RESEARCH REPORT
SWINE HEALTH

Title: Identification of protective epitopes toward developing a vaccine providing broad cross-protection against various PRRS viruses – **NPB #07-130**

Investigator: Won-Il Kim

Institution: Iowa State University

Date Submitted: December 15, 2008

Industrial Summary

Enormous genetic and antigenic variation impedes effective control and prevention of PRRS. Since PRRS virus vaccination is shown to be effective only against homologous or closely related viruses, development of a vaccine which can provide broad cross-protection against various PRRS viruses has become essential for PRRS eradication in the future. A previous study in our laboratory revealed that the significance of PRRS virus structural genes [i.e. open reading frames (ORFs) 3, 5, and 6] in cross-neutralization and their roles in protective immunity could be variable for different PRRS viruses. Our previous study also suggested that chimeric PRRS viruses generated by combining structural genes from two distinct PRRS viruses (i.e. VR2332 and JA142) in an organized manner can induce virus neutralizing antibody against both viruses. Therefore, we hypothesized that those chimeric PRRS viruses could confer broader cross-protection against the challenge of those two distinct PRRS viruses when pigs are vaccinated with the live chimeric viruses. To test the hypothesis, 114 PRRS virus-free pigs were purchased, housed in 6 different rooms, and vaccinated with either sham inoculum, two wild-type viruses (VR2332 and JA142), or one of three chimeric mutants: ORF 5, ORFs 5 and 6, or ORFs 2-6 were substituted with the corresponding gene(s) of JA142 to construct JAP5, JAP56, and JAP2-6, respectively, while the remaining virus genome used intact VR2332 sequence. At 44 days post inoculation (dpi), the pigs were challenged with either VR2332 or JA142. The pigs were bled every week until 72 dpi and necropsied at 58 dpi and 72 dpi for pathological evaluation. Pigs inoculated with chimeric PRRS viruses prior to the virus challenge produce a significantly lower level of viremia after being challenged with both VR2332 and JA142 as compared to the other groups. No significant lesion was found in the lungs from the pigs previously inoculated with the chimeric viruses after the challenge although only mild to moderate lesions were observed in the virus challenge control groups. In conclusion, these observations suggest that inoculation with a live chimeric PRRS virus can confer broader cross-protection against two different PRRS viruses, and JAP56 chimeric mutant virus is a good vaccine candidate to prevent the infection by both VR2332 and JA142. Further study remains to determine if this concept is applicable for other PRRS virus strains.

Scientific abstract

Suboptimal cross-protection between heterologous PRRS viruses is a strong obstacle to effective control of the disease by vaccination. GP5 is known to play a major role in the induction of anti-PRRS virus neutralizing antibody. Yet, our recent study has demonstrated that GP3 and M protein also significantly contribute to cross-neutralization between different PRRS viruses. Furthermore, GP3 was more critical than GP5 or M in overall virus neutralization against a strain like VR2332 whose GP5 is highly glycosylated. Therefore, it was hypothesized that a chimeric virus of 2 distinctive PRRS viruses can confer better cross-protection against those viruses if necessary genes from the 2 viruses are combined together in an organized manner. To test the hypothesis, 3 chimeric viruses designated as JAP5, JAP56 and JAP2-6, respectively, were generated from the VR2332 infectious cDNA clone by replacing its ORF5, ORFs 5 and 6 or ORFs 2-6 with that/those of the JA142 strain that is genetically and antigenically distinct from the VR2332 strain. A total of 114, 3-week-old pigs were divided into 6 groups and each group was inoculated with one of the chimeric viruses, VR2332, JA142, or a sham inoculum. At 44 days dpi, 8 pigs each within each group were randomly selected, housed separately and challenged intranasally with VR2332, JA142, or a sham inoculum to determine if protective immunity was conferred by inoculation of the chimeric viruses. All of the pigs were bled periodically until 72 dpi and tested for viremia and antibody response. Half of the pigs in each room were necropsied at 14 days after the challenge and the remaining at 28 days for pathological evaluation. Based on viremia pattern and lung pathology, the prior inoculation with JAP5 or JAP56 effectively protected the pigs from the challenge with VR2332 while the pigs inoculated with JAP56 or JAP2-6 demonstrated protection against JA142 infection. In conclusion, the JAP56 chimeric virus may be used as a vaccine candidate to induce broad cross-protection against both VR2332 and JA142.

Introduction

The existence of remarkable genetic and antigenic variability among PRRS viruses has been well documented in numerous studies and many studies have claimed that those genetic and antigenic diversities negatively affected the efficient cross-protection among the viruses. GP5 is postulated to be most important in inducing protective anti-PRRS virus antibody among membrane-associated proteins of PRRS virus since anti-GP5 virus neutralizing (VN) antibody has shown to be most potent in virus neutralization. A linear neutralizing epitope (NE) of GP5 was identified in the N-terminal ectodomain (37-45 SHLQLIYNL). Nonetheless, other determinants in GP5 or other envelope-associated proteins need to be defined to explain the mechanism of the negative effect of genetic variation on the cross-protection among PRRS viruses since the linear NE identified in GP5 of PRRS virus is highly conserved among both types of PRRS virus (i.e. European and North American) and synthesized peptides resembling the linear NE failed to induce VN antibody, suggesting that additional residues might be necessary for the induction of VN antibody. Furthermore, since recent evidence has revealed the critical involvement of minor envelope proteins (GP2, GP3, GP4) and M proteins in the interaction with target cells and in protection against virus infection, the effect of genetic variation in ORFs corresponding to those proteins need to be assessed.

In our previous studies, it was demonstrated that three sites in the N-terminal ectodomain in ORF5 were significantly involved in cross-neutralization among different PRRS viruses by conducting *in vitro* and/or *in vivo* virus neutralization assays using a VR-2332-backboned infectious clone. These sites were located in the conserved linear NE (Site I, 37-38) and two hypervariable regions [Site IV (32-34) and site II (57-59)], which were located immediately before or after the linear NE, respectively. Since some of the VN-related genetic determinants identified in ORF5 were located in the hypervariable region of ORF5, which are variable for different PRRS viruses, the development of a multivalent vaccine that comprises a cocktail of various ORF5 sequences could be considered to confer a broader range of cross-protection against various PRRS viruses as proposed in the current proposal.

In addition, it was also demonstrated that, even though GP5 played a significant role in the induction of anti-PRRS virus neutralizing antibody, envelope-associated proteins other than GP5 also contributed in an additive

manner and at varying degrees to cross-neutralization of the viruses. In particular, GP3 and M proteins played a significant role in cross-neutralization when individual ORF was assessed by gene swapping technique. Interestingly, GP3 was even more critical than GP5 or M in overall virus neutralization against a virus such as VR-2332, the prototype of North American PRRS virus, which shows the elevated resistance against VN antibody generated against other heterologous PRRS viruses. It was speculated that the enhanced resistance of the virus might be attributed to its tolerance to the virus neutralization activity of antibody directed to GP5 and M proteins because of the high glycosylation in its GP5, as the negative effect of *N*-glycosylation in the GP5 ectodomain on the induction of neutralizing antibody has been reported. In this regard, GP3 would be a better target protein to control certain PRRS viruses, such as VR-2332 whose ORF5 is highly glycosylated and subsequently, it was hypothesized that a chimeric virus of two different PRRS viruses (VR2332 and JA142) can confer better cross-protection against those two viruses based on the previous findings. Therefore, an animal experiment was conducted to confirm that *in-vitro* observations correlate to *in-vivo* evidence and determine an important basis for the development of a more efficacious vaccine which can confer broad cross-protection.

Objectives

Our long-term goal is to find a way to generate a safe and efficacious vaccine conferring excellent cross-protection. The immediate objective of the proposed study is to assess the effect of specific genetic elements of PRRS virus, which were identified from previous studies, on cross-protection against various PRRS viruses in pigs. Specific aims are to:

a) Assess the role of ORFs 3, 5 and 6 product in cross protection between viruses (donor and recipient) in pigs;
b) Evaluate the effect of structural genes (ORFs 2-6) on cross protection between viruses; and
c) Determine if a chimeric mutant derived from VR-2332 and JA142 (i.e., ORFs 2-4 of VR-2332 and ORFs 5-6 of JA142) can provide cross protection against both viruses.

Materials and Methods

***Preparation of virus inocula*.** Two field isolates (VR-2332 and JA142) and three chimeric mutants (JAP5, JAP56, and JAP23456) have been prepared for animal challenge. In order to obtain a highly homologous virus inoculum, each mutant was plaque-cloned two times in MARC145 cells and propagated once to a large volume. Each plaque-cloned mutant was sequenced for the entire structural genes to ensure maintenance of the introduced mutations at the correct positions and freedom from other inadvertent mutations. All viruses were adjusted to 10^3 $TCID_{50}$ per ml for animal inoculation or challenge.

***Animal trials*.** Two- to three-week-old crossbred pigs (n=114) of a similar genetic background and comparable health status were purchased from a herd known to be free of PRRS virus, swine influenza virus, PCV2 and *Mycoplasma hyopneumoniae*. The pigs were housed in the Large Animal Infectious Disease Facility at Iowa State University which is operated at BSL2 compliance. The pigs were randomly assigned to 6 rooms upon arrival and confirmed to be free of the pathogens listed above by testing nasal swabs and/or serum samples collected from the pigs with real-time PCR. The pigs were acclimated for one week and injected intramuscularly with one of the field or chimeric viruses as summarized in Table 1. After the first inoculation, all pigs were bled every week until 42 days post inoculation (dpi). At 42 dpi, the pigs within each inoculation group were randomly divided further into 15 rooms according to the challenge virus they were to receive (*See table 1*) and then intranasally challenged at 44 dpi. After challenge, the pigs were bled at 48, 51, 55, 58 dpi and thereafter, every 7 days until 72 dpi. PCR, ELISA and virus neutralization assay were done on the serum samples. Pigs were euthanized at 58 dpi and 72 dpi for pathological evaluation (i.e., lung score).

Table 1. Schedule of animal trial

Inoculum virus at 0 dpi (# of pigs)	Challenge virus at 44 dpi (# of pigs)	Necropsy at 58 dpi (14 days after challenge)	Necropsy at 72 dpi (28 days after challenge)
VR-2332 (24)	VR-2332 (8)	4	4
	JA142 (8)	4	4
	No inoculation (8)	4	4
JA142 (24)[a]	VR-2332 (7)	3	4
	JA142 (7)	3	4
	No inoculation (6)	3	3
No inoculation (18)[b]	VR-2332 (6)	3	3
	JA142 (6)	3	3
	No inoculation (4)	2	2
JAP5 (16)	VR-2332 (8)	4	4
	JA142 (8)	4	4
JAP56 (16)	VR-2332 (8)	4	4
	JA142 (8)	4	4
JAP2-6 (16)	VR-2332 (8)	4	4
	JA142 (8)	4	4

[a] Four pigs were dead of PRRS and streptococcal infection
[b] Two pigs were dead of streptococcal infection

Results

Detection of PRRS virus in serum after inoculation. The pigs inoculated with wild-type viruses (VR2332 and JA142) demonstrated similar levels of viremia after inoculation, showing the highest viremia (approximately, 10^5 $TCID_{50}$/ml) at 7 dpi. On the other hand, chimeric viruses (JAP5, JAP56, and JAP2-6) produced a bit lower of a level of viremia as compared to the wild-type viruses in the pigs, demonstrating peak viremia between 10^3 and 10^4 $TCID_{50}$/ml at 7dpi (Figures 1 and 2).

Detection of PRRS virus in serum after challenge with VR2332. After challenge with VR2332 at 44 dpi, the pigs previously inoculated with chimeric viruses showed significantly lower levels of viremia than the challenge control group (N) ($p<0.001$), but no different levels from the homologous challenge group (VR2332) (Figure 1). There was no statistical difference in viremia level between the JAP5, JAP56 and JAP2-6 inoculated groups. However, the JAP2-6 group showed a significantly higher viremia level than JAP5 and JAP56 at 55 dpi ($p=0.0273$), 11 days after the challenge with VR2332. Thus, the protective ability of three chimeric viruses against VR2332 virus was JAP56 = JAP5 > JAP2-6 based on viremia data.

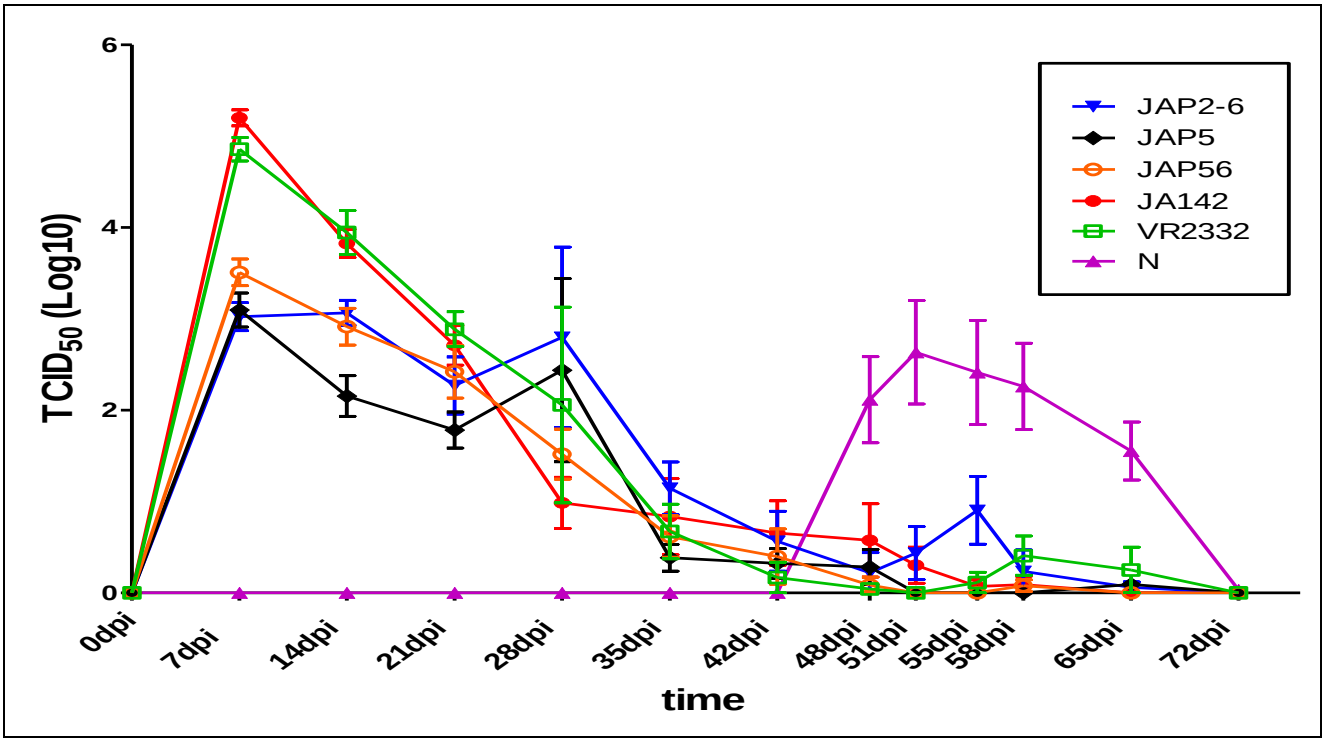

Figure 1. Detection of virus before and after challenge with VR2332

Detection of PRRS virus in serum after challenge with JA142. After challenge with JA142 at 44 dpi, all chimeric virus-inoculated groups produced significantly lower levels of viremia than the challenge control group (N) and the heterologous challenge group (VR2332) ($p<0.001$), but no different levels from the homologous challenge group (JA142) (Figure 2). The viremia level of the JAP2-6 group was significantly lower than the JAP5 group ($p=0.053$), but similar to the JAP56 group. Thus, the degree of protection of the chimeric viruses against JA142 was JAP56 = JAP2-6 >> JAP5 based on viremia data.

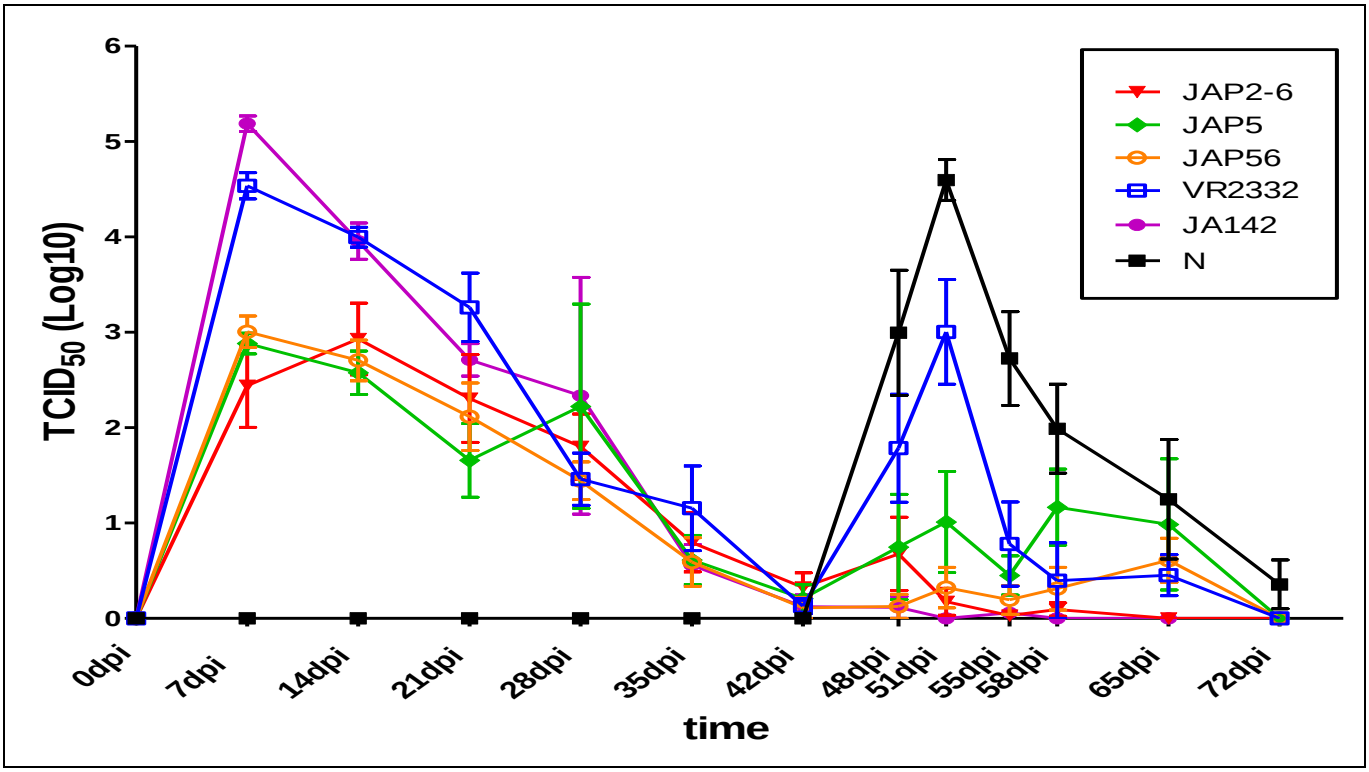

Figure 2. Detection of virus before and after challenge with JA142

Detection of PRRS virus-specific antibody. PRRS-specific antibody was detected by ELISA from 7 dpi until 72 dpi in all groups except the negative control group, which remained negative until the end of the study. No virus neutralizing (VN) antibody was detected until 28 dpi against JA142 or until 35 dpi against VR2332. VR2332- specific VN antibody was detected in 9 out of 24 pigs inoculated with VR2332 at 42 dpi (i.e., before the virus challenge), while JA142-specific VN antibody was detected in 13 out of 20 pigs inoculated with JA142 at 42 dpi. One pig in JAP5 group produced VN antibody against both VR2332 and JA142 at 42 dpi. After the virus challenge, 6 or 3 out of 16 pigs each inoculated with JAP5 or JAP56, respectively, produced VN antibody against both VR2332 and JA142 at 58 or 72 dpi although 15 out of 16 pigs inoculated with JAP2-6 induced neutralizing antibody only against JA142 at the same time points. VR2332- or JA142-inoculated pigs induced VN antibody only against each homologous virus at 58 or 72 dpi: 16 out of 24 VR2332-inoculated pigs or 19 out of 20 JA142-inoculated pigs induced VR2332- or JA142-specific VN antibody, respectively. However, 6 pigs inoculated with VR2332 and challenged with JA142 later at 44 dpi (VR2332 heterologous challenge group, n=8) also produced JA142-specific VN antibody at 58 or 72 dpi, which coincided with high levels of viremia after the challenge with JA142 in this group.

Pathological Evaluation. Gross and microscopic lesion scores and intensity of immunohistochemistry (IHC) staining for PRRS virus were determined on the lungs collected at 58 (14 days after challenge) and 72 dpi (28 days after challenge) to evaluate PRRS virus-related lesions. No significant difference in lesion score and IHC intensity was found among the groups because only mild to moderate lung lesions were observed in all groups except the negative control group.

Discussion

This study suggested that chimeric viruses which contain structural genes from two distinct PRRS viruses (i.e. VR2332 and JA142) in an organized manner could confer broader cross-protection based on the level of viremia and lung lesion after challenge with both viruses. JAP56 which contains the ORFs 5 and 6 of JA142 and the remaining genes of VR2332 demonstrated the best protective efficacy against both viruses. This study also proved the previous concept which was proposed based on *in-vitro* neutralization assay: Immunobiological role of structural genes (especially, ORFs 3, 5, and 6) of PRRS virus varies in different strains. This concept will be evaluated further with other various PRRS isolates which have been causing a consistent and persistent problem in swine production. The ultimate goal of this study is to develop a multivalent chimeric virus vaccine based on the sequences of various field-representative PRRS isolates for broader cross-protection against various PRRS viruses.