4, GP5, and M proteins were sequenced and cloned into the replicon system. This system was used to generate RPs expressing the genes of interest. The RPs are not able to replicate in the host, and can only deliver the genetic material necessary to express the vaccine antigen. This feature adds to the safety of the vaccine, because it cannot recombine with wild-type PRRSV to create new strains. Additionally, the antigens contained in this vaccine allow vaccinated animals to be differentiated from naturally infected animals by commercially available serological tests.

Forty pigs (3 weeks of age) were randomized into four groups. Each group of pigs received a combination of RPs expressing either: 1)GP3 and GP4; 2)GP5 and M; 3)GP3, GP4, GP5, and M; 4)Placebo. Vaccination occurred on Day 0 and Day 21 of the study. Pigs were challenged intranasally with a virulent strain of PRRSV on Day 42, and the study was terminated on Day 56. Blood samples were collected throughout the study to measure viremia and immune response. Vaccine 3 (GP3, GP4, GP5, M) induced neutralizing antibodies prior to challenge, and all three vaccinated groups had significantly stronger neutralizing responses post-challenge, when compared to placebo. Vaccine 2 (GP5 and M) and Vaccine 3 induced significantly stronger cell-mediated immune responses prior to challenge when compared to placebo. The three RP vaccine candidates all significantly reduced viremia on Day 51, and Vaccine 3 also reduced viremia on Day 48, when compared to placebo.

These results indicate that the RP vaccine platform is capable of inducing PRRSV-specific cell-mediated immunity and neutralizing antibodies prior to challenge. Vaccination with the three vaccine candidates tested here also reduced viremia post-challenge. Animals vaccinated in this manner remain serologically negative until after a PRRSV infection, providing an improved method for disease prevention and surveillance. These vaccine candidates are currently being evaluated in a reproductive disease model.

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Scientific Abstract:
Recent work has illuminated the role for the structural glycoproteins of PRRSV during host cell infection, suggesting novel vaccine targets for evaluation. This project utilized alphavirus-derived replicon particle (RP) vaccines to evaluate several PRRSV structural proteins as vaccine antigens. The following PRRSV structural proteins were expressed in swine using the RP vector: GP3, GP4, GP5, and M. Both the humoral and cell-mediated immune responses were measured throughout the study. Following challenge with a virulent strain of PRRSV, viremia and antibody levels were assayed by various methods.
The results indicate that RP vaccines induce specific humoral and cell-mediated responses prior to challenge. Pigs that received RPs expressing GP3, GP4, GP5, and M developed neutralizing antibody titers prior to challenge, and only RP-vaccinated pigs developed neutralizing antibodies post-challenge. Vaccinated groups that received RPs expressing either: 1) GP5 and M; or 2) GP3, GP4, GP5, and M; had significantly higher level of pre-challenge cell-mediated immune response, as measured by an interferon-γ ELISPOT assay. The vaccinated groups all had significantly reduced viremia at nine days post-challenge. The group that received RPs expressing GP3, GP4, GP5, and M also had reduced viremia at six days post-challenge.

Introduction:
The swine industry continues to struggle with PRRS, despite existing control measures and commercially available vaccines. The need for a new generation of vaccines has driven research into methods of immunizing pigs with vector-based vaccines that express PRRSV genes. Much of this work has failed to provide clear evidence of protective immunity, although partial protection from challenge has been achieved on a limited basis. Prior work by our group has demonstrated partial protection using alphavirus-derived RP expressing GP5 and M, but this approach failed to induce neutralizing antibodies. Recent work has identified the minor glycoproteins (GP2a, GP3, and GP4) of PRRSV as potential vaccine targets, due to their interactions with the host cell and immune system. This project evaluated the effect of including replicon particles (RP) expressing GP3, GP4, GP5, and M in a vaccine.

Objectives:
Evaluate the ability of RP vector vaccines expressing three of the important glycoprotein and matrix structural components of the PRRSV to induce protective immune responses and protect pigs from virulent PRRSV challenge in a vaccination-challenge model.

Materials & Methods:
Production of Vaccine Candidates
The PRRSV strain HLV349 was analyzed to determine the sequences of ORF3, ORF4, and ORF6. The PRRSV strains HLV013 and HLV093 were analyzed to determine the sequence of ORF5. These sequences were used to construct synthetic genes that were compatible with the RP vaccine platform. These genes were synthesized commercially. Completed genes were cloned into the RP platform plasmid and used to express protein. Each protein was confirmed to have proper size and antibody reactivity by Western blot analysis.
Plasmid constructs were used to transcribe RNA in vitro. The resulting RNA was purified and electroporated into Vero cells with helper RNAs. Cells were incubated for 18 hours before the RPs were harvested via affinity chromatography. The resulting RPs were titrated and safety-tested by three blind passages on Vero cells.
Vaccine Formulation
Vaccines for each immunization were formulated according to the table below. The number represents the titer of RP expressing the indicated protein included in each dose. Vaccine D is the Placebo formulation, and contained no RP components.

	HLV349 GP3	HLV349 GP4	HLV013 GP5	HLV093 GP5	HLV349 M
Vaccine A	$1x10^8$	$1x10^8$	--	--	--
Vaccine B			$5x10^8$	$5x10^8$	$1x10^9$
Vaccine C	$1x10^8$	$1x10^8$	$5x10^8$	$5x10^8$	$1x10^9$
Vaccine D	--	--	--	--	--

Table. Vaccine formulation titers of each RP component.

Pig Study
Forty pigs were acquired from a source herd free of PRRSV and swine influenza virus at three weeks of age. Pigs were randomized into four treatment groups and allowed to acclimate for one week prior to immunization. Blood samples were collected at intervals throughout the study. Vaccination (2 mL, IM) occurred on Days 0 and 21. Pigs were challenged intranasally on Day 42 with $2x10^8$ $TCID_{50}$ PRRSV strain HLV349. Pigs were euthanized and necropsied on Day 56.

	Treatment	**PRRSV components in RP vaccine**
Group A	Vaccine A	GP3, GP4
Group B	Vaccine B	GP5, M
Group C	Vaccine C	GP3,GP4, GP5, M
Group D	Vaccine D	none

Table. Pig study groups and their respective treatments.

Sample Analysis
Serum samples collected during the study were assayed for infectious virus and PRRSV-neutralizing antibodies using MARC-145 cells. Serum was also assayed for viral RNA using the Applied Biosystems AgPath NA/EU qPCR kit. Whole blood collected on the day of challenge was used to measure cell-mediated immune response via an interferon-γ ELISPOT. Lungs were examined at necropsy by a blinded pathologist for gross lesions. Serum samples were submitted to the Iowa State University Diagnostic Lab for IDEXX PRRSV ELISA. Serum samples were tested for PRRSV-specific antibody responses by Western immunoblotting. Statistical analysis was done using one-way analysis of variance (ANOVA) with $\alpha=0.05$.

Results:
Clinical signs
At necropsy there was not a significant reduction in gross lung lesions of vaccinates versus non-vaccinates. Lung lesions were generally mild in all groups.
Humoral immune response
All animals were negative for anti-PRRSV antibodies on the day of challenge, as measured by IDEXX ELISA. All animals seroconverted by this assay post-challenge, indicating successful challenge. All vaccinates that received RPs expressing GP5 or M seroconverted to these antigens prior to challenge, as determined by Western immunoblotting. Animals that did not receive RPs expressing GP5 or M remained seronegative for these antigens prior to challenge, as determined by Western immunoblotting. Four of the ten animals that received RPs expressing GP3, GP4, GP5 and M had detectable neutralizing antibody titers on the day of challenge.

	0 days post-challenge	**6 days post-challenge**	**14 days post-challenge**
Group 1 (GP3, GP4)	0	1	5
Group 2 (GP5, M)	0	3	6
Group 3 (GP3, GP4, GP5, M)	4	7	8
Group 4 (Placebo)	0	0	0

Table. Virus-neutralization assay results. Numbers represent the total animals in each group with a VN titer ≥1:8.

Cell-mediated immune response

Vaccinated animals demonstrated significantly higher ELISPOT results when PRRSV was used as the recall antigen, indicating specific induction of cell-mediated immunity.

	mean IFN-γ ELISPOT SFU/1e6 PBMC
Group 1	49.0
Group 2	81.5*
Group 3	66.3*
Group 4	14.5

Table. Results of interferon-γ ELISPOT from the day of challenge. Results are spot-forming units per $1x10^6$ PBMC after stimulation with PRRSV recall antigen. Asterisks denote significant differences from Group 4 (ANOVA, $p\leq0.05$).

Viremia

Live virus titration showed that all vaccinated groups had significantly reduced viremia compared to placebo by 9 days post-challenge. The group that received RPs expressing GP3, GP4, GP5 and M had significantly reduced viremia compared to placebo at 6 days post-challenge. By 14 days post-challenge all groups were not significantly different. The results of qPCR analysis showed a trend towards lower viremia in vaccinated groups, but the differences were not significant.

	6 days post-challenge	9 days post-challenge	14 days post-challenge
Group 1	3.30	1.91*	0.44
Group 2	2.87	1.60*	0.22
Group 3	2.60*	1.69*	0.00
Group 4	3.59	3.45	0.54

Table. Live virus titration results. Results are group mean log-transformed titers. Asterisks denote significant differences from Group 4 (ANOVA, $p\leq0.05$).

Discussion:

These results indicate that an RP vaccine expressing a combination of the GP3, GP4, GP5, and M of PRRSV can induce virus-neutralizing antibodies and a specific cell-mediated immune response. Animals that received this vaccine formulation had reduced viremia at six and nine days post-challenge compared to placebo. Additionally, groups that received either GP5 and M, or GP3 and GP4 RPs had reduced viremia at nine days post-challenge compared to placebo. However, neither of these groups had detectable neutralizing antibody titers prior to challenge. The GP5 and M RP combination was the only other vaccine formulation that induced a significant cell-mediated response compared to placebo. This suggests that the cell-mediated response in this trial was largely due to GP5 and M antigens. The induction of pre-challenge PRRSV-neutralizing antibodies in pigs receiving GP3, GP4, GP5, and M RPs, but not in the other vaccine groups, suggests that there may be a multi-antigen complex involved in mediating this response. It has been shown that the GP5-M heterodimer interacts with the GP2-GP3-GP4 heterotrimer in virus-infected cells, suggesting a mechanism to explain these results. The reduction in viremia in groups that had no detectable neutralizing antibodies could be explained in part by demonstrated cell-mediated immunity and non-neutralizing antibodies aiding in more rapid viral clearance. Our group is optimizing a virus-neutralization assay that utilizes porcine alveolar macrophages, which could potentially show neutralization in serum samples that appear negative on MARC-145 cells. This is due to receptor differences between the two cell lines that alter their susceptibility to PRRSV infection.

The induction of PRRSV-neutralizing antibodies and cell-mediated immunity using a vectored vaccine shown here is among a very limited number of such reports in the literature. As a result, the combination of GP3, GP4, GP5 and M RPs is currently being tested in a pregnant gilt challenge model. The results of this study will inform future study designs seeking to enhance the protection observed in this project. Ultimately, this work is part of progress towards an efficacious vaccine that retains DIVA capabilities, provides protection in both young pigs and pregnant animals, and does not pose a risk of reversion to virulence. This would provide benefits to both consumers and producers in the form of reduced mortalities, greater efficiencies of production, and higher profitability in the swine industry.