RESEARCH REPORT
SWINE HEALTH

Title: *In vivo* evaluation of genetic markers in the nsp2 region of PRRSV: Implications for future recombinant marker vaccine development – **NPB #06-173**

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II. Industry Summary:

Porcine reproductive and respiratory syndrome (PRRS) virus (PRRSV) continues to be a major problem to the pork industry worldwide. The limitations offered by current PRRSV vaccines require the development of a new generation of vaccines. One of the key steps in future vaccine development is to include markers for diagnostic differentiation of vaccinated animals from those naturally infected with wild-type virus. In previous study, we have constructed a marker virus, which contains a green fluorescent protein (GFP) insertion and an immunogenic epitope deletion in nsp2 region of the virus. In this study, we performed *in vivo* characterization of this marker viruse. To compliment the marker identification, we developed GFP and nsp2 epitope-based ELISAs. Pigs immunized with the recombinant virus lacked antibodies directed against the corresponding deleted epitope, while generating a high level of antibody response to GFP by 14 days post-infection. Our results demonstrated that this recombinant marker virus, in conjunction with the diagnostic tests, enable serological differentiation between marker virus-infected animals from those infected with the wild-type virus. This rationally designed marker virus will provide a basis for further development of PRRSV marker vaccines to assist with the control of PRRSV.

III. Scientific Abstract:

Porcine reproductive and respiratory syndrome virus (PRRSV) continues to be a major problem in the pork industry worldwide. The limitations of current PRRSV vaccines require the development of a new generation of vaccines. One of the key steps in future vaccine development is to include markers for diagnostic differentiation of vaccinated animals from those naturally infected with wild-type virus. Using a cDNA infectious clone of Type 1 PRRSV, we have constructed a recombinant green fluorescent protein (GFP) tagged PRRSV containing deletion of an immunogenic epitope, ES4, in the nsp2 region. In this study, we performed in vivo characterization of this marker virus in a nursery pig disease model. The results showed that the recombinant virus was attenuated with a lower level of viremia, but induced a higher level of neutralizing antibody response in comparison to that of parental virus. To compliment the marker identification, we developed GFP and ES4 epitope-based ELISAs. Pigs immunized with the recombinant virus lacked antibodies directed against the corresponding deleted epitope, while generating a high level of antibody response to GFP by 14 days post-infection. Our results demonstrated that this recombinant marker virus, in conjunction with the diagnostic tests, enable serological differentiation between marker virus-infected animals from those infected with the wild-type virus. This rationally designed marker virus will provide a basis for further development of PRRSV marker vaccines to assist with the control of PRRSV.

IV. Introduction:

Porcine reproductive and respiratory syndrome (PRRS) is the most economically significant disease of swine worldwide. It is characterized by late term reproductive failure in sows and severe pneumonia in neonatal pigs. Since its emergence in domestic swine in the 1980's, PRRS has resulted in immense economic losses to the swine industry, with recent costs in the US of at least $600 million annually [40]. The etiologic agent of PRRS is a small, enveloped virus containing a single positive-stranded RNA genome. PRRSV belongs to the family *Arteriviridae*, which includes equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV), and simian hemorrhagic fever virus (SHFV) [44]. Nucleotide sequence comparisons showed that PRRSV can be divided into distinct European (Type 1) and North American (Type 2) genotypes [1, 39]. The PRRSV genome is about 15 kb in length and contains nine open reading frames. The 3' end of the genome encodes four membrane-associated glycoproteins (GP2a, GP3, GP4 and GP5; encoded by sg mRNAs 2-5), two unglycosylated membrane proteins (E and M; encoded by sg mRNAs 2 and 6) and a nucleocapsid protein (N; encoded by sg mRNA 7) [3, 28, 31, 32, 33, 36, 57, 58]. The replicase-associated genes, ORF1a and ORF1b, situated at the 5' end of the genome, represent nearly 75% of the viral genome. The ORF1ab encoded polyprotein pp1ab is predicted to be cleaved at 12 sites to form 13 products: nsp1α, nsp1β, and nsp2 to nsp12 [1, 9, 39, 44]. Based on the study of EAV, the nsp2 plays a crucial role in the viral replication. The nsp2 contains a cysteine protease domain residing in the N-terminal. This domain induces nsp2/3 cleavage, and also functions as a co-factor with nsp4 serine protease to process the other cleavage products [9, 44, 45, 46, 55].

Modified-live attenuated vaccines against PRRSV are currently available for reduction of clinical disease associated with PRRSV (Boehringer-Ingelheim Animal Health, Inc.). However, they cannot distinguish serologically between pigs that have recovered from a natural infection and those that have been vaccinated. A genetically marked vaccine will allow the differentiation between vaccinated and naturally infected pigs, which is needed for PRRSV control and eradication programs. Previous studies on marker vaccine development for other viruses were mostly based on the manipulation of cDNA infectious clones [2, 8, 16, 27, 30], from where a foreign antigen can be inserted (positive marker) or an immunogenic epitope can be deleted (negative marker).

The antibody response to the foreign antigen or viral epitope can be used to differentiate vaccinated animals from naturally infected animals.

In this study, we performed the in vivo characterization of a marker virus to determine its potential use as marker vaccine against PRRSV infection. The GFP antigen and ES4 peptide antigen-based ELISAs were tested to determine their sensitivity and specificity as companion diagnostic assays for the marker detection and differentiation.

V. Objectives:

1. To characterize the *in vivo* biological properties of the nsp2 marker viruses.
2. To determine the genetic stability of the nsp2 marker regions.
3. To determine the ability of the nsp2 marker to differentiate vaccinated pigs from pigs infected with Type 1 field strains.
4. To determine the ability of the nsp2 marker to differentiate vaccinated pigs from pigs infected with Type 2 field strains.

VI. Materials & Methods:

Objective 1. To characterize the *in vivo* biological properties of the nsp2 marker viruses.

Cells and viruses: Baby hamster kidney cells (BHK-21 C13; American Type Culture Collection) were cultured in Minimal Eagle's medium (MEM; GIBCO BRL Life Technologies) with 5% fetal bovine serum (FBS) and antibiotics (100 units/ml penicillin, 20 ug/ml streptomycin). MARC-145 cells were maintained in MEM supplemented with 10% FBS and antibiotics. BHK cells were used for initial transfection and recovery of virus from *in vitro* transcripts. MARC-145 cells were used for virus rescue and subsequent experiments. All cells were maintained at 37 ^{0}C in a humidified 5% CO_2 incubator. The GFP/ΔES4 marker virus was constructed from US Type 1 PRRSV cDNA full-length infectious clone, pSD01-08 [12]. The second passage of the MARC-145 cell-recovered parental and marker viruses was used for *in vitro* and *in vivo* experiments.

Animal study: Eighteen four-week-old pigs were purchased from a PRRSV-free herd. The animals were randomly separated into three groups (n = 6/group) and housed under BL2 isolation conditions with an acclimation period of 7 days before starting experimental inoculations. Group 1 pigs were infected with GFP/ΔES4 marker virus, the group 2 pigs were infected with parental SD01-08 virus as the positive control, and group 3 pigs were mock-infected with the cell culture medium. Group 1 and group 2 pigs were inoculated through both intranasal and intramuscular sites with 1 x 10^6 50% tissue culture infective doses ($TCID_{50}$) of the virus (1 ml at each site). On 42 days post infection (dpi), group 1 and group 2 pigs were challenged with a heterologous Type 1 strain, SD 03-15 virus. Three pigs from group 3 were challenged with SD03-15 virus, and the other three pigs remained as mock-infected controls. Pigs were observed daily for clinical signs and body temperatures for the first 7 days after infection and the first 7 days after challenge. Blood samples were obtained once per week from all pigs. Pigs were euthanized at 21 days post challenge. The treatment and sampling schedule is detailed in Table 1. Gross lung lesions of the study animals were evaluated using a previously developed system based on the approximate volume that each lobe contributes to the entire lung: the left and right apical lobes, the left and right cardiac lobes, and the intermediate lobe each contribute 10% of the total lung volume, and the left and right caudal lobes each contribute 25%. These scores were then used to calculate the total lung lesion score based on the relative contributions of each lobe [22].

Quantification of viral load: For the detection of viral RNA and determination of viral load, serum and tissue samples were examined using a real-time, quantitative PCR (Tetracore VetAlert PRRS; 54), which is routinely performed at the South Dakota Animal Disease Research and Diagnostic Laboratory (SDSU-ADRDL).

Determination of humoral immunity: All serum samples were evaluated for anti-PRRSV antibodies using the IDEXX HerdChek® PRRS 2XR ELISA and virus neutralization assay. These tests are also routinely performed at SDSU-ADRDL under strict quality assurance guidelines.

Objective 2. To determine the genetic stability of the nsp2 marker regions.

Virus isolation and sequencing: Serum samples from 7, 14, 21, and 28 dpi were used for virus isolation as described previously [54]. The presence of virus was confirmed by IFA with PRRSV specific antibody, SDOW17 [38]. To determine the stability of the GFP insertion and ES4 epitope deletion, viral RNA was extracted from the serum-isolated virus using QIAamp Viral RNA mini kit (Qiagen) following the manufacture's instruction. The RT-PCR was performed using previously described methods [13]. The RT-PCR amplified fragment was gel purified, and the sequence was determined at the Iowa State University sequencing facility (Ames, IA). Primer pair nsp2-2144F/nsp2-2694R (Table 2) was used for RT-PCR and sequencing, and amplifies the nucleotide region (2144 to 2694 of SD01-08 genome) containing the GFP insertion and ES4 deletion.

Objective 3. To determine the ability of the nsp2 marker to differentiate vaccinated pigs from pigs infected with Type 1 field strains.

Objective 4. To determine the ability of the nsp2 marker to differentiate vaccinated pigs from pigs infected with Type 2 field strains.

ES4 and GFP antigens expression: The ES4 antigen was expressed as tandem repeat ES4 epitopes using a modified method described previously [47]. Briefly, three copies of the ES4 epitope (amino acid 736-790 of ORF1a of SD01-08), were constructed in protein expression vector, pET-28a (+) (Novagen). A flexible peptide linker, GGTGGTGGTGGTTCC, was added between the epitopes to help display the epitopes. There were two forward primers. Forward primer 1, pET-ES4F1 contained a *Bgl*II restriction site, but without a linker sequence, whereas the forward primer 2, pET-ES4F2 contained not only a *Bgl*II restriction site, but also the linker sequence. The ES4 gene fragment was first amplified with the forward primer 1 and reverse primer, pET-ES4R. The PCR product was digested with *Bgl*II and *Hind*III, and then cloned into pET-28a that was digested with *BamH*I and *Hind*III. This clone was designated as pET-28a-ES4 (+1). The second copy of ES4 was PCR amplified by forward primer 2 and reverse primer, pET-ES4R. The PCR product was digested with *Bgl*II and *Hind*III, and then cloned into pET-28a-ES4 (+1) that was digested with *BamH*I and *Hind*III. The third copy of the ES4 was inserted using the same strategy as the second copy. The final construct was

designated as pET-28a-ES4 (+3). The GFP gene was amplified from the pEGFP-N1 plasmid (Clontech) with primer pair pET-EGFP-F/pET-EGFP-R. The PCR product was digested by *BamHI* and *HindIII* restriction enzymes and ligated to the pET-28a vector that was digested with the same enzymes. Recombinant proteins were expressed in *E. coli* BL21 (DE3) to produce a fusion protein with six histidine residues at the N-terminal. The proteins were purified by nickel- affinity chromatography and analyzed by SDS-PAGE as described in our previous publication [14].

GFP and ES4 epitope-based ELISA*:* ELISAs were performed using Immulon II HB 96-well microtiter plates (Thermo Labsystems, Franklin, MA). The recombinant protein was diluted in coating buffer (15mM sodium carbonate-35mM sodium bicarbonate, pH 9.6), and the plates were coated with 100 ul of the diluted antigen in columns 1, 3, 5, 7, 9, and 11. Columns 2, 4, 6, 8, 10, and 12 were treated with 100 ul of coating buffer as a background control. Plates were incubated at 37 ^{0}C for 1 hour, and then excess protein binding sites were blocked with 10% milk in PBST buffer (1 x PBS with 0.05% Tween 20) at 4 ^{0}C overnight. The test sera were applied at 1:5 dilutions in PBST buffer with 5% milk. After 1 hour incubation at 37 ^{0}C, plates were washed with PBST and horseradish peroxidase-conjugated goat anti-swine IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added to bind to any PRRSV serum antibodies that bound to the antigen on the plates. Plates were incubated at 37 ^{0}C for another hour, washed, and the peroxidase substrate ABTS (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added for color development. The color development was quantified by reading at 405 nm with an EL800 microplate reader (BioTek Instruments Inc., Winooski, VT.) controlled by XChek Software (IDEXX Laboratories).

Serum samples from other Type 1 and Type 2 PRRSV infected animals: To determine the reactivity of ES4-epitope with various strains of PRRSV, serum samples were obtained from a group of experimental pigs that were infected with four different representative strains of Type 1 viruses: SD01-07, SD01-08, SD02-11 and SD03-15 (Lawson et al., Proc. Conf. Res. Work. Anim. Dis., abstr. 99, 2005). The SD01-07 and SD01-08 isolates were obtained from herds showing no clinical disease and SD02-11 and SD03-15 were from herds with

substantial morbidity and mortality in young pigs. These four isolates also group into different branches of the phylogenetic tree developed for Type 1 PRRSV isolates of US origin [11]. Serum samples from experimental pigs infected with Type 2 virus, VR2332 were obtained from the shared reagent resource of the PRRSV Cooperative Agriculture Project (CAP) [35].

***Statistical analysis**:* Statistical analysis was performed using GraphPad InStat version 5.1 (GraphPad Software, San Diego, California). Comparison of virus load and neutralizing antibody levels in serum samples were performed using one-way analysis of variance (ANOVA) with Turkey-Kramer multiple comparison test to determine mean significance. Differences between groups of pigs at different times post inoculation were considered statistically significant at $P \leq 0.05$ for all analysis.

VII. Results:

Objective 1. To characterize the *in vivo* biological properties of the nsp2 marker viruses.

The *in vivo* characteristics of the GFP/ΔES4 marker virus were studied in a nursery pig disease model. Group 1 to 3 of 4-week-old PRRSV negative pigs (n = 6) were initially inoculated and subsequently challenged at 42 dpi with a genetically different strain, SD03-15 as described in the Materials and Methods section. The SD03-15 is another US Type 1 strain, which was isolated from clinical samples submitted to the SD Animal Disease Research and Diagnostic Laboratory in 2003. In field reports, pigs infected with SD03-15 were experiencing a pre-weaning mortality of 80-90% for a 3-week period. Decreased performance continued through the finisher phase. In the adult sow population, there was a mild abortion storm, compared to previous US PRRSV outbreaks. Our previous experimental animal study also demonstrated the pathogenic nature of this virus (Lawson et al., Proc. Conf. Res. Work. Anim. Dis., abstr. 99, 2005).

Clinical signs, rectal temperature and gross pathologic lesions: No temperature increase was detected in any pigs after initial infection and no clinical signs were observed. After challenge, rectal temperatures were elevated in those three challenged pigs from Group 3 (initially mock infected) at one and two days post challenge (Fig. 1). Clinical signs (coughing and nasal discharge) were also observed in these three pigs. The

rest of the pigs remained asymptomatic. At necropsy, gross pathologic lesions were not observed in Group 1, Group 2, and the three strict negative control pigs. In contrast, mild gross pathologic lung lesions characteristic of PRRSV were observed in those three pigs from Group 3 that were initially mock infected and then challenged with SD03-15. The average lung lesion score was three based on a 1-10 scoring system described in the Materials and Methods section [22].

Virological and immunological properties: The duration and height of viremia was determined by real-time PCR. In comparison to Group 2 pigs infected with parental viruses (peak mean viral titer = 5.9×10^7 copies/ml), pigs that were infected with the GFP/ΔES4 marker virus had lower peak viral load (peak mean viral titer = 2.08×10^5 copies/ml, Fig. 2). At day 7 post-challenge, the viral load was two to three logs lower for the pigs vaccinated than those pigs initially mock infected and then challenged with SD03-15 virus. By day 21 post-challenge, GFP/ΔES4 marker virus infected-group pigs had a 10-fold lower viral load in comparison to the parental group, and 3/5 pigs had eliminated the virus in the serum (Fig. 2; Table 3).

By 14 dpi, all of the pigs in infected groups had seroconverted. The antibody response reached similar levels as measured by IDEXX ELISA after 21dpi (Fig. 3). Further measurement of the serum neutralizing (SN) antibody levels showed that in pigs infected with the parental virus, SN antibodies were detected from one of the six pigs by 21 days post-infection, and 3/6 pigs developed detectable SN titer that reached an average geometric mean titer (GMT) of 2 by 35 days post-infection. In contrast, neutralizing antibody responses developed faster and higher in pigs infected with GFP/ΔES4 marker virus. SN antibodies were detected from one of the six pigs by 14 days post-infection, and SN titers were detected from all of the pigs in this group, which reached an average GMT of 9.2 by 35 days post-infection. After challenge with SD03-15, an increased effect was observed, with the GMT of 18.4 from the GFP/ΔES4 marker virus infected group compared to the GMT of 5.7 from the parental virus infected group at 49 dpi (one week after challenge). Both groups reached similar SN titers at 63 dpi (three weeks after challenge) (Fig. 4). These data suggest that on the initial infection, pigs infected with GFP/ΔES4 marker virus generated higher neutralizing antibody titers than pigs infected with parental virus.

Objective 2. To determine the genetic stability of the nsp2 marker regions.

To determine stability of the GFP/ΔES4 markers, serum samples from 7 to 28 dpi were used for virus isolation on MARC-145 cells. Viruses were recovered from the serum samples collected on 7, 14, and 21 dpi, and no virus was isolated from the serum samples collected from 28 dpi. In cell culture, we only observed a small population of infected cells showing weak GFP fluorescence with the viruses isolated from 7 and 14 dpi, no GFP fluorescence was observed in infected cells with the viruses isolated from 21 dpi. However, immunofluorescent staining using nucleocapsid protein specific monoclonal antibody, SDOW17, confirmed the presence of a large population of viruses, similar to that observed in the *in vitro* study. The stability of the GFP insertion /ΔES4 deletion was determined by sequencing the corresponding regions. The results confirmed the presence of the ES4 deletion, and the GFP remained intact as a full-length gene. However, sequencing results revealed two point mutations that were located at nucleotide 144 (C to T) and nucleotide 289 (C to T) of the GFP. The nucleotide 144 mutation was silent, but the nucleotide 289 mutation caused amino acid mutation of arginine (R) to cysteine (C) at position 97 of the GFP, which is consistent with our *in vitro* sequencing analysis. Interestingly, there was still a small population of the non-mutated GFP gene detected in the viruses isolated from 7 and 14 dpi. For each dpi, we have sequenced viruses isolated from three pigs, and sequencing was performed using both forward and reverse primers, resulting in a total of six sequences for each dpi. For the viruses isolated from 7 dpi, 1/6 sequences was found to have no mutation at position 97, and the other five sequences were determined to contain the R to C mutation. For the viruses isolated from 14 dpi, 2/6 sequences identified no mutations, and these two sequences were from two different pigs. The other four sequences were also identified to contain the R to C mutation. All the sequences generated from viruses of 21 dpi contained the R to C mutation. This data was consistent with a previous report [25] that the loss of GFP fluorescence is due to the R to C mutation. The presence of small population of the non-mutated GFP would account for the weakly fluorescing cells observed in the cell culture. These results suggest that the selection may have gradually occurred to generate the mutation in favor of improved viral replication.

Objective 3. To determine the ability of the nsp2 marker to differentiate vaccinated pigs from pigs infected with Type 1 field strains.

To differentiate animals that were vaccinated with the marker vaccine from those naturally infected with the field viruses, a companion differential diagnostic assay is required. In this study, since we constructed two markers, the GFP insertion (positive marker) and ES4 deletion (negative marker), we developed both GFP and ES4 epitope-based ELISA assays for marker detection. Both GFP and ES4 epitopes were expressed as soluble recombinant proteins. We evaluated these two ELISA tests for detecting the specific antibodies. As expected, the infection of Group 1 pigs with GFP/ΔES4 marker virus did not induce a detectable antibody response against the deleted ES4 epitope (Fig. 5A), but induced a strong antibody response against the GFP antigen, starting from 14 dpi and continuing for the duration of the study to 63 dpi (Fig. 5B). In contrast, the Group 2 pigs that were infected with parental virus, antibody specific to ES4 recombinant protein could be detected at 21 dpi, and also lasted to 63 dpi (Fig. 5A), while no specific antibody response was detected against GFP antigen (Fig. 5B). After challenged with 03-15, the Group 1 pigs showed a detectable antibody response to the ES4 epitope one week after challenge, since 03-15 virus contains the ES4 epitope (Fig. 5A). No specific antibody response was detected on both GFP and ES4 epitope-based ELISAs for the serum samples from the three strict negative control pigs (Fig. 5A and B).

A basic requirement for the negative marker is that the antigenic region should be able to react with a broad array of field viruses. To ensure that the ES4 epitope can be reactive in various viral strains, we used serum samples from pigs infected with each of the four representative strains of the US Type 1 virus, SD01-07, SD01-08, SD02-11, and SD03-15 (Lawson et al., Proc. Conf. Res. Work. Anim. Dis., abstr. 99, 2005; [11]). As shown in Fig. 5C, the ES4 epitope reacted with anti-sera from all of the pigs infected with these four viral strains. The antibody response was detected by 14 dpi, and lasted more than 63 dpi.

Objective 4. To determine the ability of the nsp2 marker to differentiate vaccinated pigs from pigs infected with Type 2 field strains.

Further testing of serum samples from a group of experimental pigs infected with Type 2 prototypic strain, VR2332 showed no reactivity with the ES4 epitope on the ELISA (date not shown). Therefore, another serological test will be required to differentiate animals infected with Type 1 viruses from those animals infected with Type 2 viruses.

VIII. Discussion:

Improved protection through immunization requires a new generation of PRRSV vaccines. One of the key steps in future vaccine development is to include markers for the diagnostic differentiation of vaccinated animals from those that are naturally infected with wild-type virus. Marker vaccines are important in programs aimed at controlling or eradicating virus infections in food animals, as well as in companion animals [4, 5, 6, 50]. Herpesvirus marker vaccines were among the first proved to be effective in the field [7, 51], followed by various genetically modified RNA viruses, such as classical swine fever virus [49, 56] and Rinderpest virus [53]. In EAV eradication programs, since horses are actively involved in international trade and traffic, a marker vaccine is required by some legislative authorities. Discrimination between vaccination and infection is becoming a ruling issue [8]. Similarly, with a PRRSV elimination program, the international trade in pigs and pork will require a marker vaccine in the future. Furthermore, as the world is progressively moving toward elimination of PRRSV, serosurveillance is an essential tool to verify the disease status. The currently available conventional vaccines are unable to allow differentiation between wild-type infection and vaccination. Thus, serosurveillance is impossible in the face of ongoing vaccination or for several months after vaccination has ceased. Clearly, a marked vaccine would be of great benefit. In this study, we have evaluated two genetic markers in the nsp2 region of the virus. The positive marker (GFP insertion) will allow detection of the animals that have been vaccinated, while the negative marker (ES4 epitope deletion) would allow detecting the presence of wild-type virus in the animals.

Marker vaccines are only useful if suitable tests (companion diagnostic tests) are available to monitor the vaccination levels and to follow the spatial course of the infection. Our results showed that the GFP antigen-based ELISA detected a high level of the anti-GFP response in the group of pigs infected with the

marker virus. The ES4 epitope-based ELISA also detected a high level of antibody response in the group of pigs infected with the parental virus, but appeared to develop slower than that of the anti-GFP response. A high level, robust anti-GFP response [mean optical density (OD) = 1.74] can be detected by 14 dpi in marker virus infected pigs, while anti-ES4 antibody response (mean OD = 0.88) was detected by 21 dpi and reached higher levels by 28 dpi (mean OD = 1.38) in pigs infected with wild-type virus. One drawback of using the ES4 epitope ELISA is that the ES4 epitope is not conserved between the genotypes. The ES4 epitope is 165 nucleotides in length, and is located between AA351 to AA405 of nsp2 (AA736 to AA790 of SD01-08 ORF1a), which possesses the highest hydrophilic values [23] among the six B-cell epitopes identified on the nsp2 of Type 1 virus [41]. Analysis of the currently available nsp2 amino acid sequences of Type 1 PRRSV [11, 34] showed that this region possesses 63.6% to 100% amino acid sequence identity within the Type 1 genotype. Protein sequence analysis showed that the ES4 epitope region, AA736 - AA790, actually contains seven small B-cell epitopes (PepTool, BioTools, Inc., Edmonton, Alberta, Canada). Epitope AA745 - AA754 and AA768 – AA780 are well conserved within the Type 1 genotype. Our ES4 ELISA data was consistent with the protein sequence analysis, showing that the ES4 epitope can react with sera samples from animals infected with four representative field strains of Type 1 PRRSV. However, the ES4 epitope does not react with serum samples from animals infected with Type 2 isolates. In comparison of the identified B-cell epitopes on nsp2 region [10, 41], none of the epitopes identified in the nsp2 region was conserved between Type 1 and Type 2 isolates. Our ES4 epitope deletion mutant was constructed based on modifications of the epitope in a Type 1 PRRSV backbone. Therefore, another diagnostic assay is required to differentiate pigs vaccinated with the ES4 epitope deletion mutant from those pigs infected with Type 2 field strains. We have recently developed a nsp7-based ELISA assay to specifically identify and differentiate Type 1 and Type 2 PRRSV (manuscript in preparation), which could be another additional test adding to the future marker vaccine package.

Our results suggested that the ES4 epitope in the nsp2 region is non-essential for PRRSV replication but may play an important role in viral attenuation and pathogenesis *in vivo*. Our previous study [12] showed that insertion of the GFP alone did not substantially reduce the *in vitro* growth properties of the virus. However, when we deleted the ES4 epitope downstream of the GFP, viral titer was reduced at least two-logs in

comparison to that of parental viruses. *In vivo* characterization further demonstrated that the GFP/ΔES4 marker virus was attenuated with a lower level of viremia and higher level of neutralizing antibody response than that of wild-type virus. Protein sequence analysis showed that the ES4 epitope region contains the highest hydrophilic value on the nsp2 [23]. A high level of antibody response to ES4 epitope was observed in this study, but did not correlate with the viral neutralizing antibody response. One of the common strategies employed by the pathogens to survive is to initially present nonprotective and/or type restricted immunodominant epitopes to the host immune system, resulting in a deceptive, or decoy response and dysregulating immune responses, with bias toward nonprotective epitopes, while attenuating or preventing the recognition of other more conserved and protective epitopes. This type of "decoy response" results in type restricted protection and semiprotective immunity, which may be a mechanism responsible for limited vaccine efficacies [15, 19, 20, 26, 29, 37]. An example is hepatitis B virus, where the rapid and early antibody response to an immunodominant epitope on the Ag protein is delayed and selectively down-regulated the maturation of epitope-specific B/T cell specific responses to a less immunodominant epitope [52]. For HIV, an immunodominant determinant, the V3 loop, appears to facilitate immune escape and is involved in both isolate-restricted neutralization and limited protection [37]. In PRRSV, a panel of six B-cell epitopes was identified in the nsp2 region of Type 1 viruses [41], while eighteen B-cell epitopes were identified in the nsp2 region of Type 2 viruses [10]. Studies from our laboratory and others [10, 24, 41] also showed that the nsp2 induced a strong, early non-neutralizing antibody response. Elimination of other epitopes on the nsp2 region is on going in our laboratory. Further defining the function of these immunodominant epitopes on nsp2 and detailing the mechanisms involved in host response will be important for future vaccine designs.

Interestingly, the ES4 epitope deletion improved the stability of the GFP insertion in the nsp2. The mechanism for tolerating foreign gene insertion or epitope deletion in this region remains to be determined. The nsp2 may have size limitations for foreign gene insertion. This result is consistent with previous findings in Type 2 viruses where the replacement of a 110 amino acid region of nsp2 with GFP improved the stability of GFP expression [25]. Another interesting observation is the loss of GFP fluorescence *in vitro* and *in vivo* although the GFP gene remained intact. Sequence analysis identified the Arg-97 to Cys mutation in the GFP.

The Arg-97 to Cys mutation is exactly the same amino acid mutation identified on GFP that was inserted into the nsp2 region of a Type 2 virus [25]. As indicated by Kim et al [25] that Arg-97 plays a key role in the chromophore formation of GFP, which suggests that the chromophore formation may affect nsp2 function. In addition, since Cys is the amino acid normally involved in forming the disulfide-bond in the protein, we speculate that the additional disulfide-bond maybe required in maintaining the correct conformation of nsp2 in order to function. The defined mechanism of these observations remains to be determined. Nevertheless, the GFP retains its immunogenicity *in vivo*, and functions as an excellent positive marker for differentiation between vaccinated and wild-type virus infected animals.

VIII. References

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Table 1. Design of GFP/ΔES4 marker virus *in vivo* study.

Group (n = 6)	virus	Serum sample (dpi)	Challenge virus	Temperature records (dpi)	Necropsy (dpi)
1	GFP/ΔES4	1, 7, 14, 21, 28, 35, 42*, 49, 56, 62	SD 03-15	1-7, 42-49	62
2	Parental	1, 7, 14, 21, 28, 35, 42*, 49, 56, 62	SD 03-15	1-7, 42-49	62
3	mock	1, 7, 14, 21, 28, 35, 42*, 49, 56, 62	SD 03-15 (3 pigs) Mock (3 pigs)	1-7, 42-29	62

Table 2. Primers used for sequencing and ELISA antigen expression.

Primer name	Sequence*	Genome position in SD01-08[@]
nsp2-2144F	5’ gtc tgt gtc ctt gga cga gtg	2144 - 2164
nsp2-2694R	5’ cca agc ggc caa gga tag atc	2694 - 2714
pET-EGFP-F	5’ c*g gat cc***a tgg tga gca agg gcg agg agc**	–
pET-EGFP-R	5’ cct *aag ctt* **cct tgt aca gct cgt cca tgc cg**	–
pET-ES4F1	5’ gc *aga tct* tca gac tcc aag aga gaa	2427 - 2444
pET-ES4F2	5’ gc *aga tct* ggt ggt ggt ggt tcc tca gac tcc aag aga gaa	2427 - 2444
pET-ES4R	5’ at ccc *aag ctt* gcg g*gg atc c*cg gga caa atc ctc g	2577 - 2591

*Nucleotides of GFP are bolded, restriction enzyme sites are italized and underlined;
@ Numbers correspond to nucleotide positions within the SD01-08 genome.

Table 3. Viral load in serum measured by quantitative PCR at 21 days post-challenge with a heterologous strain, SD03-15.

Pig numbers	Viral load in serum (copies/ml)			
	GFP/ΔES4	Parental	Negative / challenged*	Negative
1	0	2.7E + 02	N/A	0
2	7.4E + 02	0	N/A	0
3	0	1.2E + 04	N/A	0
4	N/A	0	3.6E + 04	N/A
5	0	2.6E + 03	3.3E + 04	N/A
6	1.7E + 02	1.1E + 04	3.6E + 04	N/A
Mean	**1.8E + 02**	**4.3E + 03**	**3.5E + 04**	**0**

* Three of the pigs in negative group challenged with the heterologous strain, SD 03-15 at 42 dpi.

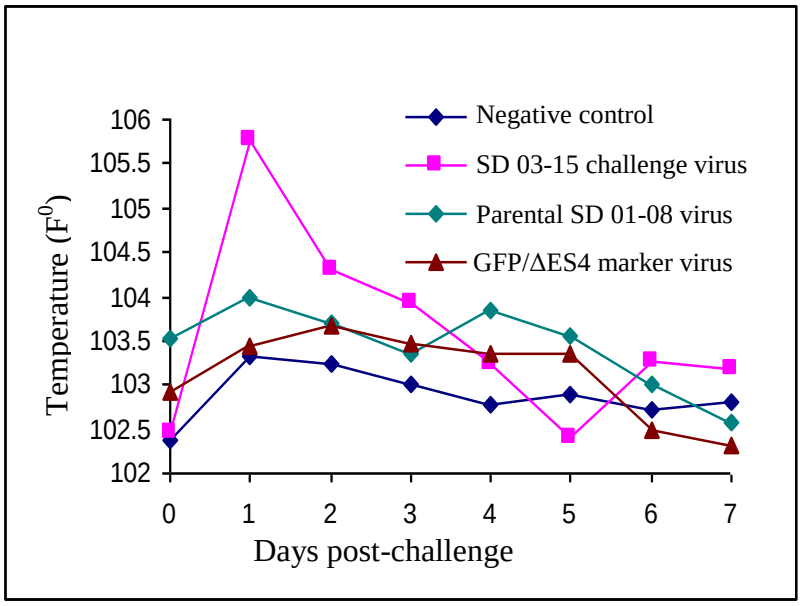

Fig. 1. Comparison of mean temperature responses between different challenge groups. Rectal temperatures were taken one day before challenge, and 7 days after challenge.

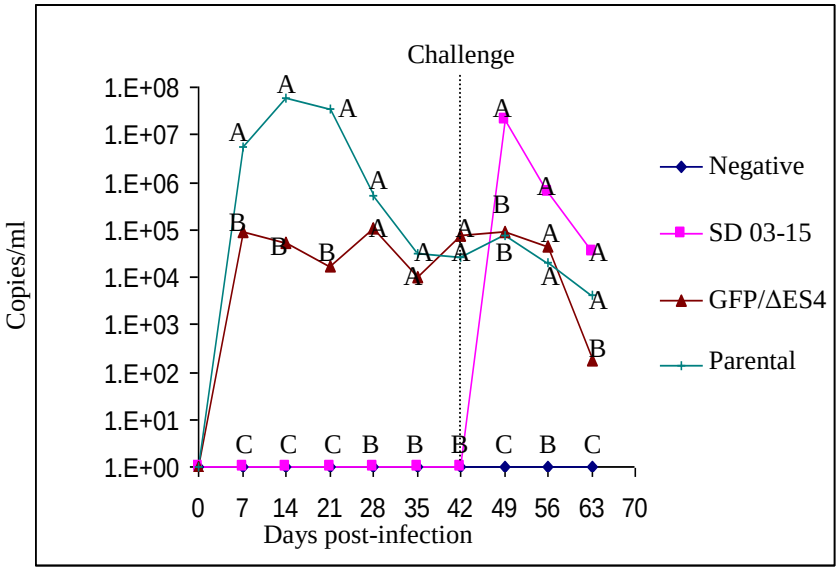

Fig. 2. Comparison of viral load in serum samples between different treatment groups. Viral load was quantified by real-time PCR, and the result was interpreted as RNA copy numbers per ml. Pigs were challenged at 42 dpi, shown as a vertical dotted line. Results are expressed as mean values (n=6). At each day post-infection, mean viral load with different capital letters (A, B or C) differ significantly ($P < 0.05$).

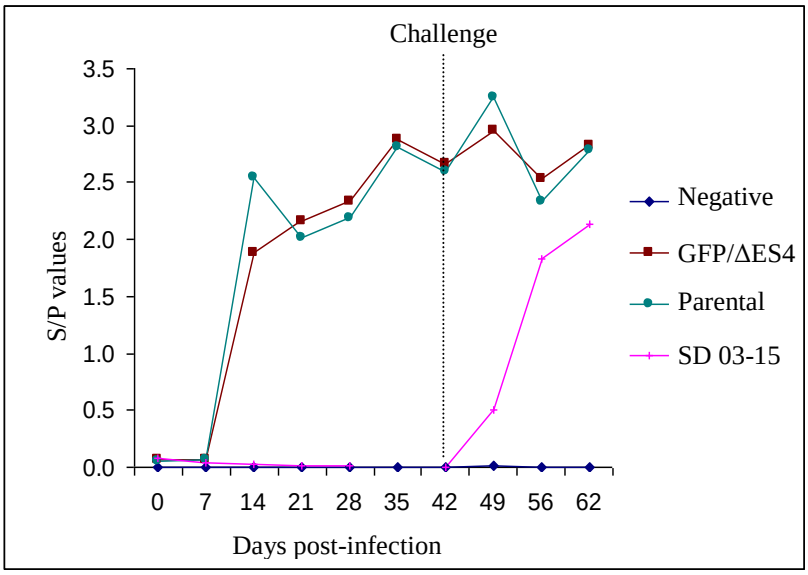

Fig. 3. Kinetics of the serum antibody response for pigs infected with GFP/ΔES4 marker virus or parental virus. PRRSV-specific serum antibodies were measured by IDEXX HerdChek® PRRSV ELISA 2XR kit. S/P ratios of greater than 0.4 are considered positive. Pigs were challenged at 42 dpi, shown as a vertical dotted line. Results are presented as mean values (n=6).

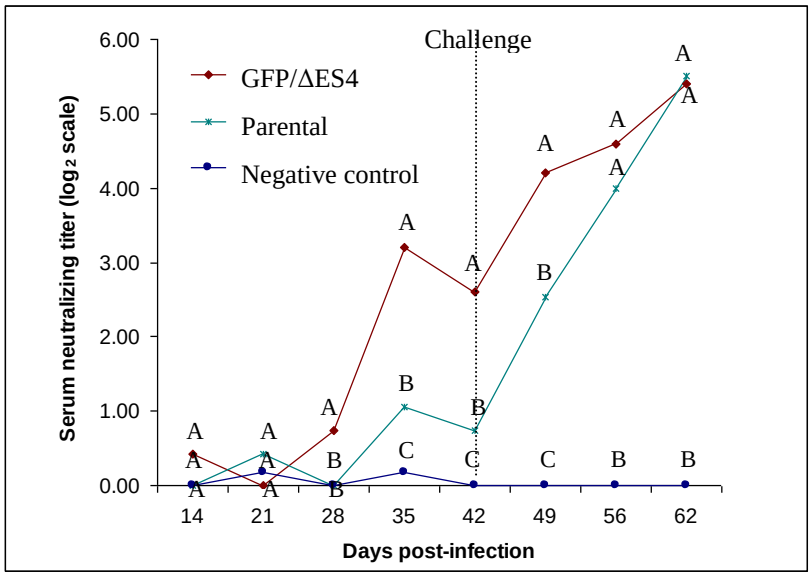

Fig. 4. Virus-neutralizing antibody responses in serum samples from different treatment groups. The neutralizing antibody response was determined by fluorescent focus neutralization assay. Results were interpreted as a 90% reduction of the viral infection, and the neutralizing antibody titers were presented as mean value (n=6) and expressed on a $\log_2$ scale. The parental SD01-08 virus was used for the viral neutralizing assay. At each day post-infection, means with different capital letters (A, B or C) differ significantly ($P < 0.05$).

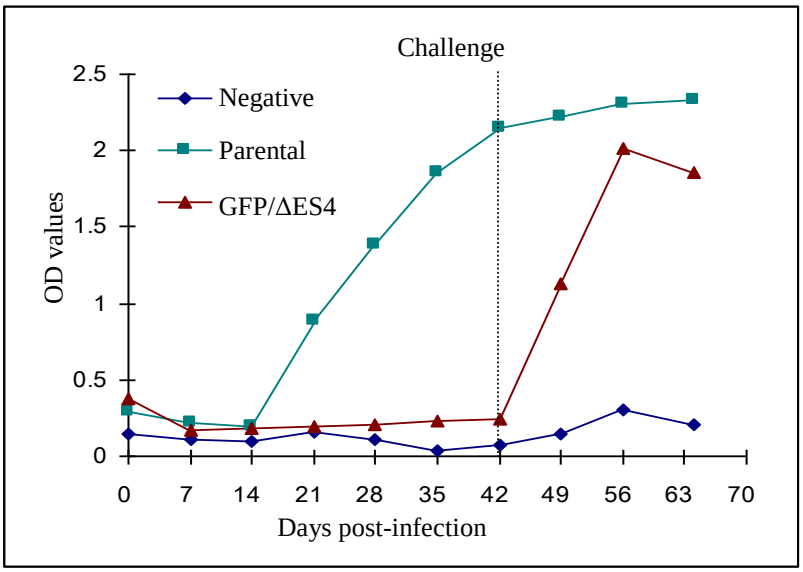

Figure 5A. ES4 epitope-based ELISA.

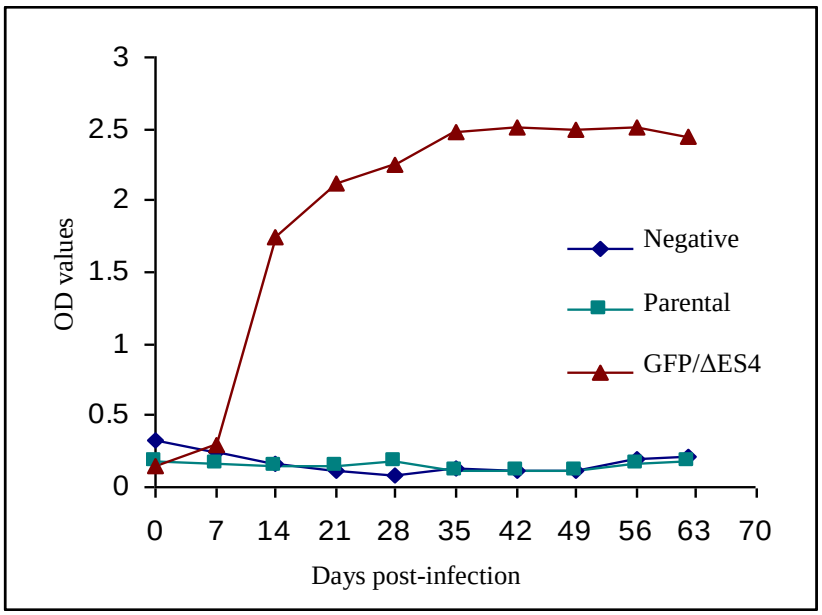

Figure 5B. GFP antigen-based ELISA.

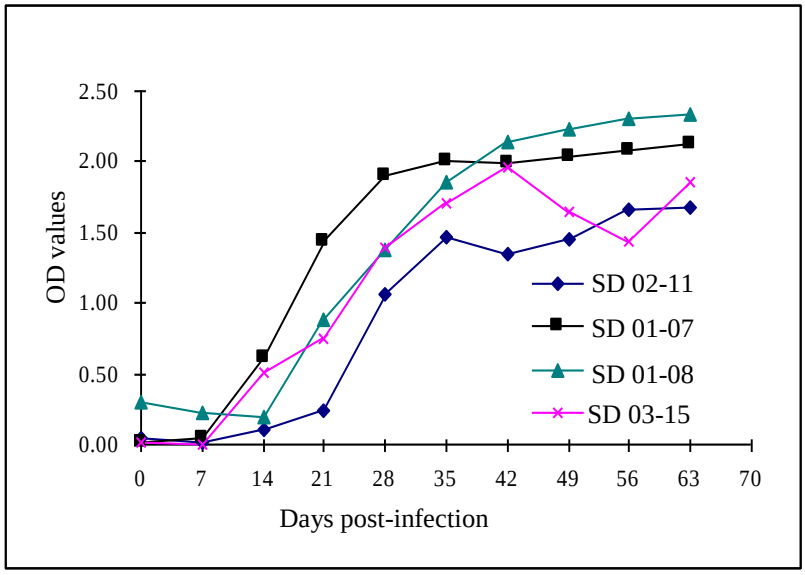

Figure 5C. ES4 epitope-based ELISA.

Fig. 5. GFP antigen or ES4 epitope-based ELISAs to differentiate animals vaccinated with GFP/ΔES4 marker virus from those pigs infected with the wild type viruses. Pigs were challenged with a heterologous virus, SD 03-15 at day 42 post-infection shown as a dotted vertical line. A & C. ES4 epitope-based ELISA; B. GFP antigen-based ELISA. Note the infection of Group 1 pigs, with the GFP/ΔES4 marker virus, did not induce a detectable antibody response against the deleted ES4 epitope (Fig. 5A), but induced strong antibody response against the GFP antigen (Fig. 5B). In contrast to the Group 2 pigs, infected with parental virus, antibody specific to ES4 recombinant protein can be detected at 21 dpi (Fig. 5A), while no specific antibody response was detected against GFP antigen (Fig. 5B); C. ES4 epitope reactivity with the anti-sera from pigs infected with four representative Type 1 PRRSV strains. Results are presented as mean values (n = 6).