RESEARCH REPORT
SWINE HEALTH

Title: Development of a broadly protective PRRS vaccine candidate: Application of non-toxic enterotoxin and *E. coli* as the adjuvant-delivery system - **NPB #12-127**

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Industry Summary:

Porcine reproductive and respiratory syndrome (PRRS) continues to be a threat to the swine industry. Currently, both modified live virus (MLV) and inactivated PRRS vaccines have been licensed for use in the field. However, these vaccines are not always efficacious in protection against infection from a wide array of heterologous PRRS virus (PRRSV) isolates in the field. Safety is also a concern for the MLV, which might be reverted to a virulent strain and subsequently shedding the reverted viruses. In this study, we developed an epitope-based candidate vaccine using a set of consensus B- and T- cell epitopes derived from PRRSV proteins of nsp9, nsp10, GP4, GP5, and N. Since epitopes expressed alone are moderately immunogenic or insufficient in inducing high level of protective immunity, these epitopes were genetically fused with a strong adjuvant, LT_{192}, which is a detoxified bacterial toxin LT (heat-labile enterotoxin produced by *E. coli*). The epitope-toxin chimera was subsequently transformed in a swine non-pathogenic *E. coli* strain to use as a live attenuated vaccine. The potential application of this epitope-toxin chimera in PRRS vaccine development was determined in a pig model. The result demonstrated that specific T-cell immune responses were stimulated after immunization. In comparison to the non-immunized pigs, pigs immunized with the candidate vaccine showed improved protection against virulent PRRSV challenge, with about 50% decrease of pneumonic lung lesions and 10-fold reduction of the viral load in serum and lung tissues at 14 days post challenge. This study establishes a vaccine construct platform and swine model system for peptide-based vaccine development against PRRSV and other swine pathogens. Advantages of our candidate vaccine in comparison to MLV or inactivated vaccines includes: 1) Epitope-based vaccines are safer to use than MLV vaccines, since there is no concern about MLV being reverted to virulent strain and subsequently shedding the viruses; 2) It includes the protective T-cell epitopes from PRRSV nonstructural proteins, which are not available from inactivated (killed) PRRS vaccines; 3) A non-pathogenic *E. coli* strain carries the epitope-toxin chimera construct to the animal, and the epitope-toxin antigen is designed to be expressed while the *E. coli* colonizes and replicates on the mucosal surface. Such type of vaccine is cheaper to produce and easy to deliver. In the future, it could be packaged with outside coating/feed materials to be produced as powder or pills and delivered as feed-additives, which would be easy to apply and prevent the traditional labor intensive immunization procedures. In addition, epitopes can be easily modified and new epitopes can be included in the construct based on the field epidemic strains. For more information, please contact Dr. Ying Fang at Department of Diagnostic Medicine and Pathobiology, Kansas State University, Manhattan, Kansas; Phone: 785-532-4452; E-mail: yfang@vet.k-state.edu.

Keywords: porcine reproductive and respiratory syndrome virus (PRRSV), heat-labile enterotoxin (LT), epitope-based vaccine, vaccine carrier, pig model.

Scientific Abstract:
Porcine reproductive and respiratory syndrome (PRRS) continues to be a threat to the swine industry. Current commercial available vaccines are not always efficacious in protection against infection from a wide array of heterologous PRRS virus (PRRSV) isolates in the field. Epitope-based vaccine represents a new approach to achieve protective immunity. The objective of this study was to construct and evaluate the immunogenicity of an epitope-based candidate vaccine for its potential application in future PRRS vaccine development. Initially, four multi-epitope antigens (chimera A-D) were constructed, which contain a set of consensus T- cell epitopes derived from PRRSV proteins of nsp9, nsp10, GP4, GP5, and N. To enhance immune responses of PRRSV T-cell epitopes, these multi-epitope antigens were genetically linked to a detoxified bacterial heat-labile enterotoxin (LT_{192}) as an adjuvant. These four epitope-toxin chimeras were transformed into a swine non-pathogenic *E. coli* strain to use as a potential live attenuated vaccine (designated as *E. coli* /epitope-toxin_mix). The *in vitro* expression of each multi-epitope antigen was detected in the supernatant of transformed *E. coli* culture. Immunogenicity of this candidate vaccine was evaluated in a PRRSV challenge pig model. The result demonstrated that specific T-cell immune responses were stimulated after immunization. At 28 days post vaccination, we observed the increased frequency of IFNγ+CD4+CD8+ cells and IFNγ+γδ T-cell populations in PBMCs stimulated by pooled synthetic peptides of T-cell epitopes. After challenge, strong IFNγ+γδ T cell response was observed. Peptide D2 (VRHHFTPSE) from N protein was identified as the epitope of γδ T cell lymphocytes; peptides A3 (CPGKNSFLDEAAYCNHL) and C3 (VRILAGGWCPGKNSFLD) from nsp10 were identified as epitopes of CD4+T lymphocytes; peptides B3 (VRGNPERVKGVLQNTRF) from nsp2 and C2 (KGRLYRWRSPVIIEK) from GP5 were identified as epitopes of CD8+T lymphocytes. In comparison to the non-immunized pigs, pigs immunized with the *E. coli* /epitope-toxin_mix showed improved protection against virulent PRRSV challenge, with about 50% decrease of pneumonic lung lesions and 10-fold reduction of the viral load in serum and lung tissues at 14 days post challenge. This study establishes a platform for future construction of epitope-based vaccines against PRRSV infection.

Introduction:
Porcine reproductive and respiratory syndrome virus (PRRSV) is a small RNA virus. It can be divided into distinct European (type I) and North American (type II) genotypes, sharing only about 63% nucleotide identity (Allende et al., 1999; Nelsen et al., 1999). Since 1999, viruses of both genotypes have been identified in North American swine herds (Ropp et al., 2004; Fang et al., 2004). 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), three unglycosylated membrane proteins (E, ORF5a and M) and a nucleocapsid protein. The replicase-associated genes, ORF1a and ORF1b, situated at the 5' end and represent nearly 75% of the viral genome. The ORF1a and ORF1b encode two long nonstructural polyproteins, which are processed into 14 nonstructural proteins (nsps) by a complex proteolytic cascade (Fang and Snijder, 2010). Certain nsp regions that encode key enzymes for viral replication, such as polymerase (nsp9) and helicase (nsp10), are highly conserved, which makes these regions as attractive targets for vaccine development.

PRRSV continues to be a threat to the swine industry, which costs the US swine industry at least $600 million annually (Miller, 2011). Currently, both live attenuated and inactivated vaccines have been licensed for use in the field. However, these vaccines are not always efficacious in protection against infection from a wide array of heterologous field isolates. Safety is also a concern for the MLV, which might be reverted to a virulent strain and subsequently shedding the reverted viruses. Therefore, development of a broadly protective PRRS vaccine has become a major challenge.

One novel approach for vaccine development is the utilization of conserved immunogenic epitopes that elicit responses by both humoral and cellular arms of the immune system. A cocktail of conserved epitopes can induce a variety of different immune responses simultaneously, which could be effectively against a heterogeneous virus population. Epitope-based vaccines have been tested in various viral pathogens that also

possess the characteristics of extensive genetic and antigenic variation, such as foot and mouth disease virus (Strohmaier et al., 1982), human papillomavirus (HPV; Van Driel et al., 1999; Muderspach et al., 2000) and HIV (Spearman et al., 2009; Gruters et al., 2002). Some of these vaccines have been used in HPV and HIV clinical trials (Van Driel et al., 1999; Muderspach et al., 2000; Spearman et al., 2009). In PRRSV, a set of immunogenic epitopes have been identified. A B-cell epitope in GP5 was determined to be the major target for induction of neutralizing antibody in both genotypes of PRRSV (Ostrowski et al., 2002). In type I PRRSV, a neutralizing epitope in GP4 was identified (Meulenberg et al., 1997; Costers et al., 2010). For T-cell epitopes, two distinct regions in GP5 of type I and type II strains were determined to contain immunodominant T-cell epitopes (Vashisht et al., 2008; Diaz et al., 2009). In addition, Diaz et al (2009) identified two T-cell epitopes in GP4 and four T-cell epitopes in nucleocapsid (N) protein of type I PRRSV; Wang et al (2011) identified four T-cell epitopes in the membrane (M) protein of type II PRRSV. Recent advanced knowledge in PRRSV nonstructural proteins (nsps) also allows us to explore the immunogenic targets in these regions (Fang and Snijder, 2010). Seven potential T-cell epitopes in nsp9, nsp10 and nsp11 regions of type II virus were identified (Parida et al., 2011). In this proposed study, two B-cell epitopes from GP4 and GP5, and ten T-cell epitopes from nsp9, nsp10, GP4, GP5 and N proteins are selected for constructing an epitope-based vaccine. This "epitope cocktail" is expected to be able to induce sufficient protective immune response to prevent PRRSV infection.

The main limitation of epitope-based vaccines, compared to conventional whole virus-based vaccine, is their low immunogenicity. To solve this problem, we conjugated the multi-epitopes to a strong adjuvant, heat-labile (LT) enterotoxin, which is produced by *E. coli.* The LT has been used successfully as mucosal adjuvants to enhance host immunity toward various pathogens that cause respiratory diseases (Lemere et al., 2002; Cheng et al., 1999; Hathaway et al., 1995). LT_{192}, an LT mutant, maintains full adjuvant function as native LT but with reduced toxicity, represents an ideal choice of adjuvant to enhance immune responses. LT_{192} adjuvant has been successfully used in our laboratory to increase antigenicity of the non-immunogenic STa and STb antigens in developing vaccines against porcine post-weaning diarrhea disease (Zhang et al., 2010 a, b). In our recent study, the LT_{192} has also been incorporated into the epitope-based vaccine construction for swine influenza virus (SIV). The SIV epitope-LT_{192} vaccinated pigs showed protection against the H1N1 virus challenge, with significant reduction of the viral load in nasal secretion and gross lung lesions (Sun et al., 2013).

Another challenge in veterinary medicine is how to deliver the vaccine into animals in a cost-effective manner. Subunit vaccines typically are not practical for pigs or other livestock animals due to high cost in subunit vaccine production, laborious vaccine administration, but marginal profits in livestock industries. Our recent study demonstrated that a non-pathogenic *E. coli* can be served as an efficient *in vivo* delivery system, and it has been successfully applied in the development of vaccines against enterotoxigenic *E. coli* infection (Ruan and Zhang, 2013). In this study, we adapted the technology and transformed the PRRSV epitope-toxin fusion construct into a non-pathogenic *E. coli* to make a candidate oral vaccine. Pigs were orally fed with this candidate vaccine and the protective immunity induced by the PRRSV epitope-toxin fusion antigen was evaluated by challenging with a virulent PRRSV.

Objectives:

Objective 1). To construct an epitope-toxin chimera using a set of consensus PRRSV epitopes and a strong adjuvant LT_{192};

Objective 2). To introduce the epitope-toxin chimeric construct into a non-pathogenic porcine *E. coli* for *in vivo* delivery;

Objective 3). To assess the efficacy of epitope-toxin antigen in prevention of PRRSV infection using a PRRSV challenge pig model.

Materials & Methods:

Obj. 1) To construct an epitope-toxin chimera using a set of consensus PRRSV epitopes and a strong adjuvant LT_{192}.

Experimental design:

As listed in Table1, a total of 9 epitopes from PRRSV nsp9, nsp10, GP4, GP5, and N proteins were used for constructing epitope-toxin antigen. These epitopes were selected based on their immunogenic properties and also the percentage of conservation within /between PRRSV genotypes. A total of five multi-epitope constructs were made with construct E contains all 9 epitopes, and each individual construct A-D contains 2 or 3 epitopes (Table 1). Each multi-epitope is genetically linked and fused with the LT_{192} gene for producing a fusion gene (Figure 1). Each fusion gene was cloned into the expression vector, pBR322.

Experimental methods:

PRRSV epitope-toxin construction: Each of the epitope was linked into a multi-epitope peptide using a method we described previously (Fang et al., 2008). A flexible peptide linker, GGGGS, was added between the epitopes to help display the epitopes. The oligonucleotide encoding each multi-epitope was synthesized as a synthetic gene and cloned into the pBR322 vector that contains the LT_{192} gene to generate a chimeric epitope-toxin construct.

Obj. 2) To introduce the epitope-toxin chimeric construct into a non-pathogenic porcine *E. coli* for *in vivo* delivery.

Experimental design:

Each of the pBR322 plasmid containing PRRSV epitope-toxin was transformed into a non-pathogenic porcine *E. coli* to generate a modified live *E. coli* carrying the PRRSV epitope-toxin chimera. Before testing in pigs, the expression of each chimeric antigen was verified *in vitro* by Western blot and immunoprecipitation. To ensure that the LT_{192} in a chimeric construct was expressed in a correct conformation and also remained its ability to bind to the ganglioside GM1 receptor on host epithelial cells, GM1 ganglioside enzyme-linked immunosorbent assay (GM1-ELISA) was performed.

Experimental methods:

Bacterial strain: The host bacterium for carrying and *in vivo* expression of PRRSV epitope-toxin antigen is an non-pathogenic porcine *E. coli* (strain 1836-2), transformed with the plasmid pDMS158 to express 987P fimbria but not containing enterotoxin (Choi et al., 1999; Francis et al., 1991; Zhang et al., 2010).

Construction of a modified live E. coli expressing the PRRSV epitope-toxin antigen: The plasmid pBR322 containing PRRSV epitope-toxin fusion gene was used to transform *E. coli* 1836-2 as described in our previous publication (Ruan and Zhang, 2012). Expression of each epitope-toxin antigen in *E. coli* /epitope-toxin was examined using Western blot, immunoprecipitation and GM1-ELISA as described below.

Recombinant protein expression and Western blot: Transformed *E. coli* containing PRRSV epitope-toxin was cultured in LB medium with ampicillin, and bacteria were harvested by high speed centrifugation. Secretory proteins were harvested from culture supernatant and cellular total proteins were extracted from the pellet. The expression of PRRSV epitope-toxin recombinant proteins were confirmed with Western blot and immunoprecipitation using anti-LT specific antibody. Detailed methods for protein purification and detection were described in our previous publications (Zhang et al., 2006; Brown et al., 2009; Fang et al., 2012)

GM1-ELISA: The experimental procedure for GM1-ELISA was described previously (Svennerholm et al., 1978). Briefly, ELISA plates were coated with GM1 ganglioside at 4^{o}C overnight and blocked with 5% BSA for 2h at 37^{o}C. The PRRSV epitope-toxin recombinant proteins were added to the plate and incubate for 1h at 37^{o}C. After washing, anti-toxin polyclonal antibody was added and HRP-conjugated goat anti-mouse IgG was used as secondary antibody. Tetramethylbenzidine (TMB) was used as a substrate for color development and the absorbance at 450 nm was measured by a microplate reader.

Obj. 3) To assess the efficacy of epitope-toxin antigen in prevention of PRRSV infection using a heterologous virus challenge pig model.

Experimental design:

Table 2 shows the experimental design of animal experiment. Pigs were immunized with the mixture of four modified live *E. coli* 1836-2 with each bacterium carries an epitope-toxin chimera (construct A-D). This mixture of *E. coli* is designated as "*E. coli*/epitope-toxin_mix" in the following description. At 28 days post immunization, pigs were challenged by a virulent PRRSV NVSL-97-7895 (GenBank accession no. AY545985). The degree of protective immunity was assessed based on the observation of clinical signs, measurement of viral load and cellular immune responses. At 14 days post challenge, animals were euthanized and pathogenic lung lesions were evaluated.

Experimental Method:

Animals / challenge groups: As presented in Table 2, twenty-four 3 weeks old pigs were randomly divided into four groups housed separately in animal isolation facilities at Kansas State University. After a 4-day acclimation period, group 3 and 4 pigs were orally immunized with 3x 10^9 CFUs of an overnight grown culture of non-transformed *E. coli* 1836-2 and the experimental vaccine - *E. coli*/epitope-toxin_mix, while other two groups of pigs remain unimmunized. At 28 days post vaccination (dpv), group 2-4 pigs were challenged with the virulent PRRSV. Group 1 pigs were remained as negative control. Challenge virus was administered intramuscularly to pigs with a dose of 3 ml per pig at 1 x 10^5 $TCID_{50}$/ml. Pigs were observed daily for clinical signs during first week after challenge. Blood samples were obtained from all pigs as scheduled in Table 2. Pigs were euthanized at 14 days after challenge to evaluate microscopic and macroscopic lung lesions.

Quantification of viral load: For the detection of viral RNA and determination of viral load, serum samples from 35 and 42 dpv and tissue samples (lung) were examined using the real-time RT-PCR as we described in our previous publications (Fang et al., 2008; Chen et al., 2010).

Flow cytometry analyses: The phenotypes and frequencies of lymphoid cell populations were determined flow cytometry analysis using immunostained PBMCs collected at 28 and 42 dpi. PBMC were stimulated with pooled or individual peptide (2μg/ml each, Pep), PRRSV virus (0.1 MOI) (virus), PHA (10μg/ml) or complete media (cell control, CC) for 48 h at 37°C in a 5% CO2 atmosphere. For the last 7 h of incubation, brefeldin A and monensin were added. PBMCs were first surface-labeled using pig lymphocyte-specific mAbs directly conjugated with different fluorochromes. Cells were fixed with 1% paraformaldehyde and permeabilized with a cell-permeabilization buffer (85.9% deionized water, 11% PBS with no Ca or Mg, 3% formaldehyde solution, and 0.1% saponin) overnight at 4°C. Cells were washed and stained with fluorochrome-conjugated pig IFNγ or its isotype control mAb (BD Biosciences) in 0.1% saponin containing fluorescence-activated cell-sorting (FACS) buffer. Immunostained cells were acquired using the FACS Aria II (BD Biosciences) flow cytometer and analyzed using FlowJo (Tree Star, Ashland, OR, USA) software. All specific immune population frequencies were presented as the percentage of total lymphocytes.

Data Analysis: Analysis of variance (ANOVA) was be used to analyze the data. Significant differences between treatment groups were evaluated using the Tukey-Kramer Honestly Significant Difference multiple comparison test. All statistical analysis was performed using JMP 5.1 (SAS Institute, Cary, NC).

Results: Report your research results by objective.

Objective 1) To construct an epitope-toxin chimera using a set of consensus PRRSV epitopes and a strong adjuvant LT_{192};

As shown in Table 1, a total of 9 epitopes from PRRSV nsp9, nsp10, GP4, GP5 and N proteins were used for constructing epitope-toxin antigen. These epitopes were selected based on their immunogenic properties and also the percentage of conservation within /between genotypes. The oligonucleotides expressing these epitopes were genetically linked with a GGGGS link and fused with the LT_{192} gene for producing a fusion gene (Figure 1). The PRRSV epitope insertion site was determined by protein structure analysis of LT, in which the N-terminal 19-149 aa encoding region of LTA1 was replaced by PRRSV epitopes, and an N-terminal porcine LT promoter and signal peptide were added (Figure 2). Such construction is designed not disrupting the conformation of LTA2 and allowing formation of the LTB pantamer structure (Figure 1). Furthermore, the PRRSV epitope-LT_{192} fusion antigen needs to be expressed as soluble secretory protein. To meet these

requirements, we constructed five chimeras with each of the chimeric genes A-D containing 2 or 3 PRRSV epitopes while chimera E contains all 9 PRRSV epitopes.

Objective 2) To introduce the epitope-toxin chimeric construct into a non-pathogenic porcine *E. coli* for *in vivo* delivery;

PRRSV epitope-LT fusion antigen construction and expression

The chimeric genes A-E were subsequently cloned into the pBR322 vector (Figure 2). Each of the plasmid pBR322 containing PRRSV epitope-toxin fusion gene was used to transform *E. coli* strain 1836-2 and the constructed bacteria were designated as *E. coli*/epitope-toxin A-E. The expression of the holotoxin-structured epitope-LT_{192} antigens were determined by Western blot using an antibody specific to His-tag. As shown in Figure 3A, fusion antigens from construct A-D were detected, but no specific fusion protein was detected from construct E, which was designed to express all 9 epitopes. This construct was not included in the following analysis.

Under the nature condition, LT will be secreted to the cell surface and LTB subunit will be subsequently cleaved from LT as a secretory protein. Therefore, the expression of LTB subunit was determined by immunoprecipitation using the supernatant harvested from transform bacterial culture. As shown in Figure 3B, the 14 kDa LTB protein was specifically precipitated by anti-LTB antibody. These results confirmed that the PRRSV epitope-LT_{192} antigens from chimeras A-D were expressed in transformed *E. coli* and these antigens were expressed as soluble proteins.

Receptor binding activity of LT from chimeras A-D

To further determine that LT in each chimeric construct maintains its biological activity, total cellular proteins and proteins from bacteria cultural supernatant were analyzed in GM1-ELISA. As shown in Figure 4, higher level of ELISA OD values were obtained from chimeric construct A-D, the OD values are comparable with that from *E. coli* expressing LT. As we expected, only background ELISA reactivity was detected in non-transformed host bacteria *E. coli* 1836-2, which does not express LT. This result further confirmed that secretory form of each chimeric epitope-LT protein was expressed and these proteins were able to maintain their biological activity by binding to the GM1 receptor.

Objective 3) To assess the efficacy of epitope-toxin antigen in prevention of PRRSV infection using a PRRSV challenge pig model.

Virus load in serum and lung tissues

Immunogenicity of PRRSV epitope-toxin chimeras were evaluated in a nursery pig model. Four groups of pigs (n=6) were used. Group 3 and 4 pigs were orally immunized with the non-transformed *E. coli* 1836-2 and the experimental vaccine - *E. coli*/epitope-toxin_mix, respectively. Groups 1-2 pigs were unimmunized. Pigs were boosted with a same dose of candidate vaccine at 14 days post vaccination (dpv). At 28 dpv, group 2-4 pigs were challenged with the virulent PRRSV strain NVSL97-7895. Group 1 pigs were remained as negative control. After challenge, viral load in serum samples was determined by real-time RT-PCR. As shown in Fig. 5A, the vaccinated group pigs had 10-fold lower amounts of virus comparison to non-vaccinated group of pigs at 14 days post challenge (dpc). There was no significant difference in viral load between these two groups of pigs at 7 dpc, although slightly lower mean viral load was detected in vaccinated group of pigs. Viral load in lung tissue samples collected at 14 dpc was further analyzed. A lower amount of virus was consistently detected in these tissues from vaccinated group of pigs in comparison to those from non-vaccinated group of pigs (Fig. 5B).

Lung pathology

At necropsy, lung pathology was evaluated. There was about 50% decrease of gross lung lesions in vaccinated group of pigs in comparison with that of non-vaccinated group of pigs (Fig. 6A). Histopathological analysis consistently showed the lower level of microscopic lung lesions in pigs immunized with the candidate vaccine

(Fig. 6B). As expected, no apparent lung lesions were observed in negative control/non-challenged group of pigs.

Enhanced frequency of IFNγ-secreting lymphocytes in candidate vaccine-immunized pigs

PBMCs isolated from pigs at 28 dpv and 14 dpc were immunostained and analyzed to determine the frequency of lymphoid immune cells, and IFNγ+ lymphocyte subsets. For PBMCs collected at 28 dpv, cells were stimulated with pooled peptide (Table 1; 2μg/ml each, **Pep**), inactivated PRRSV (stain NVSL-97 at 0.1 MOI) **(virus)**, PHA (10μg/ml) or complete media (cell control, **CC**) as a control to detect virus-specific lymphocyte responses. As shown in Table 3, PRRSV peptide stimulation in PBMCs from vaccinated pigs showed increased frequency of IFNγ+CD4+CD8+ cells comparing to unstimulated cells, and a similar result was obtained in virus-stimulated PBMCs. The IFNγ+CD4+CD8+ cells were significantly higher in PBMCs from group 4 pigs compared to that from groups 1+2 and 3 pigs. The frequency of activated γδ T cells in PBMCs from group 4 pigs was compared to that from other groups (Table 4). Consistent with the result presented in Table 3, significantly increased IFNγ+ γδ T-cell population was detected in both pooled peptide and virus-stimulated PBMCs in comparison to that in unstimulated cells (Table 4). After challenge, we further assessed the specificity of the T-cell response. Two γδ T-cell populations, IFNγ+/CD3+/γδ and IFNγ+/CD3-/γδ T cells were analyzed. Again, significantly increased IFNγ+/CD3+γδ (Table 5) and IFNγ+/CD3-/γδ (Table 6) T-cell populations were detected in pooled peptide stimulated PBMCs from group 4 pigs. We further analyzed the specificity of each individual peptide. Peptide D2 (VRHHFTPSE) from N protein was identified as the epitope of γδ T cell lymphocytes (Table 5 and 6); peptides A3 (CPGKNSFLDEAAYCNHL) and C3 (VRILAGGWCPGKNSFLD) from nsp10 were identified as epitopes of CD4+T lymphocytes (Table 7); peptides B3 (VRGNPERVKGVLQNTRF) from nsp2 and C2 (KGRLYRWRSPVIIEK) from GP5 were identified as epitopes of CD8+T lymphocytes (Table 7).

Discussion: Explain your research results and include a summary of the results that is of immediate or future benefit to pork producers.

PRRSV is one of the most important diseases for the swine industry, and causes substantial economic loss to swine producers worldwide. A safe and effective vaccine is critical to control and prevent this disease. As conventional vaccines insufficient to provide broad protection, new strategies to develop more effective vaccines have to be explored. In this study, we performed initial assessment of PRRSV epitope-toxin chimeric antigens for their feasibility in future epitope-based vaccine development. The epitope-toxin chimeric antigens contain a set of PRRSV consensus B-/T-cell epitopes, which includes epitopes from PRRSV nsp9, nsp10, GP4, GP5 and N proteins. In comparison to whole virus-based vaccines, peptide alone is generally poorly immunogenic; however, when presented with an appropriate adjuvant/carrier, it could provide sufficient level of protection. Therefore, in our study, we linked a set of PRRSV epitopes with a strong adjuvant, LT_{192} (detoxified LT) to form PRRSV epitope-toxin chimeric antigens. In previous studies, LT or LT subunit (LTB) has been used as an adjuvant to enhance the immunogenicity of viral antigens, and mostly applied as a separate component (Tamura et al., 1997; de Haan et al., 1998). The epitope-LT fusion approach used in this study presents a more efficient method for future vaccine production, since producing a single vaccine product would be more cost-effective than producing two or more separate products.

The epitope-toxin chimera was subsequently transformed into a swine non-pathogenic *E. coli* strain to use as a live attenuated vaccine. It is designed that the epitope-toxin antigen can be expressed as soluble secretory protein when the transformed *E. coli* colonizes and replicates on the host mucosal surface. Initially, we constructed an epitope-toxin chimera (construct E) by inserting a synthetic gene of 9 PRRSV epitopes into the N-terminus LTA1 region of LT. However, when expressed in *E. coli*, the epitope-toxin fusion protein could not be secreted in the cell culture supernatant. The 9 epitopes were subsequently divided into four groups (2 or 3 epitopes per group), and synthetic genes encoding each group of 2/3 epitopes were synthesized and inserted into the LTA1 region to generate PRRSV epitope-toxin constructs A-D. Transformed bacteria containing the constructs A-D were determined to be able to produce secretory epitope-toxin proteins in culture supernatant. When assayed in the GM1-ELISA, these secretory proteins exhibited GM1 binding activity, demonstrating its

ability to recognize the receptor *in vitro*. These data suggest that insertion of the large size synthetic gene, such as the gene encoding 9 epitopes, may affect the activity of LT.

The immunogenicity of the experimental vaccine - *E. coli*/epitope-toxin_mix was evaluated in a PRRSV challenge pig model. Our data demonstrate that specific T-cell immune responses were stimulated after immunization, suggesting that *E. coli* 1836-2 successfully delivered the T-cell epitopes to host immune system. At 28 dpv, we observed the increased frequency of IFNγ+CD4+CD8+ cells and IFNγ+ γδ T-cell populations. A certain level of protective immunity was observed in vaccinated animals after challenge. Although we observed significant increase of IFNγ secreting T-cell population, vaccinated pigs showed certain level of protection with one log reduction of viral load in serum, and ~50% reduction in gross lung lesions among immunized pigs. This result could be expected since our candidate vaccine only induced PRRSV specific T-cell epitopes. A complete protection requires activation of both humoral and cellular arms of the immune system. Our result showed that antibodies induced toward the PRRSV epitope-LT did not demonstrate PRRSV specific neutralizing properties. Thus, in the absence of neutralizing antibodies, immunized pigs could not prevent PRRSV infection. However, the contribution of antigen-induced T-cell immunity can be observed in the overall reduction in viral load and lung pathology scores. In the future, our candidate vaccine construct can be modified by including epitopes/antigens to stimulate neutralizing antibody response.

The ultimate goal for epitope-based antigen construction is to develop a broadly protective vaccine against PRRSV infection. Due to limited resource, this study only tested the immunogenicity of *E. coli* G58-1/epitope-toxin_mix upon immunization and challenged with a single virulent North American PRRSV strain. Future studies are needed to assess the cross protection induced by this candidate vaccine against genetically divergent PRRSV strains. Furthermore, our study was based on limited numbers of pigs/treatment group, future studies with larger number of animals and more treatment and control groups are needed to fully evaluate the protective efficacy of candidate vaccines developed from the *E. coli* /epitope-toxin platform. In summary, this study represents our initial effort towards developing an epitope-based PRRSV vaccine. The data suggest that epitope-LT chimeric antigen construction with a swine nonpathogenic *E. coli* carrier could be used as a platform for PRRSV vaccine development.

Table 1. PRRSV epitopes included in the epitope-toxin fusion antigen

Constructs	Epitope location	Sequence
Multi-epitopes construct A (PRRSV:LT-A)	A1: GP4	G V SA A Q E K I S F G
	A2: GP5	S H L Q L I Y N L
	A3: nsp10	C P G K N S F L D E A A Y C N H L
Multi-epitope construct B (PRRSV:LT-B)	B1: GP4	C L FA I L L A I
	B2: GP5	C A L A A L T C F V I R
	B3: nsp9	V R G N P E R V K G V L Q N T R F
Multi-epitope construct C (PRRSV:LT-C)	C1: GP4	F L LV G A Q Y I
	C2: GP5	K G R L Y R W R S P V I I E K
	C3: nsp10	V R I L A G G W C P G K N S F L D
Multi-epitope construct D (PRRSV:LT-D)	D1: N	I R H H L T Q T E
	D2: N	V R H H F T P S E
	D3: nsp9	K E E V A L S A Q I I Q A C D I R

Table 2. Experimental design of animal study (*dpv: days post vaccination)

Group	Vaccine construct	Challenge virus (28 dpv)	Collection of blood samples (dpv)
1 (n = 6)	No	No	0, 7, 14, 21, 28, 35, 42
2 (n = 6)	No	PRRSV NVSL-97	0, 7, 14, 21, 28, 35, 42
3 (n = 6)	*E. coli* 1836-2	PRRSV NVSL-97	0, 7, 14, 21, 28, 35, 42
4 (n = 6)	*E. coli* 1836-2/epitope-toxin	PRRSV NVSL-97	0, 7, 14, 21, 28, 35, 42

Table 3. The percentage of IFNγ expressing cells in different T cell subpopulations from pigs on 28 dpv

Groups	$CD3^+IFN\gamma^{+b}$			$CD4^+CD8^-IFN\gamma^{+c}$			$CD4^-CD8^+IFN\gamma^{+d}$			$CD4^+CD8^+IFN\gamma^{+e}$		
	CC	Pep.	Virus	CC	Pep.	Virus	CC	Pep.	Virus	CC	Pep.	Virus
1、2[a]	1.6±0.2	1.7±0.1	2.2±0.2	1.5±0.1	1.6±0.4	2.1±0.5	2.4±0.4	1.4±0.3	2.3±0.5	2.0±0.4	1.8±0.4	2.8±0.8
3	2.2±0.2	2.7±0.2	2.7±0.3	2.7±0.4	2.9±0.3	2.7±0.4	2.9±0.3	3.0±0.4	3.2±0.5	2.4±0.4	3.4±0.4	4.5±0.5
4	3.3±0.5	3.5±0.4	3.4±0.3	4.4±1.0	4.4±0.7	3.7±0.6	5.3±1.0	4.3±0.9	4.0±0.6	4.0±1.0	6.0±0.6	6.3±0.4

[a] Group 1: neg control; Group 2: neg control / challenged with PRRSV; Group 3: non-transformed *E. coli* control / challenge with PRRSV; Group 4: vaccinated / challenged with PRRSV. Group 1 and 2 were combined as they are from negative pigs at 0 dpv.
[b,c,d,e] The specific immune cell frequencies were presented as the percent of $CD3^+$ cells, $CD3^+CD4^+CD8^-$ cells, $CD3^+CD4^-CD8^+$ and $CD3^+CD4^+CD8^+$ separately.
Data is shown as mean ± SEM (n = 6).

Table 4. The percentage of IFNγ expressing cells in γδ T cell population in PBMCs from pigs on 28 dpv

Groups	$\gamma\delta\ T^+/CD3^{+b}$			$IFN\gamma^+/\gamma\delta\ T^{+c}$		
	CC	Pep.	Virus	CC	Pep.	Virus
1、2[a]	0.8±0.1	1.2±0.2	1.2±0.2	3.6±0.9	2.8±0.8	3.6±1.1
3	0.8±0.1	1.0±0.2	0.7±0.1	3.3±0.6	4.7±1.1	6.3±1.8
4	0.8±0.1	1.0±0.2	0.7±0.1	4.7±1.0	6.7±1.2	7.8±0.8

[a] Group 1: neg control; Group 2: neg control / challenged with PRRSV; Group 3: non-transformed *E. coli* control / challenge with PRRSV; Group 4: vaccinated / challenged with PRRSV. Groups 1 and 2 are combined because all were from negative pigs at 28 dpv. Three samples were taken from each group.
[b,c] The specific immune cell frequencies were presented as the percent of $CD3^+$ cells and $CD3^+\gamma\delta\ T^+$ lymphocytes separately.
Data is shown as mean ± SEM (n = 6)

Table 5. The percentages of interferon gamma cells in gamma delta T cells of PBMC at 14 DPC

Stimulus	γδ $T^+/CD3^{+\,b}$				$IFN\gamma^+/\ CD3^+\gamma\delta\ T^{+\,c}$			
	1	2	3	4	1	2	3	4
CC	1.2±0.2	0.8±0.1	0.5±0.1	0.7±0.1	2.9±0.4	3.4±0.4	0.9±0.4	2.8±0.6
A3	1.3±0.3	0.8±0.1	0.5±0.1	0.8±0.1	4.0±0.7	5.9±0.9	6.5±3.8	6.2±0.6
B3	1.4±0.2	0.9±0.1	0.5±0.1	0.9±0.1	2.7±0.7	4.3±0.4	8.8±3.3	2.9±0.6
C2	1.1±0.2	0.8±0.1	0.5±0.1	0.8±0.1	4.3±0.9	4.4±0.4	5.3±1.5	4.1±1.3
C3	1.1±0.2	0.7±0.1	0.5±0.1	0.6±0.1	4.3±1.0	3.7±0.7	3.2±0.9	3.4±0.6
D2	1.3±0.2	1.0±0.1	0.5±0.1	0.8±0.1	3.3±0.8	4.6±1.0	3.2±0.9	5.0±0.6
Pooled [a]	1.3±0.3	0.8±0.1	0.5±0.1	0.8±0.1	3.7±0.8	3.1±0.4	1.0±0.5	4.0±0.7
Virus	1.1±0.3	0.6±0.1	0.4±0.1	0.7±0.1	3.2±0.7	4.7±1.0	8.9±2.4	3.8±0.4

Group 1: neg control; Group 2: neg control / challenged with PRRSV; Group 3: non-transformed *E. coli* control / challenge with PRRSV; Group 4: vaccinated / challenged with PRRSV.

[a] Pooled peptide containing 2 ug/ml of each peptide (A3, B3, C2, C3 and D2).

[b,c] The specific immune cell frequencies were presented as the percent of $CD3^+$ cells and $CD3^+\gamma\delta\ T^+$ separately. Data is shown as mean ± SEM (n = 6)

Group 1: neg control; Group 2: neg control / challenged with PRRSV; Group 3: non-transformed *E. coli* control / challenge with PRRSV; Group 4: vaccinated / challenged with PRRSV.

[a] Pooled peptide containing 2 ug/ml of each peptide (A3, B3, C2, C3 and D2).

[b,c] The specific immune cell frequencies were presented as the percent of lymphocyte cells and γδ T^+ lymphocyte cells separately.

Data is shown as mean ± SEM (n = 6)

CC	0.8±0.1	0.5±0.1	0.3±0.1	0.4±0.1	3.3±0.3	3.2±0.3	3.0±2.0	3.4±0.6
A3	0.8±0.2	0.5±0.1	0.3±0.1	0.4±0.1	4.6±0.8	5.8±0.8	4.8±2.1	5.8±0.8
B3	0.9±0.1	0.5±0.1	0.3±0.1	0.4±0.1	3.3±1.0	4.3±0.4	6.3±1.7	3.6±0.8
C2	0.7±0.1	0.5±0.1	0.3±0.1	0.4±0.1	5.1±0.9	4.0±0.9	5.3±1.3	4.5±1.4
C3	0.7±0.2	0.4±0.1	0.3±0.1	0.3±0.0	4.6±1.0	4.2±0.9	3.3±0.7	3.4±0.9
D2	0.9±0.1	0.5±0.1	0.3±0.1	0.4±0.1	3.6±0.7	4.2±0.9	3.0±0.9	5.1±0.8
Pooled [a]	0.8±0.2	0.5±0.1	0.3±0.1	0.3±0.1	4.1±0.8	2.9±0.3	1.0±0.5	5.2±1.7
Virus	0.7±0.2	0.3±0.1	0.3±0.1	0.3±0.1	4.1±1.0	5.0±1.3	8.0±2.0	5.0±0.7

Table 7. The percentages of interferon gamma cells in different lymphocyte subpopulations at 14 DPC

Stimulus	$IFN\gamma^{+}CD4^{+}CD8^{-}$ [b]				$CD4^{-}CD8^{+}$ $IFN\gamma^{+}$ [c]				$CD4^{+}CD8^{+}$ $IFN\gamma^{+}$ [d]			
	1	2	3	4	1	2	3.	4	1	2	3	4
CC	1.9±0.1	1.8±0.2	1.3±0.2	1.6±0.2	1.6±0.3	1.9±0.2	1.3±0.2	1.6±0.2	2.1±0.4	3.0±0.4	1.7±0.3	2.1±0.2
A3	2.1±0.1	2.2±0.1	2.4±0.2	2.6±0.1	1.7±0.2	2.2±0.3	1.8±0.3	2.0±0.4	1.9±0.4	3.6±0.6	2.4±0.3	2.8±0.5
B3	1.7±0.1	1.7±0.1	2.1±0.5	2.2±0.3	1.4±0.2	1.7±0.2	1.5±0.3	1.8±0.2	1.9±0.2	2.9±0.4	2.5±0.3	3.0±0.3
C2	2.1±0.2	2.0±0.2	2.4±0.2	2.6±0.2	1.7±0.2	2.0±0.2	2.0±0.1	2.9±0.6	2.4±0.5	3.6±0.4	3.6±0.8	3.8±0.7
C3	1.9±0.2	1.9±0.1	1.5±0.2	2.1±0.2	1.6±0.3	1.9±0.2	1.4±0.2	1.7±0.3	2.4±0.4	3.3±0.5	1.8±0.3	2.1±0.4
D2	2.3±0.2	2.1±0.1	3.0±0.5	3.2±0.1	1.7±0.1	1.8±0.2	2.3±0.4	2.3±0.4	2.7±0.3	3.5±0.4	2.9±0.5	2.9±0.4
Pooled [a]	1.7±0.2	1.7±0.1	1.3±0.2	1.8±0.3	1.5±0.2	1.6±0.2	1.4±0.2	1.6±0.2	1.6±0.3	3.1±0.4	2.2±0.5	1.9±0.3
Virus	1.5±0.1	1.4±0.1	2.0±0.2	2.0±0.2	1.9±0.2	1.9±0.1	2.5±0.3	2.1±0.3	2.4±0.6	3.5±0.5	3.9±0.8	3.9±0.6

Group 1: neg control; Group 2: neg control / challenged with PRRSV; Group 3: non-transformed *E. coli* control / challenge with PRRSV; Group 4: vaccinated / challenged with PRRSV.

[a] Pooled peptide containing 2 ug/ml of each peptide(A3, B3, C2, C3 and D2).

[b,c,d,e] The specific immune cell frequencies were presented as the percent of CD3+ CD4+ CD8- cells, CD3+ CD4- CD8+ and CD3+ CD4+ CD8+ separately.

Data is shown as mean ± SEM (n = 6).

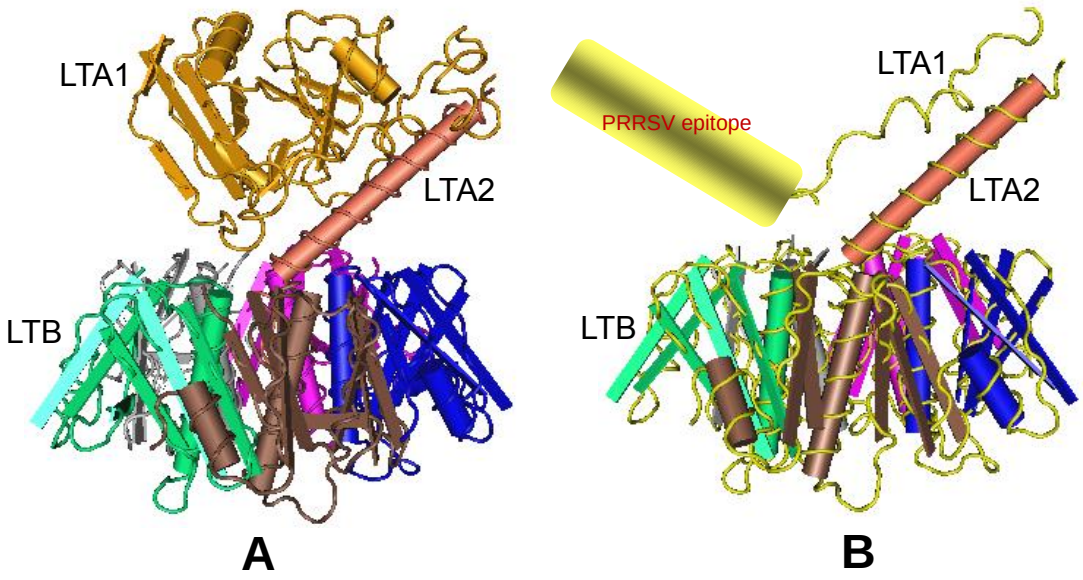

Figure 1. Predicted structure of PRRSV epitope-toxin fusion antigen. (**A**). Native structure of LT toxin. (**B**). LT toxin with PRRSV epitope inserted, in which aa 19-149 region of LTA1 is replaced by PRRSV multi-epitopes.

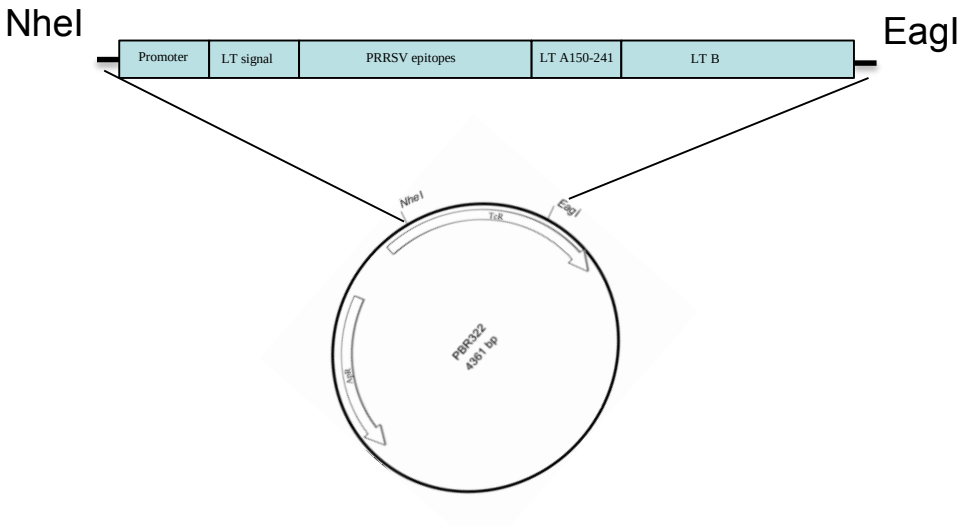

Figure 2. Plasmid map of pBR322 containing PRRSV multi-epitope-toxin fusion gene. The oligonucleotide encoding PRRSV epitopes was inserted into the LTA1 region, flanking by a promoter and LT signal at its N-terminus. The C-terminus of PRRSV epitope region is fused with rest of the LTA (aa150-241) and LTB encoding regions. This fusion gene was cloned into pBR322 vector at Nhe1/Eag1 restriction enzyme sites.

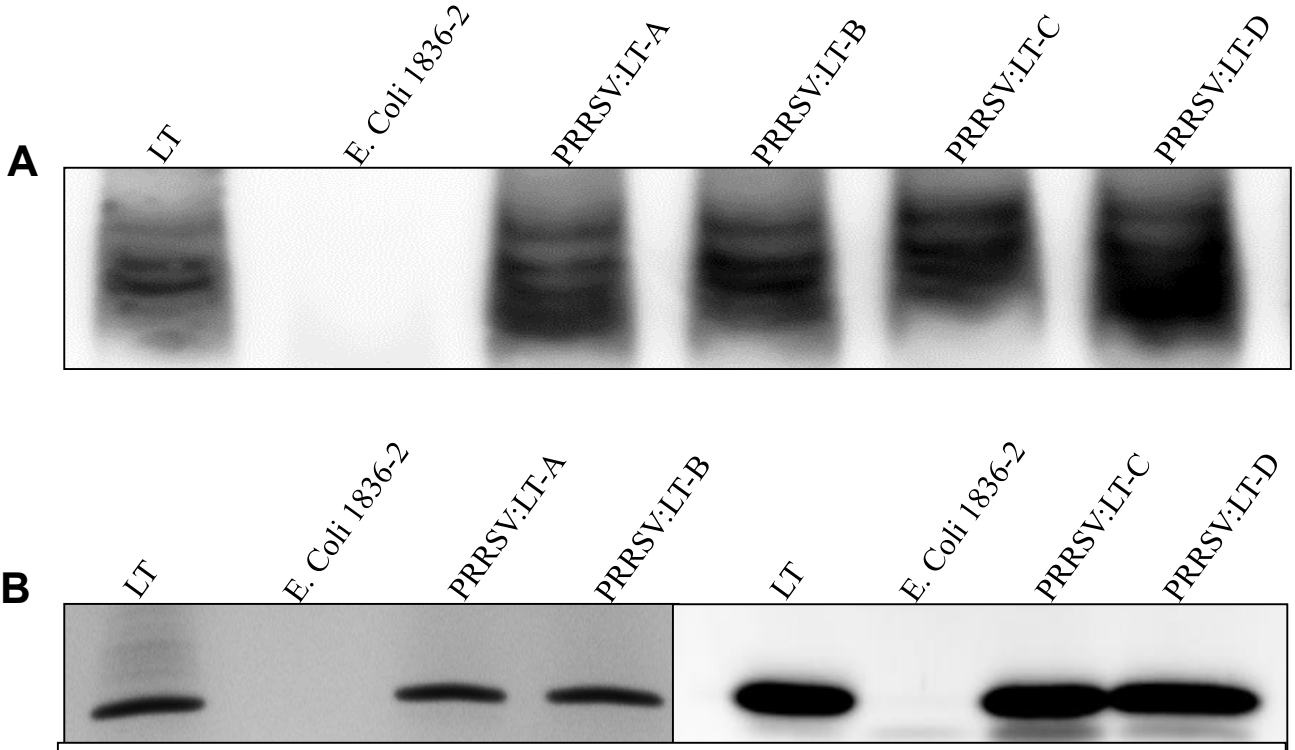

Figure 3. Expression of PRRSV epitope-LT$_{192}$ fusion proteins in transformed *E. coli* 1836-2. (A) Western blot detection for the holotoxin-structured **epitope-LT$_{192}$ fusion proteins** with the LTB pentamer using unboiled protein samples. Proteins were separated using 12% SDS-PAGE gel and detected by anti-CT antibody (recognized LT). **(B)** Immunoprecipitation detection the expression of LTB protein in bacteria cultural supernatant. Precipitated proteins were visualized using 12% PAGE gels. Transformed *E. coli* expressing only LT was using as the positive control (lane 1 in panel A; lane 1 and 5 in panel B). Non-transformed *E. coli* host strain 1836-2 was used as the negative control.

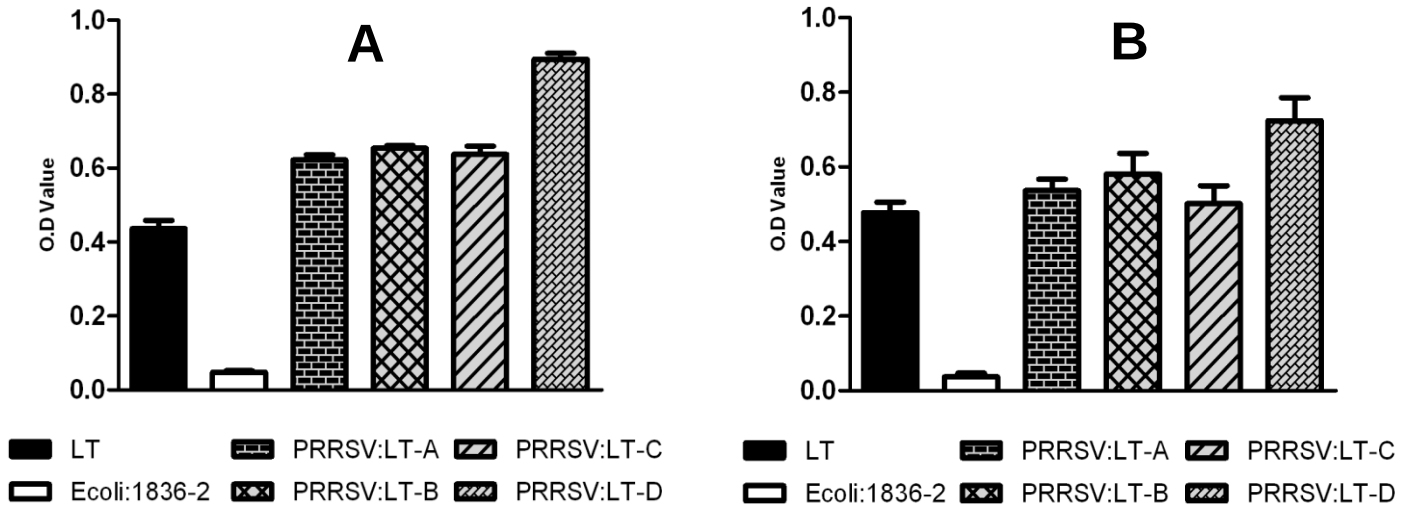

Figure 4. Binding activity of PRRSV epitope-LT chimeras to GM1 ganglioside. The ability of epitope-LT fusion proteins to recognize the GM1 receptor was measured using ELISA with GM1 as the coating antigen and anti-CT antibody (recognize LTB) as the detection antibody. Each chimera was transformed into *E. coli* strain 1836-2 that contains each individual PRRSV epitope-toxin construct. Transformed bacteria were cultured overnight in LB medium with ampicillin, and bacteria were harvested by high speed centrifugation. (**A**) Cellular total proteins extracted from the pellet; (**B**) Secretory proteins harvested from culture supernatant.

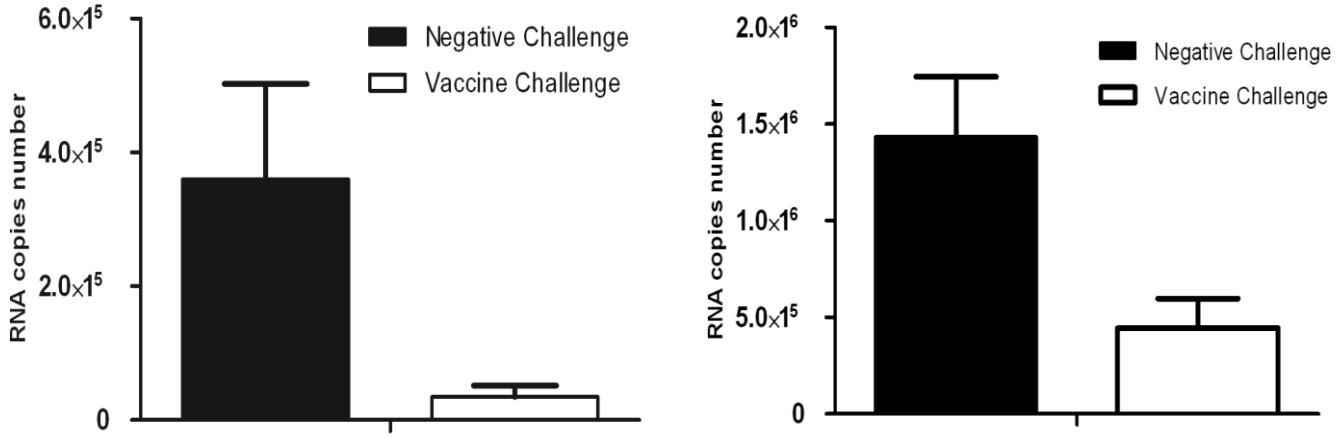

Figure 5. Viral load in serum samples and lung tissues from vaccinated (Vaccine Challenge) or non-vaccinated (Negative Challenge) groups of pigs after 14 days post challenge. The amount of virus present in serum (**A**) and lung (**B**) are shown as means ± SEM.

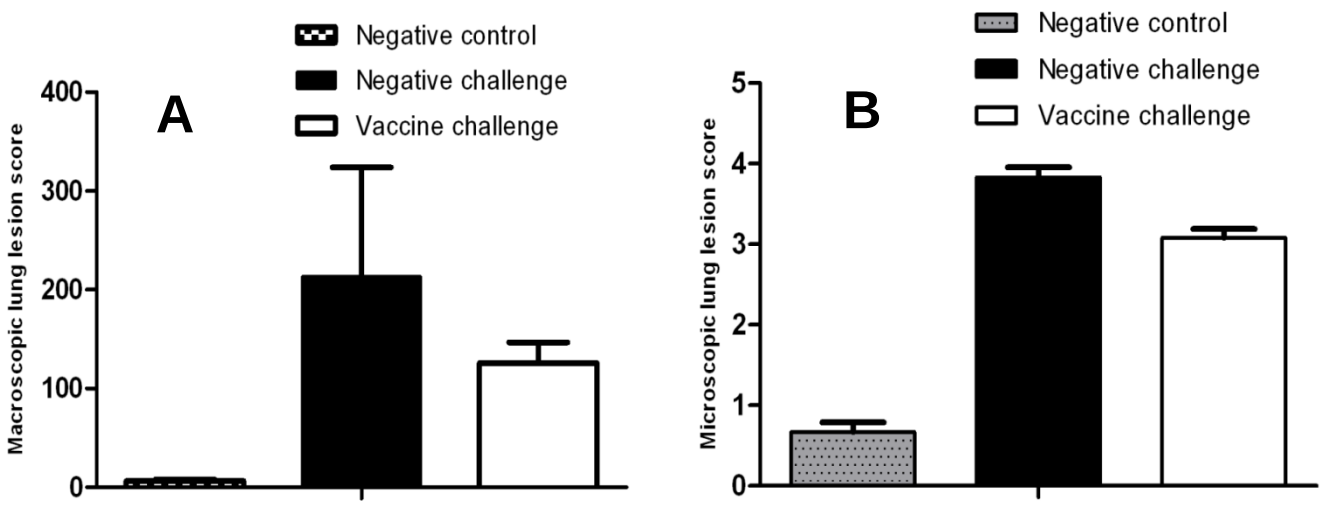

Figure 6. Comparison of lung lesion scores between groups of non-vaccinated and vaccinated pigs at 14 days post challenge. (**A**) Gross lung lesion scores based on the evaluation of percent pneumonia in each lobe and then added up for entire lung. Data points were presented as mean gross lung lesion scores for each group of pigs. (**B**) Microscopic lung lesion scores based on histopathology evaluation. Lung sections were scored for the presence and severity of interstitial pneumonia.