RESEARCH REPORT
SWINE HEALTH

Title: Development of PRRS virus-like-particles containing nanoparticle vaccine and its evaluation in pigs - **NPB # 12-166**

Investigator: Renukaradhya J. Gourapura,

Co-investigators: Ying Fang and Daral Jackwood

Institution: Food Animal Health and Research Program (FAHRP), Ohio Agricultural Research and Development Center (OARDC), The Ohio State University, and
South Dakota State University, Brookings, SD 57007

Date Submitted: 12/01/2014

Industry Summary:

Porcine Reproductive and Respiratory Syndrome (PRRS) is a chronic and economically important viral disease of pigs. Currently used PRRS virus (PRRSV) vaccines have failed to completely protect against reinfections. In addition, reports of live virus vaccine acting as a source of revertant virus resulting in catastrophic consequences is a concern. Although available killed PRRSV vaccines are safe, but their efficacy is poor. Thus, there is a pressing need of developing a safe and protective killed PRRSV vaccine to protect pigs from PRRS outbreaks. Virus-like-particles (VLPs) constitutes a specific class of subunit vaccine that mimic the structure of authentic virus particles. The VLPs function as effective and safe antigens in the absence of viral genome and potentially toxic (immunosuppressive) viral gene products. Nanotechnology based vaccine delivery is one of the important research endeavors of the 21st century. Nanoparticles made of biodegradable and biocompatible polymers [e.g. PLGA [poly(lactide co-glycolide)] are approved by FDA to use in vaccine delivery systems. *Our hypothesis is that delivery of PLGA nanoparticle-entrapped PRRSV-VLPs vaccine elicits cross-protective immune response in pigs, with increased clearance of challenged virulent heterologous PRRSV*. Our three Objectives are: *(i)* To prepare PRRSV-VLPs to one each of North American and European PRRSV strains; (ii) To develop and characterize candidate PLGA nanoparticle-entrapped PRRS-VLPs vaccine; and (iii) To evaluate cross-protective efficacy of Nano-PRRSV-VLPs vaccine in pigs. Until now only two reports on PRRS-VLPs are available which made use of only two PRRSV membrane proteins, and their efficacy as a candidate vaccine was not evaluated in pigs. Since putative PRRSV neutralizing and T-cell epitopes and are not limited to two proteins, we believe that PRRS-VLPs comprising of 5-6 viral membrane proteins serve as a potent candidate vaccine. In our study, we cloned the six PRRSV membrane protein genes in to a baculovirus vector system and generated individual recombinant baculoviruses (rBVs). To generate PRRS-VLPs, we coinfected insect cells with different combinations of rBVs and observed VLPs of approximately 50nm size only when GP5-M and E protein are in the formulation. In a pilot pig vaccine trial, PRRS-VLPs entrapped in PLGA nanoparticles and coadministered intranasally twice with a potent mucosal adjuvant, *Mycobacterium tuberculosis* whole cell lysate, and challenged with a virulent heterologous PRRSV strain 1-4-4 was evaluated. Analysis of viremia

suggested the reduced challenged viral RNA load and infectious virus in pigs received PRRS-VLPs irrespective of entrapment in NPs, while in the lungs without NPs encasing PRRS-VLPs helped to significantly reduce the viral load. In conclusion, we generated PRRS-VLPs containing all the six PRRSV membrane glycoproteins, and that could be a potential candidate vaccine when delivered with a potent adjuvant. Further, studies are required to confirm the dose-dependent vaccine efficacy and the degree of cross-protection against other heterologous PRRSV strains.

Contact: Renukaradhya J. Gourapura (Aradhya); Associate Professor, Food Animal Health Research Program, Ohio Agricultrual Research and Deverlopment Center, The Ohio State University, Phone: (330) 263-3748; Email: gourapura.1@osu.edu

Keywords: Porcine reproductive and respiratory syndrome virus (PRRSV); virus-like particles (VLPs); vaccines; immune response

Scientific Abstract:
Porcine Reproductive and Respiratory syndrome (PRRS) is an economically devastating problem plaguing the global swine industry. Since early 1990s both live attenuated and inactivated vaccines are in use, but still control and transmission of PRRS is a major problem. Most of the inactivated virus and subunit vaccines are poorly immunogenic due to their soluble nature and rapid degradation *in vivo*. Alternatively, virus like particles (VLPs) generated using viral surface proteins mimics the morphology of the native virus, and it is non-infectious as it lacks the viral genetic material. Until now only two reports on PRRS-VLPs are available and they are GP5 and M containing VLPs and influenza nucleocapsid protein and PRRSV GP5 chimeric (NA/GP5) VLPs, but their immunogenicity was not evaluated in pigs. Since putative PRRSV neutralizing epitopes are not limited to GP5 and M, we hypothesized that PRRS-VLPs comprising of all (5 or 6) viral membrane proteins (GP2a, E, GP3, GP4, GP5 and M) serve as a potent candidate vaccine. We cloned full-length genes of the six surface proteins of a type 2 PRRSV (strain SD09-28) in to a baculovirus transfer vector, transformed in to competent *E. coli,* and transfected into Sf9 cells to generate recombinant baculoviruses (rBVs). High titered rBVs stocks were used to co-infect Sf9 cells in different combinations to generate PRRS-VLPs. Our in vitro results revealed that VLPs were formed from co-infection of GP5-M, GP2a-GP3-GP4-GP5-M, GP2a-E-GP3-GP4, GP2a-GP3-GP4, as measured by transmission electron microscopy the size of the particles was in the range of 30-80nm. Nanoparticles made of biodegradable and biocompatible polymers [e.g. PLGA [poly(lactide co-glycolide)] are approved by FDA to use in vaccine delivery systems. In a pilot vaccine trial in nursery pigs, BEI inactivated PRRS-VLPs containing GP5-M and GP2a-GP3-GP4-GP5-M entrapped in PLGA nanoparticles or unentrapped were coadministered intranasally twice with a potent mucosal adjuvant, *Mycobacterium tuberculosis* whole cell lysate, and challenged with a virulent heterologous PRRSV strain 1-4-4. Analysis of viremia suggested the reduced challenged viral RNA load and infectious virus in pigs received PRRS-VLPs irrespective of entrapment in NPs, while in the lungs of pigs vaccinated with PRRS-VLPs without encasing in NPs had helped to significantly reduce the viral load. In conclusion, we generated PRRS-VLPs containing all the six PRRSV membrane glycoproteins, and that could be a potential candidate vaccine when delivered with a potent adjuvant. Further studies are required to confirm the dose-dependent PRRS-VLPs vaccine efficacy and the degree of cross-protection against other heterologous PRRSV strains. In conclusion, our results suggested that PRRSV-VLPs containing all the viral membrane proteins are beneficial in inducing the cross-protective immune response in pigs, when the candidate vaccine is used twice through intranasal route and co-administered with a potent adjuvant.

Introduction:
Development of a better cross-protective PRRSV vaccine remained as a challenge since 1990s. Although many reports have demonstrated satisfactory immunity induced by MLV-PRRS in growing pigs, others reported reversion in virulence of vaccine virus and transmission to unvaccinated pigs and sows [1-3], also

recombination between vaccine and field strains [4, 5]. Thus, prevention of virus transmission from the infected pig herd is critical to control PRRS. All these limitations made the research on development of a better cross-protective killed PRRSV vaccine a high priority. Recombinant baculovirus-mediated insect cell production technology allows rapid production of virus-like-particles (VLPs) [6]. Such technology has been proved effective for both enveloped and non-enveloped viruses like calici, influenza, parvo, polyoma, reo, paramyxo, orthomyxo, hepatitis C, Ebola, Marbug, Chikungunya, SARS corona etc., (reviewed in [7, 8]). Co-expression of multiple (2 - 4) viral surface proteins still results in VLPs that are indistinguishable from authentic viral particles [9, 10]. In addition, production of a vaccine with DIVA (differentiation of infected from vaccinated animals) potential is possible with VLPs based subunit vaccines. PRRSV-VLPs could be prepared using already published standard procedures. VLPs based vaccines have several advantages over the conventional vaccine antigens and they are as follows. (i) Extremely large-quantities of correctly folded recombinant proteins can be produced in high density cell-culture conditions in eukaryotic cells, thus baculovirus system is amenable to scale-up for large-scale vaccine production [11]. (ii) Insect cells based vaccine production can be done without the need of mammalian cell-derived supplements, thus the risks of co-culturing opportunistic pathogens is minimized. (iii) There is no threat from baculovirus in vaccinated individuals because this virus has a very narrow host-range in a few insect species. (iv) The VLPs are quite stable with no alteration of particle morphology or reduction in immunogenicity even after 9 weeks storage at room temperature [12]. (v) To further boost the vaccine potency, PRRSV-VLPs could be entrapped in PLGA nanoparticles and delivered to pigs, intranasally. (vi) Unlike MARC-145 cell derived semi-purified crude PRRSV antigens, the dose of VLPs in Nano-PRRSV-VLPs vaccine is quantifiable. Protective anti-PRRSV mucosal immunity generated with the help of innovative Nano-PRRS-VLPs vaccine will have the potential to provide better cross-protective immunity compared to existing vaccines. Importantly, success of our study in generation of PRRS-VLPs may help to design appropriate control measures to control PRRSV transmission in pregnant sows.

Objectives:

1. To prepare PRRSV-VLPs to one each of North American and European PRRSV strains
2. To develop and characterize candidate PLGA nanoparticle-entrapped PRRS-VLPs vaccine
3. To evaluate cross-protective efficacy of Nano-PRRSV-VLPs vaccine in pigs

Materials & Methods:

PRRSV isolates for candidate vaccine construction: Type II virus strain SD09-28 was used in this study, and it was originally obtained in 2009 from a PRRSV-infected farm, which represents current field circulation strains (contains 1-8-4 RFLP pattern in ORF5).

Cloning of PRRSV surface proteins genes and generation of PRRSV-VLPs in baculovirus system: Recombinant baculovirus protein expression system is established in co-investigators laboratories [15, 16]. In this study, six important PRRSV surface protein genes, GP2a, E, GP3, GP4, GP5, and M proteins were amplified by RT-PCR from the viral RNA, subcloned into a baculovirus transfer vector **'pVL1392'** using Rapid Ligation Kit (Promega Corp.). To generate PRRSV-VLPs, pVL1392 vector containing PRRSV genes constructs in the correct orientation were co-transfected to Sf9 insect cells using Linearized Baculovirus DNA Transfection Kit (BD Biosciences, BD BaculoGold^{TM}). To detect generated PRRSV-VLPs, media from the transfected Sf9 cells were examined using PCR for the presence of PRRSV nucleotide sequences in the recombinant baculovirus. Further, PRRSV-VLPs were visualized with the help of Transmission Electron Microscope (TEM) **(**Hitachi S-3500N) by comparing side-by-side with sucrose purified wildtype PRRSV.

Preparation of Nano-PRRSV-VLPs: We have standardized a protocol to prepare PLGA nanoparticle-based PRRSV vaccine with the help of previously described procedures [19, 20]. Briefly, PRRSV-VLPs were entrapped in PLGA nanoparticles by double emulsion method. The following chemicals were used to make the nanoparticles

hydrophilic and stable, such as poloxamer 188, sucrose, $Mg(OH)_2$, polyvinyl alcohol [21-23]. The PLGA (75/25) was dissolved in dichloromethane and mixed with PRRSV-VLPs, and the mixture were homogenized by sonication, then added to aqueous solution of polyvinyl alcohol and again homogenized. Nano-PRRSV-VLPs were stirred at room temperature, washed, freeze-dried, and stored at -20^0C.

Characterization of Nano-PRRSV-VLPs: The concentration of VLPs in Nano-PRRSV-VLPs were determined as described previously [22] using a BCA protein assay kit (Biorad, CA). Morphology (size and shape) of Nano-PRRSV-VLPs were determined by coating the freeze-dried vaccine powder with gold-platinum under vacuum with the help of an ion coater and examined using TEM at 10KV.

Evaluation of efficacy of Nano-PRRS-VLPs vaccine in pigs:

A pilot *in vivo* study was performed to evaluate the efficacy of PRRS-VLPs and NanoPRRS-VLPs in pigs challenged with a virulent PRRSV (1-4-4) strain. A total of 16 PRRSV antibody-free 4-6 weeks old conventional pigs were randomly assigned into one of the three groups. Pigs were vaccinated with sterile DMEM (mock vaccination) (group 1) or vaccinated with 650 µg/dose/pig of unentrapped PRRS-VLPs (group 2) or NanoPRRS-VLPs (group 3), co-administered with a potent adjuvant *Mycobacterium tuberculosis* whole cell lysate (*Mtb* WCL, 1 mg/dose/pig), twice at two weeks interval intranasally (IN). All three pig groups were challenged with a virulent heterologous PRRSV stain 1-4-4 ($4x10^6$ $TCID_{50}$ per pig in 2 ml, IN) at 2 weeks post-booster (dpv 28) by IN.

Group#	Experimental Groups	Vaccine dose	Adjuvant Dose
1	Mock (n=6)	3 ml sterile DMEM	N/A
2	PRRS-VLPs + *M. tb* WCL (n=5)	650 µg/pig/dose	1 mg/pig/dose
3	Nano-PRRS-VLPs + *M. tb* WCL (n=5)	650 µg/pig/dose	1 mg/pig/dose

Clinical monitoring, blood and tissue sampling and data analyses: Pigs were monitored daily for PRRS symptoms (temperature, respiratory distress, cough, and reduced food intake) during first 7 days post-challenge (dpc). Body weight was measured and blood samples collected at 0, 14, 28, 34, 37 and 42, and separated plasma samples were aliquoted and stored at -80 ^{0}C. Pigs were euthanized at 14 dpc (42 dpv). On the day of euthanasia, apart from plasma, bronchoalveolar lavage (BAL) fluid and homogenate of lungs were collected and stored at -80 ^{0}C. Analysis of immune correlates of protection was performed in plasma, BAL fluid, and lung homogenate samples. Statistical analysis by Kruskal-Wallis non-parametric test followed by Dunn's multiple comparison post-test, and $P<0.05$ was considered significant.

Detection of immune-correlates of protection and cross-protective immunity:

Determine humoral immunity: To compare humoral immune responses, the serum samples collected at different time points (Table 1) were evaluated by ELISA [29-31].

Quantification of viral load in tissues and viremia: To quantify PRRSV RNA and to determine the viral load, serum, BAL fluid, lung homogenate samples were analyzed by quantitative RT- PCR and cell culture immunofluorescence assay using ZMAC cells [25, 32, 33].

Results:

Objective 1: To prepare PRRSV-VLPs to one each of North American and European PRRSV strains.

The following research activities were accomplished under this Objective:

a. **Baculovirus plasmid, pVL1392, cloned separately with different genes of PRRSV structural proteins.**

The following PRRSV proteins genes cloned plasmids were received from Dr. Ying Fang, South Dakota University, adsorbed on a blotting paper: (i) SD 09-GP2 (771bp); (ii) SD 09-GP3 (765bp); (iii) SD 09-GP4 (534bp); (iv) SD 09-GP5 (603bp); (v) SD 09-M (525bp); (vi) SD 09-N (372bp) (all are from Type II PRRSV); and ((vii) SD08-GP5 (630bp) (from Type- I PRRSV).

b. **Extraction of plasmids and transformation into competent *E. coli* cells**

Plasmid DNA was extracted in RNAse free water and transformed into competent HB 101 *E. coli* following Promega quick protocol. Transformed *E. coli* were spread on LB agar plates (containing 100 µg/ml of Ampicillin) and incubated at 37 ^{0}C overnight for the growth of transformed cells.

c. Subculture of transformed cells

Next day we observed numerous colonies of transformed bacterial cells on agar plate and picked four colonies and transferred to LB broth medium (containing 100 µg/ml of Ampicillin) and incubated at 37 ^{0}C overnight.

d. Extraction of Plasmid DNA from transformed HB 101 *E. coli* cells

Plasmid DNA from transformed *E. coli* was extracted following Wizard Plus SV minipreps DNA purification protocol from Promega (Figure 1).

e. Transfection of Sf9 insect cells with different pVL1392 plasmids containing PRRSV gene inserts.

The Sf9 insect cells were transfected with pVL1392 plasmids containing the PRRSV inserts using the BD Baculogold transfection kit, and cells were incubated for 4 days at 27 ^{0}C under humid conditions to observe cytopathic effect induced by recombinant baculoviruses (recBaculovirus).

f. Passaging of recBaculovirus

Cell supernatant from the transfected Sf9 cells was collected and used for passaging of recBaculovirus using fresh Sf9 cells. Totally four passages have been done so far.

g. Confirmation of PRRSV gene inserts in the passaged recBaculovirus

During the third and fourth passages the presence of inserts was confirmed again by extracting DNA using Wizard Plus SV minipreps DNA purification protocol from Promega and insert sequences were PCR amplified using insert specific primers.

SD09-GP2 F: gcagcggccgcATGAGATGGGAGCCGCACAGAGCC
SD09-GP2R: taggatccTCACTGTGAGCTCGAAAGAAAAGTTG
SD09 GP3 F: gcagcggccgcATGGCTAATAGCTGTGCATTCCTCC,
SD09 GP3 R: ataggatccCTATCGCCGTACGGCATTGGCGGCT
SD09-GP4 F: gcagcggccgcATGGCTGCGTCCCTTCTTTTCTTCT,
SD09-GP4 R: ataggatccTCAAATTGCCAGTAGGATGGCAAAAAG
SD09-GP5 F: gcagcggccgcATGTTGGGGAAATGCTTGACCGCGG
SD09-GP5 R: ataggatccCTAAGGACGTCCCCATTGTTCCGCA
SD09- M F: gcagcggccgcATGGGGACGTCCTTAGATGACTTTTGC
SD09-M R: ataggatccTTATTTGGCATATTTGACAAGGTTC
SD09- N F: gcagcggccgcATGCCAAATAACAACGGCAAGCAGC
SD09-N R: ataggatccTCATGCTGAGGGTGATGTTGTGGC
SD0108-GP5 F: gcagcggccgcAtgaaatgttctcacaaattggggc
SD0108-GP5R: taggatccCTAgtggtggtggtggtggtgGGCCTCCCATTGCTCGGCCG AAGTTCTCG

Gel electrophoresis of amplified PRRSV insert present in the recBaculovirus was confirmed (Figure 2).

h. Infection of Sf9 cells with passage 4 (P4) of recBaculovirus for production of VLPs.

Confluent monolayer of Sf9 cells in T175 flasks were infected with P4 of one of the recombinant baculovirus (rBV) or co-infected with combinations of two to six rBVs and incubated at 27^{0}C for 4-5 days and observed satisfactory cytopathic effect of the virus infection, suggesting the replication of the

rBVs. Harvested cells and medium were subjected to differential (40% and 60%) sucrose density gradient centrifugation and the pellet was subjected to transmission electron microscopy.

Our results suggested that control Sf9 cells did not show any VLPs formation. Infection with individual rBVs also did not result in VLP production (data not shown), whereas, VLPs were generated when Sf9 cells were co-infected with rBVs expressing GP5 and M, indicating that GP5 and M proteins are essential for PRRS-VLPs (Figure 3). Further rBVs expressing five and six proteins (including E) produced VLPs. Infection with rBVs expressing three minor GPs (GP2, GP3 and GP4) also resulted in VLP formation, but the size of particles was smaller (30-40nm) compared to GP5 and M containing VLPs (50-80nm). Co-infection of Sf9 cells with all the minor GPs yielded mixed sized VLPs (30-70nm) (Figure 3).

i. **Confirmation of the presence of expressed PRRSV surface proteins in PRRSV-VLPs.** We performed ELISA using uninfected Sf9 cells supernatant and cesium chloride purified PRRS-VLPs and tested the reactivity with PRRSV hyperimmune serum. Our results suggested that Sf9 control cell supernatant gave some background OD greater than the blank OD value, but lower than the PRRSV-VLPs (Figure 4). But when the similar assay was performed using semipurified PRRSV-VLPs (using only 20% sucrose cushion), the difference in the specific reactivity was reduced (data not shown) suggesting the reactivity of insect cell proteins with the pig serum.

Objective 2: To develop and characterize candidate PLGA nanoparticle-entrapped PRRS-VLPs vaccine

Preparation and characterization of Nano-PRRSV-VLPs: We prepared PLGA nanoparticle-entrapped with PRRSV-VLPs containing either GP5-M or GP5-GP4-GP3-GP2-M (Nano-PRRS-VLPs) using the double emulsion method. The PRRSV-VLPs entrapped in NPs were circular in shape with a protein entrapment efficiency of approximately 50%. Dynamic light scattering of NPs determined their diameter based distribution, and the mean diameter ± SD of Nano-PRRS-VLPs was 310±35 nm (Figure 5).

Objective 3: To evaluate cross-protective efficacy of Nano-PRRSV-VLPs vaccine in pigs

The blood samples collected at three time points post-challenge were analyzed for the presence of replicating infectious challenged PRRSV using ZMAC (pig macrophage cell line) cells which support the growth of PRRSV. Our results suggested that both in pigs vaccinated with PRRS-VLPs either entrapped or unentrapped in NPs lower infectious virus titer at DPC 9 and 14 compared to mock-challenged pigs was observed. Also a similar trend in reduction in the viral RNA copies were observed at DPC 14 as determined RT-PCR analysis and the data was statistically significant (Figure 6). The infectious virus titer and RNA copies were estimated in the BAL fluid and lung homogenate samples from all the three experimental pig groups, and our results indicated that PRRS-VLPs unentrapped in NPs induced significantly reduced virus titers in pigs compared to both the mock-challenged and Nano-PRRS-VLPs vaccinated pigs (Figure 7).

We used PRRSV GP5 and M proteins and the challenged virus in the ELISA, and detected increased trends in the levels of antigen specific IgG antibodies production in the plasma of vaccinated (PRRS-VLPs either entrapped or unentrapped in NPs) compared to mock-challenged pigs, and the data was not statistically significant (Figure 8). In the lung homogenate of Nano-PRRS-VLPs vaccinated pigs the levels of GP5 and M proteins antigen specific IgG production was significantly higher than the mock-challenged pigs (Figure 9). While in the lungs of both the types of PRRS-VLPs vaccinated pigs the levels of antigens specific IgA production was comparable in all the three experimental pig groups (Figure 10).

Discussion

We could generate PRRSV-VLPs containing all the six viral membrane proteins and the average size of the particles was approximately 50-60nm. The specific reactivity of the VLPs *in vitro* was observed only when highly purified preparation has used in the antibody assay, suggesting the background reactivity of the insect cell proteins with the pig serum. In pigs vaccinated with the PRRS-VLPs either entrapped or unentrapped in NPs reduced infectious challenged virus titer and the total viral RNA copies in the blood was observed. But in the lungs of pigs vaccinated with PRRS-VLPs not entrapped in PLGA NPs the virus clearance was significant, suggesting that PLGA NPs entrapment is not required to boost the immune response of the PRRSV-VLPs in the lungs. However, the need of a potent adjuvant co-administration with PRRSV-VLPs should be analyzed. **In summary, our results suggested that PRRSV-VLPs containing all the viral membrane proteins are beneficial in inducing the cross-protective immune response in pigs.**

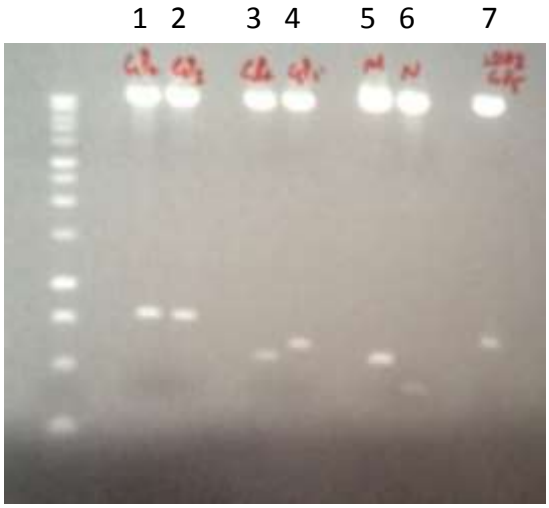

Fig. 1. Confirmation of presence of inserts in the extracted baculovirus plasmid DNA. Plasmid DNA was digested using restriction endonuclease enzymes BamH1 and Not1 and digested DNA was subjected to gel electrophoresis to detect the presence of inserts. All the inserts were present in the respective digested plasmid DNA. Lanes: Type 2 PRRSV protein genes: 1 - GP2; 2 - GP3; 3 - GP4; 4 - GP5; 5 – M; 6- N; and 7 Type 1 PRRSV protein gene GP5.

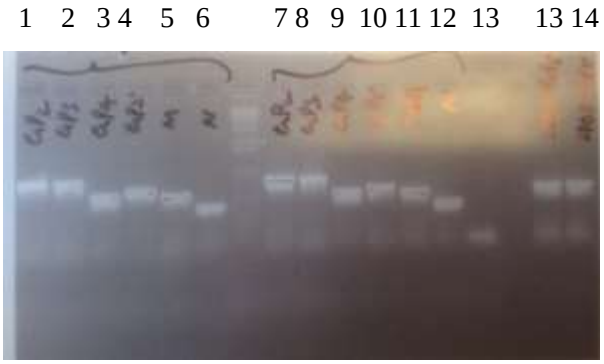

Fig. 2. Gel electrophoresis of amplified PRRSV insert present in the recBaculovirus and the respective plasmids. DNA extracted from passage 4 of recBaculovirus was subjected to PCR amplification using PRRSV gene specific primers. Left panel of lanes are for all the Type II PRRSV proteins genes specific inserts, and the middle panel has corresponding plasmids. Right panel has passage 4 of Type I PRRSV GP5 (left) and corresponding plasmid (right). Lanes: Type 2 PRRSV protein genes: 1 - GP2; 2 - GP3; 3 - GP4; 4 - GP5; 5 – M; 6- N. Lanes 7 to 13 respective plasmids. Lanes 13 Type 1 PRRSV protein gene GP5 and 14 – tis plasmid.

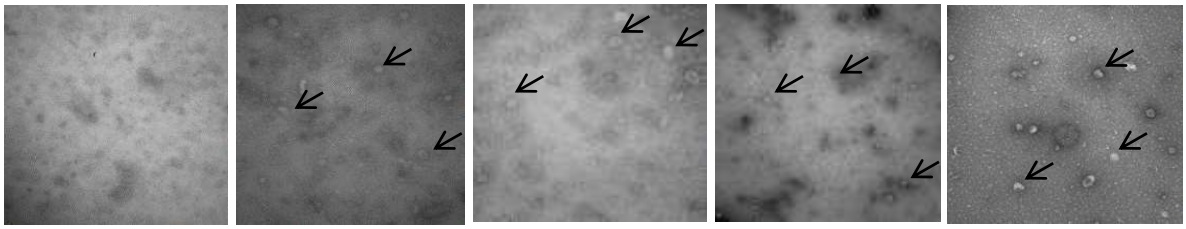

Figure 3. Transmission electron micrographs (TEM) of PRRSV-VLPs. Sf9 insect cells were infected with different combinations of recombinant baculovirus (rBVs) containing one of the PRRSV membrane protein genes, and generated PRRSV-VLPS were captured using the electron microscope. (A) Control Sf9 cells uninfected with any rBVs; Sf9 cells co-infected with rBVs expressing (B) GP5 and M; (C) GP5-GP4-GP3-GP2-M (50-80nm size particles); (D) GP2, GP3 and GP4 (30-40nm size particles); (E) GP2, E, GP3 and GP4 (30-70nm size particles).

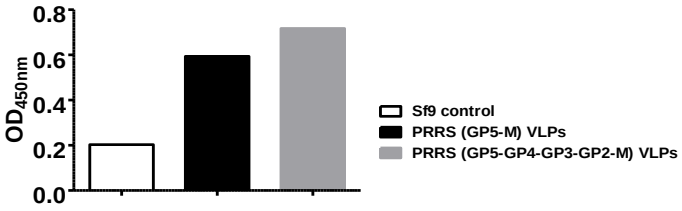

Figure 4. *In vitro* reactivity of PRRSV-VLPs. Culture supernatant harvested from Sf9 control cells uninfected with any rBVs and cesium chloride purified PRRSV-VLPs were subjected to ELISA using PRRSV hyperimmune serum. Sf9 control showed lower OD compared to either of the PRRSV-VLPs.

Physical characterization of Nano-PRRS-VLPs

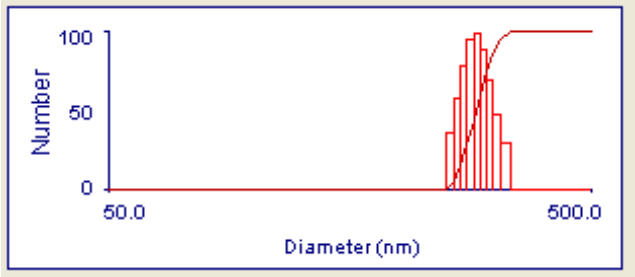

Figure 5. Size distribution of Nano-PRRS-VLPs. PRRSV-VLPs harvested from Sf9 cells infected with rBVs (GP5-GP4-GP3-GP2-M) were entrapped in PLGA nanoparticles. Entrapped nanoparticles were subjected to Dynamic light scattering (DLS) size distribution analysis to determine the average diameter distribution of nanoparticles. Results indicated that mean diameter of Nano-PRRS-VLPs was 310±35 nm.

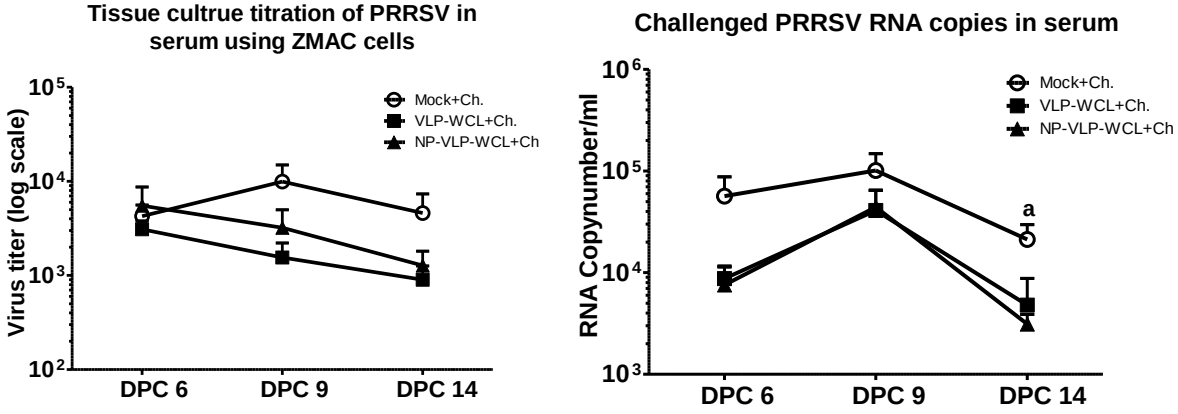

Figure 6. Clearance of challenged PRRSV from the blood of pigs

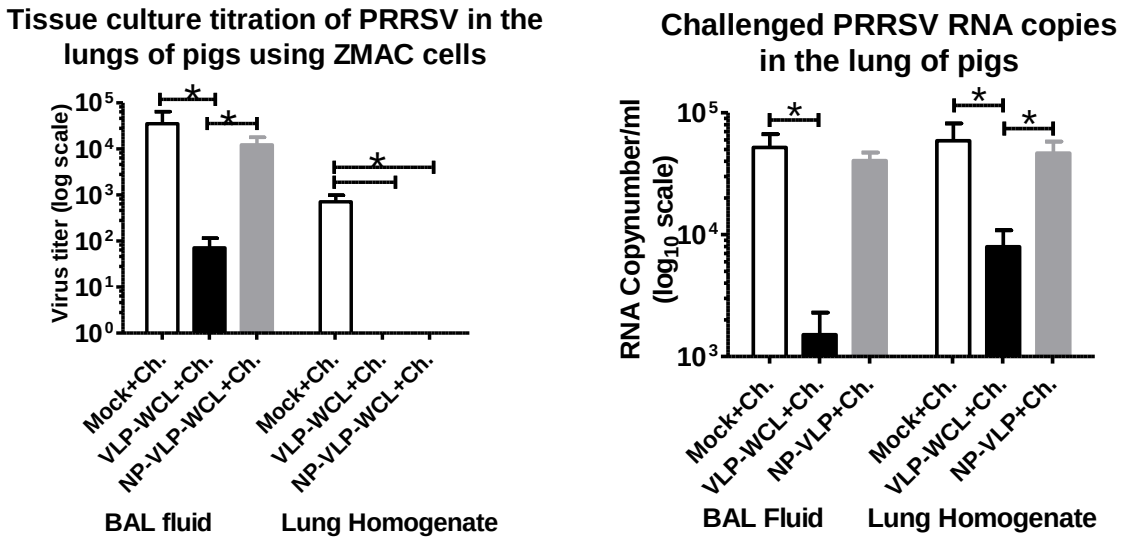

Figure 7. Clearance of challenged PRRSV from the lungs of pigs

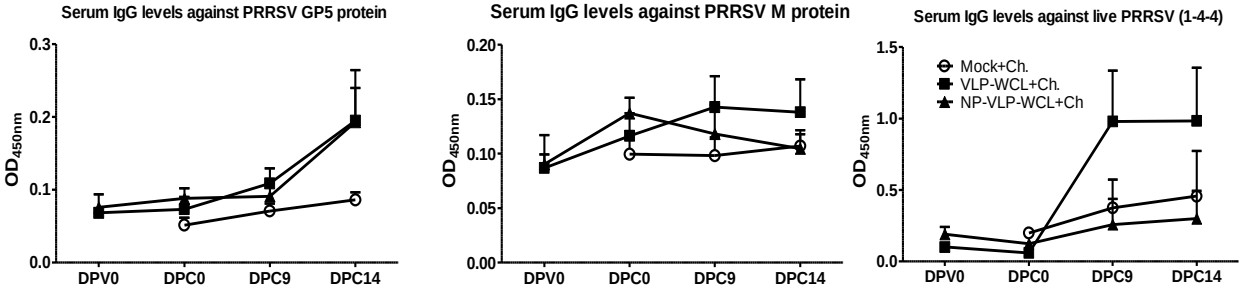

Figure 8. Levels of PRRSV specific IgG antibodies in the blood of vaccinated or unvaccinated pigs (no significant difference)

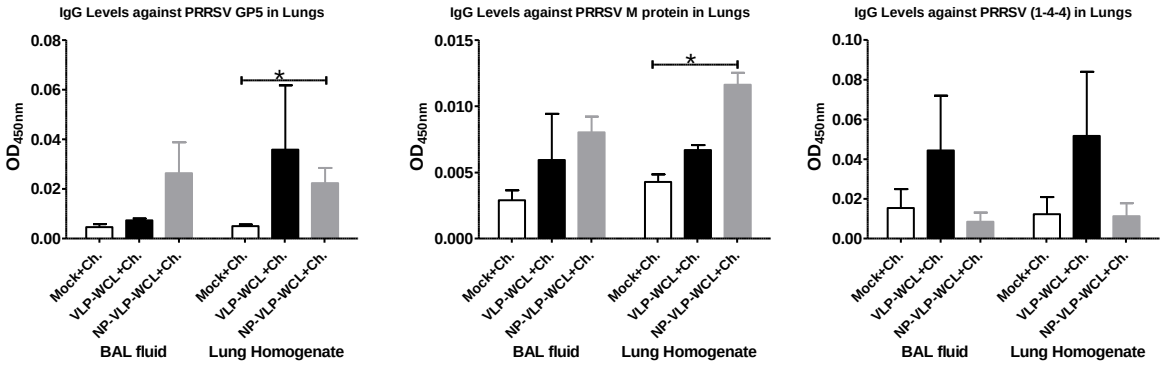

Figure 9. Levels of IgG antibodies in the Lungs of vaccinated or unvaccinated pigs

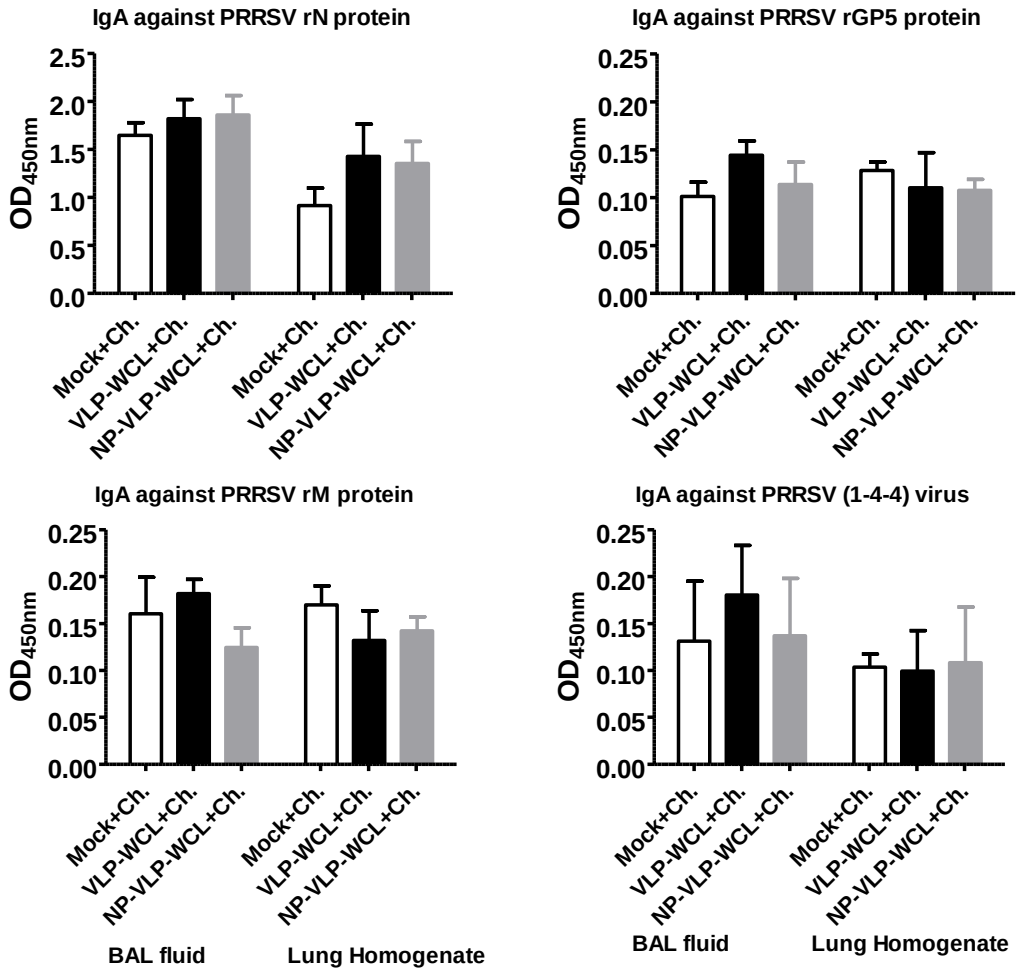

Figure 10. Levels of virus antigen specific IgA antibodies in the lungs of vaccinated or unvaccinated pigs (no significant difference).

References

[1] Madsen KG, Hansen CM, Madsen ES, Strandbygaard B, Botner A, Sorensen KJ. Sequence analysis of porcine reproductive and respiratory syndrome virus of the American type collected from Danish swine herds. Arch Virol. 1998;143:1683-700.

[2] Nielsen HS, Oleksiewicz MB, Forsberg R, Stadejek T, Botner A, Storgaard T. Reversion of a live porcine reproductive and respiratory syndrome virus vaccine investigated by parallel mutations. J Gen Virol. 2001;82:1263-72.

[3] Nielsen J, Botner A, Bille-Hansen V, Oleksiewicz MB, Storgaard T. Experimental inoculation of late term pregnant sows with a field isolate of porcine reproductive and respiratory syndrome vaccine-derived virus. Vet Microbiol. 2002;84:1-13.

[4] Murtaugh MP, Yuan S, Nelson EA, Faaberg KS. Genetic interaction between porcine reproductive and respiratory syndrome virus (PRRSV) strains in cell culture and in animals. J Swine Health Prod. 2002;10:15-21.

[5] Li B, Fang L, Xu Z, Liu S, Gao J, Jiang Y, et al. Recombination in vaccine and circulating strains of porcine reproductive and respiratory syndrome viruses. Emerg Infect Dis. 2009;15:2032-5.

[6] Cox MM. Progress on baculovirus-derived influenza vaccines. Curr Opin Mol Ther. 2008;10:56-61.

[7] Roy P, Noad R. Virus-like particles as a vaccine delivery system: myths and facts. Hum Vaccin. 2008;4:5-12.

[8] Plummer EM, Manchester M. Viral nanoparticles and virus-like particles: platforms for contemporary vaccine design. Wiley Interdiscip Rev Nanomed Nanobiotechnol. 2010.

[9] French TJ, Marshall JJ, Roy P. Assembly of double-shelled, viruslike particles of bluetongue virus by the simultaneous expression of four structural proteins. J Virol. 1990;64:5695-700.

[10] French TJ, Roy P. Synthesis of bluetongue virus (BTV) corelike particles by a recombinant baculovirus expressing the two major structural core proteins of BTV. J Virol. 1990;64:1530-6.

[11] Maranga L, Rueda P, Antonis AF, Vela C, Langeveld JP, Casal JI, et al. Large scale production and downstream processing of a recombinant porcine parvovirus vaccine. Appl Microbiol Biotechnol. 2002;59:45-50.

[12] Caparros-Wanderley W, Clark B, Griffin BE. Effect of dose and long-term storage on the immunogenicity of murine polyomavirus VP1 virus-like particles. Vaccine. 2004;22:352-61.

[13] Fang Y, Rowland RR, Roof M, Lunney JK, Christopher-Hennings J, Nelson EA. A full-length cDNA infectious clone of North American type 1 porcine reproductive and respiratory syndrome virus: expression of green fluorescent protein in the Nsp2 region. J Virol. 2006;80:11447-55.

[14] Fang Y, Christopher-Hennings J, Brown E, Liu H, Chen Z, Lawson SR, et al. Development of genetic markers in the non-structural protein 2 region of a US type 1 porcine reproductive and respiratory syndrome virus: implications for future recombinant marker vaccine development. J Gen Virol. 2008;89:3086-96.

[15] Dybing JK, Jackwood DJ. Antigenic and immunogenic properties of baculovirus-expressed infectious bursal disease viral proteins. Avian Dis. 1998;42:80-91.

[16] Dybing JK, Jackwood DJ. Expression of MD infectious bursal disease viral proteins in baculovirus. Avian Dis. 1997;41:617-26.

[17] Latham T, Galarza JM. Formation of wild-type and chimeric influenza virus-like particles following simultaneous expression of only four structural proteins. J Virol. 2001;75:6154-65.

[18] Belyaev AS, Roy P. Development of baculovirus triple and quadruple expression vectors: co-expression of three or four bluetongue virus proteins and the synthesis of bluetongue virus-like particles in insect cells. Nucleic Acids Res. 1993;21:1219-23.

[19] Semete B, Booysen L, Lemmer Y, Kalombo L, Katata L, Verschoor J, et al. In vivo evaluation of the biodistribution and safety of PLGA nanoparticles as drug delivery systems. Nanomedicine. 2010;6:662-71.

[20] Gupta RK, Chang AC, Siber GR. Biodegradable polymer microspheres as vaccine adjuvants and delivery systems. Dev Biol Stand. 1998;92:63-78.

[21] Ling J, Liao H, Clark R, Wong MS, Lo DD. Structural constraints for the binding of short peptides to claudin-4 revealed by surface plasmon resonance. J Biol Chem. 2008;283:30585-95.

[22] Rajapaksa TE, Bennett KM, Hamer M, Lytle C, Rodgers VG, Lo DD. Intranasal M cell uptake of nanoparticles is independently influenced by targeting ligands and buffer ionic strength. J Biol Chem. 2010;285:23739-46.
[23] Reischl D, Zimmer A. Drug delivery of siRNA therapeutics: potentials and limits of nanosystems. Nanomedicine. 2009;5:8-20.
[24] Kim WI, Lee DS, Johnson W, Roof M, Cha SH, Yoon KJ. Effect of genotypic and biotypic differences among PRRS viruses on the serologic assessment of pigs for virus infection. Vet Microbiol. 2007;123:1-14.
[25] Cano JP, Dee SA, Murtaugh MP, Trincado CA, Pijoan CB. Effect of vaccination with a modified-live porcine reproductive and respiratory syndrome virus vaccine on dynamics of homologous viral infection in pigs. Am J Vet Res. 2007;68:565-71.
[26] VanCott JL, Brim TA, Simkins RA, Saif LJ. Isotype-specific antibody-secreting cells to transmissible gastroenteritis virus and porcine respiratory coronavirus in gut- and bronchus-associated lymphoid tissues of suckling pigs. J Immunol. 1993;150:3990-4000.
[27] Loving CL, Brockmeier SL, Sacco RE. Differential type I interferon activation and susceptibility of dendritic cell populations to porcine arterivirus. Immunology. 2007;120:217-29.
[28] Dwivedi V, Manickam C, Patterson R, Dodson K, Weeman M, Renukaradhya GJ. Intranasal delivery of whole cell lysate of Mycobacterium tuberculosis induces protective immune responses to a modified live porcine reproductive and respiratory syndrome virus vaccine in pigs. Vaccine. 2011.
[29] Christopher-Hennings J, Nelson EA, Nelson JK, Rossow KD, Shivers JL, Yaeger MJ, et al. Identification of porcine reproductive and respiratory syndrome virus in semen and tissues from vasectomized and nonvasectomized boars. Vet Pathol. 1998;35:260-7.
[30] Benfield DA, Nelson E, Collins JE, Harris L, Goyal SM, Robison D, et al. Characterization of swine infertility and respiratory syndrome (SIRS) virus (isolate ATCC VR-2332). J Vet Diagn Invest. 1992;4:127-33.
[31] Thanawongnuwech R, Young TF, Thacker BJ, Thacker EL. Differential production of proinflammatory cytokines: in vitro PRRSV and Mycoplasma hyopneumoniae co-infection model. Vet Immunol Immunopathol. 2001;79:115-27.
[32] Wasilk A, Callahan JD, Christopher-Hennings J, Gay TA, Fang Y, Dammen M, et al. Detection of U.S., Lelystad, and European-like porcine reproductive and respiratory syndrome viruses and relative quantitation in boar semen and serum samples by real-time PCR. J Clin Microbiol. 2004;42:4453-61.
[33] Klinge KL, Vaughn EM, Roof MB, Bautista EM, Murtaugh MP. Age-dependent resistance to Porcine reproductive and respiratory syndrome virus replication in swine. Virol J. 2009;6:177.
[34] Dwivedi V, Manickam C, Patterson R, Dodson K, Murtaugh M, Torrelles JB, et al. Cross-protective immunity to porcine reproductive and respiratory syndrome virus by intranasal delivery of a live virus vaccine with a potent adjuvant. Vaccine. 2011;doi:10.1016/j.vaccine.2011.03.006.
[35] Dwivedi V, Manickam C, Patterson R, Dodson K, Murtaugh M, Torrelles JB, et al. Cross-protective immunity to porcine reproductive and respiratory syndrome virus by intranasal delivery of a live virus vaccine with a potent adjuvant. Vaccine. 2011;29:4058-66.
[36] Renukaradhya GJ, Alekseev K, Jung K, Fang Y, Saif LJ. Porcine reproductive and respiratory syndrome virus-induced immunosuppression exacerbates the inflammatory response to porcine respiratory coronavirus in pigs. Viral Immunol. 2010;23:457-66.